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Promoting plant growth using ACC deaminase-producing bacteria: insights into plant-bacterial interactions and agricultural and biotechnological applications

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"Promoting plant growth using ACC deaminase-producing bacteria: insights into plant-bacterial interactions and agricultural and biotechnological applications"

Por

Francisco Xavier Inês Nascimento

Tese julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (014/PPGBTC/2018) do Programa de Pós-Graduação em Biotecnologia e Biociências - UFSC.

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“A mente que se abre a uma nova ideia,
jamais voltará ao seu tamanho original”
Albert Einstein

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Today, I can clearly see that my scientific path was laid upon me a long time ago. This was done by my parents and family who provided me a childhood full of wonderful experiences and contact with the natural world. This was especially done by my late father, Jorge Nascimento, who took me in countless “adventures”, from fishing, hunting birds, nursering ants, collecting snails, cutting Christmas trees, and picking fruits from trees to endless walks in Nature. His naturalist and adventurous spirit was the precursor and major contributor to my development as a person and scientist, and words cannot express how I miss and feel about him. Even in his sickness he helped in the collection of material from which several of the microorganisms studied in this work (strains PLM3, PLMAX, ALCS3) were isolated. His contribution is embedded in all the written and unwritten lines of this work. My mother’s, Maria Nascimento, unconditional love and dedication trough all these years made “me” possible, and me and my work today are a true reflection of her. She also helped in the collection of material used in this work. Both their work and investment in my education is something that I dearly cherish and will never forget, and I will always try to do my best to be their “extension” and legacy in this world. In my education process, I also cannot forget and must thank to my late grandparents (Laurinda and Eduardo Nascimento, and Ivone and Francisco Inês), whom, despite all their economic and life difficulties always helped and were present for me. I also thank my sister, Aura Nascimento, for providing me two nieces (Sofia and Laura) that made my world brighter and happier. Today they call me their scientist uncle and help me being better every day.

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RESUMO

O etileno é um fitohôrmonio gasoso que apresenta um papel fundamental no desenvolvimento vegetal. Está relacionado com vários processos fisiológicos que regulam o crescimento em plantas que são importantes para seu crescimento e sobrevivência. Sob condições de estresse, as plantas aumentam sua produção de etileno, provocando efeitos adversos que podem incluir a inibição do alongamento das raízes e do processo de nodulação por rizóbios e também a aceleração dos processos de senescência e abscisão. Uma das formas de regular as concentrações de etileno consiste na utilização de bactérias produtoras da enzima ácido 1-aminociclopropano-carboxílico (ACC) desaminase. Essa enzima é responsável pela degradação de ACC, o principal precursor do etileno em plantas, transformando-o em α-cetobutirato e amônia.

Este projeto tem como objetivo principal investigar qual o papel da enzima ACC deaminase na mediação da relação entre planta e bactéria e também avaliar a eficiência de bactérias produtoras de ACC deaminase em promover o crescimento de várias espécies de plantas a serem utilizadas para diversos fins biotecnológicos.

Resultados obtidos neste trabalho demonstram o papel importante da enzima ACC desaminase na interação entre planta e bactéria, nomeadamente ao nível do processo de nodulação e promoção do crescimento de algumas espécies vegetais. Se verificou também que esta enzima é amplamente transmitida entre várias espécies de bactérias e eucariotos associados com plantas, e fatores como o próprio genótipo da planta hospedeira podem influenciar a sua seleção e transmissão em bactérias. Neste trabalho foi também criada uma metodologia permitindo o isolamento rápido de bactérias produtoras de ACC desaminase e outras enzimas envolvidas na degradação de outros fitohormônios. Seguindo essa metodologia, várias bactérias foram isoladas, caracterizadas, sendo algumas delas utilizadas em experimentos em campo onde promoveram o desenvolvimento de plantas de forma eficaz.

Palavras-chave: Etileno, ACC desaminase, bactérias promotoras do crescimento de plantas.

ABSTRACT

Ethylene is a gaseous phytohormone that plays a fundamental role in plant development, being related to several and relevant physiological processes that regulate plant growth and survival.

Under stress conditions, plants increase their ethylene production, which may result in adverse consequences, such as, inhibition of root elongation, decrease of rhizobial nodulation of roots, and, ultimately, in senescence and abscission of vegetal tissues.

One way to regulate plant ethylene levels consists on the use of bacteria that produce the enzyme 1-aminocyclopropane-1-carboxilate (ACC) deaminase, which degrades ACC (the main precursor of ethylene in higher plants) into α-ketobutyrate and ammonia.

Hence, this thesis aims to investigate the role of the enzyme ACC deaminase as a mediator of the interactions between plant and bacteria, and to evaluate the efficiency of selected ACC deaminase-producing bacteria in promoting the growth and development of several plant species to be used for agricultural and biotechnological purposes.

The results obtained in this work demonstrate the important role that the enzyme ACC deaminase plays in the interaction between plant and bacteria, namely in the nodulation process of legumes, and in promoting the growth and development of some plant species. Moreover, it has also been verified that this enzyme is widely transmitted amongst several bacteria species, as well as between eukaryotes associated with plants. Several factors such as the host plant genotype, bacterial genetic properties and others, can influence ACC deaminase selection and transmission in bacteria and eukaryotes.

Furthermore, in this work, a methodology that allows a rapid isolation of ACC deaminase-producing bacteria, as well as other phytohormone-degrading bacteria was developed and tested. Following this methodology, several bacterial strains were isolated and characterized, and some were selected to be cultivated in bioreactors and used in field experiments, where they promoted plant development effectively.

Keywords: Ethylene, ACC deaminase, bacteria, plant growth promotion

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LIST OF THE MOST SIGNIFICANT ABREVIATIONS

ET- Ethylene
ACC- 1-aminocyclopropane-1-carboxylate
SAM- S-adenosyl methionine
MTA- 5-methylthioadenosine
ACS- ACC synthase
ACO- ACC oxidase
HCN- Hydrogen cyanide
M-ACC- Malonyl-ACC
G-ACC- γ-glutamyl-ACC
JA-ACC- Jasmonoyl-ACC
AMT- ACC-N-malonyl transferase
GGT- γ-glutamyl-transpeptidase
JAR1- jasmonic acid resistance 1
MPK- Mitogen-ctivated Protein Kinase
CDPK- Calcium-dependent Protein Kinase
LRRK- Leucine-Rich Repeat Receptor Kinase
ETR- Ethylene Receptor
ERS- Ethylene Response Sensor
EIN- Ethylene Insensitive
EIL- Ethylene Insensitive-Like Protein
ERF- Ethylene Response Factor
NFs- Nodulation factors
MAMPs- Microbe-Associated Molecular Patterns
FLG- Flagellin
EF-Tu- Elongation factor-Tu
PGN- Peptidoglycan
LPS- Lipopolysaccharides
NLPs- Necrosis and Ethylene-inducing peptide 1 (Nep1)-Like Proteins
DAMPs- Damage-associated Molecular Patterns
PRR- Pattern Recognition Receptors
PTI- Pattern-Triggered Immunity
ROS- Reactive Oxygen Species
HPRGs- Hydroxyproline-Rich Glycoproteins
FLS2- Flagellin Sensitive 2
FRK1- Flg22-Induced Receptor-Like Kinase 1
EFR- EF-Tu Receptor
LRR-RP- Leucine-Rich Repeat Receptor Protein
SOBIR1- Suppressor of Brassinosteroid Insensitive 1 (BRI1)-Associated Kinase (BAK1)-interacting receptor kinase 1),
LYM- Lysin-motif Domain Proteins,
CERK1- Lysin-motif receptor kinase
LORE- Lectin S-domain-1 Receptor–Like Kinase
PROPEP1- Precursor of Peptide 1
PEPR- Pep1 Receptor Kinases
BIK1- Botrytis-Induced Kinase 1
NB-LRR- Nucleotide Binding and Leucine Rich Repeat Domains Proteins
R- Resistance Proteins
ETI- Effector-Triggered Immunity
HR- Hypersensitive Response
ETO- Ethylene-overproducing
LYK- Lysine Motif Domain-Containing Receptor-Like Kinase
NFP- Nodulation Factor Perception
NFR1- Nodulation Factor Receptor1
RLK- Receptor-Like Kinase
MKK- Mitogen-activated Protein Kinase Kinase
MCP- Methyl-Accepting Chemotaxis Protein
RTX- Rhizobitoxine
PHB- Poly-3-hydroxybutyrate
AVG- Aminoethoxyvinylglycine
MVG- Methoxyvinylglycine
FVG- Formylaminooxyvinylglycine
PAs- Polyamines
EFE- Ethylene -Forming Enzyme
SA- Salicylate
IAA- Indole-3-acetate
PAA- Phenylacetate
BA- Benzoate
rpm- rotations per minute
vvm- specific flow rate
TSB- Tryptic Soy Broth

GENERAL INTRODUCTION

Human populations well-being and economic growth are supported by ecosystems and natural resources. These provide food, water for drinking and irrigation, regulate the climate and maintain the balance in our planet. However, human society has systematically overlooked and destroyed these ecosystems and natural resources. Soils, arable lands, rivers and seas are being extensively modified and destroyed at a rate never observed before (FAO and ITPS, 2015, WWAP, 2017). As the world population continues to increase and is projected to reach 9.8 billion people by 2050 (UNDESA, 2017), more environmental damage, destruction of natural resources and ecosystems is occurring due to the unsustainable use of chemicals and the increasing production of waste and other pollutants. Furthermore, obvious climate changes are beginning to take their toll on ecosystems by altering weather patterns (FAO, 2017). Accordingly, the United Nations 2030 agenda for sustainable development (UN, 2015) aims to develop and apply sustainable measures to the use and recovery of natural resources, and conservation of ecosystems, as well as to maintain food production and economic growth.

Plants play an essential role in the development of sustainable measures to preserve and recover ecosystems and create natural resources. Plants are fundamental in practices such as agriculture, forestry, soil recovery (e.g. phytoremediation) and nutrient cycling (including biological nitrogen fixation), the maintenance of subterranean waters, rivers and sea ecosystems, the production of high-value compounds and pharmaceuticals, among others. Thus, understanding the physiology of plants and maximizing/controlling its growth/development is extremely important. In the last decades, much knowledge has been obtained in the subject of plant physiology and many aspects of plant growth and development are now understood. In this sense, the discovery of plant hormones, also known as phytohormones, and their effects, played a significant role in our understanding of plant growth and development (DAVIES, 2010).

One of the most important phytohormones regulating plant growth and development is ethylene (C_2H_4) (reviewed by DUBOIS et al. 2018). Acting as a gaseous phytohormone, ethylene is involved in multiple physiological and developmental processes of plants, such as seedling emergence, root and shoot development, leaf and flower senescence, abscission and fruit ripening, as well as, to regulating the responses to biotic and abiotic stresses. In plants, ethylene is synthesized by the action of the enzymes 1-aminocyclopropane-1-

carboxylate (ACC) synthase and ACC oxidase. The enzyme ACC synthase is responsible for the production of ACC, the direct ethylene precursor, which is converted to ethylene by the action of ACC oxidase (YANG and HOFFMAN, 1984).

Under stressful conditions, like those presented in degraded and contaminated soils (e.g. low nutrient availability, low pH, heavy metals, organic contaminants, and others) or areas affected by other environmental and biotic factors (e.g. drought, increased salinity, flooding, high and low temperatures, increased radiation, pathogen attack, and others) plants produce exaggerated levels of ethylene, termed “stress ethylene”, which are responsible for inhibiting overall plant growth (ABELES et al. 1992). Hence, controlling stress ethylene levels becomes extremely important to improve plant growth, resistance and development (GLICK, 2014). One way to accomplish this relies on the use of efficient plant-growth promoting bacteria containing the ACC deaminase enzyme. Among the production of several traits that may induce plant growth or protection, these bacteria are also able to decrease deleterious plant ethylene levels by degrading the ethylene immediate precursor, ACC, converting it into ammonia and α-ketobutyrate. By naturally associating and promoting plant growth, these bacteria can be used in several agricultural and biotechnological applications and present an alternative for the use of synthetic and chemical fertilizers, which are responsible for unacceptable pollution levels worldwide. Hence, obtaining efficient ACC deaminase-producing bacteria and understand their role in plant growth is key to the development of new strategies aiming the maximization of plant growth under a variety of conditions.

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THESIS MAIN OBJECTIVE

This thesis aims to study the role of ACC deaminase in plant-bacteria interactions, as well as to the development and optimization of strategies for increasing plant-growth through the modulation of ethylene levels by selected and efficient ACC deaminase-producing bacteria. By modulating ACC and, consequently, ethylene levels an improved plant growth is expected to be achieved, which may represent an important step in the development of sustainable and effective environmental friendly measures, as well as, to maximize many important agricultural and biotechnological applications.

SPECIFIC OBJECTIVES

- Study the role of ACC and ethylene in plant-bacterial interactions, including the nodulation process of leguminous plants
- Analyze the prevalence and evolution of ACC deaminase in Bacteria and Eukaryotes
- Isolate and characterize effective plant-growth-promoting bacteria presenting ACC deaminase activity
- Cultivation and application of selected ACC deaminase-producing bacteria under field conditions

THESIS SUMMARY

CHAPTER 1- Ethylene and ACC in plant-bacteria interactions

In this chapter, an updated view on the role of ethylene and ACC in general plant-bacterial interactions is provided. Ethylene and ACC impact the plant defense and symbiotic responses induced by pathogens and beneficial bacteria presenting different lifestyles (rhizospheric, endophytic, phyllospheric). Some of these bacteria developed intricate mechanisms to modulate plant ethylene responses. New perspectives on the impact of ethylene and ACC in microbiome assembly are discussed.

CHAPTER 2- New insights into ACC deaminase phylogeny, evolution and ecological significance

The phylogeny, evolution and ecological significance of the enzyme ACC deaminase is studied in this chapter. We demonstrate that ACC deaminase is prevalent in several plant-associated bacteria and evolved mainly by vertical transmission events but also by horizontal gene transfer. Ecological implications of ACC deaminase production are discussed.

CHAPTER 3- ACC deaminase in the nodulation process of leguminous plants

The symbiotic rhizobia-legume association is negatively affected by ethylene. In this chapter, the role of ACC deaminase in modulating deleterious leguminous plant ethylene levels is reviewed and discussed. The presence and evolution of ACC deaminase and rhizobitoxine (other mechanism leading to the reduction of plant ethylene levels) in rhizobia is also analyzed.

Furthermore, the role of ACC deaminase in the nodulation process of Alpha and Betaproteobacteria is studied.

CHAPTER 4- Characterization and expression of an exogenous ACC deaminase gene in *Serratia grimesii* BXF1, a plant-growth promoting endophyte

In this chapter, the bacterial endophyte *Serratia grimesii* BXF1 is characterized, its genome analyzed, and engineered to express an exogenous ACC deaminase gene. Consequently, the plant-growth promoting abilities of the engineered strain are tested under different experimental conditions.

CHAPTER 5- Isolation and characterization of ACC deaminase-producing bacteria

A methodology for the rapid isolation of ACC deaminase-producing bacteria is described. Additionally, methods for isolation of IAA and SA-degrading bacteria are discussed. By employing this targeted approach, several bacterial strains with phytohormone-degrading abilities were isolated and identified. Several of these strains were thoroughly characterized (including their plant-growth promoting activities) and their genomes sequenced and analysed.

CHAPTER 6- Bioreactor cultivation and field application of selected ACC deaminase-producing bacteria

An overview of the cultivation process of the ACC deaminase-producing bacteria *Pseudomonas palleroniana* MAB3 and *P. thivervalensis* SC5 in bioreactors is presented in this chapter. Additionally, the selected bacteria were used in small field experiments. Strain SC5 plant growth promotion abilities were tested in a coal-mining soil in Treviso. A mixed inoculant containing both strains, MAB3 and SC5, was tested in Xanxerê and Cachoeira do Sul, under a large scale agricultural setup using maize.

CHAPTER 1

Ethylene and ACC in plant-bacteria interactions

REVIEW MANUSCRIPT

"Ethylene and 1-aminocyclopropane-1-carboxylate (ACC) in plant-bacterial interactions"

By Francisco X. Nascimento, Márcio J. Rossi and Bernard R. Glick

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Ethylene and 1-aminocyclopropane-1-carboxylate (ACC) in plant-bacterial interactions

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Keywords: Ethylene, 1-aminocyclopropane-1-carboxylate, bacteria, microbiome, plant growth

ABSTRACT

Ethylene and its precursor 1-aminocyclopropane-1-carboxylate (ACC) actively participate in plant developmental, defense and symbiotic programs. In this sense, ethylene and ACC play a central role in the regulation of bacterial colonization (rhizospheric, endophytic and phyllospheric) by the modulation of plant immune responses and symbiotic programs, as well as by modulating several developmental processes, such as root elongation.

Plant-associated bacterial communities impact plant growth and development, both negatively (pathogens) and positively (plant-growth promoting and symbiotic bacteria). Some members of the plant-associated bacterial community possess the ability to modulate plant ACC and ethylene levels and, subsequently, modify plant defense responses, symbiotic programs and overall plant development.

In this work, we review and discuss the role of ethylene and ACC in several aspects of plant-bacterial interactions. Understanding the impact of ethylene and ACC in both the plant host and its associated bacterial community is key to the development of new strategies aimed at increased plant growth and protection.

INTRODUCTION

Plants play a vital role in the Earth's ecosystems. Their ability to photosynthesize, transforming light energy into chemical energy (in the form of sugars and other organic compounds), provides the energy (either directly or indirectly) necessary for nearly all lifeforms. Amongst these, bacteria possess a privileged relationship with plants that results from tens of millions of years of co-evolution in the Earth's soils. A huge amount (from 15% up to 70%) of a plant's photosynthetically fixed carbon is released into the rhizosphere (the portion of the soil surrounding the roots) (Neumann and Rohmeld, 2001). The bacteria present in the rhizosphere bind to external root tissues and use compounds exuded by plants as an energy sources (Philippot et al. 2013). Some bacteria, termed endophytes, not only thrive in the rhizosphere, but can also enter and colonize internal plant tissues (Hardoim et al., 2015). In addition, phyllospheric bacteria colonize aerial plant tissues (e.g. leaf) (Vorholt, 2012). Altogether, rhizospheric, endophytic and phyllospheric bacteria constitute the plant-associated bacterial community, which plays a vital role in plant growth and development. Yet, members of the plant-associated bacterial community may influence plant growth and development in different and sometimes opposing ways. Plant pathogens negatively affect plant growth and development by deleterious and parasitic actions (e.g. production of toxins and enzymes that degrade plant tissues). On the other hand, beneficial bacteria form mutualistic and symbiotic relationships with the plant host (e.g. rhizobia and leguminous plants), and promote plant growth by enhancing plant mineral uptake, nitrogen fixation, production of plant-growth promoting compounds, degradation of compounds that negatively impact plant growth (e.g. xenobiotics), and providing protection from pathogens (Glick, 2014; Santoyo et al., 2015).

Plants have developed an "immune system" composed of a series of intricate and complex mechanisms that ultimately limit and control its associated bacterial communities (Jones and Dangl, 2006). In addition, leguminous plants tightly control the symbiotic nodulation process by a mechanism termed auto-regulation of nodulation (Ferguson et al., 2010).

Plant hormones actively participate in plant developmental, defense and symbiotic programs. In the center of these processes lies ethylene (ET), a gaseous plant hormone, readily diffusible in plant tissues, that exerts its effects even in very low concentrations. ET not only regulates several aspects of plant growth (Van de Poel et al., 2015), but also participates in defense and symbiotic programs induced by bacteria (Desbrosses and Stougaard, 2011; Guinel,

2015), consequently impacting bacteriome assembly. Moreover, several reports point to the role of the direct ET precursor, 1-aminocyclopropane-1-carboxylate (ACC), in regulating plant developmental (Yoon and Kieber, 2013; Vanderstraeten and Van Der Straeten, 2017) and defense responses (Tsang et al., 2011).

As a consequence of the key role of ET and ACC, many bacteria that are closely associated with plants possess sophisticated mechanisms to sense and modulate ET and ACC levels within plant tissues and in the rhizosphere. Although many of these mechanisms are known and their effects in plant growth are documented, not much is understood about their prevalence in bacterial communities, their impact on the plant microbiome and their effect on overall plant growth.

Here, the role of ET and ACC in plant-bacterial interactions is reviewed and discussed. The impact of ET and ACC in plant development, defense and symbiotic programs, as well as, the bacterial strategies used to modulate plant ACC and ET concentrations are described in detail. Ultimately, understanding the impact of ET and ACC in plants and their associated bacteria is key to the development of new technologies aiming to maximize plant growth and protection.

1. Plant ACC and ET biosynthesis and signaling mechanisms

ET biosynthesis occurs in all higher plants via a methionine-dependent pathway (**Figure 1**), in which methionine is converted to S-adenosyl methionine (SAM) by the enzyme SAM synthase. SAM is then converted to ACC, the direct precursor of ET, and 5-methylthioadenosine (MTA), by the enzyme ACC synthase (ACS). MTA is reconverted to methionine by a series of biochemical reactions, described as the Yang cycle (Yang and Hoffman, 1984), which replenish the pool of methionine available. Finally, the enzyme ACC oxidase (ACO) converts ACC into ET, HCN and CO_2.

ACC can also be conjugated to form malonyl-ACC (M-ACC), γ-glutamyl-ACC (G-ACC) and jasmonoyl-ACC (JA-ACC) by the action of the ACC-N-malonyl transferase (AMT), γ-glutamyl-transpeptidase (GGT) and jasmonic acid resistance 1 (JAR1) enzymes, respectively (**Figure 1**). The conjugation of ACC can also regulate future ACC and ET biosynthesis (Van de Poel and Van Der Straeten, 2015).

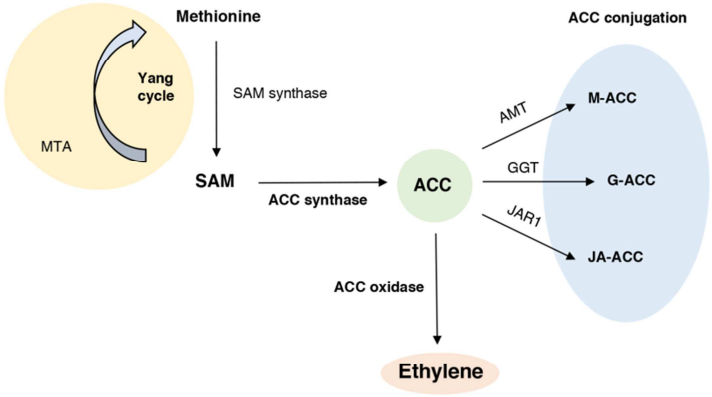

Figure 1- The ethylene biosynthetic pathway and ACC conjugation process. In this pathway methionine is converted to S-adenosyl methionine (SAM) by the enzyme SAM synthase. SAM is converted to ACC (1-aminocyclopropane-1-carboxylate) and 5-methylthioadenosine (MTA), by the enzyme ACC synthase. MTA is reconverted to methionine by a series of biochemical reactions, described as the Yang cycle. The enzyme ACC oxidase catalyzes the conversion of ACC to ET. In addition, ACC can be conjugated to M-ACC (Malonyl-ACC), G-ACC (γ-glutamyl-ACC) or JA-ACC (Jasmonoyl-ACC) by the action of the enzymes ACC-malonyl transferase, γ-glutamyl-transpeptidase and Jasmonic acid resistance 1, respectively.

1.1 ACC synthase and ACC

The ET precursor, ACC, is a nonproteinogenic α-amino acid synthesized from SAM by ACS and its production involves transcriptional, post-transcriptional, translational and post-translational regulation (Lee et al., 2017). In all higher plants that have been examined to date, the ACS enzyme is encoded by a multigene family, leading to the production of several isoforms that have specific roles in different plant cells, tissues and developmental processes (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004; Tsuchisaka et al., 2009). Furthermore, ACS can form active heterodimers that may influence their biological activity, regulation and coordination of ACC and ET production (Tsuchisaka and Theologis, 2004; Tsuchisaka et al., 2009; Lee et al., 2017).

The expression of ACS isoforms is controlled at the transcriptional level, with several internal and external cues modulating the transcription of specific ACS genes. Developmental

stages, stress conditions and the presence of phytohormones, such as auxin and ET itself, are amongst the main inducers of transcriptional changes in ACS genes (Wang et al., 2005; Vanderstraeten and Van Der Straeten, 2017).

Numerous transcription factors can bind to ACS gene promoters (Matarasso et al., 2005; Lin et al., 2009, Ito et al., 2008, Zhang et al., 2009; Li et al., 2012: Datta et al., 2015; Li et al., 2017). For example, Li et al., (2012) demonstrated that the mitogen-activated protein kinases, MPK6 and MPK3, regulate the expression of *Arabidopsis* ACS2 and ACS6 genes via the WRKY33 transcription factor that binds to the W-boxes (TTGACT/C) in the promoters of ACS2 and ACS6 genes *in vivo*.

Based on their biochemical and structural properties, namely the presence of phosphorylation sites in the C-termini, ACS can be divided in three different groups (Chae and Kieber, 2005). Group I ACS (ACS2 and ACS6) contain phosphorylation sites for both MPKs and CDPKs (calcium-dependent protein kinases). Group II ACS (ACS4, ACS5, ACS8, ACS9, and ACS11) are uniquely phosphorylated by CDPKs, and group III ACS (ACS7) does not contain any phosphorylation sites. These phosphorylation sites have an important role in the increased activation or deactivation of some ACS isoforms, in which kinases, phosphatases, and the ubiquitin-proteasome system play a pivotal role (reviewed by Xu and Zang, 2014).

1.2 ACC as an internal plant-signaling molecule

Importantly, Tsuchisaka et al., (2009) demonstrated that the disruption of all *Arabidopsis* ACS isoforms leads to lethality, further indicating the indispensable role of ACS and ACC in plant growth and development. Furthermore, the authors suggest that ACC itself, independently of ET, may play a role as a signaling molecule that controls plant growth and development. Results obtained by Xu et al., (2008) and Tsang et al., (2011) indicate that ACC takes part of a rapid signaling mechanism controlling root cell elongation that is independent of ET signaling. The *Arabidopsis fei1 fei2* mutant plants, defective in the production of the Leucine-Rich Repeat Receptor Kinases (LRRK) FEI1 and FEI2, displayed a severe defect in anisotropic root growth due to a reduced cellulose microfiber content in the cell wall at the root tip. Application of ET biosynthesis inhibitors reversed the *fei1 fei2* phenotype while ET signaling inhibitors did not. Moreover, the ET insensitive mutants *etr1* and *ein2* did not suppress the *fei1 fei2* phenotype. Interestingly, the FEI proteins interacted directly with ACS5 and ACS9 (Xu et al., 2008). Similarly, ET biosynthesis inhibitors reduced the rapid effect of cell wall stress

damage induced by isoxaben (a cellulose biosynthesis inhibitor), while the ET signaling mutants *ein3 eil1* presented similar root elongation inhibition as the wild-type plant (Tsang et al., 2011). Both the application of isoxaben and ACC led to a rapid reduction in root epidermal cell elongation in both wild-type and *ein3 eil1* mutants, however, ET signaling components were required for long-term growth responses (Tsang et al., 2011).

ACC and some of its conjugated forms can be readily transported (in a matter of minutes) within the tissues of various plants, via phloem and xylem, further indicating their importance as signaling molecules. For example, ACC can be transported from roots to leaves (long distance transport) and, in a more localized fashion, from cell to cell (short distance transport). Moreover, different cells or organs have different ACS and ACO expression profiles, and ACC may be synthesized in one cell or organ and converted to ET in another cell or organ (reviewed by Vanderstraeten and Van Der Straeten, 2017).

Curiously, the fact that ACC conjugates with other phytohormones such as jasmonic acid, a hormone closely linked to plant defense (Wasternack and Hause, 2013) suggests a role for ACC in phytohormone crosstalk and a possible effect in mediating some plant defense responses.

1.3 ACC as an external signaling molecule

The use of exogenous ACC as a mean to study ET effects on plant growth and development is a common practice amongst plant physiologists. Application of ACC to the plant growth medium often leads to the plant triple response phenotype (Merchante and Stepanova, 2017). This is possibly due to the presence of a mechanism responsible for ACC transport across the plant cell wall and membrane, leading to ACC uptake (Shin et al. 2015). Importantly, ACC can be exuded by seeds and roots (Finlayson et al., 1991; Penrose et al., 2001; Penrose and Glick, 2001), indicating the existence of a mechanism responsible for ACC exudation. Intriguingly, there is no genetic information about this mechanism. Under stressful conditions plants can produce highly elevated levels of ACC that subsequently increase ET concentrations (stress ET), leading to an inhibition of plant growth and development (Abeles et al., 1992). Hence, releasing ACC to the rhizosphere may be a useful strategy to decrease the negative effects of ACC and ET accumulation under stress conditions. Moreover, since ACC is readily diffused in water it can easily be taken up by bacteria or nearby root systems; thus, the released ACC may act as a signal to recruit beneficial bacteria and/or signal nearby plants.

1.4 ACC oxidase and ET

The plant produced ACC is converted to the gaseous phytohormone, ET, by the action of the enzyme ACO. Similar to the enzyme ACS, the enzyme ACO is also encoded by a multigene family in higher plants (Dorling and McManus, 2012, Ruduś et al., 2013). In *Arabidopsis*, a total of five ACO genes are found, however only ACO2 and ACO4 have been studied in detail (Gómez-Lim et al. 1993; Raz and Ecker, 1999; Ramonell et al. 2002; Raghavan et al. 2006; Linkies et al. 2009). These studies revealed that ACO is induced in several plant tissues by numerous treatments, such as, wounding, ethrel (a liquid compound that is transformed into ET), Fe^{2+}, 2,4-dichlorophenoxyacetic acid and ACC. Likewise, several studies have demonstrated the induction of ACO gene expression by biotic and abiotic stresses, phytohormone treatments (including ET) and developmental and ontological cues in other plant species (reviewed by Dorling and McManus, 2012).

Like ACS, the ACO enzyme isoforms are expressed under tissue specific conditions and different translational regulation mechanisms control their production (Dorling and McManus, 2012). In addition, ACO expression can also be affected by post-transcriptional and post-translational regulatory mechanisms (Datta et al., 2015).

1.5 ET signaling

Plants possess an intricate mechanism regulating ET perception and consequent ET-induced responses (**Figure 2**) (reviewed in detail by Ju and Chang, 2015). In *Arabidopsis*, ET is perceived by a five-member family of ET receptors, namely Ethylene Response 1 (ETR1), ETR2, Ethylene Response Sensor 1 (ERS1), ERS2 and Ethylene Insensitive 4 (EIN4), that are commonly located in the plant cell endoplasmic reticulum (ER). These receptors act as negative regulators of the ET signaling pathway. When ET is not present, the receptors activate a Ser/Thr kinase named Constitutive Triple Response 1 (CTR1) that suppresses the ET response by phosphorylating Ethylene Insensitive 2 (EIN2), an ER-bound protein. EIN2 is in an inactive state when it is phosphorylated by CTR1 (**Figure 2A**). On the other hand, when ET binds to the receptors it leads to the inactivation of CTR1 and as a result, EIN2 is dephosphorylated and, consequently, its C-terminal domain is released to migrate to the nucleus. There, EIN2 can, directly or indirectly, activate the transcription factors EIN3 and Ethylene Insensitive-Like Protein 1 (EIL1) that, subsequently, bind to the EIN3-binding sequence (EBS) element in the promoter region of various target genes, thus modulating their expression **(Figure 2B)**.

Some of these are the ETHYLENE RESPONSE FACTORS (ERFs) genes that further modulate the expression of a wide range of other genetic elements, including those involved in the production of other phytohormones (reviewed in detail by Müller and Munné-Bosch, 2015).

Some additional reports have shown the existence of several regulators of the ET signaling mechanism (reviewed by Wen et al., 2015), which further impact ET-induced responses.

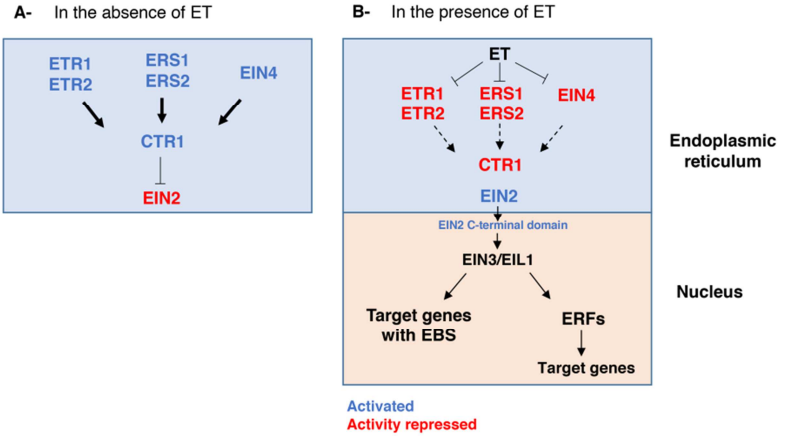

Figure 2- Overview of the ethylene signaling pathway in *Arabidopsis thaliana.* **A**- In the absence of ET, and, in the endoplasmic reticulum, ET receptors activate the CTR1 kinase that, consequently, phosphorylates EIN2, which becomes inactive and does not induce the ET response. **B**- in the presence of ET, the ET receptors bind to ET and lose their CTR1-inducing activity, which in turn leads to a diminished activation of CTR1 and, consequently, the activation of EIN2. In this way, the C terminal domain of EIN2 is cleaved and migrates to the nucleus where it induces the expression of EIN3/EIL1 transcription factors, and, subsequently, ERFs and ET-responsive genes containing EBS (EIN3-binding sequences) in their promoter regions.

Arrows indicate activation, and T-bars indicate repression of the pathway. Thick arrows represent a strong activation of CTR1 by the ET receptors; Dashed arrows denote less signaling activation of CTR1 upon ET binding to the receptors.

2. Old foes: ET and ACC act as inhibitors of bacterial colonization and the nodulation process

2.1 Pathogens

Studies using mutants impaired in ET biosynthesis and signaling demonstrated a direct role for ACC and ET in plant defense against some bacterial pathogens. *Arabidopsis ein2* mutants presented an increased susceptibility to *Erwinia carotovora* subsp. *carotovora* (now reclassified as *Pectobacterium*) infection as the number of viable bacteria was 7-10 times higher in the mutant than in the wild-type plants (Norman-Setterblad et al., 2000). Recently, Guan et al., (2015) showed that *Arabidopsis acs* mutants (that were deficient in the production of ACC) presented a higher susceptibility to *Pseudomonas syringae* infection. The authors also demonstrated that plants with reduced ACC production were colonized to a greater extent by *P. syringae*.

2.2 Rhizobial symbionts

Rhizobia can form a symbiotic relationship with leguminous plants by inducing the formation of nodules where the rhizobial nitrogen fixation process occurs. Upon the perception of plant flavonoids, rhizobia produce lipochitooligosaccharides, termed nodulation (Nod) factors (NFs) that ultimately induce the plant symbiotic response and the development of nodules. In order to colonize the plant-produced nodule, rhizobia enter the plant root hair cells, and consequently reach the nodule via infection threads, a tubular structure resulting from the invagination of the plant cell membrane. Once in the nodule, rhizobia differentiate into a specialized symbiotic organelle-like form, termed a bacteroid, which is now able to start the nitrogen fixation process, thus, providing nitrogen to the plant host (reviewed by Gage et al. 2004).

Generally, ET and ACC act as inhibitors of the nodulation process initiated by rhizobial symbionts (reviewed by Guinel, 2015). Several studies revealed that ET and ACC are involved in several phases of the symbiosis process, including, the initial response to bacterial NFs, NF signal transduction, infection thread formation, nodule development, senescence, and abscission (Penmetsa and Cook, 1997; Heidstra et al., 1997; Oldroyd et al., 2001; Prayitno et al., 2006; Larranzair et al., 2015; Guinel, 2015). For example, Penmetsa and Cook (1997) showed that the *Medicago truncatula* sickle (*skl*) mutant, insensitive to ET, formed an increased number of nodules. The *skl* mutant is defective in a gene homologous to the *Arabidopsis* EIN2 gene (Penmetsa et al., 2008). The silencing of two *Lotus japonicus* EIN2 homologous genes also resulted in increased nodule formation (Miyata et al., 2013). On the other hand, application

of exogenous ET or ACC greatly reduces nodule formation in several leguminous plants (Okazaki et al., 2004).

2.3 Bacterial endophytes

A bacterium can be considered an endophyte when isolated from internal and asymptomatic plant tissues. This definition encompasses, neutral, commensal and/or beneficial, dormant saprobes and latent bacterial pathogens (reviewed by Compant et al. 2016). In addition, two distinct classes of endophytes can be considered: obligatory endophytes, which spend its entire life cycle inside the plant host; and, non-obligatory endophytes, which spend part of their lifetime inside a plant host but may survive in different environments, such as the rhizosphere (which is the case of most studied bacterial endophytes). In this sense, it has been demonstrated that ET and ACC regulate the interaction between plants and non-obligatory bacterial endophytes. For example, Iniguez et al. (2005) demonstrated that ET acts as an inhibitor of the endophytic colonization process by the nitrogen-fixing endophytic bacterium *Klebsiella pneumoniae* 342, which presented a hyper-colonization phenotype when inoculated in the *skl* mutant of *M. truncatula*. Furthermore, the addition of ACC to the growth medium greatly reduced the *K. pneumoniae* 342 and *Salmonella typhimurium* 14028 endophytic colonization abilities in wild-type alfalfa and wheat seedlings. Conversely, addition of the ET perception inhibitor, 1-methylcyclopropane, resulted in increased endophytic colonization by these strains, in wild-type plants. Curiously, the ET-mediated inhibition of endophytic colonization was decreased in *S. typhimurium* 14028 mutant strains lacking flagellin *fliC* and *fljB* genes, and, type III secretion system genes *spaS* and *sipB,* compared to the wild-type strains, suggesting that ET responses are dependent on host perception of bacterial microbe-associated molecular patterns (MAMPs) and effectors.

2.4 Phyllospheric bacteria

The *Arabidopsis ein2* mutant displayed a modified phyllospheric bacterial community when compared to the wild-type plant, supporting the role of ET in controlling the phyllosphere microbiota (Bodenhausen et al., 2014). A higher bacterial abundance, as measured by relative 16S rRNA gene copy number, was observed in the *ein2* mutant. Moreover, *Variovorax* strains were more abundant in the phyllosphere of *ein2* mutant plants compared to wild-type plants (Bodenhausen et al., 2014). Together with the results obtained for leaf-associated pathogens, such as *P. syringae*, these results support the effect of ET and its signaling mechanism as a general inhibitor of leaf bacterial colonization.

3. Zoom in: ET and ACC regulate the plant immune and symbiotic responses

3.1 MAMPs and DAMPs: pattern triggered immunity

The first level of plant inducible defense mechanisms is activated upon recognition of bacterial colonizers and their MAMPs, like flagellin (FLG), elongation factor Tu (EF-Tu), peptidoglycan (PGN), lipopolysaccharides (LPS) and necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) (Newman et al., 2013; Bohm et al., 2014). Additionally, primary plant defenses are also activated in response to damage-associated molecular patterns (DAMPs) (Yamaguchi and Huffaker, 2011) that result from the direct action of invading bacteria (e.g. production of extracellular enzymes and peptides) or from plants' endogenous peptides and other compounds that may be released following bacterial colonization.

MAMPs and DAMPs are recognized by plant pattern recognition receptors (PRR), subsequently leading to the activation of the pattern-triggered immunity (PTI) response (Jones and Dangl, 2006; Yamaguchi et al., 2010) in which, ion fluxes, intricate MPK signaling cascades, ACC and ET biosynthesis, reactive oxygen species (ROS) production, hydroxyproline-rich glycoproteins (HPRGs), cell-wall strengthening, callose deposition, and gene transcriptional and translational reprogramming take part (Felix et al., 1999; Asai et al., 2002; Zipfel et al., 2004; Boller and Felix, 2009; Luna et al., 2010; Xu et al., 2017).

ET plays an important role in PTI, and in some cases, it acts both upstream and downstream of the PTI response (**Figure 3**). The accumulation of the LRRK FLS2 (Flagellin Sensitive 2), the receptor for the bacterial flagellin or its active epitope Flg22, is reduced in ET-insensitive *etr1* and *ein2* mutants, indicating a requirement of ET signaling for *FLS2* increased expression and consequent Flg22-induced responses (Mersmann et al., 2010; Boutrot et al., 2010; Tintor et al., 2013). Moreover, Boutrot et al., (2010) observed that FLS2 is positively regulated by EIN3 and EIN3-like transcription factors. The application of exogenous ACC also leads to an increased expression of *FLS2* (Tintor et al., 2013). Similarly, the FRK1 (Flg22-INDUCED RECEPTOR-LIKE KINASE 1) gene, which is activated in response to Flg22, and, acts downstream of FLS2 (Asai et al., 2002), is influenced by ET; transcript levels of FRK1 are reduced in *ein2-5* mutants after Flg22 treatment (Boutrot et al., 2010). Importantly, Flg22 treatment induces the activation of several defense related genes trough a MPK signaling cascade (Asai et al. 2002). Moreover, Flg22 induced the increased expression of MPK3 and MPK6, but no other MPK isoforms (Asai et al. 2002). This is

consistent with previous studies showing MPK6 activation following Flg22 treatment (Nuhse et al., 2000). The stress-responsive MPK3 and MPK6 phosphorylate the ACS2 and ACS6 isoforms, thus, leading to an increased level of ACC and, consequently, ET production (Liu and Zang, 2004; Li et al., 2012).

An increase in ET production was also observed in response to EF-Tu (Kunze et al., 2004). *Arabidopsis ein2* mutants present a decreased sensitivity to the EF-Tu epitope, elf18 (Tintor et al., 2013), however, the expression of the EF-Tu Receptor (EFR) is not affected in the *ein2* mutant, suggesting that ET acts downstream of the EFR-dependent responses. Tintor and co-workers (2013) observed that a dysfunctional ET signaling mechanism causes improper transcriptional reprogramming during EFR-triggered immunity. Recently, Xu et al., (2017) demonstrated that genes involved in the ET response were amongst the genes with translational efficiency changes in plants challenged with elf18. *Arabidopsis ein4-1*, *erf7*, and *eicbp.b* (ETHYLENE INDUCED CALMODULIN BINDING PROTEIN) mutants displayed insensitivity to elf18-induced resistance (Xu et al., 2017).

Recently, a leucine-rich repeat receptor protein (LRR-RP) RLP23 has been shown to act as the receptor for NLPs (nlp20) and act together with the SOBIR1 (Suppressor of Brassinosteroid Insensitive 1 (BRI1)-associated kinase (BAK1)-interacting receptor kinase 1), and BAK1 proteins to produce the NLP-induced defense response (Albert et al., 2015). NLPs are abundant in bacteria and can also be considered MAMPs (Bohm et al., 2014; Oome et al., 2014). *Bacillus halodurans* and *B. subtilis* nlp20 peptides trigger ET production in *Arabidopsis* (Bohm et al., 2014).

PGN from several Gram-positive and Gram-negative bacterial strains are recognized by plant lysin-motif (LysM) domain proteins, LYM1 and LYM3 (Willmann et al., 2011). Acting downstream, a membrane LysM receptor kinase (CERK1) is also required to induce transcriptional responses induced by PGN. This signal transduction mechanism leads to the activation of FRK1, whose expression has been shown to be regulated by ET, suggesting a role for ET in PGN-induced responses.

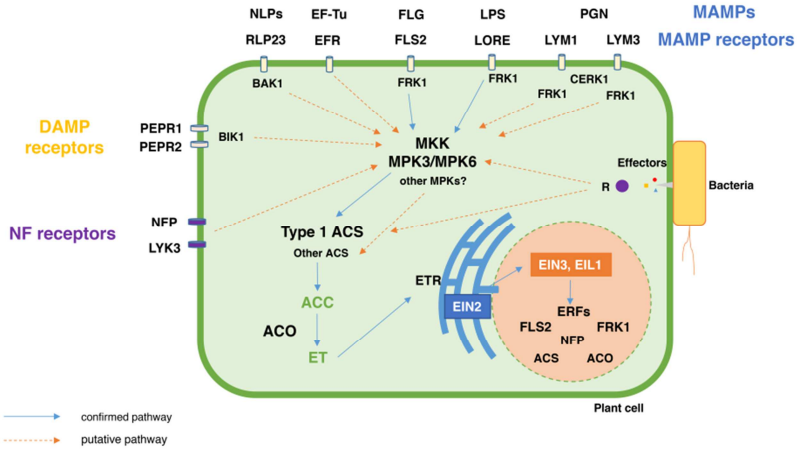

Figure 3- Schematic representation of the MAMPs, DAMPs, NF and effectors-mediated activation of the ACC and ET biosynthesis and signaling pathways.

MAMPs, DAMPs and NFs bind to their cognate receptors present in the plant cell outer membrane and, consequently, initiate the respective signal transduction pathways that lead to the production of ACC and ET. Since most MAMPs, DAMPs and NFs are known to activate MKK-MPK signaling cascades, a MAMP, DAMP and NF-triggered MKK-MPK3/6 cascade (based on *Arabidopsis* gene nomenclature) seems to play a central role in the phosphorylation, and, subsequent activation of type 1 ACS (e.g. AtACS2 and AtACS6). Nevertheless, some aspects of the MAMP, DAMP, NF, and effector induced-R protein signal transduction pathways remain elusive. For example, effector induced immunity leads to the production of ACC and ET, however, not much is understood about the role of effectors and R proteins in the activation of the ET biosynthesis and signaling pathways.

MAMP- Microbe Associated Molecular Pattern; DAMP- Damage Associated Molecular pattern; FLG-Flagellin; EF-Tu- Elongation factor-Tu; LPS-Lipopolysaccharide; PGN- Peptidoglycan; NLPs- necrosis and ethylene-inducing peptides; NF- Nodulation factors; FLS2- flagellin receptor; EFR-elongation factor-Tu receptor; RLP23- necrosis and ethylene- inducing peptides receptor; LORE- lipopolysaccharide receptor; LYM1 and LYM3-peptidoglycan receptors; NFP and LYK3- nodulation factor receptors; PEPR1-2- Pep1 receptor. R- resistance protein involved in effector recognition; CERK1- LysM receptor kinase; FRK1 -Flg22-Induced Receptor-Like Kinase 1; BAK1- Brassinosteroid insensitive 1 (BRI1)-associated kinase; Botrytis-Induced Kinase 1 (BIK1); MKK- mitogen-activated protein kinase kinase; MPK- mitogen-activated protein kinase. ACS- ACC synthase; ACO- ACC oxidase; ERF- Ethylene Response Factor.

Ranf et al., (2015) revealed that a plant lectin S-domain-1 receptor–like kinase, LORE, is responsible for the recognition of bacterial LPS. *Arabidopsis* mutants, *lore-1* and *lore-2*, present a diminished LPS-triggered accumulation of ROS, activation of MPK3 and MPK6 and expression of PTI response genes, such as FRK1 and GST1 (GLUTATHIONE-S-TRANSFERASE 1) (both of which are ET regulated). These results suggest that LPS-induced responses may modulate ACS expression in a MPK3-6 dependent manner, as previously observed by Li et al. (2012), and thereby induce the expression of ET-responsive proteins like FRK1.

DAMPs induce ET production and modulate ET responses. Nevertheless, ET itself can induce the production of DAMPs, indicating a role for ET acting upstream and downstream of the DAMP-induced response. Upon wounding, methyl jasmonate or ET application, *Arabidopsis* produces Pep1, a 23-amino acid peptide processed from PROPEP1 (Precursor of Peptide 1), which binds to the Pep1 receptor kinases PEPR1 and PEPR2 (Huffaker et al., 2006; Yamaguchi et al. 2010; Liu et al., 2013). PEPR1 and PEPR2 directly phosphorylate the BOTRYTIS-INDUCED KINASE 1 (BIK1) in response to Pep1 treatment (Liu et al., 2013). *Arabidopsis pepr1/pepr2* and *bik1* mutants present a compromised ET-induced expression of defense genes. Curiously, *pepr1/pepr2* mutants displayed a reduced sensitivity to ET, suggesting a direct effect in the ET signaling pathway (Liu et al., 2013).

Other studies have shown that the application of several bacterial extracellular enzymes that impact plant tissues (e.g. pectate lyase) induce ET production in several plant species (reviewed in Abeles et al., 1992). However, the effect of all of these applications has not been studied in detail.

3.2 Effector-triggered immunity

In addition to transmembrane PRR, plants also produce specific defense nucleotide-binding and leucine rich repeat domains (NB-LRR) proteins inside the cell (Jones and Dangl, 2006). These plant resistance (R) proteins are involved in the second layer of defense, which is induced upon recognition of specific effectors that are produced by bacteria able to suppress or evade PTI. The R proteins recognize bacterial effectors, thus, initiating effector-triggered immunity (ETI) (Jones and Dangl, 2006). The ETI response is frequently associated with hypersensitive response cell death (HR) (Jones and Dangl, 2006; Dodds and Rathjen, 2010). ET production is closely linked with ETI and HR. Some studies have revealed that following pathogen infection (*P. syringae*), ET is produced in a biphasic pattern in both *N. tabacum* and *Arabidopsis* plants (Mur et

al., 2008; 2009). The first ET peak seems to be related with PTI and it is rapidly induced. The generation of the second ET peak is dependent on ETI, as bacteria deficient in effector delivery (*hrpL* mutants) are not able to induce the second ET peak (Mur et al., 2008). Also, the bacterial *avr* gene and its ETI-inducing activity is closely related to the second ET peak production (Mur et al., 2008, 2009). Guan et al., (2015) showed that the *P. syringae2* (*rps2*) *Arabidopsis* mutant seedlings lacking the R protein and, therefore, unable to sense the *avrRpt2* effector, produced decreased effector stimulated ET levels. On the other hand, Liu et al., (2011) showed that the *Erwinia amylovora*-derived elicitor HrpNEa activates the transcription factor MYB44, which in turn enhances the expression of *EIN2*. Recently, Blüher et al., (2017) demonstrated that *Xanthomonas campestris* pv. *vesicatoria* produces a type III secretion effector, XopH, that possesses phytase activity and modulates the *Nicotiana benthamiana* defense response. The authors observed that XopH induced the expression of *N. benthamiana* ET-responsive genes encoding the pathogenesis-related proteins, PR1b, PR4 and the proteinase inhibitor PI-II. Moreover, the expression of *PR4* and *PI-II* genes were dependent on the ET signaling pathway, as silencing of ET pathway components, such as EIN2, suppressed their upregulation.

Studies using *Arabidopsis* ET-overproducing (ETO) and signaling mutants indicate that ET strongly participates in the HR response (Mur et al., 2009). For instance, *eto2-1* mutants (overproducing ACC and ET) induce an exaggerated HR, while ET insensitive mutants (*ein2-1* and *etr1-1*) present a delayed HR.

3.3 Symbiotic Nod factor-triggered response

Rhizobial NF perception by a leguminous plant leads to the initiation of the symbiotic program, which ultimately results in nodule formation and biological nitrogen fixation (reviewed by Guinel, 2015). Bacterial NFs are perceived by plant NF receptors, such as the lysine motif domain-containing receptor-like kinase 3 (LYK3) and nodulation factor perception (NFP) in *M. truncatula*, and nodulation factor receptor1 (NFR1) and NFR5 in *Lotus japonicus*. The NF receptors are plasma membrane-localized receptor-like kinase and kinase-like (RLK) proteins (Amor et al., 2003; Smit et al., 2007; Moling et al., 2014) containing an intracellular kinase domain and an extracellular region with two or three chitin-binding LysM motifs, which bind to NFs through their chitin backbone (Petutschnig et al., 2010; Broghammer et al., 2012).

The *M. truncatula skl* mutant root transcriptome revealed the important role of ET in the NF-signaling cascade and the overall

nodulation process. Larrainzar et al., (2015) observed that the *skl* mutant presented an increased expression of NFP, LYK3 and several members of the LysM kinase family, further indicating that ET impacts NF receptor gene transcription. Furthermore, ET also regulates the transcriptional response that occurs downstream of NF perception, including ACS and ACO expression, as well as other genes involved in the production of other phytohormones (Larrainzar et al., 2015). Several other events occurring after NF perception, such as calcium spiking, root hair deformation, infection thread formation and persistence, and primordium formation at sites opposite phloem poles, have been demonstrated to be affected by ET (Penmetsa and Cook, 1997; Heidstra et al., 1997; Oldroyd et al., 2001; Larrainzar et al., 2015).

Importantly, Larrainzar and colleagues (2015) also identified the presence of a NF-independent and ET-modulated response in *M. truncatula* plants challenged with rhizobial symbionts. This response likely accounts for the PTI and ETI immune response elicited by rhizobial symbionts MAMPs, DAMPs and effectors.

4. An ET and ACC-regulated mechanism controlling development and defense? The root cell elongation example

Plant developmental cues and defense responses are intrinsically related and may act synergistically to limit bacterial proliferation. Even though ET and ACC directly impact the fast and localized plant immune and symbiotic response, ET and ACC are also known for their effects in long-term plant development, especially in the modulation of root growth and development. In this sense, ET and ACC act mainly as negative regulators of the root cell elongation process.

The ACC (or ET)-induced inhibition of root cell elongation is a very fast mechanism (i.e. it occurs within minutes) and is mediated by several other players, such as ROS, HPRGs, plasma membrane H^+-ATPases and other enzymes involved in cell-wall remodeling (Le et al., 2001; De Cnodder et al., 2005, Stahl et al., 2011, Markakis et al., 2012). The crosslinking of HPRGs by ROS and the quick deposition of callose in the apoplast (the main colonization spot of bacterial endophytes) contribute to cell elongation arrest and the general inhibition of root elongation induced by ACC (axenic seedlings) (De Cnodder et al., 2005). In addition, ACC induces apoplastic alkalinization in root cells that leads to a decrease in the activity of cell-wall loosening agents which function in more acidic environments. The alkalinization occurs as a consequence of changes in H^+ efflux by the modulation of the activity state of plasma membrane H^+-ATPases (Staal et al., 2011). After a 3-hour treatment

with ACC, the expression of several genes coding for known cell-wall loosening proteins are down regulated, while genes coding for specific cell wall components together with their cross-linking enzymes (e.g. peroxidases) are upregulated (Markakis et al., 2012).

Application of ACC also leads to an increased synthesis and a modified transport of auxin, which readily impacts root developmental programs (reviewed by Muday et al., 2012). Moreover, auxin and its signaling mechanism are necessary for the ACC and ET-induced root elongation inhibition in *Arabidopsis* (Swarup et al., 2007; Stepanova et al., 2007; Ruzicka et al., 2007; Strader et al., 2010; Staal et al., 2011).

Interestingly, most of the effects identified in ACC and ET-induced responses (single application of ACC or ET) are also observed in the immune responses induced by bacteria and their MAMPs. Plant immune responses rapidly induce the production of ACC and ET, modify ion fluxes and induce growth medium alkalinization due to changes of ion fluxes across the plasma membrane, induce increased ROS production and accumulation (Boller and Felix, 2009) and increase HPRGs in the cell-wall (reviewed by Deepak et al., 2010). The crosslinking of these glycoproteins and the consequent strengthening of the cell wall in response to microbial invaders is dependent on the action of ROS (mainly H_2O_2) and peroxidase enzymes (Deepak et al., 2010). Moreover, callose deposition is induced by MAMPs (Luna et al., 2010). The plant immune response usually leads to seedling growth inhibition (Boller and Felix, 2009).

Importantly, Tsang et al., (2011) demonstrated that the application of isoxaben (an inhibitor of cellulose synthesis and general root development) or Flg22, induced root cell elongation arrest in *Arabidopsis*. The application of ET biosynthesis inhibitors reduced the negative effects of both isoxaben and Flg22, indicating a similar mechanism regulating root elongation inhibition induced by these compounds. Tsang et al., (2011) further indicated that an ACC-dependent signaling mechanism, involving auxin and ROS production acting downstream, was responsible for root elongation inhibition.

Altogether, these results are consistent with the existence of a common ET and ACC-mediated mechanism regulating root elongation (and possibly other processes) that can be activated by different elicitors, including MAMPs. The root elongation inhibition process can limit bacterial colonization and degradation of plant compounds since stronger and less elongated cells, containing more antimicrobial compounds (e.g. ROS and callose) and presenting a

modified permeability, may be less susceptible to bacterial colonization.

5. Counter attack! Bacterial modulation of plant ACC and ET levels

Bacteria have developed several mechanisms to respond and modulate plant ACC and ET levels (**Figure 4**). These mechanisms are related to bacterial physiologic adaptations upon ET perception (**Figure 4A**), and/or production of compounds and effectors that directly or indirectly impact the production and signaling of ET by the plant (**Figure 4B**), modulation of plant ET responses by degrading ACC or ET (**Figure 4C**) or, alternatively, by producing ET (**Figure 4D**).

5.1 Bacterial responses to ET

Studies on plant-associated bacteria demonstrated that ET impacts the expression of several bacterial genes involved in plant-bacterial interactions. For example, *Agrobacterium tumefaciens* virulence (*vir*) gene expression is negatively affected by exogenous ET, which leads to a decreased ability of T-DNA transfer, and, consequently, to a reduction of pathogenicity (Nonaka et al., 2008). ET induced the increased expression of the cellulose synthesis operon, as well as the CRP/FNR_{Kx} transcription factor, in the fruit-associated bacterium *Komagataeibacter xylinus,* which in turn may favor external bacterial adhesion, competitiveness and consequent production of plant-growth promoting traits (Augimeri and Strap, 2015). These results indicate that the ET impact in plant-associated bacteria responses may be strain specific and dependent on the bacterial mode of action. *Agrobacterium* is a biotrophic pathogen that colonizes internal plant tissues (mainly roots and shoots) and induces tumors. It is conceivable that upon sensing increased ET levels, *Agrobacterium* modulates its virulence through differential *vir* gene expression in order to subvert the plant defense response mediated by ET. On the other hand, *K. xylinus* is an epiphyte colonizing the external surface of fruits. ET plays an important role as a fruit ripening agent (Liu et al., 2015), so, the ET signal may indicate the ideal timing for fruit colonization by *K. xylinus,* which in turn produces a dense cellulose matrix that increases its adherence to the fruit, provides protection from environmental stresses, and provides a competitive advantage over other microorganisms (Augimeri and Strap, 2015).

A study performed by Kim et al., (2007) revealed that *Pseudomonas aeruginosa* PAO1 and many plant-associated bacteria, including *P. fluorescens*, *P. putida* and *P. syringae*, can perceive and positively respond to ET. The authors identified the

methyl-accepting chemotaxis protein (MCP), TlpQ, as the chemoreceptor responsible for ET responses in *P. aeruginosa* PAO1. Moreover, the *cheR* gene encoding a chemotaxis-specific methyl-transferase is required for strain PAO1 MCP-dependent chemotaxis towards ET (Kim et al., 2007). Homologs of the *tlpQ* gene were also identified in several other plant-associated bacteria.

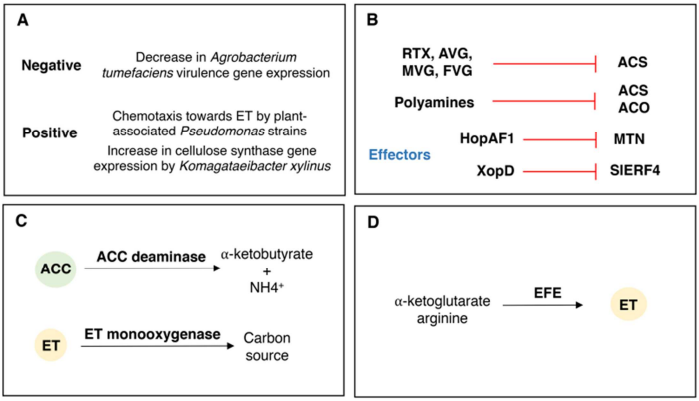

Figure 4- Bacterial mechanisms involved in the responses to ethylene and modulation of plant ACC and ethylene levels.
A- Bacterial responses to ET, both positive and negative that relate to the ET effect in the expression of several genes and traits.
B- Compounds and effectors impacting plant ACC and ET biosynthesis and signaling.
RTX-Rhizobitoxine; AVG-aminoethoxyvinylglycine;
MVG-methoxyvinylglycine; FVG-4-formylaminooxyvinylglycine;
MTN-methylthioadenosine nucleosidase.
C- Bacterial degradation of plant ACC and ET. Bacteria presenting ACC deaminase activity catabolize ACC to produce α-ketobutyrate and ammonia. Bacteria producing an ET-monoxygenase and other associated components can use ET as sole carbon source.
D- Bacterial ET production. Some bacterial pathogens produce ET by the action of an ET-forming enzyme (EFE) that uses arginine and α-ketoglutarate as substrates.

5.2 Inhibition of plant ACS by bacterial-produced vinylglycine analog compounds

5.2.1 Rhizobitoxine (RTX)

RTX is a secreted enol-ether amino acid that acts as an inhibitor of the plant ACS (Yasuta et al., 1999). The genes *rtxA* (encoding a dihydrorhizobitoxine synthase) and *rtxC* (encoding dihydrorhizobitoxine desaturase) are responsible for RTX production in *Bradyrhizobium* (Yasuta et al., 2001). Knowledge of the role of RTX in plant-bacterial interactions resulted mainly from studies of the *Bradyrhizobium*-legume symbiosis. *B. elkanii* RTX mutant strains, unable to produce RTX and, consequently, decrease plant ET levels, have decreased nodulation abilities and competitiveness in several plant hosts. Duodu et al., (1999) showed that *B. elkanii* RTX mutant strains formed fewer mature nodules than the wild-type strain in *Vigna radiata*; however, the nodulation profile of the mutant strains could be partially restored by the addition of ET biosynthesis inhibitors. Elimination of RTX production in *B. elkanii* led to increased ET production by *Macroptilium atropurpureum* and a decreased nodulation phenotype (Yuhashi et al., 2000). Similar results were obtained by Parker and Peters (2001) who showed that *Amphicarpaea edgeworthii* plants inoculated with *B. elkanii* RTX-deficient mutants RX17E and RX18E developed fewer nodules than plants inoculated with the wild-type *B. elkanii* USDA 61. Interestingly, Ratclif and Denison (2009) demonstrated that the RTX-producing *B. elkanii* increased the accumulation (by 47%) of the storage lipid poly-3-hydroxybutyrate (PHB) in root nodules of *M. atropurpureum,* compared to the *B. elkanii* RTX non-producing mutant. The synthesis of PHB supports the later reproduction of rhizobia (Ratcliff et al., 2008), suggesting that RTX-producing bacteria modulate ET levels to decrease plant sanctions against accumulation of carbon compounds at the expense of N_2 fixation.

RTX-producing bacteria can also induce disease symptoms in some plants. For example, *B. elkanii* causes foliar chlorosis in some soybean cultivars (*Glycine max*) and this effect is dependent on RTX production (Okazaki et al., 2004). Interestingly, some plant pathogens are also able to produce RTX. Mitchell and Frey (1988) showed that the plant pathogenic *Burkholderia andropogonis* strains produce RTX. *Xanthomonas oryzae* is also known to possess *rtx* genes (Sugawara et al., 2006) but its activity has never been described.

5.2.2 Aminoethoxyvinylglycine (AVG)

AVG is a powerful inhibitor of the ACS enzyme (Icekson and Apelbaum, 1983), and it has been used in many studies regarding the role of ET in plant physiology, as well as in several agricultural applications, such as, harvesting and fruit ripening delay. AVG is an unsaturated enol ether amino acid produced by *Streptomyces* sp. NRRL-5331 in fermentation broth (Pruess et al. 1973), however, not much is understood about the genetic elements involved in AVG synthesis by strain NRRL-5331, nor the biological significance of its possible interaction with a plant. Most of the studies performed with AVG resulted from the knowledge previously obtained by studying RTX.

5.2.3 Methoxyvinylglycine (MVG) and formylaminooxyvinylglycine (FVG)

P. aeruginosa strains produce another vinylglycine analog, MVG, also known as AMB (L-2-amino-4-methoxy-trans-3-butenoic acid) (Samh et al., 1973), through the expression of the *ambABCDE* gene cluster (Lee et al., 2010). Application of pure MVG decreased apple ET levels (Matoo et al., 1979). Lee et al., (2012) showed that the expression of *ambABCDE* by the biocontrol strain *P. fluorescens* CHA0 weakly interfered with the germination of several graminaceous seeds. Curiously, some rhizosphere-associated *P. fluorescens* produce the vinylglycine analog, FVG, a germination-arrest factor that has been shown to limit the germination of weedy grasses (McPhail et al., 2010; Okrent et al., 2017a). The biosynthetic cluster involved in FVG production by *P. fluorescens* WH6 has recently been described (Okrent et al., 2017b), yet, not much is understood about the biological significance of FVG in the bacterial interaction with the plant host. ET is a known inducer of seed germination (Corbineau et al., 2014), so it is possible that FVG and MVG inhibit ACS and ET production that arrests the germination of the seeds of some plants, however, this remains to be conclusively proven.

5.3 Direct decrease of plant ACC levels by ACC deaminase-producing bacteria

Bacteria that produce the enzyme ACC deaminase can directly use plant-synthesized ACC as carbon and nitrogen sources, and, at the same time, lower the ACC levels within plant tissues (Glick et al., 1998; Penrose et al., 2001; Belimov et al., 2009).

ACC deaminase is a pyridoxal phosphate (PLP)-dependent multimeric enzyme (homodimer or homotrimer) belonging to the tryptophan synthase beta superfamily, with a subunit molecular mass of approximately 35-42 kDa and it can degrade ACC and several

ACC-related substrates (reviewed by Nascimento et al., 2014). The ACC deaminase enzyme is encoded by a single gene, termed *acdS*, which is widespread in plant-associated bacteria, including symbionts like rhizobia, general rhizospheric and endophytic plant-growth-promoting bacteria such as *P. fluorescens*-group species, as well as some plant pathogens such as *P. syringae* or *Ralstonia solanacearum* (Nascimento et al., 2014).

Beneficial ACC deaminase-producing bacteria enhance plant growth and development and also increase plant tolerance to a wide variety of biotic and abiotic stresses by decreasing inhibitory ACC and ET levels (Wang et al., 2000; Grichko and Glick, 2001; Mayak et al., 2004 a,b; Belimov et al., 2005; Belimov et al., 2009; Toklikishvili et al., 2010; Nascimento et al., 2013; Gamalero and Glick, 2015; Gamalero et al., 2016).

Studies using bacterial mutants impaired in ACC deaminase production have demonstrated that the expression of ACC deaminase is extremely important for the plant-growth promoting abilities of several plant-associated bacteria, including rhizospheric (Glick et al. 1994, Li et al., 2000, Belimov et al., 2009), endophytic (Sun et al., 2009; Onofre-Lemus et al., 2008; Ali et al., 2014) and symbiotic rhizobial strains (Uchiumi et al., 2004; Ma et al., 2003).

Inoculation of leguminous plants with ACC deaminase-producing rhizobia, inoculated singly or in consortia with free-living ACC deaminase-producing bacteria, leads to an increased nodulation phenotype (reviewed in Nascimento et al., 2016). By decreasing ACC levels these bacteria diminish the inhibitory ET concentrations that affect several phases of the nodulation process (Ma et al., 2003).

ACC deaminase-producing bacteria are known to increase general root development, with special emphasis on root elongation (Glick et al., 1994; Belimov et al., 2009). Bacterial mutants impaired in ACC deaminase-production no longer promote root elongation in several plant species (Glick et al., 1994; Onofre-Lemus et al., 2008; Belimov et al., 2009). This result is consistent with the role of ET and ACC in controlling the root elongation process, as previously discussed.

ACC deaminase production also plays a role in bacterial competitiveness. The *acdS*$^-$ mutant of *Mesorhizobium* sp. MAFF303099 presented decreased nodulation and nodule occupancy abilities when compared to its wild-type counterpart (Uchiumi et al., 2004). On the other hand, rhizobial strains expressing an exogenous *acdS* gene exhibited increased nodule

occupancy compared to the wild-type strains (Ma et al., 2004; Conforte et al., 2010).

There are many studies regarding the effects of ACC deaminase in plant-growth promoting bacteria, however, not much is understood about its effect on pathogens like *P. syringae* or *R. solanacearum*, that also contain an *acdS* gene. It is conceivable that pathogens may decrease ACC levels to decrease ET-regulated plant defense responses. Alternatively, these bacteria may decrease ACC and ET levels that impact their own gene expression (e.g. *vir* gene expression in *Agrobacterium*). In fact, engineered *Agrobacterium* strains expressing ACC deaminase presented an increased ability to transfer T-DNA to different plant hosts (Hao et al., 2010; Nonaka and Ezura, 2014), however the effect of ACC deaminase in *vir* gene expression was not documented.

5.4 Bacterial effectors targeting plant ET biosynthesis and signaling pathways

The plant pathogen *Xanthomonas euvesicatoria*, produces an effector which modulates the ET response pathway in tomato. The type III secretion effector, XopD, directly targets and desumoylates the tomato ET-responsive transcription factor, SlERF4, to suppress ET production, and, consequently decrease ET-induced plant defenses (Kim et al., 2013). As a result, *X. euvesicatoria* increases its growth and delays symptom development in the host plant.

A type III effector, HopAF1, produced by *P. syringae* and encoding a deamidase-like enzyme, targets *Arabidopsis* methylthioadenosine nucleosidase proteins MTN1 and MTN2, which are involved in the Yang cycle and, consequently, ET production (Washington et al., 2016). HopAF1 inhibits the MAMP-induced increase in ET biosynthesis, leading to an increased bacterial infection. Additionally, several HopAF1 homologs are found in the genomes of other bacterial pathogens, such as *R. solanacearum* or *Acidovorax citrulli*, consistent with the suggestion that effector production targeting ET responses is an important trait in some plant pathogens (Washington et al., 2016).

5.5 Bacterial polyamines and the decrease of plant ET levels

Polyamines (PAs) are low-molecular-weight aliphatic amines commonly produced by a large number of different organisms (Miller-Fleming et al., 2015). The most abundant PAs include, spermine and spermidine, and their precursor putrescine. Importantly, the application of PAs have been shown to decrease ACC and ET levels in several plant species, apparently by limiting the action of ACS and ACO enzymes (Li et al., 1992; Li et al., 2013).

A study by Xie and colleagues (2014) showed that spermidine produced by *B. subtilis* OKB105 inhibited the expression of tobacco ACO1, consequently, reducing the ET content in root cells, and, thereby increasing tobacco root growth. Spermidine production by *B. subtilis* OKB105 is dependent on the *speB* gene encoding agmatinase. Moreover, the *yecA* gene encoding a putative amino acid/polyamine permease, is responsible for spermidine export (Xie et al., 2014).

5.6 Direct decrease of ET levels by soil bacteria expressing ET-monooxygenase

Several Actinobacteria like *Mycobacterium* and *Nocardioides*, which are common soil inhabitants, possess the ability to use ET as a sole carbon source (de Bont and Harder, 1978) by the expression of an ET-monooxygenase (Coleman and Spain, 2003). The genetic elements responsible for bacterial ET degradation have been described in detail (Coleman and Spain, 2003), however, not much is understood about the role of ET-degrading bacteria in plant development and plant-microbe interactions. Thus, more studies are necessary to elaborate the role of bacterial ET degradation in modulating plant growth.

5.7 The direct increase in ET levels by the production of a bacterial ET-forming enzyme

Pathogens like *R. solanacearum* and *P. syringae* possess the ability to produce ET, independent of a plant host (Freebairn and Buddenhage, 1964; Nagahama et al., 1991; Weingart et al., 2001). In this case, bacterial ET production is not ACC-dependent, rather, it depends on the action of an ET-Forming Enzyme (EFE) that uses α-ketoglutarate and arginine as substrates (Nagahama et al., 1991). Importantly, Weingart et al., (2001) demonstrated that a *P. syringae* pv. *glycinea efe* mutant presented a decreased pathogenicity. In addition, the expression of a bacterial EFE in transgenic tobacco plants resulted in altered plant development, with plants demonstrating a dwarf morphology. These results suggest that ET synthesis is extremely important for the action of some pathogens. This leads to intriguing questions: If a low level of ET is responsible for increased plant defenses why do some bacterial pathogenic strains produce ET? Moreover, if *P. syringae* possesses several mechanisms aimed at decreasing plant ACC and ET levels (RTX, ACC deaminase, effectors), why do the same bacterial strains sometimes produce ET?

Depending on environmental and internal cues, ET can either positively or negatively regulate stomatal opening in several plant species (Madhavan et al., 1983; Tanaka et al., 2005; Desikan et al.,

2006; Arve and Torre, 2015). Hence, under certain conditions, producing ET may lead to increased leaf colonization by *P. syringae* entering open stomata, or, alternatively, to decreased stomata opening that protects endophytic *P. syringae* from external competitors. Since ET also acts as a chemoattractant, it is also possible that ET production may act as a signaling mechanism in *P. syringae*.

ET is a major inducer of plant stress symptoms and these may be important in the later phases of the bacterial infection process. Bacteria such as *P. syringae* are transmitted mainly by soil and water (van Overbeek et al., 2010; Monteil et al., 2016), so it is possible that in the late disease stages some *P. syringae* strains produce ET to increase foliar senescence and abscission aiming for bacterial dispersal.

6. Future directions

6.1 ACC as a signaling molecule affecting microbiome assembly?

Importantly, several studies have shown that ACC deaminase-producing bacteria are enriched in the rhizosphere and seeds of stress-grown plants. Timmusk et al., (2011), showed that ACC deaminase-producing bacteria were much more abundant in the rhizosphere of wild barley growing under stressful conditions in comparison to barley grown nearby under non-stressful conditions. This result was obtained even though both sampled environments had similar soil, rock and topology characteristics. Moreover, ACC deaminase-producing bacteria were abundant in plant rhizosphere samples and almost nonexistent in bulk soil samples. Similarly, ACC deaminase-producing bacteria were more abundant in all compartments of heavy metal contaminated soils (bulk and *Brassica napus* rhizosphere) than in non-stressed soils (Croes et al., 2013).

Truyens et al., (2013) studied the cultivable endophytic population of seeds from *A. thaliana* exposed to cadmium for several generations (Cd seeds) in comparison with a population isolated from seeds of plants that were never exposed to Cd (control seeds). The authors found that metal tolerance and ACC deaminase activity were predominantly found in strains isolated from Cd seeds, while the production of siderophores, indole-3-acetic acid and organic acids was more prevalent in endophytes isolated from control seeds, further indicating a selection for ACC deaminase-producing bacteria under stress condition that is consistent with the increased ET/ACC levels induced by cadmium and other heavy-metal stresses (Thao et al., 2015).

Altogether, these results indicate that ACC and ET may act as signaling molecules under stress conditions, leading to an increased recruitment of bacteria able to decrease the elevated ACC and ET levels responsible for decreased root growth and increased plant stress. In turn, ACC and ET-modulating bacteria decrease stress ACC and ET levels, relieving the plant from its negative effects in several plant developmental cues (Glick, 2014). Nevertheless, more studies are necessary to understand the mechanism responsible for plant ACC exudation, as well as, bacterial ACC perception, and their consequent role in the plant microbiome assembly.

6.2 Is plant production and sensitivity to ET and ACC regulating the plant microbiome?

Since ET and ACC impact bacterial colonization, their role gains further importance in microbiome assembly (especially under stressful conditions). In this sense, it is conceivable that plants presenting different ET/ACC production and sensitivity abilities may possess different microbiome selection abilities. Although ET and ACC are produced by all higher plants, the timing and extent of ET/ACC production differs between plant species (Abeles et al., 1992, Wheeler et al., 2004). These differences may be explained by the abundance of genetic elements involved in ET/ACC production in various plant species. For instance, *Arabidopsis* contains 12 ACS isoforms in its genome while 6 ACS isoforms are found in *Lotus japonicus* (Desbrosses and Stougaard, 2011). Similarly, different plants possess different ET/ACC sensitivities (e.g. Woltering and Van Doorn, 1988), which is also consistent with the disparate numbers of genetic elements involved in ET perception and signaling in plant genomes (Desbrosses and Stougaard, 2011). For example, *M. truncatula* only possesses one EIN2 homolog (Penmetsa et al., 2008), while *L. japonicus* contains two EIN2 gene homologs in its genome (Miyata et al. 2013).

6.3 What is the contribution of ACC and ET-modulating bacteria to the overall plant microbiome?

The ACC deaminase-producing bacterial strain *Pseudomonas* sp. UW4, but not its *acdS*$^{-}$ mutant, increased the colonization of the arbuscular mycorrhizal fungus, *Gigaspora margarita* BEG9 in cucumber, leading to synergistic effects on plant growth (Gamalero et al., 2008). Furthermore, several reports have shown that free-living rhizospheric bacteria with ACC deaminase activity readily promote the nodulation process of several leguminous plants (Nascimento et al., 2016). These results indicate that the presence of bacteria with ACC deaminase activity can readily impact the colonization of other microorganisms present in the rhizosphere,

including symbionts. Hence, under specific conditions bacteria with the ability to modulate plant ACC and ET levels may act as regulators of the plant microbiome. New studies are necessary to assess the specific role of bacteria with ACC and ET-modulation abilities in several aspects of the microbiome assembly (e.g. bacterial endophytism, aerial tissue colonization, microbiome composition).

6.4 Strategies for the creation of inoculants with increased plant-growth promotion abilities

Bacterial inoculants aiming to increase plant growth and development are the most promising alternatives to the use of potentially polluting agrochemicals. Since plants possess different ACC and ET production/sensitivity abilities, and, stress conditions readily increase plant ACC and ET levels, new strategies need to be considered to develop specific and efficient bacterial inoculants. These strategies need to be multidisciplinary and consider not only the added bacteria but also the plant host. In this sense, it is conceivable that ET and ACC insensitive plants will benefit less from the effects of ACC and ET-modulating bacteria. On the other hand, plants producing high levels of ACC and ET (naturally or induced by stress conditions) would certainly benefit from the presence of these bacteria. In fact, Chen et al., (2013) observed that the ACC deaminase-producing *Variovorax paradoxus* 5C2 promoted the growth of the *Arabidopsis* wild-type and the *Arabidopsis* ethylene-overproducing mutant *eto1-1* but not the ethylene-insensitive mutants, *etr1-1* and *ein2-1*, even though bacterial colonization of the root systems was similar. Furthermore, *V. paradoxus* 5C2 promoted the growth of *eto1-1* plants to a greater extent compared to all other treatments (Chen et al. 2013), indicating a positive feedback between plant ACC and ET production and the beneficial effect of the ACC deaminase-producing bacteria.

Finally, since ACC and ET inhibit the nodulation process, it is expected that rhizobial inoculants will benefit from the presence of free-living bacteria with ACC and ET modulation abilities. Hence, selecting ACC and ET-modulating rhizobia in concert with ACC and ET-modulating free-living bacteria may result in increased nodulation and leguminous plant growth.

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CHAPTER 2

New insights into ACC deaminase phylogeny, evolution and ecological significance

RESEARCH MANUSCRIPT

"New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance"

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New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance

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ABSTRACT

The main objective of this work is the study of the phylogeny, evolution and ecological importance of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which its activity represents one of the most important and studied mechanisms used by plant-growth-promoting microorganisms.

The ACC deaminase gene and its regulatory elements presence in completely sequenced organisms was verified by multiple searches in diverse databases and based on the data obtained a comprehensive analysis was conducted. Strain habitat, origin and ACC deaminase activity were taken into account when analyzing the results. In order to unveil ACC deaminase origin, evolution and relationships with other closely related pyridoxal phosphate (PLP) dependent enzymes a phylogenetic analysis was also performed.

The data obtained show that ACC deaminase is mostly prevalent in some Bacteria, Fungi and members of Stramenopiles. Contrary to previous reports, we show that ACC deaminase genes are predominantly vertically inherited in various bacterial and fungal classes. Still, results suggest a considerable degree of horizontal gene transfer events, including interkingdom transfer events. A model for ACC deaminase origin and evolution is also proposed. This study also confirms the previous reports suggesting that the Lrp-like regulatory protein AcdR is a common mechanism regulating ACC deaminase expression in Proteobacteria, however, we also show that other regulatory mechanisms may be present in some Proteobacteria and other bacterial phyla.

In this study, we provide a more complete view of the role for ACC deaminase then was previously available. The results show that ACC deaminase may not only be related to plant growth promotion abilities, but may also play multiple roles in microorganism's developmental processes. Hence, exploring the origin and functioning of this enzyme may be the key in a variety of important agricultural and biotechnological applications.

INTRODUCTION

One of the key bacterial traits in facilitating plant growth is the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 3.5.99.7). This enzyme is responsible for the cleavage of the ethylene precursor, ACC, into ammonia and α-ketobutyrate [1]. By decreasing ACC levels in plants, ACC deaminase-producing organisms decrease plant ethylene levels [2,3], which when present in high concentrations can lead to a reduced plant growth and ultimately, plant death [4]. ACC deaminase was initially identified in the yeast *Hansenula saturnus* (now re-classified as *Cyberlindnera saturnus)* and the bacterium *Pseudomonas* sp. ACP [1]. Since then, many groups have reported the isolation and sometimes the manipulation of *acdS* genes (i.e. the structural gene encoding ACC deaminase) from a wide range of different organisms, mostly bacteria and fungi [5]. Moreover, several studies have addressed the detailed biochemistry of ACC deaminase and the atypical and important reaction mechanism of ACC breakdown [6]. Data obtained in these studies show that ACC deaminase is a multimeric enzyme (homodimer or homotrimer) with a subunit molecular mass of approximately 35-42 kDa and it uses one molecule of pyridoxal phosphate (PLP) per subunit. Based on its protein fold, ACC deaminase has been classified as belonging to the tryptophan synthase beta superfamily (fold type II) of PLP-binding proteins [6]. In this family are also included the ACC deaminase homolog from *Pyrococcus horikoshii* [7] and the D-cysteine desulfhydrase from *Escherichia coli* and *Salmonella typhymurium* [8,9].

ACC deaminase is central to the functional interactions of various plant-associated bacteria and fungi. The root colonizing bacteria *Pseudomonas putida* GR12-2 and *Pseudomonas* sp. UW4 no longer promote canola root elongation after its *acdS* gene is knocked out [10,11]. The symbiotic efficiency of the root nodule forming bacteria, *Rhizobium leguminosarum* bv. *viciae* and *Mesorhizobium loti* MAFF303099, is decreased upon *acdS* gene deletion [12,13]. The endophytic plant growth-promoting bacteria *Burkholderia phytophirmans* PsJN, *Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6 are less effective when their *acdS* gene is deleted [14,15]. Similarly, when ACC deaminase expression is impaired in the fungus *Trichoderma asperellum* T203, the plant growth promotion abilities of this organism are also decreased [16,17].

Bacteria and fungi that express ACC deaminase can lower the impact of a range of different stresses that affect plant growth and

development [3,17]. Using ACC deaminase-producing bacteria in association with plants subjected to different kinds of biotic and abiotic stresses resulted in enhanced plant tolerance [18–25]. The use of ACC deaminase-producing bacteria in association with plants for purposes of soil decontamination is also documented [26–28]. Increased phytoremediation potential and resistance to biotic and abiotic stresses are observed in transgenic plants expressing a bacterial ACC deaminase [29–32]. The expression of an exogenous ACC deaminase gene increases the symbiotic performance of many rhizobial strains [33–36].

Studies regarding the mechanisms regulating ACC deaminase expression have been reported for some Proteobacteria. Binding sites for CRP (cAMP receptor protein), FNR (fumarate-nitrate reduction regulatory protein) and LRP (leucine responsive regulatory protein) were present in the promoter region of the *Pseudomonas* sp. UW4 *acdS* gene and were shown to function in regulating *acdS* expression [37–39]. In addition, an LRP-like protein-coding region has been found in the immediate upstream region of many *acdS* genes. This gene was termed *acdR* (ACC deaminase regulatory protein), based on the evidence that it is necessary for optimum ACC deaminase expression in the presence of ACC. The *acdR* gene has also been demonstrated to participate in the regulation of ACC deaminase expression in *R. leguminosarum* bv. *viciae* 128C53K and *Azospirillum lipoferum* 4B [12,40]. Most other Proteobacteria that have been examined for ACC deaminase activity or *acdS* gene presence, possess an *acdR* gene in the vicinity of *acdS*, suggesting that this regulatory mechanism is widespread in *acdS*$^+$ Proteobacteria [40].

Despite the fact that many biochemical and biological features of ACC deaminase are now understood, not much is known about the origin and phylogeny of the *acdS* gene and its regulatory elements. Based upon a phylogenic analysis of a limited number of *acdS* genes partially characterized and their comparison to the phylogeny of 16S rRNA genes from the same bacteria, Hontzeas et al. [41] proposed that some ACC deaminase genes have been transmitted through horizontal gene transfer (HGT). Using the same criteria, Blaha et al. [42] suggested that ACC deaminase genes in Proteobacteria were extensively subjected to HGT. In addition, Nascimento et al. [43] suggested that in many *Mesorhizobium* spp. the *acdS* gene is transferred between strains through symbiotic island exchange. The phylogeny in Proteobacteria of *acdR* has also been investigated. Prigent-Combaret et al., [40] suggested that *acdR*, like *acdS*, may have evolved through HGT. This conclusion

notwithstanding, these authors suggest that the evolution of *acdS* and *acdR* genes might not be coupled. While phylogenetic studies of *acdS* and *acdR* genes have been focused primarily on Proteobacteria, other studies have demonstrated the presence of ACC deaminase activity in Actinobacteria [41,44–48], Firmicutes [44,48–51] and Bacteroidetes [52–55]. Furthermore, the presence of a putative functional ACC deaminase in *Phytophthora sojae* [56] further emphasizes the notion that the current view of *acdS* phylogeny and evolution is somewhat incomplete. To address this, here we have undertaken a comprehensive study of the phylogeny of *acdS* and *acdR* and the results are discussed in terms of evolutionary and ecological implications of ACC deaminase production by diverse microorganisms.

METHODS

Obtaining the sequences

To obtain bacterial ACC deaminase (AcdS) and ACC deaminase regulatory protein (AcdR) sequences, BLAST searches were performed in the NCBI databases (www.ncbi.nlm.nih.gov/) using *Pseudomonas* sp. UW4 *acdS* and *acdR* gene, as well as AcdS and AcdR protein sequences as the queries. For fungal ACC deaminase sequence retrieval, BLAST searches were performed in the NCBI database using the *Penicillium citrinum* AcdS protein sequence as the query. Default BLAST parameters were used when obtaining the sequences. A NCBI genomic BLAST search (www.ncbi.nlm.nih.gov/ sutils/genom_table.cgi) was also performed using *Pseudomonas* sp. UW4 *acdS* and AcdS sequences in order to evaluate the presence of ACC deaminase in other completely sequenced organisms. An additional BLAST search was performed in the nematode genomic database (www.nematodes.org) using *Pseudomonas* sp. UW4 or *Penicillium citrinum acdS* gene as query. Moreover, all putative AcdS sequences were analyzed for key protein residues known to be important for ACC deaminase activity, namely Lys51, Ser78, Tyr295, Glu296 and Leu322 [7,57,58] using *Pseudomonas* sp. UW4 as a reference. The AcdS sequences were aligned using MUSCLE [59] and the presence of key amino acid positions were verified. Sequences presenting different amino acids in the above-mentioned positions were discarded, as they are likely to represent related PLP dependent enzymes, such as D-cysteine desulfhydrase [58]. Sequence identities and similarities were analyzed using SIAS (http://imed.med.ucm.es/Tools/sias.html) with default parameters.

When available, the genomic regions containing the *acdS* gene were analyzed in order to identify any patterns present in the

acdS gene neighborhood. Strain information and 16S rRNA gene sequences were obtained via NCBI (http://www.ncbi.nlm.nih.gov), Goldcard (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi) and SILVA (http://www.arb-silva.de), where available. The accession numbers for sequences used in this study as well as strains descriptions are presented in **Tables S1** (Actinobacteria, Deinococcus-Thermus and Firmicutes), **S2** (α-Proteobacteria), **S3** (β-Proteobacteria), **S4** (γ-Proteobacteria), and **S5** (Eukaryotes).

ACC deaminase protein sequence analysis and comparison to closely related enzymes

Protein sequence analysis was conducted on AcdS proteins found in completely sequenced representative bacteria. The functional AcdS protein sequences of the Proteobacteria *Agrobacterium tumefaciens* D3 [60], *Az. lipoferum* 4B [40], *Bradyrhizobium japonicum* USDA110 [61], *M. loti* MAFF303099 [13], *Phyllobacterium brassicacearum* STM196 [62], *R. leguminosarum* 128C53K [12], *Sinorhizobium meliloti* SM11 [63], *Burkholderia phytofirmans* PsJN [14], *B. graminis* C4D1M [64], *Ralstonia solanacearum* GMI1000 [42], *Variovorax paradoxus* 5C2 [22], *Pseudomonas* sp. UW4 [38], *Pseudomonas* sp. ACP [1] and the Fungi, *Cyberlidnera saturnus* [1], *P. citrinum* [65], *T. asperellum* T203 [16], together with the AcdS from *Herbaspirillum frinsigense* GSF30 [66] and the putative AcdS sequences from *Agreia* sp. PHSC20C1, *Rhodococcus* sp. R04 (Actinobacteria), *Meiothermus ruber* DSM1279 (Deinococcus-Thermus) were used. Sequences were aligned using MUSCLE and the presence of conserved and variable sites was analyzed. Sequence comparisons were also performed with closely related enzymes. Therefore, D-cysteine desulfhydrase sequences from *E. coli* [8], as well as the ACC deaminase homologs from *Pyrococcus horikoshi* [7] and *Solanum lycopersicum* [58] were used and compared to the various ACC deaminase proteins.

Phylogenetic analysis

The sequences were aligned using MUSCLE and phylograms were constructed in Seaview v.4.2.12 [67] using PhyML [68]. In order to obtain the best substitution model for the construction of the phylogenetic trees, the resulting alignments were analyzed with jModeltest2 [69] and ProtTest [70]. The substitution models were chosen based on minimum BIC (Bayesian Information Criteria) values. The *acdS, acdR* and 16S rRNA gene evolutionary history was inferred by using the Maximum Likelihood method based on the GTR model with a discrete Gamma distribution (4 Gamma categories). The AcdS and AcdR phylograms were constructed using

the Maximum likelihood method based on the WAG model with a discrete Gamma distribution (4 Gamma categories). Branch support was evaluated using both aLRT (SH like) [71] and bootstrap analysis (100 replicates). Only bootstrap values above 0.75 (75%) are included in the phylograms. The resulting phylogenetic trees were plotted using FigTree v.1.4.1 (http:// tree.bio.ed.ac.uk/software/figtree).

Estimates of evolutionary divergence between *acdS* sequences or 16S rRNA sequences in groups of bacterial strains were computed using MEGA software 6.06 [72]. The number of base substitutions per site from between sequences was calculated and analyses were conducted using the Maximum Composite Likelihood model with 1000 bootstrap replications. The analysis involved 3 nucleotide sequences per group of bacterial species, previously aligned using MUSCLE. Codon positions included were 1st+2nd+3rd+Non- coding. All positions containing gaps and missing data were removed.

RESULTS AND DISCUSSION

ACC deaminase prevalence in completely sequenced organisms

After performing multiple searches in the NCBI database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) using *Pseudomonas* sp. UW4 *acdS* gene as query, it was observed that the *acdS* gene is not commonly seen in most sequenced organisms. The *acdS* gene is mainly found in Actinobacteria, members from the Deinococcus-Thermus phylum (*Meiothermus*), three classes from Proteobacteria (alpha, beta and gamma), in various Fungi classes belonging to Ascomycota and Basidiomycota, and in Stramenopiles members. These results are in agreement with previous reports, which have demonstrated ACC deaminase activity in many Actinobacteria, α, β and γ-Proteobacteria. Remarkably, putative *acdS* genes were found in *Meiothermus*, yet, there is no record of ACC deaminase activity in these thermophile strains. Putative *acdS* genes were also found many in members of Stramenopiles, mostly in *Phytophthora*. By computational analysis, Singh and Kashyap, [56] suggest that the *acdS* gene found in *Phytophthora sojae* encodes a functional ACC deaminase. Interestingly, despite the known ACC deaminase activity display by bacteria belonging to the Bacteroidetes/Chlorobi or Firmicutes, it was not possible to identify *acdS* genes in the completely sequenced bacteria belonging to these phyla. In 478 completely sequenced bacteria (accessed in July, 2013) belonging to the Bacteroidetes/Chlorobi, including many *Flavobacterium* and *Chryseobacterium* species, the *acdS* gene is not

found. Although candidate *acdS* genes are identified via BLAST, the active sites contain residues more consistent with D-cysteine desulfhydrase or a related PLP-dependent enzyme [58], such as YP_001296100 in which threonines replace residues corresponding to active site residues E296 and L322. ACC deaminase activity has been previously reported to be present in *Flavobacterium* and *Chryseobacterium* species, although at very low levels [52,55], which may represent non-specific activity of D-cysteine desulfhydrase-like enzymes. Similarly, although ACC deaminase activity has been described in many *Bacillus* and *Paenibacillus* strains [48–51], it was not possible to identify the *acdS* gene in 271 completely sequenced strains belonging to the Bacilli class (Firmicutes phylum), including many soil and plant-associated *Bacillus* and *Paenibacillus* species. It is possible that in these and many other bacterial strains the presence of an *acdS* gene may be related to a strain's specific feature in which *acdS* acquisition happened by HGT by result of a co-existence with other ACC deaminase-producing bacteria in environments where ACC deaminase production provides the bacteria with some important advantages. Other possible explanations for this inconsistency may relate to the fact that genome sequencing is biased and the sequenced strains may not be representative of bacteria that interact extensively with plants.

Analysis of ACC deaminase (putative and functional) protein sequences

In the first instance, every sequence used in this study (**Table S1–S5**) contains the previously described AcdS conserved regions that have been found to be necessary for ACC deaminase activity. Moreover, all bacterial AcdS sequences shared high sequence identity (60 to 100%) to AcdS from *Pseudomonas* sp. UW4. When comparing the putative AcdS sequences from Fungi with the functional ACC deaminase from *P. citrinum*, sequence identities ranged between 70 and 99% for the majority of fungal AcdS sequences. Exceptionally, some AcdS sequences from yeasts and some other fungi share only 52–55% identity to *P. citrinum* AcdS. Also, the Stramenopiles members share approximately 60% identity to the *P. citrinum* AcdS. Interestingly, the AcdS sequences from yeasts, some other Fungi and Stramenopiles share higher identity to *Pseudomonas* sp. UW4 AcdS sequence (70 to 85%), consistent with a relationship with Proteobacteria and the possibility of past horizontal gene transfers. A more detailed description of this issue is presented below.

Protein sequence analysis suggests that the putative *acdS* genes found in *Rhodococcus* sp. R04, *Agreia* sp. PHSC20C1 and *M.*

ruber DSM1279, encode a true ACC deaminase. By sequence comparison, it was observed that the putative AcdS contain all the conserved features present in all known functional ACC deaminases and not present in the related enzymes (**Fig. S1**). For instance, the putative AcdS sequences contain the important residues E295 and L322 known to be required for ACC deaminase activity [58] and not present in other related enzymes. These results are also supported by the fact that these Actinobacteria and *Meiothermus* AcdS protein sequences share high identity (70 to 82%) to the functional ACC deaminase from *Rhodococcus* sp. 4N-4 (partially characterized) [41]. In addition, these sequences show similar sequence identities to other β and γ-Proteobacteria AcdS sequences (~70%).

ACC deaminase phylogeny: Horizontal gene transfer or vertical transmission?

The comparison between the *acdS* phylogenetic tree (**Fig. 1**) and the 16S rRNA-based phylogeny (**Fig. 2**), suggests that ACC deaminase has evolved mainly through vertical transmission with occasional horizontal gene transfer. In the *acdS* phylogram (**Fig. 1**), it is observed that closely related strains typically have similar *acdS* gene sequences. Furthermore, many strains with different origins and isolated from different habitats (**Table S1–S5**), but belonging to the same species tend to have similar *acdS* genes.

The presence of the *acdS* gene in an organism like *M. ruber* is also consistent with the vertical transmission of this gene. It is unlikely that this bacterial thermophile (optimum growth at 60ºC) isolated from a hot spring has acquired an *acdS* gene through HGT. This is strongly supported by the *acdS* gene phylogram (**Fig. 1**) showing a well bootstrap-supported and unique cluster grouping all *Meiothermus acdS* sequences distantly from all other *acdS* genes obtained from different bacterial phyla. The presence of an *acdS* gene in the chromosome of the psychrophile marine actinobacterium, *Agreia* sp. PHSC20C1, (isolated in the Antarctic) and other soil Actinobacteria, is also consistent with the vertical transmission and ancient origin of the *acdS* gene. In *Azorhizobium* and *Bradyrhizobium* strains, the *acdS* gene is located far away from the ‘‘plastic’’ chromosomal symbiotic island containing the symbiotic genes. If these strains had acquired the *acdS* gene by HGT it might be expected that it would be present in a region that is more prone to such transfers, such as a symbiotic island or a plasmid.

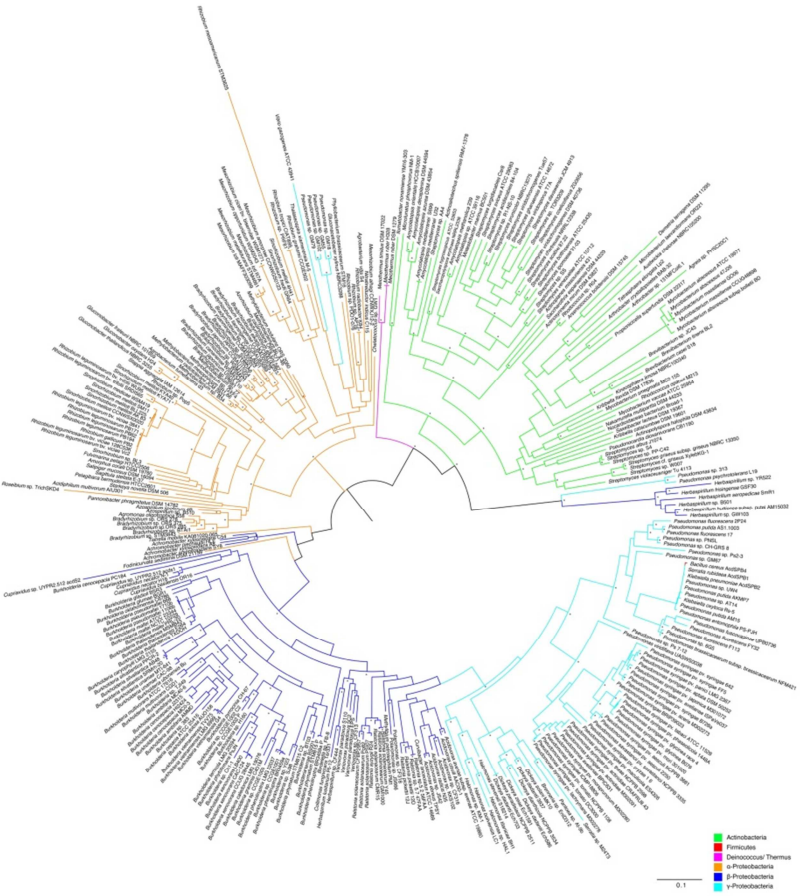

Figure 1. Phylogram based on the *acdS* gene. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). Branch support was evaluated using both aLRT (SH-like) and bootstrap analysis (100 replicates). Bootstrap values above 0.75 (75%) are displayed in the phylograms shown next to the branches as *. The analysis involved 335 nucleotide sequences and 931 patterns were found (out of a total of 1155 sites). HighRes image in DOI:10.1371/journal.pone.0099168.g001

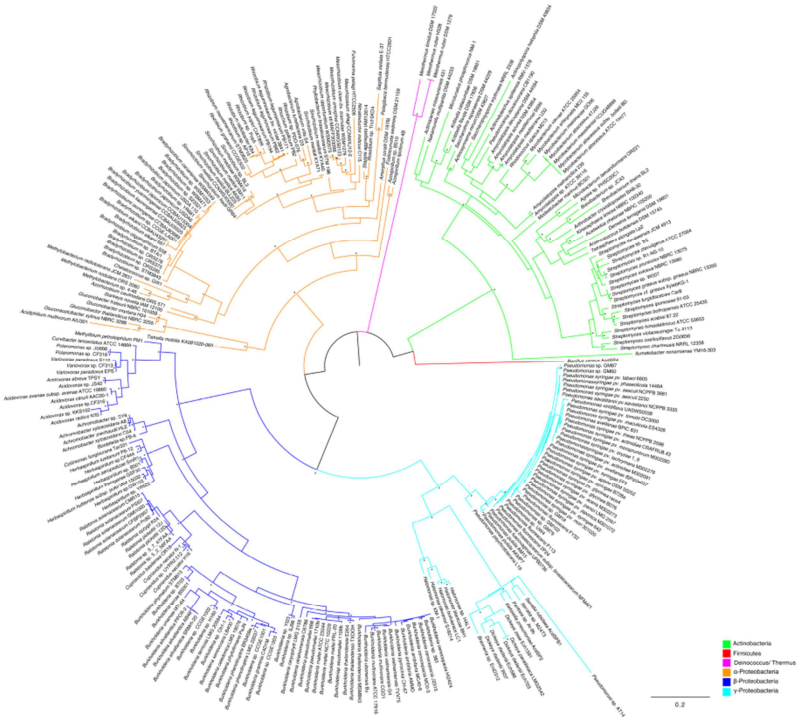

Figure 2. Phylogram based on 16S rDNA sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the GTR model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). Branch support was evaluated using both aLRT (SH like) and bootstrap analysis (100 replicates). Bootstrap values above 0.75 (75%) are displayed in the phylograms shown next to the branches as *. The analysis involved 272 nucleotide sequences and 768 patterns were found (out of a total of 1334 sites). HighRes image in DOI:10.1371/journal.pone.0099168.g002

Blaha et al. [42] and Glick et al. [3] have suggested that that the presence of *acdS* on plasmids may facilitate the lateral transfer of this gene. On the other hand, the presence of the *acdS* gene on a plasmid can also account for a different sequence divergence rate. Mobile elements and smaller replicons show higher evolutionary rates when compared to primary chromosomes [73,74]. By being present on smaller replicons, *acdS* genes may be subjected to

different evolutionary rates compared to genes present in primary chromosomes. This may help to explain the *acdS* gene phylogeny of *Burkholderia* and *Cupriavidus*. Instead of clustering together with the other β-Proteobacteria, strains belonging to the *Burkholderia* and *Cupriavidus* genus form a separate cluster (**Fig. 1**). From the available data, most *Burkholderia* and *Cupriavidus* strains have the *acdS* gene present in a second smaller chromosome. Other β-Proteobacteria possesses an *acdS* gene in the primary chromosome or in plasmids (**Table S3**). This phenomenon is also observed in *Agrobacterium* and *Rhizobium* strains, *A. vitis* S4 and *R. radiobacter* K84 which have the *acdS* gene located in a second chromosome, and therefore, cluster distantly from their *A. tumefaciens* D3 (*acdS* in plasmid) and *Rhizobium* (*acdS* in plasmid) relatives. Thus, there seems to be a connection between *acdS* phylogenetic distribution, evolution and *acdS* location in the replicon.

Environmental cues can also lead to different gene mutation rates [75]. Gene loss, acquisition, mutational rates and genome rearrangements may play a crucial role in bacterial adaptation and survival [76,77]. This is particularly important in organisms living in adverse environments like many of the organisms described here (**Table S1–S5**). It is possible that bacteria adapted to different environments may present different *acdS* divergence rates, thus being responsible for some of the variance in *acdS* genes in bacteria from the same species. When calculating the 16S rRNA and *acdS* gene evolutionary distance estimates in specific bacterial species groups it was found that the ratio between 16S rRNA and *acdS* sequence divergence is not always identical between strains and groups (**File S1**). For instance, three *Burkholderia mallei* strains isolated from three different countries show identical 16S rRNA (1200 bp) (d=0) and identical *acdS* gene (1019 bp) (d=0) sequences. In three *Burkholderia silvatlantica* strains obtained from Brazil, this is not observed; all strains present identical 16S rRNA sequences (1200 bp) (d=0) but show intraspecific differences in the *acdS* gene sequences (1019 bp) (d=0.005960.0020), sometimes accounting to up to 5 different nucleotides. Interestingly, all three *B. mallei* were obtained from human and animal blood and are known pathogens, while the three *B. silvatlantica* strains were obtained from the rhizosphere of different plants where they act like plant growth-promoting bacteria [64].

Several authors suggested HGT for *acdS* genes based on results showing a specific relative position of some *Pseudomonas* (γ-Proteobacteria) strains in the *acdS* phylogenetic tree [41,42,6]. Instead of forming a separate cluster, some *Pseudomonas* strains

clustered together with β-Proteobacteria. In this work, we obtained somewhat similar results. Although members of γ-Proteobacteria group very close to β-Proteobacteria, they form a unique cluster and are not scattered through the phylogenetic tree as observed in previous studies. A very close evolutionary relationship between these two classes has been reported [78– 81]. In fact, some bacterial strains that belonged to the *Pseudomonas* genus (γ-Proteobacteria) have been reassigned to the *Burkholderia* genus (β-Proteobacteria) [82,83]. Interestingly, the *Pseudomonas* sp. ACP AcdS sequence shares higher identity (96.7%) with *B. xenovorans* LB400 functional ACC deaminase than with *Pseudomonas* sp. UW4 AcdS (85.3%). This is also observed in the AcdS phylogram, where *Pseudomonas* sp. ACP groups closer to *B. xenovorans* LB400 (**Fig. 3**). While, Honma and Shimomura [1] tentatively identified *Pseudomonas* sp. ACP bacterium by phenotypic methods, it is conceivable that *Pseudomonas* sp. ACP is in fact a *Burkholderia* strain [64]. If this is in fact the case, then previous studies regarding the phylogeny of *acdS* may also have been influenced by the confusing relationship between *Pseudomonas* and *Burkholderia*. Furthermore, due to the recent divergent evolution and close relationship between β and γ-Proteobacteria, it is very difficult to prove *acdS* HGT in these classes.

While less prevalent than previously thought, HGT likely does occur and accounts for a portion of *acdS* gene evolution. For example, it has been shown that some *Mesorhizobium* strains may acquire a specific *acdS* gene by the means of symbiotic island transfer [43]. Nandasena et al., [84] demonstrated that *M. opportunistum* WSM2073 acquired a specific symbiotic island when it came in contact with non-endemic populations of *M. ciceri* bv. *biserrulae*, thus, gaining the ability to nodulate *Biserrula pelecinus*. The *acdS* gene was present within that symbiotic island and was therefore transferred between these strains. Moreover, the *acdS* gene sequences from those two strains share 100% identity, strongly supporting the idea of a recent transfer event. Curiously, there are some cases where *acdS* horizontal transfers seem to have occurred between strains with a more distant evolutionary relationship. This is the case of *Pseudomonas* isolates GM 18, GM 55, GM 79 and GM 102, which are found to possess *acdS* genes like those of α-Proteobacteria (**Fig. 1**). Despite belonging to the γ-Proteobacteria, *Vibrio gazogenes* ATCC43941 has an *acdS* gene resembling those of α-Proteobacteria (**Fig. 1**). Chen et al., (2013) showed that *Bacillus cereus* AcdSPB4 isolated from the casing soil of *Agaricus bisporus*

possesses an *acdS* gene highly similar to those of *Pseudomonas* (**Fig. 1**) thus, strengthening the idea of *acdS* horizontal transfer between distantly related strains.

Figure 3. Phylogram based on AcdS proteins. The evolutionary history was inferred by using the Maximum Likelihood method based on the WAG model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). Branch support was evaluated using both aLRT (SH like) and bootstrap analysis (100 replicates). Bootstrap values above 0.75 (75%) are displayed in the phylograms shown next to the branches as *. The analysis involved 431 amino acid sequences and 386 patterns were found (out of a total of 421 sites). Functional ACC deaminases are shown in bold. HighRes image in DOI:10.1371/journal.pone.0099168.g003

Interestingly, *Herbaspirillum seropedicae* SmR1, *H. frisingense* GFS30, *H. huttiense* subsp. *putei* AM15032, *Herbaspirillum* sp. B501, *Herbaspirillum* sp. GW103, *Herbaspirillum* sp. YR522, *Pseudomonas psychrotolerans* L19 and *Pseudomonas*

sp. 313 strains possess *acdS* genes that are not similar to those found in other bacteria from the same Class or even to other *Herbaspirillum* and *Pseudomonas* strains. Instead they form a unique group in the phylogenetic tree (**Fig. 1, 3**). Furthermore, these strains also possess *acdR* genes that are frequently found in *acdS*$^{+}$ Proteobacteria. In this scenario, it is possible that these strains have horizontally acquired *acdS* and *acdR* genes from a different class of bacteria yet to be determined. One may also assume that the putative *acdS* genes in these strains encode a different type of deaminase, however, ACC deaminase activity has been detected in *H. frisingense* GFS30 [66]. Moreover, the putative AcdS from *H. frisingense* GFS30 shows the conserved regions known to be important in functional ACC deaminases (i.e E295, L322) (**Fig. S1).**

Similar to what is observed in Bacteria, the AcdS phylogeny in Fungi indicates that closely related strains possess a similar ACC deaminase (**Fig. 3**). This is consistent with the notion that *acdS* genes are vertically transmitted in Fungi. However, some fungal strains like *Penicillium marneffei* and *Talaromyces stipitatus* (Ascomycota/Eurotiomycetes) are likely to have acquired the *acdS* from other Fungi belonging to the Sordariamycetes class, suggesting that like in some bacteria, fungal *acdS* genes may also be acquired by HGT. In addition, the yeasts *Cyberlindnera saturnus*, *Cyberlindnera jadinii* NBRC 0988, *Clavispora lusitaniae* ATCC 42720 (Ascomycota/ Saccharomycetes) and *Schizosaccharomyces pombe* 972h- (Ascomycota/Schizosaccharomycetes) seem to have acquired an ACC deaminase gene separately from most Ascomycota and presumably from Proteobacteria. ACC deaminase genes like those of Proteobacteria have also been detected in Fungi belonging to different classes such as *Punctularia strigosozonata* HHB-11173 (Basidiomycota/Agaricomycetes), *Fomitopsis pinicola* FP-58527 (Basidiomycota/Agaricomycetes), *Aureobasidium pullulans* AY4 (Ascomycota/Dothideomycetes), *Macrophomina phaseolina* MS6 (Ascomycota/Dothideomycetes) and *Guignardia citricarpa* CGMCC3.14348 (Ascomycota/Dothideomycetes). The Stramenopiles, *Phytophthora infestans* T30-4, *P. ramorum* Pr102, *P. sojae* P6497, *P. lateralis*, *P. kernoviae*, *P. parasitica*, *Pseudoperonospora cubensis* and *Hyaloperonospora arabidopsidis* Emoy2 also have ACC deaminase genes most similar to those of Proteobacteria (**Fig. 3**).

Searches of diverse genomic databases also have revealed the presence of putative *acdS* genes in other eukaryotic organisms like the nematode *Howardula aoronymphium* and the fly *Drosophila eugracilis*. Furthermore, these genes show high similarity to *acdS*

from Proteobacteria (**Fig. 3**). Some *acdS* genes are found in bacteria known to be associated with Eukaryotic organisms, for example, *Serratia* sp. M24T3, isolated from the nematode *Bursaphelenchus xylophilus*, and *Pantoea* sp. At-9b, the leaf cutter ant symbiont (**Table S4**). While it is possible that *Howardula aoronymphium* and *Drosophila eugracilis* may have acquired *acdS* genes from associated bacteria, it is most likely that the presence of *acdS* in these organisms results from contamination of genomic DNA.

AcdR phylogeny: Have AcdR and AcdS undergone a coupled evolution?

In the study conducted by Prigent-Combaret et al. [40], 45 of 48 studied Proteobacteria were found to possess an LRP homolog (*acdR*) near the *acdS* gene. Here, we report the presence of *acdR* in, at least, 166 of 261 Proteobacteria possessing an *acdS* gene. Still, it was not possible to obtain the *acdR* sequence in many organisms (n=78) and others only have their *acdS* gene described. The *acdR* gene was not found at least in 17 *acdS*$^{+}$ completely sequenced strains (6 *Mesorhizobium* strains, 2 *Rhizobium* strains, *Fulvimarina pelagi,* 3 root nodule *Burkholderia* strains containing the 2nd *acdS* copy in a plasmid, *Halomonas titanicae* BH1 and 4 *Pseudomonas* strains).

Moreover, the *acdR* gene is found in the opposite direction of the *acdS* gene in most studied Proteobacteria (data not shown). This is consistent with the previous reports of Grichko and Glick [37], Ma et al. [12] and Prigent-Combaret et al. [40]. This data suggests that *acdR* is a common mechanism regulating ACC deaminase expression in most Proteobacteria. Moreover, the phylogeny of *acdR* (**Fig. 4**) is related to the *acdS* gene phylogeny (**Fig. 1**), suggesting that these genes evolved in a similar and dependent manner. Closely related strains have similar *acdR* genes, as also observed in the *acdS* phylogram, suggesting that *acdR* is primarily vertically inherited. In the phylogram based on the *acdR* gene it is also observed a grouping according to the bacterial Class (taxonomy) and the gene location in the replicon (Example: 2nd chromosome location in *Burkholderia* and *Cupriavidus* vs. primary chromosome/plasmid location in other β and γ-Proteobacteria).

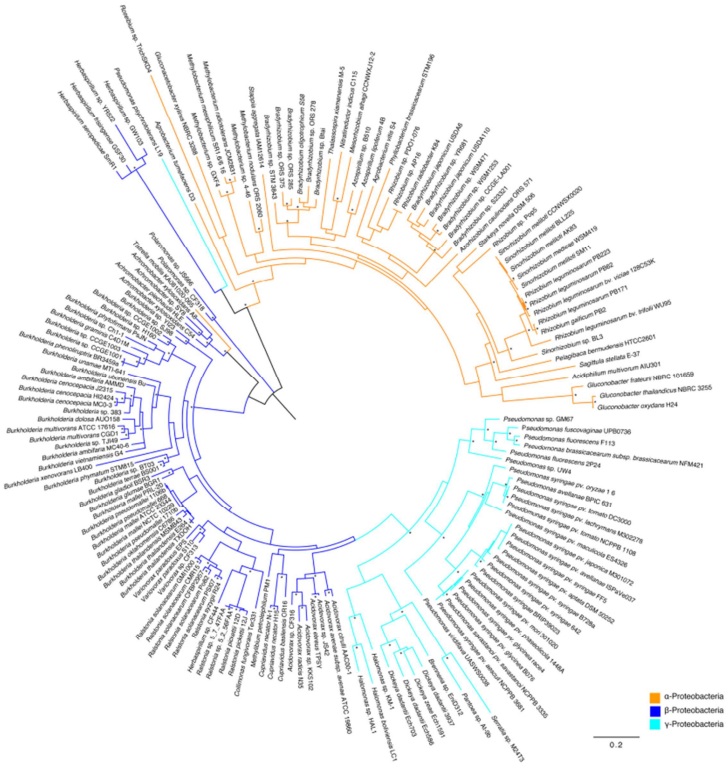

Figure 4. Phylogram based on the *acdR* gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the GTR model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). Branch support was evaluated using both aLRT (SH-like) and bootstrap analysis (100 replicates). Bootstrap values above 0.75 (75%) are displayed in the phylograms shown next to the branches as *. The analysis involved 166 nucleotide sequences and 509 patterns were found (out of a total of 594 sites). HighRes image in DOI:10.1371/journal.pone.0099168.g004

Interestingly, there are few cases where it seems that the *acdR* and *acdS* are not inherited together or may have undergone genomic rearrangements. While some strains do not have *acdR* genes in the vicinity of the *acdS* gene others do not have an *acdR* gene at all. In *Gluconacetobacter xylinus* NBRC 3288 there are various regions coding putative LRP in the upstream region of the *acdS* gene. However, they are not true *acdR* genes. A sequence

sharing high homology to the *acdR* gene is found far away (aprox. 9 kb) from the *acdS* gene. This is also observed in *B. xenovorans* LB400. In this case, despite the fact that this strain has an *acdR* gene located far from the *acdS* gene, it is still able to express ACC deaminase [64]. In *R. leguminosarum* bv. *viciae* 3841 an *acdR* gene is not found. In *M. loti* MAFF303099 the *acdR* gene is also not present, but in this case, the *acdS* gene transcription is regulated by NifA [13].

It is possible that genome rearrangements or gene insertions in smaller replicons can account for the absence of *acdR* genes in some *acdS*$^{+}$ bacterial strains. The strains *Burkholderia* sp. CCGE1002, *B. phymatum* STM815 and *B. phenoliruptrix* BR3459a (isolated from root nodules) have two copies of the *acdS* gene, one on the second chromosome and the other on a megaplasmid. The *acdS* gene copy present on the megaplasmid seems to be the result of *acdS* gene duplication and later insertion into this smaller replicon. This is consistent with the high identity between the two *acdS* copies and also the presence of transposase genes in the immediate upstream and downstream regions of the *acdS* gene. In this case, the *acdR* gene is not present and may have been lost in this process. The exceptions notwithstanding, in the majority of cases in Proteobacteria it appears that the evolution of *acdS* and *acdR* is coupled. This result is in agreement with previous reports showing that *acdR* is necessary for optimum ACC deaminase expression [38,39]. Despite being mostly inherited together, it is observed that these genes may have different evolutionary rates. Thus, for example, compared to the *Pseudomonas* sp. UW4 AcdR sequence, other AcdR sequences from Proteobacteria show identities ranging from 51% to 87%. This degree of variability is not observed in Proteobacteria AcdS sequences. By coding a regulatory protein, it is most likely that the *acdR* gene is more prone to modifications, thus, allowing fine-tuning of *acdS* transcription and expression. Previously, Nikolic et al. [85] stated that "the *acdR–acdS* gene cluster is rather rare and typically occurs in few a and b- Proteobacterial genera" based on finding the *acdR–acdS* gene cluster in four α, six β-Proteobacteria and in only three *Pseudomonas syringae* strains. They concluded that the operon is rather uncommon among γ-Proteobacteria. However, more detailed data presented by Prigent-Combaret et al. [40] and in this study, supports a widespread occurrence of *acdR–acdS* gene cluster.

The apparent lack of *acdR-acdS* clusters in Nikolic et al. [85] may be due to the inclusion of putative ACC deaminase sequences that were not confirmed by comparison with conserved protein

domains. Thus, sequences coding for D-cysteine desulfhydrases, and possibly other deaminases and aminotransferases, were considered as ACC deaminases, leading to a confusing relationship between *acdS* and *acdR* and the presence of *acdS* in some bacterial groups.

Other mechanisms regulating ACC deaminase transcription

The expression of ACC deaminase by organisms that do not possess *acdR* genes indicates that the presence of this regulator is not absolutely necessary for *acdS* transcription. The presence of CRP and FNR binding sites in the immediate upstream region of the *acdS* gene in many Proteobacteria [37,40] suggests that these elements can also account for ACC deaminase expression regulation in some Proteobacteria. It has been demonstrated directly in some instances that FNR as well as CRP regulate *acdS* transcription [37,38,40]. The NifA protein is also a known regulator of ACC deaminase expression in *M. loti* MAFF303099. In this strain, ACC deaminase expression occurs only inside of formed nodules [13,86]. In addition, a NifA binding site is found in the immediate upstream region of the *acdS* gene in this and many other *Mesorhizobium* strains, suggesting that this regulatory mechanism is widespread in this genus [43]. Interestingly, the NifA binding site (5'-TGT-N9–11-ACA-3') is quite similar to the CRP binding site (5'-TGTGA-N6-TCACA-3').

In many Actinobacteria and in *Meiothermus*, a gene encoding a protein from the GntR family of transcriptional regulators is found next to the *acdS* gene. We putatively termed it *acd-AR (*Actinobacteria) and *acd-MR* (*Meiothermus*). When performing BLAST searches using one Acd-AR protein sequence as query, the main hits are always related to other Acd-AR protein sequences found in $acdS^{+}$ Actinobacteria, suggesting a close relationship between *acdS* and *acd-AR*. The same trend is observed in *Meiothermus* despite the fact Acd-AR shares low identity to Acd-MR. There are no sequences in the database that share a high degree of similarity to Acd-MR. These results are consistent with the possibility that both *acd-AR* and *acd-MR* might be involved in the regulation of ACC deaminase expression in these organisms. Curiously, when analyzing the immediate upstream region of the *acdS* gene in various Actinobacteria it is observed that some strains appear to have no promoter regions (**Fig. S2-A**). The same is observed in *Meiothermus*. In these strains the *acdS* gene forms an operon together with the *acd-AR* gene and *acd-MR* gene, respectively. Interestingly, in Nocardioidaceae Broad-1 a leucine responsive protein is found in the vicinity of the *acdS* gene, however, it is quite

different from the Proteobacteria AcdR protein. Also, in some Actinobacteria and Proteobacteria strains the *acdS* gene is located near a transcriptional regulator belonging to the LysR family. Moreover, in *Saccharopolyspora erythraea* NRRL 233 and *Streptomyces hygroscopicus* ATCC 53653 strains the *acdS* gene also appears to be part of an operon consisting of a gene encoding a MFS family protein and another gene encoding a M20 peptidase (**Fig. S2-B**). Interestingly, a LysR transcriptional regulator is also found in the vicinity of the *acdS* gene in some Proteobacteria like *Brenneria* sp. EniD312, *B. xenovorans* LB400, *Dickeya* spp. and *Pantoea* sp. At-9b (**Fig. S2-C-H**). The presence of peptidase M20 in the vicinity of *acdS* is also observed in some of these strains. Further studies are necessary in order to characterize the importance of these regulators in ACC deaminase expression in different organisms.

ACC deaminase origin

To gain additional knowledge regarding the origin and evolution of ACC deaminase multiple searches of the database were conducted; sequences showing a high similarity to different deaminases were obtained and a phylogram was constructed (**Fig. 5**). In this instance, it was observed that ACC deaminase forms a distinct and unique group, where ACC deaminases from different organisms like Bacteria and Fungi cluster together. This is also observed with D-cysteine desulfhydrase, however only a few representatives of the considered ‘‘true’’ D-cysteine desulfhydrases (*E. coli* D-cysteine desulfhydrase) were obtained. When searching in the database, it was observed that D-cysteine desulfhydrase is an enzyme whose distribution is not widespread and it may also be not nearly as conserved as ACC deaminase. Its presence has been verified mainly in γ-Proteobacteria. Other proteins showing some homology were found in Firmicutes and other α-Proteobacteria, but in those instances showing low identity scores (39%). Interestingly, D-cysteine desulfhydrase activity has been demonstrated for *Solanum lycopersicum* [58] and *Arabidopsis thaliana* [87]. However, these enzymes form a distinct phylogenetic cluster, far away from *E. coli* and other γ-Proteobacteria D-cysteine desulfhydrase. Controversially, ACC deaminase activity has also been demonstrated for *A. thaliana*. Although Riemenschneider et al. [87] did not detect ACC deaminase activity from the product of *A. thaliana* gene ‘‘At1g48420’’, McDonell et al. [88] showed that this gene encoded a protein with the ability to breakdown ACC. Moreover, McDonnel et al. [88] suggest that the gene is responsible for regulation of *A. thaliana* endogenous ACC levels. The same authors

also suggest that this enzyme may be present in many other plant species. Curiously, Todorovic and Glick [58] did not find ACC deaminase activity in the *S. lycopersicum* At1g48420 protein homolog, sharing 70% identity and clustering together with *A. thaliana* At1g48420 protein (**Fig. 5**).

Figure 5. Phylogram constructed based on ACC deaminase and related PLP enzymes protein sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the WAG model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). Branch support was evaluated using both aLRT (SH-like) and bootstrap analysis (100 replicates). Bootstrap values above 0.75 (75%) are displayed in the phylograms shown next to the branches as *. The analysis involved 99 amino acid sequences and 570 patterns were found (out of a total of 594 sites). Sequences used for the construction of this phylogram are described in **Table S6**. HighRes image in DOI:10.1371/journal.pone.0099168.g005.

Despite showing D-cysteine desulfhydrase and ACC deaminase activity *in vitro*, it is conceivable that the at1g48420 gene product does not represent a "true" D-cysteine desulfhydrase or ACC deaminase, or at least, is only distantly related to bacterial D-cysteine desulfhydrase and ACC deaminase. The grouping that is observed in **Fig. 5** supports this latter conclusion.

Instead of clustering with bacterial ACC deaminase or D-cysteine desulfhydrase, the plant protein homologs form a distant and unique cluster with a different phylogenetic background within the broader family of these PLP-dependent enzymes. Proteins with similar origin and function often tend to be conserved. Thus, if the at1g48420 gene encoded a true ACC deaminase (similar phylogenetic background and specialization towards ACC breakdown), it would likely cluster together with bacterial and fungal ACC deaminases and have similar amino acid residues in specific sites as are present in functional ACC deaminases that are important for ACC breakdown (i.e E295, L322). Nevertheless, it is possible that proteins like At1g48420 evolved and specialized in ACC degradation in a different route than those bacterial and fungal ACC deaminases. In this case, additional studies are necessary to further unveil the characteristics of At1g48420-like proteins. It is most likely that the ability of at1g48420 gene product to use D-cysteine or ACC as substrates results from the high promiscuity that many deaminases show in cleaving multiple substrates that share similar characteristics. For example, it has been shown that ACC deaminase from *Pseudomonas* sp. ACP is able to use multiple substrates like D-cysteine and also other D-aminoacids. *E. coli* and *S. thyphimurium* D-cysteine desulfhydrases are able to efficiently use β-chloro-D-alanine (β-CDA) and other substrates (**Table 1**). Intriguingly, Todorovic and Glick [58] demonstrated that mutations in amino acids (E295S/L322T) in *Pseudomonas* sp. UW4 ACC deaminase lead to the loss of ACC deaminase activity, yet, these mutations conferred an increased D-cysteine desulfhydrase activity to the mutant enzyme. The K_m of the double mutant for D-cysteine was much lower than the K_m of the native ACC deaminase towards ACC. Moreover, the K_m of the double mutant enzyme towards D-cysteine is equivalent to that of a true D-cysteine desulfhydrase. Still, this mutant enzyme shows great inefficiency (K_{cat}= 10.9 min-1) in D-cysteine cleavage. This data shows that small amino acid changes can confer different substrate usage abilities in closely related deaminases. If ACC deaminase can use multiple substrates, it is possible that in some organisms the production of ACC deaminase can be important for cleavage of such substances, thus, giving these

organisms the ability to use other nutrient sources, or to grow under otherwise toxic conditions. This can have major implications in a microorganism's fitness, especially in organisms living under limiting conditions. For example, Soutourina et al. [89] demonstrated that the expression of D-cysteine desulfhydrase by *E. coli* relieved some of the toxic effects of D-cysteine in bacterial growth. Also, D-cysteine desulfhydrase production allowed the growth of *E. coli* in a minimal medium containing D-cysteine as the sole sulfur source, demonstrating the importance of D-cysteine desulfhydrase in bacterial growth under sulfate limitation.

Table 1- Substrate cleavage abilities of studied ACC deaminase, D-cysteine desulfhydrase and other PLP dependent (ACC deaminase or D-cysteine desulfhydrase homologs) enzymes.

Enzyme	Tested substrates	Functional substrates	K_m (mM)	Reference
Arabidopsis thaliana **"At1g48420" ACC deaminase homolog**	ACC, D-cys	ACC, D-cys	0.25 (D-cys)	[87,88]
Bradyrhizobium japonicum **USDA110 ACC deaminase**	ACC, D-ser, β-CDA	ACC, D-ser	n.a	[61]
Cyberlidnera saturnus **ACC deaminase**	ACC, DCA, D-cys, D-ser, β-CDA, OAD-ser	ACC, DCA, D-cys, D-ser	2.6 (ACC)	[1,102]
E. coli **D-cysteine desulfhydrase**	D-cys, 3-CDA, D-cyst, DLAC, DLSC, DLSCyst, Dlan, D-ala, L-ala, D-ser, L-ser, D-phen, L-phen, D-tryp, and others.	D-cys, 3-CDA, D-cyst, DLac, DLsc, DLSCyst, DLan.	0.15 (D-cys), 0.91 (3-CDA), 0.27 (D-cyst), 0.29 (DLac), 0.04 (DLsc), 0.11 (Dlan)	[8]
Methylobacterium nodulans **ACC deaminase**	ACC, D-cys, L-cys	ACC	0.8 (ACC)	[103]
Methylobacterium radiotolerans **ACC deaminase**	ACC, D-cys, L-cys	ACC	1.8 (ACC)	[103]
Penicillium citrinum **ACC deaminase**	ACC, DCA, L-ser, D-ser, DACC, DACA	ACC, DCA, D-ser	4.8 (ACC)	[65]
Pirococcus horikoshi **OT3 ACC deaminase homolog**	ACC, D-Ala, L-Ala, D- Ser, and L-Ser, D-cys	D-ser, L-ser	n.a	[7]
Pseudomonas putida **UW4 ACC deaminase**	ACC, D-cys	ACC, D-cys	3.4 (ACC)	[104]
Pseudomonas putida **UW4 ACC deaminase mutant E295S/L322T**	ACC, D-cys	D-cys	0.34 (D-cys)	[104]
Pseudomonas **sp. ACP ACC deaminase**	ACC, D-VG, β-CDA, β-FDA, D-ser, V-ACC, APC, Cys, L-hom, L-thr, L-try, L-met, L-tyr, L-cys, L-aba, DCA, D-EAC, D-TAF	ACC, D-VG, β-CDA, D-ser, β-FDA, V-ACC, OAD-ser, β-2CDA, β-2FDA, DCA, D-EAC, D-TAF	1.5 and 9.2 (ACC), 1.1 (β-FDA), 4.4 (V-ACC), 5.4 (β-CDA), 36.2 (DCA), 56 (OAD-ser), 97 (D-VG)	[1,7,105]
Salmonella typhimurium **D-cysteine desulfhydrase**	D-Cys, β-CDA, D-Ser, L-Ser, ACC, D-Ala	D-Cys, β-CDA, D-ser	0.34 (D-cys)	[9]
Solanum lycopersicum **ACC deaminase homolog**	ACC, D-cys, L-cys	D-cys	0.21 (D-cys)	[58]
Solanum lycopersicum **ACC deaminase homolog S338E/T386L**	ACC, D-cys	ACC	n.a	[58]

1-amino-2-vinylcyclopropane-l-carboxylic acid (**V-ACC**), 1-aminocyclopentane-1-carboxylate (**APC**), 1-aminocyclopropane-1-carboxylic acid (**ACC**), 3-chloro-D-alanine (**3-CDA**), Cystathionine (**Cyst**), D-alanine (**D-ala**), D-cysteine (**D-Cys**), D-cystine (**D-cyst**), D-erythro-2-amino-3-chlorobutyrate (**D-EAC**), D-methionine (**D-met**), D-phenylalanine (**D-phen**), D-serine (**D-ser**), D-threo-2-amine-3-fluorobutyrate (**D-TAF**), D-tryptophan (**D-tryp**), D-vinylglycine (**D-VG**), Dimethyl-ACC (**DACC**), DL-lanthionine (**Dlan**), DL-allocoronamic acid (**DACA**), DL-allocystathionine (**DLAC**), DL-coronamic acid (**DCA**), DL-selenocysteine (**DLSCyst**), DL-selenocystine (**DLSC**), L-alanine (**L-ala**), L-aminobutyric acid (**L-aba**), L-cysteine (**L-cys**), L-homoserine (**L-hom**), L-methione (**L-met**), L-phenylalanine (**L-phen**), L-serine (**L-ser**), L-threonine (**L-thr**), L-tryptophan (**L-try**), L-tyrosine (**L-tyr**), β -chloro-D-alanine (**β-CDA**), β-fluoro-D-alanine (**β-FDA**), β, β- dichloro-D-alanine (**β2CDA**), β, β-difluoro-D-alanine (**β2FDA**), O-acetyl-D-serine (**OAD-ser**).

It is possible that by maintaining a broad ability to cleave ACC-like substrates and some D-aminoacids, ACC deaminase genes were maintained in organisms that live in environments where ACC is not present. On the other hand, the presence of ACC deaminase in organisms that are associated with plants or other ACC-producing organisms, gave them a significant advantage in their ecology so that *acdS* genes were maintained. This may have led to the significant *acdS* gene presence in plant-associated microorganisms, especially plants grown under perennially stressful conditions [51], and increased *acdS* gene loss in microorganisms living in environments where ACC is nonexistent.

Overall, it seems that bacterial and fungal ACC deaminases (here considered to be the representatives of true ACC deaminases) belong to a large group of PLP-dependent deaminases (including bacterial D-cysteine desulfhydrase) related to tryptophan synthase beta subunit and sharing a common origin. Further, mutations and other evolutionary forces may have led to some level of substrate specialization. Yet, some conserved features appear to allow these enzymes to be able to utilize a wide range of related substrates. This is exemplified by the data presented in **Table 1**.

ACC deaminase phylogenetic distribution and evolution

A model for ACC deaminase evolution and phylogenetic distribution is proposed based on the AcdS phylogenetic analysis, AcdS protein sequence analysis, *acdS* gene location, organism habitat and origin. The evolutionary relationship among Archaea, Bacteria and Eukaryotes was also taken into account when attempting to resolve the evolution of ACC deaminase [90,91,78]. From the available sequence data, it would appear that the most ancient point for the origin of ACC deaminase in Bacteria dates to the Actinobacteria or Deinococcus-Thermus. Most Actinobacteria strains investigated (**Table S1**) possess an *acdS* gene in their primary and unique chromosome. In another ancient bacterial lineage, the Deinococcus-Thermus, the *acdS* gene is also found in the chromosome of its representatives *Meiothermus*, suggesting an *acdS* chromosomal location in a common ancestor for Bacteria. In many α-Proteobacteria, including *Azorhizobium* and *Bradyrhizobium*, the *acdS* gene is found in the primary chromosome of these strains. The *acdS* gene is also found in the primary chromosome of many marine α-Proteobacteria, and in the vinegar isolate *Gluconacetobacter xylinus* NBRC 3288. Despite the fact that ACC deaminase genes were not yet detected in any delta or epsilon-Proteobacteria (181 genome sequences available in the database), the presence of *acdS* genes in α-Proteobacterial strains that live in

environments where ACC is not present, suggests that *acdS* was present in a Proteobacteria ancestor, located in its primary chromosome and likely was acquired by vertical transmission. Many α-Proteobacteria have *acdS* genes located on plasmids, symbiotic islands or second chromosomes; this is the case of the studied Rhizobiaceae (*Rhizobium*, *Sinorhizobium*, and *Agrobacterium*), Phyllobacteriaceae (*Phyllobacterium* and *Mesorhizobium*) and *Azospirillum* strains (**Table S2**). Extensive gene transfer analysis between completely sequenced α-Proteobacteria suggested that secondary chromosomes originated from intragenomic transfers from primary chromosomes to ancestral plasmids [92]. This mechanism may have not only led to the origin of a second chromosome in some α-Proteobacteria but also in other Proteobacteria. In this regard, it is possible that *acdS* was transferred from the primary chromosome to a plasmid in some α-Proteobacteria. This possibility is consistent with the presence of *acdS* genes in the plasmids of *Rhizobium* and *Sinorhizobium* species and in the second chromosome of *R. radiobacter* K84 and *A. vitis* S4. Slater et al. [92] also suggested that some strains like *Mesorhizobium* could have evolved by plasmid gene integration into the main chromosome. This suggestion is consistent with the observation that the same gene arrangement is found between *Mesorhizobium* symbiotic islands and some *Rhizobium* and *Sinorhizobium* symbiotic plasmids, where the *acdS* gene is located. It is likely that intragenomic transfers of *acdS* genes from primary chromosomes to plasmids may have occurred in members of α-Proteobacteria as well as in β and γ-Proteobacteria. The presence of *acdS* genes in the second chromosome of *Burkholderia* and megaplasmids in *Ralstonia* and some strains of *Pseudomonas* is consistent with this idea. The occurrence of such phenomena may have led to a dispersal of *acdS* genes through plasmids that are readily transmissible between closely and more distant related strains. This leads to the puzzling phylogeny of the *acdS* gene that is observed in bacteria belonging to the same Order or Family (taxonomy) (**Fig. 1**).

In most Fungi, AcdS sequences share an average sequence identity of ~50% with Bacterial AcdS. An exception to this case is the AcdS from yeasts, *Punctularia strigosozonata* HHB-11173, *Fomitopsis pinicola* FP-58527 (Basidiomycota/Agaricomycetes), *Aureobasidium pullulans* AY4, *Macrophomina phaseolina* MS6 and *Guignardia citricarpa* CGMCC3.14348 (Ascomycota/Dothideomycetes), and Stramenopiles, which appear to have a Bacterial origin. As observed in the AcdS based phylogram (**Fig. 3**), it seems that Fungal (excluding the above-mentioned

exceptions) and Bacterial AcdS diverged long ago. At this point it's not possible to corroborate both hypothesis of AcdS monophyletic or paraphyletic origin. Still, protein sequence analysis show some conserved amino acid regions (e.g. His80 and Ala161) in ACC deaminase from Fungi, Actinobacteria, Deinococcus-Thermus, and α- Proteobacteria, suggesting a common origin for *acdS* in these organisms. Organisms belonging to β and γ-Proteobacterial classes show different amino acids in the referred positions, suggesting a later divergence from the α-Proteobacteria and the rest of ancient classes. The Fungi grouping closer to Actinobacteria is also observed in the phylogram (**Fig. 3**) suggesting a common origin for ACC deaminase in these organisms. Based on the currently available data, we suggest that *acdS* genes had an ancient origin that may date to a Eukaryote and Bacterial common ancestor that possessed this gene in its chromosome. Furthermore, it is most likely that ACC deaminase originated as a consequence of specific mutations in an already existing PLP-dependent enzyme showing high similarity to tryptophan synthase beta subunit. This is consistent with the results obtained by Todorovic and Glick [58] showing that small amino acid changes in related enzymes can be responsible for the ability to use a specific substrate. Through time, it is probable that the *acdS* gene evolved by continuous vertical transmission, in which different constraints like habitat adaptation led to *acdS* divergence and sometimes gene loss. Intragenomic transfers of *acdS* genes from primary chromosomes to plasmids may have been selected for as a consequence of the advantage of ACC deaminase production, and this probably led to HGT events and increased divergence of *acdS* genes. These intragenomic transfer events and the presence of *acdS* on plasmids may have also led to gene loss in many organisms. This is consistent with the results obtained by Prigent-Combaret et al. [40] showing that *Azospirillum lipoferum* 4B loses the plasmid containing an *acdS* gene during phenotypic variation events.

The role of ACC deaminase production in microorganism's ecology and fitness

From the available information, it is observed that many of the *acdS*$^+$ organisms here described were isolated from heavily contaminated soils or otherwise stressed environments (**Tables S1–S5**), suggesting that ACC deaminase-producing microorganisms are more prevalent and better able to live in such conditions. Organisms that produce ACC deaminase normally bind to plant tissues, and take up ACC to convert into ammonia and α-ketobutyrate [2]. The products of ACC cleavage are potential nitrogen and carbon sources

[2,33] that can play a role in the microorganism's fitness under stressful situations. Under stress conditions plants produce higher levels of the phytohormone ethylene, which means that the plants also produce higher levels of ACC [3]. Microorganisms that bind to plant tissues typically utilize plant exudates as a nutrient source. Under stress conditions, not only is the amount of ACC produced by the plant increased, the vast majority of rhizosphere microorganisms produce the phytohormone indoleacetic acid (IAA) which acts to loosen plant cell walls thereby facilitating root exudation. Bacterial IAA production has also been shown to increase ACC synthase expression in plants [93]. Thus, microorganisms that can both produce IAA and utilize ACC may have a competitive advantage over other soil microorganisms [2,94].

Importantly, a recent study by Timmusk et al. [51], showed that ACC deaminase-producing organisms were more much abundant in the rhizosphere of wild barley (*Hordeum spontaneum*) growing in a stressed environment than they were in a similar (nearby) less stressed environment. This result was obtained despite the fact that both environments had similar soil, rock and topology characteristics. In addition, ACC deaminase-producing bacteria were abundant in plant rhizosphere samples and almost nonexistent in bulk soil samples. This suggests that organisms that produce ACC deaminase more readily survive in stressed environments by the mutualistic interaction with a plant host. By degrading ACC, microorganisms decrease plant ethylene levels that under stress conditions are responsible for plant senescence and ultimately plant death [3]. Therefore, these organisms facilitate plant health under stress conditions. In turn, healthier plants provide their associated microorganisms with more nutrients thereby increasing the proliferation of these microorganisms.

Chen et al. [95] demonstrated that ACC deaminase-producing bacteria are also present in the casing soil of the ethylene-producing fungi *Agaricus bisporus*. The authors proposed a new model for the interaction between fungi and ACC deaminase producing bacteria. Bacteria possessing an *acdS* gene were able to increase fungal primordium initiation and proliferation by reducing endogenous ACC levels and consequently the inhibitory ethylene levels known to affect fungal development. These results show that ACC deaminase-producing bacteria might not only associate with plants but also with fungi, bringing significant advantages to fungal colonization in soil. On the other hand, bacteria producing ACC deaminase gain significant advantages by associating with extreme soil and plant colonizers like fungi. Being that these organisms constantly produce

ACC, bacteria able to degrade ACC may gain extra nutrient sources as previously suggested.

ACC deaminase in Fungi: Relationship with plants or regulation of endogenous ACC levels?

The production of ACC deaminase by *T. asperellum* T230 has been shown to be an important mechanism for the plant growth promotion abilities of this fungal strain [16]. When ACC deaminase production is impaired, the fungal ability to promote canola root elongation is decreased, therefore, suggesting that ACC deaminase may act in a similar way as previously described by Glick et al. [2] for plant growth-promoting bacteria. Nonetheless, it has been shown by Jia et al. [65] that ACC deaminase in *P. citrinum* is produced independently of a relationship with a plant host. This happens because *P. citrinum* is capable of producing and accumulating ACC in its tissues. That is, *P. citrinum* possesses not only an *acdS* gene but also an ACC synthase gene. Jia et al. [96] found that the ACC deaminase was induced by the presence of accumulated ACC in the intracellular spaces of *P. citrinum*, indicating that ACC deaminase may participate in the regulation of ACC levels in this strain. As a consequence, ethylene production by *P. citrinum* can also be regulated by ACC deaminase. In fact, our search of the database revealed the presence of ACC synthase homologs in most fungal strains that possess an ACC deaminase (data not shown). Together these results suggest that ACC deaminase production by Fungi can account for the regulation of endogenous ACC concentrations, and therefore regulation of ethylene levels which can inhibit primordium initiation and formation.

What is the role of ACC deaminase in pathogenic microorganisms?

Surprisingly, *acdS* and *acdR* genes are found in a wide range of plant and human pathogenic microorganisms (**Table S1-S5**), suggesting that ACC deaminase may play a role in these microrganisms' ecology. For example, the production of ACC deaminase has been reported in the human pathogenic *Burkholderia cenocepacia* J2315 [64]. However, this bacterial strain, like other pathogenic *Burkholderia* strains, is predominant in soils where it normally associates with plants [97–99]. The *acdS* gene is also found in pathogenic fungi like *Aspergillus* spp. and *Myceliophthora thermophila*. Despite causing severe diseases in immunocompromised humans, these strains are mainly found in soil [100,101]. This data suggests that the presence of *acdS* genes in human pathogenic organisms may not be related to their human pathogenesis mechanisms but rather to their possible ecological role

in soil. Also, it is possible that the presence of the *acdS* gene in these strains and in plant pathogenic bacteria is related to the continuous *acdS* vertical transmission and not to any beneficial effects of ACC deaminase production. Nevertheless, ACC deaminase production by pathogenic microorganisms may ultimately play a role in: (a) obtaining extra nutrients sources from ACC or ACC-like substrate degradation, (b) the plant or fungi-growth-promoting abilities of these organisms when they are not acting as human or plant pathogens (‘‘opportunistic’’ pathogens), (c) augmenting the ability to overcome ethylene or ACC mediated plant response systems, (d) regulation of endogenous ACC levels, or a combination of these factors.

CONCLUSIONS

The results obtained in this study provide a more complete view of the role for ACC deaminase-producing organisms then was previously available. ACC deaminase genes are not only found in plant-associated microorganisms but also in other bacterial and fungal strains isolated from a wide range of different sources (i.e. hot springs, industrial sludge, sea), hence, challenging the notion that ACC deaminase-producing organisms only interact with plants, or more interestingly, that ACC deaminase can only use ACC as a substrate. Based on multiple parameters like protein sequence analysis and phylogenetic studies we suggest that ACC deaminase belongs to a broad group of promiscuous PLP-dependent enzymes (tryptophan synthase beta subunit family) sharing a common ancestor. It is most likely that ACC deaminase originated as a consequence of specific mutations in its ancestral enzyme gene. Small amino acid mutations conferred changes in substrate specificity, however, the ability to degrade similar substrates was somehow maintained. This can account for the presence of *acdS* genes in bacteria that do not associate with ACC producing organisms. The continuous vertical transmission of *acdS* genes may also be responsible for the presence of *acdS* in these organisms. Furthermore, contrary to previous reports, here we demonstrate that the *acdS* gene is mostly vertically inherited in various bacterial and fungal classes. An ancient origin dating a Bacterial/Eukaryote ancestor is also proposed for the *acdS* gene.

Nonetheless, horizontal gene transfer does account for a wide portion of ACC deaminase evolution. For instance, some fungal classes and some members of Stramenopiles may have acquired *acdS* genes from Bacteria, suggesting that HGT events not only occur between bacteria but also may occur between distantly related organisms. The presence of the acdR gene is observed in most

Proteobacteria possessing an *acdS* gene, suggesting a coupled evolution for these genes. In other microorganisms like Actinobacteria and Deinococcus-Thermus (*Meiothermus*) the presence of genes encoding a GntR family protein are observed in the vicinity of the *acdS* gene, suggesting a different mechanism of ACC deaminase regulation. Moreover, these regulatory genes (here termed *acd-AR* and *acd-MR*) are mostly found in these *acdS*$^+$ bacteria groups, reinforcing the idea that specific regulatory elements can be found in different Bacteria classes. Additional genetic and biochemical studies are needed to gain some additional understanding of ACC deaminase functioning and its possible role(s) in the ecology of various organisms. Also, exploring the origin of ACC deaminase and related enzymes may bring new insights into the functioning of this PLP family of enzymes that may be the key to their use in a variety of important biotechnological applications.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FXN MJR CRFSS BJM BRG. Performed the experiments: FXN. Analyzed the data: FXN. Contributed reagents/materials/analysis tools: MJR CRFSS BJM BRG. Wrote the paper: FXN MJR CRFSS BJM BRG.

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CHAPTER 3

ACC deaminase in the nodulation process of leguminous plants

REVIEW MANUSCRIPT

"The role of rhizobial ACC deaminase in the nodulation process of leguminous plants"

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The role of rhizobial ACC deaminase in the nodulation process of leguminous plants

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ABSTRACT

Symbiotic rhizobia-legumes associations are extremely important in terms of sustainable agricultural practices. This symbiosis involves a complex interaction between both partners, plant and bacterium, for bacterial infection and the formation of symbiotic N-fixing nodules. In this regard, the phytohormone ethylene plays a significant role in nodule formation, acting as an inhibitor of the nodulation process. Not only does ethylene regulate nodule development but also many other plant developmental cues, including various stress responses that inhibit overall plant growth.

Some rhizobia produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, thus, being able to decrease ACC and, consequently, decrease deleterious ethylene levels that affect the nodulation process. This occurs because ACC is the immediate precursor of ethylene in all higher plants. Hence, rhizobia that express this enzyme have an increased symbiotic potential. In addition to the direct role that ACC deaminase plays in the nodulation process per se, in a limited number of instances, ACC deaminase can also modulate nodule persistence. This review focuses on the important role of rhizobial ACC deaminase during the nodulation process, emphasizing its significance to legume growth promotion.

INTRODUCTION

The symbiotic rhizobia-legume association is one of the most studied beneficial plant-microbe interactions. This symbiotic association has traditionally been used in agricultural practices to provide nitrogen to plants and, thereby, enhance plant growth [1]. This symbiosis involves legumes and a specific group of soil bacteria, collectively known as rhizobia, which are able to form root nodules and fix atmospheric N when associated with legumes. For the most part, the interest in rhizobia strains is a consequence of their ability to efficiently fix atmospheric nitrogen, making them an important component of sustainable agricultural practices.

The successful interaction between a legume and rhizobia, requires two main developmental processes for the formation of symbiotic nitrogen-fixing nodules: bacterial infection and nodule organogenesis [2,3], which must be coordinated in both a spatial and a temporal manner in order to ensure nodule formation at the site of bacterial infection on the roots [4]. The plant-bacteria symbiosis is initiated by a complex signaling dialogue between legumes and compatible rhizobia, eventually allowing the entry of rhizobia into the root. Briefly, rhizobia form an intimate symbiotic relationship with legumes by responding chemotactically to various flavonoid molecules released by the legume host. These flavonoids bind specifically and tightly to the rhizobial NodD protein, which is the major determinant of rhizobial host specificity. Each strain of rhizobia recognizes only a limited number of flavonoid structures and each species of legumes produces its own specific set of flavonoids. The flavonoid-NodD complex binds to a nodulation promoter element and induces the expression of the rhizobial nodulation genes, which in turn produces a lipochitooligosaccharide nod factor that binds to a legume root receptor and triggers mitotic cell division in roots, leading to nodule formation [3].

The development of nodules, which are plant organs wherein rhizobia reduce atmospheric nitrogen into ammonia [4], begins when the infection thread reaches the nodule primordium located in the root cortex that ultimately develops into a nodule upon release of the rhizobia [5] and after the bacteria differentiate into a specialized symbiotic organelle-like form, termed bacteroid. The process of symbiotic N_2-fixation involves a large number of rhizobial genes, namely the *nif* genes that encode the nitrogenase enzyme and its iron-molybdenum co-factor [6]. Unfortunately, many of the components of this symbiotic process, such as molecular signaling, rhizobial attachment, root hair curling, infection thread formation, as well as the nodule formation and nitrogen fixation are severely

affected by various stresses [7-14]. In addition, rhizobia also have to deal with adverse conditions within the host cells, as well as with the plant's innate immunity/response, all of which may interfere with the symbiosis [15]. Nevertheless, rhizobia possess multiple mechanisms to counteract some of the negative effects associated with environmental stresses thereby optimizing the legume-rhizobia interaction during the nodulation process.

One important strategy to counteract many of the effects of stress during the nodulation process used by rhizobia is the modulation of ethylene levels that negatively affect the nodulation process. Modulation of ethylene levels may occur either by the bacterium synthesizing rhizobitoxine [16] a competitive inhibitor of the plant enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase (the enzyme responsible for the synthesis of ACC), or by expression of the enzyme ACC deaminase which cleaves plant ACC into ammonia and α-ketobutyrate [17]. Both mechanisms are able to decrease the ethylene levels in plant root tissue, at least locally in and around the root nodules, and have beneficial effects on the symbiotic rhizobium-legume process as well as on plant growth itself. In this work we discuss the role of ACC deaminase in the nodulation process of rhizobia and its effects in legume growth promotion.

The phytohormone ethylene

Ethylene is a gaseous plant hormone produced endogenously by all higher plants and is recognized as one of the most important molecules regulating plant growth and development [18,19]. This phytohormone regulates many plant developmental processes such as germination, root and shoot elongation, abscission, senescence, flowering and fruit ripening as well as the responses to biotic and abiotic stress [19-23]. Many of the inhibitory effects of ethylene on plant growth occur as a consequence of stressful conditions. Under these conditions, the stressed plant first produces a small peak of ethylene that activates the transcription of various plant defensive genes [24]. Subsequently, the endogenous production of ethylene is substantially accelerated and generates a second and much greater peak of ethylene, which adversely affects plant growth, often turning on the transcription of genes associated with plant senescence [21]. Some of the effects of various stresses on plants are not solely attributed to the stress itself, but rather are due to the autocatalytic ethylene synthesis that ensues following the stress. Therefore, for optimal growth and development, regulation of ethylene production in plant tissues is essential [25, 26].

Ethylene biosynthesis in plants occurs via a methionine dependent pathway, which was firstly described by Adams and Yang

[27]. In this biosynthetic pathway, methionine is converted to S-adenosyl methionine (SAM) by the enzyme SAM synthase. SAM is then converted to ACC, the immediate ethylene precursor, by the action of the enzyme ACC synthase. Ultimately, ACC is converted to ethylene by the ACC oxidase enzyme. The limiting step in the plant ethylene biosynthetic pathway is the conversion of SAM to ACC by the enzyme ACC synthase, indicating the key role of ACC in plant ethylene production [28]. Nevertheless, there are some reports of limitation of ethylene formation. For instance, during hypoxia ACC cannot be converted to ethylene by ACC oxidase in the absence of oxygen [29, 30].

Ethylene effects in the nodulation process

In leguminous plants, ethylene is known for its negative role in the nodulation process initiated by rhizobia, as it inhibits the formation and functioning of nodules [31-33]. The first study of the effect of ethylene on legume nodulation was reported by Grobbelaar et al. [34]. These authors showed that exogenous ethylene (0.4 ppm) greatly inhibited the nodulation process of *Phaseolus vulgaris*. Similarly, Drennan and Norton [35] demonstrated that the application of ethephon, a liquid ethylene-releasing compound, reduced nodule number in *Pisum sativum*. Later, Goodlass and Smith [36] confirmed these results, showing that exogenous ethylene application reduced the number of nodules formed and also the nitrogen fixation abilities, in *P. sativum* and *Trifolium repens*. Application of the ACC synthase inhibitor AVG (aminoethoxyvinylglycine) induced an increase in nodule formation in *Medicago sativa* plants [37]. Similarly, Fearn and La Rue [38] showed that the application of ethylene biosynthesis and perception inhibitors restored the nodulation profile of *sym* 5 pea plants which were found to be more sensitive to normal levels of ethylene. In a more detailed study, Lee and La Rue [39], showed that exogenous ethylene application inhibited nodulation on the primary and lateral roots of *P. sativum* L. cv Sparkle and *Melilotus alba*. These authors also showed that treating the roots with 1 μM Ag+, an ethylene perception blocker, diminished the ethylene inhibitory effects. Ethylene biosynthesis inhibitors, AVG and AOA (aminooxyacetic acid) increased the nodulation, while ethephon reduced the number of nodules formed in *P. vulgaris* [40]. Although not all legumes respond similarly, addition of exogenous ethylene to most nodulating plants reduces the frequency of nodule primordia formation [41, 42].

In addition, experiments with mutant/transgenic plants have also contributed to the understanding of the role of ethylene in the regulation of the nodulation process. In this instance, Penmetsa and

Cook [43] showed that *Medicago truncatula* sickle mutants, insensitive to ethylene, formed an increased number of nodules compared to the wild-type form of this plant. Later, Penmetsa et al., [44] identified sickle mutants as being defective in a gene homologous to the *Arabidopsis* EIN2 (Ethylene Insensitive 2) gene. In agreement, the silencing of two *Lotus japonicus* EIN2 homologous genes also resulted in increased nodule formation [45]. In a study performed by Nukui et al. [46], transgenic *L. japonicus* plants expressing the mutant melon ethylene receptor gene Cm-ERS1/H70A inoculated with *Mesorhizobium loti* showed markedly higher numbers of infection threads and nodule primordia, suggesting that ethylene perception assists the negative feedback regulation of secondary nodule initiation. Likewise, the expression of the *Arabidopsis etr1-1* (a mutant ethylene receptor) gene in *L. japonicus* also leads to an increased infection thread formation [47]. Therefore, ethylene is also involved in the development of infection threads, especially infection thread initiation and elongation [2].

Several studies have shown that ethylene can inhibit numerous steps of the nodulation process (reviewed by Ferguson and Mathesius [48]). In this sense, ethylene may be involved in several phases of symbiosis, including the initial response to bacterial Nod factors, nodule development, senescence, and abscission [49, 50]. Oldroyd et al. [42] suggested that ethylene inhibits the calcium spiking process responsible for the perception of bacterial Nod factors in *Medicago truncatula*. The work of Lee and La Rue [39] indicated that exogenous ethylene did not lead to a decrease in the number of infections, but rather nearly all of the infections were blocked when the infection thread was in the basal epidermal cell or in the outer cortical cells of the plant. This leads to a reduction in infection as well as in the number of nodules in legumes. Heidstra et al. [51] postulated that a gradient of ethylene is responsible for restricting nodules radially to positions opposite to the xylem poles. Moreover, ethylene controls the epidermal responses during the nodulation process and, thus, negatively regulates multiple epidermal responses in order to inhibit rhizobial infection [46, 52]. Curiously, several studies have shown that radial restriction of nodule positioning is not observed in ethylene insensitive mutants [53, 47, 43], further confirming the hypothesis. Additionally, it was shown that endogenous ethylene production significantly increases in roots infected by *Rhizobium* or *Bradyrhizobium*, consequently decreasing the number of nodules that form on the infected plants [54, 55].

Recent studies show an intricate web of molecular mechanism underlying the plant control over nodulation. In this sense, ethylene acts as a major participant in the autoregulation of nodulation (AON) process [2, 56, 57].

Mechanisms to modulate ethylene levels in rhizobia

It is essential to regulate the ethylene level in the plant roots in order to achieve an improved symbiotic association [47]. As mentioned above, rhizobia have mechanisms that help to modulate the ethylene levels in plant roots; these include the bacterial enzyme ACC deaminase and the synthesis of the molecule rhizobitoxine.

The rhizobial enzyme ACC deaminase cleaves ACC (the immediate precursor of ethylene in plants) to ammonia and α-ketobutyrate, both of which are readily metabolized by the bacterium or the plant [26]. ACC deaminase-producing rhizobial cells can reduce ethylene concentrations in the infection threads and increase the persistence of infection threads by suppressing the defense signals in the plant cells, thus increasing the extent of nodulation of legume roots [58-62].

On the other hand, rhizobitoxine, an enol-ether amino acid, reduces the ethylene levels in plant roots in two different ways: (i) it inhibits the enzyme β-cystathionase which is necessary for methionine biosynthesis [52] and (ii) it inhibits the enzyme ACC synthase in the ethylene biosynthesis pathway [16, 52]. Rhizobitoxine-possessing strains have been found to be highly effective in enhancing nodulation and competitiveness in *Amphicarpaea edgeworthii* and *Vigna radiata* [63] and in *Macroptilium atropurpureum* [16]. In addition, rhizobitoxine is beneficial to rhizobia living inside nodules by allowing more rhizobial reproduction or by enhancing the synthesis of poly-3-hydroxybutyrate to support lateral reproduction [64-65]. Despite, the positive effect of rhizobitoxine in the nodulation process, its production appears to be limited to very few rhizobial species (mainly *Bradyrhizobium*). On the other hand, ACC deaminase genes are found in a wide range of different rhizobial species [66].

Ultimately, either ACC deaminase or rhizobitoxine production may be helpful in the nodulation process and thereby increase the nitrogen supply for legume plants due to a more effective nodulation. This may be especially important when plants are growing under stressful conditions so that ethylene may attain levels that are highly inhibitory to nodulation.

ACC deaminase in rhizobia

ACC deaminase (encoded by *acdS* genes) are prevalent in many rhizobial species, including α and β-rhizobia [66], although it is

important to note that not all strains within a particular species contain this enzyme. In α-rhizobia, *acdS* genes are found in bacteria such as *Azorhizobium, Bradyrhizobium, Methylobacterium, Mesorhizobium, Rhizobium*, and *Ensifer* (*Sinorhizobium*), *Devosia*, *Microvirga* and *Bosea*. In β-rhizobia, *acdS* genes are found in *Burkholderia* and *Cupriavidus*.

Ma et al. [67] and later Duan et al. [68] demonstrated ACC deaminase activity in several *Rhizobium* spp. (*R. leguminosarum*, *R. gallicum* and *R. hedysari*). *Sinorhizobium meliloti* SM11, carrying the *acdS* gene in a plasmid, is able to produce ACC deaminase [69]. The *Mesorhizobium* sp. MAFF303099 *acdS* gene encodes a functional ACC deaminase [70]. *Bradyrhizobium japonicum* is also able to degrade ACC trough ACC deaminase production [71]. Recently, Fedorov et al. [72] have characterized *Methylobacterium nodulans* ACC deaminase enzyme. ACC deaminase activity has also been demonstrated in members of β-rhizobia, *Burkholderia phymatum* STM815 and *Burkholderia tuberum* STM678 [73].

Despite the ability to breakdown ACC and use it as a nitrogen source, many of these strains presented different levels of ACC deaminase activity under free-living conditions. For instance, Duan and colleagues [68] showed that ACC deaminase activity ranged from 0.076 to 0.274 μmol α-ketobutyrate/mg protein/h in *Rhizobium* spp. Stiens et al. [69] and Kong et al. [74] reported a value of 0.355 and 0.180 μmol α-ketobutyrate/mg protein/h for *S. meliloti* SM11 and *S. meliloti* CCNWSX0020 ACC deaminase activity, respectively. No ACC deaminase activity was detected in *Mesorhizobium* sp. MAFF303099 under free-living conditions, however, in a bacteroid state (inside the formed nodules), *Mesorhizobium* sp. MAFF303099 showed an ACC deaminase activity of approximately 0.075 μmol α-ketobutyrate/mg protein/h) [70]. On the other hand, bacterium like *Bradyrhizobium japonicum* presented a free-living ACC deaminase activity of 1.49 μmol α-ketobutyrate/mg protein/h [71], whereas *Burkholderia* strains showed higher ACC deaminase activities, ranging from 3.55 to 4.63 μmol α-ketobutyrate/mg protein/h [73]. Although different methodologies and study conditions may account for some discrepancies between the ACC deaminase activity values, it is possible that these strains (even with similar *acdS* gene sequences) present different ACC deaminase activities due to factors, such as, *acdS* location in the replicon, *acdS* copy number and *acdS* gene transcriptional regulation. Nevertheless, rhizobia typically exhibit only a low level of enzyme activity compared with free-living plant growth-promoting bacteria (i.e., 10- to 100-fold less than free-living bacteria), suggesting the possibility that there may be

at least two types of ACC deaminase-producing bacteria [75]. There are free-living bacteria that bind relatively non-specifically to plant roots and have a high level of ACC deaminase activity, protecting plants from different stresses by lowering ethylene levels throughout the plant. Alternatively, rhizobia bind tightly to the roots of specific plants and have a low level of enzyme activity that facilitates nodulation by locally lowering ethylene levels [26].

In *Rhizobium* and *Sinorhizobium* strains, *acdS* genes are mostly found in symbiotic plasmids, while *Azorhizobium*, *Bradyrhizobium* and *Mesorhizobium* typically possess *acdS* genes in the chromosome. Still, in most *Mesorhizobium* spp. *acdS* genes are located in symbiotic islands next to the symbiotic genes [76]. This is not observed in *Azorhizobium* or *Bradyrhizobium* where *acdS* genes are found in chromosomal locations that far away from the symbiotic island [66]. In contrast, root-nodulating *Burkholderia* and *Cupriavidus* strains not only possess an *acdS* gene in a second chromosome, but also possess a second *acdS* gene copy in a symbiotic plasmid [66]. In this regard, horizontal gene transfer has been proposed for the acquisition of ACC deaminase genes by some rhizobia [66, 76].

Despite the presence of *acdS* genes in the genomes of a number of rhizobia strains, not much is understood about their functionality and role in the nodulation process. To date, some studies have reported ACC deaminase activity in some of these strains, but not all strains display enzyme activity when this activity is induced by ACC under free-living conditions, suggesting diverse types of regulation or the requirement for different elements for induction. In this sense, two different modes of regulation of the *acdS* gene have been identified. For instance, studies performed by Ma et al. [67] showed that a gene encoding an LRP-like protein (termed *acdR*) controls *R. leguminosarum acdS* transcription. *R. leguminosarum acdR* gene deletion resulted in a loss of ACC deaminase activity [67]. Analysis of completely sequenced bacterial genomes showed that *acdR* is found in most bacterial strains possessing an *acdS* gene (including *Azorhizobium, Bradyrhizobium, Methylobacterium, Rhizobium, Sinorhizobium, Burholderia and Cupriavidus*) consistent with the conclusion that *acdR* is a common regulator of *acdS* gene transcription [66]. In addition, analysis of *acdS* and *acdR* gene sequences showed that *acdR* is more prone to divergence than *acdS*, and this fact may, in part, account for a fine-tuning of *acdS* expression [66]. Curiously, most *Mesorhizobium* strains don't possess *acdR* genes [66]. In most *Mesorhizobium* spp. studied *acdS* genes are found in a chromosomal symbiotic island and are only expressed in symbiotic conditions under transcriptional

control of the NifA protein [70, 76, 77]. Analysis of the upstream regions of the *acdS* gene in many *Mesorhizobium* spp. indicate a putative NifA binding site, suggesting that NifA regulation of *acdS* expression may be common within the *Mesorhizobium* genus [76]. This is consistent with the fact that most *Mesorhizobium* strains don't produce ACC deaminase activity under free-living conditions [58, 77-79]. However, Safronova et al., [80] detected free-living ACC deaminase activity in two *Mesorhizobium loti* strains. It is possible that in some *Mesorhizobium* strains the *acdS* gene is not present in a chromosomal symbiotic island under transcriptional control of NifA, but rather it is located in a symbiotic plasmid under transcriptional control of *acdR* (as found in *Rhizobium* or *Sinorhizobium* strains). This is consistent with the presence of *acdS* and *acdR* in *M. alhagi* CCNWXJ12-2 [66]. This fact may account for the free-living ACC deaminase activity of some Mesorhizobia.

ACC deaminase effects in the nodulation abilities of rhizobia

Studies using rhizobial *acdS* deletion mutants as well as rhizobial strains expressing exogenous *acdS* genes have shown the important role of ACC deaminase in the nodulation process. For instance, Ma and co-workers [67] reported, for the first time, the effect of ACC deaminase gene in the nodulation process. Thus, an *acdS* minus mutant of *R. leguminosarum* bv. *viciae* 128C53K showed a 25% reduction of its nodulation abilities in *P. sativum* cv. Sparkle. In addition, a 23% decrease in shoot dry weight was observed in plants inoculated with the *acdS* mutant strain. The authors also showed that ACC deaminase activity did not have any influence on nitrogenase activity inside the formed nodules, further suggesting that ACC deaminase is involved only in the early stages of nodule development (infection thread formation) but not in nodule function (nitrogen fixation) *per se*. Uchiumi et al. [70] showed that a *Mesorhizobium* sp. MAFF303099 *acdS* mutant had decreased symbiotic abilities. The *acdS* mutant strain formed fewer nodules in *L. japonicus* than its wild-type counterpart, and also showed decreased nodule occupancy abilities. In both of the above mentioned cases, the ACC deaminase gene knockout resulted in a decreased ability to nodulate its host plant when compared to its respective wild-type strain, indicating that the presence of such gene plays an important role in the symbiotic efficiency and increases legume nodulation. On the other hand, rhizobial expression of exogenous ACC deaminase genes results in the increase of both nodulation efficiency and rhizobial competiveness. In this regard, Ma et al. [59] observed that *S. meliloti* Rm1021 that had been transformed to express an exogenous ACC deaminase gene had an

increased ability to nodulate *M. sativa* plants. By expressing the *acdS* and *acdR* gene from *R. leguminosarum* bv. *viciae* 128C53K, *S. meliloti* Rm1021 was able to produce 35 to 40% more nodules when compared to its wild-type form. The transformed strain also had an increased competitiveness in colonizing the nodules formed in *M. sativa* plants. Ma and co-workers [67] postulated that the transformants ability to utilize ACC as an extra nutrient source could make the bacterium proliferate better in the infection threads when compared to those that do not express ACC deaminase. Therefore, infecting cells that produce ACC deaminase are more likely to reach nodule primordia and form mature nodules. Similarly, *Mesorhizobium* sp. MAFF303099 expressing ACC deaminase under free-living conditions presented increased nodulation efficiency and competitiveness [79]. These authors integrated an extra copy of the *Mesorhizobium*. sp MAFF303099 *acdS* gene into the *Mesorhizobium* sp. MAFF303099 chromosome under the transcriptional control of a constitutive promoter. The *acdS*-transformed strain induced the formation of a higher number of nodules and was more competitive than the wild-type strain on *L. japonicus* and *L. tenuis* plants. Nascimento et al. [78] also showed that *Mesorhizobium ciceri* LMS-1 expressing an exogenous ACC deaminase was able to form an increased number of nodules in *Cicer arietinum* plants. In this instance, the plasmid pRKACC containing the *acdS* and *acdR* genes of *Pseudomonas* sp. UW4 was inserted and maintained in *M. ciceri* LMS-1, which resulted in free-living ACC deaminase activity. By expressing an exogenous ACC deaminase the pRKACC-transformed strain enhanced its nodulation activity by 127% when compared to the wild-type strain, and consequently increased chickpea biomass by 125%. Nodulation assays showed that the pRKACC-transformed strain was able to form more developed nodules in earlier stages of nodulation (31 days after inoculation-DAI), however, it was only at later nodulation stages (45 DAI) that an increased nodulation profile was verified. Also, the pRKACC-transformed strain showed similar nitrogen fixation abilities when compared to the wild-type strain. Subsequently, Nascimento et al. [81] showed that *M. ciceri* LMS-1 pRKACC maintained its increased nodulation abilities even when inoculated in different chickpea cultivars growing in soil. Using a similar approach, Brígido et al. [82] showed that a salt-sensitive *Mesorhizobium* strain was able to induce nodules in chickpea plants to the same extent as a salt-tolerant strain by expressing an exogenous *acdS* gene (pRKACC), further emphasizing the role of ACC deaminase in the nodulation abilities of these strains specially under environmental stress

(salinity) conditions. Recently, Kong et al. [74] showed that *S. meliloti* CCNWSX0020 expressing the pRKACC plasmid presented augmented nodulation abilities in *Medicago lupulina* plants. Although *S. meliloti* CCNWSX0020 possesses a functional *acdS* gene in its symbiotic plasmid and contains a moderate level of ACC deaminase activity, the expression of an exogenous (highly active) ACC deaminase still increased its nodulation abilities. Moreover, plants (*Medicago lupulina*) treated with this engineered strain displayed improved plant growth as well as copper tolerance and enhanced an antioxidant defense system.

Altogether, these data suggest that modulation of the ethylene levels in root tissues through ACC deaminase is an effective strategy to increase nodulation and competitiveness of the bacterium, supporting the previous hypothesis suggested by Ma et al. [58] as well as to increase the ability to counteract the negative effects of environmental stresses.

Contrary to what has been observed in many rhizobial strains, an *acdS* insertion mutant of *Bradyrhizobium japonicum* did not show an altered nodulation phenotype in four different hosts, rather transcriptomic analysis showed that the *acdS* gene of *Bradyrhizobium japonicum* was upregulated under symbiotic conditions [71]. The authors suggest that ACC deaminase effects in nodulation might not be common to all rhizobia. However, this study raises a number of questions. For instance, Murset et al. [71] stated that had obtained an *acdS* insertion mutant, but ACC deaminase activity was detected under free-living conditions (0.053 µmol α-ketobutyrate/mg) and inside the nodules formed by the mutant strain (0.17 to 0.59 µmol α-ketobutyrate/mg). On the other hand, Uchiumi et al., [70] have demonstrated that ACC deaminase activity of approximately 0.075 µmol α-ketobutyrate/mg protein/h was sufficient to induce increased nodulation abilities in *Mesorhizobium* sp. MAFF303099. This suggests that, in the study reportd by Murset et al. [71], the ACC deaminase activity in the mutant strain was sufficient to decrease the ethylene levels. Also, the presence of rhizobitoxine, which is present in many *Bradyrhizobium japonicum* strains [65], could decrease the ethylene levels even in the absence of ACC deaminase. The timing of the nodulation assays can also explain the absence of differences. For instance, Nascimento et al. [78] only observed differences in the nodulation profile between a wild-type and *acdS*-overproducing strain after 45 days post-inoculation.

The overwhelming weight of evidence indicates that rhizobia expressing ACC deaminase naturally or through genetically

engineering, are more competitive and increase nodulation in legumes, and consequently contribute to plant growth and development. This suggests that a relatively high level of ACC deaminase activity might be very important for developing rhizobial inocula with increased nodulation abilities even under environmental stress conditions.

Co-inoculation of legumes with rhizobia and other ACC deaminase-producing bacteria

Despite the low ACC deaminase activity in some rhizobial strains, it is generally sufficient to facilitate the nodulation process in the host plants, but not to decrease the high levels of ethylene formed in plant roots due to various environmental stresses [26]. This fact becomes more important when legumes are grown in marginal soils, where the stress conditions may be a limiting factor for the establishment of a successful rhizobium-legume symbiosis. In this sense, a strategy to increase nodulation, especially under stress conditions, may rely on the use of a combination of both rhizobial strains and other ACC deaminase-producing bacteria. In fact, co-inoculation of legumes with plant growth-promoting bacteria (PGPB) containing ACC deaminase and compatible rhizobia has proven to be a very useful approach for promoting nodulation by lowering ethylene concentrations in infected roots. For example, Shaharoona et al. [60] reported that co-inoculation with a PGPB possessing ACC deaminase activity and *B. japonicum* resulted in up to 48% better nodulation in mung bean plants compared with single inoculation of *B. japonicum*. Similarly, Remans et al. [83] verified that co-inoculation of PGPB along with rhizobia resulted in enhanced nodulation in common beans. Furthermore, inoculation of chickpea and lentil plants with a consortium of rhizobia and rhizospheric bacteria (with high ACC deaminase activity) resulted in increased nodulation and plant growth [84, 85]. Co-inoculation of ACC deaminase-producing bacteria along with respective rhizobia not only contributed to a higher nodulation ability, but it was also reported that by adjusting ethylene levels, an improvement of plant growth and yield was obtained in different plants even when grown under stress conditions. For example, co-inoculation of plants with rhizobia and ACC deaminase-containing bacteria strains enhanced nodulation and plant growth [86], even under stress conditions [87]. Another study, conducted by Belimov *et al.* [63], showed that pea plants inoculated with an inocula containing *Variovorax paradoxus* 5C-2, carrying an ACC deaminase gene, and an appropriate rhizobia strain yielded a higher level of nodulation and prevented the negative effects of drought stress compared to the plants inoculated with an

inocula containing the rhizobia and an ACC deaminase minus mutant of *V. paradoxus* 5C-2. Safronova et al., [80] demonstrated that the co-inoculation of *Mesorhizobium loti* strains and *V. paradoxus* 5C-2, both possessing ACC deaminase activity, had synergistic and additive effects on nodule number, root growth and uptake of elements (N, P, Ca, Mg, Na, Mn, Zn and Pb) in shoots of *L. edulis* and *L. ornithopodioides*. A study performed by Ahmad et al. [88] observed that co-inoculation of rhizobia and a strain of *Pseudomonas* containing ACC deaminase could be effective for reducing the deleterious effects of salinity on growth, physiology and quality of mung bean. Another study performed by Tittabutr et al. [89] revealed that co-inoculation of mungbean with *Bradyrhizobium* and a PGPB that contained a stress-induced ACC deaminase enzyme alleviates the effects of different environmental stresses. Similarly, the nodulation and growth of chickpea was increased by co-inoculation of *Mesorhizobium ciceri* and a PGPB with ACC deaminase activity, under irrigated and rainfed conditions compared to inoculation with rhizobium alone [90].

Collectively, the data clearly indicate that PGPB that contain ACC deaminase can be co-inoculated with rhizobia to improve the resistance of plants to environmental stresses by lowering the content of stress-induced ethylene in plants as well as increase the extent of nodulation of cognate legumes.

CONCLUSIONS

Rhizobia are an important component of sustainable agriculture due to their ability to fix nitrogen from atmosphere in association with legumes. However, this symbiotic rhizobium-legume association is dependent on the efficiency and competitiveness of the rhizobial strain for nodulation with indigenous soil bacteria and environmental factors. Ethylene is a phytohormone that negatively affects the nodulation process and its concentration increases significantly when plants are grown under unfavorable conditions. Thus, lowering the amount of ethylene synthesis in the nodulating roots could contribute to an improvement in legume nodulation. The rhizobial enzyme ACC deaminase is one of the mechanisms that confers a higher nodulation efficiency and competiveness ability to rhizobia and may also decrease the negative effects caused by various environmental stresses on the nodulation process. Therefore, the selection and use of rhizobial strains with high ACC deaminase activity is a promising strategy to improve the performance of rhizobia-legumes symbioses. A similar strategy consisting of the co-inoculating legumes with the combination of specific PGPB with high ACC deaminase activity and compatible

rhizobial strains is also likely to achieve a high level of nodulation, growth, and yield of the inoculated legumes; traits that are important to achieving an optimal/maximum symbiotic rhizobia-legume association.

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RESEARCH MANUSCRIPT

"Prevalence and evolution of ACC deaminase and dihydrorhizobitoxine desaturase involved in the modulation of leguminous plant ethylene levels by symbiotic rhizobia"

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Prevalence and evolution of ACC deaminase and dihydrorhizobitoxine desaturase involved in the modulation of leguminous plant ethylene levels by symbiotic rhizobia

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ABSTRACT

The phytohormone ethylene plays an important role in plant defense mechanisms as well as in the nodulation process induced by rhizobia. Consequently, several bacteria evolved the ability to modulate plant ethylene levels by the expression of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC, the direct ethylene precursor in higher plants, or via the production of rhizobitoxine by dihydrorhizobitoxine desaturase, which limits plant ACC biosynthesis.

In this work, we analyze the prevalence and evolution of the enzymes ACC deaminase and dihydrorhizobitoxine desaturase in 323 NodC$^+$ genomes from different rhizobial strains (*Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium* and *Paraburkholderia*) isolated from a wide range of leguminous plant hosts.

The results indicate that ACC deaminase and dihydrorhizobitoxine desaturase are differentially prevalent in rhizobial strains, indicating the existence of selection mechanisms, which are governed by the leguminous plant host (co-evolution), the rhizobial strain itself and the environment. In this sense, ACC deaminase is highly prevalent in ancient symbionts such as *Bradyrhizobium* and *Paraburkholderia* and mostly horizontally acquired in other rhizobia, indicating that it is subjected to a positive selection. On the other hand, dihydrorhizobitoxine desaturase is mostly present in Bradyrhizobium strains nodulating *Glycine max* and *Paraburkholderia* nodulating *Mimosa* spp., indicating a negative selection for rhizobitoxine in the symbiotic process of most leguminous plants. The factors governing the selection of these ethylene modulation genes are discussed.

Altogether, the data indicates that the ability to modulate plant ethylene levels played a significant role in the development of the symbiotic interaction between rhizobia and leguminous plants.

INTRODUCTION

The leguminous plant-rhizobia symbiosis is of one of the most specific and successful beneficial plant-microbe interactions studied to date. In this symbiotic relationship both leguminous plants and rhizobia evolved intricate signaling mechanisms, leading to a high degree of specificity in their interactions (Oldroyd and Downie, 2008). Rhizobia produce lipochitooligosaccharides, termed Nodulation (Nod) factors (NFs), encoded by nod genes, that are known to induce the plant symbiotic response. On the other hand, leguminous plants produce different flavonoids, known to induce nod gene expression in rhizobia, and perceive and respond to different rhizobial NFs, therefore, controlling the success of the bacterial internalization and nodule formation.

Importantly, leguminous plants possess mechanisms that tightly control the nodulation process (Reid et al. 2011), which can be regarded as a high energy consuming process for the plant, not only by the carbon provided to the rhizobial symbiont but also by energy necessary for the formation of the nodule structure. In this sense, hormonal regulation is one of the most important hubs in the mechanisms regulating the nodulation process (Ferguson and Mathesius, 2014). The phytohormone ethylene is central to the nodulation process (Gresshof et al. 2009; Guinel, 2015; Larranzair et al. 2015), since it is one of the key players in plant defense responses as well as in plant growth and development cues (Nascimento et al. 2018). In all higher plants, ethylene is produced from 1-aminocyclopropane-1-carboxylate (ACC) by the action of the enzymes ACC synthase and ACC oxidase. Downstream, the ethylene signaling pathway is comprised of several elements that ultimately lead to the activation of ethylene-induced transcription factors, thus, modulating the expression of several genes, including those involved in the nodulation process and plant defense responses (Nascimento et al. 2018).

Ethylene regulates the nodulation process in several plant species (**Table 1**). It modulates the initial recognition of rhizobial signals (microbial molecular associated patterns -MAMPs- and NFs), bacterial entrance to root hairs, early nodule development (infection thread and protonodule formation) and nodule functioning and senescence (Guinel, 2015). Ethylene is also involved in the modulation of abiotic stress responses, where accumulated ethylene levels (“stress ethylene”) negatively impacts plant growth (Abeles et al. 1992).

Bacteria have evolved intricate mechanisms to modulate ethylene levels, either through the production of the enzyme ACC

deaminase (Glick et al. 1998), the compound rhizobitoxine (RTX) (Sugawara et al. 2006), and, sometimes both. The enzyme ACC deaminase, encoded by the *acdS* gene, degrades ACC, the ethylene precursor, into ammonia and alpha-ketobutyrate, which bacteria readily use as nitrogen and carbon sources, respectively (Glick et al. 1998). Moreover, rhizobia expressing ACC deaminase can uptake and consume plant ACC, and, consequently, reduce the negative effects of ethylene in the nodulation process (Nascimento et al., 2016). Several studies reported that rhizobial strains impaired in ACC deaminase production present decreased nodulation abilities (Ma et al. 2003; Uchiumi et al. 2004). On the other hand, strains overexpressing ACC deaminase form more nodules, are more competitive, and possess increased plant growth promotion abilities (Ma et al. 2004; Conforte et al. 2010; Nascimento et al. 2012; Kong et al. 2015).

Rhizobitoxine is a secreted enol-ether amino acid that acts as an inhibitor of the plant enzyme ACS (Yasuta et al. 1999), leading to a reduction in ACC production and, consequently, plant ethylene levels (Yuhashi et al. 2000). RTX is synthesized through a pathway in which the dihydrorhizobitoxine desaturase enzyme, encoded by the *rtxC* gene, is involved in the ultimate step that leads to the formation of rhizobitoxine (Okazaki et al. 2004). *Bradyrhizobium* strains impaired in RTX production form fewer nodules in their host plants (Duodu et al. 1999) and are less competitive than their wild type counterparts (Yuhashi et al. 2000; Okazaki et al. 2004, Ratcliff and Denison, 2009).

The symbiotic compatibility and infection control mechanisms have led to a selection pressure on the bacterial symbionts over millions of years of evolution. In this sense, the diversity of NFs produced by rhizobia is a good indication of rhizobia adjusting to plants (Martinez-Romero, 2009) and the specificity of the interaction between rhizobia (containing *nod* genes) and their cognate leguminous plants allows us to study the leguminous host effect in rhizobial evolution. Here, the prevalence and evolution of ethylene modulation genes in *nodC*-containing rhizobial genomes is examined and discussed.

Table 1- The effect of ACC and ethylene on the nodulation process of several leguminous plants

Plant	Nodulation negatively affected by ACC	Nodulation positively affected by AVG, AOA,	Nodulation negatively affected by ET	Nodulation positively affected by STS, CoCl2	Reference
Phaseolus vulgaris cv. Pencil Podded Black Wax	n.t	n.t	Yes (ET 0.4 ppm)	n.t	Grobelaar et al., 1971
Pisam sativum cv. Sutton's Show Perfecti	n.t	n.t	Yes (Ethrel 2 ppm)	n.t	Drennan and Norton, 1972
Pisum sativum cv. Feltham First	n.t	n.t	Yes (ET 10 ppm)	n.t	Goodlass and Smith, 1979
Trifolium repens cv. Huia			Yes (ET 10 ppm)		
Medicago sativa cv. AS-R3	n.t	Yes (1 μM AVG)	n.t	n.t	Peters and Crist-Estes, 1989
Pisum sativum cv. Sparkle	n.t	n.t	Yes (ET 0.07 uL/L)	Yes (Ag 1 μM)	Lee and La Rue, 1992
Pisum sativum cv. Rondo	n.t	n.t	Yes (ET 0.07 uL/L)	Yes (Ag 1 μM)	
Melilotus alba cv. U389	n.t	n.t	Yes (ET 0.07 uL/L)	n.t	
Glycine max cv. Ransom	n.t	n.t	No (ET 0.45 ul/L)	n.t	
Glycine max cv. Tracy M	n.t	No (10 μM AVG)	**No** (100 μM Ethephon) **Yes** (1 mM Ethephon)	n.t	Hunter, 1993
Trifolium repens	n.t	Yes (0.1 mg/L AVG)	n.t	n.t	Van Workum et al., 1995
Vicia sativa ssp nigra	n.t	Yes (0.1 mg/L AVG)	n.t	n.t	
Medicago truncatula	Yes (5 μM)	nt	nt	nt	Penmetsa and Cook, 1997
Medicago truncatula sickle (ein2 mutant)	No (300 μM ACC)	nt	nt	nt	

Table 1- continued

Plant	Nodulation negatively affected by ACC	Nodulation positively affected by AVG, AOA,	Nodulation negatively affected by ET	Nodulation positively affected by STS, CoCl2	Reference
Glycine max cv. Hobbit 87	**No** (0.25 mM ACC) **Yes** (1 mM ACC)	n.t	n.t	No (10 μM STS)	Schmidt et al., 1999
Glycine max cv. Hobbit 87 etr1-1/etr1-1	No (1 mM ACC)	n.t	n.t	No (10 μM STS)	
Medicago sativa cv. Du Puits	Yes (1 μM)	Yes (10 μM AVG)	n.t	Yes (5 μM STS)	Nukui et al., 2000
Lotus japonicus B-129 Gifu	Yes (1 μM)	Yes (10 μM AVG)	n.t	Yes (5 μM STS)	
Macroptilium atropurpureum cv. Siratro	Yes (1 μM)	Yes (1 μM AVG)	n.t	Yes (5 μM STS)	
Glycine max cv. Enrei	No (1 μM)	No (10 μM AVG)	n.t	No (5 μM STS)	
Medicago truncatula cv. Jemalong	Yes (1 μM ACC)	Yes (0.1 μM AVG)	n.t	n.t	Oldroyd et al., 2001
Phaseolus vulgaris Harvester	n.t	Yes (50 μM AVG, 50 μM AOA)	Yes (Ethephon 50 μM)	Yes (10 μM CoCl2)	Tamimi and Timko, 2003

n.t- not tested.
ACC- 1-aminocyclopropane-1-carboxylate; ET- ethylene; AVG- aminoethoxyvinylglycine; AOA- aminooxyacetate; STS- Silver thiosulfate.

MATERIALS AND METHODS

Obtaining the rhizobial sequences

The protein NodC (average size of ~450 amino acids) encodes an N-acetylglucosaminyltransferase enzyme that is responsible for the production of NFs involved in the elicitation of the legume symbiotic response. A BlastP analysis (default parameters) was performed to study the presence of NodC in sequenced rhizobial genomes deposited in the NCBI database (www.ncbi.nlm.nih.gov). The NodC sequences from *Bradyrhizobium diazoefficiens* USDA 110 (WP_011084824.1), *Mesorhizobium loti* MAF303099 (WP_010913821.1), *Rhizobium leguminosarum* bv. *viciae* 3841 (WP_003540131.1), and *Paraburkholderia phymatum* STM815 (WP_012406749.1) were used as queries in the BlastP analysis.

Rhizobia possessing NodC were then selected for future studies based on the prevalence of ACC deaminase (AcdS) (average size of ~340 amino acids) and/or dihydrorhizobitoxine desaturase (RtxC) (average size of ~357 amino acids). *Bradyrhizobium diazoefficiens* USDA 110 functional AcdS (WP_011083073.1) and RtxC (WP_011084875.1) sequences were used as queries in BlastP searches (default parameters) in the NCBI database.

The DNA recombinase A (RecA) (average size of ~372 amino acids) was chosen as a representative of a housekeeping and vertically transmitted gene. RecA sequences from NodC containing rhizobia, as well as strain information (host isolation, geographical location) were obtained from the NCBI database.

Protein sequences were aligned using MUSCLE (Edgar, 2004) and used in phylogenetic and evolutionary distance analyses.

The gene location analysis (chromosome vs. plasmids) was performed in selected completely sequenced rhizobial genomes present in the NCBI database.

All sequence accession numbers and strain information is presented in **Table S1**.

Phylogenetic analysis and evolutionary distance calculations

Phylogenetic analysis and evolutionary distance calculations were performed using MEGA v.6.0.6 (Tamura et al. 2013). The best Maximum Likelihood (ML) model for each protein alignment was selected based on the lower Bayesian Information Criterion values obtained from an analysis with the Mega ML model selection tool, using default parameters.

The phylogenetic analysis was performed using the ML method, the appropriate selected model (described in the figure

caption of each phylogram), and a bootstrap method analysis with 500 replications.
The obtained newick files were uploaded in the Interactive Tree of Life (iTOL) v.3 website (http://itol.embl.de) (Letunic and Bork, 2016) and phylograms were generated, edited and analyzed.

Evolutionary distance calculations were performed in MEGA v.6.0.6, using the Bootstrap method with 500 replications and the Jones-Taylor-Thornton (JTT) matrix-based substitution model (Jones et al. 1992). The rate of variation among sites was modelled with a Gamma distribution (shape parameter=5).

Statistical correlations

Statistical correlation analyses were performed using IBM SPSS Statistics v.22 with parametric (Pearson correlation parameter, two-tailed) and non-parametric models (Kendal ‘s tau B and Spearman, two tailed). The correlations presented in this study were significant at the 0.01 level (2-tailed) by all the tested methods.

RESULTS

Phylogenetic analysis of NodC in completely sequenced rhizobia

To study the possible leguminous plant effect on the rhizobia evolution of ethylene-modulating genes we searched for rhizobial NodC-containing genomes in the NCBI database. A total of 323 NodC-containing genomes belonging to five major rhizobial groups, *Bradyrhizobium*, (n=**58)** *Mesorhizobium* (n=**91**), *Rhizobium* (n=**100)**, *Sinorhizobium/Ensifer* (n=**62**) and *Paraburkholderia* (n=**12**) were identified in the NCBI database (21/07/2017). These rhizobia were isolated from several countries and from a wide range of leguminous plant hosts (**Table S1**).

The phylogram based on NodC sequences (**Figure 1**) shows a grouping that is mostly independent of the rhizobial species. Moreover, the NodC-based phylogeny is mostly incongruent compared to the obtained RecA-based phylogeny (**Fig. S1**), further suggesting that NodC mainly evolved trough horizontal gene transfer (HGT) events, or alternatively, presents a differential evolutionary rate compared to the housekeeping RecA protein.

HGT events seem to be increased in *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* when compared to *Bradyrhizobium* and *Paraburkholderia* strains. While *Rhizobium*, *Sinorhizobium and Mesorhizobium* are dispersed throughout the NodC phylogram, *Bradyrhizobium* and *Paraburkholderia* are found clustered together in a sister clade (**Figure 1, Clade 1**).

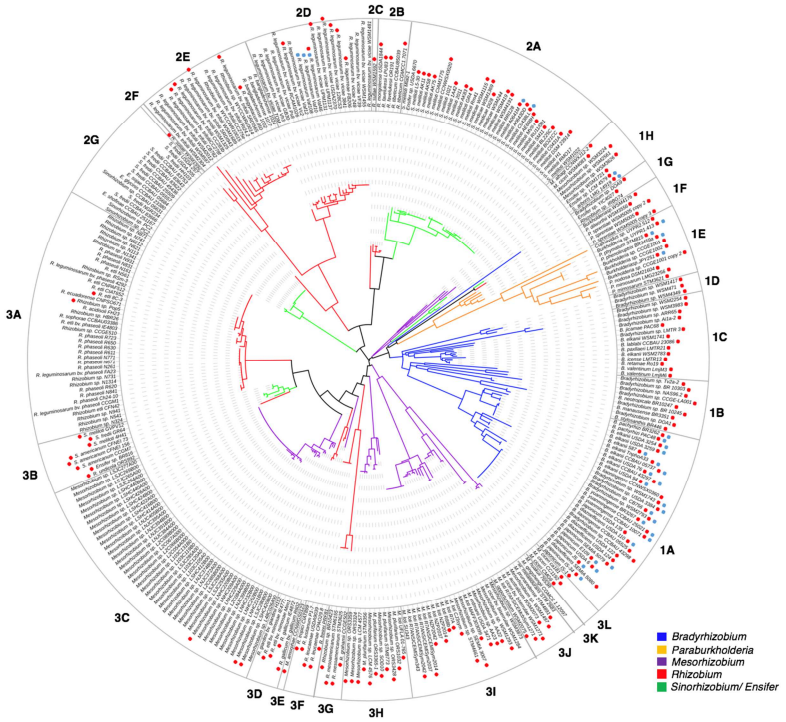

Figure 1- Phylogram based on NodC sequences from rhizobia. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (-17843.7402) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8120)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 25.5052% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Red dots indicate the presence of AcdS in the strain. Blue dots indicate the presence of RtxC in the strain.

Interestingly, NodC from *Rhizobium* and *Sinorhizobium* strains form different clusters scattered in the tree (e.g. Clade 2 vs. Clade 3) and this appears to be related to the strains' hosts and origin. Most of the strains from clade 2 were isolated from *Medicago*, *Vicia* and *Trifolium* in Europe and Asia (**Table 2; Table S1**), while strains from clade 3 were isolated from *Phaseolus*, *Acacia* and *Mimosa* from North and South America (**Table 2; Table S1**).

Table 2- Prevalence of ACC deaminase (AcdS) and dihydrorhizobitoxine desaturase (RtxC) in completely sequenced NodC-containing rhizobial genomes belonging to the different NodC groups.

NodC Group	Strains	Main hosts	AcdS/ NodC	RtxC/ NodC
1A	*Bradyrhizobium spp.*	*Glycine max*	**31/31**	**23/31**
1B	*Bradyrhizobium spp.*	*Lupinus, Phaseolus, Aeschynomene, Vigna, Centrolobium*	**9/9**	**0/9**
1C	*Bradyrhizobium spp.*	*Lupinus, Retamae*	**14/14**	**0/14**
1D	*Bradyrhizobium spp.*	*Sesbania*	**3/3**	**0/3**
1E	*Paraburkholderia spp.*	*Mimosa, Parapitadenia*	**10/10**	**4/10**
1F	*Paraburkholderia spp.*	*Lebeckia*	**3/3**	**0/3**
1G	*Ensifer* *Rhizobium* *Bradyrhizobium*	*Sesbania*	**2/3**	**0/3**
1H	*Mesorhizobium spp.* *Ensifer spp.*	*Lessertia, Alhagi, Prosopis, Indigofera*	**7/8**	**2/8**
2A	*Sinorhizobium spp.*	*Medicago, Phaseolus*	**22/35**	**2/35**
2B	*Rhizobium spp.*	*Medicago*	**4/5**	**0/5**
2C	*Rhizobium sullae*	*Hedysarum*	**1/1**	**0/5**
2D	*R. leguminosarum bv. viciae*	*Vicia, Pisum, Lens, Vavilovia*	**7/20**	**3/20**
2E	*R. leguminosarum bv. trifolii* *Rhizobium spp.*	*Trifolium*	**5/18**	**0/18**
2F	*Rhizobium sullae*	*Hedysarum*	**1/1**	**0/1**
2G	*Sinorhizobium fredii* *Sinorhizobium spp.*	*Glycine max, Lablab, broad host range*	**0/17**	**0/1**
3A	*Rhizobium spp.*	*Phaseolus vulgaris*	**2/40**	**0/40**
3B	*Sinorhizobium spp.* *Rhizobium undicola.*	*Phaseolus, Acacia, Neptunia*	**8/8**	**0/8**
3C	*Mesorhizobium spp.*	*Acmispon*	**3/42**	**0/42**
3D	*Rhizobium spp.*	*Phaseolus, Mimosa*	**4/5**	**0/5**
3E	*M. amorphae*	*Robinia*	**1/1**	**0/1**
3F	*Rhizobium spp.*	*Phaseolus vulgaris*	**5/5**	**0/5**
3G	*Rhizobium spp.*	*Mimosa, Dalea*	**4/4**	**0/4**
3H	*Mesorhizobium spp.*	*Acacia*	**11/11**	**0/11**
3I	*Mesorhizobium spp.*	*Lotus, Biserula,*	**14/20**	**0/20**
3J	*Mesorhizobium spp.*	*Biserula, Anthyllis*	**6/7**	**0/7**
3K	*Mesorhizobium spp.*	*Astragalus sinicus*	**0/2**	**0/2**
3L	*M. ciceri*	*Cicer arietinum*	**3/3**	**0/3**

AcdS/NodC- Number of AcdS-containing rhizobia/ number of NodC-containing rhizobia from the group. **RtxC/NodC**- Number of RtxC-containing rhizobia/ number of NodC-containing rhizobia from the group.

Based on the clustering and bootstrap values observed in the NodC phylogram and the original isolation host, we created NodC groups (**Figure 1, Table 2**) in which the prevalence of ethylene-modulating genes was studied. Moreover, there was a significant

correlation between the NodC grouping and the original plant host of which rhizobia was isolated.

Prevalence of ET modulation genes in completely sequenced NodC-containing rhizobial genomes

ACC deaminase coding sequences were found in NodC-containing alpha and betaproteobacteria. From 323 rhizobial genomes, 181 (56%) possessed AcdS. Yet, the prevalence of AcdS greatly varied between different bacterial groups. The AcdS was identified in 58 out of 58 (100%) *Bradyrhizobium*; 42 of 91 (46.1%) *Mesorhizobium* strains; in 35 of 100 (35%) *Rhizobium*; 34 of 72 (47.2%) *Sinorhizobium/Ensifer* strains; and 12 of 12 (100%) *Burkholderia/Paraburkholderia* strains. Moreover, there was a significant correlation between the presence of ACC deaminase, the bacterial genus and the NodC group.

The RtxC protein is not so abundantly found amongst NodC+ rhizobia. Its presence is detected in 26 of 58 (44.8%) *Bradyrhizobium*; 3 of 100 (3%) *Rhizobium*; 4 of 72 (5.5%) *Sinorhizobium/Ensifer*; and 4 of 12 (33.3%) *Paraburkholderia* strains. No RtxC is found in *Mesorhizobium*. There was a significant correlation between the presence of RtxC and the NodC group.

Prevalence and phylogenetic analysis of AcdS and RtxC in *Bradyrhizobium* spp.

AcdS is found in all NodC$^+$ *Bradyrhizobium* spp. (**Figure 1, Table 2**). The *Bradyrhizobium* spp. RecA-based phylogeny (**Fig. S2**) and AcdS-based phylogeny (**Fig. S3**) show a similar grouping where bacteria from the same species cluster together, indicating that *acdS* is mostly vertically transmitted in *Bradyrhizobia*. Moreover, strains belonging to different NodC clusters (**Fig. S4**) group together in the AcdS phylogram (**Fig. S3**), indicating that AcdS evolution in *Bradyrhizobium* is independent of that of NodC. This is consistent with the fact that no statistical correlation was found between the presence of AcdS and the NodC group in *Bradyrhizobium* spp. Additionally, evolutionary distance calculations show an increased divergence of NodC sequences (**d=0.264±0.018**) compared to AcdS (**d=0.088±0.011**). This is consistent with the fact that in *Bradyrhizobium* spp. the *nodC* gene is found in a symbiotic island, while *acdS* is present in a chromosomally stable region away from the symbiotic island region.

RtxC is found in 26 of 58 NodC$^+$ *Bradyrhizobia*, however, it is more abundant in strains belonging to the NodC cluster 1A (*G. max* symbionts) (**Figure 1, Table 2**). In fact, a statistically significant correlation between the presence of RtxC in *Bradyrhizobia* and the NodC group/plant host genus was found.

The *Bradyrhizobia* RtxC-based phylogram (**Fig. S5**) shows a grouping similar to that of NodC (**Fig. S6**), and is incongruent with a species based phylogeny, suggesting that the *rtxC* gene is transmitted between *Bradyrhizobium* strains together with the *nodC* gene. Moreover, both *nodC* and *rtxC* genes are found in the symbiotic island in *Bradyrhizobia*. Still, evolutionary distances differ between NodC (**d=0.196±0.016**) and RtxC (**d=0.092±0.011**), suggesting a latter introduction of *rtxC* into the symbiotic island, and, consequently, a less pronounced divergence. The presence of transposase genes in the direct vicinity of the *rtx* operon in *B. diazoefficiens* USDA 110 and other *Bradyrhizobia* reinforce this idea. Curiously, the *rtxC* gene is not found in non-symbiotic *Bradyrhizobia*, thus, indicating that this is a nodulation specific gene that only evolved in some $NodC^+$ strains that are mainly able to nodulate *G. max*.

Prevalence and phylogenetic analysis of AcdS and RtxC in *Mesorhizobium* spp.

Even though AcdS is well distributed in *Mesorhizobium* strains belonging to different NodC clusters, its presence is increased in *Mesorhizobium* spp. belonging to NodC clusters 3H, 3I, 3J and 3L (**Figure 1**; **Table 2**). Moreover, a statistically significant correlation between the presence of AcdS in *Mesorhizobium* spp. and the NodC group/plant host genus was found.

The *Mesorhizobium* spp. NodC (**Fig. S7)** and AcdS phylograms (**Fig. S8**) show that AcdS and NodC present a similar evolutionary history that is mostly independent of that of the housekeeping RecA protein (**Fig. S9**). This is consistent with presence of the *acdS* and *nodC* genes in the *Mesorhizobium* spp. symbiotic island, that is readily transmissible between different *Mesorhizobium* strains.

Although *nodC* and *acdS* tend to be inherited together, analysis of the evolutionary distances of NodC and AcdS indicate a different evolution for these genes. NodC sequences are more divergent (**d=0.251±0.020**) compared to AcdS (**d=0.137±0.013**). These results suggest that, even though both genes are dispersed together in a recent evolutionary history, the acquisition of *acdS* and its integration near the symbiotic genes appears to have occurred after *nodC* acquisition. Hence, the *acdS* gene seems to have been subjected to positive selection in some but not all *Mesorhizobium* strains. Alternatively, if these genes were inherited together in *Mesorhizobium* strains, then they evolved differently.

Importantly, some *Mesorhizobium* strains contain the *acdS* gene on a plasmid and not in the symbiotic island. This is the case of

Mesorhizobia in clade 1H (**Figure 1, Table 1**) nodulating *Alhagi* and *Prosopis* hosts. For example, *M. amorphae* CCNWGS0123 carries the *acdS* gene on the plasmid pM0123d. This can explain the presence of some incongruences between AcdS and NodC-based phylogenies. Analysis of the phylogram based on all rhizobia AcdS sequences (**Figure 2**), indicates that some *Mesorhizobum* strains (e.g. *M. alhagi* CCNWXJ12-2) possess an AcdS like that of *Bradyrhizobium* spp.*,* which clusters separately from the *Mesorhizobium* AcdS cluster (**Figure 2**)*,* suggesting increased HGT events between *Mesorhizobium* and *Bradyrhizobium* strains.

RtxC is not found in any *Mesorhizobium* strain, consistent with the possibility of a negative selection for this trait in the *Mesorhizobium* genus.

Prevalence and phylogenetic analysis of AcdS and RtxC in *Rhizobium* spp.

The AcdS is found in 35 of 100 NodC$^+$ strains, however, its presence is increased in specific NodC clusters 2D, 2E, 3D, 3F and 3G (**Figure 1, Table 2**), and decreased in cluster 3A (**Figure 1, Table 2**). Furthermore, two strains from NodC cluster 3G, (*R. mesoamericanum* STM6155 and *Rhizobium* sp. BR10423) possess two copies of the *acdS* gene. These strains are known to associate with *Mimosa pudica*. Interestingly, most *Paraburkholderia* species associating with *Mimosa* species also possess two *acdS* gene copies.

The *Rhizobium* spp. AcdS-based phylogeny (**Fig. S10**) indicates a clustering similar to that of NodC (**Fig S11**) and different from that of RecA (**Fig. S12**). The results obtained indicate that for most *Rhizobium* strains similar AcdS and NodC evolution occurs. In addition, a statistically significant correlation between the presence of AcdS in *Rhizobium* spp. and the NodC group/plant host genus was found. These results are consistent with the fact that both NodC and AcdS show similar evolutionary distances (**d=0.283±0.023 vs d=0.281±0.025**) and their genes are plasmid located in *Rhizobium* spp., hence, being transmitted at the same time. Nevertheless, in some cases AcdS evolution may be independent of NodC. This likely reflects the fact that in some strains the *acdS* gene is present on a different plasmid from the one containing the *nodC* genes, thereby accounting for a different evolutionary history. For example, in *R. etli* 8C-3 (NodC cluster 3A) the *acdS* gene is found on the plasmid pRsp8C3c, while the *nodC* gene is found on the plasmid pRsp8C3b.

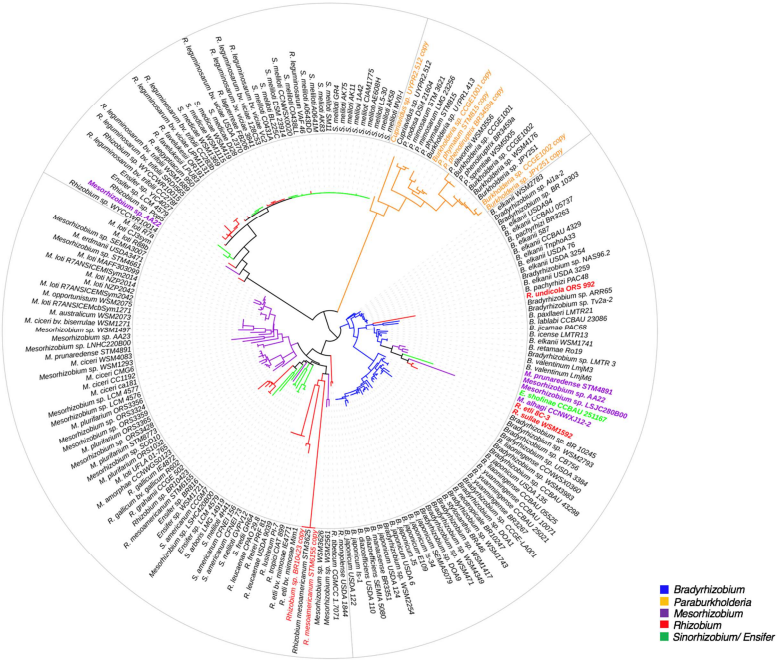

Figure 2- Phylogram based on AcdS sequences from rhizobia. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 1993). The tree with the highest log likelihood (-8579.5500) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4691)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.
Colored strain names indicate gene duplication events or gene acquisition by horizontal gene transfer.

Interestingly, analysis of the phylogram based on all rhizobia AcdS sequences (**Figure 2**) indicates that *R. etli* 8C-3 as well as *R. sullae* WSM1592 and *R. undicola* ORS992 possess an AcdS similar to that of *Bradyrhizobium* spp.*,* further suggesting HGT events between these strains.

RtxC is not commonly found in *Rhizobium* spp. Its presence is exclusive to three *R. leguminosarum* strains (Vaf10, Vaf46 and Vaf108), which have been isolated from *Vavilovia formosa* in Russia (**Figure 1, Table 2**). Curiously, *rtxC* genes are plasmid located in

these strains, suggesting an acquisition through HGT. In this regard, the RtxC from *Rhizobium* spp. share ~68% identity to the RtxC from *B. diazoefficiens* USDA 110. Analysis of the RtxC phylogeny of all rhizobia (**Figure 3**) shows that *Rhizobium* spp. RtxC are found clustered away from RtxC from other rhizobia such as *Bradyrhizobium* spp. indicating that the acquisition of RtxC is not recent.

Prevalence and phylogenetic analysis of AcdS and RtxC in *Sinorhizobium* spp.

The AcdS presence is increased in *Sinorhizobium* strains belonging to the NodC clusters 2A and 3B (**Figure 1, Table 2**). The *Sinorhizobium* spp. AcdS phylogram (**Fig. S13**) indicates a grouping that is mostly congruent to that of NodC (**Fig. S14**) and different from that of RecA (**Fig. S15**). This suggests that the evolution of AcdS is coupled to that of NodC in *Sinorhizobium/Ensifer* spp. Moreover, both NodC and AcdS show similar evolutionary distances (**d=0.199±0.016 vs. d=0.194±0.021**), which is consistent with the presence of both *nodC* and *acdS* genes in *Sinorhizobium* spp. plasmids. These results suggest that both *nodC* and *acdS* were acquired together and are transmitted via symbiotic plasmids.

RtxC is found in only four *Sinorhizobium* spp. strains, two of them belonging to the NodC cluster 1H and, and the others to cluster 2A (**Figure 1, Table 2**). *Ensifer* sp. LCM4579 and *S. arboris* LMG14919 from cluster 2A were both isolated from *Prosopis* plant hosts in Senegal and Sudan, respectively, while *S. meliloti* strains A0641M and C0438LL from cluster 2A were isolated from *Medicago sativa* cv. Oneida in Italy. The RtxC from *Sinorhizobium/Ensifer* spp. shares ~65% identity to the RtxC from *B. diazoefficiens* USDA 110.

Unfortunately, there is no information about the localization of the *rtxC* genes in these strains, mostly because the genomes are in a contig (fragmented) format. However, gene organization analysis (using *S. arboris* LMG14919 Scaffold3.5) showed the presence of *rtxC* and *nodC* genes in the same contig in *S. arboris* LMG14919, thus, suggesting that these genes are located in the same replicon and inherited together.

Analysis of the RtxC-based phylogeny of all rhizobia (**Figure 3**) shows that *Sinorhizobium* spp. RtxC is found clustering together with *Rhizobium* spp. RtxC, however, in distinct clades away from RtxC from *Bradyrhizobium* spp. and *Paraburkholderia* spp. indicating that the acquisition of RtxC by these strains is not recent.

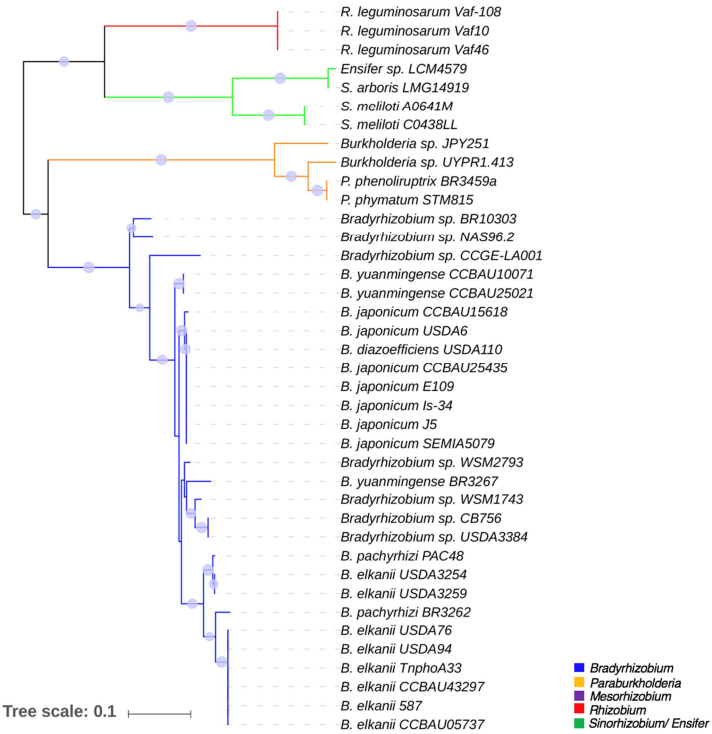

Figure 3- Phylogram based on RtxC sequences from rhizobia. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-3709.3704) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8660)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 339 positions in the final dataset.

Prevalence and phylogenetic analysis of AcdS and RtxC in *Paraburkholderia* spp.

The *Paraburkholderia* spp. AcdS-based phylogeny (**Fig. S16**) shows that AcdS evolution in these strains is similar to that of RecA (**Fig. S17**) and mostly independent of that of NodC (**Fig. S18**). These results indicate that in *Paraburkholderia* spp., AcdS mostly evolved

vertically. The divergence rate between *Paraburkholderia* spp. NodC and AcdS sequences (**d=0.235±0.018 vs. d=0.08±0.011**) indicates that AcdS was conserved to a greater extent, and, suggests the presence of this gene in a *Paraburkholderia* spp. ancestor. This is consistent with the presence of an *acdS* gene in most completely sequenced non-symbiotic *Paraburkholderia* spp. and *Burkholderia* spp. (Nascimento et al. 2014). Moreover, the *acdS* gene is found in the second chromosome of all *Paraburkholderia* spp. and *Burkholderia spp.,* while the *nodC* gene is located on a symbiotic plasmid. Interestingly of eleven strains belonging to the NodC cluster 1E (mostly nodulating *Mimosa* spp.) (**Figure 1; Table 2**). eight strains possess two AcdS sequences, while from cluster 1F (nodulating *Lebeckia*) only one (**Figure 1; Table 2**) of three strains contain two copies of AcdS. Genome analysis indicates that the second *acdS* gene copy of *Paraburkholderia* spp. is present on the symbiotic plasmids. The *acdS* gene present on the symbiotic plasmid is very similar to the chromosomal *acdS* gene, suggesting gene duplication events. This observation is consistent with a strong selection force inducing the duplication and maintenance of *acdS* genes in root-nodulating *Paraburkholderia* spp.

The *rtxC* gene is found in four strains from cluster 1E: *Burkholderia* sp. JPY251 (isolated from *Mimosa velloziana*, Brazil), *P. phenoliruptrix* BR3459a (from *Mimosa flocculosa*, Brazil), *P. phymatum* STM815 (from *Machaerium lunatum*, French Guiana) and *Burkholderia* sp. UYPR1.413 (from *Parapiptadenia rigida*, Uruguay). Genomic searches indicate that the *rtxC* gene is present on the symbiotic plasmid of *P. phymatum* STM815 (pBPHY02) and *P. phenoliruptrix* BR3459a (pSYMBR3459) near the nodulation and nitrogen fixation genes and a second copy of the *acdS* gene indicating that in this instance *nodC* and *rtxC* are inherited together. However, the divergence rate between *Paraburkholderia* spp. NodC and RtxC sequences (**d=0.203±0.018 vs d=0.113±0.013**) suggests a latter acquisition of *rtxC* genes. Alternatively, these genes may be inherited together but there is the possibility of a differential evolution. The RtxC from *Paraburkholderia* spp. shares ~66% identity to the RtxC from *B. diazoefficiens* USDA 110.

DISCUSSION

Selective pressure(s) mediate *acdS* and *rtxC* gene acquisitions

Since ACC deaminase is an ancient trait and has been shown to play a positive role in nodulation and plant growth promotion abilities of several bacterial strains (Nascimento et al. 2014; Nascimento et al. 2018), the fact that not all *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* possess *acdS* genes indicates

that its acquisition, and alternatively, loss, is subjected to selective pressure. In addition, RtxC is mostly negatively selected in rhizobia.

Therefore, we hypothesize that several factors, acting independently or in combination, may induce the positive or negative selection of the *acdS* and *rtxC* genes in rhizobia, these are:
a) the biochemistry of each ethylene modulating mechanism (ACC deaminase vs. rhizobitoxine); **b)** the plant host and its ACC levels; **c)** the plant ACC and ethylene sensitivity; **d)** the specificity of the nodulation process and the plant defense response mediated by ethylene; **e)** the genetic background of the rhizobial strain; **f)** the existence of a genetic pool of *acdS* or *rtxC* genes in the rhizosphere/endosphere/nodules.

A) The biochemistry of ACC deaminase vs. rhizobitoxine

ACC deaminase-producing organisms act as a sink, degrading plant extracellular ACC and using it as nitrogen and carbon sources (Glick, 2014). Consequently, the presence of AcdS can potentiate bacterial growth in the root system and the nodule, where ACC concentrations are increased, especially under stress conditions that naturally occur during the plant lifecycle (Glick, 2014). With the reduction of deleterious ethylene levels, the growth of both symbiont and plant host are favored, contributing to a balanced symbiotic process (positive selection). On the other hand, rhizobitoxine acts by directly limiting plant ACC synthase activity (Yasuta et al. 1999), which results in the synthesis of less plant ACC and ethylene, which are regulators of the plant defense and symbiotic mechanisms (Nascimento et al. 2018). Several studies have shown that rhizobitoxine limits plant growth (Okazaki et al. 2007), and leads to an unbalanced symbiotic relationship. For example, Ratcliff and Denison (2009) showed that RTX-producing *Bradyrhizobium* spp. modulate ethylene levels to decrease plant sanctions against accumulation of carbon compounds at the expense of N_2 fixation. This suggests that RtxC is responsible for a 'cheating' behavior and this is consistent with the presence of rhizobitoxine in plant pathogens such as *B. andropogonis*, *Xanthomonas* spp. and *P. syringae*. Thus, a negative selection by the plant host and environmental conditions limits the existence of *rtxC* in most rhizobia.

B) The plant host and its ACC levels

Several pieces of evidence indicate that plant host impacts the prevalence and evolution of ethylene modulation genes in rhizobia, suggesting a selection pressure by co-evolution. Many studies have demonstrated that the presence of plant ACC (mainly induced by persistent stressful conditions) leads to the positive selection of ACC deaminase-producing bacteria, which become more competitive by

their ability to use ACC as a nitrogen source and, consequently, benefit the plant host by reducing deleterious ethylene levels (Nascimento et al. 2018). Hence, different plant hosts with different ACC production abilities may differentially induce the selection of rhizobial ACC deaminase. The plant ACC levels depend on several factors, such as the plant's ability to produce and exude ACC (its genetic background), or the external conditions (e.g. abiotic and biotic stress). Some leguminous plants possess a relatively large number of genetic elements involved in the production of ACC compared to many other plants (Desbrosses and Stougaard, 2011). For example, *G. max* possesses 14 ACC synthase genes while most other leguminous plants possess only 6 ACC synthase genes (**Table S2**). Interestingly, most rhizobia strains that interact with *G. max* possess both ACC deaminase and rhizobitoxine.

Stress conditions are one of the main inducers of ACC and ethylene production in plants (Abeles et al. 1992), therefore the environment (e.g. geographical origin) and the natural plant resistance to stress are key determinative agents regulating plant ACC and ethylene production.

C) Plant ACC and ethylene sensitivity

Leguminous plants evolved to respond to different levels of ACC and ethylene (**Table 1**). Some studies have demonstrated that genetic differences (e.g. the number of ethylene signaling components) impact the ethylene response in different plants (Penmetsa and Cook, 1997; Miyata et al. 2013). This is consistent with the varying number of ethylene signaling components in leguminous plant species (**Table S2**). Again, *G. max* contains many copies of the ethylene signaling components compared to other leguminous plants (**Table S2**). Thus, while most leguminous plants possess 2 to 6 ETR1-like genes, *G. max* contains 16 ETR1-like isoforms (**Table S2**). Similarly, most leguminous plants contain 1 or 2 copies of the EIN2 gene and *G. max* possesses 6 EIN2 gene copies (**Table S2**). Interestingly, *G. max* presents a decreased sensitivity to exogenous ACC and ethylene (**Table 1**) which may be related to its ability to produce more ACC and ethylene compared to other leguminous plants.

D) The plant control of the nodulation process and the plant defense response, mediated by ethylene

Leguminous plants possess different symbiont selection abilities. Some plants are nodulated by a limited number of bacteria containing specific nodulation genes. On the other hand, plants like *Phaseolus vulgaris* or *Vigna unguiculata* are promiscuous and form symbiotic relationships with several rhizobia containing different

nodulation genes. Thus, the activation of the nodulation control mechanisms is disparate between leguminous plants. Therefore, it is conceivable that plant possessing different nodulation control mechanisms possess different abilities to produce nodulation-induced ACC and ethylene. Interestingly, most rhizobial strains co-evolving with *P. vulgaris* do not possess ACC deaminase or rhizobitoxine.

The plant defense mechanism(s) (independent of the nodulation process) that occurs in response to MAMPs, Damage Associated Molecular Patterns (DAMPs) and effectors also induce the production of ACC and ethylene (Nascimento et al. 2018). In this sense, the plant sensitivity to bacterial colonization (resistance to specific MAMPs from specific symbionts) may also limit the extent of plant ACC and ethylene biosynthesis, and the consequent selection of *acdS* and *rtxC* in rhizobial symbionts.

E) The genetic background of the rhizobial strain

Rhizobia possess different abilities to induce plant defense responses, mainly by their genetic differences in terms of MAMPs and NFs production, that act as stimulants or inhibitors of the plant defense response (Newman et al. 2013; Gourion et al. 2015). In addition, some rhizobia may possess other mechanisms to suppress the plant defense response (including ethylene-mediated responses), such as effectors. Some plant pathogens like *P. syringae* and *X. vesicatoria* produce type III effectors which directly modulate plant ethylene responses (Nascimento et al. 2018). Most *S. fredii* and *Sinorhizobium* spp. nodulating *G. max* (cluster 2G), and with a broad host-range, possess active type III secretion systems (T3SS) and produce a myriad of effectors (Jiménez-Guerrero et al. 2015, López-Baena et al. 2016; Ge et al. 2016). Hence, the presence of *acdS* or *rtxC* in strains that already contain other mechanisms to avoid the plant defense response could contribute to a significant decrease of ACC and ethylene, leading to an increase in bacterial colonization.

The genetic background of a rhizobial strain (e.g. the presence of mobile elements and genes involved in genetic material transfer) may also impair the frequency of HGT events, and consequently impact the acquisition of *acdS* or *rtxC*.

F) The existence of a genetic pool of *acdS* or *rtxC* genes in the rhizobial population

Since HGT events significantly impact the evolution of *acdS* and *rtxC*, the existence of a genetic pool of *acdS* or *rtxC* genes in the rhizosphere, endosphere or the root nodule, and their subsequent transmission, impact the presence of *acdS* and *rtxC* in the rhizobial

population. For example, Nandasena et al. (2007) demonstrated that the ability of *M. opportunistum* WSM2073 to nodulate *Biserula pelecinus* occurred as a consequence of the acquisition of a specific symbiotic island (containing the *acdS* gene), when it encountered non-endemic populations of *M. ciceri* bv. *biserrulae*. Hence, the increased presence of organisms that contain *acdS* or *rtxC* in transmissible elements such as plasmids or symbiotic islands may augment the frequency of HGT events and the selection of *acdS* and *rtxC*.

Are ethylene modulation genes relevant to infer the evolutionary history of the rhizobial nodulation process?

Previous studies have suggested that *Bradyrhizobium* spp. and *Paraburkholderia* spp. are precursors of the nodulation process in Proteobacteria (Martinez-Romero et al. 2010; Ormeño-Orrillo et al. 2013; Aoki et al. 2013). Several results obtained in this study support that notion. *Bradyrhizobium* spp. and *Paraburkholderia* spp. NodC sequences cluster closer together, away from most other rhizobia (**Figure 1**). NodC transmission in these genera seems to be more stable and less prone to HGT events when compared to other rhizobia.

Importantly, *acdS* genes involved in the modulation of ethylene, a common regulator of the plant immune defense system and symbiotic nodulation process (Nascimento et al., 2018), are highly prevalent and stably vertically transmitted in *Bradyrhizobium* spp. and *Paraburkholderia* spp. (including non-symbionts), but not in other rhizobia. Moreover, several *Rhizobium* spp., *Mesorhizobium* spp. and *Sinorhizobium* spp. acquired the *acdS* gene from *Bradyrhizobium* spp. (donor) by HGT. The ACC deaminase enzyme is found and is vertically inherited in ancient bacterial classes like Actinobacteria and Deinococcus-Thermus indicating its ancient origin (Nascimento et al. 2014). The presence of AcdS in *Bradyrhizobium* spp. and *Paraburkholderia* spp. likely results from a previous ancestral selection relating to the ability to internally colonize plants and promote plant growth that dates to a time before the appearance of nodulation abilities in these genera (pre-symbiotic stage). *Bradyrhizobium* spp. and *Paraburkholderia* spp. are known to promote plant growth and are commonly found in the endophytic compartment of several non-leguminous plant species (Chaintreuil et al. 2000; Onofre-Lemus et al. 2009). This is consistent with the location of *acdS* in the chromosomes of *Bradyrhizobium* spp. and *Paraburkholderia* spp., away from the nodulation genes which are usually found in mobile elements such as symbiotic islands and plasmids. Curiously, *Bradyrhizobium* spp. and *Paraburkholderia* spp.

present free-living and higher levels of ACC deaminase activity when compared to other rhizobia (Nascimento et al. 2016).

Altogether, these results suggest that the ability to modulate plant ethylene levels (mainly by producing ACC deaminase) played a significant role in the development of a beneficial plant-microbe interaction and, consequently, to the development of a symbiotic interaction.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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RESEARCH MANUSCRIPT

"Improvement of *Cupriavidus taiwanensis* nodulation and plant growth promoting abilities by the expression of an exogenous ACC deaminase gene"

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Improvement of *Cupriavidus taiwanensis* nodulation and plant-growth promoting abilities by the expression of an exogenous ACC deaminase gene

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Keywords: *Cupriavidus*, *Mimosa*, Rhizobia, Ethylene, ACC deaminase, nodulation

ABSTRACT

Several rhizobial strains possess the ability to modulate leguminous plants ethylene levels by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. While the effect of ACC deaminase has been studied in several rhizobia belonging to the Alphaproteobacteria class, not much is understood about its impact in the nodulation abilities of rhizobia belonging to the Betaproteobacteria class, which are common symbionts of Mimosa species.

In this work, we report the impact of ACC deaminase production by the Betaproteobacterium, *Cupriavidus taiwanensis* STM894, and its role in the nodulation of *Mimosa pudica*.

C. taiwanensis STM894 was studied following its transformation with the plasmid pRKACC, containing an ACC deaminase gene. The expression of the exogenous ACC deaminase led to increased nodulation and *M. pudica* growth promotion by *C. taiwanensis* STM894. These results indicate that ACC deaminase plays an important role in modulating ethylene levels that inhibit the nodulation process induced by both rhizobia belonging to the Alpha and Betaproteobacteria class.

INTRODUCTION

The phytohormone ethylene is known for its negative role in the nodulation process of leguminous plants as it inhibits the formation and functioning of root nodules [1]. In higher plants ethylene is produced via 1-aminocyclopronane-1-carboxylate (ACC), trough the action of the enzymes ACC synthase and ACC oxidase [2].

Some rhizobia can degrade the ethylene precursor, ACC, through the production of the enzyme ACC deaminase, and, consequently, decrease deleterious ethylene levels that inhibit the nodulation process [3, 4]. The enzyme ACC deaminase is responsible for the conversion of ACC to ammonia and α-ketobutyrate and is prevalent in many rhizobial strains [5, 6]. Rhizobial ACC deaminase mutant strains (*Rhizobium*, *Mesorhizobium*) present decreased nodulation abilities [3, 4]. On the other hand, rhizobial strains (*Sinorhizobium*, *Mesorhizobium*) expressing an exogenous ACC deaminase gene (*acdS*) can nodulate and promote leguminous plant growth in a greater extent [7–12]. Most of these studies have shown the importance of ACC deaminase in the nodulation process of rhizobia belonging to the Alphaproteobacteria class (hereby termed α-rhizobia), however, little is known about the role of ACC deaminase in the nodulation process of rhizobia belonging to the Betaproteobacteria class (hereby termed β-rhizobia). β-rhizobia mainly consist of *Paraburkholderia* and *Cupriavidus* strains which are frequently found associated with leguminous plants from the *Mimosa* genus [13, 14]. The β-rhizobium *Cupriavidus taiwanensis* LMG19424^T (= STM894) was isolated in Taiwan from the root nodules of *Mimosa pudica* [15] and its genome has been sequenced [16]. No ACC deaminase genes have been detected in the *C. taiwanensis* STM894 genome. The aim of this study is to assess the effect of an exogenous ACC deaminase gene in the nodulation and overall plant-growth promoting abilities of *C. taiwanensis* STM894, thus, gaining further insight into the role of ACC deaminase in β-rhizobia.

MATERIALS AND METHODS

Transformation by triparental conjugation

C. taiwanensis STM894 (STM culture collection: Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France) is a synonym of LMG19424^T and was kindly provided by Dr. Lionel Moulin (Institut de Recherche pour le Developpement, France). The bacterium was cultured in Tryptic Soy Agar (TSA) (Kasvi, Brazil) and maintained in our laboratory.

C. taiwanensis STM894 was transformed by triparental mating with plasmid pRKACC containing the *acdS* gene of *Pseudomonas* sp. UW4 cloned in pRK415 [17] as described previously [9]. *Escherichia coli* strains DH5α (pRKACC) and MT616 (pRK600) were used as donor and helper strains respectively.

C. taiwanensis STM894 was inoculated in the center of a TSA plate which was incubated for 2 days at 28°C. A loop of each of the *E. coli* strain cultures (previously grown in TSA at 37ºC) was added to the *C. taiwanensis* STM894 plate and the three cultures were gently mixed. After overnight incubation at 28°C, the *C. taiwanensis* transformants were selected based on their ability to grow in TSA medium containing 20 µg/ml tetracycline and their colony characteristics (highly mucoid).

ACC deaminase activity assay

The transformed *C. taiwanensis* STM894-pRKACC and wild-type strains were tested for ACC deaminase activity. *Pseudomonas* sp. UW4 [18] was used as a positive control. C. taiwanensis STM894 strains were grown in TSB (supplemented with 20 µg/ml tetracycline when necessary) for 2–3 days at 28°C. Cells were washed twice with 0.1 M Tris–HCl (pH 8) and then resuspended in Dworkin and Foster minimal medium [19] containing ACC as the sole nitrogen source at a final concentration of 5 mM. Cells were incubated with shaking for approximately 40h at 28°C. After induction, ACC deaminase activity was measured based on the determination of α-ketobutyrate resulting from ACC cleavage by ACC deaminase, as described by Penrose and Glick [20]. Total protein content of cells was quantified using the Bradford reagent (Sigma) according to the manufacturer's protocol. The final ACC deaminase activity was expressed in µmol α-ketobutyrate/mg protein/h.

***Mimosa pudica* nodulation assay**

Bacterial cell culture preparation

Both strains of *C. taiwanensis* STM894 (wild-type and pRKACC) were cultured in TSB medium, and supplemented with 20 *µ*g/mL tetracycline in the case of the transformed strain. After incubation for 2 days, at 28ºC on a rotary shaker with an agitation of 150 r.p.m, cell cultures were centrifuged at 6000 x g and suspended in 0.03M $MgSO_4$. Bacterial cultures were adjusted to an OD_{600} of 0.3 using a spectrophotometer.

Seed surface sterilization and germination

Seeds of *M. pudica* (Royalfleur, France) were surface sterilized with sulfuric acid to break dormancy [13]. The method consisted of submerging seeds in 98% H_2SO_4, for 10 minutes, followed by 10 minutes in a 3% sodium hypochlorite solution, and

ending with 5 washes with sterile distilled water to remove any trace of the previous solutions. After disinfection, seeds were placed on 1% agar plates and incubated in the dark, for 3 days at 25ºC. No bacterial or fungal growth was observed in the agar plates containing the seeds, indicating a successful sterilization process.

Assay conditions

After germination, one seedling was placed per pot (V= 300 ml) that was filled with a sterile mixture of sand and vermiculite (1:1).

The assay consisted of four independent treatments: negative control (without bacteria inoculation and nitrogen supplementation), positive control (without bacteria inoculation and supplemented with nitrogen), inoculation of *C. taiwanensis* STM894 (without nitrogen), and inoculation with *C. taiwanensis* STM894-pRKACC (without nitrogen). The inoculation consisted in the application of 5 mL of the respective bacterial cell culture (OD_{600}=0.3) to the plant root shoot junction. A total of eight seedlings were used per each treatment.

The assay was conducted under greenhouse conditions (average temperatures of 23ºC max. and 14ºC min.), in the Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Plants received 5 mL of a Broughton and Dillworth nutrient solution [21] (supplemented with 0.05% KNO3 in the case of the positive control) whenever necessary.

The experiment lasted for 30 days (after inoculation). Following this period, plants were harvested and parameters including root and shoot dry weight (RDW, SDW), as well as nodule number were evaluated. Roots and shoots were cut separately and dried at 60ºC for 3 days, so that dry weights could be measured on an analytical scale.

Statistical analysis

Statistical analyses were performed with SPSS v.16.0 (SPSS Inc., USA). The data was analyzed by one-way analysis of variance (ANOVA) and means were compared by the Tukey's test.

RESULTS

ACC deaminase activity of the transformed strain

C. taiwanensis STM894 incorporated the pRKACC plasmid and consequently expressed the ACC deaminase enzyme, transforming ACC into ammonia and α-ketobutyrate. The total enzymatic activity of the transformed bacterium was 7.5 *μ*mol α-ketobutyrate/mg protein/h. The ACC deaminase enzymatic activity of the transformant was slightly lower than the activity displayed by wild-type *Pseudomonas* sp. UW4 (i.e., 12.2 *μ*mol α-ketobutyrate/mg protein/h).

The expression of an exogenous ACC deaminase increased *C. taiwanensis* STM894 nodulation and plant-growth-promoting abilities

The role of the exogenous ACC deaminase in affecting the nodulation and plant growth promoting abilities of *C. taiwanensis* STM894 was studied. There was a significant increase (125%) in the number of nodules formed by the strain *C. taiwanensis* STM894-pRKACC (average of 14 nodules formed per plant) compared to the wild-type strain (average of 6 nodules formed per plant) (**Figure 1A**) indicating a positive role for ACC deaminase in the nodulation ability of *C. taiwanensis* STM894. No nodules were found in the non-inoculated plants (negative and positive controls).

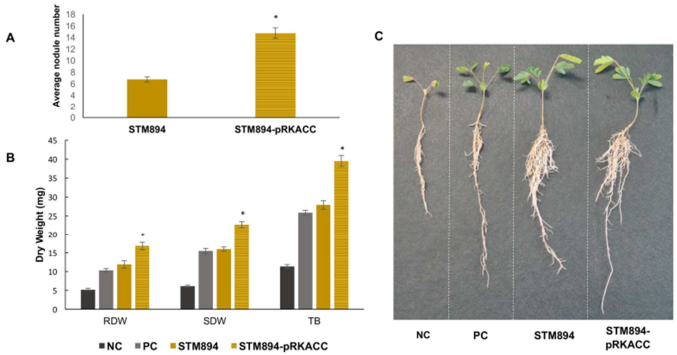

Figure 1- Results obtained from the *Mimosa pudica* assay, 30 days after inoculation.
A) Average nodule number in *M. pudica* inoculated with *Cupriavidus taiwanensis* STM894 and *Cupriavidus taiwanensis* STM894-pRKACC. **B**) Average results per plant of root dry weight (RDW), shoot dry weight (SDW), and total biomass (TB). **C**) Representative *M. pudica* plants from each treatment. **NC** – Negative Control; **PC** – Positive Control; **STM894**- plants inoculated with *Cupriavidus taiwanensis* STM894; **STM894-pRKACC**- plants inoculated with *Cupriavidus taiwanensis* STM894-pRKACC.
* represents significant statistical differences ($p<0.05$).

Plants inoculated with the wild-type strain STM894 presented a significant increase in RDW, SDW and, consequently, total biomass (TB) when compared to non-inoculated plants (negative control) (**Figure 1B, C**). A similar growth and development (RDW, SDW and TB) of both positive control (supplemented with nitrogen) and STM894 inoculated plants was observed (**Figure 1B, C**) indicating the nitrogen-fixation and overall plant-growth promoting abilities of the strain STM894. While the wild-type STM894 strain

presented significant nodulation and plant growth promotion abilities, the expression of an exogenous ACC deaminase led to an increased symbiotic and plant-growth promotion potential by strain STM894-pRKACC. The RDW, SDW and TB of plants inoculated with STM894-pRKACC was significantly higher (by approximately 42%) when compared to the plants inoculated with the wild-type STM894 strain (**Figure 1B, C**).

DISCUSSION

In this work, the β-rhizobium *C. taiwanensis* STM894 was successfully transformed with the pRKACC plasmid that conferred the ability to use ACC as a sole nitrogen source. The ACC deaminase activity presented by *C. taiwanensis* STM894-pRKACC can be considered high and within the range of those presented by free-living bacteria [22].

By expressing an exogenous ACC deaminase gene, *C. taiwanensis* STM894-pRKACC increased its ability to nodulate (by 121%) and promoted *M. pudica* growth (by 42%). Previous studies, mainly using α-rhizobia, demonstrated the important role of ACC deaminase in the nodulation process of several plants. For instance, Ma et al. [7] showed that *Sinorhizobium meliloti* Rm1021, transformed to express an exogenous ACC deaminase gene, increased its ability to nodulate *Medicago sativa* plants in 40% when compared to its wild-type form. Furthermore, Nascimento et al. [9] demonstrated that *Mesorhizobium ciceri* LMS-1-pRKACC, expressing an exogenous ACC deaminase, formed more nodules in *Cicer arietinum* plants (by 127%) when compared to its wild-type form. In addition, the expression of the plasmid pRKACC in *S. meliloti* CCNWSX0020 increased its nodulation abilities in *Medicago lupulina* plants [12].

This is the first study reporting the effect of ACC deaminase in the nodulation abilities of a β-rhizobia strain. Interestingly, ACC deaminase genes have been found in the genome of several β-rhizobia, such as *Paraburkholderia* and *Cupriavidus* [5]. In some *Paraburkholderia* strains, two copies of the *acdS* gene are found, suggesting a positive selection of *acdS* genes in these strains [5]. Moreover, ACC deaminase activity was detected in two root-nodulating *Paraburkholderia* (*P. phymatum* STM815, *P. tuberum* STM678) [23].

Altogether, these reports indicate that ACC deaminase plays an important role in modulating ethylene levels that inhibit the nodulation process induced by both α and β-rhizobia. Hence, the selection of ACC deaminase-producing rhizobia is of extreme

importance for the development of rhizobial inoculants with increased plant-growth promotion abilities.

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CHAPTER 4

Characterization and expression of an exogenous ACC deaminase gene in the plant-growth promoting endophyte, Serratia grimesii BXF1

RESEARCH MANUSCRIPT

"Non-specific transient mutualism between the plant parasitic nematode, *Bursaphelenchus xylophilus*, and the opportunistic bacterium *Serratia quinivorans* BXF1, a plant-growth promoting pine endophyte with antagonistic effects"

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Non-specific transient mutualism between the plant parasitic nematode, *Bursaphelenchus xylophilus*, and the opportunistic bacterium *Serratia quinivorans* BXF1, a plant-growth promoting pine endophyte with antagonistic effects

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ORIGINALITY-SIGNIFICANCE STATEMENT

In this work, we characterized *Serratia quinivorans* BXF1 and studied its effects in several organisms that participate in pine wilt disease (PWD) complex, namely, the pinewood nematode (PWN) *Bursaphelenchus xylophilus,* the pine host *Pinus pinaster*, and pine-associated fungi and bacteria. In our view, this is the first study reporting the non-specific and transient mutualism that may occur between plant parasitic nematodes and plant endophytic bacteria in the scale of a complex forest tree disease. Our results suggest that non-specific nematode-bacteria interactions play a significant ecological role. These results may bring new insights not only in the role of bacteria in PWD, but also to the general role of endophytic bacteria in the ecology of nematode-induced plant diseases.

SUMMARY

The aim of this study is to understand the biological role of *Serratia quinivorans* BXF1, a bacterium commonly found associated with *Bursaphelenchus xylophilus,* the plant parasitic nematode responsible for pine wilt disease. Therefore, we studied strain BXF1 effect in pine wilt disease.

We found that strain BXF1 promoted *in vitro* nematode reproduction. Moreover, the presence of bacteria led to the absence of nematode chitinase gene (*Bxcht-1*) expression, suggesting an effect for bacterial chitinase in nematode reproduction. Nevertheless, strain BXF1 was unable to colonize the nematode interior, bind to its cuticle with high affinity or protect the nematode from xenobiotic stress. Interestingly, strain BXF1 was able to promote tomato and pine plant-growth, as well as to colonize its interior, thus, acting like a plant-growth promoting endophyte. Consequently, strain BXF1 failed to induce wilting symptoms when inoculated in pine shoot artificial incisions. This bacterium also presented strong antagonistic activities against fungi and bacteria isolated from *P. pinaster.*

Our results suggest that *B. xylophilus* does not possess a strict symbiotic community capable of inducing pine wilt disease symptoms as previously hypothesized. We show that bacteria like BXF1, which possess plant-growth promoting and antagonistic effects, may be opportunistically associated with *B. xylophilus*, possibly acquired from the bacterial endophytic community of the host pine.

INTRODUCTION

The pinewood nematode (PWN) *Bursaphelenchus xylophilus,* is responsible for the development of pine wilt disease (PWD), a devastating disease affecting pine forests worldwide (reviewed in Vicente et al. 2012a). Previous studies showed that PWD symptoms and histological changes in the pine host begin to appear before the PWN population increases, which led to the hypothesis of a role for PWN-associated bacteria in the development of PWD (Oku et al., 1980; Mamiya, 1983). Moreover, Oku et al. (1980) proposed that bacteria carried by the PWN could contribute to PWD development through toxin production. Since then, many studies were conducted in order to understand the possible contribution of bacteria in PWD, and bacterial communities associated to the PWN isolated in different countries and from different pine trees were also described (reviewed in Nascimento et al. 2015). Several reports have shown that some PWN-associated bacteria were able to increase nematode reproduction, pathogenicity, and also its resistance to xenobiotics, therefore suggesting a symbiotic relationship between the PWN and its associated bacteria (Kawazu et al. 1996a; Zhao et al., 2003; Zhao and Lin, 2005; Cheng et al., 2013). However, some others reported that bacteria with nematicidal and plant-growth-promotion activities are also found associated with the PWN (Vicente et al., 2012b; Proença et al., 2012; Paiva et al., 2013), thus, challenging the previous hypothesis. At this point it is unclear if the PWN randomly acquires opportunistic bacteria throughout its life cycle inside the host tree and/or inside the insect-vector (*Monochamus* spp.), or if the PWN carries and maintains a strict vertically transmitted associated bacterial community responsible for PWD development.

In Portugal, several studies were conducted in order to characterize bacterial communities associated with the PWN (Proença et al. 2010; Vicente et al. 2011; Roriz et al., 2011). These studies revealed that bacterial communities varied depending on the sampled region (Proença et al. 2010), or from the conditions by which the PWN was obtained (Vicente et al., 2011). In our laboratory, bacteria were isolated from different *B. xylophilus* isolates obtained from various Portuguese regions and distinct sampling sources. We found that both PWN preserved in the lab and PWN directly obtained from the tree (*Pinus pinaster*, “maritime pine”) carried rich bacterial communities where the genus *Serratia* was among the main bacterial representatives in both sampling sources (Vicente et al., 2011). In this sense, the *S. liquefaciens* species group was found to be the main bacterial group associated with lab-

cultured PWN, and were also isolated from the PWN directly obtained from symptomatic *P. pinaster* trees (Vicente et al., 2011).

Here we report the characterization of *S. quinivorans* BXF1, a representative of the major *Serratia* species group found associated with the lab-cultured PWN, and the study of strain BXF1 interactions with the PWN, the pine host *P. pinaster*, and pine-associated fungi and bacteria. Understanding the characteristics of these relationships will bring new insights into plant-nematode-bacteria interactions, as well as the role of PWN-associated bacteria in PWD.

METHODS

Strain BXF1 identification and phenotypic characterization

The BXF1 strain was obtained from the *B. xylophilus* Portuguese isolate Bx7-D, which was originally acquired from a diseased *P. pinaster* tree in Oliveira do Hospital, Coimbra (Portugal) in 2009, and further maintained under laboratory conditions (growing in *Botrytis cinerea*) for several generations (Vicente et al., 2011).

Bacterial DNA was isolated using a Purelink Genomic DNA kit following the manufacturer instructions (Invitrogen, USA). Amplification of the16S rRNA gene was conducted using primers 63F-1389R under the conditions described by Hongoh et al., (2013). The PCR product was purified using the GFX kit (GE Healthcare, UK) and sequenced in Macrogen Inc. (Korea). The 16S rRNA gene sequence was analyzed using Bioedit (Hall et al., 1999) and its phylogenetic analysis was performed using MUSCLE (Edgar, 2004) and PhyML (Guindon and Gascuel, 2003). The jModeltest2 program (Darriba et al., 2012) was used in order to select the best substitution model for the phylogenetic analysis. The 16S rRNA partial sequence was submitted to Genbank (accession number KU234625). Bacterial identification via biochemical profiling was also performed using the Gram-negative identification card in the Vitek 2 system (Biomerieux) following the manufacturer's instructions. The biochemical identification was performed twice.

For all the experiments, strain BXF1 was overnight cultured in TSB medium at 28ºC and the trials repeated twice, unless otherwise stated. Indole-3-acetic acid (IAA), acetoin, ammonia, siderophore production, protease, chitinase, cellulase and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, phosphate solubilization and antibiotics resistance were tested following the methodologies described by Barry and Feeney (1969), Vicente et al., (2012b) and Rashid et al., (2012). Bacterial resistance to (+)-α-pinene, (-)-α-pinene, α-pinene (isomer mix), (+)-β-pinene, (-)-β-pinene, (+)-3-carene, 3-carene (isomer mix), R-(+)-limonene, p-cymene, mircene, 2-undecanone, citral, carvacrol, eugenol, geraniol, γ-terpinene,

linalool, xylol, toluene, phenol, benzoic acid (BA) and phenylacetic acid (PAA) was tested. The ability of BXF1 to resist each compound was tested by inoculating 20 µl of BXF1 culture in 5 ml TSB containing each of the pure compounds in the desired final concentration (0.1, 0.5, 1, 1.5% v/v). The bacterial ability to use these compounds as sole carbon source was tested by inoculating 20 µl of the BXF1 culture in filter sterilized M9 salts minimal medium (without glucose). After inoculation, the tubes were incubated at 28ºC for 5 days.

Obtaining strain BXF1 expressing the Green Fluorescence Protein

Strain BXF1 was transformed by the triparental conjugation method with the plasmid p519ngfp (Matthysse et al., 1996), containing the green fluorescence protein (GFP) by the methodology described by Nascimento et al., (2011). Briefly, overnight grown *E. coli* donor (p519ngfp) and helper (pRK600) strains were added to a culture of the recipient strain BXF1, and incubated at 28ºC. Strain BXF1 transformants were selected by its ability to grow in TSA containing 200 µg/ml kanamycin. Bacterial identity was further confirmed by 16S rDNA sequencing as described above. The strain BXF1-gfp was used in PWN attachment and plant endophytic colonization assays described below.

Screening for strain BXF1 fungal and bacterial antagonistic abilities

Antagonistic abilities of strain BXF1 were tested against *B. cinerea* and three other fungal species/strains previously obtained from diseased *P. pinaster* trees, namely, *Penicillium* sp., *Trichoderma* sp. and *Ophiostoma* sp. (Inácio et al., unpublished results).

Erlenmeyer flasks were filled with 10g of barley seeds, 10 ml distilled water and autoclaved at 120ºC for 20 min. Strain BXF1 was grown in King's B medium at 28ºC. Following overnight growth, the bacterial culture was centrifuged 2:30 min at 7800 rpm and the pellet was suspended in 0.03M $MgSO_4$ until reaching a 0.5 OD (600 nm). The flasks were inoculated with a 0.5 x 0.5 cm piece of the fungal culture previously grown on PDA for 7 days and with 2 ml of BXF1 bacterial suspension. The control consisted of fungal culture inoculation and the addition of 2 ml 0.03 M $MgSO_4$. The flasks were incubated in the dark at 25ºC for 7 days. There were five replicates for each treatment.

The *S. quinivorans* BXF1 ability to inhibit the growth of some bacterial strains obtained from Portuguese *P. pinaster* trees and from *B. xylophilus* was determined by performing an overlay test. Bacteria

were grown overnight in TSB medium at 28ºC, and 100 µl of each bacterium to be tested was incorporated in 50 ml LB. The plates were spot-inoculated (4x) with 10 µl of BXF1 suspension and incubated at 28ºC for 3 days. Antagonistic activity was considered positive by the presence of a halo around colonies. The bacterial species tested were *Chryseobacterium indologenes* 13C27, *Pseudomonas chlororaphis* 13C33, *Enterobacter* sp. 13C26 (isolated from dying *P. pinaster* trees) and *S. marcescens* PWN-146 (isolated from *B. xylophilus*; Vicente et al. 2011).

PWN preparation and disinfection

The Portuguese PWN isolate HF (Barbosa et al., 2010) was used in all the experiments performed in this study. The PWN was reproduced in Erlenmeyer flasks as described by Nascimento et al. (2013). After extraction, nematodes were washed with 3% hydrogen peroxide for 10 min (Han et al., 2003) followed by washes in sterilized 0.03M $MgSO_4$ to remove the hydrogen peroxide residue. The nematodes (mixed life stages) were finally re-suspended in 0.03M $MgSO_4$ until obtained the desired nematode concentration to be used in each posterior individual experiment.

Strain BXF1 nematicidal activity and effect on PWN resistance to xenobiotics

Nematicidal activity was determined by adding 100 µl of the PWN solution (approx. 100 nematodes) to 100 µl of bacteria solution in a 96-well sterile microplate. Two treatments were performed: Control (100 µl PWN solution + 100 µl 0.03M $MgSO_4$) and PWN+BXF1 OD_{600} 0.25 (100 µl PWN solution + 100 µl 0.5 OD_{600} bacterial solution). Five replicates (5 wells per treatment) were performed for each treatment. A similar experiment was conducted in order to test the effect of strain BXF1 in PWN xenobiotic resistance. For this assay 2-undecanone (final concentration of 1% v/v) and BA (final concentration of 0.1 mg/ml) were added individually to a suspension containing the PWN or PWN+ bacteria as described above. The sterile microplates were incubated at room temperature and nematicidal activity/mortality was determined after 24 and 48 hours as described by Barbosa et al., (2010).

Strain BXF1 attachment to the PWN cuticle

Bacterial ability to attach to the PWN was tested as described in the above section (similar to control conditions) using the BXF1-gfp strain. Five replicates were performed. After 24 and 48 hours of contact, nematodes were gently rinsed in sterile distilled water 3 times in order to remove non-specific bacteria ligation. The number of bacteria attached to the nematode was observed under a fluorescence microscope.

Strain BXF1 effect on PWN reproduction

Erlenmeyer flasks (100 ml) were filled with 20g of barley seeds, 20 ml distilled water and were autoclaved for at 120ºC for 20 min. The flasks were then inoculated with a 0.5 x 0.5 mm piece of a *B. cinerea* culture. Erlenmeyer flasks containing *B. cinerea* were inoculated with the following treatments: disinfected PWN (1 ml solution containing 1000 nematodes + 1 ml 0.03M $MgSO_4$), non-disinfected PWN (1 ml, 1000 nematodes + 1 ml 0.03M $MgSO_4$), disinfected PWN+BXF1 (1 ml, 1000 nematodes + 1 ml bacteria OD 0.5 in 0.03M $MgSO_4$) and non-disinfected PWN+BXF1 (1 ml, 1000 nematodes mixed + bacteria OD 0.5 in 0.03M $MgSO_4$). Five replicates were performed for each treatment. After inoculation the flasks were incubated at 25ºC in the dark for 10 days. At this time point, nematodes were extracted by the method described by Barbosa et al., (2010) and counted under a stereomicroscope.

Strain BXF1 effect on PWN chitinase gene expression

All PWN chitinases were predicted by a blastP search against non-redundant database. Two putative PWN chitinases were selected, BUX.s00422.469 (*Bxcht-1*) and BUX.s01092.2 (*Bxcht-2*), based on the presence of signal peptide. Nematode RNA was extracted from samples (approx. 10000 nematodes) that correspond to the four different treatments described above (PWN reproduction assay). The extraction was performed using the Qiagen RNA extraction Kit (Qiagen) according to the manufacturer's instructions. RNA was further quantified using a NanoDrop 2000 (ThermoFisher). The cDNA was synthesized from 1 μg gDNA-free total RNA by using the Superscript III reverse transcriptase (ThermoFisher) according to the manufacturer's instructions, and 5 μl (≈150 ng) were further used as template to Reverse-transcription PCR. The primers Bxcht-1F (5'-CTA TCC TTG CTG CTG GAG AG-3'), Bxcht-1R (5'-TTG GGG TCT GGA TAG AAG CC-3'), Bxcht-2F (5'-CGA ACT GGG CAC AAT ATC GT-3') and Bxcht-2R (5'-TAC CAT ACG TTG CCC TGC-3') were designed within the coding region of each gene and used in the amplification reaction. Gene amplification reaction conditions consisted of denaturing at 95°C for 2 minutes followed by 30 cycles of 15 s denaturing at 95°C, 15 s annealing at 55ºC and 30 s extension at 70°C followed by a final extension for 5 minutes at 70ºC. All PCR products were analyzed by gel electrophoresis in 1.2% agarose.

Endophytic colonization and growth promotion abilities of strain BXF1

Tomato (*Solanum lycopersicum* cv. *roma*) seeds were surface disinfected with 70% ethanol (EtOH) during 90 sec, followed by 10

min in 2% sodium hypochlorite (NaClO). Five rinses in sterilized distilled water were performed in order to remove traces of EtOH and NaClO. After disinfection, multiple seeds were immersed in a BXF1 solution (*S. quinivorans* BXF1, 0.25 OD_{600} in 0.03 M $MgSO_4$) or control solution (0.03 M $MgSO_4$) for 1 hour and posteriorly incubated in the dark at 25ºC in 1% water-agar plates until germination. After germination, two plants were sown per each pot containing sterilized commercial substrate SIRO (N, 150–250 mg/l; P2O5, 150–250 mg/l; K, 300–500 mg/l), and received further inoculation: control plants were inoculated with 2 ml sterile TSB solution, and BXF1 treatment received 2 ml of a TSB grown overnight culture. A total of 8 pots per treatment were used and incubated in a growth chamber (Aralab, Portugal) for 30 days under controlled conditions.

Pinus pinaster seedlings (1 year-old), obtained from a nursery in Portugal, were selected by their size and re-sown in unsterilized commercial substrate (2 plants per pot). Two experiments were performed: (1) inoculation of BXF1 in a shoot incision; and (2) BXF1 root inoculation. In the first experiment, pine seedlings (n=8) were inoculated with 1 ml of overnight grown TSB bacterial solution in a small shoot incision as described by Nascimento et al., (2013). Control seedlings (n=8) received 1 ml sterile TSB in the shoot incision. The root inoculation assay consisted in the application of 5 ml of an overnight grown bacterial solution in the root system of the pine seedlings (n=4). Control seedlings (n=4) received 5 ml of sterilized TSB. The plants were incubated in a growth chamber (Aralab, Portugal) for 30 days under controlled conditions. After, all seedlings were collected and parameters such as root and shoot fresh weight were measured, as well as disease symptomatology (quantified as described by Vicente et al. 2012). Statistical analysis was performed using ANOVA analysis in the SPSS statistics v.22 software (IBM)

Strain BXF1 endophytic colonization of tomato

Tomato (*Solanum lycopersicum* cv. roma) seeds were surface disinfected and inoculated with strain BXF1-gfp as described above. After germination, multiple plants were cut in small sections (root and shoot) and visualized in a Leica confocal microscope TCS SP8 STED. To confirm the endophytic behavior of strain BXF1, germinated plants were surface disinfected, washed and the procedure was repeated.

RESULTS AND DISCUSSION

Strain BXF1 phylogeny and taxonomic classification

The 16S rRNA phylogenetic analysis (Fig. S1), shows that strain BXF1 clusters together with *S. quinivorans* DSM 4597,

distantly from *S. proteamaculans* DSM 4543*, S. liquefaciens* ATCC 27592 and *S. grimesii* DSM 30063 type strains. In this sense, strain BXF1 is designated as belonging to the *S. quinivorans* species.

Curiously, based on biochemical properties, Vitek2 analysis identified *S. quinivorans* BXF1 as *S. plymuthica* with high identification scores (above 90%). This result occurred despite the fact that the Vitek2 system is able to efficiently identify other *Serratia* species such as *S. liquefaciens*, *S. grimesii* and *S. proteamaculans* (*S. liquefaciens* group) (Grimont and Grimont, 2006). Similar results have been obtained by Ashelford et al., (2002) indicating the misidentification of *S. quinivorans* species group by biochemical profile identification. These results suggest that different *S. quinivorans* strains may possess different biochemical properties, probably as a result of an increased genome plasticity.

Strain BXF1 phenotypic characterization

S. quinivorans BXF1 is a Gram-negative, rod shaped and non-sporulating bacterium, that forms pale yellow colonies in TSA medium, and is able to grow between 4 and 40ºC. BXF1 uses multiple carbon sources such as D-glucose, D-mannitol, D-mannose, D-trehalose and sucrose (Table S1), produces siderophores, extracellular proteases and chitinase, but not lipase, cellulase, ACC deaminase or other enzymes responsible for phosphate solubilization. This bacterium is also able to produce IAA, acetoin and ammonia.

Strain BXF1 is able to resist to 12 of 17 tested terpenoids (up to 1.5% v/v) (Table S2). No growth inhibition was found in the presence of α- and β-pinene, 3-carene, limonene, mircene, γ-terpinene, p-cymene and 2-undecanone, in all tested concentrations. However, terpenoids like citral, carvacrol, geraniol, linalool, and 3-eugenol inhibited bacterial growth at concentrations above 0.1%. Despite its resistance to various terpenoids, strain BXF1 does not present the ability to use any of these compounds as sole carbon sources. Remarkably, strain BXF1 is also able to grow in the presence of high concentrations of toluene, xylene, BA, PAA and phenol, and uses BA and PAA as sole carbon sources (**Table S2**). BA and PA were found to be linked to PWD development as they are found in abundance in the wilting pine (Oku, 1988; Kawazu et al., 1996 a,b; Zhang et al., 2013). Oku (1988) suggested that these compounds could act as phytotoxins since their application in healthy pine seedlings led to PWD symptoms. Subsequently, Kawazu et al. (1996a) postulated that the PWN pathogenicity was directly related to PAA production of its associated bacteria and considered PA as the major toxin responsible for the development of the PWD. The

fact that *S. quinivorans* BXF1 is able to resist/degrade these compounds suggests that this bacterium is able to live and proliferate in environments possessing high concentrations of these compounds, such as a wilting pine. By using this compounds as sole carbon sources it is possible that strain BXF1 increases its abilities to colonize the infected pine host. Similarly, strain BXF1 is also able to resist to a wide variety of antibiotics (Tetracycline 15 μg/ml; Ampicillin 50 μg/ml; Kanamycin 50 μg/ml; Chloramphenicol 30 μg/ml; Streptomycin 30 μg/ml; Rifampicin 50 μg/ml), hence, suggesting the presence of efficient drug resistance/avoidance mechanisms in its genome. Vicente et al. (2012b) showed that PWN-associated bacteria were highly resistant to antibiotics and suggested that this feature may be important for bacterial fitness and ability to subsist and proliferate inside the pine tree environment.

***S. quinivorans* BXF1 possesses fungal and bacterial antagonistic abilities**

When inoculated in barley seeds, BXF1 presents a strong fungal antagonistic activity, inhibiting colonization and development of *B. cinerea*, *Penicillium* sp., *Trichoderma* sp. and *Ophiostoma* sp. (**Fig.1 A,B,C,D**). This result is consistent with the chitinase activity demonstrated by BXF1. Not only is strain BXF1 able to degrade chitin, but it is also able to degrade N-acetylglucosamine, hence, suggesting that it possesses the ability to fully degrade chitin and use it as a carbon source. Chitinase production by *S. marcescens*, *S. proteamaculans* and *S. plymuthica* have been shown to play an important role in the biological control of phytopathogenic fungi (Frankowski et al., 2001; Mehmood et al., 2009, Wang et al., 2013; Gutierrez-Roman et al., 2014). Pine trees infected with the PWN present decreased defense systems and consequently are more susceptible to fungal infections (Futai et al. 2013). These infections can be deleterious to the tree not only by the fungal action itself but also by the increased PWN feeding and reproduction (mycophagous phase). The fact that strain BXF1 possesses strong antagonistic activities may indicate its role in pine protection against pathogenic fungi.

Anti-bacterial overlay plate tests show that strain BXF1 is able to produce metabolites able to inhibit bacterial growth. The formation of halos around BXF1 colonies was only observed in 2 of the 4 plates containing the tested strains, namely, *C. indologenes* 13C27 and *S. marcescens* PWN146. Growth inhibition was not observed in the presence of *P. chlororaphis* 13C33 or *Enterobacter* sp. 13C26 plates (**Fig. 2**). Interestingly, *C. indologenes* 13C27 was found to be one of the main species obtained directly from diseased *P. pinaster*

trees in the Comporta region (Portugal). This strain is able to produce extracellular proteases, siderophores, cellulase and is also resistant to a wide range of antibiotics. The same phenotype is observed in *S. marcescens* PWN146, which was isolated from *B. xylophilus* directly obtained from diseased *P. pinaster* trees (Vicente et al., 2011; Vicente et al., 2012b). Furthermore, *S. marcescens* PWN146 has been suggested to increase PWN infectivity and present phytotoxic effects to pine seedlings (Vicente et al., 2012b). Our results show that strain BXF1 anti-bacterial activities are only activated by specific bacterial strains, which possess competitive phenotypes. It is possible that strain BXF1 limits the proliferation of phytopathogenic bacteria, thus, limiting the extent of its deleterious actions.

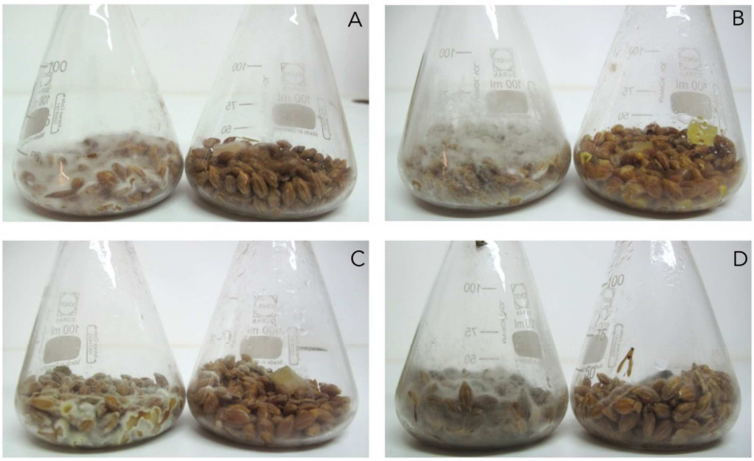

Figure 1- Antagonistic activity of *Serratia quinivorans* BXF1 towards A) *Botrytis cinerea*, B) *Penicillium* sp. C) *Trichoderma* sp. D) *Ophiostoma* sp., 7 days after incubation at 25ºC.

Anti-bacterial overlay plate tests show that strain BXF1 is able to produce metabolites able to inhibit bacterial growth. The formation of halos around BXF1 colonies was only observed in 2 of the 4 plates containing the tested strains, namely, *C. indologenes* 13C27 and *S. marcescens* PWN146. Growth inhibition was not observed in the presence of *P. chlororaphis* 13C33 or *Enterobacter* sp. 13C26 plates (**Fig. 2**). Interestingly, *C. indologenes* 13C27 was found to be one of the main species obtained directly from diseased *P. pinaster* trees in the Comporta region (Portugal). This strain is able to produce extracellular proteases, siderophores, cellulase and is also

resistant to a wide range of antibiotics. The same phenotype is observed in *S. marcescens* PWN146, which was isolated from *B. xylophilus* directly obtained from diseased *P. pinaster* trees (Vicente et al., 2011; Vicente et al., 2012b). Furthermore, *S. marcescens* PWN146 has been suggested to increase PWN infectivity and present phytotoxic effects to pine seedlings (Vicente et al., 2012b). Our results show that strain BXF1 anti-bacterial activities are only activated by specific bacterial strains, which possess competitive phenotypes. It is possible that strain BXF1 limits the proliferation of phytopathogenic bacteria, thus, limiting the extent of its deleterious actions.

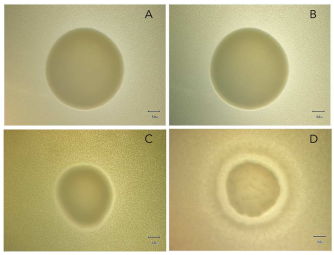

Figure 2- Antagonistic activity of *Serratia quinivorans* BXF1 towards A) *Pseudomonas chlororaphis* 13C33, B) *Enterobacter* sp. C26, C) *Chryseobacterium indologenes* 13C27, D) *Serratia marcescens* PWN-146, 3 days after incubation at 28ºC.

S. quinivorans BXF1 does not present nematicidal activity towards the PWN

The PWN mortality rate was found to be similar between control and BXF1-treated unwashed nematodes (**Table 1**) (2%, 2 dead nematodes/ 100 nematodes), after 24 and 48 hours of contact. Similarly, the disinfected PWN mortality rate was equal between control and BXF1-treated nematodes (8%), after 24 and 48 hours of contact. The disinfected PWN showed an increased mortality when compared to the non-disinfected PWN (8% vs 2%), suggesting a negative effect of the disinfection procedure (Han et al., 2003) in nematode viability. The presence of strain BXF1 did not influence nematode survival under these conditions.

In a recent report, Paiva and colleagues (2013) showed that most bacteria isolated from the PWN, including *S. plymuthica* and *S. marcescens* strains, were in fact able to kill the nematode. The authors suggested that a serine protease is responsible for *Serratia* spp. nematicidal activities. In this study we demonstrate that *S. quinivorans* BXF1 was not able to kill the PWN (under the tested conditions), although it produces extracellular proteases.

Strain BXF1 effect on PWN reproduction

Strain BXF1 promoted PWN reproduction under the tested conditions, and to a greater extent when the nematode suffered from the disinfection procedure (**Table 1**). The positive effect of BXF1 presence in PWN reproduction was found to be higher in the disinfected PWN than in the non-disinfected PWN (10.6-fold increase vs. 6.2-fold increase) treatment, 10 days after inoculation. The disinfected PWN multiplied slowly (1.7-fold increase in population) when compared to the non-disinfected PWN (4.6-fold increase in population), 10 days after inoculation. These results show that the disinfection procedure greatly influences PWN viability as previously suggested (Nascimento et al., 2015). H_2O_2 used in the disinfection procedure is a reactive oxygen species (ROS), which may induce an exaggerated oxidative stress damage to the PWN, thus, limiting its reproduction. Vicente et al. (2013) showed that *Serratia* spp. increased PWN resistance to H_2O_2 induced oxidative stress. This fact may explain the increased reproduction of the disinfected PWN in the presence of strain BXF1. Nevertheless, non-axenic PWN also benefited from the presence of strain BXF1, suggesting that promoting oxidative stress resistance is not the unique mechanism responsible for the BXF1 induced increase in PWN reproduction.

Table 1- Results obtained in the PWN reproduction, xenobiotic resistance (nematode mortality assay) and RT-PCR chitinases (*Bxcht-1* and *Bxcht-2*) expression assays.

Barley seed/*B. cinerea* assay[1]				Mortality assay (%)[2]			Gene expression[3]	
Treatment	**Mean**	**Sum**	**Fold**	**Control**	**BA**	**2-U**	***Bxcht-1***	***Bxcht-2***
Disinfected PWN	1687 ± 700	8433	1.7	8	n.t	n.t	+	+
Disinfected PWN+ BXF1	10642 ± 14203	53211	10.6	8	n.t	n.t	-	+
Non-disinfected PWN	4614 ± 2403	23071	4.6	2	65.4	98.8	-	+
Non-disinfected PWN + BXF1	6278 ± 4472	31390	6.2	2	69.6	100	-	+

[1]Meanindicates the average number of PWN (±, standard deviation); Sum indicates the sum of all nematodes per treatment; and Fold stands for fold-increase in nematode population.
[2]BA, benzoic acid; 2-U, 2-undecanone (n.t) not tested.
[3]Gene expression was determined by RT-PCR. (-) no expression; (+) expression;

Curiously, strain BXF1 presents extracellular chitinase activity and the ability to use it as a carbon source. We hypothesized that strain BXF1 increased the PWN reproduction by degrading the fungal chitin, therefore, increasing the PWN ability to feed on fungal cells. By degrading fungal chitin, the bacterium facilitates chitin acquisition by the PWN, thus, limiting the need of PWN chitinase expression. To test this hypothesis, we analyzed PWN chitinases expression in the presence/absence of strain BXF1 in both disinfected and non-disinfected PWN. We found that *Bxcht-1* was only expressed in the disinfected PWN, but not in any other treatment in the presence of bacteria (strain BXF1 or others occurring naturally) (Table 1, Fig. S2). Qiu et al. (2013) showed that the expression of *Bxcht-1* was down-regulated dramatically when *B. xylophilus* was used to inoculate *P. thunbergii* compared with the PWN culture on *B. cinerea*, suggesting that this chitinase gene is, in fact, responsible for chitin degradation when the nematode feeds on fungi. This result suggests that in the presence of bacteria the PWN does not express the *Bxcht-1* gene, thus, supporting our hypothesis. On the other hand, we found that *Bxcht-2* was expressed in every treatment independently of the absence or presence of bacteria.

Strain BXF1 does not attach to *B. xylophilus* with high affinity

Despite the positive effect in the PWN reproduction, strain BXF1 does not bind to the PWN extensively. After 24 h and 48 h of contact, the number of bacteria attached to the PWN cuticle was very low or even nonexistent (**Fig.3**). Furthermore, bacteria were not found inside the PWN. These results show that strain BXF1 does not bind specifically to the PWN and is not able to colonize its interior. Vicente et al. (2013) described similar results, showing a weak and non-specific adhesion of several *Serratia* spp. to the Japanese *B. xylophilus* Ka4. It seems that the relationship between strain BXF1 and the PWN is not as specific as other nematode-bacteria interactions. For instance, the bacteria *Xenorhabdus nematophila* is found inside the entomopathogenic nematode *Steinernema carpocapsae,* where it stays inside a vesicle until the nematode enters the insect host. Later, the bacterium is released by the nematode into the insect leading to a decrease in the insect immune response, and therefore, its death (Goodrich-Blair, 2007).

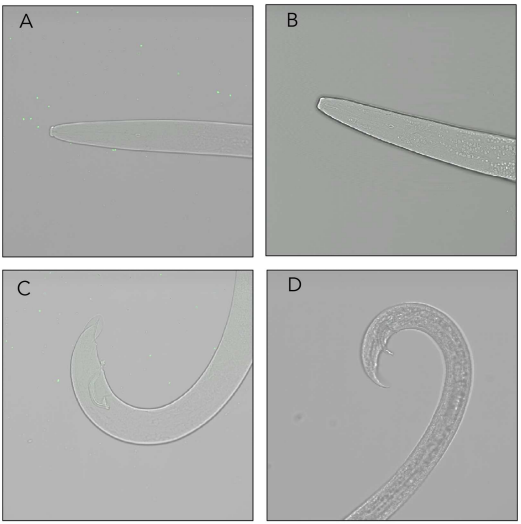

Figure 3- PWN attachment assay. A) PWN+BXF1-gfp (no resuspension after contact), head section; B) PWN+ BXF-gfp (nematode resuspended in distilled water after contact), head section; C) PWN+BXF1-gfp (no resuspension after contact), tail section; D) PWN+BXF1-gfp (nematode resuspended in distilled water after contact), tail section.

S. quinivorans BXF1 effect on PWN xenobiotics resistance

Strain BXF1 was unable to protect the PWN from BA and 2-undecanone stress (**Table 1**). The PWN mortality rate was similar in the absence or presence of strain BXF1. Nevertheless, this bacterium is able to completely degrade BA and resist high 2-undecanone concentrations. Contrary to the hypothesis of Cheng et al. (2013), our results show that PWN-associated bacteria may not generally protect the PWN from xenobiotics, even in the presence of several complete detoxification mechanisms in bacterial genomes. It is possible that these bacteria evolved independently and possess these xenobiotic detoxification mechanisms due to their endophytic nature. For example, Burnes et al. (2000) showed that bacteria able to degrade pine resins are naturally found inside a healthy pine tree.

S. quinivorans BXF1 is an endophyte which promotes tomato and pine growth and does not induce pine wilt disease symptoms

Seed and root inoculation assays show that *S. quinivorans* BXF1 is able to promote tomato plant growth under normal conditions (**Fig.4 A**).

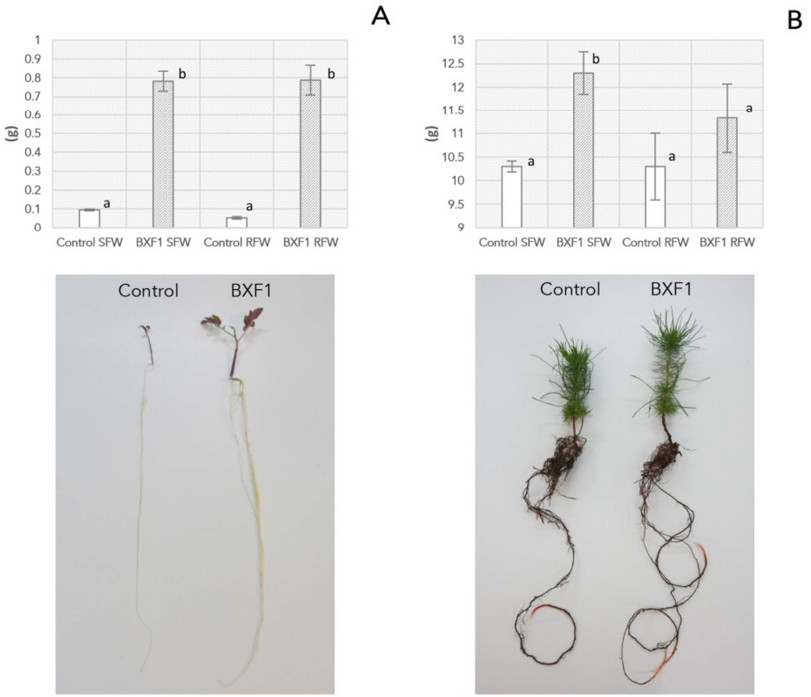

Figure 4- Results obtained from a plant-growth promotion assay in A) *Solanum lycopersicum* cv. roma and B) *Pinus pinaster* seedlings, 30 days after inoculation with *Serratia quinivorans* BXF1. White bars correspond to control and grey bars correspond to BXF1 inoculation results. Different letters correspond to statistical significant differences (P<0.05). RFW-root fresh weight; SFW- shoot fresh weight.

No disease symptoms were found in germinating tomato seedlings (data not shown). Moreover, strain BXF1 increased tomato root and shoot fresh weight by 1386 and 721%, respectively, when compared to control conditions.

Pine root inoculation assay shows that *S. quinivorans* BXF1 promotes pine seedling development. Root and shoot fresh weight was increased in the BXF1 treatment when compared to control conditions (**Fig.4 B**). However, significant statistical differences (P<0.05) were only observed in the shoot fresh weight treatment.

Pine shoot incision and inoculation assay shows that strain BXF1 was unable to induce any disease symptoms (disease incidence of 0) in *P. pinaster* seedlings (**Fig. 5**), even when inoculated in high concentrations in an artificial incision.

Figure 5- Results obtained from *Serratia quinivorans* BXF1 shoot inoculation assay in *P. pinaster,* 30 days after inoculation. Left- Control receiving sterilized TSB; Right- *Serratia. quinivorans* BXF1 inoculation.

Strain BXF1 is able to internally colonize tomato thus, supporting an endophytic behavior. The bacteria are found in the intercellular spaces of the plant cells in relative abundance (**Fig. 6**). BXF1 bacteria were also found inside the root and shoot of pine seedlings (data not shown). Overall, these results show that *S. quinivorans* BXF1 acts as an endophytic plant-growth-promoting bacterium (PGPB) and presents no phytotoxic effects to pine seedlings. The ability to produce siderophores, IAA, acetoin and ammonia may contribute for the plant-growth promoting effect of strain BXF1, which were already been demonstrated to play an important role in bacterial plant-growth promotion (Glick et al., 2014).

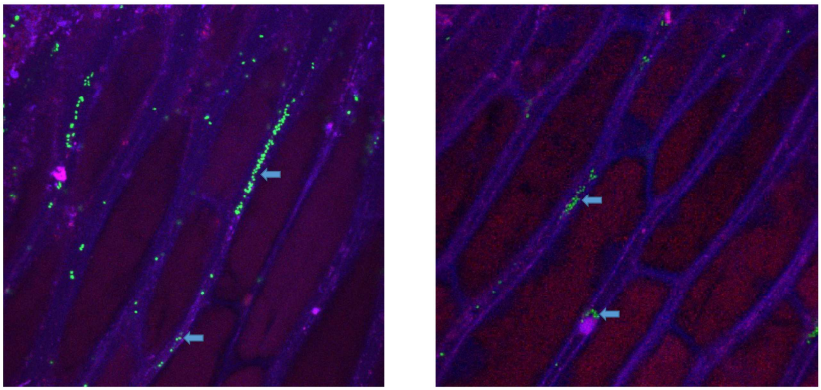

Figure 6- *Serratia quinivorans* BXF1 (gfp) endophytic colonization of tomato plant roots, 4 days after germination. Arrows indicate the presence of strain BXF1 in the plant intercellular spaces.

CONCLUSIONS

Here, we report the characterization of *S. quinivorans* BXF1, a bacterium isolated from the PWN presenting several features related to microbial antagonism and plant-growth promotion. Though found associated with the lab-cultured PWN, this bacterium had no impact in PWN viability. On the other hand, it was able to promote the PWN reproduction *in vitro*. While the PWN population increased, we showed that bacterial presence lead to an absence of the PWN *Bxcht-1* gene expression, which, on the other hand was only expressed in the surface-desinfected PWN, hence, suggesting a role in fungal cell wall degradation (complete chitinase degradation) as a potential mechanism involved in the bacterial ability to promote the PWN reproduction. Nevertheless, strain BXF1 is not able to colonize the PWN interior, attach to the PWN cuticle with high affinity or protect the PWN from xenobiotic stress. BXF1 was able to promote tomato and pine plant-growth, as well as to colonize its interior, thus, acting like a plant-growth promoting endophyte, without causing any disease symptoms. Also, BXF1 presents strong antagonistic activities against pine-associated fungi, and bacteria.

Altogether, our results suggest that the PWN does not carry a strict symbiotic bacterial community responsible for the development of PWD. In this study, we show that the PWN may carry endophytic pine PGPB, which can be acquired by the PWN inside the pine host. Bacteria like BXF1 may be positively selected (inside the pine tree) by its increased xenobiotic resistance (terpenoids) and degradation abilities (BA and PA), as well by its competition and antagonistic

abilities. Consequently, the bacterial opportunistic colonization and mycophagous capabilities may lead to a transient mutualism with the PWN when the latter is cultured on fungi. These results bring new insights into the study of nematode-bacteria interactions, suggesting that non-specific transient mutualistic interactions may occur naturally and depending on the environmental conditions.

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The authors declare that they have no competing interests.

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RESEARCH MANUSCRIPT

"From plants to nematodes: *Serratia grimesii* BXF1 genome reveals an adaptation to the modulation of multi-species interactions"

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The manuscript and the references therein are formatted according to the journal guidelines.

From plants to nematodes: *Serratia grimesii* BXF1 genome reveals an adaptation to the modulation of multi-species interactions

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Keywords: *Serratia,* endophytes, nematodes, pine wilt disease*, Bursaphelenchus xylophilus,* plant-growth-promoting bacteria

ABSTRACT

Serratia grimesii BXF1 is a bacterium with the ability to modulate the development of several eukaryotic hosts. Strain BXF1 was isolated from *Bursaphelenchus xylophilus*, the causative agent of pine wilt disease affecting pine forests worldwide. This bacterium potentiates *B. xylophilus* reproduction, act as a beneficial pine endophyte and possesses fungal and bacterial antagonistic activities, further indicating a complex role in a wide range of trophic relationships.

In this work, we describe and analyze the genome sequence of strain BXF1, and discuss several important aspects of its ecological role. Genome analysis indicates the presence of several genes related to the observed production of antagonistic traits, plant growth regulation, and the modulation of nematode development. Moreover, most of BXF1 genes are involved in environmental and genetic information processing, which is consistent with its ability to sense and colonize several niches.

The results obtained in this study provide the basis to a better understanding of the role and evolution of strain BXF1 as a mediator of interactions between organisms involved in a complex disease system. These results may also bring new insights into general *Serratia* and Enterobacteriaceae evolution towards multitrophic interactions

INTRODUCTION

Serratia species are ubiquitous to different habitats and show versatile niche occupation abilities. *Serratia* are competent soil and water colonizers and can also be found associated to a wide range of different hosts, including plants, insects, nematodes, as well as other eukaryotic organisms [1, 2]. Moreover, species and strain-specific characteristics result in different impacts in the *Serratia* hosts.

Despite much knowledge on the ecology and pathogenesis mechanisms of *S. marcescens,* the most studied bacterium from the *Serratia* genus [3], not much is understood about the ecology, evolution and general impact of other *Serratia* species.

Previous studies demonstrated that, in Portugal, *Serratia* spp. were one of the main bacterial species found associated with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, which is the causal agent of pine wilt disease (PWD) [4, 5], and, also to its insect-vector, *Monochamus galloprovinciallis* [6], hence, suggesting a role for *Serratia* species in the PWD complex. In this sense, several reports indicated that some *Serratia* strains (*marcescens* and *liquefaciens*-like) could potentiate nematode infectivity and oxidative stress resistance [7, 8] while others (*marcescens* and *plymuthica*-like) were able to kill the PWN [9].

In a recent report, we demonstrated that *Serratia* strain BXF1 (*liquefaciens*-like) could promote PWN reproduction when the latter was cultivated in *Botrytis cinerea* [10]. The presence of the chitinase-producing strain BXF1 impacted PWN chitinase gene expression, suggesting that strain BXF1 facilitates nematode chitin acquisition and consequent nematode feeding. Nevertheless, strain BXF1 was unable to internally colonize the nematode, bind to its cuticle extensively or protect the nematode from xenobiotic stress, all of which have been found to be important processes in symbiotic nematode-bacteria associations [11], hence suggesting an opportunistic and transient interaction between BXF1 and the PWN [10]. Interestingly, strain BXF1 promotes pine and tomato plant growth, colonizes internal plant tissues and do not induce PWD symptoms, thus, acting like a plant-growth promoting endophyte (PGPE). Moreover, strain BXF1 can produce the plant hormone indole-3-acetate (IAA), metabolize benzoate (BA) and phenylacetate (PAA), which are common metabolites found in wilting pine trees [12], and presents increased resistance to several plant defense compounds, such as terpenoids. Its antagonistic activities against fungi and bacteria isolated from wilting maritime pine (*Pinus pinaster*) and other PWN-associated bacteria were also observed [10].

Together with previous observations on the studies of *B. xylophilus*-associated bacteria (reviewed in Nascimento et al. [13]), these results indicated that the PWN acquires pine endophytic bacteria inside the pine tree, upon infection and consequent changes in the inner tree environment. Curiously, other eukaryotes, namely corn rootworms, can also acquire *S. grimesii* (*liquefaciens*-like) strains from plants, which seem to be a reservoir for these strains [14].

The plant microbiome has been shown to play an important role in modulating plant growth, development, as well as stress response and resistance [15]. Pine trees represent an enormous ecological niche for several micro and macro-organisms, ranging from bacteria to nematodes. Hence, the pine microbiome and its properties may influence the interaction between several organisms, including those able to induce diseases, such as the PWN. Bacteria like strain BXF1, presenting the ability to internally colonize pine trees and possessing versatile colonization abilities, opportunistically bind to the PWN and, consequently, influence some aspects of the interaction between the plant, nematode, fungi and other organisms. Nevertheless, the mechanisms responsible for this bacterial transient opportunistic multi-species colonization and its consequent effects remains elusive.

In this study, we present and comprehensively analyze the genome sequence of strain BXF1. Understanding the genetic mechanisms governing BXF1 functions may bring new insights into the evolution and ecology of *Serratia,* as well as, the role of bacterial endophytes in several aspects of complex disease systems involving several eukaryotes

METHODS

BXF1 genome sequencing

The BXF1 strain, isolated from a PWN isolate grown in *B. cinerea* plates, was previously characterized [10].

Strain BXF1 genome sequencing was conducted following genomic DNA extraction from an overnight culture using the QIAGEN Genomic DNA Purification kit (Qiagen, Germany). The obtained DNA was sequenced using Roche Titanium 454 with large-insert 3 kb paired end libraries (Centre for Genomic Research, University of Liverpool) and Illumina MiSeq (James Hutton Institute) platforms. The Initial assemblies were performed with Roche “Newbler” gsAssembler [16] and MIRA v4.0.2 [17]. The individual 454 and Miseq experiments were deposited in the European Nucleotide archive (ENA) with the accessions ERR2004553 (314,302 reads, totalling 133Mbp) and ERR2004554 (903,624 paired reads totalling 231Mbp), respectively. Ultimately, a final assembly

was constructed by combining both 454 and Miseq data using MIRA v4.0.2, which resulted in 18 contigs. Guided alignment to published complete genomes, primer walking and PCR reactions were performed to close gaps between the contigs and raise the quality of the genome. However, due to the very repetitive nature of these gap sequences (mostly 16S rRNA) only 7 regions could be effectively closed. The final 5,090,820 bp genome sequence of BXF1 is a scaffold of 11 contigs (N50= 3705947 bp), which in the final assembly were joined using 100 Ns in the repetitive gaps, based on MAUVE [18] progressive alignments against the complete genome sequence of *S. proteamaculans* 568, and as per NCBI and ENA submission guidelines. Genome comparisons to other completely sequenced *Serratia grimesii*-like strains showed the repetitive nature of these regions, further confirming the assembly.

The final genome sequence of *Serratia grimesii* BXF1 is available in the ENA under the accession LT883155.

Genome analysis

BXF1 genome annotation was performed using PROKKA [19]. Functional genome annotation of strain BXF1 was performed using BlastKOALA [20].

Genomic islands were predicted in IslandViewer 3 [21]; Phage sequences were predicted using PHAST [22]; Effector, secretion systems and secreted protein analysis were performed in EffectiveDB [23]; Transcription factors analysis was performed in the P2RP server [24]; CAZymes analysis was executed using default pfam parameters in the BESC data & tools site [25]; Protease analysis was performed using the MEROPS peptidase database analysis [26]; Antibiotics and secondary metabolites analysis were performed in antiSMASH [27].

All the sequences described in the manuscript and supporting materials were verified individually by BlastP searches (default parameters) against the UniProt/SwissProt database (The UniProt Consortium, 2017) using the Geneious software v.9.1 (http://www.geneious.com, [28]).

Comparative genomic analyses were performed using some other *Serratia grimesii*-like genome sequences (**Table 1**) available in the NCBI database.

Table 1- Genome sequences from the *S. liquefaciens* complex (SLC) strains used in this study.

Strain	Accession	Rep	Size (Mbp)	CDS	GC%	Isolation	Reference
***Serratia grimesii* BXF1**	GCA_900186025	1	5.08	4696	52.8	*Burspahelenchus xylophilus*	This work
***S. grimesii* NBRC 13537^T**	GCA_001590905.1	1	5.07	4648	52.8	Cheddar cheese	NCBI database, unpublished
***S. grimesii* A2**	GCA_000734885.1	1	5.13	4354	52.8	Actin buffer solution	Mardanova *et al.* [95]
***S. proteamaculans* 568**	CP000826.1	2	5.45	4895	55.1	Poplar endophyte	Taghavi *et al.* [96]
***S. liquefaciens* ATCC 27592^T**	CP006252.1	2	5.24	4718	55.4	Milk	Nicholson *et al.* [97]

Rep- replicons; CDS- coding sequences. GC%- average G+C content

The average nucleotide identity (ANI) and average amino acid identity (AAI) between genomes were analyzed in (http://enve-omics.ce.gatech.edu) [29], and the two-way analysis scores were presented. Analysis and comparisons of ANI values with other completely sequenced *Serratia* strains was also conducted by using pyani v0.2.3 [30]. Genome circular views and comparisons were performed using BRIG v0.95 [31].

RESULTS AND DISCUSSION

Strain BXF1 genome main features

The *S. grimesii* BXF1 genome main features of are summarized in **Table 2**.

Strain BXF1 genome is constituted by a single circular chromosome with approximately 5.09 Mbp and an average GC content of 52.8%. A total of 4787 open reading frames (ORF) were predicted, in which 4696 correspond to putative protein coding sequences (CDS). A total of 78 tRNA, 3 rRNA and 1 tmRNA were detected.

BlastKoala analysis resulted in the functional annotation of 3032 from a total of 4696 CDS (64.6%). Environmental (860) and genetic (657) information processing functions were assigned for most of the annotated CDS, followed by carbohydrate (328), amino acid (313), co-factor and vitamins (194), energy (159), nucleotide (127) and lipid (86) metabolism (**Table 2**). IslandViewer analysis indicated the presence of several genomic islands (GI) (**Table S1**) in strain BXF1 genome (**Fig. 1**). Some of these genomic islands correspond to phage sequences as found by PHAST analysis. A total of four phage sequence clusters (three complete and one incomplete phage) were found in BXF1 genome (**Table S1**).

CAZymes analysis identified 929 proteins (**Table S2**) belonging to the families of structurally-related catalytic and

carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds (**Table 2**). A total of 378 proteins were predicted as belonging to the Glycoside Hydrolase (GH) family, 361 to Glycosyl Transferases (GT), 74 to Carbohydrate Binding Modules (CBM), 60 to Carbohydrate Esterases (CE) and 55 to Auxiliary Activities (AA). Proteins belonging to the Polysaccharide Lyases (PLs) family were absent in the BXF1 genome.

Searches in the MEROPS database indicated the presence of 193 proteins (**Table S3**) with proteolytic or protease inhibition activity in the BXF1 genome (**Table 2**). The most abundant classes of proteases corresponded to metalloproteases (66, M) and serine proteases (68, S).

Transcription factor analysis indicated the presence of 406 CDS with this function, 72 CDS belonged to the two-component systems (TCS) and 21 were annotated as other DNA-binding proteins (ODP) (**Table 2**).

The complete elements for *sec* and *tat* secretion systems have been identified. The complete gene set for the protease transport system (*prsDEF*) is also present in BXF1 genome (**Table S4**). No other elements related to secretion systems were found in BXF1 genome.

Strain BXF1 phylogeny

Based on its 16S rRNA sequence, strain BXF1 was previously designated as *S. quinivorans* [10]. *Serratia quinivorans,* also known as *S. proteamaculans* subs. *quinovora,* belongs to the *S. liquefaciens* complex (SLC) [32], a group of bacteria (*S. liquefaciens*, *proteamaculans*, *quinivorans* and *grimesii*) possessing very similar phenotypic and genetic characteristics [33]. Consequently, comparative analysis show that strain BXF1 genome presents great similarity and synteny with the available genomes of other *Serratia* species belonging to the SLC (**Fig.1**), isolated from several different sources (**Table 1**). In this sense, strain BXF1 genome presents higher similarity to *S. grimesii* NBRC13537^T (ANI-99.01%, AAI-99.08%), *S. grimesii* A2 (ANI-99.04%, AAI-98.94%), followed by *S. proteamaculans* 568 (ANI-86.97%, AAI-93.88%) and *S. liquefaciens* ATCC27592^T (ANI-85.53%, AAI-93.34%) genomes (**Fig. S1-S2**).

Table 2- *Serratia grimesii* BXF1 genome main features.

Genome	**GC%**	**CDS**	**tRNA**	**tmRNA**	**GI**	**Phage**	**Secreted proteins***									
	52.8	4696	78	1	1	4	73									
Functional annotation	**EIF**	**GIF**	**CHM**	**AAM**	**CP**	**MCV**	**EM**	**NM**	**GBM**	**MOA**	**LM**	**XBM**	**MTP**	**BSM**	**OS**	**UNC**
	860	657	328	313	297	194	159	127	86	86	86	60	42	43	34	514
CAZymes	**GH**	**GT**	**CBM**	**CE**	**AA**	**PL**										
	378	361	74	60	55	0										
Merops protease	**A**	**C**	**I**	**M**	**N**	**P**	**S**	**T**	**U**							
	3	28	11	66	2	2	68	6	7							
Transcription factors	**TF**	**TCS**	**ODP**													
	406	72	21													
Secretion systems	**SecTat**		**II (*prsDEF*)**		**III**		**IV**		**V**		**VI**					
	+		+		n.f		n.f		n.f		n.f					
ANTISMASH	**NRPS**		**PKS**		**Bacteriocin**		**Siderophore**		**Arylpolyene**		**other**					
	3		1		1		1		1		1					

GI- Genomic islands;
EIF- Environmental information processing; **GIF**- Genetic Information Processing; **CHM**- Carbohydrate metabolism; **AAM**- Amino acid metabolism; **CP**- Cellular Processes; **MCV**- Metabolism of cofactors and vitamins; **EM**- Energy metabolism; **NM**- Nucleotide metabolism; **GBM**- Glycan biosynthesis and metabolism; **MOA**-Metabolism of other amino acids; **LM**- Lipid metabolism; **XBM**- Xenobiotics biodegradation and metabolism; **MTP**- Metabolism of terpenoids and polyketides; **BSM**- Biosynthesis of other secondary metabolites; **OS**- Organismal systems; **UNC**- Unclassified.
GH- Glycoside Hydrolase; **GT**- Glycosyl transferases; **CBM**- Carbohydrate Binding Modules; **CE**- Carbohydrate Esterases; **AA**- Auxiliary Activities; **PL**- Polysaccharide Lyases.
A- Aspartic; **C**- Cysteine; **I**- Inhibitors; **M**- Metallo; **N**-Asparagine; **P**- Mixed; **S**- Serine; **T**- Threonine; **U**- Unknown.
TF- Transcription factors; **TCS**- Two Component Systems; **ODP**- Other DNA-binding Proteins.
NRPS- Non-Ribosomal Polyketide Synthase; **PKS**- Polyketide Synthase.
*predicted in Effective DB

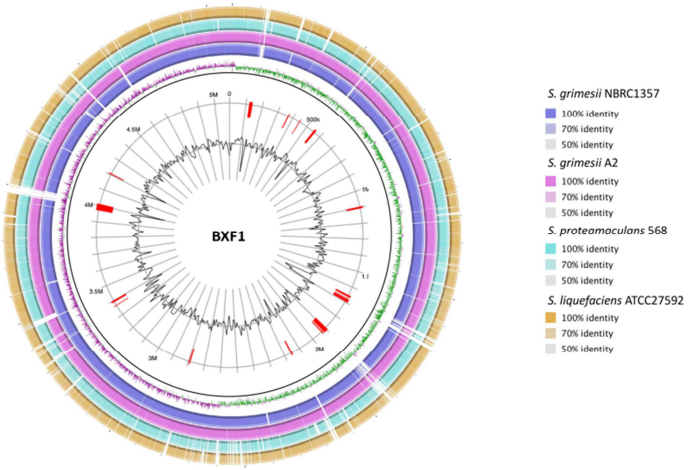

Figure 1- Circular genome representation of *Serratia grimesii* BXF1 and genome comparisons within the *Serratia liquefaciens* complex. Predicted genomic islands in BXF1 genome are represented in red.

Phenotypic analysis of *S. quinivorans* and *grimesii* type strains indicated that only *S. grimesii* can use BA as sole carbon source [32]. Strain BXF1 uses BA as sole carbon source [10] and possesses the BA degradation genes in its genome (described below). Similarly, BA degradation genes are found in *S. grimesii* NBRC13537 and *S. grimesii* A2, but not in other SLC strains. Interestingly, strain BXF1 and two *S. grimesii* strains do not possess plasmid sequences, while other SLC contain one plasmid. Moreover, the average genome GC content is similar between strain BXF1 and *S. grimesii* strains (52.8%) and higher (approx. 55%) in other SLC strains (**Table 1**). Based on the high genome similarity and phenotypic characterizations, strain BXF1 will be further designated as *S. grimesii* BXF1.

***S. grimesii* BXF1 genomic traits related to soil and plant colonization**

Strain BXF1 can colonize plant internal tissues after soil inoculation. Furthermore, strain BXF1 resisted high concentrations (up to 10 mM) of several metals and aromatic compounds (including xenobiotics like toluene and xylene) commonly found in soils [10].
Multiple genes involved in copper, silver, zinc, cadmium, mercury, cobalt, manganese, nickel, magnesium, molybdate, arsenate, chromate and tellurium transport and tolerance (**Table S5**) are present in BXF1 genome, which is consistent with its metal

resistance tolerance and its ability to survive in otherwise stressing environments.

Bacteria fiercely compete for iron acquisition in the soil, as it is a very important component for bacterial fitness [34]. Hence, competitive bacteria possess several mechanisms related to iron transport and acquisition, including siderophore production. Strain BXF1 produces siderophores [10], hence, the presence of aerobactin and an enterobactin production operons and related transport and uptake genes, and also several iron transport systems (**Table S6**). Additionally, a ferrisiderophore reductase gene homolog, which accounts for iron release from siderophores is also present.

Despite the absence of nitrogen fixation genes (*nif*), genes responsible for the assimilatory and dissimilatory nitrate reduction pathways are found in BXF1 genome (**Table S7**). The *amtB* gene and its regulator which are responsible for ammonia uptake and transport, and other genetic elements for ammonia assimilation are also found. In addition, genes involved in urea degradation trough allophanate are found in the BXF1 genome **(Table S7)**.

Strain BXF1 possesses the complete set of genes responsible for the assimilatory sulfate reduction pathway and the sulfate transport system, however, genes necessary for the dissimilatory sulfate reduction pathway are not found. The tetrathionate reduction genes are present in an operon. Strain BXF1 also contains an arylsulfatase and alkyl/arylsulfatase homologs (**Table S7**), which have been implicated in the degradation of several arylsulfate esters (including xenobiotics like SDS or 4-nitrocathecol) [35].

The phosphate and phosphonate transport systems are found in the genome of BXF1. Furthermore, an operon containing the organophosphonate degradation genes is also present (**Table S7**). This operon is related to several strains ability to degrade glyphosate [36], a common herbicide found in soils.

Interestingly, the genome of strain BXF1 harbors multiple genes related to aromatic compound degradation (**Table S8**). The complete BA and catechol degradation pathways are found clustered in a region identified as a GI. The genes responsible for the degradation of protocathecuate, homoprotocatechuate, 4-hydroxybenzoate (4-HBA), PAA and 3 and 4-hydroxyphenylacetate (HPA), which are common key intermediate metabolites in the microbial catabolic pathways of various aromatic compounds, are also present in the genome. The hydroxylated aromatic carboxylic acid exporters genes *aaeAB* may account for this strain ability to deal with stress caused by aromatic compounds.

Competition: resistance and antagonistic activities

To compete for ecological niches bacteria have developed mechanisms to limit the proliferation of competing microbes, such as antibiotic and bacteriocin production [37, 38]. Moreover, some bacteria have developed resistance mechanisms to these compounds. Several antibiotic resistance genes, antimicrobial peptide (CAMP) resistance genes and multiple multidrug efflux systems can be found in the genome of strain BXF1 (**Table S9**), which is consistent with its ability to resist to several antibiotics [10], and possibly to increase its competitiveness in several environments. Strain BXF1 has been shown to limit the growth of several bacteria and fungi [10]. AntiSMASH analysis revealed the presence of several genomic regions coding for secondary metabolites, including a bacteriocin/lantipeptide gene cluster, three non-ribosomal peptide synthase (NRPS) clusters and a type I polyketide synthase (T1PKS) gene (**Table S10**). Bacteriocins can play a role in mediating the bacterial response to competitors, potentiating its colonization abilities and possibly its ability to sense environmental changes [39]. Interestingly, BLAST searches indicate that homologs presenting high identity to BXF1 Bacteriocin/lantibiotic dehydratase gene are uniquely found in *S. grimesii* strains, thus, indicating that this is a species-specific trait.

One of the NRPS gene identified in BXF1 genome present high similarity to *S. marcescens* serrawetin W1 production gene, *srwW* [40]. Serrawettins can act like wetting agents on various surfaces, hence, increasing bacterial flagellum dependent and independent movement and swarming motility [41]. Serrawetin (previously described as serratamolide) can also act as an antibiotic [42]. Moreover, the genome of BXF1 also contains the *pswP* gene homolog involved in serrawetin W1 production [43].

Additionally, the genome of BXF1 contains several other genes related to fungal antagonism. These include four chitinase genes, a chitin and N-acetylglucosamine-binding protein and a chitobiase (**Table S10**). Other features that may account for optimal nutrient usage from chitin degradation are present, like chitoporin, the *nagBACD* operon responsible for N-acetylglucosamine metabolism, a N-acetylglucosamine transmembrane transporter and a chitooligosaccharide deacetylase. Genes encoding for pyrrolnitrin and hydrogen cyanide (HCN) production are also present in BXF1 genome. Pyrrolnitrin is a secondary metabolite with known antifungal properties [44], and HCN production is an important trait in the biological control abilities of various bacterial strains [45]. The gene cluster *prnABCD* coding for pyrrolnitrin is found in a region classified as a GI. This gene cluster shows high similarity to *S. plymuthica*

(aprox. 95-96%) functional *prnABCD* cluster. Curiously, both pyrrolnitrin and HCN production genes are only found in *S. grimesii* and not in other sequenced SLC bacteria, indicating that in the SLC these traits are specific to *S. grimesii* strains.

Root colonization

Most bacterial endophytes effectively bind to plant tissues trough the action of adhesins and other elements [46]. Strain BXF1 is motile and a competent colonizer of the rhizosphere and endosphere [10,47] **(Fig. 2a)** and leguminous root nodules (**Fig. 2b**), which is consistent with the presence of several genes related to motility, chemotaxis and attachment in its genome, including flagella, serrawetin, fimbria, lipopolysaccharide (LPS), exopolysaccharide (EPS), cellulose synthesis and quorum-sensing genes (**Table S11**). Furthermore, the genome of strain BXF1 also harbors multiple genes responsible for the main root exudates metabolism. Root exudates are composed mainly by sugars like glucose, fructose, xylose, arabinose, ribose, and maltose, and organic acids such as citrate, malate, fumarate and tartarate, which serve as important carbon sources for rhizospheric and root-associated bacteria [48–50]. Root exudates also contain, amino acids, proteins, phenylpropanoids and flavonoids that may modulate plant-microbe interactions [48].

The genome of strain BXF1 harbors multiple genes responsible for the main root exudate sugars metabolism (**Table S12**), such as fructose, xylose, arabinose, ribose and maltose degradation genes. Genes involved in mannose, trehalose, galactose, sucrose, lactose, melibiose, cellobiose, palatinose, maltulose, trehalulose, turanose, leucrose, as well as, mannitol, sorbitol, galactitol, maltitol, D-galactosamine and N-acetyl-D-galactosamine are also present, further indicating a wide range of sugar utilization by *S. grimesii* BXF1 [10]. Moreover, strain BXF1 contains several genes related to sugar transport systems, which participate in sugar acquisition (**Table S13**).

Intriguingly, we found homologs of *Sinorhizobium meliloti moc* genes in the BXF1 genome. These are responsible for the transport of rhizopines [51]. The myo-inositol degradation genes, necessary for rhizopine degradation [52] are also present in BXF1 genome (**Table S14**). Rhizopines are opine-like compounds produced by rhizobial bacteroids in the root nodule. Degradation of rhizopines favor the growth of free-living rhizobia, therefore increasing their colonization abilities [53].

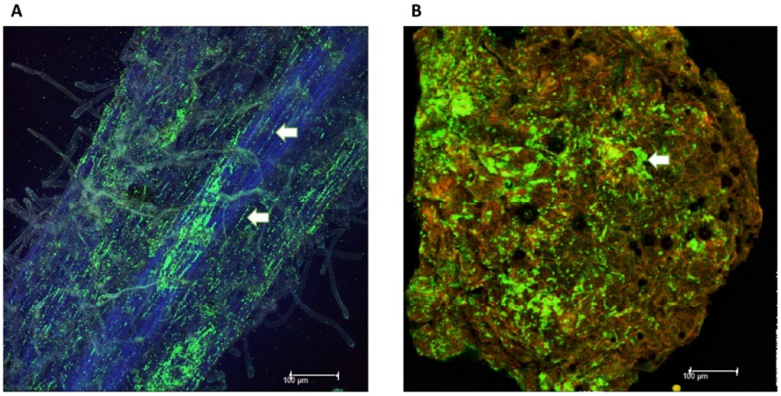

Figure 2- Colonization of common bean roots, 20 days after inoculation, by *Serratia grimesii* BXF1 pn519gfp (A), and common bean root nodules (B). Arrows indicate the presence of bacteria in intracellular spaces and root nodule surface, respectively. Images were acquired by confocal microscopy (Leica microsystems, Germany).

Strain BXF1 harbors the genetic elements of the TCA and glyoxylate cycle responsible for the degradation/transformation of major organic acids present in root exudates, such as citrate, succinate, malate, fumarate and tartrate. Moreover, the genome also contains the acetate, gluconate, oxalate and formate degradation genes, as well as other genes involved in sugar acids degradation (**Table S15**). Similarly, several amino acids metabolic pathways (**Table S16**) and transport genes (**Table S17**) are also present in the *S. grimesii* BXF1 genome.

Some plant-associated bacteria degrade plant flavonoids, which are known modulators of plant-microbe interactions [54]. These compounds are commonly degraded in a series of dihydroxylation, hydrolysis and oxidation reactions leading to the formation of several compounds, such as, protocatechuate, p-hydroxybenzoate, p-coumarate, PAA and caffeate (B-ring products); and phloroglucinol, phloroglucinol carboxylate, resorcinol and oxaloacetate (A-ring products) [54, 55]. The genome of strain BXF1 contains three quercetin dioxygenase homologs (**Table S18**), showing high homology to *E. coli* quercetin dioxygenase that converts quercetin (a major flavonoid) into 2-protocatechuoylphloroglucinolcarboxylate (2-PPC) [56]. As previously indicated, the genome of BXF1 also contains the protocatechuate, p-hydroxybenzoate, PAA and oxaloacetate degradation genes (**Table S8, S15**). In addition, homologs of *nodD*

genes, encoding the NodD protein known to bind to flavonoids, are found in BXF1 genome (**Table S18**).

Major phenylpropanoid degradation pathways (3-phenylpropionate and 3-(3-hydroxyphenyl) propionate) seem to be absent in BXF1 genome. However, a ferulate decarboxylase gene homolog is found (**Table S18**). Furuya and Kino [57] demonstrated that the HPA monooxygenase enzyme responsible for HPA degradation also demonstrates hydroxylation activity toward tyrosol and various cinnamate derivatives, catalyzing the hydroxylation of p-coumarate, caffeate, ferulate, and coniferaldehyde. Moreover, HPA monooxygenase can also oxidize phenol to catechol, and hydroxylate other phenol derivatives [58]. Hence, the presence of the HPA degradation genes in BXF1 may play a role in the degradation of phenylpropanoids and other phenolic compounds, which are linked to several aspects of plant immunity [59].

A gene encoding a NADPH-dependent curcumin reductase is also found (**Table S18**) Curcumin reductase is involved in the degradation of the phenolic compound curcumin [60]. Many of these compounds have been shown to possess antimicrobial activity, hence its degradation by BXF1 may lead to an increased bacterial resistance and root colonization abilities.

Establishment inside the plant and modulation of plant growth

Entrance, resistance and maintenance

The passage from the rhizosphere into the endosphere is a process that may naturally occur, where bacteria (opportunistic or true endophytes) can enter to internal plant tissues via root cracks [61]. Strain BXF1 can rapidly colonize internal root tissues (intercellular spaces) upon seed germination and radicle protrusion [10], however, this colonization is more preeminent in the root differentiation zone, where root crack formation is more common. This is consistent with the absence of major cell-wall-degrading enzymes (CWDE) in the BXF1 genome, as well as typical secretion systems involved in effector delivery into plant cells.

Genes involved in motility, chemotaxis and attachment have been suggested to play an important role in endophytic bacterial colonization [46]. Flagella, fimbria, LPS, EPS, cellulose synthesis and quorum-sensing genes (**Table S11**) are abundant in BXF1 genome. The genome of BXF1 also contains a gene homolog (SGBXF1_03349) to the *ndvB* gene of *S. meliloti* involved in the production of beta-(1,2)-glucan. *S. meliloti ndvB*$^-$ mutants were impaired in nodule invasion and bacteroid development [62], thus, suggesting a role for this gene in endophytic colonization.

The genome of BXF1 is rich in lytic enzymes (lipases, phospholipases, esterases, proteases, amylases, glucosidases, nucleases) that participate in the modulation of plant cell development and its organization and, consequently, facilitate bacterial entrance, colonization and maintenance. Genes coding for lipolytic enzymes, (i.e. lecithinase and other extracellular lipases) (**Table S19**), proteases, (i.e. serralysin and grymelysin) (**Table S20**), amylases and glucosidases (**Table S12**) are also found in the genome of BXF1 and are consistent with its degradative abilities [10]. Interestingly, in a search for enzymes with the ability to modulate pine metabolites we found that the BXF1 *bglC* gene showed homology to *Pinus contorta* coniferin beta-glucosidase (35.8%) and to *Arabidopsis thaliana* beta-glucosidase 46 (38%) possessing activity against monolignol glucosides like salicin, p-coumaryl alcohol glucoside, phenyl-beta-D-glucoside, coniferin, syringin and arbutin [63]. Curiously, *S. grimesii* strains were shown to be able to degrade salicin and esculin [32].

Resistance against plant defenses

Plant defense responses include the production of reactive oxygen species (ROS), reactive nitrogen species, such as nitric oxide (NO), alterations in the plant cell wall, and induction of antimicrobial compounds (e.g. secondary metabolites like terpenoids) [64]. In this sense, to colonize internal plant tissues, bacterial endophytes need to be able to cope with these stressful conditions. *S. grimesii* BXF1 genome encodes various enzymes related to ROS detoxification (**Table S21**), including three superoxide dismutases, two catalases, an alkyl peroxidase, one thyol peroxidase and a hybrid peroxiredoxin. Organic hydroperoxide resistance protein *ohrB* and its regulator *ohrR* are also present. In addition, five glutathione S-transferase genes, the glutathione ABC transporter operon, a glutathione peroxidase, glutathione synthetase, glutathione reductase and four glutaredoxin genes are found in BXF1 genome (**Table S21**). A nitric oxide dioxygenase is also present and may account to the strain ability to deal with nitrosative stress. In addition, antiSMASH analysis revealed the presence of an arylpolyene gene cluster in the genome of strain BXF1(**Table S21**). Arylpolyenes may play a role in protecting bacterial cells from exogenous oxidative stress [65].

S. grimesii BXF1 grows in the presence of high concentrations of several toxic terpenoids, however, it is unable to use these compounds as sole carbon sources [10]. Not surprisingly, limonene, pinene, geraniol and carvacrol degradation pathways are absent or incomplete in the BXF1 genome. This observation suggests that

strain BXF1 must employ different strategies to overcome the toxic effects of terpenoids. This probably occurs trough membrane integrity protection and efficient multidrug efflux systems. For instance, in *Pseudomonas aeruginosa* the *mexAB-oprM* efflux system is not only responsible for antibiotic efflux but also for terpenoid efflux [66]. Multidrug efflux systems, including *mexAB-oprM*, are abundant in the BXF1 genome (**Table S9**).

Modulation of plant growth

One of the most studied effects of PGPB relates to their ability to modulate phytohormone levels. In this sense, several studies have pointed to the importance of bacterial production and/or modulation of growth-inducing phytohormones like auxins (commonly IAA) [67] and cytokinins (CK) [68], as well as in the modulation of phytohormones related to plant defense and stress responses, such as salicylate (SA) [69] and ethylene (ET) [70].

Strain BXF1 contains multiple genetic elements involved in phytohormone production, degradation and modulation (**Table S22**).

Strain BXF1 produces the phytohormone IAA, however, in low levels (aprox. 5 μg/ml) [10]. In strain BXF1 genome an indole-3-pyruvate (IPA) decarboxylase gene is present, hence, suggesting that the demonstrated IAA production in this strain occurs via the IPA pathway. Curiously, a gene showing high similarity to *Pantoea agglomerans* IAA-aspartate hydrolase was also found. The *iaaasp* gene is responsible for the degradation of IAA-aspartate, a common plant IAA conjugate [71]. Moreover, the degradation IAA conjugates modulates free IAA levels necessary to impact plant growth [72]. PAA is also an auxin commonly found in plants [73]. Strain BXF1 possesses the PAA degradation operon.

Genes related to CK production and transformation are abundant in BXF1 genome. The *miaA* gene coding for tRNA isopentenylpyrophosphate transferase is present in BXF1. Großkinsky et al. [74] showed that *P. fluorescens* G20-18 *miaA* gene is involved in bacterial CK production. Moreover, the authors showed that CK production by *P. fluorescens* G20-18 determines biocontrol activity against *P. syringae* in *Arabidopsis.* Moreover, the genome of BXF1 also harbors the *miaB* and *miaE* genes that encode enzymes responsible for the production of 2-methylthio-N6-(dimethylallyl)adenosine and 2-methylthio-cis-ribozeatin, respectively. Strain BXF1 also possesses two LOG family proteins, one of them (SGBXF1_02759) presenting high similarity to the LOG of *Corynebacterium glutamicum* (Cg2612) responsible for the production of CK [75]. Additionally, two xanthine dehydrogenase

genes showing high similarity to *S. proteamaculans xdhA* and *xdhB* gene, linked to CK biotransformation [76], were also found.

SA plays an important role in plant defense, responses to abiotic stresses and in general plant growth and development [77]. SA can also have important roles in plant-microbe interactions, and bacterial SA production has been previously described in several bacteria [69], including *Serratia* [78]. Moreover, the production of SA has been suggested to play a role in the biocontrol abilities of several bacterial strains [69]. Two isochorismate synthase gene homologs, which convert chorismate into isochorismate (the building block for SA synthesis) were found in strain BXF1. An isochorismate-pyruvate lyase gene homolog is also present, hence, suggesting the existence of the complete pathway for SA production in strain BXF1.

Polyamines like putrescine, spermidine and cadaverine have been shown to play a significant role in bacterial plant-growth promotion, and modulation of ET biosynthesis *in planta* [79]. The complete pathways to produce putrescine, spermidine and cadaverine were identified in BXF1 genome, as well as, the genes responsible for 1,3-diaminopropane synthesis (**Table S22**). Additionally, two copies of *speG* genes, responsible for spermidine acetylation and protection against polyamine toxicity, and several spermidine export protein genes are also present. Curiously, the polyamine degradation genes found in BXF1 are involved in the formation of the proteinogenic amino acid 4-aminobutyrate (GABA), which plays an important role in plant stress response [80]. While genes responsible for GABA production are found, the GABA permease gene responsible for GABA secretion is absent from the genome of BXF1. On the other hand, strain BXF1 possesses the genes involved in GABA degradation through its transformation to succinate, which is consistent to its ability to use GABA as sole carbon source.

Serratia spp. are known to produce several volatile organic compounds (VOCs) which have a role in plant-microbe interactions [81]. For instance, *S. proteamaculans* 568 can produce at least twenty-one VOCs and sixteen of these were also found in other *Serratia* strains [82]. Most volatiles are produced as by-products of bacteria metabolism, such as fermentation, sulfur metabolism, aminoacid degradation and fatty acid biosynthesis [82, 83], which are abundant pathways in BXF1 genome. Strain BXF1 can ferment several sugars and produces acetoin [10]. For instance, strain BXF1 contains all elements necessary for the mixed acid fermentation pathways (**Table S23**) that lead to the production of several VOCs, such as ethanol, acetate, lactate and glyoxalate. The genome of BXF1 also harbors the acetolactate synthase (*budA*) and α-

acetolactate decarboxylase (*budB*) genes which are involved in acetoin and 2,3-butanediol production, both VOCs containing plant growth-promoting activities [84]. A gene encoding a pyruvate decarboxylase (*poxB*), which is involved in acetoin production is also found. Several sulfur modulation pathways are also present in the BXF1 genome (**Table S7, Table S23)**, and may account for its ability to produce sulfur based volatiles, like dimethyl sulfide (DMS) and H_2S. In addition, methionine and cysteine metabolism genes are widely present in BXF1 (**Table S16**) which are linked to the production of sulfur volatiles [82]. The genome of BXF1 harbors several elements that are involved in fatty acid biosynthesis and modification (**Table S24**), which can play a role in the production of volatile alcohols (e.g. 1-decanol) and ketones (e.g. 2-undecanone), compounds widely produced by *Serratia* species [81]. Furthermore, the genome of strain BXF1 contains several gene homologs to those of the Ehrlich pathway (**Table S24**), including several aminotransferases and alcohol dehydrogenases, playing a role in the degradation of amino acids and subsequent production of alcohols [85] (e.g. 3-methyl-1-butanol, known to be produced by *Serratia*).

What features may contribute to an association with the PWN?

Strain BXF1 can bind to the PWN cuticle [10] (**Fig. 3**). UDP-galactose and UDP-N-acetylglucosamine present in nematodes surface coat proteins play an important role in mediating the interaction between bacteria and nematode [86]. The genome of strain BXF1 contains several genes related to galactose and N-acetylglucosamine degradation, a GlNac-binding protein (SGBXF1_03542) and several N-acetylglucosamine and galactose transporters (**Table S13**). Moreover, the production of LPS, EPS and fimbria (**Table S11**) (which modulate biofilm production) play a role in strain BXF1 ability to bind to the PWN cuticle. From this ability to bind to the nematode cuticle, it is possible that strain BXF1 gains the advantage of being transported throughout the plant to other nematode feeding sites, and, other environments. In fact, nematodes may serve as important vectors for bacteria [87].

The PWN feeds on pine cells through the production of a cocktail of several extracellular pectate lyases and cellulases, which lead to plant cell disruption. The PWN genome contains 11 pectate lyase and 11 cellulase genes [88]. On the other hand, strain BXF1 does not produce any of these enzymes, however, it contains several genes encoding enzymes responsible for the degradation of compounds resulting from plant cell-wall and membrane degradation, such as phospholipids, proteins, cellobiose and glucoronate, as well as cellular contents like DNA, stored sugars and

other proteins (**Table S12, S16, S19-20**). Latter on the course of PWD, the host trees become extremely colonized by fungi which are a major feeding source for the mycophagous PWN [89]. Like the PWN, strain BXF1 presents chitinase activity [10] and possesses several genetic elements responsible for chitin degradation (**Table S10**).

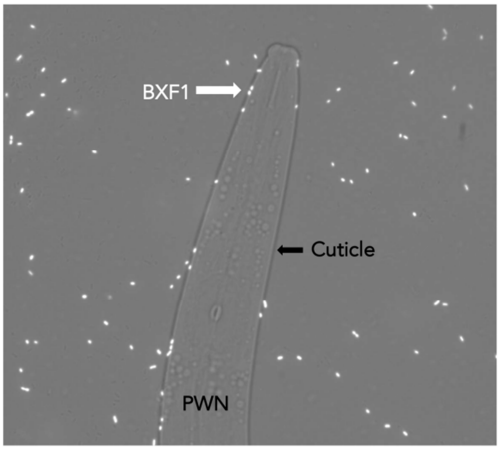

Figure 3- *Serratia grimesii* BXF1 pn519gfp attachment to the PWN cuticle. Images were acquired by fluorescence microscopy (Leica microsystems, Germany).

S. grimesii BXF1 has been found to increase PWN reproduction, however, the precise mechanism(s) responsible for this effect is/are not completely understood. Previous results suggested that strain BXF1 indirectly potentiate nematode feeding by helping the nematode to degrade fungal chitin [10]. However, there is also the possibility that strain BXF1 directly impacts nematode reproduction by the production or degradation of other compounds that regulate nematode development. The PWN suffers several transformations during its life cycle, where hatching and molting processes occur. In the hatching process the nematode secretes enzymes (lipases, chitinase, proteases) to digest the egg membranes, which facilitates rupture and consequent nematode escape. After hatching the nematode grows until becoming limited by the cuticle size. When this occurs, the moulting process initiates, which consists in the synthesis of a new cuticle and the shedding of the older cuticle. The moulting process is assisted by several proteases [90,91]. Strain BXF1 possesses a wide range of extracellular lytic enzymes, including protease and chitinase [10].

Interestingly, BXF1 lipase 1 (**Table S19**) shows high homology to *Photorhabdus* lipase, which is induced in the bacterial phase 1 (isolated from infective-stage nematodes) but not in phase 2 (bacterial free-living growth) [92]. The serralysin genes (SGBXF1_00223, SGBXF1_02407) are similar to *S. marcescens* S15 serralysin, which has been implicated in this strain ability to dissolve moths cocoon thus, allowing its better development [93]. Moreover, serralysins degrade gelatin [94], a compound containing collagen (the main component of nematode's cuticle). Strain BXF1 also encodes grimelysin, an extracellular protease able to degrade filamentous actin [95] and possibly other related compounds that also modulate nematode's cuticle.

***S. grimesii* BXF1 evolved as a multi-niche colonizer and a multi-interaction mediator?**

Overall, genomic data indicates that *S. grimesii* BXF1 evolved as a multi-niche colonizer and a multi-interaction mediator. Its genome is rich in environmental and genetic information processing pathways, clearly indicating an adaptation to several lifestyles and colonization strategies. By being a versatile colonizer, BXF1 can cope with several stresses resulting from this ecologic adaptation. Hence, BXF1 contains multiple genes involved in resource acquisition, stress protection, and competition, making it a very resilient colonizer and competitor. The carbohydrate, amino acid and lipid metabolism, allied with high chitinolytic, proteolytic and lipolytic activities of *S. grimesii* BXF1 seem to mediate a wide range of interactions with several organisms. One key factor for the neutral or beneficial nature of these interactions may be the absence of typical pathogen secretion systems, which leads to a non-pathogenic phenotype and results in an overall tolerance from its eukaryotic hosts. This is consistent with previous results which indicated that strain BXF1 is unable to kill the PWN, its insect vector, the pine and other plants, even when present in very high concentrations. Contrary to other Enterobacteriaceae and *Serratia* strains that contain a wide range of secretion systems, consequently, using "brute" force to colonize its hosts (normally inducing disease), it seems that *S. grimesii* BXF1 evolved as a tolerable bacterium colonizing its hosts in a more "friendly" manner.

Ultimately, the genomic information obtained in this study is essential for the better understanding of the specific contribution of *Serratia grimesii* BXF1 and related strains in mediating the interactions between multiple organisms involved in a complex disease system, therefore, opening new important research avenues to be explored in the future.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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RESEARCH MANUSCRIPT

"The expression of an exogenous ACC deaminase by the endophyte *Serratia grimesii* BXF1 promotes the early nodulation and growth of common bean"

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The expression of an exogenous ACC deaminase by the endophyte *Serratia grimesii* BXF1 promotes the early nodulation and growth of common bean

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Running Head
ACC deaminase increases bean nodulation

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Keywords: ACC deaminase, common bean, endophyte, ethylene, nodulation, rhizobia.

SIGNIFICANCE AND IMPACT OF THE STUDY:

In this work, we studied the effect of ACC deaminase production by the bacterial endophyte *Serratia grimesi* BXF1, and its impact on the nodulation process of common bean. The results obtained indicate that ACC deaminase is an asset to the synergetic interaction between rhizobia and the endophyte, positively contributing to the overall legume-rhizobia symbiosis by regulating inhibitory ethylene levels that might otherwise inhibit nodulation and overall plant growth. The use of rhizobia together with an ACC deaminase-producing endophyte is, therefore, an important strategy for the development of new bacterial inoculants with increased performance.

ABSTRACT

Ethylene acts as an inhibitor of the nodulation process of leguminous plants. However, some bacteria can decrease deleterious ethylene levels by the action of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which degrades ACC, the ethylene precursor in all higher plants.

Co-inoculation of rhizobia with endophytes enhances the rhizobial symbiotic efficiency with legumes, improving both nodulation and nitrogen fixation. However, not much is understood about the mechanisms employed by these endophytic bacteria. In this regard, the role of ACC deaminase from endophytic strains in assisting rhizobia in this process has yet to be confirmed.

In this study, the role of ACC deaminase in an endophyte's ability to increase *Rhizobium tropici* nodulation of common bean was evaluated. To assess the effect of ACC deaminase in an endophyte's ability to promote rhizobial nodulation, the endophyte *Serratia grimesii* BXF1, which does not encode ACC deaminase, was transformed with an exogenous *acdS* gene. The results obtained indicate that the ACC deaminase-overexpressing transformant strain increased common bean growth, and enhanced the nodulation abilities of *R. tropici* CIAT899, in both cases compared to the wild-type non-transformed strain. Furthermore, plant inoculation with the ACC deaminase-overproducing strain led to an increased level of plant protection against a seed-borne pathogen.

INTRODUCTION

The symbiosis between rhizobia and legumes is one of the most important examples of bacteria promoting plant growth, especially where sustainable agricultural practices are concerned (Nascimento et al. 2016a). Though rhizo- bia usually inhabit the rhizosphere (the portion of the soil immediately surrounding the roots), they are also able to colonize the plant roots, inducing the formation of root nodules, and consequently, fixing atmospheric nitrogen (N_2), transforming it into ammonia (NH_4^+), which can then be used by the plant (Gage 2004). Besides rhizobia, other bacteria can also colonize internal plant tissues and promote plant growth. These bacteria are known as plant growth-promoting endophytes (Reinhold-Hurek and Hurek 2011), and are able to colonize tissues such as roots, shoots, leaves, flowers and fruits (Dong et al. 2003; Compant et al. 2005). Moreover, some bacterial endophytes colonize the nodules formed by rhizobia, and this association may be beneficial to both rhizobia and endophytes (Sturz et al. 1997; Deng et al. 2011; Zgadzaj et al. 2015; Martínez-Hidalgo and Hirsch 2017).

Endophytic bacteria present several advantages when compared to obligatory rhizospheric bacteria, mostly as a consequence of their capacity to colonize the interior tissues of plants, thus, being protected from the competitive, high-stress environment of the soil (Compant et al. 2005; Hardoim et al. 2015). Moreover, endophytes modulate plant growth by mechanisms similar to those employed by rhizospheric plant-growth promoting bacteria (PGPB) (Santoyo et al. 2016). Hence, bacterial endophytes are potentially of great agricultural importance.

Serratia species have not only been described as PGPB but some strains are also endophytes capable of colonizing internal plant tissues, including root nodules (Gyaneshwar et al. 2001; Taghavi et al. 2009; Nascimento et al. 2016b; Zaheer et al. 2016).

The phytohormone ethylene, found in all higher plants, is an important modulator of normal plant growth and development (Lin et al. 2009; Van de Poel et al. 2015). Ethylene is known for its inhibition of the nodulation process initiated by rhizobia (reviewed by Guinel 2015). Some bacteria can decrease the deleterious ethylene levels through the production of the enzyme 1-aminocyclopro- pane-1-carboxylate (ACC) deaminase (Glick 2014). This enzyme, encoded by the *acdS* gene, is responsible for the breakdown of ACC, the direct precursor of ethylene in all higher plants, into NH_4^+ and α-ketobutyrate (Honma and Shimomura 1978), which bacteria can often use as both a nitrogen and carbon source. Most rhizobial strains present either low or no ACC deaminase activity (Duan et al.

2009) and have a limited capacity to decrease inhibitory ethylene levels formed in plant roots and nodules (Nascimento et al. 2016a). On the other hand, some free-living rhizospheric and endophytic bacteria, contain a high level of ACC deaminase activity and can significantly decrease overall plant ethylene levels (Glick 2014). In this way, the use of free-living bacteria with high ACC deaminase activity in conjunction with rhizobial strains may both help to promote nodulation and decrease general inhibitory ethylene levels. Nevertheless, not much is understood about the role of ACC deaminase-producing endophytes in facilitating the nodulation process. In this work, the effect of ACC deaminase production by a bacterial endophyte and its role in promoting the early nodulation and overall growth of common bean was studied. The endophytic strain *Serratia grimesii* BXF1 and its corresponding BXF1 ACC deaminase-overproducing transformant (obtained in this work) were assessed for their ability to promote the early nodulation of common beans by *Rhizobium tropici* CIAT899 (used as a commercial inoculant in several countries) and, overall common bean growth.

RESULTS AND DISCUSSION

ACC deaminase activity of the transformed BXF1 strain

Serratia grimesii BXF1 successfully incorporated the plasmid pRKACC and was subsequently able to cleave ACC into NH_4^+ and α -ketobutyrate (total enzymatic activity of 9.9 *μ*mol α-ketobutyrate per mg protein per h). However, despite this transformed strain expressed a slightly lower level of enzymatic activity than *Pseudomonas* sp. UW4 (i.e. 12.2 *μ*mol α-ketobutyrate per mg protein per h) the source of the *acdS* gene. This might be explained by the presence of a small metabolic load caused by the presence of the plasmid pRKACC in *S. grimesii* BXF1 (Glick 1995). On the other hand, strain UW4 contains the *acdS* gene in its chromosome (Duan et al. 2013). Nevertheless, the ACC deaminase activity presented by *S. grimesii* BXF1 pRKACC is much higher than that presented by most strains of rhizobia (0.07–1.49 *μ*mol α-ketobutyrate per mg protein per h) (Nascimento et al. 2016a), and is within the activity range found in many free-living bacte- ria (Glick 2014).

Expression of ACC deaminase increased the ability of strain BXF1 to promote common bean growth and *R. tropici* CIAT899 nodulation

The expression of ACC deaminase by the *S. grimesii* BXF1 transformant led to an increased ability to promote common bean growth. While the co-inoculation of both strains, BXF1 and CIAT899, slightly increased plant total biomass compared to inoculation with only strain CIAT899, the co-inoculation of CIAT899 with strain

BXF1/pRKACC, expressing ACC deaminase, resulted in a significantly increased plant biomass (**Fig. 1**). Root and shoot dry weights, and the total biomass of CIAT899 + BXF1/pRKACC inoculated plants increased by 56, 63 and 61% respectively, when compared to results obtained with the CIAT899 + the BXF1 wild-type strain (**Fig. 1**), and 68, 67 and 67% respectively, when compared to the non-inoculated control.

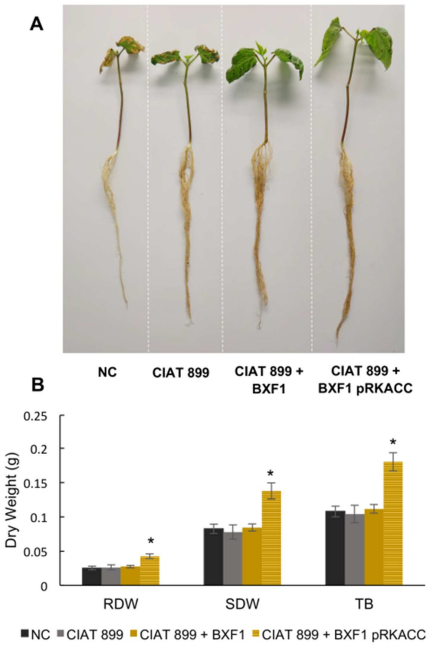

Figure 1- (a) Common bean plants obtained in the co-inoculation assay of *Rhizobium tropici* CIAT 899 and *Serratia grimesii* BXF1, wild-type and transformed with pRKACC, 20 days after inoculation. (b) Average values for root dry weight (RDW), shoot dry weight (SDW), and total biomass (TB). *Indicates significant statistical differences ($P < 0.05$)

Similarly, *S. grimesii* BXF1/pRKACC significantly enhanced rhizobial nodulation, compared to either rhizobial inoculation or co-inoculation with rhizobia and the BXF1 wild-type strain (**Fig. 2a**). Strain BXF1/ pRKACC increased the number of nodules formed by CIAT899 by 127% compared to the BXF1 wild-type strain (average of 25 vs 11 nodules formed), 20 days after inoculation (**Fig. 2a**). ACC deaminase production by the BXF1/pRKACC endophyte not only induced the formation of a greater number of nodules by CIAT899, but also increased nodule development (**Fig. 2c**), with the nodules

appearing larger and with a more pronounced pink tone (indicative of nitrogen fixation) than the nodules obtained following the co-inoculation of CIAT899 with the BXF1 wild-type (Fig. 2b). On the other hand, nodules formed by *R. tropici* CIAT899 alone, were very few in number, small and white (data not shown). No nodules were found in control plants not exposed to rhizobia.

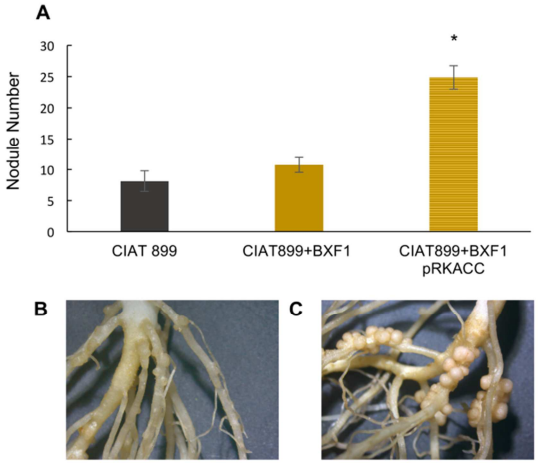

Figure 2- Number of nodules obtained in the co-inoculation assay of *Rhizobium tropici* CIAT899 and *Serratia grimesii* BXF1, wild-type and transformed with pRKACC, 20 days after inoculation (a). Developmental state of the nodules in plants inoculated with *S. grimesii* BXF1 (b) and *S. grimesii* BXF1 pRKACC (c). *Indicates significant statistical differences ($P < 0.05$).

Ethylene has been described as having an active role in the regulation of the early steps of the nodulation process by inhibiting Nod factor perception, rhizobia infection thread formation and elongation, as well as nodule pri- mordia initiation (Guinel 2015). It is likely that ACC deaminase-producing endophytes and, based on other reported results, ACC deaminase-producing rhizospheric bacteria as well, play an important role in directly assisting rhizobial nodule formation. This occurs by the effect of ACC deaminase, reducing both the inhibitory plant ethylene levels as well as the localized small rises in ethylene levels that occur as a direct consequence of the nodulation/infection process that limit the early stages of root colonization and nodule development. The reduction in plant ethylene levels may be achieved by the presence of ACC deaminase-producing bacteria either in the rhizo- sphere or the

endosphere, acting as a sink for ACC. In fact, we found that *S. grimesii* BXF1 effectively colonized common bean roots (internally and externally) (**Fig. 3a**) and nodules (externally) (**Fig. 3b**). Moreover, strain BXF1 was also recovered from the interior of surface sterilized root nodules (data not shown).

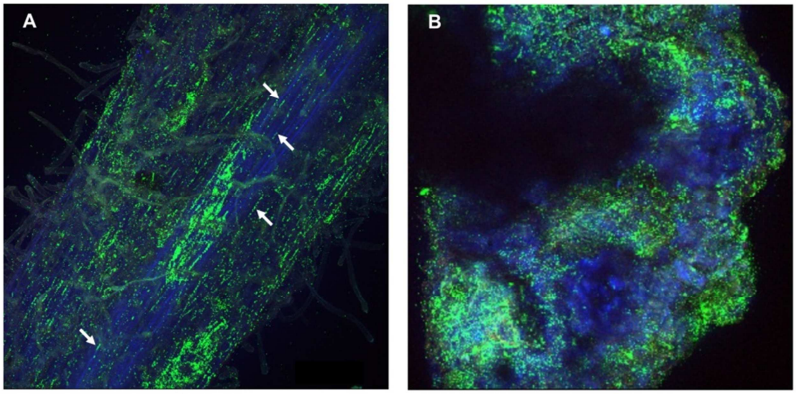

Figure 3- Confocal microscope images of the root rhizospheric and endophytic colonization of *Serratia grimesii* BXF1. (a) Colonization of the internal and external surface of the root. Arrows point to the typical endophytic colonization of intercellular spaces. (b) Colonization of the external surface of the root nodule.

The presence of the ACC deaminase-producing BXF1 endophyte not only led to increased rhizobial nodulation, but also to increased nodule development, suggesting a role for ACC deaminase in promoting nodule development and subsequent nodule nitrogen fixation. However, some other studies have suggested that ACC deaminase activity does not directly influence nitrogenase activity. For instance, an *acdS* minus mutant of *R. leguminorasum* bv. *viciae* 128C53K had a decreased ability to form nodules in *Pisum sativum*, but nodule nitrogen fixation was not affected, indicating that ethylene does not directly influence nitrogenase activity (Ma et al. 2003). Results obtained by Nascimento et al. (2012b) further confirmed this data. However, it is possible that ethylene production by nitrogen-fixing nodules plays a role in nodule senescence and abscission (Glick 2014). Hence, the presence of ACC deaminase-producing endophytes may also increase nodule persistence and the overall biological nitrogen fixation process.

Expression of ACC deaminase increased strain BXF1's ability to protect bean plants from pathogen attack

Interestingly, following their planting in pots, and after some days of growth, all cultivated bean plants (including non-inoculated plants) exhibited stress symptoms to some extent. Leaf and shoot yellowing, necrosis and reduced aerial plant growth were observed (**Fig. 4a**). No symptoms were observed in the roots (**Fig. 1a**). Some pink Gram-positive colonies were obtained from upward shoot, leaf and seed tissues (**Fig. 4b**), suggesting that infection with *Curtobacterium flacumfaciens*, a seed-borne phytopathogen that is commonly found in beans in Brazil had occurred (Harveson et al. 2015). Plants inoculated with strains CIAT899+BXF1/pRKACC demonstrated significantly fewer symptoms compared to CIAT899+BXF1, CIAT899 or non-inoculated plant treatments (**Figs 1a and 4c**). This observation represents additional evidence regarding the importance of the endophytic bacterium expressing ACC deaminase in lowering the deleterious effects of biotic stress, which would otherwise greatly increase plant ethylene levels.

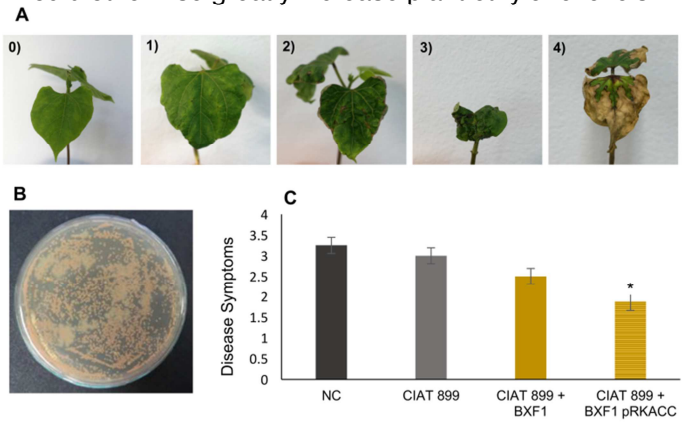

Figure 4- (a) Scale of the disease symptoms observed from least to most diseased plants. 0—no disease symptoms 1—appearance of small chlorotic spots; 2—mild levels of chlorotic spots and reduced leaf growth; 3—appearance of some necrosis spots and reduced leaf growth; 4—ample necrosis lesions and greatly reduced leaf growth. (b) Colonies of the pathogen isolated from common bean tissues. (c) Average disease symptoms (determined according to scale) presented by common beans in the co-inoculation assays (c). *Indicates significant statistical differences ($P < 0.05$)

The protective and positive impact of bacterial ACC deaminase in biotic stress control has been reported in several

studies (Wang et al. 2000; Hao et al. 2007; Toklikishvili et al. 2010; Nascimento et al. 2012b, 2013). For instance, Toklikishvili et al. (2010) showed that the ACC deaminase-producing endophyte *B. phytofirmans* PsJN could reduce the development of tumours on tomato plants infected with *Agrobacterium* strains. Furthermore, *acdS* deletion mutants of *B. phytofirmans* PsJN, were not able to significantly reduce *Agrobacterium*-induced tumours. Hence, endophytes producing ACC deaminase can act as effective biocontrol agents, and the possibility of using these bacteria to protect plants from biotic stresses is potentially of great importance in improving crop productivity.

MATERIALS AND METHODS

Strain selection and transformation with pRKACC

The endophytic strain *S. grimesii* BXF1 was selected to test the effect of ACC deaminase in its ability to promote rhizobia nodulation and common bean growth. *Serratia grimesii* BXF1 was found to be a generalist endophyte (pine, tomato, cucumber) with plant-growth promoting abilities, however, it does not contain ACC deaminase activity or the *acdS* gene (Nascimento et al. 2016b). Therefore, *S. grimesii* BXF1 was transformed with plasmid pRKACC containing the *acdS* gene of *Pseudomonas* sp. UW4 cloned into the broad-host-range plasmid pRK415 (Shah et al. 1998) by triparental conjugation, as previously reported (Nascimento et al. 2012a). Briefly, overnight grown *Escherichia coli* donor (pRKACC) and helper (containing plasmid pRK600) strains were added to a culture of the recipient strain BXF1 and incubated at 28°C.

Strain BXF1 transformants were selected by their ability to grow in Tryptic Soy Agar (TSA) medium containing both 100 μg/ml ampicillin and 15 μg/ml tetracycline.

Determination of ACC deaminase activity

Both wild-type BXF1 (negative control) and the BXF1 transformed strain were tested for ACC deaminase activity, following a method that quantifies the amount of α-ketobutyrate produced, a product resulting from the cleavage of ACC by ACC deaminase (Penrose and Glick 2003). *Pseudomonas* sp. UW4 was used as a positive control. Bacterial cells were grown at 28°C in tryptic soy broth (TSB) medium supplemented with antibiotics, when necessary. After overnight growth, bacterial cultures were centrifuged at 10 000 g for 5 min, and then suspended in 5 ml of Dworkin and Foster (Dworkin and Foster 1958) salts minimal medium containing 5 mmol L^{-1} ACC as the sole nitrogen source and incubated for 24 h at 28°C. After induction, ACC deaminase activity was measured based on the quantity of α-ketobutyrate formed (Penrose and Glick 2003). Total

protein content was quantified using the Bradford reagent (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. The final ACC deaminase activity was expressed in μmol α-ketobutyrate/ mg protein/ h. The experiment was performed in duplicate.

Plant-growth promotion assays

Bacterial cell culture preparation

Serratia strains were cultured in TSB medium supplemented with the respective antibiotics, whenever necessary, and *R. tropici* CIAT899 was cultured in yeast mannitol broth (Subbarao 1977). Bacterial cultures were grown in a rotary shaker, with agitation of 150 rev min^{-1}, for 2 days at 28°C. Following the 2-day period of growth, cultures were centrifuged at 6000 g for 3 min, and suspended in a 0.03 mol L^{-1} $MgSO_4$ solution. The cell suspension optical density was then adjusted to 0.3 at 600 nm.

Seed disinfection, germination and inoculation

Seeds of common beans (*Phaseolus vulgaris*), black turtle variety, were surface sterilized by a three-step disinfection process. This consisted of submerging the seeds for 1 min in a 70% ethanol solution, followed by 3 min in a 1% commercial sodium hypochlorite solution, and ending with five washes with sterile distilled water to remove any traces of the former solutions. Seeds were germinated on 1% agar plates, in the dark, at 25°C for 3 days. Following the germination period, one seedling was planted per pot (volume of 300 ml) filled with a sterilized mixture of sand and vermiculite (1:1 v/v), and inoculated at the root/shoot junction with 5 ml of each bacterial solution OD_{600} = 0.3 (rhizobia and endophytes respectively) when applicable.

Assay conditions

The assays were conducted, independently, under greenhouse conditions (Universidade Federal de Santa Catarina) in the period of June to August, 2016, in Florianópolis, Brazil (average temperatures of 23°C maximum, and 14°C minimum). Four treatments were employed in each assay: Negative control (without bacterial inoculation), *R. tropici* CIAT899 sole inoculation, CIAT899+ wild-type endophyte co-inoculation and CIAT899+pRKACC transformed endophyte co-inoculation. Each treatment consisted of eight plant replicates. Plants were irrigated every 2 days with 15 ml of a nitrogen-free nutrient solution (Broughton and Dilworth 1971). The plants were collected 20 days after inoculation, and root and shoot dry weight, as well as nodule number were evaluated. Roots and shoots were cut separately and dried at 60°C for 3 days, so that dry weights could be measured on an analytical scale. This experiment was conducted twice.

Plant tissue surface sterilization Root nodules and plant tissues (shoot, leaf, seed) were surface sterilized, by submerging the plant material in 70% ethanol for 1 min, 1% commercial bleach for 10 min, followed by successive washes with sterile deionized water. The washing water was subsequently inocu- lated onto TSA plates overnight to prove the effectiveness of the disinfection process. Bean tissues were ground using sterilized mortars and pestles, and inoculated onto TSA plates.

Disease symptom scoring

Bean plants presented disease symptoms that appeared following seed transfer to pots. A disease symptom scoring scale was developed based on the visualization of infected leafs presenting symptoms, as shown in **Fig. 4a**.

Root colonization imaging

Serratia transformation with pn519ngfp

Strain *S. grimesii* was transformed with plasmid p519ngfp (Mathysse et al. 1996) by the triparental conjugation method described above. Donor *E. coli* carrying the plasmid p519ngf encoding the green fluorescent protein (GFP) was grown overnight in LB medium supplemented with 50 μg/ml kanamycin, while the helper *E. coli* pRK600 and the recipient *S. grimesii* BXF1 were grown as previously described. Transconjugant strains were selected on TSA medium supplemented with 100 μg/ml ampicillin and 150 μg/ml kanamycin.

Confocal microscope visualization

To qualitatively assess the internal colonization of roots and nodules by *S. grimesii* BXF1 expressing GFP, a small assay was conducted using conditions similar to those described above. Roots were collected from plants, 20 days after inoculation, using a sterile scalpel. The plant material was visualized in a Leica TCS SP5 confocal microscope, under different fluorescent wavelengths. Images were composed in the Leica LAS X interface pro- gram (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analyses of all plant experiments were performed with SPSS Statistics ver. 22 software (SPSS Inc., IBM Company, Armonk, NY, USA), using the Student's t test.

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CONFLICT OF INTEREST

No conflict of interest declared.

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RESEARCH MANUSCRIPT

"The endophyte *Serratia grimesii* BXF1 expressing an exogenous ACC deaminase gene delays carnation flower senescence"

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The endophyte *Serratia grimesii* BXF1 expressing an exogenous ACC deaminase gene delays carnation flower senescence

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Keywords: endophyte, ethylene, ACC deaminase, flower senescence, carnation

ABSTRACT

Ethylene plays a relevant role in the promotion of flower senescence and death. Bacteria expressing the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme can decrease plant ACC, the direct ethylene precursor in higher plants, and, consequently, reduce plant ethylene levels.

In this work, the bacterial endophyte *Serratia grimesii* BXF1 and its transformed strain expressing an exogenous ACC deaminase gene were tested for their ability to reduce commercial carnation (Diantus caryophyllus) flower senescence and, consequently, increase flower shelf-life.

The results obtained in this study indicate that the expression of ACC deaminase by a bacterial endophyte increases its ability to delay the senescence of carnation, a flower known for its high sensitivity to ethylene. Ultimately, ACC deaminase-producing endophytes may be of great importance for the development of inoculants with potential use in the flower industry.

INTRODUCTION

Flower senescence can be defined as a series of events that culminate with the death of a flower, which include petals enrolling, loss of color and wilting, and gradual fading of the blossom (Tripathi and Tuteja 2007). One of the agents accountable for the senescence of a flower is the phytohormone ethylene, which is a powerful modulator of the plant growth and development (Van de Poel et al. 2015). Importantly, under stress conditions, plants produce an increased level of ethylene ("stress ethylene") which leads to an augmented senescence and, ultimately, plant death (Glick et al. 2007). In all higher plants, ethylene is synthesized via a methionine-dependent process described as the Yang Cycle (Yang and Hoffman, 1984). Methionine is converted to S-adenosylmethionine (SAM) by SAM synthase. In turn, SAM is converted to the direct ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by the action of the ACC synthase enzyme. Ultimately, ACC is transformed to ethylene by the action of the ACC oxidase enzyme (Yang and Hoffman 1984).

Different flower species respond differently to ethylene. In this sense, a flower's sensitivity to ethylene can be graded from insensitive to highly-sensitive, on a five-category scale (Ali et al. 2012). Whilst monocotyledonous plants are generally ethylene insensitive, flowers such as zinnia, carnation, rose and geranium are regarded as highly sensitive, as most of dicotyledonous plants (Woltering and Van Doorn 1988, Van Doorn 2001). Hence, decreasing ethylene levels in these species is important for an increased plant resistance and delayed senescence, which results in an increased flower shelf-life.

Some plant-associated bacteria and fungi express ACC deaminase, an enzyme that catalyzes the cleavage of ACC into ammonia and α-ketobutyrate (Nascimento et al., 2014). By producing ACC deaminase, these microorganisms can modulate plant ethylene levels, and, thus, assist the plant in overcoming some deleterious effects if this hormone (Glick, 2014). For example, bacteria expressing ACC deaminase delayed the senescence of minicarnation flowers, thus, increasing its shelf-life (Ali et al. 2012).

In our previous work, we engineered the endophytic, plant-growth promoting and antagonistic bacteria *Serratia grimesii* BXF1 (Nascimento et al. 2016), to express ACC deaminase (Tavares et al., 2018), which resulted in the increased ability to promote the nodulation profile of common bean and protected the plant against pathogen infection.

In this study, we investigate the effect of the expression of an exogenous ACC deaminase gene by *Serratia grimesii* BXF1 and its role in delaying the senescence of commercial carnation, a flower regarded as highly-sensitive to ethylene.

MATERIALS AND METHODS

Strains

Serratia grimesii BXF1 is a plant-growth promoting endophyte with the ability to successfully colonize and promote the growth of several plant species (Nascimento et al. 2016). *Serratia grimesii* BXF1 was transformed with the plasmid pRKACC, containing the plasmid pRK415 with the *acdS* gene of *Pseudomonas* sp. UW4, and its flanking regions (Tavares et al. 2018).

S. grimesii BXF1 and *S. grimesii* BXF1-pRKACC were grown in Tryptic Soy Agar (TSA) plate, at 28°C. Transformants were selected by their ability to grow in TSA containing 15 µg/mL tetracycline.

Carnation flower assay

Bacterial cell culture preparation

S. grimesii BXF1and BXF1-pRKACC strains were grown in TSB medium, supplemented with 15 µg/mL tetracycline (in the case of the transformed strain). Incubation occurred overnight, at 28ºC, in a rotary shaker at 150 r.p.m. Following this period, cells were centrifuged at 6000 g for 3 minutes, and the pellet resuspended in 0.03 M $MgSO_4$. Optical density of the bacterial cultures was then adjusted to OD_{600} = 0.15 using a spectrophotometer.

Assay conditions

Fresh carnation flowers were acquired from a commercial store in Florianópolis, Brazil, being selected by their similar size and characteristics, to decrease variability. Flowers were then cut to have 20 centimeters, from the base of the shoot to the receptacle, and immediately placed in sterile test tubes which received 5 mL of the respective bacterial solution (OD_{600} = 0.15) or 5 mL 0.03M $MgSO_4$, in the case of the control.

Ten carnation flowers were used per treatment: control (without bacteria), strain *S. grimesii* BXF1 and *S. grimesii* BXF1-pRKACC. After 48h two plants were removed for the assessment of BXF1 wild-type and BXF1-pRKACC colonization abilities by PCR (described below).

The assay was conducted for 8 days, under laboratory conditions (average temperature of 24ºC), and repeated. All flowers were irrigated at the shoot tip with 2 mL of sterile 0.03 M $MgSO_4$, whenever necessary, to maintain the shoot tip submerged at all

times. The rate of flower senescence was evaluated everyday according to a senescence scale (**Figure 1**).

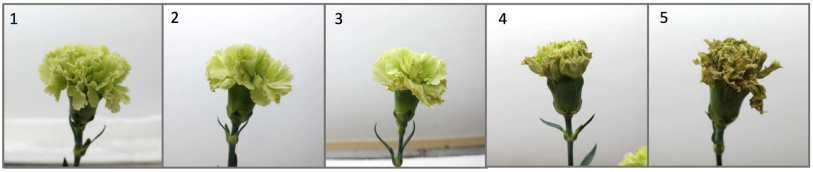

Figure 1- Scale of senescence symptoms presented by carnation flowers. 1- no symptoms of senescence (fresh cut flower); 2- Minimal yellowing in the tip of the petal. Blossom and color remain intact; 3 – Petals enrolling and minimal color loss; 4 – Petals deeply enrolled, evident loss of color and fading; 5 – Dead flower: severe petal enrolling and colorless decayed blossom

Assessment of bacterial endophytic colonization in the carnation flower shoot by PCR

In order to qualitatively assess the bacterial (*S. grimesii* BXF1 and *S. grimesii* BXF1-pRKACC) ability to endophytically colonize and migrate through the shoot of a flower, two plants of each bacterial treatment were collected 48 hours after inoculation, and had their shoots separated from the flower. Each plant shoot was surface disinfected with ethanol 70%, cut, and two radial sections of 1 cm were removed, the first section corresponding to the shoot base (0-1cm), and the second to a higher portion of the shoot (10-11 cm above the shoot tip) (**Figure 2**).

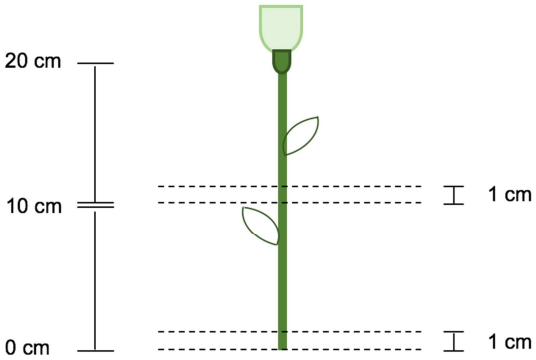

Figure 2- Schematic representation of the flower sectioning in the basis (0-1cm) and in the middle (10-11cm) of the shoot, performed for DNA extraction.

DNA extraction

Each plant section, weighting approximately 100 mg, was flash frozen using liquid nitrogen and grounded with a sterile pestle, to mechanically disrupt cells and increase the efficiency of cell lysis. After disruption, total DNA was extracted using the Plant/Fungi DNA Isolation Kit MiniPrep (Norgen, Canada) according to the manufacturer's protocol. The obtained total DNA was quantified using a NanoDrop (Thermofisher) according to the manufacturer's protocol, and adjusted to a concentration of 50 ng/µL.

Primer design and PCR conditions

PCR was performed to qualitatively confirm the presence of strains BXF1 wild-type and pRKACC inside the carnation flowers. Primer design was based on the internal region of the BXF1 *chiD* gene (single copy gene), SGBXF1_01157, (encoding a chitinase), which was found to be present in strain BXF1 genome (Genbank accession: LT883155.1) and only present in *Serratia* strains (Nascimento et al., 2018). The selected region diverged from all other chitinase genes of *Serratia* available in the NCBI database. Sequence comparisons were made using Blast analysis (http:// blast.ncbi.nlm.nih.gov), and primers were designed using Primer 3 (http://primer3.ut.ee) with default parameters, being chosen based on the region of interest (variable region only found in strain BXF1). The selected primers were: chiDF 5'-CGTCTTACCAGCAGCATTGA-3'; and chiDR 5'-CAGGCACCTTTACCACCATT-3', being able to amplify a 225 bp fragment.

For the amplification reaction, it was used a reaction volume of 25 µL, containing 2.5 µL of 10X Taq —DNA Polymerase Buffer, 2 µL of 25mM MgCl2, 0.5 µL of 10 mM dNTP's, 5 µL of each primer (5 pmol), 0.1 µl of DNA Taq Polymerase (1 U), 8.9 µl of MilliQ water, and 1 µl of DNA (50 ng/µl). PCR program ran with an initialization step of 4 minutes, at 94ºC, followed by 35 cycles of: denaturation at 94ºC (45 seconds), annealing 52ºC (1 minute), and elongation at 72ºC (1.5 minutes), and ending with the final elongation step of 10 minutes at 72ºC. PCR products were submitted to gel electrophoresis in agarose gel (1%) and visualized in a UV transilluminator.

Statistical analysis

Statistical analysis in all plant experiments was performed by T-student test by using the SPSS Statistics v.22 software (SPSS Inc., IBM Company).

RESULTS

Expression of exogenous ACC deaminase gene increased *S. grimesii* BXF1 ability to delay carnation senescence

The effect of endophytic ACC deaminase on decreasing the senescence rate of carnation, was evaluated. During 8 days, the senescence symptoms for 8 plants were evaluated (**Figure 3**). Generally, inoculation with the wild-type BXF1 did not affect the senescence rate, when compared to the control treatment (**Figure 3**). However, the presence of strain BXF1-pRKACC significantly delayed flower senescence to a considerable extent, when compared to results obtained with the wild-type strain of *S. grimesii* BXF1 or the control. The beneficial effects of strain BXF1-pRKACC were more evident 4 days after inoculation (**Figure 3**), suggesting that the effects of ethylene in senescence only start to take effect in latter periods post flower cutting.

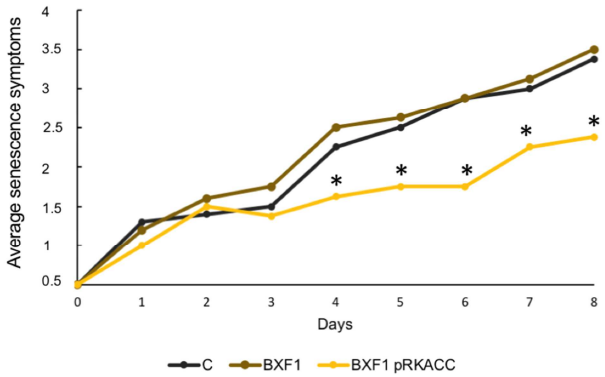

Figure 3 – Variation of the senescence symptoms presented by carnation flowers inoculated with *S. grimesii* BXF1 and *S. grimesii* BXF1-pRKACC, during 8 days. Statistical significant differences ($P < 0.05$) are marked with *

Eight days after inoculation, carnation flowers treated with BXF1-pRKACC (**Figure 4A**) presented less senescence symptoms when compared to flowers inoculated with the wild-type BXF1 (**Figure 4B**), and the non-inoculated control (**Figure 4C**), indicating the role of ACC deaminase in the regulation of flower ethylene levels.

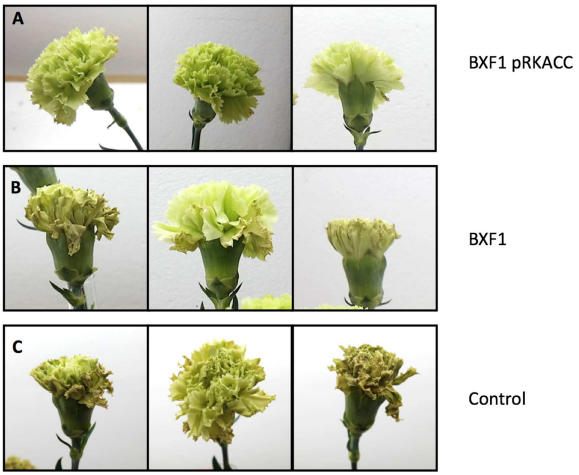

Figure 4- Differences in senescence levels of carnation flowers 8 days after inoculation with A) *S. grimesii* BXF1-pRKACC, B) *S. grimesii* BXF1, C) Non-inoculated control.

Strain BXF1 and BXF1-pRKACC migrate and colonize carnation internal plant tissues

By amplifying the *chiD* gene of strain BXF1 it was possible to ascertain the endophytic colonization abilities of both BXF1 wild-type and BXF1-pRKACC strains in carnation flowers. In this sense, both strains could be detected inside the shoot of carnation flowers 48h after inoculation and at least 10 cm away from the inoculation point (**Figure 5**), thus confirming the successful endophytic colonization abilities of these strains. No amplification product was observed in non-inoculated plants (**Figure 5**).

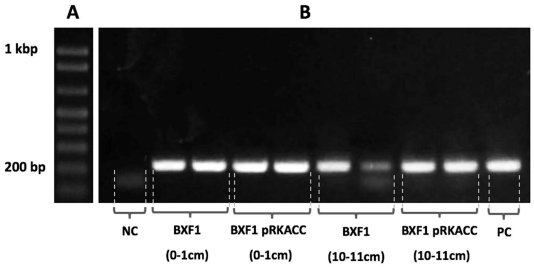

Figure 5- Agarose gel visualization of *chiD* amplification. A) DNA ladder (1 Kb Plus, Invitrogen); B) 1 – Negative control; 2,3 – BXF1 (0-1cm); 4,5 – BXF1-pRKACC (0-1cm); 6,7 – BXF1 (10-11cm); 8,9 – BXF1-pRKACC (10-11cm); 10 – Positive control.

DISCUSSION

In this work, the bacterial endophyte *Serratia grimesii* BXF1 and its transformed strain expressing an exogenous ACC deaminase gene were tested for their ability to reduce senescence levels in commercial carnation flowers.

The results obtained herein showed a significant delay of the senescence symptoms presented by carnation flowers treated with the transformed strain *S. grimesii* BXF1-pRKACC, when in comparison to the carnation flowers inoculated with the BXF1 wild-type strain and non-inoculated control, which senesced at similar rates throughout the experiment. This result indicates that ACC deaminase plays an important role in increasing the plant growth promotion potential of the BXF1 strain, which is consistent with previous studies demonstrating that the expression of exogenous ACC deaminase genes greatly improves the nodulation and plant-growth promotion abilities of a variety of bacterial strains, including biocontrol rhizospheric *Pseudomonas* (Shah et al. 1998), *Sinorhizobium* (Ma et al. 2004, Kong et al, 2015), *Mesorhizobium* (Conforte et al. 2010; Nascimento et al., 2012ab; Brígido et al. 2013) and the endophytic biocontrol agent *Serratia grimesii* BXF1 itself (Tavares et al. 2018).

In a previous study, Ali et al. (2012) demonstrated that endophytic strains expressing ACC deaminase are more effective in delaying minicarnation flower senescence than rhizospheric bacteria, since the latter cannot properly sequester and cleave ACC. As bacterial endophytes can internally colonize flower tissues, their ACC deaminase activity can perform as a sink for plant internal plant ACC concentrations, consequently decreasing the deleterious ethylene levels responsible for flower senescence.

In this work, we demonstrated that 48h after inoculation at the shoot tip, both strains BXF1 and BXF1-pRKACC were present in internal shoot tissues of carnation (10 cm away from the shoot tip), thus confirming their migratory and endophytic behavior. Considering that only the BXF1-pRKACC strain, expressing ACC deaminase, was able to delay flower senescence, it can be suggested that the presence of ACC deaminase has a great importance in reducing the available pool of ACC in flower tissues (and consequently, ethylene), thus delaying the senescence process, as previously suggested by Ali et al. (2012).

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CHAPTER 5

Isolation and characterization of ACC deaminase-producing bacteria

RESEARCH/THESIS MANUSCRIPT

"Methodology for the isolation of soil and plant-associated bacteria with phytohormone-degrading activities"

By Francisco X. Nascimento, Bernard R. Glick and Márcio J. Rossi

Manuscript in preparation

Participation: conceptualized and performed all the experimental work, writing of the manuscript.

The manuscript and the references therein are formatted according to the BMC Microbiology journal guidelines.

Methodology for the isolation of soil and plant-associated bacteria with phytohormone-degrading activities

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Keywords: Plant growth-promoting bacteria, rhizosphere, endophytes, phytohormones, ethylene, indole-3-acetate, salicylate

1. BACKGROUND

Modern agricultural practices, aiming to produce food for human and animal consumption, rely on the extensive use of environmentally polluting chemical fertilizers. This leads to environmental damage and the subsequent destruction of natural resources and ecosystems. Furthermore, as a consequence of climate change and human activities, much of the world's arable land is now degraded and presents conditions that are inhibitory to plant development. One way to address these problems includes the use of efficient plant-growth-promoting bacteria (PGPB) that tightly associate and promote plant development under a variety of stress conditions [1].

Plant growth and development is impacted by a number of different factors, including internal cues such as phytohormone biosynthesis and signaling [2]. Importantly, phytohormones not only regulate plant development but also plant-microbe interactions. In this regard, the phytohormones ethylene (ET) and salicylate (SA) act as major regulators of plant developmental programs and the bacterial colonization process due to their participation in the plant immune responses [3,4]. In addition, auxins, mainly in the form of indole-3-acetic acid (IAA), are known regulators of plant growth [5] and play an important role in plant-microbe interactions [6,7].

Bacteria that associate with plants possess the ability to modulate plant hormone levels by either producing or catabolizing phytohormones. While many aspects of bacterial phytohormone production (mainly IAA production) have been described and studied [6,7], much less is understood about the impact of bacterial phytohormone degradation in plant-microbe interactions. Nevertheless, several studies point to the important role of bacterial phytohormone degradation in modulating plant responses to bacteria colonization as well as to biotic and abiotic stresses.

Some bacteria can produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC (the immediate precursor of ethylene in all higher plants) into ammonia and α-ketobutyrate, consequently decreasing both ACC and ET levels within plant tissues and the plant rhizosphere (the area immediately surrounding plant roots) [8]. Bacteria expressing the enzyme ACC deaminase have shown to promote the growth of several plant species under a variety of stress conditions [9].

Degradation of SA is a trait found in some soil and plant-associated bacteria [4,10,11]. The degradation of SA results from the action of the SA hydroxylase enzymes which can convert SA to catechol (catalyzed by SA-1-hydroxylase) [12] or gentisate

(catalyzed by SA-5-hydroxylase) [10], and, alternatively, a salicylyl-CoA 5-hydroxylase that converts salicylyl-CoA to gentisyl-CoA [13]. SA-degrading bacteria were found to be enriched in the rhizosphere and endophytic compartment of *Arabidopsis* plants treated with SA, suggesting that SA-degradation is involved in the colonization abilities of *Arabidopsis*-associated bacteria [4]. Recently, Lowe-Power et al., [14] showed that degradation of SA protects the plant pathogenic bacterium *Ralstonia solanacearum* from SA toxicity and enhances the virulence of this bacterium toward tobacco but not tomato. Additionally, SA is an intermediate compound in the bacterial degradation of several polluting aromatic compounds found in soils, such as naphthalene and phenantrene [10].

Many plant-associated bacteria not only produce but are also able to consume IAA. This is the case of several *Bradyrhizobium*, *Azoarcus*, *Paraburkholderia*, *Pseudomonas*, *Acinetobacter*, *Arthrobacter* and *Rhodococcus* strains [15–19]. For instance, through the action of the IAA monooxygenase (and other enzymes present in the IAA catabolism -*iac*- gene cluster) IAA is converted to catechol by the plant-associated bacterium *Pseudomonas putida* 1290 [19]. In addition, several *Bradyrhizobium japonicum* strains catabolize IAA to anthranilate via isatinate [16]. *Azoarcus* degrades IAA via an anaerobic pathway that leads to the formation of either 2-aminobenzoyl-CoA or benzoyl-CoA [17]. Moreover, IAA degradation in the soil and rhizosphere mostly results from microbial activity. For example, Raczkowska-Błach et al., [20] demonstrated that IAA degradation abilities were present in 72.9% of bacterial strains that were found in the *Pinus sylvestris* rhizosphere.

IAA degradation plays an important role in the plant-growth-promoting traits of some PGPB. The *P. putida* 1290 *iacH* mutant, was significantly affected in its ability to abolish the deleterious effect of IAA on radish root elongation [19]. The wild-type *Paraburkholderia phytofirmans* PsJN but not the *iacC* mutant is able to restore primary root length in roots of *A. thaliana* in the presence of exogenous IAA [18].

The presence of phytohormone degradation genes amongst symbiotic and other soil and plant-associated bacteria indicates that the ability to modulate phytohormone concentrations in planta and in soils plays an important role in the functioning of these bacteria as well as in plant-microbe interactions. Moreover, these bacteria play a significant role in microbiome development and overall plant growth [3].

Here, we describe and discuss strategies and a targeted approach methodology for the simple isolation of soil and plant-

associated bacteria (including rhizospheric, endophytic and phyllospheric strains) with the ability to directly degrade phytohormones (SA and IAA) or its precursors (i.e. ACC the ethylene precursor). This methodology can be employed not only to isolate new bacterial strains, but also to screen for ACC, SA and IAA degradation abilities in previously obtained bacterial strain collections. The methodology described in this work was designed in order to be simple, less laborious, less expensive and, particularly, accessible for most laboratories and scientists in the developing world.

2. METHODS

2.1 Selection of the source material

2.1.1 Bacteria colonizing specific plant tissues

Different source materials can be used for the selection of bacteria colonizing plant tissues, structures (e.g. root nodules) and the rhizosphere (**Table 1**). These bacteria can have different roles in plant growth promotion and protection.

Most of the plant-growth promoting bacteria described in the literature are isolated from soil or rhizosphere of selected plants. However, this can represent a limitation since their consequent application may be limited to soil, plant roots or the external surfaces of plant seeds. Alternatively, bacteria can be isolated from within plant tissues and this may present several advantages in future applications. Thus, for example, plant growth-promoting endophytes can be used to inoculate plants at the flowering stage, which may lead to the bacterial colonization of the new produced seeds [21]. This may not only lead to increased plant growth but can also lead to an increased level of protection against some pathogens, since these endophytes may directly compete with many pathogens that are transmitted via seeds. Bacterial endophytes can also be protected from the competitive soil environment, which may impact bacterial performance (e.g. plant growth promotion abilities or degradation of xenobiotics) [22]. Therefore, the isolation and selection of endophytes may lead to the development of more efficient inoculants.

Table 1- Plant-associated bacteria and their potential applications.

Bacteria	Source	Application examples
Rhizospheric	Soil surrounding roots	General plant-growth promotion and protection, phytoremediation
Root endophyte	Root tissue	General plant-growth promotion and protection, phytoremediation
Root nodule endophyte	Root nodules	Nitrogen fixation, Rhizobia-helper bacteria, indirect increase in nodulation and nitrogen fixation
Shoot endophyte	Shoot tissue	General plant-growth promotion and protection, Nitrogen fixation
Leaf endophyte	Leaf tissue	Aerial tissue growth promotion, plant protection, Nitrogen fixation
Flower endophyte	Flower tissue	Plant reproductive organ development, regulation of flower senescence
Fruit endophyte	Fruit tissue	Regulation of fruit development and senescence, pathogen protection, seed colonization
Seed endophyte	Seeds	Seed germination, seed protection, general plant-growth promotion
Leaf epiphyte	Leaf surface tissue	Aerial tissue growth promotion, plant protection

Leaf-associated bacteria (epiphytes or endophytes) can also be extremely useful, especially at the level of field application. Most of these bacteria can cope with the stresses presented in the leaf environment, which most rhizobacteria cannot endure (e.g. temperature shifts, UV radiation, desiccation) [23]. Leaf-associated bacteria may also directly compete with many of the plant pathogens colonizing leaf tissues (e.g. *Pseudomonas syringae*, *Xanthomonas* spp.)[23]. One of the most important aspects of these bacteria resides in the possibility of their direct application onto leaves (spraying) and their subsequent ability to colonize plant tissues. Spraying is a common agricultural practice (e.g. application of pesticides or herbicides) and may facilitate the acceptance of bacterial inoculants amongst farmers.

2.1.2 Isolation of bacteria: natural versus artificial selection systems

Plant and rhizosphere samples can be obtained from plants growing in wild and natural habitats (herein termed natural conditions) or by using selected soils, conditions and trap plants (herein termed artificial conditions). Each system has its own advantages and disadvantages and its use may depend on diverse factors, such as, availability of material, reagents, equipment (e.g. growth chambers and greenhouses) and time (**Table 2**). Natural

conditions are representative of native soils and specific environments (i.e. climate adaptations) and this fact may lead to the easier isolation of bacteria naturally adapted to such situations. Artificial conditions can be created to select for desirable traits. For example, promiscuous leguminous plants such as *Phaseoulus vulgaris* or *Vigna unguiculata* are often used as trap plants to isolate rhizobia species present and adapted to certain soils. Then these strains can be tested again for their symbiotic efficiency.

Table 2- Considerations regarding the isolation of bacteria: natural versus artificial selection systems**.**

Source	Advantages	Disadvantages
Natural conditions	More representative of field conditions Increased diversity of plants to sample Climate and microclimate adaptations Less expensive Less time consuming	Lower control of conditions
Artificial conditions	Selection and control of conditions Selection of plants and specific cultivars Selection and manipulation of soil conditions Obtaining bacteria adapted to specific conditions used for commercial applications	Time consuming More expensive

2.2 Sample preparation

2.2.1 Rhizospheric bacteria

a) (Natural or artificial systems)- Remove the plant from soil, cut the shoot with a sterilized scalpel or similar cutting instrument, and store the root in a sealed sterile container (e.g. Falcon tube, sterile plate), using disinfected forceps (ethanol 70%, 2 min, or autoclaved 121ºC, 15 min). Store the root and associated soil at 4ºC until further use.

b) (Under sterile conditions) Remove the soil associated with the root system with the help of sterile forceps and store the rhizospheric soil in a sterile 50 ml falcon tube or similar container (e.g. Erlenmeyer flask, plate) containing a sterile solution of 30 mM $MgSO_4$ or 1X Phosphate Buffered Saline (PBS) (the amount may depend on the amount of soil to sample). Usually, only a small amount of soil (100 mg) is necessary to isolate bacteria. However, additional material may be used when working in marginal or degraded soils harboring fewer bacteria.

c) Alternatively, directly dip a small section of the root (i.e. 5-10 cm) several times in a sterile 10 ml solution of 30 mM $MgSO_4$ or PBS.

d) Vortex the solution for 30 secs to break soil aggregates.

e) Perform serial dilutions using 30 mM $MgSO_4$ or PBS 1X. Directly use the solutions (described below) or store them at 4ºC for further use.

2.2.2 Root and root nodule endophytes

a) Repeat step a of the procedure described in 2.2.1

b) Wash the root for several times with water to remove soil aggregates and rhizospheric bacteria. Repeat until the root system is clearly visible and free of soil particles.

c) Surface disinfect the root tissue or the root nodule by rinsing it with 70% ethanol and then with 1% bleach. This procedure may vary depending on the plant species and age. For plants with small and thin roots (e.g. tomato) a soft surface disinfection procedure is recommended. This can be accomplished by treating the roots with 70% ethanol for 1.5 min, 1% bleach solution for 10 min and 5 consecutive washes with sterile distilled water. For harder and thicker roots (e.g. tree species) and root nodules an increased time in the disinfection solutions is recommended (e.g. 2.5 min in 70% ethanol and 15 min in 1% bleach followed by 5 consecutive washes with sterile distilled water).

d) Crush a small section of the root tissue (i.e. 5-10 cm long) with the help of a sterile mortar and pestle. Add 1 ml of 30 mM $MgSO_4$ or PBS to the crushed tissue. Grind the tissue.

e) Remove the surface disinfected root nodules (3 or 4) from roots with a sterile forceps and transfer to a sterile 2 ml tube containing 500 μl of sterile 30 mM $MgSO_4$ or PBS. Crush the nodules with the help of a sterile micropestle.

f) Perform serial dilutions using 30 mM $MgSO_4$ or PBS 1X. Directly use the solutions (described below) or store them at 4ºC (for up to several days) for further use.

2.2.3 Shoot, leaf, flower and fruit endophytes

a) (Natural or artificial systems)- Cut the shoot, leaves, flower (or portions of it) with a sterile scalpel or similar cutting instrument, and place it in a sealed sterile container (e.g. Falcon tube, sterile plate), using disinfected forceps. Store the tissues at 4ºC for a short period of time (up to several days).

b) Cut small sections of shoots (i.e. 2 cm long) or leafs (i.e. 2 x 2 cm) with a sterile scalpel.

c) Repeat step c) described in procedure 2.2.2

d) Alternatively, after disinfection, cut small sections (i.e. 2 cm long) of shoot tissue and place 2 or 3 sections in sterile falcon tubes containing 5 ml of 30 mM $MgSO_4$ or PBS. Incubate overnight at room temperature with shaking (150 rpm). This procedure is useful for the isolation of endophytes from woody tissues, which are difficult to

grind. Endophytes present in tissues will be released to the liquid medium which can then be used for isolation procedures

e) Perform serial dilutions using 30 mM $MgSO_4$ or PBS 1X. Directly use the solutions (described below) or store them at 4ºC (for up to several days) for further use.

3. Targeted approach: Isolation of phytohormone-degrading bacteria from plant and soil samples

This easy and targeted methodology is based on bacterial enrichment by using a minimal medium containing the selected phytohormone as the sole carbon or nitrogen source. Any of the solutions previously employed in procedures described in section 2 can be used to isolate these bacteria. Using this simple isolation technique (**Figure 1**), a wide range of phytohormone-degrading bacteria can be easily isolated.

All growth media and solutions used in the following section are described in the supplemental materials.

3.1 Enrichment

3.1.1 ACC

a) Inoculate 20 to 50 μl of the solution obtained as described in section 2 in 5 ml of liquid Dworkin and Foster (DF), or M9 minimal medium, containing ACC (in a final concentration of 3 mM) as the sole nitrogen source, and incubate at 28ºC (or selected temperature) in an orbital shaker (150 rpm) for 4-12 days. After observing increased bacterial growth (typically ~5 days), 10 to 20 μl of the bacterial suspension can be plated onto specific media and colonies isolated.

3.1.2 SA or IAA

a) Inoculate 20-50 μl of the solution obtained as described in section 2 in 5 ml of liquid DF, or M9 minimal medium, containing SA or IAA (in a final concentration of 1 mM) as the sole carbon source, and incubate at 28ºC (or selected temperature) in an orbital shaker (150 rpm) for 4-12 days. After observing increased bacterial growth (typically ~7 days), 10 to 20 μl of the bacterial suspension can be plated onto specific media and colonies isolated.

Note: The more diluted the sample the more time that it will take the bacteria to grow in the enrichment medium. On the other hand, more concentrated samples (especially rhizosphere samples) may contain contaminating bacteria that can survive in the enrichment medium, even though these bacteria are not able to use phytohormones as sole carbon or nitrogen sources. To decrease the presence of contaminants, each of the steps 3.1.1 and 3.1.2 can be repeated.

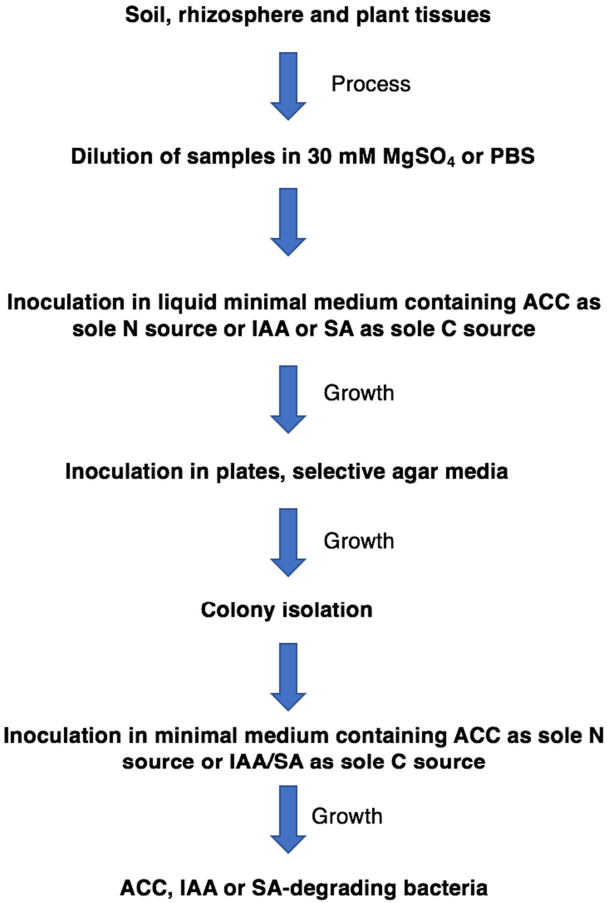

Figure 1- Schematic representation of the methodology aiming the rapid isolation of phytohormone-degrading bacteria.

3.2 Isolation

Although a generic growth medium can be used, in an effort to isolate a wide range of different phytohormone-degrading bacteria, it is also possible to use specific media and perform a second targeted approach to isolate particular bacterial groups.

Examples:

Pseudomonas: Plate 10 to 20 μl of the enrichment solution in *Pseudomonas* agar (also known as King's B -KB-) supplemented

with 100 μg/ml ampicillin. Most *Pseudomonas* strains produce fluorescent pigments in KB medium and are naturally able to resist high concentrations of ampicillin.

Actinobacteria: Plate 10 to 20 μl of the enrichment solution in Actinomycete isolation agar (AIA). This medium favors the growth of Actinobacteria.

Rhizobia: Plate 10 to 20 μl of the enrichment solution in Yeast Mannitol Agar (YMA) supplemented with Congo red (25 mg/L). Most rhizobia present whitish mucoid colonies in this medium.

Note: Additionally, plates can also be incubated at different temperatures which can promote the growth of specific bacteria (e.g. 7ºC for psychrophilic bacteria or 50ºC for thermophiles).

3.3 Confirmation of phytohormone-degradation abilities

3.3.1 Determination of ACC degradation

Qualitative ACC degradation can be easily confirmed by testing the bacteria isolated in step 3.2 for its ability to grow in minimal medium containing ACC as sole nitrogen source.

The following steps should be performed in duplicate:

a) Inoculate a colony in 5 ml DF or M9 medium containing 3 mM ACC as the sole nitrogen source (tester).

b) Inoculate a colony in 5 ml DF or M9 medium without any nitrogen source (negative control).

c) Incubate the inoculated media at 28ºC, 150-200 rpm for 5 days.

d) Measure and compare the OD_{600} of both bacterial solutions.

e) A positive ACC deaminase activity is found in strains that can grow on minimal medium containing ACC, but not in minimal media without nitrogen source.

Note: It is preferred to do this experiment in 50 ml falcon tubes or glass test tubs. In plate/well assays (e.g. 96-well plates) the growth medium often evaporates due to the long time needed to perform the experiment.

In this experiment is important to test the negative control. In some instances, some nitrogen-fixing bacteria can grow on minimal medium containing ACC as the sole nitrogen source, but can be negative for ACC deaminase activity. In dubious cases a quantitative ACC deaminase activity measurement is necessary. ACC deaminase activity can be tested using a simplified version of the method described by Penrose and Glick [24]. This can be performed either qualitatively or quantitatively, however, qualitative determination is more accessible for the standard microbiology lab.

3.3.2 Induction of ACC deaminase expression

a) Grow the selected bacteria in 5 ml of a rich medium (e.g. TSB, YMB) in a 50-ml falcon tube until luxuriant growth is achieved. This depends on the bacterial strain. Usually, *Pseudomonas* grow very well in 24 h, but other strains, such as, rhizobia or some Actinobacteria grow more slowly (48 to 72 h). Incubate at 28ºC, 150-200 rpm.

b) Centrifuge the 50-ml falcon tube at 4000 rpm in a benchtop centrifuge for 10 min and discard the supernatant.

c) Suspend and wash the cell pellet in either 5 ml DF or M9 minimal medium without a nitrogen source. Centrifuge at 4000 rpm for 10 min. Discard the supernatant.

d) Suspend the cell pellet in either 5 ml DF or M9 minimal medium containing 3 mM ACC as the sole nitrogen source. Incubate for 24h to 48h at 28ºC, 150-200 rpm. This step induces ACC deaminase activity.

e) Centrifuge the tube at 4000 rpm for 10 min and discard the supernatant.

f) Suspend the cell pellet in 1 ml 0.1M Tris-HCl pH 8.0 and transfer it to a 1.5 ml tube. Centrifuge in a micro-centrifuge the suspended cells at 10000 rpm for 1.5 min.

g) Remove the supernatant and suspend the cells in 400 μl of 0.1M Tris-HCl pH 8.0.

3.3.3. ACC deaminase activity determination

a) Add 20 μl toluene and vortex for 30 seconds (cell permeablization). This step is crucial for effectively measuring ACC deaminase activity. Some bacterial strains are more resistant to the procedure; in that case glass beads can be added to the lysate (1:2 v/v) to help disrupting the cell membrane. Vortex for additional 30 seconds. Note that ACC deaminase is a cytoplasmic enzyme [25].

b) Dispense 50 μl of lysate into 1.5 ml centrifuge tubes: Two tubes for lysate + ACC (tester); Two tubes for lysate and no ACC (negative control). Also include an internal control: one tube containing 50 μl 0.1M Tris-HCl pH 8.0 + ACC. Save the rest of the lysate at 4ºC or -20ºC (for longer periods) for protein concentration measurements or assay repetition.

c) Add 5 μl of 0.3M ACC to each 1.5 ml tube containing 50 μl of lysate (except for the negative controls of each sample) and vortex, approximately 5 secs.

d) Incubate at 30ºC for 30 min.

e) Add 500 μl 0.56M HCl and vortex, ~5 sec and centrifuge for 5 min at 10000 rpm.

f) Prepare standards 0.05-0.5 μmol α-ketobutyrate dissolved in 0.1M Tris-HCl pH 8.0 buffer.

g) Add 500 μl supernatant or standard to a glass test tube (13x100 mm) and then add 400 μl 0.56M HCl.

3.3.4 Derivatization and quantification of -ketobutyrate

a) Add 150 μl DNP reagent (0.2% 2,4-dinitrophenylhydrazine in 2 N HCl) and vortex, ~5 sec

b) Incubate at 30ºC for 30 min.

c) Add 1 ml 2 N NaOH and vortex, ~5 sec

d) Decant into cuvettes and read the OD at 540 nm. Use 50 μl Tris HCl + 900 μl 0.56 M HCl + 150 μl DNP + 1 ml 2 N NaOH as a blank.

Note: The derivatization step does not account for ACC deaminase activity. In this step, the enzyme is inactive due to the acidic pH, and the unique purpose is to derivatize phenylhydrazine to phenylhydrazone.

3.3.5 Protein content measurement

Measure protein content of 50 μl lysate. This can be achieved by using the Bradford reagent following the manufacturers specification and using a Bovine Serum Albumin (BSA) standard curve.

3.3.6 Final representation of ACC deaminase activity

The final ACC deaminase activity should be expressed in μmol α-ketobutyrate/mg protein/hour. It is calculated in the following manner:

α-ketobutyrate in sample = [OD_{540} sample (sample + ACC)] – [OD_{540} negative control (sample without ACC)].

Use the α-ketobutyrate standard curve (e.g. **Figure 2**) to calculate the correct α-ketobutyrate value.

The obtained α-ketobutyrate value is divided by the amount of protein present in 50 μl lysate. This value is then multiplied by 2 since the assay for ACC deaminase activity was determined in only half an hour.

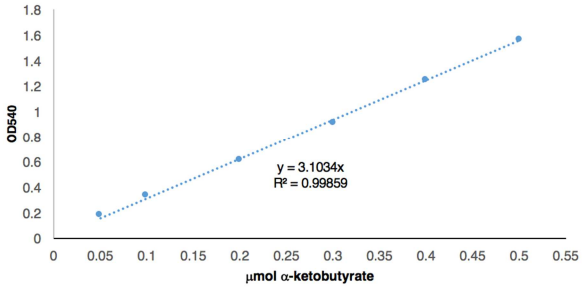

Figure 2- α-ketobutyrate standard curve.

The sole qualitative estimation of ACC deaminase activity can be made by visualizing the production of α-ketobutyrate (**Figure 3**) and by measuring ODs. This does not require either a standard curve or protein quantification. In this case only the amount of α-ketobutyrate in sample (calculated as previously described) is considered.

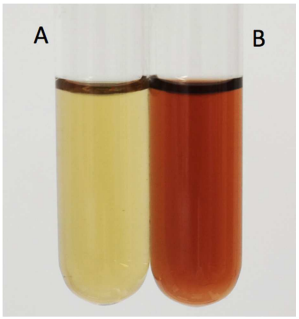

Figure 3- Quantification of α-ketobutyrate present in samples. A) Control sample (incubated without ACC); no α-ketobutyrate produced. B) Sample (incubated with ACC); α-ketobutyrate produced.

Theoretically, if the relative α-ketobutyrate in sample > 0 then the sample is positive for ACC deaminase. Nevertheless, these values need to be interpreted carefully. Most times values close to 0 (ranging from 0 to 0.08) are deemed to represent non-specific enzymatic activities from ACC deaminase related enzymes. This occurs frequently in Enterobacteriaceae, and for example, in some *Pseudomonas* and *Bacillus* spp. which possess D-cysteine desulfhydrase. In these cases, with would be necessary to ascertain the presence of true ACC deaminase genes in these strains.

3.3.7 Qualitative determination of SA and IAA degradation

Qualitative IAA or SA degradation can be easily confirmed by testing the isolated bacterial cells for their ability to grow in minimal medium containing IAA or SA as a sole carbon source.

The following is typically performed in duplicate:

a) Inoculate a small amount of a bacterial colony in 5 ml DF or M9 medium containing 1 mM IAA/SA as the sole carbon source (tester).

b) Inoculate the same bacterial colony in 5 ml DF or M9 medium without any carbon source (negative control).

c) Incubate the bacterial cell suspension at 28ºC, 150 rpm for 5 days (or more depending on the bacterium).

d) Measure and compare the OD_{600} of both bacterial solutions, i.e. a and b above.

A positive SA/IAA degradation activity is inferred from strains that can grow on minimal medium containing SA/IAA, but not in minimal media without an added carbon source.

3.3.8 SA degradation test

Alternatively, the SA degradation test can be performed in 48 or 24-well plates containing minimal medium supplemented with 1 mM SA and 0.8% agar (e.g. 24-well plates = 1 ml M9 medium containing 1 mM SA as sole carbon source per well). In this case, 5 μl of an overnight grown culture (grown in general rich medium) is inoculated in the center of the plate/well. The plate is then incubated for 24 h at 28ºC.

SA is fluorescent under UV radiation, so, SA degradation can easily be identified by examining plates under UV radiation. An UV transilluminator, commonly used in molecular biology procedures, may be employed for this purpose. The wells containing strains unable to degrade SA appear fluorescent (**Figure 4A**) while the wells inoculated with bacteria that degrade SA will not present fluorescence, (**Figure 4B**).

Alternatively, the Trinder reagent (described in the supplementary document) can be added to the medium (1 ml Trinder reagent/per well of a 24-well plate) and then incubated for 20 to 30 min. The Trinder reagent is commonly used for the detection of SA [26]. If SA is present (no degradation occurs), the medium will change color to purple (**Figure 4C**). Bacteria that can degrade SA remove it from the medium and hence no color development is observed (**Figure 4D**).

Note: Given the tendency of the Trinder reagent-SA complex to fade, the Trinder plate must be examined immediately after the incubation process.

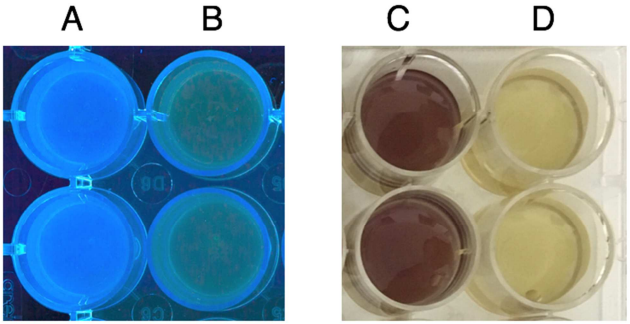

Figure 4- Results obtained in SA degradation tests. A) Medium containing 1 mM SA, which is fluorescent under UV light. B) Medium without SA (consumed by the bacteria), which does not present fluorescence under UV light. C) Medium containing 1 mM SA, which is in the presence of the Trinder reagent becomes purple. D) Medium without SA (consumed by the bacteria), which is in the presence of the Trinder reagent does not change color.

3.3.9 IAA degradation test

A IAA degradation test can be performed in 48 or 24-well plates containing minimal medium supplemented with 1 mM IAA and 0.8% agar (e.g. 24-well plates = 1 ml M9 medium containing 1 mM IAA as sole carbon source per well). In duplicate, add 5 μl of an overnight grown culture (grown in general rich medium) in the center of the well. The plate is then incubated for 24 h at 28ºC.

The detection methodology is based on the use of the Salkowski reagent that is widely used in the determination of IAA production in bacterial culture medium [27]. After incubation and growth, a solution of Salkowski reagent (described in the supplementary document) can be added to the solid medium (1 ml Salkowski reagent/per well of a 24-well plate) and further incubated for 1 hour at room temperature. Plates/wells containing bacteria unable to degrade IAA will change to a pink/reddish color (negative) (**Figure 5A**). Bacteria able to degrade IAA will consume all the available IAA and no color development will be observed (positive) (**Figure 5B**).

Figure 5- Results obtained in IAA degradation test. A) Medium containing 1 mM IAA, which in the presence of the Salkowski reagent changes color. Negative result in the IAA degradation test. B) Medium without or containing lower levels of IAA (consumed by the bacteria), which does not present color development in the addition of the Salkowski reagent (no IAA present). Positive result in the IAA degradation test.

3.3.9 General characterization of phytohormone-degrading bacterial isolates

After the isolation and confirmation of phytohormone-degrading abilities, selected bacteria can be identified by means of 16S rRNA gene sequencing and its analysis. Additionally, the selected bacteria can be tested for production of several traits involved in the plant-microbe interaction and plant-growth promotion, such as: siderophores, IAA, phosphate solubilization, protease, esterase, lipase, cellulase, amylase, acetoin, ammonia and polyamines production and nitrate reduction. A descriptive methodology of these tests can be found in the works of Vicente et al. [28] and Rashid et al. [29].

3.3.10 Plant growth promotion assays

To test the plant-growth promoting abilities of the isolated phytohormone-degrading bacteria a plant growth assay should be performed using a model plant, chosen depending on the final objectives of the study. Notwithstanding, tomato, cucumber or canola can be used as generalist plant models in these studies. These plants can be easily acquired, present a fast development, and their seeds are easy to manipulate and disinfect.

The assays can be conducted under laboratory or greenhouse conditions following the methodology of Penrose and Glick [24]. Briefly:

Seed disinfection and germination

a) Surface disinfect seeds by a treatment with 70% ethanol for 1.5 min, 1% bleach solution for 10 min and 5 consecutive washes with sterile distilled water.

b) Place the seeds in sterile plates containing 1% water agar medium. Incubate for 2 to 3 days at 24ºC in the dark.

Bacterial inocula preparation

a) Grow bacteria in rich medium (i.e. TSB) (the volume depends on the number of replicates in the plant experiment) at 200 r.p.m. at the optimum temperature of the bacterial strain.

b) Centrifuge the bacteria at 4000 g for 10 min in a benchtop centrifuge. Discard the supernatant.

c) Wash the cells with 0.03 M $MgSO_4$.

d) Centrifuge the bacteria at 4000 g for 10 min in a benchtop centrifuge. Discard the supernatant.

e) Dilute the cell suspension in 0.03 M $MgSO_4$ to a final OD_{600} of 0.5.

Preparation of the substrate, pots and inoculation

a) Mix vermiculite and sand in a 1:1 (v/v) proportion. Add distilled water to the mixture (e.g. 1L water to 4L of vermiculite sand mixture). Mix thoroughly.

b) Sterilize the mixture in an autoclave for 1 hour and 30 min, 121ºC.

c) After cooling distribute the substrate mix to the pots. Add 1 germinated plant per pot.

d) Inoculate each plant with 5 ml of the bacterial solution OD_{600}= 0.5. Perform a negative control treatment without the addition of bacteria.

Measuring plant growth

Usually results are measurable 15 to 20 days after inoculation. Plants are then removed from the pots, washed in tap water, and their roots and shoots are removed and dried at 60ºC for several days. The dry weights are measured in an analytical scaled and analyzed.

4. RESULTS AND DISCUSSION

Several phytohormone-degrading bacterial strains were successfully isolated by employing the methodology described in this work (**Table 3**). These bacteria have been isolated from different plants (e.g. *Mimosa*, *Sesbania*, *Solanum*), plant tissues (roots/rhizosphere, root nodules, shoots, leaf and fruits), as well as lower plants (e.g. Antarctic moss), and soils (agricultural, environmental, polluted) from different countries and continents, including Antarctica (**Table 3**).

Table 3- Bacteria isolated and phytohormone-degrading abilities testing following the methodology described in this work.

Bacteria	Phylum/Class	16S rRNA#	Isolation source	Country/Continent	ACC deg*	ACD **	IAA deg ***	SA deg ****
Achromobacter sp. AB2	Beta-Proteobacteria	MG602707	Antarctic soil	Antarctica	+	1.164	+	+
Achromobacter sp. SOLR10	Beta-Proteobacteria	MG602708	Solanacea rhizosphere	Brazil	+	1.392	+	+
Arthrobacter sp. PM3	Actinobacteria	MG602693	Bermuda grass rhizosphere	Brazil	+	1.467	-	-
Burkholderia sp. TRE3	Beta-Proteobacteria	MG602704	Acid mine-drainage soil	Brazil	-	n.d	+	+
Burkholderia sp. OPX	Beta-Proteobacteria	MG602709	Fungi-fruit body	Brazil	+	13.125	-	-
Lelliottia sp. AC1	Gamma-Proteobacteria	MG602700	Pine/insect	Portugal	-	n.d	+	-
Microbacterium sp. PM5	Actinobacteria	MG602705	Bermuda grass rhizosphere	Brazil	+	0.439	-	-
Pseudomonas sp. PLM1	Gamma-Proteobacteria	MG602710	Agricultural soil	Portugal	+	10.511	-	-
Pseudomonas sp. PLMAX	Gamma-Proteobacteria	MG602703	Agricultural soil	Portugal	-	n.d	+	+
P. lini ACR2	Gamma-Proteobacteria	MG602697	Cactacea rhizosphere	Portugal	+	7.679	-	-
P. mandelli ACM7	Gamma-Proteobacteria	MG602698	Moss rhizosphere	Antarctica	-	n.d	+	-
P. oryzihabitans MS8	Gamma-Proteobacteria	MG602701	Shoot of *Mimosa scabrella*	Brazil	+	3.756	-	-
P. palleroniana MAB3	Gamma-Proteobacteria	MG602696	*Amanita* sp.	Portugal	+	14.303	-	-
P. putida IAAD1	Gamma-Proteobacteria	MG602706	*Eucalyptus* rhizosphere	Portugal	-	n.d	+	-
P. thivervalensis PLM3	Gamma-Proteobacteria	MG602711	Agricultural soil	Portugal	+	15.322	-	-
P. thivervalensis SC5	Gamma-Proteobacteria	MG602695	*Solanum capsicoides* fruit/seed	Brazil	+	18.592	-	-
Pantoea cypripedii NE1	Gamma-Proteobacteria	MG602702	Root nodule	Brazil	+	5.748	-	-
Pantoea sp. MSR2	Gamma-Proteobacteria	MG602694	*Mimosa scabrella* rhizosphere	Brazil	+	0.135	-	-
Serratia marcescens DAMR1	Gamma-Proteobacteria	MG602699	Acid mine-drainage soil	Brazil	-	n.d	-	+

+ Positive; - Negative; n.d- not determined.
# 16S rRNA NCBI accession number
** ACC deaminase activity in μmol α-ketobutyrate/mg protein/hour.
*** IAA degradation test. IAA as sole carbon source.
**** SA degradation test. SA as sole carbon source.

The isolated bacteria were identified based on the partial 16S rRNA gene (approx. 1346 bp) which was amplified and sequenced (described in the supplementary information). In this sense, phytohormone-degrading bacteria belonging to different genera (and phyla) such as *Achromobacter*, *Burkholderia*, *Paraburkholderia* (β-Proteobacteria), *Lelliottia*, *Pantoea*, *Serratia*, *Pseudomonas* (γ-Proteobacteria), *Arthrobacter*, *Microbacterium* (Actinobacteria), were identified. This result indicates that the methodology described allows for the isolation of diverse bacterial groups.

The isolation of Actinobacteria and Proteobacteria presenting ACC deaminase activity is in agreement with our previous report on the wide distribution of ACC deaminase genes in these groups [9]. Curiously, we isolated two *Pantoea* strains presenting ACC deaminase activity, which is a rather uncommon trait in this genus and in Enterobacteriaceae.

Interestingly, several bacteria possessed the ability to degrade more than one tested phytohormone. For example, *Achromobacter* sp. AB2, isolated from an Antarctic soil, and *Achromobacter* sp. SOLR10, isolated from the rhizosphere of a Solanacea plant in Brazil, possessed the ability to use ACC, SA and IAA as nitrogen and carbon sources, respectively (**Table 3**). *Burkholderia* sp. TRE3 (isolated from an acid-mine drainage soil in Brazil) and *Pseudomonas* sp. PLMAX (isolated from an agricultural soil in Portugal) presented both the ability to degrade IAA and SA (**Table 3**). These results suggest that some bacteria evolved to directly modulate phytohormones concentrations in plants or in the soil (even those produced by competing bacteria). Nevertheless, it is possible that the SA degradation abilities of some bacterial strains described in this study are related to their ability to degrade other compounds, where SA acts like an intermediate in the degradative pathway [10,11].

Some of the isolated bacteria were selected and tested to produce several traits involved in the plant-microbe interactions and plant-growth promotion abilities, such as: siderophores (iron-acquisition), IAA production (phytohormone modulation), phosphate solubilization (nutrient availability, P), protease, esterase, lipase, cellulase, amylase (modulation of plant tissues and storage compounds), acetoin (volatile involved in plant growth), ammonia (nutrient availability, N) and polyamines production (phytohormone modulation, stress resistance) and nitrate reduction (nutrient cycling, plant N and NO status) (**Table 4**). In this sense, metabolic abilities differed between the isolated strains.

Table 4 – Biochemichal characterization of selected phytohormone-degrading bacteria.

Bacteria	IAA*	Sid	PO_4	Prot	Lip	Est	Amy	Cell	Acet	Poly	NO_3	NO	NH_3
Achromobacter sp. AB2	n.d	-	-	+	-	+	+	-	-	-	n.d	n.d	n.d
Achromobacter sp. SOLR10	5.60	+	+	+	+	+	+	-	-	+	+	+	+
Arthrobacter sp. PM3	2.22	-	-	-	+	-	+	-	+	-	-	-	-
Burkholderia sp. TRE3	0.28	+	+	-	+	+	+	-	-	-	+	-	+
Burkholderia sp. OPX	3.03	+	+	+	+	+	+	-	-	-	+	-	+
Lelliottia sp. AC1	7.52	+	-	-	-	-	-	-	-	+	+	-	-
Microbacterium sp. PM5	9.64	+	-	+	-	-	+	+	+	-	+	-	-
Pseudomonas mandelli ACM7	0.30	+	-	+	+	-	-	-	-	-	+	-	+
Pseudomonas sp. PLMAX	3.37	+	+	-	+	-	+	-	-	+	+	+	+
P. oryzihabitans MS8	2.71	+	+	-	+	-	+	+	-	+	+	-	+
P. palleroniana MAB3	19.06	+	+	-	+	-	-	-	-	+	+	-	+
Pseudomonas sp. PLM1	3.51	+	+	+	+	-	-	-	-	+	+	+	+
P. thivervalensis PLM3	4.61	+	+	-	+	-	-	-	-	+	+	+	+
P. thivervalensis SC5	3.20	+	+	-	+	-	-	-	-	+	+	+	+
Pantoea cypripedii NE1	69.76	+	+	+	+	-	+	-	-	-	+	-	+
Pantoea sp. MSR2	70.05	+	+	-	-	-	-	-	-	-	+	-	-
Pseudomonas lini ACR2	4.76	+	+	+	+	-	-	-	-	+	+	+	+
Serratia marcescens DAMR1	5.62	+	+	+	+	+	-	+	+	+	+	-	+

+ Positive; - Negative; n.d- not determined.

* IAA production (μg/ml); Sid- siderophores production; PO_4- Phosphate solubilization; Prot- Extracellular protease activity; Lip- Extracellular lipase activity; Est- Extracellular esterase activity; Amy-Extracellular amylase activity; Cell- Extracellular cellulase activity; Acet- Acetoin production; Poly- Polyamine production; NO_3- Nitrate reduction; NO- production of NO or N_2 gas resulting from the nitrate reduction test; NH_3- Ammonia production.

The lytic enzymatic activities differed between the tested strains, and sometimes in a strain/species-specific manner. Similar results were obtained for other traits such as phosphate solubilization, polyamine production and others. IAA production greatly varied between strains, in which *Pantoea* spp. produced the highest levels (70 μg/ml), while *Pseudomonas* strains tended to produce very low levels of this phytohormone. Altogether, these results indicate that although these strains produce ACC deaminase and/or degrade IAA and SA, their overall metabolic capacities greatly vary. This is linked to the species/genus-specific genetic background and the consequent metabolic versatility (e.g. the known fermentative and sugar degradation abilities of Enterobacteriaceae) and the bacterial ecological role in the soil and in the plant (i.e. the organic matter degradative abilities of Actinobacteria). In addition, other factors related to methodology drawbacks (e.g. growth media, growth conditions, specificity of the reagents) may impact the test results.

Selected bacteria were tested for their plant-growth promoting abilities, using cucumber as a plant model. The plant-growth promoting abilities varied amongst strains (**Figure 6**).

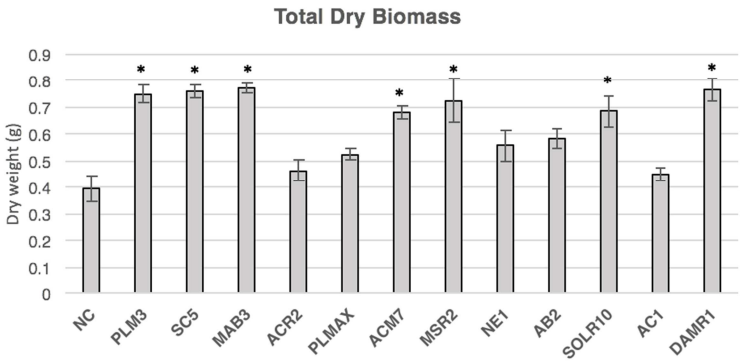

Figure 6- Total dry biomass results obtained from the cucumber growth promotion assay, 20 days after inoculation. The experiment was conducted under greenhouse conditions. Error bars represent the standard error;
* represents statistical significant differences ($p<0.05$) in comparison to non-inoculated plants (negative control-NC).

Nevertheless, most of the strains presented significant plant-growth promotion abilities. For example, *Pseudomonas* strains (PLM3, SC5, MAB3, ACM7) greatly increased cucumber total biomass (root dry weight+ shoot dry weight) resulting in growth

increments of aprox. 90% when compared to non-inoculated plants. Overall, these bacteria impacted both root and shoot development (**Figure 7**), leading to an increased plant development. Similarly, other bacterial strains such as *Achromobacter* sp. SOLR10, *Pantoea* sp. MSR2, *S. marcescens* DAMR1 also promoted cucumber plant growth in great extent. The obtained results agree with previous reports indicating the beneficial role of phytohormone-degrading bacteria in plant growth [8,18].

Figure 7- Cucumber growth promotion assay, 20 days after inoculation. A) non-inoculated plant (negative control); B) plant inoculated with bacteria (MAB3).

Some of the selected strains did not significantly promoted cucumber plant growth. While bacterial phytohormone-degradation may be linked to their plant-growth promotion abilities, other factors, such as, general bacterial metabolism (e.g. degradation of root exudates, production of plant-growth promoting compounds), colonization strategies (e.g. production of lytic enzymes and compounds involved in adherence), growth conditions (pH, temperature, humidity), the plant species (e.g. intrinsic defense and response mechanisms, exudates, tissue structure) may influence bacterial performance. Ultimately, to understand the beneficial effects of selected bacteria more studies using different plant species and growth conditions should be performed.

Additionally, to understand the genomic and general metabolic properties of selected phytohormone-degrading strains, their genome was sequenced using the Illumina Miseq platform (barcoded run). In this sense, the genomes of *Achromobacter* sp. AB2, *Achromobacter* sp. SOLR10, *Arthrobacter* sp. PM3, *Lelliottia* sp.

AC1, *Microbacterium* sp. PM5, *P. mandelli* ACM7, *Pseudomonas* sp. PLMAX, *P. oryzihabitans* MS8, *P. putida* IAAD1, *P. palleroniana* MAB3, *P. thivervalensis* PLM3, *P. thivervalensis* SC5, *Pantoea cypripedii* NE1 and *Pantoea* sp. MSR2 were obtained and analyzed (**Table 5**) (methodology described in supplementary materials).

Table 5-Overview on the sequencing and assembly of the genomes of phytohormone-degrading bacteria.

Bacteria	Reads	Contigs	Genome Size	Coverage
Achromobacter sp. AB2	2,089,996	18	6,438,278	97,4X
Achromobacter sp. SOLR10	1,234,923	40	6,520,871	56.8X
Arthrobacter sp. PM3	1,879,326	34	4,350,452	129.6X
Lelliottia sp. AC1	1,961,120	14	4,411,336	133.3X
Microbacterium sp. PM5	2,095,580	4	3,158,783	199X
P. mandelli ACM7	1,400,294	58	6,602,660	63.6X
P. oryzihabitans MS8	1,863,224	27	5,436,261	102.8X
P. putida IAAD1	1,554,554	19	5,938,605	78.5X
P. palleroniana MAB3	2,362,402	16	6,291,344	112.7X
P. thivervalensis PLM3	2,338,460	40	6,591,188	106.4X
P. thivervalensis SC5	1,480,168	37	6,592,350	67.4X
Pantoea cypripedii NE1	1,978,562	59	6,527,293	90.9X
Pantoea sp. MSR2	2,104,562	53	5,710,914	110.5X

The obtained genomes presented a high-quality (high number of multiple long reads of 300 bp, resulting in a high genome sequencing coverage), with an average low number of contigs that were easily assembled into near complete genome sequences by performing alignments against complete genomes present in the NCBI database.

Genome functional analysis demonstrated the different genetic properties of each strain (**Table 5, 6**). Genome size and coding sequences (CDS) varied depending on the strain, with Actinobacteria (PM3 and PM5) presenting smaller genomes (3 and 4.5 Mbp) and less CDS, when compared to Proteobacteria (genomes ranging from 4 to 6.5 Mbp and containing more CDS). Nevertheless, the average genome GC content (%) was higher in Actinobacteria (**Table 6**). Interestingly, *Achromobacter* spp. also presented high GC% when compared to other Proteobacteria. These results demonstrate the existence of species/group specific genomic evolutionary constraints mediating bacterial genome evolution, that, ultimately, impact the bacterial lifestyle. This is corroborated by the results obtained in the BlastKOALA functional genome annotation (**Table 6**)

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Table 6-General characteristicis of the genomes of selected phytohormone-degrading bacteria

Bacteria	GC%	CDS	BLAST KOALA*	EIP	CP	GIP	EM	CM	AM	LM	XM	MCV	AS
Achromobacter sp. AB2	65.2	5878	3136 (53.4%)	1071	616	556	210	328	419	123	135	189	48
Achromobacter sp. SOLR10	67.6	5873	3075 (52.4%)	1074	536	581	215	281	374	110	109	170	40
Arthrobacter sp. PM3	67.9	3884	1840 (47.4%)	433	224	404	124	302	275	81	64	142	45
Lelliottia sp. AC1	55.3	4184	2928 (70.0%)	866	355	665	158	341	241	69	45	177	23
Microbacterium sp. PM5	69.5	2952	1406 (47.6%)	382	153	350	75	209	185	48	21	101	24
P. mandelli ACM7	58.7	6261	3145 (50.2%)	930	410	641	214	309	388	119	114	216	47
P. oryzihabitans MS8	65.5	4929	2805 (56.9%)	839	443	595	157	287	312	81	75	188	38
P. putida IAAD1	62.9	5296	2959 (55.9%)	866	384	635	202	277	371	99	99	204	43
P. palleroniana MAB3	60.5	5677	3099 (54.6%)	974	454	647	203	283	362	112	85	209	53
P. thivervalensis PLM3	61.2	5818	3162 (54.3%)	997	481	634	197	291	362	108	79	220	48
P. thivervalensis SC5	61.2	5816	3181 (54.7%)	996	481	634	197	291	362	108	79	221	48
Pantoea cypripedii NE1	54.4	6011	3611 (60.1%)	1206	469	722	189	368	329	87	81	208	38
Pantoea sp. MSR2	54.1	5280	3357 (63.6%)	1075	419	689	180	362	296	89	71	203	32

*Total number of CDS annotated by the BlastKOALA service.
EIP- CDS involved in Environmental information processing; **CP**- CDS involved in Cellular Processes; **GIP**- CDS involved in Genetic Information Processing; **CM**- CDS involved in Carbohydrate metabolism; **AM**- CDS involved in Amino acid metabolism; **MCV**- CDS involved in Metabolism of cofactors and vitamins; **EM**- CDS involved in Energy metabolism; **LM**- CDS involved in Lipid metabolism; **XM**- CDS involved in Xenobiotics biodegradation and metabolism; **AS**- number of clusters involved in the Biosynthesis of other secondary metabolites predicted by antiSMASH.

In this sense, the studied bacteria present different genetic elements (in number and in function) involved in cell functioning and metabolic traits. For example, the genomes of Enterobacteriaceae such as *Lelliottia* sp. AC1 and *Pantoea* strains MSR2 and NE1 presented an increased number of CDS involved in carbohydrate metabolism when compared to other strains (**Table 6**). The genome of *Achromobacter* sp. AB2 presented an increased number of CDS involved in the metabolism of xenobiotics, which is consistent with its ability to degrade several phytohormones.

The presence of genes involved in the phytohormone-degradation abilities of the selected strains was also verified. The *acdS* gene (encoding for ACC deaminase) was found in the genomes of all ACC deaminase-producing bacteria. Likewise, the *iac* (IAA catabolism genes) and *sah* (SA-hydroxylase genes) were detected in the genomes of IAA and SA-degrading bacteria, further confirming the observed and previously described enzymatic activities.

Furthermore, analysis of gene clusters involved in the production of secondary metabolites (antiSMASH analysis) demonstrated that the genomes of the selected bacteria contain several CDS involved in the production of a wide range of secondary metabolites, such as bacteriocins, thiopeptides, non-ribosomal peptides, arylpolyene, terpenoids, anti-fungal compounds, and others (**Table 6**). Moreover, the presence of secondary metabolite clusters differed amongst strains. For example, a total of 48 clusters involved in the production of secondary metabolites were detected in the genome of *Pseudomonas thivervalensis* PLM3 (**Figure 8**).

One of the PLM3 genome clusters (cluster 13) encoded the enzymes involved in the biosynthesis of the 2-4-diacetylphloroglucinol (DAPG), a known anti-fungal compound involved in the fungal antagonistic abilities of several *Pseudomonas* strains [30]. Based on this fact we tested strain PLM3 for its antagonistic activities (*in vitro*) against *Botrytis cinerea*, and observed its ability to inhibit fungal growth (**Figure 9A, B**), therefore suggesting that strain PLM3 produces DAPG. Similarly, several clusters involved in the production of bacteriocins (antibacterial effects) were detected. Not surprisingly, strain PLM3 was able to inhibit the growth (*in vitro*) of the plant pathogen *P. syringae* DC3000 (**Figure 9C**). These results suggest that important bacterial functional traits can be predicted by genome analysis, thus, making it an important tool for the selection of bacteria with desired characteristics (e.g. antagonistic activities).

Cluster 1	Nrps	1	71377	Pyoverdine_biosynthetic_gene_cluster (19% of genes show similarity)	BGC0000413_c1
Cluster 2	Cf_putative	134112	142772	-	-
Cluster 3	Cf_putative	465902	470749	-	-
Cluster 4	Bacteriocin	674364	685209	-	-
Cluster 5	Cf_putative	692508	699410	-	-
Cluster 6	Cf_fatty_acid	791937	813223	-	-
Cluster 7	Cf_putative	952648	957316	-	-
Cluster 8	Cf_putative	979789	988702	-	-
Cluster 9	Nrps	996945	1059659	A33853_biosynthetic_gene_cluster (13% of genes show similarity)	BGC0001292_c1
Cluster 10	Cf_putative	1079975	1085875	-	-
Cluster 11	Cf_putative	1094643	1119745	-	-
Cluster 12	Lantipeptide	1300386	1323451	-	-
Cluster 13	Cf_putative	1344901	1357349	-	-
Cluster 14	Nrps	1543079	1628884	Pyoverdine_biosynthetic_gene_cluster (2% of genes show similarity)	BGC0000413_c1
Cluster 15	Cf_putative	1658141	1668420	-	-
Cluster 16	T3pks	1674949	1715998	2,4-Diacetylphloroglucinol_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000281_c1
Cluster 17	Cf_putative	1740199	1758087	Herboxidiene_biosynthetic_gene_cluster (2% of genes show similarity)	BGC0001065_c1
Cluster 18	Cf_putative	1859873	1872937	-	-
Cluster 19	Cf_putative	1883324	1890928	-	-
Cluster 20	Cf_fatty_acid	1973683	1994969	-	-
Cluster 21	Cf_saccharide	2063363	2098022	-	-
Cluster 22	Cf_putative	2146035	2163681	Polysaccharide_B_biosynthetic_gene_cluster (6% of genes show similarity)	BGC0001411_c1
Cluster 23	Cf_putative	2394471	2407306	-	-
Cluster 24	Nrps	2482538	2535524	Pyoverdine_biosynthetic_gene_cluster (11% of genes show similarity)	BGC0000413_c1
Cluster 25	Cf_putative	2610165	2630112	O-antigen_biosynthetic_gene_cluster (21% of genes show similarity)	BGC0000781_c1
Cluster 26	Cf_putative	2630122	2636613	-	-
Cluster 27	Cf_fatty_acid	2723846	2744775	-	-
Cluster 28	Cf_fatty_acid	2834137	2855258	-	-
Cluster 29	Butyrolactone	3045250	3058660	-	-
Cluster 30	Cf_putative	3071885	3077895	-	-
Cluster 31	Cf_putative	3124321	3143550	-	-
Cluster 32	Bacteriocin	3231404	3242273	-	-
Cluster 33	Cf_putative	3842987	3848928	-	-
Cluster 34	Cf_saccharide	4014156	4058889	Lipopolysaccharide_biosynthetic_gene_cluster (36% of genes show similarity)	BGC0000776_c1
Cluster 35	Arylpolyene	4085367	4128978	APE_Vf_biosynthetic_gene_cluster (40% of genes show similarity)	BGC0000837_c1
Cluster 36	Cf_putative	4264691	4278867	Lipopolysaccharide_biosynthetic_gene_cluster (11% of genes show similarity)	BGC0000774_c1
Cluster 37	Cf_putative	4307674	4313438	-	-
Cluster 38	Cf_putative	4318835	4331181	9-methylstreptimidone_biosynthetic_gene_cluster (9% of genes show similarity)	BGC0000171_c1
Cluster 39	Bacteriocin	4357998	4368783	Pyoverdine_biosynthetic_gene_cluster (1% of genes show similarity)	BGC0000413_c1
Cluster 40	Other	4393929	4437378	Mangotoxin_biosynthetic_gene_cluster (71% of genes show similarity)	BGC0000387_c1
Cluster 41	Cf_fatty_acid	4564252	4585496	Svaricin_biosynthetic_gene_cluster (6% of genes show similarity)	BGC0001382_c1
Cluster 42	Cf_fatty_acid	4643273	4664493	-	-
Cluster 43	Cf_putative	4699145	4716151	Enterobactin_biosynthetic_gene_cluster (8% of genes show similarity)	BGC0000343_c1
Cluster 44	Cf_putative	5200050	5222347	Alginate_biosynthetic_gene_cluster (80% of genes show similarity)	BGC0000725_c1
Cluster 45	Cf_putative	5818118	5825216	-	-
Cluster 46	Cf_putative	5856765	5876606	-	-
Cluster 47	Cf_putative	5949537	5957875	-	-
Cluster 48	Cf_saccharide	6349770	6382065	S-layer_glycan_biosynthetic_gene_cluster (12% of genes show similarity)	BGC0000795_c1

Figure 8- Results obtained from the antiSMASH analysis using strain PLM3 genome as query.

Overall, the obtained results show that the bacteria isolated in this study not only produce enzymes involved in the modulation of phytohormone levels but can also produce a wide range of important compounds (i.e. anti-fungal and anti-bacterial compounds, surfactants, exopolysaccharides, volatiles, fatty acids, and others) with relevance for agricultural and biotechnological applications.

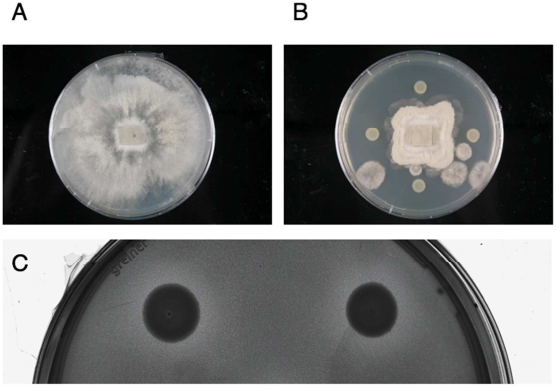

Figure 9- Antagonistic activities of strain PLM3. A) Potato dextrose media (PDA) inoculated with *Botrytis cinerea*; B) PDA inoculated with *B. cinerea* and strain PLM3. C) TSA medium incorporated with *Pseudomonas syringae* DC3000 and inoculated with strain PLM3; halos around PLM3 colonies represent antagonistic activities.

Interestingly, by employing the described methodology we also obtained several plant-associated yeasts (and yeast-like fungi) with the ability to use ACC as sole nitrogen source (**Table 7**). One of these strains (MAC1) was also able to degrade SA.

The isolated yeasts and fungi were identified by sequencing of the internal transcribed spacer (ITS) regions (described in the supplementary information). In this sense, yeast and yeast-like fungi belonging to different genus (i.e. *Candida*, *Issatchenkia*, *Saturnispora*, *Aureobasidium*, *Sarocladium*) were identified.

Several yeasts are known to associate and promote the growth of several plant species [31,32]. Curiously, one of the first ACC deaminase enzymes to be described was that of the soil yeast *Cyberlindnera saturnus* [33,34]. Moreover, our previous results, also demonstrated that several yeasts including *Aureobasidium* spp*.,* possess ACC deaminase genes [9].

Table 7- Yeasts and yeast-like fungi isolated using the methodology described in this work.

Yeasts and Yeast like fungi	ITS Genbank	Isolation source	Country	ACC*	ACD	IAA**	SA***
Aureobasidium sp. YCLL2	MG649459	Leaf of *Cistus lanadifer*	Portugal	+	n.d	-	-
Candida sp. YXB6	MG649456	Xylem of a banana tree	Brazil	+	n.d	-	-
Candida sp. YSAMBA	MG649458	Soil	Brazil	+	n.d	-	-
Candida sp. YTL3	MG649457	Leaf of a tomato plant	Brazil	+	n.d	-	-
Issatchenkia sp. YTL1	MG649460	Leaf of a tomato plant	Brazil	+	n.d	-	-
Saturnispora sp. YPM2	MG649461	External root surface of a banana tree	Brazil	+	n.d	-	-
Sarocladium sp. ALCS3	MG649463	Aluminium-contaminated soil	Portugal	+	n.d	-	-
Sarocladium sp. MAC1	MG649462	Interior of a Fuji apple	Brazil	+	n.d	-	+

+ Positive; - Negative; n.d- not determined. * ACC as sole nitrogen source. ** IAA degradation test. IAA as sole carbon source. *** SA degradation test. SA as sole carbon source.

5. CONCLUDING REMARKS

Most studies regarding the isolation of soil and plant-associated bacteria report the direct isolation of bacteria from soil and plant tissues samples by plating solutions containing the bacteria in general rich media such as TSA, NA, or R2A. Usually the isolated bacteria are characterized and tested for the production of several traits, including ACC deaminase activity, and these are

selected for posterior studies. Nevertheless, this methodology is time consuming and expensive. For example, Rashid et al., [29] isolated 174 tomato bacterial endophytes, however, only 25 of these strains (13%) demonstrated ACC deaminase activity. Similarly, Duan et al., [35] found that only 27 out of 233 (11.6%) rhizobia strains isolated from 30 different sites across Southern Saskatchewan, Canada, displayed ACC deaminase activity. To overcome this problem and easily isolate and select ACC deaminase-producing bacteria, as well as other phytohormone-degrading bacteria, a targeted approach should be considered.

In this work, we presented and demonstrated a methodology aiming the easy and fast isolation of soil and plant-associated bacteria presenting ACC deaminase activity and other phytohormone-degrading abilities. By using this methodology, we rapidly isolated several soil and plant-associated bacteria and yeasts with phytohormone-degrading abilities, without the need for an extensive and laborious isolation and maintenance of isolates. Bacterial strains belonging to different genera and phylum were obtained in this study, therefore indicating that this methodology allows for a non-biased isolation of diverse soil and plant-associated bacteria. Moreover, some of the isolated bacteria presented increased plant-growth promoting abilities and distinct genomic and metabolic signatures, which can be selected and used in several future agricultural and biotechnological applications.

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CHAPTER 6

Bioreactor cultivation and field application of selected ACC deaminase-producing bacteria

THESIS REPORT

"Bioreactor cultivation and field application of selected ACC deaminase-producing bacteria"

By Francisco X. Nascimento, Bernard R. Glick, Admir J. Giachini, Márcio J. Rossi

Participation: inoculant production in bioreactor, data analysis, Treviso field experiment, writing of the report.

Bioreactor cultivation and field application of selected ACC deaminase-producing bacteria

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Keywords: Bioreactor**,** ACC deaminase, *Pseudomonas*, field, coal-mining, maize

INTRODUCTION

The study of plant growth-promoting bacteria (PGPB) and their effects on plants has gained a considerable interest in the past few decades. This occurred because these bacteria can naturally associate with plants and promote their growth, development and productivity, hence, representing an alternative to the unsustainable use of pollutant chemicals and fertilizers on large scale agricultural practices. Moreover, due to the low production costs associated with this technology, it can be used all over the world, in developed and developing countries. This has led to development of many bacterial inoculants for use in agricultural practices (GLICK, 2015). Nonetheless, these PGPB may also be very important for the development of relevant biotechnological practices such as phytoremediation and biostimulation, aiming the recovery of polluted soils that result from industrial practices (e.g. mining). In this sense, using selected stress resistant plants and efficient bacterial inoculants is key to maximize soil recovery processes.

The recent advancements in genome sequencing and analytic technologies also allowed the discovery of new important applications for PGPB. Several studies have shown that PGPB not only directly promote plant growth but can also produce important enzymes and secondary metabolites (e.g. cellulases, phytohormones, biosurfactants, antibiotics) that can be used in a wide range of future agricultural and biotechnological applications (PREMACHANDRA et al. 2016).

Bacteria that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase have been shown to be a valuable tool in facilitating plant growth under normal and stressful conditions by their ability to decrease ACC and ethylene levels that inhibit plant growth (GLICK, 2014). Hence, developing and using bacterial inoculants containing ACC deaminase-producing bacteria is important for both agricultural and biotechnological practices.

In a recent survey (presented in chapter 5), we isolated, characterized and tested the plant-growth promotion abilities of several ACC deaminase-producing bacteria. Amongst these, the strains *Pseudomonas palleroniana* MAB3 and *Pseudomonas thivervalensis* SC5 presented an increased ability to promote plant growth and produced several compounds relevant in plant-bacterial interactions (e.g. indole-3-acetic acid, ammonia, polyamines). Additionally, the genome sequence of these bacteria was obtained and analyzed, and, revealed that these strains possess several gene clusters involved in the production of secondary metabolites such as

bacteriocins (involved in antibacterial activities) and other relevant compounds.

To efficiently create a bacterial inoculant to be applied under large-scale field conditions it is necessary to produce sizable quantities of selected bacteria in bioreactors and understand their growth kinetics and growth limitations under these conditions. At the same time, it is necessary to test the PGP abilities of selected inoculants in more realistic and representative field conditions. Therefore, in this work, the ACC deaminase-producing strains MAB3 and SC5 were cultivated in 5L bioreactors and their growth kinetics were studied. In addition, these bacteria were tested in small field experiments. In this sense, strain SC5 was tested in a field experiment in a coal-mining recovery soil (affected by low pH and heavy metal stress) in Treviso, Criciúma (Santa Catarina). A mixed inoculant containing strains MAB3 and SC5 was tested in two field experiments in Xanxerê (Santa Catarina) and Cachoeira do Sul (Rio Grande do Sul) aiming the production maize in a large scale agricultural setup.

MATERIAL AND METHODS

Pseudomonas palleroniana MAB3 cultivation in a stirred tank bioreactor

Pseudomonas palleroniana MAB3 was cultivated in a RALF 5-L stirred tank bioreactor (Bioengineering, Switzerland) (**Figure 1**) fitted with an InPro6800/12/320/T O_2 sensor and a polarographic pH sensor 405-DPAS-SC-K8S/325 (Mettler-Toledo AG, Switzerland).

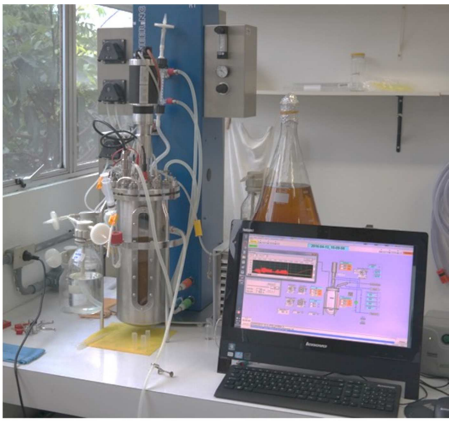

Figure1- Overview of the RALF stirred tank bioreactor used in this study.

The bioreactor containing 4.5L of Tryptic Soy Broth [casein peptone (pancreatic digest), 17 g/L; soya peptone (peptic digest of soybean), 3 g/L; glucose, 2.5 g/L; K_2HPO_4, 2.5 g/L; NaCl, 5 g/L] (**Table 1**) was sterilized in an autoclave for 30 min at 121ºC. After cooling to 28ºC, the oxygen probe was calibrated and the medium pH adjusted to 7. Consequently, 250 ml of a MAB3 solution (grown in TSB, 200 rpm, 28ºC) was aseptically introduced in the bioreactor and the batch started. The temperature (28ºC), dissolved oxygen (set point 20%) and pH (set point 7.0±0.2) were controlled and maintained during the experiment.

Table 1- Carbohydrate and nitrogen specifications of the TSB medium used in the experiments.

	Concentration g/L	Total ammount (g) in 4.5L	Total C* in 4.5L (g)	Total N in 4.5 L (g)
Casein peptone	17	76.5	0.305	10.86
Soy peptone	3	13.5	3.95	1.27
Glucose	2.5	11.25	11.25	0
K_2HPO_4	2.5	11.25	-	-
NaCl	5	22.5	-	-
Total in bioreactor	-	-	15.5	12.13

Nutrient specifications were obtained from BD Bionutrients™ Technical Manual. https://www.bdbiosciences.com/documents/bionutrients_tech_manual.pdf
*Roughly estimated.

Pseudomonas thivervalensis SC5 cultivation in an airlift bioreactor

Pseudomonas thivervalensis SC5 was cultivated in a 5L stainless steel airlift bioreactor (ROSSI et al. 2016) with external circulation (**Figure 2**), fitted with an InPro6000 O_2 sensor and a polarographic pH sensor 405-DPAS-SC-K8S/325 (Mettler-Toledo AG, Switzerland).

The bioreactor was sterilized (121 °C and 30 min) using direct steam created by a 25-L autoclave connected to the bioreactor through a derivation of the air outlet. After sterilization, the bioreactor aseptically received 4.5 L of TSB (previously sterilized for 30 min at 121ºC) and 250 ml of a SC5 solution (grown in TSB, 200 rpm, 28ºC). The bioreactor was then set for operation at 28 ± 1 °C in controlled air flow rates ranging from 0.2 and 1.2 vvm, depending of the desired O_2 saturation levels. The injected air was purified with the aid of a PTFE filtering hydrophobic membrane (Millipore Corporation, USA), with pores of 0.22 µm in diameter. The dissolved oxygen (set point 10%) and pH (set point 7.0±0.2) were controlled and maintained during the experiment.

Figure2- Overview of the 5L airlift bioreactor used in this study.

Calculation of growth kinetics parameters

During both experiments, several samples were aseptically removed from the bioreactors and the optical densities (OD) at 600 nm were recorded using a spectrophotometer. The conversion of OD_{600} to dry biomass per litre (g/L) was calculated based on previous studies with *Pseudomonas* strains performed in our lab in which an OD_{600} of 1.00 ≈ 1 g/L. The growth curves were generated by using the obtained values and several parameters were calculated.

Bacterial cell growth during the exponential phase can be mathematically described using the following equation (1):

$$\frac{dX}{dt} = \mu X \qquad \textbf{Eq.1}$$

where X is the number or mass of cells (mass/volume), t is time, and μ is the specific growth rate constant (1/time).

By using equation 1 it is possible to calculate the generation time as well as the specific growth rate using data generated from the growth curve. In this sense, the specific growth rate can be calculated from the linear portion of a semi log plot of growth versus time.

The generation time (or doubling time) can be calculated using an integrated version of equation 1:

$$X = X_0 e^{\mu t} \quad \text{or} \quad \ln X = \ln X_0 + \mu t \qquad \textbf{Eq.2}$$

in which X_0 represents the initial number or mass of cells (mass/volume).

For X to be doubled:

$$\frac{X}{X_0} = 2$$

Therefore:

$$2 = e^{\mu t} \quad \text{or} \quad \ln 2 = \mu t \qquad \textbf{Eq.3}$$

Hence:

$$t = \frac{\ln 2}{\mu} \qquad \textbf{Eq.4}$$

and t corresponds to the doubling time (h).

In the non-exponential phases of growth, the specific growth rates and doubling times were calculated by applying the geometric method described by LE DUY and ZAJIC (1973) in the modelled growth kinetics obtained.

The biomass productivity was calculated by using equation 5:

$$P_X = \frac{X - X_o}{t_{exp}} \qquad \textbf{Eq.5}$$

where P_X represents the total biomass produced by the time of the experiment ($g.L^{-1}$ per hour), and t_{exp} the total time of the experiment.

Field experiments

Treviso, Criciúma

This small field experiment was conducted in a coal-mining recovery area in Treviso (28º 28' 36'' S and 49º 27' 28" W), Criciúma (Santa Catarina). The soil in this area is known for its low pH values (pH of 3.9) and heavy metal accumulation resulting from the coal-mining activities.

A consortium of three acid-resistant plants, namely *Avena strigosa, Vicia sativa* and *Lolium multiflorum* were used in the experiment, which consisted in two treatments: non-inoculated plants (control) and plants inoculated with *P. thivervalensis* SC5. Each treatment entailed a 50x50 cm area containing hand sown plant seeds (200 kg ha^{-1} *Avena*, 100 kg ha^{-1} *Vicia*, and 62.5 kg ha^{-1} *Lolium*).

The bacterial inoculation was performed by directly applying 150 ml of a TSB grown *P. thivervalensis* SC5 solution OD_{600}= 0.5 over the soil area containing the seeds.

The experiment lasted for 3 months, and after this period, plants in each 50x50 cm area were removed, washed, dried at 60ºC and the total dry weight was measured in an analytical scale.

Xanxerê and Cachoeira do Sul

Two small field experiments using maize were conducted in Santa Catarina (SC) (Xanxerê: 26°55'09.2"S 52°22'50.8"W) and Rio Grande do Sul (RS) (Cachoeira do Sul: 30°16'35.7"S 52°53'07.4"W). These experiments were gently conducted by Prof. Admir Giachini (UFSC), as part of a larger experiment with the objective to study the plant growth promotion abilities of a commercial (endo)mycorrhiza inoculant under a large scale agricultural setup. Therefore, this experiment was conducted in agricultural soils. General information about the experiments can be found in **Table 2**.

Since P is one of the main nutrients that mycorrhiza supply to the plant, the experiment was conducted and several P fertilization strategies were tested. In this case, the experiment consisted of five treatments:

1. Non-inoculated plants without the addition of soil phosphate (NI-0P);
2. Non-inoculated plants with the addition of soil phosphate (fertilized to meet the 100% levels of the phosphate dose recommended by the Brazilian government) (NI-100P);
3. Bacterial inoculation without the addition of soil phosphate (B-0P);
4. Mycorrhiza inoculant without the addition of soil phosphate (M-0P);
5. and Mycorrhiza and bacteria inoculation without the addition of soil phosphate (MB-0P).

The mycorrhiza inoculant was mixed with seeds, with an average number of 41 fungal propagules (hyphae) per seed.

The bacterial inoculant, consisting of a mixed culture of *P. palleroniana* MAB3 and *P. thivervalensis* SC5, was tested in a small area of the larger experiments. The bacterial strains previously grown in bioreactors were mixed in a 1:1 (v/v) ratio based on OD_{600} readings. The final mixed bacterial concentration was adjusted to an OD_{600} of 0.5 (0.5 g/L). A total of 5L of this solution were distributed in each parcel (described below) directly in the soil containing the seeds.

Table 2- Field experiments general information.

EU	Sowing (2016)	Harvest (2017)	Soil P (mg/dm^3)	Maize plants and fertilization conditions		
				Cultivar	Density (plants/ Considered area)	N-K-P* (kg/ha)
RS	04/11	01/03	16.00	DOW2A401	96	110-90-110
SC	23/09	28/02	5.70	LG 6033 PRO2	68	120-115-50

EU- experimental unit

* P was added only in the NI-100 treatment.

Assay specifications and parameters analysed

Each of the bacterial treatments consisted on a single 24 m^2 (4 m x 6 m) parcel spaced from each other by 1 m. The other treatments consisted on six randomized 24 m^2 areas spaced from each other by 1m horizontally and 2m vertically. The 24 m^2 areas were divided in eight lines (for seed sowing) spaced from each other by 0.5 m.

Only the inner 10 m^2 (six inner lines of each parcel) were considered for the evaluation of plant growth parameters (considered area). In the SC and RS experiments, an average of 68 and 96 plants were present per considered area of each parcel. A total of 18 plants were taken from the considered area and their total fresh weight and total grain weight was measured in an analytical scale.

RESULTS AND DISCUSSION

Strains MAB3 and SC5 cultivation in bioreactor

The cultivation of strain MAB3 was monitored for 6 consecutive hours, and in each hour a sample was taken and OD_{600} values recorded. An overview of the experiment is presented in **Figure 3A** and **B**.

The oxygen levels were always maintained at levels above 20%, guaranteeing the presence of optimum levels of oxygen during the experiment (**Figure 3A**). The high peaks in oxygen concentrations reflect the activation of the stirrer/RPM controlling program, which were activated when the oxygen levels decreased below the selected set point.

The OD_{600} values rapidly increased during the experiment until reaching a value of 1.648 (equivalent to ~1.648 g/L) after 6 h of cultivation. Curiously, the obtained OD values did not reflect an exponential growth in all the phases of the cultivation experiment. In the first 3 hours, the bacterial growth followed an exponential growth (**Figure 3B**). During this period, the doubling time was 1.08 hr,

therefore corresponding to approximately one bacterial generation per hour.

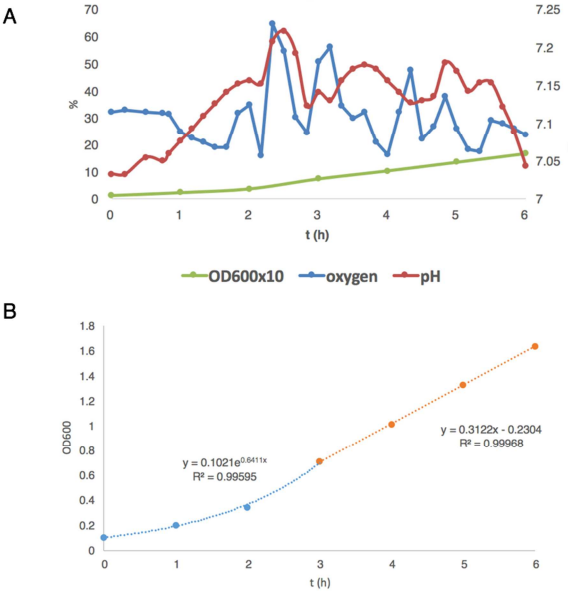

Figure 3- A) Overview of the strain MAB3 bioreactor cultivation parameters and B) strain MAB3 growth kinetics. The blue trend line indicates the exponential growth phase; the orange trend line indicates the limited growth phase.

The medium pH tended to increase during this time, thus, suggesting the production of alkaline compounds such as ammonia. *Pseudomonas* species, including strain MAB3, are known to produce ammonia following the degradation of proteins and amino acids (RHODES, 1959). This fact suggests that in the initial phases of the cultivation strain MAB3 degraded the peptones/amino acids present in the TSB medium (mainly in soy peptone, **Table 1**) thus, leading to the production of ammonia and an overall rapid growth. This result is consistent with previous studies demonstrating that amino acids, and not glucose, are the preferred carbon sources for *Pseudomonas* (ROJO, 2010). The degradation of amino acids results in metabolites that directly enter the TCA cycle, while glucose degradation is a more complex and slow pathway (more enzymes and transporters involved) (NIKEL et al., 2015). In fact, Nikel and colleagues (2015)

observed that when compared to glucose, succinate (an element of the TCA cycle) is used more rapidly by *P. putida* KT2440, however, less efficiently (less conversion into biomass). Moreover, in *Pseudomonas*, in the presence of both succinate and glucose, the glucose degradation pathway is repressed until all succinate is consumed (ROJO, 2010).

In the last 3 hours of cultivation, the bacterial growth was reduced and did not follow an exponential growth (**Figure 3 B**). During this period, a doubling time of 2.43 hours was calculated. The reduction in the bacterial growth rate indicates the presence of limiting conditions in this phase of the experiment. Interestingly, during this time, the pH values tended to decrease, suggesting the production of organic acids or other acidic compounds. Curiously, *P. putida* KT2440 and *Pseudomonas* strains (in general) are known to degrade glucose mainly via its transformation into gluconate and 2-ketogluconate, both organic acids that are released to the growth medium and subsequently taken up and transformed in the Entner-Doudoroff pathway (ROJO, 2010; NIKEL et al., 2015). This result suggests that under the limited conditions (t= 3-6 hours), glucose was the preferred source of carbon of strain MAB3, leading to the production of gluconate, and an overall slower growth.

Nevertheless, several other factors, acting individually or in group, may be involved in the growth limitation observed, amongst them: micronutrient exhaustion (e.g. vitamins), use of the nutrients to the production of secondary metabolites (e.g. surfactants and exopolysaccharides) at the expense of cell biomass, catabolite repression or population control (e.g. production of toxins). More studies are necessary in order to unveil the factors involved in the limitation of strain MAB3 growth.

The final concentration of MAB3 cells obtained was 1.648 g/L (obtained 6h after the beginning of the experiment), which in the final volume of 4.75L represented a total of 7.83 grams of cells and a productivity of 1.289 g/L/h.

The cultivation of strain SC5 was monitored for 6 consecutive hours, and in each hour a sample was taken and OD_{600} values recorded. Due to technical problems on data recording, no oxygen and pH values are available for this experiment. Nevertheless, oxygen levels were always maintained above 20% and pH values at 7.0±0.2.

The OD_{600} values increased during the experiment until reaching a final value of 1.076 (equivalent to 1.076 g/L) after 6 h of cultivation (**Figure 4**).

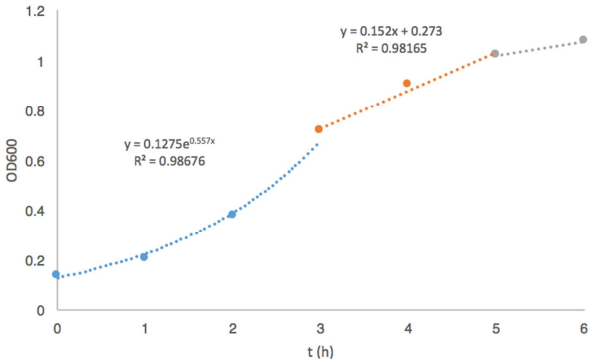

Figure 4- Strain SC5 growth kinetics when cultivated in an airlift bioreactor. The blue trend line indicates the exponential growth phase; the orange trend line indicates the limited growth phase. The grey trend line suggests the beginning of a stationary phase.

The obtained OD_{600} values for strain SC5 did not reflect an exponential growth in all the phases of the cultivation experiment. In the first 3 hours, the SC5 growth followed an exponential growth (**Figure 4**). During this period, the doubling time was 1.20 hr, therefore corresponding to approximately one bacterial generation per hour (total of 3 generations). After this period, the bacterial growth was severely affected and reached a stationary phase after 6h. This result indicates the presence of limiting conditions, that in this case, were more severe than those found in the MAB3 experiment.

The final concentration of SC5 cells obtained was 1.076 g/L (obtained 6h after the beginning of the experiment), which in the final volume of 4.75L represented a total of 5.11 grams of cells and a productivity of 0.828 g/L/h. These values are lower than those obtained in the MAB3 experiment (7.83 g, 1.289 g/L/h).

While using the same medium (TSB), the strains were grown in different types of bioreactor (MAB3 in RALF vs. SC5 in airlift). However, it is highly unlikely that the selection of the bioreactor influences the bacterial growth, since both bioreactors were able to provide optimum temperature, oxygen and pH values, and both strains grew equally in the first phase (t=0-3 h) of the cultivation (**Figure 5**).

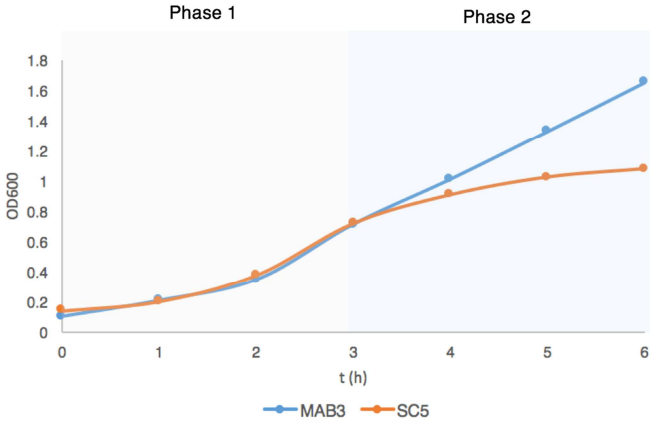

Figure 5- Growth kinetics of strains MAB3 and SC5 cultivated in TSB medium in a RALF and airlift bioreactor, respectively.

It is possible that the growth limitation observed in the SC5 experiment reflects the inability/inefficacy of this strain in using a substrate in the second phase of the experiment (t= 3-6h), that based on the previous data is presumed to be glucose and/or gluconate and/or 2-ketogluconate.

While both MAB3 and SC5 strains belong to the *Pseudomonas* genus, they are distinct species and therefore may present different genetic backgrounds. The lack of some genes involved in glucose and/or gluconate metabolism in strain SC5 would explain its slower growth in the second phase when compared to MAB3. To test this hypothesis, the genomes of strains MAB3 and SC5 were analysed for the presence of genetic elements involved in the major pathways of glucose and gluconate metabolism, namely, the Pentose phosphate (PP), Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED), and gluconate transformation/degradation (GTD) pathways (**Table 2**). The analysis revealed that both strains contain all the genetic elements involved in glucose transport and the PP, EMP and ED pathways (**Table 3**). Nevertheless, the number and presence of genetic elements involved in glucose and gluconate transformation differed between the strains (**Table 3**, **Figure 6**).

Both strains possess the glucose-1-dehydrogenase and quinoprotein glucose dehydrogenase enzymes, involved in the production of gluconate. Still, strain MAB3 possesses two genes encoding the gluconolactonase enzyme involved in the transformation of D-glucono-1,5-lactone to D-gluconate, while strain SC5 only contains one (**Table 3**). This suggests that strain MAB3 is

able to transform glucose and produce an increased level of D-gluconate when compared to strain SC5. Consistently, strain MAB3 contains three *gntP* genes encoding gluconate symporters, while strain SC5 contains two *gntP* genes, thus suggesting an increased gluconate transport to the cell of MAB3 when compared to SC5. The most striking difference between strains is at the level of 2-ketogluconate production. In this case, the gluconate-2-dehydrogenase genes involved in 2-ketogluconate production are found in strain MAB3 but absent in strain SC5, indicating that strain SC5 is unable to produce 2-ketogluconate. Hence, in the presence of glucose, it is likely that strain SC5 is only able to divert carbon from glucose itself and gluconate, while strain MAB3 is able to use glucose, gluconate and 2-ketogluconate.

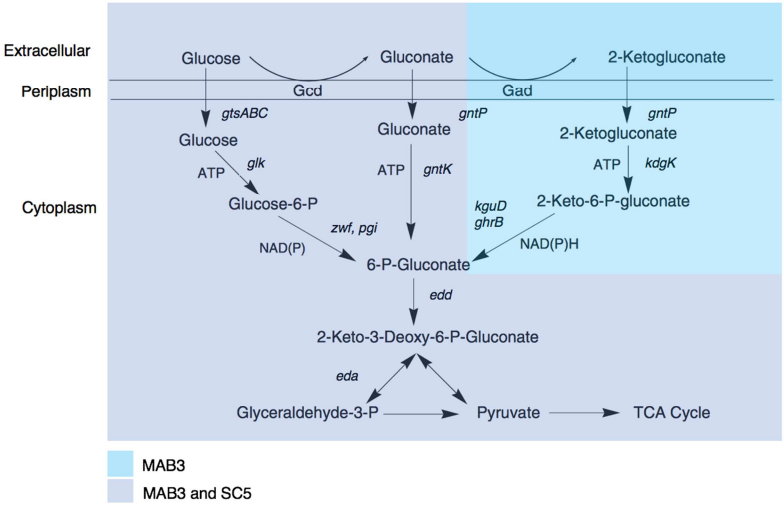

Figure 6- Potential glucose utilization routes in strains MAB3 and SC5. Pathways in dark blue are present/active in both strains. Patways in light blue are only present/active in strain MAB3. A detailed gene nomenclature can be found in Table 3. This figure was adapted from BASU and PHALE (2006).

Table 3- Genetic elements involved in glucose metabolism in *P. palleroniana* MAB3 and *P. thivervalensis* SC5.

MAB3 Locus tag	SC5 Locus Tag	Gene	Product	Pathway
CYL20_12940	CE140_13390	*gtsA*	Glucose/mannose transport system substrate-binding protein	**Glucose transport**
CYL20_12935	CE140_13385	*gtsB*	Glucose/mannose transport system permease protein	
CYL20_12930	CE140_13380	*gtsC*	Glucose/mannose transport system permease protein	
CYL20_00130	CE140_00065	*gdh*	Glucose 1-dehydrogenase	**Glucose degradation via gluconate**
CYL20_23350	CE140_22095	*gcd*	Quinoprotein glucose dehydrogenase	
CYL20_04380 **CYL20_04720**	**CE140_01430**	*gnl*	Gluconolactonase	
CYL20_12685 **CYL20_14595** **CYL20_21350**	**CE140_13245** **CE140_16015**	*gntP*	Gluconate:H+ symporter, gntp family	**Gluconate transport**
CYL20_12690	CE140_13250	*gntK*	Gluconokinase	**D-gluconate to 6-phospho-D-gluconate**
CYL20_17965	n.f	*gadh*	Gluconate 2-dehydrogenase alpha chain	**D-gluconate to 2-keto-D-gluconate (2KG)**
CYL20_17960	n.f		Gluconate 2-dehydrogenase gamma chain	
CYL20_17970	n.f		Gluconate 2-dehydrogenase cytochrome c subunit	
CYL20_02395 **CYL20_06450**	**CE140_03530** **CE140_07165** **CE140_08825**	*kdgK*	2-dehydro-3-deoxygluconokinase	**2KG to 2-keto-6-phosphogluconate**
CYL20_02385	CE140_03520	*kguD*	2-ketogluconate reductase	**2KG to 6-phospho-D-gluconate**
CYL20_22760	CE140_22880	*ghrB*	Glyoxylate/hydroxypyruvate/2-ketogluconate reductase	**2KG to 6-phospho-D-gluconate**
CYL20_12890	CE140_13345	*eda*	2-dehydro-3-deoxy-phosphogluconate aldolase	**Entner Doudoroff**
CYL20_13525	CE140_13415	*edd*	6-phosphogluconate dehydratase	
CYL20_02280 CYL20_12900	CE140_13355 CE140_06535	*zwf*	Glucose-6-phosphate 1-dehydrogenase	**Pentose phosphate**
CYL20_06185 CYL20_12895	CE140_13350 CE140_03440	*pgi*	6-phosphogluconolactonase	
CYL20_02275	CE140_06540	*gnd*	6-phosphogluconate dehydrogenase	

Table 3- continued.

CYL20_16155	CE140_18440	*rpiA*	Ribose 5-phosphate isomerase A	
CYL20_06510	n.f	*rpiB*	Ribose 5-phosphate isomerase B	
CYL20_14870	CE140_25650	*rpe*	Ribulose-phosphate 3-epimerase	
CYL20_25660	CE140_10405	*tal*	Transaldolase	
CYL20_06595 **CYL20_15590**	CE140_26365	*tkt*	Transketolase	
CYL20_04435	CE140_04470	*pgm*	Phosphoglucomutase, alpha-D-glucose phosphate-specific	**Embden-Meyerhoff-Parnas**
CYL20_16955	CE140_27700	*pmm-pgm*	Phosphomannomutase / phosphoglucomutase	
CYL20_13520	CE140_13410	*glk*	Glucokinase	
CYL20_27330	CE140_23660	*gpi*	Glucose-6-phosphate isomerase	
CYL20_21985	CE140_15460	*fruK*	1-phosphofructokinase	
CYL20_15560	CE140_26340	*fba*	Fructose-bisphosphate aldolase	
CYL20_27410	CE140_15570	*tpiA*	Triosephosphate isomerase	
CYL20_13530 **CYL20_25565**	**CE140_07805** **CE140_10495** **CE140_13420**	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	
CYL20_07815	CE140_05655	*gapN*	Glyceraldehyde-3-phosphate dehydrogenase (NADP+)	
CYL20_15575	CE140_26355	*pgk*	Phosphoglycerate kinase	
CYL20_19545	CE140_17885	*gpmI*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	
CYL20_24290	CE140_14070	*eno*	Enolase	
CYL20_13450 CYL20_26510	CE140_20110 CE140_21820	*pyk*	Pyruvate kinase	

n.f.- Not found.

*Genomic analyses were performed based on BlastKoala functional annotations.

Interestingly, Nikel et al., (2015) observed that in *P. putida* KT2440 90% of the glucose influx is channelled into the production of gluconate by glucose dehydrogenase. From the 90% of the hexose 78% was converted to gluconate-6-phosphate by gluconokinase and 12% was transformed to 2-ketogluconate by gluconate-2-dehydrogenase. Only 10% of the glucose entered the pentose phosphate pathway. This result further confirms the importance of the gluconate and 2-ketogluconate production/transformation pathways (including the Entner-Doudoroff pathway) in the *Pseudomonas* glucose metabolism.

Additionally, since high glucose concentrations can be inhibitory (catabolite repression), it is expected that a strain able to transform glucose into sub-products, in this case, gluconate and 2-ketogluconate, suffers less from the catabolite repression. This seems to be the case of strain MAB3, that by efficiently transforming glucose and gluconate to other sub products can overcome the repression/blocking of the glucose/gluconate transport and utilization pathways. On the other hand, due to its decreased ability to transform glucose, strain SC5 may suffer from glucose/gluconate accumulation and a consequent repression/blocking of glucose/gluconate transport and utilization pathways, hence leading to a decreased assimilation and a reduced growth rate. More studies are necessary to understand the regulatory genetic elements involved in glucose and other compounds metabolism in these strains.

Overall, the obtained data demonstrates that although these bacteria belong to the same genera and both present high levels of ACC deaminase activity, their genetic background and metabolic activities greatly vary. Hence, new strategies for the cultivation of these ACC deaminase-producing PGPB need to be considered and developed, to maximize their production and the consequent use in agricultural and biotechnological applications.

Field experiments

Treviso

The results obtained from the field experiment in Treviso show that when compared to the non-inoculated area (**Figure 7A**), the area receiving the *P. thivervalensis* SC5 inoculation demonstrated an increased seed germination and overall plant development (**Figure 7B**). These results also reflected on the obtained plant dry weights (**Figure 8**). In this sense, the inoculation with strain SC5 led to an

increased total plant root and shoot dry weight, and consequently, total dry biomass, when compared to the non-inoculated control.

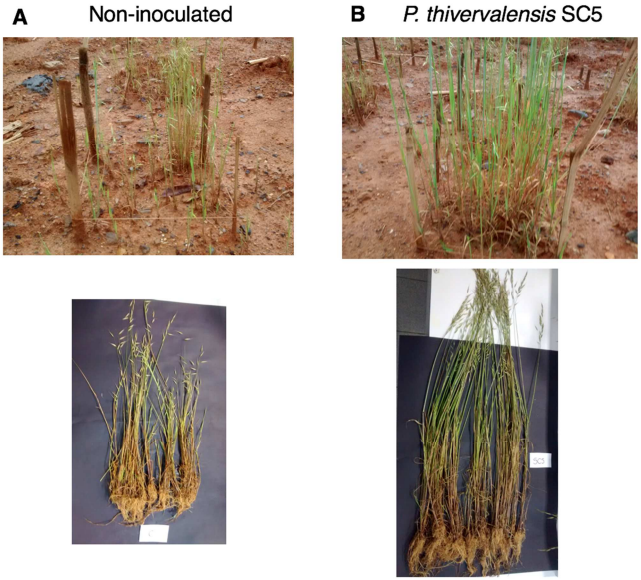

Figure 7- Results obtained from the small field experiment performed in Treviso in a coal-mining contaminated soil. A- Non-inoculated area and respective obtained plants; B- Area inoculated with *P. thivervalensis* SC5 and respective obtained plants.

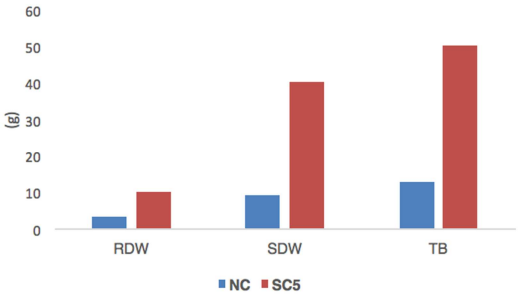

Figure 8- Plant dry biomass obtained from the small field experiment performed in Treviso in a coal-mining contaminated soil. The values were obtained from the total number of plants present in each area.
RDW- Root Dry Weight; SDW- Shoot Dry Weight; TB- Total Dry Biomass.

The conditions presented by the soils from Treviso, and, generally, from coal-mining contaminated areas are highly stressful for plants. In this case, low pH values and the presence of soluble heavy metals such as aluminium, are known inducers of the stress symptoms and growth inhibition observed in plants growing under these conditions. Ethylene is involved in the plant responses to both pH and heavy metal stress, including Al^{3+} (THAO et al. 2015). For example, SUN et al., (2010) indicated that Al^{3+}-induced ethylene production act as a signal to alter auxin distribution in roots leading to the arrest of root elongation. Hence, strain SC5 producing ACC deaminase and, consequently, decreasing inhibitory ethylene levels, may facilitate plant growth under these stressful conditions. This is consistent with previous reports showing the important role of ACC deaminase-producing bacteria in protecting plants from heavy metal stress (NIE et al. 2002; BELIMOV et al. 2005; TRUYENS et al. 2012; KONG et al., 2015). In addition, strain SC5 produces polyamines that are also known to inhibit ethylene biosynthesis and help in protecting plants from aluminium stress (YU et al. 2016). Genome analysis revealed that strain SC5 contains the genes involved in putrescine and spermidine production.

Strain SC5 is also able to transform nitrate and several amino acids into ammonia, therefore, it is possible that its presence in the rhizosphere induces soil alkalinization and reduces the negative effects of the low pH found in the Treviso soil.

Field experiments in Xanxerê and Cachoeira do Sul

Xanxerê (SC) experiment

In the SC experiment, and in the absence of added P, the bacterial inoculation resulted in a 62.2% and 36.2% increase in maize biomass and grain yield, respectively, when compared to the non-inoculated control (NI-0P) (**Figure 9AB**). In the presence of added P, only a 35.6% increase in maize biomass was observed when compared to non-inoculated control (NI-100P). A similar and comparable grain yield was obtained in the bacterial and NI-100P treatments.

The application of the mycorrhiza inoculant resulted in an increased maize biomass and grain yield when compared to the non-inoculated control (**Figure 9AB)**. Interestingly, the bacterial inoculation had a similar effect in plant development when compared to the mycorrhiza inoculant (**Figure 9A**), still, the mycorrhiza inoculant led to an increased grain yield. The application of both mycorrhiza and bacteria inoculants resulted in the maximal plant

biomass achieved, and the grain yield was comparable to that obtained from the mycorrhiza inoculant alone (**Figure 9AB**).

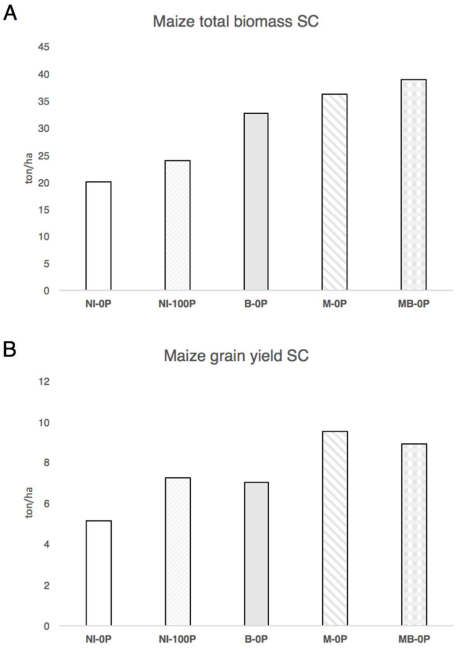

Figure 9- Results obtained from the field experiment performed in Xanxerê, Santa Catarina (SC) using maize. A) Maize total fresh biomass; B) Maize grain yield.
NI-0P- Non-inoculated plants without the addition of soil phosphate; **NI-100P**- Non-inoculated plants with the addition of soil phosphate (fertilized to meet the 100% levels of the phosphate dose recommended by the Brazilian government); **B-0P**- Bacterial inoculation without the addition of soil phosphate; **M-0P**- Mycorrhiza inoculant without the addition of soil phosphate; **MB-0P**- Mycorrhiza and bacteria inoculation without the addition of soil phosphate

The presented values correspond to the average fresh weight of 6 replicates/parcels (each one containing the average values obtained from 18 plants), and their extrapolation to ton/ha. The presented value obtained from the bacterial inoculation treatments (B-0P and MB-0P) corresponds only to the average value obtained from the 18 plants in one parcel.

Cachoeira do Sul (RS) experiment

In the RS experiment, and in the absence of added P, the bacterial inoculation resulted in a 23.0% and 31.4% increase in maize biomass and grain yield, respectively, when compared to the non-inoculated control (NI-0P) (**Figure 10AB**). In the presence of

added P, the bacterial inoculation resulted in a 6.0% and 7.6% increase in maize biomass and grain yield, respectively, when compared to the non-inoculated control (NI-100P).

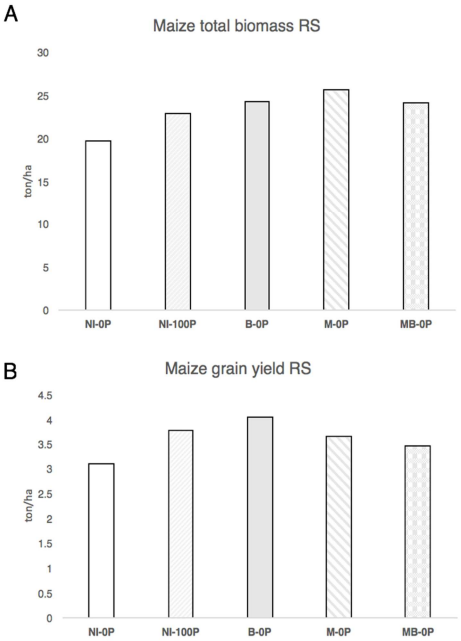

Figure 10- Results obtained from the field experiment performed in Cachoeira do Sul, Rio Grande do Sul (RS) using maize. A) Maize total fresh biomass; B) Maize grain yield.
NI-0P- Non-inoculated plants without the addition of soil phosphate; **NI-100P**- Non-inoculated plants with the addition of soil phosphate (fertilized to meet the 100% levels of the phosphate dose recommended by the Brazilian government); **B-0P**- Bacterial inoculation without the addition of soil phosphate; **M-0P**- Mycorrhiza inoculant without the addition of soil phosphate; **MB-0P**- Mycorrhiza and bacteria inoculation without the addition of soil phosphate

The presented values correspond to the average fresh weight of 6 replicates/parcels (each one containing the average values obtained from 18 plants), and their extrapolation to ton/ha. The presented value obtained from the bacterial inoculation treatments (B-0P and MB-0P) corresponds only to the average value obtained from the 18 plants in one parcel.

The application of the mycorrhiza inoculant resulted in an increased maize biomass and grain yield when compared to the non-inoculated control (**Figure 10AB**). Interestingly, in this experiment the bacterial inoculation had a similar effect in plant development

and grain yield when compared to the mycorrhiza inoculant (**Figure 10AB**). The application of both mycorrhiza and bacteria inoculants resulted in similar biomass the grain yield values when compared to the inoculation with the mycorrhiza and bacterial inoculant independently (**Figure 10AB**).

Although the lack of replicates in the bacterial inoculation treatments limits the experimental data analysis, the results obtained from the maize field experiments in SC and RS greatly suggests the existence of a beneficial effect of the mixed inoculant consisting of strain MAB3 and strain SC5. In both experiments, the bacterial inoculation resulted in an increased maize total biomass and grain biomass when compared to the non-inoculated control (sometimes even in the presence of added phosphate). These results indicate that the bacterial inoculation presented a beneficial effect of plant development under low P input, and its use may limit the application of potential pollutant P fertilizers.

Both strains MAB3 and SC5 presented the ability to solubilize phosphate *in vitro*, hence, it is possible that these strains can solubilize soil phosphate and increase plant P nutrition under field conditions. Interestingly, *Pseudomonas* strains are known to solubilize inorganic phosphate by the production of gluconate (PARK et al. 2009; OTEINO et al. 2015). As previously discussed, strains MAB3 and SC5 possess the genes involved in glucose transformation to gluconate. Therefore, it is likely that the MAB3-SC5 bacterial inoculant presents P-solubilizing activities by containing gluconate-producing strains and some levels of gluconate resulting from the bacterial growth in a medium containing glucose.

Ethylene is a known regulator of the plant response to low phosphorous stress. In this sense, under low phosphorous conditions plants tend to produce increased ACC and ethylene levels (LEI et al., 2011; ROLDAN et al., 2016), which limit root development. Thus, it is possible that the ACC deaminase-producing strains MAB3 and SC5 not only solubilize phosphate, but also decrease the plant ACC and ethylene levels resulting from low-P stress.

In general, the bacterial inoculant created in this study showed a similar beneficial effect when compared to the commercial (endo)mycorrhiza inoculant tested. Endomycorrhiza inoculants cannot be produced in bioreactors, since these symbiotic fungi need the presence of a plant host in order to complete their life cycle. This makes its production more difficult, less controllable, time-consuming and, consequently, more expensive. On the other hand, PGPB can

be easily and rapidly produced in a large scale in bioreactors, and their growth conditions can be optimized and fitted to produce selected compounds such as gluconate. Hence, the MAB3-SC5 mixed inoculant represents an alternative to both soil P application and use of expensive Endomycorrhiza inoculants under large-scale agricultural applications.

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GENERAL CONCLUSIONS

- Ethylene and ACC act as major regulators of plant-bacterial interactions including the nodulation process of leguminous plants.
- Different plants present different ACC and ethylene production abilities and sensitivities, thus, inducing a differential selection of ACC and ethylene modulation mechanisms in their associated bacterial communities and symbionts.
- Genes involved in ACC and ethylene modulation, especially, the ACC deaminase gene, are present in Bacteria and Fungi belonging to different genera and species
- ACC deaminase is present in many beneficial plant-associated bacteria and in some instances the ACC deaminase gene is horizontally transmitted between strains, possibly resulting from a selection pressure induced by the host plant.
- Engineering strains to produce ACC deaminase results in increased plant growth promotion and protection abilities, hence reflecting the importance of ACC deaminase in plant-bacteria interactions.
- Free-living bacterial strains presenting ACC deaminase activity can increase rhizobial nodulation.
- A simple methodology can be used to isolate a wide-range of ACC deaminase-producing bacteria, as well as yeasts and other fungi, from different sources. This leads to the isolation of plant-growth promoting bacteria without the need for an extensive bacterial collection.
- ACC deaminase-producing bacteria are diverse, and may present different genetic traits and metabolism. These bacteria can be selected according to the desired application.
- The genome sequence of ACC deaminase-producing bacteria and its analysis is a valuable tool, since it allows the prediction of metabolic activities.
- Selected ACC deaminase-producing bacteria are effective under field conditions, in both biotechnological (soil recovery) and agricultural setups.

FUTURE PERSPECTIVES AND STUDIES

From an applied point of view:

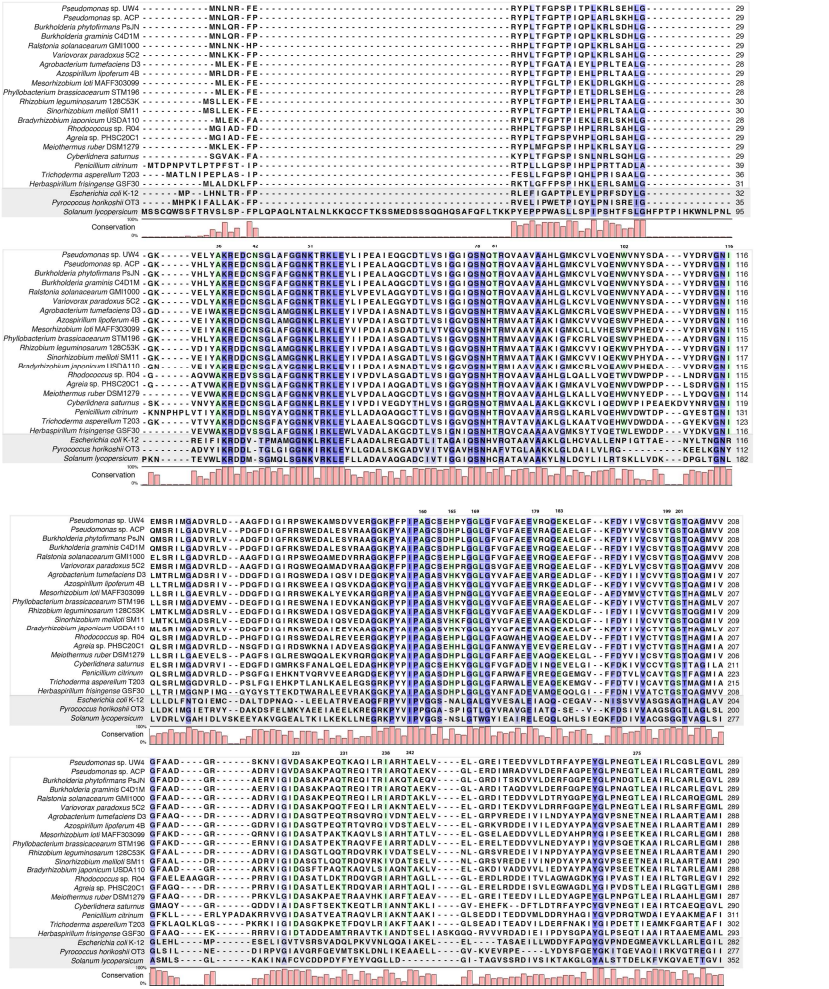
- Inoculant formulation and development based on selected ACC deaminase-producing bacteria, other phytohormone-degrading bacteria and rhizobia.
- Genome-mining of the isolated bacteria and the study of genetic clusters involved in the production of valuable compounds and enzymes (e.g. surfactants, antibiotics, novel Glycosyl hydrolases).
- Optimization of cultivation techniques in bioreactors.
- Study the effect of selected bacteria on the development of other ACC and ethylene-producing organisms such as mushrooms and algae.

From a fundamental point of view:

- Understand the role of ACC deaminase in plant microbiome assembly
- Study the role of ACC deaminase in bacterial modulation of plant defense responses and symbiotic programs
- Study the effect of ACC deaminase in bacterial endophytism
- Obtain more knowledge on the effects of ACC and ethylene in bacterial physiology (e.g. motility, chemotaxis, production of compounds, genetic responses)
- Understand the mechanism involved in ACC uptake by bacteria and ACC exudation in plants
- Ascertain the role of ACC deaminase in yeasts and other fungi
- Understand the effects of ACC deaminase in the ecology of microbes that associate with algae and mushrooms.

SUPPLEMENTARY DATA AND INFORMATION

“New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance”



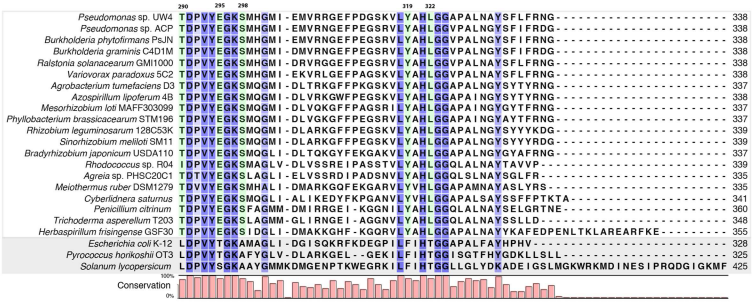


Figure S1 - Multiple sequence alignment based on functional ACC deaminases, putative ACC deaminase sequences from *Agreia* sp. PHSC20C1, *Rhodococcus* sp. R04 (Actinobacteria) and *Meiothermus ruber* DSM1279 (Deinococcus-Thermus). D-cysteine desulfhydrase from *E-coli*, PLP dependent deaminase from *Pyrococcus horikoshii* and PLP dependent deaminase from *Solanum lycopersicum* are highlighted in grey. Conserved residues between all protein groups are shown in blue. ACC deaminase conserved residues are shown in green.

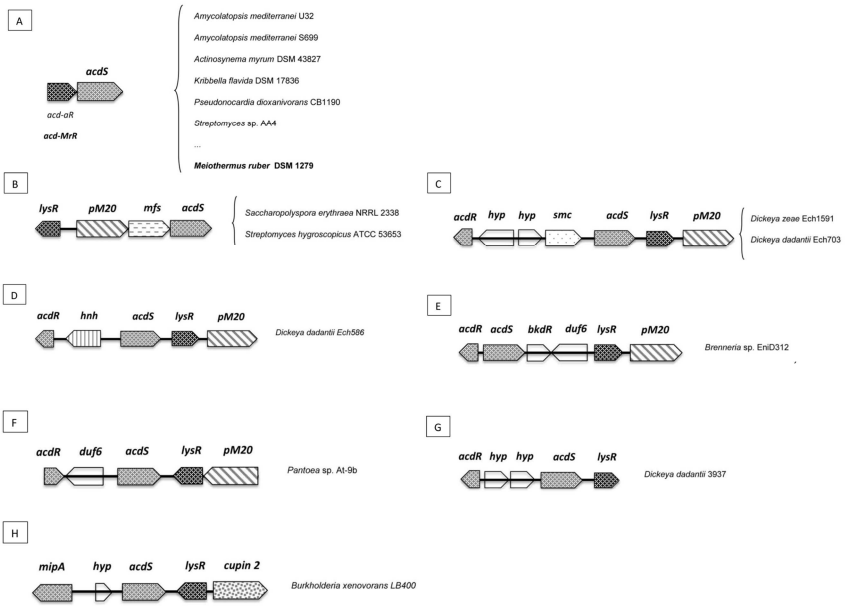


Figure S2- Putative regulators, *acdS* and neighborhood genes organization in some Actinobacteria, Deinococcus-Thermus and Proteobacteria.

Table S1- Accession numbers for Actinobacteria**,** Deinococcus-Thermus and Firmicutes 16S rRNA, *acdS* and *acdR* genes and AcdS and AcdR protein sequences. Description of the *acdS* gene location, ACC deaminase (ACCD) activity, strains relative habitat and geographical origin.

Strain	16S rRNA	*acdS*	AcdS	*acdR*(*)	AcdR(*)	*acdS* location	ACCD	Isolation/ Habitat	Origin
Actinoalloteichus spitiensis RMV-1378	AY426714.2	AGVX02000403.1	CT	n.a	n.a	n.a	n.a	Desert soil	India
Actinoplanes missouriensis 431	AJ277572.1	NC_017093.1	YP_005461921.1	NC_017093.1	YP_005461920.1	n.a	n.a	Barnyard soil	USA
Actinopolyspora halophila DSM 43834	X54287.1	AQUI01000002.1	CT	n.a	n.a	n.a	n.a	Contaminant of a culture containing 25% NaCl	n.a
Actinosynnema mirum DSM 43827	CP001630.1	CP001630.1	YP_003099210.1	CP001630.1	YP_003099209.1	C	n.a	Grass from Raritan River	USA
Agreia sp. PHSC20C1	AAOB01000003.1	AAOB01000003.1	EAR25507.1	AAOB01000003.1	EAR25508.1	n.a	n.a	Marine	Antarctic
Amycolatopsis azurea DSM 43854	AJ400709.1	NZ_ANMG01000058.1	ZP_21908980.1	NZ_ANMG01000058.1	ZP_21908979.1	n.a	n.a	Soil	Japan
Amycolatopsis decaplanina DSM 44594	AJ508237.1	NZ_AOHO01000014.1	ZP_22939816.1	NZ_AOHO01000014.1	ZP_22939815.1	n.a	n.a	Soil	India
Amycolatopsis mediterranei S699	CP002896.1	CP002896.1	AEK44688.1	CP002896.1	AEK44687.1	C	n.a	Soil sample from a pine arboretum	France
Amycolatopsis mediterranei U32	CP002000.1	CP002000.1	YP_003768201.1	CP002000.1	YP_003768200.1	C	n.a	Soil	n.a
Amycolatopsis methanolica 239	AJ249135.1	AQUL01000001.1	CT	n.a	n.a	n.a	n.a	Soil	Papua New Guinea
Amycolatopsis orientalis HCCB10007	n.a	CP003410.1	AGM07658.1	CP003410.1	AGM07657.1	C	n.a	n.a	n.a
Amycolatopsis sp. ATCC 39116	AM263202.1	NZ_JH414689.1	ZP_10050982.1	NZ_JH414689.1	ZP_10050981.1	n.a	n.a	Soil	USA
Arsenicicoccus bolidensis DSM 15745	AJ558133.2	AUFG01000015.1	CT	n.a	n.a	n.a	n.a	Lake sediment containing mine waste	Sweden
Arthrobacter crystallopoietes BAB-32	ANPE02000028.1	NZ_ANPE02000106.1	ZP_24038253.1	NZ_ANPE02000106.1	ZP_24038254.1	n.a	n.a	Soil	India
Arthrobacter sp. 131MFCol6.1	n.a	ARGT01000006.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Austwickia chelonae NBRC 105200	AJ243919.1	NZ_BAGZ01000017.1	ZP_10952085.1	NZ_BAGZ01000017.1	ZP_10952086.1	n.a	n.a	Chelonids	Australia
Bacillus cereus AcdSPB4	JN625722	JN625726.1	AEQ29826.1	n.a	n.a	n.a	n.a	*Agaricus bisporus* casing soil	China
Brevibacterium casei S18	n.a	NZ_AMSP01000011.1	ZP_18854273.1	NZ_AMSP01000011.1	ZP_18854274.1	n.a	n.a	Human healty skin	India
Brevibacterium linens BL2	NZ_AAGP01000013.1	NZ_AAGP01000039.1	ZP_05915229.1	NZ_AAGP01000039.1	ZP_05915228.1	n.a	n.a	n.a	USA
Brevibacterium sp. JC43	JF824806.1	CAHK01000051.1	CT	n.a	n.a	n.a	n.a	Host stool sample	France
Demetria terragena DSM 11295	Y14152.1	AQXW01000004.1	CT	n.a	n.a	n.a	n.a	Frozen compost soil	Germany

Ilumatobacter nonamiense YM16-303	AB360345.1	BAOL01000068.1	CT	n.a	n.a	n.a	n.a	Seashore sand	n.a
Kineosphaera limosa NBRC 100340	AB550802.1	NZ_BAHD01000034.1	ZP_10941583.1	NZ_BAHD01000034.1	ZP_10941582.1	n.a	n.a	Activated sludge	n.a
Kribbella catacumbae DSM 19601	AM778575.1	AQUZ01000035.1	CT	n.a	n.a	n.a	n.a	Tufaceous surfaces in the catacombs of St. Callistus in Rome	Italy
Kribbella flavida DSM 17836	CP001736.1	CP001736.1	ADB31588.1	CP001736.1	ADB31589.1	C	n.a	Soil	China
Meiothermus ruber DSM 1279	NC_013946.1	CP001743.1	YP_003506726.1	CP001743.1	YP_003506727.1	C	n.a	Hot spring	Russia
Meiothermus ruber H328	AB442017.1	BAOR01000002.1	CT	n.a	n.a	n.a	n.a	Hot spring	Japan
Meiothermus timidus DSM 17022	AJ871168.1	ARDL01000011.1	CT	n.a	n.a	n.a	n.a	Hot spring	Portugal
Microbacterium laevaniformans OR221	NZ_AJGR01000262.1	NZ_AJGR01000114.1	ZP_09922379.1	NZ_AJGR01000114.1	ZP_09922380.1	n.a	n.a	Subsurface sediment	USA
Microlunatus phosphovorus NM-1	AP012204.1	AP012204.1	BAK33832.1	n.p	n.p	C	n.a	EBPR activated sludge	Japan
Modestobacter marinus BC501	FO203431.1	NC_017955.1	YP_006364081.1	NC_017955.1	YP_006364080.1	C	n.a	White marble surface	Italy
Mycobacterium abscessus 47J26	AGQU01000002.1	AGQU01000002.1	EHB994390.1	AGQU01000002.1	EHB994391.1	n.a	n.a	Sputum sample from cystic fibrosis patient	UK
Mycobacterium abscessus ATCC 19977	NC_010397.1	NC_010397.1	YP_001702443.1	NC_010397.1	YP_001702444.1	C	n.a	Human knee	n.a
Mycobacterium abscessus subsp. bolletii BD	AY859681.1	NZ_AHAS01000007.1	ZP_12993124.1	NZ_AHAS01000007.1	ZP_12993125.1	n.a	n.a	Sputum of a patient	n.a
Mycobacterium massiliense CCUG 48898	AKVF01000003.1	NZ_AHAR01000007.1	ZP_09409230.1	NZ_AHAR01000007.1	ZP_09409231.1	n.a	n.a	Sputum of a patient	France
Mycobacterium massiliense GO 06	NC_018150.1	NC_018150.1	YP_006520687.1	NC_018150.1	YP_006520688.1	C	n.a	Human patient	Brazil
Mycobacterium smegmatis MC2 155	NC_008596.1	CP000480.1	YP_890948.1	CP000480.1	YP_890946.1	C	n.a	Human smegma	n.a
Mycobacterium vaccae ATCC 25954	NZ_JH814714.1	NZ_JH814693.1	ZP_11011394.1	n.p	n.p	n.a	n.a	n.a	n.a
Nakamurella multipartita DSM 44233	NC_013235.1	CP001737.1	YP_003202162.1	n.p	n.p	C	n.a	Activated sludge	Japan
Nocardioidaceae bacterium Broad-1	n.a	ADVI01000084.1	EGD41421.1	ADVI01000084.1	EGD41420.1	n.a	n.a	Contaminant of *Coccidioides* genomes	n.a
Propionicicella superfundia DSM 22317	n.a	AUIA01000003.1	CT	n.a	n.a	n.a	n.a	Contaminated groundwater	USA
Pseudonocardia dioxanivorans CB1190	CP002593.1	CP002593.1	AEA23305.1	CP002593.1	AEA23304.1	C	n.a	Industrial sludge contaminated with 1,4-dioxan	n.a
Rhodococcus opacus M213	AF095715.1	NZ_AJYC02000133.1	ZP_14483496.1	NZ_AJYC02000133.1	ZP_14483498.1	n.a	n.a	Fuel-oil contaminated soil	USA
Rhodococcus sp. R04	n.a	AFAQ01000515.1	**CT**	n.a	n.a	n.a	n.a	Oil-contaminated soil	China
Saccharopolyspora erythraea NRRL 2338	NC_009142.1	AM420293.1	YP_001104984.1	n.p	n.p	C	n.a	Soil	Philippines

Saccharothrix espanaensis DSM 44229	AF114807.1	NC_019673.1	YP_007035865.1	NC_019673.1	YP_007035864.1	C	n.a	Soil	Spain
Saxeibacter lacteus DSM 19367	n.a	AUFT01000005.1	CT	n.a	n.a	n.a	n.a	Rock	South Korea
Streptomyces acidiscabies 84-104	n.a	NZ_AHBF01000026.1	ZP_10450903.1	NZ_AHBF01000026.1	ZP_10450904.1	n.a	n.a	Potato	n.a
Streptomyces albus J1074	n.a	NZ_ABYC01000170.1	ZP_04701899.1	NZ_ABYC01000170.1	ZP_04701900.1	n.a	n.a	n.a	n.a
Streptomyces bottropensis ATCC 25435	AB026217.1	NZ_KB405067.1	ZP_23431620.1	NZ_KB405067.1	ZP_23431621.1	n.a	n.a	Soil	n.a
Streptomyces cf. griseus XylebKG-1	GL877172.1	GL877172.1	EGE42521.1	GL877172.1	EGE42520.1	C	n.a	Ambrosia beetle	n.a
Streptomyces chartreusis NRRL 12338	AGDE01000038	AGDE01000072.1	**CT**	n.a	n.a	n.a	n.a	Soil	n.a
Streptomyces coelicoflavus ZG0656	AHGS01000024	NZ_AHGS01001413.1	ZP_13043759.1	NZ_AHGS01001413.1	ZP_13043760.1	n.a	n.a	n.a	n.a
Streptomyces davawensis JCM 4913	HE971709	NC_020504.1	YP_007525103.1	NC_020504.1	YP_007525102.1	C	n.a	Soil	Phillipines
Streptomyces ghanaensis ATCC 14672	AB184662.1	NZ_ABYA01000438.1	ZP_04688956.1	NZ_ABYA01000438.1	ZP_04688955.1	n.a	n.a	Soil	Ghana
Streptomyces griseus subsp. griseus NBRC 13350	AP009493.1	AP009493.1	BAG19783.1	AP009493.1	BAG19782.1	C	n.a	Soil	n.a
Streptomyces hygroscopicus ATCC 53653	NR_044201.1	NZ_GG657754.1	ZP_07299431.1	n.p	n.p	n.a	n.a	Soil	India
Streptomyces ipomoeae 91-03	NZ_AEJC01000674.1	NZ_AEJC01000114.1	ZP_19187453.1	NZ_AEJC01000114.1	ZP_19187449.1	n.a	n.a	*Ipomoea batatas*	USA
Streptomyces prunicolor NBRC 13075	AB184294.1	BARF01000064.1	CT	n.a	n.a	n.a	n.a	Soil	n.a
Streptomyces scabiei 87.22	FN554889.1	FN554889.1	YP_003492562.1	FN554889.1	YP_003492561.1	C	n.a	Soil	n.a
Streptomyces sp. AA4	n.a	NZ_GG657746.1	ZP_07280393.1	NZ_GG657746.1	ZP_07280392.1	n.a	n.a	Soil	n.a
Streptomyces sp. PP-C42	n.a	AEWS01000972.1	**CT**	n.a	n.a	n.a	n.a	Marine	Baltic Sea
Streptomyces sp. R1-NS-10	AB808756.1	BARG01000021.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Streptomyces sp. S4	n.a	CADY01000046.1	ZP_09179036.1	CADY01000046.1	ZP_09179037.1	n.a	n.a	Leafcutter ant	n.a
Streptomyces sp. SS	AY507122.1	AKXV01000001.1	CT	n.a	n.a	n.a	n.a	Soil	China
Streptomyces sp. TOR3209	n.a	AGNH01000445.1	CT	n.a	n.a	n.a	n.a	Tomato rhizosphere	China
Streptomyces sp. W007	JN180126.1	NZ_AGSW01000117.1	ZP_09402987.1	NZ_AGSW01000117.1	ZP_09402986.1	n.a	n.a	Marine sediment	China
Streptomyces sviceus ATCC 29083	AB184559.2	CM000951.1	EDY552280.1	CM000951.1	EDY552779.1	C	n.a	Soil	n.a
Streptomyces turgidiscabies Car8	NZ_AEJB01000333.1	NZ_AEJB01000611.1	ZP_20885367.1	NZ_AEJB01000611.1	ZP_20885369.1	n.a	n.a	*Daucus carota* subsp. sativus	Japan

Streptomyces venezuelae ATCC 10712	n.a	FR845719.1	CCA54823.1	FR845719.1	CCA54824.1	C	n.a	Soil	Venezuela
Streptomyces violaceusniger Tu 4113	NZ_AEDI01000202.1	NZ_AEDI01000002.1	EFN21291.1	n.p	n.p	n.a	n.a	Soil	n.a
Streptomyces viridochromogenes DSM 40736	n.a	NZ_GG657757.1	ZP_07302930.1	NZ_GG657757.1	ZP_07302931.1	n.a	n.a	Soil	Cameroon
Streptomyces viridochromogenes Tue57	n.a	NZ_AMLP01000211.1	ZP_21112817.1	NZ_AMLP01000211.1	ZP_21112816.1	n.a	n.a	n.a	n.a
Streptomyces viridosporus T7A	n.a	AJFD01000082.1	CT	n.a	n.a	n.a	n.a	Soil	USA
Tetrasphaera elongata Lp2	NR_024735.1	CAIZ01000139.1	CCH70862.1	CAIZ01000139.1	CCH70861.1	n.a	n.a	Activated sludge	Japan

n.a- not available, unknown; **n.p**- not present; **C**- Chromosome; **CT**-conceptual translation

* at least three types of putative ACC deaminase regulators may be found in different Actinobacteria and *Meiothermus* therefore, a new nomenclature for these genes is proposed in this work. However, the reference in the table presents only *acdR* and AcdR to simplify the visualization.

Table S2- Accession numbers for α-Proteobacteria 16S rRNA, *acdS* and *acdR* genes and AcdS and AcdR proteins sequences. Description of the *acdS* gene location, ACC deaminase (ACCD) activity, strains relative habitat and origin.

Strain	16S rRNA	*acdS*	AcdS	*acdR*	AcdR	*acdS* location	ACCD	Isolation/Habitat	Origin
Acidiphilium multivorum AIU301	AP012035.1	AP012035.1	YP_004284712.1	AP012035.1	YP_004284713.1	C	n.a	Pyritic acid mine drainage	Japan
Agrobacterium tumefaciens D3	HM143942.1	AF315580.1	AAK28496.1	AF315580.1	AAK28495.1	P	Y/FL	Soil	Germany
Agrobacterium vitis S4	NC_011989.1	CP000634.1	ACM38904.1	CP000634.1	ACM38903.1	C2	n.a	Soil	Hungary
Amorphus coralli DSM 19760	DQ097300	ARFZ01000005.1	CT	n.a	n.a	n.a	n.a	*Fungia granulosa*	Israel
Azorhizobium caulinodans ORS 571	AP009384.1	AP009384.1	BAF86265.1	AP009384.1	BAF86264.1	C	n.a	Stem nodule	Senegal
Azospirillum lipoferum 4B	FQ311868.1	DQ125242.2	ABE66282.2	DQ125242.2	ACS92701.1	P	Y/FL	Soil	France
Azospirillum sp. B510	AP010946.1	AP010948.1	BAI75080.1	AP010948.1	BAI75081.1	P	n.a	Soil	Japan
Bradyrhizobium canariense WSM4349	NZ_KB890498.1	NZ_KB890498.1	CT	n.a	n.a	n.a	n.a	*Syrmatium glabrum*	USA
Bradyrhizobium elkanii 587	AJJK01001957	AJJK01000707.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	Brazil
Bradyrhizobium elkanii CCBAU 43297	AJPW01000118	AJPW01000023.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	China
Bradyrhizobium japonicum CCBAU 15354	AJPX01000844	AJPX01000049.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	China
Bradyrhizobium japonicum USDA 6	AP012206	NC_017249.1	YP_005605144.1	NC_017249.1	YP_005605143.1	C	n.a	*Glycine max*	Japan
Bradyrhizobium japonicum USDA110	NC_004463.1	NC_004463.1	BAC45506.1	NC_004463.1	BAC45505.1	C	Y/FL/BN	*Glycine max*	USA
Bradyrhizobium liaoningense CCBAU 05525	AJQC01000887	AJQC01000259.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	China
Bradyrhizobium liaoningense CCBAU 83689	AJQD01000586	AJQD01000027.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	China
Bradyrhizobium oligotrophicum S58	JQ619230	NC_020453.1	YP_007513426.1	NC_020453.1	YP_007513425.1	C	n.a	Paddy field soil	Japan
Bradyrhizobium sp. Btai1	NC_009485.1	NC_009485.1	ABQ36190.1	NC_009485.1	ABQ36191.1	C	n.a	Stem nodule	USA
Bradyrhizobium sp. CCGE-LA001	NZ_AMCQ01000411.1	NZ_AMCQ01000287.1	ZP_16040407.1	NZ_AMCQ01000287.1	ZP_16040408.1	n.a	n.a	*Phaseolus microcarpus* root nodules	Mexico
Bradyrhizobium sp. ORS 278	NC_009445.1	NC_009445.1	CAL77571.1	NC_009445.1	CAL77570.1	C	n.a	Stem nodule	Senegal
Bradyrhizobium sp. ORS 285	CAFH01000247	NZ_CAFH01000233.1	ZP_09476143.1	NZ_CAFH01000233.1	ZP_09476144.1	n.a	n.a	Stem nodule	n.a
Bradyrhizobium sp. ORS 375	CAFI01000413	NZ_CAFI01000189.1	ZP_09421036.1	NZ_CAFI01000189.1	ZP_09421035.1	n.a	n.a	*Aeschynomene indica* stem nodule	n.a

Bradyrhizobium sp. S23321	AP012279	NC_017082.1	YP_005447899.1	NC_017082.1	YP_005447900.1	C	n.a	Paddy field soil	Japan
Bradyrhizobium sp. STM 3843	CAFK01000252	NZ_CAFK01000254.1	ZP_09437058.1	NZ_CAFK01000254.1	ZP_09437057.1	n.a	n.a	n.a	n.a
Bradyrhizobium sp. WSM1253	AHMB01000041	NZ_JH600073.1	ZP_10084551.1	NZ_JH600073.1	ZP_10084552.1	n.a	n.a	Root nodule	n.a
Bradyrhizobium sp. WSM471	AHLW01000002	NZ_CM000442.1	ZP_09645255.1	NZ_CM001442.1	ZP_09645256.1	n.a	n.a	Root nodule	n.a
Bradyrhizobium sp. YR681	AKIY01000183	NZ_AKIY01000270.1	ZP_10583679.1	NZ_AKIY01000270.1	ZP_10583680.1	n.a	n.a	*Populus deltoides* root	USA
Bradyrhizobium yuanmingense CCBAU 05623	AJQJ01000234	AJQJ01000325.1	CT	n.a	n.a	n.a	n.a	*Glycine max*	China
Chelatococcus sp. GW1	ALIQ01000242	ALIQ01000170.1	CT	n.a	n.a	n.a	n.a	Wastewater of a textile dye works	n.a
Fodinicurvata sediminis DSM 21159	FJ357426	ATVH01000016.1	CT	n.a	n.a	n.a	n.a	Sediment	China
Fulvimarina pelagi HTCC2506	AY178860.1	AATP01000002.1	EAU41874.1	n.p	n.p	C	n.a	Marine	Sargasso Sea
Gluconacetobacter xylinus NBRC 3288	AP012159.1	AP012159.1	BAK82498.1	AP012159.1	BAK82490.1	C	n.a	Vinegar	n.a
Gluconobacter frateurii NBRC 101659	AB678443.1	NZ_BADZ01000001.1	ZP_11374548.1	NZ_BADZ01000001.1	ZP_11374547.1	n.a	n.a	Flower of *Monordica charantia*	Thailand
Gluconobacter oxydans H24	NC_019396.1	NC_019396.1	YP_006983155.1	NC_019396.1	YP_006983156.1	n.a	n.a	n.a	n.a
Gluconobacter thailandicus NBRC 3255	AB178396.1	NZ_BAON01000011.1	ZP_23120210.1	NZ_BAON01000011.1	ZP_23120209.1	n.a	n.a	Strawberry	Japan
Labrenzia aggregata IAM 12614	NZ_AAUW01000023.1	AAUW01000003.1	ZP_01546258.1	AAUW01000003.1	ZP_01546257.1	C	n.a	Sediment	Baltic Sea
Mesorhizobium alhagi CCNWXJ12-2	NZ_AHAM01000052.1	NZ_AHAM01000292.1	ZP_09297037.1	NZ_AHAM01000292.1	ZP_09297038.1	n.a	n.a	*Alhagi sparsifolia*	China
Mesorhizobium amorphae CCNWGS0123	NZ_AGSN01000002.1	NZ_AGSN01000010.1	ZP_09085370.1	n.p	n.p	n.a	n.a	Soil/root nodule	China
Mesorhizobium ciceri bv. *biserrulae* WSM1271	NC_014923.1	NC_014923.1	ADV14828.1	n.p	n.p	C/SI	n.a	Soil/root nodule	Italy
Mesorhizobium loti MAFF303099	NC_002678.2	NC_002678.2	BAB52295.1	n.p	n.p	C/SI	Y/BN	Soil/root nodule	Japan
Mesorhizobium loti R7a	n.a	AL672114.1	CAD31305.1	n.p	n.p	C/SI	n.a	Soil/root nodule	New Zealand
Mesorhizobium mettallidurans STM 4661	n.a	CAAF01000045.1	CCV12789.1	n.p	n.p	n.a	n.a	*Anthyllis vulneraria*	France
Mesorhizobium opportunistum WSM2075	ACZA00000000.1	ACZA00000000.1	EEW34025.1	n.p	n.p	C/SI	n.a	Soil/root nodule	Australia
Methylobacterium mesophilicum SR1.6/6	n.a	NZ_ANPA01000016.1	ZP_23995079.1	NZ_ANPA01000016.1	ZP_23995080.1	n.a	n.a	*Citrus sinensis*	Brazil
Methylobacterium nodulans ORS 2060	CP001349.1	CP001349.1	ACL60323.1	CP001349.1	ACL60322.1	C	n.a	Soil	Senegal
Methylobacterium radiotolerans JCM 2831	CP001001.1	CP001001.1	ACB23516.1	CP001001.1	ACB23515.1	C	n.a	Soil	Japan

Methylobacterium sp. 4-46	CP000943.1	CP000943.1	ACA14842.1	CP000943.1	ACA14841.1	C	n.a	*Lotononis bainesii*	n.a
Methylobacterium sp. 77	n.a	ARCS01000002.1	CT	n.a	n.a	n.a	n.a	n.a	USA
Methylobacterium sp. B34	n.a	BADE01001099.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Methylobacterium sp. GXF4	n.a	NZ_AKFK01000048.1	ZP_10354465.1	NZ_AKFK01000048.1	ZP_10354464.1	n.a	n.a	*Vitis vinifera*	USA
Nitratireductor indicus C115	AMSI01000044	NZ_AMSI01000004.1	ZP_11155157.1	NZ_AMSI01000004.1	ZP_11155156.1	n.a	n.a	Deep seawater	Indian Ocean
Pannonibacter phragmitetus DSM 14782	n.a	ARNQ01000046.1	CT	n.a	n.a	n.a	n.a	Surface of decomposing rhizomes of reed	Hungary
Pelagibaca bermudensis HTCC2601	NZ_AATQ01000003.1	AATQ01000052.1	EAU44226.1	AATQ01000052.1	EAU44227.1	n.a	n.a	Marine	Sargasso Sea
Phyllobacterium brassicacearum STM 196	AY785319.1	EF452620.1	ABO31418.1	EF452621.1	ABO31419.1	n.a	Y/FL	Soil	France
Rhizobium gallicum PB2	EF525207.1	EF525234.1	ABP88062.1	EF525261.1	ABP88045.1	n.a	Y/FL	Soil	Canada
Rhizobium grahamii CCGE 502	AEYE01000061	AEYE01000046.1	CT	n.a	n.a	n.a	n.a	*Dalea leporina*	Mexico
Rhizobium leguminosarum bv. *trifolii* SRDI565	n.a	AQUD01000006.1	CT	n.a	n.a	n.a	n.a	*Trifolium subterraneum*	Australia
Rhizobium leguminosarum bv. *trifolii* WU95	n.a	NZ_JH660657.1	ZP_18307677.1	NZ_JH660657.1 (pseudogene)	n.a	n.a	n.a	n.a	n.a
Rhizobium leguminosarum bv. *viciae* 128C53K	n.a	AF421376.1	AAL16088.1	AY172673.1	AAO17689.1	n.a	Y/FL	Soil	n.a
Rhizobium leguminosarum bv. *viciae* 3841	NC_008380.1	NC_008381.1	YP_770380.1	n.p	n.p	P	n.a	Soil	U.K
Rhizobium leguminosarum bv. *viciae* Vc2	n.a	ARDP01000039.1	CT	n.a	n.a	n.a	n.a	*Viccia cracca*	England
Rhizobium leguminosarum PB171	EF525228.1	EF525246.1	ABP88074.1	EF525273.1	ABP88057.1	n.a	Y/FL	Soil	Canada
Rhizobium leguminosarum PB223	EF525233.1	EF525250.1	ABP88078.1	EF525277.1	ABP88061.1	n.a	Y/FL	Soil	Canada
Rhizobium leguminosarum PB62	EF525210.1	EF525237	ABP88065.1	EF525264	ABP88048.1	n.a	Y/FL	Soil	Canada
Rhizobium mesoamericanum STM3625	CANI01000030	CANI01000090.1	CT	n.a	n.a	n.a	n.a	*Mimosa pudica*	French Guiana
Rhizobium radiobacter K84	NC_011985.1	CP000629.1	ACM30009.1	CP000629.1	ACM30008.1	C2	n.a	Soil	n.a
Rhizobium sp. AP16	AJVM01000087	NZ_AJVM01000009.1	ZP_10535789.1	NZ_AJVM01000009.1	ZP_10535790.1	n.a	n.a	*Populus deltoides* root	USA
Rhizobium sp. PDO1-076	AHZC01000107	NZ_AHZC01000377.1	ZP_13492557.1	NZ_AHZC01000377.1	ZP_13492558.1	n.a	n.a	*Populus deltoides* root	USA
Rhizobium sp. Pop5	AMCP01001078	NZ_AMCP01000592.1	ZP_16036138.1	NZ_AMCP01000592.1	ZP_16036139.1	n.a	n.a	*Phaseolus vulgaris* root nodule	Mexico
Rhizobium sp. PRF 81	n.a	AQHN01000086.1	CT	n.a	n.a	n.a	n.a	*Phaseolus vulgaris*	Brazil

Rhizobium tropici CIAT 899	HQ850704	NC_020061.1	YP_007336138.1	n.p	n.p	P	n.a	*Phaseolus vulgaris* root nodule	Colombia
Roseibium sp. Trich SKD4	NZ_GL476310.1	NZ_GL476315.1	ZP_07659928.1	NZ_GL476315.1	ZP_07659927.1	C	n.a	Seawater, Trichodesmium colonies	North Atlantic Ocean
Sagittula stellata E-37	NZ_AAYA00000000	AAYA01000005.1	EBA08640.1	AAYA01000005.1	EBA08641.1	C	n.a	Coastal seawater	USA
Salipiger mucosus DSM 16094	n.a	ARRM01000068.1	CT	n.a	n.a	n.a	n.a	Saline soil bordering a saltern	Spain
Sinorhizobium fredii GR64	AMCX01000136	AMCX01000095.1	CT	n.a	n.a	C	n.a	*Phaseolus vulgaris*	Spain
Sinorhizobium medicae WSM419	NC_009636.1	NC_009622	YP_001314953.1	NC_009622	YP_001314954.1	P	n.a	Soil	Italy
Sinorhizobium meliloti 4H41	n.a	AQWP01000042.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Sinorhizobium meliloti AK83	NZ_AEDH01000067.1	NZ_AEDH01000067.1	ZP_07601419.1	NZ_AEDH01000067.1	ZP_07601418.1	C3	n.a	Soil	Kazakhstan
Sinorhizobium meliloti BL225C	AEDG01000070.1	AEDG01000070.1	ZP_07593541.1	AEDG01000070.1	ZP_07593542.1	P	n.a	Soil	Italy
Sinorhizobium meliloti CCNWSX0020	AGVV01000154	NZ_AGVV01000056.1	ZP_12976532.1	NZ_AGVV01000056.1	ZP_12976531.1	n.a	n.a	*Medicago lupulina*	China
Sinorhizobium meliloti KYA40	EU603723.1	EU603722.1	ACC78287.1	n.a	n.a	n.a	n.a	Soil	Iran
Sinorhizobium meliloti KYA71	EU603721.1	EU003994.1	ABS19884.1	n.a	n.a	n.a	n.a	Soil	Iran
Sinorhizobium meliloti SM11	CP001830.1	DQ145546.1	ABA56046.1	DQ145546.1	ABA56047.1	P	Y/FL	Soil	Germany
Sinorhizobium sp. BL3	AY943949.1	EU183545.1	ABW39374.1	EU183545.1	ABW39373.1	n.a	Y/FL	Soil	Thailand
Starkeya novella DSM 506	NR_025859.1	CP002026.1	ADH87862.1	NC_014217.1	ADH87863.1	C	n.a	Soil	n.a
Thalassospira xiamenensis DSM 17429	n.a	NZ_AMRQ01000015.1	ZP_11118220.1	NZ_AMRQ01000015.1	ZP_11118219.1	n.a	n.a	Surface water of a waste oil pool	China
Tistrella mobilis KA081020-065	CP003236	CP003239.1	AFK57065.1	CP003239.1	AFK57066.1	P	n.a	Marine	Red Sea

n.a- not available; **n.p**- not present; **C**- Chromosome; **C2**- 2nd chromosome; **C/SI**- Chromosome/symbiotic island; **P**- plasmid; **Y/FL**- Yes/free living conditions; **Y/BN**- Yes/ bacteroid state (nitrogen fixing); **n.a-** unknown. **CT**- conceptual translation.

Table S3- Accession numbers for β-Proteobacteria 16S rRNA, *acdS* and *acdR* genes and AcdS and AcdR proteins sequences and description of the *acdS* gene location, ACC deaminase (ACCD) activity, strains relative habitat and origin.

Strain	16S rRNA	*acdS*	AcdS	*acdR*	AcdR	*acdS* location	AC CD	Isolation/ Habitat	Origin
Achromobacter arsenitoxydans SY8	NZ_AGUF01000004.1	NZ_AGUF01000042.1	ZP_09299986.1	NZ_AGUF01000042.1	ZP_09299985.1	n.a	n.a	Soil contaminated with arsenic	China
Achromobacter piechaudii HLE	ALJE0100011	NZ_ALJE0100	ZP_15930	NZ_ALJE0100	ZP_15930	n.a	n.a	Soil	USA

	9	0008.1	486.1	0008.1	487.1				
Achromobacter xylosoxidans A8	CP002287.1	CP002287.1	YP_003977696.1	CP002287.1	YP_003977697.1	C	n.a	Soil contaminated with polychlorinated biphenyls	Czech Republic
Achromobacter xylosoxidans C54	ACRC01000072.1	ACRC01000687.1	EFV82431.1	ACRC01000687.1	EFV82432.1	n.a	n.a	Cystic fibrosis patient	n.a
Acidovorax avenae subsp. *avenae* ATCC 19860	CP002521.1	CP002521.1	YP_004236837.1	CP002521.1	YP_004236836.1	C	n.a	Maize leaf	USA
Acidovorax citrulli AAC00-1	CP000512.1	CP000512.1	YP_972804.1	CP000512.1	YP_972803.1	C	n.a	Watermellon	n.a
Acidovorax ebreus TPSY	CP001392.1	CP001392.1	YP_002554583.1	CP001392.1	YP_002554582.1	C	n.a	Groundwater, soil	USA
Acidovorax radicis N35	NZ_AFBG01000030.1	NZ_AFBG01000023.1	ZP_08950254.1	NZ_AFBG01000023.1	ZP_08950253.1	n.a	n.a	Wheat roots	Germany
Acidovorax sp. CF316	AKJX01000236	AKJX01000025.1	EJE54366.1	AKJX01000025.1	EJE54367.1	n.a	n.a	*Populus deltoides*	USA
Acidovorax sp. JS42	CP000539.1	CP000539.1	YP_988060.1	CP000539.1	YP_988059.1	C	n.a	Nitrobenzene-contaminated sediment	USA
Acidovorax sp. KKS102	CP003872	NC_018708.1	YP_006856666.1	NC_018708.1	YP_006856665.1	C	n.a	Soil	Japan
Bordetella sp. FB-8	JN885794	ARNH01000003.1	CT	n.a	n.a	n.a	n.a	Sediment	Germany
Burkholderia ambifaria AMMD	CP000441.1	CP000441.1	YP_777038.1	CP000441.1	YP_777039.1	C2	n.a	Pea rhizosphere	USA
Burkholderia ambifaria MC40-6	NC_010552.1	NC_010552.1	YP_001809994.1	NC_010552.1	YP_001809995.1	C2	Y/FL	Soil associated with maize roots	USA
Burkholderia caledonica LMG 19076	AF215704.1	EU886299.1	ACH81521.1	n.a	n.a	n.a	Y/FL	Rhizosphere soil	Scotland
Burkholderia caryophylli LMG 2155	AB021423.1	EU886300.1	ACH81522.1	n.a	n.a	n.a	Y/FL	*Dianthus caryophyllus*	USA
Burkholderia cenocepacia HI2424	CP000459.1	CP000459.1	ABK10113.1	CP000459.1	ABK10114.1	C2	n.a	Onion field	USA
Burkholderia cenocepacia J2315	AM747721.1	AM747721.1	YP_002233009.1	AM747721.1	YP_002233010.1	C2	Y/FL	Cystic fibrosis patient	Scotland
Burkholderia cenocepacia MC0-3	CP000959.1	CP000959.1	YP_001777783.1	CP000959.1	YP_001777782.1	C2	n.a	Soil associated with maize roots	USA
Burkholderia cenocepacia PC184	n.a	NZ_CH482379.1	ZP_04944067.1	n.a	n.a	n.a	n.a	Cystic fibrosis patient	USA
Burkholderia cepacia ATCC 25416	n.a	EU886301.1	ACH81523.1	n.a	n.a	n.a	Y/FL	Onion	USA
Burkholderia dolosa AUO158	n.a	NZ_CH482381.1	ZP_04948196.1	NZ_CH482381.1	ZP_04948197.1	n.a	n.a	Cystic fibrosis patient	USA
Burkholderia gladioli BSR3	n.a	CP002600.1	AEA63740.1	CP002600.1	AEA63739.1	C2	n.a	Diseased rice sheath	South Korea
Burkholderia glumae BGR1	n.a	CP001504.1	YP_002908968.1	CP001504.1	YP_002908969.1	C2	n.a	Diseased rice panicle	Korea
Burkholderia graminis C4D1M	U96939.1	EU886302.1	ACH81524.1	NZ_ABLD01000001.1	ZP_02881625.1	n.a	Y/FL	Senescent maize roots	France
Burkholderia mallei ATCC	NC_006349.2	NC_006349.2	YP_10563	NC_006349.2	YP_10563	C2	n.a	Glanders-melioidosis patient	Burma

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Burkholderia mallei NCTC 10229	NC_008835.1	NC_008835.1	YP_001024043.1	NC_008835.1	YP_001024042.1	C2	n.a	n.a	Hungary
Burkholderia mallei PRL-20	NZ_AAZP01000007.1	AAZP010000009.1	ZP_02265430.1	AAZP010000009.1	ZP_02265429.1	n.a	n.a	Horse blood	Pakistan
Burkholderia multivorans ATCC 17616	AP009386.1	AP009386.1	YP_001585177.1	AP009386.1	YP_001585176.1	C2	n.a	Soil	n.a
Burkholderia multivorans CGD1	NZ_ACFB01000007.1	ACFB010000002.1	ZP_03583630.1	ACFB010000002.1	ZP_03583631.1	n.a	n.a	Chronic granulomatous disease patient	USA
Burkholderia oklahomensis C6786	NZ_ABBG01000575.1	NZ_ABBG01000392.1	ZP_02365950.1	NZ_ABBG01000392.1	ZP_02365951.1	n.a	n.a	Human leg wound	USA
Burkholderia phenoliruptrix BR3459a	CP003863	NC_018672.1	YP_006792732.1	NC_018672.1	YP_006792731.1	C2	n.a	*Mimosa flocculosa*	Brazil
Burkholderia phenoliruptrix BR3459a	-	NC_018696.1	YP_006836544.1	n.p	n.p	P	n.a	-	-
Burkholderia phenoliruptrix LMG 22037	AY435213.1	EU886303.1	ACH81525.1	n.a	n.a	n.a	Y/FL	Soil	n.a
Burkholderia phymatum STM815	CP001044.1	CP001044.1	YP_001861528.1	CP001044.1	YP_001861529.1	C2	n.a	Root nodule	French Guiana
Burkholderia phymatum STM815	-	CP001046.1	YP_001863743.1	n.p	n.p	P	n.a	-	-
Burkholderia phytofirmans PsJN	CP001053.1	CP001053.1	YP_001889125.1	CP001053.1	YP_001889124.1	C2	Y/FL	Onion roots	n.a
Burkholderia pseudomallei 1106b	AAMB02000036	NZ_CM000775.1	ZP_04811587.1	NZ_CM000775.1	ZP_04811063.1	C2	n.a	Human liver abcess	Thailand
Burkholderia pseudomallei 1710b	NC_007435.1	YP_335503.1	NC_007435.1	YP_335504.1	YP_335504.1	C2	n.a	Human Blood	Thailand
Burkholderia pseudomallei 668	CP000571.1	CP000571.1	YP_001062875.1	CP000571.1	YP_001062876.1	C2	n.a	Melioidosis patient	Australia
Burkholderia pyrrocinia CH-67	ALWI01000067	ALWI01000021.1	CT	n.a	n.a	n.a	n.a	Forest soil	South Korea
Burkholderia silvatlantica AB48	AF164043.2	EU886305.1	ACH81527.1	n.a	n.a	n.a	Y/FL	Pineapple roots	Brazil
Burkholderia silvatlantica PPCR-2	AY965243.1	EU886304.1	ACH81526.1	n.a	n.a	n.a	Y/FL	Sugarcane roots	Brazil
Burkholderia silvatlantica SRMrh-20	AY965240.1	EU886306.1	ACH81528.1	n.a	n.a	n.a	Y/FL	Maize roots	Brazil
Burkholderia sp. 383	CP000152.1	CP000152.1	YP_373615.1	CP000152.1	YP_373614.1	C2	n.a	Forest soil	Trinidad
Burkholderia sp. BT03	AKKD01000264	AKKD01000037.1	EJL64220.1	AKKD01000037.1	EJL64219.1	n.a	n.a	*Populus deltoides*	USA
Burkholderia sp. CCGE1001	CP002520.1	CP002520.1	YP_004230185.1	CP002520.1	YP_004230184.1	C2	n.a	n.a	n.a
Burkholderia sp. CCGE1002	NC_014118.1	NC_014118.1	YP_003607498.1	NC_014118.1	YP_003607497.1	C2	n.a	Soil, root nodule	Mexico
Burkholderia sp. CCGE1002	-	CP002016.1	ADG20824.1	n.p	n.p	P	n.a	-	-
Burkholderia sp. CCGE1003	NC_014539.1	CP002218.1	ADN59699	CP002218.1	ADN59698	C2	n.a	n.a	n.a

			.1		.1				
Burkholderia sp. Ch1-1	NZ_ADNR01000109.1	NZ_ADNR01000001.1	ZP_06838775.1	NZ_ADNR01000001.1	ZP_06838776.1	n.a	n.a	PAH contaminated soil	USA
Burkholderia sp. H160	NZ_ABYL01000300.1	NZ_ABYL01000102.1	ZP_03269041.1	NZ_ABYL01000102.1	ZP_03269042.1	n.a	n.a	n.a	n.a
Burkholderia sp. SJ98	AJHK02000001	NZ_AJHK02000011.2	ZP_11399945.1	NZ_AJHK02000011.2	ZP_11399944.1	n.a	n.a	Soil	India
Burkholderia sp. TJI49	n.a	AEXE01000286.1	EGD05602.1	AEXE01000286.1	EGD05601.1	n.a	n.a	Bark of mango tree	Pakistan
Burkholderia sp. YI23	CP003089.1	CP003089.1	AET93258.1	CP003089.1	AET93257.1	C3	n.a	Soil	South Korea
Burkholderia terrae BS001	AKAU01000146	NZ_AKAU01000015.1	ZP_10247444.1	NZ_AKAU01000015.1	ZP_10247445.1	n.a	n.a	Soil underneath mushroom foot	Netherlands
Burkholderia terricola LMG 20594	AY040362.1	EU886307.1	ACH81529.1	n.a	n.a	n.a	Y/FL	Soil	n.a
Burkholderia thailandensis E264	CP000085.1	CP000085.1	YP_439298.1	CP000085.1	YP_439297.1	C2	n.a	Environmental isolate (Soil)	Thailand
Burkholderia thailandensis MSMB43	NZ_ABBM01000203.1	NZ_ABBM01000778.1	ZP_02466645.1	NZ_ABBM01000778.1	ZP_02466646.1	n.a	n.a	Borehole	Australia
Burkholderia thailandensis TXDOH	NZ_ABBD01000779.1	NZ_ABBD01000124.1	ZP_02370572.1	NZ_ABBD01000124.1	ZP_02370571.1	n.a	n.a	Human blood	USA
Burkholderia ubonensis Bu	ABBE010007281	NZ_ABBE01001097.1	ZP_02383018.1	NZ_ABBE01001097.1	ZP_02383017.1	n.a	n.a	Rhizosphere sample from a mine site	Australia
Burkholderia unamae CAC-98	n.a	EU886308.1	ACH81530.1	n.a	n.a	n.a	Y/FL	Cofee plant rhizosphere	Mexico
Burkholderia unamae MTI-641	AY221956.1	EU886320.1	ACH81542.1	EU886320.1	ACH81543.1	n.a	Y/FL	Maize rhizosphere	Mexico
Burkholderia vietnamiensis G4	CP000615.1	CP000615.1	YP_001116376.1	CP000615.1	YP_001116377.1	C2	n.a	Industrial waste treatment facility	USA
Burkholderia vietnamiensis LMG 6999	n.a	EU886310.1	ACH81532.1	n.a	n.a	n.a	Y/FL	Human neck abcess	n.a
Burkholderia vietnamiensis TVV75	U96928.1	EU886309.1	ACH81531.1	n.a	n.a	n.a	Y/FL	Acid sulphate soil	Vietnam
Burkholderia xenovorans CAC-124	n.a	EU886312.1	ACH81534.1	n.a	n.a	n.a	Y/FL	Cofee plant rhizosphere	Mexico
Burkholderia xenovorans CCUG 28445	n.a	EU886313.1	ACH81535.1	n.a	n.a	n.a	Y/FL	Human blood	Sweden
Burkholderia xenovorans LB400	NC_007952.1	NC_007952.1	YP_554094.1	NC_007952.1	YP_554434.1	C2	Y/FL	PCB-containing landfill	USA
Collimonas fungivorans Ter331	NC_015856.1	NC_015856.1	YP_004752723.1	NC_015856.1	YP_004752724.1	C	n.a	Soil	Netherlands
Cupriavidus basilensis OR16	AHJE01000064	AHJE01000053.1	EHP41147.1	AHJE01000053.1	EHP41148.1	n.a	n.a	Pristine soil	Hungary
Cupriavidus necator N-1	NC_015726.1	CP002878.1	AEI80287.1	CP002878.1	AEI80288.1	C2	n.a	Soil	USA
Cupriavidus sp. UYPR2.512	JF683703	ARBE01000348.1	CT	n.a	n.a	n.a	n.a	*Parapiptadenia rigida* root nodule	Uruguay
Cupriavidus sp. UYPR2.512	-	ARBE010001	CT	n.a	n.a	n.a	n.a	-	-

		73.1							
Curvibacter lanceolatus ATCC 14669	AB021390.1	ARLO010000 35.1	CT	n.a	n.a	n.a	n.a	Distilled water	Canada
Herbaspirillum frisingense GSF30	AEEC010014 72	NZ_AEEC010 00097.1	ZP_11552 248.1	NZ_AEEC010 00097.1	ZP_11552 250.1	n.a	Y/F L	*Miscanthus*	Germany
Herbaspirillum huttiense subsp. putei IAM 15032	AB109890	ANJR0100001 4.1	CT	n.a	n.a	n.a	n.a	Well water	Japan
Herbaspirillum lusitanum P6-12	AJHH0100013 7	AJHH0100063 3.1	CT	n.a	n.a	n.a	n.a	*Phaseolus vulgaris* root nodule	Brazil
Herbaspirillum seropedicae SmR1	CP002039.1	CP002039.1	ADJ64675. 1	CP002039.1	ADJ64674. 1	C	n.a	*Sorghum bicolor* roots	Brazil
Herbaspirillum sp. B501	AB049133	BADJ0100113 6.1	CT	n.a	n.a	n.a	n.a	*Oryza officinalis*	Japan
Herbaspirillum sp. CF444	AKJW010001 12	NZ_AKJW010 00044.1	ZP_10721 003.1	NZ_AKJW010 00044.1	ZP_10721 002.1	n.a	n.a	*Populus deltoides*	USA
Herbaspirillum sp. GW103	AJVC0100000 4	NZ_AJVC010 00004.1	ZP_11255 386.1	NZ_AJVC010 00004.1	ZP_11255 384.1	n.a	n.a	Rhizosphere soil	South Korea
Herbaspirillum sp. YR522	AKJA0100001 6	NZ_AKJA010 00043.1	ZP_10591 596.1	NZ_AKJA010 00043.1	ZP_10591 597.1	n.a	n.a	*Populus deltoides*	USA
Methylibium petroleiphilum PM1	CP000555.1	CP000555.1	YP_00102 2786.1	CP000555.1	YP_00102 2785.1	C	n.a	Biolfilter in an oil refinery	USA
Polaromonas sp. CF318	AKIV0100005 5	NZ_AKIV0100 0015.1	ZP_10561 707.1	NZ_AKIV0100 0015.1	ZP_10561 708.1	n.a	n.a	*Populus deltoides* root	USA
Polaromonas sp. JS666	CP000316.1	CP000316.1	YP_55062 4.1	CP000316.1	YP_55062 5.1	C	n.a	Contaminated groundwater	USA
Ralstonia eutropha H16	AM260480.1	AM260480.1	YP_84088 4.1	AM260480.1	YP_84088 5.1	C2	n.a	Spring	Germany
Ralstonia pickettii 12D	NC_012856.1	NC_012856.1	YP_00298 1652.1	NC_012856.1	YP_00298 1651.1	C	n.a	Copper-contaminated sediment from a lake	USA
Ralstonia pickettii 12J	CP001068.1	CP001068.1	YP_00189 9571.1	CP001068.1	YP_00189 9570.1	C	n.a	Copper-contaminated sediment from a lake	USA
Ralstonia solanacearum CFBP2957	FP885897.1	FP885907.1	YP_00374 7920.1	FP885907.1	YP_00374 7919.1	P	n.a	Tomato	French West Indie
Ralstonia solanacearum CMR15	FP885895.1	FP885896.1	CBJ40249. 1	FP885896.1	CBJ40248. 1	P	n.a	Tomato	Cameroon
Ralstonia solanacearum GMI1000	NC_003295.1	AL646053.1	NP_52220 7.1	AL646053.1	NP_52220 6.1	P	Y/F L	Tomato	Guiana
Ralstonia solanacearum Po82	CP002819.1	CP002820.1	AEG71624 .1	CP002820.1	AEG71623 .1	P	n.a	Potato	Mexico
Ralstonia solanacearum PSI07	NC_014311.1	FP885891.2	YP_00374 9510.1	FP885891.2	YP_00374 9509.1	P	n.a	Tomato	Indonesia
Ralstonia solanacearum Y45	n.a	AFWL010003 79.1	CT	n.a	n.a	P	n.a	Tobacco plant	China
Ralstonia sp. 5_2_56FAA	ACTT0100000 8.1	ACTT0100003 5.1	EGY64300 .1	ACTT0100003 5.1	EGY64299 .1	n.a	n.a	Patient with Crohn's disease	n.a
Ralstonia sp. 5_7_47FAA	ACUF010000 76.1	NZ_ACUF010 00054.1	ZP_07677 216.1	NZ_ACUF010 00054.1	ZP_07677 217.1	n.a	n.a	Patient with Crohn's disease	n.a
Ralstonia syzygii R24	FR854086.1	FR854090.1	CCA87810	FR854090.1	CCA87809	n.a	n.a	Diseased clove tree	Indonesia

			.1		.1				
Variovorax paradoxus 5C2	n.a	AY604531.2	AAT35829.2	n.a	n.a	n.a	Y/FL	Mining waste soil	Russia
Variovorax paradoxus EPS	CP002417.1	CP002417.1	YP_004158083.1	CP002417.1	YP_004158082.1	C	n.a	Soil	n.a
Variovorax paradoxus S110	CP001635.1	CP001635.1	YP_002946967.1	CP001635.1	YP_002946966.1	C	n.a	Potato plant	USA
Variovorax sp. CF313	AKIW01000103	AKIW01000011.1	EJL79371.1	AKIW01000011.1	EJL79370.1	n.a	n.a	*Populus deltoides*	USA

n.a- not available, unknown; **n.p**- not present; **C**- Chromosome; **C2**- 2nd chromosome; **P**- plasmid; **Y/FL**- Yes/free living conditions;

Table S4- Accession numbers for γ-Proteobacteria 16S rRNA, *acdS* and *acdR* genes and AcdS and AcdR proteins sequences and description of the *acdS* gene location, ACC deaminase (ACCD) activity, strains relative habitat and origin.

Strain	16S rRNA	*acdS*	AcdS	*acdR*	AcdR	*acdS* location	ACCD	Isolation/ Habitat	Origin
Brenneria sp. EniD312	AFWW01000001	NZ_CM001230.1	ZP_09017848.1	NZ_CM001230.1	ZP_09017849.1	C	n.a	Plant	n.a
Dickeya dadantii 3937	CP002038.1	CP002038.1	YP_003881235.1	CP002038.1	YP_003881238.1	C	n.a	*Saintpaulia ionantha* plants	France
Dickeya dadantii Ech586	NC_013592.1	NC_013592.1	YP_003332054.1	NC_013592.1	YP_003332056.1	C	n.a	n.a	n.a
Dickeya dadantii Ech703	NC_012880.1	NC_012880.1	YP_002989081.1	NC_012880.1	YP_002989077.1	C	n.a	n.a	n.a
Dickeya dianthicola NCPPB 3534	n.a	AOOK01000005.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Dickeya paradisiaca NCPPB 2511	Z96096.1	AONV01000036.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Dickeya solani MK10	n.a	AOOP01000004.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Dickeya zeae Ech1591	CP001655.1	CP001655.1	YP_003005910.1	NC_012912.1	YP_003005906.1	C	n.a	n.a	n.a
Halomonas boliviensis LC1	AGQZ01000062	NZ_JH393257.1	ZP_09188008.1	NZ_JH393257.1	ZP_09188009.1	n.a	n.a	Soil around the hypersaline lake Laguna Colorada	Bolivia
Halomonas sp. HAL1	EU651835.1	AGIB01000084.1	EHA14104.1	AGIB01000084.1	EHA14105.1	C	n.a	Soil from a gold mine	China
Halomonas sp. KM-1	HD061326	NZ_BAEU01000110.1	ZP_10778750.1	NZ_BAEU01000110.1	ZP_10778748.1	C	n.a	n.a	Japan
Halomonas stevensii S18214	AJTS01000020	AJTS01000040.1	CT	n.a	n.a	n.a	n.a	Blood from a renal care patient	USA
Halomonas titanicae BH1	n.a	NZ_AOPO01000001.1	ZP_21727375.1	n.p	n.p	n.a	n.a	Rusticles of the RMS Titanic wreck	Atlantic ocean
Klebsiella oxytoca Rs-5	n.a	FJ357241.1	ACJ12921.1	n.a	n.a	n.a	n.a	n.a	n.a
Klebsiella pneumoniae AcdSPB2	JN625720	JN625725.1	AEQ29825.1	n.a	n.a	n.a	Y/FL	*Agaricus bisporus* casing soil	China
Pantoea sp. At-9b	CP002433.1	CP002436.1	ADU72453.1	CP002436.1	ADU72455.1	P	n.a	*Atta cephalotes* (leaf cutter ant)symbiont	n.a

Pseudomonas avellanae BPIC 631	AKBS01001374	NZ_JH955155.1	ZP_16386983.1	NZ_JH955155.1	ZP_16386982.1	n.a	n.a	*Corylus avellana*	Greece
Pseudomonas brassicacearum subsp. brassicacearum NFM421	CP002585.1	CP002585.1	AEA68459.1	CP002585.1	AEA68458.1	C	n.a	n.a	n.a
Pseudomonas entomophila PS-PJH	n.a	FJ882923.1	ACQ55296.1	n.a	n.a	n.a	Y/FL	Red pepper rhizosphere	Korea
Pseudomonas fluorescens 17	n.a	U37103.1	AAC44163.1	n.a	n.a	n.a	Y/FL	Soil	South Africa
Pseudomonas fluorescens 2P24	AY447045.1	EF635249.1	ABR26447.1	EF635249.1	ABR26446.1	n.a	n.a	Wheat take-all decline soil	China
Pseudomonas fluorescens F113	CP003150.1	CP003150.1	AEV63500.1	CP003150.1	AEV63499.1	n.a	Y/FL	Sugarbeet rhizosphere	n.a
Pseudomonas fluorescens FY32	FJ465156.2	FJ465155.1	ACJ69586.1	n.a	n.a	P	Y/FL	Soil	Iran
Pseudomonas fuscovaginae UPB0736	AIEU01000016	NZ_JH605158.1	ZP_10991581.1	NZ_JH605158.1	ZP_10991582.1	n.a	n.a	Sheath brown rot lesion on rice	Madagascar: Antsirabe
Pseudomonas psychrotolerans L19	NZ_AHBD01000036.1	NZ_AHBD01000009.1	ZP_09285984.1	NZ_AHBD01000009.1	ZP_09285985.1	n.a	n.a	Copper alloy coins	n.a
Pseudomonas putida AKMP7	GU396282.1	HM053973.1	ADH59751.1	n.a	n.a	n.a	n.a	Sorghum rhizosphere	India
Pseudomonas putida AM15	EF194770.1	EF011160.1	ABJ91236.1	n.a	n.a	n.a	n.a	n.a	India
Pseudomonas putida AS1.1003	n.a	EU700088.1	ACD70372.1	n.a	n.a	n.a	n.a	n.a	China
Pseudomonas savastanoi pv. savastanoi NCPPB 3335	NZ_GG774664.1	GG774632.1	EFI00387.1	GG774632.1	EFI00388.1	n.a	n.a	Diseased olive tree	France
Pseudomonas sp. 313	n.a	ANBZ01000041.1	CT	n.a	n.a	n.a	n.a	Kelp holobiont	USA
Pseudomonas sp. 6G5	n.a	M80882.1	AAA73153.1	n.a	n.a	n.a	Y/FL	Soil	n.a
Pseudomonas sp. ACP	n.a	n.a	Q00740.1	n.a	n.a	n.a	Y/FL	Soil	Japan
Pseudomonas sp. AT14	EF194771.1	EF011161.1	ABJ91237.1	n.a	n.a	n.a	n.a	n.a	India
Pseudomonas sp. CH-GRS 8	n.a	EF581137.1	ABQ10596.1	n.a	n.a	n.a	n.a	n.a	India
Pseudomonas sp. GM102	NZ_AKJB01000132.1	NZ_AKJB01000115.1	ZP_10599704.1	n.p	n.p	n.a	n.a	*Populus deltoides*	USA
Pseudomonas sp. GM18	AKJT01000077	NZ_AKJT01000072.1	ZP_10705137.1	n.p	n.p	n.a	n.a	*Populus deltoides*	USA
Pseudomonas sp. GM55	AKJJ01000082	NZ_AKJJ01000032.1	ZP_10643223.1	n.p	n.p	n.a	n.a	*Populus deltoides*	USA
Pseudomonas sp. GM67	AKJH01000024	NZ_AKJH01000180.1	ZP_10636320.1	NZ_AKJH01000180.1	ZP_10636321.1	n.a	n.a	*Populus deltoides*	USA
Pseudomonas sp. GM79	AKJE01000062	NZ_AKJE01000083.1	ZP_10616599.1	n.p	n.p	n.a	n.a	*Populus deltoides*	USA
Pseudomonas sp. PNSL	n.a	DQ830987.1	ABH03031.1	n.a	n.a	n.a	n.a	n.a	Taiwann.a

Pseudomonas sp. Ps 2-3	n.a	EU520401.1	ACA97076.1	n.a	n.a	n.a	n.a	n.a	India
Pseudomonas sp. Ps 7-12	n.a	EU520398.1	ACA97075.1	n.a	n.a	n.a	n.a	n.a	India
Pseudomonas sp. UW4	AY559493.1	AY823987.1	AAV73804.1	AY686539.1	AAU00683.1	C	Y/FL	Soil	Canada
Pseudomonas syringae BRIP39023	n.a	AMZX01000054.1	ELQ07806.1	AMZX01000054.1	ELQ07805.1	n.a	n.a	Barley	Australia
Pseudomonas syringae ICMP 18806	n.a	ANJF01000119.1	CT	n.a	n.a	n.a	n.a	n.a	New Zealand
Pseudomonas syringae pv. aceris M302273	AEAO01000730	AEAO01000438.1	CT	n.a	n.a	n.a	n.a	Maple	n.a
Pseudomonas syringae pv. actinidiae CRAFRU8.43	AFTG01000283	AFTG01000155.1	CT	n.a	n.a	n.a	n.a	*Actinidia deliciosa*	Italy
Pseudomonas syringae pv. actinidiae M302091	AEAL01000630	AEAL01000023.1	CT	n.a	n.a	n.a	n.a	*Actinidia deliciosa*	Japan
Pseudomonas syringae pv. aesculi 2250	ACXT01000186	ACXT01000276.1	CT	n.a	n.a	n.a	n.a	*Aesculus hippocastanum*	Scotland
Pseudomonas syringae pv. aesculi NCPPB3681	NZ_ACXS01000064.1	NZ_ACXS01000074.1	ZP_06457192.1	NZ_ACXS01000074.1	ZP_06457191.1	C	n.a	*Aesculus indica*	India
Pseudomonas syringae pv. aptata DSM 50252	AEAN01001255.1	AEAN01001087.1	EGH80018.1	AEAN01001087.1	EGH80019.1	n.a	n.a	Sugarbeet	n.a
Pseudomonas syringae pv. avellanae ISPaVe037	AKCK01000070	NZ_JH951881.1	ZP_17808950.1	NZ_JH951881.1	ZP_17808951.1	n.a	n.a	*Corylus avellana*	Italy
Pseudomonas syringae pv. glycinea B076	AEGG01000013.1	AEGG01000041.1	EFW79594.1	AEGG01000041.1	EFW79593.1	C	n.a	Diseased soybean leaflet	USA
Pseudomonas syringae pv. glycinea race 4	AEGH01000005.1	AEGH01000057.1	EFW85440.1	AEGH01000057.1	EFW85441.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. japonica M301072	AEAH01001400	NZ_GL384842.1	ZP_16686047.1	NZ_GL384842.1	ZP_16686046.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. lachrymans M302278	AEAM01000561.1	AEAM01000159.1	EGH95996.1	AEAM01000159.1	EGH95995.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. maculicola ES4326	AEAK01000607.1	AEAK01000297.1	EGH60475.1	AEAK01000297.1	EGH60476.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. mori 301020	AEAG01001117.1	AEAG01000549.1	EGH22588.1	AEAG01000549.1	EGH22587.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. morsprunorum M302280	AEAE01000512	AEAE01000519.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. oryzae 1_6	NZ_ABZR01000729.1	NZ_ABZR01000328.1	ZP_04588011.1	NZ_ABZR01000328.1	ZP_04588012.1	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. panici LMG 2367	ALAC01000003	ALAC01000004.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. phaseolicola 1448A	CP000058.1	CP000058.1	AAZ35072.1	CP000058.1	AAZ33570.1	C	n.a	*P. vulgaris*	Ethiopia
Pseudomonas syringae pv. syringae 642	NZ_ADGB01000251.1	NZ_ADGB01000177.1	ZP_07265622.1	NZ_ADGB01000177.1	ZP_07265623.1	n.a	n.a	Plant	USA
Pseudomonas syringae pv. syringae B728a	CP000075.1	CP000075.1	AAY36848.1	CP000075.1	AAY36849.1	C	n.a	Snap bean leaflet	USA

Pseudomonas syringae pv. syringae FF5	NZ_ACXZ01002235.1	NZ_ACXZ01004270.1	ZP_06500448.1	NZ_ACXZ01004270.1	ZP_06500449.1	n.a	n.a	Bradford pear	n.a
Pseudomonas syringae pv. tabaci 6605	AJXI01000282	AJXI01000213.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. theae NCPPB 2598	AGNN01000645	AGNN01000508.1	CT	n.a	n.a	n.a	n.a	n.a	n.a
Pseudomonas syringae pv. tomato DC3000	AE016853.1	AE016853.1	AAO57144.1	AE016853.1	AAO57143.1	C	n.a	Tomato	UK
Pseudomonas syringae pv. tomato NCPPB 1108	n.a	NZ_ADGA01000205.1	ZP_07260367.1	NZ_ADGA01000205.1	ZP_07260368.1	n.a	n.a	Tomato	UK
Pseudomonas viridiflava UASWS0038	AMQP01000017	NZ_AMQP01000152.1	ZP_11290060.1	NZ_AMQP01000152.1	ZP_11290059.1	n.a	n.a	Infected *Rhododendron* sp. leaf	Switzerland
Serratia rubidea AcdSPB1	JN625719	JN625724.1	AEQ29824.1	n.a	n.a	n.a	Y/FL	*Agaricus bisporus* casting soil	China
Serratia sp. M24T3	HQ538811	NZ_AJHJ01000044.1	ZP_09972421.1	NZ_AJHJ01000044.1	ZP_09972422.1	n.a	n.a	*Bursaphelenchus xylophilus*	Portugal
Vibrio gazogenes ATCC 43941	n.a	ASAJ01000012.1	CT	n.a	n.a	n.a	n.a	Saltwater marsh	USA

n.a- not available, unknown; **n.p**- not present; **C**- Chromosome; **P**- plasmid; **Y/FL**- Yes/free living conditions; **CT**- conceptual translation

Table S5- Accession numbers for Eukaryotes AcdS complete sequences and description of ACC deaminase (ACCD) activity, strains relative habitat and geographical origin.

Strain	AcdS	ACCD activity	Isolation/ Habitat	Origin
Arthrobotrys oligospora ATCC 24927	EGX50717.1	n.a	Dung of livestock	n.a
Arthroderma benhamiae CBS 112371	XP_003015331.1	n.a	Patient with inflammatory epidermophytosis	Switzerland
Arthroderma gypseum CBS 118893	XP_003172451.1	n.a	Soil	n.a
Arthroderma otae CBS 113480	XP_002843926.1	n.a	Human patient	Germany
Aspergillus flavus NRRL3357	XP_002378560.1	n.a	Peanut cotyledons	USA
Aspergillus fumigatus A1163	EDP53767.1	n.a	Clinical isolate	n.a
Aspergillus fumigatus Af293	XP_749239.1	n.a	Human patient	UK
Aspergillus kawachii IFO 4308	GAA91871.1	n.a	n.a	Japan
Aspergillus oryzae RIB40	XP_001823215.1	n.a	Cereal	Japan
Aureobasidium pullulans AY4	AMCU01000114.1 (CT)	n.a	Human skin sample	Malaysia
Beauveria bassiana ARSEF 2860	EJP66687.1	n.a	Insect	n.a
Chaetomium thermophilum var. thermophilum DSM 1495	EGS18697.1	n.a	Decaying wheat straw	UK
Clavispora lusitaniae ATCC 42720	XP_002616765.1	n.a	Human blood	USA
Coccidioides immitis RS	XP_001248460.1	n.a	Human patient	USA
Coccidioides posadasii C735 delta SOWgp	XP_003070780.1	n.a	Human patient	n.a
Coccidioides posadasii Silveira	EFW22190.1	n.a	Human patient	USA
Colletotrichum higginsianum IMI 349063	CCF37171.1	n.a	*Brassica rapa*	Trinidad
Colletotrichum orbiculare MAFF 240422	ENH86277.1	n.a	*Cucumis sativus*	Japan
Cordyceps militaris CM01	EGX91007.1	n.a	n.a	n.a
Cryptococcus neoformans var. neoformans B-3501[a]	XP_777022.1	n.a	n.a	Laboratory strain
Cryptococcus neoformans var. neoformans JEC21	XP_568760.1	n.a	n.a	Laboratory strain
Cyberlindnera saturnus	Q7M523.1	Y/FL	Soil	Japan

Cyberlindnera jadinii NBRC 0988	BAEL01000113.1 (CT)	n.a	n.a	n.a
Dothistroma septosporum NZE10	EME39015.1	n.a	*Pinus radiata*	New Zealand
Drosophila eugracilis	AFPQ01002869.1 (CT)	n.a	n.a	n.a
Eutypa lata UCREL1	EMR61327.1	n.a	n.a	n.a
Exophiala dermatitidis NIH/UT8656	EHY53954.1	n.a	n.a	n.a
Fomitopsis pinicola FP-58527	AEHC01000092.1 (CT)	n.a	Conifer tree	n.a
Fusarium oxysporum f. sp. cubense race 4	EMT60768.1	n.a	n.a	n.a
Fusarium pseudograminearum CS3096	EKJ74886.1	n.a	n.a	Australia
Gaeumannomyces graminis var. tritici R3-111a-1	EJT70645.1	n.a	Roots	n.a
Gibberella zeae PH-1	XP_385209.1	n.a	Wheat kernels	USA
Glomerella graminicola M1.001	EFQ25139.1	n.a	Maize	USA
Grosmannia clavigera kw1407	EFX01604.1	n.a	*Dendroctonus ponderosae* symbiont	n.a
Guignardia citricarpa CGMCC3.14348	AOTE01003224.1 (CT)	n.a	Leaf	China
Howardula aeoronymphium	CT *	n.a	*Drosophila* parasite	n.a
Hyaloperonospora arabidopsidis Emoy2	ABWE01000578.1 (CT)	n.a	*Arabidopsis thaliana*	n.a
Macrophomina phaseolina MS6	EKG09749.1	n.a	*Corchorus olitorius*	Bangladesh
Magnaporthe oryzae 70-15	XP_001522461.1	n.a	Rice	n.a
Marssonina brunnea f. sp. 'multigermtubi' MB_m1	EKD13448.1	n.a	Poplar tree	China
Metarhizium acridum CQMa 102	EFY85645.1	n.a	n.a	n.a
Myceliophthora thermophila ATCC 42464	AEO56095.1	n.a	Soil	n.a
Mycosphaerella graminicola IPO323	EGP82604.1	n.a	Wheat	Netherlands
Mycosphaerella populorum SO2202	EMF16135.1	n.a	Poplar tree	n.a
Nectria haematococca mpVI 77-13-4	XP_003045841.1	n.a	n.a	n.a
Neosartorya fischeri NRRL 181	XP_001265664.1	n.a	Canned apples	n.a
Neurospora crassa OR74A	XP_959200.2	n.a	n.a	n.a
Neurospora tetrasperma FGSC 2508	EGO61449.1	n.a	n.a	USA
Penicillium chrysogenum Wisconsin 54-1255	XP_002566393.1	n.a	n.a	n.a
Penicillium citrinum	BAA92150.1	Y/FL	n.a	n.a
Penicillium digitatum Pd1	EKV05343.1	n.a	Grapefruit	Spain
Penicillium marneffei ATCC 18224	XP_002152267.1	n.a	Bamboo rat, *Rhizomys sinensis*	Vietnam
Phytophthora infestans T30-4	XP_002999006.1	n.a	Potato	Netehrlands
Phytophthora kernoviae 00844/4	AOFK01000115.1 (CT)	n.a	*Rhododendron ponticum*	UK
Phytophthora lateralis	AOFH01000927.1 (CT)	n.a	*Chamaecyparis lawsoniana*	USA
Phytophthora parasitica P1976	ANJA01000726.1 (CT)	n.a	n.a	n.a
Phytophthora ramorum Pr102	AAQX01001189.1 (CT)	n.a	*Quercus agrifolia*	USA
Phytophthora sojae P6497	EGZ05423.1	n.a	Soybean	USA
Pseudocercospora fijiensis CIRAD86	EME86929.1	n.a	Banana	n.a
Pseudoperonospora cubensis MSU-1	AHJF01000354.1 (CT)	n.a	*Cucumis sativus*	USA
Punctularia strigosozonata HHB-11173 SS5	EIN03908.1	n.a	n.a	n.a
Schizophyllum commune H4-8	XP_003028947.1	n.a	n.a	n.a
Schizosaccharomyces pombe 972h-	NP_595003.1	n.a	n.a	Laboratory strain
Sordaria macrospora k-hell	XP_003347375.1	n.a	n.a	n.a
Talaromyces stipitatus ATCC 10500	XP_002487215.1	n.a	Rotting wood	USA

Thielavia terrestris NRRL 8126	AEO62504.1	n.a	n.a	n.a
Trichoderma asperellum T203	ACX94231.1	Y/FL	n.a	n.a
Trichoderma atroviride IMI 206040	EHK47723.1	n.a	Soil	Sweden
Trichoderma reesei QM6a	EGR46173.1	n.a	Tent canvas	Solomon Islands
Trichoderma virens Gv29-8	EHK17293.1	n.a	Agricultural soil	USA
Trichophyton equinum CBS 127.97	EGE01138.1	n.a	Human patient	Finland
Trichophyton rubrum CBS 118892	XP_003237222.1	n.a	Human patient	Germany
Trichophyton tonsurans CBS 112818	EGD96016.1	n.a	Human patient	Canada
Trichophyton verrucosum HKI 0517	XP_003021717.1	n.a	Human patient	n.a
Verticillium dahliae VdLs.17	EGY20763.1	n.a	Lettuce	California

n.a- not available, unknown**; (CT)**- conceptual translation. * Conceptual translation from the sequence obtained in *Howardula aeoronymphium* genome database.

Table S6- Accession numbers for the sequences used in Figure 5.

Organism	P	Acession
Acaryochloris sp. CCMEE 5410	Bacteria; Cyanobacteria	WP_010475890.1
Alkaliphilus metalliredigens	Bacteria; Firmicutes	YP_001318478.1
Amphimedon queenslandica	Eukaryota; Metazoa	XP_003383707.1
Arabidopsis lyrata subsp. lyrata	Eukaryota; Viridiplantae	XP_002894112.1
Arabidopsis thaliana	Eukaryota; Viridiplantae	AAF79717.1
Bacillus bataviensis	Bacteria; Firmicutes	WP_007087484.1
Bacillus cereus	Bacteria; Firmicutes	WP_016115337.1
Bacillus megaterium DSM 319	Bacteria; Firmicutes	YP_003599085.1
Bacillus thuringiensis	Bacteria; Firmicutes	WP_001046607.1
Bordetella bronchiseptica MO149	Bacteria; Proteobacteria (Beta)	YP_006902444.1
Branchiostoma floridae	Eukaryota; Metazoa	XP_002591482.1
Calditrix abyssi	Bacteria; Calditrix	WP_006929754.1
Capitella teleta	Eukaryota; Metazoa	ELT93489.1
Caulobacter crescentus CB15	Bacteria; Proteobacteria alpha	NP_420839.1
Chlamydomonas reinhardtii	Eukaryota; Viridiplantae	XP_001700834.1
Chlorella variabilis	Eukaryota; Viridiplantae	EFN58625.1
Cicer arietinum	Eukaryota; Viridiplantae	XP_004503246.1
Ciona intestinalis	Eukaryota; Metazoa	XP_002121189.1
Clostridium symbiosum	Bacteria; Firmicutes	WP_003503279.1
Coccomyxa subellipsoidea C-169	Eukaryota; Viridiplantae	EIE24755.1
Coprobacillus sp. 8_2_54BFAA	Bacteria; Firmicutes	WP_008792520.1
Cronobacter sakazakii	Bacteria; Proteobacteria (gamma)	WP_007901773.1
Deinococcus deserti VCD115	Bacteria; Deinococcus-Thermus	YP_002787455.1
Dinoroseobacter shibae DFL 12	Bacteria; Proteobacteria (alpha)	YP_001532782.1
Emiliania huxleyi	Eukaryota; Haptophyceae	EOD31262.1
Erwinia tasmaniensis Et1/99	Bacteria; Proteobacteria (gamma)	YP_001907367.1
Escherichia coli K-12	Bacteria; Proteobacteria (gamma)	YP_490176.1
Fragaria vesca subsp. vesca	Eukaryota; Viridiplantae	XP_004299485.1
Fusobacterium ulcerans	Bacteria; Fusobacteria	WP_005978024.1
Glycine max	Eukaryota; Viridiplantae	XP_003525175.1
Haliangium ochraceum	Bacteria; Proteobacteria delta	YP_003269644.1
Johnsonella ignava	Bacteria; Firmicutes	WP_005540296.1
Lactobacillus parafarraginis	Bacteria; Firmicutes	WP_008211299.1
Luminiphilus syltensis	Bacteria; Proteobacteria (gamma)	WP_009019997.1
Methylobacterium populi BJ001	Bacteria; Proteobacteria (alpha)	YP_001925011.1
Nematostella vectensis	Eukaryota; Metazoa	XP_001637312.1
Oceanibaculum indicum	Bacteria; Proteobacteria (alpha)	WP_008945246.1
Oikopleura dioica	Eukaryota; Metazoa	CBY35070.1
Pectobacterium carotovorum	Bacteria; Proteobacteria (gamma)	WP_010285227.1
Phytophthora infestans T30-4	Eukaryota; Stramenopiles	XP_002906856.1
Phytophthora sojae	Eukaryota; Stramenopiles	EGZ28718.1
Prunus persica	Eukaryota; Viridiplantae	EMJ10357.1
Pseudoalteromonas atlantica T6c	Bacteria; Proteobacteria (gamma)	YP_662233.1
Pseudomonas fluorescens F113	Bacteria; Proteobacteria (gamma)	YP_005205697.1
Pseudomonas putida UW4	Bacteria; Proteobacteria (gamma)	YP_007027206.1
Pseudomonas stutzeri	Bacteria; Proteobacteria (gamma)	WP_003291494.1
Psychrobacter arcticus 273-4	Bacteria; Proteobacteria (gamma)	YP_264886.1
Pyrococcus horikoshii OT3	Archaea; Euryarchaeota	NP_142071.2
Ramlibacter tataouinensis TTB310	Bacteria; Proteobacteria (Beta)	YP_004619850.1
Roseobacter sp. SK209-2-6	Bacteria; Proteobacteria (alpha)	EBA18139.1
Ruegeria pomeroyi DSS-3	Bacteria; Proteobacteria (alpha)	AAV95902.1
Salmonella typhimurium LT2	Bacteria; Proteobacteria (gamma)	AAL20865.1
Selaginella moellendorffii	Eukaryota; Viridiplantae	XP_002961916.1
Simiduia agarivorans	Bacteria; Proteobacteria (gamma)	YP_006915655.1
Solanum lycopersicum	Eukaryota; Viridiplantae	NP_001234368.1
Staphylococcus pettenkoferi	Bacteria; Firmicutes	WP_002470882.1
Strongylocentrotus purpuratus	Eukaryota; Metazoa;	NP_001229618.1
Syntrophobotulus glycolicus	Bacteria; Firmicutes	YP_004265300.1
Teredinibacter turnerae T7901	Bacteria; Proteobacteria (gamma)	YP_003073255.1
Thalassiosira oceanica	Eukaryota; Stramenopiles	EJK45891.1
Thermococcus sp. AM4	Archaea; Euryarchaeota	YP_002582067.2
Thermotoga maritima MSB8	Bacteria; Thermotogae	NP_228040.1
Trichoplax adhaerens	Eukaryota; Metazoa	XP_002109431.1
Triticum urartu	Eukaryota; Viridiplantae	EMS48554.1
Vibrio splendidus	Bacteria; Proteobacteria (gamma)	WP_004739369.1
Vibrio tubiashii	Bacteria; Proteobacteria (gamma)	WP_004744649.1
Volvox carteri f. nagariensis	Eukaryota; Viridiplantae	XP_002955139.1
Zea mays	Eukaryota; Viridiplantae	NP_001130254.1

Prevalence and evolution of ACC deaminase and dihydrorhizobitoxine desaturase involved in the modulation of leguminous plant ethylene levels by symbiotic rhizobia

Table S1- Accession numbers and data from the sequences used in this study.

Strain	RecA	NodC	AcdS	RtxC	Host	Country
Bradyrhizobium diazoefficiens Is-1	WP_011088499	WP_011084824	WP_011083073	n.f	*Glycine max*	Japan
B. diazoefficiens SEMIA 5080	WP_011088499	WP_011084824	WP_011083073	n.f	*Glycine max*	Brazil
B. diazoefficiens USDA 110	WP_011088499	WP_011084824	WP_011083073	WP_011084875	*Glycine max, Glycine hispida*	USA
B. diazoefficiens USDA 122	WP_011088499	WP_011084824	WP_011083073	n.f	*Glycine max*	USA
B. elkanii 587	WP_016848520	WP_016848467	WP_016842298	WP_016841944	*Glycine max*	Brazil
B. elkanii CCBAU *05737*	WP_038385971	WP_018270192	WP_038385673	WP_016841944	*Glycine max*	China
B. elkanii CCBAU *43297*	WP_016848520	WP_018270192	WP_016842298	WP_016841944	*Glycine max*	China
B. elkanii TnphoA33	WP_016848520	WP_016848467	WP_016842298	WP_016841944	*Soil*	Brazil
B. elkanii USDA3254	WP_028337774	WP_028337418	WP_028335255	WP_028336488	*Phaseolus acutifolius*	USA
B. elkanii USDA3259	WP_028337774	WP_028337418	WP_028335255	WP_028336488	*Phaseolus lunatus*	USA
B. elkanii USDA76	WP_016848520	WP_018270192	WP_016842298	WP_016841944	*Glycine max*	USA
B. elkanii USDA94	WP_028339851	WP_018270192	WP_028341533	WP_016841944	*Glycine max*	USA
B. elkanii WSM1741	WP_028350014	WP_028351282	WP_028351968	n.f	*Rhynchosia minima*	Australia
B. elkanii WSM2783	WP_028163509	WP_028168860	WP_028166584	n.f	*Leobordea carinata*	South Africa
B. icense LMTR13	WP_028350014	WP_065730101	WP_065733285	n.f	*Phaseolus lunatus*	Peru
B. japonicum E109	WP_014494106	WP_011084824	WP_014490457	WP_011084875	*Glycine max*	Argentina
B. japonicum Is-34	WP_014494106	WP_011084824	WP_041960171	WP_011084875	*Glycine max*	Japan
B. japonicum J5	WP_014494106	WP_011084824	WP_071908513	WP_011084875	*Glycine max*	Japan
B. japonicum SEMIA5079	WP_014494106	WP_011084824	WP_039155471	WP_011084875	*Glycine max*	Brazil
B. japonicum USDA 124	WP_018642760	WP_011084824	WP_026312078	n.f	*Glycine max*	USA
B. japonicum USDA 135	WP_028178852	WP_035718548	WP_028182170	n.f	*Glycine max*	USA
B. japonicum USDA 6	WP_014494106	WP_011084824	WP_014490457	WP_011084875	*Glycine max*	Japan
B. jicamae PAC68	WP_057838834	WP_057836736	WP_057839419	n.f	*Pachyrhizus erosus*	Honduras
B. lablabi CCBAU23086	WP_057860082	WP_057858623	WP_057860379	n.f	*Lablab purpureus*	China
B. liaoningense CCBAU05525	WP_028178852	WP_011084824	WP_028182170	n.f	*Glycine max*	China
B.liaoningense CCNWSX0360	WP_061882979	WP_061881388	WP_061882727	n.f	*Vigna unguiculata*	China
B. manausense BR3351	WP_057747344	WP_057750149	WP_057748155	n.f	*Vigna unguiculata*	Brazil
B.neotropicale BR10247	WP_027552595	WP_063680004	WP_027553372	n.f	*Centrolobium paraense*	Brazil
B.pachyrhizi BR3262	WP_028337774	WP_057021396	WP_057021182	WP_057018770	*Vigna unguiculata*	Brazil
B.pachyrhizi PAC48	WP_050387652	WP_050384098	WP_028335255	WP_050386098	*Pachyrhizus erosus*	Costa Rica
B.paxllaeri LMTR21	WP_065750261	WP_065756038	WP_057860379	n.f	*Phaseolus lunatus*	Peru
B.retamae Ro19	WP_028350014	WP_057847903	WP_057841440	n.f	*Retama monosperma*	Morocco
Bradyrhizobium sp. Ai1a-2	WP_027584946	WP_027584061	WP_027583079	n.f	*Andira inermis*	Costa Rica
Bradyrhizobium sp. ARR65	WP_024512643	WP_024510037	WP_024509234	n.f	*Stylosanthes viscosa*	n.a

Bradyrhizobium sp. BR10245	WP_063703308	WP_063700800	WP_063699296	n.f	*Centrolobium paraense*	Brazil
Bradyrhizobium sp. BR10303	WP_066500104	WP_066507793	WP_066514459	WP_066507726	*Centrolobium paraense*	Brazil
Bradyrhizobium sp. CB756	WP_027560271	WP_027562752	WP_027563501	WP_027562706	*Macrotyloma africanum*	*n.a*
Bradyrhizobium sp. CCBAU 43298	WP_035707474	WP_011084824	WP_035710233	n.f	*Glycine max*	China
Bradyrhizobium sp. CCGE-LA001	WP_018316624	WP_008558139	WP_008541961	WP_008558060	*Phaseolus microcarpus*	Mexico
Bradyrhizobium sp. DOA1	WP_018316624	WP_061849653	WP_061850230	n.f	*Aeschynomene Americana*	Thailand
Bradyrhizobium sp. DOA9	WP_025037325	WP_025038575	WP_025032714	n.f	*Grassland soil*	Thailand
Bradyrhizobium sp. LMTR 3	WP_065747898	WP_065745487	WP_065747876	n.f	*Phaseolus lunatus*	Peru
Bradyrhizobium sp. NAS96.2	WP_016848520	WP_074125803	WP_074125264	WP_074130136	*Lupinus albescens*	Brazil
Bradyrhizobium sp. Tv2a-2	WP_024518023	WP_024519942	WP_024516008	n.f	*Tachigali versicolor*	Panama
Bradyrhizobium sp. USDA 3384	WP_027560271	WP_027562752	WP_027563501	WP_027562706	*Crotalaria paulina*	Brazil
Bradyrhizobium sp. WSM1417	WP_026233157	WP_027515902	WP_027515111	n.f	*Lupinus sp.*	Chile
Bradyrhizobium sp. WSM1743	WP_027577058	WP_027577635	WP_027577434	WP_027577680	*Indigofera sp.*	Australia
Bradyrhizobium sp. WSM2254	WP_027548614	WP_027544415	WP_027545292	n.f	*Acacia dealbata*	Australia
Bradyrhizobium sp. WSM2793	WP_018316624	WP_026202726	WP_018320514	WP_026202344	*Rhynchosia totta*	South Africa
Bradyrhizobium sp. WSM3983	WP_027534728	WP_051383360	WP_027533435	n.f	*Kennedia coccinea*	Australia
Bradyrhizobium sp. WSM4349	WP_026233157	WP_018455476	WP_018452867	n.f	*Syrmatium glabrum*	USA
Bradyrhizobium sp. WSM471	WP_007602214	WP_007605778	WP_007604504	n.f	*Ornithopus pinnatus (Miller) Druce*	Australia
B. stylosanthis BR446	WP_063682409	WP_063690513	WP_063692803	n.f	*Stylosanthes guianensis*	Brazil
B. valentinum LmjM3	WP_057848592	WP_057853262	WP_057848434	n.f	*Lupinus mariae-josephae*	Spain
B. valentinum LmjM6	WP_057848592	WP_057853262	WP_057848434	n.f	*Lupinus mariae-josephae*	Spain
B. yuanmingense BR3267	WP_027577058	WP_057027294	WP_057030170	WP_057027263	*Vigna unguiculata*	Brazil
B. yuanmingense CCBAU 10071	WP_027577058	WP_036030696	WP_036026180	WP_036030775	*Glycine max*	China
B. yuanmingense CCBAU 25021	WP_027577058	WP_036030696	WP_036026180	WP_036030775	*Glycine Max*	China
M. amorphae CCNWGS0123	WP_040584057	WP_006201690	WP_006199545	n.f	*Robinia pseudoacacia*	China
Mesorhizobium sp. STM 4661	WP_006332853	WP_006328886	WP_006331715	n.f	*Anthyllis vulneraria*	France
M. alhagi CCNWXJ12-2	WP_008836613	WP_008840730	WP_008840027	n.f	*Alhagi sparsifolia*	China
M. loti MAFF303099	WP_010909075	WP_010913821	WP_010913628	n.f	*Lotus japonicus*	Japan
M. australicum WSM2073	WP_013895892	WP_013533535	WP_013533477	n.f	*Biserrula pelecinus*	Australia
M. ciceri bv. biserrulae WSM1271	WP_013532151	WP_013533535	WP_013533477	n.f	*Biserrula pelecinus*	Italy
M. loti R7ANS::ICEMcbSym1271	WP_010909075	WP_013533535	WP_013533477	n.f	*Biserrula pelecinus*	Australia
M.opportunistum WSM2075	WP_013895892	WP_013533535	WP_013533477	n.f	*Biserrula pelecinus*	Australia
Mesorhizobium sp. LSJC280B00	WP_023674696	WP_023678264	WP_023677571	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC420B00	WP_023717366	WP_031194121	WP_023722650	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC220B00	WP_013895892	WP_023784692	WP_023784655	n.f	*Acmispon wrangelianus*	USA
M. ciceri CC1192	WP_013532151	WP_063169399	WP_024505903	n.f	*Cicer arietinum*	Israel
M. ciceri CMG6	WP_013532151	WP_032899868	WP_024505903	n.f	*Cicer arietinum*	Tunisia
M. loti CJ3sym	WP_023669117	WP_027033365	WP_027033225	n.f	*Lotus corniculatus*	New Zealand
M. loti R7a	WP_010909075	WP_027033365	WP_027033225	n.f	*Lotus corniculatus*	New Zealand
M. loti R88b	WP_023669117	WP_027033365	WP_027033225	n.f	*Lotus corniculatus*	New Zealand
M. ciceri WSM4083	WP_027036993	WP_027038822	WP_027038814	n.f	*Bituminaria bituminosa*	Spain
M. erdmanii USDA 3471	WP_013895892	WP_027056376	WP_027056509	n.f	*Lotus corniculatus*	New Zealand

Mesorhizobium sp. WSM3626	WP_013895892	WP_027144855	WP_027144897	n.f	*Lessertia diffusa*	South Africa
Mesorhizobium sp. WSM2561	WP_008875470	WP_027155849	WP_027155251	n.f	*Lessertia diffusa*	South Africa
Mesorhizobium sp. WSM1293	WP_013532151	WP_027162814	WP_027162746	n.f	*Lotus*	Greece
M. ciceri ca181	WP_008875470	WP_029356625	WP_029356493	n.f	*Cicer arietinum*	India
Mesorhizobium sp. ORS3324	WP_040969540	WP_042645974	WP_040971464	n.f	*Acacia seyal*	Senegal
M. plurifarium STM8773	WP_040981289	WP_040985383	WP_040986735	n.f	*Acacia Senegal*	Senegal
Mesorhizobium sp. SOD10	WP_040995429	WP_040985383	WP_040998978	n.f	*Acacia senegal*	Senegal
M. plurifarium ORS3365	WP_040981289	WP_041003406	WP_041003299	n.f	*Acacia Seyal*	Senegal
M. plurifarium ORS3356	WP_040981289	WP_052467897	WP_041010489	n.f	*Acacia Seyal*	Senegal
Mesorhizobium sp. ORS3359	WP_040981289	WP_042645974	WP_042645940	n.f	*Acacia seyal*	Senegal
M. loti UFLA 01-765	WP_059185132	WP_059187590	WP_059189164	n.f	*Leucaena leucocephala*	Brazil
M. loti NZP2014	WP_013895892	WP_064987326	WP_064987150	n.f	*Lotus sp.*	New Zealand
M. loti R7ANS::ICEMlSym2014	WP_010909075	WP_064987326	WP_064987150	n.f	*Lotus sp.*	New Zealand
Mesorhizobium sp. WSM1497	WP_013532151	WP_064993562	WP_064993514	n.f	*Biserrula pelecinus*	Greece
M. loti NZP2042	WP_013895892	WP_064987326	WP_065005765	n.f	*Lotus sp.*	New Zealand
M. loti R7ANS::ICEMlSym2042	WP_010909075	WP_064987326	WP_065005765	n.f	*Lotus sp.*	New Zealand
Mesorhizobium sp. AA22	WP_023798282	WP_065010658	WP_065009191,	n.f	*Biserrula pelecinus*	Ethiopia
Mesorhizobium sp. AA23	WP_067328293	WP_067325034	WP_067322334	n.f	*Biserrula pelecinus*	Ethiopia
Mesorhizobium sp. SEMIA 3007	WP_010909075	WP_069091161	WP_069093281	n.f	Soil	Mexico
Mesorhizobium sp. LCM 4576	WP_071035268	WP_071035314	WP_071034757	n.f	*Prosopis juliflora*	Senegal
Mesorhizobium sp. LCM 4577	WP_071035268	WP_071073056	WP_071072633	n.f	*Prosopis juliflora*	Senegal
Mesorhizobium sp. ORS3428	WP_071098108	WP_071102033	WP_071101556	n.f	*Acacia senegal*	Senegal: Kamb
M. plurifarium ORS1032	WP_073988604	WP_073987798	WP_073986394	n.f	*Acacia senegal*	Senegal
M. prunaredense STM4891	WP_008875470	WP_077378855	WP_077374804	n.f	*Anthyllis vulneraria*	France
M. ciceri bv. biserrulae WSM1284	WP_013532151	WP_027162814	n.f	n.f	*Biserrula pelecinus*	Italy
M. huakuii 7653R	WP_010909075	WP_038655131	n.f	n.f	*Astragalus sinicus*	China
M. loti NZP2037	WP_010909075	WP_019856455	n.f	n.f	*Lotus japonicus*	New Zealand
M. loti R7ANS::ICEMlSym2037	WP_010909075	WP_019856455	n.f	n.f	*Lotus sp.*	New Zealand
M. loti R7ANS::ICEMlSym343	WP_010909075	WP_019856455	n.f	n.f	*Lotus sp.*	New Zealand
M. loti SU343	WP_010909075	WP_019856455	n.f	n.f	*Lotus*	Australia
M. metallidurans STM 2683	WP_008875470	WP_008878230	n.f	n.f	*Anthyllis vulneraria*	France
M. qingshengii CGMCC 1.12097	WP_010909075	WP_038655131	n.f	n.f	*Astragalus sinicus*	China
Mesorhizobium sp. L103C105A0	WP_023684868	WP_023829773	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L103C119B0	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L103C120A0	WP_023669117	WP_023829773	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L103C131B0	WP_023669117	WP_023750024	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L2C054A000	WP_023669117	WP_023806352	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L2C066B000	WP_023669117	WP_023750024	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L2C067A000	WP_023669117	WP_023806352	n.f	n.f	*Acmispon wrangelianus*	USA

Mesorhizobium sp. L2C084A000	WP_023810321	WP_023813725	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L2C085B000	WP_023669117	WP_023807976	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L2C089B000	WP_023669117	WP_023806352	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. L48C026A00	WP_023798282	WP_023802047	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC209A00	WP_013895892	WP_023795992	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC221B00	WP_013895892	WP_023778132	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC229A00	WP_013895892	WP_023770904	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC232B00	WP_013895892	WP_023766913	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNHC252B00	WP_023759787	WP_023760353	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC372A00	WP_023684868	WP_031244915	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC374B00	WP_023684868	WP_031244915	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC384A00	WP_023669117	WP_023750024	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC391B00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC394B00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC395A00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC399B00	WP_023684868	WP_023736279	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LNJC405B00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC412B00	WP_023669117	WP_023727108	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC414A00	WP_023669117	WP_023750024	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC416B00	WP_023684868	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC422A00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC424B00	WP_023684868	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC426A00	WP_023684868	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC440A00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSHC440B00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC255A00	WP_023669117	WP_023706358	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC264A00	WP_023684868	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC265A00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC268A00	WP_023669117	WP_031210697	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC269B00	WP_023669117	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC277A00	WP_023684868	WP_023687361	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. LSJC285A00	WP_023669117	WP_023829773	n.f	n.f	*Acmispon wrangelianus*	USA
Mesorhizobium sp. WSM3224	WP_027166417	WP_027168883	n.f	n.f	*Otholobium candicans*	South Africa
Mesorhizobium sp. WSM3873	WP_059185132	WP_066995472	n.f	n.f	*Biserrula pelecinus*	Eritrea
R. acidisoli FH23	WP_003586201	WP_054185963	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. aegyptiacum 950	WP_011425583	WP_064694847	WP_064694883	n.f	*Trifolium*	Egypt
R. bangladeshense 1002	WP_064685493	WP_064682971	n.f	n.f	*Trifolium*	Egypt
R. bangladeshense 1017	WP_011425583	WP_064682971	n.f	n.f	*Trifolium*	Egypt
R. bangladeshense 1024	WP_011425583	WP_064682971	n.f	n.f	*Trifolium*	Egypt
R. ecuadorense CNPSO 671	WP_003586201	WP_004679211	n.f	n.f	*Phaseolus vulgaris*	Ecuador
R. etli 8C-3	WP_074061412	WP_004679211	WP_074063931	n.f	*Phaseolus vulgaris*	Spain
R. etli bv. mimosae str. IE4771	WP_009997055	WP_040140397	WP_040140354	n.f	*Phaseolus vulgaris*	Mexico
R. etli bv. mimosae str. Mim1	WP_011425583	WP_020923593	WP_020923619	n.f	*Mimosa affinis*	Mexico

R. etli bv. phaseoli str. IE4803	WP_009997055	WP_040111869	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. etli CFN 42	WP_011425583	WP_011053464	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. etli CIAT 652	WP_004676193	WP_004679211	n.f	n.f	*Phaseolus vulgaris*	Costa Rica
R. etli CNPAF512	WP_004676193	WP_004679211	n.f	n.f	*Phaseolus vulgaris nodules*	Brazil
R. etli N561	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. favelukesii LPU83	WP_024312766	WP_024318766	WP_024319229	n.f	*Medicago sativa*	USA
R. favelukesii OR191	WP_024312766	WP_024318766	WP_024319229	n.f	*Medicago sativa*	USA
R. freirei PRF 81	WP_004108986	WP_004125966	WP_004126123	n.f	*Phaseolus vulgaris L. nodules*	Brazil
R. gallicum bv. gallicum R602	WP_026230491	WP_040114223	WP_040114282	n.f	*Phaseolus vulgaris*	France
R. gallicum IE4872	WP_028739637	WP_074070778	WP_074070854	n.f	*Phaseolus vulgaris*	Mexico
R. giardinii bv. giardinii H152	WP_018324689	WP_018328742	n.f	n.f	*Phaseolus vulgaris*	France
R. grahamii CCGE 502	WP_016554154	WP_016558502	WP_016558410	n.f	*Dalea leporine nodule*	Mexico
R. laguerreae FB 206	WP_003540131	WP_003551808	WP_077979376	n.f	*Phaseolus vulgaris*	Mexico
R. leguminosarum bv. phaseoli 4292	WP_003540131	WP_004679211	n.f	n.f	*Phaseolus vulgaris*	UK
R. leguminosarum bv. phaseoli CCGM1	WP_004676193	WP_011053464	n.f	n.f	*Phaseolus vulgaris root nodules*	Mexico
R. leguminosarum bv. phaseoli FA23	WP_003540131	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Poland
R. leguminosarum bv. trifolii CB782	WP_003568997	WP_025419125	n.f	n.f	*Trifolium semipilosum*	Kenya
R. leguminosarum bv. Trifolii CC275e	WP_003559805	WP_033181212	n.f	n.f	*Trifolium repens*	Australia
R. leguminosarum bv. trifolii CC278f	WP_026230491	WP_027681588	WP_027681608	n.f	*Trifolium nanum*	n.a
R. leguminosarum bv. trifolii CC283b	WP_003540131	WP_027690723	WP_027691130	n.f	*Trifolium ambiguum*	Caucasus
R. leguminosarum bv. Trifolii Rt24.2	WP_003540131	WP_064649245	n.f	n.f	*Trifolium pratense*	Poland
R. leguminosarum bv. trifolii SRDI565	WP_003568997	WP_017968966	WP_017968943	n.f	*Trifolium subterraneum*	Australia
R. leguminosarum bv. trifolii SRDI943	WP_017994222	WP_017996986	n.f	n.f	*Trifolium subterraneum*	Australia
R. leguminosarum bv. trifolii WSM1325	WP_012757658	WP_012755330	n.f	n.f	*Trifolium spp.*	Serifos, Greece
R. leguminosarum bv. trifolii WSM1689	WP_003568997	WP_025397940	WP_025397919	n.f	*Trifolium uniflorum*	Greece
R. leguminosarum bv. trifolii WSM2012	WP_003568997	WP_003568286	n.f	n.f	*Trifolium rueppellianum*	Ethiopia
R. leguminosarum bv. trifolii WSM2304	WP_003586201	WP_012555957	n.f	n.f	*Trifolium polymorphum*	Uruguay
R. leguminosarum bv. trifolii WSM597	WP_003586201	WP_003592722	n.f	n.f	*Trifolium pallidum L*	Uruguay
R. leguminosarum bv. viciae 128C53	WP_003540131	WP_018517098	WP_018481130	n.f	n.f	UK
R. leguminosarum bv. viciae 248	WP_003568997	WP_003551808	n.f	n.f	*Vicia faba*	UK
R. leguminosarum bv. viciae 3841	WP_003540131	WP_011654223	WP_011654145	n.f	*Vicia faba*	UK
R. leguminosarum bv. viciae	WP_003540131	WP_024323797	n.f	n.f	*Pisum sativum*	Poland

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R. leguminosarum bv. viciae Ps8	WP_003540131	WP_018068351	n.f	n.f	*Pisum sativum*	UK
R. leguminosarum bv. Viciae RCAM *1026*	WP_003540131	WP_075225990	n.f	n.f	*Pisum sativum L.*	Kazakhstan
R. leguminosarum bv. viciae TOM	WP_026158706	WP_017958628	n.f	n.f	n.f	Turkey
R. leguminosarum bv. viciae UPM1131	WP_003540131	WP_027667877	WP_027668149	n.f	*Pisum sativum*	Italy
R. leguminosarum bv. viciae UPM1137	WP_003540131	WP_028734685	n.f	n.f	*Pisum sativum*	Italy
R. leguminosarum bv. viciae USDA 2370	WP_003540131	WP_077988152	WP_077988020	n.f	n.f	n.f
R. leguminosarum bv. Viciae Vaf12	WP_003540131	WP_062944504	n.f	n.f	*Vavilovia formosa*	Russia
R. leguminosarum bv. viciae Vc2	WP_003540131	WP_018484282	WP_018481130	n.f	*Vicia cracca*	UK
R. leguminosarum bv. viciae VF39	WP_003540131	WP_003551808	n.f	n.f	*Vicia faba*	Germany
R. leguminosarum bv. viciae Vh3	WP_020572119	WP_018496572	n.f	n.f	*Vicia hirsuta*	United Kingdom
R. leguminosarum bv. viciae WSM1455	WP_003540131	WP_003551808	n.f	n.f	*Vicia faba*	Greece
R. leguminosarum bv. viciae WSM1481	WP_003540131	WP_003551808	n.f	n.f	*Vicia faba, Pisum sativum, Lens culinaris*	Greece
R.leguminosarum Vaf10	WP_012757658	WP_065284388	n.f	WP_064244960.1	*Vavilovia formosa*	Armenia
R. leguminosarum Vaf108	WP_072638300	WP_072642639	n.f	WP_064244960.1	*Vavilovia formosa*	Armenia
R. leguminosarum Vaf46	WP_003540131	WP_064251344	WP_064250327	WP_064244960.1	*Vavilovia formosa*	Armenia
R. leucaenae CPAO 29.8	WP_028753044	WP_004125966	WP_004126123	n.f	*Phaseolus vulgaris*	Brazil
R. leucaenae USDA 9039	WP_028753044	WP_004125966	WP_004126123	n.f	*Phaseolus vulgaris*	Brazil
R. lusitanum P1-7	WP_037202383	WP_004125966	WP_004126123	n.f	*Phaseolus vulgaris*	Portugal
R. mesoamericanum STM3625	WP_007532947	WP_007539206	WP_007539135	n.f	n.f	n.f
R. mesoamericanum STM6155	WP_007532947	WP_007539206	WP_028748995, WP_007539135	n.f	*Mimosa pudica*	New Caledonia
R. mongolense USDA 1844	WP_028739637	WP_022719259	WP_022719116	n.f	*Medicago ruthenica*	n.f
R. phaseoli Ch24-10	WP_004676193	WP_011053464	n.f	n.f	*Zea mays*	Mexico
R. phaseoli N161	WP_004676193	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N261	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N671	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N771	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N831	WP_004676193	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N841	WP_004676193	WP_011053464	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli N931	WP_004676193	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli R611	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli R620	WP_064825845	WP_011053464	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli R630	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli R650	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. phaseoli R723	WP_004676193	WP_029875592	n.f	n.f	*Phaseolus vulgaris*	Mexico
R. sophorae CCBAU 03386	WP_009997055	WP_040111869	n.f	n.f	*Sophora flavescens*	China
Rhizobium sp. BR10423	WP_016554154	WP_007539206	WP_028748995, WP_007539135	n.f	*Mimosa pudica*	Brazil

Rhizobium sp. CCGE 510	WP_003586201	WP_007636999	n.f	n.f	*Phaseolus albescens*	Mexico
Rhizobium sp. HBR26	WP_011425583	WP_040111869	n.f	n.f	*Phaseolus vulgaris*	Ethiopia
Rhizobium sp. IRBG74	WP_004442498	WP_022557357	n.f	n.f	*Sesbania cannabina*	Philipines
Rhizobium sp. N113	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N1314	WP_011425583	WP_011053464	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N1341	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N324	WP_003586201	WP_064842753	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N541	WP_003586201	WP_064842753	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N621	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N6212	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N731	WP_011425583	WP_011053464	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N741	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N871	WP_011425583	WP_064812010	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. N941	WP_003586201	WP_064842753	n.f	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. Pop5	WP_008525920	WP_008536482	WP_008530975	n.f	*Phaseolus vulgaris*	Mexico
Rhizobium sp. RSm-3	WP_003586201	WP_004679211	n.f	n.f	*Phaseolus vulgaris*	India
Rhizobium sp. WYCCWR10014	WP_064648314	WP_064649245	WP_064652350	n.f	*Trifolium repens*	China
Rhizobium sp. WYCCWR10015	WP_003540131	WP_063474972	WP_063474903	n.f	*Trifolium repens*	China
R. sullae WSM1592	WP_027512382	WP_027513879, WP_051336867	WP_027511977	n.f	*Hedysarum coronarium*	Italy
R. tibeticum CCBAU85039	WP_024312766	WP_072381779	n.f	n.f	*Trigonella archiducis-nicolai (Sirj.) Vassilcz.*	China
R. tibeticum CGMCC 1.7071	WP_024312766	WP_072381779	WP_072381651	n.f	*Medicago archiducis-nicolai Sirj*	China
R. tropici CIAT 899	WP_015340070	WP_004125966	WP_004126123	n.f	*Phaseolus vulgaris*	Colombia
R. undicola ORS 992	WP_027486375	WP_027487437	WP_027487279	n.f	*Neptunia natans*	Senegal
S. meliloti CCNWSX0020	WP_003533002	WP_003532849	WP_003532571	n.f	*Medicago lupulina*	China
S. medicae DI28	WP_011975674	WP_011970892	WP_011971052	n.f	*Medicago arabica*	Italy
S. medicae WSM1115	WP_011975674	WP_018210177	WP_011971052	n.f	*Medicago polymorpha*	Greece
S. medicae WSM419	WP_011975674	WP_011970892	WP_011971052	n.f	*Medicago murex*	Italy
S. meliloti A0641M	WP_010969478	WP_017266288	WP_012881248	WP_017266453	*Medicago sativa*	Italy
S. meliloti A0643DD	WP_010969478	WP_017266288	WP_012881248	n.f	*Medicago sativa*	Italy
S. meliloti C0438LL	WP_010969478	WP_017266288	WP_012881248	WP_017266453	*Medicago sativa*	Italy
S. meliloti 1A42	WP_010969478	WP_013845740	WP_013845262	n.f	*Medicago truncatula*	n.a
S. meliloti AK11	WP_010969478	WP_017269103	WP_013845262	n.f	*Medicago falcata*	Kazakhstan
S. meliloti AK58	WP_010969478	WP_017269103	WP_013845262	n.f	*Medicago falcata*	Kazakhstan
S. meliloti AK75	WP_010969478	WP_017269103	WP_013845262	n.f	*Medicago lupulina*	Kazakhstan
S. meliloti AK83	WP_010969478	WP_013845740	WP_013845262	n.f	*Medicago sativa*	Aral sea region
S. meliloti CIAM1775	WP_010969478	WP_017269103	WP_013845262	n.f	*Medicago lupulina*	Kazakhstan
S. meliloti GR4	WP_010969478	WP_013845740	WP_013845262	n.f	agricultural field	Spain
S. meliloti L5-30	WP_010969478	WP_046067373	WP_013845262	n.f	soil	Poland
S. meliloti SM11	WP_010969478	WP_014531649	WP_013845262	n.f	*Medicago sativa*	Germany
S. meliloti BL225C	WP_010969478	WP_014528577	WP_014528042	n.f	*Medicago sativa*	Italy
S. meliloti C0431A	WP_010969478	WP_014528577	WP_014528042	n.f	*Medicago sativa*	Italy
S. meliloti DSM 23914	WP_010969478	WP_014528577	WP_014528042	n.f	*Medicago*	Kazakhstan
S. meliloti AE608H	WP_010969478	WP_014531649	WP_017272414	n.f	*Medicago sativa*	Italy
S. medicae WSM1369	WP_011975674	WP_018009932	WP_018009224	n.f	*Medicago sphaerocarpa*	Italy

S. meliloti 4H41	WP_010969478	WP_018097533	WP_018099738	n.f	*Phaseolus vulgaris*	Tunisia
Ensifer sp. BR816	WP_026187508	WP_018240595	WP_018240492	n.f	*Leucaena leucocephala*	Brazil
Ensifer sp. WSM1721	WP_026613978	WP_026621872	WP_026622876	n.f	*Indigofera sp.*	Australia
S. arboris LMG 14919	WP_028000588	WP_028002360	WP_028002389	WP_028002544	*Prosopis chilensis*	Sudan
S. fredii GR64	WP_037433475	WP_028003553	WP_028003505	n.f	*Phaseolus vulgaris*	Spain
S. meliloti GVPV12	WP_003533002	WP_028003553	WP_028003505	n.f	*Phaseolus vulgaris*	Spain
S. meliloti MVII-I	WP_010969478	WP_014531649	WP_029728323	n.f	*Medicago sativa*	Germany
S. americanum CCGM7	WP_037385990	WP_037390232	WP_037377499	n.f	*Phaseolus vulgaris*	Mexico
S. americanum CFNEI 156	WP_037424417	WP_064254499	WP_064254442	n.f	*Acacia*	Mexico
S. americanum CFNEI 73	WP_037424417	WP_064254499	WP_064254442	n.f	*Acacia farnesiana*	Mexico
Ensifer shofinae CCBAU 251167	WP_065997707	WP_010875357	WP_065999868	n.f	*Glycine max*	China
Ensifer sp. YIC4027	WP_069458503	WP_069457971	WP_069461354	n.f	*Sesbania cannabina*	China
Ensifer sp. LCM 4579	WP_071015582	WP_071019928	WP_071020019, WP_071015634	WP_071018497	*Prosopis juliflora*	Senegal
E. glycinis CCBAU 23380	WP_012708181	WP_014858070	n.f	n.f	*Glycine soja*	China
E. sojae CCBAU 05684	WP_034854940	WP_014858070	n.f	n.f	*Glycine max*	China
Ensifer sp. USDA 6670	WP_029957038	WP_029964748	n.f	n.f	*Medicago sativa*	Australia
S. fredii CCBAU 05557	WP_014328498	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 25509	WP_014328498	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 45436	WP_014328498	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 83622	WP_014328498	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 83643	WP_037433475	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 83666	WP_037433475	WP_010875357	n.f	n.f	*Glycine max*	China
S. fredii CCBAU 83753	WP_037433475	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii HH103	WP_014328498	WP_014858070	n.f	n.f	soil	China
S. fredii NGR234	WP_012708181	WP_010875357	n.f	n.f	*Lablab purpureus*	New Guinea
S. fredii USDA 205	WP_014328498	WP_014858070	n.f	n.f	*Glycine max*	China
S. fredii USDA 257	WP_014764748	WP_014858070	n.f	n.f	*Glycine soja*	China
S. medicae WSM244	WP_011975674	WP_011970892	n.f	n.f	*Medicago polymorpha*	Iraq
S. meliloti 1021	WP_010969478	WP_010967454	n.f	n.f	*Medicago*	n.a
S. meliloti 2011	WP_010969478	WP_010967454	n.f	n.f	n.a	n.a
S. meliloti BO21CC	WP_010969478	WP_014528577	n.f	n.f	*Medicago spp.*	Italy
S. meliloti H1	WP_010969478	WP_014528577	n.f	n.f	*Medicago sativa*	Italy
S. meliloti Mlalz-1	WP_003533002	WP_027994117	n.f	n.f	*Medicago laciniata*	Spain
S. meliloti Rm41	WP_010969478	WP_013845740	n.f	n.f	*Medicago sativa*	n.f
S. meliloti RMO17	WP_010969478	WP_014528577	n.f	n.f	*Medicago orbicularis*	Spain
S. meliloti RRI128	WP_003533002	WP_027991113	n.f	n.f	*Medicago sativa*	Australia
S. meliloti RU11/001	WP_010969478	WP_014531649	n.f	n.f	*Medicago*	n.f
S. meliloti WSM1022	WP_003533002	WP_014528577	n.f	n.f	*Medicago orbicularis*	Greece
S. meliloti WSM4191	WP_011975674	WP_011970892	n.f	n.f	*Melilotus siculus*	Australia
Sinorhizobium sp. CCBAU 05631	WP_037424417	WP_010875357	n.f	n.f	*Glycine max*	China
Sinorhizobium sp. PC2	WP_026613978	WP_026615849, WP_046118778	n.f	n.f	*Prosopis cineraria*	India
Burkholderia sp. CCGE1001	WP_013589535	WP_062826958, WP_062827051	WP_013590235, WP_062827023	n.f	*Machaerium lunatum*	French Guiana

Burkholderia sp. CCGE1002	WP_013090445	WP_013094471	WP_013091788, WP_013094600	n.f	Rhizosphere	Mexico
Burkholderia sp. JPY251	WP_013090445	WP_018434997	WP_018437544, WP_018438156	WP_018434972	*Mimosa flocculosa*	Brazil
Burkholderia sp. UYPR1.413	WP_028364503	WP_051446721	WP_028369375	WP_028371487	*Parapiptadenia rigida*	Uruguay
Burkholderia sp. WSM4176	WP_018421721	WP_018423144	WP_018425251	n.f	*Lebeckia ambigua*	South Africa
Paraburkholderia dilworthii WSM3556	WP_027800201	WP_027802870	WP_027798506	n.f	*Lebeckia ambigua*	South Africa
Paraburkholderia mimosarum LMG 23256	WP_028211774	WP_042291076	WP_028231140	n.f	*Lebeckia ambigua*	South Africa
Paraburkholderia mimosarum STM 3621	WP_028211774	WP_042291076	WP_028212273	n.f	*Mimosa pigra*	Taiwan
Paraburkholderia nodosa DSM 21604	WP_028205964	WP_051482046	WP_028206246	n.f	*Mimosa pudica*	French Guiana
Paraburkholderia phenoliruptrix BR3459a	WP_013589535	WP_015004749	WP_014971831, WP_015004813	WP_015004789	*Parapiptadenia rigida*	Uruguay
Paraburkholderia phymatum STM815	WP_012399858	WP_012406749	WP_012404646, WP_012406832	WP_012406803	*Mimosa scabrella*	Brazil
Paraburkholderia sprentiae WSM5005	WP_027198763	WP_027193733, WP_071336652, WP_071336791	WP_027196291	n.f	Rhizosphere	Mexico

n.f- not found

Table S2 - Predicted (BlastP) number of different genetic elements involved in ethylene biosynthesis and signalling components in completely sequenced leguminous plants.

Family	Plant	ACS	ACO	ETR1*	CTR1	EIN2
Phaseolae	*Glycine max*	14	14	16	11	6
Phaseolae	*Glycine soja*	14	15	4	4	3
Phaseolae	*Vigna radiata*	9	7	2	10	4
Phaseolae	*Vigna angularis*	9	15	6	13	4
Phaseolae	*Cajanus cajan*	9	7	4	6	1
Phaseolae	*Phaseolus vulgaris*	9	9	2	2	2

Genisteae	*Lupinus angustifolius*	10	5	11	15	6
Lotea	*Lotus japonicus*	8	10	5	4	2
Cicerea	*Cicer arietinum*	8	5	5	7	1
Trifolieae	*Trifolium subterraneum*	8	4	2	3	1
Trifolieae	*Trifolium pratense*	9	4	2	3	1
Trifolieae	*Medicago truncatula*	9	8	2	3	1
Camelinae	*Arabidopsis thaliana*	8	5	1	2	1

ACS- ACC synthase; ACO- ACC oxidase; ETR1- Ethylene Receptor 1 or ETR1-like*; CTR1- Constitutive Triple Response 1 (mitogen activated kinase); EIN2- Ethylene insensitive 2.BlastP analysis were conducted using *Arabidopsis* ACS proteins as queries against the proteome of each leguminous plant present in the NCBI database.

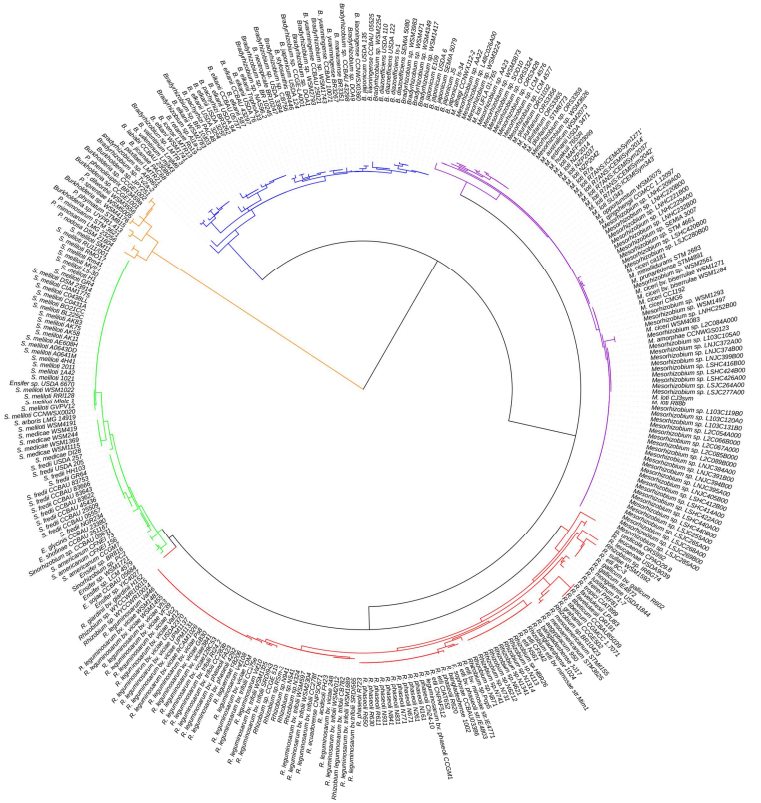

Figure S1- Molecular phylogenetic analysis based on RecA from rhizobia possessing NodC

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-4276.3633) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4757)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.6391% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 323 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset.

The color scheme is similar to that presented in Figure 1.

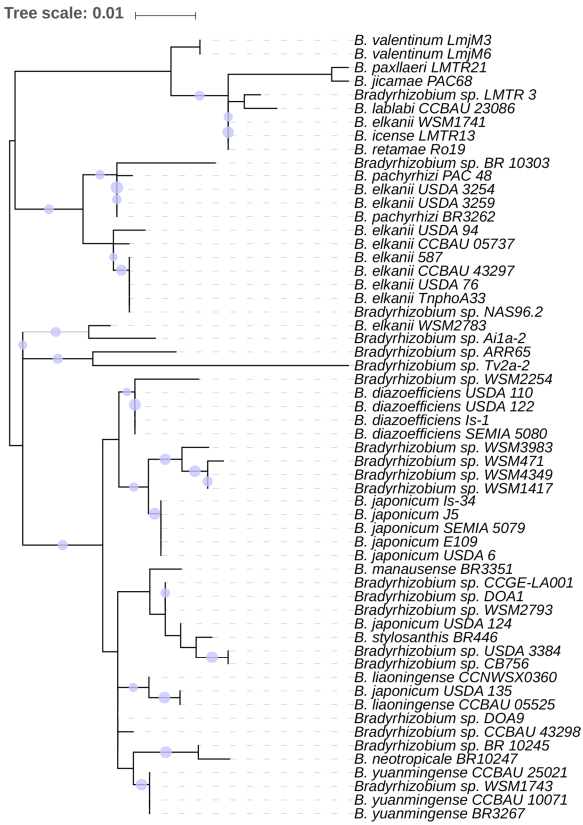

Figure S2- Molecular phylogenetic analysis based on *Bradyrhizobium* RecA
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-1813.2640) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1311)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 76.8602% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 362 positions in the final dataset. Blue circles in branches indicate a bootstrap value over 0.5 (50%).

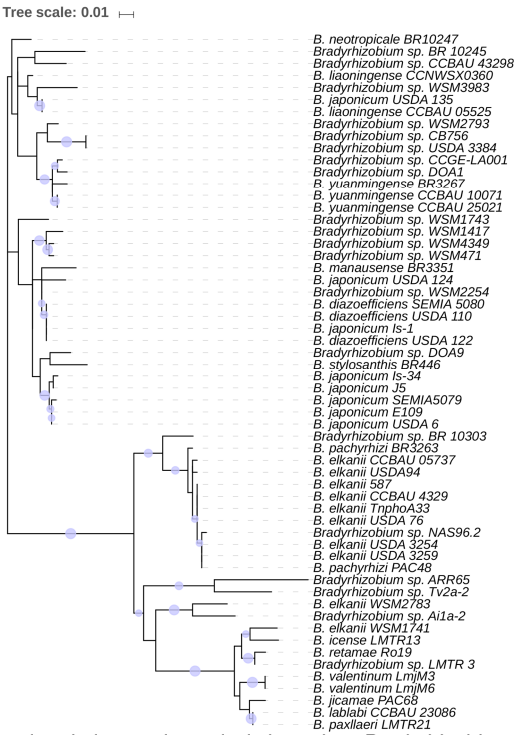

Figure S3- Molecular phylogenetic analysis based on *Bradyrhizobium* AcdS

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-2665.8723) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8024)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.4985% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 337 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

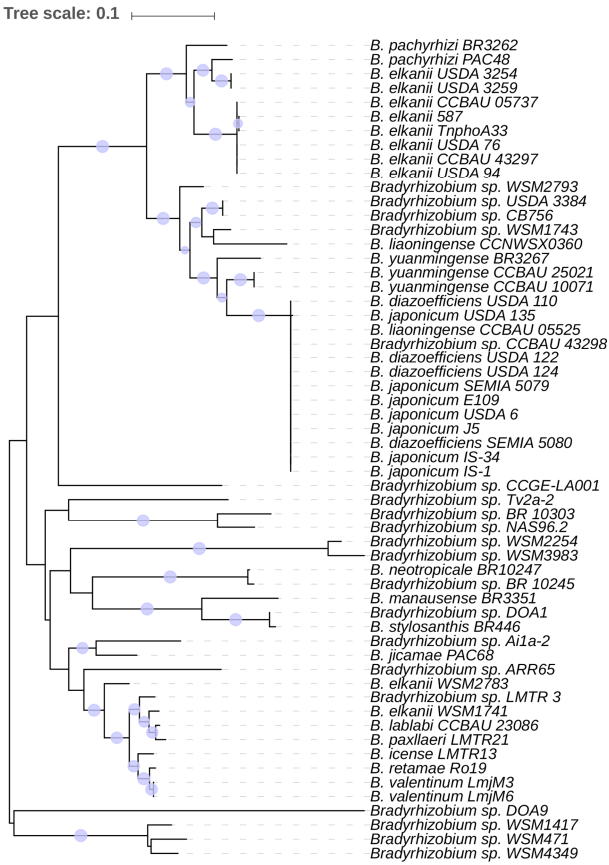

Figure S4- Molecular phylogenetic analysis based on *Bradyrhizobium* NodC

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-7671.7222) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6902)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 418 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

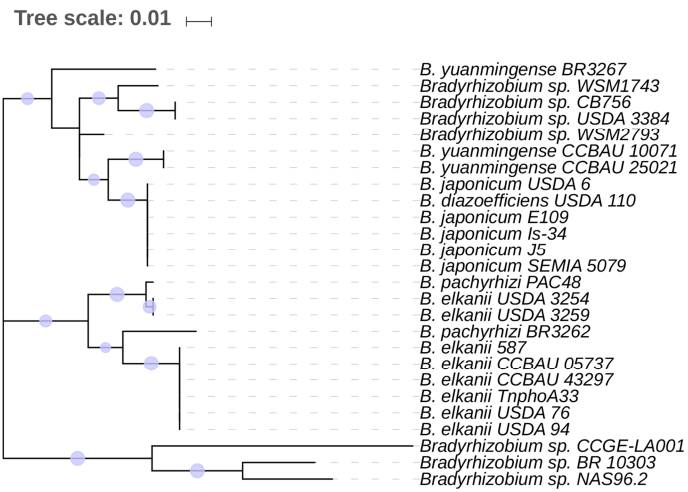

Figure S5- Molecular phylogenetic analysis based on *Bradyrhizobium* RtxC
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-2130.5993) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2933)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 351 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

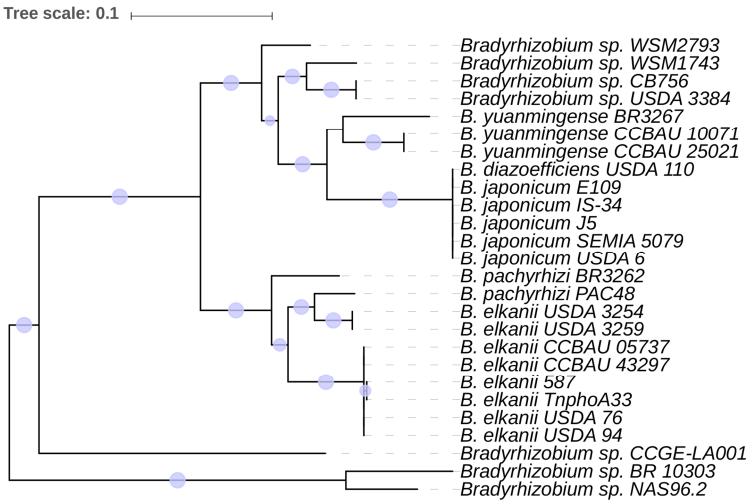

Figure S6- Molecular phylogenetic analysis based on NodC sequences from *Bradyrhizobium* strains possessing RtxC.

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-3919.3605) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3178)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 438 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

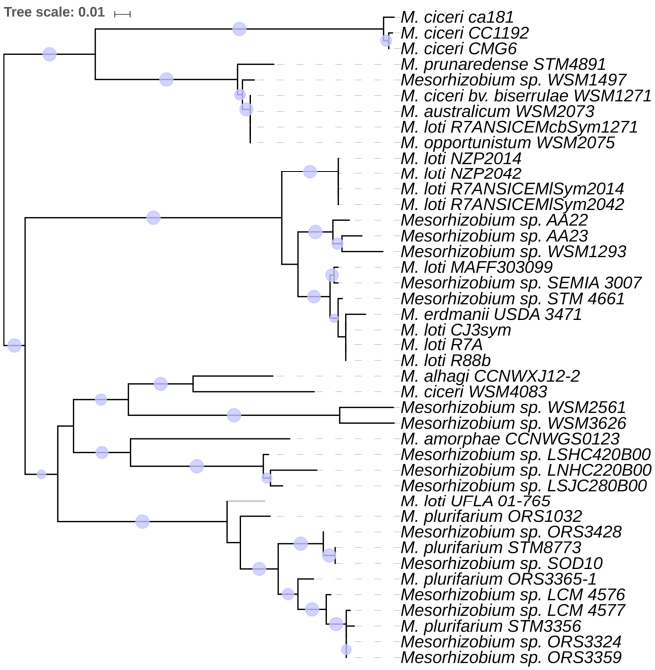

Figure S7- Molecular phylogenetic analysis based on *Mesorhizobium* NodC
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-4383.1778) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4758)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 373 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

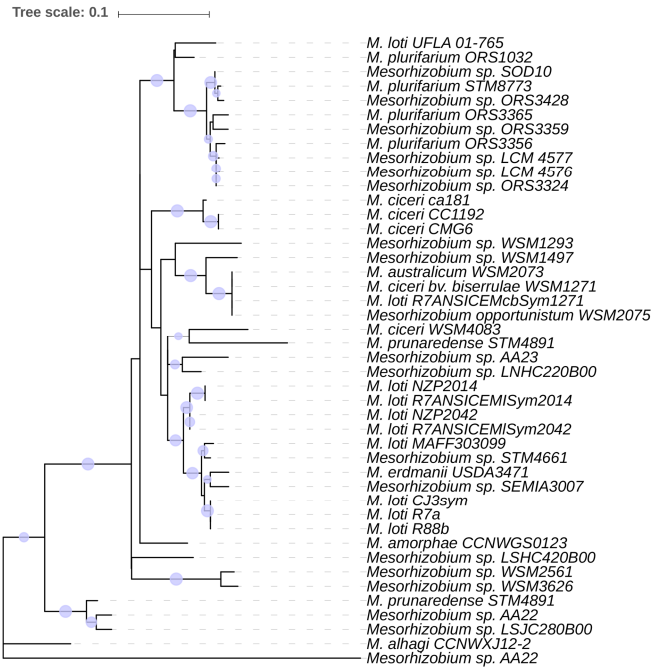

Figure S8- Molecular phylogenetic analysis based on *Mesorhizobium* AcdS

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-3804.2605) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4051)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 44 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 333 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

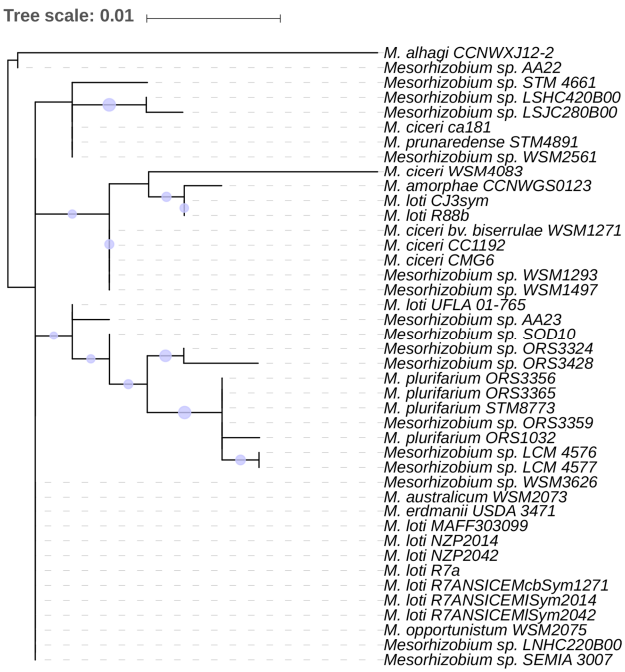

Figure S9- Molecular phylogenetic analysis based on *Mesorhizobium* RecA

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-1292.2730) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1096)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 361 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

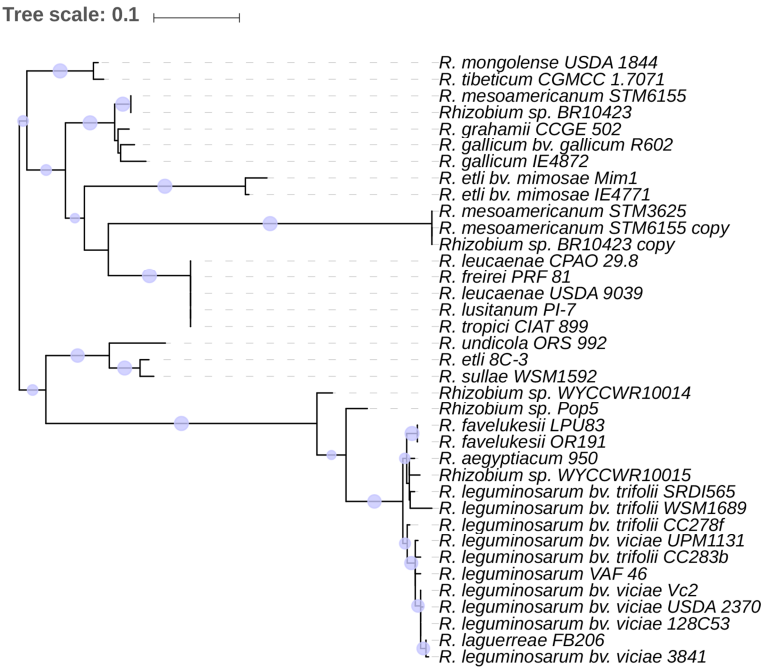

Figure S10- Molecular phylogenetic analysis based on *Rhizobium* AcdS

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-3469.1415) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4692)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 37 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 337 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

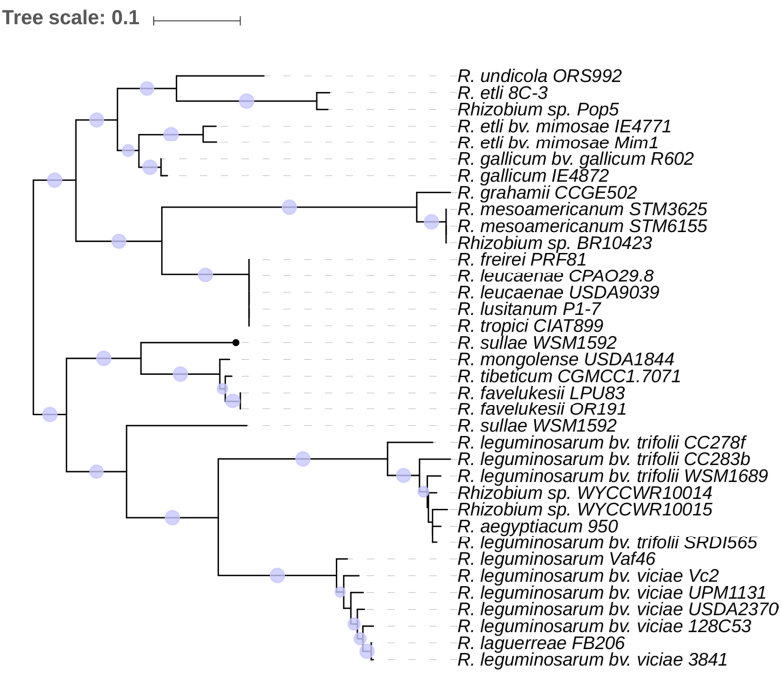

Figure S11- Molecular phylogenetic analysis based on *Rhizobium* NodC

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-5728.9047) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4634)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 424 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

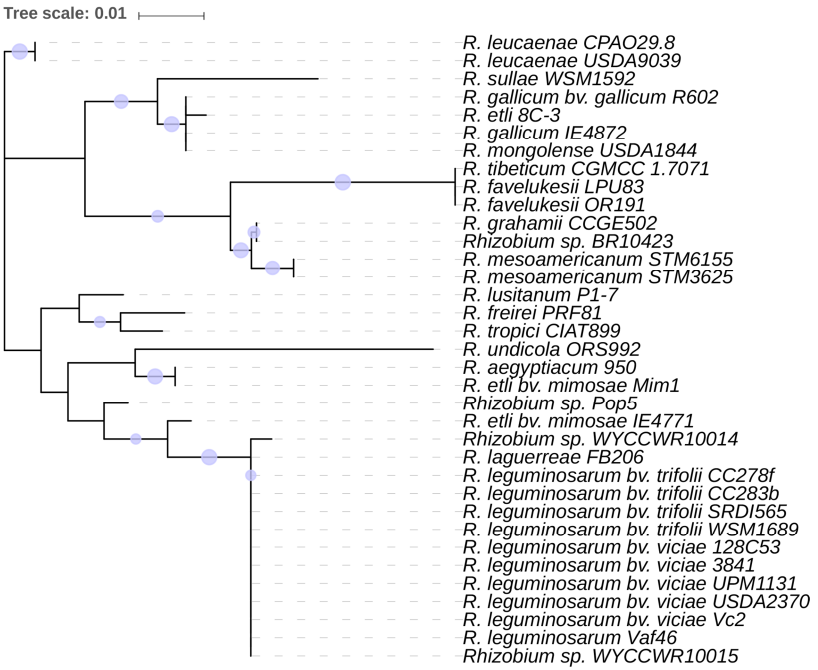

Figure S12- Molecular phylogenetic analysis based on *Rhizobium* RecA

The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-1523.49) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 359 positions in the final dataset.

Blue circles in branches indicate a bootstrap value over 0.5 (50%).

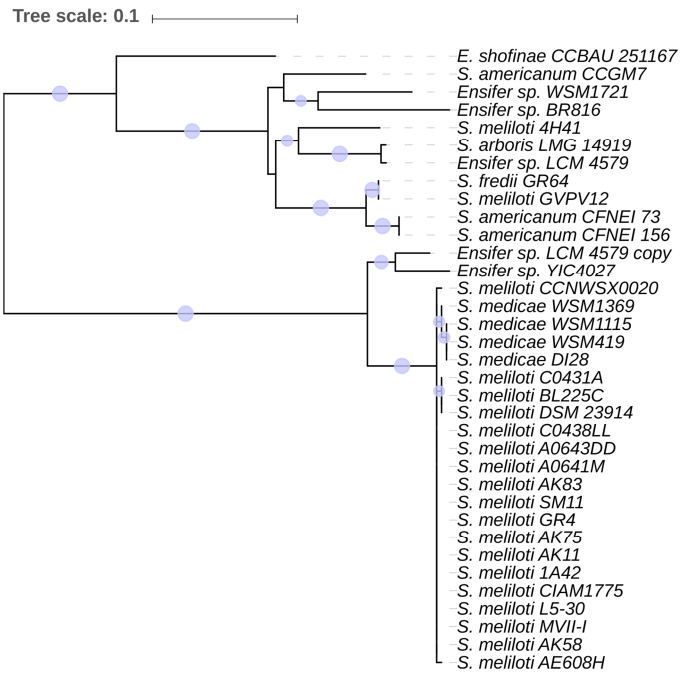

Figure S13- Molecular phylogenetic analysis based on *Sinorhizobium* AcdS
The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-2365.7835) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4777)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 300 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

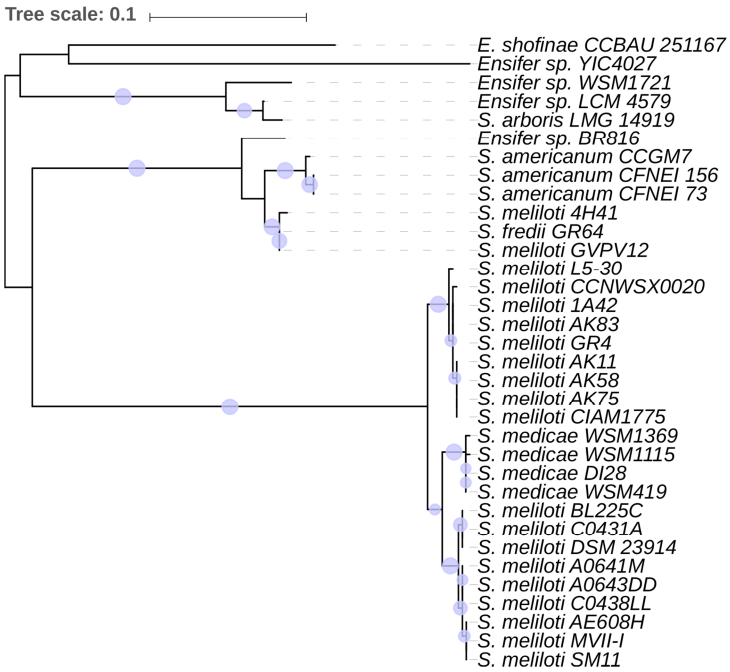

Figure S14- Molecular phylogenetic analysis based on *Sinorhizobium* NodC
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-3277.3361) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5510)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 408 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

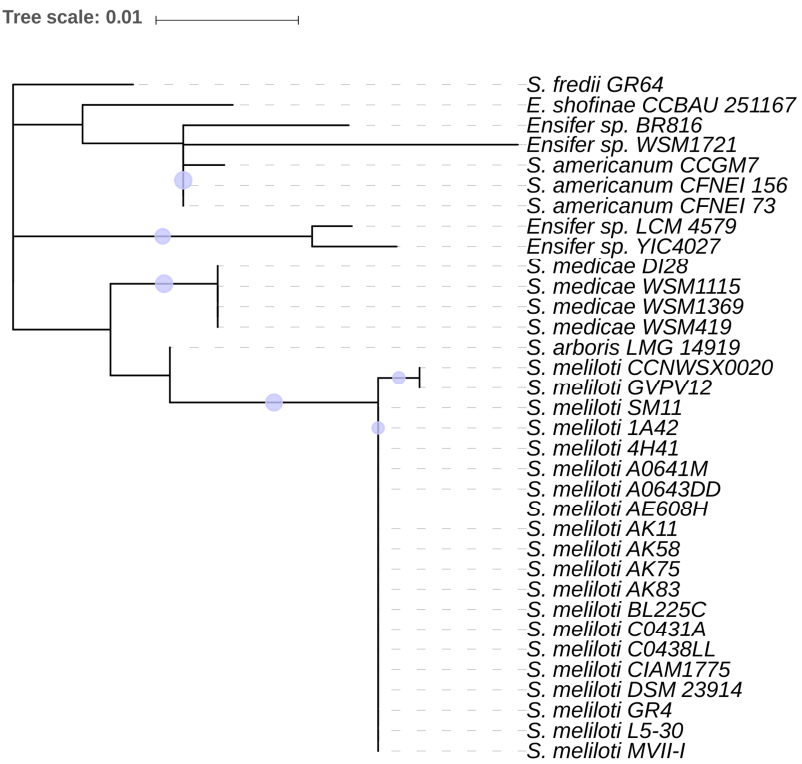

Figure S15- Molecular phylogenetic analysis based on *Sinorhizobium* RecA
The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-1317.64) is shown. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 46.25% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 360 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

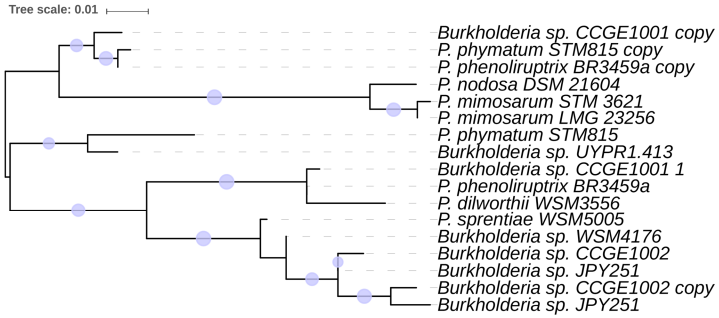

Figure S16- Molecular phylogenetic analysis based on *Paraburkholderia* AcdS
The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model. The tree with the highest log likelihood (-1681.9521) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2276)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 335 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

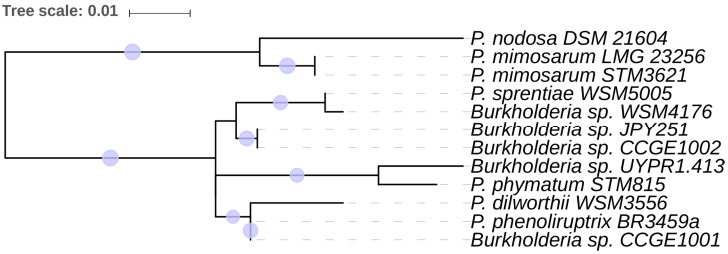

Figure S17- Molecular phylogenetic analysis based on *Paraburkholderia* RecA
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model . A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 357 positions in the final dataset.
Blue circles in branches indicate a bootstrap value over 0.5 (50%).

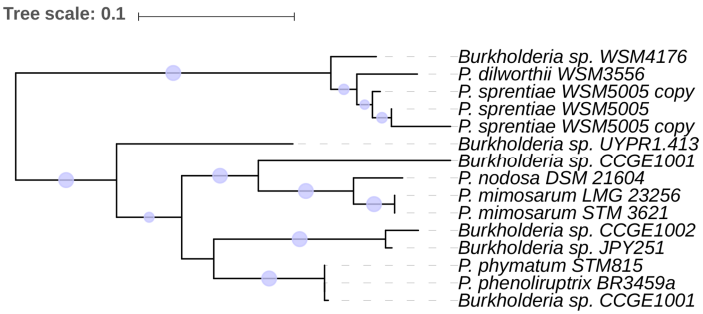

Figure S18- Molecular phylogenetic analysis based on *Paraburkholderia* NodC
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model . The tree with the highest log likelihood (-3161.2440) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3921)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 406 positions in the final dataset.

"Non-specific transient mutualism between the plant parasitic nematode, *Bursaphelenchus xylophilus*, and the opportunistic bacterium *Serratia quinivorans* BXF1, a plant-growth promoting pine endophyte with antagonistic effects"

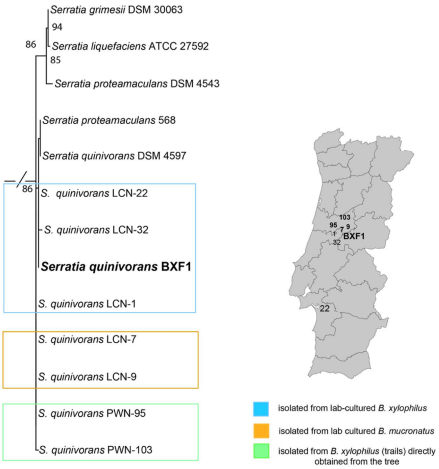

Figure S1- Phylogenetic analysis based on 16S rRNA from several *Serratia* species, including *Serratia* isolates obtained from *Bursaphelenchus xylophilus* obtained from different regions. The analysis was conducted using Maximum likelihood method, GTR+G+I, 1000 bootstrap parameters. Bootstrap values above 75 are shown in the respective branch.

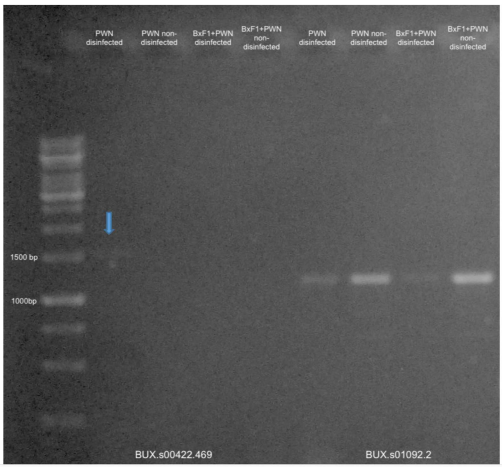

Figure S2- RT-PCR of *Bursaphelenchus xylophilus* chitinases BUX.s00422.469 (*Bxcht-1*) and BUX.s01092.2 (*Bxcht-2*).

Table S1- Biochemical profile of *Serratia quinivorans* BXF1.

Substrate/ assay	Result	Substrate/ assay	Result
Ala-Phe-Pro-Arylamidase	-	5-Keto-D-gluconate	-
Adonitol	-	Alpha-glucosidase	-
L-Pyrrolydonyl-Arylamidase	+	Succinate alkalinization	-
L-Arabitol	-	β-N-acetylgalactosaminidase	+
D-Cellobiose	-	Alpha-galactosidase	-
Beta-galactosidase	-	Phosphatase	+
H2S production	-	Glycine arylamidase	-
β-N-acetylglucosaminidase	+	L-Lactate alkalynization	-
Glutamyl arylamidase pNA	-	Ornithine decarboxylase	-
D-Glucose	+	Lysine descarboxilase	-
Gamma Glutamyl transferase	-	Decarboxylase base	-
Fermentation/glucose	-	L-Histidine assimilation	-
β-glucosidase	+	Coumarate	-
D-Maltose	-	Beta-glucoronidase	-
D-Mannitol	+	0/129 Resistance	+
D-Mannose	+	Glu-Gly-Arg-Arylamidase	+
Beta-xylosidase	-	L-Malate	-
Beta-alanine arylamidase pNA	-	ELLMAN	-
L-Proline arylamidase	+	L-Lactate assimilation	-
Lipase	-	Siderophores	+
Palatinose	-	IAA	+
Tyrosine arylamidase	-	ACC deaminase	-
Urease	-	Phosphate solubilization	-
D-sorbitol	-	Protease	+
Sucrose	+	Cellulase	-
D-Tagatose	-	Ammonia	+
D-Trehalose	+	Chitinase	+
Citrate (sodium)	-	Acetoin	+
Malonate	-		

Table S2- *Serratia quinivorans* BXF1 resistance to terpenoids and aromatic compounds (+ bacteria growth observed; - no bacterial growth observed).

Tested Compound	Growth in TSB				Growth in M9 sole carbon source
	0.1%	0.5%	1%	1.5%	
(+)-α-pinene	+	+	+	+	-
(-)-α-pinene	+	+	+	+	-
α-pinene (isomer mix)	+	+	+	+	-
(+)-β-pinene	+	+	+	+	-
(-)-β-pinene	+	+	+	+	-
(+)-3-carene	+	+	+	+	-
3-carene (isomer mix)	+	+	+	+	-
R-(+)-limonene	+	+	+	+	-
Citral	+	-	-	-	-
γ-terpinene	+	+	+	+	-
p-cymene	+	+	+	+	-
Carvacrol	-	-	-	-	-
2-undecanone	+	+	+	+	-
Geraniol	+	-	-	-	-
3-eugenol	+	-	-	-	-
Mircene	+	+	+	+	-
Linalool	-	-	-	-	-
Toluene	+	+	+	+	-
Xylene	+	+	+	+	-
	0.01 mg/ml	0.05 mg/ml	0.1 mg/ml	0.2 mg/ml	
Benzoic acid	+	+	+	+	+
Phenol	+	+	+	+	-
Phenylacetic acid	+	+	+	+	+

From plants to nematodes: *Serratia grimesii* BXF1 genome reveals an adaptation to the modulation of multi-species interactions

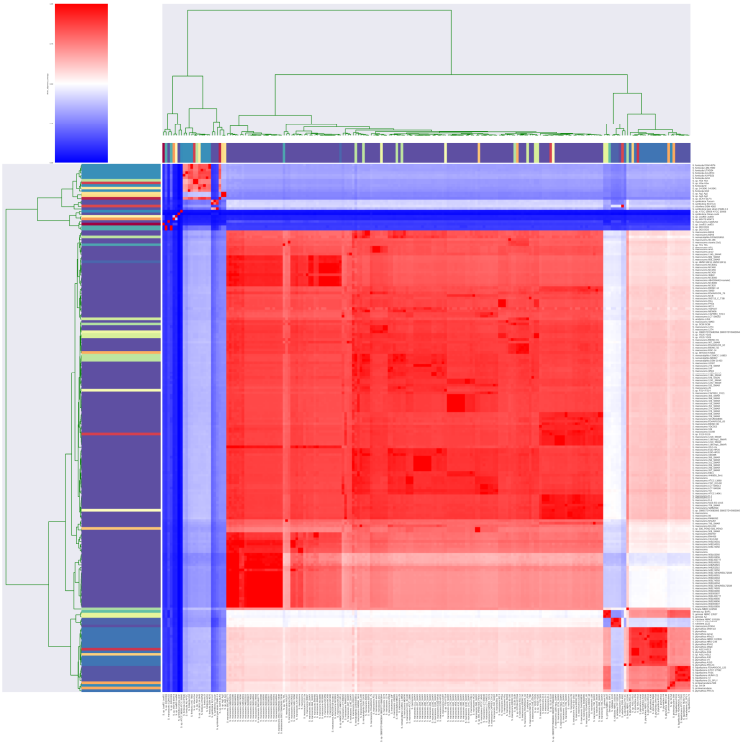

Figure S1- Results obtained from pyani analysis in the comparison of Genome alignment coverage average from over 200 *Serratia* species genomes available in the NCBI database. For purposes of easier identification, strain BXF1 is the only one where the labels list it as *Serratia*, while others are annotated as *S.*
Red colouring indicates a higher alignment coverage and percentage identity between genomes. Blue colouring indicates a lower alignment coverage and percentage identity between genomes.

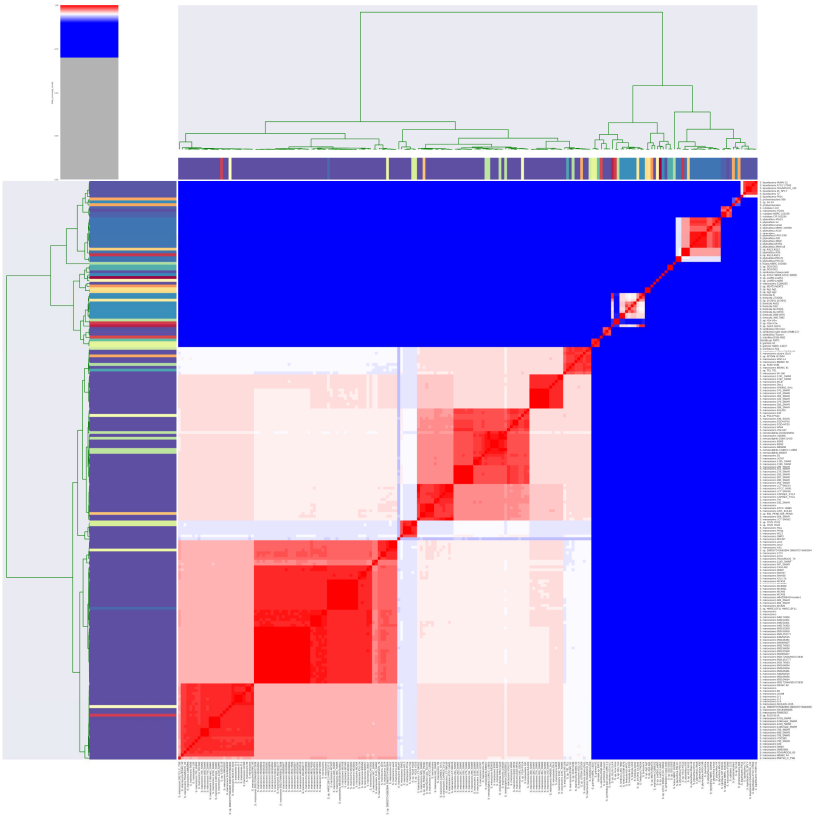

Figure S2- Results obtained from pyani analysis in the comparison of average nucleotide identity (ANI) values from over 200 *Serratia* species genomes available in the NCBI database.
For purposes of easier identification, strain BXF1 is the only one where the labels list it as *Serratia*, while others are annotated as *S.*
Red colouring indicates a higher alignment coverage and percentage identity between genomes. Blue colouring indicates a lower alignment coverage and percentage identity between genomes.

Table S1- Genomic islands predicted by Island Viewer and phage sequences predicted by Phast

Locus	Gene start	Gene end	Strand	Product	External Annotations	
SGBXF1_00106	118994	120193	1	putative transporter YycB		
SGBXF1_00107	120270	121616	1	hypothetical protein		
SGBXF1_00109	121987	123171	1	Putative prophage CPS-53 integrase		
SGBXF1_00110	123168	123998	1	hypothetical protein		
SGBXF1_00111	124119	124415	1	Prophage CP4-57 regulatory protein (AlpA)		
SGBXF1_00112	124762	124938	1	hypothetical protein		
SGBXF1_00113	124931	125284	1	hypothetical protein		
SGBXF1_00114	125329	125610	1	hypothetical protein		
SGBXF1_00115	125607	125957	1	hypothetical protein		
SGBXF1_00116	125967	128648	1	DNA primase TraC		
SGBXF1_00117	129069	129818	1	hypothetical protein	Phage	**GI1**
SGBXF1_00118	129821	130057	1	DNA-binding transcriptional regulator		
SGBXF1_00119	130359	131639	1	Reverse transcriptase (RNA-dependent DNA polymerase)		
SGBXF1_00120	131630	133651	1	Reverse transcriptase (RNA-dependent DNA polymerase)		
SGBXF1_00121	134184	135638	-1	putative HTH-type transcriptional regulator YdcR		
SGBXF1_00122	135911	136483	1	hypothetical protein		
SGBXF1_00123	136644	136967	1	Inner membrane protein YiaW		
SGBXF1_00124	136973	138109	1	Inner membrane protein YibH		
SGBXF1_00125	138674	139405	1	Transcriptional activator protein EsaR		
SGBXF1_00332	366000	366248	1	hypothetical protein		
SGBXF1_00333	366911	368521	1	Flavin-dependent tryptophan halogenase PrnA		
SGBXF1_00334	368521	369603	1	Monodechloroaminopyrrolnitrin synthase PrnB		
SGBXF1_00335	369648	371351	1	Monomeric sarcosine oxidase	Pyrrolnitrin	**GI2**
SGBXF1_00336	371373	372467	1	Aminopyrrolnitrin oxygenase PrnD		
SGBXF1_00337	372467	372637	1	FMN reductase (NADH) RutF		
SGBXF1_00338	372634	373878	1	High-affinity Na(+)/H(+) antiporter NhaS3		
SGBXF1_00410	444559	445029	-1	Arginine repressor		
SGBXF1_00411	445495	446433	1	Malate dehydrogenase		
SGBXF1_00412	446502	446762	-1	DNA-binding transcriptional regulator Nlp		
SGBXF1_00413	446944	447294	1	Mu DNA-binding domain protein	-	**GI3**
SGBXF1_00414	447343	448314	-1	Octaprenyl-diphosphate synthase		
SGBXF1_00415	448584	448895	1	50S ribosomal protein L21		
SGBXF1_00508	547614	548066	-1	DNA polymerase III subunit chi		
SGBXF1_00509	548262	549773	-1	Cytosol aminopeptidase		
SGBXF1_00510	550054	551148	1	Lipopolysaccharide export system permease protein LptF		
SGBXF1_00511	551148	552218	1	Lipopolysaccharide export system permease protein LptG		
SGBXF1_00513	552789	554051	1	Prophage CP4-57 integrase		
SGBXF1_00514	554150	556588	1	hypothetical protein		
SGBXF1_00515	557324	557593	1	hypothetical protein		
SGBXF1_00516	557705	558037	-1	hypothetical protein	-	**GI4**
SGBXF1_00517	558058	558291	-1	hypothetical protein		
SGBXF1_00518	558767	559672	1	hypothetical protein		
SGBXF1_00519	560008	560643	1	Carbonic anhydrase 1		
SGBXF1_00514	554150	556588	1	hypothetical protein		
SGBXF1_00515	557324	557593	1	hypothetical protein		
SGBXF1_00516	557705	558037	-1	hypothetical protein		
SGBXF1_00517	558058	558291	-1	hypothetical protein		
SGBXF1_00518	558767	559672	1	hypothetical protein		
SGBXF1_01012	1096443	1096757	1	transcriptional regulator BolA		
SGBXF1_01013	1097124	1098428	1	Trigger factor		
SGBXF1_01014	1098992	1099615	1	ATP-dependent Clp protease proteolytic subunit precursor		
SGBXF1_01015	1099779	1101050	1	ATP-dependent Clp protease ATP-binding subunit ClpX	-	**GI5**
SGBXF1_01016	1101245	1103599	1	Lon protease		
SGBXF1_01017	1103816	1104088	1	DNA-binding protein HU-beta		
SGBXF1_01018	1104271	1106157	1	Peptidyl-prolyl cis-trans isomerase D		
SGBXF1_01019	1106297	1106683	1	ComE operon protein 1		
SGBXF1_01020	1106840	1107256	1	Long-chain acyl-CoA		

				thioesterase FadM		
SGBXF1_01021	1107371	1108069	-1	7-cyano-7-deazaguanine synthase		
SGBXF1_01549	1653087	1654127	1	Polysaccharide biosynthesis/export protein	O-antigen	**GI6**
SGBXF1_01550	1654132	1654566	1	Low molecular weight protein-tyrosine-phosphatase wzb		
SGBXF1_01551	1654579	1656750	1	Tyrosine-protein kinase wzc		
SGBXF1_01552	1656914	1658035	1	Glycosyl transferases group 1		
SGBXF1_01553	1658113	1659531	1	hypothetical protein		
SGBXF1_01554	1659528	1660586	1	D-inositol 3-phosphate glycosyltransferase		
SGBXF1_01555	1660644	1661375	1	hypothetical protein		
SGBXF1_01556	1661952	1663385	1	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase		
SGBXF1_01557	1663567	1665174	1	hypothetical protein		
SGBXF1_01562	1674316	1675551	1	Putative O-antigen transporter		
SGBXF1_01563	1675551	1676648	1	UDP-galactopyranose mutase		
SGBXF1_01564	1676657	1677640	1	hypothetical protein		
SGBXF1_01565	1677826	1679250	1	Mannose-1-phosphate guanylyltransferase 1		
SGBXF1_01567	1680798	1681811	1	UDP-glucose 4-epimerase		
SGBXF1_01568	1681954	1683018	1	dTDP-glucose 4,6-dehydratase 2		
SGBXF1_01569	1683039	1683908	1	Glucose-1-phosphate thymidylyltransferase 2		
SGBXF1_01570	1683910	1684443	1	dTDP-4-dehydrorhamnose 3,5-epimerase		
SGBXF1_01571	1684443	1685306	1	dTDP-4-dehydrorhamnose reductase		
SGBXF1_01572	1685435	1686298	1	hypothetical protein		
SGBXF1_01573	1686861	1687394	1	dTDP-4-dehydrorhamnose 3,5-epimerase		
SGBXF1_01574	1687502	1688377	1	dTDP-4-dehydrorhamnose reductase		
SGBXF1_01575	1688399	1689187	1	Teichoic acid translocation permease protein TagG		
SGBXF1_01576	1689177	1690568	1	Teichoic acids export ATP-binding protein TagH		
SGBXF1_01577	1690561	1694577	1	?-D-glucose-1-phosphatase		
SGBXF1_01578	1694640	1695566	1	putative glycosyl transferase		
SGBXF1_01579	1695581	1696399	1	N-acetylglucosaminyl-diphospho-decaprenol L-rhamnosyltransferase		
SGBXF1_01580	1696469	1696612	1	hypothetical protein		
SGBXF1_01758	1892516	1893679	-1	Tyrosine recombinase XerD	Phage	**GI7**
SGBXF1_01759	1894061	1894729	-1	hypothetical protein		
SGBXF1_01760	1894726	1894908	-1	hypothetical protein		
SGBXF1_01761	1894924	1895313	-1	hypothetical protein		
SGBXF1_01762	1895772	1896029	-1	hypothetical protein		
SGBXF1_01763	1896088	1896453	-1	hypothetical protein		
SGBXF1_01764	1896450	1896629	-1	hypothetical protein		
SGBXF1_01765	1896632	1897036	-1	hypothetical protein		
SGBXF1_01766	1897036	1897245	-1	hypothetical protein		
SGBXF1_01767	1897242	1897670	-1	HNH endonuclease		
SGBXF1_01768	1897663	1898178	-1	hypothetical protein		
SGBXF1_01769	1898165	1898788	-1	hypothetical protein		
SGBXF1_01770	1898785	1899279	-1	hypothetical protein		
SGBXF1_01760	1894726	1894908	-1	hypothetical protein		
SGBXF1_01761	1894924	1895313	-1	hypothetical protein		
SGBXF1_01762	1895772	1896029	-1	hypothetical protein		
SGBXF1_01763	1896088	1896453	-1	hypothetical protein		
SGBXF1_01764	1896450	1896629	-1	hypothetical protein		
SGBXF1_01765	1896632	1897036	-1	hypothetical protein		
SGBXF1_01766	1897036	1897245	-1	hypothetical protein		
SGBXF1_01767	1897242	1897670	-1	HNH endonuclease		
SGBXF1_01768	1897663	1898178	-1	hypothetical protein		
SGBXF1_01769	1898165	1898788	-1	hypothetical protein		
SGBXF1_01770	1898785	1899279	-1	hypothetical protein		
SGBXF1_01771	1899282	1899425	-1	hypothetical protein		
SGBXF1_01772	1899419	1899637	-1	hypothetical protein		
SGBXF1_01773	1899918	1900016	-1	hypothetical protein		
SGBXF1_01774	1900019	1900195	-1	hypothetical protein		
SGBXF1_01775	1900232	1900570	-1	hypothetical protein		
SGBXF1_01776	1901061	1901204	1	hypothetical protein		
SGBXF1_01777	1901245	1901643	-1	hypothetical protein		
SGBXF1_01778	1901640	1902305	-1	hypothetical protein		
SGBXF1_01779	1902716	1903360	-1	putative HTH-type transcriptional regulator		

SGBXF1_01780	1903452	1903679	1	hypothetical protein		
SGBXF1_01781	1903695	1904021	1	Bacteriophage CII protein		
SGBXF1_01782	1904305	1905069	1	Phage antirepressor protein KilAC domain protein		
SGBXF1_01783	1905072	1905248	1	hypothetical protein		
SGBXF1_01784	1905245	1906267	1	hypothetical protein		
SGBXF1_01785	1906264	1907235	1	hypothetical protein		
SGBXF1_01786	1907586	1907984	1	Phage antitermination protein Q		
SGBXF1_01787	1908238	1908420	1	hypothetical protein		
SGBXF1_01788	1908464	1909096	1	hypothetical protein		
SGBXF1_01789	1909361	1909708	1	hypothetical protein		
SGBXF1_01790	1909979	1910332	1	hypothetical protein		
SGBXF1_01791	1910424	1910660	1	Lysis protein S		
SGBXF1_01792	1910663	1911148	1	Lysozyme RrrD		
SGBXF1_01793	1911145	1911519	1	hypothetical protein		
SGBXF1_01794	1912112	1912366	1	DNA polymerase III subunit theta		
SGBXF1_01795	1912330	1912446	-1	hypothetical protein		
SGBXF1_01796	1912519	1912638	1	hypothetical protein		
SGBXF1_01797	1912719	1913129	1	hypothetical protein		
SGBXF1_01798	1913229	1913906	1	hypothetical protein		
SGBXF1_01799	1913844	1914146	-1	hypothetical protein		
SGBXF1_01800	1914291	1914506	-1	hypothetical protein		
SGBXF1_01801	1914533	1915063	1	Terminase small subunit		
SGBXF1_01802	1915060	1916319	1	Phage terminase large subunit		
SGBXF1_01803	1916375	1916569	1	hypothetical protein		
SGBXF1_01804	1916625	1917953	1	hypothetical protein		
SGBXF1_01805	1917937	1918863	1	Phage Mu protein F like protein		
SGBXF1_01806	1918867	1920132	1	hypothetical protein		
SGBXF1_01816	1925504	1925713	1	hypothetical protein		
SGBXF1_01817	1925717	1926547	1	hypothetical protein		
SGBXF1_01818	1926695	1926844	1	hypothetical protein		
SGBXF1_01819	1926916	1927821	-1	hypothetical protein		
SGBXF1_01820	1927982	1928266	1	Arc-like DNA binding domain protein	-	**GI8**
SGBXF1_01821	1928376	1928657	1	hypothetical protein		
SGBXF1_01822	1928726	1931908	1	hypothetical protein		
SGBXF1_01823	1931911	1932534	1	hypothetical protein		
SGBXF1_02051	2151504	2152403	1	hypothetical protein		
SGBXF1_02052	2152657	2154159	1	hypothetical protein		
SGBXF1_02053	2154528	2154959	1	hypothetical protein		
SGBXF1_02054	2155014	2155643	1	hypothetical protein		
SGBXF1_02055	2155809	2156933	-1	hypothetical protein		
SGBXF1_02056	2156914	2157159	-1	Excisionase-like protein	-	**GI9**
SGBXF1_02057	2157159	2157656	-1	hypothetical protein		
SGBXF1_02058	2158036	2158524	1	hypothetical protein		
SGBXF1_02059	2158970	2159260	-1	hypothetical protein		
SGBXF1_02060	2159543	2159827	1	Acetyltransferase (GNAT) family protein		
SGBXF1_02639	2787422	2788144	1	ChaC-like protein		
SGBXF1_02640	2788248	2788712	1	SnoaL-like polyketide cyclase		
SGBXF1_02641	2788921	2789502	1	Bifunctional transcriptional activator/DNA repair enzyme Ada		
SGBXF1_02642	2789499	2790218	1	hypothetical protein		
SGBXF1_02643	2790221	2790871	1	Alpha-ketoglutarate-dependent dioxygenase AlkB		
SGBXF1_02644	2790947	2791141	1	hypothetical protein		
SGBXF1_02645	2791187	2791837	1	DNA-3-methyladenine glycosylase		
SGBXF1_02646	2791916	2792986	1	Bifunctional transcriptional activator/DNA repair enzyme Ada	-	**G10**
SGBXF1_02647	2793001	2793693	1	hypothetical protein		
SGBXF1_02648	2793710	2794015	1	hypothetical protein		
SGBXF1_02649	2794012	2794773	1	hypothetical protein		
SGBXF1_02650	2794989	2795411	1	LexA repressor		
SGBXF1_03207	3364807	3365022	1	hypothetical protein		
SGBXF1_03208	3365307	3366809	-1	Chaperone protein DnaJ		
SGBXF1_03209	3367295	3367891	-1	hypothetical protein		
SGBXF1_03210	3367977	3368654	1	HTH domain protein		
SGBXF1_03211	3368714	3369160	-1	universal stress protein UspC		
SGBXF1_03229	3389783	3390325	1	hypothetical protein		
SGBXF1_03230	3390383	3391300	-1	HTH-type transcriptional regulator DmlR	-	**G11**
SGBXF1_03231	3391405	3392613	1	4-hydroxybenzoate transporter PcaK		
SGBXF1_03232	3392797	3394392	-1	RNA polymerase sigma factor		

				RpoD		
SGBXF1_03233	3394994	3395920	1	hypothetical protein		
SGBXF1_03234	3396082	3396825	-1	Fatty acyl-CoA reductase		
SGBXF1_03235	3397021	3397482	-1	hypothetical protein		
SGBXF1_03236	3397878	3398270	-1	LexA repressor		
SGBXF1_03237	3398403	3398819	-1	hypothetical protein		
SGBXF1_03238	3398865	3400619	-1	Lipid A export ATP-binding/permease protein MsbA		
SGBXF1_03753	3970473	3970811	1	Outer membrane protein assembly factor BamE precursor	Phage	G12
SGBXF1_03754	3970926	3971210	-1	Persistence and stress-resistance antitoxin PasI		
SGBXF1_03755	3971191	3971637	-1	Ribosome association toxin RatA		
SGBXF1_03756	3971799	3972281	1	SsrA-binding protein		
SGBXF1_03758	3972777	3973016	-1	hypothetical protein		
SGBXF1_03759	3973723	3975378	-1	hypothetical protein		
SGBXF1_03760	3975375	3975935	-1	hypothetical protein		
SGBXF1_03761	3975910	3976632	-1	hypothetical protein		
SGBXF1_03762	3976622	3977170	-1	Caudovirales tail fiber assembly protein		
SGBXF1_03763	3977174	3980290	-1	Tail fiber protein		
SGBXF1_03764	3980296	3980901	-1	hypothetical protein		
SGBXF1_03765	3980894	3982078	-1	Baseplate J-like protein		
SGBXF1_03766	3982056	3982403	-1	hypothetical protein		
SGBXF1_03767	3982403	3984934	-1	Phage-related minor tail protein		
SGBXF1_03768	3985122	3985391	-1	hypothetical protein		
SGBXF1_03769	3985539	3985883	-1	hypothetical protein		
SGBXF1_03770	3985883	3986224	-1	hypothetical protein		
SGBXF1_03771	3986211	3986513	-1	Phage holin family 2		
SGBXF1_03772	3986523	3986978	-1	hypothetical protein		
SGBXF1_03773	3986975	3988099	-1	hypothetical protein		
SGBXF1_03774	3988096	3988806	-1	Phage virion morphogenesis family protein		
SGBXF1_03775	3988803	3989306	-1	P2 phage tail completion protein R (GpR)		
SGBXF1_03776	3989303	3989755	-1	Phage head completion protein (GPL)		
SGBXF1_03777	3989855	3990559	-1	Phage small terminase subunit		
SGBXF1_03778	3990566	3991582	-1	Phage major capsid protein, P2 family		
SGBXF1_03779	3991631	3992470	-1	Phage capsid scaffolding protein (GPO) serine peptidase		
SGBXF1_03780	3992780	3994417	1	Terminase-like family protein		
SGBXF1_03781	3994414	3995463	1	Phage portal protein		
SGBXF1_03782	3995514	3995786	1	Ogr/Delta-like zinc finger		
SGBXF1_03783	3995757	3995975	-1	hypothetical protein		
SGBXF1_03784	3996066	3998075	-1	Bacteriophage replication gene A protein (GPA)		
SGBXF1_03785	3998069	3998338	-1	hypothetical protein		
SGBXF1_03786	3998347	3998454	-1	hypothetical protein		
SGBXF1_03787	3998436	3998675	-1	hypothetical protein		
SGBXF1_03788	3998753	3999160	-1	hypothetical protein		
SGBXF1_03789	3999163	3999582	-1	hypothetical protein		
SGBXF1_03790	3999585	3999788	-1	hypothetical protein		
SGBXF1_03791	3999798	4000307	-1	Phage regulatory protein CII (CP76)		
SGBXF1_03792	4000340	4000600	-1	hypothetical protein		
SGBXF1_03793	4000757	4001320	1	Bacteriophage CI repressor helix-turn-helix domain protein		
SGBXF1_03794	4001324	4002391	1	Tyrosine recombinase XerD		
SGBXF1_03795	4002463	4002678	-1	hypothetical protein		
SGBXF1_03796	4002843	4003565	1	Prophage CP4-57 integrase		
SGBXF1_03797	4003935	4006655	1	hypothetical protein		
SGBXF1_03798	4006788	4006994	1	hypothetical protein		
SGBXF1_03799	4007347	4007694	-1	hypothetical protein		
SGBXF1_03800	4007887	4008021	-1	hypothetical protein		
SGBXF1_03801	4008067	4008762	1	hypothetical protein		
SGBXF1_03802	4009221	4009871	1	Metal-binding protein ZinT precursor		
SGBXF1_03792	4000340	4000600	-1	hypothetical protein		
SGBXF1_03793	4000757	4001320	1	Bacteriophage CI repressor helix-turn-helix domain protein	-	GI13
SGBXF1_03794	4001324	4002391	1	Tyrosine recombinase XerD		
SGBXF1_03795	4002463	4002678	-1	hypothetical protein		
SGBXF1_03796	4002843	4003565	1	Prophage CP4-57 integrase		

SGBXF1_03797	4003935	4006655	1	hypothetical protein		
SGBXF1_03798	4006788	4006994	1	hypothetical protein		
SGBXF1_03799	4007347	4007694	-1	hypothetical protein		
SGBXF1_03800	4007887	4008021	-1	hypothetical protein		
SGBXF1_03801	4008067	4008762	1	hypothetical protein		
SGBXF1_03802	4009221	4009871	1	Metal-binding protein ZinT precursor		
SGBXF1_03986	4202935	4203747	-1	Histidinol-phosphatase		
SGBXF1_03987	4204062	4204604	-1	NUDIX domain protein		
SGBXF1_03988	4204760	4205983	-1	2'-deamino-2'-hydroxyneamine transaminase		
SGBXF1_03989	4206002	4207042	-1	L-asparagine oxygenase		
SGBXF1_03990	4207749	4208918	1	Multidrug resistance protein MdtL		

Table S2- CAZymes families predicted.

Sequence ID	CAZy Families
SGBXF1_04203 Cell division protein FtsP precursor	AA1
SGBXF1_04098 Blue copper oxidase CueO precursor	AA1
SGBXF1_03554 GlcNAc-binding protein A precursor	AA10
SGBXF1_03212 Catalase-peroxidase	AA2
SGBXF1_01943 NADH dehydrogenase	AA3
SGBXF1_04535 Nitrite reductase [NAD(P)H]	AA3
SGBXF1_02157 Gluconate 2-dehydrogenase flavoprotein precursor	AA3
SGBXF1_00215 Anaerobic glycerol-3-phosphate dehydrogenase subunit A	AA3
SGBXF1_02793 D-amino acid dehydrogenase small subunit	AA3
SGBXF1_02368 Fructose dehydrogenase large subunit	AA3
SGBXF1_02080 Gamma-glutamylputrescine oxidoreductase	AA3
SGBXF1_03432 tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein MnmC	AA3
SGBXF1_03592 NADH dehydrogenase-like protein/MT1860	AA3
SGBXF1_01987 L-2-hydroxyglutarate oxidase LhgO	AA3
SGBXF1_04290 Glutamate synthase [NADPH] small chain	AA3
SGBXF1_04107 Dihydrolipoyl dehydrogenase	AA3
SGBXF1_04680 Soluble pyridine nucleotide transhydrogenase	AA3
SGBXF1_04061 Rhodocoxin reductase	AA3
SGBXF1_01737 Fructose dehydrogenase large subunit	AA3
SGBXF1_02474 4-methylaminobutanoate oxidase (formaldehyde-forming)	AA3
SGBXF1_04153 Oxygen-dependent choline dehydrogenase	AA3
SGBXF1_01906 N-methyl-L-tryptophan oxidase	AA3
SGBXF1_01848 Hydrogen cyanide synthase subunit HcnB	AA3
SGBXF1_01482 Oxygen-dependent choline dehydrogenase	AA3
SGBXF1_00504 Alcohol dehydrogenase [acceptor]	AA3
SGBXF1_04578 Aerobic glycerol-3-phosphate dehydrogenase	AA3
SGBXF1_00547 Gamma-glutamylputrescine oxidoreductase	AA3
SGBXF1_04683 Dihydrolipoyl dehydrogenase	AA3
SGBXF1_04644 Glutathione reductase	AA3
SGBXF1_01846 Hydrogen cyanide synthase subunit HcnC precursor	AA3
SGBXF1_01656 Thioredoxin reductase	AA3/CE10
SGBXF1_04258 2,4-dienoyl-CoA reductase [NADPH]	AA3/CE10
SGBXF1_02198 putative FAD-linked oxidoreductase	AA4/AA7
SGBXF1_01719 Outer membrane protein A precursor	AA5
SGBXF1_00611 Peptidoglycan-binding protein ArfA	AA5
SGBXF1_03020 Motility protein B	AA5
SGBXF1_01213 Peptidoglycan-associated lipoprotein precursor	AA5
SGBXF1_00067 putative lipoprotein YiaD precursor	AA5
SGBXF1_03905 Sulfite reductase [NADPH] flavoprotein alpha-component	AA6
SGBXF1_00039 FMN-dependent NADPH-azoreductase	AA6
SGBXF1_03180 Putative NAD(P)H-dependent FMN-containing oxidoreductase YwqN	AA6
SGBXF1_00493 Enamine/imine deaminase	AA6
SGBXF1_00014 Sulfite reductase [NADPH] flavoprotein alpha-component	AA6
SGBXF1_01961 Enamine/imine deaminase	AA6
SGBXF1_03997 Flavodoxin	AA6
SGBXF1_02256 Putative reactive intermediate deaminase TdcF	AA6
SGBXF1_02812 Putative aminoacrylate peracid reductase RutC	AA6
SGBXF1_00484 Enamine/imine deaminase	AA6
SGBXF1_01171 Flavodoxin	AA6
SGBXF1_03107 Putative reactive intermediate deaminase TdcF	AA6
SGBXF1_01704 FMN reductase (NADPH)	AA6
SGBXF1_01103 p-benzoquinone reductase	AA6
SGBXF1_01751 Putative aminoacrylate peracid reductase RutC	AA6
SGBXF1_00742 Sulfite reductase [NADPH] flavoprotein alpha-component	AA6/GH130
SGBXF1_00285 UDP-N-acetylenolpyruvoylglucosamine reductase	AA7
SGBXF1_03048 Catechol 1,2-dioxygenase	CBM12
SGBXF1_02520 Protocatechuate 3,4-dioxygenase alpha chain	CBM12
SGBXF1_02519 Protocatechuate 3,4-dioxygenase beta chain	CBM12

SGBXF1_00631 Chaperone protein DnaK	CBM13
SGBXF1_04357 Rod shape-determining protein MreB	CBM13
SGBXF1_03616 Chaperone protein DnaK	CBM13
SGBXF1_03691 Chaperone protein HscA	CBM13
SGBXF1_01901 Protein YceI	CBM2
SGBXF1_01912 Ribonuclease E	CBM20
SGBXF1_04353 Ribonuclease G	CBM20
SGBXF1_03660 Spermidine N(1)-acetyltransferase	CBM26
SGBXF1_03617 Acetyltransferase (GNAT) family protein	CBM26
SGBXF1_01872 Putative ribosomal N-acetyltransferase YdaF	CBM26
SGBXF1_01495 Putative ribosomal N-acetyltransferase YdaF	CBM26
SGBXF1_02324 hypothetical protein	CBM26
SGBXF1_02783 hypothetical protein	CBM26
SGBXF1_01454 Acetyltransferase (GNAT) family protein	CBM26
SGBXF1_00932 Acetyltransferase (GNAT) family protein	CBM26
SGBXF1_03470 Putative ribosomal N-acetyltransferase YdaF	CBM26
SGBXF1_02844 Putative ribosomal N-acetyltransferase YdaF	CBM26
SGBXF1_04161 Non-hemolytic phospholipase C precursor	CBM32
SGBXF1_01380 Non-hemolytic phospholipase C precursor	CBM32
SGBXF1_00974 Maltodextrin glucosidase	CBM34/GH13
SGBXF1_02944 putative kinase inhibitor	CBM35
SGBXF1_01247 putative kinase inhibitor protein	CBM35
SGBXF1_02134 Tail-specific protease precursor	CBM48
SGBXF1_03976 Curved DNA-binding protein	CBM48
SGBXF1_04582 Glycogen debranching enzyme	CBM48
SGBXF1_00632 Chaperone protein DnaJ	CBM48
SGBXF1_03692 Co-chaperone protein HscB	CBM48
SGBXF1_04237 CYTH domain protein	CBM48
SGBXF1_04583 1,4-alpha-glucan branching enzyme GlgB	CBM48/GH13
SGBXF1_01367 Viral enhancin protein	CBM5
SGBXF1_01620 Putrescine-binding periplasmic protein precursor	CBM50
SGBXF1_04143 2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor	CBM50
SGBXF1_01417 Lipoprotein E precursor	CBM50
SGBXF1_02561 Fe(3+)-binding periplasmic protein precursor	CBM50
SGBXF1_00532 putative L,D-transpeptidase YbiS precursor	CBM50
SGBXF1_00593 Osmotically-inducible protein Y precursor	CBM50
SGBXF1_04418 Maltose-binding periplasmic protein precursor	CBM50
SGBXF1_02207 putative L,D-transpeptidase YcfS precursor	CBM50
SGBXF1_03145 Phosphoglycerate transport regulatory protein PgtC precursor	CBM50
SGBXF1_01447 DNA protection during starvation protein	CBM50
SGBXF1_00250 sn-glycerol-3-phosphate-binding periplasmic protein UgpB precursor	CBM50
SGBXF1_04635 Trifunctional nucleotide phosphoesterase protein YfkN precursor	CBM50
SGBXF1_01325 molybdate ABC transporter periplasmic molybdate-binding protein	CBM50
SGBXF1_00028 Phosphate-binding protein PstS precursor	CBM50
SGBXF1_03924 N-acetylmuramoyl-L-alanine amidase AmiC precursor	CBM50
SGBXF1_00760 Murein hydrolase activator NlpD precursor	CBM50
SGBXF1_01242 Molybdate-binding periplasmic protein precursor	CBM50
SGBXF1_02661 murein peptide amidase A	CBM50
SGBXF1_02327 fec operon regulator FecR	CBM50
SGBXF1_04491 Bacterioferritin	CBM50
SGBXF1_02896 putative arabinose-binding protein precursor	CBM50
SGBXF1_02821 Murein DD-endopeptidase MepM	CBM50
SGBXF1_01066 Trifunctional nucleotide phosphoesterase protein YfkN precursor	CBM50
SGBXF1_03533 Thiosulfate-binding protein precursor	CBM50
SGBXF1_04713 Sulfate-binding protein precursor	CBM50
SGBXF1_01603 D-alanyl-D-alanine carboxypeptidase DacC precursor	CBM50
SGBXF1_02735 DNA protection during starvation protein	CBM50
SGBXF1_00969 Phosphate-binding protein PstS precursor	CBM50
SGBXF1_01964 Ferritin-1	CBM50
SGBXF1_02599 Putative ABC transporter substrate-binding protein YesO	CBM50
SGBXF1_04728 Murein hydrolase activator EnvC precursor	CBM50
SGBXF1_00395 2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor	CBM50
SGBXF1_00261 Tyrosine-protein phosphatase precursor	CBM50
SGBXF1_02333 putative ABC transporter-binding protein precursor	CBM50
SGBXF1_02426 sn-glycerol-3-phosphate-binding periplasmic protein UgpB precursor	CBM50
SGBXF1_00370 N-acetylmuramoyl-L-alanine amidase AmiB precursor	CBM50
SGBXF1_00330 Murein hydrolase activator NlpD precursor	CBM50
SGBXF1_00481 hypothetical protein	CBM54
SGBXF1_02511 Transglutaminase-like superfamily protein	CBM54
SGBXF1_04433 Type I phosphodiesterase / nucleotide pyrophosphatase	CBM6
SGBXF1_01126 Rare lipoprotein A precursor	CBM63
SGBXF1_03578 putative hydrolase	CE1
SGBXF1_01033 Metallo-beta-lactamase superfamily protein	CE1
SGBXF1_01693 putative metallo-hydrolase	CE1
SGBXF1_03836 putative quorum-quenching lactonase YtnP	CE1
SGBXF1_04154 Ferri-bacillibactin esterase BesA	CE1
SGBXF1_00042 Metallo-beta-lactamase superfamily protein	CE1
SGBXF1_00833 Hydroxyacylglutathione hydrolase	CE1
SGBXF1_03505 Enterochelin esterase	CE1

SGBXF1_01520 S-formylglutathione hydrolase YeiG	CE1
SGBXF1_01676 Hydroxyacylglutathione hydrolase	CE1
SGBXF1_03953 Metallo-beta-lactamase superfamily protein	CE1
SGBXF1_02568 Carboxylesterase NlhH	CE10
SGBXF1_01212 Protein TolB	CE10
SGBXF1_00843 L-lysine N6-monooxygenase	CE10
SGBXF1_02903 Carboxylesterase NlhH	CE10
SGBXF1_02394 Carboxylesterase NlhH	CE10
SGBXF1_03889 3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	CE11
SGBXF1_00663 RNA polymerase-associated protein RapA	CE11
SGBXF1_00439 ATP-dependent RNA helicase DeaD	CE11
SGBXF1_04765 ATP-dependent DNA helicase RecG	CE11
SGBXF1_01258 UvrABC system protein B	CE11
SGBXF1_01273 ATP-dependent RNA helicase RhlE	CE11
SGBXF1_00700 UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	CE11
SGBXF1_02627 ATP-dependent RNA helicase HrpB	CE11
SGBXF1_02853 ATP-dependent RNA helicase DbpA	CE11
SGBXF1_00208 ATP-dependent DNA helicase RecQ	CE11
SGBXF1_04696 Primosomal protein N'	CE11
SGBXF1_00169 ATP-dependent RNA helicase RhlB	CE11
SGBXF1_01716 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase	CE11
SGBXF1_02405 3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	CE11
SGBXF1_03293 UvrABC system protein B	CE11
SGBXF1_02028 Transcription-repair-coupling factor	CE11
SGBXF1_03744 ATP-dependent RNA helicase SrmB	CE11
SGBXF1_01435 Flavin reductase like domain protein	CE14
SGBXF1_01749 FMN reductase (NADH) RutF	CE14
SGBXF1_00572 4-hydroxyphenylacetate 3-monooxygenase reductase component	CE14
SGBXF1_00903 Flavin reductase like domain protein	CE14
SGBXF1_01372 Acetyl-/propionyl-coenzyme A carboxylase alpha chain	CE4
SGBXF1_02854 Phosphoribosylglycinamide formyltransferase 2	CE4
SGBXF1_01317 D-alanine--D-alanine ligase A	CE4
SGBXF1_00647 Carbamoyl-phosphate synthase large chain	CE4
SGBXF1_00316 Phosphoribosylamine--glycine ligase	CE4
SGBXF1_04093 Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase precursor	CE4
SGBXF1_01618 Ribosomal protein S6 modification protein	CE4
SGBXF1_03001 Basal-body rod modification protein FlgD	CE4
SGBXF1_00696 D-alanine--D-alanine ligase	CE4
SGBXF1_00860 Uric acid degradation bifunctional protein	CE4
SGBXF1_02335 Guanine deaminase	CE9
SGBXF1_00492 D-aminoacylase	CE9
SGBXF1_04322 Allantoinase	CE9
SGBXF1_02095 Imidazolonepropionase	CE9
SGBXF1_01910 Dihydroorotase	CE9
SGBXF1_00099 N-isopropylammelide isopropyl amidohydrolase	CE9
SGBXF1_01160 N-acetylglucosamine-6-phosphate deacetylase	CE9
SGBXF1_01035 Adenine deaminase 2	CE9
SGBXF1_04534 Cytosine deaminase	CE9
SGBXF1_01398 N-substituted formamide deformylase precursor	CE9
SGBXF1_00460 Alpha-D-ribose 1-methylphosphonate 5-triphosphate diphosphatase	CE9
SGBXF1_01161 Glucosamine-6-phosphate deaminase	CE9
SGBXF1_02110 Allantoinase	CE9
SGBXF1_01635 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	GH1
SGBXF1_01567 UDP-glucose 4-epimerase	GH1
SGBXF1_01082 UDP-glucose 4-epimerase	GH1
SGBXF1_01568 dTDP-glucose 4,6-dehydratase 2	GH1
SGBXF1_01168 UDP-glucose 4-epimerase	GH1
SGBXF1_01574 dTDP-4-dehydrorhamnose reductase	GH1
SGBXF1_02748 UDP-glucose 4-epimerase	GH1
SGBXF1_02359 Aryl-phospho-beta-D-glucosidase BglC	GH1
SGBXF1_02517 6-phospho-beta-glucosidase BglA	GH1
SGBXF1_00134 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	GH1
SGBXF1_04188 N,N'-diacetylchitobiose-specific phosphotransferase enzyme IIA component	GH1
SGBXF1_00138 6-phospho-beta-glucosidase BglB	GH1
SGBXF1_00771 6-phospho-beta-glucosidase GmuD	GH1
SGBXF1_04187 Aryl-phospho-beta-D-glucosidase BglC	GH1
SGBXF1_00176 dTDP-glucose 4,6-dehydratase 2	GH1
SGBXF1_02396 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	GH1
SGBXF1_01571 dTDP-4-dehydrorhamnose reductase	GH1
SGBXF1_01869 N,N'-diacetylchitobiose-specific phosphotransferase enzyme IIA component	GH1
SGBXF1_02937 GDP-6-deoxy-D-talose 4-dehydrogenase	GH1
SGBXF1_02919 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	GH1
SGBXF1_02308 Mannose-6-phosphate isomerase	GH1
SGBXF1_03476 Glucokinase	GH1
SGBXF1_04530 N,N'-diacetylchitobiose-specific phosphotransferase enzyme IIA component	GH1
SGBXF1_02231 hypothetical protein	GH10
SGBXF1_03172 NAD(P)H azoreductase	GH10
SGBXF1_04119 Protein SprT	GH10/CBM1
SGBXF1_03920 Membrane-bound lytic murein transglycosylase A precursor	GH102

SGBXF1_01229 Aldose 1-epimerase	GH103
SGBXF1_02802 Membrane-bound lytic murein transglycosylase B precursor	GH103
SGBXF1_02774 Putative glucose-6-phosphate 1-epimerase	GH103
SGBXF1_03463 Membrane-bound lytic murein transglycosylase B precursor	GH103
SGBXF1_02842 Putative oxidoreductase YceM	GH109
SGBXF1_02282 putative oxidoreductase YdgJ	GH109
SGBXF1_02603 Putative oxidoreductase YteT precursor	GH109
SGBXF1_04594 Inositol 2-dehydrogenase/D-chiro-inositol 3-dehydrogenase	GH109
SGBXF1_01397 Glucose--fructose oxidoreductase precursor	GH109
SGBXF1_03415 Thiol:disulfide interchange protein DsbD precursor	GH109
SGBXF1_03220 putative oxidoreductase YdgJ	GH109
SGBXF1_02409 Inositol 2-dehydrogenase	GH109
SGBXF1_00341 Thiol:disulfide interchange protein DsbD precursor	GH109
SGBXF1_02069 Glucose-6-phosphate 3-dehydrogenase	GH109
SGBXF1_04261 1,5-anhydro-D-fructose reductase	GH109
SGBXF1_03928 Protease 3 precursor	GH12
SGBXF1_02598 hypothetical protein	GH123
SGBXF1_03064 hypothetical protein	GH129
SGBXF1_00061 Alpha-amylase precursor	GH13
SGBXF1_02538 Oligo-1,6-glucosidase	GH13
SGBXF1_00991 Riboflavin biosynthesis protein RibD	GH13
SGBXF1_03721 tRNA-specific adenosine deaminase	GH13
SGBXF1_01337 Alpha-amylase precursor	GH13
SGBXF1_03544 transport protein TonB	GH13
SGBXF1_02729 Filamentous hemagglutinin	GH13
SGBXF1_00474 Trehalose-6-phosphate hydrolase	GH13
SGBXF1_01534 Cytidine deaminase	GH13
SGBXF1_03051 Benzoate 1,2-dioxygenase electron transfer component	GH130
SGBXF1_00879 Na(+)-translocating NADH-quinone reductase subunit F	GH130
SGBXF1_04709 Ferredoxin--NADP reductase	GH130
SGBXF1_01640 NADH oxidoreductase hcr	GH130
SGBXF1_00277 NAD(P)H-flavin reductase	GH130
SGBXF1_03094 1,2-phenylacetyl-CoA epoxidase, subunit E	GH130
SGBXF1_03704 Flavohemoprotein	GH130
SGBXF1_01178 KDP operon transcriptional regulatory protein KdpE	GH16
SGBXF1_02620 Transcriptional regulatory protein RstA	GH16
SGBXF1_02040 Transcriptional regulatory protein PhoP	GH16
SGBXF1_00967 Phosphate regulon transcriptional regulatory protein PhoB	GH16
SGBXF1_04010 Swarming motility regulation protein RssB	GH16
SGBXF1_04717 Alkaline phosphatase synthesis transcriptional regulatory protein PhoP	GH16
SGBXF1_03878 Transcriptional activator CadC	GH16
SGBXF1_03405 Transcriptional regulatory protein tctD	GH16
SGBXF1_04089 Transcriptional regulatory protein QseB	GH16
SGBXF1_02938 Heme response regulator HssR	GH16
SGBXF1_01466 DNA-binding transcriptional activator CadC	GH16
SGBXF1_01464 Transcriptional activator protein CzcR	GH16
SGBXF1_03127 DNA-binding transcriptional activator CadC	GH16
SGBXF1_04561 Transcriptional regulatory protein OmpR	GH16
SGBXF1_01474 Transcriptional regulatory protein YycF	GH16
SGBXF1_03526 Transcriptional regulatory protein OmpR	GH16
SGBXF1_03625 Transcriptional regulatory protein BaeR	GH16
SGBXF1_00620 Aerobic respiration control protein ArcA	GH16
SGBXF1_02559 Transcriptional regulatory protein QseB	GH16
SGBXF1_04000 Transcriptional regulatory protein CreB	GH16
SGBXF1_00420 Transcriptional regulatory protein BasR	GH16
SGBXF1_00962 Transcriptional regulatory protein OmpR	GH16
SGBXF1_02400 putative symporter YjmB	GH17
SGBXF1_04682 Hybrid peroxiredoxin hyPrx5	GH18
SGBXF1_01157 Chitinase D precursor	GH18
SGBXF1_00507 Valine--tRNA ligase	GH18
SGBXF1_00637 Isoleucine--tRNA ligase	GH18
SGBXF1_00976 putative peroxiredoxin	GH18
SGBXF1_04072 Thiol-disulfide oxidoreductase YkuV	GH18
SGBXF1_02770 Chitinase A1 precursor	GH18
SGBXF1_03552 Chitinase B precursor	GH18
SGBXF1_01134 Leucine--tRNA ligase	GH18
SGBXF1_03448 Thiol:disulfide interchange protein DsbE	GH18
SGBXF1_01539 Methionine--tRNA ligase	GH18
SGBXF1_02663 Thiol peroxidase	GH18
SGBXF1_03585 Putative peroxiredoxin bcp	GH18
SGBXF1_00148 Chitinase A precursor	GH18
SGBXF1_04307 Inner membrane protein YrbG	GH18
SGBXF1_03550 hypothetical protein	GH19
SGBXF1_03205 Beta-galactosidase	GH2
SGBXF1_01999 Evolved beta-galactosidase subunit alpha	GH2
SGBXF1_02721 Tryptophan synthase alpha chain	GH2
SGBXF1_03268 Endonuclease 4	GH20
SGBXF1_01166 Chitobiase precursor	GH20
SGBXF1_04601 Xylose isomerase-like TIM barrel	GH20

SGBXF1_02570 Inosose dehydratase	GH20
SGBXF1_04592 Inosose dehydratase	GH20
SGBXF1_04593 Xylose isomerase-like TIM barrel	GH20
SGBXF1_02410 Putative hydroxypyruvate isomerase YgbM	GH20
SGBXF1_01456 Putative hydroxypyruvate isomerase YgbM	GH20
SGBXF1_04038 L-ribulose-5-phosphate 3-epimerase UlaE	GH20
SGBXF1_03648 Xylose isomerase-like TIM barrel	GH20
SGBXF1_03219 Xylose isomerase-like TIM barrel	GH20
SGBXF1_02068 Inosose dehydratase	GH20
SGBXF1_00479 Beta-hexosaminidase	GH20
SGBXF1_01936 Cystine-binding periplasmic protein precursor	GH23
SGBXF1_04191 Endo-type membrane-bound lytic murein transglycosylase A precursor	GH23
SGBXF1_01141 Glutamate/aspartate periplasmic-binding protein precursor	GH23
SGBXF1_01318 Lysine-arginine-ornithine-binding periplasmic protein precursor	GH23
SGBXF1_00033 ABC transporter glutamine-binding protein GlnH precursor	GH23
SGBXF1_02967 Cystine-binding periplasmic protein precursor	GH23
SGBXF1_02155 Endo-type membrane-bound lytic murein transglycosylase A precursor	GH23
SGBXF1_04383 Membrane-bound lytic murein transglycosylase F precursor	GH23
SGBXF1_03720 Membrane-bound lytic murein transglycosylase F precursor	GH23
SGBXF1_02418 Lysine-arginine-ornithine-binding periplasmic protein precursor	GH23
SGBXF1_03391 Lysine-arginine-ornithine-binding periplasmic protein precursor	GH23
SGBXF1_03767 Phage-related minor tail protein	GH23
SGBXF1_01802 Phage terminase large subunit	GH23
SGBXF1_02433 Cystine-binding periplasmic protein precursor	GH23
SGBXF1_01703 Putative aliphatic sulfonates-binding protein precursor	GH23
SGBXF1_01629 Putative ABC transporter arginine-binding protein 2 precursor	GH23
SGBXF1_00832 Membrane-bound lytic murein transglycosylase D precursor	GH23
SGBXF1_00614 Soluble lytic murein transglycosylase precursor	GH23
SGBXF1_03244 Lysine-arginine-ornithine-binding periplasmic protein precursor	GH23
SGBXF1_01626 ABC transporter arginine-binding protein 1 precursor	GH23
SGBXF1_04394 Cyclohexadienyl dehydratase precursor	GH23
SGBXF1_01444 Glutamine-binding periplasmic protein precursor	GH23
SGBXF1_00854 Cystine-binding periplasmic protein precursor	GH23
SGBXF1_04138 Membrane-bound lytic murein transglycosylase C precursor	GH23
SGBXF1_00753 Glutamine-binding periplasmic protein precursor	GH23
SGBXF1_01224 Autoinducer 2 sensor kinase/phosphatase LuxQ	GH23/GT2
SGBXF1_01792 Lysozyme RrrD	GH24
SGBXF1_03316 Lysozyme RrrD	GH24
SGBXF1_01692 Peptidase M15	GH24
SGBXF1_04197 Lysozyme RrrD	GH24
SGBXF1_04704 Glycerol uptake facilitator protein	GH27
SGBXF1_00936 Aquaporin Z	GH27
SGBXF1_02342 Beta-lactamase	GH3
SGBXF1_01321 Periplasmic beta-glucosidase precursor	GH3
SGBXF1_03730 Holo-[acyl-carrier-protein] synthase	GH3
SGBXF1_02302 Beta-lactamase	GH3
SGBXF1_01941 Beta-hexosaminidase	GH3
SGBXF1_00573 D-alanyl-D-alanine-carboxypeptidase/endopeptidase AmpH precursor	GH3
SGBXF1_00945 Shikimate kinase 2	GH3
SGBXF1_02381 D-alanyl-D-alanine dipeptidase	GH3
SGBXF1_04545 Shikimate kinase 1	GH3
SGBXF1_02306 Esterase EstB	GH3
SGBXF1_03195 4'-phosphopantetheinyl transferase Npt	GH3
SGBXF1_02399 Thermostable beta-glucosidase B	GH3
SGBXF1_02093 D-aminopeptidase	GH3
SGBXF1_03340 Tyrosine-specific transport protein	GH31
SGBXF1_04253 Tyrosine-specific transport protein	GH31
SGBXF1_03277 Tryptophan-specific transport protein	GH31
SGBXF1_03666 Alpha-xylosidase	GH31
SGBXF1_01069 TraB family protein	GH31
SGBXF1_01871 Lichenan permease IIC component	GH32
SGBXF1_02002 Pseudouridine kinase	GH32
SGBXF1_03725 PTS system EIIBC component	GH32
SGBXF1_01928 PTS system glucose-specific EIICB component	GH32
SGBXF1_02330 putative sugar kinase YdjH	GH32
SGBXF1_00475 PTS system trehalose-specific EIIBC component	GH32
SGBXF1_04175 Sucrose-6-phosphate hydrolase	GH32
SGBXF1_01063 Inosine-guanosine kinase	GH32
SGBXF1_04193 pfkB family carbohydrate kinase	GH32
SGBXF1_02245 Pyridoxamine kinase	GH32
SGBXF1_01162 PTS system glucose-specific EIICBA component	GH32
SGBXF1_00004 Ribokinase	GH32
SGBXF1_00770 N,N'-diacetylchitobiose permease IIC component	GH32
SGBXF1_04172 2-dehydro-3-deoxygluconokinase	GH32
SGBXF1_00137 PTS system beta-glucoside-specific EIIBCA component	GH32
SGBXF1_04596 5-dehydro-2-deoxygluconokinase	GH32
SGBXF1_00064 2-dehydro-3-deoxygluconokinase	GH32
SGBXF1_00961 putative sugar kinase YdjH	GH32
SGBXF1_00075 PTS system mannitol-specific EIICBA component	GH32

SGBXF1_03270 Tagatose-6-phosphate kinase	GH32
SGBXF1_02358 PTS system beta-glucoside-specific EIIBCA component	GH32
SGBXF1_04189 Lichenan permease IIC component	GH32
SGBXF1_02299 PTS system maltose- and glucose-specific EIICB component	GH32
SGBXF1_04174 Negative regulator of SacY activity	GH32
SGBXF1_02100 Sucrose-6-phosphate hydrolase	GH32
SGBXF1_04661 2-dehydro-3-deoxygluconokinase	GH32
SGBXF1_04235 Bifunctional protein HldE	GH32/GT9
SGBXF1_01843 4-hydroxy-tetrahydrodipicolinate synthase	GH33
SGBXF1_02247 4-hydroxy-tetrahydrodipicolinate synthase	GH33
SGBXF1_03583 4-hydroxy-tetrahydrodipicolinate synthase	GH33
SGBXF1_03279 Mannonate dehydratase	GH35
SGBXF1_00531 Right origin-binding protein	GH39
SGBXF1_02566 HTH-type transcriptional activator Btr	GH39
SGBXF1_02412 Regulatory protein PchR	GH39
SGBXF1_01844 HTH-type transcriptional regulator ChbR	GH39
SGBXF1_03150 HTH-type transcriptional regulator CdhR	GH39
SGBXF1_01948 Right origin-binding protein	GH39
SGBXF1_02975 Urease operon transcriptional activator	GH39
SGBXF1_04332 HTH-type transcriptional repressor of iron proteins A	GH39
SGBXF1_03402 HTH-type transcriptional repressor of iron proteins A	GH39
SGBXF1_02281 Arabinose operon regulatory protein	GH39
SGBXF1_02271 Right origin-binding protein	GH39
SGBXF1_02398 HTH-type transcriptional activator Btr	GH39
SGBXF1_02625 HTH-type transcriptional repressor of iron proteins A	GH39
SGBXF1_03615 DNA-3-methyladenine glycosylase 2	GH39
SGBXF1_00618 Right origin-binding protein	GH39
SGBXF1_02372 Transposon Tn10 TetD protein	GH39
SGBXF1_02882 Regulatory protein SoxS	GH39
SGBXF1_00909 Transcriptional activator FeaR	GH39
SGBXF1_03818 HTH-type transcriptional activator RhaR	GH39
SGBXF1_01890 HTH-type transcriptional regulator GadX	GH39
SGBXF1_00570 HTH-type transcriptional activator Btr	GH39
SGBXF1_02261 Transcriptional activator FeaR	GH39
SGBXF1_00946 HTH-type transcriptional repressor of iron proteins A	GH39
SGBXF1_04184 HTH-type transcriptional activator RhaS	GH39
SGBXF1_01334 HTH-type transcriptional activator RhaR	GH39
SGBXF1_01491 Right origin-binding protein	GH39
SGBXF1_03981 HTH-type transcriptional activator RhaS	GH39
SGBXF1_00772 HTH-type transcriptional regulator ChbR	GH39
SGBXF1_03133 Bifunctional transcriptional activator/DNA repair enzyme AdaA	GH39
SGBXF1_02646 Bifunctional transcriptional activator/DNA repair enzyme Ada	GH39
SGBXF1_00529 HTH-type transcriptional repressor of iron proteins A	GH39
SGBXF1_01298 HTH-type transcriptional regulator CdhR	GH39
SGBXF1_04117 Right origin-binding protein	GH39
SGBXF1_02540 Alpha-glucosidase	GH4
SGBXF1_03132 Alpha-galactosidase	GH4
SGBXF1_01870 putative 6-phospho-beta-glucosidase	GH4
SGBXF1_04531 putative 6-phospho-beta-glucosidase	GH4
SGBXF1_04774 Glutamine synthetase	GH5
SGBXF1_00526 Gamma-glutamylputrescine synthetase PuuA	GH5
SGBXF1_03255 Gamma-glutamylputrescine synthetase PuuA	GH5
SGBXF1_02084 Gamma-glutamylputrescine synthetase PuuA	GH5
SGBXF1_02027 Diguanylate cyclase DosC	GH53
SGBXF1_02411 D-galactose-binding periplasmic protein precursor	GH53
SGBXF1_03946 HTH-type transcriptional regulator GalR	GH53
SGBXF1_00129 HTH-type transcriptional regulator DegA	GH53
SGBXF1_00477 HTH-type transcriptional regulator TreR	GH53
SGBXF1_02901 putative HTH-type transcriptional repressor ExuR	GH53
SGBXF1_04697 HTH-type transcriptional repressor CytR	GH53
SGBXF1_01528 HTH-type transcriptional regulator GalS	GH53
SGBXF1_02067 Catabolite control protein A	GH53
SGBXF1_04588 HTH-type transcriptional regulator GntR	GH53
SGBXF1_03668 Ribose operon repressor	GH53
SGBXF1_00003 Ribose operon repressor	GH53
SGBXF1_01538 Periplasmic binding proteins and sugar binding domain of LacI family protein	GH53
SGBXF1_03948 HTH-type transcriptional regulator AscG	GH53
SGBXF1_01529 D-galactose-binding periplasmic protein precursor	GH53
SGBXF1_01986 D-ribose-binding periplasmic protein precursor	GH53
SGBXF1_04358 RNase E specificity factor CsrD	GH53
SGBXF1_04229 Maltose regulon regulatory protein MalI	GH53
SGBXF1_01104 putative diguanylate cyclase YdaM	GH53
SGBXF1_04031 hypothetical protein	GH53
SGBXF1_00582 putative diguanylate cyclase YcdT	GH53
SGBXF1_03221 HTH-type transcriptional repressor PurR	GH53
SGBXF1_00958 D-ribose-binding periplasmic protein precursor	GH53
SGBXF1_04176 Catabolite repressor/activator	GH53
SGBXF1_01119 putative diguanylate cyclase AdrA	GH53
SGBXF1_01698 Response regulator PleD	GH53

SGBXF1_04629 Autoinducer 2-binding protein LsrB precursor	GH53
SGBXF1_00612 putative diguanylate cyclase YfiN	GH53
SGBXF1_03353 HTH-type transcriptional regulator GntR	GH53
SGBXF1_02099 HTH-type transcriptional repressor CytR	GH53
SGBXF1_00005 D-ribose-binding periplasmic protein precursor	GH53
SGBXF1_03144 HTH-type transcriptional repressor CytR	GH53
SGBXF1_02533 putative diguanylate cyclase YcdT	GH53
SGBXF1_03264 Diguanylate cyclase DosC	GH53
SGBXF1_00683 Catabolite repressor/activator	GH53
SGBXF1_00163 Phytochrome-like protein cph2	GH53
SGBXF1_03613 putative diguanylate cyclase YegE	GH53
SGBXF1_01998 HTH-type transcriptional regulator LacR	GH53
SGBXF1_01983 D-galactose-binding periplasmic protein precursor	GH53
SGBXF1_04532 HTH-type transcriptional regulator GalS	GH53
SGBXF1_04412 Catabolite control protein A	GH53
SGBXF1_04139 Phytochrome-like protein cph2	GH53
SGBXF1_02300 Maltose regulon regulatory protein MalI	GH53
SGBXF1_02220 HTH-type transcriptional repressor PurR	GH53
SGBXF1_03086 Phytochrome-like protein cph2	GH53
SGBXF1_00066 HTH-type transcriptional regulator KdgR	GH53
SGBXF1_03684 HTH-type transcriptional regulator GalS	GH53
SGBXF1_02278 L-arabinose-binding periplasmic protein precursor	GH53
SGBXF1_04653 Cyclic di-GMP phosphodiesterase Gmr	GH53
SGBXF1_02436 Cyclic di-GMP phosphodiesterase Gmr	GH53
SGBXF1_00104 Xylose operon regulatory protein	GH53/GH39
SGBXF1_02445 putative diguanylate cyclase YegE	GH53/GT5
SGBXF1_03037 Cupin superfamily protein	GH55
SGBXF1_02144 2-deoxyglucose-6-phosphate phosphatase	GH65
SGBXF1_04772 Alpha-D-glucose-1-phosphate phosphatase YihX	GH65
SGBXF1_00036 6-phosphogluconate phosphatase	GH65
SGBXF1_04540 Phosphoglycolate phosphatase	GH65
SGBXF1_00604 Phosphoserine phosphatase	GH65
SGBXF1_00200 Flavin mononucleotide phosphatase YigB	GH65
SGBXF1_03378 Sugar phosphatase YfbT	GH65
SGBXF1_01070 Copper-exporting P-type ATPase A	GH65
SGBXF1_04554 GMP/IMP nucleotidase YrfG	GH65
SGBXF1_01158 Ribonucleotide monophosphatase NagD	GH65
SGBXF1_01577 ?-D-glucose-1-phosphatase	GH65
SGBXF1_00478 Magnesium-transporting ATPase, P-type 1	GH65
SGBXF1_03062 Magnesium-transporting ATPase, P-type 1	GH65
SGBXF1_00866 Enolase-phosphatase E1	GH65
SGBXF1_02141 Phosphonoacetaldehyde hydrolase	GH65
SGBXF1_00784 Fructose-1-phosphate phosphatase YqaB	GH65
SGBXF1_01181 Potassium-transporting ATPase B chain	GH65
SGBXF1_00222 Lead, cadmium, zinc and mercury-transporting ATPase	GH65
SGBXF1_04565 Ferrous iron transport protein B	GH72
SGBXF1_03673 GTPase Der	GH72
SGBXF1_00043 tRNA modification GTPase MnmE	GH72
SGBXF1_00374 GTPase HflX	GH72
SGBXF1_03733 GTPase Era	GH72
SGBXF1_02011 Ribosome-binding ATPase YchF	GH72
SGBXF1_00418 GTPase ObgE/CgtA	GH72
SGBXF1_04780 putative GTP-binding protein EngB	GH72
SGBXF1_01924 putative aminodeoxychorismate lyase	GH73
SGBXF1_02995 Peptidoglycan hydrolase FlgJ	GH73
SGBXF1_03630 putative zinc-type alcohol dehydrogenase-like protein YjmD	GH76
SGBXF1_04397 Quinone oxidoreductase 1	GH76
SGBXF1_01350 Quinone oxidoreductase 1	GH76
SGBXF1_04388 Aldehyde reductase YahK	GH76
SGBXF1_03351 L-idonate 5-dehydrogenase (NAD(P)(+))	GH76
SGBXF1_01978 Aryl-alcohol dehydrogenase	GH76
SGBXF1_04183 Alcohol dehydrogenase	GH76
SGBXF1_02660 Zinc-type alcohol dehydrogenase-like protein	GH76
SGBXF1_02428 Alcohol dehydrogenase 1	GH76
SGBXF1_04730 L-threonine 3-dehydrogenase	GH76
SGBXF1_01521 S-(hydroxymethyl)glutathione dehydrogenase	GH76
SGBXF1_03026 NADPH-dependent curcumin reductase	GH76
SGBXF1_03075 Phthiocerol synthesis polyketide synthase type I PpsC	GH76
SGBXF1_04571 4-alpha-glucanotransferase	GH77
SGBXF1_00162 Cellulose synthase operon protein C precursor	GH8
SGBXF1_00161 Endoglucanase precursor	GH8
SGBXF1_03039 putative peptidase	GH84
SGBXF1_03901 Methionine aminopeptidase	GH84
SGBXF1_00447 Methionine aminopeptidase 1, mitochondrial	GH84
SGBXF1_01315 Methionine aminopeptidase	GH84
SGBXF1_00281 Xaa-Pro dipeptidase	GH84
SGBXF1_04019 Xaa-Pro aminopeptidase	GH84
SGBXF1_02026 hypothetical protein	GH9
SGBXF1_02509 hypothetical protein	GH9

SGBXF1_00557 D-beta-hydroxybutyrate dehydrogenase	GH92
SGBXF1_02079 Cyclopentanol dehydrogenase	GH92
SGBXF1_01410 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_02323 NADP-dependent 3-hydroxy acid dehydrogenase YdfG	GH92
SGBXF1_01393 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_04382 Cyclic-di-GMP-binding biofilm dispersal mediator protein	GH92
SGBXF1_00826 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_03638 Sorbitol-6-phosphate 2-dehydrogenase	GH92
SGBXF1_01355 Acetoacetyl-CoA reductase	GH92
SGBXF1_03494 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	GH92
SGBXF1_03052 2-(R)-hydroxypropyl-CoM dehydrogenase	GH92
SGBXF1_00579 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	GH92
SGBXF1_00767 Glucose 1-dehydrogenase 1	GH92
SGBXF1_00136 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_03834 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_02710 putative oxidoreductase YciK	GH92
SGBXF1_04057 7-alpha-hydroxysteroid dehydrogenase	GH92
SGBXF1_00559 (S)-1-Phenylethanol dehydrogenase	GH92
SGBXF1_03083 Glucose 1-dehydrogenase	GH92
SGBXF1_02440 C-factor	GH92
SGBXF1_00575 2-keto-3-deoxy-L-fuconate dehydrogenase	GH92
SGBXF1_03352 Gluconate 5-dehydrogenase	GH92
SGBXF1_01934 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_01920 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_04064 Pyridoxal 4-dehydrogenase	GH92
SGBXF1_04007 3-oxoacyl-[acyl-carrier-protein] reductase FabG	GH92
SGBXF1_04437 Acetate operon repressor	GH93
SGBXF1_02138 Pectin degradation repressor protein KdgR	GH93
SGBXF1_03041 Pca regulon regulatory protein	GH93
SGBXF1_02528 Pca regulon regulatory protein	GH93
SGBXF1_00949 Transcriptional regulator KdgR	GH93
SGBXF1_01276 HTH-type transcriptional regulator SrpS	GH93
SGBXF1_04063 Pca regulon regulatory protein	GH93
SGBXF1_04028 HTH-type transcriptional regulator YiaJ	GH93
SGBXF1_04761 Guanylate kinase	GH99
SGBXF1_03887 Lipid-A-disaccharide synthase	GT19
SGBXF1_04088 Sensor protein QseC	GT2
SGBXF1_00955 Blue-light-activated protein	GT2
SGBXF1_04287 Aerobic respiration control sensor protein ArcB	GT2
SGBXF1_01057 Chaperone protein HtpG	GT2
SGBXF1_03440 Phosphohistidine phosphatase SixA	GT2
SGBXF1_02039 Virulence sensor histidine kinase PhoQ	GT2
SGBXF1_01714 3-oxoacyl-[acyl-carrier-protein] synthase 2	GT2
SGBXF1_00819 Undecaprenyl-phosphate mannosyltransferase	GT2
SGBXF1_04524 Acetylornithine/succinyldiaminopimelate aminotransferase	GT2
SGBXF1_00160 Cyclic di-GMP-binding protein precursor	GT2
SGBXF1_02558 Sensor protein QseC	GT2
SGBXF1_03525 Sensor protein CpxA	GT2
SGBXF1_03431 3-oxoacyl-[acyl-carrier-protein] synthase 1	GT2
SGBXF1_03718 Sensor histidine kinase GlrK	GT2
SGBXF1_00824 3-oxoacyl-[acyl-carrier-protein] synthase 2	GT2
SGBXF1_01922 3-oxoacyl-[acyl-carrier-protein] synthase 2	GT2
SGBXF1_01289 hypothetical protein	GT2
SGBXF1_00827 3-oxoacyl-[acyl-carrier-protein] synthase 2	GT2
SGBXF1_00159 Cellulose synthase catalytic subunit [UDP-forming]	GT2
SGBXF1_02856 putative glycosyltransferase EpsJ	GT2
SGBXF1_03838 Sensor histidine kinase CitA	GT2
SGBXF1_01087 Cysteine--tRNA ligase	GT2
SGBXF1_02176 Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	GT2
SGBXF1_01251 Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	GT2
SGBXF1_01227 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	GT2
SGBXF1_03657 Sensor protein TorS	GT2
SGBXF1_01465 Sensor protein CzcS precursor	GT2
SGBXF1_01562 Putative O-antigen transporter	GT2
SGBXF1_03019 Chemotaxis protein CheA	GT2
SGBXF1_00419 Sensor protein BasS	GT2
SGBXF1_02536 hypothetical protein	GT2
SGBXF1_04775 Nitrogen regulation protein NR(II)	GT2
SGBXF1_04718 Sensor protein CpxA	GT2
SGBXF1_04255 4-aminobutyrate aminotransferase PuuE	GT2
SGBXF1_04737 putative glycosyltransferase EpsJ	GT2
SGBXF1_00985 Tetrathionate sensor histidine kinase TtrS	GT2
SGBXF1_00715 Glutamate-1-semialdehyde 2,1-aminomutase	GT2
SGBXF1_03472 Sensor histidine kinase YpdA	GT2
SGBXF1_04011 Swarming motility regulation sensor protein RssA	GT2
SGBXF1_03988 2'-deamino-2'-hydroxyneamine transaminase	GT2
SGBXF1_01179 Sensor protein KdpD	GT2
SGBXF1_00542 Omega-amino acid--pyruvate aminotransferase	GT2
SGBXF1_02927 putative glycosyltransferase EpsJ	GT2

SGBXF1_03331 Phosphotransferase RcsD	GT2
SGBXF1_00051 DNA gyrase subunit B	GT2
SGBXF1_02444 Diaminobutyrate--2-oxoglutarate aminotransferase	GT2
SGBXF1_03999 Sensor protein CreC	GT2
SGBXF1_00263 Alpha-ribazole phosphatase	GT2
SGBXF1_00968 Phosphate regulon sensor protein PhoR	GT2
SGBXF1_00724 Signal transduction histidine-protein kinase BarA	GT2
SGBXF1_02621 Sensor protein RstB	GT2
SGBXF1_04370 Lipid A biosynthesis lauroyl acyltransferase	GT2
SGBXF1_01919 Malonyl CoA-acyl carrier protein transacylase	GT2
SGBXF1_01578 putative glycosyl transferase	GT2
SGBXF1_02926 putative glycosyltransferase EpsJ	GT2
SGBXF1_04560 Osmolarity sensor protein EnvZ	GT2
SGBXF1_03624 Signal transduction histidine-protein kinase BaeS	GT2
SGBXF1_02940 Nitrate/nitrite sensor protein NarX	GT2
SGBXF1_02820 Lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase	GT2
SGBXF1_03404 Sensor protein QseC	GT2
SGBXF1_04646 Autoinducer 2 sensor kinase/phosphatase LuxQ	GT2
SGBXF1_02933 colanic acid exporter	GT2
SGBXF1_02929 Putative teichuronic acid biosynthesis glycosyltransferase TuaG	GT2
SGBXF1_03564 Nitrate/nitrite sensor protein NarX	GT2
SGBXF1_03333 Sensor histidine kinase RcsC	GT2
SGBXF1_04745 putative glycosyl transferase	GT2
SGBXF1_02932 hypothetical protein	GT2
SGBXF1_01473 putative sensor histidine kinase TcrY	GT2
SGBXF1_02749 UDP-glucose 6-dehydrogenase TuaD	GT2
SGBXF1_00175 UDP-N-acetyl-D-glucosamine 6-dehydrogenase	GT2
SGBXF1_02931 Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	GT2
SGBXF1_01898 Lipid A biosynthesis lauroyl acyltransferase	GT2
SGBXF1_02895 Succinylornithine transaminase	GT2
SGBXF1_00617 Phosphoserine phosphatase 1	GT2
SGBXF1_01981 Sensor histidine kinase DcuS	GT2
SGBXF1_01579 N-acetylglucosaminyl-diphospho-decaprenol L-rhamnosyltransferase	GT2
SGBXF1_04209 DNA topoisomerase 4 subunit B	GT2
SGBXF1_01885 Glucans biosynthesis glucosyltransferase H	GT2
SGBXF1_02064 Phthioceranic/hydroxyphthioceranic acid synthase	GT2/GH76/GT4
SGBXF1_02217 Cyclopropane-fatty-acyl-phospholipid synthase	GT2/GT4
SGBXF1_01075 Cyclopropane-fatty-acyl-phospholipid synthase	GT2/GT4
SGBXF1_00525 Alpha,alpha-trehalose-phosphate synthase [UDP-forming]	GT20
SGBXF1_02726 putative sulfate transporter/MT1781	GT26
SGBXF1_00183 UDP-N-acetyl-D-mannosaminuronic acid transferase	GT26
SGBXF1_04302 putative phospholipid ABC transporter-binding protein MlaB	GT26
SGBXF1_00689 UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	GT28
SGBXF1_00690 UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	GT28
SGBXF1_01127 Rod shape-determining protein RodA	GT28
SGBXF1_00407 UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	GT28
SGBXF1_00692 UDP-N-acetylmuramoylalanine--D-glutamate ligase	GT28
SGBXF1_00694 UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	GT28
SGBXF1_00693 Lipid II flippase FtsW	GT28
SGBXF1_00695 UDP-N-acetylmuramate--L-alanine ligase	GT28
SGBXF1_00174 UDP-N-acetylglucosamine 2-epimerase	GT28
SGBXF1_04135 tRNA (guanine-N(7)-)-methyltransferase	GT30
SGBXF1_01581 6-phosphogluconate dehydrogenase, decarboxylating	GT30
SGBXF1_01678 Tetraacyldisaccharide 4'-kinase	GT30
SGBXF1_04744 3-deoxy-D-manno-octulosonic acid transferase	GT30
SGBXF1_00980 Queuine tRNA-ribosyltransferase	GT34
SGBXF1_04579 Maltodextrin phosphorylase	GT35
SGBXF1_04572 Maltodextrin phosphorylase	GT35
SGBXF1_03674 Outer membrane protein assembly factor BamB precursor	GT39
SGBXF1_00528 Outer membrane protein assembly factor BamB	GT39
SGBXF1_02817 putative HTH-type transcriptional regulator YbbH	GT4
SGBXF1_03166 Inner membrane transport permease YbhS	GT4
SGBXF1_00026 Bifunctional protein GlmU	GT4
SGBXF1_04581 Glucose-1-phosphate adenylyltransferase	GT4
SGBXF1_00813 2-acyl-glycerophospho-ethanolamine acyltransferase	GT4
SGBXF1_03706 hypothetical protein	GT4
SGBXF1_02597 2-acyl-glycerophospho-ethanolamine acyltransferase	GT4
SGBXF1_01548 UTP--glucose-1-phosphate uridylyltransferase	GT4
SGBXF1_04372 Tyrocidine synthase 3	GT4
SGBXF1_04094 Inner membrane transport permease YadH	GT4
SGBXF1_04407 Diacylglycerol kinase	GT4
SGBXF1_01552 Glycosyl transferases group 1	GT4
SGBXF1_00081 putative methyltransferase YcgJ	GT4
SGBXF1_00777 Regulatory protein RecX	GT4
SGBXF1_04283 DnaA initiator-associating protein DiaA	GT4
SGBXF1_00266 Ubiquinone/menaquinone biosynthesis C-methyltransferase UbiE	GT4
SGBXF1_00211 Phospholipase YtpA	GT4
SGBXF1_01268 Inner membrane transport permease YbhR	GT4

SGBXF1_03807 Malonyl-[acyl-carrier protein] O-methyltransferase	GT4
SGBXF1_03045 3-oxoadipate enol-lactonase 2	GT4
SGBXF1_04741 Lipopolysaccharide core biosynthesis protein RfaG	GT4
SGBXF1_00871 Phosphoheptose isomerase	GT4
SGBXF1_00935 putative methyltransferase YcgJ	GT4
SGBXF1_01750 Putative aminoacrylate hydrolase RutD	GT4
SGBXF1_01569 Glucose-1-phosphate thymidylyltransferase 2	GT4
SGBXF1_01269 Inner membrane transport permease YbhS	GT4
SGBXF1_01962 Proline iminopeptidase	GT4
SGBXF1_02346 N-formylmaleamate deformylase	GT4
SGBXF1_00177 Glucose-1-phosphate thymidylyltransferase 2	GT4
SGBXF1_04568 Pimeloyl-[acyl-carrier protein] methyl ester esterase	GT4
SGBXF1_01554 D-inositol 3-phosphate glycosyltransferase	GT4
SGBXF1_01953 putative phosphatase YcdX	GT4
SGBXF1_02930 GalNAc-alpha-(1->4)-GalNAc-alpha-(1->3)-diNAcBac-PP-undecaprenol alpha-1,4-N-acetyl-D-galactosaminyltransferase	GT4
SGBXF1_04742 N-acetylgalactosamine-N,N'-diacetylbacillosaminyl-diphospho-undecaprenol 4-alpha-N-acetylgalactosaminyltransferase	GT4
SGBXF1_03885 DNA polymerase III subunit alpha	GT4
SGBXF1_02750 UTP--glucose-1-phosphate uridylyltransferase	GT4
SGBXF1_04515 putative hydrolase	GT4
SGBXF1_03165 Inner membrane transport permease YbhR	GT4
SGBXF1_04408 Glycerol-3-phosphate acyltransferase	GT4
SGBXF1_00403 Gamma-glutamylcyclotransferase family protein YtfP	GT4
SGBXF1_01575 Teichoic acid translocation permease protein TagG	GT4
SGBXF1_02367 Haloalkane dehalogenase	GT4
SGBXF1_01173 Esterase YbfF	GT4
SGBXF1_03503 Enterobactin synthase component F	GT4
SGBXF1_00549 Phospholipase YtpA	GT4
SGBXF1_02835 tRNA (mo5U34)-methyltransferase	GT4
SGBXF1_00916 Soluble epoxide hydrolase	GT4
SGBXF1_04204 1-acyl-sn-glycerol-3-phosphate acyltransferase	GT4
SGBXF1_01565 Mannose-1-phosphate guanylyltransferase 1	GT4
SGBXF1_02569 Soluble epoxide hydrolase	GT4
SGBXF1_03944 Bifunctional protein Aas	GT4
SGBXF1_01563 UDP-galactopyranose mutase	GT4
SGBXF1_01547 UTP--glucose-1-phosphate uridylyltransferase	GT4
SGBXF1_02074 Pentapeptide repeats (8 copies)	GT4
SGBXF1_03175 N-acyltransferase YncA	GT41
SGBXF1_03743 tRNA1(Val) (adenine(37)-N6)-methyltransferase	GT41
SGBXF1_00591 Mycothiol acetyltransferase	GT41
SGBXF1_00068 putative N-acetyltransferase YjaB	GT41
SGBXF1_01418 Mycothiol acetyltransferase	GT41
SGBXF1_01595 putative acetyltransferase	GT41
SGBXF1_00703 Protein translocase subunit SecA	GT41
SGBXF1_03855 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_01310 TDP-fucosamine acetyltransferase	GT41
SGBXF1_02492 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_03646 putative acetyltransferase	GT41
SGBXF1_00589 Ribosomal RNA small subunit methyltransferase C	GT41
SGBXF1_00178 dTDP-fucosamine acetyltransferase	GT41
SGBXF1_03808 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_03983 Spermine/spermidine acetyltransferase	GT41
SGBXF1_00913 putative N-acetyltransferase YycN	GT41
SGBXF1_01381 Protease synthase and sporulation negative regulatory protein PAI 1	GT41
SGBXF1_04574 putative acetyltransferase	GT41
SGBXF1_02042 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_01304 Phosphinothricin N-acetyltransferase	GT41
SGBXF1_00941 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_01341 MarR family protein	GT41
SGBXF1_04259 Ribosomal RNA large subunit methyltransferase G	GT41
SGBXF1_02534 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_00506 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_03538 Acetyltransferase YpeA	GT41
SGBXF1_04155 putative acetyltransferase	GT41
SGBXF1_01439 Mycothiol acetyltransferase	GT41
SGBXF1_03438 50S ribosomal protein L3 glutamine methyltransferase	GT41
SGBXF1_00301 Acetyltransferase (GNAT) family protein	GT41
SGBXF1_01416 Acetyltransferase	GT41
SGBXF1_04769 N-acetylglutamate synthase	GT41
SGBXF1_01968 Acetyltransferase (GNAT) family protein	GT41/CBM26
SGBXF1_03604 Spermidine N(1)-acetyltransferase	GT41/CBM26
SGBXF1_00378 Adenylosuccinate synthetase	GT47
SGBXF1_01837 Purine efflux pump PbuE	GT48
SGBXF1_04042 4-hydroxybenzoate transporter PcaK	GT48
SGBXF1_03253 Low-affinity putrescine importer PlaP	GT48
SGBXF1_04519 putative galactarate transporter	GT48
SGBXF1_04267 Hexuronate transporter	GT48
SGBXF1_00736 Proline-specific permease ProY	GT48

SGBXF1_02845 Multidrug resistance protein MdtH	GT48
SGBXF1_02734 Methyl viologen resistance protein SmvA	GT48
SGBXF1_02001 Inner membrane transporter YgjI	GT48
SGBXF1_00739 4-hydroxybenzoate transporter PcaK	GT48
SGBXF1_03858 Alpha-ketoglutarate permease	GT48
SGBXF1_01841 Aspartate-proton symporter	GT48
SGBXF1_00002 putative transport protein HsrA	GT48
SGBXF1_04656 Inner membrane metabolite transport protein YhjE	GT48
SGBXF1_02314 Inner membrane transport protein YnfM	GT48
SGBXF1_02494 D-serine/D-alanine/glycine transporter	GT48
SGBXF1_02791 Multidrug resistance protein MdtL	GT48
SGBXF1_04060 putative sulfoacetate transporter SauU	GT48
SGBXF1_02303 Inner membrane transport protein YdhP	GT48
SGBXF1_01537 Multidrug resistance protein MdtH	GT48
SGBXF1_02086 Putrescine importer PuuP	GT48
SGBXF1_03631 Hexuronate transporter	GT48
SGBXF1_01167 Sugar efflux transporter A	GT48
SGBXF1_04035 Putative tartrate transporter	GT48
SGBXF1_03291 Bicyclomycin resistance protein	GT48
SGBXF1_02515 Putative tartrate transporter	GT48
SGBXF1_00063 Putative tartrate transporter	GT48
SGBXF1_01010 muropeptide transporter	GT48
SGBXF1_00497 Arginine/ornithine antiporter	GT48
SGBXF1_04670 Putative sialic acid transporter	GT48
SGBXF1_04533 Protein TsgA	GT48
SGBXF1_01292 D-serine/D-alanine/glycine transporter	GT48
SGBXF1_00233 putative sulfoacetate transporter SauU	GT48
SGBXF1_00581 L-fucose-proton symporter	GT48
SGBXF1_02867 Multidrug resistance protein stp	GT48
SGBXF1_02402 Purine ribonucleoside efflux pump NepI	GT48
SGBXF1_03908 Putative niacin/nicotinamide transporter NaiP	GT48
SGBXF1_04600 D-xylose-proton symporter	GT48
SGBXF1_02395 Multidrug resistance protein stp	GT48
SGBXF1_01756 D-serine/D-alanine/glycine transporter	GT48
SGBXF1_00555 putative amino acid permease YhdG	GT48
SGBXF1_00119 Reverse transcriptase (RNA-dependent DNA polymerase)	GT48
SGBXF1_02874 Aromatic amino acid transport protein AroP	GT48
SGBXF1_03215 Putative multidrug resistance protein MdtD	GT48
SGBXF1_02325 putative transport protein HsrA	GT48
SGBXF1_03623 Putative multidrug resistance protein MdtD	GT48
SGBXF1_00673 Sugar efflux transporter A	GT48
SGBXF1_02617 Putative arginine/ornithine antiporter	GT48
SGBXF1_00453 Putrescine-ornithine antiporter	GT48
SGBXF1_01873 Proline/betaine transporter	GT48
SGBXF1_04707 Multidrug resistance protein D	GT48
SGBXF1_00071 Hexuronate transporter	GT48
SGBXF1_03990 Multidrug resistance protein MdtL	GT48
SGBXF1_02502 Multidrug resistance protein 3	GT48
SGBXF1_02319 Multidrug resistance protein 3	GT48
SGBXF1_01430 Multidrug resistance protein stp	GT48
SGBXF1_01664 putative MFS-type transporter YcaD	GT48
SGBXF1_03420 putative transporter	GT48
SGBXF1_04068 putative sulfoacetate transporter SauU	GT48
SGBXF1_04411 Proline/betaine transporter	GT48
SGBXF1_03845 Inner membrane transport protein YnfM	GT48
SGBXF1_01275 putative multidrug resistance protein EmrY	GT48
SGBXF1_02106 Proline/betaine transporter	GT48
SGBXF1_03821 putative transport protein HsrA	GT48
SGBXF1_04111 Aromatic amino acid transport protein AroP	GT48
SGBXF1_00146 Regulatory protein UhpC	GT48
SGBXF1_03667 Hexuronate transporter	GT48
SGBXF1_01896 Tetracycline resistance protein, class B	GT48
SGBXF1_03851 Multidrug export protein EmrB	GT48
SGBXF1_02525 4-hydroxybenzoate transporter PcaK	GT48
SGBXF1_02218 Inner membrane transport protein YdhC	GT48
SGBXF1_01065 Fosmidomycin resistance protein	GT48
SGBXF1_03951 Inner membrane transport protein YdhP	GT48
SGBXF1_03647 Inner membrane protein YihN	GT48
SGBXF1_00095 Multidrug transporter MdfA	GT48
SGBXF1_03546 Amino-acid permease RocC	GT48
SGBXF1_02339 Putative tartrate transporter	GT48
SGBXF1_01414 putative multidrug-efflux transporter/MT1670	GT48
SGBXF1_03105 putative transport protein YifK	GT48
SGBXF1_03275 Sugar efflux transporter B	GT48
SGBXF1_01004 Inner membrane transport protein YajR	GT48
SGBXF1_03877 putative cadaverine/lysine antiporter	GT48
SGBXF1_03214 Multidrug resistance protein stp	GT48
SGBXF1_00774 putative MFS-type transporter YcaD	GT48
SGBXF1_00540 putative amino acid permease YhdG	GT48

SGBXF1_00184 putative transport protein YifK	GT48
SGBXF1_00280 Inner membrane transport protein YdhC	GT48
SGBXF1_02526 4-hydroxybenzoate transporter PcaK	GT48
SGBXF1_03218 Sugar efflux transporter B	GT48
SGBXF1_00569 Putative tartrate transporter	GT48
SGBXF1_01330 Purine ribonucleoside efflux pump NepI	GT48
SGBXF1_00830 Inner membrane transport protein YdhP	GT48
SGBXF1_00971 Proline-specific permease ProY	GT48
SGBXF1_03970 Hexuronate transporter	GT48
SGBXF1_02137 Multidrug resistance protein stp	GT48
SGBXF1_02343 Putative metabolite transport protein NicT	GT48
SGBXF1_02376 Tetracycline resistance protein, class C	GT48
SGBXF1_00214 Glycerol-3-phosphate transporter	GT48
SGBXF1_03265 Lysine-specific permease	GT48
SGBXF1_04234 Inner membrane protein YjeH	GT48
SGBXF1_01426 Multidrug resistance protein stp	GT48
SGBXF1_00629 Proline/betaine transporter	GT48
SGBXF1_01834 Inner membrane transport protein RhmT	GT48
SGBXF1_03475 putative MFS-type transporter YhjX	GT48
SGBXF1_01081 Galactose-proton symporter	GT48
SGBXF1_03217 Inner membrane metabolite transport protein YdjE	GT48
SGBXF1_00329 Proton glutamate symport protein	GT49
SGBXF1_04264 Serine/threonine transporter SstT	GT49
SGBXF1_02142 L-cystine uptake protein TcyP	GT49
SGBXF1_00164 Aerobic C4-dicarboxylate transport protein	GT49
SGBXF1_04580 Glycogen synthase	GT5
SGBXF1_02464 Formate hydrogenlyase transcriptional activator	GT5
SGBXF1_01019 ComE operon protein 1	GT5
SGBXF1_03937 Phosphoenolpyruvate-protein phosphotransferase	GT5
SGBXF1_04563 30S ribosomal protein S1	GT5
SGBXF1_04080 Penicillin-binding protein 1B	GT51
SGBXF1_02389 Stage V sporulation protein D	GT51
SGBXF1_02390 Peptidoglycan synthase FtsI precursor	GT51
SGBXF1_04551 Penicillin-binding protein 1A	GT51
SGBXF1_04285 Penicillin-binding protein 2D	GT51
SGBXF1_01128 Stage V sporulation protein D	GT51
SGBXF1_00688 Peptidoglycan synthase FtsI precursor	GT51
SGBXF1_03682 Penicillin-binding protein 1F	GT51
SGBXF1_02586 Zinc transport protein ZntB	GT55
SGBXF1_02654 Zinc transport protein ZntB	GT55
SGBXF1_01594 Magnesium transport protein CorA	GT55
SGBXF1_00203 Magnesium transport protein CorA	GT55
SGBXF1_00181 4-alpha-L-fucosyltransferase	GT56
SGBXF1_00312 Endonuclease V	GT57
SGBXF1_01291 hypothetical protein	GT60
SGBXF1_04529 Peptidyl-prolyl cis-trans isomerase A precursor	GT66
SGBXF1_01086 Peptidyl-prolyl cis-trans isomerase B	GT66
SGBXF1_00032 Phosphate-specific transport system accessory protein PhoU	GT80
SGBXF1_01684 3-deoxy-manno-octulosonate cytidylyltransferase	GT82
SGBXF1_01095 hypothetical protein	GT83
SGBXF1_00908 Major phosphate-irrepressible acid phosphatase precursor	GT83
SGBXF1_01524 Major phosphate-irrepressible acid phosphatase precursor	GT83
SGBXF1_00607 hypothetical protein	GT83
SGBXF1_02702 Phosphatidylglycerophosphatase B	GT83
SGBXF1_03349 N,N'-diacetylchitobiose phosphorylase	GT84/GH94
SGBXF1_04213 ADP-ribose pyrophosphatase	GT87
SGBXF1_02858 putative Nudix hydrolase NudL	GT87
SGBXF1_02227 Isopentenyl-diphosphate Delta-isomerase	GT87
SGBXF1_04552 ADP compounds hydrolase NudE	GT87
SGBXF1_02047 Phosphatase NudJ	GT87
SGBXF1_03938 RNA pyrophosphohydrolase	GT87
SGBXF1_00310 NADH pyrophosphatase	GT87
SGBXF1_03558 GDP-mannose pyrophosphatase NudK	GT87
SGBXF1_03383 putative Nudix hydrolase YfcD	GT87
SGBXF1_03272 RNA pyrophosphohydrolase	GT87
SGBXF1_01403 Nucleoside triphosphatase NudI	GT87
SGBXF1_02761 CTP pyrophosphohydrolase	GT87
SGBXF1_02829 Dihydroneopterin triphosphate pyrophosphatase	GT87
SGBXF1_00704 8-oxo-dGTP diphosphatase	GT87
SGBXF1_00510 Lipopolysaccharide export system permease protein LptF	GT9
SGBXF1_04740 Lipopolysaccharide core heptosyltransferase RfaQ	GT9
SGBXF1_04743 Lipopolysaccharide core heptosyltransferase RfaQ	GT9
SGBXF1_04734 Lipopolysaccharide heptosyltransferase 1	GT9
SGBXF1_01131 Nicotinate-nucleotide adenylyltransferase	GT9
SGBXF1_00511 Lipopolysaccharide export system permease protein LptG	GT9
SGBXF1_04738 ADP-heptose--LPS heptosyltransferase 2	GT9
SGBXF1_04746 Phosphopantetheine adenylyltransferase	GT9
SGBXF1_04733 ADP-heptose--LPS heptosyltransferase 2	GT9
SGBXF1_03181 Lipopolysaccharide core heptosyltransferase RfaQ	GT9

SGBXF1_00170 Thioredoxin-1	GT90
SGBXF1_03853 Thioredoxin-2	GT90
SGBXF1_01076 Thioredoxin	GT90
SGBXF1_03413 Sporulation thiol-disulfide oxidoreductase A precursor	GT90/GH18

Table S3- Protease families predicted in the Merops database

Locus tag	**MEROPS family name**	MEROPS Reference	E value
SGBXF1_00638	A08	MER001313	1.20E-61
SGBXF1_02943	A24A	MER000870	3.90E-20
SGBXF1_02450	A31	MER002085	3.10E-60
SGBXF1_01193	C15	MER001424	9.10E-60
SGBXF1_03027	C15	MER001424	9.70E-51
SGBXF1_00646	C26	MER115185	1.60E-78
SGBXF1_00646	C26	MER436691	3.50E-05
SGBXF1_00727	C26	MER065554	1.30E-141
SGBXF1_01585	C26	MER065588	1.20E-93
SGBXF1_02083	C26	MER031278	1.40E-117
SGBXF1_02593	C26	MER043537	2.70E-05
SGBXF1_02717	C26	MER043392	5.40E-79
SGBXF1_03663	C26	MER045886	9.90E-53
SGBXF1_04262	C26	MER043475	2.90E-14
SGBXF1_04371	C26	MER437468	1.20E-19
SGBXF1_04525	C26	MER043394	4.40E-72
SGBXF1_02182	C40	MER002443	2.30E-56
SGBXF1_02223	C40	MER004054	6.10E-61
SGBXF1_03284	C40	MER004035	8.80E-56
SGBXF1_00027	C44	MER003327	1.90E-102
SGBXF1_01156	C44	MER034539	6.00E-112
SGBXF1_03393	C44	MER011806	9.00E-42
SGBXF1_04289	C44	MER198917	1.80E-82
SGBXF1_01001	C56	MER010992	6.00E-14
SGBXF1_01280	C56	MER031431	5.10E-15
SGBXF1_02497	C56	MER014721	4.30E-51
SGBXF1_03719	C56	MER042827	3.70E-23
SGBXF1_04286	C56	MER160094	1.00E-18
SGBXF1_00532	C82	MER107825	8.70E-71
SGBXF1_00873	C82	MER152590	7.40E-51
SGBXF1_02207	C82	MER107808	8.60E-52
SGBXF1_01612	I11	MER018254	2.60E-57
SGBXF1_02216	I13	MER029359	9.00E-05
SGBXF1_00224	I38	MER018233	5.30E-43
SGBXF1_02364	I38	MER018231	2.50E-15
SGBXF1_03683	I39	MER034541	0.00E+00
SGBXF1_01247	I51	MER028934	2.10E-10
SGBXF1_02944	I51	MER028934	8.20E-08
SGBXF1_02116	I78	MER059865	7.90E-06
SGBXF1_00375	I87	MER192051	6.20E-169
SGBXF1_00376	I87	MER191412	2.80E-137
SGBXF1_01074	I87	MER192051	1.80E-12
SGBXF1_01699	M01	MER001001	1.10E-117
SGBXF1_01433	M03A	MER001161	8.80E-07
SGBXF1_01433	M03A	MER001149	2.70E-06
SGBXF1_02341	M03A	MER001158	1.30E-253
SGBXF1_04637	M03A	MER001161	0.00E+00
SGBXF1_03669	M04	MER115298	2.90E-175
SGBXF1_04053	M10A	MER004909	6.10E-05
SGBXF1_00223	M10B	MER001096	7.50E-106
SGBXF1_02114	M10B	MER029131	1.50E-38
SGBXF1_02115	M10B	MER029131	3.40E-05
SGBXF1_02407	M10B	MER001096	7.40E-51
SGBXF1_02661	M14B	MER030306	1.70E-71
SGBXF1_03576	M15B	MER014983	8.50E-05
SGBXF1_03550	M15C	MER030246	8.30E-12
SGBXF1_03770	M15C	MER004957	4.50E-05
SGBXF1_02381	M15D	MER027775	2.30E-20
SGBXF1_03928	M16A	MER001222	4.80E-91
SGBXF1_03928	M16A	MER078753	1.10E-14
SGBXF1_03928	M16A	MER046874	9.90E-08
SGBXF1_00165	M16B	MER001233	4.20E-14
SGBXF1_00509	M17	MER001236	4.50E-269
SGBXF1_02899	M17	MER001236	3.30E-49
SGBXF1_03688	M17	MER002497	1.00E-183
SGBXF1_03575	M20A	MER001272	9.80E-87
SGBXF1_03575	M20A	MER001272	6.30E-46
SGBXF1_04689	M20A	MER001273	4.90E-86
SGBXF1_04689	M20A	MER001273	2.70E-42
SGBXF1_02036	M20B	MER001421	2.80E-188
SGBXF1_03422	M20B	MER001421	8.70E-71

SGBXF1_00884	M20C	MER001283	3.90E-232
SGBXF1_01112	M20D	MER002655	9.40E-100
SGBXF1_03243	M20D	MER002014	1.70E-69
SGBXF1_04163	M20D	MER003581	1.30E-120
SGBXF1_04164	M20D	MER014418	7.00E-14
SGBXF1_04164	M20D	MER014418	1.70E-09
SGBXF1_04518	M20D	MER002655	1.10E-79
SGBXF1_00859	M20X	MER026469	4.80E-48
SGBXF1_02111	M20X	MER026469	2.20E-50
SGBXF1_02583	M20X	MER026469	1.90E-51
SGBXF1_00330	M23B	MER015415	2.70E-25
SGBXF1_00760	M23B	MER015415	1.40E-26
SGBXF1_02821	M23B	MER003380	1.90E-121
SGBXF1_04728	M23B	MER005300	1.50E-40
SGBXF1_01315	M24A	MER001243	5.90E-74
SGBXF1_03901	M24A	MER001243	1.40E-136
SGBXF1_00281	M24B	MER001250	9.20E-132
SGBXF1_03039	M24B	MER004931	2.20E-33
SGBXF1_04019	M24B	MER001244	3.80E-122
SGBXF1_00745	M28C	MER001290	1.20E-129
SGBXF1_02622	M32	MER069021	8.80E-145
SGBXF1_01160	M38	MER033184	1.90E-47
SGBXF1_01910	M38	MER061068	1.00E-143
SGBXF1_02095	M38	MER033186	1.30E-50
SGBXF1_02110	M38	MER015112	2.00E-16
SGBXF1_02335	M38	MER037714	9.20E-43
SGBXF1_00425	M41	MER001620	2.40E-151
SGBXF1_02136	M48B	MER002637	1.60E-99
SGBXF1_03594	M48C	MER031491	2.30E-94
SGBXF1_04053	M48C	MER002639	1.20E-80
SGBXF1_03893	M50B	MER004480	3.30E-87
SGBXF1_03893	M50B	MER004480	3.00E-54
SGBXF1_01367	M60	MER042489	1.00E-113
SGBXF1_03436	M74	MER001298	5.00E-105
SGBXF1_01232	M79	MER056027	1.40E-08
SGBXF1_02688	M79	MER059868	1.70E-09
SGBXF1_03139	M90	MER217243	4.50E-108
SGBXF1_03654	N04	MER187148	2.50E-10
SGBXF1_03011	N06	MER181656	3.70E-120
SGBXF1_00539	P01	MER013629	1.50E-38
SGBXF1_04591	P01	MER035593	1.50E-14
SGBXF1_02088	S01A	MER098939	3.80E-06
SGBXF1_02624	S01A	MER005249	5.50E-83
SGBXF1_00723	S01C	MER001372	1.90E-117
SGBXF1_03893	S01C	MER001372	1.30E-05
SGBXF1_04298	S01C	MER001372	3.40E-142
SGBXF1_04299	S01C	MER001373	1.50E-70
SGBXF1_04648	S08A	MER000329	6.20E-65
SGBXF1_04649	S08A	MER000329	7.60E-58
SGBXF1_04649	S08A	MER000329	6.20E-10
SGBXF1_01956	S09A	MER000410	1.70E-120
SGBXF1_02265	S09A	MER005694	5.60E-68
SGBXF1_03543	S09A	MER000410	4.90E-103
SGBXF1_02477	S09B	MER058228	2.30E-05
SGBXF1_02476	S09C	MER034615	8.10E-07
SGBXF1_02903	S09C	MER043146	2.80E-31
SGBXF1_04210	S09C	MER107796	9.60E-85
SGBXF1_00386	S09X	MER034550	1.50E-68
SGBXF1_01440	S09X	MER037863	3.80E-06
SGBXF1_01520	S09X	MER061081	5.00E-122
SGBXF1_02394	S09X	MER031563	2.80E-31
SGBXF1_02568	S09X	MER033237	5.00E-23
SGBXF1_02568	S09X	MER034961	1.30E-06
SGBXF1_03108	S09X	MER031563	4.30E-32
SGBXF1_03578	S09X	MER065576	7.00E-26
SGBXF1_01125	S11	MER000450	3.00E-147
SGBXF1_01300	S11	MER000455	5.30E-130
SGBXF1_01603	S11	MER000450	1.70E-158
SGBXF1_02589	S11	MER043199	2.80E-65
SGBXF1_02683	S11	MER000451	4.60E-131
SGBXF1_00573	S12	MER004154	1.80E-130
SGBXF1_02093	S12	MER000457	4.20E-12
SGBXF1_02302	S12	MER000463	3.30E-85
SGBXF1_02306	S12	MER006204	3.90E-92
SGBXF1_02342	S12	MER026262	6.40E-20
SGBXF1_00421	S13	MER000472	1.30E-226
SGBXF1_01014	S14	MER000474	9.10E-113
SGBXF1_02008	S14	MER000474	6.60E-71
SGBXF1_02387	S14	MER002299	2.40E-07

SGBXF1_00605	S16	MER014135	4.90E-139
SGBXF1_01016	S16	MER000485	6.20E-133
SGBXF1_01717	S16	MER003018	3.60E-99
SGBXF1_02650	S24	MER000576	6.70E-37
SGBXF1_03236	S24	MER000576	5.50E-28
SGBXF1_03319	S24	MER037220	2.50E-08
SGBXF1_04406	S24	MER000569	1.80E-62
SGBXF1_03735	S26A	MER000589	3.70E-120
SGBXF1_00133	S33	MER036050	1.30E-05
SGBXF1_00211	S33	MER036081	1.80E-09
SGBXF1_00549	S33	MER033247	6.10E-25
SGBXF1_00916	S33	MER004146	3.00E-13
SGBXF1_01173	S33	MER031610	3.50E-42
SGBXF1_01750	S33	MER004146	1.10E-10
SGBXF1_01962	S33	MER000431	1.10E-87
SGBXF1_02346	S33	MER031610	1.00E-10
SGBXF1_02367	S33	MER017177	1.70E-11
SGBXF1_02569	S33	MER031617	4.10E-17
SGBXF1_02782	S33	MER213595	5.80E-08
SGBXF1_03045	S33	MER210990	2.60E-08
SGBXF1_03346	S33	MER034563	4.60E-59
SGBXF1_04058	S33	MER037236	9.20E-10
SGBXF1_04515	S33	MER059846	2.00E-129
SGBXF1_04568	S33	MER017142	1.10E-106
SGBXF1_02134	S41A	MER001295	2.60E-91
SGBXF1_02767	S49A	MER001299	6.20E-241
SGBXF1_02709	S49B	MER001300	8.20E-82
SGBXF1_00275	S51	MER001335	2.30E-39
SGBXF1_04576	S54	MER015468	1.30E-56
SGBXF1_03779	S73	MER150756	6.60E-18
SGBXF1_04699	T01B	MER001627	1.50E-85
SGBXF1_01514	T02	MER003338	4.60E-59
SGBXF1_02902	T02	MER017326	3.20E-28
SGBXF1_00852	T03	MER223926	5.20E-94
SGBXF1_00975	T03	MER001978	3.90E-145
SGBXF1_02507	T03	MER001978	1.30E-105
SGBXF1_00448	U32	MER037246	1.00E-126
SGBXF1_00449	U32	MER013876	1.80E-18
SGBXF1_03627	U32	MER003855	1.60E-44
SGBXF1_03627	U32	MER003855	3.30E-15
SGBXF1_04334	U62	MER016222	4.20E-206
SGBXF1_04350	U62	MER016301	3.60E-222
SGBXF1_01901	U73	MER492485	5.00E-71

Table S4- Genes involved in secretion systems

Locus/operon tag	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00703	*secA*	Preprotein translocase subunit SecA	Sec secretion system
SGBXF1_00702	*secM*	Secretion monitor	
SGBXF1_04724	*secB*	Preprotein translocase subunit SecB	
SGBXF1_00982	*secD*	Preprotein translocase subunit SecD	
SGBXF1_00983	*secF*	Preprotein translocase subunit SecF	
SGBXF1_00428	*secG*	Protein-export membrane protein	
SGBXF1_04469	*secY*	Preprotein translocase subunit SecY	
SGBXF1_00269-72	*tatABCD*	Sec-independent protein translocase protein	Twin-arginine translocation (Tat) system
SGBXF1_01120	*tatE*	Sec-independent protein translocase protein TatE	
SGBXF1_01559-61	*prsDEF*	Protease secretion system proteins	Protease secretion system

Table S5- Genes involved in metal transport and resistance

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_01070	*copA*	Copper-exporting P-type ATPase A	Copper resistance
SGBXF1_01071	*cueR*	HTH-type transcriptional regulator	
SGBXF1_01965-66	*copCD*	Copper resistance protein	
SGBXF1_04098	*cueO*	Blue copper oxidase	
SGBXF1_03425-28	*cusABCF*	Cation efflux system proteins	Copper and silver resistance
SGBXF1_00222	*zntA*	Lead, cadmium, zinc and mercury-transporting ATPase	Zinc and other metals resistance
SGBXF1_02822-24	*znuCBA*	High-affinity zinc uptake system proteins	
SGBXF1_01223	*zitB*	Zinc transporter	
SGBXF1_02654	*zntB*	Zinc transport protein	
SGBXF1_04231	*zupT*	Zinc transporter	
SGBXF1_01040-42	*znuC, mntB*	Zinc/manganese transport system elements	Zinc and manganese resistance

SGBXF1_00203	*corA*	Magnesium transport protein	Magnesium resistance
SGBXF1_02866	*mntP*	Manganese efflux pump	Manganese resistance
SGBXF1_03699	*rcnA*	Nickel/cobalt efflux protein	Nickel and cobalt resistance
SGBXF1_02446	*hoxN*	High-affinity nickel transport protein	Nickel resistance
SGBXF1_01242-44	*modABC*	Molybdenum transport system	Molybdate resistance
SGBXF1_03053-55	*arsCBR*	Arsenate reductase	Arsenate resistance
SGBXF1_00039	*chrR*	Chromate reductase	Chromate resistance
SGBXF1_00912	*tehB*	Tellurite methyltransferase	Tellurium resistance

Table S6- Genes involved in siderophore production and iron transport

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00842-46	*iutA, iucABCD*	Aerobactin production operon	Siderophore production
SGBXF1_03494-03506	*entABECF*	Enterobactin production operon	Siderophore production
SGBXF1_00277	*fre*	Ferrisiderophore reductase	Iron release from siderophores
SGBXF1_02729	*tonB*	Siderophore transmembrane transporter	Siderophore and other compounds transport
SGBXF1_02787	*foxA*	Ferrioxamine receptor	Siderophore and iron transport
SGBXF1_03569	*fpvA*	Ferripyoverdine receptor	Siderophore and iron transport
SGBXF1_02326	*fecA*	$Fe^{(3+)}$ dicitrate transport protein	$Fe^{(3+)}$ dicitrate transport
SGBXF1_04076-79	*fhuBCDA*	$Fe^{(3+)}$-hydroxamate import ABC transporter complex	Iron transport
SGBXF1_02561-63	*fbpABC*	$Fe^{(3+)}$ ABC transporter complex	Iron transport
SGBXF1_04564-66	*feoABC*	Ferrous iron transport proteins	Fe^{2+} ion uptake.
SGBXF1_02150-53	*yfeABCD*	Periplasmic chelated iron-binding proteins	Chelated iron transport, manganese transport
SGBXF1_02959-61	*efeBOU*	Iron uptake system	Fe^{2+} ion uptake
SGBXF1_02201-06	*sufABCDSE*	FeS cluster assembly proteins and cysteine desulfurase	May facilitate iron uptake from extracellular iron chelators under iron limitation

Table S7-Genes involved in nitrogen, sulfur and phosphorous metabolism

Locus tag/operon	Gene/operon name	Product/putative product	Function/Putative function
SGBXF1_02921-24	*narIJHG*	Respiratory nitrate reductase	Nitrate assimilation
SGBXF1_02939-41	*narKXL*	Nitrate/nitrite sensor and transporter	Nitrate assimilation, celular response to nitrate and nitrite
SGBXF1_03564	*narQP*	Nitrate/nitrite sensor	Nitrate assimilation
SGBXF1_03559-61	*napABC*	Periplasmic nitrate reductase	Nitrate assimilation
SGBXF1_04535-37	*nirBDC*	Nitrite reductase	Nitrate assimilation
SGBXF1_01028-29	*amtBR*	Ammonia channel	Ammonia uptake
SGBXF1_04774	*glnA*	Glutamine synthetase	Ammonia assimilation cycle
SGBXF1_04289-90	*gltBD*	Glutamate synthase [NADPH]	Ammonia assimilation cycle
SGBXF1_04775-76	*glnLG*	Nitrogen regulation sensor	Nitrogen regulation
SGBXF1_01371-72	*atzF, Dur1,2*	Allophanate hydrolase Urea amidolyase	Hydrolysis of urea to ammonia and CO2
SGBXF1_00746-49	*cysGDN*	Sulfate adenylyltransferase	Sulfate reduction
SGBXF1_00742-44	*cysJIH*	Sulfite reductase	Sulfate reduction
SGBXF1_03530-33	*cysAWTP*	Sulfate/thiosulfate import ATP-binding proteins	Sulfate transport
SGBXF1_00984-88	*ttrRSBCA*	Tetrathionate reductase and other components	Tetrathionate reduction
SGBXF1_00553-54	*atsAB*	Arylsulfatase	Arylsulfate ester degradation
SGBXF1_01700-04	*ssuBCDAE*	Alkanesulfonate monooxygenase and other components	Alkanesulfonate degradation
SGBXF1_01363-65	*ssuD*	Alkanesulfonate monooxygenase	Alkanesulfonate degradation
SGBXF1_04509-12	*tauDCBA*	Taurine dioxygenase and other components	Taurine and alkanesulfonate degradation
SGBXF1_01033	*bds1*	Aryl/alkyl sulfatase	Enables the use of SDS and 4-nitrocatechol as sulfur source
SGBXF1_02968	*dcys*	D-cysteine desulfhydrase	Use of D-cysteine as sulfur source
SGBXF1_00028 SGBXF1_00969	*pstS*	Phosphate transport system substrate-binding protein	Phosphate transport
SGBXF1_00029-31 SGBXF1_03605-07	*pstCAB*		
SGBXF1_01358-61	*phnCDE*	phosphonate transport system substrate-binding protein	Phosphonate transport system
SGBXF1_00457-67	*phnFGHIJKLMNOP*	Phosphonates utilization proteins and other elements	Phosphonate degradation

Table S8- Genes involved in the degradation of aromatic compounds and other xenobiotics

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_03049-52	*cdbABCD*	2-halobenzoate 1,2-dioxygenase	Benzoate degradation
SGBXF1_03046-48	*catBCA*	Catechol 1,2-dioxygenase	Beta-ketoadipate pathway
SGBXF1_03042-45	*pcaIJFD*	3-oxoadipate CoA-transferase	
SGBXF1_02519-20	*pcaGH*	Protocatechuate 3,4-dioxygenase	
SGBXF1_02522-23	*pcaCB*	4-carboxymuconolactone decarboxylase, 3-carboxy-cis,cis-muconate cycloisomerase	
SGBXF1_02524	*praI*	Hydroxybenzoate (4-HBA)-3-monoxygenase	Degradation of 4-hydroxybenzoate (4HBA) via protocatechuate
SGBXF1_03089-103	*paaABCDEFGHIJK*	1,2-phenylacetyl-CoA epoxidase and other elements	Phenylacetate degradation
SGBXF1_00562-68	*hpcECBDGH*	3,4-dihydroxyphenylacetate 2,3-dioxygenase and other elements	4-hydroxyphenylacetate degradation
SGBXF1_00571-72	*hpaBC*	4-hydroxyphenylacetate 3-monooxygenase oxygenase	
SGBXF1_02237-38 SGBXF1_04338-39	*aaeAB*	p-hydroxybenzoic acid efflux pump	Transport and elimination of aromatic compounds

Table S9- Genes involved in antibiotic resistance and multidrug efflux systems

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_02302	*ampC*	Beta-lactamase	Cephalosporin resistance
SGBXF1_02257	*strA*	Streptomycin 3''-kinase	Streptomycin resistance
SGBXF1_01065	*fsr*	Fosmidomycin resistance protein	Fosmidomycin resistance
SGBXF1_03291	*bcr*	Bicyclomycin resistance protein	Bicyclomycin resistance
SGBXF1_02877	*fosA*	Fosfomycin resistance protein	Fosfomycin resistance
SGBXF1_04240	*uppP*	Undecaprenyl pyrophosphate phosphatase	Bacitracin resistance
SGBXF1_02175-81	*arnBCADTEF*	UDP-4-deoxy-4-formamido-beta-L-arabinose biosynthesis proteins	Resistance to polymyxin and cationic antimicrobial peptides
SGBXF1_00419-20	*basSR*	Two-component regulatory system BasS/BasR	Resistance to polymyxin and cationic antimicrobial peptides
SGBXF1_02230	*pmrC*	Phosphoethanolamine transferase	Resistance to polymyxin
SGBXF1_01645-46 SGBXF1_01875-76 SGBXF1_03527-28	*macAB*	Macrolide export proteins	Drug export
SGBXF1_01045-46	*acrAB*	Multidrug efflux pump proteins	Drug transmembrane transport
SGBXF1_03567	*acrB*	Multidrug efflux pump subunit	
SGBXF1_02499-501	*oprM-mexAB*	Multidrug resistance proteins	
SGBXF1_01945-47	*oqxABR*	Multidrug efflux proteins	
SGBXF1_03836-37	*emrAB*	Multidrug export proteins	
SGBXF1_03620-25	*mdtABCD*	Multidrug export proteins	Confers resistance against novobiocin and deoxycholate
SGBXF1_01026-27	*mdlAB*	Multidrug resistance-like ATP-binding proteins	Xenobiotic transport and efflux
SGBXF1_02376	*mdtG*	Multidrug resistance protein	Confers resistance against fosfomycin and deoxycholate
SGBXF1_02214	*mdtK*	Multidrug resistance protein	Drug transmembrane transport, antibiotic resistance
SGBXF1_03990	*mdtL*	Multidrug resistance protein	Confers resistance to chloramphenicol
SGBXF1_02845	*mdtH*	Multidrug resistance protein	Confers resistance to norfloxacin and enoxacin
SGBXF1_00095	*mdfA*	Multidrug transporter	Drug transmembrane transport, antibiotic resistance

Table S10- Genes involved in secondary metabolites and antagonistic activities

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00226-36	-	Bacteriocin/lantibiotic production genes	Bacteriocin production
SGBXF1_00232	-	Bacteriocin/Lantibiotic dehydratase	
SGBXF1_00897-901	*nrps*	Unknown	Siderophore production?
SGBXF1_04372	*srwW*	Serrawetin W1 synthase	Serrawetin W1 production
SGBXF1_02064	*t1pks*	Type I polyketide synthase	Unknown
SGBXF1_00148 SGBXF1_02770	*chiA*	Chitinase A	Hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and chitodextrins
SGBXF1_03552	*chiB*	Chitinase B	
SGBXF1_01157	*chiD*	Chitinase D	
SGBXF1_01164	*chiP*	Chitoporin	Involved in the uptake of

			chitosugars
SGBXF1_01166	*chB*	Chitobiase	Digests the beta-1,4-glycosidic bonds in N-acetylglucosamine (GlcNAc) oligomers
SGBXF1_03554	*gbpA*	Chitin and GlNac-binding protein	Promotes bacterial attachment to GlNac residues and chitin
SGBXF1_00333-36	*prnABCD*	Tryptophan halogenase and other elements	Pyrrolnitrin production
SGBXF1_01846-48	*hcnABC*	Hydrogen cyanide synthase and others	Degradation of amino acids and production of HCN

Table S11- Genes involved in motility, chemotaxis, attachment and quorum-sensing

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_02993-03006	*flgLKJIHGFEDCBAMN*	Flagellar basal-body rod proteins	Flagella biosynthesis
SGBXF1_03009-11	*flhEAB*	Flagellar biosynthesis proteins	
SGBXF1_02969-74	*fliZACDST*	Flagellin and other flagellar proteins	Flagellum-dependent cell motility
SGBXF1_02979-92	*fliEFGHIJKLMONPQR*	Flagellar proteins	
SGBXF1_03022-23	*flhCD*	Flagellar transcriptional regulators	Flagella biosynthesis and flagellum-dependent cell motility
SGBXF1_03020-21	*motBA*	Motility proteins	Motility
SGBXF1_04010-11	*rssBA*	Regulation of swarming motility proteins	Regulation of swarming motility
SGBXF1_04372	*srwW*	Serrawetin W1 synthase	Serrawetin W1 production, motility
SGBXF1_03012-15	*cheZYBR*	Chemotaxis proteins	Chemotaxis
SGBXF1_03018-19	*cheWA*	Chemotaxis proteins	
SGBXF1_03016	*tap*	Methyl-accepting chemotaxis protein	
SGBXF1_03017	*tsr*	Methyl-accepting chemotaxis protein	
SGBXF1_00405	*tar*	Methyl-accepting chemotaxis protein	
SGBXF1_00451 SGBXF1_00083	*tsr*	Methyl-accepting chemotaxis protein	
SGBXF1_1467-72	*ecpRABCDE*	Fimbria proteins and other elements	Fimbria biogenesis
SGBXF1_01487-90	*fimA, others*	Fimbria A protein and other elements	
SGBXF1_03119-26	*smfA, others*	Fimbria A protein and other elements	
SGBXF1_04607-15	*smfA, others*	Fimbria A protein and other elements	
SGBXF1_00172-84	*wec operon*	Lipopolysaccharide biosynthesis proteins	Lipopolysaccharide (LPS) biosynthesis
SGBXF1_01562-81	*OA cluster*	dTDP-4-dehydrorhamnose reductase and other elements	O-antigen biosynthesis, LPS biosynthesis
SGBXF1_00510-11	*lptFG*	Lipopolysaccharide export system permease protein	LPS export system
SGBXF1_04308-9	*kdsCD*	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, Arabinose 5-phosphate isomerase	LPS biosynthesis
SGBXF1_04310-12	*lptCAB*	Lipopolysaccharide export system ATP-binding protein	LPS export system
SGBXF1_01547-56	*wza wzb wzc*	Tyrosine-protein kinase and glycosyl transferases	Exopolysaccharide biosynthesis
SGBXF1_02925-37	*eps, wzc*	Tyrosine-protein kinase and glycosyl transferases	Exopolysaccharide biosynthesis
SGBXF1_03349	*ndvB*	Protein NdvB	Involved in the production of beta-(1,2)-glucan
SGBXF1_00159- 64	*bcsGFEQABZC*	Cellulose synthase and other elements	Cellulose biosynthesis
SGBXF1_04088-89	*qseCB*	Sensor proteins	Quorum-sensing
SGBXF1_00787	*luxS*	S-ribosylhomocysteine lyase	Synthesis of autoinducer 2 (AI-2), quorum-sensing
SGBXF1_00210	*rhtB*	Homoserine/homoserine lactone efflux protein	Quorum-sensing
SGBXF1_00292 SGBXF1_04493	*tufA*	Elongation factor Tu	MAMP
SGBXF1_00688 SGBXF1_02390	*ftsI*	Peptidoglycan synthase	Peptidoglycan biosynthesis

Table S12- Genes involved in major carbohydrate degradation pathways

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_03118	*gcd*	Glucose dehydrogenase	Glucose degradation
SGBXF1_03269-71	*fruAKB*	1-phosphofructokinase and others	Fructose degradation
SGBXF1_00100-101	*xylAB*	Xylulose kinase, Xylose isomerase	Xylose degradation
SGBXF1_02276-77	*araAB*	L-arabinose isomerase, Ribulokinase	Arabinose degradation
SGBXF1_00003-08	*rbsRKBCAD*	Ribokinase and others	Ribose degradation
SGBXF1_04571-72	*malQP*	4-alpha-glucanotransferase, Maltodextrin phosphorylase	Maltose degradation
SGBXF1_02308	*manA*	Mannose-6-phosphate isomerase	Mannose degradation
SGBXF1_00474-5	*treAB*	Trehalose-6-phosphate hydrolase and transporter	Trehalose degradation
SGBXF1_01229-31	*galMKT*	Galactokinase and others	Galactose degradation
SGBXF1_02100	*sacA*	Sucrose-6-phosphate hydrolase	Sucrose degradation
SGBXF1_04175	*scrB*		
SGBXF1_03205	*lacZ*	Beta-galactosidase	Lactose degradation
SGBXF1_01999-2000	*ebgAC*		
SGBXF1_00960	*gatY*	D-tagatose-1,6-bisphosphate aldolase	Galactitol catabolism
SGBXF1_03961	*gatZ*		
SGBXF1_00075-77	*mtlADR*	Mannitol-1-phosphate 5-dehydrogenase and others	Mannitol degradation
SGBXF1_03635-38	*slrAEBD*	Sorbitol-6-phosphate 2-dehydrogenase and others	Sorbitol degradation
SGBXF1_01158-61	*nagBACD*	N-acetylglucosamine-6-phosphate deacetylase and others	N-acetylglucosamine degradation
SGBXF1_01166	*chb*	Chitobiase	Digests the beta-1,4-glycosidic bonds in N-acetylglucosamine (GlcNAc)
SGBXF1_03957-62	*PTS, kbaZ*	N-acetylgalactosamine permeasse, D-tagatose-1,6-bisphosphate aldolase	D-galactosamine degradation
SGBXF1_00773	*chbG*	Chitooligosaccharide deacetylase	Involved in the degradation of acetylated chitooligosaccharides chitobiose and chitotriose
SGBXF1_02604-07	*nagK*	N-acetyl-D-glucosamine kinase and others	N-acetyl-D-galactosamine degradation
SGBXF1_00061	*malS*	Amylase	Degradation of amylose, starch, amylopectin, and maltodextrins
SGBXF1_01337	*amyA*	Amylase	
SGBXF1_02540	*palH*	Alpha-glucosidase	Degrades maltose, palatinose, maltulose, trehalose, trehalulose, turanose, leucrose, sucrose and maltitol
SGBXF1_03131-32	*melBA*	Alpha-glucosidase	Melibiose degradation
SGBXF1_02538	*malL*	Maltase, Isomaltase	Maltose degradation
SGBXF1_04417	*malL*	Oligo-1,6-glucosidase, Maltase	
SGBXF1_00974	*malZ*	Maltodextrin glucosidase	Degrades maltotriose and longer maltodextrins with a chain length of up to 7 glucose units
SGBXF1_01996	*ygjK*	Glucosidase	Nigerose degradation
SGBXF1_02399	*bglB*	Cellobiase	Cellobiose, Gentibiose degradation
SGBXF1_01321	*bglX*	Beta-glucosidase	Hydrolysis of terminal, non-reducing beta-D-glucosyl residues with release of beta-D-glucose
SGBXF1_00138	*blgB*	6-phospho-beta-glucosidase	Hydrolysis of phosphorylated beta-glucosides into glucose-6-phosphate (G-6-P) and aglycone
SGBXF1_00771	*gmuD*	6-phospho-beta-glucosidase	Glucomannan degradation
SGBXF1_01870 SGBXF1_04531	*chbF*	6-phospho-beta-glucosidase	Hydrolyzes a wide variety of P-beta-glucosides including cellobiose-6P, salicin-6P, arbutin-6P and others
SGBXF1_02517	*bglA*	6-phospho-beta-glucosidase	Hydrolysis of phosphorylated beta-glucosides into glucose-6-phosphate (G-6-P) and

			aglycone
SGBXF1_02359 SGBXF1_04187	*bglC*	Aryl-phospho-beta-D-glucosidase	Hydrolysis of aryl-phospho-beta-D-glucosides

Table S13- Genes involved in sugars transport

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_04418-20	*malEFG*	Maltose/maltodextrin transport system	Maltose/maltodextrin transport system
SGBXF1_02539 SGBXF1_04416	*malK*	Multiple sugar transport system ATP-binding protein	Trehalose Maltose/maltodextrin transport system
SGBXF1_02329 SGBXF1_02900	*msmX*	Multiple sugar transport system ATP-binding protein	Maltose/maltodextrin transport system, alpha-Glucoside transport system
SGBXF1_02541	*thuGFE*	Trehalose/maltose transport system proteins	Trehalose/maltose transport system
SGBXF1_02278-80	*araFGH*	L-arabinose transport system proteins	L-Arabinose transport system
SGBXF1_01529-31	*mglBAC*	Methyl-galactoside transport system substrate-binding protein	Methyl-galactoside transport system
SGBXF1_00005 SGBXF1_00958 SGBXF1_04031	*rbsB*	Ribose transport system components	Ribose transport system
SGBXF1_00006 SGBXF1_00957 SGBXF1_04030	*rbsC*		
SGBXF1_00007 SGBXF1_00956 SGBXF1_04029	*rbsA*		
SGBXF1_01928	*ptsG*	PTS system, glucose-specific components	PTS system, glucose
SGBXF1_03524	*crr*		
SGBXF1_01162	*nagE*	PTS system, N-acetylglucosamine-specific IIA component	PTS system, N-acetylglucosamine
SGBXF1_02299	*malX*	PTS system, maltose/glucose-specific IIB component	PTS system, maltose and glucose
SGBXF1_00475	*treB*	PTS system, trehalose-specific IIB component	PTS system, trehalose
SGBXF1_03269	*fruA*	PTS system, fructose-specific components	PTS system, fructose
SGBXF1_03271	*fruB*		
SGBXF1_00075	*mtlA*	PTS system, mannitol-specific IIA component	PTS system, mannitol
SGBXF1_01869 SGBXF1_04188 SGBXF1_04530	*chbA*	PTS system N,N'-diacetylchitobiose-specific components	PTS system N,N'-diacetylchitobiose
SGBXF1_00769	*chbB*		
SGBXF1_00770	*chbC*		
SGBXF1_01868 SGBXF1_04190	*celA or licB*	PTS system, cellobiose-specific IIB component, Lichenan permease	PTS system, cellobiose, lichenan
SGBXF1_01871 SGBXF1_04189	*celB or licC*	PTS system, cellobiose-specific IIC component, Lichenan permease IIC component	
SGBXF1_03637	*srlB*	PTS system, glucitol/sorbitol-specific IIA component	PTS system, glucitol/sorbitol
SGBXF1_02862 SGBXF1_04227 SGBXF1_04226	*manX*	PTS system, mannose-specific components	PTS system, mannose
SGBXF1_02863 SGBXF1_04225	*manY*		
SGBXF1_02864	*manZ*		
SGBXF1_03957	*yadI*	PTS system	PTS system
SGBXF1_03960	*agaV*	PTS system, N-acetylgalactosamine-specific components	PTS system, N-acetylgalactosamine
SGBXF1_03959	*agaC*		
SGBXF1_03958	*agaD*		
SGBXF1_02289	*ulaC*	PTS system, ascorbate-specific component	PTS system, ascorbate
SGBXF1_02293	*ulaB*		
SGBXF1_02292	*ulaA*		
SGBXF1_02333 SGBXF1_02599 SGBXF1_02896	*ABC.MS.S*	Multiple sugar transport system substrate-binding protein	Putative multiple sugar transport system
SGBXF1_02332 SGBXF1_02600 SGBXF1_02897	*ABC.MS.P*	Multiple sugar transport system permease protein	
SGBXF1_02331	*ABC.MS.P1*	Multiple sugar transport system permease	

SGBXF1_02601 SGBXF1_02898		protein	
SGBXF1_01538 SGBXF1_04629	*ABC.SS.S*	Simple sugar transport system substrate-binding protein	Putative simple sugar transport system
SGBXF1_03259 SGBXF1_04631 SGBXF1_04632	*ABC.SS.P*	Simple sugar transport system permease protein	
SGBXF1_03258 SGBXF1_04630	*ABC.SS.A*	Simple sugar transport system ATP-binding protein	

Table S14- Genes involved in rhizopine transport and rhizopine and myo-inositol degradation

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_01983 SGBXF1_01986 SGBXF1_02411	*mocB*	Rhizopine-binding protein	Involved in rhizopine (L-3-O-methyl-scyllo-inosamine) catabolism
SGBXF1_02068	*iolE*, *mocC*	Inosose dehydratase Rhizopine catabolism protein	Dehydration of inosose, Involved in rhizopine catabolism
SGBXF1_02409	*iolX*	Scyllo-inositol 2-dehydrogenase	Oxidation of scyllo-inositol to 2,4,6/3,5-pentahydroxycyclohexanone (scyllo-inosose)
SGBXF1_04592	*iolE*	Inosose dehydratase	Myo-inositol degradation
SGBXF1_04593	*iolH*	Protein IOHL	
SGBXF1_04594	*iolG*	Inositol 2-dehydrogenase	
SGBXF1_04595	*iolD*	3D-(3,5/4)-trihydroxycyclohexane-1,2-dione hydrolase	
SGBXF1_04596	*iolC*	5-dehydro-2-deoxygluconokinase	
SGBXF1_04598	*iolB*	5-deoxy-glucuronate isomerase	
SGBXF1_04599	*iolA*	Methylmalonate semialdehyde dehydrogenase	

Table S15- Genes involved in organic and sugar acids degradation

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_01196-99	*sdhCBAD*	Succinate dehydrogenase and others	Succinate degradation
SGBXF1_00411	*mdh*	Malate dehydrogenase	Malate degradation
SGBXF1_04442-44	*aceKAB*	Isocitrate lyase	Isocitrate degradation
SGBXF1_00328	*acs*	Acetyl-coenzyme A synthetase	Acetate degradation
SGBXF1_04438 SGBXF1_04586	*gntK*	D-gluconate kinase	Gluconate degradation
SGBXF1_02156-8	*gadH*	Gluconate dehydrogenase	
SGBXF1_00355-58	*frdABCD*	Fumarate reductase and others	Fumarate degradation Oxaloacetate degradation
SGBXF1_02357	*odc*	Oxalate decarboxylase	Oxalate degradation
SGBXF1_02704	*acnA*	Aconitate hydratase	Citrate degradation
SGBXF1_04105	*acnB*	Aconitate hydratase	
SGBXF1_03197-3203	*citTGXFEDC*	Citrate lyase and transporters	
SGBXF1_02050	*icd*	Isocitrate dehydrogenase [NADP]	Oxaloacetate degradation
SGBXF1_04118	*fumA*	Fumarate hydratase class I, aerobic	Malate degradation and interconversion
SGBXF1_02310	*fumC*	Fumarate hydratase class II	
SGBXF1_02447-58	*fdhF, hyc* operon	Formate hydrogenlyase complex	Formate degradation
SGBXF1_04265	*uxaA*	Altronate dehydratase	D-glucuronate, D-altronate, D-fructuronate degradation
SGBXF1_04266	*uxaC*	Uronate isomerase	
SGBXF1_03279	*uxuA*	Mannonate dehydratase	
SGBXF1_03280 SGBXF1_03632	*uxuB*	D-mannonate oxidoreductase	
SGBXF1_03629	*rspA*	D-galactonate dehydratase family member RspA	
SGBXF1_00064 SGBXF1_04661	*kdgK*	2-dehydro-3-deoxygluconokinase	
SGBXF1_02815 SGBXF1_04441	*eda*	KHG/KDPG aldolase	

Table S16- Genes involved in amino acid metabolism

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_04023	*serA*	D-3-phosphoglycerate dehydrogenase	Serine biosynthesis, glycerate-3P
SGBXF1_01671	*serC*	Phosphoserine aminotransferase	
SGBXF1_00604	*serB*	Phosphoserine phosphatase	
SGBXF1_04425	*lysC*	Aspartate kinase	Threonine biosynthesis,

SGBXF1_00622	*thrA*	Bifunctional aspartokinase / homoserine dehydrogenase	aspartate => homoserine => threonine
SGBXF1_04692	*metL*	Bifunctional aspartokinase / homoserine dehydrogenase 2	
SGBXF1_04584	*asd*	Aspartate-semialdehyde dehydrogenase	
SGBXF1_00623	*thrB1*	Homoserine kinase	
SGBXF1_00624	*thrC*	Threonine synthase	
SGBXF1_00504 SGBXF1_01482 SGBXF1_04153	*betA*	Choline dehydrogenase	Betaine biosynthesis
SGBXF1_01481 SGBXF1_04055	*betB*	Betaine-aldehyde dehydrogenase	
SGBXF1_04722	*cysE*	Serine O-acetyltransferase	Cysteine biosynthesis, serine => cysteine
SGBXF1_01024 SGBXF1_03521	*cysK*	Cysteine synthase A	
SGBXF1_03529	*cysM*	Cysteine synthase B	
SGBXF1_02968	*dcys*	D-cysteine desulfhydrase	D-cysteine degradation
SGBXF1_01602	*cbs*	Cystathionine beta-synthase	Cysteine biosynthesis, homocysteine + serine => cysteine
SGBXF1_01601	*CTH*	Cystathionine gamma-lyase [	
SGBXF1_04425	*lysC*	Aspartate kinase	Methionine biosynthesis, apartate => homoserine => methionine
SGBXF1_00622	*thrA*	Bifunctional aspartokinase / homoserine dehydrogenase	
SGBXF1_04692	*metL*	Bifunctional aspartokinase / homoserine dehydrogenase 2	
SGBXF1_04584	*asd*	Aspartate-semialdehyde dehydrogenase	
SGBXF1_04445	*metA*	Homoserine O-succinyltransferase	
SGBXF1_04693	*metB*	Cystathionine gamma-synthase	
SGBXF1_04180	*metC*	Cystathionine beta-lyase	
SGBXF1_04436	*metH*	5-methyltetrahydrofolate--homocysteine methyltransferase	
SGBXF1_00258 SGBXF1_03163	*metE*	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	
SGBXF1_04075	*metK*	S-adenosylmethionine synthetase	Methionine salvage pathway
SGBXF1_04101	*speD*	S-adenosylmethionine decarboxylase	
SGBXF1_04100	*speE*	Spermidine synthase	
SGBXF1_00721 SGBXF1_01611	*mtnN*	Adenosylhomocysteine nucleosidase	
SGBXF1_00869	*mtnK*	5-methylthioribose kinase	
SGBXF1_00868	*mtnA*	Methylthioribose-1-phosphate isomerase	
SGBXF1_00865	*mtnB*	Methylthioribulose-1-phosphate dehydratase	
SGBXF1_00866	*mtnC*	Enolase-phosphatase E1	
SGBXF1_00867	*mtnD*	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	
SGBXF1_03106 SGBXF1_04392	*tyrB*	Aromatic-amino-acid transaminase	
SGBXF1_00667 SGBXF1_00681 SGBXF1_02340 SGBXF1_03358 SGBXF1_03508 SGBXF1_04054 SGBXF1_04669	*ilvL*	Acetolactate synthase I/II/III large subunit	Valine/isoleucine biosynthesis, pyruvate => valine / 2-oxobutanoate => isoleucine
SGBXF1_00666 SGBXF1_00682	*ilvH*	Acetolactate synthase I/III small subunit	
SGBXF1_04668	*ilvM*	Acetolactate synthase II small subunit	
SGBXF1_04662	*ilvC*	Ketol-acid reductoisomerase	
SGBXF1_04666	*ilvD*	Dihydroxy-acid dehydratase	
SGBXF1_04667	*ilvE*	Branched-chain amino acid aminotransferase	
SGBXF1_00678 SGBXF1_01889	*leuA*	2-isopropylmalate synthase	Leucine biosynthesis, 2-oxoisovalerate => 2-oxoisocaproate
SGBXF1_00676	*leuC*	3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit	
SGBXF1_00675	*leuD*	3-isopropylmalate/(R)-2-methylmalate dehydratase small subunit	
SGBXF1_00677	*leuB*	3-isopropylmalate dehydrogenase	
SGBXF1_04425	*lysC*	Aspartate kinase	Lysine biosynthesis, succinyl-DAP pathway, aspartate => lysine
SGBXF1_00622	*thrA*	Bifunctional aspartokinase / homoserine dehydrogenase 1	
SGBXF1_04692	*metL*	Bifunctional aspartokinase / homoserine dehydrogenase 2	
SGBXF1_04584	*asd*	Aspartate-semialdehyde dehydrogenase	
SGBXF1_02247 SGBXF1_03583	*dapA*	4-hydroxy-tetrahydrodipicolinate synthase	

SGBXF1_00645	*dapB*	4-hydroxy-tetrahydrodipicolinate reductase	
SGBXF1_03903	*dapD*	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	
SGBXF1_04524	*argD*	Acetylornithine/N-succinyldiaminopimelate aminotransferase	
SGBXF1_03575	*dapE*	Succinyl-diaminopimelate desuccinylase	
SGBXF1_00197	*dapF*	Diaminopimelate epimerase	
SGBXF1_03949	*lysA*	Diaminopimelate decarboxylase	
SGBXF1_00888	*proB*	Glutamate 5-kinase	Proline biosynthesis, glutamate => proline
SGBXF1_00889	*proA*	Glutamate-5-semialdehyde dehydrogenase	
SGBXF1_04127	*proC*	Pyrroline-5-carboxylate reductas	
SGBXF1_03925	*argAB*	Amino-acid N-acetyltransferase	Ornithine biosynthesis, glutamate => ornithine
SGBXF1_04687	*argB*	Acetylglutamate kinase	
SGBXF1_04688	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	
SGBXF1_04524	*argD*	Acetylornithine/N-succinyldiaminopimelate aminotransferase	
SGBXF1_04689	*argE*	Acetylornithine deacetylase	
SGBXF1_01589	*hisG*	ATP phosphoribosyltransferase	Histidine biosynthesis, PRPP => histidine
SGBXF1_01582	*hisIE*	Phosphoribosyl-ATP pyrophosphohydrolase / phosphoribosyl-AMP cyclohydrolase	
SGBXF1_01584	*hisA*	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	
SGBXF1_01585	*hisH*	Glutamine amidotransferase	
SGBXF1_01583	*hisF*	Cyclase	
SGBXF1_01587	*hisC*	Histidinol-phosphate aminotransferase	
SGBXF1_01586	*hisB*	Imidazoleglycerol-phosphate dehydratase / histidinol-phosphatase	
SGBXF1_01588	*hisD*	Histidinol dehydrogenase	
SGBXF1_00732 SGBXF1_00737 SGBXF1_02105	*hutH*	Histidine ammonia-lyase	Histidine degradation, histidine => N-formiminoglutamate => glutamate
SGBXF1_00738 SGBXF1_02107	*hutU*	Urocanate hydratase	
SGBXF1_02095	*hutI*	Imidazolonepropionase	
SGBXF1_02094	*hutG*	Formiminoglutamase	
SGBXF1_00798 SGBXF1_01226 SGBXF1_02194	*aroG*	3-deoxy-7-phosphoheptulonate synthase	Shikimate pathway, phosphoenolpyruvate + erythrose-4P => chorismate
SGBXF1_04544	*aroB*	3-dehydroquinate synthase	
SGBXF1_04362	*aroQ*	3-dehydroquinate dehydratase II	
SGBXF1_04177 SGBXF1_04450	*aroE*	Shikimate dehydrogenase	
SGBXF1_00945 SGBXF1_04545	*aroK*	Shikimate kinase	
SGBXF1_01672	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	
SGBXF1_03437	*aroC*	Chorismate synthase	
SGBXF1_02716	*trpE*	Anthranilate synthase component I	Tryptophan biosynthesis, chorismate => tryptophan
SGBXF1_02717	*trpG*	Anthranilate synthase component II	
SGBXF1_02718	*trpD*	Anthranilate phosphoribosyltransferase	
SGBXF1_02719	*trpCF*	Indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase	
SGBXF1_02721	*trpA*	Tryptophan synthase alpha chain	
SGBXF1_02720	*trpB*	Tryptophan synthase beta chain	
SGBXF1_00799	*tyrA*	Chorismate mutase / prephenate dehydrogenase	Phenylalanine biosynthesis, chorismate => phenylalanine
SGBXF1_02092	*pheA1*	Chorismate mutase	
SGBXF1_04394	*pheC*	Cyclohexadienyl dehydratase	
SGBXF1_00800	*pheA*	Chorismate mutase / prephenate dehydratase	
SGBXF1_03106 SGBXF1_04392	*tyrB*	Aromatic-amino-acid transaminase	
SGBXF1_00800	*pheA*	Chorismate mutase / prephenate dehydratase	Tyrosine biosynthesis, chorismate => tyrosine
SGBXF1_02092	*pheA1*	Chorismate mutase	
SGBXF1_00799	*tyrA*	Chorismate mutase / prephenate dehydrogenase	
SGBXF1_03106 SGBXF1_04392	*tyrB*	Aromatic-amino-acid transaminase	
SGBXF1_3113	*davD*	Glutarate-semialdehyde dehydrogenase	Lysine catabolism

Table S17- Genes involved in amino acid transport

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_01318	*argT*	Lysine/arginine/ornithine transport system substrate-binding protein	Lysine/arginine/ornithine transport system
SGBXF1_02420 SGBXF1_03389	*hisM*	Histidine transport system permease protein	

SGBXF1_03390	*hisQ*	Histidine transport system permease protein	
SGBXF1_03388	*hisP*	Histidine transport system ATP-binding protein	
SGBXF1_02418 SGBXF1_03391	*hisJ*	Histidine transport system substrate-binding protein	Histidine transport system
SGBXF1_02420 SGBXF1_03389	*hisM*	Histidine transport system permease protein	
SGBXF1_03390	*hisQ*	Histidine transport system permease protein	
SGBXF1_03388	*hisP*	Histidine transport system ATP-binding protein	
SGBXF1_01442-44	*glnOPH*	Glutamine transport system proteins	Glutamine transport system
SGBXF1_01626-30	*artJMQIP*	Arginine transport system proteins	Arginine transport system
SGBXF1_01138-41	*gltLKJI*	Glutamate/aspartate transport system protein	Glutamate/aspartate transport system
SGBXF1_02965-67	*yecCS, fliY*	Cystine transport system proteins	Cystine transport system
SGBXF1_00244 SGBXF1_02581 SGBXF1_03227	*livK*	Branched-chain amino acid transport system substrate-binding protein	Branched-chain amino acid transport system
SGBXF1_00245 SGBXF1_02577 SGBXF1_03226	*livH*	Branched-chain amino acid transport system permease protein	
SGBXF1_00246 SGBXF1_02578 SGBXF1_03225	*livM*	Branched-chain amino acid transport system permease protein	
SGBXF1_00247 SGBXF1_02579 SGBXF1_03224	*livG*	Branched-chain amino acid transport system ATP-binding protein	
SGBXF1_00248 SGBXF1_02580 SGBXF1_03223	*livF*	Branched-chain amino acid transport system ATP-binding protein	
SGBXF1_01308 SGBXF1_01345 SGBXF1_01991 SGBXF1_03864	*metQ*	D-methionine transport system substrate-binding protein	D-Methionine transport system
SGBXF1_01346 SGBXF1_03863	*metI*	D-methionine transport system permease protein	
SGBXF1_01347 SGBXF1_03862	*metN*	D-methionine transport system ATP-binding protein	
SGBXF1_00033 SGBXF1_00753 SGBXF1_00854 SGBXF1_01936 SGBXF1_02433	*ABC.PA.S*	Polar amino acid transport system substrate-binding protein	Putative polar amino acid transport system
SGBXF1_00034 SGBXF1_00035 SGBXF1_00752 SGBXF1_00855 SGBXF1_00856 SGBXF1_01109	*ABC.PA.P*	Polar amino acid transport system permease protein	
SGBXF1_00751 SGBXF1_00857 SGBXF1_01108	*ABC.PA.A*	Polar amino acid transport system ATP-binding protein	
SGBXF1_02556 SGBXF1_02658 SGBXF1_02742 SGBXF1_02743	*oppA*	Oligopeptide transport system substrate-binding protein	Oligopeptide transport system
SGBXF1_02741	*oppB*	Oligopeptide transport system permease protein	
SGBXF1_02740	*oppC*	Oligopeptide transport system permease protein	
SGBXF1_02739	*oppD*	Oligopeptide transport system ATP-binding protein	
SGBXF1_02738	*oppF*	Oligopeptide transport system ATP-binding protein	
SGBXF1_00149 SGBXF1_03507	*dppA*	Dipeptide transport system substrate-binding protein	Dipeptide transport system
SGBXF1_00150	*dppB*	Dipeptide transport system permease protein	
SGBXF1_00151	*dppC*	Dipeptide transport system permease protein	
SGBXF1_00152	*dppD*	Dipeptide transport system ATP-binding protein	
SGBXF1_00153	*dppF*	Dipeptide transport system ATP-binding protein	
SGBXF1_02675	*sapA*	Cationic peptide transport system substrate-binding protein	Cationic peptide transport system
SGBXF1_02676	*sapB*	Cationic peptide transport system permease protein	
SGBXF1_02677	*sapC*	Cationic peptide transport system permease protein	
SGBXF1_02678	*sapD*	Cationic peptide transport system ATP-binding protein	

SGBXF1_02679	*sapF*	Cationic peptide transport system ATP-binding protein	

Table S18- Genes involved in flavonoids, phenylpropanoids and other phenolics metabolism

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00927 SGBXF1_02885 SGBXF1_04589	*yhhW*	Quercetin dioxygenase	Is involved quercetin degradation, which is part of Flavonoid metabolism
SGBXF1_02439 SGBXF1_03028	*nodD*	Nodulation protein D	Regulator that binds flavonoids as inducers
SGBXF1_00893	*fdc*	Ferulate decarboxylase	Catalyzes the reversible decarboxylation of aromatic carboxylic acids like ferulic acid, p-coumaric acid or cinnamic acid
SGBXF1_00562-572	*hpaBC*	HPA monoxygenase	Hydroxylation of tyrosol and various cinnamic acid derivatives; Phenol oxidation to catechol
SGBXF1_03026	*curA*	NADPH-dependent curcumin reductase	Reduction of curcumin and other phenolics

Table S19- Genes encoding for lipolytic enzymes

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00070	*lip1*	Lipase*	Triglyceride lipase activity
SGBXF1_00549	*ytpA*	Monoacylglycerol lipase	Hydrolyzes glycerol monoesters of long-chain fatty acids
SGBXF1_02235	*lipA*	Triacylglycerol lipase	Triglyceride lipase activity
SGBXF1_03745-46	*phlAB*	Phospholipase A	Lipid degradation
SGBXF1_04161	*plcN*	Non-hemolytic phospholipase C	Hydrolyzes phosphatidylserine as well as phosphatidylcholine
SGBXF1_02568	*are*	Arylesterase	Degradation of various p-nitrophenyl phosphates, aromatic esters and p-nitrophenyl fatty acids in vitro
SGBXF1_00386	*yjfP*	Esterase	Activity toward palmitoyl-CoA and pNP-butyrate
SGBXF1_01173	*ybfF*	Esterase	Activity toward palmitoyl-CoA, malonyl-CoA and pNP-butyrate
SGBXF1_02199	*menI*	1,4-dihydroxy-2-naphthoyl-CoA hydrolase	Hydrolysis of 1,4-dihydroxy-2-naphthoyl-CoA and other acyl-CoA thioesters
SGBXF1_02306	*estB*	Esterase	Acts on short-chain (C4-C6) fatty acid esters and triglycerides, including tertiary alcohol esters
SGBXF1_04210	*yqiA*	Esterase	Activity toward palmitoyl-CoA, malonyl-CoA and pNP-butyrate.
SGBXF1_00207	*pldA*	Phosphatidylcholine 1-acylhydrolase	Lipid degradation
SGBXF1_00211	*pldB*	Lysophospholipase L2, Lecithinase B	Lipid degradation

*42% identity to *Xenorhabdus luminescens* lipase (Wang and Dowds, 1993).

Reference

Wang H, Dowds BCA. Phase variation in *Xenorhabdus luminescens*: Cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. *J Bacteriol* 1993; 175:1665–73.

Table S20- Genes encoding for extracellular proteases

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00223	*przN*	Serralysin*	Several proteolytic activities
SGBXF1_02114	*prtA*	Serralysin§	Inhibition of antibacterial peptides
SGBXF1_02115	*prtA*	Serralysin#	Inhibition of antibacterial peptides
SGBXF1_02407	*przN*	Serralysin**	Several proteolytic activities
SGBXF1_03669	*prtS*	Grimelysin	Actin degradation and possibly several proteolytic activities
SGBXF1_04648	*prtS*	Extracellular serine protease	Several proteolytic activities
SGBXF1_04649	*prtS*	Extracellular serine protease	Several proteolytic activities

*aprox. 92% identity
**aprox. 60% identity
to *S. marcescens* HR-3 serralysin with insecticidal activity (high doses) (Tao et al., 2006)

*aprox. 92% identity
**aprox. 59% identiity
to *S. marcescens* ATCC 21074 serralysin, UniProtKB - P07268 (PRZN_SERME), which allows an emerging moth to dissolve its cocoon.

*aprox. 62% identity
**aprox. 54% identity
to *Erwinia chrysanthemi* EC16 serralysin homolog which presents gelatinase activity, but is not involved in the virulence of this strain. Interestingly, high levels of serralysin decreased pectate lyase activity (Dhaler et al., 1990).

§aprox. 55% identity
#aprox. 68% identity
to *Photorhabdus* sp. Az29 proteases involved in the inhibition of insect antibacterial peptides. Reduces the antibacterial activity of *G. mellonella* hemolymph by 50%. Reduces the antibacterial activity of cecropin A by 80% and cecropin B by 75% (Cabral et al., 2004).

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Cabral CM, Cherqui A, Pereira A, Simões N. Purification and characterization of two distinct metalloproteases secreted by the entomopathogenic bacterium *Photorhabdus* sp. strain Az29.

Table S21- Genes involved in ROS stress response and protection

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_00084	*sodA*	Superoxide dismutase [Mn]	Destroys superoxide anion radicals
SGBXF1_02210	*sodB*	Superoxide dismutase [Fe]	
SGBXF1_02236	*sodC*	Superoxide dismutase [Cu-Zn]	
SGBXF1_03342	*katA*	Catalase	Protect cells from the toxic effects of hydrogen peroxide
SGBXF1_03212	*katG*	Catalase-peroxidase	
SGBXF1_03828	*ahpD*	Alkyl peroxidase	Peroxidase active against hydrogen and alkyl peroxides serves as peroxynitrite reductase
SGBXF1_02663	*tpx*	Thyol peroxidase	Removes peroxides or H2O2
SGBXF1_00141-42	*ohrBR*	Organic hydroperoxide resistance protein OhrB	Involved in organic hydroperoxide resistance
SGBXF1_04682	*hyPrx5*	Hybrid peroxiredoxin	Peroxidase and peroxiredoxin activity
SGBXF1_02246 SGBXF1_03110	*gstA*	Glutathione S-transferase	Defense against oxidative stress
SGBXF1_01597 SGBXF1_01654	*gstB*	Glutathione S-transferase	
SGBXF1_04252	*yfcF*	Glutathione S-transferase	
SGBXF1_01515-18	*gsiABCD*	Glutathione import system proteins	Glutathione import system
SGBXF1_02193	*gpx*	Glutathione peroxidase	Detoxification of Reactive Oxygen Species
SGBXF1_04122	*gshB*	Glutathione synthetase	Glutathione biosynthesis
SGBXF1_04644	*gor*	Glutathione reductase	Glutathione metabolism
SGBXF1_01616 SGBXF1_02224 SGBXF1_02847 SGBXF1_04725	*grxA*	Glutaredoxin	Cell redox homeostasis
SGBXF1_03829	*nrdH*	Glutaredoxin-like protein	
SGBXF1_00812-28	*arpe*	Arylpolyene	Involved in oxidative stress resistance
SGBXF1_03704	*hmp*	Nitric oxide dioxygenase	Nitrosative stress response
SGBXF1_02201-06	*sufABCDSE*	FeS cluster assembly proteins and cysteine desulfurase	Involved in oxidative stress resistance

Table S22- Phytohormone, polyamines and monoamine modulation genes

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_03481	*ipdC*	Indole pyruvate decarboxylase	IAA biosynthesis
SGBXF1_04163	*iaaasp*	IAA-aspartate hydrolase	Degradation of IAA-aspartate
SGBXF1_03089-03103	*paaABCDEFGHIJK*	1,2-phenylacetyl-CoA epoxidase and other elements	PAA degradation
SGBXF1_02759	*yvdD*	LOG family protein	Cytokinin production
SGBXF1_03911	*ygdH*	LOG family protein	Cytokinin production
SGBXF1_02336	*xdhA*	Xanthine dehydrogenase	Cytokinin modification

SGBXF1_02337	*xdhB*	Xanthine dehydrogenase	Cytokinin modification
SGBXF1_03348	*menF*	Isochorismate synthase	Salicylate biosynthesis
SGBXF1_03497	*entC*	Isochorismate synthase	
SGBXF1_03572	*pchB*	Isochorismate pyruvate lyase	
SGBXF1_04255	*puuE*	4-aminobutyrate aminotransferase	GABA degradation
SGBXF1_04254	*gabD*	Succinate-semialdehyde dehydrogenase	
SGBXF1_04074	*speA*	Arginine decarboxylase	Putrescine production via L-arginine
SGBXF1_04070	*speB*	Agmatinase	
SGBXF1_00454	*speC*	Ornithine decarboxylase	Putrescine production via L-ornithine
SGBXF1_04140	*speF*	Ornithine decarboxylase	
SGBXF1_04100-101	*speDE*	S-adenosylmethionine decarboxylase, Polyamine aminopropyltransferase	Spermidine biosynthesis
SGBXF1_03876-77	*cadAB*	Lysine decarboxylase Cadaverine/lysine antiporter	Cadaverine biosynthesis
SGBXF1_02442	*ddc*	L-2,4-diaminobutyrate decarboxylase	1,3-diaminopropane biosynthesis
SGBXF1_02443	*dat*	Diaminobutyrate--2-oxoglutarate aminotransferase	
SGBXF1_03604 SGBXF1_03660	*speG*	Spermidine N(1)-acetyltransferase	Protection against polyamine toxicity
SGBXF1_02813 SGBXF1_02814 SGBXF1_04618	*mdtJ*	Spermidine export protein	Protection against polyamine toxicity
SGBXF1_2080-84	*puuABCD*	Gamma-glutamyl-gamma-aminobutyrate hydrolase and others	Putrescine degradation, GABA formation
SGBXF1_03712	*patD*	Gamma-aminobutyraldehyde dehydrogenase	Putrescine degradation, GABA formation
SGBXF1_00557	*moaEF*	Monoamine oxidoreductase	Conversion of monoamine compounds or their metabolites

Table S23- Genes involved in mixed acid fermentation and VOC production

Locus tag/operon	Gene/operon	Product/putative product	Function/Putative function
SGBXF1_03381	*ackA*	Acetate kinase	Acetate formation
SGBXF1_02050	*icd*	Isocitrate dehydrogenase [NADP]	2-oxoglutarate formation
SGBXF1_04118	*fumA*	Fumarate hydratase class I, aerobic	Succinate formation
SGBXF1_02310	*fumC*	Fumarate hydratase class II	Succinate formation
SGBXF1_0274	*adhE*	Aldehyde-alcohol dehydrogenase	Ethanol formation
SGBXF1_01314	*dld*	D-lactate dehydrogenase	(*R*)-lactate formation
SGBXF1_02633	*ldhA*	D-lactate dehydrogenase	
SGBXF1_03968	*lldD*	L-lactate dehydrogenase	(*S*)-lactate formation
SGBXF1_03509	*budA*	Acetolactate synthase	Acetoin production
SGBXF1_03508	*budB*	α-acetolactate decarboxylase	
SGBXF1_04219	*dhaD*	Glycerol dehydrogenase	Glycerol fermentation Can also oxidize 1,2-propanediol and 2,3-butanediol
SGBXF1_03274 SGBXF1_03945	*dmsA*	Dimethyl sulfoxide/trimethylamine N-oxide reductase	DMS production
SGBXF1_04360-61	*mrsPQ*	Methionine-sulfoxide reductase	
SGBXF1_04667	*ilvE*	Branched-chain-amino-acid aminotransferase	Amino acid degradation
SGBXF1_00918	*ipdC*	Indole-pyruvate decarboxylase	Transforms pyruvate to acetaldehyde
SGBXF1_03481	*ipdC2*	Indole-pyruvate decarboxylase	
SGBXF1_02428	*adhP*	Alcohol dehydrogenase 1	Involved in the production of alcohols, aldehyde or ketone
SGBXF1_03723	*adhB*	Alcohol dehydrogenase 2	
SGBXF1_04185	*adh2* *ykhD*	Long-chain-alcohol dehydrogenase 2 Alcohol dehydrogenase	Involved in the production of long chain alcohols, aldehyde or ketone
SGBXF1_02745	*adhE*	Aldehyde-alcohol dehydrogenase	Involved in the production of alcohols, aldehyde or ketone
SGBXF1_01978	*xylB*	Benzyl alcohol dehydrogenase	Involved in the production of benzyl alcohols, benzyl aldehyde benzyl ketone

Table S24- Genes involved in fatty acid metabolism

Locus tag/operon	*Gene/operon*	Product/putative product	Function/Putative function
SGBXF1_04363-64	*accBC*	Acetyl-coa carboxylaseand carrier	Fatty acid biosynthesis
SGBXF1_03884	*accA*	Acetyl-coa carboxylase carboxyl transferase subunit alpha	
SGBXF1_03397	*accD*	Acetyl-coa carboxylase carboxyl transferase subunit beta	
SGBXF1_01919	*fabD*	Malonyl coa-acyl carrier protein transacylase	
SGBXF1_01918 SGBXF1_02920	*fabH*	3-oxoacyl-[acyl-carrier-protein] synthase III	
SGBXF1_00824 SGBXF1_03431	*fabB*	3-oxoacyl-[acyl-carrier-protein] synthase I	
SGBXF1_00827 SGBXF1_01714 SGBXF1_01922	*fabF*	3-oxoacyl-[acyl-carrier-protein] synthase II	
SGBXF1_00130 SGBXF1_00136 SGBXF1_00559 SGBXF1_00826 SGBXF1_01355 SGBXF1_01920 SGBXF1_03834 SGBXF1_04382	*fabG*	3-oxoacyl-[acyl-carrier protein] reductase	
SGBXF1_02405 SGBXF1_03889	*fabZ*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase [EC:4.2.1.59]	
SGBXF1_01716	*fabA*	3-hydroxyacyl-[acyl-carrier protein] dehydratase / trans-2-decenoyl-[acyl-carrier protein] isomerase	
SGBXF1_01972	*fabV*	Enoyl-[acyl-carrier protein] reductase / trans-2-enoyl-coa reductase (NAD+)	
SGBXF1_00680 SGBXF1_02496 SGBXF1_02808	*lcfH*	Long-chain acyl-coa synthetase Long-chain-fatty-acid--coa ligase	
SGBXF1_00870	*fadE*	Acyl-coa dehydrogenase	
SGBXF1_00279	*fadB*	Fatty acid oxidation complex subunit alpha	
SGBXF1_03441	fadJ	Fatty acid oxidation complex subunit alpha	
SGBXF1_00278 SGBXF1_03442	*fadA*	Acetyl-coa acyltransferase	
SGBXF1_03444	*fadL*	Long-chain fatty acid transport protein	

Methodology for the isolation of soil and plant-associated bacteria with phytohormone-degrading activities

Stock aliquots of ACC (0.3M) can be prepared by diluting ACC in water and then filter sterilizing the diluted ACC before storing the aliquots at -20ºC (for short term use) or -80ºC (for long term storage).

Stock aliquots of SA (0.1M) and IAA (0.1M) can be prepared by diluting SA or IAA in a 4:1 (v:v) solution of water and 1N NaOH and then filter sterilizing the diluted phytohormones and storing them at -20ºC.

Media, grams per liter:

Dwarkin and Foster (DF) minimal medium

Base:

KH_2PO_4 4.0 g
Na_2HPO_4 6.0 g
$MgSO_4$.$7H_2O$ 0.2g
Glucose 2.0 g
Gluconic acid 2.0 g
Citric acid 2.0 g
$(NH_4)_2SO_4$ (nitrogen source) 2.0 g (remove if testing other nitrogen sources).

Trace elements solution:

In 100 ml sterile distilled water
H_3BO_3 10 mg
$MnSO_4.H_2O$ 11.19 mg
$ZnSO_4.7H_2O$ 124.6 mg
$CuSO_4.5H_2O$ 78.22mg
MoO_3 10mg

Iron solution:

in 10 ml sterile distilled water
$FeSO_4.7H_2O$ 100 mg

Final Media:

Add **0.1 ml** of each of the solutions of trace elements and iron to the base medium, to a final volume of 1 liter.
Adjust pH to 7 with KOH.

M9 minimal medium

Na_2HPO_4 6.0 g
KH_2PO_4 3.0 g
NaCl 0.5 g
NH_4Cl 1g
pH 7.4

Tryptic Soy Agar (TSA);

Tryptone 17 g
Soy Peptone 3 g
NaCl 5 g
K_2HPO_4 2.5 g
Glucose 2.5 g
Agar 15 g

Yeast mannitol agar (YMA)

Yeast extract 1g
Mannitol 10 g
K_2HPO_4 0.5 g
$MgSO_4$ 0.2 g
NaCl 0.1
Agar 15 g

Actinomycete isolation agar (AIA)

Sodium caseinate 2.0 g
L-Asparagine 0.1 g
Sodium propionate 4.0 g
K_2HPO_4 0.5 g
$MgSO_4$ 0.1 g
$FeSO_4$ 0.001 g
Agar 15 g

***Pseudomonas* agar (King's B)**

Casein hydrolysate 10.0 g
Proteose peptone 10.0 g
K_2HPO_4 1.5 g
$MgSO_4$ 1.5 g
Agar 15.0 g
Glycerol 10 ml

Trinder's Color Reagent.

Dissolve 40g of mercuric chloride in 850 mL of water by heating. Cool the solution and add 120. mL of 1N HCL and 40g of ferric nitrate. When all the ferric nitrate has dissolved, dilute the solution to 1L with water.

Salkowski's Reagent

Mix 2 ml 0.5M $FeCl_3$ and 49 ml water and 49 ml 70% perchloric acid.

16S rRNA sequencing

The 16S rRNA gene sequencing of the bacterial strains was conducted following genomic DNA extraction from an overnight culture using the GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich, Germany) according to the manufacturer's instructions. The obtained DNA was sent to the Macrogen company (Korea), and amplified by PCR using primers 27F (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') following the Macrogen PCR amplification and sequencing protocol.

ITS sequencing

The ITS region sequencing of yeast and fungal strains was conducted following genomic DNA extraction from an overnight culture using the GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich, Germany) according to the manufacturer's instructions. The obtained DNA was sent to the Macrogen company (Korea), and amplified by PCR using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') following the Macrogen PCR amplification and sequencing protocol.

Genome sequencing and analysis

The genome sequencing of strains was conducted following genomic DNA extraction from an overnight bacterial culture using the GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich, Germany) according to the manufacturer's instructions. The DNA library was constructed using the Illumina's TruSeq DNA Nano kit (automated). The obtained libraries were sequenced using the Illumina MiSeq platform and MiSeq Reagent kit V3 (2x300 bp) (Illumina) in a barcoded run. The initial de novo genome assembly was performed using the SOAPdenovo v2.04 software (Luo et al., 2012). The final genome sequences were constructed based on a guided assembly against the complete chromosome sequences of the closest bacterial homologs using MAUVE 2.4.0 progressive alignments (Darling et al. 2004). The contigs were joined by introducing runs of 100 Ns in the assembly gap regions as indicated in the NCBI submission guidelines. The genome annotation was performed using the NCBI Prokaryotic annotation pipeline (Angiuoli et al. 2008). Functional genome annotation was performed in the BlastKOALA webservice (Kanehisa et al. 2016). Antibiotics and secondary metabolites analysis were performed in antiSMASH (Blin et al. 2017).

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