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Study of the genome of *Bacillus amyloliquefaciens* subsp. *plantarum* S499 and role of its plasmid (pS499) in rhizosphere interactions

Studio del genoma di *Bacillus amyloliquefaciens* subsp. *plantarum* S499 e ruolo del suo plasmide (pS499) nelle interazioni della rizosfera

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RIASSUNTO

La comprensione dei meccanismi che regolano le interazioni multitrofiche della rizosfera è fondamentale per migliorare l'efficacia dei biopesticidi in agricoltura. In quest'ottica, il sequenziamento dei genomi è un utile strumento per la caratterizzazione dei microrganismi di interesse agronomico. *Bacillus amyloliquefaciens* subsp. *plantarum* S499 è un rizobatterio che mostra un'attività di antagonismo diretto contro i funghi fitopatogeni e, inoltre, è particolarmente efficace come elicitore di resistenza sistemica indotta (ISR) nelle piante. Tali attività sono correlate alla produzione di metaboliti secondari bioattivi, quali i lipopeptidi ciclici appartenenti alle famiglie delle fengicine, iturine e surfattine.
Mediante sequenziamento, assembly e annotazione del genoma di S499, sono stati identificati i principali geni coinvolti nella colonizzazione radicale e nell'attività di stimolazione di crescita e di difesa delle piante. Questi geni condividono un'elevata percentuale di identità nucleotidica con i loro omologhi nel ceppo FZB42, il ceppo tipo della sottospecie batterica. Uno dei principali elementi genetici di distinzione tra S499 e FZB42 è la presenza di DNA extracromosomico: il plasmide pS499. La presenza di tale plasmide è stata evidenziata attraverso il sequenziamento del genoma, che ha permesso inoltre di identificare sul plasmide le sequenze codificanti per un sistema regolatore (Rap-Phr) coinvolto nel quorum sensing.
Attraverso un approccio di *plasmid curing*, è stata fatta una prima caratterizzazione funzionale di pS499. In primo luogo, è stato studiato l'impatto della perdita del plasmide sulla fisiologia del batterio, confrontando i comportamenti di S499, S499 P^- (ceppo senza plasmide) e FZB42 sul mezzo Luria-Bertani (LB). La crescita, l'attività proteolitica extracellulare e la modulazione della produzione dei lipopeptidi sono state significativamente influenzate dalla perdita di pS499. In accordo con un maggior rilascio di surfattina, è stato osservato anche uno *swarming* più rapido in S499 P^-, mentre la sua capacità di produzione di biofilm *in vitro* risultava ridotta. Non è stato invece osservato alcun effetto sull'evoluzione delle popolazioni batteriche *in planta* in termini di colonizzazione radicale, nonostante un'accentuata produzione di surfattine sulle radici di pomodoro da parte di S499 P^- rispetto a S499 e FZB42. I risultati della quantificazione dell'espressione relativa dei geni *srfA* e *rap* suggeriscono un effetto inibitore della sintesi di surfattina da parte del sistema Rap-Phr codificato dal plasmide. Inoltre, su LB, l'attività antagonistica contro i funghi fitopatogeni era limitata per S499 P^-, molto probabilmente a causa di una verificata riduzione della secrezione di iturine. Benché in modo meno evidente, un effetto simile è stato osservato su un mezzo (RE) che riproduce la composizione tipica degli essudati radicali. Globalmente, i risultati ottenuti mostrano che pS499 regola diversamente il fenotipo di S499 a seconda del contesto nutrizionale. Ulteriori prove sono necessarie per dimostrare che pS499 è importante per la fitness del rizobatterio nel suo habitat naturale.

ABSTRACT

Understanding how soil-borne microorganisms can modulate the plant defence responses and which factors affect rhizosphere multitrophic interactions is crucial to improve the efficacy of biopesticides in agriculture. From this perspective, whole-genome sequencing is a powerful tool to characterize the bacterial strains of agronomic interest. Among these, *Bacillus amyloliquefaciens* subsp. *plantarum* strain S499 is a plant-beneficial rhizobacterium that shows direct antagonistic properties against phytopathogenic fungi and, in addition, a remarkable efficacy as elicitor of induced systemic resistance (ISR) in plants. In these activities, the production of bioactive secondary metabolites, such as cyclic lipopeptides belonging to the fengycin, iturin and surfactin families, is involved.
By sequencing, assembling and annotating S499 genome, we identified the principal genes involved in root colonization, plant-growth promotion and biocontrol activities. These genes share a high percentage of nucleotide identity with their homologs in the strain FZB42, the type strain of the bacterial subspecies. One of the main genetic elements distinguishing S499 from FZB42 is the presence of extrachromosomal DNA (plasmid pS499). This small rolling circle plasmid was unknown before S499 genome sequencing, which also allowed to identify on pS499 the genes encoding a Rap-Phr regulatory system involved in quorum sensing.
Through a plasmid-curing approach, we carried out a functional characterization of pS499. First, we studied the impact of the plasmid loss on the bacterial physiology, by comparing the behaviours of S499, its plasmid-cured derivative, S499 P^-, and FZB42 on Luria-Bertani (LB) medium. Growth rate, extracellular proteolytic activity and the regulation of lipopeptide production were significantly affected in S499 P^-. In agreement with an increased release of surfactins, swarming motility improved after curing, whereas biofilm production was reduced *in vitro*. When the evolution of bacterial populations was compared *in planta*, pS499 seemed not to influence the root colonization ability, although we observed an over-production of surfactins by S499 P^- also on tomato roots. The quantification of the relative expression of *srfA* and *rap* genes suggested an inhibitory effect of the plasmid-encoded Rap-Phr system on surfactin synthesis. Moreover, on LB, the antagonistic effect against phytopathogenic fungi was limited for S499 P^-, most probably due to a verified reduction of iturin secretion. Although less clearly, an impact of plasmid curing on the biocontrol ability was observed also on a medium (RE) that reproduced the typical composition of plant root exudates. Globally, our results show that pS499 differently modulates S499 phenotype depending on the nutritional context. More evidences are required to prove that pS499 is relevant for the fitness of the rhizobacterium in its natural environment.

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Chapter 1

INTRODUCTION

1.1 Plant-growth-promoting rhizobacteria

The rhizosphere is the portion of soil near plant roots that is directly influenced by their growth, respiration, and nutrient exchanges. It is a highly dynamic environment that actually represents a hotspot of biodiversity compared with the surrounding bulk soil: up to 10^{11} microbial cells and, among these, more than 30 thousand bacterial species can be found around each gram of root (Egamberdieva, *et al.*, 2008; Mendes *et al.*, 2011). Plants can modulate the composition and the activity of the rhizosphere community through the exudation of metabolites and signalling molecules (Doornbos *et al.*, 2012).

Beneficial rhizosphere-associated microbes constitute the root microbiome (**Figure 1**), which contributes to the plant health as the gut microbiome does in animals (Mendes *et al.*, 2013). Indeed, rhizobacteria and mycorrhizal fungi can improve plant nutrition and often grant disease protection (Berendsen *et al.*, 2012).

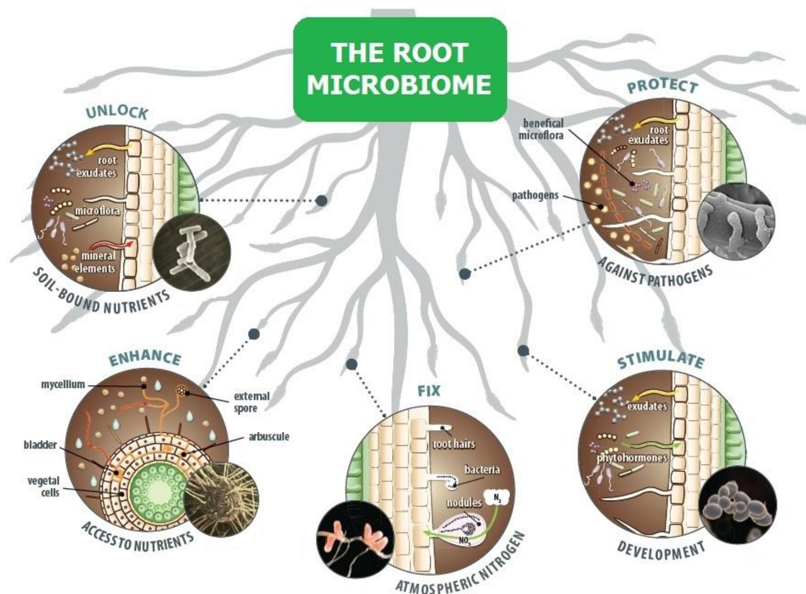

Figure 1: The root microbiome. Plant-associated microorganisms carry out multiple beneficial functions at the rhizosphere level (modified from http://www.lallemandplantcare.com).

Vice versa, soil-borne phytopathogens negatively affect plant health, by damaging the plant through production of phytotoxic substances or tissue infection. In this group, pathogenic fungi, oomycetes and bacteria are included (Mendes *et*

al., 2013). Other microorganisms establish neutral interactions, either with the plant or with the pathogens, as they occupy different ecological niches; however, these commensal microbes can influence other organisms and eventually produce an effect on the complex rhizosphere networks (Berendsen *et al.*, 2012). Such multifaceted interactions occurring below ground play a fundamental role in several aspects of the ecosystem, from soil stability to plant productivity (Haichar *et al.*, 2008).

Most of beneficial plant-microbe relationships are mutualistic or associative symbioses, as plants and microorganisms shared costs and benefits of the association (Vacheron *et al.*, 2014) The expression “plant-growth-promoting rhizobacteria” (PGPR) designates the prokaryotic microorganisms that inhabit the rhizosphere and are beneficial for the host plants (Lugtenberg and Kamilova, 2009). Indeed, PGPR can affect plant development either directly, *e.g.* by improving plant mineral uptake, or indirectly, by conferring protection against soil-borne diseases and abiotic stressors, or also in both ways (**Table 1**). In a broad sense, the well-characterized rhizobia providing nitrogen to leguminous host plants are included among PGPR (Gray and Smith, 2005).

Table 1. Principal modes of action of plant-growth-promoting rhizobacteria.

Direct mechanisms	Indirect mechanisms	
Nitrogen fixation	Detoxification	
Phosphate solubilisation	Enhancement of stress resistance	
Iron solubilisation	Biocontrol	
Phytohormones production	*Direct*	Competition
ACC[a] deaminase activity		Antagonism
Stimulation by AHLs[b]		Hyperparasitism
Stimulation by VOCs[c]	*Indirect*	ISR[d]

[a]ACC: 1-aminocyclopropane-1-carboxylate; AHLs; [b]N-acyl-L-homoserine lactones; [c]VOCs: volatile organic compounds; [d]ISR: induced systemic resistance.

Depending on their level of association with root tissues, PGPR can be classified into extracellular PGPR (ePGPR) and intracellular PGPR (iPGPR), the former locating on the rhizoplane or between the cells of root cortex, while the latter

colonizing specialized nodular structures of the root cells (Bhattacharyya and Jha, 2012). The genera *Agrobacterium*, *Arthrobacter*, *Azoarcus*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, C*hromobacterium*, *Clostridium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Micrococcus*, *Pseudomonas*, and *Serratia* encompass several beneficial strains that mostly belongs to ePGPR, whereas those belonging to the family of Rhizobiaceae (*Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, etc.) and *Frankia* spp. are regarded as iPGPR (Gray and Smith, 2005). Among all these genera, *Bacillus*, *Pseudomonas* and *Rhizobium* have been the most studied so far. The regulation of the symbiotic processes is known in the case of the rhizobia-legumes relationship, where bacteria utilize the carbohydrates synthestized by the host and, in turn, they supply ammonia and amino acids to the plant. Nonetheless, PGPR are able to exert their positive effects on many crop plants, including wheat, maize, potato and many other vegetables (Rai, 2006; Lugtenberg and Kamilova, 2009; Bhattacharyya and Jha, 2012).

1.1.1 Rhizosphere competence

1.1.1.1 The process of root colonization

Rhizosphere competence is an essential prerequisite for PGPR. It involves the active root colonization, combined with the ability to survive and multiply on the growing roots over a period of time, dealing with other microorganisms that share the same ecological niche (Kumar *et al.*, 2011). Generally, microbes are attracted by the nutritious environment created by roots through a phenomenon known as rhizodeposition (Haichar *et al.*, 2008). Plant roots secrete a variety of organic compounds, including sugars, phenolic compounds, nucleotides, amino acids, sterols, vitamins and organic acids, in different proportions. In particular, root border cells can release in the rhizosphere large amounts of carbon-rich material (Hawes *et al.* 1998). However, although this nutritious environment favours microbe proliferation, the populations of rhizobacteria do not attain the same levels than in nutrient-rich artificial media and, for this reason, their lifestyle is regarded as a starvation status (Lugtenberg and Kamilova, 2009). In this context, the species

that can better metabolize the principal exudate components, like some *Pseudomonas* spp. that are able to efficiently metabolize carbon sources and have to compete for only few compounds, achieve the best root colonization (Somers *et al.*, 2004).

Motility constitutes a major trait for root colonization. Bacteria employ several mechanisms to move, which are known as swarming, swimming, twitching, gliding and sliding (Kearns, 2010). Swarming and swimming are flagella-driven movements, involved in root colonization by PGPR, the former occurring in a multicellular way across a surface while the latter is characterising single cells in a liquid medium. Swimming motility is oriented by chemotaxis, *i.e.* the direction of the movements depends on the concentration of certain compounds. For example, malic and citric acids present in the root exudates of *Arabidopsis thaliana* and tomato (*Solanum lycopersicum* L.) are chemoattractants for *Bacillus* and *Pseudomonas* spp. (de Weert *et al.*, 2002; Rudrappa *et al.*, 2008).

As root exudates composition differs among plant species, a putative role in determining host specificity has been ascribed to chemotaxis. For example, in the bacterial genus *Azospirillum* the chemotactic responses differ from strain to strain and this specificity relies on the ecological origin of the strains (Drogue *et al.*, 2012). The relationship between PGPR and chemotaxis towards root exudate components of their original hosts was confirmed also for *Bacillus* spp. (Zhang *et al.*, 2014). However, the composition of root exudates depends not only on the plant species, but also on the growth substrate and on the developmental stage and physiological conditions of the plant. Interestingly, tomato plants recruit beneficial bacteria by enhancing the release of malic acid when attacked by pathogens (Rudrappa *et al.*, 2008; Lugtenberg and Kamilova, 2009).

However, swarming motility plays a greater role than chemotaxis in tomato root colonization, at least for some strains of *B. subtilis* (Gao *et al.*, 2016). Indeed, PGPR usually spread over the root surfaces in the form of biofilm, rather than as planktonic cells in a liquid medium (Ongena and Jacques, 2008). Biofilms are single or multispecies aggregates of microbial cells encapsulated in a matrix of extracellular polymeric substances and adherent to biotic or abiotic surfaces. In

biofilms, PGPR can better protect themselves from environmental stresses and microbial competition (Seneviratne *et al.*, 2010).

Adsorption and anchoring are two independent mechanisms used by PGPR for the adhesion on the rhizoplane. Adsorption is a weaker interaction mediated by type I and IV pili, fimbriae and specialized surface proteins that function like adhesins (*e.g.* flagellins). Lipopolysaccharides, exopolysaccharides and capsular polysaccharides are involved instead in bacterial anchoring to root surfaces, where specific receptors are located. Indeed, genetic determinants of both partners are putatively involved in these processes (Dutta and Podile, 2010; Drogue *et al.*, 2012). According to Compant *et al.* (2010), the ability to synthesize vitamin B1, NADH dehydrogenases, outer membrane proteins, and a site-specific recombinase involved in phase variation are other bacterial traits responsible for rhizosphere competence.

PGPR establish their populations mainly in the junctions between epidermal cells and the points where lateral roots emerge (Lugtenberg and Kamilova, 2009). Root tips and root hair regions, which are other zones of extensive release of exudates, are preferential sites of colonization as well. Depending on the plant species, one strain can display different colonization patterns (Drogue *et al.*, 2012). Some *Azospirillum* spp. are supposed to enter the intercellular spaces upon the enzymatic degradation of plant cell wall middle lamellae, through β-glucosidase, cellulolytic and pectinolytic activity (Khammas and Kaiser, 1991; Bekri *et al.*, 1999; Faure *et al.*, 2001;). As reported above, iPGPR colonize also internal tissues, behaving as endophytes, thus they must overcome the plant defence mechanisms. During the establishments of these associations, root-bacterium communication plays a fundamental role (Somers *et al.*, 2004).

The crosstalk between leguminous plants and symbiotic rhizobia probably represents the best-characterised example of signal exchange in the process of root colonization (Cooper, 2007). The plant releases flavonoid compounds that induce the bacterium to secrete Nod factors, in the form of lipo-chitooligosaccharides. Nod factors are perceived through specific LYK receptors by plant root hairs and stimulate the formation of root nodules, where rhizobial populations can settle and fix the atmospheric nitrogen (**Figure 2**). Different rhizobia secrete structurally

diverse Nod factors, which suggests that the plant host can recognize the signal produced by the compatible rhizobial symbiont (Van Loon, 2007; Liang *et al.*, 2014).

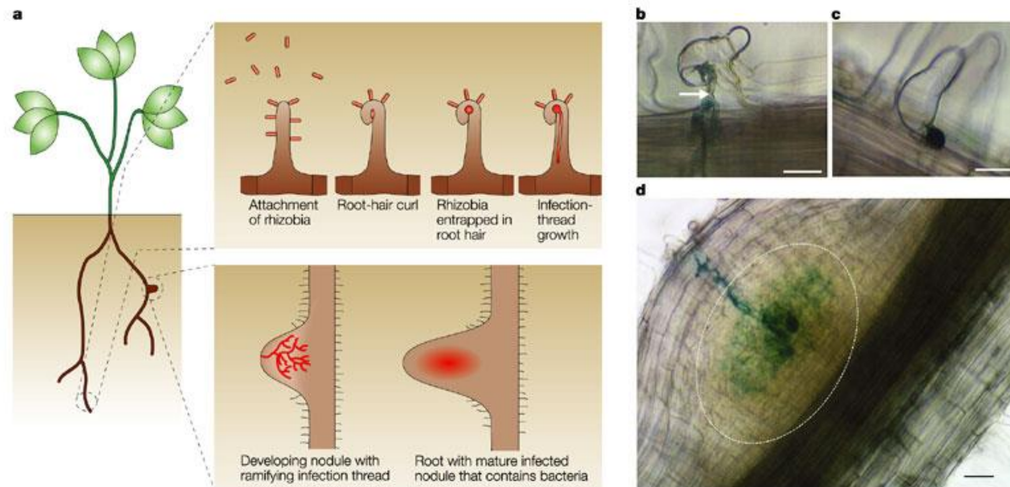

Figure 2: The formation of root nodules. **(a)** The early stages of infection of root hairs by rhizobia and the growth of a nodule on roots. Images **(b)**, **(c)** and **(d)** were taken from *Pisum sativum* or *Vicia hirsuta* inoculated with a *lacZ*-constitutively expressing strain of *Rhizobium leguminosarum*. Root samples were stained for β-galactosidase, showing bacterial cells in blue. **(b).** A normal infection thread (arrow) is shown. **(c)** A *nodO nodE* double mutant of *R. leguminosarum* that forms infection foci but not an infection thread. **(d)** Infection threads grow through root cells to the growing nodule primordium (circled). The bars represent 20 µm in **(b)** and **(c)** and 100 µm in **(d)** (Oldroyd and Downie, 2004).

1.1.1.2 Quorum sensing in the rhizosphere

Bacteria can regulate the expression of certain cellular functions in a population density-dependent way: the quorum sensing (QS; Bassler, 1999). In bacterial communication, the term “autoinducers” refers to the signalling molecules that are released in the medium and act via QS. A threshold concentration of an autoinducer is responsible for the activation or repression of a determined molecular pathway that leads to the quorum response (Bassler, 1999). In this way, bacteria can synchronize particular behaviours on a population-wide scale. Several aspects related to the rhizosphere competence of PGPR, such as biofilm formation and swarming motility, are controlled by QS (Compant *et al.*, 2010; Dutta and Podile, 2010).

The best-characterized autoinducers for Gram-negative species, like *Pseudomonas* spp., are N-acyl-L-homoserine lactones (AHLs), which vary based on the length, substitution and saturation of their acyl side chains. These differences in structure confer specificity to the signalling molecules (Auchtung, 2006). Secreted AHLs increase in concentration with increasing cell density and, once imported in the cytoplasm, they interact with their cognate LuxR receptors, which are cytoplasmic transcriptional activators or repressors (Waters and Bassler, 2005). Diketopiperazines are also implicated in QS in Gram-negative bacteria, whereas γ-butyrolactone and modified oligopeptides recognized by two-component sensor kinases are autoinducers for Gram-positive species, such as *Bacillus* spp. (Auchtung, 2006; Dutta and Podile, 2010). The best-known QS system in *B. subtilis* is the ComQXPA system, which is mainly involved in coordinating developmental pathways, producing extracellular products in effective concentration and surviving under competitive conditions (Comella and Grossman, 2005).

Rhizobacteria can perceive autoinducers produced by other bacterial species (Steidle *et al.*, 2001), interfere with their signalling processes through a quorum quenching mechanism (Dong *et al.*, 2002) or respond to QS-like molecules released by plants (Teplitski *et al.*, 2000). Indeed, besides regulating cell responses on a population-wide scale, quorum sensing allows communication within and between species. For instance, several plants can recognize AHLs and consequently modulate defence and growth responses (Ortíz-Castro *et al.*, 2008), as for *Medicago truncatula*, which produces proteins involved in isoflavone production, stress response and cytoskeleton structure (Dutta and Podile, 2010).

The rhizosphere contains a greater concentration of AHL-producing bacteria than bulk soil, suggesting that they play a role in colonization (Elasri *et al.*, 2001). The importance of QS in root colonization was demonstrated with a *LuxR*-mutant strain of *P. fluorescens* that was impaired in biofilm formation and colonization of wheat rhizosphere (Wei and Zhang, 2006). Conversely, derivative of *Serratia* spp. not producing AHLs were not impaired in colonization ability of tomato and oilseed rape roots (Schuhegger *et al.*, 2006; Müller *et al.*, 2009). However, it might be that QS is indirectly involved in rhizosphere competence, by influencing the competitive ability of PGPR under natural conditions (Compant *et*

al., 2010). For example, in *B. subtilis*, quorum responses contribute to the production of lytic enzymes and antibiotics, which plays fundamental roles in microbial competition (Comella and Grossman, 2005).

Figure 3 summarizes the discussed relevant traits involved in PGPR host recognition and colonization.

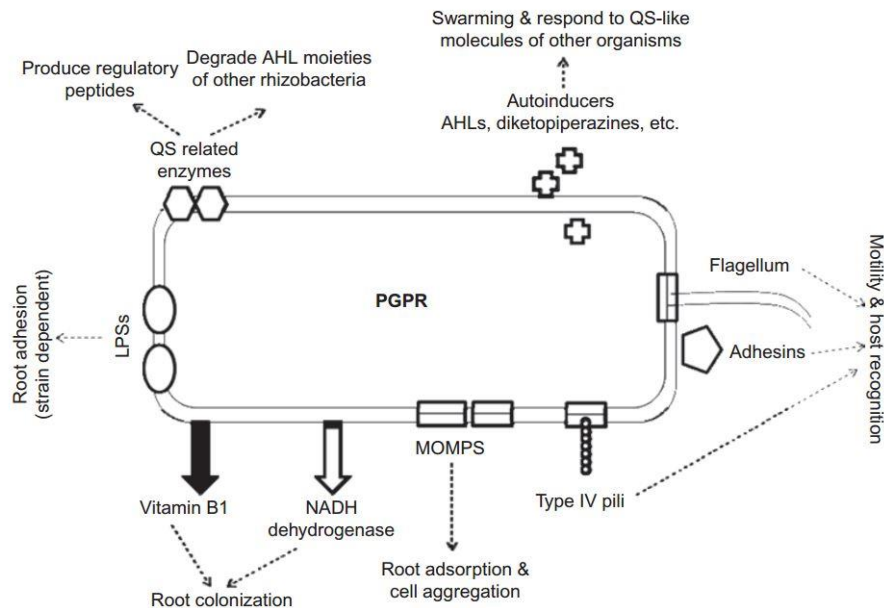

Figure 3: Different molecules produced by plant-growth-promoting rhizobacteria (PGPR) that are involved in host root recognition and colonization. QS: quorum sensing; AHLs: N-acyl-L-homoserine lactones; MOMPs: major outer membrane proteins; LPSs: lipopolysaccharides (Dutta and Podile, 2010).

1.1.2 Modes of action of PGPR

1.1.2.1 Improvement of plant nutrition

PGPR can enhance nutrient status of host plants by different mechanisms, namely the fixation of atmospheric nitrogen (N_2), the increase of the availability of minerals in the rhizosphere, the augmentation of root surface area, and the promotion of other beneficial symbioses of the host (Bhattacharyya and Jha, 2012). Often, more than one mechanisms is involved, as in the case of *Azospirillum* spp. that, besides being capable of fixing N_2, also increase water and mineral uptake by increasing root development (Lugtenberg and Kamilova, 2009).

Atmospheric nitrogen fixation and the solubilisation of inorganic nutrients, such as phosphates and iron, are widely distributed among *Allorhizobium, Azorhizobium*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Rhizobium*, *Enterobacter*, *Mesorhizobium*, *Pseudomonas*, and *Sinorhizobium* spp. (Vessey, 2003). The ability of PGPR to solubilize mineral phosphate is very attractive, as plants can absorb phosphorous in only two soluble forms, the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions, but levels of soluble phosphate are limited in soil (Glass, 1989). Secretion of organic acids or protons, chelation and exchange reactions are processes that favour phosphorous solubilisation (Bhattacharyya and Jha, 2012). Moreover, the production of enzymes, such as phytases, phosphatases, and C-P lyases, enables bacteria to release soluble phosphorus from organic compounds, like organophosphonates (Rodríguez *et al.*, 2006).

Similarly, PGPR facilitate the acquisition of iron by plant roots (Lugtenberg and Kamilova, 2009). Iron is another essential nutrient with little availability in soil. Ferric (Fe^{3+}) ions readily precipitate in oxidised forms and to avoid precipitation plants usually secrete organic chelators. Next to root surfaces, Fe^{3+} is reduced to ferrous ion (Fe^{2+}), which is immediately absorbed. Alternatively, plants can produce siderophores, which chelate Fe^{3+} and are then imported into the cells. Some bacterial species also produce siderophores and a number of plants is able to assimilate them, although most of research on microbial siderophores in the rhizosphere focuses on their role in the competition against plant pathogens (Vessey, 2003).

1.1.2.2 Production of plant growth regulators

PGPR can directly improve plant growth through the production of phytohormones and the release of enzymes, such as the 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Van Loon, 2007). By producing phytohormones PGPR can modify root morphology. For instance, *Azospirillum brasilense* produces indole-3-acetic acid, an auxin that stimulates lateral root formation (Tien *et al.*, 1979). Strains of *P. fluorescens* can induce seedling emergence and increase root length of several crop species by producing cytokinins, instead (Bent *et al.*, 2001; García de Salamone *et al.*, 2001). Moreover, the genus *Bacillus* encompasses strains

that can release high levels of gibberellins, which promote stem and shoot elongation (Gutiérrez-Mañero *et al*., 2001; Gray and Smith, 2006).

As reported above, AHLs are also elicitors of plant growth, as well as some volatile organic compounds (VOCs), like acetoin and 2,3-butanediol, which are produced by *Bacillus* spp. and have been demonstrated to have a positive effect on both plant and bacterial fitness (**Figure 4**) (Ryu *et al.*, 2003; Yi *et al.*, 2016).

Figure 4: Plant-growth-promotion through volatiles. Effect of VOCs produced by *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 on *Arabidopsis thaliana* growth (negative control below) (Giulia Molinatto, 2015).

Ethylene, on the contrary, inhibits shoot and root elongation and accelerates senescence (Abeles *et al.*, 2012). The direct precursor of ethylene in plants is 1-aminocyclopropane-1-carboxylate (ACC); therefore, PGPR able to synthesize ACC deaminases can reduce endogenous levels of this hormone in favour of root growth (Van Loon, 2007). Bacterial populations benefit from this interaction as well, since the hydrolyzation of ACC provides sources of nitrogen and carbon (ammonia and α-ketobutyrate; Doornbos *et al.*, 2012).

1.1.2.3 Biocontrol of plant diseases

PGPR can promote plant growth indirectly by enhancing stress tolerance or by detoxifying heavy metals and pathogen virulence factors (*e.g.* albicidin produced by *Xanthomonas albilineans*, fusaric acid by *Fusarium* spp.; Compant *et al.*, 2005.). Most importantly, diverse mechanisms conferring plant disease protection were reported. It is worthy to recall here that the term “biological control”, or “biocontrol”, in plant pathology refers to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations. The “biological control agent” (BCA) is the organism that antagonizes the pathogen (Pal and Gardener, 2006).

Through different kinds of interactions, such as competition, antagonism and hyperparasitism, BCAs can influence the pathogens' population density and their temporal and spatial dynamics. Alternatively, beneficial microorganisms and/or some compounds they release are perceived as elicitors of immune responses by the plant (Raaijmakers *et al.*, 2009).

1.1.2.3.1 *Competition, antagonism and hyperparasitism*

Competition takes place for space and nutrients when pathogenic and beneficial microorganisms develop in the same microbial ecological niche. For example, colonization of the rhizosphere microenvironment is a prerequisite for the efficacy of soil-borne BCAs (Lemanceau and Alabouvette, 1991). Nutritional competition for carbon compounds and for soluble iron are essential modes of action for a number of biocontrol fungi and bacteria. As already discussed, iron is a limiting factor in soil because of its low solubility, and the ability to secrete siderophores that can be assimilated through specific receptors constitutes a determining advantage for some *Pseudomonas* strains (Doornbos *et al.*, 2012). The ability to use nitrate as an alternative electron acceptor is also involved in competitiveness: being able to switch from one metabolic pathway to another, some strains can dominate in an environment that might be depleted of oxygen by root respiration (Somers *et al.*, 2004).

Antagonism is mainly based on the secretion of secondary antimicrobial metabolites (antibiosis) and lytic enzymes (predation) active on phytopathogens. A variety of antibiotics and bacteriocins is known to be involved in biocontrol, but for many of these molecules the mechanism of action has not been elucidated yet. The targets may be metalloenzymes like cytochrome c oxidases (hydrogen cyanide), the electron transport chain (phenazines, pyrrolnitrin), or cell membrane and zoospores of phytopathogenic fungi and oomycetes (2,4-diacetylphloroglucinol, cyclic lipopeptides; Raaijmakers *et al.*, 2009). The regulation of antibiotic synthesis is related to the overall metabolic status of the cell, which in turn depends on nutrient availability, pH, temperature, and various other parameters (Milner *et al.*, 1995; Duffy and Défago, 1999; Ownley *et al.*, 2003). In addition, some antagonists can attack the cell wall of pathogens through the release of cellulases, chitinases and

proteases. Otherwise, extracellular lytic enzymes can neutralize virulence factors, or degrade autoinducers, hence conferring plant disease protection in an alternative way (Compant *et al*., 2005).

Hyperparasitism is another form of direct biocontrol against soil-borne phytopathogens, even if it is better characterized in the context of fungal BCAs. For example, *Trichoderma* mycelia can penetrate the hyphae of pathogenic fungi, after inducing a cell wall damage through the production of endochitinases (Harman, 2006). Nonetheless, some bacteria can grow at the expense of living fungi through a phenomenon known as bacterial mycophagy (Leveau and Preston, 2008). For example, *Collimonas fungivorans* feeds on fungal hyphae under nutrient-poor conditions, although its biocontrol activity seems to depend mostly on a mechanism of competition (Kamilova *et al.*, 2007).

1.1.2.3.2 *Induced systemic resistance*

Systemic resistance is a broad-spectrum immune reaction that makes plants less susceptible to subsequent attacks in distal tissues. This effect is evident when the root colonization by plant-beneficial microorganisms has a positive outcome against aboveground pathogens. Induced systemic resistance (ISR) has been discovered 25 years ago when it was noticed that some beneficial *Pseudomonas* strains could reduce disease symptoms even when maintained separated from the pathogens (Van Peer *et al*., 1991; Wei *et al*., 1991). The phenomenon revealed a plant-mediated protective effect of the application of BCAs, which was later confirmed in several other genera of bacteria such as *Bacillus* and *Serratia* (Lugtenberg and Kamilova, 2009). Upon root colonization by ISR-inducing rhizobacteria, the plant falls in a unique physiological state known as "priming" (Conrath *et al.*, 2006).

In many cases, the microbial determinants involved in ISR are known. The list includes lipopolysaccharides, siderophores, flagella, some antibiotics, biosurfactants and volatile organic compounds (Bakker *et al.*, 2013). These factors are named "microbe-associated molecular patterns" (MAMPs), to be distinguished from "pathogen-associated molecular patterns" (PAMPs) and "damage-associated molecular patterns" (DAMPs); all are elicitors of plant defences. PAMPs and

DAMPs trigger systemic acquired resistance (SAR), while MAMPs induce ISR. Plant hormones play a significant role in the signalling network that coordinates plant defences: if SAR mainly depends on salicylic acid, in ISR jasmonic acid and ethylene are primarily involved. Both pathways then converge by being controlled by the same transcription factor NPR1 (Henry *et al.*, 2012).

The root-specific transcription factor MYB2 emerged as an early node of convergence in the ISR-inducing pathways (Segarra *et al.*, 2009). Interestingly, the Arabidopsis mutant *myb72* that was impaired in ISR also displayed a reduced colonization by *Pseudomonas* strains that are able to trigger ISR compared to the wild type (Doornbos *et al.*, 2009), suggesting the occurrence of a cross communication among roots and beneficial bacteria (Bakker *et al.*, 2013).

1.1.3 The use of plant-beneficial microorganisms for soil fertilization and crop protection

The demand for food production is expected to increase by 70% in 2050, because of the growth of global population and change of diet in developing countries (Popp *et al.*, 2013). The Food and Agriculture Organization (FAO) defines food security as “the possibility to have access to sufficient, safe and nutritious food to meet dietary needs and food preferences for an active and healthy life” (Flood, 2010). To guarantee global food security for a growing population we need to maximize productivity while minimizing the resources required. Soil microorganisms (bacteria, but also fungi and protozoa) promoting plant growth can help in this way, as they can be used in agriculture as biofertilizers (Vessey, 2003; Rai, 2006). Mainly by enhancing the efficacy of mineral uptake (nitrogen, phosphorous and potassium) by plants, such useful microorganisms can play a fundamental role in the crop productivity, promoting at the same time an agriculture system with a lower environmental impact (Malusà *et al.*, 2016).

Since plant pathogens represent one of the major threats to crops worldwide, plant disease protection is of crucial importance in agriculture. The global potential yield loss due to plant diseases is estimated around 16% and it is increasing, because the producers often choose to cultivate varieties that provide greater yields but are less resistant to pathogens, and also because of the more and more frequent trade

and travel exchanges (Anderson, 2004; Oerke, 2006). Therefore, a reduction of crop losses through efficient strategies of plant disease protection is now more than ever required.

When pathogens cannot be controlled by agronomic practices or the use of resistant varieties, the application of pesticides becomes necessary. Chemical pesticides are applied on many crops as the main method of protection from pathogens and from their vectors (insects and nematodes) (Strange and Scott, 2005). However, this extensive use of targeted products may lead to the emergence of resistance in pathogen's population, which compromises their efficacy. Moreover, a reduction of chemical pesticides would meet the demand for a sustainable agriculture, meaning the adoption of farming techniques that preserve the environment and the health of growers and consumers, allowing future generations to do the same. Biological control, especially in a context of integrated pest management strategy, is a valid alternative to chemicals, although much effort is still required to improve the efficacy and the applicability of biopesticides (Leadbeater and Gisi, 2010).

Although the use of plant-beneficial microorganisms may have great advantages in terms of lowering the negative impact of plant disease control, it has also several limitations, as inconsistent efficacy under field conditions and costs of development and registration (Fravel, 2005). A better understanding of the biology of the complex interactions occurring in soil is an important step towards an improved use of such valuable tools.

1.2 *Bacillus* genus as source of plant-beneficial strains

The genus *Bacillus* is one of the most important genera of PGPR and encompasses a wide variety (in terms of genetics and ecology) of Gram-positive, aerobic, rod-shaped bacteria, which can form robust dormancy structures called endospores (Turnbull, 1996). This is an important trait in the development of biopesticides, because spore formation facilitates the storage of the product by extending the shelf life and makes the inoculants more resistant to environmental stress. Moreover, some *Bacillus* spp. are facultative anaerobes and are able to adapt to oxygen limitation, which is another advantage for their survival in soil (Nakano and Hulett, 1997).

Several species, like *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. pumilus*, *B. subtilis* and *B. sphaericus*, include strains known for their biocontrol and/or plant-growth-promoting potential, among which some have already been commercialized as foliar or soil inoculants (Pérez-García *et al.*, 2011). *Bacillus thuringiensis* has been used as biopesticide since the beginning of 20th century, thanks to its insecticidal toxins (Cry proteins). It later became the source of genes for the genetically modified "Bt" crops resistant to insects. Nowadays, it shares more than 70% of the total market of BCAs *sensu lato* (Sanauhja *et al.*, 2011). Other *Bacillus*-based products account for about half of the remaining sales (Choudhary and Johri, 2009; Cawoy *et al.*, 2011). Several of these bioformulates (*e.g.* Ballad® and Sonata® with *B. pumilus*, Serenade® with *B. subtilis*) are used to control diseases, such as rusts, downy and powdery mildews, on many vegetables and fruit crops.

The plant-beneficial effects of *Bacillus* spp. rely on the combined action of multiple mechanisms, from the improvement of plant nutrition to the biocontrol activity in both ISR-independent and dependent way (**Figure 5**). These mechanisms mostly depend on the efficient production of a broad range of secondary metabolites, which are involved not only in the antibiotic activity of the bacteria but also in the interactions with the host plant and in the processes that favour niche colonization (Ongena and Jacques, 2008; Kumar *et al.*, 2011).

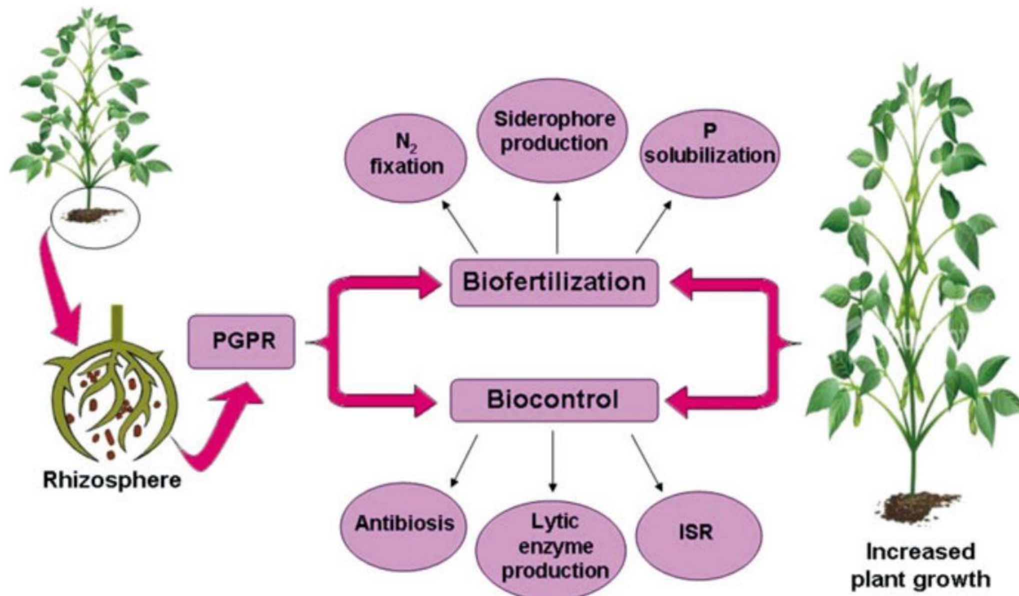

Figure 5: Modes of action of *Bacillus* PGPR. Plant growth promotion by rhizosphere *Bacillus* spp. results from a combination of multiple mechanisms (Kumar *et al.*, 2011).

1.2.1 Structures and roles of *Bacillus* secondary metabolites

Members of *Bacillus* genus are known to be good producers of bioactive molecules. Indeed, up to 8.5% of the bacterial genome can be devoted to the synthesis of secondary metabolites, as for *B. amyloliquefaciens* subsp. *plantarum* FZB42 (Chen *et al.*, 2009). A large part of those molecules is non-ribosomally synthesized by complex enzymatic machineries (Sieber and Marahiel, 2003). Some antibiotics are synthesized by ribosomes as linear precursor peptides and subsequently processed through proteolysis and post-translational modifications (Stein, 2005).

1.2.1.1 Bacteriocins

The term “bacteriocins” refers to a heterogeneous group of antimicrobial peptides produced by ribosomal synthesis (Riley and Wertz, 2002), which can be important in competitive interactions of PGPR, especially against phytopathogenic bacteria and nematodes (Chowdhury *et al.*, 2015). *Bacillus* bacteriocins represent the second biggest group after those produced by lactic acid bacteria, and they display a variety of different chemical structures. They have been divided into three

classes: post-translationally modified peptides (class I, mainly lantibiotics), non-modified peptides (class II) and large proteins (class III; Abriouel *et al.*, 2011).

Lanthionine-containing antibiotics are known as lantibiotics and distinguished by the presence of inter-residual thioether bonds, which are formed after the dehydration of L-serine and L-threonine and the addition of cysteine thiol groups (Stein, 2005). The gene clusters involved in their synthesis (10-15 kb) include structural genes and genes responsible for the modifications, the regulation, the export, and the immunity of the producer strain (Fickers, 2012).

Subtilin is the best-characterized lantibiotic produced by *B. subtilis* and it is structurally related to the biopreservative nisin produced by *Lactococcus lactis* (**Figure 6**). Its mature form is a 32-residue pentacyclic peptide that is active against a broad range of Gram-positive bacteria. Subtilin causes the dissipation of the transmembrane proton motive force following the formation of pores in the cytoplasmic membrane (Stein, 2005).

Ericin A and ericin S are other lantibiotics having antimicrobial activity comparable to subtilin and similar structure (**Figure 6**) (Stein, 2005). On the contrary, mersacidin has a more globular structure and acts by preventing cell wall biosynthesis upon binding to the cell wall precursor lipid II (Brötz *et al.*, 1995). Subunits of haloduracin, lichenicidin, amylolysin, and other *Bacillus* lantibiotics show the same pattern of lanthionine bridges of mersacidin. Sublancin 168 and subtilisin A are also included in class I even if structurally different from typical lanthipeptides (Abriouel *et al.*, 2011; Arguelles-Arias *et al.*, 2013).

Class II of non-modified peptides includes antibiotics characterised by a molecular weight below 5 kDa. Among these ones, coagulin displays antilisterial activity (Le Marrec *et al.*, 2000) and thuricin 17 has an additional function of plant-growth promotion (Lee *et al.*, 2009).

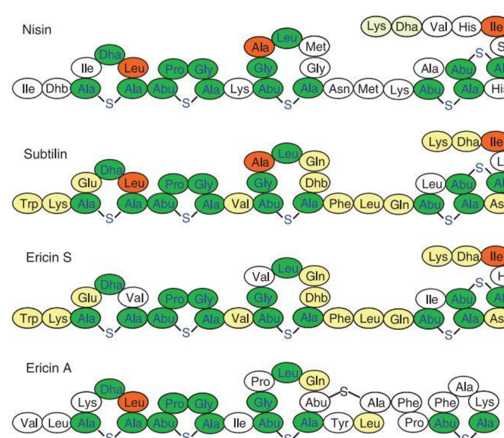

Figure 6: Structures of nisin A, subtilin and ericins. Conserved residues at identical positions to all four bacteriocins are highlighted in green, while those conserved only in subtilin and ericins are in yellow; other conserved residues are in light red (modified from Abriouel *et al.*, 2010).

Megacins belong instead to class III that groups heat sensitive proteins with a molecular weight higher than 30 kDa. These proteins have phospholipase A2 activity, as they convert phospholipids to lysophospholipids (Abriouel *et al.*, 2011; Fickers, 2012).

1.2.1.2 Polyketides and non-ribosomally synthesized peptides

Polyketides macrolactin, difficidin and bacillaene are produced by *B. amyloliquefaciens* and *B. subtilis* strains, which possess the corresponding large gene clusters (Chen *et al.*, 2009). Indeed, these molecules are synthesized in a non-ribosomal way by polyketides synthases (PKS), which are modularly organized enzymes that coordinate the assembly of a large class of secondary metabolites, through the condensation of acil-CoA monomers and subsequent modifications by dehydratase, methylation, ketoreductase, enoylreductase, oxidation, methyltransferase, and cyclase domains (Fickers, 2012).

The functions of polyketydes span from antibacterial to immunosuppressive and antitumor activities. For example, difficidin has received special attention for its suppressive action against *Erwinia amylovora*, a devastating plant pathogen causing fire blight disease of apple, pear and other rosaceous plants (Fickers, 2012).

Cyclic lipopeptides of surfactin, fengycin and iturin families, whose production is widespread among *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. subtilis*, are non-ribosomally synthesized peptides. Due to their amphiphilic structure composed of a polar peptidic ring linked to a hydrophobic lipid tail (**Figure 7**), these molecules have potent biosurfactants properties and thus they have multiple biotechnological applications (Ongena and Jacques, 2008).

Surfactins are heptapeptides interlinked with a β-hydroxy fatty acid (C12-C17) to form a cyclic lactone ring structure (**Figure 7**). They display haemolytic, antibacterial, antimycoplasma and antiviral activity but they are not fungitoxic (Peypoux *et al.*, 1999). Surfactins are also involved in swarming motility (Kinsinger *et al.*, 2003) and biofilm formation on roots (Bais *et al.*, 2004), besides playing a fundamental role as elicitors of ISR in plants (Ongena *et al.*, 2007; Cawoy *et al.*, 2014). Interestingly, a recently published study revealed that structural variants of

surfactins act individually on the respective producing strain as paracrine signals for biofilm formation and root colonization (Aleti *et al.*, 2016).

Fengycins (also called plipastatins) are lipodecapeptides with an internal lactone ring in the peptidic moiety, plus a saturated or unsaturated β-hydroxy fatty acid chain (C14-C19) (**Figure 7**), and display antifungal activity (Vanittanakom *et al.*, 1986). Iturins include seven variants, among which bacillomycins and mycosubtilin are the best known; all of them are heptapeptides linked to a β-amino fatty acid chain (C14-C17) (**Figure 7**). Iturins have limited antibacterial activity but strong haemolytic and antifungal effect (Maget-Dana and Peypoux, 1994). The effectiveness of several *Bacillus* strains in controlling fungal soil-borne, foliar and post-harvest pathogens has been related mainly to fengycin and iturin production. Their proposed mode of action is pore formation in cell membranes, leading to an imbalance of transmembrane ion fluxes and to a general disorganization of cytoplasm (Pérez-García *et al.*, 2011).

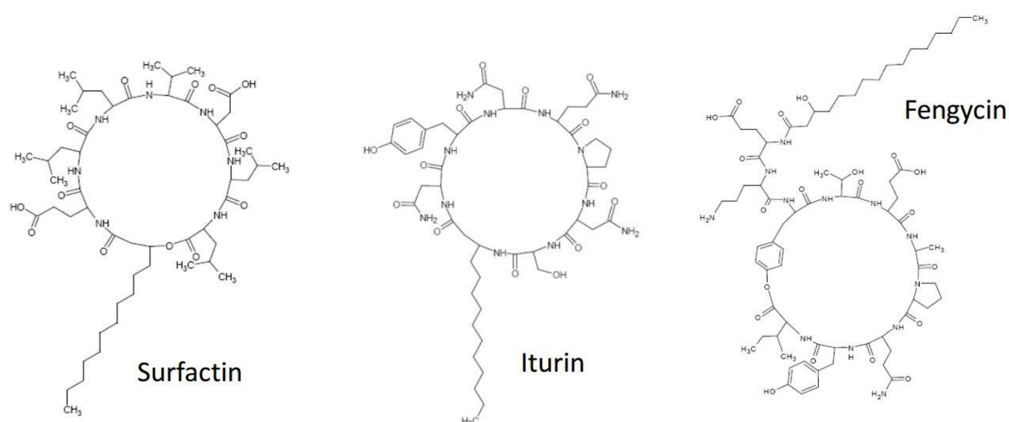

Figure 7: Representative structures of the three main families of *Bacillus* cyclic lipopeptides.

A multicarrier thiotemplate mechanism is responsible for the production of non-ribosomally synthesized peptides, in which megaenzymes termed non-ribosomal peptide synthetases (NRPS) participate. NRPS are composed of modular catalytic domains that are involved in adenylation, thiolation and condensation cycles of the peptide elongation process (**Figure 8**). The loading module is involved in the incorporation of the first amino acid, while the termination module releases the newly synthesized peptide through a thioesterase domain (Sieber and Marahiel,

2003). Moreover, NRPS often include other domains for specific modifications, such as oxidation, reduction, methylation, formylation, epimerisation, and heterocyclisation. This leads to the generation of a broad diversity of structures, which is directly linked to the variety of biological activities of these molecules (Fickers, 2012). The gene clusters encoding NRPS modules can be longer than 150 kb and their assembly follows the colinearity rule (Chen *et al.*, 2009).

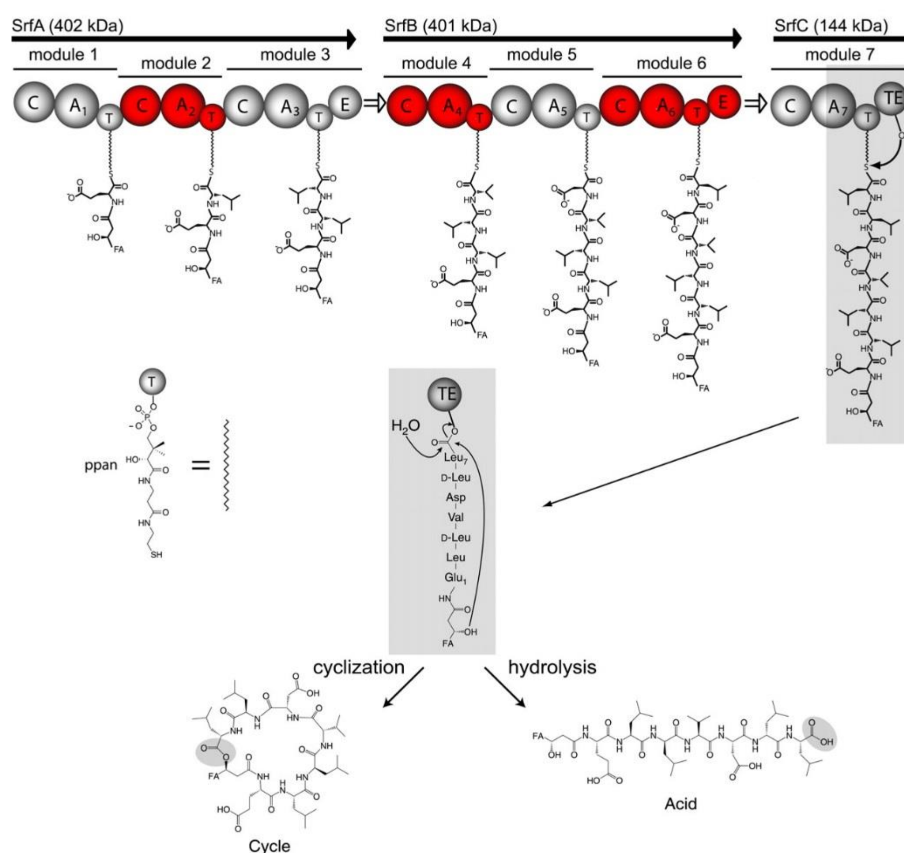

Figure 8: Surfactin synthetase. Example of non-ribosomal peptide synthetases: the surfactin synthetase consists of 24 individual domains responsible for 24 chemical reactions. These domains catalyse activation (A), covalent binding (T), elongation (C), epimerization (E), and release (TE) by either cyclization or hydrolysis. The domains are organized in modules, which incorporate the building blocks into the growing peptide chain. The peptide chain is covalently tied to the multienzyme via the cofactor phosphopantetheine (ppan). FA, fatty acid chain (Sieber and Marahiel, 2003).

Among antibiotics, bacilysin is a dipeptide active against bacteria and yeast, whereas bacillibactin is another non-ribosomally synthesized peptide that acts as a siderophore. Indeed, it is involved in a particular transport system that allows *Bacillus* cells to accumulate and import iron ions present in the environment at low

concentrations (Chen *et al.*, 2009). *Bacillus cereus* UW85 produces zwittermicin A, a fungistatic antibiotic, whose biosynthesis is a hybrid of NRPS and PKS pathway. It controls some Gram-positive and Gram-negative bacteria, besides being involved in the suppression of the fruit rot of cucumber and the damping-off disease of alfalfa induced by *Phytophthora medicaginis* (Silo-Suh, *et al.*, 1998).

The synthesis of many polyketides and lipopeptides depends on the membrane protein YczE (Chen *et al.*, 2007) and on a functional phospho-pantetheinyl transferase Sfp, an enzyme that activates PKS and NRPS by transferring 4'-phosphopantetheine from coenzyme A to the carrier proteins (Nakano *et al.*, 1992; Lambalot *et al*., 1996). Indeed, the domesticated strain *B. subtilis* 168 is not able to produce surfactin due to a frameshift mutation in *sfp* gene, but the introduction of a native *sfp* allele induced surfactin synthesis in *B. subtilis* 168 (Nakano *et al.*, 1992; Stein, 2005).

Surfactin synthesis usually occurs in the transition from the exponential to the stationary growth phase, whereas the production of fengycins and iturins begins later in the stationary phase (Raaijmakers *et al.*, 2010). Surfactin expression is related to population density and with the development of cellular competence, *i.e.* the ability of exogenous DNA uptake, as the gene of the competence regulator *comS* is embedded within the surfactin synthetase gene cluster, which is controlled by the quorum sensing via ComX (Stein, 2005). ComX is an extracellular signalling peptide that, upon reaching a critical concentration, activates the histidine kinase ComP, which phosphorylates the transcriptional activator ComA. Phosphorylated ComA regulates the expression of various genes, including the surfactin synthetase operon (Roggiani and Dubnau, 1993; Comella and Grossman, 2005). The activity of ComA is also modulated by Rap-Phr quorum-sensing systems (Boguslawski *et al.*, 2015), and the expression of the surfactin synthetase genes is regulated by several other transcription factors such as AbrB, CodY, DegU and PerR (Raaijmakers *et al.*, 2010). The two-component response regulator DegU is also required for the expression of the fengycins and iturins operons (Koumoutsi *et al.*, 2007; Tsuge *et al.*, 2007), although little is known about the modulation of their expession.

1.2.1.3 The primary role of cyclic lipopeptides in the antagonistic activity of *Bacillus amyloliquefaciens/subtilis* against fungal phytopathogens

A comparative study that included several strains of *B. amyloliquefaciens/subtilis* group showed that the secretion of cyclic lipopeptides (CLPs) plays a key role in the direct inhibition of fungal phytopathogens (Cawoy *et al.*, 2015). Those strains were selected according to their lipopeptide signatures and divided into three groups: producers of the three families of CLPs (surfactin, fengycin and iturin), producers of surfactin and fengycin but not iturin, and non-producers. The first group, which included among others *B. amyloliquefaciens* subsp. *plantarum* S499, was the most efficient in the growth inhibition of some foliar (*Cladosporium cucumerinum*, *Botrytis cinerea*) and soil-borne (*Fusarium oxysporum*, *Pythium aphanidermatum*) pathogens confronted on an artificial rich medium. Iturin production seemed to be determinant in the antagonistic ability, as the second group was significantly less active compared with the first (**Figure 9**). Inhibition size against *B. cinerea* and *F. oxysporum* well correlated with total amounts of fengycins and iturins detected in the medium, while the inhibition of *C. cucumerinum* mainly depended on iturin production. The biocontrol effect against *P. aphanidermatum* was limited, probably due to the different composition of oomycete cell walls compared with true fungi.

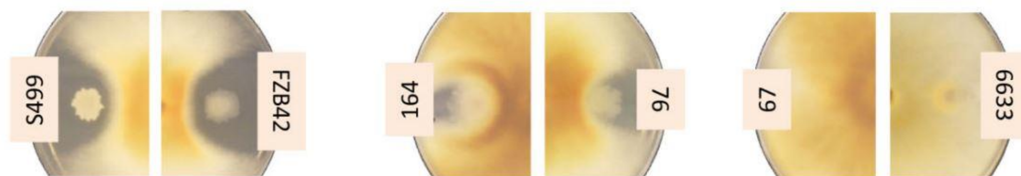

Figure 9: Role of cyclic lipopeptides in *Bacillus* antagonistic activity. Inhibition of phytopathogenic fungi by some representative strains of three groups of *Bacillus amyloliquefaciens/subtilis*: producers of the three families of CLPs (left), producers of surfactin and fengycin but not iturin (middle), and non-producers (right) (Cawoy *et al.*, 2015).

Cawoy *et al*. (2015) evaluated also the antagonistic potential and the production of CLPs by selected *Bacillus* strains, including *B. amyloliquefaciens* subsp. *plantarum* S499, when grown in natural root exudates. Bacteria were still

active in inhibiting mycelial growth and, interestingly, MALDI-MS imaging showed that mostly iturins accumulated at the front of fungal arrest. Other antibiotics, such as bacteriocins and polyketides, were not detected in the assays, confirming a primary function of CLPs in the biocontrol activity of the tested isolates.

1.2.1.4 Surfactin as elicitor of induced systemic resistance in plants

Among CLPs, surfactin is the most efficient in stimulating plant immunity (Henry, 2013). Previously, Ongena *et al.* (2007) demonstrated that pure surfactins induced ISR in bean at similar levels of living cells of *B. amyloliquefaciens* subsp. *plantarum* S499. They also showed that the overexpression of surfactin synthetase gene in the naturally poor producer *B. subtilis* 168 enhanced its protective effect, since a significant disease reduction was observed in treated plants. Indeed, lipoxygenase and lipid hydroperoxidase activities were stimulated in bacterized tomato plants. Moreover, strains of *B. amyloliquefaciens/subtilis* identified as reliable producers of surfactins (S499, 98S) were more effective in reducing disease incidence in tobacco plants challenged by *B. cinerea* compared with medium (FZB42, QST713) and non-producers (56, BN01; **Figure 10**) (Cawoy *et al.*, 2014).

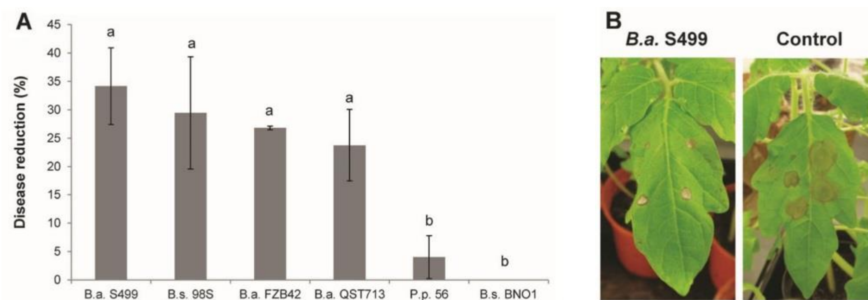

Figure 10: Role of surfactins in ISR. (A) Disease reduction on tomato leaves inoculated with *Botrytis cinerea* after root inoculation with the six selected *Bacillus* strains compared with non-inoculated control plants. **(B)** Containment of typical spreading lesions in plants inoculated with *B. amyloliquefaciens* subsp. *plantarum* S499 before infection with *B. cinerea* (modified from Cawoy *et al*., 2014).

The role of surfactin as MAMP was studied *in vitro* as well, by adding the CLPs in micromolar concentrations to tobacco cell suspensions (Jourdan *et al.*,

2009). Surfactin (but not fengycin nor iturin) triggered extracellular medium alkalization, ion fluxes and the production of reactive oxygen species, which are well-known defence-related early events. An induction of phenylalanine ammonia-lyase and lipoxygenase gene expression, coupled with an increased activity of these enzymes and with the accumulation of phenols, was also observed in the elicited cells. These responses are strictly linked to Ca^{2+} influx and to dynamic changes in protein phosphorylation. At the same time, a phytotoxic effect of surfactins could be excluded for concentrations up to 10 µM. Indeed, such amphiphilic molecules are putatively involved in a temporary destabilization of plant plasma membranes, which does not induce irreversible pores formation, but may prompt a signalling cascade leading to defensive responses (Jourdan *et al.*, 2009).

Protein receptors for rhizobacterial MAMPs has not been identified yet. Henry and colleagues (2011) focused on various approaches to study the mechanism governing the perception of surfactins by plant cells. They observed oxidative burst induction in tobacco cells, structure/activity relationship, competitive inhibition, insertion kinetics within plant membranes, and they combined those data with a thermodynamic determination of binding parameters on model membranes. Their results confirmed that surfactin perception more likely relies on a lipid-driven process at the plasma membrane level, namely a transient channelling, rather than on the presence of receptors. Considering the hypothesis of dynamic membrane compartmentalization, the induction of ISR could be related to the ability to temporally and spatially organize protein complexes, which would in turn activate the signalling pathway (Henry, 2013).

1.2.2 *Bacillus amyloliquefaciens* subsp. *plantarum*, model PGPR for unravelling plant-microbe interactions: genomics and taxonomy

Bacillus spp. were originally classified according to their ecophysiology and their metabolic diversity into three main groups: pathogenic, environmental and industrial species (Hamdache *et al.*, 2013). In 1997, a consortium of 46 laboratories sequenced the complete genome sequencing of *B. subtilis* 168, which was selected as a paradigm of Gram-positive bacteria. Indeed, its genetic, physiology and biochemical features had been extensively studied for several decades (Kunst *et al.*,

1997). Later on, the progress in next generation sequencing (NGS) technologies allowed easier, cheaper and faster analyses that led to the publication of thousands of prokaryotic genomes (Loman *et al.*, 2012). Among these, several genomes of PGPR, including strains of *Bacillus* spp., have been deposited in public databases, either in the form of a draft (*i.e.* a set of fragmented sequences covering the most part of the genome) or as a sequence representing the whole genome. Comparative analyses of genomic data within *Bacillus* genus indicated that *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* are closely related species, while *B. anthracis*, *B. cereus* and *B. thuringiensis* form a distinct phylogenetic group, as for *B. clausii* and *B. halodurans* (Hamdache *et al.*, 2013).

Initially, *B. amyloliquefaciens* species received attention for their abundant release of α-amylases and proteases (Priest, 1987). Indeed, the name *B. amyloliquefaciens* was chosen by the Japanese scientist who discovered the species (Fukumoto, 1943) because it produced ("-faciens") a liquefying ("-lique-") amylase ("amylo-"). To group all the plant-associated *B. amyloliquefaciens* strains, a subgroup named "*Bacillus amyloliquefaciens* subspecies *plantarum*" was instituted and *B. amyloliquefaciens* FZB42 was elected as the type strain of the taxon (**Figure 11**) (Borriss *et al.*, 2011). The rhizobacteria belonging to *B. amyloliquefaciens* subsp. *plantarum* are known for improving plant growth and for their antagonistic activity against plant pathogens. The strain FZB42 is particularly efficient against phytopathogens bacteria and fungi and already present on the market as BCA (Borriss, 2011). Its ability in the root colonization of *Arabidopsis thaliana*, *Zea mays* and *Lemna minor* was assessed with a FZB42 derivative labelled with the green fluorescent protein through a confocal laser scanning microscopy (Fan *et al.*, 2011). Moreover, the activities of plant growth stimulation and disease protection were reported for FZB42 and many other strains of *B. amyloliquefaciens* subsp. *plantarum* (Chowdhury *et al*., 2015). For these reasons, this taxon can be regarded as a model to study plant-beneficial interactions in *Bacillus* spp.

The genome of the strain FZB42 was the first to be sequenced and analysed to identify the key genes involved in the plant-associated lifestyle (Koumoutsi *et al.*, 2004; Chen *et al.*, 2007). A big portion (8.5%) of FZB42 genome is devoted to the synthesis of secondary metabolites and, more precisely, nine gene clusters

involved in the production of bioactive peptides and polyketides were described (**Figure 12**) (Chen *et al.*, 2009). Based on genotype and phenotype coherence, the taxon of *B. amyloliquefaciens* subsp. *plantarum* has undergone two reclassifications, first by being identified as a later heterotypic synonym of *B. methylotrophicus* (Dunlap *et al.*, 2015) and then as a later heterotypic synonym of *B. velezensis* (Dunlap *et al.*, 2016).

The annotation and the functional characterization of PGPR genomes can contribute to an improved understanding of their ecology and of the mechanisms involved in biocontrol and in plant-growth promotion activities. For instance, a comparative study that included the genomes of 12 *Bacillus* spp. with PGPR activity identified specific features involved in root colonization, growth promotion and biocontrol, among which specific genes linked with carbon usage, transport systems, signalling and production of secondary metabolites (Hossain *et al.*, 2015). Moreover, genome mining enables to identify the gene clusters encoding ribosomal and non-ribosomal bioactive secondary metabolites that are generally silent, which can generate an underestimation of the biosynthetic potential of some isolates (Hamdache *et al.*, 2013).

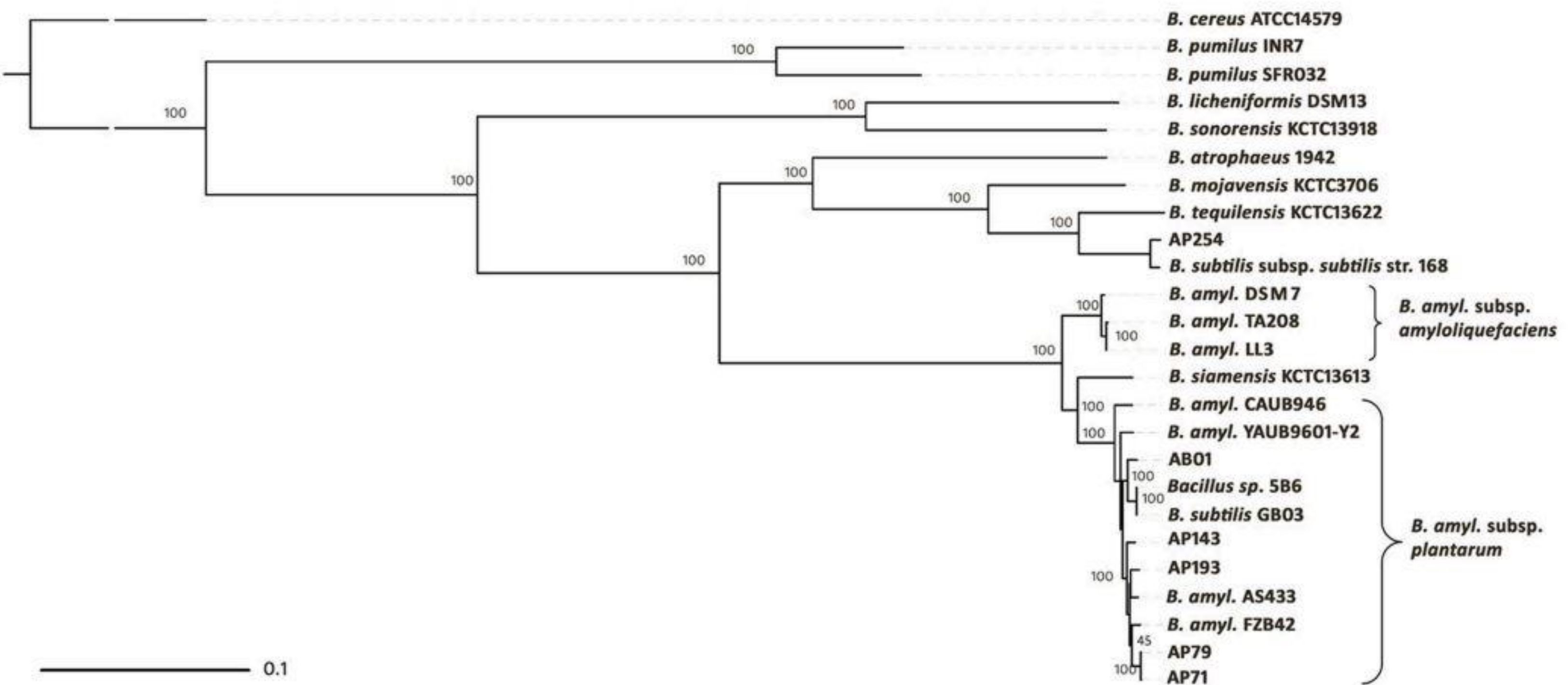

Figure 11: *Bacillus* spp. phylogenetic tree. Maximum-likelihood phylogenetic tree of 25 *Bacillus* strains based on the 729,383 bp sequence of their core genome (Hossain *et al.*, 2015).

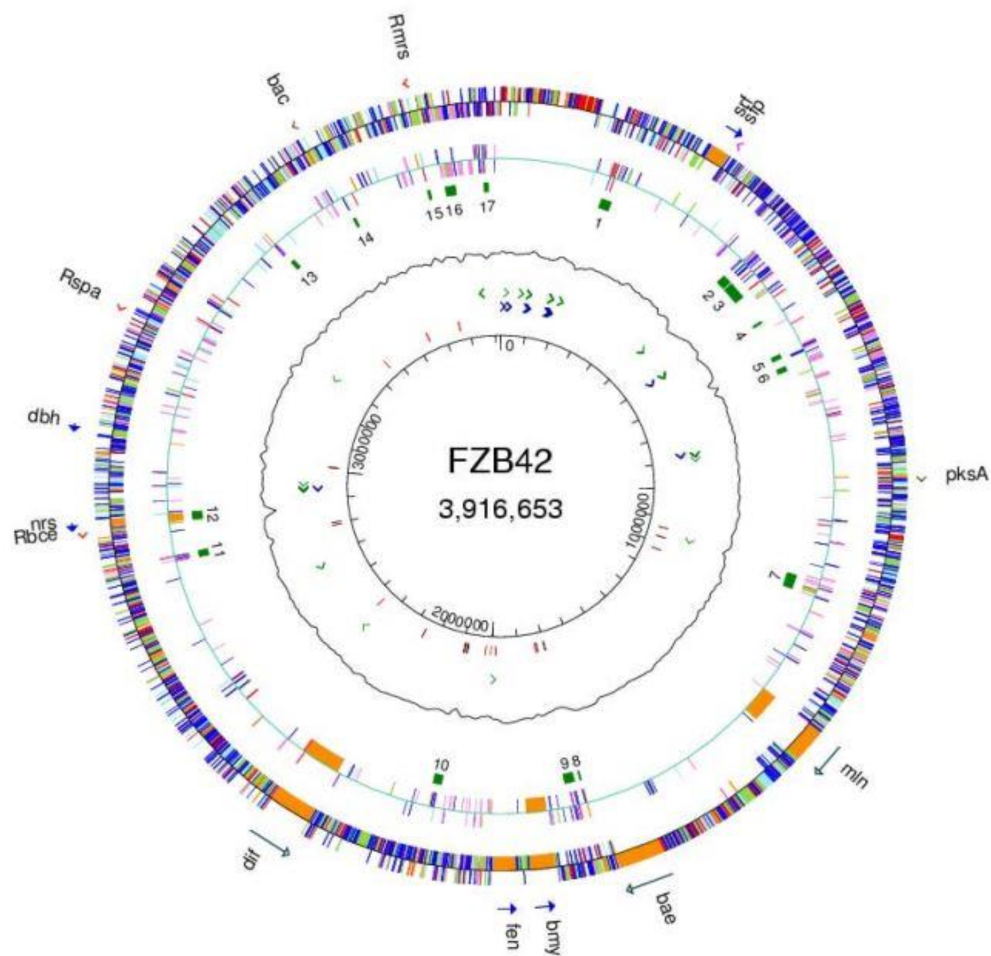

Figure 12: The genome of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42. Outmost circle: genes and gene clusters involved in synthesis and export (detoxification) of secondary metabolites: *srf*, surfactin; *sfp*, phospho-panthetheinyl-transferase; *pksA*, regulator polyketide synthesis; *mln*, macrolactin; *bae*, bacillaene, *bmy*, bacillomycin D; *fen*, fengycin; *dif*, difficidin; *Rbce*, bacitracin export; *nrs*, hybrid polyketide/cysteine-containing peptide; *dbh*, bacillibactin; *Rspa*, subtilin immunity; *bac*, bacilysin; *Rmrs*, mersacidin immunity. First circle: all genes in colour code according to their functions: cell envelope and cellular processes, green; information pathways, orange; intermediary metabolism, pink; other functions, red; unknown, black. Second circle: genes not conserved in *Bacillus subtilis* including four gene clusters involved in synthesis of secondary metabolites (orange); Third circle: the numbered 17 DNA islands (green), 4th circle: GC content profile, 5th circle: rRNAs (green), 6th circle: tRNAs (cyan), 7th circle: prophages (black), transposons, and IS elements (red), 8th circle: scale (bp) (Chen *et al.*, 2007).

1.2.3 Impact of environmental factors on lipopeptide production in the rhizosphere

Root exudates not only act as chemoattractants for PGPR, but they are also the primary nutrients that support their growth in the rhizosphere. Therefore, the host plant imposes to the associated bacteria a particular nutritional environment, which influences their cellular physiology, besides determining the selection of specific microbial populations (Hartmann *et al.*, 2009). Other factors, such as the interaction with niche competitors or physicochemical parameters like humidity, pH, temperature, mineral content, and oxygen availability, contribute to shape the size and the behaviour of rhizobacterial communities. Studying which and how environmental factors affect the physiology of plant-beneficial *Bacillus*, including the production of secondary metabolites, is necessary to improve their efficacy (Nihorimbere *et al.*, 2009).

1.2.3.1 Modulation by plant determinants and rhizosphere competitors

Several works showed that the pattern of CLPs secreted by *B. amyloliquefaciens* subsp. *plantarum* S499 is substantially modulated in favour of surfactins upon root colonization. In laboratory conditions (*i.e.* planktonic cells grown in rich medium), this strain efficiently produces all the three families of CLPs in their various forms, but mainly surfactins were detected on *Arabidopsis*, bean, corn, lettuce, tobacco, tomato, and wheat roots (**Figure 13**) (Nihorimbere *et al.*, 2012; Debois *et al.*, 2014; Debois *et al.*, 2015). Indeed, surfactin synthesis is stimulated by the presence of organic acids that are abundant in plant root exudates (Nihorimbere *et al.*, 2012; Kamilova *et al.*, 2006).

Time-of-flight secondary ion mass spectrometry imaging revealed that higher quantities of C_{14} and C_{15} surfactins compared to C_{12} and C_{13} homologues are released by root-adhering colonies (**Figure 13**). Similar proportions were found in bacteria developing as biofilm, where surfactins were still the main CLPs produced (Nihorimbere *et al.*, 2012). Interestingly, long-chain surfactins are also the most active as ISR elicitors (Jourdan *et al.*, 2009).

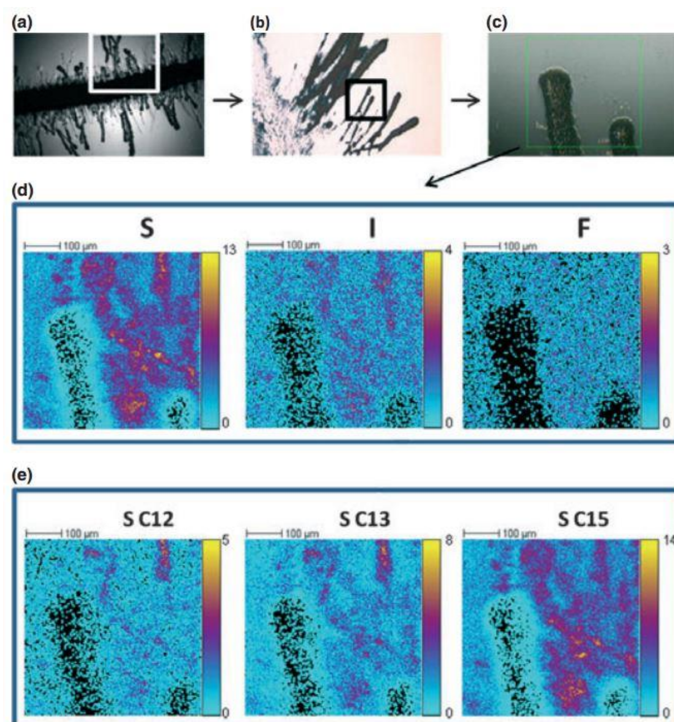

Figure 13: TOF-SIMS imaging of lipopeptides produced by *Bacillus amyloliquefaciens* subsp. *plantarum* S499 colonizing tomato roots. Optical microscope images of **(a)** the colonized root and **(b)** part of the colonization pattern after transfer onto the silicon wafer. **(c)** Video image of the scanned zone. **(d)** High-definition scan of the lipopeptide distribution: images of the sum of surfactins (S), iturins (I) and fengycins (F) ions and **(e)** relative distribution of surfactin homologues in the same pattern are shown. The maximum ion count recorded in a pixel in the image is indicated on the colour scale bar (Nihorimbere *et al.*, 2012).

A recent study demonstrated that C_{14} and C_{15} homologues are secreted very early by root-colonizing cells and that the presence of root exudates is not essential for surfactin accumulation. Moreover, certain plant cell wall polysaccharides, such as xylan and arabinogalactan, are perceived by S499 and closely related strains (FZB42, QST713) as signals that trigger surfactin production (Debois *et al.*, 2015). The same polysaccharides have been shown to induce the formation of biofilm in several strains of *Bacillus amyloliquefaciens/subtilis*, suggesting that they may play a key role in the crosstalk that leads to root colonization (Beauregard *et al.*, 2013).

In parallel, the pattern of *Bacillus* CLPs is modulated by the presence of other microorganisms in the same ecosystem. Certain phytopathogenic fungi and not others boost the production of fengycins and iturins in *B. amyloliquefaciens*. Since this occurs without any physical contact among the microbial colonies, it is conceivable that the bacteria perceive specific chemicals signals emitted by some pathogens, which might up-regulate the expression of antibiotics (Cawoy *et al.*, 2014). Analyses of gene expression confirmed that different soil-borne fungal pathogens induce varying levels of transcription of the genes involved in the

synthesis of CLPs, indicating that *B. amyloliquefaciens* evolved to give species-specific responses to competitors in the environment (Li *et al.*, 2014).

1.2.3.2 Effect of temperature, water and oxygen availability

Physicochemical factors such as temperature, pH and water availability can influence the physiology of both host plants and associated rhizobacteria. In this context, the study by Pertot *et al.* (2013) assessed the impact of low and high temperatures and drought on the interaction between *B. amyloliquefaciens* subsp. *plantarum* S499 and plants (bean, tomato and zucchini). Regarding surfactin productivity, a positive effect of low temperatures (15°C) was observed. In those conditions, a slower population growth could end in a higher accumulation of the CLPs, in agreement with the fact that a reduced growth rate enhances surfactin synthetase gene expression in *B. subtilis* (Nihorimbere *et al.*, 2009). Consistently, the ISR effect was more evident in bean and tomato plants grown at 15°C than on plants cultivated at 25°C. An increased surfactin production could therefore compensate the adverse effect of cold stress on other traits involved in rhizosphere fitness, *e.g.* motility and biofilm formation, which resulted impaired in cold conditions. By contrast, no differences in root colonization nor in disease protection activity emerged between bacterized plants treated with low water regime and those regularly irrigated (Pertot *et al.*, 2013).

The effect of pH and temperature on iturin production was studied by Leães *et al.* (2013). By qRT-PCR, they demonstrated that *ituD* gene expression increased more than 20 times upon growing *Bacillus* sp. P11 at pH 6 compared to pH 7.4, and almost ten times at the temperature of 37°C compared to bacterial growth at 30°C. On the contrary, by increasing the temperature to 42°C and the pH to 8 *ituD* gene expression in the strain decreased. However, Fickers *et al.* (2008) showed that lowering growth temperature from 37°C to 25°C fostered the biosynthesis of mycosubtilin (CLP of the iturin family) in *B. subtilis* ATCC6633 and its derivative strain BBG100 without visible changes in gene expression, indicating that temperature may also affect mycosubtilin synthetase turnover.

Oxygen concentration was another parameter monitored for its effect on lipopeptide production. Oxygen is often a limiting factor in soil and especially in

the rhizosphere, because it is consumed by root and root-associated microorganisms. Soil-borne aerobic bacteria, such as *Bacillus* spp., may thus be affected by oxygen depletion in their natural habitat. When *B. amyloliquefaciens* subsp. *plantarum* S499 was grown under different aeration rates, surfactins, fengycins and iturins were more efficiently produced under low oxygen concentrations. Anyway, changing the aeration conditions did not change the relative proportion of the three families of CLPs released, therefore this factor cannot explain the surfactin-enriched lipopeptide signature *in planta* (Nihorimbere *et al.*, 2012). The impact of oxygen concentration on lipopeptide biosynthesis was also demonstrated with other strains, such as *B. subtilis* ATCC6633 (Guez *et al.*, 2008) and *B. subtilis* BBG21 (Fahim *et al.*, 2012).

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Chapter 2

OBJECTIVES OF THE THESIS

A sustainable agriculture aims at reducing the release of synthetic chemical products in the environment. For this reason, the use of alternatives as microbial biopesticides is increasing worldwide. Several PGPR, and in particular the strains of *B. amyloliquefaciens* subsp. *plantarum*, display biofertilizer activity and/or have the ability to contain plant diseases. However, field efficacy of most of these bacterial strains is still unsatisfying. Indeed, as the rhizosphere is a complex and dynamic system, the efficacy of PGPR inoculants can be impaired by several biotic and abiotic factors. In many cases, molecular crosstalk determining the colonization of the host plant, or allowing to survive over niche competitors, or yet the mechanisms underlying adaptation to environmental stresses, are poorly known. Therefore, a deeper understanding of major traits regulating rhizosphere interactions may help in improving the efficacy of plant beneficial strains in crop systems.

The general aim of this thesis is to broaden the knowledge on biological mechanisms underlying the crosstalk between PGPR belonging to *B. amyloliquefaciens* subsp. *plantarum*, host plants and phytopathogenic fungi, especially focusing on the bacterial features. Hence, the specific objectives of the thesis are:

(*I*) *B. amyloliquefaciens* subsp. *plantarum* strain S499 genome sequencing, assembly and annotation, and S499 genome mining for genes putatively involved in rhizosphere interactions;

(*II*) search for strain-specific genetic features of S499, in comparison with closely related strains, and in particular with the type strain *B. amyloliquefaciens* subsp. *plantarum* FZB42;

(*III*) characterization of a peculiar extrachromosomal element (plasmid pS499), encoding a quorum-sensing regulatory system, under nutrient-rich growth conditions;

(*IV*) elucidating the role played by the plasmid pS499 in the rhizosphere, by growing bacteria on tobacco and tomato roots and on a “recomposed root exudates” medium.

Chapter 3

COMPLETE GENOME SEQUENCE OF *Bacillus amyloliquefaciens* subsp. *plantarum* S499, A RHIZOBACTERIUM THAT TRIGGERS PLANT DEFENCES AND INHIBITS FUNGAL PHYTOPATHOGENS

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Short Genome Communications

Complete genome sequence of *Bacillus amyloliquefaciens* subsp. *plantarum* S499, a rhizobacterium that triggers plant defences and inhibits fungal phytopathogens

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ABSTRACT

Bacillus amyloliquefaciens subsp. *plantarum* S499 is a plant beneficial rhizobacterium with a good antagonistic potential against phytopathogens through the release of active secondary metabolites. Moreover, it can induce systemic resistance in plants by producing considerable amounts of surfactins. The complete genome sequence of *B. amyloliquefaciens* subsp. *plantarum* S499 includes a circular chromosome of 3,927,922 bp and a plasmid of 8,008 bp. A remarkable abundance in genomic regions of putative horizontal origin emerged from the analysis. Furthermore, we highlighted the presence of genes involved in the establishment of interactions with the host plants at the root level and in the competition with other soil-borne microorganisms. More specifically, genes related to the synthesis of amylolysin, amylocyclicin, and butirosin were identified. These antimicrobials were not known before to be part of the antibiotic arsenal of the strain. The information embedded in the genome will support the upcoming studies regarding the application of *B. amyloliquefaciens* isolates as plant-growth promoters and biocontrol agents.

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1. Introduction

Since its isolation from a cultivated soil in the Iturie region (Democratic Republic of Congo; Delcambe, 1965), the rhizobacterium *Bacillus amyloliquefaciens* subsp. *plantarum* S499 (available at BCCM/LMG Bacteria Collection, LMG 29676) has been extensively described due to its plant beneficial properties shown in greenhouse and field trials (Nihorimbere et al., 2010; Pertot et al., 2013). The protection provided by S499 against phytopathogens relies on its potential to produce multiple antimicrobial metabolites (Cawoy et al., 2015) and on its ability to induce systemic resistance in plants (Ongena et al., 2005a,b, 2007). This strain is an excellent producer of the surfactin-type lipopeptide, which not only contributes to the high rhizosphere competence of the bacterium but which also acts as the main elicitor of host immunity (Cawoy et al., 2014). It most probably explains why in a comparative study, which included 16 root-associated *Bacillus* isolates, S499 was the most efficient in disease reduction (Cawoy et al., 2014). For these reasons, S499 has often been used as model to investigate the impact of biotic and abiotic factors on rhizosphere fitness and antibiotic production in *B. amyloliquefaciens*, but also to study the molecular interactions established with the host plant (Debois et al., 2015; Henry et al., 2011; Nihorimbere et al., 2012; Pertot et al., 2013). Furthermore, expression of its biocontrol-related antibiome has been characterised in details, both *in vitro* and *in planta* (Debois et al., 2014). The availability of the S499 complete genome can provide an additional tool for in-depth investigation of the mechanisms involved in biocontrol of plant diseases and be the basis for the development of novel more effective biofungicides based on bacteria belonging to *B. amyloliquefaciens* subsp. *plantarum*.

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Table 1
Features of *Bacillus amyloliquefaciens* subsp. *plantarum* S499 genome.

Features	Chromosome	Plasmid
Genome size (bp)	3,927,922	8,008
G+C content (%)	46.6	40.40
Total predicted CDS[a]	3,974	7
rRNA operons	24	–
tRNA genes	81	–
Insertion sequence	1	–
Phage-associated genes	154	–

[a] CDS: Coding DNA Sequences.

2. Materials and methods

2.1. DNA extraction and genome sequencing

Genomic DNA was extracted from S499 cultures using a PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Invitrogen, USA) according to the manufacturer's instructions. A 10-kb PacBio® RS II single-molecule real-time (SMRT) cell (Chin et al., 2013) was used to sequence the whole genomic DNA of S499 through PacBio technology at Baseclear B.V. (Leiden, Netherlands).

2.2. Genome assembly and annotation

Assembly of subreads obtained with the PacBio® RS II SMRT was carried out using the RS hierarchical genome assembly process (HGAP) protocol version 3.0, as available in SMRT Portal v2.0 (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/HGAP-in-SMRT-Analysis). The SMRT Portal was configured and used with a public machine image that Pacific Biosciences maintains and upgrades on Amazon Cloud (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/%22Installing%22-SMRT-Portal-the-easy-way—Launching-A-SMRT-Portal-AMI). Whole genome assembly was achieved with a comparative method, which combined *de novo* assembly and mapping through the MAUVE aligner tool (Darling et al., 2010). Annotation was carried out using Rapid Annotation Subsystem Technology (RAST; Aziz et al., 2008). Tandem repeats were detected with Tandem Repeats Finder (Benson, 1999) and genomic islands were screened using IslandViewer (Dhillon et al., 2013; Langille and Brinkman, 2009). IS Finder (Siguier et al., 2006) and PHAST (Zhou et al., 2011) were used to identify insertion sequences and prophage regions. Genome mining for bioactive secondary metabolites was performed via antiSMASH 3.0 (Weber et al., 2015).

3. Results and discussion

The genetic equipment of S499 consists of a 3,927,922 bp circular chromosome and an 8,008 bp plasmid (Fig. 1). The plasmid contains only seven coding DNA sequences (CDS) while the circular chromosome has 3,974 CDS, and 106 predicted RNA genes (Table 1). The circular chromosome includes 99 tandem repeats and one insertion sequence element of 1,275 bp identified as "ISBsu1", encoding a transposase and a tail length tape measure protein. Furthermore, we detected four prophage regions, containing 154 putative phage-related genes. Screening for genomic islands revealed the presence of five additional regions of hypothetical horizontal origin, which include the CDS involved in antibiotic resistance, detoxification and stress responses (Table 1). This finding could reflect the S499 adaptation to its natural soil ecosystem. In such habitat, the evolution of bacterial populations continuously exposed to environmental stresses could largely depend on a high rate of horizontal gene exchanges (Aminov, 2011).

Through RAST classification into subsystems (Table 2), we highlighted the importance of the coding portion of the S499 genome

Table 2
Distribution of *Bacillus amyloliquefaciens* subsp. *plantarum* S499 coding DNA sequences (CDS) in subsystems according to RAST Server.

Subsystem	CDS
Cofactors, Vitamins, Prosthetic Groups, Pigments	229
Cell Wall and Capsule	138
Virulence, Disease and Defense	60
Potassium metabolism	9
Photosynthesis	0
Miscellaneous	47
Phages, Prophages, Transposable elements, Plasmids	25
Membrane Transport	69
Iron acquisition and metabolism	30
RNA Metabolism	155
Nucleosides and Nucleotides	114
Protein Metabolism	162
Cell Division and Cell Cycle	55
Motility and Chemotaxis	86
Regulation and Cell signaling	64
Secondary Metabolism	6
DNA Metabolism	105
Fatty Acids, Lipids, and Isoprenoids	138
Nitrogen Metabolism	31
Dormancy and Sporulation	117
Respiration	74
Stress Response	108
Metabolism of Aromatic Compounds	12
Amino Acids and Derivatives	444
Sulfur Metabolism	39
Phosphorus Metabolism	31
Carbohydrates	414

that supports bacterial development on roots. Indeed, as much as 15% of the classified CDS were assigned to categories related to the ability of S499 to effectively colonize plant roots and produce secondary metabolites responsible for the control of phytopathogens ("Motility and Chemotaxis", "Membrane Transport", "Virulence, Disease and Defense", "Secondary Metabolism" and "Stress Responses"). Regarding the intrinsic plant growth promotion function of the strain (Nihorimbere et al., 2010), we assessed the presence of the genes necessary for synthesis of auxin, phytase, and volatile compounds (e.g. 2,3-butanediol and acetoin) known to be implicated in this activity (Idriss et al., 2002; Ryu et al., 2004; Table 2). In the context of phytopathogen biocontrol, the antiSMASH analysis allowed to identify gene clusters encoding the enzymatic machinery for synthesis of nonribosomal peptides (surfactins, iturins, fengycins, bacillibactin, and bacilysin) and polyketides (bacillaene, difficidin, and macrolactin, Table 3). The antiSMASH analysis identified additional genes related to the production of other antimicrobials, such as the lantibiotic amylolysin (Arguelles-Arias et al., 2013), the bacteriocin amylocyclicin (Scholz et al., 2014), and the aminoglycoside antibiotic butirosin (Llewellyn et al., 2007), which have not been detected using chemical analysis in culture media of the strain to date (Debois et al., 2014).

The genomic features of S499 thus clearly reflect its root-associated lifestyle and its biocontrol potential. Most importantly, the complete genome of this efficient and peculiar *Bacillus* strain is being used for developing transcriptomic studies with the aim of understanding how the antibiome expression profile in this bacterium is modulated upon interaction with a variety of host plants and with the numerous competitors present in the rhizosphere microbiome.

4. Nucleotide sequence accession number

The complete nucleotide genome sequence of *B. amyloliquefaciens* subsp. *plantarum* S499 has been deposited at

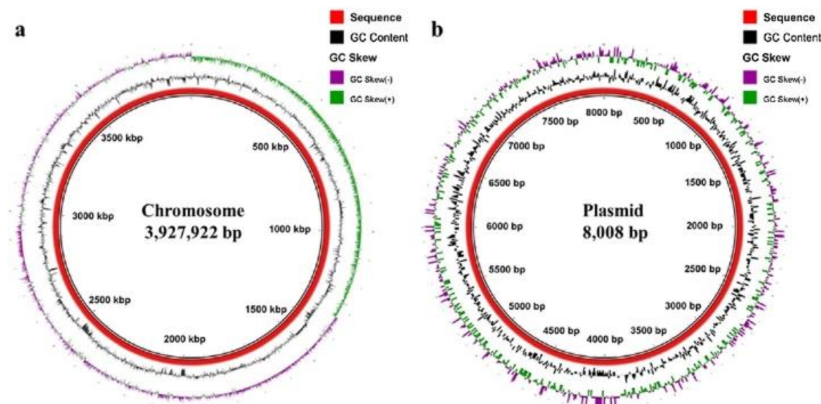

Fig. 1. Genomic map representation of the *Bacillus amyloliquefaciens* subsp. *plantarum* S499 complete genome. (a) Circular chromosome, (b) Plasmid. Rings show (from the inside): (1) nucleotide sequence; (2) GC percent; (3) GC skew. Genomic maps were constructed using the BLAST Ring Image Generator (BRIG, version 0.95; Alikhan et al., 2011).

Table 3
Identification of gene clusters potentially involved in the synthesis of secondary metabolites by *Bacillus amyloliquefaciens* subsp. *plantarum* S499.

Cluster[a]	Type[b]	From[c]	To[c]	Secondary metabolite[d]
1	Trans-AT PKS cluster	852,198	952,636	Difficidin
2	Type III PKS cluster	1,068,971	1,110,071	Unknown
3	Terpene	1,160,436	1,182,319	Unknown
4	Nrps-Transatpks	1,204,945	1,342,774	Fengycin Iturin
5	Transatpks-Nrps	1,399,613	1,502,314	Bacillaene
6	Transatpks	1,728,554	1,814,435	Macrolactin
7	Lantipeptide	1,981,950	2,010,839	Amylolysin
8	Terpene	2,169,549	2,190,289	Unknown
9	Other types of PKS cluster	2,272,301	2,313,545	Butirosin
10	Nonribosomal peptide synthetase cluster	2,589,001	2,654,407	Surfactin
11	Nrps-Bacteriocin	3,025,102	3,091,892	Bacillibactin Amylocyclin
12	Other	3,599,548	3,640,966	Bacilysin

[a] Clusters identified using default settings of antiSMASH 3.0.
[b] Class of gene cluster according to antiSMASH 3.0.
[c] Location of gene clusters in the *B. amyloliquefaciens* subsp. *plantarum* S499 genome.
[d] Secondary metabolites potentially produced based on the gene clusters.

DDBJ/EMBL/GenBank under the accession numbers CP014700 (chromosome) and CP014701 (plasmid).

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The PhD candidate actively participated in S499 genome assembly and annotation. Subsequently, she carried out the genome analysis and wrote the paper.

Chapter 4

KEY IMPACT OF AN UNCOMMON PLASMID ON *Bacillus amyloliquefaciens* subsp. *plantarum* S499 DEVELOPMENTAL TRAITS AND LIPOPEPTIDE PRODUCTION

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Key Impact of an Uncommon Plasmid on *Bacillus amyloliquefaciens* subsp. *plantarum* S499 Developmental Traits and Lipopeptide Production

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The rhizobacterium *Bacillus amyloliquefaciens* subsp. *plantarum* S499 (S499) is particularly efficient in terms of the production of cyclic lipopeptides, which are responsible for the high level of plant disease protection provided by this strain. Sequencing of the S499 genome has highlighted genetic differences and similarities with the closely related rhizobacterium *B. amyloliquefaciens* subsp. *plantarum* FZB42 (FZB42). More specifically, a rare 8008 bp plasmid (pS499) harboring a *rap-phr* cassette constitutes a major distinctive element between S499 and FZB42. By curing this plasmid, we demonstrated that its presence is crucial for preserving the typical physiology of S499 cells. Indeed, the growth rate and extracellular proteolytic activity were significantly affected in the cured strain (S499 P^-). Furthermore, pS499 made a significant contribution to the regulation of cyclic lipopeptide production. Surfactins and fengycins were produced in higher quantities by S499 P^-, whereas lower amounts of iturins were detected. In line with the increase in surfactin release, bacterial motility improved after curing, whereas the ability to form biofilm was reduced *in vitro*. The antagonistic effect against phytopathogenic fungi was also limited for S499 P^-, most probably due to the reduction of iturin production. With the exception of this last aspect, S499 P^- behavior fell between that of S499 and FZB42, suggesting a role for the plasmid in shaping some of the phenotypic differences observed in the two strains.

Keywords: *Bacillus*, genome comparison, plasmid, surfactin, biological control

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INTRODUCTION

Some strains of the *Bacillus amyloliquefaciens* species have been described as beneficial rhizobacteria, because of their ability to promote growth and/or protect plants from infection by multiple pathogens (Lugtenberg and Kamilova, 2009; Cawoy et al., 2011; Kumar et al., 2011). This protective effect against disease is achieved through multiple mechanisms, of which competition for space/nutrients, direct antibiosis against pathogens, and induction of systemic resistance (ISR) in the host plant are the most relevant (Nihorimbere et al., 2011; Bakker et al., 2013). Borriss et al. (2011) separated this bacterial species into two taxa "*B. amyloliquefaciens* subspecies *amyloliquefaciens*" and "*B. amyloliquefaciens* subspecies *plantarum,*" which grouped together all the plant-associated *B. amyloliquefaciens* strains. Recently, the

B. amyloliquefaciens subspecies *plantarum* has undergone two reclassifications as a later heterotypic synonym of *B. methylotrophicus* (Dunlap et al., 2015), and then as *B. velezensis* (Dunlap et al., 2016). The commercially available strain *B. amyloliquefaciens* FZB42 (FZB42; RhizoVital® 42, Abitep GmbH) is considered as the type strain of this "*plantarum*" subspecies. This is based on its genetic richness in key genes or clusters involved in its plant-associated lifestyle and the synthesis of bioactive secondary metabolites (BSM) acting as signals for intra- or inter-species cross-talks (e.g., stimulation of ISR), and/or as antimicrobials for suppressing competitors in the rhizosphere (Koumoutsi et al., 2004; Chen et al., 2007, 2009; Borriss et al., 2011; Chowdhury et al., 2015).

As part of this BSM arsenal, surfactins, fengycins, and iturins are the three main families of cyclic lipopeptides (CLPs) produced by the *Bacillus amyloliquefaciens* species, synthesized in an mRNA-independent way by modular enzymes (non-ribosomal peptide synthetases, NRPS, or hybrid polyketide synthases/non-ribosomal peptide synthetases, PKS–NRPS; Walsh, 2004). These compounds have multiple functions that are crucial both for rhizosphere fitness of the producing strains but also for their biocontrol potential (Ongena et al., 2005a; Ramarathnam et al., 2007; Romero et al., 2007; Ongena and Jacques, 2008; Kim et al., 2010; Raaijmakers et al., 2010; Cawoy et al., 2015). Surfactins are heptapeptides linked to a β-hydroxy fatty acid (various homologs from C12 to C17) which display some ISR-eliciting activity and some antibacterial and antiviral activity, but are not fungitoxic (Peypoux et al., 1999; Ongena et al., 2007). As wetting agents, surfactins also help the movement of producing cells along the roots by facilitating swarming motility (Kinsinger et al., 2003; Julkowska et al., 2005; Leclère et al., 2006). Moreover, an essential role in the formation of biofilm on roots has been recognized for this CLP (Bais et al., 2004). Fengycins are lipodecapeptides with an internal lactone ring in the peptide moiety, plus a saturated or unsaturated β-hydroxy fatty acid chain (C14–C19), and show strong antifungal activity (Vanittanakom et al., 1986). Iturins include seven variants, of which bacillomycins and mycosubtilin are the best known; all of them are heptapeptides linked to a β-amino fatty acid chain (C14–C17). Iturins have limited antibacterial activity, but a strong antifungal effect (Maget-Dana and Peypoux, 1994). Fengycins and iturins have been shown to be essential for the ISR-independent biocontrol provided by several *Bacillus* strains (Ongena and Jacques, 2008; Raaijmakers et al., 2010).

Bacillus amyloliquefaciens subsp. *plantarum* S499 (S499) represents another strain that has been widely investigated due to its biocontrol potential, and more broadly in the context of molecular interaction with the host plant and other soil-borne microorganisms. S499 also synthesizes the three CLP families, but in quite different proportions compared to FZB42 and other *Bacillus amyloliquefaciens* subsp. *plantarum* strains, suggesting a divergent regulatory pathway in the synthesis of secondary metabolites (Cawoy et al., 2015). For instance, S499 is a very efficient producer of surfactins *in vitro* and *in planta*, which correlates with a higher potential for ISR induction compared to FZB42 and other strains belonging to the same subspecies (Jacques et al., 1999; Cawoy et al., 2014). S499 is also quite distinct in terms of other phenotypic traits such as biofilm formation, motility and root colonization (Cawoy et al., 2014). In addition, some environmental factors and plant determinants influence CLP production in S499, making this strain a good model for studying multitrophic interaction in the rhizosphere ecosystem (Nihorimbere et al., 2012; Pertot et al., 2013; Debois et al., 2015). The genome of S499 has recently been sequenced, assembled, and annotated with the scope of pointing out some genetic determinants possibly related to its relatively specific behavior (CP014700–CP014701, Molinatto et al., 2016).

In the work presented here, we performed further comparative genomics, revealing some peculiarities in terms of genetic equipment and organization at chromosome level compared to the type strain FZB42. However, an additional feature of S499 is the presence of a plasmid (pS499) containing a *rap-phr* cassette encoding the response regulator aspartate phosphatase (Rap) and its putative Rap regulatory peptide (Phr). As Rap-Phr systems have pleiotropic regulatory effects on a number of cellular processes (Pottathil and Lazazzera, 2003), we pursued the functional characterisation of pS499 by evaluating how its loss affects S499 behavior compared to the type strain FZB42. Our data provide some evidence regarding the crucial impact of this plasmid on several traits related to rhizosphere competence, such as substrate utilization, cell motility, biofilm formation, and antagonism against fungal phytopathogens.

MATERIALS AND METHODS

Bacterial and Fungal Strains

All the bacterial strains used in this work (Table S1) were stored at length in glycerol 30% at −80°C and routinely grown at 28°C in Luria-Bertani broth (LB; tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, pH 7) and on LB broth amended with agar 16 g l^{-1} (LBA). The phytopathogenic fungi used in this work (Table S1) were grown on potato dextrose agar (PDA) at 28°C and stored at length on PDA slants at room temperature.

Genome Comparative Analysis

Phylogenetic analysis was carried out with Gegenees software 2.2.1 (Ågren et al., 2012) through fragmented all-against-all comparison (fragment size = 500; sliding step size = 500) performed on the sequences of *B. amyloliquefaciens* subsp. *plantarum* and closely related strains whose complete genome was present on NCBI (http://www.ncbi.nlm.nih.gov) on 14 March 2016. In particular, 19 strains belonging to different *Bacillus* species were included in the analysis. The heat plot tab generated by Gegenees was used to build the Neighbor-Joining phylogenetic tree with the Neighbor and DrawGram applications of the Phylogeny Inference Package (PHYLIP) version 3.695 (Felsenstein, 1989).

Genome alignment of the S499 genome against FZB42 was done with the sequence-based comparison tool on SEED Viewer version 2.0 (Overbeek et al., 2005). On the same platform, the BLAST tool was used to perform gene by gene sequence alignments. The genomes of FZB42 and S499 were also aligned by applying the progressive algorithm

and maintaining the default settings implemented in open-source MAUVE aligner v2.3.1 (Darling et al., 2010). BRIG application (Alikhan et al., 2011) was used to display circular comparisons.

To assess the frequency of plasmids similar to the S499 plasmid (pS499), the NCBI Genome Assembly, and Annotation reports for *B. amyloliquefaciens* subsp. *plantarum* and *B. amyloliquefaciens* subsp. *amyloliquefaciens,* and the NCBI Plasmid Annotation report for *B. subtilis* were examined (included sequences are listed in Table S2). Moreover, strains 23, 76, 98R, 98S, 104, and GA1 belonging to different *Bacillus* spp. were tested using PCR with Rap1 primers (Table S3) after isolation of plasmid DNA using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific Inc.). The PCR program was as follows: 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 68°C. Annotation of the pS499 sequence (CP014701) was done using Prokka (Seemann, 2014). The sequence encoding the Phr peptide was retrieved by aligning the *phrQ* gene (BAPNAU_RS20550, Wu et al., 2013) against the pS499 sequence. Alignments of the plasmid-encoded *rap* genes were performed on NCBI blastn suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and with EMBOSS Needle Pairwise Sequence Alignment tools (http://www.ebi.ac.uk/Tools/psa/).

Plasmid Curing

Bacillus amyloliquefaciens subsp. *plantarum* S499 was cured of its native plasmid according to the procedure described by Feng et al. (2013), with some modifications. Briefly, S499 was grown for 16 h at 30°C (180 rpm) in sterile 15 ml tubes containing 5 ml of LB broth. The resulting cell culture was diluted 10 times in LB broth and 50 μl were transferred to sterile 15 ml tubes containing 5 ml of LB broth amended with 0.005% sodium dodecyl sulfate. Inoculated tubes were incubated at 42°C (180 rpm) for 12 h. This step was repeated 14 times. After each 12 h incubation, serial dilutions of the cell cultures were streaked on LBA dishes and incubated at 30°C for 24 h. Selected colonies were picked up and total DNA was extracted using a PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The extracted total DNA was amplified in PCR reactions, where Rep primers (Table S3) specific for the pS499 sequence encoding *rep* gene were used. To rule out the possibility that potential plasmid cured derivatives were the result of contaminations, 16S rDNA, *cheA*, *gyrA* genes from S499 and its derivatives were amplified using primer pairs reported in Table S3. In all the cases, the PCR programs consisted of a first step at 95°C for 5 min and 30 cycles in series of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C and finally 5 min at 72°C. The presence/absence of the *rep* amplicons was checked on a 1% agarose gel to select plasmid cured derivatives. Amplicons from 16S rDNA, *cheA*, *gyrA* genes were purified with illustra ExoProStar 1-Step (GE Healthcare Europe GmbH), and sequenced with an ABI PRISM 3730xl DNA analyzer (Applied Biosystems, Thermo Fisher Scientific Inc., USA). To determine the level of nucleotide sequence identity, sequences of 16S rDNA, *cheA*, *gyrA* amplicons deriving from S499 and its derivative were subsequently aligned with EMBOSS Needle Pairwise Sequence Alignment tools (http://www.ebi.ac.uk/Tools/psa/).

Growth Curves

A SpectraMax M2E Multi-Mode Microplate Reader (Molecular Devices LLC, USA) was used to determine the growth rates of S499, and the plasmid-cured S499 strain (S499 P^-). FZB42 was included in all the following assays as a comparison. The test was carried out in sterile 48-well plates. A volume of 10 μl of a cell suspension [optical density at 600 nm (OD_{600}) = 0.001 corresponding to the 10^3 colony forming units (CFU)] was inoculated into LB broth (1 ml) and a modified LB broth (1 ml), where tryptone (10 g l^{-1}) was replaced by casamino acids (10 g l^{-1}). Non-inoculated LB and modified LB broths were used as a control. The plate was incubated with continuous shaking for 40 h at 28°C and OD_{600} was measured every 30 min. Three wells were used for each strain (replicates) and the experiment was repeated.

The supernatants of the 48-well LB cultures were filtered through a 0.2 μm membrane (Sartorius AG, Germany) at the end of the incubation period (40 h). To identify and quantify CLPs (surfactins, fengycins, and iturins), the culture filtrates of each strain were analyzed with ultra-performance liquid chromatography—electrospray ionization mass spectrometry (UPLC-ESI-MS) according to the procedure described below. Three repetitions of the assay were used to calculate the mean values of production.

Extracellular Proteolytic Activity

The FZB42, S499 and S499 P^- strains were inoculated into 15 ml sterile tubes containing 5 ml of LB broth and grown overnight (16 h) at 28°C (180 rpm). The resulting cell cultures were diluted 100 times by transferring 50 μl into sterile 15 ml tubes containing 5 ml of LB broth (three replicates for each strain). Once inoculated, 15 ml tubes were incubated for 6 h in the same conditions reported above. OD_{600} was recorded at the end of the incubation period. Subsequently, tubes were centrifuged at 4000 rpm for 10 min to remove the cells and the supernatants were filtered through a 0.2 μm membrane (Sartorius AG). A volume of 225 μl of culture filtrates (three replicates for each tube) was transferred to 1.5 ml sterile microfuge tubes and mixed with 150 μl of 1% azocasein stock solution (50 mM Tris-HCl, pH 8.8). After 4 h of incubation at 37°C, the undigested substrate was precipitated by adding 375 μl of 5% trichloroacetic acid and centrifuged for 3 min at 13,200 rpm. Supernatants were transferred to new 1.5 ml sterile microfuge tubes containing 400 μl of 1 M NaOH, and absorbance at 405 nm (OD_{405}) was then recorded. Relative proteolytic activity was calculated as the ratio of the OD_{405}/OD_{600}. The experiment was repeated.

The production of extracellular proteases was also assessed on skimmed milk dishes. For this purpose, a volume of 5 μl of a cell suspension (OD_{600} = 1) corresponding to 5×10^5 CFU for each strain was inoculated onto LBA amended with 1% (w/v) of skimmed milk. Once inoculated, Petri dishes were incubated at 28°C and the diameter of the clarification halo was measured after 48 h. Five Petri dishes (replicates) were used for each strain and the experiment was repeated.

Kinetics of Cyclic Lipopeptide Production

The FZB42, S499, and S499 P^- strains were grown overnight on LBA at 30°C in Petri dishes. Bacterial cells were scraped

from LBA surface and collected with a loop in 1 ml of sterile distilled H_2O, washed three times in sterile distilled H_2O, and the OD_{600} was adjusted to 1. A volume of 1 ml of the deriving cell suspensions was inoculated into Erlenmeyer flasks containing 100 ml of LB broth. Flasks were then incubated for 24 h at 28°C (110 rpm). The samples were collected every hour from 0 to 12 h with final sampling after 24 h. From each flask, 3 ml of cell culture were transferred to three microfuge tubes (1 ml each), which were centrifuged for 5 min at 12,600 rpm. Supernatants were used for the identification and quantification of cyclic lipopeptides with UPLC-ESI-MS. Cell pellets were stored at −20°C for analysis of gene expression (*srfA* and *rap*) using RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and measurement of bacterial growth with flow cytometry. The experiment was carried out three times.

Samples were analyzed using reverse phase UPLC (Acquity class H, Waters Corp., USA) coupled with a single quadrupole MS (SQ Detector, Waters Corp.) on an Acquity UPLC BEH C18 2.1 × 50 mm, 1.7 μm column (Waters Corp.). Elution started at 30% acetonitrile (flow rate of 0.60 ml min^{-1}). After 2.43 min, the percentage of acetonitrile was brought up to 95% and held for 5.2 min. Then, the column was stabilized at 30% acetonitrile for 1.7 min. Compounds were identified based on their retention times compared to authentic standards (98% purity; Lipofabrik Society, France) and the masses detected in the SQDetector. Ionization and source conditions were set as follows: source temperature = 130°C; desolvation temperature = 400°C; nitrogen flow = 1000 l h^{-1}; cone voltage = 120 V.

Quantification of Relative Gene Expression

Total RNA was extracted with a NucleoSpin® RNA kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions. Quantification of *srfA* and pS499 *rap* relative gene expression was done using reverse transcription Real-time PCR (StepOnePlus™, Thermo Fisher Scientific Inc.), with a qPCRBIO SyGreen 1-Step Hi-ROX kit (PCR Biosystems Ltd, UK). Primers used in the reactions were designed by Primer3web version 4.0.0 (Untergasser et al., 2012) and are listed in Table S3. The qRT-PCR program consisted of a first step of 10 min at 48°C, followed by 2 min at 95°C, and 40 cycles in series of 5 s at 95°C and 30 s at 60°C. The housekeeping gene *gyrA* was used as an endogenous control.

The relative gene expression was calculated according to the comparative C_T method (Livak and Schmittgen, 2001). At each time point, ΔC_T was determined by subtracting the threshold cycle (C_T) value of *gyrA* from the C_T value of the target gene; then the ΔC_T of sample "time 0" was subtracted from the ΔC_T values of the following sampling times, obtaining $\Delta\Delta C_T$ values. Finally, the relative quantity (RQ) of gene expression was calculated according to this formula:

$$RQ = 2^{-\Delta\Delta CT}$$

Measurement of Bacterial Growth with Flow Cytometry

A first sonication step was used to dissolve cellular aggregates. Cell pellets were suspended in 500 μl of staining solution S1 (72 g l^{-1} tartaric acid; 3.89 g l^{-1} Na_2HPO_4; 2.85 g l^{-1} EDTA; 0.0375 g l^{-1} sucrose monohydrate; pH 3) over three cycles of gentle sonication (15–20 s at 25–30% of the power of the device, Sonopuls HD 2070, Bandelin GmbH, Germany). Subsequently, 500 μl of solution S2 (1.95 g l^{-1} citric acid; 5.8 g l^{-1} NaCl; 1.45 g l^{-1} Na_2HPO_4; pH 3.8) supplied with 0.2% acridine orange were added. Samples were then mixed by vortexing thoroughly, before being analyzed with flow cytometry (BD Accuri™ C6, Becton, Dickinson and Company, USA) for cell counting.

Swarming Motility and Biofilm Formation

The swarming motility of FZB42, S499, and S499 P^- was evaluated according to Pertot et al. (2013). The diameter of the bacterial macrocolonies was measured at 12, 16, and 20 h after inoculation. Four Petri dishes (replicates) were used for each strain and the experiment was repeated.

The production of biofilm by FZB42, S499, and S499 P^- in 24-well polystyrene plates was determined according to Pertot et al. (2013). Specific biofilm formation (SBF) was calculated according to Yaryura et al. (2008) with the following formula:

$$SBF = (B - NC)/BG$$

where B is the amount of crystal violet attached to the well surfaces measured at 590 nm (OD_{590}), NC is the OD_{590} of the negative control and BG is the bacterial growth measured at 600 nm (OD_{600}). Eight wells (replicates) were used for each strain and the experiment was repeated.

Antagonism against Fungal Phytopathogens

The antifungal activity of FZB42, S499, and S499 P^- was tested against *Cladosporium cucumerinum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Cladosporium cucumerinum* was streaked over the whole LBA surface in Petri dishes, and subsequently a volume of 5 μl of bacterial cell suspension (OD_{600} = 1) corresponding to 5 × 10^5 CFU was inoculated onto it. Petri dishes were incubated at 28°C for 72 h. To test for *F. oxysporum* f. sp. *radicis-lycopersici*, bacterial cells were inoculated onto LBA, 2 cm from the edge of the Petri dishes. Once inoculated, dishes were incubated at 28°C for 72 h. Plugs of *F. oxysporum* f. sp. *radicis-lycopersici* mycelium (5 mm) were cut away from the edge of young (5-day-old) colonies grown on PDA and placed 2.5 cm from the bacterial colonies. Petri dishes were incubated at 28°C for 72 h. Dishes not inoculated with the bacterial strains were used as a control. At the end of the incubation period, the inhibition zone (distance between bacterial colonies and mycelia) was measured. Moreover, two plugs (5 mm) of medium were removed from the inhibition zone and transferred to 1.5 ml microfuge tubes containing 1 ml of 50% acetonitrile and 0.1% formic acid. Cyclic lipopeptides were extracted by regular vortexing for 2 h at room temperature. Then, samples were centrifuged and filtered through a 0.2 μm membrane (Sartorius AG) before being injected into UPLC-ESI-MS columns according to the procedure reported above. Three dishes were used for each combination (replicates) and the experiment was repeated.

Statistical Analysis

As the *F*-test ($\alpha = 0.05$) revealed non-significant differences between repeated experiments ($p > 0.5$), the data were pooled. For proteolytic activity, CLP production, swarming motility, and biofilm formation assays, data were subjected to one-way ANOVA. The data obtained in the antagonism experiments were subjected to multifactorial ANOVA. Tukey's test ($\alpha = 0.05$) was used to perform mean pairwise comparisons. Statistical analysis was carried out using Excel (Microsoft Corp., USA) and Statistica (Dell Inc., USA).

RESULTS

Specific Genetic Traits of the *Bacillus amyloliquefaciens* subsp. *plantarum* S499 Chromosome

Comparative genomics first confirmed that S499 belongs to the taxonomic group of the *B. amyloliquefaciens* subsp. *plantarum*. Indeed, its genome shares the highest level of sequence similarity with the NJN-6, JJ-D34, CAU B946, and B25 strains, forming a subclade of the branch including FZB42 and all other strains classified under the "*plantarum*" subspecies. This branch is separated from those of the closely related *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7, *B. subtilis* subsp. *subtilis* 168, and *B. licheniformis* ATCC 14580 (**Figure 1**). However, comparison of general genomic features (genome size, G+C content, the number of coding sequences, RNA operons, tRNAs, and insertion sequence elements) showed high similarity with these species. A significant difference emerged in the content of phage-related genes. Indeed, the S499 genome has 154 phage-related genes, while only 44 and 71 of these genes are present in the FZB42 and *B. licheniformis* ATCC 14580 genomes, respectively. However, the phage-related gene content in the S499 genome is lower than that of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 (273 genes) and *B. subtilis* subsp. *subtilis* 168 (268 genes) (**Table 1**).

Assembly of the S499 genome sequence revealed that its chromosome has a different arrangement, including a large inversion compared to the FZB42 genome (**Figure 2A**). Alignment against the FZB42 genome showed 98% of nucleotide identity out of 94% query cover (**Figure 2B**). The S499 genome shares 46 and 88% of coding sequences (CDS), with respectively >99% and >90% identity at amino acid level with the FZB42 genome. Their core genomes include 3547 CDS. As

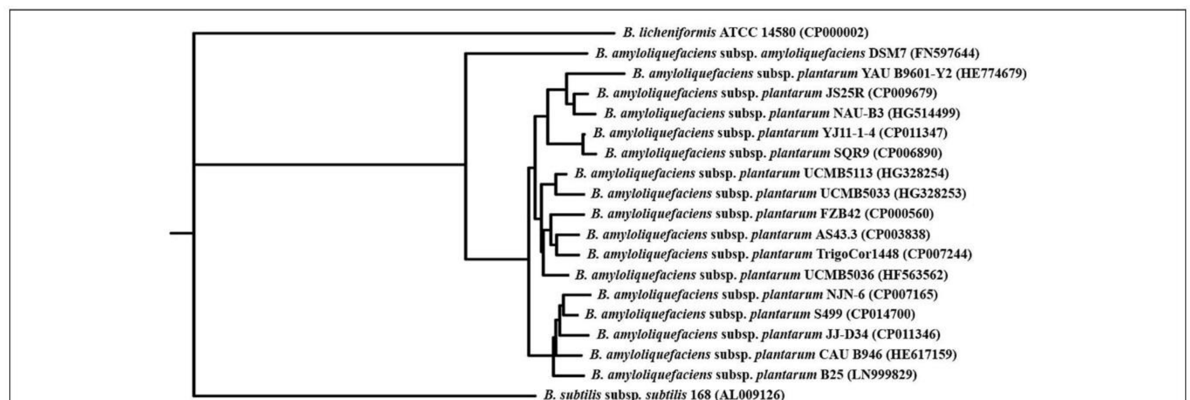

FIGURE 1 | Phylogenetic tree of *Bacillus amyloliquefaciens* subsp. *plantarum*. The Neighbor-Joining phylogenetic tree was obtained with PHYLIP applications after fragmented all-against-all comparison performed using Gegenees software 2.2.1 with the complete genome sequences of 19 strains belonging to the *Bacillus* genus (accession numbers are reported in brackets).

TABLE 1 | Principal genomic features of *Bacillus amyloliquefaciens* subsp. *plantarum* S499, in comparison with the genomes of the closely related *Bacillus* spp.

	B. amyloliquefaciens subsp. *plantarum* S499	*B. amyloliquefaciens* subsp. *plantarum* FZB42	*B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7	*B. subtilis* subsp. *subtilis* 168	*B. licheniformis* ATCC 14580
Genome size (bp)	3,927,922	3,918,589	3,980,199	4,214,814	4,222,645
G+C contents (%)	46.6	46.4	46.1	43.5	46.2
Coding sequences (CDS)	3974	3863	3924	4114	4199
Ribosomal RNA operons	8	10	10	10	7
Number of tRNAs	81	89	94	86	72
Plasmids	1	–	–	–	–
Insertion sequence elements	1	9	18	–	10
Phage-associated genes	154	44	273	268	71

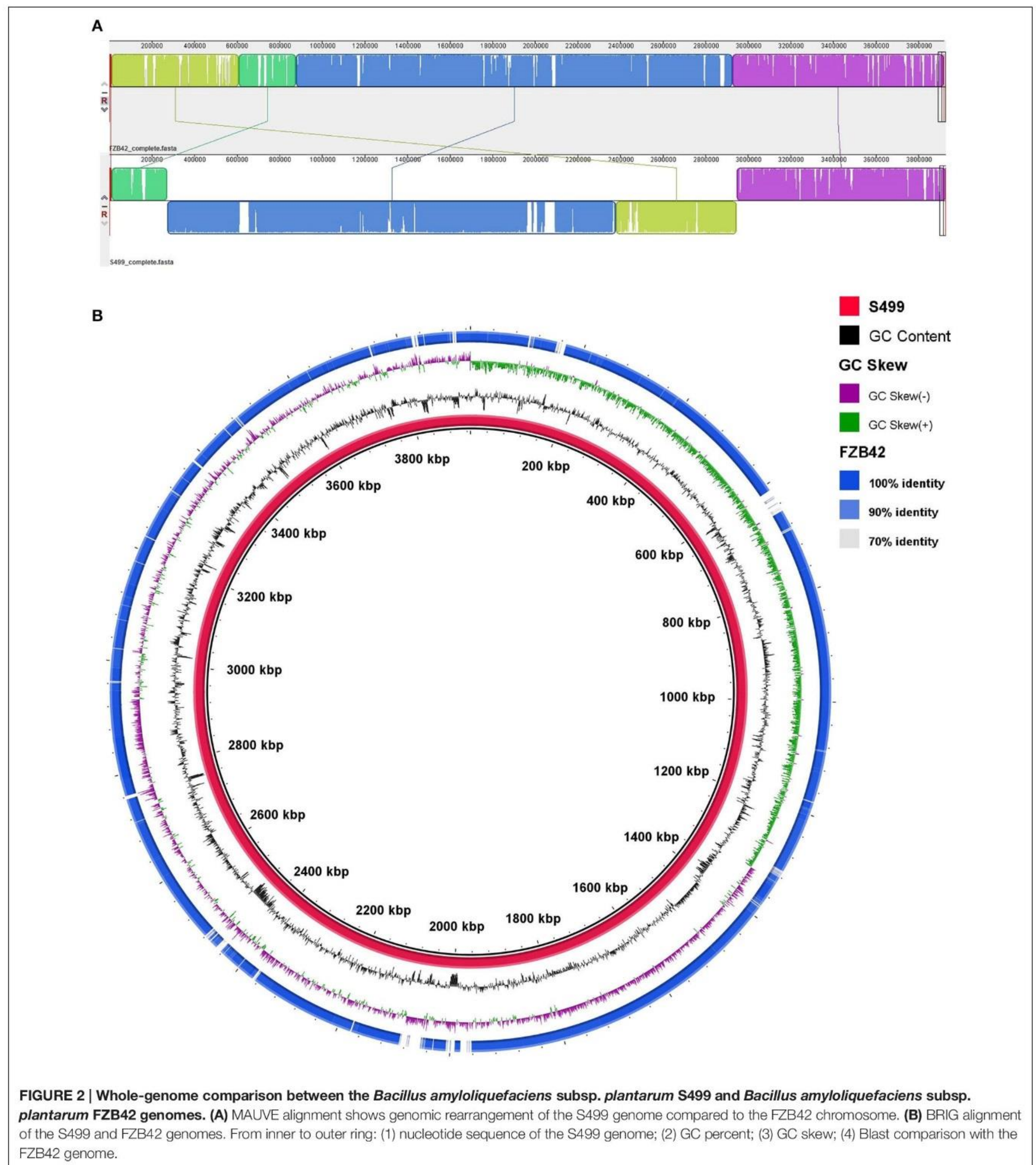

FIGURE 2 | Whole-genome comparison between the *Bacillus amyloliquefaciens* subsp. *plantarum* S499 and *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 genomes. (A) MAUVE alignment shows genomic rearrangement of the S499 genome compared to the FZB42 chromosome. **(B)** BRIG alignment of the S499 and FZB42 genomes. From inner to outer ring: (1) nucleotide sequence of the S499 genome; (2) GC percent; (3) GC skew; (4) Blast comparison with the FZB42 genome.

regards homologous genes with functions related to rhizosphere competence, plant-growth promotion and antimicrobial activity, nucleotide identity is always above 95%, with one case of 100% sequence identity for the *abrB* gene (**Table 2**).

A large number (46%) of S499 CDS not shared with FZB42 are annotated as hypothetical proteins, whereas 30% are phage-related genes. Moreover, genes putatively involved in primary metabolism (10%), transcriptional regulation (4%),

TABLE 2 | Genes involved in root colonization, plant growth promotion and biocontrol common to *Bacillus amyloliquefaciens* subsp. *plantarum* S499 and *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 (sequence similarity is expressed as the percentage of nucleotide identity).

Gene name	Locus tag		Product	Identity (%)
	S499	FZB42		
MOTILITY AND BIOFILM FORMATION				
abrB	AS588_RS09635	RBAM_RS05945	Transition state regulator	100
ecsA	AS588_RS10440	RBAM_RS05140	ABC transporter ATP-binding protein	98
ecsB	AS588_RS10435	RBAM_RS05145	ABC transporter permease	98
ecsC	AS588_RS10430	RBAM_RS05150	ABC transporter-associated protein	98
Efp	AS588_RS04045	RBAM_RS11340	Elongation factor P	98
epsA-O	AS588_RS15840-AS588_RS15910	RBAM_RS15740-RBAM_RS15810	Operon for capsular polysaccharides biosynthesis	96–98
degU	AS588_RS16430	RBAM_RS16295	Two-component response regulator	99
fla-che	AS588_RS07105-AS588_RS07250	RBAM_RS07995-RBAM_RS08140	Operon for flagellar synthesis and chemotaxis	98–99
lytS	AS588_RS02230	RBAM_RS12940	Sensor histidine kinase	98
motA	AS588_RS08495	RBAM_RS06710	Flagellar motor rotation protein	98
motB	AS588_RS08500	RBAM_RS06705	Flagellar motor rotation protein	98
pgsA-C	AS588_RS16615-AS588_RS16625	RBAM_RS16480-RBAM_RS16490	Operon for poly-γ-glutamate synthesis	99
resE	AS588_RS10795	RBAM_RS04785	Sensor histidine kinase	98
sacB	AS588_RS17695	RBAM_RS17620	Levan sucrose	98
Sfp	AS588_RS12395	RBAM_RS01880	Phosphopantetheinyl transferase necessary for surfactin synthesis	98
sigH	AS588_RS13505	RBAM_RS00625	Sigma factor H	98
sigW	AS588_RS13075	RBAM_RS01130	ECF sigma factor W	99
sinI	AS588_RS03975	RBAM_RS11410	SinR antagonist	99
sinR	AS588_RS03970	RBAM_RS11415	Master regulator of biofilm formation	99
spo0A	AS588_RS04160	RBAM_RS11225	Master regulator of initiation of sporulation	98
srfABCD	AS588_RS12405-AS588_RS12420	RBAM_RS01840-RBAM_RS01855	Surfactin synthetases	97–98
swrA	AS588_RS16295	RBAM_RS16170	Swarming protein	95
swrB	AS588_RS07095	RBAM_RS08150	Swarming protein	96
swrC	AS588_RS00450	RBAM_RS03555	Multidrug efflux pump	98
yqxM-sipW-tasA	AS588_RS03955-AS588_RS03965	RBAM_RS11420-RBAM_RS11430	Operon essential for biofilm formation	97–98
ycbA	AS588_RS12820	RBAM_RS01415	Sensor histidine kinase	96
ycdH	AS588_RS12675	RBAM_RS01565	High affinity zinc ABC transporter lipoprotein	98
yfiQ	AS588_RS01060	RBAM_RS04220	Putative surface adhesion protein	98
ylbF	AS588_RS07835	RBAM_RS07405	Positive regulator of ComK	99
ymcA	AS588_RS06825	RBAM_RS08420	Protein involved in community development	99
yqeK	AS588_RS03495	RBAM_RS11910	Putative HD phosphatase	98
yusV	AS588_RS15075	RBAM_RS15000	Protein involved in swarming/biofilm formation	98
CARBOHYDRATE CATABOLISM				
abnA	AS588_RS02280	RBAM_RS12890	Endo 1,5-alpha-L-arabinase	98
bglC	AS588_RS06260	RBAM_RS09035	Endo-1,4-beta-glucanase	96
bglS	AS588_RS18110	RBAM_RS18065	Endo-beta-1,3-1,4 glucanase	97
eglS	AS588_RS06260	RBAM_RS09035	Endo-1,4-beta-glucanase	96
galE1	AS588_RS09290	RBAM_RS06070	UDP-glucose 4-epimerase	97
galK1	AS588_RS09285	RBAM_RS06075	Galactokinase	97
galT1	AS588_RS09295	RBAM_RS06065	Galactose-1-phosphate uridyltransferase	97
ganA	AS588_RS09300	RBAM_RS06060	Arabinogalactan endo-1,4-beta-galactosidase	97
kdgA	AS588_RS06305	RBAM_RS08975	2-dehydro-3-deoxyphosphogluconate aldolase	98
kdgK	AS588_RS06315	RBAM_RS08965	2-dehydro-3-deoxygluconate kinase	97

(Continued)

TABLE 2 | Continued

Gene name	Locus tag		Product	Identity (%)
	S499	FZB42		
lacE	AS588_RS09280	RBAM_RS06080	Phosphotransferase system	97
lacF	AS588_RS09275	RBAM_RS06085	Phosphotransferase system	99
lacG	AS588_RS09270	RBAM_RS06090	Putative 6-phospho-beta-galactosidase	98
lacR	AS588_RS09265	RBAM_RS06095	Lactose phosphotransferase system repressor protein	97
Pel	AS588_RS00695	RBAM_RS03860	Pectate lyase	97
pelB	AS588_RS18195	RBAM_RS18130	Pectin lyase	97
Pgm	AS588_RS09260	RBAM_RS06100	Predicted phosphoglucomutase	97
yhfE	AS588_RS10370	RBAM_RS05210	Glucanase/aminopeptidase	99
ysdC	AS588_RS02275	RBAM_RS12895	Putative endo-1,4-beta-glucanase	98
xylA	AS588_RS06580	RBAM_RS08680	Xylose isomerase	97
xylB	AS588_RS06575	RBAM_RS08685	Xylulose kinase	97
xynA	AS588_RS17000	RBAM_RS16860	Xylanase	96
xynD	AS588_RS06235	RBAM_RS09060	Xylanase	97
xynP	AS588_RS06595	RBAM_RS08665	Hypothetical symporter of oligosaccharides	97
xynB	AS588_RS06590	RBAM_RS08670	Xylan beta-1,4-xylosidase	96
PLANT GROWTH PROMOTION				
alsD	AS588_RS16685	RBAM_RS16550	Acetolactate decarboxylase	98
alsS	AS588_RS16690	RBAM_RS16555	Acetolactate synthase	99
alsR	AS588_RS16695	RBAM_RS16560	LysR transcriptional regulator	98
bdhA	AS588_RS00220	RBAM_RS03320	2,3-butanediol dehydrogenase	99
dhaS	AS588_RS05800	RBAM_RS09505	Putative indole-3-acet-aldehyde dehydrogenase	98
Phy	S588_RS05510	RBAM_RS09795	Phytase	98
yhcX	AS588_RS10830	RBAM_RS04750	Nitrilase	98
ysnE	AS588_RS17725	RBAM_RS17660	Putative IAA acetyl-transferase	96
ywkB	AS588_RS17210	RBAM_RS17070	Putative auxin efflux carrier	97
ANTIMICRIOBAL ACTIVITY (GENE CLUSTERS FOR NON-RIBOSOMALLY SYNTHESIZED PEPTIDES AND POLYKETIDES)				
bacA-E	AS588_RS17515- AS588_RS17535	RBAM_RS17420- RBAM_RS17440	Bacilysin	97–99
baeB-S	AS588_RS06740- AS588_RS06800	RBAM_RS08445- RBAM_RS08505	Bacillaene	97–98
bmyA-D	AS588_RS06215- AS588_RS06230	RBAM_RS09065- RBAM_RS09080	Iturin	96–97
dfnA-M	AS588_RS04400- AS588_RS04470	RBAM_RS10910- RBAM_RS10980	Difficidin	96–98
dhbA-F	AS588_RS14595- AS588_RS14615	RBAM_RS14490- RBAM_RS14510	Bacillibactin	96–97
fenA-E	AS588_RS06080- AS588_RS06100	RBAM_RS09195- RBAM_RS09215	Fengycin	96
mlnA-I	AS588_RS08035- AS588_RS08075	RBAM_RS07160- RBAM_RS07200	Macrolactin	96–97
srfA-D	AS588_RS12405- AS588_RS12420	RBAM_RS01840- RBAM_RS01855	Surfactin	97–98

transport systems (2%), tetracycline resistance, and lanthionine biosynthesis are included in the remaining unique S499 CDS. Similarly to S499, a considerable number of FZB42 unique CDS (44%) are also classified as hypothetical proteins. However, among function-annotated CDS there are ribosomal genes (12%), genes encoding enzymes involved in transport, and detoxification (6%), genes related to restriction systems (5%), transcriptional regulation (3%), and acriflavine resistance (Table S4).

A Rare Plasmid Is Present in the Genome of *Bacillus amyloliquefaciens* subsp. *plantarum* S499

An additional important genetic feature distinguishing S499 from FZB42 is the presence of plasmid DNA (8008 bp). Based on the plasmid:chromosome sequence coverage ratio, we estimate that there are at least two copies of this plasmid (pS499) per cell. The few genes located on pS499 (**Figure 3**)

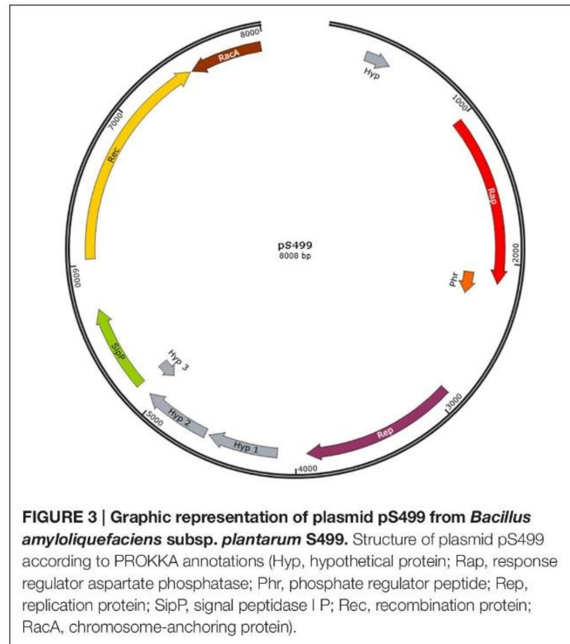

FIGURE 3 | Graphic representation of plasmid pS499 from *Bacillus amyloliquefaciens* subsp. *plantarum* S499. Structure of plasmid pS499 according to PROKKA annotations (Hyp, hypothetical protein; Rap, response regulator aspartate phosphatase; Phr, phosphate regulator peptide; Rep, replication protein; SipP, signal peptidase I P; Rec, recombination protein; RacA, chromosome-anchoring protein).

encode proteins involved in replication (AS588_19065, Rep) and mobilization (AS588_19090, Rac; AS588_19095, RecA) of the plasmid, a signal peptidase (AS588_19085, SipP), and a response regulator aspartate phosphatase (AS588_19060, Rap). Moreover, we identified a plasmid region encoding the putative Rap regulatory peptide (Phr) located downstream of the *rap* gene. In addition to this plasmid *rap-phr* cassette, nine genes encoding different members of the Rap family are located on the chromosome of S499. Seven of these genes show 98–99% nucleotide identity with the homologous genes of FZB42 (**Table 3**).

Comparison of the 32 available genome sequences of other *B. amyloliquefaciens* subsp. *plantarum* strains revealed that three of them (JS25R, NAU-B3, B25) have a plasmid of similar size also containing a *rap* sequence (**Table 4**). The *rap* genes encoded by the plasmids of JS25R (CP009680, 8438 bp) and NAU-B3 (HG514500, 8439 bp) showed 99% identity with the *rap* gene of pS499 at nucleotide level. In contrast, the *rap* gene located on the plasmid of B25 (LN999830, 8138 bp) does not share homology with that located on pS499. However, the B25 Rap protein shows 47% identity with the chromosome-encoded RapI of S499 at amino acid level.

The very low occurrence of a plasmid similar to pS499 also extends to the taxon *B. amyloliquefaciens* subsp. *amyloliquefaciens*, where only 2 out of 36 strains possess a plasmid. Specifically, the plasmid of strain MBE1283 (CP013728, 13,003 bp) has no *rap* genes, while the plasmid of strain LL3 (CP002635, 6758 bp) includes a *rap* gene that is not homologous to the one encoded by pS499 (**Table 4**) even if it shares 38% identity with the S499 chromosome-encoded RapI at amino acid level.

TABLE 3 | Genes encoding Rap proteins identified in the genome of *Bacillus amyloliquefaciens* subsp. *plantarum* S499 and sequence similarity with the homologous genes of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42.

Gene name	Locus tag		Product	Identity at nucleotide level (%)
	S499	FZB42		
CHROMOSOME				
rapA_2	AS588_RS09075	RBAM_RS06230	RapA	99
rapI_1	AS588_RS11695	–	RapI	–
rapF_1	AS588_RS12260	RBAM_RS02015	RapF/RapC	99
rapJ	AS588_RS12690	RBAM_RS01550	RapJ	99
rapH	AS588_RS14175	RBAM_RS02305	RapH	53
rapF_2	AS588_RS16895	RBAM_RS16755	RapF	98
rapA_4	AS588_RS17040	RBAM_RS16900	RapA/RapB	99
rapF_3	AS588_RS17360	RBAM_RS17235	RapF	98
rapI_3	AS588_RS18595	RBAM_RS18570	RapI/RapX	98
PLASMID				
rapA_5	AS588_19060	–	RapA/RapQ	–

In *B. subtilis*, out of 11 small plasmids (ranging from 2246 to 8737 bp), only two (pTA1040, 7837 bp, and pTA1060, 8737 bp) include *rap* sequences (*rap40* and *rap60*) displaying respectively 65 and 71% identity with the *rap* gene of pS499 at nucleotide level (**Table 4**). Other *rap* genes encoded by bigger plasmids (75–85 Kb) do not show identity with the *rap* gene of pS499.

In addition, we were also able to rule out the presence of analogous plasmids in six other *Bacillus* spp. strains from our lab collection (23, 76, 98R, 98S, 104, GA1), since no bands were observed in gel electrophoresis after plasmid DNA extraction and no pS499 *rap* gene was amplified by PCR (data not shown).

The Presence of Plasmid PS499 Affects Growth Kinetics and Proteolytic Activity

An S499 colony that had lost the plasmid was isolated after 14 culture cycles in sub-inhibitory conditions. On LBA, the colonies of the cured strain, named S499 P^-, were morphologically different and more precisely, smoother, and larger compared to colonies of the wild-type S499. The 16S rDNA and the sequences of *gyrA* and *cheA* genes of S499 P^- were amplified, sequenced, and aligned to the respective sequences of S499 to prove that S499 P^- was a derivative of S499. For each gene, the PCR products shared 100% nucleotide sequence identity (Supplementary Data Sheet).

Following cultivation in LB broth, the growth dynamics of S499 P^- clearly differed from the parental strain and were actually more similar to the kinetics observed for FZB42, with a reduced lag phase and an increased growth rate. The logarithmic phase lasted 5.3 ± 0.2 h (average ± standard error) for S499 P^-, while for S499 and FZB42 it lasted 6.3 ± 0.3 h and 6.3 ± 0.2 h respectively. However, S499 P^- had a lower final OD_{600} compared to S499 and FZB42 (**Figure 4**, black lines). Given these trends, we tested whether the faster cellular growth of S499 P^- could be linked to higher proteolytic activity, liberating amino acids as substrates from the proteinaceous source. First,

TABLE 4 | Plasmids similar to pS499 present in *Bacillus amyloliquefaciens* and *Bacillus subtilis* strains.

Strain	Plasmid name	Plasmid (accession number)	*Rap* gene (locus tag)	Identity (%)
B. amyloliquefaciens subsp. *plantarum* JS25R	pBMJS25R	CP009680	NG74_RS19355	99
B. amyloliquefaciens subsp. *plantarum* NAU-B3	pBAMMD1	HG514500	BAPNAU_RS20545	99
B. amyloliquefaciens subsp. *plantarum* B25	II	LN999830	BAMMD1_RS18490	–
B. amyloliquefaciens subsp. *amyloliquefaciens* LL3	pMC1	CP002635	LL3_RS20265	–
B. subtilis IAM 1232	pTA1040	NC_001764	pTA1040_p6	65
B. subtilis IFO 3022	pTA1060	NC_001766	pTA1060_p7	71

The plasmids harboring a rap gene are reported in the table (sequence similarity with the pS499 gene is expressed as the percentage of nucleotide identity).

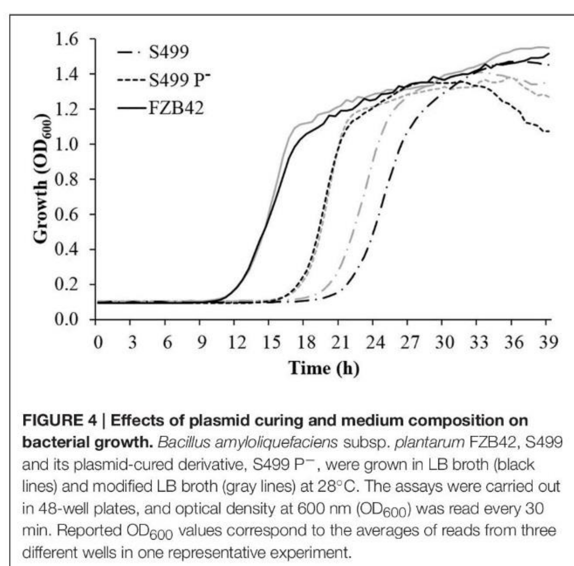

FIGURE 4 | Effects of plasmid curing and medium composition on bacterial growth. *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P$^-$, were grown in LB broth (black lines) and modified LB broth (gray lines) at 28°C. The assays were carried out in 48-well plates, and optical density at 600 nm (OD_{600}) was read every 30 min. Reported OD_{600} values correspond to the averages of reads from three different wells in one representative experiment.

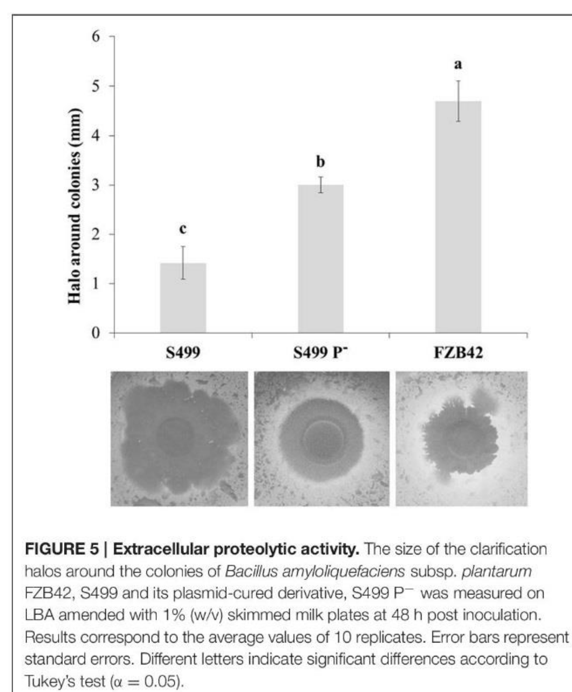

FIGURE 5 | Extracellular proteolytic activity. The size of the clarification halos around the colonies of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P$^-$ was measured on LBA amended with 1% (w/v) skimmed milk plates at 48 h post inoculation. Results correspond to the average values of 10 replicates. Error bars represent standard errors. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

we observed that substitution of tryptone by casamino acids in LB broth did not affect the cell growth of FZB42 and S499 P$^-$, whereas it led to faster entry in the logarithmic phase in S499 (**Figure 4**, gray lines). The hypothesis of higher proteolytic activity of S499 P$^-$ compared to S449 was then confirmed by testing the size of clarification halos forming around the colonies on LBA amended with skimmed milk. Indeed, after 48 h of incubation, the clarification halo around S499 P$^-$ (3.0 ± 0.2 mm; average ± standard error) was larger than that of S499 (1.4 ± 0.3 mm), but smaller than the halo around FZB42 colonies (4.7 ± 0.4 mm) (**Figure 5**). These results were also supported by the fact that, after 6 h growth in LB medium, the release of extracellular proteases was greater in S499 P$^-$ than in the wild type, determining slightly higher digestion of azocasein (Figure S1).

Plasmid PS499 Influences Lipopeptide Production

UPLC-ESI-MS analysis of culture filtrates at the end of the bacterial growth revealed that S499 P$^-$ produced more surfactins (120.35 ± 8.29 μg ml^{-1}, average ± standard error) and fengycins (39.60 ± 7.41 μg ml^{-1}), but fewer iturins (32.30 ± 10.08 μg ml^{-1}) than S499 (95.54 ± 1.33 μg ml^{-1}, 22.29 ± 7.02 μg ml^{-1} and 56.65 ± 13.99 μg ml^{-1}, respectively), although not significantly. FZB42 instead produced many more fengycins (238.10 ± 14.43 μg ml^{-1}) and iturins (211.05 ± 24.01 μg ml^{-1}), but fewer surfactins (84.10 ± 7.05 μg ml^{-1}) than S499 and S499 P$^-$ (Figure S2).

To better understand the role of the plasmid in CLP production, we set up a new growth assay that allowed us to follow the kinetics of production during culture. In line with the OD_{600} results, CFU counts indicated that S499 P$^-$ grew faster than S499, showing significant differences compared to the growth curve of S499 at 11 and 12 h, but not differently from FZB42 in these conditions (**Figure 6A**). The production of surfactins by S499 P$^-$, similarly to FZB42, started earlier compared to S499, resulting in a significant increase in surfactin

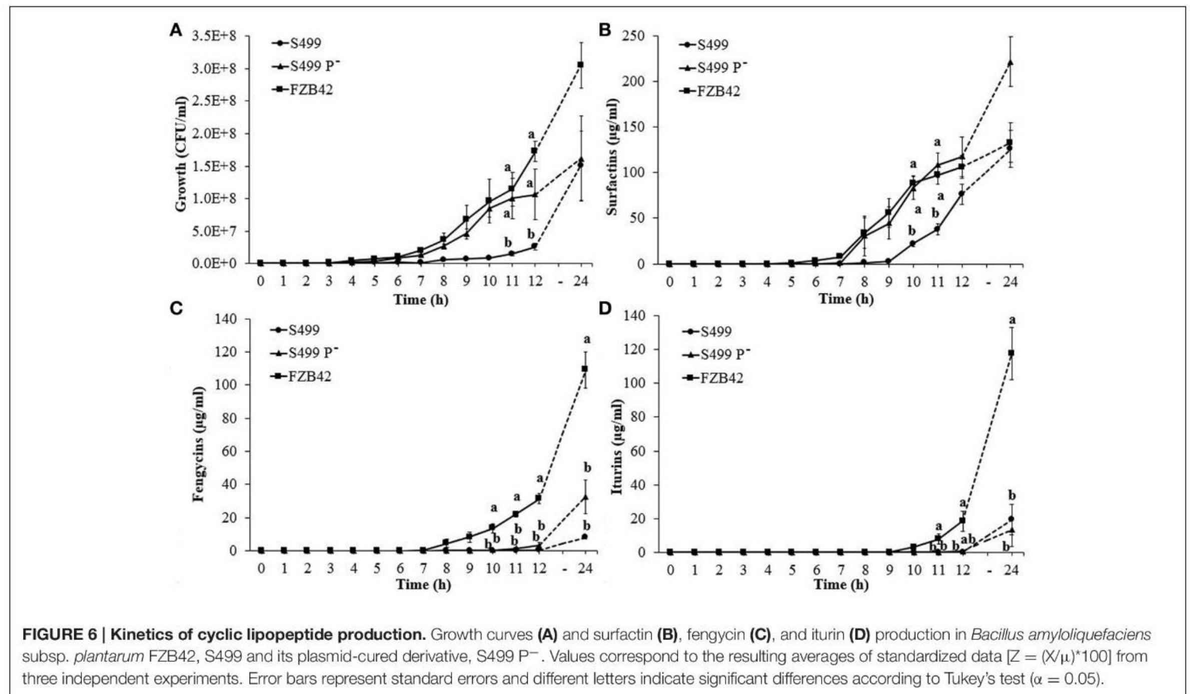

FIGURE 6 | Kinetics of cyclic lipopeptide production. Growth curves **(A)** and surfactin **(B)**, fengycin **(C)**, and iturin **(D)** production in *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P^-. Values correspond to the resulting averages of standardized data [$Z = (X/\mu)*100$] from three independent experiments. Error bars represent standard errors and different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

concentrations measured after 10 and 11 h of incubation. At 12 h, S499 P^- and FZB42 had released 118 ± 21 $\mu g\ ml^{-1}$ (average ± standard error) and 106 ± 12 $\mu g\ ml^{-1}$ of surfactins respectively, while S499 produced only 76 ± 11 $\mu g\ ml^{-1}$. At the final time-point (24 h), the trend for surfactin production by S499 P^- (222 ± 27 $\mu g\ ml^{-1}$) was also higher than for FZB42 (133 ± 22 $\mu g\ ml^{-1}$), although not significantly (**Figure 6B**).

Surfactins were detected in the culture filtrates of the three strains 3 h after *srfA* gene expression started to increase. Regression analysis of surfactin production and *srfA* gene expression levels assessed 3 h before was highly significant ($p < 0.01$, $y = 1.7059x + 15.624$, $R^2 = 0.283$). In accordance with surfactin production rates, *srfA* gene expression in S499 P^- and FZB42 increased earlier than in S499. Specifically, transcription levels of the *srfA* gene increased after 5 h of incubation and production started after 8 h in FZB42 and S499 P^-, whereas in S499 gene expression increased after 7 h and surfactins were detected after 10 h (Figure S3). When the expression of the plasmid-encoded *rap* gene was assessed in S499, its transcripts were detected at similar level of *gyrA* gene expression during early ($\Delta C_T = -0.69 \pm 0.66$; average ± standard error), middle ($\Delta C_T = 0.61 \pm 0.65$) and late growth phases ($\Delta C_T = 1.80 \pm 1.12$).

Kinetic tests showed no significant differences in the production of fengycins (detected from 10 h after incubation) in S499 (0.04 ± 0.03 $\mu g\ ml^{-1}$, average ± standard error) and S499 P^- (0.55 ± 0.18 $\mu g\ ml^{-1}$), and production was significantly lower than in FZB42 (13.53 ± 2.70 $\mu g\ ml^{-1}$) (**Figure 6C**). Similar behavior was observed for iturins, which were only detected in the culture filtrates of S499 and S499 P^- starting from 12 h after incubation, and in lower quantities (0.10 ± 0.08 and 0.70 ± 0.43 $\mu g\ ml^{-1}$) compared to the amounts detected in FZB42 supernatants (18.49 ± 5.85 $\mu g\ ml^{-1}$) (**Figure 6D**). However, at the end of the experiment (24 h) we observed an increasing although not significant trend for the production of fengycins and lower production of iturins by S499 P^- compared to S499, in accordance with previous assays.

The Impact of Plasmid PS499 on Motility and Biofilm Formation

Given the key role of surfactin in motility and biofilm formation in *Bacillus* (Raaijmakers et al., 2010), earlier and more abundant release of this CLP by S499 P^- could have an impact on these two phenotypic traits. Indeed, after 12 h of incubation, the diameter of S499 P^- macrocolonies (27.3 ± 0.2 mm, average ± standard error) was significantly larger than that observed for the parental strain (18.3 ± 0.4). The difference between S499 P^- and S499 was retained during the experiment, with their colonies measuring 67.0 ± 0.6 mm and 55.5 ± 1.5 mm respectively after 16 h. On the other hand, FZB42 was significantly faster than both S499 P^- and S499. Indeed, its average macrocolony diameter was 46.5 ± 0.4 mm after 12 h of incubation, and the colonies already reached the edge of the dishes after 16 h. After 20 h of incubation, the surface of each dish was fully covered by the bacterial cells for all strains (**Figure 7A**).

Conversely, the ability to form biofilm was reduced in S499 P^- compared to both S499 and FZB42. Indeed, after 72 h of static incubation, the SBF values of S499 P^- (1.51 ± 0.12, average ± standard error) were significantly lower compared to S499 (2.14

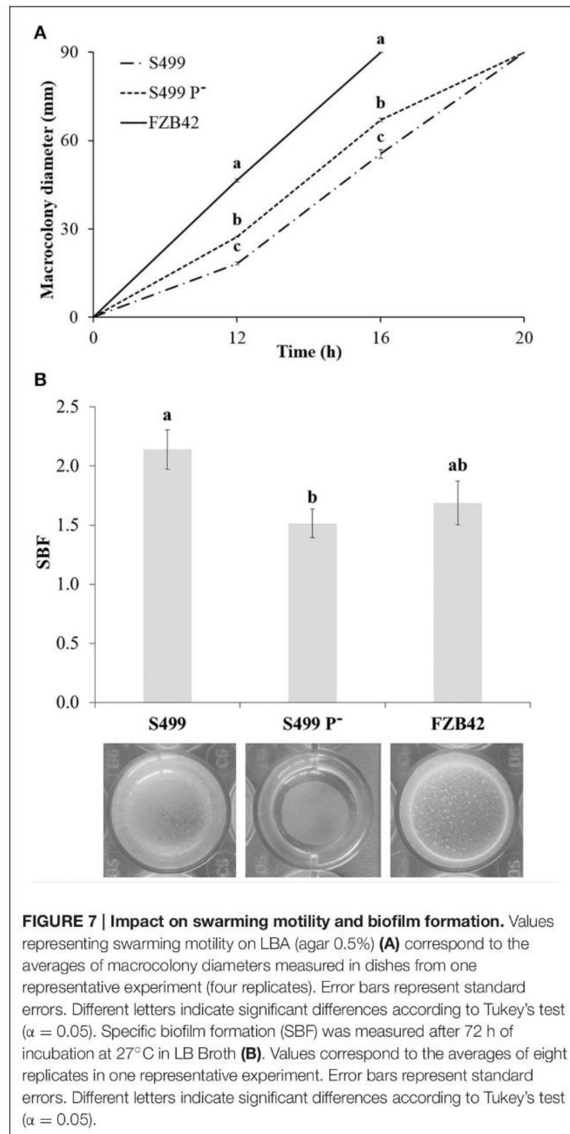

FIGURE 7 | Impact on swarming motility and biofilm formation. Values representing swarming motility on LBA (agar 0.5%) **(A)** correspond to the averages of macrocolony diameters measured in dishes from one representative experiment (four replicates). Error bars represent standard errors. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$). Specific biofilm formation (SBF) was measured after 72 h of incubation at 27°C in LB Broth **(B)**. Values correspond to the averages of eight replicates in one representative experiment. Error bars represent standard errors. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

$\pm$ 0.17) and FZB42 (1.69 $\pm$ 0.19). S499 P$^-$ produced a pellicle at the liquid-air interface that was not attached to the edge of the wells, as it was for S499 and FZB42 (**Figure 7B**).

Plasmid PS499 Influences Fengycin and Iturin-Dependent Antifungal Activity

Since S499 produces fengycins and iturins, which show direct antifungal activity, we further investigated the effect of pS499 on fungal growth inhibition potential *in vitro*. Multifactorial ANOVA indicated a significant effect of the pathogen type ($p < 0.01$), the *Bacillus* strain ($p < 0.01$) and the pathogen $\times$ *Bacillus* strain ($p < 0.01$) on the inhibition zones. The S499 P$^-$ strain was less effective than S499 and FZB42 in inhibiting the growth of *C. cucumerinum* and *F. oxysporum* f. sp. *radicis-lycopersici,* S499 and FZB42 were significantly more effective against *F. oxysporum* f. sp. *radicis-lycopersici* than against *C. cucumerinum*. Indeed, the inhibition zone was smaller for S499 P$^-$ (1.6 $\pm$ 0.2 mm against *C. cucumerinum* and 0.7 $\pm$ 0.3 mm against *F. oxysporum* f. sp. *radicis-lycopersici*) compared to S499 (4.0 $\pm$ 0.5 mm/6.5 $\pm$ 0.5 mm) and FZB42 (4.5 $\pm$ 0.2 mm/7.0 $\pm$ 0.4 mm) (**Figure 8A**).

In these confrontation assays performed on gelified LB medium, bacterial cells evolve as microcolonies that can be viewed as a kind of biofilm-structured non-motile community. Their physiology may thus be quite different compared to the planktonic state, with a possible impact on CLP synthesis. Plugs of medium were removed from the inhibition zone to quantify the CLP pattern through UPLC-ESI-MS analysis. Regression analysis indicated a significant effect of iturin (positive, $p < 0.01$, $y = 0.256x + 2.522$, $R^2 = 0.245$) and surfactin concentration (negative, $p < 0.01$, $y = -0.618x + 6.710$, $R^2 = 0.541$) on the size of inhibition zone, but not for fengycins.

Multifactorial ANOVA indicated a significant effect of the *Bacillus* strain ($p < 0.01$) on surfactin production, of the pathogen type ($p < 0.01$), the *Bacillus* strain ($p < 0.01$), and the pathogen $\times$ *Bacillus* strain ($p < 0.01$) on fengycin production, and of the *Bacillus* strain ($p < 0.01$) on iturin production. Surfactin production by S499 P$^-$ (7.47 $\pm$ 0.84 μg plug^{-1} from the inhibition zone of *C. cucumerinum* and 7.69 $\pm$ 0.75 μg plug^{-1} from the inhibition zone of *F. oxysporum* f. sp. *radicis-lycopersici*) was significantly higher than by S499 (3.36 $\pm$ 0.63 μg plug^{-1} and 3.94 $\pm$ 1.05 μg plug^{-1} respectively) and FZB42 (2.18 $\pm$ 0.35 μg plug^{-1} and 1.12 $\pm$ 0.14 μg plug^{-1} respectively) (**Figure 8B**). Fengycin production seemed to be stimulated by the presence of *C. cucumerinum*, but only in the case of FZB42. Indeed, in FZB42—*C. cucumerinum* dual cultures we detected 12.80 $\pm$ 3.50 μg plug^{-1} of fengycins, whereas in FZB42—*F. oxysporum* f. sp. *radicis-lycopersici* dual cultures we detected 3.37 $\pm$ 0.67 μg plug^{-1} of fengycins. The quantity of fengycins extracted from the inhibition zones around the colonies of S499 P$^-$ and S499 was similar and comprised between 1.03 $\pm$ 0.23 and 1.97 $\pm$ 0.46 μg plug^{-1} (**Figure 8C**). Iturin production was lower in S499 P$^-$ compared to S499, in line with the trend observed in liquid cultures. Indeed, we detected only 2.93 $\pm$ 0.84 μg plug^{-1} of iturins in S499 P$^-$— *C. cucumerinum* dual cultures and 1.76 $\pm$ 0.30 μg plug^{-1} in S499 P$^-$—*F. oxysporum* f. sp. *radicis-lycopersici* dual cultures, while we extracted 6.83 $\pm$ 1.71 μg plug^{-1} and 8.85 $\pm$ 3.01 μg plug^{-1} from the respective inhibition zones of S499 (**Figure 8D**).

DISCUSSION

The efficacy of some *B. amyloliquefaciens* subsp. *plantarum* strains available on the market as biofungicides (Borriss, 2011) can be improved after elucidating the molecular mechanisms that govern the multiple interactions occurring among beneficial bacteria, pathogenic microorganisms, host plants and the environment (Raaijmakers et al., 2009; Dutta and Podile, 2010; Berendsen et al., 2012). Convinced that the availability of the

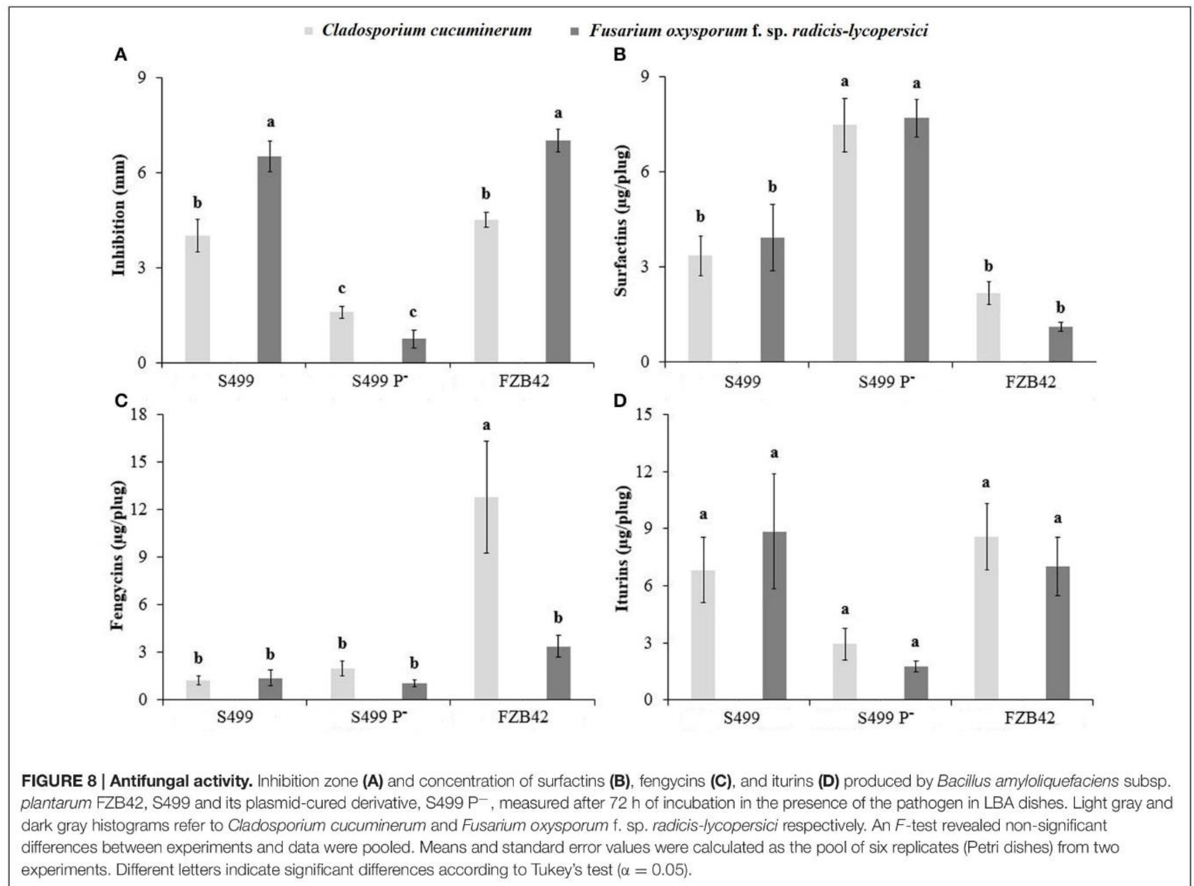

FIGURE 8 | Antifungal activity. Inhibition zone **(A)** and concentration of surfactins **(B)**, fengycins **(C)**, and iturins **(D)** produced by *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P$^-$, measured after 72 h of incubation in the presence of the pathogen in LBA dishes. Light gray and dark gray histograms refer to *Cladosporium cucuminerum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* respectively. An *F*-test revealed non-significant differences between experiments and data were pooled. Means and standard error values were calculated as the pool of six replicates (Petri dishes) from two experiments. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

bacterial genomes may foster this elucidation, we compared the genomes of S499 and the well-characterized FZB42 (Chen et al., 2007) to find unique characteristics that may explain the S499 distinctive phenotype (Ongena et al., 2005b; Cawoy et al., 2014, 2015).

The genome comparison showed a high degree of genetic conservation between the two strains. A large percentage of sequence identity was found for the genes responsible for root colonization, plant growth promotion, and biocontrol. Therefore, we hypothesize that divergences in regulatory elements could explain the different behavior of the bacteria, especially influencing their sensing of and adaptation to the environment. Indeed, some specific genes were identified as transcriptional factors or annotated as hypothetical proteins and it would be challenging to analyse how they contribute to determining the phenotypic differences observed between S499 and FZB42. Some other unique CDS encode transport proteins, such as ABC transporter permeases (e.g., AS588_RS00475, AS588_RS09125, RBAM_RS18310) and other transmembrane proteins (e.g., RBAM_RS01165, RBAM_RS02920), that might play a role in perceiving and responding to the environment in different ways for S499 and FZB42. In general, cell membrane transport systems are crucial for the survival of many microorganisms in natural conditions (Konings, 2006). For example, surface colonization relies on the efficient transfer of potassium ions (K^+) in *B. subtilis*, since a mutation in the K^+ transporter KtrAB prevents cell spreading (Kinsinger et al., 2005).

Within unique CDS in the S499 genome, phage-related sequences were also more numerous. Phages represent one of the main channels in horizontal gene transfer, therefore genetic variability among bacterial strains is significantly dependant on prophage acquisition (Brüssow and Hendrix, 2002). According to Wang et al. (2010), cryptic prophages help the host to overcome adverse environmental conditions (oxidative, osmotic, and acid stress). An abundance of prophage regions could reflect a higher plasticity of the S499 genome. This hypothesis is also supported by the presence of genomic rearrangements and plasmid DNA, which are other features contributing to genetic variation in bacteria and their rapid evolution (Van Elsas et al., 2000; Dobrindt and Hacker, 2001).

The presence of a plasmid (pS499) emerged as a distinctive feature of S499, as shown by our comparative analysis. This is an interesting aspect, given the fact that plasmids are not frequent either in the available sequenced strains or in other *Bacillus* spp. strains (23, 76, 98R, 98S, 104, GA1) also identified as good antibiotic producers in our laboratory (Cawoy et al., 2015). Based on its size and structural organization, pS499 can be ascribed to the class of small rolling circle replicons (Guglielmetti et al., 2007). These natural plasmids have a typical organization that includes a replication module, a gene responsible for plasmid mobilization and one or more modules not involved in plasmid metabolism but encoding traits related to the host physiology. For instance, in some strains the plasmid encodes a heat shock protein putatively involved in stress responses, which may represent an advantage for the bacterium in its natural environment (Thorsted et al., 1999). Some impact of pS499 on the physiology of S499 can be expected from the presence of a *rap-phr* cassette. Indeed, some plasmid-encoded response regulator aspartate phosphatases have already been proven to control extracellular protease production, biofilm architecture, sporulation, and the genetic competence of *B. subtilis* and *B. amyloliquefaciens* (Meijer et al., 1998; Koetje et al., 2003; Parashar et al., 2013; Boguslawski et al., 2015). As the *rap* gene located on pS499 was expressed in S499 cells from an early growth phase, a role in regulating the cellular physiology of the bacterium can be foreseen.

Chromosome-encoded Rap-Phr systems have been extensively studied in *B. subtilis*, where they control cellular processes regulated by two-component systems, such as competence development, antibiotic synthesis, protein secretion, and sporulation (Perego et al., 1994; Bongiorni et al., 2005; Auchtung et al., 2006). Rap proteins (11 members in *B. subtilis*) counteract kinases by dephosphorylating intracellular response regulators (e.g., Spo0F) or alternatively, they inhibit transcriptional factors such as ComA and DegU in their DNA binding activity (Bongiorni et al., 2005). Seven of the 11 characterized *rap* genes are followed by a *phr* gene, encoding a phosphatase regulator (Phr) precursor peptide. Phr peptides are extracellularly processed to pentapeptides. Once they reach a critical concentration, therefore acting as quorum sensing signals, the mature peptides are reimported through an oligopeptide permease (Opp) into the cytoplasm, where they inhibit Rap proteins (Pottathil and Lazazzera, 2003). Besides the *rap-phr* cassette located on pS499, we also identified 10 *rap* genes in the S499 chromosome, with *rapI_1* representing another difference between S499 and FZB42. Having *rapI_1* and an additional *rap-phr* cassette encoded by a plasmid, which can further increase its copy number, could constitute an ecological advantage for S499 compared to FZB42. Interestingly, Thorsted et al. (1999) highlighted that the presence of plasmid-encoded *rap* genes is more diffuse among Russian strains isolated from the soil rather than in strains selected to be used in the Japanese fermentation industry. By curing S499 of its plasmid, we aimed to elucidate the role played by the Rap-Phr system encoded by pS499 in the phenotypic differences that have so far been characterized in S499 and FZB42.

The absence of the *rap-phr* cassette located on pS499 caused faster growth of S499 P$^-$ compared to the wild strain, which could be linked to improved substrate utilization ability by the cured cells. Indeed, increased activity of secreted proteases was observed for S499 P$^-$. The increase in proteolytic activity is in accordance with previous studies showing that the pTA1060-encoded Rap60-Phr60 system controls the secretion of proteolytic enzymes. More precisely, *rap60* is involved in down-regulation of the *aprE* gene (responsible for production of the extracellular protease subtilisin) during post-exponential growth (Koetje et al., 2003; Boguslawski et al., 2015) through a complex molecular pathway. The *rap* gene of pS499 displays 71% sequence identity with *rap60* at nucleotide level. Nevertheless, in our case, increased proteolytic activity of S499 P$^-$ was also observed during the early growth phase (6 h), with very low biomass. Thus, it is conceivable that the *rap-phr* cassette of pS499 may behave differently from pTA1060-encoded Rap60-Phr60, although the same negative control on the secretion of extracellular proteases can be assumed. Consistently with the earlier start of exponential growth phase for FZB42, both azocasein and skimmed milk assays showed that FZB42 extracellular proteolytic activity was higher compared to S499 P$^-$. Therefore, in addition to the plasmid, other genetic features are responsible for the different phenotypes of FZB42 and S499.

The higher growth rate of S499 P$^-$ can only partially be explained by the increased production of proteases. Indeed, in modified LB cultures the S499 curve did not overlap that of S499 P$^-$, but still rose later and showed a milder slope. Another possible explanation for the observed growth shift is related to the energy cost of pS499 replication in S499 cells. Indeed, the presence of plasmids is frequently associated with reduced growth rates, especially in the case of large and high copy number plasmids (Smith and Bidochka, 1998; Diaz Ricci and Hernández, 2000). For example, Trautwein et al. (2016) recently reported that deletion of the 262 Kb native plasmid in *Phaeobacter inhibens* DSM 17395 improved the growth efficiency of the strain. They hypothesized that either the plasmid delays cell division by slowing down the DNA replication process, or that it has a metabolic weight that compromises growth efficiency. Even if pS499 is considerably reduced in size, we can assume a similar fitness cost for the host cells. However, it is worth noting that S499 tended to keep its plasmid during cell multiplication, despite the hypothesized energy cost. In fact, although exposed to sublethal conditions during our curing procedure, plasmid loss was a rare event in S499. This corroborates the hypothesis that pS499 could provide a real selective advantage in natural environments, as in the case of pQBR103 from *Pseudomonas fluorescens* for example (Lilley and Bailey, 1997) and many other bacterial plasmids (Thomas, 2004).

Plasmid curing considerably affected the CLP production by S499. Kinetic assay showed that S499 P$^-$ behaves similarly to FZB42 as far as surfactin is concerned, suggesting a role of the plasmid in regulating surfactin synthesis. However, it remains unclear whether earlier production by S499 P$^-$ is due to earlier entrance in the exponential phase or to divergences

in transcriptional regulation. Indeed, it is known that *srfA* gene expression is cell-density dependent, being controlled by the ComP-ComA signal transduction system (Nakano et al., 1991). Given the differences observed in growth rates, earlier surfactin production could be related to faster multiplication of S499 P^{-} and FZB42 populations. Nevertheless, we noted that the cured strain accumulated many more surfactins than FZB42 and S499 over time. Consistently, *srfA* gene expression was down-regulated in *B. subtilis* OKB105 transformed with the plasmid-encoded *rap* gene from *B. amyloliquefaciens* subsp. *plantarum* NAU-B3 (99% nucleotide sequence identity with pS499 *rap*; Yang et al., 2015). Furthermore, it has been demonstrated that the Rap phosphatase modulates *srfA* transcription by forming a ternary complex with ComA and *srfA* promoter (Yang et al., 2015). Similarly, we can postulate that loss of the pS499 *rap* gene enhanced *srfA* expression because of the removal of inhibition in the cured strain. Conversely, no significant differences between the cured strain and its parental strain were observed in terms of the release of fengycins and iturins. We can therefore assume that the differences between S499 and FZB42 observed in the production of these CLPs mostly depend on a different regulatory pathway, not involving pS499.

As a consequence of different surfactin production, in S499 P^{-} some phenotypic traits related to rhizosphere competence were affected, e.g., the speed of surface colonization. *Bacillus* spp. are capable of multicellular behavior known as swarming motility, a common bacterial way of moving across surfaces powered by rotating flagella (Kearns and Losick, 2003). Swarming motility is highly dependent on the secretion of surfactins, which reduce surface tension given their amphiphilic nature (Kinsinger et al., 2003; Leclère et al., 2006; Kearns, 2010). Therefore, a boost in surfactin production by S499 P^{-} can explain the increase in its swarming ability. Similarly, the faster surface spreading of FZB42 can be related to higher production of surfactins compared to S499, but not to S499 P^{-}. Indeed, according to our kinetic assays, the surfactin production rates of S499 P^{-} and FZB42 were similar. Here we can speculate that the phenotypes were influenced by the different growth rates of the strains. Even without reaching a final conclusion on the mechanism involved, we can assume that the presence of the plasmid could be necessary in the process of root surface colonization by S499, because swarming motility is a major factor favoring root colonization, even more than chemotaxis (Gao et al., 2016).

Bacillus spp. develop in the rhizosphere in the form of biofilms, which are also crucial for the biocontrol of plant pathogens (Bais et al., 2004; Chen et al., 2012, 2013). SBF values were lower for FZB42 compared to S499 and on curing S499 we observed a further reduction in SBF in S499 P^{-}. These results suggest a role for the plasmid in determining the difference found between S499 and FZB42. Surfactins function as paracrine signals that induce the differentiation of biofilm matrix producer cells (López et al., 2009). By regulating the phosphorylation state of DegU, ComA, and Spo0A, different Rap phosphatases control multiple signaling cascades. This redundant network can integrate exogenous and endogenous signals, leading to the formation of distinct biofilm subpopulations (motile cells, matrix producers, competent cells, cannibals, etc.; Mielich-Süss and Lopez, 2015). In the light of the increased and earlier surfactin production associated with plasmid curing, we would have expected an increase rather than a reduction in SBF. However, it is likely that very small amounts of this lipopeptide are sufficient to trigger biofilm formation and that other factors become more important later. It has indeed been observed that the surfactin-producing subpopulation is actually restrained in biofilm layers (Mielich-Süss and Lopez, 2015). Although considerable effort is required to understand which specific pathway is targeted by the pS499-encoded Rap, we can assume that its lack is most probably the cause of the altered phenotype observed for S499 P^{-}. Likewise, McLoon et al. (2011) showed that RapP encoded by an 80 Kb plasmid from *B. subtilis* NCIB 3610 is required for the formation of robust biofilms typical of wild-type strains. Later studies revealed that RapP is a Spo0F phosphatase and that it is involved in the phosphorelay modulating the expression of *epsA-O* and *yqxM-sipW-tasA* biofilm operons (Parashar et al., 2013).

Similarly to previous results (Cawoy et al., 2015), regression analysis of the antagonism assay showed that limited iturin production affected the intrinsic ability of S499 P^{-} to inhibit the mycelial growth of *C. cucumerinum* and *F. oxysporum* f. sp. *lycopersici*. Furthermore, it is conceivable that the negative correlation with surfactin concentration resulted from a physiological imbalance: the more resources were allocated to the synthesis of surfactins, in particular by S499 P^{-}, the fewer were available for synthesis of iturins, which are known to be produced in the stationary phase of bacterial growth (Jacques et al., 1999). Plasmid pS499 is therefore indirectly relevant for the antifungal activity of the strain, being involved in the modulation of lipopeptide production. At all events, the fact that S499 and FZB42 produced similar quantities of iturins in dual culture with the pathogens suggests that other genetic traits are also involved.

In conclusion, our data show that the plasmid-encoded *rap* gene of *B. amyloliquefaciens* subsp. *plantarum* S499 has a role in controlling several traits like protease secretion, production of surfactins and biofilm formation. Growth and motility are also influenced, either indirectly by the pS499 Rap-Phr system and/or by the presence of the plasmid itself. To our knowledge, we provide here the first report on the relationship between a plasmid, or control of Rap phosphatase on fengycin and iturin production, and the related impact on biocontrol. To illustrate these molecular pathways more exhaustively, further studies on the cellular mechanisms are necessary. Finally, by comparing the behavior of FZB42 and the S499 plasmid-cured derivative S499 P^{-}, we can conclude that pS499 plays a significant role in the phenotype of the two strains, although other genetic differences merit additional investigation.

AUTHOR CONTRIBUTIONS

GM carried out all the experiments, analyzed the data and wrote and edited the manuscript. LF carried out UPLC-ESI-MS analysis and wrote and edited the manuscript. SS carried out

qRT-PCR and wrote and edited the manuscript. GP, IP, and MO conceived the work, designed the experiments, analyzed the data and wrote and edited the manuscript. All the authors have read the manuscript and agreed to its content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00017/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Supplementary Table 1: Microorganisms used in this study.

Phylum	Strain	ID
Bacteria	*Bacillus amyloliquefaciens* subsp. *plantarum* S499	S499
	Bacillus amyloliquefaciens subsp. *plantarum* S499 P^-	S499 P^-
	Bacillus amyloliquefaciens subsp. *plantarum* FZB42	FZB42
	Bacillus amyloliquefaciens GA1	GA1
	Bacillus subtilis/amyloliquefaciens 23	23
	Bacillus subtilis/amyloliquefaciens 76	76
	Bacillus subtilis/amyloliquefaciens 98R	98R
	Bacillus subtilis/amyloliquefaciens 98S	98S
	Bacillus subtilis/amyloliquefaciens 104	104
Fungi	*Cladosporium cucumerinum*	
	Fusarium oxysporum f. sp. *radicis-lycopersici*	

Supplementary Table 2: Genetic sequences analysed to describe the frequency of plasmids similar to pS499. Sequences were present on the NCBI Genome Assembly and Annotation reports for *Bacillus amyloliquefaciens* subsp. *plantarum* and *B. amyloliquefaciens* subsp. *amyloliquefaciens,* and the Plasmid Annotation report for *B. subtilis* (http://www.ncbi.nlm.nih.gov).

Species	Strain / Plasmid	Accession no.
Bacillus amyloliquefaciens subsp. *plantarum*	FZB42	CP000560
	CAU B946	HE617159
	YAU B9601-Y2	HE774679
	M27	AMPK00000000
	AS43.3	CP003838
	UCMB5036	HF563562
	UCMB-5033	HG328253
	UCMB5113	HG328254
	NAU-B3	HG514499
	SK19.001	AOFO00000000
	TrigoCor1448	CP007244
	AH159-1	JFBZ00000000
	SQR9	CP006890
	W2	JOKF00000000
	JS25R	CP009679
	AP183	JXAM00000000
	GR4-5	JYGH00000000
	KACC 13105	JTKJ00000000
	NJN-6	CP007165
	JJ-D34	CP011346
	YJ11-1-4	CP011347
	G341	CP011686
	OB9	LGAU00000000
	B26	LGAT00000000
	KCTC 13012	LHCC00000000
	NBIF-003	LJJY00000000
	NRRL B-4257	LLZC00000000
	KACC 18228	LLZA00000000
	NRRL B-4257	LLZB00000000
	FKM10	LNTG00000000
	RC218	LQCL00000000
	B25	LN999829

Bacillus amyloliquefaciens subsp. *amyloliquefaciens*	DSM7	FN597644
	TA208	CP002627
	LL3	CP002634
	XH7	CP002927
	Y2	CP003332
	DC-12	AMQI00000000
	EGD-AQ14	AVQH00000000
	UASWS BA1	AWQY00000000
	CC178	CP006845
	LFB112	CP006952
	EBL11	JCOC00000000
	B1895	JMEG00000000
	CMW1	BBLH00000000
	X1	JQNZ00000000
	HB-26	AUWK00000000
	JJC33M	JTJG00000000
	LPL-K103	JXAT00000000
	TF28	JUDU00000000
	L-H15	CP010556
	KHG19	CP007242
	12B	JZDI00000000
	L-S60	CP011278
	516_BAMY	JVEA00000000
	Lx-11	AUNG00000000
	629	LGYP00000000
	Bs006	LJAU00000000
	XK-4-1	LJDI00000000
	RHNK22	LMAG00000000
	Jxnuwx-1	LMAT00000000
	MBE1283	CP013727
	11B91	LPUP00000000
	B4140	LQYO00000000
	B425	LQYP00000000
	UMA56639	CP006058
	UMAF6614	CP006960
	B15	CP014783

Bacillus subtilis	pIM13	M13761
	pTA1040	U32378
	pTA1015	U32379
	pTA1060	U32380
	p1414	AF091592
	pBS608	AY836798
	pLS30	AB243053
	pPL1	DQ140187
	pLS20	AB615352
	pLS32	AB615353
	pBS32	KF365913
	pSU01	ANIP01000001
	unnamed1	CP014472
	unnamed2	CP014473
	pBEST195S	AP011542

Supplementary Table 3. Primers used in this study. Oligonucleotides were designed by Primer3web version 4.0.0 (Untergasser *et al.*, 2012).

Target gene	Primers	Sequence (5'-3')	References
16S rRNA	9-F	GAGTTTGATCCTGGCTCAG	Weisburg et al.(1991)
	1512-R	ACGGCTACCTTGTTACGACTT	
cheA	CheA-F	AGAGCTGCCCATACGCTGAAAGGCATGAGC	This study
	CheA-R	GGTTTCTACCGGCACCATCCGCATATTAAG	
gyrA	Gyr-F	GAGACGCACTGAAATCGTGA	This study
	Gyr-R	GCCGGGAGACGTTTAACATA	
	GyrA-F	CAGTCAGGAAATGCGGACATCCTT	This study
	GyrA-R	CAAGATAATGCTCCAGACATTGTT	
rap	Rap-F	AGGACATGGAAGAGGACCAA	This study
	Rap-R	GTCCGGTCCCTTCAGATTTT	
	Rap1-F	ATACGAATTCATTATCGTTGCGGCATGTCG	This study
	Rap1-R	ATTAGGATCCTCTAAGAGTCCGCCCCATT	
rep	Rep-F	CATAGAATTCGAGGACTAGCATCAGAAGGAGT	This study
	Rep-R	ATTAGGATCCTGAAGTCCAAGCCTTTCCG	
srfA	Srf-F	ATTGTTTACGGTGGCTCTGG	Debois et al.(2015)
	Srf-R	CGCTGCGATAGTCAAAATCA	

Supplementary Table 4: Genes present in the *Bacillus amyloliquefaciens* subsp. *plantarum* S499 genome not shared by the FZB42 genome and vice versa. Unique CDS were identified using the sequence based comparison tool on SEED Viewer version 2.0 (Overbeek *et al.*, 2005) and verified with NCBI annotations (http://www.ncbi.nlm.nih.gov) on S499 (CP014700) and FZB42 (CP000560) genomes.

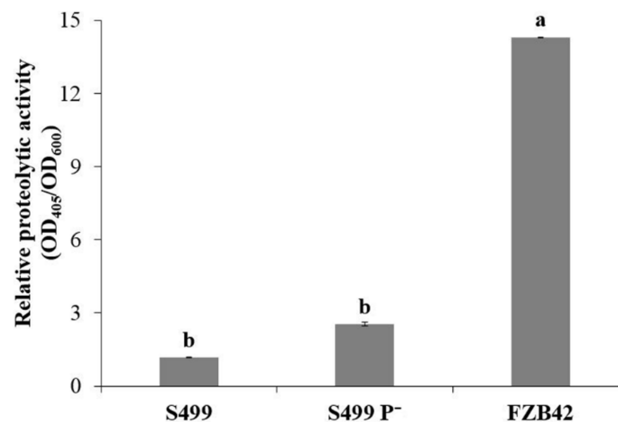
S499 specific genes		FZB42 specific genes	
Locus tag	**Function**	**Locus tag**	**Function**
AS588_RS00475	ABC transporter, permease protein	RBAM_RS00685	30S ribosomal protein S12
AS588_RS00490	hypothetical protein	RBAM_RS00710	30S ribosomal protein S10
AS588_RS00500	tRNA-guanine transglycosylase (EC 2.4.2.29)	RBAM_RS00735	30S ribosomal protein S19
AS588_RS00505	putative aminoglycoside 6-adenylyltansferase	RBAM_RS00740	50S ribosomal protein L22
AS588_RS00730	collagen adhesion protein	RBAM_RS00745	30S ribosomal protein S3
AS588_RS00735	sortase A, LPXTG specific	RBAM_RS00765	50S ribosomal protein L14
AS588_RS03020	phage protein	RBAM_RS00830	50S ribosomal protein L36
AS588_RS03025	hypothetical protein	RBAM_RS00840	30S ribosomal protein S11
AS588_RS03040	phage replication initiation	RBAM_RS00850	50S ribosomal protein L17
AS588_RS03045	helicase loader DnaI	RBAM_RS00880	30S ribosomal protein S9
AS588_RS03050	hypothetical protein	RBAM_RS01165	membrane protein
AS588_RS03060	hypothetical protein	RBAM_RS01170	hypothetical protein
AS588_RS03065	hypothetical protein	RBAM_RS01175	hypothetical protein
AS588_RS03070	hypothetical protein	RBAM_RS01205	acriflavin resistance protein
AS588_RS03075	hypothetical protein	RBAM_RS01865	hypothetical protein
AS588_RS03080	C-5 cytosine-specific DNA methylase family protein	RBAM_RS02315	hypothetical protein
AS588_RS03085	methyltransferase	RBAM_RS02595	ester cyclase
AS588_RS03090	hypothetical protein	RBAM_RS02600	hypothetical protein
AS588_RS03095	dimeric dUTPase (EC 3.6.1.23)	RBAM_RS02650	TetR family transcriptional regulator
AS588_RS03105	hypothetical protein	RBAM_RS02670	transcriptional regulator
AS588_RS03115	phage-related protein	RBAM_RS02685	CarD family transcriptional regulator
AS588_RS03125	hypothetical protein	RBAM_RS02795	hypothetical protein
AS588_RS03135	hypothetical protein	RBAM_RS02805	hypothetical protein
AS588_RS03145	phage terminase-like protein, small subunit	RBAM_RS02830	hypothetical protein
AS588_RS03150	phage terminase, large subunit	RBAM_RS02890	SAM-dependent methyltransferase
AS588_RS03155	phage portal protein	RBAM_RS02905	TVP38/TMEM64 family protein
AS588_RS03160	phage head maturation protease	RBAM_RS02920	DUF6 transmembrane transporter
AS588_RS03165	phage major capsid protein	RBAM_RS03570	type I restriction-modification system, DNA-methyltransferase subunit M
AS588_RS03170	phage tail fiber protein	RBAM_RS03575	type I restriction-modification system, DNA-methyltransferase subunit S
AS588_RS03175	hypothetical phagelike protein	RBAM_RS03580	type I restriction-modification system, DNA-methyltransferase subunit R
AS588_RS03180	FIG01228293: hypothetical protein	RBAM_RS03585	hypothetical protein
AS588_RS03185	FIG01225884: hypothetical protein	RBAM_RS03590	McrA protein

AS588_RS03190	FIG01229968: hypothetical protein	RBAM_RS03620	RNA-binding protein, RRM domain
AS588_RS03195	phage major tail protein	RBAM_RS03700	membrane protein, putative
AS588_RS03200	FIG01246408: hypothetical protein	RBAM_RS03725	putative hydroxylase
AS588_RS03205	phage tail length tape-measure protein	RBAM_RS03730	hypothetical protein
AS588_RS03210	putative tail or base plate protein gp17 [Bacteriophage A118]	RBAM_RS03735	plantazolicin synthase D
AS588_RS03230	phage protein	RBAM_RS03740	dehydrogenase
AS588_RS03235	phage protein	RBAM_RS03745	caax amino protease family
AS588_RS03250	hypothetical protein	RBAM_RS03750	SAM-dependent methyltransferase
AS588_RS03255	hypothetical protein	RBAM_RS03885	collagen like triple helix with GXT repeats
AS588_RS03260	hypothetical protein	RBAM_RS04120	membrane protein
AS588_RS03330	hypothetical protein	RBAM_RS04525	hypothetical protein
AS588_RS05315	hypothetical protein	RBAM_RS04720	metallophosphatase
AS588_RS06535	hypothetical protein	RBAM_RS05095	hypothetical protein
AS588_RS06555	transcriptional regulator	RBAM_RS05320	mep operon protein MepB
AS588_RS06565	hypothetical protein	RBAM_RS05540	cytochrome P450
AS588_RS06685	DnaJ-class molecular chaperone CbpA	RBAM_RS05910	sporulation protein YjcZ
AS588_RS08085	sporulation protein YjcZ	RBAM_RS05990	hypothetical protein
AS588_RS08315	hypothetical protein	RBAM_RS05995	hypothetical protein
AS588_RS08915	FIG01238565: hypothetical protein	RBAM_RS06000	DUF3037 domain-containing protein
AS588_RS08920	phage-like element PBSX protein xkdU	RBAM_RS06030	hypothetical protein
AS588_RS08925	phage baseplate	RBAM_RS06105	Kelch repeat protein
AS588_RS08930	phage-like element PBSX protein xkdS	RBAM_RS06110	hypothetical protein
AS588_RS08935	FIG01234021: hypothetical protein	RBAM_RS07145	sporulation protein YjcZ
AS588_RS08940	phage-like element PBSX protein xkdQ	RBAM_RS07155	hypothetical protein
AS588_RS08945	phage-like element PBSX protein xkdP	RBAM_RS07270	hypothetical protein
AS588_RS08950	phage tail length tape-measure protein	RBAM_RS07275	hypothetical protein
AS588_RS08955	phage-like element PBSX protein xkdN	RBAM_RS07275	hypothetical protein
AS588_RS08960	phage tail fibers	RBAM_RS07925	50S ribosomal protein L19
AS588_RS08965	phage-like element PBSX protein xkdK	RBAM_RS08250	30S ribosomal protein S15
AS588_RS08970	hypothetical protein	RBAM_RS08560	hypothetical protein
AS588_RS08975	phage-like element PBSX protein xkdJ	RBAM_RS19045	phage integrase
AS588_RS08980	Lin1275 protein	RBAM_RS08720	YoaW
AS588_RS08985	phage-like element PBSX protein xkdH	RBAM_RS08745	DUF4944 domain-containing protein YoaO
AS588_RS08990	hypothetical protein	RBAM_RS08750	hypothetical protein
AS588_RS08995	phage-like element PBSX protein xkdG	RBAM_RS08780	hypothetical protein
AS588_RS09000	FIG01230357: hypothetical protein	RBAM_RS09015	hypothetical protein
AS588_RS09005	phage-like element PBSX protein xkdE	RBAM_RS09020	hypothetical protein
AS588_RS09010	phage terminase, large subunit [SA bacteriophages 11, Mu50B]	RBAM_RS09025	hypothetical protein
AS588_RS09090	lanthionine biosynthesis protein LanM	RBAM_RS09320	IS231-related transposase
AS588_RS09100	lanthionine biosynthesis protein LanM	RBAM_RS09325	predicted short chain dehydrogenase

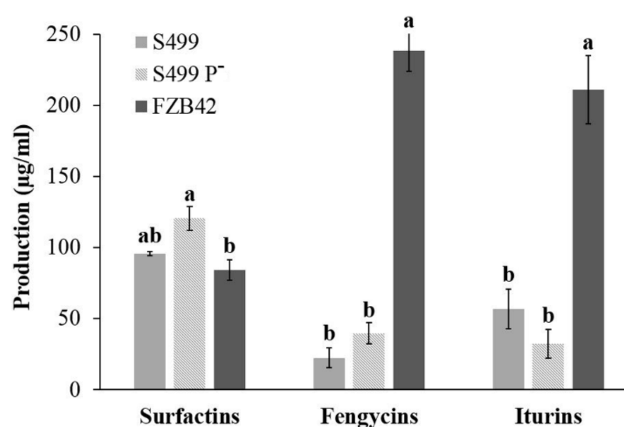
AS588_RS09105	hypothetical protein	RBAM_RS09370	YoaF
AS588_RS09120	hypothetical protein	RBAM_RS09840	FAD-dependent pyridine nucleotide-disulphide oxidoreductase, GBAA2537 homolog
AS588_RS09125	bacitracin ABC transporter, permease protein, putative	RBAM_RS09865	hypothetical protein
AS588_RS09240	hypothetical protein	RBAM_RS09870	hypothetical protein
AS588_RS09255	hypothetical protein	RBAM_RS09875	site-specific recombinase
AS588_RS09325	lactoylglutathione lyase and related lyases	RBAM_RS10990	DNA-binding protein
AS588_RS09350	hypothetical protein	RBAM_RS11795	30S ribosomal protein S21
AS588_RS09370	hypothetical protein	RBAM_RS11960	GCN5-related N-acetyltransferase
AS588_RS09375	gene 22	RBAM_RS11985	phosphate-starvation-inducible protein PsiE
AS588_RS09380	phage minor structural protein	RBAM_RS11990	two component system histidine kinase (EC 2.7.3.-)
AS588_RS09385	phage tail protein	RBAM_RS12000	protein of unknown function DUF418
AS588_RS09390	phage tail length tape-measure protein	RBAM_RS12005	GCN5-related N-acetyltransferase
AS588_RS09395	hypothetical protein	RBAM_RS12840	hypothetical protein
AS588_RS09400	phage protein	RBAM_RS12915	50S ribosomal protein L35
AS588_RS09405	phage major tail protein, TP901-1 family	RBAM_RS13270	hypothetical protein
AS588_RS09410	tail protein	RBAM_RS13275	hypothetical protein
AS588_RS09415	phage capsid and scaffold	RBAM_RS13285	hypothetical protein
AS588_RS09420	phage capsid and scaffold	RBAM_RS13295	hypothetical protein
AS588_RS09430	hypothetical protein	RBAM_RS13300	hypothetical protein
AS588_RS09435	hypothetical protein	RBAM_RS13305	hypothetical protein
AS588_RS09440	phage major capsid protein	RBAM_RS13525	transcriptional regulator, DeoR family
AS588_RS09445	phage protein	RBAM_RS13675	predicted NRPS adenylation domain
AS588_RS09450	phage minor capsid protein	RBAM_RS19050	amino acid adenylation domain-containing protein(EC:3.1.2.14,EC:5.1.1.3)
AS588_RS09455	portal protein, phage associated	RBAM_RS13695	conserved domain protein
AS588_RS09460	phage terminase, large subunit	RBAM_RS16640	hypothetical protein
AS588_RS09465	YqaS	RBAM_RS16650	hypothetical protein
AS588_RS09475	cysteine protease	RBAM_RS17225	hypothetical protein
AS588_RS09480	transcriptional regulator	RBAM_RS17230	hypothetical protein
AS588_RS09490	hypothetical protein	RBAM_RS17255	hypothetical protein
AS588_RS09495	DNA Methyltransferase	RBAM_RS17365	hypothetical protein
AS588_RS09500	hypothetical protein	RBAM_RS17370	hypothetical protein
AS588_RS09505	hypothetical protein	RBAM_RS17375	hypothetical protein
AS588_RS09525	hypothetical protein	RBAM_RS17380	hypothetical protein
AS588_RS09535	prophage Lp1 protein 19	RBAM_RS17485	protein liaG
AS588_RS09540	hypothetical protein	RBAM_RS17490	bacitracin transport permease protein BCRB
AS588_RS09545	hypothetical protein	RBAM_RS17810	hypothetical protein
AS588_RS09555	chain A, Nmr Structure Of Bacillus Subtilis Protein Yqai, Northeast Structural Genomics Target Sr450	RBAM_RS18100	hypothetical protein
AS588_RS09560	DNA, complete sequence	RBAM_RS18305	MrsG
AS588_RS09565	transcriptional regulator	RBAM_RS18310	lantibiotic ABC transporter permease
AS588_RS09570	phage regulatory protein	RBAM_RS18410	hypothetical protein

AS588_RS09575	DNA-binding protein
AS588_RS09580	transcriptional regulator
AS588_RS09590	hypothetical protein
AS588_RS09600	phage integrase
AS588_RS09670	sporulation protein YjcZ
AS588_RS10115	hypothetical protein
AS588_RS10325	hypothetical protein
AS588_RS11480	hypothetical protein
AS588_RS11485	hypothetical protein
AS588_RS11525	MFS transporter, tetracycline resistance protein
AS588_RS11550	serine transporter
AS588_RS11645	hypothetical protein
AS588_RS11685	hypothetical protein
AS588_RS11690	FRG domain-containing protein
AS588_RS11700	hypothetical protein
AS588_RS11710	FtsK/SpoIIIE family protein
AS588_RS11975	hypothetical protein
AS588_RS12450	hypothetical protein
AS588_RS13030	adenine-specific DNA modification methyltransferase
AS588_RS13035	hypothetical protein
AS588_RS13045	chromosome segregation ATPase
AS588_RS15260	SAM-dependent methyltransferase
AS588_RS15340	hypothetical protein
AS588_RS15925	hypothetical protein
AS588_RS16115	FIG01238735: hypothetical protein
AS588_RS16285	hypothetical protein
AS588_RS16730	hypothetical protein
AS588_RS16755	FIG01242153: hypothetical protein
AS588_RS16760	NAD(P)H-dependent oxidoreductase
AS588_RS16780	hypothetical protein
AS588_RS16795	hypothetical Cytosolic Protein
AS588_RS16995	hypothetical protein
AS588_RS17325	respiratory nitrate reductase alpha chain (EC 1.7.99.4)
AS588_RS17365	phosphatase
AS588_RS17375	pXO1-41
AS588_RS17380	hypothetical protein
AS588_RS17440	hypothetical protein
AS588_RS17460	hypothetical protein
AS588_RS18150	hypothetical protein
AS588_RS18155	hypothetical protein
AS588_RS18160	hypothetical protein
AS588_RS18165	hypothetical protein
AS588_RS18170	hypothetical protein
AS588_RS18190	hypothetical protein
RBAM_RS18465	hypothetical protein
RBAM_RS19055	hypothetical protein
RBAM_RS19060	resolvase domain-containing protein
RBAM_RS18520	hypothetical protein
RBAM_RS18530	hypothetical protein
RBAM_RS18535	hypothetical protein
RBAM_RS18660	hypothetical protein
RBAM_RS18815	transporter, LysE family
RBAM_RS18890	YnaF
RBAM_RS18895	response regulator aspartate phosphatase
RBAM_RS18945	30S ribosomal protein S18

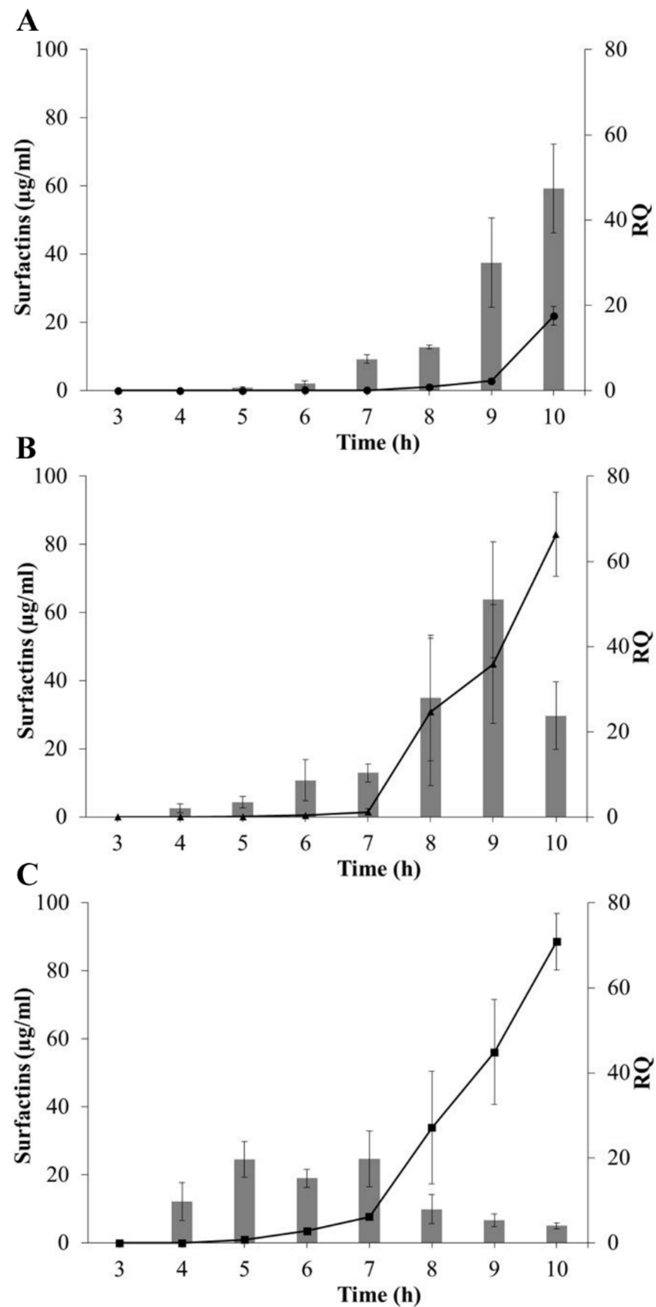
AS588_RS18410	hypothetical protein	
AS588_RS18525	fructose-1,6-bisphosphatase, Bacillus type (EC 3.1.3.11)	
AS588_RS18530	superfamily I DNA/RNA helicase protein	
AS588_RS18535	FIG01240834: hypothetical protein	
AS588_RS18540	nucleotide pyrophosphohydrolase	
AS588_RS18555	DEAD-like helicase	
AS588_RS18570	hypothetical protein	
AS588_RS18615	FIG038982: hypothetical protein	
AS588_RS18695	hypothetical protein	
AS588_RS18865	LysR family transcriptional regulator	
AS588_RS18920	transcriptional regulator, Cro/CI family	
AS588_19070	Rep protein	
AS588_19090	mobilization protein	



Supplementary Figure 1: Extracellular proteolytic activity. Bacterial culture filtrates of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P^-, tested with azocasein assay. Absorbance at 600nm (OD_{600}) indicates cell growth after 6 h incubation at 28°C in LB medium. Absorbance at 405nm (OD_{405}) indicates the quantity of digested casein by the extracellular protease released in culture filtrates. Average values of three replicates from one representative experiment are shown. Error bars represent standard errors. Different letters indicate significant differences according to Tukey's test (α = 0.05). The experiment was repeated



Supplementary Figure 2: Production of cyclic lipopeptides. Surfactin, fengycin and iturin concentrations detected through UPLC-ESI-MS in the bacterial culture filtrates of *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42, S499 and its plasmid-cured derivative, S499 P^-, upon 24 h growth at 28°C in LB medium. Production values correspond to the resulting averages of standardised data [Z=(X/µ)*100] from three independent experiments. Error bars represent standard errors. Different letters indicate significant differences according to Tukey's test (α = 0.05).



Supplementary Figure 3: Surfactin production and relative quantity of *srfA* gene expression. Surfactin production (lines) and fold-increase of *srfA* gene expression compared to "time 0" (histograms) in *B. amyloliquefaciens* subsp. *plantarum* S499 **(A)**, its plasmid-cured derivative, S499 P^- **(B)** and FZB42 **(C)**. Production values correspond to the resulting averages of standardised data [Z=(X/µ)*100] from three independent experiments. Average RQ values of the three experiments are shown. Error bars represent standard errors.

Supplementary Data Sheet

Supplementary Data 1. Alignment of 16SrDNA. Sequences were aligned with EMBOSS Needle Pairwise Sequence Alignment tools (http://www.ebi.ac.uk/Tools/psa/).

```
#=======================================
#
# Aligned_sequences: 2
# 1: S499_16S
# 2: S499P-_16S
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1399
# Identity:    1399/1399 (100.0%)
# Similarity:  1399/1399 (100.0%)
# Gaps:           0/1399 ( 0.0%)
# Score: 6995.0
#
#
#=======================================

S499_16S           1 TCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTG     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S         1 TCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTG     50

S499_16S          51 AGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S        51 AGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC    100

S499_16S         101 GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       101 GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGG    150

S499_16S         151 TGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGG    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       151 TGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGG    200

S499_16S         201 TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTG    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       201 TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTG    250

S499_16S         251 ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       251 ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC    300

S499_16S         301 AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       301 AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT    350

S499_16S         351 GAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       351 GAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA    400

S499_16S         401 GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCAC    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S       401 GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCAC    450
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S499_16S        451 GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT   500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      451 GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT   500

S499_16S        501 CCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATG   550
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      501 CCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATG   550

S499_16S        551 TGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTG   600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      551 TGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTG   600

S499_16S        601 AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA   650
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      601 AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA   650

S499_16S        651 GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC   700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      651 GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC   700

S499_16S        701 GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGT   750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      701 GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGT   750

S499_16S        751 CCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAG   800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      751 CCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAG   800

S499_16S        801 TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA   850
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      801 TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA   850

S499_16S        851 CTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG   900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      851 CTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG   900

S499_16S        901 GTTTAATTTGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG   950
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      901 GTTTAATTTGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG   950

S499_16S        951 ACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTG   1000
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      951 ACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTG   1000

S499_16S       1001 CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC   1050
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S     1001 CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC   1050

S499_16S       1051 GAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGG   1100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S     1051 GAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGG   1100

S499_16S       1101 TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA   1150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S     1101 TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA   1150

S499_16S       1151 TGCCCCCTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGG   1200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S     1151 TGCCCCCTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGG   1200

S499_16S       1201 GCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCG   1250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S     1201 GCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCG   1250
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```
S499_16S        1251 GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGC   1300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      1251 GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGC   1300

S499_16S        1301 GGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC   1350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      1301 GGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC   1350

S499_16S        1351 GTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTA   1399
                     |||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_16S      1351 GTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTA   1399


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Supplementary Data 2. Alignment of partial *gyrA* gene. Sequences were aligned with EMBOSS Needle Pairwise Sequence Alignment tools (http://www.ebi.ac.uk/Tools/psa/).

```
#=======================================
#
# Aligned_sequences: 2
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# 2: S499P-_GyrA
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 925
# Identity:     925/925 (100.0%)
# Similarity:   925/925 (100.0%)
# Gaps:           0/925 ( 0.0%)
# Score: 4625.0
#
#
#=======================================

S499_GyrA          1 ATCCCGGGCGCTTCCGGATGTGCGTGACGGTCTGAAGCCGGTTCACAGAC     50
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S499P-_GyrA        1 ATCCCGGGCGCTTCCGGATGTGCGTGACGGTCTGAAGCCGGTTCACAGAC     50

S499_GyrA         51 GGATTTTGTACGCAATGAATGATTTAGGCATGACCAGTGACAAACCATAT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       51 GGATTTTGTACGCAATGAATGATTTAGGCATGACCAGTGACAAACCATAT    100

S499_GyrA        101 AAAAAATCTGCCCGTATCGTCGGTGAAGTTATCGGTAAGTACCACCCGCA    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA      101 AAAAAATCTGCCCGTATCGTCGGTGAAGTTATCGGTAAGTACCACCCGCA    150

S499_GyrA        151 CGGTGACTCAGCGGTTTACGAATCAATGGTCAGAATGGCGCAGGATTTTA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA      151 CGGTGACTCAGCGGTTTACGAATCAATGGTCAGAATGGCGCAGGATTTTA    200

S499_GyrA        201 ACTACCGCTACATGCTTGTTGACGGACACGGCAACTTCGGTTCGGTTGAC    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA      201 ACTACCGCTACATGCTTGTTGACGGACACGGCAACTTCGGTTCGGTTGAC    250

S499_GyrA        251 GGCGACTCAGCGGCCGCGATGCGTTACACAGAAGCGAGAATGTCAAAAAT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA      251 GGCGACTCAGCGGCCGCGATGCGTTACACAGAAGCGAGAATGTCAAAAAT    300
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S499_GyrA         301 CGCAATGGAAATTCTGCGTGACATTACGAAAGACACGATTGACTATCAAG     350
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       301 CGCAATGGAAATTCTGCGTGACATTACGAAAGACACGATTGACTATCAAG     350

S499_GyrA         351 ATAACTATGACGGTTCAGAAAGAGAGCCTGCCGTCATGCCTTCGAGATTT     400
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       351 ATAACTATGACGGTTCAGAAAGAGAGCCTGCCGTCATGCCTTCGAGATTT     400

S499_GyrA         401 CCGAATCTGCTCGTAAACGGGGCTGCCGGTATTGCGGTCGGAATGGCGAC     450
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       401 CCGAATCTGCTCGTAAACGGGGCTGCCGGTATTGCGGTCGGAATGGCGAC     450

S499_GyrA         451 AAACATTCCCCCGCATCAGCTTGGGGAAGTCATTGAAGGCGTGCTTGCCG     500
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       451 AAACATTCCCCCGCATCAGCTTGGGGAAGTCATTGAAGGCGTGCTTGCCG     500

S499_GyrA         501 TAAGTGAGAATCCTGAGATTACAAACCAGGAGCTGATGGAATACATCCCG     550
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       501 TAAGTGAGAATCCTGAGATTACAAACCAGGAGCTGATGGAATACATCCCG     550

S499_GyrA         551 GGCCCGGATTTTCCGACTGCAGGTCAGATTTTGGGCCGGAGCGGCATCCG     600
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       551 GGCCCGGATTTTCCGACTGCAGGTCAGATTTTGGGCCGGAGCGGCATCCG     600

S499_GyrA         601 CAAGGCATATGAATCCGGACGGGGATCAATCACGATCCGGGCTAAGGCTG     650
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S499P-_GyrA       601 CAAGGCATATGAATCCGGACGGGGATCAATCACGATCCGGGCTAAGGCTG     650

S499_GyrA         651 AAATCGAAGAGACTTCATCGGGAAAAGAAAGAATTATTGTCACGGAACTT     700
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S499P-_GyrA       651 AAATCGAAGAGACTTCATCGGGAAAAGAAAGAATTATTGTCACGGAACTT     700

S499_GyrA         701 CCTTATCAGGTGAACAAAGCGAGATTAATTGAAAAAATCGCGGATCTTGT     750
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S499P-_GyrA       701 CCTTATCAGGTGAACAAAGCGAGATTAATTGAAAAAATCGCGGATCTTGT     750

S499_GyrA         751 CCGAGACAAAAAAATCGAAGGAATTACCGATCTGCGAGACGAATCCGACC     800
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S499P-_GyrA       751 CCGAGACAAAAAAATCGAAGGAATTACCGATCTGCGAGACGAATCCGACC     800

S499_GyrA         801 GTAACGGAATGAGAATCGTCATTGAGATCCGCCGTGACGCCAATGCTCAC     850
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S499P-_GyrA       801 GTAACGGAATGAGAATCGTCATTGAGATCCGCCGTGACGCCAATGCTCAC     850

S499_GyrA         851 GTCATTTTGAATAACCTGTACAAACAAACGGCCCTGCAGACGTCTTTCGG     900
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_GyrA       851 GTCATTTTGAATAACCTGTACAAACAAACGGCCCTGCAGACGTCTTTCGG     900

S499_GyrA         901 AATCAACCTGCTGGCGCTCGTTGAC    925
                      |||||||||||||||||||||||||
S499P-_GyrA       901 AATCAACCTGCTGGCGCTCGTTGAC    925


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Supplementary Data 3. Alignment of partial *cheA* gene. Sequences were aligned with EMBOSS Needle Pairwise Sequence Alignment tools (http://www.ebi.ac.uk/Tools/psa/).

```
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# 1: S499_CheA
# 2: S499P-_CheA
# Matrix: EDNAFULL
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# Extend_penalty: 0.5
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# Length: 850
# Identity:     850/850 (100.0%)
# Similarity:   850/850 (100.0%)
# Gaps:           0/850 ( 0.0%)
# Score: 4250.0
#
#
#=======================================

S499_CheA          1 ATCTGGCGCATTTAACCCACCTGATGGAAAATGTGCTGGACGCCATCCGC     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA        1 ATCTGGCGCATTTAACCCACCTGATGGAAAATGTGCTGGACGCCATCCGC     50

S499_CheA         51 AACGGAGAAATGCCCGTTACATCGGATTGGCTGGACGTGCTGTTTGAAGC    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA       51 AACGGAGAAATGCCCGTTACATCGGATTGGCTGGACGTGCTGTTTGAAGC    100

S499_CheA        101 GCTTGATCATCTTGAAGAGATGGTGCAGTCCATTATTGACGGAGGAGACG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      101 GCTTGATCATCTTGAAGAGATGGTGCAGTCCATTATTGACGGAGGAGACG    150

S499_CheA        151 GCAAACGTGATATTTCTGAAGTAAGTGCGAAGCTCGACGTAAATGCGGTG    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      151 GCAAACGTGATATTTCTGAAGTAAGTGCGAAGCTCGACGTAAATGCGGTG    200

S499_CheA        201 CATGAGACTGCGGCTTCAGCCGAAACAGCAGAACCGCCGGCTTCAAAACA    250
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S499P-_CheA      201 CATGAGACTGCGGCTTCAGCCGAAACAGCAGAACCGCCGGCTTCAAAACA    250

S499_CheA        251 ACAGACTTCAACTGAATGGAATTATGATGAGTTCGAACGGACTGTTATTG    300
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S499P-_CheA      251 ACAGACTTCAACTGAATGGAATTATGATGAGTTCGAACGGACTGTTATTG    300

S499_CheA        301 AAGAGGCGGAAGAGCAAGGATTCAGCCGCTATGAAATAACGGTTTCCCTG    350
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S499P-_CheA      301 AAGAGGCGGAAGAGCAAGGATTCAGCCGCTATGAAATAACGGTTTCCCTG    350

S499_CheA        351 AATGAAAGCTGCATGCTCAAAGCCGTGCGCGTGTACATGATATTTGAAAA    400
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S499P-_CheA      351 AATGAAAGCTGCATGCTCAAAGCCGTGCGCGTGTACATGATATTTGAAAA    400

S499_CheA        401 GCTGAATGAAGCCGGGGAAGTCGCAAAAACGATACCTGCCGCGGAAGTGC    450
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S499P-_CheA      451 TTGAGACGGAAGATTTCGGAACGGATTTTCAGGTGTGTTTCTTGACAAAG    500

S499_CheA        501 CAGCCTGCTGAAGAAATTAAAGAACTCATCAGCGGCATTTCAGAAGTGGA    550
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S499P-_CheA      501 CAGCCTGCTGAAGAAATTAAAGAACTCATCAGCGGCATTTCAGAAGTGGA    550
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S499_CheA        551 GAATGTCGAAATTTCTGCCGGTGCGCCGTTAAAAACGGCCGAAAAACCGC    600
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S499P-_CheA      551 GAATGTCGAAATTTCTGCCGGTGCGCCGTTAAAAACGGCCGAAAAACCGC    600

S499_CheA        601 AAGCAGCTGAACCGGTGAAGGAAACTCCGGTTAAAAAGGCTGAAAAACAG    650
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S499P-_CheA      601 AAGCAGCTGAACCGGTGAAGGAAACTCCGGTTAAAAAGGCTGAAAAACAG    650

S499_CheA        651 CCGAAACCGCAAGCGAAAACGGAAGAGCAGCCGAAGCATCACAGCGGCTC    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      651 CCGAAACCGCAAGCGAAAACGGAAGAGCAGCCGAAGCATCACAGCGGCTC    700

S499_CheA        701 GAAAACGATTCGCGTCAACATTGAAAGACTGGATTCTTCAATGAACCTTT    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      701 GAAAACGATTCGCGTCAACATTGAAAGACTGGATTCTTCAATGAACCTTT    750

S499_CheA        751 TTGAAGAACTTGTCATTGACCGCGGACGTCTTGAGCAGATCGCCAAAGAG    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      751 TTGAAGAACTTGTCATTGACCGCGGACGTCTTGAGCAGATCGCCAAAGAG    800

S499_CheA        801 CTTGACCACAATGAGCTGACTGAAACCGTTGAACGCCTGACCAGAATTTC    850
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
S499P-_CheA      801 CTTGACCACAATGAGCTGACTGAAACCGTTGAACGCCTGACCAGAATTTC    850


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The PhD candidate carried out all the experiments, analysed the data and wrote the manuscript (refer also to “Author contributions” in the text).

Chapter 5

PLASMID pS499 AFFECTS *Bacillus amyloliquefaciens* subsp. *plantarum* S499 SURFACTIN PRODUCTION ON PLANT ROOTS AND GROWTH AND BIOCONTROL ACTIVITY ON RECOMPOSED ROOT EXUDATES MEDIUM

5.1 Introduction

Genome sequencing of the plant-beneficial rhizobacterium *B. amyloliquefaciens* subsp. *plantarum* S499 (S499) allowed to identify for the first time a small plasmid (pS499; 8,008 bp) endogenous to this bacterial strain (Molinatto *et al.*, 2016). In soil, rhizobacteria can benefit from the acquisition of small rolling circle plasmids in multiple ways. Plasmids can confer niche-specific functions (*e.g.* antibiotic or metal resistances), which are useful under selective pressure, or they can improve particular metabolic functions, by increasing the copy number of related genes (Thomas, 2004). Moreover, an increased gene copy number correspond to an accelerated mutation rate, resulting in a greater adaptive ability (Metzgar and Wills, 2000). Additionally, plasmids can favour the adaptation to the environment by promoting recombination in the host cells through their rolling circle replication mechanism (Guglielmetti, 2007).

To functionally characterise pS499, a plasmid-cured derivative of S499 (S499 P$^-$) was obtained. On a nutrient-rich medium (Luria-Bertani, LB), pS499 is involved in regulating growth, extracellular proteolytic activity and cyclic lipopeptide (CLP) production, most probably through the expression of the *rap-phr* cassette located in its sequence (Molinatto *et al.*, 2017). Indeed, Rap-Phr quorum-sensing regulators are known to have pleiotropic effects on several cellular processes in *Bacillus* spp. (Pottahil and Lazazzera, 2003). In particular, other plasmid-encoded Rap-Phr systems had already been proven to be involved in controlling the production of exoproteases and surfactins in *B. subtilis* and *B. amyloliquefaciens* (Koetje *et al.*, 2003; Parashar *et al.*, 2013, Yang *et al.*, 2015). In addition, our results suggested that pS499 is involved in the regulation of some important features related to rhizosphere competence, such as swarming motility, biofilm production and antifungal activity (Molinatto *et al.*, 2017). However, to our knowledge, the role of a plasmid-encoded Rap-Phr system have never been investigated *in planta* or under growth conditions that reproduce the rhizosphere environment.

According to our genome comparative analysis, pS499 represents a major genetic difference between S499 and the type strain of *B. amyloliquefaciens* subsp. *plantarum*, FZB42 (FZB42) (Molinatto *et al.*, 2017). Moreover, S499 P$^-$ showed an

intermediate behaviour between S499 and FZB42 when some bacterial features, such as growth rate, extracellular proteolytic activity, surfactin production and swarming ability, were compared in LB medium (Molinatto *et al.*, 2017). Therefore, we can speculate that the presence of the plasmid could contribute to determine the differences observed in the behaviour of S499, when compared to FZB42.

In this work, we aim at understanding the role of pS499 in the rhizosphere and, at the same time, evaluating the contribution of pS499 in shaping S499 distinctive phenotype under growth conditions that mimic the natural habitat of the rhizobacterium. To this end, we compare S499, S499 P^- and FZB42 behaviours on tomato and tobacco roots and on a substrate containing the main components of root exudates ("recomposed root exudates" medium, RE; Nihorimbere *et al.*, 2012).

5.2 Materials and methods

5.2.1 Bacterial and fungal strains

Bacillus amyloliquefaciens subsp. *plantarum* S499, its plasmid-cured derivative S499 P^- and *B. amyloliquefaciens* subsp. *plantarum* FZB42 were stored at length in glycerol 30% at -80°C and routinely grown at 28°C on LB broth (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, pH 7) amended with agar 16 g l^{-1} (LBA). The phytopathogenic fungi *Cladosporium cucumerinum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* were grown on Potato Dextrose Agar (Sigma-Aldrich Corp, USA) 39 g l^{-1}, pH 7 (PDA) at 28°C and stored at length on PDA slants at room temperature.

5.2.2 Colonization of tobacco and tomato roots in gnotobiotic systems

Root colonization ability of the bacterial strains S499, S499 P^- and FZB42 was assessed on tobacco (*Nicotiana tabacum* L. cv. "Xanthi") and tomato (*Solanum lycopersicum* L. cv. "Moneymaker") plantlets grown in a gnotobiotic system.

Tobacco and tomato seeds were surface sterilized by dipping them in 75% ethanol for 2 min and subsequently in a 30% commercial bleach solution amended

with 0.1% Tween 80 for 15 min. Seeds were then rinsed thoroughly with sterile distilled water and laid on Hoagland medium [$Ca(NO_3)_2 \cdot 4H_2O$ 825 mg l^{-1}, $CuSO_4 \cdot 5H_2O$ 0.05 mg l^{-1}, EDTA 5.20 mg l^{-1}, $FeSO_4 \cdot 7H_2O$ 3.90 mg l^{-1}, H_3BO_3 1.40 mg l^{-1}, KH_2PO_4 170 mg l^{-1}, KNO_3 316 mg l^{-1}, $MgSO_4 \cdot 7H_2O$ 513 mg l^{-1}, $MnCl_2 \cdot 4H_2O$ 0.90 mg l^{-1}, $NaMoO_4 \cdot 2H_2O$ 0.02 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.10 mg l^{-1}, pH 6.5] amended with agar 14 g l^{-1} and solidified in square Petri dishes (120 ×120 × 16 mm).

Tobacco seeds were bacterized with S499, S499 P^- or FZB42 by dipping them in 1 ml of a bacterial cell suspension [Optical Density at 600 nm (OD_{600}) = 1] corresponding to 1×10^8 CFU ml^{-1}, whereas tomato plantlets were inoculated two days after germination by pipetting on the root collar a volume of 4 µl of a cell suspension (OD_{600} = 1) corresponding to 1×10^8 CFU ml^{-1}. Once the inoculum had dried, dishes were incubated in a vertical position at 25°C with a photoperiod 14/10 (light/dark).

Tobacco roots were sampled at 14 and 21 days post-inoculation (dpi), by removing two plugs (10 mm), including the root segment and the surrounding medium, from each plantlet. Tomato roots were sampled at 24, 48 and 72 hours post-inoculation (hpi). Samples were collected at 1 and 4 cm from the point of inoculation by removing a plug (10 mm) that included the root segment and the surrounding medium. Two plugs from different tomato roots were pooled in each sample. Bacterial cells were harvested from the plugs through three cycles of gentle sonication (15-20 s at 25-30% of the power of the device, Sonopuls HD 2070, Bandelin GmbH, Germany) in 1 ml of sterile dH_2O. Subsequently, serial dilutions of the cell suspensions were plated on LBA and colonies were counted after 24 h of incubation at 30°C. At least five and three samples per strain were used as replicates to estimate the bacterial population densities on tobacco and tomato roots, respectively.

5.2.3 Surfactin productivity on tomato and tobacco roots

In planta surfactin productivity was evaluated on additional samples (plugs) collected in the experiments of root colonization. At each time of sampling, ten and

six samples per strain were collected from tobacco and tomato roots, respectively, as described above.

Lipopeptides were extracted from the plugs in 1 ml of 50% acetonitrile and 0.1% formic acid by regular vortexing for 2 h at room temperature. Then, samples were centrifuged and filtered through a 0.2 µm membrane (Sartorius AG, Germany) before being injected in ultra-performance liquid chromatography - electrospray ionization mass spectrometry (UPLC-ESI-MS) columns. Samples were analysed using reverse phase UPLC (Acquity class H, Waters Corp., USA) coupled with a single quadrupole MS (SQ Detector, Waters Corp.) on an Acquity UPLC BEH C18 2.1 × 50 mm, 1.7 µm column (Waters Corp.). Elution started at 30% acetonitrile (flow rate of 0.60 ml min^{-1}). After 2 min and 26 s, the percentage of acetonitrile was brought up to 95% and held for 5 min and 12 s. Then, the column was stabilised at 30% acetonitrile for 1 min and 42 s. Surfactins were identified based on their retention times compared to authentic standards (98% purity; Lipofabrik Society, France) and the masses detected in the SQDetector. Ionization and source conditions were set as follows: source temperature was 130°C, desolvation temperature was 400°C, nitrogen flow was 1000 l h^{-1} and cone voltage was 120 V.

Surfactin productivity was calculated as the ratio between the concentrations of extracted surfactins and the average bacterial population density estimated at the same time of sampling.

5.2.4 Relative gene expression of *srfA* and *rap* in bacterial cells colonizing tomato roots

Bacterial gene expression was analysed on tomato roots in a gnotobiotic system. Tomato plantlets (*Solanum lycopersicum* L. cv. "Moneymaker") were grown and inoculated as described above (*5.2.2*). Roots were sampled at 24, 48 and 72 hpi. At each time point, ten roots per strain were cut below the point of inoculation and pooled; bacterial cells were collected by vortexing the roots in 10 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The suspensions were centrifuged for 5 min at 16,000 g and cell pellets were used for RNA extraction.

Total RNA was extracted with NucleoSpin® RNA kit (Macherey-Nagel GmbH & Co. KG, Germany) according to manufacturer's instructions. Relative

expression of *srfA* and *rap* genes was quantified by reverse transcription Real-time PCR (StepOnePlus^{TM}, Thermo Fisher Scientific Inc., USA) with qPCRBIO SyGreen 1-Step Hi-ROX kit (PCR Biosystems Ltd, UK). The housekeeping gene *gyrA* was used as endogenous control. Primers used in the reactions (**Table 1**) were designed by Primer3web version 4.0.0 (Untergasser *et al.*, 2012). The qRT-PCR program consisted of a first step of 10 min at 48°C followed by 2 min at 95°C and 40 cycles in a series of 5 s at 95°C and 30 s at 60°C.

Table 1. Primers used in this study.

Target gene	Primers	Sequence (5'-3')
gyrA	Gyr-F	GAGACGCACTGAAATCGTGA
	Gyr-R	GCCGGGAGACGTTTAACATA
rap	Rap-F	AGGACATGGAAGAGGACCAA
	Rap-R	GTCCGGTCCCTTCAGATTTT
srfA	Srf-F	ATTGTTTACGGTGGCTCTGG
	Srf-R	CGCTGCGATAGTCAAAATCA

The relative gene expression was calculated according to the comparative C_T method (Livak and Schmittgen, 2001). At each time point, ΔC_T was determined by subtracting the threshold cycle (C_T) value of *gyrA* from the C_T value of the target gene; then the ΔC_T of sample "time 0" was subtracted from the ΔC_T values of the following times of sampling, obtaining $\Delta\Delta C_T$ values. Finally, the relative quantity (RQ) of gene expression was calculated according to the formula: $RQ = 2^{-\Delta\Delta CT}$.

5.2.5 Antifungal activity and lipopeptide production on recomposed root exudates medium

The antifungal activity of S499, S499 P^- and FZB42 was tested against two phytopathogenic fungi, *C. cucumerinum* and *F. oxysporum* f. sp. *radicis-lycopersici*, on RE medium adapted from Nihorimbere *et al.* (2012) [$(NH_4)_2SO_4$ 1 g l^{-1}, $CuSO_4$ 1.6 mg l^{-1}, $Fe_2(SO_4)_3$ 1.2 mg l^{-1}, KCl 0.5 g l^{-1}, KH_2PO_4 0.7 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, $MnSO_4$ 0.4 mg l^{-1}, MOPS 21 g l^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 4 mg l^{-1}, casamino acids 0.5 g l^{-1}, citrate 2 g l^{-1}, fructose 1.7 g l^{-1}, fumarate 0.5 g l^{-1}, glucose

1 g l^{-1}, malate 0.5 g l^{-1}, maltose 0.2 g l^{-1}, oxalate 2 g l^{-1}, ribose 0.3 g l^{-1}, succinate 1.5 g l^{-1}, yeast extract 1 g l^{-1}, pH 6.5] amended with agar 16 g l^{-1}.

A loopful of a 5-day-old mycelium of *C. cucumerinum* was collected from PDA plates and streaked over the whole RE medium surface in Petri dishes. Subsequently, a volume of 5 µl of bacterial cell suspension (OD_{600} = 1) corresponding to 1×10^8 CFU ml^{-1} was inoculated onto it. Once inoculated, dishes were incubated at 28°C for 72 h. At the end of the incubation period, the inhibition zone (distance between mycelium and bacterial colonies) was measured.

To test *F. oxysporum* f. sp. *radicis-lycopersici*, the bacterial inoculum (same volume and concentration as above) was spotted on RE surface, 2 cm from the edge of Petri dishes. Once inoculated, dishes were incubated at 28°C for 72 h. Then, plugs (5 mm) of mycelium were cut away from the edge of 5-day-old colonies of *F. oxysporum* f. sp. *radicis-lycopersici* grown on PDA and placed at 2.5 cm from the bacterial colonies. Petri dishes not inoculated with the bacterial strains were used as a control. After a new incubation at 28°C for 72 h, the radius of mycelia was measured. The inhibition zone was calculated as the reduction of the radius of mycelium facing the bacteria compared to the untreated control.

In both confrontation assays, two plugs (5 mm) of medium were removed from the zones comprised between the bacterial colonies and the mycelia and subsequently transferred to 1.5 ml microfuge tubes containing 1 ml of 50% acetonitrile and 0.1% formic acid. Lipopeptides were extracted by regular vortexing for 2 h at room temperature. Then, samples were centrifuged and filtered through a 0.2 µm membrane (Sartorius AG) before being injected in UPLC-ESI-MS columns to identify and quantify the CLPs, according to the procedure described above (*5.2.3*). Three dishes for each combination were used as replicates and the experiment was repeated.

5.2.6 Growth and lipopeptide production in recomposed root exudates broth

A SpectraMax M2E Multi-Mode Microplate Reader (Molecular Devices LLC, USA) was used to determine the cell growth rates of S499, S499 P^- and FZB42 in RE broth (1:1, diluted 1:2, 1:5, 1:10 or deprived of casamino acids and

yeast extract). For the growth curves in the different RE dilutions, a volume of 10 µl of a bacterial cell suspension (OD_{600} = 0.001) corresponding to 1×10^5 CFU ml^{-1} was inoculated in 1 ml of medium. The assays were carried out in sterile 48-well plates. For the growth curves in RE without yeast extract and casaminoacids, a volume of 25 µl of a bacterial cell suspension (OD_{600} = 0.1) corresponding to 1×10^7 CFU ml^{-1} was inoculated in 250 µl of medium. The assays were carried out in sterile 96-well plates. Plates were incubated with continuous shaking for 40 h at 28°C and OD_{600} was measured every 30 min. Three wells were used for each strain and the experiments were repeated.

The supernatants of RE (1:1) cultures were filtered through a 0.2 µm membrane (Sartorius AG) at the end of the incubation period (40 h), after pooling the content of three wells (repetitions) for each strain. To identify and quantify the CLPs, the culture filtrates were analysed through UPLC-ESI-MS according to the procedure reported above (*5.2.3*).

5.2.7 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA), except for the data of mycelial growth inhibition obtained in the antagonism experiments, which were subjected to multifactorial ANOVA. Data obtained in the root colonization and surfactin productivity assays on tomato roots were analysed with Duncan's test ($\alpha = 0.05$) to detect significant differences. Tukey's test ($\alpha = 0.05$) was applied in mean pairwise comparisons of bacterial population density and surfactin productivity on tobacco roots, mycelial growth inhibition and CLP production in RE medium. Statistical analysis was carried out with Excel (Microsoft Corp., USA) and Statistica (Dell Inc., USA).

5.3 Results

5.3.1 Impact of the plasmid pS499 on the root colonization ability

On tobacco roots, the bacterial population densities of *B. amyloliquefaciens* subsp. *plantarum* S499, its plasmid-cured derivative S499 P$^-$ and the type strain FZB42 were not significantly different at 14 dpi ($p > 0.05$). Conversely, at 21 dpi, S499 P$^-$ population was significantly reduced ($p < 0.05$) compared to FZB42 but not compared to its parental strain S499 (**Figure 1**).

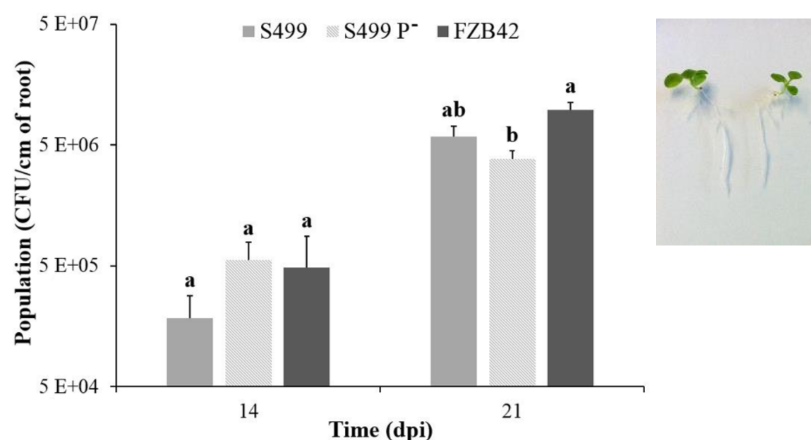

Figure 1: Colonization of tobacco roots at 14 and 21 days post inoculation (dpi). For each time of sampling, mean values of population density measured on at least five inoculated roots are shown. Vertical bars represent standard errors. Different letters indicate significant differences within values of each time of sampling according to Tukey's test ($\alpha = 0.05$).

Under the same experimental conditions, tomato seedlings grew much faster and formed larger roots allowing a more detailed assessment of the evolution of populations over the first 72 hpi. On tomato roots, FZB42 was the fastest strain in colonization, whereas S499 and S499 P$^-$ showed similar behaviours. Indeed, at 24 hpi, FZB42 population was greater in the zone of the root closer to the point of inoculation (a) compared to both S499 and S499 P$^-$ ($p < 0.05$). Conversely, at 48 hpi, FZB42 showed a major growth ($p < 0.01$) in the zone more distant from the point of inoculation (b), indicating that the strain was faster in spreading along the root. At 72 hpi, the three strains reached the same level of population density in both zones of the roots ($p > 0.05$) (**Figure 2**).

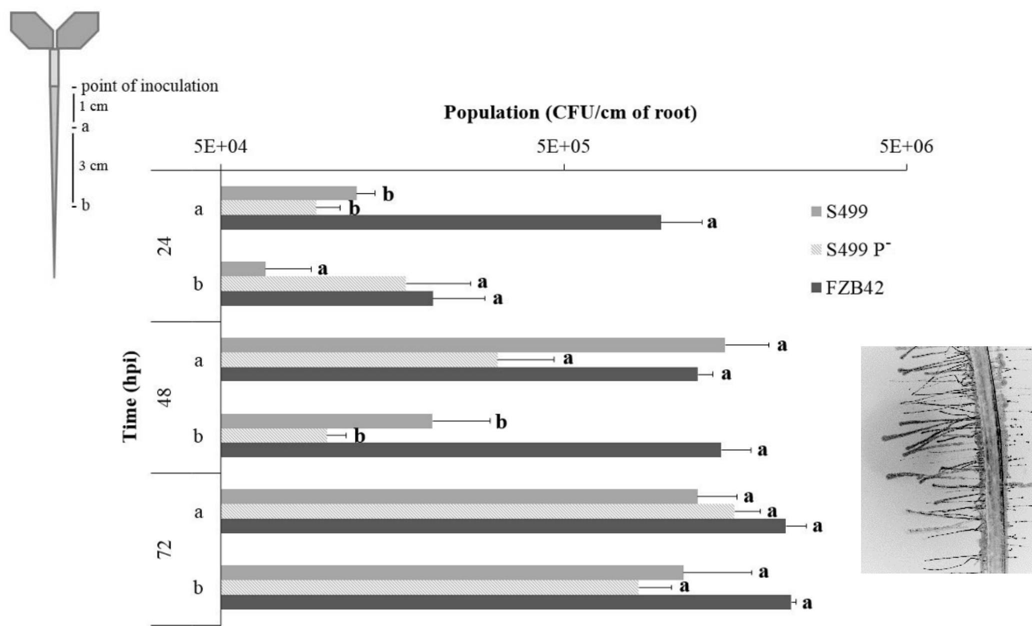

Figure 2: Colonization of tomato roots at 24, 48 and 72 hours post inoculation (hpi). Samples were collected at 1 (a) and 4 (b) cm from the point of inoculation (root collar). For each zone and time of sampling, mean values of population density measured on six inoculated roots are shown. Vertical bars represent standard errors. Different letters indicate significant differences within values of each zone and time of sampling according to Duncan's test (α = 0.05).

5.3.2 Influence of pS499 on the surfactin productivity of bacterial cells colonizing tomato and tobacco roots

The production of CLPs by *B. amyloliquefaciens* subsp. *plantarum* S499, its plasmid-cured derivative S499 P^- and the type strain FZB42 was assessed *in planta* on tobacco and tomato roots growth under gnotobiotic conditions. In both plant systems and for all the three strains, only surfactins were detected in considerable quantities after extraction and analysis by UPLC-ESI-MS.

More specifically, on tobacco roots, S499 and S499 P^- did not display significant differences in surfactin productivity at 14 dpi. However, S499 was significantly more efficient compared to FZB42 ($p < 0.01$). At 21 dpi, the values of S499 and S499 P^- productivity were similar and for both strains significantly higher than FZB42 ($p < 0.01$) (**Figure 3**). On tomato roots, S499 and S499 P^- productivity was higher compared to FZB42 at 24 hpi ($p < 0.05$). Conversely, at 48 hpi and 72 hpi, S499 P^- resulted more efficient than S499 and FZB42. More precisely, S499 P^-

productivity was significantly higher compared to S499 and FZB42 at 48 h ($p < 0.01$), but only compared to FZB42 at 72 h ($p < 0.05$) (**Figure 4**).

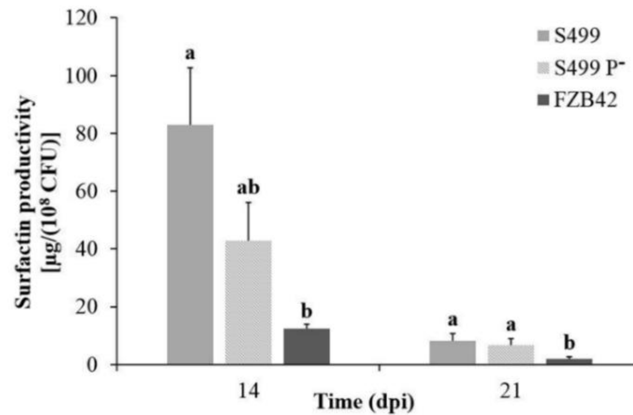

Figure 3: Surfactin productivity on tobacco roots at 14 and 21 days post inoculation (dpi). The quantity of surfactins extracted from each plug was divided by the average value of population of the corresponding strain and time of sampling to obtain surfactin productivity. For each time point, mean values calculated on ten inoculated roots are shown. Vertical bars represent standard errors. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

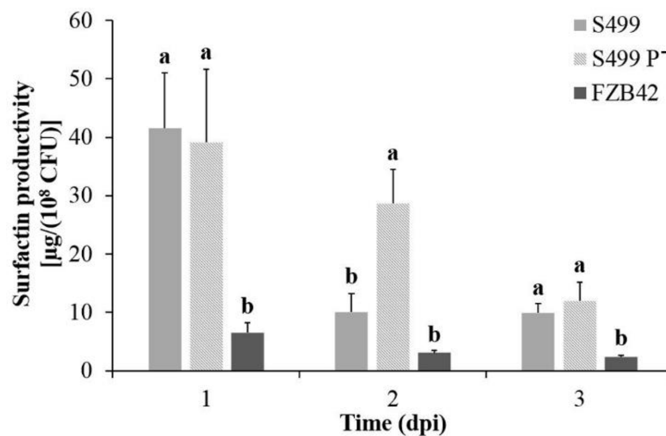

Figure 4: Surfactin productivity on tomato roots at 24, 48 and 72 hours post inoculation (hpi). Average quantities of surfactins detected in the plugs removed at 1 and 4 cm from the point of inoculation were divided by the average value of population of the corresponding strain and time of sampling to obtain surfactin productivity. For each time point, mean values calculated on 12 inoculated roots are shown. Vertical bars represent standard errors. Different letters indicate significant differences according to Duncan's test ($\alpha = 0.05$).

5.3.3 Modulation of *srfA* and *rap* gene expression in bacterial cells colonizing tomato roots

Relative *srfA* gene expression was evaluated in bacterial cells developing on tomato roots to compare the kinetics of surfactin synthesis. As expected, relative quantification (RQ) values of *srfA* gene expression were higher in S499 P^-, especially at 48 hpi and 72 hpi. Conversely, RQ levels were similar for S499 and FZB42 (**Figure 5A**).

In parallel, to understand how the plasmid-encoded Rap-Phr system could be involved in the regulation of surfactin production *in planta*, the expression of the *rap* gene was assessed in S499 cells. Interestingly, a higher RQ of *rap* gene expression was associated with a down-regulation of *srfA* gene at 72 hpi (**Figure 5B**).

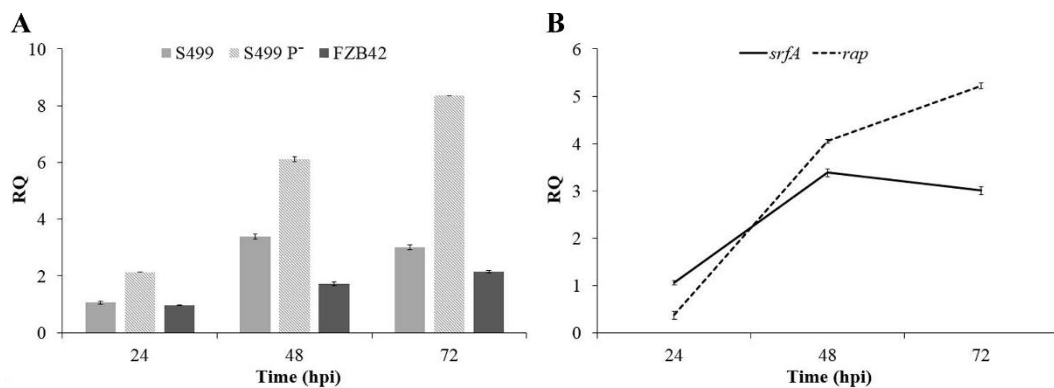

Figure 5: Relative gene expression of *srfA* and *rap* in bacterial cells colonizing tomato roots. Relative quantities (RQ) of *srfA* transcripts in S499, S499 P^- and FZB42 (**A**) and *srfA* and *rap* (**B**) transcripts at 24, 48 and 72 hours post inoculation (hpi) compared to "time 0". At each time of sampling, the cell material collected from ten inoculated tomato roots was pooled for RNA extraction and Real-time qRT-PCR. Gene expression of *gyrA* was used as endogenous control. Average RQ values of two technical replicates are shown and vertical bars represent standard deviations.

5.3.4 Impact of pS499 on the antifungal activity on recomposed root exudates medium

To understand the role that pS499 could play in the biocontrol activity against phytopathogenic fungi, such as *C. cucumerinum* and *F. oxysporum* f. sp. *radicis-lycopersici*, the antagonistic ability of S499, S499 P^- and FZB42 was compared on RE solid medium. Mycelial growth inhibition was significantly

affected by the pathogen type ($p < 0.01$), the *Bacillus* strain ($p < 0.01$) and the pathogen × *Bacillus* strain ($p < 0.05$), according to the multifactorial ANOVA.

More specifically, the size of the inhibition zones was significantly lower for S499 P^- (2.4 ± 0.5 mm, average ± standard error) compared to S499 (5.1 ± 0.4 mm) against *C. cucumerinum*. In these assays, FZB42 showed an intermediate behaviour (4.2 ± 0.5 mm). Conversely, mycelial growth inhibition was similar for S499 and S499 P^- (6.1 ± 0.4 mm and 5.9 ± 0.7 mm, respectively) against *F. oxysporum* f. sp. *radicis-lycopersici*, whereas FZB42 displayed a stronger antagonistic activity against this pathogen (8.6 ± 0.6 mm) (**Figure 6**).

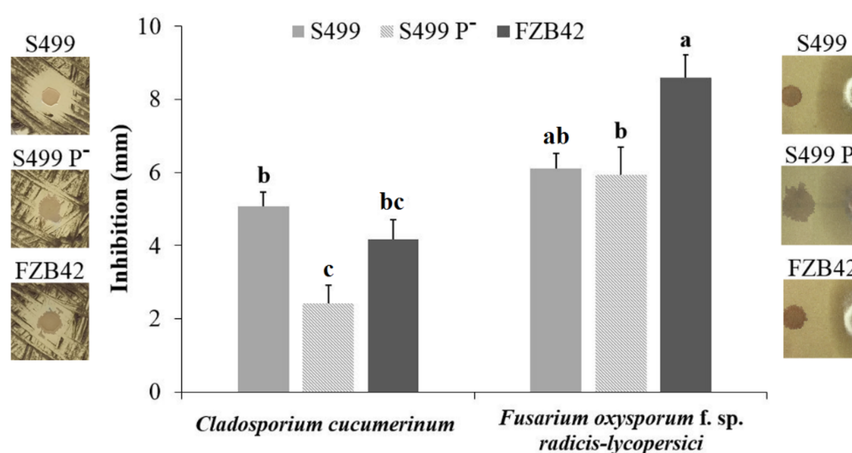

Figure 6: Antifungal activity on recomposed root exudates (RE) medium. Values correspond to the averages of the inhibition zones measured after 72 hours of incubation in the presence of the pathogen in two independent experiments. In each experiment, three replicates were used for each combination. Vertical bars represent standard errors. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

To study the lipopeptide production pattern of the bacteria facing the pathogens, plugs of RE medium were removed from the inhibition zones and the extracted CLPs were identified and quantified through UPLC-ESI-MS analysis. In the plugs removed from the inhibition zone of *C. cucumerinum*, iturins were detected in lower amounts for S499 P^- and FZB42 (1.64 ± 0.38 µg $plug^{-1}$ and 1.51 ± 0.27 µg $plug^{-1}$, respectively) than for S499 (2.14 ± 0.11 µg $plug^{-1}$), but the statistical analysis did not find any significant difference ($p > 0.05$). In these extractions, fengycin were not detected and the production of surfactins was limited and similar for the three strains (1.58 ± 0.64 µg $plug^{-1}$, 1.07 ± 0.40 µg $plug^{-1}$ and 0.97 ± 0.61 µg $plug^{-1}$ for S499, S499 P^- and FZB42, respectively; $p > 0.05$).

In the confrontation assays against *F. oxysporum* f. sp. *radicis-lycopersici*, S499 P⁻ produced much more surfactins (11.65 ± 1.23 µg $plug^{-1}$) than S499 and FZB42 (1.66 ± 0.35 µg $plug^{-1}$ and 1.53 ± 0.25 µg $plug^{-1}$, respectively; $p < 0.01$). The production of fengycins ($p < 0.05$) and iturins ($p > 0.05$) was higher for FZB42 (1.64 ± 0.54 µg $plug^{-1}$ and 6.07 ± 2.52 µg $plug^{-1}$, respectively) than for S499 (0.29 ± 0.14 µg $plug^{-1}$ and 2.53 ± 0.71 µg $plug^{-1}$) and S499 P⁻ (0.32 ± 0.11 µg $plug^{-1}$ and 3.54 ± 0.89 µg $plug^{-1}$), although not always significantly higher (**Figure 7**). However, regression analyses did not highlight any effect of the concentration of the released CLPs on the mycelial growth inhibition ($p > 0.05$).

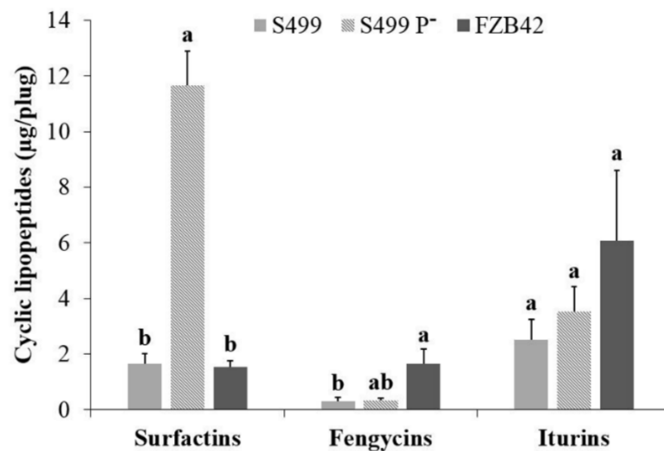

Figure 7: Cyclic lipopeptides detected in the confrontation assays against *Fusarium oxysporum* f. sp. *lycopersici*. Surfactins, fengycins and iturins were quantified after 72 hours of incubation in the presence of the pathogen in RE dishes. Means and standard error values were calculated on the pool of six replicates per strain from two independent experiments. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

5.3.5 Growth and lipopeptide production in recomposed root exudates broth

The influence of pS499 under nutritional conditions that mimic the natural environment of *B. amyloliquefaciens* subsp. *plantarum* was evaluated also in liquid cultures of S499, S499 P⁻ and FZB42. However, in RE broth, the plasmid-cured strain S499 P⁻ showed a very limited growth. FZB42 was faster than S499 in entering the logarithmic phase, consistently with its behaviour *in planta*, nevertheless S499 reached a higher OD at the end of the incubation period (**Figure 8**).

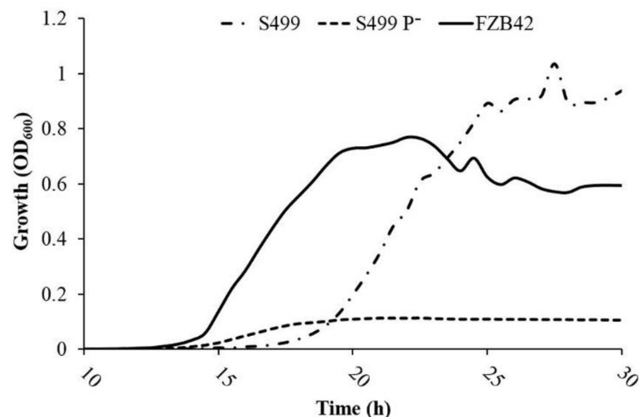

Figure 8: Growth of S499, S499 P⁻ and FZB42 in recomposed root exudates (RE) medium. For each curve, values correspond to the average of Optical Density (600 nm) reads from three wells of one representative experiment.

Lipopeptide production was assessed in RE broth. Strain S499 significantly produced more surfactins than FZB42, while FZB42 significantly produced more fengycins and iturins than S499 ($p < 0.01$). As expected, surfactin production by S499 P⁻, which was impaired in growth, was very low and the other CLPs were not detected at all (**Figure 9**).

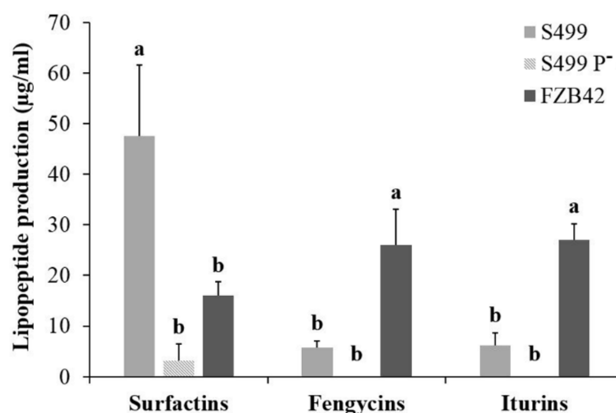

Figure 9: Lipopeptide production in recomposed root exudates (RE) medium. Values of CLP production correspond to the average of four repetitions, in which quantities were obtained by pooling three wells per strain. Vertical bars represent standard deviations. Different letters indicate significant differences according to Tukey's test (α = 0.05).

To exclude a toxic effect of some components of RE medium on S499 P⁻ growth, several assays were carried out in different dilutions of RE broth (1:2, 1:5 and 1:10), in order to dilute the putative inhibitor. However, S499 P⁻ did not grow

in these media, suggesting a deficiency in the nutrient assimilation. The differences observed between FZB42 and S499 growth curves were conserved in the diluted RE media (**Figure 10**).

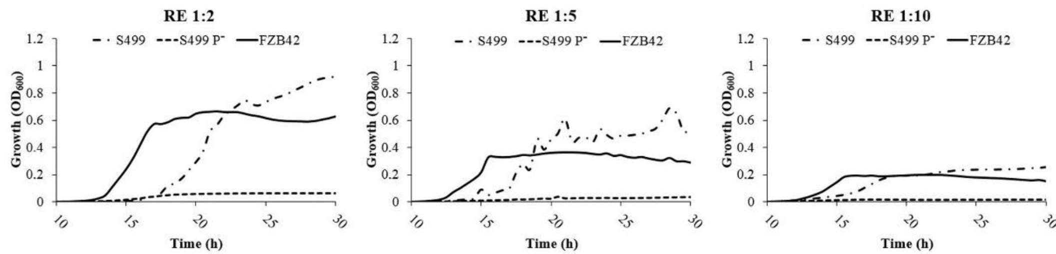

Figure 10: Growth of S499, S499 P$^-$ and FZB42 in diluted RE media. For each curve, values correspond to the average of Optical Density (600 nm) reads from three wells of one representative experiment.

Further assays were carried out in RE broth deprived of yeast extract and casamino acids. When these complex nitrogen sources were removed from RE, S499 P$^-$ grew reaching an OD similar to that of S499. Conversely, FZB42 started to grow very late (**Figure 11**).

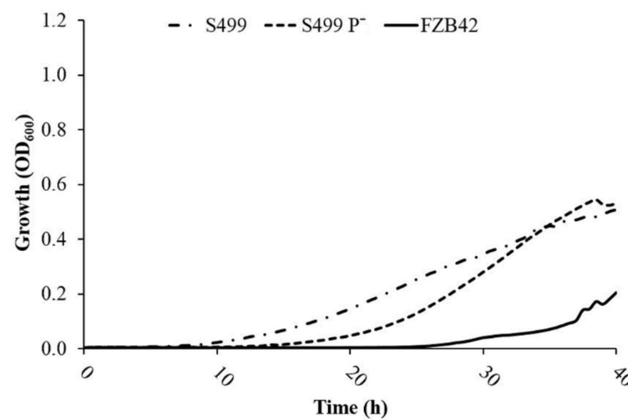

Figure 11: Growth of S499, S499 P$^-$ and FZB42 in recomposed root exudates (RE) medium without complex nitrogen sources. For each curve, values correspond to the average of Optical Density (600 nm) reads from three wells of one representative experiment.

5.4 Discussion

In this work, we investigated on the putative role of a small rolling circle plasmid (pS499; Molinatto *et al*., 2017) of *B. amyloliquefaciens* subsp. *plantarum* S499 under growth conditions closer to the natural environment of the rhizobacterium. Moreover, we studied the impact that pS499 may have on the distinct behaviour shown by S499 in comparison with FZB42, regarded as the type strain of the subspecies (Borriss *et al.*, 2011).

According to our results, pS499 seems not to play a fundamental role in the process of colonization. Indeed, although the plasmid-cured strain (S499 P^-) had previously shown a faster growth and an improved swarming ability in LB medium (Molinatto *et al*., 2017), we could not appreciate any significant difference between S499 and S499 P^- in the evolution of bacterial populations on tobacco and tomato roots. Therefore, the differences observed between S499 and FZB42 in the colonization ability are unlikely related to the presence of pS499. Considering the growth curves obtained in RE broth, we could ascribe the faster root colonization shown by FZB42 to a higher cell growth rate in presence of root exudates. In the long term, S499 reached similar levels of population density on roots, as well as its biomass lately increased in RE broth.

In LB broth, the faster growth of FZB42 and S499 P^- compared to S499 was associated to a higher extracellular proteolytic activity (Molinatto *et al.*, 2017). Similarly, an earlier entrance in the logarithmic phase by FZB42 might be ascribed to a higher production of exoproteases in RE, given the presence of some complex nitrogen sources (yeast extracts and casamino acids) in this medium. Although S499 P^- was impaired in growing in RE broth, it is worth noting that its limited increase of biomass occurred before the entrance in the logarithmic phase by S499. In other words, S499 P^- displayed an intermediate behaviour between S499 and FZB42, as previously observed in LB medium (Molinatto *et al.*, 2017). Hence, we can hypothesize that pS499 contributes to the control of exoprotease production under multiple growth conditions.

To verify if the anticipated growth of FZB42 could be explained by a greater proteolytic activity, we set up new assays in RE broth deprived of yeast extract and casamino acids. Besides confirming that FZB42 growth mainly depends on the

presence of the complex nitrogen sources, the results of these assays indicate that S499 is more efficient in the utilization of sugars and organic acids, representing the main components of RE broth (Nihorimbere *et al.*, 2012). This could explain why in the complete RE medium S499 produced more biomass than FZB42.

Moreover, in absence of yeast extract and casamino acids, S499 P^- restored the ability of its parental strain to efficiently grow on the carbon sources. Therefore, it is tempting to speculate that in S499 cells the plasmid-encoded Rap-Phr regulatory system may slow down the production of exoproteases favouring the utilization of sugars and organic acids. Similarly, Koetje *et al.* (2003) put forward the hypothesis that *B. subtilis* strains may benefit from the presence of a plasmid-borne Rap-Phr quorum-sensing system suppressing the production of exoproteases. At low cell densities, this inhibition may promote the utilization of the readily available energy sources. However, further data in support of these preliminary considerations are needed.

Surfactins were the only family of CLPs that we could detect on inoculated tobacco and tomato roots under gnotobiotic conditions. This is in line with previous observations (Nihorimbere *et al.*, 2012; Debois *et al.*, 2014) and confirms the importance of surfactins in plant-bacteria interactions from the early stages of root colonization. Nonetheless, our results suggest that the plasmid-encoded Rap-Phr system could be involved in limiting S499 surfactin production *in planta*. In fact, we observed an abnormal synthesis of surfactins in the plasmid-cured strain (S499 P^-), especially during the first hours of tomato root colonization. Moreover, our data suggest that the *rap* gene expression could reduce *srfA* transcription, confirming an inhibitory effect already demonstrated *in vitro* for a similar plasmid-encoded Rap protein (Yang et al., 2015).

At all events, pS499 seems not to be involved in the mechanisms governing the divergent regulation of surfactin production in S499 and FZB42 when cells are growing on roots. Indeed, globally both S499 and S499 P^- released surfactins more efficiently than FZB42 *in planta*. These results are consistent with previous studies correlating in a dose-response manner a higher surfactin production with the higher ability of certain strains, including S499, in triggering early-defence responses in tobacco cells and ISR in tomato plants (Cawoy *et al.*, 2014).

In RE broth, CLP production by S499 was clearly modulated in favour of surfactins, consistently with previous observations (Nihorimbere *et al.*, 2012). Conversely, CLP production by FZB42 was less affected by the nutritional context, as similar relative proportions of the three families were retrieved in RE and LB media (Molinatto *et al.*, 2017). A reduced surfactin production by FZB42 compared to S499 in RE broth was already reported by Cawoy *et al.* (2014). Although we could not study the impact of pS499 on this phenomenon, our data confirmed that these two strains differently modulate their CLP profiles in presence of sugars and organic acids typically found in root exudates.

The release of surfactins is important for root colonization, because they help the bacteria in swarming motility and induce biofilm formation (Kinsinger *et al.*, 2003; Bais *et al.*, 2004). However, the higher surfactin productivity by S499 P$^-$ on tomato roots did not improved its colonization ability compared to S499 and FZB42. On the contrary, a faster colonization by FZB42 was associated with a lower surfactin productivity compared to S499 P$^-$ and S499, suggesting that limited amounts of surfactins can grant an efficient colonization. In fact, Cawoy *et al.* (2014) reported that different *Bacillus* isolates, distinguished for their surfactin productivity, formed similar consistent biofilms on roots.

A surfactin overproduction by S499 P$^-$ was observed also on RE medium, in the confrontation assays against *F. oxysporum* f. sp. *radicis-lycopersici*. Conversely, the effects of plasmid curing on the modulation of fengycin and iturin production were not visible under these nutritional conditions. In agreement with this, the growth reduction of *Fusarium* mycelium by S499 and S499 P$^-$ was similar in size, whereas we can ascribe the greater antagonistic ability of FZB42 to a higher release of the antifungal CLPs, as previously reported (Cawoy *et al.*, 2015). In the confrontation assays against *C. cucumerinum*, a general low lipopeptide production did not allow finding a relation between the antifungal activity and the concentration of a specific kind of CLPs, which was found for iturins on LB medium (Molinatto *et al.*, 2017). As S499 P$^-$ was anyhow impaired in the antagonism against *C. cucumerinum*, the impact of pS499 on the biocontrol activity merits additional investigation.

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The PhD candidate carried out all the experiments with the precious help of Brieuc Van Hassel (graduate student), Sébastien Steels and Aurélien Legras (laboratory technicians). She analysed the data under the supervision of Dr. Marc Ongena, Dr. Gerardo Puopolo, Prof. Carlo Viti and Prof. Ilaria Pertot.

Chapter 6

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Bacillus amyloliquefaciens subsp. *plantarum* strain S499 (S499) is a plant-beneficial rhizobacterium that revealed direct antagonistic properties against phytopathogenic fungi and, in addition, a remarkable efficacy as elicitor of plant ISR (Ongena *et al.*, 2005a; Ongena *et al.*, 2005b). These features were mainly ascribed to the production of cyclic lipopeptides (CLPs) belonging to the fengycin, iturin and surfactin families (Ongena *et al.*, 2007; Cawoy *et al.*, 2014; Cawoy *et al.*, 2015). Surfactins play also a major role in determining the rhizosphere competence of *Bacillus* spp. (Ongena and Jacques, 2008). For its attractive features, S499 was previously elected as a model bacterial strain for the molecular characterization of the interactions between plant roots and *Bacillus* spp. (Henry *et al.*, 2011; Nihorimbere *et al.*, 2012; Debois *et al.*, 2015). Furthermore, it was also studied to determine the impact of environmental factors on the bacterial fitness, which is crucial for improving the efficacy of biofungicides (Pertot *et al.*, 2013).

To achieve an in-depth genetic characterization of this plant-beneficial rhizobacterium, we carried out the S499 genome sequencing, assembly and annotation. We found that S499 is equipped with a chromosome of 3,927,922 bp and a considerable portion of the identified genes is putatively involved in cellular functions related to its root-associated lifestyle (Molinatto *et al.*, 2016). In addition, we identified genes involved in the synthesis of amylolysin, amylocyclicin and butirosin, antimicrobials that were not previously known to be part of S499 arsenal. These compounds are active mainly against other bacteria (Lewellyn *et al.*, 2007; Arguelles-Arias *et al.*, 2013; Scholz *et al.*, 2014), an aspect of S499 biocontrol properties that have received less attention so far. It would be interesting to understand in which conditions S499 expresses those genes and what are the environmental factors that drive their activation.

Through comparative genomics, we confirmed that S499 belongs to *B. amyloliquefaciens* subsp. *plantarum*, sharing a high degree of genetic conservation with strain FZB42 (FZB42), the type strain of this bacterial taxon. Noteworthy, the genes known to be involved in the rhizosphere competence displayed a high level of nucleotide identity (Molinatto *et al.*, 2017). On the other hand, the majority of unique coding sequences of S499 and FZB42 genomes was annotated as hypothetical proteins, which hampered to elucidate their contribution in shaping the

phenotypes of these bacterial strains. Indeed, although belonging to the same subspecies, S499 and FZB42 showed different behaviours, especially regarding the modulation of CLP production (Cawoy *et al.*, 2014; Cawoy *et al.*, 2015). Such differences were confirmed by our results. In fact, S499 produced more surfactins than FZB42 in the recomposed root exudates (RE) medium and *in planta*, whereas, FZB42 produced more fengycins and iturins than S499 in Luria-Bertani (LB).

The sequencing of S499 genome allowed to identify a plasmid of 8,008 bp (pS499), which was unknown before and represented one of the most evident genetic features distinguishing S499 and FZB42 (Molinatto *et al.*, 2017). Interestingly, this small plasmid encodes a response regulator aspartate phosphatase (Rap) and a phosphatase regulatory peptide (Phr). We verified that the plasmid-encoded *rap* gene was expressed in both LB and RE media, as well as in S499 cells colonizing tomato roots, suggesting an active role in the regulation of the bacterial physiology. Indeed, Rap-Phr systems are well-characterized quorum-sensing regulatory systems in *B. subtilis* (Pottathil and Lazazzera, 2003) and, besides, they are involved in the regulation of surfactin production in *B. amyloliquefaciens* (Yang *et al.*, 2015). For this reason, we hypothesized that the presence of pS499 could contribute to explain the differences observed between S499 and FZB42 regarding CLP production and rhizosphere competence.

To elucidate the role played by pS499 in S499 cells, a plasmid-cured derivative of S499 (S499 P^-) was obtained (Molinatto *et al.*, 2017). In LB medium, S499 P^- was similar to FZB42 in several aspects: faster growth linked to an increased proteolytic activity, improved swarming motility, enhanced lipopeptide production and anticipated *srfA* gene expression compared to S499 (Molinatto *et al.*, 2017). However, different results were obtained *in planta*. Indeed, S499 and S499 P^- showed similar patterns of root colonization, despite S499 P^- had a reduced ability to form biofilm in LB broth. This result underlines the importance of the nutrient sources in determining the behaviour of the bacterial strains. Moreover, the cell physiology may be affected by the different growing conditions (planktonic cultures vs. populations devoloping as microcolonies on roots). Thus, depending on the environmental conditions, the impact of pS499 in the regulation of cell metabolism may be more or less evident.

Similarly to what observed in LB medium, surfactin synthesis in the cured strain was enhanced also on tomato roots. As FZB42 showed an opposite behaviour compared to S499 P^- in terms of surfactin productivity *in planta*, it is hard to find a direct relation between the presence of pS499 and the different phenotypes of S499 and FZB42 observed on plant roots. In addition, the increased surfactin productivity of S499 P^- did not produce any visible effect on rhizosphere competence. It might be of interest to verify how the colonized plants respond in terms of ISR, since surfactins are the main elicitors of plant immunity among CLPs (Cawoy *et al.*, 2014).

Lipopeptide production by S499 P^- could not be evaluated in RE broth, since its growth was drastically impaired. The reason of this metabolic restraint have not been understood yet, but further investigation on this intriguing aspect is being carried out. However, in the antagonism assays on gelified RE medium, which was anyhow conducive for S499 P^- growth, we could not detect any significant difference with the wild-type S499 in fengycin and iturin production. Conversely, S499 P^- produced less iturins than S499 on LB agar, which correlated with a reduced antifungal activity (Molinatto *et al.*, 2017). As a reduced pathogen inhibition by S499 P^- was also observed on RE, further analyses are necessary to elucidate how pS499 may affect the biocontrol ability of S499 in the rhizosphere.

From the growth curves obtained for S499 and FZB42 in RE broth, it is clear that these two strains of *B. amyloliquefaciens* subsp. *plantarum* differently regulates the assimilation and utilization of nitrogen and carbon sources. This may explain the different early colonization rates shown by S499 and FZB42 on tomato roots. Additional studies are in progress in order to confirm this hypoyhesis and better understand the regulation of the bacterial metabolism.

Globally, our results reveal that pS499 differently affects the S499 behaviours depending on the nutritional context. This modulation is likely related to the expression of the plasmid-encoded *rap-phr* cassette. Nonetheless, more evidences are required to prove that pS499 is relevant for the fitness of the rhizobacterium in its natural environment. If a direct effect on the interactions between S499 and plant roots can be ultimately excluded, more attention should be addressed to the putative regulatory role of pS499 in the antagonistic activity. From

this perspective, the plasmid may provide S499 with an ecological advantage in terms of niche occupation.

In conclusion, from S499 genome sequencing we collected more information on the biotechnological potential of this bacterial strain, besides providing a complete genetic background that can support the future investigation on S499 interactions in the rhizosphere. Moreover, we were able to explore novel genetic features, also by obtaining a useful tool (S499 P^{-}) to investigate on their functions. Although the data did not allow to have a clear picture of pS499 influence on S499 physiology, our study provided some bases to direct further researches in this field.

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