RESEARCH ARTICLE

Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499

Venant Nihorimbere[1], Hélène Cawoy[1], Alexandre Seyer[2], Alain Brunelle[2], Philippe Thonart[1] & Marc Ongena[1]

[1]Walloon Center for Industrial Biology, University of Liège/Gembloux Agro-Bio Tech, Gembloux, Belgium; and [2]Centre de recherche de Gif, Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette, France

Correspondence: Marc Ongena, Walloon Center for Industrial Biology, University of Liege/Gembloux Agro-Bio Tech, Passage des déportés 2, B-5030 Gembloux, Belgium. Tel.: +32 8 1622305; fax: +32 8 1614222; e-mail: marc.ongena@ulg.ac.be

Present address: Venant Nihorimbere, Faculty of Agronomy and Bio-engineering, University of Burundi, P.O. Box 7268, Bujumbura, Burundi.

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Abstract

Cyclic lipopeptides (cLPs) of the surfactin, iturin and fengycin families synthesized by plant-associated *Bacilli* represent an important class of antibiotics as they may be tightly involved in the protective effect of selected strains against phytopathogens. However, their production by *Bacillus* cells developing on roots under rhizosphere conditions is still poorly understood. In this work, we combined electrospray and imaging mass spectrometry-based approaches to determine the detailed pattern of surfactins, iturins and fengycins produced *in planta* by *Bacillus amyloliquefaciens* S499. Very different production rates were observed for the three cLPs families. Whereas surfactin accumulated in significant amounts, much lower quantities of iturins and fengycins were detected in the environment of colonized roots in comparison with laboratory medium. In addition, the surfactin pattern produced by strain S499 evolving on roots is enriched in homologues with long fatty acid chains (C15) compared with the chains typically secreted under *in vitro* conditions. Additional experiments revealed that lipopeptide production by root-associated S499 cells is qualitatively and quantitatively dictated by the specific nutritional context of the rhizosphere (exudates enriched in organic acids, oxygen limitation) but also by the formation of biofilm-related structures around root hairs. As surfactins, iturins and fengycins retain specific functions and bioactivities, the biological relevance of their differential production observed *in planta* is discussed in the context of biocontrol of plant diseases.

Introduction

Bacterial cyclic lipopeptides (cLPs) are composed of a lipid tail linked to a short cyclic oligopeptide and can be synthesized by various genera and species such as *Streptomyces*, *Pseudomonas* and *Bacillus* via non-ribosomal peptide synthetases forming or not forming hybrid complexes with polyketide synthases (Finking & Marahiel, 2004). This leads to a remarkable heterogeneity among cLP structures which vary in the type and sequence of amino acid residues, in the nature of the peptide cyclization and in the length, saturation and branching of the fatty acid chain (Raaijmakers *et al.*, 2006; Ongena & Jacques, 2008). The cLPs secreted by *Bacillus* strains encompass structural variants depending on the genetic background of the considered strain. Although other classes have been identified recently (Hagelin *et al.*, 2007; Lee *et al.*, 2007), *Bacillus* cLPs may be classified in three main families: surfactins, iturins and fengycins (Ongena & Jacques, 2008). Surfactins are heptapeptides interlinked with a β-hydroxy fatty acid (length C_{12}–C_{16}) to form a cyclic lactone ring structure. The group of iturins encompasses seven variants including bacillomycins and mycosubtilins. They are also heptapeptides but are linked to a β-amino fatty acid chain with a length that usually varies from C_{14} to C_{17}. Fengycins A and B, also called plipastatins, are lipodecapeptides with an internal lactone ring in the peptidic moiety. The β-hydroxy fatty acid (C_{14}–C_{19}) can be mono-unsaturated, in contrast to the fatty acids of the two other families, which are in all cases saturated.

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cLPs display multiple versatile functions in the ecology of the producing strains and notably in interactions with co-existing organisms, including bacteria, fungi, oomycetes, protozoan predators and plants (Raaijmakers *et al.*, 2010). In the context of biological control of plant diseases, the contribution of cLP production to the global efficacy of some selected *Bacillus* strains at reducing infection caused by phytopathogens has been clearly established. They may represent key factors for efficient root colonization and are involved in direct pathogen inhibition and stimulation of the host plant's immune system (Ongena & Jacques, 2008).

Plant roots usually release soluble carbon compounds, pH and redox-modulating factors, complexing agents (siderophores, phenols, carboxylates), antimicrobials (antibiotics, quorum-sensing inhibitors) and specific stimulatory compounds (Uren, 2007). They may also physically shape the soil surrounding roots. The combination of all these events creates specific habitat conditions suitable for microbial development. This is the so-called rhizosphere effect, which may result in the selection of particular microbial populations by the host plant and/or in a drastic modification of the cellular physiology of root-associated species (Hartmann *et al.*, 2009). Other biotic factors inherent to the rhizosphere ecology such as auxiliary microflora or abiotic parameters as humidity, pH, temperature or dissolved oxygen availability, may have a marked influence on the size and physiology of particular microbial populations (Hartmann *et al.*, 2009; Hinsinger *et al.*, 2009). The influence of these conditions on the inhabiting microflora is being extensively studied at the population level because of the interest in engineering this environment to improve plant health. Less information is available about the impact of rhizosphere factors on the biosynthesis of specific metabolites involved in the biocontrol activity of root-associated beneficial microorganisms in general and of antibiotics secreted by rhizobacteria in particular. Such impact can be appreciated using non(over)-producing mutants or derivatives with reporter systems for biosynthetic gene expression, but direct antibiotic detection in the micro-environment is a complementary approach especially when dealing with bacterial species or strains that are difficult to engineer genetically, such as some natural *Bacillus* isolates. However, accurate identification and quantification of rhizobacterial antibiotics have been successfully performed only in a limited number of works, probably because of the inherent difficulties in extraction and measurement of the small amounts produced (Thomashow *et al.*, 2008).

In this context, some studies have dealt with lipopeptide secretion by *Bacillus* isolates growing in the phytosphere (Asaka & Shoda, 1996; Touré *et al.*, 2004; Romero *et al.*, 2007; Kinsella *et al.*, 2009; Nihorimbere *et al.*, 2009). However, a better assessment of the extent of changes that may occur in the global cLP pattern produced by *Bacillus* during interaction with plants compared to the one observed after growth in laboratory conditions deserves further investigation and was the main objective of the present work. To this end, we developed HPLC-coupled and imaging mass spectrometry methods to perform a detailed characterization of the cLPs secreted by *Bacillus amyloliquefaciens* S499 evolving on the roots of tomato plants. To explain the observed changes in the relative proportions of surfactins, iturins and fengycins produced *in planta* compared with *in vitro* conditions, we also investigated to what extent the cLP signature may be modulated both qualitatively and quantitatively by some rhizosphere-specific factors such as nutritional source, low oxygen status and biofilm formation.

Materials and methods

Bacterial strain

The *Bacillus* strain S499 used in this study was formerly identified as *Bacillus subtilis* based on basic bacteriological tests, but *gyrA* sequencing revealed that it most probably belongs to the closely related *B. amyloliquefaciens* species. Detailed results and methods are presented in Supporting Information, Appendix S1.

Plant cultures and determination of S499 populations on roots

For tomato cultures under hydroponic conditions, surface-sterilized tomato seeds were allowed to germinate for 5 days at room temperature in the dark on gelified (agar 15 g L^{-1}) sterile nutrient medium containing 5 mM Ca $(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and micronutrients. They were transferred in sterilized 50-mL tubes filled with nutrient solution, inoculated with strain S499 and incubated at 25 ± 2 °C in the greenhouse with a 16-h photoperiod alternating sunlight and fluorescent light. For inoculum preparation, the bacteria were grown on solidified Luria–Bertani (LB) medium for 24 h and harvested cells were resuspended in NaCl 0.85%. The concentration was determined by measuring turbidity at 600 nm. S499 cells were inoculated at a final concentration of 5×10^7 cells mL^{-1}.

Monitoring of colonization by strain S499 was performed on root samples of approximately 1 g that were directly transferred and incubated under shaking for 15 min in 10 mL of sterile peptone water (bacto-peptone 0.5 g L^{-1}; NaCl 5 g L^{-1}, Tween 80) before serial dilutions. Vegetative cell concentrations were determined by

plate-counts on (i) LB medium on the basis of typical morphology of the colonies and (ii) blood agar (containing, (L^{-1}): $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 14 g; KH_2PO_4, 6 g; $Na_3citrate{\cdot}2H_2O$, 1 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; agar, 15 g; glucose, 5 g; defibrinated sheep blood, 30 mL) on the basis of hemolytic halo formed around colonies after 3 days of incubation at 28 °C.

For cultures of tomato plantlets under gnotobiotic conditions, sterilized tomato seeds were immersed in a suspension of S499 cells at a concentration of 10^8 CFU mL^{-1} for 10 min. The seeds were then transferred on agar plates containing sterile plant nutrient solution as described above. The plates were incubated in a growth chamber at 28 °C for 2 weeks under appropriate fluorescent light illumination.

For colonization measurements, roots (approximately 50 mg) were gently detached and immersed in 5 mL sterile peptone water. Cells still adhering to the gelified medium were taken up in the same volume of peptone water until visual assessment of complete resuspension. The two samples were combined and the resulting 10-mL volume served as the basis for serial dilutions. In these conditions, no contaminating microflora was observed (detection limit 100 CFU g^{-1} of root) and *Bacillus* cells were counted by plating on LB medium.

Analyses of lipopeptides from hydroponic cultures

To evaluate *in planta* production of cLPs, roots (approximately 2 g, fresh weight) were cut into 2–3-cm segments and immersed in 10 mL of 90% acetonitrile (ACN), 0.1% HCl, 0.9% Triton-X 100, ultrasonicated for 2 min and extracted for 2 h under regular vortexing. This extract was combined with the one obtained after submitting the hydroponic liquid collected at the same time to solid phase extraction on a C_{18} cartridge (10 g; Alltech). The matrix with loaded material was washed with 20% ACN before desorption of the lipopeptides with pure ACN. The volume of the combined sample was reduced to approximately 2 mL (aqueous phase) and the resulting material was again loaded on a C_{18} cartridge (900 mg) for further washing of salts and hydrophilic compounds. Lipopeptides were desorbed with 1 mL ACN and the solution was concentrated five times using a vacuum concentrator.

The resulting samples were analyzed by reverse phase HPLC (HPLC Waters Alliance 2695/diode array detector) coupled with a single quadrupole mass spectrometer (Waters SQD mass analyser) on an X-terra MS (Waters) 150 × 2.1 mm, 3.5 μm column. Specific LC-MS methods were used for analyses of the three cLP surfactin, iturin and fengycin families. In some instances, however, a single method allowing the simultaneous measurement of all three families was used. Surfactins were eluted in the isocratic mode (78% ACN in water acidified with 0.1% formic acid) at 0.5 mL min^{-1} and 40 °C. Iturins and fengycins were selectively desorbed using ACN gradients from 35% to 65% in 35 min and from 40% to 60% in 40 min, respectively. Compounds were first identified on the basis of their retention times compared with authentic standards (supplied by AIBI asbl, Gembloux, Belgium). The identity of each cLP homologue in every family was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization source conditions in the MS as source temperature, 130 °C; desolvation temperature, 250 °C and nitrogen flow 500 L h^{-1}. Specific cone voltages of 70, 60 and 100 V were used for surfactins, iturins and fengycins, respectively. The positive ion mode was used for analysis of all three families because a higher signal/background ration was obtained compared to negative ion recording.

Analyses of lipopeptides produced on plantlets in gnotobiotic conditions

Generally, the time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging method used for sample processing is the same as described by Debois *et al.* (2008), including detailed characteristics and settings for the mass spectrometer. Briefly, lipopeptide material present at the surface of the plate was directly transferred onto a polished silicon wafer by applying a manual press for 30 s. The wafer was then allowed to dry for 30 min under vacuum at room temperature before introducing it in the mass spectrometer. A TOF-SIMS IV (ION-TOF GmbH, Münster, Germany) reflectron-type TOF mass spectrometer was used for imaging experiments. The primary ion source is a bismuth LMIG which delivers Bi_3^+ cluster ions. The secondary ions are accelerated to a kinetic energy of 2 keV and are post-accelerated to 10 keV before hitting a hybrid detector. Details concerning image processing are given in the legend of Fig. 4. Methanolic solutions (1 mg mL^{-1} or serial dilutions of 0.3 mg mL^{-1}) of authentic standards (95% pure) were prepared and 10-μL drops were deposited on the same gelified medium as the one used for plantlet growth and colonization. After incubation at room temperature for 2 h, the plates were processed for imaging as described for those used for analyzing cLP production by S499.

Core sampling in gelified medium was performed with a Pasteur pipette and lipopeptides contained in the 200-μL agar piece were then extracted by adding 100 μL acidified (HCl 0.1%) ACN/water 80/20 v/v. The mixture was ultrasonicated for one minute, extracted under regular stirring for 30 min and incubated overnight at −20 °C. Extracts

were then centrifuged at 18 000 ***g*** for 10 min and the supernatant was analyzed in LC-MS as described above.

Growth conditions for cLP production by S499 cells in liquid cultures

Lipopeptide production by S499 was studied in various media. The optimized medium contained (L^{-1}): bactopeptone, 30 g; saccharose, 20 g; yeast extract, 1.9 g; KH_2PO_4, 1.9 g; $CuSO_4$, 0.001 mg; $FeCl_3.6H_2O$, 0.005 mg; $NaMoO_4$, 0.004 mg; KI, 0.002 mg; $MnSO_4{\cdot}H_2O$, 3.6 mg; $MgSO_4$, 0.45 g; $ZnSO_4{\cdot}7H_2O$, 0.014 mg; H_3BO_3, 0.01 mg; citric acid, 10 mg. To evaluate the production of cLPs in the recomposed exudate (RE) medium and in the presence of individual carbon sources, the same salt composition was used: 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KCl, 1.0 g L^{-1} yeast extract, 1.2 mg L^{-1} $Fe_2(SO_4)_3$, 0.4 mg L^{-1} $MnSO_4$, 1.6 mg L^{-1} $CuSO_4$, 2 g L^{-1} $(NH_4)_2SO_4$. The composition of RE medium regarding carbon sources is as follows: 0.8 g L^{-1} glucose, 1.3 g L^{-1} fructose, 0.2 g L^{-1} maltose, 0.02 g L^{-1} ribose, 5.6 g L^{-1} citrate, 1.4 g L^{-1} succinate, 0.2 g L^{-1} malate, 0.8 g L^{-1} casamino acids. To test the effect of carbon sources, individual substrates were added at a final concentration of 5 g L^{-1}.

All the S499 cultures were performed in 12-well plates by dispensing 2.5 mL of medium per well. Plates were incubated under agitation on a rotary shaker for 72 h at 28 °C. Biofilm cultures were performed in 96-well polystyrene microtiter plates under static incubation and wells were filled with 200 μL of OM medium. In all cases, the pH of the different media was adjusted to 6.8 before filter-sterilization. At the end of incubation time, bacterial density was monitored by measuring turbidity (OD) at 600 nm and converting to cell concentration, calculated using the relation one unit $OD_{600\ nm}$ corresponding to 1.5×10^8 CFU mL^{-1}. At the end, cultures were centrifuged (15 min, 13 000 ***g***) and supernatant samples were processed for lipopeptide extraction and analysis by LC-MS as described above. Three wells per plate were used for each medium but corresponding supernatants were combined to yield a unique sample, which was submitted to extraction and LC-MS analysis. The experiments were repeated at least three times to calculate mean values and standard deviations mentioned in the results.

cLP production as a function of oxygen concentration

These experiments were conducted in a 2-L bioreactor using the RE medium described above. The 1.5-L working volume of the fermentor was inoculated with a 16-h-old preculture (25 mL) prepared in the same medium. Temperature was maintained at 30 °C and pH at a value of 6.8. For aerobic conditions, aeration rate and agitation were fixed respectively at 0.5 VVM (volume of air per volume of medium per minute) and 150 r.p.m. In these conditions, the oxygen concentration was not limiting for the main part of the culture. Oxygen-depleted condition was created by replacing air with nitrogen, flushing the reactor through the sparger until pO_2 value reached zero. This nearly anaerobic condition was maintained over the culture time by subsurface injection of nitrogen at a low flow rate.

Results

Lipopeptide pattern of strain S499 developing on roots of hydroponic plants

Bacillus amyloliquefaciens strain S499 (see Appendix S1 for species identification) efficiently secretes various forms of surfactins, iturins and fengycins upon growth under laboratory conditions in a medium previously established for enhanced production of such compounds (referred to as OM medium below). These cLP species are listed in Appendix S2, which also provides further details about the LC-ESI-MS method (reverse phase HPLC coupled with electrospray ionization mass spectrometry) we have optimized for both sensitive detection and reliable identification/quantification of these molecules.

In a first approach to investigate *in planta* cLP production, experiments were conducted on S499-inoculated tomato plants grown in hydroponic culture mode to facilitate measurement of bacterial populations established on roots and recovery of the metabolites secreted by these colonizing cells. S499 cell density on roots and lipopeptide production were determined at three different timepoints using tomato plants colonized for 13, 18 and 23 days (Table 1). *Bacillus* colonies were detected among the other root-adhering microorganisms on the basis of their typical morphology on LB medium. In several experiments, this was combined with the observation of hemolytic activity due to cLP secretion as revealed after plating dilution series on sheep blood agar. In these assays, more than 90% of the CFUs resembling *Bacillus* S499 colonies also induced a clear hemolysis halo similar to the one formed by cells of the same age plated from a pure culture of the strain. Moreover, in one representative experiment, some *Bacillus* S499-resembling colonies were picked up and subcultured in optimized liquid medium under standardized conditions as described above. This allowed verification of the similarity of their cLP signatures compared to the one classically obtained with pure cultures of the strain. We thus considered that under our experimental conditions, the very large

Table 1. Colonization and lipopeptide production by strain S499 developing on roots of tomato plants grown in hydroponic culture mode

Colonization time (days)	S499 population on roots (CFU g^{-1} root FW)*	Lipopeptide production (ng g^{-1} root FW)†		
		Surfactin	Iturin	Fengycin
13	$3.9 \pm 0.4 \times 10^6$	61 ± 15	nd	nd
18	$8.1 \pm 3.0 \times 10^5$	186 ± 55	13 ± 9	nd
23	$5.6 \pm 2.2 \times 10^5$	294 ± 85	17 ± 5	nd

*Average values ± SD calculated from four individual plants.
†Average values ± SD calculated from three (analyses at 13 and 18 days) or five samples each obtained in independent experiments but consisting of approximately 2 g FW of combined root material corresponding to three plants.
nd, not determined.

majority of colonies with typical *Bacillus* morphology corresponded to the inoculated S499 strain. The S499 population on tomato roots grown for 23 days was slightly higher than 5×10^5 CFU g^{-1} root FW (Table 1). However, higher concentrations of the bacteria were measured in the rhizosphere after shorter periods of incubation of the bacterized plants. This suggests a colonization process quite similar to the one commonly reported for rhizobacteria, with a first phase of increasing cell density followed by a decrease of the population to reach an almost stable level (Rainey, 1999; Espinosa-Urgel *et al.*, 2002; Nihorimbere *et al.*, 2009). These results show that an efficient root colonization by S499 occurred which is a key event required for optimal expression of biocontrol activity by beneficial rhizobacteria (Lugtenberg *et al.*, 2001; Lugtenberg & Kamilova, 2009).

At the same time points, lipopeptides secreted by the *Bacillus* cells colonizing tomato roots were recovered. To that end, material directly extracted from the nutrient solution was combined with the material recovered by repeated extraction from root tissues using ACN and detergent. This is justified by the fact that lipopeptides secreted by root-adhering *Bacillus* cells may remain tightly associated with the membrane structure of plant cells. Such an assumption is based on our results from an independent study showing that surfactin in the presence of cultured cells or root tissues, spontaneously binds with high affinity to plant cell membrane. Such binding is long-lasting, as it was possible to recover most of the initial quantities when extraction was performed 5 days later (on both cells and roots). Moreover, surfactin was extracted from the membranes in its intact form as revealed by LC-MS, suggesting that possible degradation by plant enzymes at the cell wall level is not obvious (M. Ongena and G. Henry, unpublished data).

The extracted lipopeptide material was then analyzed using the optimized LC-ESI-MS method (Appendix S2). It revealed that, at any time-point, members of the surfactin family were mainly secreted by root-colonizing S499 cells, whereas iturins were detected but in much lower concentrations and fengycins were not produced at a measurable level. These data are presented in Table 1. Figure 1a presents the typical LC-MS trace obtained for analysis of the three families of cLPs recovered after 23 days of colonization by S499, together with quantitative data. No traces of any lipopeptide could be detected in extracts prepared from control noninoculated plants (data not presented). Considering a biomass density of 5×10^5 cells g^{-1} of root, it corresponds to surfactin and iturin productivities of 26 μg per 10^8 cells and 1.7 μg per 10^8 cells, respectively. Based on these results, it appears that surfactin is produced in much larger quantities than the two other cLP families when S499 grows on roots. Interestingly, this lipopeptide pattern produced *in planta* clearly differs from the one observed upon *in vitro* cultivation of the strain in the artificial OM medium. Figure 1b shows that under these laboratory conditions, iturins and fengycins are produced in similar amounts, each constituting more than half of the surfactins formed in terms of relative quantities.

Strain S499 grown in an agitated flask in OM medium usually co-produces the main $C_{12}/C_{13}/C_{14}/C_{15}$ surfactin homologues in the proportion 7/17/48/33% (Fig. 2b). Interestingly, the proportion of C_{15} markedly increased in the pattern secreted by the strain growing on roots of hydroponically grown tomato plants (Fig. 2a). The very low amounts of iturins and fengycins produced *in planta* precluded such comparative analysis of homologue patterns for these two families.

Mass spectrometry imaging of lipopeptide signature under gnotobiotic conditions

We wanted to confirm this differential secretion of the various cLPs by S499 under more controlled conditions regarding the auxiliary microflora. The presence of co-habiting microorganisms might induce some misinterpretation of the results due to (i) possible degradation of some cLPs via hydrolytic enzymes (differential susceptibility of members of the three families), (ii) interference in the regulation of their biosynthetic apparatus, or (iii) any other indirect effect on *Bacillus* cell physiology. In a second

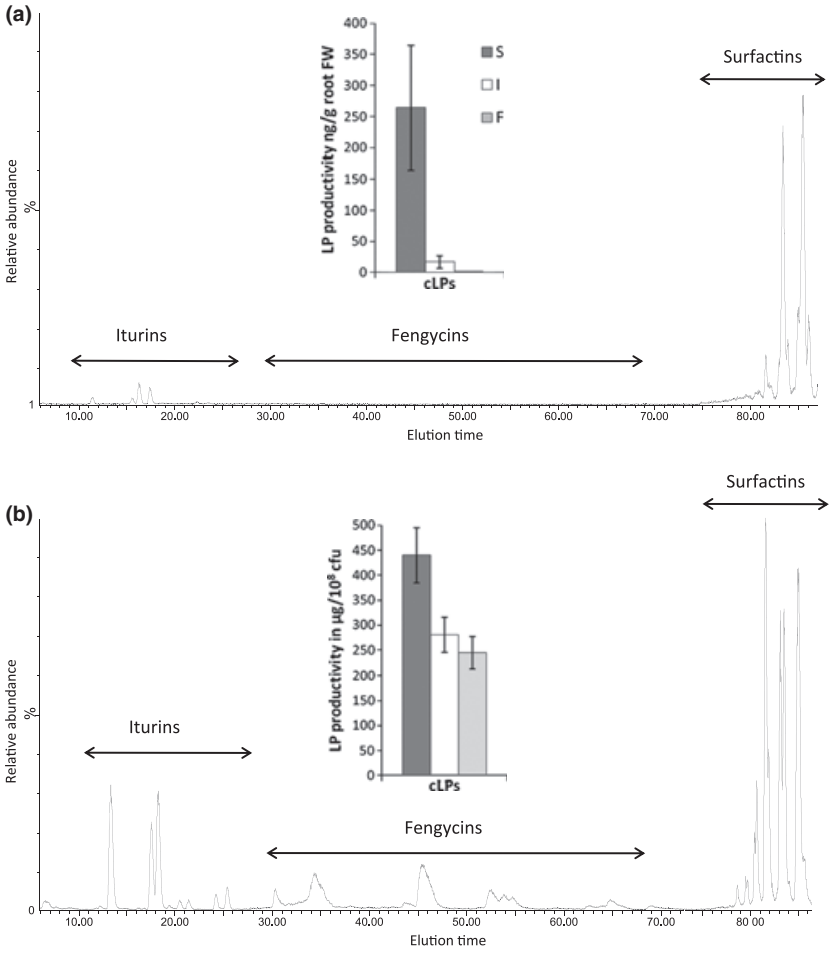

Fig. 1. Comparison of representative LC-ESI-MS profiles of lipopeptides produced *in planta* by strain S499 colonizing roots of hydroponically-grown tomato plants (a) with those produced under laboratory conditions in agitated optimized medium (b). Detection was performed in the SCAN mode (*m/z* range 900–1550). For clarity, *Y* axes of LC-MS traces in a and b were not linked to the same scale but absolute values of relative abundance of cLP ions detected in (b) are higher in several orders of magnitude compared to (a). Elution time periods for each cLP family are indicated and every single peak represents a specific homologue. The relative proportions of total surfactins, iturins and fengycins were calculated using authentic standards and are shown in the boxed graphs. Data and standard errors were calculated from five samples obtained in five different experiments but each consisting of approximately 2 g fresh weight of combined root material coming from three plants.

approach to evaluate *in planta* cLP production, we thus used a gnotobiotic system and took advantage of the recent developments in time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging for two-dimensional analysis of lipidic compounds in various biological samples (Debois *et al.*, 2008). With this imaging technique it is possible to analyze the lipopeptides secreted by S499 cells developing on roots of tomato plantlets grown from sterilized seeds on gelified medium. S499 readily colonizes the root system of 15-day-old plantlets by forming microcolonies that cover many root hairs (Fig. 3). Under these conditions, the mean value for the S499 population established on roots after approximately 2 weeks was 2.3×10^7 CFU g^{-1} root FW. No contaminating microflora was detected on control or bacterized plantlets after 2 weeks of incubation (detection limit of 10^2 CFU g^{-1} root FW).

We first analyzed lipopeptides secreted by S499 at the microcolony level at the extremity of two root hairs (Fig. 4). Material present at the surface of the plate was carefully transferred onto a silicon wafer (image of the imprint in Fig. 4b), allowed to dry for 30 min and then submitted to scanning by TOF-SIMS over the small area (500×500 µm) around S499-coated root hair tips (Fig. 4c) corresponding to the video image recorded with an integrated camera in the TOF-SIMS mass spectrometer. Based on the color-scale from dark (no count) to yellow (maximal number of counts corresponding to cLP molecular ions) in Fig. 4d, this imaging MS analysis revealed the relative amounts of surfactins, iturins and fengycins that were secreted by microcolony-forming S499 cells. Very few cLPs remained associated with the microcolonies, but rather diffused into the surrounding gelified medium (Fig. 4d). It also clearly appears from the density of red-to-yellow spots in images S, I and F in Fig. 4d that the production of surfactin was significantly more efficient than that of iturin or fengycin. Based on total counts corresponding to the more abundant homologues of the three families (surfactin C_{14}, iturin C_{15} and fengycin C_{16}), the quantity of surfactins is respectively 11 times and 34 times higher than the amounts of iturins and fengycins. Figure 4e presents images displaying the

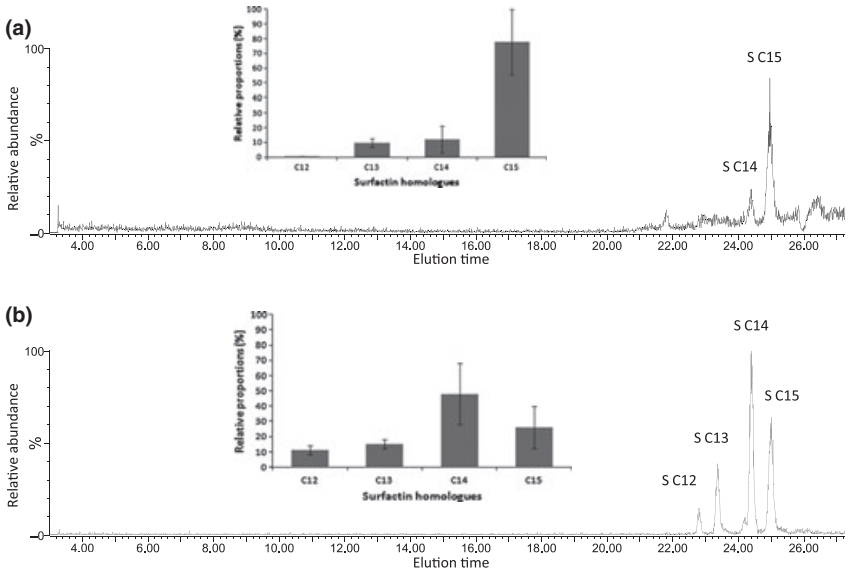

Fig. 2. Comparison of representative LC-ESI-MS profiles of surfactin homologues produced *in planta* by strain S499 colonizing roots of hydroponically grown tomato plants (a) with those produced under laboratory conditions in agitated optimized medium (b). Detection was performed in the SCAN mode (*m/z* range 900–1550). As mentioned in legend of Fig. 1, *Y* scales of LC-MS traces in a and b were not linked. The various homologues of surfactin with fatty acid chain lengths of 12, 13, 14 and 15 carbons are indicated as S C12, S C13, S C14 and S C15, respectively. The relative proportions of these homologues are shown in the boxed graphs in terms of percentages of total surfactins. Data and standard errors were calculated from three samples obtained in three different experiments but each consisting of approximately 2 g fresh weight of combined root material coming from three plants.

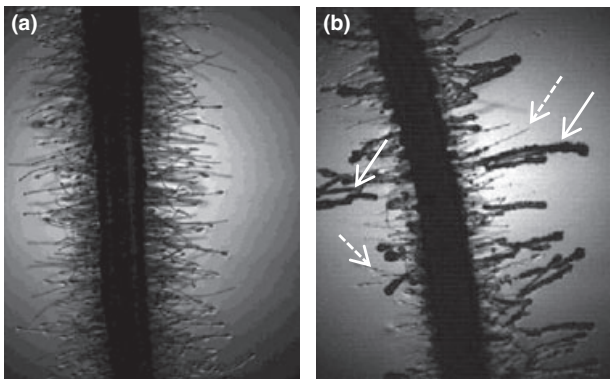

Fig. 3. Illustration of microcolonies surrounding lateral root hairs typically observed upon colonization of tomato roots by strain S499. (a) Root section of non-bacterized plants grown for 2 weeks under gnotobiotic conditions on gelified medium. (b) Root section of tomato plants 2 weeks after seed treatment with strain S499. Plain and doted arrows show respectively fully-colonized and non-colonized root hairs.

distributions and relative intensities of selected ions corresponding to the C12, C13 and C15 homologues of surfactin as extracted from the 2-D spectrum of total surfactins presented in Fig. 4d, S. Much higher signals corresponding to C_{15} surfactin compared to C_{12} and C_{13} homologues are clearly visible. This supports data from hydroponic experiments suggesting that long acyl-chain homologues are secreted in a large majority *in planta*. The relative abundances of these ions corresponding to surfactin homologues but also of those corresponding to surfactins, iturins and fengycins (Fig. 4d) are well illustrated in spectra presented in Fig. 4f where the Y scale used for the fengycins (right) is 200 times lower than the scale for surfactin/iturin (left). Analysis of concentrated solutions of authentic standards spotted on the same gelified medium and processed in the same way as described above, showed a detection limit of approximately 3 μg mL^{-1}.

TOF-SIMS imaging of cLPs was also performed over a larger surface (5 × 5 mm) of another colonized-root system (Fig. 5). The scanned area is represented in the boxed zone in Fig. 5a and the imprint obtained after transfer on silicon wafer is shown in Fig. 5b. It includes a section of the main root (delimited by the dotted lines) and extends over a few millimeters covering the root hair zone (delimited by the dotted zig-zag line) and part of the gelified medium. TOF-SIMS analysis revealed that some surfactins remained associated with bacterial cells present along the main root but again it was clear that this lipopeptide readily diffuses into the medium to accumulate in distant noninoculated zones (yellow zone in Fig. 5c, S). By comparing S, I and F in Fig. 5c,

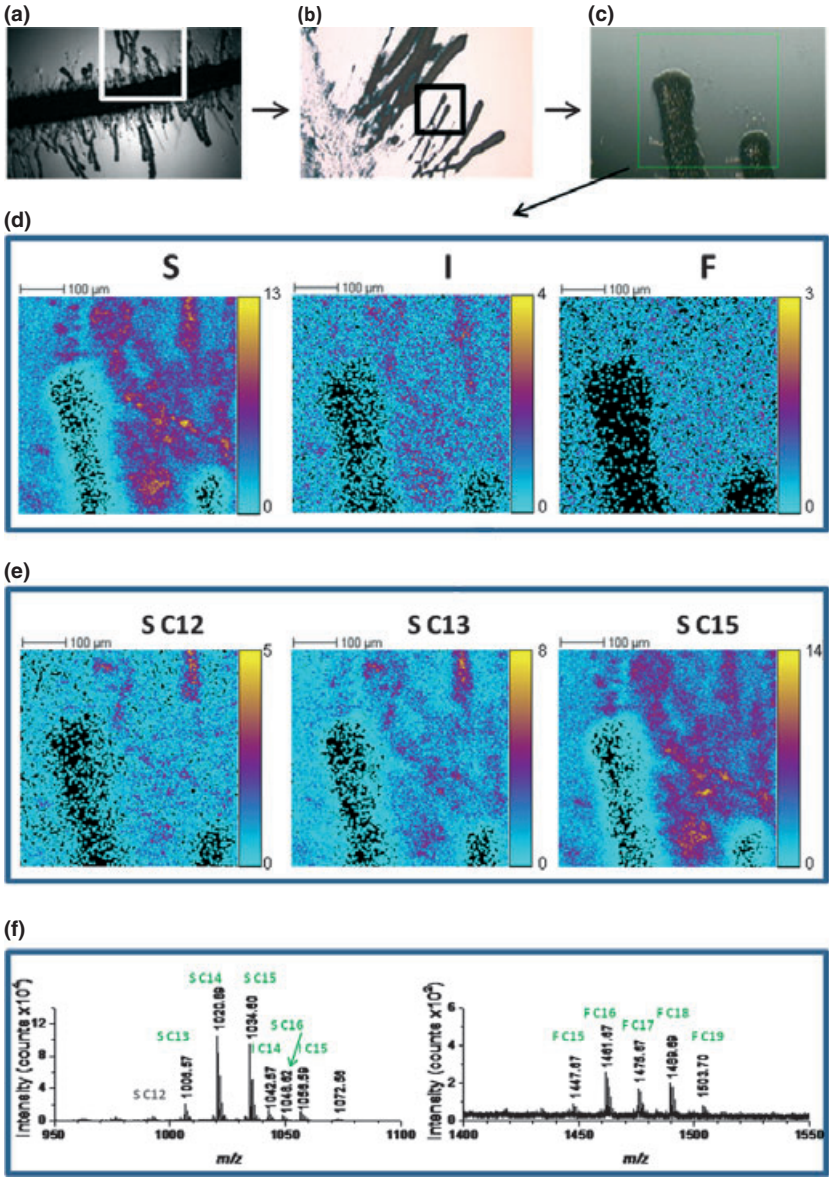

Fig. 4. TOF-SIMS imaging of lipopeptides produced by S499 colonizing roots of tomato plantlets grown on gelified medium in Petri plates. (a) Optical microscope image of the main root and root hairs colonized by S499 cells forming microcolonies. (b) Microscope image of a part of the colonization pattern after transfer onto the silicon wafer. (c) Video image of the scanned zone (delimited by the green square) recorded with an integrated camera inside the TOF-SIMS instrument. (d) High-definition scan of the lipopeptide distribution at the extremity of biofilm-embedded root hairs. TOF-SIMS images of the sum of surfactin (S), iturin (I) and fengycin (F) ions are shown. The color scale indicates the relative amounts of total surfactin/iturin/fengycin ions detected. The maximum ion count recorded in a pixel in the image is indicated on this colour scale bar. (e) Relative distribution of surfactin homologues in the same pattern. (f) Corresponding partial mass spectra of surfactins (S C12-S C16)/iturins (I C14, I C15) (left) and fengycins (F C15-F C19) (right). Similar images and results were obtained by analyzing two other root zones from plantlets inoculated and grown independently.

surfactins are clearly seen in much higher quantities compared to iturins and fengycins. This imaging MS technique provides useful information to study *in situ* production of cLPs, but only molecules present at the surface of the gelified medium are transferred on the silicon wafer and can thus be detected. This technique only allows semi-quantitative measurements (Appendix S3). To better determine the actual concentrations of the three types of cLPs, we have extracted the compounds contained in small agar cylinders collected by core-sampling from the medium in the close vicinity of S499-colonized roots. LC-ESI-MS analysis of ACN extracts corresponding to three core-samples collected along the roots revealed concentrations of 5.6 ± 2.2, 0.8 ± 0.3 and 0.2 ± 0.2 μM for surfactins, iturins and fengycins, respectively. This is, respectively, 83%, 12% and 4% in terms of proportions relative to total cLP amounts, which is globally in agreement with data from imaging showing much higher relative quantities for surfactin compared to the iturin and fengycin.

Influence of plant exudates as nutrients on S499 lipopeptide signature

The strain S499 is highly recalcitrant to transformation and we were unable to generate mutants with reporter systems for cLP gene expression that would have been useful for *in planta* investigations. We alternatively developed *in vitro* assays to further evaluate the impact of some rhizosphere factors on lipopeptide production by

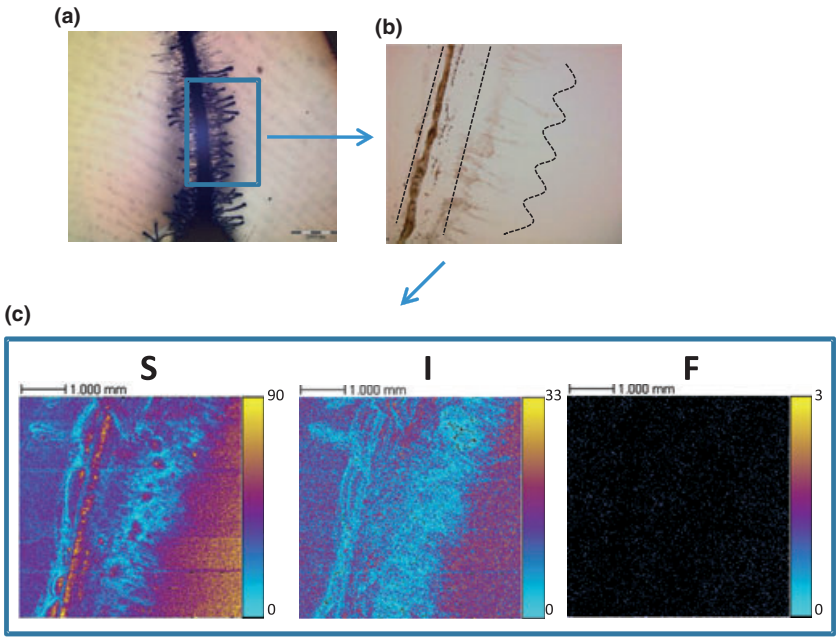

Fig. 5. Analysis of the S499 lipopeptide pattern by TOF-SIMS imaging over a surface of 5 × 5 mm of the colonized-root system of a tomato plantlet. (a) Microscope image of the main root and root hairs colonized by S499 cells. (b) Microscope image of a part (boxed zone in a) of the colonization pattern transferred onto silicon wafer. It includes a section of the main root (delimited by the dotted lines) and extends over a few millimeters covering the root hair zone (delimited by the dotted zig-zag line) and part of the gelified medium. (c) High-definition scan of the lipopeptide distribution at the extremity of biofilm-embedded root hairs. TOF-SIMS images of the sum of surfactins (S), iturins (I) and fengycins (F) ions are shown. The color scale indicates the relative amounts of total surfactin/iturin/fengycin ions detected. The maximum ion count recorded in a pixel in the image is indicated on this color scale bar. This experiment was repeated once with similar results.

cells colonizing root tissues. More specifically, we first tested the effect on cLP signature of the nutritional status imposed by the plant. To that end, S499 was grown in agitated culture performed in 12-well plates in the presence of root exudates as sole carbon source (NE medium). These exudates were collected from hydroponic cultures of non-bacterized tomato plants grown for 4 weeks in the greenhouse in the same hydroponic conditions as described for S499-inoculated plants. This medium was supplemented with 2 g L^{-1} $(NH_4)_2SO_4$ to compensate for the lack of available nitrogen. Significant growth of the strain occurred in these cultures but final biomass densities were quite low ($OD_{600\ nm}$ between 0.2 and 0.3) and consequently, much lower cLP concentrations were measured in the medium than in rich medium such as OM (Table 2). However, cLP production rate upon growth in these natural exudates corresponds to one-third of the one calculated under optimal conditions. These data confirm that exudate components are extremely conducive to cLP synthesis by strain S499. Qualitatively, the relative proportion of iturins compared to surfactins is similar to the one observed in optimized medium but the formation of fengycins appears to be significantly less efficient. Concerning the relative proportions of surfactins with different acyl chains, high levels of C_{14} and C_{15} homologues were detected after growth on these natural root exudates and together they represent more than 80% of the total surfactins produced under these conditions (Fig. 6).

The nature and concentration of components in the naturally produced exudates could not be reliably determined because of amounts close to the detection limits of the methods we used. However, the content of carbon sources typically found in tomato exudates and suitable for microbial growth such as sugars, organic acids and amino acids was previously determined by Kamilova and colleagues (2006). On that basis, a minimal medium containing these compounds added in the reported proportions as sole carbon source (RE medium) was created and used to cultivate S499. In a total of 10 g L^{-1} of glucose equivalent, this RE medium contained 72% organic acids, 23% sugars and 5% amino acids. Upon growth in the presence of such higher concentrations of nutrients in the medium compared to natural exudates, approximately 10-fold increased amounts of both biomass and lipopeptides were measured (Table 2). However, the relative proportions of the three cLP families produced in recomposed medium and natural exudates are similar

Table 2. Comparison of production levels and patterns of lipopeptides produced by strain S499 in various *in vitro* conditions and media

Medium	cLP, mg L^{-1}* (%)†			cLP, μg 10^{-8} CFU‡
	Surfactin	Iturin	Fengycin	Total three families
NE	32 ± 7 (58)	15 ± 8 (27)	8 ± 6 (14)	147
RE	364 ± 112 (61)	175 ± 65 (27)	70 ± 24 (11)	304
OM-b	179 ± 46 (80)	27 ± 18 (13)	17 ± 11 (7)	Nc
OM	628 ± 108 (51)	321 ± 55 (26)	272 ± 71 (22)	436
RE-O_2^+	556 (66)	212 (26)	73 (8)	341
RE-O_2^-	252 (67)	108 (29)	10 (4)	740

Nc, non-calculated due to inaccurate biomass quantification.

NE, natural exudates collected from tomato plants supplemented with $(NH_4)_2SO_4$ 2 g L^{-1}.

RE, recomposed exudates containing as nutritive sources (i) sugars 2.3 g L^{-1}: glucose 34%, fructose 57%, maltose 8%, ribose 0.75%; (ii) organic acids 7.2 g L^{-1}: citrate 77%, succinate 19%, malate 2%; (iii) amino acids 0.8 g L^{-1} added as casamino acids in addition to the inorganic nutrients described in M&M.

OM, optimized medium containing as nutritive sources saccharose 20 g L^{-1}, casein peptone 30 g L^{-1} and yeast extract 1.9 g L^{-1} in addition to the inorganic nutrients described in M&M.

OM-b, cLP production under conditions conducive for biofilm formation in OM medium (growth in 96-well plates under static conditions) by contrast with OM, RE, NE, where S499 was grown as planktonic cells in agitated cultures (12-well plates).

O_2^+ and O_2^- mean cLP production in RE medium after growth respectively in well aerated and in nitrogen flushed/oxygen-depleted bioreactor.

*Concentrations of lipopeptides in the culture supernatant. Data are means and standard errors calculated from at least four repeats of the experiment, except for data from RE-O_2 assays which represent the average values from two independent assays.

†Percentage of each of the three families in total lipopeptide amounts.

‡Lipopeptide production rate calculated at the end of the culture time.

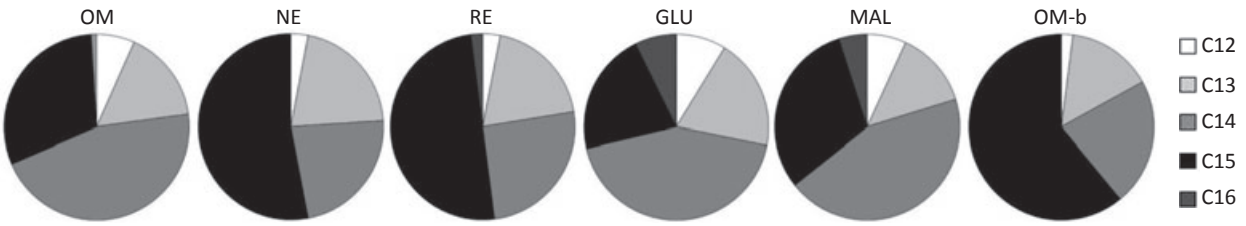

Fig. 6. Relative proportions of surfactin homologues varying in the length of the fatty acid chain produced by S499 during *in vitro* growth upon various conditions and as determined by LC-ESI-MS. OM, cLPs produced in optimized medium; NE, in natural exudates; RE, in recomposed exudates; GLU and MAL, in the presence of glucose and malic acid as sole carbon sources; OM-b, under biofilm-forming conditions in optimized medium. Data are mean values calculated from three independent cultures.

(Table 2) as also observed for the distribution of surfactin homologues (Fig. 6).

The main representatives of substrates used in RE medium were then tested individually to better appreciate whether some specific sugars or organic acids may constitute a more adequate nutritional basis for cLP production than others. All the C sources supported growth to an almost similar level (data not shown) but clear differences were observed concerning cLP productivities. As shown in Fig. 7a, surfactin secretion is more efficient in the presence of organic acids such as citrate and malate than in the presence of sugars. Interestingly, citrate is by far the most abundant substrate released by noninoculated tomato roots (Kamilova *et al.*, 2006). By contrast, iturin production rate is slightly higher on growth in the presence of sugars compared to organic acids (Fig. 7b) and fengycins are only poorly produced in the presence of citrate, succinate or malate (Fig. 7c). The nature of the carbon source did not markedly influence the relative quantities of various homologues co-produced within the surfactin family, as shown for glucose and malate in Fig. 6.

Influence of biofilm formation on cLP production

Rhizobacterial microcolonies such as those formed by strain S499 developing on tomato roots (Fig. 3) may be considered biofilm-related structures (Bais *et al.*, 2004; Ramey *et al.*, 2004). We therefore wanted to evaluate the effect of such biofilm formation on the pattern of cLPs produced by the strain. To that end, S499 was grown in

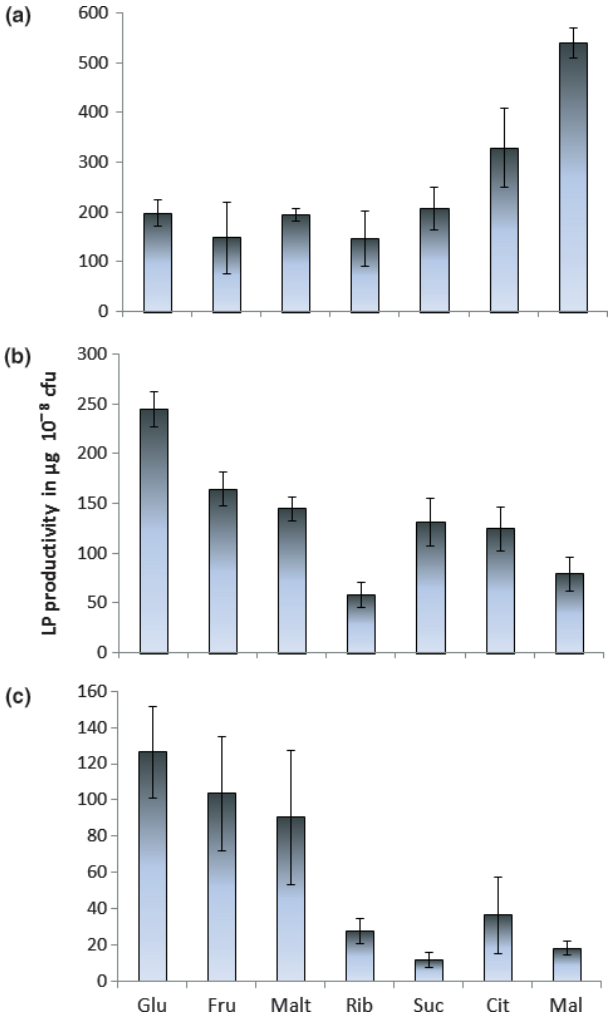

Fig. 7. Lipopeptide productivities of S499 in minimal medium in the presence of various substrates (Glu, glucose; Fru, fructose; Malt, maltose; Rib, ribose; Suc, succinate; Cit, citrate; Mal, malate) typically found in tomato exudates and used as sole carbon sources. (a) Surfactins; (b) iturins; (c) fengycins. Data are mean values and standard errors calculated from five independent experiments.

static liquid cultures (OM medium) performed in wells of microtiter plates. In these conditions, cells readily aggregate to form pellicles/biofilms at the liquid–air interface (Fig. 8). LC-MS profiling of the lipopeptides produced under these conditions revealed clear differences compared to the pattern observed in agitated cultures in the same medium. Indeed, the results presented in Table 2 show that surfactin secretion by S499 cells evolving in biofilm structures is very effective compared to secretion of iturins and fengycins, which together represent no more than 20% of total cLPs. These relative proportions of the three cLP families are similar to those secreted upon root colonization of hydroponically grown tomatoes and clearly differ from the cLP signature of

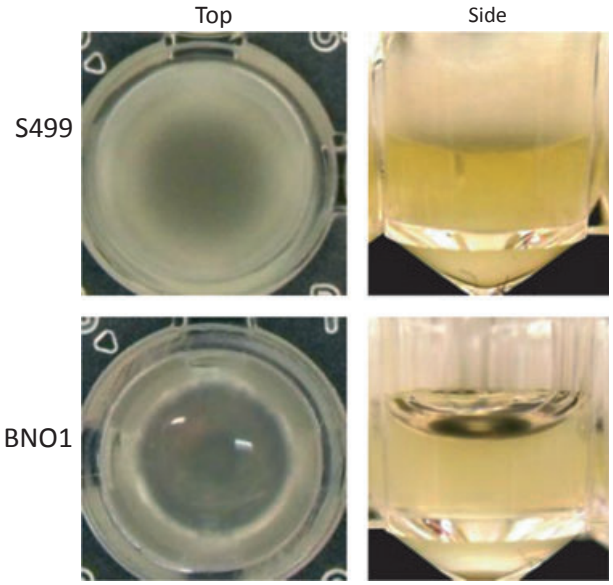

Fig. 8. Pellicle/biofilm formation at the liquid-air interface by strain S499 as compared to the non-biofilm-forming *Bacillus* strain BNO1 upon growth in static cultures in optimized medium.

planktonic cells, which typically secrete much higher amounts of iturins and fengycins corresponding to approximately half of the quantities of surfactin produced (Table 2, Fig. 1). Looking more specifically at the distribution of surfactin homologues, it also clearly appears that biofilm formation favors the production of peptides containing a C_{15} fatty acid chain, which represent about 60% of total surfactins (Fig. 6).

Influence of oxygen limitation on cLP production

Oxygen depletion is common in many soils and especially in the rhizosphere, notably due to root- and microbe-associated consumption (Højberg *et al.*, 1999). This factor may thus potentially impact basic physiological processes, including antibiotic synthesis in aerobic *Bacillus* species. The effect of oxygen concentration was evaluated by cultivating the strain in RE medium in a bioreactor at two different aeration rates, one corresponding to non-limiting oxygen concentration throughout the entire culture time (except a period of 2–3 h during the mid-exponential growth phase when the pO_2 dropped to zero, data not shown) and the other corresponding to a nearly anaerobic condition reached by flushing the medium with nitrogen prior to inoculation. Final cell densities were much lower in the anaerobic conditions ($OD_{600\ nm}$ 0.9) compared to aerated culture ($OD_{600\ nm}$ 2.2) but a significant biomass was still measured. S499 could thus develop with limited availability or absence of molecular oxygen

as a terminal electron acceptor. As other *Bacillus* strains, S499 may utilize nitrate as an alternative electron acceptor or may be able to grow anaerobically on minimal media in the absence of terminal electron acceptors (Hoffmann *et al.*, 1988; Mach *et al.*, 1988; Nakano *et al.*, 1988; Glaser *et al.*, 1995; Ramos *et al.*, 2000). S499 produces iturin, surfactin and fengycin lipopeptides upon growth in the two conditions of oxygen availability tested, but in terms of production rate, cLPs are more efficiently produced under air-starved conditions (Table 2). However, oxygen concentration in the medium during growth does not seem to impact the relative proportions of the three cLP families. These are very similar under the two conditions applied and also similar to the values obtained in the same medium after growth in 12-well plates (see above, Table 2).

Discussion

An efficient production of cLPs is important for the biocontrol activity of *Bacillus* isolates as well as for their global fitness in natural soil habitat (Ongena & Jacques, 2008; Raaijmakers *et al.*, 2010). This arises from several lines of indirect evidence but further information is still needed about qualitative and quantitative aspects of lipopeptide production by *Bacillus* cells growing in the rhizosphere. In the present work, we used *B. amyloliquefaciens* strain S499 to show that natural conditions are conducive to the biosynthesis of some of these cLPs in the phytosphere as occasionally demonstrated previously (Asaka & Shoda, 1996; Touré *et al.*, 2004; Romero *et al.*, 2007; Kinsella *et al.*, 2009). However, the lipopeptide signature of the bacteria is substantially modulated upon living in the rhizosphere compared to laboratory conditions. Indeed, much higher proportions of surfactins than fengycins and iturins were found in the tomato rhizosphere. The surfactin-enriched pattern was demonstrated via LC-ESI-MS analysis of cLPs produced on hydroponically grown plants and also illustrated by TOF-SIMS imaging after root colonization of plantlets growing on gelified medium. This MS imaging technique thus appears to be a very powerful method to study the production of lipid-containing molecules by microorganisms developing on solid supports.

Such an *in planta* lipopeptide signature is of relevance in the context of biocontrol as each family of *Bacillus* lipopeptides displays specific biological activities and is thus involved in different mechanisms for combating phytopathogens (Ongena & Jacques, 2008; Raaijmakers *et al.*, 2010). By contrast with surfactins, iturins and fengycins retain strong antifungal action but our data show that these cLPs are poorly secreted by S499 growing on tomato roots. Whether these low amounts of iturins detected in our assays are sufficient to provide any consistent antagonism by S499 towards fungal phytopathogens under real soil conditions is questionable. It usually requires concentrations in the 20–100 μM range depending on the target organism. There is a clear dilution effect inherent to our hydroponic experimental conditions. The diffusion of iturins released by biofilm-forming *Bacillus* cells in normal soil is certainly much more limited and their concentration in the microenvironment surrounding roots may be sufficient to provide some biological effect. This is in agreement with other reports demonstrating the involvement of iturins in the control of fungal diseases (Asaka & Shoda, 1996; Leclère *et al.*, 2005). Nevertheless, correlation of biocontrol activity with iturin production in these studies was mainly established using non-producing or overproducing derivatives but the level of iturin production *in planta* has not been determined.

Surfactins have been identified as an elicitor stimulating the host plant immune system and the efficient surfactin production *in planta* demonstrated here, supports the high systemic resistance-triggering potential of strain S499 (Ongena *et al.*, 2002, 2005, 2007; Jourdan *et al.*, 2009). The surfactin concentrations measured in hydroponic experiments may appear low (approximately 0.12 μM) but could be underestimated due to possible enzymatic degradation by auxiliary microflora and/or incomplete extraction. However, in plate experiments, surfactin concentration reached 50 μM within the diffusion zone close to the colonized roots. Because cLP destruction by other organisms cannot occur in this gnotobiotic system and because the dilution effect is reduced under these conditions compared to the hydroponic system, such concentration probably better reflects the amounts of surfactin that may be actually secreted by S499 evolving on tomato roots. Moreover, in a previous study conducted with *B. subtilis* BGS3, we observed a cLP concentration of 1.8 μM in the hydroponic medium surrounding tomato roots (Nihorimbere *et al.*, 2009). Even higher quantities of cLPs were detected in the cucumber rhizosphere colonized by strain QST713 (Kinsella *et al.*, 2009). The concentration of surfactin in the tomato rhizosphere may thus be biologically relevant, considering its activity as an inducer of resistance in the host plant (2–10 μM) (Ongena *et al.*, 2007; Jourdan *et al.*, 2009). Interestingly, we show in this work that the cLP signature in the rhizosphere may also vary compared to laboratory conditions regarding the relative proportions of homologues within the surfactin family. Long-chain C_{14} and C_{15} surfactins are indeed mainly produced *in planta* and they are also the most active as elicitors of plant defense reactions (Jourdan *et al.*, 2009) and as antiviral/antibacterial or hemolytic compounds (Kracht *et al.*, 1999; Bonmatin *et al.*, 2003; Dufour *et al.*, 2005). Such an important

role for the length of the acyl chain may be related to the fact that the chain readily inserts into phospholipid bilayers (Carrillo *et al.*, 2003; Eeman *et al.*, 2006; Heerklotz & Seelig, 2007).

The influence of some factors inherent to the development of *Bacillus* in the rhizosphere has been further investigated to better explain the differential cLP synthesis *in planta*. Our data suggest that the cLP pattern can be markedly affected by nutritional factors as surfactin synthesis by S499 is apparently favored in the presence of organic acids, the main components of tomato exudates (Kamilova *et al.*, 2006). To what extent this increase in surfactin production is related to the drastic metabolic reorientation observed in *Bacillus* upon shift from sugars to organic acids as source of carbon and energy (Schilling *et al.*, 2007) is not known and certainly deserves further examination. The surfactin-related lychenisin also accumulates differently in the culture broth upon growth in various media differing in C source (Li *et al.*, 2008). Akpa *et al.* (2001) have also demonstrated the influence of the nature of the carbon source on the pattern of surfactins but not mycosubtilins produced by *B. subtilis*. The influence of the non-polar fraction of natural exudates (hydrophobic material containing phenolics, flavonoids, fatty acids, sterols, vitamins) was also tested but no effect on the cLP pattern was seen (data not shown). Our data show that production rate for all three cLP families was higher under oxygen-starved conditions which prevail in soil. Such a positive influence of O_2 limitation on surfactin production has been occasionally demonstrated by modifying the oxygenation method or by modulating the oxygen transfer rate in a bioreactor (Kim *et al.*, 1997; Lee *et al.*, 2001; Yeh *et al.*, 2006). However, oxygen availability had no marked effect on the relative proportions of the three cLP families and therefore this parameter does not contribute to explaining the surfactin-enriched lipopeptide signature *in planta*.

Several studies performed with *B. subtilis* strains showed that cLPs could be involved in formation of the biofilm/pellicle or fruiting bodies (Branda *et al.*, 2001; Bais *et al.*, 2004; Hofemeister *et al.*, 2004). Conversely, little is known about the impact of biofilm formation on lipopeptide production. Some previous studies have reported the consequences of growing on solid support on cLP synthesis by *Bacillus* and it is clear that lipopeptide production by biofilm-aggregated cells developing on gelified media may be qualitatively and quantitatively modified compared to cells living freely in liquid cultures (Leenders *et al.*, 1999; Nihorimbere *et al.*, 2009). In another approach, Gancel *et al.* (2009) have also demonstrated an enhancement of surfactin production upon cell immobilization on polymer particles. We show here that surfactin synthesis by S499 cells forming biofilm is very effective compared to iturins and fengycins as observed for the cLP pattern secreted *in planta*. The development of *Bacillus* cells as root-adhering microcolonies may thus be a crucial factor *per se* to explain such differential production of the three cLP families. Regulation of surfactin biosynthesis is under the control of a complex network that governs cellular differentiation and quorum sensing via ComX and other pheromones of the Phr family (Rainey, 1999; Espinosa-Urgel *et al.*, 2002; Duitman *et al.*, 2007). Therefore, population-driven responses may play an important regulatory role regarding cLP synthesis in cells evolving in root-associated biofilms.

In addition, due to low root exudation rate and nutrient diffusion constraints through the multilayered biofilm structure, rhizobacterial cells evolving on roots are obviously in a nutrient-starved state, which drives their physiology to slow growth rate (Lugtenberg *et al.*, 2001; Bais *et al.*, 2006; Spormann, 2008). Such an effect of growth rate/phase on surfactin synthesis was previously illustrated with two different strains by experiments in chemostat bioreactors that allow the study of metabolite synthesis upon fixed growth rate and constant cellular physiological state. For both strains, these assays showed that *srfA* surfactin gene expression significantly decreased with increasing growth rate (Kakana, 2005; Nihorimbere *et al.*, 2009). More generally, results from this work illustrate that root surface colonization, biofilm formation and secondary metabolite secretion are intricately related and there is a need for continued effort in elucidating the mechanisms that govern interactions between these phenomena.

In conclusion, our data show that the *in situ* lipopeptide signature does not reflect the broad panoply of cLPs that may be produced *in vitro* in a laboratory medium. Even if some *B. subtilis* or *B. amyloliquefaciens* strains are well equipped genetically to produce a vast array of antibiotics (Stein, 2005; Chen *et al.*, 2009), our results suggest that only a limited part of this antibiotic-devoted genetic background may be expressed readily in rhizosphere. Whether such differential cLP production is specific for S499 or is conserved upon colonization of other plants than tomato remains to be established. However, this work contributes to enhancing our knowledge on *Bacillus* fitness in natural living conditions regarding its antibiotic-producing potential. This certainly represents an essential step for improving the level and reliability of the efficacy of such beneficial strains as biological agents for the control of plant diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. S499 strain identification.
Appendix S2. Optimization of LC-ESI-MS analysis for determination of lipopeptide pattern produced by strain S499 *in vitro.*

Appendix S3. TOF-SIMS imaging analysis of authentic standards (mixtures of homologues) of the three lipopeptide families.

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