RESEARCH ARTICLE

Role of secondary metabolites in the interaction between *Pseudomonas fluorescens* and soil microorganisms under iron-limited conditions

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One sentence summary: *Pseudomonas fluorescens* produces several secondary metabolites in its interactions with soil fungi and bacteria, and shifts from being mutualistic to antagonist under iron-limited conditions.

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ABSTRACT

Microorganisms can be versatile in their interactions with each other, being variously beneficial, neutral or antagonistic in their effect. Although this versatility has been observed among many microorganisms and in many environments, little is known regarding the mechanisms leading to these changes in behavior. In the present work, we analyzed the mechanism by which the soil bacterium *Pseudomonas fluorescens* BBc6R8 shifts from stimulating the growth of the ectomycorrhizal fungus *Laccaria bicolor* S238N to killing the fungus. We show that among the three secondary metabolites produced by the bacterial strain—the siderophores enantio-pyochelin and pyoverdine, and the biosurfactant viscosin—the siderophores are mainly responsible for the antagonistic activity of the bacterium under iron-limited conditions. While the bacterial strain continues to produce beneficial factors, their effects are overridden by the action of their siderophores. This antagonistic activity of the strain *P. fluorescens* BBC6R8 in iron-depleted environments is not restricted to its influence on *L. bicolor*, since it was also seen to inhibit the growth of the actinomycete *Streptomyces ambofaciens* ATCC23877. We show that the strain *P. fluorescens* BBc6R8 uses different strategies to acquire iron, depending on certain biotic and abiotic factors.

Keywords: mutualism; antagonism; mycorrhiza helper bacteria; iron; siderophores; viscosin

INTRODUCTION

Historically, microorganisms have been classified according to their effects on other organisms as pathogens, antagonists, parasites, mutualistics and commensals. While some microorganisms, such as biotrophic pathogens and symbionts, have a strict and specific lifestyle, most are versatile in their behaviors and can vary from antagonistic to neutral or beneficial and vice versa depending on various biotic and abiotic parameters. Opportunistic pathogens are often commensal microorganisms that take advantage of the weaknesses of their host defenses. For example, the opportunistic human pathogen *Candida albicans* is a commensal microorganism, commonly found as part of the normal flora of the digestive track (Jenkinson and Douglas 2002). Similarly, saprophytic fungi such as *Alternaria* or *Fusarium* that normally live on dead organic matter can become pathogenic when they encounter debilitated, stressed or senescent plants (Prell and Day 2001). Most documented cases of opportunistic infections are related to deficiencies of the immune system of the host, whether a plant or animal (Whipps 2001; Berg, Eberl and Hartmann 2005). Less is known regarding the potential shift of behavior of mutualistic microorganisms during their lifetime. In the present work, we address this point by focusing on the interaction between an ectomycorrhizal (EcM) fungus and a mycorrhiza helper bacterium.

EcM fungi establish a symbiotic relationship with trees in which they provide water and inorganic nutrients for their host in exchange for sugars (Smith and Read 2008). These exchanges take place in ectomycorrhiza, a mixed organ composed of root and fungal tissues. EcM symbiosis is widespread across the world, with most trees from temperate and boreal forests having been found to evidence its working. EcM symbiosis is not restricted to the mutualistic association between symbiotic fungi and plant roots, since mycorrhizal root tips are colonized by a complex microbiome with which plant roots and EcM fungi interact (Deveau 2016). Within this complex microbiome, some bacteria, called mycorrhiza helper bacteria (MHB), can be beneficial in stimulating the formation or function of mycorrhiza (Frey-Klett, Garbaye and Tarkka 2007; Deveau and Labbé 2016). A number of genera of both Gram-negative and Gram-positive bacteria constitute the range of MHB. Mechanisms of action differ between strains and can involve spore germination, or stimulation of pre-symbiotic growth, plant defenses or short root production (Frey-Klett, Garbaye and Tarkka 2007). MHB have aroused the interest of the scientific community due to their potential to improve health and growth of tree seedlings (Garbaye 1994). However, despite several significant successes of field inoculations of MHB to improve mycorrhiza formation and tree growth (Garbaye and Bowen 1989; Poole *et al.* 2001; Founoune *et al.* 2002; Frey-Klett, Garbaye and Tarkka 2007; Zhao *et al.* 2014), no MHB is currently routinely used to inoculate tree nurseries, and none has been commercialized yet. This is partly due to the seeming adaptive nature of these strains where, for reasons largely unknown, their beneficial effects are not always observed. Thus, they can be good models to study the mechanisms by which a beneficial strain can shift to being either harmless or harmful towards other organisms. In our own work, we applied the EcM fungus *Laccaria bicolor* S238N and the MHB strain *Pseudomonas fluorescens* BBc6R8 as model organisms.

Pseudomonas fluorescens BBc6R8 is an MHB strain that has been isolated from a sporocarp of *L. bicolor* and which promotes the establishment of EcM symbiosis between *L. bicolor* and Douglas fir (Frey-Klett, Pierrat and Garbaye 1997). Its MHB effect is found in its ability to stimulate the survival and the pre-symbiotic growth of *L. bicolor* S238N mycelium under unfavorable growth conditions, notably thanks to nutrient complementation (Brulé *et al.* 2001; Deveau *et al.* 2010). This MHB strain also appears to benefit from the interaction with the fungus, since its survival in the soil is increased in the presence of the EcM fungus (Deveau *et al.* 2010). However, the helper effect of the strain *P. fluorescens* BBc6R8 is lost when the bacterial strain is inoculated at elevated concentrations (Frey-Klett *et al.* 1999). The objective of the present work was to decipher the mechanism by which *P. fluorescens* BBc6R8 shifts from having a beneficial influence towards an antagonist one. In addition, we analyzed the specificity of this antagonistic activity through the analysis of the interaction between *P. fluorescens* BBc6R8 and the soil bacterium *Streptomyces ambofaciens* ATCC23877.

MATERIALS AND METHODS

Fungal–bacterial and bacterial–bacterial confrontation bioassays

All strains used in this work are listed in Table 1. The EcM basidiomycete *L. bicolor* S238N was maintained on Pachlewski agar medium P5 (Di Battista *et al.* 1996) at 25°C for 3 weeks. *Pseudomonas fluorescens* BBc6R8 WT and the mutants $\Delta viscB$, Tn::foxA, 7B3 and 7C3 were stored at –80°C in LB Medium (Sambrook, Fritsch and Maniatis 1989) with 20% glycerol added and kanamycin (50 μg mL^{-1}) when appropriate. To prepare the bacterial inoculum for the in vitro bioassays with *L. bicolor*, the bacterial strains were first grown on 10% TSA plates (3 g L^{-1} Tryptic Soy Broth from Difco and 15 g L^{-1} of agar) supplemented with kanamycin when appropriate at 25°C for 65 h. The bioassay was prepared as described in Deveau *et al.* (2007), except that King's B (KB) agar medium (20 g L^{-1} proteose peptone #3 from Difco, 1.5 g L^{-1} K$_2$HPO$_4$, 15 g L^{-1} of agar, supplied after autoclaving with 10 mL L^{-1} 100% glycerol and 6 mL L^{-1} MgSO$_4$ 1M) was used instead of P20Th- for iron starvation conditions. Briefly, four droplets of bacterial solution of a single strain (OD$_{600 nm}$ 0.7 in sterile water) were distributed at 1.2 cm from the center of a fungal plug. Control treatments in which bacteria were replaced by sterile water droplets were performed. Plates were incubated in the dark at 10°C to reproduce soil temperature. The diameter of fungal colonies was measured every 5–8 days. For each treatment, seven replicates were performed. The full experiment was performed four times independently.

The *S. ambofaciens* ATCC23877–*P. fluorescens* BBc6R8 interaction was analyzed using the following setup. Five-microliter drops of *S. ambofaciens* (10^9 spores mL^{-1}) and of *P. fluorescens* BBc6R8 (10^9 cfu mL^{-1}) were deposited 4 mm apart on KB agar plates and were incubated at 28°C for 24–72h. Control experiments with *P. fluorescens* BBc6R8 or *S. ambofaciens* alone were performed for each set of incubation conditions. The full experiment was performed three times independently.

Transposon mutagenesis of P. fluorescens BBc6R8 and screen of the library for the loss of siderophore production

Transposon mutagenesis was carried using the transposon mini Tn5::*gfp*::*lux* cloned into pGP704 (Fones *et al.* 2010). The transposon was introduced into *P. fluorescens* BBc6R8 by triparental mating using the helper plasmid pRK2013. The resultant bacterial mixture was spread onto M9 agar containing 50 μg mL^{-1} kanamycin. Resulting colonies were inoculated into 100 μL of KB broth in 42 96-well plates and incubated at 28°C overnight.

Table 1. List of strains used in this study.

Strains	Characteristics	References
L. bicolor S238N	EcM basidiomycete fungus	Deveau *et al.* (2007)
S. ambofaciens ATCC23877	Reference strain isolated from soil	Pinnert-Sindico (1954)
P. fluorescens BBc6R8	Mycorrhiza helper bacterium isolated from sporocarps of *L. bicolor* S238N. Wild-type strain	Frey-Klett, Pierrat and Garbaye (1997)
P. fluorescens BBc6R8 Tn5::foxA	Mutant derived from *P. fluorescens* BBc6R8, Tn5 insertion in *foxA*	Galet *et al.* (2015)
P. fluorescens BBc6R8 7B3	Mutant derived from *P. fluorescens* BBc6R8 by Tn5 insertion	This study
P. fluorescens BBc6R8 7C3	Mutant derived from *P. fluorescens* BBc6R8 by Tn5 insertion	This study
P. fluorescens BBc6R8 Δ*viscB*-(P448) Δ*viscB*-(P450)	Knock-out isogenic mutants of gene *viscB* derived from *P. fluorescens* BBc6R8	This study
P. tolaasii LMG 2342[T]	White line agar assay indicator strain	Wong and Preece (1979)
P. reactans LMG5329	WLIP producer	Wong and Preece (1979)
P. fluorescens SBW25	Viscosin producer	de Bruijn *et al.* (2007)

A total of 75 μL of 50% (v/v) glycerol were then added to each well, and plates were stored at –80°C as a mutant library.

Mutants for siderophore production were screened by replication of the Tn5 bacterial library on KB plates. After 48 h of growth at 28°C, plates were observed under UV light, and colonies that did not produce fluorescence were selected.

Direct mutagenesis of *P. fluorescens* BBc6R8 *viscB* gene

Since *viscB* is a very large gene (12.9 kb), partial as opposed to full deletion of the gene 2562998405 (locus MHB_001840; Deveau *et al.* 2014) was carried out (between positions 146 and 7113), with a stop codon introduced at the site of the deletion. The deletion was created using the two-step allelic exchange strategy described by Zhang and Rainey (2007) and previously used to generate T3SS secretion mutants in *P. fluorescens* BBc6R8 (Cusano *et al.* 2011). Flanking regions were cloned into the suicide integration vector pUIC3 using the In-fusion BioBrick assembly system (Clontech, TakaraBio Europe, Saint Germain en Laye, France). Cycloserine enrichment was used to enrich strains that had lost the chromosomally integrated pUIC3-based vector. Strains were grown overnight in 100 mL LB broth; 500 μL of the overnight culture were inoculated into 30 mL pre-warmed LB broth and cultivated at 28°C with shaking (200 rpm) for 2 h. Tetracycline (Tc) was added at the final concentration of 10 μg mL^{-1} to inhibit the growth of cells that had lost pUIC3. After growth for 3 h, cycloserine was added at 3 mg mL^{-1} and growth was continued for another 3 h (during this step the growing Tc-resistant cells were killed). The cells were then washed in sterile water, diluted and inoculated onto M9 plus X-Gal plates. Double recombinants were verified by loss of lacZY activity, loss of Tc resistance, PCR screening for recombinants in which the deletion had occurred and finally sequencing across the deleted region. Two confirmed mutants, designated Δ*viscB*-(P448) and Δ*viscB*-(P450), were chosen for use in subsequent assays to control for possible non-specific changes (such as point mutations elsewhere in the genome) which could potentially have been introduced during the mutagenesis procedure and affected bacterial fitness.

Extraction, isolation and identification of enantio-pyochelin and desferrioxamine E

Pseudomonas fluorescens BBc6R8 and *S. ambofaciens* ATCC23877 were grown on separate KB agar plates at 26°C. After 72 h of incubation, cells and agar media were recovered and transferred to glass scintillation vials. One milliliter of ethyl acetate/methanol/formic acid (65:35:0.1, v/v) were added to the samples. Samples were then sonicated for 10 min. The extraction procedure was repeated three times. The resulting crude extracts were centrifuged at 10 000 rpm for 2 min. The supernatants were recovered and dried *in vacuo*. Samples were then resuspended in 100 μL of methanol for further analysis. Samples were directly infused into the mass spectrometer using a Triversa nanomate-electrospray ionization source (Advion Biosystems, Ithaca, NY, USA) coupled to a 6.42 T Thermo LTQ-FT-ICR mass spectrometer (Thermo-Electron Corporation, San Jose, CA, USA). FT-MS and ion trap MS/MS spectra were acquired using Tune Plus software version 1.0 and Xcalibur software version 1.4 SR1 (Thermo-Electron Corporation). The instrument was tuned on *m/z* 816, the 15+ charge state of cytochrome C.

Extraction, isolation and identification of pyoverdine

Pseudomonas fluorescens BBc6R8 was grown in 1.5 L of Davis Minimum Broth without dextrose (Becton, Dickinson and Company), supplemented with 20 mmol glycerol (DMBgly) with shaking (130 rpm) at 28°C for 90 h. Cells were removed by centrifugation (20 min, 4200 *g*, 4°C). The pH of the supernatant was adjusted to 6, Amberlite XAD-4 adsorbent resin (50 g/L) was added and the mixture was agitated for 4 h at 70 rpm at 28°C. The XAD-4 resin was then passed through a fritted funnel, and the bound metabolites were eluted with MeOH/H$_2$O (50:50) under agitation, for 4 h. The combined eluates were dried using a rotary evaporator, resuspended in 1 mL of deionized water and a second chromatography step was performed on an open column of Sephadex LH-20 (15 g). After loading, the column was rinsed with deionized water and colored bands were systematically collected. Four fractions were obtained, lyophilized and subsequently analyzed by LC/MS. The LC/MS system consisted of an Agilent HPLC 1100 Series system coupled to an AB Sciex 3200 Q TRAP mass spectrometer. The HPLC separation was carried out on reversed phase using a linear gradient of 2:98–100:0 MeOH/H$_2$O over a period of 30 min, followed by isocratic elution with 100% MeOH for 30 min (Macherey-Nagel Nucleodur PolarTec column; 250 × 4.6 m; particle size 5 μm; flow rate 1 mL min^{-1}; UV-DAD monitoring).

Extraction, isolation and identification of viscosin

Pseudomonas fluorescens BBc6R8 was grown with shaking (140 rpm) at 26°C in six 5 L Erlenmeyer flasks, each containing 1.5

L of DMBgly. After 72 h of incubation, cell and supernatant fractions of the cultures were separated by centrifugation at 4000 rpm for 10 min. The supernatant was acidified to pH 3 with HCl and extracted twice with ethyl acetate (1:1, v/v). The resulting crude extract was fractionated by vacuum liquid chromatography over reversed-phase silica gel (Macherey-Nagel Polygoprep 50–60) using a stepwise gradient of methanol–water and finally dichloromethane to give six fractions. ^{1}H NMR profiling of these fractions indicated the 100% MeOH fraction to be of further interest because its ^{1}H NMR spectrum exhibited three clusters of resonances typical of peptides, i.e. downfield amide signals, α- proton resonances and upfield side chain protons. RP-HPLC separation of this fraction using a Waters HPLC system (consisting of a 600 pump, a 996 photodiode array detector, a Rheodyne 7161 injector and a Perkin Elmer Series 200 vacuum degasser) and gradient elution from 80%–100% MeOH in H_2O (0.05% TFA) over 45 min, followed by isocratic elution at 100% MeOH for an additional 10 min (column: Phenomenex Luna C18(2)-100A, 10 × 250 mm, 5-μm column in combination with a Phenomenex SecurityGuard Luna-C18(2) 10 × 10 mm pre-column; 2.0 mL min^{-1} flow rate; UV monitoring at 215 nm) gave viscosin in a semi pure form. The compound was subsequently purified twice by RP-HPLC; first by employing a Macherey-Nagel Nucleosil 120–5, 4 × 250 mm column (1.0 mL min^{-1}, 40 min gradient elution from 60% to 100% MeOH-H_2O (0.05% TFA) followed by a 20-min hold) and second by using a Waters SymmetryShield RP18, 4.6 × 250 mm column (1.0 mL/min, 40 min gradient elution from 50% to 100% ACN-H_2O (0.05% TFA) followed by a 20-min hold) to yield 13.5 mg of pure viscosin: ESI-TOF-MS: [M+H]$^+$ = 1126.7, [M+Na]$^+$ = 1148.7; HR-ESI-TOF-MS: [M+Na]$^+$ = 1148.6798 *m/z* consistent with the molecular formula $C_{54}H_{95}N_9O_{16}$Na. HR-ESI-TOF-MS spectra were recorded on a Bruker Daltonic's micro TOF-Q instrument. NMR experiments were carried out on a Bruker Avance 300 DPX and on a Bruker DRX-250 spectrometer, respectively. Spectra were referenced to the residual solvent signal of d_4-MeOH with resonances at $\delta_{H/C}$ 3.35/49.0.

White-line-in-agar test

The white line test was performed according to the method of Wong and Preece (1979). *Pseudomonas fluorescens* BBc6R8 was streaked on KB medium at 1 cm distance next to *P. tolaasii* LMG 2342^T and the formation of a white precipitate in the agar was evaluated after 24–72 h growth at 26°C. *Pseudomonas reactans* LMG 5329 (WLIP producer) and *P. fluorescens* SBW25 (viscosin producer) (de Bruijn *et al.* 2007) were included as positive and negative controls, respectively. Assays were carried out in duplicate and repeated twice.

MALDI imaging mass spectrometry

The experiment was performed as previously described (Phelan *et al.* 2014). Briefly, a region of agar including the microorganisms grown alone or in interaction was excised with a razor blade and laid upon a MALDI MSP 96 anchor target plate (Bruker Daltonics). A photograph was taken of the MALDI plate holding the agar samples. Universal MALDI matrix (Sigma-Aldrich) was applied on top of the samples using a 53-μm sieve, and samples were dried at 37°C overnight. A second photograph was taken of the MALDI plate. All microbial colonies were subjected to MALDI-TOF MS in positive reflectron mode using a 400–500 μm spatial resolution in XY by a Bruker Daltonics Microflex. Data were analyzed using FlexImaging 3.0.

Statistical analyses

Statistical analyses were performed using the R software. Normality of datasets was tested using the Shapiro–Wilk test followed by appropriate tests depending on the normality of data.

RESULTS

Pseudomonas fluorescens BBc6R8 stimulates *L. bicolor* S238N radial growth *in vitro* on P20Th- medium but inhibits it on KB.

As previously reported (Deveau *et al.* 2007), the ability of *P. fluorescens* BBc6R8 to stimulate the growth of *L. bicolor* S238N (Brulé *et al.* 2001) can be reproduced *in vitro* when the two microorganisms are co-cultivated on P20Th- medium (Fig. 1A). However, the growth of *L. bicolor* was completely inhibited by the strain BBc6R8 when P20Th- medium was replaced by the iron-limited KB medium in a similar set up (Fig. 1B).

Identification of the secondary metabolites produced by strain BBc6R8 through genome mining and imaging mass spectrometry

In order to identify the metabolites potentially involved in this inhibitory effect, the genome sequence of strain *P. fluorescens* BBc6R8 (Deveau *et al.* 2014) was explored to identify gene clusters involved in the production of secondary metabolites. None of the genes commonly responsible for the production of secondary metabolites involved in *Pseudomonas* antagonism towards fungi and other eukaryotes, such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), pyrrolnitrin, pyoluteorin or rhizoxins (Loper *et al.* 2008, 2012; Gross and Loper 2009), was found in the genome of strain BBc6R8. However, complete gene clusters coding for the non-ribosomal peptide synthetases responsible for the biosynthesis of the two siderophores enantio-pyochelin (Youard *et al.* 2007) and a pyoverdine, and for the production of a cyclic lipopeptide, were found (Table 1, Supporting Information). In addition, a truncated gene cluster coding for enzymes involved in the production of mangotoxin was found, but it is expected to be non-functional due to the absence of the corresponding *mbo*-like operon (Carrión *et al.* 2012). The production of the two siderophores was confirmed by mass spectrometry. The MS2 profile of the ion with a precursor mass of *m/z* 325 [M+H]$^+$ completely matched with the fragmentation profile of enantio-pyochelin (Fig. 1, Supporting Information). The capability of strain BBc6R8 to produce a pyoverdine is in line with the bioinformatic analysis and was already proven in previous studies by UV- and MS-based experiments with the purified compound (Gamalero *et al.* 2003). At that time, a mass of 1305.7 Da was determined using ESI-MS. In this study, we detected this pyoverdine as a minor metabolite along with a suite of four major pyoverdine derivatives, ranging in size from 1276.5 to 1306.6 Da (Fig. 2A, Supporting Information). Empirically, pyoverdines of one strain differ in the nature of the dicarboxylic acid side chain. So far glutamic acid, α-ketoglutaric acid, succinic acid (amide) and malic acid (amide) were found (Budzikiewicz 2004) and the relative amounts of the various side chains of a pyoverdine depend on the cultivation time, iron concentration and pH of the culture broth (Schäfer, Tarak and Budzikiewicz 1991). Therefore, the occurrence of the compounds can be readily attributed to the presence of the succinic acid amide (*Pyo-Suca*, *m/z* 1277.7 [M+H]$^+$), succinic acid
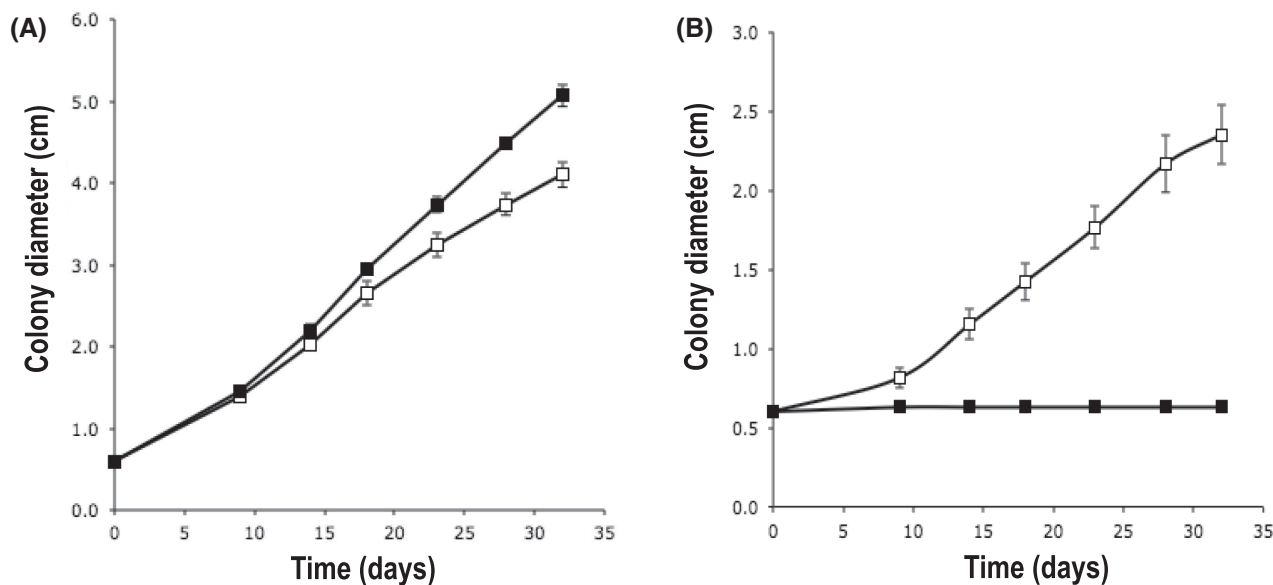
Figure 1. Effect of *P. fluorescens* BBc6R8 on the radial growth of *L. bicolor* S238N on P20Th- (**A**) and KB (**B**) agar media. Open squares: *L. bicolor* S238N alone, filled squares: *L. bicolor* S238N co-cultivated with *P. fluorescens* BBc6R8. Each point is the mean value of seven biological replicates ±SD. The experiment was reproduced three times independently and similar results were obtained each time.

(*Pyo-Suc*, *m/z* 1278.7 [M+H]$^+$), malic acid (*Pyo-Mal*, *m/z* 1295.8 [M+H]$^+$), α-ketoglutaric acid (*Pyo-Kgl*, *m/z* 1306.6 [M+H]$^+$) and glutamic acid (*Pyo-Glu*, *m/z* 1307.7 [M+H]$^+$) congeners (Fig. 2A, Supporting Information). All pyoverdine derivatives showed the pyoverdine-characteristic UV-absorption maxima at 365 and 380 nm (Fig. 2B, Supporting Information), which result from their common fluorescent chromophore (Abdallah 1991) and indicated in addition that they were detected as deferrated free pyoverdines. Lastly, the structure elucidation of the lipopeptide was obtained by NMR and MS analyses. The purified lipopeptide gave an [M+Na]$^+$ peak at *m/z* 1148.6798 in the high-resolution mass spectrum (Fig. 3, Supporting Information), appropriate for a molecular formula of $C_{54}H_{95}N_9O_{16}$ which corresponded according to a database search to the structures of either viscosin or WLIP. These two structures represent epimers, i.e. the only structural difference between viscosin and WLIP is the D versus L configuration of one leucine residue. The white-line-agar assay was used to discriminate between the two lipopeptides. The absence of a white line in this assay when BBc6R8 was grown alongside *P. tolaasii* supports the deduction that the lipopeptide of BBc6R8 is viscosin (Fig. 4, Supporting Information) because WLIP is known to produce a white precipitate in combination with tolaasin, whereas viscosin does not (Wong and Preece 1979). Furthermore, the 1D-NMR experiments confirmed the overall lipopeptidic nature of the isolated compound (Fig. 5, Supporting Information), and demonstrated that the observed chemical shift values were in good agreement with the reported ^{1}H NMR data for viscosin (Laycock *et al.* 1991).

Next, imaging mass spectrometry (IMS) was used to follow the production of enantio-pyochelin, pyoverdine and viscosin during the interaction between *L. bicolor* S238N and *P. fluorescens* BBc6R8 on P20Th- and KB medium. None of the three compounds was detected when the microorganisms were cultured alone or together on P20Th- medium (data not shown). In contrast, the three compounds were produced by the bacterial strain on KB medium when the bacterium was cultivated alone or in the presence of *L. bicolor* S238N (Fig. 2).

Both enantio-pyochelin and viscosin were seen diffusing from the bacterial colonies into the external medium as soon as 24 h post-inoculation, and reached the fungal colonies at 72 h.

Laccaria bicolor S238N is not sensitive to viscosin but to siderophores

Viscosin and other similar cyclic lipopetides have been reported to have antibiotic and antifungal activities (Raaijmakers *et al.* 2010). To determine whether viscosin could be responsible for the inhibitory effect of BBc6R8, two knock-out isogenic mutants were constructed by deletion of the *viscB* gene encoding for a non-ribosomal peptide synthetase. The non-polar deletions were checked by PCR, and the inability of the mutants to produce viscosin was confirmed by LC-MS (data not shown). No difference in the inhibition of the growth of *L. bicolor* S238N was observed between the WT bacterial strain and the *viscB* mutants (Fig. 6, Supporting Information). In addition, to test whether viscosin can have an impact on *L. bicolor* S238N growth in conditions where no other secondary metabolite is produced, purified viscosin was added to P20Th- prior inoculation with *L. bicolor* S238N. Two concentrations were tested: one corresponding to the concentration measured in the extracellular medium of pure cultures of *P. fluorescens* BBc6R8 (1.5 mg L^{-1}) and a 10-fold higher concentration close to that produced by the soil biocontrol strain *P. fluorescens* SBW25 (de Bruijn *et al.* 2007). Strain SBW25 is phylogenetically closely related to strain BBc6R8 (Jun *et al.* 2016), and has been shown to induce lysis of zoospores of the oomycete *Phytophtora infestans* through the production of viscosin. A slight inhibition of the radial growth of *L. bicolor* S238N was measured (–12%, $P < 0.01$ Kruskal–Wallis test) at the highest concentration, but not at the level that is produced by BBc6R8 (Table 2). Thus, we conclude that *L. bicolor* S238N is very slightly sensitive to viscosin, and that the lipopeptide is not responsible for the growth inhibition observed on KB medium in the presence of *P. fluorescens* BBc6R8.
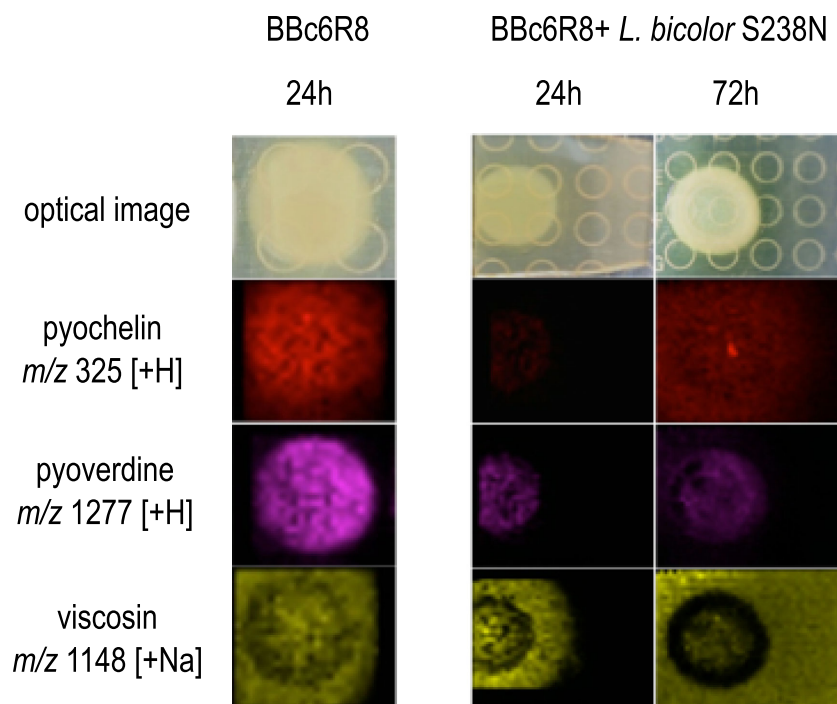
Figure 2. Microbial IMS images of secondary metabolites produced by *P. fluorescens* BBc6R8 grown alone (left images) and side by side with *L. bicolor* S238N (right images) on KB medium. *Laccaria bicolor* S238N was grown for 3 weeks on KB medium to allow hyphal development before the inoculation of *P. fluorescens* BBc6R8. Optical images are displayed in the top row, all other images are overlays of falsely colored *m/z* distributions over optical images.

Table 2. Effect of viscosin on the radial growth of *L. bicolor* S238N after 5 and 10 days of co-culture. Each value corresponds to the mean diameter of six biological replicates ±SD. 'a' indicates values which are statistically significantly different according to a Kruskal–Wallis test ($P < 0.01$) followed by a Mann–Whitney post-hoc test. dpi: days post-inoculation.

	Control (cm)	Viscosin 1.5 mg L^{-1} (cm)	Viscosin 15 mg L^{-1} (cm)
5 dpi	1.7 ± 0.08	1.6 ± 0.06	1.3 ± 0.08[a]
10 dpi	2.8 ± 0.06	2.8 ± 0.11	2.5 ± 0.1[a]

In the second step, we tested whether the production of siderophores by *P. fluorescens* BBc6R8 could inhibit the growth of the EcM fungus. To do so, we isolated two clones from a Tn5 mutant library of strain BBc6R8 that did not produce fluorescence under UV excitation. Neither pyoverdine nor enantio-pyochelin could be detected by IMS when the two mutants were grown on KB medium (Fig. 3A). The effect of the two clones on the growth of *L. bicolor* S238N was tested *in vitro* on iron-deficient and non-deficient media. Mutants 7B3 and 7C3 harbored the same phenotype as the wild type when confronted with *L. bicolor* S238N on P20Th- medium (data not shown). In contrast, none of the mutants inhibited the growth of *L. bicolor* S238N on KB medium, suggesting that competition for iron is responsible for the antagonistic effect of BBc6R8 (Fig. 3B). Unfortunately, we were unable to identify the position of the Tn5 insertion in the genome sequences of the mutant 7B3 and 7C3, for unknown technical reasons. Thus, we cannot exclude the possibility that mutants 7B3 and 7C3 have lost other functions in addition to siderophore production. Therefore, we tested whether competition for iron was responsible for the growth inhibition observed on KB medium by supplementing KB medium with iron(III) chloride (20 mg L^{-1})

and by monitoring the radial growth of *L. bicolor* cultivated alone or together with BBc6R8 WT. If competition for iron is involved in the inhibition of growth of *L. bicolor* in the presence of *P. fluorescens* BBc6R8, then the addition of iron should suppress this inhibition. Under these conditions, BBc6R8 no longer inhibited the radial growth of *L. bicolor* S238N, but rather stimulated it (+16% after 9 days of incubation, t-test $P < 0.05$). Altogether, our data suggest that the production of siderophores is very likely to be responsible for the inhibitory effect of BBc6R8. In addition, our data suggest that *P. fluorescens* BBc6R8 also produces beneficial compounds on KB medium, but that their beneficial effect is masked by the antagonistic activity of the siderophores.

Siderophores of *P. fluorescens* BBc6R8 also inhibit the growth of *S. ambofaciens* ATCC23877 on KB medium

We demonstrated in a previous study that *P. fluorescens* BBc6R8 ceases production of pyoverdine and enantio-pyochelin when desferrioxamine and coelichelin, two siderophores produced by the actinomycete *S. ambofaciens*, are present in the environment (Galet *et al.* 2015). Instead of using its own siderophores to scavenge iron, *P. fluorescens* BBc6R8 captures *S. ambofaciens* siderophores thanks to the receptor FOXA (Galet *et al.* 2015). This observation was made when the two microorganisms were cultivated side by side on 26A agar medium (Galet *et al.* 2015). However, when we replaced 26A agar medium by KB agar medium using the same co-cultivation assay, *P. fluorescens* BBc6R8 continued to produce enantio-pyochelin and pyoverdine in the presence of *S. ambofaciens* as indicated by IMS (Fig. 4A). *Streptomyces ambofaciens* also produced a siderophore—desferrioxamine E—when grown alone on KB medium (Fig. 4B). The identity of desferrioxamine E was confirmed by tandem mass spectrometry (Fig. 7, Supporting Information). A very low amount of desferrioxamine E was detected when the two
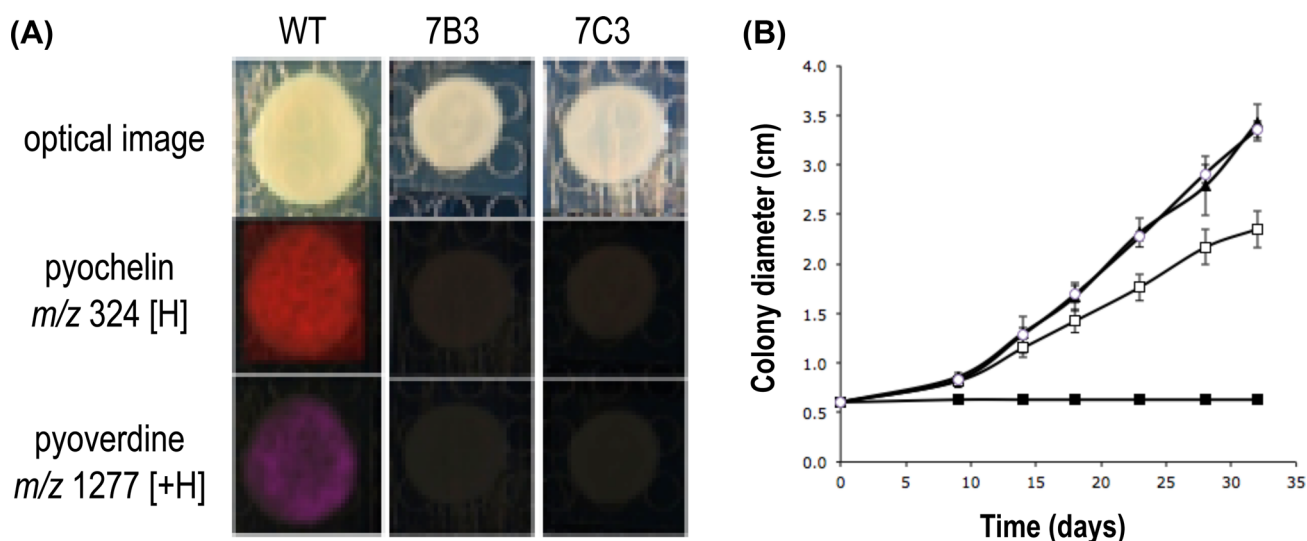
(A)

(B)

Figure 3. Suppression of pyochelin and pyoverdine production in *P. fluorescens* BBc6R8 and its effects on the interaction with *L. bicolor* S238N. **(A)** IMS images of secondary metabolites produced by *P. fluorescens* BBc6R8 WT and by the mutants 7B3 and 7C3 grown on KB medium for 24 h. Similar results were obtained at 72 h and 7 days. Optical images are displayed in the top row, all other images are overlays of falsely colored *m/z* distributions over optical images. **(B)** Effects of *P. fluorescens* BBc6R8 WT and of the mutants 7B3 and 7C3 on the radial growth of *L. bicolor* S238N on KB agar medium. Open squares: *L. bicolor* S238N alone, closed squares: *L. bicolor* S238N co-cultivated with *P. fluorescens* BBc6R8 WT, closed triangles: *L. bicolor* S238N co-cultivated with 7B3, open circles: *L. bicolor* S238N co-cultivated with 7C3. Each point is the mean value of seven biological replicates ±SD. The experiment was reproduced three times independently and similar results were obtained each time.

microorganisms were co-cultured on KB medium (Fig. 4B). This reduction of desferrioxamine E production was independent of siderophore piracy by FOXA and of the siderophore production by *P. fluorescens* BBc6R8, since both mutants Tn5::foxA and 7B3 induced the same phenotype as that of the WT strain (Fig. 4C). In addition to its effect on siderophore production, *P. fluorescens* BBc6R8 inhibited the development of *S. ambofaciens* colonies (Fig. 4A, C). This inhibition was not due to *S. ambofaciens* siderophore piracy by *P. fluorescens* BBc6R8, since the Tn5::foxA mutant similarly inhibited the growth of *S. ambofaciens* (Fig. 4C). In contrast, mutant 7B3 and 7C3 that lost the ability to produce siderophores did not seem to inhibit the growth of *S. ambofaciens*, suggesting that *P. fluorescens* siderophores are also responsible, at least in part, for the inhibition of the growth of *S. ambofaciens* on KB medium (Fig. 4C).

DISCUSSION

MHB have aroused interest due to their potential application in improving seedling growth and survival in tree nurseries (Frey-Klett, Garbaye and Tarkka 2007; Deveau and Labbé 2016), and to reduce the use of fertilizers in intensive tree culture systems such as short rotation coppices or Poplar culture (Zhao *et al.* 2014). However, the transition from laboratory to field use has not yet been successfully proven, partly because of the changeable effects of MHB. Their application requires an understanding of their mechanisms of action and a delineation of the precise conditions in which these bacteria exert their beneficial activities. Indeed, as most MHB are non-obligate mutualists, the outcome of the interaction with EcM fungi may be strongly influenced by external parameters. Here, we show that the beneficial activity of the MHB strain *P. fluorescens* BBc6R8 on the growth of the EcM fungus *L. bicolor* S238N *in vitro* is lost in iron starvation conditions. Determinants responsible for the stimulatory activity are most probably still produced during iron starvation, since mutants for siderophore synthesis recovered their ability to stimulate the growth of the fungus *in vitro*. However, these

beneficial determinants would be nullified by the deleterious impact of competition for iron. A similar pattern has been described for the MHB strain *Paenibacillus* sp. EJP73: the bacterial strain produces a mixture of compounds among which some inhibit the growth of the EcM fungus *Lactarius rufus*, while others are stimulatory (Aspray *et al.* 2013). The set of inhibitory compounds produced by the strain BBc6R8 is likely to be reduced, since genes linked to the synthesis of antibiotic compounds classically produced by *P. fluorescens* strains (e.g. DAPG, HCN, pyrrolnitrin, pyoluteorin, rhizoxins; Gross and Loper 2009) were absent from the genome of the MHB strain. In addition, the biosurfactant viscosin produced by the MHB strain BBc6R8 does not appear to be involved in its antagonistic activity, in contrast to other *Pseudomonas* strains (Laycock *et al.* 1991; de Bruijn *et al.* 2007; Alsohim *et al.* 2014). In this regard, the MHB strain BBc6R8 is very close to the plant growth-promoting rhizobacteria (PGPR) WGS358, 374 and 417 that also lack genes for antibiotic synthesis and rely solely on their high competiveness for iron to exert their PGPR activity (Berendsen *et al.* 2015).

The high efficiency of strain BBc6R8 in the capture of iron strongly contrasts with the limited ability of the fungus to achieve the same. *Laccaria bicolor* produces several siderophores of the hydroxamate family in the presence of low iron levels, the main representative being linear fusigen (Haselwandter *et al.* 2013). However, the quantity produced by the fungus is very low, since linear fusigen, as well as the two other siderophores produced by the fungus, ferricrocin and triacetylfusarinine, cannot be detected by LC-UV methods, and high-sensitivity LC-MS is required to detect them (Haselwandter *et al.* 2013). In accordance with this observation, we were not able to detect any of the *L. bicolor*-derived siderophores on KB medium by MALDI-IMS when grown alone or in the presence of *P. fluorescens* BBc6R8 (data not shown). In addition, Haselwandter *et al.* (2013) reported that the release of siderophores by *L. bicolor* into the medium is greatly delayed, only occurring after several weeks of growth in low-iron medium. This contrasts strongly with the behavior of *P. fluorescens* BBc6R8, which releases massive quantities of the high-affinity siderophore pyoverdine a few hours after
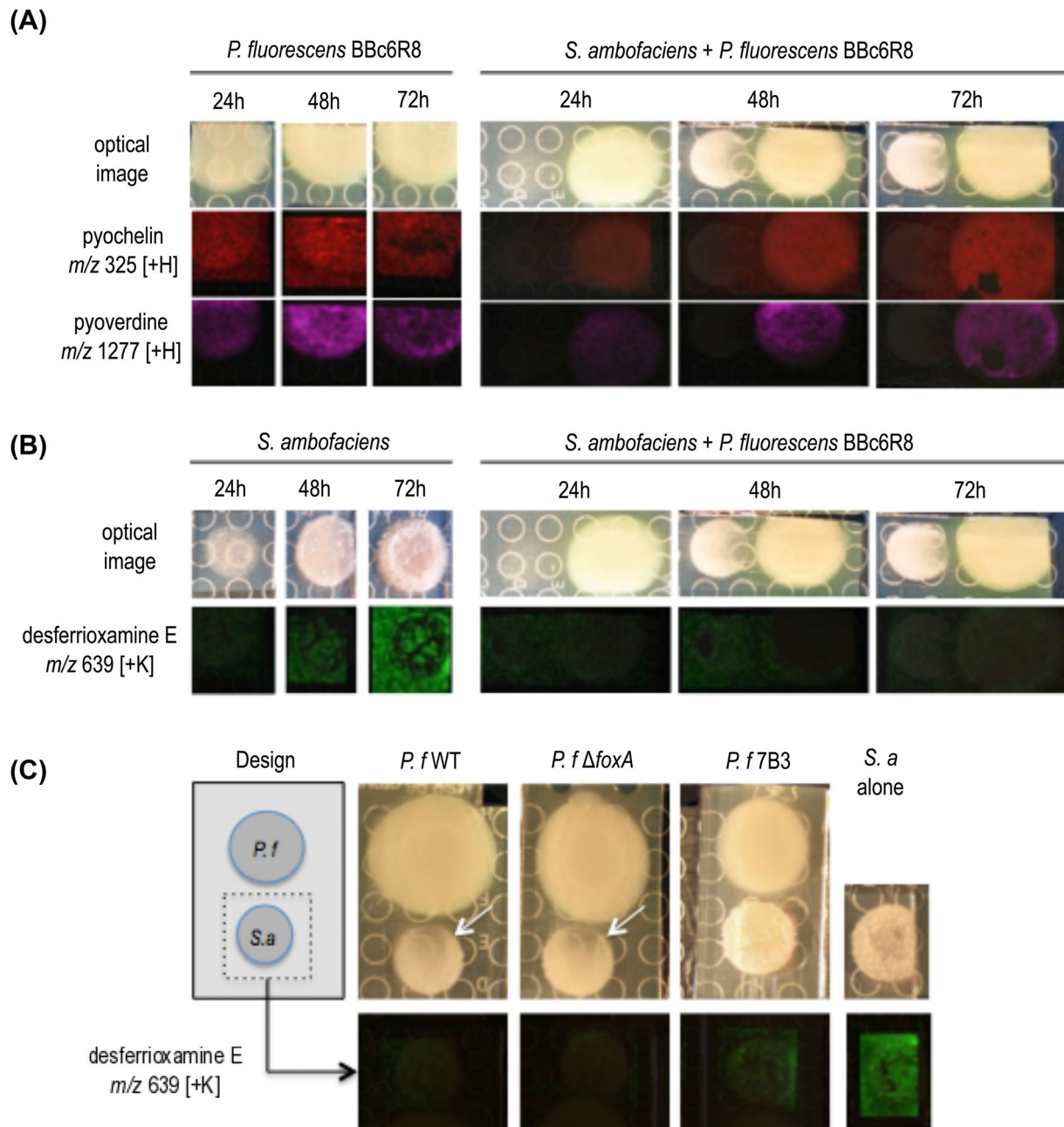
(A)

(B)

(C)

Figure 4. Microbial IMS images of secondary metabolites produced by *P. fluorescens* BBc6R8 (**A**) and *S. ambofaciens* ATCC23857 (**B**) when cultured alone or side by side on KB medium. Optical images are displayed in the top row, all other images are overlays of falsely colored *m/z* distributions over optical images. (**C**) Production of desferrioxamine E by *S. ambofaciens* ATCC23857 when cultured alone or side by side with *P. fluorescens* BBc6R8 WT or mutants Tn5::*foxA* or 7B3. The white arrows on the optical images point at the inhibition zone on the colony of *S. ambofaciens*.

encountering low-iron conditions (Galet *et al.* 2015). In the present case, we also observed an important production and diffusion of the low affinity siderophore enantio-pyochelin. In addition to the production of siderophores, the bacterium possesses a wide range of receptors to uptake xenosiderophores, among which hydroxamate siderophores are included (Galet *et al.* 2015). However, this potential piracy of fungal siderophores, if it exists, would probably play a minor role in the antagonistic activity of BBc6R8, as pyoverdine mutants recovered their stimulatory ability. Nevertheless, it is likely that most of the iron present is taken up by the bacterium, and that the fungus cannot obtain enough to sustain its own growth. Iron is essential

to the growth of the majority of microorganisms due to its involvement in a wide variety of housekeeping processes, such as respiration or DNA synthesis. Very few organisms can live in the absence of bioavailable iron by substituting iron with other transition metals such as cobalt, manganese or copper. Despite its relatively high abundance in soil, iron is generally poorly bioavailable to organisms, existing mainly in its ferric form which is insoluble in water and highly inaccessible to biological ligands (Kosman 2003). To overcome this problem, many microorganisms produce siderophores. Fe^{3+}-siderophore complexes are then taken up by cells through specific membrane receptors. Competition for iron is often a component of

antagonistic interactions between fungi and fluorescent pseudomonads (Cornelis and Matthijs 2002). It is one of the well-known mechanisms of biocontrol of plant root pathogenic fungi (Haas and Défago 2005). Pyoverdines can sequester iron from the rhizosphere and render it unavailable for the nutrition of rhizospheric fungi producing less potent siderophores. Our data suggest that this antagonism may not be restricted to phytopathogenic fungi, but that it could also affect plant mutualistic fungi. Whether the antagonism described here *in vitro* also occurs in natural soil remains to be tested. However, previous analyses regarding the availability of iron to *P. fluorescens* in the rhizosphere and in soil indicated that other *P. fluorescens* strains actively transcribe genes involved in siderophore production within hours after inoculation in the soil (Loper and Henkels 1997). We observed that mutants 7B3 and 7C3 poorly survived in soil compared to the WT (data not shown), thus suggesting that *P. fluorescens* also produces siderophores in soil. Frey-Klett *et al.* (1999) showed that high inocula of *P. fluorescens* BBc6R8 inhibited mycorrhiza formation, and they hypothesized that high bacterial density may lead to harmful competition. Competition for iron may be part of the antagonistic interaction occurring at high bacterial density in soil.

The high efficiency of *P. fluorescens* in scavenging iron does not only specifically affect the EcM fungus *L. bicolor* S238N but also the actinomycete bacterium *S. ambofaciens*. Therefore, we can expect that it has a broad influence in natural environments. Interestingly, *P. fluorescens* did not use the same strategy to obtain iron when it was co-cultured with *S. ambofaciens*, depending on the composition of the medium. In one case, *P. fluorescens* BBc6R8 massively produced its two siderophores, while in another instance, it stopped producing its own siderophores and captured the siderophores produced by *S. ambofaciens* thanks to its receptor for xenosiderophore FOXA (Galet *et al.* 2015). The behavior of *S. ambofaciens* in the presence of *P. fluorescens* also altered depending on the growth conditions: the actinomycete strongly reduced the production of the siderophores ferrioxamine in the presence of *P. fluorescens* on KB medium. The physiological consequences of such a reduction in the biosynthesis of desferrioxamine may not be limited to the acquisition of iron, since desferrioxamine production has been also linked to morphogenesis in *Streptomyces* (Lambert *et al.* 2014). The two media mainly differed in their source of sugar (glucose versus glycerol) and peptides (tryptone versus peptone). Whether these changes in behavior are related to nutrient quality or to other factors remains to be investigated. Nevertheless, this indicates that we cannot predict the behavior of bacterial strains in natural environments on the basis of their genomic contents, since a single strain has numerous strategies to solve a single issue, such as in the well-studied case of iron starvation, and the factors that determine which strategy is used await elucidation. This issue is not restricted to the environment, since the human opportunistic pathogen *P. aeruginosa* also uses multiple pathways to acquire iron during the infection of lungs (Konings *et al.* 2013) and adapts its strategies to acquire iron depending on the type of infection at hand (Cornelis and Dingemans 2013). Therefore, despite our extensive knowledge on the mechanisms of iron scavenging by microorganisms, a lot remains to be discovered regarding the regulation of the process *in situ*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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