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Pseudomonas fluorescens pirates both ferrioxamine and ferri-coelichelin siderophores from Streptomyces ambofaciens

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Running Head: P. fluorescens pirates Streptomyces siderophores

FOOTNOTES

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xenosiderophore, Streptomyces ambofaciens, Pseudomonas fluorescens, iron chelation, TonB-dependent receptor
Abstract

Iron is essential in many biological processes. However, its bioavailability is reduced in aerobic environments such as soil. To overcome this limitation, microorganisms have developed different strategies, such as iron chelation by siderophores. Some bacteria have even gained the ability to detect and utilize xenosiderophores, i.e. siderophores produced by other organisms. We illustrate an example of such an interaction between two soil bacteria, *Pseudomonas fluorescens* strain BBc6R8 and *Streptomyces ambofaciens* ATCC23877, which produce the siderophores pyoverdine and enantio-pyochelin and the siderophores desferrioxamines B, E and coelichelin, respectively. During pairwise cultures on iron-limiting agar medium, no induction of siderophore synthesis by *P. fluorescens* BBc6R8 was observed in presence of *S. ambofaciens* ATCC23877. Co-cultures with a *Streptomyces* mutant strain that produced either coelichelin or desferrioxamines, as well as culture in a medium supplemented with desferrioxamine B, resulted in the absence of pyoverdine production; however, culture with a double mutant deficient in desferrioxamines and coelichelin production did not. This strongly suggests that *P. fluorescens* BBbc6R8 utilizes the ferrioxamines and ferri-coelichelin produced by *S. ambofaciens* as xenosiderophores, and therefore no longer activates the production of its own siderophores. A screening of a library of *P. fluorescens* BBc6R8 mutants highlighted the involvement of the TonB-dependent receptor FoxA in this process: the expression of foxA and genes involved in the regulation of its biosynthesis was induced in presence of *S. ambofaciens*. In a competitive environment such as soil, siderophore piracy could well be one of the driving forces that determines the outcome of microbial competition.
Introduction

Bacteria detect, assimilate and integrate different environmental signals in order to better adapt to their habitat and to cope with changes in environmental conditions. Multiple signaling pathways allow them to communicate with each other within the same species or between different species (1). This can be achieved through the production and detection of diffusible molecules in the environment. In response to these interactions, the microorganisms have developed complex metabolic and physiological responses. One of the essential environmental factors vital for organisms is iron. It plays an essential role in many biological processes such as DNA synthesis, respiration and photosynthesis. Iron can adopt two different ionic forms Fe$^{2+}$ or Fe$^{3+}$. This property makes it an important player in the oxidation-reduction reactions in the cell. However, while iron is an abundant element on earth, its bioavailability is reduced in aerobic environments, as in soil. Ferric iron (Fe$^{3+}$) forms insoluble ferric hydroxides (solubility product of $\sim 10^{-39}$) in the presence of oxygen (2–4). Therefore, iron is a limiting factor for the growth of microorganisms.

To overcome the limitation of iron bioavailability, aerobic bacteria have developed several highly specialized strategies to acquire this metal from different sources. One of them consists in producing siderophores under conditions of iron deficiency (5). Siderophores are low molecular weight molecules (200 to 2000 Da) with diverse iron affinities and to date, more than 500 different chemical structures of siderophores have been identified. A single bacterial species can produce different siderophores although in general, not all are produced at the same time. For example, Pseudomonas aeruginosa is able to switch between the synthesis of its siderophores, pyoverdine and pyochelin, depending on environmental conditions. In a severe iron depletion environment, pyoverdine, the most effective but metabolically expensive siderophore, is produced. However in environments moderately depleted
in iron, pyochelin, a less metabolically expensive siderophore, is used to take up iron (6).

In addition to acquiring iron via specific receptors for their own siderophores, many bacteria possess uptake systems for xenosiderophores, i.e. siderophores produced by other organisms. Thus, in competitive environments like soil, this allows them to utilize exogenous siderophores, in a strategy known as siderophore piracy (7, 8). A study, published by Cornelis and Bodilis (2009), revealed that the majority of siderophore receptors are conserved in the different representatives of a species (the core receptor) while others are acquired by horizontal gene transfer. For example, *Pseudomonas fluorescens* SBW25 possess 23 other putative siderophore receptors in addition to the receptor of its own pyoverdine. This allows the bacterium to take up 19 heterologous pyoverdines from 25 different *Pseudomonas* isolates (9).

*Pseudomonas fragi*, which does not produce siderophores, is able to use enterobactin, pyoverdine and desferrioxamine B produced by the bacterial species *E. coli*, *P. fluorescens* or *P. aeruginosa* and *Pseudomonas stutzeri*, respectively (10).

Other genera of bacteria, like *Yersinia* (11), *Erwinia* (12), *Vibrio* (13), *Amycolatopsis* (14), are also able to detect and to take up xenosiderophores in addition to the use of their own siderophore. The wide distribution of xenosiderophore uptake genes in many bacteria species suggests that siderophore piracy is a common process in multispecies communities. However, most of these studies were performed *in vitro* using purified siderophores and little is known regarding the occurrence of siderophore piracy during biotic interactions.

Actinomycetes and Pseudomonads represent two of the major groups of bacteria found in soils and rhizospheres (15, 16) and are likely to simultaneously utilize similar resources such as iron or even to compete for them. Both groups produce siderophores to take up this essential element but different types of molecules are
produced by the two groups. Many Pseudomonads produce pyoverdines, a family of high-affinity catecholate-hydroxamate siderophores while the actinomycetes of the genus *Streptomyces*, for instance, secrete hydroxamate siderophores such as desferrioxamines and coelichelin. In this study, we show, through a pairwise interaction between the two soil bacteria *Streptomyces ambofaciens* ATCC23877 (17) and *Pseudomonas fluorescens* BBc6R8 (18, 19) that the *P. fluorescens* strain uses the Streptomycte’s siderophores and does not induce the production of its own siderophores pyoverdine and enantio-pyochelin in the presence of *S. ambofaciens* ATCC23877 when grown on iron limiting conditions. Our study reveals that *P. fluorescens* recognizes *Streptomyces* siderophores through FoxA, a TonB-dependent receptor, whose biosynthesis is induced in the presence of *S. ambofaciens* ATCC23877 siderophores.

**Materials and methods**

**Bacterial strains, media and culture conditions**

All *Streptomyces*, *Pseudomonas* and *Escherichia coli* strains used in this work are listed in Table 1. *Streptomyces* strains were manipulated as described by Kieser et al. (20). *Pseudomonas* cell stocks were prepared by streaking each strain on Trypticase soy agar (TSA) medium (containing kanamycin at 20 μg/ml for the P18B10 and P28H6 mutants) and incubating plates for 48 hours at 26.5°C or at 37°C for *P. aeruginosa* PA01. A single clone was resuspended in 50 μl water and spread on TSA medium then grown for 48 hours. Cells were collected, centrifuged at 13,500 g for 2 minutes and the pellet was washed twice with sterile water. The pellet was then resuspended with sterile water to an OD₆₀₀ 0.7 (10⁹ Colony-Forming Units -CFU-per ml).
To analyse the effect of the *S. ambofaciens* ATCC23877 strain on the production of siderophores by *P. fluorescens* BBC6R8, a bioassay was set up on 26A agar medium (for 400 mL, Glucose: 0.4 g; Tryptone: 6 g; NaCl: 2 g; pH 7.2). *Streptomyces* and *Pseudomonas* were streaked side by side at 3 mm apart from each other and were incubated at 26.5°C (37°C with *P. aeruginosa* PA01) during 2 days. The streaks were made of 2 μl of a spore suspension of *S. ambofaciens* ATCC23877 at 10^9 CFU/ml and 2 μl of *Pseudomonas* at 10^7 CFU/ml. A control experiment with *Pseudomonas* alone was done for each incubation condition. When mentioned, the iron chelator 2,2'-bipyridyl (200 μM), desferrioxamine B mesylate (200 μM) or FeSO₄·7H₂O (12.5 μM) were added in 26A agar medium. For fluorescence detection, a Chemidoc XRS (Biorad) was used with UV transmission (302 nm).

To test the ability of the *S. coelicolor* ΔdesΔcch mutant (strain W13) to take up exogenous siderophores, two different experiments were carried out. The first experiment was as follows: 100 μl of a spore suspension of *S. coelicolor* M512 at 2x10^8 CFU/ml or 100 μl of a cell suspension of *P. fluorescens* BBC6R8 at 2x10^7 CFU/ml were spread on 26A agar plate supplemented or not with 2,2'-bipyridyl at 100 or 200 μM. After 3 days of growth at 26.5°C, plugs (8 mm of diameter) were obtained from confluent regions and then placed on 26A agar plates containing 200 μM of 2,2'-bipyridyl and evenly spread with spores of the W13 strain (10^6 or 10^9 CFU). Plates were incubated at 26.5°C for 3 days. The halo of growth around the plugs was then analyzed. In the second experiment, the M512 and BBC6R8 strains (10^4 CFU) were streaked on both side of a 26A agar plate supplemented with 200 μM 2,2'-bipyridyl. Plates were incubated at 26.5°C for 3 days and the W13 mutant (2 μl at 10^9 CFU/ml) was then streaked at about 3 mm alongside the M512 or BBc6R8 strains.
DNA manipulation and transcriptional analysis

Isolation, cloning, and manipulation of DNA were carried out as previously described for *Streptomyces* (21, 22), *Pseudomonas* (23) and *E. coli* (24). Amplification of DNA fragments by PCR was performed with Dreamtaq DNA polymerase (Fermentas). All primers are described in Table 2. Transposon insertion site in *P. fluorescens* BBc6R8 was determined using a double-round nested PCR-based sequencing approach. First round PCR was performed using the primers gfptns2, nCEKG2A, nCEKG2B, nCEKG2C with a ratio of 3:1:1:1. PCR conditions were as follows: 94°C for 5 minutes, followed by cycles of 94°C for 30 seconds, 62°C for 30 seconds lowered in successive cycles by 1°C until reaching 54°C and 72°C for 3 minutes. A further 27 cycles annealing at 54°C were performed and followed by a final extension of 72°C for 3 minutes. A second round PCR was performed using the primers gfptns2 and CEG4, followed by sequencing using the primer gfptnS2.

For RNA extraction, cells were collected from streaks grown on 26A agar medium for 2 days at 26.5°C. *Streptomyces* was grown on cellophane membranes. The extraction was performed with Aurum™ Total RNA Mini kit (Biorad) according to the manufacturer's instructions, except for the addition, during cell lysis, of a sonication step (3x 10 s) at high frequency using the Bioruptor apparatus (Diagenode). RNAs were eluted in a final volume of 30 µl and quantified with the NanoDrop-1000 apparatus. Before reverse transcription, RNA samples were treated with Dnase I (1 U of Dnase I (Fermentas) in presence of 10 U of Ribolock Rnase Inhibitor (Fermentas) per 1 µg of RNA). DNase I was then inactivated at 65°C during 10 min in presence EDTA (2.3 mM). The absence of residual genomic DNA was verified by a 35 cycle PCR using RNAs as templates and the primer pairs hrdB-F/hrdB-R and selR-F /selR-R for *Streptomyces* and *Pseudomonas*, respectively. Reverse transcription was performed with iScript Advanced cDNA synthesis kit for RT-qPCR (Biorad) according
to the manufacturer's instructions. The sequences of primer pairs used to amplify cDNAs and their target genes are listed in Table 2. Real-time quantitative PCR (qPCR) were carried out on CFX96 (Biorad) with microplates (Multiplate™ 69-Well Unskirted PCR plates, Low-Profil Biorad) covered with a film (Microseal'Bb'Adhesive Seals, Biorad). The reaction mixture was composed of 5 µl of SYBR Green Supermix (Biorad), 0.2 µM of each primer pairs and 4 µl of cDNA diluted 1/10. The qPCR conditions were as follow: 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C. To verify the absence of secondary products, melting curves were realized from 65 to 95°C with an increase of 0.5°C/cycle. Total RNA levels were normalized using transcripts from the housekeeping genes hrdB (25) for Streptomyces and selR (MHB_002629, (26)) for Pseudomonas as a control. The gene hrdB encodes the major sigma factor of Streptomyces and was used as an internal control to quantify the relative expression of target genes as it is expressed fairly constantly throughout growth (25, 27). selR was defined, with two other genes, as a potential housekeeping gene based on microarray data (accession number GSE38243 on the Gene Expression Omnibus at NCBI, (26)) and was chosen as a reference after checking the stability of its expression in the present setup. Both hrdB and selR transcripts levels were experimentaly confirmed to be stable in our growth conditions (data not shown).

For each treatment, at least three biological replicates were performed and the average and standard deviation of the relative expression to the reference genes hrdB or selR of each transcript were calculated (28). One factor ANOVA analyses were made with R to test for differences of transcript levels between treatments.
Construction of *S. coelicolor* M512 desD and cchH mutant strains

The cosmids C105.2.E01 and F76.2.F08 from the *S. coelicolor* transposon insertion single-gene knockout library (29) were used to replace the wild type alleles of desD and cchH, respectively, in *S. coelicolor* M512. Mutated cosmids were introduced in *S. coelicolor* M512 by intergenic conjugation from *E. coli* ET1567/pUZ8002 (30). Gene replacements were confirmed by PCR analysis using the flanking and the internal primers, desC-F and EZR1 for the mutant *S. coelicolor* M512 desD, sam0552-R and EZL2 for the mutant *S. coelicolor* M512 cchH.

Results

*P. fluorescens* BBc6R8 does not produce its siderophore pyoverdine in the vicinity of *S. ambofaciens* ATCC23877

*P. fluorescens* BBc6R8 produced a green-yellow pigment and fluoresced under UV when grown on iron depleted 26A agar medium (Fig. 1a, b). When the medium was supplemented with FeSO₄·7H₂O (12.5 μM), *P. fluorescens* BBc6R8 did not fluoresce anymore (Fig. 1d). This green-yellow pigmentation reflects the presence of the pyoverdine siderophore produced by *P. fluorescens* BBc6R8 under conditions of iron limitation (31). During the screening of pairwise interactions between *S. ambofaciens* ATCC23877 and other soil bacteria, we observed that when grown on 26A agar medium in close proximity to *S. ambofaciens*, *P. fluorescens* BBc6R8 partially lost its ability to fluoresce (Fig. 1c, e, f). Similar results were observed in the presence of the high affinity iron chelator 2, 2’-bipyridyl (200 μM) in the agar medium (Fig. S1). Interestingly, a stronger effect was observed when *S. ambofaciens* ATCC23877 was cultivated on 26A agar for 30 hours before the Petri dish was inoculated with the BBc6R8 strain. Under this condition, neither the green-yellow pigment nor the fluorescence could be observed (Fig. 1g). Similar results were obtained when 2,2’-
bipyridyl was present in the agar medium (Fig. S1). The negative effect on pyoverdine production was proportional to the lag between the inoculation of the two bacteria on the plate. In addition, streaking the bacterial partners perpendicularly to one another way revealed that the effect of S. ambofaciens on the pyoverdine production by P. fluorescens occurs only in the area surrounding the streak of Streptomyces (Fig. 1h, i). Altogether, these data show that in the presence of S. ambofaciens ATCC23877, the BBc6R8 strain does not produce its own siderophore.

Transcription of pyoverdine and enantio-pyochelin synthesis genes is no longer induced in the presence of S. ambofaciens ATCC23877

The biosynthesis and uptake of pyoverdine as well as their regulation have been thoroughly studied in P. aeruginosa (32–35) and in P. fluorescens SBW25 (9). A search in the P. fluorescens BBc6R8 genome sequence (26) indicated that all the genes involved in these processes are conserved in this strain (Table S1), suggesting similar mechanisms of biosynthesis, uptake and regulation in the BBc6R8 strain as in P. aeruginosa. Therefore, we tested the influence of S. ambofaciens on the expression of these genes by varying the duration of preincubation of Streptomyces on the plate before the addition of Pseudomonas. Messenger RNAs from cell lysates of P. fluorescens BBc6R8 grown on 26A agar medium in absence or presence of S. ambofaciens were quantified using RT-qPCR. The duration of preincubation of Streptomyces varied between 0 to 54h before the BBc6R8 strain was streaked on the plate. The expression of genes involved in the biosynthesis of pyoverdine (pvdD), its regulation (pvdO, pvdS, pvdQ) and export (pvdE), and in the biosynthesis of the second type of siderophore of P. fluorescens BBc6R8, enantio-pyochelin (phcF), were monitored after two days of growth. As expected, all siderophore-related genes were transcribed when Pseudomonas was cultivated.
alone, reflecting the iron limitation in the 26A medium. In contrast, transcript levels of the pyoverdin genes were significantly reduced when *P. fluorescens* BBc6R8 was grown in the presence of *S. ambofaciens* (Fig. 2, p<0.01, one factor ANOVA) except for *pvdD* and *pvdS* when the two bacterial strains were simultaneously streaked on the agar plate. As for the production of pyoverdine, the decrease in transcript levels was proportional to the time lag between the seeding of the two bacterial strains on the plate for all genes analysed. The expression of the enantio-pyochelin gene *phcF* was similarly affected by the presence of *S. ambofaciens* ATCC23877. Altogether, these data confirm our initial observation that *P. fluorescens* BBc6R8 reduces and even stops producing its own siderophores on iron-limited medium in the presence of *S. ambofaciens* ATCC23877.

**S. ambofaciens** ATCC23877 expresses genes necessary for desferrioxamine and coelichelin production on 26A medium

*S. ambofaciens* ATCC23877 produces two types of tris-hydroxamate siderophores: desferrioxamine (B and E) and coelichelin (36). We hypothesized that *S. ambofaciens* produces these siderophores when grown on 26A agar medium, and that these siderophores would then be accessible to the BBc6R8 strain. To test this hypothesis, we analysed the expression of the genes of the biosynthesis pathways of these two siderophores by RT-qPCR. The transcription of *desC* (acyl-CoA acyltransferase, desferrioxamine, (36)) and *samR0552/cchH* (NRPS, coelichelin, (36)) was monitored over a two and a half day time-course. Both genes were expressed under these conditions and their transcription peaked around 30 hours (Fig. 3). This indicates that *S. ambofaciens* also detects iron deficiency on 26A medium and induces the expression of its siderophore biosynthetic genes, strongly suggesting that desferrioxamine and coelichelin are produced.
**P. fluorescens BBc6R8 utilizes siderophores produced by Streptomyces**

Consequently, *P. fluorescens* BBc6R8 could use either ferrioxamine(s) and/or ferri-coelichelin as xenosiderophores to cope with the lack of iron of the 26A medium. To test this hypothesis, *P. fluorescens* BBc6R8 was cultivated on 26A agar plate supplemented with desferrioxamine B mesylate at 200 µM. After 48 hours of culture, *P. fluorescens* BBc6R8 did not fluoresce in presence of the exogenous purified siderophore in contrast to the control (Fig. 4a, b). The addition of 8 µM of desferrioxamine B in the medium was sufficient to inhibit pyoverdine production (Fig. 4c). This indicates that *P. fluorescens* BBc6R8 stops producing its own siderophore in presence of desferrioxamine B, likely utilizing this compound as a xenosiderophore.

*Streptomyces coelicolor* A3(2) produces the same siderophores as *S. ambofaciens* ATCC23877 (36–38). Since cosmids mutated in the biosynthetic desD (type C siderophore synthetase,(36)) and cchH (coelichelin NRPS, (38)) genes are available from the *S. coelicolor* transposon insertion single-gene knockout library (29), we decided to disrupt these genes in *S. coelicolor* M512 (a derivative of the *S. coelicolor* A3(2) M145 strain unable to produce the pigmented antibiotics actinorhodin and undecylprodigiosine (39)). Pairwise cultures between *P. fluorescens* BBc6R8 and *S. coelicolor* cchH and desD mutants revealed that *Pseudomonas* did not fluoresce in the presence of either of the two single mutants after 2 days of culture (Fig. 4d, e). However, in the presence of the *S. coelicolor* ΔdesΔcch strain, a double mutant deficient in the biosynthesis of desferrioxamine and coelichelin (36), the production of the green-yellow pigment by *P. fluorescens* was not affected (Fig. 4f). Therefore, our data suggest that *P. fluorescens* BBc6R8 has the ability to recognize and to take up...
both ferrioxamine and ferri-coelichelin and consequently no longer produces its own siderophores, pyoverdine and enantio-pyochelin.

**Streptomyces is unable to take up *P. fluorescens* BBc6R8 siderophores**

The question arose if *Streptomyces* conversely to *Pseudomonas* was able to use pyoverdin and/or enantio-pyochelin as xenosiderophores. It has been reported that the *S. coelicolor* ΔdesΔcch strain (W13 strain) cannot grow on agar medium supplemented with 200 μM 2', 2'-bipyridyl due to an extreme iron deficiency (36). Indeed, in our experimental setup, it was unable to grow on a 26A plate containing the iron chelator (data not shown). Therefore, we examined whether *P. fluorescens* BBc6R8 could compensate for this deficiency by providing its own siderophores. A plug was collected from a 3 days culture of BBc6R8 on a 26A agar plate with or without 2', 2'-bipyridyl (200 μM) and placed onto a 26A agar plate supplemented with the iron chelator (200 μM) and evenly spread with the W13 strain. Alternatively, the W13 strain was streaked on a 26 A plate containing 2', 2'-bipyridyl (200 μM) alongside the BBc6R8 strain inoculated on plate 3 days earlier. As a control similar experiments were carried out with *S. coelicolor* M512 instead of *P. fluorescens* BBc6R8. While M512 efficiently promoted the growth of W13, BBc6R8 had no effect (Fig. S2). Therefore, we conclude that *S. coelicolor* is unable to use pyoverdine and enantio-pyochelin as xenosiderophores. By extension we expect a similar behavior for *S. ambofaciens* since the two species encode the same extracellular siderophore binding proteins ((40); data not shown).
*P. fluorescens* BBc6R8 uses a TonB-dependent receptor to detect *S. ambofaciens* ATCC23877 ferri-siderophores

To identify the genes of *P. fluorescens* BBc6R8 involved in the uptake of ferrioxamines and ferri-coelichelin, pairwise cultures were performed on solid 26A medium between *S. ambofaciens* ATCC23877 and 4,400 clones from a transposon mutant library of *P. fluorescens* BBc6R8 (P. Burlinson and A. Deveau, pers. com.). *S. ambofaciens* was streaked on plate 30 hours before the *Pseudomonas* mutants to allow for accumulation of ferrioxamines and ferri-coelichelin in the medium. Two mutants, P28H6 and P18B10, still fluoresced under UV in these conditions (Fig. 5a-d). Similar results were observed when the P28H6 and P18B10 clones were grown near the *S. coelicolor* M512 desD and cchH mutants unable to produce desferrioxamine and coelichelin, respectively (Fig. 5e, f). In addition, production of pyoverdine was also observed in the two *Pseudomonas* mutants when cultivated on 26A agar plate containing 200 μM of desferrioxamine B (Fig. 5g, h). Therefore, we concluded that the mutants P28H6 and P18B10 of *P. fluorescens* BBc6R8 are no longer able to recognize and/or to take up the *Streptomyces* siderophores. Analysis of the mutants revealed that both P28H6 and P18B10 are mutated in the same gene, the gene MHB_05767 (Fig. S3). A BLASTp search showed that the product of this gene shares respectively 88%, 84% and 67% identity with FoxA, a TonB-dependent ferrioxamine B receptor, of *P. fluorescens* Pf0-1, *Pseudomonas protegens* Pf-5 and *P. aeruginosa* PAO1 (41, 42) (Fig. S4). By analogy, we named the gene of *P. fluorescens* BBc6R8 foxA. From this set of experiments, we conclude that *P. fluorescens* BBc6R8 would be able to detect and to take up the siderophores ferrioxamine B and ferri-coelichelin via the outer membrane receptor FoxA.
S. ambofaciens ATCC23877 induces expression of genes involved in the TonB-dependent receptor synthesis

In P. fluorescens Pf0-1, P. protegens Pf-5 and P. aeruginosa PAO1, foxA belongs to an operon with the genes foxI (encoding a ECF sigma factor), foxR (encoding an anti-sigma factor) and pepSY (encoding a PepSY TM helix protein) which is under the regulation of the Fur protein (42). Analysis of the genome sequence of P. fluorescens BBc6R8 indicates that the homologs of genes foxI, foxR and pepSY were also present in BBc6R8 (Table S1) and organized in a similar cluster as in P. fluorescens Pf0-1, P. protegens Pf-5 and P. aeruginosa PAO1. A homolog of the fur gene was also retrieved in the genome sequence of the BBc6R8 strain (Table S1). This suggests that the mechanism of regulation of the desferrioxamine-mediated iron uptake system in P. fluorescens BBc6R8 is similar to that of P. aeruginosa (32, 34).

After 24 h of incubation with S. ambofaciens, the expression level of foxA, foxI, foxR and pepSY genes increased compared to the control treatment (p<0.01, one factor (R8 alone) ANOVA, Fig 6). The transcription level of fur was significantly modified by the presence of S. ambofaciens although the fold change was much smaller than for the other transcripts (Fig 6, p<0.01 one factor (R8 alone) ANOVA). These data show that in the presence of S. ambofaciens, and in conditions stimulating siderophore production, the P. fluorescens BBc6R8 foxA transcriptional regulation cascade is induced, most likely resulting in the production of the FoxA receptor and in the uptake of the Streptomyces siderophores.

Utilization of desferrioxamine is shared among other fluorescent pseudomonads and P. aeruginosa

As similar biosynthetic and uptake systems are present in P. fluorescens BBc6R8, P. aeruginosa PAO1 (41, 43), P. protegens Pf-5 and P. fluorescens Pf0-1 (42), we
expected that these bacterial strains might also be able to react to the presence of
the siderophores produced by *S. ambofaciens* ATCC23877. Indeed, we found that *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1 and *P. protegens* Pf-5 no longer produced a
green pigment in presence of *S. ambofaciens* ATCC23877 and did not fluoresce
under UV. Interestingly, *P. fluorescens* SBW25, which does not have the foxA gene,
fluoresced even in presence of *S. ambofaciens* (Fig. S5). In the presence of purified
desferrioxamine B, *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1 and *P. protegens* Pf-5
no longer fluoresced, again in contrast to the strain SBW25 (Fig. S5). In co-cultures
with *S. coelicolor* M512 desD, *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1 and
*P. protegens* Pf-5 fluoresced less than in single culture, suggesting that they are also
able to utilize coelichelin (Fig. S5). Interestingly, a screening of a library of sixty
fluorescent pseudomonad strains isolated from forest soil (Table 1) revealed that all
these strains were also able to utilize *S. ambofaciens* siderophores (data not shown).

**Discussion**

Our study reports interspecies adaptative behaviour during common utilization of
limited iron ressources between the two soil inhabitants *Pseudomonas* and
*Streptomyces*. In particular, we show that, in iron deficient conditions, *P. fluorescens*
BBc6R8 does not induce the production of the fluorescent siderophore pyoverdine in
the presence of *S. ambofaciens* ATCC23877. Instead, *P. fluorescens* very likely
utilizes the *S. ambofaciens* siderophores desferrioxamine B and coelichelin as
xenosiderophores thanks to its FoxA receptor. In contrast to most studies in which
the use of xenosiderophores was revealed indirectly through the addition of purified
siderophore in the growth medium (44–47), binding affinity assays (10, 48), native
PAGE and surface plasmon resonance (49) or with labeled iron (10, 50, 51), we
revealed potential iron piracy through a direct interaction between a Gram-positive
and a Gram-negative bacterium. This potential piracy likely occurs only in one direction. Indeed, the growth of *S. coelicolor ΔdesΔcch* mutant under iron deficient conditions could not be rescued in the presence of *P. fluorescens* BBc6R8 (Fig. S1).

Similar behaviour is expected from *S. ambofaciens* ATCC23877 since this strain is extremely close phylogenetically to *S. coelicolor* and they encode the same extracellular siderophore binding proteins. Similar piracy involving streptomycetes has previously been reported but only with other actinomycetes (e.g. *Streptomyces* and *Amycolatopsis* (14, 44, 52). It is interesting to note that these examples also involved desferrioxamines, and that competition for iron could either stimulate (44, 52) or curtail (14) the growth and/or development of *Streptomyces*. The effect of the siderophore piracy by *P. fluorescens* on *S. ambofaciens* remains to be identified. Indeed, we could not observe any effect on the morphological differentiation of *S. ambofaciens* as it only forms vegetative mycelium on the 26A medium.

Although our model bacteria were isolated from independent ecological niches (*Laccaria bicolor* sporocarp for *P. fluorescens* BBc6R8 (53); soil in Picardie Region, France, for *S. ambofaciens* (17)) and experiments were carried out in laboratory growth conditions, this interaction for iron capture between *Streptomyces* and *Pseudomonas* likely occurs in natural environments. Indeed, several studies have reported that these bacterial genera share common ecological niches within soils including environments such as the rhizosphere or bulk soils (54–56). Therefore, they are expected to use common pools of scarce but essential elements such as iron.

The piracy would occur through the production by *P. fluorescens* BBc6R8 of the TonB-dependent receptor FoxA. Interestingly, the BBc6R8 strain does not produce ferrioxamine which is the most effective compound for iron scavenging, followed by pyoverdine and enantio-pyochelin both of which it does produce (56). In a competitive environment such as soil, it is certainly more advantageous to use
efficient chelators produced by its neighbors rather than to produce its own siderophores especially if they are less effective and metabolically costly as demonstrated in (6). In addition, since desferrioxamines are synthesized by many soil organisms, \textit{P. fluorescens} BBc6R8 strain could obtain in this way iron at low energetical cost in many competitive situations. Interestingly, it should be noted that purified desferrioxamine added in the culture medium had a positive effect on the growth of \textit{P. fluorescens} BBc6R8 (data not shown). In contrast, \textit{S. ambofaciens} impacted negatively the growth of BBc6R8 in co-cultures, presumably because of the secretion of secondary metabolites or/and through nutrient competition (data not shown).

The production of desferrioxamines seems to be conserved throughout Streptomyces (57, 58). The \textit{desABCD} genes that direct the synthesis of these chelating agents (37, 59) have been found in all \textit{Streptomyces} sequenced genomes and in other related genera, such as \textit{Salinispora} (60). Furthermore, Kobayakawa and coworkers detected by HPLC the production of desferrioxamines in 78\% of their \textit{Streptomyces} collection (61). Desferrioxamines are also produced by other actinomycetes and some Gram-negative bacteria (62, 63).

Our data indicate that the BBc6R8 outer membrane receptor FoxA would not only allow the uptake of desferrioxamines of \textit{S. coelicolor} but also of the hydroxamate siderophore ferri-coelichelin. The ability seems to be widespread amongst fluorescent Pseudomonads. Indeed, the FoxA receptor is present in three different subclades of the \textit{P. fluorescens} genus (64) and based on our screening of sixty \textit{P. fluorescens} environmental strains, it is likely that they also possess a \textit{foxA} gene and even a \textit{foxA} operon. A BLASTp analysis also showed that FoxA homologues are present in other \textit{Pseudomonas} species such as \textit{P. stutzeri}, \textit{P. aeruginosa}, \textit{P. fulva}, \textit{P. putida} or \textit{P. resinovorans} (data not shown) and also in different genera of bacteria
like *Yersinia enterocolitica* (11) and *Erwinia Herbicola* (12). In these latter, FoxA is known to bind a collection of ferrioxamine derivatives with different chain lengths or bridges (65). The tris-hydroxamate siderophore coprogen is also recognized to a certain extent by FoxA (65). Desferrioxamine B, coelichelin and coprogen are linear siderophores (36, 65). These data suggest that FoxA could bind other linear ferric-tris-hydroxamate siderophores (65). Therefore, *P. fluorescens* BBc6R8 may have the ability to obtain a wider variety of siderophores than investigated in this study through the production of the FoxA receptor and thus very efficiently compete with other bacteria. FoxA could then be considered as an outer membrane receptor with a broad spectrum, at least for siderophores of the tris-hydroxamate family, and as a widespread receptor. Other bacteria, such as *Streptomyces* for example, also possess broad-spectrum tris-hydroxamate receptors. Indeed, in *S. ambofaciens* ATCC23877 and *S. coelicolor* A3(2), CdtB, a siderophore binding protein involved in iron-siderophore transport, is able to bind ferrioxamines and ferri-coelichelin with high affinity (36, 40). Moreover, DesE, a second siderophore binding protein binds different ferric-tris-hydroxamates, with the exception of ferri-coelichelin (40). To possess some broad spectrum siderophore receptors may be a frequent strategy among microorganisms selected during evolution to be more competitive in soil. The capability to take up xenosiderophores can be amplified if bacteria possess multiple xenosiderophore receptors. This property is particularly well developed in the genus *Pseudomonas*. Indeed, several *Pseudomonas* species possess numerous TonB-dependent receptors, usually more than 20 with *P. protegens* Pf-5 encoding the highest number (45) of TonB-dependent receptors (43). Twenty nine have been identified so far in the draft genome sequence of the BBc6R8 strain (26). Several bacteria possessing multiple siderophore binding proteins, like *Pseudomonas fragi*, have even lost the ability to produce siderophores and rely exclusively on...
siderophore piracy (10). Whether siderophore piracy results from the loss of the
ability to synthesize the cognate siderophores during evolution or the acquisition of
receptor genes through horizontal transfer remains an open question. Nevertheless,
our data and others strongly support the idea that the capacity for siderophore piracy
is widespread among bacteria and that piracy by "cheaters" does happen *in vivo* (66).
What is unclear is to which extent does this piracy really occur in natural ecosystems
such as soils and how it impacts community dynamics. Metagenomic combined with
metatranscriptomic may help answering this question in the future by providing a
more complete picture of who is producing what and who is "cheating" in natural
complex microbial communities.

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Gregor (Ben-Gurion University of the Negev) for proofreading the manuscript. JG
was supported by a CJS (Contrat Jeune Scientifique) grant from INRA.
References


**Figure legends**

**Fig. 1** Effect of *S. ambofaciens* ATCC23877 on green-yellow fluorescent pigment production by *P. fluorescens* BBc6R8. a and b *P. fluorescens* BBc6R8 (R8); c *S. ambofaciens* ATCC23877 (Sa); d *P. fluorescens* BBc6R8 cultivated on medium supplemented with FeSO₄; e and f *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877; g *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 which has been streaked on plate 30 h before the BBc6R8 strain; h *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 co-cultivated in a perpendicular way; i *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 (streaked on plate 30 h before the BBc6R8 strain) co-cultivated in a perpendicular way. For parts b, c, d, f, g, h and i, plates were visualized under UV. Photographs were taken from below the plates after a two day (co-)culture of incubation at 26.5°C on 26A agar medium.

**Fig. 2** Effect of *S. ambofaciens* ATCC23877 on the expression levels of *P. fluorescens* BBc6R8 genes involved in the biosynthesis of pyoverdine and enantio-pyochelin as measured by RT-qPCR. Expression levels are expressed as...
the transcript levels of target genes relative to the transcript level of the housekeeping gene selR which is stable in the tested growth conditions. Data are expressed as the mean value of three biological replicates. Error bars denote standard error. For each transcript, values with the same letter are not significantly different according to a one-factor ANOVA (p>0.01). R8: *P. fluorescens* BBc6R8 grown on 26A agar medium in absence of *S. ambofaciens*. 0h to 54h: *P. fluorescens* BBc6R8 grown in presence of *S. ambofaciens* ATCC23877 and *S. ambofaciens* was streaked on 26A plate 0 h, 24 h, 30 h, 48 h or 54 h before the BBc6R8 strain. Total RNAs were extracted 48 h after the plates were inoculated with *P. fluorescens* and incubated at 26.5°C.

Fig. 3 Expression levels of *S. ambofaciens* ATCC23877 genes controlling the synthesis of desferrioxamine B and E and coelichelin as measured by RT-qPCR. The desC gene (A) is involved in the synthesis of desferrioxamines and samR0552 gene (B) in the coelichelin biosynthesis. Total RNAs were extracted after 24, 30, 48 and 54 h of growth on 26A agar medium at 26.5°C. Expression levels are expressed as the transcript levels of target genes relative to the transcript level of the housekeeping gene hrdB which is stable in the tested growth conditions. Data are expressed as the mean value of three biological replicates. Error bars denote standard error. For each transcript, values with the same letter are not significantly different according to a one-factor ANOVA (p>0.01).

Fig. 4 Effect of desferrioxamine and coelichelin on the production of the pyoverdine by *P. fluorescens* BBc6R8. a *P. fluorescens* BBc6R8 (R8); b *P. fluorescens* BBc6R8 cultivated in presence of 200 µM of desferrioxamine B mesylate; c *P. fluorescens* BBc6R8 cultivated in presence of 8 µM of desferrioxamine B mesylate; d *P.
fluorescens BBc6R8 + S. coelicolor M512 cch; e P. fluorescens BBc6R8 + S. coelicolor M512 desD; f P. fluorescens BBc6R8 + S. coelicolor ΔdesΔcch. The Streptomyces strains were streaked on plate 30 h before the inoculation of P. fluorescens. Photos were taken under UV from below the plates after a two day (co-)culture on 26A agar medium at 26.5°C.

Fig. 5 Effect of the disruption of the foxA gene on the pyoverdine production by P. fluorescens BBc6R8. a P. fluorescens P28H6; b P. fluorescens P18B10; c P. fluorescens P28H6 or d P. fluorescens P18B10 cultivated with S. ambofaciens ATCC23877 (Sa); e P. fluorescens P28H6 cultivated with S. coelicolor M512 cchH and f with S. coelicolor M512 desD; g P. fluorescens P28H6 and h P. fluorescens P18B10 cultivated in presence of 200 µM of desferrioxamine B mesylate. The Streptomyces strains were streaked on plate 30 h before the inoculation of P. fluorescens. Photos were taken under UV from below the plates after a two day (co-)culture at 26.5°C on 26A agar medium.

Fig. 6 Effect of S. ambofaciens ATCC23877 on the expression levels of P. fluorescens BBc6R8 genes controlling the synthesis of FoxA receptor. Analysis was done by RT-qPCR. R8 : P. fluorescens BBc6R8 grown on 26A agar in absence of S. ambofaciens. 0h to 54h: P. fluorescens BBc6R8 grown in presence of S. ambofaciens ATCC23877 which was streaked on solid 26A plate 0h, 24h, 30h, 48h or 54h before the BBc6R8 strain. Expression levels are expressed as the transcript levels of target genes relative to the transcript level of the housekeeping gene selR which is stable in the tested growth conditions. Data are expressed as the mean value of three biological replicates. Error bars denote standard error. For each
transcript, values with the same letter are not significantly different according to a one-factor ANOVA (p>0.01).

Table 1  Bacterial strains used in this work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
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<td><em>S. ambofaciens</em> ATCC23877</td>
<td>Reference strain [wild type]</td>
<td>(17)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 ΔredD ΔactI-ORF4 from <em>S. coelicolor</em> M145; deficient in undecylprodigiosin and actinorhodin production</td>
<td>(39)</td>
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<td><em>S. coelicolor</em> M512 desD</td>
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<td>This study</td>
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<tr>
<td><em>S. coelicolor</em> M512 desD::Tn5062</td>
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<td></td>
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<td><em>S. coelicolor</em> ΔdesΔcch</td>
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<td>(36)</td>
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<td><em>E. coli</em> ET12567(pUZ8002)</td>
<td>Donor strain in intergeneric conjugation, a methylation-defective strain</td>
<td>(30)</td>
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<td><em>P. fluorescens</em> BBc6R8</td>
<td>Isolated from <em>Laccaria bicolor</em></td>
<td>(18)</td>
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<tr>
<td><em>P. fluorescens</em> SBW25</td>
<td>Isolated from rhizosphere of sugar beet</td>
<td>(67)</td>
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<td><em>P. fluorescens</em> Pf0-1</td>
<td>Isolated from agricultural soil</td>
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<td>Human opportunistic pathogen</td>
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<td><em>P. fluorescens</em> P18B10</td>
<td>Mutant derived from BBc6R8, Tn5 insertion in foxA</td>
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<tr>
<td>Library of 60 <em>P. fluorescens</em> strains</td>
<td>Strains isolated from the bulk soil of a forest nursery, the ectomycorrhizosphere and the ectomycorrhizas of <em>Laccaria bicolor/Douglas fir</em></td>
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### Table 2 Primer pairs used in qPCR analyses or to determine transposon insertion sites in the P. fluorescens BBc6R8 genome

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**Round of PCR**

- *gfptns2*: ATCACCTTCAGGCTCCACAAC For first and second round PCR of transposon mutants
- *nCEKG2A*: gcgaagaattcatggaagagatc For first round PCR of transposon mutants
- *nCEKG2B*: gcgaagaattcatggaagagatc For first round PCR of transposon mutants
- *nCEKG2C*: gcgaagaattcatggaagagatc For first round PCR of transposon mutants
- *CEKG4*: GCCACCGCGCTGACATGAC For second round PCR of transposon mutants