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Fitness in soil and rhizosphere of *Pseudomonas fluorescens* C7R12 compared with a C7R12 mutant affected in pyoverdine synthesis and uptake

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Abstract

Fluorescent pseudomonads have evolved an efficient strategy of iron uptake based on the synthesis of the siderophore pyoverdine and its relevant outer membrane receptor. The possible implication of pyoverdine synthesis and uptake on the ecological competence of a model strain (*Pseudomonas fluorescens* C7R12) in soil habitats was evaluated using a pyoverdine minus mutant (PL1) obtained by random insertion of the transposon Tn5. The Tn5 flanking DNA was amplified by inverse PCR and sequenced. The nucleotide sequence was found to show a high level of identity with *pvs*B, a pyoverdine synthetase. As expected, the mutant PL1 was significantly more susceptible to iron starvation than the wild-type strain despite its ability to produce another unknown siderophore. As with the wild-type strain, the mutant PL1 was able to incorporate the wild-type pyoverdine and five pyoverdines of foreign origin, but at a significantly lower rate despite the similarity of the outer membrane protein patterns of the two strains. The survival kinetics of the wild-type and of the pyoverdine minus mutant, in bulk and rhizosphere soil, were compared under gnotobiotic and non-gnotobiotic conditions. In gnotobiotic model systems, both strains, when inoculated separately, showed a similar survival in soil and rhizosphere, suggesting that iron was not a limiting factor. In contrast, when inoculated together, the bacterial competition was favorable to the pyoverdine producer C7R12. The efficient fitness of PL1 in the presence of the indigenous microflora, even when coinoculated with C7R12, is assumed to be related to its ability to uptake heterologous pyoverdines. Altogether, these results suggest that pyoverdine-mediated iron uptake is involved in the ecological competence of the strain *P. fluorescens* C7R12.

Keywords: Pyoverdine; Outer membrane protein; Population dynamics; Soil; Rhizosphere; Tn5 mutagenesis; Inverse PCR; *Pseudomonas fluorescens*

1. Introduction

Fluorescent pseudomonads can suppress various soilborne plant diseases [1]. Their efficacy has been related to their antagonistic activities and to their rhizosphere competence [2,3]. A clear relationship has been established between the suppression of soilborne diseases by fluorescent pseudomonads and their densities in the rhizosphere [4,5]. However, since the microbial inoculations would mainly be performed in soils before the plant is grown up, the strains should also be able to survive in the soil and show a good saprophytic ability. To fulfill these requirements, progress must be made in our knowledge of which bacterial traits affect the soil- and rhizosphere-colonizing ability of fluorescent pseudomonads.

Despite the abundance of iron in soils, the concentration of ferric iron available to the soilborne microflora is very low [6]. Since Fe^{3+} is an essential element for most microorganisms, this ion is often a limiting factor for microbial growth and activity in soil habitats [7]. Most microorganisms have developed an active strategy for iron acquisition based on the use of siderophores and of the corresponding ferrisiderophore membrane receptors [8]. The major siderophores of the fluorescent pseudomonads, called pyoverdines, show a very high affinity for Fe^{3+} [9]

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and their membrane receptors are usually very specific [10]. These features enable the fluorescent pseudomonads to compete efficiently with the soilborne microflora and pyoverdine-mediated iron competition was shown to play a determinative role in the microbial antagonism performed by biocontrol strains against some pathogens [7,11–13]. Compared to the numerous studies dealing with microbial antagonism, little attention has been given to the possible implication of pyoverdine-mediated iron uptake on the ecological competence of fluorescent pseudomonads in soil [14–16].

The aim of the present study was to determine if pyoverdine synthesis and incorporation could be involved in the soil and rhizosphere competence of a model strain of *Pseudomonas fluorescens* C7R12. For that purpose, a Tn5 mutant of C7R12, affected in pyoverdine synthesis and uptake, was obtained and the fitness of the wild-type strain and the mutant was compared both in bulk and rhizosphere soils.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. fluorescens strain C7R12 is a spontaneous mutant resistant to rifampicin (100 mg l^{-1}) of strain C7 [17]. The rifampicin resistance is used as a specific marker to allow the recovery of the strain when introduced into soil. The wild-type strain C7 was previously isolated from the rhizosphere of flax cultivated in the Châteaurenard soil that suppresses fusarium wilts [18]. The strain C7R12 was shown (i) to improve the suppression of fusarium wilts achieved by non-pathogenic *Fusarium oxysporum* [19] and (ii) to be rhizosphere competent [17].

Escherichia coli 1830 (pJB4JI) [20] was used as the donor strain for the Tn5 mutagenesis. *E. coli* MC1022 [21] and *E. coli* JM109 (Promega, Grenoble, France) were used as hosts for plasmid maintenance.

Depending on the experiments, the fluorescent pseudomonad strains, wild-type and Tn5 mutant, were grown either in King's B medium (KB) [22], Luria Broth medium (LB) [23], synthetic succinate medium (SM) [9] or in semi synthetic casamino acids medium (CAA) [24] at 25°C. *E. coli* strains were grown in LB medium at 37°C.

Bacterial inoculants were produced on KB agar plates supplemented with rifampicin (100 mg l^{-1}) for the wild-type or with rifampicin (100 mg l^{-1}) plus kanamycin (50 mg l^{-1}) for the Tn5 mutant, and incubated at 25°C for 48 h. Bacteria were scraped from the medium and suspended in sterile distilled water, pelleted by centrifugation ($5000 \times g$, 20 min) and washed twice with sterile distilled water. The bacterial density of the suspensions was determined using a calibration curve assessed by turbidity ($\lambda = 600$ nm).

All strains were stored at −80°C in 50% glycerol.

2.2. Transposon mutagenesis

Transposon Tn5 was introduced into C7R12 by conjugation. *E. coli* 1830 containing Tn5 transposon in pJB4JI [20] and *P. fluorescens* C7R12 were grown in LB broth for 4 h. Mating was performed by mixing approximately 10^9 cells of donor and recipient on membrane filters after two washing steps in fresh LB broth. Filters were incubated overnight at 25°C on LB plates. Cells were resuspended in 0.1 M $MgSO_4 \cdot 7H_20$, plated on LB supplemented with kanamycin (50 mg l^{-1}) and rifampicin (100 mg l^{-1}), and incubated at 25°C for 24 h. One conjugant (PL1) was selected for its absence of fluorescence under UV illumination (365 nm) after growth on solid KB for 48 h at 25°C. The presence of Tn5 in the non-fluorescent PL1 transconjugant was checked by probing blots of *Eco*RI digests of total DNA with pRZ104 [21] used as a probe. Digoxigenin labeling of plasmid pRZ104, DNA hybridization and probe detection were done using a non-radioactive DNA labeling and detection kit (Boehringer, Meylan, France) according to the supplier's instructions.

2.3. Inverse PCR (IPCR) and sequencing

In order to identify the sequences flanking the inserted Tn5 in the selected mutant (PL1), an IPCR was performed [25]. Total genomic DNA of PL1 mutant was digested with *Sal*I that cuts the Tn5 (sequence L19385, 5818 bp) only once (position 2684). After separation by agarose gel electrophoresis, the restriction fragments were transferred onto a nylon membrane (Biodyne Plus, Polylabo, Strasbourg, France) and hybridized with two different DNA probes: a *Hpa*I 5445-bp fragment (position 187–5631 of the sequence L19385) of Tn5 obtained after digestion of pRZ104 (probe DIG-Tn5) and a 691-bp fragment of the 3′ end of Tn5 (position 3487–4177 of the sequence L19385) obtained by PCR (probe DIG-KMTn5) using the following primers: Tn5KMU (5′-TGG-ACC-CCT-TGG-CGT-CAT-CAA-3′) and Tn5KML (5′-TTG-TCG-GCA-GCC-TGG-TTC-ATC-3′). The PCR reaction was conducted in a 50-μl reaction volume in a programmable thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The reaction mixture contained 50 ng of pRZ104 plasmid DNA and 0.05 U μl^{-1} of *Taq* DNA polymerase in the corresponding buffer (Appligene-Oncor, Illkirch, France). Final concentrations of each primer (Oligo Express, Grenoble, France) and of dNTPs were 0.5 μM and 200 μM, respectively. Thermal cycling consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63.7°C for 1 min, and elongation at 72°C for 1 min, with a final elongation step at 72°C for 3 min. Probe labeling, hybridization and detection were performed as described above. Only one hybridization band, the 3′ end of the Tn5 and its flanking region, was obtained with DIG-KMTn5 (data not shown). Two hybridization bands were obtained with DIG-Tn5, both

Tn5 ends with their respective flanking regions (data not shown). The 5′ end of the Tn5 and its flanking region have been identified by comparison of the blots obtained with both probes. The DNA fragment containing the 5′ end of the Tn5 was extracted from agarose (Agarose gel DNA extraction kit, Boehringer, Meylan, France), and self-circularized at 4°C overnight using T4 DNA ligase (1 ng DNA, 1 U T4 ligase). The ligation mixture was then amplified with the following primers: UpTn5fa (5′-ATG-CTG-GAG-TTC-TTC-GCC-3′) (position 2495–2512 of the Tn5 sequence L19385) and LowTn5fa (5′-CTG-GAA-AAC-GGG-AAA-GGT-3′) (position 52–35 of the Tn5 sequence L19385) after an initial denaturation of 3 min at 95°C, 35 cycles of 95°C, 58°C and 72°C for 1 min each, and a final elongation step of 5 min at 72°C were performed. The resulting PCR product was cloned into the pGEMTeasy vector system (Promega, Grenoble, France) according to the procedure described by the manufacturer. DNA sequencing was performed by Genome Express (Grenoble, France) using the universal primers T7 and SP6. The sequence obtained (946 bp) has been deposited in the GenBank database under accession number AF172448. Nucleotide sequence homology searches against major sequence databases were done using the program FASTA, alignments were performed using the programs CLUSTALW and BESTFIT (GCG Wisconsin package, version 10.0).

2.4. Growth and siderophore measurements

Growth and pyoverdine measurements were made from cultures of C7R12 and PL1 in 250-ml Erlenmeyer flasks containing 75 ml of SM and placed on a rotary shaker at 150 rpm. Growth was estimated turbidimetrically at 600 nm. The pyoverdine production was evaluated by measuring the absorbance at 400 nm of supernatants obtained from stationary phase cultures (48 h) after centrifugation (10 000 × *g*, 10 min) and adjustment to pH 7.0 by addition of Tris–HCl buffer (1 M, pH 7.0) [9]. The ability of C7R12 and PL1 to grow under iron-stress conditions was compared by determining the minimal concentration of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDHA) inhibiting (MIC, minimal inhibitory concentration) their bacterial growth on solid KB. Possible synthesis of other siderophores than pyoverdine by PL1 was evaluated using the chrome-azurol-S (CAS) assay developed by Schwyn and Neilands [26].

2.5. Purification of the outer membrane (OM) proteins

The OMs from pseudomonad cells grown in CAA medium or in iron-supplemented CAA medium (100 μM) were prepared as described by Cornelis et al. [27]. Briefly, the cells were harvested by centrifugation (10 000 × *g*, 5 min) and suspended in 5 ml Tris–HCl buffer (10 mM, pH 7.5). The cells were then broken by three cycles of 30 s sonication at 4°C. Unbroken cells were pelleted by centrifugation (9000 × *g*, 7 min). The supernatant containing the membrane material was submitted to a series of centrifugation (30 000 × *g*, 30 min) and resuspension steps in successively Tris–HCl buffer (10 mM, pH 7.5), Tris–HCl buffer supplemented with $MgCl_2$ (2 mM) and Tris–HCl buffer supplemented with Triton X-100 to a final concentration of 2% (v/v). The last suspension was agitated at 40°C for 30 min, centrifuged (30 000 × *g*, 60 min) and washed twice with Tris–HCl buffer (10 mM, pH 7.5). The resulting OM samples were kept at −20°C. The protein content of these samples was determined by the Lowry method [28]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [29] with 10% polyacrylamide gels, and protein bands were detected by staining with Coomassie brilliant blue R250.

2.6. [^{59}Fe]Pyoverdine uptake and [^{59}Fe]pyoverdine binding to OMs

Purification of pyoverdines was performed by filtration of 48-h culture supernatants through an amberlite XAD-4 column (Sigma Chemical Co., St. Louis, MO, USA) as described in Meyer et al. [24].

The wild-type strain C7R12 and the Tn5 mutant PL1 were compared for (i) their ability to incorporate homologous pyoverdine ([^{59}Fe]PVD_{C7R12}) and to incorporate 34 structurally different heterologous pyoverdines chelated to ^{59}Fe (listed in the legend of Fig. 2), and for (ii) the ability of their OM proteins to bind [^{59}Fe]PVD_{C7R12}. In both experiments, the labeling mixtures contained each 5 μl of the commercially available $^{59}FeCl_3$ solution (Amersham, Little Chalfont, UK), 50 μl of a 1-mM solution of each purified pyoverdine, and 945 μl of an incubation medium (SM with the nitrogen source omitted).

The samples were prepared as follows. For iron uptake experiments, cells harvested by centrifugation of late exponential phase SM culture (40 h) were washed twice with distilled water and suspended in the incubation medium to a final absorbance (600 nm) of 0.33. Two hundred μl of each labeling mixture made of a different purified pyoverdine was added to 1.8 ml of cell suspension and the resulting samples were incubated for 20 min. For OM binding experiments, 200 μg OM proteins were diluted to a final volume of 1.8 ml in Tris–HCl buffer (10 mM, pH 7.5). Two hundred μl of labeling mixture was added to the OM proteins and incubated at 37°C for 10 min. Cell suspension and OM protein samples were pre-incubated at 37°C for 10 min in glass tubes with agitation (100 rpm). One ml of the cell suspensions or OM protein samples was then filtered through membranes (HAWP, pore size 0.45 μm, Millipore, Bedford, MA, USA) and washed twice with 2 ml of incubation medium.

Radioactivity associated with cells or OM, remaining on the filter, was measured in a Beckman Gammamatic 4000

counter, whereas total radioactivity was measured from 1 ml non-filtered samples (controls). Comparison of the radioactivity remaining on the filter after filtration of cell suspension or OM samples to that of the corresponding controls gave an indication on the [^{59}Fe]pyoverdine uptake and on the affinity of the OM for [^{59}Fe]pyoverdine, respectively.

2.7. Soil and plant experiments

The experiments were performed in the calcic silt–clay soil from Châteaurenard (France). This soil is known for its natural suppressiveness to fusarium wilts and its main characteristics have previously been described [30]. The soil was air-dried and sieved (<2 mm).

Survival of the wild-type C7R12 and of the Tn5 mutant PL1 was compared in the Châteaurenard soil kept uncultivated and in the rhizosphere of tomato grown in this soil. These experiments were performed under gnotobiotic and non-gnotobiotic conditions.

Briefly, 131 g of soil was filled into containers (102 ml) corresponding to an apparent density of 1.06. These containers were sterilized by γ-radiation (40 kGy; Conservatome, Dagneux, France) for the experiments performed under gnotobiotic conditions. The water potential of the soil was adjusted and kept at pF = 2 with sterile water. Some containers were kept uncultivated and others were cultivated with tomato (*Lycopersicon esculentum* Mill., cv. H63-5). Seeds of tomato were sterilized in a 1.25% (v/v) solution of NaOCl for 20 min, washed three times with sterile distilled water and placed on sterile filters at 25°C for 48 h. Five tomato seedlings were transferred per container. The cultivated and uncultivated containers were placed in a flow cabinet used as a growth chamber on a cycle of 16 h light (25°C) and 8 h dark (22°C) in order to keep the atmosphere sterile.

Immediately after sowing, a 5-ml inoculum of the wild-type C7R12 and the Tn5 mutant PL1 was introduced at the surface of each container either separately or in combination to obtain a bacterial density of 10^7 colony forming units (CFU) per gram of dry soil.

The extraction of the bacteria for enumeration was performed as follows. From the containers without plants, 15 ml of soil (bulk soil), corresponding to 14.1 g of dry soil, were collected with a punch and suspended in 135 ml sterile water by mixing with a Waring blender (Polylabo, Strasbourg, France) for 60 s. From the containers with plants, the soil that remained at the root surface of three root systems, after a gentle brushing, was suspended in 10 ml of sterile distilled water (rhizosphere soil). The dry weight of the rhizosphere soil was measured. The suspensions of bulk soil and rhizosphere soil were diluted in distilled sterile water and plated (three plates per suspension) on KB supplemented with rifampicin (100 mg l^{-1}) or with both rifampicin and kanamycin (50 mg l^{-1}) for the samples from gnotobiotic treatments, and on KB supplemented with these antibiotics and with cycloheximide (100 mg l^{-1}), ampicillin (40 mg l^{-1}) plus chloramphenicol (13 mg l^{-1}) [31] for the samples from non-gnotobiotic treatments. In addition, the absence of contaminants in non-inoculated controls was tested by dilution-plating onto unamended solid KB. Bacterial enumeration was performed 0, 2, 5, 9 and 19 days after inoculation on five independent replicates (containers) per experimental treatment and date. The bacterial densities were expressed as CFU per gram of dry soil. Since populations of bacteria approximate a log normal distribution [32], values were exponential transformed before analysis. Transformed values of microbial enumeration were submitted to variance analysis and then to Fisher's least significant difference test. All experiments were duplicated and the results shown represent one representative experiment.

3. Results

3.1. Characteristics of the mutant PL1 related to pyoverdine synthesis and uptake

Compared to the wild-type, the absorbance spectrum of the supernatant from the Tn5 mutant did not show any peak at 400 nm indicating that pyoverdine was not produced and, therefore, that the mutant PL1 was affected in the pyoverdine synthesis (pvd$^-$) (data not shown). Accordingly, the MIC of EDDHA was significantly lower for PL1 (<16 μg ml^{-1}) than for C7R12 (>1000 μg ml^{-1}). However, a positive reaction induced by PL1 on CAS medium indicated that the mutant affected in pyoverdine synthesis was still able to produce at least one other siderophore.

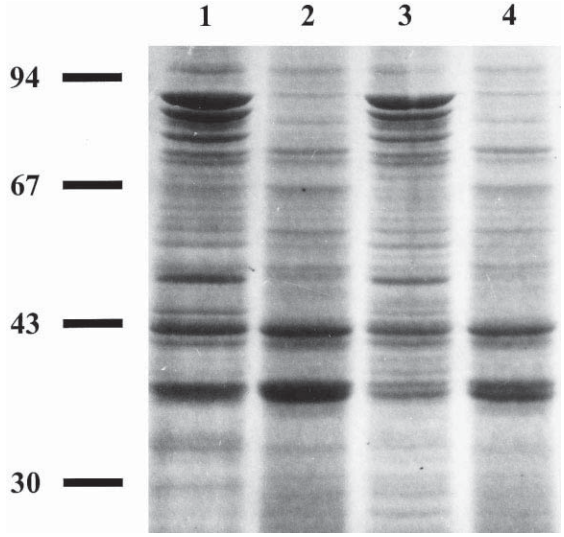

Fig. 1. OM protein patterns of C7R12 (lanes 1 and 2) and PL1 (lanes 3 and 4) grown in CAA medium supplemented (lanes 2 and 4) or not (lanes 1 and 3) with 100 μM $FeCl_3$. The positions of standard proteins are shown on the left: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

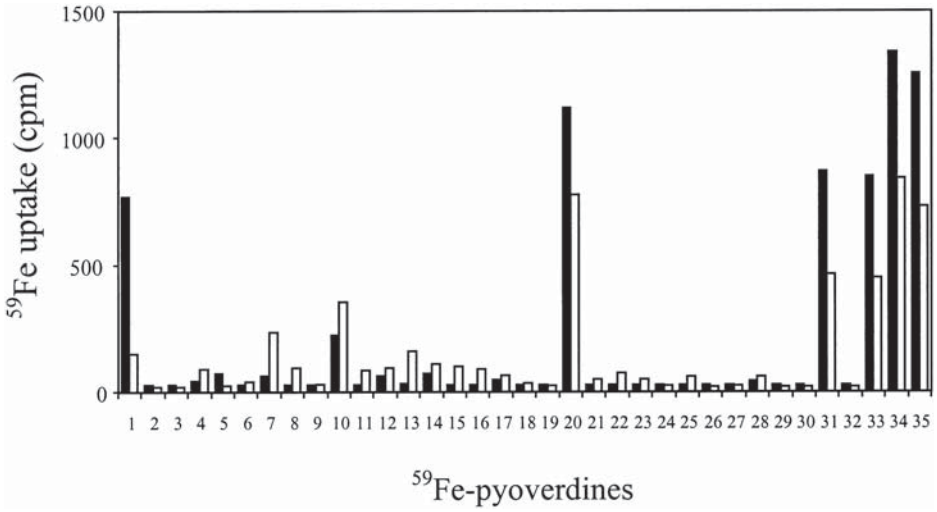

Fig. 2. ^{59}Fe uptake in C7R12 (■) and PL1 (□) as mediated by pyoverdines of: 1, *P. fluorescens* E8; 2, *P. syringae* ATCC 19310; 3, *P. fluorescens* 9AW; 4, *P. putida* ATCC 12633; 5, *P. fluorescens* 51W; 6, *P. aeruginosa* Pa6; 7, *P. fluorescens* CCM 2998; 8, *P. fluorescens* CHAO; 9, *P. tolaasii* NCPPB 2192; 10, *P. aeruginosa* 27853; 11, *P. fluorescens* ii; 12, *P. fluorescens* SB8.3; 13, *P. fluorescens* ATCC 17400; 14, *P. fluorescens* 1.3; 15, *P. fluorescens* 267; 16, *P. fluorescens* ATCC 13525; 17, *P. aeruginosa* ATCC 15692; 18, *P. fluorescens* 18.1; 19, *P. fluorescens* 12; 20, *P. fluorescens* CFBP 2392; 21, *P. putida* CFBP 2461; 22, *Pseudomonas* sp. ATCC 15915; 23, *Pseudomonas* sp. 9054; 24, *Pseudomonas* sp. 9077; 25, *Pseudomonas* sp. 92104; 26, *P. putida* 9033; 27, *P. putida* 9040; 28, *P. putida* 9042; 29, *P. putida* 9051; 30, *P. putida* 9052; 31, *P. fluorescens* CTR1015; 32, *P. fluorescens* 2908; 33, *P. fluorescens* A214; 34, *P. tolaasii* Ps3a; 35, *P. fluorescens* C7R12.

The OM protein patterns of mutant PL1 and wild-type strain C7R12 grown in iron-enriched and iron-depleted media were compared by SDS–PAGE. The protein patterns of the cells grown under iron-stress conditions (lanes 1 and 3) and under non-iron-stress conditions (lanes 2 and 4) are displayed in Fig. 1. A set of proteins was only present under iron-limiting conditions. The apparent molecular mass of these iron-regulated OM proteins (IROMPs) was 46, 50, 77, 82, 84 and 86 kDa, four of them (77, 82, 84 and 86 kDa) having a size range in agreement with ferrisiderophore receptor function [33]. These proteins were present for both the wild-type and the pvd$^-$ mutant during iron-stress.

The affinity of the respective IROMP-containing OMs of C7R12 and PL1 for the purified pyoverdine of C7R12 (PVD_{C7R12}) was compared during binding experiments. Although both OMs from C7R12 and PL1 showed an affinity for PVD_{C7R12}, this affinity was lower in PL1 than in C7R12: binding of [^{59}Fe]PVD_{C7R12} to purified OMs represented 4266 cpm per 100 μg of proteins (standard deviation 148) for C7R12 and only 3211 cpm per 100 μg of proteins (standard deviation 112) for PL1.

In the same way, the cells from both strains incorporated [^{59}Fe]PVD_{C7R12} but at a significant lower rate in PL1 than in C7R12 (Fig. 2). Besides the homologous pyoverdine PVD_{C7R12} (35), C7R12 and PL1 were shown to incorporate five other pyoverdines of foreign origins (1, 20, 31, 33 and 34). Three of these were structurally different and did not resemble the pyoverdine produced by C7R12 (20, 31 and 33) ([34,35], Meyer, J.M. and Budzikiewicz, H., unpublished results). The two others (1 and 34), as well as PVD_{C7R12} (35), remain structurally unknown. As for PVD_{C7R12}, the rate of incorporation for each of these heterologous pyoverdines was lower in PL1 than in C7R12. To assess the iron status of PL1, growth of this mutant was compared with that of strain C7R12 when grown at different levels of iron availability. The growth of both strains was higher in SM supplemented with 100 μM iron (Fig. 3B) than in non-amended SM (Fig. 3A), indicating, as previously described [36], that the SM medium is iron-limiting for fluorescent pseudomonads. Under these iron-stress conditions (SM), the growth of PL1 was delayed compared to the wild-type C7R12 (Fig. 3A) and pyoverdine was only synthesized by C7R12 (data not shown). In contrast, under non-iron-stress conditions (SM supplemented with 100 μM iron), the growth kinetics of C7R12 and PL1 were similar (Fig. 3B). The addition of 50 μM PVD_{C7R12} to SM led (i) to an increase of the growth rates of both strains and (ii) to a decrease of the delay of PL1 growth compared to C7R12 (Fig. 3C).

3.2. IPCR sequence analysis

In order to identify the DNA sequence flanking the inserted Tn5 transposon, an IPCR was performed. The PCR resulted in the amplification of a single DNA fragment of the expected size of 1.2 kb. Subsequent sequence analysis of the IPCR product showed the presence of a 250-bp sequence belonging to Tn5 which validates the procedure. The remaining 946-bp sequence flanking the 5′ end of the inserted Tn5 shows a high level of identity (73.5%) to the pyoverdine synthetase B (*pvs*B, position 4431–5385) of *P. fluorescens* 17400 recently registered by Mossialos et al. (EMBL accession no. AF237701).

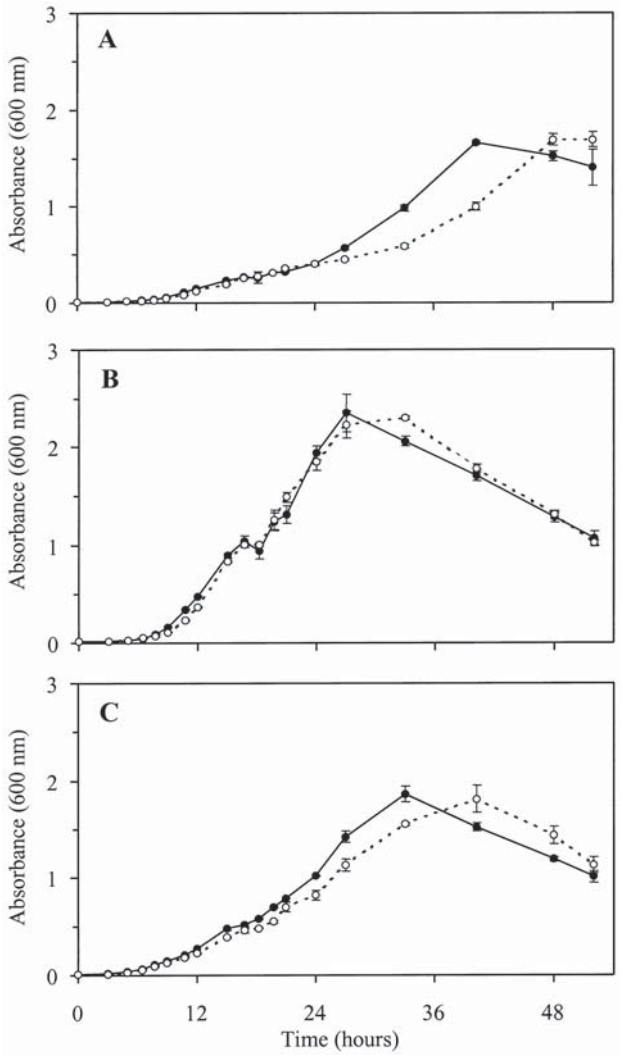

Fig. 3. Growth kinetics of C7R12 (-●-) and of PL1 (- -○- -) in the iron-limiting SM (A), in SM supplemented with 100 μM $FeCl_3$ (B) or with 50 μM PVD_{C7R12} (C). Error bars not displayed are included in the symbols.

3.3. Survival kinetics in soil and rhizosphere

The growth kinetics of C7R12 and PL1, either inoculated separately or in combination, were compared in bulk soil and in rhizosphere soil under non-gnotobiotic conditions (Fig. 4) and under gnotobiotic conditions (Fig. 5).

In non-gnotobiotic systems (Fig. 4), the density of the introduced strains increased during the first 2 days and then decreased at further sampling points. The initial increase was more strongly expressed in the rhizosphere (Fig. 4C,D) than in the bulk soil (Fig. 4A,B). The survival kinetics of the wild-type and the mutant were similar under all experimental conditions.

In gnotobiotic systems (Fig. 5), where the density of contaminants was below the detection limit ($< 10^2$ CFU g^{-1} of dry soil) as indicated from the uninoculated containers, the density of the introduced strains increased during the first 2 days and then reached a plateau. No difference could be recorded between the growth kinetics of the wild-type and the mutant when inoculated separately in both bulk and rhizosphere soil (Fig. 5A,C). In contrast, the plateau reached by PL1 was significantly lower than that of C7R12 when inoculated in combination into both bulk and rhizosphere soils (Fig. 5B,D). The lower level of the plateau for PL1 compared to C7R12 was ascribed to a lower growth rate of the mutant. The difference between the plateau of C7R12 and of PL1 was more pronounced in the bulk soil than in the rhizosphere. Thus the growth rate of PL1 in competition with C7R12 was more affected in the bulk soil than in the rhizosphere. When C7R12 and PL1 were inoculated separately or in combination, the carrying capacities of the rhizosphere and bulk soils were similar.

4. Discussion

Pyoverdine has been shown to be involved in the microbial antagonism performed by fluorescent pseudomonads against some pathogens. Therefore, pyoverdine-mediated antagonism may also be involved in the bacterial competitivity in soil habitats where Fe^{3+} is a limiting element [7].

In order to assess this hypothesis, a mutant, affected in the pyoverdine synthesis, of a rhizosphere competent model strain was selected after random insertion of Tn5. The similarity of the growth kinetics in vitro of the wild-type and of the pvd^- mutant, under non-iron-limiting conditions (Fig. 3B), suggests that besides the pyoverdine-mediated iron uptake no other bacterial trait was affected by the mutagenesis. This observation is in agreement with the single insertion of the Tn5 in a biosynthetic gene of pyoverdine as indicated by the high level of identity of the flanking DNA with a pyoverdine synthetase (*pvs*B).

PL1 was able to synthesize at least one other siderophore as shown by the positive reaction on CAS, indicating that C7R12 too is able to produce at least an additional siderophore. Other strains of fluorescent pseudomonads have previously been shown to produce siderophores other than pyoverdine such as pyochelin [39], salicylic acid [40] or quinolobactin [41]. Despite the initial lag in growth of PL1, the similar final density of both strains, when cultivated in iron-limiting succinate medium (Fig. 3A), could be due to the synthesis of a second siderophore by the mutant PL1. However, the efficacy of iron uptake mediated by these other siderophore(s) is significantly lower than the one mediated by pyoverdine. Indeed, PL1 is a lot more susceptible to iron starvation, as evaluated by EDDHA MIC, than the pyoverdine-producing wild-type. Besides having an impaired synthesis of pyoverdine, PL1 was shown to be affected in the rate of Fe-pyoverdine uptake, despite the similarity of its iron-regulated protein pattern compared to the wild-type. Iron-regulated phenotypes were previously shown to be

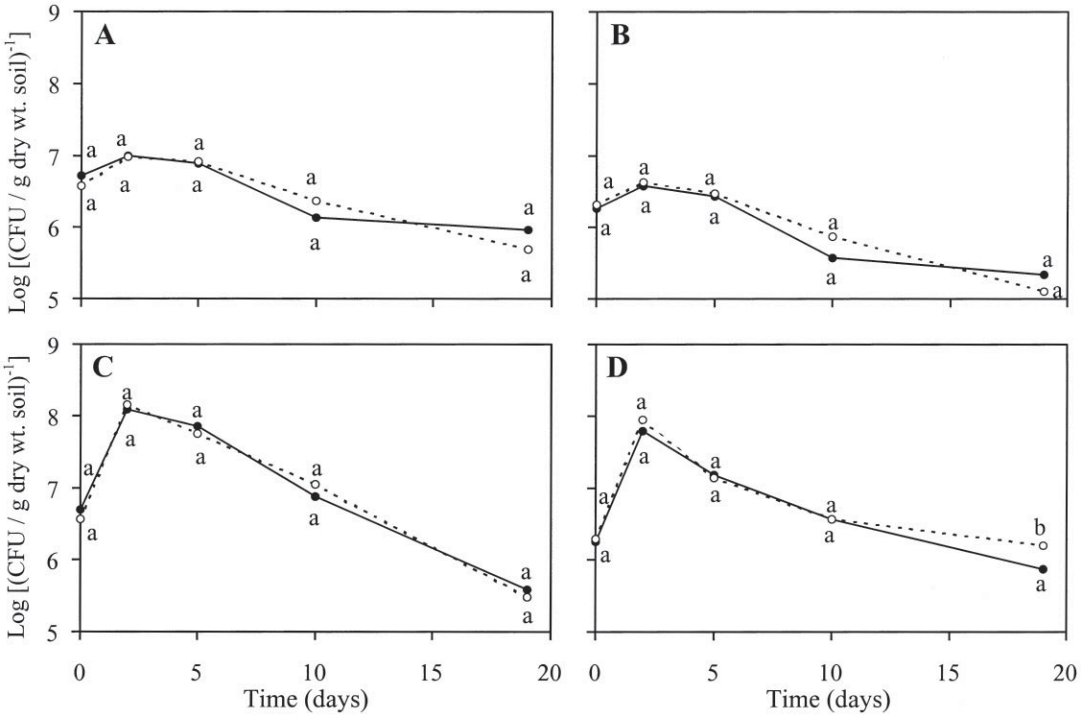

Fig. 4. Growth kinetics of C7R12 (-●-) and PL1 (- -○- -) in the bulk soil separately (A) or in combination (B), and in the rhizosphere separately (C) or in combination (D), under non-gnotobiotic conditions. For the same date, means designated with the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test.

affected by the inactivation of genes required for the pyoverdine biosynthesis [38]. In the present study, the reduced rate of Fe-pyoverdine uptake could be ascribed to a decreased expression of the iron-regulated genes encoding membrane receptor proteins, these genes are furthermore known to be located closely to the pyoverdine biosynthetic genes [37].

Under non-gnotobiotic conditions, the survival kinetics

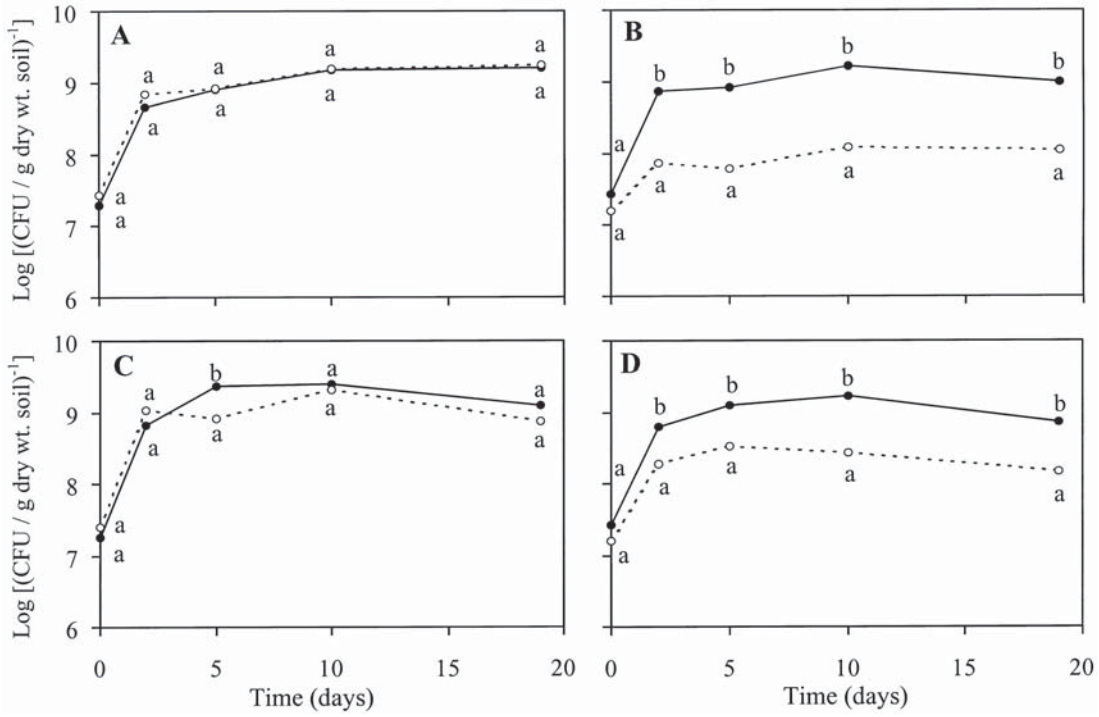

Fig. 5. Growth kinetics of C7R12 (-●-) and PL1 (- -○- -) in the bulk soil separately (A) or in combination (B), and in the rhizosphere separately (C) or in combination (D), under gnotobiotic conditions. For the same date, means designated with the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test.

of C7R12 and of PL1 in soil and rhizosphere, when introduced separately or in combination, were similar (Fig. 4). These results suggest that the iron uptake in soil of PL1 was as efficient as the one by C7R12. Since PL1 produces no pyoverdine and since the iron uptake mediated by the alternative siderophore(s) is less efficient than the one mediated by pyoverdine, as indicated by the MIC of ED-DHA of PL1 compared to C7R12, the similar survival of PL1 and C7R12 could only be ascribed to the ability of PL1 to incorporate heterologous pyoverdines. Indeed, PL1 and C7R12 were shown to have the exceptional ability to incorporate five heterologous pyoverdines of different structures (Fig. 2). Since protein membrane receptors of pyoverdines are known to be usually highly specific [10], the ability of C7R12 and PL1 to use a wide range of pyoverdines could be related to the presence of multiple iron-regulated membrane proteins as shown in the present study (Fig. 1). The ability of PL1 to incorporate several heterologous pyoverdines would enable the pvd$^-$ mutant to compete as efficiently as the wild-type C7R12 with the indigenous microflora, despite the lower rate of Fe-pyoverdine uptake by PL1 compared with C7R12. The possible ecological advantage to bacteria conferred by the ability to utilize a large variety of siderophores was previously suggested by Jurkevitch et al. [42]. This hypothesis was recently supported by the data of Loper and Henkels [43], showing, with the help of the ice nucleation reporter gene *inaZ*, that bacteria producing heterologous pyoverdines enhanced the iron availability for an introduced mutant fluorescent pseudomonad impaired in pyoverdine synthesis but still able to use heterologous pyoverdines. These authors further reported a lower transcription of pyoverdine genes in an introduced strain of *Pseudomonas putida* (N1R) in the presence than in the absence of the indigenous microflora. As shown in the present study, under non-gnotobiotic conditions, iron uptake would then be mostly based on the incorporation of heterologous pyoverdines so that the ability to synthesize pyoverdine does not give a competitive advantage to the wild-type over the pvd$^-$ mutant. Although the similarity of the survival of the pvd$^-$ mutant PL1 compared to the wild-type strain C7R12 could be ascribed to its ability to incorporate iron from heterologous pyoverdines in a soil known for its low iron availability [44], the possible implication of limiting factors other than iron such as carbon cannot be ruled out.

In order to exclude the possible presence of any heterologous pyoverdines, further experiments were performed in gnotobiotic experimental systems (Fig. 5). When inoculated separately, the survival of the two strains was similar (Fig. 5A,C), suggesting that, in the absence of any microbial competitor, the iron was available at a sufficient level to allow for its uptake by the non-identified siderophore(s) of PL1. In contrast, when inoculated together with C7R12 (Fig. 5B,D), the initial growth of PL1 was significantly lower than that of the wild-type strain. Subsequently, the mutant was maintained at a significantly lower level than the wild-type strain. The lower competitivity of PL1 could be ascribed to the lack of synthesis of pyoverdine which was shown to mediate a more efficient iron uptake than the non-identified siderophore(s) produced by both strains. But, since PL1 is able to incorporate the pyoverdine from C7R12, PL1 would be expected to survive in a similar way as C7R12. Indeed, several studies have reported an increased survival of pvd$^-$ mutants when combined with strains producing pyoverdines that can be incorporated by the mutants [45,46]. Raaijmakers et al. [47] have even suggested from their results the possibility to increase the fitness of fluorescent pseudomonads by including, in their genomes, genes encoding additive OM proteins. In the present study, although the pvd$^-$ mutant PL1 remained able to incorporate the homologous pyoverdine from C7R12, the rate of this incorporation was significantly lower in the pvd$^-$ mutant than in the wild-type (Fig. 2). Consequently, the growth kinetics of PL1, in iron-limiting succinate medium, was delayed compared to that of the wild-type even in the presence of large amounts of purified pyoverdine from C7R12 (Fig. 3C). The lower fitness of the mutant PL1 compared to the wild-type C7R12 when coinoculated under gnotobiotic conditions could therefore be ascribed to the lower rate of incorporation of the homologous pyoverdine PVD_{C7R12}.

Altogether, our data indicate the importance of the pyoverdine-mediated iron uptake and more specifically pyoverdine incorporation on the fitness of the biocontrol agent *P. fluorescens* C7R12. Indeed, this strain produces at least another siderophore in addition to pyoverdine and moreover C7R12 displays the exceptional ability to incorporate at least five heterologous pyoverdines. These traits enabled a pvd$^-$ mutant to display a competitivity similar to the wild-type under natural conditions. Other strains of fluorescent pseudomonads such as *P. putida* WCS358 were also shown to utilize pyoverdines from other strains of pseudomonads [46]. However, to our knowledge, very few strains have been demonstrated to be able to incorporate as many heterologous pyoverdines exhibiting a range of different structures as *P. fluorescens* C7R12.

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