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PLANT MICROBE INTERACTIONS

Identification of Traits Shared by Rhizosphere-Competent Strains of Fluorescent Pseudomonads

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Abstract Rhizosphere competence of fluorescent pseudomonads is a prerequisite for the expression of their beneficial effects on plant growth and health. To date, knowledge on bacterial traits involved in rhizosphere competence is fragmented and derived mostly from studies with model strains. Here, a population approach was taken by investigating a representative collection of 23 *Pseudomonas* species and strains from different origins for their ability to colonize the rhizosphere of tomato plants grown in natural soil. Rhizosphere competence of these strains was related to phenotypic traits including: (1) their carbon and energetic metabolism represented by the ability to use a wide range of organic compounds, as electron donors, and iron and nitrogen oxides, as electron acceptors, and (2) their ability to produce antibiotic compounds and *N*-acylhomoserine lactones (*N*-AHSL). All these data including origin of the strains (soil/rhizosphere), taxonomic identification, phenotypic cluster based on catabolic profiles, nitrogen dissimilating ability, siderovars, susceptibility to iron starvation, antibiotic and N-AHSL production, and rhizosphere competence were submitted to multiple correspondence analyses. Colonization assays revealed a significant diversity in rhizosphere competence with survival rates ranging from approximately 0.1 % to 61 %. Multiple correspondence analyses indicated that rhizosphere competence was associated with siderophore-mediated iron acquisition, substrate utilization, and denitrification. However, the catabolic profile of one rhizosphere-competent strain differed from the others and its competence was associated with its ability to produce antibiotics phenazines and N-AHSL. Taken together, these data suggest that competitive strains have developed two types of strategies to survive in the rhizosphere.

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Introduction

Several strains of fluorescent *Pseudomonas* species are known to improve plant health and/or growth [24, 26, 35]. They have been shown to play an important role in the natural suppressiveness of soils to *Fusarium* wilts, to take all disease of wheat and to tobacco root rot [39, 77]. Hence, fluorescent pseudomonads are considered as potential biocontrol agents of soilborne diseases, and several greenhouse and field studies have demonstrated their efficacy as microbial inoculants [16, 36, 70]. Despite the positive effects

reported in these and other studies, overall biological control of soilborne diseases achieved by fluorescent pseudomonads is often inconsistent [37, 76]. This inconsistency has been associated with the lack of expression of the key biocontrol traits at the right place and time and with inefficient root colonization by the introduced strains [42]. Indeed, a clear relationship has been established between the level of disease suppression and the population densities of these bacteria in the rhizosphere of the corresponding host plant [7, 42, 61, 63]. To improve and expand the consistency of biological control, selection of rhizosphere-competent strains is essential. Consequently, progress must be made toward a better understanding of bacterial traits and genes involved in rhizosphere-competence of fluorescent pseudomonads.

Many efforts have been made over the past decades to identify traits involved in rhizosphere competence. One of the most commonly used strategies involves generation and characterization of mutants of *Pseudomonas* strains that are defective in specific phenotypic traits [reviewed in 42]. Other strategies adopted are the in vivo expression technology (IVET) [64, 65] and microarray-based expression profiling [43, 44]. Collectively, these and other studies have revealed a number of traits, including flagella, O-antigenic side chain of lipopolysaccharides, nitrate reductase, phenazines, siderophores, surfactants, amino-acids transport and metabolism, and oxidative stress resistance that are important in the rhizosphere competence of specific *Pseudomonas* strains. These studies were mostly conducted in gnotobiotic conditions. Furthermore, whether the traits identified are specific for these model *Pseudomonas* strains only or also apply to other beneficial *Pseudomonas* species and strains has not been addressed in much detail. To examine this question, a population-based approach to uncover traits involved in rhizosphere competence in the presence of indigenous microflora is required. This type of approach was initiated in the early nineties by comparing the diversity of indigenous populations of fluorescent pseudomonads associated with different soils and roots of different plant species [8, 33, 34, 41]. The strategy as summarized by Latour et al. [33] first consisted in comparing indigenous populations associated with roots and bulk soils in order to identify traits that discriminate these two types of populations [8, 34, 41]. These traits are expected to be involved in the rhizosphere competence. Comparable studies were performed by Mavingui et al. [45], Glandorf et al. [23], Frey et al. [17], Garbeva et al. [21], Bergsma-Vlami et al. [4], and several other research groups [reviewed in 3]. Collectively, these studies showed that host plant and even cultivars within a given plant species [49, 59] harbor or select for specific populations of fluorescent pseudomonads.

The aforementioned approach allowed us to show that, compared to those from soils, pseudomonads from the rhizosphere were (1) more efficient in mobilizing ferric iron, (2) more frequently nitrate reducers and denitrifiers, and (3) able to use specific organic compounds as carbon and energy sources [33]. Since the two first traits were shared by populations selected by two plants species in two soils [34], their implication in rhizosphere adaptation was then evaluated using a model strain approach that consisted of comparing rhizosphere competitiveness of the model strain *Pseudomonas fluorescens* C7R12 to that of mutants affected in their ability to synthesize pyoverdine and/or nitrate reductase [56, 57]. The use of these mutants confirmed the involvement of the two above traits in the rhizosphere competence of the model strain. However, since some discrepancies occurred between studies with different strains in the past [10, 28, 69], conclusions drawn from our model strain *P. fluorescens* C7R12 may well be not applicable to others. More recent studies [1, 30] support the fact that the ability of bacteria to utilize specific root exudate constituents determines, in part, their rhizosphere competence. Again, these studies refer to model strains and therefore may not necessarily be representative of bacterial traits involved in rhizosphere competence of other pseudomonads.

The overall objective of this study was to take a population approach and to search for traits shared by rhizosphere competent strains of fluorescent pseudomonads. To this end, we investigated a collection of *Pseudomonas* species and strains for their ability to colonize the rhizosphere of tomato plants grown in non-sterile soil. The strains tested were selected from our early diversity studies and were chosen to be representative of the populations associated with soils and roots of different crops [34, 41]. The rhizosphere competence of these strains was related to a number of phenotypic traits, including substrate utilization, nitrogen dissimilation, siderophore-mediated iron acquisition, and the production of antibiotics and *N*-acylhomoserine lactones.

Materials and Methods

Bacterial Strains

The *Pseudomonas* strains used in this study are listed in Table 1 and include: (1) two reference strains, i.e., *P. fluorescens* strains C7R12 and A6; and (2) 21 strains including six strains isolated from soils (Dijon or Châteaurenard, France), nine strains from the rhizosphere of flax (*Linum usitatissimum* L. cv. Opaline), and six strains from the rhizosphere of tomato (*Lycopersicon esculentum* Mill., cv. H63-5) cultivated in these soils [34, 41]. The two reference strains have biocontrol and plant growth-promoting activities [5, 20, 36, 68]. The 21 strains were selected from genotypes defined previously on a whole cell rep-PCR

Table 1 Presentation of the fluorescent pseudomonad*s* analyzed in this study and of the variables and modalities submitted to multiple correspondence analysis

Strain	Origin	Species and biovars	Phenotypic cluster[a]	Dissimilating ability[b]	Siderovar[c]	MIC of EDDHA ppm[d]	N-AHSL production[e]	Phenazine production[f]	Survival rate[g] %
DTR133	Rhizosphere (tomato)	*P. chlororaphis*	1	NR	16	1,000 (3)	C6-HSL (+) 3-oxo-C6HSL	+	15.95 (G3)
C7R12	Rhizosphere (flax)	*P. fluorescens* bv. II	2	TDe	1	125 (2)	(−)	−	2.3 (G2)
CTR212	Rhizosphere (tomato)	*P. fluorescens* bv. II	8	TDe	9	1,000 (3)	(−)	−	61.04 (G5)
CTR1015	Rhizosphere (tomato)	*P. fluorescens* bv. II	2	TDe	9	500 (3)	(−)	−	32.98 (G4)
A6	Rhizosphere (bean)	*P. fluorescens* bv. VI	3	ND	2	500 (3)	(−)	−	0.42 (G1)
CLR711	Rhizosphere (flax)	*P. fluorescens* bv. VI	3	ND	7	1,000 (3)	C6-HSL (+)	−	1.28 (G2)
CTRp112	Rhizosphere (tomato)	*P. fluorescens* bv. VI	3	ND	8	500 (3)	(−)	−	0.11 (G1)
DLR228	Rhizosphere (flax)	*P. jessenii*	7	NR	21	125 (2)	(−)	−	3.40 (G2)
DLE3216	Rhizosphere (flax)	*P. jessenii*	7	NR	21	125 (2)	(−)	−	0.39 (G1)
DLR223	Rhizosphere (flax)	*P. jessenii*	5	ND	21	31.25 (1)	(−)	−	3.5 (G2)
DS131	Bulk soil	*P. jessenii*	3	ND	21	4 (1)	(−)	−	2.66 (G2)
DS824	Bulk soil	*P. jessenii*	6	ND	21	62.5 (1)	(−)	−	4.06 (G2)
DS1026	Bulk soil	*P. jessenii*	5	ND	21	31.25 (1)	(−)	−	3.6 (G2)
DTRp621	Rhizosphere (tomato)	*P. jessenii*	7	NR	21	125 (2)	(−)	−	5.04 (G3)
CLRp812	Rhizosphere (flax)	*P. lini*	2	TDe	9	125 (2)	(−)	−	12.54 (G3)
CLE513	Rhizosphere (flax)	*P. lini*	2	TDe	9	62.5 (1)	(−)	−	25.52 (G4)
CS611	Bulk soil	*P. lini*	2	TDe	9	125 (2)	(−)	−	33.38 (G4)
DLE411J	Rhizosphere (flax)	*P. lini*	2	TDe	31	125 (2)	(−)	−	7.2 (G3)
DLR426	Rhizosphere (flax)	*P. lini*	2	TDe	9	125 (2)	(−)	−	7.4 (G3)
DLRp214	Rhizosphere (flax)	*P. lini*	4	TDe	9	125 (2)	(−)	−	8.58 (G3)
DTR335	Rhizosphere (tomato)	*P. lini*	2	TDe	9	31.25 (1)	Unidentified (+)	−	39.28 (G4)
CS111	Bulk soil	*P. putida bv. A*	3	ND	5	250 (2)	(−)	−	0.20 (G1)
CS413	Bulk soil	*P. putida bv. A*	3	ND	5	250 (2)	(−)	−	1.82 (G2)

[a] The seven clusters as identified in Fig. 1 correspond to the modalities of this variable

[b] The modalities of this variable are *ND*, non dissimilator; *NR*, nitrate reducer; *TDe*, total denitrifier

[c] The eight siderovars as defined in Table 2 correspond to the modalities of this variable

[d] Values in brackets correspond to the three modalities of this variable defined as follows 1, [4–62.5 μg ml^{-1}]; 2, [125–250 μg ml^{-1}]; 3, [500–1,000 μg ml^{-1}]

[e] The two modalities of this variable correspond to the ability (+) or the inability (−) of the strains to synthetize N-AHSL

[f] Based on both PCR and RP-HPLC analysis

[g] The modalities (competitivity groups) of this variable are indicated in brackets and are defined as follows: group 1 (G1)<1 %, group 2 (G2) [1–5 %[, group 3 (G3) [5–20 %[, group 4 (G4) [20–40 %[, group 5 (G5)≥40 %

fingerprinting method and 16S rRNA PCR-RFLP profiles of a collection of 340 strains isolated from two soil types and two rhizospheres [34, 41]; these strains are therefore considered as representative of the diversity of this collection. Taxonomic classification of the collection of strains and their ability to dissimilate, nitrogen, and to produce *N*-acyl-homoserine lactones (N-AHSL) were described previously [11, 13, 34, 41] and are presented in Table 1.

Catabolic Profiling

Carbon utilization profiles of the bacterial strains were evaluated by using the API-Biotype 100 strips (API Systems, La Balme les Grottes, Montalieu-Vercieu, France). The tests were performed according to the recommendation of the manufacturer. Briefly, strains were cultivated for 48 h on King's B (KB) agar medium [31], cells were harvested in sterile distilled water, and suspensions were prepared in API Biotype Medium to turbidities equivalent to the no. 3 McFarland standard and then inoculated into test strips. Assimilation reactions at 25 °C were scored 1 (for positive) and 0 (for negative) after 48 h. The similarity matrix between bacterial isolates was calculated by using the Jaccard coefficient [29]. Cluster analysis was performed by applying the unweighted pair-group method with averages [UPGMA; 73].

Siderophore Typing

Pyoverdine isoforms produced during bacterial growth (24 h at 25 °C, 200 rpm) in casamino-acid medium (CAA; 5 g/l of casamino acids with low chloride and iron content (Difco, Lawrence, KS), 1.2 g/l of K_2HPO_4, and 0.25 g/l of $MgSO_4$, $7H_2O$), were subjected to isoelectrophoresis according to the method of Koedam et al. [32] and adapted by Fuchs et al. [19]. Culture supernatants (400 µl) were lyophilized, and the dried residue was dissolved in 20 µl sterile distilled water. The BioRad model 111 mini IEF cell apparatus was used with electrophoresis conditions as recommended by the manufacturer. Polyacrylamide gels (5 % *w*/*v*) containing ampholines (BioLyte 3/10; BioRad Hercules, CA) were prepared according to the manufacturer's recommendations and loaded with 1 µl samples of 20-fold concentrated CAA culture supernatants. Pyoverdines were visualized under UV light and analyzed for their corresponding isoelectric pH values (pHi) as previously described [19].

Purification of pyoverdines for iron uptake studies was done through the XAD-4 Amberlite chromatographic procedure [53]. Preparation of the [^{59}Fe]-pyoverdine complexes from the XAD purified pyoverdines was performed as described by Meyer et al. [55] by mixing a dilution of a commercial solution of $^{59}FeCl_3$ (Amersham) with a solution of XAD-purified pyoverdine solution (PVD:iron ratio >20). Experimental conditions of the iron uptake experiments are similar to those described by Meyer et al. [55]. Briefly, bacterial strains were cultivated for 40 h at 25 °C in succinate medium [52], harvested by centrifugation, washed, and resuspended at OD600=0.3 in succinate medium without the nitrogen source. Cell suspension was preincubated for 10 min at 25 °C before the addition of the labelling mixture. After 20 min, 1 ml samples were filtered onto membranes (0.45 µm, Whatman) and washed twice with 2 ml nitrogen-free succinate medium. Cell-associated radioactivity was counted in a Gamma 4000 Beckman counter.

Grouping of strains into siderovars was based on the two criteria of Meyer et al. [54], i.e., strains that have an identical pyoverdine system for iron uptake, as attested by an identity in pyoverdine-IEF pattern, and are highly efficient in acquisition of iron from the same pyoverdine(s) were classified in the same siderovar group. For strains that were deficient in pyoverdine production (i.e., strains CTR212 and DS824 in the present study), siderovar classifications was done based on the results of the iron uptake studies only.

Susceptibility to Iron Stress

The ability of the strains to grow under iron stress conditions was assessed with a test adapted from Lemanceau et al. [40]. In short, the minimal concentration of EDDHA at which bacterial growth was completely suppressed was determined for each strain. KB agar medium was supplemented with 0, 4, 8, 16, 31.25, 62.5, 125, 250, 1,000 µg/ml of EDDHA (Sigma, Saint Louis, Missouri), chelated or not with iron. For each strain and each EDDHA concentration, 20 µl of a bacterial suspension (10^7 CFU/ml) were inoculated and incubated for 72 h at 25 °C after which growth was assessed visually. Experiments were duplicated.

Antibiotic Production

The presence of the phenazine biosynthesis genes *phzCD* was investigated both by PCR and Southern hybridization according to the methods described by de Souza et al. [9]. PCR reactions were conducted in a 25 µl-reaction volume. Reaction mixtures contained 3 µl of cell suspension [41] and 1 U of Taq DNA polymerase (Q-Biogen, Illkirch, France) in the corresponding buffer (10 mM Tris–HCl, pH 9.0 at 25 °C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 0.2 mg/ml BSA, Q-Biogen, Illkirch, France). Final concentrations of each primer and of dNTPs were 0.8 and 200 µM, respectively. DMSO was added to a final concentration of 3.5 % (*v*/*v*). Amplification were performed in a thermal cycler (GeneAmp PCR system 9600, Perkin-Elmer, Applied Biosystems, Forster City, CA), with an initial denaturation step (3 min at 94 °C) followed by 30 cycles (1.5 min at 94 °C; 1 min at 68 °C; and 1.5 min at 72 °C),

and a final extension step (3 min at 72 °C). *P. fluorescens* strain 2–79, a well-known phenazine producer [46], was included as a positive control. Aliquots of the amplification products were analyzed by electrophoresis in 0.8 % agarose gel, stained with ethidium bromide, and photographed under UV illumination. Southern hybridization on total genomic DNA and on amplified PCR products was performed according to the methods described by Souza et al. [9] using a digoxigenin-labelled probe prepared from the PCR product obtained from *P. fluorescens* strain 2–79.

Production of phenazine compounds was investigated according to the methods described by de Souza et al. [9]. Strains were grown in 4.0 ml of liquid KB medium for 4 days at 25 °C. Cultures were acidified to pH 2.0 with trifluoroacetic acid (TFA, 0.1 % *v/v* final concentration) and the phenazine compounds were extracted twice with two volumes of ethylacetate. Organic fractions were pooled and evaporated to dryness under vacuum, and resuspended into 1 ml of 100 % MeOH. Phenazines were separated by HPLC, on a C_{18}-reverse phase column fitted onto a Waters HPLC 600 system coupled to a Waters 996 photodiode detector. In the designated solvent system, phenazines exhibit typical peak maxima at 248 and 367 nm. In addition to phenazine antibiotics, extracts of the culture supernatants were also screened for other known antibiotics, including monoacetylphloroglucinol (MAPG; peak maxima at 285 and 330 nm), 2,4-diacetylphloroglucinol (2,4-DAPG; peak maxima at 270 and 330 nm), and pyoluteorin (PLT; peak maxima at 255 and 308 nm).

Rhizosphere Competence Assays

Twenty spontaneous rifampin resistant derivatives were obtained for each of the 23 strains. Amongst each series of 20 mutants, one rifampin resistant mutant was selected for each strain following a general strategy previously applied [15, 18, 22]. Briefly, this selection was based on (1) the similarity of the growth kinetics of the rifampin-resistant derivative and the corresponding wild-type strain, when cultivated in succinate liquid medium [52], (2) the similarity of the catabolic profiles of the rifampin-resistant derivative and the corresponding wild-type strain as assessed by the Biotype 100 strips (see above); (3) the stability of the rifampin resistance, as evaluated by following 15 successive sub-cultures of the derivative on KB agar medium supplemented or not with rifampin (100 mg/l); and (4) the competitive ability of the rifampin resistant derivative when co-inoculated with the corresponding wild-type strain, as measured by the survival kinetics of both strains in KB broth.

Evaluation of the rhizosphere competence of each of the 23 rifampin-resistant derivatives was tested on tomato seedlings (*Lycopersicon esculentum* Mill., cv. H63-5) cultivated in the soil of Châteaurenard (France). The physico-chemical characteristics of this soil have been described previously [34]. The bioassay conditions described by Steinberg et al. [74] were slightly modified. Briefly, 11 g of non-sterile Châteaurenard soil were introduced into a 10-ml syringe fitted with a cotton plug. The syringe was transferred into a test tube containing water; the cotton plug allowed provision to the soil sample of sufficient water to maintain constant soil moisture (near its water-holding capacity). Tomato seeds were surface-sterilized in 1.25 % (*v/v*) solution of NaOCl for 20 min, washed three times with sterile distilled water, and germinated on wet sterile filter paper at 25 °C for 48 h. Five tomato seedlings were transferred per microcosm. Each bacterial mutant was introduced in the microcosms at a density of 10^7 CFU/g dry soil. Plants were grown for 22 days in a growth chamber (16 h light, 25 °C; 8 h dark, 22 °C) and population densities of each of the introduced strains were enumerated on 0 and 22 days after inoculation for five independent replicates (microcosms) per mutant and date, as follows. Bacteria were extracted by blending the content of the syringe which was fully colonized by roots at day 22, therefore corresponding to rhizosphere soil and roots, for 1 min in 89 ml of sterile water with a Warring blender. Appropriate dilutions of the suspensions were spread onto KB agar medium supplemented with rifampin (100 mg/l) and cycloheximide (100 mg/l). The bacterial densities were expressed as CFU/g dry soil. For each strain, the densities at day 22 were expressed as the percentage of the initial densities measured at day 0. In the Châteaurenard soil, indigenous populations naturally resistant to rifampin were smaller than 10^2 CFU/g and did not interfere in monitoring the population densities of the introduced strains.

Data and Statistical Analyses

Survival rates (percent) calculated as described above were ranked in groups arbitrarily defined as follows: group 1 (G1)<1 %, group 2 (G2) [1–5 %[, group 3 (G3) [5–20 %[, group 4 (G4) [20–40 %[, group 5 (G5) ≥40 %. Multiple correspondence analysis (MCA) [2, 25] was applied to relate the rhizosphere competence groups to eight parameters tested: i.e., origin, species, phenotypic cluster, nitrogen dissimilation, siderovar, susceptibility to iron stress, N-AHSL production, antibiotic production (Table 1). The statistical analyses were performed with SAS 8.2 software (SAS/STAT Version 8. Cary, NC). The multiple correspondence analysis (PROC CORRESP) carried out distinguished (1) seven active variables: origin, phenotypic cluster, dissimilating ability, siderovar, MIC of EDDHA, N-AHSL, phenazines, for which two, nine, three, nine, three, two, two modalities were identified, respectively, and (2) two supplementary variables (taxonomic identification and competitivity group) for which five modalities were identified. All the data from each type of measurements, for each strain were displayed in a matrix shown in Table 1.

Results

Substrate Utilization

Full information on substrate utilization by the strains tested is provided in Table S1 (supplementary material). Numerical analysis showed that the level of similarity between the 23 strains tested ranged from 0.62 to 0.97 (Fig. 1). At a similarity of 0.82 or higher, eight phenotypic clusters were delineated: four clusters included at least two strains, while the other four only included one strain. Two clusters (1 and 8) were distant from the others and from each other, whereas the remaining clusters could distributed in two clades at a similarity level 0.76. One (A) included clusters 2, 3, and 4 and the other (B) clusters 5, 6, and 7. The clade A differed mainly from the other and from the two distant clusters by its ability to use D-glucuronate (12 out of 15 strains) and D-galacturonate (14 out of 15 strains). Cluster 3 could be differentiated from the other two (clusters 2 and 4) by its unability to use sorbitol; and cluster 4 by its unability to use D(+)-arabinol and D-glucosamine. The clade B only encompassed strains belonging to *Pseudomonas jessenii*. Within this clade, cluster 7 could be differentiated for the other two (5 and 6) by its ability to use L(+)-arabinose and the unability to use myo-inositol.

The two distant clusters (1 and 8) only included one strain each, *Pseudomonas chlororaphis* DTR133 and *P. fluorescens* CTR212, respectively. Cluster 8 differed from all the other clusters by its ability to use a limited range of compounds (33) among the 99 tested and was especially the only one to not use D-manitol, D-saccharate, protocacechuate, parahydroxybenzoate, and serine. Cluster 1, including the only strain of *P. chlororaphis*, was the sole cluster to use i-erythritol, 5-keto-D-gluconate, L-tryptophan, gentisate, and m-hydroxybenzoate.

Siderophore Typing

Strains were classified in eight different groups (PL1, PL5, PL7, PL8, PL9, PL16, PL21, PL31) according to their pyoverdine IEF patterns (Table 2). Four of these IEF correspond to the siderovars described previously by Meyer et al. [55]: A6 syn. PL1, PL7, PL8, and PL9. The two groups with the highest number of strains were PL9 (7 strains) and PL21 (6 strains), which mostly gathered *Pseudomonas lini* and *P. jessenii* strains, respectively. The six remaining groups included only one or two strains each. Two strains (*P. fluorescens* CTR212 and DS824) produced pyoverdines in amounts too low to enable classification based on IEF patterns.

Grouping of strains according to their IEF patterns was supported by pyoverdine-mediated iron uptake studies: strains belonging to a same group incorporated iron sequestered to siderophores of the other strains in their IEF group, whereas this was not the case for siderophores produced by other groups. For these uptake studies, one strain of each IEF group was selected for pyoverdine purification and the corresponding pyoverdines were tested as [^{59}Fe]-pyoverdine complexes in uptake experiments with all 23 strains. Based on these uptake studies, the pyoverdine-deficient strains CTR212 and DS824 were classified in siderovar groups 9 and 21, respectively. Iron incorporation data correlated well with the grouping obtained through isoelectrophoresis with, however, some exceptions. Strains CLR711 and CTRp112 were assigned to different siderovars (7 and 8) on the basis of differences in their IEF patterns despite their ability to incorporate their respective [^{59}Fe]-pyoverdines. This discrepancy between IEF and [^{59}Fe]-pyoverdine incorporation was previously ascribed to the presence of a common receptor-binding motif in these structurally different pyoverdines [51]. Other strains were also able to incorporate other [^{59}Fe]-pyoverdines than their own although at a lower rate. This was the case for *P. fluorescens* C7R12, as previously reported [57], and for strains CS111 and C413 (Table 2). Because of its ability to incorporate others [^{59}Fe]-pyoverdines than *P. fluorescens* A6, strain C7R12 was ranked in a different siderovar (1) than A6 (siderovar 2) despite the similarity of their IEF patterns. Therefore, integration of data on IEF patterns and [^{59}Fe]-pyoverdine incorporation allowed the delineation of nine siderovars for the 23 strains, most of them belonging to siderovar 9 (eight strains) and to siderovar 21 (seven strains).

Susceptibility to Iron Stress

The Minimal Inhibitory Concentration (MIC) of EDDHA that completely inhibited bacterial growth ranged from 4 to 1,000 μg/ml (Table 1), indicating a high degree of polymorphism for this trait. All 23 strains were able to grow at the highest concentration of EDDHA when chelated with iron (data not shown), indicating that the sensitivity of the bacterial strains to EDDHA was related to iron deprivation induced by this chelator. The strains were organized in three groups according to their MIC values. Group 1 included strains with MICs from 4 to 62.5 μg/ml, with strain *P. jessenii* DS131 having the lowest MIC. Group 2 strains had MIC values between 125 and 250 μg/ml, and group 3 contains strains with MIC values between 500 and 1,000 μg/ml. *P. chlororaphis* DTR133 and *P. fluorescens* strains CTR212 and CLR711 had a MIC value of 1,000 μg/ml, indicating a remarkable ability to grow under iron stress conditions.

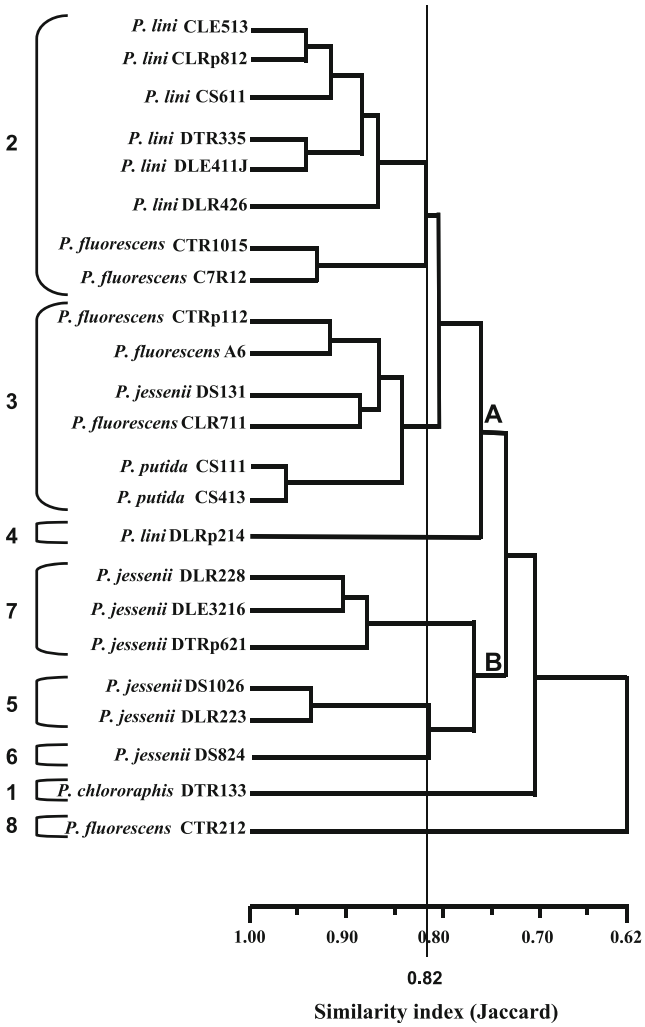

Figure 1 Dendrogram showing the similarities between trophic profiles obtained for the 23 strains. Phenotypic clusters encompassing strains showing a level of similarity at least equal to 0.82 are indicated on the *left inside* of the figure. *A* and *B* designate two clades grouping clusters showing a level of similarity at least equal to 0.76

Antibiotic Production

For all 23 strains, phloroglucinols (MAPG and 2,4-DAPG) and pyoluteorin were not detectable by RP-HPLC and also PCR-based analyses with primers specific for genes involved in the biosynthesis of these antibiotics was negative. PCR analysis with phenazine specific primers resulted in an amplification product of the expected size for control strain *P. fluorescens* 2–79, and for strains CLR711, C7R12, A6, DTR133, DS131, and DLE411J. Subsequent hybridization of the obtained PCR products and of BamHI-digested genomic DNA of these strains with the *phzCD* probe (from control strain 2–79) only yielded a positive signal for strains 2–79, DTR133, and DLE411J. HPLC analysis confirmed the presence of phenazine compounds in the culture supernatants of *P. fluorescens* 2–79 (control strain) and *P. chlororaphis* DTR133. None of these compounds were detected in any other strain culture, including DLE411J and those from which a positive PCR signal was obtained.

Rhizosphere Competence

The survival rate in tomato rhizosphere varied considerably among the strains and ranged from 0.11 % to 61.04 % (Fig. 2; Table 1). Four strains belonging to rhizosphere competence group G1 showed a survival rate below 1 %. Eight strains belonging to group G2 showed a survival rate between 1.28 % and 4.06 %, and six strains belonging to group G3 showed an intermediate level of rhizosphere competence with survival rates between 5.04 % and 15.95 %. Four strains belonging to group G4 exhibited survival rates between 25.52 % and 39.28 %, and only one strain (CTR212) had a survival rate higher than 40 % (61.04 %) and was classified in rhizosphere competence group G5.

Relationships between Competitiveness and Bacterial Traits

The multiple correspondence analyses of the competitivity groups as a function of the seven variables tested (origin, phenotypic cluster, dissimilating ability, siderovar, MIC of EDDHA, N-AHSL, phenazine) is shown in Fig. 3. The first horizontal axis, which explains 17.9 % of the total inertia, is explained by a specific strain DTR133 differing from the others by its taxonomic identification (*P. chlororaphis*), phenotypic cluster (1), siderovar (16), and ability to produce phenazines. Furthermore, this strain is one of the three shown to produce N-AHSL. The second vertical axis, which explains 15 % of the total inertia, opposes groups (G1 and G2) corresponding to a low competitivity (survival rate below 5 %) and groups (G3, G4, and G5) corresponding to a high competitivity (survival rate up to 61.04 %). The opposition between competitivity groups is mostly explained by the phenotypic clusters, dissimilating abilities, siderovars, and to a lower extent by the origins and MIC of EDDHA. More specifically, phenotypic clusters 2, 4, and 8, total denitrifier (TDe), siderovars 9 and 31, rhizospheric origin, MIC of EDDHA 2 (125–250 ppm) are associated with the competitive groups whereas clusters 3, 5, 6, non-dissimilator (ND), siderovars 2, 5, 8, 21, bulk soil origin, MIC of EDDHA 1 (4–62.5 ppm) are with the less competitive ones. Differences in the species (modalities of the supplementary variable taxonomic identification) associated with competitive and less competitive groups were recorded. *P. lini* was associated with the competitive groups G3, G4, and G5, whereas *P. jessenii* and *P. putida* were associated with the less competitive groups G1 and G2. *P. fluorescens* showed an intermediate position.

Statistical significance of the average survival rate for the different modalities was further tested for each variable by

Table 2 Pyoverdine isoelectrofocusing (IEF) patterns and [^{59}Fe]–pyoverdine incorporation in the eight IEF groups of fluorescent pseudomonads as mediated by their pyoverdines

Strains		Pyoverdine		Iron-incorporation as mediated by pyoverdine								Siderovars
		Designation	Isoforms Isoelectric pH	PL1	PL5	PL7	PL8	PL9	PL16	PL21	PL31	
P. chlororaphis	DTR133	PL16	7.75–7.65–5.8–5.6–5.2	−	−	−	−	−	+	−	−	16
P. fluorescens bv. II	C7R12	PL1	9–8.95–7.95	+	−	+/−	+/−	+/−	−	−	−	1
P. fluorescens bv. II	CTR212	PVD (−)	Not detectable	−	−	−	−	+	−	−	−	9
P. fluorescens bv. II	CTR1015	PL9	5.4–4.05	−	−	−	−	+	−	−	−	9
P. fluorescens bv. VI	A6	PL1	9–8.95–7.95	+	−	−	−	−	−	−	−	2
P. fluorescens bv. VI	CLR711	PL7	7.9–5.55	−	−	+	+	−	−	−	−	7
P. fluorescens bv. VI	CTRp112	PL8	9–7.85	−	−	+	+	−	−	−	−	8
P. jessenii	DLR228	PL21	9.1–8	−	−	−	−	−	−	+	−	21
P. jessenii	DLE3216	PL21	9.1–8	−	−	−	−	−	−	+	−	21
P. jessenii	DLR223	PL21	9.1–8	−	−	−	−	−	−	+	−	21
P. jessenii	DS131	PL21	9,1–8	−	−	−	−	−	−	+	−	21
P. jessenii	DS824	PVD(−)	not detectable	−	−	−	−	−	−	+	−	21
P. jessenii	DS1026	PL21	9.1–8	−	−	−	−	−	−	+	−	21
P. jessenii	DTRp621	PL21	9.1–8	−	−	−	−	−	−	+	−	21
P. lini	CLRp812	PL9	5.4–4.05	−	−	−	−	+	−	−	−	9
P. lini	CLE513	PL9	5,4–4.05	−	−	−	−	+	−	−	−	9
P. lini	CS611	PL9	5.4–4,05	−	−	−	−	+	−	−	−	9
P. lini	DLE411J	PL31	7.55–5.3–5.2	−	−	−	−	−	−	−	+	31
P. lini	DLR426	PL9	5.4–4.05	−	−	−	−	+	−	−	−	9
P. lini	DLRp214	PL9	5.4–4.05	−	−	−	−	+	−	−	−	9
P. lini	DTR335	PL9	5.4–4.05	−	−	−	−	+	−	−	−	9
P. putida bv. A	CS111	PL5	9–8.8–7.8	+/−	+	+/−	+/−	−	−	−	−	5
P. putida bv. A	CS413	PL5	9–8.8–7.8	+/−	+	+/−	+/−	−	−	−	−	5

ANOVA. Highly significant differences were found between survival means according to the phenotypic clusters, siderovars, and dissimilating abilities ($P<0.0001$). Although being not significant, differences were also found according to the origin ($P=0.07$), MIC of EDDHA ($P=0.0739$), and NAHL ($P=0.06$), with P values close to the held cut of 0.05 value. In contrast, no significant difference was recorded for phenazine ($P=0.31$). As suggested by the MCA, the survival mean was significantly the highest in strains belonging to the phenotypic cluster 8 (Table 3). In contrast, the strains belonging to the phenotypic clusters 6, 5, 7, and 3 did not differ significantly and showed the lowest survival rates. Phenotypic clusters 2, 1, and 4 had intermediate survival means. Strains with the ability to totally denitrify (TDe) exhibited a significantly higher survival rate compared to the nitrate reducer (NR) and non-dissimilator (ND) strains (Table 3). Strains belonging to siderovars 9 and 16 survived significantly better in the rhizosphere than all the other strains. Strains belonging to siderovars 1, 7, 5, 2, and 8 did not differ significantly and showed the lowest survival. Strains belonging to siderovars 31 and 21 showed an intermediate survival (Table 3). Finally, the significantly highest survival was found in strains with a MIC of EDDHA comprised between 500 and 1,000 μg ml^{-1} (modality 3), while strains belonging to the two other modalities did not significantly differ.

Discussion

Adaptation to the rhizosphere of 21 strains representative of a larger collection of soil and rhizosphere pseudomonads plus that of two additional reference strains was assessed by measuring their survival in non-sterile conditions, in the presence of the indigenous microbiota. Competitiveness of

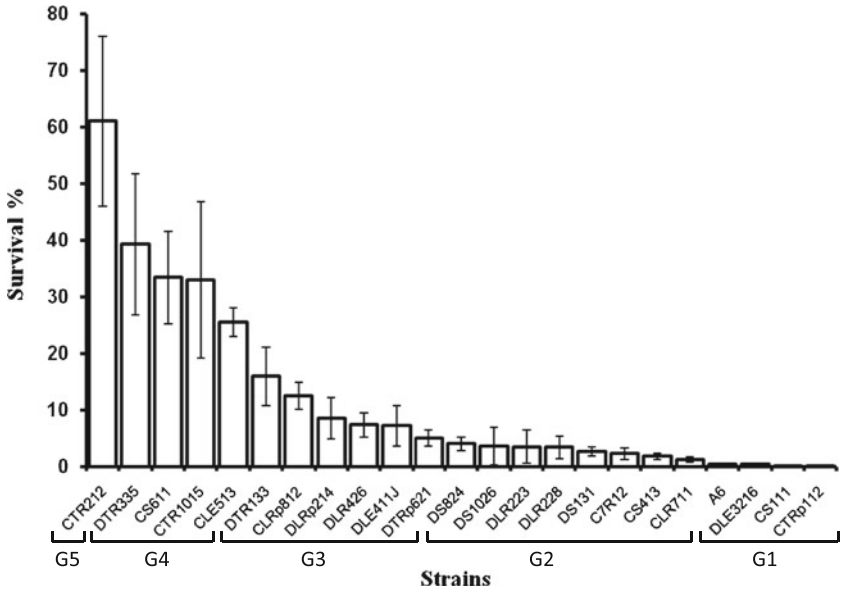

Figure 2 Compared survival of the 23 rifampin resistant mutants in the rhizosphere of tomato cultivated in the non-sterile soil of Châteaurenard. The survivals are expressed as percentages of the densities measured at days 22 and 0. The competitivity groups are indicated as follows: group 1 (G1)<1 %, group 2 (G2) [1–5 %[group 3 (G3) [5–20 %[group 4 (G4) [20–40 %[, group 5 (G5)≥40 %

introduced bacteria was previously shown to be a major component of the rhizosphere competence [56] especially in the presence of the indigenous microbiota [50]. Since an introduced strain should survive in the presence of the resident microbial communities, rhizosphere, competence was assessed under non-gnotobiotic conditions in contrast with previous studies [71, 72].

Possible relationships between these rhizospheric competence and physiological traits of the bacterial strains tested were searched by appropriate statistical methods. Multiple correspondence analyses were first applied to identify possible traits explaining the bacterial competitiveness and were then followed by mean multiple comparisons and variances analyses to determine if these traits were indeed involved in the rhizosphere competence of the fluorescent pseudomonads. Such a strategy was followed for identifying traits shared by antagonistic fluorescent pseudomonads [14] and more recently by Rezzonico et al. [66] to determine if the 2,4-diacetylphloroglucinol enhanced the ability of strains to suppress soilborne diseases.

Results evidenced a high diversity among the bacterial strains tested with respect for their competitivity in tomato rhizosphere. This high diversity is in agreement with previous reports [23, 62].

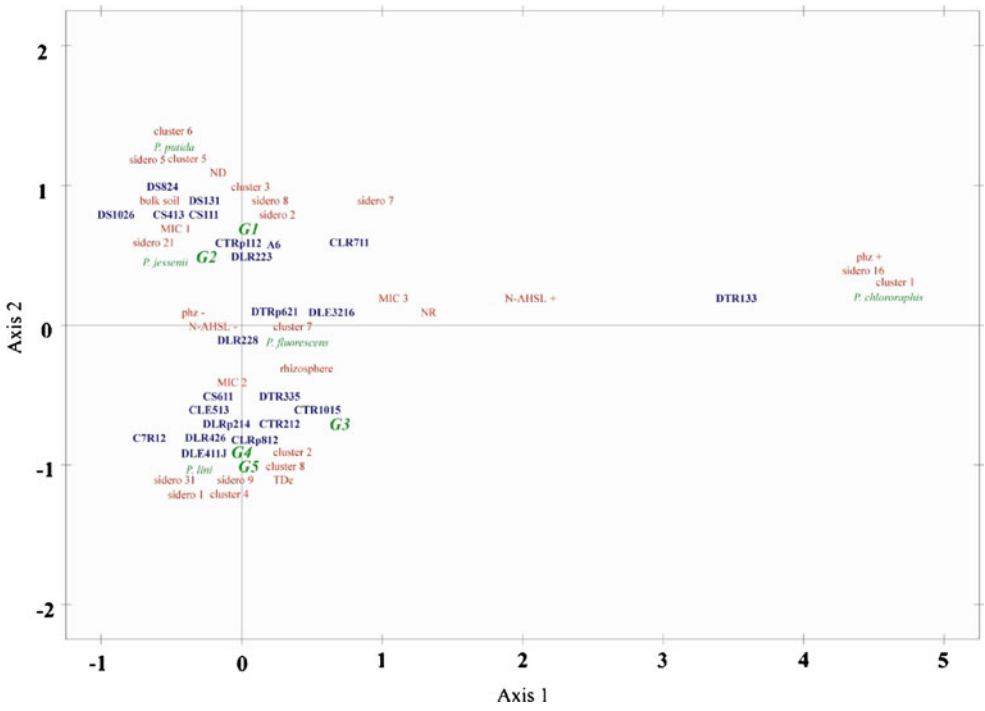

Figure 3 Multiple correspondence analysis in which active variables (origin, phenotypic cluster, dissimilating ability, siderovar, MIC of EDDHA, N-AHSL, phenazine) appear in *red letters*, supplementary variables (taxonomic identification and competitivity groups) appear in *italic green letters*, and strains appear in *blue letters*

Table 3 Multiple comparisons by ANOVA of survival means in relation to phenotypic clusters (A), dissimilating abilities: *ND* non dissimilator, *NR* nitrate reducer, *TDe*, total denitrifier (B), siderovars (C), MIC of EDDHA (D)

	Survival rate (%)	Statistical groups
A. Phenotypic clusters		
Cluster 8	61.04	a
Cluster 2	20.08	b
Cluster 1	15.95	bc
Cluster 4	8.58	cd
Cluster 6	4.06	de
Cluster 5	3.55	e
Cluster 7	2.94	e
Cluster 3	1.08	e
B. Dissimilating abilities		
TDe	23.02	a
NR	5.68	b
ND	1.96	b
C. Siderovars		
Siderovar 9	27.59	a
Siderovar 16	15.95	a
Siderovar 31	7.20	b
Siderovar 21	3.24	bc
Siderovar 1	2.30	bcd
Siderovar 7	1.28	cd
Siderovar 5	1.01	cd
Siderovar 2	0.42	cd
Siderovar 8	0.11	d
D. MIC of EDDHA		
500–1000	18.72	a
4–62.5	13.10	ab
125–250	7.48	b

Means with the same letter are not significantly different ($\alpha \leq 0.1$)

Multiple correspondence analysis revealed two major strategies explaining the above-mentioned variations in tomato rhizosphere competence.

The first strategy relies upon trophic traits that include the ability to use various electron acceptors and donors, as discriminated on the axis 2 of the Fig. 3. Some fluorescent pseudomonads only use oxygen as a final electron acceptor while others also use nitrogen oxides; ferric iron being an intermediate electron acceptor in the respiratory chain [6]. The best colonizers showed both a low susceptibility to iron starvation and produce specific pyoverdine as indicated by their siderovars (9, 16, 31), pointing out a contribution of the siderophore mediated iron uptake in the adaptation to the rhizosphere (Fig. 3, Table 3). Competitiveness experiments were performed in Châteaurenard soil which has a low iron bioavailability [40]. As a consequence iron competition in the rhizosphere of plants cultivated in this soil is high and was shown to be involved in the natural suppressiveness to *Fusarium* wilts [38]. Furthermore, pyoverdine mediated iron uptake was previously shown in this soil to be involved the rhizosphere competence of the *P. fluorescens* model strain C7R12 [57]. The low susceptibility to iron starvation of the efficient colonizers is in agreement with previous data indicating the significant higher values of MIC of isolates from rhizosphere than from bulk soil [40, 67]. As stated above, the best colonizers belonged to a limited number of siderovars; it is tempting to speculate that the corresponding pyoverdines would be more efficient iron chelators than those from other siderovars. This remains to be tested.

Although being susceptible to iron starvation, some strains (*P. lini* CLE513 and DTR335) appeared to be efficient colonizers. These strains were total denitrifiers. The fact that these TDe strains showing a low ability to chelate iron, remained efficient colonizers could be related to the competitive advantage given by their ability to reduce nitrogen oxides in situation of low oxygen relative pressure resulting from the high humidity content of the experimental soil. This hypothesis is supported by the previous demonstration of the contribution of the hydric potential on the competitive advantage given by nitrate reductase vs. pyoverdine to a wild-type strain [56]. More generally, the ability to totally denitrify was property shared by all the efficient colonizers among the strains tested. This observation concurs with former observations indicating a frequency of nitrogen dissimilating pseudomonads higher in rhizosphere than in bulk soil [8]. Interestingly, the strains harboring only a nitrate reductase activity (NR) were not significantly better colonizers that the non-denitrifers (Table 3). The nitrate reduction to nitrite shows the highest energetic yield and is expected to contribute to the rhizosphere competence as previously shown by Mirleau et al. [56]. However, the downstream reactions that lead to nitrogen contribute to further increase the energetic yield and the global metabolic efficiency of the bacteria.

Aside from electron acceptors, discrimination of the phenotypic clusters indicates that an expansive substrate utilization profile contributes to bacterial rhizosphere competence (Table 3). Strains included in phenotypic clusters 8 and 2 were in overall significantly better colonizer than those from the other clusters. In contrast, the less efficient colonizers were distributed in the clusters 6, 5, 7, and 3. Only few compounds allow the discrimination of the phenotypic clusters. Among them, sorbitol utilization is shared by all strains of phenotypic cluster 2, irrespectively of their colonizing efficiency (five efficient colonizers out of the eight strains). The five efficient colonizers belonged to siderovar 9 which is a characteristic trait of rhizosphere competent strains (Table 3). Interestingly, a possible contribution of sorbitol utilization to root colonization ability was previously suggested in a survey of the diversity of *Bacillus*

polymyxa in soil and wheat rhizosphere [45]. Additionally, ability to use trehalose is globally shared by the members of phenotypic clusters 2, 1, and 8. Trehalose utilization was previously found in populations preferentially associated with roots and ectomycorrhiza [17, 34, 41]. Among the 13 isolates able to use trehalose, four were rhizosphere competent (G5 and G4), five were intermediate (G3); however, three were a poor colonizer (G1, G2). Among these strains, *P. fluorescens* C7R12 (G2) is known to have a specific life style in the rhizosphere as indicated by its endophytic behavior [68] and the presence of a type three secretion system [47], possibly accounting for its dose independent efficiency in biological control [58]. The two other poor colonizers (*P. fluorescens* A6, CTRp112) appeared to be ND, suggesting that the inability to reduce nitrogen oxides was a more important limiting factor. Remarkably, the very efficient root colonizer *P. fluorescens* CTR212, the only strain of phenotypic cluster 8, differed from the others by its inability to use the following compounds: D-mannitol, D-saccharate, protocacechuate, parahydroxibenzoate, serine. However, this strain belonged to siderovar 9, was TDe and showed a very low susceptibility to iron starvation. Taken together these data clearly favor an involvement of the energetic metabolism in the rhizosphere competence of fluorescent pseudomonads. More specifically, ability to efficiently use different electron acceptors appeared to be a more discriminating trait of rhizosphere competent strains than their ability to use specific organic compounds. This supports and extends previous data indicating that ability to use ferric iron and nitrogen oxides as electron acceptors is a discriminating trait of rhizospheric populations isolated from different soils [33], whereas the ability to use specific organic compounds was less consistent. Since the composition of root exudates is known to differ according to plant genotypes, contribution of the ability to use specific organic compounds to rhizosphere competence may differ between rhizospheres of different species. Carbon utilization profiles of fluorescent pseudomonads were indeed previously shown to differ according to the rhizosphere (tomato and flax) from which they were isolated [41]. However, data from this study indicate that competitivity of fluorescent pseudomonads isolated, from flax and tomato rhizosphere, did not significantly differ when tested in tomato rhizosphere.

The second strategy relies upon the synthesis of antibiotic phenazines and its regulation by N-AHSL, as discriminated on the axis 1 of the Fig. 3. This strategy is represented by the only strain (DTR133) producing phenazines and one of the two producing N-AHSL. Phenazines were indeed previously shown to be involved in the competitiveness of a model strain [50] and their synthesis to be regulated by N-AHSL [78]. Strain DTR133 belongs to the species *P. chlororaphis* which is known to produce phenazines [46]. Interestingly, this strains, which is NR, has a very low susceptibility to iron stress which might be related to the specific siderophore produced (siderovar 16) but also to its ability to synthesize phenazines. Indeed, as recently suggested [48], the ability of pseudomonads to produce phenazines may give them a competitive advantage under the iron-limiting conditions prevailing in the Châteaurenard soil [38] since they are redox-active antibiotics [12, 27] and may contribute to iron mobilization in soils [60, 75]. Phenazine synthesis could then be involved in the competition against other organisms via its antibiotic activity but also give a competitive advantage to DTR133 via its contribution to iron acquisition.

In conclusion, two major strategies were identified to explain variations of rhizosphere competitiveness of a set of fluorescent pseudomonads representative of wider collection of isolates from soils and rhizosphere. One relied on specific energetic and carbon metabolism and especially on the bacterial ability to efficiently use electron acceptors such as iron and nitrogen oxides. The second relied on the synthesis of phenazines which might at the same time contribute to microbial antagonism but also to iron acquisition.

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