Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp.

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Abstract: In this study, the potential of different *Pseudomonas* strains to utilize heterologous siderophores was compared with their competitiveness in the rhizosphere of radish. This issue was investigated in interactions between *Pseudomonas putida* WCS358 and *Pseudomonas fluorescens* WCS374 and in interactions between strain WCS358 and eight indigenous *Pseudomonas* strains capable of utilizing pseudobactin 358. During four successive plant growth cycles of radish, strain WCS358 significantly reduced rhizosphere population densities of the wild-type strain WCS374 by up to 30 times, whereas derivative strain WCS374(pMR), harboring the siderophore receptor PupA for ferric pseudobactin 358, maintained its population density. Studies involving interactions between strain WCS358 and eight different indigenous *Pseudomonas* strains demonstrated that despite the ability of these indigenous isolates to utilize pseudobactin 358, their rhizosphere population densities were significantly reduced by strain WCS358 by up to 20 times. Moreover, rhizosphere colonization by WCS358 was not affected by any of these indigenous strains, even though siderophore-mediated growth inhibition of WCS358 by a majority of these strains was demonstrated in a plate bioassay. In conclusion, it can be stated that siderophore-mediated competition for iron is a major determinant in interactions between WCS358 and WCS374 in the rhizosphere. Moreover, our findings support the common assumption that cloning of siderophore receptor genes from one *Pseudomonas* strain into another can confer a competitive advantage in interactions in the rhizosphere. Interactions between WCS358 and the selected indigenous rhizosphere isolates, however, indicate that other traits also contribute to the rhizosphere competence of fluorescent *Pseudomonas* spp.

Key words: siderophore, siderophore receptors, root colonization, fluorescent *Pseudomonas*.

Résumé : La présente étude porte sur l'aptitude de différentes souches de *Pseudomonas* à utiliser des sidérophores hétérologues, comparée à leur compétitivité dans les rhizosphères de radis. Dans ce but, des recherches ont été menées sur les interactions entre le *Pseudomonas putida* WCS358 et le *Pseudomonas fluorescens* WCS374 ainsi que sur les interactions entre la souche WCS358 et huit souches indigènes de *Pseudomonas* capables d'utiliser la pseudobactine 358. Au cours de quatre cycles successifs de culture de radis, la souche WCS358 a réduit significativement, jusqu'à 30 fois, la densité des populations de la souche indigène type WCS374 dans les rhizosphères, tandis qu'une souche dérivée de WCS374(pMR), porteuse du récepteur de sidérophores PupA pour la pseudobactine ferrique 358, a maintenu la densité de ses populations. Les études portant sur les interactions entre la souche WCS358 et huit différentes souches indigènes de *Pseudomonas* ont montré qu'en dépit de l'aptitude des isolats indigènes à utiliser la pseudobactine 358, les densités de leurs populations ont été significativement réduites, jusqu'à 20 fois, par la souche WCS358. De plus, la colonisation des rhizophères par la souche WCS358 n'a été affectée par aucune des souches indigènes même si, via les sidérophores, l'inhibition de la croissance de WCS358 par une majorité de ces souches a été démontrée dans des bio-essais sur plaques. En conclusion, il peut être avancé que la compétition pour le fer via les sidérophores dans les rhizosphères est un déterminant majeur dans les interactions entre le WCS358 et le WCS374. D'autre part, cette étude appui l'opinion actuelle que le clonage des gènes récepteurs de sidérophores d'une souche de *Pseudomonas* dans une autre peut conférer un avantage compétitif dans les interactions au niveau des rhizosphères. Toutefois, ces interactions entre la WCS358 et les isolats indigènes sélectionnés des rhizosphères indiquent que d'autres caractères contribuent également à la compétence des *Pseudomonas* spp. fluorescents dans les rhizosphères.

Mots clés : sidérophore, récepteurs de sidérophores, colonisation des racines, *Pseudomonas* fluorescents. [Traduit par la Rédaction]

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Introduction

Inefficient root colonization by selected strains of plant growth promoting fluorescent *Pseudomonas* spp. is generally accepted as one of the major causes for the inconsistency of biological control of soilborne plant pathogens under field conditions (Bull et al. 1991; Schippers 1992; Weller 1988). In this context, many bacterial traits have been studied for their possible involvement in root colonization, including adherence (Anderson et al. 1988; Glandorf 1992), motility (De Weger et al. 1987*a*; Howie et al. 1987), tolerance to adverse environmental conditions (Loper et al. 1985), and the production of antibiotics (Mazzola et al. 1992) and of siderophores (Bakker et al. 1990; Loper and Buyer 1991). Siderophores are low molecular mass compounds that are excreted by microorganisms under iron-limited conditions. These compounds have a high binding affinity and specificity for iron(III) and facilitate its transport into the cell (Neilands 1981). Plant growth promoting strain *Pseudomonas putida* WCS358 produces a fluorescent pseudobactin type of siderophore, which consists of a dihydroxyquinoline chromophore linked to a linear nonapeptide (Van der Hofstad et al. 1986). All of the pseudobactins determined thus far share common structural features but differ especially in the length and composition of the peptide part (Abdallah 1991). Because of this structural diversity, most *Pseudomonas* strains can metabolize iron only via a limited number of these compounds (Bakker et al. 1990). *Pseudomonas putida* WCS358 is remarkable in this respect as it can exploit a large variety of heterologous siderophores, whereas its own siderophore, designated pseudobactin 358, can only be utilized by a very small number of indigenous pseudomonads (Bakker et al. 1990; Raaijmakers et al. 1994).

Ferric siderophore transport into the bacterial cell is initiated by binding of the ferric siderophore complex to specific outer membrane receptor proteins. These receptors tend to be of high molecular mass (75–90 kDa) and generally their expression is regulated by iron availability (Neilands 1982). By exploiting the specific nature of the receptor and the relatively nonspecific nature of proteins related to iron uptake in the periplasm and cytoplasmatic membrane, many genes encoding ferric pseudobactin receptors of fluorescent pseudomonads have been cloned (Bitter et al. 1991; Magazin et al. 1986; Marugg et al. 1989; Morris et al. 1992). In *P. putida* WCS358, ferric pseudobactin 358 transport is initiated by binding to the specific receptor PupA (pseudobactin uptake protein A) (Bitter et al. 1991). The structural gene of this 85-kDa PupA protein has been sequenced (Bitter et al. 1991). Introduction of the *pupA* gene into *P. fluorescens* WCS374, via cosmid pMR, enabled derivative WCS374(pMR) to metabolize ferric pseudobactin 358 but not other ferric pseudobactins (Bitter et al. 1991). In addition to this specific receptor protein, strain WCS358 harbors multiple iron-regulated and also siderophore–inducible genes for outer membrane proteins, of which at least one mediates iron uptake via two distinct heterologous siderophores (Koster et al. 1993). Also, for *Pseudomonas* strain M114 an additional ferric siderophore receptor gene has been identified (O'Sullivan et al. 1990).

With respect to siderophore-mediated competition for iron(III) between different *Pseudomonas* strains in the plant rhizosphere, the ability to exploit heterologous siderophores has been postulated to confer a competitive advantage (Bakker et al. 1990; Jurkevitch et al. 1992). This hypothesis is compatible with the observation that mutants of *Pseudomonas* strains defective in siderophore production colonized the rhizosphere as well as their siderophore-producing wild-type strains (Bakker et al. 1987; Höfte et al. 1991; Loper 1988). However, so far the determinative role of receptors of fluorescent pseudomonads in siderophore-mediated interactions in rhizosphere environments has not been investigated in detail. If siderophore-mediated competition for iron is a determinant of interactions between strains of *Pseudomonas* spp., then rhizosphere colonization may be enhanced by cloning siderophore receptor genes from one strain into the other.

In this study, the potential of different strains of fluorescent *Pseudomonas* spp. to utilize specific heterologous siderophores was compared with their competitiveness in the rhizosphere of radish. Interactions between plant growth promoting strains *P. putida* WCS358 and *P. fluorescens* WCS374 were studied during four successive plant growth cycles of radish. The utilization of pseudobactin 358 was studied in detail by introducing the gene encoding the PupA receptor for uptake of ferric pseudobactin 358 into strain WCS374. The utilization of heterologous siderophores in competition for iron in the rhizosphere was also investigated for interactions between *P. putida* WCS358 and eight different indigenous pseudomonads, which were selected from radish rhizosphere for their ability to utilize pseudobactin 358.

Materials and methods

Bacterial strains

Wild-type strains *P. putida* WCS358 and *P. fluorescens* WCS374 are plant growth promoting and disease suppressing rhizobacteria isolated from the roots of potato (Bakker et al. 1986; Duijff et al. 1993; Geels and Schippers 1983). In strain WCS374(pMR), cosmid pMR encodes resistance to tetracyclin and harbors the *pupA* gene of strain WCS358 (Bitter et al. 1991; Marugg et al. 1989). The siderophore receptor PupA enables strain WCS374(pMR) to metabolize iron complexed to pseudobactin 358. For production of inoculum, all strains were cultured on King's medium B (KB) agar (King et al. 1954) for 48 h at 27 °C, harvested in 0.1 M $MgSO_4 \cdot 7H_2O$, and washed twice by centrifugation (10 min, 5000 × *g*). In soil and rhizosphere studies, population densities of indigenous *Pseudomonas* spp. were enumerated using KB agar supplemented with 100 μg cycloheximide/mL, 40 μg ampicillin/mL, and 13 μg chloramphenicol/mL (KB^+) (Geels and Schippers 1983).

Isolation and characterization of indigenous pseudomonads that utilize pseudobactin 358

Isolation

Indigenous *Pseudomonas* spp. were isolated from roots of radish plants (*Raphanus sativus* cv. Saxa Nova) that were grown for 14 days in a sandy soil (5.5% organic matter, pH_{H_2O} 6.8) collected from a commercial greenhouse (Leeman et al. 1991). Rhizosphere suspensions were dilution plated on KB^+ agar supplemented with 300 μM pseudobactin 358 (KB^+Ps358) (Raaijmakers et al. 1994). The ability of these rhizosphere isolates, designated RDR (radish rhizosphere) isolates, to utilize pseudobactin 358 was subsequently checked

in a plate assay based upon reversal of iron starvation induced by ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) (Buyer and Leong 1986). Rifampicin-resistant derivatives of these RDR isolates were obtained by transferring colonies successively onto KB agar plates containing an increasing concentration (25, 50, 100, and 150 μg/mL) of rifampicin (Serva). Rifampicin resistance was stable in vitro as was tested by subculturing these strains in KB four times for 24 h at 27°C, and by comparing the number of colony-forming units (cfu) after dilution plating of suspensions on KB and on KB supplemented with rifampicin (150 μg/mL).

Cell envelope protein and lipopolysaccharide patterns

All RDR isolates were characterized by analysis of their lipopolysaccharide (LPS) and cell envelope protein (CEP) patterns according to the method described by De Weger et al. (1987*b*). Cell envelopes containing LPS and CEP were obtained from cells grown in KB medium for 24 h at 27°C by differential centrifugation after disruption of the cells by ultrasonic treatment. Samples were solubilized by incubation for 15 min at 95°C in a standard sample mixture (Lugtenberg et al. 1975). Solubilized cell envelope samples (5 μL) containing approximately 1 mg CEP/mL were applied to a 3% stacking – 17% resolving gel. After electrophoresis (Mini Protean 2, Bio-Rad), proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. For the analysis of LPS, cell envelope samples were first treated with proteinase K to degrade proteins, after which 5 μL of 10-fold dilutions were applied to a 3% stacking 20% resolving gel. After electrophoresis, LPS was stained with silver reagent (Bio-Rad).

Siderophore-mediated antagonism between bacterial strains

The production of siderophores by these RDR isolates was determined in a chrome-azurol-S (CAS) assay (Schwyn and Neilands 1987). Antagonism between the RDR isolates and the three strains WCS358, WCS374, and WCS374(pMR) was determined in a plate bioassay according to the method described by Bakker et al. (1990). Isolates were spot inoculated on KB agar plates and on KB agar plates supplemented with 200 μM Fe^{3+} (KBFe), added as $FeCl_3$. After incubation for 48 h at 27°C, a suspension of WCS358, WCS374, or WCS374(pMR) (10^7 cfu/mL) was atomized over both types of plates. Suspensions of the atomized strains were prepared in 0.01 M $MgSO_4$. After incubation for 24 h at 27°C, growth inhibition of the atomized strains was scored visually. This plate bioassay, using both KB and KBFe plates, was also performed using suspensions of the atomized strains prepared in 0.01 M $MgSO_4$ supplemented with 10 mM of $FeCl_3$. Addition of 10 mM of Fe^{3+} to the atomized suspension complexed the siderophores produced by the spot-inoculated strains on KB plates and created iron-rich conditions for subsequent growth of the atomized strain.

Interactions between *P. putida* WCS358 and *P. fluorescens* WCS374

Interactions between *P. putida* WCS358 and *P. fluorescens* WCS374 were studied during four successive growth cycles of radish of approximately 21 days. Bacterial strains were introduced onto the roots of radish seedlings only at the beginning of the first plant growth cycle, and not in successive cycles. Radish seedlings with roots of approximately 2 cm in length were obtained after 4 days of growth in nonsterile river sand. Prior to treatment with the bacterial strains, the roots were washed twice with sterile water to remove adhering soil particles. The roots were dipped for 5 min in mixed suspensions (1:1) of WCS358 (10^8 cfu/mL) and WCS374 or WCS374(pMR) (10^8 cfu/mL), or in a suspension of WCS358, WCS374, or WCS374(pMR) (10^8 cfu/mL). Excess of bacterial suspension was removed by blotting the treated roots with sterile filter paper. Treated seedlings were planted in polyvinyl chloride (PVC) tubes (16 cm high and 5 cm in diameter) containing non-sterile sandy soil (5.5% organic matter, pH_{H_2O} 6.8) collected from a commercial greenhouse (Leeman et al. 1991). Each treatment consisted of 10 replicates of two plants. After each plant growth cycle of approximately 21 days, population densities of WCS374, WCS374(pMR), and WCS358 were determined in rhizosphere and in soil. Plant roots and soil were harvested from the PVC tube. The soil was sieved (2 mm mesh) to remove all root segments. Rhizosphere suspensions were prepared from root segments sampled at random at a depth of 0–8 cm from the stem base. These segments were suspended in 5 mL sterile 0.1 M $MgSO_4 \cdot 7H_2O$, and shaken vigorously for 30 s in glass test tubes containing 2.5 g glass beads (0.17 mm diameter). Soil suspensions were prepared by suspending a portion of the soil (1 g) in 5 mL sterile 0.1 M $MgSO_4 \cdot 7H_2O$, and by shaking it vigorously for 30 s in glass test tubes containing 2.5 g of glass beads (0.17 mm diameter).

For the subsequent plant growth cycle, PVC tubes were filled with the remaining soil of the earlier cycle and radish was sown and grown again for approximately 21 days. This procedure was performed for four plant growth cycles.

Interactions between *P. putida* WCS358 and RDR isolates

Radish seedlings with roots approximately 2 cm in length were obtained as described previously. The roots were treated with suspensions of WCS358, an RDR isolate, or with mixed suspensions (1:1) of WCS358 (10^8 cfu/mL) and an RDR isolate (10^8 cfu/mL). Treated seedlings were planted in PVC tubes containing nonsterile sandy soil as described previously. Each treatment consisted of 10 replicates of two plants. Rhizosphere population densities of the applied bacterial strains were determined after a plant growth period of 14 days on root segments sampled at random at a depth of 0–8 cm from the stem base as described previously.

Plant cultivation and growth conditions

Radish plants were grown under controlled conditions with a 16-h light period (irradiance 60 $W \cdot m^{-2}$) at 24°C and 70% relative humidity followed by an 8-h dark period at 20°C and 85% relative humidity. Plants were watered twice a week, once with tap water and once with 30 mL of a nutrient solution containing (per litre) 0.51 g KNO_3, 0.16 g KH_2PO_4, 0.084 g $Mg(NO_3)_2 \cdot 6H_2O$, 1.2 mg Borax, 0.5 mg $MnSO_4$, and 0.5 mg $NaMoO_4$. This solution was formulated by us based upon chemical analysis of the soil used elsewhere in this study.

Enumeration of applied bacterial strains

Soil and rhizosphere population densities of strains WCS358, WCS374, and derivative WCS374(pMR) were enumerated by

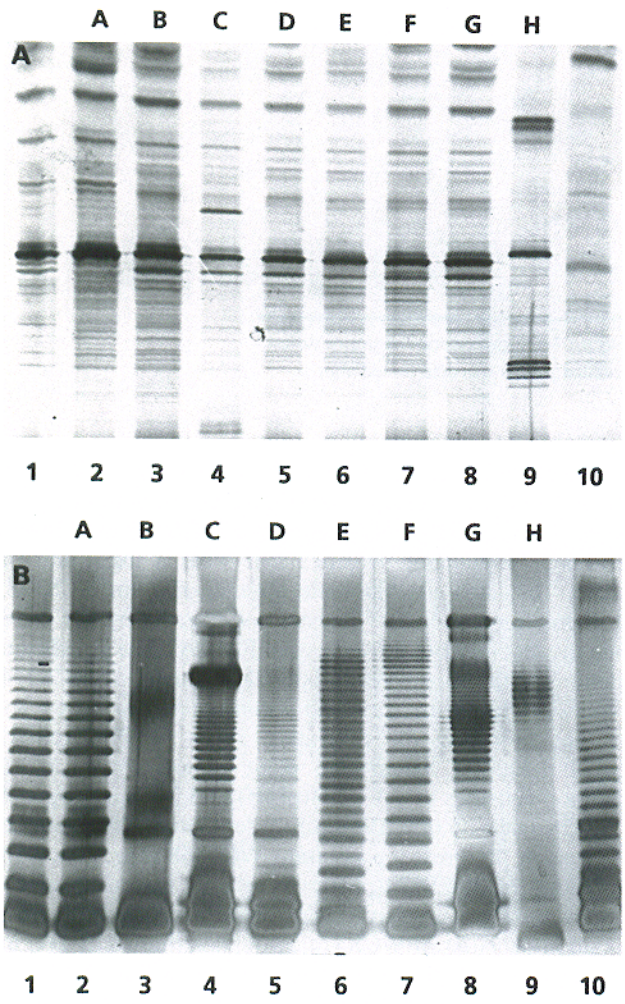


Fig. 1. (A) CEP patterns of *P. putida* WCS358 (lane 1), *P. fluorescens* WCS374 (lane 10), and eight indigenous bacteria selected for their ability to utilize pseudobactin 358 (lanes 2–9). (B) Silver-stained proteinase K treated cell envelope patterns of the same isolates described in A. Labels above the lanes refer to the LPS group.

immunofluorescence colony staining (IFC) according to the method described by Van Vuurde and Roozen (1990) and modified by Leeman et al. (1991). Aliquots of soil or rhizosphere suspensions (0.1 mL) were mixed homogeneously with agar medium (0.4 mL, 40°C) in 24-well tissue plates on a reciprocal shaker. After incubation for 24 h at 27°C, the colonies that had developed in the agar media were stained with 50-fold diluted fluorescein isothiocyanate-conjugated antibodies. Immunoglobulins were obtained against whole cells of WCS358 or WCS374 in rabbit as described by Vruggink and Maas-Geesteranus (1975). They were kindly provided by J.M. van der Wolf and J.W.L. van Vuurde from the Research Institute for Plant Protection at Wageningen (IPO-DLO), the Netherlands. Polyclonal antisera Ab9059 and Ab9061 are highly specific for WCS358 (Raaijmakers et al. 1994) and for WCS374 (Bakker et al. 1989), respectively. For enumeration of population densities of strain WCS358 by IFC, KB^+Ps358 agar medium (Raaijmakers et al. 1994) was used. For strain WCS374 and its derivative WCS374(pMR), KB^+ agar medium was used. To determine the maintenance of cosmid pMR, encoding resistance to tetracyclin and harboring the *pupA* gene, population densities of derivative strain WCS374(pMR) were also determined by IFC in KB^+ supplemented with tetracyclin (100 μg/mL) and in KB^+Ps358. Stained colonies were enumerated using a Zeiss Axioskop microscope with fluorescence illumination.

Rhizosphere population densities of rifampicin-resistant derivatives of the RDR isolates were determined by mixing rhizosphere suspensions homogeneously with KB^+ supplemented with rifampicin (150 μg/mL). Colonies of the RDR isolates that had developed in the agar medium were stained with ethidium bromide (1 mg/mL). Stained colonies were enumerated using a Zeiss Axioskop microscope with fluorescence illumination.

Statistical analysis

Populations of *Pseudomonas* spp. introduced on seed or planting material approximate a lognormal distribution along growing roots (Loper et al. 1984). Therefore, enumerated values were submitted to logarithmic transformation prior to analysis. Transformed values were analyzed by analysis of variance followed by Student's *t* test. Experiments were performed at least twice.

Results

Isolation and characterization of indigenous pseudomonads that utilize pseudobactin 358

Isolation

Twenty-one pseudomonads were isolated from the microbial population present on roots of radish plants by dilution plating rhizosphere suspensions on KB^+Ps358. Their ability to utilize pseudobactin 358 was confirmed in a plate assay based upon reversal of iron starvation induced by EDDA. All 21 isolates produced a fluorescent pigment on KB agar as viewed under uv light. The number of cfu enumerated on KB^+Ps358 represented 0.02% of the number of fluorescent cfu enumerated on KB^+ agar.

Cell envelope protein and lipopolysaccharide patterns

To identify these 21 isolates we analyzed their CEP and LPS patterns. Analysis of the CEP patterns revealed that all of these isolates except one had a very similar profile, which was almost identical to that of strain WCS358 (Fig. 1A). Subsequent analysis of their LPS structure revealed eight unique patterns, of which only pattern A was identical to that of strain WCS358 (Fig. 1B). LPS patterns A, C, and D each represented four isolates or more, whereas the other LPS patterns each represented one isolate only. For further characterization of these 21 isolates, we used one isolate of each distinct LPS pattern. They were designated radish rhizosphere isolates RDR-A to RDR-H.

Siderophore-mediated antagonism between bacterial strains

CAS assays demonstrated that all isolates produced siderophores under iron-limited conditions. Siderophore-mediated antagonism between isolates RDR-A to RDR-H and WCS358 was determined in a plate bioassay using both KB

Table 1. Antagonism between different strains of fluorescent *Pseudomonas* spp. Strains were spot-inoculated on KB agar and on KBFe plates.

Spot-inoculated strain	Atomized strain					
	WCS358		WCS374		WCS374pMR	
	KB	KBFe	KB	KBFe	KB	KBFe
WCS358	−	−	+	−	−	−
WCS374	−	−	−	−	−	−
WCS374pMR	−	−	−	−	−	−
RDR-A	−	−	+	−	−	−
RDR-B	+	−	+	−	+	−
RDR-C	+	−	+	−	+	−
RDR-D	+	−	+	−	+	−
RDR-E	−	−	+	−	−	−
RDR-F	+	−	+	−	+	−
RDR-G	+	−	+	−	+	−
RDR-H	−	−	−	−	−	−

NOTE: Strains were spot inoculated on KB agar or KBFe agar plates. After incubation (48 h, 27°C), suspensions of strains were atomized over these plates. Growth inhibition (+) of the atomized strains by the spot-inoculated strains was determined after incubation for 24 h at 27°C.

and KBFe plates (Table 1). Strain WCS358 did not inhibit growth of any of the RDR isolates on either KB or KBFe plates (data not shown). However, RDR isolates inhibited growth of strain WCS358 in some combinations on KB but not KBFe plates (Table 1). Growth inhibition of strain WCS358 by isolates B, C, D, F, and G could be overcome by the addition of 10 mM $FeCl_3$ to the atomized suspension of strain WCS358 (data not shown). Growth of derivative strain WCS374(pMR) was not inhibited by WCS358, RDR-A, RDR-E, or RDR-H on KB plates, whereas strain WCS374 was inhibited by WCS358 and almost all RDR isolates, including RDR-A and RDR-E (Table 1). Growth inhibition of both WCS374 and WCS374(pMR) could be overcome by the addition of 10 mM $FeCl_3$ to the atomized suspensions of both strains (data not shown).

Interactions between *P. putida* WCS358 and *P. fluorescens* WCS374

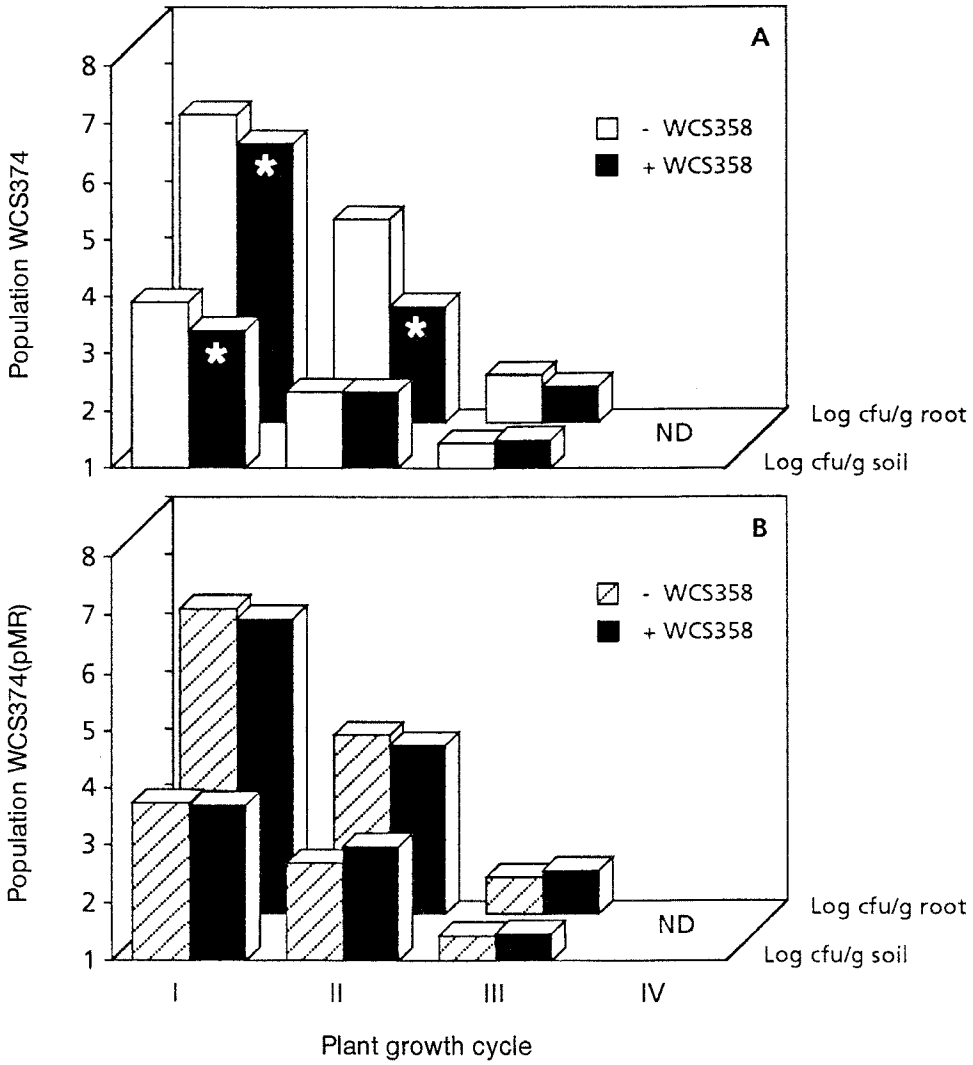
Roots of radish seedlings were treated with suspensions of WCS358, WCS374, or WCS374(pMR), or with mixed suspensions (1:1) of WCS358 and WCS374 or WCS374(pMR). For all treatments, the number of cells of each strain that adhered to the roots at the beginning of the experiment was approximately 5×10^6 cfu/g. Bacterial strains were applied only at the beginning of the first of four successive plant growth cycles. After the first plant growth cycle, the rhizosphere population density of wild-type WCS374 was reduced significantly by a $\log_{10}$ unit of 0.5 when coinoculated with WCS358 (Fig. 2A). This threefold reduction in rhizosphere population density of WCS374 after the first plant growth cycle was reflected in the population densities of WCS374 in soil (Fig. 2A). These soil populations served as inoculum for rhizosphere colonization during the second plant growth cycle. When strain WCS374 was not coinoculated with strain WCS358, its initial soil population density of 3.9 $\log_{10}$ cfu/g soil led to a rhizosphere population density of 4.5 $\log_{10}$ cfu/g root at the end of the second plant growth cycle (Fig. 2A). However, when coinoculated with WCS358, initial population densities of WCS374 of 3.4 $\log_{10}$ cfu/g soil only resulted in a density of 3.0 $\log_{10}$ cfu/g root (Fig. 2A). Therefore, in the presence of WCS358, wild-type strain WCS374 established rhizosphere population densities that were a $\log_{10}$ unit of 1.5 lower (i.e., 30-fold) than in the absence of strain WCS358. Soil populations of WCS374 were not affected by WCS358 during the second plant growth cycle (Fig. 2A). The significant reductions in the rhizosphere population density of WCS374 by WCS358 during the first and second plant growth cycles were not observed for derivative strain WCS374(pMR) (Fig. 2B). During the second plant growth cycle, initial population densities of WCS374(pMR) of 3.7 $\log_{10}$ cfu/g soil resulted in a density of 4.2 $\log_{10}$ cfu/g root, both in the absence as well as in the presence of strain WCS358 (Fig. 2B). Soil populations of WCS374(pMR) were also not affected by WCS358 during successive plant growth cycles (Fig. 2B). Population densities of WCS374(pMR) determined by IFC in KB^+, in KB^+ supplemented with tetracyclin, and in KB^+Ps358 were identical (data not shown), indicating that the cosmid pMR harboring the *pupA* gene was maintained at 100% in strain WCS374(pMR) throughout the experiment.

The differences in rhizosphere colonization between WCS374 and WCS374(pMR) in the presence of strain WCS358 were not due to different rhizosphere population densities of the coinoculated strain WCS358. When coinoculated with either strain WCS374 or WCS374(pMR), rhizosphere population densities of WCS358 decreased after the first plant growth cycle from 6.0 $\log_{10}$ cfu/g root down to 3.3 $\log_{10}$ cfu/g root in the second cycle. During the third and fourth plant growth cycles, rhizosphere population densities of strain WCS358 further decreased to a density of 2.8 $\log_{10}$ cfu/g root. Similar results were obtained when strain WCS358 was inoculated alone.

Interactions between *P. putida* WCS358 and RDR isolates

Roots of radish seedlings were treated with suspensions of WCS358, or with RDR isolates A–H, or with mixed suspensions (1:1) of WCS358 and one of each of the RDR

Fig. 2. Population dynamics of *P. fluorescens* WCS374 (A) and its derivative WCS374(pMR) (B) during four successive 21-day plant growth cycles. Strains WCS374 (open bars) and derivative WCS374(pMR) (hatched bars) were inoculated on roots of radish seedlings at the beginning of the first cycle, either alone or as a mixture with *P. putida* WCS358 (solid bars). Population densities were determined in both rhizosphere and soil. An asterisk (*) indicates statistically significant differences ($p = 0.05$).



isolates. For all treatments, the number of cells of each strain that adhered to the roots at the beginning of the experiment was approximately 5×10^6 cfu/g root. Rhizosphere population densities of the applied bacterial strains were determined after 14 days. Since all RDR isolates were able to grow in KB$^+$Ps358 medium, IFC was used to discriminate between these isolates and wild-type strain WCS358. However, since isolates RDR-A and RDR-E also reacted positively with the WCS358 antiserum, we used rifampicin-resistant derivatives of the RDR isolates, which exhibited the same antagonistic properties as described previously for their parental strains. Although iron-regulated growth inhibition of WCS358 by RDR isolates B, C, D, F, and G was demonstrated in vitro (Table 1), rhizosphere population densities of strain WCS358 were not affected by any of the RDR isolates (Fig. 3A). Rhizosphere population densities of most of the RDR isolates were reduced significantly by strain WCS358 with a $\log_{10}$ unit ranging from 0.4 to 1.3, which accounts for a 2- to 20-fold reduction (Fig. 3B). Only the population densities of RDR-A and RDR-H were not affected by strain WCS358 (Fig. 3B).

Discussion

The efficacy of specific strains of *Pseudomonas* spp. to control soilborne plant diseases depends on successful establishment in the rhizosphere (Bowen 1991; Bull et al. 1991). In this context, the ability to exploit a wide variety of siderophores has been postulated to confer a competitive advantage (Bakker et al. 1990; Jurkevitch et al. 1992). In the present study, the potential of different strains of *Pseudomonas* spp. to utilize

heterologous siderophores was compared with their competitiveness in the rhizosphere. Interactions between *P. putida* WCS358 and *P. fluorescens* WCS374 were studied during four successive plant growth cycles of radish. During the first plant growth cycle, strain WCS358 reduced the rhizosphere population density of wild-type strain WCS374 by three times (Fig. 2A). Especially during the second plant growth cycle, interactions between WCS358 and WCS374 were most obvious (Fig. 2A). In the presence of strain WCS358, wild-type strain WCS374 established rhizosphere population densities that were 30 times lower than those established in the absence of strain WCS358 (Fig. 2A). These significant reductions in the rhizosphere populations of wild-type strain WCS374 during the first and second plant growth cycle were not observed for its derivative strain WCS374(pMR) harboring the siderophore receptor PupA for ferric pseudobactin 358 (Fig. 2B). The population densities of strain WCS358 were not affected by strain WCS374 nor by derivative WCS374(pMR). These results demonstrate that siderophore-mediated competition plays a major role in the interaction between strains WCS358 and WCS374, and confirm the in situ production of pseudobactin 358 by strain WCS358, as was suggested by Bakker et al. (1990). Moreover, these results support the common assumption that the rhizosphere competence of particular strains of fluorescent *Pseudomonas* spp. can be enhanced by cloning siderophore receptor genes from one strain into another. Siderophore-mediated interaction between the two strains was more predominant in the rhizosphere than in soil (Fig. 2), confirming results obtained previously in bioassays using auxotrophic mutants (Bossier et al. 1988; Powell et al. 1980). Interactions between WCS358 and WCS374 could not be demonstrated any more during the third plant growth cycle, probably because of the low population densities of the applied bacterial strains (Fig. 2). At these densities, however, the effect of siderophore-mediated interactions may still be there, resulting in local dominance of WCS358 on the root. This, however, cannot be measured by the sampling and isolation techniques used. In situ localization of these strains by immunofluorescence colony staining (Van Vuurde and Roozen 1990) may provide a highly specific and sensitive technique to study interactions between low rhizosphere populations.

The utilization of heterologous siderophores in competition for iron in the rhizosphere was also investigated for interactions between *P. putida* WCS358 and indigenous pseudomonads capable of utilizing pseudobactin 358. Twenty-one pseudomonads were isolated from the roots of radish plants on low-iron KB$^+$Ps358 medium. Their ability to utilize pseudobactin 358 was confirmed in EDDA assays. Subsequent analysis of their CEP patterns revealed that all of these isolates except one have a similar profile that is almost identical to that of strain WCS358 (Fig. 1A). These results may suggest that these isolates are very similar to strain WCS358. However, in subsequent analysis of their LPS patterns, eight unique LPS patterns were observed, of which only pattern A was identical to the LPS pattern of strain WCS358 (Fig. 1B). These results indicate that most of these isolates are different from strain WCS358. LPS patterns A, C, and D each represent four isolates or more, whereas the other LPS patterns represent one isolate only. For subsequent characterization and for rhizosphere studies we used one isolate of each LPS pattern, designated isolates RDR-A to RDR-H.

Fig. 3. Interactions between *P. putida* WCS358 and isolates RDR-A to RDR-H in radish rhizosphere. These RDR isolates were inoculated onto roots of radish seedlings either alone (-WCS358) or in a 1:1 ratio with strain WCS358 (+WCS358). Population densities of strain WCS358 (A) and of isolates RDR-A to RDR-H (B) were determined after 14 days of plant growth. For each treatment, an asterisk (*) indicates statistically significant differences ($p = 0.05$).

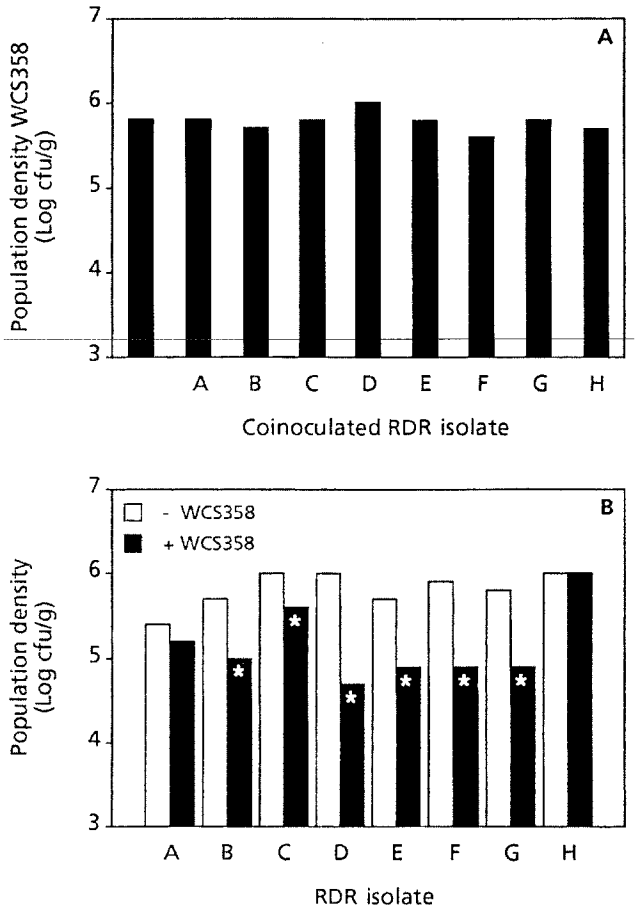


Plate bioassays revealed that a majority of the RDR isolates, except RDR-A, RDR-E, and RDR-H, can inhibit growth of strain WCS358 (Table 1). This growth inhibition of strain WCS358 is not only iron regulated (Table 1) but also iron antagonized because it could be overcome by the addition of 10 mM $FeCl_3$ to the atomized suspension of strain WCS358. These results strongly indicate that the growth inhibition of strain WCS358 by a majority of the RDR isolates is mediated by the production of siderophores. A possible role of iron-regulated and iron-antagonized antibiotics (Gill and Warren 1988), however, cannot be excluded. As was demonstrated for strain WCS358, growth of derivative strain WCS374(pMR) was also not inhibited by isolates RDR-A, RDR-E, and RDR-H. In contrast, wild-type strain WCS374 was inhibited by almost all RDR isolates, including RDR-A and RDR-E (Table 1). These results indicate that strains

WCS358 and WCS374(pMR) can use the siderophores produced by isolates RDR-A, RDR-E, and RDR-H, whereas wild-type strain WCS374 can only use the siderophore produced by RDR-H. Because derivative WCS374(pMR) harbors the PupA receptor of strain WCS358, which has a high specificity for pseudobactin 358 (Bitter et al. 1991), these results suggest that both isolates RDR-A and RDR-E apparently produce siderophores with a chemical structure that is similar to that of pseudobactin 358. Analysis of the chemical structure of the siderophores produced by RDR-A and RDR-E will be required to support this suggestion.

In view of the results obtained in the rhizosphere studies conducted with strains WCS358, WCS374, and derivative WCS374(pMR) (Fig. 2), we expected that strain WCS358 would not reduce the rhizosphere population densities of all RDR isolates, because of their ability to utilize pseudobactin 358. Moreover, most of the RDR isolates, except RDR-A, RDR-E, and RDR-H, may even adversely affect the rhizosphere population densities of strain WCS358 by competition for iron via specific siderophores that cannot be utilized by strain WCS358 (Table 1). However, the results demonstrate that rhizosphere population densities of strain WCS358 were not affected by any of the RDR isolates (Fig. 3A), whereas the population densities of most RDR isolates, except RDR-A and RDR-H, were significantly reduced by strain WCS358 (Fig. 3B). As was stated by Buyer and Sikora (1990) and by Jurkevitch et al. (1992), siderophore-mediated competition for iron between microorganisms can be regarded as occurring in two steps: (*i*) competition between the excreted siderophores for iron(III) and (*ii*) competition between microorganisms for the ferric siderophore complexes. The former is controlled by the proton dissociation and formation constants of each siderophore, as well as by their concentrations and kinetics of exchange, while the latter is governed by the existence of an uptake mechanism for, and its affinity to, the ferric siderophore complexes. In this context, strain WCS358 may have maintained its population density in the presence of the competing RDR isolates as a result of a more efficient uptake of ferric pseudobactin 358. This assumption is supported by previous studies (Raaijmakers et al. 1994) that demonstrated that for the uptake of ferric pseudobactin 358, these RDR isolates harbor receptors other than PupA, possibly with a lower affinity. Analysis of the uptake rate of iron complexed to pseudobactin 358 by these RDR isolates will be necessary to confirm this supposition. Nevertheless, this explanation does not account for the significant reductions in rhizosphere population densities of most of the RDR isolates, because they still can acquire iron via their own specific siderophores that cannot be utilized by strain WCS358. Therefore, these results suggest that also mechanisms other than competition for iron contribute to the interactions between *P. putida* WCS358 and most of the RDR isolates.

For example, competition for nutrients present in root exudates has been postulated as an important aspect of microbial interactions in the rhizosphere (Bowen 1991). In this context, Bowen (1991) suggested that microorganisms that are able to utilize specific substrates that are little used by other microorganisms obviously will have a competitive advantage. Furthermore, microorganisms with high growth rates will have an advantage in competition for substrates that are used by a broad range of other microorganisms (Bowen 1991). Preliminary analysis of growth on 12 carbon sources (API-20NE, Biomerieux) demonstrated that most of the RDR isolates utilize exactly the same carbon sources as strain WCS358. However, isolates RDR-A and RDR-H can utilize other carbon sources in addition to those utilized by strain WCS358, including arabinose, mannitol, and maltose (data not shown). Since RDR-A and RDR-H were the only isolates that were not reduced in rhizosphere interactions with strain WCS358 (Fig. 3B), these results suggest that utilization of these specific carbon sources gives them a competitive advantage. However, it should be emphasized that the use of only 12 carbon sources is entirely inadequate and we do not know the significance of their utilization in terms of rhizosphere competence. Future research will focus on elucidating the mechanisms involved in interactions between plant growth promoting strain *P. putida* WCS358 and these specific rhizosphere isolates.

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