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Siderophore-Mediated Uptake of Fe^{3+} by the Plant Growth-Stimulating *Pseudomonas putida* Strain WCS358 and by Other Rhizosphere Microorganisms

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Under iron-limited conditions, *Pseudomonas putida* WCS358 produces a siderophore, pseudobactin 358, which is essential for the plant growth-stimulating ability of this strain. Cells of strain WCS358, provided that they have been grown under Fe^{3+} limitation, take up $^{55}Fe^{3+}$ from the $^{55}Fe^{3+}$-labeled pseudobactin 358 complex with K_m and V_{max} values of 0.23 μM and 0.14 nmol/mg of cell dry weight per min, respectively. Uptake experiments with cells treated with various metabolic inhibitors showed that this Fe^{3+} uptake process was dependent on the proton motive force. Furthermore, strain WCS358 was shown to be able to take up Fe^{3+} complexed to the siderophore of another plant-beneficial *P. fluorescens* strain, WCS374. The tested pathogenic rhizobacteria and rhizofungi were neither able to grow on Fe^{3+}-deficient medium in the presence of pseudobactin 358 nor able to take up $^{55}Fe^{3+}$ from $^{55}Fe^{3+}$-pseudobactin 358. The same applies for three cyanide-producing *Pseudomonas* strains which are supposed to be representatives of the minor pathogens. These results indicate that the extraordinary ability of strain WCS358 to compete efficiently for Fe^{3+} is based on the fact that the pathogenic and deleterious rhizosphere microorganisms, in contrast to strain WCS358 itself, are not able to take up Fe^{3+} from Fe^{3+}-pseudobactin 358 complexes.

Frequent cultivation of monocultures on the same field is a practical demand of modern agriculture. However, frequent cultivation of, e.g., potato in the same field results in yield decreases of up to 30% (11, 12, 22). The causal agents of these yield decreases are assumed to be deleterious, cyanide-producing *Pseudomonas* spp. (1, 23). The rhizosphere also harbors various pathogenic microorganisms which influence the potato yield, e.g., bacteria like *Erwinia carotovora*, which can cause rotting of the potato tubers, and fungi like *Verticillium* spp., which may cause wilting of the potato plants (12, 23).

Bacterization of seed potatoes with certain fluorescent *Pseudomonas* spp. has a beneficial effect on potato yield (2, 11). These plant-beneficial *Pseudomonas* strains have been selected after screening of large numbers of fluorescent, root-colonizing *Pseudomonas* spp. on antibiosis activity against a series of rhizosphere microorganisms (10). For some *Pseudomonas* spp. this antibiosis activity is primarily based on the production of antibiotic compounds (5), while for other *Pseudomonas* spp., like *Pseudomonas putida* WCS358, antibiosis is based on successful competition for Fe^{3+} by strain WCS358 in comparison with that by the pathogenic or deleterious microorganisms (9, 22, 23). Under Fe^{3+} limitation, the beneficial *Pseudomonas* cells produce powerful fluorescent siderophores (7, 17, 18, 25), Fe^{3+}-chelating compounds, which are part of high-affinity Fe^{3+} uptake systems. Recently, it has been demonstrated that these beneficial *Pseudomonas* strains actually produce these siderophores in the rhizosphere (3, 23). Also the ability of the beneficial *Pseudomonas* strain to produce siderophores was shown to be a prerequisite for the increase in potato tuber yield in the field (2). These results suggest that the iron metabolism in soil plays an essential role in plant growth stimulation. Supposedly, the outcome of the competition for limiting Fe^{3+} in soil between the plant, the deleterious (or pathogenic) organisms, and the beneficial microorganisms is of prime importance. This paper focuses on the Fe^{3+} uptake mediated by the siderophore of strain WCS358, pseudobactin 358, in plant growth-stimulating *Pseudomonas* strains and in deleterious or pathogenic rhizosphere microorganisms. The results strongly support previous assumptions that the plant-beneficial *P. putida* strain WCS358 acts as a microbial pesticide.

MATERIALS AND METHODS

Bacteria, fungi, and growth conditions. The plant growth-stimulating strains *P. putida* WCS358 and *P. fluorescens* WCS374 and their siderophore-negative mutants have been described elsewhere (7, 10, 18). Cyanide-producing *Pseudomonas* strains A11, A14, and A63 were isolated from potato roots by A. W. Bakker. Pathogenic *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* were obtained from the Plant Protection Service in Wageningen, The Netherlands. All strains were maintained on King B medium (16). Cells to be used in uptake assays were grown in half-strength standard succinate medium (SSM) (19) after inoculation with approximately 10^7 bacteria per ml by incubation on a rotary shaker at 200 rpm for 16 h at 28°C. When appropriate, the medium was supplemented with 50 μM $FeCl_3$ from a 100 mM $FeCl_3$ stock solution in 1 N HCl.

The pathogenic fungi *Verticillium dahliae* and *V. albo-atrum* were obtained from the Phytopathological Laboratory Willy Commelin Scholten in Baarn, The Netherlands. These fungi were grown on solid YMG medium, which contains 0.4% yeast extract, 1% malt extract, and 0.4% glucose, at 28°C (*V. dahliae*) or 23°C (*V. albo-atrum*). The conidia were harvested with PBS (10 mM sodium phosphate [pH 7.2], 155 mM NaCl) and washed three times with PBS (26). Subse-

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quently, SSM was inoculated with 5×10^7 conidiospores per ml and incubated for 20 h on a rotary shaker (150 rpm) at 28°C, after which more than 80% of the conidiospores had germinated. For uptake assays, the young mycelia were harvested by centrifugation and resuspended in fresh half-strength SSM.

Antibiosis assay. To test the antagonistic activity of one microorganism towards another, the method described by Geels and Schippers (10) was slightly modified. The antagonistic *Pseudomonas* strain or its siderophore-negative mutant was spot-inoculated on SSM agar. After incubation for 48 h at 28°C, the cells were killed by chloroform vapor. Subsequently a suspension of the test organism (10^8 bacterial CFU per ml of 10^7 fungal spores per ml) was sprayed over the agar surface. After incubation at 28°C for 20 h (bacteria) or 4 days (fungi), the inhibition zones were judged.

Purification of siderophores. Siderophores were isolated from culture supernatants of 64-h-old cultures in SSM, as described by van der Hofstad et al. (25). Briefly, contaminating proteins in the culture supernatant were removed by precipitation with 100% ammonium sulfate. Pseudobactins were extracted from the resulting supernatant fluid with phenol-chloroform (1:1, wt/vol) and subsequently precipitated with diethylether. Pseudobactin 358 was further purified to homogeneity by DEAE-Sephadex chromatography, and the structure of pseudobactin 358 was determined (G. A. J. M. van der Hofstad, A. M. M. van Pelt, G. M. G. M. Verjans, C. A. van der Mast, R. Amons, B. Schippers, and P. J. Weisbeek, manuscript in preparation). The concentration of pseudobactin 374 in the preparation was determined by its specific absorbance at 400 nm, with pseudobactin 358 used as a standard.

Fe^{3+} uptake. Logarithmically growing bacteria (A_{620}, 0.4) were harvested by centrifugation at 3,000 × *g* for 15 min at room temperature. The cells were resuspended in fresh half-strength SSM to an A_{620} of 0.2 (approximately 7×10^8 cells per ml and 0.15 mg of cell dry weight per ml) and incubated for 1 h at 28°C on a rotary shaker at 200 rpm prior to use. When appropriate, inhibitors were added from concentrated stock solutions and incubated with the cells for 20 min at 28°C prior to the start of the uptake experiment. The influence of arsenate was tested in phosphate-free succinate medium, in which the phosphate buffer was replaced by an equimolar concentration of morpholinopropanesulfonate, pH 7.2. To study whether these compounds interact with the energy metabolism, their influence on bacterial motility was determined as described by Shoesmith (24). Motility was quantified by counting the number of bacteria moving across a small aperture in a microscope lens. Motility was reduced by 10 μM nigericin, 10 μM valinomycin, and 1 mM sodium azide by 49, 63, and 45%, respectively, and abolished by 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 50 μm 2,4-dinitrophenol (DNP) (both more than 90% inhibition).

$^{55}FeCl_3$ (specific activity, 10 to 35 mCi/mg) in 0.5 N HCl was purchased from New England Nuclear Corp., Boston, Mass. The $^{55}Fe^{3+}$-labeled pseudobactin 358 and $^{55}Fe^{3+}$-labeled pseudobactin 374 stock solutions (50 nmol of Fe^{3+} per ml; 25 μCi/ml) were prepared with a 20% excess of pseudobactin and passed through polyvinylidene difluoride membrane filters of 0.45 μm pore size (Millex disposable filter units, 4 mm; Millipore).

Uptake was started by adding 0.5 to 1.0 μM $^{55}Fe^{3+}$-pseudobactin (final concentration) from the stock solution to the cell suspension in a waterbath at 28°C under continuous stirring. At regular time intervals, 0.5-ml samples of the cell suspension were taken in duplicate and treated as described below. Initially, membrane filters were used to trap the bacteria as described by Cox (6). However, high background levels (10 to 20% of the total ^{55}Fe added) were found with membrane filters of a variety of compositions and origins: cellulose nitrate from Sartorius (type SM113), Nalgene (type 200), and Schleicher & Schuell (type BA85), cellulose acetate from Sartorius (type SM111), filters of mixed cellulose nitrate and cellulose acetate from Millipore (type HA), and polysulfone filters from Gelman (type HT). With membrane filters of polyvinylidene difluoride from Millipore (type HVLP), background levels were lower (3 to 8%) but very variable within one experiment, resulting in differences of 8 to 20% between duplicate measurements.

Excellent reproducibility was observed in an assay in which bacteria are separated from the medium by centrifugation through a layer of silicone oil as described by Kashket (15) with minor modifications. Samples (0.5 ml) were layered on 0.3 ml of a silicone oil mixture (type AR20–type AR200, 9:6; Wacker silicone, Wacker Chemie, Munich, Federal Republic of Germany) in an Eppendorf vial at 4°C. The cells were immediately separated from the medium by centrifugation for 3 min in an Eppendorf centrifuge (type 5414S), which resulted in a cell pellet below the silicone oil layer. The vial was turned upside down, after which the bottom part containing the bacterial pellet was cut off and mixed vigorously with 0.5 ml of water until the cell pellet was resuspended. Scintillation fluid (8 ml; Quickszint212; Zinsser Analytic) was added, and the radioactivity of the mixture was determined by using the tritium channel of an LKB type 1214 Rackbeta liquid scintillation counter with 34% efficiency. Background levels, i.e., radioactivity migrating with cells unable to take up the ^{55}Fe (e.g., cells of strain WCS374, see Results), were below 1.5% of the input radioactivity, and the variation between duplicate measurements was usually below 3%. The results shown are representative of at least three separate experiments which yielded essentially the same results.

RESULTS

Characteristics of pseudobactin 358-mediated Fe^{3+} uptake by *P. putida* WCS358. When $^{55}Fe^{3+}$-pseudobactin 358 was supplied to cells of strain WCS358 grown under Fe^{3+} limitation, $^{55}Fe^{3+}$ was taken up rapidly at 28°C (Fig. 1). Bacteria grown with excess Fe^{3+} were hardly able to take up the Fe^{3+} from the $^{55}Fe^{3+}$-pseudobactin 358 complex. At 4°C, Fe^{3+} uptake in Fe^{3+}-limited cells was drastically reduced (Fig. 1). Determination of rates of pseudobactin 358-mediated Fe^{3+} uptake at temperatures ranging from 4 to 42°C and at pH values from 6.0 to 8.0 showed that temperatures between 28 and 32°C and pH values between 7.0 and 7.5 were optimal for uptake. Therefore, all subsequent experiments were carried out at 28°C and pH 7.2.

From a Lineweaver-Burke plot, a K_m value of 0.23 μM and a V_{max} value of 0.14 nmol/mg of cell dry weight per min were calculated. The rate of uptake of the $^{55}Fe^{3+}$ from $^{55}Fe^{3+}$-pseudobactin 358 (0.6 μM) was not influenced at all by the presence of a threefold excess of unlabeled desferri-pseudobactin 358 (1.8 μM), while a similar excess of unlabeled Fe^{3+}-pseudobactin 358 reduced the initial rate of uptake of the labeled Fe^{3+} approximately threefold (Fig. 2).

Energy source for pseudobactin 358-mediated Fe^{3+} uptake. The low rate of uptake at 4°C (Fig. 1) suggests that the uptake of Fe^{3+} from the $^{55}Fe^{3+}$-pseudobactin 358 is an energy-dependent process. Several inhibitors of energy me-

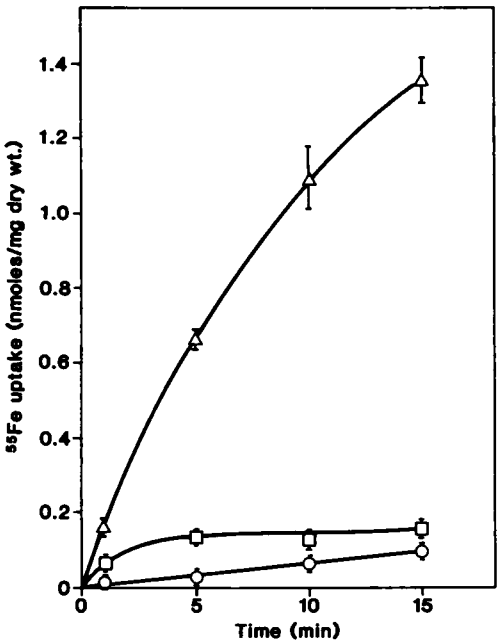

FIG. 1. Pseudobactin 358-mediated ^{55}Fe uptake by *P. putida* WCS358. Cells grown under Fe^{3+} limitation were used in uptake experiments at 28°C (△) or 4°C (□). Cells grown in excess Fe^{3+} were used in an uptake assay at 28°C (○). Data are presented as the means and standard errors of duplicate samples.

tabolism were tested to get information about the energy source which drives the uptake process (Table 1). Neither in phosphate-containing nor in phosphate-free medium was the rate of Fe^{3+} uptake influenced by the presence of arsenate, which reduces intracellular ATP concentrations without

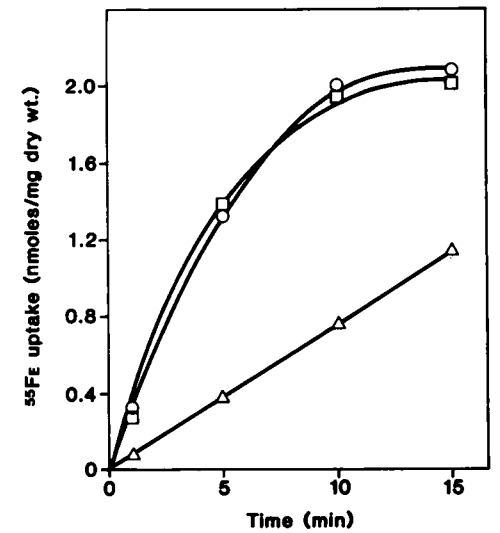

FIG. 2. Effect of the addition of excess desferripseudobactin 358 or Fe^{3+}-complexed pseudobactin 358 on the pseudobactin 358-mediated Fe^{3+} uptake by cells of *P. putida* WCS358. Fe^{3+} uptake was measured in cell suspensions containing 0.6 μM $^{55}Fe^{3+}$-pseudobactin 358 without further additions (○), supplemented with 1.8 μM desferripseudobactin 358 (□), and supplemented with 1.8 μM Fe^{3+}-pseudobactin 358 (△). Data are presented as the means of duplicate samples. Standard errors (not shown) are within the size of the symbols.

TABLE 1. Effect of various inhibitors on the pseudobactin 358-mediated uptake of Fe^{3+} by cells of *P. putida* WCS358

Inhibitor	Final concn (mM)	% Inhibition[a]
Sodium azide	1	>95
DNP	0.05	>95
CCCP	0.05	81
Arsenate[b]	10	<3
Nigericin	0.01	<3
Valinomycin	0.01	36
$HgCl_2$	0.01	>95
N-Ethylmaleimide	0.1	>95

[a] Percent inhibition was calculated from values measured 15 min after the addition of 0.5 μM $^{55}Fe^{3+}$-pseudobactin 358.
[b] Identical results were obtained in phosphate-containing and phosphate-free medium.

decreasing the proton motive force. Sodium azide, which inhibits generation of the proton motive force, appeared to be a very potent inhibitor of Fe^{3+} uptake. The uncouplers CCCP and DNP also markedly reduced Fe^{3+} uptake (Table 1). These results indicate that the proton motive force drives the uptake process. To establish which component of the proton motive force is the actual driving force, the influence of the presence of the ionophores nigericin and valinomycin was determined. Nigericin did not influence uptake of Fe^{3+}, but valinomycin reduced the uptake of Fe^{3+} substantially. The sulfhydryl reagents *N*-ethylmaleimide and $HgCl_2$ abolished the uptake of Fe^{3+} (Table 1).

Interactions of pseudobactin 358 with deleterious and pathogenic rhizosphere microorganisms. In an antibiosis assay (Fig. 3), the cyanide-producing *Pseudomonas* strains A11, A14, and A63, the pathogenic bacteria *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*, and the pathogenic fungi *V. dahliae* and *V. albo-atrum* were unable to grow around the spot of inoculation of the wild-type strain WCS358. Since no or only a small inhibition zone was observed around the inoculation spot of the siderophore-negative mutant of strain WCS358, the growth inhibition observed around the wild-type strain must have been caused by the presence of pseudobactin 358. The small zone of inhibition sometimes observed around the siderophore-negative mutant was most likely caused by nutrient limitation around the bacterial inoculation spot, since no growth inhibition was observed by the siderophore-negative mutant in growth assays performed on nutrient-rich agar surfaces (e.g., King B medium or YMG). For the cyanide-producing *Pseudomonas* spp. and the two *E. carotovora* subsp., the reciprocal experiment (inhibition of *P. putida* WCS358 by the plant-deleterious strain) was performed, but no inhibition of growth of strain WCS358 was observed (data not shown).

All these bacterial and fungal strains were tested for pseudobactin 358-mediated uptake of Fe^{3+} for 25 min. Only background levels of ^{55}Fe label (<1.5%) were found to be associated with the cells or the young mycelia (data not shown).

Interactions of the high-affinity Fe^{3+} uptake systems of two plant growth-stimulating *Pseudomonas* strains. As described previously (3), strain WCS358 is able to grow on solid King B medium in the presence of the siderophore of strain WCS374, pseudobactin 374, while strain WCS374 is unable to grow on these plates in the presence of pseudobactin 358. In order to be able to correlate inhibition on plates with uptake, the experiment was repeated on solid SSM medium. Results similar to those on solid King B medium were obtained (data not shown). The observed difference in anti-

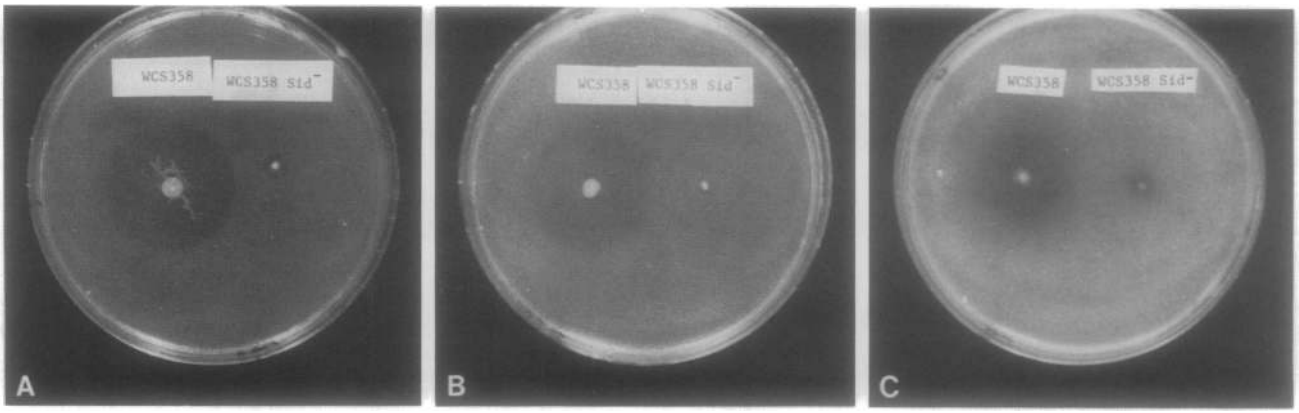

FIG. 3. Antibiosis assay on solid SSM medium with *P. putida* WCS358 and its siderophore-negative (Sid^-) mutant spot-inoculated at the left and right of the plate, respectively. The plates were later sprayed with suspensions of the cyanide-producing strains A63 (A), the pathogenic strain *E. carotovora* subsp. *carotovora* (B), or the pathogenic fungus *V. albo-atrum* (C).

biosis between these two strains appeared to be caused by differences in uptake characteristics: strain WCS358 was able to take up Fe^{3+} from Fe^{3+}-pseudobactin 374 complexes, even to a level similar to that reached by strain WCS374 with its own pseudobactin. In contrast, strain WCS374 was unable to incorporate Fe^{3+} from the Fe^{3+}-pseudobactin 358 complex (Fig. 4). Similar results were obtained when siderophore-negative mutants were used instead of the wild-type strains (data not shown).

DISCUSSION

Under the optimal condition of a temperature of 28°C and a pH value of 7.2, pseudobactin 358-mediated Fe^{3+} uptake in strain WCS358 had a K_m value of 0.23 μM and V_{max} value of 0.14 nmol/mg of cell dry weight per min. No kinetic parameters are available for Fe^{3+} uptake in *Pseudomonas* spp. mediated by a pyoverdine-type siderophore, but our results strongly resemble published uptake profiles (14, 20). Since Fe^{3+}-pseudobactin 358, and not the free form of pseudobactin 358, competes with $^{55}Fe^{3+}$-pseudobactin 358 for uptake (Fig. 2), complex formation of pseudobactin 358 with Fe^{3+} apparently transforms the former molecule into a form recognizable for the uptake system.

Pseudobactin 358-mediated Fe^{3+} uptake in WCS358 requires an energized membrane, as demonstrated by the strong inhibition of Fe^{3+} uptake by sodium azide, DNP, and CCCP. Phosphate bond energy does not seem to be involved, since arsenate had no effect on uptake (Table 1). Nigericin, which reduces the proton gradient (ΔpH) over the cytoplasmic membrane by exchange of K^+ ions for H^+ ions (21), did not significantly influence Fe^{3+} uptake. Valinomycin, however, a potassium ionophore reducing the electrochemical potential ($\Delta\psi$) over the cytoplasmic membrane (21), reduced Fe^{3+} uptake substantially (Table 1). These

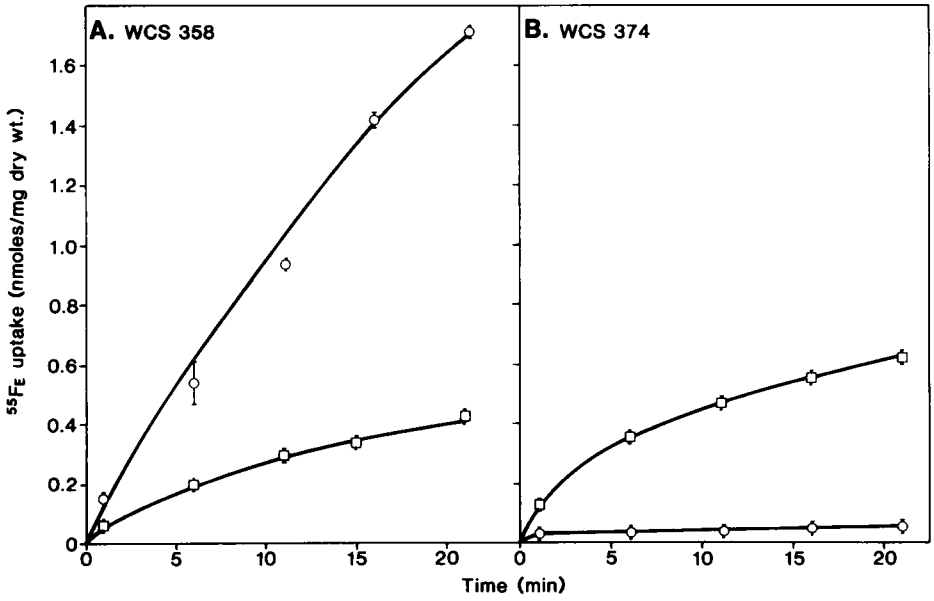

FIG. 4. ^{55}Fe uptake mediated by pseudobactin 358 (○) or pseudobactin 374 (□) by cells of strains *P. putida* WCS358 (A) and *P. fluorescens* WCS374 (B) grown under Fe^{3+} limitation. $^{55}Fe^{3+}$-pseudobactins were used at a final concentration of 1 μM. Data are presented as the means and standard errors of duplicate samples.

results indicate that for the pseudobactin 358-mediated Fe^{3+} uptake, the electrochemical gradient ($\Delta\psi$) is important, while the proton gradient (ΔpH), at least under our standard conditions (pH 7.2), is of minor importance.

This Fe^{3+} uptake system of strain WCS358 seemed to be highly efficient, allowing not only uptake from its own Fe^{3+} siderophore complex, but also from the Fe^{3+} siderophore complex of another plant growth-stimulating strain, *P. fluorescens* WCS374. Furthermore, strain WCS358 was able to take up Fe^{3+} from the Fe^{3+}-pseudobactin complexes of at least four other root-colonizing *Pseudomonas* strains (unpublished results), showing that this strain can make use of various pseudobactins for its iron nutrition. This latter property can be useful in the rhizosphere, where a variety of pseudomonads are present (7, 8). The ability to use the siderophores of neighboring species for its iron nutrition may be an important factor in the competition of strain WCS358 with other microorganisms in the rhizosphere (3, 23).

In order to study the reaction of deleterious and pathogenic rhizosphere microorganisms on the siderophore of the plant growth-stimulating *P. putida* strain WCS358, seven representatives (five bacterial and two fungal species) were chosen from the group of plant-deleterious or plant-pathogenic microorganisms. None of these organisms was able to grow on Fe^{3+}-limited medium in the presence of pseudobactin 358 (Fig. 3). Furthermore, using Fe^{3+} uptake assays, we showed that these fungal and bacterial species were unable to incorporate Fe^{3+} complexed by pseudobactin 358. This latter result demonstrates that the inability to grow in the presence of pseudobactin 358 (Fig. 3) (4) is determined at the level of Fe^{3+} uptake from the Fe^{3+}-pseudobactin 358 complex. So far, models on the role of pseudomonads in competition for Fe^{3+} were based only on growth inhibition experiments on Fe^{3+}-deficient solid medium (4, 10, 17). In our experience, the results of antibiosis assays obtained on different media (e.g., King B medium and SSM) are much more variable than the results of uptake experiments. Therefore, conclusions about the ability of a strain to antagonize other microorganisms by virtue of its siderophore are more accurate when they are based on antibiosis assays in combination with Fe^{3+} uptake experiments. Another reason to interpret antibiosis assays carefully is the recent finding of a fluorescent *Pseudomonas* strain whose antibiotic activity towards fungal growth is due to an antibiotic which is only active under low-iron conditions (13).

Recently, the production of siderophores in the rhizosphere by strain WCS358 was demonstrated (3, 23). This result, combined with those of the antibiosis on Fe^{3+}-deficient medium (Fig. 3) and of Fe^{3+} uptake, indicates that the presence of pseudobactin 358 in the rhizosphere deprives other plant-deleterious and -pathogenic rhizosphere microorganisms of the essential element Fe^{3+}, resulting in a reduction of their activity or growth. Consequently, these results indicate that competition for Fe^{3+}, at the level of uptake of Fe^{3+} from Fe^{3+}-siderophore complexes, is the basis for the action of *P. putida* WCS358 as a microbial pesticide.

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