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A Method for Detection of Pseudobactin, the Siderophore Produced by a Plant-Growth-Promoting *Pseudomonas* Strain, in the Barley Rhizosphere

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Detection in the rhizosphere of the siderophore produced by an inoculated microorganism is critical to determining the role of microbial iron chelators on plant growth promotion. We previously reported the development of monoclonal antibodies (MAb) to ferric pseudobactin, the siderophore of plant-growth-promoting *Pseudomonas* strain B10. One of these MAb reacted less strongly to pseudobactin than to ferric pseudobactin. The MAb reacted to Al(III), Cr(III), Cu(II), and Mn(II) complexes of pseudobactin at a level similar to the level at which it reacted to ferric pseudobactin and reacted less to the Zn(II) complex, but these metals would make up only a small fraction of chelated pseudobactin in soil on the basis of relative abundance of metals and relative binding constants. Fourteen-day-old barley plants grown in limed and autoclaved soil were inoculated with 10^9 CFU of *Pseudomonas* strain Sm1-3, a strain of *Pseudomonas* B10 Rif^r Nal^r selected for enhanced colonization, and sampled 3 days later. Extraction and analysis of the roots and surrounding soil using the MAb in an immunoassay indicated a concentration of 3.5×10^{-10} mol of ferric pseudobactin g^{-1} (wet weight). This is the first direct measurement of a pseudobactin siderophore in soil or rhizosphere samples.

Siderophores are low-molecular-weight iron-chelating agents produced by most bacteria and fungi under iron-limiting conditions (12). The role of siderophores in biocontrol of plant pathogens was first demonstrated with pseudobactin, the siderophore produced by plant-growth-promoting *Pseudomonas* strain B10 (6, 7). Siderophore-mediated interactions between plant-associated microorganisms have been reviewed (8–10, 13, 18).

Siderophores have not yet proven successful in biocontrol of plant pathogens on a routine basis, particularly in field studies. Analysis of the parameters controlling siderophore concentration in natural ecosystems requires a sensitive and specific assay for each siderophore to be studied.

We have previously reported (4) the production of monoclonal antibodies (MAb) reactive to ferric pseudobactin, their specificities, and a competitive enzyme-linked immunosorbent assay (ELISA) (5). In this paper, we further describe the specificity of one MAb, a method for extracting pseudobactin and ferric pseudobactin from soil, and an improved ELISA for use with rhizosphere extracts. We present data on the population of an antibiotic-resistant mutant of *Pseudomonas* strain B10, *Pseudomonas* strain Sm1-3, selected for enhanced colonization, and the concentration of ferric pseudobactin in the rhizosphere of barley plants inoculated with *Pseudomonas* strain Sm1-3. In this paper, we use the term "rhizosphere" broadly to include the rhizoplane and the roots as well.

MATERIALS AND METHODS

Media. Rhizosphere medium (RSM) was previously described (3). RSM rifampin-nalidixic acid (RIF-NAL) plates were supplemented with 100 μg each of RIF and NAL per ml and, in some cases, with 50 μg of cycloheximide per ml. Plant nutrient solution consisted of 2 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 1 mM $MgSO_4$, 3.18 mM $CaNO_3$, 1 mM KH_2PO_4, 1 μM $ZnSO_4$, 2 μM $MnCl_2$, 0.5 μM $CuSO_4$, 0.2 μM $NiSO_4$, 0.2 μM $CoSO_4$, 0.2 μM Na_2MoO_4, and 10 μM H_3BO_3 and was adjusted to pH 6.8 with NaOH. ACES-buffered saline contained (per liter) 8.5 g of NaCl and 3.644 g of ACES and was adjusted to pH 6.8 with NaOH.

Siderophores. Ferric pseudobactin and pseudobactin were isolated as previously described (4), except that instead of a benzyl alcohol extraction the supernatant was passed through a column of Amberlite XAD-16. The column was washed with 2 column volumes of deionized water, and the red-brown material was eluted with 1:1 water-methanol. The solvent was removed by rotary evaporation, and the ferric siderophore was then purified by gel filtration as previously described (4). Chromic pseudobactin was prepared as previously described (19). Al(III), Cu(II), Mn(II), and Zn(II) complexes of pseudobactin were prepared by combining pseudobactin with a 10% molar excess of the metal.

Ferrichrome, ferrichrome A, and rhodotorulic acid were provided by J. B. Neilands. Pseudobactin 589 A was provided by M. Persmark. A chloroform-phenol extract of culture supernatant from *Pseudomonas putida* WCS 358 was provided by P. Weisbeek. Free ligands were converted to iron complexes by adding $Fe(ClO_4)_3$, neutralizing the solution, and centrifuging to remove excess ferric hydroxide.

ELISA. The procedure for quantitation of ferric pseudobactin by a competitive ELISA was previously described (4). For incubation, 96-well plates that had been blocked with nonfat dry milk to prevent adsorption of protein were used, while for ELISA, 96-well plates containing immobilized chromic or ferric pseudobactin (see below) were used. Standards or samples were combined with MAb and diluted to a total volume of 250 μl in each well of an incubation plate. The plate was incubated for 1 h at 37°C to allow pseudobactin-antibody complexes to form. Two aliquots (100 μl each) were transferred from each well of the incubation plate to two wells of an ELISA plate and incubated for

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18 h at 4°C to allow the MAb that was not complexed to solution-phase pseudobactin to bind with immobilized (solid-phase) pseudobactin. The plate was then washed to remove solution-phase MAb. Anti-mouse immunoglobulin conjugated to alkaline phosphatase (Zymed Laboratories, South San Francisco, Calif.) was added and incubated for 1 h at 37°C. The plate was washed to remove solution-phase antibody-alkaline phosphatase conjugate, and the alkaline phosphatase substrate, *p*-nitrophenyl phosphate, was added. The plate was incubated for 1 to 4 h to allow the reaction product, *p*-nitrophenol, to form. Plates were read on a ThermoMax 96-well plate reader (Molecular Devices Corp., Menlo Park, Calif.) at 405 nm.

Assay specificity. Hybridoma strain 5D4-C3 was grown as previously described (4). MAb 5D4-C3 was purified by ammonium sulfate precipitation followed by chromatography on protein G (GammaBind Plus; Genex Corp., Gaithersburg, Md.). ELISA plates were prepared by coating Polysorb 96-well plates (Nunc, Roskilde, Denmark) with chromic pseudobactin-keyhole limpet hemocyanin conjugate as previously described (4).

The specificity of the ELISA was tested by assaying various ferric siderophores and metal complexes of pseudobactin in the competitive ELISA. The reactivity of each siderophore or pseudobactin complex, at a concentration of 5×10^{-9} mol per well, was compared with a standard curve for ferric pseudobactin ranging from 10^{-8} to 1.2×10^{-12} mol per well. Controls for pseudobactin complexes consisted of 5×10^{-9} mol of ferric pseudobactin plus an equimolar quantity of the metal being tested. The recognition of pseudobactin by MAb 5D4-C3 was assayed by adding 1 mM ethylenediamine di(*o*-hydroxy-phenylacetic acid) (EDDHA) or 1 mM *N*,*N*′-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED) to each well in order to keep the pseudobactin from scavenging iron. Controls consisted of ferric pseudobactin in the presence of each chelating agent. All experiments were performed twice.

Strains. *Pseudomonas* strain B10 was obtained from John Leong. *Pseudomonas* strain B10 Rif^r Nal^r was prepared by spreading *Pseudomonas* strain B10 on RSM RIF-NAL plates. A single colony was transferred 10 times on RSM plates and then grown on RSM RIF-NAL plates to confirm the stability of the mutation. In order to maximize colonization, washed cells of *Pseudomonas* strain B10 Rif^r Nal^r were used to inoculate barley grown in sand, isolated from rhizosphere extracts on RSM RIF-NAL plates supplemented with cycloheximide to inhibit fungal growth, and reinoculated on barley. After eight cycles of isolation and inoculation, several single colonies were evaluated for colonization. The strain giving the greatest colonization, designated *Pseudomonas* strain Sm1-3, was selected for future studies.

Plant inoculation. Forty to fifty grams of field-moist sieved (<0.5 cm) Sassafras sandy loam soil, limed to pH 7 and autoclaved twice, was placed in 25-mm-diameter 120-mm-long plastic tubes with conical bottoms (RLC-3 Cone-tainers; Stuewe and Sons, Corvallis, Oreg.). A single surface-sterilized barley seed (*Hordeum vulgare* cv. Minorimugi) was planted 1 cm deep in each tube. After germination at 27°C, the plants were grown for 14 days at 22°C by using a 14-h-day–10-h-night cycle. Plants were watered daily with 5.5 ml of plant nutrient solution. *Pseudomonas* strain Sm1-3 was grown overnight in RSM at 22°C, washed with plant nutrient solution, and resuspended in plant nutrient solution, and 2.5 ml was pipetted onto the soil at a concentration of 4×10^8 CFU/ml. Plants were harvested 3 days later. For each experiment, three plants treated with *Pseudomonas* strain Sm1-3 and one untreated control plant were used.

Sample collection. The roots and soil from each Cone-tainer were removed, cut into 1-cm segments with a razor blade, and thoroughly mixed. Approximately one-half of the sample was transferred into a previously tared sterile 125-ml Erlenmeyer flask for colonization studies, and the other half was transferred into a previously tared 50-ml Teflon centrifuge tube equipped with a sealing cap (Nalgene) for siderophore extraction. All manipulations were performed under sterile conditions.

Colonization assay. After the samples were weighed, 25 ml of cold sterile plant nutrient solution was added and the flasks were shaken at 4°C for 15 min. The slurry was diluted in cold plant nutrient solution in sterile test tubes, and an aliquot was spread on RSM RIF-NAL plates in a laminar flow hood by using a SpiralPlater (Spiral Systems Inc., Cincinnati, Ohio). Plates were incubated at 30°C, and colonies were counted.

Siderophore extraction. The extraction method was developed by adding known quantities of pseudobactin and ferric pseudobactin to samples of barley roots and soil and extracting with various aqueous and organic solvents. Liquefied phenol provided quantitative (100%) recovery of pseudobactin and ferric pseudobactin in soil. A variety of other extractants, including 1 M NaCl, 1 M ammonium acetate, 10% MeOH, 25% MeOH, 0.1 M tetraethylammonium acetate in 10% MeOH, 1 mM Fe-EDTA in ACES-buffered saline, 2 M pyridine-acetic acid (pH 5.5), pyridine, and 1:1 chloroform-phenol, gave low yields (data not shown).

Twenty milliliters of liquefied phenol was added to each weighed centrifuge tube. The tubes were rotated for 1 h at room temperature and centrifuged for 20 min at $7{,}600 \times g$. Fourteen milliliters was removed, frozen, and dried by sublimation under vacuum. The extract was resuspended in 10 ml of ACES-buffered saline containing 1 mM Fe-EDTA and ultrafiltered by using a 3,000-molecular-weight cutoff filter (Centriprep 3; Amicon). The filtrate was analyzed by ELISA.

ELISA for rhizosphere samples. ELISA plates were prepared by covalent cross-linking of pseudobactin to the surface of 96-well plates (Covalink; Nunc). Fifty microliters of 0.5 mM pseudobactin in 0.1 M borate buffer, pH 7.5, was added to each well. Fifty microliters of 1 mM disuccinimidyl suberate (Pierce) in 1:1 dimethyl sulfoxide-0.1 M borate, pH 7.5, was then added and mixed. After incubation overnight at room temperature, the plate was washed five times with deionized water. Two hundred microliters of 1% casein in phosphate-buffered saline (PBS) containing 1 mM Fe-EDTA was added, and the plates were incubated at 37°C for 2 h. The plates were washed five times in PBS-Tween and stored at 4°C.

Samples were quantitated by mixing 200 μl of rhizosphere extract, 25 μl of ACES-buffered saline, and 25 μl of MAb 5D4-C3 in an incubation plate and comparing the absorbance with a standard curve constructed with 25 μl of the ferric pseudobactin standards, 200 μl of a control plant rhizosphere extract, and 25 μl of MAb 5D4-C3. In order to identify interferences in rhizosphere samples, 14-point standard addition curves were constructed by combining 25 μl of the ferric pseudobactin standards, typically ranging from 10^{-8} to 10^{-13} mol per well, with 200 μl of rhizosphere extract and 25 μl of MAb. These curves were compared with ones for identical amounts of the ferric pseudobactin standards and MAb but with the sample replaced by ACES-buffered saline.

TABLE 1. Reactivities of metal complexes of pseudobactin with MAb 5D4-C3 in a competitive ELISA[a]

Siderophore	% Reactivity for siderophore[b]	Control	% Reactivity for the control
Al(III) Ps	108	FePs + Al^{3+}	96
Cr(III) Ps	106	ND[c]	
Cu(II) Ps	83	FePs + Cu^{2+}	98
Mn(II) Ps	106	FePs + Mn^{2+}	107
Zn(II) Ps	44	FePs + Zn^{2+}	30

[a] Abbreviations: Ps, pseudobactin; FePs, ferric pseudobactin.
[b] With the absorbance of 5×10^{-9} mol of FePs being equal to 100% and the absorbance corresponding to the upper plateau of the FePs standard curve (high absorbance; low concentration of FePs) being equal to 0%. These values were obtained from a four-parameter curve fit with a correlation coefficient of 0.952 (Softmax software; Molecular Devices Corporation, Menlo Park, Calif.).
[c] ND, not determined.

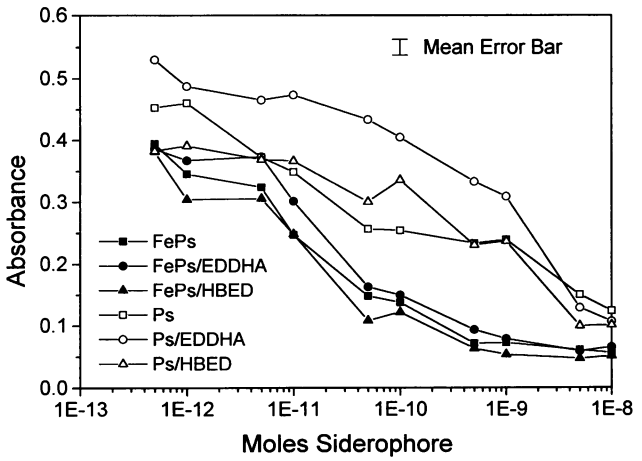

FIG. 1. Results of a competitive ELISA comparing pseudobactin (Ps) and ferric pseudobactin (FePs) in the presence and absence of 1 mM EDDHA or HBED. Each datum point represents the mean of two wells in an ELISA plate.

RESULTS

In the ELISA procedure, the A_{405} is proportional to the amount of *p*-nitrophenol and, therefore, to the amount of immobilized alkaline phosphatase. The amount of immobilized alkaline phosphatase is proportional to the amount of immobilized MAb, while the amount of immobilized MAb is inversely proportional to the amount of solution-phase ferric pseudobactin. Therefore, the A_{405} is inversely proportional to the amount of ferric pseudobactin in the sample being analyzed.

It seemed possible that siderophores produced by other organisms might react in the ELISA, thus making it impossible to quantitate ferric pseudobactin. Ferrichrome, ferrichrome A, rhodotorulic acid, ferric pseudobactin 358, and ferric pseudobactin 589 gave no response in our assay (data not shown), indicating that MAb 5D4-C3 binds very weakly, if at all, to these siderophores. Pseudobactin 358 and pseudobactin 589 have structures similar, but not identical, to pseudobactin from *Pseudomonas* strain B10 (15, 20). We previously reported that MAb 5D4-C3 did not bind to several other siderophores produced by fluorescent pseudomonads (4).

Siderophores in soil might be expected to bind other metals besides iron, although given the large amount of iron in soil and the selectivity of siderophores for iron (12), ferric siderophore should be the dominant species. The Al(III), Cr(III), Cu(II), and Mn(II) complexes of pseudobactin gave responses similar to that of ferric pseudobactin, while the Zn complex was less reactive (Table 1). However, the control which had Zn^{2+} plus ferric pseudobactin also had a decreased response, indicating that Zn^{2+} interferes with our assay. An assay using serial dilutions of Zn^{2+} and a constant concentration of ferric pseudobactin indicated that Zn^{2+} interfered only at concentrations greater than 12.5 μM, equivalent to 1 nmol per well (data not shown).

It seemed possible that MAb 5D4-C3 would not react with pseudobactin, in which case it would be possible to assay for ferric pseudobactin alone. Standard curves for pseudobactin and ferric pseudobactin in the presence and absence of two strong chelating agents, EDDHA and HBED, were compared. As shown in Fig. 1, the chelating agents alter the pseudobactin response, presumably by preventing formation of ferric pseudobactin. They do not alter the response of ferric pseudobactin, indicating that they do not interfere with the assay. Pseudobactin is somewhat reactive in our assay but not as reactive as ferric pseudobactin. While it might be possible to quantitate each one separately, by splitting a sample and adding iron to one portion and not to the other, in this study all assays of rhizosphere samples used additions of iron, as Fe-EDTA, in order to convert any pseudobactin to ferric pseudobactin. Results, therefore, represent the sum of pseudobactin and ferric pseudobactin.

The colonization of barley by *Pseudomonas* strain B10 was assayed by using an antibiotic-resistant mutant. Preliminary experiments suggested that colonization was poor, so a strain exhibiting improved colonization, *Pseudomonas* strain Sm1-3, was isolated. When barley grown in sand was inoculated, *Pseudomonas* strain B10 Rif^r Nal^r colonization averaged 2.2×10^5 CFU/g while *Pseudomonas* strain Sm1-3 averaged 2.6×10^6 CFU/g.

Extraction of rhizosphere samples with liquefied phenol followed by sublimation gave 100% yield when either pseudobactin or ferric pseudobactin was added in known quantities. While addition of ethyl ether to the liquefied phenol (1:10 phenol-ether) followed by extraction with H_2O did not give quantitative recovery, the removal of phenol by sublimation under vacuum did give 100% yield.

The assay for ferric pseudobactin was tested by a standard addition protocol. Without ferric pseudobactin present in the extract, a sigmoid-like curve results, as shown by the solid line for the control plant in Fig. 2. With ferric pseudobactin present in the extracts of plants treated with *Pseudomonas* strain Sm1-3, the curve (dashed line) plateaus when the ferric pseudobactin concentration of the extract, which is constant from well to well, exceeds the ferric pseudobactin concentration of the standard. A well containing sample extract, MAb, and buffer but no ferric pseudobactin standard should have the absorbance corresponding to the plateau if no interferences exist. As shown in Fig. 2, in which the absorbance and calculated concentration of the well are indicated by dotted lines, this was the case. Therefore, samples could be analyzed by comparing their absorbances in the assay to a standard curve for a control plant extract, ferric pseudobactin standard, and MAb 5D4-C3. By increasing the dilution of MAb 5D4-C3 from 1:40 to 1:300, we were able to enhance the sensitivity of the assay and produce a straight line when the data were plotted semilogarithmically over a narrow range of concentrations, as shown in Fig. 3.

This assay was used in two separate experiments with

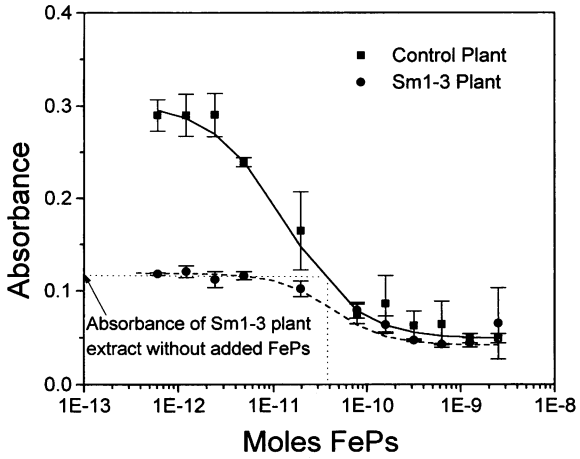

FIG. 2. Competitive ELISA for detection of ferric pseudobactin (FePs) in barley rhizosphere samples. Rhizosphere extracts were added to each well on a standard curve, producing a curve from which the concentration of FePs in the extract can be determined. Curves plateau for the control when the sensitivity of the assay is exceeded or, for the inoculated plant, when the amount of FePs in the sample exceeds that in the added standard. Additionally, absorbance of a single-point assay containing no FePs standard (dotted lines) can also be used to determine the concentration of FePs in extracts. Each datum point represents the mean of two wells in an ELISA plate. Bars, standard deviations.

three treated plants plus one control plant. In the first experiment, the mean concentration of ferric pseudobactin in three rhizosphere samples was 2.28×10^{-10} mol/g (wet weight), with a standard deviation of 0.92×10^{-10}. In the second experiment, the ferric pseudobactin concentration was $4.80 \times 10^{-10} \pm 0.62 \times 10^{-10}$ mol/g (wet weight). Colonization data were also collected in the second experiment, and the mean for the three plants was 6.91 ± 0.97 log CFU/g (wet weight). The moisture content of the samples at harvest in the second experiment was 14%.

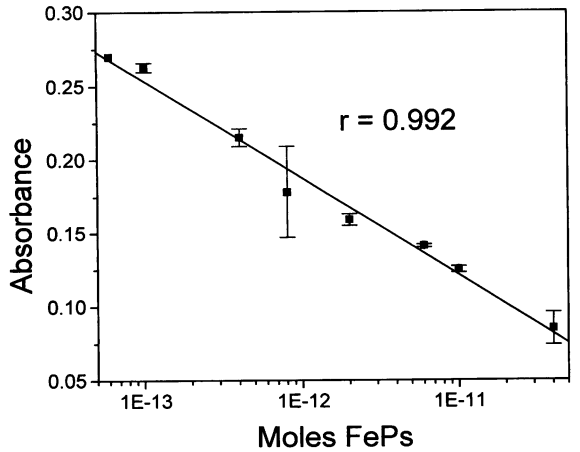

FIG. 3. Linear standard curve for ferric pseudobactin (FePs) over a narrow range produced by using a more dilute MAb concentration in the competitive ELISA. Each datum point represents the mean of two wells in an ELISA plate. Bars, standard deviations.

DISCUSSION

We previously reported that three MAb to ferric pseudobactin did not recognize the siderophores produced by seven other pseudomonads (4). Those results are extended in this paper, in which we have shown that three hydroxamate siderophores as well as the siderophores from two more pseudomonads are not recognized by MAb 5D4-C3. Mihashi et al. (11) used a similar competitive assay to demonstrate cross-reactivity among similar phytosiderophores to a MAb raised to nicotianamine. The high specificity of MAb 5D4-C3, clearly, is advantageous in an assay for ferric pseudobactin in rhizosphere samples. There may well be other rhizosphere pseudomonads producing siderophores that do cross-react in the ELISA. If the control plant extracts have the same concentration of cross-reacting siderophores as the treated plants, the cross-reacting siderophores will not affect the data.

It seemed possible that pseudobactin would form other metal complexes in soil which, when extracted, might be unreactive in our assay. While Al(III), Cr(III), Cu(II), Fe(III), and Mn(II) complexes do react, the Zn(II) complex was relatively unreactive. Because Zn^{2+} itself interferes with the assay, we are unable to say whether zinc pseudobactin reacts with MAb 5D4-C3. However, the high concentration (12.5 μM) of Zn^{2+} required to interfere with the ELISA is unlikely to occur except in highly contaminated soils and, therefore, should not pose a problem under most circumstances.

The phenol extraction system reported here is the first method we know of to quantitatively extract any siderophore from soil. We have not tested it on other siderophores, so we cannot say whether it will be generally useful. We also have not tested it on other soils, and it seems possible that in certain soils it might be less effective.

In our previous work, we first covalently linked pseudobactin to latex-coated polystyrene plates, but high variation from plate to plate rendered this method almost useless (4). We next coated polystyrene plates with chromic pseudobactin-keyhole limpet hemocyanin conjugate, and this was more satisfactory. Covalent linking of pseudobactin to Covalink plates appears to be a superior method, since using these plates in conjunction with reduced antibody concentration has resulted in a detection limit unobtainable by our previous methods.

Data from two separate studies using three treated plants plus one control plant indicate that the extraction and immunoassay provide the means for detecting ferric pseudobactin in barley rhizosphere from plants inoculated with *Pseudomonas* strain Sm1-3. This is the first time that a siderophore produced by a fluorescent pseudomonad has been directly measured in a rhizosphere sample. However, the soil was limed and autoclaved, so the data reported here may be quantitatively different from *Pseudomonas* colonization and pseudobactin concentration in natural soils.

Bioassays for detection of a variety of siderophores are available (1, 14, 16, 17), but no one has reported a bioassay for siderophores produced by fluorescent pseudomonads. Presumably this is due to the ability of fluorescent pseudomonads to use each other's siderophores for iron transport (2), thus rendering bioassays nonspecific. The immunoassay reported here is more sensitive and requires a smaller sample than bioassays (1, 14, 16, 17).

Extraction of rhizosphere samples with liquefied phenol followed by immunoassay provides a method for the determination of ferric pseudobactin in soil. We are currently

applying this method to study the effects of the inoculation method, inoculum density, and soil pH on colonization and pseudobactin concentration in the rhizosphere of barley plants.

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