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Utilization of Heterologous Siderophores Enhances Levels of Iron Available to *Pseudomonas putida* in the Rhizosphere

JOYCE E. LOPER* AND MARCELLA D. HENKELS

Agricultural Research Service, U.S. Department of Agriculture, and Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon

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***Pseudomonas* spp. have the capacity to utilize siderophores produced by diverse species of bacteria and fungi, and the present study was initiated to determine if siderophores produced by rhizosphere microorganisms enhance the levels of iron available to a strain of *Pseudomonas putida* in this natural habitat. We used a previously described transcriptional fusion (*pvd-inaZ*) between an iron-regulated promoter (*pvd*) and the ice nucleation reporter gene (*inaZ*) to detect alterations in iron availability to *P. putida*. Ice nucleation activity (INA) expressed from the *pvd-inaZ* fusion by *P. putida* N1R or N1R Pvd$^-$, a derivative deficient in the production of a pyoverdine siderophore, was inversely related to the concentration of ferric citrate in a culture medium. In culture, INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was reduced in the presence of the ferric complex of pseudobactin-358, a pyoverdine siderophore produced by *P. putida* WCS358 that can be utilized as a source of iron by N1R Pvd$^-$. In the rhizosphere of cucumbers grown in sterilized soil, N1R Pvd$^-$ (*pvd-inaZ*) expressed INA, indicating that iron availability was sufficiently low in that habitat to allow transcription of the iron-regulated *pvd* promoter. Coinoculation with WCS358 or N1R significantly decreased INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere, whereas coinoculation with a pyoverdine-deficient mutant of WCS358 did not reduce INA expressed by N1R Pvd$^-$ (*pvd-inaZ*). These results indicate that iron availability to N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere was enhanced by the presence of another strain of *P. putida* that produces a pyoverdine that N1R Pvd$^-$ (*pvd-inaZ*) was able to utilize as a source of iron. In culture, strain N1R Pvd$^-$ also utilized ferric complexes of the siderophores enterobactin and aerobactin as sources of iron. In the rhizosphere of cucumbers grown in sterilized soil, INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was reduced in the presence of strains of *Enterobacter cloacae* that produced enterobactin, aerobactin, or both siderophores, but INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was not altered in the presence of a mutant of *E. cloacae* deficient in both enterobactin and aerobactin production. Therefore, the iron status of *P. putida* was altered by siderophores produced by an unrelated bacterium coinhabiting the rhizosphere. Finally, we demonstrated that INA expressed by N1R containing *pvd-inaZ* in the rhizosphere differed between plants grown in sterilized versus nonsterilized field soil. The results of this study demonstrate that (i) *P. putida* expresses genes for pyoverdine production and uptake in the rhizosphere, but the level of gene expression is influenced by other bacteria that coexist with *P. putida* in this habitat, and (ii) diverse groups of microorganisms can alter the availability of chemical resources in microbial habitats on root surfaces.**

In environments in which iron is limited, fluorescent pseudomonads produce pyoverdines, a class of siderophores comprised of a dihydroxyquinoline chromophore linked to a peptide of variable length and composition (1). Pyoverdines are secreted from the bacterial cell and can chelate ferric iron in the environment via the hydroxamate and hydroxyacid groups present within the peptide moiety of the molecule (1). Strains of *Pseudomonas* spp. have outer membrane receptor proteins that transport ferric iron complexed to their cognate pyoverdines into the bacterial cell (3), where the iron becomes available for metabolic processes. Certain strains can also utilize ferric complexes of pyoverdines produced by other strains of *Pseudomonas* spp. due to the presence of multiple outer membrane receptors that recognize heterologous pyoverdines (16). Furthermore, *Pseudomonas* spp. can utilize iron complexes of a variety of different siderophores produced by fungi and bacteria (references 12 and 25 and references therein), including the catechol enterobactin (29) and the hydroxamate aerobactin, which are produced by members of the *Enterobacteriaceae*.

* Corresponding author. Mailing address: Horticultural Crops Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 3420 N.W. Orchard Ave., Corvallis, OR 97330. Phone: (541) 750-8771. Fax: (541) 750-8764. E-mail: loperj@bcc.orst.edu.

Pseudomonas putida and *Pseudomonas fluorescens* are common rhizosphere and soil inhabitants, and certain strains promote plant growth and health by suppressing diseases caused by soilborne pathogens. The capacity of these strains to produce pyoverdines is linked, in some cases, with disease suppression; mutants deficient in pyoverdine production can be less effective than parental strains in biological control (17). Pyoverdines produced in situ by *Pseudomonas* spp. are thought to chelate iron in a form that is unavailable to pathogens, thereby preventing the pathogens' access to the already limited pool of soluble iron in the rhizosphere. We previously described an iron sensor (*pvd-inaZ*) that is useful in assessing levels of biologically available iron in the rhizosphere, in soil, and on leaf surfaces (18, 20). The iron sensor is comprised of an iron-regulated promoter of a pyoverdine production and uptake (*pvd*) region from *Pseudomonas syringae* fused to an ice nucleation reporter gene (*inaZ*). In culture and in natural habitats, ice nucleation activity (INA) expressed by *Pseudomonas* spp. containing *pvd-inaZ* is inversely related to iron availability (18, 20). Our previous results indicated that INA expressed by *P. fluorescens* containing *pvd-inaZ* was not static on root surfaces but instead varied over time (18). Numerous factors, such as the solubilization of iron by organic acids exuded from plant roots (23) or the presence of phytosiderophores

TABLE 1. Bacterial strains

Strain	Description[a]	Relevant characteristics[b]	Source or reference
P. putida			
N1R	Rhizosphere bacterium	Pvd^+ Ice^- Rif^r	7
N1R Pvd^-	Tn*5* derivative of N1R designated JL4316	Pvd^- Ice^- Rif^r Km^r	27
N1R (*pvd-inaZ*)	N1R containing fusion plasmid *pvd-inaZ*	Pvd^+ Ice^+ Rif^r Km^r	This study
N1R Pvd^- (*pvd-inaZ*)	N1R Pvd^- containing fusion plasmid *pvd-inaZ*	Pvd^- Ice^+ Rif^r Km^r	This study
WCS358	Rhizosphere bacterium	Pvd^+ Ice^-	9
WCS358 Pvd^-	Tn*5* derivative of WCS358 designated JM218	Pvd^- Ice^- Km^r	24
WCS358 Pvd^- (*pvd-inaZ*)	WCS358 Pvd^- containing fusion plasmid *pvd-inaZ*	Pvd^- Ice^+ Km^r	This study
B10	Rhizosphere bacterium	Pvd^+ Ice^-	15
B10 (*pvd-inaZ*)	B10 containing fusion plasmid *pvd-inaZ*	Pvd^+ Ice^+ Km^r	This study
E. cloacae			
EcCT-501	Biological control agent	Ent^+ Iuc^+	26
JL1157	Rif^r derivative of EcCT-501	Ent^+ Iuc^+ Rif^r	19
LA122	Derivative of JL1157, Δ*iuc*	Ent^+ Iuc^- Rif^r	19
LA266	Derivative of JL1157, Δ*ent*	Ent^- Iuc^+ Rif^r	6
LA235	Derivative of JL1157, Δ*ent* Δ*iuc*	Ent^- Iuc^- Rif^r	6

[a] Δ*ent*, deletion of gene(s) required for enterobactin production; Δ*iuc*, deletion of gene(s) required for aerobactin production.
[b] Pvd^+ and Pvd^-, pyoverdine producer or nonproducer, respectively; Ice^+ and Ice^-, ice nucleation active or not ice nucleation active, respectively; Rif^r, rifampin resistant; Km^r, kanamycin resistant; Ent^+ and Ent^-, enterobactin producer or nonproducer, respectively; Iuc^+ and Iuc^-, aerobactin producer or nonproducer, respectively.

(13) or microbial siderophores (4), are likely to influence concentrations of iron available to *Pseudomonas* spp. on root surfaces, and these could contribute to temporal variations in INA expressed in situ by *Pseudomonas* spp. containing *pvd-inaZ*.

The present study was initiated to determine if levels of iron available to *P. putida* are altered by siderophores produced by its microbial coinhabitants in the rhizosphere. The capacity to utilize heterologous siderophores produced by other members of the rhizosphere microflora is a proposed fitness factor, conferring a selective advantage on strains of *Pseudomonas* spp. (2, 12, 30). In support of this hypothesis, Bakker et al. (2) demonstrated that the rhizosphere population size of a mutant of *P. putida* WCS358 deficient in pyoverdine production (Pvd^-) is enhanced in the presence of the parental strain or another strain that produces a pyoverdine that WCS358 can utilize. Furthermore, a strain of *P. fluorescens* that cannot utilize the ferric complex of pseudobactin-358 is suppressed by WCS358 in the rhizosphere, but this suppression is eliminated by introduction of the *pupA* gene, which encodes the outer membrane receptor for the ferric complex of pseudobactin-358 (30). These experiments indicate that the capacity to utilize heterologous siderophores can alter the outcome of interactions among strains of *Pseudomonas* spp. in the rhizosphere, as evaluated by the relative population sizes of coinoculated strains. Because bacterial population size can be altered by many factors other than iron availability, we initiated this study to determine if the presence of heterologous siderophores can alter the iron status of a strain of *P. putida* in the rhizosphere. We demonstrated that the rhizosphere bacterium *P. putida* N1R can utilize ferric complexes of pseudobactin-358, enterobactin, and aerobactin as sources of iron. Furthermore, INA expressed by N1R containing *pvd-inaZ* was reduced in the presence of strains producing these siderophores in the rhizosphere, indicating that microbial coinhabitants alter iron availability to N1R in this natural habitat. Finally, we demonstrated that INA expressed by N1R containing *pvd-inaZ* in the rhizosphere differs between plants grown in sterilized versus nonsterilized field soil. The results of these experiments indicate that the iron status of *P. putida* is altered by its coinhabitants in the rhizosphere.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains are listed in Table 1. The *pvd-inaZ* construct consists of a stable plasmid containing an iron-regulated promoter, obtained from a cluster of pyoverdine biosynthesis and uptake genes from *P. syringae*, fused to a promoterless ice nucleation reporter gene (20). Introduction of *pvd-inaZ* confers iron-regulated INA on *Pseudomonas* spp. (18, 20). N1R, N1R Pvd^-, WCS358 Pvd^-, and B10 do not produce detectable ice nuclei without an introduced ice nucleation gene. *pvd-inaZ* was introduced into these strains by conjugation, as described previously (20).

Transcriptional activity of the *pvd* promoter in culture. The effect of iron on INA expressed by *Pseudomonas* spp. containing *pvd-inaZ* was evaluated in 5-ml shake cultures of modified RSM medium (5). For 1 liter of medium, 0.75 g of $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 18.22 g of ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid], and 2.00 g of NaOH were dissolved in 879 ml of deionized water, and the pH was adjusted to 7.0. After autoclaving, the following sterile stock solutions were added: 1 ml of 1 M KH_2PO_4 (pH 7.0), 20 ml of 50% glycerol, and 100 ml of 10% (wt/vol) Casamino Acids. The medium was supplemented with different concentrations of ferric citrate ($Fe_3C_6H_5O_7$). To equalize treatments for citrate, citric acid trisodium salt dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$) was added to give a total citrate concentration of 10^{-3} M. After 24 h of shaking at 25°C, samples were evaluated for INA and numbers of culturable bacteria (CFU). The number of ice nuclei was determined by the droplet freezing assay (21), and the number of CFU was determined by spreading diluted samples on King's medium B (KMB) agar (14). INA, expressed as $\log_{10}$ (ice nuclei/CFU), was calculated as previously described (21). Reported values are the means of three replicate cultures. The results from duplicate experiments were similar, and results of a representative experiment are presented.

Evaluation of siderophore production and utilization by *P. putida* N1R. Siderophore production was detected by the formation of orange halos surrounding bacterial colonies on CAS agar (32) after 48 h of incubation at 27°C. The capacity of N1R Pvd^- to utilize siderophores produced by test strains of *P. putida* or *Enterobacter cloacae* was detected in a cross-feeding assay. KMB agar was autoclaved, cooled to 50°C, amended with 600 μg of 2,2′-dipyridyl per ml, and seeded with 10^5 CFU of N1R Pvd^- per ml. After solidification, test strains were inoculated by spotting 5-μl drops on the surface of the seeded agar. Plates were incubated for 48 h, and growth of N1R Pvd^- surrounding colonies of test strains was considered indicative of cross-feeding. The experiment was done three times with the same results.

Effect of the ferric complex of pseudobactin-358 on INA expressed by *Pseudomonas* spp. containing *pvd-inaZ* in culture. Pseudobactin-358 complexed with iron at 80% (to avoid free iron in the system) was kindly provided by Peter A. H. M. Bakker (Utrecht University, Utrecht, The Netherlands). The effects of different concentrations of the ferric complex of pseudobactin-358 on INA of N1R Pvd^- (*pvd-inaZ*), WCS358 Pvd^- (*pvd-inaZ*), and B10 (*pvd-inaZ*) were evaluated in 5-ml shake cultures containing modified RSM medium amended with 10^{-6} M ferric citrate. After 24 h of incubation at 25°C, samples were evaluated for INA (21) and CFU. Reported values are the means of three replicate cultures. The results from duplicate experiments were similar, and results of a representative experiment are presented.

Cross-feeding of N1R Pvd^- (*pvd-inaZ*) in the rhizosphere. Cross-feeding of N1R Pvd^- (*pvd-inaZ*) in the rhizosphere of cucumbers was evaluated in enclosed soil chambers. Cucumber seeds (*Cucumis sativus* L. cv. Marketmore) were surface sterilized in a 95% ethanol solution (30 s) followed by a 1% hypochlorite solution (10 min). Seeds were rinsed thoroughly in sterile deionized water and

TABLE 2. Utilization of heterologous siderophores by *P. putida* N1R Pvd$^-$

Test strain	Siderophore production phenotype	Cross-feeding of N1R Pvd$^-$ [a]
P. putida		
N1R	Pvd$^+$	+
N1R Pvd$^-$	Pvd$^-$	−
WCS358	Pvd$^+$	+
WCS358 Pvd$^-$	Pvd$^-$	−
E. cloacae		
JL1157	Ent$^+$ Iuc$^+$	+
LA122	Ent$^+$ Iuc$^-$	+
LA266	Ent$^-$ Iuc$^+$	+
LA235	Ent$^-$ Iuc$^-$	−

[a] The capacity of N1R Pvd$^-$ to utilize siderophores produced by test strains was detected in a cross-feeding assay. Molten KMB amended with 600 μg of 2,2′-dipyridyl per ml was seeded with 10^5 CFU of the indicator strain *P. putida* N1R Pvd$^-$ per ml. +, zone of growth surrounding colonies of test strains indicated siderophore cross-feeding; −, no detectable zone. The experiment was done three times with identical results.

placed in covered sterile beakers containing moist filter papers for 2 days at 27°C. Chambers consisted of centrifuge tubes (Beckman Polyallomer [38 by 102 mm]; Beckman Instruments Inc., Palo Alto, Calif.) containing 80 ml of Warden sandy silt loam (pH 7.4; 6.0 mg of Fe per kg) at a matric potential of −0.03 MPa. Chambers were capped with 50-ml glass beakers and, except where noted, autoclaved for 60 min. The bacterial inoculum was prepared by growing strains for 24 h at 27°C in 5-ml shake cultures containing either SM medium (20) amended with 10^{-4} M ferric citrate for *Pseudomonas spp.* or Luria-Bertani medium (31) for *E. cloacae*. Cells were harvested by centrifugation and resuspended in sterile deionized water to an optical density of 0.1 at 600 nm, which corresponded to approximately 10^8 CFU/ml. The inoculum consisted of these suspensions (to achieve approximately 10^6 CFU/root system), 100-fold dilutions of these suspensions (to achieve approximately 10^4 CFU/root system), or 1:1 (vol:vol) mixtures of suspensions from two bacterial strains. Ten minutes before planting in the chambers, seedlings were dipped in bacterial suspensions. One seedling was planted in each chamber. Following planting, capped chambers were incubated at 25°C with a photoperiod of 12 h. At various times after planting, root systems were retrieved from each of five replicate tubes. Root systems were removed, gently shaken to remove loose soil, and placed in culture tubes containing wash buffer (22). Tubes containing root systems were placed in a bath-style sonicator for 5 min prior to serial dilution of root washings. Samples from each dilution series were evaluated by the droplet freezing assay to estimate INA (21) and spread on KMB agar supplemented with antibiotics to estimate CFU. Antibiotics were rifampin at 100 μg/ml for strains of *E. cloacae*, *P. putida* N1R, and *P. putida* WCS358 and rifampin at 100 μg/ml and kanamycin at 50 μg/ml for N1R Pvd$^-$, N1R (*pvd-inaZ*), N1R Pvd$^-$ (*pvd-inaZ*), WCS358 Pvd$^-$, and WCS358 Pvd$^-$ (*pvd-inaZ*). Petri plates were incubated at 27°C for 48 h before colonies were counted. Colonies of Pvd$^+$ and Pvd$^-$ strains of *P. putida* could be distinguished by the presence or absence of fluorescence under UV light (λ = 366 nm). Nonamended KMB was used in all experiments for total bacterial counts and to detect any contaminants in the rhizosphere of plants grown from untreated control seed in sterilized soil. All experiments were done twice, with similar results. Results of a representative experiment of each are presented.

Statistical analysis. The SAS (Statistical Analysis Systems Institute, Cary, N.C.) General Linear Models procedure was used for statistical analysis of INA data. Mean bacterial population sizes in the rhizosphere were calculated by averaging the logarithm (base 10) of values obtained for five individual root systems, each of which served as a replicate for statistical analysis. Where statistical differences among treatments are specified, Fisher's protected least significant difference at P of 0.05 was used to separate mean values.

RESULTS

***P. putida* N1R Pvd$^-$ utilizes ferric complexes of siderophores produced by other strains of rhizosphere bacteria.** A Pvd$^-$ mutant of N1R (N1R Pvd$^-$) did not produce a zone on CAS agar, indicating a lack of siderophore production on this medium. N1R Pvd$^-$ grew very poorly on KMB containing 600 μg of the iron chelator 2,2′-dipyridyl per ml, except within zones surrounding colonies of bacteria that produced siderophores that N1R Pvd$^-$ could utilize. Zones of growth of N1R Pvd$^-$ were observed surrounding colonies of strains N1R and WCS358, but no zone was observed surrounding colonies of WCS358 Pvd$^-$ (Table 2). Zones of growth of N1R Pvd$^-$ also were observed surrounding colonies of *E. cloacae* JL1157, which produces the siderophores enterobactin and aerobactin; LA122, a mutant that produces only enterobactin; and LA266, a mutant that produces only aerobactin. No zones of N1R Pvd$^-$ growth were observed surrounding colonies of LA235, a mutant of *E. cloacae* JL1157 that produces neither aerobactin nor enterobactin.

INA expressed by *Pseudomonas* spp. containing *pvd-inaZ* is inversely related to iron availability in culture. INA expressed by *P. putida* N1R (*pvd-inaZ*), *P. putida* N1R Pvd$^-$ (*pvd-inaZ*), *P. putida* WCS358 Pvd$^-$ (*pvd-inaZ*), and *P. fluorescens* B10 (*pvd-inaZ*) decreased by 5 to 8 orders of magnitude as the concentration of ferric citrate in RSM medium was increased from 10^{-7} M to 10^{-3} M (Fig. 1). INA expressed by N1R did not differ significantly from that expressed by N1R Pvd$^-$, indicating that the mutation in the Pvd$^-$ derivative did not alter INA expressed from the *pvd-inaZ* fusion.

INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) in culture is diminished in the presence of ferric pseudobactin-358. In RSM medium containing 10^{-6} M ferric citrate, INA was expressed by virtually every cell of N1R Pvd$^-$ (*pvd-inaZ*), WCS358 Pvd$^-$ (*pvd-inaZ*), or B10 (*pvd-inaZ*) (i.e., approximately 0 $\log_{10}$ [ice nuclei/cell]) (Fig. 2). When the medium was amended with 10^{-4} M of the ferric complex of pseudobactin-358, the pyoverdine produced by WCS358, INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) decreased by 2 orders of magnitude (Fig. 2). INA expressed by WCS358 Pvd$^-$ (*pvd-inaZ*) decreased by almost 5 orders of magnitude in the presence of 10^{-4} M ferric pseu-

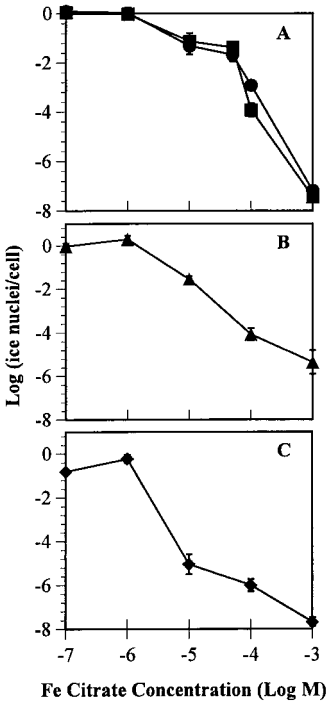

FIG. 1. INA [$\log_{10}$ (ice nuclei/cell)] expressed by *Pseudomonas* spp. containing *pvd-inaZ* in RSM medium amended with ferric citrate. *P. putida* N1R (*pvd-inaZ*) (●) and the pyoverdine-deficient mutant N1R Pvd$^-$ (*pvd-inaZ*) (■) (A), *P. putida* WCS358 Pvd$^-$ (*pvd-inaZ*) (▲) (B), and *P. fluorescens* B10 (*pvd-inaZ*) (◆) (C) were grown for 24 h in RSM medium amended with 10^{-3} to 10^{-7} M ferric citrate. INA values are means of three replicate cultures. Error bars represent standard errors of the means; most error bars are obscured by graph symbols.

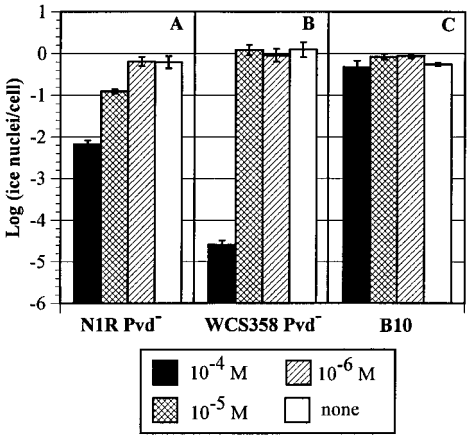

FIG. 2. Influence of ferric pseudobactin-358 on INA [$\log_{10}$ (ice nuclei/cell)] expressed by *Pseudomonas* spp. containing *pvd-inaZ*. *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) (A), *P. putida* WCS358 Pvd$^-$ (*pvd-inaZ*) (B), and *P. fluorescens* B10 (*pvd-inaZ*) (C) were grown for 24 h in a medium amended with 10^{-4}, 10^{-5}, or 10^{-6} M ferric pseudobactin-358 or a nonamended medium. INA values are means of three replicate cultures. Error bars represent standard errors of the means.

dobactin-358 (Fig. 2). In contrast, INA expressed by B10 (*pvd-inaZ*) did not change in the presence of ferric pseudobactin-358 (Fig. 2), a siderophore that B10 could not utilize as a source of iron (data not shown). Therefore, exogenous sources of ferric pseudobactin-358 decreased the INA of only those strains that could utilize the siderophore as a source of iron.

INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumbers decreases in the presence of WCS358 or N1R. One day after inoculation of roots, N1R Pvd$^-$ (*pvd-inaZ*) expressed -1.4 $\log_{10}$ (ice nuclei/cell) in the rhizosphere of cucumbers (Fig. 3A and C). When coinoculated with 10^6 CFU of WCS358 or the wild-type strain N1R per root, both of which produce pyoverdines that N1R Pvd$^-$ could utilize, INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was only ca. -2.6 $\log_{10}$ (ice nuclei/cell) (Fig. 3A). In contrast, coinoculation with WCS358 Pvd$^-$ did not decrease INA expressed by N1R Pvd$^-$ (*pvd-inaZ*). A similar pattern of INA expression by N1R Pvd$^-$ (*pvd-inaZ*) was observed at 6 days following its inoculation onto cucumber roots (Fig. 3B), although INA at 6 days was less than that at 1 day for all treatments. When coinoculated with 10^4 CFU of WCS358 or N1R per root, INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was decreased significantly at 6 days following inoculation (Fig. 3D).

When inoculated at 10^6 CFU/root, all of the strains (WCS358, WCS358 Pvd$^-$, and N1R) decreased the population size of N1R Pvd$^-$ (*pvd-inaZ*) to similar degrees (Table 3). When inoculated at 10^4 CFU/root, none of the strains consistently decreased the population size of N1R Pvd$^-$ (*pvd-inaZ*) in two replicated experiments.

INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumbers decreases in the presence of *E. cloacae* JL1157. When inoculated at 10^4 CFU/root, *E. cloacae* JL1157 decreased INA expressed by coinoculated cells of N1R Pvd$^-$ (*pvd-inaZ*) at 1 day after planting (Fig. 4A). In contrast, mutants of JL1157 deficient in aerobactin production (LA122), enterobactin production (LA266), or production of both siderophores (LA235) did not decrease significantly INA expressed by coinoculated cells of N1R Pvd$^-$ (*pvd-inaZ*). Five days following its inoculation onto cucumber roots, N1R Pvd$^-$ (*pvd-inaZ*) expressed less INA in rhizospheres coinoculated with JL1157 or the aerobactin-producing derivative LA266 than in the rhizosphere of control plants (Fig. 4B). INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was greater in rhizospheres coinoculated with LA235, which produces neither enterobactin nor aerobactin, than in the rhizosphere of control plants. Coinoculation with LA235 also decreased the population size of N1R Pvd$^-$ (*pvd-inaZ*) in one experiment (Table 4), but none of the strains of *E. cloacae* had a consistent influence on the population size of N1R Pvd$^-$ (*pvd-inaZ*) in both replicated experiments.

INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) and N1R (*pvd-inaZ*) in the rhizosphere of cucumbers grown in field soil. Because the results described above indicated that siderophores produced by specific bacteria could alter iron availability to N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere, we evaluated the influence of the native microflora on INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) and N1R (*pvd-inaZ*). One day after inoculation of cucumber roots grown in sterilized soil, N1R Pvd$^-$ (*pvd-inaZ*) expressed 34-fold-greater INA than that expressed by N1R (*pvd-inaZ*) (Fig. 5A). In sterilized soil, INA expressed by N1R (*pvd-inaZ*) was not altered significantly by coinoculation with WCS358 or WCS358 Pvd$^-$, whereas INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was de-

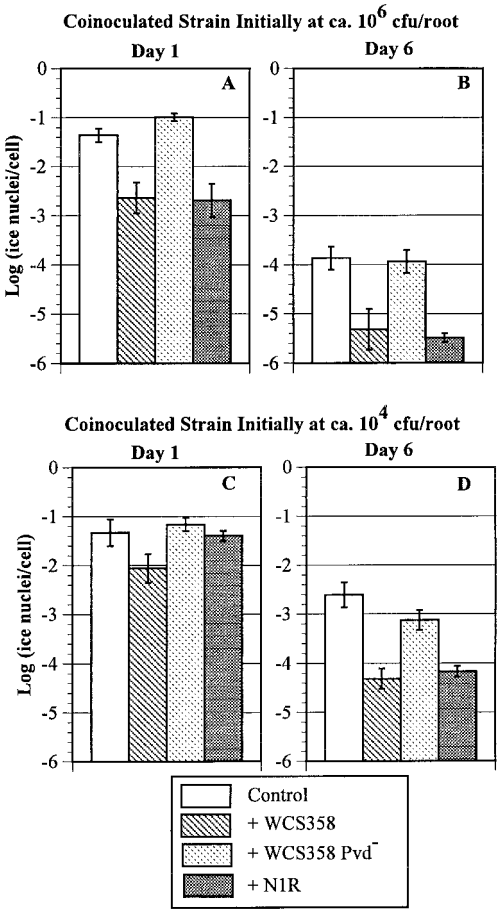

FIG. 3. Influence of coinoculated strains of *P. putida* on INA [$\log_{10}$ (ice nuclei/cell)] expressed by *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumber grown in sterilized soil. Surface-sterilized cucumber roots were inoculated with 10^4 CFU of N1R Pvd$^-$ (*pvd-inaZ*) per root system alone (Control) or in combination with 10^6 CFU (A and B) or 10^4 CFU (C and D) of a coinoculated strain (WCS358, WCS358 Pvd$^-$, or N1R) per root system and planted in sterilized soil. INA expressed by *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) was assessed at 1 day (A and C) or 6 days (B and D) after planting. INA values are means of five replicate root systems. Error bars represent standard errors of the means.

TABLE 3. Influence of *P. putida* strains differing in pyoverdine production on population size of *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumber

Coinoculated strain[a]	Rhizosphere population size of N1R Pvd$^-$ (*pvd-inaZ*)[b] ($\log_{10}$ [CFU/root system] ± SEM) for coinoculated strain initially at:			
	ca. 10^6 CFU/root system		ca. 10^4 CFU/root system	
	Day 1	Day 6	Day 1	Day 6
None	6.42 ± 0.20	7.33 ± 0.06	5.66 ± 0.21	7.04 ± 0.07
WCS358	5.09 ± 0.29	6.11 ± 0.18	5.52 ± 0.25	6.80 ± 0.13
WCS358 Pvd$^-$	5.11 ± 0.06	6.19 ± 0.11	5.57 ± 0.25	6.88 ± 0.07
N1R	4.78 ± 0.09	5.49 ± 0.09	5.86 ± 0.19	6.70 ± 0.13

[a] Root systems were dipped in aqueous suspensions of N1R Pvd$^-$ (*pvd-inaZ*) alone or in combination with a coinoculated strain (WCS358, WCS358 Pvd$^-$, or N1R) before planting in sterilized soil. Coinoculated strains applied at ca. 10^6 CFU/root system established rhizosphere populations of 6.5 to 7.0 $\log_{10}$ (CFU/root system) at day 1 and 7.5 to 7.6 $\log_{10}$ (CFU/root system) at day 6. Coinoculated strains applied at ca. 10^4 CFU/root system established populations of 5.9 to 6.4 $\log_{10}$ (CFU/root system) at day 1 and 6.9 to 7.0 $\log_{10}$ (CFU/root system) at day 6.

[b] Initial populations of N1R Pvd$^-$ (*pvd-inaZ*) were approximately 10^4 CFU/root system. Mean rhizosphere population sizes were estimated at 1 or 6 days after root systems were inoculated with bacterial strains.

creased in the presence of WCS358. INA expressed by both strains was lower at 6 days than at 1 day following inoculation, but the influence of WCS358 on INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) was statistically significant on both days.

One day after inoculation, INA expressed by N1R (*pvd-inaZ*) and N1R Pvd$^-$ (*pvd-inaZ*) was lower in the rhizosphere of plants grown in nonsterilized field soil than in sterilized field soil (Fig. 5). INA expressed by the strains did not decrease markedly over time in the rhizosphere of plants grown in nonsterilized field soil. Furthermore, WCS358 did not reduce INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) significantly in the rhizosphere of cucumbers grown in nonsterilized field soil.

Populations of all strains were approximately 100-fold larger in the rhizosphere of plants grown in sterilized soil than in nonsterilized field soil (Table 5). In one experiment, the population size of N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of plants grown in sterilized soil was decreased when coinoculated with WCS358 Pvd$^-$ (Table 5). In this experiment, coinoculation with strain WCS358 altered the population size of N1R (*pvd-inaZ*) but not of N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of plants grown in sterilized soil. These effects were not observed in the rhizosphere of plants grown in sterilized soil in a second experiment (data not shown) or in nonsterilized field soil in either of the two experiments.

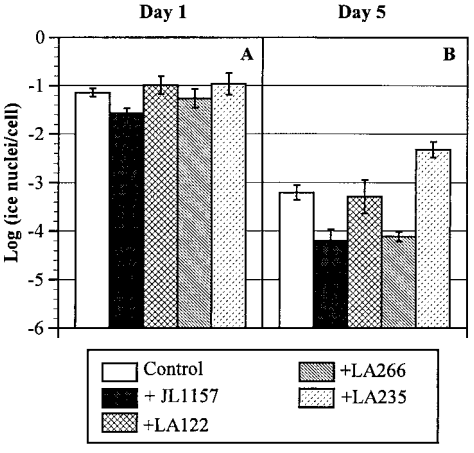

FIG. 4. Influence of coinoculated strains of *E. cloacae* on INA [$\log_{10}$ (ice nuclei/cell)] expressed by *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumber grown in sterilized soil. Surface-sterilized cucumber roots were inoculated with 10^4 CFU N1R Pvd$^-$ (*pvd-inaZ*) per root system alone (Control) or in combination with 10^4 CFU of a strain of *E. cloacae* (JL1157 [Ent$^+$ Iuc$^+$], LA122 [Ent$^+$ Iuc$^-$], LA266 [Ent$^-$ Iuc$^+$], or LA235 [Ent$^-$ Iuc$^-$]) per root system and planted in sterilized soil. INA expressed by *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) was assessed at 1 day (A) or 5 days (B) after planting. INA values are means of five replicate root systems. Error bars represent standard errors of the means.

TABLE 4. Influence of *E. cloacae* strains differing in enterobactin and aerobactin production on population size of *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumber

E. cloacae strain[a]	Rhizosphere population size of N1R Pvd$^-$ (*pvd-inaZ*)[b] ($\log_{10}$ [CFU/root system] ± SEM)	
	Day 1	Day 5
None	5.40 ± 0.25	7.04 ± 0.12
JL1157	5.88 ± 0.24	6.79 ± 0.14
LA122	4.75 ± 0.24	6.43 ± 0.13
LA266	5.21 ± 0.17	6.72 ± 0.15
LA235	4.26 ± 0.15	5.97 ± 0.35

[a] Root systems were dipped in aqueous suspensions of N1R Pvd$^-$ (*pvd-inaZ*) alone or in combination with a strain of *E. cloacae* (JL1157, LA122, LA266, or LA235) before planting in sterilized soil. Initial populations of *E. cloacae* on inoculated roots were approximately 10^4 CFU/root system. Mean population sizes of *E. cloacae* on five replicate root systems were 6.3 to 6.6 $\log_{10}$ (CFU/root system) at day 1 and 7.6 to 7.9 $\log_{10}$ (CFU/root system) at day 5.

[b] Initial population size of N1R Pvd$^-$ (*pvd-inaZ*) on inoculated roots was approximately 10^4 CFU/root system. Mean rhizosphere population sizes were estimated from five replicate root systems at 1 or 5 days after inoculation with bacterial strains.

DISCUSSION

The results of this study demonstrate that the iron status of *P. putida* in the rhizosphere is altered by the presence of siderophores produced by other bacterial strains occupying that habitat. These results complement those of a previous study demonstrating that the rhizosphere population size of *P. putida* is influenced by its capacity to utilize pyoverdines produced by coinoculated strains (2, 30). The ice nucleation reporter gene system described here provides a complementary approach to the assessment of population size for studies evaluating the importance of siderophore utilization patterns in microbial interactions in the rhizosphere. In this study, certain bacterial strains influenced INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) while having minimal effects on the population size of N1R Pvd$^-$ (*pvd-inaZ*). We speculate that INA may be sensitive to alterations in iron availability that are too small to have immediate effects on population size. Because iron availability affects many metabolic processes of bacteria, alterations in iron availability that are not associated with immediate and detectable changes in population size are nonetheless likely to have ultimate effects on the fitness of a bacterial strain. The *pvd-inaZ* construct provides a tool for assessing specific effects of bacteria on the physiology of their microbial coinhabitants in natural habitats.

Pseudomonas spp. are known to have the capacity to utilize siderophores produced by diverse species of microorganisms (12, 16, 25, 29). In this study, we provide evidence that *E. cloacae* produces enterobactin and aerobactin in the rhizosphere in concentrations that are recognized as iron sources by a coinoculated strain of *P. putida*. Strains of *E. cloacae* that produced aerobactin, even in the absence of enterobactin production, were particularly effective in enhancing iron availability to *P. putida*. Although the affinity of aerobactin for iron is lower than that of enterobactin (10, 11), aerobactin is more stable

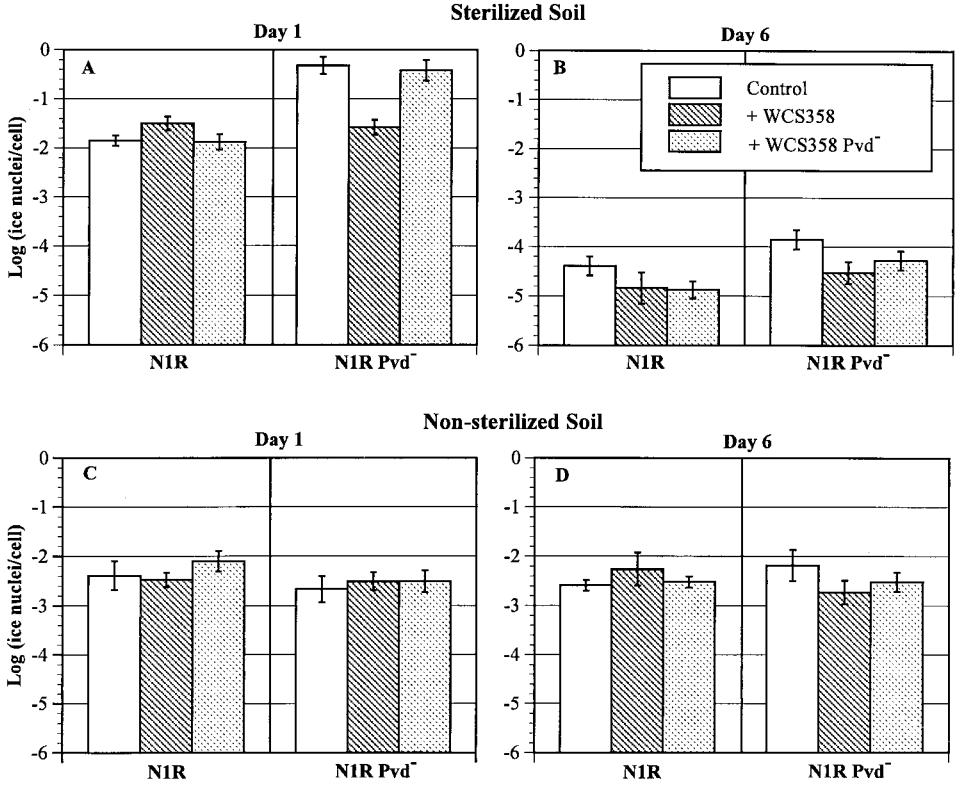

FIG. 5. Influence of soil sterilization on INA [$\log_{10}$ (ice nuclei/cell)] expressed by *P. putida* N1R (*pvd-inaZ*) or N1R Pvd$^-$ (*pvd-inaZ*) in the rhizosphere of cucumbers. Surface-sterilized cucumber roots were inoculated with 10^4 CFU of N1R (*pvd-inaZ*) or N1R Pvd$^-$ (*pvd-inaZ*) per root system alone (Control) or in combination with 10^4 CFU of a second strain (WCS358 or WCS358 Pvd$^-$) per root system and planted in sterilized soil (A and B) or nonsterilized soil (C and D). INA expressed by *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) and N1R (*pvd-inaZ*) was assessed at 1 day (A and C) or 6 days (B and D) after planting. INA values are means of five replicate root systems. Error bars represent standard errors of the means.

and water soluble than enterobactin and is active over a range of pH values (28). Aerobactin's stability and other chemical properties contribute to its importance as a virulence factor in *Escherichia coli* (28) and also are likely to be important factors in determining its role in ecological interactions of bacteria in the rhizosphere. Other factors, such as the relative levels of aerobactin and enterobactin produced in situ by *E. cloacae*, could also account for the differential contribution of these siderophores to the iron status of *P. putida*. Irrespective of the relative importance of enterobactin and aerobactin in the rhizosphere, the contribution of both siderophores to the iron nutrition of *P. putida* suggests that diverse groups of microorganisms can alter the chemical composition of microbial habitats on root surfaces.

Previously, we demonstrated that the iron-regulated promoter evaluated in this study is not expressed uniformly over time by *P. putida* in the rhizosphere or the spermosphere. Instead, transcriptional activity reached a maximum within approximately 24 h after *P. putida* was inoculated onto root or seed surfaces and decreased thereafter. One possible explanation for this decrease in transcription was that iron complexed by microbial siderophores became more available to *P. putida*

TABLE 5. Influence of *P. putida* strains differing in pyoverdine production on population size of *P. putida* N1R Pvd$^-$ (*pvd-inaZ*) or *P. putida* N1R (*pvd-inaZ*) in the rhizosphere of cucumber grown in sterilized or nonsterilized soil

Coinoculated strain[a]	Rhizosphere population size[b] ($\log_{10}$ [CFU/root system] ± SEM)							
	Sterilized soil				Nonsterilized soil			
	N1R Pvd$^-$ (*pvd-inaZ*)		N1R (*pvd-inaZ*)		N1R Pvd$^-$ (*pvd-inaZ*)		N1R (*pvd-inaZ*)	
	Day 1	Day 6	Day 1	Day 6	Day 1	Day 6	Day 1	Day 6
None	6.54 ± 0.17	7.50 ± 0.05	6.61 ± 0.15	7.43 ± 0.08	4.09 ± 0.24	4.23 ± 0.16	4.26 ± 0.13	4.39 ± 0.19
WCS358	6.40 ± 0.10	7.37 ± 0.08	5.85 ± 0.27	7.06 ± 0.08	3.75 ± 0.25	4.35 ± 0.28	3.81 ± 0.29	3.98 ± 0.18
WCS358 Pvd$^-$	5.99 ± 0.33	7.17 ± 0.04	6.30 ± 0.15	7.22 ± 0.09	4.05 ± 0.19	4.40 ± 0.17	3.40 ± 0.22	4.49 ± 0.09

[a] Before planting in sterilized or nonsterilized soil, root systems were dipped in aqueous suspensions of N1R Pvd$^-$ (*pvd-inaZ*) or N1R (*pvd-inaZ*) alone or in combination with a coinoculated strain (WCS358 or WCS358 Pvd$^-$). Immediately following inoculation, the population size of coinoculated strains on roots was approximately 10^4 CFU/root system. In sterilized soil, mean population sizes of WCS358 and WCS358 Pvd$^-$ on five replicate root systems were 6.0 to 6.5 $\log_{10}$ (CFU/root system) at day 1 and 6.8 to 7.5 $\log_{10}$ (CFU/root system) at day 6. In nonsterilized soil, mean population sizes of these strains were 3.0 to 4.3 $\log_{10}$ (CFU/root system) at day 1 and 3.6 to 4.7 $\log_{10}$ (CFU/root system) at day 6.

[b] Immediately following inoculation, the population sizes of N1R (*pvd-inaZ*) and N1R Pvd$^-$ (*pvd-inaZ*) were approximately 10^4 CFU/root system each. Mean rhizosphere population sizes were estimated at 1 or 6 days after root systems were inoculated with bacterial strains.

in the rhizosphere or spermosphere over the course of an experiment. The results of the present study argue that siderophores produced by other rhizosphere bacteria enhance iron availability to *P. putida*. Nevertheless, siderophores produced by indigenous soil microorganisms could not account for the temporal patterns of *pvd* gene expression by *P. putida* in the rhizosphere of plants grown in nonsterilized field soil. INA expressed by N1R (*pvd-inaZ*) and N1R Pvd$^-$ (*pvd-inaZ*) was stable in nonsterilized field soil relative to sterilized soil, a result opposite that which would be expected if the decrease in *pvd* gene expression were caused by siderophores produced by the native soil microflora. The population sizes of N1R and N1R Pvd$^-$ increased to much higher levels in the rhizosphere of plants grown in sterilized than in nonsterilized field soil, and the relative physiological statuses of the bacteria in the two environments provide one possible explanation of the temporal patterns of *pvd* gene expression. In the field soil, competition for resources other than iron could have limited the population size of *P. putida* such that the *pvd* promoter was transcribed at a stable, moderate level throughout the experiment. In contrast, sterilized soil may be transiently replete in carbon, because the organic matter content of soil is enhanced (8) and few microbial competitors are present immediately after autoclaving. With adequate carbon, iron may have been the limiting resource to *P. putida* immediately after it was inoculated onto the root surfaces of plants grown in sterilized soil, but carbon or other resources could then become limiting as they were utilized by the inoculated bacterium.

The production of pyoverdines by *Pseudomonas* spp. in the rhizosphere is one mechanism by which these bacteria suppress soilborne bacteria and fungi that cause plant disease (17). The results of this study confirm that *P. putida* expresses genes for pyoverdine production and uptake in the rhizosphere, but the level of gene expression is influenced by other bacteria that coexist with *P. putida* in this habitat. Furthermore, siderophore-mediated interactions between microorganisms can be altered by the indigenous microflora, as evidenced by the differential effects of WCS358 on INA expressed by N1R Pvd$^-$ (*pvd-inaZ*) in sterilized and nonsterilized field soils. These data provide an example of how the expression of genes involved in biological control can be altered by biotic components of the environment and point to microbial community context as a factor influencing the in situ activities of biological control agents.

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