APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 1934–1941 Vol. 60, No. 6
0099-2240/94/$04.00+0

A Biological Sensor for Iron Available to Bacteria in Their Habitats on Plant Surfaces

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Received 16 November 1993/Accepted 15 March 1994

A sensor responsive to iron was constructed by fusing a promoterless ice nucleation activity gene (*inaZ*) to an iron-regulated promoter of a genomic region involved in pyoverdine (fluorescent siderophore) (*pvd*) production in *Pseudomonas syringae*. Cells of *Pseudomonas fluorescens* and *P. syringae* that contained the *pvd-inaZ* fusion expressed iron-responsive ice nucleation activity in the bean rhizosphere and phyllosphere, respectively, and in culture. Addition of Fe(III) to leaves or soil reduced the apparent transcription of the *pvd-inaZ* reporter gene, as shown by a reduction in the number of ice nuclei produced, indicating that Fe(III) was primarily responsible for mediating transcription of the *pvd-inaZ* gene even in natural environments. A *Pseudomonas* sp. strain having an intact *iceC* gene, which conferred Fe-insensitive expression of ice nucleation activity, was included in all studies to account for small strain- or environment-dependent differences in the ability of bacterial cells to produce ice nuclei. Thus, a comparison of the ice nucleation activity conferred by *pvd-inaZ* with the activity conferred by *iceC* revealed the bioavailability of iron in culture or natural habitats. The relative ice nucleation activities expressed by strains containing *iceC* or *pvd-inaZ* indicated that, while not abundant, Fe(III) is not present at extremely low concentrations at all microsites colonized by bacteria on plant surfaces. Biological sensors that are constructed by fusing *inaZ* to chemically responsive promoters provide a novel way to characterize chemical constituents of microbial habitats.

A variety of environmental factors, including the availability of nutrients, influence the growth, activity, and gene expression of microorganisms in culture and presumably in natural habitats. One of these factors, iron availability, profoundly affects gene expression in prokaryotes and is particularly important in the activity and metabolism of bacteria (54). Although iron is abundant, the extreme insolubility of ferric hydroxide limits the equilibrium concentration of free iron available in aerobic aqueous environments at pH 7 to approximately 10^{-18} M (44). Iron exists in the form of insoluble oxides in soils at neutral or alkaline pH values (28, 29). Although iron is essential to virtually all forms of life, excessive concentrations are toxic (53). Thus, most organisms have systems for specific chelation and regulated transport of Fe(III) into the cell. Most microorganisms use siderophores and corresponding membrane receptors for iron acquisition (35). Siderophores, which are low-molecular-weight compounds produced under iron-limiting conditions, chelate the ferric ion with a high specific activity and serve as vehicles for the transport of Fe(III) into microbial cells (36). Transport of iron into the cells is mediated by a membrane receptor that specifically recognizes a ferric-siderophore complex (37).

Fluorescent pseudomonads are common inhabitants of the plant phyllosphere and rhizosphere and are important as phytopathogens, as incitants of frost injury, and as agents for biological control of plant disease. The fluorescent pseudomonads are characterized by the production of yellow-green pigments, called pyoverdines or pseudobactins, that fluoresce under UV light and function as siderophores (1). Pyoverdines inhibit fungal growth by sequestering iron as ferric-pyoverdine complexes (30, 42, 48). Although pyoverdine-mediated competition for Fe(III) has been proposed as a mechanism for biological control of certain plant diseases caused by soilborne fungi (30, 42, 48), there is no evidence indicating how commonly the growth of fungi or bacteria is limited by the lack of available iron in soil or on plant surfaces. Bacteria do not form uniform populations spatially on leaf and root surfaces, but instead are often found in localized assemblages where the resources provided by the plant are more favorable for growth or survival (9, 21). Iron availability at the sites colonized by bacteria, in contrast to the iron contents of bulk environmental samples, cannot be quantified by conventional methods. A goal of this study was to develop a method by which the levels of iron available in microbial habitats could be assessed.

In this study, a reporter gene conferring a phenotype that is readily detected and quantified in environmental samples was placed under the regulatory control of an iron-regulated promoter; the resulting gene fusion provided a tool to estimate the availability of iron in habitats occupied by bacteria on plant surfaces. Of the available reporter genes, we selected *inaZ* (23), which confers ice nucleation activity, for use in this study. The *inaZ* reporter is composed of an ice nucleation gene from *Pseudomonas syringae* (12) that is devoid of its native promoter and encodes an outer membrane protein (InaZ) that catalyzes ice formation at −2 to −10°C, thereby limiting the supercooling of water (11, 27, 55). Ice nucleation activity is related quantitatively to the InaZ protein content of a cell (23, 50) and can be quantified conveniently by a droplet-freezing assay of unprocessed aqueous suspensions of environmental samples (24). Because neither plant tissue nor soil contains significant numbers of ice nuclei active at −5°C or above (2, 26), even low

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levels of *inaZ* gene expression by an introduced ice nucleation-active bacterium can be detected in environmental samples. Furthermore, the ice nucleation phenotype is expressed in many gram-negative bacteria (23) and can be quantified from cells grown under a wide range of environmental conditions (24, 39). Because ice nucleation activity does not depend on rapid cell growth (2, 26), it is a useful reporter of gene expression in quiescent cells, such as those that may be found in natural habitats.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. syringae* 31 is an ice nucleation-active (Ina^+ or Ice^+), epiphytic bacterium that was originally isolated from a corn leaf surface (2). Strain 31R1, a spontaneous mutant of strain 31, was selected for its resistance to rifampin. Strain 31R1-P6, an Ina^- mutant of strain 31R1 derived by deletion of a region within the *inaZ* gene, was obtained from J. Lindemann (DNA Plant Technologies, Oakland, Calif.). *Pseudomonas fluorescens* Pf-5 (14), which was isolated from the cotton rhizosphere, was obtained from C. R. Howell (Agricultural Research Service, U.S. Department of Agriculture, College Station, Tex.). Plasmid pSFL12 (32) contains genes involved in pyoverdine production in *P. syringae* 31 cloned in broad-host-range cosmid pLAFR1 (10), which confers tetracycline resistance. Plasmid pVSP61 (obtained from William Tucker, DNA Plant Technologies) confers kanamycin resistance and contains replicons of pVS1 (15) and pACY184 (6). In preliminary experiments, pVSP61 was maintained stably in *P. syringae* 31R1-P6 and *P. fluorescens* Pf-5 inhabiting phyllospheres and rhizospheres, respectively, for at least 7 days after inoculation. The *inaZ* gene of *P. syringae* S203 (12) was provided by G. J. Warren (DNA Plant Technologies). The *iceC* gene of *P. syringae* Cit7R1 (41) is virtually identical to *inaZ* in its amino acid sequence (25). *Pseudomonas* spp. were grown routinely on nutrient agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) glycerol; *Escherichia coli* was grown on Luria-Bertani medium (46). When it was necessary, the media were supplemented with tetracycline (25 μg/ml), kanamycin (50 μg/ml), rifampin (100 μg/ml), spectinomycin (50 μg/ml), or ampicillin (100 μg/ml).

DNA biochemistry. Plasmid DNA was isolated by the alkaline lysis procedure, and ligations, restriction digestions, agarose gel electrophoresis, and Southern hybridizations were performed by standard methods (46). Mobilizable plasmids were introduced into *Pseudomonas* spp. by triparental matings, using pRK2013 as a helper plasmid (8).

Identification of an iron-sensitive promoter. Plasmid pSFL12 was mutagenized with Tn*3*-HoHo1 by using previously described methods (51). Following matings with *E. coli* donors harboring pSFL12::Tn*3*-HoHo1, colonies of *P. syringae* 31R1 that harbored fusion plasmids were selected on medium 925 (20) containing *myo*-inositol (10 g/liter) as a sole carbon source, tetracycline, ampicillin, and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). After 3 days of incubation at 27°C, the colonies were screened for a blue color, which indicated that there was a Tn*3*-HoHo1 insertion downstream of a promoter.

Assessment of transcriptional activity and pyoverdine production in culture. *Pseudomonas* spp. were grown at 25°C in a minimal salts medium (SM) (31) supplemented with different concentrations of $FeCl_3$. The effects of other micronutrients were evaluated in SM supplemented with $ZnSO_4$ (10^{-5} to 10^{-7} M), $CuCl_2$ (2×10^{-6} to 10^{-7} M), or $MnCl_2$ (10^{-5} to 10^{-7} M); the maximum concentrations of the elements used were the greatest concentrations that did not reduce cell growth significantly. After 48 h of growth, β-galactosidase activity was assessed by the method of Miller (34). The ice nucleation activity of cells was quantified by the droplet-freezing assay (24). The concentrations of pyoverdine in culture supernatants were estimated by determining the A_{405} of the ferric-pyoverdine complex. $FeCl_3$ was added to a culture supernatant to a final concentration of 10^{-3} M, the preparation was mixed for 30 min, diluted 1:1 in 1 M potassium phosphate buffer (pH 7.5), and centrifuged to remove the precipitate, and the A_{405} was measured. Values for β-galactosidase activity, ice nucleation activity, and pyoverdine concentration were normalized for the number of bacterial cells, as determined by the optical density at 560 nm of the cultures. The values reported below are the means obtained from three replicate cultures.

Assessment of transcriptional activity of *Pseudomonas* spp. on plant surfaces. The ice nucleation activities of *Pseudomonas* spp. inhabiting rhizospheres and phyllospheres of beans (*Phaseolus vulgaris* L. cv. Bush Blue Lake) were determined. Except where noted below, the bacterial inoculum was grown in SM supplemented with 10^{-3} M $FeCl_3$. Cells were harvested from cultures by centrifugation and resuspended in sterile deionized water to an optical density at 560 nm of 0.1. The suspensions were diluted to final densities of approximately 10^5 and 10^6 CFU/ml for inoculation of leaves and roots, respectively. For rhizosphere studies, bean seeds were surface sterilized in a 1% hypochlorite solution for 10 min, rinsed thoroughly in deionized water, and placed on moist paper towels for 3 days. The roots of the resulting bean seedlings were dipped in aqueous suspensions of *P. fluorescens* Pf-5 and planted in pots containing Warden sandy-silt loam soil (−0.3 bar, pH 7.2, 14 mg of Fe per kg). In some experiments, a solution of the ferric-sodium salt of EDTA (FeEDTA) was mixed into the soil at a concentration of 300 μg/kg prior to planting. Soil amendment with FeEDTA, which has a stability constant of 25 (28), is thought to increase the biological availability of iron in the rhizosphere (19, 47). For phyllosphere studies, bean seeds were planted in acid-washed sand and watered with Hoagland's solution. Leaf surfaces were inoculated by dipping an entire plant into a bacterial suspension and covering it loosely with a plastic bag. After inoculation, the plants were grown for 5 days in environmental growth chambers maintained at 25°C with a 12-h photoperiod. Individual leaflets from trifoliate leaves or individual root systems were placed in a washing buffer (40) and sonicated for 5 min to remove bacterial cells from tissue surfaces or rhizosphere soil. The washing buffer containing suspended bacterial cells was diluted, and bacterial population size was determined by spreading aliquots of dilutions on King's medium B (17) containing rifampin, using previously described methods (33, 40). The ice nucleation activities of bacteria inhabiting the rhizosphere or phyllosphere were determined by the droplet-freezing assay (24). In an experiment to evaluate the effect of the rhizosphere environment and harvesting procedures on ice nucleation activity, the roots of bean seedlings were inoculated with a suspension of ice nucleation-active *P. fluorescens* cells, and the seedlings were planted in soil and immediately harvested. The ice nucleation activity of the inoculated cells and population size were determined as described above. For each treatment we used five plants, and each experiment was repeated.

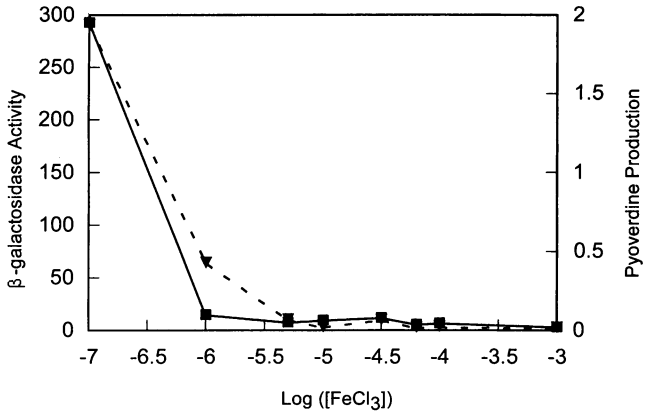

FIG. 1. β-Galactosidase activity and pyoverdine production by *P. syringae* 31R1(pJEL1395) grown in SM containing different concentrations of iron. Symbols: ■, β-galactosidase activity, in Miller units (34); ▼, pyoverdine production, as determined by A_{405} of the ferric-pyoverdine complex. The β-galactosidase activity and pyoverdine production values were normalized for the number of bacterial cells, as determined by measuring the turbidity of cultures at 560 nm. Values are the means obtained from three replicate cultures.

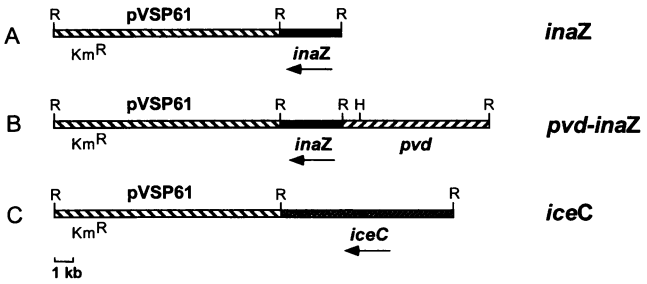

FIG. 2. Ice nucleation reporter gene construction. (A) *inaZ* (12) devoid of its native promoter and cloned as a 3.4-kb *Eco*RI fragment in the opposite orientation to the *plac* promoter of the pUC8 polylinker. (B) *pvd-inaZ*, consisting of the promoterless *inaZ* gene cloned downstream from an 8.0-kb *Eco*RI fragment involved in pyoverdine production (*pvd*) in *P. syringae*. (C) *iceC* (41) driven by its native, iron-constitutive promoter and contained within a 9.5-kb *Eco*RI fragment. The three constructions were cloned in plasmid vector pVSP61, which is maintained stably in *Pseudomonas* spp. Restriction endonuclease cut site abbreviations: R, *Eco*RI; H, *Hin*dIII.

RESULTS

Identification of an iron-regulated promoter. A genomic region whose transcription was regulated strongly by iron was identified by Tn*3*-HoHo1 mutagenesis of plasmid pSFL12 (32), which contains cloned genes involved in pyoverdine biosynthesis (*pvd*) in *P. syringae* 31. Colonies of *P. syringae* 31R1 containing pSFL12 with random insertions of Tn*3*-HoHo1 were evaluated for β-galactosidase activity. We identified 40 colonies that were blue on medium 925 containing X-Gal but were white on medium 925 containing X-Gal and 10^{-4} M $FeCl_3$. Plasmid pJEL1395, which was isolated from one of the 40 colonies, did not confer β-galactosidase activity on *E. coli* DH5α or HB101. The level of pyoverdine production and the level of β-galactosidase activity of *P. syringae* harboring pJEL1395 decreased by 300-fold as the concentration of added $FeCl_3$ increased from 10^{-7} to 10^{-6} M (Fig. 1). In SM amended with 10^{-6} M $FeCl_3$, the level of β-galactosidase activity was at the lower limit of detection. Therefore, further decreases in the level of β-galactosidase activity with increases in the $FeCl_3$ concentration to values greater than 10^{-6} M would not have been detectable. The Fe(III)-mediated change in transcription of the region involved in *pvd* biosynthesis was apparent in cells at all stages of growth in culture (data not shown). Other trace elements (Mn^{2+}, Zn^{2+}, Cu^{2+}) had only small effects on the β-galactosidase activity conferred by pJEL1395 or on pyoverdine production in *P. syringae*. Thus, of the elements evaluated, only iron substantially affected the transcriptional activity of the *pvd* promoter; this effect paralleled the specific effect of Fe(III) on pyoverdine production.

The single Tn*3*-HoHo1 insertion of pJEL1395 was located on an 8-kb *Eco*RI fragment of pSFL12. Plasmid pSFL12 restores fluorescence to nine mutants of *P. syringae* 31R1 that are deficient in pyoverdine production (32). A subclone of pSFL12, which contained the 8-kb *Eco*RI fragment cloned into plasmid pVSP61, restored fluorescence to three of the nine pyoverdine-deficient mutants of *P. syringae* 31R1. Thus, the 8-kb region was involved in pyoverdine production in *P. syringae* 31. The Tn*3*-HoHo1 insertion of pJEL1395 was located 1.0 kb from the left side of the 8-kb *Eco*RI fragment and was oriented such that the *lacZ* gene was transcribed from right to left, as the fragment is shown in Fig. 2. We therefore suspected that the iron-regulated promoter from which *lacZ*

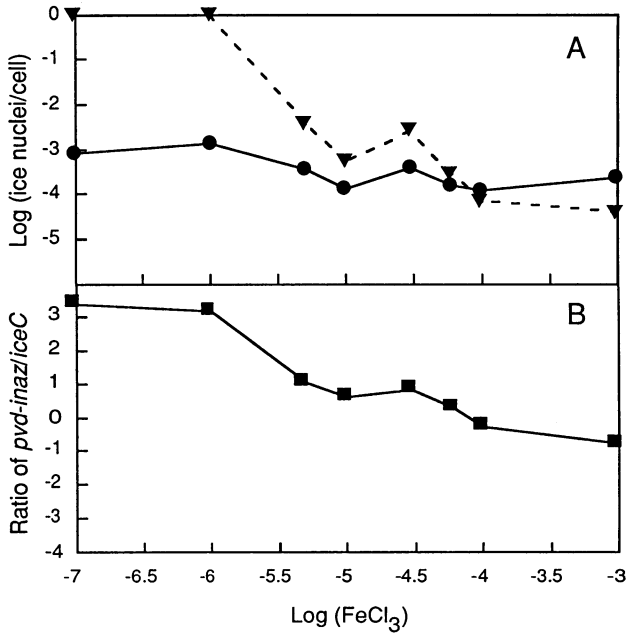

FIG. 3. (A) Iron-regulated ice nucleation activity expressed by *P. syringae* 31R1-P6 containing *iceC* (●) or *pvd-inaZ* (▼). The ice nucleation activities of cells that were cultured at 25°C with shaking in SM (31) amended with various concentrations of $FeCl_3$ were quantified by performing the droplet-freezing assay at temperature of −5°C (24). Ice nucleation activity values were normalized for the number of bacterial cells, as determined by measuring the turbidity of cultures at 560 nm. (B) Difference in ice nucleation activities expressed by cells of *P. syringae* 31R1-P6 containing *pvd-inaZ* and cells containing *iceC* grown as described above.

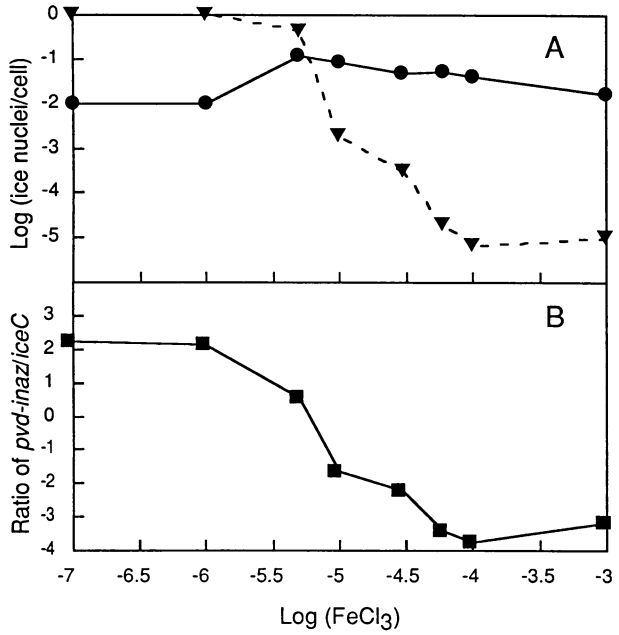

FIG. 4. Iron-regulated ice nucleation activity expressed by *P. fluorescens* Pf-5 containing *iceC* (●) or *pvd-inaZ* (▼). The ice nucleation activities of cells that were cultured at 25°C with shaking in SM (31) amended with various concentrations of $FeCl_3$ were quantified by performing the droplet-freezing assay at temperature of −5°C (24). Ice nucleation activity values were normalized for the number of bacterial cells, as determined by measuring the turbidity of cultures at 560 nm. (B) Difference in ice nucleation activities expressed by cells of *P. fluorescens* Pf-5 containing *pvd-inaZ* and cells containing *iceC* grown as described above.

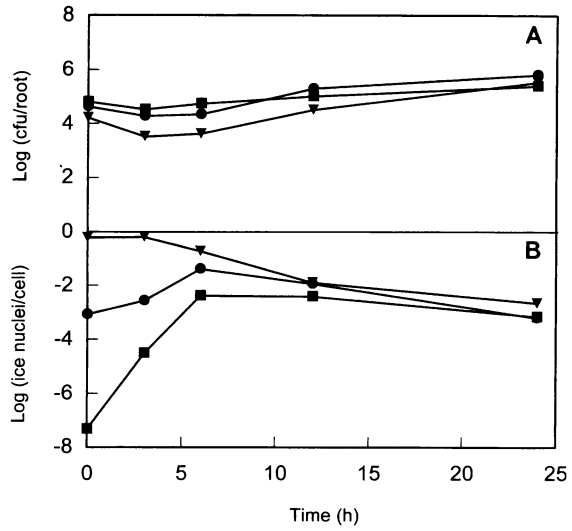

FIG. 5. Ice nucleation activities and rhizosphere populations of *P. fluorescens* Pf-5 containing *pvd-inaZ*. Root systems of beans were dipped in suspensions of bacterial cells grown in SM amended with 10^{-7} M $FeCl_3$ (▼), 10^{-5} M $FeCl_3$ (●), or 10^{-4} M $FeCl_3$ (■), and then the beans were planted in Warden sandy-silt loam soil. The rhizosphere population size (A) and the in situ ice nucleation activity (B) of *P. fluorescens* Pf-5 containing *pvd-inaZ* were estimated periodically for 24 h following inoculation of root systems.

was transcribed was present on the 8-kb *Eco*RI fragment of pSFL12.

Construction of an iron sensor. A *pvd-inaZ* fusion was constructed by fusing the 8-kb *Eco*RI fragment of pSFL12 to a promoterless *inaZ* gene (Fig. 2). Constructions were introduced by conjugation into *P. fluorescens* Pf-5 and *P. syringae* 31R1-P6, neither of which produced detectable ice nuclei in the absence of an introduced ice nucleation gene. The construction containing the promoterless *inaZ* gene (Fig. 2) did not confer ice nucleation activity on either strain. The ice nucleation activities of *P. syringae* and *P. fluorescens* strains conferred by *pvd-inaZ* were regulated dramatically by iron (Fig. 3 and 4). Transcription of the iron-regulated *pvd* gene(s) was detected as high levels of ice nucleation activity in cells grown in media containing low levels of Fe(III) (i.e., $\leq 10^{-6}$ M added $FeCl_3$) (Fig. 3 and 4). At such low $FeCl_3$ concentrations, every cell produced at least one ice nucleus active at −5°C, the maximum level of ice nucleation activity that can be quantified by the droplet-freezing assay. Cells containing *pvd-inaZ* expressed 10,000- to 100,000-fold-greater ice nucleation activity at low concentrations of $FeCl_3$ ($\leq 10^{-6}$ M) than at high concentrations of $FeCl_3$ ($\geq 10^{-4}$ M). In contrast, the ice nucleation activity of cells containing *iceC*, the ice nucleation gene of *P. syringae* transcribed from its native promoter, was not affected substantially by the $FeCl_3$ concentration in the culture medium, indicating that InaZ protein production and function were not affected greatly by iron availability. Thus, a comparison of the ice nucleation activities of cells containing *pvd-inaZ* and cells containing *iceC* provided a way to assess iron availability to *Pseudomonas* spp. It is important to note that at low $FeCl_3$ concentrations ($\leq 10^{-6}$ M added $FeCl_3$), the ice nucleation activity expressed by cells containing *pvd-inaZ* was greater than the activity expressed by cells containing *iceC*, whereas at high $FeCl_3$ concentrations ($\geq 10^{-4}$ M), the ice nucleation activity expressed by cells containing *pvd-inaZ* was less than the activity expressed by cells containing *iceC* (Fig. 3B and 4B). The low levels of ice nucleation activity expressed by strains containing *pvd-inaZ* in iron-replete media ($\geq 10^{-4}$ M added $FeCl_3$) were attributed to low levels of constitutive transcriptional activity of the *pvd* promoter.

Transcriptional activity in the rhizosphere. The ice nucleation activity of *P. fluorescens* was not influenced by the rhizosphere environment or by the methods used to obtain bacterial samples from rhizosphere soil; the cultured cells used as the inoculum expressed levels of ice nucleation activity equivalent to the levels expressed by similar cells retrieved

TABLE 1. Ice nucleation activity expressed by *P. fluorescens* Pf-5 cells containing *pvd-inaZ* or *iceC* constructions in the bean rhizosphere

Construction	Soil treatment[a]	Population size (log [CFU/g])[b]	Ice nucleation activity (log [ice nuclei/cell])[b]
iceC	None	5.4 a	−1.1 a
iceC	FeEDTA	5.4 a	−0.9 a
pvd-inaZ	None	5.5 a	−2.9 b
pvd-inaZ	FeEDTA	5.5 a	<D

[a] For the FeEDTA treatment a solution of FeEDTA was mixed into the soil to a concentration of 300 μg/kg soil prior to planting.

[b] Population size and ice nucleation activity were assessed 5 days after plants were inoculated with bacteria containing the constructions. Each value is the mean of five replicates. Values followed by the same letter are not statistically different as determined by the Waller-Duncan test at $P = 0.05$. <D, below the limit of detection, which was −5.5 log (ice nuclei per cell) in this experiment.

TABLE 2. Ice nucleation activity expressed by *P. syringae* 31R1-P6 cells containing *pvd-inaZ* or *iceC* constructions in the bean phyllosphere

Construction	Plant treatment[a]	Population size (log [CFU/g])[b]	Ice nucleation activity (log [ice nuclei/cell])[b]
iceC	None	6.6 b	−1.7 b
pvd-inaZ	None	6.5 b	−2.1 b
pvd-inaZ	$FeCl_3$	5.8 a	−2.8 a

[a] For the $FeCl_3$ treatment, plants were sprayed 2 days before inoculation with 10^{-4} M $FeCl_3$; other plants were not sprayed.

[b] Population size and ice nucleation activity were assessed 5 days after plants were inoculated with bacteria containing the constructions. Each value is the mean of five replicates. Values followed by the same letter are not statistically different as determined by the Waller-Duncan test at $P = 0.05$.

from the rhizosphere shortly after inoculation. Furthermore, cells of *P. fluorescens* Pf-5 containing *pvd-inaZ* that were grown in media amended with various concentrations of $FeCl_3$ and then applied to plant roots initially expressed levels of ice nucleation activity equivalent to the levels expressed in culture (Fig. 5). By 12 h after inoculation of plant roots, however, cells retrieved from the rhizosphere expressed equivalent levels of ice nucleation activity regardless of the $FeCl_3$ concentration in the initial inoculum. Therefore, the levels of ice nucleation activity expressed by bacteria that had inhabited the rhizosphere for at least 12 h reflected the transcriptional activity of the *pvd* promoter in the rhizosphere and not the transcriptional activity expressed in the initial inoculum. The rate at which the ice nucleation activity of cells grown in the presence of 10^{-7} M $FeCl_3$ decreased following inoculation of bean roots was similar to the rate observed when stationary-phase cultures of *P. fluorescens* containing *pvd-inaZ* were amended with 10^{-4} M $FeCl_3$. This rate, approximately 2 log (nuclei per cell) in 12 h, presumably reflected the rate at which the ice nucleation protein turned over in *P. fluorescens* cells.

P. fluorescens cells that contained *iceC* served as positive controls in rhizosphere experiments. Any environmental or biological factors that might have influenced the activity or production of the ice nucleation protein (39) could be identified by their effects on the ice nucleation activity expressed by cells containing *iceC*. In the rhizosphere of bean, *P. fluorescens* cells containing promoterless *inaZ* expressed only background levels of ice nucleation activity (i.e., 10^{-8} ice nucleus per cell). Cells that contained *pvd-inaZ* produced ice nuclei, indicating that the concentrations of available iron were sufficiently low to allow transcriptional activity of the *pvd* promoter by *P. fluorescens* occupying the rhizosphere. Nevertheless, cells that contained *pvd-inaZ* expressed lower levels of ice nucleation activity than cells containing *iceC* did (Table 1). Because cells containing *pvd-inaZ* expressed higher levels of ice nucleation activity than cells containing *iceC* expressed in a low-iron culture medium (SM containing $<10^{-5}$ M added $FeCl_3$) (Fig. 3), we concluded that available Fe(III) was present in microsites colonized by *P. fluorescens* in the rhizosphere. Amendment of soil with FeEDTA had no effect on the ice nucleation activity of cells containing *iceC*, indicating that this treatment had no direct effect on the production or activity of the ice nucleation protein. In contrast, cells containing *pvd-inaZ* expressed much less ice nucleation activity on roots of plants in soil amended with FeEDTA than on roots of plants in nonamended soil, suggesting that the levels of soluble iron in the soil influenced the transcriptional activity of the *pvd* promoter by *P. fluorescens* in the rhizosphere.

Transcriptional activity on leaf surfaces. In the bean phyllosphere, the ice nucleation activity expressed by *P. syringae* cells containing *pvd-inaZ* was similar to the activity expressed by cells containing *iceC* (Table 2). The level of ice nucleation activity expressed by cells containing *pvd-inaZ* was significantly lower on plants to which $FeCl_3$ was applied as a foliar spray prior to inoculation with bacteria than on unsprayed plants. Thus, iron affected expression of ice nucleation activity by *P. syringae* containing *pvd-inaZ* on leaf surfaces as well as in culture.

DISCUSSION

An iron-regulated promoter was transcribed by *P. fluorescens* in the bean rhizosphere and by *P. syringae* inhabiting bean leaf surfaces, as detected by an ice nucleation reporter gene system. Amending of the soil with iron or adding iron to leaf surfaces resulted in a reduction in the ice nucleation activity expressed by cells containing *pvd-inaZ*, indicating that Fe(III), rather than the other constituents of the rhizosphere and phyllosphere environments evaluated, was the primary factor affecting the transcriptional activity of the *pvd* promoter. On the basis of the relative ice nucleation activities expressed by cells containing the *pvd-inaZ* or *iceC* constructions, we estimated that the *Pseudomonas* spp. examined sensed a concentration of iron on plant surfaces that was intermediate between the concentration in iron-replete culture media and the concentration in low-iron culture media. Because only a fraction of the iron added in the form of $FeCl_3$ is actually present in a soluble form in a neutral aqueous solution (44), however, it is difficult to estimate the exact concentration of Fe(III) available in a culture or on plant surfaces. Nevertheless, bacteria inhabiting the rhizosphere or phyllosphere may not experience the extreme iron deprivation that has been suggested by chemical models which estimate the availability of the ferric ion in soil (29). It is likely that the availability of iron in bulk soil differs from the availability of iron in microbial habitats in the rhizosphere, which may be influenced profoundly by iron acquisition systems of plants (45), oxygen depletion caused by microbial respiration (13), and other factors.

The mean transcriptional activity of a population of bacterial cells containing *pvd-inaZ* was evaluated in this study. Therefore, the possibility that a small subset of the population experienced iron deprivation cannot be excluded. A rhizosphere cell population expressing a mean ice nucleation activity of −2.0 log (ice nucleus per cell) may be, in two extreme examples, a population in which each cell expresses the same level of ice nucleation activity or a population in which 1% of the cells fully express ice nucleation activity (one nucleus per cell) and 99% of the cells express only background levels of ice nucleation activity (−8 log [ice nucleus per cell]). In the latter hypothetical case, 1% of the cells containing *pvd-inaZ* would occupy habitats in which iron is not available, whereas 99% of the cells would occupy habitats in which iron is available. The method described in this paper does not differentiate between these extreme examples or intermediate cases that may exist. If microbes are not greatly limited by the available Fe(III) in the microhabitats that they occupy, however, the current models of microbial population dynamics based on competition for available Fe(III) (30, 42, 48) may require reassessment.

A clear concept of the nature of microbial habitats is fundamental to the field of microbial ecology. Although it is widely appreciated that microorganisms exist in microhabitats with resources that differ greatly from the resources of the bulk

samples accessible to scientific study, characterization of these microhabitats has eluded scientific investigation. Concentrations of elements or chemical compounds that influence biological systems are currently assessed primarily by chemical procedures, usually by analyzing samples that are large relative to the size of bacteria. Although such analytical methods are sensitive indicators of chemical concentrations in bulk environmental samples, they do not assess whether a chemical is present in a form that is available biologically to the organisms that occupy natural habitats. Numerous physicochemical factors influence the form of a chemical compound or element in solution or soil (28); because the chemical form has a profound effect on the biological activity of a chemical, methods that evaluate only the concentration of a chemical do not provide an accurate assessment of the biological relevance of the chemical to an ecosystem. Thus, it is sometimes observed that chemical concentrations that are determined do not have the expected effects on biological communities or indicator organisms (52). There is a need for new methods to determine biologically meaningful levels of chemicals present in disturbed and natural ecosystems. Sensors based on a bioluminescence (*lux*) reporter system have been developed to assess biologically available forms of Hg(II) (49) and naphthalene (18) in environmental samples such as surface water amended with substrates required for bacterial growth or soil slurries. Because the ice nucleation activity expressed by bacterial cells inhabiting natural substrates can be quantified directly, the *inaZ* gene was selected as a reporter system for the sensor assessing the iron available to bacteria occupying natural habitats. In the future, *inaZ* sensors responsive to a variety of chemical signals may allow characterization of many chemical components of microbial habitats on plant surfaces, in soil, or in aqueous environments.

The ice nucleation reporter system provided a sensitive, convenient, and inexpensive tool for the study of bacterial gene expression in natural habitats. Because of its sensitivity, the ice nucleation reporter gene system may be uniquely suited to studies evaluating the chemical environment of microbial habitats. For example, β-galactosidase activity was a useful reporter of the *pvd* promoter in culture only when the level of transcriptional activity was relatively high (i.e., in *P. syringae* cells grown in media containing $\leq 10^{-6}$ M added $FeCl_3$) (Fig. 1). In fact, progressive decreases in the level of transcription of this gene with increases in the $FeCl_3$ concentration to values greater than 10^{-6} M were not observed with *lacZ* fusions because of undetectably low levels of β-galactosidase activity (Fig. 1). In contrast, the ice nucleation reporter system provided a more sensitive way to assess transcriptional activity; ice nucleation activity was detected in *P. syringae* cells containing *pvd-inaZ* grown in media containing up to 10^{-3} M added $FeCl_3$ (Fig. 3). Because the transcriptional activities of the *pvd* promoter expressed by *Pseudomonas* spp. occupying habitats in the phyllosphere or rhizosphere were in the range detectable with the *inaZ* reporter system, such activity may not have been detected with a less sensitive reporter gene, such as *lacZ*. A phosphate sensor, constructed by fusing a phosphate-responsive promoter to a promoterless *lacZ* gene, provided a way to assess phosphate availability to *Pseudomonas putida* occupying the rhizosphere of plants grown in sand or sterile soil, but this sensor was not sensitive enough to assess phosphate availability to the same bacteria occupying the rhizosphere of plants grown in a nonsterile soil (7). The ice nucleation reporter gene system is particularly useful in habitats where the transcriptional activity of target genes is low or only a small number of cells are present. Both situations are likely to occur in natural environments such as the rhizospheres of plants grown in native soils.

Many phenotypes expressed by microorganisms are readily detected and quantified in culture, whereas the same phenotypes are difficult to detect in natural environments. Pyoverdine production by *Pseudomonas* spp. is an example of such a phenotype. Typically, siderophore concentrations in soil are estimated by performing a bioassay with indicator organisms that grow only if a specific ferric siderophore or a general class of ferric siderophores (i.e., ferric siderophores with catechol or hydroxamate groups) provides an exogenous source of iron (3, 38, 43). Bioassays have not been useful for specifically assessing pyoverdines in soil, however, because no indicator strain that specifically utilizes a single ferric pyoverdine as an iron source has been identified. *Pseudomonas* spp. commonly utilize a ferric pyoverdine(s) produced by other strains of *Pseudomonas* spp. (5, 22) and ferric siderophores produced by members of diverse genera of bacteria and fungi (16). Pseudobactin, a pyoverdine produced by *P. fluorescens* B10, was detected recently in a rhizosphere at a concentration of 3.5×10^{-10} M by an immunoassay in which specific monoclonal antibodies were used (4). Because the transcriptional activity of the *pvd* promoter is proportional to pyoverdine production in *Pseudomonas* spp. (Fig. 1), ice nucleation activity conferred by the *pvd-inaZ* fusion may provide a reliable way to assess pyoverdine production by *Pseudomonas* spp. Because of the relative convenience of ice nucleation activity assays, in situ expression of pyoverdine biosynthesis genes can be readily assessed as an estimate of pyoverdine production by *Pseudomonas* spp. occupying microhabitats. Future studies using the complementary approaches of assessing the transcriptional activity of the *pvd* promoter and estimating pseudobactin concentration with an immunoassay should provide considerable insight into factors that influence pyoverdine production by *Pseudomonas* spp. occupying natural habitats.

ACKNOWLEDGMENTS

We are grateful to M. D. Henkels for assisting with the growth chamber experiments and for preparing the figures; S. Carnegie for assisting with the construction and testing of the gene fusions; C. R. Howell, J. Lindemann, W. Tucker, and G. J. Warren for providing strains; and E. Clark and M. Wilson for reviewing the manuscript.

This research was supported in part by grant 91-37030-6802 from the U.S. Department of Agriculture Competitive Grants Program.

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