Iron Stress and Pyoverdinin Production by a Fluorescent *Pseudomonas* in the Rhizosphere of White Lupine (*Lupinus albus* L.) and Barley (*Hordeum vulgare* L.)

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Induction of high-affinity iron transport during root colonization by *Pseudomonas fluorescens* Pf-5 (*pvd-inaZ*) was examined in lupine and barley growing in microcosms. *P. fluorescens* Pf-5 (*pvd-inaZ*) contains a plasmid carrying *pvd-inaZ*; thus, in this strain, ice nucleation activity is regulated by pyoverdinin production. Lupine or barley plants were grown for 18 or 8 days, respectively, in soil amended with 2% calcium carbonate and inoculated with *P. fluorescens* Pf-5 (*pvd-inaZ*) at a density of 4×10^8 CFU g (dry weight) of soil⁻¹. A filter paper blotting technique was used to sample cells from the rhizosphere in different root zones, and then the cells were resuspended for enumeration and measurement of ice nucleation activity. The population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere decreased by one order of magnitude in both lupine and barley over time. The ice nucleation activity ranged from -3.4 to -3.0 log ice nuclei CFU⁻¹ for lupine and -3.0 to -2.8 log ice nuclei CFU⁻¹ for barley, was similar in all root zones, and did not change over time. An in vitro experiment was conducted to determine the relationship between ice nucleation activity and pyoverdinin production in *P. fluorescens* Pf-5 (*pvd-inaZ*). An ice nucleation activity of approximately -3.0 log ice nuclei CFU⁻¹ was measured in the in vitro experiment at 25 to 50 μ M FeCl₃. By using the regression between ice nucleation activity and pyoverdinin production determined in vitro and assuming a *P. fluorescens* Pf-5 (*pvd-inaZ*) population density of 10^8 CFU g of root⁻¹, the maximum possible pyoverdinin accumulation by *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere was estimated to be 0.5 and 0.8 nmol g of root⁻¹ for lupine and barley, respectively. The low ice nucleation activity measured in the rhizosphere suggests that nutritional competition for iron in the rhizosphere may not be a major factor influencing root colonization by *P. fluorescens* Pf-5 (*pvd-inaZ*).

Under iron-limiting conditions, many organisms have been shown to produce low-molecular-weight iron-chelating agents called siderophores, which facilitate transport of Fe(III) into the cells (19). Since iron bioavailability is extremely low in aerobic, neutral- to high-pH environments, nutritional competition for this trace element has been presumed to be important in plant root colonization by microorganisms and in disease suppression. Based on current models, the competitiveness of an individual strain in acquiring iron is dependent on the efficacy of its own siderophore as well as the capacity to utilize siderophores produced by other microorganisms (2, 4, 6, 25). Most transport systems are highly specific for individual siderophore types (20). Hence, the plant-growth-promoting effect of some introduced bacteria is thought to be in part due to siderophores that sequester iron into a form unavailable to microorganisms in the rhizosphere that are deleterious to plants (10, 13). The role of siderophores in disease suppression by fluorescent pseudomonads is controversial. While they seem to be important in suppression of *Fusarium* spp. and *Erwinia carotovora* (13), control of *Gaeumannomyces graminis* var. *tritici* is mainly due to the antibiotic phenazine, while siderophores play only a minor role (8, 26, 27).

One of the problems in elucidating the role of siderophores in microbial competition has been the inability to isolate and purify these compounds from soil. During growth under iron stress conditions in vitro, pyoverdinin, one of the siderophores produced by fluorescent pseudomonads, accumulates to con-

centrations of up to 300 μ M within 48 h (18). Although these very large quantities are produced in vitro, microbiologists have largely been unable to isolate siderophores from natural systems and have instead been forced to rely on indirect methodology for detection and quantification of different siderophore types in soil. By using bioassays, concentrations of bacterial siderophores in soil have been estimated to be between 0.04 and 0.3 nmol g of soil⁻¹ (20), and the concentration of fungal siderophores may range between 30 and 240 μ g kg⁻¹ or between 0.09 and 0.75 nmol g of soil⁻¹ (1). In another approach employing monoclonal antibodies, the pyoverdinin concentration was estimated to be between 0.2 and 0.5 nmol g of root⁻¹ (5). All of these assays require sampling large quantities of soil and preclude localization of siderophore production in specific microsites or analysis of their dynamic rates of production over time. Additionally, siderophore production may be underestimated due to siderophore degradation during sample preparation.

Recently, Loper and Lindow (15) developed an ice nucleation reporter system to detect induction of the high-affinity iron transport system in *Pseudomonas fluorescens* Pf-5 (*pvd-inaZ*). This strain carries a plasmid in which the *inaZ* gene cassette is fused to an iron-regulated promoter involved in pyoverdinin production. The *inaZ* reporter encodes an outer membrane protein (InaZ) that catalyzes ice formation at -2 to -10° C. Ice nucleation activity can be quantified by a droplet-freezing assay (14). In vitro ice nucleation of *P. fluorescens* Pf-5 (*pvd-inaZ*) strongly increased with decreasing Fe concentration. The maximal ice nucleation was measured at 0 to 1 μ M Fe, but ice nucleation was very low at 0.1 to 1 mM Fe (15). Addition of Fe-EDTA to soil strongly decreased ice nucleation

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by *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere of bean (15).

We speculated that one of the primary factors which could significantly influence the bioavailability of iron to microorganisms in the rhizosphere is iron uptake by plants. In this research, studies were conducted in a soil low in available Fe planted with an iron-efficient dicot, lupine (*Lupinus albus* L.), or with an iron-efficient grass, barley (*Hordeum vulgare* L.), to examine the induction of the iron stress response in *P. fluorescens* Pf-5 (*pvd-inaZ*) in different root zones over time. An in vitro experiment was conducted to calibrate pyoverdinin production in relation to ice nucleation activity. The in vitro data were then used to estimate the maximum potential pyoverdinin production by *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere.

MATERIALS AND METHODS

Strain description. *P. fluorescens* Pf-5 (*pvd-inaZ*) contains a plasmid carrying *pvd-inaZ*. Thus, in this strain ice nucleation activity is regulated by a promoter for pyoverdinin, the siderophore of fluorescent pseudomonads (15). The plasmid also confers kanamycin resistance.

Calibration of siderophore production with ice nucleation activity. *P. fluorescens* Pf-5 (*pvd-inaZ*) was grown in 50% MG medium (5 g of mannitol liter⁻¹, 1 g of glutamic acid liter⁻¹, 0.25 g of KH₂PO₄ liter⁻¹, 0.1 g of NaCl liter⁻¹, 0.1 g of MgSO₄ liter⁻¹, pH 7.0) with 10 μ M Fe as FeCl₃ for 18 h. After centrifugation, the cells were resuspended in sterile P-saline buffer (8.5 g of NaCl liter⁻¹, 0.3 g of KH₂PO₄ liter⁻¹, 0.6 g of Na₂HPO₄ liter⁻¹, pH 7.0). Acid-washed 20-ml vials containing 5 ml of 50% MG medium with 5, 10, 25, or 50 μ M Fe as FeCl₃ were then inoculated with the washed cells to give a final cell density of 1.6×10^7 CFU ml⁻¹. The vials were placed on an orbital shaker at an ambient temperature of 20°C.

After 48 h of growth, viable-cell numbers, siderophore production, and ice nucleation activity were determined. For the viable-cell numbers, 10 μ l of an appropriate dilution was plated on King's medium B agar (12) with 150 μ g of streptomycin ml⁻¹, 50 μ g of ampicillin ml⁻¹, and 50 μ g of kanamycin ml⁻¹. The concentration of pyoverdinin in culture supernatants was determined colorimetrically. After FeCl₃ was added to the cell suspension to a final concentration of 10^{-3} M, the suspension was centrifuged, and the absorbance of the supernatant was measured at 450 nm. The siderophore concentration was calculated by using an extinction coefficient of 6,800 liters (mol cm)⁻¹ (18).

Determination of ice nucleation activity. Ice nucleation activity was measured as described by Lindow (14). Briefly, 60 μ l droplets of the cell suspension or an appropriate dilution were placed on a paraffin-coated aluminum boat floating on an ethanol bath with a temperature of -10°C . After 1 min, the number of frozen droplets was counted. The ice nucleation activity was calculated by dividing the ice nucleation frequency by the number of CFU per droplet. It is given as log ice nuclei per CFU.

Determination of ice nucleation activity and population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere. Ice nucleation activity and population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere were measured in lupine and barley in two separate experiments. *P. fluorescens* Pf-5 (*pvd-inaZ*) was grown in MG medium (10 g of mannitol liter⁻¹, 2 g of glutamic acid liter⁻¹, 0.5 g of KH₂PO₄ liter⁻¹, 0.2 g of NaCl liter⁻¹, 0.2 g of MgSO₄ liter⁻¹, pH 7.0) for 24 h. After centrifugation and resuspension in sterile P-saline buffer, the inoculum was added to a final density of 4×10^8 CFU g (dry weight) of soil⁻¹ to a sandy soil amended with 2% (wt/wt) CaCO₃ and mixed in thoroughly. The Fe-deficient soil (Dello loamy sand, typical Psammaque, sandy alluvium [pH 7.1], 4.9 mg of diethylenepentaacetic acid [DTPA]-extractable Fe kg⁻¹) was placed into black acrylic microcosms (295 mm high, 175 mm long, and 15 mm wide), each with a removable transparent acrylic front, and covered with a sheet of black acrylic to keep the root system dark. To avoid preferential water percolation along the soil-plate interface, a polyester cloth (thickness, 1 mm) was placed in the back of the microcosms. A small piece of this cloth emerged from the top of the box and was inserted into two 20-ml plastic vials. The cloth was moistened by filling the vials with 30% Hoagland's solution (9). With this wick system, the soil in the microcosms was maintained at a uniform moisture content of 7% (wt/wt).

Two 9-day-old white lupine seedlings or two 8-day-old barley seedlings (cv. Steptoe) were transplanted into each microcosm (three replicates). The roots were placed flat on the soil surface facing the acrylic front. The microcosms were inclined at an angle of 55° with the front facing down. Thus, most of the roots grew along the transparent acrylic front plate. The experiments were conducted under controlled environmental conditions (a regimen of 15 h of light and 9 h of darkness at 22°C, a relative humidity of 65%, and a photosynthetic photon flux density of 370 microeinsteins mm⁻² s⁻¹).

The rhizosphere was sampled nondestructively by using filter paper to lift cells from the root surface (17). Nitrocellulose filter paper (NitroBind; 0.45- μ m pore size; Micron Separations Inc., Westboro, Mass.) was cut into small rectangular pieces (2 by 10 mm) and autoclaved. After the Perspex front was removed from the microcosms, these strips were picked up individually with forceps, moistened

TABLE 1. Ice nucleation activity and siderophore production by *P. fluorescens* Pf-5 (*pvd-inaZ*) in vitro at 5 to 50 μ M Fe^a

Fe concn (μ M)	Ice nucleation activity (log ice nuclei CFU ⁻¹)	Siderophore production (log mol CFU ⁻¹)
5	-1.62 d	-16.73 c
10	-2.50 c	-16.80 c
25	-3.00 b	-17.16 b
50	-3.57 a	-17.45 a

^a Values are means of three replicates. Different letters in the same column indicate significant differences ($P < 0.05$).

with sterile P-saline buffer, and placed onto the root surface by applying slight pressure with the forceps. As the diameter of the roots is 0.5 to 1 mm, the filter paper also covered the soil adjacent to the roots. The filter paper pieces were placed on different sections of the root system: 0 to 1 or 1 to 2 cm from the root tip or in the lateral root zone for lupine and 0 to 1, 1 to 2, or 2 to 3 cm from the root tip for barley. For the bulk soil samples, filter paper was placed on the soil surface at least 3 cm from the roots. The filter paper was removed from the roots or the soil after approximately 5 min and placed into a 1.5-ml microcentrifuge tube containing 1 ml of sterile P-saline buffer. Soil particles adhering to the filter paper were not removed. Eight replicate filter paper pieces from the same root zone and one microcosm were all transferred into the same microcentrifuge tube. The tubes were vortexed for 1 min to dislodge bacteria from the filter paper. For the bacterial enumeration, 10 μ l of the suspension or the appropriate dilution was plated onto King's medium B agar with 150 μ g of streptomycin ml⁻¹, 50 μ g of ampicillin ml⁻¹, and 50 μ g of kanamycin ml⁻¹. The remaining suspension or an appropriate dilution was used for the determination of the ice nucleation activity.

The rhizosphere of white lupine was sampled 2, 6, 10, 14, and 18 days after transplanting. The rhizosphere of barley was sampled 2, 4, 6, and 8 days after transplanting.

Statistical analysis. The population density was log transformed. The effects of the Fe concentration in the in vitro experiment were examined by a one-way analysis of variance. The significance of differences in ice nucleation and population density between the root zones over time in the microcosm experiments was determined by a two-way analysis of variance followed by a Student-Newman-Keuls test with Sigmapstat (Jandel Corporation, San Rafael, Calif.).

RESULTS

Calibration of ice nucleation with siderophore production in vitro. An in vitro experiment was conducted to determine the relationship between ice nucleation activity and pyoverdinin production by *P. fluorescens* Pf-5 (*pvd-inaZ*). The ice nucleation activity and pyoverdinin concentration in the medium decreased significantly with increasing Fe concentration (Table 1). When the strain was grown at 50 μ M Fe, the ice nucleation activity was -3.57 log ice nuclei CFU⁻¹, which corresponds to a siderophore production of -17.45 log mol CFU⁻¹. Under low-Fe conditions with 5 μ M Fe, a siderophore production of -16.73 log mol CFU⁻¹ and an ice nucleation activity of -1.62 log ice nuclei CFU⁻¹ were measured. The final cell densities after 2 days of growth were similar at all four Fe concentrations (data not shown). The results of this experiment were used to calculate a regression to characterize the relationship between ice nucleation activity and siderophore production (Fig. 1).

Ice nucleation activity and population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere. The ice nucleation activities in the rhizosphere of lupine were similar in all three root zones examined and did not change over time (Fig. 2A). The population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere of lupine was consistently lower in the lateral root zone than in the first 2 cm from the root tip over the 18-day period of the experiment (Fig. 2B). The population density decreased significantly from day 10 to day 14 in all root zones by approximately one order of magnitude.

Both ice nucleation activity and population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere of barley were similar throughout the first 3 cm from the root tip (Fig. 3A).

FIG. 1. Relationship between ice nucleation activity of *P. fluorescens* Pf-5 (*pvd-inaZ*) and siderophore production in vitro at 5, 10, 25, or 50 μM Fe.

While the ice nucleation activity did not change with time, the population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) decreased significantly from day 4 to 6 (Fig. 3B).

In both experiments, the ice nucleation activity of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the bulk soil varied from -3.0 to -3.5 log ice nuclei CFU^{-1} . Induction of the iron stress response was slightly lower in the rhizosphere of lupine (-3.0 to -3.4 log ice nuclei CFU^{-1}) than in the rhizosphere of barley (-2.8 to -3.0 log ice nuclei CFU^{-1}).

Assuming a surface area of $10^5 \text{ mm}^2 \text{ g}$ (dry weight) of root $^{-1}$, the population density of approximately 100 CFU mm^{-2} , determined for *P. fluorescens* Pf-5 (*pvd-inaZ*) by the filter paper technique, corresponds to 10^7 CFU g (dry weight) of root $^{-1}$. Previous experiments (17) have shown that the filter paper technique lifts approximately 10% of the cells obtainable

FIG. 3. Ice nucleation activity (A) and population density (B) of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere of barley over time in three different root zones. Values are means of three replicates. Lowercase a's indicate insignificant differences between the root zones ($P < 0.05$).

by destructive sampling. The actual population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere would therefore be approximately 10^8 CFU g (dry weight) of root $^{-1}$. By using this population density, the ice nucleation activity in the rhizosphere, and the regression obtained in the in vitro experiment, the siderophore concentration in the rhizospheres of lupine and barley can be estimated to range between 0.5 and 0.8 nmol g (dry weight) of root $^{-1}$ (Table 2).

DISCUSSION

This study shows that *P. fluorescens* Pf-5 (*pvd-inaZ*) is only mildly iron stressed in the rhizospheres of lupine and barley in a soil low in available iron (Fig. 2 and 3). An ice nucleation activity similar to that in the rhizosphere (approximately -3.0 log ice nuclei CFU^{-1}) was measured in the in vitro experiment at 25 to 50 μM FeCl_3 . This in vitro experiment was conducted to establish a relationship between ice nucleation activity and pyoverdinin production (Fig. 1). The range of Fe concentrations was chosen to obtain ice nucleation activities similar to those measured in the rhizosphere. Additionally, Loper and Lindow (15) showed that within this range (-2 to -4 log ice nuclei CFU^{-1}), the relationship between Fe concentration and ice nucleation activity is linear, while this is not the case at higher ice nucleation activities.

The measured ice nucleation activity in the rhizosphere corresponds well with the results of a short-term study (15). The mild iron stress of *P. fluorescens* Pf-5 (*pvd-inaZ*) may be in part due to the capacity of fluorescent pseudomonads to use plant iron chelators as iron sources (11). Iron stress of a given strain may also be decreased by the utilization of siderophores produced by other rhizosphere microorganisms (22, 25). However, pyoverdinin is only one of several siderophores produced by fluorescent pseudomonads (19). Thus, *P. fluorescens* Pf-5 (*pvd-inaZ*) may produce other siderophores not detectable in this ice nucleation assay.

FIG. 2. Ice nucleation activity (A) and population density (B) of *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere of lupine over time in three different root zones. Values are means of three replicates. Different lowercase letters indicate significant differences between the root zones ($P < 0.05$).

TABLE 2. Minimum and maximum levels of ice nucleation activity of *P. fluorescens* Pf-5 (*pvd-inaZ*) and minimum and maximum concentrations of siderophores in the rhizospheres of lupine and barley^a

Plant	Ice nucleation (log ice nuclei CFU ⁻¹)		Siderophore production ^b					
			log mol CFU ⁻¹		log mol g of root ⁻¹		nmol g of root ⁻¹	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
Lupine	-3.4	-3.0	-17.31	-17.16	-9.31	-9.16	0.49	0.69
Barley	-3.0	-2.8	-17.16	-17.08	-9.16	-9.08	0.69	0.83

^a Estimated by regression between ice nucleation and siderophore concentration obtained in vitro.^b Assuming a population density of 10⁸ CFU g of root⁻¹.

Using pyoverdine promoter-driven ice nucleation in combination with the filter paper technique provides a novel method to assess iron stress in the rhizosphere. In contrast to extraction of siderophores from entire root systems (5, 20), our method allows nondestructive sampling of the same root system over time in different root zones and can therefore increase our knowledge of Fe stress and siderophore production on a microsite level. However, the filter paper lifts only cells that are easily removed from the root surface. Iron stress of these cells may differ from that of cells tightly adhering to the roots. To test this, we conducted another experiment in which cells were sampled either by the filter paper method or by homogenizing root pieces. The measured ice nucleation levels were -2.6 and -1.9 log ice nuclei CFU⁻¹ for the filter paper method and destructive sampling, respectively (unpublished data). Thus, cells tightly adhering to the roots may be more Fe stressed than those that are easily removed. After sonicating roots, Loper and Lindow (15) also measured similar ice nucleation activities in the rhizosphere.

Many factors may influence the availability of iron in the rhizosphere (6). An important factor is plant iron uptake, which represents a major sink for iron depletion. Two responses to iron limitation have been identified for plants (16). Iron-efficient grasses like barley excrete phytosiderophores to acquire Fe (16). These are very effective iron chelators and may serve as an iron source for pseudomonads (11). Iron-efficient dicots like lupine respond to iron stress by rhizosphere acidification and reductases that convert Fe(III) to more soluble Fe(II) (7). Since the root tip is the main site of chelate release and iron uptake by the plant (16), it was thought that *P. fluorescens* Pf-5 (*pvd-inaZ*) may be more iron stressed at the root tip than in the other root parts, particularly because the Fe requirement of microorganisms is higher than that of plants (21). Surprisingly, ice nucleation activity levels were similar in all root zones examined and were only slightly higher in barley than in lupine (Fig. 2 and 3). By the end of the experiments, the plants were slightly chlorotic, and the lupine plants had formed proteoid roots in response to the low Fe availability (7). Thus, both plants and *P. fluorescens* Pf-5 (*pvd-inaZ*) were slightly iron stressed in this soil.

Although this research examined only one strain of *P. fluorescens*, the low Fe stress measured in this study raises the question of whether siderophores play an important role in competition between microorganisms in the rhizosphere. However, the low Fe stress may be a result of population dynamics of this bacterium. Iron demand is likely to be highest during exponential growth, but the population density of *P. fluorescens* Pf-5 (*pvd-inaZ*) decreased considerably over time (Fig. 2 and 3), which is quite common for introduced bacterial strains. A declining population is likely to have a low Fe demand. This suggests that competition for Fe has a minimal impact on root colonization by *P. fluorescens* Pf-5 (*pvd-inaZ*). This impact may

not be limited to the soil used in this study, as a similar ice nucleation activity was measured in the rhizosphere of bean growing in another soil (15).

The estimated pyoverdine production by *P. fluorescens* Pf-5 (*pvd-inaZ*) in the rhizosphere (0.5 to 0.8 nmol g [dry weight] of root⁻¹ [Table 2]) is in the same range as in other studies on bacterial siderophores in the rhizosphere (5, 20, 23) but very low compared to the pyoverdine production measured in vitro under extreme Fe stress (18). The results of the present study suggest that pyoverdine production by a given strain may be estimated by ice nucleation in the rhizosphere and the regression between ice nucleation and pyoverdine concentration obtained in vitro. However, such estimates must be viewed with caution, since the promoter controlling *inaZ* is only one of a number of promoters initiating pyoverdine biosynthesis. To be useful as an indicator of pyoverdine synthesis, it would have to be representative of the other promoters or the one promoter that limits pyoverdine production. The correlation between ice nucleation and pyoverdine production in vitro ($r^2 = 0.78$ [Fig. 1]) indicates that this could be the case. However, in the rhizosphere, pyoverdine synthesis may be limited by expression of other promoters. Furthermore, in vitro, only Fe limited the growth of *P. fluorescens* Pf-5 (*pvd-inaZ*), while in the rhizosphere other nutrients (especially carbon) could limit the amount of pyoverdine produced by a cell. The pyoverdine concentration in the rhizosphere is further decreased by absorption of siderophores to soil particles (24) and degradation. Studies with plants have shown that degradation of phytosiderophores by rhizosphere microorganisms can be substantial (28). Additionally, some microbial siderophores can be taken up and utilized as iron sources by plants (3, 24, 29).

Based on the findings of this research, *P. fluorescens* Pf-5 (*pvd-inaZ*) was only mildly Fe stressed in the rhizosphere. This could be due to the capacity of this strain to utilize siderophores produced by other microorganisms or by plants or due to a declining population. The study suggests that nutritional competition for iron in the rhizosphere may not be a major factor influencing root colonization by *P. fluorescens* Pf-5 (*pvd-inaZ*).

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