Lack of a Role for Fluorescent Siderophore Production in the Biological Control of Pythium Damping-Off of Cucumber by a Strain of *Pseudomonas putida*

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ABSTRACT

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Hyphal growth but not sporangial germination of *Pythium ultimum* was inhibited by the synthetic iron chelating compound ethylenediamine (di-o-hydroxyphenylacetic acid) (EDDHA) on water agar and by strain N1R of *Pseudomonas putida* on King's medium B. Eight prototrophic derivatives of N1R (deficient in fluorescent siderophore [pyoverdine] biosynthesis [Pvd$^-$]) were obtained by Tn*5* mutagenesis. Pvd$^-$ derivatives did not inhibit hyphal growth of *P. ultimum* on King's medium B, but protected cucumber seedlings from Pythium damping-off in three different agricultural soils at levels statistically indistinguishable from the parental strain, N1R. Therefore, pyoverdine production by strain N1R was not critical to biocontrol of Pythium damping-off of cucumber under the conditions of this study.

Fluorescent pseudomonads affect biocontrol of certain soilborne plant pathogens due, in part, to the production of secondary metabolites, including antibiotics and siderophores, in the rhizosphere of agricultural plants (21,22,41,42). Siderophores are biosynthetic compounds that are produced under iron-limiting conditions (chelate the ferric ion [Fe^{3+}] with high specific activity) and serve as vehicles for the transport of iron (III) into microbial cells (27).

Fluorescent pseudomonads are characterized by their production of yellow-green pigments, termed pyoverdines, which fluoresce under ultraviolet irradiation and function as siderophores (6). Some researchers postulate that pyoverdines are produced in the plant rhizosphere where they deprive pathogens of iron required for spore germination, hyphal growth, and pathogenesis (18,35,36). Involvement of iron competition in biological control has been inferred from experiments in which purified siderophores or synthetic iron chelators were tested for activity in soil systems (15,16,35) or mutants deficient in siderophore production were compared with parental strains with respect to biocontrol activity (3,4,21).

Although, by definition, all fluorescent pseudomonads produce pyoverdines, these siderophores are not universally implicated in the biocontrol efficacy of fluorescent pseudomonads. For example, pyoverdines contribute only minimally to the biocontrol of take-all of wheat or black root rot of tobacco by *Pseudomonas* spp. (1,9,43). The role of pyoverdines in the suppression of soilborne plant diseases by fluorescent pseudomonads may vary with plant host, pathogen, soil type, and possibly the strain of *Pseudomonas* evaluated.

Pythium ultimum Trow, an oomycete fungus, is widely distributed in agricultural soils and induces preemergence and postemergence damping-off and root rot of many agronomic and horticultural crops. Seed treatment with certain fluorescent pseudomonads controls Pythium diseases of several crops (4,10,14, 21,28). Pyoverdine production is important in the biocontrol of Pythium root rot of wheat by *Pseudomonas fluorescens* (Migula) (4) and in the biocontrol of Pythium damping-off of cotton by *P. fluorescens* strain 3551 (21). Iron deprivation imposed by synthetic chelators or a purified pyoverdine inhibits mycelial growth of *P. ultimum* isolates in culture (21,25), although effects on sporangium or oospore germination are unknown. Mechanisms other than iron competition, including nutrient or site competition and antibiotic production, are also implicated in the biocontrol of Pythium damping-off diseases by fluorescent pseudomonads (10,13,40).

Our study was initiated to evaluate the role of pyoverdine production by *Pseudomonas putida* (Migula), strain N1R, in biocontrol of Pythium damping-off of cucumber. Strain N1R is a biocontrol agent of Fusarium wilt of cucumber, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cucumerinum* J. H. Owen (7,29), and of Pythium damping-off of cucumber, caused by *P. ultimum* (32). Baker and colleagues (7,8,35) proposed that strain N1R and certain other fluorescent pseudomonads produce a pyoverdine in the cucumber rhizosphere in concentrations adequate to antagonize *F. o. cucumerinum*. Our hypothesis was that pyoverdine production of strain N1R may play a role in biocontrol of Pythium damping-off. Our approach was to evaluate the iron sensitivity of an isolate of *P. ultimum* by examining the effects of iron chelators on sporangium germination, germ tube elongation, hyphal growth, and biomass production by *P. ultimum* in culture; derive a series of pyoverdine production-deficient mutants of N1R; and evaluate the biocontrol activity of such mutants against *P. ultimum* damping-off of cucumber.

MATERIALS AND METHODS

Organisms, media, and growth conditions. *P. ultimum*, isolate N1 (19), was maintained at 23 C on 2% water agar (WA) and transferred monthly. Isolate N1 does not produce oospores, but produces sporangia on WA after 2–3 wk. Germ tube elongation and radial growth of *P. ultimum* were determined on buffered water agar (pH 7.0), which contained 0.01M PIPES [piperazine-N-N'-bis(2-ethane-sulfonic acid), dipotassium salt] (Sigma Chemical, St. Louis, MO). Sporangial germination and biomass of *P. ultimum* were evaluated in a glucose-asparagine medium (GAM), composed of 10 g glucose, 0.5 g L-asparagine, 0.5 g $MgSO_4 \cdot 7H_20$, 1.5 g KH_2PO_4, 0.1 g $CaCl_2$, 0.1 g NaCl, and 1.2 g tris buffer in 1 L of distilled water. One milliliter of the following micronutrient solution was also added to each liter: 420 mg $ZnCl_2$,

7 mg $MnCl_2 \cdot 4H_20$, and 25 mg $CuSO_4 \cdot 5H_20$ in 100 ml of distilled water. The pH was adjusted to 5.5 with 1N HCl.

Strain N1R of *P. putida*, a spontaneous rifampicin-resistant mutant of a field isolate, was provided by R. Baker (Colorado State University, Fort Collins, CO) (7). *P. putida* was routinely cultured at 27 C in nutrient broth (NB), (Difco Laboratories, Detroit, MI) or on nutrient agar (NA) (Difco) supplemented with 1% glycerol. Fluorescence of strain N1R was observed under UV light (366 nm) after 24–48 h of growth at 27 C on King's medium B (KMB), which promotes pyoverdine production by fluorescent pseudomonads (14). Population doubling times of *P. putida* were determined from cultures growing in NB + 1% glycerol by change of optical density at 640 nm (A_{640}) with time. Prototrophy was determined by evaluating bacterial growth on 925 medium (17). Strains of *Escherichia coli* (Migula) Castellani & Chalmers were grown at 37 C in Luria Bertani medium (LB) (23). Strains SM10 (pSUP1011) and S17-1 (pSUP1021) (38) were obtained from R. Simon (University of Bielefeld, West Germany). Strain C600 (pLG221) (5) was obtained from G. J. Boulnois (University Medical Centre, Geneva, Switzerland). Bacterial strains were stored at −80 C in NB supplemented with 10% (w/v) glycerol. Antibiotics were used when specified at the following concentrations ($\mu g\ ml^{-1}$): rifampicin (Sigma), 100; kanamycin (Sigma), 50; cycloheximide (Sigma), 100. All solutions and media were made in deionized water.

Chelators. Iron was removed from EDDHA [ethylenediamine (di-o-hydroxyphenylacetic acid)] (Sigma) according to the methods of Rogers (33). Stock solutions of EDDHA (140 mM), deferoxamine mesylate (Sigma, 100 mM), and 2,2′ dipyridyl (Sigma, 200 mM) were sterilized by filtration, stored at 4 C in polycarbonate containers, and added to autoclaved media. Media amended with EDDHA were kept at 4 C for 24 h before use to allow slow chelation of iron. When specified, $FeCl_3$, $CuSO_4$, or $ZnSO_4$ were added to media at concentrations equimolar to that of added chelators.

Effect of synthetic chelators on hyphal growth of *P. ultimum*. Buffered water agar was amended with 0, 10, 20, 40, 80, 160, 320, 630, and 12,601 μM EDDHA or with 300 μM 2,2′ dipyridyl. Single hyphal tips, obtained from a 24-h WA culture of *P. ultimum*, were placed on the medium in petri dishes. Radial growth was measured after 19, 27, and 42 h. Five replicate plates were used for each treatment.

The effect of EDDHA on biomass production of *P. ultimum* was evaluated in GAM. EDDHA or $FeCl_3$ was added at 0, 100, 200, 400, or 800 μM to GAM in 18- × 150-mm test tubes. In another experiment, 0, 125, 250, 500, 1,000, 2,000, or 4,000 μM of EDDHA or $FeCl_3$ was added to GAM containing 1,000 μM of $FeCl_3$ or EDDHA. An agar plug (4 mm in diameter) of *P. ultimum* from 24-h WA cultures was added to each tube. Ten agar plugs were also dried at 70 C and weighed. Tubes were placed on a rotary shaker in a completely randomized design at 27 C in the dark. Mycelial mats were removed from the tubes after 3 days growth, dried for 24 h at 70 C, and weighed. The average weight of an agar plug was subtracted from the total weight of the mycelial mats. Mycelial mats from three replicate tubes were evaluated for each treatment.

Effect of strain N1R and synthetic chelators on sporangial germination and germ tube elongation of *P. ultimum*. Sporangial suspensions were prepared by comminuting a 1- to 3-month-old water agar culture of *P. ultimum* in a Waring blender with 60 ml of sterile deionized water. After 30–60 sec of comminuting, the suspension was filtered though sterile cheesecloth and collected in two sterile 2.5- × 10-cm polypropylene centrifuge tubes. Sporangia were pelleted by centrifugation at 2,000 *g* for 15 min and resuspended in 3 ml of sterile deionized water to a concentration of 10^4–10^5 sporangia per milliliter. A 0.5-ml sample of the sporangial suspension was spread on WA amended with chelators (EDDHA at 0, 700, 1,400, or 2,100 μM; deferoxamine mesylate at 0 or 10,000 μM; and 2,2′ dipyridyl at 0, 10, 50, 100, or 200 μM). Sporangial germination was assessed after 4 h of incubation at 23 C.

Sporangial germination in the presence of EDDHA was also tested in GAM, a liquid medium. One-hundred microliters of GAM and 40 μl of sporangial suspension were added to 1.5-ml microcentrifuge tubes. EDDHA was added to final concentrations of 0, 62, 125, 250, 500, 1,000, or 2,000 μM. Tubes were incubated for 4 h at 23 C. Germination of sporangia in a 30-μl sample of each tube was evaluated under a compound microscope. Five replicate tubes were evaluated from each treatment.

Germ tube growth was evaluated on WA containing 0 or 1,000 μM EDDHA. A sample of sporangial suspension (0.5 ml) was spread on the medium and incubated for 5, 7, 10, 12, 24, and 29 h at 23 C. Lengths of germ tubes on the surface of agar samples from each plate were measured with the aid of an ocular micrometer. Five replicate plates were evaluated from each treatment.

Tn*5* mutagenesis and selection of Pvd⁻ mutants. Strains of *E. coli* (SM10 [pSUP1011], S17-1 [pSUP1021], and C600 [pLG221]) were used as donors in matings with strain N1R for Tn*5*-mediated mutagenesis, as described previously (5,38); Donor and recipient strains were grown at 27 C with shaking to late log phase (approximately 10^8 cfu/ml). Cells were pelleted by centrifugation and resuspended in LB. Recipient and donor cells were mixed (10:1, v:v), spread on LB agar, and incubated at 27 C for 18 h. Putative Tn*5* insertion mutants of N1R were detected on KMB amended with kanamycin (Km) and ampicillin (Ap). Nonfluorescent (Pvd^-), Km^r, Ap^r colonies were detected under UV light (366 nm), purified through three consecutive transfers on KMB, and tested for the production of oxidase (34) and for growth on KMB amended with 500 μM 2-2′dipyridyl. The mating experiment was repeated once.

Genetic characterization. Genomic DNA was isolated from 1.5-ml cultures of N1R and Pvd^- mutants at late log phase. A standard detergent lysis was followed by extraction with hexadecyltrimethylammonium bromide and chloroform to remove exopolysaccharides (2). DNA then was purified with standard phenol:chloroform extraction and ethanol precipitation. Genomic DNA was digested with the restriction endonucleases *Eco*R1 and *Bam*H1 (BRL, Bethesda Research Laboratories, Rockville, MD) according the manufacturer's instructions and analyzed by electrophoresis on 0.7% agarose gels with tris-phosphate EDTA buffer (23) for 3 h at 30 volts, followed by 12 h at 10 volts. For Southern hybridizations, the DNA was transferred from the gel to nylon membranes (Nytran, Schleicher, & Schuell) by standard techniques (23). The 3.3 kb *Hind*III fragment internal to Tn*5* and cloned in pUC8 was nick-translated with biotinylated-TTP (BRL), purified with a D50 column (IBI, International Biotechnologies, New Haven, CT), and hybridized with the blot by standard techniques (23). Hybridizations were visualized by development with a nonradioactive nucleic acid detection kit (Blu GENE R [BRL]).

Effect of N1R and Pvd^- mutants on growth of *P. ultimum* in dual culture. Bacterial suspensions of N1R and Pvd^- mutants were adjusted to $A_{640} = 0.1$ (approximately 10^9 cfu/ml). Two 50-μl drops of bacterial suspension were placed on either edge of the KMB agar surface in each 100- × 15-mm plastic petri dish. After incubation at 27 C for 48 h, a 4-mm-diameter plug of *P. ultimum* from a water agar culture was placed in each dish. Petri dishes were sealed with Parafilm (American Can, Greenwich, CT) and incubated at 26 C for 72 h. Dishes without bacterial colonies served as controls. Three replicate plates were used for each treatment.

To determine whether the observed inhibition by the siderophore strain N1R was reversible by iron, the following experiment was performed. Dishes containing two 50-μl drops of bacterial suspension, as above, were incubated for 48 h at 27 C, then inverted over a glass petri dish cover containing a paper towel saturated with chloroform. After 30 min, the dishes were vented in a laminar flow hood for 1 h, and 5 ml of KMB agar with or without 100 μM $FeCl_3$ was poured on top. The dishes were incubated at 27 C for 24 h, and a plug (4 mm in diameter) of *P. ultimum* was placed in the center of the dish. Hyphal growth was assessed after 72 h. Two replicate dishes were used for each treatment.

Biological control assay. Biological control of Pythium damp-

ing-off was evaluated in three soils. Newberg fine sandy loam (initially pH 6.0) was mixed 1:1 (v/v) with washed river sand and adjusted to pH 7.4 with $CaOH_2$. Warden sandy-silt loam, collected from a field near Prosser, WA, had a pH of 7.2 and contained 0.77% organic matter, 9.6 mg kg^{-1} Fe, 14 mg kg^{-1} P, 300 mg kg^{-1} K, 50 mg kg^{-1} nitrate, 11 cmol kg^{-1} g Ca, and 1.1 cmol kg^{-1} Mg. Oceano loamy sand (Moss Landing soil [21]) had a pH of 7.2.

Inoculum of *P. ultimum*, prepared as described by Paulitz and Baker (31), was quantified by dilution plating on a medium selective for *Pythium* spp. (26). Newberg fine sandy loam river sand mix and Warden soil were infested with *P. ultimum* to a final level of 30 cfu/g. Oceano loamy sand had indigenous populations of *Pythium* spp. of 372 cfu/g and was not infested with additional inoculum. Bacterial inoculum was prepared by washing cells grown in KMB for 48 h in 0.1M $MgSO_4$ and resuspending in 0.1M $MgSO_4$ to an optical density of $A_{640} = 0.1$ (approximately 10^9 cells per milliliter). Pelgel (2% w/w, Nitragin, Milwaukee, WI) was added to the adjusted bacterial suspension. Sixty cucumber (*Cucumis sativus* L. 'Straight Eight') seeds were soaked in 6 ml of the cell suspension for 1 h and planted in plastic pots 6.5 cm in diameter, containing 150 g of soil (10 seeds per pot, six pots per treatment). Control seed was treated with Pelgel only. A nondiseased treatment was prepared for the Oceano loamy sand by mixing metalaxyl (50% EC, 30 μg/ml). Pots were placed in a completely randomized design in a growth chamber (12 h light/dark cycle) where the soil temperature was maintained at 23 C. Pots were watered daily with distilled water. After 7 days, the seedling emergence from six pots was evaluated for each treatment.

Rhizosphere population densities of N1R and derivative strains were evaluated 8 days after planting seed, treated as above, in 70 g of Newberg fine sandy loam river sand mix in 16- × 2.5-cm in diameter plastic pine cells (Cone-Tainer, Canby, OR), six cells per treatment. Root systems were removed from each cell and shaken to remove loosely adhering soil. The 1-cm section of the root closest to the seed was discarded. Remaining roots were placed in 99 ml of sterile water, sonicated for 2 min, and dilution-plated on KMB amended with rifampicin and cycloheximide. Bacterial colonies were counted after 2 days of incubation at 27 C. The experiment was repeated once and analyzed by one-way analysis of variance.

Statistical analysis. All experiments were arranged in a completely randomized design and were repeated once with similar results. Only the first trials are presented. All data were analyzed with one-way analysis of variance. Comparisons among treatment means and means of appropriate control treatments were made with *t* tests. Tukey's multiple range test was used to separate treatment means in the biological control assay experiments.

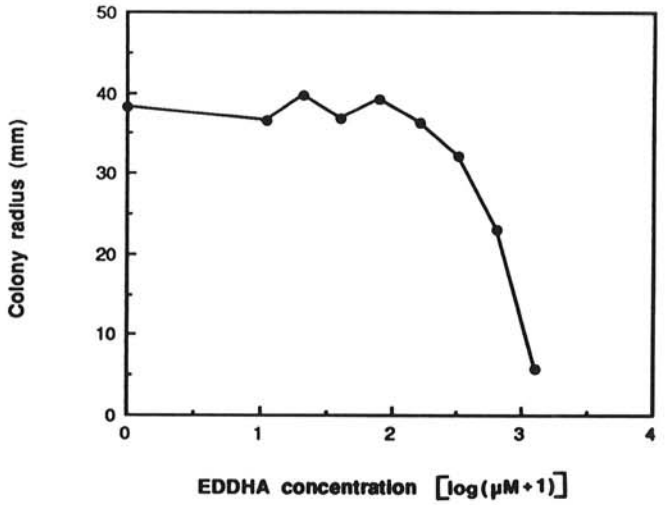

Fig. 1. Radial growth of *Pythium ultimum* on water agar amended with the chelator EDDHA [ethylenediamine (di-o-hydroxyphenylacetic acid)]. Radii were measured 42 h after a hyphal tip of *P. ultimum* was transferred to the medium. Values presented are the means of five replicates. Mean standard error = 1.65 mm.

RESULTS

Effect of synthetic chelators on hyphal growth of *P. ultimum* in culture. Radial growth of *P. ultimum* on WA amended with more than 630 (Log 2.8) μM EDDHA was significantly less than on unamended WA (Fig. 1). Similarly, biomass accumulation of *P. ultimum* in GAM containing 800 μM EDDHA was significantly less than in GAM without EDDHA (Fig. 2A). $FeCl_3$ was also inhibitory at 800 μM. Growth inhibition imposed by amendment of GAM with 1,000 μM EDDHA was reversed by the addition of 125 or 250 μM $FeCl_3$, although greater concentrations of $FeCl_3$ were toxic (Fig. 2B). The growth inhibition with 1,000 μM $FeCl_3$ in GAM was reversed by the addition of 4,000 μM EDDHA (Fig. 2B). No radial growth of *P. ultimum* was observed on water agar amended with 300 μM 2,2'dipyridyl; however, growth was observed when WA-dipyridyl medium was amended with 300 μM $FeCl_3$, $CuSO_4$, or $ZnSO_4$ (data not shown).

Effect of strain N1R and synthetic chelators on sporangial germination of *P. ultimum*. The frequency of sporangial germination of *P. ultimum* on KMB (84%) was not influenced by proximity to 24-h-old colonies of N1R. Sporangium germination was not influenced by amendment of GAM with 2,000 μM EDDHA. Eighty-six percent of sporangia germinated in GAM that was either unamended or amended with 2,000 μM EDDHA.

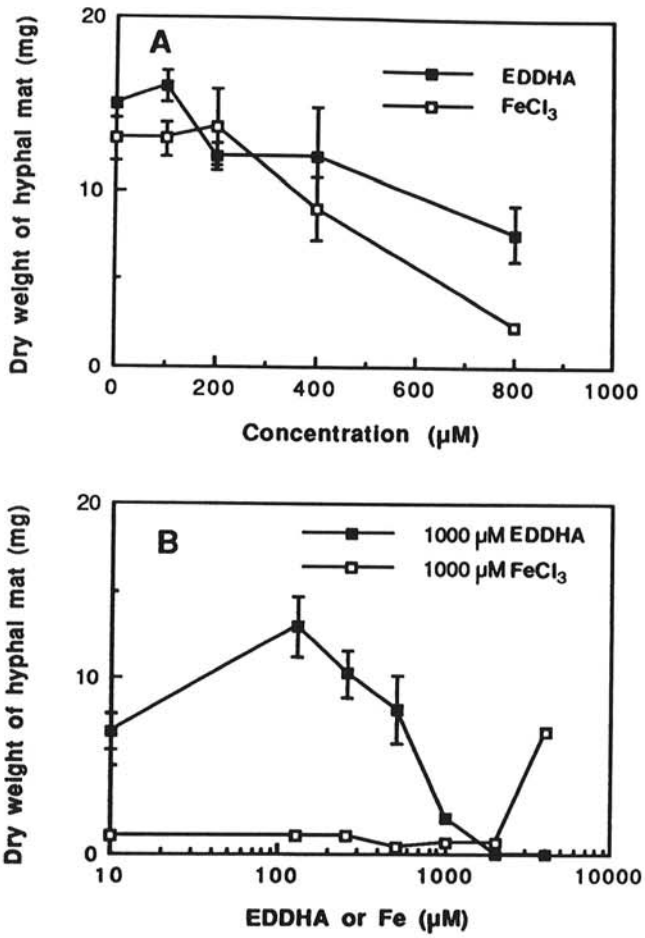

Fig. 2. Effects of EDDHA (■) and $FeCl_3$ (□) on biomass accumulation of *Pythium ultimum* grown in liquid glucose asparagine medium (GAM) buffered to pH 5.5 with 10 mM tris. **A**, Effect of EDDHA and $FeCl_3$, when added separately. **B**, Effect of $FeCl_3$ and EDDHA when added at various concentrations to treatments containing 1000 μM EDDHA (■) or 1000 μM $FeCl_3$ (□), respectively. Dry weight of unamended GAM control = 13.3 mg. Bars represent standard error of the mean.

EDDHA, deferoxamine mesylate, or 2,2′ dipyridyl (200 μM) did not inhibit the frequency of sporangial germination of *P. ultimum* on WA. In all treatments, 60–77% of the sporangia germinated on WA after 4 h. Germ tube elongation on water agar amended with 1,000 μM EDDHA was not inhibited until 12 h after sporangial germination and then was reduced by 60% (Fig. 3).

Tn*5* mutagenesis and selection of Pvd⁻ mutants. Putative Tn*5* mutants of strain N1R were obtained at a frequency of 3.6×10^{-8}, 5.4×10^{-8}, and 1.0×10^{-8} per recipient after matings with strains SM10 (pSUP1011), S17-1 (pSUP1021), and C600 (pLG221) of *E. coli*, respectively. Of 4,000 individual Ap^r, Km^r colonies that were observed under UV light for fluorescence, 10 (0.25%) were nonfluorescent (Pvd⁻). Nine of the 10 Pvd⁻ mutants were prototrophic, as determined by growth on 925 medium. None of the Pvd⁻ mutants grew on KMB supplemented with 1,600 μM 2,2′dipyridyl, although parental strain N1R grew on this medium. Pvd⁻ mutants grew on KMB containing 1,600 μM 2,2′dipyridyl and 1,600 μM $FeCl_3$ but not on KMB containing 1,600 μM 2,2′dipyridyl and 1,600 μM $CuSO_4$ or $ZnSO_4$. Eight of the nine prototrophic Pvd⁻ mutants grew in nutrient broth + 10% glycerol at a rate similar to N1R, with generation times of 72 min. However, strain JL4327 grew more slowly, with a generation time of 94 min.

Southern analysis of genomic DNA identified a single *Eco*R1 fragment containing Tn*5* in each of the nine prototrophic Pvd⁻ mutants. The nine mutants fell into four classes, which were defined by the size of the *Eco*R1 fragment containing Tn*5* (Table 1). Seven of the nine mutants had unique sites of Tn*5* insertion, based on Southern analysis of *Bam*H1 digested genomic DNA. The fragments of *Bam*H1 digested genomic DNA of strains JL4314 and JL4317 with homology to Tn*5* were identical in size, suggesting that these strains may be siblings. The genomic DNA of strain JL4315 had only a single observable *Bam*H1 fragment with homology to Tn*5*. The lack of a second homologous *Bam*H1 fragment in strain JL4315 was not investigated further, but may have been due to a deletion of a portion of Tn*5* after the insertion event, or to the presence of two *Bam*H1 fragments of similar size that were not resolved by gel electrophoresis.

Effect of strain N1R and Pvd⁻ mutants on hyphal growth of *P. ultimum* in dual culture. Strain N1R of *P. putida* inhibited mycelial growth of *P. ultimum* on KMB agar. Radial growth of *P. ultimum* from an agar plug placed on KMB overlay agar was greater in directions away from than toward an underlying N1R colony. In contrast, rapid and concentric radial growth was observed when the overlay agar was amended with 100 μM $FeCl_3$. *P. ultimum* was not inhibited on KMB by any of the Pvd⁻ mutants listed in Table 1; the fungus grew directly over colonies of the Pvd⁻ strains.

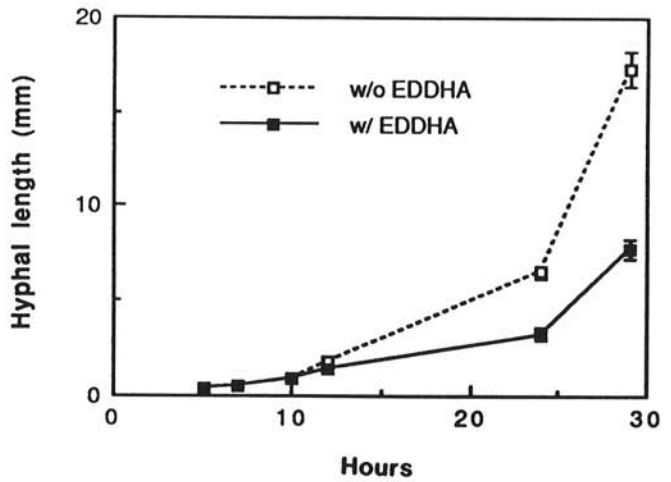

Fig. 3. Germ tube elongation of *Pythium ultimum* on water agar amended with 1 mM EDDHA. Germ tube and hyphal length of each of 25 germinated sporangia were measured at 5, 7, 10, 12, 24, and 29 h. After 12 h, germ tube length differed significantly between treatments, according to *t* test, ($P < 0.05$). Bars represent standard error of the mean.

Biological control of Pythium damping-off of cucumber by Pvd⁻ mutants. Treatment of cucumber seeds with *P. putida* (N1R) improved emergence of cucumber seedlings in three different soils infested with Pythium (Fig. 4). Five Pvd⁻ mutants improved emergence as well as the parental strain in the Newberg fine sandy loam river sand mix and in the Warden sandy silt loam. In the Oceano loamy sand, which had a high concentration of indigenous *Pythium* spp., all Pvd⁻ mutants improved emergence as well as the parental strain, but only three out of five Pvd⁻ mutants gave control equivalent to the fungicide treatment.

The rhizosphere populations of strain N1R and the nine prototrophic Pvd⁻ mutants did not differ significantly ($P > 0.05$). The bacteria established rhizosphere population sizes of 6.9–7.3 log units per gram of rhizosphere soil 7 days after planting in the three soils tested.

DISCUSSION

Hyphal growth of isolate N1 of *P. ultimum* was dependent on the iron availability of the growth medium, which was manipulated by amendment with EDDHA or by paired culture with strain N1R of *P. putida*. Inhibition of hyphal growth of *P. ultimum* was attributed to pyoverdine production of strain N1R, because inhibition was reversed with 100 μM $FeCl_3$, and Pvd⁻ mutants of strain N1R were not inhibitory. The observation that growth of isolate N1 was inhibited by EDDHA is consistent with those of Loper (21) and Becker and Cook (4), who studied other isolates of *P. ultimum*. Difficulties encountered in quantifying hyphal growth on agar surfaces were overcome by measuring biomass accumulation in a defined, buffered liquid medium (GAM). Concentrations of EDDHA and $FeCl_3$ exceeding 400 μM inhibited the growth of *P. ultimum*. Growth inhibition imposed by one of these compounds was reversed by the other. The iron chelator, 2,2′dipyridyl, was not useful to assess the sensitivity of *P. ultimum* to iron deprivation due to lack of specificity for the ferric ion. Inhibition of hyphal growth by 2,2′dipyridyl was reversed by $ZnSO_4$, $CuSO_4$, and $FeCl_3$.

Iron availability clearly influences spore germination of certain fungal phytopathogens. For example, iron deprivation inhibits germination of chlamydospores of *F. o. cucumerinum* (8,37). In other cases, supraoptimal concentrations of iron are toxic to spore germination (1,24). Sporangial germination of isolate N1 of *P. ultimum*, however, was not dependent on iron availability of the growth medium, as manipulated in our study. Frequency of sporangial germination in culture was not altered by amendment of the growth medium with EDDHA or by proximity to strain N1R of *P. putida*. Sporangia germinated rapidly, within 1–2 h after exposure to nutrients. Many of the biochemical processes requiring iron and necessary for germination may be completed before sporangia are triggered to germinate. Sporangia may also utilize internal reserves of iron for germination and initial germ tube growth and may not require exogenous iron, as was suggested with the chlamydospores of *F. solani* (9).

In *P. ultimum*, the transition between independence and dependence of germ tube elongation on exogenous iron occurred

TABLE 1. Characteristics of *Pseudomonas putida* (strain N1R) and Pvd⁻ mutants

Stain	Phenotype	Size of fragments with Tn*5* insert		
		*Eco*R1	*Bam*H1	
N1R	Pvd⁻, Rif^r, Km^r	...	...	...
JL4313	Pvd⁻, Rif^r, Km^r	15	25.0	10.0
JL4314	Pvd⁻, Rif^r, Km^r	28	22.0	7.5
JL4315	Pvd⁻, Rif^r, Km^r	17	20.0	...
JL4316	Pvd⁻, Rif^r, Km^r	28	11.7	18.5
JL4317	Pvd⁻, Rif^r, Km^r	28	22.0	7.5
JL4318	Pvd⁻, Rif^r, Km^r	25	28.0	11.7
JL4319	Pvd⁻, Rif^r, Km^r	28	6.2	4.5
JL4320	Pvd⁻, Rif^r, Km^r	28	7.7	3.7
JL4327	Pvd⁻, Rif^r, Km^r	25	26.0	10.5

within 12 h after sporangial germination. If soilborne sporangia germinate immediately in response to seed exudates, we envision that *P. ultimum* will not be sensitive to iron deprivation for 12 h after a seed is planted. In contrast, sporangia of *P. ultimum* germinate and colonize the seed coat of cucumber (20) or the pericarp of sugar beet (28) within a few hours after seed is planted in soil infested with *Pythium*. Thus, sporangia of *P. ultimum* may escape iron deprivation imposed by fluorescent pseudomonads on the seed surface due to rapid germination and colonization of internal tissues of the germinating seed.

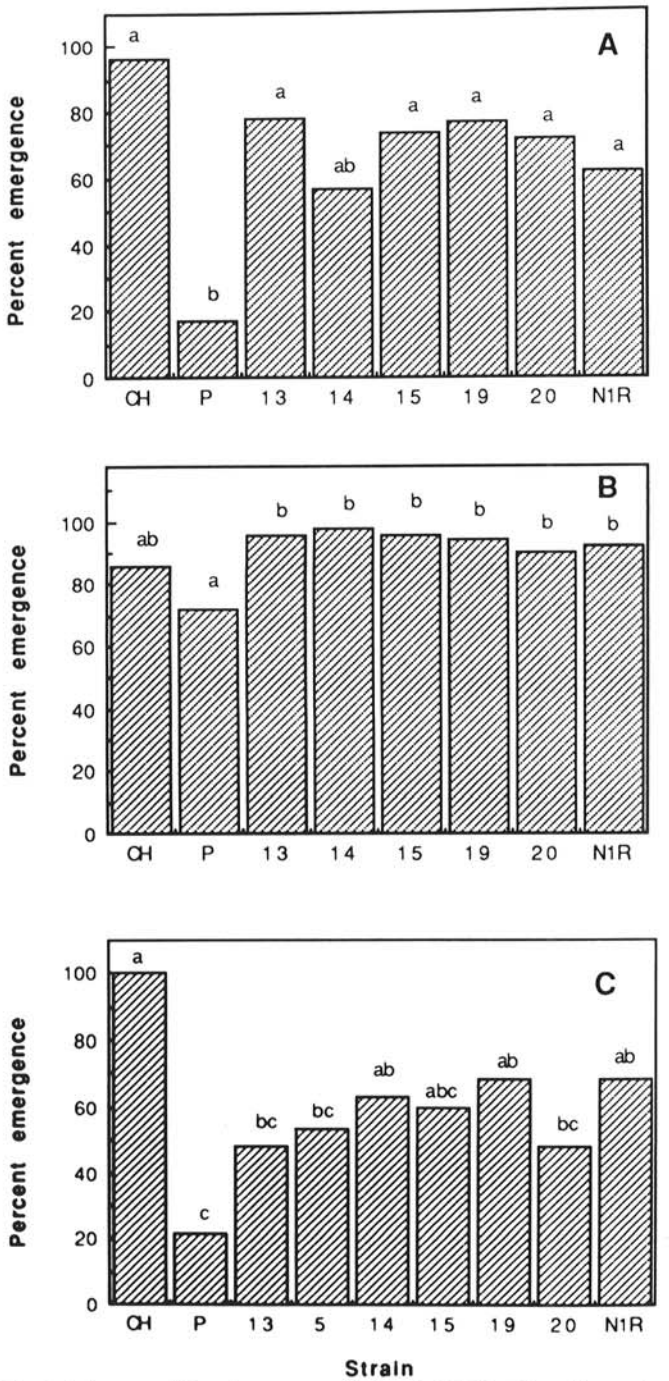

Fig. 4. Influence of *Pseudomonas putida* (strain N1R) and nonfluorescing derivative strains on emergence of cucumber seedlings from soils with indigenous or introduced populations of *P. ultimum*. Seed treated with bacterial strains (10^8 cfu/seed) or untreated seed (P) were planted in Newberg fine sandy loam river sand mix, **A**, Warden sandy silt loam, **B**, or Oceano loamy sand, **C**. Soil in **A** and **B** was artificially infested with *P. ultimum* (30 cfu/g). Soil in **C** was naturally infested with *Pythium* spp. at 373 cfu/g. CH = untreated seeds planted in uninfested soil, **A** and **B**, or soil treated with metalaxyl, **C**. Numbers specify Pvd$^-$ derivatives of strain N1R and correspond to the last two digits of strain designations given in Table 1. Strain 5 is a Pvd$^+$ derivative of strain N1R, with a Tn*5* insertion. Bars with a common letter do not differ significantly according to Tukey's multiple range test, $P < 0.05$. Each treatment had six replicates.

Pyoverdine production did not affect the biological control activity of strain N1R of *P. putida* against Pythium damping-off of cucumber grown in the three soils of this study. Strain N1R and five Pvd$^-$ derivatives obtained following Tn*5* mutagenesis were equivalent in biocontrol of Pythium damping-off of cucumber. Each of the five Pvd$^-$ mutants contained a single and unique insertion of Tn*5*, as determined by Southern analysis. Strain N1R and the five Pvd$^-$ derivatives also established equivalent population densities in the cucumber rhizosphere. Thus, pyoverdine production of strain N1R was not critical to its establishment in the cucumber rhizosphere under conditions of this study.

Previously, Loper (21) suggested that pyoverdine production contributed to the biocontrol activity of *P. fluorescens* (strain 3551) against Pythium damping-off of cotton. The previous and present studies differ with respect to the host plant, soil type and temperature, species and strain of *Pseudomonas*, isolate of *P. ultimum* tested, and the duration observed between planting and seedling emergence (3–5 days for cucumber and 7–14 days for cotton). Pyoverdine production contributed more to the effect of *P. fluorescens* 3551 on emergence of cotton from soils infested with *Pythium* observed 7–14 days following planting than to cotton seed colonization by *P. ultimum* observed 24 h after planting (21). Pyoverdine concentration in the cotton spermosphere 24 h after bacterial seed treatment may not be sufficient to decrease seed colonization by *P. ultimum*. Additionally, *P. ultimum*, which persists in agricultural field soils as sporangia or oospores (12,39), may not be susceptible to iron competition during the initial stages of pathogenesis. From our results, we suggest that sporangia of *P. ultimum* (isolate N1) were not sensitive to iron competition by strain N1R of *P. putida*. In contrast, hyphal growth of *P. ultimum* during the later stages of seed coat and endosperm colonization may be quite sensitive to pyoverdine-mediated iron competition. We speculate that the opportunity for such competition is greater on slower-emerging cotton than on cucumber seed. Controlled studies clearly are needed to resolve the contribution of pyoverdine production of *Pseudomonas* spp. to biocontrol of Pythium damping-off diseases of plant hosts grown under various conditions.

Properties common to strain N1R and Pvd$^-$ derivatives, such as growth in the spermosphere of cucumber or the production of unidentified antifungal metabolites, determined their common activities against Pythium damping-off of cucumber. *P. putida* (N1R) may utilize substrates, such as sugars, amino acids, or ethanol, that trigger sporangial germination or saprophytic growth of *P. ultimum* before colonization of the seed, as was proposed to explain the biocontrol of *Pythium aphanidermatum* (Edson) Fitzp. by other bacterial biocontrol agents on cucumber (10). In preliminary work (30), strain N1R reduced the concentration of ethanol and acetaldehyde in the spermosphere of pea and soybean 2–12 h after seed imbibition, which led to the suggestion that the bacterium may compete for seed volatiles that stimulate germination and growth of *P. ultimum*.

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