Role of Fluorescent Siderophore Production in Biological Control of *Pythium ultimum* by a *Pseudomonas fluorescens* Strain

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ABSTRACT

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Pseudomonas fluorescens Migula strain 3551, isolated from cotton rhizosphere soil, protected cotton from seed colonization and preemergence damping-off caused by *Pythium ultimum*. The role of fluorescent siderophore production by strain 3551 in its antagonism against *P. ultimum* was investigated. Fourteen nonfluorescent (Flu−) Tn*5* insertion mutants of *P. fluorescens* 3551 were obtained following matings with *Escherichia coli* SM10 (pSUP1011). Strain 3551 grew on an iron-deficient medium, whereas the 14 Flu− derivative strains did not. Southern analysis of *Eco*RI digested genomic DNA from the Flu− mutants probed with nick-translated λ:Tn*5* confirmed the presence of single insertions of Tn*5* in the chromosomal DNA of each derivative strain. Representative mutants from five of nine *Eco*RI fragment size classes were compared to the parental strain with respect to antagonism against *P. ultimum* in the cotton spermosphere. Cotton seed treated with metalaxyl, strain 3551, or Flu− derivative strains were evaluated for colonization by *P. ultimum* 24 hr after planting into infested soil and for their subsequent emergence. Although the parental strain 3551 decreased colonization of cotton seed by *P. ultimum* and increased seedling emergence to a level statistically equivalent to metalaxyl treatment, Flu− derivative strains did not. Differences between 3551 and Flu− derivative strains were greater with respect to emergence than *P. ultimum* colonization. No consistent differences were observed between strain 3551 and Flu− derivative strains with respect to their growth rates or population sizes in the cotton spermosphere. Results indicate that fluorescent siderophore production by *P. fluorescens* 3551 contributes to but does not account for all of its antagonistic activity against *P. ultimum*.

Certain strains of fluorescent pseudomonads increase yields or control biologically one or more soilborne plant pathogens when applied as seed or seed piece inoculants to agricultural crops (3,22,23,26). Several reports suggest that beneficial pseudomonads enhance plant growth and affect biological control by producing fluorescent siderophores that sequester iron in the root environment, making it less available to competing deleterious microflora (10,11,21,25). This hypothesis had its genesis with the finding that mutants of plant growth-promoting rhizobacteria that did not exhibit in vitro antibiosis were deficient in fluorescent siderophore production (Flu−) and no longer promoted plant growth (12). The Flu− mutants also failed to cause a detectable alteration in the composition of the root microflora as was observed with parental strains. Siderophores produced by *Pseudomonas* spp. have since been implicated in the biological control of potato seed piece decay caused by *Erwinia carotovora* (Jones) Bergey et al (29), in suppression of several wilt diseases caused by *Fusarium* spp. (11,21,25), and in growth responses of wheat grown in *Pythium*-infested soils (2). The suggested involvement of fluorescent siderophores in biological control has been inferred from experiments in which siderophores, purified from culture filtrates, were tested for activity in soil systems, or mutants deficient in siderophore production were compared to parental strains with respect to biocontrol activity. In all cases, however, Flu− mutants were obtained after chemical mutagenesis and their single-site nature was not established. The availability of genetic tools for analysis of plant-associated pseudomonads now facilitates the derivation of strains with well-characterized single-site mutations. Confirmation of the above experiments with single-site insertion mutants will provide more definitive evidence for the role of siderophores in biological control. The present study was initiated to identify naturally occuring rhizosphere bacteria that control biologically preemergence damping-off of cotton caused by *Pythium ultimum* Trow and to determine the role of siderophore production in biological control of *P. ultimum* by derivation of well-characterized, single-site deficiency mutants for comparison to parental strains with respect to biocontrol activity in a soil system.

MATERIALS AND METHODS

Media and growth conditions. *Pseudomonas* spp. were routinely grown in nutrient agar or on nutrient broth (Difco Laboratories, Detroit, MI) supplemented with 1% (w/v) glycerol (NAG or NBG, respectively). Population doubling times were determined by change of optical density at 640 nm (A_{640nm}) with time from cultures grown in NBG at 30 C. Fluorescence of strains of *Pseudomonas* was observed under UV light (λ = 366 nm) on King's Medium B (KBM) (9) after 24–48 hr of incubation at 25 C. Prototrophy was evaluated by observation of growth on a minimal medium containing (grams per liter): K_2HPO_4, 3; NaH_2PO_4, 1; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.4; glycerol, 15; and agar, 15. *Escherichia*

coli strains were grown in Luria Bertani medium (LB) (15). Antibiotics were used when specified at the following levels (micrograms per milliliter): rifampicin (Sigma), 100; kanamycin (Sigma), 50; cycloheximide (Sigma), 150. *P. ultimum* was maintained on hemp seed in sterile water and grown on water agar (WA, 2% agar) or potato-dextrose agar (PDA, Difco). Sporangia were harvested from oatmeal agar slant plates (8) flooded with sterile water and incubated at 21 C for 5–7 days.

Soils. Soils used as sources for bacterial isolations were obtained from the top 15 cm of agricultural fields in Iowa, Mississippi, Florida, and California. Soils were air dried, sieved, and stored at 4 C for up to 2 wk. Three field sites, in Fresno, Pinole, and Moss Landing, CA, were sources of soils used in cotton seed assays, emergence assays, and growth chamber experiments. Textures of Fresno, Pinole, and Moss Landing soils were loam, sandy loam, and loamy sand, respectively. Chemical characteristics were determined by a commercial testing laboratory (A & L Midwest Agricultural Laboratories, Inc., Omaha, NE) as follows: pH: 7.5, 6.8, 6.7; phosphorus: 28, 93, 164 μg/g; nitrate: 32, 68, 17 μg/g; potassium: 93, 167, 431, μg/g; magnesium: 254, 431, 192 μg/g; calcium: 860, 1,230, 600 μg/g; DPTA extractable iron: 7, 141, 47 μg/g; DPTA extractable zinc: 2.2, 3.8, 15.0 μg/g; DPTA extractable manganese: 10, 141, 17 μg/g; DPTA extractable copper: 0.4, 1.4, 1.4 μg/g; DPTA extractable boron: 0.8, 0.9, 1.2 μg/g for Fresno, Pinole, and Moss Landing soils, respectively. The Fresno and Moss Landing soils had indigenous populations of *P. ultimum* at levels varying from 6 to 150 propagules per gram of air-dried soil. The Pinole soil was pasteurized before use and subsequently infested with the sporangia (approximately 20 propagules per gram of air-dried soil) of an isolate of *P. ultimum* obtained from R. Lukens (Chevron Chemical Co., Richmond, CA). Propagule levels of *P. ultimum* reported in the results were determined by the soil drop method (28) immediately before each experiment.

Isolation of bacteria from the cotton rhizosphere. Soils were adjusted gravimetrically to approximately −0.01 MPa matric potential (−0.01 MPa = −0.1 bar) by interpolation from their individual moisture release curves. Cotton seed (Acala SJ2, mechanically delinted) was planted to a depth of 1.5 cm in a 400-ml plastic beaker. Beakers were covered with plastic wrap and placed in a growth chamber (19 C, 16 hr light). On emergence, root systems were retrieved from the soil. Three centimeters of roots were excised from the apical section of the taproot, placed in 20 ml of washing buffer (0.1 M phosphate buffer [pH = 7.0] supplemented with 0.1% [w/v] peptone [Difco]) in a 125-ml Erlenmeyer flask, and shaken (200 rpm) for 20 min. Root washings were plated on KBM, 10% tryptic soy agar (Difco), and NAG. Five individual colonies were selected from each medium/soil combination. They were streaked to purity on the medium of isolation, then stored immediately at −70 C in 15% glycerol. Strains were individually characterized with respect to oxidase reaction, gram stain reaction, fluorescence on KBM, and anaerobic growth by standard methods (7). Fluorescent pseudomonads were further characterized according to the criteria and methods of Stanier et al (27) for arginine dihydrolase, denitrification and lecithinase activity, levan production, gelatin liquification, growth at 41 C, and growth on minimal medium containing one of the following as a sole carbon source: erythritol, galactose, hippuric acid, benzoic acid, trehalose, creatine, benzylamine, and *m*-inositol.

Assay for cotton seed colonization by *P. ultimum* and seedling emergence. The cotton seed assay was an adaption of the method described by Osburn and Schroth (18). Sixty milliliters of soil was placed in a brass ring (6 cm diameter, 6 cm height) on a ceramic plate. The soil was saturated via capillary action and equilibrated to −0.01 MPa matric potential in a pressure plate apparatus (Soil Moisture Inc., Santa Barbara, CA) for 24 hr at 21 C. Rings containing soil were placed on one-half of a petri dish (85 cm diameter). Four cotton seeds were planted to a depth of 1 cm in each ring. The ring and petri dish were placed inside a sealed plastic bag and incubated at 20 C for 24 hr. Seeds were removed from soil with forceps, rinsed carefully to remove all soil particles, placed on water agar plates, and incubated at 25 C for 24 hr. *P. ultimum* colonization was identified by observation of characteristic hyphae (28) growing out from cotton seed 18–24 hr following plating. Four individual seeds from each of five replicate rings were evaluated for the presence or absence of *P. ultimum* colonization per treatment. Where specified (emergence assays), subsequent seedling emergence was recorded daily from 7 to 14 days after planting of seed into rings. Rings containing soil were incubated at 20 C with 12 hr light (900 ft candles) and 12 hr darkness. Emergence of four seeds from each of five replicated rings was evaluated.

Growth chamber experiments. Conetainers (Ray Leach Nursery, Canby, OR) were filled with Moss Landing soil (49 propagules of *P. ultimum* per gram of air-dried soil). Three seeds were planted to a depth of 1 cm in each Conetainer. Six replications of two Conetainers each were planted for each treatment. Conetainers were placed in growth chambers maintained at 20 C (±1 C) with 16 hr light, 8 hr darkness. Emergence was recorded 11–14 days after planting.

Seed treatment. Bacterial growth from a 24-hr culture on NAG was suspended in water and adjusted to a cell density of approximately 5×10^8 colony-forming units (cfu)/ml (A_{640nm} = 0.45). Cotton seed was dipped in the bacterial suspension for 5–10 min before planting. The population size of fluorescent pseudomonads on the seed was 10^5–10^6 cfu per seed when applied by this method. Metalaxyl (Ciba-Geigy, Greensboro, NC) was applied to seed at 1.3 g/kg of seed.

Spermosphere colonization. *Pseudomonas fluorescens* 3551 was obtained as described above from the rhizosphere of cotton grown in soil from Modesto, CA. Spermosphere colonization by *P. fluorescens* 3551 was estimated by following the population size of its rifampicin-resistant derivative, strain 3793. Individual seeds were retrieved from the soil with sterile forceps, shaken gently to remove all but the closely adhering soil, and placed in 20 ml of washing buffer in a 125-ml Erlenmeyer flask. Flasks were placed on a rotary shaker (200 rpm) for 30–60 min. Spermosphere population sizes were quantified by dilution plating of the seed washings on KBM supplemented with rifampicin and cycloheximide. Rifampicin-resistant colonies were enumerated after 2 days of incubation at 27 C. No naturally occurring rifampicin-resistant colonies were observed when seed washings of uninoculated controls were plated.

Data analysis. Statistical analysis was done using the general linear models and analysis of variance procedures of Statistical Analysis Systems (SAS release 82.3, SAS Institute, Inc., Cary, NC). Colonization and emergence data were transformed to the arcsin of the square root of proportion values. Because of the lognormal distribution of these and other data sets (14) describing rhizosphere bacterial populations, the logarithmic transformation was applied to all population values before statistical analysis.

Transposon mutagenesis. *E. coli* strain SM10 (pSUP1011), obtained from R. Simon (University of Bielefeld, West Germany) under contract from Agrigenetics (Denver, CO), was used for Tn*5*-mediated mutagenesis as described by Simon et al (24). Cells of donor *E. coli* SM10 harboring pSUP1011 and recipient *P. fluorescens* 3793 were grown to late log phase (approximately 10^9 cfu/ml), centrifuged for 1 min in a microfuge, carefully resuspended in LB broth, mixed in a ratio of 1 donor:10 recipients, and 100 μl of the mating mixture was spread onto a NAG plate. After 8–12 hr of incubation at 25 C, the cells were suspended in 5 ml of sterile water, and 100-μl aliquots were spread on KBM plates supplemented with rifampicin and kanamycin. After 48 hr of incubation at 25 C, colonies were enumerated and observed for fluorescence under UV irradiation (λ = 366 nm).

Conditions of iron-limited growth. KBM or WA was supplemented with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (EDDA, 1,000 μg/ml, Sigma) resulting in media (KBM-EDDA or WA-EDDA, respectively) with low levels of available iron. The EDDA was prepared before incorporation into the media as described by Ong et al (17) after removing iron as described by Rogers (20). KBM-EDDA and WA-EDDA were kept at 4 C for 24 hr before use to allow slow chelation of iron.

Bacterial suspensions were adjusted to a uniform density of 0.1

A_{640nm} units (approximately 1×10^8 cfu/ml) and streaked with a calibrated 1-μl loop on KBM-EDDA medium. Only four suspensions were streaked on each plate to minimize cross-feeding of Flu− strains. An agar plug (5 mm diameter) from the margin of an actively growing culture of *P. ultimum* on water agar was transferred to the center of a WA-EDDA plate. Radial growth was monitored for 7 days.

Inhibition of *E. coli* in culture. *E. coli* strains AN194 (proC, leu, trp, thi) and AN193 (as AN194 but deficient in enterobactin production) were received from J. B. Neilands (Department of Biochemistry, University of California, Berkeley, CA). These strains were used as indicators for iron-dependent antibiosis of *P. fluorescens* 3551 and its Flu− derivatives. Twenty microliters of a turbid water suspension of each *P. fluorescens* strain was spotted onto the center of individual plates containing either KBM or KBM supplemented with 10^{-4} M FeCl. Plates were incubated for 48 hr at 25 C, then sprayed with a turbid water suspension of *E. coli* AN193 or AN194. Plates were incubated another 48 hr before observation. Inhibition was scored if a clear zone in which no *E. coli* growth was visible surrounding the *Pseudomonas* colony.

DNA isolation and Southern analysis. Genomic DNA from *P. fluorescens* strains was isolated from 5 ml of a late log phase culture. Cells were harvested by centrifugation, resuspended in 2 ml of Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0), mixed with 1 ml of 0.1 M Tris-HCl, 9% SDS, pH 8.5, and incubated for 30 min at 0 C. The lysate was then extracted twice with an equal volume of phenol:chloroform (1:1), once with chloroform, and dialyzed against TE buffer (0.01 mM Tris HCl, 0.001 mM EDTA, pH 8.0). The genomic DNA was digested with the restriction enzyme *Eco*RI (IBI, New Haven, CT) and run on a 0.7% agarose gel in Tris-acetate buffer at 25 mAmp for 12 hr. DNA fragments were transferred from the gel to Gene-Screen Plus (New England Nuclear Corp. [NEN], Boston, MA) by an adaptation of the method of Southern as specified by NEN. Phage particles of λ:Tn*5* were received from S.-T. Liu (Chevron Chemical Co., Richmond, CA). Phage DNA was isolated as described by Maniatis (15). One microgram of λ:Tn*5* DNA was nick-translated according to the method of Rigby et al (19) with [^{32}P]-dCTP (410 Ci/mmole, Amersham, Arlington Heights, IL) and a nick translation kit (Amersham). The specific activity was 1.8×10^8 cpm/μg. DNA was purified by the spun column method and hybridized as described by Maniatis (15).

RESULTS

Effect of bacterial strains on cotton seed colonization by *P. ultimum* and on seedling emergence. Ninety-six individual bacterial strains obtained from rhizosphere soils of cotton were tested for their effects on colonization of cotton seed by *P. ultimum*. Of the 96 strains, 27 were characterized as fluorescent pseudomonads, five as other gram negative bacteria, and 64 as gram positive bacteria. Only nine of the strains protected cotton seed from colonization by *P. ultimum* at a level statistically similar to that of metalaxyl seed treatment in at least two independent seed assays (data not shown). All nine strains were fluorescent pseudomonads characterized as *P. fluorescens* or *P. putida* Migula. *P. fluorescens* 3551 was selected for further study because it could be manipulated genetically (discussed below) and it successfully protected cotton seed from colonization by *P. ultimum* in nine of 10 assays done in three soil types with indigenous or introduced populations of *P. ultimum* (Table 1). In the one assay in which protection was not observed, the metalaxyl standard was also ineffective. In the remaining nine assays, strain 3551 protected seed at a level comparable to the metalaxyl standard in six cases. These results indicate that strain 3551 consistently provided protection against seed colonization by *P. ultimum*.

Strain 3551 was further tested for its effect on emergence of cotton seedlings grown in soil with indigenous or introduced populations of *P. ultimum*. In three emergence assays and two growth chamber experiments, seedling emergence from seeds treated with strain 3551 was significantly greater than that of untreated seeds (Table 2). In the three cases where metalaxyl was also included, seedling emergence from seeds treated with 3551 was similar to that of the metalaxyl treatment. These results indicate that seed treatment with strain 3551 consistently increased cotton seedling emergence.

Strain 3793, a spontaneous rifampicin-resistant derivative of

TABLE 1. Influence of *Pseudomonas fluorescens* seed treatments on colonization of cotton seed by *Pythium ultimum* in three soil types

	Percent[x] of seeds colonized by *P. ultimum*									
	Pinole soil				Moss Landing soil			Fresno soil		
Seed treatment	Exp.[y] 1 (9 p/g)	Exp. 2 (16 p/g)	Exp. 3 (16 p/g)	Exp. 4 (25 p/g)	Exp. 1 (49 p/g)	Exp. 2 (49 p/g)	Exp. 3 (49 p/g)	Exp. 1 (6 p/g)	Exp. 2 (6 p/g)	Exp. 3 (19 p/g)
Untreated control	50 a	100 a	88 a	100 a	100 a	81 a	93 a	44 a	44 a	28 a
Metalaxyl	0 b	0 b	0 c	0 c	0 c	0 b	0 c	0 b	13 ab	0 b
P. fluorescens 3551	6 b	19 b	19 bc	62 b	37 b	6 b	56 b	0 b	19 ab	3 b
P. fluorescens 3793	NT	NT	44 b	NT	NT	63 b	NT	NT	NT	0 b

[x] Means in a given column followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan test. An F value representing significance of treatment effects at $P = 0.05$ was observed in every case.
[y] Exp = Experiment.
[z] p/g = Propagule of *Pythium ultimum* per gram of soil. The propagule level of each soil was determined for individual experiments.

TABLE 2. Influence of *Pseudomonas fluorescens* seed treatments on seedling emergence of cotton

	Percent emergence[x] of cotton seeds				
	Seed assays[y]			Growth chamber experiments	
Seed treatment	Experiment 1 (81 p/g)	Experiment 2 (19 p/g)	Experiment 3 (19 p/g)	Experiment 1	Experiment 2
Untreated control	4 a	20 a	20 a	0 a	46 a
Metalaxyl	50 b	NT	NT	42 ab	88 b
P. fluorescens 3551	25 b	45 b	55 b	54 b	88 b
P. fluorescens 3793	NT	NT	NT	50 b	88 b

[x] Emergence means in a given column followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan test. An F value representing significance of treatment effects at $P = 0.05$ was observed in every case.
[y] Seed assays were done in Fresno soil. Propagule levels were determined for each experiment and are given as propagule/gram soil (p/g).

strain 3551, was selected as single colony growing on KBM-rifampicin following a 3-day incubation of a plate that had been spread with 100 μl of a log-phase culture (approximately 5×10^8 cfu/ml) of strain 3551. Strain 3793 was fluorescent, prototrophic, had the same growth rate as its parental strain in NBG, and exhibited stable resistance to rifampicin following more than 100 generations of growth without selection. In seed assays (Table 1), emergence assays, and growth chamber experiments (Table 2), the effects of seed treatment with strain 3793 were statistically similar to those with strain 3551. The spontaneous mutation to rifampicin resistance was not associated with loss of efficacy in strain 3793.

Isolation and characterization of nonfluorescent Tn*5* mutants of 3793. Tn*5* mutants of strain 3793 were obtained at a frequency of 6×10^{-7} per recipient following matings with *E. coli* strain SM10 (pSUP1011). Individual Km^r, Rif^r colonies were observed under UV light for fluorescence and 22 of 8,017 (0.3%) were nonfluorescent (Flu−). Of the 22 nonfluorescent strains analyzed, 14 had single bands with homology to the λ:Tn*5* probe following Southern analysis. However, eight strains had two or more bands with homology to λ:Tn*5*. The nature of these multiple regions of homology was not determined but may have been due to multiple transpositions of Tn*5* or to secondary transpositions of IS50 sequences into the bacterial genome. The 14 strains with single insertions of Tn*5* fell into nine classes on the basis of the size of the *Eco*RI fragment containing Tn*5* (Table 3). The 14 strains were prototrophic but some differed from parental strains with respect to growth rate in NBG (Table 3). These 14 strains were unable to grow on the iron-deficient medium, KBM-EDDA, but their growth was restored when 10^{-3} M FeCl was incorporated into the KBM-EDDA medium. Parental strains 3551 and 3793 produced a zone of inhibition against *E. coli* AN193 on KBM but not on KBM supplemented with 10^{-4} M FeCl. No zone of inhibition was observed against the enterochelin-producing parental strain, AN194. None of the 14 Flu− derivative strains produced a zone of inhibition against either AN193 or AN194. The Flu− mutants were deficient in siderophore production as determined by their iron-limited growth and lack of iron-regulated antibiosis against *E. coli* AN194.

Influence of iron on growth of *P. ultimum* in culture. *P. ultimum* did not grow on WA-EDDA although hyphal growth covering the entire surface of the plate was observed on WA alone after 24 hr. The inhibition of hyphal growth due to EDDA was reversed by addition of 10^{-3} M FeCl to the agar medium. No growth was observed when WA-EDDA was supplemented with $CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ at concentrations ranging from 10^{-3} to 10^{-5} M. These results indicate that the growth inhibition observed on WA-EDDA was specifically attributed to the unavailability of the ferric ion.

TABLE 3. Characteristics of *Pseudomonas fluorescens* strains

Strain	Phenotype	Size of *Eco*R1 fragment with Tn*5* insert (kb)	Generation[x] time (min)
3551	Flu+	none	42
3793	Flu+, Rif^r	none	43
3820	Flu−, Rif^r	6.9	52
3831	Flu−, Rif^r	7.6	45
3830	Flu−, Rif^r	8.0	35
3819	Flu−, Rif^r	8.7	40
3825	Flu−, Rif^r	9.6	59
3811	Flu−, Rif^r	12.5	32
3813	Flu−, Rif^r	12.5	67
3815	Flu−, Rif^r	12.5	73
3817	Flu−, Rif^r	12.5	75
3818	Flu−, Rif^r	12.5	56
3829	Flu−, Rif^r	12.5	NT[y]
3824	Flu−, Rif^r	14.3	41
3810	Flu−, Rif^r	17.0	58
3826	Flu−, Rif^r	20.0	39

[x] Values are means of three replications (SEM = 5).
[y] NT = Not tested.

Effect of nonfluorescent mutants in seed assay and emergence experiments. The 14 Flu− mutants were compared to parental strains 3551 and 3793 and to metalaxyl seed treatment with respect to their abilities to protect cotton seed from colonization by *P. ultimum*. Although no statistically significant differences were observed between individual Flu− mutants and the parental strains with respect to seed colonization by *P. ultimum*, a significant difference was observed when the pooled mean of the Flu− mutants was compared with the mean of the parental strain 3793 (28 and 12% colonization, respectively, Table 4). These data suggest that the fluorescence phenotype has a detectable effect on colonization of cotton seed by *P. ultimum*.

Significant differences were observed between six representative Flu− mutants and the parental strains with respect to seedling emergence, i.e., the parental strains were statistically similar to metalaxyl seed treatment, whereas the Flu− derivative strains were statistically similar to untreated controls (Table 5). These observations were confirmed through class comparisons showing a significant effect of the fluorescence phenotype on efficacy of bacterial strains for improving emergence of cotton seed.

Spermosphere colonization by *P. fluorescens* 3793 and Flu− derivative strains. No consistent differences were observed between spermosphere populations of *P. fluorescens* 3793 and the Flu− mutants described in Table 3 (Fig. 1). Whereas the spermosphere population sizes of some Flu− derivatives (3819, 3815) were less than that of 3793, population sizes of other strains (3824, 3830) were greater. In the cases of 3815 and 3830, spermosphere population sizes correlated to the relative growth rates of these strains in culture which were less and greater, respectively, than that of 3793 (Table 1). However, this correlation was not observed for 3819 and 3824.

TABLE 4. Influence of *Pseudomonas fluorescens* 3793 and Flu− derivatives on colonization of cotton seed by *Pythium ultimum*

Seed treatment	Seed colonization (%) Experiment 1	Experiment 2	Mean[x]
Untreated control	50	63	56 a
Metalaxyl	6	0	3 d
P. fluorescens strains			
3551	...	18	...
3793	12	12	12 cd
3810	25	18	22 bcd
3811	38	19	28 bc
3813	56	6	31 bc
3815	44	31	38 ab
3817	31	19	25 bc
3818	19	19	19 bcd
3819	31	38	35 b
3820	31	25	28 bc
3824	19	13	16 bcd
3825	19	25	22 bcd
3826	38	44	41 ab
3829	25	38	31 bcd
3830	31	38	35 b
3831	19	25	22 bcd

Analysis of variance

Source of variation	Degrees of freedom	Sums of squares	Observed F^y
Total	135	14.60	
Experiment	1	0.10	1.13
Treatment	16	4.20	3.01**
Control vs. 3793	1	1.39	15.90**
3793 vs. Flu− mutants	1	0.39	4.49*
3793 vs. Metalaxyl	1	0.15	1.77
Error	118	10.30	

[x] Mean values followed by a common letter do not differ significantly at $P = 0.05$ as determined by the Waller-Duncan test. Fresno soil with indigenous levels of *P. ultimum* at 156 and 144 propagules per gram of soil was used for experiments 1 and 2, respectively.
[y] Indicated F values are significant at $P = 0.01$ (**) or $P = 0.05$ (*).

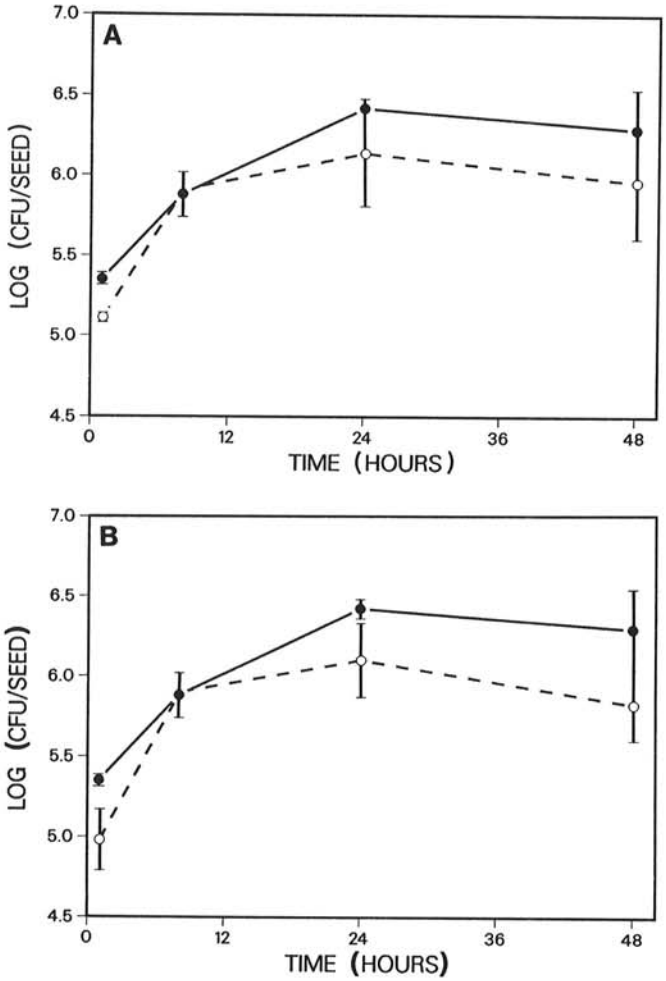
TABLE 5. Influence of *Pseudomonas fluorescens* 3551 and nonfluorescent derivative stains on emergence of cotton seed grown in Fresno field soil

Seed treatment	Percent emergence Experiment 1	Experiment 2	Mean[x]
Untreated control	35	10	23 de
Metalaxyl	80	85	83 a
P. fluorescens strains:			
3551	70	60	65 ab
3793	50	60	55 bc
3815	20	25	23 de
3819	40	30	35 cd
3826	20	35	28 de
3830	5	20	13 e
3824	15	20	18 de
3813	40	10	25 de

Analysis of variance

Source of variation	Degrees of freedom	Sums of squares	Observed F^y
Total	98	24.36	
Treatment	9	11.07	8.15**
Control vs. 3551	1	1.97	13.07**
3551 vs. 3793	1	0.09	0.57
3551 vs. Metalaxyl	1	0.49	3.27
3793 vs. Flu− mutants	1	1.89	12.54**
Experiment	1	0.01	0.01
Error	88	13.29	

[x] Values followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan test. Statistical analysis considered the arcsin of the square root of the values presented above.
[y] Indicated F values are significant at $P = 0.01$ (**).



DISCUSSION

The fluorescent siderophore production by *P. fluorescens* 3551 was implicated as a mechanism by which bacterial seed treatment increased seedling emergence of cotton. The role of the fluorescent siderophore in increasing seedling emergence was suggested by comparison of effects associated with producing strains to those of nonproducing (Flu−) derivative strains. In every case, Flu− derivative strains obtained through single-hit Tn*5* mutagenesis did not increase seedling emergence as did the parental Flu+ strains. In

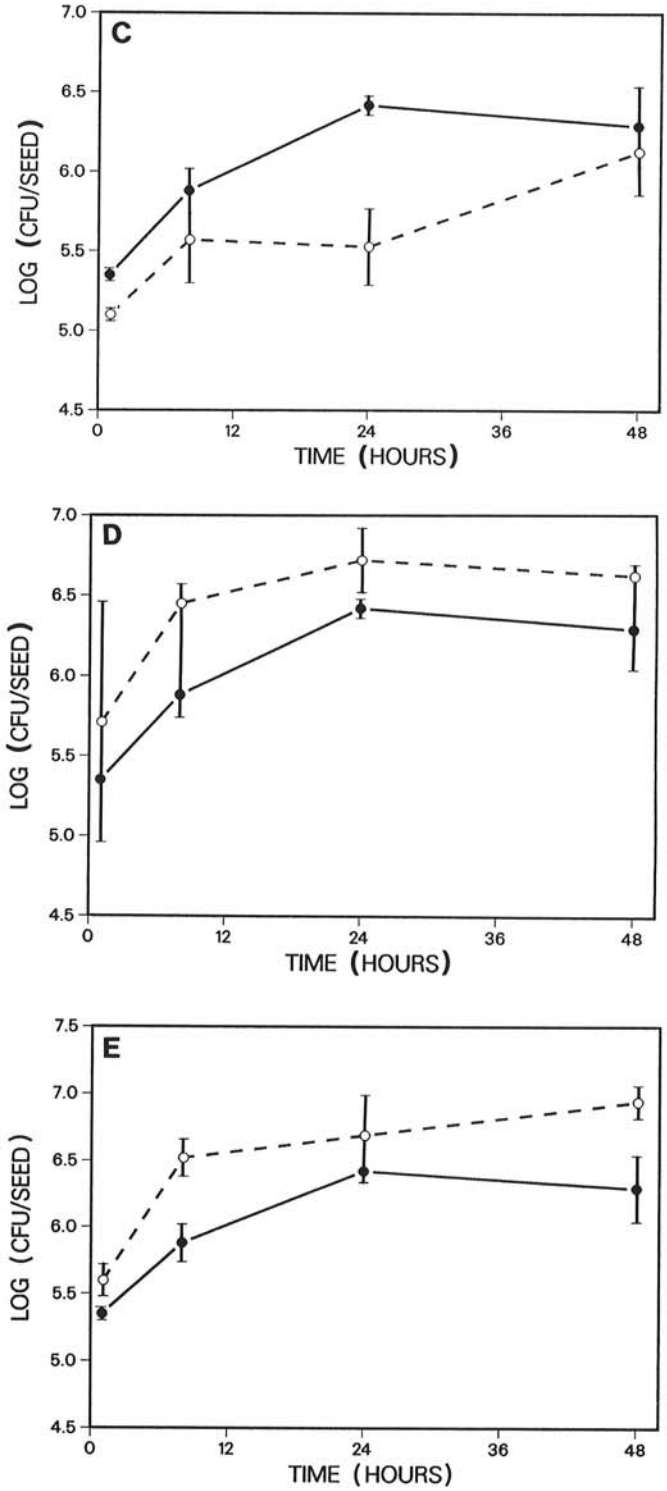


Fig. 1. Spermosphere populations ($\log_{10}$[cfu per seed]) of strain 3793 (•) and Flu− derivatives (o) determined over time from cotton seed in Fresno soil: **A**, strain 3826, **B**, strain 3819, **C**, strain 3815, **D**, strain 3824, and **E**, strain 3830.

some cases, the Tn*5* insertion inactivating fluorescent siderophore production was also associated with a quantitative change in spermosphere colonization. However, neither the direction nor the magnitude of this quantitative change were consistent among the Flu− derivative strains. Therefore, no singular role of fluorescent siderophore production in determining the spermosphere population size of *P. fluorescens* 3551 was suggested. The observed correlation between the Flu+ phenotype and the ability to increase seedling emergence could not be explained simply by a consistently high spermosphere population size. Rather, the effect of the Flu+ phenotype on seedling emergence appears to be independent of its potential effects on cotton seed colonization by *P. fluorescens* 3551.

Flu− mutants differed markedly from parental Flu+ strains in their influences on seedling emergence of cotton but differed less in their initial effects on cotton seed colonization by *P. ultimum*. While Flu− derivatives were not as effective as the Flu+ parent with respect to protecting seed from colonization by *P. ultimum*, they did reduce seed colonization relative to the untreated control. The concentration of fluorescent siderophore in the spermosphere 24 hr after bacterial seed treatment may not be sufficient to decrease seed colonization by *P. ultimum*. Continued in situ siderophore production subsequent to this 24-hr period appeared important in control of damping-off of cotton caused by *P. ultimum* since strain 3551 and its Flu− derivative strains differed with respect to their effects on seedling emergence. Other properties common to *P. fluorescens* 3551 and its Flu− derivative strains, such as the ability to grow competitively in the spermosphere of cotton or the production of other unidentified metabolites, may explain their common activities against initial colonization of cotton seed by *P. ultimum*. As primary colonizing sugar fungi (6), *Pythium* species are poor saprophytes on organic matter previously colonized by other microbes (1). Spermosphere colonization and nutrient utilization by *P. fluorescens* 3551 and its derivatives may contribute to the observed reduction in seed colonization by *P. ultimum*, independent of the involvement of fluorescent siderophores. Nutrient competition is a proposed mechanism for biological control of *Pythium* damping-off by rhizosphere bacteria (5).

The siderophore production phenotype of *P. fluorescens* 3551 influenced preemergence damping-off more than initial seed colonization by *P. ultimum*, presumably due to its effect on stages of disease development subsequent to saprophytic seed colonization. For example, strain 3551 may influence fungal infection or may associate with hyphae after fungal penetration of the cotton seed. In this study, hyphal growth of *P. ultimum* was inhibited in culture by iron-starvation imposed by EDDA. Presumably, iron starvation could also occur in the plant or on the seed surface. Alternatively, the fluorescent siderophore may trigger a host response which then decreases infection or slows disease progress. This interpretation suggests that more than one mechanism is operating in this *P. fluorescens-P. ultimum* biological control system. A fluorescent siderophore produced by strain 3551 contributes to, but does not completely determine, its biocontrol activity against *Pythium ultimum*.

At least several genes or groups of genes are involved in the production of fluorescent siderophores by the fluorescent pseudomonads (4,13,16). Some of these genes may also be involved in aspects of primary metabolism and in the production of other secondary metabolites. The genetic complexity of fluorescent siderophore production complicates the interpretation of studies that compare biocontrol efficacy of deficiency mutants to that of a wild type strain. This is particularly true when uncharacterized chemically induced mutants are compared to parental strains. Even in this report, where mutations were associated with a single transposon insertion, one cannot discount the possibility that the production of other unidentified metabolites was also affected. However, the probability of misinterpretation caused by pleiotrophic effects of the Tn*5* insertions was lessened by testing mutants with insertions located in five different *Eco*RI fragments of genomic DNA. It is unlikely that all five classes of mutants would be deficient in the same second unidentified metabolite. It is even less likely in this case, where tested mutant strains were not debilitated in growth in the cotton spermosphere. Nevertheless, the possibility exists that an unidentified metabolite, having biosynthetic steps common to fluorescent siderophore production, may be responsible for the effects attributed here to the fluorescent siderophore.

Single Tn*5* insertions in the genomic DNA of *P. fluorescens* strain 3551 inactivated this strain in biocontrol of preemergence damping off caused by *P. ultimum*. These insertions provide a physical marker that will facilitate the cloning of genes determining biocontrol activity and the subsequent identification of those gene products.

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