Lack of Evidence for a Role of Antifungal Metabolite Production by *Pseudomonas fluorescens* Pf-5 in Biological Control of Pythium Damping-Off of Cucumber

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ABSTRACT

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Pseudomonas fluorescens Pf-5 protected cucumber from preemergence damping-off caused by *Pythium ultimum* and produced a number of antifungal metabolites in culture, including a fluorescent siderophore (pyoverdine), pyoluteorin, pyrrolnitrin, cyanide, and an uncharacterized compound termed antibiotic 3. Of these, the roles of pyoluteorin and pyoverdine in the biocontrol activity of Pf-5 against Pythium damping-off of cucumber were investigated. Tn*5* insertion mutants of Pf-5 were screened for loss of pyoluteorin production (Plt^-) or pyoverdine production (Pvd^-). Thirteen Plt^- mutants, 13 Pvd^- mutants, and six antibiotic production (Apd^-) mutants, which were deficient in pyoluteorin, pyrrolnitrin, and antibiotic 3 production, were identified. Three Plt^{++} mutants, which produced greater quantities of pyoluteorin in culture than did Pf-5, were identified. The parental strain, Pf-5, inhibited hyphal growth of *P. ultimum* in culture, whereas the Plt^- and Apd^- mutants did not inhibit *P. ultimum* on certain growth media. In contrast, the Plt^{++} mutants produced inhibition zones against *P. ultimum* that were larger than those produced by Pf-5 on an agar medium. Nevertheless, the Plt^-, Pvd^-, Apd^-, and Plt^{++} mutants were similar to Pf-5 in suppression of Pythium damping-off of cucumber. These results are consistent with the suggestion that antifungal metabolite production by Pf-5 did not contribute substantially to its biocontrol activity against Pythium damping-off of cucumber.

Certain fluorescent pseudomonads, when applied to agricultural plants as seed inoculants, suppress plant diseases caused by soilborne fungi (4,8,38,39,46). Metabolites with antifungal activity, including antibiotics (9,10,43), cyanide (45), and fluorescent siderophores (24,28,38), are produced by *Pseudomonas* spp. that inhabit the rhizosphere. The importance of metabolite production to the biocontrol activities of these strains is supported by the following findings: 1) purified compounds inhibit fungal growth in culture and suppress disease when added to soil (19,21,22) or seed (12,13); 2) *Pseudomonas* spp. produce antifungal compounds in the rhizosphere (17,44) or spermosphere (14); and 3) mutants, deficient in metabolite production, suppress disease less effectively than do wild-type strains (14,17,27,42,45). Thus, certain antifungal metabolites produced by bacteria inhabiting the rhizosphere mediate significant disease control.

Strain Pf-5 of *P. fluorescens,* which was isolated from the rhizosphere of cotton (*Gossypium hirsutum* L.), suppresses damping-off diseases of cotton caused by *Rhizoctonia solani* Kühn (12) and *Pythium ultimum* Trow (13). Pf-5 is known to produce two antibiotics in culture, pyrrolnitrin and pyoluteorin, which inhibit hyphal growth of *R. solani* (12) and *P. ultimum* (13), respectively. Seed treatment with purified pyrrolnitrin suppresses damping-off of cotton caused by *R. solani* (12), whereas treatment with pyoluteorin suppresses damping-off caused by *P. ultimum* (13). The finding that the antibiotics, when applied to the seed surface at concentrations of 66 μg per seed, mimic the biocontrol activity of Pf-5 is suggestive of a role for these antibiotics in disease suppression. Nevertheless, the in situ production of antibiotics by strain Pf-5 in concentrations adequate to suppress disease in the spermosphere or rhizosphere of cotton has not been demonstrated.

Strain Pf-5 also produces a fluorescent pigment, termed a pyoverdine or pyoverdin, that is characteristic of fluorescent pseudomonads. The pyoverdines function as siderophores (30), defined as low molecular weight, virtually ferric-specific ligands that solubilize extracellular iron(III) and are transported by specific outer membrane receptors into the bacterial cell (32). Pyoverdines are thought to suppress certain soilborne diseases by sequestering iron that is required for growth of target pathogens in the spermosphere and rhizosphere of host plants (24,28,38). The involvement of iron competition in biological control has been inferred from experiments in which the addition of purified pyoverdines or synthetic iron chelators to soil suppresses disease (21,22,37). Additionally, mutants deficient in pyoverdine production are less suppressive than are parental strains (2,27). Although convincing evidence supports a role for pyoverdines in biological control of certain soilborne plant diseases, pyoverdines are not implicated universally in biocontrol activity of fluorescent pseudomonads (18,35,43).

The purpose of this study was to determine the role of pyoluteorin and pyoverdine production by Pf-5 in its suppression of Pythium damping-off of cucumber (*Cucumis sativus* L.). The ubiquitous oomycete, *P. ultimum,* is the causal agent of pre- and postemergence damping-off and root rot of many agronomic, vegetable, and nursery crops. Pyoverdine production is important in the biocontrol of Pythium root rot of wheat (*Triticum aestivum* L.) (2) and Pythium damping-off of cotton (27) by *P. fluorescens.* The production of antibiotics, such as oomycin A (10,14) or pyoluteorin (13), also is implicated in the biocontrol of Pythium damping-off diseases by *P. fluorescens.* We derived single-insertion transposon mutants of Pf-5 that were defective in the production of pyoluteorin or a pyoverdine and compared these mutants with parental strains with respect to suppression of preemergence damping-off of cucumber caused by *P. ultimum.*

MATERIALS AND METHODS

Media, organisms, and growth conditions. *P. fluorescens* Pf-5, which was isolated from the cotton rhizosphere, was received from C. Howell (USDA, Cotton Research Laboratory, College Station, TX) (12). Strain Pf-5 is resistant to streptomycin and ampicillin. Pf-5 and derivatives were cultured routinely at 27 C on King's Medium B (KMB) (20), on nutrient agar (Difco Laboratories, Detroit, MI) containing 1% (w/v) glycerol (NAGly), or in nutrient broth (Difco) containing 1% (w/v) glycerol (NBGly). Doubling times of Pf-5 and derivatives were determined by monitoring the absorbance at 600 nm of cultures grown while being shaken at 27 C in KMB broth. Replicate flasks were included in each experiment, and each experiment was repeated. Auxotrophy was evaluated on minimal medium 925 (23) containing 4% (w/v) glycerol. Antibiotics were extracted from bacteria grown for 5 days on the surface of medium 523 (16) or nutrient agar amended with 2% (w/v) glucose (NAGlc), or for 3 days in a modified KMB broth that contained reduced amounts of glycerol (5 g/L) and $K_2HPO_4 \cdot 3H_2O$ (1.5 g/L). *Escherichia coli* C600(pLG221) (3), which was obtained from G. Boulnois (University Medical Centre, Geneva, Switzerland), was grown at 37 C in Luria-Bertani medium (LB) (29). Antibiotics were used as specified at concentrations of 50, 50, and 100 mg/L for kanamycin (Km), streptomycin (Sm), and ampicillin (Ap), respectively.

Isolate N1 of *P. ultimum,* which was obtained from T. Paulitz (Macdonald College, McGill University, Quebec, Canada), produces sporangia but does not produce oospores in culture (25). Sporangia were produced by isolate N1 at 22 C on water agar composed of 2% (w/v) Bacto agar (Difco). The sporangia on the surface of a 3-wk-old culture were suspended in 10 ml of potato-dextrose broth (PDB) (Difco). Isolate J1 of *R. solani* was obtained from C. Howell (USDA, Cotton Research Laboratory, College Station, TX). Hyphal suspensions were produced by growing isolate J1 in PDB with shaking for 3 days at 27 C, then homogenizing the hyphal mat and spent broth in a sterile blender.

Isolation of antibiotics. Pyoluteorin (13) and pyrrolnitrin (12) were extracted from bacterial cells and spent agar media of bacterial cultures by a modification of published procedures. Bacteria were spread on the surface of 523 or NAGlc agar contained in a petri plate (15-cm diameter) and incubated at 27 C for 5 days. Cells on the agar surface of each petri plate were suspended in sterile deionized water and collected by centrifugation. Antibiotics were extracted from pelleted cells with 6 ml of 80% acetone. The spent agar (approximately 20 ml) was diced and extracted twice with an equal volume of acetone. The acetone was removed under vacuum in a Speed Vac (Savant, Farmingdale, NY), and the residual aqueous phase was extracted with an equal volume of chloroform. The chloroform phase was dried under vacuum, and the residue was dissolved in 50 μl of acetone. Silica gel (GHLF) thin-layer chromatography (TLC) plates (Analtech, Newark, DE) were spotted with 5-μl aliquots of the final acetone solution, developed with chloroform/acetone (9:1), and observed under UV light (254 nm). Pyoluteorin and pyrrolnitrin standards were observed as UV-absorbing spots with R_f values of 0.36 and 0.86, respectively.

Antagonism tests. Cells of *P. fluorescens* were inoculated in a 1-cm-diameter spot onto the surface of 523 or NAGlc medium. After 3 days of incubation at 27 C, cultures were exposed to chloroform vapors for 30 min to kill bacterial cells and then vented for 30 min. Molten agar containing sporangial or hyphal suspensions of *P. ultimum* or *R. solani* was poured over the agar surface. An overlay of *R. solani* consisted of 1 ml of mycelial suspension, prepared from isolate J1 as described above, and 5 ml of molten potato-dextrose agar (PDA; Difco). An overlay of *P. ultimum* consisted of 2 ml of sporangial suspension, prepared from isolate N1 as described above, and 7 ml of molten PDA amended with 0.5 mM $FeCl_3$. Hyphal growth of *P. ultimum* is inhibited by pyoverdines when the fungus is grown under iron-limiting conditions (27,35). The pyoverdine-mediated inhibition of *P. ultimum* in PDA overlays often masked inhibition by other antifungal compounds produced by Pf-5. Thus, overlays of *P. ultimum* in PDA were amended with 0.5 mM $FeCl_3$, which decreased the sensitivity of *P. ultimum* to the pyoverdine but not to other antifungal metabolites produced by Pf-5. Inhibition zone diameters were measured after incubating plates at 27 C for 2 days. Duplicate plates were evaluated in each experiment, and the experiment was done twice. Volatile inhibitors produced by *P. fluorescens* were detected in a split plate experiment. Bacteria were grown for 3 days on tryptic soy agar (TSA; Difco) in one side of a split plate before inoculation of the TSA in the other side with a 4-mm-diameter plug of a water agar culture of *P. ultimum.* Radial growth of *P. ultimum* on the fungal side was measured after 1 day of incubation at 27 C. Cyanide production was detected with a picric acid assay (5).

Transposon mutagenesis and screening. *E. coli* C600, containing the Tn*5* delivery plasmid pLG221 (3), was grown overnight while being shaken at 37 C in LB Km. *P. fluorescens* Pf-5 was grown overnight while being shaken at 27 C in NBGly. Equal volumes of donor *E. coli* and recipient *P. fluorescens* were washed twice in sterile water. Aqueous suspensions were combined 1:1 (v/v), spread on the surface of NAGly, and incubated at 37 C for 6 h. Cells on the agar surface then were suspended in 0.1 M $MgSO_4$ and diluted serially. Aliquots of the diluted suspensions were spread on media selective for donor, recipient, and putative Tn*5* mutant strains. Fluorescence of putative Tn*5* mutants was observed under UV light (366 nm) after 2 days of growth on KMB Km-Sm. Putative Tn*5* insertion mutants were screened for defects in pyoluteorin and pyrrolnitrin production by an antibiotic miniprep procedure. Each transconjugant was grown for 3 days while being shaken at 27 C in modified KMB Km-Sm. Cells were pelleted by centrifugation and the supernatant discarded. The cells were resuspended in the residual supernatant, transferred to microfuge tubes, vortexed for 30 s with 500 μl of ethyl acetate, and centrifuged. The ethyl acetate phase was collected and dried in a Speed Vac. The residue was dissolved in 10 μl of methanol and spotted on a silica gel (GHLF) TLC plate. Plates were developed and spots visualized as described above. Mutants that

appeared deficient in the production of pyoluteorin or pyrrolnitrin by the antibiotic miniprep procedure were evaluated again for antibiotic production on 523 agar medium, as described above.

Southern analysis of Tn*5* mutants. Total genomic DNA was isolated from saturated KMB broth cultures of strain Pf-5 and derivatives by the CTAB method (1). The DNA was digested with the restriction enzymes, *Eco*R1 or *Bam*H1 (Bethesda Research Laboratories, Gaithersburg, MD: BRL). Restriction fragments were separated by electrophoresis in 0.7% agarose gels in Tris-phosphate EDTA (29) and transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH). The 2.3-kb *Xho*1 fragment of Tn*5* that contains the kanamycin phosphotransferase gene was cloned into pBR322 (29) to form the plasmid pJEL1605, which served as a Tn*5* probe. pJEL1605 was nick-translated with biotinylated-TTP (BRL) and purified with a D50 column (International Biotechnologies Inc., New Haven, CT). Membranes were incubated with the biotinylated probe and washed with high stringency (0.16× SSC, 68 C), as suggested by the supplier. Hybridizations were visualized with the Blue Gene kit (BRL). Since Tn*5* has no restriction site recognized by *Eco*R1, a mutant was considered to contain a single insertion of Tn*5* if only one fragment of *Eco*R1-digested genomic DNA hybridized to the probe. Since Tn*5* has one restriction site recognized by *Bam*H1, a mutant strain with a single insertion of Tn*5* was expected to have two fragments of *Bam*H1-digested genomic DNA that hybridized to the probe.

Biocontrol tests. A 1:1 (v/v) mixture of Newberg fine sandy loam and river sand was used for all experiments. The soil, pH 6.0, contained 0.02% total nitrogen, 9 mg/kg of phosphorus, 82 mg/kg of potassium, and 87 meq/kg of calcium. Inoculum of *P. ultimum* for infestation of soil was prepared as described by Paulitz and Baker (34). Five mycelial mats from 5-day-old 50-ml PDB cultures of isolate N1 were added to 6 kg of twice-autoclaved soil-sand mix, which had been amended with 1% (w/w) ground oats. The soil inoculum was incubated and stirred occasionally under axenic conditions at room temperature for 3 wk. Air-dried inoculum was stored for up to 6 mo. Propagules of *P. ultimum* were enumerated from dry soil inoculum immediately before use by spreading aliquots of serial dilutions of soil inoculum onto the surface of Mircetich medium (31). Raw soil was infested with soil inoculum to obtain 100 propagules of isolate N1 of *P. ultimum* per gram of soil. Infested soil was placed in plastic cups (300 g per cup) and amended with a volume of deionized water to bring the final soil moisture to −0.01 MPa. The cups were covered with plastic wrap and incubated in a growth chamber at 21 C and 12-h daylength for 3 days to allow the soil moisture to equilibrate. Bacterial cells from KMB broth cultures were washed, suspended in 1.5% carboxy-methylcellulose (2,250–4,500 centipoises, Sigma Chemical Company, St. Louis, MO), and applied at 2×10^8 cells per cucumber seed (cv. Marketmore). As a fungicide standard for comparison with bacterial seed treatments, metalaxyl (Ciba-Geigy, Greensboro, NC) was applied as an aqueous drench at a rate of 0.2 mg/g of soil. Ten replicate cups, each with three seeds, were planted for each treatment. Seedling emergence was scored 10 days after planting. Rhizosphere population sizes of Pf-5 and derivatives, which were naturally resistant to streptomycin and ampicillin, were estimated from entire root systems of three replicate plants by dilution-plating of root washings on KMB Sm-Ap by published methods (27). Each experiment was done two or three times, as specified in Results.

Data analysis. Seedling emergence and bacterial rhizosphere population data for bacterial treatments were analyzed statistically by the general linear models and analysis of variance procedures of Statistical Analysis Systems (SAS Institute Inc., Cary, NC). To achieve homogeneity of variances, the emergence data from the control and metalaxyl treatments, which generally were more uniform than the bacterial treatments, were removed from the data set before analysis of the seedling emergence data. Interactions between treatment and experiment repetition were evaluated for each experimental design. Where no significant interactions were found, experiments with a common design were pooled for analysis. In these cases, repeated experiments were treated as blocks over time. Where a significant interaction between treatment and experiment repetition was observed, repeated experiments were analyzed and presented separately. The logarithmic (base ten) transformation was applied to individual estimations of rhizosphere bacterial population size before statistical analysis.

RESULTS

Antifungal compounds produced in culture by Pf-5. Pf-5 grown on 523 medium inhibited hyphal growth of *P. ultimum* and *R. solani* in overlays of PDA and in PDA amended with 0.5 mM $FeCl_3$. On 523 medium, Pf-5 produced concentrations of pyoluteorin and pyrrolnitrin that were detectable by TLC. Pf-5 grown on NAGlc inhibited hyphal growth of *P. ultimum* and *R. solani* in overlays of PDA, but it did not produce detectable levels of pyoluteorin or pyrrolnitrin. On NAGlc, Pf-5 produced a compound, termed antibiotic 3, that inhibited *P. ultimum* and *R. solani* and migrated on TLC plates with an R_f that differed from those of pyrrolnitrin or pyoluteorin. Antibiotic 3 was neither detected in cells or spent culture medium of Pf-5 grown on 523 medium nor in modified KMB broth. Cultures of Pf-5 grown on TSA or KMB produced cyanide and inhibited growth of *P. ultimum* or *R. solani* grown on TSA separated from that of Pf-5 in split plates.

Isolation of Tn*5* insertion mutants of Pf-5. Putative Tn*5* mutants were obtained at frequencies ranging from 3×10^{-7} to 5×10^{-5} following six independent matings between Pf-5 and *E. coli* C600 (pLG221). Of the 6,286 Km^r exconjugants examined, 54 (0.9%) were auxotrophs with at least 13 different nutritional requirements. Thirteen (0.2%) mutants did not fluoresce on KMB and were considered deficient in pyoverdine production (Pvd^-).

Antibiotics were extracted from modified KMB broth cultures of each of the 6,286 transconjugants and separated by TLC to identify mutants of Pf-5 that were defective in pyoluteorin or pyrrolnitrin production. Thirteen (0.2%) Plt^- mutants, which did not produce detectable pyoluteorin but produced wild-type levels of pyrrolnitrin, were identified. Three (0.05%) Plt^{++} mutants, which produced increased levels of pyoluteorin and wild-type levels of pyrrolnitrin, were identified. Six (0.1%) *a*ntibiotic *pro*duction (Apd^-) mutants, which did not produce detectable pyoluteorin, pyrrolnitrin, or antibiotic 3, were identified. No mutants were identified that were deficient solely in pyrrolnitrin production.

Characterization of Pvd^- mutants. The 13 Pvd^- derivatives were prototrophic, contained single Tn*5* insertions, and grew at rates similar to that of the parental strain in culture (Table 1). Southern analysis of *Bam*H1-digested genomic DNA of the Pvd^- mutants demonstrated that each of the Pvd^- mutants was unique in the site of the Tn*5* insertion. The Pvd^- mutants produced pyrrolnitrin and pyoluteorin in modified KMB broth and on 523 medium. On 523 medium, Pf-5 produced inhibition zones that averaged 48 or 36 mm in diameter against *P. ultimum* or *R. solani,* respectively. The Pvd^- mutants grown on 523 agar produced inhibition zones against *P. ultimum* and *R. solani,* respectively, that were similar in size to those produced by Pf-5 (Table 1). On NAGlc agar, Pf-5 and Pvd^- derivatives produced inhibition zones that averaged 25 mm in diameter against *P. ultimum* (Table 1). All Pvd^- derivatives produced cyanide when grown on TSA.

Characterization of Plt^- and Plt^{++} mutants. The 13 Plt^- mutant strains were prototrophic, produced a fluorescent pigment on KMB, produced pyrrolnitrin on 523, contained single Tn*5* insertions, and defined five Plt^- linkage groups based on the size of the *Eco*R1 fragment into which the Tn*5* inserted (Table 2). Southern analysis of *Bam*H1-digested DNA of the 13 Plt^- mutants demonstrated that 12 of the 13 mutants were unique in the site of the Tn*5* insertion. Two mutant strains, JL4134 and JL4139, had Tn*5* insertions in indistinguishable locations in the genome,

based on Southern analysis of *Bam*H1-digested genomic DNA. These strains were obtained from the same mutagenesis experiment and were probably siblings. Plt$^-$ mutants grown on 523 agar did not inhibit growth of *P. ultimum* in overlays of PDA containing 0.5 mM $FeCl_3$ (Table 2), but the mutants did inhibit growth in overlays without added $FeCl_3$. Thus, on 523 medium, pyoluteorin production by Pf-5 was responsible for antagonism of *P. ultimum* in overlays of PDA containing 0.5 mM $FeCl_3$. Zones of inhibition against *R. solani* surrounding Plt$^-$ mutants were equivalent in size to those surrounding Pf-5 (Table 2), presumably because the Plt$^-$ mutants produced wild-type levels of pyrrolnitrin, as detected by TLC. When grown on NAGlc agar, Pf-5 and Plt$^-$ mutants in the Plt-I, Plt-II, and Plt-IV linkage groups were similar with respect to inhibition of *P. ultimum* (Table 2). Plt$^-$ mutants grown on NAGlc also produced wild-type levels of antibiotic 3, as detected by TLC. Of the phenotypes evaluated, mutants in the Plt-I, Plt-II, and Plt-IV linkage groups differed from Pf-5 only in pyoluteorin production and antagonism of *P. ultimum* on 523 agar.

In contrast to the specific Plt$^-$ phenotype of mutants in the Plt-I, Plt-II, and Plt-IV linkage groups, mutants in the Plt-III and Plt-V groups were deficient in several characteristics. Doubling times of Plt-V mutants in KMB broth were approximately twice that of Pf-5 (Table 2). Mutants in both Plt-III and Plt-V grew so poorly on NAGlc that their inhibition of *P. ultimum* could not be evaluated on this medium. Plt-III and Plt-V mutants produced lower levels of cyanide on TSA medium than did Pf-5 (Table 2). The single Tn*5* insertions present in the genomic DNA of these mutants were associated with multiple phenotypic effects and were not specific to pyoluteorin production.

The three pyoluteorin overproducers (Plt^{++}) were prototrophic, produced a fluorescent pigment on KMB, produced pyrrolnitrin on 523, produced antibiotic 3 on NAGlc, contained single Tn*5*

TABLE 1. Pyoverdine production mutants of *Pseudomonas fluorescens* Pf-5

Strain	Size (kb) of Tn*5* containing fragments		Doubling time[x]	Inhibition of fungi on agar media[y]			Mean percent emergence[z]
				523		NAGlc	
	*Eco*R1	*Bam*H1		*Rs*	*Pu*	*Pu*	
Pf-5			100 ± 5	100 ± 3	100 ± 2	100 ± 2	70 ab
JL3975	10.5	24.2, 7.5	95	100	105	95	58 ab
JL3979	13.8	18.2, 10.8	108	84	103	101	62 ab
JL4268	13.8	18.0, 10.5	100	101	114	96	63 ab
JL4187	17.5	20.2, 11.9	96	91	112	104	61 ab
JL4177	18.5	16.8, 4.3	98	92	86	98	72 ab
JL4127	21.5	18.6, 16.5	111	94	105	105	70 ab
JL4182	21.5	22.2, 9.2	109	92	100	103	64 ab
JL4185	21.5	19.5, 15.0	108	96	115	105	62 ab
JL4142	24.5	14.5, 7.4	96	96	136	99	63 ab
JL4147	24.5	11.4, 4.7	100	90	122	105	73 a
JL4180	24.5	7.1, 7.1	90	89	120	105	57 b
JL4186	25.3	14.3, 14.3	103	103	86	105	69 ab
JL4126	25.3	26.5, 6.2	119	106	120	104	67 ab

[x]The doubling time of each mutant is expressed as the average percentage of the doubling time of Pf-5, determined as an internal control in each experiment, ± SEM. The average doubling time of Pf-5 in King's Medium B broth was 60 min.
[y]The inhibition zone diameter of each mutant is expressed as the average percentage of the zone diameter produced by Pf-5, measured as an internal control on each plate, ± SEM. *Rs*, inhibition zone diameters with *Rhizoctonia solani* added as an overlay in potato-dextrose agar (PDA); *Pu*, inhibition zone diameters with *Pythium ultimum* added as an overlay in PDA ± 0.5 mM $FeCl_3$.
[z]The mean of three experiments with cucumber seedlings is presented. Values followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan *k*-ratio *t* test. Mean percent emergence of the carboxy-methylcellulose and metalaxyl treatments was 10 and 90, respectively.

TABLE 2. Pyoluteorin production mutants of *Pseudomonas fluorescens* Pf-5

Tn*5* linkage group	Strain	Size (kb) of Tn*5* containing fragment		Doubling time[x]	Inhibition of fungi on agar media[y]			Cyanide production[z]
					523		NAGlc	
		*Eco*R1	*Bam*H1		*Rs*	*Pu*	*Pu*	
...	Pf-5			100 ± 5	100 ± 3	100 ± 2	100 ± 2	+
Plt-I	JL4296	8.4	19.6, 14.0	99	94	0	91	+
	JL4236	8.4	23.5, 8.6	96	105	0	106	+
Plt-II	JL4140	10.1	5.2, 4.6	98	103	0	121	+
	JL4138	10.1	5.8, 4.0	100	102	0	93	+
Plt-III	JL4171	12.7	9.4, 6.2	113	101	0	NG	+/−
Plt-IV	JL4175	18.3	22.5, 7.8	107	100	0	109	+
	JL4274	18.3	18.3, 12.1	112	105	0	101	+
	JL4128	18.3	17.0, 13.8	99	104	0	104	+
	JL4211	18.3	22.3, 9.9	102	92	0	111	+
Plt-V	JL4237	24.8	10.5, 7.8	205	91	0	NG	+/−
	JL4293	24.8	10.9, 7.2	189	97	0	NG	+/−
	JL4134	24.8	12.1, 7.2	162	93	0	NG	+/−
	JL4139	24.8	12.1, 7.2	172	102	0	NG	+/−

[x]The doubling time of each mutant is expressed as the average percentage of the doubling time of Pf-5, determined as an internal control in each experiment, ± SEM. The average doubling time of Pf-5 in King's Medium B broth was 60 min.
[y]The inhibition zone diameter of each mutant is expressed as the average percentage of the zone diameter produced by Pf-5, measured as an internal control on each plate, ± SEM. *Rs*, inhibition zone diameters with *Rhizoctonia solani* added as an overlay in potato-dextrose agar (PDA); *Pu*, inhibition zone diameters with *Pythium ultimum* added as an overlay in PDA ± 0.5 mM $FeCl_3$. NG = No growth of *P. fluorescens*.
[z]+/− = These strains produced less cyanide than did Pf-5.

insertions, and defined two linkage groups, based on the size of the *Eco*R1 fragment into which the Tn*5* inserted (Table 3). Two mutant strains, JL4239 and JL4292, had Tn*5* insertions in indistinguishable locations in the genome, based on Southern analysis of *Bam*H1-digested genomic DNA. These strains were obtained from different mutagenesis experiments, however, and were not siblings. The Plt^{++} mutants grown on 523 agar produced zones of inhibition against *P. ultimum* that were two to three times larger than those produced by Pf-5 (Table 3). This result is consistent with the conclusion that pyoluteorin production was responsible for antagonism of *P. ultimum* by Pf-5 on 523 medium.

Characterization of Apd^- mutants. The Apd^- mutants were prototrophic, produced a fluorescent pigment on KMB, contained single Tn*5* insertions, and defined two Apd^- linkage groups, based on the size of the *Eco*R1 fragment into which the Tn*5* inserted (Table 3).

The Apd-I mutants formed colonies that were larger and lighter in color than those of Pf-5. Apd-I mutants grown on 523 or NAGlc agar did not inhibit growth of *P. ultimum* or *R. solani* in overlays of PDA (Table 3). The Apd-I mutants produced no detectable pyoluteorin, pyrrolnitrin, antibiotic 3, or cyanide.

The Apd-II mutant JL4170 secreted a brown pigment in broth and agar cultures and inhibited the growth of *P. ultimum* and *R. solani* (Table 3). JL4170 produced no detectable pyoluteorin, pyrrolnitrin, or antibiotic 3 on any medium, but it produced cyanide on TSA. Thus, inhibition of fungi by JL4170 grown on 523 or NAGlc was not attributed to its production of pyoverdine or the three antibiotics. The antifungal activity of the brown

TABLE 3. Pyoluteorin overproduction and antibiotic production mutants of *Pseudomonas fluorescens* Pf-5

Phenotype	Strain	Size (kb) of Tn*5* containing fragment		Doubling time (min)[w]	Inhibition of fungi on agar media[x]			Cyanide production	Mean percent emergence[y]
					523		NAGlc		
		*Eco*R1	*Bam*H1		*Rs*	*Pu*	*Pu*		
Wild type	Pf-5			100 ± 5	100 ± 3	100 ± 2	100 ± 2	+	82 ab
Plt^{++}	JL4297	10.2	6.3, 3.7	90	98	197	95	+	83 ab
	JL4239	11.4	21.0, 8.9	112	103	301	130	+	72 bc
	JL4292	11.4	21.0, 8.9	129	107	235	113	+	83 ab
Apd-I	JL4106	15.5	13.0, 6.0	143	0	0	0	−	ND[z]
	JL4210	15.5	13.4, 5.7	110	0	0	0	−	87 ab
	JL4097	15.5	14.0, 5.1	101	0	0	0	−	90 a
	JL4209	15.5	14.5, 4.3	94	0	0	0	−	83 ab
	JL4135	15.5	ND	160	0	0	0	−	63 c
Apd-II	JL4170	27.0	8.5, 6.5	94	65	192	86	+	73 bc

[w]The doubling time of each mutant is expressed as the average percentage of the doubling time of Pf-5, determined as an internal control in each experiment, ± SEM. The average doubling time of Pf-5 in King's Medium B broth was 60 min.

[x]The inhibition zone diameter of each mutant is expressed as the average percentage of the zone diameter produced by Pf-5, measured as an internal control on each plate, ± SEM. *Rs*, inhibition zone diameters with *Rhizoctonia solani* added as an overlay in potato-dextrose agar (PDA); *Pu*, inhibition zone diameters with *Pythium ultimum* added as an overlay in PDA ± 0.5 mM $FeCl_3$.

[y]The mean of two experiments is presented. Values followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan *k*-ratio *t* test. Mean percent emergence of the carboxy-methylcellulose and metalaxyl treatments was 30 and 97, respectively.

[z]Not determined.

TABLE 4. Biocontrol of Pythium damping off of cucumber by *Pseudomonas fluorescens* Pf-5 and Plt^- derivatives

Treatment		Percent emergence[z] of cucumber seedlings			
		Experiment			
Tn*5* linkage group	Strain	1	2	3	Mean
...	Pf-5	80 a	57 a–d	87 a	74
Plt-I	JL4296	70 a	53 a–d	77 ab	67
	JL4236	77 a	43 b–d	73 ab	65
Plt-II	JL4140	63 ab	74 a	87 a	75
	JL4138	57 ab	70 ab	83 a	70
Plt-III	JL4171	73 a	43 b–d	70 ab	62
Plt-IV	JL4175	70 a	63 a–c	73 ab	69
	JL4274	60 ab	60 a–c	57 b	59
	JL4128	60 ab	53 a–d	83 a	66
	JL4211	70 a	40 cd	80 ab	63
Plt-V	JL4237	73 a	30 d	63 ab	56
	JL4293	40 b	40 cd	73 ab	51
	JL4134	37 b	50 a–d	73 ab	53
	Metalaxyl	93	97	100	97
	None	10	0	20	10

Analysis of variance

Source of variation	Degrees of freedom	Experiment 1			Experiment 2			Experiment 3		
		Sums of squares	*F*	*P*	Sums of squares	*F*	*P*	Sums of squares	*F*	*P*
Total	129	10.75			10.34			7.26		
Treatment	12	2.14	2.42	0.008	1.94	2.25	0.013	0.95	1.47	0.147
Plt-I, -II, -IV vs. Pf-5	1	0.18	2.43	0.121	0.00	0.00	0.946	0.09	1.66	0.200
Plt-III, -V vs. Pf-5	1	0.47	6.39	0.013	0.20	2.75	0.100	0.22	4.14	0.044
Error	117	8.61			8.4			6.31		

[z]Values followed by a common letter do not differ significantly ($P = 0.05$) as determined by the Waller-Duncan *k*-ratio *t* test.

pigment produced by JL4170 was not determined.

Biocontrol of Pythium damping-off of cucumber. Treatment of seeds with Pf-5 increased emergence of cucumber seedlings by an average of 60% from soil infested with *P. ultimum*. The 13 Pvd$^-$ mutants tested were as effective as Pf-5 in increasing cucumber seedling emergence (Table 1). No significant difference ($P = 0.305$) was observed between the pooled mean of emergence values of the Pvd$^-$ mutant treatments and the mean of the Pf-5 treatment (65 and 70% emergence, respectively). The mean rhizosphere population sizes of the Pvd$^-$ mutant strains, which ranged from 6.0 to 6.7 log(cfu/root system), did not differ significantly ($P < 0.05$) from that of Pf-5, which was 6.1 log(cfu/root system).

Mutants that were deficient in pyoluteorin production also did not differ consistently from Pf-5 in biocontrol activity (Table 4). Whereas certain mutants in the Plt-I, Plt-II, and Plt-IV groups differed from Pf-5 in individual tests, these differences were not observed in repeated experiments. For example, whereas strain JL4274 of the Plt-IV group was less effective than Pf-5 in the third experiment, it was similar to Pf-5 in the first and second experiments (Table 4). No significant differences were observed between the pooled means of the emergence values of the Plt-I, Plt-II, and Plt-IV treatments and the mean of the Pf-5 treatment (Table 4). In contrast, the pooled means of the emergence values of the Plt-III and Plt-V treatments differed significantly from that of the Pf-5 treatment in two of the three experiments. The mean rhizosphere population sizes of the Plt$^-$ mutant strains, which ranged from 6.2 to 7.0 log(cfu/root system), did not differ significantly from those established by Pf-5, which ranged from 6.1 to 7.0 log(cfu/root system) among experiments. Thus, the reduced biocontrol activities of Plt-III and Plt-V mutants could not be explained by impaired ability of these strains to establish rhizosphere populations. Nevertheless, because of the pleiotropic natures of the Plt-III and Plt-V mutants, reduction in biocontrol activity could not be attributed to the Plt$^-$ phenotype.

The three Plt^{++} mutants did not differ significantly from Pf-5 with respect to biocontrol of Pythium damping-off of cucumber (Table 3). No significant differences ($P = 0.715$) were observed between the pooled means of the emergence values of the Plt^{++} treatments and the mean of the Pf-5 treatment. The mean rhizosphere population sizes of the Plt^{++} mutant strains on surviving plants, which ranged from 5.8 to 6.3 log(cfu/root system), did not differ significantly from that of Pf-5, which was 6.5 log(cfu/root system).

Five of six Apd$^-$ mutants were as effective as Pf-5 in suppression of Pythium damping-off of cucumber (Table 3). Strain JL4135 was less effective than Pf-5 in biocontrol of *P. ultimum* (Table 3). No significant differences ($P = 0.686$) were observed between the pooled means of the emergence values of the Apd$^-$ treatments and the mean of the Pf-5 treatment. Three of the Apd$^-$ mutants established rhizosphere population sizes on surviving plants that were not significantly different from that of Pf-5. Strains JL4209 and JL4135, however, established mean rhizosphere population sizes of 5.4 and 5.5 log(cfu/root system), respectively, which differed significantly from that of Pf-5, which was 6.5 log(cfu/root system).

DISCUSSION

The major conclusion of this study is that two antifungal metabolites produced by Pf-5, pyoluteorin and a pyoverdine, were not required for biological control of Pythium damping-off of cucumber. Pyoverdine production by *P. fluorescens* Pf-5 was not required for biological control of Pythium damping-off of cucumber because biocontrol activities of Pf-5 and Pvd$^-$ derivatives were indistinguishable. All 13 Pvd$^-$ mutant strains improved cucumber seedling emergence from soils infested with *P. ultimum* as well as the Pvd$^+$ parental strain did. Several lines of evidence support the conclusion that pyoluteorin production played, at most, a minor role in the biocontrol of *P. ultimum* by Pf-5. Mutants derived from strain Pf-5 that were deficient specifically in pyoluteorin biosynthesis (Plt-I, Plt-II, and Plt-IV) were similar to the wild-type strain in suppression of Pythium damping-off of cucumber. Mutants that overproduced pyoluteorin in culture did not suppress disease to a greater extent than did the wild-type strain. Finally, mutants that produced no detectable antibiotics or hydrogen cyanide in culture (Apd$^-$) were similar to the wild-type strain with respect to disease suppression. Thus, while pyoluteorin inhibits *P. ultimum* in culture and on seed surfaces (13) and was responsible for antagonism of *P. ultimum* by Pf-5 on an agar medium (Table 2), pyoluteorin was not responsible for suppression by Pf-5 of preemergence damping-off of cucumber caused by *P. ultimum*. We suggest from these results that mechanisms other than pyoverdine or pyoluteorin production were responsible for the suppression of Pythium damping-off of cucumber under the conditions tested. The mechanism(s) by which Pf-5 suppressed disease may have been physical exclusion, or competition for nutrients required for germination of sporangia or subsequent mycelial growth, rather than antifungal metabolite production.

Mutants in Plt-I, Plt-II, and Plt-IV linkage groups may have Tn*5* insertions in genes involved in the pyoluteorin biosynthetic pathway, since these mutants were indistinguishable from Pf-5 in phenotypes other than pyoluteorin production. In contrast, Plt-III and Plt-V mutants were distinguished from Pf-5 by extremely poor growth on media containing glucose and reduced production of cyanide, in addition to the Plt$^-$ phenotype. We speculate that the Plt-III and Plt-V mutants have defects in glucose metabolism that also influence pyoluteorin production. A linkage of glucose metabolism and antifungal metabolite production has been observed in other *Pseudomonas* spp.; oomycin A production by *P. fluorescens* Hv37a, a biocontrol agent of Pythium damping-off of cotton, is regulated by the glucose concentration of the growth medium (15). Mutations in the *afu*A and *afu*B loci of Hv37a are deficient in glucose dehydrogenase and in the production of the antifungal compound, oomycin A (11). Whereas the *afu*A and *afu*B loci of strain Hv37a are known to be required for the glucose induction of oomycin A production (11), potential regulatory effects of the Plt-III and Plt-V mutations on pyoluteorin production of strain Pf-5 have not been investigated.

Pyoluteorin overproduction mutants produced inhibition zones against *P. ultimum* that were two to three times larger than those produced by Pf-5. Nevertheless, these mutants were no more effective than strain Pf-5 in suppression of Pythium damping-off of cucumber. Although these mutants produced more pyoluteorin than did Pf-5 in culture, they may not overproduce pyoluteorin in the spermosphere of cucumber. Certain derivatives of other biocontrol agents that overproduce key metabolites also do not exhibit enhanced biocontrol activity. For example, a derivative of *Agrobacterium radiobacter* K84 that overproduces agrocin 84 in culture is no more effective than strain K84 in biocontrol of crown gall disease (40). Thus, the overproduction of key metabolites by biocontrol agents grown in culture may not always correlate with enhanced in situ activity of a biological control agent.

Antibiotic production (Apd$^-$) mutants produced no detectable pyoluteorin, pyrrolnitrin, antibiotic 3, or hydrogen cyanide in culture. Southern analysis confirmed the presence of a single Tn*5* insertion in each Apd$^-$ mutant. One explanation of the pleiotropic nature of the Apd$^-$ phenotype is that a single genetic lesion resulted in the loss of an enzyme common to the biosynthetic pathways of multiple antifungal metabolites. The diversity of primary precursors of the metabolic pathways influenced by the Apd$^-$ phenotype, however, argues against this possibility. Proline or a related molecule is the primary precursor of the pyoluteorin-biosynthetic pathway, which proceeds by the serial addition of acetyl groups, followed by unsaturation and chlorination (7). Tryptophan is the primary precursor of pyrrolnitrin biosynthesis in *Pseudomonas* spp.; tryptophan is rearranged to form an aminophenyl pyrrole (6), which is subsequently chlorinated (6,36). The successive chlorination steps are thought to be mediated by

discrete enzymes with specific substrate requirements (36). Hydrogen cyanide is released by the decarboxylation of glycine in *Pseudomonas* spp. (47). Therefore, the distinct pathways for pyoluteorin, pyrrolnitrin, and hydrogen cyanide biosynthesis preclude the possibility that the Apd^- strains have a mutation in a gene encoding an enzyme that is common to the biosynthetic pathways of these three antifungal compounds. If the genes encoding biosynthetic enzymes of these pathways are closely linked, the pleiotropic Apd^- phenotype may be explained by polar effects of a Tn*5* insertion on downstream genes involved in antifungal metabolite biosynthesis. No linkage of genes of these biosynthetic pathways was detected in this study. Therefore, we speculate that the *apd* loci are involved in an aspect of primary metabolism or regulation of the multiple antifungal metabolite biosynthetic pathways of Pf-5.

Similar biocontrol activities of Pvd^+ and Pvd^- strains were observed here (Table 1) and in a recent study in which pyoverdine production contributed little to the biocontrol activity of *P. putida* N1R against Pythium damping-off of cucumber (35). The results of both studies differ, however, from an earlier study that demonstrated an important role for pyoverdine production in the biocontrol activity of *P. fluorescens* 3551 against Pythium damping-off of cotton (27). The two cucumber studies were done in soils with lower pH and higher iron content than that of the cotton study. Thus, microbial competition for iron may have differed among the studies. In addition, the plant hosts of these studies differ in the rapidity of seedling emergence, which was 3–5 days for cucumber (34) and 7–14 days for cotton (27). Thus, the opportunity for pyoverdine-mediated iron competition between *Pseudomonas* spp. and *P. ultimum* may be greater on the more slowly emerging cotton than on cucumber seed. Finally, different composition and quantities of seed exudates may differentially influence expression of pyoverdine biosynthesis genes of *Pseudomonas* spp. inhabiting the spermosphere of cotton versus cucumber. Controlled studies clearly are needed to resolve the contribution of pyoverdine production of *Pseudomonas* spp. to suppression of Pythium damping-off diseases of different plant hosts.

For a metabolite to protect a seed from infection by *P. ultimum,* it must be present in concentrations adequate for fungal inhibition before seed infection. *P. ultimum* persists in agricultural soils as sporangia or oospores (41), which germinate rapidly in response to seed exudates, and colonizes pea seed coats (26) or sugarbeet pericarps (33) within 10 h of seed imbibition. In contrast to the rapid colonization of seeds by *P. ultimum,* the in situ production of antifungal metabolites by *Pseudomonas* spp. inhabiting the spermosphere may be slow. For example, production of the antifungal compound, oomycin A, by strain Hv37a of *P. fluorescens* inhabiting the cotton spermosphere is not expressed fully until 12 h after seed imbibition (10). Oomycin A production is expressed more rapidly on seed surfaces by derivatives of Hv37a that produce oomycin A constitutively than by the wild-type strain. Constitutive producers are more effective than Hv37a in suppression of Pythium damping-off of cotton, presumably because the rapid accumulation of oomycin A in the spermosphere reduces colonization of cotton seeds by *P. ultimum* immediately after imbibition (10). Similarly, the concentrations of pyoluteorin and pyoverdine that are adequate for inhibition of *P. ultimum* may not be produced by Pf-5 before fungal infection of cucumber seeds.

No evidence for the role of antifungal metabolite production in the biocontrol of Pythium damping-off of cucumber was obtained by the mutagenesis approach employed in this study. Nevertheless, antifungal metabolite production may contribute to biocontrol activity of Pf-5 on other host plants, such as cotton, or to supression of postemergence damping off or root rot caused by *P. ultimum.* The genes for pyoluteorin biosynthesis that were identified here will be useful in future studies evaluating gene expression of Pf-5 in the spermosphere of various host plants, and in construction of derivative strains of Pf-5 that constitutively produce pyoluteorin.

LITERATURE CITED

1. Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley & Sons, New York.
2. Becker, J. O., and Cook, R. J. 1988. Role of siderophores in suppression of *Pythium* species and production of increased-growth response of wheat by fluorescent pseudomonads. Phytopathology 78:778-782.
3. Boulnois, G. J., Varley, J. M., Sharpe, G. S., and Franklin, F. C. H. 1985. Transposon donor plasmids, based on ColIb-P9, for use in *Pseudomonas putida* and a variety of other Gram negative bacteria. Mol. Gen. Genet. 200:65-67.
4. Burr, T. J., and Caesar, A. 1984. Beneficial plant bacteria. Crit. Rev. Plant Sci. 2:1-20.
5. Castric, P. A. 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa.* Can. J. Microbiol. 21:613-618.
6. Chang, C. J., Floss, H. G., Hook, D. J., Mabe, J. A., Manni, P. E., Martin, L. L., Schröder, K., and Shieh, T. L. 1981. The biosynthesis of the antibiotic pyrrolnitrin by *Pseudomonas aureofaciens.* J. Antibiot. 34:555-566.
7. Cuppels, D. A., Howell, C. R., Stipanovic, R. D., Stoessl, A., and Stothers, J. B. 1986. Biosynthesis of pyoluteorin: A mixed polyketide-tricarboxylic acid cycle origin demonstrated by [1,2-$^{13}C_2$]acetate incorporation. Z. Naturforsch. 41c:532-536.
8. Davison, J. 1988. Plant beneficial bacteria. Bio/Technology 6:282-286.
9. Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. 26:75-91.
10. Gutterson, N. 1990. Microbial fungicides: Recent approaches to elucidating mechanisms. Crit. Rev. Biotechnol. 10:69-91.
11. Gutterson, N., Ziegle, J. S., Warren, G. J., and Layton, T. J. 1988. Genetic determinants for catabolite induction of antibiotic biosynthesis in *Pseudomonas fluorescens* Hv37a. J. Bacteriol. 170:380-385.
12. Howell, C. R., and Stipanovic, R. D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.
13. Howell, C. R., and Stipanovic, R. D. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70:712-715.
14. Howie, W. J., and Suslow, T. V. 1991. Role of antibiotic biosynthesis in the inhibition of *Pythium ultimum* in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescens.* Mol. Plant-Microbe Interact. 4:393-399.
15. James, D. W., and Gutterson, N. I. 1986. Multiple antibiotics produced by *Pseudomonas fluorescens* Hv37a and their differential regulation by glucose. Appl. Environ. Microbiol. 52:1183-1189.
16. Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas.* Phytopathology 60:969-976.
17. Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., and Défago, G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Interact. 5:4-13.
18. Keel, C., Voisard, C., Berling, C. H., Kahr, G., and Défago, G. 1989. Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. Phytopathology 79:584-589.
19. Keel, C., Wirthner, P., Oberhänsli, T., Voisard, C., Burger, U., Haas, D., and Défago, G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: Role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis 9:327-341.
20. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
21. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886.
22. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. *Pseudomonas* siderophores: A mechanism explaining disease-suppressive soils. Curr. Microbiol. 4:317-320.
23. Langley, R. A., and Kado, C. I. 1972. Studies on *Agrobacterium tumefaciens.* Conditions for mutagenesis by n-methyl-n′-nitro-n-

nitrosoguanidine and relationships of *A. tumefaciens* mutants to crown gall induction. Mutat. Res. 14:277-286.
24. Leong, J. 1986. Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. Annu. Rev. Phytopathol. 24:187-209.
25. Lifshitz, R., Sneh, B., and Baker, R. 1984. Soil suppressiveness to a plant pathogenic *Pythium* species. Phytopathology 74:1054-1061.
26. Lifshitz, R., Windham, M. T., and Baker, R. 1986. Mechanism of biological control of preemergence damping-off of pea by seed treatment with *Trichoderma* spp. Phytopathology 76:720-725.
27. Loper, J. E. 1988. Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Phytopathology 78:166-172.
28. Loper, J. E., and Buyer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Interact. 4:5-13.
29. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. Meyer, J. M., and Abdallah, M. A. 1978. The fluorescent pigment of *Pseudomonas fluorescens:* Biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. 107:319-328.
31. Mircetich, S. M., and Kraft, J. M. 1973. Efficiency of various selective media in determining *Pythium* populations in soil. Mycopathol. Mycol. Appl. 50:151-161.
32. Neilands, J. B. 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. 1:27-46.
33. Osburn, R. M., Schroth, M. N., Hancock, J. G., and Hendson, M. 1989. Dynamics of sugar beet seed colonization by *Pythium ultimum* and *Pseudomonas* species: Effects on seed rot and damping-off. Phytopathology 79:709-716.
34. Paulitz, T. C., and Baker, R. 1987. Biological control of Pythium damping-off of cucumbers with *Pythium nunn:* Population dynamics and disease suppression. Phytopathology 77:335-340.
35. Paulitz, T. C., and Loper, J. E. 1991. Lack of a role for fluorescent siderophore production in the biological control of Pythium damping-off of cucumber by a strain of *Pseudomonas putida.* Phytopathology 81:930-935.
36. Roitman, J. N., Mahoney, N. E., and Janisiewicz, W. J. 1990. Production and composition of phenylpyrrole metabolites produced by *Pseudomonas cepacia.* Appl. Microbiol. Biotechnol. 34:381-386.
37. Scher, F. M., and Baker, R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. Phytopathology 72:1567-1573.
38. Schippers, B., Bakker, A. W., and Bakker, P. A. H. M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25:339-358.
39. Schroth, M. N., and Hancock, J. G. 1982. Disease-suppressive soil and root-colonizing bacteria. Science 216:1376-1381.
40. Shim, J.-S., Farrand, S. K., and Kerr, A. 1987. Biological control of crown gall: Construction and testing of new biocontrol agents. Phytopathology 77:463-466.
41. Stanghellini, M. E., and Hancock, J. G. 1971. The sporangium of *Pythium ultimum* as a survival structure in soil. Phytopathology 61:157-164.
42. Thomashow, L. S., and Weller, D. M. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici.* J. Bacteriol. 170:3499-3508.
43. Thomashow, L. S., and Weller, D. M. 1990. Application of fluorescent pseudomonads to control root diseases of wheat and some mechanisms of disease suppression. Pages 109-122 in: Biological Control of Soilborne Plant Pathogens. D. Hornby, ed. CAB International, Wallingford, UK.
44. Thomashow, L. S., Weller, D. M., Bonsall, R. F., and Pierson, L. S. III. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56:908-912.
45. Voisard, C., Keel, C., Haas, D. and Défago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8:351-358.
46. Weller, D. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.
47. Wissing, F. 1974. Cyanide formation from oxidation of glycine by a *Pseudomonas* species. J. Bacteriol. 117:1289-1294.