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# Mutations Affecting Hyphal Colonization and Pyoverdine Production in Pseudomonads Antagonistic toward *Phytophthora parasitica*

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**In previous studies, *Pseudomonas putida* 06909 and *Pseudomonas fluorescens* 09906 suppressed populations of *Phytophthora parasitica* in the citrus rhizosphere, suggesting that these bacteria may be useful in biological control of citrus root rot. In this study we investigated the mechanisms of antagonism between the bacteria and the fungus. Both bacteria colonized *Phytophthora* hyphae and inhibited the fungus on agar media. A hyphal column assay was developed to measure the colonization of bacteria on fungal hyphae and to enrich for colonization-deficient mutants. In this way we identified Tn*5* mutants of each pseudomonad that were not able to colonize the hyphae and inhibit fungal growth in vitro. Colonization-deficient mutants were nonmotile and lacked flagella. Survival of nonmotile mutants in a citrus soil was similar to survival of a random Tn*5* mutant over a 52-day period. Additional screening of random Tn*5* mutants of both pseudomonads for loss of fungal inhibition in vitro yielded two distinct types of mutants. Mutants of the first type were deficient in production of pyoverdines and in inhibition of the fungus in vitro, although they still colonized fungal hyphae. Mutants of the second type lacked flagella and were not able to colonize the hyphae or inhibit fungal growth. No role was found for antibiotic production by the two bacteria in the inhibition of the fungus. Our results suggest that both hyphal colonization and pyoverdine production are important in the inhibition of *Phytophthora parasitica* by *P. fluorescens* and *P. putida* in vitro.**

Fluorescent pseudomonads have received much attention in recent years as potential biological control agents for suppression of root pathogens. Bacterial colonization of the rhizosphere, substrate competition, niche exclusion, and the production of secondary metabolites, including antibiotics and siderophores, may contribute to control of soilborne pathogens by these bacteria (15, 48). We are investigating factors influencing biological control by fluorescent pseudomonads that have shown some effectiveness in the control of citrus root rot caused by *Phytophthora parasitica* (45). Previous results suggested that copper resistance was important for competitive survival of these bacteria in citrus grove soils, since copper-sensitive transposon mutants of *Pseudomonas fluorescens* survived well in sterile soil but not in nonsterile soil (50). In this study, transposon mutagenesis was used to isolate mutants of *P. fluorescens* and *Pseudomonas putida* defective in antagonism toward *Phytophthora parasitica*.

Competition for iron by the production of siderophores has been extensively studied as a mechanism of biological control and was considered in this study, since iron-limiting conditions can occur in the alkaline soils of southern California citrus groves. Siderophores are iron(III) transport molecules which are excreted in large amounts in culture media by many microbes under iron-limiting conditions (8, 31). The production of siderophores and efficient transport of iron by fluorescent pseudomonads may contribute to the antagonism of these organisms toward root pathogens by depriving the pathogens of iron and suppressing spore germination, hyphal growth, and pathogenicity (5, 9, 11, 21, 23–26, 38, 51). However, not all data have supported the hypothesis that siderophore production plays a role in biological control of fungal pathogens (1, 14, 34, 49). In this paper we describe the isolation and preliminary analysis of mutants defective in pyoverdine production in the pseudomonads that we are investigating with citrus.

Antagonistic bacteria are thought to protect against root pathogens more effectively if they have a strong ability to colonize the root system (43, 48). Various factors, including soil matric potential, soil temperature, chemotaxis, bacterial motility, cell attachment, and plant genotypes, have been discussed in relation to root colonization (2, 10, 16, 39, 46). In addition to the colonization of plant roots, the colonization of fungal hyphae could be an important mechanism for maintaining a close association between antagonistic bacteria and fungal pathogens. Although there have been a few reports of hyphosphere (4, 32) or mycosphere (13) interactions between bacteria and fungal pathogens, little is known about the importance or mechanisms of bacterial colonization of fungal hyphae in biological control. The first bacterium that we found to be effective in biological control of citrus root rot was isolated with *Phytophthora parasitica* from citrus rhizosphere soil. This bacterium, *P. putida* 06909, grew extensively on *Phytophthora* hyphae and inhibited, but did not kill, the fungus in vitro. Subsequently, other fluorescent pseudomonads which were effective in biological control of citrus root rot were isolated from citrus rhizosphere soil by baiting with *Phytophthora* hyphae to recover hypha-colonizing strains (45). In this study, mutants defective for hyphal colonization were isolated by transposon mutagenesis and enrichment through hyphal columns. Random Tn*5* mutants were also screened for any other defect that reduced in vitro antagonism against *Phytophthora parasitica*.

## MATERIALS AND METHODS

**Microorganisms and cultural conditions.** *P. putida* 06909 was first detected as a contaminant in a culture of *Phytophthora parasitica* that was isolated from citrus rhizosphere soil, and

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the bacterium was subsequently purified from the fungal culture. *P. fluorescens* 09906 was isolated by baiting citrus rhizosphere soil with mycelial mats of *Phytophthora parasitica* (45, 50). Both bacterial strains reduced fungal propagule densities in the rhizosphere of citrus seedlings when they were tested as biological control agents for *Phytophthora* root rot (45). All bacteria were stored in 40% glycerol at −80°C. For routine work, *Pseudomonas* strains were grown on mannitol-glutamate (MG) medium (19) or MG medium supplemented with 0.25 g of yeast extract per liter (MGY medium). *Escherichia coli* cultures were grown on Luria-Bertani agar (29). The following antibiotic concentrations were used: 100 μg of kanamycin per ml and 30 μg of tetracycline per ml for *P. fluorescens*, 10 μg of kanamycin per ml and 100 μg of tetracycline per ml for *P. putida*, and 50 μg of kanamycin per ml and 12.5 μg of tetracycline per ml for *E. coli* cultures. *Phytophthora parasitica* 1-91, obtained from infected citrus roots near Thermal, Calif., was grown on pseudomonas agar F (PAF) (Difco Laboratories, Detroit, Mich.) at 28°C for 5 days. Agar discs (diameter, 3.5 mm) were cut from the edge of the mycelium and used as inocula for broth cultures or for inhibition assays on agar plates.

**Tn*5* mutagenesis.** Either *E. coli* C600 (37) containing suicide vector pGS9::Tn*5* (41) or *E. coli* S17.1 (42) containing suicide vector pSUP2021::Tn*5* (42) was used as the donor in matings with *P. fluorescens* or *P. putida*. The mating procedures used have been described previously (50). After mating, single colonies of *P. fluorescens* 09906 and *P. putida* 06909 which grew on MG agar selection plates containing kanamycin were transferred to new MG agar plates in a grid pattern for further screening.

**Hyphal column assay.** An agar disc containing hyphae of *Phytophthora parasitica* was dropped into a 500-ml flask containing 250 ml of V8-C broth (30). The flask was incubated by shaking it slowly at room temperature. After 2 days of growth, the preparation containing the agar disc was blended with a homogenizer, and incubation was continued for another 5 days. The hyphal mass was then washed with sterile water to remove the V8-C broth. A 4-g portion of the washed hyphal mass was placed into a 5-ml polypropylene syringe which was plugged with glass wool. This amount of hyphae filled the syringe to a volume of about 4.5 ml. The hyphal column was then washed with 20 ml of 0.01 M phosphate buffer (pH 7.0) before the bacteria were added.

Bacteria were grown for about 15 h in MGY broth. The cells were centrifuged, washed with phosphate buffer, and resuspended in phosphate buffer. A 2-ml sample of the bacterial suspension (approximately $10^8$ CFU/ml) was added slowly to the hyphal column; this additional buffer volume passed through the column in a few seconds. The actual number of total bacteria added to the column was quantified in each experiment by dilution plating. After 5 min of equilibration, the hyphal column was washed with 2 ml of phosphate buffer. Three additional washes, each with 2 ml of phosphate buffer, were performed to remove most of the free bacteria (Fig. 1). The number of cells removed from the column after each wash was determined by dilution plate counting and was subtracted from the total number applied to the column to calculate the percentage of cells retained in the hyphal column.

To select hyphal colonization-deficient mutants of *P. fluorescens* and *P. putida*, we used an enrichment method that was essentially the same as that described for isolation of mutants of *P. fluorescens* that did not adhere to sand columns (7). A total of 96 Tn*5* insertion mutants were individually grown in 10 ml of MGY broth at 28°C for 15 h. The cultures were then pooled and centrifuged. The cells were suspended in phosphate buffer, and the concentration was adjusted to approximately $10^8$ CFU/ml. A 2-ml sample of the bacterial suspension was added to a hyphal column. Cells which passed through the column after washing with phosphate buffer were dilution plated onto MG agar plates containing kanamycin to obtain single colonies. A total of 96 of the colonies were then grown in MGY broth, pooled, and passed through a second hyphal column for further enrichment of colonization-deficient mutants. If the percentage of cells that were retained in the hyphal column decreased more than 40% after three subsequent washes, the culture was considered enriched for colonization deficiency. The culture was then dilution plated, and single colonies of the Tn*5* mutants were retested for hyphal colonization and compared with the wild-type cultures in replicated hyphal column assays.

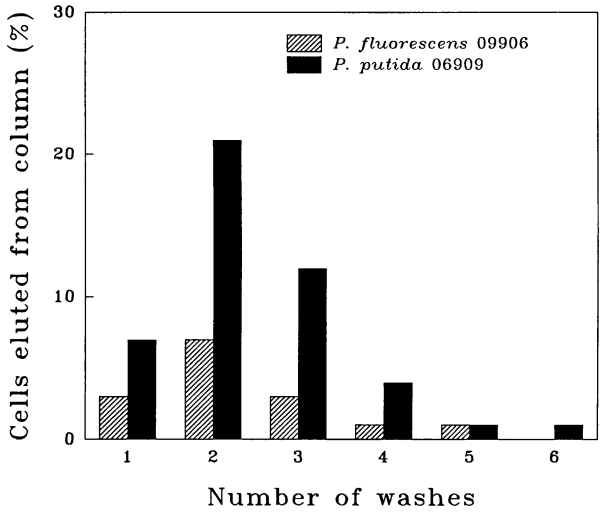


FIG. 1. Removal of free *P. fluorescens* and *P. putida* cells from columns of *Phytophthora parasitica* hyphae with phosphate buffer washes. The values are the percentages of the total numbers of bacterial cells applied to the columns.

**Fungal inhibition in vitro.** To test for diffusible inhibitory factors, the bacteria and fungus were inoculated side by side on PAF, MG agar, or corn meal agar (Difco) plates. Agar discs containing mycelium of *Phytophthora parasitica* were transferred to the centers of the agar plates, and the bacterial strains were spot inoculated onto the agar surfaces at a distance of 10 mm. The plates were incubated at 28°C and observed for several days to determine any effects that the bacterial colonies had on the growth of the fungal mycelium.

A second method for observing fungal growth inhibition involved the direct inoculation of bacteria onto fungal mycelia. An agar disc containing mycelium of *Phytophthora parasitica* was transferred to the center of a PAF plate. Wild-type *P. fluorescens* 09906, wild-type *P. putida* 06909, or Tn*5* mutants were individually transferred from MG agar plate cultures to the center of the mycelium at the surface of the disc with a pin. All plates were incubated for 7 to 8 days at 28°C, and the diameter of each fungal colony was measured. The control plates contained *Phytophthora* discs without bacteria or only bacteria inoculated at the centers of plates. Three replicate plates were used for each treatment.

In addition to isolation of mutants that were defective for hyphal colonization by the column assay, Tn*5* mutants were screened at random for defects in the inhibition of fungal growth to identify other mechanisms that might be important in the antagonistic interaction. A total of 1,440 Tn*5* mutants of

*P. fluorescens* 09906 and 1,268 Tn*5* mutants of *P. putida* 06909 were individually tested by the fungal inhibition assay described above. Mutants that did not inhibit *Phytophthora parasitica* were retested and compared with the wild-type strains for fungal inhibition. The fluorescent pigmentation (pyoverdine production) of these bacterial strains was observed under UV light (366 nm) after 48 h of growth at 28°C on MG agar plates. Motility was observed by phase-contrast microscopy, and the presence of flagella was determined by transmission electron microscopy, using both negative staining and shadow-casting methods (18). The negative stain used was 2% aqueous uranyl acetate, and the heavy metal used in unidirectional shadowcasting was a 13-mm length of 0.02-gauge wire (80% platinum, 20% palladium) evaporated at a 20° angle in a Denton model DV-502 vacuum evaporator. Bacterial samples were examined with a Hitachi model H-600 transmission electron microscope operating at 75 kV.

**Competition for iron in fungal inhibition.** To study the effect of iron on the inhibition of *Phytophthora parasitica* by the bacteria, $FeCl_3 \cdot 6H_2O$ (Sigma) was added to autoclaved PAF to a final concentration of 136, 272, or 1,360 μg/ml to produce conditions of high iron availability. An iron-deficient medium was produced by adding an iron chelator to PAF. Iron was removed from ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDA) (Sigma) as described by Rogers (36), and the EDDA solution was prepared as described by Ong et al. (33). PAF was supplemented with iron-deficient EDDA at a concentration of 200 μg/ml as described by Loper et al. (27), which resulted in PAF-EDDA medium containing low levels of available iron. Transposon mutants defective in inhibition of *Phytophthora parasitica* were grown overnight in MG broth supplemented with kanamycin at 28°C. The bacterial suspensions were then washed twice with sterile distilled water, and the concentration of each suspension was adjusted to an optical density ($A_{600}$) of 0.1. Four 10-μl samples of the bacterial suspensions were spotted onto each PAF-EDDA plate. The same mutants were also spotted onto PAF-EDDA medium supplemented with $FeCl_3 \cdot 6H_2O$ (272 μg/ml) and compared with the wild-type bacteria for growth to confirm that the mutants were specifically iron deficient. Three replicate plates were used for each strain.

**Genetic characterization of mutants.** Plasmid and total DNAs of the bacteria were isolated as described previously (6). Plasmid pGS9::Tn*5* was digested with restriction enzyme *Hin*dIII, and the entire plasmid was used as a probe to verify the Tn*5* insertions. The resulting linearized pGS9::Tn*5* was labeled by random-primed labeling with digoxigenin-11-dUTP and used as described in the instructions for the Genius DNA labeling and detection kit (both obtained from Boehringer Mannheim Biochemicals). Southern blotting of *Eco*RI and *Bam*HI digests of genomic DNAs from agarose gels onto nylon membranes, the hybridization wash conditions, and detection of labeled DNA were done essentially as described by the manufacturer of the detection kit. The sizes of the DNA fragments hybridizing with the Tn*5* probe were calculated by comparing the relative mobilities of the fragments with the relative mobilities of linear size standards.

**Cloning and marker exchange.** Total DNAs of colonization-deficient mutants 09906.10 and 06909.15 were completely digested with *Eco*RI. The *Eco*RI-digested fragments were cloned into the *Eco*RI site of cloning vector pRK415 (20), which was cut with *Eco*RI and pretreated with alkaline phosphatase to prevent self-ligation of the vector (28). The vectors containing the isolated DNAs were then transformed into competent cells of *E. coli* DH5α (3, 37). The transformed cultures were plated onto Luria-Bertani agar plates containing kanamycin and tetracycline to select transformants harboring pRK415 with chromosomal DNA insertions containing Tn*5*.

Plasmid DNAs from *E. coli* DH5α transformants were isolated and transformed into *E. coli* S17.1 (42). Plasmids from selected *E. coli* S17.1 transformants were mobilized into wild-type strains 09906 and 06909 by biparental mating on YDC plates as described previously (50). The YDC plates were incubated at 28°C for 24 h, and the cells were suspended in sterile distilled water, serially diluted, and plated onto MG agar plates containing kanamycin and tetracycline. Several transconjugants were selected, grown in MG broth overnight, and tested for retention of kanamycin resistance and loss of tetracycline resistance. The total DNAs of two kanamycin-resistant, tetracycline-sensitive transconjugants, 09906.10MK and 06909.15MK, were digested with *Eco*RI, separated by agarose gel electrophoresis, and hybridized with the Tn*5* probe described above to confirm that the homologous Tn*5*-containing DNA had recombined with the wild-type bacterial chromosome. Recombinants 09906.10MK and 06909.15MK were then tested for inhibition and colonization of *Phytophthora parasitica* mycelium.

**Bacterial survival in soil.** The survival of hyphal colonization-deficient mutant 09906.10 in soil was compared with that of a random Tn*5* mutant of *P. fluorescens* 09906. The random mutant, 09906.1, had been shown in a previous study to survive well in soils and served as a convenient control strain that had the same kanamycin resistance as other Tn*5* mutants (50). A clay loam soil (pH 6.7) used in the previous study was collected, air dried, and sieved through a 1-mm mesh. Samples (100 g) of the soil were then placed in 500-ml screw-cap bottles (Corning Glass Works, Corning, N.Y.). The bacteria were grown, washed, and mixed with the soil as described previously (50). Two final concentrations of each bacterial strain ($4.5 \times 10^6$ and $4.5 \times 10^7$ CFU/g for 09906.1; $6.1 \times 10^6$ and $6.1 \times 10^7$ CFU/g for 09906.10) were used in this experiment. The final water content of the soil was adjusted to 10 ml/100 g of air-dried soil. The caps of the bottles were sealed with Parafilm, and the water content of each bottle was checked and adjusted as described previously (50). The bottles were kept at room temperature (22°C) in the dark. At different times, a 10-g sample was taken from each bottle, and the number of bacteria was determined by dilution plating on MG agar containing kanamycin (100 μg/ml). A control experiment, in which only sterile distilled water was added to the air-dried soil, showed that the MG agar plates containing kanamycin selected against the indigenous bacteria in the soil.

## RESULTS

**Hyphal colonization.** An assay was developed to quantitatively measure the colonization of pseudomonads on hyphae of *Phytophthora parasitica* and to be used for enrichment for noncolonizing mutants of the bacteria. When bacteria were applied to a column of *Phytophthora* hyphae, large proportions of the cells of *P. fluorescens* 09906 and *P. putida* 06909 were retained by fungal hyphae (Fig. 1). Most of the free cells of these strains were removed from the column by four washes with phosphate buffer. Additional washes removed only an additional 1 to 2% of the total amount of bacteria added to the column. Therefore, in all subsequent experiments, four washes were used to determine the amounts of bacteria that were retained in columns. When *E. coli* JM83 (47) was added to a hyphal column, an average of only 5% of the bacterial cells were retained by the hyphae (Table 1). Wild-type *E. coli* K-12 was also tested in a single (nonreplicated) column experiment,

TABLE 1. Colonization of *Phytophthora parasitica* hyphal columns by *P. fluorescens*, *P. putida*, and *E. coli*

| Strain | % of bacteria retained in column[a] | No. of expts |
|---|---|---|
| *P. fluorescens* 09906 | 65 ± 13 | 5 |
| *P. putida* 06909 | 69 ± 10 | 3 |
| *P. fluorescens* 09906.10 | 13 ± 10 | 3 |
| *P. putida* 06909.15 | 14 ± 12 | 3 |
| *E. coli* JM83 | 5 ± 7 | 4 |
| *E. coli* K-12 | 5 | 1 |

[a] Means ± standard deviations of the values from replicate experiments.

and only 5% of the cells were retained in the column after four washes.

The hyphal columns were used to enrich for colonization-deficient mutants of the pseudomonads in pools of random Tn*5* mutants. After four pools (96 mutants per pool) of Tn*5* mutants of *P. putida* 06909 were screened, one colonization-deficient Tn*5* mutant (06909.15) was found. Another Tn*5* mutant (*P. fluorescens* 09906.10), which lacked flagella, was also found to have reduced retention in fungal columns. The percentages of bacteria that were retained in the hyphal columns were almost as low for the mutants as for *E. coli* (Table 1). Inoculation of the colonization-deficient mutants onto discs containing *Phytophthora parasitica* mycelia on PAF plates showed that the mutants were not able to colonize the fungal mycelium or inhibit fungal growth (Fig. 2 and Table 2). The colonization-deficient mutants were nonmotile, and both of the mutants lacked flagella (Fla$^-$) (Fig. 3).

**Competition for iron in fungal inhibition.** No fungal inhibition was observed when either *P. fluorescens* 09906 or *P. putida* 06909 was coinoculated side by side with *Phytophthora parasitica* on PAF, MG agar, or corn meal agar (Difco) plates. Thus, no evidence for antibiotic production by these two strains capable of inhibiting fungal growth was observed. However, after strains 09906 and 06909 were inoculated individually onto the tops of discs containing *Phytophthora parasitica* mycelia on PAF plates, the bacteria colonized the fungal mycelium and inhibited fungal growth (Table 2). When PAF plates were supplemented with $FeCl_3 \cdot 6H_2O$, the bacteria still colonized the fungal mycelium but did not inhibit fungal growth. The growth of bacterial strains 09906 and 06909 on iron-supplemented PAF was slightly enhanced, but no fluorescent pigment was produced by either of the bacteria when iron was added to PAF. These data suggested that pyoverdines were involved in the inhibition of *Phytophthora parasitica* on PAF plates.

**Characteristics of inhibition-defective mutants.** In addition to specific selection of colonization-deficient mutants with the hyphal column assay, random Tn*5* mutants of both *P. fluorescens* 09906 and *P. putida* 06909 were tested for the loss of the ability to inhibit *Phytophthora parasitica* in vitro. We detected

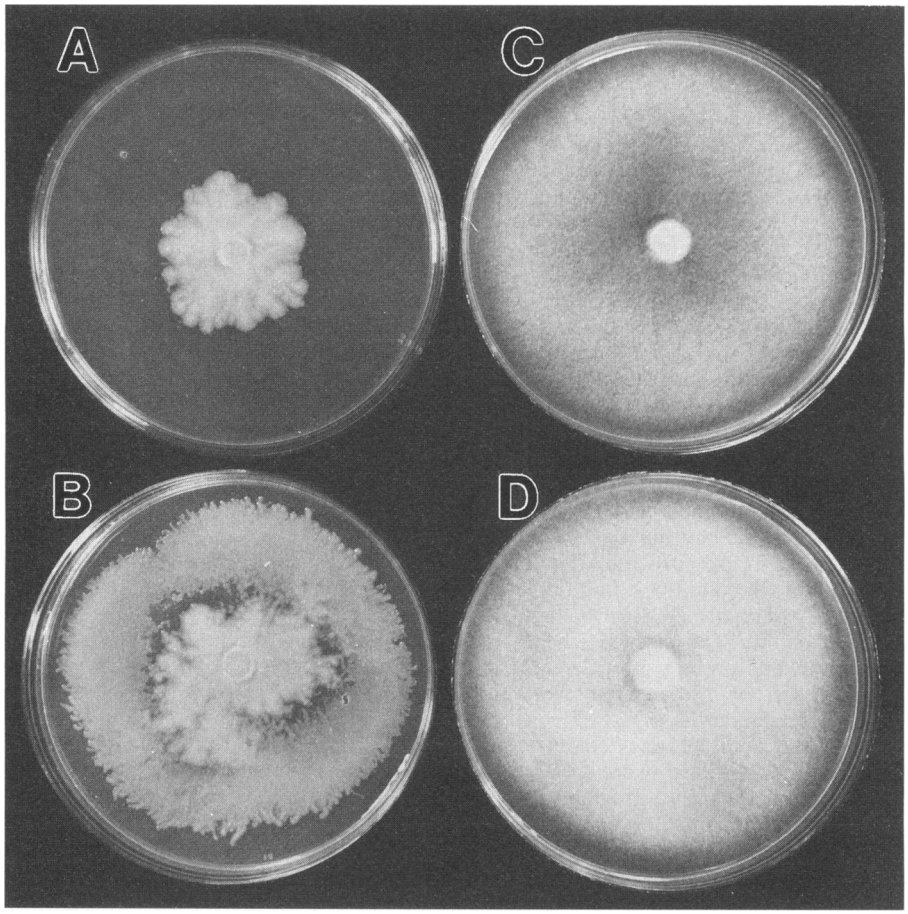


FIG. 2. Colonization and inhibition of colonies of *Phytophthora parasitica* by *P. fluorescens* 09906 (A), *P. fluorescens* 09906.5 (Pvd$^-$) (B), and *P. fluorescens* 09906.11 (Fla$^-$) (C). (D) Control without bacteria.

TABLE 2. Bacterial strains and the ability to colonize and inhibit colonies of *Phytophthora parasitica*

| Bacterial strain | Relevant characteristics[a] | Bacterial colonization of fungal mycelium | Diam of fungal colony (mm)[b] |
|---|---|---|---|
| *P. fluorescens* | | | |
| Control | | | 82.2[c] |
| 09906 | Wild type | + | 38.0[d] |
| 09906.5 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 76.5[c,e] |
| 09906.6 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 76.3[c,e] |
| 09906.7 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 70.3[e] |
| 09906.8 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 80.0[c,e] |
| 09906.9 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 74.7[c,e] |
| 09906.10 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 77.8[c,e] |
| 09906.11 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 80.3[c,e] |
| 09906.12 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 79.8[c,e] |
| *P. putida* | | | |
| Control | | | 82.0[c] |
| 06909 | Wild type | + | 29.5[f] |
| 06909.1 | Flu(weak) Km$^r$, Tn*5* insertion mutant | + | 28.8[f] |
| 06909.2 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 82.2[c] |
| 06909.3 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 80.3[c] |
| 06909.4 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 75.0[c,e] |
| 06909.5 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 79.5[c] |
| 06909.6 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 82.3[c] |
| 06909.7 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 78.2[c,e] |
| 06909.8 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 72.5[c,d,e] |
| 06909.9 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 60.8[g] |
| 06909.10 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 82.5[c] |
| 06909.11 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 78.0[c,e] |
| 06909.12 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 77.8[c,e] |
| 06909.13 | Pvd$^-$ Km$^r$, Tn*5* insertion mutant | + | 80.8[c] |
| 06909.14 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 55.2[g] |
| 06909.15 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 66.5[d,e,g] |
| 06909.16 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 73.7[c,d,e] |
| 06909.17 | Fla$^-$ Km$^r$, Tn*5* insertion mutant | − | 62.2[d,g] |

[a] Km$^r$, kanamycin resistance; Pvd$^-$, deficient in pyoverdine production; Fla$^-$, lacking flagella; Flu, fluorescence on PAF.

[b] Diameter of fungal colony on PAF measured 7 days (*P. fluorescens* experiment) or 8 days (*P. putida* experiment) after placement of a mycelial disc at the center of a plate with or without bacteria inoculated onto the disc. Data are averages of the values from three replicate experiments.

[c–g] For each species, values with the same superscript letter are not significantly different as determined by Duncan's multiple-range test ($P = 0.05$).

two types of mutants of each pseudomonad which exhibited reduced inhibition of fungal growth on PAF plates (Table 2). The mutants of the first type were nonfluorescent but were still able to colonize the fungal mycelium (Fig. 2B). The mutants of the second type produced fluorescent pigments but were not able to colonize the fungal mycelium (Fig. 2C). The nonfluorescent Tn*5* mutants were not able to grow on an iron-limited PAF-EDDA medium, while the wild-type strains grew well on this medium. The nonfluorescent mutants did grow when the PAF-EDDA medium was supplemented with 272 μg of $FeCl_3 \cdot 6H_2O$ per ml. These results suggested that the nonfluorescent mutants were defective for pyoverdine production (Pvd$^-$). A weakly fluorescent mutant (06909.1), which had the same ability as wild-type strain 06909 to inhibit *Phytophthora parasitica* on PAF, was also detected among the Tn*5* mutants (Table 2). Mutant 06909.1 was able to grow on both PAF-EDDA plates and plates containing PAF-EDDA agar supplemented with $FeCl_3 \cdot 6H_2O$, indicating that a functional pyoverdine was produced.

The second type of mutants that were defective for colonization and inhibition of *Phytophthora parasitica* produced functional pyoverdines, since these mutants were fluorescent and grew on PAF-EDDA medium (Table 2). However, these mutants were nonmotile and lacked flagella, as determined by electron microscopy.

**Southern blot analysis of Tn*5* mutants.** A Southern blot analysis of *Eco*RI- and *Bam*HI-digested DNAs of Tn*5* mutants indicated that each mutant had a single Tn*5* insertion. Tn*5* has no *Eco*RI sites, and the expected single *Eco*RI fragments were observed for each mutant when blots were probed with labeled Tn*5*. *Bam*HI cuts once near the center of Tn*5*, and two *Bam*HI fragments with Tn*5* homology were observed in each mutant. No hybridization of the pGS9::Tn*5* probe was observed with DNAs from wild-type strains 09906 and 06909. The sizes of DNA fragments with homology to Tn*5* indicated that transposon insertions in at least two different *Eco*RI fragments resulted in the pyoverdine-deficient (Pvd$^-$) phenotype of 09906. The Tn*5* insertions of 12 Pvd$^-$ mutants of 06909 were also in *Eco*RI fragments of at least two different sizes. However, the sums of the sizes of the two *Bam*HI fragments in all of the Pvd$^-$ mutants of 06909 were similar (36.3 to 43.9 kb), which may indicate that Tn*5* was inserted into one large *Bam*HI fragment of strain 06909. This amount of variation in fragment sizes calculated on the basis of gel mobility is not unusual for conventional electrophoresis, which gives poor resolution of large fragments. However, the sizes of the smaller *Bam*HI fragments in the 12 Pvd$^-$ mutants of strain 06909 varied from 6.3 to 20.4 kb, indicating that Tn*5* insertions in a DNA region that is several kilobases long affected pyoverdine production.

In nonmotile Tn*5* mutants of both strain 09906 and strain 06909 that were isolated by lack of fungal inhibition or by loss of colonization of fungal hyphae, the transposon insertions causing these phenotypic changes were in several different sites, as determined by the sizes of *Eco*RI and *Bam*HI fragments that exhibited homology to Tn*5*. In nonmotile mutant

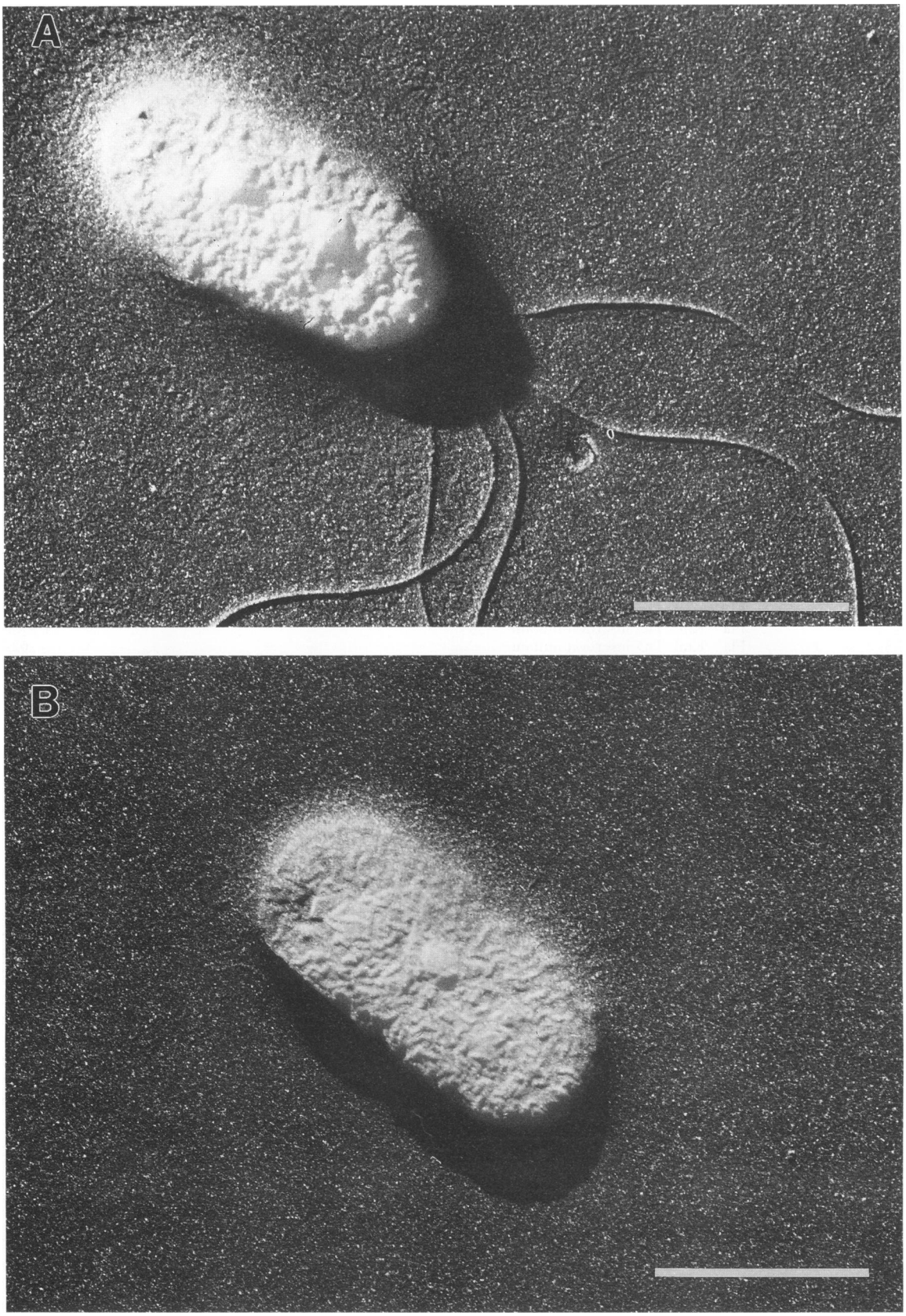


FIG. 3. Electron micrograph of wild-type ($Fla^+$) *P. putida* 06909 (A) and Tn*5* mutant 06909.15 ($Fla^-$) that was defective in colonization of hyphae of *Phytophthora parasitica* and inhibition of fungal growth (B).

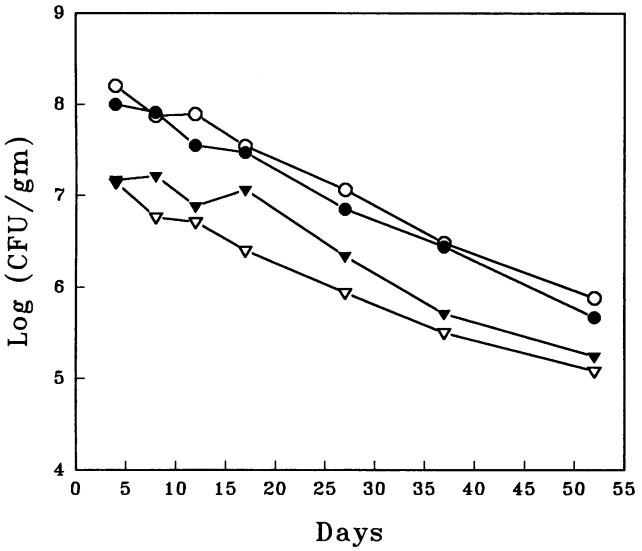


FIG. 4. Survival of Tn*5* mutants 09906.1 and 09906.10 of *P. fluorescens* in nonsterile soil from a citrus field. Symbols: ●, strain 09906.1 inoculated at a concentration of $4.5 \times 10^7$ CFU/g of soil; ▼, strain 09906.1 inoculated at a concentration of $4.5 \times 10^6$ CFU/g of soil; ○, strain 09906.10 inoculated at a concentration of $6.1 \times 10^7$ CFU/g of soil; ▽, strain 09906.10 inoculated at a concentration of $6.1 \times 10^6$ CFU/g of soil.

06909.15, which was isolated by the enrichment procedure for noncolonizing mutants, the Tn*5* insertion appeared to be in the same *Eco*RI and *Bam*HI fragment as the Tn*5* insertion in nonmotile mutant 06909.16, which was selected on the basis of its defect in fungal colonization and inhibition in the agar plate assay.

**Marker exchange.** Vector pRK415 containing cloned *Eco*RI fragments with Tn*5* insertions from mutants 09906.10 and 06909.15 was conjugated into wild-type strains 09906 and 06909, respectively. After overnight growth of the transconjugants in MG broth, 69% of 09906 transconjugants and 96% of 06909 transconjugants were kanamycin resistant and tetracycline sensitive, indicating that the desired homologous recombinations had occurred in a portion of cells in each culture. Two of the recombinants, 09906.10MK and 06909.15MK, were selected for further analysis. The recombinants were nonmotile and were not able to colonize fungal mycelium and inhibit fungal growth in the agar plate assay, thus confirming the hypothesis that the single Tn*5* insertions in the original mutants were responsible for the loss of motility and fungal colonization and inhibition. Probing of *Eco*RI digests of DNAs from 09906.10MK and 06909.15MK with Tn*5* indicated that the Tn*5* insertions were in the same position as they were in 09906.10 and 06909.15.

**Survival in soil.** Survival of hyphal colonization-deficient mutant 09906.10 was similar to that of random Tn*5* mutant 09906.1 over a 52-day period in soil from a citrus grove (Fig. 4). The populations of both strains gradually decreased over time at about the same rate, as observed in previous experiments with *P. fluorescens* in this soil (50).

## DISCUSSION

The colonization and inhibition of *Phytophthora parasitica* by *P. fluorescens* 09906 and *P. putida* 06909 in vitro were disrupted by Tn*5* insertions which caused (i) the loss of flagella and colonization of fungal hyphae and (ii) the loss of pyoverdine production. There was no evidence that either bacterium produced antifungal compounds other than pyoverdines that inhibited *Phytophthora parasitica* in vitro, since all antagonism-defective mutants lacked flagella or pyoverdine production.

Wild-type *P. fluorescens* 09906 and *P. putida* 06909 were retained by the hyphae of *Phytophthora parasitica* in the hyphal column assay, but *E. coli* was not retained in significant numbers. The *E. coli* strains tested were shown to have peritrichous flagella by electron microscopy, and *E. coli* cells are slightly larger than the cells of the *Pseudomonas* species tested (22). Therefore, the hyphal columns did not retain pseudomonads because of simple filtration, and it is likely that the bacteria attached directly to the hyphae. Hyphal colonization-deficient Tn*5* mutants of *P. fluorescens* and *P. putida* were isolated by enrichment through hyphal columns. These mutants were nonflagellated and were defective in colonization and inhibition of colonies of *Phytophthora parasitica* in vitro. Additional nonflagellated mutants were isolated by randomly screening Tn*5* mutants for loss of hyphal colonization and inhibition on agar plates. The characteristics of these additional mutants, together with the results of marker exchange mutagenesis, confirmed the linkage between the loss of flagella and the loss of the ability to colonize and inhibit fungal mycelia. Whether the loss of motility or the loss of attachment to mycelia was responsible for the inability to colonize fungal mycelia is not yet known. Motility-defective mutants that have flagella could be isolated to address this question.

The loss of flagella in Tn*5* mutants of *P. fluorescens* has also been associated with an inability to colonize potato roots, which was attributed to the loss of motility rather than to adhesion to roots (10). However, nonmotile mutants derived from chemical mutagenesis of three strains of *P. fluorescens* were not affected in their ability to colonize wheat roots (16), and a nonflagellated Tn*5* mutant of *P. putida* RW3 colonized soybean roots as well as the wild-type strain did (39). In another study, nonflagellated Tn*5* mutants of *P. fluorescens* were defective in adhesion to sand, suggesting that the flagella, or other outer membrane proteins associated with flagella, were involved in adhesion to surfaces (7). Flagella of *P. fluorescens* have also been observed to adhere to soil amoebae (35). Pili are more commonly associated with bacterial attachment, and pili of *P. fluorescens* have been associated with binding to corn roots (46). Whether our nonflagellated mutants were also defective in pilus formation was not determined, as pili of these bacteria were not observed in our electron microscopic studies.

The loss of flagella did not affect the survival of *P. fluorescens* 09906 in a citrus soil. Similarly, nonflagellated Tn*5* mutants of *P. fluorescens* Pf0-1 survived in soil as well as the wild-type strain did, except in soil columns that were watered over a period of 5 days (7). Whether the observed loss of retention in hyphal columns and in vitro colonization of hyphae in $Fla^-$ mutants affect biological control of citrus root rot by *P. fluorescens* 09906 and *P. putida* 06909 is under investigation. The results of a preliminary study indicated that the $Fla^-$ mutants inhibited propagule production by *Phytophthora parasitica* in the citrus rhizosphere as well as the wild-type strains did (45).

Antibiotic production can be an important factor in biological control of fungal pathogens by bacteria (12, 17, 44), but no antibiotic production by either *P. fluorescens* 09906 or *P. putida* 06909 was observed. When inoculated directly onto fungal colonies, however, both bacteria colonized the fungus and prevented further spread of the mycelium. The addition of iron to the medium decreased this inhibition, suggesting that com-

petition for iron by pyoverdine production was involved. The reason for the lack of fungal inhibition by pyoverdine production in side-by-side inoculations with the bacteria is not known, but perhaps the fungal hyphae transport enough iron to the hyphal tips from other areas of the agar plate to compensate for reduced iron availability induced locally by pyoverdine production. However, when the bacteria contact the fungal hyphae, they rapidly colonize the entire mycelial mat, and pyoverdine production may then have a more general effect on fungal growth.

Pyoverdine-deficient Tn*5* mutants were observed to colonize fungal hyphae, but they were defective for inhibition of the spread of fungal mycelia in vitro. In preliminary experiments, a Pvd$^-$ mutant of *P. putida* 06909 was less effective in reducing the propagule densities of *Phytophthora parasitica* in the citrus rhizosphere than the wild-type strain was, but a Pvd$^-$ mutant of *P. fluorescens* 09906 was not defective for inhibition of fungal propagule production (45). Siderophore production has been associated with bacterial suppression of several fungal diseases (23, 26, 40), but this is not true in all cases (1, 14, 34, 49). Different soil types, pathogens, host plants, and pseudomonad strains may influence the effect of siderophores on disease suppression (26). A larger study of the effect of Pvd$^-$ mutations in *P. fluorescens* 09906 and *P. putida* 06909 on biological control of citrus root rot is in progress.

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