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Role of Copper Resistance in Competitive Survival of *Pseudomonas fluorescens* in Soil

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A copper-resistant strain (09906) of *Pseudomonas fluorescens* that was isolated from a citrus grove soil is being investigated as a biological control agent for *Phytophthora* root rot. Since citrus grove soils in California are often contaminated with copper from many years of copper fungicide applications, the role of copper resistance in survival of strain 09906 was investigated. Three copper-sensitive Tn*5* mutants were obtained with insertions in different chromosomal DNA regions. These insertions were not in the chromosomal region that hybridized with the copper resistance operon (*cop*) cloned from *Pseudomonas syringae*. A copper-sensitive mutant survived as well as the wild type in a sterile loamy sand without added copper, but with 10 and 15 μg of $CuSO_4$ added per g of soil, populations of the copper-sensitive mutant were 27- and 562-fold lower, respectively, than that of the wild type after a 25-day period. In a sterilized citrus grove soil, populations of the copper-sensitive mutant and wild-type strain were similar, but in nonsterile citrus soil, populations of the copper-sensitive mutant were 112-fold lower than the wild type after 35 days. These data suggest that copper resistance genes can be important factors in persistence of *P. fluorescens* in soil contaminated with copper. In addition, these genes appear to play a role in competitive fitness, even in soils with a low copper content.

Microbial systems for regulating trace-metal uptake can be important factors in competition with other microbes and in their survival when the metal ions are either limiting (17) or present at toxic levels (30). Copper is an essential trace element that is also toxic at high levels. Bacteria exposed to toxic levels of copper have evolved a number of mechanisms to regulate copper uptake and resist copper toxicity (32). An energy-dependent efflux of copper has been described in *Escherichia coli* strains from pigs fed a dietary supplement of copper (27, 28), and sequestration of copper in the periplasm and outer membrane confers resistance in strains of *Pseudomonas syringae* exposed to agricultural copper applications (11). Plasmid-determined copper resistance in *E. coli* is also linked to a chromosomally encoded copper uptake and intracellular transport system (25, 26).

Fitness advantages associated with metal resistance systems in natural isolates of bacteria are often assumed rather than directly tested. However, there is considerable experimental evidence that copper resistance in plant pathogenic bacteria enhances their pathogenic and epiphytic fitness on crops sprayed with copper compounds (1, 2, 13, 19). In addition, copper resistance can enhance the effectiveness of nonpathogenic bacteria used for biological control of foliar diseases (12). The common association of copper resistance with highly competitive bacterial predators in soil (8, 10) also suggests a role for copper resistance in fitness of certain soil bacteria.

In studying factors important for the effectiveness of pseudomonads in biological control of citrus root rot disease caused by *Phytophthora parasitica* (37), we noticed that our most effective antagonistic strains were copper resistant (36). The soils of California citrus groves are often contaminated with copper from many years of copper fungicide applications. The objective of this study was to determine whether copper resistance was important for survival and competitiveness of these bacteria in soil.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas fluorescens* 09906 was isolated from lemon rhizosphere soil near Ventura, Calif., and was used as a biological control agent for *Phytophthora* root rot (33). *P. fluorescens* 09906 was stored frozen at −80°C in 40% glycerol and grown on mannitol-glutamate (MG) medium (16) or MG supplemented with yeast extract at 0.25 g/liter (MGY). *E. coli* cultures were grown on LB agar (24). Antibiotic concentrations used were as follows: 100 μg of kanamycin and 30 μg of tetracycline per ml for *P. fluorescens* or 50 μg of kanamycin and 12.5 μg of tetracycline per ml for *E. coli* cultures.

Mutagenesis. *E. coli* C600 containing the suicide vector pGS9::Tn*5* was used as a donor in matings with *P. fluorescens*. A loopful of strain 09906 and a loopful of strain C600 were mixed on YDC agar plates (35). The plates were incubated at 28°C for 24 h, and cells were suspended in sterile distilled water, serially diluted, and plated onto MG agar plates containing kanamycin. After incubation of plates for 48 h at 28°C, single colonies were transferred to new MG agar plates in a grid pattern. The MIC of $CuSO_4$ for *P. fluorescens* 09906 was determined as described before (14). Cells of 09906 were suspended in sterile water to about 5×10^8 CFU/ml and spotted in duplicate (10 μl/spot) on MGY agar containing various concentrations (0 to 4 mM) of cupric sulfate. To screen for copper-sensitive Tn*5* mutants from mutant pools of 09906, all mutants were replica plated onto MGY and MGY plates containing 1.2 mM $CuSO_4$. Copper-sensitive mutants were selected, and the MICs were further determined.

Detection of homology to *cop*. The plasmids and total genomic DNAs were isolated as described previously (14). The *Pst*I-*Pst*I fragment from pCOP2 (5), which contains the entire *cop* operon (21, 22), was gel purified with a GENECLEAN KIT (catalog no. 3105; BIO 101, San Diego, Calif.) as recommended by the manufacturer, labeled by random primed labeling with digoxigenin-11-dUTP, and used as

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	$CuSO_4$ MIC (mM)	Source or reference
Bacteria			
P. fluorescens			
09906	Cu^r, wild type	1.6	This study
09906.1	Cu^r Km^r, Tn*5* insertion mutant	1.6	This study
09906.2	Cu^s Km^r, Tn*5* insertion mutant	0.16	This study
09906.3	Cu^s Km^r, Tn*5* insertion mutant	0.16	This study
09906.4	Cu^s Km^r, Tn*5* insertion mutant	1.0	This study
E. coli			
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacI tonA21*		18
Plasmids			
pCOP2	Cu^r Tc^r, *cop* operon from *P. syringae* PT23 cloned in pRK404		5
pGS9::Tn*5*	Km^r, Tn*5*-containing suicide vector		29

[a] Cu^r, copper resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

outlined in the Genius DNA Labeling and Detection Kit (both from Boehringer Mannheim Biochemicals). Southern blotting of *Eco*RI, *Bam*HI, and *Hin*dIII complete digests of genomic DNAs from agarose gels to nylon membranes, hybridization wash conditions, and detection of labeled DNA were done essentially as described by the manufacturer in the detection kit. In molecular characterization of Tn*5* mutants, pGS9::Tn*5* was used as a probe to verify the Tn*5* insertion. Southern blotting was performed as described above.

Soil preparation and bacterial inoculation. The loamy sand (23) used in this experiment was collected from Santa Ana River, Bloomington, San Bernardino, Calif., with a soil pH of 6.7 (31). The clay loam soil (pH 6.7) was collected from a citrus field in Fallbrook, San Diego, Calif. Before use, the soils were sieved through a 1-mm-pore-size screen. The concentration of extractable copper in citrus soil and sand was determined by a DTPA (diethylenetriaminepentaacetic acid) method described by Baker and Amacher (3). Soil was sterilized by autoclaving 500-ml screw-cap bottles (Corning Glass Works, Corning, N.Y.), each with 100 g of soil for 40 min. Test bacteria were grown in MG broth at 28°C to mid-log phase. The cells were then centrifuged at 6,000 × *g* for 7 min and resuspended in sterile distilled water, and their concentrations were adjusted to an optical density of 0.6 at A_{600}. One milliliter of the bacterial suspension was diluted with sterile distilled water before addition to the soil. The final water content of the soil was 10 ml/100 g of air-dried soil. The bacterial suspension was mixed thoroughly in the soil. The bottles were left at room temperature (22°C) in the dark. The caps of the bottles were further sealed with Parafilm, and water loss from samples, though minimal, was routinely checked by changes in sample weight and adjusted with sterile distilled water when necessary. To study survival in soil containing different levels of copper sulfate, the copper sulfate solutions were added and mixed into loamy sand 1 day before bacteria were added.

Bacterial survival in soils. At different time intervals, a 10-g sample was taken from each bottle, and the number of bacteria was determined by decimal dilution of the soil with sterile distilled water and plating of 0.1 ml of each soil suspension on triplicate plates of MG agar containing 100 μg of kanamycin per ml. After 48 h of incubation at 28°C, colonies on plates with 30 to 300 colonies were counted. A survival comparison of a copper-resistant strain, 09906.1, and a copper-sensitive mutant, 09906.2, inoculated individually to soil amended with 0, 5, 10, or 15 μg of copper sulfate per g of soil was performed in the first trial. In the second trial, 09906.1 and 09906.2 were individually added into citrus soil and sterile citrus soil to investigate competitive effects with other microbes. The population dynamics of two copper-sensitive mutants, 09906.2 and 09906.3, were detected in citrus soil in the third experiment. Each treatment consisted of three replicates.

RESULTS

Mutagenesis. The MIC of cupric sulfate for wild-type *P. fluorescens* 09906 was 1.6 mM (Table 1). Of 1,440 Tn*5* insertion mutants of *P. fluorescens* 09906, 3 did not grow when screened on MGY agar containing 1.2 mM $CuSO_4$. Two mutants, 09906.2, and 09906.3, were 10-fold more sensitive to copper (MIC = 0.16 mM) than the wild type. One mutant (09906.4) had an intermediate sensitivity (MIC = 1.0 mM). A random Tn*5* insertion mutant, 09906.1, which had the same MIC as the wild-type bacterium was selected as a kanamycin-resistant strain with wild-type copper resistance for soil survival studies.

Molecular characterization of Tn*5* mutants. Southern blot analysis of *Eco*RI-digested genomic DNA of the copper-sensitive Tn*5* insertion mutants 09906.2, 09906.3, and 09906.4, using Tn*5* as a probe, indicated that each mutant had a single Tn*5* insertion (Fig. 1). However, Southern analysis of *Bam*HI and *Hin*dIII digests of DNA from these mutants suggested that the three insertions were not closely clustered in the *P. fluorescens* genome. The *Eco*RI fragments containing Tn*5* in these three mutants were measured as 18.8, 20.0, and 15.3 kb, respectively. The *Bam*HI fragments containing Tn*5* totalled 38.5, 19.4, and 30.4 kb, respectively, and the *Hin*dIII fragments containing Tn*5* totalled 34.8, 35.2, and 14.3 kb, respectively. A single Tn*5* insertion was also detected in the copper-resistant strain 09906.1 by Southern analysis, using pGS9::Tn*5* as a probe. No hybridization of Tn*5* to wild-type 09906 DNA was observed. No plasmids were detected in *P. fluorescens* 09906, and the Tn*5* insertions therefore appeared to be in chromosomal genes.

Detection of homology to *cop*. Homology between the *cop* operon from *P. syringae* and the chromosomal DNA of wild-type *P. fluorescens* was detected by Southern blot hybridization (Fig. 2). The chromosomal DNA of three copper-sensitive Tn*5* insertion mutants, 09906.2, 09906.3, and 09906.4, digested with *Eco*RI, *Bam*HI, and *Hin*dIII showed the same homology pattern with the *cop* operon as the wild-type *P. fluorescens*, indicating that the Tn*5* insertions were not in this *cop* homolog region of the genome.

Survival in sterile loamy sand. Population levels of the copper-resistant strain 09906.1 and the copper-sensitive mutant 09906.2 showed no statistical differences ($P = 0.05$, by Duncan's multiple range test) over a 25-day period (Fig. 3) in sterile loamy sand with no added cupric sulfate. The concentration of extractable copper measured from this sand was low (6.0×10^{-3} μg/g). Similarly, no statistical differences ($P = 0.05$) were observed among populations of the copper-resistant strain 09906.1 in sand amended with 0, 5,

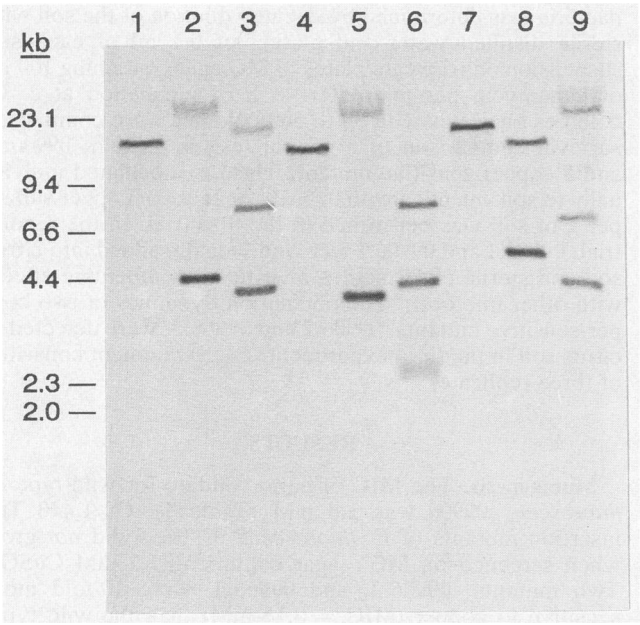

FIG. 1. Hybridization of digoxigenin-labeled plasmid pGS9::Tn*5* to chromosomal DNA of *P. fluorescens* strains. Lane 1, Tn*5* mutant 09906.2 DNA digested with *Eco*RI; lane 2, 09906.2 DNA digested with *Bam*HI; lane 3, 09906.2 DNA digested with *Hin*dIII; lane 4, Tn*5* mutant 09906.4 DNA digested with *Eco*RI; lane 5, 09906.4 DNA digested with *Bam*HI; lane 6, 09906.4 DNA digested with *Hin*dIII; lane 7, Tn*5* mutant 09906.3 DNA digested with *Eco*RI; lane 8, 09906.3 DNA digested with *Bam*HI; lane 9, 09906.3 DNA digested with *Hin*dIII.

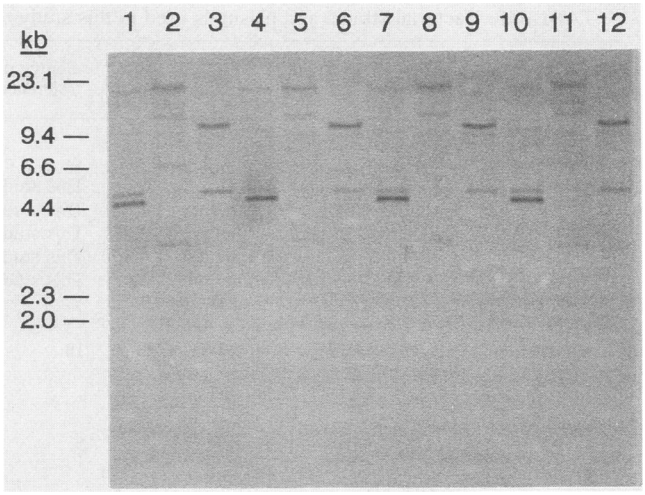

FIG. 2. Hybridization of the digoxigenin *cop* operon of *P. syringae* to chromosomal DNA of *P. fluorescens* strains. Lane 1, wild-type 09906 DNA digested with *Eco*RI; lane 2, 09906 DNA digested with *Bam*HI; lane 3, 09906 DNA digested with *Hin*dIII; lane 4, Tn*5* mutant 09906.2 DNA digested with *Eco*RI; lane 5, 09906.2 DNA digested with *Bam*HI; lane 6, 09906.2 DNA digested with *Hin*dIII; lane 7, Tn*5* mutant 09906.3 DNA digested with *Eco*RI; lane 8, 09906.3 DNA digested with *Bam*HI; lane 9, 09906.3 DNA digested with *Hin*dIII; lane 10, Tn*5* mutant 09906.4 DNA digested with *Eco*RI; lane 11, 09906.4 DNA digested with *Bam*HI; lane 12, 09906.4 DNA digested with *Hin*dIII.

10, and 15 μg of cupric sulfate per g. When the sand was amended with 10 and 15 μg of cupric sulfate per g, populations of the copper-resistant strain 09906.1 were maintained at 1.2×10^8 and 6.2×10^7 CFU/g of soil, respectively, after 25 days. In contrast, population levels of the copper-sensitive mutant 09906.2 were 27- and 562-fold less than that of the copper-resistant strain 09906.1 in sand amended with 10 and 15 μg of $CuSO_4$ per g of soil, respectively.

Survival in citrus soil. In sterile citrus soil, populations of the copper-resistant strain 09906.1 and the copper-sensitive mutant 09906.2 were 1.5×10^9 and 5.4×10^8 CFU/g of soil, respectively, 35 days after the bacteria were added (Fig. 4). In nonsterile soil, however, the population of copper-resistant strain 09906.1 was 112-fold higher than that of the copper-sensitive mutant 09906.2. When the copper-resistant strain 09906.1 and the two copper-sensitive Tn*5* insertion mutants 09906.2 and 09906.3 were individually added into nonsterile citrus soil, populations of the copper-resistant strain declined less than 10-fold over a 30-day period, from 3.2×10^7 to 4.1×10^6 CFU/g of soil (Fig. 5). However, populations of the two copper-sensitive mutants declined more than 200-fold during the same period. The concentration of extractable copper in the autoclaved citrus soil (3.0×10^{-2} μg/g) was similar to that of the nonsterile citrus soil (2.6×10^{-2} μg/g).

DISCUSSION

Root colonization by introduced antagonistic bacteria is important for biological control of root pathogens (34). However, the maintenance of populations of antagonists in the soil should also be important for long-term root colonization and biological control. Our data suggest that copper resistance can be an important factor in persistence of *P. fluorescens* in soil contaminated with copper. Copper has been widely used as an antimicrobial agent in agriculture for decades, and in some areas copper has accumulated to levels that are toxic to plants and microorganisms (15, 20). Copper resistance in *P. fluorescens* 09906 may have evolved to overcome the selective pressure from many years of copper applications in citrus groves.

In addition, copper resistance in *P. fluorescens* 09906 appears to be an important factor in competitive fitness, even in soils with a low copper content. The Tn*5* mutants

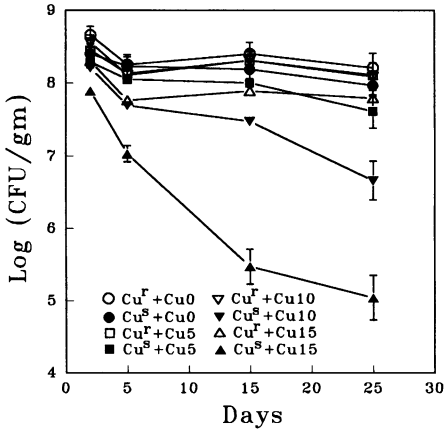

FIG. 3. Differential survival of copper-resistant (Cu^r) strain 09906.1 and copper-sensitive (Cu^s) strain 09906.2 of *P. fluorescens* in a sterile loamy sand amended with 0, 5, 10, or 15 μg of $CuSO_4$ per g of soil.

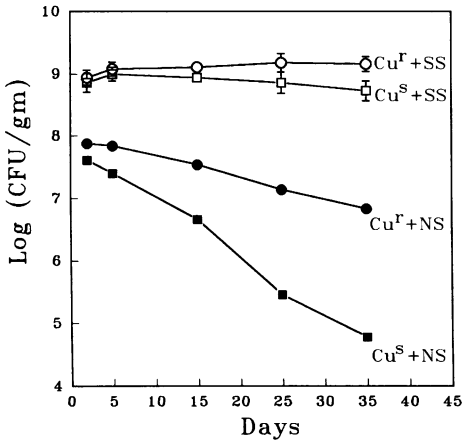

FIG. 4. Differential survival of copper-resistant (Cu^r) strain 09906.1 and copper-sensitive (Cu^s) strain 09906.2 of *P. fluorescens* in sterile (SS) versus nonsterile (NS) soil from a citrus grove.

may be more sensitive to toxic effects of other microbes, such as bacterial predators. Several kinds of nonobligate bacterial predators attack other bacteria when they are not able to obtain enough soluble nutrients in soil (10). *Cupriavidus necator* produces a factor that chelates copper and is toxic to microbes that are copper sensitive (7–9).

The loss of competitive fitness in the copper-sensitive mutants may also reflect a basic physiological importance of the copper resistance genes in this strain. Unlike *P. syringae* (13), copper resistance in *P. fluorescens* 09906 is apparently determined by chromosomal rather than plasmid-borne genes. A preliminary experiment (data not shown) suggested that copper-sensitive Tn*5* mutants of 09906 may be defective in copper uptake, since they did not grow well under copper-limiting conditions created by the addition of the specific chelator 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid. This chelator has been used to induce copper deficiency in plants (4). Copper resistance in 09906 may therefore have evolved from genes similar to the chromosomal *cut* genes of *E. coli*, which are essential for normal copper uptake and storage (6, 25, 26). The loss of competitive

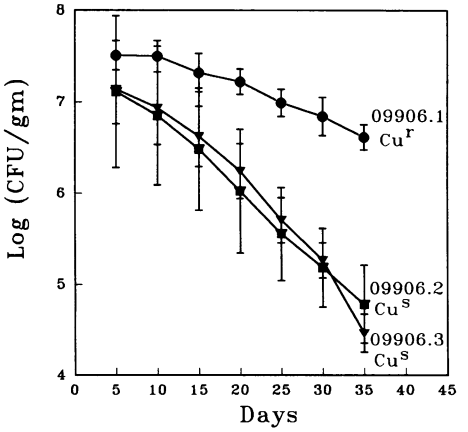

FIG. 5. Differential survival of copper-resistant (Cu^r) strain 09906.1 (●) of *P. fluorescens* and two copper-sensitive (Cu^s) mutants, 09906.2 (■) and 09906.3 (▼), in nonsterile soil from a citrus field.

survival in the copper-sensitive mutants of 09906, but not survival in sterile soil, suggests that if the resistance genes are involved in copper transport, then competition for copper uptake may be a factor in competitive survival of soil microbes.

Many species of *Pseudomonas*, including *P. fluorescens*, contain chromosomal genes that hybridize with the *P. syringae cop* operon, but the Tn*5* insertions that disrupted copper resistance in our mutants of 09906 were not in the *cop* homolog region. The insertions could have disrupted genes that regulate the *cop* homologs, but the different locations of independent Tn*5* insertions causing the same loss of resistance makes this seem unlikely. For the same reason, it is unlikely that we have mutated a single global regulon that could have caused a loss of many functions in addition to copper resistance, with subsequent effects on survival. These mutants survived well in sterile, low-copper soils, suggesting that the effects of the Tn*5* insertions were more specific. Cloning and characterization of these copper resistance genes and investigations of their physiological functions are in progress.

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