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Relationship of in vitro Antibiosis of Plant Growth-Promoting Rhizobacteria to Plant Growth and the Displacement of Root Microflora

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ABSTRACT

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In preliminary experiments, plant growth-promoting rhizobacteria (PGPR) genetically marked for resistance to rifampicin and nalidixic acid caused significant ($P = 0.01$) increases ranging from 300 to 500% in total weight of potato plants grown in field soils in the greenhouse. The five PGPR strains used were fluorescent *Pseudomonas* spp. that exhibited antibiosis on King's B medium against 10 or more of 40 rhizosphere bacterial isolates. Mutants that caused no antibiotic effects did not induce plant growth increases. Growth rates of mutants were similar to that of wild-type strains, and the mutants colonized plant roots with population densities similar to the wild-types. In field tests, wild-type PGPR caused increases in stolon development, but no increases were detected in plants grown from potato seed pieces treated with the mutants. Treatment of seed pieces with wild-type PGPR resulted in reductions in root zone fungal and Gram-positive bacterial population densities ranging from 23 to 64% and 25 to 93%, respectively. In contrast, no differences were detected in microbial populations on roots of control plants and plants treated with mutants having no antibiosis activity. These results indicate that the ability of PGPR to induce increased plant growth is related in part to antibiosis that occurs in root zones and the subsequent displacement of certain root colonizing microorganisms.

Plant growth-promoting rhizobacteria (PGPR) were recently used to increase plant growth in greenhouse and field tests (3,7,9,10,12,13) and dried inocula have been developed (7,13,14) to facilitate their use in the field. Experiments with radish, sugar beet, and potato indicated that the PGPR enhanced plant growth by changing the composition of rhizosphere microflora rather than by producing growth-stimulating substances which directly affected the plants (6,8,15). A general screening of the rhizobacteria for antibiosis activity showed that they were inhibitory to a wide variety of microorganisms (3,16). Although the PGPR exhibited antibiosis to native microflora (3,16), it was not known whether the antagonism in soil resulted from antibiosis, competition, or both. It is also unknown whether antibiosis by PGPR in culture is related to production of inhibitory substances on root surfaces.

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Microbial production of antibiotics was demonstrated in soil organic matter (17), but determining the role of antibiosis in the rhizosphere remains difficult. It is reasonable to expect that antibiosis should enhance the capacity of the producers to colonize root surfaces and to compete for nutrients in the rhizosphere.

This report contrasts the ability of antibiotic-negative mutants and wild-type PGPR to colonize the rhizospheres of potato plants, to displace root microflora, and to cause an increase in plant growth.

MATERIALS AND METHODS

Antibiotic activity by rhizobacteria. PGPR from radish and potato were tested for antibiosis against *Erwinia carotovora* subsp. *carotovora* (*Ec*) and a range of bacteria, including rhizosphere isolates from roots of healthy radish plants. The tested PGPR strains included fluorescent pseudomonads A1, B10, BK1, TL3B1, and TL3B2 from potato (10), E2, E6, E8, 14, and 15 from celery (9), and unidentified nonfluorescent Gram-negative strains 17, 111, 117, 121, 124, and E10 from radish (9). A 0.01-ml drop of a 10^9 cfu

(colony forming units) per milliliter of suspension of the test PGPR strain was spotted onto King's medium B (KB) agar plates and incubated 24 hr at 28 C. A suspension of the challenged bacterium ($\sim 10^8$ cfu/ml) was then sprayed over the KB plates, which were incubated for an additional 24 hr.

Isolation of mutants lacking in vitro antibiosis. Fluorescent *Pseudomonas* spp. PGPR resistant to rifampicin and nalidixic acid (rif, nal PGPR) at 100 μg/ml were stressed to mutate by exposure to *N*-methyl-*N*'-nitro-nitrosoguanidine (NTG), ultraviolet light at 254 nm, or ethyl methane sulphonate (EMS). For the NTG treatment, bacterial cells were centrifuged for 30 min at 6,000 *g*, washed, and suspended in sterile 0.1 M citrate buffer, pH 5.5.

TABLE 1. Colonization of roots by antibiosis-negative mutants is not substantially different than with wild-type plant growth-promoting rhizobacteria on potato in field tests

Field location and date	Seed piece treatment[a]	Population 2 wk after plant emergence (cfu/cm root)[b]
Tenneco ranch, 1978	TL3B2	1.9×10^2
	TL3B2 AB^{-1}	2.3×10^2
	A1	2.2×10
	A1 AB^{-1}	9.4×10^2
Shafter, CA, 1978	A1	4.2×10^3
	A1 AB^{-1}	4.0×10^3
Tenneco ranch, 1979	A1	9.6×10^5
	A1 AB^{-6}	8.0×10^5
	A1 AB^{-7}	5.6×10^4
Shafter, CA, 1979[c]	A1	6.0×10^0
	A1 AB^{-1}	6.3×10^2
	A1 AB^{-2}	5.3×10^2
	A1 AB^{-3}	2.0×10^3
	A1 AB^{-4}	0
	A1 AB^{-5}	2.0×10^1

[a] Strains A1 and TL3B2 are wild-type, antibiosis-positive fluorescent *Pseudomonas* spp. Strains A1 AB^- and TL3B2 AB^- are antibiosis-negative mutants, AB^{-1} is a nitrosoguanidine mutant, and AB^- to AB^{-7} are mutants induced by ultraviolet light.
[b] Average populations of three to five replications, three plants per replication, 50 cm per plant root.
[c] Field was planted under dry conditions and maintained in a dry state during the season.

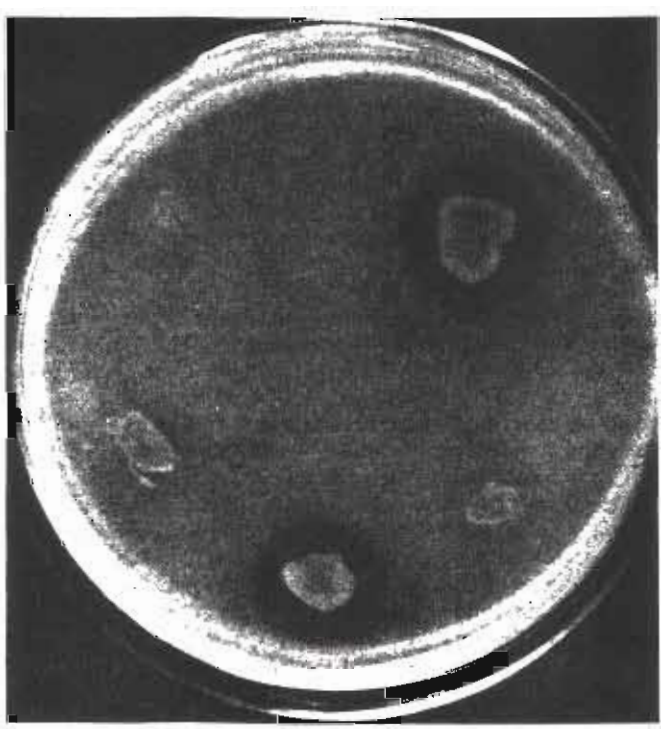

Fig. 1. Growth of three antibiosis-negative mutants, following mutagenesis with nitrosoguanidine, on medium seeded with *Erwinia carotovora* subsp. *carotovora*. Two strains retained in vitro antibiosis.

TABLE 2. Comparison of weights of 1-wk-old field-grown potato plants grown from seed pieces treated with wild-type plant growth-promoting rhizobacteria or with antibiosis-negative mutants

Seed piece treatment[a]	Average plant weight[b] (g)
A1	50*[c]
TL3B2	33*
Water	25
A1 AB^-	26
TL3B2 AB^-	22

[a] A1 and TL3B2 are wild-type Fluorescent *Pseudomonas* spp. and AB^- are nitrosoguanidine mutants lacking in vitro antibiosis.
[b] Average of five replications, per treatment, with three randomly sampled plants per replication.
[c] * indicates significant difference ($LSD_{0.05} = 4$).

TABLE 3. No increase in stolon length of field grown potato plants grown from seed pieces treated with antibiosis-negative mutants of plant growth-promoting rhizobacteria[a]

Seed piece treatment[b]	Average length of stolon[c] (cm)
TL3B2	50*[d]
A1	47*
Water	27
TL3B2 AB^-	21
A1 AB^-	24

[a] Stolon lengths were measured 2 wk after plant emergence.
[b] A1 and TL3B2 are wild-type fluorescent *Pseudomonas* spp. and AB^- are nitrosoguanidine mutants lacking in vitro antibiosis.
[c] Average of five replications, per treatment, with three randomly sampled plants per replication.
[d] * indicates significant difference ($LSD_{0.05} = 8$).

TABLE 4. No increase in plant growth in a greenhouse assay using antibiosis-negative mutants of plant growth-promoting rhizobacteria (PGPR) as seed treatments

Mutagenic agent[a]	Seed piece treatment[b]	Average weight per plant (g)[c,d]
NTG	A1	1.5
	BK1	1.1
	TL3B1	1.1
	TL3B2	1.0
	Water (control)	1.3
UV light	A1-2	1.0
	A1-3	0.5
	A1-4	0.7
	A1-5	0.7
	A1-6	0.9
	Water (control)	0.5
EMS	A1-7	3.2
	BK1-2	2.1
	TL3B1-2	4.2
	Water (control)	2.8
EMS	A1-8	1.4
	A1-9	3.3
	BK1-3	3.4
	B10-2	3.1
	Water (control)	5.3

[a] NTG = nitrosoguanidine, and EMS = ethyl methane sulphonate.
[b] Seed pieces were dipped into bacterial suspensions prior to planting in field soils; controls were dipped into water. All wild-type PGPR significantly increased growth in a similar assay.
[c] Average of six to eight replications per treatment with three plants per replication. There were no significant differences between means ($P = 0.05$).
[d] Wild-type PGPR consistently caused significant plant growth increases in a previous study (10).

TABLE 5. Displacement of rhizoplane fungi by plant growth-promoting rhizobacteria on potato roots in field tests

Field location	Seed piece treatment[a]	PGPR population[b] (cfu/cm root)	Rhizoplane fungal population[c] (cfu/cm)			
			3 days	% Change	7 days	% change
Tenneco Vedura	Water	0	1.1	...	2.0	...
	A1	9.6×10^5	0.4	−64	1.3	−35
	B10	3.8×10^4	0.5	−55	1.3	−35
	E6	8.5×10^5	0.4	−64	1.2	−40
Tenneco Rosedale	Water	0	1.2	...	1.3	...
	A1	2.8×10^5	0.5	−58	0.8	−38
	B10	3.3×10^4	0.7	−42	1.0	−23

[a] Fluorescent pseudomonads A1 and B10 were from potato periderms and E6 was from celery roots.
[b] Amount of root colonization by plant-growth-promoting rhizobacteria at sample time.
[c] Sample was taken 2 wk after plant emergence and represents six replications, each with 40 cm of roots. Populations were recorded 3 and 7 days after planting.

Nutrient broth (0.05%) and 200 μg/ml NTG were then added to each tube. After incubation for 30 min at 28 C, cultures were centrifuged, washed with sterile distilled water, resuspended in nutrient agar broth, and incubated 2 hr at 28 C. Serial 10-fold dilutions up to 10^{-5} were prepared from each tube and 0.1-ml aliquots were spread on KB plates, which were incubated for 48 hr at 28 C. Plates were sprayed with *Ec*, incubated 24 hr, and examined for colonies not exhibiting antibiosis against *Ec*. These colonies were removed; purified on rif, nal KB; transferred to normal KB; and challenged with *Ec* to confirm lack of antibiotic activity (Fig. 1).

For the UV treatment, bacterial suspensions containing 10^8 cfu/ml were prepared in 0.1 M $MgSO_4$. Five milliliters of each suspension was placed in glass petri dishes, and exposed 35 cm below a germicidal ultraviolet lamp (254 nm) for 40 sec. Serial 10-fold dilutions were prepared, and 0.1-ml aliquots were spread on KB. Antibiosis-negative mutants were selected as described above.

The EMS treatment was done by adding 3 ml of sterile nutrient broth inoculated with 0.1 ml of 24-hr-old PGPR broth cultures to tubes and incubating 6 hr at 28 C. EMS (0.15 per tube) was added and, following incubation for 1 hr, cultures were centrifuged and the pellets were washed and resuspended in sterile water. Antibiosis-negative mutants were selected as previously described. Mutants without antibiotic activity in vitro (AB^-) retained resistance to rifampicin and nalidixic acid and had similar generation times, reactions on Hugh-Liefson's O/F (4) and oxidase (11) tests that compared to wild-type PGPR.

Root colonization by antibiosis-negative mutants and their effects on plant growth. The ability of antibiosis-negative mutants to colonize plant roots in field tests was examined by dipping potato seed pieces (cultivar White Rose) at planting time in suspensions containing 10^9 cfu/ml of mutants in 0.1 M $MgSO_4$. The treated seed pieces were mechanically planted in four fields of sandy loam soil type near Shafter, CA. Eight different mutants were used, and one mutant (A1 AB^{-1}) was used in three fields. Experiments were designed as randomized blocks with five replications per treatment. Two weeks after plant emergence, the extent of rhizosphere colonization by the mutants was examined as previously described (10).

The effect of mutants on increased early-season plant growth was studied in the greenhouse and in the same fields described above, and rhizosphere populations were measured. Four greenhouse tests were done with 16 mutants. Seed pieces were dipped in 10^9 cfu/ml suspensions and planted in moist sandy loam field soil in 12.7-cm (5-inch)-diameter clay pots. Treatments were replicated six to eight times with three plants per replication. Effects of mutants on potato growth in the field were determined in one field by selecting and weighing plants at random 2 wk after emergence. Weights of plants treated with the two mutants and with two wild-type PGPR were compared. In a different field, the length of stolons 2 wk after emergence was measured on plants treated with two mutants and two wild-type PGPR.

Displacement of root microflora by PGPR. The effect of PGPR in displacing many commonly occurring fungi and Gram-positive bacteria on potato roots was determined with greenhouse and field-grown plants. Root zone fungal populations were measured on plants treated with PGPR strain A1 or one of four AB^- mutants in the greenhouse using the technique of Huisman and Ashworth (5). Eight replicate plates, each containing 40 cm of roots, were used for each treatment. Colony counts of rhizoplane fungi were recorded after 3 and 7 days of incubation on Cellophane extract agar (5). With field-grown plants, the rhizoplane fungal populations were measured in two tests on plants grown from seed pieces inoculated with wild-type PGPR A1, B10, and E6. Samples were taken and treated the same as in the greenhouse study.

TABLE 6. Displaeement of rhizoplane Gram-positive bacteria by plant growth-promoting rhizohacteria on potato roots in field tests

Field location	Seed piece treatment[a]	Root population of Gram-positive bacteria[b] (cfu/cm)	% Change compared to control
Tenneco Rosedale	Water	4.1×10^6	...
	A1	6.4×10^5	−84
	E6	3.6×10^5	−91
Tulelake, CA	Water	2.4×10^4	...
	B10	1.8×10^4	−25
	E6	2.4×10^3	−90
Tulelake, CA	Water	3.7×10^4	...
	A1	1.3×10^4	−65
	B10	2.6×10^3	−93

[a] Fluoreseent pseudomonads A1 and B10 were from potato periderms and E6 was from celery roots.
[b] Average of five replieatives with three plants per replieation; 50 cm of root were sampled per plant.

The effect of wild-type PGPR and AB^- mutants on population densities of Gram-positive bacteria on roots of greenhouse-grown plants was determined by using the previously described experimental design and the same strains. With field-grown plants, the changes in Gram-positive population densities were evaluated only with plants that were inoculated with wild-type PGPR. Three field tests were done, each with five replications per treatment, with three randomly selected plants per replication. A 50-cm root sample from each plant was placed in 10 ml of sterile 0.01 M $MgSO_4$, agitated, and serial 10-fold dilutions were prepared. Aliquots (0.1-ml) were spread on nutrient agar containing 30 μg/ml each of dicloran (botran), benomyl, mystatin, and 100 μg/ml cycloheximide, and potassium dichromate, which selectively inhibited Gram-negative organisms. Colony counts of Gram-positive bacteria were recorded after 48 hr of incubation at 28 C. The selectivity of the potassium dichromate nutrient agar (PDC-NA) for Gram-negative organisms was tested by streaking media with the wild-type PGPR (all Gram-negative) and by randomly conducting a Gram stain on 30 isolates which grew from root washings. No attempt was made to identify the bacteria further.

TABLE 7. No substantial reduction of root microflora by treatment with antibiosis-negative mutants of plant growth-promoting rhizobacteria in field soils in the greenhouse[a]

Seed piece treatment[b]	Population of Gram-positive bacteria (cfu/cm root)	Change compared to control (%)	Population of fungi (cfu/cm root)	Change compared to control[c] (%)
AB^{-1}	3.2×10^6	+88	2.1	+24
AB^{-2}	3.1×10^6	+82	2.1	+24
AB^{-3}	1.3×10^6	−24	1.7	0
AB^{-4}	1.2×10^6	−29	1.5	−12
Wild-type	4.7×10^5	−72	0.9	−47
Water	1.7×10^6	...	1.7	...

[a] Experiment was repeated twice with similar results.
[b] Four antibiosis negative mutants and the antibiosis-positive wild-type strain A1 were used. Mutants 1 and 2 were mutagenized with ethyl methane sulphonate, number 3 with ultraviolet light, and number 4 with nitrosoguanidine.
[c] Fungal populations on roots were determined after 7 days incubation at 26 C.

RESULTS

Antibiotic activity by rhizobacteria and isolation of mutants. All 16 PGPR had antibiotic activity in vitro against *Ec* and at least 10 or more of the 40 challenged rhizosphere bacteria. Diameters of zones of inhibition ranged from 3 to 35 mm on KB plates.

Mutants without antibiosis activity: root colonization and effects on plant growth. Sixteen mutants without antibiosis activity (AB^-) were obtained following mutagenesis of wild-type fluorescent pseudomonad PGPR. None of the AB^- mutants was fluorescent, but all retained resistance to rifampicin and nalidixic acid.

Rhizosphere colonization of the AB^- mutants was not substantially different from the wild-type PGPR with mutant populations ranging from 1.5 log units less than the wild-type PGPR to 1.5 greater in one field (Table 1). However, colonization of roots by the antibiosis-negative mutants did not result in an increase in total plant weight 2 wk after emergence as occurred with wild-type A1 and TL3B2 (Table 2). The mutants A1 AB and TL3B2 AB^- in contrast to wild-type PGPR also did not cause an increase in stolon length of field plants (Table 3). Furthermore, none of the 16 AB^- mutants caused an increase in plant growth in greenhouse assays (Table 4), while wild-type PGPR consistently increased plant growth in a previous study (10).

Displacement of root microflora by PGPR. Potato rhizosphere colonization by wild-type fluorescent pseudomonad PGPR resulted in reduced populations of both native rhizoplane fungi and Gram-positive bacteria in field tests. Fungal population reductions ranged from 23 to 64% on roots colonized by three PGPR in comparison to controls (Table 5). No attempt was made to identify all of the rhizoplane fungi; however, most of the displaced fungi were *Penicillium, Aspergillus*, and *Fusarium* spp. Common plant pathogens such as *Rhizoctonia solani* and *Pythium* spp. were infrequently encountered on either PGPR-treated or nontreated plants in these studies when fungal root colonization on Cellophane extract agar was measured.

Reductions in Gram-positive bacteria on roots colonized with PGPR ranged up to 93% and were greater than the reduction in fungal populations (Table 6). The PDC-NA medium used to determine populations of Gram-positive bacteria inhibited Gram-negative rhizosphere bacteria, and none was identified from platings of root washings on the medium.

Treatment of potato seedpieces with AB^- mutants did not result in a substantial reduction in the number of root microflora in greenhouse trials in contrast to the wild-type A1 (Table 7). The Gram-positive bacterial population densities on roots colonized by mutants ranged from 88% greater to 24% less than controls, while the wild-type caused a 72% reduction in numbers of Gram-positive bacteria. The mutants also did not substantially alter population densities of fungi on roots as did the wild-type strain A1.

DISCUSSION

The comparison of wild-type PGPR which exhibit antibiosis with AB^- mutants showed that the ability to produce antibiotics in vitro was directly related to the capacity of rhizobacteria to significantly increase plant growth. However, antibiosis was not related to the root-colonizing capacity of these bacteria since there was no substantial difference between wild-type and mutants, at least during the early stages of plant growth in the field. Although we did not determine if the AB^- mutants persisted on roots throughout the season (as do the wild-type PGPR [10]), they were present in approximately equal populations at the time when wild-type PGPR had already induced significant plant growth increases. It is unlikely that the AB^- mutants failed to increase plant growth as a result of multiple mutations, since 16 different mutants were used. The similarity of mutant growth rates with wild-type PGPR and the root colonization capacity of the AB^- mutants provides evidence that they were not "crippled."

The role of antibiotics is controversial since detection of antibiotics in soils is difficult. Our evidence for antibiotic production in the root zone by use of mutants and the evaluation of plant response and microbial displacement supports claims (17) of other workers that antibiotics are produced and are functionally active in the soil. It also supports suggestions that the mode of action of growth-promoting bacteria involves root zone antagonism. Various researchers (1–3,15) have suggested that PGPR produce antibiotics inhibiting growth of "saprophytic pathogens," which may be ubiquitous in root zones.

The capacity of rhizobacteria to colonize and inhibit certain components of the root zone microflora suggests that they have great potential for beneficially altering the composition of the rhizosphere leading to improved plant health. PGPR appear to be a relatively heterogeneous group since they are antagonistic to a different spectrum of rhizosphere organisms. Therefore, the treatment of plants with a combination of rhizobacteria antagonistic to various soilborne plant pathogens and quasipathogens could have a marked effect in reducing root disease if the rhizobacteria are not mutually inhibitory.

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