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Antagonism of *Pseudomonas putida* Strain PP22 to Phytopathogenic Bacteria and Its Potential Use as a Biocontrol Agent

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ABSTRACT

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Twenty-seven of 963 strains of epiphytic bacteria isolated from fruits of bell pepper and tomato were found to be antagonistic to *Erwinia carotovora* subsp. *carotovora* on KB medium. All but two of the antagonistic strains were characterized and identified: eight as *Pseudomonas fluorescens*, 16 as *P. putida*, and one as *Flavobacterium* sp. One strain of *P. putida*, PP22, inhibited the growth of a broad spectrum of phytopathogenic bacteria on media, including 24 strains of soft rot bacteria (in the genera of *Erwinia, Pseudomonas, Xanthomonas*, and *Cytophaga*), one strain of *P. solanacearum*, four pathovars of *X. campestris*, and five pathovars of *P. syringae*. Strain PP22 produced iron-chelating siderophores and an antibacterial compound that was heat- and trypsin-resistant. This strain suppressed the growth of *E. c.* subsp. *carotovora* on potato slices and survived on the tubers and roots of potato plants for more than 5 wk. Application of strain PP22 to potato tubers reduced the severity of bacterial soft rot caused by *E. c.* subsp. *carotovora* and *X. campestris* by an average of 21 and 44%, respectively, in four separate trials.

Bacterial soft rot is the leading cause of decays of many vegetables after harvest. It accounts for over 80% of disorders of several vegetables at transit or in markets where the produce is sold (14). *Erwinia cartovora* is generally thought to be the principal cause of the problem (1,14), but pectolytic bacteria belonging to the genera of *Pseudomonas* (5,13), *Xanthomonas* (12), *Cytophaga* (11), *Clostridium* (3), *Flavobacterium* (15), and *Bacillus* (6) are also involved. Control measures generally available for postharvest decays of vegetables are not always effective for control of bacterial soft rot. Moreover, available measures have little systemic activity and cannot reduce the development of diseases in most inoculated produce (1). A new control strategy that aims at this important problem awaits development (7).

Biological control of plant diseases by microbial agents has been extensively investigated during the past few years (2,19). Some success in using bacteria as biocontrol agents of postharvest pathogens has been reported. Application of *Bacillus subtilis* (B-3) reduced Monilinia brown rot of stone fruits in laboratory tests (17). Colyer and Mount (4) isolated a strain of *P. putida* from tomato fruits and showed that this strain was able to reduce the soft rot of potato tubers by 75%. Xu and Gross (21,22) reported that strains of *P. fluorescens* and *P. putida* isolated from potato plants and soil suppressed seed piece decay and increased the yield of potato tuber in the field by 10–11%. Rhodes and Logan (18) recently developed a method for selecting fluorescent pseudomonads with potential for controlling blackleg disease of potato. Although the use of microbial agents for controlling bacterial soft rot appears promising, the commercial feasibility of this practice has not been proved.

This report concerns the isolation and characterization of a strain of *P. putida* that inhibits the in vitro growth of a broad spectrum of phytopathogenic bacteria and actively colonizes the tuber and roots of potato plants. A preliminary account of this work has been presented (9).

MATERIALS AND METHODS

Isolation and characterization of antagonists. Healthy bell pepper and tomato fruits purchased from local supermarkets were used. The surface of fruit (5 cm^2) was washed three to five times with 1 ml of sterile water. Samples of the wash fluid were streaked on crystal violet-pectate medium (CVP) (5). Nonpectolytic colonies were selected and tested for ability to inhibit the growth of *Erwinia carotovora* subsp. *carotovora* (strain SR319) on King's medium B (KB). Activity was determined by the overlay-agar techniques previously described for the assay of bacteriocin (20). Nonpectolytic colonies were transferred from CVP medium onto KB agar plates at four colonies per plate. After incubation at 28 C for 2 days, colonies were killed by chloroform vapor and each culture plate was immediately overlaid with 3 ml of a suspension of *E. c.* subsp. *carotovora* (10^6 cfu/ml) prepared in 0.6% warm (50 C) water agar. Two days later, zones of inhibition, if present, were measured from the edge of the producing colony to the perimeter of the clear zone. Antibiosis-positive strains were further identified and characterized on the basis of results of conventional bacteriological tests. Strains of fluorescent *Pseudomonas* spp. were identified according to the determinative scheme of Palleroni (16), and *Flavobacterium* sp. was identified according to the descriptive scheme of Lund (15).

Screening for siderophore and antibiotic production. Antagonists obtained from the initial screening were tested for ability to inhibit the growth of *E. c.* subsp. *carotovora* on three agar media: nutrient agar (NA), KB, and KB + 1 mM $FeCl_3$ (KB-Fe^{+++}). Presumptively, strains that inhibited *E. c.* subsp. *carotovora* on KB but not on NA or KB-Fe^{+++} medium were considered to produce siderophores, whereas those that inhibited *E. c.* subsp. *carotovora* on all three media were considered to produce antibiotics or a combination of antibiotics and siderophores. The antagonistic strains were later tested for antibacterial activity against 34 strains of phytopathogenic bacteria seeded on KB, NA, and KB-Fe^{+++} media.

Detection of antibacterial activity in broth medium. *P. putida* strain PP22 isolated during the study was grown in Difco nutrient broth supplemented with 0.1% yeast extract at 28 C for 2 days. The supernatant from centrifugation (10,000 *g* × 10 min) of the culture was concentrated to one-tenth of the original volume in a rotatory evaporator at 58 C. Next, the concentrated supernatant was heated at 100 C for 20 min or digested with trypsin at a concentration of 100 μg/ml at 37 C for 1 hr. A 20-μl sample of the treated culture extract was deposited onto filter disks (7 mm in diameter) that had been placed on NA plates seeded with *E. c.* subsp. *carotovora*. Zones of inhibition were measured around the filter disks after 2 days of incubation at 28 C.

Isolation of mutants. To facilitate the study of interactions between the pathogen and the antagonist on plant tissues, two mutants of *P. putida* strain PP22 and one of *E. c.* subsp. *carotovora* strain SR319 were isolated. The mutant

E. c. subsp. *carotovora*, designated as Ecc (Cm^r Gm^r) and resistant to both chloramphenicol and gentamicin, was isolated from the parent strain by a spontaneous, two-stepped mutation. A 0.1-ml sample of a bacterial suspension containing approximately 7×10^{10} cfu/ml of cells of *E. c.* subsp. *carotovora* strain SR319 was spread over a NA plate supplemented with chloramphenicol (30 μg/ml). A colony growing on the NA-chloramphenicol plate was selected, purified, and designated as Ecc (Cm^r). The Ecc (Cm^r) suspension containing 7×10^{10} cfu/ml of cells was subsequently spread over a NA plate supplemented with both chloramphenicol (30 μg/ml) and gentamicin (20 μg/ml). A colony growing on the NA-chloramphenicol-gentamicin plate was isolated, purified, and designated as Ecc (Cm^r Gm^r). A mutant PP22 (Rm^r Sm^r), resistant to both rifampicin (50 μg/ml) and streptomycin (100 μg/ml), was isolated from the parent strain, *P. putida* PP22, by the same procedure as described above. This mutant PP22 (Rm^r Sm^r) was further mutagenized with Tn*5* by using *E. coli* SM10 (pSUP1011) as a vector (10). Transconjugants growing on KB supplemented with rifampicin (50 μg/ml) and kanamycin (30 μg/ml) were isolated and screened for absence of activity against *E. c.* subsp. *carotovora* on NA medium. The antibiosis-negative mutant obtained from Tn*5* transposition was designated as PP22 (Rm^r Sm^r Ab^-).

Colonization on potato tubers and roots by strain PP22. To determine whether strain PP22 was able to colonize or to survive on the surface of plants, untreated potato tubers were exposed to an aqueous suspension of strain PP22 (Rm^r Sm^r) and bacterial populations were monitored weekly over a 5-wk period. Potato tubers (cv. Russet Burbank) were submerged in an aqueous suspension of strain PP22 (Rm^r Sm^r) cfu/ml) for 20 min. Tubers were removed from the bacterial suspension and allowed to air-dry at room temperature. Forty tubers were incubated in a controlled-environment chamber (15 C, 70% humidity) and 50 were planted in sandy loam soil in the greenhouse. Two or three tubers were removed periodically from the chamber, and 10 g of the periderm was peeled and ground in 10 ml of sterile water. The resulting suspensions were serially diluted, and 0.1-ml portions were spread on KB medium supplemented with rifampicin and streptomycin. Similarly, 10 g of roots was collected from potato plants grown in the greenhouse 2 wk after plant emergence. The roots were placed into a flask containing 100 ml of water and incubated for 1 hr. The flask was vigorously shaken for 2 min, and a sample of liquid phase was serially diluted; 0.1-ml portions of the dilutions were spread on KB-rifampicin-streptomycin medium.

Effect of strain PP22 on growth of *E. c.* subsp. *carotovora* in vivo. Sterile potato disks, 18 mm in diameter and 3 mm deep, were prepared as previously described (12) and placed on water agar plates, three disks per plate. Disks on the same plate were inoculated with one of the following five bacterial suspensions: Ecc (Cm^r Gm^r), PP22 (Rm^r Sm^r), PP22 (Rm^r Sm^r Ab^-), Ecc (Cm^r Gm^r) + PP22 (Rm^r Sm^r), and Ecc (Cm^r Gm^r) + PP22 (Rm^r Sm^r Ab^-). Bacteria grown to log phase in nutrient broth were pelleted from the broth by centrifugation and resuspended in sterile water to make a final concentration of about 6×10^6 cfu/ml. The suspension containing both *E. c.* subsp. *carotovora* and PP22 was prepared by mixing an equal volume of each strain in the tube. Each disk was inoculated with 50-μl portions of suspension. Bacterial populations were determined immediately after inoculation and again after 3 days of incubation at 28 C. At these times, single disks were removed and placed into 10 ml of water in tubes. The tubes were vigorously shaken for 3 min, then samples of the liquid phase of the resulting suspensions were serially diluted and spread on the selective medium. A NA medium supplemented with chlorampenicol and gentamicin was used to determine the population of *E. c.* subsp. *carotovora*, and one with rifampicin and streptomycin was used to determine the population of strain PP22.

Effect of strain PP22 on the development of soft rot in potato tubers. Two similar batches of potato tubers (Russet Burbank) were removed from storage and artificially wounded (6 mm deep) with a 26-gauge syringe needle at eight sites per tuber. One batch of eight to 10 tubers was placed in a basket and submerged in 4 L of an aqueous suspension of strain PP22 (10^7 cfu/ml), and another batch was placed in water alone. The tubers were removed 20 min later and allowed to air-dry at room temperature. Each batch of tubers was further divided into two groups; one was sprayed with a water suspension of *E. c.* subsp. *carotovora* (10^5 cfu/ml) and the other was sprayed with an aqueous suspension of a soft-rotting strain of *X. campestris*, Xc5 (10^5 cfu/ml) (12). Tubers receiving the same treatment were allowed to air-dry, then were placed in polyethylene bags, weighed, and incubated at room temperature (about 20 C) for 5 days. After incubation, tubers were removed from the bag and immediately washed under a steady stream of water to remove rotted tissue. The weight of soft-rotted tubers was determined by calculating the difference between the weight of tubers before the treatment and the weight of unrotted tissue after incubation. The percentage of weight loss was calculated by dividing the weight of soft-rotted tubers by the weight of tubers before treatment.

Table 1. Comparison of inhibitory effects of eight antibiotic-producing strains of antagonists on the growth of *Erwinia carotovora* subsp. *carotovora* on three agar media[a]

Antagonists	Inhibition zone (mm) on:		
	KB[b]	NA[c]	KB-Fe^{+++}[d]
Pseudomonas putida			
PP6	11.2	12.1	8.1
PP11	8.6	6.3	7.9
PP22	16.1	13.7	15.1
PP23	14.5	7.3	9.2
Pseudomonas fluorescens			
PF9	9.3	5.8	7.1
Flavobacterium sp.			
FB3	6.2	4.6	6.3
Unidentified			
UD1	12.7	12.3	11.2
UD2	5.2	4.2	4.2

[a] Antagonists were grown on King's medium B (KB), Difco nutrient agar (NA), and KB + 1 mM $FeCl_3$ (KB-Fe^{+++}). The indicator bacterium, *E. c.* subsp. *carotovora*, was added at the concentration of 10^6 cfu/ml. Values are means of three replicates repeated on two occasions ± the standard deviation.
[b] LSD ($P \leq 0.05$) = 1.0.
[c] LSD ($P \leq 0.05$) = 0.6.
[d] LSD ($P \leq 0.05$) = 0.5.

RESULTS

Of 963 strains of epiphytic bacteria isolated from fruits of bell pepper and tomato, 27 were antagonistic to *E. c.* subsp. *carotovora* grown on KB medium. After extensive characterization, these antagonists were identified as *P. fluorescens* (eight strains), *P. putida* (sixteen strains), and *Flavobacterium* sp. (one strain); two strains remained unidentified. Although all 27 strains were antagonistic to *E. c.* subsp. *carotovora* and formed inhibition zones ranging from 5 to 11 mm in diameter on KB medium, the majority of strains (seven of *P. fluorescens* and 12 of *P. putida*) failed to do so on NA or KB medium supplemented with $FeCl_3$. Eight strains (one of *P. fluorescens*, four of *P. putida*, one of *Flavobacterium* sp., and the two unidentified strains) inhibited the growth

of *E. c.* subsp. *carotovora* to various degrees on NA, KB, and KB-Fe^{+++} agar media (Table 1). Strain PP22 of *P. putida* caused the highest inhibition zone in each medium and was chosen for further investigation. Thirty-four strains of phytopathogenic bacteria belonging to four genera were inhibited by strain PP22 (Table 2). The pattern of inhibition was affected by the composition of the medium on which test bacteria were grown. On KB medium, inhibition zones ranging from 13 to 23 mm in diameter were observed for all phytopathogenic bacteria tested. When KB-Fe^{+++} and NA media were used, one strain each of *E. c.* subsp. *atroseptica, E. chrysanthemi, P. marginalis*, and *P. solanacearum* were not inhibited by PP22. Attempts were made to isolate antibiosis-negative mutants by transposon mutagenesis. A mutant that produced a normal level of fluorescence but showed no activity against *E. c.* subsp. *carotovora* on KB medium was obtained. This mutant PP22 (Rm^r Sm^r Ab^-) retained full capacity to inhibit *P. solanacearum* and *P. marginalis* (one strain) grown on KB medium; the ability to inhibit *E. chrysanthemi* and *E. atroseptica* grown on NA or KB-Fe^{+++} medium was partially reduced, however. The antibacterial compound produced by strain PP22 was not inactivated by heat treatments (100 C, 20 min) or by digestion with trypsin (100 μg/ml, 37 C, 1 hr), which suggests that the antibiotic produced by strain PP22 is most likely not proteinaceous.

Strain PP22 was tested for ability to colonize and survive on the surface of tubers and roots of potato plants. Bacterial counts on tubers and roots declined (Fig. 1), but the population of PP22 (Rm^r Sm^r) remained relatively high (10^4 cfu/gm of tissue) throughout the 5-wk period monitored. Thus, strain PP22 is able to colonize, or at least to survive on, the surface of plants for at least 5 wk.

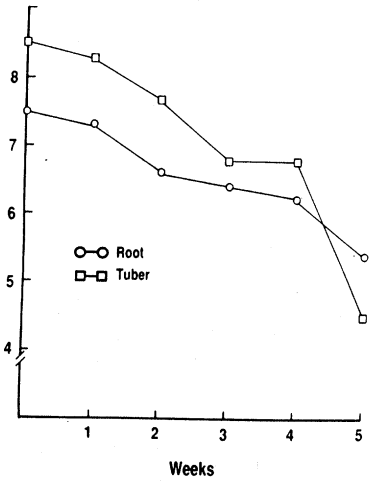

Fig. 1. Populations of *Pseudomonas putida* strain PP22 (Rm^r Sm^r) on the surface of potato tubers and potato roots for a 5-wk period after plant emergence.

Strain PP22 suppressed the growth of *E. c.* subsp. *carotovora* and the development of soft rot on potato disks (Table 3). When Ecc (Cm^r Gm^r) alone was inoculated onto potato disks, the pathogen multiplied normally. The population increased from 4.8×10^6 to 5.5×10^9 cfu per disk, and soft rot was observed. When Ecc (Cm^r Gm^r) was inoculated in combination with PP22 (Rm^r Sm^r) onto potato disks, the population of Ecc (Cm^r Gm^r) decreased from 2.1×10^6 to 5.2×10^5 cfu per disk, and the development of soft rot in potato disks was reduced. When disks were coinoculated with antibiosis-negative mutant PP22 (Rm^r Sm^r Ab^-) and Ecc (Cm^r Gm^r), however, the *E. c.* subsp. *carotovora* multiplied normally and soft rot developed. Thus, the ability of strain PP22 (Rm^r Sm^r) to retard the growth of Ecc (Rm^r Sm^r) appears due to the production of an inhibitory substance rather than to competition of nutrients or attachment sites. Moreover, strain PP22 is capable of multiplication to some degree on potato disks irrespective of the presence or absence of the pathogen.

To determine whether it could be used as a biocontrol agent, strain PP22 was tested for ability to reduce the development of soft rot in potato tubers artificially inoculated with *E. c.* subsp. *carotovora* or with a soft-rotting strain of *X. campestris* (Xc5). The reduction in severity of soft rot over four separate tests ranged from −12 to 50% for *E. c.* subsp. *carotovora* and from 0 to 73% for Xc5 (Table 4). Strain PP22 appears to be more antagonistic to Xc5 than to *E. c.* subsp. *carotovora*. The average reduction was 44% for Xc5 and 21% for *E. c.* subsp. *carotovora*.

Table 2. Inhibitory effects of *Pseudomonas putida* strain PP22 on phytopathogenic bacteria[a]

Bacteria	Sources	Range of inhibition zone[b] (mm) on: KB	KB-Fe^{+++}	NA
Soft rot bacteria				
Erwinia carotovora subsp. *carotovora* (8 strains)	(13)	13–19	11–17	8–11
E. c. subsp. *atroseptica* (SR-8)	A. Kelman	15–21	13–18	0
E. chrysanthemi (120A)	A. Kelman	17–19	0	11–13
Xanthomonas campestris (5 strains)	(12)	17–23	14–19	9–18
Pseudomonas marginalis (5 strains)	(13)	13–18	0–12	0–14
P. viridiflava (3 strains)	(13)	15–19	13–16	5–13
Cytophaga johnsonae (1 strain)	(11)	19–22	6–10	8–13
P. solanacearum (1 strain)	T. Denny	14–17	0	0
X. campestris pvs. *phaseoli, vesicatoria, campestris, citri* (1 strain each)	E. Civerolo	18–21	17–23	11–19
P. syringae pvs. *lachrymans, savastanoi, syringae, glycinea, phaseolicola* (1 strain each)	W. Fett	16–18	9–19	13–16

[a]Strain PP22 was grown on King's medium B (KB), KB + 1 mM $FeCl_3$ (KB-Fe^{+++}), or Difco nutrient agar (NA). Experiments were repeated twice, one duplication each time.
[b]Lowest and highest inhibition zone observed with each specie.

Table 3. Effects of *Pseudomonas putida* strain PP22 and its antibiotic-defective (Ab^-) mutant on multiplication of *Erwinia carotovora* subsp. *carotovora* (Ecc) on potato disks

Treatments[a]	Bacterial counts (log cfu/disk): Initial	3 Days after incubation	Rot[b]
1. Ecc (Cm^r Gm^r) alone	6.68	9.74	+
2. PP22 (Rm^r Sm^r) alone	6.80	7.86	−
3. PP22 (Rm^r Sm^r Ab^-) alone	6.76	7.83	−
4. 1 + 2			±
Ecc (Cm^r Gm^r)	6.32	5.72	...
PP22 (Rm^r Sm^r)	6.65	7.97	...
5. 1 + 3			+
Ecc (Cm^r Gm^r)	6.36	8.23	...
PP22 (Rm^r Sm^r Ab^-)	6.58	7.34	...

[a]Experiments were repeated twice, two duplications each treatment. Data represent the mean of two replications repeated on two occasions.
[b]+ = Total maceration, − = no maceration, ± = reduced maceration.

Table 4. Reduction of bacterial soft rot of potato tubers by treatment with *Pseudomonas putida* strain PP22

Treatments[a]	Percent tubers soft-rotted[b]					Av. percent reduction[c]
	Test 1	Test 2	Test 3	Test 4	Av.	
Ecc + H_2O	14[d]	38	25	20	24	···
Ecc + PP22	10	29	28	10	19	21
Xc5 + H_2O	11	18	21	23	18	···
Xc5 + PP22	3	6	7	23	10	44

[a]Tubers were wounded with a needle and treated with strain PP22 or with H_2O as a control and subsequently challenged with either *Erwinia carotovora* subsp. *carotovora* (Ecc) or *Xanthomonas campestris* (Xc5).

[b]Calculated by the formula: [(gram weight of tubers soft-rotted)/(gram weight of tubers before treatment)] × 100.

[c]Calculated by the formula: [(percent loss in control − percent loss in treated)/(percent loss in control)] × 100.

[d]Data were analyzed by analysis of variance using a factorial experimental design within experiments. All components of the analysis were significant at the 1% level.

DISCUSSION

Epiphytic bacteria antagonistic to soft rot pathogens of harvested fruits and vegetables are commonly present on the surface of plants. Twenty-seven strains antagonistic to *E. c.* subsp. *carotovora* on KB medium were isolated and characterized; 24 were identified as either *P. fluorescens* or *P. putida*. Fluorescent pseudomonads usually coexist with other microorganisms in diverse environments, including soil, water, and biomaterials (16). The prevalence of this group in nature may be due to their catabolic versatility (16) and their ability to produce a broad spectrum of secondary metabolites that may be toxic to other microorganisms (8). Two species, *P. putida* and *P. fluorescens*, are frequently isolated from plant tissue or soil and reported as potential biocontrol agents of phytopathogens (4,21,22). Three lines of evidence presented in this study suggest that antibacterial activity of *P. putida* strain PP22 may have been associated with the production of both fluorescent siderophores and antibiotics: 1) Antibacterial activity of strain PP22 against most strains of phytopathogenic bacteria can be detected in media limiting the siderophore production; 2) the Tn*5*-generated mutant PP22 (Rm^r Sm^r Ab^-) produces a normal level of fluorescence but fails to inhibit the growth of *E. c.* subsp. *carotovora* on KB medium; and 3) this mutant retains the full capacity to inhibit the growth of *P. solanacearum* and one strain of *P. marginalis* on KB medium, although the ability to inhibit *E. c.* subsp. *carotovora* and other phytopathogenic bacteria has been inactivated by Tn*5* insertion.

In addition to ability to produce an antibacterial compound, strain PP22 possesses several other properties that meet the requirements of a biocontrol agent. It colonized the tubers and roots of potato plants and reduced the development of soft rot on potato tubers. The variation in the efficacy of control as shown in Table 4 may be due to several factors. One possibility is the presence of natural antagonists of *P. putida* strain PP22 on the surface of the plants. Previous studies (6,18,21,22) and the one reported here have focused on how artificially introduced antagonists affect pathogens or plants. Almost no information is available on the interaction between potential antagonists and natural epiphytic microflora that might occupy the same ecologic niche on plants. An effective biocontrol agent may not be discovered or constructed until the dynamics of the five-way interaction of antagonist, pathogen, plant, epiphytic microflora, and environment are fully understood.

Several strains of *P. putida* have been previously isolated and shown to be potential biocontrol agents of the soft rot bacterium *E. carotovora* (2,4,19,21,22). The relatedness of *P. putida* strain PP22 examined in this study to *P. putida* strain M17 described by Colyer and Mount (4) and *P. putida* strain W4P63 described by Xu and Gross (22) is presently unknown. Strains M17 and W4P63 were targeted only for control of *E. carotovora*, and their activity against other phytopathogenic bacteria has not been reported (4,21,22). Strain PP22 has been shown to be antagonistic, at least in vitro, to an extremely broad spectrum of bacteria. This strain is therefore potentially useful for control of not only diverse groups of soft rot bacteria but also other field pathogens such as *P. syringae* and *X. campestris*.

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