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Hrp⁻ Mutants of *Pseudomonas solanacearum* as Potential Biocontrol Agents of Tomato Bacterial Wilt

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There have been many attempts to control bacterial wilt with antagonistic bacteria or spontaneous nonpathogenic mutants of *Pseudomonas solanacearum* that lack the ability to colonize the host, but they have met with limited success. Since a large gene cluster (*hrp*) is involved in the pathogenicity of *P. solanacearum*, we developed a biological control strategy using genetically engineered Hrp⁻ mutants of *P. solanacearum*. Three pathogenic strains collected in Guadeloupe (French West Indies) were rendered nonpathogenic by insertion of an Ω-Km interposon within the *hrp* gene cluster of each strain. The resulting Hrp⁻ mutants were tested for their ability to control bacterial wilt in challenge inoculation experiments conducted either under growth chamber conditions or under greenhouse conditions in Guadeloupe. Compared with the colonization by a pathogenic strain which spread throughout the tomato plant, colonization by the mutants was restricted to the roots and the lower part of the stems. The mutants did not reach the fruit. Moreover, the presence of the mutants did not affect fruit production. When the plants were challenge inoculated with a pathogenic strain, the presence of Hrp⁻ mutants within the plants was correlated with a reduction in disease severity, although pathogenic bacteria colonized the stem tissue at a higher density than the nonpathogenic bacteria. Challenge inoculation experiments conducted under growth chamber conditions led, in some cases, to exclusion of the pathogenic strain from the aerial part of the plant, resulting in high protection rates. Furthermore, there was evidence that one of the pathogenic strains used for the challenge inoculations produced a bacteriocin that inhibited the in vitro growth of the nonpathogenic mutants.

Bacterial wilt caused by *Pseudomonas solanacearum* E. F. Smith is a serious disease of numerous commercially valuable plant species of tropical and subtropical countries. To date, the means available for controlling the disease are still limited. Among them, biological control has been investigated with an increased interest and has been recently reviewed (31). In the last decade, numerous studies have been devoted to bacteria showing a capacity to control *P. solanacearum* either by producing antibiotics or bacteriocins which inhibit growth of the pathogen within the rhizosphere or by inducing host plant resistance. These bacteria are distributed among different genera and species including *Pseudomonas fluorescens* (1, 9, 22), *Pseudomonas glumae* (15), *Pseudomonas cepacia* (2), *Bacillus* spp. (1, 14), *Erwinia* spp. (14), and spontaneous nonpathogenic mutants of *P. solanacearum* (7, 20, 22, 24, 29).

Although such studies frequently gave promising results under in vitro or controlled conditions, the use of bacteria antagonistic towards *P. solanacearum* in naturally infested soils has met with limited success. Poor competition ability and poor edaphic adaptation of the introduced organism that is in competition with the indigenous microflora, particularly within the rhizosphere, are cited as the most probable causative explanations.

In this respect, recent consideration has been given to Tn*5*-induced nonpathogenic mutants obtained by random insertions within the *hrp* gene cluster of *P. solanacearum* (5). These Hrp⁻ mutants, though nonpathogenic on young tomato plants, retained the ability to infect from the soil and to multiply to some extent within the host plant (30). This limited invasion was advantageous, as the most invasive Hrp⁻ mutants were capable of excluding pathogenic strains from the susceptible host (32). This suggested that Hrp⁻ mutants could be used as endophytic biocontrol agents over a longer period, i.e., up to fruit harvest.

Ultimately, this approach must be tested under field conditions. However, such experimentation using Tn*5*-induced mutants is not ecologically acceptable because (i) the transposon could be a source of genetic instability in the mutants and (ii) transposition of Tn*5* might lead to its transfer to other bacteria, including human pathogens. It was therefore necessary to construct ecologically acceptable mutants.

Among the original Tn*5*-induced mutants of *P. solanacearum*, strain GMI1353 was one of the mutants which showed the greatest potential as biocontrol agents (32) and was therefore chosen as the prototype strain. DNA sequencing has shown that the Tn*5* insertion in GMI1353 was located within *hrpO* (16). We decided to construct a new *hrpO* mutant by inserting the nontransposable Ω-Km interposon (11) within *hrpO*. Since the *hrp* region is conserved among all strains of *P. solanacearum* so far tested (4), we subsequently transformed a series of pathogenic strains by marker exchange mutagenesis.

In this paper, we report the construction and characterization of these Ω-Km interposon-induced mutants. The objectives of this work were (i) to study the temporal and spatial patterns describing the establishment of an Hrp⁻ mutant in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli DH5α	*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* φ80d*lacZ* ΔM15	Bethesda Research Laboratory
P. solanacearum		
GMI1000	Wild type, isolated from tomato in French Guyana, 1966, race 1, bv. IV	25
GA2	Wild type, isolated from eggplant in Guadeloupe, 1986, race 1, bv. III	28
GA4	Wild type, isolated from eggplant in Guadeloupe, 1983, race 1, bv. III	28
GT4	Wild type, isolated from tomato in Guadeloupe, 1984, race 1, bv. III	28
GT1	Wild type, isolated from tomato in Guadeloupe, 1985, race 1, bv. I	28
GMI1353	Tn*5*-induced *hrp* mutant of GMI1000	32
GMI8171	Ω-Km-induced *hrp* mutant of GA2	This work
GMI8172	Ω-Km-induced *hrp* mutant of GA4	This work
GMI8173	Ω-Km-induced *hrp* mutant of GT4	This work
GMI8174	Ω-Km-induced *hrp* mutant of GMI1000	This work
GMI8217	Spontaneous Sm^r Rif^r derivative of GT1	This work
GMI8220	Spontaneous Sm^r derivative of GA2	This work
GMI8224	Spontaneous Sm^r derivative of GA4	This work
Plasmids		
pHP45Ω-Km	pHP45 (2.4 kb) carrying the Ω-Km interposon (2.3 kb)	11
pGMI885	pBR322 carrying a 19-kb *Eco*RI-*Eco*RI fragment from GMI1000, with the *hrpO*::Tn*5* mutation	6
pVir2	pLAFR3 carrying part of the *hrp* gene cluster	6
p2-17	Bluescript (3.0 kb) carrying the 8-kb *Eco*RI-*Eco*RI fragment from pVir2 plasmid	C. Boucher
pPMV137	Deletion of a 1.3-kb *Cla*I-*Cla*I fragment from p2-17	This work
pPMV138	Insertion of the Ω-Km interposon at the *Eco*RV site of pPMV137	This work

tomato tissues, (ii) to determine whether these mutants affected fruit production, (iii) to assess the protective effect of these mutants on bacterial wilt over the entire length of a growing season, as is required for management of this disease in production agriculture, and (iv) to evaluate the potential transfer of the Ω-Km interposon from the Hrp^- mutants to a coinoculated strain of *P. solanacearum* in the stem tissues of tomato plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The origin and relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria medium (23). *P. solanacearum* was streaked on Kelman's medium (21) and grown at 30°C in peptone broth (5). All Hrp^- mutants of *P. solanacearum* were resistant to kanamycin (50 mg $liter^{-1}$), and the pathogenic strains were resistant to streptomycin (200 mg $liter^{-1}$) or streptomycin and rifampin (50 mg $liter^{-1}$).

DNA methods. The standard methods (plasmid DNA extraction, restriction enzyme digestion, DNA ligation, agarose gel electrophoresis, transformation of *E. coli* by electroporation, Southern blotting, and hybridization) were performed as described by Ausubel et al. (3). Isolation of genomic DNA from *P. solanacearum* and transformation of *P. solanacearum* by linearized plasmid DNA were performed as described by Boucher et al. (5).

Construction of plasmid pPMV138. Physical localization of the Tn*5* insertion in GMI1353 was determined by restriction analysis of the plasmid pGMI885. The restriction map of pGMI885 was compared with that of plasmid p2-17 carrying the corresponding wild-type fragment of *P. solanacearum* GMI1000. This showed that the Tn*5* insertion in GMI1353 was within 100 bp of a unique *Eco*RV site. Furthermore, DNA sequencing has shown that the *Eco*RV site and thus the Tn*5* insertion in GMI1353 are located within *hrpO* (16). Thus, the Ω-Km interposon was inserted at this *Eco*RV site of plasmid p2-17. The Ω-Km interposon confers kanamycin resistance to *P. solanacearum* and encodes transcriptional and translational terminators in both orientations (12, 26). Thus, insertion of the interposon creates a polar mutation. Furthermore, it is unable to transpose or promote illegitimate recombination.

The *Eco*RV site of the Bluescript vector was removed by deletion of a 1.3-kb *Cla*I-*Cla*I fragment. The resulting plasmid, pPMV137, was digested by *Eco*RV. The Ω-Km interposon was purified as a 2.4-kb *Eco*RI-*Eco*RI fragment from the pHP45Ω-Km plasmid, and the extremities of the fragment were filled in by treatment with the Klenow fragment. This fragment was then ligated with the *Eco*RV-digested pPMV137. The ligation product was then used to transform *E. coli* DH5α, and the kanamycin- and ampicillin-resistant clones were selected. This allowed us to obtain plasmid pPMV138.

Plant material and growth conditions. The experiments were conducted in a growth chamber in a P3 containment laboratory (16 h of light, 200 microeinsteins m^{-2} s^{-1}, 30°C, 50% relative humidity; 8 h of dark, 23°C, 95% relative humidity). Additional experiments were carried out in seminatural conditions in a greenhouse in Guadeloupe (French West Indies). These experiments were performed in accordance to the recommendations of the French Biosafety Committee, during two seasons (cool season: November to April, 20 and 27°C (night and day, respectively); warm season: May to October, 23 and 30°C (night and day, respectively); photoperiod, 12 ± 1 h). Tomato plants of the susceptible cultivars Floradel (Petoseed, Saticoy, Calif.) and Supermarmande (Vilmorin, La Ménitré, France) were grown in 8-cm pots containing steam-sterilized compost.

Plant inoculation. *P. solanacearum* was grown in peptone broth (5) on a rotary shaker at 30°C for 24 h. Bacterial density was measured as the optical density of the suspension at 670 nm, with reference to a standard curve quantified by plate enumeration. The inoculum concentration was adjusted with distilled water. Tomato plants were inoculated with each Hrp^- mutant at the three full-leaf stage, i.e., about 4 weeks old and 15 cm tall, by pouring 100 ml of a bacterial suspension adjusted to 10^8 CFU ml^{-1} into each pot (10^{10} CFU per plant). Challenge inoculation with a pathogenic strain was performed 1 to 4 weeks after inoculation of the nonpathogenic mutant by pouring 100 ml of a bacterial suspension adjusted to 10^7 CFU ml^{-1} into each pot (10^9 CFU per plant). Each experiment was repeated at least twice, with two replications of 14 to 16 plants for each treatment, randomly arranged in order to minimize the environmental effects in the growth chamber or in the greenhouse. The ability to induce a hypersensitive response was tested in tobacco leaves, cv. Bottom special, infiltrated as previously described (5).

Disease and control rating. Disease development, expressed as a disease index on a scale of 0 to 5 (34) was recorded for each treatment at specific times after the challenge inoculation. Disease indices within each Hrp^--Hrp^+ combination were compared by Fisher's exact test (SAS software; Statistical Analysis System, Inc., Cary, N.C.). The relative protection rate (%P) was calculated according to the following formula (32): $\%P = [1 - (DI \text{ of } Hrp^-\text{-}Hrp^+ \text{ treatment}/DI \text{ of } Hrp^+ \text{ control})] \times 100$ where DI is the disease index.

Bacterial isolation and quantification. To qualitatively assess the invasiveness of nonpathogenic and/or pathogenic strains in tomato plants, stems were surface sterilized with ethanol and cut into 2-cm segments, and each segment was imprinted onto a petri dish containing Kelman's medium amended with the appropriate antibiotic. After incubation at 30°C for 48 h, the maximum height of colonization was recorded for each plant. Colonization was expressed as the percentage of positive isolations at a specific level in each treatment. Percentages of colonization within each Hrp^--Hrp^+ combination were compared by Fisher's exact test.

To quantify bacterial populations in planta, 2-cm stem segments were sampled at specific levels of the plant, weighed, and incubated in test tubes containing 4 ml of sterile distilled water at 10°C for 12 h, to allow exudation and sedimentation of bacteria from the vessels while stopping their multiplication (18). The suspensions were serially diluted and plated onto Kelman's medium amended with kanamycin or streptomycin by using a Spiral Plate Maker (Interscience, Saint Nom La Breteche, France). After incubation at 30°C for 48 h, bacterial densities were calculated from positive isolations and expressed as log(CFU g [dry weight] of stem $tissue^{-1}$). The mean bacterial densities in each Hrp^--Hrp^+ combination were compared by Student's *t* test.

Potential in planta recombination between mutants and wild-type strains was assessed by plating stem extracts from coinfected plants onto Kelman's medium amended with kanamycin, streptomycin, and rifampin.

Yield assessment. Characteristics of yield (number of flowers per plant, number of fruits per plant, and mean weight per fruit) were recorded during cool and warm seasons in Guadeloupe for plants inoculated with the nonpathogenic mutants GMI8171 and GMI8172, respectively, and compared with those of noninoculated plants.

Antibiosis. Direct antagonism between pathogenic and nonpathogenic strains of *P. solanacearum* was studied with a modified technique of Gross and Vidaver (19). A log-phase aliquot (25 μl) of each indicator strain grown in peptone broth (approximately 10^9 CFU ml^{-1}) was added to 3 ml of molten peptone soft agar (0.75% [wt/vol] agar) cooled to 45°C and poured over a 2,3,5-triphenyltetrazolium chloride-agar plate. The soft agar layer was allowed to solidify, and 10 μl of filtrate (filter pore size, 0.45 μm) of the culture supernatant of each producer strain grown in peptone broth was spotted onto the plate. Plates were incubated at 30°C for 24 h to allow observation of inhibition zones.

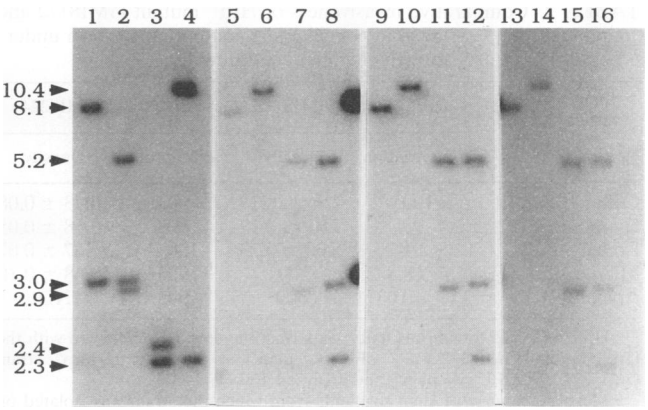

FIG. 1. Southern analysis of wild-type and mutant strains of *P. solanacearum*. Genomic DNA of the strains was transferred onto a nylon membrane after electrophoresis in a 0.8% agarose gel. The probe was DNA from pPMV138 plasmid, ^{32}P-labeled by nick translation. The final washes of the nylon membrane were performed at 65°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. Lanes: 1, *Eco*RI-digested p2-17; 2, *Eco*RI-*Eco*RV-digested p2-17; 3, *Hin*dIII-digested pHP45Ω-Km; 4, *Hin*dIII-digested pPMV138; 5, *Eco*RI-digested genomic DNA of GMI1000; 6, *Eco*RI-digested genomic DNA of GMI8174 mutant; 7, *Eco*RI-*Eco*RV-digested genomic DNA of GMI1000; 8, *Eco*RI-*Hin*dIII-digested genomic DNA of GMI8174; 9, *Eco*RI-digested genomic DNA of GA2; 10, *Eco*RI-digested genomic DNA of GMI8171 mutant; 11, *Eco*RI-*Eco*RV-digested genomic DNA of GA2; 12, *Eco*RI-*Hin*dIII-digested genomic DNA of GMI8171; 13, *Eco*RI-digested genomic DNA of GA4; 14, *Eco*RI-digested genomic DNA of GMI8172 mutant; 15, *Eco*RI-*Eco*RV-digested genomic DNA of GA4; 12, *Eco*RI-*Hin*dIII-digested genomic DNA of GMI8172. Assignment of the bands: 8.1 kb, *Eco*RI-*Eco*RI fragment from *P. solanacearum* cloned in p2-17; 10.4 kb, 2.3-kb-long Ω-Km interposon inserted into the previous fragment; 5.2 kb and 2.9 kb, the EcoRV-digested 8.1-kb EcoRI-EcoRI fragment; 3.0 kb, vector moiety of p2-17; 2.4 kb, vector moiety of pHP45Ω-Km; 2.3 kb, *Eco*RI- or *Hin*dIII-digested Ω-Km interposon.

RESULTS

Construction of the Hrp^- mutants of *P. solanacearum*. The plasmid pPMV138, carrying the Ω-Km interposon at the *Eco*RV site of *hrpO*, was linearized by *Kpn*I digestion and introduced by transformation into four different wild-type strains of *P. solanacearum*, GA2, GA4, GT4, and GMI1000. Kanamycin-resistant clones were obtained from these four strains; all of the clones were ampicillin-sensitive, indicating that they had lost the plasmid and integrated the Ω-Km interposon into their megaplasmid by marker exchange. This was confirmed by Southern analysis of genomic DNA of the three mutants, GMI8171, GMI8172, and GMI8174 (Fig. 1).

Unlike spontaneous nonpathogenic mutants that are typically nonfluidal (7, 20, 22, 24, 29), the Ω-Km-induced mutants retained a fluidal colony morphology on Kelman's medium. In addition, they induced neither the hypersensitive response upon leaf infiltration in tobacco cv. Bottom special nor the

TABLE 2. Comparative invasiveness of Hrp⁻ mutant GMI8172 and parental strain GMI8224 in 6-week-old tomato plants grown under growth chamber conditions[a]

Stem segment (vertical distance from soil surface [cm])	Hrp⁻ mutant GMI8172		Hrp⁺ strain GMI8224	
	Colonization[b]	Density[c]	Colonization	Density
Collar (0)	100.0	7.35 ± 0.17	100.0	10.73 ± 0.08
Hypocotyl (5)	93.7	7.01 ± 0.17	100.0	10.68 ± 0.05
Epicotyl (10)	81.2	6.00 ± 0.23	100.0	10.67 ± 0.07
Middle stem (15)	18.8	ND[d]	100.0	10.68 ± 0.10
Apex (30)	0.0	ND	90.6	ND

[a] Four-week-old tomato plants cv. Supermarmande were inoculated with the Hrp⁻ mutant GMI8172 (10^{10} CFU per plant) or the parental Hrp⁺ strain GMI8224 (10^9 CFU per plant) and analyzed 2 weeks later.
[b] Mean percentage of stem segments from which the strain was isolated on selective medium (32 plants for each treatment).
[c] Density expressed as log(CFU gram [dry weight]$^{-1}$), calculated on positive isolations (means ± standard errors of the means).
[d] ND, not determined.

disease upon stem inoculation in tomato cv. Supermarmande. These Ω-Km-induced nonpathogenic mutants exhibited the typical Hrp⁻ phenotype that was observed with the original Tn*5* mutants (5).

Invasiveness of Hrp⁻ mutants in tomato plants. Although the four Hrp⁻ mutants were unable to induce irreversible disease symptoms in susceptible tomato plants, they caused, in some experiments, rapid and transitory wilt symptoms on some leaves or leaflets on a very limited number of inoculated plants.

The invasiveness of the Hrp⁻ mutant GMI8172 in 6-week-old tomato plants was compared with that of the parental Hrp⁺ strain GMI8224 under growth chamber conditions (Table 2). In the case of the nonpathogenic mutant, the colonization within the aerial part of the plants exhibited a gradient in bacterial density that decreased with distance from collar to middle stem. The average maximum height of colonization was approximately 15 cm above soil level, corresponding to the third leaf axil. In comparison, the pathogenic strain GMI8224 colonized the majority of the plants to the apex, with a constant bacterial density which was much higher than that achieved by the nonpathogenic mutant. All plants inoculated with strain GMI8224 showed symptoms of wilt 2 weeks after inoculation.

Invasiveness of the Hrp⁻ mutant GMI8172 was also assessed in mature tomato plants grown under greenhouse conditions. Four-week-old tomato plants were inoculated and analyzed at the end of the fruit harvest, i.e., 3 months later. The density of bacteria recovered at the taproot level (8.51 ± 0.18 log CFU g [dry weight]$^{-1}$) and epicotyl level (5.66 ± 0.25 log CFU g [dry weight]$^{-1}$) was consistent with that obtained 2 weeks after inoculation in the previous experiment, indicating that colonization of these mutants is long lasting. No bacteria were isolated from the middle stem level (60 cm above soil level) or from the fruit peduncles.

Effect of Hrp⁻ mutants on fruit production. Two experiments were carried out under greenhouse conditions in Guadeloupe to assess the effect of colonization by the nonpathogenic mutants GMI8171 and GMI8172 on plant growth and fruit production. These experiments were conducted during two different seasons, as climatic conditions influence plant growth, fruit production, and bacterial multiplication in planta. Analysis of these data indicates that neither the number of fruit per plant nor the mean weight per fruit was affected by the presence of the nonpathogenic bacteria within the plants, during both of the seasons in which the tests were done (Table 3). In comparison, control plants inoculated with the pathogenic strain GMI8224 during the warm season were all wilted within 6 weeks and yielded no fruit.

TABLE 3. Effect of Hrp⁻ mutants on fruit production under greenhouse conditions[a]

Treatment	No. of flowers per plant	No. of fruit per plant	Mean wt per fruit (g)
Cool season			
GMI8171	12.5 ± 0.27	6.1 ± 0.25	101 ± 3.75
Water (control)	13.4 ± 0.28	6.2 ± 0.33	88 ± 3.92
Warm season			
GMI8172	17.1 ± 0.78	3.2 ± 0.36	101 ± 6.64
Water (control)	17.8 ± 1.01	3.3 ± 0.36	88 ± 4.20

[a] Four-week-old tomato plants cv Floradel were inoculated with Hrp⁻ mutants GMI8171 or GMI8172 (10^{10} CFU per plant) or water (control). Results are the means ± standard errors of the means for 90 plants (cool season) and 30 plants (warm season) for each treatment.

Challenge inoculation under growth chamber conditions. Plants inoculated with the Hrp⁻ mutant GMI8171 or GMI8172 were challenge inoculated with Hrp⁺ strain GMI8220 or GMI8224, respectively (Sm^r derivatives of the parental strains of the mutants) or with strain GMI8217 (Sm^r Rif^r derivative of strain GT1). Data presented in Table 4 indicate that the percentage of plants colonized by the Hrp⁻ mutants at the collar level was comparable to that obtained with the mutant alone (Table 2). In the combinations GMI8171 versus GMI8220 and GMI8172 versus GMI8217, the presence of the Hrp⁻ mutants within the stem was correlated with a significant reduction of the percentage of plants colonized by the pathogenic strain, compared with the control. This exclusion of the pathogenic strain from the aerial part of the plants has been expressed as a relative exclusion rate. The highest exclusion rates were correlated to a significant reduction in the disease index and thus to the highest protection rates.

Challenge inoculation under greenhouse conditions. Plants inoculated with the Hrp⁻ mutant GMI8171, GMI8172, or

TABLE 4. Challenge inoculation under growth chamber conditions[a]

Hrp⁻-Hrp⁺ combination and corresponding control	Colonization (%)[b]		Relative exclusion rate (%)[c]	Disease index[d]	Relative protection rate (%)
	Hrp⁻ mutant	Hrp⁺ strain			
GMI8171-GMI8220	82.1	14.3 S	80.7	0.53 S	71.1
Control GMI8220		74.1		1.85	
GMI8172-GMI8224	92.6	50.0 NS	44.0	2.32 NS	27.8
Control GMI8224		89.3		3.22	
GMI8172-GMI8217	100	46.4 S	51.9	1.43 S	70.3
Control GMI8217		96.4		4.82	

[a] Four-week-old tomato plants, cv. Supermarmande, were inoculated with Hrp⁻ mutants (10^{10} CFU per plant), challenge-inoculated 7 days later with Hrp⁺ strains (10^9 CFU per plant), and analyzed 3 weeks later.
[b] Mean percentage of stem segments at the collar level from which the strain was isolated on selective medium (28 plants for each treatment). S, significantly different, and NS, not significantly different, according to Fisher's exact test (P = 0.05).
[c] Percent exclusion = [1 − (% colonization in the Hrp⁻-Hrp⁺ treatment/% colonization in the Hrp⁺ control)] × 100.
[d] Disease index recorded 3 weeks after challenge inoculation. S, significantly different, and NS, not significantly different, according to Fisher's exact test (P = 0.05).

TABLE 5. Challenge inoculation under greenhouse conditions[a]

Hrp⁻-Hrp⁺ combination and corresponding control	Hrp⁻ mutant		Hrp⁺ strain		Disease index[d]	Relative protection
	Colonization[b]	Density[c]	Colonization	Density		
GMI8171-GMI8217	75.5	5.82 ± 0.15	100.0	9.93 ± 0.18 S	0.87 S	54.0
Control GMI8217			100.0	10.83 ± 0.05	1.89	
GMI8172-GMI8217	83.0	6.32 ± 0.15	98.1	10.39 ± 0.11 S	1.15 S	53.2
Control GMI8217			100.0	10.89 ± 0.04	2.46	
GMI8173-GMI8217	82.7	6.04 ± 0.13	98.2	10.76 ± 0.10 NS	2.06 NS	32.4
Control GMI8217			100.0	10.75 ± 0.13	3.02	

[a] Four-week-old tomato plants, cv. Floradel, were inoculated with Hrp⁻ mutants (10^{10} CFU per plant), challenge inoculated 4 weeks later with the Hrp⁺ strain GMI8217 (10^9 CFU per plant), and analyzed 4 weeks later.

[b] Mean percentage of stem segments at the epicotyl level from which the strain was isolated on selective medium (56 plants for each treatment).

[c] Density expressed as log(CFU gram [dry weight]$^{-1}$) calculated on positive isolations (means ± standard errors of the means). S, significantly different, and NS, not significantly different, according to Student's t test ($P = 0.05$).

[d] Disease index recorded 4 weeks after challenge inoculation. S, significantly different, and NS, not significantly different, according to Fisher's exact test ($P = 0.05$).

GMI8173 were challenge inoculated 4 weeks later with the pathogenic strain GMI8217. There was a noticeable heterogeneity of disease indices within the different controls inoculated with the pathogenic strain GMI8217 (Table 5). Nevertheless, disease indices of each Hrp⁻-Hrp⁺ treatment and each Hrp⁺ control were compared, and relative protection rates could be established for each combination. One month after challenge inoculation, all plants were analyzed for the presence of bacteria at the epicotyl level. Interestingly, the rates of colonization and the bacterial density of the Hrp⁻ mutants at the epicotyl level were similar to those obtained with the mutants alone (Table 2).

The presence of the Hrp⁻ mutants within the stem tissues was not associated with an exclusion of the Hrp⁺ strain: almost all the plants were colonized at the epicotyl level by strain GMI8217, with a density much higher than that of the Hrp⁻ mutants. However, presence of the Hrp⁻ mutants GMI8171 and GMI8172 within the stem was correlated with a significant decrease in the density of the coinoculated pathogenic strain, compared with that of the pathogenic strain alone. This observation was consistent with the higher protection rate achieved with these mutants than with GMI8173. Furthermore, no Km^r Sm^r Rif^r bacteria were isolated from the 122 coinfected plants analyzed. The potential recombination rate between mutants and wild-type strains in planta could thus be estimated as less than 3×10^{-10}.

Antibiosis. In order to study direct antibiosis between the nonpathogenic mutants and the pathogenic strains used for this study, in vitro antibiosis tests were conducted. Strain GT1 and its Sm^r Rif^r derivative GMI8217 produce a substance that inhibited the growth of strains GA2, GA4, and GT4; of their Hrp⁻ derivatives, GMI8171, GMI8172, and GMI8173; and of their Sm^r derivatives, GMI8220 and GMI8224. To determine whether the substance produced by strain GMI8217 specifically affects *P. solanacearum*, 19 other bacterial species were tested. No growth inhibition was seen in any of these strains (13). This substance was further identified as a bacteriocin (19), since dilutions of the supernatant filtrate showed a diffuse thinning of growth. If bacteriophages were responsible for the antibiosis, a decreasing number of discrete phage plaques would have been found. Furthermore, the bacteriocin was detected in extracts from tomato stems colonized by strain GMI8217. Conversely, no antibiosis against strain GMI8217 was detected in the supernatant of the three Hrp⁻ mutants tested.

DISCUSSION

The mutants used in this study were constructed to (i) test their ability as potential biocontrol agents against wild-type strains of *P. solanacearum* in natural conditions and (ii) avoid dissemination of an antibiotic resistance gene in bacterial populations. This last requirement implies that no horizontal transfer of the resistance gene is possible. In fact, in the mutants, the Ω-Km interposon should behave like a genomic gene. Hence, it can be transferred to another strain of *P. solanacearum* via homologous recombination. Indeed, our experiments have shown that it is possible to obtain homologous recombination of the *hrp* region between different wild-type strains of *P. solanacearum* in artificial conditions. This result is consistent with other findings that have shown that the nucleotide sequence of this region is conserved in this species (4) and among several bacterial species (17). In natural conditions, we did not detect any transfer of the Ω-Km interposon between coinoculated strains, suggesting that the potential transfer rate in planta was low. However, if such a transfer occurs, the receptor strain would become nonpathogenic and therefore, arguably, would not represent an additional threat to the environment.

The invasiveness of Ω-Km induced Hrp⁻ mutants was similar to that observed in young tomato plants with the previously described Tn*5*-induced Hrp⁻ mutant GMI1353 (30), but the analysis was continued up to fruit harvest in the present paper. The ability of the nonpathogenic mutants to colonize the host tissues was reduced compared with that of a pathogenic strain, although they persisted at low levels in the plant. Hrp⁻ mutants did not reach the fruit peduncles, suggesting that the fruit were free of bacteria. This may be advantageous for the environment, as it could reduce the dissemination of genetically modified microorganisms, and may also alleviate consumer concern. The survival of Hrp⁻ mutants over a period of 3 months after inoculation within plants showing no wilt symptoms mimicks the survival of Hrp⁺ strains in latent infections of resistant host plants (8, 18, 27). Although the similarity between both situations is striking, the mechanisms of survival may not be the same, since, for example, Hrp⁻ mutants induce lower levels of electrolyte leakage in tomato leaf discs than do Hrp⁺ strains (16).

Hrp⁻ mutants of *P. solanacearum* produce amounts of exopolysaccharides comparable to those of Hrp⁺ strains, and the *hrp* gene cluster is thus not involved in exopolysaccharide

biosynthesis (33). The major exopolysaccharide is thought to act as an invasive factor which protects the bacteria from plant agglutinins (10) and to prevent the bacteria from sticking to plant cell walls (35). The combination of exopolysaccharide production and a disabled *hrp* region probably confers to Hrp$^-$ mutants a unique ability to invade and to survive within the xylem vessels.

When tomato plants were inoculated with Hrp$^-$ mutants, the presence of the mutants within the plant tissues had no apparent effects on growth and fruit production over a 3-month period. However, as the culture conditions in the greenhouse were different from those for tomato production, the yield obtained was much lower than that generally obtained by a tomato producer. Small-scale field experiments are in progress to confirm these preliminary results.

Under challenge inoculation with pathogenic strains, the Hrp$^-$ mutants demonstrated protective ability, as observed by the reduced incidence of disease under treatment conditions compared with the controls. The results obtained under growth chamber conditions were the most consistent with the results observed with the original Tn*5* mutants, in which near total exclusion of the pathogenic strain was demonstrated (32). It is probably significant that this work was similarly performed under growth chamber conditions. Higher protection rates were obtained when the Hrp$^-$ mutant was able to exclude the pathogenic strain from the aerial part of the plant. The control rates achieved by the different combinations may suggest some strain specificity in the effectiveness of the biological control.

Cohabitation of pathogenic and nonpathogenic strains was observed in greenhouse conditions, resulting in lower protection rates. Comparison between the control rates obtained in the growth chamber with those in the greenhouse may indicate that the protection afforded by these Hrp$^-$ mutants could be short-term. The question of the applicability of this approach to production agriculture, in which protection is needed for the duration of a growing season, is currently being studied in Guadeloupe, where small-scale field experiments with Hrp$^-$ mutants are in progress.

Several possible mechanisms may be proposed to explain the protective ability of the Hrp$^-$ mutants: (i) induced plant defense mechanisms, (ii) antibiosis between strains, or (iii) competition for space in planta. Although induced resistance has been cited as a possible mechanism of biocontrol involving spontaneous nonpathogenic mutants of *P. solanacearum* (22), our data cannot yet confirm this hypothesis.

The study of direct antibiosis between strains demonstrated that the pathogenic strain GMI8217 produced, in vitro and in planta, a bacteriocin that inhibited the in vitro growth of the three Hrp$^-$ mutants tested. Whether this antibiosis occurs in the vascular tissues colonized by both pathogenic and nonpathogenic strains has not been established yet. The fact that the bacterial density of the mutants was comparable in plants colonized by the mutant alone and in plants colonized by both strains may indicate that the bacteriocin produced in planta by strain GMI8217 is not effective on the mutant population. Anyhow, antibiosis may not be involved in the observed protection, since the Hrp$^-$ mutants did not inhibit the growth of the pathogenic strains used in this study.

Many bacteriocin-producing spontaneous nonpathogenic mutants of *P. solanacearum* have been tested for their protective effect against pathogenic strains with inconsistent success (7, 20). However, the limitations of such afluidal mutants may be attributed to their inability to multiply in planta. Since Hrp$^-$ mutants are able to survive within host plant tissues over a long period, a bacteriocin-producing Hrp$^-$ mutant could be a biocontrol agent sufficiently robust to work under field conditions. Work is in progress to transform strain GT1 and other bacteriocin-producing strains by insertion of the Ω-Km interposon within the *hrp* gene cluster.

Competition for space within the xylem vessels leading to exclusion of the pathogenic strain from the plants colonized by the nonpathogenic mutant is another possible hypothesis. The simultaneous presence of both Hrp$^-$ and Hrp$^+$ strains within the tomato stem, as revealed by positive isolations, does not indicate that both strains are located in the same xylem vessels or in the same vascular bundles. Two different bacterial populations may colonize distinct xylem vessels without interference. Therefore, cytological studies are in progress to determine the histological localization of both strains.

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REFERENCES

1. **Anuratha, C. S., and S. S. Gnanamanickam.** 1990. Biological control of bacterial wilt caused by *Pseudomonas solanacearum* in India with antagonistic bacteria. Plant Soil **124:**109–116.
2. **Aoki, M., K. Uehara, K. Koseki, K. Tsuji, M. Iijima, K. Ono, and T. Samejima.** 1991. An antimicrobial substance produced by *Pseudomonas cepacia* B5 against the bacterial wilt disease pathogen, *Pseudomonas solanacearum*. Agric. Biol. Chem. **55:**715–722.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Green Publishing Associates and Wiley Interscience Publisher, New York.
4. **Boucher, C. A., P. A. Barberis, and M. Arlat.** 1988. Acridine orange selects for deletion of *hrp* genes in all races of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. **1:**282–288.
5. **Boucher, C. A., P. A. Barberis, A. P. Trigalet, and D. A. Demery.** 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn*5*-induced avirulent mutants. J. Gen. Microbiol. **131:**2449–2457.
6. **Boucher, C. A., F. Van Gijsegem, P. A. Barberis, M. Arlat, and C. Zischek.** 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. **169:**5626–5632.
7. **Chen, W. Y., and E. Echandi.** 1984. Effects of avirulent bacteriocin-producing strains of *Pseudomonas solanacearum* on the control of bacterial wilt of tobacco. Plant Pathol. **33:**245–253.
8. **Ciampi, L., and L. Sequeira.** 1980. Multiplication of *Pseudomonas solanacearum* in resistant potato plants and the establishment of latent infections. Am. Potato J. **57:**319–329.
9. **Ciampi-Panno, L., C. Fernandez, P. Bustamante, N. Andrade, S. Ojeda, and A. Contreras.** 1989. Biological control of bacterial wilt of potatoes caused by *Pseudomonas solanacearum*. Am. Potato J. **66:**315–332.
10. **Duvick, J. P., and L. Sequeira.** 1984. Interaction of *Pseudomonas solanacearum* lipopolysaccharide and extracellular polysaccharide with agglutinin from potato tubers. Appl. Environ. Microbiol. **48:**192–198.
11. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene **52:**147–154.
12. **Frey, J., and H. M. Krisch.** 1985. Omega mutagenesis in gram-negative bacteria: a selectable interposon which is strongly polar in a wide range of bacterial species. Gene **36:**143–150.
13. **Frey, P.** Unpublished data.
14. **Fucikovsky, L., I. Luna, and C. Lopez.** 1990. Bacterial antagonists to *Pseudomonas solanacearum* in potatoes and some other plant pathogens, p. 201–206. *In* Z. Klement (ed.), Proceedings of the 7th

International Conference on Plant Pathogenic Bacteria. Akademiai Kiado, Budapest.

15. **Furuya, N., Y. Kushima, K. Tsuchiya, N. Matsuyama, and S. Wakimoto S.** 1991. Protection of tomato seedlings by pre-treatment with *Pseudomonas glumae* from infection with *Pseudomonas solanacearum* and its mechanisms. Ann. Phytopathol. Soc. Jpn. **57**:363–370.
16. **Gough, C. L., S. Genin, V. Lopes, and C. A. Boucher.** 1993. Homology between the HrpO protein of *Pseudomonas solanacearum* and bacterial proteins implicated in a signal peptide-independent secretion mechanism. Mol. Gen. Genet. **239**:378–392.
17. **Gough, C. L., S. Genin, C. Zischek, and C. A. Boucher.** 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. **5**:384–389.
18. **Grimault, V., and P. Prior.** 1993. Bacterial wilt resistance in tomato associated with tolerance of vascular tissues to *Pseudomonas solanacearum*. Plant Pathol. **42**:589–594.
19. **Gross, D. C., and A. K. Vidaver.** 1990. Bacteriocins, p. 245–249. *In* Z. Klement, K. Rudolph, and D. C. Sands (ed.), Methods in phytobacteriology. Akademiai Kiado, Budapest.
20. **Hara, H., and K. Ono.** 1991. Effect of weakly-virulent bacteriocin-producing strain of *Pseudomonas solanacearum* on the protection of tobacco plant from bacterial wilt. Ann. Phytopathol. Soc. Jpn. **57**:24–31.
21. **Kelman, A.** 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology **44**:693–695.
22. **Kempe, J., and L. Sequeira.** 1983. Biological control of bacterial wilt of potatoes: attempts to induce resistance by treating tubers with bacteria. Plant Dis. **67**:499–503.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Mc Laughlin, R. J., L. Sequeira, and D. P. Weingartner.** 1990. Biocontrol of bacterial wilt of potato with an avirulent strain of *Pseudomonas solanacearum*: interactions with root-knot nematodes. Am. Potato J. **67**:93–107.
25. **Message, B., P. Boistard, M. Pitrat, J. Schmit, and C. Boucher.** 1978. A new class of fluidal avirulent mutants of *Pseudomonas solanacearum* unable to induce a hypersensitive reaction, p. 823–833. *In* Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. Station de Pathologie Végétale, Institut National de la Recherche Beaucouzé, France.
26. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
27. **Prior, P., M. Beramis, M. Chillet, and J. Schmit.** 1990. Preliminary studies for tomato bacterial wilt (*Pseudomonas solanacearum* E.F.Sm.) resistance mechanisms. Symbiosis **9**:393–400.
28. **Prior, P., and H. Steva.** 1990. Characteristics of strains of *Pseudomonas solanacearum* from the French West Indies. Plant Dis. **74**:13–17.
29. **Tanaka, H., H. Negishi, and H. Maeda.** 1990. Control of tobacco bacterial wilt by an avirulent strain of *Pseudomonas solanacearum* M4S and its bacteriophage. Ann. Phytopathol. Soc. Jpn. **56**:243–246.
30. **Trigalet, A., and D. Demery.** 1986. Invasiveness in tomato plants of Tn*5*-induced avirulent mutants of *Pseudomonas solanacearum*. Physiol. Mol. Plant Pathol. **28**:423–430.
31. **Trigalet, A., P. Frey, and D. Trigalet-Demery.** 1993. Biological control of bacterial wilt caused by *Pseudomonas solanacearum*: state of the art and understanding, p. 225–233. *In* A. C. Hayward and G. L. Hartman (ed.), Bacterial wilt: the disease and its causative agent *Pseudomonas solanacearum*. C.A.B. International, Wallingford, United Kingdom.
32. **Trigalet, A., and D. Trigalet-Demery.** 1990. Use of avirulent mutants of *Pseudomonas solanacearum* for the biological control of bacterial wilt of tomato plants. Physiol. Mol. Plant Pathol. **36**:27–38.
33. **Trigalet-Demery, D., H. Montrozier, G. Orgambide, V. Patry, O. Adam, L. Navarro, V. Cotelle, and A. Trigalet.** 1993. Exopolysaccharides of *Pseudomonas solanacearum*: relation to virulence, p. 312–315. *In* G. L. Hartman, and A. C. Hayward (ed.), Bacterial wilt. ACIAR Proceedings, no. 45. Australian Centre for International Agriculture Research, Canberra.
34. **Winstead, N. N., and A. Kelman.** 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology **42**:628–634.
35. **Young, D. H., and L. Sequeira.** 1986. Binding of *Pseudomonas solanacearum* fimbriae to tobacco leaf cell walls and its inhibition by bacterial extracellular polysaccharides. Physiol. Mol. Plant Pathol. **28**:393–402.