Xylem Colonization by an HrcV⁻ Mutant of *Ralstonia solanacearum* Is a Key Factor for the Efficient Biological Control of Tomato Bacterial Wilt

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Microscopic studies of the colonization of the vascular tissues of tomato by an HrcV⁻ (formerly HrpO⁻) mutant strain of *Ralstonia solanacearum* were carried out after either root inoculation of the mutant strain alone or delayed challenge inoculation by a pathogenic strain. The use of two different marker genes, *lacZ* and *uidA*, introduced into either mutant or wild-type strains, respectively, permitted histological observation for the presence of both strains simultaneously. In roots, both strains could be found together in infected root tips and in lateral root emergence sites (lateral root cracks), but these bacterial strains subsequently invaded separate xylem vessels in the root system. At the hypocotyl level, a novel staining procedure, in conjunction with bacterial isolation and counting, showed three vascular colonization patterns: exclusive colonization by each of the competitors or simultaneous presence of each strain in separate xylem vessels. The relative frequencies of these patterns depended upon the root inoculation techniques used. The presence of one population always influenced the density of the other challenge-inoculated population. In plants inoculated with both wild-type and mutant strains, the population of the wild-type strain is lower than in plants inoculated with the wild type alone. In contrast, growth of the HrcV⁻ mutant strain was significantly increased in the presence of the pathogenic strain. Two agriculturally acceptable techniques for plant inoculation were tested. Inoculation of plants by transplanting them into soil amended with clay microgranules impregnated with the HrcV⁻ mutant strain gave higher and more reproducible colonization of the plants than inoculation by watering a bacterial suspension on the roots. Significant percentages of exclusive colonization by the HrcV⁻ mutant strain were only obtained after the clay microgranule inoculation technique. Competition for space in xylem vessels is one of the possible explanations for the protective ability of the HrcV⁻ mutant strain against subsequent invasion by a pathogenic strain.

Additional keywords: granular inoculant.

Hrp⁻ mutants of *Ralstonia (Pseudomonas) solanacearum* have already been described as potential biocontrol agents of tomato bacterial wilt (Trigalet and Trigalet-Demery 1990; Frey et al. 1994). The biocontrol agent was first root inoculated and allowed to establish itself in the xylem vessels for a week before a challenge inoculation with the pathogenic strain was done, and the ratio of pathogenic strain to mutant strain in the inoculum was 0.1 (Trigalet and Trigalet-Demery 1990). This sequence of inoculations in a growth chamber was purposely chosen to mimic the natural situation of field experiments in which tomato plants are root inoculated with the biocontrol agent in the nursery at the two-leaf stage, and planting them a week later into infested soils constitutes the start of a challenge inoculation by the natural inoculum.

The protective ability of certain Hrp⁻ mutants against further invasion by a pathogenic strain has been correlated with their abilities to invade and survive within host plants; the more invasive the mutant strain the more protective it is (Trigalet and Demery 1986; Trigalet and Trigalet-Demery 1990). More than 70% of relative protection has been recorded 3 or 4 weeks after the challenge inoculation with the pathogenic strain under controlled conditions (Frey et al. 1994).

HrcV⁻ (formerly HrpO⁻; Bogdanove et al. 1996) mutant strains are the most invasive and the most protective of all Hrp⁻ mutant strains of *R. solanacearum* so far tested. HrcV⁻ mutant strains are unable to elicit the hypersensitive response when infiltrated into leaf panels of tolerant tobacco cv. Bottom Special and are unable to induce wilting of the susceptible tomato cv. Supermarmande after root inoculation. HrcV⁻ mutant strains of *R. solanacearum* are still able to infect tomato roots and to multiply within xylem vessels. However, colonization by the HrcV⁻ mutant strain is restricted to the roots and lower part of the stems, and concentrations of the mutant strain measured at collar level are several orders of magnitude lower than that of the pathogenic strain (Frey et al. 1994).

Cohabitation in symptomless plants was demonstrated by simultaneous isolation of both competitors, the Hrp⁻ mutant strain and the pathogenic strain, and correlated with poor relative protection. In contrast, higher relative protection rates were correlated with the exclusive isolation of the Hrp⁻ mutant strains (Trigalet and Trigalet-Demery 1990; Frey et al. 1994).

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Several mechanisms have been proposed to explain the protective ability of these Hrp⁻ mutants (Trigalet et al. 1994). Bacteriocin-mediated antibiosis is observed within the rhizosphere or within the plant tissues (Frey et al. 1994). However, spontaneous bacteriocin-resistant mutant strains arose with high frequency (Frey et al. 1996). In addition, a high level of protection has been recorded in the absence of any direct antagonism between the Hrp⁻ mutant strain and the pathogen (Frey et al. 1994). Thus, bacteriocin-mediated antibiosis may not be responsible for the observed protection. Besides, little is known about other potential mechanisms such as induced host resistance by the Hrp⁻ mutants or competition for space and/or nutrients within xylem vessels between the Hrp⁻ mutant and pathogenic strains.

In Guadeloupe, biocontrol field experiments have met with limited success and no more than 45% relative protection could be recorded 9 weeks after planting (A. Trigalet and P. Prior, *unpublished*). However, these results motivated further research on the mechanisms involved in the protection and on the efficiency of the root inoculation technique under agricultural conditions.

In this paper, we describe construction of an HrcV⁻ mutant strain and a pathogenic strain that harbor different gene fusions that constitutively express β -galactosidase and β -glucuronidase (GUS) activities, respectively. With these engineered strains the objectives of this work were (i) to cytologically investigate the infection process of the HrcV⁻ mutant alone at the root and hypocotyl levels of tomato plants and to compare it with that of the pathogenic type strain, (ii) to assess the potential influence of each bacterial population upon invasiveness of the other after delayed inoculation by the pathogen, and (iii) to compare the efficiency of different inoculation techniques with the HrcV⁻ mutant in order to find out which of them ensures the best invasion of the mutant and the best protection toward subsequent invasion by the pathogen.

RESULTS

Construction of bacterial strains expressing reporter gene activities.

The Tn5-*gusA3* transposon was introduced into the GMI 1000 pathogenic strain by triparental mating and 65 transconjugants were screened for their GUS activities. Two of them, namely GMI 1557 and GMI 1559, were not affected in their pathogenicity and exhibited activities of GUS about 58.5 ± 6.5 or 24.1 ± 5.6 (nmol hydrolyzed PNPG substrate $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹), respectively. The GMI 1557 recombinant strain was used in this study. GMI 1557 induces total wilting of tomato plants (disease index 5) 24 days after root inoculation (Table 1). A disease index of 2.5 was recorded as soon as 7 days (not shown). Thus, aggressiveness of the GMI 1557 strain is comparable to that of the reference GMI 1229 or GMI 8217 pathogenic strains (Trigalet and Demery 1986; Trigalet and Trigalet-Demery 1990; Frey et al. 1994).

Insertion of the nontransposable Omega-Cm interposon within the *hrcV* gene of the pathogenic GMI 1485 strain, in which a Tn5-B20 transposon is already inserted (Table 2), was successfully carried out and transformants were selected for their β -galactosidase activity. The GMI 1556 strain was an *hrcV::* Ω -Cm mutant strain that expressed a high level of β -galactosidase activity, comparable to that of the parental GMI 1485 strain in minimal medium complemented with glucose, i.e., about 3,000 Miller units (Arlat et al. 1992). The GMI 1556 mutant strain is unable to induce either the hypersensitive response in tobacco cv. Bottom Special (not shown) or the disease of tomato (Table 1). Thus, the GMI 1556 mutant strain exhibits the typical Hrp⁻ phenotype that has already been observed after insertion of the omega cassette in the *hrcV* gene of pathogenic strains (Frey et al. 1994).

Table 1. Invasiveness of the HrcV⁻ GMI 1556 mutant and GMI 1557 pathogenic strains

Inoculations ^u	Days after inoculation ^v	Disease index ^w	Isolation		Positive xylem bundles (%) ^z	
			Positive isolations (%) ^x	Bacterial densities ^y	β -galactosidase	β -glucuronidase
1W	21	0	15.5 ab	3.05 $\pm$ 0.72	7.0 a	
3W	21	0	27.5 ab	3.45 $\pm$ 0.52	22.5 bc	
mG	21	0	47.5 bc	5.42 $\pm$ 0.63	83.5 f	
1W	31	0	32.5 abc	3.08 $\pm$ 0.25	26.0 bc	
3W	31	0	30.0 abc	3.55 $\pm$ 0.27	36.0 cd	
mG	31	0	82.5 d	4.97 $\pm$ 0.23	84.2 fg	
1W	41	0	35.5 abc	3.12 $\pm$ 0.21	29.0 bcd	
3W	41	0	34.0 abc	3.72 $\pm$ 0.26	39.0 cde	
mG	41	0	82.5 d	5.12 $\pm$ 0.31	87.4 fgh	
Control	11	3.9	97.5 de	10.89 $\pm$ 0.43		63.0
Control	24	5.0	NA	NA		NA

^u Inoculation by watering (W) with a calibrated inoculum of the mutant strain: 1W, unique inoculation; 3W, three successive inoculations at 2-day intervals; mG, clay microgranules. Twenty plants were sampled at three different times for each analysis and three independent replicates were carried out. Control: inoculation (1W) with the pathogenic strain.

^v Days after the first inoculation with the Hrp⁻ mutant strain (three techniques: 1W, 3W, or mG), or by watering with the pathogenic strain (control).

^w Disease index (scale 0 to 5).

^x Percentage of positive isolations by imprinting onto agar culture medium supplemented with kanamycin and chloramphenicol for the mutant strain, and with tetracycline for the pathogenic strain. NA = not analyzed, totally wilted. Data within columns followed by the same letters are not significantly different according to the 95% confidence limits of frequencies (Sokal et al. 1969).

^y Bacterial densities expressed as log(CFU per gram of dry weight⁻¹) of positive isolations (mean $\pm$ standard error of the mean) in three replicates (60 plants).

^z Percentages of vascular bundles containing blue-stained HrcV⁻ mutant strain (β -galactosidase) or magenta-red-stained pathogenic strain (β -glucuronidase) out of 270 vascular bundles examined in 60 plants.

The root infection process and vascular colonization of the HrcV⁻ GMI 1556 mutant strain and the GMI 1557 pathogenic strain.

The process of infection and xylem invasion by the HrcV⁻ GMI 1556 mutant or the GMI 1557 pathogenic strain was first assessed in roots of 2-week-old tomato plants grown in test tubes (Vasse et al. 1995). In addition, xylem invasion was also investigated in transverse slices at the hypocotyl level of 6-week-old tomato plants grown in peat pots. Both samples, the roots and the hypocotyl slices, were observed by light microscopy after staining of the β -galactosidase (mutant strain) or GUS (pathogenic strain) activity.

In test-tube-grown plants, the surfaces of root extremities and of axils of the emerging lateral roots (hereafter referred to as lateral root cracks) were colonized by the GMI 1556 HrcV⁻ mutant strain 3 days after inoculation, and xylem invasion was detected as soon as 7 days after inoculation (data not shown). Colonization of root tips and of lateral root cracks by the pathogenic strain was detected as soon as 1 day after inoculation and xylem invasion was noticeable as early as 3 days after inoculation (data not shown). Time course of infection of tomato by the GMI 1557 pathogenic strain is similar to that of the reference GMI 1000 pathogenic strain (Vasse et al. 1995). Thus, microscopic examination revealed that both the HrcV⁻ mutant and the pathogenic strains colonize the same sites, namely root tips and lateral root cracks, but that the HrcV⁻ mutant colonized and invaded xylem vessels more slowly than the pathogenic strain.

In peat-pot-grown tomato, the pathogenic strain was detected in xylem vessels at the hypocotyl level of all plants sampled as soon as 5 days after inoculation. In contrast, xylem colonization by the HrcV⁻ mutant was first detected 8 days after inoculation in 10 to 30% of sampled plants depending on the inoculation technique. Inoculation with the microgranule technique led to the highest values of invasion by the HrcV⁻ mutant as demonstrated by percentages of positive isolations

and percentages of invaded vascular bundles that were significantly (95% confidence limits) higher than those obtained after the watering inoculation technique (Table 1). At the hypocotyl level the highest bacterial densities of the HrcV⁻ mutant also resulted from the microgranule technique of inoculation (Table 1).

Thus, the HrcV⁻ GMI 1556 mutant and the GMI 1557 pathogenic strains should both be referred to as infective and invasive according to the phenotypes that we have formerly described (Vasse et al. 1995). However, the invasiveness of the pathogenic strain is significantly more rapid and more extensive than that of the HrcV⁻ mutant strain.

The root infection process and vascular colonization by the GMI 1557 pathogenic strain in competition with the HrcV⁻ GMI 1556 mutant strain.

The influence of the pathogenic strain on the HrcV⁻ mutant strain was investigated following challenge inoculation by the pathogenic strain 7 days after inoculation with the HrcV⁻ mutant strain. In test tubes and with the two-step staining procedure, we observed that both the pathogenic and the HrcV⁻ mutant strains infected root tips and lateral root cracks. While both strains could simultaneously be present on these sites (Fig. 1A, B), they subsequently invaded separate xylem vessels in all cases examined so far (Fig. 1C, D, and E). Thus, the pathogenic strain may invade some xylem vessels in the main root through lateral root infection (Fig. 1C), when the HrcV⁻ mutant strain is already established in a separate xylem vessel as the result of infection from another infection site. The reverse can also be observed (Fig. 1D), where the mutant strain has infected a lateral root crack while the pathogenic strain is already fully established in a xylem vessel in the main root. Even when the mutant strain is fully established in a root tip and in xylem vessels of lateral and main roots (Fig. 1B), a faint additional infection by the pathogenic strain may occur on the same root tip. To summarize, the pathogenic strain is

Table 2. Bacterial strains, plasmids, and transposons

Designation	Relevant characteristics	Source
Bacteria		
<i>Escherichia coli</i> DH5 α		Bethesda Life Technologies, Cergy Pontoise, France
<i>E. coli</i> LE392		Sharma and Singer 1990
<i>Ralstonia solanacearum</i>		
<i>R. solanacearum</i> GMI 1000	Wild-type strain pathogenic on tomato, isolated in French Guyana 1966, race 1, biovar IV	Boucher et al. 1985
<i>R. solanacearum</i> GMI 1485	GMI1000::Tn5B20 pathogenic, Km ^r	Arlat et al. 1992
<i>R. solanacearum</i> GMI 556	Ω -Cm induced <i>hrcV</i> mutant of GMI 1485 Km ^r , Cm ^r	This work
<i>R. solanacearum</i> GMI 1557	GMI1000::Tn5-gusA3 mutant pathogenic, Tc ^r	This work
Plasmids		
pHP45 Ω -Cm	pHP45 (2.4kb) carrying the Ω -Cm interposon (3.7 kb)	Prentki and Krisch 1984
pRK2013	Km ^r Tra RK2+ Δ rep RK2 repE1+	Figurski and Helinski 1979
pVIR2	pLAFR3 carrying part of the <i>hrp</i> gene cluster	Boucher et al.
1987		
p2-17	Bluescript (3 kb) carrying a 8-kb <i>EcoRI</i> - <i>EcoRI</i> fragment of pVIR2 plasmid	Boucher et al. 1987
pPMV137	Deletion of a 1.3-kb <i>ClaiI</i> - <i>ClaiI</i> fragment from p2-17	Frey et al. 1990
pPMV138	Insertion of the Ω -Km interposon at the <i>EcoRV</i> site of pPMV137	Frey et al. 1990
pGMI2037	Substitution of the 2.1-kb <i>BamHI</i> - <i>BamHI</i> fragment of pPMV138 by the 3.7-kb <i>BamHI</i> - <i>BamHI</i> fragment of pHP45 Ω -Cm	This work
pSB467	ColE Ω ::Tn5-gusA3 Tc ^r	Sharma and Signer 1990
Phage		
T4	Specific for <i>E. coli</i>	Arlat et al. 1992
Transposons		
Tn5-gusA3	Tn5 carrying a promoterless <i>uidA</i> gene into IS50L	Sharma and Singer 1990

still able to infect a root tip even when the HrcV⁻ mutant strain has established itself at this site some days before.

Another staining procedure applied to hypocotyl slices from tomato grown in peat pots indicated that the pathogenic and the HrcV⁻ mutant strains always colonize separate xylem vessels (Fig. 2C). However, because xylem vessels are assembled in vascular bundles, and because staining may diffuse into the surrounding medium, it was in some cases difficult to clearly distinguish separate colonization by the two competitors in adjacent xylem vessels within the same vascular bundle. Three distinct colonization patterns could be distinguished: (i) extensive and sole colonization by the pathogenic strain (Fig. 2A) in the absence of any detectable HrcV⁻ mutant strain; (ii)

exclusive colonization by the HrcV⁻ mutant strain (Fig. 2B) with no detectable pathogenic bacteria; and (iii) simultaneous vascular colonization of separate xylem vessels within the same vascular bundle by either strain (Fig. 2C). Extensive colonization by the pathogenic strain (pattern [i]) was detected in protoxylem, metaxylem, and secondary xylem vessels at the hypocotyl level of partially wilted plants (Fig. 2A). Simultaneous colonization by both competitors was mainly observed in partially wilted plants (Fig. 2C) where xylem colonization by the pathogenic strain was not massive, though sufficient to induce disease. The relative occurrence of the three vascular colonization patterns was strongly dependent upon the inoculation technique used, as described below.

Fig. 1. Cytological analyses of infection by wild-type and mutant bacterial strains in tomato grown in test tubes. Light microscopy of infection and vascular colonization of tomato roots by the HrcV⁻ GMI 1556 mutant strain, 9 days after the challenge inoculation by the GMI 1557 pathogenic strain. Bacteria were localized by histochemical staining of the β -galactosidase activity (blue) produced by the HrcV⁻ mutant strain in contrast to the β -glucuronidase activity (magenta red) of the pathogenic strain. **A**, Simultaneous infections by the HrcV⁻ mutant and pathogenic strains at the tip of a secondary root. **B**, Infection at the tip of a secondary root and colonization of xylem vessels in secondary and main roots by the HrcV⁻ mutant strain. There is a faint localized infection by the pathogenic strain (black arrow). **C**, Progression of the HrcV⁻ mutant strain in xylem vessels of the main root, which appears to be independent of infection and colonization by the pathogenic strain originating from the tip of the secondary root. **D**, Infection by the HrcV⁻ mutant strain at the site of emergence of a secondary root, and colonization in the main root xylem by the pathogenic strain resulting from a different invasion site. **E**, Vascular colonization by the HrcV⁻ mutant and pathogenic strains in separate xylem vessels in the main root. Bars represent 100 μ m.

Efficiency of exclusion of the pathogenic strain by the HrcV⁻ mutant strain.

Inoculations by watering with the HrcV⁻ mutant strain were unable to prevent tomato from total wilting upon delayed challenge inoculation by the pathogenic strain. At most, 11 days after challenge inoculation, time course of wilting is slackened by a previous watering with the HrcV⁻ mutant strain when compared with that of the control (Tables 1 and 3). However, 24 days after the pathogen inoculation all plants exhibited a high level of wilt symptoms (Table 3). All plants that were first inoculated by watering with the HrcV⁻ mutant strain wilted within 24 and 34 days after the challenge inoculation, i.e., 31 and 41 days after the mutant inoculation, respectively (Table 3). In contrast, previous inoculation of the HrcV⁻ mutant strain by the microgranule technique led to significant protection against the challenge inoculation by the pathogenic strain. This is illustrated by the noticeable reduc-

tion of disease, which remains stable at index 1.55 as soon as 24 days after the challenge inoculation (Table 3).

Invasiveness by the pathogenic strain following the challenge inoculation is consequently reduced, as seen by the significant decrease in the percentage of the strain isolated, and the reduced percentage of invaded vascular bundles, as well as reduced bacterial densities (Table 3), when compared with that of the pathogenic strain inoculated alone (Table 1). At 11 days after challenge inoculation, the percentage of positive isolations of the pathogenic strain is about 97.5 in the control (Table 1); this percentage dropped down to 47.5 following the HrcV⁻ mutant-microgranule treatment. At 31 and 41 days after the mutant-microgranule inoculation, i.e., 24 and 34 days after challenge inoculation, the pathogenic strain can be still isolated from symptomless plants at a frequency of about 30% (Table 3). At the hypocotyl level and depending on the inoculation technique, this modified invasiveness was illustrated by

Fig 2. Cytological analyses of infection by wild-type and mutant bacterial strains in tomato grown in peat pots. Light microscopy of infection and colonization of tomato stems at the hypocotyl level: 2-mm-thick slices at lower part of hypocotyl near collar zone. **A**, Portion of stem section from a partially wilted plant, 6 days after inoculation by the pathogenic strain. There is abundant colonization by the pathogenic strain of protoxylem (1), metaxylem (2), and secondary xylem (3) vessels. **B**, Entire stem section of a symptomless tomato plant that exhibits exclusive colonization of whole xylem vascular bundles by the Hrp⁻ mutant. **C**, Entire stem section of a partially wilted tomato plant that harbors both competitors, the HrcV⁻ mutant strain (arrow heads) and the pathogenic strain (arrows). Bars represent 250 μ m.

Table 3. Invasiveness of the pathogenic GMI 1557 strain inoculated 1 week after previous inoculation with the HrcV⁻ GMI 1556 mutant strain

Inoculations ^u	Days after mutant strain inoculation ^v	Disease index ^w	Isolation		β -Glucuronidase staining
			Positive isolations (%) ^x	Bacterial densities ^y	Positive bundles (%) ^z
1W	18	2.25	90.0 d	10.63 $\pm$ 0.41	71.0 cde
3W	18	1.90	62.5 abc	10.37 $\pm$ 0.39	69.0 cd
mG	18	1.55	47.5 ab	8.78 $\pm$ 0.42	23.0 ab
1W	31	5.0	NA	NA	NA
3W	31	5.0	97.5 de	10.43 $\pm$ 0.37	71.0 cde
mG	31	1.55	30.0 a	6.72 $\pm$ 0.41	18.0 a
mG	41	1.55	30.0 a	6.16 $\pm$ 0.67	16.0 a

^u For definitions see Table 1.

^v Days after the mutant strain inoculation, i.e., 11, 24, and 34 days after the challenge inoculation with the pathogenic strain, respectively.

^w Disease index (scale 0 to 5).

^x Percentages of positive isolations of the pathogenic strain after imprinting onto agar medium complemented with tetracycline.

^y Bacterial densities, see Table 1. NA = not analyzed, totally wilted. Data within columns that are followed by the same letter are not significantly different according to the 95% confidence limits of percentages (Sokal et al. 1969).

^z Percentages of vascular bundles invaded by the pathogenic strain.

a significant lowering of the density of the pathogenic strain or even by total exclusion of the pathogenic strain. In cases in which both strains were simultaneously present, the HrcV⁻ mutant strain noticeably reduced the average densities of the pathogenic strain. The average density of the pathogenic strain was between 11^{10} and 10^{11} CFU · g (dry weight)⁻¹ at 11 days in control plants (Table 1) and at 18 days after the watering treatments (Table 3), whereas the density dropped down to 10^9 CFU · g (dry weight)⁻¹ after the microgranule (mG) treatment (Table 3). The contrast is even clearer at 31 days in partially wilted plants, in which the pathogenic strain was about $4 \cdot 10^{10}$ CFU · g (dry weight)⁻¹ in the three successive waterings (3W) treatment and only $5 \cdot 10^6$ CFU · g (dry weight)⁻¹ in the mG treatment (Table 3), whereas control and unique watering (1W) treatments were totally wilted and no longer usable for counting (Tables 1 and 3). Cases in which the pathogenic strain was absent and the HrcV⁻ mutant strain was present were only recorded from symptomless plants after the mG treatment. In these cases, and whatever the detection technique used, presence of the pathogenic strain could no longer be detected while the HrcV⁻ mutant strain was exclusively and abundantly present in 37.5 (18 days) to 62.5% (41 days) of symptomless plants (Table 4). The mean disease index is maintained at 1.55 even 34 days after the challenge inoculation, which corresponds to a 31% disease index and a 69% protection index.

The invasiveness of the HrcV⁻ mutant strain was significantly enhanced after challenge inoculation with the pathogenic strain, compared with inoculation by the mutant strain alone. Percentages of positive vascular bundles that exhibited the blue β-galactosidase activity were noticeably increased, as well as densities of the mutant strain that reached more than 8 in log · CFU · g (dry weight)⁻¹ at 31 days, i.e., 24 days after the challenge inoculation (Table 4), whereas densities of the mutant strain inoculated alone never exceeded 4.97 log · CFU · g (dry weight)⁻¹ at 31 days, whatever the inoculation technique (Table 1). In symptomless plants protected by the microgranule treatment at 31 and 41 days, and in the case of cohabitation by both competitors, mean bacterial densities of the HrcV⁻ mutant strain even became significantly higher (Table 4) than that of the pathogenic strain (Table 3).

DISCUSSION

The HrcV⁻ GMI 1556 mutant strain is both infective and invasive in roots of tomato, and at collar level it reached densities of between $5 \cdot 10^4$ and $5 \cdot 10^5$ CFU · g (dry weight)⁻¹. These densities are lower than those previously attributed to the Hrp⁻ phenotype (Frey et al. 1994). However, in previous work the HrcV⁻ GMI 8172 mutant strain used originated from a different pathogenic strain, namely, the highly aggressive GA4 isolate; in addition, inoculation and bacterial numeration techniques as well as the plant stage at inoculation were also different. Nevertheless, these data confirm that HrcV⁻ mutant strains are poor colonizers, compared with pathogenic strains.

Competition clearly modifies the densities of both competitors. We previously described how Hrp⁻ mutants of *R. solanacearum* are poor colonizers (Trigalet and Demery 1986; Trigalet and Trigalet-Demery 1990; Frey et al. 1994). This reduced invasiveness is probably due to their altered ability to secrete exocellular proteins that are active in plants (Arlat et al. 1994). This could explain the increased density of the HrcV⁻ mutant strain in the presence of the pathogenic strain in symptomless plants, but the nature of this complementation remains unknown.

Which mechanism(s) could explain the exclusive colonization by the HrcV⁻ mutant strain? Our results illustrate that the mutant and pathogenic strains do actually compete to invade xylem vessels. No direct antagonism has ever been demonstrated between these two strains, which are both derived from the same parental GMI 1000 pathogenic strain. Thus, prevention of invasion of the pathogenic strain by the mutant strain is probably not due to direct antagonism caused by a bacteriocin or a bacteriophage. Competition for space is strongly suggested by microscopic examination of roots or hypocotyl slices where invasion of the competitors always occurred in separate xylem vessels: the greater the number of vascular bundles invaded by the HrcV⁻ mutant strain the greater the prevention of the pathogenic strain. The exclusive colonization by the HrcV⁻ mutant was always associated with the presence of the mutant in all vascular bundles of hypocotyl

Table 4. Invasiveness of the HrcV⁻ GMI 1556 mutant strain after delayed challenge inoculation with the GMI 1557 pathogenic strain

Inoculations ^a	Days after mutant strain inoculation ^b	Isolation		β-Galactosidase staining	
		Positive isolations (%) ^w	Bacterial densities ^x	Positive bundles (%) ^y	Exclusion (%) ^z
1W	18	37.5 a	7.79 ± 0.57	38.0 a	0 a
3W	18	70.0 ab	8.05 ± 0.64	39.0 ab	0 a
mG	11	92.5 bc	7.93 ± 0.48	91.0 de	37.5 b
1W	31	NA	NA	NA	NA
3W	31	95.0 bcd	8.17 ± 0.52	51.0 abc	0 a
mG	31	100 cd	8.04 ± 0.59	89.0 d	57.5 bc
mG	41	100 cd	8.78 ± 0.61	92.0 def	62.5 bcd

^a For definitions see Table 1.

^b Days after the first inoculation with the GMI 1556 mutant strain, i.e., 11, 24, and 34 days after the challenge inoculation with the pathogenic strain, respectively.

^w Percentages of positive isolations of the mutant strain onto agar medium supplemented with kanamycin and chloramphenicol. NA = not analyzed, totally wilted. Data within columns that are followed by the same letter are not significantly different according to the 95% confidence limits of percentages (Sokal et al. 1969).

^x Bacterial densities as in Table 1.

^y Percentages of vascular bundles containing the mutant strain, see Table 1.

^z Percentages of exclusion: percentages of symptomless plants from where the pathogenic strain could neither be isolated on specific medium nor detected in vascular bundles.

slices, which reinforces the hypothesis of competition for space. However, the prevention of colonization of the pathogenic strain at the hypocotyl level seems somewhat contradictory with the increased density of the HrcV⁻ mutant strain, unless we admit that nutritional complementation has already occurred at a lower level in the roots. Prevention of the pathogenic strain at the hypocotyl level is all the more puzzling as infection and subsequent invasion of xylem vessels by the pathogenic strain appeared to be independent of the presence of the Hrp⁻ mutant strain in root tissues, and that invasiveness by the pathogenic strain has always been described as faster and more massive than that of Hrp⁻ mutant strains (Trigalet and Demery 1986; Trigalet and Trigalet-Demery 1990; Frey et al. 1994). It is unlikely that any technique for inoculation of the HrcV⁻ mutant strain, even if it is highly efficient, could ensure a complete establishment of the mutant in all of the xylem vessels, because as roots grow they give the pathogenic strain the opportunity of infecting newly emerged root tips and lateral root cracks that are not yet colonized by the HrcV⁻ mutant strain.

Local resistance of tomato tissue induced by the presence of the Hrp⁻ mutant strain is another possible mechanism that may be involved in the observed protection. Formation of tyloses and browning of cells in the vicinity of invaded xylem vessels of tomato have already been observed after inoculation by the HrcV⁻ mutant strain GMI8172 alone (P. Frey, *unpublished data*). Both competition for space within xylem vessels and induction of a local, nonspecific resistance induced by the HrcV⁻ mutant strain appear to be the most probable explanations that could account for the protective effect against a challenge infection by the pathogenic strain. Induced resistance resulting from the presence within host tissues of non-pathogenic mutants of *Ralstonia solanacearum* has long been suggested (Sequeira and Hill 1974; Sequeira et al. 1977; Kempe and Sequeira 1983) and complex, different mechanisms are probably involved in the induced resistance, the nature of which is not yet elucidated.

Work is in progress to assess the potential of the local resistance induced by previous establishment of a protective Hrp⁻ mutant strain upon multiplication of the mutant strain itself or upon a subsequent, pathogenic invader strain. In cauliflower leaves the growth deficiency of an HrpXc⁻ mutant of *Xanthomonas campestris* pv. *campestris* can be complemented by the presence of the wild-type strain (Kamoun and Kado 1990). A possible explanation for this complementation lies in the ability of the wild-type strain to suppress the vascular hypersensitive response (VHR) of the plant, hence enabling the HrpXc⁻ mutant to grow, whereas the mutant cannot develop when the VHR normally occurs (Kamoun et al. 1992). The latter explanation does not seem to be relevant to the GMI 1485 pathogenic strain, which induces browning of cells in the vicinity of invaded xylem vessels and vascular coating (Vasse et al. 1995). However, because the nature of the localized resistance induced by both competitors is only based upon microscopic observations (tyloses and cell browning) we need additional quantitative information about the molecular mechanism that is involved in the VHR to assess the potential suppression of localized induced resistance by the pathogenic strain.

The double-staining technique involving a two-step procedure has already been usefully applied to study the expression of a plant promoter-GUS gene fusion in the presence of either symbiotic or phytopathogenic bacteria (Pichon et al. 1994;

Pontier et al. 1994). The staining procedure described in this work and used in hypocotyl slices embedded into soft agar is an original, one-step, and easy to handle procedure. This technique is also very well adapted to the simultaneous detection of two different bacterial strains in vascular tissues of the host plant, which to our knowledge is described for the first time here. The endogenous β -galactosidase activity of tomato tissues can be inactivated by incubation of tomato fragments in 1.25% glutaraldehyde for 1 h. However, this endogenous activity was weak compared with that of the Hrp⁻ GMI 1556 mutant strain and the prior inactivation step was not actually necessary.

Our present results show that the microgranule inoculation technique is more efficient than watering for optimal colonization of the vascular system by the HrcV⁻ mutant strain and may lead to significant protection against tomato bacterial wilt. The use of comparable granular inoculants amended with nutrients has recently been employed to improve efficiency of inoculation of *Bradyrhizobium japonicum* in soils (Fouilleux et al. 1994, 1996). Work is in progress on technical improvements that are necessary to extend the use of enriched clay microgranules as an optimal carrier for Hrp⁻ protective mutant strains in field experiments with a view to applying biological control of *R. solanacearum* under natural conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, transposons, and culture conditions.

Origins and relevant characteristics of bacterial strains, transposons, and plasmids used in this study are listed in Table 2. *Escherichia coli* was grown at 37°C in Luria broth (LB) (Maniatis et al. 1982). *R. solanacearum* was streaked on Kelman's solid medium (Kelman 1954) and grown at 30°C in peptone broth (Boucher et al. 1985).

Plant material, growth conditions, and root inoculations.

Tomato cv. Supermarmande (Vilmorin, La Ménétrée, France) seedlings were grown in test tubes under axenic conditions. Seeds of tomato were surface sterilized and germinated as previously described (Boucher et al. 1985). Five germinated seeds were deposited onto the surface of 75 ml of 0.7% soft agar containing 0.46% (wt/vol) Murashige and Skoog plant nutrient solution (Collect, ICN Biomedicals, Costa Mesa, CA) in large, sterile test tubes (33 mm diameter, 220 mm long). Tubes were kept in a growth chamber (16 h light, 400 μ E s⁻¹ m⁻², 30°C; 8 h dark, 26°C). Plants were inoculated by adding 5 ml of the mutant strain inoculum adjusted to 2.10⁸ CFU · ml⁻¹ into each test tube at the first leaf stage. Challenge inoculation, with 5 ml of the pathogen inoculum adjusted to 2.10⁷ CFU · ml⁻¹, was done 7 days after the first inoculation with the Hrp⁻ mutant strain. Small tomato plants inoculated in test tubes were further examined to visualize the very first stages of root infection and invasion of xylem vessels.

Tomato plants were also grown in peat pots and inoculated at the two-leaf stage (3 weeks old) either by watering with a calibrated inoculum or by planting tomato seedlings (cotyledon, one-leaf stage) in friable horticultural humus that had previously been amended with clay microgranules (Natural Plant Protection, Noguères, France) containing carbon and nitrogen nutrients and the Hrp⁻ calibrated inoculant. Watering inoculation was carried out by pouring 30 ml of

2.10^8 CFU $\cdot$ ml $^{-1}$ mutant strain suspension onto each peat pot, once (1W) or three times with a 2-day interval (3W). The clay microgranules (mG) were previously enriched with carbon and nitrogen sources with a modified procedure from Foulleux et al. (1996) that enabled survival of the HrcV $^{-}$ mutant inoculum at a concentration of 10^5 CFU per g of dry soil after 3 months in naturally infested soils in Guadeloupe (data not shown). Enriched microgranules were air dried at 37°C and stored in a container, with silica gel as desiccator. Before inoculation, dried microgranules were moistened with a 40% (vol/wt) of a 3.10^9 CFU $\cdot$ ml $^{-1}$ mutant strain suspension. Moistened microgranules were then incorporated in the peat-mold into which tomato plants had been transplanted. Five grams of microgranule-inoculum was used for each plant. Whatever the technique used, the HrcV $^{-}$ mutant suspension was calibrated to obtain a mean inoculum of about 6.10^9 CFU for each individual plant. The pathogenic strain was inoculated at a concentration of 6.10^8 CFU for each individual plant, directly by watering control plants, and 7 days after the mutant strain had been inoculated, in the case of dual inoculation. Inoculated plants were kept in a growth chamber (16 h light, 400 μ E s $^{-1}$ m $^{-2}$, 31°C; 8 h dark, 26°C). Three independent replications were carried out and 60 plants were used in each treatment. Twenty plants were sampled for each replicate at three different times after the inoculation with the pathogenic strain.

DNA methods.

Standard methods (plasmid DNA extraction, restriction enzyme digestion, DNA ligation, agarose gel electrophoresis, transformation of *E. coli*, Southern blotting, and hybridization) were performed as already described (Ausubel et al. 1990). Isolation of genomic DNA and transformation of *R. solanacearum* by linearized plasmid DNA were performed as previously described (Boucher et al. 1985).

Introduction of the *gus* gene into *R. solanacearum* strains.

The Tn5-*uidA* transposon was kindly provided by E. R. Signer (Sharma and Signer 1990). Mutagenesis was performed with triparental matings with *R. solanacearum* GMI 1000, *E. coli* LE392 containing the pSB467 plasmid (Table 2), and the pRK2013 plasmid as helper, as previously described by Figurski and Helinski (1979).

Ten milliliters of each exponentially grown culture was put onto a Millipore conjugation membrane. After overnight incubation on Kelman's solid medium, bacteria were resuspended in LB medium and incubated with T4 bacteriophage for 2 h at 37°C with agitation to eliminate *E. coli* cells. Bacteria were centrifuged and streaked onto minimal medium containing glycerol and tetracycline. Tetracycline-resistant transconjugants (10μ g $\cdot$ ml $^{-1}$) were purified on the same minimal medium and their GUS activity was assessed as described below.

Introduction of the *lacZ* gene into *R. solanacearum* strains.

Because the pathogenic GMI 1485 (Arlat et al. 1992) was resistant to kanamycin due to the presence of the transposon Tn5-B20 (Simon et al. 1989) we could not use the pPMV138 plasmid (Table 2) to select a mutant containing the *hrcV*:: Ω -Km interposon. Therefore, we substituted the Km resistance gene for the chloramphenicol resistance gene of the Ω -Cm interposon (Fellay et al. 1987). DNA of plasmid pPMV138 (Table 2) was partially digested by *Bam*HI to eliminate the Ω -

Km interposon located at the *Eco*RV site of the *hrcV* gene (Frey et al. 1994). The resulting 9.8-kb fragment was separated by electrophoresis and purified with a GeneClean kit (BIO 101, La Jolla, CA). Then, a 3.7-kb purified *Bam*HI fragment of an Ω -Cm interposon (Prentki and Krisch 1984) was cloned into the 9.8-kb fragment and the ligation product was used to transform *E. coli* DH5 α . Kanamycin (50 μ g $\cdot$ ml $^{-1}$) and chloramphenicol (10 μ g $\cdot$ ml $^{-1}$) resistant transformants were purified and some individual clones were physically mapped. Purified recombinant DNA, prepared by the Qiagen method (Qiagen, Courtaboeuf, France), was further used to transform the GMI 1485 strain (Table 2). Integration of the Ω -Cm interposon into the *hrcV* gene was checked by Southern blot analysis of total genomic DNA with an enhanced chemiluminescence (ECL) direct nucleic acid labeling system (plasmid pPMV138) probe (Amersham Pharmacia Biotech, Courtaboeuf, France). The gene name *hrcV* is now used instead of *hrpO* in agreement with the unified nomenclature of the *hrpO* genes of phytopathogenic bacteria (Bogdanove et al. 1996).

GUS and β -galactosidase activity assays.

GUS enzyme activity was spectrophotometrically quantified by Jefferson's procedure (Jefferson et al. 1986), slightly modified. Briefly, bacteria were grown overnight in Kelman's broth medium. One milliliter of each exponential culture was permeabilized with 2 drops of toluene and 10 s of intense vortexing. In a microtitration well, 30 μ l of these suspensions was mixed with 120 μ l of a reaction buffer (50 mM NaHPO $_4$, pH 7.0, 1 mM EDTA, 5 mM dithiothreitol (DTT), 100 mM *p*-nitrophenyl- β -D-glucoside [PNPG]) and incubated at 37°C. Absorbance was measured at 405 nm in an automatic plate reader (MR 5000, Dinattech Lab., Saint-Cloud, France).

Histochemical assays for GUS and β -galactosidase activities were performed on tomato roots as follows: (i) for GUS activity, tomato roots were rinsed in 50 mM phosphate buffer pH 7.0, and prefixed in a 0.5% buffered *p*-formaldehyde solution for 45 min at 0°C, rinsed four times in 50 mM phosphate buffer, incubated in the GUS reaction solution (50 mM phosphate buffer, pH 7.0, 5 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 mg of Magenta-gluc [Byosynth, Staad, Switzerland] per ml), 0.02% sodium dodecyl sulfate (SDS) for 2 h at 37°C; (ii) for β -galactosidase activity, the roots were rinsed in phosphate buffer and fixed 1 h with 1.25% (vol/vol) glutaraldehyde to eliminate the endogenous plant β -galactosidase activity, rinsed again twice in 0.2 M cacodylate buffer pH 7.2, and incubated overnight at 20°C in the 0.2 M cacodylate buffer containing 5 mM potassium ferro and ferricyanide and 0.08% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate. Then, whole root samples were examined by microscopy with a Leitz photomicrographic system (WILD MPS 46/52, Leitz, Germany).

A second staining procedure was developed with transverse sections taken at the hypocotyl level of tomato plants that have previously been inoculated by the bacterial strains of interest. Tomato stems were surface sterilized with a 70% ethanol solution and cut into 2-mm slices. These slices were deposited onto a 0.8% soft agar layer, solidified onto a microscope slide. The 0.8% sterile soft agar contained the following sterile reaction buffer: NaHPO $_4$ 50 mM, potassium ferricyanide 5 mM, potassium ferrocyanide 5 mM, Magenta-gluc (Biosynth) 1 mg/ml, X-Gal (Biosynth) 0.04% (wt/vol). Stem slices were

immediately covered with a thin layer of the same soft agar, then incubated overnight in a moist chamber at 30°C. Segments included in this reactive agar were observed by microscopy.

Bacterial isolation and quantification.

Stems of sampled plants were surface sterilized with ethanol and cut into 1-cm-long segments at the hypocotyl level. Each segment was left standing overnight on the selective agar medium before the segment was removed. The plates were incubated at 30°C for 2 days to observe bacterial growth. Two-centimeter stem segments, originally adjacent to the imprinted segments, were incubated in test tubes containing 4 ml of distilled water at 10°C for 3 h. Bacterial suspensions were serially diluted and plated onto specific agar medium with a Spiral Plate Maker (Interscience, Saint Nom la Breteche, France). After incubation at 30°C for 48 h, bacterial densities were calculated and expressed as log CFU · g (dry weight)⁻¹ of stem tissue.

Disease and exclusion rating.

Disease development, expressed as a disease index on a scale of 0 to 5 (Winstead and Kelman 1952) was recorded for each treatment at different times after challenge inoculation with the GMI 1557 pathogenic strain. Relative exclusion index was expressed as the percentage of plants from which the pathogenic strain could not be isolated either by imprinting or counting, or detected by staining for GUS activity in agar inclusions.

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