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Polygalacturonases are required for rapid colonization and full virulence of *Ralstonia solanacearum* on tomato plants

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Ralstonia solanacearum causes bacterial wilt, a soil-borne vascular disease of many crop plants. Plant cell wall-degrading polygalacturonases (PGs) are significant virulence factors for this pathogen. *R. solanacearum* mutants lacking PehA (an endo-PG), or PehB (an exo-PG), or both PehA and PehB are all less virulent than the wild-type strain on wounded eggplants. To more closely mimic the natural infection process, unwounded tomato plants were soil-soak inoculated with wild-type and PG mutant strains. All three PG mutants were significantly reduced in virulence on intact tomato plants, despite surviving well in potting mix. Over the course of disease development, populations of PG mutant and wild-type bacteria in plants were significantly different. The PG mutants, especially the two *pehA* mutants, colonized fewer tomato stems, colonized stems more slowly, and had lower mean bacterial populations in stems than the wild-type strain. These results suggest that PehA and PehB are necessary for rapid host colonization, and that production of these enzymes contributes quantitatively to the ability of *R. solanacearum* to colonize host vascular tissue and wilt plants. A threshold bacterial population of around 1×10^8 cfu in the centimetre of tomato stem directly above the cotyledons correlated with the appearance of wilt symptoms.

Keywords: bacterial wilt; *Ralstonia solanacearum*; *Pseudomonas solanacearum*; *Lycopersicon esculentum*; polygalacturonase.

INTRODUCTION

Ralstonia solanacearum (Smith) Yabuuchi *et al*. causes bacterial wilt, a devastating plant disease common in tropical, subtropical and warm-temperate regions of the world [*5*, *15*]. This bacterium has a very wide host range, attacking over 450 different plant species, including such economically important crops as tomato, tobacco, potato, peanut and banana [*15*, *23*]. A soil-borne pathogen, *R. solanacearum* normally enters plant roots through wounds or natural openings where lateral roots emerge. It first colonizes the host's root cortex, then infects the vascular parenchyma, and finally invades the xylem elements, which transport water and nutrients up from the soil [*30*, *31*]. Once inside the xylem, the bacteria multiply and spread rapidly throughout the plant's vascular system. Susceptible plants respond to these high bacterial populations by wilting, yellowing and dying. Little is known about the early events in the development of this disease and what specific factors contribute to the rapid systemic colonization of host plants by the bacterium. Moreover, *R. solanacearum* can thrive in plant vascular systems without causing disease [*11*, *15*]. Although latent infections are epidemiologically important, the traits required to establish and maintain bacterial populations in symptomless hosts are not understood [*23*].

R. solanacearum produces several known virulence factors, including extracellular polysaccharide (EPS), and a consortium of plant cell wall-degrading enzymes such as endoglucanase (EG) and polygalacturonase (PG). Mutants deficient in the production of EPS and EG suffer reduced virulence [*8*, *19*, *25*], but the precise roles of these factors in disease development are not yet known. A recent study of tomato root invasion and stem colonization by *R. solanacearum* mutants deficient in the production of EG and EPS I (high molecular mass acidic EPS) found that neither EPS I nor EG are necessary for root invasion [*26*]. However, EPS I and EG are required for rapid stem colonization by *R. solanacearum*. EPS I also facilitates spread of the bacterium within tomato stems [*26*].

Pectic compounds consist largely of polymerized galacturonic acid residues and are major components of higher plant primary cell walls and middle lamellae [*10*, *12*]. *R. solanacearum* produces three PGs, which degrade pectic polymers by hydrolysis. PehA is an endo-PG, which cleaves the polymer internally at random, releasing mostly galacturonic acid trimers and larger oligomers.

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PehB and PehC are both exo-PGs. They are different, however, in that PehB is an exo-poly-α-D-galacturonosidase that releases digalacturonic acid, while PehC is likely an exopolygalacturonase that releases only galacturonic acid monomers [17]. Site-directed *pehA*, *pehB* and *pehA pehB* double mutant strains are all significantly reduced in virulence relative to wild-type strain K60, and the *pehA pehB* double mutant is less virulent than either single mutant [17, 27]. Thus, PehA and PehB are both required for full virulence of *R. solanacearum*, and they contribute additively to host plant wilting and mortality.

However, we do not know just how PGs act during disease development. These enzymes could conceivably play many roles in bacterial wilt disease and/or pathogen survival. PGs may aid bacterial root invasion by digesting pectic gels around wounds or lateral root emergence points, and by degrading root cortical cell walls; by digesting pectin they may provide nutrients facilitating rapid bacterial multiplication; they may block xylem vessels by generating gels of partially digested pectic materials and weakening cell walls to release tyloses [3]; they may contribute to bacterial spread and colonization by breaking down the pectin-containing pit membranes that separate adjacent xylem vessels; the oligogalacturonides generated by PGs may induce the production of other bacterial virulence factors [16, 32]; and finally, PGs may macerate plant tissue in the terminal stages of wilt disease and during saprophytic growth [17, 18, 28]. These various hypothesized biological roles are not mutually exclusive.

To understand more precisely how PGs contribute to the virulence of *R. solanacearum*, we tested the hypothesis that PG facilitates bacterial colonization of host stems by conducting a time-course study on the colonization of tomato stems by *R. solanacearum pehA*, *pehB* and *pehA pehB* mutants. We also studied the progression of the pathogen along tomato stems as disease developed, and identified a correlation between threshold bacterial population levels in stems and the appearance of disease symptoms.

MATERIALS AND METHODS

Bacterial strains and growth

We used a wild-type and three mutant strains of *R. solanacearum* in this study (Table 1). To prepare bacterial inoculum, *R. solanacearum* strains were freshly streaked from frozen stocks onto TZC plates [20]. Streptomycin and gentamicin were added at 30 and 10 mg ml^{-1}, respectively, as appropriate. Strains were grown in Boucher's minimal medium [4] containing 0·1 % (w/v) yeast extract and 0·2 % (w/v) citric acid (pH adjusted to 7·0). Appropriate concentrations of the bacterial suspensions were made in sterile water using OD_{600} as an initial measurement of cell density. Final inoculum cell density was confirmed by dilution plating. To detect and quantify bacteria in plant stems, tissue homogenates were dilution plated on modified semi-selective medium (SM) [11] in which TZC agar was amended with cycloheximide (100 $\mu g\ ml^{-1}$), bacitracin (25 $\mu g\ ml^{-1}$), polymyxin B sulfate (50 $\mu g\ ml^{-1}$), chloramphenicol (6·25 $\mu g\ ml^{-1}$) and ampicillin (12·5 $\mu g\ ml^{-1}$).

Plant growth and inoculation

Tomato (*Lycopersicon esculentum* Mill. cv. "Bonny Best") seeds were planted in Peat-lite mix (Scotts-Sierra Horticultural Company, Marysville, OH, U.S.A.) grown in a growth chamber at 28 °C with 14 h of light. Twelve days after planting, the seedlings were transplanted to 100 g (dry wt) of mix in four pots with saucers and watered daily with half-strength Hoagland's solution. Plants were inoculated 3 days after transplanting with *R. solanacearum* by pouring 50 ml of bacterial inoculum into each pot. Water was used as a negative control. Plants were coded and plant location was randomized across treatments. For virulence assays, the plants were rated daily using a five-point disease index (DI) scale as follows: 0, no wilted leaves; 1, ⩽25 % wilted; 2, 26–50 % wilted; 3, 51–75 % wilted; and 4, 76–100 % wilted or dead [25].

Bacterial survival in potting mix

To test the survival of *R. solanacearum* strains in Peat-lite mix, 50 ml of bacterial suspension were poured into pots prepared as above to yield an initial population of 3·10–3·75 × 10^7 cfu g^{-1} soil; pots were incubated in a 28 °C growth chamber. To determine the number of live bacterial cells in the mix, core samples from each infested pot were taken using a No. 6 cork borer 3 and 15 days after potting mix infestation, put into preweighed flasks containing 50 ml of sterile water, and weighed again. The resulting suspensions were agitated and dilution plated on SM for wild-type strain K60, and on SM plus streptomycin and gentamicin for strain K60-309.

TABLE 1. *Bacterial strains used in this study*

Strain	Relevant characteristics*	Reference
Ralstonia solanacearum		
K60	Wild-type race 1, biovar 1	[20]
K60-06	K60 *pehA*::Ω Ap^r Cm^r Sm^r	[2]
K60-209	K60 *pehB*::*aacC1* Ap^r Cm^r Gm^r	[17]
K60-309	K60 *pehA*::Ω *pehB*::*aacC1* Ap^r Cm^r Sm^r Gm^r	[17]

*Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin; and superscript r, resistant.

Sampling of plant stems

To determine bacterial populations in the stems of inoculated tomato plants, the plants were removed from the pots at different times after inoculation. The roots and tops of the plants were cut off, and the remaining stems were washed under running water. A 3 cm stem segment spanning the crown was sterilized in 10 % household bleach for 1 min, followed by two 1 min washes in sterile water. A 1 cm internal segment, either 0·5 cm above the crown (lower stem) or immediately above the cotyledon (midstem), was then excised from the 3 cm stem piece and ground in 200 μl sterile water in a 1·7 ml microcentrifuge tube. The homogenates were dilution plated on TZC with appropriate antibiotics using Autoplate model 3000 (Spiral Biotech). Plates were incubated at 28 °C until colonies were apparent. Our theoretical threshold for detection was 10 cfu cm^{-1} stem.

Statistical analysis

We $\log(x + 1)$ transformed the bacterial population data as recommended by Steel and Torrie [29]. Data on virulence, potting mix survival and tomato stem colonization of *R. solanacearum* strains were analysed by ANOVA using the SAS, version 6.10 (SAS Institute, Cary, NC, U.S.A.) general linear models procedure. Means were compared using the least significant difference (LSD) test.

RESULTS

Virulence of R. solanacearum *PG mutants on tomato plants*

To establish the relative virulence of the PG mutants K60-209 (PehB$^-$), K60-06 (PehA$^-$), and K60-309 (PehA$^-$ PehB$^-$) and the wild-type strain on tomato plants, we inoculated unwounded tomato plants by pouring bacterial inoculum into the soil. All the PG mutants were significantly reduced ($P < 0{\cdot}001$) in virulence relative to the wild-type strain when tomatoes were inoculated using this biologically representative procedure. The *pehA pehB* double mutant strain K60-309 was the least virulent of all, with an average final disease index (DI) of less than 2, compared with a DI of 3·94 for wild-type strain K60 (Fig. 1).

Survival of R. solanacearum *PG mutant strains in artificially infested potting mix*

To determine whether similar populations of the wild-type and PG mutant strains were present in the potting mix under our experimental conditions, we compared the survival of *pehA pehB* double mutant strain K60-309 to that of wild-type strain K60. We measured the number of viable *R. solanacearum* cells recovered from potting mix 3 days after infestation (immediately before disease symptoms appear) and 15 days after inoculation (just after the end of the virulence assay). Populations of both strains in potting mix declined quickly after initial infestation, dropping approximately 40-fold from around 2×10^7 cfu g^{-1} on day 3 to around 8×10^5 cfu g^{-1} on day 15. However, there was no significant difference in survival between the wild-type and PG mutant strains at either 3 or 15 days after infestation.

Spread of R. solanacearum *PG mutants along the stem*

To measure pathogen progress up the stems, we quantified bacterial populations in the lower and midstems of inoculated plants. Unsurprizingly, cells of this root-infecting pathogen were detected first in the lower stem (data not shown). In addition, if bacterial cells were present in the midstem before wilt symptoms appeared, then the midstem population was always 10- to 1000-fold lower than that in the lower stem. However, once plants showed any symptoms (DI > 0), pathogen populations in the lower and midstem were similar.

Colonization of tomato stems by PG mutants

Rapid bacterial invasion and colonization of host plants could depend on the ability to degrade plant pectic substances. To determine if the low virulence of PG mutants is associated with reduced plant infection and/or to lower bacterial populations *in planta*, we compared the presence and density of wild-type and PG mutant strains in stems of infected tomato plants. Loss of PG production slowed *R. solanacearum* plant colonization significantly

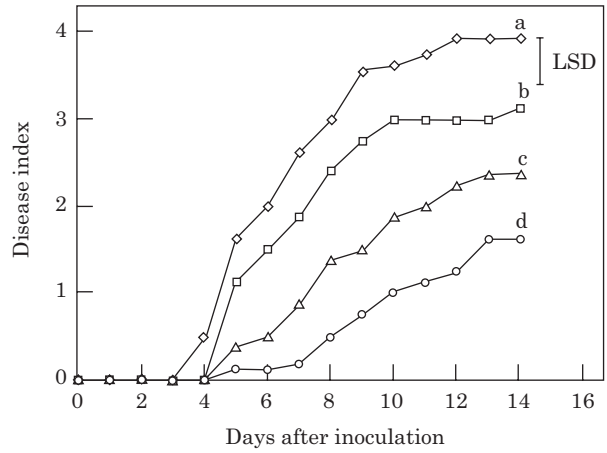

FIG. 1. Virulence of *R. solanacearum* PG mutants on tomato plants. Fifteen day old tomato plants were inoculated by pouring $2{\cdot}5 \times 10^7$ bacteria g^{-1} soil into each pot. Strains are K60 (wild-type, ◇); K60-209 (PehB$^-$, □); K60-06 (PehA$^-$, △); and K60-309 (PehA$^-$PehB$^-$, ○). Plants were rated on a disease index ranging from 0 (healthy) to 4 (76–100 % leaves wilted). Points shown are the means of three separate experiments, each containing 16 plants per treatment. Means with different letters are significantly different at day 14 according to the LSD test ($P < 0{\cdot}001$). The vertical line indicates the LSD value.

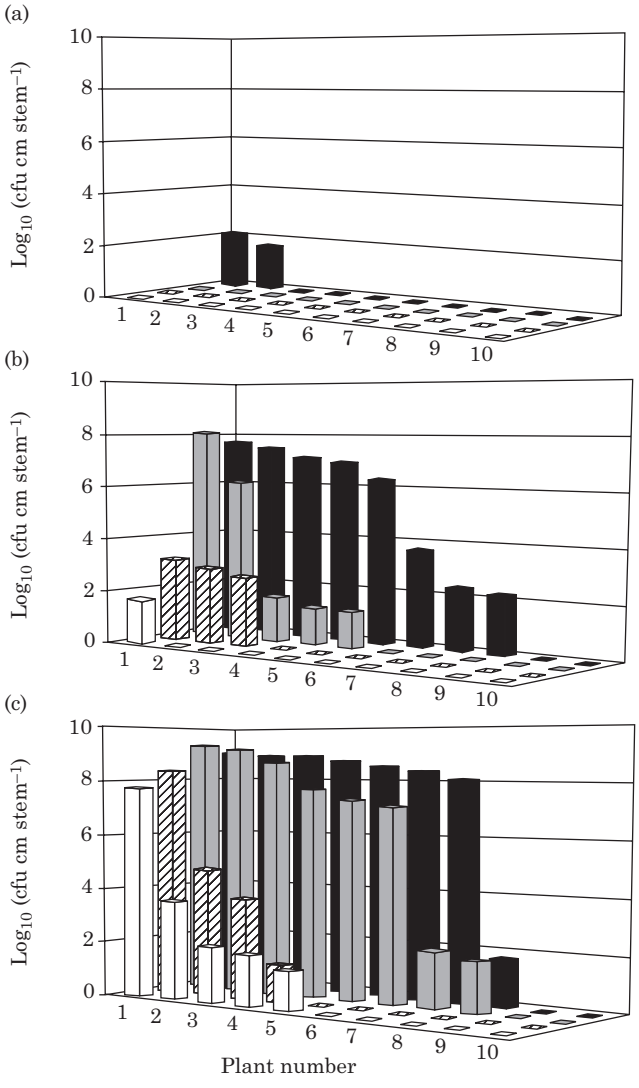

FIG. 2. Colonization of individual tomato stems by *R. solanacearum* PG mutants. Bacterial populations were assayed in the 1 cm of stem immediately above the cotyledon in 10 plants per strain 3 (a), 4 (b), and 5 (c) days after inoculation. Plants were inoculated by pouring a bacterial suspension onto the soil surface to a final concentration of 5–6·25 × 10^7 cfu g^{-1} potting mix. Each vertical bar denotes the bacterial population in a single plant inoculated with strains K60 (wild-type, ■), K60-209 (PehB⁻, ▩), K60-06 (PehA⁻, ▨) and K60-309 (PehA⁻PehB⁻, □).

TABLE 2. *Bacterial populations* in stems of tomato plants inoculated with* R. solanacearum *PG mutants*

Strain	Day 3	Day 4	Day 5
K60 (wild-type)	0·38 a†	4·34 b	6·22 b
K60-209 (PehB⁻)	ND a‡	1·87 a	5·31 b
K60-06 (PehA⁻)	ND a	0·87 a	1·81 a
K60-309 (PehA⁻PehB⁻)	ND a	0·17 a	1·66 a

*Average bacterial populations from the 10 plants shown individually in Fig. 2, expressed as $\log_{10}$ cfu cm^{-1} stem.

†Means ($n = 10$) with different letters are significantly different according to the LSD test ($P < 0·001$).

‡Not detectable, considered as 0 for statistical purposes.

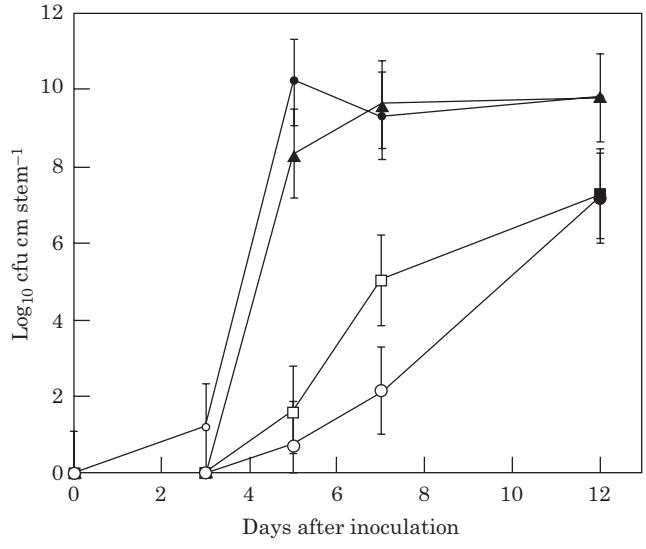

FIG. 3. Average *R. solanacearum* populations in tomato stems. For each time point, the 1 cm section of stem above the cotyledons was ground and dilution plated separately as described in Methods ($n = 16$). (●) K60 (wild-type); (▲) K60-209 (PehB⁻); (□) K60-06 (PehA⁻); (○) K60-309 (PehA⁻PehB⁻). Open symbols indicate that the majority of plants at this timepoint showed no disease symptoms; closed symbols indicate that the majority of plants showed disease symptoms (DI > 1). Bars indicate LSD groupings ($P < 0·001$). Inoculum concentrations (cfu g^{-1} potting mix) were 9·5 × 10^7 for K60, 1·23 × 10^8 for K60-209, 1·05 × 10^8 for K60-06, and 1·10 × 10^8 for K60-309.

(Fig. 2). Three days after inoculation, the pathogen was already present in 20 % of the stems of plants inoculated with wild-type strain K60, but in none of the PG mutant-inoculated plants. Four days after inoculation, *R. solanacearum* was detected in 80 % of K60-inoculated stems, as compared with 50, 30 and 10 % of the stems from plants inoculated with the *pehB*, *pehA* and *pehA pehB* mutants, respectively. The average population of the wild-type strain was significantly different from those of all three PG mutants on day 4 (Table 2). By day 5, both the wild-type and *pehB* mutant were present in 80 % of tomato plants, while the *pehA* and *pehA pehB* mutants had infected only 40–50 % of the plants. At this point, K60 stem populations were significantly different from those of the two *pehA* mutants, K60-06 and K60-309, although not from populations of *pehB* mutant K60-209 (Table 2).

To determine if tomato colonization by the PG mutants differed from the wild-type strain over the full course of disease, we also measured stem population levels in a longer time frame (Fig. 3). As above, after 3 days,

bacteria were detected only in plants inoculated with the wild-type strain. The wild-type population increased to approx. 10^8 cfu cm^{-1} stem by day 5, levelled off to around 10^9 at day 7, and then increased only slightly by day 12. Average populations in plants inoculated with *pehB* mutant K60-209 were two orders of magnitude lower than those of the wild-type strain at day 5, although, as above, the two strains were not significantly different. K60 and K60-209 populations were similar at days 7 and 12. However, stem populations of the *pehA* and *pehA pehB* mutants remained significantly lower than those of K60 and the *pehB* mutant throughout the entire experiment. The *pehA pehB* double mutant did not reach 10^2 cfu cm^{-1} stem until 7 days after inoculation.

Threshold bacterial populations for wilt symptoms

The appearance of wilt symptoms reliably correlated with pathogen population level in the stem (Fig. 3). Without exception, whenever plants had a disease index of 1 or greater, we detected at least $1{\cdot}18 \times 10^8$ cfu cm^{-1} stem immediately above the cotyledon. This relationship held true for both the wild-type strain and all three PG mutant strains tested. Stems of asymptomatic plants contained fewer than $1{\cdot}18 \times 10^8$ cfu cm^{-1} in all but two of the 42 tomato stems analysed.

DISCUSSION

Three previously constructed *R. solanacearum* mutants deficient in production of extracellular PGs PehA, PehB, and both PehA and PehB, respectively, cause significantly less disease than the wild-type strain on artificially wounded eggplants [2]. Here, we found that these three PG mutants were also significantly reduced in virulence on a different host, tomato, under more biologically representative inoculation conditions. We then used these inoculation conditions to determine whether PGs are necessary for rapid host plant invasion and colonization.

We found that *R. solanacearum* populations in potting mix decreased significantly over time, which is consistent with previous data on survival of this species in natural soil [13, 22, 24]. Under our conditions, after 15 days we detected about 8×10^5 cfu g^{-1} of both the wild-type and PG double mutant strains, a population level documented to cause disease in naturally infested soil [9, 15]. Thus, both strains were equally available for infection well past the point when all plants inoculated with the wild-type strain were either symptomatic or dead, demonstrating that the reduced virulence of the PG mutants was not caused by their poor survival in potting mix. A logical alternative hypothesis is that PG mutants were less virulent because they did not invade the root and/or systemically colonize host plants as well as the wild-type strain.

We chose to measure colonization as bacterial populations in stems rather than roots because the presence of large bacterial populations in the stem represents a well-established infection, while passive entry into the root cortex can only be distinguished from successful colonization of xylem vessels by histological analysis [26]. In addition, quantifying root-associated bacterial populations is complicated by the contaminating microflora in rhizosphere soil [14]. Finally, because stem colonization is unquestionably prerequisite to wilt disease development [26], populations at this stage may be most biologically informative.

Our data identify a bacterial population threshold in tomato stems for the onset of wilt symptoms. Saile *et al.* [26] reported that *R. solanacearum* had to colonize the entire stem before tomato plants began to wilt. We found that the bacteria not only had to colonize the stems, but also to attain a population level above $1{\cdot}18 \times 10^8$ cfu cm^{-1} in order to cause wilting. It appears that Bonny Best, the susceptible tomato cultivar used here, can tolerate only a certain level of *R. solanacearum*. It would be interesting to determine if disease-resistant cultivars can tolerate higher bacterial populations without wilting, although some work in this area suggests that resistant tomato cultivars do not allow high stem populations to develop [24].

Once bacteria entered the host stem, their populations increased very rapidly, often by four orders of magnitude in 48 h. During this very early window in colonization, we observed large differences in bacterial titre among individual plants within the same treatment, ranging, for example, from undetectable to 10^9 cfu cm^{-1} stem in tomatoes inoculated with strains K60 on day 5. However, 10 days after inoculation all K60-treated plants were fully wilted or dead, suggesting that although there may be variability in determining events early in colonization, all plants were eventually overwhelmed by the pathogen.

These stem colonization studies show that *R. solanacearum* wild-type and PG mutant strains differ significantly in their ability to spread and multiply in tomato stems. To summarize: (1) the PG mutants reached the 1 cm of stem above the cotyledon more slowly than wild-type strain K60; (2) the PG mutants colonized fewer tomato stems overall than K60, and the two *pehA* mutants, K60-06 and K60-309, colonized fewer tomato stems than the *pehB* mutant K60-209 at all sampling dates; and (3) the average bacterial population was significantly lower in PG mutant-infected tomato stems than in those infected by the wild-type strain K60. Populations of the two *pehA* mutants remained different from those of K60 and the *pehB* mutant even at day 12, while stem colonization by the *pehB* mutant differed from wild-type only until 4 days after inoculation. Therefore,

the absence of either the endo-PG, PehA, or the exo-PG, PehB, resulted in both reduced virulence (disease) and reduced ability to colonize tomato stems (as determined by bacterial populations in stems). Since the two *pehA* mutants were more affected than the *pehB* mutant, it appears that PehA plays a more important role in wilting plants and colonizing host stems.

Previous work demonstrated that EPS I and EG also affect tomato stem colonization by *R. solanacearum* [*26*]. Our results further suggest that the several extracellular virulence factors produced by this pathogen each contribute incrementally to host plant colonization and to the development of bacterial wilt disease. Importantly, however, none of these factors are absolutely required for colonization or pathogenicity (although EPS mutants are very severely attenuated in virulence). The virulence of a strain lacking all four known extracellular virulence factors remains to be determined.

Although we have found that PG mutants did not colonize tomato stems as well as the wild-type strain, we still do not know why. Gene regulation patterns suggest indirectly that PGs are important early in bacterial wilt disease development. Expression of the PehA and PehB structural genes is controlled by a regulatory cascade involving PhcA, a global regulator of several virulence functions, and PehSR, a positive two-component regulator of both PG production and bacterial motility [*1*, *7*]. In culture, PhcA represses *pehSR* expression at bacterial populations over about 10^6 cfu ml^{-1}, which in turn reduces PG production and cell motility [*1*]. Thus PG is probably expressed at its highest level at the low pathogen cell densities occurring early in disease development.

PG may help *R. solanacearum* invade roots by digesting the pectin gels surrounding lateral root emergence points, although some studies suggest that root invasion may be largely nonspecific [*6*, *21*, *26*]. It therefore seems most likely that PG enhances bacterial spread and colonization once bacteria are inside a host plant. These enzymes may digest pit membranes separating adjacent xylem vessels or contribute to wilting by generating the gels and tyloses observed in xylem vessels of wilted plants [*3*]. Endo-PG degrades pectin more quickly than exo-PG, which could explain why PehA makes a larger contribution than PehB to wilting plants and colonizing stems. Histopathological and ultrastructural studies comparing plants infected by *R. solanacearum* wild-type and PG mutant strains will help us to determine more precisely the roles of PG in host plant colonization.

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