Ralstonia solanacearum Pectin Methylesterase Is Required for Growth on Methylated Pectin but Not for Bacterial Wilt Virulence

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Ralstonia (Pseudomonas) solanacearum causes bacterial wilt, a serious disease of many crop plants. The pathogen produces several extracellular plant cell wall-degrading enzymes, including polygalacturonases (PGs) and pectin methylesterase (Pme). Pme removes methyl groups from pectin, thereby facilitating subsequent breakdown of this cell wall component by PGs, which are known bacterial wilt virulence factors. *R. solanacearum* PGs could not degrade 93% methylated pectin unless the substrate was first demethylated by Pme, but as the degree of methylation of the pectin substrate decreased, PG activity increased. Primers derived from a published *pme* sequence generated an 800-bp DNA probe fragment, which identified Pme-encoding plasmids from a *R. solanacearum* genomic library. A *pme* chromosomal mutant had no detectable Pme activity in vitro and no longer grew on 93% methylated pectin as a carbon source. Curiously, the *pme* mutant, which had no detectable PG activity on highly methylated pectin, was just as virulent as the wild-type strain on tomato, eggplant (aubergine), and tobacco. Since PG activity is required for full virulence, this result suggests that the pectin in these particular hosts may not be highly methylated, or that the breakdown of highly methylated pectin is not a significant factor in the disease process in general. A positive response regulator of PG production called PehR was not required for wild-type Pme production. However, a mutant strain lacking PhcA, which is a global regulator of several virulence genes, produced no detectable Pme activity. Thus, *pme* expression is directly or indirectly regulated by PhcA but not by PehR.

Bacterial wilt is a devastating plant disease that affects economically important hosts, such as potatoes, tomatoes, bananas, and tobacco (14). *Ralstonia* (formerly *Pseudomonas*) *solanacearum* (Smith) Yabuuchi et al., which causes the disease, has an extensive host range, including over 450 plant species in tropical and warm temperate zones worldwide (27). In many parts of the world, this disease is a primary constraint on crop production (10, 14). The bacterium is generally soil borne: it enters host plant roots through wounds or at lateral root emergence points, colonizes the root cortex, and subsequently invades the developing xylem vessels (37, 39). Once established in the xylem, the pathogen spreads rapidly throughout the plant, inducing yellowing, stunting, wilting, necrosis, and death.

Bacterial wilt disease appears to be the result of multiple virulence factors working in concert. *R. solanacearum* produces extracellular polysaccharide (EPS) that is important for disease development, although its precise mechanism of action is uncertain (17, 21, 32).

Other known virulence factors belong to an extracellular enzyme consortium that breaks down plant cell walls. These enzymes apparently facilitate bacterial invasion and spread by digesting cortical cell walls, the pectic gels surrounding lateral root emergence points, and the pit membranes that separate adjacent xylem vessels. Enzymatic cell wall degradation probably creates the gels and tyloses typically found in the vessels of wilting plants (6). In addition, these enzymes may also release nutrients that enable rapid bacterial multiplication.

Members of this consortium include an endoglucanase (Egl), which breaks down cellulose (30), and three polygalacturonases (PGs), which hydrolytically degrade the pectic compounds that are a major constituent of the primary plant cell wall and middle lamella (3). Pectins are complex polysaccharides, primarily chains of α -1,4-linked galacturonic acid; they also contain significant amounts of other sugars (12). A variable but substantial fraction of galacturonate residues in plant pectins is methylated (22, 26, 38). In contrast, polygalacturonate is a simple polymer of unmethylated galacturonate.

R. solanacearum produces one endo-PG, PehA (also known as PglA); an exo-poly- α -D-galacturonosidase, PehB; and an exo-PG, PehC. PehA cleaves polygalacturonate internally, yielding trimers and larger galacturate oligomers. PehB releases digalacturonic acid, while PehC generates only monogalacturonic acid from the same substrate. Site-directed mutants lacking PehA, PehB, or both are all significantly reduced in virulence, demonstrating that PG activity contributes quantitatively to bacterial wilt disease development (19, 31).

However, PGs cannot attack highly methylated pectins, so methyl groups must be removed from pectin substrates before hydrolytic cleavage. *R. solanacearum* produces one known pectin methylesterase, or Pme (E.C. 3.1.1.11), which cleaves methoxyl groups from methylated pectin, thereby making it susceptible to PG activity. The gene encoding this enzyme was cloned from *R. solanacearum* 50905 (35), but its role in pectin degradation and pathogenesis has not been studied.

Little is known about the biological function of Pme in plant pathogenesis. *Erwinia chrysanthemi*, which causes soft rot disease, produces two very different Pme's, PemA and PemB (24, 33). PemA, which is extracellular, is required for wild-type systemic spread of the bacterium in African violets (7). The role in virulence of PemB, which is an outer membrane lipoprotein, has not been determined.

To better understand the role of Pme in bacterial wilt disease, we cloned and characterized the *pme* gene from *R. so-*

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TABLE 1. Strains and plasmids used in this study

Strain	Relevant characteristics ^a	Reference or source
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 relA</i> ϕ 80 <i>lacZ</i> Δ M15 <i>hsdR17 supE44 thi-1 recA1</i> <i>gyrA96</i>	13
<i>R. solanacearum</i>		
K60	Wild-type race 1, biotype 1	23
K61-B	<i>pme::sacB</i>	This study
K60-309	<i>pehA::Ω pehB::aacC1</i> Sm ^r Gm ^{rb}	19
K60-509	<i>pehA::Ω pehB::aacC1</i> <i>pme::sacB</i>	This study
K71	<i>pehR::Tn3-uidA</i> Km ^r	1
K60- <i>phcA</i>	<i>phcA::Ω</i> Sm ^r	1
K71- <i>phcA</i>	<i>pehR::Tn3-uidA phcA::Ω</i> Km ^r Sm ^r	1
Plasmids		
pBluescript KS+	Ap ^r	Stratagene
pT7Blue	Ap ^r	Novagen
pLAFR3	Tc ^r	36
pMeB1	2.7-kb <i>ClaI</i> fragment of <i>pme</i> in pBluescript	This study
pMe71	1.8-kb <i>EcoRI</i> fragment of <i>pme</i> in pT7Blue	This study
pMeL1	1.8-kb <i>EcoRI</i> fragment of <i>pme</i> in pLAFR3	This study
pMe71 Δ SacII	Internal 0.55-kb deletion of <i>pme</i> in pMe71	This study
pUM24	<i>NptI sacB sacR</i> Km ^r	28
pMe7SB	3.8-kb <i>SacB-Kan</i> cartridge in- serted into <i>SacII</i> deletion of pMe71 Δ SacII	This study

^a Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Gm, gentamicin; Sm, streptomycin.

^b *pehA:: Ω* denotes the endo-PG-encoding *pehA* gene interrupted by the Ω gene cartridge, which encodes streptomycin resistance. *pehB::aacC1* denotes the *pehB* exo-poly- α -D-galacturonidase gene interrupted by a gentamicin resistance gene cartridge.

lanacearum K60. We then constructed a chromosomal *pme* mutant strain to determine how the loss of this activity affected bacterial virulence and ability to use highly methylated pectin as a sole carbon source. In addition, we studied the regulation of Pme expression by measuring Pme activity in various regulatory mutant backgrounds.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this research are listed in Table 1. *R. solanacearum* strains were cultured at 28°C in CPG broth (1 g of Casamino acids/liter, 10 g of peptone/liter, 5 g of glucose/liter) (15) or on TZC plates containing CPG plus 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride (23). Boucher's minimal medium (BMM) (8) supplemented with 0.2% (wt/vol) citric acid was used to grow strains for the PG reaction product analysis or the quantitative Pme assay. Antibiotics were added at standard concentrations as required.

Growth on various carbon sources was assayed in BMM supplemented with 0.2% (wt/vol) carbon source. Pectin or polygalacturonic acid used as a sole carbon source or for reaction product analysis was washed four times in 70% ethanol to remove low-molecular-weight soluble nutrients or contaminants. For carbon source utilization experiments, cultures were inoculated in 250-ml Erlenmeyer flasks containing 25 ml of medium to an initial optical density at 600 nm (OD₆₀₀) of 0.020 and incubated in a 300-rpm shaker at 28°C; bacterial growth was measured spectrophotometrically as culture OD₆₀₀.

Tobacco extract for plant induction studies was obtained by a method similar to that of Beaulieu and Van Gijsegem (5). Fully expanded tobacco leaves (cv. Bottom Special) were surface sterilized, rinsed with sterile water, cut into 1-cm strips, and shaken overnight in BMM without carbon. The resulting extract was

FIG. 1. Subcloning and deletion mutagenesis of *pme*. A 2.7-kb *ClaI* fragment of strain K60 DNA encoding Pme activity was cloned into pBluescript to create pMeB1. From this, an internal 1.8-kb *EcoRI-ClaI* fragment encoding Pme activity was subcloned into pLAFR3 and pT7Blue to create pMeL1 and pMe71, respectively. A 3.8-kb DNA fragment containing an *NptI-sacB-SacR* cartridge (28) was cloned into the remaining *SacII* site of *SacII* deletion construct pMe71 to create pMe7SB.

filter sterilized. Bacterial cultures were then grown in BMM supplemented with 0.2% carbon source plus 0.5% (vol/vol) tobacco extract.

Chemicals and enzymes. Growth medium components were purchased from Difco Laboratories (Detroit, Mich.). Highly methylated pectin (P9561; 93% methylated from citrus) was from Sigma Chemical Co. (St. Louis, Mo.); apple pectin (76282; 70 to 75% methylated) and citrus pectin (76280; 63 to 66% methylated) were from Fluka Biochemika (Buchs, Switzerland). DNA restriction and modification enzymes were from Promega Corp. (Madison, Wis.). Radiochemicals were from NEN (Boston, Mass.). Electrophoresis chemicals and nylon transfer membranes were from Bio-Rad Laboratories (Richmond, Calif.). Oligonucleotides were purchased from the University of Wisconsin Biotechnology Center (Madison, Wis.). All other chemicals were from Sigma Chemical Co.

DNA manipulations. Recombinant DNA manipulations were performed in accordance with standard procedures (4). *R. solanacearum* strains were transformed by electroporation as previously described (2). To clone the strain K60 *pme* gene, an internal DNA fragment of the *pme* structural gene was amplified by PCR using primers derived from the strain 50905 *pme* gene (35). The primer sequences were 5'-GCCGGCACCTACAACGAATC3' and 5'-GCCCGGTGTGTTGTACTC3'. The resulting 800-bp fragment was radiolabeled and used to probe a *R. solanacearum* K60 chromosomal DNA library (2).

Construction of *pme* mutant and PG-*pme* triple mutant strains. A 0.55-kb *SacII* fragment was deleted from the *pme* coding sequence by using the three internal *SacII* sites in the gene. This deletion generated plasmid pMe71 Δ SacII (Fig. 1). We inserted a 3.8-kb fragment of the pUM24 *NptI-sacB-sacR* gene cartridge, which encodes resistance to kanamycin (28), into the remaining *SacII* site of pMe71 Δ SacII to create pMe7SB. This disabled *pme::sacB* construct was exchanged into the genomes of wild-type strain K60 and K60-309 (*pehA pehB* double mutant) by gene replacement as previously described (19).

Enzyme activity assays. Pme and PG activities were measured qualitatively by using a modification of the pectin overlay method, which allows detection of both activities on a single petri plate (33). Colonies grown 24 to 36 h on CPG were overlaid with two successive layers of substrate containing 0.5% 93%-methylated pectin, 20 mM EDTA (pH 8.0), 50 mM Tris (pH 8.0), 0.2% Triton X-100, and 0.8% agarose. The overlaid plates were incubated overnight at 28°C, stained with 0.5% (wt/vol) ruthenium red with gentle shaking, and destained with distilled water. Pme activity appeared as a dark purplish-red halo surrounding colonies; PG activity was manifested as a clear zone surrounding the colonies.

Pme activity was measured quantitatively as production of methanol from methylated pectin (40). Pme activity was calculated as micrograms of methanol produced per minute per CFU for each strain, based on a methanol standard curve.

Apoptotic fluids from tobacco leaves infused with 5×10^4 bacteria/cm² were obtained after 18 h of bacterial growth in planta as previously described (2); Pme activity in apoptotic fluids was measured quantitatively as described above.

PG reaction product analysis. Three hundred microliters of cleared supernatant from cultures grown to an OD₆₀₀ of >1.0 was reacted with 700 μ l of 0.4% (wt/vol) pectin or polygalacturonic acid; 25 μ l of each reaction was separated by thin-layer chromatography (TLC) and enzyme reaction products were detected with phosphomolybdate spray as previously described (25). Pure mono-, di-, and trigalacturonic acid (Sigma G2125, D4288, T7407) were used as standards.

FIG. 2. Overlay activity assay for Pme production. Twenty-four-hour colonies grown on CPG were overlaid with two successive layers of highly methylated pectin substrate and stained with 0.5% (wt/vol) ruthenium red. This assay detected both PG and Pme activity. Clear zones correspond to low-molecular-weight galacturonate oligomers degraded by extracellular endo-PG. Dark halos indicate the formation of polygalacturonic acid from highly methylated pectin by Pme.

Additional cell-associated or plant-inducible Pme activities. K61B (*pme*) cells grown in the presence of tobacco extract were sonicated in 50 mM MES (morpholineethanesulfonic acid) buffer (pH 5.7) containing 0.1% (wt/vol) PMSF (phenylmethylsulfonyl fluoride) and centrifuged at $10,000 \times g$ for 10 min. Enzyme reaction product analysis was performed on both the supernatant (cytoplasmic fraction) and the residual pellet (membrane, cell wall, and periplasm-enriched fractions), using highly methylated pectin as the substrate. Next, 0.1% Triton X-100 was added to the cell membrane-cell wall-periplasm reaction to dissociate membrane-bound enzymes.

Virulence assays. The virulence of *R. solanacearum* strains was tested on 21-day-old eggplants (aubergines) (cv. Black Beauty), 15-day-old tomatoes (cv. Bonnie Best), or 40-day-old tobacco (cv. Bottom Special). Plants were grown in 80 g (dry weight) of Jiffy Mix per 4-in. pot. Bacteria from freshly streaked plates were grown overnight in BMM supplemented with 0.2% glucose, centrifuged to pellet the cells, and resuspended in sterile water. Eggplant and tobacco roots were wounded by cutting vertically down across each pot 1 cm away from the base of the stem. Tomato roots were not wounded. Fifty milliliters of a water suspension of washed bacteria was poured over the dry soil to yield a final bacterial concentration of 5×10^5 to 1×10^6 CFU/g of soil for eggplants and tobacco or 6.25×10^7 CFU/g of soil for tomatoes. For each assay, inoculum concentrations were determined by dilution plating. Plants were coded and inspected daily for signs of wilting by a rater blind to treatment identity and were rated on a zero-to-four disease index scale as follows: 0, no wilting; 1, 1 to 25% wilting; 2, 26 to 50% wilting; 3, 51 to 75% wilting; and 4, 76 to 100% wilted or dead. Each assay contained 16 plants per treatment, and was repeated three times. Results were analyzed using Systat Version 5.2 (Systat, Inc., Evanston, Ill.).

RESULTS

Cloning and analysis of the *pme* gene. The sequence of an 800-bp DNA fragment amplified from *R. solanacearum* K60 chromosomal DNA was 96% identical to an internal region of the *pme* locus from strain 50905 (data not shown). This *pme* probe hybridized to 10 cosmids in an *R. solanacearum* K60 genomic library; one cosmid including a 2.7-kb *Cla*I fragment encoding Pme activity in *Escherichia coli* was chosen for further subcloning and study (Fig. 1). Plasmid constructs pMe71 and pMeL1 were electroporated into *E. coli* DH5 α and screened for Pme production on activity overlay plates. Both plasmids conferred on *E. coli* the ability to demethylate pectin; in addition, pMeL1 in trans restored Pme production to chromosomal *pme* mutant K61B (see below) (Fig. 2).

TABLE 2. Pme activity produced by various *R. solanacearum* strains

Strain	Relevant phenotype	% Endo-PG activity ^a	Pme activity ^b
K60	Wild type	100	3.32 ± 0.03
K61B	<i>Pme</i> ⁻	100	0.06 ± 0.05
K71	<i>PehR</i> ⁻	5	3.26 ± 0.05
K60- <i>phcA</i>	<i>PhcA</i> ⁻	180	0.09 ± 0.07
K71- <i>phcA</i>	<i>PehR</i> ⁻ <i>PhcA</i> ⁻	5	0.13 ± 0.08

^a Endo-PG activity determined previously (1) expressed as a percentage of that of the wild type.

^b Pme activity expressed in micrograms of methanol produced per minute per cell $\times 10^{-10} \pm$ standard error.

Characterization of *R. solanacearum pme* mutants. We used gene replacement to construct a *pme* mutant strain, called K61B, and a *pme pehA pehB* triple mutant called K60-509. Both mutants had wild-type colony morphology when grown on TZC plates and a wild-type growth curve when grown in CPG broth (data not shown). Southern blot analysis confirmed that the mutants carried a single interrupted copy of *pme* (data not shown).

Neither mutant displayed Pme activity in the qualitative plate overlay, although Pme was easily detected in the wild-type strain and in *E. coli* carrying the cloned *pme* gene (Fig. 2). Similarly, both strains carrying interrupted chromosomal copies of *pme* produced only background levels of methanol in the quantitative Pme assay (Table 2).

***pme* mutants cannot grow on highly methylated pectin.** Wild-type strain K60 grew equally well in minimal medium containing either polygalacturonic acid or 93% methylated pectin as a carbon source. However, although *pme* mutant K61B grew as well as K60 on polygalacturonic acid, K61B could not utilize highly methylated pectin as a carbon source. In fact, the growth curve of K61B on highly methylated pectin

FIG. 3. Growth of wild-type and Pme mutant strains on various substrates. Wild-type strain K60 (open squares) and K61B (*pme*) (closed squares) grown in minimal medium plus 93% methylated pectin carbon source, K60 (open triangles) and K61B (closed triangles) grown in minimal medium plus polygalacturonic acid, and K60 (open circles) and K61B (closed circles) grown in minimal medium with no added carbon source. Results shown are the means of three replicated experiments; within each experiment, every strain was replicated four times.

FIG. 4. PG reaction product analysis. Culture supernatant from either wild-type strain K60 or *pme* mutant K61B was combined with polygalacturonic acid or with pectin of differing methylations. Twenty-five microliters of each reaction mixture was separated by TLC. Lanes: 1, K60 with 93% methylated pectin; 2, K60 with 70 to 75% methylated pectin; 3, K60 with 63 to 66% methylated pectin; 4, K60 with polygalacturonic acid; 5, monogalacturonic acid standard; 6, digalacturonic standard; 7, trigalacturonic standard; 8, K61B (*pme*) with 93% methylated pectin; 9, K61B with 70 to 75% methylated pectin; 10, K61B with 63 to 66% methylated pectin; 11, K61B with polygalacturonic acid.

was indistinguishable from that of either strain in medium lacking a carbon source (Fig. 3).

Pme is required for PG activity on highly methylated pectin. TLC analysis of PG reaction products indicated that wild-type strain K60 culture supernatant degraded the following substrates equally well: 93% methylated pectin, 70 to 75% methylated apple pectin, 63 to 66% methylated citrus pectin, and polygalacturonic acid (Fig. 4, lanes 1 to 4). In each case, the reaction product profiles were identical, showing mostly digalacturonic acid but also mono- and trigalacturonic acid and small amounts of higher-molecular-weight products, visible as a faint smear below the trimer band.

In contrast, supernatants of the *pme* mutant K61B generated no reaction products from 93% methylated pectin (Fig. 4, lane 8), indicating that without Pme activity, the strain's PGs could not act on this substrate. K61B could generate some faint mono- and digalacturonic acid and higher-molecular-weight products from 70 to 75% methylated pectin (lane 9); when the substrate was only 63 to 66% methylated, this strain produced noticeably more reaction products (lane 10). On nonmethylated polygalacturonic acid, the reaction profile of K61B was similar to that of the wild-type strain, although the relative concentrations of the reaction products were slightly different (lane 11).

Virulence of *pme* mutant strains is unaffected. Soil inoculation virulence assays of wild-type strain K60, PG mutant strains, and the *pme* mutant strain were conducted in eggplants (aubergines), tomatoes, and tobacco. Wild-type strain K60 wilted eggplants completely by day 6 postinoculation (Fig. 5). The disease induced by *pme* mutant K61B was statistically indistinguishable from that caused by the wild-type strain. As previously reported (19), the K60-309 (*pehA pehB*) double mutant was significantly less virulent than K60, with an average disease index of 3 by the end of the assay. Loss of Pme activity also had no effect on virulence in this background; there was no difference between the disease progress curves of K60-309 and K60-509 (*pehA pehB pme*). Similar results were obtained when tobacco and tomatoes were inoculated with these strains (data not shown). The absence of a functional *pme* locus did not reduce virulence in either host.

Search for additional Pme genes or activities. We did not detect any other gene with homology to the 800-bp *pme* probe in the strain K60 genome with a low stringency Southern blot

analysis (data not shown). We also detected no inducible or cell-bound Pme activity in the culture supernatant, cytoplasm, or cell membrane-cell wall-periplasm fractions of the *pme* mutant when cells were grown in the presence of tobacco leaf extract or highly methylated pectin (data not shown).

We compared Pme activity in bacterial culture supernatants and in apoplastic fluids of tobacco leaves in which *R. solanacearum* cells had grown for 18 h. For wild-type strain K60, Pme levels were substantially higher in planta (around 3×10^{-8} U/CFU) than in culture (around 3×10^{-10} U/CFU). However, although *pme* mutant K61B produced only background Pme activity in culture, apoplastic fluids from tobacco plants infused with K61B cells contained the same level of Pme activity as

FIG. 5. Virulence of *R. solanacearum* mutant strains in eggplants. Roots of 21-day-old eggplants (aubergines) were wounded and then inoculated with 5×10^5 bacteria per g of soil. Plants were rated daily on a disease index from 0 (no wilt) to 4 (76 to 100% wilted). Points shown are means of three experiments, each containing 16 plants per treatment. K60 (wild type), open square; K61B (*pme*), closed square; K60-309 (*pehA pehB*), open triangle; K60-509 (*pme, pehA pehB*), closed triangle.

those from plants infused with the wild-type strain. Apoplastic fluids from water-infused control plants contained about half as much Pme activity as fluids from plants infused with either bacterial strain. It seems probable that most, if not all, in planta Pme activity was of host plant origin.

Pme expression is regulated by PhcA. To better understand regulation of Pme production, we measured Pme activity produced by three different regulatory mutants. K60-*phcA* lacks a LysR-type global regulator of virulence genes called PhcA (9); K71 carries a mutation in *pehR*, which encodes a positive response regulator controlling expression of PG genes (1), and K71-*phcA* carries both mutations. Both strains lacking *phcA* produced only trace Pme activity, similar to that of K61B (Pme⁻). Conversely, K71 produced wild-type levels of Pme activity (Table 2). These results demonstrate that *pme* expression in vitro is directly or indirectly regulated by PhcA, but not by PehR, and thus is not coordinately regulated with PGs.

DISCUSSION

We have isolated a *pme* gene from the bacterial wilt pathogen, *R. solanacearum*. The DNA sequence of an 800-bp fragment of the cloned gene was 96% identical to an internal region of a published *pme* sequence from a different strain of the same species (35), and a plasmid carrying the cloned gene conferred Pme activity on *E. coli*. Moreover, the plasmid-borne cloned gene restored Pme activity to a *pme* chromosomal mutant. To better understand the biological role of this gene in *R. solanacearum*, we constructed a *pme* chromosomal mutant, K61B, and a *pme* *pehA* *pehB* triple mutant, K60-509.

R. solanacearum PGs cannot act on highly methylated pectin unless Pme first removes methoxyl groups from the galacturonic acid polymer. K61B could not break down highly methylated pectin into its component galacturonic acid moieties, even though this strain was able to effectively degrade unmethylated polygalacturonic acid into mono-, di-, and trigalacturonic acid. Further, K61B did not grow on highly methylated pectin as a sole carbon source, although it did grow on polygalacturonic acid. In contrast, wild-type strain K60 grew well on highly methylated pectin media. Thus, functional PGs were present in K61B, but they were unable to depolymerize highly methylated pectin without Pme activity.

Loss of Pme activity had no measurable effect on pathogen virulence. Strain K61B, which carries an inactivated *pme* locus, caused bacterial wilt disease on three different host plants in a manner indistinguishable from the wild-type strain, and a *pme* *pehA* *pehB* triple mutant was just as virulent as a *pehA* *pehB* double mutant. This result was not expected, because Pme activity is required for PG activity on highly methylated pectin (Fig. 4), and loss of PG activity significantly reduces virulence (2, 19, 31). While it is possible that our virulence assay is not sufficiently stringent to detect a small but potentially ecologically significant reduction in virulence, we do not believe this is likely because our assay unambiguously discriminates virulence differences among strains lacking various PGs.

Another plausible reason for the apparent unimportance of Pme in virulence is that host plant pectic compounds may be sufficiently unmethylated that bacterial PGs can easily degrade them. We found that even in the absence of Pme activity, *R. solanacearum* PGs retained detectable, although much reduced, activity on 70 to 75% and 63 to 66% methylated pectin in vitro. The degree of methylation of native plant pectins varies, ranging from 13 to approximately 70% (38). Pectin from the potato, a solanaceous plant related to the three hosts we used, is between 53 and 61% methylated (22). It may be that the pectic compounds in eggplants, tomatoes, and tobacco are

not methylated to such a degree that Pme is required for breakdown of pectin by the bacterial PGs.

Surprisingly, similar levels of Pme activity were present in apoplastic fluid from tobacco leaves infused with wild-type and *pme* mutant strains. Fluids from water-infused control plants contained only about half as much Pme activity. It seems most likely that this apoplastic Pme was produced by the plant. Plant Pmes are often cell wall-bound in an inactive form (29). They may be released or activated during infection, as a result of enzymatic degradation of plant cell walls by bacteria. If this were the case, these plant Pmes could demethylate the plant's own pectic compounds enough to make them vulnerable to bacterial PGs.

It is also possible that *R. solanacearum* produces more than one *pme*, and that a second Pme activity, expressed only when the bacterium grows in planta, can compensate for the absence of the enzyme encoded by the mutated *pme* locus. Pectic enzymes in other plant-associated bacteria are often highly redundant and in some cases are expressed only after induction by plant signals (20). However, we were unable to detect such an additional Pme gene or activity in *R. solanacearum*. No additional sequences with homology to the *pme* locus were identified by Southern blot under very low stringency conditions. Moreover, we did not detect additional Pme activities in vitro, either in the supernatant or in association with the cell, even when bacteria were grown on highly methylated pectin as a sole carbon source or in the presence of potentially inducing crude tobacco leaf extracts. Nevertheless, without in planta studies that can distinguish among specific Pme isozymes, we cannot definitively conclude that *R. solanacearum* produces no other Pme(s).

If bacterial Pme does not contribute to bacterial wilt virulence, why does *R. solanacearum* produce and secrete it? Perhaps the ability to degrade highly methylated pectin substrates contributes to the organism's fitness during the saprophytic part of its life cycle, when it often survives in association with latent hosts, decaying plant debris, or plant roots (14). This hypothesis could be tested by comparing root colonization, latent infection, and soil survival by wild-type and Pme⁻ strains.

Expression of virulence factors in *R. solanacearum* is controlled by a complex hierarchical regulatory network (32). In response to the accumulation of an autoinducer at high bacterial cell densities, a LysR-type global regulator called PhcA positively regulates expression of virulence factors EPS and Egl but represses expression of a two-component type regulator called *pehSR*, which in turn positively regulates PG production and motility (1, 11, 16). Thus, a *phcA* mutant strain has about twice the wild-type level of PG activity and is hypermotile, but produces dramatically reduced levels of Egl and EPS and is essentially nonvirulent. The current model proposes that *R. solanacearum* cells are highly motile and produce PG at low population densities, presumably corresponding to saprophytic life and the early stages of host plant infection and colonization. Then at high cell density, presumably corresponding to ideal conditions for full-blown disease, PhcA represses expression of *pehSR*, thus reducing motility and PG production. At the same time, PhcA induces expression of EPS and Egl.

We found that in culture, production of Pme is positively regulated by PhcA, and is independent of PehR. These results are not consistent with the current model for regulation of *R. solanacearum* virulence genes. First, Pme, which was not required for virulence in our assays, appears to be coregulated with the known virulence factors Egl and EPS. Moreover, although Pme presumably acts on methylated pectins in concert with or even prior to the PGs, if *pme* is regulated by PhcA,

it would be expressed at high population density, while the PGs are expressed earlier, at low population density. Thus, in a sense our regulatory findings are consistent with the lack of a role in virulence for *Pme*, but leave us without an understanding of *Pme*'s true biological function. It must be noted that all these results were obtained in culture, and a very different pattern of gene expression may prevail in the host (34). Further, while *PhcA* may be the ultimate regulator in the hierarchical control of *pme* expression, it is likely that, as has been shown for EPS (17, 18), *pme* expression is also affected by a number of intervening regulators responsive to additional signals. The current regulatory model may need to be re-evaluated in light of in planta gene expression studies.

ACKNOWLEDGMENTS

This research was supported by USDA NRICGP 94-37303-0950, University of Wisconsin Hatch Project 3735, and by the University of Wisconsin College of Agricultural and Life Sciences.

We gratefully acknowledge Mark Schell, Qi Huang, and Mark Kainz for helpful discussions.

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