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An Exo-Poly-α-D-Galacturonosidase, PehB, Is Required for Wild-Type Virulence of *Ralstonia solanacearum*

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***Ralstonia solanacearum*, which causes bacterial wilt disease of many plant species, produces several extracellular plant cell wall-degrading enzymes that are suspected virulence factors. These include a previously described endopolygalacturonase (PG), PehA, and two exo-PGs. A gene encoding one of the exo-PGs, *pehB*, was cloned from *R. solanacearum* K60. The DNA fragment specifying PehB contained a 2,103-bp open reading frame that encodes a protein of 74.2 kDa with a typical N-terminal signal sequence. The cloned *pehB* gene product cleaves polygalacturonic acid into digalacturonic acid units. The amino acid sequence of *pehB* resembles that of *pehX*, an exo-PG gene from *Erwinia chrysanthemi*, with 47.2% identity at the amino acid level. PehB also has limited similarity to plant exo-PGs from *Zea mays* and *Arabidopsis thaliana*. The chromosomal *pehB* genes in *R. solanacearum* wild-type strain K60 and in an endo-PG PehA$^-$ strain were replaced with an insertionally inactivated copy of *pehB*. The resulting mutants were deficient in the production of PehB and of both PehA and PehB, respectively. The *pehB* mutant was significantly less virulent than the wild-type strain in eggplant virulence assays using a soil inoculation method. However, the *pehA* mutant was even less virulent, and the *pehA pehB* double mutant was the least virulent of all. These results suggest that PehB is required for a wild-type level of virulence in *R. solanacearum* although its individual role in wilt disease development may be minor. Together with endo-PG PehA, however, PehB contributes substantially to the virulence of *R. solanacearum*.**

Ralstonia (*Pseudomonas* or *Burkholderia*) *solanacearum* E. F. Smith causes bacterial wilt, a soil-borne vascular disease that is distributed worldwide and limits the production of such economically important crops as tobacco, tomato, potato, peanut, banana, and eggplant (7). The bacterium normally enters plants from the soil through wounds in the roots and then multiplies in the xylem vessels and spreads through the plant's vascular system (62, 66).

Although substantial research effort has been devoted to this disease since its causal agent was first identified by E. F. Smith a century ago (55), the mechanism of wilting caused by *R. solanacearum* is still not fully understood. Molecular genetic approaches have identified several important virulence factors, including extracellular polysaccharides (11, 29) and extracellular plant cell wall-degrading enzymes such as endoglucanase (46) and polygalacturonase (PG) (2, 50).

PG is a hydrolytic enzyme that degrades the pectic polymers that are the major components of higher-plant middle lamellae and primary cell walls. There are two types of PG, endo-PG and exo-PG. Both types cleave pectic polymers by hydrolysis, but they release different products; endo-PGs (EC 3.2.1.15) cleave the pectic polymer at random, releasing large fragments and rapidly decreasing substrate viscosity, while exo-PGs release either galacturonate monomer in the case of exopolygalacturonase (EC 3.2.1.67) or dimer in the case of exo-poly-α-D-galacturonosidase (EC 3.2.1.82) (9).

Pectolytic enzymes are produced by a wide range of fungal and bacterial plant pathogens causing symptoms including leaf spot, soft rot, and wilt (8). The role of pectolysis, however, has been studied most extensively in the soft rot erwiniae, where research has focused primarily on the endo-pectolytic enzymes that are responsible for the massive tissue maceration symptoms typical of soft rot (8, 43). Little is known about the function of pectolytic enzymes, especially exo-pectolytic enzymes, in plant diseases with different modes of pathogenesis. So far, only one bacterial exo-PG gene, *pehX* from *Erwinia chrysanthemi*, whose gene product is an exo-poly-α-D-galacturonosidase, has been cloned, sequenced, and characterized with respect to its role in virulence (19). Maize and *Arabidopsis thaliana* have exo-PG genes that are specifically expressed in pollen (4, 41). Additional pollen-specific genes from *Oenothera*, *Brassica*, tobacco, cotton, and alfalfa appear to be exo-PGs based on sequence homology, but no enzymatic assays have confirmed they encode exo-PG activity (6, 28, 40, 45, 61).

Unlike erwiniae, *R. solanacearum* K60 causes primarily wilt symptoms rather than tissue maceration. This strain produces one endo-PG, PehA, and two exo-PGs, PehB and PehC (1). PG has long been suspected of contributing to the virulence of *R. solanacearum*, probably by releasing nutrients for the pathogen, contributing to host vessel blockage, and/or aiding in initial invasion, bacterial spread, and colonization (27, 49, 66). However, how and to what extent the endo- or exo-PG or both the endo- and exo-PGs contribute to virulence are still not completely understood. To date only *pehA*, which encodes the endo-PG, has been cloned from *R. solanacearum* K60 (2). *pglA*, a *pehA* homolog from *R. solanacearum* AW, has also been cloned and sequenced (50). PehA and PglA mutants are reduced in virulence but still cause some disease symptoms, suggesting that endo-PG is important although not absolutely required for wilting (2, 50). Expression of *pehA*, *pehB*, and *pehC* is positively regulated by a two-component regulator, PehSR (2). In addition, *pehA* expression is also regulated, probably indirectly, by VsrB (25) and the global regulator PhcA (24).

To better understand the contribution of exo-PG to bacterial wilt, we cloned and sequenced *pehB*, one of the exo-PG genes from *R. solanacearum* K60. We then constructed *R. solanacearum* mutants deficient in either PehB only or both PehA and PehB and studied the virulence of these mutants. We found

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TABLE 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	F$^-$ *endA1 relA* ϕ80 *lacZ*Δ*M15 hsdR17 supE44 thi-1 recA1 gyrA96*	17
DH5αF′	F′ *endA1 relA* ϕ80 *lacZ*Δ*M15 hsdR17 supE44 thi-1 recA1 gyrA96*	17
BL21(DE3)	*hsdS gal* (λ*c*I *ts*857 *ind*1 S*am*7 *nin*5 *lac*UV5-T7 gene 1)	60
R. solanacearum		
K60	Wild-type race 1, biotype 1	32
AW103	AW *pehB*::Tn*phoA* Km^r	51a
K60-06	K60 *pehA*::Ω Sm^r	3
K60-209	K60 *pehB*::*aacC1* Gm^r	This study
K60-309	K60 *pehA*::Ω *pehB*::*aacC1* Sm^r Gm^r	This study
Plasmids		
pBS KS$^+$	Ap^r	Stratagene
pBS KS$^+$ΔBam	Ap^r, no *Bam*HI site	3a
pBS SK$^+$	Ap^r	Stratagene
pLAFR3	Tc^r	57
pUCGM	Gm^r	52
pT7Blue	Ap^r	Novagen
pET29b(+)	Km^r	Novagen
M13mp18/19		38, 69
pQHBL1	pLAFR3 with a 29-kb K60 DNA insert, PehB$^+$ Tc^r	This study
pQHBB4	pBS SK$^+$ with a 10-kb *Cla*I-*Eco*RI fragment from pQHBL1, PehB$^+$ Ap^r	This study
pQHBB8	pBS SK$^+$ with a 3.3-kb *Sau*3AI fragment from pQHBB4, PehB$^+$ Ap^r	This study
pQHBL9	pLAFR3 with a 2.8-kb *Sau*3AI fragment from pQHBB4, PehB$^+$ Tc^r	This study
pQHBM1	pBS KS$^+$ΔBam with a 1.1-kb *Kpn*I fragment from pQHBB8, Ap^r	This study
pQHBM2	pQHBM1 with a 0.8-kb Gm^r cassette from pUCGM, Gm^r Ap^r	This study
pQHBM3	pQHBB8 with a 0.8-kb Gm^r cassette from pUCGM, PehB$^-$ Ap^r	This study
pQHBT7	pT7Blue with *pehB* ORF DNA, Ap^r	This study
pQHBET	pET29b(+) with a 2.1-kb *Nde*I-*Hin*dIII fragment from pQHBT7, PehB$^+$ Km^r	This study

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

that although it is required for wild-type level of virulence, PehB alone plays a minor role in the development of wilt symptoms. In combination with PehA, however, PehB contributes significantly to the virulence of *R. solanacearum.*

(A preliminary report of portions of this study has been presented elsewhere [1].)

MATERIALS AND METHODS

Materials. All materials used in DNA manipulation were purchased from Promega Corporation (Madison, Wis.), New England BioLabs, Inc. (Beverly, Mass.), Novagen Inc. (Madison, Wis.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Stratagene (La Jolla, Calif.). [α-^{35}S]CTP (>1,000 Ci/mmol) was purchased from Du Pont-NEN (Boston, Mass.). Electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, Calif.). Growth medium components were from Difco Laboratories (Detroit, Mich.) or Fisher Scientific (Pittsburgh, Pa.). Other chemicals were from Sigma Chemical Co. (St. Louis, Mo.) or Mallinckrodt Chemical Inc. (Paris, Ky.).

Bacterial strains and plasmids. Bacterial strains and plasmids used and constructed in this study are listed in Table 1.

Media and growth of bacterial strains. Except where specified, *R. solanacearum* strains were grown at 28°C in CPG broth (20) or on TZC plates (32). *Escherichia coli* strains were cultured at 37°C in Luria-Bertani medium (39). When needed, antibiotics were added at 50 μg/ml for ampicillin, 30 μg/ml for streptomycin, 25 μg/ml for kanamycin, 15 μg/ml for tetracycline, and 10 μg/ml for gentamicin. A modified pectate semisolid agar (PEC-SSA) medium (pH 5.5) (56) containing 3% sodium polygalacturonate, 0.06% $CaCl_2 \cdot 2H_2O$, 0.5% yeast extract, and 0.3% agar was used to screen *E. coli* clones containing the *pehB* gene. PG-producing clones formed pits around bacterial colonies after 96 h at room temperature.

DNA manipulations. Chromosomal DNA of *R. solanacearum* strains was isolated by using Qiagen (Chatsworth, Calif.) columns as specified by the supplier. *R. solanacearum* was transformed by electroporation as described previously (2). Isolation of plasmid DNA, restriction mapping, cloning, subcloning, transformation of *E. coli* strains, Southern blot hybridization, and genomic library construction were performed by standard procedures (48). DNA labeling and detection were performed nonradioactively, using a DIG High Prime labeling and detection kit (Boehringer Mannheim Biochemicals).

Cloning of *pehB* from strain K60. We used Tn*phoA* as a probe to identify an *Eco*RI fragment containing the *pehB*::Tn*phoA* insertion in mutant AW103. AW103, a Tn*phoA* mutant of wild-type *R. solanacearum* AW, was a generous gift from Mark Schell, University of Georgia. The labeled *pehB*::Tn*phoA* fragment was used as a probe to screen a 1,500-clone K60 genomic library constructed in cosmid pLAFR3 (68). Positive clones were screened for both the ability to cause pitting on PEC-SSA plates and the ability to restore PehB production to *pehB*-deficient strains. Assorted restriction enzymes were used to generate a series of subclones in pBluescript (pBS); the subclones were screened for exo-PG activity as described above. The minimal functional *pehB* structural gene was cloned by partially digesting pQHBB4 with *Sau*3A1 and cloning the resulting fragments into the *Bam*HI site of pBS or pLAFR3, followed by screening on PEC-SSA plates.

DNA sequencing and computer analysis. Plasmids for DNA sequencing were constructed by subcloning the 2.5-kb *Nru*I-*Kpn*I fragment of pQHBB8 into vectors M13mp18 and M13mp19. Single-stranded DNA template was prepared from *E. coli* DH5α F′ infected with the helper phage M13K07. Larger subclones and the junction sites of the subclones were sequenced by using synthesized oligonucleotides purchased from Cruachem Inc. (Dulles, Va.). The sequences of both strands of the *Nru*I-*Kpn*I fragment were determined by the dideoxy-chain termination method using *Taq* polymerase at 45°C with both GTP and 7-deaza-GTP to avoid compressions caused by the high G+C content of the DNA. DNA and protein sequence data were analyzed by using the Genetics Computer Group (GCG; University of Wisconsin—Madison) package (13).

Subcloning of the *pehB* gene into T7 expression vector and expression of the *pehB* gene product in *E. coli*. We used PCR to amplify the *pehB* open reading frame (ORF) DNA starting immediately after the ATG start codon at nucleotide 120 and ending immediately before the TAG stop codon at nucleotide 2219. Synthetic primers were generated containing *Nde*I and *Hin*dIII sites in the places of the ATG start and the TAG stop codons, respectively (5′-GTGTACATATG CGACAAAAAAGAATGCGCCG-3′ and 5′-TTCCCGAAGCTTGGGTGCCG GCTTCACGTT-3′). The PCR product was first cloned into pT7Blue vector to generate pQHBT7. The 2.1-kb *Nde*I-*Hin*dIII fragment from pQHBT7 was then cloned into T7 expression vector pET29b(+) to create pQHBET. Sequencing using the T7 promoter primer confirmed the precise insertion of the *pehB* ORF DNA at the *Nde*I site of pET29b(+). The expression of the recombinant PehB

protein, which utilized the ATG start codon contained in the *Nde*I restriction sequence and the stop codon following the His tag sequence in the vector, was analyzed by induction of mid-log-phase *E. coli* BL21(DE3) with 2 mM isopropylthiogalactopyranoside (IPTG). One-milliliter cell suspensions were withdrawn before and 90 min after IPTG induction and centrifuged, and the pellets were resuspended in 100 μl of 1× Laemmli sample buffer (34) and boiled for 5 min. The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels (34).

Construction of *pehB* and *pehA pehB* mutants by site-directed mutagenesis. To generate a site-directed *pehB* mutation for gene replacement, we first constructed pQHBM1 by cloning the internal 1.1-kb *Kpn*I fragment of the *pehB* gene from pQHBB8 into pBS KS$^+$ΔBam. The gentamicin resistance (Gm^r) gene cassette from pUCGM (*aacC1*) was excised with *Bam*HI and inserted into the unique *Bam*HI site in the 1.1-kb *Kpn*I fragment of pQHBM1. The resulting plasmid, pQHBM2, conferred an Ap^r (Ampicillin resistant) Gm^r phenotype. We transformed pQHBM2 into wild-type strain K60 and *pehA* mutant K60-06 by electroporation. This was followed by selection on gentamicin-containing media for strains that had undergone homologous double recombination between *pehB*::*aacC1* on pQHBM2 and the chromosomal *pehB* locus. Gene replacement was confirmed by Southern blot analysis and by screening for loss of PehB production on isoelectric focusing–thin-layer activity stain overlay (IEF-ASO) gels (42).

Protein preparations and enzyme assays. Extracellular protein preparations were made by collecting the supernatants from 72- to 96-h *R. solanacearum* cultures grown in Boucher's minimal medium (5) supplemented with 0.4% (wt/vol) galacturonic acid as the sole carbon source (pH adjusted to 7.0). For endo-PG assays and for running IEF-ASO gels, these supernatants were concentrated 30- to 100-fold in a Centricon-50 concentrator (Amicon, Beverly, Mass.). Exo-PG activity was measured quantitatively as reducing sugar ends released by the method of Lever (35), modified as follows. A 300-μl aliquot of the supernatant was mixed with 700 μl of 50 mM morpholinethanesulfonic acid (MES; pH 5.7) containing 0.5% polygalacturonic acid (PGA; potassium salt) and incubated at 45°C. At various time intervals, 300-μl aliquots were removed, added to 1 ml of 0.5% *p*-hydroxybenzoic acid hydrazide in 0.5 M NaOH, and boiled for 5 min. The absorbance at 410 nm was read after cooling. One unit of exo-PG activity was defined as the amount of enzyme that releases 1 μmol of oligogalacturonate from PGA per min. Endo-PG activity was measured as rate of viscosity change in a substrate solution containing 1% sodium polygalacturonate in 50 mM sodium acetate, pH 5.0 (30, 54). Fifty microliters of concentrated culture supernatant was added to 1 ml of substrate solution in a Brookfield model DV-I viscometer at 28°C, and viscosity was measured over 20 min. Endo-PG activity is reported as a percentage of the activity of wild-type strain K60. Pectolytic activity was also determined qualitatively on pI 3.0 to 10.0 IEF-ASO gels (42).

Analysis of PG reaction products. For analysis of the PG reaction products, *E. coli* strains containing pQHBB8 (PehB$^+$) and pBS SK$^+$ (vector alone) were grown in King's B medium (33) to an optical density at 600 nm of about 1.0. The cultures were then adjusted to the same cell density, and cell pellets were fractionated by the method of Cornelis et al. (10). *R. solanacearum* strains were grown overnight in modified EG medium (51) containing 1% glycerol instead of sucrose. Their cell densities were also adjusted before supernatants were collected by centrifugation. The cytoplasmic fraction of the *E. coli* strains or the supernatant of the *R. solanacearum* strains was incubated with 0.5% PGA (potassium salt) in 50 mM MES (pH 5.7) (3:7, vol/vol) at 45°C. Reaction product samples were taken at appropriate times after incubation and analyzed by thin-layer chromatography (TLC) according to the method of Lojkowska et al. (36).

Virulence assays. *R. solanacearum* strains, freshly streaked from frozen stocks onto TZC plates with appropriate antibiotics, were grown in CPG broth overnight. Appropriate concentrations of the bacterial suspensions were made with sterile water. Virulences of *R. solanacearum* strains were tested by using both petiole and soil inoculation methods on 22-day-old eggplants (*Solanum melongena* L. cv. "Black Beauty") grown in Jiffy mix in 4-in. pots. Eighty grams (dry weight) of Jiffy mix was used in the soil inoculation experiments. For petiole inoculation, 2.5×10^6 bacteria were injected into the petiole of the second true leaf, and the plants were coded and blindly rated daily, using a disease index of 0 to 4 (46). For soil inoculation, the roots were wounded by cutting across each pot with a scalpel 1.5 cm from the base of the stems. Bacteria (2×10^7/g of soil) were then poured into the pots in a 50-ml volume, and the plants were coded and rated blindly as described above. Inoculum cell density was confirmed by dilution plating each bacterial suspension on TZC plates. Water was used in both inoculation methods as a negative control. There were 16 plants per treatment in each experiment, and each experiment was repeated four times. Virulence assay results were analyzed by using SYSTAT version 5.2.

Nucleotide sequence accession numbers. The *pehB* DNA sequence has been submitted to GenBank and given accession no. U60106. The accession numbers for the fungal, plant, and bacterial PG sequences used in the dendrogram are as follows (in the order of the branches in the dendrogram of Fig. 8, from top to the bottom): *Sclerotinia sclerotiorum*, L29040, L29041, and L12023; *Aspergillus* spp., U05020, X58892, X54146, X52903, X58893, X58894, U05015, D14282, and U17167; *Cochliobolus carbonum*, M55979; *Aspergillus niger*, X64356; *Fusarium moniliforme* L02239; *Lycopersicon esculentum*, A15981, M20269, X04583, X05656, X14074, and M37304; *Persea americana*, L06094 and X66426; *Actinidia deliciosa*, L12019; *Malus domestica*, L27743; *Zea mays*, X57743, X57575, X57627, X62384, X66692, X62385, X65844, X65845, X62383, X64408, X64422, X65854, and X65852; *A. thaliana* A, X73222 and X72292; *Gossypium hirsutum*, U09805 and U09717; *Nicotiana tabacum*, X71016, X71017, X71018, X71019, and X71020; *Medicago sativa*, U20431; *Brassica napus*, L19879; *A. thaliana* B, X72291; *Prunus persica*, X76735; *E. chrysanthemi*, M31308; *E. carotovora*, L32172, M83222, X52944, and X51701; and *R. solanacearum pglA*, M33692.

RESULTS

Cloning and analysis of the *pehB* gene. To isolate the *pehB* gene from *R. solanacearum* K60, we first cloned an *Eco*RI fragment containing the *pehB*::Tn*phoA* insertion from AW103, a PehB-deficient mutant of strain AW. This *pehB*-containing fragment was used as a probe to screen a strain K60 genomic library in order to find the K60 *pehB* homolog. From 1,500 cosmids screened, 6 positive cosmids were identified. All six cosmids conferred pectolytic ability on *E. coli*, as indicated by pitting on PEC-SSA plates. When transformed into mutant AW103, the cosmids restored PehB production, as detected on IEF-ASO gels, suggesting that they contained the *pehB* structural gene (data not shown). One of the cosmids was designated pQHBL1 and chosen for further study. The *pehB* gene of pQHBL1 was mapped and isolated by subcloning analysis followed by PG detection on PEC-SSA plates. Plasmid pQHBB4, which contains a 10-kb *Cla*I-*Eco*RI fragment of pQHBL1, conferred PG activity (Fig. 1). This plasmid was partially digested with *Sau*3AI and ligated into the *Bam*HI site of either pBS SK$^+$ or pLAFR3 to obtain the smallest clone that encoded a functional *pehB* product. The shortest PG-encoding fragment cloned in pBS SK$^+$ was 3.3 kb in length and was designated pQHBB8 (Fig. 1). When a 0.8-kb *Bam*HI fragment containing the Gm^r gene cassette from pUCGM was inserted into the unique *Bam*HI site of pQHBB8, the resulting plasmid pQHBM3 (Fig. 1) did not confer pectolytic activity, indicating that inserting the Gm^r gene cassette into the *Bam*HI site disrupted *pehB* expression. A shorter 2.8-kb *Sau*3A1 DNA fragment was cloned into the broad-host-range cosmid vector pLAFR3 to generate pQHBL9 (Fig. 1). Cosmid pQHBL9 conferred pectolytic activity on *E. coli* (data not shown) and restored PehB production to mutant AW103 (Fig. 2), confirming that it contains a functional *pehB* gene.

Nucleotide sequence of the *pehB* gene and comparison with other PG genes. We sequenced both strands of the 2.5-kb *Nru*I-*Kpn*I fragment of pQHBL9 (Fig. 3). This sequence contained only one ORF that could encode an estimated 80-kDa PehB (49). There are four potential ATG translational start codons in this ORF, at positions 117, 132, 150, and 165, and two potential TTG start codons, at positions 153 and 180. The ORF ends with UAG at position 2220. Of the potential start codons, only ATG-117 and ATG-150 have potential ribosome-binding sites and likely −35 and −10 *E. coli*-like promoter regions at appropriate positions (18). PehB is a secreted protein since the enzyme is abundant in culture supernatants but is scant in periplasmic shock fluids and not detectable in lysed cells (data not shown). In addition, AW103 with a *pehB*::Tn*phoA* fusion is blue on 5-bromo-4-chloro-3-indolyl phosphate (X-phos) plates, indicating that the fusion protein is secreted (data not shown). It seems most likely that PehB starts at ATG-117 because only translation initiating at this point produces a protein with a characteristic N-terminal secretion signal sequence containing a positively charged N-terminal region, an adjacent hydrophobic core, and a more polar C-terminal region, similar to the signal sequences of many extracellular proteins (15, 63–65). The ORF starting at ATG-117 encodes a protein of 74.2 kDa (701 amino acids) with a calculated pI of 6.9. Six bases after the UAG stop codon,

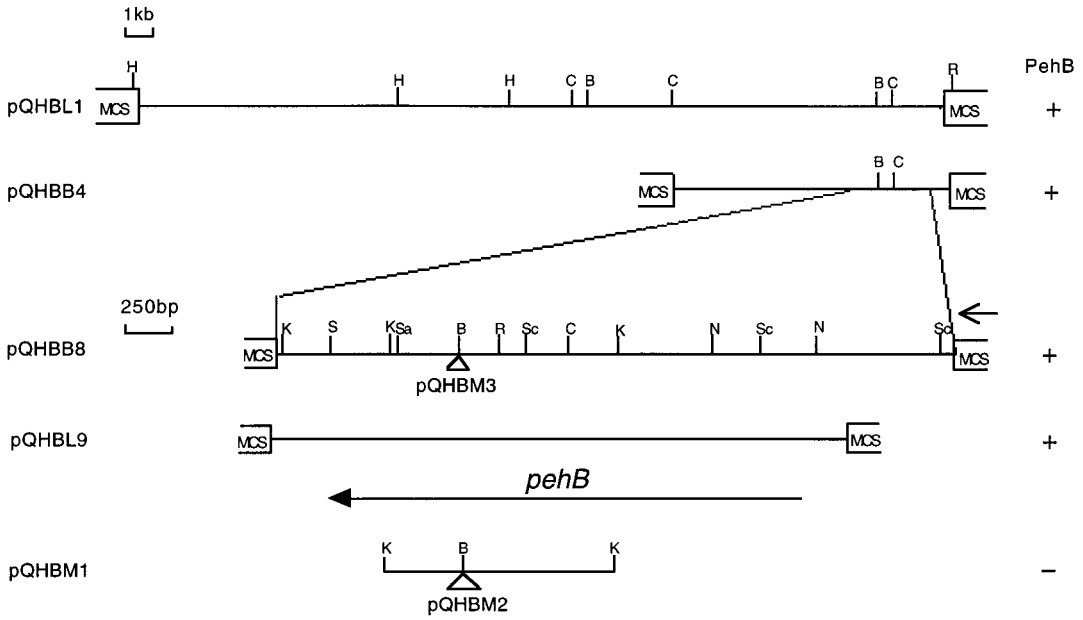

FIG. 1. Restriction maps of plasmids containing *pehB*. Restriction endonuclease sites: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; R, *Eco*RI; S, *Sma*I; Sa, *Sal*I; Sc, *Sac*II. MCS, multiple cloning sites. PehB activity is indicated by the presence (+) or absence (−) of pitting on PEC-SSA plates by *E. coli* DH5α carrying the plasmid. Open arrows indicate the direction of the *lac* promoter of the vector. The solid arrow illustrates the direction and approximate size of the *pehB* transcript. Open triangles indicate the location of the Gm^r gene cassette inserted in pQHBB8 and pQHBM1 to obtain pQHBM3 and pQHBM2, respectively.

between bases 2229 and 2257, there is a possible transcriptional terminator, a near-perfect G+C-rich palindrome consisting of an 11-bp stem with a single mismatch separated by a 6-base loop. The next potential ORF starts 115 bp downstream from the stop codon. These structural elements suggest, but do not prove, that *pehB* is transcribed independently and is not part of an operon. The G+C content of the *pehB* gene is 63.3%, in general agreement with the high G+C content of the *R. solanacearum* chromosome (23). Codon usage in the *pehB* gene resembles that of other *R. solanacearum* genes sequenced to date (data not shown).

A GenBank database search found significant nucleotide sequence similarities between the *pehB* gene and *pehX* of *E. chrysanthemi*, the only other sequenced bacterial exo-PG gene (19). *pehB* is also similar to two eukaryotic exo-PGs from *Z. mays* and *A. thaliana* (4, 41). At the amino acid level, PehB is 47.2, 26.2, and 21.6% identical to PehX and the exo-PGs from *Z. mays* and *A. thaliana*, respectively, based on the BestFit program of the GCG package (13). We compared the amino acid sequences of the four exo-PGs and one representative endo-PG, PglA of *R. solanacearum* (26), using the PileUp program of the GCG package. We also compared our alignment with one comparing five endo-PGs from bacteria, fungi, and plants (21) to see how the exo-PGs relate to each other and to the endo-PGs. The results are presented in Fig. 4. We found that the highly conserved regions in endo-PGs, NTD G(or A), G(or Q)DD, GHG.S.GS, and NG(or A)L(or V)RIK (21), corresponding to amino acids 282 to 285, 304 to 306, 330 to 337, and 362 to 367 in PglA, were also similarly conserved among all exo-PGs as N.DG (447 to 450), GDD (469 to 471), GHG....GS (502 to 510), and N(or V)GL(or V)R.K (531 to 536) (numbers in parentheses refer to amino acid positions in PehB). The M and Y found near the C termini of all endo-PGs (21) (M^{386} and Y^{399} in PglA) were also present near the C termini of all exo-PGs examined. However, a number of conserved amino acid residues and short highly conserved regions, including GA.GD(or N)G.T (PehB positions 195 to 202) and KG(or A)..DG.G (PehB positions 329 to 336), were found only in the exo-PGs. The T and G near the N terminus and WW found in all endo-PGs (21) (PehA positions 121, 165, and 188 to 189) were not conserved among the exo-PGs.

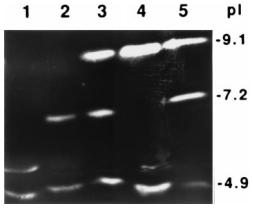

FIG. 2. Isoelectric focusing (pI 3.0 to 10.0)–thin-layer activity stain overlay gel of the extracellular proteins from *R. solanacearum* strains. Two microliters of 100-fold-concentrated extracellular protein preparations was loaded onto each lane at a position corresponding to approximately a pI of 5.0; the faint clearing at this position is a loading artifact. Lanes: 1, K60-309 ($PehA^-$ $PehB^-$); 2, K60-06 ($PehA^-$); 3, K60 (wild type); 4, K60-209 ($PehB^-$); 5, AW103 (pQHBL9). AW103 ($PehB^-$) (not shown) has the same PG profile as K60-209. Numbers at the right indicate the locations of PehA, PehB, and PehC corresponding to pIs of approximately 9.1, 7.2, and 4.9, respectively.

Characterization of the PehB protein. On IEF-ASO gels, PehB migrated at a pI about 7.2 (Fig. 2), similar to the calculated pI of 6.9. To determine the molecular weight of the *pehB* gene product, we subcloned the PehB ORF into *E. coli* expression vector pET29b(+) to generate pQHBET. The recombinant protein, including PehB and a 1.2-kDa C-terminal histidine tag from the vector, was approximately 80 kDa (Fig. 5). The apparent size of PehB, therefore, is about 79 kDa, in general agreement with the size of 74.2 kDa predicted by DNA sequencing. When transformed into *E. coli* BL21(DE3), pQHBET caused pitting on PEC-SSA plates after 7 days of incubation at room temperature, further confirming that the subcloned gene product contains PehB. To confirm that PehB is an exo-PG, and to further determine whether mono- or

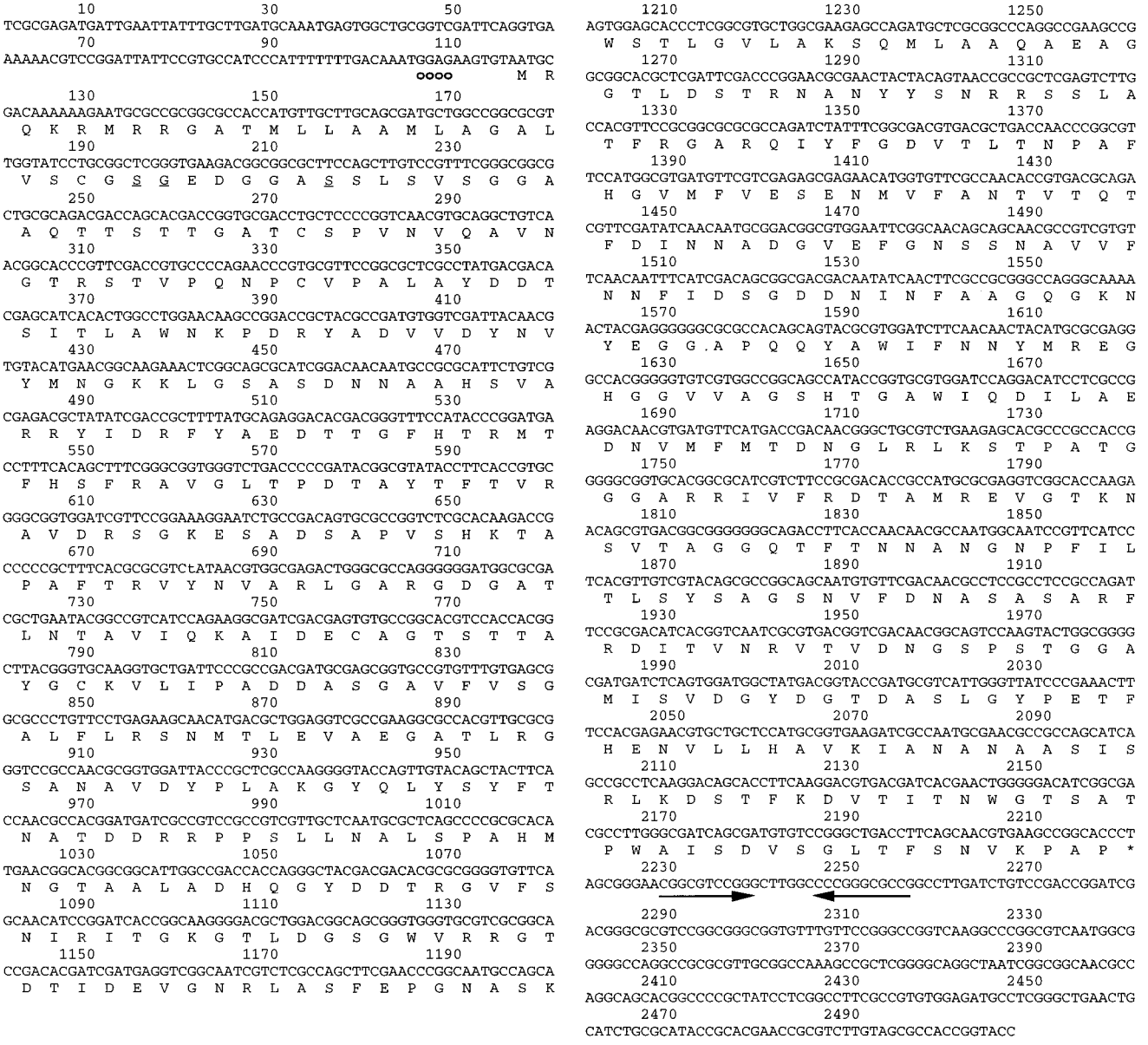

FIG. 3. Nucleotide sequence of the 2.5-kb *Nru*I-*Kpn*I *R. solanacearum* DNA fragment containing the *pehB* gene. A potential ribosome-binding site is marked by circles. Potential starting amino acids of the mature PehB, as predicted by the method of von Heijne (63–65), are underlined. The stop codon is indicated by the asterisk. An 11-base palindrome that could form a stem-loop structure immediately after the stop codon is indicated by arrows.

digalacturonic acid is its reaction product, we characterized the reaction products of PehB contained in the cell-bound fraction of *E. coli* by TLC. As shown in Fig. 6, lane 8, the reaction product of PehB was exclusively digalacturonic acid; therefore, the enzyme is an exo-poly-α-D-galacturonosidase.

Construction and characterization of *R. solanacearum pehB* and *pehA pehB* mutant strains. We initially tried to construct *pehB* and *pehA pehB* mutants by marker exchange-eviction mutagenesis involving the use of an *nptI-sacB-sacR* cartridge (44) but were not successful. We later learned that this approach also did not work in *Pseudomonas aeruginosa* or *P. syringae* (67). As an alternative, we used a Gm^r gene cassette for site-directed mutagenesis of *pehB* (52).

We constructed a plasmid, pQHBM2, containing the internal 1.1-kb *Kpn*I fragment of *pehB* interrupted by a Gm^r gene cassette inserted in the unique *Bam*HI site (Fig. 1). This plasmid, which cannot replicate in *R. solanacearum*, was electroporated into wild-type strain K60 and *pehA* mutant K60-06; potential *pehB* mutants were selected on gentamicin and gentamycin plus streptomycin, respectively. One $PehB^-$ transformant was designated K60-209, and one $PehA^-$ $PehB^-$ transformant was named K60-309. Southern blot analysis confirmed that the *pehB* gene in the two strains was disrupted in the *Bam*HI site by a single Gm^r gene cassette and that the vector plasmid was not present in the genome (data not shown).

Both K60-209 and K60-309 had wild-type colony morphology when grown on TZC plates. They also grew at a rate indistinguishable from that of the wild-type strain in CPG broth and in tobacco leaves (data not shown). The PG activities of the extracellular protein preparations from strains K60-

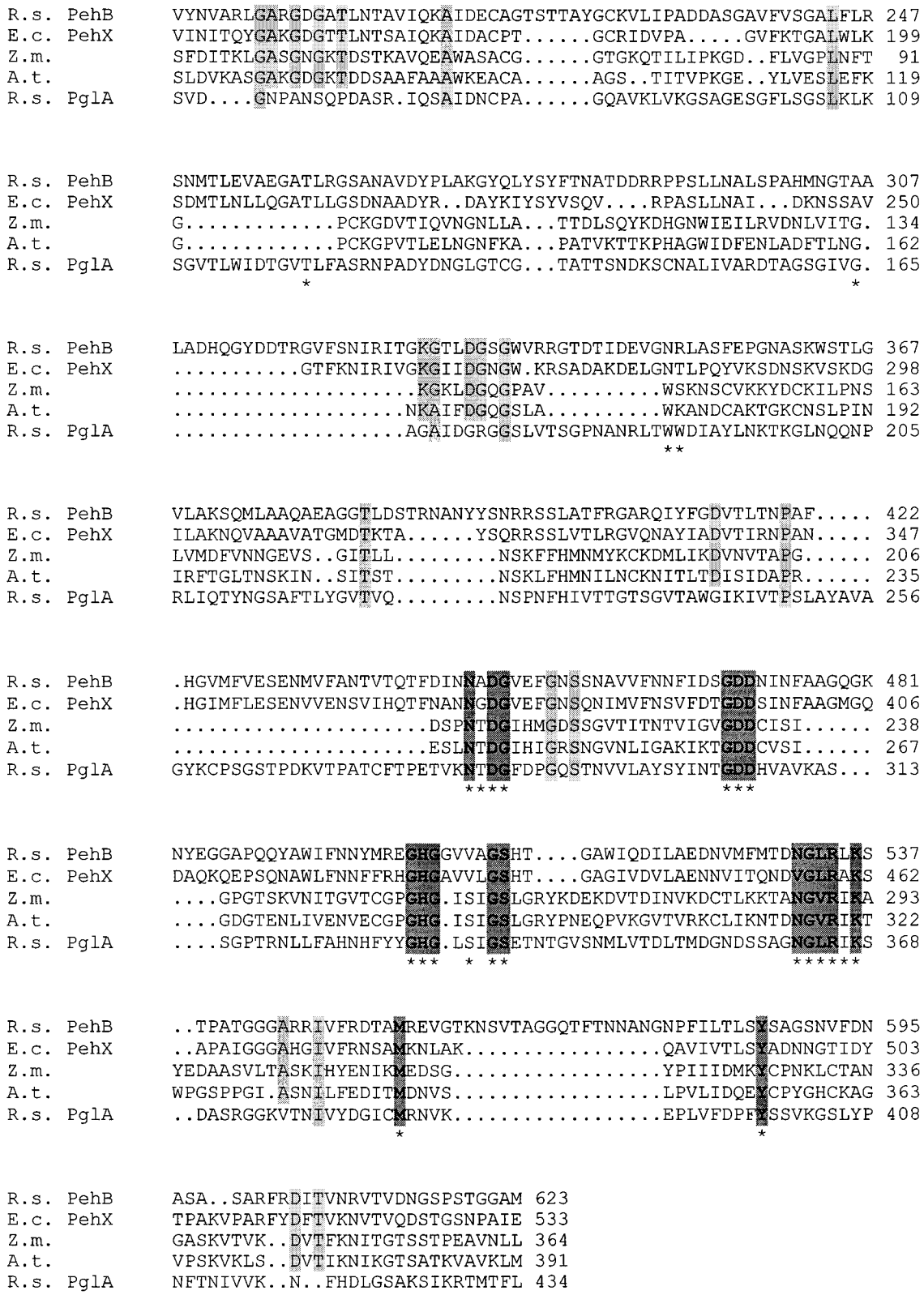

FIG. 4. Comparison of the deduced amino acid sequences of four exo-PGs from *R. solanacearum* (R.s. PehB), *E. chrysanthemi* (E.c. PehX) (19), *Z. mays* (Z.m.) (4), and *A. thaliana* (A.t.) (41) and one endo-PG from *R. solanacearum* (R.s. PglA) (26) aligned with the GCG PileUp program. The N- and C-terminal alignment results were omitted because these regions contained no substantial similarity. Residues conserved among all endo-PGs revealed by Herlache et al. (21) are marked by asterisks under the PglA sequence. Highly conserved residues among all four exo-PGs and the one endo-PG, PglA, as well as among other endo-PGs, are shaded in dark gray. Residues conserved among all four exo-PGs and the one endo-PG but not among other endo-PGs are shaded in light gray. Numbers refer to the position of the amino acids in those proteins.

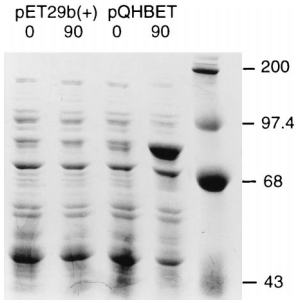

FIG. 5. Sodium dodecyl-sulfate-polyacrylamide gel of PehB recombinant protein expression in *E. coli*. The indicated plasmids were grown in *E. coli* BL21(DE3) at 37°C to mid-log phase. Expression was induced with 2 mM IPTG. Numbers at right indicate the sizes of (in kilodaltons) molecular weight markers. Numbers at top indicate minutes after IPTG induction.

TABLE 2. PG activities of various *R. solanacearum* mutants

Strain	PG activity measured by:	
	Change of substrate viscosity[a] (% of wild-type level)	Reducing sugar ends generated[b] (U of enzyme/10^7 bacterial cells)
K60 (wild type)	100	2.68
K60-06 ($PehA^-$)	8.1	1.62
K60-209 ($PehB^-$)	93.8	1.55
K60-309 ($PehA^-$ $PehB^-$)	2.7	0.47

[a] Measured by change in viscosity of a pectate substrate solution (30, 54); indicates primarily *endo*-PG activity.

[b] Measured by a modification of the method of Lever (35) as described in Materials and Methods; detects reducing sugar ends generated as a measure of *exo*-PG activity.

209 and K60-309 were measured by viscometric and reducing sugar assays and compared with those of K60 and K60-06. The results are summarized in Table 2. The *pehB* mutant decreased the polygalacturonate substrate viscosity (an indication of endo-PG activity) almost as rapidly as the wild-type strain K60, while it was only 58% as effective as K60 in generating reducing sugar ends (an indication of exo-PG activity). A *pehA pehB* double mutant decreased substrate viscosity slightly less than the *pehA* mutant, and its ability to generate reducing sugar ends was also dramatically reduced.

Analysis of the extracellular proteins from strains K60, K60-06, K60-209, and K60-309 on IEF-ASO gels confirmed the absence of PehA, PehB, and $PehA^+$ PehB from K60-06, K60-209, and K60-309, respectively (Fig. 2).

When the reaction products of PGs from the supernatants of the four *R. solanacearum* strains were analyzed by TLC (Fig. 6, lanes 1 to 4), we found that the extracellular enzymes from wild-type K60 released a mixture of products ranging from galacturonate monomer, dimer, and trimer to larger oligomers of galacturonic acid. Unlike K60, the PehA mutant produced no trimer or larger oligomers whereas the PehB mutant released much less dimer, confirming that the reaction product of PehB is galacturonate dimer and that PehB is an exo-poly-α-D-galacturonosidase. Interestingly, the PehA PehB double mutant produced only monomer, suggesting that the remaining pectolytic enzyme, PehC, may be an exopolygalacturonase.

Role of PGs in the virulence of *R. solanacearum*. The virulence of mutant strains K60-209 ($PehB^-$), K60-06 ($PehA^-$), and K60-309 ($PehA^-$ $PehB^-$) was compared with that of the wild-type strain K60. Virulence on 22-day-old eggplants was assayed first by means of a petiole inoculation method. In these assays, *pehB* mutant K60-209 was consistently less virulent than the wild-type strain, although it was more virulent than the *pehA* mutant. The least virulent of all was the *pehA pehB* double mutant (Fig. 7A).

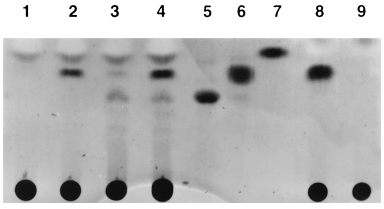

FIG. 6. Characterization of the reaction products of PGs from *R. solanacearum* and *E. coli* by TLC. Supernatants of the *R. solanacearum* strains or cytoplasmic fractions of the *E. coli* strains were incubated with 0.5% PGA (potassium salt) in 50 mM MES (pH 5.7) (3:7, vol/vol) at 45°C for 20 min or 2 h, respectively. Twenty microliters of the reaction mixture for *R. solanacearum* strains or 15 μl for *E. coli* strains was loaded on each lane. Lanes: 1, K60-309 ($PehA^-$ $PehB^-$); 2, K60-06 ($PehA^-$); 3, K60-209 ($PehB^-$); 4, K60 (wild type); 5, trigalacturonic acid standard (4 μg); 6, digalacturonic acid standard (4 μg); 7, monogalacturonic acid standard (4 μg); 8, *E. coli* DH5α(pQHBB8); 9, *E. coli* DH5α(pBS SK^+ vector).

We also tested the virulence of these strains by using a soil inoculation method that more closely mimicked natural bacterial wilt infection conditions (Fig. 7B). All mutant and wild-type strains had the same relative virulence in the soil inoculation assay as in the petiole inoculation assay, although the difference between virulence levels among strains was much greater after soil inoculation. Further, in the soil assay there was an increased lag period between inoculation and appearance of wilt symptoms. After petiole inoculation, it took approximately 3.5 days for wild-type strain K60 to cause a disease index of 2 (50% of leaf area wilted); after soil inoculation, however, it took 5.5 days for K60 to reach the same disease index. *pehA pehB* double mutant K60-309 was unable to wilt 50% of leaf area on most plants even 14 days after soil inoculation.

We conducted a post hoc test of differences in disease index due to strain, using Tukey's honestly significant difference multiple-comparison test. In soil inoculation, the *pehB* mutant was significantly less virulent than the wild-type strain by day 4 of the assay ($P = 0.001$). Furthermore, the *pehA* and *pehA pehB* mutant strains also differed from the wild type, from the *pehB* mutant, and from each other by day 8 ($P = 0.001$). However, in the petiole inoculation experiments, while the *pehA* and *pehA pehB* mutant strains were less virulent than wild-type strain K60 ($P = 0.001$), the *pehB* mutant was not significantly different from the wild type in virulence (Fig. 7). An analysis of variance of the soil and the petiole data indicated that bacterial strains caused the only significant experimental effect in both assays.

DISCUSSION

We cloned and sequenced the *pehB* gene of *R. solanacearum* and found that it was required for the wild-type level of virulence in the bacterium, as determined by soil inoculation assays. We concluded that the cloned gene encoded the exo-poly-α-D-galacturonosidase PehB based on the following lines of evidence. (i) This gene conferred pectolytic activity on *E. coli*, which normally does not produce pectolytic enzymes. (ii) When introduced into a PehB-deficient mutant, this gene restored PehB production. (iii) The *pehB* mutant was barely affected in its ability to rapidly decrease substrate viscosity (an

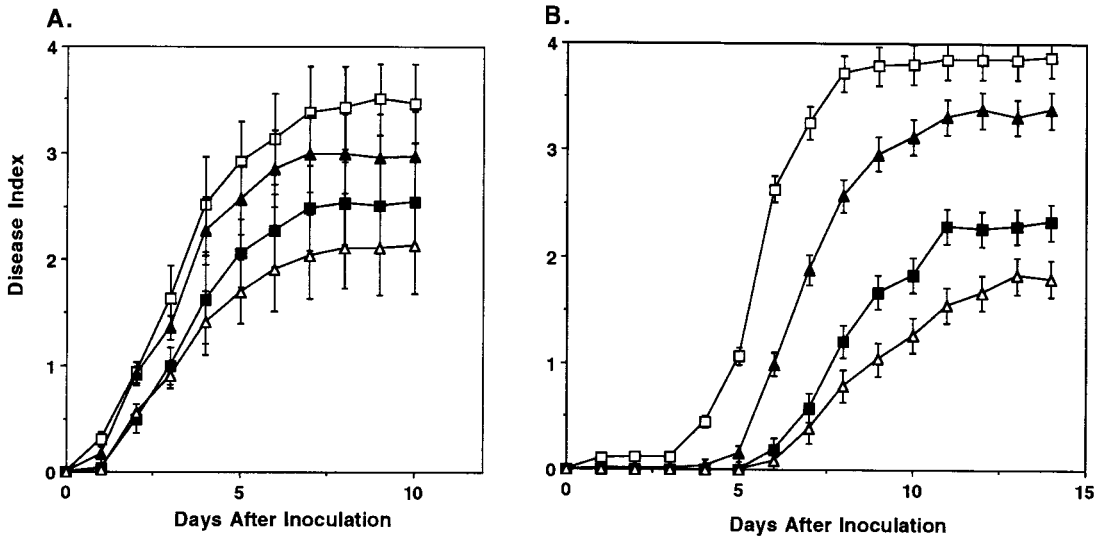

FIG. 7. Virulence of *R. solanacearum* mutants affected in production of PG isozymes on eggplants. (A) Petiole inoculation. A total of 2.5×10^6 bacteria were injected into the petiole of the second leaf of 22-day-old eggplants. (B) Soil inoculation. Roots of 22-day-old eggplants were wounded by a single scalpel slice through the soil 1.5 cm from the base of the stem and then inoculated with 2×10^7 bacteria/g of soil. Strains K60 (wild type), K60-209 (PehB$^-$), K60-06 (PehA$^-$), and K60-309 (PehA$^-$ PehB$^-$) are indicated by open squares, closed triangles, closed squares, and open triangles, respectively. Plants were rated on a disease index ranging from 0 (healthy) to 4 (76 to 100% leaves wilted) (46). Points shown are means of four separate experiments, each containing 16 plants per treatment. Bars indicate standard errors.

indication of endo-PG activity), but it generated only 58% as much reducing sugar ends as the wild-type strain. (iv) A GenBank database search revealed that the amino acid sequence of *pehB* is similar to those of an assortment of bacterial and plant exo-PG genes. (v) Finally, the reaction product of this gene product on polygalacturonic acid was exclusively digalacturonic acid.

The ORF of *pehB* encodes a predicted protein of 74.2 kDa, which was confirmed by overexpressing the PehB protein in *E. coli*. Sequence analysis of the *pehB* gene suggests the presence of a typical N-terminal secretion signal sequence, which has been found in all bacterial endo- and exo-PGs sequenced to date (16, 19, 22, 26, 47, 53, 59). We believe that PehB is a secreted protein because PehB activity is found primarily in the extracellular fraction and mutants carrying a *pehB*::Tn*phoA* fusion are blue on X-phos plates (37).

Our amino acid sequence comparison among four exo-PGs identified a number of regions conserved in all the exo-PGs that were also similarly present in all endo-PGs, suggesting they may be required for a common function of both the endo- and exo-PGs, such as substrate binding and hydrolysis. Our analysis also confirmed the presence of the conserved tyrosine residue (position 586 in PehB) in the C termini of all four exo-PGs, although our full-length PileUp comparison identifies this tyrosine residue in PehX as Y^{494}, nine amino acid residues ahead of the one designated by Stratilova et al. (58). In addition, several highly conserved amino acid residues and regions are present in the exo-PGs but not in the endo-PGs, suggesting that they may be specific to substrate recognition and terminal polymer cleavage by exo-PGs. Site-specific mutagenesis could demonstrate that these regions are indeed required for either endo- or exo-PG activity or for both. A dendrogram of 58 diverse PG amino acid sequences constructed by the GCG PileUp program indicates that prokaryotic exo-PGs are more similar to prokaryotic endo-PGs than to eukaryotic exo-PGs (Fig. 8). This dendrogram is based on pairwise alignments of sequences along their entire lengths and is not a phylogenetic reconstruction. However, this analysis of PG sequences does sort out along phylogenetic lines and may suggest that exo-PG genes diverged from endo-PG genes separately in the evolution of eukaryotes and prokaryotes.

We generated *R. solanacearum* K60 mutants deficient in PehB and both PehA and PehB. The virulence of these mutants on eggplants was tested either by injecting 2.5×10^6 bacteria directly into the petiole or by soaking the soil with 2×10^7 bacteria/g of soil. Since *R. solanacearum* is a soil pathogen that causes infection through wounds on plant roots, injecting bacteria into the petiole bypasses the natural infection site of the bacterium. Thus, the petiole inoculation method cannot test the hypothesis that PGs are required for natural entry of *R. solanacearum* into host plants. We therefore also used a more natural soil inoculation method. Virulence assays using both inoculation methods revealed that *R. solanacearum* strains unable to produce PehB were less virulent than the wild-type strain, although the difference is statistically significant only in the soil inoculation assay. The *pehB* mutant was, however, more virulent than a strain lacking the endo-PG gene, *pehA*. A *pehA pehB* double mutant was substantially reduced in virulence, suggesting that these two PGs may function additively or synergistically during bacterial wilt disease development. The differences in virulence between the mutants and the wild-type strain were more striking in the soil inoculation assay than in the petiole inoculation, suggesting that both PehA and PehB contribute to primary infection involving the entry of the bacterium from the wounds into the vascular systems. This also appears to be the case for another extracellular plant cell wall-degrading enzyme produced by *R. solanacearum*, the endoglucanase Egl (12).

It is not possible to conclusively determine whether the cloned *pehB* gene in pQHBL9 can restore wild-type virulence in *trans*, since the vector pLAFR3 is not stably maintained during bacterial growth in plants (3a). It is therefore possible that the *pehB*::*accCI* construct in K60-209 has a polar effect on some other gene. However, K60-209 is not affected in production of any known virulence factors and grows normally in planta. Further, the stem-loop structure at the end of the *pehB*

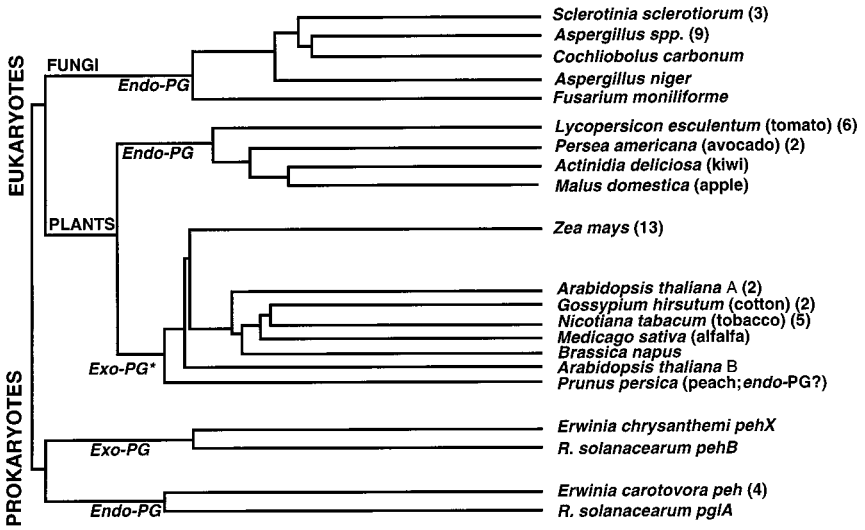

FIG. 8. Dendrogram of plant, fungal, and bacterial PG amino acid sequences constructed by the GCG PileUp program. The number of PG sequences used for each species is indicated in parentheses. Accession numbers for the PG sequences are given in Materials and Methods. *Not all the sequences in this cluster have been enzymatically proven to be exo-PGs.

ORF and the 115-bp gap before the next possible ORF suggest strongly that no downstream gene is cotranscribed with *pehB*.

Since *pehA pehB* double mutant K60-309 still produces at least one exo-PG, PehC, we plan to generate a mutant that produces no PG activity to find out how exo-PGs and total PGs contribute to the virulence of *R. solanacearum*. Even when a triple *pehA pehB pehC* mutant is constructed, it is possible that *R. solanacearum* will produce additional plant-induced pectolytic enzymes like the soft rot pathogen *E. chrysanthemi* (31). Our finding from the reaction product analysis that PehC released galacturonate monomer instead of dimer like PehB may be useful for *pehC* cloning and identification.

Although we now know that both endo- and exo-PGs contribute to bacterial wilt development, we still do not know exactly what role they play in disease. They may aid initial invasion, as suggested by the differences between the results of our soil and petiole inoculation virulence assays. Pectolytic enzymes may contribute to early infection by digesting pectic gels formed around wounds on roots; later in disease development they may block xylem vessels by generating gels and tyloses from hydrolysis of the pectic materials in the vessel and adjacent cell walls. They may also help bacterial spread and colonization by breaking down the pectin-containing pit membranes that separate adjacent xylem vessels (14). We can test the hypothesis that PG is required for infection and spread of *R. solanacearum* by monitoring the movement of mutant and wild-type cells in host plants.

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