

Characterization of the *Agrobacterium vitis* *pehA* Gene and Comparison of the Encoded Polygalacturonase with the Homologous Enzymes from *Erwinia carotovora* and *Ralstonia solanacearum*

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DNA sequencing of the *Agrobacterium vitis* *pehA* gene revealed a predicted protein with an M_r of 58,000 and significant similarity to the polygalacturonases of two other plant pathogens, *Erwinia carotovora* and *Ralstonia* (= *Pseudomonas* or *Burkholderia*) *solanacearum*. Sequencing of the N terminus of the PehA protein demonstrated cleavage of a 34-amino-acid signal peptide from pre-PehA. Mature PehA accumulated primarily in the periplasm of *A. vitis* and *pehA*⁺ *Escherichia coli* cells during exponential growth. *A. vitis* PehA released dimers, trimers, and monomers from polygalacturonic acid and caused less electrolyte leakage from potato tuber tissue than did the *E. carotovora* and *R. solanacearum* polygalacturonases.

Agrobacterium vitis (formerly *A. tumefaciens* biovar 3) is the primary causal agent of crown gall, a hyperplastic disease, on grapevines (8, 31). Crown gall is an economically important disease in all major viticultural regions of the world (13). *A. vitis* has been detected only in infected grape plants and infected vineyard soils and has been shown in greenhouse experiments to survive poorly in nongrape rhizospheres and in bulk soil (9, 14). Although *A. vitis* is tumorigenic on other plants, it shows a great degree of specificity for grape plants, where it also causes sunken necrotic lesions on the roots (9, 12, 31).

The necrotic lesions caused by *A. vitis* are reminiscent of disease symptoms produced by pectolytic plant pathogens (15). The bacterium produces polygalacturonase (PG), an enzyme that catalyzes the hydrolytic cleavage of pectic polymers in plant cell walls, and the enzyme can be isolated from necrotic lesions on grape roots (29). PG is present in culture supernatants of both tumorigenic pTi-carrying strains and pTi-lacking nontumorigenic strains, indicating that the enzyme is chromosomally encoded (29). The *A. vitis* PG is unique among known bacterial PGs in having an acidic pI (29).

Compared with the wild type on juvenile grape roots, an *A. vitis* derivative carrying a Tn5 insertion in the *pehA* gene produces smaller tumors on grape stems (but not potato disks), attaches poorly, multiplies at a reduced rate, and fails to form necrotic lesions at 10^5 CFU ml⁻¹ (11, 35). These observations suggest that PehA may have multiple functions in *A. vitis* pathogenesis that are not relevant to the diseases caused by other pathogenic bacteria known to produce PG, such as *Erwinia carotovora*, a soft-rot pathogen, and *Ralstonia* (= *Pseudomonas* or *Burkholderia*) *solanacearum*, a vascular wilt pathogen. For example, galacturonic acid has been shown to enhance *vir* gene expression in *A. tumefaciens* (3), suggesting that a PG which releases monomers from pectic polymers in the cell wall could enhance *Agrobacterium* tumorigenicity.

The PG-encoding *peh* and *pgl* genes from *E. carotovora* and *R. solanacearum*, respectively, have been cloned and extensively characterized (1, 21, 24, 26, 27, 40, 42, 49), and the

A. vitis *pehA* gene was previously cloned (35). We have now determined the *pehA* DNA sequence and characterized its product. This is the first molecular characterization of a pectic enzyme from a member of the Rhizobiaceae. This has permitted direct comparison of the primary structures, enzymological properties, and effects on plant tissue of the PGs from these disparate plant pathogens.

***pehA* DNA sequence analysis.** Plasmids were manipulated in vitro and transformed according to standard methods (41) into *Escherichia coli* DH5 α (Life Technologies, Inc., Grand Island, N.Y.). *E. coli* cultures were grown at 37°C in Luria-Bertani medium supplemented with 100 μ g of ampicillin ml⁻¹. pCPP2068 carries the *A. vitis* *pehA* gene in pBluescript (35). Double-stranded pCPP2068 DNA templates were sequenced by the dideoxy chain termination method (41). The first strand was sequenced by using nested exonuclease III deletions, and then gaps were filled in by using synthetic primers. The second strand was sequenced at the Cornell Biotechnology Center by using *Taq* polymerase and a model 370 ABI DNA Sequencer with primers complementary to the first strand. DNA sequences were analyzed by using the DNASTAR Lasergene software package (DNASTAR, Madison, Wis.) and the Genetics Computer Group Sequence Analysis software package (16). Sequences in the GenBank database with similarities to open reading frames (ORFs) in pCPP2068 were identified by searches with the BLASTX and FASTA programs (2, 32).

The 1.6-kb *pehA* ORF (Fig. 1) was identified by its similarity to PGs from *E. carotovora* and *R. solanacearum*. Portions of two other ORFs were also found within pCPP2068, flanking *pehA*. Three potential in-frame start methionines for *pehA* are present at bp 338, 362, and 413. A consensus Shine-Dalgarno ribosome-binding site occurs between bp 351 and 354. No ribosome-binding site is apparent in the proper location upstream of the other potential start codons; therefore, the *pehA* coding sequence is thought to extend from bp 362 to 2008. This ORF would encode a 549-amino-acid protein with an M_r of 58,000. No rho-independent transcriptional terminator is apparent at the 3' end of the *pehA* gene (data not shown).

Most of the pectate-inducible pectolytic enzymes of *Erwinia chrysanthemi* are repressed in the absence of pectate by the

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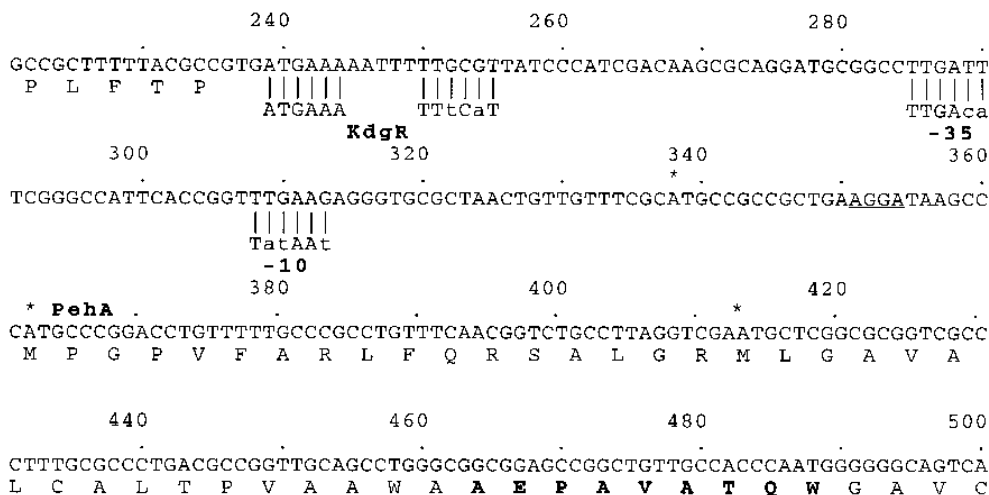


FIG. 1. Sequence of the 5' region of PehA. The 3' end of ORF1 is shown along with the putative transcriptional regulatory sequences aligned with their respective consensus sequences. Matches with the consensus sequence are indicated by an uppercase letter. Three potential *pehA* start methionines are denoted by asterisks above the DNA sequence at positions 338, 362, and 413. The consensus ribosome-binding site preceding the putative *pehA* start methionine is underlined. The sequenced N-terminal amino acids of mature PehA are shown in boldface type. Numbering starts with the *Hind*III site used to clone *pehA* into pCPP2068 (35). The GenBank accession number for *pehA* is U73161.

KdGR repressor, and putative KdGR-binding sites have been identified in the upstream regions of the pectate lyase *pel* genes of *E. carotovora* (6). A region with similarity to the KdGR consensus repressor-binding site (33) is present 115 bp upstream of the *pehA* start methionine at bp 239 to 255 (Fig. 1). This putative KdGR-binding site matches the consensus at 10 of 12 positions and has the proper spacing between elements of the repressor-binding site. This observation suggests that KdGR-mediated control of pectic enzyme production may be broadly conserved in gram-negative bacteria.

Many of the known bacterial endolytic pectate-degrading enzymes are targeted to the extracellular milieu by the Sec-dependent, type II secretion pathway and, consequently, are synthesized with N-terminal signal peptides which are required for the first step of secretion (6). The N-terminal 34 amino acids of PehA have two features characteristic of signal peptides (48), a hydrophobic region and a conserved Ala-Ala cleavage site; however, no positively charged amino acids are located near the start methionine.

ORF1 terminates with two TGA stop codons 125 bp upstream of the putative PehA start site (Fig. 1). No rho-independent transcriptional terminator is apparent in the 3' end of ORF1, but the putative KdGR-binding site overlaps a short poly(A)-poly(T) inverted repeat following this ORF. No genes with similarity to ORF1 could be identified in the GenBank database by using the BLASTX or FASTA program.

Comparison of the PehA sequence with representative PG sequences of bacterial, fungal, and plant origins. Figure 2 shows a Clustal alignment of the *A. vitis* PehA with the PGs from *R. solanacearum*, *E. carotovora*, *Aspergillus niger*, and *Lycopersicon esculentum* (tomato). The gap weight and length parameters (penalties of 10 for each) were chosen to preserve alignment of the conserved regions identified in endo- and exo-PGs, including the GHG putative active site (40). The *A. vitis* PG is most similar to PglA from *R. solanacearum* (50.0%), followed by Peh from *E. carotovora* (35.1%), as determined by the DNASTAR MEGALIGN program. Similarities are much lower for the PGs from *A. niger* (14.2%) and tomato plants (15.0%) but are clearly evident in the four domains that are conserved in prokary-

otic and eukaryotic PGs (centered at PehA residues 278, 300, 328, and 363) (46). It has been suggested that bacterial pathogens, which are in close ecological association with plants, may have acquired the PG gene by horizontal transfer (21, 40). Since *Agrobacterium* pathogenesis involves interkingdom DNA transfer (bacterium to plant), we were particularly interested in the relative similarity of the *A. vitis* and tomato PGs. However, the level of similarity to the tomato PG is no higher for *A. vitis* than for the other two pathogens and provides no support for a role of *A. vitis* in this process.

We similarly compared the *A. vitis* PehA with *E. chrysanthemi* PehX and *A. tumefaciens* Pgl (18, 38). *E. chrysanthemi* *pehX* encodes exo-poly- α -D-galacturonidase, and *A. tumefaciens* *pgl* encodes a protein with no known enzymatic activity but with some similarity to *E. carotovora* *peh*. PehA was only weakly similar to PehX (15.4%) and Pgl (15.3%).

PehA purification and N-terminal sequencing. PG activity in 36-h *A. vitis* CG49 cultures grown in Kado 523 medium at 25°C is located primarily in the culture supernatant (29). However, as discussed above, the deduced amino acid sequence revealed an atypical N-terminal signal peptide. To determine if this putative signal peptide is appropriately processed, PehA was purified from the culture supernatant of a 4-liter overnight culture of *E. coli* KS476(pCPP2068), an *lky207 degP41* leaky-

TABLE 1. Subcellular localization of PG activity in *A. vitis*

Cell fraction	Relative activity (%) (mean \pm SE)		
	Malate dehydrogenase ^a	β -Lactamase ^b	PG ^c
Cytoplasm	81.3 \pm 2.6 ^d	24.3 \pm 7.1	2.0 \pm 0.8
Periplasm	21.0 \pm 6.4	66.9 \pm 2.1	67.0 \pm 9.6
Extracellular	0	13.1 \pm 5.0	31.0 \pm 9.9

^a Malate dehydrogenase activity was measured as described previously (45).

^b β -Lactamase activity was assayed by the loss of nitrocefin absorbance at 540 nm.

^c PG activity was assayed by the 2-cyanoacetamide assay (4).

^d Values represent the mean and standard error for three replications.

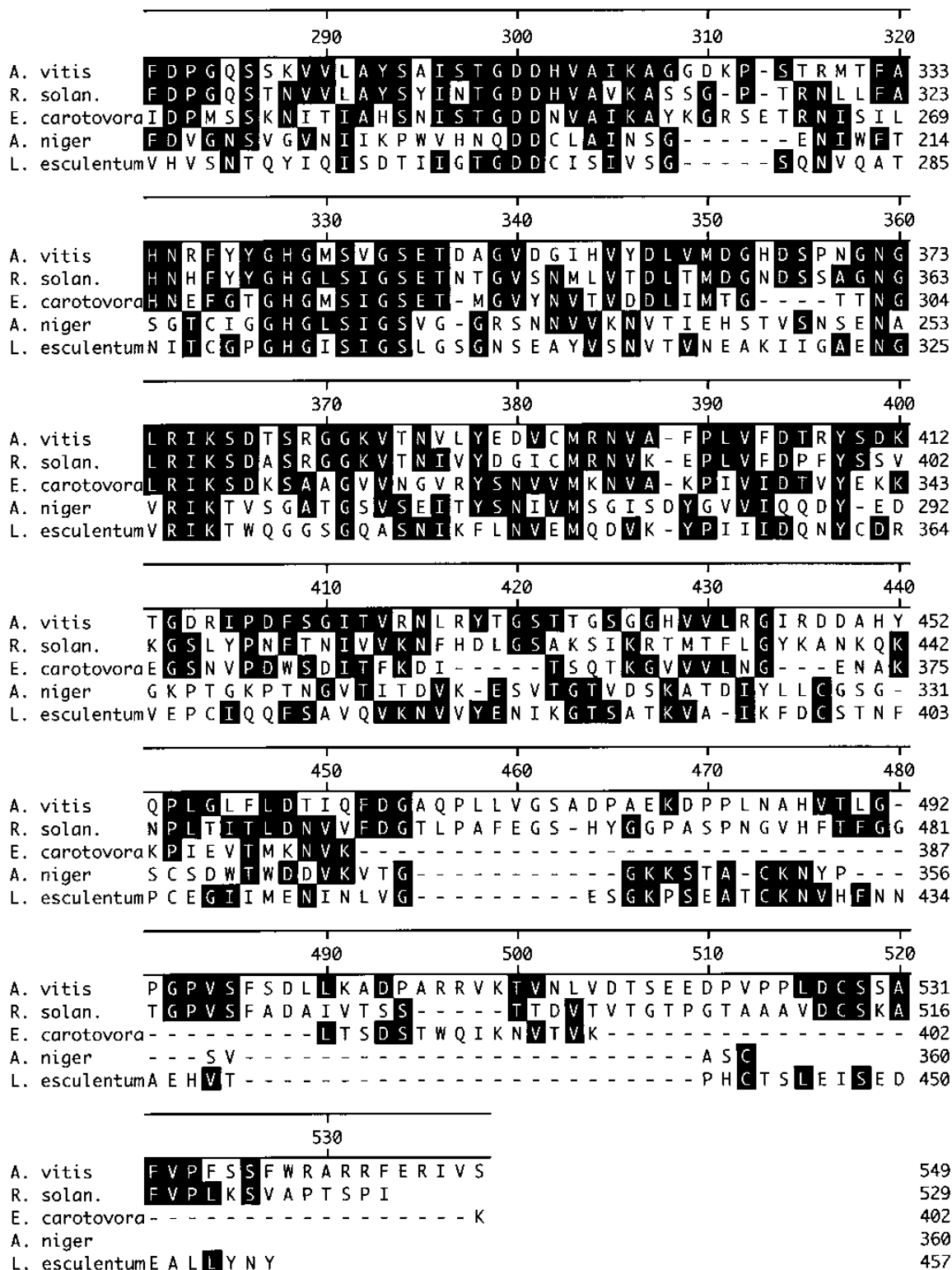


FIG. 2—Continued.

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ily in the 250 mM NaCl fraction. The buffer was exchanged with 20 mM bis-Tris, pH 5.8, and proteins were further separated with a MemSep DEAE convective liquid chromatography cartridge (Millipore, Bedford, Mass.) by using a complex

NaCl gradient (0 to 100 mM NaCl in 3 min with a convex curve gradient followed by a linear NaCl gradient from 100 to 250 mM, in which PehA eluted). The gradient was delivered by using a ConSep LC100 liquid chromatography apparatus (Mil-

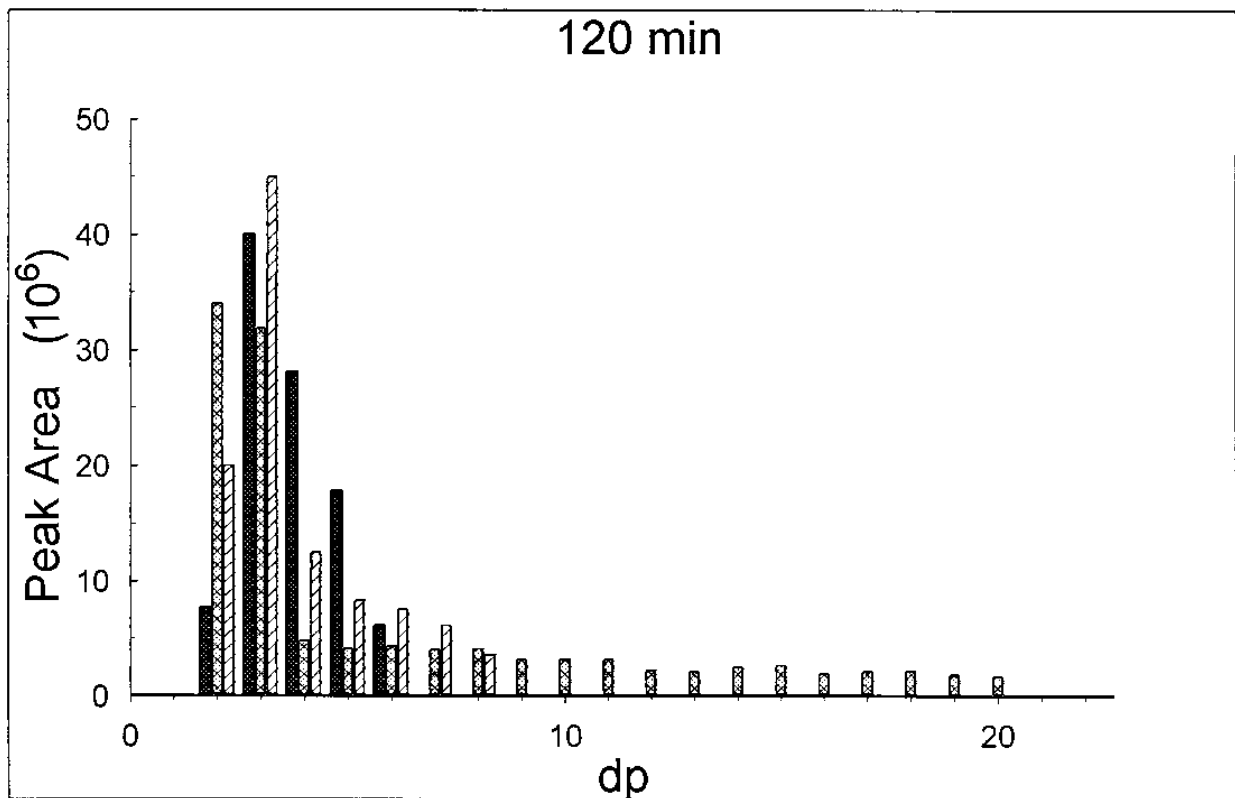
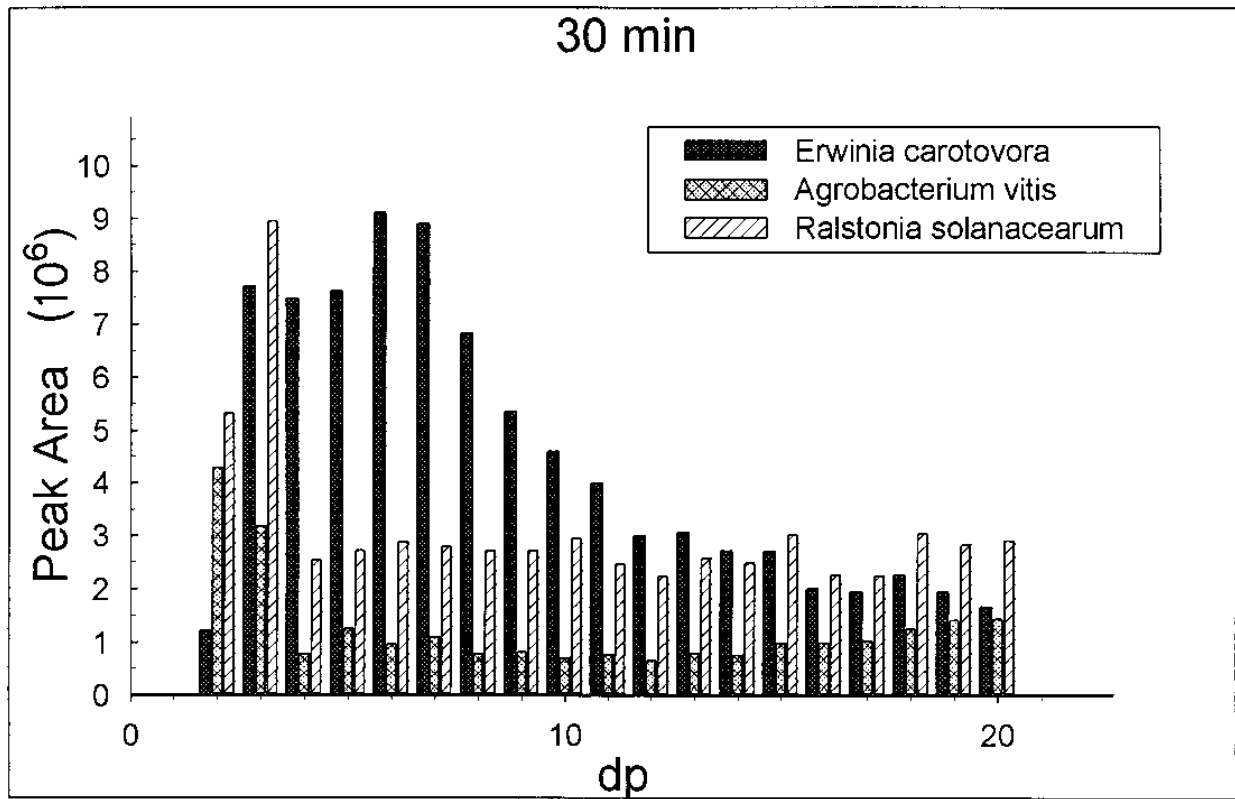


FIG. 3. HPAEC-PAD analysis of products released from pectate by partial digestion with PGs from three plant pathogenic bacteria. Reaction mixtures contained 0.2% polygalacturonic acid and equivalent levels of the three enzymes. Reactions were stopped at the times indicated on the graphs by titrating the reaction mixture to pH 10 with 0.01 M KOH, and proteins were removed with StrataClean resin (Stratagene, La Jolla, Calif.). Reaction products are specified as dp of oligogalacturonic acid length versus peak area for each enzyme and time point. Chromatographic conditions included a CarboPac PA1 column and a nonlinear potassium oxalate (pH 6) mobile phase gradient (25 to 500 mM in 100 min). Alkali (500 mM KOH) was added post-chromatography column to enhance PAD detection of reaction products. Minimal run-to-run variation in peak area was observed. For example, the following mean peak area ($\times 10^6$) and standard error values ($\times 10^6$) were observed for the *R. solanacearum* 16-h runs from two separate hydrolysis experiments: dp 2, 25.8 ± 2.3 ; dp 3, 99.2 ± 5.3 ; dp 4, 6.6 ± 1.3 ; and dp 5, 0.7 ± 0.1 .

lipore). PG activity was determined by the increase in reducing sugars in reaction mixtures containing 0.2% polygalacturonic acid (Sigma Chemical Co., St. Louis, Mo.) and 50 mM potassium acetate, pH 5.25, as assayed with the 2-cyanoacetamide reagent (4). A unit of PG activity is defined as that releasing 1 μ mol of reducing sugar per min at 30°C. The purity of the PG-containing fractions was determined with sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis gels stained with ProtoGold (Research Diagnostics, Flanders, N.J.).

Purified PehA has an apparent molecular weight of 58,000 on SDS-polyacrylamide gels (data not shown), which is in agreement with the predicted molecular weight of 54,000 deduced from the DNA sequence for the mature protein. Recombinant PehA has the same pI as PehA produced by *A. vitis* CG49, indicating that the enzyme is processed identically in *E. coli* and *A. vitis* (reference 35 and data not shown). The N-terminal sequence of the first 9 amino acids was determined by Edman degradation at the Cornell University Biotechnology

Program Protein Analysis Facility. These amino acids (residues 35 to 43) are shown in boldface type in Fig. 1 and match the deduced sequence, indicating that the signal peptide is appropriately processed.

Subcellular localization of PehA. The type II secretion pathway, which in *E. carotovora* and *R. solanacearum* is used to secrete PGs to the extracellular medium (21, 25, 47), has not been demonstrated to be present in *Agrobacterium* spp. However, PehA appears to have a functional signal peptide, which is required for type II secretion because the type II pathway is an extension of the Sec (general protein export) pathway. The subcellular localization of PehA was observed in overnight cultures of both *E. coli* DH10B (Life Technologies) carrying pCPP2068 and *A. vitis* CG49 carrying pSM243cd (*bla*⁺) to determine if PehA is secreted (20). Since *E. coli* appears to lack a functional type II secretion system, Sec-processed PehA should accumulate in the periplasm. If PehA is actively secreted from *A. vitis*, the majority of enzyme activity should be found in the supernatant fraction of the CG49 culture. This, in

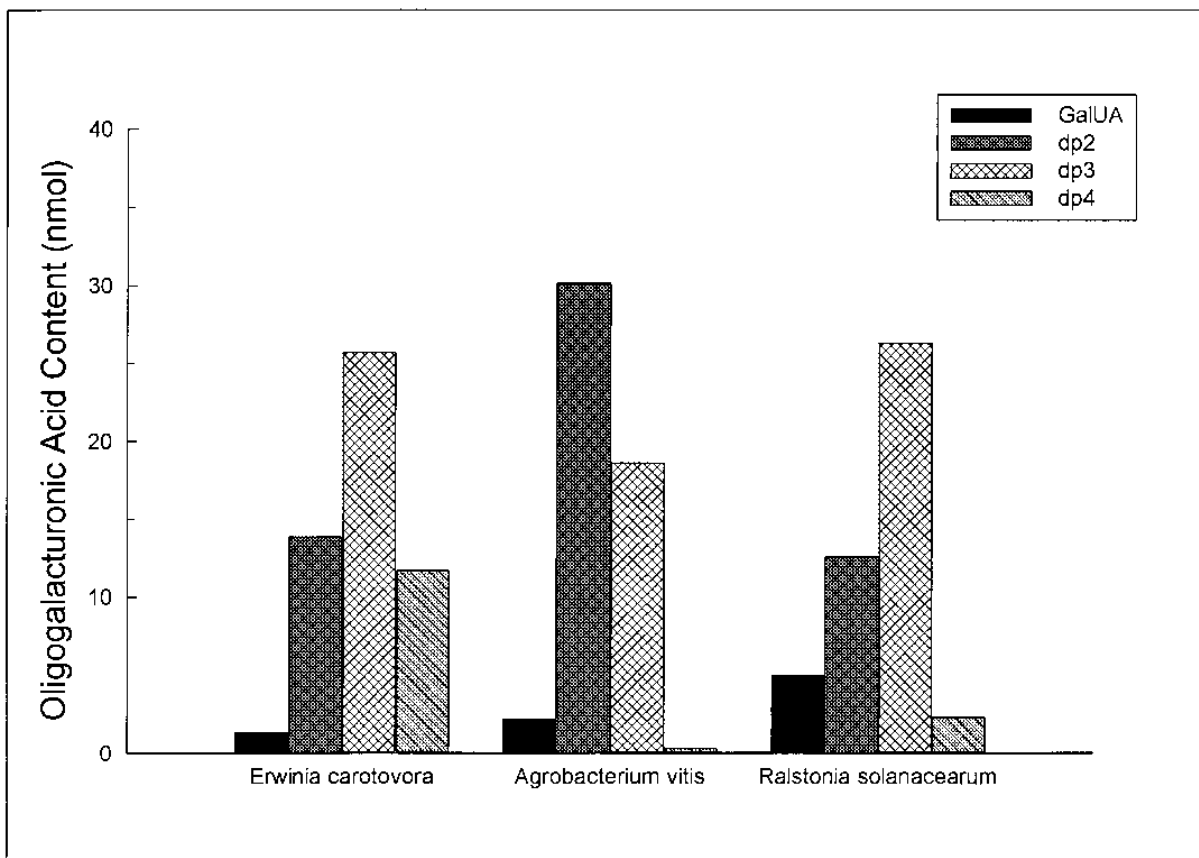


FIG. 4. HPAEC-PAD analysis of the near-limit products released from pectate by PGs from three plant-pathogenic bacteria. Chromatographic conditions included a linear sodium acetate (pH 12.8) mobile phase gradient (5 to 500 mM sodium acetate in 100 mM sodium hydroxide lasting 120 min). Galacturonic acid (GalUA) and oligogalacturonic acid contents are expressed as the amount detected in the 25- μ l sample injected relative to commercial (Sigma Chemical Co.) standards and a standard (dp 4) purified by preparative high-performance liquid chromatography (23).

turn, would suggest the presence of a type II secretion system in *A. vitis*.

Subcellular fractionation studies with *E. coli* DH10B (pCPP2068) showed that PehA in early-stationary-phase cultures was 66% periplasmic and 31% extracellular. The extracellular pool presumably resulted from leakage across the outer membrane, since 30% of the periplasmic marker β -lactamase was also released, as determined by β -lactamase assays with nitrocefin (Glaxo, Greenford, Middlesex, England) at 540 nm. The distribution of PehA activity in late-log-phase cultures of CG49(pSM243cd) was similar to the distribution in *E. coli*, with most of the PG activity found in the periplasmic fraction (Table 1). More than 80% of the activity of the cytoplasmic marker malate dehydrogenase was found in the cytoplasmic fraction (45), with the remainder in the periplasmic fraction. These results, taken together with the results of N-terminal sequencing, indicate that PehA is recognized by the Sec machinery, efficiently processed, and transported to the periplasm.

However, the higher proportion of PG activity relative to β -lactamase activity in the supernatant of *A. vitis* cultures raises the possibility that PehA is actively, but inefficiently, secreted by a type II protein secretion system in *A. vitis*. An example of this is found in the inefficient secretion of PehX by the type II system of *E. chrysanthemi* (19). On the other hand, PehA may simply leak out of the periplasm, especially from older cultures such as those used by McGuire et al. (29). The possibility of active PehA secretion will have to be resolved in the future by identifying (if present) and mutating any genes encoding the type II pathway in *A. vitis* and then determining the effects of the mutation on PehA secretion.

Characterization of the enzymological properties of PehA and comparison with the PGs of *R. solanacearum* and *E. carotovora*. To conveniently prepare sufficient quantities of PehA, PglA, and Peh for activity assays, the enzymes were partially purified from transformed cultures of *E. coli* KS476 expressing at high levels the cognate genes from vector promoters. The plasmids used were pCPP2068, pTM5 (*R. solanacearum* pglA⁺ [42]), and pSS2001 (*E. carotovora* peh⁺ [26]). The anion-exchange chromatography protocol described above for PehA purification was not useful for the other two PGs because of their basic pIs. Hence, affinity chromatography on cross-linked pectate was used to partially purify all three of the PGs from supernatants of overnight 2-liter cultures of *E. coli* KS476 transformants (28, 36). The proteins were adsorbed to the matrix following ammonium sulfate precipitation and dialysis against 50 mM potassium acetate buffer, pH 3.5–1 mM EDTA. The matrix and bound proteins were washed with 1 bed volume of the same buffer, and then proteins were eluted by using single bed volumes of buffer in steps of pH 4.5, 5.5 (50 mM potassium acetate, 1 mM EDTA), and 7.5 (20 mM Tris buffer, 1 mM EDTA). PG activity eluted in the pH 7.5 fraction. Collected fractions were dialyzed against 50 mM potassium acetate, pH 5.25, overnight.

PehA has a pH optimum of 5.25 (data not shown), which is similar to the pH optima for the homologous PGs from *R. solanacearum* (5.7) (42) and *E. carotovora* (5.5) (49). The buffers used were 50 mM potassium acetate for pH 3.5 to 6.4 and Tris-HCl for pH 7.0 to 8.5. The temperature optimum for PehA is somewhat higher than that for its homologs and occurs between 45 and 55°C, but there is very little activity above 60°C (data not shown).

To confirm that PehA is an endo-PG and to identify any patterns of pectate depolymerization that might yield clues to enzyme function in pathogenesis, we analyzed the reaction products of the PGs from *A. vitis*, *R. solanacearum*, and *E. ca-*

rotovora by high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) (22), as shown in Fig. 3 and 4. This analytical technique was used because of its state-of-the-art ability to both separate oligosaccharide positional isomers and determine the widest possible size range of underivatized products of pectolytic depolymerization (23). PGs were added to substrate (0.2% sodium pectate in 50 mM potassium acetate, pH 5.25) to a final concentration of 56 U ml⁻¹ (*A. vitis* PehA), 65 U ml⁻¹ (*R. solanacearum* PglA), and 60 U ml⁻¹ (*E. carotovora* Peh). Reaction products were analyzed at 30 min, 2 h, and 16 h as described elsewhere (22) and shown in Fig. 3 and 4. To optimally analyze the different ranges of products released during the course of the digestion, a nonlinear potassium oxalate mobile phase gradient was used for the 30-min and 2-h digests (Fig. 3), whereas a linear sodium acetate, high-pH mobile phase gradient was used for the 16-h, near-limit digest (Fig. 4).

A. vitis PehA is an endo-PG, as demonstrated by its ability to release dimers, trimers, and larger oligomers of galacturonic acid from pectate (Fig. 3). While data are shown only up to a degree of polymerization (dp) of 20, peaks corresponding to oligogalacturonic acids in excess of dp 40 were detected in the 30-min hydrolysate. Each of the three PGs produced a different near-limit digest profile of oligogalacturonic acids (Fig. 4). While PehA generates primarily dimers, the other two PGs are primarily trimer-generating enzymes. Galacturonic acid levels produced by PehA are intermediate between those produced by the other two PGs (Fig. 4). The near-limit products of all three PGs contained trace quantities of the pentamer. In agreement with previously published results (40, 42, 49), both the *R. solanacearum* and *E. carotovora* PGs were found to be endolytic.

In overnight digests, all three PGs were able to hydrolyze pectin with up to 68% esterification (Sigma Chemical Co.) as detected by clear halos on buffered agarose gels containing substrate (34), but none of the PGs could hydrolyze 98% esterified pectin (Sigma Chemical Co.) (data not shown). The activities of all three enzymes on partially esterified pectin are much lower than they are on pectate as measured by the increase in reducing-sugar concentration by a modified Nelson-Somoygi assay (30), an assay chosen to avoid alkaline degradation of the esterified polymer. Thus, all three PGs are pectate hydrolases and are classified as EC 3.2.1.15. Although it does not appear that PehA would be efficient at releasing galacturonic acid monomers from pectic polymers for *vir* gene induction, the observation that the *picA* locus of *A. tumefaciens* is more strongly induced by complex pectic polysaccharides than by simple oligogalacturonates leaves open the possibility that PehA may still function in releasing signals from host cell walls (37).

Comparison of the effects of the PGs from *A. vitis*, *R. solanacearum*, and *E. carotovora* on plant tissue. Tissue maceration was measured by tactile assessment of potato tuber disks treated with 60 U of each PG (7). Plant cell damage was measured as the increase in conductivity of the tuber disk bathing solution (7). The enzymes used were prepared by affinity chromatography on cross-linked pectate as described above. The relationship between tissue maceration activity and enzyme pI followed the pattern previously observed with bacterial pectate lyases, where maceration increases with enzyme pI (5). The pI 10.6 *E. carotovora* PG macerated potato disks very efficiently, while the pI 9.0 *R. solanacearum* PG was intermediate and the pI 4.9 *A. vitis* PG macerated weakly. However, the ability to macerate potato tuber tissue did not correlate with the ability to damage plant cells for the PGs of *E. carotovora* and *R. solanacearum*. Figure 5 presents the data

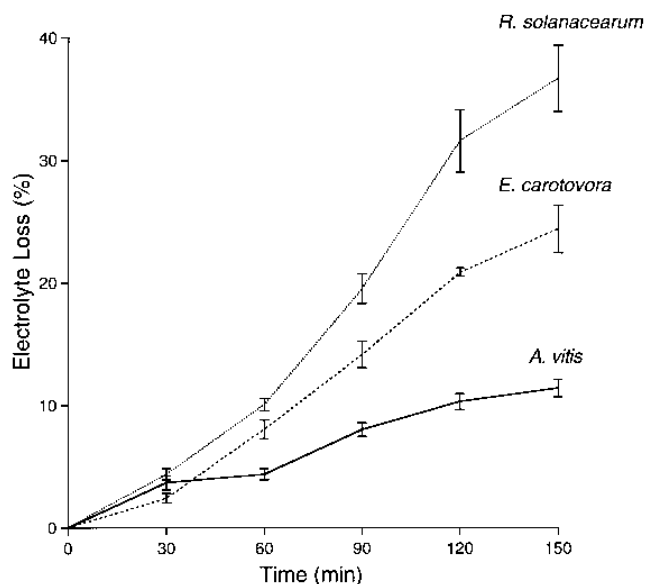


FIG. 5. Comparison of cell damage to potato disks (0.5 by 8 mm) caused by 60-U amounts of recombinant PG from *R. solanacearum*, *E. carotovora*, and *A. vitis*. The data are the means and standard errors (error bars) from three sets of three disks cut from a single potato tuber. Cell damage is expressed as the percentage of total electrolytes released into the bathing medium by three freeze-thaw cycles minus that observed with a heat-denatured enzyme control. Measurements of electrolyte leakage were made every 30 min for 2.5 h by using a conductivity bridge (YSI Instruments, Yellow Springs, Ohio).

obtained in a representative experiment measuring cell damage. The experiment was repeated four times with similar results, although the overall magnitude of electrolyte leakage in each replication varied. For example, the percentage of electrolytes released by *R. solanacearum* PglA varied between 16 and 38%. This variability was probably due to physiological differences in the potato disks.

Our results indicate that the *A. vitis* PG efficiently releases oliguronides from polygalacturonic acid and is less damaging to potato tuber tissue than are the PGs from *E. carotovora* and *R. solanacearum*. Thus, the *A. vitis* PG is similar to *E. chrysanthemi* PelA in having an acidic pI and little effect when applied exogenously to potato tuber tissue but a significant role in pathogenesis (5, 10). Further experiments in which the *A. vitis* *pehA* is replaced with its homologs from *R. solanacearum* and *E. carotovora* will reveal whether the enzymological properties of PehA are important for pathogenesis.

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