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Analysis of the *Pseudomonas solanacearum* Polygalacturonase Encoded by *pglA* and Its Involvement in Phytopathogenicity

MARK A. SCHELL,[1,2]* DANIEL P. ROBERTS,[1,2]† AND TIMOTHY P. DENNY[2]

Departments of Microbiology[1] and Plant Pathology,[2] University of Georgia, Athens, Georgia 30602

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A major endopolygalacturonase excreted by *Pseudomonas solanacearum* was purified to >95% homogeneity and shown to have an isoelectric point of 9.0 and a subunit molecular mass of 52 kilodaltons (kDa). The gene encoding this enzyme (*pglA*) was isolated from a genomic library of *P. solanacearum* DNA based on its expression in *Escherichia coli* and shown to be contained on a 1.8-kilobase DNA fragment. The identity of the *pglA* gene product and the 52-kDa polygalacturonase was demonstrated by immunoadsorption and isoelectric focusing experiments. The cloned *pglA* gene was apparently expressed from its own promoter in *E. coli* and its product was partially secreted into the periplasm. The *pglA* gene was insertionally inactivated in vitro and used to mutate the chromosomal *pglA* gene of *P. solanacearum* by marker exchange mutagenesis. The resulting mutant strain was deficient in production of the 52-kDa polygalacturonase and took twice as long to wilt and kill tomato plants as the wild-type parent in plant bioassay experiments. Complementation in *trans* with the wild-type cloned *pglA* gene restored virulence to near wild-type levels. The data indicate that the *pglA* gene is important, but not absolutely necessary, for pathogenesis.

Pseudomonas solanacearum is a soil-borne plant pathogen that causes important wilting diseases in many crop plants throughout the world (4). Although this pathogen produces several types of cell wall-degrading enzymes (13), it is unclear exactly how and to what extent these enzymes function in disease. Ultrastructure and light microscopy studies have shown that *P. solanacearum* degrades plant cell wall material (13, 16, 40, 42), suggesting that cell wall-degrading enzymes may be involved in the progression of the pathogen through plant tissues (13, 16, 42). Husain and Kelman (13) concluded that cell wall-degrading enzymes may also play a minor role in inducing wilt due to their extensive degradation of the vascular system. Further evidence for the role of cell wall-degrading enzymes in pathogenesis comes from previous marker exchange mutagenesis experiments in which a strain of *P. solanacearum* deficient in β-1,4-endoglucanase production was shown to be less virulent on tomato plants than the wild-type parent (32).

The plant cell wall is a polymeric mesh consisting primarily of cellulose, hemicellulose, pectic substances, and protein (21). Cellulose and hemicellulose are integral components of the cell wall, but pectic substances are located mainly in the outer wall regions within the middle lamella (21). Because they are more exposed than other cell wall components, pectic substances are especially susceptible to enzymatic degradation (5). Therefore, the action of pectin-degrading enzymes may expose underlying structures to other cell wall-degrading enzymes (3, 37). Thus, pectin-degrading enzymes may play a central role in the penetration of plant tissue by *P. solanacearum*.

Pectin-degrading enzymes have been shown to be important in pathogenesis by a number of bacterial plant pathogens (6, 8), most notably the soft rot erwinias. Purified *Erwinia* pectic enzymes macerate plant tissue and cause plant cell death (2, 23), and cloned *Erwinia* pectic enzyme genes confer the ability to macerate plant tissue on the non-plant pathogen *E. coli* (7, 15, 27, 31). Biochemical and genetic data (5) indicate that the excreted *Erwinia* enzymes pectate lyase, polygalacturonase, and pectin methylesterase act in a concerted manner to degrade the pectic portion of plant cell walls and provide nutrition for the pathogen. Dow et al. (11) have shown that mutants of *Xanthomonas campestris* pv. *campestris* that are markedly reduced in the ability to excrete several extracellular enzymes are also nonpathogenic in black rot disease of crucifers. Some *P. solanacearum* strains also excrete polygalacturonase, pectate lyase, and pectin methylesterase (13, 25), and it is possible that these enzymes also act in a coordinate fashion that is important during pathogenesis.

Despite their potential importance in pathogenesis, studies of the pectic enzymes of *P. solanacearum* have been limited to partial physical characterization (13, 25). To better understand the pectic enzymes of *P. solanacearum* and evaluate their importance in pathogenesis, a major excreted polygalacturonase was chosen for study. This enzyme was purified and characterized, and its structural gene (*pglA*) was cloned and used in marker exchange mutagenesis experiments to generate a *pglA*-deficient mutant that was reduced in virulence on tomato plants. Full virulence was restored by complementation in *trans* with the wild-type *pglA* gene. This demonstrates that the *pglA* gene is important, but not absolutely required, for pathogenesis.

MATERIALS AND METHODS

Chemicals. Molecular biology enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), Boerhinger-Mannheim (Indianapolis, Ind.), or New England Biolabs (Beverly, Mass.). Medium components were from Difco Laboratories (Detroit, Mich.). Electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, Calif.). Isoelectric focusing chemicals were from Pharmacia (Piscataway, N.J.). Polygalacturonic acid was purchased from Sigma Chemical Co; before use in activity stain overlays, it was first washed with 0.9 M acetic acid in 70% ethanol. Most

* Corresponding author.
† Present address: Soil-Borne Disease Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description[a]	Source or reference
Strains		
P. solanacearum		
AW	Wild-type race 1 tomato pathogen	34
AR	Spontaneous nonmucoid mutant of *P. solanacearum* AW	34
PG3	*pglA*::*nptI* Km^r	This work
GH-2W	Race 1 tomato pathogen	34
203-1W	Race 2 banana pathogen	34
106-W	Race 1 tobacco pathogen	34
E. coli		
HB101	*hsdS20 recA13 proA2 lacY1 rpsL20*	20
JM83	Δ(*lac-proAB*) *rpsL lacZΔM15*	J. Messing (45)
C600	*thi-1 leuB6 lacY1*	20
JM107	*endA1 hsdR17* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	R. Geever (45)
Plasmids		
pLAFR3	pLAFR1 derivative, Tc^r	J. Jones (12, 36)
pUC4K	Ap^r Km^r	G. H. Lacy (39)
pRK404	Tc^r	G. Ditta (9)
pRK2013	Km^r	G. Ditta (10)
R751	Tp^r	A. Summers (14)
pJE8, pCB15-5, pCB15-4	*pglA*$^+$ Tc^r; Fig. 1	This work
pCB2, pPA56, pTM5, pTM7	*pglA*$^+$ Ap^r; Fig. 1	This work
pPA569	*pglA*::*nptI* Ap^r Km^r	This work
pDW7	*pglA*::*nptI* Tc^r Km^r; Fig. 1	This work

[a] Tc^r, Km^r, Ap^r, and Tp^r designate resistance to tetracycline, kanamycin, ampicillin, and trimethoprim, respectively. *pglA*$^+$ produces PGA.

other chemicals were from Sigma Chemical Co. and were of reagent grade purity.

Bacterial strains and plasmids. Descriptions of bacterial strains and plasmids used are given in Table 1.

Media. Polygalacturonase detection plates (PG plates) were prepared as described previously (1) and developed by flooding with 1% hexadecyltrimethylammonium bromide or 2 N HCl. Basal salts medium (BSM) used for selection in bacterial matings consisted of 50 mM sodium-potassium phosphate, pH 7.0, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgCl_2$, 2 μM $FeSO_4$, 0.2 mM $CaCl_2$, 8 μM Na_2MoO_4, 5 μM $MnCl_2$, and 0.5% glucose. Medium for crude protein preparations for enzyme analysis (EG medium) was 50 mM sodium-potassium phosphate, pH 7.0, 0.07% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.0003% $ZnSO_4$, 0.0005% $Ca(NO_3)_2$, 0.0002% $MnSO_4$, 0.0003% $FeCl_3$, 0.1% dialyzed Casamino Acids, 0.1% dialyzed yeast extract, and 1% sucrose (34). Antibiotic levels used to maintain or select strains and plasmids were kanamycin (50 μg/ml), ampicillin (100 μg/ml), tetracycline (20 μg/ml), or trimethoprim (300 μg/ml).

Construction of plasmids. Cosmid pJE8 was isolated from a previously constructed genomic library (in pLAFR3) of *P. solanacearum* DNA (32) by screening the 1,000 members for *Escherichia coli* JM107 clones exhibiting polygalacturonase activity on PG plates. Plasmid pCB2 (Fig. 1) was constructed by ligating *Eco*RI-digested pJE8 DNA with *Eco*RI-digested pUC9 DNA, transforming into *E. coli*, and screening Ap^r transformants for activity on PG plates. Plasmids pCB15-5 and pCB15-4 were constructed by ligating the 7.6-kilobase (kb) *Eco*RI fragment from pCB2 into *Eco*RI-digested pLAFR3 in both orientations. Plasmid pPA56 was constructed by digesting pCB2 with *Xho*I and *Hin*dIII, converting cohesive ends to blunt ends with DNA polymerase I, and recircularization of the 7.0-kb vector-containing fragment. To construct pTM5 and pTM7, the 1.8-kb *Pst*I fragment from pPA56 was cloned in both orientations into *Pst*I-digested pUC9. To construct pPA569, the ends of the 1.1-kb *Pst*I fragment from pUC4K (containing *nptI* [Km^r]) (39) were made blunt with T4 DNA polymerase, the *Cla*I ends of pPA56 were rendered blunt with DNA polymerase I, and the two resultant fragments were ligated and transformed into *E. coli*. Plasmid pDW7 was constructed by ligating *Pst*I-digested pPA569 DNA with *Pst*I-digested pRK404 DNA, followed by Tc^r Km^r selection.

Bacterial matings and marker exchange mutagenesis. Bacterial matings were performed by growing washed, mid-log-phase *E. coli* and *P. solanacearum* cells together on nutrient agar plates, followed by transconjugant selection on BSM plates as described previously (32). pCB15-5 was transferred into *P. solanacearum* PG3 from *E. coli* JM83 by triparental mating with *E. coli* HB101(pRK2013) followed by Tc^r Km^r selection on BSM plates.

Marker exchange mutagenesis of *pglA* was accomplished by first transferring pDW7 (containing the Km^r determinant from pUC4K [*nptI*] inserted in *pglA*) from *E. coli* C600 into *P. solanacearum* AW by triparental mating with *E. coli* HB101(pRK2013), followed by Km^r Tc^r selection on BSM plates. *P. solanacearum* merodiploid strains that had undergone homologous recombination between the plasmid-borne mutant *pglA* gene and the chromosomal *pglA* gene were selected by conjugation with *E. coli* R751 (Tp^r and incompatible with the pRK404 replicon of pDW7), followed by Km^r Tp^r selection for marker exchange mutants.

Protein preparations and enzyme assays and excretion analysis. Crude intra- and extracellular protein preparations from stationary-phase *P. solanacearum* strains grown at 30°C in EG medium were made as previously described (32) and in some cases concentrated 10-fold with a Spin-vac concentrator. For excretion analysis, *E. coli* and *P. solanacearum* cells were fractionated into cytosolic, periplasmic, and extracellular preparations as described by Yanagida et al. (44).

Polygalacturonase activity was quantified by measuring reducing sugar production (24) in reactions (40°C) containing 0.2% polygalacturonic acid and 50 mM sodium-potassium phosphate, pH 6.5. One unit of polygalacturonase activity is defined as the amount of enzyme that releases 1 μmol of galacturonic acid reducing equivalents per min. Pectate

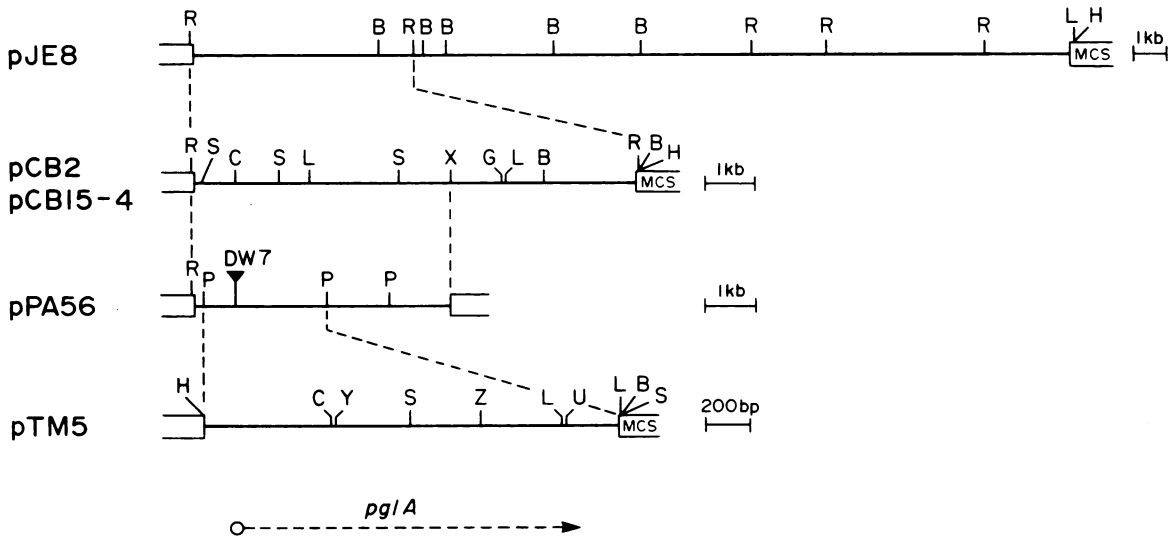

FIG. 1. Physical maps of plasmid inserts containing *pglA*. Restriction endonuclease cleavage sites: B, *Bam*HI; C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; L, *Sal*I; Z, *Sph*I; U, *Stu*I; Y, *Sty*I; S, *Sma*I; P, *Pst*I; R, *Eco*RI; X, *Xho*I. Cloning vectors used were pLAFR3 for pJE8, pCB15-4, and pCB15-5 and pUC9 for pCB2, pPA56, and pTM5. pCB15-5 contains the same insert as pCB15-4 but in the opposite orientation. The solid triangle shows the location of the *nptI* fragment of pUC4K inserted in pPA56 to create the *pglA*::*nptI* insert on plasmids pPA569 and pDW7; pDW7 contains the same *pglA*::*nptI* insert as pPA569 but in a pRK404 vector. Dotted arrow at the bottom illustrates the probable location and extent of the *pglA* transcript. MCS, Multicloning site.

lyase activity was determined by monitoring the increase in absorbance at 235 nm (22) in reactions containing 0.1% sodium polypectate, 50 mM Tris hydrochloride (pH 8.5), and 1 mM $CaCl_2$. Glucose-6-phosphate dehydrogenase was assayed by the method of Langdon (18); β-lactamase was assayed by the method of Schindler and Huber (35). Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as the standard.

Electrophoretic analysis of proteins. Protein preparations were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (17) stained with Coomassie brilliant blue or on isoelectric focusing gels with pH 3 to 10 ampholytes prepared and run as described by Reid and Collmer (29). SDS-polyacrylamide renaturation gels were prepared and run as described previously (7), except that samples were incubated at 37°C for 15 min (not boiled) in sample buffer without β-mercaptoethanol prior to gel loading. Polygalacturonase activity in SDS-polyacrylamide renaturation gels and in isoelectric focusing gels was detected with agarose overlays containing polygalacturonic acid (pH 6.5) and stained with ruthenium red (29).

Purification of polygalacturonase. Four liters of 20 mM sodium-potassium phosphate, pH 7.0, containing 0.1% $(NH_4)_2SO_4$, 0.3% sodium succinate, 0.1% dialyzed yeast extract, and 0.1% dialyzed Casamino Acids was inoculated with *P. solanacearum* AR (10^7 cells per ml) and shaken at 200 rpm at 32°C for 48 h. *Pseudomonas solanacearum* AR (34) was used because it does not produce viscous extracellular polysaccharide, which would complicate purification. Cells were removed by centrifugation at 10,000 × *g* for 20 min, the culture supernatant (CF) was concentrated 60-fold by ultrafiltration, (Amicon, UM10 membrane), and insoluble material was removed by centrifugation at 10,000 × *g* for 15 min. The concentrated CF was dialyzed overnight (1/50 against 5 mM Tris hydrochloride pH 7.0), loaded onto a 100-ml carboxymethyl cellulose column (equilibrated with 5 mM Tris hydrochloride, pH 7.0), washed with 100 ml of 5 mM Tris hydrochloride, pH 7, and eluted with a 500 ml of 0 to 0.25 M NaCl linear gradient in 5 mM Tris hydrochloride, pH 7.0. The main polygalacturonase activity peak eluted at 0.15 M NaCl and was further purified by gel filtration on a Bio-Gel A0.5M column (1 by 50 cm) eluted with 5 mM Tris hydrochloride, (pH 7.0)–0.1 M NaCl.

Immunological methods. Rabbit (New Zealand White) antiserum against the purified polygalacturonase was prepared by previously described methods (33) and analyzed by immunodiffusion (26). For immunoadsorption of the 52-kilodalton (kDa) polygalacturonase, culture supernatants were added to 200 μl of 50 mM sodium-potassium phosphate (pH 7.5)–0.15 M NaCl (PS buffer) containing antiserum and incubated for 1 h at 25°C and then overnight at 4°C. After addition of 20 μl of 10% insoluble protein A (Sigma, P-9151) in PS buffer, incubation at 4°C for 1 h, and centrifugation for 2 min at 12,000 × *g*, unadsorbed polygalacturonase activity in the supernatant was determined.

Analysis of polygalacturonase production in planta. Proteins from tomato plants infected with *P. solanacearum* were prepared by vortexing carefully excised internal sections of plant stems in 0.5 ml of water for 2 to 3 min, incubation at 25°C for 15 min, revortexing, centrifuging at 10,000 × *g* for 10 min, and then concentrating the water extract 10-fold with a Spin-Vac concentrator.

Virulence assays. Growth of Marion tomato plants, stem inoculation with *P. solanacearum* strains, and other experimental details and conditions of the virulence bioassay were described previously (32). A total of 20 plants (in four individual assays) were inoculated per strain at each of two cell concentrations, and the results for each inoculum concentration were analyzed as a single experiment. The plants were coded and scored in a single-blind experiment with a disease index modified from Winstead and Kelman (41): 0, no leaves wilted; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; 4, 76 to 100% of leaves wilted.

Recombinant DNA techniques. Restriction mapping of cosmid DNA was performed by a modification of the Southern cross method (28); restriction mapping of other plasmids was performed by standard methods (20). Methods used for

ligation, restriction enzyme digestion, transformation, filling-in of cohesive ends, plasmid DNA and fragment isolation, and electrophoretic analysis have been described previously (20, 32).

RESULTS

Cloning and analysis of the *pglA* gene. Two polygalacturonase-producing *E. coli* cosmid clones were obtained from a *P. solanacearum* AW cosmid library by screening for polygalacturonase activity on PG plates. The two cosmids contained identical DNA inserts, as determined by restriction endonuclease mapping. One cosmid, pJE8, was selected for further study (Fig. 1).

The location of the *pglA* gene, which encodes the polygalacturonase activity (PGA), on pJE8 was determined by subclone and mutation analysis. Plasmid pCB2, which contained a 7.6-kb *Eco*RI fragment from pJE8, also mediated the production of polygalacturonase in *E. coli* transformants on PG plates. *E. coli* strains containing smaller subclones of this fragment (pPA56 and pTM5; Fig. 1) also had activity on PG plates. Plasmid pPA568, which contained a 2-base-pair (bp) insertion at the *Cla*I site of pPA56 (produced by filling in the cohesive ends with DNA polymerase I and religation), did not mediate the production of PGA. Therefore, the *pglA* gene is totally contained on the 1.8-kb *Pst*I fragment of pTM5 and its coding region spans the *Cla*I site.

The 1.8-kb *Pst*I fragment from pPA56 was inserted in both orientations into pUC9 to make pTM5 and pTM7, both of which mediated the production of PGA in *E. coli*, indicating that the *pglA* promoter was probably located on the 1.8-kb *Pst*I fragment. In addition, the PGA activity produced by plasmid pTM5 was induced over 10-fold by isopropylthiogalactoside (IPTG), whereas the PGA activity of pTM7 was less than that of pTM5 and was not IPTG inducible (data not shown). The higher IPTG-induced levels of PGA associated with pTM5 were probably due to transcription directed by the *lac* promoter on the vector, suggesting the direction of transcription of *pglA* indicated in Fig. 1.

Characterization of the *pglA* gene product. Isoelectric focusing gels coupled with polygalacturonase activity staining revealed a single polygalacturonase species with a pI of 9.0 in culture supernatants from *E. coli* strains containing either pCB15-4 or pJE8 (Fig. 2). Culture supernatants from *P. solanacearum* AW contained an apparently identical pI 9.0 polygalacturonase as well as an additional polygalacturonase with a pI of 8.0 (Fig. 2). Further confirmation that pJE8 and pTM5 contained only a single polygalacturonase-encoding gene (*pglA*) was obtained from SDS-polyacrylamide gel analysis and immunoadsorption experiments. Culture supernatants of *E. coli*(pJE8) and *P. solanacearum* AW each showed only a single polygalacturonase species of 52 kDa molecular mass (52-kDa PGA) (Fig. 3, lanes 1 and 2) when electrophoresed on SDS-polyacrylamide gels, and the location of polygalacturonase activity in the gel was determined by an activity stain overlay. In addition, immunoadsorption experiments with antiserum against the 52-kDa PGA (see below) removed all polygalacturonase activity from *E. coli* JM107(pJE8) and *E. coli* JM83(pTM5) culture supernatants (Table 2). These results indicate that (i) the *pglA* gene encodes a major 52-kDa polygalacturonase (PGA) produced by *P. solanacearum* and (ii) only a single polygalacturonase gene (*pglA*) is contained on cosmid pJE8.

Purification and characterization of a major excreted polygalacturonase. The culture supernatant from *P. solanacearum* AW contained at least two polygalacturonase species

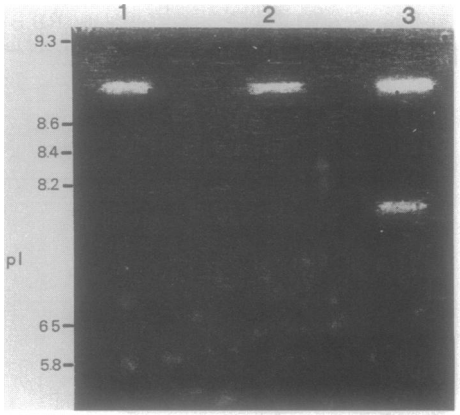

FIG. 2. Isoelectric focusing analysis of cloned polygalacturonase from *P. solanacearum*. Culture supernatants from each strain were concentrated 25- to 75-fold and applied to an isoelectric focusing gel (pH 3.0 to 10.0 ampholytes). After focusing to equilibrium, the gel was analyzed with a polygalacturonase activity stain overlay and photographed. Lane 1, *E. coli* JM83(pCB15-4); lane 2, *E. coli* JM83(pJE8); lane 3, *P. solanacearum* AW. Numbers at left indicate the migration positions of the isoelectric point standards.

(Fig. 2) but no detectable pectate lyases. These polygalacturonases were separated by cation-exchange chromatography. A major polygalacturonase activity peak eluted with 0.15 M NaCl, and a minor polygalacturonase activity peak eluted at approximately 0.10 M NaCl. Further purification of the major polygalacturonase activity by gel filtration on a Biogel A0.5M column resulted in a preparation of a 52-kDa polypeptide that was greater than 95% pure as judged by SDS-polyacrylamide gel electrophoresis (Fig. 4). The final specific activity of this purified polygalacturonase preparation had increased from 20 U/mg in culture supernatants to 140 U/mg for the Biogel-purified PGA. Elution position during gel filtration (ca. 45 kDa) under conditions in which this enzyme was fully active suggest that it is a single-subunit enzyme. The purified polygalacturonase was apparently free of other polygalacturonases, as determined by isoelectric

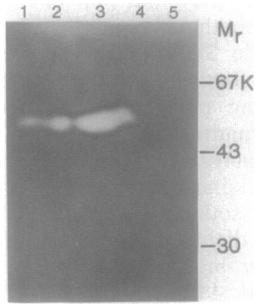

FIG. 3. Analysis of polygalacturonase production by *P. solanacearum* and *E. coli* by activity stain after SDS-polyacrylamide gel electrophoresis. A 50-μl amount of 20-fold-concentrated, dialyzed culture supernatant from each strain was adjusted to 0.1% SDS, incubated at 37°C for 1 h, and electrophoresed on SDS–10% polyacrylamide gel at room temperature. The gel was soaked for 2 h in 50 mM Tris hydrochloride, pH 7.5, to remove SDS and then analyzed for polygalacturonase activity by in situ activity stain overlay. Lane 1, *E. coli* JM107(pJE8); lane 2, *P. solanacearum* AW; lane 3, 1 μg of purified 52-kDa PGA; lane 4, concentrated sterile medium; lane 5, *P. solanacearum* PG3. Numbers at right indicate the migration positions of molecular weight standards (in thousands).

TABLE 2. Immunoadsorption analysis of production of the 52-kDa PGA by *P. solanacearum* and *E. coli* strains[a]

Addition	Residual PGA (U/ml)				
	AW	PG3	PG3(pCB15-5)	JM107(pJE8)	JM83(pTM5)
None	0.70	0.45	0.64	0.22	2.55
AS					
6 μl	0.46	0.46	0.47	<0.02	<0.02
12 μl	0.46	0.45	ND[b]	ND	ND
PI					
6 μl	0.62	0.46	0.65	0.23	2.32
12 μl	0.70	0.45	ND	ND	ND

[a] Culture supernatants from *P. solanacearum* strains AW, PG3, and PG3(pCB15-5) and *E. coli* strains JM107(pJE8) and JM83(pTM5) were incubated with PGA antiserum (AS) or preimmune serum (PI) and then treated with protein A-Sepharose to absorb antibody-bound PGA. After centrifugation, residual PGA not removed by the immunoadsorption was determined.
[b] ND, Not determined.

focusing, and exhibited an isoelectric point of 9.0 (data not shown).

Analysis of the catalytic properties of the 52-kDa PGA indicated that it had a pH optimum of 5.7 and a temperature optimum of 45°C and was inactive above 60°C. The simultaneous measurement of viscosity reduction and reducing sugar production during incubations at 30°C with 0.1% solutions of sodium polygalacturonate showed that the purified PGA enzyme reduced viscosity 50% after a 10-min incubation but that less than 10% of the glycosidic bonds were cleaved (data not shown). This suggests that the 52-kDa polygalacturonase is probably an α-1,4-endopolygalacturonase (EC 3.2.1.15).

Localization of polygalacturonases in *P. solanacearum* and *E. coli*. *P. solanacearum* cells grown to mid-log phase (OD_{600} of 0.5) produced 0.07 U of polygalacturonase activity per ml of culture supernatant; cells grown to stationary phase (OD_{600} of 3.0) produced 0.7 U of polygalacturonase activity per ml in the culture supernatant. In contrast, the intracellular levels of polygalacturonase activity in both cultures were very low (<0.005 U/ml of culture). Since greater than 90% of the polygalacturonase activity was found in the culture supernatant, it is likely that the majority of the

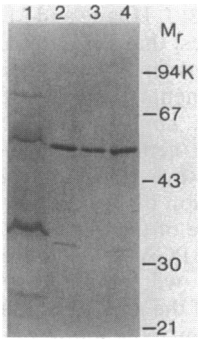

FIG. 4. SDS-polyacrylamide gel analysis of PGA from *P. solanacearum*. Samples from each purification step were denatured and run on an SDS–10% polyacrylamide gel. The gel was stained with Coomassie blue and photographed. Lane 1, 40 μg of protein from concentrated culture supernatant (CF); lane 2, 6 μg of protein from the carboxymethyl cellulose column, 0.15 M NaCl elution peak; lane 3, purified PGA (6 μg of protein) from the Biogel A0.5M column elution; lane 4, 12 μg of protein of the Biogel-purified PGA preparation. The numbers at the right indicate the migration of the molecular weight markers (in thousands).

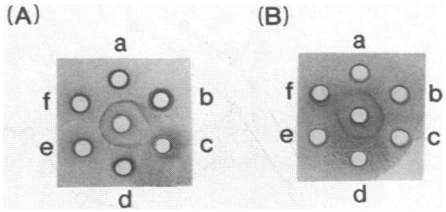

FIG. 5. Coomassie blue-stained immunodiffusion gel analysis of 52-kDa PGA production in vitro and in planta. (A) The center well contains antiserum against the 52-kDa pI 9.0 polygalacturonase. Peripheral wells contained 10 μl of (a) water extract (0.2 ml/g) of tomato plant stem tissue taken from a section 1.0 cm below inoculation site 7 days after inoculation with *P. solanacearum* AW, (b) extract from the same infected plant from a region 15 cm below the inoculation site, (c) water extract (0.2 ml/g of plant tissue) of uninfected tomato plant stem, (d) 20-times-concentrated culture supernatant of *P. solanacearum* AW, (e) 0.5 μg of purified PGA, and (f) 3.0 μg of PGA. (B) Center well contains anti-PGA antiserum. Peripheral wells contained 10 μl of 20-times-concentrated culture supernatants from the following *P. solanacearum* strains (a) 106-W, (b) AW, (c) 203-1W, (d) GH-2W and (f) PG3. Well e contained 0.3 μg of purified 52-kDa PGA.

polygalacturonases of *P. solanacearum* (including the 52-kDa PGA) are excreted (extracellular) proteins. In comparison, 45 and 20% of the PGA activity produced by late-log-phase (OD_{600} of 0.8) *E. coli*(pPA56) cells was found in the periplasmic and extracellular (i.e., culture supernatant) fractions, respectively. Similarly, 74 and 20% of the β-lactamase activity of the same cells was found in the periplasm and culture supernatant, respectively. However, 36% of the PGA activity was found in the cytoplasmic fraction, compared with 5% for the β-lactamase. Greater than 99% of the glucose-6-phosphate dehydrogenase activity was contained in the cytoplasmic fractions. These results suggest that the PGA enzyme is specifically secreted into the *E. coli* periplasm but that excretion into the extracellular medium occurs by nonspecific leakage.

Construction of a *P. solanacearum* strain with a *pglA* mutation. The fact that polygalacturonase can degrade plant cell walls and is produced by a variety of plant pathogens suggests a possible involvement of the *pglA* gene product in pathogenesis by *P. solanacearum*. Immunodiffusion analysis of extracts of tomato plants infected with *P. solanacearum* AW showed that the 52-kDa PGA was produced in the stems of infected plants (Fig. 5A). Further analysis by immunodiffusion showed that the same *pglA* gene product (or a very similar antigen) was apparently produced by different strains of *P. solanacearum* (races 1 and 2) isolated from various geographical regions and host plants (Fig. 5B). This conclusion was also confirmed by Southern hybridization analysis (data not shown).

To assess the importance of the *pglA* gene in phytopathogenicity, a *pglA*-deficient strain of *P. solanacearum* was constructed by marker exchange mutagenesis. To accomplish this, pDW7 (Km^r Tc^r) containing *nptI* inserted in the *ClaI* site of the *pglA* gene (Fig. 1) was allowed to recombine with the chromosomal copy of the *pglA* gene. Marker exchange recombinants were obtained after conjugation of the *pglA*$^+$/*pglA*::*nptI* merodiploid with *E. coli* containing R751 (which is incompatible with pDW7) and selection for Km^r and Tp^r exconjugants. The resultant *pglA*::*nptI* strain produced wild-type levels of extracellular polysaccharide, as judged by colony morphology, but had significantly reduced polygalacturonase activity on PG plates. One colony, designated PG3, was chosen for further study.

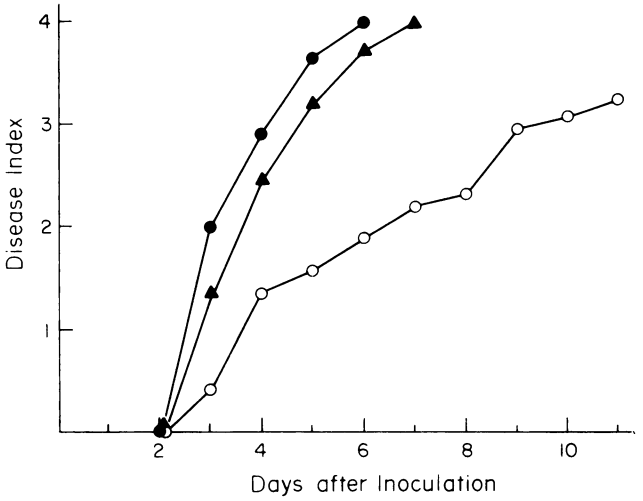

FIG. 6. Plant bioassay of virulence of various *P. solanacearum* strains. Marion tomato plants were stem-inoculated with 10^7 cells of strains AW (●), PG3 (○), and PG3(pCB15-5) (▲). A total of 20 plants were inoculated with each strain during the course of four separate experiments (five plants per experiment). Development of disease was monitored daily and assigned a plant disease index value from 0 to 4 (see text for definitions). Graph points represent the combined average disease index values for all plants inoculated with that strain for all four experiments.

P. solanacearum PG3 extracellular protein preparations contained 0.42 U of polygalacturonase per ml, compared with the wild-type strain that produced 0.74 U of polygalacturonase per ml. Only low levels of polygalacturonase were detected in intracellular *P. solanacearum* PG3 extracts. Only one polygalacturonase species (pI = 8.0) was detected in *P. solanacearum* PG3 extracellular protein preparations (data not shown). Analysis with SDS-polyacrylamide gels and the activity stain overlay showed that the 52-kDa PGA was absent from preparations of the mutant strain (Fig. 3). Furthermore, polygalacturonase activity in culture supernatants from *P. solanacearum* PG3 was not reduced by immunoadsorption with PGA antiserum, whereas activity from wild-type *P. solanacearum* AW was reduced to the level found with the PG3 mutant (Table 2). Immunodiffusion analysis of concentrated culture supernatants from strain PG3 also failed to detect production of PGA (Fig. 5B). In summary, these data indicate that the 52-kDa PGA is not produced by *P. solanacearum* PG3 and that the *pglA* gene was effectively inactivated by marker exchange mutagenesis.

The *pglA* mutation derived by marker exchange was complemented in *trans* by transfer of pCB15-5 (containing *pglA* on a 7.6-kb *Eco*RI fragment) into *P. solanacearum* PG3. This *pglA*$^+$/*pglA*::*nptI* merodiploid strain produced 45% more polygalacturonase activity per ml of culture than did PG3. The extra activity produced by introduction of pCB15-5 was completely removed by immunoadsorption with antiserum against the 52-kDa PGA (Table 2).

Analysis of the importance of the 52-kDa PGA in plant pathogenicity. The rate at which plants wilted and died from *P. solanacearum* infection was affected by the *pglA*::*nptI* mutation. Tomato plants inoculated with 10^7 *P. solanacearum* AW cells reached a disease index rating of 3 (i.e., greater than 51% wilted) after 4 days (Fig. 6). In comparison, tomato plants inoculated with 10^7 cells of *P. solanacearum* PG3 achieved a disease index rating of 3 after 9 days. Complementation of the *pglA* mutation with the 52-kDa PGA-encoding plasmid pCB15-5 produced a merodiploid strain that wilted tomato plants to a disease index rating of 3 ca. 4.5 days after inoculation with 10^7 cells (Fig. 6), nearly identical to wild-type behavior. At lower cell densities, results were essentially the same. Tomato plants inoculated with 10^4 cells of *P. solanacearum* AW wilted to a disease index rating of 3 after 6 days, while plants inoculated with *P. solanacearum* PG3 wilted to a disease index rating of 3 after 11 days (data not shown). When 10^4 cells of *P. solanacearum* PG3(pCB15-5) were used, tomato plants wilted to a disease index rating of 3 after 7 days. Approximately 20% of the tomato plants inoculated with *P. solanacearum* PG3 at either inoculum level failed to develop any visible disease symptoms, while the remaining 80% of the plants eventually wilted and died. In comparison, 100% of the tomato plants were killed when inoculated with 10^7 or 10^4 cells *P. solanacearum* strain AW or PG3(pCB15-5).

The phenotypes of *P. solanacearum* strains PG3 and PG3(pCB15-5) were highly stable in planta as determined by analysis of the antibiotic resistance of cells isolated from infected plants. The growth rate of *P. solanacearum* strains AW, PG3, and PG3(pCB15-5) at 30°C in M9 salts supplemented with 0.05% Casamino Acids and 0.05% sucrose were 1.6, 1.8, and 1.6 h, respectively. Since there was no substantial difference in growth rate between these strains, the decreased virulence of *P. solanacearum* PG3 was not due to a drastic reduction in in vitro growth rate.

DISCUSSION

A major polygalacturonase excreted by *P. solanacearum* was purified and shown to be a 52-kDa single-subunit endopolygalacturonase with a pI of 9.0. The gene encoding this enzyme (*pglA*) was isolated from a cosmid library of *P. solanacearum* genomic DNA and was apparently expressed in *E. coli* from its own promoter at low levels. In addition, the *pglA* gene product (PGA) appeared to be partially secreted into the *E. coli* periplasm, although we could not clearly demonstrate active excretion into the extracellular medium as is found in *P. solanacearum*. In contrast, the *egl* gene product of *P. solanacearum* is not secreted into the periplasm or extracellular medium by *E. coli*, but rather accumulates in the membrane as a higher-molecular-weight precursor protein (32; J. Huang and M. Schell, unpublished). We tentatively suggest that PGA and Egl may be excreted by different mechanisms in *P. solanacearum* and that some biochemical components necessary for excretion of PGA may be present in *E. coli*.

The cloned *pglA* gene was used in marker exchange mutagenesis to make a *P. solanacearum* strain that was deficient in production of the 52-kDa PGA enzyme. Bioassays of the virulence of this mutant *P. solanacearum* strain showed that loss of PGA doubled the time required by the pathogen to totally wilt and kill infected tomato plants. Complementation of the *pglA*::*nptI* mutation in *trans* with a 7.6-kb DNA fragment containing the wild-type *pglA* gene restored virulence of the mutant to near wild-type levels. Therefore, *pglA* (as well as the previously described *egl* gene [32]) of *P. solanacearum* apparently encodes a product that enhances the aggressiveness of the pathogen, since its absence slowed the rate of disease development rather than totally eliminating the ability to wilt tomato plants. Nonetheless, the *pglA*-deficient strain was still capable of killing tomato plants (albeit taking twice as long), indicating that PGA is not absolutely required for disease production.

The observation that the loss of PGA in *P. solanacearum* PG3 had about the same effect on virulence as the loss of Egl (32) was surprising. The 52-kDa PGA accounts for only half of the total polygalacturonase activity produced by *P. solanacearum*, with the remainder of the activity resulting from at least one other species of polygalacturonase. In contrast, Egl is the only detectable endoglucanase activity produced by *P. solanacearum* (32). This suggests that the *pglA* gene product is as important in disease production as the *egl* gene product. However, since we used stem inoculation to measure virulence, the relative role of these gene products during natural infection via the roots may be different. It is also possible that the *egl* and *pglA* gene products may be more important to the disease process in specific types of host plants or under specialized conditions or environments not present in our assay system.

Because neither *pglA* nor *egl* is essential for disease production but each instead enhances pathogenicity, it appears that *P. solanacearum* has a complex mechanism of pathogenesis by which loss of an individual gene product may have only a minor effect on its ability to wilt a host plant. Similar results have been reported for *Erwinia chrysanthemi*, in which deletion of genes encoding four pectolytic enzymes did not completely eliminate its ability to cause soft rot disease (30). Furthermore, Xu et al. (43) have described transposon Tn*5* mutants of *P. solanacearum* that do not produce extracellular polysaccharide slime but can still wilt and kill an infected plant. These data support the suggestion that genes encoding extracellular macromolecules can enhance virulence but that the primary genetic determinants absolutely required for pathogenesis reside in some as yet uncharacterized gene products.

The importance of the *pglA* and *egl* genes to disease, as well as microscopy studies (13, 40) and isolation of Tn*5* mutants reduced in invasiveness, (38), indicate that penetration of plant tissues by *P. solanacearum* may be important in wilt disease. The enzymatic action of PglA and Egl may facilitate penetration of the cortical tissue at wound sites, aid in the progression of the bacterium throughout the plant, or enhance the effect of other pathogenic molecules. It is clear that these proteins do not directly function in nutrition, since *P. solanacearum* AW cannot utilize cellulose, cellobiose, or polygalacturonic acid as sole carbon and energy sources. Precise definition of the molecular role of PGA and Egl awaits comparative physiological, biochemical, and microscopic studies of the effects of *pgl*- and *egl*-deficient *P. solanacearum* strains on tomato plants.

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