MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 5, pp. 293-300, 1990

Inactivation of Multiple Virulence Genes Reduces the Ability of *Pseudomonas solanacearum* to Cause Wilt Symptoms

Timothy P. Denny,[1] Brian F. Carney,[1] and Mark A. Schell[1,2]

Departments of Plant Pathology[1] and Microbiology[2], University of Georgia, Athens 30602 U.S.A.
Received 13 February 1990. Accepted 17 April 1990.

Previous research on *Pseudomonas solanacearum* had shown that endoglucanase (encoded by *egl*), endopolygalacturonase (encoded by *pglA*), and extracellular polysaccharide (encoded by multiple *eps* genes) are each virulence factors involved in wilt disease of tomato. We have now constructed *egl pglA* and *egl eps* double mutants of *P. solanacearum* and compared them to the single mutants for production of extracellular enzymes in culture and for virulence by both stem and root inoculation procedures. The *egl* and *egl eps* mutants produced no endoglucanase activity, but unexpectedly overproduced polygalacturonase (PG) activity by 30 to 140%. Inactivation of *pglA* in either a wild-type or *egl9*::Tn*5* background reduced total PG activity approximately 25 to 50%. Viscometry data indicated that the *pglA* mutants were deficient in endo-PG activity, but retained exo-PG activity. Virulence of the *pglA* and *egl pglA* mutants was not affected when 10^6 bacteria were injected into stems of tomato plants or when 10^9 bacteria were poured onto the soil; when stems were inoculated with 10^4 bacteria of the mutants, the plants wilted 1 to 2 days slower than those inoculated with the parent strains. In contrast, inactivation of *egl* consistently reduced the ability of *P. solanacearum* to wilt tomato plants regardless of the strain, the type of assay, or the inoculum concentration tested. The *egl eps* mutant, which did not wilt plants in two-thirds of the stem inoculation experiments and appeared not to infect plants when added to the soil, was the least virulent of the defined mutants. The root inoculation assay was also used to show the close relationship between the appearance of wilt symptoms and reduced water use by the plant, strengthening the hypothesis that wilt is the result of vascular dysfunction.

Additional keywords: *Lycopersicon esculentum*, marker exchange mutagenesis.

Pseudomonas solanacearum (Smith) Smith probably produces a variety of virulence factors that increase the speed and severity of the wilting disease which appears soon after infection of a susceptible host plant (Buddenhagen and Kelman 1964). Early research results suggested that the extracellular polysaccharide (EPS) slime and the extracellular enzymes which are capable of degrading components of plant cell walls might be important in causing wilt symptoms on young tomato plants (Husain and Kelman 1958a, 1958b; Kelman and Cowling 1965).

We recently showed that two extracellular enzymes, a 43-kDa endoglucanase (EG) and a 52-kDa endopolygalacturonase (endo-PG) encoded by *egl* and *pglA*, respectively, are minor virulence factors. When either of these genes was site-specifically inactivated, the resulting mutants, PS6 (*egl9*::Tn*5*) and PG3 (*pglA1*::*nptI*), wilted tomato plants 55 to 65% slower than did the wild type, but still killed most of the plants within 2 wk (Roberts *et al.* 1988; Schell *et al.* 1988). Another mutant, AW1-1, with Tn*5* in an uncharacterized *eps* gene produces no EPS slime, but is referred to as EPS impaired (EPS^i) because colonies have what is thought to be a thin coating of EPS (Denny *et al.* 1988; T. P. Denny and S.-R. Baek, unpublished). AW1-1 wilted tomato plants 85% slower than did the wild-type parent, and the mutant killed few of the plants during the 2-wk experiments (Denny *et al.* 1988). Each of these mutant strains regains full virulence when it is complemented by the wild-type gene in *trans* or when reverted to the wild type (Roberts *et al.* 1988; Schell *et al.* 1988; T. P. Denny and S.-R. Baek, unpublished). In contrast, spontaneous, pleiotropic mutants of *P. solanacearum* such as AW1-PC that are EPS^- and have less than 5% of normal EG activity (in addition to other phenotypic alterations) do not cause typical wilt symptoms even though they can still infect plants and cause stunting and adventitious root formation (Denny *et al.* 1988; Kelman 1954).

A logical extension of our earlier work was to determine what effect inactivating multiple virulence genes would have on the ability of *P. solanacearum* to wilt tomato plants. A double mutant that is EPS^i and EG^- was of special interest, since it should resemble the spontaneous mutant strain AW1-PC. We also wished to know whether the observed virulence of the mutants would be altered if the bacteria were required to infect plants via the roots. This natural route of infection by *P. solanacearum* appears to require penetration through root tissues (Wallis and Truter 1978), and mutants deficient in cell wall degrading enzymes might be less virulent under these circumstances than when the bacteria are injected directly into stem tissue as in the standard bioassay.

MATERIALS AND METHODS

Bacterial strains and plasmids. Descriptions of the bacterial strains and plasmids used in this study are listed in Table 1. We call the spontaneous change of a wild-type strain such as AW1 to the pleiotropic strain AW1-PC "phenotype conversion", and we refer to these mutants

Address correspondence to T. P. Denny: Department of Plant Pathology, University of Georgia, Athens, GA 30602 U.S.A.

as PC-type strains. Strain AW1-PC was previously designated AW1-A (Denny *et al.* 1988).

Culture conditions. Strains of *P. solanacearum* (stored at −70° C) were routinely grown at 30° C on BGT agar medium or in BG broth (Boucher *et al.* 1985); strains of *Escherichia coli* (Migula) Castellani and Chalmers were grown at 37° C in Luria-Bertani medium (Maniatis *et al.* 1982). Antibiotics were added when necessary as follows: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 25 or 100 μg/ml; kanamycin (Km), 40 μg/ml; nalidixic acid (Nx), 20 or 60 μg/ml; tetracycline (Tc), 15 μg/ml; and trimethoprim (Tp), 200 μg/ml. The lower of the two antibiotic concentrations was used for *P. solanacearum*. BSM plates (Schell *et al.* 1988) were sometimes used to counterselect *E. coli* donors after matings. EG activity was detected on carboxymethylcellulose (CMC) plates (Andro *et al.* 1984; Roberts *et al.* 1988). For enzyme assays, strains of *P. solanacearum* were grown in EG broth with 0.5% sucrose (Schell *et al.* 1988).

Recombinant DNA techniques. Unless otherwise specified, the methods for restriction enzyme digestion, plasmid DNA and restriction fragment isolation, ligation, transformation, and electrophoretic analyses have been described previously (Maniatis *et al.* 1982; Roberts *et al.* 1988; Schell *et al.* 1988).

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Pseudomonas solanacearum		
AW1	Nx^r derivative of wild-type strain AW, PG$^+$ EG$^+$ EPS$^+$	Denny *et al.* 1988
PG3	AW *pglA1*::*nptI* Km^r	Schell *et al.* 1988
PS6	AW *egl9*::Tn*5* Km^r	Roberts *et al.* 1988
PS66	PS6 *pglA1*::CAT Km^r Cm^r	This study
AW1-1	AW1 *eps-1*::Tn*5* EPS^i Nx^r Km^r	Denny *et al.* 1988
AW1-2	AW1-1 *egl3*::Tn*3*-HoHo1 Nx^r Km^r Ap^r	This study
AW1-PC	Spontaneous phenotype conversion mutant of AW1, EPS$^-$ EG^i Nx^r	Denny *et al.* 1988
Escherichia coli		
HB101	*hsdS20 recA13 lacY1 rpsL20*	Maniatis *et al.* 1982
C2110	*polA* Nx^r	Stachel *et al.* 1985
JM83	Δ(*lac-proAB*) *rpsL lacZΔM15*	Yanisch-Perron *et al.* 1985
Plasmid		
pHE3	*egl*$^+$ IncP (*polA*-independent replicon) Tc^r	Roberts *et al.* 1988
pHE31	*egl3*::Tn*3*-HoHo1 derivative of pHE3, Tc^r Ap^r	This study
pTM7	pUC9::*pglA* Ap^r	Schell *et al.* 1988
pMB204	pTM7(*pglA1*::CAT) Ap^r Cm^r	This study
pMB205	pMB204::pRK404	This study
pRK404	Broad host range, Tc^r	Ditta *et al.* 1985
pHoHo1	Tn*3*-HoHo1 donor, *tnpA* ColE1 Ap^r	Stachel *et al.* 1985
pSShe	Transposon helper plasmid, *tnpA*$^+$ ColE1 Cm^r	Stachel *et al.* 1985
pRK2013	Conjugation helper plasmid (*tra*$^+$), ColE1 Km^r	Figurski and Helinski 1979
R751	IncP Tp^r	Jobanputra and Datta 1974

[a] Ap^r, Cm^r, Km^r, Nx^r, Tc^r, and Tp^r designate resistance to ampicillin, chloramphenicol, kanamycin, nalidixic acid, tetracycline, and trimethoprim, respectively. PG, polygalacturonase activity; EG, endoglucanase activity; EPS, extracellular polysaccharide; CAT, chloramphenicol acetyltransferase; and *tnpA*, Tn*3* transposase activity. EPS^i and EG^i, impaired in EPS production and EG activity, respectively.

Generation of double mutants. Marker exchange mutagenesis (Ruvkun and Ausubel 1981) used to generate strain PS66, the *egl pglA* mutant, began with the construction of pMB204 by ligating a chloramphenicol acetyltransferase (CAT) gene cartridge (on a 1.8-kilobase [kb] *Acc*I fragment derived from Tn*9*) into the unique *Cla*I site in the *pglA* gene of pTM7, and selecting Ap-resistant (Ap^r) Cm^r transformants of *E. coli* JM83. Next, pMB205 was created by ligating pMB204 digested with *Hin*dIII and *Bam*HI with similarly digested pRK404, and selecting Ap-sensitive (Ap^s) Cm^r Tc^r transformants of *E. coli* JM83. pMB205 was transferred into *P. solanacearum* PS6 (*egl9*::Tn*5*) by triparental mating with *E. coli* HB101(pRK2013) as a helper, followed by Km^r Cm^r selection. Strains of *P. solanacearum* that had undergone homologous recombination between the plasmid-borne *pglA1*::CAT gene and the genomic *pglA* gene were recovered after mating with *E. coli* HB101(R751) followed by selection for Km^r Cm^r Tp^r on BSM plates (Roberts *et al.* 1988). Colonies were passed on BGT-Cm plates, and the three fastest growing Tc^s colonies were selected for further study.

To create strain AW1-2, the *egl eps* mutant, the *egl* gene on pHE3 was first mutagenized with Tn*3*-HoHo1 essentially as described by Stachel *et al.* (1985). Briefly, *E. coli* HB101 that has the Tn*3*-HoHo1 donor plasmid (pHoHo1) and the transposition helper plasmid (pSShe) was transformed (Cohen *et al.* 1972) with pHE3 plasmid DNA (Table 1). pHE3 was then transferred into *E. coli* C2110 (which is *polA*) by triparental mating followed by Nx^r Tc^r Ap^r selection; only pHE3::Tn*3*-HoHo1 can replicate in C2110 under these selection conditions. Colonies were replica-plated onto CMC plates, and one EG$^-$ derivative, designated pHE31 (*egl3*::Tn*3*-HoHo1), was transferred into *P. solanacearum* AW1-1 (*eps-1*::Tn*5*) by triparental mating followed by Km^r Tc^r Ap^r selection. The marker exchange mutant, AW1-2 (*eps-1*::Tn*5*, *egl3*::Tn*3*-HoHo1), recovered after the transfer of R751 as above, was EPS^i on BGT plates and EG$^-$ on CMC plates.

Enzyme assays. Cultures of *P. solanacearum* were grown for 3 days at 30° C in EG broth without antibiotics. After centrifugation (13,000 × *g*, 5 min, 4° C), the supernatants from 1.0-ml samples were carefully removed and stored at −20° C; the cells were washed twice with 0.1 M NaCl, suspended in 1.0 ml of distilled water, and stored at −20° C. To solubilize the bacteria, 25-μl volumes of the washed cell suspensions were mixed with 75 μl of 1.33% (w/v) sodium dodecyl sulfate (SDS) plus 0.133 M NaOH and heated for 10 min at 65° C. Protein in the solubilized sample was quantified with the BCA reagent (Smith *et al.* 1985; Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol; the standard was bovine serum albumin similarly treated with SDS and NaOH.

The presence of the 52-kDa *pglA* gene product in culture supernatants was determined on SDS-10% polyacrylamide renaturation gels by an *in situ* PG activity stain overlay (Ried and Collmer 1985; Schell *et al.* 1988). Total PG activity was quantified by measuring the reducing sugars (Nelson 1944) released during incubation at 37° C with 0.5% (w/v) polygalacturonic acid (PGA) in 50 mM sodium-potassium phosphate buffer, pH 6.5, plus 2 mM EDTA. PGA (Sigma Chemical Co., St. Louis, MO) was washed

with 0.9 M acetic acid in 70% ethanol before use. EG activity was quantified after incubation at 50° C with 1.5% (w/v) CMC (Sigma) in 100 mM sodium-potassium phosphate buffer, pH 7.0. Insoluble material was removed by low-speed centrifugation before absorbance readings were taken. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μmole of α-D-galacturonic acid or glucose per minute for PG and EG, respectively.

Viscometry. Samples were adjusted to 0.25 U of PG per milliliter by dilution with AW1 culture supernatant that had been heated at 100° C for 15 min to inactivate PG activity. Boiled culture supernatant was used as a diluent to maintain equal amounts of viscous EPS in all samples. Two-milliliter samples were warmed to 37° C in a size 100 Cannon-Fenske-type viscometer (Fisher Scientific, Pittsburgh, PA) held in a temperature-controlled water bath, and 4.0 ml of 0.75% (w/v) PGA in assay buffer (see above), previously warmed to 37° C, was added to initiate the reaction. The time required for the solution to flow through the viscometer was determined at frequent intervals, and the results are reported as a percentage of the maximum viscosity of PGA. The endo-PG enzyme encoded by *pglA* was purified to homogeneity (Schell *et al.* 1988) from a *P. solanacearum* PC-type strain engineered to overexpress this gene more than 100-fold.

Virulence assays. Virulence of the strains of *P. solanacearum* on tomato (*Lycopersicon esculentum* Mill.) cultivar Marion, measured by the rate at which leaves wilted, was assessed using two different inoculation methods. The primary method was the stem inoculation procedure described in detail elsewhere (Roberts *et al.* 1988). In each experiment, three to five plants received approximately 1–5 $\times 10^6$ or 1–2 $\times 10^4$ cells of each strain (in 20 μl of water) via a stab wound in the stem. The number of leaves wilted was recorded for each plant daily, and the percentage of leaves wilted was calculated for each treatment. The data reported are averages of three or more independent experiments. The time required for pairs of strains to cause 25 or 50% wilt was compared using the nonparametric Mann-Whitney U test (Sokal and Rolf 1969).

An alternate inoculation procedure required that *P. solanacearum* infect tomato plants through undisturbed roots. Standard 25-mm diameter glass culture tubes wrapped in aluminum foil were filled three-fourths full with 35 g of a dry, sterile soil-peat-perlite mixture and brought to field capacity by adding 18 ml of water. Single tomato plants were grown in each tube under artificial lighting until they were 8–10 cm tall. Each strain of *P. solanacearum* was tested on 10 seedlings by pipeting onto the soil 1.8 ml of water suspensions containing approximately 1×10^9 cells per milliliter (to give about 10^8 cells per milliliter of soil water). The plants were incubated in a growth chamber (30° C daytime temperature, 25° C nighttime temperature) with a 13-hr photoperiod. The number of leaves wilted was recorded daily, and the rate of wilt was determined as it was for the stem inoculation. Infection of symptomless plants was determined qualitatively at the end of each experiment by checking for growth of *P. solanacearum* in sap squeezed from the base of cut stems onto BGT-Nx plates.

Water consumption. At approximately 24-hr intervals the weight of each tube was recorded before and after the addition of water (or occasionally liquid fertilizer) necessary to reestablish field capacity. The grams of water used each hour were calculated (to correct for variation in the length of the daily interval), and the hourly water consumption 3 days prior to complete wilt was set as 100% for each plant. All of the plants were asymptomatic at this time. The percentage of water use by each plant was then calculated for the period covering 2 days before (days −2 and −1) to 1 day after (day +1) complete wilt (set at day 0) regardless of when during the 14-day experiment wilt occurred.

RESULTS

Construction of double mutants. Specific mutagenesis of the desired genes was accomplished following a standard approach (Ruvkun and Ausubel 1981) in which a cloned gene is first inactivated by insertion of an antibiotic resistance marker gene and then used to replace the wild-type gene in *P. solanacearum*. An *egl pglA* double mutant was created by first inactivating *pglA* with a CAT gene insert and then moving this mutation into strain PS6 (*egl9*::Tn*5*). Three Km^r Cm^r Tc^s strains were examined for PG activity after SDS-polyacrylamide renaturation gel electrophoresis (Schell *et al.* 1988) and found to lack the 52-kDa endo-PG protein encoded by *pglA* (results not shown). Furthermore, Southern blot analysis of *Eco*RI-digested genomic DNA of these mutants showed that the CAT gene was inserted into the 8-kb fragment containing *pglA* (results not shown). We concluded that *egl pglA* double mutants had been generated, and one such isolate, designated PS66 (*egl9*::Tn*5*, *pglA1*::CAT), was selected for further study.

The CAT gene was not expressed well in PS66, so we opted to use Tn*3*-HoHo1 mutagenesis to inactivate *egl* when constructing an *eps egl* double mutant. Of the 480 pHE3::Tn*3*-HoHo1 insertion mutations examined, 15 were EG^- on CMC plates. The *egl3*::Tn*3*-HoHo1 mutation was marker-exchanged into AW1-1 (*eps-1*::Tn*5*) to create the double mutant, AW1-2, which retained the EPS^+ mutant phenotype on BGT plates, was EG^- on CMC plates, and was Lac^+ on BG plates supplemented with X-gal due to fusion of the promoterless *lacZ* gene with *egl*.

Enzyme production in culture. Both the original single mutants and the double mutants were tested repeatedly for PG and EG activity (Table 2). The enzyme levels in culture supernatants are reported as specific activities (that is adjusted for the amount of cellular protein) to account for variation between cultures. The PG activity of PG3 (*pglA1*::*nptI*) was reduced 25–30% as observed previously (Roberts *et al.* 1988). Unexpectedly, the presence of *egl9*::Tn*5* (in PS6) or *egl3*::Tn*3*-HoHo1 (in AW1-2) mutations increased the PG activity 142 and 33%, respectively, when compared to the immediate parent strain. The elevated PG activity in PS6 was reduced 50% by the subsequent inactivation of *pglA* to create PS66 (*egl9*::Tn*5*, *pglA1*::CAT), which had total PG activity similar to the wild type. In contrast to the variation observed in PG activity, the defined single and double mutants were either normal or negative for EG activity. The spontaneous, phenotype conversion mutant, AW1-PC, had the expected

low EG activity (Denny *et al.* 1988), which was near the limits of detection for the assay.

Purified endo-PG enzyme (0.5 U) reduced the viscosity of PGA 50% in 4.5 min by cleaving approximately 1.6% of the glycosidic bonds (Fig. 1). This pattern of digestion is typical of an enzyme that cleaves PGA in an endolytic fashion (Zink and Chatterjee 1985; Crawford and Kolattukudy 1987) and confirms our previous report (Schell *et al.* 1988). AW1 and PS6 (*egl9*::Tn*5*) did not produce pure endo-PGs, because samples of their supernatants with 0.5 U of PG activity reduced the viscosity of the PGA substrate by 50% in about 8 min, and by 70–80% in 30 min. An equal amount of PG activity from PG3 (*pglA1*::*nptI*) and PS66 (*egl9*::Tn*5*, *pglA1*::CAT) reduced the viscosity by less than 20% in 10 min and by only 20–30% in 30 min, suggesting that they produce little or no endo-PG activity.

Table 2. EPS production and enzyme activity of strains of *Pseudomonas solanacearum*

Strain	Mutated loci	Enzyme activity[a] (U/mg) PG	EG	EPS[b]
AW1	None	0.45 ± .06	0.30 ± .02	Wild type
PG3	*pglA*	0.33 ± .02	0.32 ± .03	Wild type
PS6	*egl*	1.09 ± .08	ND[c]	Wild type
PS66	*egl pglA*	0.52 ± .03	ND	Wild type
AW1-1	*eps*	0.51 ± .04	0.30 ± .05	Impaired
AW1-2	*eps egl*	0.68 ± .11	ND	Impaired
AW1-PC	Unknown	0.54 ± .39	0.005 ± .004	Negative

[a] Enzyme-specific activity determined as described in the text. PG, polygalacturonase; EG, endoglucanase. One unit (U) of enzyme activity released 1 μmole of reducing sugar per minute. Cellular protein varied from 0.76 to 1.13 mg per milliliter of supernatant. Enzyme activities are the averages of three to six experiments ± standard deviation.

[b] Phenotype of extracellular polysaccharide (EPS) production on BGT agar plates. EPS-impaired strains produce no fluidal EPS, but they appear to have a coating of EPS, which is different from the EPS-negative strain.

[c] ND, not detected.

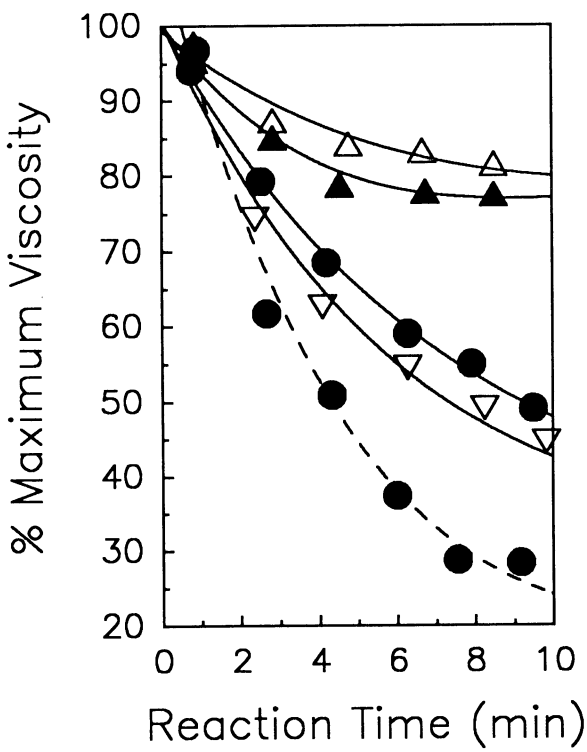

Fig. 1. Change in viscosity of the polygalacturonic acid (PGA) substrate due to action of polygalacturonases (PGs). Reactions contained 6.0 ml of 0.5% PGA and 0.5 units of PG activity from culture supernatants of strains AW1 (●), PG3 (△), PS6 (▽), and PS66 (▲), or from purified endo-PG (●- - - -●). Fully polymerized PGA increased the viscosity of the reaction mixture approximately 22 sec. The results shown are representative of two or more experiments.

Effect of single and double mutations on virulence. When tomato plants were stem inoculated with 1–5 × 10^6 cells of the single mutants (Fig. 2A), all of the mutants except PG3 wilted 50% of the leaves significantly slower ($\alpha \leq 0.025$) than wild-type AW1. The reduced rates at which PS6 (*egl9*::Tn*5*) and AW1-1 (*eps-1*::Tn*5*) induced wilt symptoms appeared to be similar to those observed previously (Denny *et al.* 1988; Roberts *et al.* 1988). In contrast to our earlier results when using a high inoculum level (Schell *et al.* 1988), PG3 (*pglA1*::*nptI*) was consistently as virulent as AW1 in this set of four experiments. It was not surprising then that there was no difference between the *egl* mutant, PS6, and the *egl pglA* mutant, PS66. Unlike with AW1, however, plants inoculated with PS66 routinely had bacterial ooze appear on the surface of unwounded areas of the stems and petioles, and plants inoculated with PG3 occasionally had this. AW1-1 wilted 25% of the leaves significantly faster

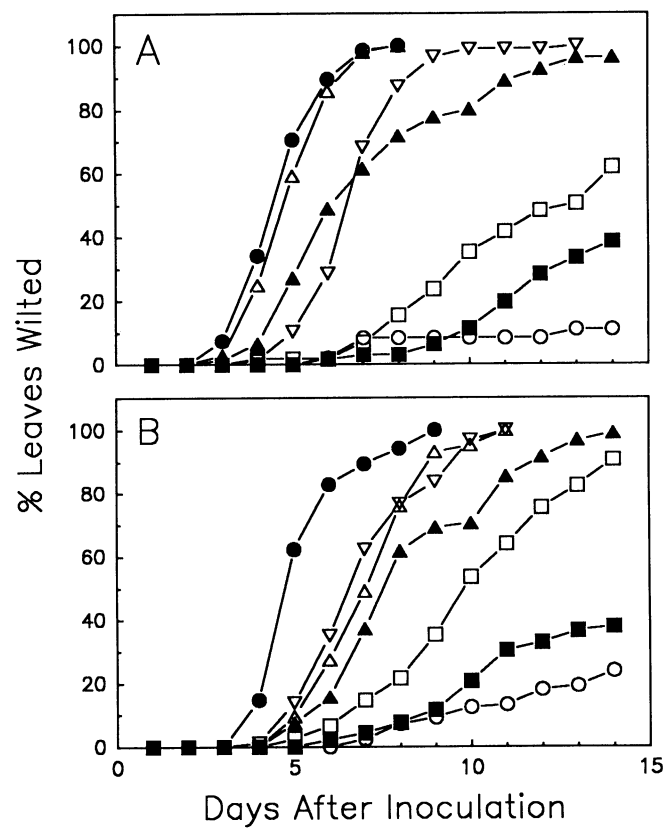

Fig. 2. Virulence of strains of *Pseudomonas solanacearum* on tomato plants when inoculum was applied via a stem wound. **A**, Plants were inoculated with 1–5 × 10^6 cells. **B**, Plants were inoculated with 1–2 × 10^4 cells. AW1, ●; AW1-PC, ○; PG3, △; PS6, ▽ ; PS66, ▲; AW1-1, □; and AW1-2, ■.

($\alpha = 0.1$) than AW1-2 (*eps-1*::Tn*5*, *egl3*::Tn*3*-HoHo1). On average, AW1-2 was more virulent than the PC-type strain, AW1-PC, but in four of six experiments these two strains behaved the same.

Additional stem inoculations were performed to see if a lower inoculum level would enhance the differences between AW1 and the mutants (Fig. 2B). Reducing the inoculum concentration 100-fold had almost no effect on the ability of AW1, which wilted 50% of the leaves significantly faster ($\alpha < 0.025$) than all the other strains. Likewise, there was little change in the ability of PS6 (*egl9*::Tn*5*) or AW1-2 (*eps-1*::Tn*5*, *egl3*::Tn*3*-HoHo1) to cause wilt. In this set of four experiments, PG3 (*pglA1*::*nptI*) was significantly less virulent than AW1, and PS66 (*egl9*::Tn*5*, *pglA1*::CAT) was slightly less virulent than either PS6 or PG3 (but the difference was not significant). AW1-1 (*eps-1*::Tn*5*) caused 25% wilt significantly faster than AW1-2 ($0.05 < \alpha < 0.1$); the difference between AW1-1 and AW1-2 was enhanced in Figure 2B, because AW1-1 wilted tomato plants faster in these experiments at the lower inoculum level than at the higher inoculum level (Fig. 2A). Plants infected with the low concentration of either PG3 or PS66 routinely had bacteria oozing from the stems and petioles, and greasy lesions on petioles and leaflets were also commonly observed.

The ability of *P. solanacearum* to infect and wilt tomato plants via unwounded roots was examined in two experiments by adding water suspensions of the bacteria to the soil of seedlings grown in large glass tubes. In this assay, reduced virulence of the mutants could be manifested by a low percentage of plants becoming infected, a delay in the beginning of wilt symptoms, or a slow rate of wilting. In the first experiment, AW1, PG3 (*pglA1*::*nptI*), and PS6 (*egl9*::Tn*5*) each infected and killed all 10 plants, and similar to the results with stem inoculation, PG3 killed plants almost as fast as AW1, whereas PS6 was distinctly slower (Fig. 3, expt. 1). The PC-type strain, AW1-PC, caused stunting and adventitious root formation on 40% of the plants, but no typical wilt symptoms (data not shown). The remaining plants that were treated with AW1-PC in the first experiment remained asymptomatic, and *P. solanacearum* was not recovered from the cut stems at the end of the experiment, suggesting that the plants had not been infected. For the second experiment, the tomato seedlings were grown under a higher light regime prior to inoculation and were more robust. In this case, PG3 and AW1 infected seven and eight plants, respectively, and caused wilt and death at the same rate (Fig. 3, expt. 2). PS6 was again less virulent, infecting and killing only three plants, and strain PS66 (*egl9*::Tn*5*, *pglA1*::CAT) behaved similarly. AW1-2 (*eps-1*::Tn*5*, *egl3*::Tn*3*-HoHo1) and AW1-PC were also examined in the second experiment, but all of the plants inoculated remained asymptomatic and none of the plants was found to be infected. Therefore, the mutants deficient in either EG alone or EG and EPS appeared to be less virulent than the wild type in the soil inoculation assay primarily because they failed to infect the plants.

Relationship of water use to wilt symptoms. The amount of water consumed by each plant during the root inoculation assays was recorded daily to evaluate whether the onset of wilt symptoms was associated with reduced water use. Only the results for the strains with normal production of EPS (AW1, PG3, and PS6) were analyzed. Plotting the average percentage of wilt and the average percentage of water use together (Fig. 4A) shows that increased wilt was closely matched by decreased water use, but that decreased water use lagged behind the appearance of wilt symptoms. Plotting water use versus wilt for the 124 pairs of data from days −2, −1, and 0 confirmed an inverse linear relationship between these two parameters (Fig. 4B). This treatment of the data, however, shows that some plants were severely or completely wilted while still consuming nearly normal amounts of water, and that a few plants used water at a rate greater than 100% in the 2 days before wilting.

DISCUSSION

It is presumed that phytopathogens rely on a variety of virulence factors during pathogenesis. For example, wilting is thought to be the result of increased resistance to water flow through the xylem due to pathogen-produced cell wall degrading enzymes creating embolisms in vessels and EPS plugging pit membranes (Van Alfen 1989). We previously constructed strains of *P. solanacearum* with single insertion mutations in *egl*, *pglA*, and *eps* genes and showed that their gene products are involved in causing

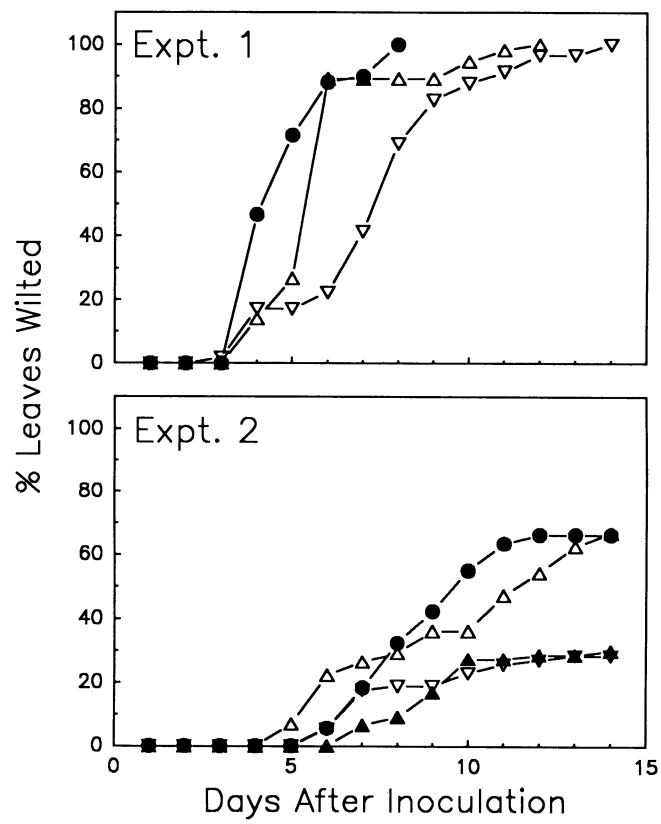

Fig. 3. Virulence of strains of *Pseudomonas solanacearum* on tomato plants when inoculum was added to the soil. Sufficient inoculum was added to give approximately 1×10^8 cells per gram of soil water. Each strain was tested on 10 plants whose roots were not artificially wounded. The results are from two separate experiments (expt. 1 and 2). AW1, ●; PG3, △; PS6, ▽ ; and PS66, ▲.

wilt symptoms on tomato (Denny *et al.* 1988; Roberts *et al.* 1988; Schell *et al.* 1988). When the single mutants were stem inoculated in the present study, strains PS6 (*egl9*::Tn*5*) and AW1-1 (*eps-1*::Tn*5*) behaved the same as previously described. In contrast to prior results, however, at the higher inoculum level (10^6 cells) the single mutant strain PG3 (*pglA1*::*nptI*) was as virulent as the wild-type strain AW1. We believe that this apparently normal virulence of PG3 at a high inoculum level was not seen before because 1) previous experiments were performed during the fall, a season when our tomato plants are less susceptible to wilt by *P. solanacearum* than those tested during the spring and early summer for this work, and 2) this time PG3 killed all of the plants inoculated, whereas in the earlier work 20% of the plants remained asymptomatic (Schell *et al.* 1988). Nevertheless, when plants were inoculated with the lower concentration of PG3 (10^4 cells), either previously or in this study, it consistently wilted plants slower than AW1. Therefore, endo-PG is a virulence factor if plants are not challenged with an overwhelming amount of inoculum, although it seems to contribute less to the ability of *P. solanacearum* AW1 to wilt tomato plants than do EG activity and EPS.

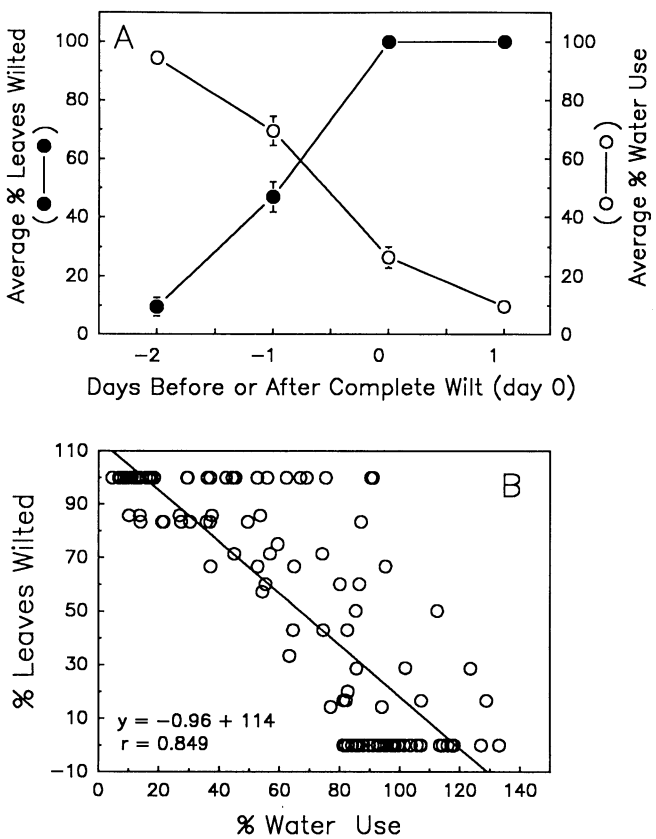

Fig. 4. The relationship between wilt symptoms and water consumption after tomato plants were infected by strains AW1, PG3, and PS6 of *Pseudomonas solanacearum*. Data for water consumption were recorded daily for each plant during the soil inoculation experiments shown in Figure 2. **A**, Results from a 4-day period when the day of complete wilt was set at zero. Percent water use was calculated for each plant relative to its hourly consumption on day −3 when the plants were asymptomatic. The means ± standard error are shown. **B**, Wilt versus water use for the 124 pairs of data from days −2, −1, and 0.

We extended our investigation of the virulence factors of *P. solanacearum* by generating two mutant strains, AW1-2 (*eps-1*::Tn*5*, *egl3*::Tn*3*-HoHo1) and PS66 (*egl9*::Tn*5*, *pglA1*::CAT), that are each deficient in two of the three factors. Simultaneous inactivation of multiple virulence factors has been reported only a few other times (Daniels *et al.* 1988; Kotoujansky 1987; Ried and Collmer 1988). AW1-2 was consistently less virulent than AW1-1 (*eps-1*::Tn*5*), but PS66 was less virulent than PS6 (*egl9*::Tn*5*) only when the inactivation of *pglA* alone had an effect (that is in stem inoculation with 10^4 cells). The effects of the double mutations appeared to be at best additive rather than synergistic. AW1-2 was on average slightly more virulent than AW1-PC, the spontaneous PC-type mutant, but these two strains behaved virtually the same in four of the six separate experiments in which they were both tested. It appears that the simultaneous loss of EPS production and EG activity can, for the most part, account for why PC-type strains like AW1-PC do not cause wilt.

The assays for enzyme production were intended simply to confirm the phenotypes of the double mutants, but uncovered a surprising 30 to 140% increase in PG activity associated with the two mutations in *egl* (*egl3*::Tn*3*-HoHo1 and *egl9*::Tn*5*). Because the two different insertions in *egl* did not increase PG activity equally, it seems likely that this effect was due to the type of insertion element or to their positions within *egl* rather than to inactivation of the *egl* gene per se. This unpredictable increase in PG activity illustrates how even simple mutations can have unintended side effects. The increased PG activity of PS6 did not appear to have affected the spectrum of activity (that is ratio of endo vs exo), because equal units of PG activity from AW1 and PS6 reduced the viscosity of the PGA substrate at the same rate. Therefore, the *egl9*::Tn*5* insertion in PS6 increased the production of all types of PGs equally.

Inactivation of *pglA* in AW1 and PS6 (to create PG3 and PS66, respectively) reduced total PG activity 30 to 50%, and the viscometry data suggest that PG3 and PS66 produced mainly exo-PGs. It is often presumed that cell wall degrading enzymes with exo-activity have minor roles in pathogenesis, because they degrade substrates relatively slowly. However, the importance of pectolytic enzymes cannot always be predicted from studies with model substrates like PGA (Barras *et al.* 1987; Boccara *et al.* 1988). In the case of *P. solanacearum*, it is possible that PG3 and PS66 remained fully virulent when tested at the higher inoculum concentrations, because the large bacterial population produced enough exo-PG activity to compensate for the loss of the endo-PG. In addition, it might be that the oozing of the *pglA* mutants, PG3 and PS66, through unwounded areas of stems and petioles was due to the exclusive production of exo-PGs. That *P. solanacearum* produces several PG isozymes whose individual contribution to virulence varies is reminiscent of the multiple pectate lyase isozymes produced by *Erwinia chrysanthemi* Burkholder *et al.* (Collmer and Keen 1986; Kotoujansky 1987). Inactivation of individual pectate lyase genes in *E. chrysanthemi* may or may not reduce maceration of potato tubers (Roeder and Collmer 1985, 1987; Payne *et al.* 1987) or systemic infection of leaf-inoculated saint-

paulia plants (Boccara *et al.* 1988). These results show that the function and importance ascribed to different types of cell wall degrading enzymes can depend on the bioassay system.

The goal of the soil inoculation assay was to test whether the enzyme-deficient mutants of *P. solanacearum* would be even less virulent than wild-type AW1 when required to infect tomato plants via roots that were not artificially wounded. When the inoculum was poured onto the soil, it was impossible to ensure that the bacteria became evenly distributed or that they encountered a natural wound or site of secondary root emergence which was susceptible to infection. Thus, there was an element of chance as to whether a plant was effectively inoculated. We reduced, but could not eliminate, this inherent source of variation in the soil inoculation assay by using an inoculum concentration high enough to give almost 100% infection with wild-type AW1. However, even if most of the plants were successfully inoculated, it was still uncertain how efficiently the mutant strains of *P. solanacearum* could initiate infection. Consequently, the results for the mutants must be examined within each experiment relative to the wild type. PG3 (*pglA1*::*nptI*) behaved essentially the same as AW1 in the two soil inoculation experiments, both in terms of the percent plants infected and the rate at which plants wilted. The plants successfully infected by PS6 (*egl9*::Tn*5*) and PS66 (*egl9*::Tn*5*, *pglA1*::CAT) in the soil inoculation experiments wilted at a rate relative to AW1 that was about the same as in the stem inoculation assay. Therefore, the rates at which the wild type and the mutants wilted plants were largely independent of whether roots or stems had been infected. However, the EG^- mutants (PS6 and PS66) and, especially, the mutants deficient in EG and EPS (AW1-2 and AW1-PC) generally infected fewer plants than did AW1. These results suggest that EG and EPS are important virulence factors required for successful infection of roots, although reduced survival of the mutants in the soil could be a contributing factor. Understanding the importance of these and other possibilities will require further research.

In addition to testing mutants for virulence, the root inoculation assay gave us the opportunity to examine the relationship between water use and the appearance of wilt symptoms. There are two major hypotheses of how *P. solanacearum* might affect extracellular water potential and thus cause tomato plants to wilt (Van Alfen 1989). The most popular theory is that this bacterium and the EPS slime it produces obstruct the vascular elements and inhibit transpiration, but it is also possible that toxins released by *P. solanacearum* could induce stomatal dysfunction and cause the plant to wilt because it transpires too quickly. We used water consumption as an indirect measure of transpiration to show that there is a strong association between decreased water use and the appearance of wilt symptoms. That decreased water consumption was not observed to precede wilt and that some plants wilted while still using large amounts of water were probably due to the long sampling interval. Whereas many plants went from no symptoms to 50–100% wilt in less than 1 day, their water use was measured for the entire daily interval. Thus, a plant that suddenly wilted near the end of the interval would have already used a nearly normal amount of water. In addition, because so few plants appeared to have increased water consumption just before wilting, we feel that our results provide no good evidence for stimulated transpiration being responsible for wilt. The reduction in water consumption coincident with the appearance of wilt symptoms supports the hypothesis that vascular dysfunction is responsible for the wilt of tomato plants infected with *P. solanacearum*, and is compatible with our genetic studies that implicate EPS as the major wilt-inducing factor (Denny *et al.* 1988; T. P. Denny and S.-R. Baek, unpublished).

ACKNOWLEDGMENTS

We thank Mandhana Bijaisoradat for technical assistance and J. Huang for providing the partially purified endo-PG enzyme.

This research was supported by state and Hatch funds allocated to the Georgia Agricultural Experiment Stations, and by USDA grants 86-CRCR-1-2242 and 87-CRCR-1-2314.

LITERATURE CITED

Andro, T., Chambost, J.-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. J. Bacteriol. 160:1199-1203.

Barras, F., Thurn, K. K., and Chatterjee, A. K. 1987. Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. Mol. Gen. Genet. 209:319-325.

Boccara, M., Diolez, A., Rouve, M., and Kotoujansky, A. 1988. The role of individual pectate lyases of *Erwinia chrysanthemi* strain 3937 in pathogenicity on saintpaulia plants. Physiol. Mol. Plant Pathol. 33:95-104.

Boucher, C. A., Barberis, P. A., Trigalet, A. Ph., and Demery, D. A. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn*5*-induced avirulent mutants. J. Gen. Microbiol. 131:2449-2457.

Buddenhagen, I., and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 2:203-230.

Cohen, S., Chang, A. C. Y., and Hsu, L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69:2110-2114.

Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. 24:383-409.

Crawford, M. S., and Kolattukudy, P. E. 1987. Pectate lyase from *Fusarium solani* f. sp. *pisi*: Purification, characterization, *in vitro* translation of the mRNA, and involvement in pathogenicity. Arch. Biochem. Biophys. 258:196-205.

Daniels, M. J., Dow, J. M., and Osbourn, A. E. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Annu. Rev. Phytopathol. 26:285-312.

Denny, T. P., Makini, F. W., and Brumbley, S. M. 1988. Characterization of *Pseudomonas solanacearum* Tn*5* mutants deficient in extracellular polysaccharide. Mol. Plant-Microbe Interact. 1:215-223.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, W.-X., Finlay, D. R., Guiney, D., and Helinski, D. R. 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid 13:149-153.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Husain, A., and Kelman, A. 1958a. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology 48:155-165.

Husain, A., and Kelman, A. 1958b. The role of pectic and cellulolytic enzymes in pathogenesis by *Pseudomonas solanacearum*. Phytopathology 48:377-386.

Jobanputra, R. S., and Datta, N. 1974. Trimethoprim R factors in

enterobacteria from clinical specimens. J. Med. Microbiol. 7:169-177.
Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.
Kelman, A., and Cowling, E. B. 1965. Cellulase of *Pseudomonas solanacearum* in relation to pathogenesis. Phytopathology 55:148-155.
Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. 25:405-430.
Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
Payne, J. H., Schoedel, C., Keen, N. T., and Collmer, A. 1987. Multiplication and virulence in plant tissues of *Escherichia coli* clones producing pectate lyase isozymes PLb and PLe at high levels and of an *Erwinia chrysanthemi* mutant deficient in PLe. Appl. Environ. Microbiol. 53:2315-2320.
Ried, J. L., and Collmer, A. 1985. Activity stain for rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. Appl. Environ. Microbiol. 50:615-622.
Ried, J. L., and Collmer, A. 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. Mol. Plant-Microbe Interact. 1:32-38.
Roberts, D. P., Denny, T. P., and Schell, M. A. 1988. Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. J. Bacteriol. 170:1445-1451.
Roeder, D. L., and Collmer, A. 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. 164:51-56.
Roeder, D. L., and Collmer, A. 1987. Marker-exchange mutagenesis of the *pelB* gene in *Erwinia chrysanthemi* CUCPB 1237. Pages 218-223 in: Plant Pathogenic Bacteria: Proc. Internatl. Conf. on Plant Pathogenic Bacteria, 6th; 1985 June 2–7; Maryland. E. L. Civerolo, A. Collmer, A. G. Gillaspie, and R. E. Davis, eds. Martinus Nijhoff, Dordrecht.
Ruvkun, G. B., and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.
Schell, M. A., Roberts, D. P., and Denny, T. P. 1988. Analysis of the *Pseudomonas solanacearum* polygalacturonase encoded by *plgA* and its involvement in phytopathology. J. Bacteriol. 170:4501-4508.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.
Sokal, R. R., and Rolf, F. J. 1969. Pages 387–395 in: Biometry. W. H. Freeman and Co., San Francisco.
Stachel, S. E., An, G., Flores, C., and Nester, E. W. 1985. Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium*. EMBO J. 4:891-898.
Van Alfen, N. K. 1989. Reassessment of plant wilt toxins. Annu. Rev. Phytopathol. 27:533-550.
Wallis, F. M., and Truter, S. J. 1978. Histopathology of tomato plants infected with *Pseudomonas solanacearum*, with emphasis on ultrastructure. Physiol. Plant Pathol. 13:307-317.
Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.
Zink, R. T., and Chatterjee, A. K. 1985. Cloning and expression in *Escherichia coli* of pectinase genes of *Erwinia carotovora* subsp. *carotovora*. Appl. Environ. Microbiol. 49:714-717.