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Characterization of *Pseudomonas solanacearum* Tn*5* Mutants Deficient in Extracellular Polysaccharide

Timothy P. Denny, Felister W. Makini, and Stevens M. Brumbley

Department of Plant Pathology, University of Georgia, Athens 30602 U.S.A.
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Pseudomonas solanacearum **produces copious amounts of extracellular polysaccharide (EPS) that may be largely responsible for the wilt symptoms typical of infections by this phytopathogenic bacterium. To test this hypothesis, we generated multiple Tn*5* mutants of strains 82N and AW1 that were either impaired in production of EPS (EPS^i) or were EPS$^-$ in culture, and tested them for a variety of cultural traits, for virulence on tomato, and for EPS production *in planta*. The Tn*5* mutants were compared with the virulent parental strains and their pleiotropic, spontaneous EPS$^-$ mutants that do not wilt tomato. Southern blot analysis confirmed that each mutant contained only one Tn*5* insertion, and transformation with genomic DNA from each Tn*5* mutant demonstrated that each Tn*5* insertion was linked to the EPS deficiency. The mutants of strain 82N varied in the amount of EPS they produced in culture and in their ability to wilt tomato plants. These mutants, however, appeared to be pleiotropic, which precluded making conclusions regarding the involvement of EPS in symptom expression. Four EPS^i mutants of strain AW1 could not be differentiated in culture from the parental strain except by their lack of EPS. Three of these mutants retained their ability to wilt tomato plants, but also produced almost wild-type levels of EPS *in planta*. The fourth EPS^i mutant of AW1 produced little EPS in tomato and was reduced in its ability to cause wilt symptoms. One Tn*5* mutant of AW1, which was EPS$^-$ in culture and *in planta*, mimicked the pleiotropic, spontaneous EPS$^-$ mutant in every way, including the inability to wilt tomato plants. The results from the EPS^i mutants of strain AW1 support the hypothesis that EPS production by this pathogen has a significant role in pathogenesis.**

Additional keywords: *Lycopersicon esculentum*, pleiotropic mutants, Tn*5* mutagenesis.

Pseudomonas solanacearum E. F. Sm., responsible for a number of wilt diseases around the world, is one of the most intensively studied phytopathogenic bacteria. Much of the research concerning this pathogen's interaction with plants has focused on secreted and cell-surface macromolecules that may have a role in pathogenesis. One such macromolecule is the extracellular polysaccharide (EPS) that most wild-type strains produce in large quantities when grown in culture (Buddenhagen and Kelman 1964; Sequeira 1985). The EPS of *P. solanacearum* forms a fluid, amorphous slime layer around the cells that is easily removed by washing with water, rather than being organized in a distinct capsule (Buddenhagen and Kelman 1964; Sequeira 1985).

The EPS produced in culture by wild-type strains is a viscous, high molecular weight, neutral compound, composed of greater than 80% galactosamine that is probably *N*-acetylated (Dudman 1959; Whatley *et al.* 1980; Drigues *et al.* 1985; Akiyama *et al.* 1986). Glucose and rhamnose are the other sugars most frequently reported. The presence of *N*-acetylgalactosamine in a bacterial polysaccharide is unusual, and may be unique (Sutherland 1985). There is no information regarding either the structure of the polysaccharide produced in culture or the nature of the EPS produced by *P. solanacearum in planta*.

Biochemical and physiological investigations into the role of the EPS in pathogenesis have been inconclusive. The hypothesis receiving the most attention is that the production of viscous EPS by *P. solanacearum* in host plants interferes with movement of water through vessels or pit membranes and results in the typical wilt symptoms (Buddenhagen and Kelman 1964; Van Alfen 1982). Kelman (1954) documented that spontaneously occurring nonfluidal variants of *P. solanacearum* do not cause wilt. Despite the ability of these nonfluidal variants to cause stunting, vascular discoloration, and adventitious root formation, they are commonly referred to as being avirulent (Buddenhagen and Kelman 1964; Sequeira 1985). We will refer to the nonfluidal variants as "spontaneous avirulent mutants" (Staskawicz *et al.* 1983) to distinguish them from the Tn*5* induced mutants.

Subsequent research has shown that spontaneous avirulent mutants produce very little or no EPS slime (Husain and Kelman 1958; Drigues *et al.* 1985). However, the EPS$^-$ phenotype can be accompanied by changes in many other phenotypic traits, such as increased motility, piliation and indole acetic acid production, reduced secretion of endoglucanase and pectinase enzymes, and modification of the lipopolysaccharide (Morales *et al.* 1985). Therefore, the inability of the spontaneous avirulent mutants to cause wilt cannot be attributed solely to the loss of EPS production (Buddenhagen and Kelman 1964). The observations that tomato cuttings wilt in culture supernatants, crude EPS preparations, and sap from infected plants (Husain and Kelman 1958; Akiyama *et al.* 1986) provide only indirect evidence to support the role of EPS in wilt. The hypothesis that the EPS of *P. solanacearum* might interfere with attachment of bacteria to plant cells during compatible interactions also has been investigated extensively, but the recent results do not support this idea (Sequeira 1985).

A number of researchers have begun to apply genetic techniques to study the pathogenic mechanisms of *P. solanacearum*. In preliminary research, Staskawicz *et al.*

Present address of second author: National Agricultural Laboratories, P.O. Box 14733, Nairobi, Kenya.

(1983) created an EPS-deficient mutant of strain S82 by IS*50* insertion and found that it was no longer virulent on potato. These results suggest that EPS is important in pathogenesis, but again, because the possibility of a pleiotropic mutation was not considered, the conclusion cannot be made that the loss of EPS production was completely responsible for the change in virulence. Boucher *et al.* (1985, 1987) and Xu *et al.* (1988) used transposon mutagenesis to create nonpathogenic (Vir^-) mutants of the strains GMI1000 and K60, respectively, and subsequently isolated wild-type genes required for pathogenesis and induction of the hypersensitive response. All of the Vir^- mutants of GMI1000 and six of eight Vir^- mutants of K60 produced normal amounts of EPS, indicating that EPS production alone is not sufficient for pathogenesis. Furthermore, mutants of both strains were found that were deficient in EPS production on agar medium but still wilted host plants. Although detailed results were not presented for the EPS^- Vir^+ mutants, these preliminary findings suggest that EPS may not have a major role in pathogenesis (Boucher *et al.* 1985; Xu *et al.* 1988).

Our research with *P. solanacearum* was prompted by the inconclusive and apparently contradictory results regarding the involvement of EPS in inducing wilt. The major weakness of the research reported to date is the poor characterization of the EPS-deficient strains. Although most Tn*5* mutants of *P. solanacearum* contain only a single transposon insertion (Staskawicz *et al.* 1983; Boucher *et al.* 1985; Xu *et al.* 1988), and additional transposition does not appear to be a problem (Boucher *et al.* 1987; Roberts *et al.* 1988), this does not preclude the possibility of a pleiotropic mutation due to effects on genes adjacent to the insertion (Berg *et al.* 1980). Furthermore, it is possible that EPS production could be affected indirectly by mutations in unrelated pathways or processes that are required for pathogenesis.

We selected a large number of independent Tn*5* mutants of two strains of *P. solanacearum*, one of which (strain 82) was the same as used by Staskawicz *et al.* (1983). In addition to assaying for the ability to wilt tomato plants, the amount of EPS produced in culture and *in planta* was quantified, and several additional cultural characteristics were examined. Many of the mutants were pleiotropic, but the results for the remaining EPS-deficient mutants support the hypothesis that production of EPS by *P. solanacearum in planta* may be responsible for wilt symptoms.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. Bacterial strains are listed in Table 1. The spontaneous nalidixic acid (Nal) resistant strains (Miller 1972) were EPS^+ and fully virulent on tomato plants. Typical spontaneous avirulent mutants were generated during still culture (Kelman and Hruschka 1973). *Escherichia coli* strain SM10(pSUP2021) was grown on Luria-Bertani (LB) agar medium (Maniatis *et al.* 1982) supplemented with ampicillin (Ap, 100 μg/ml) and kanamycin (Km, 50 μg/ml), or in LB broth with one-half the antibiotic concentrations. *P. solanacearum* was routinely grown at 28–30° C on BGT medium (Boucher *et al.* 1985) containing 1.6% agar and Nal (20 μg/ml) or with Nal and Km. When appropriate, 25 μg/ml of Km was add to BG broth. Dialyzable BG broth was prepared by dissolving the peptone, yeast extract, and casamino acids in a small volume of water and dialyzing three times in 330 ml of water (4° C for 24 hr each), pooling the 1 L of dialysate, adding glucose, and autoclaving. Minimal medium contained 1× M9 salts (without $CaCl_2$ in all cases) (Miller 1972), 0.2% glucose, and 1.5% agar. For isolation of DNA and the determination of extracellular enzyme activity, *P. solanacearum* cells were grown in broth containing 1× M9 salts, 0.1% yeast extract, and 0.5% (v/v) glycerol. Generation times were determined according to Miller (1972) by serial plating on BGT medium from log phase cultures of cells growing in 1× M9 salts plus 0.05% casamino acids and 0.05% sucrose.

Transposon mutagenesis. The transposon Tn*5* was introduced into *P. solanacearum* on the suicide vector pSUP2021 during conjugation with the *E. coli* donor by a modification of the method of Boucher *et al.* (1981). Donor and recipient strains were grown in appropriate broth media at 30° C to an $OD_{600} = 1.0$. Donor cells were washed twice with water in a microcentifuge, mixed with recipient cells at a 1:5 ratio (v/v), and the mixed cells were pelleted. The cell pellet was suspended in water at one-fifth its original volume, and 50 μl drops were applied to BGT plates lacking antibiotics. After incubating at 28° C for 16 hr, the cells in each spot were suspended in 0.5 ml sterile water and 0.1-ml samples spread on BGT selection plates containing Nal and Km. The selection plates were incubated for 48 hr, and putative EPS-deficient colonies, which appeared red or purple rather than white, were streaked for single colonies, restreaked, and then stored at −80° C in 15% glycerol (Silhavy *et al.* 1984). The number of *P. solanacearum* recipient cells was determined for nine matings by plating dilutions on BGT plus Nal, and the efficiency of mutagenesis calculated as the number of mutants per recipient. To test for spontaneous Km resistance, mock matings with *P. solanacearum* were performed without adding the *E. coli* donor.

Table 1. Bacterial strains[a]

Designation	Relevant characteristics	Origin
Pseudomonas solanacearum		
82	EPS^+ wild-type potato isolate	L. Sequeira
82N	EPS^+ Nal^r derivative of 82	This work
82N-A	EPS^- Nal^r, spontaneous avirulent mutant	This work
82N-1	EPS^- 82N::Tn*5* Nal^r Km^r	This work
82N-2, 82N-3, 82N-7, 82N-10, 82N-31	EPS^i 82N::Tn*5* Nal^r Km^r	This work
AW	EPS^+ wild-type tomato isolate	Schell 1987
AW1	EPS^+ Nal^r derivative of AW	This work
AW1-A	EPS^- Nal^r, spontaneous avirulent mutant	This work
AW1-1, AW1-41, AW1-71, AW1-74	EPS^i AW1::Tn*5* Nal^r Km^r	This work
AW1-80	EPS^- AW1::Tn*5* Nal^r Km^r, spontaneous avirulent mimic	This work
Escherichia coli SM10(pSUP2021)	Ap^r Cm^r Km^r, Tn*5* donor	Simon *et al.* 1983

[a] Abbreviations: EPS, extracellular polysaccharide; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid.

DNA manipulations and bacterial transformation. Total DNA was isolated from *P. solanacearum* cells grown to stationary phase in medium selected to reduce EPS production. Cells were washed twice with water to remove most of the EPS, and then DNA was isolated by the procedure of Silhavy *et al.* (1984) with the modifications previously described (Denny *et al.* 1988). For Southern blot analysis, DNA was digested to completion with *Eco*RI or *Bam*HI, electrophoresed through a 0.7% agarose gel, and transferred to a nitrocellulose membrane (Smith and Summers 1980). The 3.4 kilobase pair *Hind*III internal fragment of Tn*5*, which includes 320 base pairs of each IS*50* element, was isolated from an agarose gel, nick-translated to incorporate ^{32}P-dATP (Maniatis *et al.* 1982), and used as a hybridization probe as described previously (Denny 1988). Tn*5* DNA will be contained in one or two restriction fragments when genomic DNA containing a single insertion of this transposon is completely digested with *Eco*RI or *Bam*HI, respectively (Berg and Berg 1983). Transformation of *P. solanacearum* with the DNA from the Tn*5* mutants was performed essentially as described by Boucher *et al.* (1985). The results of Boucher *et al.* (1986) suggest that the Tn*5* and flanking genomic DNA recombines site-specifically during this process, and we have confirmed this specificity in our laboratory.

Production of EPS in culture. The *P. solanacearum* strains were examined to determine if EPS was present as a capsule by removing cells from BGT plates and examining smears with Tyler's modification of Anthony's method (Conn *et al.* 1957). *Klebsiella pneumoniae* was used as a positive control.

The viscosity of culture supernatants was an indication of the relative amount of EPS produced in culture. Cells were removed by centrifugation from cultures grown 3 or 4 days in BG broth (with antibiotics), and the viscosity of the supernatant, measured in seconds, determined with a size-100 Fenske-Ostwald viscometer. Measurements were made at room temperature and corrected for the viscosity of water. In some experiments cells were grown in BG broth containing a crude extract of tomato stems. The extract was prepared by blending stem tissue with water (about 1 g/ml), removing most of the insoluble material by centrifugation (48,000 × *g* for 30 min), and filter sterilizing the supernatant (0.45 μm filter with a Whatman GF/A prefilter). The complete medium was prepared by mixing equal volumes of the plant extract and autoclaved 2× BG broth.

The concentration of nondialyzable hexosamines in culture supernatants was estimated with a modified Elson and Morgan reaction (Gatt and Berman 1966). Cells were grown in dialyzable BG broth for 3 days at 28° C, and the culture supernatant collected after the cells were removed by centrifugation at 13,800 × *g* for 30 min. The cell pellet was washed once with water (one-half the original volume), and the supernatant from the wash combined with the culture supernatant. The samples were then loaded into dialysis bags (12,000 M_r cut-off) and dialyzed against large volumes of water at 4° C for 3 days. Appropriately diluted samples (0.45 ml) were mixed with 0.15 ml of concentrated HCL, hydrolyzed at 110° for 30 min in sealed tubes (Akiyama *et al.* 1986), and the colormetric assay performed. The results were read at OD_{530}, the background due to residual media components subtracted, and the hexosamine concentration determined from an *N*-acetylgalactos amine standard curve. The *N*-acetylgalactosamine standards were subjected to the entire analysis procedure beginning with the hydrolysis step.

Hydrophobicity and agglutination assays. Hydrophobicity of bacterial cells can be inferred from the extent that organic solvents decrease the turbidity of cell suspensions when the cells partition into the organic phase (Lichtenberg *et al.* 1985). Cells of *P. solanacearum* grown in BG broth were tested by vortexing 3-ml bacterial suspensions (with a predetermined OD_{600} between 0.5 and 1.0) with 3 ml of xylene or hexadecane, allowing the phases to separate, and measuring the change in OD_{600} of the aqueous phase. Autoagglutination of *P. solanacearum* cells (Sequeira 1985) was evaluated by preparing cell suspensions in water (OD_{600} = 1) from cultures grown on BGT agar plates, adding 0.5 M $CaCl_2$ to a final concentration of 10 mM, and rating the extent of agglutination after more than 4 hr at room temperature without agitation. Autoagglutination in the presence of Ca^{2+} has been associated with increased piliation of some spontaneous avirulent mutants (Sequeira 1985).

Enzyme assays. The enzyme activities of polygalacturonase and endoglucanase in culture supernatants were quantified by the method of Nelson (1944). Polygalacturonase assays containing 4 mg/ml of polygalacturonic acid (previously washed with 0.9 M acetic acid in 70% ethanol), 40 mM phosphate buffer (pH 6.3), 2.0 mM EDTA, and 20% (v/v) culture supernatant were conducted for 2 hr at 37° C. Endoglucanase assays containing 4 mg/ml of carboxy-methylcellulose, 120 mM phosphate buffer (pH 7.0), and 20% (v/v) culture supernatant were conducted for 4 hr at 50° C. One unit of enzyme activity was defined as releasing 1 nmole/min of α-D-galacturonic acid or glucose for polygalacturonase or endoglucanase, respectively.

Virulence assays. Tomato (*Lycopersicon esculentum* 'Marion') plants were grown and stem inoculated as described in detail elsewhere (Roberts *et al.* 1988). Each of three to five plants received approximately 5×10^6 cells in 20 μl of water, or 20 μl of water alone, applied into a stab wound in the stem. After inoculation, the plants were incubated at 30° C in a growth chamber. Virulence, as measured by the degree of wilt, was recorded for each plant during daily examinations with a wilt index as follows: 0 = no leaves wilted, 1 = 1–25% leaves wilted, 2 = 26–50% leaves wilted, 3 = 51–75% leaves wilted, and 4 = 76–100% leaves wilted. The average daily wilt index for each treatment was calculated for each experiment, and the averages of three or more experiments reported.

Characterization and quantification of hexosamines produced *in planta*. Stems of tomato plants infected with *P. solanacearum* or treated with water were cut off at ground level and the leaves were removed. Stems were weighed and chopped into small pieces with a razor blade, and each sample was homogenized with 8 ml of water in a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) for 30 sec while maintaining the sample at about 4° C. Plant debris was removed by centrifugation at 13,800 × *g* for 30 min at 4° C, and five volumes of 95% ethanol were added immediately. The sample was held overnight at 4° C, and the precipitate collected by centrifugation as above. A volume of water equal to the original volume of supernatant was thoroughly mixed with the pellet, the sample was incubated in a 65° C water bath for 5 min, and insoluble material removed by centrifugation. The final supernatant, which contained ethanol-precipitable polysaccharides, was stored at 4° C.

Hexosamines present were quantified by the Elson and Morgan reaction as described above. Extracts from tomato plants treated with water served as the background control.

Hexosamines in the plant extracts were characterized by gas-liquid chromatography (GC) as follows. Ethanol precipitable polysaccharides were prepared as above but dissolved in water at 5% of the original volume. The samples were hydrolyzed with HCL as for the Elson and Morgan reaction, and the HCL removed by evaporation during three cycles of alternately adding 0.5 ml of water and drying the samples under a stream of air. The alditol acetate derivatives were prepared (Albersheim *et al.* 1967) and, after decomposition of the acetic anhydride by shaking with 1 ml of water, derivatized sugar residues were removed from interfering compounds by five extractions with benzene (1:1, v/v). The benzene fractions were pooled, evaporated to dryness, and dissolved in 100 μl of chloroform immediately before analysis of 5 μl samples on a 1.5-m, 0.32-cm inner diameter glass column packed with 3% Silar 10C (Milton Roy Laboratory Group, State College, PA). The injector was at 260° C, the flame ionization detector at 240° C, and the column temperature programmed to increase from 220° C to 255° C at 2° C per min. The linear velocity of the nitrogen carrier gas was 1.4 cm/sec. Internal standards were used to identify the glucosamine and galactosamine peaks.

RESULTS

Transposon mutagenesis. The 18 matings performed between *E. coli* SM10(pSUP2021) and *P. solanacearum* strain 82N generated Km^r colonies at an average frequency of 1.8×10^{-7} per recipient. Nine of the approximately 5,000 Km^r colonies examined were deficient in EPS production on BGT selection plates. Mutagenesis of AW1 occurred at a frequency of 1.1×10^{-7} per recipient, and 10 EPS-deficient colonies were selected from the 8,500 Km^r colonies examined from 84 matings. Spontaneous Km^r colonies of *P. solanacearum* appeared at a frequency of less than 10^{-9}. Some matings produced multiple EPS-deficient colonies, but Southern blot analysis showed that three mutants of 82N and five mutants of AW1 contained Tn*5* in the same restriction fragments as a strain given in Table 1, and these duplicates were discarded. All of the mutants studied except for 82N-1, 82N-2, and 82N-3 were from separate matings. All of the Km^r EPS-deficient mutants in Table 1 were prototrophic when tested on minimal medium, did not express either of the other two antibiotic resistance markers on pSUP2021, and had generation times in broth culture that were not significantly different from the EPS^+ parents (Table 2).

Colony morphology. Colonies of the parental strains 82N and AW1 grown on BGT plates were round or irregular in shape, opaque, and white with pink or red centers. Both strains produced large amounts of opaque white slime, but strain 82N appeared to produce less than did AW1. Colonies of 82N-A and AW1-A, the spontaneous avirulent mutants, were round, opaque with a narrow clear margin, and dark red or purplish red, respectively; these strains were classified as EPS^- (Tables 1 and 2). Colonies of the mutants 82N-1 and AW1-80 were indistinguishable from the spontaneous EPS^- mutants. The remaining four Tn*5* mutants of AW1 were all round, opaque, grayish lavender with a narrow white margin, glistening, and often slightly domed. When viewed through the agar, colonies of these mutants were dark purplish red with a white margin. Even though strains AW1-1, AW1-41, AW1-71, and AW1-74 did not produce slime, they appeared to have retained some EPS production on BGT plates (Table 2) and were classified as impaired in EPS production (EPS^i) (Table 1). These four mutants did not produce slime even in areas of confluent growth. The EPS^i mutants of strain 82N were variable in appearance and slime production on BGT plates (Table 2). Except for strain 82N-2, isolated young colonies of the EPS^i mutants of strain 82N resembled the EPS^i mutants of AW1. However, some white slime was observed in areas of confluent growth of strain 82N-10, and mature colonies of strains 82N-3 and 82N-31

Table 2. Selected cultural characteristics of *P. solanacearum* parental strains, spontaneous avirulent mutants, and Tn*5*-induced, EPS-deficient mutants[a]

Strain	Generation time (hr)[b]	EPS rating[c]	Viscosity (sec)[d]	Hexosamine concentration (μg/ml)[e]	Agglutination by $CaCl_2$	Enzyme activity (U/ml)[f]	
						PGA	EGL
82N	1.7 ± 0.3	+++	106.0 a	798.9 ± 189.2	−	1.5 b,c	19.5 a
82N-A	Nt	−	2.8 e	4.8 ± 2.9	+	2.4 b,c	0.6 b
82N-1	1.8 ± 0.2	−	4.4 e	6.7 ± 6.5	+	24.3 a	1.3 b
82N-2	1.7 ± 0.3	±	33.3 d	98.6 ± 11.5	±	3.5 b,c	22.1 a
82N-3	1.8 ± 0.2	++	60.5 b,c	638.0 ± 64.0	−	8.1 b	18.4 a
82N-7	2.0 ± 0.2	+	5.8 e	4.2 ± 2.5	+	22.1 a	18.4 a
82N-10	1.5 ± 0.1	+±	72.6 b	689.7 ± 62.4	−	Nd	Nd
82N-31	1.7 ± 0.3	++	46.9 c,d	736.7 ± 124.2	−	Nd	21.6 a
AW1	1.5 ± 0.4	+++	116.9 a	332.6 a	−	68.5 b	17.7 a
AW1-A	Nt	−	9.3 b	21.2 b	+	108.3 a	2.0 b
AW1-1	1.4 ± 0.1	+	4.3 b	6.0 c	−	85.2 a,b	15.3 a
AW1-41	1.1 ± 0.1	+	8.4 b	26.6 b	−	75.0 a,b	17.3 a
AW1-71	1.4 ± 0.3	+	10.4 b	29.4 b	−	98.9 a,b	15.7 a
AW1-74	1.1 ± 0.3	+	6.0 b	25.4 b	−	79.0 a,b	19.1 a
AW1-80	1.1 ± 0.1	−	4.5 b	19.3 b,c	+	105.7 a	2.3 b

[a] Values followed by the same letter were not significantly different in the Waller-Duncan multiple range test ($P = 0.05$). Other values are ± SD. Nt, not tested. Nd, not detected.
[b] Averages of two experiments.
[c] Ratings of EPS produced by colonies growing on BGT plates: +, mucoid; −, nonmucoid; ±, mucoid in areas of confluent growth.
[d] Time for supernatants of cultures grown in BG broth to pass through a size-100 viscometer; averages of four experiments.
[e] Dialyzed culture supernatants assayed with an Elson and Morgan reaction. Results for 82N and mutants are from one experiment, and results for AW1 and mutants are from two experiments.
[f] PGA, polygalacturonase; EGL, endoglucanase. One unit (U) released 1 nmole/min of product. Averages of three experiments.

were moderately mucoid. Isolated colonies of strain 82N-2 were similar to 82N-A, but a small amount of transparent slime was seen where colonies of 82N-2 were close together. The only remarkable differences observed when the strains were grown on BG plates (i.e., without tetrazolium chloride) were that colonies of all the EPS$^-$ mutants were transparent and pale tan, whereas all of the other strains studied were opaque and white.

Genetic analysis of the EPS deficient mutants. Southern blots of total DNA from the mutants were probed with the internal *Hind*III fragment of Tn*5* (Fig. 1). This probe did not hybridize to either of the parental strains or their spontaneous avirulent mutants (data not shown). Digestion of DNA from the mutants with *Eco*RI or *Bam*HI generated one or two hybridizing restriction fragments, respectively, as would be expected for a single insertion of Tn*5*. The additional, fainter bands present in the *Bam*HI digests of some strains were partial digestion products. All of the *Eco*RI fragments, which ranged in size from 6.7 kb to greater than 23 kb, were larger than the size of Tn*5* alone (5.7 kb). Two mutants of 82N and four mutants of AW1 appeared to have Tn*5* in the same 8 kb and 25 kb *Eco*RI fragments, respectively, but the results of the *Bam*HI digestions showed that all of the mutants had unique Tn*5* insertions. However, the two strongly hybridizing *Bam*HI fragments of strains AW1-41, AW1-71, and AW1-74 all had the combined size of 8.2 kb, suggesting three independent insertions in one *Bam*HI restriction fragment.

The same DNA used in the Southern blot analysis above was also used in transformation experiments to test the linkage of the Tn*5* insertions with the phenotypic changes observed for the mutants. Competent cells of the parental strains were incubated with total DNA from the mutants and then spread on BGT Km plates. All of the Km^r transformants had also acquired the EPS-deficient colony morphology of the mutant strain from which the transforming DNA originated. Selected transformants were tested for production of viscous EPS in broth culture and extracellular polygalacturonase and endoglucanase activity and found to be indistinguishable from their analogous mutants. Southern blot analysis demonstrated that, for the three mutants of AW1 examined, the transformants had Tn*5* insertions in identical *Eco*RI and *Bam*HI fragments (Fig. 2).

Production of EPS in culture. No capsules were detected for any of the *P. solanacearum* strains when grown on BGT plates. Because even the EPS^i strains apparently had only EPS that was loosely attached to the cell, the viscosity of the culture supernatants are likely to be directly related to the amount of EPS produced by each strain. Viscosity

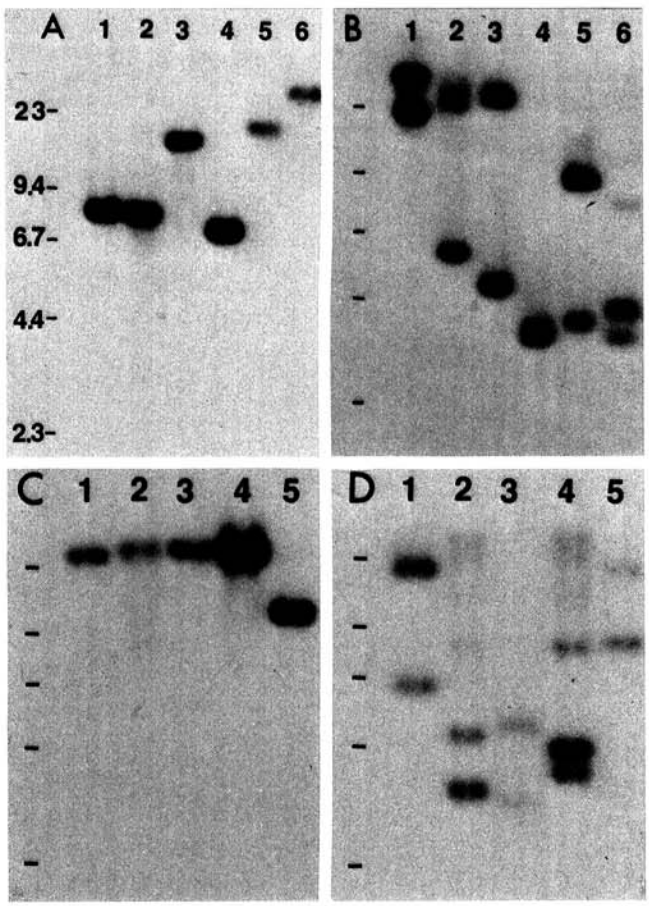

Fig. 1. Characterization of the Tn*5* insertions in the *P. solanacearum* EPS-deficient mutants by Southern blot analysis. Total genomic DNA was isolated from mutants of strain 82N (A and B) or strain AW1 (C and D) and digested with either *Eco*RI (A and C) or *Bam*HI (B and D) before electrophoresis, transfer to nitrocellulose, and hybridization to the *Hind*III fragment of Tn*5*. **A** and **B**, lane 1, 82N-1; lane 2, 82N-2; lane 3, 82N-3; lane 4, 82N-31; lane 5, 82N-7; lane 6, 82N-10. **C** and **D**, lane 1, AW1-1; lane 2, AW1-41; lane 3, AW1-71; lane 4, AW1-74; lane 5, AW1-80. Lambda DNA digested with *Hind*III was used as the DNA size marker (given in kilobases).

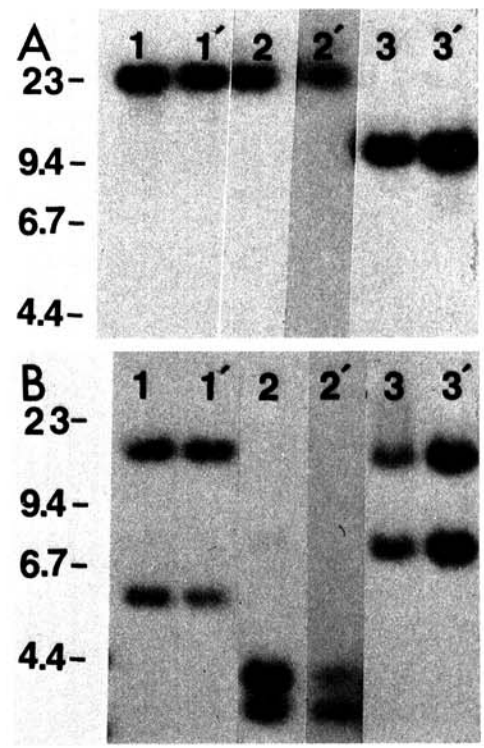

Fig. 2. Southern blot analysis of the Tn*5* insertions in selected mutants of *P. solanacearum* and strain AW1 that had been transformed with DNA from EPS deficient mutants. **A**, Total DNA from strains AW1-1, AW1-74, and AW1-80 (lanes 1, 2, and 3, respectively) and from their corresponding transformed strains (Lanes 1′, 2′, and 3′, respectively) digested with *Eco*RI. **B**, Total DNA from the same strains as in A, but digested with *Bam*HI. The probe was the *Hind*III fragment from Tn*5*.

measurements reliably separated the parental strains from all of the EPS-deficient mutants (Table 2), and reflected the intermediate production of EPS (seen on agar medium) by strains 82N-2, 82N-3, 82N-10, and 82N-31. None of the other EPS^i mutants, however, could be differentiated from the EPS$^-$ strains by using this procedure. The addition of an aqueous extract of tomato stems to the broth medium had no effect on the viscosity of stationary phase cultures (data not shown).

A preliminary GC analysis showed that the EPS from strains 82N and AW1 contained primarily galactosamine with minor amounts of rhamnose and glucose. Hexosamines in the culture supernatants were quantified with the Elson and Morgan colormetric assay, which is specific for these sugars (Gatt and Berman 1966). The moderate to large amounts of hexosamines produced by the EPS^i mutants 82N-2, 82N-3, 82N-10, and 82N-31 supported the conclusion that these strains still produced varying amounts of EPS on agar medium and in broth culture. In agreement with the viscosity data, the EPS^i mutants AW1-41, AW1-71, AW1-74, and 82N-7 produced the same low amount of hexosamines in culture as the EPS$^-$ mutants, and the mutant AW1-1 produced distinctly less hexosamines than any other mutant of strain AW1. The difference in colony morphology between these EPS^i mutants and the EPS$^-$ strains was, therefore, not explained by these results.

Additional cultural characteristics. A further attempt was made to differentiate between the EPS^i and EPS$^-$ mutants by using additional cultural characteristics. Neither the parental strains nor the mutants were measurably hydrophobic. In contrast, some of the mutants autoagglutinated after the addition of $CaCl_2$. The biochemical basis of the agglutination was not determined, but the assay clearly separated the EPS^i and the EPS$^-$ mutants of AW1 (Table 2). The assay was not particularly helpful for the strains of 82N because, although the three EPS^i mutants that retained substantial EPS production were not agglutinated, the other two mutants could not be clearly distinguished from the EPS$^-$ mutant.

We were concerned that an EPS^i phenotype might be due to a general deficiency in protein secretion, so we measured the activities in culture supernatants of polygalacturonase (PGA) and endoglucanase (EGL), two secreted enzymes (Schell 1987; Schell *et al.* 1988) (Table 2). Spontaneous avirulent strains generally secrete reduced amounts of EGL (Kelman and Cowling 1965; Schell 1987), and this was true for strains 82N-A and AW1-A. All of the EPS^i mutants of 82N, except 82N-2, had enzyme activity patterns that were different from 82N, with almost all combinations of increased or decreased activity observed. The two EPS$^-$ mutants of strain 82N had similarly low EGL levels, but 82N-1 had 10 times the PGA activity of 82N-A. In contrast, all four EPS^i mutants of AW1 were essentially the same as the parental strain, and the two EPS$^-$ mutants of strain AW1 were alike in having slightly increased PGA activity and substantially reduced EGL activity.

Virulence assays. None of the mutants were able to wilt tomato plants as rapidly as the parental strains (Fig 3). The EPS^i mutants of strain 82N varied considerably in the rate at which they caused wilt symptoms, but most were almost as virulent as the parent strain. All of these mutants except 82N-10 eventually killed all of the inoculated plants during the course of the 14-day assay. The least virulent strains were the two EPS$^-$ mutants, 82N-A and 82N-1. Although about 35% of the leaves of plants inoculated with these mutants eventually died, the plants did not exhibit typical wilt symptoms. In some cases the one or two affected leaves slowly became chlorotic and desiccated without ever really wilting. Some of the other plants developed a stem rot centered at the point of inoculation that caused the leaves above that point to wilt. We believe that the stem rot was the result of secondary pathogens, but the data from these plants was still included in the results. All of the plants inoculated with 82N-A and 82N-1 were stunted, and adventitious root buds appeared on large areas of the stems.

Similar to the results for mutants of 82N, three of the four EPS^i mutants of AW1 were almost as virulent as the parent strain and killed all of the inoculated plants. However, strain AW1-1 was distinctly less virulent than the other AW1 EPS^i mutants. Some plants infected with AW1-1 slowly developed severe wilt symptoms, although only one of 10 died during the assay period, whereas others wilted only a little, but were stunted and developed many adventitious root buds. Like strain AW1, all four of the EPS^i mutants of this strain caused the pith of infected plants to become soft

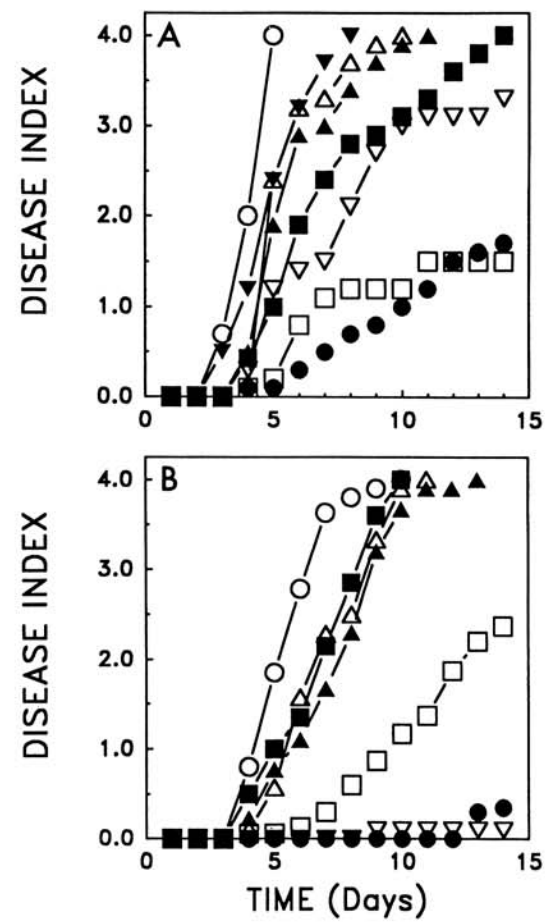

Fig. 3. Virulence of *P. solanacearum* strains on tomato plants. In each experiment three to five plants were stem inoculated with each strain at day 0. The degree of wilt was rated by using the disease index described in the text, and daily averages were calculated. The results shown are the averages of three or more experiments. **A**, strains 82N (o), 82N-A (●), 82N-1 (□), 82N-2 (■), 82N-3 (△), 82N-7 (▲), 82N-10 (▽), and 82N-31 (▼). **B**, Strains AW1 (o), AW1-A (●), AW1-1 (□), AW1-41 (■), AW1-71 (△), AW1-74 (▲), and AW1-80 (▽).

and discolored. The EPS⁻ mutants, AW1-A and AW1-80, did not wilt any plants and killed very few leaves, but both stunted inoculated plants and induced extensive development of adventitious root buds.

Pure cultures of *P. solanacearum* were easily recovered from stem tissue infected with AW1 or the EPS^i mutants of AW1 when samples were streaked on BGT plates with antibiotics. Each stem yielded cultures that contained colonies like those of the strain used as the inoculum and colonies that resembled spontaneous EPS⁻ (avirulent) mutants. No colonies with a mucoid phenotype were recovered from any of the plants inoculated with AW1 EPS^i mutants. In one experiment, samples from two plants killed by AW1-41 were also streaked on BGT plates without antibiotics. Almost pure cultures of *P. solanacearum* were recovered, but no mucoid revertants were observed (frequency less than 0.1%).

Production of hexosamine containing polysaccharide *in planta*. The final step in analysis of the EPS-deficient mutants of AW1 was to determine if they produced EPS in infected tomato plants. Plants were harvested after they were completely wilted or after 14 days. For strains AW1-1, AW1-80, and AW1-A, which did not kill the plants, particular care was taken to ensure that the plants had been extensively colonized by these mutants. In the first experiment, extracts from three stems were pooled before precipitation, and hexosamines were quantified with an Elson and Morgan reaction and also characterized by GC analysis. In the second and third experiments, samples prepared from individual stems were analyzed only with the colormetric assay. Hexosamines were not detected in extracts from healthy tomato plants (data not shown). Hexosamines were consistently recovered from plants wilted by AW1 (Table 3), and GC analysis indicated a composition of 83% galactosamine and 17% glucosamine. Similar amounts of hexosamine also were recovered from plants wilted by the EPS^i mutants AW1-41, AW1-71, and AW1-74, and shown to consist of 69–75% galactosamine, with the remainder as glucosamine. Much less hexosamine was recovered from plants infected with the EPS^i mutant AW1-1, and even less from the EPS⁻ mutants AW1-80 and AW1-A, and galactosamine made up only 34–42% of these samples. Even plants in which AW1-1 had macerated stems and caused almost complete wilt had low amounts of hexosamines.

Table 3. Amount of hexosamines recovered from stems of tomato plants infected with strain AW1 of *P. solanacearum* or its mutants[a]

Strain	Hexosamine (μg/g of tissue)[b]			
	Expt. 1	Expt. 2	Expt. 3	Average
AW1	238	90	138	155
AW1-A	14	6	Nt	10
AW1-1	28	39	14	27
AW1-41	149	161	77	129
AW1-71	148	62	97	102
AW1-74	61	110	73	81
AW1-80	7	6	Nt	7

[a] Hexosamines were precipitated from aqueous extracts of stem tissue by the addition of ethanol and quantified with an Elson and Morgan reaction. Results from the individual experiments are given to show the full extent of the variation observed.

[b] Values are the averages from three to five infected stems in each experiment. Stem samples were combined before analysis in experiment 1. Nt, not tested.

DISCUSSION

A number of Tn*5* mutants of *P. solanacearum* were generated that produced less EPS in culture than the wild-type parents. Southern blot analysis showed that each strain had a single copy of Tn*5*, and transformation experiments demonstrated that the changes in phenotype were the result of Tn*5* insertions. The most common mutation was to a phenotype that was considered to be impaired in EPS production (EPS^i) rather than EPS⁻. Some mutants of strain 82N were observed to produce EPS on agar medium only in areas of high colony density or when the culture was incubated for an extended period of time. In this case, misleading conclusions would have been made if only isolated young colonies had been examined for EPS production. A single Tn*5* mutant of each strain used in this study appeared identical to the spontaneous avirulent, EPS⁻ mutants. Careful examination of colony morphology and other cultural characteristics indicated that the EPS^i strains are different from the EPS⁻ strains. The spontaneous mutation of the EPS^i mutants of AW1 to an EPS⁻ form (without loss of Km^r) confirmed that these are two distinct phenotypes.

Other researchers have stated that their Tn*5* mutants resemble spontaneous avirulent mutants on agar medium (Boucher *et al.* 1985; Staskawicz *et al.* 1983; Xu *et al.* 1988). Without more complete descriptions of their mutants, however, we cannot tell if their strains are like our EPS^i or EPS⁻ mutants. That spontaneous mutants of *P. solanacearum* can have a variety of colony morphologies (Husain and Kelman 1958; Buddenhagen and Kelman 1964; Denny, unpublished observations), and presumably produce different amounts of EPS, further complicates the comparison of our work to that of others.

The results for the EPS^i mutants of the two strains must be considered separately from each other and from the results for the EPS⁻ mutants. The EPS^i mutants of strain 82N varied greatly in the amount of EPS that they produced in culture and also varied in the other cultural characteristics examined, suggesting that they are pleiotropic mutants. There was no consistent correlation between the amount of EPS produced and the additional phenotypic changes. The altered expression of the secreted enzymes is particularly significant, because the level of PGA and EGL activity can affect the virulence of *P. solanacearum* (Roberts *et al.* 1988, Schell *et al.* 1988). There did not appear to be a correlation between the ability of the EPS^i mutants of 82N to wilt tomato and the production of EPS in culture (production of EPS *in planta* was not examined), but due to the multiple phenotypic changes of these mutants, no conclusions can be made regarding the role of EPS during pathogenesis by strain 82N.

The results with the EPS^i mutants of strain AW1 were more informative. All four mutants produced uniformly low amounts of EPS *in vitro* regardless of the culture conditions and could not be differentiated from the EPS⁻ mutants in this regard. In addition, none of the EPS^i mutants were significantly different from the wild type based on the other cultural characteristics, although they all had slightly elevated levels of PGA activity. The EPS^i mutants AW1-41, AW1-71, and AW1-74 were almost as virulent on tomato as the wild type, but infected plants also had almost wild-type amounts of galactosamine containing polysaccharide.

We believe that the amount of ethanol precipitable galactosamine is indicative of the level of EPS produced *in planta* because the amount of galactosamine recovered was strain dependent, whereas the recovery of glucosamine was constant. No mucoid *P. solanacearum* were recovered from plants infected with these EPS^i mutants, indicating that there was not contamination or reversion of the mutants *in planta*. Furthermore, no revertants of the EPS^i mutants to the wild type were found after they were grown without antibiotics in culture conditions that favor the generation of spontaneous avirulent mutants (Denny, unpublished results). Therefore, it appears that the production of EPS by AW1-41, AW1-71, and AW1-74 may be induced *in planta*. The almost identical behavior of these three mutants is in agreement with the genetic analysis that suggested they all may have Tn*5* inserted in the same *Bam*HI fragment. Steinberger and Beer (1988) have similarly reported finding mutants of *Erwinia amylovora* that are EPS$^-$ in culture but appear to produce EPS *in planta*.

The EPS^i mutant AW1-1 was much less virulent on tomato than the other EPS^i mutants. Its reduced ability to wilt tomato plants was not due to mutation to EPS$^-$ forms during pathogenesis, because comparable numbers of EPS$^-$ forms were recovered for the other three EPS^i mutants of AW1 that remained highly virulent. It also does not appear likely that failure to multiply *in planta* can explain the reduced virulence of AW1-1. The induction of adventitious root buds over much of the stem, the high numbers of bacteria recovered in numerous random samples of infected stems, and the oozing of bacteria from the base of cut stems (10 cm from the inoculation site) all suggest that AW1-1 grows well in tomatoes. These observations are supported by a preliminary experiment, which showed that strain AW1-1 is not affected in its ability to colonized tomato stems (Denny, unpublished observation). Besides its greatly reduced virulence, the only way that AW1-1 was observed to differ from the other EPS^i mutants was that it consistently produced less than one-fourth as much hexosamine both in culture and *in planta*. The correlation between the severity of wilt symptoms and the amount of EPS produced *in planta* by the EPS^i mutants of AW1 supports the hypothesis that EPS production has an important role in pathogenesis. Further genetic studies of these mutants will be required to confirm this conclusion.

Two Tn*5* mutants, 82N-1 and AW1-80, were considered to be EPS$^-$. Besides the differences in colony morphology, their autoagglutination by $CaCl_2$ and reduced secretion of EGL activity distinguished the EPS$^-$ mutants from the EPS^i mutants in culture. These traits are associated with the spontaneous mutation to the avirulent, EPS$^-$ phenotype (Schell 1987; Sequeira 1985). It was not surprising, therefore, to find that strain AW1-80 was identical to AW1-A (the corresponding spontaneous EPS$^-$ mutant) in every cultural characteristic examined, and only the elevated PGA activity secreted by 82N-1 differentiated this strain from 82N-A. The pairs of EPS$^-$ mutants of strains AW1 and 82N were also similar in their inability to wilt tomato plants, and AW1-80 and AW1-A produced the least EPS *in planta* of any strains tested. There was the possibility that a random Tn*5* insertion could have occurred at the same time as a spontaneous mutation to the EPS$^-$ phenotype. This was shown not to be the case for AW1-80, because DNA from this mutant transformed *P. solanacearum* strain AW1 to the typical EPS$^-$ phenotype. The Tn*5* induced EPS mutants are another example of a pleiotropic mutation in *P. solanacearum*, and as such, the inability to wilt tomato plants cannot be attributed only to poor EPS production.

The genetic basis of the spontaneous mutation to the EPS$^-$, avirulent form is unknown. Although the mutant has not been observed to revert to the wild type, which suggests that DNA is deleted, no evidence for a deletion has been found (Morales *et al.* 1985; Schell *et al.* 1987). The Tn*5* insertions in the EPS$^-$ mutants AW1-80 and 82N-1 could be directly responsible for the multiple phenotypic changes observed or might have inactivated a locus that regulates the spontaneous event. In either case, it appears that the loss of gene function at a single locus can mimic the spontaneous mutation. Furthermore, now that the region involved in this process has been tagged by Tn*5*, it can be cloned and analyzed to determine the genetic basis for the spontaneous mutation.

This study showed that, unless great care is taken to characterize EPS-deficient mutants of *P. solanacearum* both in culture and *in planta*, it would be easy to make a misleading conclusion concerning the involvement of EPS in pathogenesis. Our results with EPS-deficient Tn*5* mutants of *P. solanacearum* strain AW1 suggest that EPS production in tomato plants is required for typical wilt symptoms. Further proof of this conclusion and insight into the exact role of the EPS will require additional genetic, biochemical, and physiological research.

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