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Genetic and Biochemical Characterization of a *Pseudomonas solanacearum* Gene Cluster Required for Extracellular Polysaccharide Production and for Virulence

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Infection of host plants by *Pseudomonas solanacearum* results in wilting, which is thought to be due largely to the occlusion of xylem vessels by the *P. solanacearum* extracellular polysaccharide (EPS) that primarily consists of *N*-acetylgalactosamine (GalNAc). By means of Tn*3* mutagenesis, we identified a 6.5-kb gene cluster that contains five complementation units required for EPS production and virulence in this bacterium. There was positive correlation between the amount of EPS produced in culture and (i) in planta growth and (ii) virulence. Based on analysis of β-glucuronidase–gene fusions, these genes are expressed both in broth cultures and in planta and may be constitutive. Both wild-type and mutant strains contained similar amounts of UDP-GalNAc, the predicted primary substrate for EPS synthesis. Thus, the EPS mutants we obtained should be useful in the analysis of steps in the assembly of the polysaccharide and how this process is related to virulence.

Virulence in *Pseudomonas solanacearum* (E.F.Sm.) is a complex and multifaceted phenomenon. Most of the known or presumed virulence factors in *P. solanacearum* are extracellular products, including the plant cell wall-degrading enzymes polygalacturonase (3, 27) and endoglucanase (26) and the plant hormones auxin, cytokinin, and ethylene (9). Although this array of extracellular products would seem to constitute a formidable arsenal for virulence, the most intensively studied virulence factor from *P. solanacearum* is a relatively inert product, extracellular polysaccharide (EPS) (1, 11, 13, 19, 31, 36, 38). This high-molecular-weight polymer is thought to be responsible for induction of the wilt symptoms that characterize the disease caused by *P. solanacearum*.

Wild-type virulent strains of *P. solanacearum* produce EPS that forms a loosely associated slime both in culture and in planta. Since the pathogen is limited primarily to plant xylem vessels, EPS is thought to cause wilt symptoms by obstructing water flow. This was first suggested by Hussain and Kelman (19), who observed that EPS-containing culture supernatants could wilt tomato cuttings and that spontaneous mutants lacking EPS were nonvirulent. The mechanism of wilt induction may involve occlusion of xylem pit membranes (34). However, EPS may also contribute to virulence by other means. For example, Young and Sequeira (38) observed that fimbrae of *P. solanacearum* are rapidly agglutinated by plant cell wall fragments and the EPS can prevent this agglutination. It is possible, therefore, that EPS prevents binding of bacteria to the plant cell wall and thereby facilitates systemic movement of the bacterium.

Very little is known about the structure of EPS of *P. solanacearum*. Akiyama et al. (1) suggested that EPS is a homopolymer of *N*-acetylgalactosamine (GalNAc). Glucose and rhamnose have been reported to be minor components (1, 11, 13), but the presence of these sugars is probably the result of incomplete purification. Instead, two amino sugars, bacillosamine (2,4-diamino-2,4,6-trideoxyglucose) and galactosaminuronic acid, probably are integral components of the *P. solanacearum* EPS. The latter sugar is apparently responsible for the acidic nature of the molecule (33a).

Most of the early work on the relationship of EPS to virulence in *P. solanacearum* was based on spontaneous EPS$^-$ mutants. However, interpretation of results obtained with these mutants is confounded by the fact that such mutants are pleiotropic and are affected in other characteristics that also are likely to be important for virulence (9, 25). More recently, Denny et al. (11) obtained Tn*5* mutants of *P. solanacearum* with two distinct colony morphologies: those producing no visible EPS (EPS$^-$) and those producing an intermediate, EPS-impaired colony morphology (EPS^i). Virulence was impaired to differing degrees in both of these mutant types. Although colorimetric analysis of culture supernatants for total hexosamine content did not reveal a clear and consistent difference between EPS$^-$ and EPS^i strains, culture supernatants of both mutant types were generally less viscous and contained fewer nondialyzable hexosamines than wild type, suggesting that both classes represent legitimate EPS mutants. The occurrence of two types of morphologically distinct EPS mutants has also been reported for *Erwinia stewartii* (12).

Although most of the current evidence suggests that EPS is important for virulence of *P. solanacearum*, a recent report of EPS$^-$ mutants that retain nearly wild-type levels of virulence (36) poses a serious challenge to this hypothesis. Thus, it is possible that EPS is not strictly required for virulence of wild-type strains of *P. solanacearum*, but this conclusion needs to be examined further because these EPS$^-$ virulent mutants have not been characterized fully and may themselves have a pleiotropic phenotype. For example, overproduction of polygalacturonase and endoglucanase is associated with the EPS$^-$ phenotype of some mutants and the corresponding increase in maceration potential may be sufficient to produce disease symptoms, particularly in stem inoculation assays. Critical evidence for the role of EPS in

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virulence of *P. solanacearum* requires a more detailed characterization of both virulent and nonvirulent EPS-affected mutants. The purpose of this study, therefore, was to analyze in more detail some of the genes required for both EPS production and virulence. To this end, we have used saturation mutagenesis to study the region of the genome tagged by Tn*5* in the EPS$^-$ nonvirulent mutants KD500 and KD600 (37), which map to the same 12.5-kb *Eco*RI fragment (37a). We have characterized the resulting mutants chemically, by EPS and nucleotide sugar content, and by their virulence in a normally susceptible host.

MATERIALS AND METHODS

Bacterial strains and plasmids. The origins and characteristics of bacterial strains and plasmids used in this study are listed in Table 1.

Media and antibiotics. *P. solanacearum* strains were cultured routinely at 28°C in liquid CPG medium (17) or on TZC medium (CPG containing 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride) (21). Minimal medium was one-fourth-strength M63 (5) with 0.4% glycerol and 0.5% glucose. *Escherichia coli* strains were grown on LB medium (24) at 37°C. The following antibiotics were added when required: kanamycin (Km), 25 μg/ml; ampicillin (Ap), 50 μg/ml; tetracycline (Tc), 15 μg/ml; and nalidixic acid (Nal), 20 μg/ml.

DNA manipulations. General procedures for DNA manipulations, including agarose gels electrophoresis, Southern blotting, nick translation, and plasmid DNA isolation, have been described elsewhere (5, 23). Chromosomal DNA was isolated from *P. solanacearum* as described previously (10). Hybridization of Southern blots and subsequent washes were completed by the method of Amasino (4) except that the hybridization solution was modified to omit NaCl and polyethylene glycol (22a).

Transposon mutagenesis of cosmid DNA in *E. coli*. The Tn*3-gus* transposon, Tn*3*-HoGus, was kindly provided by B. Staskawicz. In this construct the *uidA* (*gus*) gene, which encodes β-glucuronidase, has been inserted into transposon Tn*3*-HoHo1 (9) in place of the *lacZ* reporter gene. Reporter gene fusions created upon transposition of Tn*3*-HoGus can be used to monitor expression of genes into which Tn*3*-HoGus has inserted and may also be used to determine the direction of transcription of these genes.

The cosmid clone pL500 was mutagenized after transformation into the transposon donor strain *E. coli* HB101 (pSShe, pHoGus) by the method of Stachel et al. (29). pL500 can replicate in a *polA*-deficient background, but plasmids pSShe and pHoGus cannot. Therefore, pL500 plasmids were conjugated into *polA* strain C2110 by triparental mating with the HB101 donor (pSShe, pHoGus, pL500) and the HB101 helper (pRK2013) strains. Following triparental matings, C2110 cells containing pL500::Tn*3*-HoGus were selected on LB agar supplemented with nalidixic acid, kanamycin, tetracycline, and ampicillin. Plasmid DNA was isolated from these strains, digested with restriction enzymes, and analyzed by agarose gel electrophoresis to verify the presence of Tn*3*. The location of each Tn*3* insertion was determined by restriction mapping.

Transformation and marker exchange in *P. solanacearum*. To prepare competent cells, log-phase *P. solanacearum* cultures were harvested when they reached optical density (OD) values of between 0.4 and 0.8 at 600 nm. The cells were washed four times by centrifuging and resuspending them in Milli-Q water. After the final wash, they were resuspended

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
P. solanacearum		
K60	Wild-type EPS$^+$	Kelman
KD500	K60::Tn*5* EPS$^-$	37
KD600	K60::Tn*5* EPS$^-$	37[a]
P. solanacearum K60::Tn*3* (Km^r Ap^r)		
500197	*epsA*::Tn*3* EPS$^-$	This work
50045	*epsB*::Tn*3* EPS$^-$	This work
500131	*epsB*::Tn*3* EPS$^-$	This work
500168	*epsB*::Tn*3* EPS$^-$	This work
500154	*epsB*::Tn*3* EPS$^-$	This work
500169	*epsC*::Tn*3* EPS$^+$	This work
50071	*epsC*::Tn*3* EPS$^-$	This work
500127	*epsC*::Tn*3* EPS$^+$	This work
50041	*epsD*::Tn*3* EPS^i	This work
500140	*epsD*::Tn*3* EPS^i	This work
50010	*epsD*::Tn*3* EPS^i	This work
50064	*rvrA* ::Tn*3* EPS^i	This work
500107	*rvrA* ::Tn*3* EPS^i	This work
50078	Tn*3* EPS$^+$	This work
50039	Tn*3* EPS$^+$	This work
50040	Tn*3* EPS$^+$	This work
50013	Tn*3* EPS$^+$	This work
E. coli		
HB101	*recA leu thi thr hsdR hsdM pro* St^r	5
C2110	Nal^r *polA1 rha his*	29
Plasmids		
pSShe	Cm^r *tnpA*$^+$	29
pHoGus	Tn*3*::*gus tnpA tnp*$^+$	Staskawicz
pRK2013	Km^r Tra$^+$ ColE1	15
pLAFR3	Tc^r IncP	30
pL500	pLAFR3 + 28-kb insert	This work
pL5001	pLAFR3 + 12.5-kb pL500 subclone	This work
pL500197	pL5001 *epsA*::Tn*3*	This work
pL50039	pL500::Tn*3*	This work
pL500168	pL5001 *epsB*::Tn*3*	This work
pL500131	pL5001 *epsB*::Tn*3*	This work
pL500200	pL5001 *epsB*::Tn*3*	This work
pL50045	pL500 *epsB*::Tn*3*	This work
pL500163	pL5001 *epsB*::Tn*3*	This work
pL500154	pL5001 *epsB*::Tn*3*	This work
pL500169	pL5001 *epsC*::Tn*3*	This work
pL50071	pL500 *epsC*::Tn*3*	This work
pL500127	pL5001 *epsC*::Tn*3*	This work
pL50013	pL500::Tn*3*	This work
pL500167	pL5001::Tn*3*	This work
pL50078	pL500::Tn*3*	This work
pL500160	pL5001::Tn*3*	This work
pL50041	pL500 *epsD*::Tn*3*	This work
pL50010	pL500 *epsD*::Tn*3*	This work
pL50019	pL500 *epsD*::Tn*3*	This work
pL500140	pL5001 *epsD*::Tn*3*	This work
pL50064	pL500 *rvrA*::Tn*3*	This work
pL500107	pL5001 *rvrA*::Tn*3*	This work
pL50040	pL500::Tn*3*	This work

[a] KD600 was mistakenly referred to as EPS$^+$ in reference 37.

in 1/100 volume of 20% glycerol before storage at −80°C. For transformation, 40 μl of competent cells was mixed with 0.1 to 1 μg of plasmid DNA and electroporated in an electric field of 10 Kv/cm, with a 400-Ω external resistor and a capacitance of 14 μF. Following electroporation, the bacteria were mixed with 0.5 ml of CPG and incubated at 28°C on a rotary shaker for 4 h before plating on TZC medium containing the appropriate antibiotics. Typical transformation efficiencies with pL500 or its Tn*3* derivatives were 10^3 to 10^4 transformants per μg of DNA. Marker exchange mutants, resulting from double homologous recombination, were identified by screening individual Km^r colonies for loss of pLAFR3 based on tetracycline sensitivity. Potential marker exchange mutants were verified by Southern blot analysis of their genomic DNAs.

Virulence assays. Virulence of wild-type and mutant *P. solanacearum* strains was assessed by means of an eggplant (*Solanum melongena* L. cv. Black Beauty) seedling bioassay (37). In this assay, wild-type strain K60 caused wilting of the seedlings by 6 days and systemic necrosis and collapse by 9 days. Each strain was tested on a minimum of 10 seedlings.

The growth of wild-type and mutant strains in planta was determined after infiltrating fully expanded tobacco leaves (28) with bacterial inoculum prepared as described above. Disks of infiltrated tissue were cut with a cork borer and ground with a tissue homogenizer. Bacterial CFU in the homogenate were determined by dilution plating on TZC medium.

Complementation analysis. Complementation analysis was completed by transforming mutant strains with the wild-type cosmid (pL500) or selected Tn*3* derivatives, as described above. Complementation was defined as the restoration of wild-type colony morphology in an EPS-affected mutant following transformation with test DNA. Typically, 100 to 1,000 colonies of each transformed strain were assessed visually for the complemented phenotype.

β-Glucuronidase assays. β-Glucuronidase activity was measured fluorimetrically by a procedure modified from Jefferson (20). Briefly, bacterial cells from broth cultures or Miracloth-filtered homogenates from infected leaf tissues were centrifuged and the pellet was resuspended in an extraction buffer (20). β-Glucuronidase was released from intact cells by sonication on ice (5), with three 10-s bursts at a power setting of 30 W. Enzyme activity was calculated by determining the rate of 4-methylumbelliferone accumulation in the extraction buffer supplemented with 6.25 mM 4-methylumbelliferyl-β-D-glucuronide as substrate. 4-Methylumbelliferone was quantified by fluorimetry at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Standard curves were prepared with authentic 4-methylumbelliferone. For in planta expression studies, corrections for fluorescence quenching due to plant extracts were made by preparing a second standard curve with leaf tissue homogenate in the diluent.

Generation of spontaneous mutants. Spontaneous mutants were generated by prolonged stationary culture of strain K60 in TGY broth (22). After incubating for 7 days at 28°C, serial dilutions of 20 separate stationary cultures were plated on TZC agar medium and maintained at 28°C for 2 days. Spontaneous mutants were easily identified because of their red, butyrous colony morphology.

Preparation of crude EPS. Wild-type and mutant strains were grown to mid-log phase (OD_{600} of 1.1 to 1.5) in CPG broth and the culture supernatants were concentrated 10-fold on a rotary evaporator at 55°C. The concentrated supernatants were extracted twice with Tris-equilibrated phenol (5) prewarmed to 68°C and once with chloroform. The aqueous phase was dialyzed extensively against double-distilled water, lyophylized, and then stored at −20°C until further analysis.

Quantification of *N*-acetylgalactosamine. Crude EPS was hydrolyzed in 2 N trifluoroacetic acid at 121°C for 1 h, and the hydrolysate was used to prepare alditol acetate derivatives of the component sugars (2, 6). The alditol acetates were separated on a Varian model 3740 gas-liquid chromatograph (Varian Instruments Division, Palo Alto, Calif.) equipped with a flame ionization detector and a glass column packed with 3% OV-275 on Gas-Chrom Q. Two chromatography programs were used: (i) temperature was increased from 160 to 230°C (2°C/min) and then held at 230°C for 6 min, and (ii) temperature held at 200°C for 15 min and then increased from 200 to 230°C (1°C/min). Peaks were quantified with a Hewlett-Packard 3390A integrator. *N*-Acetylgalactosamine (GalNAc) was identified by cochromatography with derivatized, authentic *N*-acetylgalactosamine and quantified by reference to an inositol internal standard. For comparison of EPS produced by different strains, cultures were grown to mid-log phase (OD_{600} of 1.1 to 1.5) and GalNAc content was normalized to an OD of 1.0.

With this procedure, calculated amounts of EPS represent maximum possible values since alditol acetate derivatization of galactosamine (GalN) and GalNAc yield the same product. Thus, any GalN or non-EPS-associated GalNAc present in crude EPS would cause an overestimation of the amount of EPS.

Extraction and analysis of nucleotide sugars. Sugar nucleotides were released from CPG-grown bacteria by resuspending cell pellets in 2.6 N formic acid and incubating them for 10 min at 28°C on a rotary shaker (8). Insoluble cellular material was removed by centrifugation, and the supernatant was lyophilized before storing it at −20°C. Prior to analysis, samples were dissolved in Milli-Q water and passed through a 0.45-μm membrane filter (Millipore Corp.). Sugar nucleotides were separated by high-pressure liquid chromatography (HPLC) with a Beckman model 420/332 instrument and eluted at a flow rate of 1.0 ml/min from an Ultrasphere-ODS column (4.6 by 15 cm) in an ion pair buffer system consisting of 40 mM H_3PO_4 adjusted to pH 6.5 with triethylamine. Column effluent was monitored at 262 nm with a Gilson Holochrome system or with a Hewlett-Packard model 1040A diode array UV detector. To ensure the reproducibility of retention times, it was necessary to wash the column with acetonitrile between samples. Authentic UDP-*N*-acetylgalactosamine and UDP-*N*-acetylglucosamine were used as standards.

RESULTS

pL500 complements the EPS⁻ Tn*5* mutant KD500 but not the spontaneous EPS⁻ mutants. The cosmid pL500 was obtained from an *Eco*RI library of strain K60, prepared in pLAFR3, by homology with the Tn*5*-containing *Eco*RI DNA fragment of the EPS⁻ nonvirulent mutant strain KD500 (35a). When transformed into KD500, pL500 restored both EPS production and virulence, thus demonstrating that it contains information essential for both of these phenotypes. However, when pL500 was used to transform 20 independent spontaneous mutants that had EPS-affected colony morphologies, the resulting transformants remained EPS affected.

Tn*3* insertions affecting EPS and virulence are clustered. To identify the portion of pL500 required for normal EPS

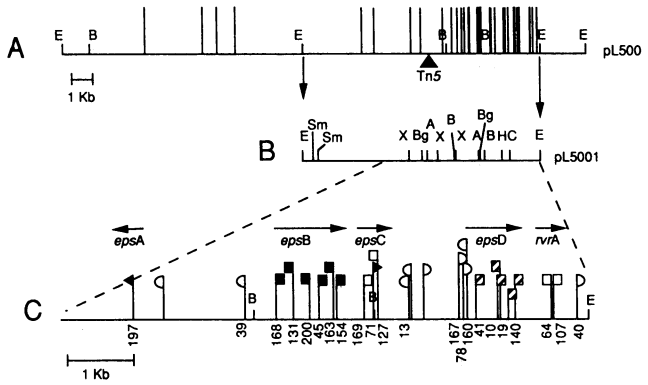

FIG. 1. Clustered Tn*3* insertions affect virulence and colony morphology. (A) Distribution of Tn*3* insertions (vertical lines) within pL500. The single Tn*5* symbol indicates the approximate position of the adjacent Tn*5* insertions KD500 and KD600 (37). Note that the insertions in KD500 and KD600 are separated from *epsA-epsD* and *rvrA* (panel 1C) by Tn*3* insertions that have no effect on colony morphology or virulence. (B) Restriction map of the 12.5-kb *Eco*RI subclone, pL5001. E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; Sm, *Sma*I; X, *Xho*I; A, *Apa*I; C, *Cla*I; H, *Hin*dIII. (C) Phenotypes associated with Tn*3* mutants. Mutations referred to in the text are numbered. Symbols: Virulence— ɑ, wild type; □, reduced; △, nonvirulent; colony morphology—○, wild type; ●, minus (no visible slime); ⊘, impaired (intermediate between wild type and minus). The direction of each symbol corresponds to the orientation of Tn*3* insertion relative to *uidA* (*gus*) transcription. Within *epsB* and to the right, all rightward-facing insertions, but none of the leftward-facing insertions, expressed β-glucuronidase. Five complementation units (*epsA*, *epsB*, *epsC*, *epsD*, and *rvrA*) are indicated by the horizontal arrows, the direction of which corresponds to the direction of transcription as determined by analysis of β-glucuronidase expression.

production and virulence, pL500 was mutagenized with Tn*3*. Tn*3* insertions, selected to give an even distribution of mutations, were used for site-directed mutagenesis of the K60 genome by marker exchange. Marker exchange was confirmed by Southern blot analysis of genomic DNAs. Only those Tn*3* insertions clustered in a 6.5-kb region affected colony morphology or virulence or both (Fig. 1). This region was subcloned in a 12.5-kb *Eco*RI fragment to create pL5001 which was remutagenized with Tn*3*, resulting in 17 additional K60 mutants, most of which were also affected in colony morphology or virulence or both (Fig. 1).

At least five complementation units are required for normal colony morphology and/or virulence. Five separate complementation units were identified by the inability of plasmid-borne Tn*3* insertions to complement certain chromosomal mutations (Table 2). For example, *epsB* was defined by the fact that plasmids containing *epsB*::Tn*3* insertions failed to complement the *epsB* mutant, 50045. But, when transformed into an *epsC* mutant, 50071, these same plasmids readily restored EPS production. Of the five complementation units identified, four of them, *epsA–epsD*, were required for both fluidal colony morphology and virulence, and the fifth, termed *rvrA* (for reduced virulence), was required for virulence only (Fig. 1). With the exception of the Tn*3* insertions in *epsC*, all mutations in the same complementation unit had similar effects on colony morphology and virulence. Insertions in *epsA*, *epsB*, and insertion 71 in *epsC* yielded mutants which produced no visible slime and were classified as EPS$^-$; *epsD* mutants formed what appeared to be an intermediate amount of slime and were classified as EPS^i. Plas-

TABLE 2. Results from complementation analysis[a]

strain phenotype	strain genotype	plasmid: 500197	50039	500168	500131	500200	50045	500163	500154	500169	50071	500127	50013	500167	50078	500160	50041	50010	50019	500140	50064	500107	50040	500 c
●	*epsA*-197	●					○			○	○						○					○		○
●	*epsB*-168		○	●	●	●	●	●	●	○	○													○
●	*epsB*-45		○	●	●	●	●	●	●	○	○						○	○			○			○
○	*epsC*-169									○	○[b]													
●	*epsC*-71		○	○			○		○	●	●[b]	●	○	○	○	○	○	○						○
○	*epsC*-127								○	○	●	○	○											○
⊘	*epsD*-41									○	○	○	○		○	⊘	⊘	⊘	⊘	⊘	○		○	○
⊘	*epsD*-10														○			⊘		⊘	○		○	○
○	*rvrA*-64														○		○	○			○		○	○

[a] Complementation is indicated by the restoration of wild-type (○) colony morphology in mutants with EPS$^-$ (●) or EPS^i (⊘) colony morphologies.
[b] 50071(pL50071) and 500169(pL50071) grew slowly in culture.
[c] Plasmid 500 (pL500), the wild-type clone, served as a positive control.

mid-borne insertions 169 and 127 had no obvious effect on colony morphology after marker exchange into the K60 chromosome, yet they failed to complement the EPS$^-$ strain 50071 (*epsC*). In addition, when pL50071 was used to transform strain 500127 or 500169, it had a negative effect on their phenotype: 500127 (pL50071) was EPS$^-$, like strain 50071, and 500169 (pL50071) grew poorly in culture. Other Tn*3*-containing plasmids had no effect on the colony morphology or growth of 500169 or 500127, including pL500169 and pL500127, thus underscoring the unique interdependence of these three mutations. In other respects these mutants behaved normally. For example, the mutation in 50071 was readily complemented by plasmids bearing insertions in other complementation units, and both pL500169 and pL500127 complemented mutations in other complementation units. Similarly, when pL50071 was used to transform strains that had mutations in other complementation units, it restored their colony morphology to wild type. Based on these observations, we have defined *epsC* to include insertions 169 and 127.

All of the mutants that were affected in colony morphology were also affected in virulence, as measured by their

TABLE 3. Virulence of K60 Tn*3* mutants

Strain/genotype	Colony morphology[a]	Eggplant seedlings	
		Total no. assayed	% Killed[b]
K60	+	41	96.4 ± 8.7
epsA::Tn*3*	−	10	0
epsB::Tn*3*	−	82	33.7 ± 11.2
epsC::Tn*3*	−	21	2.5 ± 4.3
epsC::Tn*3*	+	20	70
epsD::Tn*3*	i	70	39.8 ± 19.9
rvrA::Tn*3*	+	27	36.0 ± 11.2
Tn*3*[c]	+	72	93.3 ± 11.1

[a] +, Wild type; −, no visible slime; i, impaired (intermediate between + and −).
[b] Calculated by combining the data obtained from analysis of several independent mutants in each complementation unit. At least four separate trials were used for each calculation, except for the *epsA*::Tn*3* mutant (one trial) and the *epsC*::Tn*3* mutants that exhibited wild-type colony morphology (two trials).
[c] Tn*3* mutants exhibiting wild-type colony morphologies and virulence (50078, 50039, 50040, and 50013).

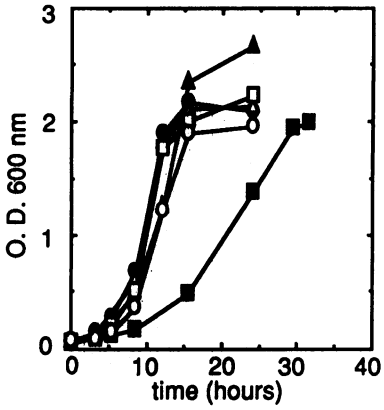

FIG. 2. Growth of mutant and wild-type strains in CPG broth cultures. Symbols: ○, K60 (EPS$^+$); ■, 500197 (*epsA* EPS$^-$); ▲, 50071 (*epsC* EPS$^-$); □, 50041 (*epsD* EPS^i); ●, 50045 (*epsB* EPS$^-$); △, 500107 (*rvrA* EPS^i). The data represent observations from a single experiment, but are typical of those obtained in similar experiments with the same strains.

capacity to kill eggplant seedlings (Table 3). Furthermore, all EPS$^-$ mutants were less virulent than EPS^i mutants. With few exceptions, insertions that did not affect colony morphology also did not reduce the capacity of these mutants to kill eggplant seedlings. Two exceptions are the *rvrA* mutants 50064 and 500107, which had wild-type colony morphologies but were substantially reduced in virulence (Table 3). However, chemical analysis (see below) revealed that the *rvrA* mutant 50064 was also EPS deficient. Similarly, the *epsC* mutants 500169 and 500127 had reduced virulence yet appeared to have wild-type colony morphologies. Reversion of mutant strains to wild type apparently was not a factor during the virulence assays since all colonies reisolated from killed seedlings had antibiotic sensitivities and colony morphologies indistinguishable from those of the original strains.

Mutants affected in EPS and virulence are impaired in their ability to grow in tobacco leaves. With the exception of the *epsA* mutant 500197, EPS-affected mutants grew as well as, or better than, wild type in CPG broth cultures (Fig. 2) and in minimal medium (data not shown). Their growth in tobacco leaves, however, was substantially impaired when compared with wild type. The mutants could be separated into two classes: (i) EPS$^-$ mutants, typified by three strains with insertions in *epsB* (Fig. 3A), which grew poorly and were characterized by an initial decline in bacterial numbers; (ii) EPS^i mutants, including *epsD* and *rvrA* mutants, which exhibited an extended lag phase, but eventually reached populations close to that of wild type (Fig. 3B). Although two of the *epsC* mutants, 500169 and 500127, were slightly impaired in the ability to kill eggplant seedlings (Table 3), they grew like the wild-type strain in tobacco leaves (data not shown); this was in contrast to the third *epsC* mutant, 50071, which was only weakly virulent and grew poorly in tobaccco leaves.

***eps* genes are expressed in planta and in broth cultures.** Tn*3-gus* insertions within and to the right of *epsB* expressed β-glucuronidase activity only when they were oriented in a left-to-right direction (5′-3′ with respect to *gus*), regardless of their effects on colony morphology or virulence (Fig. 1). Conversely, to the left of *epsB*, those insertions expressing β-glucuronidase were transcribed from right to left.

When mutants of each of the complementation units were grown in CPG broth, β-glucuronidase activity paralleled cell density during the lag and log phases of growth (Fig. 4A). However, when net cell growth decreased at stationary phase, β-glucuronidase activity consistently exhibited a continuing increase in all mutants examined. Substantial expression of β-glucuronidase from *eps* and *rvr* gene fusions was also observed in minimal medium, but the relationship to growth was not examined. β-Glucuronidase activity was detected in homogenates from tobacco leaves inoculated with mutant strains, and there was no enzyme activity detected in homogenates obtained from leaves inoculated with either wild type or mutants harboring Tn*3-gus* in the inactive orientation. EPS^i mutants (*epsD* and *rvrA*) and 500169 (*epsC*) produced detectable β-glucuronidase only after bacterial populations had increased substantially (Fig. 4B). Similarly, EPS$^-$ mutants, which grew poorly in the plant, produced detectable levels of β-glucuronidase only after bacterial populations began to increase (data not shown). The ability to detect β-glucuronidase activity in planta only after prolonged periods of infection, when bacterial populations have reached maximum, probably results from the insensitivity of the β-glucuronidase assay at low bacterial populations. However, it remains formally possible that these genes are expressed poorly, or not at all, early in infection.

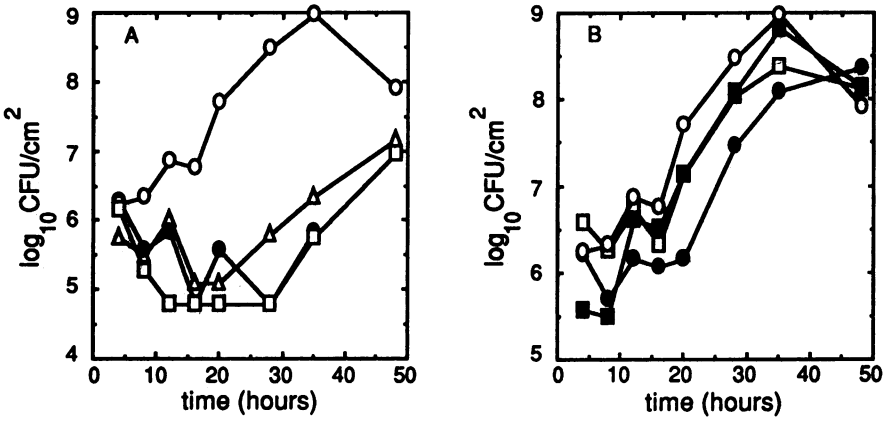

FIG. 3. Growth of mutant and wild-type strains in tobacco leaves. (A) Wild-type (EPS$^+$) and *epsB* (EPS$^-$) mutants: ○, K60; ●, 50045; △, 500131; □, 500168. (B) Wild-type (EPS$^+$) and *epsD* (EPS^i) mutants: ○, K60; □, 50041; ■, 500140; ●, 50010.

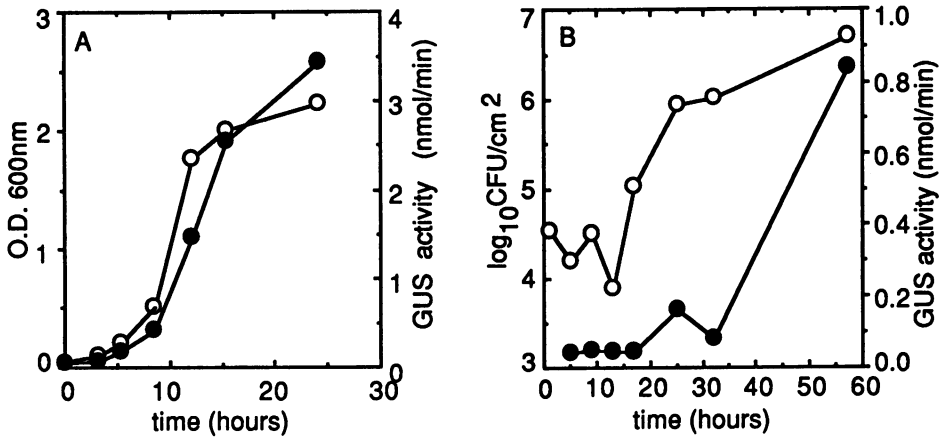

FIG. 4. Growth and β-glucuronidase activity in the *rvrA* mutant 500107. (A) CPG broth. (B) Tobacco leaves. Symbols: ○, bacterial growth; ●, β-glucuronidase activity.

Tn*3* mutants affected in colony morphology and/or virulence have decreased levels of nondialyzable GalNAc in culture supernatants. Arabinose, mannose, galactose, glucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (GalNAc) were detected in the hydrolyzed polysaccharide fraction of supernatants from cultures of mutant and wild-type strains. Except for differences in the GalNAc peak, gas-liquid chromatography profiles obtained with the two temperature programs were similar for all strains. When the amount of nondialyzable GalNAc found in culture supernatants was normalized to cell density and compared with the wild-type strain (100%), EPS$^-$ mutants of *epsA-epsC* averaged 19.3% and the EPS^i mutants, 50041 (*epsD*) and 50064 (*rvrA*), averaged 65 and 50% of wild-type levels, respectively (Table 4). The *epsC* mutant 500169, which had wild-type colony morphology and retained a high level of virulence, produced 94% of the wild-type level of GalNAc.

UDP-GalNAc is present in wild-type and mutant cells. UDP-GalNAc was one of the dominant nucleotide sugars in formic acid extracts from wild-type and mutant strains. Based on integration of the peak area from HPLC chromatograms, the concentration of UDP-GalNAc was not significantly different among mutants (500197, *epsA*; 50045, *epsB*; 500169 *epsC*; 50041, *epsD*; and 50064, *rvrA*) or between mutants and wild type. In addition to cochromatography with authentic UDP-GalNAc, the UV absorption spectrum of the peak that coelutes with authentic UDP-GalNAc was characteristic of UDP, with an absorption maximum at 262 nm. However, with this HPLC system, UDP-GalNAc coelutes with its C_4 epimer, UDP-*N*-acetylglucosamine (UDP-GluNAc). To determine the relative proportions of these two sugars, fractions were collected from the HPLC, hydrolyzed (as described for analysis of EPS) to remove the UDP moiety, and derivatized for separation and quantification of alditol acetates by gas-liquid chromatography, as described previously. With this procedure, UDP-GalNAc accounted for 87.3 ± 2.8% of the total UDP-GalNAc/UDP-GluNAc peak in both mutant and wild-type extracts, except for 500197 (*epsA*), which was not analyzed.

TABLE 4. *N*-Acetylgalactosamine content in culture supernatants of mutant and wild-type strains

Strain	GalNAc/GlcNAc	GalNAc (μg/liter)[a]	% Wild type	Colony morphology[b]	Genotype
K60	82.3 ± 0.36	805	100	+	Wild type
500197	15.3 ± 0.2	150	18.6	−	*epsA*::Tn*3*
50045	15.9 ± 1.4	156	19.3	−	*epsB*::Tn*3*
50071	16.5 ± 0.3	161	20	−	*epsC*::Tn*3*
50041	53.5 ± 3.0	523	65	i	*epsD*::Tn*3*
50064	41.3 ± 2.4	404	50	+	*rvrA*::Tn*3*
500169	77.4	757	94	+	*epsC*::Tn*3*

[a] GalNAc was calculated by first normalizing to GlcNAc, which was constant between strains, and then by comparison with an inositol internal standard. For purposes of comparison, bacterial cultures were grown to mid-log phase (OD_{600}, 1.1 to 1.5) and GalNAc content was normalized to an OD of 1.0.

[b] +, Wild type; −, no visible slime; i, impaired (intermediate between + and −).

DISCUSSION

Tn*3* mutagenesis was used to identify a 6.5-kb gene cluster required for both fluidal colony morphology and virulence in *P. solanacearum*. Five complementation units were identified. These appear to encode functions that are distinct from those affected in the EPS$^-$ virulent Tn*5* mutant KD700, based on comparisons of restriction map data (36). They are probably also distinct from the function(s) affected in the EPS$^-$ nonvirulent Tn*5* mutants KD500 and KD600 (37), because Tn*3* insertions that have no effect on colony morphology or virulence separate *epsA-epsD* and *rvrA* from the Tn*5* insertions of KD500 and KD600 (Fig. 1). Furthermore, in preliminary complementation studies, both KD500 and KD600 were strongly complemented by plasmids bearing *epsC*, *epsD*, and *rvrA* mutations and poorly complemented by plasmids bearing *epsA* or *epsB* mutations (unpublished data). In addition, pL500 probably does not contain the *phcA* gene identified by Denny (10b) because *phcA*, which is required for EPS production in *P. solanacearum* AW1, complements some spontaneous EPS$^-$ mutants of K60, while in this study pL500 complemented none of 20 independently derived spontaneous EPS$^-$ mutants of K60.

The apparently small sizes of *epsB-epsD* and *rvrA*, combined with the results from the complementation experiments, suggest that these are probably single genes under the control of separate promoters, similar to the situation in some strains of *X. campestris* (16, 18) in which the EPS gene cluster contains several complementation units. These *P. solanacearum* genes may be directly involved in EPS bio-

synthesis since all five complementation units are necessary for EPS production and clustering is typical of genes for acidic heteropolysaccharide synthesis in other bacteria (12, 14, 16). It is possible that this *P. solanacearum* gene cluster contains additional genes because several Tn*3* insertions that have no obvious affect on colony morphology or virulence, but are located within the cluster, are actively transcribed to produce β-glucuronidase. Furthermore, the uneven distribution of Tn*3* insertions outside of the cluster leaves open the possibility that adjacent DNA sequences also encode genes required for EPS production or virulence or both.

Complementation unit *epsC* was unusual because it was defined by three separate Tn*3* insertions that produced two opposing phenotypes: mutants 500169 and 500127, which had wild-type colony morphologies, and 50071, which was EPS^-. The interdependence of these three insertions was indicated by the fact that pL50071 acted as a dominant negative mutation only in strains harboring chromosomal insertion 169 or 127: it changed the EPS^+ colony morphology of 500127 to EPS^- and caused 500169 to grow slowly in culture. Similarly, when strain 50071 was transformed with pL50071, creating a strain with multiple copies of the mutation, the transformants grew slowly in culture (data not shown). One interpretation of these results is that the *epsC*::71 gene produces an abnormal product that interferes with EPS production in the absence of a wild-type *epsC* gene product and that adversely affects growth when present in multiple copies.

Complementation analysis was conducted in the recombination-proficient strain K60 because recombination-deficient mutants of *P. solanacearum* are not available. It is possible, therefore, that complementation may have resulted from double homologous crossover events between chromosomal and plasmid sequences. However, this seems unlikely for several reasons. First, double crossover events were generally difficult to obtain in K60; often more than 1,000 colonies had to be screened to obtain a single marker exchange mutant. Second, the restoration of wild-type colony morphologies in complementation experiments was always all or none; if double crossover events were responsible for the observed phenotypes, then one would expect to see both complemented and noncomplemented colonies in the transformed population. Third, complementation occurred between and not within clusters of insertions. These clusters had distinct mutant phenotypes and were separated by insertions with no apparent effect on colony morphology, indicating that the functions they affect are not contiguous. Fourth, when complemented strains were removed from tetracycline selection, thus allowing loss of the introduced plasmid, mutant colonies reappeared (data not shown). This indicates that, for these colonies, the transient restoration of wild-type colony morphology was not due to homologous recombination.

The two Tn*3* insertions in *rvrA*, 64 and 107, complemented strains harboring mutations in *epsA-epsD*, thus indicating the presence of a different complementation unit. However, complementation between 50064 and 500107 was not tested because both strains had wild-type colony morphologies, and the complementation assay was useful only for strains with altered colony morphologies. The alternative complementation test, based on the restoration of virulence, is unreliable because the trans-merodiploids were unstable, and tetracycline selection could not be imposed in planta. Consequently, insertions 64 and 107 have been tentatively placed in the same complementation unit (*rvrA*) based on their similar phenotypes and close proximity.

The expression of *epsA-epsD* and *rvrA* may be constitutive, since β-glucuronidase activity derived from Tn*3*-mediated gene fusions was detected in cells grown under a variety of conditions. In addition, expression of these gene fusions in culture appeared to be growth phase independent: β-glucuronidase activity was correlated with cell density during the lag and log phases of growth but continued to increase as the cultures entered stationary phase. This observation is consistent with the fact that the most abundant source of EPS is very old (72 to 96 h) shake cultures.

Since *N*-acetylgalactosamine is the main component of *P. solanacearum* EPS, calculation of its abundance in the crude polysaccharide fraction of culture supernatants provided a useful measure of how mutations in all five complementation units affected EPS. It was interesting that the *rvrA* mutant 50064, which had a wild-type (EPS^+) colony morphology, produced only one-half the wild-type level of GalNAc and therefore must also be considered EPS affected. This result demonstrates that visual assessment of colony morphology on plates is not a reliable indicator of EPS production and raises the possibility that other complementation groups, required for wild-type EPS production but not for wild-type colony morphology, may be present within this gene cluster. For example, three of the six Tn*3* insertions that separate *epsC* from *epsD* and that affect neither colony morphology nor virulence express β-glucuronidase (data not shown), thus indicating that this region is actively transcribed.

The majority of galactosamine in semipurified EPS from strain K60 is N-acetylated, but the methods used in this study could not resolve galactosamine from its N-acetylated derivative. It remains possible, therefore, that some of these *P. solanacearum* mutants are defective in acetylation of EPS. Acetylation-defective EPS mutants have been identified from *X. campestris* (11a). Such mutants produce EPS that has altered rheological properties.

Denny et al. (11) could not detect a consistent difference in amounts of EPS produced by EPS^- and EPS^i mutants of *P. solanacearum* AW1 in BGT (CPG supplemented with yeast extract) broth cultures. Their analysis of EPS was based on a colorimetric assay for total hexosamines; it is likely that the presence of other, non-EPS-associated amino sugars in the supernatant interfered with calculations of GalNAc. For instance, we found that glucosamine (or *N*-acetylglucosamine), presumably derived from the hydrolysis of lipopolysaccharide, was more abundant than GalNAc even after partial purification by hot phenol extraction (data not shown). Consequently, any method not capable of resolving GalNAc and GlcNAc would provide misleading results.

The occurrence of two classes of EPS-affected mutants, similar to the EPS^- and EPS^i mutants of *P. solanacearum*, has also been reported for *E. stewartii* (12). EPS^i mutants might result from the production of variant polysaccharides with altered physical properties. In *X. campestris*, such variant polymers result from mutations in genes coding for the glycosyltransferases involved in side-chain assembly and in acetylases and ketolases involved in polymer modification (11a). It is possible that the EPS^i phenotype of *epsD* and *rvrA* mutants in *P. solanacearum* results from the synthesis of such variant polymers.

Virtually nothing is known concerning the biosynthesis of EPS in *P. solanacearum*; however, based on our results, it seems likely that UDP-GalNAc is a key intermediate in this process. This interpretation is based on two facts: first, UDP sugars are the most common substrates for bacterial polysaccharide synthesis (32), and second, lysed K60 cells rapidly incorporate [^{14}C]UDP-GalNAc into a lipophilic frac-

tion (10a), which is characteristic of the formation of lipid-linked intermediates of polysaccharide synthesis (7, 32, 33). In *X. campestris*, genes for nucleotide sugar formation are physically separated from the cluster of *gum* genes that encode the enzymes for EPS assembly (11a). This may also be the case for *P. solanacearum* since we have found that mutations in *epsA-epsD* and *rvrA* do not affect the level of the nucleotide sugar, UDP-GalNAc.

Most of the current evidence supports the conclusion that EPS is important for virulence of *P. solanacearum*, including the results reported here. We have established strong positive correlations between the amount of EPS produced in culture and (i) in planta growth and (ii) virulence. Nevertheless, critical evidence for the role of EPS in virulence of *P. solanacearum* requires a more detailed analysis of the various EPS$^-$ mutants and, in particular, a better understanding of the genetics and biochemistry of EPS synthesis. For example, while quantitative changes in EPS production seem to affect virulence, qualitative changes, including those affecting polymer structure, size, and rheological properties, are also likely to affect virulence. Without detailed knowledge of the structure of EPS produced by the wild-type strain and by EPS mutants with reduced virulence, it is difficult to draw firm conclusions about the role of EPS in the development of disease symptoms. In *P. solanacearum*, mutations affecting specific glycosyltransferases may provide the least ambiguous assessment of the role of EPS in disease; glycosyltransferases are highly specific enzymes whose functions are usually limited to a single step in the assembly of a single polysaccharide (35), and the effect of such mutations on polymer structure can be predicted and subsequently verified. The data we present constitute the first step in a continuing effort to define the biochemical and genetic requirements of EPS production.

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