JOURNAL OF BACTERIOLOGY, July 1990, p. 3946–3951
0021-9193/90/073946-06$02.00/0

Vol. 172, No. 7

Highly Virulent Strains of *Pseudomonas solanacearum* That Are Defective in Extracellular-Polysaccharide Production

PEILIN XU,[1] MICHIAKI IWATA,[1] SALLY LEONG,[2] AND LUIS SEQUEIRA[1]*

Department of Plant Pathology[1] and Plant Disease Resistance Unit, U.S. Department of Agriculture Agricultural Research Station,[2] University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 30 October 1989/Accepted 10 April 1990

Extracellular polysaccharide (EPS) has long been regarded as one of the most important factors involved in wilting of plants by *Pseudomonas solanacearum*. By means of transposon Tn*5* mutagenesis, we have isolated a class of mutants that have an afluidal colony morphology but retain the ability to cause severe wilting and death of tobacco plants. One such mutant, KD700, was studied in detail. By marker exchange mutagenesis, the altered colony morphology was shown to be the result of a single Tn*5* insertion in a 14.3-kilobase *Eco*RI fragment. This defect could be corrected by introducing a homologous clone from a cosmid library of the wild-type, parental strain K60. The Tn*5*-containing fragment was introduced into other *P. solanacearum* wild-type strains by marker exchange, and these altered strains had the same afluidal phenotype as KD700. *N*-Acetylgalactosamine (GalNac), the major constituent of EPS of all wild-type strains of *P. solanacearum*, was not detected by gas chromatography-mass spectrometry analysis of vascular fluids from wilting plants infected by KD700. In contrast, GalNac was readily detected in similar fluids of plants infected by K60. Polysaccharides extracted from culture filtrates of KD700 contained approximately one-fifth of the GalNac present in polysaccharides from K60. No differences in growth rates in culture or in planta between the mutant and the parental strains were observed. Since strains that are deficient in EPS production can remain highly virulent to tobacco, we conclude that EPS, or at least its GalNac-containing component, may not be required for disease development by *P. solanacearum*.

Wilting of plants is a common symptom associated with vascular invasion by diverse groups of plant-pathogenic bacteria (22). Although the mechanism of wilting is not understood, the production of extracellular polysaccharides (EPS) by bacteria growing within the vascular system of the plant is thought to contribute to plugging of xylem vessels, interfering with water transport. The production of EPS in planta has been demonstrated for several plant-pathogenic bacteria, but the direct relationship between EPS production and virulence has been established only in a few instances (22).

Pseudomonas solanacearum E.F.Sm. is the causal agent of bacterial wilt of numerous economically important crops worldwide. In the 1950s, Kelman first reported the correlation between the colony morphology of this organism on a tetrazolium medium and its virulence in tobacco (16). Virulent strains invariably produced copious amounts of slime, while spontaneous avirulent mutants retained a butyrous, slimeless appearance. By means of an eosin-serum staining technique, Husain and Kelman (15) demonstrated the presence of bacterial slime around cells of the virulent strain in culture and in bacterial exudate from vascular tissues of diseased plants. Tomato cuttings wilted in tracheal sap obtained from diseased tobacco and freed of bacteria by ultrafiltration. The substance responsible for wilting was a complex polysaccharide; it is now known that the acidic fraction of *P. solanacearum* EPS is composed mostly of a polymer of *N*-acetylgalactosamine (GalNac) and small amounts of rhamnose and glucose (1, 12). That the composition of EPS can vary among strains was shown by Drigues et al. (12).

The rapid development of molecular genetic techniques for *P. solanacearum*, especially the use of transposon Tn*5* mutagenesis, has allowed the generation of site-specific mutants with desired phenotypes and the cloning of the corresponding wild-type DNA fragments for molecular analysis (7, 11, 27). Tn*5* mutants with defects in EPS production have been very useful in evaluating the role of EPS in virulence. For example, Staskawicz et al. (20) used transposon mutagenesis to identify and characterize a virulence locus involved in EPS production in *P. solanacearum*. All mutants with afluidal colony types were avirulent. The authors concluded that IS*50* elements from Tn*5* were independently transposing in these mutants and were genetically linked to the afluidal phenotype. More recently, Denny et al. (11) described a series of Tn*5*-generated mutants of *P. solanacearum* in which the ability to wilt tomato plants was closely associated with the ability to synthesize EPS in culture.

We report here the isolation and characterization of a class of afluidal Tn*5* mutants of *P. solanacearum* that retain high virulence to tobacco. This finding suggests that EPS may not be essential for virulence in *P. solanacearum*.

MATERIALS AND METHODS

Media, strains, and plasmids. *P. solanacearum* strains were grown in CPG medium (14) or in CPG medium containing 2,3,5-triphenyltetrazolium chloride and 1.8% agar (TZC medium) (16). A selective medium, Sm-1, modified as described previously (27), was used to isolate Tn*5* mutants. For transformation, bacteria were grown in minimal medium by the method of Boucher et al. (7). MANY medium was used for matings, as described by Anderson and Mills (3). Strains and plasmids relevant to this work are listed in Table 1.

Chemicals and reagents. Restriction endonucleases, enzymes and buffers for DNA ligation, and a kit for nick translation were from Bethesda Research Laboratories.

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
TB1	$r_K^- m_K^+$ *lacZ*(M15)	J. Jessee
HB101	*proA2 recA13 hsdS20 ara-4* $r_K^- m_K^-$ F^- *galK2 rpsL20* (Str) *supE44 leu-6*	18
SM10	*chr* RP4-2 Tc::Mu	19
P. solanacearum		
K60	Vir^+ EPS^+ Km^s wild type	16
26	Vir^+ EPS^+ Km^s wild type	10
81	Vir^+ EPS^+ Km^s wild type	10
82	Vir^+ EPS^+ Km^s wild type	10
134	Vir^+ EPS^+ Km^s wild type	10
139	Vir^+ EPS^+ Km^s wild type	10
147	Vir^+ EPS^+ Km^s wild type	10
S210	Vir^+ EPS^+ Km^s wild type	10
B1	Vir^- EPS^- HR^+ Km^s	15
KD400	K60::Tn*5* Vir^+ EPS^- Km^r	27
KD700	K60::Tn*5* Vir^+ EPS^- Km^r	27
KD702	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD704	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD705	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD711	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD713	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD714	K60::Tn*5* Vir^+ EPS^- Km^r	This study
KD300	K60::Tn*5* Vir^- EPS^- Km^r	27
KD500	K60::Tn*5* Vir^- EPS^- Km^r	27
KD712	K60::Tn*5* Vir^- EPS^- Km^r	This study
C700-A,-B,-C	KD700 carrying pL700, Km^r Tc^r	This study
Plasmids		
pBR322	Ap^r Tc^r	4
pLAFR3	IncP1 Tc^r *rlx*$^+$	21
pSUP2021	(pBR325 *mob*)::Tn*5* Km^r Amp^r	19
ColE1::Tn*5*	Km^r	18
pKD700	pBR322 carrying a 14.3-kb *Eco*RI fragment from KD700, Ap^r Km^r	This study
pL700-A,-B,-C	pLAFR3 containing K60 genomic DNA, Tc^r	This study

Sources of lysozyme, alkaline phosphatase, RNase, Gigapack Plus extract, Zeta-probe membrane, and nitrocellulose were described previously (27).

Molecular genetics techniques. The methods for transposon mutagenesis, DNA isolation, cloning of Tn*5* flanking sequences, construction of a genomic library of *P. solanacearum* K60, transformation, Southern blot analysis, and other related techniques were reported previously (18, 27).

Plant inoculation techniques. Tobacco (*Nicotiana tabacum* L. 'Bottom Special') seedlings were grown individually in pots containing muck soil at 28°C under Sylvania Gro-Lux and General Electric Cool White fluorescent lights in a growth chamber. Plants were inoculated 5 to 6 weeks after transplanting, when they were approximately 30 cm tall. Stem inoculation was carried out as described by Bowman and Sequeira (8). A 15-μl portion of a bacterial suspension containing 10^8 CFU/ml was injected into plants at the axil of the third fully expanded leaf from the top. Each strain was tested on five plants. Wilting index ratings (8) were recorded at 2-day intervals for 10 days and thereafter on the 15th day after inoculation.

To determine the number of viable bacteria in inoculated plants, 1-cm lengths of stem tissue were ground with a mortar and pestle in sterile water, and appropriate dilutions were spread on TZC plates. Numbers of *P. solanacearum* colonies were determined after 48 h of growth at 28°C.

Isolation and analysis of EPS. To extract EPS from infected tobacco plants, 1-cm pieces of stem just below the inoculation site were collected at various intervals after inoculation and immersed in sterile distilled water at room temperature for 1 h. Under these conditions, bacteria exuded from the plant tissues and formed a cloudy suspension. The plant tissues were then removed, the suspension was centrifuged to remove bacteria, and the supernatant was lyophilized. EPS in this lyophilized material was extracted by the procedure of Duvick and Sequeira (13). Gas chromatography (GC) analysis for the hexosamine content of the purified EPS preparations was completed by the methods of Albersheim et al. (2) and Hendrick and Sequeira (14). Alditol acetate derivatives were analyzed with a Varian model 3740 gas chromatograph (Varian Instruments, Palo Alto, Calif.) equipped with a flame ionization detector. Derivatives of glucosamine and galactosamine were separated on a glass column (200 cm by 2 mm) of 3% OV-275 on 100–120 mesh Gas Chrom Q. Peak areas were quantified with a Hewlett-Packard 3390A integrator. Inositol was used as an internal standard. Sugar standards were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Purified EPS preparations also were analyzed by GC-mass spectrometry (GC/MS) at the Complex Carbohydrate Research Center, University of Georgia. Samples were *N*-acetylated with acetic anhydride, reduced with $NaBD_4$, and acetylated with acetic anhydride-pyridine overnight (26). These reagents were evaporated, and trimethylsilyl derivatives were prepared with Tri-Sil (Pierce Chemical Co.). Analysis was done with a Hewlett Packard 5890 GC with a 30-m, DB-1 capillary column (J & W Scientific). For GC/MS, a 15-m column rather than a 30-m column was used. A standard galactosamine sample was analyzed in the same manner. All samples contained inositol as an internal standard.

To isolate EPS from culture filtrates, each strain of *P. solanacearum* was grown in 1 liter of CPG broth on a rotary shaker at 28°C for 72 h. Cells were removed by centrifuging the cultures at 10,000 × *g*, and then EPS in the supernatant fluid was purified by the procedure of Duvick and Sequeira (13). Samples were analyzed for hexosamine content by GC and GC/MS as described above.

RESULTS

In a previous study, we used the suicide plasmid pSUP2021 to obtain avirulent mutants of *P. solanacearum* (27) and found that several Tn*5* transconjugants had an afluidal colony morphology on TZC medium that was distinctly different from that of the fluidal parental strain K60 (Fig. 1). Colonies of the transconjugants were small, deep red, butyrous, and similar in appearance to strain B1, a spontaneous avirulent derivative of K60 that has been studied extensively (14–16, 24). Derivatives such as B1 simultaneously lose the abilities to produce EPS and to cause wilting of tobacco plants (16, 20). Consistent with these results, we found that two of the Tn*5* mutants we generated were no longer virulent on tobacco (KD300 and KD500, Table 1). The remaining seven mutants, however, retained a high level of virulence on this host.

Total genomic DNA from all the afluidal mutants was isolated and digested with *Eco*RI or *Eco*RI plus *Bam*HI, electrophoresed in agarose gels, transferred to Zeta-probe membrane, and probed with ^{32}P-labeled ColE1::Tn*5* DNA.

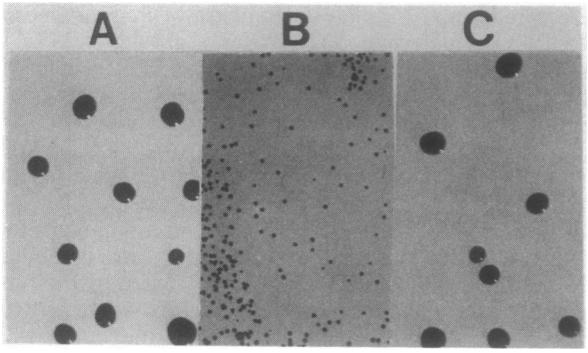

FIG. 1. Colony morphology of 3-day-old *P. solanacearum* cells on a tetrazolium medium (TZC). (A) Wild-type K60; (B) EPS$^-$ Tn*5* mutant KD700; (C) complemented strain C700.

All of the mutants were shown to carry a single Tn*5* insertion (Fig. 2). One such mutant, KD700, was used in further molecular and pathogenicity studies. The 14.3-kilobase (kb) *Eco*RI fragment that contains DNA sequences flanking the Tn*5* insertion in KD700 was isolated from the K60 genomic library and cloned directly into the *Eco*RI site of pBR322 to yield the plasmid pKD700.

To verify that the altered colony morphology was caused by the Tn*5* insertion, marker exchange and gene complementation experiments were done. pKD700 was transformed into the wild-type strain K60 as described previously (27). All the Km^r Tc^s transformants had afluidal colony morphology and showed the same level of virulence on tobacco as the original mutant KD700. The Southern analysis of 10 randomly chosen transformants, probed with ^{32}P-labeled pKD700, revealed that Tn*5* was inserted at the same genomic location as in the original mutant KD700 (data not shown). Thus, a homologous double cross-over event apparently was responsible for the observed marker exchange. These results indicate that Tn*5* insertion in the 14.3-kb *Eco*RI fragment was responsible for altering the colony morphology of KD700.

In addition to the parental strain K60, pKD700 was also introduced into other *P. solanacearum* strains, some of them with different host specificities. These strains included representatives of race 1 (strains 26, 147, and 134), race 2 (strains 139 and S210), and race 3 (strains 81 and 82), which

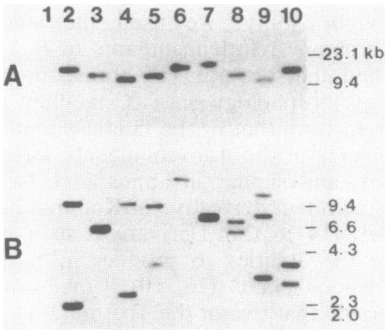

FIG. 2. Southern analysis of *P. solanacearum* Tn*5* mutants with afluidal colony morphology. DNA was digested with *Eco*RI (A) or *Eco*RI plus *Bam*HI (B), electrophoresed in 0.5% agarose gels, transferred to Zera-probe membranes, probed with ^{32}P-labeled ColE1::Tn*5*, and autoradiographed. The lanes contain DNA from *P. solanacearum* strains: 1, K60; 2, KD500; 3, KD700; 4, KD702; 5, KD704; 6, KD705; 7, KD711; 8, KD712; 9, KD713; 10, KD714.

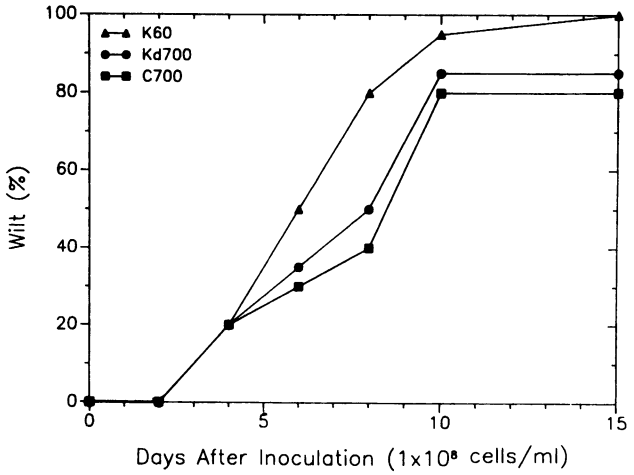

FIG. 3. Symptom development on tobacco plants stem-inoculated with wild-type strain K60, Tn*5* mutant KD700, and complemented strain C700 of *P. solanacearum*. Plants were inoculated when 6 weeks old. Each point represents the average value of five replicates, based on wilting index of each plant.

originated from widely different geographic areas and from several different host plants. The *Eco*RI fragment of pKD700 was exchanged in all these strains, and as in the case of K60, the marker-exchanged transformants had afluidal colony morphology but remained highly virulent on the appropriate host plants. When chromosomal DNAs from these wild-type strains were probed with pKD700, there was strong homology, although there were evident restriction fragment length polymorphisms among them. As expected, afluidal pKD700 transformants of these strains all carried Tn*5* insertions in the homologous regions.

Using pKD700 as a probe, we identified three homologous cosmid clones, pL700A, pL700B, and pL700C, from a pLAFR3 cosmid gene library of *P. solanacearum* K60 (27). The *Eco*RI genomic DNA insertions into pLAFR3 of pL700A, pL700B, and pL700C were 25.5, 21.4, and 20.5 kb in size, respectively. All three cosmids had a 12-kb DNA fragment in common (data not shown). The three cosmids were introduced individually into KD700 by transformation (27), and the Tc^r transformants were examined for colony morphology on TZC plates. All the Tc^r transformants of pL700A reverted to the fluidal colony type (Fig. 1). This indicated that pL700A was able to complement the mutation (e.g., strain C700). This, combined with the results from marker exchange experiments, established conclusively that the Tn*5* insertion in KD700 was responsible for altering colony morphology and for the defect in EPS production. In contrast, the transformants obtained with pL700B or pL700C retained the afluidal colony morphology. Thus, it appears that these two cosmids do not encode sufficient genetic information to restore EPS production.

Virulence assays. The virulence of the wild-type strain K60, the afluidal strain KD700, and the complemented strain C700 was estimated from the wilting index of inoculated tobacco plants (8). Development of disease was recorded over a period of 15 days after inoculation (Fig. 3). The patterns of disease development in plants inoculated with either KD700 or C700 closely resembled that of K60. Wilting occurred most rapidly between 4 and 10 days after inoculation. Symptom development with KD700 and C700A initially was slower than with K60, but by 10 days, nearly all plants had wilted completely regardless of the strain used for

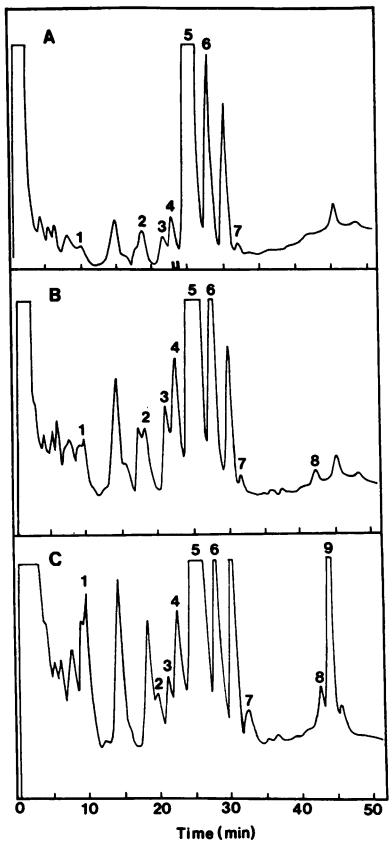

FIG. 4. GC analysis of carbohydrates in extracts from tobacco plants infected with *P. solanacearum*. Samples were prepared and analyzed as described in Materials and Methods. (A) Extract from healthy plant; (B) extract from plant infected with KD700; (C) extract from plant infected with the wild-type strain K60. Extracts of infected tissues were obtained after the tobacco plants had wilted completely (10 days after inoculation). Inositol (peak no. 6) was the internal standard. The peaks correspond to: 1, rhamnose; 2, unidentified; 3, mannose; 4, galactose; 5, glucose; 6, inositol; 7, unidentified; 8, glucosamine; 9, galactosamine.

inoculation. It is evident that the afluidal strain KD700 is an aggressive pathogen and is only slightly less virulent than the parental wild-type strain. In the absence of tetracycline selection in the plant, the cosmid pL700A was not stable in *P. solanacearum*, and up to 50% of the complemented cells became sensitive to the antibiotic after 4 days. This may account for the fact that the virulence of C700 was not restored entirely to the level of K60 (Fig. 3).

Detection of EPS in infected plant tissue. Since previous investigations have shown that EPS of *P. solanacearum* is composed mostly of a linear polymer of *N*-acetylgalactosamine (1, 12) and this hexosamine is absent from lipopolysaccharide and other polysaccharides produced by this organism in culture (12, 14), estimates of EPS in exudates from infected plants were obtained by GC analysis of galactosamine. The results of GC analysis of tobacco stem exudates collected 10 days after inoculation (when plants were completely wilted) established that KD700 did not produce detectable amounts of EPS in host tissues (Fig. 4B). A large peak of galactosamine was detected in exudates from plants infected by K60 (Fig. 4C). Similar results were obtained at the Complex Carbohydrate Research Center at the University of Georgia after GC/MS analysis of the same

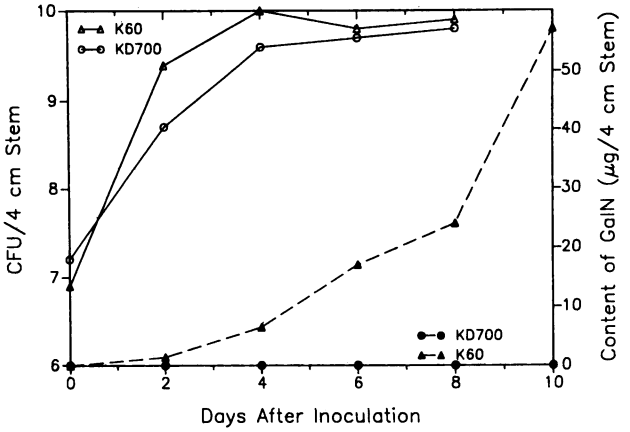

FIG. 5. Growth patterns (open symbols) and EPS production (solid symbols) of the *P. solanacearum* wild-type (K60) and the EPS$^-$ mutant (KD700) in tobacco plants at various times after stem inoculation. The experimental procedures were as described in Materials and Methods. GalN, Galactosamine.

samples of exudates. There was detectable GalNac only in the exudates from plants inoculated with K60.

In further tests, EPS production in planta was monitored at different intervals after inoculation of tobacco plants with KD700 and, for comparison, with K60. The growth patterns of the two strains in infected tobacco plants were very similar (Fig. 5). Both grew exponentially during the first 2 days after inoculation, but populations leveled off thereafter. As measured by galactosamine content, EPS could be detected in plant exudates by 2 days after inoculation with K60 and accumulated rapidly during the stationary phase of bacterial growth (Fig. 5). No EPS was detected in exudates from tissues infected by KD700 at any time after inoculation, even though populations of this strain were comparable to those of K60.

Detection of EPS in culture. To confirm that the afluidal colony morphology in KD700 resulted from a defect in EPS synthesis, assays for EPS were completed with culture fluids of strains K60, KD700, and C700. The supernatant of a 1-liter culture of K60, grown at 28°C under constant shaking for 72 h, yielded about 300 mg of polysaccharide after purification by the method of Duvick et al. (13). Under the same culture conditions, KD700 and C700 yielded about 50 and 320 mg of polysaccharide, respectively. These differences reflect the inability of KD700 to synthesize large amounts of EPS in a rich medium and the apparent restoration of this ability by complementation in C700.

Initial GC analysis of alditol acetate derivatives from hydrolysates of partially purified EPS from culture fluids of K60, C700, and KD700 indicated that galactosamine was present in extracts from both K60 and C700 but was not detectable in that from KD700, even when analyzed at twice the concentration used for the other two strains (data not shown). The minimum detectable amount of galactosamine with our system was 0.1 μg/mg of purified extract. With extracts from K60 and C700, galactosamine constituted approximately 50% of the total identifiable sugars. This is consistent with previous reports (1). The results provided additional evidence that pL700 completely restored the ability of KD700 to produce EPS.

With our GC system, the peaks of glucosamine and galactosamine derivatives were close, and there was a high baseline value at high retention times. It seemed likely,

therefore, that small amounts of galactosamine would not be detected with our analytical procedures. For this reason, single samples of culture extracts from strains K60, KD700, and B1 were sent for GC/MS analysis at the Complex Carbohydrate Research Center at the University of Georgia. The results of GC analysis of trimethylsilyl derivatives of methyl glycosides confirmed that extracts from both KD700 and B1, which have a slimeless colony phenotype, contained 25 and 12 μg of GalNac per mg, respectively, compared with 114 μg/mg for K60, the wild-type mucoid strain. On this basis, KD700 and B1 produced approximately 22 and 10%, respectively, of the amount of GalNac produced by K60 in a liquid culture medium.

The MS analysis of trimethylsilyl methyl glycoside of authentic GalNac (molecular weight 451) showed ions at 436 and 392, which were due to the loss of a methyl radical (−15) and acetamide (−59), respectively. The GC/MS analysis of all *P. solanacearum* culture extracts corroborated the presence of GalNac (data not shown).

DISCUSSION

We have demonstrated that KD700 is a Tn*5*-induced mutant of *P. solanacearum* K60 that apparently is unable to produce EPS in planta. Despite this defect, KD700 retained the ability to cause severe wilting of tobacco. Our results do not exclude the possibility that KD700 produces polysaccharides other than the acidic fraction that has a very high content of GalNac and that is referred to as EPS for this organism in the literature (1, 11–13). Assuming that hexosamine content provides a reasonable estimate of EPS production in planta, however, our data indicate that EPS is not essential for wilting by *P. solanacearum*. This conclusion challenges the view dominating this area of research for the last three decades (11, 15, 16, 20).

The strongest genetic evidence linking EPS production to pathogenicity in *P. solanacearum* stems from the work done with the spontaneous mutant B1 (16). B1 is a slimeless variant of strain K60 that was selected because of its characteristic butyrous colony appearance on TZC medium (16). B1 is consistently avirulent in tobacco, tomato, potato, and other hosts of the parental fluidal strain. Further investigations have shown, however, that the shift to the B1 phenotype is highly pleiotropic and involves changes not only in EPS production, but in lipopolysaccharide composition, induction of the hypersensitive reaction (HR) in plants, piliation, indole acetic acid synthesis, cellulase production, etc. (9, 13, 14, 24).

Thus, the early reports of an absolute correlation between EPS production and virulence in *P. solanacearum* are questionable because of the possible involvement of other virulence factors that are also altered in spontaneous mutants. More recently, Staskawicz et al. (20) and Denny et al. (11) have reported on the isolation of Tn*5*-generated mutants of *P. solanacearum* that combine EPS$^-$ and avirulent phenotypes. We also have obtained Tn*5* mutants of this type (e.g., KD300 and KD500) that carry single Tn*5* insertions in their genomes (Fig. 2). This class of mutants is in contrast to KD700, which retained high virulence but lost the ability to produce large amounts of EPS. These conflicting results might be reconciled if we assume that in mutants such as KD500, the EPS$^-$ Vir$^-$ phenotype is the result of a defect in a function, possibly regulatory, that controls both the production of EPS and the expression of virulence. In KD700, however, the defect may result from the direct inactivation of a gene responsible for catalyzing EPS biosynthesis. Several factors favor this hypothesis. First, the majority of EPS$^-$ Tn*5*-induced mutants that we isolated remained virulent, since only two of seven were avirulent. EPS is a rather complex macromolecule, requiring many enzymes for its biosynthesis. Consequently, there should be multiple targets for Tn*5* insertion. Second, in addition to the parental strain K60, the introduction by marker exchange of pKD700 into strains with different host specificity always resulted in mutants that carried the EPS$^-$ Vir$^+$ phenotype. Finally, a connection between the EPS$^-$ phenotype and pathogenicity-regulating functions has been observed in other systems involving bacteria-plant interactions. For example, in *Rhizobium phaseoli*, two genes control both EPS production and nodulation (5, 6). These findings demonstrate what may be a common feature among a number of microbes, namely, a common control mechanism for both EPS production and the expression of genes involved in the interaction with the host plant.

The results we report here on EPS production in culture by strains of *P. solanacearum* that were classified as EPS$^-$ on the basis of colony appearance on TZC medium are interesting and demonstrate that strains such as B1 and KD700 are EPS deficient rather than EPS$^-$. On the basis of analysis for GalNac, these strains, which have the same slimeless colony phenotype on TZC medium, produce small amounts of EPS when grown in liquid culture in a rich medium. These amounts range from 10 to 22% of the amounts produced by the wild-type parental strain, K60, as determined by GC/MS analysis. That the mutant strains produce a smaller, defective polymer that does not contribute to the slimy appearance in culture is a distinct possibility that merits further study.

The growth rate of KD700, both in culture and in the plant, was very similar to that of the parental wild-type strain K60. Since KD700 was slightly less virulent than K60, the role of EPS in the virulence of *P. solanacearum* cannot be ruled out completely. Previous investigators have provided some data that both support and refute this possibility. We have already mentioned the genetic data that establish a strong correlation between EPS production and virulence (11, 20) in several strains. Contrary to this view, Walls and Truter (23) monitored the progressive destruction of conducting tissues of tomato infected with *P. solanacearum* by means of light and electron microscopy and concluded that watersoaking was caused by a combination of several factors, EPS being only one of them. Similarly, Liao and Hsu (17) found that differences in virulence among *P. solanacearum* strains were not correlated with variation in the amounts of EPS produced in culture. More recently, Woods (25) reported on the isolation of a group of *P. solanacearum* (race 2) variants that formed small, afluidal colonies on TZC medium and that could induce wilting of banana plants, although less aggressively than the fluidal types. It is evident that the role of EPS in the mechanism of wilting caused by bacterial invasion of plants is still unresolved.

Recently, workers in several laboratories, including our own, have successfully cloned genes that control virulence in *P. solanacearum* (7, 10, 28). The detailed molecular analysis of these genes and the biochemical characterization of the products of these genes should provide a much better understanding of the role of EPS in the physiology of wilting caused by *P. solanacearum*.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison (Project 3078), and by

grants from the International Potato Center (Lima, Peru) and the National Science Foundation. Also supported in part by Department of Energy grant DE-FG09-87-ER13810 as part of the USDA/DOE/NSF Plant Science Centers program.

The assistance of Caitilyn Allen in the preparation of the manuscript is gratefully acknowledged.

LITERATURE CITED

1. **Akiyama, Y., S. Eda, S. Nishikawaji, H. Tanaka, and A. Ohnishi.** 1986. Comparison of extracellular polysaccharide produced by 17 virulent strains of *Pseudomonas solanacearum*. Ann. Phytopathol. Soc. Jpn. **52**:741–744.
2. **Albersheim, P., D. J. Nevins, P. D. English, and A. Karr.** 1967. A method for analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohydr. Res. **5**:340–345.
3. **Anderson, D. M., and D. Mills.** 1985. The use of transposon mutagenesis in isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. Phytopathology **75**: 104–108.
4. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Hyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
5. **Borthakur, D., J. A. Downie, A. W. B. Johnston, and J. W. Lamb.** 1985. *psi*, a plasmid-linked *Rhizobium phaseoli* gene that inhibits exopolysaccharide production and which is required for symbiotic nitrogen fixation. Mol. Gen. Genet. **200**:278–282.
6. **Borthakur, D., and A. W. B. Johnston.** 1987. Sequence of *psi*, a gene on the symbiotic plasmid of *Rhizobium phaseoli* which inhibits exopolysaccharide synthesis and nodulation and demonstration that its transcription is inhibited by *psr*, another gene on the symbiotic plasmid. Mol. Gen. Genet. **207**:149–154.
7. **Boucher, C., P. Barberis, A. Trigalet, and D. Demery.** 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn*5*-induced avirulent mutants. J. Gen. Microbiol. **131**:2449–2457.
8. **Bowman, J., and L. Sequeira.** 1982. Resistance to *Pseudomonas solanacearum* in tobacco: infectivity titration in relation to multiplication and spread of pathogen. Am. Potato J. **59**:155–164.
9. **Buddenhagen, I. W., and A. Kelman.** 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. **2**:203–230.
10. **Cook, D., E. Barlow, and L. Sequeira.** 1989. Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol. Plant-Microbe Interact. **2**:113–121.
11. **Denny, T. P., F. W. Makini, and S. M. Brumbley.** 1988. Characteristics of *Pseudomonas solanacearum* Tn*5* mutants deficient in extracellular polysaccharide. Mol. Plant-Microbe Interact. **1**:215–223.
12. **Drigues, P., D. Demery-Lafforgue, A. Trigalet, P. Dupin, D. Samain, and J. Asselineau.** 1985. Comparative studies of lipopolysaccharide and exopolysaccharide from a virulent strain of *Pseudomonas solanacearum* and from three avirulent mutants. J. Bacteriol. **162**:504–509.
13. **Duvick, J. P., and L. Sequeira.** 1984. Interaction of *Pseudomonas solanacearum* lipopolysaccharide and extracellular polysaccharide with an agglutinin from potato tubers. Appl. Environ. Microbiol. **48**:192–198.
14. **Hendrick, C. A., and L. Sequeira.** 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. **48**:94–101.
15. **Husain, A., and A. Kelman.** 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology **48**:155–165.
16. **Kelman, A.** 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology **44**:693–695.
17. **Liao, Y., and S. Hsu.** 1980. Comparison of extracellular polysaccharide from different strains of *Pseudomonas solanacearum*, the pathogen of bacterial wilt of solanaceous plants in Taiwan. J. Agric. Res. China **29**:265–271.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad-host-range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1**:784–790.
20. **Staskawicz, B. J., D. Dahlbeck, J. Miller, and D. Damm.** 1983. Molecular analysis of virulence gene(s) in *Pseudomonas solanacearum*, p. 345–352. *In* A. Puhler (ed.), Molecular genetics of bacterial-plant interactions. Springer-Verlag, Berlin.
21. **Staskawicz, B. J., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169**:5789–5794.
22. **Van Alfen, N. F.** 1982. Wilts: concepts and mechanisms, p. 459–474. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 1. Academic Press, Inc., New York.
23. **Walls, F. M., and S. J. Truter.** 1978. Histopathology of tomato plants infected with *Pseudomonas solanacearum*, with emphasis on ultrastructure. Physiol. Plant Pathol. **13**:307–317.
24. **Whatley, M. H., N. Hunter, M. A. Cantrell, C. Hendrick, K. Keegstra, and L. Sequeira.** 1980. Lipopolysaccharide composition of the wilt pathogen, *Pseudomonas solanacearum*. Plant Physiol. **65**:557–559.
25. **Woods, A. C.** 1984. Moko disease: atypical symptoms induced by afluidal variants of *Pseudomonas solanacearum* in banana plants. Phytopathology **74**:972–976.
26. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1986. Isolation of plant cell walls and cell wall components. Methods Enzymol. **118**:3–40.
27. **Xu, P. L., S. A. Leong, and L. Sequeira.** 1988. Molecular cloning of genes that specify virulence in *Pseudomonas solanacearum*. J. Bacteriol. **170**:617–622.