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Extracellular Polysaccharide Is Required for Wild-Type Virulence of *Pseudomonas solanacearum*

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Several *Pseudomonas solanacearum* strains which produced no detectable extracellular polysaccharide (EPS) in planta had been reported to remain highly virulent when tested at high inoculum concentrations (P. Xu, M. Iwata, S. Leong, and L. Sequeira, J. Bacteriol. 172:3946–3951, 1990; P. Xu, S. Leong, and L. Sequeira, J. Bacteriol. 170:617–622, 1988). Two of these mutants, KD700 and KD710, have now been molecularly and genetically mapped to the EPSI gene cluster described by Denny and Baek (Mol. Plant-Microbe Interact. 4:198–206, 1991). When a range of inoculum concentrations was used, these two mutants and all other EPS-defective mutants tested were found to be reduced in virulence to eggplants and tobacco relative to the wild-type strain. Thus, EPS consistently is required for the wild-type level of virulence in *P. solanacearum*.

Despite many years of investigation, the role of extracellular polysaccharide (EPS) in the pathogenesis of plant-pathogenic bacteria remains unclear. EPS is present around the bacterial cell either as a loosely organized slime or in the form of a capsule. In pathogenic microbe-plant interactions, the slime is thought to play a role in virulence by occluding the vascular system and reducing water transport and by protecting bacteria from plant defense compounds (5). However, EPS is not the only factor necessary for pathogenesis, since nonmucoid mutants which are highly virulent have been isolated (8).

There is a large body of evidence supporting the importance of EPS in the virulence of *Pseudomonas solanacearum* E.F. Sm., the causal agent of bacterial wilt in solanaceous plants. Kelman and associates (4, 12) found that spontaneous slimeless mutants, which produce butyrous colonies of a deep red color on media containing 2,3,5-triphenyltetrazolium chloride (TZC), are always decreased in virulence. In contrast, cultures that produce spreading, slimy colonies on TZC were highly virulent. Spontaneous mutants arise at a high frequency when cells are grown in nonaerated broth. The correlation between virulence and EPS production is clouded because spontaneous mutants may be affected in several other traits, including lipopolysaccharide (LPS) structure (11), polygalacturonase activity (1), piliation (17), and indole 3-acetic acid synthesis (14), etc. There is also evidence that some spontaneous mutants have gross chromosomal rearrangements (3, 12a).

The correlation between EPS and virulence was better defined when transposon-induced mutants of *P. solanacearum* were isolated. Most EPS-defective mutants were found to be affected in virulence (7, 9, 10, 13, 16). At least three clusters of genes participating in EPS production have been identified. The *ops* gene cluster (7, 13, 19) contains at least seven distinct complementation units within a 6.5-kb sequence in strain K60. Mutations in each complementation unit affect EPS production (7) as well as LPS structure (13). Two additional clusters of EPS genes were identified in strain AW1 by Denny et al. (9, 10). These clusters, EPSI and EPSII, are located in adjacent regions of the chromosome but are regulated differently. Mutations in EPSI prevent EPS expression in planta and on minimal medium plates. Mutations in EPSII prevent EPS expression only on rich media (9). In all these instances, there was a good correlation between loss of EPS and reduced virulence.

Several *P. solanacearum* EPS mutants which, quite unexpectedly, remained highly virulent when assayed by stem inoculation of tobacco plants were isolated (18, 19). Only one mutant, KD700, was characterized biochemically (18). This mutant produced no detectable amount of EPS in planta and only 20% of the wild-type amount of EPS in culture. Since this mutant appears to contradict the dogma that EPS is required for virulence in *P. solanacearum*, Xu et al. cloned out the Tn*5* and flanking DNA from the KD700 genome and used the flanking DNA as a hybridization probe to identify the corresponding wild-type DNA in a cosmid library (18). The cosmid named pL700A hybridized to the flanking DNA and was able to complement KD700 to the wild-type level of EPS production and virulence (18).

In this communication, we report that KD700 and KD710, obtained by Xu et al. (19), map to the EPSI region previously identified by Denny and Baek (9). Since EPSI mutants are affected in virulence, we have reexamined the virulence of KD700 and related mutants at a wide range of inoculum concentrations. Contrary to previous findings, our results demonstrate that a reduction of EPS synthesis is consistently associated with a reduction in virulence of *P. solanacearum* KD700.

Many of the 700 series of EPS mutants (Table 1) originally isolated by Xu et al. (18, 19) remained uncharacterized. We determined whether any of these mutations map to the region defined by the Tn*5* insertion in KD700 by transforming them with either pL700A or the vector pLAFR3 and assaying for complementation of EPS production on TZC plates. Four mutants (KD703, KD708, KD710, and KD713) in addition to KD700 produced slimy colonies when harboring pL700A, in contrast to controls. Of these four Tn*5*-generated mutants, however, only KD710 harboring pL700A was restored to the wild-type level of EPS production. Also, in Southern blot analyses, only KD710 appeared to have the Tn*5* insertion within the DNA contained by pL700A (Fig. 1C and data not shown). The other three mutants probably were not directly affected in the genetic region covered by pL700A and were not characterized further.

We were interested in determining whether the 700 series

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TABLE 1. *P. solanacearum* strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
K60	Wild type	A. Kelman
B1	Spontaneous mutant	A. Kelman
KD500, 600,	Tn*5* EPS$^-$ Vir^i CH154$^-$	19
KD400	Tn*5* EPS$^-$ Vir^i CH154$^+$	19
500155, 50037	Tn*5* EPS$^+$ Vir$^+$	7
700 series[b]	Tn*5* EPS^i Vir^i CH154$^+$	18, 19
T700A	K60 with pKD700 integrated in the chromosome; EPS^i Vir^i Tc^r	This work
C700	KD700 transformed with pL700A; EPS$^+$ Vir$^+$ Tc^r Kan^r	18
KD700A	C700 cured of pL700A; EPS^i Vir^i Kan^r	This work
AW1-1	Tn*5* in EPSI; EPS^i Vir^i Kan^r	9
AW1-41	Tn*5* in EPSII; EPS^i Vir$^+$ Kan^r	9
Plasmids		
pLAFR3	Tc^r	15
pKD700	pLAFR3 with KD700 genomic DNA fragment, containing Tn*5* and flanking sequences; Kan^r Tc^r	18
pL700A	Cosmid isolate from K60 genomic library which hybridized to pKD700; Tc^r	18
pQF4	Cosmid containing EPSI and EPSII DNA	9
pPF12	Cosmid containing a portion of EPSI	9
pL5001	Cosmid subclone of *ops* complementation units	13

[a] Vir, virulent to tobacco; −, mutant; +, wild type; i, impaired relative to wild type; Tc^r, resistant to 15 μg of tetracycline per ml; Kan^r, resistant to 50 μg of kanamycin sulfate per ml; CH154$^+$, lysed by bacteriophage CH154; CH154$^-$, not affected.

[b] 700 series: KD700, KD702, KD703, KD704, KD705, KD708, KD710, KD711, KD712, KD713, and KD714.

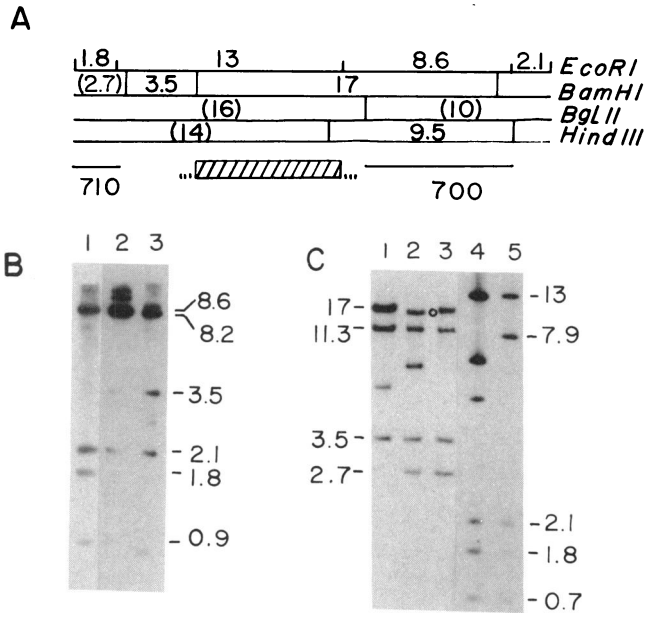

FIG. 1. Molecular mapping of KD700 and KD710. (A) Restriction map of pL700A. The numbers indicate the estimated sizes of the restriction fragments in kilobase pairs. Numbers in parentheses indicate that one end of the restriction fragment endpoint lies outside the DNA cloned in pL700A. The hatched box represents the position of the EPSI gene cluster in *P. solanacearum* AW1 (9). The positions of the Tn*5* insertions in KD700 and KD710 are underlined. (B) Comparison of the *Eco*RI and *Bam*HI digestion restriction patterns of pL700A (lane 2), pQF4 (lane 1), and pPF12 (lane 3). The blot was probed with the radiolabeled insert from pL700A. (C) Genomic Southern blot of KD710 (lane 1), KD700 (lane 2), and K60 (lane 3). DNA was isolated by the protocol of Cook et al. (6), cut with *Bam*HI, and probed with radiolabeled pL700A insert DNA. The restriction fragment containing the Tn*5* in KD700 (lane 2) is indicated with a circle. To demonstrate more clearly the location of the Tn*5* insertion, another blot of *Bgl*II and *Eco*RI digestion of K60 (lane 4) and KD700 (lane 5) chromosomal DNA was probed as described above. The numbers indicate the molecular size (in kilobase pairs) of each restriction fragment.

of mutations mapped to previously defined EPS gene clusters. Since bacteria containing mutations in the *ops* gene cluster were resistant to lysis by the LPS-specific phage, CH154, we examined the ability of the phage to form plaques on these strains. All of the 700 series of mutants we tested were lysed by CH154. Therefore, none of these mutations belong to the *ops* complementation groups, because mutations at these loci would have resulted in LPS-defective strains.

To determine whether the EPS genes affected in KD700 and KD710 mapped to the EPSI or the EPSII cluster, we radiolabeled pL700A with a random primer kit (Promega) and probed plasmid DNAs from pQF4 and pPF12 (9). pL700A hybridized strongly with the inserts from the two plasmids (Fig. 1B). In addition, the restriction pattern of pL700A was very similar to the published restriction patterns of EPSI and EPSII (Fig. 1B) (9).

To determine whether pL700A can functionally complement EPSI and/or EPSII mutations, we transformed pL700A into the EPSI mutant AW1-1 and the EPSII mutant AW1-41. pL700A restored EPS production to AW1-1 but not to AW1-41, suggesting that pL700A contained only EPSI genes. This conclusion is supported by the observation that KD700 and KD710, like other EPSI mutants, did not produce EPS when grown on minimal media. The plasmid pQF4 was also able to complement the mutation in KD710. It did not complement EPS production in KD700, but this result will be clarified below. In any case, pL700A contains genes of the same function as the EPSI cluster.

The locations of Tn*5* in KD700 and KD710 were determined by Southern blot analyses of chromosomal DNAs digested with restriction enzymes and probed with pL700A. In KD700, Tn*5* is located within a 7.9-kb *Bgl*II-*Eco*RI restriction fragment (Fig. 1C, lane 4). In KD710, Tn*5* is in a 2.7-kb *Bam*HI fragment (Fig. 1C, lane 1). These locations are not within the borders established for the previously mapped EPSI cluster (9). Instead, they lie to either side of this cluster. Also, the insert in pQF4 does not include the area where the Tn*5* of KD700 was located; hence, pQF4 could not complement KD700. These results suggest that the EPSI region may be more extensive than previous mapping suggested.

The molecular and genetic characterizations revealed an inconsistency between the results of Denny et al. (9) and those of Xu et al. (18, 19). Mutants such as AW1-1 were affected in virulence (9), whereas KD700 and KD710 were reported to be highly virulent. Since the mutations occurred in the same region where the EPSI cluster is located, we reexamined the virulence of KD700 and KD710.

Preliminary virulence assays were completed with axenic

TABLE 2. Virulence assays of *P. solanacearum* strains by the axenic eggplant seedling assay

Strain	No. killed/total no. of plants	
	Day 8[a]	Day 16[a]
K60	35/36	36/36
500155	8/10	10/10
50037	9/11	9/11
KD400	0/12	3/12
KD500	0/12	0/12
KD600	0/16	2/16
KD700	0/16	4/16
KD700A	1/29	20/29
T700	0/16	5/16
KD702	0/16	2/16
KD703	0/12	4/12
KD704	0/16	2/16
KD705	0/16	0/16
KD710	0/17	13/17
KD711	0/16	0/16
KD712	0/16	1/16
KD713	1/12	4/12
KD714	4/16	9/16

[a] Days after inoculation.

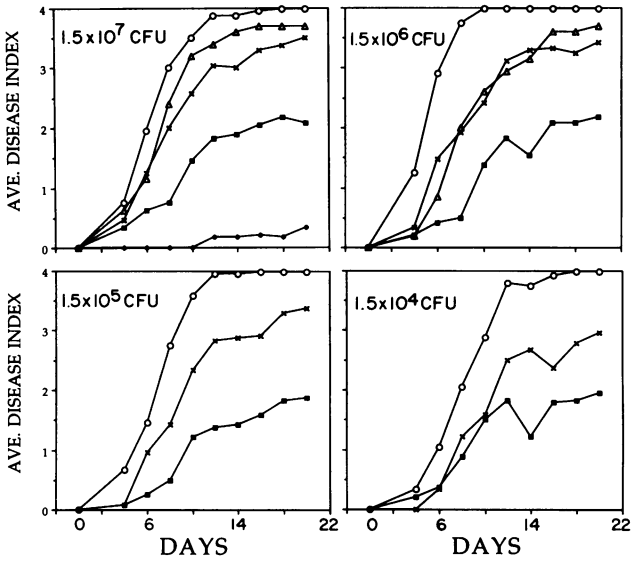

FIG. 2. Infectivity titration of tobacco plants with strains K60 (○), KD700A (△), KD710 (×), KD400 (■), and B1 (◆). The estimated number of bacteria injected into each plant is indicated in the upper left corner of each panel. B1 was tested only at 1.5×10^7 CFU per plant. KD710 was tested at 1.5×10^7 and 1.5×10^6 CFU per plant. Each datum point represents an average disease index for a minimum of five inoculated plants. Index values: 0, no disease symptoms; 1, inoculated leaf showing wilt symptoms; 2, approximately 25% of leaves with wilt; 3, approximately 50% of leaves wilted; 4, plant completely wilted.

seedlings of eggplant (*Solanum melongena* cv. Black Beauty) and tomato (*Lycopersicon esculentum* cv. Bonny Best) as previously described (13, 19). Plants were inoculated by clipping primary leaves with scissors dipped in a bacterial suspension of 3×10^8 CFU/ml (Table 2). The parental strain K60 killed 35 of 36 eggplant seedlings by the eighth day. Two strains (500155 and 50037) which harbor Tn*5* insertions but were not affected in EPS production also killed the majority of the inoculated plants. In contrast, most EPS mutants were able to kill only some of the eggplant seedlings by the 16th day. Similar results were obtained in assays with tomato seedlings (data not shown).

To be certain that the KD700 mutant had not spontaneously lost virulence during or following storage as lyophilized cells, two additional strains were constructed. The first strain was derived from C700 (KD700 transformed with pL700A) made by Xu et al. (18). We cured C700 of pL700A by selecting for kanamycin-resistant colonies which were sensitive to tetracycline. The resulting strain, KD700A, was no longer able to produce EPS on plates but could be restored to the wild-type phenotype when transformed again with pL700A. A second strain was made by integrating pKD700, the original cosmid subclone from KD700 containing the Tn*5* insertion, into the *P. solanacearum* K60 genome. An EPS$^-$ Kan^r strain, T700, was isolated. When transformed with pL700A, T700 was restored to wild-type EPS production on plates. Both KD700A and T700 were tested for virulence by the axenic seedling assay and were found to be reduced in virulence in comparison with K60 (Table 2).

An infectivity titration was completed with 3-week-old tobacco plants stem inoculated with strains KD700A, KD710, KD400, K60, and B1 (avirulent). The cells were grown to exponential phase in CPG broth (18) amended with 1 g of yeast extract per liter, centrifuged, and washed with sterile water once before being resuspended in water at various concentrations ranging from 10^6 to 10^9 cells per ml. These suspensions were used to inoculate *Nicotiana tabacum* cv. Bottom Special plants. Each plant was injected at the axil of the first fully developed leaf with 15 μl of each cell concentration. At least five plants were used for each dilution. Symptoms were recorded on a scale of 0 to 4, ranging from no symptoms to complete wilting, respectively, as described previously (19). The avirulent B1 strain did not produce disease symptoms, as expected, but K60 caused complete wilting of all plants by 10 days after inoculation when 1.5×10^6 cells per plant were injected. At the same cell concentration, KD710 caused complete wilting of only one of five plants, while KD700A and KD400 did not cause complete wilting in any of the plants after 10 days. By the 16th day, KD710 and KD700A had wilted two of five plants and two of six plants, respectively. KD400 still did not cause complete wilting of any plants (Fig. 2). The results of this infectivity titration were confirmed in an independent experiment (data not shown).

It is worthwhile to note that while KD700 and KD700A were reduced in virulence in comparison with K60 at all concentrations tested, they were more pathogenic at the higher inoculum concentration than at lower inoculum doses (compare panels at top left and bottom right in Fig. 2). This may explain the discrepancy between our results and those of Xu et al. (18). Because Xu et al. (18) used a high inoculum dose, they may have missed the reduction in virulence that would have been more obvious with a lower-concentration inoculum.

There is one additional Tn*5*-induced EPS mutant of *P. solanacearum* that may not be affected in virulence (2). This mutant, however, remains uncharacterized. Some Vir$^+$ mutants may produce reduced amounts of EPS in culture but produce wild-type levels of EPS in planta, as reported by Denny and Baek (9). It is possible that the mutant isolated by Boucher et al. (2) belongs in this category.

Although we confirmed that there is a consistent require-

ment for production of EPS for wild-type virulence in *P. solanacearum*, it is clear that several mutants that are reduced in EPS production can wilt plants but at a lower rate than K60. At present, we do not know whether this ability to wilt plants can be attributed to partial EPS production in planta or to the compensatory effects of other virulence factors produced by the pathogen.

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