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Genetic Evidence that Extracellular Polysaccharide Is a Virulence Factor of *Pseudomonas solanacearum*

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To test whether the extracellular polysaccharide (EPS) produced by *Pseudomonas solanacearum* is responsible for the wilt symptoms caused by this plant pathogen, two classes of Tn*5*-induced, EPS-impaired mutants were further characterized and manipulated. In comparison with wild-type AW1, the class I mutant AW1-1 produced about 95% less EPS on rich and minimal media and slowly wilted one third as many tomato plants in stem and root inoculation assays. In contrast, class II mutants produced nearly wild-type amounts of EPS on minimal medium and largely retained virulence. Eight cosmid clones were identified in a genomic library of wild-type strain AW1 that restored EPS production by the mutants to varying degrees, with one cosmid fully complementing both class I and class II mutants. Southern blot analysis, restriction mapping, subcloning, and Tn*3*-HoHo1 mutagenesis demonstrated the existence of two neighboring regions involved in EPS production. The minimum sizes for regions I and II were 9 and 2.6 kilobases (kb), respectively. Phenotype conversion, which results in spontaneous EPS$^-$ mutants, decreased expression of *lacZ* fusions in both regions. EPS$^+$ merodiploids of AW1-1 were more virulent than AW1-1, but plasmid instability *in planta* hindered our interpretation of these experiments. However, allelic replacement in certain AW1-1 merodiploids generated strains that were EPS$^+$ and kanamycin sensitive, and these wild-type recombinants were invariably as virulent as AW1. These results provide genetic evidence that EPS is an important virulence factor required by *P. solanacearum* strain AW1 to wilt tomato.

Additional keywords: Lycopersicon esculentum.

Pseudomonas solanacearum (Smith) Smith is a soilborne plant pathogenic bacterium that causes lethal wilting diseases in many cultivated and wild plants throughout the world (Buddenhagen and Kelman 1964; Persley 1986). Early research suggested that extracellular polysaccharide (EPS) might be the most important virulence factor contributing to the ability of *P. solanacearum* to induce wilt (Husain and Kelman 1958). The EPS produced by wild-type strains growing in a rich culture medium is a water-soluble, high molecular weight polymer composed mostly of *N*-acetylgalactosamine (Akiyama *et al.* 1986; Drigues *et al.* 1985) that is released as an amorphous slime (Buddenhagen and Kelman 1964; Denny *et al.* 1988). Production of this EPS within xylem vessels, which *P. solanacearum* colonizes extensively (Wallis and Truter 1978), probably would reduce water movement and cause wilt symptoms (Buddenhagen and Kelman 1964; Van Alfen 1989). However, the EPS produced by *P. solanacearum in planta* has not been characterized, and, although wilting of infected tomato plants is associated with reduced water uptake (Denny *et al.* 1990), the cause of the vascular dysfunction was not determined. Experiments showing that cuttings of tomato seedlings wilt when placed in solutions of crude EPS produced in culture or in sap from infected plants (Akiyama *et al.* 1986; Husain and Kelman 1958) are not very informative, because they do not directly address the role of EPS during pathogenesis. Therefore, the physiological and biochemical data are insufficient to conclude that EPS is responsible for the wilt symptoms caused by *P. solanacearum*.

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In investigating the role of EPS in wilt, the best approach is to use defined mutants that are altered only in their production of EPS (Van Alfen 1989). Unfortunately, generating the desired mutants of *P. solanacearum* has proved difficult. Spontaneous phenotype conversion results in "PC-type" mutants that are EPS$^-$ and do not wilt tomato plants (Boucher *et al.* 1985; Brumbley and Denny 1990; Husain and Kelman 1958). However, because PC-type mutants are pleiotropic (Buddenhagen and Kelman 1964; Brumbley and Denny 1990), it is unclear whether the loss of EPS production is solely responsible for their reduced ability to cause wilt symptoms. It should be noted that, because the degree of wilt induced in a susceptible host is usually considered to be commensurate with the virulence of *P. solanacearum* (a convention that we follow), PC-type strains are commonly referred to as being avirulent (Buddenhagen and Kelman 1964; Sequeira 1985). Nevertheless, PC-type mutants remain pathogenic and cause other disease symptoms such as stunting, stem necrosis, and proliferation of adventitious roots (Denny *et al.* 1988; Husain and Kelman 1958). We recently found that the multiple traits affected by phenotype conversion appear to be regulated by the *phcA* gene, with a functional *phcA* being necessary for the maintenance of the wild-type phenotype (Brumbley and Denny 1990).

Early research results with poorly characterized Tn*5*-induced EPS-deficient mutants were contradictory, with Staskawicz *et al.* (1983) finding an EPS$^-$ mutant that did not wilt potato and Boucher *et al.* (1985) reporting EPS-deficient mutants that still wilted tomato plants. Recently, Xu *et al.* (1990) described Tn*5* mutants of strain K60 that produce very little galactosamine-containing EPS in culture or in tobacco plants and that are almost as virulent as

the wild type. However, the effect of the Tn*5* insertions on other known virulence factors was not determined, and they were unable to restore full virulence to the mutant strains. Therefore, their conclusion that EPS may not be required for disease development by *P. solanacearum* must be examined further (Coplin and Cook 1990).

Preliminary genetic evidence in support of EPS as a virulence factor was presented by Denny *et al.* (1988), who described three classes of Tn*5*-induced EPS-deficient mutants of strain AW1. Class I and class II mutants were designated as EPS-impaired (EPS^i), because their butyrous colonies are lavender colored on tetrazolium chloride-containing media rather than the dark red typical of EPS^- PC-type mutants. Activity of endoglucanase and polygalacturonase, two extracellular enzymes that contribute to virulence (Denny *et al.* 1990), was normal for both class I and class II mutants. The class I and class II mutants had single Tn*5* insertions in two different *Eco*RI fragments and differed in virulence. The rate at which tomato plants were wilted by the single class I strain (AW1-1) was 16% that of the wild-type parent, compared with 78% for the three class II strains (AW1-41, AW1-71, and AW1-74); this difference was correlated with the amount of EPS produced *in planta*. The single Tn*5*-induced class III mutant (AW1-80) is EPS^-, but is indistinguishable from spontaneous, pleiotropic PC-type mutants (Brumbley and Denny 1990).

The present study extends the research with the class I and class II EPS^i mutants of strain AW1 by cloning the wild-type regions that complement these mutations in culture and that partially restore virulence to AW1-1. The cloned DNA also allowed EPS^+ wild-type recombinants of strain AW1-1 to be recovered, and they were all fully virulent. These results are strong genetic evidence that EPS is an important virulence factor required by *P. solanacearum* strain AW1 to cause wilt symptoms on tomato.

MATERIALS AND METHODS

Bacterial strains and plasmids. Descriptions of the bacterial strains and plasmids used in this study are given in Table 1. Strain AW1-PC is a spontaneous, pleiotropic PC-type mutant that was previously designated AW1-A (Denny *et al.* 1988). All strains were stored at −70° C in 15% glycerol.

Culture conditions. *P. solanacearum* strains were routinely grown at 30° C on BGT agar medium or in BG broth (Boucher *et al.* 1985), and *Escherichia coli* strains were grown at 37° C on Luria-Bertani medium (Maniatis *et al.* 1982). Medium containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) had isopropyl-β-D-thiogalactopyranoside, but lacked tetrazolium chloride (Carney and Denny 1990). The minimal medium (MM) for *P. solanacearum* was one-quarter-strength M63 salts (without thiamine or $MgSO_4$) (Brumbley and Denny 1990) plus 0.5% glucose, solidified with agar when required. Antibiotics were added when necessary as follows: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 40 μg/ml; nalidixic acid (Nal), 40 μg/ml; tetracycline (Tc), 15 μg/ml.

Virulence assays. Virulence of the *P. solanacearum* strains on tomato (*Lycopersicon esculentum* Mill. 'Marion'), measured by the rate at which leaves wilted, was assessed by using two different inoculation methods. The primary method was the stem inoculation procedure described in detail elsewhere (Roberts *et al.* 1988). In each experiment, three to five plants received a water suspension of each strain via a stab wound in the stem. The number of leaves wilted was recorded for each plant on a daily basis and the percentage of leaves wilted was calculated for each treatment. The time required for pairs of strains to cause 50% wilt

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Pseudomonas solanacearum		
AW1	Derivative of wild-type strain AW, EPS^+ Nal^r	Denny *et al.* 1988
AW1-1	Class I mutant of AW1 (*eps-1*::Tn*5*), EPS^i Nal^r Km^r	Denny *et al.* 1988
AW1-128, -129, -130, -131	Class I mutants of AW1 (*eps-128*, *-129*, *-130*, *-131*::Tn*3*-HoHo1), EPS^i Nal^r Ap^r	This study
AW1-41, -71, -74	Class II mutants of AW1 (*eps-41*, *-71*, *-74*::Tn*5*), EPS^i Nal^r Km^r	Denny *et al.* 1988
AW1-PC	Spontaneous phenotype conversion mutant of AW1, EPS^- Nal^r	Denny *et al.* 1988
AW1-80	Class III induced PC-type (*phcA80*::Tn*5*), EPS^- Nal^r Km^r	Denny *et al.* 1988
Escherichia coli		
DH5α	φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17 supE44 gyrA96* Δ(*lacZYA-agrF*)U169	BRL[b]
Plasmids		
pLAFR3	Broad host range cosmid vector, Tc^r	Staskawicz *et al.* 1987
pRK2013	Conjugation helper plasmid, Km^r	Figurski and Helinski 1979
pUC9	ColE1 Ap^r	Vieira and Messing 1982
pBS1	13.7-kb *Eco*RI fragment containing *eps-1*::Tn*5* in pUC9, Ap^r	This study
pBS410	16-kb *Eco*RI fragment containing *eps-41*::Tn*5* in pLAFR3, Tc^r	This study
pCB5, pOF6, pQG10, pIH3, pOE10, pSG5, pQF4, pPF12	Cosmid clones containing AW1 DNA in pLAFR3, Tc^r	This study
pQF40/41	13.7-kb *Eco*RI fragment with most of region I in pLAFR3, opposite orientations, Tc^r	This study
pQF42	8.3-kb *Bam*HI-*Eco*RI fragment from region I in pLAFR3, Tc^r	This study
pQF43	8.1-kb *Bam*HI-*Hin*dIII fragment from region I in pLAFR3, Tc^r	This study
pQF44	6.3-kb *Hin*dIII-*Eco*RI fragment with region II in pLAFR3, Tc^r	This study
pQF45	1.9-kb *Bam*HI fragment from region II in pLAFR3, Tc^r	This study
pGA93	2.2-kb *Eco*RI-*Bgl*II fragment with *phcA* in pLAFR3, Tc^r	Brumbley and Denny 1990

[a] Ap^r, Km^r, Nal^r, and Tc^r designate resistance to ampicillin, kanamycin, nalidixic acid, and tetracycline, respectively. EPS, extracellular polysaccharide; EPS^i, impaired in EPS production. EPS^i strains produce butyrous colonies on BGT plates, but they have a thin white coating that distinguishes them from the EPS^- strains.

[b] Bethesda Research Laboratories, Gaithersburg, MD.

was compared with the nonparametric Mann-Whitney U test (Sokal and Rolf 1969).

An alternate inoculation procedure required that *P. solanacearum* infect tomato plants through undisturbed roots (Denny *et al.* 1990). Each strain of *P. solanacearum* was tested on 10 seedlings grown in 25-mm glass culture tubes by pipeting water suspensions of the pathogen onto the soil. Plants were watered daily by bringing the soil to field capacity. The percentage of leaves wilted was determined as in the standard assay. Infection of plants was determined at the end of each experiment by checking for growth of *P. solanacearum* from sap squeezed from the base of cut stems onto BGT plates supplemented with Nal and cycloheximide (50 μg/ml).

Growth of *P. solanacearum* in tomato stem tissue. Tomato plants were stem inoculated as above. At each time interval stems from three or four plants were surface disinfested, and 1.0-cm transverse sections were finely chopped and then crushed in 1.0 ml of phosphate-buffered saline (0.1 M KPO_4, 0.15 M NaCl, 3 mM KCl, adjusted to pH 7.3). Distribution of *P. solanacearum* within the stems was qualitatively assessed by applying 5-μl drops of the supernatant from each section to BGT plates supplemented with Nal and cycloheximide (50 μg/ml). Multiplication was quantified by pooling samples for the 9-cm region centered on the site of inoculation and applying 10-μl drops of a dilution series to modified BGT (0.1× normal peptone) supplemented as above. These plates were incubated at 37° C, which reduced EPS production, but had little effect on the viability of the *P. solanacearum* strains.

Table 2. Production of extracellular polysaccharide (EPS) in culture by wild-type, mutant, and merodiploid strains of *Pseudomonas solanacearum*

	Plasmid in merodiploid[a]						
Strain	**None**	**pPF12**	**pCB5**	**pIH3**	**pOE10**	**pSG5**	**pQF4**
AW1	+++	+++	+++	+++	+++	+++	+++
	640(380)	520	990	670			1,210
Class I							
AW1-1	i	i	i	i	+	++	+++
	40(20)	80	60	210	280	420	670
Class II							
AW1-41	i	±	+++	+++	+++	+++	+++
	40(300)	180	930	890	670	750	1,330
AW1-71	i	±	+++	+++	+++	+++	+++
	70(320)	230	1,060	1,050			990
AW1-74	i	±	+++	+++	+++	+++	+++
	40(200)	231	820	880			1,730
Class III							
AW1-PC	neg	neg	±	±	±	±	±
	60(50)	60	90	120			90
AW1-80	neg	neg	±	±	±	±	±
	60(30)	60	210	420			330

[a] The first line for each strain gives the score for EPS produced during growth on BGT plates. Qualitative values for EPS slime were as follows (in descending order): +++, copious; ++, moderate; +, little; ±, very little (only in areas of confluent growth). No fluidal EPS was produced by EPS-impaired (i) and EPS^- negative (neg) strains. The second line for selected strains gives the micrograms of hexosamine per milligram of cell protein produced during growth in BG broth or MM broth (values in parentheses). Data are the averages of two or more experiments. The results for pOF6 and pQG10, which are not shown, were similar to those for pCB5 and pIH3, respectively. EPS was recovered from broth cultures and quantified as described in the test.

Quantification of EPS. EPS in culture supernatants of *P. solanacearum*, grown in BG or MM broth for 4 days at 30° C, was quantified as previously described (Brumbley and Denny 1990). Briefly, the EPS was precipitated with acetone and the concentration of hexosamines, which is a reliable indication of EPS content (Denny *et al.* 1988; Xu *et al.* 1990), was estimated with a modified Elson and Morgan reaction. The results were normalized by correcting for the amount of total protein in solubilized cell pellets as determined using the bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL).

In two stem inoculation experiments, the EPS produced by *P. solanacearum* growing in tomato plants was recovered from homogenized stem tissue as described previously (Denny *et al.* 1988), except that 4 volumes of acetone was

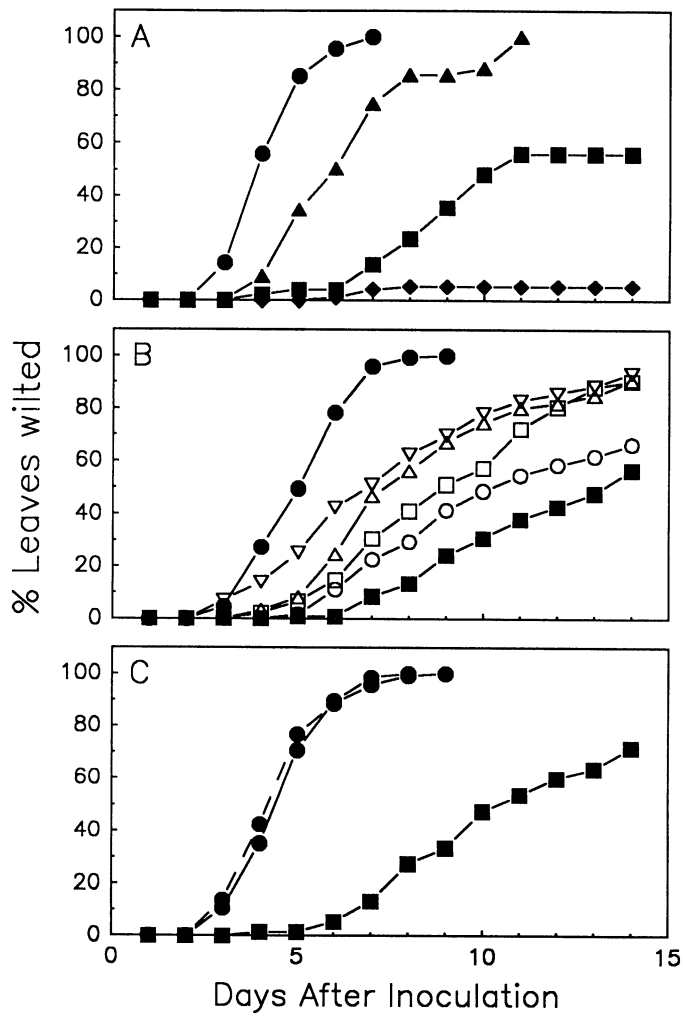

Fig. 1. Virulence of *Pseudomonas solanacearum* strains on tomato plants when inoculum was injected into the stem. **A,** A comparison of the wild-type strain AW1 (●) with EPS^i class I strain AW1-1 (■), EPS^i class II strain AW1-41 (▲), and EPS^- class III strain AW1-PC (◆). Data are the average of two or more experiments. **B,** Virulence of AW1 (●), AW1-1 (■), and AW1-1 merodiploids containing the cosmids pSG5 (▽), pOE10 (△), pQF4 (□), and pIH3 (○). Data are the average of three to five experiments. **C,** Virulence of AW1 (●), AW1-1 (■), and five independent EPS^+ wild-type recombinants of AW1-1 (●- - -●). Each wild-type recombinant strain was tested two or three times; they were equally virulent, so the results were combined.

used instead of ethanol to precipitate the EPS. Hexosamines in the plant extracts were quantified as above and corrected for background levels recovered from healthy controls. Previous results established that only small quantities of hexosamine other than galactosamine are present in healthy or diseased stem tissue (Denny *et al.* 1988; Xu *et al.* 1990).

DNA manipulation and bacterial matings. Total DNA was isolated from *P. solanacearum* cells as previously described (Denny et al. 1988), and an alkaline lysis procedure was used to isolate plasmid DNA from *E. coli* (Birnboim 1983). Bacterial matings were performed as described by Carney and Denny (1990). Mutagenesis of cosmid DNA with Tn*3*-HoHo1 to create *lacZ* fusions, and subsequent transfer of these mutations into the genome of AW1, were performed as described elsewhere (Denny *et al.* 1990). Standard procedures were followed for transformation of *E. coli*, digestion with restriction enzymes, electrophoresis, Southern blots, nick translation, and hybridization (Carney and Denny 1990; Maniatis *et al.* 1982). DNA fragments were isolated from low-temperature melting agarose gels by the freeze and squeeze method (Benson 1984).

RESULTS

Characterization of EPS-deficient mutants. The two classes of EPS^i mutants are indistinguishable when grown on BGT agar plates (Denny *et al.* 1988). In contrast, when cultured on MM agar plates, the class I strain (AW1-1) remained EPS-deficient, whereas the class II strains (AW1-41, AW1-71, and AW1-74) resembled the wild-type AW1 (data not shown). This difference between the two EPS^i classes was confirmed by quantifying EPS recovered from broth cultures (Table 2). The EPS^i and EPS$^-$ strains grown in BG broth produced only 6–11% of the EPS recovered from AW1, which is similar to our previous results (Denny *et al.* 1988), even though this study used a different method to recover the EPS. Consistent with the change in colony morphology seen on MM agar, growth in MM broth did not affect EPS production by AW1-1, but stimulated the class II strains to make near wild-type amounts of EPS. Assaying supernatants of the EPS-deficient strains before acetone precipitation did not reveal a detectable level of nonprecipitable hexosamine. In addition, the EPS precipitated with acetone required hydrolysis before hexosamines could be detected, indicating a largely polymeric form.

As was reported previously (Denny *et al.* 1988), when tomato plants were stem inoculated with 1–5 × 10^6 cells, AW1-41 was more virulent than AW1-1, and AW1-PC (an EPS$^-$, pleiotropic PC-type) was essentially incapable of causing wilt (Fig. 1A). A 100-fold reduction in the inoculum concentration of AW1, AW1-1, and AW1-41 had little effect on the behavior of these strains (data not shown). Over the course of 10 experiments, AW1-1 killed 62% fewer tomato plants than did AW1. Reducing the inoculum concentration of AW1-PC usually reduced the severity of stunting and adventitious root bud formation, and sometimes the inoculated plants had no disease symptoms. The marked reduction in virulence of some EPS-deficient strains was not due to a failure to multiply within stems of tomato plants (Fig. 2). All three strains tested migrated throughout

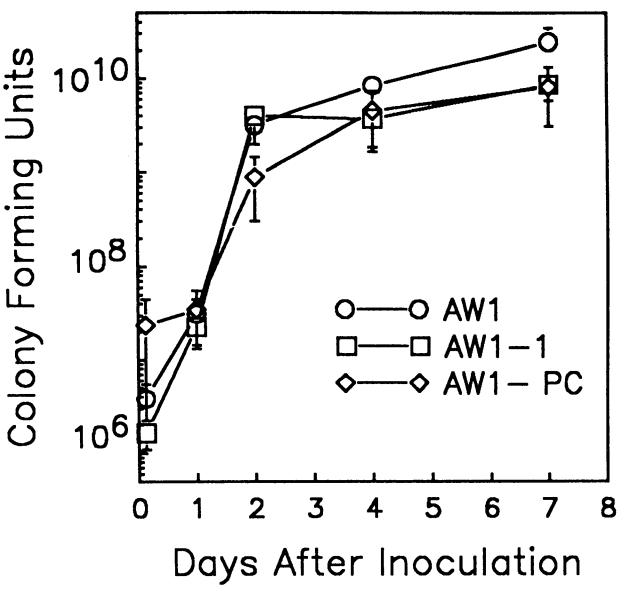

Fig. 2. Multiplication of *Pseudomonas solanacearum* within stems of tomato plants. Plants were inoculated via a stab wound with strains AW1, AW1-1, or AW1-PC, and viable bacteria were recovered from the 9-cm stem segment centered on the site of inoculation. Data are the mean of two or three independent experiments ± SE.

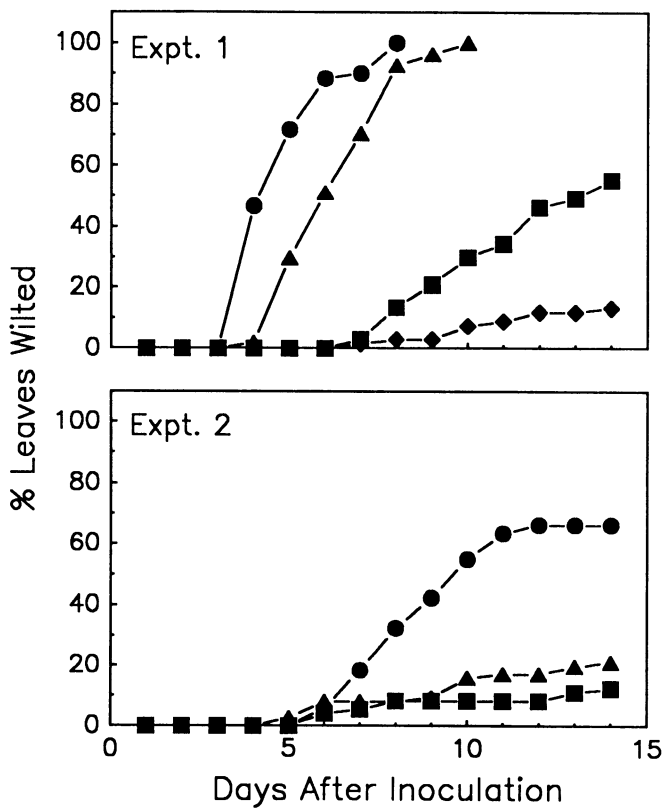

Fig. 3. Virulence of *Pseudomonas solanacearum* strains on tomato plants when inoculum was added to the soil. The results are from two separate experiments (experiments 1 and 2), for which strains AW1 (●), AW1-1 (■), AW1-41 (▲), or AW1-PC (◆) were tested. Each strain was tested on 10 plants whose roots were not artificially wounded. AW1-PC did not infect any of the plants in the second experiment.

the entire length of the stems within 48 hr, but because AW1-1 and AW1-PC did not appear to have reached the same density as AW1 in the base of the stems, these EPS-deficient strains may be slightly reduced in their ability to move within the xylem (data not shown).

The ability of the EPS-deficient mutants to infect and wilt tomato plants via undisturbed roots was examined in two experiments (Fig. 3). In the first experiment, AW1-1 and AW1-41 infected all of the plants and caused wilt about as they did when stem inoculated. In contrast, AW1-PC infected 40% of the plants, but only a few of the cotyledons wilted. For the second experiment, the tomato seedlings were grown under a higher light regime before inoculation and were more robust. In this case, AW1 infected 80% of the plants and killed 70% of them. AW1-1 and AW1-41 behaved similarly, infecting 30 and 20% of the plants and killing 10 and 20% of them, respectively. AW1-PC was not recovered from the sap of any of the 10 plants in the second experiment.

Cloning of wild-type DNA required for production of EPS. A 13.7-kb *Eco*RI fragment containing the Tn*5* insertion from AW1-1 (*eps-1*::Tn*5*) was cloned in pUC9 and designated as pBS1 (Fig. 4B). The *eps-1*::Tn*5* mutation mapped to a point 5.0 kb from the *Hin*dIII site at the right end of the fragment and 3.0 kb from the nearest *Bam*HI site. Colony blots of a previously prepared genomic library of AW1 (Carney and Denny 1990) were screened for homology to the *P. solanacearum* DNA cloned in pBS1, and eight cosmids that hybridized were identified. Restriction mapping showed that these cosmids spanned a 55-kb stretch of the wild-type genome (Fig. 4A, C).

Each of the eight cosmids was transferred individually into AW1 and into the EPS-deficient strains to assess their effect on EPS production (Table 2). Although not apparent on BGT plates, several cosmids enhanced EPS production by AW1. The five cosmids containing most or all of the 13.7-kb *Eco*RI fragment complemented AW1-1 to varying degrees; pQG10 and pIH3 that have the P*lac* promoter

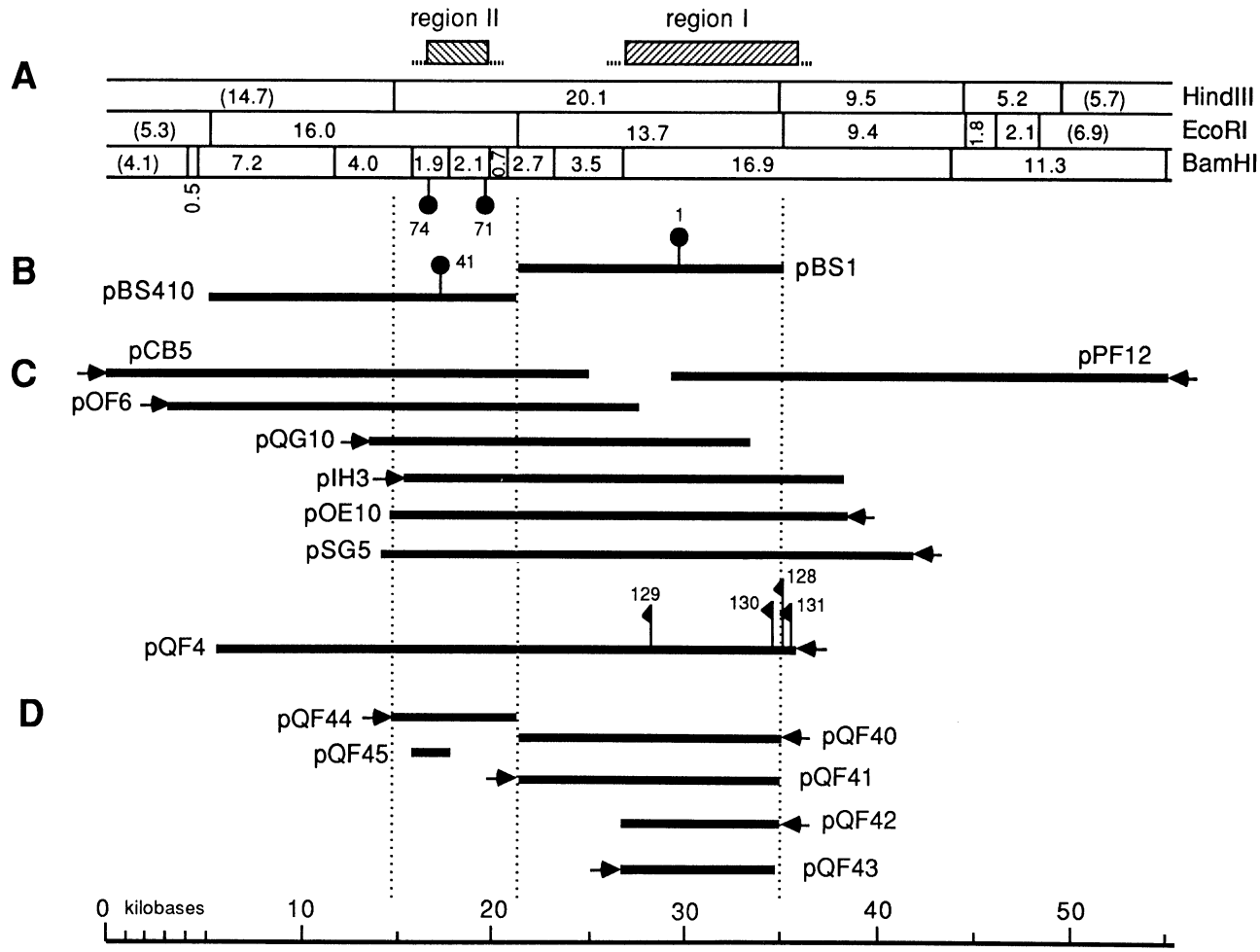

Fig. 4. The cloned portion of *Pseudomonas solanacearum* strain AW1 that has regions I and II involved in production of EPS. **A,** The restriction map of the 55 kb of strain AW1 DNA contained in the eight cosmids. The numbers are fragment sizes in kilobases (those in parentheses are the distance to the end of the cloned DNA, not to the next restriction site). The positions of *eps-71*::Tn*5* and *eps-74*::Tn*5* in the genome are indicated by the filled circles. The crosshatched boxes designate minimum sizes for regions I and II, the endpoints of which are unknown. **B,** The *Eco*RI fragments with *eps-1*::Tn*5* and *eps-41*::Tn*5* cloned in pBS1 or pBS410, respectively; filled circles show the position of the insertions. **C,** The wild-type DNA cloned in eight cosmids that share homology with pBS1. The arrows designate the direction of transcription from the P*lac* promoter in the pLAFR3 vector. The positions of *eps-128*, *eps-129*, *eps-130*, and *eps-131*::Tn*3*-HoHo1 in pQF4 are indicated; the flags point in the direction of transcription of *lacZ*. **D,** The insert DNA of the subclones that helped define regions I and II. See Table 1 for a description of the restriction fragments that were subcloned into pLAFR3.

on the left (as shown in Fig. 4C) restored less EPS production than pOE10, pSG5, and pQF4 that have P*lac* on the right. Only pQF4 completely restored EPS production by AW1-1 to a wild-type level. All of the cosmids except pPF12 completely restored EPS production to the three EPS^i class II strains, suggesting that the class II mutations are clustered close to *eps-1*::Tn*5* (see next section for details). All of the cosmids except for pPF12 also restored limited EPS production to the PC-type EPS$^-$ class III strains.

Portions of pQF4 were subcloned into pLAFR3 (Fig. 4D) and found to vary in their ability to complement the EPS^i class I strain. The 13.7-kb fragment was cloned in the same (pQF40) or opposite (pQF41) orientation with respect to P*lac* as in pQF4. The AW1-1(pQF40) merodiploid was extremely mucoid on BGT plates and produced an almost normal amount of EPS in BG broth culture (610 μg of EPS per milligram of cell protein compared with 800 μg/mg for AW1 grown at the same time). The AW1-1(pQF41) merodiploid was moderately mucoid on BGT plates and produced 410 μg of EPS per milligram of protein in BG broth. Neither pQF40 nor pQF41 had any effect on EPS production by AW1-41. The 8.3-kb *Bam*HI-*Eco*RI fragment in pQF42 and the 8.1-kb *Bam*HI-*Hin*dIII fragment in pQF43 (Fig. 4D) both failed to complement AW1-1, suggesting that the mutated transcriptional unit extends beyond the *Bam*HI site 3 kb to the left of the Tn*5* insertion.

Preliminary characterization of two regions involved in EPS production. One region involved in EPS production was identified using four Tn*3*-HoHo1 insertions in pQF4 that abolished the ability of this cosmid to complement the *eps-1*::Tn*5* mutation in AW1-1. Restriction mapping showed that the *eps-129*::Tn*3*-HoHo1 insertion was 1.2 kb to the left of *eps-1*::Tn*5*, whereas the other three Tn*3*-HoHo1 insertions were clustered within the terminal 1 kb at the right end of pQF4 (Fig. 4C). All four insertions in pQF4 created Lac$^+$ fusions that were oriented with the promoterless *lacZ* transcribed from right to left (as shown in Fig. 4C). Each of the Tn*3*-HoHo1 insertions was transferred into the genome of AW1, and the resulting mutants, designated AW1-128 to -131, were all EPS^i on BGT plates and Lac$^+$ on BG-X-Gal plates. pQF40 complemented AW1-129 but not AW1-128, AW1-130, or AW1-131, whereas complementation by pPF12 was exactly the reverse. Together with the complementation data for AW1-1, these results indicate that 1) region I spans at least 9 kb, with the left border in the 2.7- or 3.5-kb *Bam*HI fragments and the right border somewhere beyond the right end of pQF4 (Fig. 4A); 2) there are at least two genes in region I and they are likely to be transcribed from right to left.

Because spontaneous PC-type derivatives of AW1 are EPS$^-$ due to mutation of the positive-acting *phcA* regulatory gene (Brumbley and Denny 1990), we tested whether *eps* genes in region I are regulated by phenotype conversion. The EPS^i Lac$^+$ strains AW1-129 and AW1-130 were allowed to undergo phenotype conversion during 5 days in stationary culture (Brumbley and Denny 1990), and the resulting EPS$^-$PC-type derivatives (AW1-129PC and AW1-130PC) were found to be Lac$^-$ on BG-X-Gal plates. Phenotype conversion had not directly inactivated the *lacZ* fusions, because the merodiploids AW1-129PC(pGA93) and AW1-130PC(pGA93), which carry a wild-type copy of *phcA* on a plasmid, were EPS^i and Lac$^+$. Therefore, it seems that at least two *eps* genes in region I require a functional *phcA* gene for normal expression.

The similar phenotype of the class II mutants and their complementation by pIH3 (and other cosmids) suggested that these Tn*5* insertions mark a second region for EPS production that is close to region I. This possibility was confirmed by subcloning the 6.3-kb *Hin*dIII-*Eco*RI fragment in pQF44 (Fig. 4C) that restored EPS production to all three class II mutants on BGT plates (data not shown). In addition, using pQF44 to probe Southern blots of *Bam*HI or *Hin*dIII digested AW1-41, AW1-71, and AW1-74 genomic DNA, mapped the insertions to either the 1.9- or 2.1-kb *Bam*HI fragments (Fig. 4A, B). The position of *eps-41*::Tn*5* in the 1.9-kb *Bam*HI fragment was confirmed by restriction mapping pBS410 (Fig. 4B). pBS410 did not complement either AW1-71 or AW1-74, suggesting that the three class II Tn*5* insertions are in a single complementation unit. The 1.9-kb *Bam*HI fragment in pQF45 (Fig. 4D) also did not complement any of the class II mutants. These results indicate that region II has a minimum size of 2.6 kb (the distance between the *eps-71*::Tn*5* and *eps-74*::Tn*5* insertions) (Fig. 4A) and likely contains a single gene. Region II also appeared to be regulated by phenotype conversion, because expression of a Tn*3*-HoHo1 Lac$^+$ fusion that resulted in a typical class II EPS^i mutant was similar to the *lacZ* fusions in region I (data not shown).

Performance of AW1-1 merodiploids *in planta*. Four cosmids that fully or partially restored EPS production

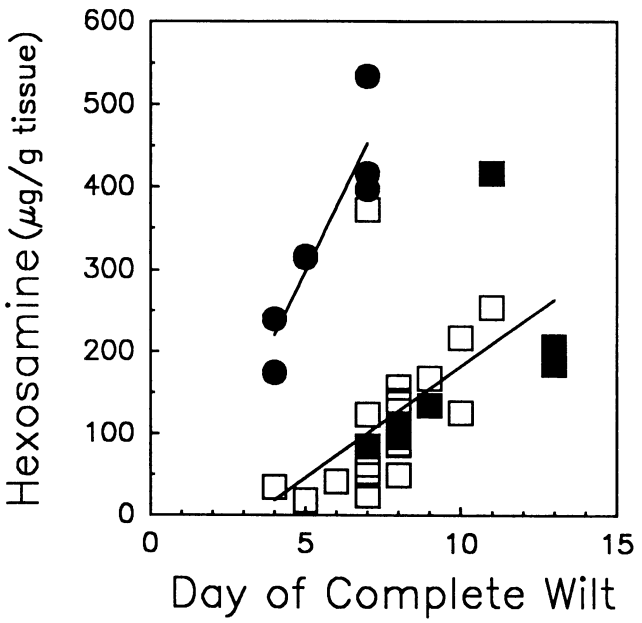

Fig. 5. The relationship between the time tomato plants completely wilted and the amount of extracellular polysaccharide (EPS) produced *in planta*. Data are from two experiments where tomato plants were inoculated with AW1 (●) or four different AW1-1 merodiploids (□) via stem wounds; data for AW1-1(pQF4) are shown by (■). Each plant was harvested when 100% of the leaves were wilted, and total hexosamines in the stems estimated as described in the text. Regression lines: AW1, $y = -91.9 + 77.8x$, ($r = 0.912$); AW1-1 merodiploids, $y = -89.9 + 27.1x$, ($r = 0.585$).

to AW1-1 in culture (pQF4 > pSG5 > pOE10 > pIH3; see Table 2) were tested to determine whether they similarly enhanced the virulence of AW1-1. Although all of the recombinant cosmids increased the ability of AW1-1 to wilt tomato plants, none of them completely restored virulence (Fig. 1B). Statistically, every merodiploid except AW1-1(pIH3) induced 50% wilt sooner than AW1-1 ($P = 0.1$), and all but AW1-1(pSG5) induced wilt later than AW1 ($P = 0.05$). The cosmid vector alone had no effect on AW1-1 (data not shown). Although variation among the merodiploids was not significant, the degree to which the cosmids improved virulence by AW1-1 was consistent (pSG5 > pOE10 > pQF4 > pIH3; Fig. 1B). Therefore, EPS production in culture by the merodiploids was generally correlated with virulence except for the case of AW1-1(pQF4), which was distinctly less virulent than AW1, even though it resembled the wild type in culture.

When considering the involvement of EPS in wilt, the amount of EPS that the strains produce *in planta* is a more relevant factor than their behavior in culture. Between 175 and 535 μg of EPS per gram of stem tissue was recovered from tomato plants killed by wild-type AW1, whereas the plants killed by the merodiploids had about one third as much EPS on any given day (Fig. 5). Plants infected with AW1-1, most of which were not completely wilted within 14 days, averaged 44 μg of EPS per gram of tissue. The amount of EPS recovered from the plants generally increased with the time required for complete wilt to occur for both AW1 and the merodiploids. In contrast to its behavior in culture, AW1-1(pQF4) produced essentially the same amount of EPS as the other merodiploids *in planta* (Fig. 5). Therefore, the low virulence of the merodiploids, including AW1-1(pQF4), was coincident with their producing substantially less EPS *in planta* than AW1.

Isolates of Nal^r *P. solanacearum* recovered from plants inoculated with AW1-1 were invariably Km^r and either EPS^i or EPS$^-$ (like spontaneous PC-type mutants). When Nal^r *P. solanacearum* were recovered from tomato plants killed by the AW1-1 merodiploids there were five distinguishable phenotypes. A minority of the isolates were Km^r Tc^r and had a colony morphology either like the original merodiploids that were inoculated (i.e., EPS^i or various degrees of EPS$^+$) or were EPS$^-$. A majority of the isolates recovered were Km^r Tc^s and either EPS^i or EPS$^-$; these presumably arose from loss of the cosmid during growth in the absence of antibiotic selection *in planta*. Unexpectedly, isolates that were fully EPS$^+$ and Km^s Tc^s were occasionally recovered. That EPS$^+$ derivatives were found when the merodiploid used as the inoculum was EPS^i (i.e., AW1-1[pIH3]); that antibiotic resistance markers for both the cosmid and the Tn*5* insertion were always lost suggested that allelic replacement had produced wild-type recombinants.

Characterization of AW1-1 EPS$^+$ wild-type recombinants. To produce EPS$^+$ derivatives under controlled conditions, AW1-1 and selected EPS^i merodiploids were grown in BG broth without antibiotics (Table 3). Only EPS^i Km^r colonies were recovered from cultures of AW1-1, AW1-1(pLAFR3), or AW1-1(pCB5). In contrast, EPS$^+$ Km^s isolates were recovered only from those merodiploids that carried cosmids with DNA overlapping the *eps-1*::Tn*5* insertion. No EPS$^+$ Km^r isolates were recovered in these experiments, and except for most of the derivatives of AW1-1(pQF42), the EPS$^+$ isolates were Tc^s. Similar EPS$^+$ Km^s isolates were recovered when the merodiploids were cultured in MM broth without antibiotics (data not shown). Sixteen independent EPS$^+$ Km^s isolates, which came from plants and broth cultures, produced wild-type levels of EPS during growth in BG broth (data not shown). Southern blot analysis of four EPS$^+$ Km^s Tc^s isolates that were generated *in vitro* (and that were used in plant inoculations, see below) showed that the Tn*5* insert was either mostly or completely lost (Fig. 6A). More importantly, the native 8.3-kb *Bam*HI-*Eco*RI restriction fragment from region I was regenerated (Fig. 6B). These results indicate that allelic replacement, presumably via homologous recombination, results in EPS$^+$ wild-type recombinants. We cannot readily explain how two of the EPS$^+$ strains retained a portion of the Tn*5* insertion.

Five independent EPS$^+$ Km^s Tc^s wild-type recombinants were selected for virulence assays: Three were from AW1-1(pIH3), and one each was from AW1-1(pQF42) and AW1-1(pQF43). One wild-type recombinant from AW1-1(pIH3) was recovered from an infected plant, but the other four strains were produced *in vitro* (see above). The five EPS$^+$ wild-type recombinants were tested multiple times for virulence in the stem inoculation assay and they always wilted tomato plants as quickly as AW1 (Fig. 1C).

DISCUSSION

Further characterization of the EPS-deficient mutants of strain AW1 strengthened the likelihood that EPS is a virulence factor of *P. solanacearum*. The EPS^i class I strain and the PC-type strain both grew well and spread rapidly throughout the stems of tomato plants, so the failure to multiply can be ruled out as a reason for their reduced virulence in the standard stem inoculation assay. The earlier suggestion that EPS production can be induced in the class II strains (Denny *et al.* 1988) was confirmed by the observation that growth in minimal medium stimulated normal levels of EPS. The almost normal amount of EPS that the class II strains produce *in planta* (Denny *et al.* 1988; T. P. Denny, unpublished) is now understandable. The

Table 3. Recovery of EPS$^+$ wild-type recombinants from EPS^i AW1-1 merodiploids after growth in culture without antibiotics[a]

Plasmid	Overlap with *eps-1*::Tn*5*	EPS$^+$ colonies recovered[b]	
		Frequency	Antibiotic resistance
None	...	$<1 \times 10^{-6}$	NA
pLAFR3, pCB5	None	$<1 \times 10^{-6}$	NA
pPF12	0.2 kb	$\sim 1 \times 10^{-4}$	Km^s Tc^s
pQF42	3–5 kb	$\sim 5 \times 10^{-3}$	Km^s (Tc^s or Tc^r)
pIH3	>5 kb	$\sim 1 \times 10^{-3}$	Km^s Tc^s

[a] The merodiploids were EPS^i due to insufficient overlap with the mutated region of AW1-1 or improper orientation of the cloned fragment. Cultures were grown to early stationary phase twice in succession in BG broth without antibiotics before analysis on BGT plates containing various combinations of antibiotics.

[b] No EPS$^+$ colonies were recovered from cultures of AW1-1, AW1-1 (pLAFR3), or AW1-1(pCB5). In all cases, the remaining colonies were EPS^i Km^r and usually Tc^s. NA, not applicable. (See Table 1 for antibiotic abbreviations; ^{s}, sensitive.)

enhanced expression of genes when bacteria are transferred from rich culture medium to conditions of nutrient limitation has recently been observed for pathogenicity (*hrp*) and avirulence (*avr*) genes (Hunyh *et al.* 1989; Lindgren *et al.* 1989). It cannot be assumed, however, that *P. solanacearum* makes the same EPS in the two different media (or *in planta*), because some bacteria have the capacity to synthesize several different EPSs (Fett *et al.* 1989; Glazebrook and Walker 1989; Rudolph et al. 1989; Whitfield 1988). Future efforts to characterize the EPS of *P. solanacearum* should concentrate on the EPS made in minimal medium or isolated from sap of infected plants.

Inoculation of undisturbed roots is a more rigorous test of the ability of *P. solanacearum* to colonize a plant than is the stem inoculation assay. Despite the inherent variability of this type of bioassay (Denny *et al.* 1990), when the mutants are compared within experiments relative to the wild type, it is clear that AW1-1 was consistently reduced in its ability to wilt tomato plants. In addition, both AW1-1 and AW1-41 infected fewer plants than did the wild type in the second experiment, when the plants were less susceptible. This observation supports the suggestion that EPS may have an important role during the infection of roots by *P. solanacearum* or that it may increase survival of the bacterium in the soil (Denny *et al.* 1990).

The identification of two neighboring regions involved in EPS production, one of which spanned greater than 9 kb, was not surprising, because genes for EPS production by bacteria are usually clustered (Coplin and Cook 1990; Frosch *et al.* 1989; Long *et al.* 1988; Wang *et al.* 1987; Whitfield 1988). Our interest in these regions has been further stimulated by the finding that genes in both regions are regulated by phenotype conversion. Detailed complementation studies will be required to determine how many genes for EPS production by *P. solanacearum* are in and around regions I and II. However, regions I and II do not contain all the genes for EPS production in *P. solanacearum* strain AW1, because we have additional EPS^i mutants with Tn*5* insertions in different *Eco*RI fragments (T. P. Denny, unpublished). There are also the genes for EPS production that are induced by nutrient limitation, which would have been missed when Tn*5* mutants were screened on BGT plates (Denny *et al.* 1988).

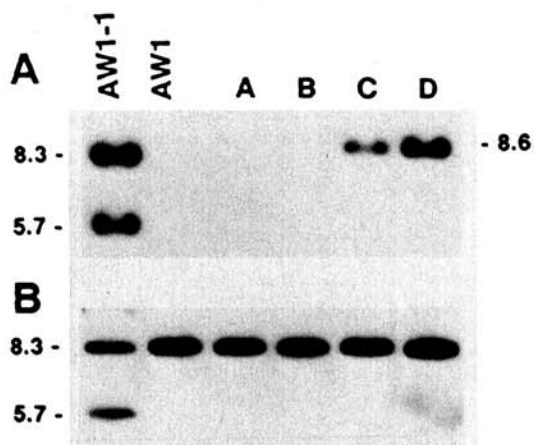

Fig. 6. Southern blot analysis of region I in *Pseudomonas solanacearum*. Total genomic DNA from the wild-type AW1, the EPS^i Tn*5* mutant AW1-1, and four EPS$^+$ Km^s Tc^s derivatives of AW1-1 (in lanes A–D) was digested with *Bam*HI and *Eco*RI. The EPS$^+$ derivatives were generated *in vitro*, and were four of the five wild-type recombinants used in virulence assays. The hybridization probes were, **A**, the *Hin*dIII fragment of Tn*5* and, **B**, the 8.3-kb *Bam*HI-*Eco*RI fragment within region I isolated from pQF42.

If EPS is required by *P. solanacearum* to cause typical wilt symptoms, then fully complementing the mutation in AW1-1 should have restored virulence. Unfortunately, although AW1-1(pQF4) produced wild-type levels of EPS in culture, it made much less EPS than AW1 in tomato stems and was not as virulent as the wild type. Similar results were found for AW1-1(pQF40) (data not shown). The failure of these merodiploids to complement *in planta* was probably due to plasmid instability, because a minority of the *P. solanacearum* bacteria recovered from the stems 1–2 wk after infection with the merodiploids were Tc^r and resembled the strain inoculated. Xu *et al.* (1990) also observed plasmid instability and the failure of merodiploids of *P. solanacearum* strain K60 to complement *in planta*. In addition, a sizable percentage of the bacteria recovered were PC-types, which are much less virulent than the AW1-1 parent strain (Buddenhagen and Kelman 1964; Denny *et al.* 1988) and can reduce the apparent virulence of a wild-type strain (Averre and Kelman 1964).

Even though plasmid instability in the merodiploids complicated the complementation tests for virulence, the results presented in Figure 5 reveal several notable aspects of the relationship between EPS production and wilt. First, the merodiploids wilted tomato plants without producing wild-type amounts of EPS. It may be that there is a threshold level of EPS production that is necessary for complete wilt or that the merodiploids produced locally high concentrations of EPS that our examination of whole stems did not discern. Either explanation could account for why the mutant studied by Xu *et al.* (1990), which produced 22% of the wild-type level of galactosamine in culture, largely retained virulence. Second, more EPS was recovered from plants that wilted slowly than from those that wilted quickly. It may be that, besides the amount of EPS, its rate of production is a factor in virulence. These two observations are consistent with why the wild type, which rapidly produced "excess" EPS, wilted plants faster than the merodiploids. It should also be apparent that it would be difficult to resolve the uncertainties that these observations raise when standard physiological approaches are used.

The recovery of EPS$^+$ wild-type recombinants from some merodiploids of AW1-1 (that were not complemented) provided an alternate genetic approach to investigate the relationship between production of EPS and wilt symptoms. Characterization of the EPS$^+$ strains showed that they were invariably Km^s and, for the four strains examined, had regained a wild-type region I. Because the process also required that the plasmid carry homologous wild-type sequences that spanned the Tn*5*-mutated region, these results strongly suggest that site-specific recombination resulted in allelic replacement. This process, which can be thought of as "reverse" marker-exchange, was unusual only in that the high frequency and easily recognizable EPS$^+$ phenotype allowed wild-type recombinants to be recovered without applying positive selection (see Russell and Dahlquist 1989).

It is unlikely that additional mutations would have occurred, and in any event, testing five independent EPS^+ wild-type recombinants for virulence would preclude random second mutations from being a factor. Most significantly, the five EPS^+ wild-type recombinants tested in the stem inoculation assay all wilted tomato plants as fast as the wild-type strain. These results fulfill the primary set of "molecular Koch's postulates" as defined by Falkow (1988) and indicate that EPS has a significant role in the ability of *P. solanacearum* strain AW1 to wilt tomato plants. Better proof of this hypothesis will require knowledge of the precise biochemical functions of the mutated genes and the regulatory network that controls their expression.

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