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 Tn5-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 cosmids 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 Tn3-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 (Walls 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*.

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; Brumblay and Denny 1990; Husain and Kelman 1958). However, because PC-type mutants are pleiotropic (Buddenhagen and Kelman 1964; Brumblay 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; Sequiera 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 (Brumblay and Denny 1990).

Early research results with poorly characterized Tn5-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 Tn5 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 Tn5 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 Tn5-induced EPS-deficient mutants of strain AW1. Class I and class II mutants were designated as EPS-impaired (EPS'), because their butyryl colonizers are lavender colored on tetrazolium chloride-containing media rather than the red dark 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 Tn5 insertions in two different *EcoRI* fragments and differed in virulence. The rate at which tomato plants were wilted by the single class I strain (AWI-I) was 16% that of the wild-type parent, compared with 78% for the three class II strains (AWI-41, AWI-71, and AWI-74); this difference was correlated with the amount of EPS produced in planta. The single Tn5-induced class III mutant (AWI-80) is EPS', but is indistinguishable from spontaneous, pleiotropic PC-type mutants (Brumley and Denny 1990).

The present study extends the research with the class I and class II EPS' 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-I 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 MgSO4) (Brumley 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

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pseudomonas solanacearum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AW1</td>
<td>Derivative of wild-type strain AW, EPS' Nal'</td>
<td>Denny et al. 1988</td>
</tr>
<tr>
<td>AWI-1</td>
<td>Class I mutant of AW1 (eps-1::Tn5), EPS' Nal' Km'</td>
<td>Denny et al. 1988</td>
</tr>
<tr>
<td>AW1-128, -129, -130, -131</td>
<td>Class I mutants of AW1 (eps-128, -129, -130, -131::Tn3-HoH01), EPS' Nal' Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>AW1-41, -71, -74</td>
<td>Class II mutants of AW1 (eps-41, -71, -74::Tn5), EPS' Nal' Km'</td>
<td>Denny et al. 1988</td>
</tr>
<tr>
<td>AWI-PC</td>
<td>Spontaneous phenotype conversion mutant of AW1, EPS' Nal'</td>
<td>Denny et al. 1988</td>
</tr>
<tr>
<td>AW1-80</td>
<td>Class III induced PC-type (phecA80::Tn5), EPS' Nal' Km'</td>
<td>Denny et al. 1988</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Δ80IacZAM15 endA1 recA1 hsdR17 supE44 gyrA96 Δ(lacZYA-argF)U169</td>
<td>BRL&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLAFR3</td>
<td>Broad host range cosmid vector, Tc'</td>
<td>Staskawicz et al. 1987</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Conjugation helper plasmid, Km'</td>
<td>Figurski and Helinski 1979</td>
</tr>
<tr>
<td>pUC9</td>
<td>ColEl Ap'</td>
<td>Vieira and Messing 1982</td>
</tr>
<tr>
<td>pBS1</td>
<td>13.7-kb EcoRI fragment containing eps-1::Tn5 in pUC9, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pBS410</td>
<td>16-kb EcoRI fragment containing eps-41::Tn5 in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pCB5, pOF6, pQO10, pFH3, pOE10, pSG5, pQF4, pPF12</td>
<td>Cosmid clones containing AW1 DNA in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pQF40/41</td>
<td>13.7-kb EcoRI fragment with most of region I in pLAFR3, opposite orientations, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pQF42</td>
<td>8.3-kb BamHI-EcoRI fragment from region I in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pQF43</td>
<td>8.1-kb BamHI-HindIII fragment from region I in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pQF44</td>
<td>6.3-kb HindIII-EcoRI fragment with region II in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pQF45</td>
<td>1.9-kb BamHI fragment from region II in pLAFR3, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pGA93</td>
<td>2.2-kb EcoRI-BglII fragment with plc4 in pLAFR3, Tc'</td>
<td>Brumley and Denny 1990</td>
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</tbody>
</table>

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

<sup>a</sup> 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 pipetting 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 KPO₄, 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*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid in merodiploid</th>
<th>None</th>
<th>pPF12</th>
<th>pCB5</th>
<th>pHE3</th>
<th>pOE10</th>
<th>pSG5</th>
<th>pQF4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW1</td>
<td></td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Class I</td>
<td></td>
<td>640(380)</td>
<td>520</td>
<td>990</td>
<td>670</td>
<td>1,210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AW1-1</td>
<td>i</td>
<td>i</td>
<td>i</td>
<td>i</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td>40(20)</td>
<td>80</td>
<td>60</td>
<td>210</td>
<td>280</td>
<td>420</td>
<td>670</td>
</tr>
<tr>
<td>AW1-41</td>
<td>i</td>
<td>±</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>AW1-71</td>
<td>i</td>
<td>±</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>AW1-74</td>
<td>i</td>
<td>±</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td>70(320)</td>
<td>180</td>
<td>930</td>
<td>890</td>
<td>670</td>
<td>750</td>
<td>1,330</td>
</tr>
<tr>
<td>AW1-PC</td>
<td>neg</td>
<td>neg</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>AW1-80</td>
<td>neg</td>
<td>neg</td>
<td>60</td>
<td>90</td>
<td>120</td>
<td>90</td>
<td>330</td>
<td></td>
</tr>
</tbody>
</table>

*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 pQF6 and pQG10, which are not shown, were similar to those for pCB5 and pHE3, 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 **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⁺ class I strain AW1-1 (■), EPS⁺ 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 pHE3 (○). 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 quantitated 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 cosmids DNA with Tn3-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* strains 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* classes was confirmed by quantitating EPS recovered from broth cultures (Table 2). The EPS* 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 × 10⁶ 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.](image)

![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.](image)
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 EcoRI fragment containing the Tn5 insertion from AW1-1 (eps-I::Tn5) was cloned in pUC9 and designated as pBS1 (Fig. 4B). The eps-I::Tn5 mutation mapped to a point 5.0 kb from the HindIII site at the right end of the fragment and 3.0 kb from the nearest BamHI 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 EcoRI fragment complemented AW1-1 to varying degrees; pQG10 and pIH3 that have the Plac promoter

![Figure 4](image-url)

**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::Tn5 and eps-74::Tn5 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 EcoRI fragments with eps-I::Tn5 and eps-I::Tn5 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 Plac promoter in the pLAFR3 vector. The positions of eps-128, eps-129, eps-130, and eps-131::Tn3-HoH1 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 pOElO, pSG5, and pQF4 that have Plac on the right. Only pQF4 completely restored EPS production by AW-1 to a wild-type level. All of the cosmids except pPF12 completely restored EPS production to the three EPS class II strains, suggesting that the class II mutations are clustered close to eps-1::Tn5 (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 class I strain. The 13.7-kb fragment was cloned in the same (pQF40) or opposite (pQF41) orientation with respect to Plac as in pQF4. The AW-1-l(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 AW-1-l(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 BamHI-EcoRI fragment in pQF42 and the 8.1-kb BamHI-HindIII fragment in pQF43 (Fig. 4D) both failed to complement AW1-1, suggesting that the mutated transcriptional unit extends beyond the BamHI site 3 kb to the left of the Tn5 insertion.

**Preliminary characterization of two regions involved in EPS production.** One region involved in EPS production was identified using four Tn3-HoH01 insertions in pQF4 that abolished the ability of this cosmid to complement the eps-1::Tn5 mutation in AW1-1. Restriction mapping showed that the eps-129::Tn3-HoH01 insertion was 1.2 kb to the left of eps-1::Tn5, whereas the other three Tn3-HoH01 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 Tn3-HoH01 insertions was transferred into the genome of AW1, and the resulting mutants, designated AW1-128 to -131, were all EPS+ 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 BamHI 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+ 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. Pheno-

type 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+ 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 pI3 (and other cosmids) suggested that these Tn5 insertions mark a second region for EPS production that is close to region I. This possibility was confirmed by subcloning the 6.3-kb HindIII-EcoRI 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 BamHI or HindIII digested AW1-41, AW1-71, and AW1-74 genomic DNA, mapped the insertions to either the 1.9- or 2.1-kb BamHI fragments (Fig. 4A, B). The position of eps-41::Tn5 in the 1.9-kb BamHI 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 Tn5 insertions are in a single complementation unit. The 1.9-kb BamHI 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::Tn5 and eps-74::Tn5 insertions) (Fig. 4A) and likely contains a single gene. Region II also appeared to be regulated by phenotype conversion, because expression of a Tn3-HoH01 Lac+ fusion that resulted in a typical class II EPS+ 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(l)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.](image)

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to AW-1 in culture (pQF4 > pSG5 > pOE10 > pI3; see Table 2) were tested to determine whether they similarly enhanced the virulence of AW-1. Although all of the recombinant cosmid isolated increased the ability of AW-1 to wilt tomato plants, none of them completely restored virulence (Fig. 1B). Statistically, every merodiploid except AW-1-(pI3) induced 50% wilt sooner than AW-1 (P = 0.1), and all but AW-1-(pSG5) induced wilt later than AW-1 (P = 0.05). The cosmid vector alone had no effect on AW-1 (data not shown). Although variation among the merodiploids was not significant, the degree to which the cosmid improved virulence by AW-1 was consistent (pSG5 > pOE10 > pQF4 > pI3; Fig. 1B). Therefore, EPS production in culture by the merodiploids was generally correlated with virulence except for the case of AW-1-(pQF4), which was distinctly less virulent than AW-1, 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 AW-1, whereas the plants killed by the merodiploids had about one third as much EPS on any given day (Fig. 5). Plants infected with AW-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 AW-1 and the merodiploids. In contrast to its behavior in culture, AW-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 AW-1-(pQF4), was coincident with their producing substantially less EPS in planta than AW-1.

Isolates of Na1 P. solanacearum recovered from plants inoculated with AW-1 were invariably Km' and either EPS' or EPS' (like spontaneous PC-type mutants). When Na1 P. solanacearum were recovered from tomato plants killed by the AW-1 merodiploids there were five distinguishable phenotypes. A minority of the isolates were Km' Te' and had a colony morphology either like the original merodiploids that were inoculated (i.e., EPS' or various degrees of EPS'') or were EPS'. A majority of the isolates recovered were Km' Te' and either EPS' or EPS''; these presumably arose from loss of the cosm id during growth in the absence of antibiotic selection in planta. Unexpectedly, isolates that were fully EPS' and Km' Te' were occasionally recovered. That EPS' derivatives were found when the merodiploid used as the inoculum was EPS' (i.e., AW-1-(pI3)); that antibiotic resistance markers for both the cosm id and the Tn5 insertion were always lost suggested that allelic replacement had produced wild-type recombinants.

Characterization of AW-1 EPS' wild-type recombinants. To produce EPS' derivatives under controlled conditions, AW-1 and selected EPS' merodiploids were grown in BG broth without antibiotics (Table 3). Only EPS' Km' colonies were recovered from cultures of AW-1, AW-1(pLAFR3), or AW-1-(pCB5). In contrast, EPS' Km' isolates were recovered only from those merodiploids that carried cosmids with DNA overlapping the eps-l::Tn5 insertion. No EPS' Km' isolates were recovered in these experiments, and except for most of the derivatives of AW-1-(pQF42), the EPS' isolates were Te'. Similar EPS' Km' isolates were recovered when the merodiploids were cultured in MM broth without antibiotics (data not shown). Sixteen independent EPS' Km' 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' Te' isolates that were generated in vitro (and that were used in plant inoculations, see below) showed that the Tn5 insert was either mostly or completely lost (Fig. 6A). More importantly, the native 8.3-kb BamH1-EcoRI 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 Tn5 insertion.

Five independent EPS' Km' Te' wild-type recombinants were selected for virulence assays: Three were from AW-1-(pI3), and one each was from AW-1-(pQF42) and AW-1-(pQF43). One wild-type recombinant from AW-1-(pI3) 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 AW-1 (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 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 EPS1 AW-1 merodiploids after growth in culture without antibiotics

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Overlap with eps-l::Tn5</th>
<th>EPS' colonies recovered</th>
<th>Frequency</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>...</td>
<td>&lt;1 x 10^-6</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>pLAFR3, pCB5</td>
<td>None</td>
<td>&lt;1 x 10^-6</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>pPF12</td>
<td>0.2 kb</td>
<td>~1 x 10^-4</td>
<td>Km' Te'</td>
<td></td>
</tr>
<tr>
<td>pQF42</td>
<td>3.5 kb</td>
<td>~5 x 10^-3</td>
<td>Km' (Tc' or Te')</td>
<td></td>
</tr>
<tr>
<td>pI3</td>
<td>&gt;5 kb</td>
<td>~1 x 10^-3</td>
<td>Km' Te'</td>
<td></td>
</tr>
</tbody>
</table>

*The merodiploids were EPS' due to insufficient overlap with the mutated region of AW-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.

*No EPS' colonies were recovered from cultures of AW-1, AW-1 (pLAFR3), or AW-1-(pCB5). In all cases, the remaining colonies were EPS' Km' and usually Te'. NA, not applicable. (See Table 1 for antibiotic abbreviations; ', 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 (Huny et al. 1989; Lindgren et al. 1989). It cannot be assumed, however, that _P. solanacearum_ makes the same EPS in the two different media (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

![Fig. 6. Southern blot analysis of region I in Pseudomonas solanacearum.](image)

Total genomic DNA from the wild-type AW1, the EPS Tn5 mutant AW1-1, and four EPS+ Km' Tc' derivatives of AW1-1 (in lanes A-D) was digested with _BamHI_ and _EcoRI_. 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 _HindIII_ fragment of Tn5 and, B, the 8.3-kb _BamHI-EcoRI_ 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' 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' 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 Tn5-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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LITERATURE CITED


