Dramatically Reduced Virulence of Mutants of *Pseudomonas solanacearum* Defective in Export of Extracellular Proteins Across the Outer Membrane

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***Pseudomonas solanacearum* is an important wilt-inducing pathogen that infects a wide variety of important crop plants throughout the world. Studies using artificial inoculation methods suggest that some of its extracellular proteins play a significant, but auxiliary role in production of wilt disease. We isolated mutants of race 1 and race 3 strains of *P. solanacearum* with Tn*5* insertions at a single locus (*eep*) whose culture supernatants lack all of its known extracellular enzymes and most other detectable extracellular proteins (EXPs). Analysis of subcellular fractions of *eep*::Tn*5* mutants showed that they still synthesized many of these EXPs but accumulated them inside the cell. Experiments with PhoA fusion proteins showed that export of proteins across the inner membrane was not affected by the *eep* mutation, suggesting that *eep* functions only in protein export across the outer membrane. Production of extracellular polysaccharide was not obviously affected by the *eep* mutation. Analysis of *eep* mutants in stem-inoculated tomato plants showed that they had lost the ability to cause wilt symptoms or kill the plant, possibly because they colonized stems much more slowly than wild types. Plants grown in soil inoculated with the *eep* mutants did not develop any visible disease symptoms over a 20-day period, and their stems contained fewer than 10^3 *P. solanacearum* cells, whereas wild types killed plants in 14 days, and more than 10^{10} cells were found in their stems. These results suggest that an individual or group of extracellular proteins of *P. solanacearum* is required for infection via the roots, as well as wilting and killing of host plants.**

Additional keywords: endoglucanase, *out* genes, pectin methylesterase, polygalacturonase, wilt disease.

Pseudomonas solanacearum produces a large amount and variety of extracellular proteins (EXPs) (Schell 1987; Schell *et al.* 1993a). Many of these are polysaccharidases (e.g., polygalacturonases, endoglucanase), which can degrade plant cell wall components. Thus EXPs are likely to somehow be involved in the wilt disease caused by *P. solanacearum* on its many host plants (Hayward 1991). However, only a few of the EXPs in culture supernatants of race 1 strains have been purified, characterized, or assessed for their role in disease: endopolygalacturonase, PglA (Schell *et al.* 1988); endoglucanase, Egl (Roberts *et al.* 1988; Schell 1987); and exopolygalacturonases, PglB (Schell *et al.* 1993a), PehB, and PehC (C. Allen and L. Sequeira, University of Wisconsin, personal communication). In general, when directly injected into the stem, race 1 mutants deficient in production of one or two polysaccharidases still wilt and kill host plants but more slowly than wild types. The onset of symptoms and death can be delayed by 25 to 80% depending on the type of enzyme missing, inoculum size, and age of plants (Denny *et al.* 1990). The role of these enzymes in root infection (the more likely mode of infection in the field) and entry into the vascular system is largely unknown and may be crucial. In the few cases where more natural infection methods have been used to study bacterial plant pathogens (Denny *et al.* 1990; Dow *et al.* 1990), the magnitude of virulence reduction caused by EXP deficiency has been enhanced. Other EXPs (e.g., pectin methylesterase [Spok *et al.* 1991]), may also enhance virulence but have not yet been analyzed in this regard. Analysis of the number and types of EXPs produced by race 2 and race 3 strains of *P. solanacearum* or of their role in producing disease has been minimal.

The importance of EXPs in other bacterially caused plant diseases has been investigated in two other pathogens. *Xanthomonas campestris* pv. *campestris* also produces multiple extracellular enzymes capable of degrading plant cell wall components: one or two endoglucanases (Gough *et al.* 1988), three polygalacturonate lyases (Dow *et al.* 1989), and two proteases (Dow *et al.* 1990). *X. campestris* mutants defective in production of one polygalacturonate lyase produced black rot symptoms on crucifers just like the wild type (Dow *et al.* 1989). Mutants deficient in production of the major extracellular endoglucanase grew like the wild type in plants and caused full black rot symptoms but at a 40% slower rate (Gough *et al.* 1988). Protease-deficient mutants of *X. campestris* were somewhat reduced in virulence (Dow *et al.* 1990) but not under all conditions examined (Tang *et al.* 1987).

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Similar analyses of *Erwinia chrysanthemi* show, for the most part, that only three of its five major extracellular pectate lyase isozymes appear to be important for pathogenesis (Boccara *et al.* 1988; Roeder and Collmer 1985). However, the relative importance of individual enzymes is variable, depending on host plant or tissue type (Beaulieu *et al.* 1993). Mutants deficient in all five pectate lyase enzymes were 80–98% reduced in virulence but still rotted potato tubers, albeit at reduced rates (Ried and Collmer 1988). However, *E. chrysanthemi* (and perhaps other plant pathogens) produces new, additional enzymes *in planta* (Kelemu and Collmer 1993; Beaulieu *et al.* 1993). *E. carotovora* mutants lacking polygalacturonase were reported to retain virulence (Willis *et al.* 1987). Thus, most studies of prokaryotic phytopathogens producing cell-wall-degrading EXPs suggest that individually the EXPs are not absolutely required for disease but rather enhance or accelerate its development.

On the other hand, mutants of *X. campestris* (Dow *et al.* 1987) or *Erwinia* (Andro *et al.* 1984; Thurn and Chatterjee 1986), which produce all their EXPs but retain them inside the cell, are nonpathogenic, suggesting that EXPs as a group (or some as yet unidentified individual EXP) are absolutely required for disease production on their respective host plants. These mutants result from inactivation of homologous, highly conserved genes encoding an export apparatus responsible for translocation of many EXPs across the outer membrane of both animal and plant pathogens (Dums *et al.* 1991; Lindeberg and Collmer 1992; Bally *et al.* 1992; Pugsley 1993). Inactivation of another cluster of genes, the *hrp* genes, found in many bacterial plant pathogens (Willis *et al.* 1991), also leads to complete loss of pathogenicity. Recent evidence strongly suggests that *hrp* genes encode a different type of protein export apparatus (Fenselau *et al.* 1992; Gough *et al.* 1992) that transports the harpin protein and perhaps other pathogenicity factors out of the cell (Wei *et al.* 1992; He *et al.* 1993; Huang *et al.* 1992). Thus, the magnitude and details of the importance of EXPs in virulence of plant pathogens still need further definition.

Here we describe the identification of the *eep* locus of *P. solanacearum*; when this locus is inactivated, at least six major EXPs of *P. solanacearum* are not produced, apparently because their export across the outer membrane is blocked. This loss of EXPs outside the cell leads to complete loss of ability to infect, cause disease, and efficiently colonize plant stems. Our results suggest that as a group EXPs are required for infection and wilt disease, possibly because they disrupt tissue and enhance *P. solanacearum's* ability to grow *in planta,* allowing dissemination throughout the plant.

RESULTS

Isolation of *P. solanacearum* mutants that are defective in export of most major extracellular proteins.

By mating *P. solanacearum* strain PO41 with *P. aeruginosa* strain 1826, which carries the suicide plasmid pMO75::Tn*5*, we obtained 8,000 Tn*5* insertion mutants. When these were screened for production of polygalacturonase (PG) or endoglucanase (EG) activity on indicator plates, we found 19 *P. solanacearum* mutants that simultaneously produced dramatically lower activity of both PG and EG. Nonetheless, these mutants retained wild-type mucoid colony morphology and growth characteristics on both BG and BSM minimal plates. *In vitro* enzyme assays for EG or PG activity showed negligible levels of either enzyme in culture supernatants of all the mutants; however, cellfree extracts from eight of the mutants contained high levels of both

Table 1. Bacterial strains used in this study

Pseudomonas strain	Relevant characteristics	Source or reference
P. solanacearum PO41	Race 3, wild type potato pathogen, Rif^r	L. Y. He
P. solanacearum AW	Race 1, wild type tomato pathogen	Schell 1987
P. solanacearum GH2W	Race 1, wild type tomato pathogen	Schell 1987
P. solanacearum UW9	Race 2, banana pathogen	L. Sequeira
P. solanacearum UW150	Race 3, potato pathogen	L. Sequeira
P. solanacearum D4	*eep*-4::Tn*5* mutant of PO41, Km^r	This study
P. solanacearum AD4	*eep*-4::Tn*5* mutant of AW, Km^r	This study
P. solanacearum D4.1	*eep*-4::Tn*5* mutant transformant of PO41, Km^r	This study
P. aeruginosa 1826 (pMO75::Tn*5*)	Km^r	B. Holloway; Whitta *et al.* 1985

Table 2. Distribution of extracellular enzymes in cultures of *eep*::Tn*5* mutants of *Pseudomonas solanacearum*

	Specific activity[a]								
	Polygalacturonase			Endoglucanase			Pectin methylesterase		
Strain	Sol[b]	Mem	Ext	Sol	Mem	Ext	Sol	Mem	Ext
AW	und[c]	und	384	und	und	224	und	und	2.5
AD4	144	192	und	24	124	und	2.6	0.1	und
PO41	und	und	48	und	und	21	und	und	und
D4.1	16	24	und	5	23	und	und	und	und

[a] Activity of polygalacturonase (PG) and endoglucanase (EG) given in nanomoles of reducing sugar released per minute per milligram of total cell protein. Pectin methylesterase (PME) activity given in nanomoles of H^+ released per hour in units per milligram of total cell protein.

[b] Sol = soluble (cytoplasmic + periplasmic) fraction; Mem = membrane fraction (pellet from centrifuging at 150,000 × *g* for 1 hr); Ext = extracellular fraction (culture supernatant).

[c] Undetectable: for PG and EG <0.10; for PME <0.01.

activities. One of these mutants, designated D4, was further analyzed.

To ensure that the phenotype of the D4 mutant was due to a single Tn*5* insertion, chromosomal DNA was prepared from strain D4 and, using transformation, the genomic segment with the Tn*5* insertion (Km^r marker) was recombined into the genome of the wild-type race 3 parent strain PO41 and into the genome of our more extensively characterized *P. solanacearum* race 1 strain AW (Schell *et al.* 1993a,b). The phenotype of the resultant Km^r transformants (strains D4.1 and AD4, respectively; Table 1) was identical to the original mutant (i.e., showed very low PG and EG activity on indicator plates). *In vitro* analysis showed that both transformants produced nearly wild-type levels of EG and PG activity but, in complete contrast to their wild-type parents, failed to export it. All detectable activity was found inside the cells and none in the culture supernatants (Table 2). Similar analysis of culture supernatants and cellfree extracts of strain AD4 for activity of another extracellular enzyme, pectin methylesterase (PME), showed it too was retained in the cell (Table 2). These results suggest that a single Tn*5* insertion at a homologous locus in both race 1 and race 3 strains of *P. solanacearum* blocks extracellular export of at least three major EXPs. We designated this locus *eep* (for export of extracellular proteins). Wild-type strain PO41 (and its *eep* mutant) did not produce detectable PME activity, nor did another race 3 *P. solanacearum* strain (UW150). However, PME was present at high levels (>1.6 units/mg of protein) in supernatants of a race 2 strain (UW9) and another race 1 strain (GH2W).

Next we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to investigate the effect of the *eep*::Tn*5* insertion mutation on production of other proteins (Fig. 1 and other gels not shown). Comparison of the culture supernatants of wild-type strains AW (race 1) and PO41 (race 3) as well as a race 2 isolate (UW9) showed they all contain at least six major species of EXPs (Fig. 1A; lanes 3–5); however, the relative amounts of individual polypeptides in each supernatant varied somewhat with culture conditions. Nonetheless, based on results of multiple analyses like that shown in Figure 1, several common species of polypeptide appear to be present in supernatants of all three strains (e.g., 28, 35, and

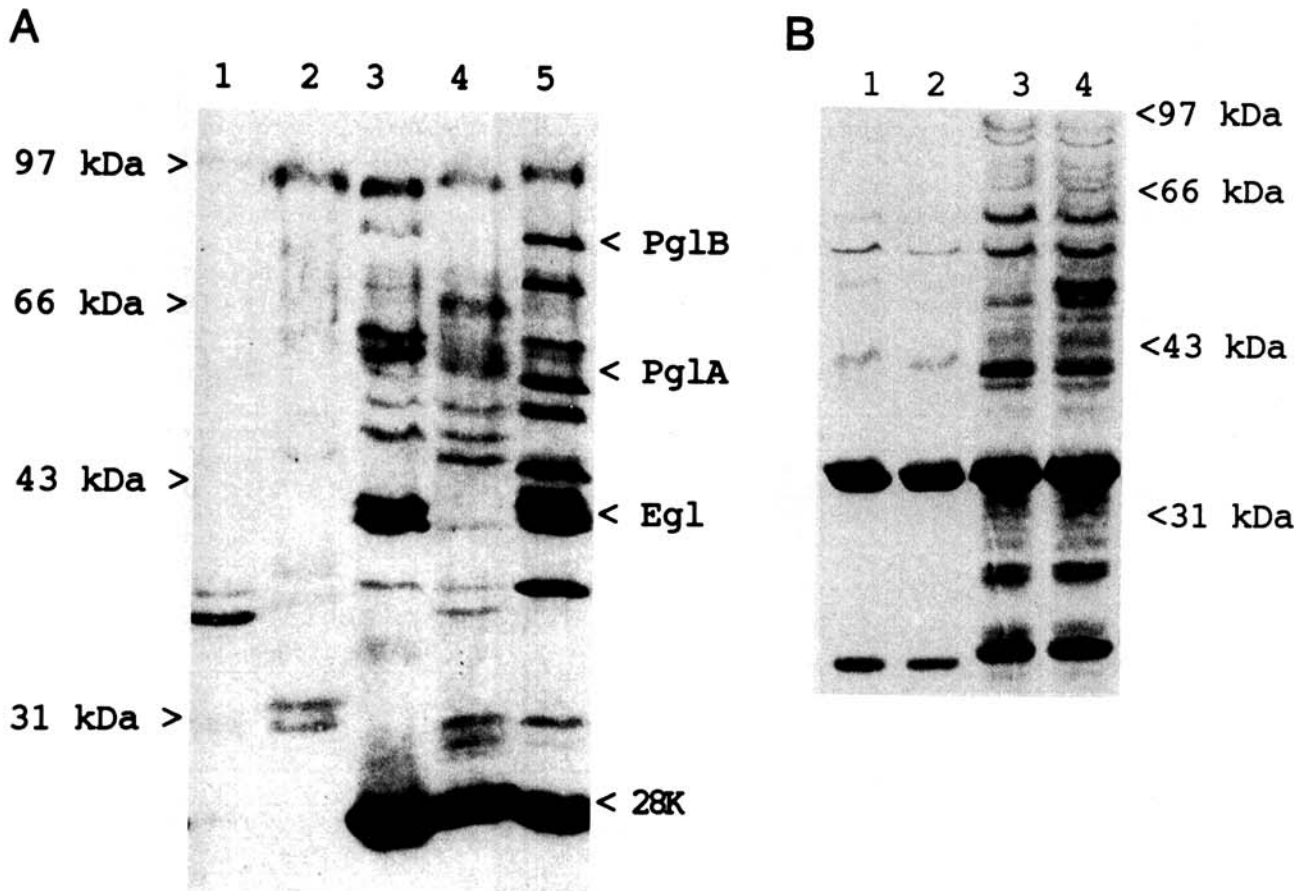

Fig. 1. Extracellular and membrane protein profiles of wild type and *eep*::Tn*5* mutants of *Pseudomonas solanacearum.* **A,** Culture supernatants of *P. solanacearum* strains: D4.1 race 3 *eep*::Tn*5* (lane 1); AD4 race 1 *eep*::Tn*5* (lane 2); AW race 1 wild type (lane 3); PO41 race 3 wild type (lane 4); UW9 race 2 wild type (lane 5) grown in BSM salts medium (Roberts *et al.* 1988) with 0.1% dialyzed yeast extract, 0.1% dialyzed casamino acids, and 1% sucrose to stationary phase (5×10^9 cells per ml) were prepared and analyzed by SDS-10% PAGE stained with Coomassie blue. For strains AD4, D4.1, and PO41, 3-ml culture equivalents were electrophoresed; for AW and UW9, only 1-ml culture equivalents were used. Migration of molecular weight standards is indicated at the left; migration position of identified EXPs of strain AW are indicated at the right. **B,** Cells were grown in BG, harvested, sonicated, and the membrane fraction recovered by centrifugation (pellet from 150,000 × *g* for 1 hr); 0.05 ml culture equivalents of membrane fraction were electrophoresed on SDS-10% PAGE and stained with Coomassie blue. Lane 1, AD4; lane 2, AW; lane 3, PO41; lane 4, D4.1 Migration of molecular weight markers is shown at the right.

43 kDa) and thus could be homologous. In support of this possibility, experiments with anti-Egl antiserum suggest that the enzyme responsible for most of the extracellular EG activity of PO41 and the race 2 strain is very similar, if not identical to, the 43-kDa Egl of race 1 strain AW (Schell 1987, and not shown).

With few exceptions, all species of EXPs were missing from culture supernatants of the respective *eep* mutants (Fig. 1A; lanes 1 and 2), including the major one, the 28-kDa EXP that correlates with extracellular polysaccharide (EPS) production by strain AW (Schell *et al.* 1993b). In contrast, the profiles of membrane-associated and soluble proteins of both *eep* mutants on SDS-PAGE were very similar to those of their respective wild-type parents (Fig. 1B and not shown). The only major differences from wild types were that for both *eep* mutants a new 49-kDa polypeptide became prominent in the membrane fraction (Fig. 1B), and a new 30-kDa polypeptide appeared in the soluble fraction (not shown). These polypeptides may represent EXPs that aberrantly accumulate in these compartments because they cannot be exported. Normal production of most membrane proteins by *eep* mutants suggests that the general export pathway (i.e., Sec-dependent export across the inner membrane) has not been affected.

eep Mutants are defective only in export of proteins across the outer membrane.

The above results suggest that the *eep* mutation causes a specific defect in export of proteins across the outer membrane or their release from the cell. To further establish where the *eep* mutation blocks the export pathway of *P. solanacearum*, the intracellular location of several accumulated extracellular enzymes was determined. Analysis of the soluble (cytoplasmic + periplasmic) and membrane fractions of AD4 and D4.1 cells (Table 2) showed that more than 80% of the EG activity was in the membrane fraction, while the PG activity was nearly equally divided between the soluble and membrane fractions. Most of the PME activity of AD4 was in the soluble (presumably periplasmic) fraction. It should be noted that the total PG activity (and possibly PME activity) derives from the combined activities of multiple isozymes (Schell *et al.* 1988), whereas EG activity derives only from the Egl protein (Roberts *et al.* 1988). A similar localization pattern for EG and PG activity was observed when the *egl* and *pglA* genes were expressed in *Escherichia coli,* which lacks the system required for their extracellular export (Roberts *et al.* 1988; Huang and Schell 1990b). Localization of Egl in the membrane fraction likely requires its processing and export across the inner membrane by the Sec-encoded general export pathway, suggesting that the *eep* mutation has specifically blocked export across the outer membrane without affecting export across the inner membrane.

This hypothesis was further confirmed by analysis of the ability of *P. solanacearum* strain AD4 to export two previously characterized PhoA hybrids: one encoded on pJH123 (Huang and Schell 1990a) and comprising the signal sequence and 177 residues of mature PglA fused to mature PhoA, and another encoded on pJH113 (Huang and Schell 1992) and comprising the signal sequence and 65 residues of mature Egl fused to mature PhoA. In wild-type *P. solanacearum* AW, these hybrids are processed and exported across the inner membrane by the Sec-encoded pathway, resulting in high levels of PhoA (alkaline phosphatase) activity. However, further export of the hybrids across the outer membrane does not occur: the PglA-PhoA hybrid localizes to the periplasm, and the Egl-PhoA hybrid localizes to the membrane (Huang and Schell 1990a, 1992). AD4 with either the *pgl*A-*phoA* or the *egl-pho*A fusion gene produced the same levels of PhoA activity as wild-type strain AW with the same genes. Moreover, the PhoA activity was distributed between the soluble and membrane fractions in the same proportion as it was in wild-type cells (Table 3). Since PhoA (and its hybrids) requires export across the inner membrane to attain catalytic activity (Manoil *et al.* 1990), these results suggest that in the *eep* mutants the Sec-dependent pathway for export across the inner membrane is intact. The behavior of PhoA hybrids and unaltered membrane protein profiles of *eep* mutants are consistent with the conclusion that *eep* inactivation affects only export across the outer membrane.

eep Mutants cannot cause wilt disease.

Some EXPs of *P. solanacearum*, such as Egl and PglA, although not absolutely required to cause disease, do play supporting roles in disease by decreasing the time needed to wilt and kill a host plant (Roberts *et al.* 1988; Schell *et al.* 1988; Denny *et al.* 1990). To determine if loss of ability to export most major EXPs causes a more dramatic effect on virulence, we compared disease development in tomato plants that were stem-inoculated with *eep*::Tn*5* strains or their wild-type parents (Table 4). The race 3 strain PO41 (isolated from potato) was clearly less virulent on tomato than the race 1 strain

Table 3. Localization of active PglA-PhoA and Egl-PhoA fusion proteins in *Pseudomonas solanacearum eep* mutants

Strain	Fusion	PhoA activity[a]	
		Membrane fraction[b]	Soluble fraction[b]
AW(pJH123)	PglA-PhoA	13	105
AD4(pJH123)	PglA-PhoA	11	115
AW(pJH113)	Egl-PhoA	71	26
AD4(pJH113)	Egl-PhoA	66	19
AW	None	<1	<1
AD4	None	<1	<1

[a] Activity expressed in nanomoles of *p*-nitrophenol released per minute per 10^9 cells.
[b] Membrane fraction (pellet from 150,000 × *g* for 1 hr). Soluble fraction contains cytoplasmic and periplasmic contents.

Table 4. Virulence of *eep*::Tn*5* mutants of *Pseudomonas solanacearum* on tomato plants

Strain	No. of cells inoculated into stem	Disease index[a] after			
		3 days	7 days	10 days	15 days
Wild-type AW	10^4	0	85	D[b]	D
AW *eep*::Tn*5*	10^4	0	0	0	0
Wild-type AW	10^7	45	100	D	D
AW *eep*::Tn*5*	10^7	0	0	0	0
Wild-type PO41	10^4	0	26	30	60
PO41 *eep*::Tn*5*	10^4	0	0	0	0
Wild-type PO41	10^7	0	28	50	90
PO41 *eep*::Tn*5*	10^7	0	0	0	0

[a] Evaluated as described previously (Roberts *et al.* 1988) and approximately equal to the percentage of wilted leaves in the inoculated population (five plants).
[b] All plants dead.

AW, taking over twice as long to totally wilt and kill a majority of the plants (15 vs. 7 days; Table 4). The *eep* mutants AD4 and D4.1 were severely reduced in virulence. Neither *eep* mutant wilted nor killed any plants over a 15-day period, whereas wild-type AW wilted and killed all plants in less than 8 days. However, plants inoculated with AD4 did show low levels of some disease symptoms such as minor chlorosis, stunting, and appearance of adventitious root nodules. These symptoms were more pronounced at the higher inoculation levels (10^7 cells/plant); dissection of these plant stems 15 days after inoculation with AD4 revealed some internal damage (discoloration and maceration of the pith), but in microscopic observations of stem sections, the vascular bundles appeared largely intact. The extent of the internal stem damage was variable but in general was confined to a 3-cm zone above and below the inoculation site. In contrast, internal components of stems of plants infected with wild types were extensively rotted and dissolved. Plants infected with high levels of the race 3 *eep* mutant D4.1 showed very little obvious internal damage to the stem. These results suggest that normal production of EXPs is necessary for *P. solanacearum* to successfully wilt and kill host plants.

When tomato plants were infected with the *eep* mutant AD4 via the roots by soil-inoculation (a situation more closely resembling the natural infection route of *P. solanacearum*), no disease symptoms were ever observed in any of 18 treated plants (three separate experiments, six plants each), even 20 days after inoculation. Under these same conditions, the wild-type strain AW killed 80% of the plants in less than 14 days. Also in complete contrast to its wild-type parent, when the race 3 *eep* mutant D4.1 was tested on its natural host (potato) by soil inoculation, it failed to cause disease symptoms on any of the plants. Dissection of stems of these *eep*-mutant-treated tomato or potato plants after 20 days revealed no internal damage or evidence of *P. solanacearum* infection. These results suggest that extracellular proteins and/or enzymes are required for successful infection of host plants (via the roots) by *P. solanacearum*.

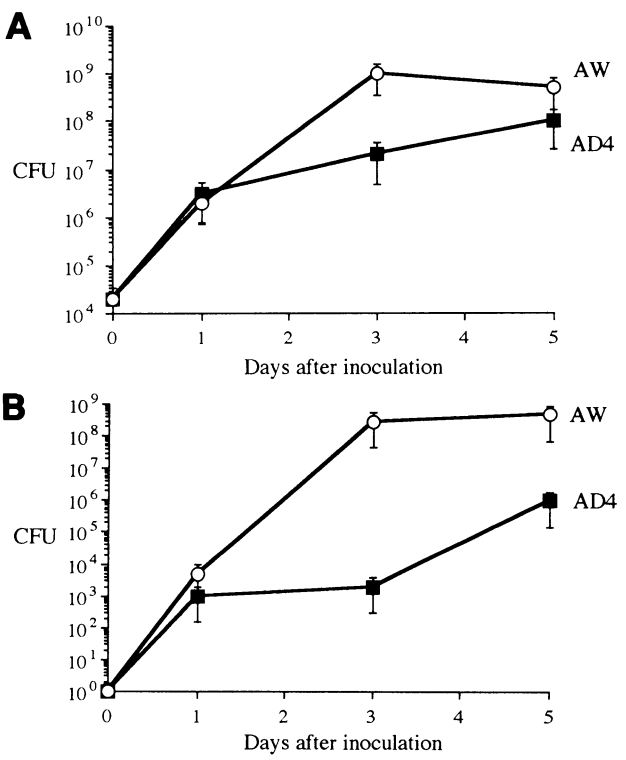

Fig. 2. Multiplication and dissemination of *eep*::Tn*5* mutants in tomato plants. Six-week-old tomato plants were stem-inoculated with 10^4 cells of strain AW (O, wild type) or AD4, (■, *eep*::Tn*5*). At the indicated times, duplicate plants were sacrificed, and the number of viable cells present in duplicate stem sections was determined as described in Materials and Methods. **A,** Number of cells found in 1.5-cm sections containing the inoculation site; **B,** number of cells found in 1.5-cm sections taken from a point 5 cm above the inoculation site. Values represent the average of at least 3 independent experiments. CFU = colony-forming units.

eep Mutants are reduced in ability to grow and spread *in planta*.

To further investigate why race 1 *eep* mutants did not wilt or kill plants, we compared them with wild types for their ability to infect, multiply, and disseminate *in planta*. At 10- and 20-day intervals after soil-inoculation of tomato plants with strains AD4 or AW, 1.5-cm stem sections were taken from a point 2 cm above the soil surface and analyzed for viable cells by plate counts. In three separate experiments with five plants each we found fewer than 10^2 viable cells in the stem sections of plants inoculated with the *eep*::Tn*5* mutant AD4, whereas more than 10^9 cells were found in the stem sections of plants soil-inoculated with wild-type AW. This suggests that some EXPs are required for infection via the roots. Analysis of the plant's roots at 10 days showed that similar numbers of both strains (approximately 10^5) were associated with the root system, largely ruling out viability loss as an explanation for failure of AD4 to infect.

To determine if the AD4 *eep* mutant is altered in its ability to grow *in planta* and colonize plant stems, we injected 10^4 cells into the stems of tomato plants and monitored the number of viable cells at the inoculation site and at a point 5 cm above the inoculation site over a 5-day period (Fig. 2). In the 1.5-cm segment of the stem containing the inoculation site, both the wild type and *eep* mutant multiplied rapidly, increasing by 200-fold during the first 24 h (Fig. 2A). After 3 days, the number of wild-type AW cells reached approximately 10^9 in this segment, whereas the number of AD4 cells (2.5×10^7) was 40-fold lower. However, by 5 days the levels of AD4 were only fivefold less than the wild type. Thus AD4 can clearly multiply to high levels at the inoculation site but at a rate significantly slower than the wild type. While the *in vitro* growth rates of AD4 and its parent AW in BSM minimal or rich (BG) media appeared identical, it is possible that a subtle, nonspecific inhibition of *in vitro* growth rate caused by EXP accumulation could be magnified *in planta*. Reduced *in planta* growth is probably unrelated to loss of *hrp* functions since AD4 gave a normal hypersensitive response in tobacco leaves.

Analysis of the number of viable cells in stem sections located 5 cm above the inoculation site showed a more striking difference between wild types and *eep* mutants (Fig. 2B). Three days after stem-inoculation with 10^4 cells, the number of viable *eep* mutant cells at this remote point was at least 10^5-fold less than the number of AW cells in similar remote sections of stems from plants inoculated with wild types. After 5 days, the number of *eep* mutants at this point was still

100-fold less than for wild types. Similar results were obtained from analyses of stem sections located 5 cm below the inoculation site (not shown). These data suggest that *eep* mutants colonize stems much more slowly than wild-type cells.

Inactivation of *eep* does not affect EPS I production.

EPS is an important virulence factor of *P. solanacearum* (Denny and Baek 1991; Kao and Sequeira 1991). If EPS synthesis involves extracellular proteins whose levels or localization are altered by the *eep* mutation, then the resultant reduced EPS production alone could be responsible for the lower virulence of *eep* mutants. However, colonies of both *eep* mutants were as mucoid as wild types, and additionally synthesis of EPS I, the major component of *P. solanacearum* EPS (Orgambide *et al.* 1991; Schell *et al.* 1993b) (as quantified by the amount of polymeric hexosamine in BG culture supernatants), was not affected by inactivation of *eep*. Race 1 strains AW and AD4 both produced 300 μg of EPS I per milligram of cell protein, whereas race 3 strains PO41 and D4.1 produced 1,500 μg of EPS I per milligram of protein. Analysis of EPS I levels in stems of tomato plants 7 days after inoculation with 10^6 cells showed that wild-type AW and the *eep* mutant AD4 apparently produced similar amounts of EPS I *in planta* (130 and 75 μg per centimeter of stem, respectively). These data suggest that the reduced virulence of *eep* mutants is not a result of reduced EPS I synthesis and that EPS I production in culture or *in planta* does not require any of the major EXPs whose export is affected by *eep* inactivation, including the 28-kDa EXP whose levels usually correlated with EPS I levels (Schell *et al.* 1993b).

DISCUSSION

We constructed and characterized mutants of two different races of *P. solanacearum* that are defective in normal production of most major EXPs. Both mutants result from inactivation of a similar, if not identical, locus (*eep*) and accumulate wild-type levels of several extracellular enzymes (and likely most other major EXPs) inside the cell, because they cannot export them across the outer membrane. The *eep* mutants of either race 1 or race 3 *P. solanacearum* strains were dramatically reduced in virulence, causing almost no external symptoms when 10^4 cells were injected into stems of their respective hosts (tomato and potato), despite the fact that *in planta eep* mutants apparently can produce more than 60% of the wild-type level of the major virulence factor EPS I. Thus, by itself, the ability to produce high levels of EPS I *in planta* appears insufficient for *P. solanacearum* to wilt and kill a host. However, because we do not know the exact function of EPS I *in planta* and did not perform structural analysis, we cannot rule out the possibility that the EPS I produced by the *eep* mutants may differ from the wild type in structure (e.g., decorations) and/or activity.

Studies of race 1 strains in tomato plants showed that at lower inoculation levels (10^4 cells per plant) the *eep* mutation clearly reduced the ability of *P. solanacearum* to multiply *in planta*, since maximal levels of *eep* mutants in stems were consistently five- to 20-fold lower than for the wild type. However, when plants were inoculated at 100-fold higher levels, the difference between final numbers of wild-type and mutant *P. solanacearum* in the stems at 7 days was reduced to only threefold (Huang and Schell, unpublished data); nonetheless these mutant-infected plants still did not wilt or die. In contrast, 100% of the plants inoculated with the wild type (at 10^6 or 10^4) wilted and died within 7 days.

The most dramatic effects of *eep* inactivation on *in planta* behavior of *P. solanacearum* were the loss of ability to infect via the roots and the dramatic reduction in ability to rapidly colonize remote points in the stem. In the latter aspect, *eep* mutants were reduced by two to four orders of magnitude depending on initial inoculum size (Fig. 2B and not shown). This may result from their reduced *in planta* growth rates and/or from a reduced ability to disseminate throughout plant tissues. Why are *eep* mutants so dramatically affected in ability to infect and colonize the stem? Most likely they lack some EXP(s) that is essential for these processes. One obvious possibility is that some of *P. solanacearum's* extracellular enzymes (e.g., PG, EG) are involved in penetration and/or degradation of root and vascular tissues and thereby facilitate infection and stem colonization. Such enzymes may also help to release nutrients from plant cells for rapid *in planta* growth. Whatever the reason, it is clear that EXPs are critical for efficient, rapid infection and stem colonization, both of which are required for wilt disease. EPS I, on the other hand, appears to be of much lesser importance for stem colonization and of much greater importance in wilt induction (Denny and Baek 1991; Denny *et al.* 1990).

The exact molecular nature of the *eep* locus is unclear; however, the phenotype of *eep* mutants (i.e., accumulating most EXPs inside the cell and complete loss of virulence) is largely identical to that of *out* mutants of *Erwinia* (Lindeberg and Collmer 1992), *xcp* mutants of *P. aeruginosa* (Bally *et al.* 1992), and *xps* mutants of *X. campestris* (Dow *et al.* 1987). All these mutants result from insertions at a homologous, multigenic locus, which encodes an export apparatus that translocates some extracellular proteins across the outer membrane (Lindeberg and Collmer 1992; Bally *et al.* 1992; Dums *et al.* 1991). Preliminary experiments with cosmids containing genomic fragments of *P. solanacearum* DNA that complemented the *eep*::Tn*5* mutation show that they specifically hybridize to fragments containing the *outDEF* genes of *E. chrysanthemi*. These data suggest that *eep* or sequences near it may encode part of an *out*-like export apparatus. This is interesting because the *eep*-dependent Egl protein of *P. solanacearum*, unlike all other known proteins exported via *out*-type systems, uses an unusual 45-residue two-part leader sequence for export (Huang and Schell 1992). The first 19 residues are a lipoprotein signal sequence that directs export of an Egl precursor with a lipid-modified N-terminus across the inner membrane. The remaining 26 residues and lipid at the N-terminus of this Egl precursor are removed during export across the outer membrane (Huang and Schell 1992), possibly in conjunction with the *eep* system. However, the extent and significance of similarity between the *eep* locus and *out* genes remains to be clarified by DNA sequence analysis and further biochemical studies.

The wild-type race 3 strain PO41 was much less virulent on tomato than the wild-type race 1 strain AW, even though *in vitro* PO41 produced fivefold more of the primary virulence determinant EPS I than AW. On the other hand, PO41 pro-

duced at least eightfold less extracellular PG and EG activity and dramatically less of the 28-kDa EXP. In addition, PO41 (and another race 3 strain) produced no detectable PME activity, in marked contrast to race 1 and race 2 strains, which produced at least 100-fold more PME than PO41. It is tempting to speculate that production of very low levels of PME activity may be a distinguishing characteristic of *P. solanacearum* strains in race 3, and that the reduced virulence and aggressiveness of race 3 strains on tomato may in part derive from reduced production of PME, Egl, and other extracellular enzymes. However, it is possible that relative production of these EXPs *in planta* and *in vitro* is very different, and thus analysis of extracellular enzyme production of these and other representative strains *in planta* is necessary to further support this correlation.

MATERIALS AND METHODS

Bacterial strains, growth, and media.

Strains used are listed in Table 1. Except where stated otherwise, *P. solanacearum* was grown at 30° C in BG (B-broth with 0.5% glucose [Huang *et al.* 1993]). BSM minimal medium and indicator plates for detection of EG or PG production *in situ* were prepared and developed as described previously (Roberts *et al.* 1988; Schell *et al.* 1988). Antibiotic levels used were rifampicin, 100 μg/ml; tetracycline, 20 μg/ml; and kanamycin, 50 μg/ml.

Transposon mutagenesis.

Eight milliliters of washed, mid-log-phase cells of *P. solanacearum* PO41 (2×10^9), and 1.5 ml (4×10^8) of *P. aeruginosa* 1826 (pMO75::Tn*5*) were mixed together; 0.3 ml aliquots were spread on BG agar plates, and then incubated at 30° C for 6 hr. Cells were removed from plates, and Tn*5* insertion mutants were selected by plating on BG plates with rifampicin and kanamycin. Colonies arising after 2 days at 30° C were analyzed *in situ* for EG and PG activity.

Transformation of *P. solanacearum* with genomic DNA.

Genomic DNA was prepared from *P. solanacearum* strain D4 by the method of Carney and Denny (1990) and transformed (recombined) into the genome of strains AW and PO41 by the method of Boucher *et al.* (1987) by selecting for Km^r colonies on BG plates.

Analysis of extracellular macromolecules.

EXPs in culture supernatants were prepared and analyzed by SDS-PAGE essentially as described previously (Schell 1987; Schell *et al.* 1988). Methods for preparation of culture supernatants, cellfree extracts, and further fractionation into soluble (cytoplasmic + periplasmic) and membrane-enriched fractions of *P. solanacearum* cells were described previously (Huang and Schell 1992). PG, EG, and PhoA activities were assayed as described previously (Huang and Schell 1990b; Roberts *et al.* 1988). PME activity was measured by incubating samples at 33° C with 3 ml of 0.5% citrus pectin (Sigma P-9153, adjusted to pH 6.0 with NaOH and then to pH 7.0 with Tris-HCl pH 7.0) and monitoring the rate of decrease in pH until it dropped by 0.5 units. One unit is defined as the amount of PME that hydrolyzes 1 nmol of ester bonds per hour (assuming each ester hydrolyzed releases 1 H^+). EPS I in culture supernatants and total cell protein in cultures were quantified as described previously (Huang *et al.* 1993). EPS I levels *in planta* were determined as described by Denny and Baek (1991).

Analysis of virulence, multiplication, and spread *in planta.*

Six-week-old tomato plants (*Lycopersicon esculentum* Mill. 'Marion') were stem-inoculated with 10^4 or 10^7 cells at the third leaf node as described previously (Denny and Baek 1991; Roberts *et al.* 1988). For soil inoculation, 20 ml of a freshly grown *P. solanacearum* culture (approximately 10^{11} cells) was mixed with 300 ml of sterile potting soil in a 10-cm diameter pot. Two 4-wk-old tomato plants were immediately transplanted into the inoculated soil. Disease index (wilting) was assessed as described previously (Roberts *et al.* 1988; Denny *et al.* 1990).

To analyze multiplication and spread *in planta*, 6-wk-old tomato plants were stem-inoculated with 10^4 cells or soil-inoculated with 10^{11} cells as above. At subsequent times, two plants were surface-sterilized with ethanol, and 1.5-cm stem sections were cut from the inoculation site and from a point 5 cm above the inoculation site. Duplicate stem sections were crushed in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, homogenized in a stomacher blender, and plated on BG agar plates for enumeration of bacterial cells in the tissue homogenate.

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