

Cloning and Characterization of *tek*, the Gene Encoding the Major Extracellular Protein of *Pseudomonas solanacearum*

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Susceptible plants infected by *Pseudomonas solanacearum* usually wilt, largely due to extracellular proteins (EXPs) and the high-molecular-mass extracellular polysaccharide (EPS I) this pathogen produces. Circumstantial evidence suggested that a 28-kDa protein, the single most abundant EXP made by *P. solanacearum* in culture, is associated with production of EPS I, and thus might have a role in pathogenesis. The 28-kDa EXP was purified and, based on its N-terminal amino acid sequence, an oligonucleotide mixture was made and used as a hybridization probe to clone the gene encoding it. DNA sequence analysis suggested that the coding sequence for the 28-kDa EXP is within a gene, designated *tek*, that encodes a 58-kDa membrane-associated precursor protein that is processed by signal peptidase II during export. Analysis of radiolabeled polypeptides expressed from *tek* confirmed that it encodes a 58-kDa precursor protein, which is exported out of the cells as a 55-kDa preprotein and processed extracellularly to release the very basic 28-kDa EXP from its C terminus. The position, transcriptional direction, and regulated expression of *tek* suggest that it is cotranscribed with *xpsR*, a gene essential for regulating biosynthesis of EPS I, and reinforces the association of the 28-kDa EXP with virulence. However, since *P. solanacearum* mutants lacking only the 28-kDa EXP produced wild-type amounts of EPS I and were fully virulent, the function of this protein remains unclear.

Additional keywords: gene regulation, protein export, protein processing.

Pseudomonas solanacearum causes lethal wilting diseases of many plants worldwide. During pathogenesis it produces a variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms (Denny and Schell, 1994; Schell et al. 1994a). The rapid wilting of plants infected by *P. solanacearum* is related to its production of copious amounts of the high-molecular-mass, acidic extracellular polysaccharide EPS I (Denny and Baek 1991; Kao et al. 1992). The unusual, nitrogen-rich EPS I (Orgambide et al. 1991) is encoded for by the 18-kb *eps* gene cluster that has

more than eight genes involved in biosynthesis (Schell et al. 1993; Huang and Schell 1995). Both EPS I and lipopolysaccharide biosynthesis involve the *ops* gene cluster, which probably encodes for synthesis of some sugar precursors (Kao and Sequeira 1991; Kao and Sequeira 1994). The relatively uncharacterized Region II locus, located about 3 kb downstream of *eps*, is conditionally involved in EPS I production; for undetermined reasons, Region II mutants make normal levels of EPS I in MM (a minimal medium supplemented with glucose) and in planta, but only low levels in medium containing peptone (Denny and Baek 1991; Schell et al. 1993). Biosynthesis of EPS I also is controlled by an exceptionally complex network that regulates transcription of *eps* (Huang et al. 1995). This network has three known signal transduction modules (*phcA*, *vsrA/D*, and *vsrB/C*) that are interconnected by the unique XpsR protein. Interestingly, *xpsR* is located just upstream of Region II (see Figure 1).

Pseudomonas solanacearum also makes a variety of extracellular proteins (EXPs) that participate in pathogenesis. Among the more abundant EXPs that have been characterized are an endoglucanase, a pectin methylesterase, and three polygalacturonases, which presumably help to degrade plant cell walls (Denny and Schell 1994; Schell et al. 1994a). Mutants lacking individual cell-wall-degrading enzymes are still pathogenic, but generally cause wilt symptoms more slowly than do the wild type (Denny et al. 1990), whereas *eep* mutants defective in export of most major EXPs do not cause wilt and seem to be debilitated in their ability to invade or colonize plants (Kang et al. 1994). Although less abundant, PopA1 and other "phytotoxic" proteins made by *P. solanacearum* may play a critical role in pathogenesis due to their elicitation of self-destructive defense responses by plant cells (Arlat et al. 1994). Although encoded by nonhomologous genes, the biological activity of PopA1 is similar to that of harpins produced by other phytopathogenic bacteria (Wei et al. 1992; He et al. 1993), and both require conserved *hrp*-encoded processes for export (Arlat et al. 1994; Salmond 1994).

The single most abundant EXP (constituting ≥30% of the total) in the culture supernatants of several diverse *P. solanacearum* isolates has an apparent molecular mass of 28 kDa when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Schell 1987; Schell et al. 1994a). No enzymatic or other biological activity has been

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ascribed to the 28-kDa EXP, but several observations suggested that it is associated with EPS I. Unlike several extracellular enzymes, when EXPs were fractionated by nondenaturing gel filtration the 28-kDa peptide eluted in a high-molecular-mass complex with EPS I (Schell et al. 1994a). SDS-PAGE analysis of culture supernatants of five nonallelic EPS-deficient mutants of *P. solanacearum* strain AW showed that they all produce dramatically less 28-kDa EXP (Schell et al. 1994b; Huang et al. 1993; Schell et al. 1994a; Huang et al. 1995). However, *eep* mutants that do not secrete EXPs (including the 28-kDa EXP) and are very low in virulence made normal amounts of EPS I, suggesting that the 28-kDa EXP is not essential for EPS I production (Kang et al. 1994). From these results it seemed logical that this EXP might have an important role during pathogenesis by *P. solanacearum*. As a first step in studying the function of the 28-kDa EXP, we report here the identification, cloning, and characterization of *tek* (for twenty eight kilodalton protein), the structural gene that encodes production of the 28-kDa EXP. Although insertional inactivation of *tek* and concomitant loss of the 28-kDa EXP did not obviously affect virulence, we found that this protein is unexpectedly produced as part of a 58-kDa precursor protein that is exported and proteolytically processed outside the cell.

RESULTS

Cloning of *tek*, the gene encoding the 28-kDa EXP.

The 28-kDa EXP was purified from *P. solanacearum* culture supernatants by phosphocellulose chromatography and

gel filtration (see Materials and Methods). Both high-performance liquid chromatography and high-performance gel electrophoresis suggested that there was only a single 28-kDa polypeptide present in the Bio-Gel-purified preparation (results not shown). Subsequently, this preparation was run on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and the sequence of the first 17 amino acid residues determined to be H₂N-XNKQKNFLFVDTVSVD-COOH. The region encoding residues 2 to 7 was selected as the best candidate for design of an oligonucleotide probe to find the *tek* gene because these amino acids are encoded by the least degenerate DNA sequence (64-fold). However, *P. solanacearum* uses GC-rich codons for these amino acids more than 88% of the time (data not shown), so by not considering some of the AT-rich codons we designed the fourfold degenerate TEK4 oligonucleotide mixture with a high probability of containing a sequence encoding the N terminus of the 28-kDa EXP. A search of the PIR and Swiss-Prot databases revealed that the mixture should also hybridize to the *pglA* gene in *P. solanacearum*, which has an internal amino acid sequence (NKQKN) identical to that in the 28-kDa EXP (Huang and Schell 1990a).

When the ³²P-labeled TEK4 probe was hybridized to colony blots of a *P. solanacearum* genomic DNA library (Carney and Denny 1990) eight clones (0.8% of total) gave positive signals. Dried agarose gels with *Bam*HI- or *Eco*RI-digested cosmid DNA prepared from these clones showed that four of the cosmids hybridized to both the TEK4 probe and an internal fragment from *pglA*; these clones were not examined further. The TEK4 probe alone hybridized to a 2.6-kb *Eco*RI

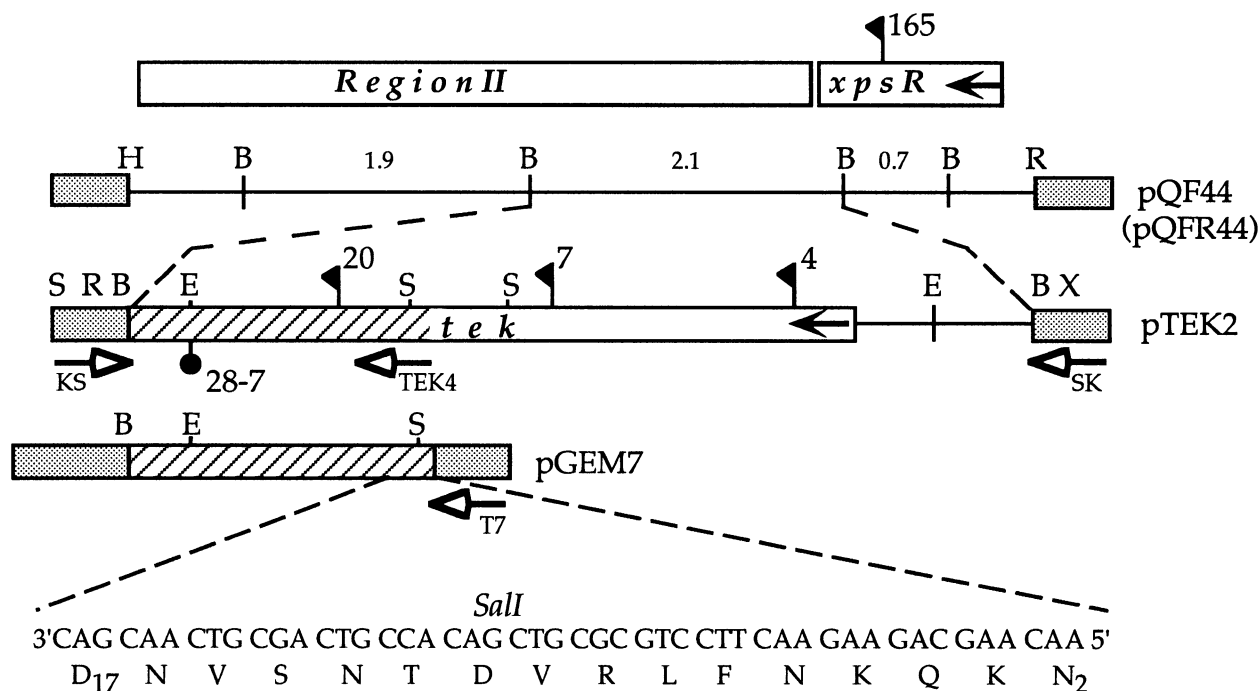


Fig. 1. Physical and genetic maps of plasmids containing *xpsR*, *tek*, and the adjoining Region II locus. Open or cross-hatched boxes represent genes or loci and stippled boxes the flanking vector sequences; filled arrows within boxes denote direction of transcription. Numbers above the line for pQF44 are the sizes, in kilobases, for the *Bam*HI fragments. Open arrows denote annealing sites of the oligonucleotides used for polymerase chain reaction amplification and sequencing. Flags 4, 7, and 20 show the position and transcription direction of plasmidborne and genomic Tn5-B20(*lacZ*) reporter insertions in *tek*; flag 165 represents a nonpolar, genomic Tn5-B20(*lacZ*) insertion in *xpsR*; solid circle 28-7 shows the position of the *nptI* cartridge insertion in *tek*. The DNA sequence and corresponding translated amino acids are those determined using the T7 primer on pGEM7. Restriction site abbreviations are: E, *Bst*EII; S, *Sal*I; R, *Eco*RI; X, *Xba*I; B, *Bam*HI, H, *Hind*III.

fragment from cosmids pDC1 and pHA8, and a 2.1-kb *Bam*HI fragment from cosmids pCB5 and pHD10; the bands were of equal intensity after a 37°C post-hybridization wash, but a subsequent 45°C wash removed most of the probe from the 2.6-kb *Eco*RI fragment. Interestingly, pCB5 had previously been found to contain the Region II locus and the adjacent *xpsR* gene (Denny and Baek 1991; Schell et al. 1993). Bona fide homology of TEK4 to this region was confirmed by its hybridization to the 2.1-kb *Bam*HI fragment in pQF44, which has a 6.4-kb fragment containing only Region II and *xpsR* (Fig. 1).

The 2.6-kb *Eco*RI fragment from pDC1 (likely identical to the 2.6-kb fragment in pHA8) and the 2.1-kb *Bam*HI fragment from pQF44 were subcloned to generate pTek1 (not shown) and pTek2 (Fig. 1), respectively. To map the location and orientation of the TEK4 oligonucleotide hybridization sequence within these cloned fragments, the polymerase chain reaction (PCR) was performed using TEK4 as one primer in conjunction with either a KS or SK primer, which anneal to opposite ends of the multicloning site. For both plasmids amplification was successful only with the TEK4/KS primer pair; five bands (0.3 to 1.3 kb) were amplified from the pTek1 template, but only a single 0.7-kb band was generated from the left-hand end of pTek2 (as shown in Figure 1). Suspecting that pTek2 was more likely to contain part of the *tek* gene, we cloned the 0.7-kb PCR product to create pGEM7 and used a T7 primer site in the vector to determine the DNA sequence of the region containing the TEK4 hybridization site and adjacent DNA. DNA sequence analysis revealed a region complementary to one of the oligonucleotides in the TEK4 mixture. Most importantly, translation of this and the flanking region revealed a predicted amino acid sequence identical to that determined for the N terminus of the 28-kDa EXP (except that arginine and not phenylalanine was found at position 9). These results indicated that pTek2 contains part or all of *tek*, the gene encoding the 28-kDa EXP.

DNA sequence analysis of *tek*.

The DNA sequence for both strands of pTek2 was determined (GenBank number BSU40655). Sequence analysis revealed a 1,737-base pair, 579-residue open reading frame (ORF) starting 147 bases downstream from the stop codon of *xpsR* and ending 25 bases from the *Bam*HI site at the other end of the cloned fragment (Fig. 1). The first possible translation initiation codon is a GTG at base 165 (numbered from first nucleotide after the termination codon of *xpsR*), which is preceded by a likely ribosomal binding site (152-ACGGAG-157); an ORF starting here gives a 58-kDa, 572-residue protein (Fig. 2). Although several other potential translation initiation codons were found downstream of base 165, none was preceded by a reasonable ribosomal binding site. A BLAST search of GenBank did not reveal any proteins with good homology to this ORF, but limited homology (44% similarity, 27% identity) was found with the S-layer protein of *Caulobacter crescentus*, encoded by *rsaA* (Gilchrist et al. 1992), and several other S-layer genes (results not shown). However, the primary structure of proteins that form paracrystalline surface layers is not conserved, so this finding had little predictive value.

The amino acid composition of the putative 58-kDa protein does not vary markedly from the average prokaryotic protein (Doolittle 1986) except for having low levels of His (0.35%) and aromatic amino acids (no Trp, 1.6% Phe, 1.0% Tyr), and high levels of Ser (9.8%) and Thr (10.1%). The putative GTG translation start codon is followed by a short charged region and then a 17-residue hydrophobic region that ends with the sequence LVAC (Fig. 2). This is the typical structure of a lipoprotein signal peptide, which is recognized and cleaved from the N terminus of some proteins by signal peptidase II during their export across the inner membrane of Gram-negative bacteria (Wu and Tokunaga 1986). Surprisingly, the N-terminal sequence of the 28-kDa EXP determined empirically and confirmed by the PCR analysis was located far

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1                               45
MQPQFKKRALVLGVGASAIIVLVACGGGGGSESSATLSPSQVQGVQKAVDFYLSQANVVFTDCNKTTTTDTEGNFTVPSGCAKSAIKVSGGT
                               ↑
91                               135
DIGTGLPFGGVLQAPATDLTQGGTVLVSPMTTLLSQVGTQSSALAGKLGQASDLLSKDPMNDSGLLQNVVATQQQLIEQIAKALTGMSQ
181                               225
STGGTLTPDAAAVAVAAVASALIGATGSTDVSDPPLITPTIVTTVKNSAASLPASVVTNVDAIAANLAALIAPVLAGYVANVNVGLDSV
271                               315
ERVELSATPAETLAALKNAGSMHAVVDSVQSDASSLLAETVTPASVCDTTLVDSLSSLGNAVAEGDDDTINEAATTLGSNVNSGNLSGLI
361                               405
NRNKQKNFLRVDTVSVNDTVVPVANAITVRADTISTLKTSVTQVGSPFGYGNSEIRAGVRYRYNGNELSAVIQRIVLTFNSSNKLIVAGRL
↑
451                               495
PAGTNFDSVLKGATNTRLSTGDNLLDGSTGELVLPIAKLQAKLKNSGILTAQVQDALTPKAPARVTMALALADTSGQMVRVRAATGH
541
GNRTKSLPVIRINAGDSSVVGYGKRSVVTLLP

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Fig. 2. Predicted amino acid sequence of Tek. The amino acid sequence of the putative 58-kDa protein encoded by the *tek* open reading frame was derived from DNA sequence analysis of pTek2 (GenBank accession number BSU40655). The double-underlined region is the predicted 24 amino acid lipoprotein signal peptide; the single-underlined region is the experimentally determined N-terminal sequence of the 28-kDa EXP. The arrows indicate the sites where Tek is processed.

downstream of the beginning of the 58-kDa ORF, between residues 362 and 379. There are no possible translational start codons within 20 residues of this region, suggesting that the 28-kDa EXP is derived by post-translational cleavage of a larger polypeptide, possibly a precursor protein. Thus, it appeared that the *tek* gene could be much larger than was anticipated originally.

Production of the 28-kDa EXP by *P. solanacearum* involves at least three steps.

The DNA sequence analysis predicted that *tek* encodes a 58-kDa precursor that is processed during export to generate a 55-kDa preprotein that may be membrane-associated due to lipid modification. We therefore monitored synthesis, processing, and fate of ^{35}S -labeled Tek-related polypeptides in *P. solanacearum* by immunoprecipitation/SDS-PAGE analysis using antiserum raised against a homogeneously pure preparation of the 28-kDa EXP. Two polypeptides (with molecular masses of 58 kDa and 55 kDa) in the whole cell fraction were specifically immunoprecipitated, whereas identical immunoprecipitations using preimmune serum gave no major products (Fig. 3, lanes 1 and 2). These results indicate that the 58- and 55-kDa cellular polypeptides are encoded by *tek*, contain antigenic determinants found on the 28-kDa EXP, and are thus likely precursors of the 28-kDa EXP. Chasing with cold methionine for 5 min caused complete disappearance of the 58-kDa polypeptide from the whole cell fraction and somewhat increased the amount of labeled 55-kDa polypeptide (Fig. 3, lane 3), suggesting that the 58-kDa polypeptide is the

primary translation product of *tek* and the precursor of the 55-kDa polypeptide. No 28-kDa EXP-related products were detected initially in immunoprecipitates of pulse-labeled culture supernatants (not shown). However, after chasing with cold methionine for 30 min, a 26-kDa polypeptide (likely the 28-kDa EXP) and a 55-kDa polypeptide (likely an extracellular preprotein form of the 28-kDa EXP) were detected in the culture supernatants (Fig. 3, lanes 4 and 5).

To determine the intracellular location of the 58-kDa and 55-kDa precursors, we fractionated pulse-labeled *P. solanacearum* cells into soluble and membrane-enriched fractions and analyzed their content of Tek-related products. The large majority of both the 55-kDa and 58-kDa polypeptides were found in the membrane fraction (Fig. 4A), consistent with the proposed lipoprotein modification of Tek polypeptides predicted from DNA sequence analysis. The 55-kDa preprotein was likely bound to a membrane(s) inside the cell, rather than on the cell surface, because pronase E treatment of intact cells (at 4°C to block further export) did not affect the amount of 55-kDa preprotein present (Fig. 4B). However, sonication before pronase addition caused complete disappearance of both the 58-kDa and 55-kDa polypeptides (Fig. 4B, lane 4), indicating that cell breakage renders them accessible to protease. Interestingly, after protease treatment, a small amount of a 28-kDa Tek-related polypeptide remained, indicating that the 28-

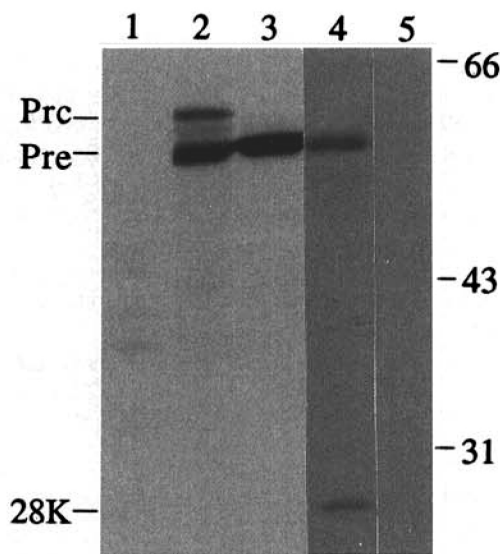


Fig. 3. Immuno detection of *tek*-encoded products from *P. solanacearum*. Cultures were pulse labeled with ^{35}S -methionine for 1 min, chased with unlabeled methionine for various times, and separated into culture supernatants and cell pellets. Fractions (0.3-ml culture equivalent) were immunoprecipitated with anti-28-kDa extracellular protein (EXP) antiserum (lanes 2 to 4) or preimmune serum (lanes 1 and 5) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography. Lanes 1 and 2, pellets from cells before chasing; lane 3, pellets from cells chased for 5 min; lanes 4 and 5, culture supernatants chased for 30 min. Positions of molecular mass standards are at the right (in kilodaltons). Prc, putative Tek precursor; Pre, putative C-terminal 210 residues of pre-protein Tek (i.e., the 28-kDa EXP).

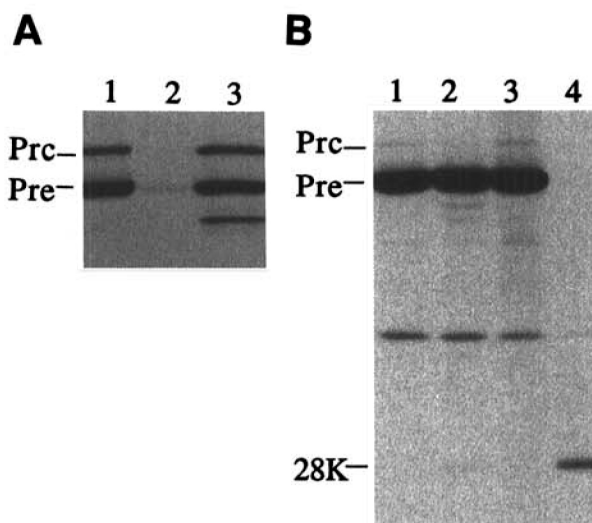


Fig. 4. Localization of cell-pellet-associated Tek. **A**, *Pseudomonas solanacearum* cultures were pulse labeled with ^{35}S -methionine for 1 min and immediately chilled in ice. All subsequent manipulations were carried out at 4°C. The culture supernatants were removed and cell pellets fractionated into water-soluble and membrane fractions. Fractions (0.3-ml culture equivalent) were immunoprecipitated with anti-28-kDa extracellular protein (EXP) antiserum and analyzed as Figure 3. Lane 1, cell pellets; lane 2, water soluble; lane 3, membranes. **B**, Cultures were pulse labeled with ^{35}S -methionine for 1 min and chased with unlabeled methionine for 2 min. Culture supernatants were removed and cell pellets (0.15-ml culture equivalent) suspended (lanes 1 and 2) or sonicated (lanes 3 and 4) in the labeling medium and treated with 100 µg of pronase E per ml on ice for 30 min. Cell suspensions were centrifuged at the end of pronase E treatment to remove any secreted or pronase E-released Tek polypeptides. Trichloroacetic acid-insoluble materials were analyzed by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis as above for panel A. Lane 1, untreated intact cells; lane 2, intact cells treated with pronase E; lane 3, untreated broken cells; lane 4, broken cells treated with pronase E. Figure labels Prc, Pre, and 28K are as in Figure 3.

kDa EXP can be derived from the 55-kDa preprotein by proteolysis.

The above results are consistent with predictions from the DNA sequence analysis of *tek* and support our hypothesis that the 28-kDa EXP is one end product of proteolytic processing of the 58-kDa primary translation product. However, the fate of the N-terminal 337 residues of the 55-kDa Tek preprotein is unclear, since the preprotein was not observed in immunoprecipitated culture supernatants (probably because it cannot react with the antiserum generated against the 28-kDa EXP). However, because the 28-kDa EXP constitutes $\geq 30\%$ of the total extracellular protein of *P. solanacearum*, we were able to monitor directly synthesis and processing of all extracellular Tek-related products without immunoprecipitation by simply analyzing total [35 S]-methionine-labeled proteins by SDS-PAGE. After a 1-min chase with cold methionine, the only Tek-related product detected in a pulse-labeled culture was the 55-kDa polypeptide, and it was exclusively located inside the cell (Fig. 5, lanes 2 and 6). With continued chasing (5 to 60 min), the amount of 55-kDa polypeptide inside the cell decreased concomitant with an increase in the amount found in the culture supernatant (Fig. 5, lanes 3 to 5 and 7 to 9). Simultaneously, levels of two other prominent protein species began to increase in the culture supernatant: the 28-kDa EXP and a new 33-kDa species. Because similarly labeled culture supernatants of a *tek* insertion mutant (see below; Fig. 5, lanes 1, 10, and 11) lacked these two proteins, it is likely that they represent the C-terminal and N-terminal portions, respectively, of the 55-kDa preprotein that has been processed (cleaved) extracellularly.

Expression of *tek* is coordinated with *eps* and some other virulence genes.

xpsR encodes a protein essential for the regulated expression of the *eps* biosynthetic gene cluster and is transcribed

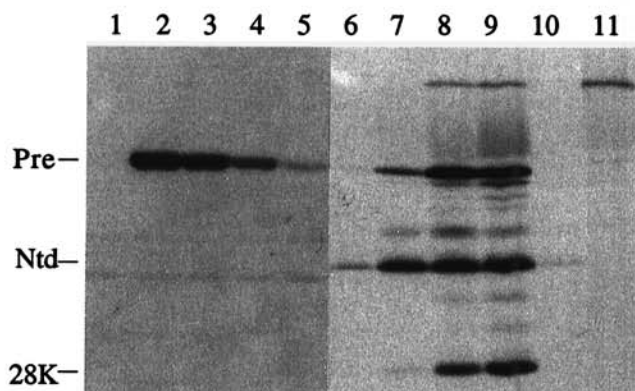


Fig. 5. Synthesis and fate of Tek-related polypeptides. *Pseudomonas solanacearum* cultures were pulse labeled with [35 S]-methionine, chased, immunoprecipitated with the anti-28-kDa extracellular protein (EXP) antiserum, and analyzed as described in the legend to Figure 3 except that trichloroacetic acid-precipitated proteins from pulse-chase labeled culture supernatants (lanes 6 to 11) were directly analyzed without immunoprecipitation. Lane 1, cell pellets of AW28-7 (*tek::nptI*) chased for 1 min; lanes 2 to 5, cell pellets of AW chased for 1, 5, 25, and 60 min, respectively; lanes 6 to 9, culture supernatants of AW chased for 1, 5, 25, and 60 min, respectively; lanes 10 and 11, culture supernatants of AW28-7 (*tek::nptI*) chased for 5 and 25 min, respectively. Labels Pre, Ntd, and 28K are as in Figure 3; Ntd, N-terminal domain (33-kDa) of Tek preprotein.

tionally controlled by both *phcA* and *vsrD* (Huang et al. 1995). Because *tek* is less than 170 bases downstream of *xpsR* and is transcribed in the same direction, we investigated whether these two genes are similarly regulated. pTek2 was mutagenized with Tn5-B20, and three plasmids (pTek2-4, -7, and -20) with *lacZ* fusions (Fig. 1) were isolated, characterized, and used in an allele replacement procedure to create *P. solanacearum* strains with corresponding genomic *tek::lacZ* fusions (AW1-T4, -T7, and -T20). Individual regulatory genes in each *tek* mutant were inactivated by recombining *vsrD::Ω* and *phcA::Ω* alleles into the genome; the same alleles were also recombined into strain AW-R165, which carries a nonpolar *xpsR::lacZ* fusion (Huang et al. 1995). When grown in BG broth (BG is a rich medium containing peptone, yeast extract, casamino acids, and glucose), inactivation of *vsrD* reduced expression of *tek* and *xpsR* about threefold, whereas inactivation of *phcA* reduced expression of both genes about 30-fold (Table 1); the results for *xpsR* are similar to those reported previously (Huang et al. 1995). Similar results were obtained when using MM except that, unlike AW-R165, *lacZ* activity for AW1-T4 (and the other mutants with *tek::lacZ* in a wild-type genetic background) was higher in MM than in BG broth.

We further examined the coordinated regulation of *xpsR* and *tek* by creating plasmids pRG-PXT and pRG-XT that have, respectively, a promoter-plus or promoter-minus copy of *xpsR* followed by the 5' end of *tek* fused to the promoterless *lacZ* gene in pRG970 (Table 2). These plasmids were moved into wild-type and *phcA* mutant strains of *P. solanacearum* and grown in BG broth to test whether the presence of the *xpsR* promoter was required for regulated expression of *tek*. The level of β -galactosidase activity encoded by pRG-PXT was 20-fold greater in the wild-type than in a *phcA* mutant (4,168 versus 213 Miller units), whereas pRG-XT encoded equally low levels of β -galactosidase in both genetic backgrounds (182 versus 243 Miller units). Thus, not only are *xpsR* and *tek* co-regulated by VsrD and PhcA, they appear to be cotranscribed from the promoter immediately upstream of *xpsR* (Huang et al. 1995).

The 28-kDa EXP is not required for EPS production or virulence.

Typical of Region II mutants (Denny and Baek 1991), the *tek::Tn5-B20* mutants were EPS⁻ (nonmucoid) on BG plates and EPS⁺ (mucoid) on MM plates, and EPS production on

Table 1. Effect of inactivation of various regulatory genes on expression of *tek* and *xpsR* grown in BG or MM liquid media^a

Relevant genotype ^b	β -galactosidase activity ^c	
	BG	MM
<i>tek4::Tn5-B20</i>	1,833	2,745
<i>tek4::Tn5-B20, vsrD::Ω</i>	568	360
<i>tek4::Tn5-B20, phcA::Ω</i>	60	40
<i>xpsR165::Tn5-B20</i>	1,777	607
<i>xpsR165::Tn5-B20, vsrD::Ω</i>	592	202
<i>xpsR165::Tn5-B20, phcA::Ω</i>	56	29

^a BG is a rich medium containing peptone, yeast extract, casamino acids, and glucose; MM is a minimal medium supplemented with glucose.

^b Results for *tek4* are similar to those for *tek7* and *tek20*.

^c Enzyme assays used cells from cultures grown to OD₆₀₀ = 0.9 to 1.2; data are representative of three or more assays.

BG plates was restored by pQF44, which carries *xpsR* and Region II. However, because mutants with transposons downstream of *tek* have the same phenotype (Denny and Baek 1991) it seemed likely that the medium-conditional EPS production by the *tek* mutants, and possibly other traits, could have been due to polar effects of the transposons. We created *P. solanacearum* mutants that lacked only the 28-kDa peptide either by inserting a nonpolar *nptI* cartridge into the *Bst*EII site near the 3' end of *tek* (AW-28-7; Fig. 1) or by replacing the *Bst*EII fragment that encodes most of *tek* with the *nptI* cartridge (AW-28-11). SDS-PAGE of total EXPs made by these mutants showed that production of the 28-kDa peptide was apparently eliminated (results not shown). Moreover, immunoprecipitation of labeled, solubilized whole cell proteins of AW-28-7 using anti-28-kDa EXP antiserum yielded no detectable proteins (Fig. 5, lane 1). Unlike the strains with Tn5-B20 insertions in *tek*, both AW-28-7 and AW-28-11 had a wild-type, mucoid colony appearance on plates regardless of the growth medium, and chemical assay for EPS I showed wild-type levels (results not shown). When stem inoculated into susceptible tomato plants, both nonpolar *tek* mutants caused wilt symptoms as rapidly as wild-type AW1. We determined that AW-28-7 did not frequently revert to wild type in planta because all *P. solanacearum* recovered from wilted plants (650 colonies from 7 plants) retained Km resistance, and analysis of EXP production by several of these showed that they were deficient in the 28-kDa protein. Therefore, the 28-kDa EXP did not appear to be essential for EPS production or virulence.

DISCUSSION

We discovered that the sequence encoding the N terminus of the 28-kDa EXP, the major extracellular protein of *P. sola-*

nacearum, is located near the middle of *tek*, a gene that DNA sequence analysis predicted to encode a 58-kDa polypeptide. SDS-PAGE analysis of *tek*-encoded polypeptides of pulse-labeled *P. solanacearum* proteins immunoprecipitated with anti-28-kDa EXP antiserum confirmed that the *tek* primary translation product is a 58-kDa polypeptide whose C-terminal portion becomes the 28-kDa EXP via multi-step processing. The first processing event, which generates a 55-kDa membrane-associated preprotein, is probably the removal of a signal peptide during export of the Tek precursor across the inner membrane. This hypothesis is based on the observation that the first 24 residues of Tek have all the essential features of a lipoprotein-type signal sequence: an N-terminal charged region, followed by a 16-residue hydrophobic region with a possible β -sheet structure (Chou and Fasman 1978), and ending with a near-consensus lipoprotein signal peptide modification and cleavage site (LVA↓C) flanked by a β -turn structure (Wu and Tokunaga 1986; Pugsley and Schwartz 1985). Moreover, the 2.5-kDa molecular mass difference between the Tek primary product and the derived, exported preprotein is consistent with removal of a 24-residue sequence. Thus, like the precursor of the extracellular endoglucanase enzyme made by *P. solanacearum* (Huang and Schell 1990b), the primary translation product of Tek is probably modified by addition of glycerol and a fatty acyl chain at Cys-24 before cleavage by signal peptidase II. It is interesting to note that following the putative lipoprotein signal peptidase II cleavage site in both endoglucanase and Tek is a homologous, conserved glycine + serine-rich sequence (LAG↓CGGGDGDPS LSTASVSAT and LVA↓CGGGGG—SESSATLSPS, respectively). The role of this conserved sequence is unclear, but one possibility is that it is involved in directing further export across the outer membrane, possibly interacting with the *eep* system of *P. solanacearum*, a type II export system (Salmond

Table 2. Bacterial strains and plasmids^a

Strains or Plasmids	Relevant characteristics	Source
<i>Pseudomonas solanacearum</i>		
AW, AW1	Wild type and Nal ^r , respectively	Schell 1987; Denny and Baek 1991
AW1-T4, -T7, -T20	<i>tek4</i> ::Tn5-B20, <i>tek7</i> ::Tn5-B20, <i>tek20</i> ::Tn5-B20, respectively; EPS ^c , Lac ⁺ Nal ^r Km ^r	This work
AW28-7, AW28-11	<i>tek</i> :: <i>nptI</i> and Δ <i>tek</i> :: <i>nptI</i> , respectively, EPS ⁺ Km ^r	This work
AW-R165	<i>xpsR165</i> ::Tn5-B20, EPS ⁻ Lac ⁺ Km ^r	Huang et al. 1995
AW-D4	<i>vsrD4</i> :: Ω , EPS ⁻ Sp ^r	Huang et al. 1995
Plasmids		
pBluescript KS II	ColEI, Ap ^r	Stratagene (La Jolla, CA)
pTZ19U	ColEI, Ap ^r	Mead et al. 1985
pLAFR3	Broad-host-range vector, Tc ^r	Staskawicz et al. 1987
pRG970	Unclassified IncP, promoterless <i>lacZ</i> and <i>gusA</i> , Sp ^r	Van den Eede et al. 1992
pDC1, pHA8, pCB5, pQF4	Cosmid clones containing AW1 genomic DNA, Tc ^r	This work; Denny and Baek 1991
pQF44	6.3-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pQF4 in pLAFR3, Tc ^r	Denny and Baek 1991
pQFR44	6.3-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pQF4 in pTZ19U, Ap ^r	This work
pJH162	2.1-kb <i>Eco</i> RI- <i>Sty</i> I fragment from pQF44	Huang et al. 1995
pTek1	2.6-kb <i>Eco</i> RI fragment from pDC1 in pBluescript KS, Ap ^r	This work
pTek2	2.1-kb <i>Bam</i> HI fragment from pQF44 in pBlueScript KS, Ap ^r	This work
pTek2-4, -7, -20	pTek2 with Tn5-B20 insertions in <i>tek</i> , Ap ^r Km ^r	This work
pMJ28, pMJ11	pQFR44 with <i>nptI</i> cartridge in <i>Bst</i> EII site of <i>tek</i> or replacing the 1.6-kb <i>Bst</i> EII fragment, respectively, Ap ^r Km ^r	This work
pRG-PXT, pRG-XT	Promoter-plus and promoter-minus, respectively, <i>xpsR</i> - <i>tek</i> '- <i>lacZ</i> in pRG970, Sp ^r	This work
pGEM7	700-bp fragment polymerase chain reaction-amplified from pTek2 using TEK4 and KS primers cloned in pGEM-T (Promega), Ap ^r	This work
pGA952 Ω	pGA952 with Ω cartridge in <i>Eco</i> RV site of <i>phcA</i> , Ap ^r Sp ^r	Brumbley et al. 1993; This work

^a EPS^c, conditional EPS production: EPS on BG (a rich medium containing peptone, yeast extract, casamino acids, and glucose), and EPS⁺ on MM (a minimal medium supplemented with glucose).

1994), that is required for extracellular export of Tek, and the endoglucanase (Kang et al. 1994).

After export across the outer membrane the 55-kDa preprotein form of Tek is proteolytically processed either at the cell surface or in the culture supernatant to generate the 28-kDa EXP and a "residual" 33-kDa polypeptide. Hydrophathy analysis showed that the processing site at amino acid 361 (Fig. 2) is within the most hydrophilic region of Tek, suggesting that it is on the surface of the extracellular Tek protein. A hypersensitivity of this region to proteolytic action was observed during *in vitro* pronase treatment, which released an approximately 28-kDa peptide. Whether *in vivo* processing of the Tek preprotein is normally accomplished by a specific extracellular protease(s) or is due to autocatalytic activity by Tek is currently under investigation.

Despite our success in identifying *tek* and in characterizing its products, their function(s) remained obscure. Although the 55-kDa Tek preprotein might be the biologically active unit of Tek, for the most part this preprotein exists only transiently as a membrane-associated form. Alternatively, its cleavage products, the 28-kDa EXP and 33-kDa polypeptide, may be the functional units. However, the 33-kDa N-terminal portion of Tek is also transient and short lived, since it disappears rather than accumulating in culture supernatants, presumably due to degradation. The 33-kDa polypeptide is very acidic (pI 3.85) and could function to "neutralize" the very basic (pI 11.4) 28-kDa EXP portion of the Tek preprotein until after export and cleavage. Only the C-terminal portion of Tek, i.e. the 28-kDa EXP, stably accumulates to high levels in culture supernatants, suggesting it is the likely functional unit of Tek. Since the 28-kDa EXP cofractionates with EPS I during Bio-Gel gel filtration, and its stable accumulation in *P. solanacearum* culture supernatants requires EPS I (M. A. Schell, unpublished), it is likely that it has some physical and possibly functional association with this important virulence factor. Given the highly basic nature of the 28-kDa EXP, a strong binding affinity for the acidic EPS I is plausible. Loss of production of only the 28-kDa EXP by insertion of a nonpolar, antibiotic resistance cartridge in *P. solanacearum* did not affect either the amount or gross properties of EPS I produced, or the virulence as measured by stem inoculation. It may be that the 28-kDa EXP modifies the properties of EPS I in subtle but important ways, although preliminary tests did not reveal any major changes in the size or intrinsic viscosity of EPS I made by the mutants lacking this EXP (T. P. Denny and M. A. Schell, unpublished). However, we do not know or understand all of the functions that EPS I may have in the various niches that *P. solanacearum* occupies, and so may have overlooked an important role for the 28-kDa EXP.

To our surprise, we found that *tek* is located 3 kb downstream of *eps* and immediately following *xpsR*, an integral part of the network regulating expression of the *eps* biosynthetic gene cluster, which is itself regulated by *VsrD* and *PhcA*. We believe that *xpsR*, *tek*, and perhaps additional genes downstream are in the same transcription unit (i.e., part of an operon) because (i) both *xpsR* and *tek* are transcribed in the same direction, (ii) the 5' end of *tek* is only 147 nucleotides downstream of the *xpsR* termination codon but no promoter or termination loop sequences were found in the small intergenic region, (iii) inactivation of the *phcA* and *vsrD* regulatory genes similarly decreased transcription of genomic *lacZ*

fusions in both *xpsR* and *tek*, and (iv) *PhcA*-regulated expression of *tek::lacZ* on a plasmid required the *xpsR* promoter. Although the β -galactosidase activity encoded by genomic *xpsR* and *tek* reporter fusions in a wild-type background was differentially affected by the medium, this may be because the *xpsR165::lacZ* allele is nonpolar whereas the *tek::lacZ* fusions inactivate both *tek* and additional genes downstream that might affect gene expression (see below). Therefore, even though an obvious function for *tek* was not found in this study, its presence in the *xpsR* operon suggests that it may have an undiscovered role in virulence.

The *tek* gene resides entirely on the 2.1-kb *Bam*HI fragment that along with the downstream 1.9-kb *Bam*HI fragment had been defined as the Region II locus (Denny and Baek 1991). Confirming our previous findings, all of the numerous Tn5-B20 insertions within *tek* resulted in *P. solanacearum* mutants that were EPS⁻ on medium with 1% peptone (i.e., BG medium) and EPS⁺ on MM. However, inactivation of *tek* alone did not result in a Region II phenotype, since the *tek::nptI* mutants were fully mucoid on BG medium. These results suggest that the conditional EPS production of the *tek::Tn5-B20* mutants is due to polar effects of the transposon on one or more downstream genes. Therefore, contrary to our earlier conclusion (Denny and Baek 1991), it is now clear that the Region II locus is limited to the 1.9-kb *Bam*HI fragment, which contains one or more genes that are likely to be cotranscribed with *xpsR* and *tek*. We are currently characterizing Region II to investigate its role in regulating production of EPS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The *P. solanacearum* strains and plasmids used are described in Table 2. Plasmids were maintained in *Escherichia coli* strain DH5 α (GIBCO BRL, Gaithersburg, MD). The standard culture conditions for *P. solanacearum* and *E. coli* are described elsewhere (Carney and Denny 1990), as is the MM for *P. solanacearum* (Clough et al. 1994). Antibiotics were added when necessary as follows: ampicillin (Ap), 100 μ g/ml; kanamycin (Km), 50 μ g/ml; nalidixic acid (Nal), 20 μ g/ml; Spectinomycin (Sp), 50 μ g/ml; tetracycline (Tc), 15 μ g/ml.

Purification of the 28-kDa EXP and preparation of antiserum.

Culture supernatant from wild-type strain AW was prepared and mixed with phosphocellulose as described previously (Schell 1987). After centrifugation (10 min, 10,000 \times g) to remove the phosphocellulose, 670 g of ammonium sulfate was dissolved in each liter of the supernatant fraction and stirred for 2 h at 4°C to precipitate remaining EXPs. After centrifugation (15 min, 10,000 \times g), the precipitate was dissolved in 200 ml of 25 mM Tris-HCl (pH 8.0) and dialyzed twice for 12 h against the same buffer. The dialysate was concentrated to 20 ml by ultrafiltration (Amicon UM10 membrane) and 5 ml (13 mg of protein) loaded on a Bio-Gel A 0.5M column (1 \times 50 cm). Proteins eluting with the void volume were pooled, concentrated by ammonium sulfate precipitation (90% saturation) as above, dissolved in 25 mM Tris-HCl (pH 6.8), and stored at -20°C. For antiserum production,

2 mg of the preparation was electrophoresed on a 15 × 0.2-cm preparative SDS–10% polyacrylamide gel (Huang et al. 1989); the 28-kDa EXP band was visualized by soaking the gel in cold 0.5 M KCl, cut out, crushed, and soaked overnight in water. The eluted 28-kDa peptide, which was homogeneously pure, was used to produce antisera in a New Zealand White rabbit as described previously (Schell 1987).

Determination of the N-terminal amino acid sequence of the 28-kDa EXP.

Twelve micrograms of the concentrated Bio-Gel A 0.5 M eluate containing the purified 28-kDa EXP was electrophoresed on a SDS–15% polyacrylamide gel as recommended by Applied Biosystems Inc. (ABI, Foster City, CA; User Bulletin No. 42). Modifications to minimize N-terminal blockage and alteration of amino acid side chains were to polymerize the gel overnight, to heat the sample in solubilization buffer at 60°C for 15 min before loading, and to include 0.1 M sodium thioglycolate in the cathode reservoir buffer (Hunkapiller et al. 1983). Subsequently, proteins were electroblotted to a ProBlott (ABI) polyvinylidene difluoride membrane and stained with Coomassie blue, according to the manufacturer's recommendations. The prominent 28-kDa band was cut out and the N-terminal amino acid sequence determined on an ABI Model 470A sequencer at the University of Georgia Molecular Genetics Instrumentation Facility (UGA MGIF).

Design and use of the TEK4 oligonucleotide mixture.

The partially degenerate, 18-base TEK4 oligonucleotide mixture (5'-AAC AAG CAG/A AAG/A AAC TTC-3') corresponded to the probable coding sequence for amino acids 2 to 7 (NKQKNF) of the 28-kDa EXP. TEK4 was synthesized at the UGA MGIF on an ABI model 394 DNA synthesizer and purified using an ABI oligo purification cartridge. The four oligonucleotides in the TEK4 mixture were estimated to have dissociation temperatures of 46 to 50°C (Miyada and Wallace 1987).

For hybridizations, TEK4 was labeled with [³²P]-dATP (3,000 µCi/mmol) and terminal deoxynucleotidyl transferase using a 3'-end labeling kit (Promega, Madison, WI). This reaction, which was modified by using 10 pmoles of DNA and incubating for 3 h, added a poly-A tail to TEK4 but should not have affected its dissociation temperature (Collins and Hunsaker 1985). Free nucleotides were removed using a Sephadex G-25 spin column (Sambrook et al. 1989). The TEK4 probe was hybridized to colony blots (see below) or dried agarose gels for 16 h at 25°C as described by Miyada and Wallace (1987). The blots or gels were then washed for 3 to 5 min in 0.9 M NaCl, 90 mM sodium citrate (pH 7.0), 0.1% SDS, twice at 25°C and once at 37 and then 45°C.

The TEK4 oligonucleotide was also used as a primer for PCR in combination with the KS or SK primers (Stratagene Cloning Systems, La Jolla, CA). The PCR reaction mixtures contained (in 50 µl total): 5 µl of 10× reaction buffer, 8 µl of dNTP mix (1.25 mM each nucleotide), 3 µl of 25 mM MgCl₂, 2.5 µl of KS or SK primer (22.5 pmoles), 2 µl of TEK4 primer (100 pmoles), 0.5 ng of linearized plasmid template, and 0.25 µl of *Taq* DNA polymerase (1.25 units, Perkin-Elmer Cetus, Norwalk, CT). Except as noted, reagents were from the Repli-pack reagent kit (Boehringer Mannheim Corp., Indianapolis, IN). PCR was performed as follows: an

initial 5 min at 94°C; then 3 cycles of 30 s at 94°C, 1 min at 37°C, 2 min ramp to 72°C, and 1 min at 72°C; then 32 cycles of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C; then a final 4 min at 72°C. Amplified products were electrophoresed through 1.5% agarose gels or purified using Magic PCR preps (Promega). The purified 700-bp fragment amplified from pTek2 was ligated to the pGEM-T vector (Promega) using the manufacturer's protocol and reagents, and transformed into *E. coli* DH5α competent cells.

General DNA methods.

Plasmid DNA was prepared using Magic Mini-preps (Promega) or by the boil mini-prep method (Sambrook et al. 1989). Isolation of and transformation with genomic DNA from *P. solanacearum* have been described (Clough et al. 1994). DNA manipulation in vitro, agarose gel electrophoresis, transformation or electroporation, triparental matings, and making colony blots on nitrocellulose or nylon membranes are described elsewhere (Carney and Denny 1990; Brumbley and Denny 1990; Sambrook et al. 1989; Ausubel et al. 1989). DNA fragments were isolated from agarose gels using the Prep-a-Gene matrix (BioRad, Hercules, CA) as described by the manufacturer. DNA sequencing of pTek2 and pTek2::Tn5-B20 used double-stranded plasmid templates, and was performed at the UGA MGIF on an ABI Model 373A DNA sequencer. To sequence the DNA flanking the Tn5-B20 insertions we employed a primer (5'-GTAAAACGACGGATCC AT-3') that hybridized near the 5'-end of *lacZ* in the transposon. DNA sequences were analyzed with programs (Devreux et al. 1984) in the Wisconsin Genetic Computer Group (GCG) or Intelligenetics Group packages, and databases searched with BLAST (Altschul et al. 1990).

Construction of 28-kDa EXP deficient mutants and virulence tests.

pTek2 in *E. coli* was mutagenized with Tn5-B20, which carries a promoterless *lacZ* gene, as described by Simon et al. (1989), and the position and orientation of the transposon insertions determined by restriction mapping. Selected transposon insertions that had *lacZ* within the 2.1-kb *Bam*HI fragment and transcribed in the same direction as *tek*, were site-specifically recombined into the genome of *P. solanacearum* AW1 as described (Huang et al. 1995). Southern blot analysis confirmed that each strain had the *lacZ*-containing transposon inserted within the 2.1-kb *Bam*HI fragment that contains *tek*. To create nonpolar insertions within the genomic copy of *tek*, plasmid pQFR44 was partially digested with *Bst*EII and the cohesive ends filled in with dNTPs and DNA polymerase I (Klenow). This preparation was ligated with *Hind*II-digested pSB315 (Galan et al. 1992) and then transformed into *E. coli*. Ap^r Km^r transformants were screened for pQFR44 derivatives that had the nonpolar *nptI* cartridge of pSB315 inserted into the *Bst*EII site within the coding sequence of the 28-kDa EXP and in the same transcription direction (Fig. 1). Plasmid pMJ28 had the *nptI* cartridge inserted near the C terminus of *tek*, whereas in pMJ11 the *nptI* cartridge replaced the 1.6-kb *Bst*EII fragment that contains all but the C terminus of *tek*. These plasmids were electroporated into wild-type *P. solanacearum* and marker-exchange recombinants, AW-28-7 and AW-28-11, were selected by their Km^r Ap^s phenotype.

Virulence on tomato (*Lycopersicon esculentum* Mill. 'Marion') plants, measured by the rate at which leaves wilted, was assessed by stem inoculation with 2×10^4 cells in 20 μ l of sterile water as described (Roberts et al. 1988).

Expression of *tek* and *xpsR*.

Strains with *tek::lacZ* or *xpsR::lacZ* fusions in a wild-type genetic background (Table 2) were site-specifically mutagenized with pGA952 Ω plasmid DNA or AW-D4 genomic DNA to introduce *phcA::\Omega* and *vsrD::\Omega* mutations, respectively. The single mutants and related double mutants were grown in BG or MM broth and then assayed for β -galactosidase activity (Miller 1972; Clough et al. 1994). To create pRG-PXT, the promoter-plus *xpsR-tek'-lacZ* construct, pJH162 was digested with *EcoRI* (620 nucleotides upstream of the *xpsR* start codon) and *EcoRV* (270 bases downstream of the *tek* start codon), and the ends made blunt with Klenow enzyme. The resulting fragments were ligated to pRG970 previously digested with *SmaI*, and transformed into DH5 α . Plasmid DNA from β -galactosidase-positive, ampicillin-sensitive transformants was examined by restriction digestion, and one clone that contained the desired 1.9-kb insertion in the correct orientation was selected. A similar approach was used to create pRG-XT, the *xpsR* promoterless construct, except that pJH162 was digested with *BspEI* (20 nucleotides upstream of the *xpsR* start codon but downstream of the promoter) (Huang et al. 1995) and *EcoRV* to create a 1.4-kb fragment that was subsequently cloned into pRG970.

Pulse-chase labeling and immunoprecipitation analysis.

Pseudomonas solanacearum cells were grown to OD₆₀₀ = 1.5 in a pulse-labeling medium, pulse-labeled with 30 μ Ci/ml of [³⁵S]-methionine and then chased with 200 μ g of unlabeled methionine per ml as described previously (Huang et al. 1989). Cultures were centrifuged at the end of the pulse or during the chase to obtain culture supernatants and cell pellets. In some experiments, pellets were further fractionated into water-soluble and membrane fractions as described elsewhere (Huang et al. 1993). After fractionation, trichloroacetic acid was added to 10% and insoluble materials were recovered, solubilized, immunoprecipitated, electrophoresed on an SDS-10% polyacrylamide gel, and analyzed by fluorography as described previously (Huang et al. 1989).

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