Characterization of a Negative Regulator of Exopolysaccharide Production by the Plant-Pathogenic Bacterium *Pseudomonas solanacearum*

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Wild-type strains of the bacterial wilt pathogen *Pseudomonas solanacearum* exhibit reduced exopolysaccharide production and virulence when transformed with plasmids carrying the *epsR* locus. To understand the function of *epsR*, we used mutagenesis and DNA sequencing to identify the gene responsible for the shutoff of exopolysaccharide production. The *epsR* gene encodes a 236-amino-acid polypeptide that, based on polypeptide sequence homology, has significant similarity to other proteins of the luxR family of environmentally responsive, two-component regulatory systems. When a mutated copy of the *epsR* gene was marker-exchanged into the wild-type *P. solanacearum* chromosome, however, we observed no effect on growth in culture or on exopolysaccharide production. This suggests that the EpsR phenotype becomes apparent only via overproduction of the EpsR protein. By means of an antiserum directed against the EpsR protein, we detected the overproduction of EpsR in cell lysates of a strain of *P. solanacearum* harboring a multicopy plasmid with an active *epsR* gene but not in one harboring the same plasmid with a mutated *epsR* gene.

Virtually all plant-pathogenic and symbiotic bacteria produce exopolysaccharides (EPS) either as an organized, covalently bound capsule or as a loosely associated slime. The production of EPS has been implicated in protecting bacteria from toxic chemicals and desiccation, in attachment to specific surfaces, and as virulence factors in plant and animal pathogens (Coplin and Cook 1990; Ferris and Beveridge 1985; Van Alfen 1982).


*Pseudomonas solanacearum* (E. F. Sm.) is the causal agent of bacterial wilt of numerous economically important crops worldwide. Although the mechanism of wilting caused by this bacterium is not completely understood, it is known that EPS play an important role, perhaps by contributing to plugging of xylem vessels and interfering with water transport in the plant (Husain and Kelman 1958; Kelman 1954). Mutations that prevent EPS expression by the pathogen *in planta* consistently result in decrease in virulence (Kao et al. 1992). EPS may also contribute to virulence by other means; for example, they may prevent binding of bacteria to the plant cell wall (Young and Sequeira 1986).

Transposon mutagenesis of *P. solanacearum* has led to the identification of several clusters of EPS genes (Cook and Sequeira 1990; Denny et al. 1988; Denny and Baek 1991; Kao and Sequeira 1991; Kao et al. 1992). The regulation of EPS synthesis in *P. solanacearum* is relatively less understood, but both positive and negative regulatory factors have been reported (Brumbley and Denny 1990; Huang and Sequeira 1990; Negishi et al. 1993). A positive regulator of EPS production named *phcA* has been identified by Brumbley and Denny (1990). Negishi et al. (1993) have reported the isolation of a small plasmid from a spontaneously nonpathogenic strain of *P. solanacearum* that can reduce EPS production and pathogenicity of wild-type strains. Huang and Sequeira (1990) had previously isolated a cosmid clone containing a chromosomal DNA fragment from the spontaneous, avirulent, B1 strain. When introduced into wild-type *P. solanacearum* strains, this cosmid reduced EPS production, increased polgalacturonase production, and decreased virulence to inoculated plants (Huang and Sequeira 1990). Transposon mutagenesis of the cosmid led to the suggestion that a single transcriptional unit named *epsR* was responsible for these various phenotypes (Huang and Sequeira 1990; Gosti et al. 1992). However, many questions remain about the *epsR* locus. For example, since the original *epsR* locus was cloned from the mutant B1 genome, is its effect due to a mutant gene product? Also, does the shutoff of EPS production result from the effect of titrating nucleic acid sequence, or from the activity of a polypeptide? To answer these and other questions, we cloned the *epsR* homolog from the wild-type K60 strain and performed extensive mutagenesis and DNA sequencing of both the K60 and B1 *epsR* alleles. In this article, we report the molecular and genetic characterization of the *epsR* gene and immunological detection of the EpsR protein. We also report that deletion of

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the chromosomal *epsR* gene did not have an observable effect on the growth of *P. solanacearum* in culture.

**RESULTS**

Cloning of a functional *epsR* locus from strain K60.

Plasmid pBE6 (Table 1), previously found to interfere with EPS production in wild-type strains, was initially isolated from the genome of the spontaneous avirulent mutant strain, B1. Before cloning the *epsR* homolog from the wild-type strain K60, we probed a Southern blot of restriction-digested K60 and B1 genomic DNAs with the 1.6-kb BamHI-HindIII restriction fragment from pBE6 that contains the sequence necessary for EpsR activity (Huang and Sequeira 1990; Fig. 1A). The genomes of both K60 and B1 contain two non-overlapping regions that hybridized to the pBE6 probe (Fig. 1B, and data not shown). Therefore, it was necessary to clone DNA fragments containing both sequences.

*Escherichia coli* colonies harboring a cosmid library of the K60 genome were screened by colony hybridization, using the radiolabeled 1.6-kb BamHI-HindIII fragment as probe. Of approximately 1,400 colonies tested, 10 hybridized with the probe. Restriction analysis of these positive clones revealed two sets of nonidentical patterns, as represented by pKL4 and pKL6. Thus, the two sequences from the K60 genome that hybridized to the probe in Southern blots were cloned. To determine whether the clones were functional in shutting off EPS production, plasmids from each class, as well as negative (pLAFR3) and positive (pBE6) controls, were electroporated into K60 cells. The tetracycline-resistant transformants harboring pBE6 or pKL4 had a colony morphology similar to that of the spontaneous variant, B1, whereas cells harboring either pLAFR3 or pKL6 gave rise to fluidic colonies. Several days after transfection, small amounts of EPS began to accumulate in the EpsR+ strains as determined by colony appearance (Fig. 2 shows an example of the EpsR phenotype). Intact plasmids were recovered from these transmerodiploid strains (data not shown), confirming that the plasmids were stably maintained under selective conditions. Since pKL4 contained genomic DNA from the wild-type K60 library and had a restriction pattern that was indistinguishable from that of the B1 *epsR* gene, we concluded that functional *epsR* genes are present in both B1 and K60 strains. Also, since the pKL6 cosmid did not have EpsR activity, we did not analyze it further.

**Mapping of the *epsR* functional unit.**

The inserts from pKL4 and the B1 cosmid pBE6, were subcloned to define the sequences necessary for EpsR activity. A subclone named pKL44, derived from pKL4, and the comparable subclone derived from pBE6 both contained a 1.3-kb Stul to NsiI restriction fragment that decreased EPS expression when introduced in either pLAFR3 or pLAFR6 vectors (Fig. 3). It was previously known that Tn5-gus insertions in this region of pBE6 abolished the EpsR phenotype (Huang and Sequeira 1990).

The 1.3-kb Stul to NsiI restriction fragments encompassing the functional *epsR* unit from both K60 and B1 strains were sequenced by the dyeoxy chain termination method (Sanger et al. 1977). The nucleotide sequences derived from both strains were identical, which was consistent with the observation that both clones had identical effects on EPS expression.

Analyses of the sequence from the insert of pKL44 revealed an open reading frame (ORF) starting at nucleotide 215 and terminating with a TGA codon at nucleotide 1072 (Fig. 4). The sequence in this area does not possess obvious homologies to previously defined pseudomonad promotors.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant characteristics and construction</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5a</td>
<td>F−, endA1, hsdR17(rK− mK+) recA1</td>
<td>BRL</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F−, ompT, cI, mK−, ΔADE3</td>
<td>Novagen</td>
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<tr>
<td><em>P. solanacearum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K60</td>
<td>Wild-type virulent, EPS+ Tc'</td>
<td>A. Kelman</td>
</tr>
<tr>
<td>B1</td>
<td>Spontaneous, avirulent, EPS− Tc'</td>
<td>A. Kelman</td>
</tr>
<tr>
<td>K60.4</td>
<td>K60::pBE6.4 marker exchange mutant EPS+ Km'</td>
<td>This work</td>
</tr>
<tr>
<td>B1.4</td>
<td>B1::pBE6.4 marker exchange mutant EPS− Km'</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
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<td>pLAFR3</td>
<td>IncP1, Tc' r'/1'</td>
<td>Peet et al. 1986</td>
</tr>
<tr>
<td>PLAFR5</td>
<td>IncP1, Tc' r'/1'</td>
<td>Keen et al. 1988</td>
</tr>
<tr>
<td>PLAFR6</td>
<td>IncP1, Tc' r'/1'</td>
<td>B. Staskawicz</td>
</tr>
<tr>
<td>pBE6</td>
<td>pLAFR3 containing an 8-kb B1 chromosomal DNA fragment, Tc'</td>
<td>Huang and Sequeira 1990</td>
</tr>
<tr>
<td>pBE6.4</td>
<td>pBE6::Tn5-gus Ap Tc' Km'</td>
<td>Huang and Sequeira 1990</td>
</tr>
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<td>pKL4</td>
<td>pLAFR5 containing a 20-kb Sau3A K60 chromosomal DNA fragment cloned into the <em>BamHI</em> site</td>
<td>This work</td>
</tr>
<tr>
<td>pKL44</td>
<td>pLAFR6 containing a 1.3-kb Stul−NsiI DNA fragment from pKL4 cloned between the <em>Xmal</em> and the <em>PstI</em> sites</td>
<td>This work</td>
</tr>
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<td>pKL48</td>
<td>pKL44 with a filled-in <em>BamHI</em> site in the <em>epsR</em> locus</td>
<td>This work</td>
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<td>PKL50</td>
<td>pKL44 with deletion of nt 1–286</td>
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</tr>
<tr>
<td>pKL52</td>
<td>pKL44 with deletion of nt 892–1185</td>
<td>This work</td>
</tr>
<tr>
<td>pKL53</td>
<td>pKL44 with deletion of nt 892–1338</td>
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<tr>
<td>pKL54</td>
<td>pKL44 with an in-frame deletion of nt 746–892</td>
<td>This work</td>
</tr>
<tr>
<td>pKL58</td>
<td>Subclone containing sequences from nt 215–1290 cloned in; pLAFR3</td>
<td>This work</td>
</tr>
<tr>
<td>pepsRA</td>
<td>Subclone containing sequences from nt 365–1290; pLAFR3</td>
<td>This work</td>
</tr>
<tr>
<td>pG-epsRA</td>
<td>Insert from pepsRA fused to the <em>epsG</em> promoter and cloned in pLAFR3</td>
<td>This work</td>
</tr>
<tr>
<td>pG-epsRB</td>
<td>DNA fragment from nt 458–1290 fused to the <em>epsG</em> promoter; in pLAFR3</td>
<td>This work</td>
</tr>
<tr>
<td>pT7-epsRA</td>
<td>Insert from pepsRA cloned behind a T7 promoter in pET11</td>
<td>This work</td>
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</table>
(Deretic et al. 1989b). Within this ORF is a potential ATG initiation codon at nucleotide 644, which would allow a coding capacity of 143 amino acids. However, another potential initiation codon, GTG, exists at nucleotide 365. The putative polypeptide initiating translation at this position would result in a product of approximately 25 kDa, in good agreement with the 25-kDa protein expressed in maxicells from a plasmid containing the epsR sequence (Huang and Sequiera 1990).

Deletion mutations derived from pKL44, which contains a functional subclone of the K60 epsR gene, were constructed to determine whether the identified ORF encodes EpsR activity (Fig. 3). The mutants were electroporated into strain K60, and the effect on EPS production was compared visually with that of cells electroporated with control plasmids. A plasmid (pKL48) that had the BamHI site filled in with Klenow enzyme and deoxynucleotides and that was shown to have lost the BamHI site by restriction analysis was EpsR⁺. Also, a polymerase chain reaction (PCR)-derived DNA fragment containing sequences from nucleotides 215–1290 and cloned in pLFR3 (pKL58) was EpsR⁺. However, deletion of the first 287 nucleotides from the BamHI site caused the resulting plasmid (pKL50) to lose EpsR activity and resulted in a wild-type level of EPS production. Thus the 5′ end of the ORF, including sequences upstream of the GTG codon at nucleotide 365, is required for activity.

At the 3′ end of the ORF, deletions that removed the sequence from the Spol site at nucleotide 892 to the NsiI site at nucleotide 1338 (pKL53) and deletions of sequences between the two internal Spol sites (pKL52) caused a loss of EpsR activity. Finally, a translationally in-frame deletion (pKL54) of the 49 codons between nucleotides 745 and 892 made by digestion with Bal31 nuclease and religation did not have EpsR activity. In summary, much of the 5′ and 3′ ends of the ORF in pKL44 is necessary for EpsR activity.

The 5′ end of the putative epsR ORF was thought to contain the promoter of the epsR gene because EpsR activity was not affected when the BamHI site was filled in, but activity was abolished when the sequence up to the BamHI site was deleted. A potential initiation codon in this region is the GTG at nucleotide 365. To determine whether translation initiating at nucleotide 365 can lead to a functional epsR gene, we made fusions with the promoter of the P. solanacearum opsG gene, which directs the synthesis of UDP-rhamnose, a sugar nucleotide that is required for both EPS and LPS synthesis in P. solanacearum (Kao and Sequiera 1991; C. Kao, unpublished). Prior experiments had indicated that the opsG fragment was able to drive the expression of a promoterless gus gene (C. Kao, unpublished). The opsG promoter was synthesized by PCR as a fragment with a NdeI site (CATATG) at its 3′ end. A DNA fragment of the epsR gene from nucleotides 365–1290 was generated by PCR with an added 5′ NdeI site, thus replacing the original GTG sequence with the ATG from the NdeI restriction site. This PCR fragment of the epsR sequence, when cloned in pLFR3 to generate the plasmid pepsRA, had no EpsR activity. However, when the same fragment was fused to the opsG promoter via the respective NdeI sites and cloned in pLFR3 (Fig. 3), the resultant fusion construct, pG-epsRA, was able to shut off EPS production in strain K60. Colonies transformed with pG-epsRA remained devoid of EPS for approximately 4 days after electroporation.
A second construct, pG-epsRB, which contains nucleotides 458–1290 of epsR fused to the opaG promoter and cloned into pLAFR3, was not able to shut off EPS expression (Fig. 3). Therefore, the sequence between nucleotides 365 and 458 was necessary for EpsR activity. Apparently, translation cannot start downstream of nucleotide 458 and result in a functional epsR product, and the sequence upstream of the BamHI site must have promoter activity.

**Analysis of epsR nucleotide sequence.**

To obtain clues to the function of the EpsR protein, we compared the 236-amino-acid residue sequence encoded by the epsR ORF to entries in the GenBank database (version 65). FastA search revealed that 13 bacterial proteins have significant homology to EpsR. Several of these polypeptides have been identified as members of a family of bacterial signal-transducing systems, including luxR, that share extended homology, especially at their C-terminal ends (Deretic et al. 1989a; Gross et al. 1989; Stout and Gottesman 1990). An alignment of all these polypeptides revealed 22 residues, mostly at the C-terminal end of each polypeptide, that were highly conserved (Fig. 5). The C-terminal segment of the putative EpsR protein sequence had 16 residues that were identical to the sequence of the 22 highly conserved residues. Of the six residues in EpsR that were different from the consensus sequence, five were conserved in terms of the residue’s charge.

**Genetic comparison of epsR activity in K60 and B1 strains.**

The epsR genes cloned from either B1 or K60 were identical in nucleotide sequence and phenotype (see above). To determine whether the genetic background affects expression of the epsR gene, the Tn3-gus insertion, pBE6.4, was marker-exchanged into both K60 and B1 chromosomes. Marker-exchange mutants resulting from double homologous recombination were identified by screening individual Km' colonies for spontaneous loss of pLAFR3 based on tetracycline sensitivity. Homologous recombination in the resulting strains, designated K60.4 and B1.4, was verified by Southern blot analysis (Fig. 1C). Both strains contained Tn3-gus insertions in the 3.6-kb band and resulted in a larger band of approximately 12 kb (Fig. 1C, lanes 2 and 4). The recombinant strains, however, did not appear to be affected in EPS production, as determined by colony morphology, i.e., K60.4 produced wild-type amounts of slime, and B1.4 remained defective in EPS production. From these results, we concluded that the EpsR phenotype is observed only when multiple copies of the epsR gene are present on plasmids. Furthermore, the lack of a functional epsR gene apparently does not affect the viability of the recombinant strains in culture.

**Detection of EpsR protein in P. solanacearum.**

Since the shutoff of EPS production was observed only when EpsR was expressed from multicopy plasmids, we used...
Western blots to determine the amount of EpsR protein produced in *P. solanacearum* strains carrying either EpsR*+* or EpsR- plasmids (Fig. 6). K60 transformed with either pLAFR3 (Fig. 6, lane 1) or the inactive pepsRA (Fig. 6, lane 4, and Table 1) produced wild-type levels of EPS and had no detectable reaction to the antiserum raised against EpsR protein produced in *E. coli* (lane 5). However, signals from two K60 cells independently transformed with pBE6 (lanes 2 and 3) had signals that were at least 10-fold higher than background. In addition, the protein detected by the anti-EpsR serum was of approximately 25 kDa, the same size as the protein produced in *E. coli*. This suggests that the EpsR translation initiation site in *P. solanacearum* is likely the GTG codon at nucleotide 365. The signals above the major band of 25 kDa were not visible proteins in the original Coomassie Blue stained gels, and probably represent minor amounts of partially denatured proteins.

**DISCUSSION**

In this article, we have presented a molecular genetic characterization of *epsR*, a gene that regulates multiple functions in *P. solanacearum*, including EPS biosynthesis (Huang and Sequeira 1990). We conclude that *epsR* encodes a trans-acting negative regulator of EPS synthesis, the effects of which are apparent only when it is present on a multicopy plasmid (Huang and Sequeira 1990). The *epsR* genes cloned from a spontaneous avirulent mutant strain (B1) and a wild-type strain (K60).

![Diagram](Fig. 3. Mutagenesis of the *epsR* functional unit. The position of the Tn3-gus insertions in the plasmid pBE6 are indicated by flags, the orientations of which refer to the direction of translation of the reporter gene. Dark flags indicate that the insertion affected *epsR* function in strain K60 and resulted in normal exopoly saccharide (EPS) production. Deletions within the *epsR* gene in pKL44 and its derivatives are indicated by the schematics, and the effects on the EpsR phenotype are listed on the right. + indicates that the plasmid electroporated into strain K60 was able to shut off EPS production; – indicates that Eps expression was not affected. Restriction sites are indicated by the following codes: B: *BamHI*, E: *EcoRI*, H: HindIII, N: *NsiI*, S: *SphI*, St: *StuI*. The cross-hatched boxes indicate the position of the putative EpsR translation sequence. The filled-in triangle denotes the filled-in *BamHI* site in plasmid pKL48. The lines in pKL52 and pKL54 indicate that the sequences were deleted. The black box represents the 250-bp *opsG* promoter DNA fragment that was derived by polymerase chain reaction (PCR) from the *opsG* gene and includes a putative Shine-Delgarno sequence. The restriction fragments used to construct fusions with the *opsG* promoter were generated by PCR reactions using 5' oligonucleotides that contain a novel Ndel restriction site and a 3' oligonucleotide that contains a novel HindIII restriction site. The two 5' oligonucleotides were: EpsRA (5' ATACATATGAATCTAATT- CGTAGTCGAA; Ndel site underlined) and EpsRB (5' ATACATATGGAACCGGCGAAGGCTT 3'). The 3' oligonucleotide was EpsR3'H (5' ATAAAGCTTGGCAGCCAACGCTGCTACT 3'; HindIII site underlined) that hybridizes to the sequence between nucleotides 1273 and 1290. All three oligonucleotides contain an extra three nucleotides at the 5' end to facilitate eventual cloning.)
Fig. 4. Nucleotide sequence of epsR. A, Sequencing strategy employed. The arrows represent the direction and extent of the sequencing reaction. The black bar locates the minimal epsR functional unit, and the cross-hatched box indicates the EpsR open reading frame. B, Nucleotide sequence of the epsR region extending for 1,338 bp from the Stul site to the NsiI site. The predicted amino acid (aa) sequence for the entire 286-aa open reading frame is overlined. The flags indicate the insertion positions of the Tn3-gus in the B1 epsR gene. Black flags those that did not. Location and orientation of the oligonucleotide primers used for polymerase chain reactions are indicated by arrows. The stretch of amino acids that are underlined have sequence homology to proteins belonging to a family of environmentally responsive regulators (see Fig. 5). The nucleotide sequence data reported in this article will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number M61197.
Fig. 5. Alignment of *Pseudomonas solanacearum* EpsR residues 181–236 with the *Escherichia coli* DnaY-ORF (Maramatsu and Mixuno 1990), UhpA (Friedrich and Kaden 1987), UvrC-ORF2 (Moolenar et al. 1987), NarL (Gonzalez et al. 1989; Nohno et al. 1989), RcsB (Stout and Gottesman 1990), and MalT (Cole and Ribaud 1986); the *Bordetella pertussis* BvgA (Arico et al. 1989); the *B. subtilis* GerE (Henner et al. 1988; Kunst et al. 1988), DegU (Henner et al. 1988), and ComA (Weinrauch et al. 1989); the *Rhizobium melliloti* FixD (David et al. 1988); the *P. oleovorans* A1K (Egink et al. 1990); and the V. *fischeri* LuxR (Engberts et al. 1987). A consensus sequence is defined by the residues conserved in identity or charge at more than nine residues out of 14 for each position.

![Consensus](image)

**Fig. 6.** A Western blot that demonstrates that the EpsR protein is expressed in EpsR+ cells. Lanes 1–4 represent *Pseudomonas solanacearum* extracts from approximately 200 μl of mid-log cultures. The plasmids harbored by each strain are noted above each lane. Lanes 2 and 3 represent two independent K60 strains transformed with plasmid pBES. Lane 5 contains partially purified EpsR protein produced in *Escherichia coli*. By comparison with molecular weight markers, this band is approximately 25 kDa. Antiserum to EpsR was used at a 1:1,000 dilution, and the secondary serum (goat antirabbit conjugated to horseradish peroxidase) was used at a 1:3,000 dilution. The reaction was visualized by chemiluminescence (ECL kit, Amersham).

of *P. solanacearum* were shown to have the same activities and nucleotide sequences. Nucleotide sequencing and mutagenesis studies suggest that the putative EpsR protein consists of 236 amino acid residues encoded in a 1.3-kb StuI to NsiI restriction fragment. The amino acid sequence translated from the putative EpsR polypeptide bears striking resemblance to a class of bacterial regulatory proteins that includes the RcsB polypeptide, a positive regulator of capsule production in enteric bacteria (Stout and Gottesman 1990; Stout et al. 1991). Results from Western blots using anti-EpsR serum suggest that cells that are reduced in EPS production are expressing the EpsR protein.

**Mechanism of EpsR activity.**

The EpsR phenotype is mediated by the expression of the EpsR protein and is not merely a nucleotide sequence that titrates out factors that positively regulate EPS synthesis. We base this conclusion on the following results. First, the intact ORF encoding the putative EpsR polypeptide is required for shutdown of EPS biosynthesis; mutations at the 5' or 3' ends or internal to the ORF abolished EpsR activity. Second, the epsR gene must be transcribed, either from its endogenous promoter, or from the opsG promoter, for activity. Third, the homology of the putative EpsR polypeptide sequence to other regulatory proteins is consistent with the concept that the protein plays a regulatory role in EPS synthesis. Finally, we observed in Western blots of cell lysates carrying EpsR+ plasmids, the production of a polypeptide of the size predicted by DNA sequence data.

The EpsR phenotype is expressed in cells for about 4 days after electroporation. Thereafter, the cells begin to produce some EPS. When these leaky colonies were streaked out onto fresh plates, the transformants again exhibited an approximately 4-day lag in EPS production in comparison to wild-type strains. We do not know whether the structure of the EPS produced by the transformants is chemically identical to...
that produced in the first 4 days after transformation. It is also possible that the effect of the EpsR protein is modulated by growth conditions or other cellular regulatory mechanisms. However, since EpsR expressed from the constitutive opg promoter also eliminated EPS production for approximately 4 days, it is likely that modulation of EpsR activity occurs via a posttranscriptional mechanism. In E. coli, for example, the regulation of capsule polysaccharide production can be mediated through the Lon-dependent degradation of the RcsA protein (Stout et al. 1991). Whether this type of regulation exists in P. solanacearum remains to be explored.

The epsR gene.

The promoter for the epsR gene apparently is present in the 1.3-kb Stu–NsiI restriction fragment since subclones containing the fragment were functional even when we used a vector (pLAFR6) that contained transcriptional termination signals on both sides of the cloning site (B. Staskawicz, personal communication). Since the EpsR phenotype requires nucleotides 365–458 even in the presence of an active opg promoter, translation must initiate in this region, perhaps at the GTG codon at nucleotide 365. GTG has been reported as a translational start signal for several proteins, including bacteriophage MS2 (Gold 1988). The proposed epsR translational sequence is also preceded at an appropriate distance by a potential Shine-Dalgarno sequence (Shine and Dalgarno 1974). In addition, the codon following the putative initiation start site (GTG) is AAA, one of the most abundant second codons (Gold 1988).

The deduced primary amino acid sequence of EpsR had significant homology with all members of a subfamily of regulators (DegU, BvgA, NarL, UvrC-ORF2, UhpA, GerE, ComA, RcsB, and MalT). The homology was found principally at the C terminus of these polypeptides, as it is in the case of EpsR (Fig. 4). Furthermore, deletions of this region of the EpsR sequences in the constructs pKL52, pKL53, and pKL54 (Fig. 3) all abolished EpsR activity. Thus, we suggest that the EpsR protein is another member of this subgroup. We used the computer to derive, from the aligned sequence, a profile characteristic of this family. This profile was successfully used to identify sites in the database (NBRF version 25) proteins already known—FixJ, (David et al. 1988) and LuxR, (Engbrecht and Silverman 1987)—or not known—AlkK (Eggink et al. 1990) and dnaY-ORF (Maramatsu and Mixuno 1990)—as members of this family group (Fig. 4). Particularly intriguing is the fact that RcsB, which positively regulates capsular synthesis in enteric bacteria, is a member of this subgroup (Stout and Gottesman 1990).

The concept that EPS production is regulated by environmental stimuli has been well established for E. coli capsular synthesis (Stout et al. 1991), P. aeruginosa alginate synthesis (Deretic et al. 1989b), and X. campestris EPS synthesis (Daniels et al. 1989; Ferris and Beveridge 1985; Osbourn et al. 1990; Tang et al. 1990). If epsR is a member of a plus-minus regulatory switch, the putative positive regulator remains to be identified. The phca gene of P. solanacearum, encoding a potential positive regulator of EPS production (Brumbley and Denny 1990), cannot overcome the shutoff of EPS expression caused by epsR (C. Allen, unpublished). We have also determined that a functional epsR plasmid does not affect the expression of gus reporter fused to the opg genes that are required for both EPS and LPS production (C. C. Kao, unpublished). Therefore, the target genes regulated by the EpsR protein and the previous observation that epsR affects polygalacturonase activities are areas for future research.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1.

Media and antibiotics.

P. solanacearum strains were routinely cultured at 28°C in CPG medium (Kao et al. 1992) or on TZC medium (CPG containing 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride [Kelman 1954]). Recombinants were grown in the same media containing appropriate antibiotics at the following concentrations: kanamycin (Km), 25 µg/ml; ampicillin (Ap), 50 µg/ml; tetracycline (Tc), 15 µg/ml.

Molecular techniques.

General DNA manipulations were performed by means of established protocols (Ausubel et al. 1988). Mutagenesis was generally performed with epsR subclones in the pBluescript vector (Stratagene), after which the inserts were cloned into pLAFR3 plasmid for phenotype assays. The cosmid library of K60 genomic DNA in E. coli DH5A was prepared by the same protocol as described in Xu et al. (1988) and was a kind gift of M. Atkinson. Transfections of P. solanacearum cells and colony hybridization were performed as previously described (Kao et al. 1992; Grunstein and Hogness 1975, respectively). Polymerase chain reactions were performed for 35 cycles at a denaturing temperature of 94°C (1 min), a hybridization temperature of 45°C (30 sec), and an extension temperature of 72°C (1 min).

DNA sequencing and computer analysis.

Nucleotide sequencing by the dideoxy chain termination method was completed by the Novagen Corporation (Madison, WI) and by the Cetus Corporation (Emeryville, CA). The Genetic Computer Group Sequence Analysis Software Package (Devereux et al. 1984), obtained from the Physical Science Laboratory (Stoughton, WI) and run on a VAX/VMS computer, was used for protein and nucleic acid sequence analysis, database searches, and homology assessment. To allow for the high GC content (68%) of P. solanacearum (Palleroni and Doudoroff 1971), a comparison table derived from the codon usage of Streptomyces (GC content about 72% [Bibb et al. 1984]) was used for the codon preference and GC third-position bias studies. The FastA search (Pearson and Lipman 1988) was conducted through the GenBank services provided by the European Molecular Biology Laboratory (Fuchs et al. 1990).

Antiserum against the EpsR protein.

Rabbit serum specific for the EpsR polypeptide was generated against EpsR produced in E. coli. The EpsR antigen was used as the insert of the pepsRA (Table 1) cloned downstream of the T7 promoter in the pET11 expression vector (Novagen). In this construct, the original GTG codon at nt
365 is exchanged by an ATG codon that is a part of the NdeI recognition site (CATATG). This plasmid was then transformed into E. coli strain BL21(DE3) (Table 1). EpsR synthesis was induced with 1 mM isopropyl B-D-thiogalac-topyranoside for 3 hr at 37°C. The cells were harvested and lysed by sonication (Branson Instruments) with three 20-sec bursts alternating with 1-min incubations on ice. The lysate was spun at 5,000 g for 10 min to yield an insoluble pellet containing EpsR. This pellet was washed three times with 1 M urea to remove other E. coli proteins, and then the pellet was solubilized by the addition of sample buffer containing sodium dodecyl sulfate (SDS) (Laemmli 1970). The solubilized material was electrophoresed on a 12% polyacrylamide-SDS gel (Laemmli 1970) and visualized by staining with Coomassie blue R250 (0.1% solution in H2O); gel fragments containing EpsR were excised and dried overnight. The gel fragments were ground into a fine powder, and material containing approximately 100 µg of EpsR was emulsified with complete Freund’s adjuvant and injected into each of two female New Zealand white rabbits at the University of Wisconsin Animal Care Facility. The rabbits were each boosted three times with approximately 75 µg of antigen in incomplete Freund’s adjuvant before serum was obtained for use in Western blots. Western blots were performed as previously described (Kao and Ahlquist 1992).

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