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).

EPS synthesis requires the coordinated activity of numerous structural and regulatory gene products. In *Xanthomonas campestris* pv. *campestris*, and in various species of *Rhizobium*, EPS and other pathogenicity factors seem to be modulated both positively and negatively by two-component regulatory systems (Gray *et al.* 1990; Osbourn *et al.* 1990; Tang *et al.* 1990; Zhan and Leigh 1990; Zhan *et al.* 1990).

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 polygalacturonase 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 *Bam*HI-*Hin*dIII 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 *Bam*HI-*Hin*dIII 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 fluidal 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 *Stu*I to *Nsi*I restriction fragment that decreased EPS expression when introduced in either pLAFR3 or pLAFR6 vectors (Fig. 3). It was previously known that Tn*3-gus* insertions in this region of pBE6 abolished the EpsR phenotype (Huang and Sequeira 1990).

The 1.3-kb *Stu*I to *Nsi*I restriction fragments encompassing the functional *epsR* unit from both K60 and B1 strains were sequenced by the dideoxy 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 promoters

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics and construction	Source
E. coli		
DH5α	F$^-$, *endA*1, *hrdR*17($r_K^-m_K^+$) *recA*1	BRL
BL21(DE3)	F$^-$, *omp*T, r_K^-,m_B^-::λDE3	Novagen
P. solanacearum		
K60	Wild-type virulent, EPS$^+$ Tc^s	A. Kelman
B1	Spontaneous, avirulent, EPS$^-$ Tc^s	A. Kelman
K60.4	K60::pBE6.4 marker exchange mutant EPS$^+$ Km^r	This work
B1.4	B1::pBE6.4 marker exchange mutant EPS$^-$ Km^r	This work
Plasmids		
pLAFR3	IncP1, Tc^r r/l$^+$	Peet *et al.* 1986
PLAFR5	IncP1, Tc^r r/l$^+$	Keen *et al.* 1988
PLAFR6	IncP1, Tc^r r/l$^+$	B. Staskawicz
pBE6	pLAFR3 containing an 8-kb B1 chromosomal DNA fragment, Tc^r	Huang and Sequeira 1990
PBE6.4	pBE6::Tn*3-gus*4 Ap^r Tc^r Km^r	Huang and Sequeira 1990
pKL4	pLAFR5 containing a 20-kb *Sau*3A K60 chromosomal DNA fragment cloned into the *Bam*HI site	This work
pKL44	pLAFR6 containing a 1.3-kb *Stu*I–*Nsi*I DNA fragment from pKL4 cloned between the *Xma*I and the *Pst*I sites	This work
pKL48	pKL44 with a filled-in *Bam*HI site in the *eps*R locus	This work
PKL50	pKL44 with deletion of nt 1–286	This work
pKL52	pKL44 with deletion of nt 892–1185	This work
pKL53	pKL44 with deletion of nt 892–1338	This work
pKL54	pKL44 with an in-frame deletion of nt 746–892	This work
pKL58	Subclone containing sequences from nt 215–1290 cloned in; in PLAFR3	This work
pepsRA	Subclone containing sequences from nt 365–1290; pLAFR3	This work
pG-epsRA	Insert from pepsRA fused to the *ops*G promoter and cloned in pLAFR3	This work
pG-epsRB	DNA fragment from nt 458–1290 fused to the *ops*G promoter; in pLAFR3	This work
pT7-epsRA	Insert from pepsRA cloned behind a T7 promoter in pET11	This work

(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 Sequeira 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 *Bam*HI site filled in with Klenow enzyme and deoxynucleotides and that was shown to have lost the *Bam*HI site by restriction analysis was EpsR$^+$. Also, a polymerase chain reaction (PCR)-derived DNA fragment containing sequences from nucleotides 215–1290 and cloned in pLAFR3 (pKL58) was EpsR$^+$. However, deletion of the first 287 nucleotides to the *Bam*HI 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 *Sph*I site at nucleotide 892 to the *Nsi*I site at nucleotide 1338 (pKL53) and deletions of sequences between the two internal *Sph*I 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 *Bal*31 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 *Bam*HI site was filled in, but activity was abolished when the sequence up to the *Bam*HI 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 Sequeira 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 *Nde*I 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′ *Nde*I site, thus replacing the original GTG sequence with the ATG from the *Nde*I restriction site. This PCR fragment of the *epsR* sequence, when cloned in pLAFR3 to generate the plasmid pepsRA, had no EpsR activity. However, when the same fragment was fused to the *opsG* promoter via the respective *Nde*I sites and cloned in pLAFR3 (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.

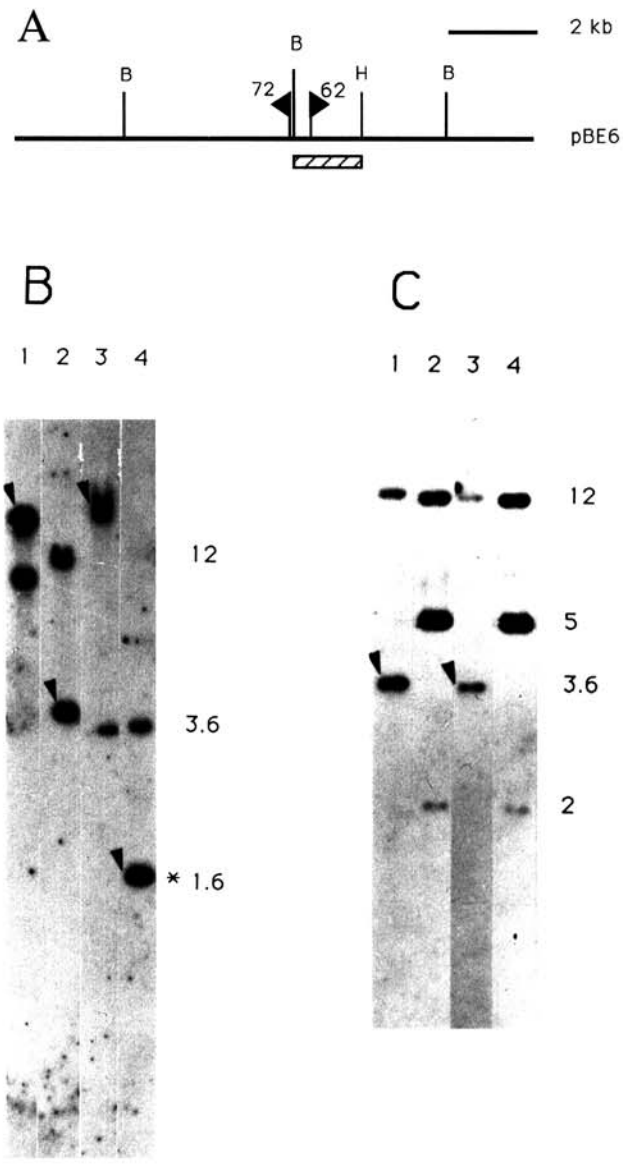

Fig. 1. Molecular characterization of the *epsR* unit. **A,** Partial restriction map of plasmid pBE6 containing the *eps*R sequence isolated from *Pseudomonas solanacearum* B1. Borders of the *epsR* unit are defined by Tn*3-gus* insertions affecting its function (Huang and Sequeira 1990). These insertions are indicated by flags pointing in the direction of *gus* transcription. The cross-hatched box indicates the 1.6-kb *Bam*HI-*Hin*dIII restriction fragment (1.6BH) used to screen the K60 library. B, *Bam*HI; H, *Hin*dIII. **B,** Two K60 genomic DNA fragments hybridized to the 1.6BH probe in Southern blot analysis. Filters containing restriction enzyme-digested K60 genomic DNA were probed with the [^{32}P]-labeled 1.6BH restriction fragment shown in A. Digestions were performed with: *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), and *Bam*HI and *Hin*dIII (lane 4). Arrows indicate the fragment that was eventually demonstrated to contain a functional *epsR* unit, and the asterisk identifies the restriction fragment corresponding to the 1.6BH probe. An identical hybridization profile was obtained with the genomic DNA of B1 strain of *P. solanacearum.* The size of the bands (in kb) are indicated on the right of the autoradiograph. **C,** To determine that the *epsR* genes in K60 and B1 were disrupted in marker-rescued mutants, genomic DNAs from K60 (lane 1), K60.4 (lane 2), B1 (lane 3), and B1.4 (lane 4) were digested with *Bam*HI and probed with the [^{32}P]-labeled 1.6BH fragment shown in A. The approximately 3.6-kb restriction fragment contains the functional *epsR* unit (arrow). Molecular weights are indicated in kilobases.

A second construct, pG-epsRB, which contains nucleotides 458–1290 of *epsR* fused to the *opsG* 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 *Bam*HI 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 Tn*3-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^r 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

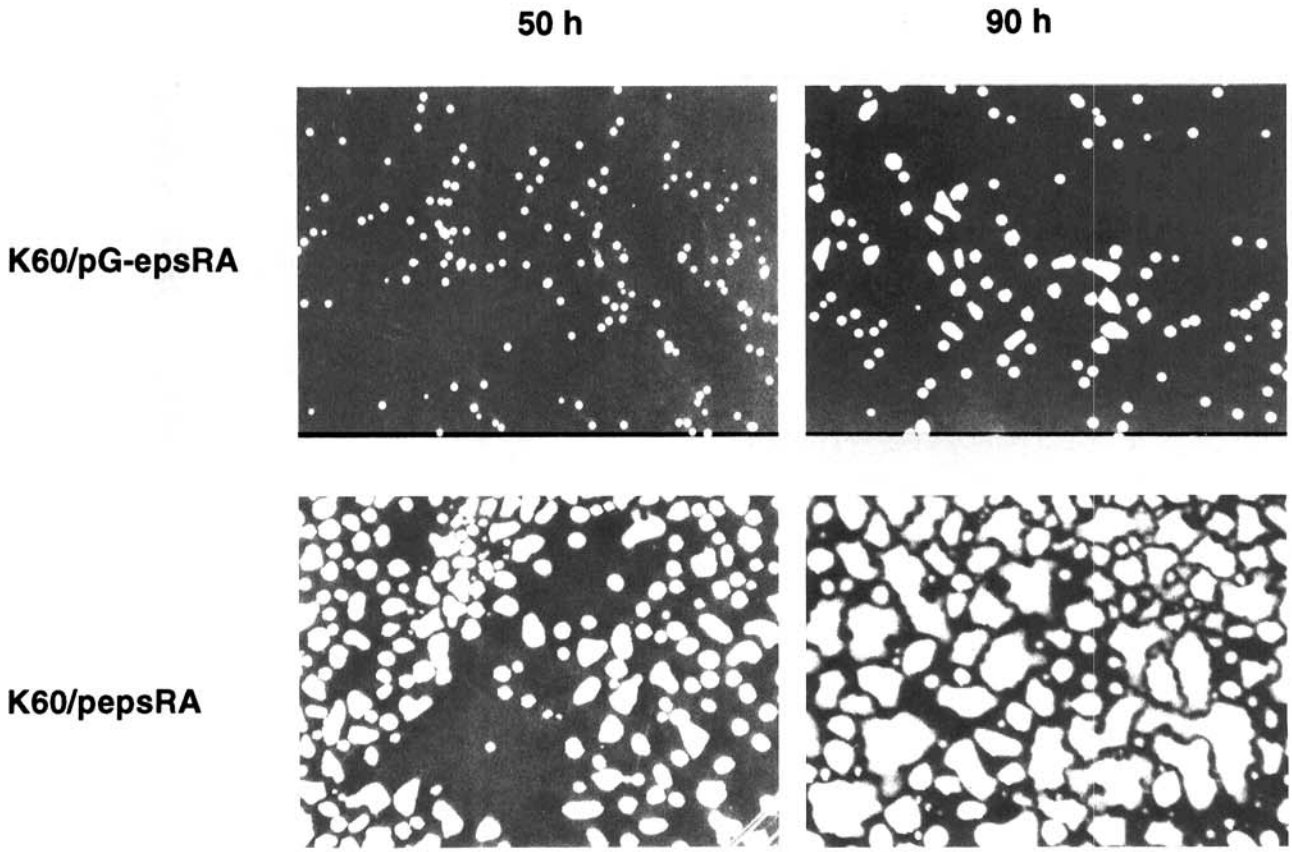

Fig. 2. The EpsR phenotype. K60 cells were electroporated with the $EpsR^+$ plasmid pG-epsRA (top row) or with the $EpsR^-$ plasmid pepsRA (bottom row). The photographs were taken at either 50 or 90 hr after electroporation, as indicated.

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)

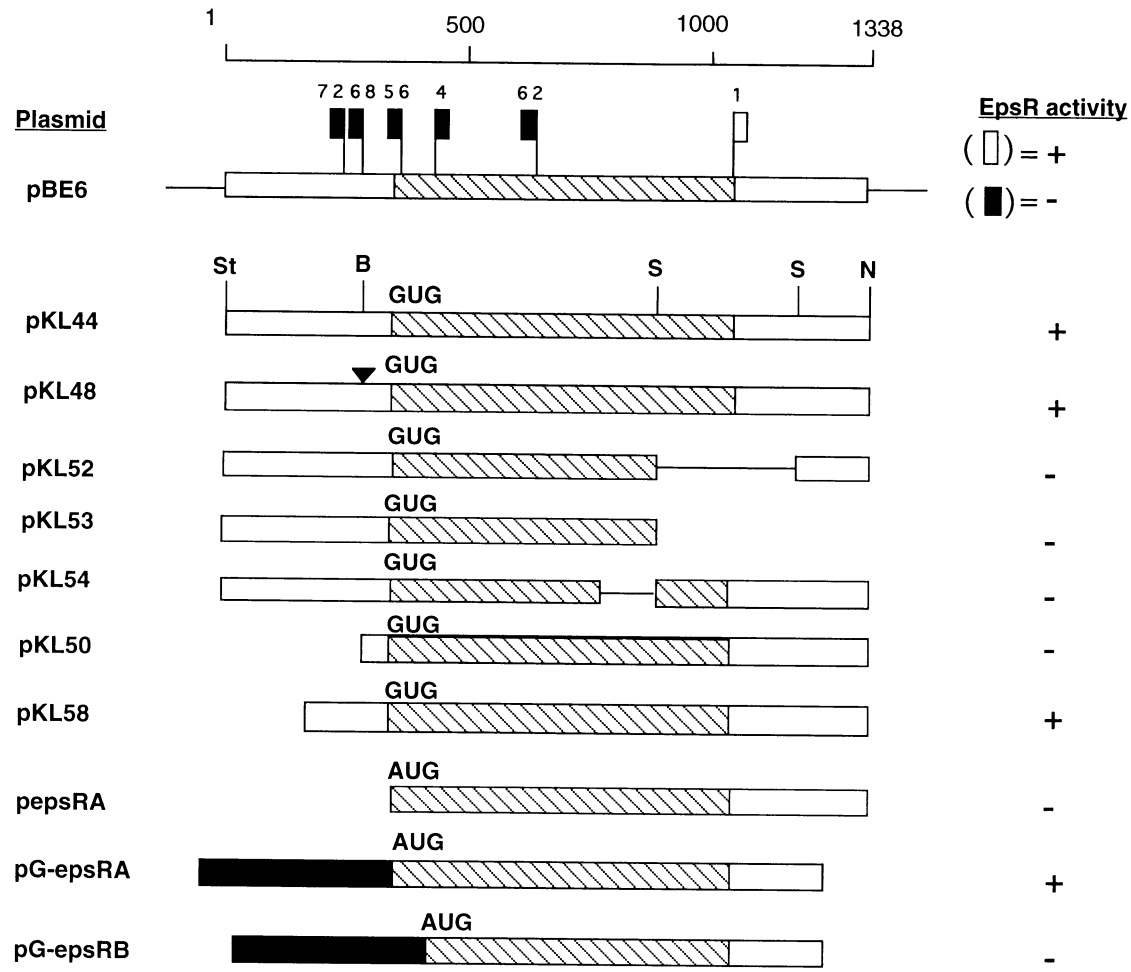

Fig. 3. Mutagenesis of the *epsR* functional unit. The position of the Tn*3-gus* insertions in the plasmid pBE6 are indicated by flags, the orientations of which refer to the direction of translation of the reporter gene. Dark flags indicate that the insertions affected *epsR* function in strain K60 and resulted in normal exopolysaccharide (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: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, N: *Nsi*I, S: *Sph*I, St: *Stu*I. The cross-hatched boxes indicate the position of the putative EpsR translation sequence. The filled-in triangle denotes the filled-in *Bam*HI 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 *Nde*I restriction site and a 3′ oligonucleotide that contains a novel *Hin*dIII restriction site. The two 5′ oligonucleotides were: EpsRA (5′ ATACATATGAAATCAATCGTAGTCGAAC; *Nde*I site underlined) and EpsRB (5′ ATA-CATATGGAACCGGGCGAAGCCCTT 3′). The 3′ oligonucleotide was EpsR3′H (5′ ATAAAGCTTGAGCCATCGGCTACGACT 3′; *Hin*dIII 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.

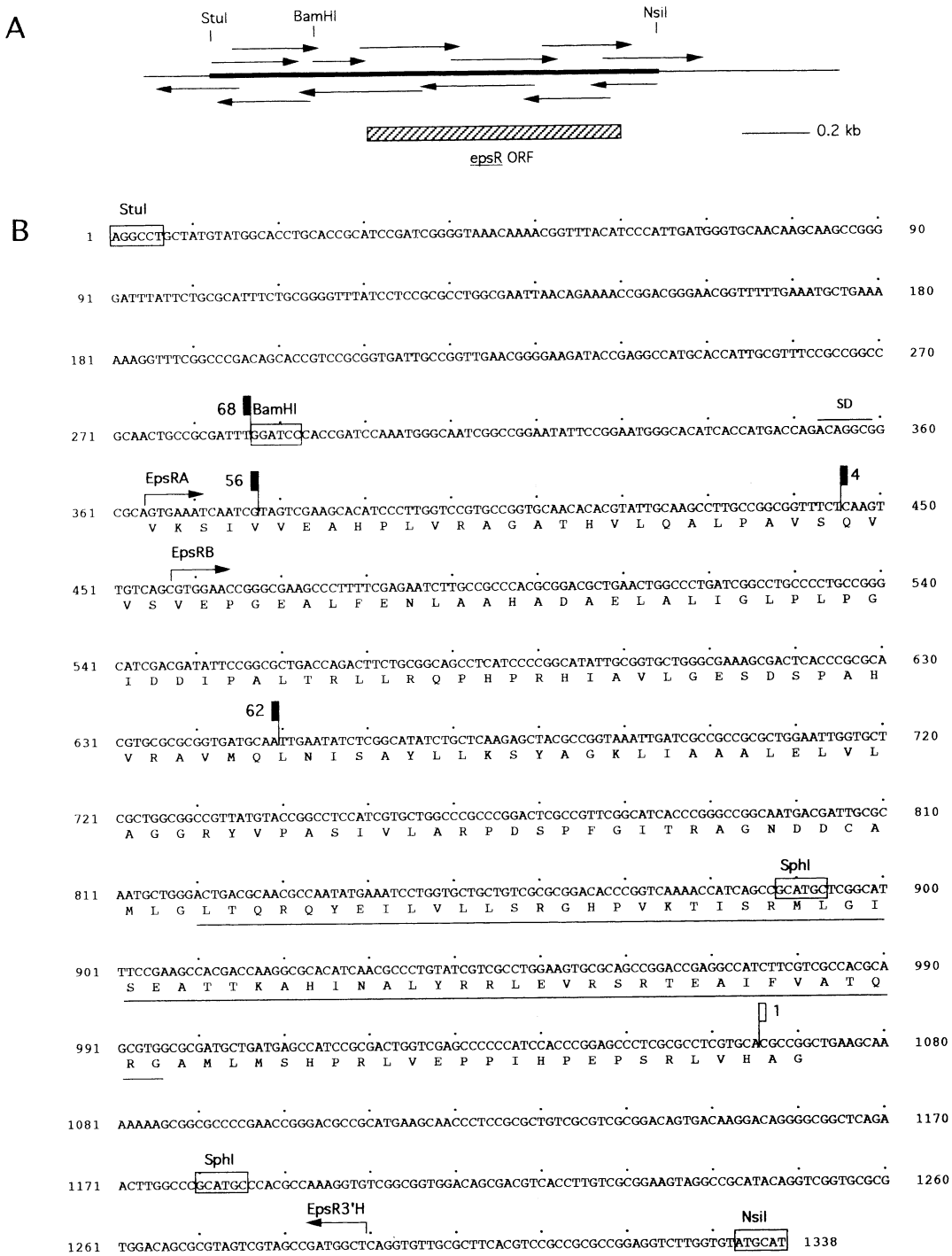

Fig. 4. Nucleotide sequence of *epsR*. **A,** Sequencing strategy employed. The arrows represent the direction and extent of the sequencing reaction. The black bar locates the minimal *epsR* functional unit, and the cross-hatched box indicates the EpsR open reading frame. **B,** Nucleotide sequence of the *epsR* region extending for 1,338 bp from the *Stu*I site to the *Nsi*I site. The predicted amino acid (aa) sequence for the entire 286-aa open reading frame (described in the text) is indicated in one-letter code. The proposed 236-aa sequence for EpsR starts at position 365. A potential Shine and Dalgarno sequence is overlined. The flags indicate the insertion positions of the Tn*3-gus* in the B1 *epsR* gene. Black flags denote insertions that abolished the EpsR phenotype, white 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.

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BvgA        STLISVLSNR ELTVLQLLAQ GMSNKDIADS MFLSNKTVST YKTRLLQKLN ATSLVELIDL AKRNN
Dna5-ORF    SSTVTVLSNR EVTILRYLVS GLSNKEIADK LLLSNKTVSA HKSNIYGKLG LHSIVELIDY AKLYE
UhpA        DDANDILTKR ERQVAEKLAQ GMAVKEIAAE LGLSPKTVHV HRANLMEKLG VSNDVELARR MF.DG
UvrC-ORF2   ESPFASLSER ELQIMLMITK GQKVNEISEQ LNLSPKTVNS YRYRMFSKLN IHGDVELTHL AIRHG
GerE        FQSKPSLTKR EREVFELLVQ DKTTKEIASE LFISEKTVRN HISNAMQKLG VKGRSQAVVE LLRMG
DegU        RRPLHILTRR ECEVLQMLAD GKSNRGIGES LFISEKTVKN HVSNILQKMN VNDRTQAVVV AIKNG
NarL        ERDVNQLTPR ERDILKLIAQ GLPNKMIARR LDITESTVKV HVKHMLKKMK LKSRVEAAVW VHQER
FixJ        RARLQTLSER ERQVLSAVVA GLPNKSIAYD LDISPRTVEV HRANVMAKMK AKSLPHLVRM ALAGG
RcsB        GYGDKRLSPK ESEVLRLFAE GFLVTEIAKK LNRSIKTISS QKKSAMMKLG VENDIALLNY LSSVT
ComA        QKEQDVLTPR ECLILQEVEK GFTNQEIADA LHLSKRSIEY SLTSIFNKLN VGSRTEAVLI AKSDG
MalT        LIRTSPLTQR EWQVLGLIYS GYSNEQIAGE LEVAATTIKT HIRNLYQKLG VAHRQDAVQH AQQLL
AlkT        NKADALLTRK QIAVLRLVKE GCSNKQIATN MHVTEDAIKW HMRKIFATLN VVNRTQATIE AERQG
LuxR        NKSNNDLTKR EKECLAWACE GKSSWDISKI LGCSERTVTF HLTNAQMKLN TTNRCQSISK AILTG

Consensus   ------LT-R E--VL-L--- G-----IA-- L--S--TV-- H------KL- V-----AV-- A---G
EpsR        DCAMLGLTQR QYEILVLLSR GHPVKTISRM LGISEATTKA HINALYRRLE VRSRTEAIFV ATQRG
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Fig. 5. Alignment of *Pseudomonas solanacearum* EpsR residues 181–236 with the *Escherichia coli* DnaY-ORF (Maramatsu and Mixuno 1990), UhpA (Friedrich and Kadner 1987), UvrC-ORF2 (Moolenaar *et al.* 1987), NarL (Gunzalus *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 meliloti* FixJ (David *et al.* 1988); the *P. oleovorans* AltK (Eggink *et al.* 1990); and the *V. fischeri* LuxR (Engebrecht *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.

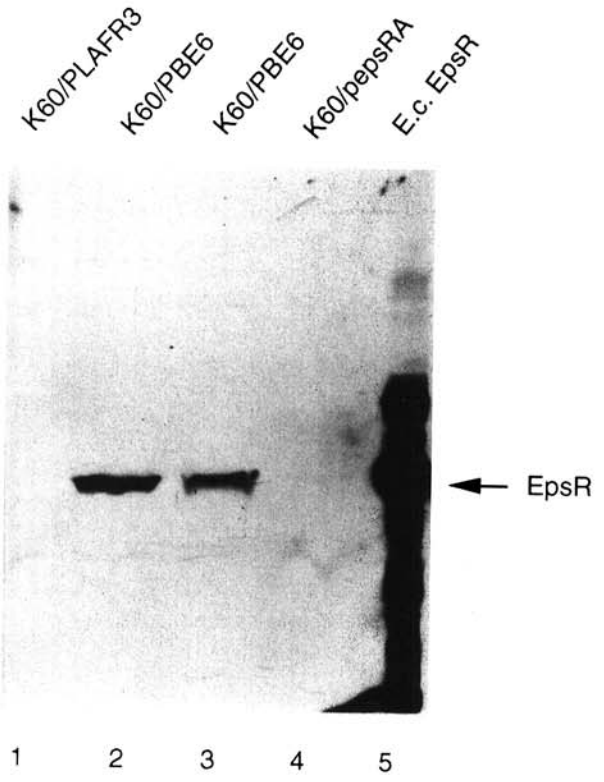

Fig. 6. A Western blot that demonstrates that the EpsR protein is expressed in EpsR+ cells. Lanes 1–4 represent *Pseudomonas solanacearum* extracts from approximately 200 µl of mid-log cultures. The plasmids harbored by each strain are noted above each lane. Lanes 2 and 3 represent two independent K60 strains transformed with plasmid pBE6. 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 *Stu*I to *Nsi*I 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 *opsG* 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 *StuI–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 *opsG* 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 Delgarno 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 from the data bank (NBRF version 25) proteins already known—FixJ, (David *et al.* 1988) and LuxR, (Engbrecht and Silverman 1987)—or not known—AltK (Eggink *et al.* 1990) and *dnaY*-ORF (Maramatsu and Mixuno 1990)—as members of this gene family (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 *ops* 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* DH5α 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 used was encoded by the insert of 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 *Nde*I 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-thiogalactopyranoside 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 H_2O); 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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