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EpsR Modulates Production of Extracellular Polysaccharides in the Bacterial Wilt Pathogen *Ralstonia* (*Pseudomonas*) *solanacearum*

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***Ralstonia solanacearum* is the causal agent of bacterial wilt of many agriculturally important crops. Exopolysaccharide synthesized by products of the *epsI* operon is the major virulence factor for *R. solanacearum*. Expression of *epsI* has been demonstrated to be under the control of several proteins, including several two-component regulators. Overexpression of EpsR was found previously to reduce the amount of synthesis specifically from the *epsI* promoter. Here we present data that a single chromosomal copy of *epsR* activates the *epsI* promoter, suggesting that EpsR is a concentration-dependent effector of *epsI* gene expression. Furthermore, the ability of EpsR to modulate *epsI* expression is dependent on the phosphorylation state of EpsR. Gel mobility shift assays suggest that EpsR can specifically bind the *epsI* promoter and that this binding requires a phosphorylated form of EpsR.**

Many prokaryotes produce extracellular polysaccharides that have important roles in pathogenesis (8). In the plant pathogen *Ralstonia solanacearum*, extracellular polysaccharides (EPS) are major virulence factors required to cause the agriculturally important disease bacterial wilt (15). Although the exact role of EPS has not been demonstrated, it may interfere with water transport in the plant by plugging the xylem vessels, leading to wilt (20).

Several genes involved in EPS production in *R. solanacearum* have been identified. Structural gene clusters include *opsI* (7, 22), *opsII* (29), *rgnII* (10), and *epsI* (10). The *opsI* and *opsII* gene clusters are important for both EPS and lipopolysaccharide syntheses since mutations in them affect the production of both macromolecules. The *opsI* cluster consists of at least seven genes, some of which are important for nucleotide sugar synthesis (7, 22). The *rgnII* cluster is largely uncharacterized since it is required for EPS production only in culture, not in plants (10). Mutational analyses of *epsI* suggest that it encodes proteins responsible for synthesis of the acidic component of EPS, which is absolutely required for *R. solanacearum* infection of plants (10, 23, 32).

Sequence analysis revealed that *epsI* encodes polypeptides transcribed from a single promoter (18). Regulation of *epsI* is complex, involving at least seven proteins, including the highly basic XpsR, which likely affects expression of *epsI* directly (17). The two-component regulatory systems VsrB-VsrC (19) and VsrA-VsrD (37) have been shown genetically to positively regulate *epsI* and *xpsR* expression, respectively. The LysR-like transcriptional regulator PhcA has been demonstrated to positively regulate *xpsR* expression (4, 5, 17). Finally, EpsR overexpressed in plasmids of four to six copies per cell can specifically reduce synthesis from the *epsI* promoter, decreasing EPS production from colonies (16, 21, 29). EpsR has sequence similarity to effector proteins of two-component regulatory systems (21). A thorough Tn*5*::*lacZ* mutagenesis of *R. solanacearum* selecting for EPS-defective strains resulted in the identification and *lacZ* tagging of 12 complementation groups of EPS genes (29). Overexpression of EpsR affected the expression of only *epsI*::*lacZ* genes, suggesting a specific interaction between EpsR and the *epsI* promoter. In this work, we provide evidence that the chromosomal copy of *epsR* encodes a positive regulator of *epsI*. An *epsR* insertional mutation also reduced the virulence of *R. solanacearum*. We show that a mutation at the putative phosphorylation site renders EpsR unable to regulate synthesis of *epsI* and prevents an EpsR-dependent gel mobility shift of the *epsI* promoter.

MATERIALS AND METHODS

Growth and maintenance of bacterial strains. The strains and plasmids used in the experiments described herein are listed in Table 1. *R. solanacearum* was routinely cultured in CPG medium (per liter, 10 g of tryptone, 5 g of glucose, 1 g of Casamino Acids, 1 g of yeast extract, and 15 g of agar as appropriate) at 30°C. Antibiotics, where used, were at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; ampicillin, 100 μg/ml; streptomycin, 25 μg/ml.

Molecular techniques. Plasmids were isolated from *Escherichia coli* by using Qiagen (Chatsworth, Calif.) columns. Chromosomal DNA isolation and Southern hybridizations were done as previously described (21). To transform *R. solanacearum*, 2-ml cultures at an optical density at 600 nm (OD_{600}) of approximately 1.0 were washed three times with sterile water and finally resuspended in 100 μl of water. The cells were then electroporated with 0.5 μg of plasmid with a Gene Pulser (Bio-Rad, Hercules, Calif.) set at 25 μF with a field strength of 6,000 V/cm. After electroporation, the cells were incubated for 3 h in CPG broth before being plated onto selective medium.

Site-directed mutagenesis. The aspartate at residue 47 of EpsR was changed to alanine by amplifying *epsR* in two halves by using PCR with pGepsR as a template. The 5′ half of *epsR* was generated with the following primers: PUC19PC (5′ GCCTGCAGGTCGACTCTAG 3′), which hybridizes with sequence in the plasmid vector of pGepsR, and *epsRD-A5′* (5′ CAGCGGCTGC GTGGGCGGCAAG 3′), which hybridizes to nucleotides (nt) 486 to 508 of *epsR* (21). The 3′ half of *epsR* was amplified by using *epsRD-A3′*, which hybridizes to nt 500 to 523 and contains an *Alw*NI site (5′ CAGCCGCTGAACTGGCCGTG ATC 3′), and *epsR*3′ *Eco*RI, which hybridizes to nt 1125 to 1142 and contains an *Eco*RI site (5′ GAATTCCCGCGACGCGACAGCGCG 3′). The PCR products encoded by the 5′ and 3′ halves of *epsR* were individually cloned into PCRII (Novagen, Milwaukee, Wis.), creating TAepsRD-A5′ and TAepsRD-A3′ respectively. Inserts from TAepsRD-A5′ and TAepsRD-A3′ were released with *Hin*dIII-*Alw*NI and *Eco*RI-*Alw*NI digestions, respectively. The fragments were ligated to $pBSKS^+$ linearized with *Eco*RI and *Hin*dIII to reconstitute *epsR* encoding the amino acid change at residue 47, creating pBepsRD-A. Clones which contained *epsRD-A* mutations were verified by screening for the presence of the *Alw*NI site. Since ampicillin selection is ineffective in *R. solanacearum* K60, we cloned the Ω fragment (encoding streptomycin resistance) into the *Hin*dIII site in the polylinker region of pBepsRD-A, creating pepsRD-AΩ. This plasmid was then transformed by electroporation into the *epsI* mutant S49, selecting for streptomycin resistance. To investigate the effect of overexpression of EpsRD-A in *R. solanacearum*, the *Eco*RI-*Hin*dIII fragment from pepsRD-A was cloned into the *Eco*RI and *Hin*dIII sites of pLAFR3, creating pepsRD-A.

In vivo labeling and Western blot analysis. Strains grown in 2 ml of CPG broth to an OD_{600} of 0.5 were washed three times in low-phosphate medium (M9

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source
Escherichia coli DH5α	F^- *endA1 hrdR17* (R_k^- m_K^+) *recA1*	14
Ralstonia solanaceaum		
K60	Wild type	26
S49	EPS^-; *epsI*::*lacZ* fusion; Kan^r	29
S70	EPS^-; *epsI*::*lacZ* fusion; Kan^r	29
S90	EPS^-; *rgnII*::*lacZ* fusion; Kan^r	29
S112	EPS^-; *vsrB*::*lacZ* or *vsrC*::*lacZ* fusion; Kan^r	29
S50	EPS^-; *ops*::*lacZ* fusion; Kan^r	29
K60::*epsR*Ω	EPS^+; $EpsR^-$	This work
S49::*epsR*Ω	EPS^-; *epsI*::*lacZ* fusion; *epsR* Kan^r Sm^r	This work
S70::*epsR*Ω	EPS^-; *epsI*::*lacZ* fusion; *epsR* Kan^r Sm^r	This work
S90::*epsR*Ω	EPS^-; *rgnII*::*lacZ* fusion; *epsR* Kan^r Sm^r	This work
S112::*epsR*Ω	EPS^-; *vsrB*::*lacZ* or *vsrC*::*lacZ* fusion *epsR* Kan^r Sm^r	This work
S50::*epsR*Ω	EPS^-; *opsG*::*lacZ* fusion; *epsR* Kan^r Sm^r	This work
S49::*epsRD-A* 1	EPS^-; *epsI*::*lacZ* fusion; *epsR* EpsRD-A^+ Kan^r Sm^r	This work
S49::*epsRD-A* 10	EPS^-; *epsI*::*lacZ* fusion; *epsR* EpsRD-A^+ Kan^r Sm^r	This work
Plasmids		
pLAFR3	Tc^r	25
pGL10	Kan^r	Y. Brun
pGL10lacZ	Promoterless *lacZ* gene from mini-Tn*5*::*lacZ* cloned into pGL10; Kan^r	This work
pGL10Ω	Ω fragment from pHP45Ω cloned into the *Bgl*II site of pGL10; Kan^r Sm^r	This work
pGepsI::lacZ	Nucleotides 1 to 260 of the *epsI* promoter cloned into the *Eco*RI and *Bam*HI sites of pGL10lacZ; Kan^r Sm^r	This work
pGepsR::lacZ	Nucleotides 25 to 365 of *epsR* cloned into the *Eco*RI and *Kpn*I sites of pGL10lacZ; Kan^r Sm^r	This work
pGopsG::lacZ	360-bp fragment containing the *opsG* promoter cloned into the *Eco*RI site of pGL10lacZ; Kan^r Sm^r	This work
Mini-Tn5lacZ2	Amp^r Kan^r	28
pHP45Ω	Sm^r Amp^r	Y. Brun; 34
pepsRΩ	Ω cassette cloned into the *Sma*I site of *epsR* contained on pET11epsR	This work
pTAepsI	Nucleotides 1 to 260 of *epsI* cloned into PCRII as a PCR product; Kan^r Sm^r	This work
pKLS44	Amp^r	21
pET11epsR	Amp^r	21
pGepsR	Nucleotides 25 to 1290 of *epsR* cloned into pGL10	This work
pepsR	Nucleotides 25 to 1142 of *epsR* cloned into pLAFR3; Tet^r	This work
pepsRD-A	Nucleotides 25 to 1142 of *epsRD-A* cloned into *Eco*RI and *Hin*dIII sites of pLAFR3; Tet^r	This work
pBepsRD-A	Nucleotides 25 to 1142 of *epsRD-A* cloned in pBSKS	This work
pepsRD-AΩ	Nucleotides 286 to 1142 of *epsRD-A* cloned in pBSKS	This work
pKL4	Tc^r	16
pL112	Tc^r	29
pL90	Tc^r	29

[a] Amp^r, ampicillin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Kan^r, kanamycin resistance.

medium containing 30 μM Na_2HPO_4 and KH_2PO_4) (36) and then resuspended in 2 ml of low-phosphate medium and incubated at 30°C for an additional 4 h. One-half millicurie of orthophosphate (200 mCi/mmol; Amersham, Arlington Heights, Ill.) was added to 1-ml aliquots of cells. Orthophosphate can freely diffuse into bacterial cells, be incorporated into nucleotides, and subsequently be used as a substrate for phosphorylation. After a 20-min incubation at 30°C, the samples were washed twice with sterile water and resuspended in 60 μl of 1× Laemmli loading dye (27). Ten microliters of each sample was loaded on a sodium dodecyl sulfate–10% polyacrylamide gel. After electrophoresis, the gel was wrapped in plastic wrap and exposed to X-ray film for 1 h at −80°C. After autoradiography, the gel was washed extensively with 1× Western transfer buffer (10% methanol, 200 mM glycine, 25 mM Tris [pH 8.3]). The gel was blotted onto a nitrocellulose membrane and probed with anti-EpsR antibodies as previously described (21).

Gel mobility shift. DNA probes used for gel mobility shift assays contained a 240-bp fragment (nt −140 to +100) of the *epsI* promoter. The 240-bp fragment was produced by digesting pTAepsI with *Eco*RI and then end labeling with Klenow polymerase, [α-^{32}P]dATP (3,000 Ci/mmol), and dTTP. In competition experiments, a 320-bp fragment containing the *opsG* promoter was made by PCR as previously described (21). *epsI* promoter used in competition experiments was also synthesized by PCR by using K60 chromosomal DNA as the template and the following primers: *epsI* 5′, which hybridizes to nt 1 to 20 (5′ GAATTCTCT GTCGAATTGGG) and *epsI* 3′, which hybridizes to nt 218 to 238 (5′ GGATC CGCTTACGAACATGAATGCG 3′) (18). Protein extracts were made from 50 ml of *R. solanacearum* culture grown to an OD_{600} of approximately 1.0. The cells were harvested, washed once in extraction buffer (50 mM Tris-HCl [pH 7.9], 10 mM EDTA, 10% glycerol, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM sodium pyrophosphate, 0.4 mg of phenylmethylsulfonyl fluoride per ml), and finally resuspended in 1 ml of extraction buffer. Lysates were prepared by sonication with three 10-s bursts with a model 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, Pa.) set to 30% output. Protein concentrations were determined by the Bradford assay with bovine serum albumin as the standard (3). DNA binding reaction components, including 50-μg protein extracts, competitor DNAs (when appropriate), and binding buffer [50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 30 mM KCl, 12% glycerol, 0.5 μg of poly(dI-dC) per ml, 1 mM dithiothreitol] were incubated for 15 min at room temperature before the addition of 5 nM end-labeled *epsI* promoter. Reaction mixtures were incubated for a further 20 min at room temperature before electrophoresis in a 3.5% (wt/vol) polyacrylamide (Tris-glycine [pH 7.9]) native gel at 70 V for 12 h at 4°C.

RESULTS

EpsR can act as both a positive and negative regulator of *epsI* expression. We previously determined that overexpression of EpsR in plasmids of four to six copies per cell resulted in decreased expression from the *epsI* promoter (29). To examine the effect of inactivation of the chromosomal copy of *epsR*, we inserted an Ω cassette carrying streptomycin resistance into the unique *Sma*I site in *epsR* contained in PET11epsR, creating pepsRΩ. This plasmid contains a ColEI replication origin which is not utilized in *R. solanacearum*. Exchange of *epsR*::Ω

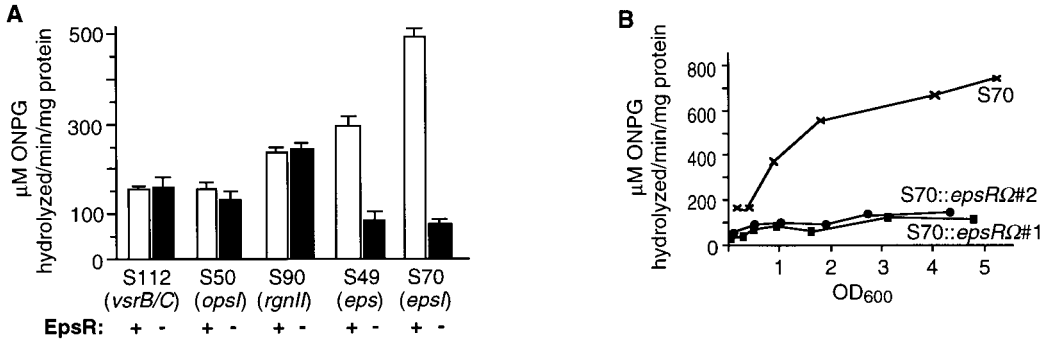


FIG. 1. (A) Effect of inactivation of EpsR on expression from different EPS genes. Cells used were grown to an OD_{600} of 1.5 and then assayed as described previously (29, 30). The bars represent an average of three independent trials. (B) Effect of the *epsR*Ω mutation on expression from the *epsI* promoter at different cell densities. Cells used for the assay were collected at the indicated optical densities and then frozen at −70°C until use. β-Galactosidase activities (micromoles of ONPG [*o*-nitrophenyl-β-D-galactopyranoside] hydrolyzed per minute per milligram of protein) were determined and plotted.

with the chromosomal copy of *epsR* was made in the wild type, K60, and the EPS mutants S49, S50, S70, and S112 by homologous recombination. The homologous integration of *epsR*::Ω was checked by Southern blotting by using labeled *epsR* DNA as a probe (data not shown). Mutations in *epsR* were obtained in several strains, S70 and S49, which have *epsI*::*lacZ* fusions, S50, which has an *opsI*::*lacZ* fusion, S112, which has a *vsrB*::*lacZ* or *vsrC*::*lacZ* fusion, and finally S90, which contains a *rgnII*::*lacZ* fusion (29).

Expression of EPS genes in the absence of EpsR was assayed by measuring β-galactosidase activity. In two S70 and two S49 strains with independently derived *epsR*Ω insertional mutations, *epsI* expression was reduced four- to sixfold (Fig. 1A). Since overexpression of EpsR repressed *epsI* expression (29), we were surprised to find that an *epsR*Ω mutation also reduced *epsI* expression. Expression of other genes involved in EPS biosynthesis, including *opsI*, *rgnII*, and *vsrB* or *vsrC*, were unaffected by the inactivation of *epsR* (Fig. 1A), consistent with the previous report that EpsR specifically affected the *epsI* promoter. Since *epsI* expression increases with culture density (29), we assayed *epsI* expression during growth of the culture in strains which contain an *epsR*Ω insertional mutation. Samples of S70 (*epsR*$^+$) and two independently derived S70::*epsR*Ω mutant strains were collected at the indicated optical densities and assayed for β-galactosidase activity. Strains lacking EpsR had a reduced, but not a complete lack of, β-galactosidase activity throughout the growth of the culture, with the greatest difference from the activity of strain 70 at the higher optical densities (Fig. 1B). These data suggest that EpsR acts as positive regulator of *epsI* synthesis when present in one copy and that the negative effect on *epsI* expression was due to overexpression by multicopy plasmids.

In planta analysis of EpsR. The effect of single and multiple copies of *epsR* on the ability of *R. solanacearum* to kill eggplant seedlings was assayed as previously described (7, 21, 22, 40). At least 10 plants were inoculated with either the wild type, K60, K60/pKL4 (overexpressing EpsR), or K60::*epsR*Ω (*epsR* insertional mutant), and the results of two independently performed assays are presented (Table 2). The wild type, K60, caused death in a majority of the plants by 11 days postinoculation (Table 2). Overexpression of EpsR slowed the wilting process, with only approximately half of the plants killed at 11 days postinoculation (Table 2). However, at 14 days postinoculation, more than half of the plants inoculated with K60/pKL4 died, possibly due to the loss of pKL4, since there is no antibiotic selection in the plant. Strains with an *epsR*Ω insertional mutation showed reduced wilting, with only approximately half of the inoculated plants killed at 11 days postinoculation. This result is consistent with reduction but not abolition of *epsI* expression due to the lack of EpsR. As expected for a stable genetic change, no significant increase in plant death occurred by 14 days postinoculation.

Regulation of EPS genes. To facilitate a more convenient analysis of the effects of *epsR*, we fused the promoters of *epsI*, *opsG*, and *epsR* to pGL10::lacZ, which contains a promoterless *lacZ* gene. The *epsI* promoter contained nt −140 to +120 in plasmid pGepsI::lacZ, the *opsG* promoter contained nt −360 to +1 in pGopsG::lacZ, and the *epsR* promoter contained nt −280 to +40 in pGepsR::lacZ. Promoter activities when EpsR is overexpressed or absent were assayed by measuring β-galactosidase activity. Transformation of strain K60 with pGepsI::lacZ and pKL4 resulted in a reduction of *epsI* expression to 15% of that of K60 transformed with pGepsI::lacZ and pLAFR3 (Table 3). Furthermore, β-galactosidase activity resulting from strains transformed with pGopsG::lacZ was unchanged, as expected, whether EpsR was overexpressed or absent (Table 3). The *epsI* and *opsG* promoters on plasmids, thus, mimicked the phenotype of their chromosomal counterparts with regards to EpsR regulation and demonstrated the efficacy of the system (29). Strains transformed with pGepsR::lacZ had approximately 150 U of β-galactosidase activity, demonstrating that the *epsR* promoter is expressed when contained on a plasmid (Table 3). However, the expression of the *epsR* promoter was not affected when strain K60 harbored both pGepsR::lacZ and pKL4 or when K60 contained pGepsR::

TABLE 2. Effect of *epsR*Ω mutation and overexpression on the ability of *R. solanacearum* to kill eggplant seedlings

Strain and trial no.	No. of seedlings killed/no. inoculated[a]	
	Day 11	Day 14
K60		
Trial 1	12/13	12/13
Trial 2	9/10	10/10
K60/PKL4		
Trial 1	6/13	9/13
Trial 2	5/13	8/13
K60::*epsR*Ω		
Trial 1	6/13	7/13
Trial 2	7/12	7/12

[a] Inoculated seedlings were scored for death at 11 and 14 days postinoculation.

TABLE 3. Activity of the *epsI*, *opsG*, and *epsR* promoters in the presence or absence of EpsR

Strain	EpsR[a]	Plasmids	β-Galactosidase activity (Miller U[b])	Relative activity[c]
K60	+	pGepsI::lacZ + pLAFR3	337.3	1.0
K60	+++	pGepsI::lacZ + pKL4	50.4	0.2
K60	+	pGL10::lacZ + pLAFR3	36.5	0.1
K60	+	pGopsG::lacZ + pLAFR3	533.4	1.0
K60::*epsR*Ω	−	pGopsG::lacZ + pLAFR3	612.9	1.2
K60	+++	pGopsG::lacZ + pKL4	600.9	1.1
K60	+	pGepsR::lacZ + pLAFR3	177.8	1.0
K60::*epsR*Ω	−	pGepsR::lacZ + pLAFR3	169.7	0.9
K60	+++	pGepsR::lacZ + pKL4	157.5	0.9

[a] +, produced from one copy of *epsR*; +++, produced from pKL4; −, not produced.
[b] β-Galactosidase activity is expressed in Miller units (micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein [30]). Values shown are the average of two independent trials.
[c] Activity of each promoter in the presence of wild-type levels of EpsR is adjusted to 1.0.

lacZ and an *epsR*Ω insertion mutation (Table 3). Therefore, EpsR does not regulate its own expression.

EpsR is phosphorylated in vivo. Most effector proteins of two-component regulatory systems are modified at a conserved aspartic acid by phosphorylation, which can modulate a variety of the protein's activities (33). EpsR contains an aspartate (D) at amino acid 47, which is the most likely site of phosphorylation (Fig. 2A). We changed D47 to an alanine by using the scheme diagrammed in Fig. 2A. The 5′ and 3′ halves of *epsR* were amplified by PCR by using primers which changed codon 47 to an alanine while adding a unique *Alw*NI site (Fig. 2A). The alanine codon created is one which is commonly used in *R. solanacearum* (6). DNA carrying the mutant version of *epsR* was cloned into pLAFR3, creating pepsRD-A.

Western blot analysis using antibodies directed against EpsR detected a protein of the size predicted for EpsR in extracts of strain S70 containing pKL4, the EpsR-overexpressing plasmid (Fig. 2B). A protein of similar size and abundance is produced in S70 transformed with pepsRD-A, demonstrating that the D-A mutation did not noticeably affect the stability of the resultant protein. This protein band was not readily apparent in lysates made from strains which contained only the chromosomal copy of *epsR* (Fig. 2B). However, a longer exposure revealed the lower level of EpsR made from this strain (data not shown) (6).

To determine if EpsR is phosphorylated in vivo, radiolabeled orthophosphate was added to cells adapted to growth in low-phosphate medium. Lysates made from S70/pKL4 produced a band of the same size as that of EpsR (Fig. 2C). The covalent bond formed between aspartate and the phosphate

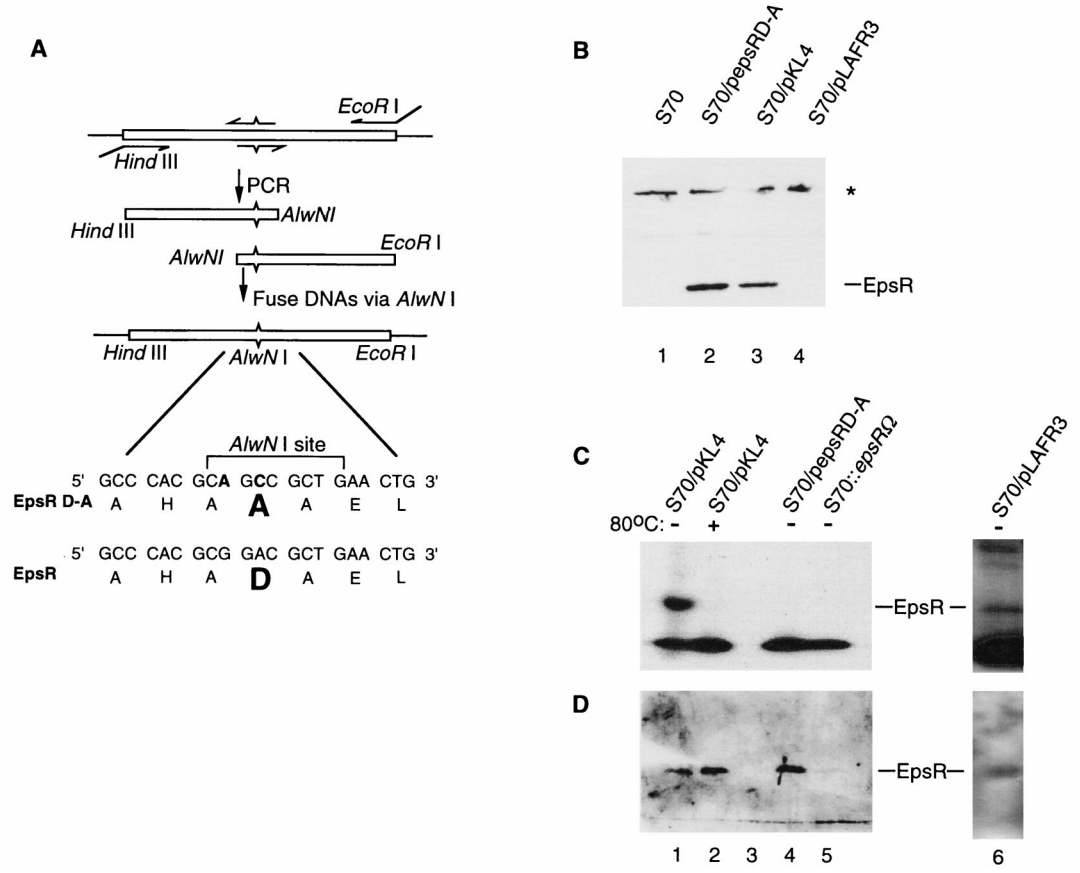


FIG. 2. (A) A schematic diagram of site-directed mutagenesis of the conserved aspartate (D47) in *epsR* is shown. Oligonucleotide primers are indicated with arrows. The internal pair of primers contains changes in the *epsR* sequence, indicated by ˆ or ˇ. Changes made in the *epsR* sequence are shown at the bottom of the figure, and the encoded amino acids are in the standard one-letter code. The original aspartate in the EpsR sequence and the alanine in the EpsRD-A sequence are shown in bold type. (B) Western blot analysis shows that EpsRD-A is expressed in *R. solanacearum* (S70) when cells are transformed with pepsRD-A. The star designates nonspecific recognition of protein by the anti-EpsR antibodies. (C) EpsR (lane 1), but not EpsRD-A (lane 3), is phosphorylated. A longer exposure of this gel (lane 6) reveals the phosphorylation of chromosomally derived EpsR. (D) After autoradiography, the gel in Fig. 3C was subjected to Western blot analysis with anti-EpsR serum (21). The results show that EpsR and EpsRD-A are present in cell lysates in similar quantities.

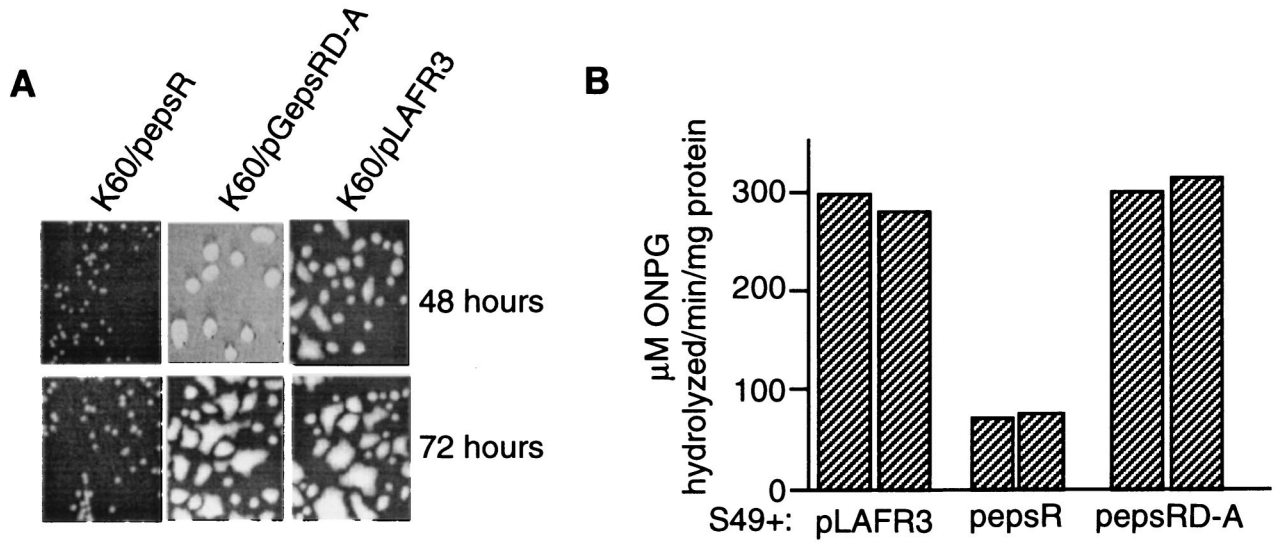


FIG. 3. (A) Colony morphology of K60 transformed for 48 and 72 h with pepsR, pGepsRD-A, and pLAFR3. (B) Overexpression of EpsRD-A does not affect expression of the *epsI* promoter as measured in strain S49, containing the *epsI*::*lacZ* fusion. Cells containing the plasmids indicated at the bottom of the graph were grown to an OD_{600} of 1.2 and assayed for β-galactosidase activity (see legend to Fig. 1 for clarification of values) as described in Materials and Methods.

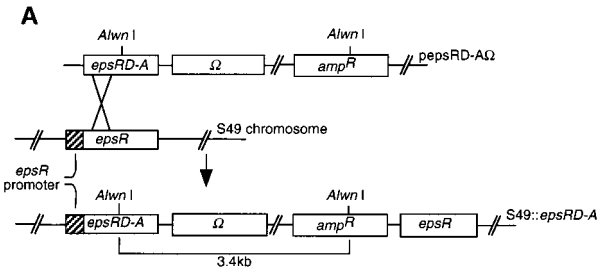
group in response regulators is inherently unstable (38). In lysates heated to 80°C for 15 min prior to electrophoresis, the band corresponding to EpsR was no longer detectable. Moreover, this band was absent in *epsR* insertion mutant strains and was apparent in strains which contained the chromosomal copy of *epsR* only after prolonged exposure (Fig. 2C, lane 6), suggesting that EpsR is phosphorylated in the absence of overexpression. No band corresponding to EpsRD-A can be detected in lysates made from cells which contain pepsRD-A (Fig. 2C). The same gel was stripped of labeled phosphate, transferred to a nitrocellulose membrane, and probed with anti-EpsR antibodies to show that EpsRD-A was being expressed in the labeled cells (Fig. 2D). EpsRD-A was present at levels comparable to those of wild-type EpsR (compare lanes 4 and 2). Therefore, D47 of EpsR is critical for phosphorylation.

EpsRD-A cannot modulate expression of *epsI*. To examine the effect of EpsRD-A on EPS production, K60 was transformed with pepsRD-A. The wild type, K60, became visibly mucoid approximately 48 h after plating (Fig. 3A). Overexpression of EpsRD-A did not grossly affect the abundance of EPS produced by K60, in contrast to overexpression of wild-type EpsR from plasmid pepsR, which visibly reduced the amount of EPS produced by K60 colonies (Fig. 3A). The plasmid pepsR contains a PCR-derived clone of *epsR* and demonstrates that EpsR is sufficient to reduce production of EPS. As previously reported, the effect of EpsR lessens as the culture ages, since colonies transformed for 72 h are noticeably more mucoid than those transformed for 48 h (21).

The effect of EpsRD-A on the expression of the *epsI* promoter was measured in strain S49. Consistent with previous results, wild-type EpsR showed *epsI* expression reduced fivefold in comparison to that of cells which contained pLAFR3. Overexpression of EpsRD-A had no effect on *epsI* expression (Fig. 3B). Therefore, phosphorylation of EpsR is required for repression of *epsI* expression.

The effect of one copy of *epsRD-A* on *epsI* expression was examined. To replace wild-type *epsR* with *epsRD-A*, S49 was transformed with pepsRD-AΩ, which contained a promoterless *epsRD-A* gene (Fig. 4A). Streptomycin- and ampicillin-resistant colonies were selected. Southern blotting confirmed that 2 of 20 isolates (strains 1 and 10) examined had recombination events which occurred before codon 47 of *epsR*. The resulting strain should express only the *epsRD-A* allele, since the other *epsR* copy is left without a functional promoter (Fig. 4A). Promoter activity in strains which contain one copy of *epsRD-A* is reduced between four- and sixfold, based on measurements of two independently isolated *epsRD-A* integrants (Fig. 4B). Since this is approximately the same reduction of expression of *epsI* observed in strain S49 lacking a functional copy of *epsR*, phosphorylation is required for activation of *epsI* expression.

EpsR participates in a complex with the *epsI* promoter. All of the results described above suggest that phosphorylation



B

Strain	Miller Units
S49	235.2
S49::*epsRD-A* #1	50.2
S49::*epsRD-A* #10	62.3
S49::*epsRΩ*	55.6

FIG. 4. (A) Schematic diagram for construction of S49 chromosomes with one copy of *epsRD-A*. The desired integration event and the resulting chromosome are shown. (B) Expression from the *epsI* promoter is reduced in S49 strains which contain *epsRD-A*.

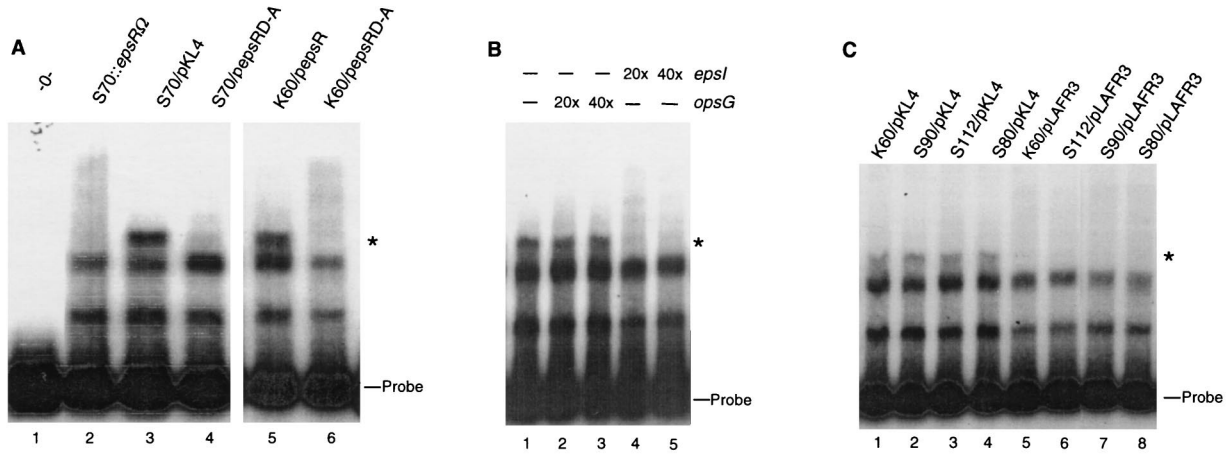


FIG. 5. (A) Gel mobility shift assay shows that EpsR mediates a gel shift of the *epsI* promoter. Strains used to make each extract are indicated above the lanes. The EpsR-dependent shifted band is designated with a star. S70::*epsR*Ω containing pLAFR3 serves as a negative control since it lacks a functional copy of *epsR*. (B) Extracts overexpressing EpsR specifically shift the *epsI* promoter. A molar excess of nonradiolabeled DNA containing the *opsG* (nt −310 to +10) or *epsI* (nt −140 to +100) promoters was added as indicated above the autoradiogram. (C) The EpsR-mediated gel shift is detected in extracts made from strains S90 (*xpsR*), S112 (*vsrB* or *vsrC*), and S80 (*phcA*) overexpressing EpsR from pKL4 (29). Free probe is indicated to the right of each gel.

plays a critical role in the activity of EpsR. We used a gel mobility shift assay to examine the interaction of EpsR with the *epsI* promoter region and the role of phosphorylation in this interaction. Clarified extracts made from strains K60 and S70 overexpressing wild-type EpsR from either pKL4 or pepsR were incubated with a labeled 240-bp probe containing a functional *epsI* promoter. Three predominant bands were observed in a gel mobility shift assay, two of which were present in all extracts, including S70::*epsR*Ω, which does not contain a functional copy of *epsR*, and the third band, with the slowest mobility, which was present only in extracts from cells overexpressing EpsR (Fig. 5A, compare lane 2 to lanes 3 and 5). This band likely contains EpsR, while the two lower bands are not specific to EpsR, since they appear in all reactions, including ones which used lysates lacking EpsR (Fig. 5A, lane 1). In addition, extracts made from K60 and from S70, which overexpresses EpsRD-A, did not give rise to the specific band (Fig. 5A, lanes 4 and 6). These results strongly suggest that EpsR expressed in either wild-type *R. solanacearum* or a mutant lacking the acidic exopolysaccharide can form a complex with the *epsI* promoter. Furthermore, phosphorylation of EpsR is required for complex formation with the *epsI* promoter.

To further determine whether the unique shifted band represents a complex specific to *epsI*, we performed reactions with cell extracts preincubated with a 20- or 40-fold molar excess of nonradiolabeled *epsI* and *opsG* promoter sequences (Fig. 5B). Unlabeled *epsI* promoter abolished the band corresponding to the EpsR-induced complex, while preincubation with the *opsG* promoter region had no effect on the integrity of this band, suggesting that the proteins in the shifted complex bind the *epsI* promoter specifically (Fig. 5B). The lack of an EpsR-specific gel shift with extracts containing wild-type levels of EpsR may be due to a lack of sensitivity in the gel mobility shift assay or that the shifted complex we observed contains EpsR in a multimeric form.

To address the possibility that any of the known *epsI* regulators, XpsR, VsrB or VsrC, and PhcA, play a role in the EpsR-associated gel shift, we made clarified extracts from mutant strains S90 (*xpsR*), S112 (*vsrB* or *vsrC*), and S80 (*phcA*) containing pKL4 and tested them for the ability to form the EpsR-associated shifted complex. The EpsR-specific gel shift of the *epsI* promoter was still detected in these mutant backgrounds at levels similar to those of strain K60 containing pKL4, suggesting that PhcA, XpsR, and VsrB or VsrC are not required for the EpsR-induced gel shift of the *epsI* promoter (Fig. 5C).

DISCUSSION

An acidic form of EPS produced by the *epsI* gene cluster is a major virulence factor of the bacterial wilt pathogen, *R. solanacearum*. Disruption of the *epsI* structural genes will reduce or eliminate the ability of *R. solanacearum* to kill plants. Three signal transduction systems, VsrA-VsrD (37), VsrB-VsrC (19), and PhcA (4, 5, 17), positively affect *epsI* expression. EpsR has been reported to be a negative regulator of EPS when present in multicopy plasmids, decreasing EPS production and expression from the *epsI* promoter (16, 21, 29). In this work, we have extended the characterization of EpsR and found that a single copy of *epsR* in the *R. solanacearum* chromosome is required for wild-type levels of *epsI* expression and virulence. Thus, the level of EpsR protein in the cell can result in different phenotypes. Furthermore, both the repressive and stimulatory effects of EpsR and the formation of an EpsR-specific complex with the *epsI* promoter require an aspartate residue, which is important for the phosphorylation of EpsR.

After visual inspection of colonies with an *epsR* mutation, it was previously reported that one copy of EpsR was not affecting EPS expression (21). However, when expression from the *epsI* promoter was examined with the more sensitive and quantitative *lacZ* promoter fusions, expression was reduced in strains with an *epsR*Ω mutation. This observation, coupled with the fact that *epsR*Ω mutant strains are reproducibly affected in virulence, suggests that the chromosomal copy of *epsR* is positively regulating *epsI* synthesis, and only when overexpressed from multicopy plasmids can EpsR act as a repressor of *epsI*. We note that *epsI* expression and virulence are not abolished in *epsR*Ω mutant strains, suggesting that EpsR is contributing to, but not essential for, expression of *epsI*.

Together, our results suggest that EpsR directly regulates *epsI* expression. First, EpsR does not affect the expression of any of the other regulators of *epsI*. Second, overexpression of EpsR and the absence of the chromosomal copy of *epsR* affected expression from only *epsI*. Third, multicopy plasmids

carrying the known positive regulators of *epsI* did not alleviate EpsR-mediated repression of *epsI* expression, suggesting that EpsR does not simply titrate one of these regulators (data not shown) (6). Our data does not eliminate the possibility that overexpression of EpsR interferes with the ability of another positive regulator to associate with the *epsI* promoter.

It is possible, although unlikely, that EpsR does not interact directly with the *epsI* promoter. Since our binding assays were done with crude cell extracts, we cannot rule out the possibility that overexpression of EpsR may in some way stimulate another protein to bind the *epsI* promoter, which in turn accounts for the observed *epsI* gel shift in extracts overexpressing EpsR. However, the EpsR-specific gel shift of the *epsI* promoter was observed with lysates putatively lacking PhcA, XpsR, and VsrB or VsrC, suggesting that these proteins are not required for the EpsR-induced gel shift of the *epsI* promoter. The C terminus of EpsR is homologous to the DNA-binding domains of other response regulators which have been shown to bind DNA (21). EpsR is highly basic (predicted pI of 8.5), which is consistent with the idea that EpsR has a natural affinity for DNA. All of these results are consistent with the working model that the activity of EpsR is mediated through formation of a complex with the *epsI* promoter.

EpsR-specific gel shift of the *epsI* promoter requires phosphorylation of EpsR. For *R. solanacearum*, this is the first direct demonstration of transcriptional regulation by protein modification. Most response regulators are thought to be phosphorylated at a conserved aspartate found in the N terminus. Several proteins have been shown experimentally to be phosphorylated, which in turn regulates many different aspects of the protein's activity, including dimerization, DNA binding, and transcriptional regulation (2, 11–13, 24, 33, 39). EpsR has extensive homology with response regulators in the OmpR class; however, its phosphorylation domain is unique. EpsR is missing two conserved aspartate residues at amino acid positions 13 and 14, which are present in nearly all response regulators. In NarL, these residues are thought to form an acid pocket which facilitates phosphorylation (1). This suggests that the mechanism of phosphorylation for EpsR may be different from that of proteins such as NarL and NarP. Although EpsR is missing these residues, D47 is clearly required for phosphorylation, likely serving as the phosphate acceptor. We were unable to detect an EpsR-mediated gel shift of the *epsI* promoter by using extracts made from *E. coli* overexpressing EpsR. Coincident with this, preliminary results indicate that EpsR is not phosphorylated in *E. coli*, providing further proof that phosphorylation is required for the EpsR-mediated gel shift observed when *R. solanacearum* extracts are used (data not shown) (6). For *E. coli* strains transformed with either pKL4 or pepsR, we were unable to identify a phosphorylated band corresponding to EpsR. The identity of the kinase is not known; however, a functional homolog appears to be one that is absent in *E. coli.*

Approximately 1% of the nonessential genes in *R. solanacearum* are directly or indirectly involved in EPS production (29). At present, all regulators of EPS production affect expression from the *epsI* promoter. In *E. coli*, the *epsI* promoter is not expressed when carried on the plasmid pGepsI::lacZ, although other promoters involved in EPS production (*opsG*) are expressed (6). Since expression of *epsI* in *R. solanacearum* is dependent on several regulatory proteins, it is not surprising that this gene is not expressed in *E. coli*. Determining the signals required for activation of *epsI*, how each of the regulators interacts with each of the other regulators, and what conditions in planta might result in overexpression of EpsR are potential topics of future study.

The paradigm for differential regulation of a promoter is the lambda phage protein, which regulates its own expression by binding to different operator sequences located in its promoter (35). Several other bacterial proteins have been shown to act as both activators and repressors of transcription. In *E. coli*, the response regulators NarL and NarP can both positively and negatively affect transcription by the location of their DNA binding sites with respect to the transcriptional start site (9). The flagellar genes of *Caulobacter crescentus* are also both positively and negatively regulated by the response regulator FlbD (31). Should EpsR levels be regulated in the cell, then EpsR may interact specifically with the *epsI* promoter at multiple sites and with differing affinities. While it is possible that the repressive effect of EpsR is due to artificial overexpression, an abundance of EpsR retains the ability to specifically interact with the *epsI* promoter. Perhaps, with normal cellular levels of EpsR, a high-affinity binding site will be recognized, leading to activation of *epsI*. However, in our in vitro experiments, we are unable to detect an EpsR-mediated gel shift of the *epsI* promoter by using extracts containing wild-type levels of EpsR.

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