JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6659–6666 Vol. 182, No. 23
0021-9193/00/$04.00+0
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Multicomponent Transcriptional Regulation at the Complex Promoter of the Exopolysaccharide I Biosynthetic Operon of *Ralstonia solanacearum*

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Received 18 May 2000/Accepted 6 September 2000

High-level transcription of *eps*, an operon encoding biosynthesis of an exopolysaccharide virulence factor of the phytopathogen *Ralstonia* (*Pseudomonas*) *solanacearum*, requires the products of at least seven regulatory genes (*phcA*, *phcB*, *xpsR*, *vsrA-vsrD*, and *vsrB-vsrC*), which are organized in three converging signal transduction cascades. Because *xpsR* and the *vsrB-vsrC* two-component system are the most downstream cascade components required for activation of *eps*, we explored how these components control transcription from the *eps* promoter (P_{eps}). Deletion and PCR mutagenesis identified an upstream region of P_{eps} (nucleotides −82 to −62) that is critical for transcription activation by VsrB-VsrC and XpsR and also is required for negative control of P_{eps} by the putative *eps* regulator EpsR. Using PCR mutagenesis we generated the *vsrC1* allele that encodes a response regulator that constitutively activates P_{eps} in the absence of its cognate sensor, VsrB. However, activation of P_{eps} by *vsrC1* still required *xpsR*. Unexpectedly, the amino acid substitution conferring the constitutive phenotype on VsrC1 is 12 residues from its C terminus, outside the known functional domains of response regulators. Finally, a modified DNase I footprinting method was used to demonstrate specific binding of both VsrC1 and VsrC to the −72 to −62 upstream region of P_{eps}.

Ralstonia (*Pseudomonas*) *solanacearum*, which causes a lethal wilting disease of solanaceous and many other types of other plants (15, 16), enters hosts via natural openings or wounds in roots and then proceeds to extensively colonize xylem vessels of the vascular system (37, 44). Although secreted plant cell wall-degrading exoenzymes enhance virulence (possibly by facilitating invasion and vascular colonization [23, 24, 37]), it is exopolysaccharide I (EPS I), a large, nitrogen-rich, acidic exopolysaccharide (34), that is the primary virulence factor of *R. solanacearum*. EPS I is produced in copious amounts and is required for wilting and killing of hosts (8, 29). EPS I apparently causes wilting by restricting water flow through xylem vessels (6). It also markedly enhances the speed and extent of stem colonization (37).

In *R. solanacearum*, production of EPS I (as well as some exoenzymes) is stringently controlled by a cascading network of more than 10 regulatory genes (5, 11, 20, 41). Inactivation of any of seven genes in this network causes a >85% reduction in transcription from the *eps* promoter (P_{eps}), leading to loss of EPS I production and the ability to wilt and kill. However, inactivation of all but two of these genes (*vsrB* and *vsrC*) can be suppressed by constitutive expression of *xpsR* from a vector promoter (20). These and other data showed that VsrB, VsrC, and XpsR are the most downstream components in the *eps* regulatory cascade and suggested that they may directly affect interaction of RNA polymerase with P_{eps}.

The predicted amino acid sequences of VsrB and VsrC imply that they comprise a two-component system in which VsrB is a sensor kinase and VsrC is its cognate response regulator (19, 20). However, no homologs of the very basic XpsR protein have been found. How these three proteins interact to control P_{eps} is not clear. Nonetheless, analogy to other two-component systems (17) implies that, in response to some unknown signal, VsrB phosphorylates VsrC, thereby stimulating it to turn on transcription, possibly via direct binding to P_{eps}. EpsR, a putative DNA-binding protein, is another potential regulator of P_{eps}, since its overproduction strongly represses EPS I synthesis by *R. solanacearum* strain K60 (18, 25).

Since there was no clear or direct evidence for physical interactions between P_{eps} and VsrC, XpsR, or EpsR, we first used deletion and PCR mutagenesis to define an upstream region of P_{eps} that is absolutely required for its transcription activation by VsrB-VsrC and XpsR. Then we generated a *vsrC* allele that activated P_{eps} independently of *vsrB* and used DNase I footprinting to show that both this constitutively active VsrC protein and wild-type VsrC directly bind to the region of P_{eps} that was identified by mutagenesis to be important in transcription activation. However, since the affinity of VsrC for P_{eps} was weak and since the constitutively active VsrC protein was unresponsive to VsrB and still required XpsR for activation of P_{eps}, we speculate that XpsR may facilitate or stabilize binding of VsrC to P_{eps}.

MATERIALS AND METHODS

Bacteria and plasmids. Most strains and plasmids constructed and used in this work are described in Table 1. *R. solanacearum* was grown in BG or BSM medium (36) at 30°C, while *Escherichia coli* was grown in LB (33) at 37°C. The host strain and vectors used for cloning were *E. coli* DH5α (14) and pTZ19U/18U (31), respectively. pPF12 (7) and pEPS1 (21) were described previously. *R. solanacearum* strain AW22 was constructed by transposon mutagenesis with Tn*3lacZ* (7). Derivatives of AW22 were constructed by natural transformation (20) with genomic DNA from AW-R164, AW-C, and AW-MG2 (19). Concentrations (micrograms per milliliter) of antibiotics used to select and maintain plasmids were kanamycin (Km), 50; spectinomycin (Sp), 50; ampicillin (Amp), 100; and tetracycline (Tc), 20.

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TABLE 1. Important *R. solanacearum* strains and plasmids used

Strain/plasmid	Relevant characteristics	Reference or source
Strain		
AW	Wild-type pathogen, biovar 1	38
AW201	*epsA1*::*npt1*	21
AW-C	*vsrC*::*cat*	20
AW-R164	*xpsR*::*nptI*	20
AW22	*eps*::Tn*3-lacZ*	This study
AW22B	AW22 *vsrB*::Ω	This study
AW22BC	AW22 *vsrC*::*cat vsrB*::Ω	This study
AW22RBC	AW22 *vsrC*::*cat vsrB*::Ω *xpsR*::*nptI*	This study
AW19A	*epsB*::Tn*5lacZ*	4
Plasmid		
pXPS1	8-kb *Pst*I fragment of pPF12 in pTZ18U	This study
pXPS2	900-bp *Bam*HI fragment of pEPS1 in pTZ19U	This study
pXPS5	320-bp *Sph*I fragment of pXPS2 in pTZ18U	This study
pTZSZ12	176-bp *Bam*HI fragment of pPSZ12 in pTZ18U	This study
pPSZ22	Nucleotides −44 to +23 of P_{eps} fused to *lacZ* via *Bam*HI site of pRG970	This study
pPSZ15, pPSZ17, pPSZ20, pPSZ12, pPSZ19, pPSZ21	Same as pPSZ22, but with longer lengths of P_{eps} fused to *lacZ* (see Fig. 1)	This study
pPSZ15-2, pPSZ15-7	pPSZ15 with −67 A to G and −72 G to T, respectively	This study
pEPSM2, pEPSM7	pPSZ12 with −67 A to G and −72 G to T, respectively	This study
pKVC3	*Rsa*I-*Bam*HI fragment of *vsrC* in pTZ19U	This study
pRKVC3	*vsrC* from pKVC3 in pRK415	This study
pVSRC1	*vsrC* with H147R and S209L mutations in pRK415	This study
pVSRC2	*vsrC* with S209L mutation in pRK415	This study
pVSRCH, pCB92	*vsrC* and *vsrC1*, respectively, in pTrc-His	This study
pEPSR	*epsR* from AW in *Bam*HI and *Eco*RI sites of pTZ19U	This study
pEPSR-T	*Sty*I fragment of *epsR* in *Eco*RV site of pTOK2	This study

Construction of P_{eps} reporter plasmids. P_{eps} fragments with various lengths of upstream sequences were made by PCR using primers EPS1 (5′-CCGGATCC CCAACTGTAAATCGTA-3′), EPS2 (5′-CCGGATCCAAACGAAATATGCA TT-3′), EPS3 (5′-CCGGATCCTTTCGGTATTGAAGC-3′), EPS4 (5′-CCGGA TCCATGCACAACCGTATC-3′), EPS5 (5′-TCGGATCCATCGCCACCGGT ACTG-3′), EPS6 (CCGGATCCAGAACGATCCATGTTTC-3′), EPS7 (5′-CC CCGGGTTGGCGTTCTGCCTAT-3′), EPS9 (5′-GAGGATCCAGTTGCAGA AACGGCCA-3′), M13F (TGTAAAACGACGGCCAGT-3′), M13R (5′-AGC GAATAACAATTTCACACAGGA-3′), and T7 (5′-TAATACGACTCACTAT AGGG-3′). PCR mixtures (100 μl) contained 10 mM Tris (pH 8.3), 50 mM KCl, 1 mM of each deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, 0.1 nmol of primers, 1 to 5 ng of template, and 2 U of *Taq* polymerase (Perkin-Elmer). Each amplification cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. After 30 cycles, there was a final extension at 72°C for 10 min. The P_{eps} DNA fragments from nucleotides −538 to +23, −337 to +23, and −243 to +23 were amplified using pXPS1 as template and primer pairs EPS6-EPS4, EPS7-EPS4, and EPS9-EPS4, respectively. The P_{eps} DNA fragments from −143 to +23, −101 to +23, −68 to +23, and −44 to +23 were amplified using primer pairs M13F-EPS4, EPS1-EPS4, EPS2-EPS4, and EPS3-EPS4, respectively, and pXPS5 as template. PCR products were digested with *Bam*HI and/or *Sma*I and ligated into the promoterless *lacZ* fusion vector pRG970 (43) digested in the same manner. After transformation into *E. coli* and plating on LB agar with Sp and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), plasmid DNA was isolated from blue colonies, and the orientation and identity of the insert were confirmed.

PCR mutagenesis of P_{eps} and *vsrC*. To mutagenize P_{eps}, sequences between nucleotides −143 and +23 were PCR amplified essentially as above using a pTZSZ12 template. However, the dATP concentration was 0.2 mM instead of 1 mM, and $MnCl_2$ was added to 0.5 mM. *Bam*HI-digested PCR products were ligated with *Bam*HI-digested pRG970 and transformed into *E. coli*, and ~10,000 blue-colored transformants arising on LB plates with Sp and X-Gal were individually picked and pooled, and plasmid DNA was isolated from them. Pooled plasmids were electroporated into *R. solanacearum* strain AW201 (*epsA1*::*nptl*). After plating on BG agar with Sp and X-Gal, white or pale-blue transformants (i.e., those with decreased LacZ activity) were observed at 0.3%. Plasmids from these colonies were individually isolated, transformed into *E. coli*, reisolated, and analyzed with restriction enzymes to confirm P_{eps} inserts. Sequence alterations were determined after recloning inserts into pTZ19U.

To mutagenize *vsrC*, PCRs were performed under mutagenic conditions as above, using pKVC3 as template and an M13F-T7 primer pair. After digestion with *Hin*dIII and *Eco*RI, the PCR products were ligated with similarly digested pTZ19U and transformed into *E. coli*. Plasmid DNA was isolated en masse from ~20,000 transformant colonies that were washed from plates. The *vsrC* alleles in this plasmid pool were released by digestion with *Hin*dIII and *Eco*RI and ligated with similarly digested pRK415 (26), followed by transformation into *E. coli*. Plasmid DNA was isolated from ~20,000 pooled Tc-resistant colonies and electroporated into *R. solanacearum* strain AW22B (*vsrB*::Ω *eps*::*lacZ*). After selection on BG plates with X-Gal and Tc, transformants showing increased expression of the *eps*::*lacZ* reporter (i.e., darker-blue color) were analyzed.

To construct the *vsrC2* allele, *vsrC* sequences between nucleotides 523 and 1,194 (GenBank U18134) were amplified using a pRKVC3 template, a T7 primer, and a primer containing *vsrC* sequences between nucleotides 1,162 and 1,194 but having a C1185T mutation (S209L) and a same-sense C1171G silent mutation that introduced a *Bam*HI site (5′-TCCATGCGCAAGATGATCTGG ATCCGCGTGCGC-3′). The resultant PCR product and a plasmid with sequences 1,145 to 1,290 of *vsrC* were mixed, and PCR amplification was performed with T7 and M13F primers. The 800-bp *vsrC* PCR product was digested with *Hin*dIII and *Eco*RI and cloned into pRK415. Plasmids from transformants were isolated and screened for the introduced *Bam*HI site. The *vsrC* allele from one candidate plasmid (pVSRC2) was sequenced to confirm that it contained the S209L mutation.

Purification of His-tagged VsrC proteins. Wild-type *vsrC* was PCR amplified using pKVC3 as template and primers M13F and VSRCN (5′-ACGGATCCA CGAGCTCGCTGCG-3′; complementary to sequences encoding the N terminus of VsrC). The product was digested with *Bam*HI and cloned in frame to the hexahistidine-encoding tag of *Bam*HI-digested pTrc-HisA (Invitrogen), yielding plasmid pVSRCH. To generate pCB92, the *Sac*I-*Kpn*I fragment of pVSRCH was replaced with the analogous fragment from pVSRC1.

E. coli JM109 (45) cells containing pVSRCH or pCB92 were shaken at 37°C in LB plus Amp until the optical density at 600 nm (OD_{600}) was 0.6. The 100-ml culture was shifted to 28°C, and isopropyl-β-D-thio-galactoside was added to 0.4 mM. After 18 h, cells were harvested, suspended in 10 ml of buffer B (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 8.0]) and sonicated five times for 10 s. The sonicate was centrifuged at 30,000 × *g* for 25 min, and the supernatant was passed through a 3-ml column containing 1 ml of Ni-nitriloacetic acid resin (Sigma) equilibrated with buffer B. The column was washed with 10 ml of buffer B followed by 10 ml of buffer B with 50 mM imidazole. VsrCs were eluted with buffer B plus 0.5 M imidazole; sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated >90% purity.

Construction of an *epsR* null mutant. The *epsR* homolog of *R. solanacearum* strain AW was PCR amplified from genomic DNA as above except that annealing and extension were at 70°C. Primers (EPSRN, 5′-AAGGATCCAGG CGGCGCAGTG-3′ [nucleotides 355 through 381 with an added *Bam*HI site] and EPSRC, 5′-ATGAATTCAGCCCGGCGTGCACGAGGCG-3′ [nucleotides 1,055 through 1,075 with an added *Eco*RI site]) were based on the sequence of *epsR* from *R. solanacearum* strain K60 (GenBank M61197). The resultant PCR

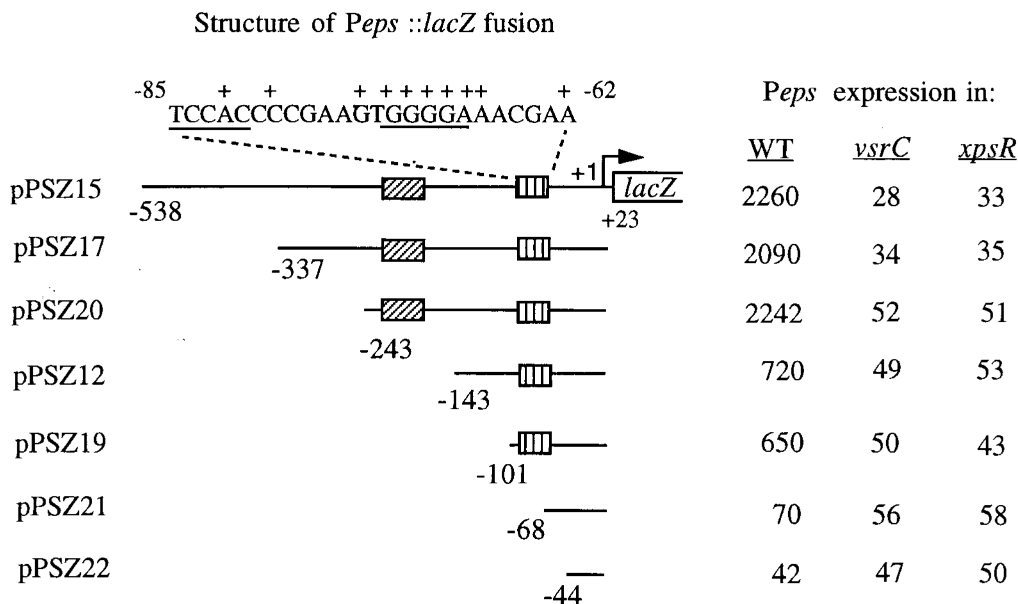

FIG. 1. Identification of upstream regions involved in transcriptional regulation of P_{eps} by VsrC and XpsR. The *eps* promoter and various lengths of upstream sequences were fused to *lacZ* on pRG970, generating plasmids pPSZ12 through pPSZ22. Plasmids were transferred into *R. solanacearum* wild-type (WT) strain AW, into strain AW-C, a *vsrC* mutant, and into strain AW-R164, an *xpsR* mutant. P_{eps} expression (i.e., transcription directed by the P_{eps} fragment) was monitored by measuring LacZ activity (given in Miller units [33]) in cells from cultures grown overnight in BG medium as described previously (4, 20). LacZ activity from cells harboring an empty pRG970 vector was 15. Nucleotide numbering is relative to the transcription start site of *eps* (21). The hatched boxes indicate the two important regions for activation of *eps*. The sequence at the top is that of the region identified by PCR mutagenesis as essential for transcription activation by *xpsR* and *vsrC*. +, positions of substitution mutations that reduced or eliminated activation of P_{eps} (see Table 2). Underlined sequences, putative palindromic recognition sequences for VsrC. Values are averages from three experiments with <25% variation.

product was digested with *Bam*HI and *Eco*RI and cloned into pTZ18U. DNA sequencing showed that *epsR* from strain AW is >97% identical to *epsR* from strain K60.

To construct the *epsR* null mutant, an internal *Sty*I fragment lacking sequences encoding the first 12 N-terminal residues and last 52 C-terminal residues of EpsR was made blunt ended with T4 DNA polymerase and then cloned into the *Sma*I site of suicide vector pTOK2 (27). The resultant plasmid (pEPSR-T) was electroporated into *R. solanacearum* AW, and strains in which the plasmid integrated into *epsR* by a single recombination event were selected by Tc resistance. Genomic DNA from two strains was prepared and disruption of the genomic copy of *epsR* was confirmed by PCR; no PCR products could be obtained using EPSRN and EPSRC primers, whereas a PCR product of the predicted size was obtained with wild-type DNA as template. An EPSRN and M13R vector primer gave a PCR fragment of the size predicted for the truncated *epsR* gene.

Measurement of PglA. Assay samples were spotted onto nitrocellulose and after blocking for 2 h at 25°C with 5% skim milk (Difco) in TBS (10 mM Tris HCl [pH 7.4], 140 mM NaCl), the membrane was washed three times with TBST (TBS with 0.2% Tween 20) and submerged for 1 h in 10 ml of TBST containing a 1:1,000 dilution of anti-PglA antiserum (39). After three washes with TBST, one with TBST containing 0.85 M NaCl, and one with TBST, bound PglA antibodies were detected with anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Jackson Immunologicals), 5-bromo-4-chloro-3-indolyl-phosphate, and nitroblue tetrazolium (32).

DNase I footprinting. Target DNA fragments were prepared by PCR essentially as described above and previously (46) using 0.2 mM dNTPs, pPSZ17 as template, and primers *M13L (5′-[6-FAM]-CACGACGTTGTAAAACGACG GCCAGT-3′; PE Applied Biosystems) and T7. The resultant PCR product (containing sequences between −337 and +23 of P_{eps} and labeled at one 5′ end with the 6-FAM fluorescent tag) was gel purified, and 40 ng (10 nM) was used in 10-μl footprinting-reaction mixtures that contained 10 mM Tris-HCl (pH 7.5), 5 mM KCl, 1 mM EDTA, 8% glycerol, and 0.5 to 4 μm (0.4 to 3.5 μg) of purified His-tagged VsrC proteins. The total protein concentration was maintained at 4.5 mg/ml using bovine serum albumin. After 30 min at 30°C, reaction mixtures were placed at 26°C, and 5 μl of RNase-free DNase I (1.2×10^{-5} U/μl, freshly diluted [Boehringer Mannheim]) were added. After 4 min, 15 μl of 0.5 M EDTA (pH 8.0) was added; reactions were extracted with phenol-chloroform and passed through a CENTRI-SEP column (Princeton Separations). Digestion products were vacuum dried, dissolved in 12 μl of deionized formamide, 0.2 μl of GS-500-ROX size standards (PE Biosystems) was added, and the fragmentation patterns were analyzed with an ABI 310 Genetic Analyzer as described previously (46).

General molecular and genetic techniques. Methods for plasmid isolation from *E. coli* or *R. solanacearum* and subsequent transfer into *E. coli* using the $CaCl_2$-treated competent cells or into *R. solanacearum* via electroporation were described earlier (21, 22). Restriction enzymes, DNA ligase, Klenow fragment, and other DNA enzymes were used according to the manufacturer's recommendations. DNA sequences were obtained using an ABI 377 sequencer. Other general molecular genetic techniques used are described elsewhere (1, 28).

RESULTS

Two regions of the *eps* promoter are involved in its transcriptional activation. To define the extent of upstream sequences that are required for transcriptional activation of P_{eps}, we constructed a series of reporter plasmids with various lengths of P_{eps} fused to *lacZ*. Expression of these reporter genes was assayed in an *R. solanacearum* wild-type strain and in strains lacking key regulators. A reporter plasmid with sequences between −538 and +23 of P_{eps} fused to *lacZ*(pPSZ15) gave a high level of P_{eps} expression that was strongly dependent on both *xpsR* and *vsrC*, since P_{eps} expression was reduced at least 50-fold by inactivation of either regulator (Fig. 1). Deletion of sequences upstream of −337 or −243 did not dramatically alter expression or regulation, indicating that sequences between −243 and +23 are sufficient for wild-type expression from P_{eps}. When sequences between −243 and −143 were deleted (pPSZ12 [Fig. 1]), expression was reduced about threefold, suggesting that a site in the region between nucleotides −243 and −143 stimulates P_{eps} expression. Residual expression from this reporter construct, however, remained strongly dependent on both *vsrC* and *xpsR*. Deletion of sequences between −143 and −101 did not significantly reduce transcription below that observed with a reporter having the −143 to +23 region of P_{eps} fused to *lacZ*. However, when sequences between nucleotides −101 and −68 were deleted, P_{eps} expres-

TABLE 2. Expression and regulation of mutant *eps* promoters[a]

Mutation	P_{eps} expression (Miller units) in		
	WT	*vsrC*	*xpsR*
None	780	49	53
−82 A to G	148	37	48
−79 C to T	151	36	42
−74 G to A	65	45	52
−72 G to T	160	46	37
−71 G to A	75	59	65
−70 G to A	48	40	37
−69 G to A	70	41	39
−68 A to G	142	43	58
−67 A to G	151	59	55
−62 A to G	214	49	51
−38 T to A	326	30	32
−32 A to C & −29 T to C	10,200	NT	1,340
−12 T to C	82	31	29
−9 A to G	69	NT	NT
−7 T to C	45	37	31

[a] Derivatives of $P_{eps::lacZ}$ fusion plasmid pPSZ12 (Fig. 1) containing the indicated mutations were placed in *R. solanacearum* strains AW (wild-type [WT]), AW-C (*vsrC*), and AW-R164 (*xpsR*). Expression (transcription) from P_{eps} was measured by assay of LacZ (given in Miller units [33]) as described in Fig. 1. NT, not tested. Values are averages of three independent determinations with less than 25% variation.

TABLE 3. Effect of selected mutations on expression directed by P_{eps} fragments with different upstream lengths

Plasmid	P_{eps} sequences fused to *lacZ*	Mutation	Expression from P_{eps}[a]
pPSZ15	−538 to +23	None	2,260
pPSZ15-2	−538 to +23	−67 A to G	195
pEPSM2	−143 to +23	−67 A to G	164
pPSZ15-7	−538 to +23	−72 G to T	262
pEPSM7	−143 to +23	−72 G to T	190

[a] Expression from P_{eps} in wild-type *R. solanacearum* AW cells carrying the indicated $P_{eps::lacZ}$ fusion plasmids with the indicated mutations was measured as in Fig. 1 and Table 2.

sion was reduced to less than 5% of the levels observed with the −243 to +23 fusion. Expression was only marginally further reduced when P_{eps} sequences down to −44 were deleted (pPSZ22 [Fig. 1]), regardless of the genetic background. These data show that the −101 to −68 region contains sequences that are absolutely required for activation of P_{eps} by these two regulators.

Determination of nucleotides required for activation and regulation of P_{eps}. To delineate more precisely the sequences that are critical for P_{eps} regulation, a fragment with P_{eps} nucleotides −143 to +23 was subjected to mutagenic PCR, and the products were joined to *lacZ* on pRG970 to generate transcriptional fusions. The resultant pooled reporter constructs were introduced into wild-type *R. solanacearum*, and colonies were screened for altered $P_{eps::lacZ}$ expression by using X-Gal. Sixteen mutant plasmids were obtained and characterized further by DNA sequencing and quantitative LacZ assays (Table 2). $P_{eps::lacZ}$ expression from plasmids with single-nucleotide substitutions at P_{eps} nucleotides −74, −71, −70, and −69 was reduced ~10-fold, almost to the basal levels given by plasmids in which all upstream activation sequences had been deleted (compare to pPSZ22 and pPSZ21 [Fig. 1]). When assayed in *xpsR* or *vsrC* mutants of *R. solanacearum*, expression directed by these mutant promoters was only marginally reduced, indicating that these mutations largely eliminate activation of P_{eps} transcription by VsrC and XpsR. Fusion plasmids with single nucleotide substitutions at P_{eps} nucleotides −82, −79, −72, −68, and −67 were reduced about fivefold. However, when assayed in *xpsR* or *vsrC* mutant backgrounds of *R. solanacearum*, P_{eps} expression from these promoters was reduced an additional three- to fourfold to basal levels, indicating that these mutant promoters are inefficiently activated by VsrC and XpsR. In summary, these results suggest that nucleotides between −82 and −62, and in particular the sequence GTGGG GAA between −74 and −67 (Fig. 1), are important for activation of P_{eps}. It is plausible that this region contains the binding sites for P_{eps} activators, perhaps VsrC and/or XpsR.

When P_{eps} sequences between nucleotides −538 and −143 were restored in the proper orientation and position to the $P_{eps::lacZ}$ fusion plasmids with substitutions at −67 or −72 and the resultant plasmids placed in wild-type *R. solanacearum*, P_{eps} expression was largely the same as observed with shorter (−143 to +23) P_{eps} fragments (Table 2 versus Table 3). These results support the conclusion that the primary P_{eps} regulatory sequences required for transcription activation by VsrC and XpsR lie downstream of nucleotide −143, while sequences upstream of −143 can only enhance activation by these regulators.

Three other single-nucleotide substitutions (at nucleotides −12, −9, and −7) also dramatically reduced P_{eps} expression (Table 2). The position and nature of these mutations are consistent with the presumed role of this region as the −10 consensus hexamer of the promoter (21). A single-nucleotide substitution at position −38 reduced P_{eps} expression threefold, while a double mutation changing nucleotides at both −32 and −29 dramatically increased P_{eps} expression (Table 2). These data are consistent with the presumed role of this region as the −35 consensus hexamer that comprises a σ^{70}-type RNA polymerase recognition site (21).

EpsR can inhibit transcription activation of P_{eps} by XpsR and VsrC. *epsR* encodes a putative DNA-binding protein that inhibits EPS production by *R. solanacearum* strain K60, but only when plasmid borne (18, 25, 30). Moreover, the two reported phenotypes of *epsR* mutants of strain K60 are contradictory (3, 25). Therefore, we needed to explore a possible role for *epsR* in regulating P_{eps} transcription in our *R. solanacearum* strain. To do this we transferred plasmid pKL4 containing the K60 *epsR* gene (25) into our wild-type AW strain harboring a genomic *eps*::*lacZ* fusion (AW19A). The addition of pKL4 reduced expression of *eps* by sevenfold (Table 4). To determine what P_{eps} sequences are required for this effect, pKL4

TABLE 4. Effect of plasmid-borne *epsR* on expression from wild-type and mutant *eps* promoters in *R. solanacearum*

Strain[a]	*eps* expression (Miller units) in presence of:	
	pLAFR3	pKL4
AW19A (*epsB*::*lacZ*)	1,980	253
AW (pPSZ12) [−143 to +23]	710	60
AW (pPSZ19) [−101 to +23]	568	57
AW (pPSZ22) [−44 to +23]	34	31
AW (pEPSM9) [−143 to +23; −70 G to A]	55	44
AW (pEPSM3) [−143 to +23; −74 G to A]	51	37

[a] *R. solanacearum* strains contained either the pLAFR3 vector or pKL4 (pLAFR3 carrying *epsR*). AW19A is a wild-type strain with a genomic *epsB*::*lacZ* reporter. AW strains harbored the indicated $P_{eps::lacZ}$ reporter plasmids (see Fig. 1); numbers in brackets indicate region of P_{eps} fused to *lacZ* and mutation, if any. *eps* expression was measured as in Fig. 1.

TABLE 5. Activation of *eps* expression by wild-type and mutant alleles of *vsrC* in various *R. solanacearum* regulatory mutants

Strain[a]	*eps* expression (Miller units) in presence of:			
	No plasmid	pRKVC3	pVSRC1	pVSRC2
AW22B	3.6	5.2	32.0	28.6
AW22C	4.1	44.0	36.1	41.4
AW22BC	3.2	4.5	29.0	24.2
AW22RBC	3.3	3.1	5.8	NT
AW22	37.2	38.3	33.0	NT

[a] Plasmid pVSRC1 (*vsrC1* with H146R and S209L mutations), pVSRC2 (*vsrC2* with only an S209L mutation), or pRKVC3 (wild-type *vsrC*) was placed in *R. solanacearum* strains harboring a genomic *eps*::*lacZ* reporter and various regulatory-gene mutations. Expression of *eps* was monitored as in Fig. 1. AW22, wild type; AW22B, *vsrB* mutant; AW22C, *vsrC* mutant; AW22BC, *vsrB vsrC* mutant; and AW22RBC, *xpsR vsrB vsrC* mutant. NT, not tested.

was transferred into strain AW harboring reporter plasmids with different lengths of P_{eps} sequences fused to *lacZ* (Fig. 1). Expression from reporters with P_{eps} sequences between −143 and +23 or between −101 and +23 fused to *lacZ* was specifically reduced by greater than ninefold by the presence of pKL4 (Table 4). However, deletion of P_{eps} sequences between nucleotides −101 and −44 eliminated this effect. When pKL4 was placed in a strain harboring fusion plasmid pEPSM9 or pEPSM3, which cannot be transcriptionally activated due to mutations in the −82 to −62 regulatory region, expression from P_{eps} was not significantly decreased. Thus, *epsR* affects P_{eps} only if it has been activated, suggesting that *epsR* interferes with transcriptional activation of P_{eps} mediated by *vsrC* and *xpsR* via the −82 to −62 upstream region.

To further investigate P_{eps} regulation by *epsR*, we constructed an *epsR* null mutant of strain AW. Similar to some results with strain K60 (25), inactivation of *epsR* did not obviously affect EPS production. Moreover, when genomic (*eps130*::*lacZ*) or plasmid-borne (pPSZ20 or pSZ21) $P_{eps::lacZ}$ fusions were transferred into the AW strain that lacks EpsR, expression from P_{eps} was the same as in the wild type (data not shown). Thus, *epsR* affects P_{eps} expression in strain AW only when plasmid-borne, probably due to the 10-fold overproduction of EpsR caused by an elevated copy number (25).

Isolation and characterization of *vsrB*-independent *vsrC* alleles. Previous genetic studies (19, 20) and analogy to other two-component systems (17) suggest that, in response to some signal, VsrB phosphorylates VsrC, converting it into a form that can activate transcription from P_{eps}. However, since XpsR is also required, this assumption is tentative. To explore the dependency of VsrC on the VsrB sensor kinase, as well as XpsR, we set out to isolate *vsrC* alleles that activate P_{eps} independently of VsrB and/or XpsR. A pool of 20,000 plasmids containing heavily PCR-mutagenized *vsrC* alleles was transferred into *R. solanacearum* strain AW22B (*eps*::*lacZ vsrB*::Ω), and transformants with elevated *eps*::*lacZ* expression were selected. After reisolation and retransformation into AW22B, only one plasmid (pVSRC1) consistently and strongly increased P_{eps} expression in the absence of VsrB. LacZ assays showed that in either AW22B or AW22BC (*vsrB vsrC eps*::*lacZ*) double mutants, pVSRC1 caused a greater than ninefold increase in transcription of *eps*::*lacZ* (Table 5). In contrast, pRKVC3 with wild-type *vsrC* only slightly increased *eps* expression. This suggested that the *vsrC1* allele on pVSRC1 harbors a mutation that makes it nearly fully active in the absence of phosphorylation by VsrB. When pVSRC1 was placed in the wild-type reporter strain AW22 or strain AW22C (*vsrC eps*::*lacZ*), P_{eps} activation was similar to that observed in strain AW22BC (Table 5), suggesting that the activity of VsrC1 cannot be dramatically increased by VsrB. When pVSRC1 was placed in a strain lacking VsrC, VsrB, and XpsR (AW22RBC), transcription of *eps*::*lacZ* showed only a small (less than twofold) increase. Restoring *vsrB* to this strain (i.e., converting it to a *vsrC xpsR* mutant) had no effect on its *vsrC1*-mediated activation of P_{eps} (data not shown). Thus, although VsrC1 protein is very active without VsrB, it still requires *xpsR* for P_{eps} activation. Not surprisingly, all of our attempts to isolate *vsrC* alleles that functioned independently of *xpsR* by screening the pool of mutant *vsrC* alleles in *xpsR* mutants were unsuccessful.

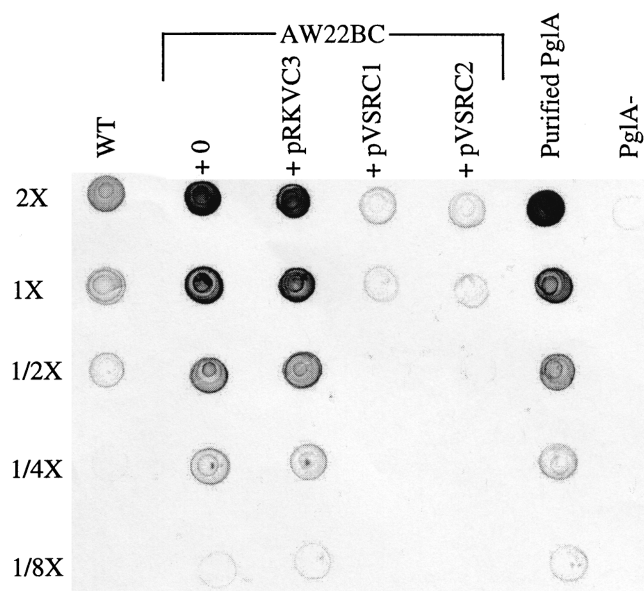

FIG. 2. Regulation of *pglA* by wild-type *vsrC* and mutant alleles of *vsrC*. Concentrated supernatants of 24-h-old BSM cultures of strain AW22 (wild-type background [WT]) or from double mutant strain AW22BC (*vsrB vsrC*) containing the indicated plasmids were serially diluted twofold in BSM and 5 μl of each dilution (2× through 1/8×) spotted on nitrocellulose. The amount of PglA was assayed using anti-PglA antiserum and alkaline phosphatase-conjugated secondary antibody. pRKVC3 contains wild-type *vsrC*; pVSRC1 and pVSRC2 contain the *vsrB*-independent alleles *vsrC1* and *vsrC2*, respectively. PglA−, supernatant from *R. solanacearum* PG3 (*pglA*::*nptI* [39]). Purified PglA (50, 25, 12, 6, and 3 μg, respectively, for each of the 5 dilutions listed at the left) was used for calibration.

Inactivation of *vsrB* or *vsrC* increases PglA production by about sevenfold (19, 20; Fig. 2), indicating that, in addition to positive regulation of *eps*, the VsrB-VsrC two-component system negatively regulates production of polygalacturonase PglA. Placing the *vsrC1* allele in an AW22BC mutant caused its derepressed PglA level to be reduced back to wild-type levels (Fig. 2); in contrast, introduction of wild-type *vsrC* into the same strain did not affect PglA levels. Thus, *vsrC1* exhibits both positive and negative regulation of appropriate targets without the input of VsrB.

DNA sequence analysis of the *vsrC1* allele showed two nucleotide substitutions: A996→G and C1185→T, causing substitution of His146 with Arg and of Ser 209 with Leu. To explore the contribution of each amino acid substitution to the *vsrC1* phenotype, splice overlap PCR was used to construct a *vsrC* allele encoding a VsrC with only the S209L substitution. When cloned into pRK415 and assayed in regulatory mutants of *R. solanacearum*, this allele (*vsrC2*) had essentially the same effect on expression of *eps* and *pglA* as the *vsrC1* allele (Table 5 and Fig. 2) indicating that the *vsrB*-independent phenotype of *vsrC1* and *vsrC2* is largely the result of the S209L substitu-

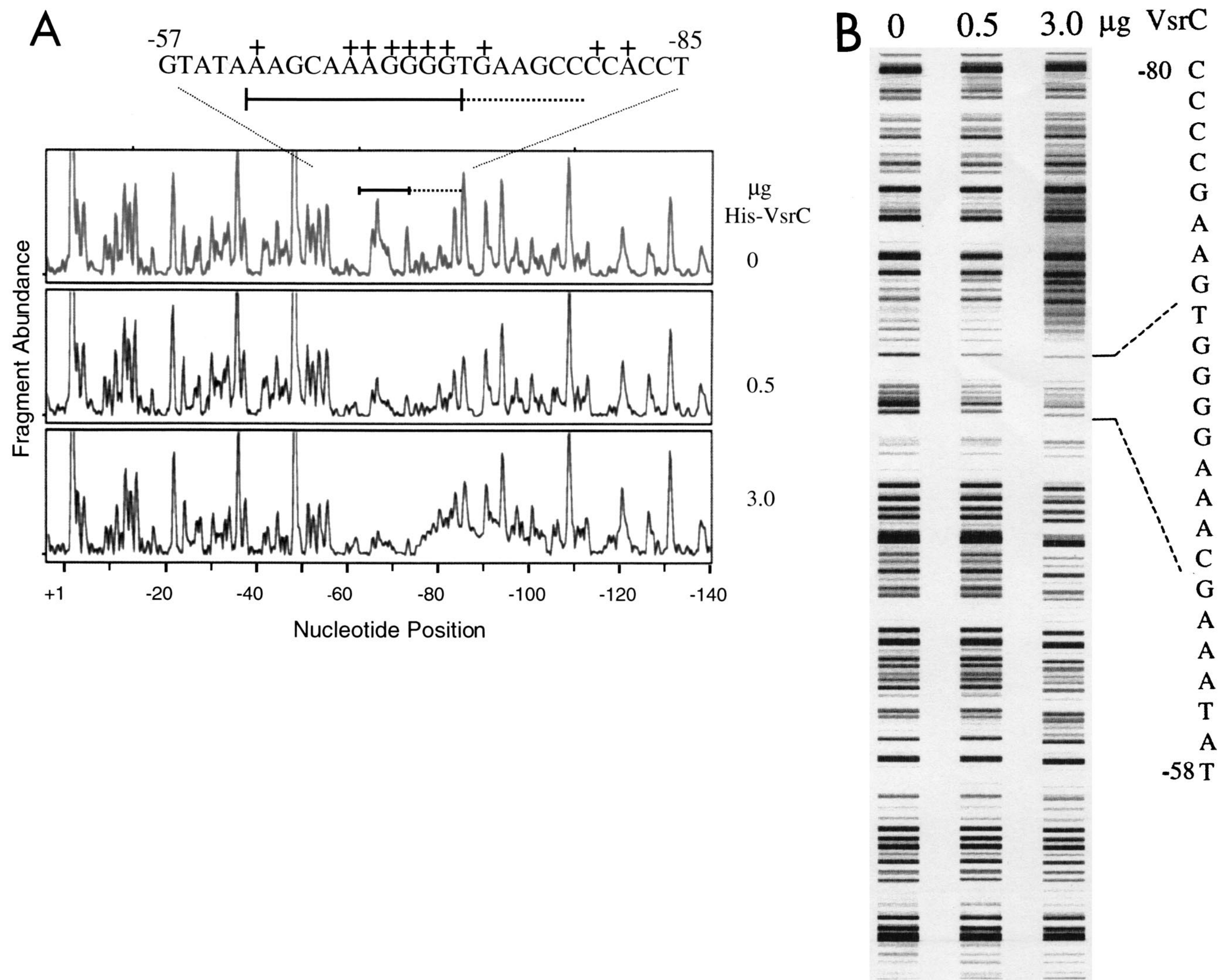

FIG. 3. DNase I footprinting analysis of VsrC binding to P_{eps}. Reactions were set up, processed, and analyzed using the ABI 310 as described in Materials and Methods and previously (46). (A) Electropherograms from reactions with increasing amounts of His-VsrC. The *y*-axis gives fluorescence intensity, which is proportional to fragment abundance; the *x*-axis gives elution position of fragments, which is proportional to their size. Bottom scale gives nucleotide position relative to the *eps* transcription start site (21) determined using the elution positions of internal size standards. Solid bar, upstream region of P_{eps} that is protected from DNase I digestion by His-VsrC. Dashed line, region made hypersensitive by VsrC. The DNA sequence of the protected region, marked with the same bars, is shown above with nucleotides identified by mutagenesis as critical for transcription activation (see Table 2) marked +. (B) "False gel image" representation of electropherograms in panel A generated by Genotyper 2.5 software, which converts the fluorescence intensity of peaks into proportional gray-scale bands. The sequence of the VsrC-protected region is given on the right.

tion. The position of this altered residue is unexpected and striking, since the analogous region in all other response regulators is outside of the helix-turn-helix DNA-binding domain and other regions implicated in their function (17).

In vitro analysis of VsrC binding to P_{eps}. Genetic data could not distinguish whether VsrC directly binds to and activates P_{eps} or works via an intermediate, so we investigated the ability of VsrC to bind to P_{eps} in vitro. First we constructed expression plasmids harboring either wild-type *vsrC* or the constitutively active *vsrC1* with their N termini translationally fused in frame to a hexahistidine encoding tag. Both His-tagged alleles were nearly as active as wild-type *vsrC*; when cloned on pRK415 and placed in an *R. solanacearum vsrC* mutant, both alleles increased *eps*::*lacZ* expression by >10-fold, to ca. 50% of wild-type levels (data not shown). After both His-tagged proteins were purified to >90% homogeneity, up to 20 µg of each was used in gel mobility shift assays with appropriate ^{32}P-labeled P_{eps} fragments. However, no consistent specific mobility shifting was detected under a variety of conditions (data not shown).

Next we tried a new, rapid footprinting analysis (46) to detect VsrC binding to P_{eps}. A fragment with the −337 to +23 region of P_{eps} that was fluorescently labeled at one 5′-end with 6-FAM was briefly incubated with His-VsrC and then DNase I. Fragmentation patterns were analyzed on an ABI 310 Genetic Analyzer. Run outputs, displayed as electropherograms or "false gel images" (Fig. 3), clearly show an upstream region of P_{eps} where the abundance of certain fragments decreases with increasing amounts of His-VsrC protein in the reaction. This is likely due to a VsrC-specific hindrance of the access of DNase I to this region (i.e., protection). Supporting this, incubation of the P_{eps} fragment with a control protein preparation or incu-

bation of purified His-VsrC with a promoter fragment of a gene not controlled by VsrC did not affect their DNase I fragmentation patterns (data not shown). We also observed that similar to other DNA binding proteins, incubation of the P_{eps} fragment with 3 μg of His-VsrC specifically caused increased DNase I cleavage (i.e., hypersensitivity) adjacent to the protected region (Fig. 3A). Footprinting reactions using the constitutively active His-VsrC1 protein gave essentially the same results (data not shown). From the size of the affected fragments, the positions of nucleotides specifically protected (bound) by VsrC were determined to be between −62 and −72, the same P_{eps} region that harbors many nucleotides important for transcription activation by VsrC and XpsR (Table 2). The hypersensitive region is upstream between nucleotides −76 and −90.

DISCUSSION

Transcription of *eps*, an operon encoding biosynthesis of the EPS I virulence factor of *R. solanacearum*, is controlled by a complex network composed of at least three interacting and environmentally responsive systems (41). We previously found that activation of transcription driven by P_{eps} sequences downstream of nucleotide −143 was completely dependent on *xpsR* and the *vsrB-vsrC* two-component system (21). Extending that work here, we showed that sequences downstream of nucleotide −243 are required and sufficient for wild-type P_{eps} expression and regulation. However, when P_{eps} sequences between −243 and −143 were deleted, expression was reduced threefold but remained fully dependent on *xpsR* and *vsrB-vsrC*. Thus, the −243 to −143 region enhances the transcription activation that is mediated by these regulators. The global virulence regulator of *R. solanacearum*, PhcA (5, 41), may play a role in this enhancement, because purified PhcA binds to and protects the −185 to −140 region of P_{eps} from DNase I digestion and because the enhanced activation that requires the −243 to −143 region is absent in *phcA* mutants (our unpublished data).

Further promoter deletion experiments clearly showed that nucleotides below −101 are absolutely critical for activation of P_{eps}. Subsequent mutagenesis studies (Table 2) revealed that many of the critical nucleotides lie between −82 and −62, in particular within the GTGGGGAA located between −74 and −67. Inactivation of either *vsrC* or *xpsR* did not further reduce expression from P_{eps} fragments with mutations in this region, suggesting that VsrC and/or XpsR may directly bind to this site to mediate transcription activation. Using DNase I footprinting we confirmed that VsrC does indeed directly bind to and protect the −74 to −67 region. Recent footprinting experiments (W. Yindeeyoungyeon and M. Schell, unpublished data) have identified another VsrC-protected binding site upstream of a new *eps* gene. The sequences of these two VsrC-protected sites show extensive similarity and suggest that VsrC may recognize a conserved palindromic consensus sequence found in both regions (TCCNC-N_8-GGGGA; Fig. 1). However, in contrast to similar footprinting experiments with another DNA-binding protein (46), a >100-fold excess of wild-type or constitutively active VsrC did not fully protect either site from DNase I, implying that the affinity of VsrC for these sites is relatively low. Perhaps XpsR (or another factor) is required for strong binding. In support of this hypothesis, we found that transcriptional activation by both wild-type and the constitutively active *vsrC1* allele still requires *xpsR*. Although enhancement of DNA binding by an auxiliary protein is not a common property of response regulators (17), another *R. solanacearum* response regulator, VsrD, also requires an auxiliary protein for its transcriptional regulation (22). Unfortunately, in vitro testing for the effect of XpsR on VsrC binding has not been possible due to the insolubility of our purified XpsR preparation. Alternatively, it is plausible that XpsR may be involved in the phosphorylation status of VsrC; however, the activity of VsrC1 in vivo and in vitro was essentially wild type regardless of the presence of the VsrB sensor kinase. While these data suggest that VsrC1 is fully active in the absence of phosphorylation, other possibilities remain. Site-directed mutagenesis of Asp-58 in VsrC's receiver domain, the presumed site of phosphorylation by VsrB, should better define the role of VsrB and XpsR in phosphorylation and/or activation of VsrC .

The position of the substitution in VsrC that conferred independence from VsrB (residue 209, 12 residues from the C terminus) is interesting and novel, but not surprising considering that *vsrC1* was the only bona fide VsrB-independent allele found in a population of >20,000 heavily mutagenized alleles. VsrC, a FixJ-type response regulator (17), is very similar to NarL, the crystal structure of which has been determined (2). Alignment of the C termini of VsrC and NarL (which are 80% similar) suggests that the S209L substitution is located in the middle of helix 10 which follows the helix-turn-helix DNA-binding domain. The region containing helix 10 has not been implicated in transcription activation by response regulators, nor is its amino acid sequence highly conserved. However, circumstantial evidence that the C terminus of a response regulator may interact with RNA polymerase has been reported: deletion of the last two or three residues of BvgA severely inhibited growth of *Bordetella pertussis*, but this effect was suppressed by mutations affecting the α-subunit of RNA polymerase (42). We have found that *vsrC1* also can cause growth inhibition in *R. solanacearum*. Since genetic and biochemical evidence suggests that interactions between transcriptional regulators and the α-subunit of RNA polymerase are sometimes important in transcriptional activation (35), it is plausible that the C-terminal region of some response regulators (e.g., helix 10) may interact with RNA polymerase to stimulate transcription. Site-directed mutagenesis and chemical cross-linking studies are required to further investigate this possibility.

In contrast, most mutations conferring sensor independence on response regulators are N terminal (e.g., V88L for NarL [10] and some N-terminal deletions [13]). These mutations are thought to mimic changes caused by sensor kinase-mediated phosphorylation; i.e., they cause a conformational change that alleviates occlusion of the C-terminal helix-turn-helix DNA-binding domain (9, 12). Similarly, the substitution at the C terminus of VsrC1 may also alter its conformation in a way that relieves or prevents inhibitory interactions of its helix-turn-helix DNA-binding domain with other parts of the polypeptide that block or reduce its function.

The role of EpsR in P_{eps} regulation and its relationship to *xpsR* and *vsrC* remain unclear, because we found that inactivation of *epsR* did not dramatically affect expression from P_{eps} or EPS I biosynthesis, whereas when present on a multicopy plasmid, *epsR* did reduce P_{eps} expression by greater than sevenfold. This inhibitory effect occurred only when the VsrC-binding site at P_{eps} was intact. Although indirect evidence for the binding of EpsR to P_{eps} has been reported (3), we were unable to confirm this. Preliminary analyses with *lacZ* reporters show that elevated levels of EpsR slightly (threefold) reduce expression of *xpsR* but not of *vsrC*. Although this implies that environmentally directed overproduction of EpsR could reduce levels of XpsR and hence shut down EPS production, further in vivo and in vitro studies are needed to clarify the

physiological role and mechanism of action of EpsR and XpsR with VsrC at the −82 to −62 region of P_{eps}.

ACKNOWLEDGMENTS

This research was supported in part by grant MCB 97-27921 from the National Science Foundation.

The authors thank Tim Hoover and Ellen Neidle for critical reading of the manuscript.

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