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A Complex Network Regulates Expression of *eps* and Other Virulence Genes of *Pseudomonas solanacearum*

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We have discovered an unusual and complex regulatory network used by the phytopathogen *Pseudomonas solanacearum* to control transcription of *eps*, which encodes for production of its primary virulence factor, the exopolysaccharide EPS I. The major modules of this network were shown to be three separate signal transduction systems: PhcA, a LysR-type transcriptional regulator, and dual two-component regulatory systems, VsrA/VsrD and VsrB/VsrC. Using *lacZ* fusions and RNA analysis, we found that both PhcA and VsrA/VsrD control transcription of another network component, *xpsR*, which in turn acts in conjunction with *vsrB*/*vsrC* to increase transcription of the *eps* promoter by >25-fold. Moreover, gel shift DNA binding assays showed that PhcA specifically binds to the *xpsR* promoter region. Thus, the unique XpsR protein interconnects the three signal transduction systems, forming a network for convergent control of EPS I in simultaneous response to multiple environmental inputs. In addition, we demonstrate that each individual signaling system of the network also acts independently to divergently regulate other unique sets of virulence factors. The purpose of this complex network may be to allow this phytopathogen to both coordinately or independently regulate diverse virulence factors in order to cope with the dynamic situations and conditions encountered during interactions with plants.

Pseudomonas solanacearum causes a lethal wilt disease of over 200 different plants (19). All virulent strains produce large amounts of an unusual extracellular polysaccharide (EPS) slime; the major portion of this slime is EPS I, a >1,000-kDa acidic, unbranched polymer of *N*-acetylgalactosamine, *N*-acetylgalactosaminuronic acid, and *N*-acetylbacillosamine decorated with 3-OH butyric acid (37, 46). Early in vitro studies (26) and recent in planta studies of EPS-deficient mutants (11, 29) suggest that at least one major function of EPS I is to cause wilting of infected plants, probably by blocking water flow in the xylem (12); EPS I, however, is not required for growth in planta. Production of EPS I requires the 18-kb *eps* gene cluster, which encodes several membrane-associated and soluble polypeptides involved in its biosynthesis and export (11, 22, 46). Synthesis of EPS I and O antigen of *P. solanacearum* also involves portions of the *ops* gene cluster, which may encode synthesis of a common sugar precursor (10, 29, 30).

P. solanacearum also produces many extracellular proteins (EXPs) that are likely or proven virulence factors; some are plant cell wall-degrading enzymes such as endoglucanase Egl (44), polygalacturonases PglA and PglB (47, 50), and pectin methylesterase Pme (53). Studies of mutants lacking Egl or PglA (12, 47) suggest that once inside the stem, *P. solanacearum* does not absolutely require these individual enzymes for wilting; more likely, they function in root invasion or acceleration of disease development. Mutants defective in export of most major EXPs outside of the cell (but not EPS) poorly infect plants via the roots and do not wilt or kill, even when large numbers are injected directly into the stem (28). Thus, although EPS I is the primary known virulence factor, some EXP or group of EXPs are also important for rapid wilting and killing.

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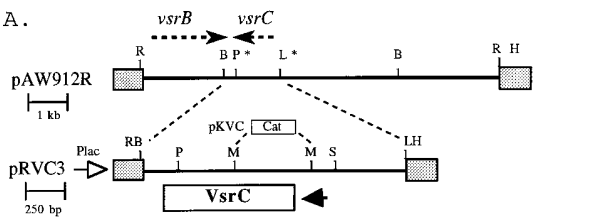
Previously we reported that *eps* is positively controlled by PhcA (5), a member of the LysR family of transcriptional regulators (45), and also by VsrA and VsrB (21, 48), two distinct sensors of the type found in the two-component regulatory family (39). Each of these regulators appears to additionally and differentially control production of some EXPs and other virulence factors in an independent fashion (6, 47). Here we report identification of three new *eps* regulatory genes: *vsrC* and *vsrD*, encoding distinct response regulators for VsrB and VsrA, respectively, and *xpsR*, encoding a unique basic protein. We also show that because *xpsR* transcription is controlled by both PhcA and VsrA/VsrD, and because XpsR is required by VsrB/VsrC for activation of *eps*, XpsR serves to link together three separate signal transduction systems into a complex virulence control network.

MATERIALS AND METHODS

Bacteria, plasmids, media. *Escherichia coli* strains used were DH5α (17), HB101(pRK2013) (14), and BL21 DE3 (57). *P. solanacearum* strains used were AW (wild type) (44), AW1-80 (*phcA80*::Tn*5*) (6), AW1-130 (*eps-130*::*lacZ*) (11), AW91 (*vsrB91*::Tn*phoA*), AW-MG2 (*vsrB2*::Ω) and AW1-130B (*eps-130*::*lacZ*/*vsrB91*::Tn*phoA*) (21), and AW120 (*vsrA120*::Tn*phoA*) and AW1-135 (*eps-130*::*lacZ*/*vsrA120*::Tn*phoA*) (48). Plasmid vectors used were pTZ18U or pTZ19U (34) and pRK415 (31). *P. solanacearum* and *E. coli* were grown at 30°C in B broth (4) containing 0.5% glucose (BG medium) and at 37°C in LB (35), respectively. Minimal medium contained BSM salts (50) and 1% sucrose. Antibiotics were used at 50 μg/ml for kanamycin, 100 μg/ml (20 μg/ml for *P. solanacearum*) for ampicillin, 50 μg/ml for spectinomycin, 25 μg/ml for tetracycline, and 40 μg/ml for chloramphenicol.

Plasmid constructions. Plasmids were constructed as follows: pVC3 by inserting the 1.4-kb *Bam*HI-*Sal*I fragment of pAW912R (21) into *Bam*HI-*Sal*I-digested pTZ18U; pVD211 by ligating the 1.4-kb *Fsp*I fragment of pHM20 (48) into *Sma*I-digested pTZ18U; pJH161 and pJH162 by cloning the 2.1-kb *Eco*RI-*Sty*I fragment of pQF44 (11) in both orientations into *Sma*I-digested pTZ19U; pOX1 by religating *Bsp*EI-*Xba*I-digested pJH161 under dilute conditions; and pJH163 by filling in cohesive ends of *Xho*I-digested pJH161 and religation. The *Eco*RI-*Hin*dIII fragments of pVC3, pVD211, pJH161, pJH162, pJH163, and pOX1 were recloned into *Eco*RI-*Hin*dIII-digested pRK415 to produce pRVC3 (Fig. 1A), pRVD211 (Fig. 3A), pRJH161, pRJH162, pRJH163, and pROX1 (Fig. 4A), respectively. The 1.2-kb *Nco*I-*Bam*HI fragment of pGA952 (5), which



B.

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                                                                    -35
1    CGCGCACAAGCAAAAACCCTGCCGCGTCATCAAAAACGGCCGCGCGGACGTTGGCGCGCCCTGCCGCGGTC
       -10          +1-->                SmaI
72   TGATAATCTCGACACCATCATAATGTTCCGCACACCCGGGGTCGGGTGCGCAGTCGGGCAGGCCGAGGCAC

143  GCATCGCCACCGCCGACACCGCGCTGCAACAACCTGCGGAACACGACCGTGAAGGCCGTCGGCATCAGGCC
                                 MluI                                RBS
214  CGACAGCCATCGCCCGCGCCGGGGCGGCACGCGTACAACAACAAGAGGGCTCTGCCTCACCGGGGAATAGA
     vsrC-->
285  ATG ACG AGC TCG CTG CGT AGC CTG CTG GTG GTC GAT GAC CAC CTC TCG CCT TAT
1     M   T   S   S   L   R   S   L   L   V   V   D   D   H   L   S   P   Y
                                                          #
339  CTG GCA CGA CCA CCT TTC TTG CGC ACG CCC TGC CTG AAA TCC AAG TCC ATG CGG
19    L   A   R   P   P   F   L   R   T   P   C   L   K   S   K   S   M   R

393  CCA TCG CCG GCG CAG GCC CTG GCG CTG ATC GAC GAG GGC ATC CGC CCG GAC GTC
37    P   S   P   A   Q   A   L   A   L   I   D   E   G   I   R   P   D   V

447  GTC TTG CTG GAT GTC TGG CTG TCC GAT TGC ACG GGT TTC GAC GCG ATG CGC GAA
55    V   L   L   D   V   W   L   S   D   C   T   G   F   D   A   M   R   E
                 #
500  CTG CGT GGC AAG CTG CCC GAG GCA CGC TTC GCC TTC ATG TCG GCC GAG AAC ACG
73    L   R   G   K   L   P   E   A   R   F   A   F   M   S   A   E   N   T

554  CCG GAG ATC GTC GCG AAG GCG CAG GCG CTG GGC GCC ATC GGG TTC ATC GGT AAA
91    P   E   I   V   A   K   A   Q   A   L   G   A   I   G   F   I   G   K
                 MluI                                                    #
608  CAT GCC GAC GCG CAC GCG TTC TCG CAG GCG GTG GCA GGC ATT TTC GGC GGA GCC
109   H   A   D   A   H   A   F   S   Q   A   V   A   G   I   F   G   G   A

662  TCG TCG TTC CCG TCG GAA GAC GAC CTG GGC ACG GCC AAC GGC AAG AGC GGC GCC
127   S   S   F   P   S   E   D   D   L   G   T   A   N   G   K   S   G   A

716  TCC CAC GGT ATC CCC ATC ACG CCG GCC GAG CTG GGC CTG ACG CCG CGT CAG GGT
145   S   H   G   I   P   I   T   P   A   E   L   G   L   T   P   R   Q   G
                                                    O       O
770  TCG GTG CTG GCG CTG CTG CTC GAA GGA CTG CCG AAC AAA TCC ATC GCA CGC CAG
163   S   V   L   A   L   L   L   E   G   L   P   N   K   S   I   A   R   Q
          O   O               O                       O   O  PstI
824  TTG GGC CTG ACG GAA AAC ACC GTC AAG GAA CAC GTC TCG GCG ATC CTG CAG CGG
181   L   G   L   T   E   N   T   V   K   E   H   V   S   A   I   L   Q   R
          O               O   O           O
878  CTG GGC GTG CGC ACG CGC ATC CAG ATC ATC TCG CGC ATG GAG CGC TTC CGC CTG
199   L   G   V   R   T   R   I   Q   I   I   S   R   M   E   R   F   R   L
          O   O
932  ACC GGC TCG GCC TGA CCCTGCCGCACACGACGCCGCGCTCTGCGGCGCCGTGCACAACAACGTCCG
217   T   G   S   A  STOP

998  TCGT TCA GTA GGC CAC TTG CGG CTC AGC GGC TCG GCC...<--vsrB
       STOP Y   A   V   Q   P   E   A   A   R   G
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FIG. 1. Mutational and sequence analysis of *vsrC*. (A) Physical and genetic maps of broad-host-range plasmids carrying *vsrC*. Dashed arrows represent transcripts. pKVC carrying the inactivated *vsrC* gene was constructed by replacing the 400-bp *Mlu*I fragment of pVC3 (containing the same insert as pRVC3 but on pTZ19U) with a *cat* (Cm^r) cartridge (41). The large box represents the *vsrC* ORF; the solid arrowhead shows its direction of transcription. Here and in other diagrams, the arrow labeled Plac shows transcription direction from the *lac* promoter of the pRK415 vector. Here and in Fig. 3A and 4A, the stippled box represents the vector polylinker showing only relevant restriction endonuclease cleavage sites: B, *Bam*HI; H, *Hin*dIII; L, *Sal*I; M, *Mlu*I; P, *Pst*I; R, *Eco*RI; S, *Sma*I; *, not unique site. (B) Nucleotide and predicted amino acid sequences of *vsrC*. The transcription start point (+1→; see Fig. 6B) and −35 and −10 consensus sequences of the promoter (18) are marked. RBS, possible ribosome-binding site. Conserved residues of receiver domains in response regulator proteins are marked (#). The LuxR/FixJ/MalT-type DNA-binding domain is double underlined, and its conserved residues are marked (○).

contains *phcA*, was cloned between the *Nco*I and *Bam*HI sites of the T7 expression vector pET3d (57) to generate pET3231.

pKVC (Fig. 1A) was constructed by inserting the 2.7-kb *Hin*cII fragment containing the *cat* gene (41) into *Mlu*I-digested pVC3. pD9 and pX10 (Fig. 3A) were constructed by inserting the 2-kb *Hin*dIII fragment containing Ω (42) into pHM20 partially digested with *Stu*I. pJH164 (Fig. 4A) was constructed by inserting the 0.9-kb *Sal*I fragment containing a derivative of *nptI* (15) into *Xho*I-digested pJH161. pJH165 was derived by mutagenesis of pJH162 with λ::Tn*5B20*(*lacZ*) (52). pRJH166 was derived by mutagenesis of pRJH162 with pHoHo and pSShe (54).

***P. solanacearum* strain constructions.** Regulatory mutants AW-C1 (*vsrC1*::*cat*), AW-D9 (*vsrD9*::Ω), AW-X10 (*orfX10*::Ω), AW-R164 (*xpsR164*::*nptI*), and AW-R165 (*xpsR165*::*lacZ*) were constructed by electroporation of wild-type AW with plasmids pKVC, pD9, pX10, pJH164, and pJH165 DNA, respectively. Since all of these plasmids are ColE1 based and hence unable to replicate in *P. solanacearum*, marker-exchanged recombinants are directly selected by plating on the appropriate antibiotic. Bona fide replacement of the wild-type genomic alleles was always confirmed by Southern blot analysis and complementation with a cloned wild-type gene in *trans* on pRK415. pRK415 derivatives were transferred from *E. coli* to *P. solanacearum* by mobilization with pRK2013 as described previously (50). Strains with the *eps-130*::*lacZ* reporter and a regulatory mutation(s) were constructed by transformation of strain AW1-130 or its derivatives with genomic DNA from the appropriate regulatory mutant by the procedure of Boucher et al. (4). A *phcA* mutation was introduced into strain AW-R165 as described by Brumbley and Denny (6); other derivatives of AW-R165 were constructed transforming it with genomic DNA.

In planta analysis. Virulence (i.e., rate of wilting and killing) or growth of *P. solanacearum* in planta was assessed three times by stem inoculation of at least four tomato plants with 10^4 cells of each strain as described previously (11).

Analysis of EPS I and EXPs. Culture supernatants from 48-h cultures were prepared, dialyzed, and analyzed as described previously (21), either for amount of EPS I by assaying for hexosamine content or for EXPs by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme assays for endoglucanase and polygalacturonases.

DNA sequence analyses. A modified dideoxy-chain termination protocol (51) with double-stranded plasmid templates, Sequenase 2.0 (U.S. Biochemical), [^{35}S]dATP, and deaza-dGTP was used. DNA sequences were analyzed with programs (13) in the Wisconsin Genetics Computer Group and Intelligenetic Group packages or FASTA (40).

Primer extension analysis. Total cellular RNA was isolated as described by Williams and Rogers (59). Primers (5′TTCGGTAATTGCCCTCCGGAT3′ for *xpsR*, 5′GTCATTCTATTCCCCGGTGA3′ for *vsrC*, and 5′GCGAATCATGCT GCTCCTTGT3′ for *vsrA*) were 5′ end labeled with [γ-^{32}P]ATP and T4 DNA polynucleotide kinase (33), and 200,000 cpm was then annealed with 50 μg of RNA in 20 μl of 50 mM Tris-HCl (pH 8.0)–100 mM KCl by heating at 90°C for 1 min, 60°C for 2 min, and then 4°C for 15 min. Hybrids were extended at 42°C for 50 min in 50 μl of 50 mM Tris-HCl (pH 8.0)–60 mM KCl–4 mM $MgCl_2$–0.5 mM deoxynucleoside triphosphates containing 6 U of RNasin and 10 U of avian myeloblastosis virus reverse transcriptase. Products were analyzed on 5% polyacrylamide sequencing gels (33).

Gel retardation analysis of DNA binding. *E. coli* BL21 DE3(pET3231) was grown to mid-log phase, and PhcA production was induced for 3 h with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested, and crude cell-free protein extracts were prepared by sonication and incubated with ca. 3,000 cpm of DNA fragments (labeled by filling in with Klenow enzyme and [α-^{32}P]dATP [33]). Binding was analyzed by electrophoresis as described previously (49).

Molecular genetic techniques. Cloning, transformation, fill-in of cohesive ends, electroporation, assay of β-galactosidase, and methods for DNA preparation and analysis were standard (21, 33).

Nucleotide sequence accession numbers. The sequences of *vsrC*, *vsrD*, and *xpsR* have been deposited in GenBank under accession numbers U18134, U18135, and U18136, respectively.

RESULTS

Identification and characterization of VsrC, a response regulator of *eps* and other virulence genes. *vsrB* encodes a two-component sensor that controls expression of multiple virulence factors of *P. solanacearum* (21). Since two-component sensors usually regulate by phosphorylating a closely linked response regulator (58), we sequenced regions flanking *vsrB*; downstream we found a 220-residue (24-kDa) open reading frame (ORF) designated VsrC (Fig. 1). Consistent with this, a plasmid containing a fragment with the ORF fused to the *lac* promoter specifically caused *E. coli* to overproduce a new 24-kDa polypeptide (not shown). The VsrC amino acid sequence has ca. 25% identity to members of the RO_{III} group of two-component response regulators, including NarL, FixJ, and BvgA (39). Moreover, VsrC has all of the characteristics expected for a response regulator (Fig. 1B): an N-terminal receiver domain and a C-terminal region similar to the DNA-binding domain found in the LuxR/FixJ/MalT regulator family (20, 27).

To confirm that VsrC is the partner response regulator of VsrB, we inactivated *vsrC* by replacing its 400-bp *Mlu*I fragment with a *cat* gene (pKVC; Fig. 1A) and then exchanged this mutant allele (*vsrC1*::*cat*) for the wild-type one in the genome of *P. solanacearum*. The resultant inactivation of *vsrC* in *P. solanacearum* caused (i) a dramatic reduction in virulence (i.e.,

no wilt symptoms seen in stem-inoculated tomato plants), (ii) a 30-fold reduction in EPS I production (Table 1), (iii) a 30-fold decrease in *eps* transcription (as measured with an *eps*::*lacZ* reporter gene; Table 1), (iv) reduced production of EXPs of 28 and 97 kDa (Fig. 2A), and (v) elevated production of the 52-kDa PglA polygalacturonase (Fig. 2A and enzyme assays not shown). In *trans*, the cloned *vsrC* gene on pRVC3 (Fig. 1A) fully restored *vsrC* mutants to wild type (Table 1 and data not shown). Since the changes caused by *vsrC* mutation are identical in nature and magnitude to those observed for the *vsrB* mutant AW91 (Table 1 and Fig. 2A), it is likely that VsrC is the partner response regulator of VsrB.

Identification and analysis of VsrD, a second response regulator controlling *eps* and different virulence genes. VsrA is another two-component sensor that also controls *eps*; it is physically and functionally very different from VsrB (48). Sequence analysis of the region downstream of *vsrA* (Fig. 3) revealed two ORFs, VsrD (210 residues) and OrfX (146 residues). The VsrD protein (24 kDa) was observed during maxicell analysis (not shown); OrfX was not (see below). In an end-to-end alignment with only two one-residue gaps, the sequence of the VsrD ORF is 40% identical to the sequence of GacA, a RO_{III}-type response regulator that controls production of several distinct antifungal agents by *Pseudomonas fluorescens* (32). VsrD also shows 25 to 39% sequence identity to other RO_{III}-type response regulators such as UhpA, BvgA, FixJ, and NarL (39) but only 20% identity to the VsrC ORF. Thus, while both VsrD and VsrC are apparently RO_{III}-type response regulators, they are not closely related.

To evaluate the function of *vsrD*, we inactivated it by inserting an Ω fragment into its *Stu*I site on pHM20 (D9; Fig. 3A) and exchanged this mutant allele (*vsrD9*::Ω) into the genome of *P. solanacearum*. The resultant *vsrD* mutant had a phenotype largely identical to that of *vsrA* mutants, as indicated by its (i) loss of virulence and reduced growth in planta (i.e., causing no disease symptoms and exhibiting 30-fold-reduced growth in stem-inoculated tomato plants), (ii) 20-fold-reduced *eps*::*lacZ* expression and EPS I production (Table 1), and (iii) altered EXP profile (Fig. 2B). EPS I production, *eps* transcription, and altered EXP profile of *vsrD* mutants were fully restored to wild-type levels by cloned *vsrD* on pRVD211 in *trans* (Table 1 and Fig. 2B). These results strongly suggest that VsrD is the partner response regulator of VsrA.

orfX (between *vsrA* and *vsrD*; Fig. 3B) appears to encode a polypeptide with amino acid sequence similarity to response regulators that contain only a receiver domain (e.g., CheY and SpoOF [39]). However, *orfX* lacks a significant ribosome-binding site and when placed in *E. coli* maxicells did not direct synthesis of any proteins of the size expected for OrfX (15 kDa; not shown). Moreover, insertional inactivation of *orfX* in *P. solanacearum* (X10; Fig. 3A) had no obvious affect on its phenotype (including virulence or production of EPS I and EXPs), indicating *orfX* is dispensable for expression of all known *vsrA*/*vsrD*-regulated genes.

***xpsR*, a nonallelic suppressor of *vsrA* or *vsrD* mutations, is the sixth regulatory gene required for *eps* expression.** While characterizing *vsrA*, we discovered that in *trans*, pQF44 (containing the 6.4-kb *Hin*dIII-*Eco*RI fragment from just downstream of *eps*; Fig. 4A) restored the wild-type, EPS^+ (mucoid) phenotype to the EPS-deficient *P. solanacearum vsrA* mutant AW120 but not to *vsrB* mutants. Quantitative measurements showed that pQF44 increased expression of *eps*::*lacZ* reporters and EPS I production by *vsrA* mutants over 15-fold to nearly wild-type levels (Table 2). While this result suggested that pQF44 complements the *vsrA* mutation, Southern blot analysis and later restriction mapping and characterization of *vsrA* (48) showed that *vsrA* was not on pQF44 or anywhere else in the 10-kb region downstream of *eps*. Thus, pQF44 contains a nonallelic suppressor of *vsrA* that we designated *xpsR*.

Subclones with the 2.1-kb *Eco*RI-*Sty*I fragment of pQF44 in either orientation (pRJH161 and pRJH162; Fig. 4A) also restored the EPS^+ phenotype to *vsrA* mutants. In *trans*, pRJH161 restored production of EPS I and expression of *eps*::*lacZ* reporters to *vsrA* mutants exactly like pQF44 (Table 2). Similarly, pRJH161 suppressed mutations in *vsrD* (Table 2), encoding the partner response regulator of VsrA, but not those in *vsrC* (not shown).

TABLE 1. Effects of inactivation of various regulatory genes on *eps* expression in *P. solanacearum*

Relevant genotype[a]	EPS I production (μg of polymeric hexosamine/mg of total cell protein)[b]	*eps*::*lacZ* expression[c]
Wild type	393	230
vsrC1::*cat*	<10	7
vsrC1::*cat*/*vsrC*$^+$	281	228
vsrB91::Tn*phoA*	<10	7
vsrA120::Tn*phoA*	<10	9
vsrD9::Ω	<10	13
vsrD9::Ω/*vsrD*$^+$	324	238
xpsR164::*nptI*	<10	8
xpsR164::*nptI*/*xpsR*$^+$	351	219
vsrC1::*cat xpsR164*::*nptI*	NT	8
vsrD9::Ω *xpsR164*::*nptI*	NT	7
vsrC1::*cat vsrD9*::Ω	NT	4

[a] *vsrC*$^+$ was carried on pRVC3, *vsrD*$^+$ was carried on pRVD211, and *xpsR*$^+$ was carried on pRJH162.

[b] Measured from 48-h-old dialyzed culture supernatants, using Erlich's reagent as described by Brumbley and Denny (6). NT, not tested.

[c] Expressed as β-galactosidase activity determined with cell extracts of overnight cultures of strains additionally carrying *eps-130*::Tn*3*HoHo1(*lacZ*) as described by Miller (35). Values are from at least three independent experiments with <20% variation.

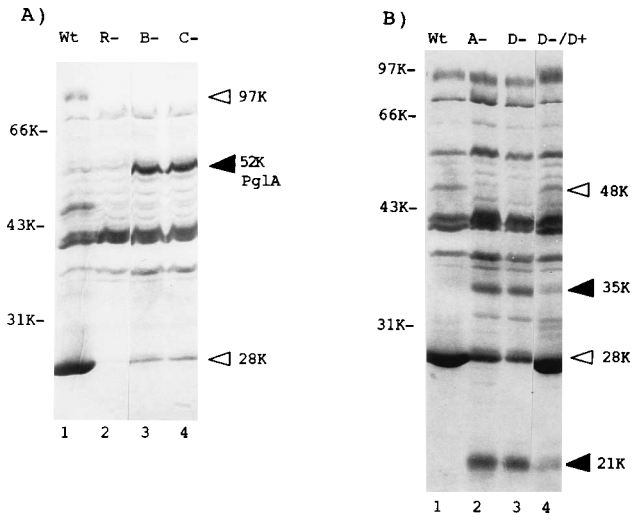
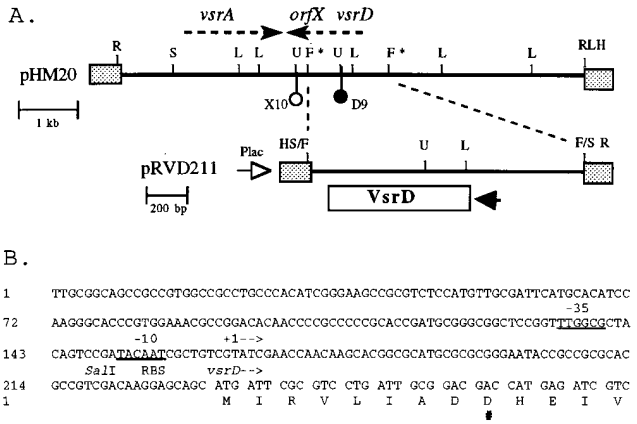


FIG. 2. EXP profiles of *P. solanacearum* mutants. One-milliliter culture supernatants were prepared and analyzed by SDS-PAGE and staining with Coomassie blue as described previously (21). Polypeptides whose production is decreased by each mutation are marked by open arrowheads with their sizes; those whose production is increased are marked by solid arrowheads. (A) Lanes: 1, AW (wild type); 2, AW-R164 (*xpsR*); 3, AW91 (*vsrB*); 4, AW-C3 (*vsrC*). (B) Lanes: 1, AW (wild type); 2, AW120 (*vsrA*); 3, AW-D9 (*vsrD*); 4, AW-D9(pRVD211) (*vsrD vsrD*$^+$).



B.

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                -10          +1-->
143   CAGTCCGATACAATCGCTGTCGTATCGAACCAACAAGCACGGCGCATGCGCGCGGGAATACCGCCGCGCAC
        SalI  RBS      vsrD-->
214   GCCGTCGACAAGGAGCAGC ATG ATT CGC GTC CTG ATT GCG GAC GAC CAT GAG ATC GTC
1                         M   I   R   V   L   I   A   D   D   H   E   I   V
                                                          #
272   CGC GCC GGT CTT CGG CAG TTC CTG TCG GAA GAG CGG GAC ATC GAG GTG GCC GGC
14     R   A   G   L   R   Q   F   L   S   E   E   R   D   I   E   V   A   G
                                                      StuI
326   GAG GCG GCC AGC GGG GAA GAG GTG ATG GAG CAA CTC CGC ACC GAG GCC TTC GAC
32     E   A   A   S   G   E   E   V   M   E   Q   L   R   T   E   A   F   D

380   GTG GTG GTG CTG GAC ATC TCC ATG CCC GAC CGC AAC GGC ATC GAC ACG CTC AAG
50     V   V   V   L   D   I   S   M   P   D   R   N   G   I   D   T   L   K
                  #
434   CTG GTA CGG CAG CGT CAC CCC GAC CTG CCG GTG CTG ATC CTG TCC ACC TTT CCG
68     L   V   R   Q   R   H   P   D   L   P   V   L   I   L   S   T   F   P

488   GAA GAC CAG TAC GCG ATC AAC CTG ATC CGG GCC GGC GCC TCG GGC TAC CTG ACC
86     E   D   Q   Y   A   I   N   L   I   R   A   G   A   S   G   Y   L   T

542   AAG GAA AGC GCG CCG GAC GAA CTC GTC AAG GCC ATC CGC ACC GTC TCG CAA GGC
104    K   E   S   A   P   D   E   L   V   K   A   I   R   T   V   S   Q   G
      #
596   CGC CGC TAT GTG AGC CCG ACG GTC GCC GAG CTG CTG ATC GGC GGG CTG GAG AAG
122    R   R   Y   V   S   P   T   V   A   E   L   L   I   G   G   L   E   K

650   CCG ACC GAC CAG CCG CTG CAC CAG ACC CTG TCC AAG CGC GAG TTC CAG ATC TTC
140    P   T   D   Q   P   L   H   Q   T   L   S   K   R   E   F   Q   I   F
                                          O   O       O   O
704   TGC AAG CTG GCG CGC GGC CAG TCT GTT TCG ATC ATC GCC GAG GAG CTG TTC CTG
158    C   K   L   A   R   G   Q   S   V   S   I   I   A   E   E   L   F   L
                        O                   O   O           O
758   AGC GTC AAG ACG GTC AGT ACG TAC CGC ACC CGC ATC CTG GAA AAG ATG GGC ATG
176    S   V   K   T   V   S   T   Y   R   T   R   I   L   E   K   M   G   M
      O           O   O                                   O
812   AAG TCG AAC GCC GAC CTG ACG TAT TAC GCC ATC AAG AAC GGA CTG GTG GAA TGA
194    K   S   N   A   D   L   T   Y   Y   A   I   K   N   G   L   V   E STOP

866   TGCCGCAGCGGCCGGACAGC GGGGTCCCGATACCCGGCAT CGGCCGCAAACCCTGTTGGC ATGTAGGA
                                                  orfX-->
934   CAACATCCGATA GCACCCGCTAAACCCGCTGG CTACCATCAACGC ATG CTG GAT CAA GCC GTC
1                                                   M   L   D   Q   A   V

997   ACC CTC TCC ACC CGT GGC TTG CGC GTC CTA TTG ATC GAG GAT TCC CCC GTG CTG
7      T   L   S   T   R   G   L   R   V   L   L   I   E   D   S   P   V   L
                                          StuI                  #
1051  CGC AGC ATG GTG CTG GAG TAC CTC AAG GCC TCC GCC TTC GTG GCG GTG GTG GAA
25     R   S   M   V   L   E   Y   L   K   A   S   A   F   V   A   V   V   E

1105  TGG GCA GAC ACG GAA GAC CTG GCA CTG CGC CTG TTG GCG CAA GGC CCG TAC GAC
43     W   A   D   T   E   D   L   A   L   R   L   L   A   Q   G   P   Y   D

1159  GTC GTC ATC GTG GAC CTG CAA CTG CGC CAG GGA AAC GGC TTC AAG GTG CTG CAG
61     V   V   I   V   D   L   Q   L   R   Q   G   N   G   F   K   V   L   Q
                  #
1213  TCG CTG CGC GAC CAG GCA TCG CCG AGC GTG CGC ATC GTC TAT ACC AAC CAC GCT
79     S   L   R   D   Q   A   S   P   S   V   R   I   V   Y   T   N   H   A

1267  CAG GTC CCG ACC TAC CGG CAA CGC TGC TTC GAA GCC GGT GCC AAC TAC TTC TTC
97     Q   V   P   T   Y   R   Q   R   C   F   E   A   G   A   N   Y   F   F

1321  GAC AAA TCG CTT GAG CTC GAC AAG GTG TTC GAG GTC ATC GAA GAG CGC GCG GGC
115    D   K   S   L   E   L   D   K   V   F   E   V   I   E   E   R   A   G
      #
1375  ATG GTC CGC CCG CGG CCC CAG GCA GCC CAC CAC CCG CAC CAC TGA GCGGCACGGGC
133    M   V   R   P   R   P   Q   A   A   H   H   P   H   H   STOP

1431  ATCGCCCCGGACGGCACCCCCATCCCTCATCCCTTTTCCCCACGACCGAGGAGTCTGCGATGAAAACCCTG

1502  TGTGCCGTCTTCGCCCTGTCCTGCATGCTTCTGGCCGGCTGCAACACCATGGCCGGCGCCGGCAAGGACAT

1573  CCAGAACGGCGGCCAGAAACTGGAAAACGCGGCCGACAACGCCAAACAGAAGATGTAAGCGGCCTCGGGCG

1644  GGTACGACCGACGAGCCCCGCCACGGGGCTTTTTGCTTTTTATCGGGCGACGTGCA     TCA GAC CAG
                                                                  STOP V   L

1709  CGC GTT GCC CTT GCC CGC CAC GGG CAT GTC TCC CGC...<--vsrA
       A   N   G   K   G   A   V   P   M   D   G   A
```

FIG. 3. Mutational and sequence analysis of *vsrD*. (A) Physical and genetic maps of plasmids containing *vsrD*, *orfX*, or mutant alleles. Dashed arrows represent transcripts. The solid circle D9 and the open circle X10 show the location of Ω fragments (42) insertion in *vsrD* and *orfX*, respectively. The large box represents the *vsrD* ORF; the solid arrowhead indicates its transcription direction. The arrow labeled Plac and restriction site abbreviations are as in Fig. 1A and additionally F (*Fsp*I) and U (*Stu*I). pHM20 uses a pTZ18U vector. pVD211 contains the same insert as pRVD211 but on pTZ19U vector. (B) Nucleotide and predicted amino acid sequences of *vsrD* and *orfX*. The transcription start point (+1→; see Fig. 6C) and −35 and −10 consensus sequences of the promoter are indicated. RBS, possible ribosome-binding site. Conserved residues of receiver domains of response regulators are marked (#). The LuxR/FixJ/MalT-type DNA-binding domain is double underlined, and its conserved residues are marked (○).

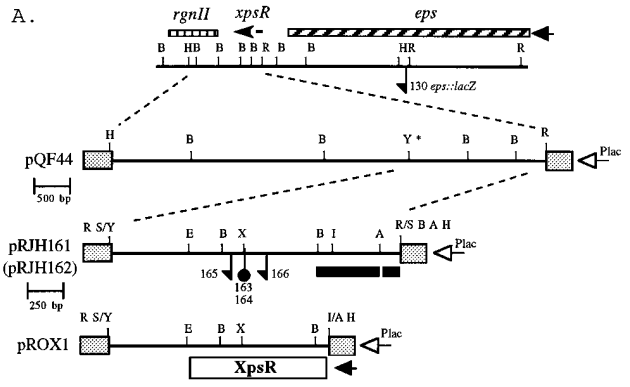
Since cloned *xpsR* suppresses mutations in either *vsrA* or *vsrD*, it likely encodes a new regulator of *eps* that acts below the level of *vsrA* or *vsrD*. To test this, we inactivated *xpsR* by inserting a nonpolar *nptI* cartridge (15) into its unique *Xho*I site and exchanged this mutant allele (*xpsR164*::*nptI*; Fig. 4A) into the genome of *P. solanacearum*. Inactivation of *xpsR* caused a dramatic reduction in virulence, *eps*::*lacZ* expression, and production of EPS I and EXPs of 28 and 97 kDa (Table 1 and Fig. 2A). Cloned *xpsR* on either pRJH162 or pRJH161 (Fig. 4A) restored all of these mutant traits to wild-type levels (Table 1 and data not shown), but cloned *vsrA* or *vsrD* or overexpression of *vsrC* did not (not shown). *xpsR* mutants clearly differed from *vsrB* or *vsrC* mutants in that they did not overproduce the 52-kDa PglA and differed from *vsrA* or *vsrD* mutants in EXP profile (Fig. 2 and enzyme assays not shown) and symptom production in tomato plants.

While inactivation of *xpsR* lowered *eps*::*lacZ* expression by 20-fold, additional inactivation of either *vsrC* or *vsrD* in *xpsR* mutants did not further reduce *eps* expression (Table 1). This and the observation that *vsrC vsrD* double mutants are reduced in *eps* expression almost to the same extent as single mutants (Table 1) implies that none of these regulators act independently on *eps*.

Molecular analysis of *xpsR*. The DNA sequence of the region containing the *xpsR* mutation was determined, and a 307-residue (35-kDa) ORF was identified as XpsR (Fig. 4B). This was confirmed by maxicell analysis since pJH161 containing the ORF directed synthesis of a 33-kDa polypeptide, whereas pJH163, an identical plasmid except for a 4-bp insertion at the unique *Xho*I site within the ORF, or pTZ19U vector did not (Fig. 5 and data not shown). Furthermore, when the insert with the 4-bp insertion mutation in the XpsR ORF was recloned onto pRK415 and placed in *trans* in *P. solanacearum* mutants, it no longer complemented *xpsR* mutants or suppressed *vsrA* or *vsrD* mutants. The predicted amino acid sequence of XpsR (Fig. 4B) did not show significant homology to any ORF in GenBank, nor did any other of the possible translation products of the *xpsR* sequence. Thus, XpsR may be a new type of regulatory protein. The putative XpsR protein is very basic (pI 10.6) because of two regions that are rich in basic amino acids (Fig. 4B).

Both *phcA* and *vsrA*/*vsrD* control *eps* via *xpsR*. Previous studies (5, 6, 47) showed that the LysR-type transcriptional regulatory PhcA controls production of EPS and many other known and potential virulence determinants (e.g., Egl, PglA, and Pme). Consistent with this, inactivation of *phcA* caused a 50-fold reduction in *eps* transcription as measured with an *eps*::*lacZ* reporter (Table 3). Placing the *xpsR*-containing plasmid pROX1 (Fig. 4A) in a *phcA* mutant largely suppressed this lowered *eps* expression (Table 3) but did not affect the coordinately reduced levels of EPS I, Egl, or Pme (not shown). Thus, cloned *xpsR* on pROX1 apparently suppressed only the effect of a *phcA* mutation on *eps* transcription, but not mucoidy, indicating that EPS I production requires other loci independently controlled by PhcA but not by *xpsR*.

On pROX1, transcription of *xpsR* is high, since its coding region beginning at the ribosome-binding site (*Bsp*EI site; Fig. 4B) is fused to the *lac* promoter of the pRK415 vector, which is constitutive and strong in *P. solanacearum* (24). In contrast, pRJH162 (on which *xpsR* transcription is directed by its own



B.

```
                                                    XbaI
1     GAATTATTGACGCAATCCTTAGGTCTAGACTGTTTTTGGGTATCGGATGGGACTTTTGTGTGATATGGGCG
                                                                  000-------000
72    CCGTCCGGGAGGGCAGGGGCCGGCTGTGCGACATTTACAAAGCTGGCGTGATGTTAAAAAATCTTTACTCT
                                                -35          -10          +1-->
143   CCTTTAGTGGTATTTCAGGCAAGCATTGGTGAAAGGGGTCTGGCAAACGGGCATCATGTGCCCTATCTGTG

214   TAGTGTCCTTTGTTGTTGCACGGATTCAATAATGATATTTCGGGGGATTTTGTGGAGATTTTAGCGATGCT
                                                                  BspEI
285   TGTTGGGCGCATCGCTGGCTGGGCTGTGAACAGGCGCGCGCGCCTGGATGCGGCCGGCATTCAAAATCCGG
      RBS
356   AGGGCAATTACCGAA ATG GAA CAG AAG CTC ATC TTT TCG CGC GAA CAA TTG GAC AGA
1                     M  E  Q  K  L  I  F  S  R  E  Q  L  D  R

413   TAC TTC GCT TCT TAT GTC GGC ATG GAA GGG GGC AAC CCG GCG GGC GCC GTC TGG
15     Y  F  A  S  Y  V  G  M  E  G  G  N  P  A  G  A  V  W
                  BamHI
467   TTC TGC GAC AAG GAT CCG CAC CCC TGG ACG GAA TCG CTG GTG GCG CCG CTG GGG
33     F  C  D  K  D  P  H  P  W  T  E  S  L  V  A  P  L  G

521   CCG CGT GAG CAG CCC AAC GCC TGG GGC AAG GTC TTT CGT GCA CGC CAC CGC GAC
51     P  R  E  Q  P  N  A  W  G  K  V  F  R  A  R  H  R  D

575   AGC ATG GAG CGA TGG CAG TCG CAC CAG AAG ATC GCA AGG ATC ATG GCG GCC GCA
69     S  M  E  R  W  Q  S  H  Q  K  I  A  R  I  M  A  A  A

629   CGC GCC GAG ACC CAT CGG CGG CCG CAG AGC GAG TGC GAT TGG AAG CAT TAC TTC
87     R  A  E  T  H  R  R  P  Q  S  E  C  D  W  K  H  Y  F

683   GCG GAT CTG CTC TAC GGG CCG AAC GGC GCC GAG TTC AAG CTC AGC CTG TTT CCG
105    A  D  L  L  Y  A  P  N  G  A  E  F  K  L  S  L  F  P

737   CTG CCT GCA CAG CAG ATC GGC GAC ACG CCG TGG TCG CGG GCA TTT CGC GGA CAG
123    L  P  A  Q  Q  I  G  D  T  P  W  S  R  A  F  R  G  Q

791   CCC GCG CTG GTT CCG AAG CAG CGC TAC GTT GAT CTG TGC CGG ACC GGC AGC CGG
141    P  A  L  V  P  K  Q  R  Y  V  D  L  C  R  T  G  S  R

845   TTC CGT TTC ATC TCG AAG ATT CGC GAG CGC TGG CGG CCC AAG ATC GTG TTG TGC
159    F  R  F  I  S  K  I  R  E  R  W  R  P  K  I  V  L  C

899   TTC GGC GAG CGG CAC ACG GAT GAC TAC GTG CAC GCC TTC GGC CTG CAG ATG GCC
177    F  G  E  R  H  T  D  D  Y  V  H  A  F  G  L  Q  M  A

953   TCG ACC CAC GAG TTC ATC CTG CAG CCG GCC GAT CTC GCC AAG ACG CTG CAG GTG
195    S  T  H  E  F  I  L  Q  P  A  D  L  A  K  T  L  Q  V
          XhoI
1007  CTC GAG CAC GAC GGT ACC ACC TGG ATC ATC TGT CCG CCG CTG GCG GGC GCC TCG
213    L  E  H  D  G  T  T  W  I  I  C  P  P  L  A  G  A  S

1061  GGG TTG ACC TCC GAC GTG CTG CTC GAA GCG ATG GGG CGC TAC ATC TCG CGA TGG
231    G  L  T  S  D  V  L  L  E  A  M  G  R  Y  I  S  R  W

1115  CTG GTG CCC GCT GAT TTT CCC CGG CTG CAG GAT GGC GCC GTG GTT CGC AGC CTG
249    L  V  P  A  D  F  P  R  L  Q  D  G  A  V  V  R  S  L
                        BamHI
1169  GTG CAG GAG CGG GGG GCG GAT CCG TTC GGC GAC GGC CGG GAG ACC CGG GCA ACG
267    V  Q  E  R  G  A  D  P  F  G  D  G  R  E  T  R  A  T

1223  TAT CCG GCA ACC GGA CGC GTA CCG TTT CCG CCG CCG GGC AGG GAC GAC ATC GCA
285    Y  P  A  T  G  R  V  P  F  P  P  P  G  R  D  D  I  A

1277  GGG CGG GTT GCC TAC TAA TTCAACAGGTTCAACAGGATGCCGGCACGCTCCCGGGTATGCCGCGA
303    G  R  V  A  Y  STOP
             BstEII
1342  TAGCGGCTGCCCGGGTCACCGGGTATGGGTGGCGCAGCGTGGTGGAAAGGGCGTGGCGAAAAGCCGCAGTG
```

FIG. 4. Mutational and sequence analysis of *xpsR*. (A) Physical and genetic maps of genomic region and plasmids containing *xpsR* or its mutant alleles. The upper portion shows the segment of the *P. solanacearum* genome containing region II (*rgnII*), *eps*, (hatched boxes [11, 46]), and *xpsR*; the dashed arrow represents the *xpsR* transcript. Flag 130 shows the position and transcription direction of genomic Tn*3*-HoHo1(*lacZ*) reporter in *eps* in strain AW1-130 (11); solid circle 164 or 163 shows the position of the *nptI* cartridge (15) insertion or site of frameshift mutation in *xpsR*; flags 165 and 166 show positions and transcription direction of Tn*5B20*(*lacZ*) and Tn3-HoHo1(*lacZ*) reporters in *xpsR* on pJH165 and pRJH166, respectively. Solid bars below pRJH161 indicate fragments used in the gel retardation assay. The large box represents the *xpsR* ORF; the solid arrowhead shows its transcription direction. Arrows labeled Plac and restriction site abbreviations are as in Fig. 1A and additionally A (*Xba*I), E (*Bst*EII), I (*Bsp*EI), X (*Xho*I), and Y (*Sty*I). pRJH162 contains the same insert as pRJH161 but in the opposite orientation. pJH161 and pJH162 also contain the same insert but on a TZ19U vector instead of pRK415. (B) Nucleotide and predicted amino acid sequences of *xpsR*. The transcription start point (+1→) (see Fig. 6A) and putative −35 and −10 sequences of the promoter are marked.

promoter, because it is in the opposite orientation relative to the vector promoter; Fig. 4A) did not suppress the effect of *phcA* mutation on *eps* expression. Thus, in *phcA* mutants, suppression of reduced *eps* transcription by *xpsR* apparently requires constitutive, high-level expression. However, the lower levels of XpsR produced from pRJH162 were sufficient to cause a major suppressive effect on the lowered *eps*::*lacZ* expression caused by either a *vsrD* or *vsrA* mutation (Table 2). *phcA vsrD* double mutants showed the same levels of reduced *eps* expression as a *phcA* mutant and were restored to near wild-type levels by pROX1 but not pRJH162, indicating that neither *phcA* or *vsrD* is essential for *eps* transcription when *xpsR* is constitutively expressed (Table 3 and data not shown).

One explanation for the suppressive effect of *xpsR* on *phcA* would be that PhcA positively controls expression of *xpsR*. To test this, we inserted a transcriptional *lacZ* reporter into *xpsR* on pJH162 and exchanged the reporter allele into a wild-type genome (AW-R165; Fig. 4A). When a *phcA* mutation was also introduced into this genome, expression of *xpsR*::*lacZ* was decreased 40-fold (Table 4), suggesting that *phcA* is required for positive control and high-level transcription of *xpsR*.

Since cloned *xpsR* also suppressed the effect of *vsrD* mutations on *eps*::*lacZ* expression, we tested if VsrD also controls expression of *xpsR*. A *vsrD* mutation caused a 5-fold reduction in expression of the genomic *xpsR*::*lacZ* reporter (Table 4) or of a plasmid-borne one on pRJH166 (not shown), much less than the 40-fold reduction caused by *phcA* mutations. However, this is consistent with the above observation that a moderate increase in *xpsR* expression is sufficient to largely suppress the effect of *vsrA* or *vsrD* mutations on *eps*, whereas a much higher level of XpsR is required to similarly suppress the effect of *phcA* mutation on *eps*. Inactivation of *vsrB* or *vsrC* did not affect *xpsR*::*lacZ* expression. Thus, it appears that both *vsrD* and *phcA* exert control over *eps* transcription indirectly by transcriptionally controlling *xpsR*. However, since inactivation of *vsrD* in a *phcA* background did not further reduce the expression of *xpsR*::*lacZ* (Table 4), *phcA* is epistatic to *vsrD* for *xpsR* regulation.

Transcriptional control of *xpsR* by *phcA* was confirmed by primer extension analysis. After annealing of purified *P. solanacearum* RNA with an *xpsR*-specific primer and extension with reverse transcriptase, two major cDNAs were detected with RNA from the wild type, but none were detected with RNA from *phcA* mutants (Fig. 6A). Thus, while all detectable *xpsR* transcription clearly requires active PhcA, it may begin at two different sites (nucleotides 208 and 219; Fig. 4B). Possible *E. coli* σ^{70} −35 and −10 promoter consensus sequences are correctly positioned upstream of nucleotide 208 but not upstream of nucleotide 219 (Fig. 4B), suggesting that nucleotide 208 is a bona fide transcription start site of *xpsR* and that the apparent additional start site at 219 may be an RNA processing site or termination site for reverse transcriptase. Analogous primer extensions showed that *vsrC* and *vsrD* are transcribed independently of *phcA*, since levels of *vsrC*- or *vsrD*-specific cDNA detected after extension of RNA from the wild type and *phcA* mutants were equivalent (Fig. 6B and C).

PhcA specifically binds to the *xpsR* promoter region. If PhcA directly activates transcription of *xpsR*, then it would be expected to bind to the *xpsR* promoter. As with other LysR-type regulators (45), PhcA binding should be detectable by a gel

RBS, possible ribosome-binding site. Sequence with characteristics consistent with consensus binding site of LysR-type regulators (45) is marked (000– – – 000) near −75. Regions rich in basic amino acids are double underlined.

TABLE 2. *xpsR* suppresses the effects of mutations in *vsrA* or *vsrD* on *eps*

Relevant genotype[a]	EPS I production[b]	*eps*::*lacZ* expression[b]
Wild type	393	230
vsrA120::Tn*phoA*		
Alone	<10	9
+pQF44 (*xpsR*$^+$)	165	130
+pRJH161 (*xpsR*$^+$)	181	145
+pRJH162 (*xpsR*$^+$)	96	85
vsrD9::Ω		
Alone	<10	13
+pRJH161 (*xpsR*$^+$)	142	143

[a] On plasmids pQF44 and pRJH161, *xpsR* expression is driven by its own promoter and the strong *lac* promoter of pRK415 vector; on pRJH162, it is expressed only from its own promoter.
[b] Expressed as in Table 1.

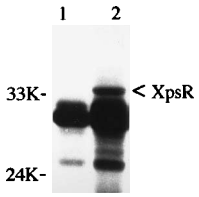


FIG. 5. Maxicell analysis of *xpsR*. *E. coli* SK6501 maxicells (1) containing pTZ19U vector (lane 1) or pJH161(*xpsR*$^+$) (lane 2) were UV irradiated, recovered, labeled with [^{35}S]methionine, and analyzed by SDS-PAGE and fluorography as described previously (24). Positions of migration of molecular weight standards are shown at the left.

mobility retardation assay. Therefore, a crude protein extract (20% PhcA as estimated from SDS-PAGE) of *E. coli* harboring the *phcA*-overexpressing plasmid pET3231 was preincubated with a ^{32}P-labeled 610-bp fragment containing the *xpsR* promoter (Fig. 4A) and electrophoresed. Migration of all of the labeled 610-bp fragment was retarded after preincubation with 3 μg of crude extract from *E. coli*(pET3231), whereas none was retarded after preincubation with extracts from *E. coli* harboring the pET3d vector lacking *phcA* (Fig. 7). To better localize the *xpsR* DNA sequences responsible for the specific binding of PhcA, the labeled 610-bp fragment was cleaved into 160- and 450-bp fragments before use as a binding substrate. The majority of the 450-bp fragment containing the *xpsR* promoter (positions −177 to +274), but none of the 160-bp fragment lacking it, was specifically retarded by preincubation with 3 μg of the PhcA-containing extract (Fig. 7). These results suggest that PhcA directly and specifically binds to sequences near the *xpsR* promoter, possibly at the 13-bp sequence near −75 (Fig. 4B), which is consistent with the sequence organization and position proposed as a consensus for the binding sites of LysR-type regulators (45).

DISCUSSION

Previous studies (6, 21, 48) identified several genes (*phcA*, *vsrA*, and *vsrB*) that differentially affected production of EPS I and other virulence factors of *P. solanacearum*, but they did not investigate interactions between them. As illustrated in the model in Fig. 8 and discussed below, our new studies show that these genes, in combination with two new ones (*vsrD* and *vsrC*), comprise three separate signal transduction systems that are linked into a complex regulatory network by the newly discovered and unusual *xpsR* gene product. These data also imply that the network modulates transcription from the *eps* promoter (and likely others) in simultaneous response to multiple signals.

The network contains at least three distinct signal transduction systems. Using mutational and DNA sequence analysis, we found that *vsrC* and *vsrD* encode the partner response regulators of the two-component sensors VsrB and VsrA, respectively, although in vitro phosphorylation studies are required for absolute confirmation. By analogy to other two-component systems (39, 56), the VsrA and VsrB sensors should respond to environmental stimuli by phosphorylating their partner response regulator, which in turn activates (or represses) transcription of the proper target genes. The VsrA/VsrD and VsrB/VsrC two-component systems are clearly separate and likely respond to different stimuli. This conclusion is based on the observations that (i) the predicted amino acid sequences of VsrD and VsrC show only low similarity; (ii) the amino acid sequences, sizes, and numbers of periplasmic domains, and other structural features of sensors VsrA and VsrB, also are very different (21, 48); and (iii) although inactivation of either system reduces *eps* transcription (Table 1), other aspects (e.g., EXP profiles and in planta growth) of the phenotypes of their mutants differ, indicating that they control only partially overlapping sets of targets. Only two other dual two-component systems with common target genes have ever been clearly documented (NarX/NarL-NarQ/NarP [55] and ComP/ComA-DegS/DegU [36]). The Nar system differs from the Vsr system because the amino acid sequences of its two response regulators (and sensors) are very similar to one another and because either response regulator can communicate with either sensor. The superficial similarities between the Vsr system and the Com-Deg system remain to be clarified.

PhcA (5) is a member of the large, diverse LysR family of prokaryotic regulators (45), nearly all of which activate tran-

TABLE 3. Vector-directed expression of *xpsR* suppresses the effect of a *phcA* mutation on *eps*

Relevant genotype[a]	*eps*::*lacZ* expression[b]
Wild type	230
phcA80::Tn*5*	
Alone	3
+pRJH162 (*xpsR*$^+$)	5
+pROX1 (*xpsR*$^+$)	94
phcA80::Tn*5 vsrD9*::Ω	
Alone	3
+pROX1 (*xpsR*$^+$)	93

[a] On pROX1, *xpsR* is expressed from the *lac* promoter on pRK415; on pRJH162, it is expressed from its own promoter.
[b] Expressed as in Table 1.

TABLE 4. Regulation of *xpsR* by *phcA* and *vsrD*

Relevant genotype[a]	*xpsR*::*lacZ* expression[b]
Wild type	1,035
phcA	24
vsrD9::Ω	221
phcA vsrD9::Ω	22
vsrB2::Ω	1,038
vsrC1::*cat*	1,038

[a] *phcA* is a spontaneous mutation obtained as described by Brumbley and Denny (6).
[b] Expressed as β-galactosidase activity in cell extracts of overnight cultures of strains of the indicated genotypes additionally carrying *xpsR165*::Tn*5B20*(*lacZ*).

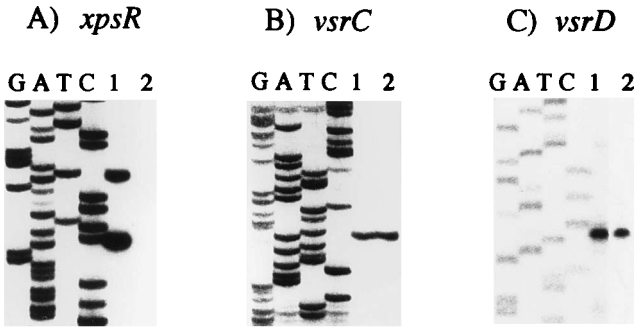


FIG. 6. Primer extension analysis of transcription of regulatory genes. Total cellular RNA purified from the wild type (lane 1) and *phcA* mutants (lane 2) was annealed with gene-specific labeled primers and extended with reverse transcriptase, and the products were analyzed by electrophoresis and autoradiography along with a dideoxy-chain termination sequence ladder (G-A-T-C) generated with the same primers.

scription in response to small signal molecules. Thus, *phcA* transcription activation also likely requires a signal molecule. This signal may relate to cell density, because transcription rates of all PhcA-regulated genes tested (*eps*, *egl*, and *xpsR*) increase 50-fold during growth from 10^7 to 10^9 cells per ml (8). While other data implicate fatty acid methyl esters as possible signal molecules for *phcA*-regulated genes (9), the precise nature of how signals are perceived by *phcA* (as well as VsrA and VsrB) remains to be defined.

XpsR interconnects several signal transduction systems to bring about convergent control of *eps* transcription. The most interesting and novel part of the network is XpsR, a very basic protein with no known homologs. On the basis of the following observations we have concluded that XpsR mediates indirect control of the *eps* promoter by both the VsrA/VsrD and PhcA signal transduction systems: (i) the reduced *eps* transcription caused by inactivation of either PhcA or VsrA/VsrD was completely abolished by constitutively expressed *xpsR* (Table 3), (ii) analysis of *xpsR* transcription by using primer extension and

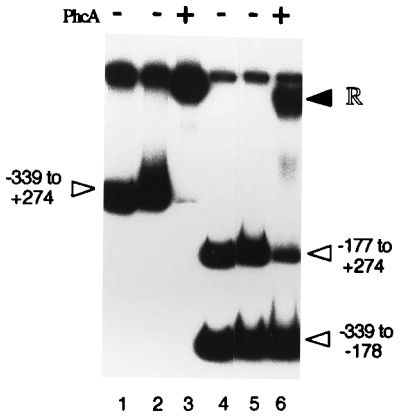


FIG. 7. Gel retardation assay for binding of PhcA to *xpsR*. Crude cell-free protein extracts [lanes 1 and 4, no protein extract; lanes 2 and 5, 3 μg of extract protein from *E. coli*(pET3d); lanes 3 and 6, 3 μg of extract protein from *E. coli*(pET3231)] were prepared and incubated with ^{32}P-labeled DNA fragments containing the *xpsR* promoter, electrophoresed on a 5% polyacrylamide gel, and autoradiographed. pET3231 contains *phcA* with its translation initiation codon fused in frame to the *Nco*I site of the T7 expression vector pET3d (57). Labeled DNA fragments: lanes 1 to 3, 610-bp *Eco*RI-*Bam*HI fragment of pRJH161 (Fig. 4) containing positions −339 to +274 of *xpsR* (+1 = transcription start); lanes 4 to 6, the same fragment cut at −177 with *Xba*I, giving fragments of 450 bp (−177 to +274) and 160 bp (−339 to −178). R, position of retarded fragment.

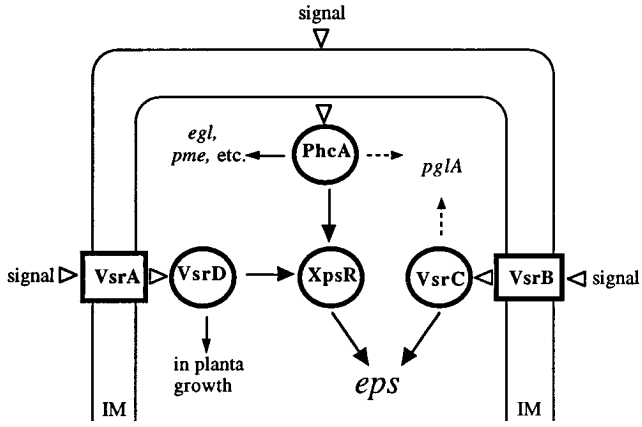


FIG. 8. Model for the organization of the *P. solanacearum* virulence regulatory network. Details of the model are presented in Discussion. Symbols: boxes, membrane-bound two-component sensors; circles, transcriptional regulators; arrowheads, putative signal transduction path; solid arrows, positive transcriptional control; and dashed arrows, negative transcriptional control. IM, inner membrane; *egl*, endoglucanase; *pme*, pectin methylesterase; *pglA*, polygalacturonase A.

lacZ fusions (Table 4) showed that both PhcA and VsrA/VsrD are required for maximum activation of *xpsR* transcription, and (iii) gel shift assays (Fig. 7) suggested that PhcA directly binds to the *xpsR* promoter. Since VsrD also controls *xpsR* (but not via *phcA* [23]), it too may bind to the *xpsR* promoter. This would be unusual, since only a few of the more than 50 LysR-type regulators have ever been reported to simultaneously control a promoter in conjunction with another activator (45). However, the phenotype of double *phcA vsrD* mutants suggested that active PhcA is a prerequisite for modulation of transcription of *xpsR* by VsrA/VsrD. Thus at *xpsR*, PhcA acts like a transcriptional switch, while VsrA/VsrD acts as a modulator of the *phcA*-activated transcription. Consistent with this scenario, the 10-fold increase in transcription of *xpsR* effected by PhcA is not enough to turn on *eps* transcription, but rather the further 5-fold increase in *xpsR* transcription caused by VsrA/VsrD is also required.

Activation of *eps* also required VsrC (and likely its signal-dependent phosphorylation by VsrB), because no significant *eps* transcription was observed in *vsrC* or *vsrB* mutants (Table 1), even when *xpsR* is overexpressed (43). Since *xpsR* does not control transcription of *vsrB* or *vsrC* and vice versa (Table 4, Fig. 6B, and reference 21), and since overexpression of *vsrC* did not suppress *xpsR* mutations, it is likely that active XpsR and VsrB/VsrC are simultaneously required and sufficient for activation of the *eps* promoter. Thus, XpsR differs from other ancillary proteins that act in concert with a two-component system (e.g., RcsA [16] and ChvE [60]), because in these other cases, the ancillary proteins are not absolutely required for transcription but rather only enhance expression.

How XpsR and VsrB/VsrC interact to activate *eps* transcription is unclear. One possibility is that via its basic domains, XpsR directly binds to the 150-bp regulatory region of the *eps* promoter (22) to facilitate binding or activation by VsrC; however, XpsR contains no obvious DNA-binding motifs. Alternatively, it could promote signal transfer from VsrB to VsrC or protect VsrC activity; this seems unlikely because inactivation of XpsR did not affect all VsrB/VsrC-regulated processes. Thus, experiments probing the biochemical mode of action of XpsR are essential and may reveal new but common mechanisms used to interconnect signal transduction systems into

networks. Although many prokaryotes (56) and some eukaryotes (7, 38) use two-component or similar systems to control fundamental cellular processes in response to environment, it remains to be seen if any of these other systems are interconnected into networks by XpsR-like proteins.

The network also divergently regulates many other virulence factors. Another unusual feature of the network is that, in addition to convergently controlling *eps*, each of its signal transduction systems independently regulates other known or potential virulence factors. (i) Only the PhcA component positively controls expression of *egl*, *pme*, and several other EXP genes (47); in the case of *egl* (and likely others), PhcA directly binds to and activates its promoter (23, 25). (ii) VsrA and VsrD are the only components that appear to regulate genes required for efficient, rapid growth in planta and negatively control production of some EXPs (Fig. 2B). (iii) PhcA and VsrB/VsrC (but not VsrA/VsrD) independently exert negative control over *pglA* (21). This control of *pglA* may involve the PehR/PehS two-component system because its expression is affected by PhcA (2, 3). Thus, it is plausible that many other regulatory components, targets, and interconnections of the network remain to be discovered.

What is the purpose of such an elaborate network? Conserved signal transduction systems that control genes in response to single, common environmental parameters (e.g., cell density, osmolarity, and nitrogen levels) are widespread in bacteria. However, in certain situations, the ability to adjust expression of a set of genes in simultaneous response to several environmental signals would confer a major advantage. To convergently control production of the metabolically expensive virulence factor EPS I in response to multiple signals, *P. solanacearum* has apparently linked several preexisting monotonic signal transduction systems into a network rather than evolving a dedicated system to control *eps*. Since all network components must be active (i.e., detecting the appropriate signal) for *eps* expression, the likely purpose of the network is to ensure that high-level EPS I production occurs only if most environmental parameters are favorable. This prevents spurious production of EPS I when it may be disadvantageous or even deleterious. However, retaining the ability to divergently control other targets permits the same network to independently turn on and/or turn off other types of virulence genes in response to individual signals. The network system appears to be designed for maximum economy and utility. It presumably allows this pathogen to monitor its progress during pathogenesis, to deal with challenges from the host defenses, and in general to cope with the dynamic and diverse situations encountered inside or outside of its host plants. Although the hypothesized purpose(s) of the network derives largely from in vitro data, current in planta studies to determine where and when in disease the regulated virulence factors are maximally expressed and in response to what signals should help to verify this model.

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