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A Two-Component System in *Ralstonia* (*Pseudomonas*) *solanacearum* Modulates Production of PhcA-Regulated Virulence Factors in Response to 3-Hydroxypalmitic Acid Methyl Ester

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Expression of virulence factors in *Ralstonia solanacearum* is controlled by a complex regulatory network, at the center of which is PhcA, a LysR family transcriptional regulator. We report here that expression of *phcA* and production of PhcA-regulated virulence factors are affected by products of the putative operon *phcBSR*(*Q*). *phcB* is required for production of an extracellular factor (EF), tentatively identified as the fatty acid derivative 3-hydroxypalmitic acid methyl ester (3-OH PAME), but a biochemical function for PhcB could not be deduced from DNA sequence analysis. The other genes in the putative operon are predicted to encode proteins homologous to members of two-component signal transduction systems: PhcS has amino acid similarity to histidine kinase sensors, whereas PhcR and OrfQ are similar to response regulators. PhcR is quite unusual because its putative output domain strongly resembles the histidine kinase domain of a sensor protein. Production of the PhcA-regulated factors exopolysaccharide I, endoglucanase, and pectin methyl esterase was reduced 10- to 100-fold only in mutants with a nonpolar insertion in *phcB* [which express *phcSR*(*Q*) in the absence of the EF]; simultaneously, expression of *phcA* was reduced fivefold. Both a wild-type phenotype and *phcA* expression were restored by addition of 3-OH PAME to growing cultures. Mutants with polar insertions in *phcB* or lacking the entire *phcBSR*(*Q*) region produced wild-type levels of PhcA-regulated virulence factors. The genetic data suggest that PhcS and PhcR function together to regulate expression of *phcA*, but the biochemical mechanism for this is unclear. At low levels of the EF, it is likely that PhcS phosphorylates PhcR, and then PhcR interacts either with PhcA (which is required for full expression of *phcA*) or an unknown component of the signal cascade to inhibit expression of *phcA*. When the EF reaches a threshold concentration, we suggest that it reduces the ability of PhcS to phosphorylate PhcR, resulting in increased expression of *phcA* and production of PhcA-regulated factors.

Bacteria sense a variety of extracellular signals via cell-membrane-associated or intracellular sensor proteins (32, 35, 38). Signal perception usually results in the sensor protein directly altering transcription of target genes or initiating a signal cascade that terminates in altered gene expression. A very common type of signal cascade is the two-component system, typically consisting of a membrane-associated sensor protein (with a variable input domain and a conserved histidine kinase transmitter domain) and a cytoplasmic response regulator protein (with a conserved receiver domain and a variable output domain) (32). In the case of pathogens, environmental signals can modulate expression of pathogenicity or virulence factors so that they are expressed at specific stages of parasitism (14, 48).

Ralstonia solanacearum, a vascular pathogen of many plants, including many economically important crops (7, 17), utilizes a complex regulatory network (Fig. 1) to activate production of the major virulence factor, exopolysaccharide fraction I (EPS I) (39). This network includes at least two two-component systems (VsrA/D [19, 40] and VsrB/C [19, 20]) and a LysR-type transcriptional regulator (PhcA) (5, 6), all of which must be activated for production of EPS I. The signals that VsrA, VsrB, and PhcA respond to are still unknown. Connecting PhcA and VsrA/VsrD to the regulation of *eps* expression is XpsR, which appears to work with VsrC to activate the *eps* promoter (19, 22). PhcA is also pivotal in regulating production of plant cell-wall-degrading enzymes and motility (6). Besides lacking EPS I, *phcA* mutants produce 50-fold less endoglucanase (EGL) activity (6), and 10-fold less pectin methyl esterase (PME) activity (41); simultaneously, endopolygalacturonase A activity increases 10-fold (41), and the percentage of cells that are motile during the late-exponential phase increases nearly 100-fold (6, 10). The mechanism by which PhcA modulates motility is unknown, but it appears to involve another two-component system encoded by *pehSR* (39).

Expression of some PhcA-regulated genes in wild-type *R. solanacearum* appears to be associated with high cell densities during exponential multiplication (10). For example, expression of *xpsR* and *egl*, which are directly regulated by PhcA (19, 22), is low in batch cultures at cell densities below 10^7 CFU/ml, but expression increases 20- to 50-fold before the culture reaches 3×10^8 CFU/ml (10). Motility is also differentially expressed; motility-track photography revealed that wild-type cells are motile only between 10^7 and 10^8 CFU/ml. In contrast, expression of *phcA* did not vary more than twofold from 10^5 to 10^9 CFU/ml in batch cultures, suggesting that posttranscriptional activation or stabilization of PhcA may be partially responsible for the differential expression of virulence and motility. Thus, *R. solanacearum* may have a biphasic life cycle: one phase (motile, low-virulence phase) may be more adapted for

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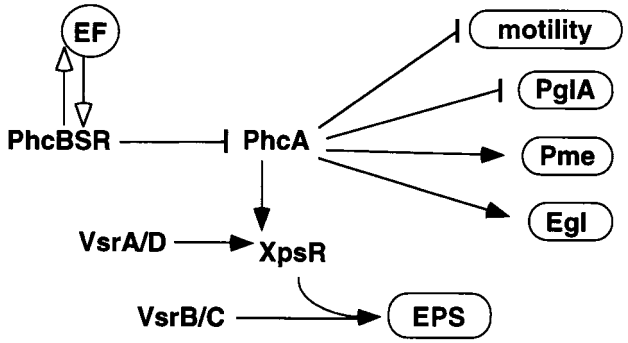

FIG. 1. Simplified model of the network regulating virulence factors and motility in *R. solanacearum*. Lines with solid arrowheads or bars represent positive or negative control, respectively, of gene expression. Lines with open arrowheads represent synthesis or sensing of the EF that has tentatively been identified as 3-OH PAME. PglA and Pme denote polygalacturonase A and PME activities, respectively. Egl and EPS denote EGL activity and EPS production, respectively. Not shown are PehS, PehR, and EpsR (reviewed in reference 39).

survival outside a host, and the other phase (nonmotile, high-virulence phase) may be more adapted for survival and colonization in a plant host (10, 39).

The *phcB* locus, which also affects expression of PhcA-dependent traits, was originally identified in the mutant strain AW1-83 (11). Although it has an insertion mutation about 14 kb away from *phcA*, AW1-83 has a phenotype nearly identical to that of *phcA* mutants (6, 11). Unlike *phcA* mutants, however, AW1-83 is extracellularly complemented by an endogenous extracellular factor (EF) that diffuses into the medium and the air surrounding wild-type cells (11). Preliminary genetic analyses demonstrated that the *phcB* locus is required for production of the EF (11), which has tentatively been identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME) (15). In contrast to expression of PhcA-regulated genes, *phcB* is maximally expressed in batch cultures at cell densities below 10^7 CFU/ml, and its expression decreases 20-fold by 10^8 CFU/ml (10). These results suggested that the EF might serve as an autoinducer for the biphasic expression of PhcA-regulated traits. However, addition of excess 3-OH PAME to batch cultures only slightly altered the differential expression of *xpsR* or *egl*, indicating that an additional condition or factor is required (10).

To investigate the role of the EF and the *phcB* locus in controlling production of PhcA-regulated factors, we further characterized the *phcB* gene, as well as two additional downstream genes (*phcS* and *phcR*) and an open reading frame (ORF [*orfQ*]) that appear to be cotranscribed in an operon with *phcB*. PhcS, PhcR, and OrfQ show amino acid sequence similarities to members of two-component systems for signal transduction. We report here that PhcS and PhcR, together with the EF, modulate production of selected PhcA-regulated factors by altering the function of PhcA or the expression of *phcA*.

MATERIALS AND METHODS

Strains, culture conditions, and assay methods. *R. solanacearum* wild-type strain AW1 (13) and *R. solanacearum* mutants were routinely grown at 30°C in BG medium (1% Bacto peptone, 0.1% Casamino Acids, 0.1% yeast extract, 0.5% glucose) or BGT agar (BG medium plus 1.6% agar and 0.005% tetrazolium chloride). AW1-80 (*phcA80*::Tn*5*) and AW1-83 (*phcB83*::Tn*5*$^+$) have been described previously (6, 11); these strains have the same pleiotropic phenotype, except that AW1-83 is restored to wild type by the EF. The *Escherichia coli* strains used were HB101 (4), SK6501 (*recA56* [1]), GM48 (*dam dcm* [30]), DH5α (Gibco BRL), and XL1-Blue (Stratagene); they were grown at 37°C in Luria-Bertani medium (29). 3-OH PAME (Sigma Chemical Corp.) was added to cultures to a final concentration of 20 nM (from a 20 μM stock solution in methylene chloride), which fully restores a *phcB* mutant to the wild-type phenotype (15). The antibiotics used were ampicillin (100 μg/ml for *E. coli*, 10 μg/ml for *R. solanacearum*), kanamycin (50 μg/ml), nalidixic acid (20 μg/ml), spectinomycin (50 μg/ml), and tetracycline (15 μg/ml).

To determine levels of β-galactosidase activity, cells were grown in BG medium to an optical density at 600 nm of ≈1.0, permeabilized with chloroform and sodium dodecyl sulfate, and assayed with the substrate *o*-nitrophenyl-β-D-galactopyranoside (11, 29). To quantify EPS I production, EGL activity, and PME activity, cells were cultured in EG medium (37) for 72 h, and supernatants were assayed as previously described (6, 11). Production of EF was assessed qualitatively in lid agar assays (11) by testing whether a strain could, via the vapor phase, induce AW1-83 to become mucoid (visible EPS slime); this assay can detect ≤5 pmol of 3-OH PAME (15).

Molecular genetic techniques. Standard methods for cloning, $CaCl_2$-mediated transformation of *E. coli*, triparental matings, DNA analysis, and DNA preparation have been described previously (3, 8, 28). Natural transformation of *R. solanacearum* occurred as described elsewhere (10). Transposon mutagenesis with Tn*5*-B20 (42), which carries a promoterless *lacZ* gene, was used to create transcriptional reporter fusions by infecting *E. coli* SK6501 harboring pLMO5 with λ::Tn*5*-B20 as described previously (20). The Tn*5*-B20 insertions were restriction mapped, and selected insertions were transferred into the genome of AW1 by allelic replacement (19).

Proteins encoded by *phcBSR* were characterized with maxicells (21, 36). Briefly, cells of *E. coli* SK6501 harboring the desired genes on a plasmid were UV irradiated and then labeled with [^{35}S]methionine. Labeled proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by fluorography after the gel was treated with 2,5-diphenyloxazole (25).

DNA sequences were determined from double-stranded plasmid DNA on an automated DNA sequencer (Applied Biosystems, Inc.) at the Molecular Genetic Instrumentation Facility at the University of Georgia. Universal primers were used to sequence cloned fragments in pUC or pBluescript vectors. To sequence DNA flanking transposon insertions, we used the primers 5′-GTAAAACGACGGGATC CAT-3′ and 5′-GCCGCACGATGAAGAGCAG-3′, which hybridized to the left and right IS*50* elements, respectively, of Tn*5*-B20. DNA flanking the left end of Tn*3*-HoHo1 inserts was sequenced with the primer 5′-AAAAGAGGCGTCAGA GGC-3′ Specific primers were made to sequence gaps. Sequences were analyzed with the Wisconsin Package (version 8; Genetics Computer Group), Gene Jockey Sequence Processor (BioSoft), and MacDNAsis Pro (version 3.5; Hitachi Software Engineering). The BLAST program (2) was used for homology searches of amino acid sequences within the SWISS-PROT, GenBank/EMBL, and PIR databases.

Construction of plasmids and *R. solanacearum* strains. Plasmids were constructed as described in Table 1 and are illustrated in Fig. 2.

To create *R. solanacearum* mutants, we first cloned either the 2.0-kb polar Ω cartridge (34) or the 0.9-kb nonpolar *nptI* cartridge (16) into existing restriction sites in various plasmids (Fig. 2) as follows. In each case, constructs with the promoter of the *nptI* cartridge aligned with the transcription direction of *phcBSR*(*Q*) were selected. The region upstream of *phcB* was disrupted (i) by inserting Ω into the *Bam*HI site in pKS11E and (ii) by inserting Ω or *nptI* into the *Msc*I and *Eag*I sites of pUC660. *phcB* was disrupted by inserting *nptI* or Ω into the *Sma*I site of pKS-B. *phcS* was disrupted (i) by inserting *nptI* or Ω into the *Bcl*I site of pLMO5 (that had been replicated in *E. coli* GM48 to prevent methylation) or (ii) by replacing the three internal *Msc*I fragments in pSK-S with the *nptI* cartridge. *phcR* was disrupted (i) by inserting *nptI* or Ω into the *Eco*RI site of pSK-BSR or (ii) by replacing the region between *Stu*I and *Eco*RV of pKS-R with the *nptI* cartridge. *orfQ* was disrupted by inserting Ω into the *Sma*I site of pSK-Q. To create a Δ*phcBS* mutation, the 3-kb *Nco*I fragment in pKS-BSR was replaced with the *nptI* cartridge. To create Δ*phcBSR*, the 2.6-kb *Stu*I fragment in pLMO5 (replicated in GM48) was replaced with the *nptI* cartridge. To create Δ*phcBSR*(*Q*), the following steps were done sequentially: the 3.4-kb *Bst*EII fragment from pBBgl8 was deleted and religated; the 6.4-kb *Pst*I fragment from pBBgl8Δ*Bst*EII was cloned into the *Pst*I site of pUC19; the 0.84-kb *Eco*RI fragment was deleted to remove the *Hin*cII site of the polylinker and religated; *phcB* and *orfQ* were deleted by *Hin*cII digestion and religated; and the Ω cartridge was cloned into the remaining *Hin*cII site. This deletion of *phcBSR*(*Q*) left 218 bp of the 5′ end of *phcB* and 78 bp of the 3′ end of *orfQ* flanking the Ω cartridge. Each mutant allele created as outlined above was recombined into the genome of AW1 as described previously (10), and proper allelic replacement was confirmed by Southern blot analysis.

Nucleotide sequence accession number. The sequence of the 5,852 bases from *Bam*HI to *Eco*RI of pBBgl8 (Fig. 2), containing 646 bases upstream through 265 bases downstream of *phcBSR*(*Q*), was deposited in GenBank under accession no. U6193. Nucleotides are numbered beginning at the *Bam*HI site at the left end of pBBgl8 (Fig. 2).

RESULTS

***phcB* is the first gene of an operon.** Previous attempts to create mutants with the same phenotype as AW1-83 with Tn*3*-HoHo1 as well as new attempts with Tn*5*-B20 were mostly unsuccessful (9, 10). Despite inactivation of *phcB*, which elim-

TABLE 1. Plasmids used in this study

Designation	Relevant characteristics or construction[a]	Source or reference
Vectors		
pSK, pKS	pBluescriptSKII$^+$ and pBluescriptKSII$^+$, respectively; ColE1, Ap^r	Stratagene
pUC9	ColE1 Ap^r	46
pLAFR3	Broad host range; Tc^r	43
pSL1180	ColE1, large multicloning site, Ap^r	Pharmacia
Recombinants		
pHB9	pLAFR3 cosmid clone containing *phcA* and *phcBSR*(*Q*) from AW1	6
pGA91-95	4.0-kb *Eco*RI fragment in pLAFR3 with a Tn*3*-HoHo1 (*lacZ*) insertion, Lac$^+$ Ap^r Tc^r	5
pLMO4, pLMO5	4.0-kb *Bam*HI-*Eco*RI fragment containing *phcB* and *phcS* on pLAFR3 and pUC9, respectively	11
pKS11E	11.1-kb *Eco*RI fragment of pHB9 ligated into the *Eco*RI site of pKS	This study
pBBgl8	8.0-kb *Bam*HI-*Bgl*II fragment from pHB9 ligated into the *Bam*HI site of pSK	This study
pSL-BSR, pSK-BSR, pKS-BSR, pLA-BSR	4.9-kb *Bam*HI-*Sac*I fragment of pHB9 cloned into similarly digested pSL1180 to create pSL-BSR; this fragment then recovered by *Bam*HI-*Hin*dIII digestion and ligated into similarly digested pSK, pKS, and pLAFR3 to create pSK-BSR, pKS-BSR, and pLA-BSR, respectively	This study
pSK1.6	1.6-kb *Eco*RI fragment isolated from pLMO4::Tn-*3*-HoHo1-165 (with the *Eco*RI site at the left end of the transposon) cloned into the *Eco*RI site of pSK	This study
pUC660	0.66-kb *Hin*cII fragment of pSK1.6 ligated into the *Hin*cII site of pUC19	This study
pSK963, pLA963	0.96-kb fragment from pSK1.6 partially digested with *Sma*I ligated into the *Sma*I site of pSK to create pSK963; this fragment then recovered by *Bam*HI-*Hin*dIII digestion and ligated into similarly digested pLAFR3 to create pLA963	This study
pKS-B, pLA-B	2.9-kb *Nco*I fragment from pKS-BSR ligated into the *Sma*I site of pKS; 0.5-kb *Pst*I fragment then removed with a *Pst*I site in the polylinker to create pKS-B; 2.4-kb *Bam*HI fragment from pKS-B ligated into the *Bam*HI site of pLAFR3 to create pLA-B	This study
pKS-B2.2	2.2-kb *Bam*HI fragment isolated from pLMO5::Tn*5*-B20-152 (with the *Bam*HI site at the left end of the transposon) ligated into the *Bam*HI site of pKS	This study
pKS-BS, pLA-BS	3.6-kb *Bam*HI fragment isolated from pLMO5::Tn*5*-B20-157 (with the *Bam*HI site at the left end of the transposon) ligated into the *Bam*HI site of pKS to create pKS-BS; this fragment recovered by *Bam*HI-*Hin*dIII digestion and ligated into similarly digested pLAFR3 to create pLA-BS	This study
pSK-S, pLA-S	1.7-kb *Hin*cII fragment of pLMO5 ligated into the *Sma*I site of pSK to create pSK-S; this fragment recovered by *Bam*HI-*Eco*RI digestion and ligated into similarly digested pLAFR3 to create pLA-S	This study
pKS-R	pKS-BSR digested with *Bam*HI-*Nco*I and religated after filling in cohesive ends with the Klenow enzyme	This study
pSL-R, pLA-R	1.6-kb *Sac*I fragment recovered from pKS-R (with a *Sac*I site in the polylinker) and cloned into the *Sac*I site of pSL1180 to create pSL-R; this fragment recovered from pSL-R with the *Bam*HI-*Hin*dIII sites of the polylinker and ligated into similarly digested pLAFR3 to create pLA-R	This study
pSK-SR	pSK-BSR digested with *Bam*HI-*Bst*EII to delete a 2.0-kb fragment and religated after filling in cohesive ends with Klenow enzyme	This study
pUC-SR, pLA-SR	2.9-kb insert recovered from pSK-SR with the *Xho*I and *Xba*I sites of the polylinker, cohesive ends filled with Klenow enzyme, and ligated into the *Hin*cII site of pUC9 to create pUC-SR; this fragment recovered with *Bam*HI and *Hin*dIII sites in the polylinker and ligated into similarly digested pLAFR3 to create pLA-SR	This study
pSK-Q	1.8-kb *Eco*RI fragment from partially-digested pBBgl8 ligated into the *Eco*RI site of pKS; 1.5-kb *Eco*RI-*Eco*RV fragment removed by *Eco*RV digestion (with the *Eco*RV site of the polylinker) and ligated into the *Sma*I site of pSK	This study
pLA-Q	1.5-kb insert from pSK-Q recovered by *Bam*HI-*Hin*dIII digestion and ligated into similarly digested pLAFR3	This study

[a] Ap^r, ampicillin resistance; Tc^r, tetracycline resistance. The transcriptional direction of all genes subcloned into pLAFR3 is in the same orientation as P_{lac} of this vector.

inated production of EF, among the resulting 11 Tn*5*-B20 mutants, only one (strain AW1-150) did not produce intermediate amounts of PhcA-regulated factors. Surprisingly, strain AW1-150, which has a phenotype indistinguishable from that of AW1-83, has Tn*5*-B20 inserted at exactly the same location (as determined by DNA sequencing) as the Tn*5*-B20 in AW1-149, a strain with an intermediate phenotype. The transposons in these two strains, however, are in opposite orientations (Fig. 2). These results indicated that both the position and the orientation of the transposon were critical and suggested to us that the phenotype of AW1-83 and AW1-150 might be the result of infrequent nonpolar mutations.

To test whether *phcB* might be in an operon with genes downstream that affect phenotype, *phcB* was mutated by insertion of the polar and nonpolar insertional cartridges Ω and *nptI*, respectively, into the *Sma*I site at position 963 (Fig. 2), and the resultant alleles were site specifically exchanged into AW1. The polar *phcB*::Ω mutant was EF negative; it produced colonies on BGT plates that slowly became mucoid, but made nearly wild-type levels of PhcA-regulated factors in broth cul-

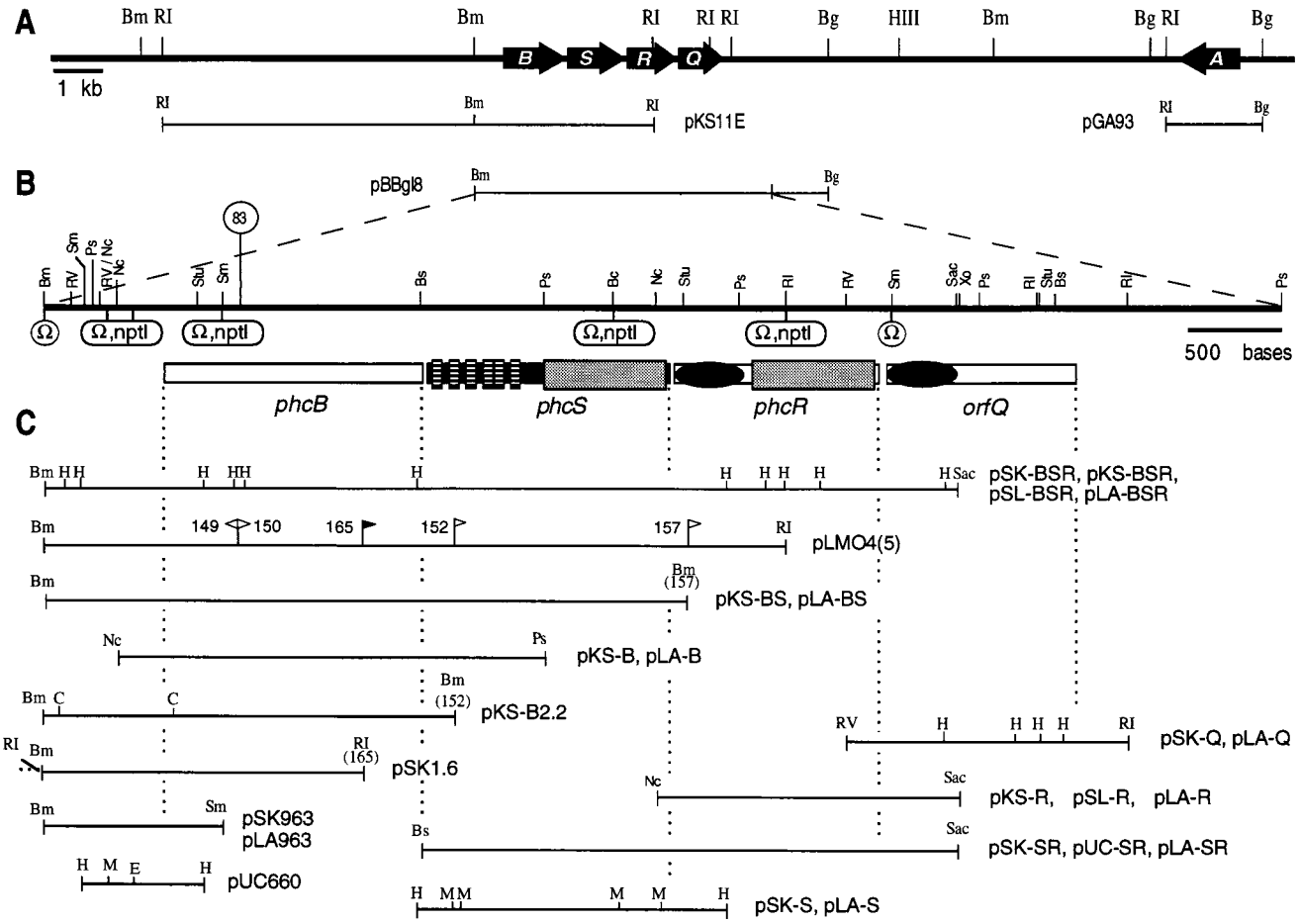

FIG. 2. (A) Partial map of AW1 cosmid clone pHB9 and subclones. *phcBSR*(*Q*) and *phcA* are indicated on pHB9. (B) Enlargement of subclone pBBgl8 to indicate restriction sites, site of insertion that created AW1-83 (circled 83), site of Ω and *nptI* insertions, and architecture of identified domains within PhcS, PhcR, and OrfQ (hydrophobic domains of PhcS indicated by hatched rectangles, histidine-kinase transmitter domain indicated by grey rectangles; receiver domain of response regulators indicated by black ovals). (C) Subclones that originated from pHB9. White flags on pLMO5 indicate the position and orientation of Tn*5*-B20 inserts, and the black flag on pLMO4 indicates the position and orientation of Tn*3*-HoHo1-165. Bc, *Bcl*I; Bg, *Bgl*I; Bm, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; E, *Eag*I; H, *Hin*cII; HIII, *Hin*dIII; M, *Msc*I; Nc, *Nco*I; Ps, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; Sac, *Sac*I; Sm, *Sma*I; Stu, *Stu*I; and Xo, *Xho*I.

tures (Table 2). In contrast, the nonpolar *phcB::nptI* mutant had a phenotype identical to that of AW1-83 and AW1-150: it produced no detectable EF; its colonies were nonmucoid on BGT plates; in broth culture it produced about 60-fold-less EPS I, 20-fold-less EGL activity, and 10-fold-less PME activity; and it was restored to wild type by the EF or 3-OH PAME (Table 2 and results not shown). As seen before (11), the phenotype of a *phcA* mutant was similar to that of the nonpolar *phcB::nptI* mutant, with the exception that 3-OH PAME did not restore it to wild type. The *phcB::nptI* mutant was complemented to wild type by *phcB* in *trans* on pLA-B (Table 2). These results, as well as additional data presented below, are consistent with the hypothesis that *phcB* is in an operon and that expression of genes downstream results in reduced production of PhcA-regulated factors if PhcB (and thus EF) is absent.

TABLE 2. Effect of *phcA* and *phcB* mutations and 3-OH PAME on production of three PhcA-regulated virulence factors

Mutation	Effect of mutation on production of[a]:		
	EPS I	EGL	PME
None	329	371	40
phcA80::Tn*5*	12 (12)	<5 (<5)	5 (5)
phcB::Ω	190	182	36
phcB83::Tn*5*$^+$	5 (285)	22 (254)	7 (59)
phcB150::Tn*5*-B20	6	27	5
phcB::nptI	<5 (329)	18 (263)	3 (60)
phcB::nptI (pLA-B)	311	297	37

[a] EPS I, micrograms of extracellular galactosamine polysaccharide per milligram of cell protein; EGL, nanomoles per minute per milligram of cell protein; PME, units per milligram of cell protein. Values are the means of two or more cultures; variation was <20%. Values in parentheses are those obtained when 3-OH PAME was added to cultures to a final concentration of 20 nM.

Characterization of *phcB*, a gene required for EF production. Defined as a locus essential for production of the EF, *phcB* was previously delineated by Tn*3*-HoHo1 insertions and complementation (11). The nucleotide sequence of *phcB* predicts a single ORF from nucleotide 647 to nucleotide 2050 in pBBgl8 (Fig. 2). This ORF, whose start is a GTG codon with a putative ribosome binding site (GGCAGA) eight nucleotides upstream, is predicted to encode a protein of 467 amino acids with a molecular mass of 52.9 kDa. Maxicells harboring pKS-B or pKS-B2.2 produced a single labeled protein with a size of about 53 kDa (Fig. 3B and data not shown), which was also the

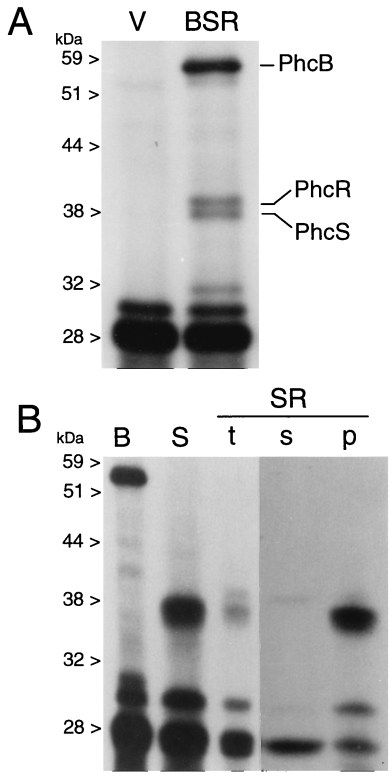

FIG. 3. Maxicell analysis of proteins encoded by *phcB*, *-S*, and *-R*. (A) *E. coli* SK6501 maxicells contained pSK and pSK-BSR in the left and right lanes, respectively. The bands corresponding to PhcB, PhcS, and PhcR are labeled on the right. (B) Maxicells contained (from the left) pKS-B, pSK-S, pSK-SR (total proteins [t]), pSK-SR (soluble proteins [s]), and pSK-SR (pelleted proteins [p]). Soluble and pelleted proteins were separated by ultracentrifugation after the labeled maxicells were lysed by sonication. Molecular mass standards migrated as indicated to the left. The bands at 27 and 29 kDa are β-lactamase and pre-β-lactamase, respectively, encoded by the vector.

largest of three proteins produced by maxicells with pSK-BSR (Fig. 3A). The approximately 31-kDa protein encoded by pSK-BSR (Fig. 3A) is believed to be due to a fusion of vector and insert sequences; this band was not present from maxicells containing pKS-B, which has a different region juxtaposed to the P_{lac} of the vector. Database searches found no significant homologs of PhcB.

It was not clear from earlier work whether there is a gene upstream of *phcB* that affects EF production (11), so we investigated the function of this region by mutagenesis with Ω and *nptI* cartridges. Insertion of a polar Ω cartridge into the genome at the *Bam*HI site 646 bases upstream of *phcB* (nucleotide 1 of pBBgl8) had no effect on production of EPS and EF or colony morphology. Likewise, insertion of the nonpolar *nptI* cartridge into the genome at the *Msc*I or *Eag*I sites 286 or 167 bases, respectively, upstream of *phcB* (Fig. 2B) did not affect production of EPS and EF or colony morphology. In contrast, insertion of Ω into the same *Msc*I and *Eag*I sites produced mutants with reduced EF and intermediate amounts of EPS (based on colony morphology). Plasmid pLA963, which contains the 5′ end of *phcB* and 646 bases upstream (Fig. 2C), did not restore wild-type production of the EF or colony morphology despite containing all of the predicted ORFs that span both the mutated *Msc*I and *Eag*I restriction sites. These results suggest that this region contains no functional genes involved in EF production and are consistent with it containing the promoter and/or transcriptional regulatory sequences for *phcB*.

Three ORFs downstream of *phcB* are related to members of two-component systems. DNA sequence analysis of about 4 kb downstream from *phcB* suggested that there are three closely spaced ORFs transcribed in the same direction as *phcB*. The first of these ORFs (bases 2060 to 3388), designated *phcS*, is predicted to start at an ATG codon 10 nucleotides from the end of *phcB* (Fig. 2); 5 nucleotides upstream of this start codon is a putative ribosome binding site (AAGGGA). Database searches revealed that the carboxyl end of the predicted PhcS shares significant amino acid homology with the histidine kinase transmitter domain of two-component systems (Fig. 4A and C) (32); the sensor protein with the greatest homology (37.4% identical, 57.4% similar) to the carboxyl-terminal 240 amino acids of PhcS was HupT from *Rhodobacter capsulatus*. The putative histidine kinase domain of PhcS has all the conserved residues and blocks of amino acids associated with this family of proteins (Fig. 4C) (44). The amino terminus of sensors often contains hydrophobic domains that anchor them to the cytoplasmic membrane. Hydrophobicity analysis (24) showed that the amino terminus of PhcS has up to six potential membrane-spanning, hydrophobic domains of 17 to 24 amino acids each (Fig. 4D). PhcS is predicted to be a protein of 442 amino acids with a molecular mass of 48.5 kDa, but maxicells containing pSK-S produced a protein with a size of about 36 kDa (Fig. 3B). A protein of similar size was also produced by maxicells containing pKS-SR and pSK-BSR (Fig. 3). We believe that the 36-kDa protein is either an aberrantly migrating PhcS protein, partially degraded PhcS, or the product of a shorter ORF preferred in *E. coli*. Lysis and ultracentrifugation of labeled maxicells to separate soluble (cytoplasmic) from insoluble (membrane) proteins suggested that PhcS is membrane associated (Fig. 3B).

Following the *phcS* stop codon by nine nucleotides is *phcR*, an ORF spanning bases 3397 to 4515 (Fig. 2); it begins with an ATG codon preceded by a putative ribosome binding site (AG GAG) five nucleotides upstream. The nucleotide sequence of *phcR* predicts a protein of 372 amino acids with a molecular mass of 40.4 kDa. Although maxicell analyses with pKS-R repeatedly failed to give a labeled protein for PhcR, plasmids pSK-BSR and pKS-SR directed synthesis of a soluble, approximately 38-kDa protein that could be the *phcR* product (Fig. 3). Database searches with the amino acid sequence of PhcR revealed that it is similar to both response regulators and histidine kinase sensors of two-component systems for signal transduction (Fig. 4A). The amino-terminal 130 amino acids of PhcR share significant homology (up to 59% similar) to the amino-terinal receiver domain of response regulators, with all of the conserved blocks and residues present (Fig. 4B) (32, 47). The carboxyl-terminal 240 amino acids of PhcR have significant homology (about 50% similar) to the histidine kinase domain of sensors (Fig. 4C); however, it is missing one of the two conserved asparagine residues in the N block and lacks both conserved phenylalanine residues in the F block (32, 44). The only protein found to have the same chimeric response regulator-histidine kinase sensor architecture is AsgA from *Myxococcus xanthus* (33), which is 27.2% identical (51.7% similar) over the entire length of PhcR (Fig. 4A to C).

There is a gap of 62 nucleotides between *phcR* and *orfQ*, which spans bases 4577 to 5587 (Fig. 2). There is no obvious transcriptional terminator in this gap, so it seems likely that *orfQ* is cotranscribed with *phcBSR*; this conclusion was supported by the finding that expression of a genomic *orfQ::lacZ* reporter was reduced sevenfold when the reporter was placed downstream of the polar *phcB*::Ω mutation (10). *orfQ* has a

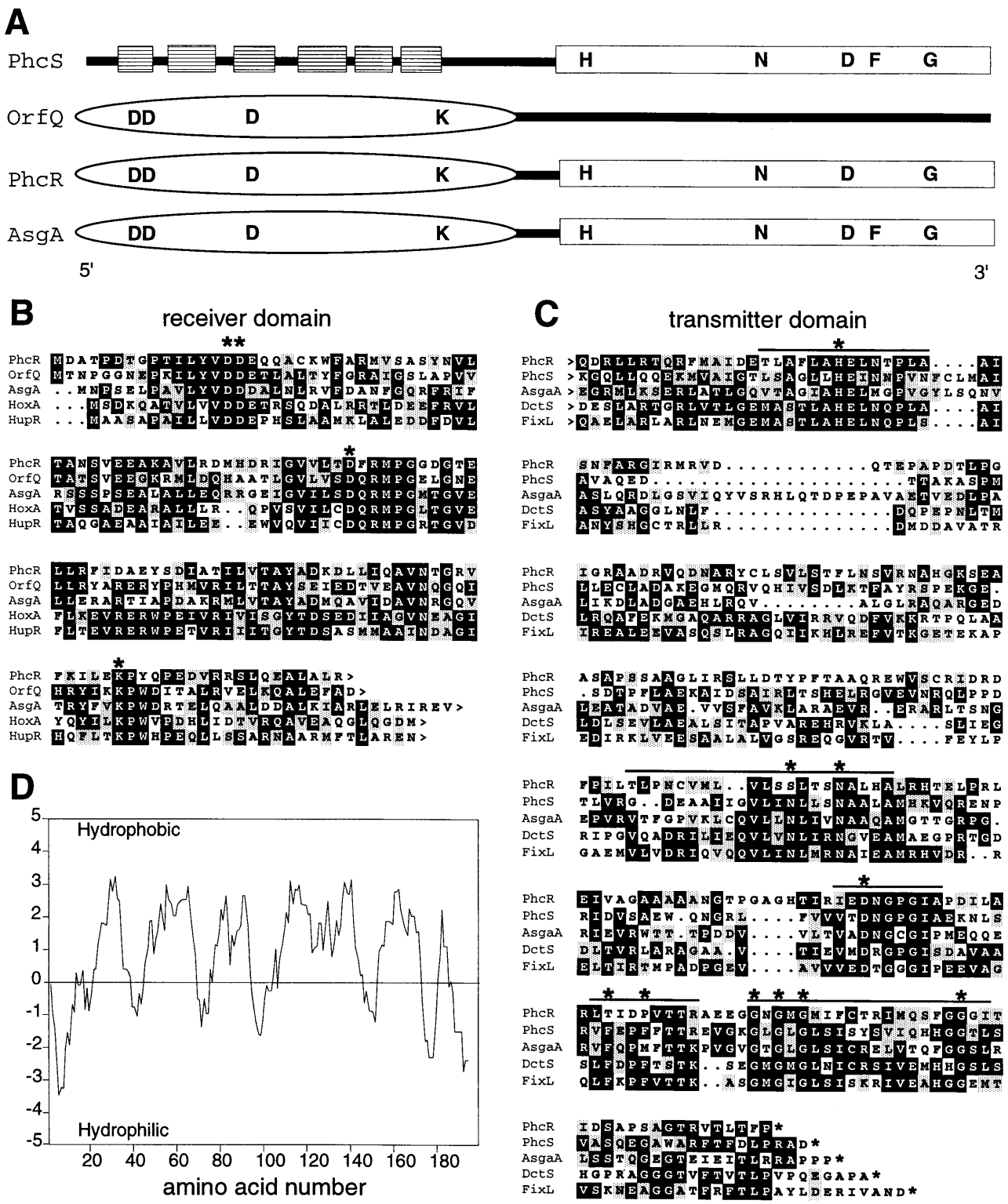

FIG. 4. Sequence analysis of PhcS, PhcR, and OrfQ from *R. solanacearum*. (A) Schematic of the proteins predicted to be encoded by *phcS*, *phcR*, and *orfQ* and comparison to AsgA from *Myxococcus xanthus*. Open ovals denote the two-component response regulator receiver domains, whereas open rectangles indicate histidine kinase transmitter domains; letters indicate the presence of the most highly conserved blocks of amino acids (45, 48). Hatched rectangles represent the putative membrane-spanning domains in the sensor protein input domain. (B) Alignment of the amino-terminal receiver domains of PhcR, OrfQ, AsgA from *M. xanthus* (U20214); HoxA from *Alcaligenes eutrophus* (P29267); and HupR from *R. capsulatus* (P26408). The symbol > indicates that the amino acid sequence continues. The most common amino acid at each position is boxed in black; conserved substitutions are shaded gray. The highly conserved D and K residues are indicated by asterisks above them. (C) Alignment of the carboxyl-terminal histidine kinase transmitter domains of PhcS, PhcR, and AsgA (U20214); DctS from *Rhodopseudomonas capsulata* (P37739); and FixL from *Rhizobium meliloti* (P10955). Each aligned sequence begins with an internal residue (indicated by >) and ends with the carboxyl-terminal residue. The PhcR and AsgA sequences in panel C are contiguous with the amino-terminal sequences in panel B. The important conserved residues and blocks (H, N, D, F, and G) are indicated by asterisks and lines, respectively, above them. (D) Kyte-Doolittle hydrophobicity plot of PhcS showing the six hydrophobic, putative membrane-spanning domains in the amino-terminal 180 amino acids.

TABLE 3. Effect of various mutations in *phcBSR*(*Q*) on expression of PhcA-regulated virulence factors

phc genotype				Mutation[a]	Effect of mutation on expression of[b]:		
B	*S*	*R*	*Q*		EPS	EGL	PME
+	+	+	+	None	329 ± 30	371 ± 19	40 ± 2
−	+	+	+	*B*::*nptI*	<5	16 ± 1	<3
+	−	+	+	*S*::*nptI*	295 ± 3	269 ± 9	38 ± 1
+	+	−	+	Δ*R*	297 ± 21	170 ± 15	44 ± 7
+	+	+	−	*Q*::Ω	438 ± 1	358 ± 27	59 ± 2
−	−	+	+	Δ*BS*	312 ± 12	204 ± 12	17 ± 1
−	+	−	+	Δ*BSR* (pLA-S)	281 ± 2	231 ± 17	46 ± 5
−	+	+	−	*B*::*nptI*/*Q*::Ω	10 ± 1	<5	<3
+	−	−	+	Δ*BSR* (pLA-B)	273 ± 5	343 ± 20	43 ± 0
+	−	+	−	Δ*S*/*Q*::Ω	347 ± 12	268 ± 12	60 ± 3
+	+	−	−	*R*::Ω	305 ± 10	324 ± 8	46 ± 0
+	−	−	−	*S*::Ω	304 ± 20	319 ± 4	56 ± 1
−	+	−	−	*B*::*nptI*/*R*::Ω	340 ± 3	339 ± 7	56 ± 3
−	−	+	−	Δ*BS*/*Q*::Ω	296 ± 6	216 ± 11	21 ± 2
−	−	−	+	Δ*BSR*	287 ± 11	274 ± 20	54 ± 2
−	−	−	−	Δ*BSRQ*::Ω	230 ± 25	145 ± 5	36 ± 3

[a] All deleted regions (except Δ*BSRQ*) were replaced with the nonpolar *nptI* cartridge to allow transcription of downstream genes.

[b] EPS, micrograms of extracellular galactosamine polysaccharide per milligram of protein; EGL, nanomoles per minute per milligram of cell protein; PME, units per milligram of cell protein. Values are the means ± standard errors of two or more cultures.

TABLE 4. Effect of mutations in the *phcBSR*(*Q*) operon and 3-OH PAME on the production of PhcA-regulated virulence factors

phc genotype				Mutation[a]	PAME[b]	Effect of mutation on expression of[c]:		
B	*S*	*R*	*Q*			EPS I	EGL	PME
+	+	+	+	None	−	329 ± 30	371 ± 19	40 ± 2
−	−	+	+	Δ*BS*	−	312 ± 12	204 ± 12	17 ± 1
−	+	+	+	Δ*BS* (pLA-S)	−	<5	19 ± 0.5	<3
−	+	+	+	Δ*BS* (pLA-S)	+	324 ± 18	266 ± 5	39 ± 1
−	+	−	−	*B*::*npntI*/*R*::Ω	−	340 ± 3	339 ± 7	56 ± 3
−	+	+	−	*B*::*nptI*/*R*::Ω (pLA-R)	−	<5	22 ± 1	<3
−	+	+	−	*B*::*nptI*/*R*::Ω (pLA-R)	+	331 ± 3	239 ± 15	51 ± 8
−	−	−	+	Δ*BSR*	−	287 ± 11	274 ± 20	54 ± 2
+	−	−	+	Δ*BSR* (pLA-B)	−	273 ± 5	343 ± 20	43 ± 0
−	+	−	+	Δ*BSR* (pLA-S)	−	281 ± 2	231 ± 17	46 ± 5
−	−	+	+	Δ*BSR* (pLA-R)	−	295 ± 11	315 ± 17	26 ± 5
−	+	+	+	Δ*BSR* (pLA-SR)	−	5 ± 1.2	13 ± 0.2	<3
−	+	+	+	Δ*BSR* (pLA-SR)	+	293 ± 10	275 ± 25	42 ± 1

[a] All deleted regions were replaced with the nonpolar *nptI* cartridge to allow transcription of downstream genes.

[b] Cultures were supplemented with 3-OH PAME at a final concentration of 20 nM.

[c] EPS I, micrograms of extracellular galactosamine polysaccharide per milligram of cell protein; EGL, nanomoles per minute per milligram of cell protein; PME, units per milligram of cell protein. Values are means ± standard errors of two or more cultures.

potential ribosome binding site (AAGAGGAG) six nucleotides upstream of an ATG start codon and is predicted to encode a protein with 336 amino acids and a molecular mass of 37.2 kDa; plasmids with *orfQ* were not analyzed with maxicells. Database searches with OrfQ revealed that the amino-terminal 130 amino acids are homologous to a response-regulator receiver domain (Fig. 4A and C). The response regulator with the highest homology over the amino-terminal 130 amino acids was AsgA of *M. xanthus* (41.6% identical, 61.6% similar); this region of OrfQ is also 37.7% identical (60% similar) to the corresponding region in PhcR. Most response regulators have a putative helix-turn-helix DNA-binding motif in the carboxyl-terminal output domain (31), but we were unable to identify such a motif in OrfQ. Amino acid homology with only the carboxyl-terminal 210 amino acids of OrfQ found no significant homologs in the databases.

PhcS and PhcR function together to reduce production of PhcA-regulated factors. As previously mentioned, only nonpolar mutations in *phcB* dramatically reduced production of EPS I, EGL, and PME (Table 2). Since production of these virulence factors is positively regulated by PhcA (10, 39), we hypothesized that *phcSR*(*Q*) encodes a signal transduction system that in the absence of the EF, negatively regulates production or activity of PhcA. To test this hypothesis, we first inactivated each ORF in the operon, either singly or in combination, by site-specific incorporation of Ω or *nptI* cartridges and determined the mutants' production of EPS I, EGL, and PME. Mutants with *phcS*, *phcR*, or *orfQ* inactivated in any combination still produced mucoid colonies on BGT plates; however, isolated single colonies of most mutants became mucoid at least 1 day later than the wild type, suggesting slower production of EPS. When grown in broth for 3 days, only the mutants with a *phcB* S^+R^+ combination did not make wild-type amounts of EPS I, EGL, and PME (Table 3). Even elimination of the entire *phcBSR*(*Q*) region did not reduce production of any of the PhcA-regulated virulence factors tested by more than 50%, which is consistent with our hypothesis that functional genes in this locus negatively affect production of these factors.

To demonstrate the negative effect of PhcS and PhcR, we cloned the genes encoding these proteins and expressed them from the *lac* promoter of a broad-host-range vector. As predicted, complementation of *phcBS*, *phcB*/*R*, and *phcBSR* mutants with plasmid-borne *phcS*, *phcR*, and *phcSR*, respectively, strongly reduced production of PhcA-regulated factors, and the repressive activity of PhcS and PhcR was reversed by addition of 3-OH PAME (Table 4). Additional conclusions that can be drawn from these results are that (i) functional *phcS* and *phcR* genes were cloned that correspond to the ORFs found by sequencing and that (ii) since they functioned in *trans*, PhcS and PhcR must interact as proteins, presumably to transduce a signal. Therefore, PhcS and PhcR appear to function together to reduce production of PhcA-regulated virulence factors, and 3-OH PAME somehow interferes with this activity.

PhcS and PhcR affect virulence factors by interfering with expression of *phcA*. PhcS and PhcR might reduce production of PhcA-regulated virulence factors by reducing expression of *phcA* or the function of PhcA (Fig. 1). We examined expression of *phcA* by moving pGA91-95, a plasmid with a *phcA95*::*lacZ* reporter expressed from its native promoter (5), into various genetic backgrounds and assessed β-galactosidase activity. As predicted, expression of *phcA* was markedly lower (ca. fivefold) in the *phcB*::*nptI* mutant background (*phcB* S^+R^+) than in the wild type, and this effect was largely negated by addition of 3-OH PAME (Table 5). Indeed, the effect of the nonpolar *phcB* mutation on expression of *phcA* was comparable to that seen when *phcA* itself was inactivated (*phcA* is positively autoregulating [5]). Similar results were obtained when the *phcA95*::*lacZ* reporter was recombined into the genome of AW1, AW1-83 (*phcB83*::*Tn5*$^+$), and AW1-80 (*phcA80*::*Tn5*) (15). Furthermore, providing AW1-83 (and other nonpolar *phcB* mutants) with *phcA* in *trans* on pLAFR3, a low-copy-number plasmid, increased EPS I production, as well as EGL and PME activities, to one-fourth to one-half that

TABLE 5. Expression of plasmid-borne *phcA*::*lacZ* in *R. solanacearum* strains with different mutations and effect of 3-OH PAME[a]

Mutation	Effect of mutation on *phcA* expression (Miller units)[b]	
	Solvent	3-OH PAME
None	158 ± 3	173 ± 8
phcB::*nptI*	31 ± 1	114 ± 5
phcA::*Tn5*	32 ± 1	19 ± 1

[a] Strains contained plasmid pGA91-95, which carries the *phcA95*::*lacZ* reporter allele expressed from a native promoter. BG cultures contained tetracycline and either 20 nM 3-OH PAME in methylene chloride (diluted from a 1,000× stock) or a comparable amount of the solvent.

[b] *phcA* expression was monitored by measuring β-galactosidase activity; values are reported as Miller units (29) and are means ± standard errors of four cultures grown to an optical density at 600 nm of ≈1.0.

of the wild type; a nonfunctional allele of *phcA* did not have this effect (9). Therefore, in the absence of the EF, PhcS and PhcR somehow reduced expression of *phcA*, and hence the expression of PhcA-regulated virulence genes, and this effect was partially reversed by overexpression of *phcA*.

DISCUSSION

We reported previously that mutants of *R. solanacearum* AW1 with random Tn*3*-HoHo1 insertions in *phcB*, although invariably EF negative, had confusing, intermediate phenotypes (11). With one exception, we observed similar results when *phcB* was mutated with Tn*5*-B20. Inactivation of *phcB* by site-specific insertion of defined polar and nonpolar antibiotic resistance cartridges revealed that only nonpolar mutations resulted in a phenotype like that of the original AW1-83 mutant. Therefore, strains AW1-83 and AW1-150 must contain atypical nonpolar Tn*5* insertions, and other *phcB* transposon mutants contain insertions that are intermediate or fully polar. The lack of polarity in AW1-83 is most likely due to the out-facing *nptII* promoter in the extra $IS50_L$ of the complex Tn*5* insertion, which is fused to genomic sequences within *phcB* (11, 23a). Other researchers have also documented examples in which Tn*5* or its derivatives can have nonpolar effects (12, 49). Because only nonpolar mutations in *phcB* drastically reduced production of multiple PhcA-regulated virulence factors, we hypothesized and then proved that (i) expression of one or more genes downstream of *phcB* is essential for this phenotype, (ii) these genes encode proteins that can negatively affect PhcA-regulated genes, and (iii) these proteins probably act by interfering with expression of *phcA* or the function of PhcA. Our earlier finding that expression of a PhcA-regulated *eps*::*lacZ* reporter is reduced 100-fold when introduced into the genome of AW1-83 (11) further supports this regulatory scheme (Fig. 1).

It is clear that *phcB* is essential for production of the EF, but its role is still obscure. Invariably, inactivation of *phcB* resulted in mutants that made undetectable amounts of the EF in lid agar assays. Even extracts of AW1-83 culture supernatants that were concentrated 1,000-fold did not contain detectable EF activity (15), whereas supernatants from wild-type cultures are fully active without concentration (11). The biochemical function of PhcB is unknown, and database searches revealed no significant homologs. However, if the EF is indeed 3-OH PAME, then it seems likely that PhcB somehow diverts an intermediate in lipopolysaccharide biosynthesis to produce this 3-hydroxylated fatty acid (27).

Each of the remaining proteins encoded by the putative *phcBSR*(*Q*) operon (PhcS, PhcR, and OrfQ) contains domains that resemble those found in members of two-component systems for signal transduction. PhcS has an amino acid sequence that is typical of sensor proteins, but PhcR appears to be a very unusual response regulator because, besides a conserved phosphate receiver domain, its output domain is homologous to the histidine kinase transmitter region of sensor proteins. Although multiple two-component sensor proteins (e.g., VirA, BvgS, and LemA) have a transmitter domain followed by an apparent receiver domain (32), only PhcR and AsgA have the reverse combination. Although OrfQ is predicted to resemble a response regulator because it has an amino-terminal receiver domain, it lacks an identifiable output domain and did not appear to affect production of PhcA-regulated factors. We believe that *orfQ* is the last gene in this putative operon, because preliminary sequencing of DNA downstream suggests that the adjacent ORF is transcribed in the opposite direction.

The only other protein known to have the same unusual architecture as PhcR is AsgA (33), which is essential for *M. xanthus* cells to produce the extracellular A signal required for proper development of fruiting bodies (23). Because AsgA has both a receiver and a transmitter domain, Plamann et al. (33) postulated that it may function in a phosphorelay system to control A signal production. Subsequent tests were consistent with in vitro autophosphorylation of a histidine residue in AsgA by [γ-^{32}P]ATP, suggesting that this protein has a functional transmitter module (26). The precise role of AsgA in production of A signal is still unknown, because no cognate sensor protein (or other phosphorylating agent) or phosphoryl-receiving protein(s) has been found, and genes directly regulated by AsgA have yet to be identified.

PhcR also likely functions as an intermediary in a signal cascade. Our genetic data indicate that PhcS is likely to be the cognate sensor for PhcR, because, in the absence of PhcB (and thus the EF), both PhcS and PhcR must be present to reduce expression of *phcA* and production of PhcA-regulated factors. PhcS and PhcR must interact as proteins and not as *cis*-acting elements because they function when expressed in *trans* from separate transcriptional units. Presently, we can only speculate on the role of PhcR in signal transduction. One possibility is that phosphorylation of the PhcR receiver domain modulates autophosphorylation of its transmitter domain, which in turn affects an unidentified downstream component of the cascade that (directly or indirectly) reduces expression of *phcA*. It is also possible that phosphorylated PhcR (or another protein phosphorylated by PhcR) interacts directly with PhcA to reduce its ability to activate transcription; since *phcA* is autoregulatory, its expression would also decrease. This model of post-transcriptional modification is consistent with the observation that expression of PhcA-regulated genes varies up to 100-fold despite the relatively constant expression of *phcA* (10). Both scenarios differ from a true phosphorelay system, such as that controlling initiation of sporulation in *Bacillus subtilis* (18).

When contemplating the role of the EF in the PhcSR regulatory system in *R. solanacearum*, there are two likely options. One is that the EF initiates the signal cascade by activating autophosphorylation of PhcS. However, models based on this premise do not fit the data because they incorrectly predict that *phcS* mutants will not produce PhcA-regulated factors. The alternative is that the EF interacts with PhcS to inhibit its autophosphorylation (or to enhance its dephosphorylation), thereby interrupting the signal cascade by preventing subsequent phosphorylation of PhcR. We know of only one other example in which an endogenous fatty acid was suggested to negatively affect the phosphorylation status of a two-component sensor. In that case, in vitro autophosphorylation of KinA,

the major protein kinase that phosphorylates Spo0F in *B. subtilis* (18), was inhibited by the addition of 1 to 10 μM concentrations of certain fatty acids (45). C_{16} to C_{20} *cis*-unsaturated fatty acids (including palmitoleic acid) were the most potent, whereas saturated fatty acids (including palmitic acid) had no effect. Strauch et al. (45) hypothesized that a fatty acid in *B. subtilis* may serve as a growth-dependent inhibitor of KinA, but they presented no data that this occurs in vivo. In contrast, our genetic data suggest that 3-OH PAME and PhcS interact in vivo, justifying a future examination of the nature of that interaction in vitro.

In conclusion, our investigation of the *phcB* locus in *R. solanacearum* revealed that it contains some of the major components of what appears to be a signal cascade that regulates expression of *phcA* or the function of PhcA and thus modulates virulence. Some of the interesting and unusual aspects of this system are as follows. (i) It both produces and responds to the endogenous EF. (ii) This EF likely is a novel signal molecule, because it appears to be a fatty acid methyl ester. (iii) This signal molecule may act by perturbing the phosphorylation status of a two-component sensor protein. (iv) The cognate response regulator has an almost unique architecture that suggests it has an unusual role as an intermediary in signal transduction (and that there may be additional, unidentified system components). (v) The system controls expression of a global regulator of virulence in this phytopathogen. Therefore, further investigation of the *phcBSR*(*Q*) regulatory system in *R. solanacearum* should increase our knowledge of the diverse mechanisms and functions of two-component systems and signal transduction.

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