The Global Virulence Regulator PhcA Negatively Controls the *Ralstonia solanacearum* *hrp* Regulatory Cascade by Repressing Expression of the PrhIR Signaling Proteins^{▽†}

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PhcA positively and negatively regulates many genes responsible for pathogenicity of *Ralstonia solanacearum*. The type III secretion system-encoding *hrp* regulon is one of the negatively controlled operons. PhcA bound to the promoter region of *prhIR* and repressed its expression, demonstrating that PhcA shuts down the most upstream component of a signal transfer system for *hrpB* activation.

Ralstonia solanacearum, a soilborne phytopathogen, causes wilt disease in a wide range of plant hosts throughout the tropical and subtropical world (14, 25). After invasion through root openings, *R. solanacearum* colonizes, multiplies in intercellular spaces, and then invades xylem vessels. The bacteria produce extracellular polysaccharide (EPS) in xylem vessels, and this is believed to be responsible for wilt symptom because large amounts of EPS slime reduce sap flow in xylem vessels (8).

Many gram-negative pathogenic bacteria that infect plants and animals use a type III secretion system (TTSS) to interact with their respective hosts (11, 17). Virulence proteins, so-called type III effectors, are injected from pathogens into the host cytosol via the TTSS needle structure (18). Proteins that compose TTSS are encoded by *hrp* (hypersensitive reaction and pathogenicity) genes, which are clustered and form the *hrp* regulon (29). TTSS is a key pathogenicity determinant, as *R. solanacearum* with TTSS mutations cannot cause disease (2).

Growth in a minimal medium or cocultivation with plant cells induces the expression of the *R. solanacearum* *hrp* regulon via an AraC-type transcriptional regulator, HrpB (12). Plant signals, which are presumed to reside on the surface of plant cells, are perceived by PrhA, which is located on the outer membrane of *R. solanacearum* (20). PrhA transduces signals to the periplasmic domain of the inner membrane protein PrhR, which is an anti-sigma factor. Once PrhR receives the signals, an extracytoplasmic function sigma factor, PrhI, is released from PrhR to the cytoplasm and directs RNA polymerase to transcribe *prhJ* (3). Subsequently, PrhJ, which is a member of the LuxR/UhpA family of transcriptional activators, activates transcription of *hrpG*, and then HrpG induces expression of *hrpB* (4). Moreover, it has been suggested that HrpG activity is increased by metabolic signals perceived in the minimal medium through an as-yet-unknown mechanism (28).

PhcA is a LysR-type transcriptional regulator of EPS synthesis and other virulence factors (6), and its activity is controlled by a quorum-sensing system with a unique autoinducer, 3-hydroxy palmitic acid methylester (10). At high cell densities, activated PhcA induces transcription of *xpsR*, which encodes a positive regulator of EPS production (16). PhcA regulates other virulence-associated activities in addition to EPS production. Synthesis of β -1,4-endoglucanase and pectin methylesterase are positively regulated at high cell densities (27), while synthesis of endopolygalacturonase, a siderophore, and motility by flagella or type IV pili are negatively regulated (1, 5, 9, 26). The expression of the *hrp* regulon is also negatively regulated by PhcA at high cell densities (13). Therefore, *R. solanacearum* has two cell density-dependent phenotypes which are regulated by PhcA, and virulence gene expression controlled by PhcA plays a critical role in successful host plant infection (22). The focus of this work was to analyze regulation mechanism of PhcA for the *hrp* regulon in detail.

PhcA binds to the promoter region of the *prhIR* operon in the *hrp* gene regulatory cascade. The PhcA regulator has been genetically demonstrated to negatively regulate the *hrp* regulon (13). HrpB, the AraC family transcriptional regulator, positively regulates the entire *hrp* regulon (7). Plant signals perceived during the pathogen-host plant interaction are speculated to activate *hrpB* expression through a six-gene regulatory cascade (Fig. 1) (3). We used promoter binding assays to determine the step in the *hrpB* cascade at which PhcA exerts its negative regulation of the TTSS regulon.

An ~1-kb DNA fragment containing *phcA* was amplified from chromosomal DNA of *R. solanacearum* OE1-1 (race 1, biovar 3 [19]) grown in BG medium at 28°C (2) by using PCR with a pair of primers, *phcAA5* and *phcAB4* (see Table S1 in the supplemental material). The amplified fragment was cloned into pET22b(+) (Novagen, Madison, WI) to construct *pphcA5*. *Escherichia coli* BL21(DE3) cells transformed with *pphcA5* were incubated at 37°C in 500 ml of fresh LB medium supplemented with ampicillin. When the cell optical density at 600 nm reached 0.6, IPTG (isopropyl- β -D-thiogalactopyranoside; final concentration, 1 mM) was added, and incubation was continued for two more hours at 28°C. PhcA was purified from these cells using the His tag on its C terminus with

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FIG. 1. Model of *hrp* gene regulatory cascade in *R. solanacearum* induced in response to bacterium-plant cell contact. PhcA is a negative regulator of *hrp* regulon. Lines with arrows represent positive regulation of gene expression. Open arrowheads represent protein translation. A solid arrow indicates cell nutrient status related to growth conditions. O.M., outer membrane; I.M., inner membrane. (Modified from reference 4 with permission of the publisher.)

Ni-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) and HiTrap Q FF (5 ml; GE Healthcare Bio-Sciences, Piscataway, NJ) columns by using ÄKTA prime (GE Healthcare Bio-Sciences). At the final step, PhcA was eluted with a linear gradient of 0.5 M Na_2SO_4 in 10 mM Tris-HCl (pH 7.5), concentrated, and dialyzed against a dialysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 10% glycerol). Three proteins with molecular mass of ca. 40 kDa were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Purified proteins were transferred to a polyvinylidene difluoride membrane. Each of three protein bands was cut out from the membrane and subjected to a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA). The N-terminal amino acid sequences of all three proteins were MVN VDTKLLV, which were identical to the sequence for PhcA. Since PhcA is activated in proportion to cell density in the quorum-sensing system, these proteins might reflect the modified forms of PhcA, although it was heterologously expressed and purified from *E. coli*.

PhcA is demonstrated to bind to the *xpsR* promoter (16). Two biotinylated *xpsR* promoter fragments were used. Biotin-labeled DNA fragments were prepared with a 5'-biotinylated oligonucleotide primer, *xpsRB3B* (see Table S1 in the supplemental material). PCR amplification was performed at 95°C for 4 min followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 2 min. Purified PhcA (4 to 18 pmol) was mixed with biotinylated DNA fragments, and binding to DNA was analyzed using the LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The larger *xpsR* fragment contained the promoter region to which PhcA can bind, and a smaller fragment lacked this region (Fig. 3A) (30). As expected, the purified PhcA bound only to the larger fragment in a concentration-dependent manner (Fig. 3B), indicating that the His-tagged PhcA indeed showed the expected promoter-

FIG. 2. Purification of PhcA. Overexpressed His-tagged proteins were separated by 12.5% SDS-PAGE. Arrows indicate PhcA identified by N-terminal amino acid sequence analysis. Lane 1, purified PhcA; lane M, molecular mass marker. Molecular masses are on the right in kDa.

binding activity. Biotinylated DNA fragments containing promoter regions of *prhA*, *prhIR*, *prhJ*, *hrpG*, and *hrpB* were prepared with primer pairs *prhAB2B/prhAA5*, *prhIB2B/prhIA1*, *prhJB2B/prhRA1*, *hrpGB2B/hpaBB1*, and *hrpBB4B/hrpCA1*, respectively (Fig. 3A; see Table S1 in the supplemental material). Even when an excess amount of PhcA was used, no PhcA binding to promoter regions of *hrpB*, *hrpG*, *prhJ*, and *prhA* was observed. PhcA bound only to the promoter region of *prhIR* in a concentration-dependent manner (Fig. 3C). This result clearly demonstrated that PhcA targets the *prhIR* operon in the *hrp* regulatory cascade. Since the *phcA* mutation had no effect on expression of *prhA* (13) and since *prhA* is constitutively expressed (unpublished data), this conclusion makes sense. PrhIR are the first proteins receiving the host cell signal from PrhA. Once *prhIR* expression is repressed, expression of all the downstream genes, including *hrpB*, could be shut down (Fig. 1), resulting in the absence of *hrp* regulon expression.

Two shifted bands were observed depending on the amount of PhcA, indicating that two possible PhcA binding sites could exist in the promoter region of *prhIR*. The *xpsR* promoter region also contains two PhcA binding sites, one main site and another very weak site (16, 30). Judging from the band density, the two binding sites in the *prhIR* promoter appeared to have identical affinities to PhcA. PhcA regulates several target promoters, including the *xpsR* and *prhIR* promoters (9), and its regulatory actions on each might be different.

Determination of the PhcA binding site in the *prhIR* promoter region. Although we have not determined the precise transcriptional start site, we demonstrated that PhcA bound to a 514-bp fragment upstream from the start codon of *prhIR*. In order to narrow down the PhcA binding site in the *prhIR* promoter region, a series of fragments that had 50-bp deletions and were biotinylated on the 3' end were constructed with

FIG. 3. Gel mobility shift assay for PhcA. (A) Biotinylated promoter DNA fragments are as follows: P_{xpsR1} , *xpsR* containing the PhcA binding region; P_{xpsR2} , *xpsR* lacking this region; P_{hrpB} , *hrpB*; P_{hrpG} , *hrpG*; P_{prhJ} , *prhJ*; P_{prhIR} , *prhIR*; P_{prhA} , *prhA*. The length of the fragment (bp) is shown at the left end of each line. Next to the 3' end is a start codon marked ATG or GTG. (B) Biotinylated *xpsR* promoter fragments were mixed with purified PhcA with C-terminal His tag (0 to 18 pmol), separated on a polyacrylamide gel, and transferred onto a positively charged nylon membrane. The membrane was treated with streptavidin-horseradish peroxidase conjugate, and labeled DNA fragments were visualized with Chemi-Lumi One (Nakarai Tesque, Kyoto, Japan). One microgram of poly(dI · dC) was added as nonspecific competitor DNA. (C) Biotinylated promoter fragments of genes belonging to the *hrp* gene regulatory cascade were incubated with His-tagged PhcA. Promoters P_{hrpB} , P_{hrpG} , P_{prhJ} , P_{prhIR} , and P_{prhA} are shown on the top. Open triangles indicate the DNA fragments forming complexes with PhcA.

primer pairs *prhIB2B/prhIA3*, *prhIB2B/prhIA4*, *prhIB2B/prhIA5*, *prhIB2B/prhIA6*, *prhIB2B/prhIA7*, *prhIB2B/prhIA8*, *prhIB2B/prhIA9*, and *prhIB2B/prhIA10* (Fig. 4A; see Table S1 in the supplemental material), and PhcA binding was analyzed by electrophoretic mobility shift assay. While PhcA bound to *prhIR* promoter fragments ranging from 514 bp (P_{prhIR}) to 153 bp (P_{prhIR8}), no binding to the DNA fragment 106 bp in size (P_{prhIR9}) was observed (Fig. 4B), indicating that the PhcA binding site in the *prhIR* promoter region is located at least between nucleotides -153 and -106 upstream from the start codon (Fig. 4A). We found that more free 153-bp fragment (P_{prhIR8}) remained than free 206-bp fragment (P_{prhIR7}) when these fragments were mixed with PhcA (Fig. 4C). This may reflect, as mentioned before, a possible second PhcA binding site or that the PhcA binding site extends beyond the 153-bp fragment.

Typical LysR-type transcriptional regulators usually repress transcription from their own promoter and activate transcription from a divergent primer. They generally need coinducers, which include extracellular signaling molecules, metabolites, and ions, and are highly specific for a particular LysR-type regulator (24). On the other hand, PhcA does not autoregulate its own promoter and may not require a coinducer. PhcA instead requires an additional activator, the two-component regulator VsrD in the case of positive regulation of *xpsR* (16). A consensus sequence for the binding site of LysR-type regulators, a T-N₁₁-A motif, is located in the promoter region of *xpsR* (15). PhcA is unique among LysR-type regulator proteins because it controls multiple and unlinked genes positively and

negatively (25). In this study, we demonstrated for the first time PhcA binding to the promoter of negatively regulated genes. Although no significant overall similarity between the promoter sequences of positively regulated *xpsR* and negatively regulated *prhIR* was found, the hypothetical recognition site sequence (the interrupted dyadic sequence and T-N₁₁-A motif) was found in the *prhIR* promoter (Fig. 4D). Symmetrical guanines in the two dyad arms that are expected to be directly involved in binding (24) were also identified. Putative -35 and -10 sequences of σ^{70} were found in the promoter region of *prhIR*. However, unlike the positively regulated *xpsR*, the *prhIR* T-N₁₁-A motif overlapped with the -35 sequence (Fig. 4D), suggesting that PhcA binding may prevent formation of active complexes between RNA polymerase and promoters.

The PhcA binding region of *prhIR* promoter is required for inhibition of gene expression. Since PhcA bound to the promoter region of *prhIR* operon in vitro, we investigated whether binding of PhcA to the promoter region would repress *prhIR* gene expression in vivo. First, a *phcA* deletion mutant, RK5035, was constructed as follows. A ~1.7-kb DNA fragment containing the *phcA* open reading frame was amplified from OE1-1 chromosomal DNA by using a pair of primers, *phcAA4* and *phcAB5* (see Table S1 in the supplemental material). The resulting amplified fragment was cloned into a pK18mobsacB vector (23) to construct pphcA21. Since two PstI sites were located in the *phcA* gene, pphcA21 was digested and religated to delete the internal PstI fragment, creating plasmid pphcA22, which was used to construct a *phcA* mutant by using allelic replacement double crossover. DNA fragments containing dif-

FIG. 4. Gel mobility shift assay for PhcA binding to the *prhIR* promoter region. (A) Schematic drawing of *prhIR* promoter deletion. 1, *P_{prhIR}*; 2, *P_{prhIR2}*; 3, *P_{prhIR3}*; 4, *P_{prhIR4}*; 5, *P_{prhIR5}*; 6, *P_{prhIR6}*; 7, *P_{prhIR7}*; 8, *P_{prhIR8}*; 9, *P_{prhIR9}*. The length of fragment (bp) is shown at the left end of each line. Next to the 3' end is a start codon marked GTG. The putative PhcA binding site is indicated by a circle. (B) Biotinylated *prhIR* fragments with several deletions were mixed with 18 pmol of purified His-tagged PhcA and separated by SDS-PAGE. DNA bands were visualized as described for Fig. 3. Numbers above the lanes indicate the promoter fragments represented in panel A. All DNA fragments were incubated with 18 pmol of PhcA. (C) Biotinylated *prhIR* fragments *P_{prhIR7}*, *P_{prhIR8}*, and *P_{prhIR9}* were mixed with either 0 or 18 pmol of purified His-tagged PhcA. Numbers above the lanes indicate the promoter fragments represented in panel A. (D) Nucleotide sequence of the *prhIR* promoter region. 8> and 9> indicate the beginnings of *P_{prhIR8}* and *P_{prhIR9}*, respectively. Putative -35 and -10 sequences of σ^{70} are boxed. A hypothetical recognition site sequence is shown below to illustrate the interrupted dyadic sequence and T-N₁₁-A motif (outlined letters); guanines expected to be directly involved in binding are underlined. The initiation codon is shown in italics.

ferent amounts of the *prhIR* promoter region, *P_{prhIR}*, *P_{prhIR8}*, and *P_{prhIR9}* (Fig. 4A), were cloned into a broad-host-range *lacZ* transcriptional fusion vector, pHRP309 (21), to construct plasmids pprhIR1, pprhIR8, and pprhIR9, respectively. These plasmids, along with the empty vector pHRP309, were put into *R. solanacearum* strains OE1-1 and RK5035 (*phcA*), and promoter activities were measured (Table 1). Background β -galactosidase activities with and without *phcA* were similar (pHRP309; Table 1). When cells carried pprhIR1 or pprhIR8, *prhIR* promoter activity was derepressed only in the *phcA* mutant and increased two- to threefold. In contrast, the promoter activity of pprhIR9-carrying cells was derepressed even in the

TABLE 1. *prhIR* promoter activity in the wild type and the *phcA* mutant

Plasmid	Promoter size (bp) ^a	β -Galactosidase activity (Miller units) ^b			
		OE1-1		RK5035 ^c	
		Mean	Standard error	Mean	Standard error
pprhIR1	514	3,047	689	8,061	1,316
pprhIR8	153	3,272	854	5,726	1,614
pprhIR9	106	9,140	3,561	7,638	1,919
pHRP309	None	1,429	310	1,482	361

^a Size refers to the region upstream from the start codon. The promoter region was fused to *lacZ*.

^b Cells were grown in BG medium up to a cell optical density at 600 nm of 1, and the enzyme activity was measured with SDS-chloroform-treated cells. Mean values and standard errors are in Miller units.

^c RK5035 is a *phcA* deletion mutant of OE1-1.

wild-type background, and no increase in expression was observed for RK5035 (Table 1). This is in good agreement with our in vitro result showing that PhcA bound to promoter fragments in pprhIR1 and pprhIR8 but did not bind to the fragment in pprhIR9 (Fig. 4B). Taken together, these two results suggest that PhcA binds to the promoter region of *prhIR* operon and represses *prhIR* expression in *R. solanacearum*.

Overexpression of *phcA* inhibits TTSS function (9) and reduces *hrp* regulon expression (unpublished data). *hrp* regulon expression and TTSS formation is necessary for the early stage of *R. solanacearum* infection (9), at which point PhcA is inactive because of low pathogen cell density in the intercellular spaces. With the aid of effector proteins secreted by the TTSS, *R. solanacearum* can proliferate and increase cell numbers. At that time, TTSS is no longer required, and in turn, EPS is synthesized. Active PhcA, depending on the quorum-sensing system (10), upregulates EPS production and concomitantly represses the *hrp* regulon. Expression of *prhA*, whose product is speculated to sense signals, is likely to be constitutive (unpublished data). As we have demonstrated in this study, the most efficient way to stop the signal cascade and repress the *hrp* regulon is to reduce the early components PrhIR. This is consistent with the observation that strains with mutations in *prhIR* have no detectable expression of *hrpB* and severely reduced virulence on host plants (3).

Nucleotide sequence accession numbers. The sequences of strain OE1-1 *phcA* and *prhIR*, including the promoter regions, have been deposited in DDBJ under accession numbers AB450919 and AB450920, respectively.

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