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Negative Regulation of *hrp* Genes in *Pseudomonas syringae* by HrpV

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Mutations in the five *hrp* and *hrc* genes in the *hrpC* operon of the phytopathogen *Pseudomonas syringae* pv. syringae 61 have different effects on bacterial interactions with host and nonhost plants. The *hrcC* gene within the *hrpC* operon encodes an outer membrane component of the Hrp secretion system that is conserved in all type III protein secretion systems and is required for most pathogenic phenotypes and for secretion of the HrpZ harpin to the bacterial milieu. The other four genes (in order), *hrpF*, *hrpG*, (*hrcC*), *hrpT*, and *hrpV*, appear to be unique to the group I *hrp* clusters found in certain phytopathogens (e.g., *P. syringae* and *Erwinia amylovora*) and are less well understood. We initiated an examination of their role in Hrp regulation and secretion by determining the effects of functionally nonpolar *nptII* cartridge insertions in each gene on the production and secretion of HrpZ, as determined by immunoblot analysis of cell fractions. *P. syringae* pv. syringae 61 *hrpF*, *hrpG*, and *hrpT* mutants were unable to secrete HrpZ, whereas the *hrpV* mutant overproduced and secreted the protein. This suggested that HrpV is a negative regulator of HrpZ production. Further immunoblot assays showed that the *hrpV* mutant produced higher levels of proteins encoded by all three of the major *hrp* operons tested—HrcJ (*hrpZ* operon), HrcC (*hrpC* operon), and $HrcQ_B$ (*hrpU* operon)—and that constitutive expression of *hrpV* in *trans* abolished the production of each of these proteins. To determine the hierarchy of HrpV regulation in the *P. syringae* pv. syringae 61 positive regulatory cascade, which is composed of HrpRS (proteins homologous with σ^{54}-dependent promoter-enhancer-binding proteins) and HrpL (alternate sigma factor), we tested the ability of constitutively expressed *hrpV* to repress the activation of HrcJ production that normally accompanies constitutive expression of *hrpL* or *hrpRS*. No repression was observed, indicating that HrpV acts upstream of HrpRS in the cascade. The effect of HrpV levels on transcription of the *hrpZ* operon was determined by monitoring the levels of β-glucuronidase produced by a *hrpA′*::*uidA* transcriptional fusion plasmid in different *P. syringae* pv. syringae 61 strains. The *hrpV* mutant produced higher levels of β-glucuronidase than the wild type, a *hrcU* (type III secretion) mutant produced the same level as the wild type, and the strain constitutively expressing *hrpV* in *trans* produced low levels equivalent to that of a *hrpS* mutant. These results suggest that HrpF, HrpG, and HrpT are all components of the type III protein secretion system whereas HrpV is a negative regulator of transcription of the Hrp regulon.

The characteristic ability of many phytopathogenic bacteria to elicit the hypersensitive response (HR) in nonhost plants or to be pathogenic in host plants is dependent on *hrp* and *hrc* genes (2). *hrc* genes represent a subset of the *hrp* genes that have been renamed to reflect their conservation among the type III protein secretion systems of both plant and animal pathogens (6). Among these, *hrcC* has been particularly well studied; it encodes an outer membrane protein that is essential for type III protein secretion and has a primary role in protein translocation across the outer membrane (2, 7, 32). The *hrcC* genes of *Erwinia amylovora* and *Pseudomonas syringae* are flanked by four small genes, which together form the *hrpC* operon. These four genes, *hrpF*, *hrpG*, *hrpT*, and *hrpV*, appear to be characteristic of group I *hrp* clusters, such as those of *P. syringae* and *E. amylovora*, and they are absent from the group II *hrp* clusters of *Ralstonia solanacearum* and *Xanthomonas campestris* pv. vesicatoria (9, 19). Group I and II *hrp* clusters also differ notably in their regulatory components, with group I *hrp* genes being activated by an alternate sigma factor and group II *hrp* genes being activated by an AraC homolog (2).

Essential activities in type III secretion can be ascribed to many of the Hrc proteins, such as HrcC, but less is known about the functions of the Hrp proteins. Notable exceptions are the HrpA, -L, -R, -S, and -Z proteins of *P. syringae*. HrpA is a Hrp-specific pilin (26). HrpR and -S show similarity with σ^{54}-dependent promoter-enhancer-binding proteins, and both are required to activate the σ^{54}-dependent production of HrpL, a sigma factor in the ECF (extracytoplasmic factor) family which activates the expression of other *hrp* genes and many *avr* genes (17). HrpZ is a harpin, a type of protein first reported from *E. amylovora* (31), which can elicit an apparent programmed cell death when infiltrated into the leaves of tobacco and several other plants (15). HrpZ is secreted in culture in a *hrp*-dependent manner from *P. syringae* (15), but the protein does not appear to be the physiological elicitor of the HR: mutations in *hrmA*, an *avr*-like gene, completely block the ability of a functional cluster of *P. syringae hrp* genes to function in *Escherichia coli* to elicit the HR, but they have no effect on HrpZ secretion (1, 3). Avr (avirulence) proteins appear to be the actual elicitors of the HR, and there is compelling evidence that many of these function inside plant cells following delivery by the *P. syringae* Hrp system (11, 22, 28, 30). Whether HrpZ has a primary role as an extracellular component of the Avr protein delivery system is unknown, but its secretion in culture provides an assay for the functioning of the Hrp secretion pathway in *P. syringae*.

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In the accompanying paper, we have shown that mutations in the *P. syringae* pv. syringae 61 *hrpF*, *hrpG*, *hrpT*, and *hrpV* genes result in altered plant reaction phenotypes, with the effects of each mutation being quantitatively different (9). Unexpectedly, expression of *hrpV* in *trans* reduced the ability of wild-type *P. syringae* pv. syringae 61 to elicit the HR, suggesting that HrpV may be a negative regulator of the Hrp regulon. To test this hypothesis and to investigate further the functions of the other genes in the *hrpC* operon, we have determined the effects of mutations in these genes on the production of several Hrp marker proteins and on the secretion of the HrpZ harpin. Subsequently, we also investigated the place of HrpV inhibition in the HrpRS-HrpL regulatory cascade.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Most of the bacterial strains and plasmids used in this study are described in the accompanying paper (9). Additional strains used were *P. syringae* pv. syringae 61–2074 (*hrpL*::Tn*phoA*), *P. syringae* pv. syringae 61–2088 (*hrcU*::Tn*phoA*), and *P. syringae* pv. syringae 61–2095 (*hrpS*::Tn*phoA*) (16). *E. coli* was routinely grown in Luria broth or Terrific broth at 37°C (4). *Pseudomonas* strains were grown in King's B (KB) medium (20) or LM medium (13) at 28 to 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (18), adjusted to pH 5.5, was used at 25°C. Antibiotics were used in selective media at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 50; tetracycline, 10; spectinomycin 50; gentamicin 10; and nalidixic acid, 20.

Recombinant DNA techniques. Restriction endonuclease digestion, agarose gel electrophoresis, DNA fragment preparation, plasmid extraction, DNA ligation, and transformation by the $CaCl_2$ procedure were performed according to standard procedures (4). Plasmids were introduced into bacteria by transformation, electroporation (Gene Pulsar; Bio-Rad, Richmond, Calif.), or triparental mating (10).

Construction of pCPP2385, pCPP2389, and pCPP2383. pCPP2385, which contains a constitutively expressed *hrpL*, was constructed by inserting an ΩSp^r fragment (7) downstream of *hrpL* in pCPP2311. This provided a selectable marker that would be effective in *P. syringae* for pCPP2311, which contains *hrpL* subcloned downstream of the *lac* promoter in pUCP18 (27). pCPP2389 constitutively expresses *hrpRS* and was made by subcloning a 2.2-kb *Bam*HI-*Bgl*II fragment derived from pHIR11 downstream of the *lac* promoter in pCPP30 (5). The 190-bp promoter-active *Sac*I-*Hin*cII fragment upstream of *hrpA* was cloned into a promoterless *uidA* gene in pCPP45 (5) to create the *hrpA′*::*uidA* transcriptional fusion in plasmid pCPP2383.

Preparation of anti-HrcC and anti-$HrcQ_B$ antibodies. HrcC and $HrcQ_B$ were purified from *E. coli* NovaBlue (λDE3) (Novagen, Madison, Wis.) carrying pNCHU316 and pNCHU366, respectively, according to previously described procedures (7). Before immunoblot analysis the antisera were preabsorbed with cell lysate mixtures of *E. coli* and *P. syringae* pv. syringae 61 mutants according to the following modifications of previously used procedures (15). Five-milliliter cultures of *E. coli* and *P. syringae* pv. syringae 61 mutants (*P. syringae* pv. syringae 61–N393 and *P. syringae* pv. syringae 61–N322 for incubation with HrcC and $HrcQ_B$ antibodies, respectively) were grown overnight to stationary phase, washed several times with the same volume of 1× phosphate-buffered saline (0.058 M Na_2HPO_4, 0.017 M $NaH_2PO_4 \cdot H_2O$, 0.068 M NaCl), sonicated, incubated at 100°C for 10 min, and then allowed to cool to room temperature. Two hundred microliters of the corresponding antiserum and sodium azide at a final concentration of 0.02% were added into the cell lysate and incubated at room temperature for 2 h. The mixtures were centrifuged at 20,000 × *g* for 30 min, and the preabsorbed antisera were collected from the supernatant.

Immunoblot analysis of the expression of HrpZ, HrcC, HrcJ, and $HrcQ_B$ proteins. Wild-type *P. syringae* pv. syringae 61 and *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants were grown in 5 ml of KB broth at 30°C to an optical density at 600 nm (OD_{600}) of 0.5. The cells were collected by centrifugation, washed once in 5 ml of Hrp minimal medium, resuspended in 5 ml of the same medium, and incubated with shaking for 5 h. The cell and supernatant fractions were then separated by centrifugation. HrpZ expression was analyzed as described previously (24) or as outlined below. The supernatants were precipitated with trichloroacetic acid at a final concentration of 5%, washed with acetone, dissolved in 30 μl of 10 mM Tris buffer (pH 8.0), and boiled for 5 min after an equal volume of 2× loading buffer (0.625 M Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 2% β-mercaptoethanol) was added. The cell pellets were washed with 10 mM Tris buffer (pH 8.0), resuspended in 125 μl of 10 mM Tris buffer, and boiled with an equal volume of 2× loading buffer for 5 min. A 20-μl sample of each fraction was subjected to SDS-10% polyacrylamide gel electrophoresis in a 0.75-mm-thick gel in a Mighty Small apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The prestained protein markers—ranging from 175.0 to 6.5 kDa—were from Bio-Rad. After separation, the protein bands were transferred to Immobilon-P membranes (Millipore Inc., Bedford, Mass.) in a TE70 semidry transfer unit (Hoefer Scientific Instruments) for 40 to 60 min. The membranes were probed with polyclonal anti-HrpZ, anti-HrcJ, anti-HrcC, anti-$HrcQ_B$, or anti-NPTII antibodies individually. Anti-NPTII antibodies (5 Prime→3 Prime, Inc., Boulder, Colo.) were used as an internal control to standardize the protein concentration of each lane. Immunodetection of the bands was performed by alkaline phosphatase-based chromogenic and chemiluminescent assays with Sigma Fast BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium tablets (0.15 mg of BCIP/ml and 0.3 mg of nitroblue tetrazolium/ml) or 0.25 mM disodium 2-chloro-5(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo(3.3.1.1^{3,7})decan}-4-yl)-1-phenyl phosphate (CDP-Star; Tropix Inc., Bedford, Mass.), respectively. Membranes were exposed for 2 to 15 min to OMAT X-ray film (Kodak, Rochester, N.Y.) for visualization of chemiluminescence.

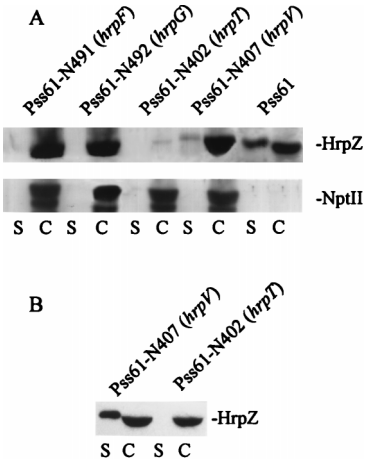

FIG. 1. Effects of *nptII* cartridge insertions in the *P. syringae* pv. syringae 61 (Pss61) *hrpF*, *hrpG*, *hrpT*, and *hrpV* genes on the production and secretion of HrpZ. (A) Bacteria in early-logarithmic-phase growth in KB medium were harvested by centrifugation and resuspended at an OD_{600} of 0.5 in Hrp minimal medium. After a further 5 h of incubation, the cultures were fractionated into cell (C) and supernatant (S) fractions by centrifugation and assayed for the accumulation of HrpZ and NptII by SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with appropriate antibodies and chemiluminescent immunodetection. (B) Bacteria were grown to stationary phase in KB medium and then handled as described above.

Measurement of β-glucuronidase activity. Transcription of the *hrpZ* operon was determined by measuring the expression of β-glucuronidase from the *hrpA′*::*uidA* transcriptional fusion plasmid pCPP2383. *P. syringae* pv. syringae 61 derivatives carrying pCPP2383 were grown in KB medium to stationary phase and then transferred to Hrp minimal medium at an initial OD_{600} of 0.5. The bacteria were harvested 0, 4, and 10 h later for determination of β-glucuronidase activity and total protein concentration. β-Glucuronidase activity was estimated with 4-methylumbelliferyl-β-D-glucuronide and a TKO 100 minifluorometer (Hoefer Scientific Instruments) according to the procedure outlined in the operating instructions. Total protein concentration was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.). Specific activity is presented in nanomoles of methylumbelliferone production per milligram of total protein per minute.

RESULTS

***nptII* cartridge insertions in *hrpF*, *hrpG*, *hrpT*, and *hrpV* result in three classes of mutants that are altered in their HrpZ production and HrpZ secretion phenotypes.** Functionally nonpolar *nptII* cartridge insertions were constructed previously in each gene in the *P. syringae* pv. syringae 61 *hrpC* operon (9). To determine the effects of mutations in *hrpF*, *hrpG*, *hrpT*, and *hrpV* on HrpZ production and secretion, we performed an immunoblot analysis of HrpZ levels in the cell and supernatant fractions of cultures that were in logarithmic growth in Hrp minimal medium 5 h after being shifted from logarithmic growth in complex KB medium (Fig. 1A). Three classes of mutants could be discerned: (i) *hrpF* and *hrpG* mutants were unable to secrete HrpZ, (ii) the *hrpT* mutant had strongly reduced HrpZ production, and (iii) the *hrpV* mutant overproduced HrpZ and secreted at least some of the protein.

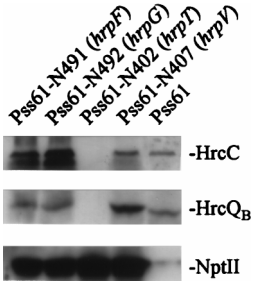

FIG. 2. Effects of *nptII* cartridge insertions in the *P. syringae* pv. syringae 61 (Pss61) *hrpF*, *hrpG*, *hrpT*, and *hrpV* genes on *hrcC* and *hrcQ*$_B$ expression. Cell fractions of cultures grown for 5 h in Hrp minimal medium, as described in the legend to Fig. 1A, were assayed for the accumulation of HrcC and HrcQ$_B$ by SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with appropriate antibodies and chemiluminescent immunodetection.

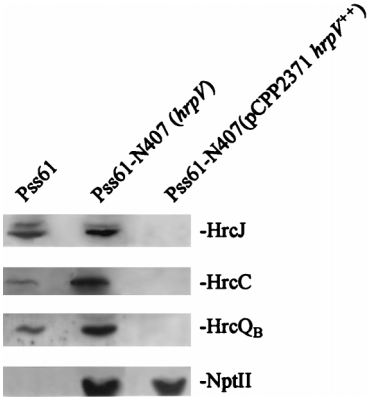

FIG. 3. Effects of mutating or overexpressing *hrpV* on the accumulation of Hrc proteins encoded by three different *hrp* operons. Bacteria grown for 5 h in Hrp minimal medium, as described in the legend to Fig. 1A, were assayed for the accumulation of HrcJ, HrcC, and HrcQ$_B$ by SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with appropriate antibodies and chemiluminescent immunodetection. Pss61, *P. syringae* pv. syringae 61.

NptII expressed from its cognate promoter in the cartridge inserted in each mutant was used as a control for constitutive gene expression and for nonspecific leakage of cytoplasmic proteins to the medium.

In repeated experiments we observed variability in the phenotypes of the *hrpV*, *hrpT*, and *hrpG* mutants that appeared to correlate with the duration of previous growth in complex media. When grown to higher culture densities, the *hrpG* mutant produced low levels of HrpZ whereas the *hrpT* and *hrpV* mutants produced and secreted more HrpZ, respectively. The phenotypes of *hrpT* and *hrpV* mutants grown to stationary phase in KB medium before being shifted to Hrp minimal medium are shown in Fig. 1B. The *hrpG* mutant produced no detectable HrpZ in this experiment (not shown), and we chose to employ the culture conditions described in the legend to Fig. 1A in subsequent experiments. In light of this variability, it is important to note that regardless of culture conditions, the *hrpV* mutant always secreted at least some HrpZ whereas the *hrpT* mutant did not. These data suggested that HrpT contributes to HrpZ secretion, whereas HrpV does not. The reduced production of HrpZ by the Δ*hrpT*::*nptII* mutant suggested that HrpV was a negative regulator that was being overexpressed from the proximal, upstream *nptII* promoter. The apparent overexpression of HrpZ that was observed in the *hrpV* mutant (Fig. 1A) further supported this hypothesis.

Effects on HrcC and HrcQ$_B$ accumulation of *nptII* cartridge insertions in *hrpF*, *hrpG*, *hrpT*, and *hrpV* further indicate that HrpV is a negative regulator of *hrp* expression. To determine the effects of *nptII* cartridge insertions within the *hrpC* operon on the production of other components of the Hrp system, immunoblot analysis of HrcC and HrcQ$_B$ (encoded by the *hrpU* operon) was performed on the cell fractions of various mutants grown to early logarithmic phase in complex medium and then in minimal medium (Fig. 2). Again, three classes of mutants could be discerned: (i) *hrpF* and *hrpG* mutants (with *nptII* insertions upstream of *hrcC*) produced higher levels of HrcC but wild-type levels of HrpQ$_B$, (ii) the *hrpT* mutant produced no detectable HrcC or HrpQ$_B$, and (iii) the *hrpV* mutant produced higher levels of both HrcC and HrcQ$_B$ than did wild-type *P. syringae* pv. syringae 61. NptII levels provided an internal control against protein loading variability that would account for these differences. These observations indicated that in early-logarithmic-phase growth in Hrp minimal medium, certain *P. syringae* pv. syringae 61 *hrpC* operon mutants—*P. syringae* pv. syringae 61–N491 (Δ*hrpF*::*nptII*) and *P. syringae* pv. syringae 61–N492 (*hrpG*::*nptII*)—accumulate more HrcC through expression of the *nptII* promoter than through expression of the native promoter, and they further support the notion that overproduction of HrpV represses *hrp* expression whereas disruption of *hrpV* increases *hrp* expression.

Constitutive expression of *hrpV* in *trans* represses production of proteins encoded by three of the major *hrp* operons. To directly test the effects of altered levels of *hrpV* expression on regulation of the *hrp* system, we examined the accumulation of the products of three of the major *hrp* secretion operons in *P. syringae* pv. syringae 61 cells that were either wild type, deficient in *hrpV*, or overexpressing *hrpV* (Fig. 3). Overexpression of *hrpV* was achieved by transforming Δ*hrpV*::*nptII* mutant *P. syringae* pv. syringae 61–N407 with pCPP2371, which has the *hrpV* gene expressed from the *nptII* promoter in vector pML122. HrcC, HrcQ$_B$, and HrcJ, which are products of the *hrpC*, *hrpU*, and *hrpZ* operons, respectively, were equally affected by these changes in *hrpV* expression. That is, accumulation of the three Hrc proteins was higher in *P. syringae* pv. syringae 61–N407 than in the wild type and almost undetectable in *P. syringae* pv. syringae 61–N407(pCPP2371).

Constitutive expression of *hrpL* or *hrpRS* blocks the repressive effects of *hrpV* overexpression. To determine at what level in the *hrp* regulatory cascade HrpV acts, *hrpV* was overexpressed in cells containing constitutively expressed *hrpL* or *hrpRS*, and the resulting levels of HrcJ production were determined (Fig. 4). HrcJ was analyzed because it is encoded by the *hrpZ* operon but its accumulation is not subject to apparent posttranscriptional regulation, as has been observed with HrpZ (7). Constitutive expression of *hrpL* or *hrpRS* results in constitutive expression of *hrp* genes under the normally repressive conditions of growth in complex media (34). To rigorously test the possibility that HrpV was an anti-sigma factor acting on HrpL we introduced plasmids into *P. syringae* pv. syringae 61–2074 (*hrpL*::Tn*phoA*) that were designed to produce much higher levels of HrpV than HrpL. *hrpL* was expressed from the *lac* promoter in pUCP18, which is relatively weak in *Pseudomonas* spp., while *hrpV* was expressed from the *nptII* promoter in pML122, which is much stronger (21). This combination of copy number and promoter strength resulted in high production of HrpV, which was visible as a unique band of the predicted size on a Coomassie blue-stained SDS-polyacrylamide gel (data not shown). Nevertheless, HrpV did not reduce HrcJ production in the presence of HrpL, indicating that HrpV acts upstream of HrpL in the regulatory cascade (Fig. 4A). Simi-

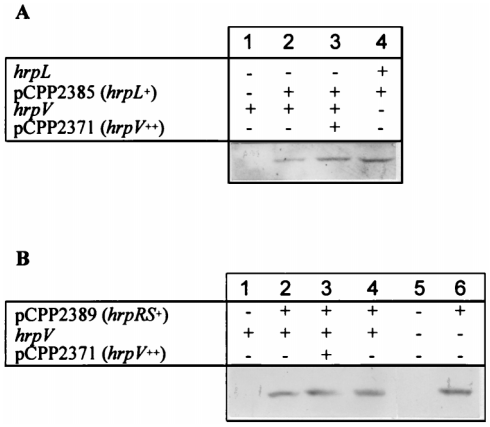

FIG. 4. Prevention of *hrpV*-dependent inhibition of HrcJ accumulation by constitutive expression of *hrpL* and *hrpRS*. All bacteria were grown in LM medium and assayed for the accumulation of HrcJ by immunoblot analysis, as described in the legend to Fig. 1, but with chromogenic immunodetection. Cultures denoted *hrpL*− carried a chromosomal *hrpL*::Tn*phoA* mutation; those denoted *hrpV*− carried a Δ*hrpV*::*nptII* mutation. (A) Lanes: 1, *P. syringae* pv. syringae 61–2074; 2, *P. syringae* pv. syringae 61–2074(pCPP2385); 3, *P. syringae* pv. syringae 61–2074(pCPP2385/pCPP2371); 4, *P. syringae* pv. syringae 61–N407(pCPP2385). (B) Lanes: 1, *P. syringae* pv. syringae 61; 2, *P. syringae* pv. syringae 61(pCPP2389); 3, *P. syringae* pv. syringae 61(pCPP2389/pCPP2371); 4, *P. syringae* pv. syringae 61(pCPP2389/pML122); 5, *P. syringae* pv. syringae 61–N407; 6, *P. syringae* pv. syringae 61–N407(pCPP2389).

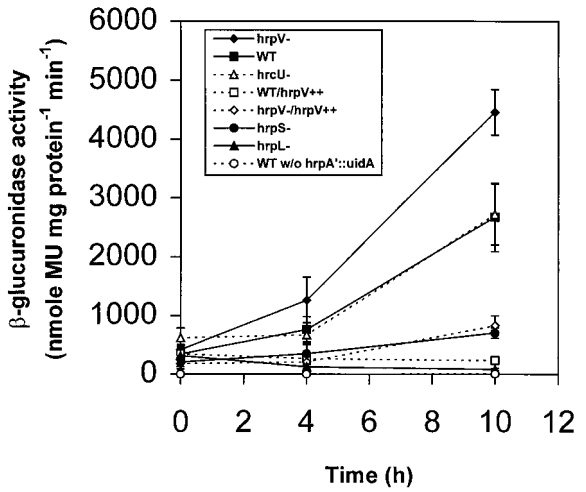

FIG. 5. Effects on *hrpA′*::*uidA* expression of altering the levels of expression of *hrpV*, *hrpL*, and *hrpS*. Bacteria were grown in KB medium to stationary phase and transferred to Hrp minimal medium at an initial OD_{600} of 0.5. β-Glucuronidase activity and total protein concentration were measured at the indicated times as described in the text. The values represent the means of three replicates. The vertical lines indicate standard errors, and where they are absent, the limits were within the symbol dimensions. Except where noted, all strains carried pCPP2383 (*hrpA′*::*uidA*). The strains tested were *P. syringae* pv. syringae 61–N407 (*hrpV*−), *P. syringae* pv. syringae 61–2088 (*hrcU*−), *P. syringae* pv. syringae 61 (WT), *P. syringae* pv. syringae 61–N407(pCPP2371) (*hrpV*−/*hrpV*++), *P. syringae* pv. syringae 61–2095 (*hrpS*−), *P. syringae* pv. syringae 61(pCPP2371) (*hrpV*++), *P. syringae* pv. syringae 61–2074 (*hrpL*−), and *P. syringae* pv. syringae 61 without pCPP2383 (WT w/o *hrpA′*::*uidA*). MU, methylumbelliferone.

larly, *hrpV* failed to repress the accumulation of HrcJ that resulted from constitutive expression of *hrpRS* (Fig. 4B). Constitutive expression of *hrpL* or *hrpRS* also overcame the repressive effects of *hrpV* overexpression by pCPP2371 when the bacteria were grown in Hrp minimal medium rather than LM medium (data not shown). It is also important to note that Δ*hrpV* mutant *P. syringae* pv. syringae 61–N407 produced no detectable HrcJ in Hrp-repressive LM medium (Fig. 4B). These observations suggest that the step at which *hrpV* interferes with *hrp* gene expression lies upstream of the *hrpRS* and *hrpL* induction cascade.

Constitutive expression of *hrpV* represses transcription of the *hrpZ* operon. The previous experiments were based on the differential accumulation of HrpZ and three Hrc proteins, particularly HrcJ, which is in the *hrpZ* operon. To verify that overexpression of HrpV represses transcription of the *hrpZ* operon, as opposed to acting posttranscriptionally, we constructed plasmid pCPP2383 (*hrpA′*::*uidA*), in which the promoter of the *hrpZ* operon is transcriptionally fused to a *uidA* gene, and analyzed β-glucuronidase activity in *P. syringae* pv. syringae 61 derivatives that carried the plasmid and were altered in their production of HrpV and other components of the Hrp system (Fig. 5). Deletion of *hrpV* resulted in levels of *hrpA′*::*uidA* expression significantly higher than that in the wild type, whereas overexpression of *hrpV* resulted in a reduction in *hrpA′*::*uidA* expression to a level equivalent to that of *hrpL* and *hrpS* mutants. *P. syringae* pv. syringae 61–2088, a *hrcU*::Tn*phoA* mutant that fails to export HrpZ out of the cytoplasm (7), expressed *hrpA′*::*uidA* at wild-type levels. Thus, the effects of *hrpV* on the accumulation of two products of the *hrpZ* operon, HrpZ and HrpJ, that were observed as described above can be attributed to the transcription of the operon.

DISCUSSION

By examining the effects of *nptII* cartridge insertions in the four small genes that flank *hrcC* in the *hrpC* operon, we have learned that *hrpF*, *hrpG*, and *hrpT* have roles in HrpZ secretion, whereas *hrpV* encodes a negative regulator of *hrp* gene expression. These observations raise several questions regarding the plant reaction and biochemical phenotypes of these genes, the functions of their products in Hrp secretion and regulation, and the global control of the *hrp* regulon.

To properly interpret the phenotypes of these mutants, it is necessary to consider the effects of the *nptII* cartridge that was used to construct them. The cartridge lacks a transcription terminator, thus permitting *nptII* and downstream genes to be driven by the cartridge promoter and enabling the construction of mutants in complex media that support robust bacterial growth but poor *hrp* expression. The efficacy of this approach for testing the functions of individual genes is supported by the observation that appropriate single-gene subclones can restore wild-type HR elicitation activity to *nptII*-marked *hrpF*, *hrpG*, *hrpT*, and *hrcC* mutants (9). These subclones can similarly restore the ability to secrete HrpZ (data not shown). However, constitutive expression of downstream genes can have unanticipated effects, as we have found here with *hrpV* and will discuss further below.

It is also noteworthy that the plant reaction phenotypes of some of these *hrpC* operon mutations, reported in the accompanying paper (9), are not as strong as their HrpZ secretion phenotypes. We anticipated that any mutation preventing HrpZ secretion would have a strong Hrp phenotype, as was observed with *hrpF* and *hrcC*. However, the *hrpG* and *hrpT* mutants retained some ability to elicit the HR in tobacco and disease in beans. Since the physiological elicitors of the HR are now thought to be Avr proteins that are transferred into plant cells by the Hrp system, one explanation is that these mutations do not completely inhibit that process. At present, this possibility is not testable because there is no assay for *P. syringae* Avr protein secretion in culture or for quantitative Avr protein transfer in planta.

The functions of HrpF, HrpG, and HrpT in the Hrp secretion pathway are unknown. Although they are not present in animal-associated bacteria, they are conserved among different pathovars of *P. syringae* (9) and are found in other phytopathogenic bacteria, such as *E. amylovora* (19), suggesting that they have specific functions in plant pathogenesis. The amino acid sequence of HrpT reveals that it is a putative lipoprotein, and it may have a role as a HrcC chaperone. This notion is based on two observations: (i) homologs of HrcC that are involved in type II secretion require a similarly small lipoprotein chaperone for insertion into the outer membrane (14) and (ii) group II *hrp* clusters lack a lipoprotein like HrpT, and their HrcC proteins lack the C-terminal region that is thought to interact with these chaperones, whereas the HrcC proteins of group I *hrp* systems possess this region (8). However, the biological function of HrpT is yet to be determined in these bacteria.

HrpV appears to be a negative regulator of the HrpR/S-HrpL activator cascade and is the first negative regulator reported for the *P. syringae* Hrp system. However, in assessing its role it is important to note that overexpression of *hrpV* eliminates expression of the Hrp regulon, whereas deletion of *hrpV* results only in moderate increases in *hrp* expression. One explanation for this would be the presence of a functionally equivalent copy of *hrpV* elsewhere in the genome. This would be analogous to the two recently reported negative regulators, YscM1 and YscM2, of the Yop virulon (which includes the type III secretion system) in *Yersinia enterocolitica* (29). To seek a second copy of *hrpV*, we probed *P. syringae* pv. syringae 61 total DNA with PCR-amplified *hrpV* genes in a DNA gel blot at moderate stringency, but we observed no extra hybridizing bands (data not shown).

We can postulate three potential functions for HrpV as a negative regulator. First, HrpV may be a negative-feedback regulator preventing overproduction of HrcC. The location of *hrpV* at the end of the *hrpC* operon is consistent with this hypothesis, as is the observation that the *X. campestris* pv. vesicatoria HrcC (HrpA1) protein induces the phage shock protein operon when expressed in *E. coli* (32).

A second potential function for HrpV is to delay the expression of other *hrp* operons until the channel-forming HrcC multimers have formed in the outer membrane. This model is based on the concept that the type III protein secretion system has evolved through the recruitment of two separate translocators: a flagellum export-derived system for translocation across the inner membrane (encoded by the *hrpJ* and *hrpU* operons) and HrcC for translocation across the outer membrane. We have previously shown that *P. syringae* pv. syringae 61 *hrcC* mutants accumulate some HrpZ in the periplasm, whereas mutants affected in the *hrpJ* and *hrpU* operons accumulate HrpZ only in the cytoplasm (7). This suggests that premature expression of the *hrpJ* and *hrpU* operons could result in deleterious localization of some Hrp and Avr proteins in the periplasm. Supporting this hypothesis is the observation that *hrcC* (*hrpA1*) in *X. campestris* pv. vesicatoria is activated by HrpG, which is higher in the regulatory cascade than HrpX, which in turn activates the other *hrp* genes (33). If HrpV preferentially represses either the *hrpC* operon or the *hrpJ* and *hrpU* operons, this must be determined by factors in addition to HrpL that affect the expression of these operons.

A third potential function for HrpV is to increase expression of the Hrp regulon upon contact with host cells through removal of the protein from the bacterial cell. This would be analogous to the host contact-dependent secretion of LcrQ, a negative regulator of *yop* and *ysc* expression, by *Yersinia* spp. (23). If HrpV can be secreted, this does not appear to happen in culture, because mutations blocking Hrp secretion do not inhibit *hrp* expression, as would be expected with accumulation of a normally secreted negative regulator. Furthermore, there is no consensus that *P. syringae hrp* expression is higher in planta than in minimal media that mimic plant intercellular fluids (25, 35). However, any increase in *hrp* expression in planta may be hard to detect if it is transient or of moderate magnitude. Support for the hypothesis that HrpV can be secreted is found in the puzzling observation that HrpV overexpression has a relatively minor effect on elicitation of the HR in tobacco leaves (9) whereas it virtually abolishes *hrp* expression in culture. This would be explainable if the HrpV pool could be secreted in planta but not in culture. Regardless of which hypothesis is correct, it is important to note that the *hrpV* mutation does have an effect on the pathogenesis of *P. syringae* pv. syringae 61 in beans (9).

Our data indicate that HrpV acts upstream of HrpRS. Several aspects of HrpRS remain puzzling. There is controversy regarding whether HrpS alone or both HrpR and HrpS are required to activate *hrp* expression (12, 34) and whether the *hrpRS* operon is expressed at a higher level in planta than in culture under Hrp-derepressing conditions (25, 35). The observation that the N-terminal domains associated with regulation in related proteins are missing from HrpR and HrpS suggests that the levels of active HrpRS may be key in regulating *hrp* expression, and our data provide indirect evidence that high levels of HrpV inhibit HrpRS production. Key questions for the future are whether HrpV directly controls expression of the *hrpR* promoter, whether bacteria in planta secrete HrpV and thereby increase *hrp* gene expression, and why *hrpV* mutants overexpressing the Hrp system are impaired in pathogenesis. Given the complex functions of the Hrp pathway in protein secretion and pathogenesis, it is not surprising that there is at least one negative regulator controlling the system.

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