The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*

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Bacterial surface appendages called pili often are associated with DNA and/or protein transfer between cells. The exact function of pili in the transfer process is not understood and is a matter of considerable debate. The Hrp pilus is assembled by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000. In this study, we show that the *hrpA* gene, which encodes the major subunit of the Hrp pilus, is required for secretion of putative virulence proteins, such as HrpW and AvrPto. In addition, the *hrpA* gene is required for full expression of genes that encode regulatory, secretion, and effector proteins of the type III secretion system. *hrpA*-mediated gene regulation apparently is through effect on the mRNA level of two previously characterized regulatory genes, *hrpR* and *hrpS*. Ectopic expression of the *hrpRS* gene operon restored gene expression, but not protein secretion, in the *hrpA* mutant. Three single amino acid mutations at the HrpA carboxyl terminus were identified that affect the secretion or regulatory function of the HrpA protein. These results define an essential role of the Hrp pilus structural gene in protein secretion and coordinate regulation of the type III secretion system in *Pst* DC3000.

Many Gram-negative bacterial pathogens, including *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, possess a unique protein secretion system called the type III protein secretion system that transfers virulence proteins directly into the host cell (1–3). This system has been shown to play a critical role in bacterial infection of plants, animals, and humans. Genes involved in type III protein secretion have been characterized extensively in several human, animal, and plant bacteria (1–3). However, the actual mechanism by which virulence proteins are transferred into the host cell is poorly understood. Several general features of type III protein secretion have been revealed: (*i*) type III protein secretion appears to be activated fully upon contact with the host cells *in vivo* (4, 5); (*ii*) extracellular filamentous appendages often are associated with type III protein secretion (6–8); (*iii*) a secretion signal is localized in the 5′ region of the mRNA of the secreted protein (9–11); and (*iv*) the secretion apparatus is genetically and morphologically similar to the bacterial flagellum (12).

In plant pathogenic bacteria the type III protein secretion system (also called the Hrp secretion pathway or system) is encoded by *hrp* (for hypersensitive reaction and pathogenicity) genes (2, 13, 14). Nine *hrp* genes have been renamed *hrc* (for *hrp* genes conserved) because of their broad conservation among all bacteria that harbor type III protein secretion systems (15). The Hrp secretion system of *Pseudomonas syringae* has been shown to secrete two families of proteins that elicit host responses: harpins, such as HrpZ and HrpW (16–18), and Avr proteins (19, 20). The expression of *P. syringae hrc/hrp* genes is tightly controlled. Most *hrp* genes are expressed at a very low level in standard, nutrient-rich medium. The expression of *hrc/hrp* genes is induced in infected plant tissues or in artificial *hrp*-inducing minimal media that presumably mimic the *in planta* conditions (21–23). Three intracellular positive regulatory proteins are required for expression of *hrc/hrp* genes: HrpR and HrpS, which belong to the NtrC family of two-component regulatory proteins (24–26), and HrpL, a member of the ECF (extracytoplasmic factor) family of alternate σ factors (27). The HrpS, HrpR, and HrpL proteins appear to function as a regulatory cascade in which HrpS and HrpR activate the expression of HrpL in response to a signal in host tissue or in *hrp*-inducing minimal medium (25, 26). HrpL is presumed to activate all *hrp* and *avr* genes by recognizing a consensus sequence motif (“harp box”) present in the upstream regions of many *hrp* and *avr* genes (26, 27). Recently, a putative negative regulator encoded by the *hrpV* gene has been identified in *P. syringae* (28). In *hrp*-inducing minimal medium, overexpression of the *hrpV* gene down-regulates *hrp/hrc* gene expression, whereas a *hrpV* mutant is elevated in *hrp/hrc* gene expression (28).

In a previous study, we found that *Pst* strain DC3000 assembles a *hrp*-dependent pilus (the Hrp pilus) (6). We showed that the Hrp pilus structural protein, HrpA, is required for *Pst* DC3000 to cause disease in *Arabidopsis* and to elicit the hypersensitive response (HR) in tobacco and tomato (6). Pili also have been shown to be required for bacterial conjugation (29) and for transfer of T-DNA to plant cells by *Agrobacterium tumefaciens* (30). The conjugative F pilus plays a major role in mediating contact between donor and recipient bacteria during mating (29). However, whether pili have other functions in protein and/or DNA transfer is not clear and is a matter of considerable debate. In this study, we show that the *hrpA* gene plays a key role in secretion of Hrp and Avr proteins in culture.

Materials and Methods

Bacterial Strains and Culture Conditions. *Pst* DC3000 and four *hrp* mutant derivatives (*hrpA*$^-$, *hrpS*$^-$, *hrcC*$^-$, and *hrcC*$^-$ *hrpT*$^-$*V*$^-$) were used in this study. The *hrpA*, *hrpS*, and *hrcChrpTV* mutants were made in previous studies (17). The *hrpA* mutant does not make the major structural protein of the Hrp pilus (6). The *hrpS* regulatory mutant is defective in expression of *hrc/hrp* genes (17). The *hrcChrpTV* mutant is defective in protein secretion (17). The *hrcChrpTV* mutant was used as a secretion mutant

Abbreviations: *Pst, Pseudomonas syringae* pv. *tomato*; hrp, hypersensitive reaction and pathogenicity; HR, hypersensitive response.

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control in early experiments (Fig. 1). Because the Tn*5Cm* insertion has a polar effect on the downstream negative regulatory gene *hrpV* in the *hrcChrpTV* mutant, a *hrcC* deletion mutant was constructed in this study and was used in most experiments presented here.

pHRPA (6) and pHRPCT (this study) contain the *hrpA* gene and the *hrcC* and *hrpT* genes, respectively, downstream of the *lac* promoter of pUCP18 (31). pHRPRS1 carries the *P. syringae* pv. *syringae* 61 *hrpRS* ORFs under the control of the salicylic acid-inducible P_G promoter in pKMY299 (32), and it complemented the *hrpS* mutation in *Pst* DC3000 (Table 1). pHRPRS2 carries the *Pst* DC3000 *hrpRS* ORFs under the control of P_{lac} in pUCP18, and it also complemented the *hrpS* mutation (Table 1).

Pst strains were grown at 22–28°C in King's medium B (33) or LB (34). For induction of *hrp* genes, bacteria were grown at 20°C in *hrp*-inducing minimal liquid or agar medium (6). *Escherichia coli* DH5α was used for all cloning experiments; it was grown in LB at 37°C. Antibiotics used were 100 mg/liter rifampicin, 100 mg/liter ampicillin, 34 mg/liter chloramphenicol, and 50 mg/liter kanamycin.

Protein and RNA Analysis. For immunoblot analysis of Hrp/Hrc proteins in culture, bacteria first were grown at 28°C to an OD_{600} of 0.5–0.8 in 5 ml of LB broth supplemented with appropriate antibiotics. Bacteria then were pelleted and resuspended in 5 ml of *hrp*-inducing broth or LB and incubated with shaking (250 rpm) at 20°C for 12 hr. Cultures then were separated into cell (C) and supernatant (S) fractions by centrifugation. The cell fraction was resuspended in 0.5 ml of sterile water, whereas the supernatant fraction was concentrated 10-fold with Microcon 10 microconcentrators (Amicon). Hrp/Hrc proteins in these fractions were analyzed by SDS/PAGE followed by immunoblotting with appropriate antibodies as described before (17). For immunoblot analysis of Hrp/Hrc proteins expressed *in planta*, bacteria were grown in LB at 28°C to an OD_{600} of 0.8. Bacterial suspensions (OD_{600} of 1.5) prepared in distilled water were infiltrated into leaves of tobacco by using needleless syringes. Six hours after infiltration, the infiltrated leaf tissue was excised and bacteria were expelled from the infiltrated leaf tissue by reinfiltrating the leaf tissue with an excess amount of distilled water. Bacteria were collected by centrifugation, and the levels of Hrp/Hrc proteins in the expelled bacteria were analyzed by immunoblotting with appropriate antibodies. Immunoblot analysis of AvrPto followed the protocol of van Dijk *et al.* (20). In all immunoblotting experiments, gel staining with Coomassie brilliant blue R-250 was used to ensure equal loading of samples. For determination of steady-state levels of *hrp* and *avr* transcripts, total RNA was isolated from bacteria grown in LB or *hrp*-inducing minimal medium for 4 hr at 20°C by following a standard protocol (34). Five micrograms of total RNA isolated from each bacterial strain was analyzed by Northern blot.

Mutagenesis of the *hrpA* Gene. For site-directed mutagenesis, the *hrpA* gene from pHRPA (6) was cloned into pAlter1 (Promega). Six amino acid residues (i.e., G^{23}, A^{54}, K^{93}, D^{95}, I^{101}, and I^{111}) that are conserved between the HrpA proteins of *P. syringae* pv. *tomato* and *Erwinia amylovora* and/or among the HrpA proteins of *P. syringae* pvs. *tomato*, *syringae*, and *glycinea* (35, 36) were replaced individually by residues with different properties (A^{23}, E^{54}, I^{93}, S^{95}, T^{101}, and Pro^{111}). The mutagenized *hrpA* inserts then were recloned into pUCP18 (31) and examined for complementation of the *hrpA* deletion mutation (see ref. 6 for procedures). For random mutagenesis of the *hrpA* gene, pHRPA was transformed into the *E. coli* mutator strain XL1-Red (Stratagene). After being subcultured four times, each for 12 hr at 37°C, pHRPA was isolated from XL1-Red and introduced *en masse* into the *Pst* DC3000 *hrpA* nonpolar mutant by electroporation. Transformants were grown individually in LB in microtiter plate wells overnight at 28°C ($OD_{600} > 1.0$). The overnight bacterial cultures were diluted 10-fold in distilled water to an OD_{600} of 0.1–0.2, and the diluted cultures were infiltrated into tobacco and *Arabidopsis* leaves for HR and pathogenesis tests, respectively (see ref. 6 for procedures).

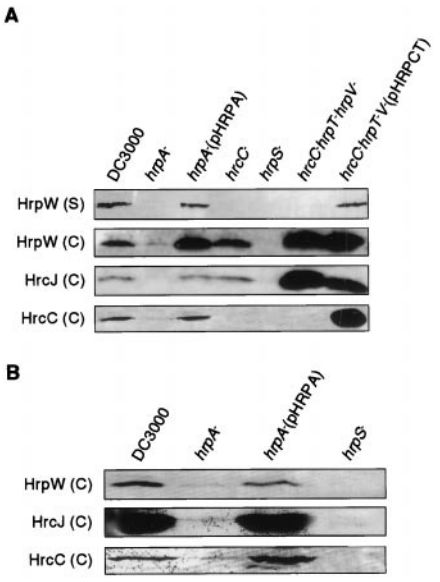

Fig. 1. Immunoblot analysis of HrpW, HrcJ, and HrcC proteins in *Pst* DC3000 and *hrp* mutants in *hrp*-inducing medium (*A*) and *in planta* (*B*). Conditions for bacterial growth and immunoblot analysis are described in *Materials and Methods*. For analysis of HrpW expression in *hrp*-inducing cultures, the levels of HrpW in cell-associated (C) and supernatant (S) fractions were examined.

Pathogenesis Assays. Pathogenesis assay procedures were the same as described before (6).

Results

Initial Observations of the Effect of a *hrpA* Deletion Mutation on the Secretion of HrpW. To examine a role of the Hrp pilus in secretion of proteins, we investigated the effect of a *hrpA* deletion mutation on the secretion of HrpW in culture, in which a possible involvement of the Hrp pilus in mediating bacterial attachment to host cells is excluded. The cellular distribution of HrpW in *Pst* DC3000 and *hrpA*, *hrcC*, *hrcChrpTV*, and *hrpS* mutants was determined. As shown in Fig. 1*A*, HrpW was produced and secreted in DC3000 and in the *hrpA* mutant complemented by pHRPA, which carries the wild-type *hrpA* gene (6); produced but not secreted in the *hrcC* and *hrcChrpTV* secretion mutants; and not detectable in the *hrpS* regulatory mutant. In the *hrpA* deletion mutant, HrpW was not secreted in the medium and only barely detectable in the cell fraction (Fig. 1*A*). However, the amount of HrpW was higher in the *hrpA* mutant than in the *hrpS* mutant. The same expression and secretion patterns were observed when bacteria were grown in liquid or agar medium. For all subsequent experiments, liquid cultures were used. The higher level of HrpW in the *hrcChrpTV* mutant, compared with that in the wild-type DC3000, is due to the polar effect of the Tn*5Cm*-induced mutation in the *hrcC* gene on the downstream *hrpV* gene, which was shown previously to encode a putative negative regulator in *P. syringae* pv. *syringae* (28). Consistent with this prediction, the HrpW was not overproduced in a nonpolar *hrcC* mutant, whereas it was overproduced in the *hrcChrpTV* mutant carrying pHRPCT, which is equivalent to the *hrpV* mutant (Fig. 1*A*). Overproduction of HrpW in the *hrcChrpTV* mutant carrying pHRPCT suggests that HrpV is also a negative regulator in *Pst* DC3000.

***hrpA* Is Required for Full Expression of Other Hrp Proteins.** The unexpected down-regulation of the secreted protein HrpW in the *hrpA* deletion mutant prompted us to examine a possible effect of the *hrpA* mutation on the expression of those Hrp proteins that are components of the type III secretion machinery. Specifically, we examined expression of HrcC and HrcJ (37, 38), for which we have antibodies. As expected, in *hrp*-inducing medium HrcJ was highly expressed in DC3000, the *hrcC* and *hrcChrpTV* mutants, and the *hrpA* mutant complemented by pHRPA, but not in the *hrpS* mutant. However, the expression of HrcJ was suppressed significantly in the *hrpA* mutant (Fig. 1*A*). The same expression and localization pattern was observed for HrcC, except that HrcC was not detected in the *hrcC* or *hrcChrpTV* mutant (Fig. 1*A*). To rule out the possibility that the effect of the *hrpA* mutation on expression of *hrc/hrp* genes is due to an artifact in *hrp*-inducing culture, we determined the expression patterns of HrpW, HrcJ, and HrcC in DC3000 and the *hrp* mutants *in planta*. We found an expression pattern similar to that in *hrp*-inducing culture (Fig. 1*B*). Specifically, HrpW, HrcJ, and HrcC were produced highly in DC3000, but not in the *hrpS* mutant. The amounts of these proteins were very low in the *hrpA* mutant, but higher than in the *hrpS* mutant. pHRPA restored the expression of these proteins in the *hrpA* mutant (Fig. 1*B*). Thus, we conclude that the *hrpA* gene is required for full expression of both secreted proteins (HrpW) and components of the Hrp secretion machinery (HrcC and HrcJ) in *hrp*-inducing minimal medium and *in planta.*

The *hrpA* Deletion Mutation Affects the Expression of *hrp*, *hrc*, and *avr* Genes at the RNA Level. To determine whether the *hrpA* gene controls the expression of *hrc/hrp* genes at the RNA as well as the protein level, we directly measured the steady-state mRNA level of the *hrpW* operon in DC3000 and various mutants. Consistent with the immunoblot results, this operon was significantly down-regulated in the *hrpA* and *hrpS* mutants, as compared with DC3000, the *hrpA* deletion mutant complemented by pHRPA, and the *hrcC* mutant (Fig. 2). We also determined the effect of the *hrpA* mutation on the expression of five (i.e., *hrpL*, *hrpJ*, *hrpP*, *hrpF*, and *hrpA*; see Fig. 2) of the six core *hrc/hrp* gene operons and *avrPto*, which encodes a protein presumably delivered into the plant cell via the Hrp secretion system (40, 41). As shown in Fig. 2, the steady-state mRNA levels of all five *hrc/hrp* gene operons and *avrPto* were suppressed significantly in the *hrpA* and *hrpS* mutants, although the basal level of the *avrPto* transcript in the *hrpA* mutant was higher than those of other transcripts. Thus, the effect of the *hrpA* mutation is at the transcript level.

Three Amino Acid Residues Located at the Carboxyl Terminus Are Essential for the Secretion and/or Regulation Function of HrpA. We next attempted to define amino acid substitution mutations that affect the secretion and/or regulatory function of the HrpA protein. An HR assay was used to screen for these mutations. As reported previously, the wild-type *hrpA* gene cloned in pHRPA restored the ability of a *hrpA* deletion mutant to elicit an HR in tobacco (6). We reasoned that a mutation that affects the regulatory or secretion function of the HrpA protein would eliminate the ability of the *hrpA* gene (pHRPA) to complement the genomic *hrpA* deletion mutation. We used both site-directed and random mutagenesis procedures in the screen. Site-directed mutagenesis was facilitated by the relatively few residues (six were substituted in this study) conserved among the hypervariable HrpA proteins of *P. syringae* pathovars and *E. amylovora* (35, 36). Residue substitutions in two positions (D^{95} to S^{95} and I^{111} to P^{111}) were found to eliminate the ability of the *hrpA* gene to complement the *hrpA* mutation for the elicitation of HR in tobacco (Fig. 3*A*). Random mutagenesis enabled us to identify a third mutant *hrpA* gene that fails to complement the *hrpA*

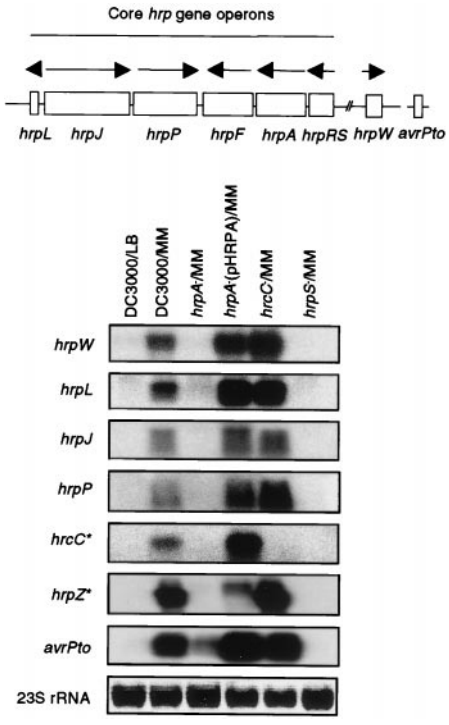

Fig. 2. RNA blot analysis of *hrp* and *avr* transcripts in *Pst* DC3000 and *hrp* mutants. *hrp* gene operons, named by their first genes, are diagrammed at the top. The directions of transcription are indicated by arrows. The *hrpW* gene is linked to the core *hrp* cluster, whereas *avrPto* is not. Conditions for bacterial growth and RNA analysis are described in *Materials and Methods*. The genes indicated on the left were used as probes. The 23S rRNA visualized after ethidium bromide staining was used as loading control. RNA was isolated from bacteria grown in *hrp*-repressing LB or *hrp*-inducing minimal medium (MM). *, The *hrcC* and *hrcZ* genes are located within the *hrpF* and *hrpA* gene operons, respectively. See ref. 39 for a more detailed description of the *P. syringae hrp* gene cluster.

mutation. Sequence analysis revealed an amino acid substitution at position 94 (from E^{94} to K^{94}) in the HrpA protein (Fig. 3*A*). Thus, all three identified mutations are at the carboxyl terminus of the HrpA protein. The E94K mutation appears primarily to affect the regulatory function of HrpA, whereas the D95S and I111P mutations primarily affect the secretion function (Fig. 3*B*). However, the absolute amounts of Hrc and Hrp proteins in these mutant strains varied somewhat from experiment to experiment.

The *hrpA* Mutation Affects the Transcript Level of the *hrpRS* Operon. To examine further the effect of the *hrpA* deletion mutation on gene expression, we determined the steady-state message levels of the *hrpRS* positive regulatory operon in DC3000 and the *hrpA* mutant. As expected, the expression of the *hrpRS* operon was barely detectable in DC3000 grown in LB or in the *hrpS* mutant grown in the *hrp*-inducing medium, but was induced when DC3000 and the *hrc* mutant were grown in *hrp*-inducing minimal medium. The expression of *hrpRS* operon was significantly down-regulated in the *hrpA* mutant, whereas overexpression of the *hrpA* gene from pHRPA elevated the expression of the *hrpRS* operon (Fig. 4*A*). Furthermore, when the *hrpRS* operon was expressed ectopically from a plasmid (pHRPRS1), the repression of *hrc/hrp* gene expression in the *hrpA* mutant was completely relieved. Specifically, HrpW and HrcC were produced in both DC3000 and the *hrpA* mutant when *hrpRS* genes were ectopically expressed in the otherwise *hrp*-repressing LB (Fig. 4*B*). Similarly, ectopic expression of the *hrpRS* gene operon allowed expression of HrpW and HrcC proteins in the *hrpA*

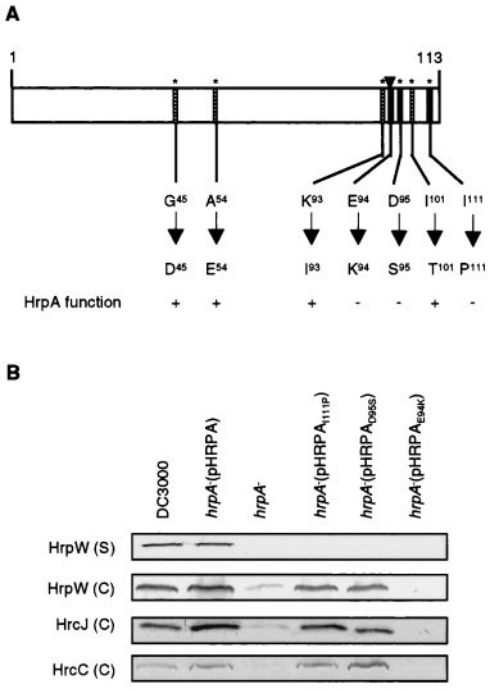

Fig. 3. (*A*) A diagram of the *P. syringae* pv. *tomato* DC3000 HrpA protein (113 aa in length). Six amino acid residues (*) conserved among HrpA proteins of *P. syringae* pvs. *tomato*, *syringae*, and *glycinea* and *E. amylovora* were mutated by site-directed mutagenesis. The E^{94}-to-K^{94} mutation, indicated by an arrowhead, was obtained by random mutagenesis. Amino acid residue substitutions that did not affect the HrpA function are indicated by hatched bars. Those substitutions that eliminated the ability of pHRPA to complement the genomic *hrpA* mutation are indicated by solid bars. The HrpA function was assayed by the ability (+) or inability (−) of the corresponding pHRPA derivatives to complement the genomic *hrpA* deletion mutation for HR elicitation in tobacco leaves and disease causation in *Arabidopsis thaliana* leaves. (*B*) Immunoblot analysis of the effect of single amino acid mutations of HrpA on the production of HrpW, HrcJ, and HrcC in *hrp*-inducing medium. Both cell-associated (C) and supernatant (S) fractions were analyzed for HrpW. Conditions for bacterial growth and immunoblot analysis are described in *Materials and Methods*.

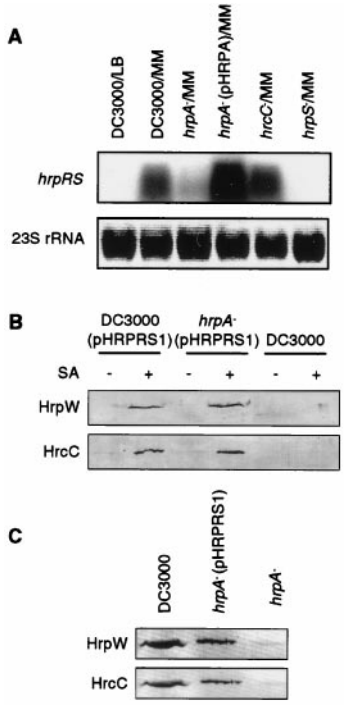

Fig. 4. (*A*) Effect of the *hrpA* mutation on the steady-state message abundance of *hrpRS*. The procedures for bacterial growth and RNA preparation and blotting are the same as in the legend to Fig. 2, except that the RNA gel blot was hybridized to the *hrpRS* gene probe. (*B*) Effects of ectopic expression of *hrpRS* on the accumulation of Hrp/Hrc proteins in the *hrpA* mutant. Bacteria were grown in LB supplemented with (+) or without (−) 35 μM salicylic acid (SA). The original culture was used directly for analysis by SDS/PAGE followed by immunoblot analysis with antibodies against HrpW and HrcC, respectively, without further fractionation. (*C*) Effects of SA-induced expression of *hrpRS* from pHRPRS1 on the accumulation of Hrp/Hrc proteins in the *hrpA* mutant *in planta*. Bacteria were supplemented with 35 μM SA before infiltration. Constitutive expression of *Pst* DC3000 *hrpRS* genes from pHRPRS2 also restored the expression of HrpW in the *hrpA* mutant (see Fig. 5).

mutant *in planta* (Fig. 4*C*). The same results were obtained for the HrcJ protein (data not shown). These experiments suggest that the *hrpA* mutation affects expression of *hrc*, *hrp*, and *avr* genes through a reduction of the transcript level of the *hrpRS* operon.

Secretion of HrpW and AvrPto Is Blocked in the *hrpA* Deletion Mutant Even When Gene Expression Is Restored. The complete restoration of gene expression in the *hrpA* deletion mutant by the ectopically expressed *hrpRS* gene operon enabled the testing of a direct role of HrpA in protein secretion again. As shown in Fig. 5*A*, when grown in the *hrp*-inducing medium, DC3000 produced and secreted HrpW to the medium with or without constitutive expression of the *hrpRS* gene operon from pHRPRS2. However, HrpW was not detected in the culture medium of the *hrpA* mutants even when the *hrpRS* gene operon was constitutively expressed. Instead, the expressed HrpW was recovered exclusively in the cell fraction. Similarly, secretion of AvrPto was blocked by both the *hrpA* deletion mutation and the $hrpA_{D95S}$ point mutation (Fig. 5*B*). pHRPA restored the secretion of AvrPto. Ectopic expression of the *hrpRS* operon from pHRPRS1 and pHRPRS2 increased the level of AvrPto, but did not result in the secretion of AvrPto in the *hrpA* mutant. Consistent with a defect in protein secretion, the *hrpA* mutant ectopically expressing the *hrpRS* genes from pHRPRS1 or pHRPRS2 did not elicit HR in tobacco or cause disease in *Arabidopsis* (Table 1). Thus, in addition to the involvement of the *hrpA* gene in the regulation of the Hrp secretion system, the Hrp pilus structural protein is required for the secretion of HrpW and AvrPto in culture.

Discussion

In this study, we have attempted to define the function of the *hrpA* gene, which encodes the Hrp pilus structural protein, in

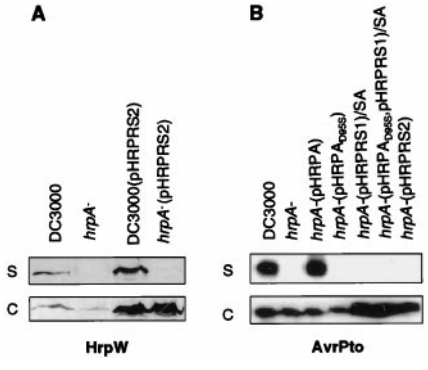

Fig. 5. Effect of ectopic expression of the *hrpRS* operon in the *hrpA* mutant on HrpW and AvrPto secretion. Bacteria were grown in *hrp*-inducing minimal medium. Bacterial supernatant (S) and cell (C) fractions were analyzed by SDS/PAGE followed by immunoblotting with HrpW (*A*) or AvrPto antibody (*B*).

Table 1. Plant reactions to DC3000 and various *hrp* mutants

Bacteria	Reaction	
	Arabidopsis thaliana (ecotype Columbia)	Tobacco (cv. Samsun NN)
DC3000 ± SA	D	HR
hrpS$^-$	Null	Null
hrpA$^-$	Null	Null
hrpA$^-$/pHRPA	D	HR
hrpS$^-$/pHRPRS1 + SA	D	HR
hrpS$^-$/pHRPRS2	D	HR
hrpA$^-$/pHRPRS1 + SA	Null	Null
hrpA$^-$/pHRPRS2	Null	Null
hrpA$^-$/pHRPA$_{D95S}$, pHRPRS1 + SA	Null	Null

HR, rapid, localized tissue collapse in the infiltrated area within 24 hr; D, disease symptoms (slowly developing necrosis and spreading tissue chlorosis) observed 3 days after infiltration; Null, no visible plant reactions; SA, 35 μM salicylic acid.

type III protein secretion in *P. syringae* pv. *tomato* DC3000. We show that a functional HrpA protein is required for secretion of HrpW and AvrPto in culture. In addition, we found that the *hrpA* mutation affects the full expression of all six core *hrc/hrp* gene operons as well as *hrpW* and *avrPto* that reside outside the core *hrc/hrp* gene cluster. We identified 3 aa residues at the carboxyl terminus that affect the secretion and/or regulatory function of the HrpA protein. Finally, we show that the *hrpA* mutation affects the transcript level of the two positive regulatory genes *hrpR* and *hrpS* and that ectopic expression of the *hrpRS* gene operon can completely restore gene expression in the *hrpA* mutant. We suggest that the Hrp pilus is an integral component of a supramolecular protein secretion structure that enables *Pst* DC3000 to deliver virulence proteins at the right place and time during bacterial infection of plants.

Pathogenic bacteria devote a large number of genes to type III protein secretion. In the *P. syringae hrp* gene cluster alone, for example, about 27 genes are coregulated and they encode either regulatory, secretion, or effector proteins (42). In addition, several *hrp*-regulated genes that encode effector (Avr) proteins have been shown to be unlinked to the *hrp* gene cluster (43, 44). Thus, turning on the type III protein secretion system is an energy-consuming process. Because the final outcome of turning on the type III secretion system is delivery of some virulence proteins into the host cell, it would be beneficial for bacteria to prevent full induction of type III-secretion-associated genes until host cells are available for protein injection. This prediction is consistent with the observed "contact-dependent activation" (transcriptionally or posttranscriptionally) of type III protein secretion in *Yersinia pseudotuberculosis*, *Salmonella typhimurium*, *Shigella flexneri*, and *E. coli* (7, 8, 45–47). Expression of *hrp* genes also has been shown to be induced in infected plant tissues (21–23). Host–bacterium contact appears to be important for bacterial elicitation of an HR (48) and for expression of *hrp* genes in *Ralstonia solanacearum* (5), although evidence for contact-dependent activation of the *P. syringae* Hrp system has yet to be obtained. The molecular basis of bacterial sensing of host–bacterium contact is not understood in any bacterium. In *Yersinia*, the current hypothesis for contact-dependent activation assumes that host–bacterium contact removes bacterial surface sensors, such as YopN (49), LcrG (50, 51), and TyeA (52), that normally prevent secretion (53). However, an involvement of secreted proteins (e.g., LcrV) as part of an extracellular secretion appendage in the positive regulation of Yop secretion is a formal possibility (53). In *R. solanacearum*, host cell-dependent *hrp*

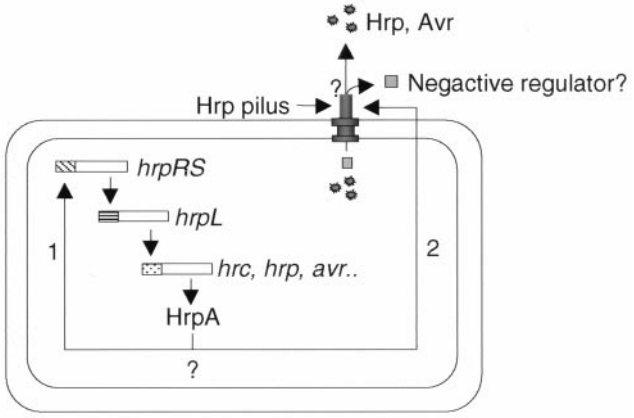

Fig. 6. A hypothetical model depicting *hrpA*-mediated coupling of protein secretion and gene regulation. Arrow 1 indicates that *hrpA* directly affects the transcription or RNA stability of the *hrpRS* operon independent of its effects on secretion of Hrp and Avr proteins. Arrow 2 indicates that *hrpA* indirectly regulates the *hrpRS* operon through its involvement in secretion of a negative regulator (HrpV?) as well as other Hrp and Avr proteins. The question marks indicate uncertainties in the model regarding whether (*i*) *hrpA* affects *hrpRS* expression directly and/or indirectly through its effects on secretion, (*ii*) Hrp and Avr proteins are secreted through the pilus, and (*iii*) a negative regulator (e.g., HrpV) is secreted.

gene expression is controlled by a novel signaling pathway involving *prhA* and *prhJ* (54, 55).

The significant down-regulation of *hrp*, *hrc*, and *avr* genes in the *hrpA* deletion mutant *in vivo* suggests that assembly of the Hrp pilus, secretion of effector proteins, and expression of type III-secretion-associated genes are coordinately regulated in *Pst* DC3000 and that the HrpA protein, as part of a supramolecular secretion structure, may participate in sensing host cells by *Pst* DC3000. In support of a role of a pilus protein in contact-based gene regulation, the adhesive P pilus of *E. coli* has been shown to sense the host–bacterium contact signal, resulting in the induction of bacterial iron starvation-response genes (56). In *Yersinia* spp., an artificial minimal medium has been shown to mimic the host–bacterium contact signal (by presumably changing the conformation of surface sensors) to induce the Ysc type III secretion system (45, 53). It is therefore likely that the composition of the *hrp*-inducing minimal medium and growth conditions *in vitro* mimic the plant–bacterium contact signal *in vivo*.

Whether pili are involved directly in the transfer of DNA or protein or indirectly in mediating cell–cell contact has been a long-standing and unresolved question. We show here that the HrpA protein is necessary for secretion of HrpW and AvrPto in culture, in which the possible function of pilus-mediated bacterial attachment to the host cell is excluded. The demonstrated role of HrpA in the secretion of AvrPto and HrpW therefore is consistent with a hypothesis that the Hrp pilus is an integral component of the Hrp secretion structure. The Hrp pilus could be the functional equivalent of the extracellular part (called the short-needle extension) of the *S. typhimurium* type III secretion supramolecular structure (12), presumably providing a conduit for protein secretion. Assembly of the longer pilus in *Pst* DC3000 may reflect a need for this bacterium to deliver proteins through the thick plant cell wall, which is lacking in animal cells. Alternatively, HrpA, as part of the secretion structure, is required for maintaining the integrity of the Hrp secretion apparatus. Finally, the HrpA protein may function as a chaperone protein that pilots HrpW, AvrPto, and other secreted proteins through the Hrp secretion machinery (and along the Hrp pilus).

The observed dual function of HrpA in gene regulation and protein secretion suggests that HrpA-mediated gene regulation may be linked to the HrpA function in protein secretion. The simplest explanation would be that HrpA, as a component of the Hrp secretion structure, is required for secretion of a negative regulator, such as HrpV. Secretion of such a negative regulator would derepress the *hrc*, *hrp*, and *avr* genes through activation of the *hrpRS*/*hrpL* regulatory cascade (Fig. 6). Gene regulation based on export of a negative regulator (i.e., LcrQ) via the type III protein secretion system, but not a pilus, has been shown in *Y. pseudotuberculosis* (46). However, our isolation of mutant *hrpA* genes that primarily affect protein secretion without significantly affecting gene expression (Fig. 3*B*) does not appear to support this hypothesis, unless the mutant HrpA proteins can counteract the action of a negative regulator in the bacterial cell. It therefore is possible that the regulatory function of the *hrpA* gene is independent of its role in protein secretion. The *hrpA* mutation may directly or indirectly affect the transcription and/or RNA stability of the *hrpRS* operon (Fig. 6). Our future research is aimed at resolving the exact mechanism by which the *hrpA* gene controls type III protein secretion and affects gene regulation.

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