

Characterization of the *hrpC* and *hrpRS* Operons of *Pseudomonas syringae* Pathovars *Syringae*, *Tomato*, and *Glycinea* and Analysis of the Ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* Mutants To Elicit the Hypersensitive Response and Disease in Plants

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The species *Pseudomonas syringae* encompasses plant pathogens with differing host specificities and corresponding pathovar designations. *P. syringae* requires the Hrp (type III protein secretion) system, encoded by a 25-kb cluster of *hrp* and *hrc* genes, in order to elicit the hypersensitive response (HR) in nonhosts or to be pathogenic in hosts. DNA sequence analysis of the *hrpC* and *hrpRS* operons of *P. syringae* pv. *syringae* 61 (brown spot of beans), *P. syringae* pv. *glycinea* U1 (bacterial blight of soybeans), and *P. syringae* pv. *tomato* DC3000 (bacterial speck of tomatoes) revealed that the 13 genes comprising the right half of the *hrp* cluster (including those in the previously sequenced *hrpZ* operon) are conserved and identically arranged. The *hrpC* operon is comprised of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV*. *hrcC* encodes a putative outer membrane protein that is conserved in all type III secretion systems. The other four genes appear to be characteristic of group I Hrp systems, such as those possessed by *P. syringae* and *Erwinia amylovora*. The predicted products of these four genes in *P. syringae* pv. *syringae* 61 are HrpF (8 kDa), HrpG (15.4 kDa), HrpT (7.5 kDa), and HrpV (13.4 kDa). HrpT is a putative outer membrane lipoprotein. HrpF, HrpG, and HrpV are all hydrophilic proteins lacking N-terminal signal peptides. The HrpG, HrcC, HrpT, and HrpV proteins of *P. syringae* pathovars *syringae* and *tomato* (the two most divergent pathovars) had at least 76% amino acid identity with each other, whereas the HrpF proteins of these two pathovars had only 36% amino acid identity. The HrpF proteins of *P. syringae* pathovars *syringae* and *glycinea* also showed significant similarity to the HrpA pilin protein of *P. syringae* pathovar *tomato*. Functionally nonpolar mutations were introduced into each of the genes in the *hrpC* operon of *P. syringae* pv. *syringae* 61 by insertion of an *nptIII* cartridge lacking a transcription terminator. The mutants were assayed for their ability to elicit the HR in nonhost tobacco leaves or to multiply and cause disease in host bean leaves. Mutations in *hrpF*, *hrcC*, and *hrpT* abolished or greatly reduced the ability of *P. syringae* pv. *syringae* 61 to elicit the HR in tobacco. The *hrpG* mutant had only weakly reduced HR activity, and the activity of the *hrpV* mutant was indistinguishable from that of the wild type. Each of the mutations could be complemented, but surprisingly, the *hrpV* subclone caused a reduction in the HR elicitation ability of the Δ *hrpV*:*nptIII* mutant. The *hrpF* and *hrcC* mutants caused no disease in beans, whereas the *hrpG*, *hrpT*, and *hrpV* mutants had reduced virulence. Similarly, the *hrcC* mutant grew little in beans, whereas the other mutants grew to intermediate levels in comparison with the wild type. These results indicate that HrpC and HrpF have essential functions in the Hrp system, that HrpG and HrpT contribute quantitatively but are not essential, and that HrpV is a candidate negative regulator of the Hrp system.

Many gram-negative plant-pathogenic bacteria elicit a rapid, localized necrosis in infiltrated tissues of plants that are outside their host range. This defense-associated apparent programmed cell death is known as the hypersensitive response (HR) (34). The ability of these bacteria to elicit the HR in nonhost plants, or to be pathogenic in their hosts, is dependent on *hrp* genes, which may be universal in plant-pathogenic

Pseudomonas, *Xanthomonas*, *Erwinia*, and *Ralstonia* spp. (3, 34). The *hrp* genes are clustered, and many encode components of a type III protein secretion system that appears to be dedicated to the secretion of virulence proteins in both plant and animal pathogens. Nine of the *hrp* genes have homologs in animal-pathogenic *Yersinia*, *Shigella*, and *Salmonella* spp., and these have been renamed *hrc* (for HR and conserved) (8).

The species *Pseudomonas syringae* is divided into pathovars largely on the basis of host specificity (42). *hrp* genes have been studied in the *P. syringae* pathovars *syringae* (brown spot of beans), *phaseolicola* (halo blight of beans), *tomato* (bacterial speck of tomatoes), and *glycinea* (bacterial blight of soybeans) (3, 10). The *hrp* cluster of *P. syringae* pv. *syringae* 61, cloned on cosmid pHIR11, has been studied most extensively because it has the useful property of conferring on nonpathogenic bacteria, such as *Pseudomonas fluorescens* and *Escherichia coli*, the

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ability to elicit the HR in tobacco and several other plants (25). pHIR11 contains four major operons (*hrpJ*, *hrpU*, *hrpC*, and *hrpZ*), which encode all of the type III pathway components, one harpin (HrpZ), and one pilus subunit (HrpA) (3, 21, 22, 24, 26, 36, 46, 51). This cluster also contains *hmmA*, which is an apparent *avr* (avirulence) gene (4), and several *hrp* genes of unknown function that either have no homologs or have homologs only in the closely related *Erwinia amylovora* (9, 24, 32, 45). pHIR11 also carries three regulatory genes that encode the positive regulators *hrpR* and *hrpS* and the *hrp*-activating alternate σ factor HrpL (51).

The sequence of the *hrpZ* operon, which encodes HrpA, HrpZ, and secretion pathway components such as HrcJ, has been analyzed in *P. syringae* pv. *syringae* 61, *P. syringae* pv. tomato DC3000, and *P. syringae* pv. *glycinea* U1 (45). The comparison suggests that the arrangement of *hrp* genes is conserved among *P. syringae* pathovars and that HrpZ does not directly control host range. The actual role of HrpZ in elicitation of the HR or pathogenesis remains uncertain (1, 43), and a primary function of the Hrp system may be the delivery of Avr effector proteins directly into plant cells (3, 18). Whether any components of the Hrp system itself affect host specificity is not known.

Eight of the Hrc proteins show similarity to a group of proteins involved in flagellar basal body biogenesis and flagellum-specific secretion (3, 8). HrcC (formerly known as HrpH), the remaining Hrc protein, is a member of the PulD-pIV superfamily of secretins, which are outer membrane proteins involved in macromolecular traffic across the bacterial outer membrane (22, 47). HrcC has been shown to be required for both HrpZ secretion and the delivery of Avr signals (18, 21). *hrcC* is carried in the *hrpC* operon and is preceded by *hrpF* and *hrpG*, two small open reading frames (ORFs) of unknown function that were found as a byproduct of our previous analysis of the *hrpZ* operons of *P. syringae* pv. *syringae* 61, *P. syringae* pv. tomato DC3000, and *P. syringae* pv. *glycinea* U1 (24, 45). Recently, Kim et al. (32) reported the presence in *E. amylovora* of ORFs similar to *hrpF* and *hrpG* upstream of *hrcC* and two new ORFs, *hrpT* and *hrpV*, downstream of *hrcC*, and they confirmed the products of all four ORFs by T7 expression.

We have focused our analysis of *hrp* genes on three *P. syringae* strains: *P. syringae* pv. *syringae* 61 (the source of pHIR11), *P. syringae* pv. tomato DC3000 (a model pathogen of *Arabidopsis* spp. as well as the tomato), and *P. syringae* pv. *glycinea* U1 (a strain in race 4, which is used extensively in *avr* gene studies). Here we report two results. One is the sequence of the *hrpC* and *hrpRS* operons of *P. syringae* pv. *syringae* 61, *P. syringae* pv. tomato DC3000, and *P. syringae* pv. *glycinea* U1, which reveals the complete conservation of *hrp* gene arrangement in the right half of the *hrp* clusters of these three pathovars and the relative variation among sets of homologous genes. The other is the construction, complementation, and phenotypic analysis of functionally nonpolar mutations in *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* in *P. syringae* pv. *syringae* 61, which reveals that these genes differ significantly in their contributions to plant reaction phenotypes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was routinely grown in Luria-Bertani medium or Terrific broth at 37°C (6). *Pseudomonas* strains were routinely grown in King's B (KB) medium (33) at 28 to 30°C, but for certain experiments the *hrp*-derepressing minimal medium containing fructose (28), adjusted to pH 5.5, was used. Antibiotics were used in selective media at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 50; tetracycline, 20; and nalidixic acid, 20.

Recombinant DNA techniques. Restriction endonuclease digestion, agarose gel electrophoresis, DNA fragment preparation, plasmid extraction, DNA ligation, and transformation by CaCl₂ followed standard procedures (6). Plasmids were introduced into bacteria by transformation, electroporation (Gene Pulsar; Bio-Rad, Richmond, Calif.), or triparental mating (14).

DNA sequencing and analysis. The *hrcC* operon of *P. syringae* pv. tomato DC3000, carried on plasmid pCPP2201 (45), and the *hrpF*, *hrpG*, *hrpT*, and *hrpV* genes of *P. syringae* pv. *glycinea* U1, carried on plasmid pCPP2200 (45), were sequenced with the ABI 373A DNA sequencer at the Cornell Biotechnology Center DNA-sequencing facility, with specific primers synthesized by Integrated DNA Technologies (Coralville, Iowa). Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group sequence analysis software package (13) and DNASTAR (DNASTAR Inc., Madison, Wis.). Homology searches against major sequence databases were done with the BLAST program (5). BESTFIT alignments were considered significantly similar if the score determined with default parameters was at least five times the standard deviation above the mean quality score of 100 randomized alignments (13, 15).

Construction of functionally nonpolar mutations in the *hrpC* operon. To create nonpolar mutations in the *P. syringae* pv. *syringae* 61 *hrpC* operon, a 1.5-kb *nptII* cassette lacking a rho-independent transcription terminator (1, 7) was used to disrupt *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV*. The cassette marked deletions in *hrpF*, *hrcC*, *hrpT*, and *hrpV* and was used for insertional inactivation of *hrpG*. These recombinant constructions were cloned into vector pRK415 (30) (see Fig. 4). The DNA fragments used in the construction were amplified by PCR with *Pfu* polymerase (Stratagene, La Jolla, Calif.), and the corresponding primers are shown in Table 2. Inactivation of *hrpF* was achieved with two PCR-generated fragments by using pHIR11 as a template and *prs5-prs8* and *prs7-prs9* as primers. The amplified 1.1-kb DNA fragment of *prs5* plus *prs8* was treated with *XbaI* and *BamHI* and then cloned into pCPP2988 to generate a 2.6-kb *XbaI-KpnI* fragment. This 2.6-kb fragment was then subcloned into a pRK415 derivative, which had previously received the 1.3-kb *prs7-prs9*-generated fragment in the *KpnI* and *SsrI* sites, to produce pNCHU491. To mutate *hrpG*, a 1.8-kb *BamHI-HindIII* fragment from pNCHU329 was cloned into pCPP2988 containing an insertion of the 4-kb *SalI-KpnI* fragment isolated from pCPP2107, and the total 7.3-kb fragment was subsequently cloned into pRK415 at the *BamHI-KpnI* sites to produce pNCHU492. The 5' (ca. 2-kb)- and 3' (ca. 1-kb)-flanking sequences of the *hrpT* gene were obtained from PCR-amplified DNA fragments by using *prs1-prs2* and *prs3-prs4*, respectively, as primers. These DNA fragments were cloned into pCPP2988 at appropriate restriction sites. This construct resulted in a 34-bp deletion of *hrpT* that was replaced by the *nptII* gene. The 4.7-kb *BamHI-KpnI* fragment isolated as described above was cloned into pRK415 to produce pNCHU402. Primer *prs1* was also used in the construction of the *hrpV* mutation. A 1,040-bp *EcoRV-KpnI* fragment and *prs1-prs6*-generated fragments were cloned in two steps into pCPP2988. The 4.7-kb *BamHI-KpnI* fragment containing *nptII* was subsequently ligated with pRK415 to produce pNCHU407. pNCHU393 ($\Delta hrcC::nptII$), pNCHU404 ($\Delta hrpT::nptII$), pNCHU407 ($\Delta hrpV::nptII$), pNCHU491 ($\Delta hrpF::nptII$), and pNCHU492 (*hrpG::nptII*) (see Fig. 4) were introduced into *P. syringae* pv. *syringae* 61 by triparental mating, using *E. coli* DH10B (carrying the constructed plasmids) as the donor and the helper strain *E. coli* HB101(pRK2013) (14). The mating mixtures were spotted on KB agar supplemented with nalidixic acid, tetracycline, and kanamycin at 30°C for 2 to 3 days. Cells from single transconjugant colonies were inoculated in 5 ml of KB broth supplemented with nalidixic acid and kanamycin. The bacteria were subcultured for 5 days, and then the final cultures were diluted and spread on KB agar plates containing nalidixic acid and kanamycin. Mutants were identified by screening on KB agar for kanamycin resistance and tetracycline sensitivity (23).

Complementation of mutations. The *hrpF*, *hrpG*, *hrcC*, and *hrpT* genes from the *P. syringae* pv. *syringae* 61 *hrpC* operon were subcloned from available constructs (Table 1) or amplified by PCR as described above and were cloned individually into pRK415. The primers used are listed in Table 2. Resultant plasmids pNCHU515 (*hrpF*_{PS8}), pNCHU 513 (*hrpG*_{PS8}), pNCHU421 (*hrcC*_{PS8}), and pNCHU451 (*hrpT*_{PS8}) were then transformed into the corresponding mutants by triparental mating as described above.

Plant assays. HR assays were performed in tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants that were grown under greenhouse conditions at 23 to 25°C with a photoperiod of 16 to 24 h and transferred to the laboratory for the assays. Bacterial samples were prepared by suspending them in distilled water at a density of 10⁸ to 10⁹/ml. The cells were then grown for 24 h on KB agar plates. Inoculations were performed by pricking leaves with a dissecting needle and then infiltrating the bacterial suspension with a 1-ml syringe lacking a needle. The development of the HR at room temperature was scored within 24 h. Virulence assays were performed in bean (*Phaseolus vulgaris* cv. Eagle) plants that were grown under greenhouse conditions at 23 to 25°C with a photoperiod of 16 to 18 h. Bacteria were grown overnight on KB agar plates and suspended in 5 mM MES (morpholinoethanesulfonic acid), pH 5.5, at a density of 10⁵ CFU/ml. Inoculations were performed by infiltration as described above. Plants were incubated at high humidity, and the appearance of disease symptoms was scored after 5 days. Multiplication assays were performed by grinding 0.6-cm-diameter leaf discs from infiltrated leaves in 1 ml of 5 mM MES (pH 5.5), followed by serial dilution and plating of the samples onto agar plates with 1 μ g of cycloheximide/ml and appropriate antibiotics.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
HB101	F' <i>hsd20 recA13 thr leu thi pro</i> Sm ^r	6
DH10B	<i>endA1 hsdR17 recA1 relA Δ(argF-lacZYA)U169 f80d lacZΔM15</i>	Life Sciences Technologies (Gaithersburg, Md.)
<i>P. syringae</i> pv. <i>syringae</i> 61	Wild type isolated from wheat; Nal ^r	25
61-N393	Derivative carrying <i>ΔhrcC::nptII</i> nonpolar mutation	12
61-N402	Derivative carrying <i>ΔhrpT::nptII</i> nonpolar mutation	This study
61-N407	Derivative carrying <i>ΔhrpV::nptII</i> nonpolar mutation	This study
61-N491	Derivative carrying <i>ΔhrpF::nptII</i> nonpolar mutation	This study
61-N492	Derivative carrying <i>hrpG::nptII</i> nonpolar mutation	This study
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Wild type; Rp ^r	D. E. Cuppels
<i>P. syringae</i> pv. <i>glycinea</i> Race 4 U1	Wild type	C. J. Baker
Plasmids		
pRK2013	IncP Tra RK2 ⁺ <i>ΔrepE1</i> ⁺ Kan ^r	14
pBluescript KS or SK	ColE1 Ap ^r <i>mcs-lacZ</i>	Stratagene
pT7-6	Ap ^r , ϕ 10 T7 RNA polymerase promoter	48
pCPP30	IncP LacZ' Tc ^r	D. W. Bauer (Cornell University)
pCPP2988	pBluescript II SK(-) carrying 1.5-kb <i>HindIII-SalI</i> fragment from pRZ102 with <i>nptII</i> lacking terminator	1
pRK415	Broad-host-range vector unstable in absence of selection; Tc ^r	30
pHIR11	25-kb <i>hrp</i> cluster fragment from <i>P. syringae</i> pv. <i>syringae</i> 61 in pLAFR3	25
pNCHU7	5.4-kb <i>P. syringae</i> pv. <i>syringae</i> 61 <i>EcoRI-BamHI</i> fragment in pCPP30; contains <i>hrpRS-hrpD</i>	This study
pNCHU162	3.6-kb <i>P. syringae</i> pv. <i>syringae</i> 61 <i>SmaI-HindIII</i> fragment in pBluescript SK(-); contains <i>hrpB</i> , <i>hrcI</i> , and <i>hrpDEFG</i> genes	This study
pNCHU169	6-kb <i>P. syringae</i> pv. <i>syringae</i> 61 <i>BglII-EcoRI</i> fragment in pBluescript SK(-); contains <i>hrcRSTU</i> and <i>hrpVT</i> genes	This study
pNCHU316	prs1-prs2-generated 2.1-kb <i>BamHI-HindIII</i> fragment subcloned in pET29a; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrcC</i> with N-terminal S · Tag ^a fusion	This study
pNCHU329	1.8-kb <i>SalI</i> fragment containing <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrpG</i> subcloned in pT7-6	This study
pNCHU366	0.4-kb <i>NcoI-EcoRI</i> fragment subcloned in pET29a; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrcQ_B</i> with N-terminal S · Tag fusion	C.-J. Chang
pNCHU421	prs1-prs2-generated 2.1-kb <i>BamHI-HindIII</i> fragment subcloned in pRK415; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrcC</i>	This study
pNCHU451	250-bp <i>EcoRV-HindIII</i> fragment from pNCHU407 subcloned in pRK415; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrpT</i>	This study
pNCHU513	750-bp <i>KpnI-EcoRI</i> fragment from pNCHU491 subcloned in pRK415; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrpG</i>	This study
pNCHU515	400-bp <i>PvuII-HindIII</i> fragment from pNCHU329 subcloned in pRK415; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrpF</i>	This study
pCPP2107	a 7.2-kb <i>P. syringae</i> pv. <i>syringae</i> 61 <i>KpnI</i> fragment from pCPP2145 subcloned in pBluescript KS(-); contains <i>hrpB</i> , <i>hrcJ</i> , <i>hrpD</i> , <i>hrpE</i> , and complete <i>hrpC</i> operon	22
pCPP2145	10.6-kb <i>BglII</i> fragment from pHIR11 subcloned in pCPP30; contains complete <i>hrpZ</i> and <i>hrpC</i> operons and <i>hrcRSTU</i> genes	22
pCPP2200	pUCP19 carrying 10-kb <i>Sau3A1</i> partial fragment of <i>P. syringae</i> pv. <i>glycinea</i> U1 DNA containing <i>hrpRS-hrcU</i>	45
pCPP2201	pUCP19 carrying 10-kb fragment of <i>P. syringae</i> pv. <i>tomato</i> DNA containing <i>hrpRS-hrpV</i>	45
pCPP2372	0.3-kb fragment from prs10-prs11-generated PCR product in pRK415; contains <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrpV</i> for complementation	This study

^a S · Tag, a peptide encoded by the sequences of pET29a (Novagen Inc., Madison, Wis.).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in GenBank under accession no. AF051694 (*P. syringae* pv. *syringae* 61 *hrpTV*), AF061028 (*P. syringae* pv. *tomato* DC3000 *hrpRS*), AF061029 (*P. syringae* pv. *tomato* DC3000 *hrpF* to -*V*), AF069650 (*P. syringae* pv. *glycinea* U1 *hrpRS*), AF069651 (*P. syringae* pv. *glycinea* U1 *hrpJ* to -*G*), and AF069652 (*P. syringae* pv. *glycinea* U1 *hrp TVU*).

RESULTS

Sequence analysis of the *hrpRS* and *hrpC* operons of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* U1 and the *hrpT* and *hrpV* genes of *P. syringae* pv. *syringae* 61. The

complete sequence of the *hrpRS* operons of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* U1, the *hrpC* operon of *P. syringae* pv. *tomato* DC3000, and portions of the *hrpC* operon of *P. syringae* pv. *glycinea* U1 were obtained by using a series of specific oligonucleotide primers to sequence pCPP2200 (*P. syringae* pv. *glycinea* U1) and pCPP2201 (*P. syringae* pv. *tomato* DC3000), each of which possesses an approximately 10-kb insert containing the *hrpZ* operon and flanking DNA (45). The sequenced DNA displayed the same organization as the equivalent region from *P. syringae* pv. *syringae* 61, as shown in Fig. 1, except for the *hrpT* and *hrpV* genes, which

TABLE 2. Primers used in this study

Primer	Sequence (5'-3') ^a	Restriction enzymes
prs1	5'-TATGGATCCATGCGCAAGGCCTTGATG TG-3'	<i>Bam</i> HI
prs2	5'-TAATAAGCTTCATCACGCATGTCCGGAGG CCA-3'	<i>Hind</i> III
prs3	5'-ACTAGGTACCGTCGTGAAGT-3'	<i>Kpn</i> I
prs4	5'-GCGCGAATTCATGCGAAAA-3'	<i>Eco</i> RI
prs5	5'-CTGGATCTAGAGCAGCGCAT-3'	<i>Xba</i> I
prs6	5'-TGAGAAGCTTCTCCTCGACCTCG-3'	<i>Hind</i> III
prs7	5'-TGAGGTCGGTACCAACATCGC-3'	<i>Kpn</i> I
prs8	5'-ATTGCGATCCTCGATGTCGTGCGCGGT-3'	<i>Bam</i> HI
prs9	5'-AACGCATGGTATCGAGG-3'	
prs10	5'-ACATTCTAGATGATCGAGGTCGAG-3'	<i>Xba</i> I
prs11	5'-TGCTGAATTCCTAAGCCAGATGA-3'	<i>Eco</i> RI

^a Restriction sites are indicated in boldface.

were not observed previously in the *P. syringae* pv. *syringae* 61 *hrcC* (*hrpH*) region (22). Upon resequencing this region in *P. syringae* pv. *syringae* 61, we discovered a missing nucleotide (G) (Fig. 2). The corrected *hrcC* sequence predicts a protein that is 47 amino acids smaller than that originally reported and has a different sequence for the last 23 amino acids, and the corrected *hrcC* is followed by the *hrpT* and *hrpV* genes.

Each of the subclones from *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *glycinea* U1 contained the entire *hrpRS*, *hrpZ*, and *hrpC* operons. In addition pCPP2201 contained 1 kb of DNA upstream of *hrpR*, which displayed no significant ORFs or similarity to sequences in the databases, while pCPP2200 contained very little DNA upstream of *hrpR* but contained all of *hrcU* and part of *hrcT*. A detailed analysis of the sequences of the *hrpC* and *hrpRS* operons is presented below (Fig. 3).

HrpF and HrpG. HrpF is a 74-amino-acid (8-kDa), putatively cytoplasmic protein which exhibited a high degree of divergence between *P. syringae* pathovars *syringae* and tomato. Sequence homology searches identified two extensive homologies. The first of these was between the HrpF proteins of *P. syringae* and *E. amylovora* (32). The HrpF proteins of *P. sy-*

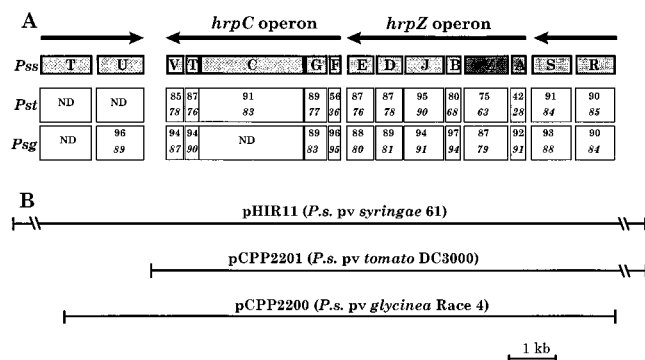


FIG. 1. Organization of the right half of the *hrp-hrc* clusters of *P. syringae* pathovars *syringae*, tomato, and *glycinea* and primary clones used in this study. (A) Organization of the *P. syringae* *hrp* cluster from *hrpRS* to *hrpT*. Conserved type III secretion genes (*hrc* genes) are indicated by the crosshatched boxes, genes encoding proteins known to be secreted are shaded dark gray, and the remaining *hrp* genes are shown as light-gray boxes. The identity (italic) and similarity scores for the predicted proteins from *P. syringae* pv. tomato DC 3000 (*Pst*) and *P. syringae* pv. *glycinea* U1 (*Psg*), relative to homologous proteins in *P. syringae* pv. *syringae* 61 (*Pss*), are shown in the open boxes beneath the relevant genes, except for those not done (ND). (B) Primary clones are aligned beneath the operon maps. *P.s.*, *P. syringae*.

1064	CCCTTTGAAACCGACAGCCTGTGTGACCCGGGCCAGGCGCTGAGCCTCGATGCCAGCGC
	P F E T D S L C D P G Q G L S L D G Q R
1124	TGCAATGGTATGCCCGCAGGACTGGGGTGTGGCTGCTGGTTCGCGGCAACATACC
	S Q W Y A R K D W G V A V V A R N N T
1184	GACAAACCGGTGCGGATCGACGAGAGTCGCTGCGGTGGTCTGCGGTCATCGCGTCGCC
	D K P V R I D E S R C G G R W V I G V A
1244	GCCTGGCCCATGATGGCTGCAACCGGGCAGGAAAGCGAAGTGTATATCGCGGTACGT
	A W P H A W L Q P G E E S E V Y I A V R
1304	CAGCCACAGATATCGAAAATGCGCAAAGAGAGCGCCATCTACTCTCCGGGGCAAG
	Q P Q I S K M A K E S R P S L L R G A K
	→ <i>hrpT</i>
1364	CCATGAAGATCAGCAGCGTTCAGCTCGTGTGGTGGTGTTCGCTACCCCTGACCGGGTGG
	P *
	M K I S S V A V V L V V F A T L T G C
1424	CCACCCAGGTTGTTTCGGGAAGCTGCTGCAACCGCCGACTCCACAGCCGCGAAGTGG
	A T H G C S G T A C K R P D S T S R E L
1484	CATCTGGTGGCTCCGGACATGCGTGACCGTCTTGACGACAGGACCAACCGCGTATT
	V I W W P P D M R D G L D D Q D H E R D
	→ <i>hrpV</i>
1544	ACACCGTCTGTAAGTTAAGGGACTAGACATGATCAGGTCGAGGAGGAAAACAGCGTCTTA
	Y T V V K L R D * M I E V E E K T A F Y
1604	CTACAAGCTTGGCGCAGAGTCTCGCGTCTGGCCGGTGGCAAACGGAGTCTCTTCGT
	Y N V A A Q S P A V W P V A N G V S F V
1664	CAGCCGCGCAGCATCATGACTGGGCATTGCGTGCACATGAGGGTTCGAGCGTGGC
	S R R E H H D W G I A L H I E G R A L R
1724	TCCGAAACACTCAGAGGGCGCTGCACTGAGTTCCTCACAAGCCGAACGATCAATGA
	P E Q L R E A L Q L R P S Q A E R F N D
1784	CTACTTCTGTTCTCGATATCGCGGTGATTTCGTGGTGGCGCAGCGGTGAGCGACGC
	Y F L F L D M R D F V V W H A V S D A
1844	CCCCTATGCGTCACCAACTGGATGATTCGCGGAAACGAGTGTATCGAGCGGCT
	P Y A V T N L D D I R R N E L I L A G L
1904	GGATCATCTGGCTTAGGAGATAACACCCCGTGAAGAAGACCGCCCATCTGGGGCGG
	D H L A *
1964	TCTTTTTTGGCAGATGCAATCCAAAAGCATCAGGCGTACTGTCTATCGGAGGCTC

FIG. 2. Sequence of nucleotides 1064 to 2022 from the 2,022-bp *Sst*I fragment containing the *hrcC*, *hrpT*, and *hrpV* genes of *P. syringae* pv. *syringae* 61. Potential ribosome-binding sites and the potential signal peptide of HrpT are underlined. The inverted repeat, marked by opposing arrows, forms the putative terminator for the *hrpC* operon. The italicized amino acids at the C terminus of HrcC represent the revised amino acid sequence predicted from the corrected sequence of *hrcC*, which has an additional G, indicated by boldface. The beginning of coding regions and stop codons are indicated by broken arrows and asterisks, respectively.

ringae pv. *syringae* 61 and *E. amylovora* Ea321 possess 27% identity and 47% similarity and are homologous according to BESTFIT alignment randomization analysis (15). The search also identified similarity between HrpF from *P. syringae* pathovars *syringae* and *glycinea* and HrpA from *P. syringae* pathovar tomato. HrpA has been identified as a secreted protein in *P. syringae* pv. tomato DC3000 and is thought to be involved in the assembly of a pilus-like structure (46, 52). HrpF_{Pss} and HrpF_{Psg} possessed 31% identity and 55% similarity, and 32% identity and 58% similarity, respectively, to HrpA_{Pst}. Both alignments passed the randomization test for significance but did incorporate gaps. The similarity was most marked within a region of 30 amino acids at the amino terminus of the protein.

Curiously, HrpF and HrpA did not show significant similarity to each other when compared within each of the three pathovars. Although there was limited similarity between the amino-terminal amino acids, the overall scores for the proteins did not pass the randomization test. This reflects the fact that the HrpA proteins of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *syringae* 61 are only 27% identical and 43% similar and the HrpF proteins of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *syringae* 61 are only 36% identical and 56% similar. In contrast the HrpA and HrpF proteins of *P. syringae* pv. *syringae* 61 are highly similar to their homologs in *P. syringae* pv. *glycinea* U1. Therefore, HrpA and HrpF share the attribute of being highly divergent in *P. syringae* pathovar tomato relative to the other two pathovars, in addition to being small hydrophilic proteins encoded by the first ORFs of polycistronic operons.

HrpG is predicted to be a 143-amino-acid (15.4-kDa) cytoplasmic protein. It is highly conserved among *P. syringae* patho-

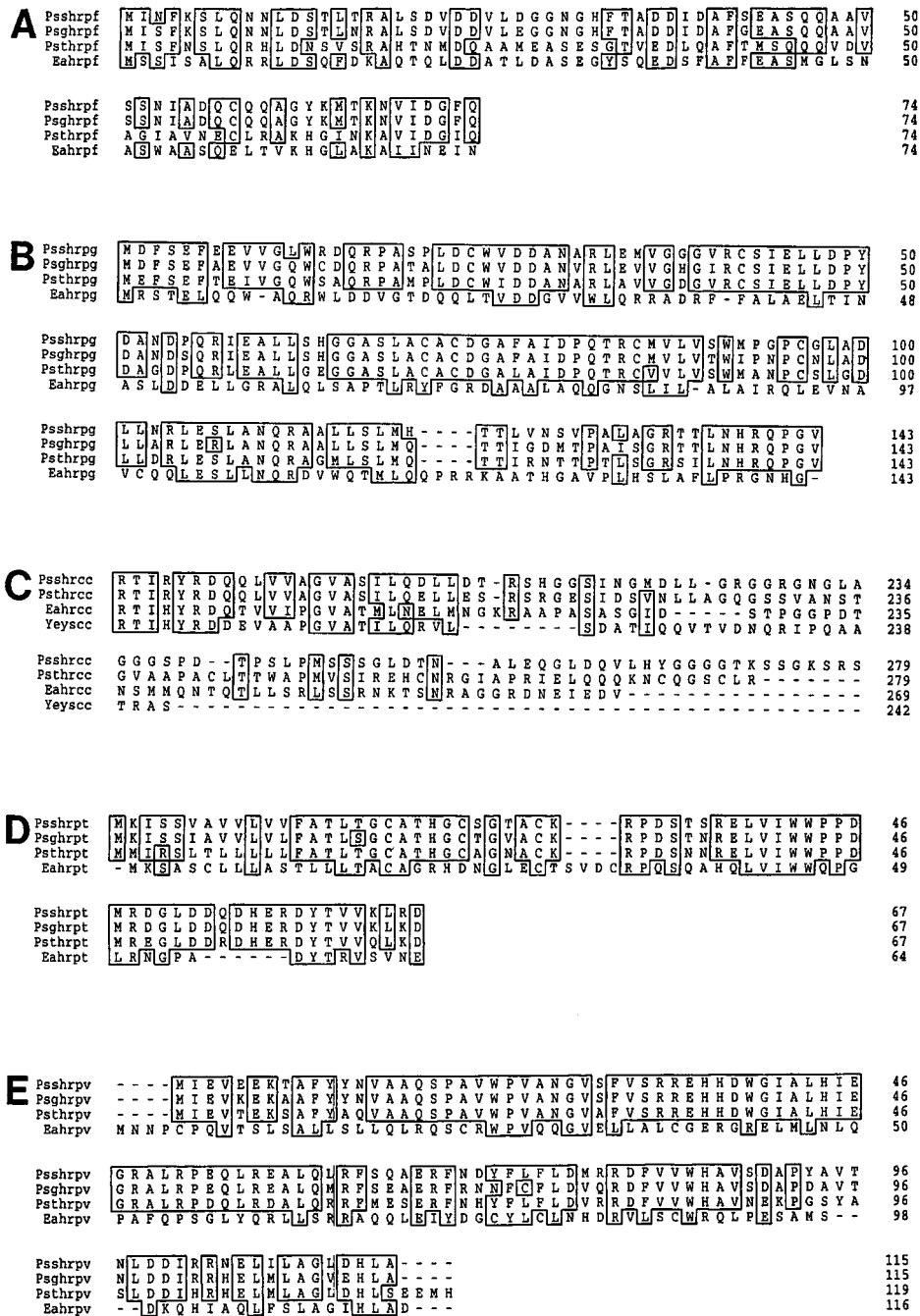


FIG. 3. Alignments of the predicted amino acid sequences for HrpF, HrpG, HrcC, HrpT, and HrpV from *P. syringae* pv. *syringae* 61 (Pss) with those of homologous proteins from other bacteria possessing the type III secretion system. (A) HrpF; (B) HrpG; (C) an internal portion of HrcC containing amino acids 186 to 279; (D) HrpT; (E) HrpV. Sequences are from *P. syringae* pv. tomato DC 3000 (Pst), *P. syringae* pv. glycinea U1 (Psg), *E. amylovora* Ea321 (Ea), and *Yersinia enterocolitica* (Ye). Alignments were made by using the Genetics Computer Group PILEUP algorithm. The accession numbers are U56662 (*E. amylovora* HrpF to -V), U25813 (*P. syringae* pv. *syringae* 61 HrpF to -G), L01064 (*P. syringae* pv. *syringae* 61 HrcC), and M74011 (*Y. enterocolitica* YscC). Open boxes indicate identical amino acids.

vars *syringae*, tomato, and glycinea. However, HrpG of *P. syringae* does not show significant homology to its counterpart in *E. amylovora* or to any other proteins in the database.

HrcC. Many of the features and homologies of HrcC and related proteins have been described previously (17, 27). Here we will focus mostly on the new information derived from the corrected sequence of *P. syringae* pv. *syringae* 61 *hrcC* and from the comparison of HrcC in *P. syringae* pathovars *syringae* and

tomato. Homology searches show that HrcC is a member of the PulD-pIV superfamily of secretins, which are outer membrane proteins involved in macromolecular traffic across the bacterial outer membrane (47). The superfamily includes the type III-specific proteins HrcC (*E. amylovora*, *Xanthomonas campestris*, and *Ralstonia solanacearum*), YscC (*Yersinia* spp.), PscC (*Pseudomonas aeruginosa*), SpiA and InvG (*Salmonella typhimurium*), MxiD (*Shigella flexneri*), and SepC (*E. coli*) (27).

The N-terminal portion of HrcC also exhibits homology to the NolW protein of *Rhizobium fredii*, which is involved in host specificity (40). Comparing HrcC proteins from *P. syringae* pathovars syringae and tomato with each other and with homologs from other bacteria revealed that there is a region of approximately 70 amino acids, which begins about 230 amino acids into the 700-amino-acid HrcC protein, that is highly divergent between the two pathovars. This region is also divergent between HrcC in *Erwinia*, *Xanthomonas*, and *Ralstonia* and is largely absent in YscC from *Yersinia*. Moreover, although the C-terminal end of HrcC is highly conserved between *P. syringae* pathovars syringae and tomato, and moderately conserved between *P. syringae* and *E. amylovora*, the *P. syringae* HrcC is significantly longer than the corresponding proteins from *Yersinia*, *Xanthomonas*, and *Ralstonia*, and the C-terminal ends of the proteins from these different species are not highly conserved.

HrpT and HrpV. The predicted product of *P. syringae* pv. syringae 61 *hrpT* is a 67-amino-acid, 7.5-kDa outer membrane lipoprotein and that of *hrpV* is a 115-amino-acid, 13.4-kDa hydrophilic protein. The predicted product of *P. syringae* pv. tomato DC 3000 *hrpV* is a slightly larger protein of 119 amino acids (13.9 kDa). HrpT and HrpV both have homologs with a significant degree of homology in *E. amylovora* but show no homology to other proteins in the database.

HrpR and HrpS. The sequence of the operon encoding HrpR and HrpS has previously been reported for *P. syringae* pathovars phaseolicola and syringae (19, 20, 51). The *hrpR* and *hrpS* genes from *P. syringae* pv. tomato DC 3000 and *P. syringae* pv. glycinea U1 are more highly conserved than those of the other two pathovars, and as in *P. syringae* pathovars syringae and phaseolicola, they are also highly similar to each other (data not shown). The similarity between HrpR and HrpS in each of the *P. syringae* pathovars glycinea, tomato, and phaseolicola is significantly stronger than the similarity between the reported sequences of HrpR and HrpS from *P. syringae* pv. syringae 61. Furthermore, reexamination of the HrpR sequence from *P. syringae* pv. syringae 61 following comparison with those of the other pathovars suggested that there is a frameshift resulting from a sequencing error between amino acids 235 and 249. Resequencing of this region revealed an additional nucleotide corresponding to amino acid 239. After correction, the deduced amino acid sequences show even greater identity between HrpR_{PSS} and HrpS_{PSS} (data not shown).

In *P. syringae* pv. tomato DC 3000 and *P. syringae* pv. syringae 61 there is a region of approximately 1 kb of AT-rich, noncoding DNA upstream of *hrpRS*, which forms the "right" boundary of the conserved *hrp* cluster. The flanking DNA beyond this noncoding region encodes putative virulence-associated proteins, including AvrE, and other proteins involved in the Hrp system, such as HrpW (11, 38).

Construction of functionally nonpolar mutations in the *P. syringae* pv. syringae 61 *hrpC* operon. To investigate the role of each gene in the *hrpC* operon, individual ORFs were disrupted by insertion of a 1.5-kb *nptII* (neomycin phosphotransferase II) cassette lacking a rho-independent transcription terminator, followed by marker exchange recombination with the *P. syringae* pv. syringae 61 chromosome. The construction of the individual mutations is outlined in Fig. 4, and the plasmids and primers used are listed in Tables 1 and 2. In brief, recombinant plasmids pNCHU402, pNCHU407, pNCHU491, and pNCHU492 were each transformed into *P. syringae* pv. syringae 61 by triparental mating, and kanamycin-resistant transformants were screened for loss of tetracycline resistance. The

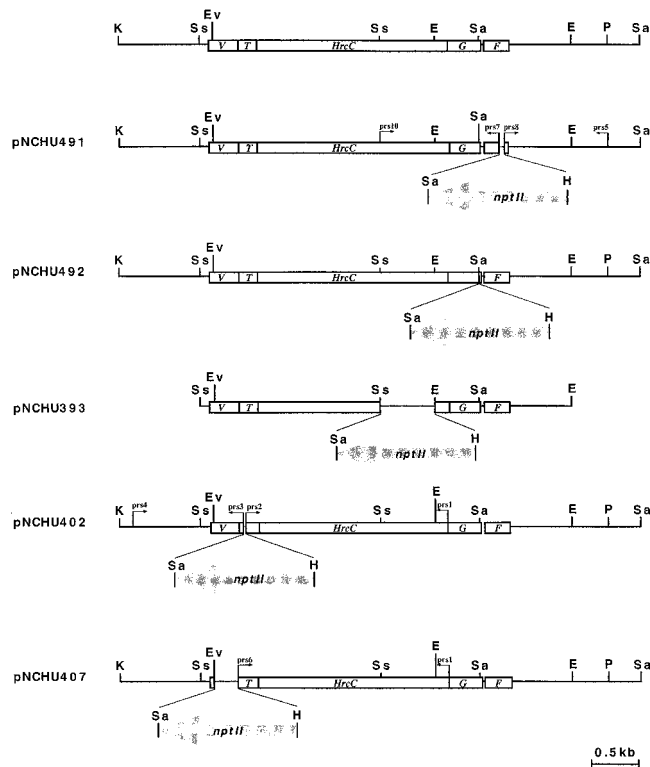


FIG. 4. Construction of functionally nonpolar mutations in the *P. syringae* pv. syringae 61 *hrpC* operon. In each construction, the gray arrow represents the *nptII* cassette lacking a terminator and its orientation; the dotted line represents the internal deletion that is replaced by the *nptII* cassette. Primers involved with the PCR-amplified DNA fragments are indicated by small arrows. F, G, T, and V represent the *hrpF*, *-G*, *-T*, and *-V* genes, respectively. The restriction enzymes are abbreviated as follows: K, *KpnI*; E, *EcoRI*; Sa, *Sall*; EV, *EcoRV*; H, *HindIII*; P, *PstI*; Ss, *SstI*; Bg, *BglII*.

mutations were confirmed by DNA gel blotting and hybridization, using the *nptII* gene as a probe (data not shown).

Altered abilities of *P. syringae* pv. syringae 61 *hrpF*, *hrpG*, *hrpC*, *hrpT*, and *hrpV* mutants and complemented strains to elicit HR in tobacco leaves. To evaluate the effect of mutations on the ability of *P. syringae* pv. syringae 61 to elicit an HR, the mutants—*hrpF*, *hrpG*, *hrpC*, *hrpT*, and *hrpV*—were infiltrated individually into tobacco leaves at a range of inoculum levels. The HR was evaluated for rapid tissue collapse at 24 h postinoculation. Nonpolar mutations in *hrpF*, *hrpG*, and *hrpT* abolished or greatly reduced the ability of *P. syringae* pv. syringae 61 to elicit an HR in tobacco. The *hrpG* mutant retained significant HR-eliciting ability, but the HR observed was weaker than that caused by the wild-type strain. However, mutation of the *hrpV* gene had no observable effect on the timing or intensity of the HR. The results of the HR elicitation experiments are summarized in Table 3. The ability of *hrpF*, *hrpG*, *hrpC*, and *hrpT* mutants to elicit the HR in tobacco plants was restored to the wild-type phenotype by complementation with the corresponding subclones (Table 3). Curiously, complementation of the *hrpV* mutation, which lacked any obvious HR phenotype, resulted in a significant reduction in HR-eliciting activity when relative levels of activity were assessed by serial dilution.

Altered abilities of *P. syringae* pv. syringae 61, *hrpF*, *hrpG*, *hrpC*, *hrpT*, and *hrpV* mutants and complemented strains to multiply and produce disease symptoms in bean leaves. The ability of *hrpC* operon mutants to cause disease was assessed by

TABLE 3. Phenotypes of *P. syringae* pv. *syringae* 61 *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants in planta

<i>P. syringae</i> strain	Genotype	HR in tobacco ^a	Disease in beans ^b	Multiplication in beans ^c	Complementing plasmid	HR in tobacco (complemented strain)
61	Wild type	I	I	21,005 ^A	pNCHU515NA	NA ^d
61-N491	$\Delta hrpF::nptIII$	IV	III	367 ^C	pNCHU515	I
61-N492	$\Delta hrpG::nptIII$	II	II	1,411 ^B	pNCHU513	I
61-N393	$\Delta hrcC::nptIII$	IV	III	4 ^D	pNCHU421	I
61-N402	$\Delta hrpT::nptIII$	III	II	1,020 ^B	pNCHU451	I
61-N407	$\Delta hrpV::nptIII$	I	II	320 ^C	pCPP2372	II

^a Phenotypic classes: I, wild-type HR; II, HR slightly reduced, with difference observable mostly at lower inoculum levels; III, HR very weak and spotty, with some inoculations producing no response; IV, no HR.

^b Phenotypic classes: I, wild-type symptoms of spreading lesion and chlorotic halo; II, symptoms reduced; III, no symptoms.

^c Fold increase in bacterial population (CFU) in 1 cm² of leaf tissue 2 days after inoculation; mean of four tests. The superscript capital letters indicate that the values are significantly different from each other ($P = 0.05$) by the analysis of variables test (JMP version 2.0.5 [1989]; SAS Institute, Carry, N.C.).

^d NA, not applicable.

infiltrating bacteria into bean leaves at 10⁵ CFU/ml and determining bacterial multiplication after 2 days and symptom expression after 5 days (Table 3). Conditions of high humidity were used to favor disease development. The strains tested fell into three classes with regard to their ability to multiply: (i) the *hrcC* mutant multiplied the least; (ii) *P. syringae* pv. *syringae* 61 multiplied the most; and (iii) the *hrpF*, *hrpG*, *hrpT*, and *hrpV* mutants multiplied to an intermediate level. The strains could be divided into three different classes with regard to the production of symptoms on bean leaves: (i) the *hrpF* and *hrcC* mutants were symptomless; (ii) *P. syringae* pv. *syringae* 61 produced necrotic, water-soaked lesions; and (iii) the *hrpG*, *hrpT*, and *hrpV* mutants produced significantly smaller lesions. Thus, although the ability of the *hrpV* mutant to elicit the HR was indistinguishable from that of wild-type *P. syringae* pv. *syringae* 61, its ability to multiply and produce disease symptoms was impaired.

DISCUSSION

We have characterized the *hrpC* operons of three *P. syringae* pathovars and compared the effects on bacterium-plant interactions of mutations in the *P. syringae* pv. *syringae* 61, *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* genes. Our findings reveal that the structure of the *hrpC* operon is conserved in these *P. syringae* pathovars and in *E. amylovora* and that each of the five genes in the *hrpC* operon contributes differently to the ability of *P. syringae* pv. *syringae* 61 to interact with plants. The structure of the *hrpC* operon and the differential phenotypes of the genes within it are significant for several reasons.

The *P. syringae* HrcC protein is a member of a superfamily of outer membrane proteins that are thought to multimerize and form a channel for translocation of proteins or filamentous phages across the outer membrane (17, 22, 29). The HrcC (formerly HrpH) protein of *P. syringae* pv. *syringae* 61 is essential for secretion of the HrpZ harpin (21). HrcC (formerly HrpA1) of *X. campestris* pv. *vesicatoria* has been shown to be localized to the outer membrane, and the protein also induces the *psp* operon when produced in *E. coli* (50), which is indicative of multimerization in the outer membrane (37). *P. syringae* pv. *syringae* 61 *hrcC* mutants accumulate HrpZ in the periplasm, which provides further evidence for a role of HrcC in protein translocation across the outer membrane (12). HrcC and its homologs are conserved components of all known type III secretion systems, but the flanking genes differ widely in many of those systems. For example, *hrcC* and *yscC* are flanked by different genes in *R. solanacearum*, *X. campestris* pv. *vesicatoria*, and *Y. enterocolitica*, respectively (41, 49, 50). In contrast, the *hrcQ*, *-R*, and *-S* homologs are present in the same

order in all of these bacteria, as are their flagellar biogenesis homologs (49).

The *hrp* clusters of plant-pathogenic bacteria have been divided into two groups based on their regulatory components and *hrp* gene compositions (3). Group I contains *P. syringae* and *E. amylovora*, and group II contains *R. solanacearum* and *X. campestris*. Conservation of the *hrpC* operon appears to be characteristic of group I *hrp* clusters, a notion that is further supported by the recent finding that *Erwinia chrysanthemi* also carries the *hrpC* operon (31). Since the *hrpF*-*hrpG*-*hrcC*-*hrpT*-*hrpV* arrangement is not widely conserved (in contrast, for example, to the *hrcQ*-*hrcR*-*hrcS* arrangement), the existence of the *hrpC* operon suggests a close relationship among the group I *hrp* clusters, probably as a result of horizontal transfer to these diverse bacteria.

Further evidence for conservation of the *hrp* gene clusters among *P. syringae* pathovars was found by comparing the *hrpRS*, *hrpZ*, and *hrpC* operons of *P. syringae* pv. *syringae* 61, *P. syringae* pv. tomato DC 3000, and *P. syringae* pv. glycinea U1. Three taxonomic groups have been identified among the *P. syringae* pathovars on the basis of PCR-restriction fragment length polymorphism analysis of rRNA operons, and *P. syringae* pathovars *syringae*, tomato, and glycinea each belong to a different group (39). The divergence of *P. syringae* pathovars tomato and *syringae* is further supported by DNA-DNA hybridization studies (16). The finding of an identical gene arrangement for the 13 *hrp* and *hrc* genes comprising the right half of the *hrp*-*hrc* gene clusters in these divergent pathovars supports the hypothesis that the type III pathways of the different *P. syringae* pathovars are similar in function and that the differing pathogenic properties of these bacteria are determined by proteins that travel the pathway and are encoded in more variable regions (2).

The functions of the four Hrp proteins encoded by the *hrpC* operon are unclear, although mutations affecting each of them alter interactions of the bacterium with diagnostic plants. It should be noted that these genes are all designated *hrp*, even if they do not have a typical Hrp phenotype, because they are in a *hrp* operon (8) and mutations in them have at least some effect on the Hrp system. As expected, the *hrcC* mutation completely abolished all plant reaction phenotypes tested. The *hrpF* mutation had a similarly strong effect. The possibility that HrpF is secreted is suggested by the significant similarity between the *P. syringae* pv. *syringae* 61 and *P. syringae* pv. glycinea U1 HrpF proteins and the *P. syringae* pv. tomato DC 3000 HrpA protein and by the sequence divergence of HrpF in the three pathovars. HrpA is a Hrp-secreted pilin (46), and the degree of divergence has been reported to be higher for extracellular components of the type III secretion system (35). In

contrast, HrpG and HrpT were more conserved and the corresponding mutations had intermediate effects in each of the plant reaction assays. The phenotypes of the *hrpF*, *hrpG*, *hrcC*, and *hrpT* mutants were all consistent with the hypothesis that these genes encode components of the Hrp pathway, but the *hrpV* mutation was puzzling in two ways. First, it had a strong effect on multiplication in beans but not on the HR in tobacco. Second, when complemented with a *hrpV* subclone in trans, the *hrpV* mutant acquired a reduced HR phenotype. One explanation for this is that HrpV is a negative regulator of the Hrp system. In the accompanying paper (44), we further explore this issue and the *hrpF*, *hrpG*, *hrpT*, and *hrpV* mutations by examining the effects of these mutations on the expression of the Hrp regulon and on the secretion of the HrpZ harpin and we further discuss the role of each of these proteins in the Hrp system.

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