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# The *hrpK* Operon of *Pseudomonas syringae* pv. tomato DC3000 Encodes Two Proteins Secreted by the Type III (Hrp) Protein Secretion System: HopB1 and HrpK, a Putative Type III Translocator

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*Pseudomonas syringae* is a gram-negative bacterial plant pathogen that is dependent on a type III protein secretion system (TTSS) and the effector proteins it translocates into plant cells for pathogenicity. The *P. syringae* TTSS is encoded by *hrp-hrc* genes that reside in a central region of a pathogenicity island (Pai). Flanking one side of this Pai is the exchangeable effector locus (EEL). We characterized the transcriptional expression of the open reading frames (ORFs) within the EEL of *P. syringae* pv. tomato DC3000. One of these ORFs, PSPTO1406 (*hopB1*) is expressed in the same transcriptional unit as *hrpK*. Both HopB1 and HrpK were secreted in culture and translocated into plant cells via the TTSS. However, the translocation of HrpK required its C-terminal half. HrpK shares low similarity with a putative translocator, HrpF, from *Xanthomonas campestris* pv. vesicatoria. DC3000 mutants lacking HrpK were significantly reduced in disease symptoms and multiplication in planta, whereas DC3000 *hopB1* mutants produced phenotypes similar to the wild type. Additionally, *hrpK* mutants were reduced in their ability to elicit the hypersensitive response (HR), a programmed cell death associated with plant defense. The reduced HR phenotype exhibited by *hrpK* mutants was complemented by *hrpK* expressed in bacteria but not by HrpK transgenically expressed in tobacco, suggesting that HrpK does not function inside plant cells. Further experiments identified a C-terminal transmembrane domain within HrpK that is required for HrpK translocation. Taken together, HopB1 is a type III effector and HrpK plays an important role in the TTSS and is a putative type III translocator.

*Pseudomonas syringae* is a host-specific gram-negative bacterial pathogen that requires a type III protein secretion system (TTSS) to be pathogenic (4, 29). Its TTSS secretes two classes of proteins: helper or accessory proteins that assist in the translocation or “injection” of the other class of proteins, termed effectors. Collectively, both types are called Hop proteins (for Hrp outer proteins) because they are secreted by the *P. syringae* TTSS. The TTSS apparatus is encoded by *hrp* genes (hypersensitive response [HR] and pathogenicity genes). *hrp* genes were named as such because mutants identified in genetic screens lost their pathogenic ability and failed to elicit the HR, a programmed cell death associated with plant defense. Recently, the *P. syringae* pv. tomato DC3000 genome sequence was completed (9). Genomic approaches to identify additional Hops in DC3000 have increased the total Hop inventory to more than 30 (18). The role that most of these proteins play in bacterial plant interactions is unknown. However, several effectors have recently been shown to be capable of suppressing plant defense responses (1, 16, 22, 25, 37, 46, 72).

A subset of *P. syringae* effectors are named avirulence (Avr) proteins because they were originally isolated due to their ability to stop a virulent pathogen from being pathogenic on

specific host plants that contained a corresponding resistance (R) gene, a phenomenon referred to as gene-for-gene resistance (50). The nature of the host specificity displayed by *P. syringae* pathovars is not completely understood, but it is at least partially due to the number of type III effectors that are recognized as Avr proteins by the R protein surveillance system of the host plant's innate immune system.

To better understand host specificity and pathogenicity of *P. syringae* pv. tomato DC3000, it is important to define the complete effector inventory. One logical region of the genome to search was within the pathogenicity island that encoded the TTSS apparatus (3). The central region of this pathogenicity island contains the *hrp-hrc* genes that encode the TTSS apparatus. Flanking this central region are the conserved effector locus (CEL) and the exchangeable effector locus (EEL), which appear rich in effector and helper genes (3). DC3000 CEL mutants are reduced in their ability to grow in plants and cause disease symptoms (3). Recently, this phenotype has been shown to be due to the effectors AvrE and HopPtoM, which are encoded by genes within the CEL (7, 22). In contrast, DC3000 EEL mutants have a subtle reduction in the production of disease symptoms and growth in planta (3). However, the DC3000 EEL contains several candidate effector genes, including two that possess active HrpL-dependent promoters (27, 76). The EEL is variable even between closely related strains of *P. syringae*, and analyzing EELs from different *P. syringae* strains has been useful as a strategy to identify new effector genes as well as to provide insights into differences,

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similarities, and evolutionary relatedness of *P. syringae* strains (3, 13, 24).

The *hrpK* gene is located in the conserved *hrp-hrc* cluster at the border of the EEL in *P. syringae* (3, 13, 24, 57). The role HrpK plays in type III secretion is unknown. *hrpK* mutants show variable HR phenotypes on plants (8, 57). *P. syringae* *hrpK* mutants retain their ability to secrete the HrpZ harpin in culture, suggesting that HrpK is not an essential component of the *P. syringae* TTSS apparatus (17). Additionally, the predicted N-terminal end of HrpK shares the characteristics of *P. syringae* type III secreted proteins, suggesting that HrpK is a secreted protein (63).

Animal pathogen TTSSs utilize accessory proteins called translocators to deliver or translocate effector proteins across the plasma membrane into mammalian host cells. In the prototypical *Yersinia* TTSS, YopB, YopD, and LcrV act as translocators (19). *Yersinia* mutants defective in these proteins secreted Yop effectors in culture at a level similar to that of the wild type, but they failed to deliver effectors into host cells (35, 68, 71). Other data are consistent with these proteins acting as a translocon complex. For example, they are all capable of interacting with each other (61, 68), each can form pores in liposomes (35, 39, 61), and they cause the release of small-molecular-weight dyes (but not larger-molecular-weight dyes) from mammalian cells (58). Other well-studied animal pathogen TTSSs also have translocators identified on the basis of similar criteria (10, 28, 43, 45).

Less is known about type III translocators in plant pathogens. Translocating effectors into plant cells may be significantly different than effector translocation into animal cells because the plant cell wall may act as an additional barrier. Harpin helper proteins secreted by a number of TTSS-containing plant pathogens may act as translocators, since the *P. syringae* HrpZ harpin forms pores in artificial lipid bilayers (5, 56). However, *P. syringae* *hrpZ* mutants are not significantly altered in their ability to grow in planta or in effector (*Avr*) protein translocation (2), which would be predicted if the bacterium lacked a major component required for translocation. However, DC3000 is known to contain multiple harpins, which may mask a translocator phenotype (9, 14).

A putative translocator, HrpF, has been identified in *Xanthomonas campestris* pv. vesicatoria. *X. campestris* pv. vesicatoria *hrpF* mutants secrete proteins via the TTSS in culture but fail to translocate proteins into plant cells (66). Moreover, HrpF can form pores in artificial lipid bilayers, consistent with it acting as a translocator (11). HrpF has homology with NolX, a type III secreted protein from *Sinorhizobium fredii* whose function is unknown (42, 54).

Here, we report the identification of two additional proteins, HrpK and HopB1, that are secreted via the DC3000 TTSS. Both HrpK and HopB1 are translocated into plant cells. HopB1 contributes little to virulence, whereas HrpK contributes to symptom production and growth in planta. We present evidence that indicates that HrpK is a type III translocator for the *P. syringae* TTSS.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, primers, and media.** Bacterial strains and plasmids used in this work are listed in Table 1. All primers used for plasmid constructions are listed in Table 2. *Escherichia coli* strains DH5 $\alpha$  and DH5 $\alpha$ F'

*lacI*<sup>H</sup> were grown in Luria-Bertani broth at 37°C. *P. syringae* pv. tomato DC3000 and *Pseudomonas fluorescens* 55 strains were grown in King's B broth (KB) at 30°C or *hrp*-inducing fructose minimal medium at 22°C (44, 52). Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 20; gentamicin, 10; kanamycin, 50; rifampin, 100; spectinomycin, 50; and tetracycline, 20.

**Plasmid construction.** The Gateway cloning system (Invitrogen, Carlsbad, Calif.) was used to generate many of the constructs used in the experiments described, following the manufacturer's instructions. Briefly, entry constructs were made in pENTR with the pENTR/D-TOPO Gateway cloning kit. The following entry constructs were generated: pLN293 (*hrpK*), pLN420 (*hopB1*), pLN916 (*hrpK*<sub>1-382</sub>), pLN1157 (*hrpK*<sub>1-467</sub>), pLN1158 (*hrpK*<sub>1-582</sub>), pLN1159 (*hrpK*<sub>1-698</sub>), pLN1160 (*hrpK* <sub>$\Delta$ TM</sub>), pLN1161 (*hrpK*<sub>382-TM</sub>), and pLN1162 (*hrpK*<sub>80-TM</sub>). The primer sets used to amplify the inserts recombined into pENTR for each of the constructs are as follows: P638–P639, pLN293; and P1245–P1246, pLN420. For pLN916, the PCR product obtained with primers P638 and P1073 was digested with EcoRV and the 1.1-kb fragment (corresponding to amino acids 1 to 382 of HrpK) isolated was recombined into pENTR. The remaining constructs were generated with the QuikChange Site-Directed mutagenesis kit (Stratagene, La Jolla, Calif.) with pLN293 as template DNA with the following primer sets: P1509–P1510, resulting in pLN1157; P1511–P1512, resulting in pLN1158; P1513–P1514, resulting in pLN1159; P1281–P1282, resulting in pLN1160; P1373–P1374, resulting in pLN1161; and P1392–P1393, resulting in pLN1162.

For HR complementation studies, we constructed a broad-host-range Gateway destination vector by cloning the Gateway cassette from pLN461 with a hemagglutinin (HA) epitope tag into pBBR1MCS-5 (53) as a XhoI-SacI fragment generating construct pLN677.

For complementation of the pHIR11 *hrpK* mutation, we first PCR amplified a gentamicin resistance cassette from pBBR1MCS-5 (53) with primers P278 and P279 and cloned it as a BamHI-EcoRI fragment into pRG930, resulting in pLN1155 (73). Afterwards, *hrpK* with its native promoter was PCR cloned into pLN1155 with primers P101 and P277 and BamHI, generating construct pLN174.

**Generation of *hopB1* and *hrpK* mutants.** The DC3000 nonpolar *hrpK* mutant (UNL111) and the DC3000 *hopB1* mutant (UNL130) were generated by similar strategies. Fragments (each, 2 kb) were PCR amplified upstream and downstream of each gene. For *hopB1*, we used P35–P36 and P37–P38 primer sets for the upstream and downstream fragments, respectively. For *hrpK*, we used P209–P210 and P355–P356 primer sets for the upstream and downstream fragments, respectively. The adjacent fragments for each gene were separately cloned into pCPP2988 (2) in the same orientations on either side of an *nptII* gene lacking transcriptional terminators. The DNA fragments containing each mutation and *nptII* were cut out with XbaI and KpnI and cloned into the broad-host-range vector pRK415 (51), resulting in constructs pLN825, which carries an *hopB1* mutation, and pLN826, which carries an *hrpK* mutation. These constructs were separately electroporated into DC3000 and homologous recombination was selected for by selecting for retention of the antibiotic marker (Km) linked to the mutation and loss of the plasmid marker (Tc). Each mutant was confirmed by PCR and Southern analysis.

The *hrpK* polar mutation, UNL132, was generated in a way similar to the above mutations, except that 2-kb fragments from *hrpK* were PCR cloned on either side of a Sp<sup>+</sup>/Sm<sup>r</sup> omega fragment from pHP45 (26) with the primer sets P139–P140 and P141–P142, generating construct pLN884. The fragment containing this mutation was cut out of pLN884 as an XbaI-KpnI fragment and cloned into pRK415, resulting in pLN882, which was subsequently electroporated into DC3000. The *hrpK* polar mutation was recombined into the chromosome as described above.

**Constructing the *hrpK* mutation in cosmid pHIR11.** A BamHI-SacI fragment from pLN877 that carried a part of the *hrp-hrc* cluster containing the *P. syringae* pv. *syringae* 61 *hrpK* gene was digested with SalI. A 1.6-kb fragment starting from within the coding region of *hrpK* and extending downstream was cloned into pCPP2988. A 2.2-kb EcoRI fragment, which represented a *hrpK* upstream fragment, was cut out of pLN877 with XbaI and EcoRV and then cloned adjacent to the SalI fragment, resulting in pLN827. This construct was electroporated into *E. coli* C2110 (pHIR11). C2110 is a *polA* temperature-sensitive mutant. Thus, incubation of C2110 (pHIR11) (pLN827) at 42°C forced pLN827 to recombine into pHIR11 because ColE1 plasmids cannot replicate without *polA* (48). Lowering the temperature to 30°C allowed the ColE1 plasmid pLN827 to recombine back out of pHIR11. A recombinant pHIR11 derivative containing an *hrpK* deletion mutation was identified by PCR and named pLN468. This construct was subsequently conjugated into *P. fluorescens* 55 for the experiments described.

**RNA isolation and RT-PCR.** DC3000 was grown in either *hrp*-inducing fructose minimal medium at 22°C or KB at 30°C overnight. RNA was isolated with

TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference or source
<b>Strains</b>		
<i>E. coli</i>		
C2110	PolA <sup>ts</sup> , Nal <sup>r</sup>	48
DB3.1	F <sup>-</sup> <i>gyrA462 endA1 Δ(sr1-recA) mcrB nmr hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>supE44 ara-14 galK2 lacY1 proA2 rpsL20</i> (Sm <sup>r</sup> ) <i>xyl-5λ<sup>-</sup> leu mut-1</i>	Invitrogen
DH5α	<i>supE44 ΔlacU169</i> (φ80 <i>lacZΔM15</i> ) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> Nal <sup>r</sup>	36; Life Technologies
DH5αF' <i>lacI<sup>q</sup></i>	F' <i>proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15 zzf::Tn5 supE44 ΔlacU169</i> (φ80 <i>lacZΔM15</i> ) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> Nal <sup>r</sup>	Life Technologies
<i>P. fluorescens</i>		
55	Wild type, Nal <sup>r</sup>	41
<i>P. syringae</i> pv. phaseolicola		
NPS3121	Wild type, spontaneous Rif <sup>r</sup>	49
<i>P. syringae</i> pv. tomato		
DC3000	Wild type, spontaneous Rif <sup>r</sup>	21
DC3000- <i>hrcC</i>	<i>hrcC::TnphoA</i> , type III-deficient mutant, Rif <sup>r</sup> Crn <sup>r</sup>	75
UNL111	DC3000 nonpolar <i>hrpK</i> deletion, Rif <sup>r</sup> Km <sup>r</sup>	This work
UNL130	DC3000 <i>hopB1</i> deletion, Rif <sup>r</sup> Km <sup>r</sup>	This work
UNL132	DC3000 containing a polar omega fragment in <i>hrpK</i> , Rif <sup>r</sup> Sp <sup>r</sup> /Sm <sup>r</sup>	This work
<b>Plasmids</b>		
pAvrRpt2-600	pDSK600 derivative containing <i>avrRpt2</i> , Sp <sup>r</sup> /Sm <sup>r</sup>	64
pBBR1MCS-5	Broad-host-range cloning vector, Gm <sup>r</sup>	53
pBC SK(+)	Cloning vector, Cm <sup>r</sup>	Stratagene
pBluescript II KS(+)	Cloning vector, Ap <sup>r</sup>	Stratagene
pBluescript II SK(+)	Cloning vector, Ap <sup>r</sup>	Stratagene
pCPP2089	pHIR11 derivative containing <i>TnphoA</i> insert into <i>hrcC</i> , Tc <sup>r</sup> Km <sup>r</sup>	40
pCPP2318	pCPP30 derivative carrying <i>blaM</i> lacking signal peptide sequences, Tc <sup>r</sup>	15
pCPP2988	pBluescript II KS(-) vector carrying the <i>nptII</i> cassette lacking a transcriptional terminator, Ap <sup>r</sup> Km <sup>r</sup>	2
pCPP3234	Gateway destination pVLT35 derivative containing the adenylate cyclase ( <i>cyoA</i> ) gene for C-terminal fusions, Sp <sup>r</sup> /Sm <sup>r</sup> Cm <sup>r</sup>	69
pCPP3297	pLN18 derivative containing unmarked <i>hrcC</i> mutation, Tc <sup>r</sup> Km <sup>r</sup>	69
pDSK600	Broad-host-range cloning vector, Sp <sup>r</sup> /Sm <sup>r</sup>	60
pENTR/D-TOPO	Gateway system donor vector, Km <sup>r</sup>	Invitrogen
pFLAG-CTC	FLAG expression vector, Ap <sup>r</sup>	Sigma Chemical Co.
pHIR11	pLAFR3 containing a functional TTSS from <i>P. s. syringae</i> 61	40
pHP45Ω Sp/Sm	Vector containing an Sp <sup>r</sup> /Sm <sup>r</sup> omega fragment with transcriptional and translational terminators, Ap <sup>r</sup> Sp <sup>r</sup> /Sm <sup>r</sup>	26
pML123	Broad-host-range cloning vector, Gm <sup>r</sup> Km <sup>r</sup>	55
pPZP212	<i>Agrobacterium tumefaciens</i> binary vector, Km <sup>r</sup>	35
pQE30	N-terminal His <sub>6</sub> -tag expression vector, Ap <sup>r</sup>	Qiagen
pRG930	Broad-host-range cloning vector lacking a vector promoter, Sp <sup>r</sup> /Sm <sup>r</sup>	73
pRK415	Broad-host-range vector, unstable in absence of selection, Tc <sup>r</sup>	51
pUC18	Cloning vector, Ap <sup>r</sup>	74
pVLT35	Broad-host-range cloning vector containing <i>lacI<sup>q</sup></i> , Sp <sup>r</sup> /Sm <sup>r</sup>	23
pLN18	pHIR11 derivative with <i>shcA</i> and <i>hopPsyA</i> mutations, Tc <sup>r</sup> Km <sup>r</sup>	47
pLN57	pQE30 derivative containing <i>hopB1</i> <sub>189-456</sub> , Ap <sup>r</sup>	This work
pLN60	pBBR1MCS-5 derivative carrying a <i>hopB1-avrRpt2</i> <sub>81-255</sub> fusion, Gm <sup>r</sup>	This work
pLN62	pBBR1MCS-5 derivative carrying <i>avrRpt2</i> <sub>81-255</sub> with an RBS, Gm <sup>r</sup>	This work
pLN63	Derivative of pML123 that contains <i>hopB1</i> , Gm <sup>r</sup> Km <sup>r</sup>	This work
pLN64	Derivative of pML123 that contains a <i>hrpK</i> -FLAG gene fusion, Gm <sup>r</sup> Km <sup>r</sup>	This work
pLN65	pBBR1MCS-5 derivative carrying a <i>hrpK</i> <sub>1-399</sub> - <i>avrRpt2</i> <sub>81-255</sub> fusion, Gm <sup>r</sup>	This work
pLN174	pRG930 derivative that contains <i>hrpK</i> with its native promoter and a Gm <sup>r</sup> cassette, Sp <sup>r</sup> /Sm <sup>r</sup> Gm <sup>r</sup>	This work
pLN265	pPZP212 derivative containing <i>hrpK-ha</i> , Km <sup>r</sup>	This work
pLN293	pENTR/D-TOPO derivative carrying a <i>hrpK</i> PCR-amplified fragment, Km <sup>r</sup>	This work
pLN377	pCPP3234 derivative obtained by recombination with pLN293 carrying <i>hrpK</i> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN420	pENTR/D-TOPO derivative carrying <i>hopB1</i> , Km <sup>r</sup>	This work
pLN421	pCPP3234 derivative obtained by recombination with pLN420 carrying <i>hopB1</i> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN468	pHIR11 derivative with a <i>hrpK</i> mutation, Tc <sup>r</sup> Km <sup>r</sup>	This work
pLN677	Gateway destination pBBR1MCS-5 derivative containing a hemagglutinin (HA) tag for C-terminal fusions, Gm <sup>r</sup> Cm <sup>r</sup>	This work
pLN774	pLN677 derivative obtained by recombination with pLN293 carrying <i>hrpK</i> , Gm <sup>r</sup>	This work
pLN825	pRK415 derivative containing a <i>hopB1</i> mutation, Tc <sup>r</sup> Km <sup>r</sup>	This work

Continued on following page

TABLE 1—Continued

Strain or plasmid	Characteristics	Reference or source
pLN826	pRK415 derivative containing a <i>hrpK</i> nonpolar mutation, Tc <sup>r</sup> Km <sup>r</sup>	This work
pLN827	pCPP2988 derivative containing a <i>hrpK</i> mutation, Ap <sup>r</sup> Km <sup>r</sup>	This work
pLN877	pUC18 derivative containing a BamHI-SacI fragment from the left side of pHIR11, Ap <sup>r</sup>	This work
pLN882	pRK415 derivative containing the <i>hrpK</i> polar mutation, Tc <sup>r</sup> Km <sup>r</sup>	This work
pLN883	pFLAG-CTC containing a PCR-amplified <i>hrpK</i> fragment, Ap <sup>r</sup>	This work
pLN884	pBC SK(+) derivative containing the <i>hrpK</i> polar mutation, Cm <sup>r</sup> Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN915	pBBR1MCS-5 derivative carrying a <i>hrpK-avrRpt2</i> <sub>81-255</sub> fusion, Gm <sup>r</sup>	This work
pLN916	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>1-382</sub> , Km <sup>r</sup>	This work
pLN917	pCPP3234 derivative obtained by recombination with pLN916 carrying <i>hrpK</i> <sub>1-382</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN921	pBBR1MCS-5 derivative carrying <i>avrRpt2</i> <sub>81-255</sub> , Gm <sup>r</sup>	This work
pLN1157	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>1-467</sub> , Km <sup>r</sup>	This work
pLN1158	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>1-582</sub> , Km <sup>r</sup>	This work
pLN1159	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>1-698</sub> , Km <sup>r</sup>	This work
pLN1160	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>ΔTM</sub> , Km <sup>r</sup>	This work
pLN1161	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>382-TM</sub> , Km <sup>r</sup>	This work
pLN1162	pENTR/D-TOPO derivative carrying <i>hrpK</i> <sub>80-TM</sub> , Km <sup>r</sup>	This work
pLN1163	pLN677 derivative obtained by recombination with pLN917 carrying <i>hrpK</i> <sub>1-382</sub> , Gm <sup>r</sup>	This work
pLN1164	pLN677 derivative obtained by recombination with pLN1157 carrying <i>hrpK</i> <sub>1-467</sub> , Gm <sup>r</sup>	This work
pLN1165	pLN677 derivative obtained by recombination with pLN1158 carrying <i>hrpK</i> <sub>1-582</sub> , Gm <sup>r</sup>	This work
pLN1166	pLN677 derivative obtained by recombination with pLN1159 carrying <i>hrpK</i> <sub>1-698</sub> , Gm <sup>r</sup>	This work
pLN1167	pLN677 derivative obtained by recombination with pLN1160 carrying <i>hrpK</i> <sub>ΔTM</sub> , Gm <sup>r</sup>	This work
pLN1168	pLN677 derivative obtained by recombination with pLN1161 carrying <i>hrpK</i> <sub>382-TM</sub> , Gm <sup>r</sup>	This work
pLN1169	pLN677 derivative obtained by recombination with pLN1162 carrying <i>hrpK</i> <sub>80-TM</sub> , Gm <sup>r</sup>	This work
pLN1170	pCPP3234 derivative obtained by recombination with pLN1157 carrying <i>hrpK</i> <sub>1-467</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN1171	pCPP3234 derivative obtained by recombination with pLN1158 carrying <i>hrpK</i> <sub>1-582</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN1172	pCPP3234 derivative obtained by recombination with pLN1159 carrying <i>hrpK</i> <sub>1-698</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN1173	pCPP3234 derivative obtained by recombination with pLN1160 carrying <i>hrpK</i> <sub>ΔTM</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN1174	pCPP3234 derivative obtained by recombination with pLN1161 carrying <i>hrpK</i> <sub>382-TM</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work
pLN1175	pCPP3234 derivative obtained by recombination with pLN1162 carrying <i>hrpK</i> <sub>80-TM</sub> , Sp <sup>r</sup> /Sm <sup>r</sup>	This work

guanidium thiocyanate-saturated hot phenol (pH 6.6) (Ambion, Austin, Tex.) (67) at 60°C and extracted twice with phenol:chloroform:isoamyl alcohol and twice with chloroform:isoamyl alcohol. RNA was precipitated with ammonium acetate and ethanol and resuspended in water. Samples were treated with DNase I (Ambion) according to the manufacturer's instructions. DNA was made from the isolated RNA with the Pro-Star HF Single Tube reverse transcriptase PCR (RT-PCR) kit (Stratagene). DNA fragments were amplified with PCR under the following conditions: 25 cycles of 95°C for 30 s, 52°C for 2 min, and 68°C for 4 min. After amplification, PCRs were run on a DNA agarose gel to determine the genes that were transcribed. The primer sets used for RT-PCR and the expected product size were as follows: *hrpL-hrpJ* junction (780 bp), P350-P340; *hrpK* (2.3 kb), P128-P129; *hrpK-hopB1* junction (430 bp), P141-P327; *hopB1* (1.4 kb), P338-P339; *impA'* (840 bp), P333-P334; PSPTO1409 (846 bp), P325-P326; PSPTO1410 (411 bp), P331-P332; and PSPTO1411 (405 bp), P323-P324. Southern analysis was separately performed on RT-PCR products to confirm that each PCR product corresponded to the genes from the DC3000 EEL.

**Generation of anti-HopB1 antibodies.** To purify HopB1 for antibody production, we PCR cloned a fragment corresponding to amino acids 189 to 456 of HopB1 with primers P132 and P133 and cloned it into pQE30 (QIAGEN, Valencia, Calif.) using BamHI and SalI restriction enzymes generating construct pLN57. This construct produces a product containing an N-terminal six-histidine residue (His<sub>6</sub>) fused to HopB1<sub>189-456</sub>.

*E. coli* DH5αF' *lacI*<sup>r</sup> carrying pLN57 was grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, Mo.) for 3 h. His<sub>6</sub>-HopB1<sub>189-456</sub> was purified under denaturing conditions in 8 M urea with the Ni-nitrilotriacetic acid metal chelate resin as described in the His-tag expression and purification manual (QIAGEN). Purified HopB1<sub>189-456</sub> was sent to the University of Illinois at Ur-

bana-Champaign Immunological Research Center, where the samples were injected into a rabbit to generate anti-HopB1<sub>189-456</sub> polyclonal antibodies. The crude antiserum we received was preabsorbed against extracts of *E. coli* DH5α and DC3000, which was grown under conditions that do not express type III-related products.

**Type III secretion experiments.** To detect type III secreted proteins in the supernatants of DC3000 cultures, we cloned *hrpK* and *hopB1* into broad-host-range vectors. *hopB1* was PCR cloned into pML123 (55) with primers P17 and P18 as a BamHI-HindIII fragment, generating construct pLN63. The *hrpK*-containing construct was made by PCR amplification with primers P224 and P226 and was cloned into pFLAG-CTC (Sigma-Aldrich) as an XhoI-KpnI fragment, generating construct pLN883, which produces HrpK fused to a C-terminal FLAG peptide. The *hrpK-flag* gene fusion was amplified from pLN883 with primers P224 and P225 and cloned into pML123 as an XhoI-BamHI fragment, creating construct pLN64.

To perform type III secretion assays, pLN63 and pLN64 were mobilized into DC3000, the DC3000 *hrcC* mutant pLN63 was mobilized into UNL130, and pLN64 was mobilized into the *hrpK* mutant UNL132. *Pseudomonas* strains were grown overnight on KB plates containing appropriate antibiotics. DC3000 and DC3000 carrying either pLN63 or pLN64 were inoculated at an OD<sub>600</sub> of 0.3 in *hrp*-inducing minimal medium at 22°C and grown for 6 h. All cultures also contained plasmid pCPP2318 (14), which encodes the mature β-lactamase lacking its signal peptide and thus remains in the cytoplasm. Cell and supernatant fractions were separated, and the protein was precipitated as previously described (63). Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% gels and transferred to membranes for immunoblotting with each of the following primary antibodies: anti-FLAG (Sigma-Aldrich), anti-β-lactamase (Chemicon International, Temecula, Calif.), or

TABLE 2. Primer information

Primer no.	Primer nucleotide sequences <sup>a</sup>	Enzyme sites
P17	5'-AGTA <b>AAGCTT</b> ATGATGCTGTTTCCAGTA-3'	HindIII
P18	5'-AGTAG <b>GGATCC</b> TGAAATGTAGGGGCCCGG-3'	BamHI
P35	5'-AGTA <b>CTCGAG</b> GAATCCAGACGTGAGGCT-3'	XhoI
P36	5'-AGTAG <b>GGTACC</b> TCGTCGGACCTGATTGTG-3'	KpnI
P37	5'-AGTA <b>TCTAGA</b> ATCGCCCGATTGCAGCGG-3'	XbaI
P38	5'-AGTAG <b>GGATCC</b> TCCTGCAGCTTGCAGAGT-3'	BamHI
P46	5'-ATGCG <b>GGATCC</b> ATGCGCCTTCGCAATCCC-3'	BamHI
P101	5'-ATGCG <b>GGATCC</b> TCAATGCGCCTGTCAAT-3'	BamHI
P128	5'-ATCG <b>AAGCTT</b> TGGCAACTCACCATGCGTA-3'	HindIII
P129	5'-AGTC <b>GAATTC</b> ATGCGCCTTCGCAATCCC-3'	EcoRI
P132	5'-AGTAG <b>GTCGAC</b> TCACGAGACAACAACGTC-3'	SalI
P133	5'-AGTAG <b>GGATCC</b> TCGGAAGGTCGGGACCAC-3'	BamHI
P139	5'-ATGCT <b>TCTAGA</b> GTGTGGCATCGCGCTGAA-3'	XbaI
P140	5'-ATGT <b>GGATCC</b> GGTACTTTGGAATCGGGG-3'	BamHI
P141	5'-ACGCG <b>GAATTC</b> TGAGGGCGGACCGTCCAT-3'	EcoRI
P142	5'-ATT <b>CGGTACC</b> ACCTCAGTAGCGAGGCCG-3'	KpnI
P209	5'-TGCG <b>TCTAGA</b> GAGCTGCTTTTGTTCCTG-3'	XbaI
P210	5'-ATGT <b>GGATCC</b> AGGAGTGGGTTGGTTCAC-3'	BamHI
P219	5'-AGCG <b>AAGCTT</b> TGCTGTAAGACTTGGGTTT-3'	HindIII
P224	5'-GCAC <b>CTCGAG</b> TGGAACCACTTGCACCT-3'	XhoI
P225	5'-ATGT <b>GGATCC</b> TCACTTGTTCATCGTCGTC-3'	BamHI
P226	5'-ATGT <b>GGTACC</b> ATGCGCCTTCGCAATCCC-3'	KpnI
P277	5'-ATGCG <b>GGATCC</b> TGCCTGAGTGGTTATCTG-3'	BamHI
P278	5'-ATGCG <b>GGATCC</b> CTGTTTCCCTGTGTGAAAT-3'	BamHI
P279	5'-ATGCG <b>GAATTC</b> CACATTTCCCGAAAAGT-3'	EcoRI
P313	5'-AGTA <b>AAGCTT</b> TGAAAATGTCGGGGCCCGG-3'	HindIII
P316	5'-AGTAG <b>GGATCC</b> GACCTTATAGGAAAGCCT-3'	BamHI
P318	5'-AGTAG <b>GGATCC</b> CACGAGACGGGCGGTTCA-3'	BamHI
P319	5'-AGTA <b>AAGCTT</b> CACGAGACGGGCGGTTCA-3'	HindIII
P320	5'-AGTA <b>TCTAGA</b> CTTAGCGGTAGAGCATTG-3'	XbaI
P323	5'-AACAAAGATCGTCTACGTA-3'	
P324	5'-GCTTGCAGACGACTGAAA-3'	
P325	5'-TCTCGAAGGAATGGAGCA-3'	
P326	5'-CGTGAAGATGCATTTTCGC-3'	
P327	5'-TCACGAGACAACAACGTC-3'	
P331	5'-CGCGCTATAAAAAACCTG-3'	
P332	5'-CTTTTATAGCGGCAGACGG-3'	
P333	5'-ATGGGATTTTACGTCCG-3'	
P334	5'-TTACAGGCGCACCTCTCC-3'	
P338	5'-ATGATGCTGTTTCCAGTA-3'	
P339	5'-CGCGGAGATTCAATCATG-3'	
P340	5'-AACTTGTGCTCGTTACGC-3'	
P350	5'-CCAGAACCTGTTTCAGAT-3'	
P355	5'-GCAT <b>GGTACC</b> CATCAGGATCGCCAGATG-3'	KpnI
P356	5'-GCAT <b>CTCGAG</b> CATAACGTTGATCCGGTG-3'	XhoI
P366	5'-ATGAA <b>AAGCTT</b> TGGAGGCCTAATCATGCACGAGACGGGCGGTTCA-3'	HindIII
P391	5'-AGCT <b>GGATCC</b> TTAGCGGTAGAGCATTGC-3'	BamHI
P562	5'-AGTC <b>GAATTC</b> AAACAATGCGTATATCCAGTTCTC-3'	EcoRI
P638	5'-CACCACTCTCGGAGGCAACTAACAATGCGT-3'	
P639	5'-ATGCGCCTTCGCAATCCCGAA-3'	
P754	AGTC <b>GGATCC</b> TCATGCATAATCAGGTACATCATAAGGATAATGCGCCTTCGCAATCCC	BamHI
P1073	5'-ATGAG <b>GGATCC</b> ACCCTGTAAAGACTTGGG-3'	BamHI
P1245	5'-CACCCGTACGCGGAGATTCAACAATGAGACC-3'	
P1246	5'-GACCTTATAGGAAAGCCTCACGCTCTGG-3'	
P1281	5'-GGGCGTCTGGGCGGACAGATGGATGCTGTGAAAAAGCATAAGGC-3'	
P1282	5'-GCCTTATGCTTTTTCACAGCATCCATCTGTCGCCCAGACGCC-3'	
P1373	5'-GCGGTGGCCGGGATTGATATCGGGC-3'	
P1374	5'-CATCTGTCCGCCAGACGCCCGATAT-3'	
P1392	5'-GGCGGGCAGCTGCCAATGGGCGTCTGGGCGGACAGATG-3'	
P1393	5'-CATCTGTCCGCCAGACGCCCATTTGGCAGCTGCCCGGCC-3'	
P1509	5'-GGTCCGCAGTGATGCCGGTGGGCGCGCCGACC-3'	
P1510	5'-GGTCGGCGGCCACCCGCATCACTGCGGACC-3'	
P1511	5'-GCCGATAACCAGAAAGGGTGGGCGCGCCGACC-3'	
P1512	5'-GGTCGGCGGCCACCCCTTCTGGTTATCGGC-3'	
P1513	5'-GCCAAGCCTCAGCCGGGTTGGGCGCGCCGACC-3'	
P1514	5'-GGTCGGCGGCCACCCCGCTGAGGCTTCGC-3'	

<sup>a</sup> Restriction enzyme sites are in boldface type.

anti-HopB1. Primary antibodies were recognized by goat anti-mouse or anti-rabbit immunoglobulin G-alkaline phosphatase conjugate secondary antibodies (Sigma-Aldrich) and visualized on autoradiographs with the Western-Light chemiluminescence system (Tropix, Bedford, Mass.).

**AvrRpt2 translocation assays.** To perform AvrRpt2 translocation assays, we made a plasmid construct to facilitate making effector fusions to AvrRpt2, which lacks its own secretion signals. A fragment corresponding to amino acids 81 to 255 of AvrRpt2 (AvrRpt2<sub>81-255</sub>) was PCR amplified from pAvrRpt2-600 (64) with primers P318 and P320 and cloned into pBBR1MCS-5 (53) with BamHI-XbaI, generating construct pLN921. Constructs containing *hopB1-avrRpt2*<sub>81-255</sub> and *hrpK-avrRpt2*<sub>81-255</sub> gene fusions were generated in a similar manner. *hopB1* was PCR amplified from DC3000 with primers P313 and P316 and cloned as a HindIII-BamHI fragment into pLN921, generating construct pLN60. *hrpK* was PCR amplified from DC3000 with primers P224 and P102 and cloned into pLN921 as an XhoI-BamHI fragment, generating construct pLN915. To make a construct that encoded a fusion of the first 399 amino acids of HrpK with AvrRpt2<sub>81-255</sub>, we PCR cloned *hrpK*<sub>1-399</sub> from DC3000 with primer set P224-P219 into pBluescript II SK(+), with XhoI and HindIII. We next PCR cloned *avrRpt2*<sub>81-255</sub> from pAvrRpt2-600 template DNA into the pBluescript II SK(+) derivative containing *hrpK*<sub>1-399</sub> such that they were in frame with each other by using the primer set P319-P391 and HindIII and BamHI restriction enzymes. The *hrpK*<sub>1-399-avrRpt2<sub>81-255</sub> fusion was cut out of pBluescript II SK(+) as an XhoI-BamHI fragment and cloned into pBBR1MCS-5, generating construct pLN65. We made an AvrRpt2<sub>81-255</sub> construct that included a functional ribosomal binding site upstream of the *avrRpt2*-truncated gene for a negative control in translocation assays. This construct was generated by PCR amplification of *avrRpt2*<sub>81-255</sub> from pAvrRpt2-600 with primers P366 and P320. The fragment was ligated into pBBR1MCS-5 as HindIII-XbaI, generating construct pLN62.</sub>

Constructs that encoded different AvrRpt2 fusions were electroporated into *P. syringae* pv. phaseolicola NPS3121. *P. syringae* pv. phaseolicola strains were resuspended in 5 mM MES (morpholineethanesulfonic acid), pH 5.6, at an OD<sub>600</sub> of 0.1 and infiltrated into *Arabidopsis thaliana* Col-0 with a needleless syringe. Plants were observed for the elicitation of the HR within 24 h.

**Pathogenicity and HR assays.** pENTR constructs pLN293 (*hrpK*), pLN916 (*hrpK*<sub>1-382</sub>), pLN1157 (*hrpK*<sub>1-467</sub>), pLN1158 (*hrpK*<sub>1-582</sub>), pLN1159 (*hrpK*<sub>1-698</sub>), pLN1160 (*hrpK*<sub>ΔTM</sub>), pLN1161 (*hrpK*<sub>382-TM</sub>), and pLN1162 (*hrpK*<sub>80-TM</sub>) were used for LR recombination reactions with the Gateway LR reaction cloning kit (Invitrogen) with pLN677, a Gateway destination vector that produces products fused to a C-terminal HA tag generating pLN774, pLN1163, pLN1164, pLN1165, pLN1166, pLN1167, pLN1168, pLN1169, respectively. These constructs were used for complementation experiments with the DC3000 *hrpK* mutant UNL111.

To perform pathogenicity assays and in planta bacterial growth assays, tomato (*Lycopersicon esculentum* cv. MoneyMaker) and *A. thaliana* Col-0 were dip-inoculated into bacterial suspensions at an OD<sub>600</sub> of 0.2 in 10 mM MgCl<sub>2</sub> with 0.02% silwet L-77 (Lehle Seeds, Round Rock, Tex.) and sampled as previously described (25). Symptoms were recorded 4 days after infection. To perform HR assays, DC3000 strains were infiltrated into *Nicotiana tabacum* cv. Xanthi at an OD<sub>600</sub> of 0.2 ( $2 \times 10^8$  cells/ml) in 5 mM MES (pH 5.6) along with 10-fold serially diluted samples with a needleless syringe and observed for the elicitation of an HR at 24 h. *P. fluorescens* 55 (pHIR11) strains were infiltrated into *N. tabacum* cv. Xanthi plants with a needleless syringe at an OD<sub>600</sub> of 0.4 and observed for the elicitation of an HR at 18 h.

**Adenylate cyclase (CyaA) translocation assay.** All constructs used in CyaA assays were generated with the Gateway cloning system (Invitrogen). The pENTR constructs pLN293 (*hrpK*), pLN916 (*hrpK*<sub>1-382</sub>), pLN1157 (*hrpK*<sub>1-467</sub>), pLN1158 (*hrpK*<sub>1-582</sub>), pLN1159 (*hrpK*<sub>1-698</sub>), pLN1160 (*hrpK*<sub>ΔTM</sub>), pLN1161 (*hrpK*<sub>382-TM</sub>), and pLN1162 (*hrpK*<sub>80-TM</sub>) were used for LR recombination reactions with pCPP3234 (69) with the Gateway LR reaction cloning kit (Invitrogen), resulting in constructs pLN377, pLN917, pLN1170, pLN1171, pLN1172, pLN1173, pLN1174, and pLN1175, respectively. These constructs made HrpK or HrpK truncations fused to CyaA fusions at their C termini and were used in CyaA translocation experiments.

The procedure for CyaA translocation assays was described in Schechter et al. (69). Briefly, DC3000 and the *hrcC* type III-deficient mutant carrying the appropriate constructs were infiltrated into *N. benthamiana* plants at an OD<sub>600</sub> of 0.6 in 5 mM MES (pH 5.6) containing 100 μM IPTG. Plants were sampled at 7 h with a 0.8-cm cork borer. Leaf disks were ground in liquid nitrogen and resuspended in 300 μl of 0.1 M HCl. Protein concentrations were measured by the Bio-Rad (Hercules, Calif.) protein assay. Cyclic AMP (cAMP) was quantified using the Correlate-EIA Direct cAMP Enzyme Immunoassay kit (Assay Designs, Ann Arbor, Mich.) according to the manufacturer's instructions.

**Generation of HrpK transgenic plants.** To make transgenic tobacco plants that constitutively express HrpK, the 35S promoter and the 35S terminator were

cut out of pRTL2 with EcoRI-HindIII and HindIII-BamHI, respectively. *hrpK* fused at its 3' end with a nucleotide sequence corresponding to an HA tag was PCR amplified with primers P754 and P562. *hrpK-ha* was cloned downstream of the 35S promoter in the binary vector pPZP212 (34), resulting in construct pLN265. *Agrobacterium tumefaciens* C58C1 carrying pLN265 was used to transform *N. tabacum* cv. Xanthi via the leaf disk method. Transformants were selected on plates containing 150 mg of kanamycin/liter. T1 generation plants used in our experiments were assayed for production of HrpK-HA by grinding 1-cm-diameter leaf disks in liquid nitrogen and resuspending the plant tissue in 200 μl of 1× SDS tracking buffer. Samples were run on SDS-PAGE, and an immunoblot analysis was performed with high-affinity anti-HA antibodies (Roche, Indianapolis, Ind.) as described above.

## RESULTS

### RT-PCR analysis of the DC3000 exchangeable effector locus.

Sequence analysis of the EEL in DC3000 found several open reading frames (ORFs) that could be type III related (Fig. 1A) (3). Two ORFs in this region, PSPTO1408 and *hrpK*, were preceded by Hrp box-containing promoters, suggesting that they might be HrpL regulated (3). Downstream of *hrpK* in the DC3000 EEL is PSPTO1406. Based on its proximity to *hrpK*, we suspected that *hrpK* and PSPTO1406 were part of the same transcriptional unit. To test this and to determine which ORFs within the EEL were transcribed, we used RT-PCR. RNA was isolated from DC3000 grown in KB, a rich medium, which is known to suppress *hrp* gene expression and in *hrp*-inducing conditions (Fig. 1B). In rich medium, primer sets for each ORF produced RT-dependent PCR products corresponding to every ORF, with the exception of PSPTO1408, PSPTO1409, *hrpK*, and PSPTO1406 (Fig. 1B). All of these ORFs are downstream of potential Hrp boxes, which may be repressed in rich conditions. When RNA was isolated from DC3000 grown in *hrp*-inducing conditions, RT-dependent PCR products were obtained for all ORFs including PSPTO1408, PSPTO1409, *hrpK*, and PSPTO1406, indicating that the Hrp promoters upstream of *hrpK* and PSPTO1408 were active (Fig. 1C). Additionally, based on RT-dependent amplification of DNA spanning the *hrpK* and PSPTO1406 junction, these two genes are cistronic (Fig. 1C). We confirmed that all RT-PCR products corresponded to the EEL by noting by Southern analysis that they hybridized to an EEL probe (Fig. 1D). Taken together, our results indicate that all of the ORFs are transcribed. However, only the ORFs downstream of Hrp boxes were dependent on *hrp*-inducing conditions for their expression, and *hrpK* and PSPTO1406 are together in an operon.

### HrpK and PSPTO1406 (HopB1) are secreted via the TTSS.

To establish whether *hrpK* and PSPTO1406 encoded proteins secreted by the TTSS, we performed culture secretion assays. Antibodies were raised against the product of PSPTO1406, and these were used to determine whether natively expressed PSPTO1406 was secreted. However, the product of PSPTO1406, while expressed in *hrp*-inducing conditions, was detected only in small amounts, which precluded us from determining whether the native product was type III secreted (Fig. 2A). The PSPTO1406 product was not detected in extracts made from DC3000 grown in KB, a rich medium (data not shown). These experiments provided further evidence that *hrpK* and PSPTO1406 were in an operon, since we were not able to detect PSPTO1406 protein in extracts from the *hrpK* polar mutant UNL132 (Fig. 2A).

To test whether PSPTO1406 and HrpK were secreted via the

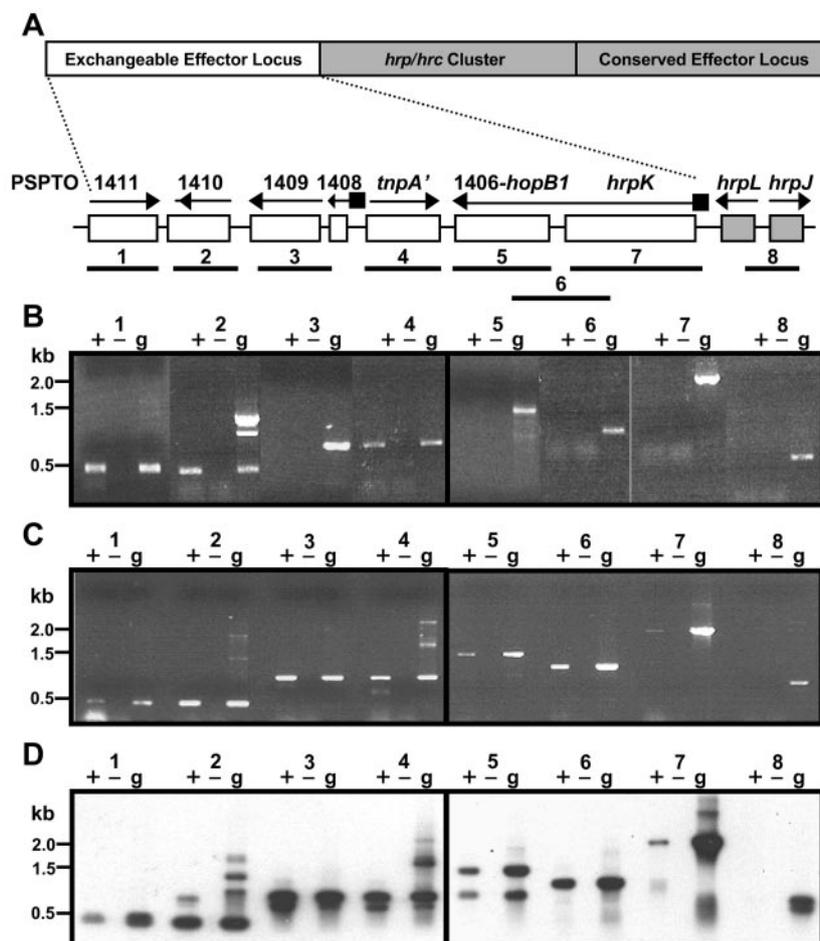


FIG. 1. RT-PCR analysis of the *P. syringae* pv. tomato DC3000 EEL confirms that *hrpK* and PSPTO1406 are polycistronic and that both predicted *hrp* promoters in the EEL region are active. (A) Organization of the EEL in DC3000. White boxes depict the ORFs of the EEL along with *hrpK*, which is part of the *hrp-hrc* cluster. Other *hrp-hrc* cluster genes, *hrpL* and *hrpJ*, which are not dealt with here are depicted as grey boxes. Arrows above ORFs indicate the predicted direction of transcription, and the black boxes indicate putative *hrp* promoters. The numbered lines beneath the ORFs represent PCR products produced by specific primer sets used below. (B to D) The numbers above each figure correspond to the PCR primer sets shown in panel A. +, experimental PCRs containing RT; -, control PCRs lacking RT; g, control PCRs without RT and DC3000 total DNA. Primer set 8 was used as a negative control because its PCR product spans two genes transcribed in opposite orientations, which would not produce RNA. (B) RT-PCR analysis of RNA isolated from DC3000 grown in KB, a rich medium. Primer sets 1, 2, and 4 produced an RT-dependent PCR product, indicating that the ORFs spanning these regions are transcribed. Notably, primer sets 3 and 5 to 7, corresponding to PSPTO1408, PSPTO1409, PSPTO1406, and *hrpK*, did not produce an RT-dependent PCR product. (C) RT-PCR analysis of RNA isolated from DC3000 grown under *hrp*-inducing conditions. Under these conditions, all primer sets produced an RT-dependent PCR product, indicating that the ORFs corresponding to primer sets 3 and 5 to 7 were only transcribed under *hrp*-inducing conditions. Primer set 6 produced an RT-dependent PCR product, which confirmed that *hrpK* and PSPTO1406 were in an operon. (D) High-stringency Southern blot analysis showed that the EEL region hybridized to all of the PCR products, confirming that the PCR products corresponded to the EEL.

TTSS, their corresponding genes were cloned into broad-host-range plasmids. Since we do not have antibodies raised to HrpK, the *hrpK* construct produced HrpK fused to a C-terminal FLAG epitope, which can be recognized by commercially available antibodies. These constructs were electroporated into DC3000 and grown in *hrp*-inducing medium. The cultures were separated into cell and supernatant fractions by centrifugation, and the samples were analyzed by SDS-PAGE and immunoblotting. As seen in Fig. 2, both HrpK and PSPTO1406 were secreted from DC3000 in a manner dependent on the TTSS. Therefore, both HrpK and PSPTO1406 are Hrp outer proteins (Hops). Because *hrpK* was classically defined as an *hrp* gene, its *hrp* prefix was retained. PSPTO1406 has been referred

to in other publications as EEL ORF1 (3) or HopPtoB1 (27) in data not shown. The nomenclature for *P. syringae* Hops has recently been revised, and we will follow this new naming system. Thus, the protein product of PSPTO1406 will be referred to hereafter as HopB1.

**HopB1 and HrpK are translocated into the plant cell.** To test whether HopB1 and HrpK were translocated into plant cells, we used two reporter systems. In one, the gene of interest is fused to an *avrRpt2* derivative lacking its amino-terminal secretion signal and therefore is not secreted and translocated unless the candidate gene encodes sufficient type III secretion signals (32). If the *AvrRpt2* fusion is translocated into plant cells, it elicits an HR on *Arabidopsis* Col-0, which contains the

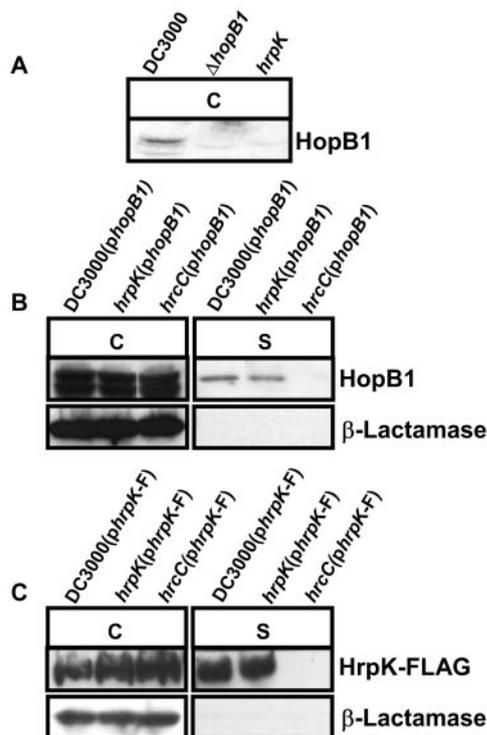


FIG. 2. HrpK and HopB1 (PSPTO1406) are secreted via the DC3000 TTSS. (A) Immunoblot analysis using anti-HopB1 antibodies showed that HopB1 is made in small amounts in cell (C) extracts of DC3000 grown under *hrp*-inducing conditions. HopB1 was not made in the DC3000 *hopB1* mutant, UNL130, or the *hrpK* polar mutant, UNL132, suggesting that the protein band observed was due to the *hopB1* gene and that *hopB1* is in the same transcriptional unit as *hrpK*. (B) *P. syringae* pv. tomato DC3000 strains carrying the *hopB1*-containing plasmid pLN63 (*hopB1*) were grown under *hrp*-inducing conditions and separated into cell (C) and supernatant (S) fractions, concentrated 13.3 and 133 fold, respectively, and analyzed by SDS-PAGE and immunoblots with anti-HopB1 antibodies. The results clearly showed that HopB1 is secreted via the TTSS. (C) *P. syringae* pv. tomato DC3000 strains carrying a construct, pLN64 (*phrpK-F*), that made HrpK C-terminally fused to the FLAG epitope tag were grown under *hrp*-inducing conditions, and secretion experiments were performed as above. Anti-FLAG antibodies were used as the primary antibody in the immunoblot to detect HrpK-FLAG. HrpK-FLAG is abundantly found in the supernatant fractions of DC3000 and the *hrpK* mutant. (B and C) A DC3000 *hrcC* mutant (*hrcC*), which is defective in the TTSS, was used as a negative control. All strains also carried pCPP2318, which encodes mature  $\beta$ -lactamase that remains in the cytoplasm and acts as a lysis control.

RPS2 R protein. Constructs containing either the full-length *hopB1*, full-length *hrpK*, or a 3' truncated version of *hrpK* (corresponding to the first 399 amino acids of HrpK) fused to *avrRpt2* lacking its type III secretion signals were electroporated into *P. syringae* pv. phaseolicola. Strains carrying these fusions produced stable fusion proteins based on immunoblotting (data not shown). These strains were infiltrated into *Arabidopsis* Col-0. As seen in Fig. 3A, strains containing the HopB1- $\Delta$ AvrRpt2 fusion produced an HR, as did the full-length HrpK- $\Delta$ AvrRpt2 fusion. However, the HrpK- $\Delta$ AvrRpt2 fusion produced an HR that was reduced and delayed by about 7 h, suggesting that HrpK did not deliver AvrRpt2 as well as HopB1. Interestingly, strains expressing the HrpK<sub>1-399</sub>-

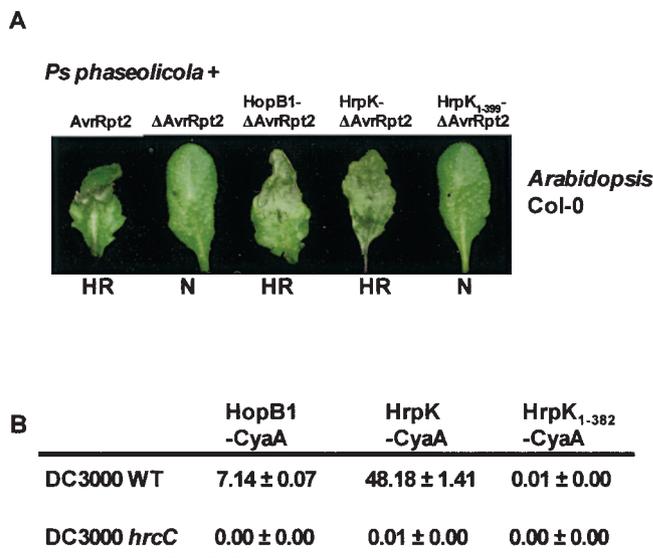


FIG. 3. HopB1 and HrpK are translocated into plant cells based on reporter assays; however, HrpK requires its C-terminal half to translocate. (A) AvrRpt2 translocation assays with HopB1 and HrpK AvrRpt2 fusions. *P. syringae* pv. phaseolicola NPS3121 carrying constructs that encoded either full-length AvrRpt2, an 80-amino-acid N-terminal deletion of AvrRpt2 ( $\Delta$ AvrRpt2), full-length HopB1 fused to  $\Delta$ AvrRpt2 (HopB1- $\Delta$ AvrRpt2), full-length HrpK fused to  $\Delta$ AvrRpt2 (HrpK- $\Delta$ AvrRpt2), and the first 399 amino acids of HrpK fused to  $\Delta$ AvrRpt2 (HrpK<sub>1-399</sub>- $\Delta$ AvrRpt2) were infiltrated into *A. thaliana* Col-0 at an OD<sub>600</sub> of 0.5 and scored after 12 to 19 h for elicitation of the HR. N, no visible HR. (B) CyaA translocation assays with HopB1 and HrpK CyaA fusions. DC3000 and a DC3000 *hrcC* mutant defective in TTSS carrying constructs that encoded either full-length HopB1 (HopB1-CyaA), HrpK (HrpK-CyaA), or the first 382 amino acids of HrpK (HrpK<sub>1-382</sub>-CyaA) fused to CyaA were infiltrated into *N. benthamiana* and assayed for cAMP production 7 h after infiltration as described in Materials and Methods. cAMP levels are reported in picomoles of cAMP per micrograms of protein with standard errors. The levels of cAMP indicated that the full-length HopB1-CyaA fusion and the full-length HrpK-CyaA fusion were translocated. However, the N-terminal HrpK-CyaA fusion had background levels of cAMP, indicating that it was not translocated.

$\Delta$ AvrRpt2 fusion that contained the first 399 amino acids of HrpK did not elicit an HR. Typically, type III secretion signals are contained within the first 100 amino acids of a TTSS substrate, but the results with the HrpK<sub>1-399</sub>- $\Delta$ AvrRpt2 fusion suggested that the C terminus of HrpK provided secretion and/or translocation information.

To further test HopB1 and HrpK translocation, we used a different translocation reporter system, which utilizes CyaA fusions (69, 70). In this assay, a candidate type III secreted protein is fused to CyaA. If the CyaA fusion is translocated into eukaryotic cells, it indicates that the test effector provided type III secretion signals to CyaA. Translocation of CyaA can be detected because it produces cAMP. Since CyaA is dependent on calmodulin, cAMP is produced only in eukaryotic cells. We made constructs that fused full-length HopB1 and HrpK to CyaA, as well as a construct that produced the first 382 amino acids of HrpK fused to CyaA (HrpK<sub>1-382</sub>-CyaA). These constructs were electroporated into DC3000 and confirmed to make stable CyaA fusions by performing immunoblotting with anti-CyaA antibodies (data not shown). Each strain was infil-



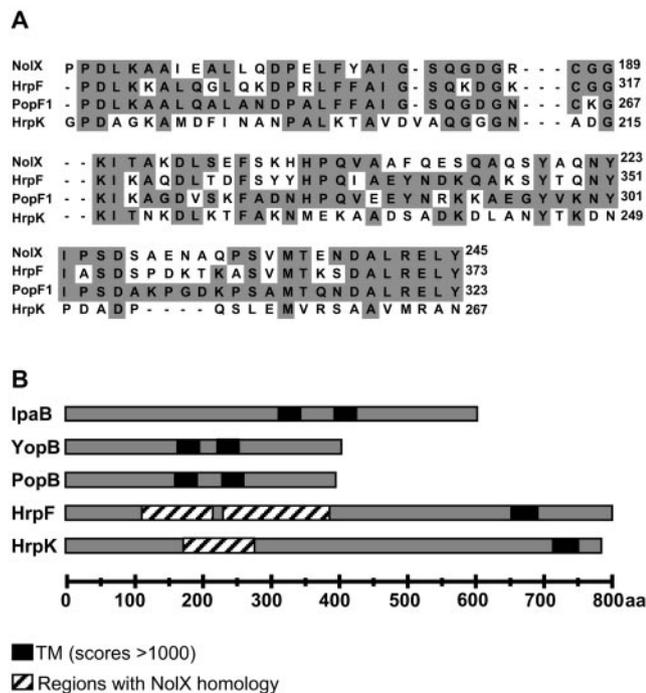


FIG. 5. PSI-BLAST searches show that HrpK is similar to HrpF, NolX, and PopF1. (A) Regions that were identified in PSI-BLAST searches were aligned with DNASTar MegAlign. Shown are *S. fredii* NolX, accession no. AAB17674; *X. campestris* pv. vesicatoria HrpF, accession no. AAB86527; *R. solanacearum* PopF1, accession no. NP\_523114; and DC3000 HrpK, accession no. AE016860. (B) TM regions in HrpK and other putative type III translocators from plant-associated bacteria. TM regions were predicted with the TMPred program ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)). Only scores of > 1,000 are shown. See the text for additional details.

the type III secreted PopF1 (*E* value,  $7e-7$ ) and PopF2 (*E* value, 0.27) from *Ralstonia solanacearum*, and NolX (*E* value,  $2e-7$ ), a type III secreted protein from *S. fredii* (54). Huguet and colleagues (42) identified two regions in HrpF with similarity to a single region in NolX. HrpK, PopF1, and PopF2 each have regions that share similarity with this same region in NolX (Fig. 5A).

Because there is evidence that HrpF is a translocator and it has been noted to share similar structural characteristics with the *Yersinia* YopB translocator and other translocators from animal pathogens (10), we compared HrpK with HrpF, YopB, and other translocators for transmembrane (TM) domains, a structural feature that has been associated with type III translocators. We used a TM prediction program TMPred ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) to identify TM domains within type III translocators and candidate translocators such as HrpK. We found the previously identified TM domains in IpaB (score, 1923 and 2461), PopB (score, 1910 and 2362), YopB (score, 1356 and 2516), and HrpF (score, 1150) (Fig. 5B) (11). We also found a TM domain at the C terminus of HrpK (score, 1980) (Fig. 5B). Taken together, HrpK shares similarities with HrpF, a putative type III translocator, and possesses predicted TM domains, a structural feature consistent with it acting as a type III translocator.

**DC3000 *hrpK* mutants are reduced in their ability to grow in planta and to cause disease symptoms, while *hopB1* mutants lack any apparent virulence phenotype.** Since both HrpK and HopB1 were type III secreted and translocated proteins (Fig. 2 and 3), we determined their effect on virulence. We constructed a DC3000 nonpolar *hrpK* mutant, UNL111, and a DC3000 *hopB1* mutant, UNL130, as described in Materials and Methods. These mutant strains were dip inoculated into tomato (*L. esculentum* cv. Moneymaker) and *A. thaliana* Col-0 plants and observed for symptoms and bacterial growth for a period of 4 days. The DC3000 *hrpK* nonpolar mutant UNL111 was reduced in disease symptom development and bacterial multiplication in planta (Fig. 6). In contrast, the DC3000 *hopB1* mutant had little or no effect on disease symptom production and was only slightly reduced in its ability to grow in planta (Fig. 6). The weak growth phenotype observed for *hopB1* mutants is consistent with growth phenotypes of other *P. syringae* effector mutants and suggests that many *P. syringae* effectors are functionally redundant.

**A DC3000 *hrpK* mutant and *P. fluorescens* carrying a pHIR11 derivative with a *hrpK* mutation are reduced in their ability to translocate Avr proteins.** The pathogenicity assays and in planta bacterial multiplication assays indicated that HrpK performed an important role in type III-related plant interactions. HrpK is not a required component of the type III secretion apparatus (Fig. 2B and C) (17) and in silico data suggested that HrpK may be a type III translocator. To test whether the DC3000 *hrpK* mutant UNL111 was defective in its ability to deliver Avr proteins, we performed HR assays by infiltrating different dilutions of the wild type and UNL111 into tobacco (*N. tabacum* cv. Xanthi) (Fig. 7A). These experiments showed that the wild-type strain elicited an HR at  $2 \times 10^7$  cells/ml, whereas UNL111 required  $2 \times 10^8$  cells/ml. This phenotype was complemented when *hrpK* was provided in *trans* (Fig. 7A). Because this HR is due to the translocation of type III effectors, it suggests that HrpK either functions as a translocator for the DC3000 TTSS or that HrpK is a type III effector that acts as an Avr protein.

We tested whether HrpK was an Avr protein by transiently delivering *hrpK* using *A. tumefaciens*. When *hrpK* was delivered transiently into tobacco, it failed to elicit an HR, indicating that HrpK was not an Avr protein in tobacco (data not shown). We also tested whether the *X. campestris* pv. vesicatoria *hrpF* gene that encodes a putative TTSS translocator could complement the reduced HR phenotype produced by UNL111. pDhrpF, which carries *hrpF*, was electroporated into UNL111; it failed to complement the reduced HR UNL111 exhibited in tobacco (data not shown). These data suggested that the *hrpK* mutant was reduced in its ability to translocate Avr proteins into plant cells and that HrpK might have a role in translocation.

To further test whether HrpK was a type III translocator, we used cosmid pHIR11, which encodes a functional *P. syringae* pv. *syringae* 61 TTSS (40). When pHIR11 is expressed by nonpathogens such as *P. fluorescens* it confers the ability to elicit an HR on tobacco and other plants because it translocates one Avr protein, HopPsyA, into plant cells (6). We made a pHIR11 derivative, pLN468, that was defective in *hrpK*. When *P. fluorescens* (pLN468) was infiltrated into tobacco plants, it showed a greatly diminished ability to elicit an HR

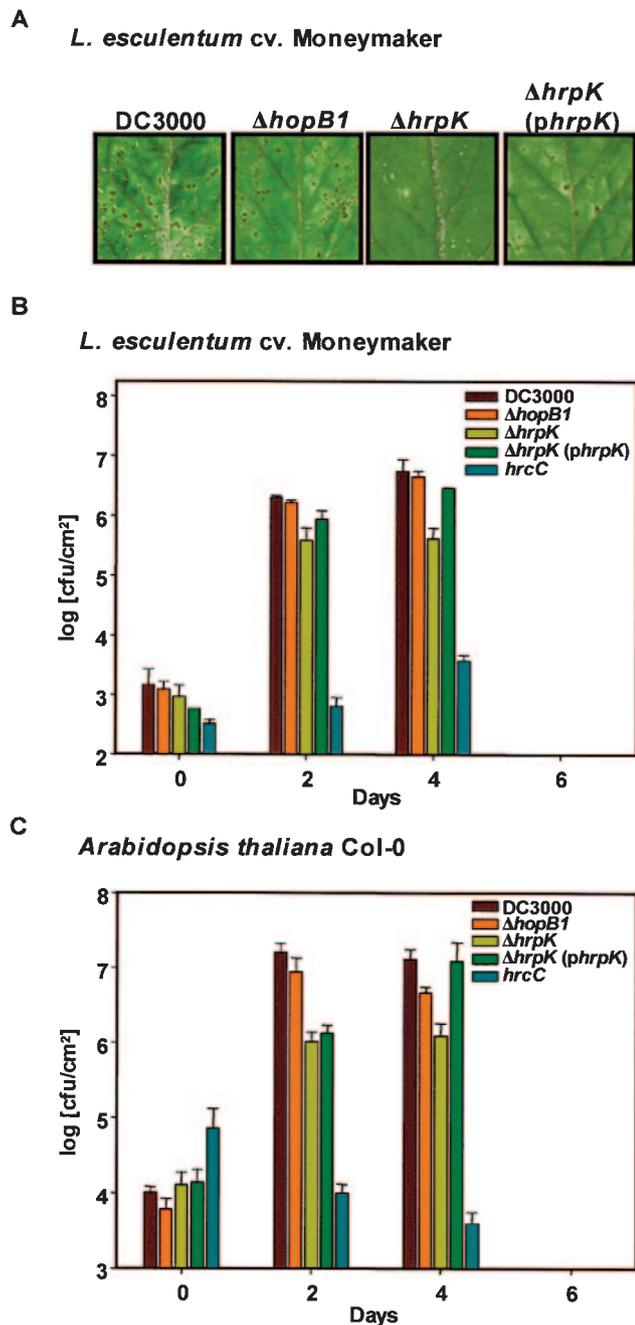


FIG. 6. Pathogenicity assays and bacterial multiplication in planta indicate that a DC3000 *hrpK* mutant but not a DC3000 *hopB1* mutant is significantly reduced in virulence on tomato and *Arabidopsis*. The following strains were used in pathogenicity and bacterial growth assays: wild-type DC3000; the *hopB1* mutant, UNL130 ( $\Delta hopB1$ ); the DC3000 nonpolar *hrpK* mutant, UNL111 ( $\Delta hrpK$ ); UNL111 carrying pLN64 encoding HrpK-FLAG [ $\Delta hrpK$ (phrpK)]; and a DC3000 *hrcC* mutant. Three- to 4-week-old *L. esculentum* cv. Moneymaker plants and *A. thaliana* Col-0 were dip inoculated into different bacterial suspensions at an OD<sub>600</sub> of 0.2 with 0.02% Silwet L-77. Disks measuring 0.64 cm<sup>2</sup> for tomato plants and 0.4 cm<sup>2</sup> for *Arabidopsis* were excised, ground in water, serially diluted, and plated on KB plates containing appropriate antibiotic markers. (A) Disease symptoms produced on tomato on day 4. Bacterial growth was tracked for a period of 4 days in tomato (B) and *Arabidopsis* (C). The results show that the *hrpK* mutant was reduced in symptom production and bacterial growth in planta, whereas the *hopB1* mutant was similar to the wild type. The *hrpK* mutant phenotype was complemented by pLN64 carrying *hrpK*-flag.

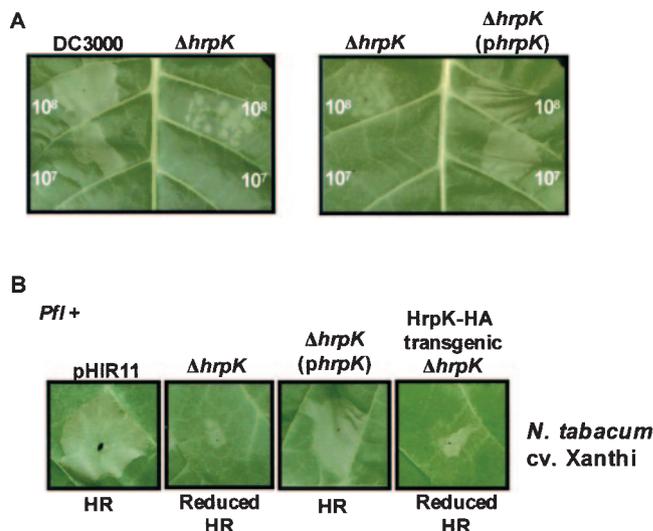


FIG. 7. *hrpK* mutants are reduced in their ability to translocate Avr proteins. (A) *N. tabacum* cv. Xanthi was infiltrated at an OD<sub>600</sub> of 0.2 (2 × 10<sup>8</sup> cells/ml) and 10-fold serial dilutions of DC3000, the DC3000 *hrpK* mutant UNL111 ( $\Delta hrpK$ ), and UNL111 (pLN64), which carried *hrpK* in trans [ $\Delta hrpK$ (phrpK)]. The *hrpK* mutant showed a visible repeatable reduction in its ability to elicit the HR when compared to the wild type. (B) *N. tabacum* cv. Xanthi or transgenic *N. tabacum* cv. Xanthi lines expressing HrpK-HA were infiltrated with *P. fluorescens* 55 (Pfl) carrying cosmid pHIR11, a pHIR11 derivative (pLN468) lacking *hrpK* ( $\Delta hrpK$ ), or Pfl (pLN468) with pLN174, which carried *hrpK* (phrpK), each at an OD<sub>600</sub> of 0.4 (4 × 10<sup>8</sup> cells/ml). Production of the HR was documented at 18 h postinfiltration.

relative to *P. fluorescens* (pHIR11) (Fig. 7B), consistent with HrpK acting as a type III translocator. In complementation experiments, we found that *P. fluorescens* (pLN468) was complemented when *hrpK* was provided in trans, but it was not complemented if HrpK was provided inside plant cells when these experiments were carried out on transgenic tobacco expressing HrpK (Fig. 7B). The requirement for HrpK to be outside of the plant cell for complementation is consistent with HrpK acting as a type III translocator.

**The C terminus of HrpK is required for translocation of the CyaA reporter.** The HrpK translocation experiments indicated that HrpK was translocated into plant cells (Fig. 3), which could suggest that HrpK was an effector or that it acted inside plant cells. However, both translocation assays required full-length HrpK for translocation of the reporters (Fig. 3). Translocation of an effector-reporter fusion generally only requires the N-terminal half of the effector for efficient translocation. We were interested in whether the C-terminal portion of HrpK possessed a region that would be required for reporter translocation.

To explore the requirement of the C-terminal half of HrpK and the TM domain for Cya translocation, we fused *hrpK* deletions to *cyaA* (Fig. 8A). As noted above, a C-terminal HrpK deletion that corresponded to the first 382 amino acids of HrpK fused to CyaA (HrpK<sub>1-382</sub>-CyaA) was not translocated (Fig. 3 and 8A). Indeed, three other HrpK C-terminal deletions, HrpK<sub>1-467</sub>-CyaA, HrpK<sub>1-582</sub>-CyaA, and HrpK<sub>1-698</sub>-CyaA, were not translocated based on low levels of cAMP production (Fig. 8A). Interestingly, when we generated a con-

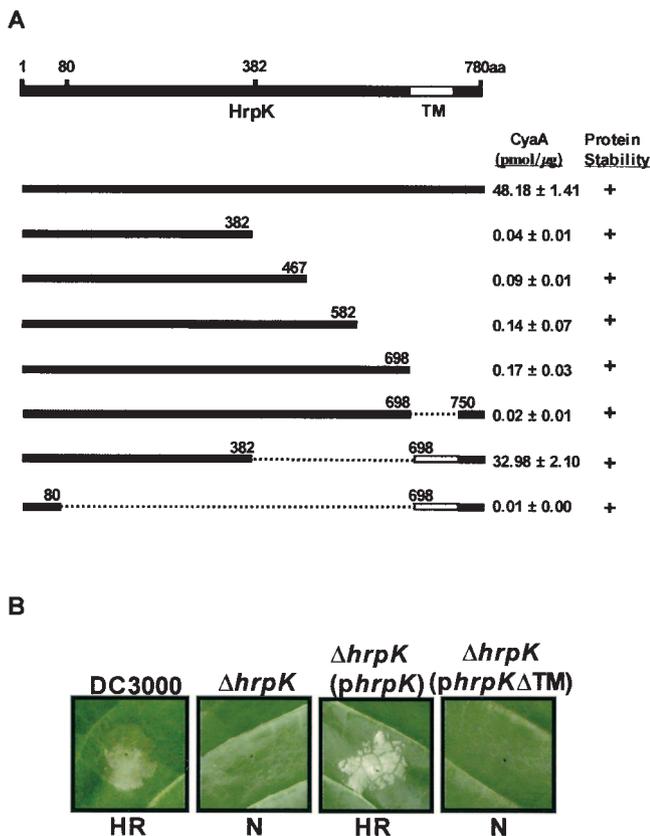


FIG. 8. HrpK requires a TM domain for proper function. (A) Schematic representation of HrpK truncations and deletions. Numbers indicate amino acid positions in HrpK. The CyaA column indicates detection of cAMP production in the CyaA translocation assay. All constructs were checked for protein stability by SDS-PAGE and immunoblotting with anti-CyaA antibodies. The constructs and their corresponding products are listed as follows: pLN377, full-length HrpK-CyaA; pLN917, HrpK<sub>1-382</sub>-CyaA; pLN1170, HrpK<sub>1-467</sub>-CyaA; pLN1171, HrpK<sub>1-582</sub>-CyaA; pLN1172, HrpK<sub>1-698</sub>-CyaA; pLN1173, HrpK<sub>1-698,750-780</sub>-CyaA; pLN1174, HrpK<sub>1-382,750-780</sub>-CyaA; and pLN1175, HrpK<sub>1-80,750-780</sub>-CyaA. (B) HrpK<sub>1-698/750-780</sub> failed to complement the reduced HR produced by the DC3000 *hrpK* mutant UNL111. *N. tabacum* cv. Xanthi was infiltrated at an OD<sub>600</sub> of 0.2 ( $1 \times 10^8$  cells/ml), and 10-fold serial dilutions were performed. The pictures show leaves that were infiltrated with bacteria at a cell density of  $1 \times 10^7$  cells/ml. The following strains were infiltrated: wild-type DC3000; the DC3000 *hrpK* mutant UNL111 ( $\Delta hrpK$ ); UNL111 carrying pLN774 expressing full-length HrpK-HA (*phrpK*); and UNL111 carrying pLN1167 expressing the TM domain deletion (*phrpK* $\Delta$ TM). The construct pLN1167 carrying the TM domain deletion was unable to complement the reduced HR phenotype exhibited by UNL111.

struct that produced an HrpK-CyaA fusion that corresponded to the first 382 amino acids fused to amino acids 698 to 780 of HrpK, which included the predicted TM domain of HrpK, we found that this fusion protein was translocated, based on significant cAMP production (Fig. 8A). A smaller N-terminal portion of HrpK fused to HrpK<sub>689-780</sub>-CyaA was not translocated (Fig. 8A). Taken together, the first 382 amino acids of HrpK fused to its last 82 amino acids is sufficient to translocate the C-terminal CyaA moiety into the plant cytoplasm. These results are consistent with HrpK acting as a type III translocator and the TM domain is required for HrpK translocation.

To test the importance of the TM domain for the function of

HrpK, we made construct pLN1167, which encodes a HrpK variant (fused to the HA epitope) that lacked amino acids 698 to 750, which corresponded to the predicted TM domain. This construct was electroporated into DC3000 and the DC3000 *hrpK* mutant UNL111. We tested whether this HrpK variant would complement the reduced HR phenotype that is associated with UNL111 (Fig. 7A). Both strains were confirmed to make the HrpK variant by immunoblotting with anti-HA antibodies. The reduced HR phenotype exhibited by UNL111 was not complemented by pLN1167 when these strains were infiltrated into tobacco (Fig. 8B). Testing this construct for complementation was useful because it is the smallest deletion that removes the TM domain. Since this HrpK variant was unable to complement the *hrpK* mutant, it suggests that the TM domain is necessary for HrpK function.

## DISCUSSION

Here we present transcriptional expression data for the DC3000 EEL. Two Hrp promoters, one upstream of the *hrpK* gene and the other upstream of PSPTO1408, were active in conditions that transcribe the DC3000 TTSS (Fig. 1). We were unable to show that PSPTO1408 or PSPTO1409 encoded proteins secreted by the TTSS of DC3000 (data not shown). PSPTO1409 is carried in the EEL region of *P. syringae* pv. delphinii PDDCC529 (24), which suggests that it may encode a TTSS protein. However, the predicted N terminus of the products of both PSPTO1408 and PSPTO1409 does not resemble other *P. syringae* Hops (33, 63). Additional secretion and translocation assays are in progress to determine whether either of these ORFs encode TTSS substrates.

We showed that two genes downstream of the other Hrp promoter in the DC3000 EEL, *hrpK* and *hopB1*, were transcribed as an operon (Fig. 1). It is important to emphasize that *hrpK* is not part of the EEL, but instead it is a conserved *hrp-hrc* gene flanking the EEL. Indeed, *hrpK* resides in this EEL-flanking location in all *P. syringae* strains tested (3, 13, 24, 57). HopB1 and HrpK were secreted in culture and translocated into plant cells by the DC3000 TTSS (Fig. 2 and 3).

We know little about the role HopB1 plays in bacterium-plant interactions. Based on the fact that the *hopB1* mutant's growth phenotype resembles wild-type DC3000 (Fig. 6), HopB1 does not appear to contribute significantly to virulence. This is consistent with HopB1 belonging to the effector class because most *P. syringae* effectors contribute subtly to pathogenesis, but collectively they are required (5). The weak individual contribution to virulence displayed by most effectors is likely due to their functionally redundant activities inside plant cells. *hopB1* is present in multiple *P. syringae* EELs (13, 24), which suggests that it does play an important role. If it did not, it would probably be lost due to the apparent instability of the EEL.

A hint to HopB1's function may be provided by the similarity HopB1 shares with other ORFs in *P. syringae* and ORFs in other bacterial species (Fig. 4). The region within HopB1 that shows similarity is similar to two repeated regions in these other proteins (Fig. 4A). At first glance, the *P. syringae* ORFs that share similarity to HopB1 suggests that these ORFs, *holPtoAC*<sub>Psy</sub> and PSPTO4996 (*hopPtoB2*), may encode additional type III effectors. However, there are several facts that

argue against this. First, their predicted N-termini do not resemble *P. syringae* Hops; second, they do not have identifiable TTSS-related Hrp promoters; and third, they share similarity to ORFs contained in bacteria not known to possess TTSSs. Thus, the identification of the shared region in other ORFs may not identify additional type III secreted proteins as originally thought (9, 27, 30), but instead it may reveal clues to the biochemical activity of HopB1 and the role it plays in the *P. syringae* TTSS.

There is now substantial evidence, much of it reported here, that HrpK acts as a translocator for the *P. syringae* TTSS. DC3000 *hrpK* mutants were significantly reduced in virulence (Fig. 6); HrpK defective strains were less efficient at effector translocation than wild-type strains, based on their reduced HR phenotypes (Fig. 7); PSI-BLAST searches revealed that HrpK shared weak similarity with HrpF, a putative type III translocator from *X. campestris* pv. vesicatoria (Fig. 5); *P. syringae* pv. *syringae* B728a *hrpK* mutants retained the ability to secrete proteins in culture via the TTSS (17); and the conservation of *hrpK* in *P. syringae* strains argues for an accessory role in the TTSS. Taken together, these data strongly support that HrpK is a type III translocator. We are currently testing whether HrpK can form pores in membranes and allow for the release of low-molecular-weight dyes from eukaryotic cells, two criteria that are shared by several animal pathogen type III translocators (10).

The *P. syringae* HrpZ harpin is capable of forming pores in lipid bilayers, which suggest that it may be a type III translocator (56). One distinction that can be made between HrpK and HrpZ is that HrpK contributes to virulence more significantly than HrpZ, based on pathogenicity assays with *P. syringae* mutants (Fig. 6) (2). However, this may reflect that HrpZ's activity is redundant in *P. syringae*. Indeed, the N-terminal half of HrpW from *P. syringae* shares characteristics with HrpZ, and both proteins have the ability to induce the HR when purified and infiltrated into leaf tissue (14, 38). The elicitation of the HR by HrpZ and HrpW is different from the HR elicited by Avr proteins because it appears to be independent of R proteins and host specificity (65). In fact, the HrpZ- and HrpW-elicited HR may be analogous to the ability of animal pathogen type III translocators to lyse erythrocytes, both of which may be due to pore formation in the eukaryotic plasma membrane. One prediction that will be tested is whether HrpZ and HrpW interact with HrpK, which (if they are all translocators) might occur because animal pathogen translocators often interact with each other to form a translocon complex (10, 20).

An interesting observation made here was the requirement of the C-terminal half of HrpK for translocation of the HrpK reporter fusions into plant cells (Fig. 3). Translocation of bacterial plant pathogen effectors is thought to require the N-terminal half of the effector, by both the AvrRpt2 and the Cya translocation assays (12, 32, 59). Therefore, HrpK exhibited an unusual translocation requirement. To further investigate this phenomenon, we made HrpK-CyaA fusions that had C-terminal truncations, which identified the requirement of a predicted TM domain (Fig. 8). Our present hypothesis is that HrpK is not completely translocated into plant cells, rather only that a C-terminal portion gains access to the plant cytoplasm, allowing for the C-terminal AvrRpt2 and CyaA reporters to indicate translocation. Consistent with this is that HrpK-

CyaA fusions that have the N-terminal half of HrpK fused to a C-terminal portion containing the TM domain were translocated (Fig. 8A).

TM domains are also present in the *X. campestris* pv. vesicatoria HrpF putative translocator and in several animal pathogen translocators (10). Hume et al. (43) suggested that two TM domains present in the *Shigella* IpaB translocator insert themselves into the lipid bilayers with the hydrophilic region between the two domains traversing the membrane. Additionally, mutational analyses of IpaB have shown that the putative TM domains are important for IpaB function (31). In an extensive mutational analysis of the *Yersinia* YopD translocator, the TM domain was required for translocation of the YopE effector (62). Interestingly, the YopD derivative lacking the TM domain was still able to efficiently form pores in artificial lipid bilayers (62). These data suggest that the YopD TM domain is important for translocation in a manner that is uncoupled from the ability to form pores and that pore formation is not sufficient for the translocation of effectors. Continuing to analyze other translocators will help reveal the similarities and differences between the different translocon complexes. Moreover, comparing translocators from animal pathogens to putative translocators like HrpK will likely facilitate the characterization of these proteins. However, even closely related translocators appear to function differently in their respective TTSSs (43). Likewise, because the plant cell wall amounts to an additional barrier to translocation there are likely to be significant differences between type III translocators that deliver effector proteins into plant cells and their animal pathogen counterparts.

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#### REFERENCES

1. Abramovitch, R. B., Y. J. Kim, S. Chen, M. B. Dickman, and G. B. Martin. 2003. *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J.* **22**:60–69.
2. Alfano, J. R., D. W. Bauer, T. M. Milos, and A. Collmer. 1996. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally nonpolar *hrpZ* deletion mutants, truncated HrpZ fragments, and *hmrA* mutations. *Mol. Microbiol.* **19**:715–728.
3. Alfano, J. R., A. O. Charkowski, W. Deng, J. L. Badel, T. Petnicki-Ocwieja, K. van Dijk, and A. Collmer. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* **97**:4856–4861.
4. Alfano, J. R., and A. Collmer. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* **179**:5655–5662.
5. Alfano, J. R., and A. Collmer. 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* **42**:385–414.
6. Alfano, J. R., H.-S. Kim, T. P. Delaney, and A. Collmer. 1997. Evidence that the *Pseudomonas syringae* pv. *syringae* *hrp*-linked *hmrA* gene encodes an Avr-like protein that acts in an *hrp*-dependent manner within tobacco cells. *Mol. Plant Microbe Interact.* **10**:580–588.

7. Badel, J. L., K. Nomura, S. Bandyopadhyay, R. Shimizu, A. Collmer, and S. Y. He. 2003. *Pseudomonas syringae* pv. tomato DC3000 HopPtoM (CEL ORF3) is important for lesion formation but not growth in tomato and is secreted and translocated by the Hrp type III secretion system in a chaperone-dependent manner. *Mol. Microbiol.* **49**:1239–1251.
8. Bozso, Z., P. G. Ott, and Z. Klement. 1999. HR-positive phenotype of the *Pseudomonas syringae* pv. *syringae* *hrpK* mutant and *hrp* gene superinduction in tobacco leaves treated with protein synthesis inhibitors. *Physiol. Mol. Plant Pathol.* **55**:215–223.
9. Buell, C. R., V. Joardar, M. Lindeberg, J. Selengut, I. T. Paulsen, M. L. Gwinn, R. J. Dodson, R. T. Deboy, A. S. Durkin, J. F. Kolonay, et al. 2003. The complete sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA.* **100**:10181–10186.
10. Buttner, D., and U. Bonas. 2002. Port of entry—the type III secretion translocon. *Trends Microbiol.* **10**:186–192.
11. Buttner, D., D. Nennstiel, B. Klusener, and U. Bonas. 2002. Functional analysis of HrpF, a putative type III translocon protein from *Xanthomonas campestris* pv. *vesicatoria*. *J. Bacteriol.* **184**:2389–2398.
12. Casper-Lindley, C., D. Dahlbeck, E. T. Clark, and B. J. Staskawicz. 2002. Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells. *Proc. Natl. Acad. Sci. USA* **99**:8336–8341.
13. Charity, J. C., K. Pak, C. F. Delwiche, and S. W. Hutcheson. 2003. Novel exchangeable effector loci associated with the *Pseudomonas syringae* *hrp* pathogenicity island: evidence for integron-like assembly from transposed gene cassettes. *Mol. Plant Microbe Interact.* **16**:495–507.
14. Charkowski, A. O., J. R. Alfano, G. Preston, J. Yuan, S. Y. He, and A. Collmer. 1998. The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J. Bacteriol.* **180**:5211–5217.
15. Charkowski, A. O., H.-C. Huang, and A. Collmer. 1997. Altered localization of HrpZ in *Pseudomonas syringae* pv. *syringae* *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. *J. Bacteriol.* **179**:3866–3874.
16. Chen, Z., A. P. Kloek, A. Cuzick, W. Moeder, D. Tang, R. W. Innes, D. F. Klessig, J. M. McDowell, and B. N. Kunkel. 2004. The *Pseudomonas syringae* type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on *Arabidopsis thaliana*. *Plant J.* **37**:494–504.
17. Collmer, A., J. L. Badel, A. O. Charkowski, W.-L. Deng, D. E. Fouts, A. R. Ramos, A. H. Rehm, D. M. Anderson, O. Schneewind, K. van Dijk, and J. R. Alfano. 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. USA* **97**:8770–8777.
18. Collmer, A., M. Lindeberg, T. Petnicki-Ocwieja, D. Schneider, and J. R. Alfano. 2002. Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. *Trends Microbiol.* **10**:462–469.
19. Cornelis, G. R. 2002. *Yersinia* type III secretion: send in the effectors. *J. Cell Biol.* **158**:401–408.
20. Cornelis, G. R. 2002. The *Yersinia* Ysc-Yop 'type III' weaponry. *Nat. Rev. Mol. Cell Biol.* **3**:742–752.
21. Cuppels, D. A. 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol.* **51**:323–327.
22. DebRoy, S., R. Thilmony, Y. B. Kwack, K. Nomura, and S. Y. He. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. USA* **101**:9927–9932.
23. de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis. 1993. Analysis of *Pseudomonas* gene products using *lacI<sup>q</sup>/P<sub>trp</sub>-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123**:17–24.
24. Deng, W. L., A. H. Rehm, A. O. Charkowski, C. M. Rojas, and A. Collmer. 2003. *Pseudomonas syringae* exchangeable effector loci: sequence diversity in representative pathovars and virulence function in *P. syringae* pv. *syringae* B728a. *J. Bacteriol.* **185**:2592–2602.
25. Espinosa, A., M. Guo, V. C. Tam, Z. Q. Fu, and J. R. Alfano. 2003. The *Pseudomonas syringae* type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. *Mol. Microbiol.* **49**:377–387.
26. Fellay, R., J. Frey, and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. *Gene* **52**:147–154.
27. Fouts, D. E., R. B. Abramovitch, J. R. Alfano, A. M. Baldo, C. R. Buell, S. Cartinhour, A. K. Chatterjee, M. D'Ascenzo, M. Gwinn, S. G. Lazarowitz, N.-C. Lin, G. B. Martin, A. H. Rehm, D. J. Schneider, K. van Dijk, X. Tang, and A. Collmer. 2002. Genomewide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. USA* **99**:2275–2280.
28. Frithz-Lindsten, E., A. Holmström, L. Jacobsson, M. Soltani, J. Olsson, R. Rosqvist, and Å. Forsberg. 1998. Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. *Mol. Microbiol.* **29**:1155–1165.
29. Galán, J. E., and A. Collmer. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**:1322–1328.
30. Greenberg, J. T., and B. A. Vinatzer. 2003. Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. *Curr. Opin. Microbiol.* **6**:20–28.
31. Guichon, A., D. Hersh, M. R. Smith, and A. Zychlinsky. 2001. Structure-function analysis of the *Shigella* virulence factor IpaB. *J. Bacteriol.* **183**:1269–1276.
32. Guttman, D. S., and J. T. Greenberg. 2001. Functional analysis of the type III effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the use of a single-copy genomic integration system. *Mol. Plant Microbe Interact.* **14**:145–155.
33. Guttman, D. S., B. A. Vinatzer, S. F. Sarkar, M. V. Ranall, G. Kettler, and J. T. Greenberg. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science* **295**:1722–1726.
34. Hajdukiewicz, P., Z. Svab, and P. Maliga. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* **25**:989–994.
35. Hakansson, S., K. Schesser, C. Persson, E. E. Galyov, R. Rosqvist, F. Homble, and H. Wolf-Watz. 1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J.* **15**:5812–5823.
36. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
37. Hauck, P., R. Thilmony, and S. Y. He. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. USA* **100**:8577–8582.
38. He, S. Y., H.-C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* harpin<sub>PS</sub>: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**:1255–1266.
39. Holmstrom, A., J. Olsson, P. Cherepanov, E. Maier, R. Nordfelth, J. Pettersson, R. Benz, H. Wolf-Watz, and A. Forsberg. 2001. LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. *Mol. Microbiol.* **39**:620–632.
40. Huang, H.-C., S. W. Hutcheson, and A. Collmer. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and TnphoA tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol. Plant Microbe Interact.* **4**:469–476.
41. Huang, H.-C., R. Schuurink, T. P. Denny, M. M. Atkinson, C. J. Baker, I. Yucel, S. W. Hutcheson, and A. Collmer. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J. Bacteriol.* **170**:4748–4756.
42. Huguet, E., and U. Bonas. 1997. hrpF of *Xanthomonas campestris* pv. *vesicatoria* encodes an 87-kDa protein with homology to NoIX of *Rhizobium fredii*. *Mol. Plant Microbe Interact.* **10**:488–498.
43. Hume, P. J., E. J. McGhie, R. D. Hayward, and V. Koronakis. 2003. The purified *Shigella* IpaB and *Salmonella* SipB translocators share biochemical properties and membrane topology. *Mol. Microbiol.* **49**:425–439.
44. Huynh, T. V., D. Dahlbeck, and B. J. Staskawicz. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**:1374–1377.
45. Ide, T., S. Laarmann, L. Greune, H. Schillers, H. Oberleithner, and M. A. Schmidt. 2001. Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cell. Microbiol.* **3**:669–679.
46. Jackson, R. W., E. Athanassopoulos, G. Tsiamis, J. W. Mansfield, A. Sesma, D. L. Arnold, M. J. Gibbon, J. Murillo, J. D. Taylor, and A. Vivian. 1999. Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar phaseolicola. *Proc. Natl. Acad. Sci. USA* **96**:10875–10880.
47. Jamir, Y., M. Guo, H.-S. Oh, T. Petnicki-Ocwieja, S. Chen, X. Tang, M. B. Dickman, A. Collmer, and J. R. Alfano. 2004. Identification of *Pseudomonas syringae* type III effectors that suppress programmed cell death in plants and yeast. *Plant J.* **37**:554–565.
48. Kahn, M. L., and P. Hanawalt. 1979. Size distribution of DNA replicative intermediates in bacteriophage P4 and *E. coli*. *J. Mol. Biol.* **128**:501–525.
49. Kang, L., J. Li, T. Zhao, F. Xiao, X. Tang, R. Thilmony, S. He, and J. M. Zhou. 2003. Interplay of the *Arabidopsis* nonhost resistance gene NHO1 with bacterial virulence. *Proc. Natl. Acad. Sci. USA* **100**:3519–3524.
50. Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* **24**:447–463.
51. Keen, N. T., S. Tamaski, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**:191–197.
52. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Med.* **22**:301–307.
53. Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson. 1995. Four new derivatives of the broad-host-

- range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**:175–176.
54. **Krishnan, H. B.** 2002. NolX of *Sinorhizobium fredii* USDA257, a type III-secreted protein involved in host range determination, is localized in the infection threads of cowpea (*Vigna unguiculata* [L.] Walp) and soybean (*Glycine max* [L.] Merr.) nodules. *J. Bacteriol.* **184**:831–839.
  55. **Labes, M., A. Puhler, and R. Simon.** 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for gram-negative bacteria. *Gene* **89**:37–46.
  56. **Lee, J., B. Klusener, G. Tsiamis, C. Stevens, C. Neyt, A. P. Tampakaki, N. J. Panopoulos, J. Noller, E. W. Weiler, G. R. Cornelis, and J. W. Mansfield.** 2001. HrpZ<sub>PspH</sub> from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore in vitro. *Proc. Natl. Acad. Sci. USA* **98**:289–294.
  57. **Mansfield, J., C. Jenner, R. Hockenhull, M. A. Bennett, and R. Stewart.** 1994. Characterization of *avrPphE*, a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY*, a new *hrp* gene identified in the halo-blight bacterium. *Mol. Plant Microbe Interact.* **7**:726–739.
  58. **Marenne, M. N., L. Journet, L. J. Mota, and G. R. Cornelis.** 2003. Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: role of LcrV, YscF and YopN. *Microb. Pathog.* **35**:243–258.
  59. **Mudgett, M. B., O. Chesnikova, D. Dahlbeck, E. T. Clark, O. Rossier, U. Bonas, and B. J. Staskawicz.** 2000. Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. *Proc. Natl. Acad. Sci. USA* **97**:13324–13329.
  60. **Murillo, J., and N. T. Keen.** 1994. Two native plasmids of *Pseudomonas syringae* pathovar *tomato* strain PT23 share a large amount of repeated DNA, including replication sequences. *Mol. Microbiol.* **12**:941–950.
  61. **Neyt, C., and G. R. Cornelis.** 1999. Role of SycD, the chaperone of the *Yersinia* Yop translocators YopB and YopD. *Mol. Microbiol.* **31**:143–156.
  62. **Olsson, J., P. J. Edqvist, J. E. Broms, A. Forsberg, H. Wolf-Watz, and M. S. Francis.** 2004. The YopD translocator of *Yersinia pseudotuberculosis* is a multifunctional protein comprised of discrete domains. *J. Bacteriol.* **186**:4110–4123.
  63. **Petnicki-Ocwieja, T., D. J. Schneider, V. C. Tam, S. T. Chancey, L. Shan, Y. Jamir, L. M. Schechter, M. D. Janes, C. R. Buell, X. Tang, A. Collmer, and J. R. Alfano.** 2002. Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA* **99**:7652–7657.
  64. **Pirhonen, M. U., J. C. Lidell, D. L. Rowley, S. W. Lee, S. Jin, Y. Liang, S. Silverstone, N. T. Keen, and S. W. Hutcheson.** 1996. Phenotypic expression of *Pseudomonas syringae* *avr* genes in *E. coli* is linked to the activities of the *hrp*-encoded secretion system. *Mol. Plant Microbe Interact.* **9**:252–260.
  65. **Preston, G., H.-C. Huang, S. Y. He, and A. Collmer.** 1995. The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. *Mol. Plant Microbe Interact.* **8**:717–732.
  66. **Rossier, O., G. Van den Ackerveken, and U. Bonas.** 2000. HrpB2 and HrpF from *Xanthomonas* are type III-secreted proteins and essential for pathogenicity and recognition by the host plant. *Mol. Microbiol.* **38**:828–838.
  67. **Sambrook, J., and D. W. Russell.** 2001. *Molecular cloning: a laboratory manual*, 3rd. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  68. **Sarker, M. R., C. Neyt, I. Stainier, and G. R. Cornelis.** 1998. The *Yersinia* Yop virulon: LcrV is required for extrusion of the translocators YopB and YopD. *J. Bacteriol.* **180**:1207–1214.
  69. **Schechter, L. M., K. A. Roberts, Y. Jamir, J. R. Alfano, and A. Collmer.** 2004. *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. *J. Bacteriol.* **186**:543–555.
  70. **Sory, M.-P., A. Boland, I. Lambermont, and G. R. Cornelis.** 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cya4* gene fusion approach. *Proc. Natl. Acad. Sci. USA* **92**:11998–12002.
  71. **Sory, M. P., and G. R. Cornelis.** 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol. Microbiol.* **14**:583–594.
  72. **Tsiamis, G., J. W. Mansfield, R. Hockenhull, R. W. Jackson, A. Sesma, E. Athanassopoulos, M. A. Bennett, C. Stevens, A. Vivian, J. D. Taylor, and J. Murillo.** 2000. Cultivar-specific avirulence and virulence functions assigned to *avrPphF* in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO J.* **19**:3204–3214.
  73. **Van den Eede, G., R. Deblaere, K. Goethals, M. Van Montagu, and M. Holsters.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. *Mol. Plant Microbe Interact.* **5**:228–234.
  74. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
  75. **Yuan, J., and S. Y. He.** 1996. The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J. Bacteriol.* **178**:6399–6402.
  76. **Zwiesler-Vollick, J., A. E. Plovianich-Jones, K. Nomura, S. Bandyopadhyay, V. Joardar, B. N. Kunkel, and S. Y. He.** 2002. Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. *Mol. Microbiol.* **45**:1207–1218.