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Pseudomonas syringae HrpJ Is a Type III Secreted Protein That Is Required for Plant Pathogenesis, Injection of Effectors, and Secretion of the HrpZ1 Harpin

Zheng Qing Fu, Ming Guo, and James R. Alfano*

Plant Science Initiative and Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68588-0660

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The bacterial plant pathogen *Pseudomonas syringae* requires a type III protein secretion system (TTSS) to cause disease. The *P. syringae* TTSS is encoded by the *hrp-hrc* gene cluster. One of the genes within this cluster, *hrpJ*, encodes a protein with weak similarity to YopN, a type III secreted protein from the animal pathogenic *Yersinia* species. Here, we show that HrpJ is secreted in culture and translocated into plant cells by the *P. syringae* pv. tomato DC3000 TTSS. A DC3000 *hrpJ* mutant, UNL140, was greatly reduced in its ability to cause disease symptoms and multiply in *Arabidopsis thaliana*. UNL140 exhibited a reduced ability to elicit a hypersensitive response (HR) in nonhost tobacco plants. UNL140 was unable to elicit an AvrRpt2- or AvrB1-dependent HR in *A. thaliana* but maintained its ability to secrete AvrB1 in culture via the TTSS. Additionally, UNL140 was defective in its ability to translocate the effectors AvrPto1, HopB1, and AvrPtoB. Type III secretion assays showed that UNL140 secreted HrpA1 and AvrPto1 but was unable to secrete HrpZ1, a protein that is normally secreted in culture in relatively large amounts, into culture supernatants. Taken together, our data indicate that HrpJ is a type III secreted protein that is important for pathogenicity and the translocation of effectors into plant cells. Based on the failure of UNL140 to secrete HrpZ1, HrpJ may play a role in controlling type III secretion, and in its absence, specific accessory proteins, like HrpZ1, may not be extracellularly localized, resulting in disabled translocation of effectors into plant cells.

Pseudomonas syringae is a host-specific bacterial plant pathogen that is capable of infecting many different plant species (3, 38). *P. syringae* causes a variety of diseases on susceptible plants, typically producing symptoms that manifest as necrotic and chlorotic lesions on aerial plant parts. On resistant plants, *P. syringae* often triggers plant innate immune responses, including the hypersensitive response (HR), a programmed cell death response associated with plant resistance (36).

A central pathogenicity factor for *P. syringae* is a type III protein secretion system (TTSS) called the Hrp TTSS, which is encoded by the *hrp-hrc* cluster within the Hrp pathogenicity island (2). The *P. syringae* Hrp TTSS translocates or injects many type III secreted proteins, known as effectors, into plant cells. *P. syringae* mutants defective in the Hrp TTSS are severely compromised in pathogenicity and are unable to elicit an HR on nonhost plants (53, 54). This indicates that collectively, these injected effectors are required for pathogenesis and that the nonhost HR is likely due to plant recognition of a subset of injected effectors in resistant plants. This is consistent with the well-documented recognition of bacterial type III effectors historically referred to as avirulence (Avr) proteins by plant resistance (R) proteins (16, 19).

The availability of the complete genomes of *P. syringae* pv. tomato DC3000 (9), *P. syringae* pv. syringae B728a (23), and *P. syringae* pv. phaseolicola 1448a (44) facilitated the identification of many *P. syringae* type III secreted proteins (12, 17, 30). Recently, a unified naming system for *P. syringae* type III secreted proteins was established, and these names will be used for the proteins described in this paper (52). Evidence that many of the *P. syringae* type III effectors injected into plant cells act as suppressors of the plant's innate immune system is accumulating (21, 57, 58). However, the enzymatic activities of the majority of *P. syringae* effectors and their plant targets remain unknown.

Bacterial TTSSs secrete other helper or accessory proteins that make up the extracellular portion of the type III apparatus and other proteins that function to help the type III apparatus deliver effector proteins into host cells. These include proteins that make up the type III-related needles, sheaths, or pili (depending on the TTSS) needed for the extracellular conduit that transports effectors. In the *P. syringae* TTSS, the HrpA1 protein has been shown to be the main component of the Hrp pilus (43, 51, 63). Other accessory proteins include translocator proteins that assist effectors in crossing the eukaryotic plasma membrane (10). Another group of proteins, called harpins, which are glycine-rich, heat-stable proteins predicted to modify the plant cell wall and/or act as translocators, have been identified (39, 50, 67). *P. syringae* has two harpins identified thus far, HrpZ1 and HrpW1. HrpZ1 was the first protein shown to be secreted via the *P. syringae* Hrp TTSS (34) and has been shown to form pores in artificial membranes, suggesting a role in translocation (50). HrpW1 has an N-terminal harpin domain and a C-terminal pectate lyase domain, suggesting that it acts on the plant cell wall (13). More recently, type III secreted HrpK1 has been shown to be required for pathogenicity and for effector translocation (60). Because of these phenotypes and other circumstantial evidence, HrpK1 appears to be a type III translocator.

The *P. syringae* type III apparatus is made up of about 20

* Corresponding author. Mailing address: Plant Science Initiative, The Beadle Center for Genetic Research, University of Nebraska, 1901 Vine St., Lincoln, NE 68588-0660. Phone: (402) 472-0395. Fax: (402) 472-3139. E-mail: jalfano2@unl.edu.

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proteins. Ten of these proteins, the so-called Hrc proteins (for *HR* and *c*onserved), are conserved in all nonflagellar TTSSs, and nine of the Hrc proteins are conserved in flagellar TTSSs (4, 40). The lone exception is HrcC, which belongs to the secretin family of outer-membrane proteins present in several macromolecule transport systems in gram-negative bacteria (27). The TTSSs of bacterial plant pathogens fall into two different groups based on the degree of conservation of their protein components. Group I includes the model TTSSs of *P. syringae* and *Erwinia amylovora*, and group II includes the model TTSSs of *Ralstonia solanacearum* and *Xanthomonas campestris* (4, 35). Several *P. syringae* Hrp proteins encoded by the *hrp-hrc* cluster are conserved in a subset of nonflagellar TTSSs, several are conserved only in bacterial plant pathogen TTSSs, and others appear to be unique to *P. syringae* and other group I Hrp TTSSs (4, 35).

One *P. syringae* protein, HrpJ, appears to be conserved in a subset of nonflagellar TTSSs and possesses clear homologs in plant pathogenic group I TTSSs but is not noticeably similar to any proteins in plant pathogenic group II TTSSs (4, 40). HrpJ is encoded by a gene within a five-gene operon in the *P. syringae hrp-hrc* cluster, and it has been reported to share similarity with YopN, a protein secreted via the *Yersinia* sp. Ysc TTSS (4, 40). *Yersinia* spp. grown in culture secrete Yop proteins via their Ysc TTSS at 37°C in the absence of calcium but not in the presence of calcium (8, 55). Thus, the absence of calcium appears to act as an environmental cue for the contact-dependent injection of Yop proteins by the Ysc TTSS into animal cells (61). *Yersinia yopN* mutants secrete Yop proteins in the presence or absence of calcium, a phenotype referred to as "calcium blind" (25). Thus, YopN is viewed as a control protein that prevents inappropriate type III secretion.

To address the role that HrpJ has in the DC3000 TTSS, we tested whether HrpJ was secreted in culture and translocated by the TTSS and determined its effect on the secretion and translocation of other *P. syringae* type III secreted proteins. Here, we report that HrpJ is secreted in culture and translocated into plant cells by the *P. syringae* pv. tomato DC3000 TTSS. A DC3000 *hrpJ* mutant was greatly reduced in its ability to cause disease and multiply in plant tissue. Moreover, the DC3000 *hrpJ* mutant was reduced in its ability to elicit the nonhost HR, suggesting that it was less competent in the translocation of type III effectors. This was confirmed by the finding that individual DC3000 effectors were translocated at very low levels, if at all, by the DC3000 *hrpJ* mutant. Interestingly, the DC3000 *hrpJ* mutant retained its ability to secrete type III effectors in culture but was unable to secrete the HrpZ1 extracellular accessory protein. These findings allowed us to propose that HrpJ functions as a control protein for the *P. syringae* TTSS and that its activities are required for the translocation of effectors into plant cells and for the secretion of the HrpZ1 harpin in culture.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strain DH5α was used for general cloning (Table 1) and was grown in Luria-Bertani broth at 37°C. *Pseudomonas syringae* pv. tomato DC3000 was grown in King's B broth at 30°C or type III-inducing fructose minimal medium at 20°C (41, 46). Antibiotics were used at the following concentrations (μg ml^{-1}): rifampin, 100; ampicillin, 100; gentamicin, 10; kanamycin, 50; chloramphemiol, 20; tetracycline, 20; and spectinomycin, 50.

General DNA manipulations. Restriction enzymes, T4 ligase, and DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). The thermostable DNA polymerase used in PCRs was *Pfu* polymerase (Stratagene, La Jolla, Calif.). The oligonucleotide primers used for plasmid constructions were ordered from Integrated DNA Technologies (Coralville, Iowa), and these primers or information about them will be made available upon request. For cloning using Gateway technology, we amplified desired target genes using PCR and *Pfu* polymerase and cloned the amplified fragments into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, Calif.). The resulting pENTR constructs were recombined with Gateway destination vectors by LR reaction using LR clonase (Invitrogen) following the manufacturer's instructions. We used standard cycling conditions for PCRs. Plasmids were introduced into *P. syringae* strains by electroporation. General DNA sequence analysis was performed with Lasergene software (DNAstar Inc., Madison, Wis.). Database searches were done with the BLASTN, BLASTP, BLASTX, and PSI-BLAST programs at NCBI (http://www.ncbi.nlm.nih.gov/blast/index.shtml) (5).

Construction of plasmids. *hrpJ*, *avrPto1*, and *avrB1* were cloned into the pENTR/D-TOPO vector (Invitrogen) by PCR with primers P759 and P760, P689 and P690, and P1134 and P1135, resulting in constructs pLN375, pLN307, and pLN820, respectively. pLN820, pLN323, and pLN307 were recombined into the CyaA Gateway destination vector pCPP3234, resulting in pLN918, pLN1979, and pLN1985, respectively. The *hrpJ* entry construct pLN375 was recombined into the destination vectors pCPP5040, pLN705, and pLN677, resulting in constructs pLN426, pLN736, and pLN726, respectively. The adenylate cyclase coding region lacking its start codon was amplified by PCR with primers P1963 and P1710 from pCPP3234 and ligated into pBluescript KS(−) by use of SacI and NotI restriction enzymes, resulting in construct pLN2043. Gateway cassette frame A was digested with EcoRV and ligated into the NotI restriction site of pLN2043, resulting in construct pLN2190. The Gateway CyaA cassette from pLN2190 was isolated and ligated into the HindIII and SacI restriction sites of pML123, resulting in the broad-host-range Gateway destination vector pLN2193. The *hrpJ* entry construct pLN375 was recombined into pLN2193, resulting in pLN2234.

Construction of the DC3000 *hrpJ* nonpolar mutant UNL140. To construct a DC3000 nonpolar *hrpJ* mutant, DNA upstream of *hrpJ* was amplified by PCR with primers P801 and P802. This PCR product was ligated into the XbaI and HindIII restriction enzyme sites of pCPP2988, which is a pBluescript derivative that contains an *nptII* gene lacking a transcriptional terminator, resulting in pLN2179. A DNA region downstream of *hrpJ* was amplified by PCR with primers P803 and P804 and ligated into the XhoI and KpnI sites of pLN2179, resulting in construct pLN2180. The insert in pLN2180, which contained an *nptII* gene flanked by the upstream and downstream fragments of *hrpJ*, was isolated by digestion with restriction enzymes KpnI and XbaI and ligated into pRK415, resulting in construct pLN2302. This construct was electroporated into DC3000. Putative mutants were identified by selection for retention of the antibiotic marker linked to the mutation and loss of the plasmid marker. A colony with this phenotype was confirmed to have the *hrpJ* gene replaced with *nptII* by PCR with primer sets P759 and P760 and P988 and P986. This DC3000 mutant was designated UNL140. Additionally, UNL140 was confirmed to carry an *hrpJ* deletion mutation by Southern analysis by probing with DNA flanking *hrpJ*.

Type III secretion assays. *P. syringae* strains were grown overnight on King's B plates containing appropriate antibiotics. Test strains were inoculated at an optical density at 600 nm (OD_{600}) of 0.3 in type III-inducing minimal medium at 20°C (41) (or at 30°C in experiments to test for temperature regulation) and grown for 6 h. Cell and supernatant fractions were separated by centrifugation, and the protein in the supernatant fraction was precipitated with 12.5% trichloroacetic acid. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to membranes for immunoblotting. The following primary antibodies were used: anti-AvrPto1, anti-HrpZ1, anti-HrpA1, anti-hemagglutinin (HA) (Roche Diagnostics Corp., Indianapolis, Ind.), anti-β-lactamase (Chemicon International, Temecula, Calif.), anti-FLAG (Sigma Chemical Co.), and anti-NptII (Cortex Biochem, San Leandro, Calif.). Primary antibodies were recognized by anti-mouse, anti-rabbit, or anti-rat immunoglobulin G-alkaline phosphatase conjugate secondary antibodies (Sigma Chemical Co.) and visualized on autoradiographs with the Western-Light chemiluminescence system (Tropix, Bedford, Mass.). NptII or β-lactamase was used as an indicator of nonspecific cell lysis in secretion assays.

Pathogenicity and HR assays. DC3000 strains were assessed for their ability to cause disease symptoms and multiply in planta by dipping of *Arabidopsis thaliana* ecotype Col-0 plants into bacterial suspensions that were adjusted to an OD_{600} of 0.2 in 10 mM $MgCl_2$ containing 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) and enumerated as previously described (22). DC3000 strains were tested for the ability to elicit an HR on *Nicotiana tabacum* cv. Xanthi by infiltration of plant tissue with strains adjusted to an OD_{600} of 0.2 along with 10-fold serially

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TABLE 1. Strains and plasmids

Designation	Main feature	Characteristics	Reference and/ or source
Bacterial strains			
E. coli DH5α	General use	*supE44* Δ*lacU169*(φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nai^r	33; Life Technologies
P. syringae pv. tomato			
DC3000	Wild-type strain	spontaneous Rif^r	18
DC3000 *hrcC* mutant	TTSS defective	DC3000 *hrcC* mutant, Rif^r Cm^r	70
UNL140	*hrpJ* mutant	DC3000 nonpolar *hrpJ* deletion, Rif^r Km^r	This work
Plasmids			
pAvrRpt2-600	Broad-host-range construct encoding AvrRpt2	pDSK600 derivative, Sp^r/Sm^r	62
pBBR1MCS1/5	Broad-host-range vector	Cm^r (MCS1) or Gm^r (MCS5)	47
pBluescript-II KS(−)	Cloning vector	Ap^r	Stratagene
pCPP2308	Broad-host-range construct encoding HrpL	pML122 derivative, Gm^r	Alan Collmer
pCPP2318	Broad-host-range construct encoding mature β-lactamase	pCPP30 derivative carrying *blaM* lacking signal peptide sequences, Tc^r	14
pCPP2330	Broad-host-range construct encoding AvrB1-FLAG	pML123 derivative, Gm^r	28
pCPP2988	Construct carrying *nptII*	pBluescript II KS(−) vector carrying 1.5-kb HindIII-SalI fragment with *nptII* lacking transcriptional terminator, Ap^r Km^r	1
pCPP3234	Gateway destination vector for CyaA fusions	Sp^r Sm^r	64
pCPP5040	Broad-host-range Gateway destination vector for HA fusions	pML123 derivative, Gm^r	42
pENTR/D-TOPO	Gateway entry vector	Km^r	Invitrogen
pLN307	*avrPto1* entry construct	Km^r	This work
pLN323	*avrPtoB* entry construct	Km^r	42
pLN375	*hrpJ* entry construct	Km^r	This work
pLN420	*hopB1* entry construct	pENTR/D-TOPO derivative, Km^r	60
pLN421	Broad-host-range construct encoding HopB1-CyaA	pCPP3234 derivative obtained by recombination with pLN420, Sp^r/Sm^r	60
pLN426	Broad-host-range construct encoding HrpJ-CyaA	pML123 derivative obtained by recombination with pLN375, Gm^r	This work
pLN677	Broad-host-range Gateway destination vector for HA fusions	pBBR1MCS-5 derivative, Gm^r	60
pLN705	Broad-host-range Gateway destination vector for HA fusions	pBBR1MCS-1 derivative, Cm^r	31
pLN726	Broad-host-range construct encoding HrpJ-HA	pLN677 derivative obtained by recombination with pLN375, Gm^r	This work
pLN736	Broad-host-range construct encoding HrpJ-HA	pLN705 derivative obtained by recombination with pLN375, Cm^r	This work
pLN820	*avrB1* entry construct	pENTR/D-TOPO derivative, Km^r	This work
pLN918	Broad-host-range construct encoding AvrB1-CyaA	pCPP3234 derivative obtained by recombination with pLN820, Sp^r/Sm^r	This work
pLN1979	Broad-host-range construct encoding AvrPtoB-CyaA	pCPP3234 derivative obtained by recombination with pLN323, Sp^r/Sm^r	This work
pLN1985	Broad-host-range construct encoding AvrPto1-CyaA	pCPP3234 derivative obtained by recombination with pLN307, Sp^r/Sm^r	This work
pLN2043	Construct carrying *cyaA*	pBluescript II KS(−) derivative containing *cyaA*, Ap^r	This work
pLN2179	Construct carrying DNA upstream of *hrpJ* adjacent to an *nptII* cassette	pBluescript II KS(−) derivative, Ap^r Km^r	This work
pLN2180	Broad-host-range *hrpJ* deletion construct	pBluescript II KS(−) derivative, Ap^r Km^r	This work
pLN2190	Construct carrying *cyaA* with flanking Gateway recombination sites	pBluescript II KS(−) derivative, Ap^r Cm^r	This work
pLN2193	Gateway destination vector for CyaA fusions	pML123 derivative, Gm^r Cm^r	This work
pLN2234	Broad-host-range construct encoding HrpJ-CyaA	pLN2193 derivative obtained by recombination with pLN375, Gm^r	This work
pLN2302	*hrpJ* deletion construct	pRK415 derivative carrying insert from pLN2180, Tc^r	This work
pML122/23	Broad-host-range vector	Gm^r	49
pRK415	Broad-host-range vector	Tc^r	45

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diluted samples by use of a needleless syringe. The DC3000 *hrpJ* mutant UNL140 was complemented with pLN426 for the HR and pathogenicity assay. For AvrB1- and AvrRpt2-dependent HR assays, pCPP2330 (encoding AvrB1-FLAG) and pAvrRpt2-600 (encoding AvrRpt2) were electroporated into DC3000 or the DC3000 *hrpJ* mutant UNL140. *A. thaliana* ecotype Col-0 was infiltrated with these strains at an OD_{600} of 0.1 in 5 mM MES (morpholineethanesulfonic acid). HR production was assessed 12 h after infiltration. The DC3000 *hrpJ* mutant UNL140 was complemented with pLN736 (in strains expressing AvrB1-FLAG) or pLN726 (in strains expressing AvrRpt2).

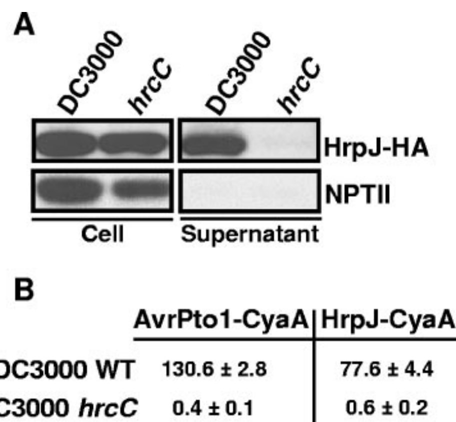
Adenylate cyclase (CyaA) translocation assays. Constructs that encoded CyaA fusions were electroporated into DC3000 for translocation assays. These included constructs pLN2234, pLN421, pLN1985, pLN918, and pLN1979, which express HrpJ-CyaA, HopB1-CyaA, AvrPto1-CyaA, AvrB1-CyaA, and AvrPtoB-CyaA, respectively. *Nicotiana benthamiana* leaves were infiltrated with test strains at an OD_{600} of 0.6 in 5 mM MES (pH 5.6). After 10 h, the leaf samples were taken 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 with Bio-Rad total protein assays. Cyclic AMP (cAMP) was quantified with a direct cAMP corrected enzyme immunoassay kit (Assay Design, Ann Arbor, MI).

RESULTS

HrpJ shares similarity with YopN homologs from animal pathogens and contains secretion signal characteristics of *P. syringae* TTSS substrates. The *Pseudomonas syringae* pv. tomato DC3000 *hrpJ* gene is the first gene of a five-gene operon within the *hrp-hrc* cluster (2). HrpJ protein was previously reported to share similarity with *Yersinia* sp. YopN homologs (4, 40). PSI-BLAST searches using DC3000 HrpJ identified HrpJ homologs from *Erwinia amylovora* and other bacterial plant pathogens that contain similar group I Hrp TTSSs in the first PSI-BLAST iteration. Also identified in iteration 1 were YopN homologs from *Bordetella bronchiseptica* and other *Bordetella* species. YopN and YopN homologs from other TTSS-containing bacteria were identified in the second and third iterations of PSI-BLAST. The amino acid sequence identity between HrpJ and the YopN homologs was low (between 18 and 22% identity). The only proteins that were clear homologs of *P. syringae* HrpJ were the HrpJ homologs in other group I Hrp TTSSs. Interestingly, we were unable to identify any proteins similar to HrpJ in *Ralstonia solanacearum* and *Xanthomonas campestris*, two bacterial plant pathogens that have group II Hrp TTSSs (4). Our analyses further support that the DC3000 HrpJ protein is similar to the large family of YopN proteins identified in many bacteria that contain TTSSs.

The DC3000 HrpJ protein also contained several characteristics identified in the N termini of other *P. syringae* type III secreted substrates (32, 59). For example, the predicted amino acid sequence of HrpJ contains an isoleucine in position 3, 6% serine in the first 50 amino acids, and no aspartate or glutamates in the first 12 residues, consistent with the N-terminal biochemical characteristics of other *P. syringae* TTSS substrates. In sum, HrpJ appears to be similar to YopN and contains biochemical characteristics in its N terminus, consistent with it being a type III secreted protein.

HrpJ is secreted in culture and translocated into plant cells via the *P. syringae* TTSS. To determine whether HrpJ is secreted in culture by the DC3000 TTSS, we made a plasmid construct, pLN426, which expressed HrpJ fused to an HA epitope. This construct was introduced by electroporation into wild-type DC3000 and a DC3000 *hrcC* mutant defective in type III secretion. Type III secretion assays were performed with



	AvrPto1-CyaA	HrpJ-CyaA
DC3000 WT	130.6 ± 2.8	77.6 ± 4.4
DC3000 *hrcC*	0.4 ± 0.1	0.6 ± 0.2

FIG. 1. HrpJ is secreted in culture and translocated into plant cells by the DC3000 type III system. (A) DC3000 and the DC3000 *hrcC* mutant, both carrying pLN426, which encodes HrpJ fused to the hemagglutinin epitope (HrpJ-HA), were grown under conditions that induce type III secretion and separated into cell-bound and supernatant fractions as described in Materials and Methods. Samples were subjected to SDS-PAGE and immunoblot analysis using anti-HA or anti-NPTII antibodies. The NPTII protein is also encoded by pLN426 and is used here as a control for nonspecific cell lysis. HrpJ-HA was detected in supernatant fractions of DC3000 but not in supernatant fractions from the *hrcC* mutant, indicating that HrpJ is secreted by the DC3000 TTSS. (B) Adenylate cyclase (CyaA) assays with HrpJ-CyaA and AvrPto1-CyaA fusions were carried out by infiltration of *Nicotiana benthamiana* with DC3000 strains carrying construct pLN2234 or pLN1985, which produced either HrpJ-CyaA or AvrPto1-Cya (a type III effector known to be injected into plant cells), respectively. Plant tissue was harvested 10 h after infiltration, and cAMP levels were determined as described in Materials and Methods. Levels of cAMP are reported in picomoles of cAMP per microgram of protein, with standard errors. WT, wild type.

these strains, and HrpJ-HA was localized to supernatant fractions from wild-type DC3000 cultures, indicating that HrpJ is secreted in culture via the *P. syringae* TTSS (Fig. 1A). HrpJ-HA remained cell bound in cultures from the DC3000 *hrcC* mutant, confirming that it required a functional TTSS to be extracellularly localized (Fig. 1A).

To determine whether HrpJ is translocated or injected into plant cells, we used the adenylate cyclase (CyaA) translocation assay (11, 65). In this assay, C-terminal CyaA fusions are made to a candidate type III translocated protein. The CyaA fusion is expressed in a TTSS-containing bacterium and exposed to eukaryotic cells. Because CyaA activity is dependent on calmodulin, only fusions that are injected into the eukaryotic cell produce cAMP. *Nicotiana benthamiana* leaves were infiltrated with DC3000 and the DC3000 *hrcC* mutant, each carrying construct pLN2234, which encoded HrpJ-CyaA. After 10 h, levels of cAMP were determined with a commercially available enzyme-linked immunosorbent assay kit as described in Materials and Methods. Plant tissue infiltrated with wild-type DC3000 expressing HrpJ-CyaA had significantly higher cAMP levels than plant tissue infiltrated with the type III defective mutant expressing HrpJ-CyaA (Fig. 1B). These results clearly indicate that HrpJ-CyaA is injected into plant cells by the DC3000 TTSS.

The DC3000 *hrpJ* mutant is greatly reduced in disease symptom production, multiplication in planta, and ability to elicit an HR on nonhost plants. To determine the extent to

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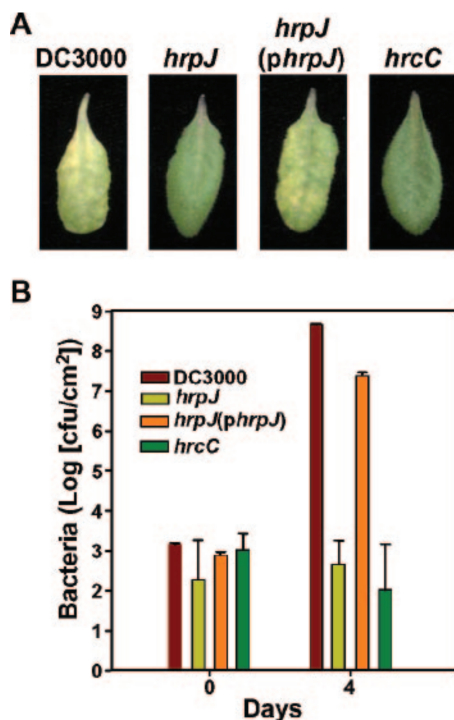

FIG. 2. HrpJ is required for symptom production and bacterial growth in *Arabidopsis*. (A) Leaves from *A. thaliana* Col-0 plants that were dip inoculated into DC3000 suspensions at a concentration of 1×10^8 cells/ml. The strains used were wild-type DC3000, a DC3000 *hrcC* mutant, UNL140 (a nonpolar *hrpJ* mutant), and UNL140 carrying pLN426, a plasmid containing *hrpJ-HA*. Photographs were taken 4 days after inoculation. (B) Bacterial growth in *A. thaliana* Col-0 leaves of the strains in panel A was monitored over a 4-day period. Results show that DC3000 *hrpJ* mutants are greatly reduced in their ability to grow in planta and in disease symptom production, and these phenotypes are complemented when *hrpJ* is provided in *trans*.

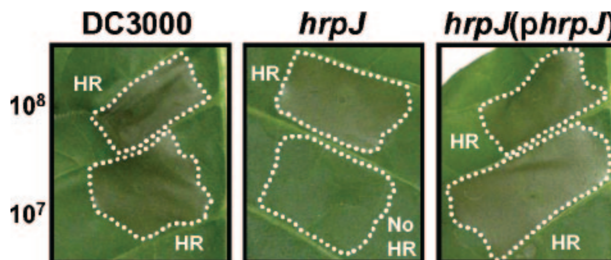

FIG. 3. DC3000 *hrpJ* mutant is reduced in its ability to elicit a nonhost HR, suggesting that it is impaired in effector translocation. Tobacco leaves (*N. tabacum* cv. Xanthi) were infiltrated with DC3000 strains at 1×10^8 cells/ml (top of each panel) or 1×10^7 cells/ml (bottom of each panel). The strains used for infiltration were wild-type DC3000 (left panel), the DC3000 *hrpJ* mutant UNL140 (middle panel), and UNL140 carrying pLN375 (right panel), which contains *hrpJ*. UNL140 was unable to elicit an HR at 1×10^7 cells/ml, and this phenotype was complemented when pLN375 was introduced into UNL140.

which HrpJ contributed to plant-microbe interactions, a nonpolar *hrpJ* mutant was constructed by marker exchange recombination as described in Materials and Methods. Briefly, we PCR amplified DNA fragments 2 kb upstream and 2.2 kb downstream of *hrpJ* and cloned these in the same orientation on either side of a 1.5-kb neomycin phosphotransferase II (*nptII*) cassette that lacked a rho-independent transcription terminator. A construct carrying this *nptII* cassette and *hrpJ* flanking DNA was introduced into DC3000, and marker exchange was selected for by loss of the plasmid marker and retention of the *nptII* cassette marker. The resulting mutant would have the *hrpJ* gene replaced by the *nptII* cassette except for the first 2 codons and the last 2 codons of the 368-codon *hrpJ* gene. The *hrpJ* mutation needed to be nonpolar because *hrpJ* is the first gene of an apparent five-gene operon. A nonpolar *hrpJ* mutant designated UNL140 was confirmed to contain an *nptII* cassette insertion by PCR and Southern analyses. Pathogenicity assays were carried out by dip inoculation of *A. thaliana* Col-0 plants with wild-type DC3000, the DC3000 *hrcC* mutant defective in TTSS, the DC3000 *hrpJ* mutant UNL140, and UNL140 carrying pLN426, which encodes HrpJ-HA. UNL140 was greatly reduced in its ability to produce disease symptoms on *A. thaliana* leaves compared to wild-type DC3000 (Fig. 2A). Indeed, the symptoms produced by UNL140 were similar to those produced by the type III defective *hrcC* mutant, indicating that HrpJ is required for the bacterium to be pathogenic and benefit from possessing a TTSS. Wild-type levels of symptom production were restored when pLN426, which carries *hrpJ*, was introduced into UNL140 (Fig. 2A), demonstrating that the defect was due to the absence of *hrpJ* and that the mutation was nonpolar.

The DC3000 *hrpJ* mutant UNL140 was monitored for its ability to multiply in *A. thaliana* leaves over a 4-day period. UNL140 was greatly reduced in its ability to grow in planta, exhibiting bacterial titers that were similar to those in the DC3000 *hrcC* mutant (Fig. 2B). Near-wild-type DC3000 growth levels were restored when UNL140 carried *hrpJ* in *trans* (Fig. 2B). Taken together, the production of reduced disease symptoms and the reduction of growth in planta indicate that HrpJ plays an important role in the TTSS and plant pathogenicity.

Type III translocation of effectors into plant cells is impaired in the DC3000 *hrpJ* mutant. The pathogenicity phenotypes of the DC3000 *hrpJ* mutant UNL140 suggested that HrpJ was required for pathogenicity. Because the central function of the TTSS is to translocate proteins into eukaryotic cells, we sought to determine the extent to which UNL140 was affected in its ability to translocate type III effector proteins into plant cells. One indication that a *P. syringae* mutant is affected in effector translocation is the reduction or abrogation of its ability to elicit a nonhost HR (60). We infiltrated *N. tabacum* cv. Xanthi (tobacco) leaves with different titers of wild-type DC3000, UNL140, or UNL140(pLN426), which expressed HrpJ-HA. DC3000 elicited an HR at or above a cell titer of 1×10^7 cells/ml. The DC3000 *hrpJ* mutant UNL140 was unable to elicit an HR at a titer of 1×10^7 cells/ml (Fig. 3). UNL140 was capable of eliciting an HR at 1×10^8 cells/ml. The reduced HR phenotype displayed by UNL140 was complemented when *hrpJ* was provided in *trans*. Since the elicitation of this HR requires translocation of effectors into plant cells, a reduced ability to elicit an HR suggests that UNL140 is impaired in its ability to translocate effectors into plant cells. However, it is important to note that the residual HR-eliciting ability dis-

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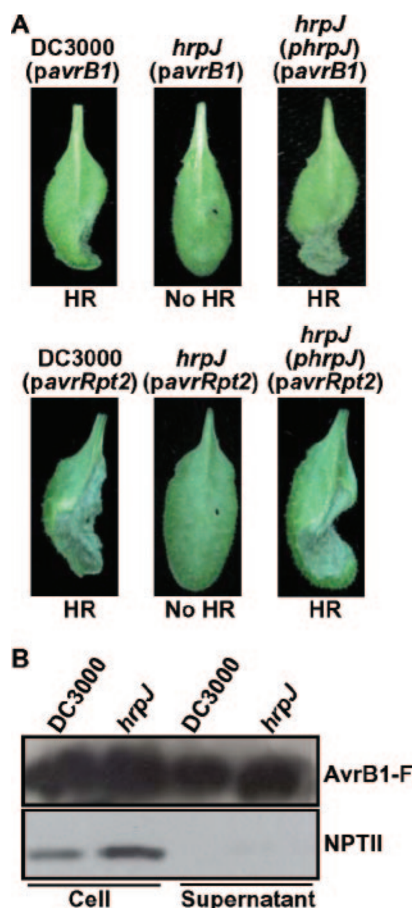

FIG. 4. DC3000 *hrpJ* mutant does not translocate Avr proteins into *Arabidopsis* but maintains the ability to secrete AvrB1 in culture. (A) The well-characterized type III effector genes *avrB1* and *avrRpt2*, which encode proteins recognized by the innate immune system of *A. thaliana* Col-0 (i.e., Avr proteins), were introduced into wild-type DC3000 and the DC3000 *hrpJ* mutant UNL140 on plasmids pCPP2330 and pAvrRpt2-600, respectively. *A. thaliana* Col-0 was infiltrated with these strains separately, and 12 h later, they were assessed for HR production. UNL140 was unable to elicit an AvrB1- or AvrRpt2-specific HR unless plasmid-encoded HrpJ was provided. (B) DC3000(pCPP2330) and UNL140(pCPP2330) were grown in type III-inducing conditions and separated into cell-bound and supernatant fractions. These samples were subjected to SDS-PAGE and immunoblot analysis using anti-FLAG or anti-NPTII antibodies, and these immunoblots are shown. NPTII was used as a control for nonspecific cell lysis. The DC3000 *hrpJ* mutant UNL140 secreted AvrB1-FLAG in culture, indicating that HrpJ was not required for type III secretion of this effector into culture supernatants.

played by UNL140 suggests that it is not completely disabled in translocation.

To directly test whether UNL140 is affected in its ability to translocate specific effectors into plant cells, two different assays were used. First, we infiltrated *A. thaliana* Col-0 with DC3000 and UNL140 expressing the *P. syringae* type III effector AvrB1 (fused to a FLAG epitope) or AvrRpt2 in *trans*. AvrB1 and AvrRpt2 are recognized by the resistance proteins RPM1 and RPS2, respectively, of the innate immune system of *A. thaliana* Col-0 and result in the induction of defense responses, including an HR (6, 29, 56). As shown in Fig. 4A, DC3000 expressing either AvrB1-FLAG or AvrRpt2 elicited an HR in *A. thaliana* Col-0 plants within 12 h. In contrast, UNL140 expressing AvrB1-FLAG or AvrRpt2 did not elicit an HR in these plants unless *hrpJ* was also provided in *trans* (Fig. 4A).

Failure to elicit an Avr protein-dependent HR may be due to the inability to inject the Avr protein (i.e., effector protein) into plant cells, or it may be due to the failure of the Avr protein to be exported from the bacterial cell. To determine whether the DC3000 *hrpJ* mutant UNL140 was competent to secrete AvrB1 via the TTSS, we performed secretion assays with DC3000 and UNL140 expressing AvrB1-FLAG. Immunoblot analysis of culture supernatant fractions confirmed that AvrB1-FLAG was secreted via the TTSS by both DC3000 and UNL140 (Fig. 4B). Collectively, these data indicate that the DC3000 *hrpJ* mutant cannot translocate AvrB1 or AvrRpt2 in the amounts required to elicit an HR. Because the *hrpJ* mutant secreted AvrB1 in culture, the translocation defect exhibited by this mutant appears to be at the level of translocation and not in the secretion of these proteins from the bacterial cell.

We used CyaA translocation assays to test whether UNL140 was defective in the translocation of other *P. syringae* type III effectors that have previously been shown to be translocated into plant cells by *P. syringae*. Constructs that encoded AvrPto1-CyaA, AvrB1-CyaA, HopB1-CyaA, and AvrPtoB-CyaA were electroporated into wild-type DC3000 and the DC3000 *hrpJ* mutant UNL140. *N. benthamiana* was infiltrated with these strains, and cAMP levels in plant tissue were determined at 10 h postinfiltration. In each case, cAMP levels were significantly elevated in tissue infiltrated with DC3000 expressing the effector-CyaA fusions whereas cAMP levels in tissue infiltrated with the corresponding UNL140 strains were extremely low, indicating that UNL140 is defective in translocation (Table 2). It is important to note that even though cAMP levels were low in samples infiltrated with UNL140 expressing AvrB1-CyaA and AvrPtoB-CyaA, they were higher than the levels produced by UNL140 infiltrated with HopB1-CyaA and AvrPto1-CyaA (Table 2). This suggests that the translocation of specific effectors was affected differently by the absence of HrpJ. This is consistent with UNL140 retaining residual nonhost HR-eliciting ability (Fig. 3). It is not clear whether the very low levels of cAMP produced by all of the effector-CyaA fusion strains correspond to biologically relevant translocation. Indeed, the fact that UNL140 expressing AvrB1 did not elicit an HR when infiltrating *A. thaliana* (Fig. 4A) suggests that the

TABLE 2. Adenylate cyclase (CyaA) translocation assays of effector CyaA fusions in wild-type DC3000 and the DC3000 *hrpJ* mutant UNL140

Effector-CyaA fusion[a]	cAMP activity (pmol/μg protein)[b] for:	
	DC3000	UNL140
AvrPto1	123.3 ± 3.5	0.8 ± 0.1
AvrB1	58.2 ± 0.0	4.8 ± 0.0
HopB1	50.1 ± 5.1	0.1 ± 0.0
AvrPtoB	51.0 ± 5.2	8.2 ± 1.3

[a] Wild-type DC3000 or the *hrpJ* mutant UNL140 carrying pLN1985 (AvrPto1), pLN918 (AvrB1), pLN421 (HopB1), or pLN1979 (AvrPtoB) was used in CyaA assays.
[b] cAMP was quantified in triplicate for each sample, and the values are means ± standard deviations.

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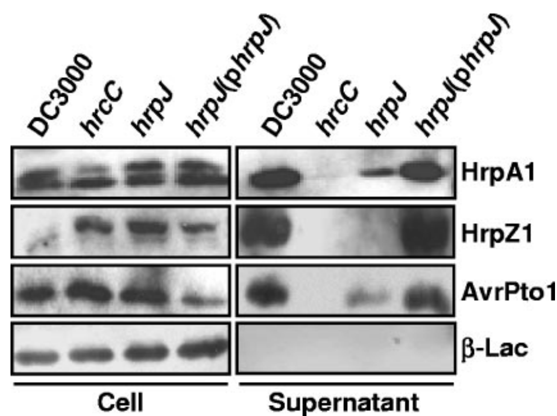

FIG. 5. DC3000 *hrpJ* mutant maintains the ability to secrete HrpA1 and AvrPto1 in culture but cannot secrete detectable amounts of HrpZ1. DC3000 strains were grown in type III-inducing conditions, and secretion assays were performed to determine whether natively expressed TTSS substrates were secreted in culture. The strains used were as follows: wild-type DC3000, a DC3000 *hrcC* mutant defective in TTSS, the DC3000 *hrpJ* mutant UNL140, and UNL140 carrying pLN726, which contains *hrpJ*. The cultures were separated into cell-bound and supernatant fractions, and these were subjected to SDS-PAGE and immunoblot analysis. HrpA1, HrpZ1, and AvrPto1 were detected with anti-HrpA1, -HrpZ1, and -AvrPto1 antibodies, respectively. Each strain also contained pCPP2318, which encodes β-lactamase (β-Lac) lacking its export sequence and therefore remains cell bound unless significant nonspecific cell lysis occurs. Reduced levels of HrpA1 and AvrPto1 were detected in *hrpJ* supernatant fractions. In contrast, HrpZ1 was not detected in supernatant fractions of the *hrpJ* mutant.

translocation of AvrB1 was not at levels needed for detection by the RPM1-dependent innate immune system. In sum, HrpJ plays an important role in the translocation of specific effectors but is not required for the type III secretion of effectors from the bacterial cell.

DC3000 *hrpJ* mutants can secrete the HrpA1 pilus protein and the AvrPto1 effector in culture but cannot secrete the HrpZ1 harpin. The failure of effectors to be translocated by the DC3000 *hrpJ* mutant may be related to the failure of the mutant to secrete to the milieu accessory proteins needed for translocation. To investigate more closely the effect that HrpJ has on secretion in culture of other type III substrates, we performed type III secretion assays that monitored the secretion of three different types of type III secreted proteins from the DC3000 *hrpJ* mutant UNL140: HrpA1, which is the major component of the Hrp pilus (63); HrpZ1, which is an accessory protein belonging to a group of proteins called harpins, which are secreted in high abundance in culture (34); and AvrPto1, which is a member of the effector class and known to be secreted in culture (66). DC3000 and UNL140 were grown in type III-inducing conditions, separated into cell-bound and supernatant fractions, and subjected to SDS-PAGE and immunoblot analysis using antibodies that recognized natively expressed proteins. HrpA1 and AvrPto1 were detected in the supernatant fraction of DC3000 and UNL140, although in somewhat reduced amounts (Fig. 5). Surprisingly, we were unable to detect HrpZ1 in supernatant fractions of UNL140 cultures even though the experiment was repeated several times, indicating that HrpJ plays an important role in the secretion of HrpZ1 that is different from its role in the type III secretion of either HrpA1 or AvrPto1 (Fig. 5).

Yersinia mutants defective in YopN constitutively secreted Yop effectors even under conditions that normally inhibit type III secretion (i.e., in the presence of 2.5 mM calcium at 37°C) (25, 69). The addition of calcium to DC3000 cultures did not inhibit type III secretion (data not shown). However, the *P. syringae* TTSS does secrete TTSS substrates in a temperature- and pH-dependent manner (66). To determine whether the DC3000 *hrpJ* mutant UNL140 displayed a deregulation phenotype analogous to that of *Yersinia yopN* mutants, we tested whether UNL140 could secrete TTSS substrates in temperature and pH conditions reported to inhibit *P. syringae* type III secretion (66). We performed type III secretion assays with wild-type DC3000 and the DC3000 *hrpJ* mutant UNL140 at 20°C, a permissive temperature for secretion, and at 30°C, a nonpermissive temperature for type III secretion. At 20°C, we detected HrpA1 and AvrPto1 in the supernatant fractions of both wild-type DC3000 and UNL140, indicating that these proteins were secreted from both strains. However, at 30°C, neither strain secreted detectable amounts of HrpA1 or AvrPto1 to the supernatant fraction. As observed in Fig. 5, HrpZ1 was not detected in the supernatant fractions of UNL140. In analogous experiments with varied pH, secretion assays were performed with a pH of 6.0, which is permissive for type III secretion, and a pH of 7.0, which is nonpermissive. HrpA1 and AvrPto1 were found to be secreted by both strains under the permissive-pH condition but not under the nonpermissive-pH condition (data not shown). To ensure that the TTSS was fully induced, we repeated the temperature and pH experiments with strains constitutively expressing HrpL, which is an alternate sigma factor required for the transcription of *P. syringae* type III-related genes (68). These experiments produced results identical to those described above with strains that did not express HrpL in *trans* (data not shown). Thus, UNL140 did not exhibit a detectable deregulation phenotype and maintained a temperature- and pH-dependent TTSS.

DISCUSSION

We have shown here that HrpJ is secreted and translocated by the *P. syringae* pv. tomato DC3000 TTSS (Fig. 1). A DC3000 mutant defective in HrpJ was severely affected in its ability to grow in planta and cause disease symptoms in *A. thaliana* (Fig. 2). This demonstrates that HrpJ is important for the DC3000 TTSS and plant pathogenesis. Additionally, the DC3000 *hrpJ* mutant possessed phenotypes that suggested that it was impaired in effector translocation into plant cells (Fig. 3 and 4; Table 2). Interestingly, this defect appeared to be at the level of translocation because the *hrpJ* mutant secreted AvrPto1 and AvrB1, two type III effectors, in culture (Fig. 4B and 5) but was unable to translocate these and other effectors into plant cells (Fig. 4 and Table 2). The DC3000 *hrpJ* mutant was also capable of secreting HrpA1 (Fig. 5), the main component of the Hrp type III pilus (63), suggesting that the *hrpJ* mutant could secrete type III effectors and at least one class of extracellular accessory protein. However, the *hrpJ* mutant could not secrete the HrpZ1 harpin (Fig. 5), a protein that is a candidate translocator and normally secreted in high abundance by *P. syringae* in culture (1, 34, 50).

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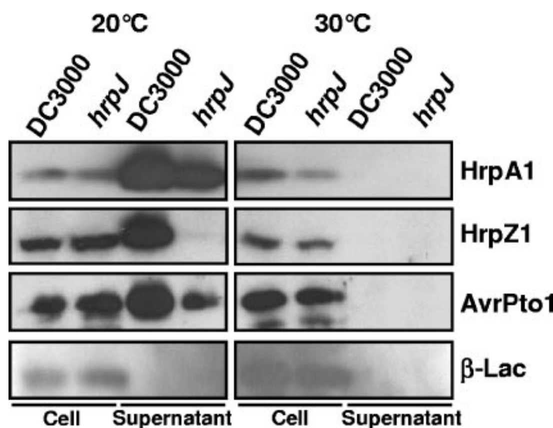

FIG. 6. DC3000 *hrpJ* mutant retains a TTSS that functions in a temperature-dependent manner. DC3000 and the DC3000 *hrpJ* mutant UNL140 were grown in type III-inducing medium at temperatures that are known to induce wild-type DC3000 type III secretion (20°C) or inhibit it (30°C). These cultures were separated into cell-bound and supernatant fractions and subjected to SDS-PAGE and immunoblot analysis. HrpA1, HrpZ1, and AvrPto1 were detected with anti-HrpA1, -HrpZ1, and -AvrPto1 antibodies, respectively. Both strains also carried pCPP2318, which encodes β-lactamase (β-Lac) without its export signal and acted as a control for nonspecific cell lysis. An immunoblot from a representative experiment is shown. UNL140 type III secretion maintained regulation by temperature as previously shown for DC3000 (66).

What is the role of HrpJ in the DC3000 TTSS? One important clue is that HrpJ shares weak similarity with YopN from *Yersinia* spp. as well as other apparent YopN homologs in other TTSS-containing bacteria (4, 40). YopN acts as a control protein for the Ysc TTSS because *Yersinia* sp. *yopN* mutants constitutively secrete Yop type III effectors in culture, even under conditions (e.g., in the presence of calcium) that normally prevent Yop secretion (25). The possibility of identifying a DC3000 mutant that constitutively secretes effectors was one of the reasons that we initiated this study. However, as we show here, the DC3000 *hrpJ* mutant did not secrete type III effectors under temperature and pH conditions known to inhibit type III secretion (Fig. 6). Thus, we failed to detect a constitutive secretion phenotype similar to the phenotype exhibited by *Yersinia yopN* mutants. It may be that a *hrpJ* mutant would exhibit such a phenotype under other repressive conditions that as of yet have not been identified.

We found that HrpJ-CyaA was translocated into plant cells (Fig. 1B). Since *hrpJ* resides in a DNA region that encodes the Hrp type III apparatus and the *hrpJ* mutant is defective in translocation, we suspect that HrpJ plays a role in controlling the secretion of other type III secreted proteins. It does appear that type III secreted proteins that act outside the plant cell can be found to be translocated. For example, Guttman et al. (32) found that HrpW1 was translocated into plant cells by *P. syringae* even though its pectate lyase domain suggests that this protein is active on the plant cell wall. The *Yersinia* YopN protein has been reported to be translocated into animal cells even though no function in host cells has been determined (15, 20, 26). Thus, the translocation of a type III secreted protein inside eukaryotic cells does not necessarily indicate that it acts within eukaryotic cells.

The DC3000 *hrpJ* mutant was severely reduced in its ability to translocate type III effectors into plant cells (Fig. 4 and Table 2). However, it retained a reduced ability to elicit a nonhost HR on tobacco plants (Fig. 3), and strains expressing AvrB1-CyaA and AvrPtoB-CyaA produced cAMP amounts that, while very small, were still significantly larger than cAMP production by strains expressing AvrPto1-CyaA and HopB1-CyaA. This suggests that the *hrpJ* mutant retained a weak ability to translocate specific effectors. Because the *hrpJ* mutant is essentially nonpathogenic (Fig. 2), the very low level of translocation must be insufficient to support pathogenicity. The translocation defect in the DC3000 *hrpJ* mutant appears more severe than those in *Yersinia yopN* mutants, which retain the ability to translocate effectors (7, 20). This suggests that these proteins, while sharing similarities, may not have identical roles in their respective TTSSs.

The implications of the failure of the *hrpJ* mutant to secrete the HrpZ1 harpin in culture deserve additional comment. Unlike the *hrpJ* mutant, *hrpZ1* mutants are not greatly affected in pathogenicity (37). Therefore, it seems likely that the *hrpJ* phenotype is not due solely to the failure to secrete HrpZ1. The secretion of other extracellular accessory proteins may be blocked in *hrpJ* mutants. Interestingly, *Salmonella enterica* InvE, a type III-related protein that has been reported to share similarity with YopN (40), is required for effector translocation and the secretion of wild-type amounts of the translocators SipB, SipC, and SipD (48). As noted above, there is indirect evidence that HrpZ1 acts as a translocator (28, 50), and our results provide additional circumstantial evidence that this is the case. Therefore, an attractive hypothesis is that the *P. syringae hrpJ* mutant is unable to translocate type III effectors due, at least in part, to its inability to secrete translocators.

The *hrpJ* mutant phenotype seems more similar to the *S. enteric invE* mutant phenotype than it does to the phenotype of *Yersinia yopN* mutants in that *yopN* mutants can secrete all proteins known to be secreted by their wild-type strains. It should be noted that one important difference between InvE and HrpJ (and YopN) is that InvE remains cell bound and is not a type III secreted protein (48). This leaves open the possibility that YopN and HrpJ exert their function from within the bacterial cell. Indeed, recent models for YopN function suggest that YopN blocks the secretion of other type III secreted proteins by plugging the type III pore from within the bacterial cell (15, 24).

Our current model for HrpJ is that it acts as a control protein that may determine which type III proteins are secreted and the order of their secretion. For example, there may be a requirement for HrpZ1 and other accessory proteins to be secreted prior to the secretion of the effectors and, in the absence of HrpJ, type III effectors are inappropriately released before contact with the plant cell is established, leading to severely reduced translocation. This translocation defect may be further exacerbated by the failure of HrpZ1 to be extracellularly localized. Future experiments will identify the inventory of type III secreted proteins that are not secreted in culture from the *hrpJ* mutant. Identifying this group of proteins should shed additional light on why the *hrpJ* mutant is defective in translocation. We will also test whether the *hrpJ* mutant phenotypes are complemented by a cell-bound HrpJ derivative lacking type III secretion signals. This will allow us to address

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whether there is a requirement for HrpJ to be secreted before effectors can be translocated or for HrpZ1 to be secreted. Determining the molecular basis for the role of HrpJ in type III secretion will likely lead to a better understanding of how type III protein traffic is deployed during bacterium-plant interactions.

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