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The *Pseudomonas syringae* HopPtoV Protein Is Secreted in Culture and Translocated into Plant Cells via the Type III Protein Secretion System in a Manner Dependent on the ShcV Type III Chaperone

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The bacterial plant pathogen *Pseudomonas syringae* depends on a type III protein secretion system and the effector proteins that it translocates into plant cells to cause disease and to elicit the defense-associated hypersensitive response on resistant plants. The availability of the *P. syringae* pv. tomato DC3000 genome sequence has resulted in the identification of many novel effectors. We identified the *hopPtoV* effector gene on the basis of its location next to a candidate type III chaperone (TTC) gene, *shcV*, and within a pathogenicity island in the DC3000 chromosome. A DC3000 mutant lacking ShcV was unable to secrete detectable amounts of HopPtoV into culture supernatants or translocate HopPtoV into plant cells, based on an assay that tested whether HopPtoV-AvrRpt2 fusions were delivered into plant cells. Coimmunoprecipitation and *Saccharomyces cerevisiae* two-hybrid experiments showed that ShcV and HopPtoV interact directly with each other. The ShcV binding site was delimited to an N-terminal region of HopPtoV between amino acids 76 and 125 of the 391-residue full-length protein. Our results demonstrate that ShcV is a TTC for the HopPtoV effector. DC3000 overexpressing ShcV and HopPtoV and DC3000 mutants lacking either HopPtoV or both ShcV and HopPtoV were not significantly impaired in disease symptoms or bacterial multiplication in planta, suggesting that HopPtoV plays a subtle role in pathogenesis or that other effectors effectively mask the contribution of HopPtoV in plant pathogenesis.

Pseudomonas syringae causes a variety of diseases on many different susceptible plant species, generally producing chlorotic and necrotic lesions on leaves or fruits (4, 43). Resistant plants respond to a high level of *P. syringae* inoculum by producing a defense-associated programmed cell death known as the hypersensitive response. The ability of *P. syringae* both to cause disease and to elicit a hypersensitive response are dependent on the type III protein secretion system (TTSS) encoded by the *hrp* and *hrc* genes (5). The *hrp* and *hrc* genes are contained within the Hrp pathogenicity island, and the flanking regions are rich in genes that encode TTSS substrates (3). TTSS substrates belong to two broad classes of type III-secreted proteins: effectors, which are translocated into plant cells, and extracellular accessory proteins, which aid in the translocation of effectors.

P. syringae TTSS effectors are generally named Avr (for avirulence), Vir (for virulence), or Hop (for Hrp-dependent outer protein) proteins, based on how they were originally identified (5, 73). The completed genome sequencing of *P. syringae* pv. tomato DC3000 has greatly facilitated the identification of effector genes in DC3000 (21, 32, 37, 39, 61, 79). The majority of *P. syringae* TTSS substrates have common characteristics in their N-terminal regions, including a high serine content, a leucine, isoleucine, or valine at residue 3 or 4, and the absence of aspartate or glutamate within the first 12 amino acids (21, 37). The roles that these effectors play inside plant cells remain largely unknown. However, several have recently been shown to suppress innate plant immunity (1, 6, 15, 27, 41, 48, 57).

TTSSs are found in many plant, animal, and insect pathogens and other eukaryote-associated gram-negative bacteria (22, 24, 34, 45). Many of the TTSS substrates from animal pathogens utilize customized type III chaperones (TTCs) to assist in their secretion (29, 60). These proteins bind to specific TTSS substrates often preventing association with other proteins and/or aggregation. They have also recently been implicated in affecting the order of secretion of effectors from TTSS-containing bacteria (13, 69). With the exception of clear homologs, generally TTCs do not show sequence similarity with each other. However, they have several general characteristics: they tend to be small proteins (ca. 15 kDa in mass), possess acidic isoelectric points (pI < 6), and have an amphipathic region in their C termini, and the gene that encodes the TTC is usually next to its cognate effector gene (75). Moreover, the crystal structures of several TTCs have been determined, and their three-dimensional configurations are similar (10, 11, 28, 56, 68). Based on the crystal structure of TTC-effector pairs, the TTC binds to the effector so that the bound region of the effector is in a nonglobular state (11, 68). Maintaining an effector in a nonglobular state may make the effector competent for type III secretion (67).

Bacterial plant pathogen TTSSs also utilize TTCs (35, 72).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
E. coli DH5α	*supE44* Δ*lacU169*(φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nal^r	40; Life Technologies
P. fluorescens 55	Nal^r	44
P. syringae pv. phaseolicola NPS3121	Wild type; Rp^r	49
P. syringae pv. tomato		
DC3000	Wild type; Rp^r	23
DC3000 *hrcC*	Lacks *hrcC*, secretion-deficient mutant, Rp^r Cm^r	78
UNL120	DC3000 mutant containing a polar insertion in *shcV*, Rp^r Sp^r	This work
UNL125	DC3000 mutant containing an insertion mutation in *hopPtoV*, Rp^r Sp^r	This work
Plasmids		
pCPP2318	pCPP30 derivative carrying *blaM* lacking signal peptide sequences, Tc^r	20
pEG202	Yeast two-hybrid vector, creates fusion to LexA DNA binding domain, Ap^r	36
pHIR11	pLAFR3 derivative carrying *P. snyringae* pv. syringae 61 *hrp*/*hrc* cluster, Tc^r	44
pJG4-5	Yeast two-hybrid vector, creates fusion to B42 activation domain, Ap^r	36
pKnockout-Ω	Vector used to make Campbell insertion mutations, Sp^r	76
pLN30	pKnockout-Ω derivative for interruption of *shcV*, Sp^r	This work
pLN31	pKnockout-Ω derivative for interruption of *hopPtoV*, Sp^r	This work
pLN127	pML123 derivative carrying *hopPtoV* with an HA tag, Gm^r	This work
pLN517	pML123 derivative carrying *shcV* and *hopPtoV*, Gm^r	This work
pLN290	pBBR1-MCS5 derivative containing *′avrRpt2* lacking N-terminal 40 residues and allowing fusion of protein of interest at SpeI site, Gm^r	This work
pLN679	pEG202 derivative carrying *hopPtoV*, Ap^r	This work
pLN680	pJG4-5 derivative carrying *shcV*, Ap^r	This work
pLN681	pJG4-5 derivative carrying *hopPtoV*, Ap^r	This work
pLN688	pQE30 derivative carrying *shcV*, Ap^r	This work
pLN702	pLN290 derivative carrying *hopPtoV*, Gm^r	This work
pLN703	pLN290 derivative carrying *shcV* and *hopPtoV*, Gm^r	This work
pLN709	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{1–194}$, Ap^r	This work
pLN710	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{195–391}$, Ap^r	This work
pLN728	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{1–97}$, Ap^r	This work
pLN730	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{98–194}$, Ap^r	This work
pLN739	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{76–125}$, Ap^r	This work
pLN765	pEG202 derivative carrying *hopPtoV* fragment corresponding to HopPtoV$_{51–150}$, Ap^r	This work
pML123	Broad-host-range cloning vector, Gm^r	53
pQE30	Expression vector for affinity purification, IPTG inducible, N-terminal six-His tag, Ap^r	Qiagen

The first TTC identified in a bacterial plant pathogen was the *P. syringae* ShcA protein (for specific Hop chaperone), which assists in the type III secretion of the HopPsyA effector (72). This report also identified several other candidate *P. syringae* TTC genes flanking effector genes whose nucleotide sequences were deposited in the databases (72). Recently, one of these candidate chaperones, ShcM, was confirmed to function as a TTC for the HopPtoM effector (8). Both ShcA and ShcM were shown to bind to their cognate effectors (8, 72). The binding site of ShcA was within the amino-terminal 166 amino acids of HopPsyA, similar to the binding site location of TTCs in animal pathogens, whereas the binding site of ShcM was localized between amino acids 100 and 400 of the 732-amino-acid-long HopPtoM (8, 72).

Here we report a novel TTC-effector pair, ShcV and HopPtoV, from *P. syringae* pv. tomato DC3000. We show that the HopPtoV effector is secreted in culture and translocated via the TTSS into plant cells in a manner dependent on ShcV. Moreover, based on yeast two-hybrid analysis, we show that ShcV binds to a site within the amino-terminal third of HopPtoV. Collectively, these data demonstrate that HopPtoV is a type III effector and that the ShcV TTC facilitates its secretion via the DC3000 TTSS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown routinely in LM (40) or Terrific broth (64) at 37°C. *P. syringae* strains were grown in King's B broth at 28°C (51). For type III secretion assays, *P. syringae* pv. tomato DC3000 strains were grown in *hrp*-inducing fructose medium at 22°C (46). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; gentamicin, 10 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}.

General DNA manipulations. For standard molecular biological manipulations, well-described protocols were used (65). Restriction enzymes, T4 ligase, and DNA polymerase were all purchased from New England Biolabs (Beverly, Mass.). The thermostable DNA polymerase used in PCRs was *Pfu* polymerase (Stratagene, La Jolla, Calif.). Oligonucleotide primers were ordered from Invitrogen (Grand Island, N.Y.) and Integrated DNA Technologies (Coralville, Iowa). Information on the primers used in PCRs is provided in Table 2. The standard cycling conditions used for PCRs were 2 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and 10 min at 72°C. Plasmids were introduced into *Pseudomonas* strains by electroporation. DNA sequence analysis was performed with Lasergene software (DNAstar Inc., Madison, Wis.). Database searches were done with BLASTN, BLASTP, BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/index.html), and 3D-PSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm/).

Type III protein secretion assays. To clone *shcV* and *hopPtoV* into pML123 (53), we used primer P409, which contained the ribosome-binding site of *shcV*, and P413, which annealed to the 3′ end of *hopPtoV* and added a nucleotide sequence encoding the hemagglutinin (HA) epitope. The resulting construct was named pLN517. Primers P412, which contained the ribosome-binding site of

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TABLE 2. Primers used for plasmid constructions

Primer	Nucleotide sequence	Enzyme site
P248	5′-CTATCTACGGATGTAATC-3′	
P249	5′-AAAATGTGTCGCTGTGTC-3′	
P251	5′-TACTTCACTAATAGCCTG-3′	
P259	5′-GGGAACAGTGCTCGTGTAG-3′	
P409	5′-AGTAGGATCCCAGATATTGCTATCTACG-3′	BamHI
P412	5′-AGTAAAGCTTGGAAAGAGTATGTTGGAT-3′	HindIII
P413	5′-AGTATCTAGATCAAGCGTAATCTGGAACATCGTATGGGTACTTTCTATCCGGAATAAC-3′	XbaI
P511	5′-AGTCTCTAGACTTAGCGGTAGAGCATTG-3′	XbaI
P516	5′-AGTCACTAGTAGAAAAAGTAGCGCTTCAAG-3′	SpeI
P578	5′-AGTCAAGCTTCAGATATTGCTATCTACG-3′	HindIII
P892	5′-AGTGAATTCATGAGCTTATCGCCGACG-3′	EcoRI
P893	5′-CCGCTCGAGTCAGTTAAAACGAAAATG-3′	XhoI
P894	5′-ACTGAATTCATGCGGTTTGATGCTGCC-3′	EcoRI
P895	5′-TATGCGGCCGCTTACTTTCTATCCGGAAT-3′	NotI
P1043	5′-GATCACTAGTCTTTCTATCCGGAATAAC-3′	SpeI
P1047	5′-ATAGGATCCAGCTTATCGCCGACGCTG-3′	BamHI
P1048	5′-GGCCGGTACCGTTAAAACGAAAATGTGT-3′	KpnI
P1054	5′-TATGCGGCCGCGTGCGGATGTTTTATATG-3′	NotI
P1055	5′-GCGGAATTCTGGAAAGCCCTGAACGAA-3′	EcoRI
P1086	5′-AGTCCTCGAGTCATGCTGCCCATACACTGCCTC-3′	XhoI
P1087	5′-AGTCGAATTCATCAATCTGCAGCATCGCTCG-3′	EcoRI
P1088	5′-AGTCGTCGACTCAGTGCGGATGTTTTATATGGCGTTC-3′	SaII
P1089	5′-AGTCGAATTCGTTCGTCCAGAAATGGGTGTAG-3′	EcoRI
P1090	5′-AGTCGTCGACTCAGTTCCACTGCCCTGCCAC-3′	SalI
P1091	5′-AGTCGAATTCCGAGTCCGTTTTGCAAGATACCC-3′	EcoRI
P1092	5′-AGTCGTCGACTCATGGAATATGTGCACCGAAACGC-3′	SalI

hopPtoV, and P413 were used to clone *hopPtoV* into pML123, resulting in pLN127.

Type III protein secretion assays were performed on DC3000 cultures as previously described (61, 71). In brief, DC3000 strains were grown in *hrp*-inducing fructose medium at 22°C for 4 to 6 h. The cell-bound proteins β-lactamase (encoded by pCPP2318) and neomycin phosphotransferase II (NptII; encoded by pML122) were used as indicators of nonspecific lysis. Cultures were separated into cell-bound and supernatant fractions by centrifugation, and total protein present in the supernatant fraction was precipitated with 12.5% trichloroacetic acid. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by standard procedures (64), transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-HA (Roche Diagnostics Corp., Indianapolis, Ind.), anti-NPTII (Cortex Biochem, San Leandro, Calif.), or anti-β-lactamase (Chemicon International, Temecula, Calif.) primary antibodies. Anti-β-lactamase and anti-NPTII primary antibodies were recognized with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.), and anti-HA primary antibodies were recognized with goat anti-rat immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Co.). Membrane-bound secondary antibodies were visualized with the Western Star chemiluminescence detection system (Applied Biosystems, Foster City, Calif.) on X-ray film.

AvrRpt2 translocation assays. To determine if HopPtoV was translocated into plant cells, we used the AvrRpt2 translocation assay (38, 59). *avrRpt2*, which lacked 5′ nucleotides corresponding to the amino-terminal 40 amino acids of AvrRpt2, was PCR cloned with primers P516 and P511 and pAVRRPT2-600 (47) as a template. This fragment was cloned into pBBR1MCS-5 (52) with SpeI and XbaI restriction sites, generating pLN290. *hopPtoV* was PCR cloned into pLN290 with primers P412 and P1043, producing pLN702. A DNA fragment containing *shcV* and *hopPtoV* was amplified with PCR with primers P578 and P1043 and cloned into pLN290, resulting in construct pLN703. These constructs were electroporated into *P. syringae* pv. phaseolicola NPS3121 and infiltrated into *Arabidopsis thaliana* Col-0 plants at an optical density at 600 nm (OD_{600}) of 0.4 in 5 mM morpholineethanesulfonic acid (MES, pH 5.6) with a needleless syringe. The production of a hypersensitive response was scored after 48 h.

Yeast two-hybrid interactions. Interactions between proteins were tested with the pEG202/pJG4-5 yeast two-hybrid system (14). The *shcV* fragment was cloned into the vector pJG4-5 with primers P892 and P893, resulting in construct pLN680. *hopPtoV* was cloned into pEG202 with primers P894 and P895, making construct pLN679. *hopPtoV* DNA fragments corresponding to the N-terminal 97 and 194 amino acids of HopPtoV were cloned into pEG202 with primers P894 and P1086 and primers P894 and P1054, respectively, producing constructs pLN728 and pLN709, respectively. A *hopPtoV* DNA fragment corresponding to the portion of the protein from residue 195 to the C terminus of HopPtoV was cloned into pEG202 with primers P1055 and P895, resulting in pLN710. Fragments corresponding to internal regions of HopPtoV were cloned into pEG202 with primer pairs P1087 and P1088 (resulting in pLN730), P1089 and P1090 (resulting in pLN765), and P1091 and P1092 (resulting in pLN739). Interaction analyses were performed in *Saccharomyces cerevisiae* strain EGY48 (pSH18-34) by standard protocols (14, 36).

Coimmunoprecipitation experiments with ShcV and HopPtoV. We cloned *shcV* by PCR with primers P1047 and P1048 into pQE30 (Qiagen, Valencia, Calif.), resulting in construct pLN688. This construct produces ShcV fused to six histidine residues at its amino terminus. For coimmunoprecipitation experiments, we expressed pLN688 and pLN127, which encodes HopPtoV fused to an HA epitope at its C terminus in *E. coli*. Briefly, *E. coli*(pLN127, pLN688) overnight cultures were used to inoculate 50-ml *E. coli* cultures at a 1:10 dilution in LM broth, which were grown to an OD_{600} of 0.8. Bacterial cultures were centrifuged, resuspended in 10 ml of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), and centrifuged again. Cell pellets were resuspended in 10 ml of cold lysis buffer (20 mM Tris [pH 8.0], 100 mM NaCl) containing lysozyme at 1 mg ml^{-1} and 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 10 min. Cells were sonicated three times for 30 s each, and the cell lysates were centrifuged at 12,000 rpm at 4°C. The aqueous phase was transferred to a new tube, and aliquots were saved separately for analysis.

To immunoprecipitate HopPtoV-HA, a sample of 100 μl of anti-HA affinity matrix (Roche Diagnostics Corp., Indianapolis, Ind.) was added to a sample containing 250 μg of total protein in cold lysis buffer. Samples were mixed on a rotary shaker at 4°C overnight. The affinity matrix was pelleted and washed three times with cold lysis buffer. The washed matrix was resuspended in 100 μl of SDS-PAGE sample buffer, boiled for 5 min, centrifuged again, and loaded on an SDS-PAGE gel. After the samples were separated by SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes, and immunoblot analysis was done with anti-HA (Roche Diagnostics Corp.) and anti-His (Qiagen) antibodies to determine whether His-ShcV coimmunoprecipitated with HopPtoV-HA.

Construction of DC3000 *hopPtoV* and *shcV*/*hopPtoV* mutants. We used a suicide vector, pKnockout-Ω (76), to generate insertion mutations in both *shcV* and *hopPtoV*. An internal fragment within the *shcV* coding region was amplified with primers P248 and P249 and cloned into pKnockout-Ω that had been digested with XcmI, resulting in construct pLN30. Likewise, an internal fragment within *hopPtoV* was PCR amplified with primers P251 and P259 and cloned into XcmI-

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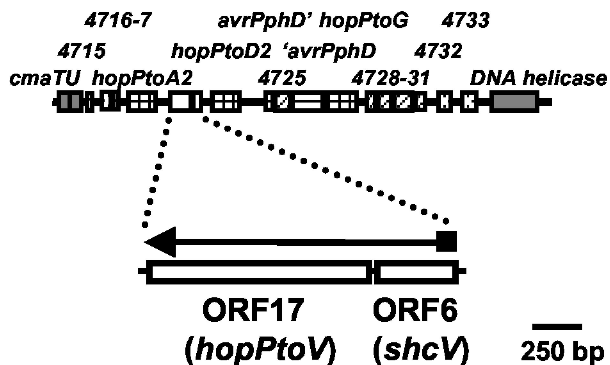

FIG. 1. Organization of ORF6 (*shcV*) and ORF17 (*hopPtoV*) within a pathogenicity island in the chromosome of *P. syringae* pv. tomato DC3000. ORF6 (*shcV*), a candidate chaperone gene, and ORF17 (*hopPtoV*), its cognate effector gene, are predicted to be part of the same transcriptional unit and are within a pathogenicity island containing other type III-related genes. ORF6 and ORF17 are depicted as white boxes, and the confirmed effector genes *hopPtoA2* (7), *hopPtoD2* (27), and *hopPtoG* (61) are represented as hatched boxes. An insertion sequence-interrupted *avrPphD* gene is depicted as horizontally striped boxes, and the apparent borders of this type III-related region are delimited by *cmaTU* and a DNA helicase gene, both represented as grey boxes. Other ORFs in the region are represented as stippled boxes. With the exception of ORF6 and ORF17, the numbers represent the ORF numbers given when the genome was annotated and should be preceded by the prefix PSPTO. Insertion sequences are depicted as diagonally striped boxes. The arrow indicates the predicted direction of ORF6 and ORF17 transcription, and the black box represent a putative HrpL-dependent (type III) promoter.

digested pKnockout-Ω, producing construct pLN31. Campbell insertion mutations were made in DC3000 by introducing pLN30 and pLN31 separately into DC3000 via triparental matings. Because pKnockout-Ω cannot replicate in *P. syringae*, the majority of DC3000 colonies that became spectinomycin resistant likely represented *shcV* or *hopPtoV* mutants. Putative mutants were confirmed to have insertions in *shcV* or *hopPtoV* by PCR with primers that flanked each gene. The DC3000 *shcV* and *hopPtoV* mutants were designated UNL120 and UNL125, respectively.

***Arabidopsis* pathogenicity assays.** *A. thaliana* ecotype Col-0 cells used in pathogenicity assays were grown in a growth chamber at 23°C with 8 h of light per day. Disease symptoms and bacterial multiplication in planta were determined by dip-inoculating plants in *P. syringae* suspensions adjusted to an OD_{600} of 0.2 in 10 mM $MgCl_2$ containing 0.02% Silwet L-77 (Lehle Seeds, Round Rock, Tex.). Bacterial growth was determined as previously described (3). Briefly, 0.3-cm^2 leaf disks were harvested from infected plants on days 0, 2, and 4 postinoculation and macerated with a mortar and pestle in microcentrifuge tubes. Different dilutions were spotted on KB plates with the appropriate antibiotics after a 2- to 3-day incubation period at 30°C and enumerated.

RESULTS

Protein product of ORF6 has the general properties of type III chaperones. A candidate chaperone-effector pair encoded by ORF6 and ORF17, respectively, was identified during a genomewide effector gene search (61). ORF6 and ORF17 are downstream of a typical type III (Hrp) promoter and within a pathogenicity island (Pai) that contains the effector genes *hopPtoA2*, *hopPtoD2*, and *hopPtoG* (Fig. 1) (7, 27, 61). Upon completion of the annotation of the DC3000 genome, ORF6 and ORF17 were given the names PSPTO4721 and PSPTO4720, respectively (16).

Generally, we identified candidate chaperone genes based on their proximity to genes that encode *P. syringae* TTSS substrates (21, 37, 72). However, we identified ORF17 as a candidate effector gene because it was adjacent to ORF6, an ORF predicted to encode a protein that has many of the characteristics of TTCs. The product of ORF17 does not possess many of the general characteristics of *P. syringae* effectors (39, 61). For example, there is an aspartate residue at position 4, there is no isoleucine, leucine, or valine in position 3 or 4, and the overall serine content in the first 50 residues is low (6%), all features that would be inconsistent with the export-associated patterns found in confirmed *P. syringae* TTSS substrates (39, 61). The lack of export-associated patterns within the ORF17 product is likely to be unrelated to whether it uses a TTC, because other *P. syringae* effectors that are dependent on TTCs have typical export-associated patterns (8, 72).

ORF6 encoded a predicted product that was relatively small (14.7 kDa), had an acidic pI (6.49), and had a predicted amphipathic region in its C-terminal region, which suggested that it might be a TTC (75). Moreover, with a protein-fold recognition program (3D-PSSM) (50), the ORF6 product showed a predicted fold similar to that of *Salmonella* SigE (PSSM *E* value, 5.16) and *Yersinia* SycE (PSSM *E* value, 12.7), both TTCs (26, 74).

ORF17 encodes a product that is secreted into culture supernatants via the TTSS, and its secretion is enhanced by overexpression of ORF6. Because ORF17 was adjacent to a candidate chaperone gene and within a Pai rich in effector genes, we wanted to test whether the ORF17 product was secreted by DC3000 into culture supernatants. To address this, we PCR cloned ORF17 with nucleotide sequences added to the 3′ primer that encoded an HA affinity tag into pML123, producing pLN127. This construct was introduced into DC3000 and a DC3000 *hrcC* mutant, which is defective in type III secretion. DC3000(pLN127) secreted a small amount of the ORF17 protein product into the supernatant fraction (Fig. 2A). To determine whether ORF6 would increase the amount of the ORF17 product found in culture supernatants, ORF6 and ORF17 were PCR cloned into pML123, resulting in construct pLN517. This construct, which produces the ORF6 and ORF17 (HA-tagged) products, was electroporated into DC3000 and the DC3000 *hrcC* mutant. The amount of ORF17 product found in the DC3000 supernatant fractions increased dramatically when ORF6 was also present (Fig. 2A), which clearly showed that ORF17 is a TTSS substrate and that ORF6 likely encoded a TTC. Hereafter, we refer to ORF6 and ORF17 as ShcV and HopPtoV, respectively.

DC3000 mutant defective in *shcV* does not secrete detectable amounts of HopPtoV in culture. To determine how dependent HopPtoV secretion was on ShcV, two different mutations were made with a suicide vector called pKnockout-Ω (76). *shcV* and *hopPtoV* were interrupted via integration of pKnockout-Ω, generating the DC3000 mutants UNL120 and UNL125, respectively. Based on the orientation of pKnockout-Ω in the DC3000 chromosome, UNL120 is predicted to produce a polar mutation in *shcV*, and because *hopPtoV* is likely in the same operon (Fig. 1), *hopPtoV* transcription is probably blocked in UNL120. UNL125 was disrupted within *hopPtoV* but possessed an intact *shcV* gene.

When the *hopPtoV* mutant UNL125 was transformed with pLN127 (which carries *hopPtoV-ha*) or pLN517 (which carries *shcV* and *hopPtoV-ha*), HopPtoV-HA was present in the supernatant fraction of wild-type DC3000 cultures, as expected

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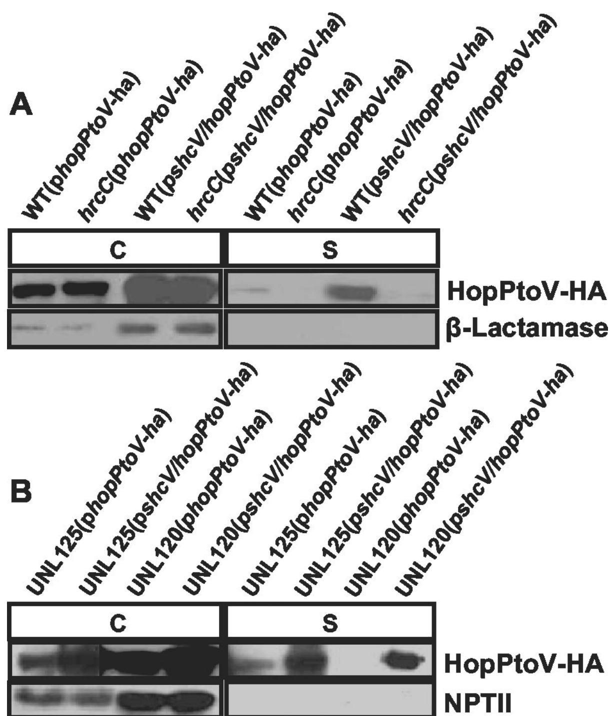

FIG. 2. HopPtoV is secreted in culture via the DC3000 TTSS in an ShcV-dependent manner. (A) Cultures of wild-type (WT) *P. syringae* pv. tomato DC3000 and a DC3000 TTSS-defective mutant (*hrcC*) were grown in *hrp*-inducing medium and separated into cell-bound (C) and supernatant (S) fractions. The cell-bound and supernatant fractions were concentrated 13.3-fold and 133-fold, respectively, relative to the initial culture volumes. The samples were separated by SDS-PAGE and immunoblotted. HopPtoV and β-lactamase, which was used as a lysis control, were detected with anti-HA and anti-β-lactamase antibodies, respectively. Wild-type DC3000 strains containing plasmid pLN127 (p*hopPtoV-ha*) or pLN517 (p*shcV*/*hopPtoV-ha*) secreted HopPtoV-HA into the supernatant fraction. However, much more HopPtoV-HA was found in the supernatant fractions from DC3000(pLN517), which overexpressed ShcV. (B) Cultures of DC3000 *shcV* (UNL120) and *hopPtoV* (UNL125) insertion mutants containing plasmid pLN127 (p*hopPtoV-ha*) or pLN517 (p*shcV*/*hopPtoV-ha*) were grown in *hrp*-inducing medium, separated into cell-bound (C) and supernatant (S) fractions, and separated by SDS-PAGE. Immunoblotted proteins were detected with anti-HA or anti-NPTII antibodies. Plasmid-encoded NPTII should remain cell bound and was used as a lysis control. The *shcV* mutant UNL120 was unable to secrete HopPtoV-HA unless *shcV* was also provided in *trans*.

(Fig. 2B). Interestingly, much more HopPtoV-HA was found in the supernatant fraction of UNL125(pLN127) than was present in DC3000(pLN127) (Fig. 2), perhaps due to the absence of native HopPtoV in UNL125, allowing more HopPtoV-HA (i.e., detectable HopPtoV) to be type III secreted into the supernatant fraction. Detectable amounts of HopPtoV-HA were not secreted into supernatant fractions of the DC3000 *shcV* mutant UNL120(pLN127) (Fig. 2B), which demonstrated the requirement of ShcV for the type III secretion of HopPtoV. However, when *shcV* and *hopPtoV-ha* were provided in *trans* to UNL120, HopPtoV-HA was again found in large amounts in the supernatant fraction (Fig. 2B).

HopPtoV is translocated into plant cells and ShcV is required for HopPtoV translocation. To determine whether HopPtoV was a secreted accessory protein or an effector translocated into plant cells and, if so, whether ShcV was required for the translocation of HopPtoV, we used a translocation assay that tests whether a TTSS substrate is translocated into plant cells (38, 59). In this assay, a candidate TTSS effector that possesses potential type III secretion signals is fused to a truncated AvrRpt2 Avr protein lacking its own type III secretion signals. The truncated AvrRpt2 moiety retains its Avr domain, which is recognized by *Arabidopsis* Col-0, resulting in production of a hypersensitive response. We made *hopPtoV-′avrRpt2* fusion constructs with and without the *shcV* gene and electroporated these constructs into *P. syringae* pv. phaseolicola NPS3121, which is nonpathogenic on *Arabidopsis*. NPS3121 carrying pLN702, which encoded HopPtoV-′AvrRpt2 alone, did not elicit a hypersensitive response when infiltrated into *Arabidopsis* Col-0 plants, indicating that this fusion protein was not translocated into plant cells (Fig. 3). In contrast, NPS3121 with pLN703, which encoded ShcV and HopPtoV-′AvrRpt2, did elicit a hypersensitive response on *Arabidopsis* Col-0 (Fig. 3). These experiments demonstrated that HopPtoV is translocated into plant cells and that the translocation of HopPtoV into plant cells is dependent on the ShcV TTC.

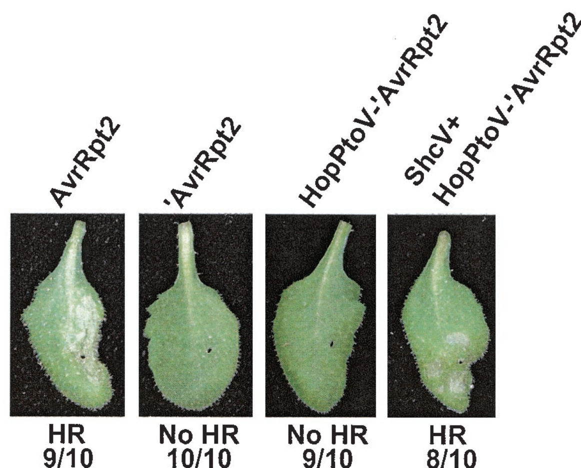

FIG. 3. AvrRpt2 translocation assays indicate that HopPtoV is translocated into plant cells in an ShcV-dependent manner. *P. syringae* pv. phaseolicola NPS3121 carrying plasmids that encoded either full-length AvrRpt2, N-terminally truncated AvrRpt2 (′AvrRpt2), or a HopPtoV-truncated AvrRpt2 fusion (HopPtoV-′AvrRpt2) with and without ShcV were infiltrated into *A. thaliana* Col-0 (*RPS2*) plants at an OD_{600} of 0.4. Plants were scored for production of a hypersensitive response (HR) after 48 h, and representative leaves were photographed. Ten leaves were infiltrated for each bacterial strain, and the number of times that the pictured result was observed over the total number of samples is indicated as a fraction under each picture.

ShcV type III chaperone interacts with its cognate effector HopPtoV. To determine whether ShcV binds to HopPtoV, we made constructs that contained either *hopPtoV* with a C-terminal HA tag (pLN127) or *shcV* with an N-terminal six-His tag (pLN688). These plasmids were transformed together into *E. coli*, and this strain was grown to an OD_{600} of 0.8. The cells were sonicated, and the soluble proteins were incubated with anti-HA high-affinity matrix that binds to the HA epitope. Samples from the total soluble protein, proteins not bound to the matrix, proteins washed off in the final wash, and proteins bound to the matrix were separated on SDS-PAGE gels and

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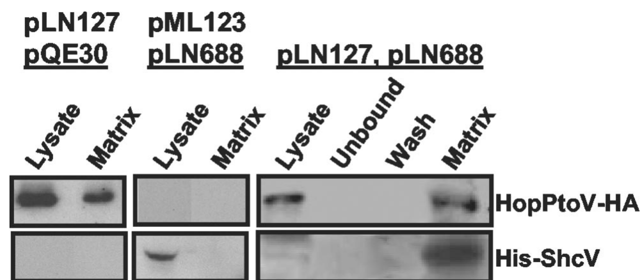

FIG. 4. HopPtoV interacts with ShcV in coimmunoprecipitation experiments. Soluble protein samples from sonicated *E. coli* DH5α(pLN127, pLN688), which expressed HopPtoV-HA and His-ShcV, were mixed with anti-HA affinity matrix as described in Materials and Methods. Aliquots from the soluble total protein (lysate), the protein sample post-anti-HA matrix treatment (unbound), the final wash, and the proteins bound to the matrix (matrix) were separated by SDS-PAGE and immunoblotted. HopPtoV-HA and His-ShcV were detected with anti-HA and anti-His antibodies, respectively. Also included were samples that contained constructs that expressed either ShcV-HA or His-ShcV and a vector control (either pQE30 and pML123), demonstrating that both proteins needed to be present to detect His-ShcV in the matrix sample.

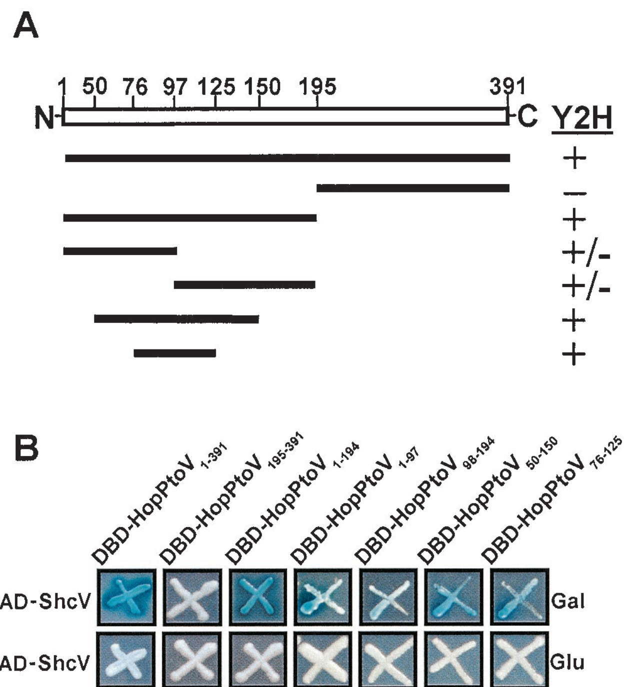

FIG. 5. ShcV interacts with the N-terminal third of HopPtoV in LexA yeast two-hybrid assays, with the strongest binding occurring between amino acids 76 and 125 of HopPtoV. (A) Schematic representation of the HopPtoV fragments that were fused to the DNA-binding domain (DBD) and used in the LexA yeast two-hybrid interaction assay. The top white box represents the full-length HopPtoV protein (391 amino acids). The lower black bars represent a series of HopPtoV fragments that were fused to the DBD and tested in the yeast two-hybrid assay to determine if they interacted with ShcV fused to the transcriptional activation domain (AD). A representation of the results is shown: +, strong interaction; −, no detectable interaction; +/−, weak interaction. (B) Yeast strains carrying pJG4-5::*shcV* (producing AD-ShcV) and pEG202 with different *hopPtoV* fragments (producing DBD fusions) were grown at 30°C for 2 days on the appropriate selective medium containing X-Gal and either galactose (Gal) or glucose (Glu). Results were scored based on the amount of blue pigmentation produced by the colonies: dark blue, strong interaction; pale blue, weak interaction; and white, no detectable interaction. Analogous experiments measuring the rescue of leucine auxotrophy, the other reporter of this yeast two-hybrid system, showed similar results (data not shown).

analyzed by immunoblotting with antibodies that recognize either HopPtoV-HA or His-ShcV. As expected, HopPtoV-HA was detected in the total soluble protein fraction and the matrix fraction (Fig. 4). ShcV was detected weakly in the lysate fraction and in large amounts in the resin fraction in the presence of HopPtoV-HA, indicating that ShcV binds to HopPtoV, which is consistent with ShcV's acting as a TTC for HopPtoV (Fig. 4). ShcV did not bind to the anti-HA high-affinity matrix in the absence of HopPtoV-HA, nor did anti-His antibodies recognize any proteins similar in size to His-ShcV in the absence of His-ShcV (Fig. 4), two important controls to validate the specificity of this interaction. The wash fraction shown in Fig. 4 represents the last wash before bound proteins were eluted from the matrix. Thus, our results demonstrate that His-ShcV and HopPtoV-HA bind to each other, a prerequisite for a TTC-effector pair.

ShcV interacts with HopPtoV in yeast two-hybrid assays, and its binding site is most likely within amino acid residues 76 to 125 of HopPtoV. The secretion, translocation, and coimmunoprecipitation experiments support the notion that ShcV acts as a TTC and is needed for the type III secretion and translocation of HopPtoV. To further delimit the interaction of ShcV with HopPtoV, we tested the ability of these proteins to interact in LexA-based yeast two-hybrid interaction assays (36). The *shcV* gene was cloned into the activation domain vector pJG4-5, which created a fusion between ShcV and the activation domain. The full-length *hopPtoV* and a series of *hopPtoV* gene portions were cloned into the LexA DNA-binding domain (DBD) vector pEG202, producing protein fusions with the DBD (Fig. 5A).

Full-length (391 amino acids) DBD-HopPtoV interacted strongly with AD-ShcV in our yeast two-hybrid experiments, as indicated by the production of blue pigmentation on galactose-containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium, which activated the pJG4-5 *GAL1* promoter upstream of *shcV* (Fig. 5B). These proteins also interacted when they were expressed in the opposite yeast two-hybrid vector, producing DBD-ShcV and AD-HopPtoV fusion proteins (data not shown). None of the protein fusions produced any blue pigmentation when they were expressed individually with vector controls, indicating that they did not activate transcription on their own (data not shown). DBD-HopPtoV$_{195\text{-}391}$, the C-terminal HopPtoV portion encompassing amino acids 195 to 391, produced white colonies on galactose-containing plates, indicating that this HopPtoV portion did not contain an ShcV binding site (Fig. 5B). Yeast cells expressing the fusion protein containing the N-terminal half of HopPtoV, DBD-HopPtoV$_{1\text{-}194}$, produced deep blue pigmentation on galactose medium, indicating that the ShcV binding site is contained within this moiety of HopPtoV (Fig. 5B). Yeast cells expressing the fusion proteins DBD-HopPtoV$_{1\text{-}97}$ and DBD-HopPtoV$_{98\text{-}194}$ produced only pale blue colonies, indicating that they interacted weakly with AD-ShcV (Fig. 5B).

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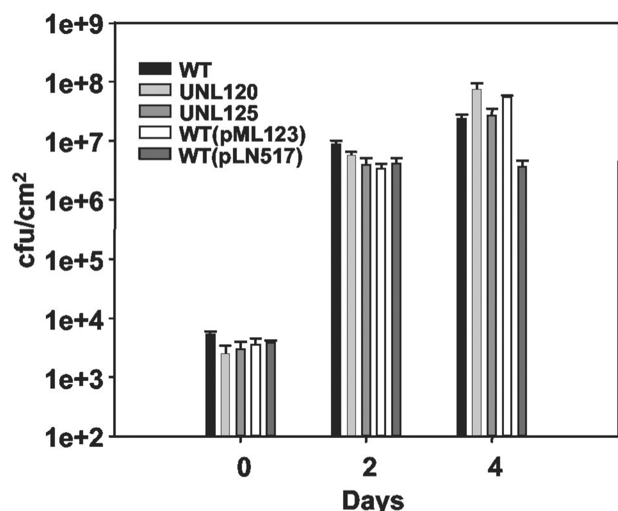

FIG. 6. ShcV and HopPtoV do not contribute measurably to the ability of DC3000 to grow in *Arabidopsis* leaf tissue. The following strains were dip inoculated into *A. thaliana* Col-0 as described in Materials and Methods: wild-type DC3000 (WT); wild-type DC3000 with the empty vector pML123; DC3000(pLN517) expressing *shcV* and *hopPtoV*; the DC3000 *shcV* mutant UNL120; and the DC3000 *hopPtoV* mutant UNL125. Leaf tissue was harvested at days 0, 2, and 4 and enumerated by plating dilutions on KB plates with the appropriate antibiotics. Each assay was done at least three times, and error bars indicate standard deviations. These results suggest that ShcV and HopPtoV do not contribute significantly to growth in planta. The disease symptoms produced by these strains were similar (data not shown).

Because these fusions combined extend over the region of HopPtoV that strongly interacted with ShcV, this suggests that the junction between these two portions is likely to contain the ShcV binding site. Yeast cells expressing AD-ShcV and either DBD-HopPtoV$_{76\text{-}125}$ or DBD-HopPtoV$_{51\text{-}150}$ produced blue colonies nearly as dark as those produced by the fusion DBD-HopPtoV$_{1\text{-}194}$, supporting the idea that the ShcV binding site resides within amino acids 76 to 125 of HopPtoV (Fig. 5). In summary, the ShcV binding site on HopPtoV resides within the N-terminal 194 amino acids with the strongest binding occurring within 76 to 125 amino acids of HopPtoV. However, due to the weak binding of AD-ShcV to DBD-HopPtoV$_{1\text{-}97}$, we cannot exclude that there may be additional ShcV binding sites within the first 97 amino acids of HopPtoV.

DC3000 *hopPtoV* and *shcV/hopPtoV* mutants show no significant reduction in disease symptoms or bacterial multiplication in planta. We were also interested in determining the importance of HopPtoV in the pathogenic ability of DC3000. To test this, we dip-inoculated the *shcV* polar mutant UNL120 and the *hopPtoV* mutant UNL125 into *Arabidopsis* Col-0 plants and surveyed disease symptom production and bacterial growth in planta over a 4-day period. Both mutants produced disease symptoms similar to those produced by wild-type DC3000 (data not shown), and their growth in plant tissue was not impaired (Fig. 6). Moreover, ectopic overexpression of *shcV* and *hopPtoV* in DC3000 did not enhance bacterial growth in planta (Fig. 6). Therefore, the contribution of HopPtoV to virulence in DC3000 does not appear to be significant. However, the lack of a virulence phenotype can also be explained by the presence of other effectors in DC3000 with similar activities.

We also tested whether HopPtoV was an Avr protein on the plants that we commonly use in our laboratory. To do this, we PCR cloned *hopPtoV* into an *Agrobacterium* binary vector (pPZP212) and infiltrated *Agrobacterium* strains carrying this construct into the leaves of tomato (*Lycopersicon esculentum* cv. Moneymaker), *Arabidopsis* (Col-0), tobacco (*Nicotiana tabacum* cv. Xanthi), and *Nicotiana benthamiana*. None of the infiltrated leaves showed a hypersensitive response (data not shown). This indicates that these plants do not have innate immune systems that can recognize HopPtoV. However, it remains a possibility that HopPtoV acts as an Avr protein on other plants.

DISCUSSION

This report describes experiments that show that HopPtoV is a type III effector that is secreted in culture (Fig. 2) and translocated (Fig. 3) into plant cells by the *P. syringae* pv. tomato DC3000 TTSS. Both the type III secretion and translocation of HopPtoV are dependent on ShcV (Fig. 2 and 3), a protein that possess the general characteristics of a TTC. As part of a larger survey study, HopPtoV was recently shown to be translocated into plant cells with a different translocation assay than we described here (66). However, this report did not demonstrate that HopPtoV translocation was dependent on ShcV. ShcV binds directly to HopPtoV, based on immunoprecipitation and yeast two-hybrid experiments (Fig. 4 and 5), and the binding site is within the amino-terminal third of HopPtoV, with the strongest binding occurring between residues 76 and 125 of the 391-amino-acid-long HopPtoV (Fig. 5). We found that DC3000 *hopPtoV* mutants were not significantly impaired in either disease symptom production or bacterial growth in planta (Fig. 6). However, given the number of type III effectors present in DC3000 and their apparently functionally redundant roles, this result does not mean that HopPtoV is unimportant for plant pathogenesis. In fact, the number of *P. syringae* effector mutants that show a reduction in symptom production and growth in plant tissue is limited to only a few effectors, illustrating why the large inventory of *P. syringae* effectors were not isolated in genetic screens assessing virulence reduction (55, 62).

The identification of ShcV as a TTC brings the total number of demonstrated bacterial plant pathogen TTCs to four: ShcA (72), DspB/F (35), ShcM (8), and ShcV. DspB/F is from *Erwinia amylovora* CFPB1430, and ShcA is from *P. syringae* pv. syringae 61, while ShcM and ShcV are both from DC3000. When we identified ShcA as a TTC, we performed a search of the nucleotide databases to identify other putative TTC genes adjacent to *P. syringae* effector and *avr* genes and identified several additional candidate *P. syringae* TTCs (72). The availability of the entire DC3000 genome sequence and the identification of many confirmed and predicted DC3000 TTSS substrates provided an opportunity to identify TTC genes in DC3000. We searched the adjacent regions of the confirmed and predicted TTSS substrates described by Collmer et al. (21) and found nine TTCs and candidate TTCs in DC3000, which are listed in Table 3. Several of the candidates on this list likely represent functional TTCs. For example, ShcA$_{Pto}$ is a homolog

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TABLE 3. Confirmed and predicted type III chaperones from *P. syringae* pv. tomato DC3000

Chaperone or ORF	Size (kDa)	pI	Effector or ORF	Accession no.	Reference(s)
$ShcA_{Pto}$	14.4	5.3	$HopPsyA_{Pto}$	AE016875	16, 72
ShcM	18.0	5.3	HopPtoM	AE016860	8, 72
ShcV	14.7	6.5	HopPtoV	AAO58159	This work, 16
PSPTO0503	15.6	6.1	HopPtoF	AE016857	16, 70
PSPTO1369	19.9	6.8	HopPtoN	AF232006	3, 72
PSPTO1376	14.6	5.3	AvrE	AF232006	3, 72
PSPTOA0017	15.6	5.5	HopPtoS1	AE016855	16, 39, 61
PSPTO4589	17.1	5.9	PSPTO4588	AE016872	16
PSPTO4599	17.2	5.3	PSPTO4597	AE016872	16

of ShcA (72), PSPTO1376 (also referred to as AvrF) and AvrE are homologs of DspB/F and DspA/E, respectively, a chaperone-effector pair from *Erwinia amylovora* (12, 35), and PSPTO0503 and HopPtoF are homologs of AvrPphF ORF1 and ORF2, respectively, both of which are required for AvrPphF-induced defense responses, suggesting that AvrPphF ORF1 encodes a TTC (61, 70).

TTCs from animal pathogens belong to two main classes (22, 60). Class I consists of chaperones that bind to effectors, and these are separated further if they interact with only one effector (class IA) or multiple effectors (class IB). Class II chaperones assist in the secretion of translocators, which are secreted proteins that form pores in the eukaryotic plasma membrane that allow effector delivery. The TTCs that function in the evolutionarily related flagellum biogenesis system appear to constitute a separate class (class III) (9). The DC3000 chaperones (and candidates) listed in Table 3 are likely to be class I chaperones because the TTSS substrates that utilize them are effectors instead of translocators. Translocators in bacterial plant pathogen TTSSs have yet to be well characterized. However, the putative translocators HrpZ from *P. syringae* and HrpF from *Xanthomonas campestris* pv. vesicatoria do not appear to require TTCs for their secretion (2, 17, 42, 54, 63).

The confirmed DC3000 chaperones ShcA, ShcM, and ShcV have several similarities and some differences with each other and with animal-pathogen TTCs. For example, there is no indication that ShcA, ShcM, or ShcV stabilizes it cognate effector. However, only in the case of ShcA were the experiments carried out with natively expressed effector (72). In experiments with ShcM (8) and ShcV (Fig. 2), the effector was overexpressed from multicopy plasmids, which may mask a reliance on the chaperone for effector stability. The majority of animal-pathogen TTCs stabilize their cognate effectors (29, 60), as does the *E. amylovora* DspB/F TTC (35). The instability of effectors may be due to aggregation-prone domains present in the effectors, as was shown for the *Yersinia* YopE effector, which possesses an aggregation domain that is masked by the SycE TTC (13, 30). We are currently testing whether DC3000 effectors possess domains that cause interactions with other proteins that might interfere with their type III secretion in the absence of a TTC.

An interesting difference between the DC3000 TTCs is that while the secretion of *P. syringae* pv. syringae HopPsyA is dependent on the ShcA TTC, when *hopPsyA* is provided in *trans* the overexpression of HopPsyA overrides the need for ShcA (72). This is in contrast to the DC3000 effectors HopPtoM and HopPtoV, which are dependent on their TTCs even when they are overexpressed (8) (Fig. 2). In practice, this may indicate that HopPtoM and HopPtoV would be better proteins than HopPsyA with which to elucidate chaperone-associated domains. However, it may also provide an important clue to why certain effectors require TTCs while many others apparently do not. For example, perhaps HopPtoM and HopPtoV interact more strongly with cytosolic proteins than HopPsyA, blocking their secretion more stringently. Alternatively, the secretion signals for HopPtoM and HopPtoV may not be available for recognition by the type III apparatus unless a chaperone is bound. Indeed, the similarities of the protein structures of the SicP-SptP and SycE-YopE chaperone-effector complexes in the absence of sequence similarity make the hypothesis that the bound TTC acts as part of a type III secretion signal an attractive one (11, 68).

Given the prevalence of TTCs in TTSS-containing organisms and the extent to which animal-pathogen TTCs have been studied, a reasonable question to ask is what can be learned from studying bacterial plant pathogen TTCs. Even though significant advances have been made on TTCs from animal pathogens, it is still not known how they facilitate the secretion of TTSS substrates. Isolating and studying TTCs from more distantly related TTSSs such as plant pathogens should provide insight into their activities. A recent phylogenetic analysis of TTSSs places the TTSS of *P. syringae* in a different group from animal-pathogen TTSSs (31). Initial reports of plant-pathogen TTCs do indicate they may have different properties than their animal-pathogen counterparts.

Another practical reason to catalog the DC3000 effectors that require TTCs is the possibility that effectors that utilize TTCs are the first ones to be translocated into host cells and are important for early events in pathogenesis. Several recent papers provide evidence that TTCs may help set up a hierarchy for TTSS substrates to gain access to the type III apparatus, either by helping their cognate effectors compete for the type III secretion machinery (13), by their involvement in regulation of the TTSS through feedback regulation (18, 19, 33, 77), or by acting as coactivators (25, 58). Moreover, the *Yersinia* effectors that require TTCs, YopE, YopH, and YopT, play antiphagocytic roles that are required early during the infection process (29). Thus, DC3000 effectors that utilize chaperones may play important early roles in plant-bacterium interactions. The identification of these effectors, their TTCs, and the mecha-

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nisms used for their delivery will provide a more complete picture of plant pathogenesis by *P. syringae*.

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