Molecular Microbiology (2002) **44**(6), 1469–1481

The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of *Pseudomonas syringae*

Karin van Dijk,[1,2] Vincent C. Tam,[2] Angela R. Records,[1] Tanja Petnicki-Ocwieja[1,2,3] and James R. Alfano[1,2]*

[1]*Department of Biological Sciences, University of Nevada, Las Vegas, NV 89154-4004, USA.*
[2]*The Plant Science Initiative and the Department of Plant Pathology, University of Nebraska, Lincoln, NE 68588-0660, USA.*
[3]*School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0118, USA.*

Summary

Pseudomonas syringae **uses a type III protein secretion system encoded by the Hrp pathogenicity island (Pai) to translocate effector proteins into plant cells. One of these effector proteins is HopPsyA. A small open reading frame (ORF), named *shcA*, precedes the *hopPsyA* gene in the Hrp Pai of *P. s. syringae* 61. The predicted amino acid sequence of *shcA* shares general characteristics with chaperones used in type III protein secretion systems of animal pathogens. A functionally non-polar deletion of *shcA* in *P. s. syringae* 61 resulted in the loss of detectable HopPsyA in supernatant fractions, consistent with ShcA acting as a chaperone for HopPsyA. Cosmid pHIR11 carries a functional set of type III genes from *P. s. syringae* 61 and confers upon saprophytes the ability to secrete HopPsyA in culture and to elicit a HopPsyA-dependent hypersensitive response (HR) on tobacco. *P. fluorescens* carrying a pHIR11 derivative lacking *shcA* failed to secrete HopPsyA in culture, but maintained the ability to secrete another type III-secreted protein, HrpZ. This pHIR11 derivative was also greatly reduced in its ability to elicit an HR, indicating that the ability to translocate HopPsyA into plant cells was compromised. Using affinity chromatography, we showed that ShcA binds directly to HopPsyA and that the ShcA binding site must reside within the first 166 amino acids of HopPsyA. Thus, ShcA represents the first demonstrated chaperone used in a type III secretion system of a bacterial plant pathogen. We searched known *P. syringae* type III-related genes for neighbouring ORFs that shared the general characteristics of type III chaperones and identified five additional candidate type III chaperones. Therefore, it is likely that chaperones are as prevalent in bacterial plant pathogen type III systems as they are in their animal pathogenic counterparts.**

Accepted 14 March, 2002. *For correspondence at the University of Nebraska. E-mail jalfano2@unl.edu; Tel. (+1) 402 472 0395; Fax (+1) 402 472 3139.

Introduction

Type III protein secretion systems are present in many Gram-negative pathogens of both plants and animals (Hueck, 1998; Galán and Collmer, 1999; Cornelis and van Gijsegem, 2000). These secretion systems are particularly noteworthy because they can translocate effector proteins directly into eukaryotic cells (Cornelis and Wolf-Watz, 1997). In bacterial plant pathogens belonging to the genera *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas*, type III systems (also referred to as Hrp systems) are encoded by *hrp/hrc* genes (Lindgren, 1997; He, 1998). Hrp systems were originally discovered because phytopathogens require them for elicitation of the hypersensitive response (HR) on non-host plants and pathogenesis on host plants (Alfano and Collmer, 1996). The HR is a programmed cell death of plant cells associated with plant defence (Klement, 1982). The effectors secreted by Hrp systems have been referred to as Hop proteins (for Hrp-dependent outer protein) (Alfano and Collmer, 1997). A subset of the Hops was identified because they were recognized by the resistance (*R*) gene-based surveillance systems of plants and have historically been referred to as avirulence (Avr) proteins. Indirect evidence supports the view that at least a subset of Avr proteins are translocated into plant cells by the type III system, where they initiate an HR (Alfano and Collmer, 1997; Kjemtrup *et al*., 2000). Recently, several Avr proteins have been shown to be secreted in culture by the type III secretion systems of plant pathogens (Ham *et al*., 1998; van Dijk *et al*., 1999; Mudgett and Staskawicz, 1999; Rossier *et al*., 1999).

Pseudomonas syringae pathovars cause diseases on many different agricultural crops, which produce a wide

variety of symptoms including foliar spots, specks and blights. *P. syringae* interactions with plants are specific: certain pathovars can only infect certain plant species. Much of the specificity seen in these interactions results from the presence of *avr* genes and the gene-for-gene interactions that they have with plant *R* genes (Keen, 1990; Dangl, 1994). Relatively recently, the pathogenicity island (Pai) that encodes the Hrp system of *P. syringae* strains has been reported (Alfano *et al*., 2000). The Hrp Pai has a tripartite structure: the centre of it carries the conserved *hrp/hrc* gene cluster that encodes the main components of the secretion apparatus; one flank contains another conserved region termed the conserved effector locus (CEL) because it encodes several secreted products; and the other flank carries a variable region designated the exchangeable effector locus (EEL) because it is dissimilar even between closely related strains of *P. syringae* and appears to carry *hop* genes and *avr* genes.

The EEL of *P. s. syringae* 61 carries only two discernible open reading frames (ORFs): ORF1 and *hopPsyA* (formerly known as *hrmA*) (Heu and Hutcheson, 1993; Alfano *et al*., 1997). The HopPsyA protein is secreted in culture by *P. syringae* and, when expressed transiently in tobacco, it elicits an HR, indicating that its site of action is inside plant cells (Alfano *et al*., 1997; van Dijk *et al*., 1999; Collmer *et al*., 2000). The predicted product of ORF1 shares several of the general characteristics of chaperones used in the type III systems of animal pathogens (Wattiau *et al*., 1996; Cornelis *et al*., 1998).

Type III chaperones have been identified in several animal pathogens (Ménard *et al*., 1994; Day and Plano, 1998; Fu and Galán, 1998; Wainwright and Kaper, 1998; Page *et al*., 2001) and, other than between homologues, they share little amino acid sequence similarity with each other. However, they do share several general structural characteristics such as a small size, an acidic isoelectric point and predicted α-helical secondary structure (Wattiau *et al*., 1996; Bennett and Hughes, 2000; Plano *et al*., 2001). Chaperones have been shown to prevent premature aggregation of effectors in the bacterial cytoplasm and/or to prevent cytoplasmic proteolysis of effectors (Ménard *et al*., 1994; Wattiau *et al*., 1994). One of the better defined chaperone/effector pairs is SycE/YopE from *Yersinia* spp. (Wattiau and Cornelis, 1993). SycE has been shown to bind directly to amino acids 15–50 of the YopE effector (Schesser *et al*., 1996; Woestyn *et al*., 1996), stabilizing YopE in the bacterial cytoplasm (Frithz-Lindsten *et al*., 1995; Cheng *et al*., 1997) and apparently giving YopE a competitive advantage over other effectors for access to the type III apparatus (Boyd *et al*., 2000). The recent determination of the crystal structure of the *Salmonella* SicP/SptP chaperone/effector pair indicates that the SptP effector is partially unfolded when the SicP chaperone is bound to it, which may indicate that SicP helps to maintain SptP in a secretion-competent state (Stebbins and Galán, 2001).

To date, the presence of type III chaperones in plant pathogens has not been demonstrated, and a recent review noted that the presence of customized chaperones in animal pathogen type III systems may illustrate a substantial difference between animal and plant pathogen type III systems (Staskawicz *et al*., 2001). However, it has been reported that ORF1 upstream of *hopPsyA* (Alfano *et al*., 1997) and DspB resemble type III chaperones (Gaudriault *et al*., 1997). Moreover, DspB is required for the type III secretion of DspA from *Erwinia amylovora* (Gaudriault *et al*., 1997), consistent with DspB acting as a type III chaperone. We describe here the identification and characterization of ShcA and demonstrate that it is a type III chaperone for the *P. syringae* effector HopPsyA.

Results

ORF1 has chaperone-like characteristics

The predicted amino acid sequence of the ORF preceding *hopPsyA*, ORF1, in the EEL of *P. s. syringae* 61 shares general characteristics with chaperones used in

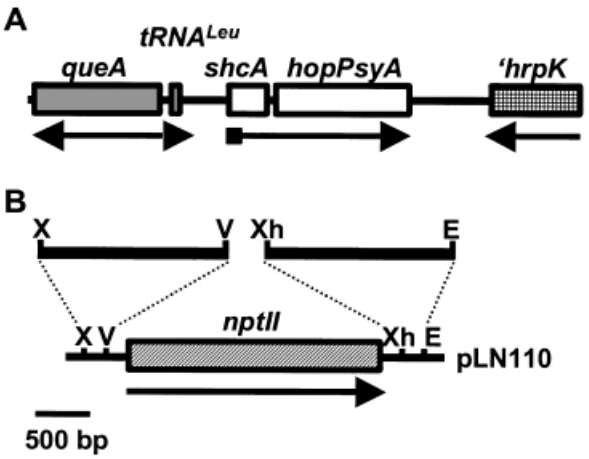

Fig. 1. Organization of the *shcA* and *hopPsyA* operon of *P. s. syringae* 61.
A. The *shcA* ORF and *hopPsyA* are depicted as white boxes. At the border of the Hrp Pai are the *tRNA*^{Leu} and *queA* genes depicted as grey boxes. A 5′-truncated *hrpK* gene is represented as a hatched box. The arrows indicate the predicted direction of transcription, and the black box denotes the presence of a putative HrpL-dependent promoter upstream of the *shcA* ORF.
B. Construction of the deletion mutation in the *shcA* ORF marker exchanged into *P. s. syringae* 61. Black bars depict regions that were amplified along with added restriction enzyme sites, and each is aligned with the corresponding DNA region represented in (A). The striped box depicts the *nptII* cassette that lacks transcriptional and translational terminators used in making the functionally nonpolar *shcA P. s. syringae* 61 mutant. *Eco*RI, E; *Eco*RV, V; *Xba*I, X; and *Xho*I, Xh.

type III protein secretion systems of animal pathogens (Wattiau *et al*., 1996). The predicted product of ORF1 is relatively small (12.6 kDa), has an acidic pI (4.8) and a predicted amphipathic region in its C-terminal region, all characteristics consistent with it encoding a type III chaperone. The proximity of ORF1 to *hopPsyA* and the presence of a Hrp promoter immediately upstream of both *hopPsyA* and ORF1 indicates that they are probably part of the same transcriptional unit. The organization of the predicted operon containing ORF1 and *hopPsyA* is shown in Fig. 1A. To test whether ORF1 encodes a protein product, two ORF1-FLAG constructs were made, pLN84 and pLN100. Translation of ORF1 carried on pLN84 was dependent on a vector ribosome binding site (RBS), and translation of ORF1 carried on pLN100 was dependent on the native RBS upstream of ORF1 in the *P. s. syringae* 61 chromosome. *Escherichia coli* strains expressing either construct produced an ORF1-FLAG protein detected by anti-FLAG antibodies, indicating that the native RBS was functional and that ORF1 does encode a protein (data not shown). The product of ORF1 will hereafter be referred to as ShcA (for specific Hop chaperone).

A P. s. syringae *61* shcA *mutant fails to secrete the HopPsyA effector*

Because ShcA shares general similarities with type III chaperones, we tested whether a loss-of-function mutation in *shcA* would alter the stability and the type III secretion of HopPsyA. A functionally non-polar deletion mutation was constructed in the *shcA* coding region and transferred to the chromosome of *P. s. syringae* 61 (see Fig. 1B and *Experimental procedures*). The resulting mutant was designated UNL131. *P. s. syringae* 61 cultures were grown in Hrp-inducing fructose medium at 22°C and separated into cell-bound and supernatant fractions. HopPsyA was found in the supernatant fraction of the wild-type *P. s. syringae* 61 as reported previously (van Dijk *et al*., 1999), but could not be detected in the supernatant fraction from the *shcA* mutant, UNL131, consistent with ShcA acting as a HopPsyA chaperone (Fig. 2). The mutation carried by UNL131 is non-polar, as demonstrated by the presence of HopPsyA in the cell-bound fraction of UNL131. The ability of UNL131 to secrete HopPsyA was restored by supplying *shcA in trans* on pLN106 (Fig. 2). The secretion of HopPsyA by UNL131 was also complemented by pLN92, which carries both *shcA* and *hopPsyA*, and pLN1, which only carries *hopPsyA*. Thus, whatever the role of ShcA, the production of more HopPsyA inside the bacterial cell can apparently compensate for ShcA's absence. However, when *shcA* and *hopPsyA* are both supplied *in trans* on construct pLN92, more HopPsyA is detected in the supernatant fraction than when *hopPsyA* is supplied alone (Fig. 2). There are several examples of mutations in genes predicted to encode type III chaperones that dramatically affect the stability of their cognate effector (Wattiau and Cornelis, 1993; Fu and Galán, 1998; Niebuhr *et al*., 2000). However, the *P. s. syringae shcA* mutant UNL131 did not dramatically alter the stability of HopPsyA based on the presence of HopPsyA in the cell-bound fraction of

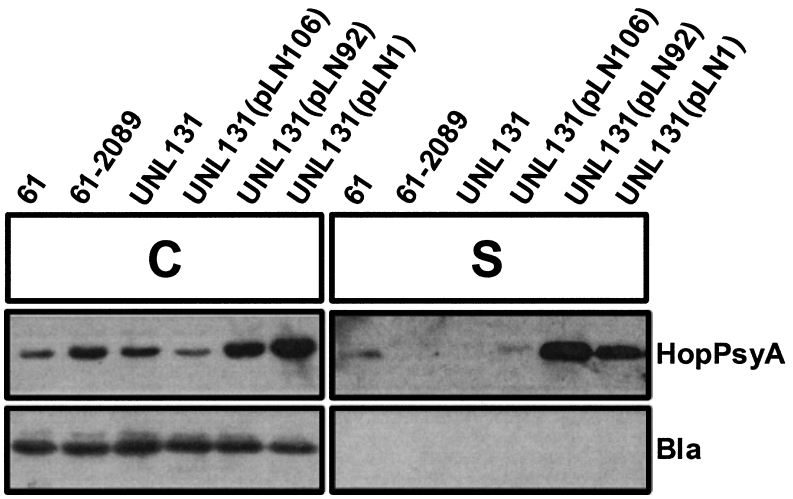

Fig. 2. A *P. s. syringae* 61 *shcA* mutant UNL131 does not secrete HopPsyA, and *shcA* provided *in trans* complements this defect. *P. s. syringae* 61 cultures were grown at 22°C in *hrp*-inducing medium and separated into cell-bound (C) and supernatant fractions (S). The cell-bound fractions were concentrated 13.3-fold, and the supernatant fractions were concentrated 100-fold relative to the initial culture volumes. The samples were subjected to SDS–PAGE and immunoblot analysis, and HopPsyA and β-lactamase (Bla) were detected with either anti-HopPsyA or anti-β-lactamase antibodies followed by secondary antibodies conjugated to alkaline phosphatase as described in *Experimental procedures*. The image of the immunoblot was captured using a Hewlett-Packard 6200C ScanJet scanner with the accompanying software. For figure construction, the image was manipulated with Microsoft POWERPOINT 2000 to meet the publisher's specifications. 61, wild type; 61-2089, type III secretion mutant; UNL131, *shcA* mutant; pLN106, construct carrying *shcA*; pLN92, construct carrying *shcA* and *hopPsyA*; pLN1, construct carrying *hopPsyA*. All the bacterial strains carried pCPP2318, which encodes the mature β-lactamase.

UNL131 (Fig. 2). Moreover, pulse–chase experiments comparing HopPsyA stability in the *shcA* mutant UNL131 and wild-type *P. s. syringae* 61 were unable to detect any decrease in HopPsyA stability in the absence of ShcA (data not shown).

A functional type III secretion system encoded by cosmid pHIR11 requires ShcA for HopPsyA secretion but not for the type III secretion of HrpZ

Cosmid pHIR11 carries a functional set of type III genes from *P. s. syringae* 61 and *shcA* and *hopPsyA*, which collectively confer upon non-plant pathogens such as *P. fluorescens* the ability to secrete HopPsyA in culture and to elicit an HR on tobacco (van Dijk *et al*., 1999; Collmer *et al*., 2000). To investigate ShcA's role in HopPsyA secretion further, we marker exchanged a non-polar *shcA* mutation into pHIR11 constructing pLN85. *P. fluorescens* cultures grown in Hrp-inducing conditions were separated into cell-bound and supernatant fractions. *P. fluorescens*(pLN85) was unable to secrete detectable amounts of HopPsyA into the supernatant fraction, indicating that, like the *P. s. syringae shcA* mutant, bacteria carrying a pHIR11 derivative lacking *shcA* were unable to secrete HopPsyA in culture (Fig. 3). *P. fluorescens*(pLN85) also carrying pLN105, which contains *shcA*, regained the ability to secrete HopPsyA to the supernatant fraction. Interestingly, the type III secretion defect possessed by *P. fluorescens*(pLN85) was not a general defect in type III secretion because this strain was still capable of secreting HrpZ, another protein that travels the type III pathway (Fig. 3). Moreover, *P. fluorescens*(pLN85) was capable of secreting the Avr protein AvrPto in culture, further supporting the view that the *shcA* mutation specifically affected HopPsyA secretion (data not shown).

ShcA affects the translocation of HopPsyA into plant cells

Bacteria carrying pHIR11 require *hopPsyA* for HR elicitation on tobacco, and HopPsyA expressed transiently in tobacco cells is sufficient to elicit an HR, indicating that HopPsyA is translocated to the interior of tobacco cells where it is recognized by the plants defence system, resulting in the triggering of defences including the HR (Alfano *et al*., 1996; 1997; Collmer *et al*., 2000). To determine whether *shcA* was required for the translocation of HopPsyA inside plant cells, we tested the ability of *P. fluorescens*(pLN85) to elicit a HopPsyA-dependent HR on tobacco (*Nicotiana tabacum* cv. Xanthi). *P. fluorescens*(pLN85) was often completely unable to elicit an HR, producing a similar response to bacteria carrying pHIR11 derivatives that encode disabled type III secretion systems. This suggests that ShcA is required for the translocation of HopPsyA into plant cells. However, occasionally, a greatly reduced HR was visible on tobacco that was infiltrated with *P. fluorescens*(pLN85), indicating that ShcA dramatically affected the translocation of HopPsyA into plant cells, but apparently some HopPsyA can still be translocated into plant cells from bacterial cells lacking ShcA (Fig. 4). The reduced HR phenotype produced by *P. fluorescens*(pLN85) was complemented when *shcA* was supplied *in trans* (Fig. 4). The fact that we were unable to detect any HopPsyA in supernatant fractions in type III secretion assays using strains lacking ShcA (Figs 2 and 3), but were able variably to detect a weak HopPsyA-dependent HR in plant experi-

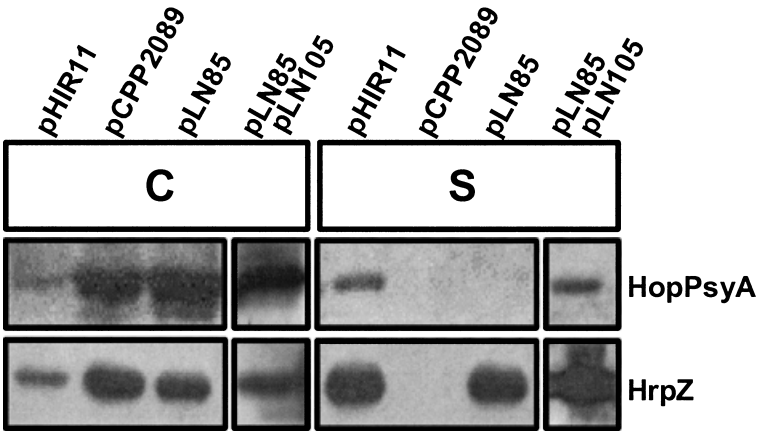

Fig. 3. *shcA* is required for the type III secretion of HopPsyA, but not HrpZ secretion. *P. fluorescens* 55 cultures were grown in *hrp*-inducing medium and separated into cell-bound (C) and supernatant (S) fractions. The cell-bound fractions were concentrated 13.4-fold, and the supernatant fractions were concentrated 133-fold relative to the initial culture volumes. The samples were subjected to SDS–PAGE and immunoblot analysis, and HopPsyA and HrpZ were detected with either anti-HopPsyA or anti-HrpZ antibodies followed by secondary antibodies conjugated to alkaline phosphatase as described in *Experimental procedures*. The image of the immunoblot was captured using a Hewlett-Packard 6200C ScanJet scanner with the accompanying software. For figure construction, the image was manipulated with Microsoft POWERPOINT 2000 to meet the publisher's specifications. pHIR11, wild-type Hrp system; pCPP2089, encodes a defective Hrp system; pLN85, pHIR11 derivative with *shcA* mutation; pLN105, broad-host-range plasmid carrying *shcA*.

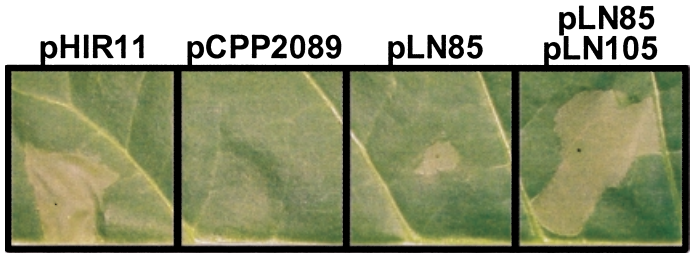

Fig. 4. Tobacco leaves showing that *P. fluorescens* 55 carrying a pHIR11 derivative with a functionally non-polar *shcA* mutation is impaired in its ability to translocate HopPsyA into plant cells. *P. fluorescens* 55 cultures were grown overnight in King's Broth, suspended in 5 mM MES, pH 5.6, to an OD_{600} of 1.0 and infiltrated into tobacco leaf panels. Because the pHIR11-induced HR results from the translocation of HopPsyA inside plant cells, a reduced HR indicates that HopPsyA is not delivered well enough to induce a confluent HR. The leaf panels were photographed with incident light 24 h later. pHIR11, HR^+ wild-type Hrp system; pCPP2089, HR^- defective Hrp system; pLN85, pHIR11 derivative with *shcA* mutation; pLN105, broad-host-range plasmid carrying *shcA*.

ments, indicates that the translocation assay appears to be more sensitive than our secretion assays in detecting HopPsyA.

ShcA interacts with HopPsyA

One criterion that any protein suspected of being a chaperone must fulfil is that it must bind to the protein that it is predicted to chaperone. To determine whether ShcA binds to HopPsyA *in vitro*, we made constructs using polymerase chain reaction (PCR) that contained either *hopPsyA* alone (pLN1) or both *hopPsyA* and *shcA-flag* (pLN2). These plasmids were electroporated into the *P. s. syringae shcA* mutant UNL131, and strains containing both constructs were grown separately in King's B broth medium to an optical density of 1.0 (see *Experimental procedures*). The cells were sonicated, and the soluble proteins were incubated with anti-FLAG affinity gel that binds to the FLAG epitope. The sonicate, wash and gel fractions were separated on SDS–PAGE gels and analysed with immunoblots using antibodies that recognize either HopPsyA or ShcA-FLAG. HopPsyA was only detected in the gel fraction in the presence of ShcA-FLAG, indicating that HopPsyA binds to ShcA, which is consistent with ShcA acting as a molecular chaperone for HopPsyA (Fig. 5). It is important to note that the wash fractions presented in Fig. 5 represent the last wash of the gel samples before eluting bound proteins from the gel. Thus, the gels were washed sufficiently such that the detected HopPsyA was interacting specifically with ShcA-FLAG bound to the anti-FLAG affinity gel. The absence of HopPsyA in the gel fraction from the sample that lacked ShcA-FLAG further supports the fact that HopPsyA was binding specifically to ShcA-FLAG (Fig. 5).

ShcA interacts with the N-terminal 166 amino acids of HopPsyA

To determine what portion of HopPsyA interacts with ShcA, we carried out similar experiments to those described above (Fig. 5). We PCR cloned fragments of *hopPsyA* that corresponded to the N-terminal 166 amino acids, C-terminal 276 amino acids and the C-terminal 256 amino acids of HopPsyA into a pML123 derivative containing *shcA-flag*, constructing pLN67, pLN68 and pLN69 respectively. These constructs were electroporated into the *P. s. syringae* 61 *shcA* mutant UNL131 and grown as described above. Soluble protein samples were isolated from sonicated cultures and mixed with anti-FLAG affinity gel. After several washes, the gel fractions were loaded onto SDS–PAGE gels and analysed with immunoblots with antibodies that recognized either HopPsyA or ShcA-FLAG. Only the cultures carrying pLN67 produced a

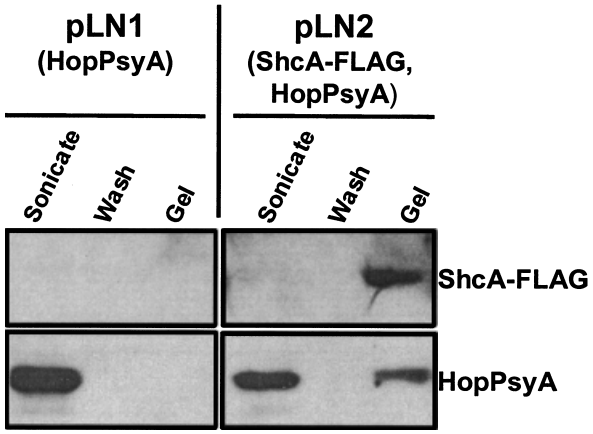

Fig. 5. Immunoblot showing that ShcA binds to HopPsyA. Soluble protein samples from sonicated cultures of *P. s. syringae* 61 *shcA* mutant UNL131 carrying pLN1 (HopPsyA) or pLN2 (ShcA-FLAG, HopPsyA) were mixed with anti-FLAG M2 affinity gel. The gel was washed with TBS buffer, mixed with SDS–PAGE buffer and, along with the sonicate and wash samples, subjected to SDS–PAGE and immunoblot analysis. HopPsyA and ShcA-FLAG were detected with anti-HopPsyA or anti-FLAG antibodies followed by secondary antibodies conjugated to alkaline phosphatase as described in *Experimental procedures*. The immunoblot image was scanned in to generate a digital image and imported into Microsoft POWERPOINT 2000 to construct the figure.

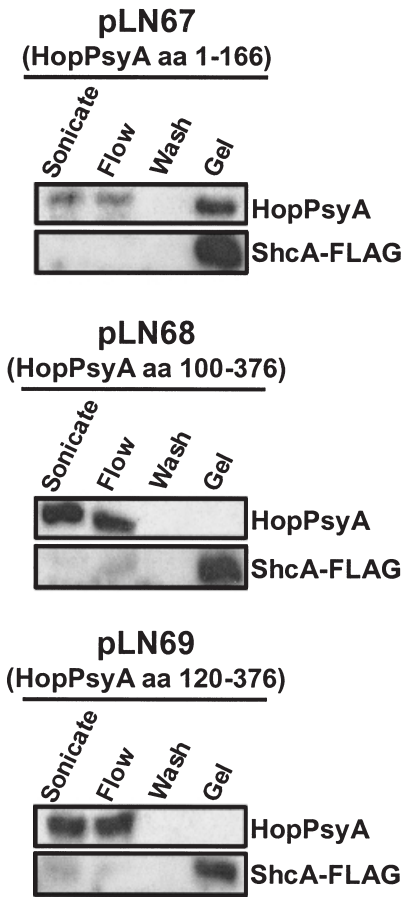

Fig. 6. Immunoblot showing that ShcA binds within the N-terminal 166 amino acids of HopPsyA. Soluble protein samples from sonicated cultures of UNL131 carrying pLN67, pLN68 and pLN69 were mixed with anti-FLAG M2 affinity gel. The gel was washed with TBS buffer, mixed with SDS–PAGE buffer and subjected to SDS–PAGE and immunoblot analysis. Proteins not binding to the gel were represented in a flowthrough fraction. pLN67, pLN68 and pLN69 contain *shcA-flag* and fragments of *hopPsyA*, which correspond to the N-terminal 166 amino acids (pLN67), the C-terminal 276 amino acids (pLN68) or the C-terminal 256 amino acids (pLN69) of the 376-amino-acid HopPsyA protein. HopPsyA and ShcA-FLAG were detected with anti-HopPsyA or anti-FLAG antibodies followed by secondary antibodies conjugated to alkaline phosphatase as described in *Experimental procedures*. The immunoblot image was scanned in to generate a digital image and imported into Microsoft POWERPOINT 2000 to construct the figure.

HopPsyA fragment that interacted with ShcA-FLAG, indicating that the ShcA binding site of HopPsyA resides in the N-terminal 166 amino acids (Fig. 6). The constructs pLN68 and pLN69 produced the C-terminal 276 and 256 amino acids of the 376-amino-acid HopPsyA protein respectively. Because both these truncated proteins failed to bind in detectable amounts to the ShcA-FLAG-containing affinity gel, the ShcA binding site probably resides within the first 100 amino acids of HopPsyA (Fig. 6).

Other putative type III chaperones associated with characterized type III-related P. syringae *genes*

To investigate the prevalence of chaperones in *P. syringae* type III systems, we searched nucleotide sequences adjacent to known *avr* genes and type III-related genes for neighbouring ORFs whose predicted products share the general characteristics of type III chaperones. We limited our search to nucleotide sequences from any published *P. syringae avr* gene and genes within the *P. syringae* Hrp pathogenicity island likely to encode effectors (Alfano *et al.*, 2000). DNA sequences for all the deposited *avr* and *hrp*-related sequences of *P. syringae* were retrieved from GenBank and analysed with DNASTAR software for ORFs adjacent to putative effector genes, a common characteristic of type III chaperone genes. In some cases, there was not enough adjacent DNA to search effectively for ORFs. However, we were able to identify five ORFs neighbouring different *avr* or putative effector genes that represent good candidate chaperone genes. These candidate chaperone/effector pairs are listed in Table 1 along with salient features of the predicted chaperones. Therefore, it is likely that other type III chaperones exist in *P. syringae* and that plant pathogenic type III systems are similarly reliant on chaperones for the secretion of specific effectors as their animal pathogenic counterparts.

Discussion

Here, we report the characterization of the *shcA* gene of *P. s. syringae* 61 and its involvement in the secretion of the HopPsyA effector from the *P. s. syringae* type III

Table 1. Putative chaperones associated with characterized *P. syringae avr* and type III-related genes.

Chaperone or ORF name	Size (kDa)	pI	Effector or ORF name	*P. syringae* pathovar and strain	Accession number	Reference
EEL ORF6	13.2	6.3	EEL ORF5 (AvrBsT)	*syringae* B728a	AF232005	Alfano *et al.* (2000)
CEL ORF2	14.6	5.3	AvrE	*tomato* DC3000	AF232006	Alfano *et al.* (2000)
CEL ORF4	18.0	5.3	CEL ORF3	*tomato* DC3000	AF232006	Alfano *et al.* (2000)
CEL ORF8	19.9	6.8	CEL ORF7	*tomato* DC3000	AF232006	Alfano *et al.* (2000)
AvrPphF ORF1	15.6	6.1	AvrPphF ORF2	*phaseolicola* 1375A	AF231452	Tsiamis *et al.* (2000)

system. We found that *P. s. syringae* mutants and pHIR11 derivatives lacking ShcA are defective in the ability to secrete HopPsyA via the type III system (Figs 2 and 3). Interestingly, this defect appears specifically to affect the secretion of HopPsyA, because a derivative of pHIR11 with a *shcA* mutation maintained the ability to secrete HrpZ, another type III-secreted protein (Fig. 3). *shcA* mutants maintained the ability to translocate other Avr proteins into plant cells (data not shown); however, we cannot exclude the possibility that ShcA is required for the secretion of other *P. syringae* effectors. These results are consistent with ShcA acting as a type III chaperone for HopPsyA. The acid test to determine whether a protein is acting as a type III chaperone has been to determine whether the protein binds specifically to the effector it is suspected of chaperoning. Indeed, using affinity chromatography, we showed that ShcA interacts *in vitro* with HopPsyA and that the ShcA-binding domain must reside within the N-terminal 166 amino acids of HopPsyA (Figs 5 and 6). Based on the criteria used for defining type III chaperones in animal pathogenic type III systems, ShcA does appear to be a molecular chaperone for HopPsyA and represents the first demonstrated type III chaperone used in a type III secretion system of a bacterial plant pathogen.

Type III chaperones often increase the stability of the effector that they interact with (Wattiau and Cornelis, 1993; Fu and Galán, 1998; Iriarte and Cornelis, 1998), but there are several examples where effector stability is not affected by the chaperone (Ménard *et al*., 1994; Woestyn *et al*., 1996). Based on our findings, ShcA does not affect the stability of HopPsyA (Fig. 2). The significance of why some effectors need chaperones to maintain their stability is presently not understood. It is possible that the chaperones that stabilize their cognate effector perform a different role in the bacterial cell than chaperones that do not.

One model that may explain why certain effectors need chaperones for their secretion, while others do not, is that a chaperone may help its cognate effector to compete against other type III traffic for entry into the type III pathway (Boyd *et al*., 2000). Our result showing that overexpression of HopPsyA compensates for the absence of ShcA in secretion assays by restoring HopPsyA secretion is consistent with such a model because additional HopPsyA molecules would increase the likelihood that a fraction of the HopPsyA pool would assume a secretion-competent state in the absence of its chaperone. Also consistent with this model is the finding that less HopPsyA remains in the cell fraction in secretion experiments using *P. fluorescens* carrying pHIR11 than when *P. s. syringae* strains are tested (van Dijk *et al*., 1999). The former encodes the effectors HopPsyA (Alfano *et al*., 1997) and HrpK (K. van Dijk and J. R. Alfano, unpublished), whereas the latter carries the full suite of effectors present in that strain of *P. syringae*.

Our search for type III chaperone-like genes adjacent to known *avr* or candidate effector genes in *P. syringae* identified five ORFs in *P. syringae* that may encode type III chaperones (Table 1). There are several additional points worth noting that increase the likelihood that these candidate chaperone genes encode bona fide chaperones. First, the EEL ORF6 present in *P. s. syringae* B728a is upstream of EEL ORF5, which is a homologue of AvrBsT and YopJ/P (Hardt and Galán, 1997; Ciesiolka *et al*., 1999). Although this does not mandate that EEL ORF6 encodes a chaperone, the predicted product of EEL ORF6 does contain a leucine-rich repeat similar to the type found in SycE and SycH, the chaperones for the *Yersinia* spp. effectors YopE and YopH respectively (Cornelis *et al*., 1998). Moreover, adjacent to an AvrBsT homologue in *E. amylovora* (NCBI accession no. AAF63399) is an ORF that shares high sequence identity (55.4%) with EEL ORF6. Interestingly, chaperones have not been implicated in the secretion of AvrBsT or other YopJ/P homologues (Ciesiolka *et al*., 1999; Cornelis and Van Gijsegem, 2000). Secondly, the predicted product of CEL ORF2 shares amino acid sequence identity (42.1%) with DspB/F, a protein required for the type III secretion of DspA/E, an AvrE homologue present in *E. amylovora* (Gaudriault *et al*., 1997). Thirdly, CEL ORF4 and ORF8 are both adjacent to genes that encode proteins predicted to be type III effectors, and CEL ORF3 was recently shown to be a type III-secreted protein (A. Collmer, personal communication). Lastly, the AvrPphF Avr activity from *P. s. phaseolicola* has been shown to be dependent on two adjacent ORFs, designated ORF1 and ORF2 (Tsiamis *et al*., 2000). Because ORF1's predicted product shares several of the characteristics associated with chaperones, it is likely that ORF1 encodes a chaperone, thus making ORF2 a better candidate for the AvrPphF effector. It is important to note that our chaperone search was limited by the fact that many *avr* gene nucleotide sequences present in the databases do not contain enough adjacent sequence to determine whether a chaperone gene was located nearby. Nevertheless, we did identify candidate chaperone genes. Thus, it is likely that chaperones are as common in bacterial plant pathogen type III systems as they are in animal pathogens.

We feel that identifying *P. syringae* chaperone/effector pairs will be important in dissecting the molecular basis of plant pathogenesis mediated by type III secretion. Recent research on other type III secretion systems suggests that there exists a hierarchy for effector secretion. For example, the flagellar biogenesis system clearly shows that proteins are secreted in a temporal manner from this archetypal type III system for proper assembly of flagel-

lar substructures, and type III chaperones appear important for this process (Fraser *et al*., 1999; Minamino and Macnab, 1999). Moreover, *Yersinia* researchers have provided evidence suggesting that effectors required early in pathogenesis may require chaperones to compete for access to the type III secretion apparatus against effectors that are used later (Boyd *et al*., 2000; Lloyd *et al*., 2001). If this hypothesis proves correct, then identifying chaperone/effector pairs in *P. syringae* may help to identify a subgroup of effectors that is used early during plant pathogenesis. Alternatively, the need for a chaperone may reflect the instability of a specific effector or the tendency of effectors to aggregate in the absence of their chaperones. With the ongoing genome sequencing project on *P. s. tomato* DC3000 nearing completion, continuing to search for ORFs that share general characteristics with chaperones may help in the identification of as yet unidentified effector genes in the genome of *P.* s*. tomato* DC3000.

We are continuing our characterization of the ShcA chaperone by delineating the ShcA binding site on HopPsyA more specifically, determining whether ShcA has a regulatory role as noted recently for *Salmonella* and *Yersinia* type III chaperones (Darwin and Miller, 2000; Tucker and Galán, 2000; Francis *et al*., 2001) and integrating the chaperone-binding domain of HopPsyA with other secretion signals required for HopPsyA secretion. Our long-term goal is to understand the secretion signals that successfully target HopPsyA to the type III secretion system and to determine how the ShcA chaperone facilitates this process.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 2. *E. coli* strains were grown routinely in LM (Hanahan, 1983) or Terrific broth (Sambrook *et al*., 1989) at 37°C. *P. s. syringae* 61 and *P. fluorescens* 55 were grown routinely in King's B broth at 30°C (King *et al*., 1954). For type III secretion assays, *P. fluorescens* 55 and *P. s. syringae* 61 were grown in Hrp-inducing fructose minimal medium described by Huynh *et al*. (1989) at 22°C. Antibiotics were used at the following concentrations ($\mu g\,ml^{-1}$): ampicillin, 100; gentamicin, 10; kanamycin, 50; spectinomycin, 50; and tetracycline, 20.

Plant materials and HR assay

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) were grown in a greenhouse with a photoperiod of 16 h. Infiltration of tobacco leaves with *P. fluorescens* carrying pHIR11 derivatives was done by resuspending cultures grown on King's B plates in 5 mM MES (pH 5.6) at an OD_{600} of 1.0 and infiltration into leaf punctures with needleless syringes. HRs were evaluated after 24 h.

General DNA manipulations and plasmid constructions

Restriction enzymes, T4 ligase and DNA polymerase were purchased from either New England Biolabs or Life Technologies. The thermostable DNA polymerase used in PCRs was *Pfu* polymerase (Stratagene). The cycling parameters used for PCRs were: 1 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 55°C and 3 min at 72°C; and 10 min at 72°C. Standard molecular biological techniques were used according to well-described protocols (Sambrook *et al*., 1989).

The plasmid constructs that produce ShcA fused to the FLAG epitope were made in the following way. *shcA* was PCR amplified from pHIR11 using the primers 5′-ATGAAA GCTTCCCGCCTTGGCGTTTGAC-3′ and 5′-ATGAAGATCT CGTGCGGGCTTCTCGCCA-3′, which have *Hin*dIII and *Bgl*II restriction sites, respectively, and subcloned into the *Hin*dIII and *Bgl*II sites of pFLAG-CTC resulting in pLN84. The construct that contains the native *shcA* ribosome binding site (RBS), pLN100, was made by PCR amplifying *shcA* from pHIR11 with 5′-ATGAAAGCTTGGCGAACCGATTATGAGT-3′, which anneals upstream of the *shcA* RBS and contains a *Hin*dIII site, and 5′-CGCCTCTAGATTATTTGTATAGTTCATC CATGCCATG-3′, which contains the eight codons that encode the FLAG epitope and a *Xho*I site, and cloning it into the corresponding sites in pBluescript-II SK+. For complementation experiments that needed the native *shcA* gene, the *shcA* coding region along with its RBS was PCR amplified with 5′-ATGAAAGCTTGGCGAACCGATTATGAGT-3′, which contains a *Hin*dIII site, and 5′-ATGACCCGGGTCACGTG CGGGCTTCTCG-3′, which contains a *Sma*I site, and subcloned into two different broad-host-range vectors, pBBR1MCS-5 and pMB393, resulting in pLN105 and pLN106 respectively.

pLN1 was made by PCR amplifying *hopPsyA* with the primers 5′-ATATAAGCTTAGGAGCTTTTAATGAACCCT-3′, which contains the RBS of *hopPsyA* and a *Hin*dIII site, and 5′-CGCCTCTAGATTATTTGTATAGTTCATCCATGCCATG-3′, which contains an *Xba*I site, and subcloning the product into the corresponding sites in pML123. The *shcA*-FLAG DNA fragment from pLN100 was isolated by cutting with *Xho*I, filling in the 3′ recessed end and cutting with *Bam*HI. This fragment was cloned into pLN1 and pML123, both of which were previously digested with *Hin*dIII, filled in and digested with *Bam*HI resulting in constructs pLN2 and pLN3 respectively. Plasmids pLN67, pLN68 and pLN69, which contain *shcA-flag* and a truncated *hopPsyA* fragment, were made by cloning the appropriate amplified PCR product into pLN3 that was digested with *Xba*I and *Sac*I. pLN67 was made by PCR amplifying the 5′ 498 nucleotides of *hopPsyA* with 5′-CGTGTCTAGAAGGAGCTTTTAATGAACC-3′, which contains a *Xba*I site and RBS, and 5′-ATTGGAGCTCTCATCTG CCGCATTCATAGGC-3′, which contains a *Sac*I site and a stop codon; pLN68 was made by PCR amplifying nucleotides 300–1128 of *hopPsyA* with 5′-GCTGTCTAGAAGGAGCTTTT AATGGATCTGGAGAAGGGCGGA-3′, which contains an *Xba*I site, an RBS and a start codon, and 5′-ATATG AGCTCTCAGTTTCGCGCCCTGAG-3′, which contains a *Sac*I site; and pLN69 was made using the PCR primers 5′-GCTGTCTAGAAGGAGCTTTTAATGACATCAAAACAGAC ATTT-3′, which contains an *Xba*I site, an RBS and a start codon, and the same reverse primer as used for construct pLN68.

Table 2. Strains and plasmids.

Designation	Characteristics	Reference or source
Strain		
E. coli		
DH5α	*supE*44 Δ*lacU*169(ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1, Nal^R	Hanahan (1983); Life Technologies
DH5αF′ *lacI*^{q}	F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 *zzf*::Tn*5* *supE*44 Δ*lacU*169(ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1, Nal^R	Life Technologies
MC4100	F^- Δ(*lac*)169 *araD*136 *relA rpsL thi*, Str^R	Oliver and Beckwith (1981)
C2110	$PolA^{ts}$ Nal^R	Kahn and Hanawalt (1979)
HB101	F^- Δ(*gpt-proA*)62 *leuB*6 *supE*44 *ara*-14 *galK*2 *lacY*1 Δ(*mcrC-mrr*) *rpsL*20 (Str^R) *xyl*-5 *mtl*-1 *recA*13	ATCC
P. fluorescens 55	Nal^R	Huang *et al*. (1988)
P. s. syringae		
61	Wild type; spontaneous Nal^R	Huang *et al*. (1988)
61–2089	*hrcC*::Tn*phoA*	Huang *et al*. (1991)
UNL131	*P. s. syringae* 61 mutant containing a non-polar *nptII* cassette in *shcA*	This work
Plasmids		
pBluescript-II KS–	Cloning vector, Ap^R	Stratagene
pBluescript-II SK+	Cloning vector, Ap^R	Stratagene
pBBR1MCS-5	Broad-host-range cloning vector, Gm^R	Kovach *et al*. (1995)
pCPP2089	pHIR11 derivative containing Tn*phoA* insert into *hrcC*, Tc^R Km^R	Huang *et al*. (1991)
pCPP2318	pCPP30 derivative carrying *blaM* lacking signal peptide sequences, Tc^R	Charkowski *et al*. (1997)
pCPP2324	pBluescript-II KS^- carrying a 2.4 kb *Bam*HI–*Avr*II *shcA*$^+$, *hopPsyA*$^+$ fragment	Alfano *et al*. (1997)
pCPP2988	pBluescript-II KS^- vector carrying 1.5 kb *Hin*dIII–*Sal*I fragment with *nptII* lacking transcriptional terminator	Alfano *et al*. (1996)
pFLAG-CTC	FLAG expression vector, Ap^R	Sigma Chemical
pHIR11	pLAFR3 derivative carrying *P. s. syringae* 61 *hrp/hrc* cluster, Tc^R	Huang *et al*. (1988)
pLN1	Derivative of pML123 containing a PCR-amplified fragment carrying *hopPsyA*	This work
pLN2	Derivative of pLN1 that contains a PCR-amplified fragment carrying *shcA*-flag gene fusion	This work
pLN67	pML123 construct containing a *shcA*-flag gene fusion and a fragment of *hopPsyA* corresponding to the N-terminal 166 amino acids	This work
pLN68	pML123 construct containing a *shcA*-flag gene fusion and a fragment of *hopPsyA* corresponding to the C-terminal 276 amino acids	This work
pLN69	pML123 construct containing a *shcA*-flag gene fusion and a fragment of *hopPsyA* corresponding to the C-terminal 256 amino acids	This work
pLN82	Derivative of pBluescript-II SK+ carrying a PCR-amplified 5 kb fragment representing DNA upstream of *shcA* on pHIR11	This work
pLN83	Derivative of pCPP2988 containing a 1.5 kb PCR-amplified fragment upstream of *shcA* on pHIR11	This work
pLN84	pFLAG-CTC derivative containing PCR-amplified *shcA* cloned into the *Hin*dIII and *Bgl*II sites	This work
pLN85	pHIR11 derivative that contains a non-polar *shcA* deletion mutation	This work
pLN92	Derivative of pML123 containing the insert in pCPP2324 cut out with and cloned using *Sac*I and *Hin*dIII	This work
pLN100	Derivative of pBluescript-II KS– that carries a PCR-amplified fragment containing a *shcA*-FLAG fusion cloned into the *Hin*dIII and *Xho*I restriction enzyme sites	This work
pLN101	Derivative of pLN83 containing 1.5 kb PCR-amplified fragment downstream of *shcA*	This work
pLN105	Derivative of pBBR1MCS-5 containing a PCR-amplified fragment carrying *shcA* cloned into the *Hin*dIII and *Sma*I restriction enzyme sites	This work
pLN106	Derivative of pMB393 containing a PCR-amplified fragment carrying *shcA* cloned into the *Hin*dIII and *Sma*I restriction enzyme sites	This work
pLN107	Derivative of pCPP2988 containing a PCR-amplified *Xba*I–*Eco*RV fragment representing DNA upstream of *shcA*	This work
pLN108	Derivative of pLN107 containing a 1.5 kb PCR-amplified *Xho*I–*Kpn*I fragment	This work
pLN110	Derivative of pRK415 that contains the *shcA* deletion mutation and upstream and downstream DNA contained in pLN108	This work
pMB393	Stable broad-host-range vector, Sp^R	Gage *et al*. (1996)
pML123	Broad-host-range cloning vector, Gm^R	Labes *et al*. (1990)
pRK415	Broad-host-range vector, unstable in absence of selection, Tc^R	Keen *et al*. (1988)
pRK2013	Tra^+, Km^R	Ruvkin and Ausubel (1981)

Construction of a deletion mutation in shcA *and marker exchange into* P. s. syringae *61*

A schematic diagram of the *P. s. syringae* 61 *shcA* mutation construction is shown in Fig. 1B. To isolate a PCR product upstream of *shcA*, we relied on the sequence of the *queA* gene available in another strain of *P. s. syringae*, B728a, and designed the primer 5′-ATGATCTAGATAGCCGTGCTC GATGGCC-3′. Using this primer, which contains an engineered *Xba*I site along with a primer complementary to the region near the start of *shcA*, 5′-ATGAGATATCGTC AAACGCCAAGGCGGG-3′, which contains an *Eco*RV site, a 1.5 kb fragment representing the DNA upstream of the *shcA* gene was cloned into pCPP2988 upstream of a terminatorless *nptII* cassette resulting in pLN107. The 1.5 kb region immediately downstream of the *shcA* gene was PCR amplified from pHIR11 using the primers 5′-ATGACTCGAGT CAATCTTCCGACGTATT-3′ and 5′-ATGAGGTACCTCATATT GATTGCTCCTG-3′, which contain the restriction sites *Xho*I and *Kpn*I respectively. This product was subcloned into pLN107 using *Xho*I and *Kpn*I constructing pLN108. The DNA insert of pLN108 was subcloned into the *Xba*I and *Kpn*I sites of the broad-host-range plasmid pRK415 constructing pLN110. This construct was electroporated into *P. s. syringae* 61 and plated on King's B plates containing kanamycin and tetracycline. Loss of the plasmid and retention of the *nptII* marker was achieved by growing *P. s. syringae* 61(pLN110) under Km^R selection. Fresh cultures were inoculated daily with a small aliquot of the culture over a 3 day period. The last culture grown was plated on King's B plates containing kanamycin, and isolated colonies were picked onto King's B plates containing kanamycin with or without tetracycline. Total DNA was made from Km^R Tc^S colonies, and the *P. s. syringae* 61 *shcA* mutant, UNL131, was identified with PCR using primers that flanked the deletion site.

Construction of deletion mutation in shcA *and recombination into pHIR11*

To construct pLN85, a pHIR11 derivative that lacks the *shcA* gene, a 5 kb *Bgl*II–*Bam*HI fragment was isolated from pHIR11 and subcloned into pBluescript-II SK+, resulting in pLN82. pLN82 carries vector sequences, *shcA*, *hopPsyA* and downstream DNA and was used as the template DNA in a PCR using the primers 5′-GGAAACAGCTATGACCATG-3′ and 5′-ATGAGAATTCGCATCTCCATGCATCTT-3′. The isolated 1.5 kb PCR product representing the DNA upstream of *shcA* on pHIR11 was subcloned upstream of a terminatorless *nptII* cassette in pCPP2988 using *Xba*I and *Eco*RI restriction enzyme sites constructing pLN83. The 1.5 kb region immediately downstream of the *shcA* gene was PCR amplified using the same primer set used to PCR amplify the 1.5 kb fragment downstream of *shc*A used in making the *P. s. syringae* 61 *shcA* mutant described above. This product was subcloned into pLN83 using *Xho*I and *Kpn*I restriction enzyme sites constructing pLN101.

The *shcA* deletion mutation carried on pLN101 was transferred to pHIR11 using a strategy based on the principle that ColE1 plasmids, such as pBluescript-II, cannot replicate in *polA E. coli* mutants, whereas broad-host-range plasmids, such as pHIR11, can. The procedure used has been described previously (Alfano *et al*., 1996). Briefly, pLN101 was electroporated into *E. coli* C2110(pHIR11), and transformants were plated on LM plates selecting for the markers of both plasmids. Ap^R and Tc^R transformants were grown at 30°C in 5 ml of LM broth containing tetracycline for four consecutive days. Each day, a small amount of the grown-up culture was transferred to fresh LM broth containing tetracycline. Dilutions (1:1000) were plated on LM media, and Tc^R colonies were picked onto media containing tetracycline with and without ampicillin. DNA was isolated from Ap^S colonies, and a pHIR11 derivative with a *shcA* deletion, named pLN85, was identified using PCR. pLN85 was conjugated by triparental mating, first into *E. coli* MC4100 and from that strain into *P. fluorescens* 55 with either DH5α or HB101 carrying the helper plasmid pRK2013.

Preparation of protein samples, SDS–PAGE and immunoblot analysis

The protein samples were made as described previously (van Dijk *et al*., 1999) with some modification. Briefly, *Pseudomonas* spp. were grown overnight on King's B plates at 30°C. Cells were washed and resuspended in *hrp*-inducing fructose minimal medium (Huynh *et al*., 1989) and grown at 22°C in a refrigerated shaking incubator. For *P. s. syringae* 61 cultures, cells were adjusted to an initial OD_{600} of 0.15 and grown to an OD_{600} of 0.3. For *P. fluorescens* 55 cultures, the starting OD_{600} was 0.3, and the cultures were grown to an OD_{600} of 0.5. Cultures (80 ml) were separated into cell-bound and supernatant fractions by centrifugation at 4°C. The cell pellets were resuspended in 4 ml of H_2O. The total protein present in the cell-bound fraction was determined according to the method of Bradford (1976). SDS–PAGE tracking buffer was added to a 100 µl aliquot of the cell-bound fraction for analysis with SDS–PAGE. To prepare the supernatant fractions, 40 ml of the crude supernatant fractions were recentrifuged at 4°C, and the top 20 ml was transferred to a new tube, and proteins were precipitated by adding trichloroacetic acid to 9.4% with incubation at 4°C for 3 h. The protein pellets were gently washed with 5 ml of acetone and resuspended in 200 µl of SDS–PAGE tracking buffer.

Approximately 100 µg of total protein from each cell-bound fraction was added to SDS–PAGE gels. Based on the amount of protein in each cell-bound fraction, the amount of supernatant fraction loaded onto SDS–PAGE gels was adjusted to reflect total protein present in each culture. Proteins were separated with SDS–PAGE by standard procedures (Sambrook *et al*., 1989), transferred to polyvinylidene difluoride membranes and immunoblotted using anti-HopPsyA, -HrpZ, -β-lactamase or -FLAG as primary antibodies. The production of anti-HopPsyA and -HrpZ antibodies was described previously (He *et al*., 1993; van Dijk *et al*., 1999). The anti-β-lactamase antibodies were purchased from 5 Prime → 3 Prime, and the anti-FLAG antibodies were purchased from Sigma Chemical. Primary antibodies were recognized by goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Sigma Chemical), which were visualized by chemiluminescence using a Western light chemiluminescence detection system (Tropix) and X-Omat X-ray film (Eastman Kodak).

Assay to determine ShcA–HopPsyA interaction

Samples of 100 ml of *P. s. syringae* UNL131 cultures were inoculated at a 1:100 dilution with fresh overnight cultures in King's B broth and grown to an OD_{600} of 1.0. Cultures were centrifuged at 4°C, resuspended in 5 ml of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), centrifuged again and cell pellets were stored at –80°C. Cell pellets were resuspended in 10 ml of cold extraction buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing 1 mg ml^{-1} lysozyme and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and incubated on ice for 10 min. Cells were sonicated three times for 30 s, 1 ml of a high-salt buffer (1.5 M NaCl, 100 mM $CaCl_2$, 100 mM $MgCl_2$) was added, and the cell lysates were centrifuged at 1200 r.p.m. at 4°C. The aqueous phase was transferred to a new tube, and aliquots were saved separately for later analysis.

A sample of 120 μl of anti-FLAG gel, prepared according to the manufacturer's (Sigma Chemical) instructions, was mixed with 1.8 ml of each sonicate fraction and allowed to mix on a rotating shaker for 2 h at 4°C. The anti-FLAG gel mixtures were centrifuged at low speeds and washed four times with 250 μl of TBS buffer. The last wash was saved for SDS–PAGE analysis. The washed anti-FLAG gel was resuspended in 50 μl of TBS, mixed with SDS–PAGE sample buffer and subjected to SDS–PAGE and immunoblot analysis as described above.

Acknowledgements

We thank Karen Kesterson for assistance in constructing UNL131, and members of the Alfano laboratory for reviewing the manuscript. This work was supported by National Research Initiative Competitive Grants Program, US Department of Agriculture grant 01-35319-10019 and National Science Foundation grant IBN-0096348.

References

Alfano, J.R., and Collmer, A. (1996) Bacterial pathogens in plants: life up against the wall. *Plant Cell* **8:** 1683–1698.

Alfano, J.R., and Collmer, A. (1997) The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J Bacteriol* **179:** 5655–5662.

Alfano, J.R., Bauer, D.W., Milos, T.M., and Collmer, A. (1996) Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally nonpolar *hrpZ* deletion mutants, truncated HrpZ fragments, and *hrmA* mutations. *Mol Microbiol* **19:** 715–728.

Alfano, J.R., Kim, H.-S., Delaney, T.P., and Collmer, A. (1997) Evidence that the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in an *hrp*-dependent manner within tobacco cells. *Mol Plant–Microbe Interact* **10:** 580–588.

Alfano, J.R., Charkowski, A.O., Deng, W., Badel, J.L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. (2000) The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretions genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc Natl Acad Sci USA* **97:** 4856–4861.

Bennett, J.C., and Hughes, C. (2000) From flagellum assembly to virulence: the extended family of type III export chaperones. *Trends Microbiol* **8:** 202–204.

Boyd, A.P., Lambermont, I., and Cornelis, G. (2000) Competition between the Yops of *Yersinia enterocolitica* for delivery in eukaryotic cells: role of the SycE chaperone binding domain of YopE. *J Bacteriol* **182:** 4811–4821.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Charkowski, A.O., Huang, H.-C., and Collmer, A. (1997) Altered localization of HrpZ in *Pseudomonas syringae* pv. syringae *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. *J Bacteriol* **179:** 3866–3874.

Cheng, L.W., Anderson, D.M., and Schneewind, O. (1997) Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. *Mol Microbiol* **24:** 757–765.

Ciesiolka, L.D., Hwin, T., Gearlds, J.D., Minsavage, G.V., Saenz, R., Bravo, M., *et al.* (1999) Regulation of expression of avirulence gene *avrRxv and* identification of a family of host interaction factors by sequence analysis of *avrBsT*. *Mol Plant–Microbe Interact* **12:** 35–44.

Collmer, A., Badel, J.L., Charkowski, A.O., Deng, W.-L., Fouts, D.E., Ramos, A.R., *et al.* (2000) *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc Natl Acad Sci USA* **97:** 8770–8777.

Cornelis, G., and van Gijsegem, F. (2000) Assembly and function of type III secretion systems. *Annu Rev Microbiol* **54:** 734–774.

Cornelis, G.R., and Wolf-Watz, H. (1997) The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23:** 861–867.

Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., *et al.* (1998) The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* **62:** 1315–1352.

Dangl, J.L. (1994) The enigmatic avirulence genes of phytopathogenic bacteria. In *Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals – Molecular and Cellular Mechanisms*, Vol. 192. Dangl, J.L. (ed.). Berlin: Springer-Verlag, pp. 99–118.

Darwin, K.H., and Miller, V.L. (2000) The putative invasion protein chaperone SicA acts together with InvF to activate the expression of *Salmonella typhimurium* virulence genes. *Mol Microbiol* **35:** 949–959.

Day, J.B., and Plano, G.V. (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol Microbiol* **30:** 777–788.

van Dijk, K., Fouts, D.E., Rehm, A.H., Hill, A.R., Collmer, A., and Alfano, J.R. (1999) The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature and pH sensitive manner. *J Bacteriol* **181:** 4790–4797.

Francis, M.S., Lloyd, S.A., and Wolf-Watz, H. (2001) The type

III secretion chaperone LcrH co-operates with YopD to establish a negative, regulatory loop for control of Yop synthesis in *Yersinia pseudotuberculosis*. *Mol Microbiol* **42:** 1075–1093.

Fraser, G.M., Bennett, J.C.Q., and Hughes, C. (1999) Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum. *Mol Microbiol* **32:** 569–580.

Frithz-Lindsten, E., Rosqvist, R., Johansson, L., and Forsberg, A. (1995) The chaperone-like protein YerA of *Yersinia pseudotuberculosis* stabilizes YopE in the cytoplasm but is dispensable for targeting to the secretion loci. *Mol Microbiol* **16:** 635–647.

Fu, Y., and Galán, J.E. (1998) Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of *Salmonella typhimurium*. *J Bacteriol* **180:** 3393–3399.

Gage, D.J., Bobo, T., and Long, S.R. (1996) Use of Green Fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *J Bacteriol* **178:** 7159–7166.

Galán, J.E., and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284:** 1322–1328.

Gaudriault, S., Malandrin, L., Paulin, J.-P., and Barny, M.-A. (1997) DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol Microbiol* **26:** 1057–1069.

Ham, J.H., Bauer, D.W., Fouts, D.E., and Collmer, A. (1998) A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proc Natl Acad Sci USA* **95:** 10206–10211.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580.

Hardt, W.-D., and Galán, J.E. (1997) A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. *Proc Natl Acad Sci USA* **94:** 9887–9892.

He, S.Y. (1998) Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu Rev Phytopathol* **36:** 363–392.

He, S.Y., Huang, H.-C., and Collmer, A. (1993) *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73:** 1255–1266.

Heu, S., and Hutcheson, S.W. (1993) Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. *Mol Plant–Microbe Interact* **6:** 553–564.

Huang, H.-C., Schuurink, R., Denny, T.P., Atkinston, M.M., Baker, C.J., Yucel, I., *et al.* (1988) Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J Bacteriol* **170:** 4748–4756.

Huang, H.-C., Hutcheson, S.W., and Collmer, A. (1991) Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*pho*A tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol Plant–Microbe Interact* **4:** 469–476.

Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62:** 379–433.

Huynh, T.V., Dahlbeck, D., and Staskawicz, B.J. (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245:** 1374–1377.

Iriarte, M., and Cornelis, G.R. (1998) YopT, a new *Yersinia* Yop effector protein, affects the cytoskeleton of host cells. *Mol Microbiol* **29:** 915–929.

Kahn, M.L., and Hanawalt, P. (1979) Size distribution of DNA replicative intermediates in bacteriophage P4 and *E. coli*. *J Mol Biol* **128:** 501–525.

Keen, N.T. (1990) Gene-for-gene complementarity in plant–pathogen interactions. *Annu Rev Genet* **24:** 447–463.

Keen, N.T., Tamaski, S., Kobayashi, D., and Trollinger, D. (1988) Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70:** 191–197.

King, E.O., Ward, M.K., and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Med* **22:** 301–307.

Kjemtrup, S., Nimchuk, Z., and Dangl, J.L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Curr Opin Microbiol* **3:** 73–78.

Klement, Z. (1982) Hypersensitivity. In *Phytopathogenic Prokaryotes*, Vol. 2. Mount, M.S., and Lacy, G.H. (eds). New York: Academic Press, pp. 149–177.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166:** 175–176.

Labes, M., Puhler, A., and Simon, R. (1990) A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for gram-negative bacteria. *Gene* **89:** 37–46.

Lindgren, P.B. (1997) The role of *hrp* genes during plant–bacterial interactions. *Annu Rev Phytopathol* **35:** 129–152.

Lloyd, S.A., Forsberg, A., Wolf-Watz, H., and Francis, M.S. (2001) Targeting exported substrates to the Yersinia TTSS: different functions for different signals? *Trends Microbiol* **9:** 367–371.

Ménard, R., Sansonetti, P., Parsot, C., and Vasselon, T. (1994) Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell* **79:** 515–525.

Minamino, T., and Macnab, R.M. (1999) Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J Bacteriol* **181:** 1388–1394.

Mudgett, M.B., and Staskawicz, B.J. (1999) Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. *Mol Microbiol* **32:** 927–941.

Niebuhr, K., Jouihri, N., Allaoui, A., Gounon, P., Sansonetti, P.J., and Parsot, C. (2000) IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Mol Microbiol* **38:** 8–19.

Oliver, D.B., and Beckwith, J. (1981) *E. coli* mutant pleiotropically defective in the export of secreted proteins. *Cell* **78:** 1089–1099.

Page, A.-L., Fromont-Racine, M., Sansonetti, P., Legrain, P., and Parsot, C. (2001) Characterization of the interaction partners of secreted proteins and chaperones of *Shigella flexneri*. *Mol Microbiol* **42:** 1133–1145.

Plano, G.V., Day, J.B., and Ferracci, F. (2001) Type III export: new users for an old pathway. *Mol Microbiol* **40:** 284–293.

Rossier, O., Wengelnik, K., Hahn, K., and Bonas, U. (1999) The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. *Proc Natl Acad Sci USA* **96:** 9368–9373.

Ruvkin, G.B., and Ausubel, F.M. (1981) A general method for site-directed mutagenesis in prokaryotes. *Nature* **289:** 85–88.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning*, 2nd edn. Cold Spring Harbor, NY: Cold Spring. Harbor Laboratory Press.

Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. (1996) Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J Bacteriol* **178:** 7227–7233.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galán, J.E. (2001) Common and contrasting themes of plant and animal diseases. *Science* **292:** 2285–2289.

Stebbins, C.E., and Galán, J.E. (2001) Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. *Nature* **414:** 77–81.

Tsiamis, G., Mansfield, J.W., Hockenhull, R., Jackson, R.W., Sesma, A., Athanassopoulos, E., *et al.* (2000) Cultivar-specific avirulence and virulence functions assigned to *avrPphF. Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO* **19:** 3204–3214.

Tucker, S.C., and Galán, J.E. (2000) Complex function for SicA, a *Salmonella enterica* serovar *typhimurium* type III associated chaperone. *J Bacteriol* **182:** 2262–2268.

Wainwright, L.A., and Kaper, J.B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Mol Microbiol* **27:** 1247–1260.

Wattiau, P., and Cornelis, G.R. (1993) SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. *Mol Microbiol* **8:** 123–131.

Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G.R. (1994) Individual chaperones required for Yop secretion by *Yersinia*. *Proc Natl Acad Sci USA* **91:** 10493–10497.

Wattiau, P., Woestyn, S., and Cornelis, G.R. (1996) Customized secretion chaperones in pathogenic bacteria. *Mol Microbiol* **20:** 255–262.

Woestyn, S., Sory, M.P., Boland, A., Lequenne, O., and Cornelis, G.R. (1996) The cytosolic SycE and SycH chaperones of *Yersinia* protect the region of YopE and YopH involved in translocation across eukaryotic cell membranes. *Mol Microbiol* **20:** 1261–1271.