

***Salmonella* type III secretion-associated chaperones confer secretion-pathway specificity**

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Summary

Type III protein secretion systems (TTSSs) are ancestrally related to the flagellar export system and are essential for the virulence of many bacteria pathogenic for humans, animals and plants. Most proteins destined to travel the TTSS pathway possess at least two domains that specifically target them to the secretion apparatus. One of the domains is located within the amino terminal first ~20 amino acids and the second domain, located within the first ~140 amino acids, serves as a binding site for specific chaperones. It has been previously proposed that these two secretion signals are capable of operating independently of one another to facilitate secretion into the extracellular environment. We have found that in the absence of their chaperone-binding domains, the *Salmonella typhimurium* TTSS-secreted proteins SptP and SopE are no longer targeted for secretion through their cognate TTSS and, instead, are secreted through the flagellar export pathway. These results indicate the existence of an ‘ancestral’ flagellar secretion signal within TTSS-exported proteins that is revealed in the absence of the chaperone-binding domain. Furthermore, we found that secretion into culture supernatants as well as translocation into host cells by the cognate TTSS require both, the amino terminal and chaperone-binding domains. We conclude from these studies that a critical function for the TTSS-associated chaperones is to confer secretion-pathway specificity to their cognate secreted proteins.

Introduction

A number of Gram-negative pathogenic bacteria have evolved a specialized protein secretion system termed type III to deliver effector virulence proteins into host cells

(Galán and Collmer, 1999; Cornelis and Van Gijsegem, 2000). The core components of type III secretion systems (TTSSs) are an envelope-associated organelle known as the ‘needle complex’, several inner-membrane proteins, and a membrane-associated ATPase that presumably energizes the secretion process. These components are largely conserved even allowing the secretion of heterologous substrates (Ginocchio and Galán, 1995; Rosqvist *et al.*, 1995).

Despite intense scrutiny, the mechanisms by which TTSS substrates are recognized by the secretion machinery remain poorly understood and the subject of some controversy. Two signals have been identified in TTSS substrates that are important for their routing into the secretion pathway (Michiels and Cornelis, 1991; Sory *et al.*, 1995; Cheng *et al.*, 1997). One of the signals is generally located within the first ~20 amino acids of the secreted proteins (Michiels and Cornelis, 1991; Sory *et al.*, 1995). Studies have shown that addition of this domain to various reporter proteins is enough to mediate their secretion into the culture supernatant (Michiels and Cornelis, 1991). The apparent promiscuity of the actual amino acid sequences capable of mediating secretion has led to the hypothesis that the mRNA and not the polypeptide serves as secretion signal (Anderson and Schneewind, 1997; 1999). This assertion was largely based on two observations: (i) in general, there is little amino acid sequence similarity amongst the sequences that target the different secreted proteins; and (ii) introduction of frame-shift mutations that alter the primary amino acid sequence without altering the mRNA sequence often results in polypeptides that can be secreted at apparently wild-type levels. However, this hypothesis has been recently challenged and evidence has been presented that secondary structural features of the secretion signal such as amphipathicity serve as recognition motif by the TTSS (Lloyd *et al.*, 2001; 2002). The issue has been further complicated by the finding that the secretion signal of at least one component of the *Salmonella* pathogenicity island-1 (SPI-1) TTSS apparatus, InvJ, whose targeting to the ‘secretion’ is dependent on this TTSS, does not appear to depend on secondary structure features of the secretion signal or even its mRNA sequence (Rusmann *et al.*, 2002). In this case it has been hypothesized that the essential requirement for the amino terminal secretion signal of InvJ is a polypeptide sequence that is disordered

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Dedicated to the memory of Dr Robert Macnab, a wonderful colleague and a brilliant scientist.

and unable to acquire secondary or tertiary structural features (Russmann *et al.*, 2002).

The second secretion signal is located within the first ~140 amino acids of at least some of the secreted proteins (Cheng *et al.*, 1997). This region serves as binding site for specific cytoplasmic chaperones that form highly specific and tight complexes with their cognate secreted proteins (Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994; 1996; Sory *et al.*, 1995; Schesser *et al.*, 1996; Woestyn *et al.*, 1996). Although long recognized as essential components of TTSSs, the actual function of these chaperones had remained poorly understood until recently. The availability of several crystal structures of chaperones and chaperone-effector complexes has significantly enhanced the understanding of the function of this protein family (Birtalan and Ghosh, 2001; Luo *et al.*, 2001; Stebbins and Galán, 2001; Birtalan *et al.*, 2002; Evdokimov *et al.*, 2002). These structures suggest that these chaperones function, at least in part, to maintain extended portions of the effectors in a significantly non-globular, secretion competent state that is primed for transfer through the needle complex as an unfolded polypeptide. Comparison of the crystal structures of several chaperones and chaperone/effector complexes suggests a second potential function for these chaperones. Despite their lack of overall primary sequence similarity, all chaperones whose structures are known display virtually identical folds and have similar hydrophobic regions that could serve as targeting features recognized by the secretion machinery. Furthermore, comparison of the structures of the TTSS-secreted proteins SptP and YopE in complex with their respective chaperones, reveals that the main-chain path across the chaperones is strikingly similar, suggesting that these features may serve as additional recognition signals for targeting the complexes to the TTSS (Birtalan *et al.*, 2002).

Salmonella enterica encodes two TTSSs that play important roles at different stages of the infection cycle (Galán, 2001). One of the systems, encoded within the *Salmonella* pathogenicity island 1 (SPI-1), mediates the initial interaction of *Salmonella* with the intestinal epithelium, allowing bacterial entry and the stimulation of the production of pro-inflammatory cytokines (Galán and Curtiss, 1989). The second TTSS is encoded within the *Salmonella* pathogenicity island 2 (SPI-2) and is essential for systemic infection (Hensel *et al.*, 1995; Ochman *et al.*, 1996). In this study, we demonstrate that TTSS-associated chaperones serve to confer secretion-pathway specificity to their cognate secreted proteins. We show that the *Salmonella* SPI-1 TTSS effector proteins SopE and SptP devoid of their chaperone-binding domains are targeted to the flagellar-associated export apparatus rather than to the SPI-1 TTSS. In addition, we show that contrary to what has been previously shown in other systems (Sory *et al.*, 1995; Cheng *et al.*, 1997; Lloyd *et al.*, 2001), secretion

through the SPI-1 TTSS requires both the amino terminal signal sequence and the chaperone-binding domain.

Results

The amino terminus of SptP mediates SPI-1 TTSS-independent secretion

To gain insight in the mechanisms of substrate recognition by TTSSs, we sought to define the domains of SptP (Kang *et al.*, 1996; Fu and Galán, 1998; 1999) that are necessary for its secretion through the *Salmonella* SPI-1 TTSS. We chose to focus on SptP because its crystal structure, alone and in complex with its chaperone SicP, has become recently available (Stebbins and Galán, 2000; 2001), allowing the precise definition of the different structural domains of this secreted protein. Of relevance to the present study, the crystal structure of the SptP-SicP complex established that the chaperone-binding domain of SptP is located between amino acids 35 and 139 (Stebbins and Galán, 2001). Furthermore, protease susceptibility analysis indicated that the amino-terminal 35 residues of SptP were not protected by the chaperone from proteolytic degradation (Stebbins and Galán, 2000; 2001). We therefore sought to investigate whether the first 35 amino acids of SptP were sufficient to mediate the secretion through the SPI-1 TTSS of the secretion-signalless reporter protein alkaline phosphatase (PhoA). This *phoA*-deletion mutation has been previously utilized in our laboratory to report TTSS-mediated secretion (Russmann *et al.*, 2002). To ensure the appropriate levels of expression and stoichiometry, a bicistronic plasmid was constructed expressing the cognate chaperone, SicP (Fu and Galán, 1998), and the SptP₁₋₃₅-PhoA fusion under the transcriptional control of its native promoter in the low copy plasmid pWSK29 (Wang and Kushner, 1991) (see Fig. 7). The resulting plasmid was introduced into a Δ sptP *Salmonella* strain with a functional SPI-1 TTSS or into an isogenic derivative containing a non-polar insertion mutation in the SPI-1 TTSS essential gene *invA* (Galán *et al.*, 1992). The two strains were examined for their ability to express and secrete the SptP₁₋₃₅-PhoA chimeric protein. Expression of SptP₁₋₃₅-PhoA was comparable in both strains (Fig. 1). Surprisingly, SptP₁₋₃₅-PhoA was efficiently secreted not only by the strain with a functional SPI-1 TTSS but also by the strain carrying an *invA* mutation (Fig. 1). The presence of SptP₁₋₃₅-PhoA in the culture supernatant of the *invA* mutant strain was not the result of bacterial lysis or non-specific leakage as the cytoplasmic protein fumarate dehydrogenase was not detectable in the same culture supernatant preparations (Fig. 1).

The unexpected secretion of SptP₁₋₃₅-PhoA chimeric protein in the *invA*-null mutant background suggested that the amino terminal secretion signal of SptP is either able

The chaperone-binding domain of SptP confers specificity of secretion through the SPI-1 TTSS

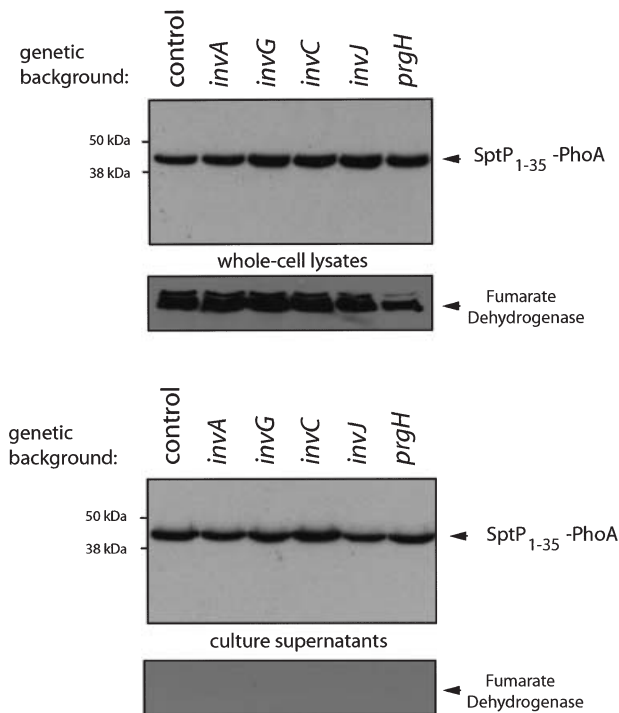


Fig. 1. The first 35 amino acids of SptP mediate the SPI-1 TTSS-independent secretion of the reporter protein alkaline phosphatase. A chimeric protein consisting of the first 35 amino acids of SptP and alkaline phosphatase devoid of its secretion signal sequence was expressed in a $\Delta sptP$ *S. typhimurium* mutant strain (indicated as 'control' in the figure panels) and isogenic derivatives carrying mutations in different essential components of the SPI-1 TTSS (*invA*, *invG*, *invC*, *invJ* and *prgH*) as indicated. Whole-cell lysates and culture supernatants of these strains were examined for the presence of the SptP₁₋₃₅-PhoA by Western immunoblot with an antibody directed to PhoA or the cytoplasmic protein fumarate dehydrogenase as described in *Experimental procedures*.

to circumvent the requirement for the InvA protein but is maintained as a substrate for secretion through the SPI-1 TTSS or it is secreted by a SPI-1 TTSS-independent pathway. To discriminate between these possibilities, we examined the secretion of SptP₁₋₃₅-PhoA in *Salmonella* strains carrying loss-of-function mutations in genes that encode structural proteins of the needle complex (*invG* and *prgH*) (Kubori *et al.*, 1998), or other essential components of the SPI-1 TTSS such as the membrane-associated ATPase InvC (Eichelberg *et al.*, 1994), or the needle-length control protein InvJ (Kubori *et al.*, 2000). SptP₁₋₃₅-PhoA was secreted in all mutant backgrounds (Fig. 1). In all cases, the cytoplasmic protein fumarate dehydrogenase was not detected in the same culture supernatant preparations (Fig. 1), indicating that the presence of SptP₁₋₃₅-PhoA in this fraction was a result of its specific secretion and not to bacterial cell lysis or non-specific leakage. Taken together, these results argue that the first 35 amino acids of SptP mediate secretion in a SPI-1 TTSS independent manner.

Previous studies have demonstrated the ability of TTSSs to engage heterologous substrates (Rosqvist *et al.*, 1995). Therefore, the observation that the first 35 amino acids of SptP were capable of mediating secretion in a SPI-1 TTSS-independent manner prompted us to investigate the possibility that this secretion signal could direct substrates to either the SPI-2 TTSS (Hensel *et al.*, 1995; Ochman *et al.*, 1996) or the related flagellar-associated export system (Macnab, 1992). To eliminate any possibility that the secretion-signal-less PhoA itself was responsible for mediating the secretion of the SptP₁₋₃₅-PhoA chimeric protein through a hitherto unknown secretion pathway, we use the effector domain region of SptP (SptP₁₆₂₋₅₄₃) as a surrogate reporter of secretion. The resulting construct (SptP_{Δ36-161}) lacks amino acids 36 through 161, which contains the chaperone-binding domain (Fu and Galán, 1998; Stebbins and Galán, 2001). Similar to what we observed with SptP₁₋₃₅-PhoA, SptP_{Δ36-161} was readily detected in culture supernatants of the *S. typhimurium invA* mutant strain, indicating that it was secreted in a SPI-1-independent manner (Fig. 2A). Furthermore, SptP_{Δ36-161} was secreted by a *S. typhimurium* strain simultaneously carrying loss-of-function mutations in *invA* and *spiA*, which encodes for an essential component of the SPI-2-encoded TTSS (Ochman *et al.*, 1996) (Fig. 2A). In all cases, secretion of SptP_{Δ36-161} in these strains was not caused by bacterial cell lysis because the cytoplasmic protein fumarate dehydrogenase was not detectable in the same culture supernatant preparations (Fig. 2A). Furthermore, secretion was not a result of overexpression as this construct produced SptP_{Δ36-161} at equivalent level to that of wild-type protein. These results indicate that the first 35 amino acids of SptP direct the reporter protein to a pathway distinct from the TTSSs encoded in either SPI-1 or SPI-2. Remarkably, SptP_{Δ36-161} was not secreted in a *fliGHI*-null strain (detection limit <0.1% of wild-type levels) (Lockman and Curtiss, 1990), which is defective in the flagellar-associated export system (Macnab, 1992), although expression of this protein was unaffected in this mutant strain (Fig. 2A). Similarly, secretion of SptP₁₋₃₅-PhoA was also abolished in the *fliGHI*-null mutant strain (Fig. 2C). These results indicate that the first 35 amino acids of SptP are sufficient to direct secretion through the flagellar-associated export pathway but are not able to direct secretion through the TTSSs encoded in either SPI-1 or SPI-2. In contrast and as previously shown (Kaniga *et al.*, 1996), the secretion profile of full-length SptP (and thus containing its chaperone-binding domain) was consistent with its status as an SPI-1-TTSS effector protein: secretion was detected only in the presence of a functional SPI-TTSS regardless whether there was a func-

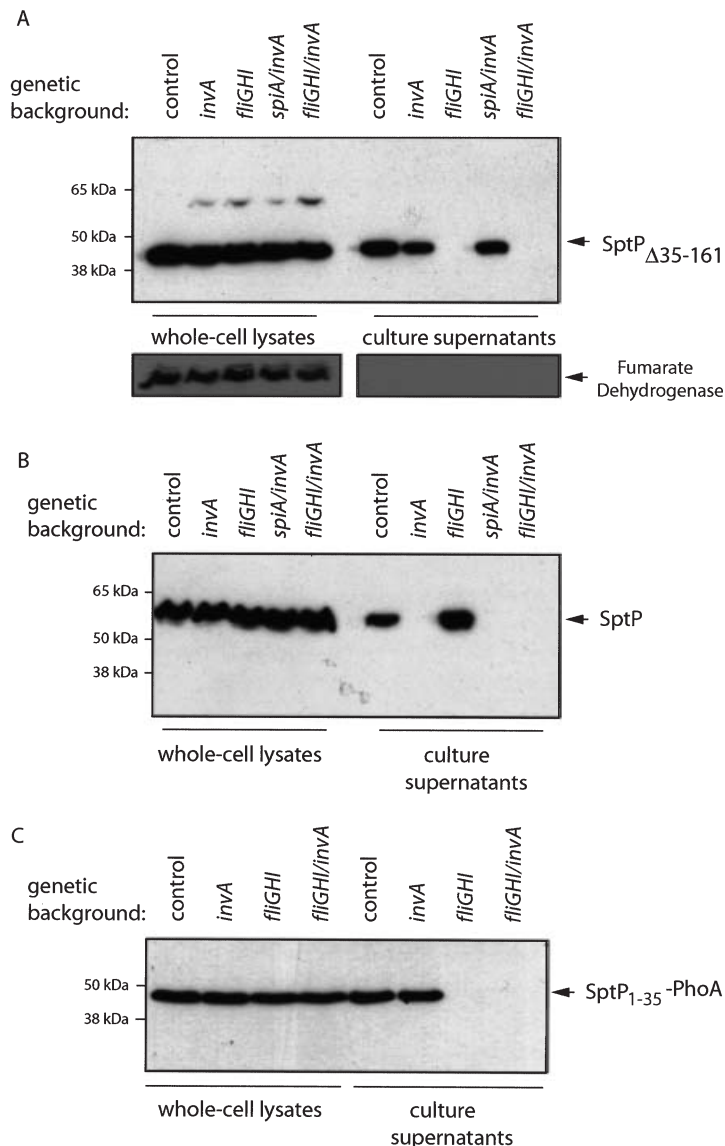


Fig. 2. The chaperone-binding domain of SptP confers secretion-pathway specificity. A deletion mutant (SptP_{Δ35-161}) lacking the chaperone-binding domain (A), full-length SptP (B) or SptP₁₋₃₅-PhoA (C) were expressed in a $\Delta sptP$ *S. typhimurium* strain (indicated as 'control' in the figure panels) and isogenic derivatives deficient in the SPI-1 TTSS (*invA*), flagellar export apparatus (*fltGH*), the SPI-2 TTSS (*spiA*) or a combination of these mutations as indicated. Whole-cell lysates and culture supernatants of these strains were examined for the presence of SptP_{Δ35-161} (A), wild type SptP (B) or SptP₁₋₃₅-PhoA (C) by western immunoblot with a monoclonal antibody directed to SptP (A and B), PhoA (C) or fumarate dehydrogenase (A) as described in *Experimental procedures*.

tional flagellar and/or SPI-2 TTSS export apparatus or not (Fig. 2B).

Taken together, these results indicate that the first 35 amino acids of SptP contain an 'ancestral' secretion signal that, by itself, is capable of directing secretion through the flagellar export apparatus. However, in the presence of the chaperone-binding domain, this signal is no longer able to direct secretion through the flagellar export system, indicating that TTSS-associated chaperones confer secretion-specificity by targeting substrates to their cognate TTSSs.

The amino-terminus and chaperone-binding domains of SptP act synergistically to mediate secretion through the SPI-1 TTSS

It has been previously proposed that the amino terminus

and the chaperone-binding domains of TTSS secreted proteins serve as secretion signals that are able to independently target substrates to a specific TTSS (Cheng *et al.*, 1997; Lloyd *et al.*, 2001). Our observation that the amino terminus of SptP by itself is indeed able to mediate secretion although through a heterologous pathway prompted us to re-examine the generalization of the 'two-independent-signal hypothesis' to the SPI-1 TTSS substrates. More specifically, our observation that the chaperone-binding domain of SptP confers specificity for secretion through the SPI-1 TTSS raised the question whether the amino-terminus and the chaperone-binding domain serve as two independent secretion signals or whether they act in a synergistic manner. To investigate the requirement of the amino-terminus of SptP in targeting secretion through the SPI-1 TTSS, the amino terminus of SptP was sequentially deleted to generate the truncated

forms SptP_{Δ25-35}, SptP_{Δ15-35} and SptP_{Δ10-35}, and SptP_{Δ1-35}. All deletion mutants retained the chaperone-binding and effector domains intact. Of all the deletions tested, only SptP_{Δ25-35} was secreted in a SPI-1 TTSS-dependent manner, although at significantly reduced levels (~30% of wild type). In contrast, SptP_{Δ15-35}, SptP_{Δ10-35} and SptP_{Δ1-35} were not secreted (<0.1% wild type) even though they were produced at levels equivalent to that of wild type (Fig. 3). These results suggest that the amino-terminal ~35 residues of SptP are required to mediate SPI-1-dependent secretion in the presence of the chaperone-binding domain. This secretion signal is slightly longer than the previously identified signals in other systems (Galán and Collmer, 1999; Cornelis and Van Gijsegem, 2000). More importantly, our results demonstrated that the chaperone-binding domain alone is not sufficient to mediate secretion into the culture supernatant through the type III secretion system. Therefore, we conclude from these studies that the amino-terminus and the chaperone-binding domain of SptP do not function independently to mediate secretion but rather they act in a cooperative manner to mediate specific secretion through the SPI-1 TTSS.

The flagellar-associated export apparatus can secrete a SopE mutant lacking its chaperone-binding domain

To investigate whether our findings on the nature of the secretion signals of SptP were applicable to other SPI-1 TTSS effector proteins, we examined the secretion signals of SopE (Wood *et al.*, 1996; Hardt *et al.*, 1998b). A chaperone for SopE was recently identified as InvB (Sang Ho Lee and J. E. Galán, in press), a previously identified SPI-

1 encoded protein (Eichelberg *et al.*, 1994) that also serves as chaperone for SipA (Bronstein *et al.*, 2000). Consistent with the observations made with SptP, secretion of the deletion mutant SopE_{Δ39-77}, which lacks its chaperone-binding site, was secreted in an SPI-1 TTSS-independent manner (Fig. 4). More importantly, secretion of SopE_{Δ39-77} was not observed in a *Salmonella fliGHI* mutant strain (<0.1% of wild type) indicating that the SPI-1 independent secretion of this mutant is also mediated by the flagellar export system (Fig. 4). This was in contrast to the secretion of wild-type SopE, which as previously reported (Hardt *et al.*, 1998b) was strictly dependent on the presence of a functional SPI-1 TTSS (Fig. 4). These results further demonstrate that the chaperone-binding domain of TTSS-effector proteins is required to confer TTSS specificity.

The flagellar-associated export apparatus cannot mediate the delivery into host cells of a SopE mutant lacking its chaperone-binding domain

The observation that SPI-1 TTSS effector proteins lacking their respective chaperone-binding domains can be secreted by the flagellar apparatus prompted us to investigate whether this system could deliver these mutant proteins into host cells. To address this issue we examined whether expression of SopE_{Δ39-77}, which is secreted through the flagellar export apparatus (see above), was capable of complementing the invasion phenotype of a strain of *S. typhimurium* carrying loss-of-function mutations in *sopE*, *sopE2* and *sopB*. This strain is unable to enter into cells as it lacks the three effector proteins that

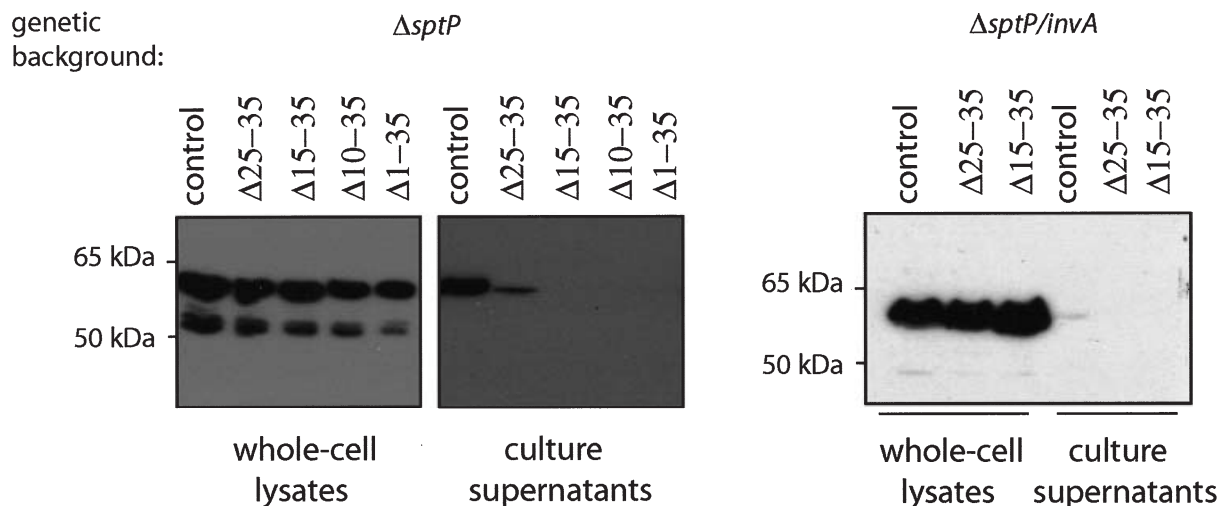


Fig. 3. The amino terminal secretion signal and the chaperone-binding domain of SptP act synergistically to mediate secretion through the SPI-1 TTSS. Full-length SptP (labelled as 'control' in the figure panels) or different deletion mutants of SptP (SptP_{Δ25-35}, SptP_{Δ15-35}, SptP_{Δ10-35} and SptP_{Δ1-35}) were expressed in a $\Delta sptP$ *S. typhimurium* strain or an isogenic mutant derivative deficient in the SPI-1 TTSS (*invA*). Whole-cell lysates and culture supernatants of these strains were examined for the presence of SptP or the deletion mutants by western immunoblot with a monoclonal antibody directed to SptP as described in *Experimental procedures*.

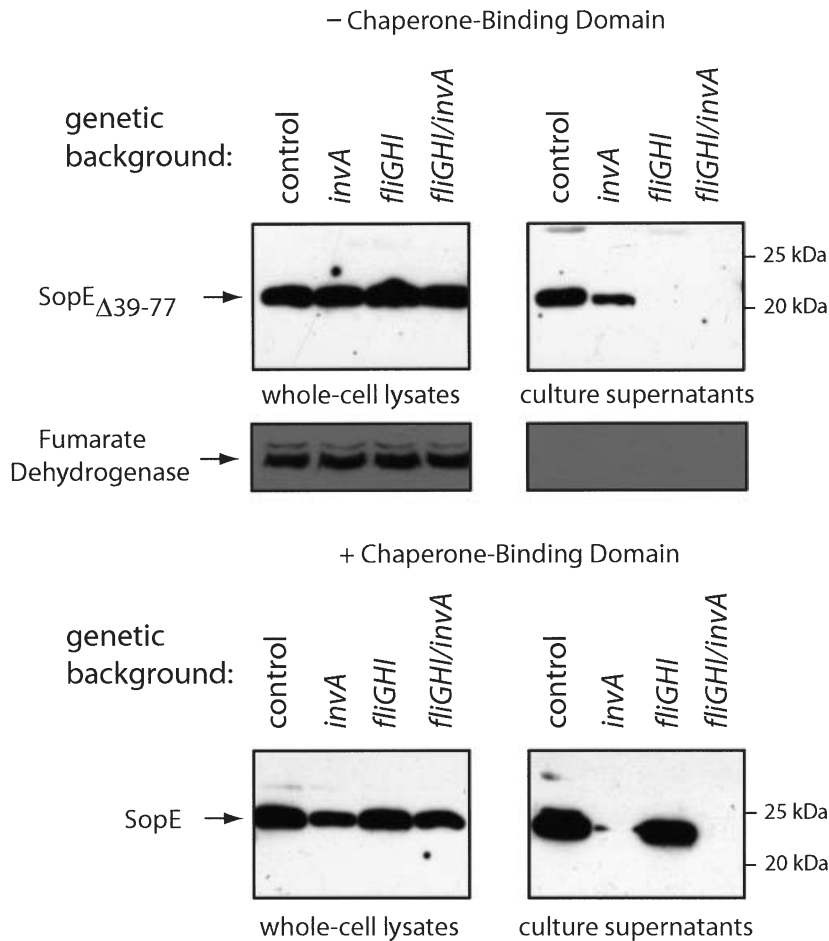


Fig. 4. A SopE mutant lacking its chaperone-binding domain is secreted through the flagellar export apparatus but not through the SPI-1 TTSS. M45-epitope-tagged full-length SopE (lower panel) or a deletion mutant (SopE $_{\Delta 39-77}$) lacking the chaperone-binding domain (upper panel) were expressed in wild-type *S. typhimurium* (indicated as 'control' in the figure panels) and isogenic mutant derivatives deficient in the SPI-1 TTSS (*invA*), flagellar export apparatus (*fliGHI*), the SPI-2 TTSS (*spiA*) or a combination of these mutants, as indicated. Whole-cell lysates and culture supernatants of these strains were examined for the presence of the SopE or SopE $_{\Delta 39-77}$ by western immunoblot with a monoclonal antibody directed to the M45 epitope as described in *Experimental procedures*.

mediate actin cytoskeleton rearrangements required for bacterial entry (Zhou *et al.*, 2001). Because the effector function of SopE is not dependent on the presence of its chaperone-binding domain when delivered into cells by transfection or microinjection (Hardt *et al.*, 1998a), we reasoned that SopE $_{\Delta 39-77}$ should be able to complement the invasion defect of the entry deficient triple mutant if its secretion through the flagellar apparatus led to its translocation into host cells. The *sopE*, *sopE2* and *sopB* triple mutant's ability to invade cells is readily completable by a wild-type copy of SopE (Zhou *et al.*, 2001) (Fig. 5) and therefore served as an ideal surrogate for the potential ability of SopE $_{\Delta 39-77}$ to complement such a defect. Indeed, induction of actin cytoskeleton reorganization and the mediation of bacterial entry constitutes the most sensitive assay available for SPI-1 TTSS effector protein translocation because the cellular responses can be observed before the bacterial proteins can be detected within cells by any biochemical or microscopy means (T. Kubori and J. E. Galán, unpubl. obs.). As shown in Fig. 5, SopE $_{\Delta 39-77}$ was not able to complement the invasion phenotype of the *sopE sopE2 sopB* triple mutant with or

without the presence of a functional SPI-1 TTSS. As previously shown (Zhou *et al.*, 2001) (Fig. 5), expression of wild-type SopE was able to complement the invasion defect of this strain. These results indicate that although the flagellar export apparatus is capable of efficiently secreting SPI-TTSS substrates lacking their chaperone-binding domain, this system is not capable of delivering them into host cells. Furthermore, these results showed that in the absence of the chaperone-binding domain, the SPI-1 TTSS effector proteins cannot be injected into cells even in the presence of a functional cognate TTSS. These observations may also provide an explanation for previous results indicating the requirement of the chaperone-binding domain for the translocation of Yop protein into host cells (Sory *et al.*, 1995).

Characterization of the amino terminal secretion signal of SptP

Although it is well established that all proteins secreted through TTSSs possess a discrete 'secretion signal' encoded within the first ~20 codons, the nature of such a

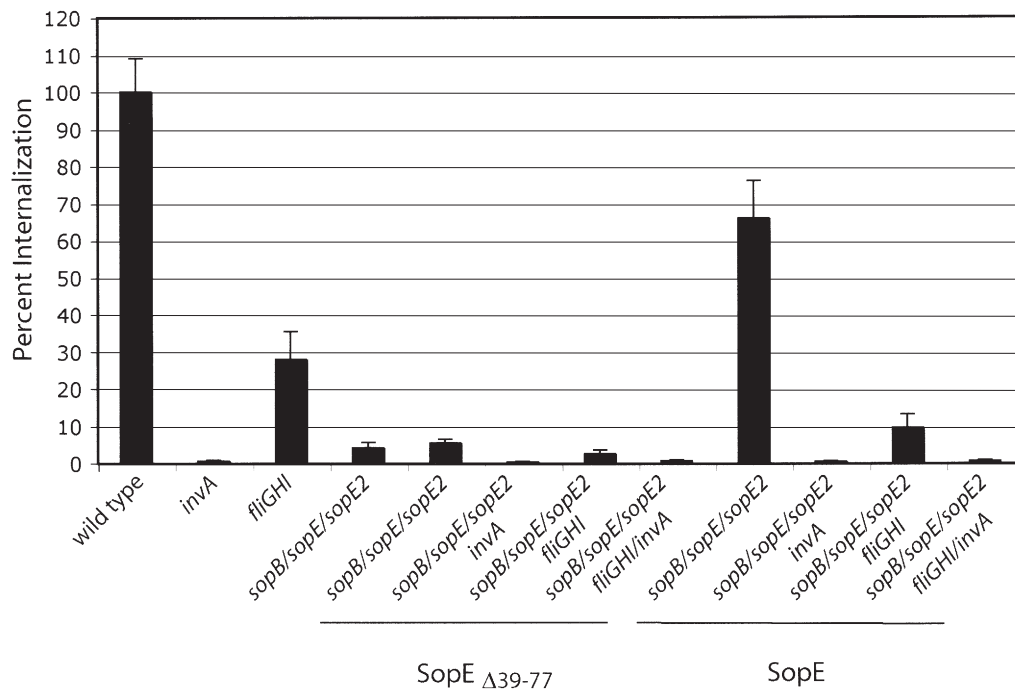


Fig. 5. A SopE mutant lacking its chaperone-binding domain cannot complement a *sopE* loss-of-function mutation despite its secretion through the flagellar-associated export apparatus. Plasmids encoding full-length SopE or its mutant derivative SopE $_{\Delta 39-77}$ (lacking its chaperone-binding domain) were introduced into a non-invasive (*sopE/sopE2/sopB*) strain and the SPI-1 TTSS-defective (*invA*), and flagellar-export-defective (*fliGHI*) isogenic derivatives (Eichelberg and Galán, 2000). Notice that as previously reported (Eichelberg and Galán, 2000), absence of flagella indirectly affects bacterial entry therefore internalization values of strains carrying the *fliGHI* mutation are relatively lower. As a measure of their ability to translocate SopE or SopE $_{\Delta 39-77}$, the different strains were tested for their ability to enter into cultured epithelial cells. Values, normalized to those of wild type (100%), are the percentage of the initial inoculum that survived the gentamicine treatment (see *Experimental procedures*) and represent the mean \pm standard deviation of three independent determinations. The actual invasion value for wild-type *S. typhimurium* was 38 ± 3.1 .

secretion signal has been the subject of some controversy (Anderson and Schneewind, 1997; 1999; Lloyd *et al.*, 2001). We conducted experiments aimed at further characterizing the amino terminal sequence of SptP. We examined whether there were some common features in the requirements of the amino terminal sequence to target a protein substrate to the flagellar apparatus or the SPI-1 TTSS. In particular, we examined the effect of introducing frame-shifting mutations to the secretion signal of SptP in the absence of its chaperone-binding domain. Introduction of two base pairs immediately after the 10th codon of sptP $_{\Delta 36-161}$ completely abrogated (<0.1% of wild type) the secretion of the resulting protein although it did not alter its synthesis (Fig. 6A). As introduction of this frame shift drastically altered the primary sequence of amino acids 11th through 35th of SptP $_{\Delta 36-161}$ while preserving the mRNA sequence, these results are consistent with the notion that the polypeptide sequence is an essential element of the 'secretion signal'. Furthermore, because SptP $_{\Delta 36-161}$ lacks the chaperone-binding domain and is therefore secreted by the flagellar-associated export apparatus, these results indicate that the primary amino acid sequence of the 'secretion signal' is essential for its secretion through this ancestral pathway. We then exam-

ined the effect of introducing the same frame shifting mutations to the secretion signal of SptP in the presence of its chaperone-binding domain. Similarly to what we observed with SptP $_{\Delta 36-161}$, introduction of one (+1) or 2 (+2) base pairs immediately after the 10th codon of sptP abrogated (<0.1% of wild type) the secretion of the resulting proteins although their expression levels in whole-cell lysates were equivalent to wild type (Fig. 6B). These results indicate that the primary amino acid sequence of the secretion signal is essential for secretion through both the flagellar export system and the SPI-1 TTSS. In addition, these results further substantiate the notion that both an intact amino terminal secretion signal and the chaperone-binding domain are necessary for secretion through the SPI-1 TTSS, and that either of these signals by themselves are not sufficient to mediate secretion through the cognate TTSS.

Discussion

The mechanisms by which proteins destined to travel through TTSSs are recognized by the secretion machinery are poorly understood. Two elements of the secreted proteins have been proposed to play critical roles in this

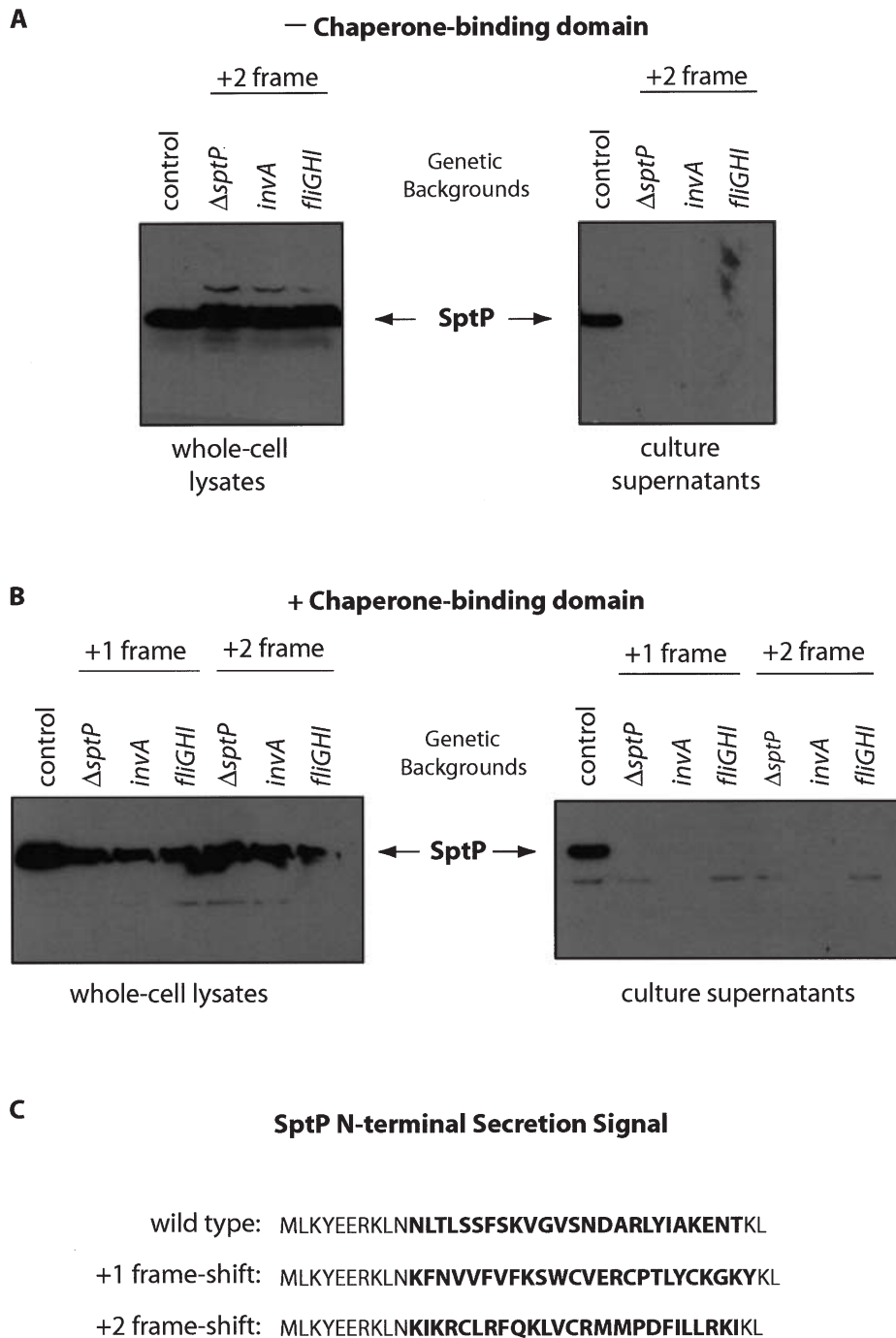


Fig. 6. Introduction of frameshift mutations in the amino terminal secretion signal of SptP and SptP_{Δ36-161} abrogate their secretion through the SPI-1 TTSS and the flagellar export pathway respectively. Frameshift mutations (+1 and/or +2) were introduced within the amino terminal secretion signal of SptP (B) or SptP_{Δ36-161} (A), which lacks the chaperone-binding domain. The resulting constructs were expressed in a *S. typhimurium* *sptP*-deficient mutant or its isogenic derivatives deficient in the SPI-1 TTSS (*invA*) or flagellar export apparatus (*fljGHl*). Whole-cell lysates and culture supernatants of these strains were examined for the presence of the SptP or SptP_{Δ36-161} by western immunoblot with a monoclonal antibody directed to SptP as described in *Experimental procedures*. Lanes labelled as 'controls' denote whole-cell lysates or culture supernatants (as indicated) of $\Delta sptP$ *S. typhimurium* expressing full-length SptP or SptP_{Δ36-161}, which contains the wild-type amino terminus sequence. The amino acid sequence of the relevant region of the signal sequence is shown in C. The amino acid sequence of the region mutated by frame-shifting is shown in bold.

process: a domain located within the first ~20 amino acids (or codons) and a second domain located within the first ~140 amino acids that bind specific cognate chaperones (Cornelis and Van Gijsegem, 2000). It has been previously reported that the amino terminal secretion signal and the chaperone-binding domain are capable of mediating secretion into the extracellular environment independently from one another (Cheng *et al.*, 1997; Lloyd *et al.*, 2001). Consequently, it has been shown that the amino terminal first ~20 amino acids of TTSS-secreted effectors can mediate the secretion of reporter proteins (Michiels and Cornelis, 1991; Sory *et al.*, 1995), and that some *Yersinia* spp. TTSS effector proteins can be secreted in the absence of their chaperone-binding domains (Sory *et al.*, 1995; Cheng *et al.*, 1997) or in the absence of the amino terminal secretion signal (Cheng *et al.*, 1997; Lloyd *et al.*, 2001). Our findings are not consistent with these previous observations. We have shown here that the amino terminal secretion signal of the *Salmonella* SPI-1 TTSS effector protein SptP is capable of mediating the secretion of the reporter protein alkaline phosphatase. However, we found that secretion of the reporter protein was not through the SPI-1 or SPI-2 encoded TTSSs. Surprisingly, we found that the amino terminal secretion signal of SptP targeted the reporter protein to the flagellar export apparatus instead. Targeting of the reporter protein by the secretion signal of the SPI-1 TTSS effector proteins to the flagellar export apparatus was not due to intrinsic properties of the reporter itself as identical results were obtained when the effector domains of SptP or SopE were used as reporters of secretion. Despite efficient secretion through the flagellar export pathway, in the absence of the chaperone-binding domain the *Salmonella* the SPI-1 TTSS effector protein SopE was not translocated into host cells, further demonstrating the significant differences between these two ancestrally related secretion systems. In the presence of the chaperone-binding domain, however, we found that SptP and SopE were secreted only in the presence of a functional SPI-1 TTSS, consistent with previous observations.

Our results indicate that the amino terminal secretion signal of SptP is not sufficient to mediate secretion through its cognate TTSS. These results differ with previous observations indicating that the amino terminal secretion signal of *Yersinia* spp. Yop proteins was sufficient to mediate the secretion of reporter proteins (Michiels and Cornelis, 1991; Sory *et al.*, 1995; Cheng *et al.*, 1997). The different results may reflect differences in the experimental systems. Alternatively, it is possible that export through the flagellar apparatus may have occurred in the *Yersinia* studies as this possibility was not specifically tested, which would have required the examination of secretion in the absence of a functional flagellar export apparatus. Indeed, studies have previously reported the surprising

finding that in the absence of the chaperone-binding domain, Yop proteins can be efficiently secreted but cannot be translocated into host cells (Sory *et al.*, 1995) [except in the context of a mutant strain lacking all Yop proteins, in which low efficiency translocation could be observed (Boyd *et al.*, 2000)]. It is possible that the failure of Yop proteins devoid of chaperone-binding domain to be translocated into host cells despite their efficient secretion may have been due to their targeting to the flagellar export apparatus, which is unable to mediate protein translocation into host cells. More experiments will be required to clarify this issue.

The flagellar export apparatus is widely considered to be 'ancestral' to TTSS (Nguyen *et al.*, 2000). In this context, we hypothesize that TTSS effector proteins may have an 'ancestral' secretion signal that can be recognized by the flagellar export apparatus in the absence of the chaperone-binding domain. In the presence of the chaperone-binding domain, however, the chaperone effector complex is no longer recognized by the flagellar export apparatus but only by its cognate TTSS, indicating that an important function of TTSS-associated chaperones is to confer pathway specificity to the secreted proteins. Indeed, previous studies have shown that the *Yersinia* spp. flagellar export system is capable of secreting proteins that are not associated with the flagellar apparatus itself (Young *et al.*, 1999). One of these proteins, YplA, has also been shown to be exported by the two *Yersinia* TTSS systems under certain growth conditions (Young and Young, 2002). No chaperone for this protein has yet been identified. Although it remains formally possible that such a chaperone may yet be discovered, it is possible that proteins that lack specific chaperones may exhibit a more promiscuous secretion behaviour and may be capable of engaging more than one secretion pathway. Indeed, under certain experimental growth conditions, the TTSS-exported protein InvJ, which lacks a cognate chaperone, can be secreted by either the SPI-1 TTSS or the flagellar export apparatus (Sang Ho Lee and J. E. Galán, unpubl. obs.), which would be consistent with this hypothesis. Although it is unlikely that the secretion of InvJ through the flagellar system is physiologically significant as this protein is a component of the SPI-1 TTSS and not an effector of virulence, it is possible that as previously proposed for other proteins such as YplA (Young and Young, 2002), this flexibility may allow them to be secreted under vastly different conditions and therefore exert their function in a wider range of environments. Previous studies have demonstrated that substrates of type III secretions systems can be secreted in heterologous bacteria provided that they are expressed in the presence of their cognate chaperones (Rosqvist *et al.*, 1995). In addition, a very small number of TTSS secreted proteins can be targeted by more than one TTSS system in the case of pathogens

encoding more than one such system (Miao and Miller, 2000). It is not known whether proteins that can be targeted by more than one TTSS display less stringent recognition mechanisms.

Our findings indicate that both, the amino terminal secretion signal and the chaperone-binding domain of SptP are required for secretion through the cognate TTSS. Disruption of either of these domains effectively precluded secretion of this effector protein through its cognate TTSS. These observations are also apparently at odds with previous studies that have indicated that both signals are capable of operating as 'independent' secretion signals (Cheng *et al.*, 1997; Lloyd *et al.*, 2001). We are uncertain about the reasons for this discrepancy. It is possible that the different findings may reflect intrinsic differences between these TTSSs. Alternatively and as we have shown for SptP and SopE, secretion through another pathway could have confounded the results in other experimental systems.

The identification of the existence of a secretion signal within the amino terminus of SptP that can be recognized both by the cognate SPI-1 TTSS and, in the absence of the chaperone-binding domain, by the flagellar export apparatus, prompted us to examine the composition of such a signal in more detail. We identified a domain located between amino acids 10 and 25 that are essential for secretion. Amino acids 1 through 10 may also be required for secretion but as their coding sequence overlaps with that of the carboxyl terminus of the upstream SicP chaperone and/or are required for efficient expression of SptP, their specific involvement in secretion could not be unambiguously established. We found that introduction of frame-shifting mutations within the amino terminal secretion signal of SptP completely abolished secretion through both, the flagellar export and SPI-1 TTSS, suggesting that structural features of this polypeptide are essential for recognition by these two secretion systems. More studies will be required to ascertain whether secretion through flagella and the SPI-1 TTSS require the same features in the signal sequence.

In summary, our results demonstrate that TTSS-associated chaperones confer secretion pathway specificity, provide further support for the ancestral relationship between TTSSs and flagellar export systems, and demonstrate that both the amino terminus and the chaperone-binding domains of effector proteins are required for their proper targeting to their cognate TTSS.

Experimental procedures

Bacterial strains and growth conditions

All strains used in this study are isogenic derivatives of *S. enterica* serovar *typhimurium* SL1344 (Hoiseh and Stocker, 1981). Strains carrying mutations in *invA* (Galán *et al.*, 1992),

prgH (Kubori *et al.*, 1998), *invC* (Eichelberg *et al.*, 1994) (*invG* (Kaniga *et al.*, 1994), *invJ* (Collazo *et al.*, 1995), *fliGH* (Lockman and Curtiss, 1992), *sptP* (Kaniga *et al.*, 1996), or *sopE/SopE2/sopB* (Zhou *et al.*, 2001) have been previously described. A strain carrying a Δ *sptA* mutation was constructed following standard procedures (Kaniga *et al.*, 1994) (L. M. Chen and J. Galán, unpubl. results). Bacterial strains were grown in L-broth or L-agar supplemented with appropriate antibiotics. The following antibiotics were used at the indicated concentrations: streptomycin 50 μ g ml⁻¹; ampicillin 100 μ g ml⁻¹; kanamycin 50 μ g ml⁻¹; tetracycline 5 μ g ml⁻¹.

Plasmid constructions

The schematic representation of the various fusion constructs used in this study is detailed in Fig. 7. The plasmid expressing the SptP₁₋₃₅-PhoA chimeric protein was constructed by polymerase chain reaction (PCR) amplification of a ~800 base pair (bp) fragment containing the open reading frame (ORF) of *sicP* (cognate chaperone), 105 bp of *sptP* encoding the amino-terminal 35 residues, and the native *sicP-sptP* promoter using primers (*Xba*I) 5'-GCTCTAGAAG GATCTTTACGCTGACT-3' and (*Hind*III) 5'-CCCAAGCTTAG TATTTCTTAGCAAT-3'. Restriction endonuclease cleavage sites were incorporated into the primers for directional cloning into the *Xba*I and *Hind*III sites of the low copy plasmid pWSK29 (Wang and Kushner, 1991). A DNA fragment encoding a secretion signal-less PhoA (Russmann *et al.*, 2002) was cloned into the resulting plasmid generating a translational fusion of PhoA to the amino-terminal 35 residues of SptP. This plasmid expresses the cognate chaperone SicP and the chimeric protein under the control of its native promoter.

To construct *sptP* _{Δ 36-161} plasmid, the *phoA* fragment of the plasmid encoding SptP₁₋₃₅-PhoA (see above) was replaced with a PCR-amplified DNA fragment encoding the effector domains of SptP (residues 162 through 541) using primers 5'-CCCAAGCTTAACGATGTTGGAGCAGAAAGT-3' and 5'-CCGCTCGAGATCAGCTTGCCGTCGTCATAA-3'. The resulting construct encodes StpP _{Δ 36-161}, which lacks the chaperone-binding domain. As a control, a plasmid encoding full-length SptP was constructed by replacing *phoA* from the plasmid encoding SptP₁₋₃₅-PhoA (see above) with a PCR amplified DNA fragment encoding *sptP*₃₆₋₅₄₁ using primers 5'-CCCAAGCTTGATAAGGCATATGTTGCGCCT-3' and 5'-CCGCTCGAGATCAGCTTGCCGTCGTCATAA-3'. Plasmids encoding amino-terminal deletions of SptP (*sptP* _{Δ 25-35}, *sptP* _{Δ 15-35}, *sptP* _{Δ 10-35}, *sptP* _{Δ 1-35}) were constructed by inverse PCR using the following set of primers: (5'-CCCAAGCTTA TCATTCGACACACCAAC-3'); (5'-CCCAAGCTTAGACAACG TAAATTATT-3'); (5'-CCCAAGCTTATTCAATTTCTCTCC TC-3'); and (5'-CCCAAGCTTGATAAGGCATATGTTGCGC CT-3'). A plasmid encoding *sopE*₃₃₉₋₇₇ was generated by inverse PCR using primers 5'-CCCAAGCTTTACTGCGAG AATACTTTTTGCTAA-3' and 5'-CCCAAGCTTTTGACAAAT AAAGTCG TAAAGATT-3' on a template plasmid containing full-length *sopE* tagged with the M45 tag (DRSRDRLPPE TETRIL) (Obert *et al.*, 1994) at the C-terminus under the regulatory control of the native *sopE* promoter.

The *sptP* frame-shift mutations were introduced by replacing the nucleotide sequences corresponding to

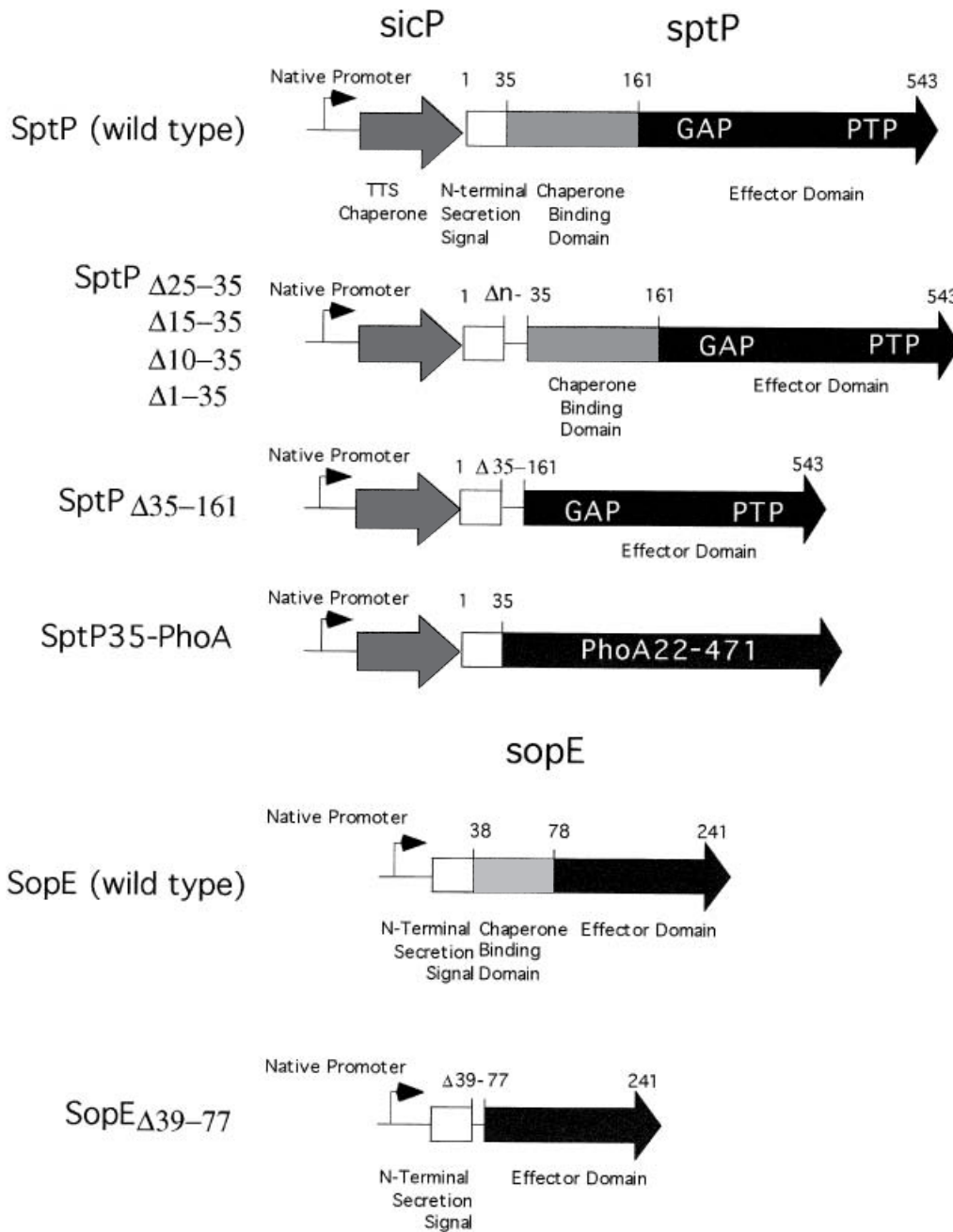


Fig. 7. Schematic diagram of SptP and SopE deletion constructs used in this study.

residues 10–35 with the following sequences: (+1) 5'-AAATT AACGTTGTCTTCGTTTTCAAAGTTGGTGTGTCGAAAG ATGCCCGACTTTATATTGCATAAGGAAAATAC-3', or (+2) 5'-AAAATTAACGTTGTCTTCGTTTTCAAAGTTGGTGTGTCGAAATGATGCCCGACTTTATATTGCTAAGGAAAATA-3'. Complementary sequences were annealed and ligated into a plasmid encoding *sptP* _{$\Delta 10-35$} .

All plasmids expressed the different SptP or SopE constructs under the control of their native promoters. All SptP constructs encode its cognate chaperone within the same context as the chromosomal locus. In all cases,

plasmid constructs were verified by nucleotide sequence analysis.

In vitro secretion assay

Salmonella strains harbouring various expression constructs were grown in L-broth supplemented with 0.3 M NaCl under conditions that stimulate the expression of the SPI-1 TTSS (Eichelberg and Galan, 1999). Whole cells and culture supernatants were separated by centrifugation. Whole cells were

resuspended in appropriated volume of SDS-PAGE loading buffer. Culture supernatants were passed through a 0.45- μ m filtered and precipitated in the presence of 10% trichloroacetic acid (TCA) and 0.1% deoxycholic acid overnight at 4°C. Precipitated culture supernatant pellets were washed with acetone after centrifugation and resuspended in SDS-PAGE loading buffer. Whole-cell and culture supernatant samples were run on 10% SDS-PAGE and transferred to nylon membrane using semidry transfer apparatus (Bio-Rad). Western blot analysis was performed using chemiluminescence detection system (Pierce). SptP₃₅-PhoA, SptP (including deletion constructs) and SopE were detected with monoclonal antibodies directed to PhoA, SptP and the epitope tag M45 (Obert *et al.*, 1994) respectively.

Gentamicin protection assay

The ability of *Salmonella* to enter into cultured epithelial cells was evaluated by the gentamicin protection assay as previously described (Galán and Curtiss, 1989).

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