Hydrophobic Residues of the Autotransporter EspP Linker Domain Are Important for Outer Membrane Translocation of Its Passenger*

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Jorge J. Velarde‡§ and James P. Nataro¶**‡‡

From the Center for Vaccine Development, Departments of ‡Biochemistry, §Pediatrics, ¶Medicine, and **Microbiology & Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201

The autotransporter family of proteins is an important class of Gram-negative secreted virulence factors. Their secretion mechanism comprises entry to the periplasm via the Sec apparatus, followed by formation of an outer membrane β barrel, which allows the N-terminal passenger domain to pass to the extracellular space. Several groups have identified a region immediately upstream of the β domain that is important for outer membrane translocation, the so-called linker region. Here we characterize this region in EspP, a prototype of the serine protease autotransporters of enterobacteriaceae. We hypothesized that the folding of this region would be important in the outer membrane translocation process. We tested this hypothesis using a mutagenesis approach in conjunction with a series of nested deletions and found that in the absence of a complete passenger, mutations to the C-terminal helix, but not the upstream linker, significantly decrease secretion efficiency. However, in the presence of the passenger mutations to the amino-terminal region of the linker decrease secretion efficiency. Moreover, amino acids of hydrophobic character play a crucial role in linker function, suggesting the existence of a hydrophobic core or hydrophobic interaction necessary for outer membrane translocation of autotransporter proteins.

However, pore formation in planar lipid bilayers and the crystal structure of NaIP suggest a diameter between 8.4 and 10 Å (6). Remarkably, a passenger domain over 100 kDa in size can translocate through the outer membrane into the extracellular space utilizing this pore. Recently, however, Oomen et al. (6) have suggested that an accessory protein in the outer membrane may aid the translocation process, perhaps acting with the β pore (6, 12). In either case, proper barrel insertion into the outer membrane appears to be crucial, but events taking place in the periplasm and at the outer membrane remain obscure.

Several laboratories have shown that in various autotransporters (namely BrkA (13, 14), Ssp (15), IgA protease (11), and AIDAI (16–18)), a region immediately upstream of the β domain is important for the secretion process. Sequential deletions in this region, termed by some the linker domain, result in a diminution of translocation efficiency of either a heterologous epitope (16, 17) or a portion of the natural autotransporter passenger domain (13). This linker, along with the β domain, has been termed the translocation unit (16). The exact contribution of the linker domain is still under investigation, and importantly, few studies have addressed secretion of native passengers. The C-terminal portion closest to the β domain was shown for NaIP to be helical and to interact with residues extending into the hydrophilic pore of the β domain (6). The same secondary structure has been strongly predicted for the majority of autotransporters (2, 19, 20). In addition to translocation, Konieczny et al. (18) have suggested that the linker may have a role in stabilization of the β domain in the outer membrane. Other groups studying BrkA from Bordetella pertussis (14) and Ssp from Serratia marcescens (15) have suggested that the more N-terminal region of the linker, upstream of the putative α-helix, may function as an autochaperone. This function could be important for the autotransporter to fold into its proper conformation in the extracellular space and to confer resistance to outer membrane proteases. Oliver et al. (14) suggest that the chaperone function is widespread among autotransporters based on in silico homologies, although it has been shown experimentally only for BrkA and Ssp (14, 15).

Recognizing the critical importance of and uncertainty regarding the autotransporter linker domain, we here define the linker region of the autotransporter EspP and characterize its properties as they relate to outer membrane translocation. EspP is a member of the serine protease autotransporters of enterobacteriaceae (SPATE)1 subfamily of autotransporters (2). After translocation, it is cleaved away from its β domain between residues 1023 and 1024 and secreted from enterobacteriaceae; MHT, epitope containing the Myc epitope, His epitope, and TEV protease recognition sequence; MBP, maltose-binding protein.

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‡‡ To whom correspondence should be addressed: Center for Vaccine Development, University of Maryland, Baltimore, 685 W. Baltimore St., HSF 444, Baltimore, MD 21201. E-mail: jnataro@medicine.umaryland.edu.

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morrhagic E. coli as a 104-kDa mature toxin (21, 22). Although its in vivo target and mechanism of action are unknown, it has been shown to cleave coagulation factor V in vitro (21). It has significant overall homology and shares an active serine protease motif with other members of the SPATE subfamily of autotransporters (2). We employed a mutagenesis approach to first define a putative linker domain for EspP; we then investigated the nature of its primary and secondary structure, as well as its localization (38). This involved (i) generating linker constructs and deletions, transformed into chemically competent HB101 (24) once proper construction was confirmed. Secretion analysis of these mutants was performed by osmotic cold shock (37). Pellets from a secretion experiment were utilized and resulted in an extraction of periplasmic contents. 500 μl of supernatant was filtrated through a 0.45-μm filter, then subjected to anti-His6 immunoblot analysis as described above. Whole-cell limit tryptic digest (12, 36) was done by resuspending cell pellets from a secretion experiment in 500 μl of 10 mM Tris, pH 7.4, 0.5 mM MgCl₂, 100 μg/ml trypsin, and incubating on ice for 15 min. The reaction was stopped by adding soybean trypsin inhibitor to 200 μg/ml. The cells were centrifuged at 8500 × g for 10 min at 4 °C. The wash was removed and filtered through a 0.2-μm filter, and kept at −70 °C for as many rounds of experiments as needed. The cell pellets were resuspended in 500 μl of 10 mM Tris, pH 7.4, 15 μl was added to an equal volume of 2× Laemmli buffer and subjected to anti-His6 immunoblot analysis as described above. Whole-cell limited tryptic digest (12, 36) was done by resuspending cell pellets from a secretion experiment in 500 μl of 10 mM Tris, pH 7.4, 0.5 mM MgCl₂, 100 μg/ml trypsin, and incubating on ice for 15 min. The reaction was stopped by adding soybean trypsin inhibitor to 200 μg/ml. The cells were centrifuged at 8500 × g for 10 min at 4 °C. The wash was removed and filtered through a 0.2-μm filter, and kept at −70 °C for as many rounds of experiments as needed. The cell pellets were resuspended in 500 μl of 10 mM Tris, pH 7.4, 15 μl was added to an equal volume of 2× Laemmli buffer and subjected to anti-His6 immunoblot analysis as described above. Whole-cell limited tryptic digest (12, 36) was done by resuspending cell pellets from a secretion experiment in 500 μl of 10 mM Tris, pH 7.4, 0.5 mM MgCl₂, 100 μg/ml trypsin, and incubating on ice for 15 min. The reaction was stopped by adding soybean trypsin inhibitor to 200 μg/ml. The cells were centrifuged at 8500 × g for 10 min at 4 °C, then resuspended by adding anti-His6 antisera (Qiagen) at a dilution of 200 ng/ml; primary anti-EspP antiserum was used at a 1:5000 dilution; and primary MBP antiserum (New England Biolabs) was used at a 1:17,000 dilution. Secondary anti-mouse antibody (KPL) for the anti-His6 immunoblot was used at a concentration of 40 ng/ml, secondary anti-rabbit antibody (KPL) for the EspP and MBP immunoblot was used at a concentration of 28.6 ng/ml. Results were visualized with ECL+ (Amersham Biosciences) and after exposure to Kodak autoradiography film for an appropriate time.

**RESULTS**

**EspP Contains a Functional Linker Domain as Part of Its Translocation Unit**—To characterize the secretion of SPATE proteins, we first asked whether EspP contained a linker region required for outer membrane translocation, as has been shown for numerous other autotransporters (11, 13–18). To this end, we utilized inverse PCR to construct a series of deletion mutants in the C terminus of the passenger domain, corre-
responding to the putative EspP linker as determined by homologies to other autotransporters (14, 16). Interestingly, Dutta and Nataro2 have shown that there is a proteinase K-sensitive site at the N terminus of this region in the closely related autotransporter Pet, proposing that this site separates two discrete domains. The EspP gene was manipulated in clone PB28-5 (21). Primers were designed to delete the passenger domain from residue 74 to 821 (Fig. 1A) and then sequentially toward the C terminus in the suspected linker domain; this leaves the signal sequence and 19 downstream residues intact. An MHT epitope was introduced into a NotI site created at the deletion point during PCR. Resulting constructs were transformed into HB101.

To assay secretion, cultures at equal cell densities were pelleted, resuspended in fresh L-broth, and incubated for 3 h. EspP in supernatants was quantitated by ELISA; secretion of deletion mutants was expressed as percentage secretion of a Δ242–291 deletion harboring the MHT epitope at the deletion site (essentially the full-length passenger). Both immunoblot analysis using anti-His antibodies and ELISA using anti-Myc antibodies (Fig. 1B) revealed that with successive passenger deletions extending past residue 868, secretion efficiency was decreased. Translocation was essentially undetectable when only 4 residues prior to the known EspP β cleavage site remained. Outer membrane preparations of HB101 expressing the deletion constructs were prepared by differential Triton X-100 solubilization of the inner and outer membranes (32, 33). All constructs displayed comparable amounts of β domain in the outer membrane, suggesting that the difference in secretion efficiency was not due to differences in transcription, translation, inner membrane secretion, β domain outer membrane insertion, or processing from the β domain (Fig. 1C). We did not detect maltose-binding protein (MBP) in the supernatants of these mutants (data not shown), suggesting that detection of the passenger was not due to outer membrane permeabilization. These data agree with the prediction of a putative linker domain for EspP, the minimal functional length of which is between 27 and 5 residues upstream of the β processing site. As previously shown with AIDA-I (16), we clearly observed a decrease in secretion efficiency with deletions of increasing size, suggesting that the upstream regions of the linker domain may have a role in the secretion process.

The Putative C-terminal α-Helical Region of the Linker Is Important for Secretion of a Small Foreign Epitope—The extreme C terminus of the linker domain immediately upstream of the β domain has been predicted to be an α-helix in numerous autotransporters (2, 19, 20) and was elucidated as such for NalP (6). This region may play a role in the translocation of the passenger domain by providing the necessary length to traverse the barrel toward the extracellular space (17). Konieczny et al. (18) have also suggested that this putative helix may help to stabilize the β domain in the outer membrane, and Oomen et al. (6) have inferred, from the crystal structure, its interaction with the inner face of the β domain. We hypothesized that in EspP this predicted helical region would contribute to translocation and that disruption of this putative secondary structure would significantly diminish secretion efficiency. We chose a deletion construct (Δ74–928) that was still easily detectable by both immunoblot and ELISA (Fig. 2A). This construct maintains 95 residues upstream of the β processing site. A series of nonconservative site-directed mutations were introduced starting at residue 933 and extending to residue 1031. The secretion efficiency for these mutations was analyzed by ELISA in the same manner as for the deletion constructs above; the data were normalized against construct Δ74–928 containing no site-directed mutations and are reported as a percentage thereof (Fig. 2B). The most significant effects were seen for residues D1014K, F1018P, and E1021K. These residues are all found in the C terminus of the linker within the putative α-helix. The outer membrane preparations showed comparable levels of β domain for all constructs except D1014K (Fig. 2C). We did not detect MBP in the supernatants (data not shown). The absence of the D1014K β domain in the outer membrane is not attributed to improper cleavage of the β domain from the passenger, since the unprocessed species could not be detected. These data suggest a role for the C-terminal helical region in secretion of a small epitope.

The N-terminal Region of the Linker Is Important for Secretion of a Full-length Passenger Domain—We presumed that if mutations to the putative α-helix of the linker were detrimental for secretion of Δ74–928, then the same would be true for a construct containing the larger passenger domain. To test this, we removed the active protease site from EspP (Δ242–291, Fig. 2A) in order to prevent autodegradation. At the deletion site, we inserted the MHT epitope and mutagenized the passenger C terminus using the same primers employed in mutagenesis of Δ74–928. Secretion efficiency was analyzed by anti-Myc ELISA (Fig. 2B). Surprisingly, we found that from the putative helical region (residues 1005–1031), only D1014K still conferred a significant decrease in secretion efficiency. However, a number of mutations upstream of D1014 now also demonstrated a significant effect on secretion (Fig. 2B). Again, the only construct showing a significant decrease in outer membrane β domain insertion was D1014K (Fig. 2C), which did not yield an unprocessed passenger/β domain. We were unable to detect MBP in the supernatant for the nonpermissive mutants (data not shown). In light of these results, we hypothesized that small nested deletions in the linker region would yield a similar effect on secretion efficiency. Deletions were again constructed (Δ928–946, Δ946–971, Δ971–997, and Δ997–1019) by inverse PCR, and an MHT epitope was inserted at the deletion site (Fig. 3A). Secretion was analyzed by ELISA and reported as a percentage of Δ242–291 secretion (Fig. 3B). All of the nested deletions displayed a clear decrease in secretion efficiency. Outer membrane preparations showed equivalent outer membrane β domain insertions and the MBP lysis controls were negative (data not shown). These data support our inference that the N-terminal region of the linker plays a role in translocation of a full-length passenger domain, although not necessarily a truncated construct.

Amino Acids of Hydrophobic Character Are Crucial to Linker N-terminal Domain Function—Upon closer examination, we found that the large majority of mutations affecting secretion in the upstream linker region of EspP were to hydrophobic or aromatic amino acids. We hypothesized that the hydrophobic amino acids upstream of the putative helix were important in the secretion process of a full-length passenger domain and tested this hypothesis via site-directed mutagenesis. We chose various amino acids and created both conservative and nonconservative mutations in the Δ242–291 construct. Secretion analysis was accomplished as above. When the hydrophilic amino acids were mutated to either hydrophobic or hydrophilic residues (Y995E, Y995T, Y995F, N948D, N948L, T958Y, and T958L), the mutations were tolerated with respect to secretion (Fig. 4). However, when the hydrophobic amino acids were mutated to aspartic acid residues (L951D, L992D, and F963D), there was a substantial diminution of secretion efficiency (10–20% of wild type), which was not seen for conservative mutations of the same residues (L951I, F963W, F963Y, and L992I). All outer membrane preparations showed comparable amounts of

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1 P. R. Dutta and J. P. Nataro, unpublished observations.
FIG. 1. Definition of the EspP linker. Sequential deletions of the passenger and linker domain were designed by inverse PCR (A). An MHT epitope was inserted in the deletion site and utilized for secretion analysis (B) both by α-His$_6$ immunoblotting and α-Myc ELISA. The outer membranes of HB101 cells expressing these constructs were analyzed by SDS-PAGE (C) and shown to contain comparable amounts of β domain.
FIG. 2. Mutational analysis of linker domain. A, two constructs were selected for mutational analysis of the linker region. Δ74–928 maintains the signal sequence and 19 amino acids downstream as well as 26 residues of the linker domain previous to the β cleavage site. Δ242–291 is missing the active serine motif but maintains a majority of the passenger domain. B, secretion efficiency was analyzed as described by anti-Myc ELISA. Mutations significantly affecting Δ242–291 secretion are marked with a star. The putative α-helical region of the EspP linker is noted below the graph. C, the outer membrane of HB101 cells expressing all nonsecreting constructs was isolated and analyzed by SDS-PAGE. D1014K was the only mutation in either Δ74–928 or Δ242–291 to exhibit a diminution in outer membrane β domain.
of β domain insertion and all of the MBP lysis controls were negative (data not shown). We attempted to detect nonsecreted mutant constructs within whole-cell HB101 lysates by anti-His6 immunoblot analysis but were unable to do so, suggesting that the nonsecreted protein is being rapidly degraded.

Nonsecreted Linker Mutants Can Be Recovered from the Periplasm of UT5600 (OmpT/H11002) Cells—Oliver et al. (14) have described extracellular recovery of constructs harboring deletions in the analogous linker region of BrkA when they were expressed in strain UT5600, an OmpT(−) strain. OmpT is an outer membrane amphipathic β barrel that has been shown to function as an extracellular protease (40). We chose three of our nonsecreted linker mutations (L951D, F963D, and L992D) and assayed their secretion from UT5600, as previously described. The results were normalized to secretion of nonmutated 242–291 from strain UT5600. In contrast to Oliver et al. (14), we were unable to rescue secretion of our mutants from this strain (data not shown). In an attempt to localize the nonsecreted protein, we washed the cell pellet in 0.1% Triton X-100 for 15 min at room temperature, previously shown to release misfolded SPATEs (35). We observed a small amount of nonmutated ∆242–291 in the cell wash solution, whereas the mutated nonsecreted constructs could not be detected (Fig. 5A). A limited tryptic digest of the solvent exposed surface proteins on UT5600 also demonstrated recovery of a small amount of nonmutated ∆242–291 tryptic fragments, yet tryptic fragments of the nonsecreted mutants could not be found (Fig. 5B). However, these species were detected in the whole-cell lysates (Fig. 5C) and the periplasmic fraction (Fig. 5D) of UT5600 cells, whereas wild-type ∆242–291 was barely detectable. These data would suggest that the nonsecreted mutants were not localized on the surface of the bacteria but are transiently present in the periplasm.

Hydrophobic Residues in the SPATE Linker Domain Are Conserved—The SPATE family has previously been studied for homology (2), clearly showing an increased amino acid conservation in the C-terminal region of the autotransporters, with the β domain displaying the highest homologies. Based on our observations suggesting the existence of a hydrophobic core, we wished to examine the conservation of amino acid hydrophobicity and hypothesized that this region would have a significant conservation of amino acid character at the residues we mutated. Alignment of the SPATEs in the linker region was accomplished using ClustalW (39) (EMBL-EBI; available on the World Wide Web at www.ebi.ac.uk/clustalw/) and GeneDoc (available on the World Wide Web at www.psc.edu/biomed/genedoc/). As predicted, this analysis revealed substantial conservation of hydrophobic character, including residues we had targeted for mutagenesis (Fig. 6).

DISCUSSION

A growing body of literature implicates the C-terminal linker of the autotransporter passenger domain in secretion, yet the precise function of this region is not well characterized. We first sought to perform deletion experiments in the putative EspP linker domain to demonstrate the presence and role of this
region in the translocation of EspP. We constructed a series of deletions, removing a majority of the passenger domain and successively decreasing the size of the linker domain. With successive deletions closer to the C terminus, we observed a decrease in translocation efficiency until secretion was essentially undetectable (Fig. 1). As has been shown for other auto-transporters (11, 13–18), EspP therefore possesses a linker as an integral component of the translocation unit, with a minimal length between 27 and 5 residues upstream of the processing site (1023/1024). We also found notable the consistent diminution of detectable epitope with successive deletions after residue 868. Although there is a minimal linker region crucial

Fig. 4. Hydrophobic residues of the linker domain have a significant effect on secretion. Both conservative and nonconservative mutations were made to select hydrophobic and hydrophilic amino acids in construct Δ242-291, and secretion was analyzed by α-MyC ELISA as described.

Fig. 5. Nonsecreted mutants can be localized to the periplasmic space of UT5600 cells. A, UT5600 cells were washed with 10 mM Tris, pH 8.0, 0.1% Triton X-100, 5 mM MgCl₂, and the cell wash was analyzed by anti-His₆ immunoblotting. B, UT5600 cells were subjected to whole-cell limited tryptic digest and only wild-type Δ242-291 was detected on anti-EspP immunoblot. C, anti-His₆ immunoblots of whole-cell lysate demonstrated that the nonsecreted proteins could be found intracellularly. D, UT5600 cells expressing the various constructs were then subjected to cold osmotic shock, and the nonsecreted mutants could be localized by anti-His₆ immunoblot in the periplasmic extract.
for outer membrane translocation, this observation was a first indication of the involvement of the SPATE upstream linker in translocation efficiency.

The C-terminal region of the linker has been shown to be helical for NalP and is inferred from the crystal structure to electrostatically interact with the inner face of the β barrel (6); this helical motif is predicted to be conserved across a large number of autotransporters (2, 19, 20). We hypothesized that this putative α-helix would be the essential motif in the linker domain. To test this hypothesis, we designed mutations in a construct that was still well secreted and easily detected by ELISA and immunoblot (Δ74–928). This deletion includes the signal sequence and 19 residues downstream of the signal cleavage site, and the β domain. Mutations were designed to be disruptive of secondary structures throughout the linker region. As predicted, D1014K, F1018P, and E1021K had the most detrimental effects on translocation efficiency. These data suggest that proper secretion of the upstream region to be different from that of the helix. To characterize this effect further, we designed a series of small nested deletions from residue 928 to residue 1019 and found that all of the nested deletions in this region decreased translocation efficiency. These data suggest that proper secretion of the complete passenger domain (but not the truncated construct) requires, at least in part, the portion of the linker region upstream of the putative helix, and call into question the relevance of prior studies utilizing truncated reporter constructs.

Upon further inspection of secretion-deficient mutations, we found that the critical amino acids were consistently hydrophobic in character. We therefore designed both conservative and nonconservative mutations to select residues and observed their effects on secretion (Fig. 4). None of the mutations involving any of the hydrophilic residues (Y995E, Y995T, Y995F, N948D, N948L, T958Y, and T958L) had a significant effect on translocation of the MHT epitope in Δ242–291; the effect of conservative mutations to hydrophobic amino acids (L951I, F963W, F963Y, F972W, F972Y, and L992I) was also insignificant. On the other hand, nonconservative mutations to hydrophobic amino acids (L951D, F963D, and L992D) significantly decreased the efficiency of translocation. We and others have observed that SPATE autotransporters share significant homology throughout the passenger domain, especially at the C terminus of the passenger (2). Pileup analysis revealed conservation of hydrophobic amino acids in this region including residues we had targeted for mutagenesis (Fig. 6). Our data therefore suggest the existence of a hydrophobic core necessary for proper conformation of this region or a site of either intramolecular or intermolecular hydrophobic interaction. In any case, it is likely not only that this region is important for efficient secretion in the presence of the passenger domain but that the hydrophobic amino acids in this region are crucial. We have termed this region the hydrophobic secretion facilitation domain except for a deletion from residues 242–291 where the MHT epitope was inserted (Δ242–291). This deletion removes the serine protease motif and thereby prevents autoligation.5 To our surprise, secretion effects were significantly different from those observed for the smaller construct (Fig. 2). First, aside from D1014K, mutations to the C-terminal α-helix that had resulted in diminution of secretion in Δ74–928 no longer affected secretion. However, mutations between residue 928 and residue 995 now resulted in a significant decrease in translocation efficiency (Fig. 3), suggesting the function of the upstream region to be different from that of the helix. To characterize this effect further, we designed a series of small nested deletions from residue 928 to residue 1019 and found that all of the nested deletions in this region decreased translocation efficiency. These data suggest that proper secretion of the complete passenger domain (but not the truncated construct) requires, at least in part, the portion of the linker region upstream of the putative helix, and call into question the relevance of prior studies utilizing truncated reporter constructs.

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**Fig. 6.** The putative linker domains of the SPATEs have a conservation of hydrophobic residues. Sequence alignments were accomplished using ClustalW and analyzed using GeneDoc. Hydrophobic residues are shaded. Mutated residues that significantly affected secretion efficiency of Δ242–291 are indicated with an asterisk.
domain (HSF) and are currently working on defining its exact limits and function.

We attempted to determine the location of three nonsecreted HSF mutants in HB101 and UT5600. Although we could recover neither the wild-type nor the mutants from HB101, each of the nonsecreted mutants could be recovered from the periplasmic space of UT5600 cells, but none from the surface of the bacteria or supernatant (Fig. 5). This suggests that alterations to the HSF region have a significant effect on some aspect of the EspP outer membrane translocation process. The β domains for all three nonsecreted constructs could be localized to the outer membrane, verifying that the event affected does not perturb barrel insertion.

Current models of autotransporter secretion still leave some important questions unanswered. Foremost, the conformation of the passenger domain during the secretion process and its relationship to barrel insertion are still unclear. Several investigators have proposed size and conformational limitations to the polypeptides capable of being efficiently secreted by the autotransporter barrel (41, 42), and the dimensions of the barrel aperture are suggested to be 2 nm or less by several methods, including crystallography (6, 9, 10). Yet experimental evidence has also suggested that some folding of both native passengers and foreign polypeptides is tolerated (43–45). This folding may result in a secretion-competent, protease-resistant state (45), which would allow the passenger to efficiently traverse through the available pore. Indeed, the small size of the pore is in seeming contradiction to the ability of folded passengers to be secreted through the channel. One model that has not been adequately addressed in the autotransporter field would entail coupled folding and insertion of the barrel with passenger translocation, possibly assisted by an outer membrane chaperone as well, necessitating a hydrophobic interaction with the remaining passenger domain. The result of HSF disruption would then be similar to the disruption of interactions with a heterologous chaperone, namely a decrease in secretion efficiency. Future work on autotransporter secretion will be needed to address these various hypotheses, including the identification of critical intermediates.

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Jorge J. Velarde and James P. Nataro

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