Dautin et al., Supplementary Fig. 1
Supplementary Fig. 1: The introduction of TEV sites into EspP*Δ1 does not impair passenger domain translocation. AD202 producing His-tagged EspP*Δ1 or a derivative of His-tagged EspP*Δ1 containing the indicated TEV site insertion was subjected to pulse-chase labeling after the addition of IPTG, and proteinase K was added to half of each sample. EspP-containing polypeptide were then immunoprecipitated with the C-terminal antipeptide antiserum. In this experiment the accessibility of the passenger domain to protease digestion reported the kinetics and efficiency of passenger domain translocation. Although the TEV5 insertion appears to slightly delay passenger domain translocation, the presence of the His tag and the other TEV insertions does not significantly affect protein biogenesis (for a comparison with unmodified EspP*Δ1, see Skillman et al., 2005). Lanes 1-4, no proteinase K added (-PK); lanes 5-8, proteinase K added (+PK).
Dautin et al., Supplementary Fig. 2
Supplementary Fig. 2: Protease inhibitors do not significantly affect the processing of EspPΔ1. AD202 transformed with pJH62 was grown in the presence of the indicated protease inhibitors and subjected to pulse-chase labeling after the addition of IPTG. EspP-containing peptides were immunoprecipitated using a C-terminal antipeptide antiserum. In this experiment the appearance of the discrete β domain fragment reported the kinetics and efficiency of passenger domain cleavage.
Dautin et al., Supplementary Fig. 3
Supplementary Fig. 3: Mutation of Asp$^{1120}$ does not affect passenger domain translocation.

AD202 producing EspPΔ1 or a derivative of EspPΔ1 harboring the indicated mutation was subjected to pulse-chase labeling after the addition of IPTG, and proteinase K was added to half of each sample. EspP-containing polypeptides were then immunoprecipitated with the C-terminal antipeptide antiserum. Lanes 1-4, no protease added (-PK); lanes 5-8, proteinase K added (+PK).
Dautin et al., Supplementary Fig. 4
Supplementary Fig. 4: Passenger domain cleavage does not involve interactions between EspP monomers. (A) To determine if two EspP molecules are necessary to create a functional protease, EspPΔ1(D1120N) and wild-type EspPΔ1 were co-expressed. If EspP dimerization is necessary for proteolytic activity, then the synthesis of the mutant protein would generate catalytically inactive heterodimers and consequently inhibit the processing of the wild-type protein. AD202 was transformed with RB11-EspPΔ1-6His, a plasmid encoding a His-tagged version of EspPΔ1 under the control of the lac promoter and/or pBAD-HA-EspPΔ1(D1120N), a plasmid encoding EspPΔ1(D1120N) under the control of the araBAD promoter. Cells were grown in LB, and expression of the plasmid-borne genes was induced by adding 100 μM IPTG and/or 0.2% arabinose to cultures for 30 min as appropriate (0.2% glucose was added to cultures of doubly-transformed cells that were not supplemented with arabinose). Passenger domain cleavage was then analyzed by Western blot using antisera directed against the HA tag (top panel) and the His tag (bottom panel). Western blot analysis using the C-terminal anti-EspP antiserum indicated that >20-fold more mutant EspPΔ1 was synthesized than wild-type protein (data not shown). The results show that even though the catalytically inactive D1120N mutant was produced in vast excess, it did not inhibit the processing of the wild-type protein (compare bottom panel, lanes 2 and 4). The lack of a dominant negative effect indicates that dimerization of EspP is not required for passenger domain cleavage. (B) AD202 was transformed with pJH62, pBAD-EspP*Δ1, a plasmid encoding EspP*Δ1 under the control of the araBAD promoter, and/or pTrc-HA-EspPΔ1(D1120N). If passenger domain cleavage occurs through an intermolecular reaction, then EspP*Δ1, which contains the putative catalytic D1120 residue but lacks a functional cleavage site, should be able to process the passenger domain of EspPΔ1(D1120N), which is catalytically inactive but which still has an intact cleavage site. Expression of the plasmid-borne genes was induced by adding 5 μM IPTG and/or 0.2% arabinose to cultures for 30 min. Passenger domain cleavage was then analyzed by Western blot using antisera directed against the HA tag (top panel) and the EspP C-terminal peptide (bottom panel). The observation that none of the ~30 kDa fragment corresponding to the cleaved β barrel was detected in cells producing both forms of the protein indicates that passenger domain cleavage does not involve an intermolecular reaction (compare bottom panel, lanes 2 and 4).
Dautin, et al., Supplementary Fig. 5
Supplementary Fig. 5: Mass spectrum of the cleaved EspPΔ1 passenger domain reveals two species that differ in mass by 18 Da. (A) A detail of the electrospray mass spectrum of the purified EspPΔ1 passenger domain for charge states +17 and +16. Dashed lines show the calculated positions of two distinct species. Similar twinned peaks were apparent throughout the spectrum (data not shown). (B) The masses of the two species observed in part A were calculated from the whole mass spectrum using the vendor’s software. The smaller peak in each pair has a mass of 15027.2 Da, which is essentially identical to the theoretical mass of the polypeptide (15028.7 Da). The more intense peak has a mass of 15,009.4. The mass difference of 18 Da suggests that the larger peak could be a mono-dehydrated version of the EspPΔ1 passenger domain.
Dautin, et al., Supplementary Fig. 6
Supplementary Fig. 6: The loss of 18 Da in the smaller EspPΔ1 passenger domain species is due to a modification of the C-terminal residue. The purified EspPΔ1 passenger domain was digested with trypsin and analyzed by nanoscale LS/MS/MS. Initial experiments revealed the presence of both a 806.4 Da peptide, which corresponds to the C-terminal tryptic fragment (AFLNEVN), and another peptide which is smaller by 18 Da (788.4 Da). These results provided evidence that the 18 Da difference in the two EspPΔ1 passenger domain species is attributable to a modification of one of the last seven residues of the polypeptide. Two MS/MS spectra illustrating the results of fragmentation of the 806.4 ion (top) and the 788.4 ion (bottom) are shown. B-series ions (which contain the N-terminus of the peptide), y-series ions (which contain the C-terminus of the peptide) and internal fragments are indicated. The perfect correspondence of a nearly complete set of b-series ions (b2-b6) in both spectra indicates that the first six residues of the 806.4 and 788.4 Da species are identical and places the modification at the final residue. In addition, the presence of unique ions in the bottom spectrum whose masses are 18 Da smaller than the expected y4 and y5 ions (labeled “y4-18” and “y5-18”) provides further evidence of a modification at the last residue of the EspPΔ1 passenger domain. This modification is likely due to the presence of a succinimide group (which is a dehydrated asparagine).
A

Trypsin

-  +  -  +  -  +  -  +

HAPr

HAPr(D733E)

HAPr(D733N)

HAPr(N631A)

ProPrn (P.93)

B

Trypsin

-  +  -  +  -  +  -  +

BrkA-6His

BrkA(D833E)-6His

BrkA(D833N)-6His

BrkA(N731A)-6His

BrkA(N731Q)-6His

ProBrkA

Dautin et al., Supplementary Fig. 7
Supplementary Fig. 7: Mutations in pertactin and BrkA that abolish processing do not affect passenger domain translocation. BL21(DE3)codon+RIL transformed with a plasmid encoding HA-tagged pertactin or the indicated mutant (panel A) or His-tagged BrkA or the indicated mutant (panel B) was grown as described in the legend to Fig. 8. After the induction of autotransporter synthesis, 1 ml culture aliquots were placed on ice and either treated with 40 μg/ml trypsin for 20 min or mock-treated. Samples were then TCA precipitated and analyzed by Western blot using anti-HA or anti-His tag antisera. The nearly complete susceptibility of the mutant forms of pertactin and BrkA to protease treatment demonstrates that the mutations did not affect passenger domain translocation.
Dautin, et al., Supplementary Fig. 8
Supplementary Fig. 8: Mutations that inhibit the cleavage of EspPΔ1 have the same effect on full-length EspP. AD202 was transformed with a plasmid encoding full-length EspP or the indicated EspP mutant, and autotransporter synthesis was induced by the addition of 100 µM IPTG. Passenger domain cleavage was then analyzed by Western blot using the C-terminal antipeptide antiserum (top panel) or an antiserum directed against the N-terminus of the passenger domain (bottom panel).