SUPPLEMENTARY MATERIALS AND METHODS

Plasmid construction

To make pC6H1, a BamHI site was first introduced into pJH62 at the last codon of *espPΔ1* using the oligonucleotides Bam1F and GCBam1F (the sequence of all oligonucleotides is listed in Supplementary Table 1). The complementary oligonucleotides Bam6H1 and Bam6H2, which encode a hexahistidine tag, were then ligated into the BamHI site. To construct pBAD-EspP*Δ1, we amplified *esp*PΔ1* with primers DirXbaIEspP and RevSEQTrc using pKMS3 as a template and cloned the resulting PCR fragment into the Xba I and Hind III sites of pBAD33 (Guzman et al., 1995). To generate RB11-EspPΔ1−6His, in which *espPΔ1-6His* is expressed under the control of a *lac* promoter, an EcoR I-Hind III fragment was excised from pC6H1 and cloned into the corresponding sites of RB11 (Newitt and Bernstein, 1998). To make pTrcHA-EspPΔ1(D1120N), two complementary oligonucleotides (HA1 and HA2) were hybridized and inserted into the Eag I site of pJH62 containing the D1120N mutation. To construct pBAD-HA-EspPΔ1(D1120N), we first amplified *espPΔ1(D1120N)* with primers DirXbaIEspP and RevSEQTrc using pTrc-EspPΔ1(D1120N) as a template and cloned the PCR fragment into the Xba I and Hind III sites of pBAD33. The oligonucleotides HA1 and HA2 were then ligated into the Eag I site of the resulting plasmid. Specific point mutations were introduced into the β domain of full-length EspP by subcloning an ~800 bp Kpn I-Hind III fragment from the appropriate mutant version of pJH62 into pRLS6 (Szabady et al., 2005). The N1023A and N1023Q mutations were introduced into pRLS6 using the PCR overlap extension method.

To construct pTrcBrkA-6His and pTrcHAPrn, *brkA* and *prn* were amplified using genomic DNA from *B. pertussis* strain Tomaha I as a template and the oligonucleotides DirBrkA-1 and RevBrkA-1 or DirPrn-1 and RevPrn. The resulting PCR fragments were inserted into pCR-BluntII-TOPO (Invitrogen) to generate pCRBluntIIBrkARev and pCRBluntPrnRev. These plasmids were then digested with Xba I and Hind III, and the fragment containing *brkA* or *prn* was cloned into the cognate sites of pTrc99A (Pharmacia) to make pTrcBrkA and pTrcPrn. Subsequently, a BstB I site was introduced into pTrcBrkA and pTrcPrn at positions corresponding to the C terminus of BrkA or the N-terminus of the Prn passenger domain using the oligonucleotides DirBrkABstBI and RevBrkABstBI or DirPrn-BstBI and RevPrn-BstBI. Ligation of the complementary oligonucleotides Dir6His-BstBI and Rev6His-BstBI or DirPrn-HA and RevHA-Prn into the BstB I site of the resulting plasmids generated pTrcBrkA-6His and pTrcHAPrn. To place a Shine-Dalgarno sequence upstream of *brkA*, we first made pTrcOmpA-ΔNdeI by cloning the ~1 kb EcoR I-Hind III fragment of pJH36 (Lee and Bernstein, 2002) into pHDB66 (Szabady et al., 2005). We then cloned the Nde I-Hind III fragment of pTrcBrkA-6His containing *brkA* into the cognate sites of pTrcOmpA-ΔNdeI to generate pTrcBrkA-6His*. Specific point mutations were introduced into pTrcBrkA-6His* and pTrcHAPrn using the PCR overlap extension method.

Purification of the cleaved EspPΔ1 passenger domain

To purify the His-tagged EspPΔ1 passenger domain, BL21 transformed with pND2 was grown in M9 medium supplemented with all 20 amino acids, and 10 μM IPTG was added at OD_{550}=0.2. After 3 h, cells were pelleted (5000 x g, 4°C, 30 min), and the supernatant was passed through a 0.2 μm membrane and applied to a 3 ml Ni-NTA column equilibrated with 50 mM K_2HPO_4 pH

7.5. The column was then washed with buffer E (50 mM K_2HPO_4 pH 7.5, 200 mM NaCl) containing 20-50 mM imidazole, and the protein was eluted in buffer E containing 200 mM imidazole.

Whole protein mass spectrometry

His-tagged EspPΔ1 passenger domain purified as described above was first further purified on an Agilent HP1100 HPLC instrument. Approximately 50 pmol of protein was applied to a reversed phase column (Zorbax 300SB-C3 2.3 x 100 mm with a 10 mm pre-column) at 0.2 ml/min in 5% acetic acid, 5% acetonitrile at 40ºC and eluted with a 5-80% acetonitrile gradient. The outlet of the column was plumbed into an Agilent MSD (single quad) mass spectrometer which scanned between 300 and 1700 M/Z. Multiple peaks originating from different charge states of the protein were transformed into a neutral mass spectrum using the vendor's software.

Trypsin digestion of the purified EspPΔ1 passenger domain

His-tagged EspPΔ1 passenger domain purified as described above (~3 pmol) was diluted into 50 mM ammonium bicarbonate, 20% acetonitrile and incubated at 37ºC overnight with ~100 fmol porcine trypsin (Invitrogen). The digestion was then quenched by adding 3 volumes 1% formic acid. Samples were stored at –80ºC if not used immediately.

Analysis of EspPΔ1 passenger domain 806.4 Da and 788.4 Da fragments by LC/MS/MS

Initial experiments performed using a LC/MS/MS system comprised of a CapLC system (Waters) interfaced with a LTQ-FT (Thermo-Finnegan) revealed the presence of both the EspPΔ1 passenger domain C-terminal tryptic fragment (806.4 Da) and a peptide whose mass was

18 Da smaller (788.4 Da). Subsequent experiments were performed using a Nanoacquity HPLC system (Waters) interfaced with a QTOF-2 instrument (Waters). A pre-column (5 μm Symmetry C18, 180 μm x 20 mm, Waters) was used in a vented association with an analytical column (3.5 μm Symmetry C18, 75 μm x 100 mm, Waters). Peptides isolated from the MS that had masses of 788.4 and 806.4 were fragmented by collision-induced dissociation and analyzed by MS/MS.

Test for the presence of C-terminal iso-asparagine in the cleaved EspPΔ1 passenger domain

All experiments were performed using the Nanoacquity HPLC/QTOF-2 LC/MS/MS system described above. The mass spectrometer was set up to alternately collect MS data for 0.3 s between the masses of 700 D and 900 D and then MS/MS data for 1 s from a parent mass of 806.4 D. A method that would allow optimal separation of the synthetic peptides AFLNEVN and AFLNEVN(iso) was established. This method involved a 5 min trap loading at 10 μl/min in 0.1% formic acid, 1% acetonitrile followed by an elution at 0.6 μl /min. After an initial flow of 0.1% formic acid, 15% acetonitrile, a > 2 min separation of the two peptide isoforms was achieved by running a 15-19.5% acetonitrile gradient over the column in 39.5 min. A mixture of the two synthetic peptides, a sample of the tryptic digest of the EspPΔ1 passenger domain, and the individual peptides were run in series; the mixture of synthetic peptides was typically run both before and after the tryptic digest to ensure consistency of results. The first run in any given series always showed altered elution behavior and was discarded. Samples contained ~0.5 picomoles of a single peptide or a mixture of the two peptides. Since the capacity of the trap column was relatively small, the amount of the tryptic digest that would provide a sufficient signal for a summed spectrum without causing peak distortions or peak displacement due to the presence of other peptides in the sample was determined empirically.

SUPPLEMENTARY REFERENCES

Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J Bacteriol **177**:4121-4130

Lee HC, Bernstein HD (2002) Trigger factor retards protein export in *Escherichia coli*. J Biol Chem **277**:43527-43535

Newitt JA, Bernstein HD (1998) A mutation in the *Escherichia coli* secY gene that produces distinct effects on inner membrane protein insertion and protein export. J Biol Chem **273**:12451-12456