Supporting Information

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SI Materials and Methods

Purification of an EspP β Domain-Lipid Crosslinking Adduct. Plasmid pC6H encodes a truncated version of EspP (EspPΔ1) containing a C-terminal His_6 tag (1). An amber mutation was introduced into pC6H at the codon that is equivalent to codon 1149 in full-length *espP* to create pJH110. AD202 cells transformed with pJH110 and pDULEBpa were grown at 37 °C in M9 medium overnight, washed, and added to 600 mL fresh medium at OD_{550} = 0.05. When the culture reached OD_{550} = 0.4, 10 μM isopropyl-β-D-thio-galactoside (IPTG) and 1 mM p-benzoyl-L-phenylalanine (Bpa) were added, and the cells were grown for an additional 40 min. Subsequently the culture was chilled on ice, and half the cells were subjected to UV irradiation for 10 min. Both UV-irradiated and untreated cells were collected by centrifugation (4,100 × *g*, 15 min, 4 °C) and resuspended in 5 mL buffer A [62.5 mM $K_2H(PO)_4$ (pH 7.5), 250 mM NaCl, 25 mM imidazole (pH 8), 1.25 mM $MgCl_2$, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride] containing 10 μg/mL DNase I. Cells then were lysed by sonication, and 1.25 mL of a 25% (vol/vol) Elugent (Calbiochem) solution was added to the cell extracts. The cell extracts were rotated at 4 °C for 2 h and then centrifuged (20,800 × *g*, 15 min, 4 °C) to remove insoluble material. The supernatant was mixed with 100 μL nickel-nitrilotriacetic acid resin (Invitrogen), equilibrated in buffer A, and rotated at 4 °C for 30 min. The slurries were transferred to columns and washed with 5 mL buffer B [50 mM $K_2H(PO)_4$ (pH 7.5), 200 mM NaC, 0.1% *N*-dodecyl-β-D-maltoside, 10% glycerol) containing 20 mM imidazole] and 5 mL buffer B containing 50 mM imidazole. His_6-tagged proteins then were eluted with buffer B containing 250 mM imidazole.

1. Barnard TJ, Dautin N, Lukacik P, Bernstein HD, Buchanan SK (2007) Autotransporter structure reveals intra-barrel cleavage followed by conformational changes. *Nat Struct Mol Biol* 14:1214–1220.

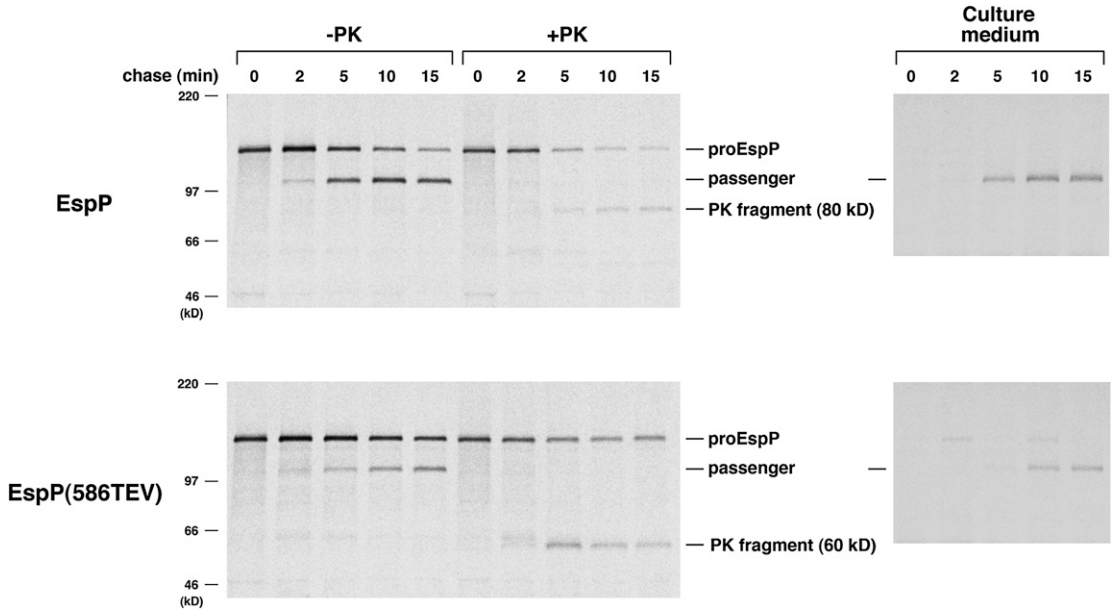

Fig. S1. Biogenesis of EspP and the EspP mutant that contains a short linker insertion at residue 586 (EspP) at 25 °C. AD202 cells transformed with pRLS5 (P_{trc}-*espP*) or pJH97 pRLS5 [P_{trc}-*espP(586TEV)*) were shifted to 25 °C when cultures reached OD_{550} = 0.2, and 100 μM IPTG was added 30 min later. After an additional 30-min incubation, cells were subjected to pulse-chase labeling. Cell-free culture medium fractions were obtained as described previously (1). Half of the cells were treated with PK, and EspP-containing polypeptides were immunoprecipitated from cell and culture medium fractions using an anti-EspP N-terminal antiserum. It has been shown previously that the ~60-kDa PK fragment isolated from cells that produce EspP(586TEV) results from the stalling of passenger-domain translocation near the site of the linker insertion (1).

1. Ieva R, Bernstein HD (2009) Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. *Proc Natl Acad Sci USA* 106: 19120–19125.

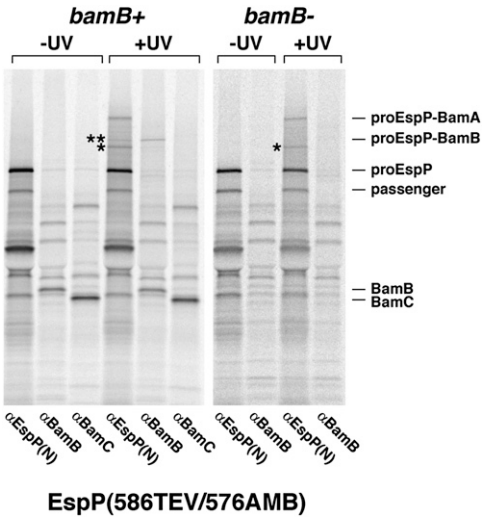

Fig. S2. Crosslinking of residue 575 to BamB. AD202 and HDB133 (AD202 *bamB::kan*) cells were transformed with pDULEBpa and a derivative of pRI23 harboring an amber codon at residue 575. Cells were pulse labeled at 37 °C and subjected to a 1-min chase after the addition of 200 μM IPTG. Half of each sample was UV irradiated, and equal portions were used for immunoprecipitations with the indicated antisera. The data show that a relatively weak, ∼180-kDa crosslinking adduct (denoted here and in Fig. 1*A* by a double asterisk) contains BamB, but a stronger, slightly smaller adduct (denoted here and in Fig. 1*A* by an asterisk) contains neither BamB nor BamC.

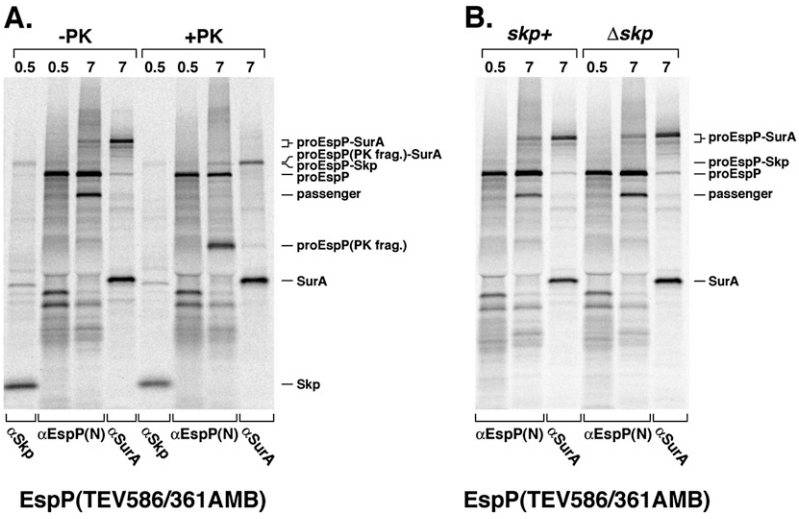

Fig. S3. Skp and SurA interact with residue 361 at distinct stages of EspP biogenesis. (*A*) AD202 cells were transformed with pDULEBpa and a derivative of pRI23 harboring an amber codon at residue 361. Cells were subjected to pulse-chase labeling at 25 °C after the addition of 200 μM IPTG, were UV irradiated, and were collected by centrifugation. Half of each sample was treated with PK, and equal portions were used for immunoprecipitations with the indicated antisera. (*B*) AD202 (*skp+*) and HDB131 (AD202 *Δskp*) cells were transformed with pDULEBpa and a derivative of pRI23 harboring an amber codon at residue 361. Cells were subjected to pulse-chase labeling at 25 °C after the addition of 200 μM IPTG and were UV irradiated. Equal portions of each sample were used for immunoprecipitations with the indicated antisera.

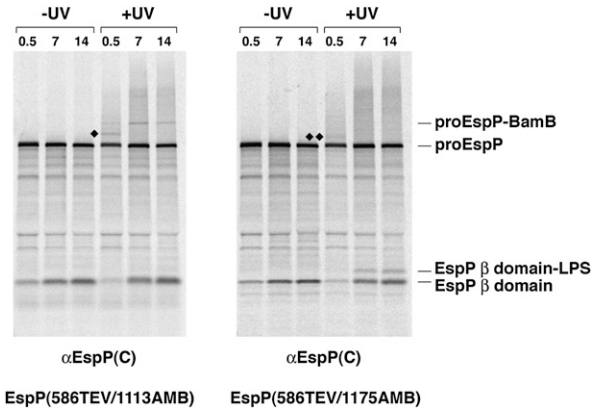

Fig. S4. Crosslinking of EspP β domain residues to ~15- to 20-kDa proteins in a *Δskp* strain. HDB131 cells were transformed with pDULEBpa and a derivative of pRI23 harboring an amber codon at residue 1113 or 1175. Cells were subjected to pulse-chase labeling at 25 °C after the addition of 200 μM IPTG. Half of each sample was UV irradiated, and equal portions were used for immunoprecipitations with the EspP C-terminal antiserum. The diamond and double diamonds indicate crosslinking adducts that contain proEspP and an unidentified protein(s) that is about the same size as Skp. The absence of Skp in HDB131 originally was confirmed by Western blot (1) and is demonstrated here by the absence of a proEspP-Skp crosslinking adduct in Fig. S3*B*.

1. Ieva R, Bernstein HD (2009) Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. *Proc Natl Acad Sci USA* 106: 19120–19125.

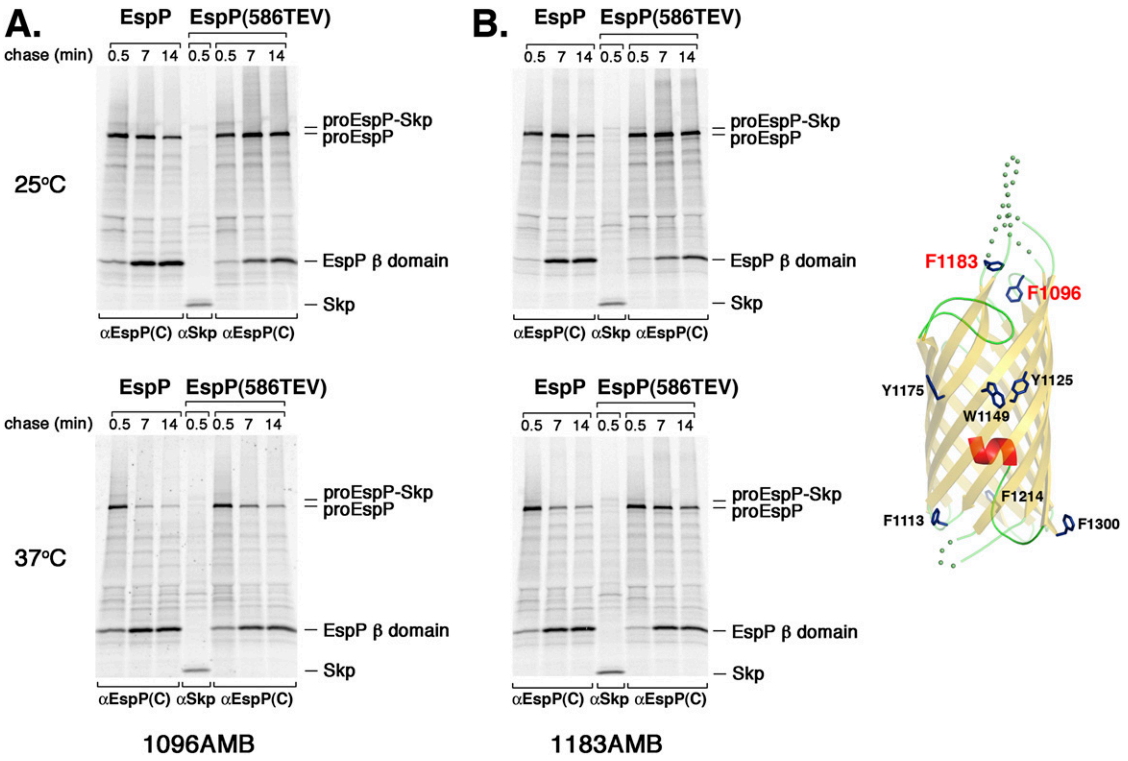

Fig. S5. Crosslinking of EspP β domain residues 1096 and 1183 to Skp. AD202 cells were transformed with pDULEBpa and a derivative of pRI22 or pRI23 harboring an amber codon at residue 1096 (*A*) or 1183 (*B*). Cells were subjected to pulse-chase labeling at 25 °C or 37 °C after the addition of 200 μM IPTG. Half of each sample was UV irradiated, and equal portions were used for immunoprecipitations with the indicated antisera. The crystal structure of the β domain (1) and the location of each residue are shown in the diagram on the right.

1. Barnard TJ, Dautin N, Lukacik P, Bernstein HD, Buchanan SK (2007) Autotransporter structure reveals intra-barrel cleavage followed by conformational changes. *Nat Struct Mol Biol* 14: 1214–1220.

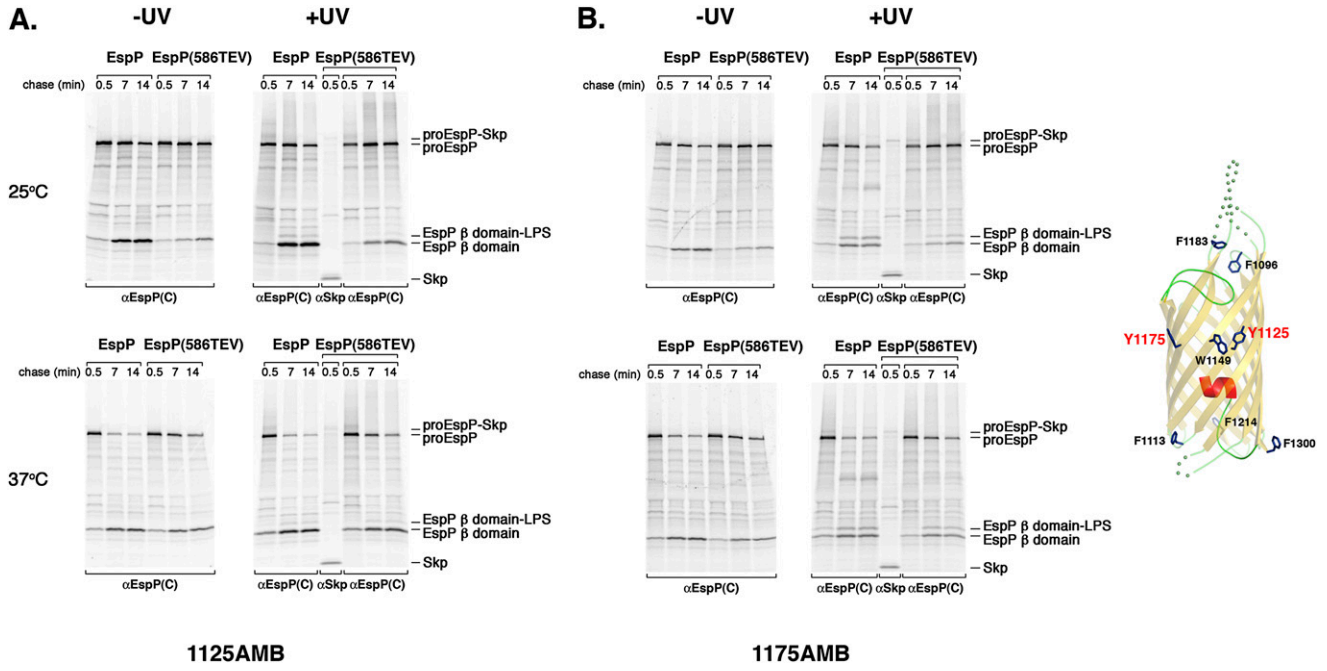

Fig. S6. Crosslinking of EspP β domain residues 1125 and 1175 to Skp and LPS. AD202 cells were transformed with pDULEBpa and a derivative of pRI22 or pRI23 harboring an amber codon at residue 1125 (*A*) or 1175 (*B*). Cells were subjected to pulse-chase labeling at 25 °C or 37 °C after the addition of 200 μM IPTG, and samples were processed as described in the legend of Fig. S2. The crystal structure of the β domain and the location of each residue are shown on the right.

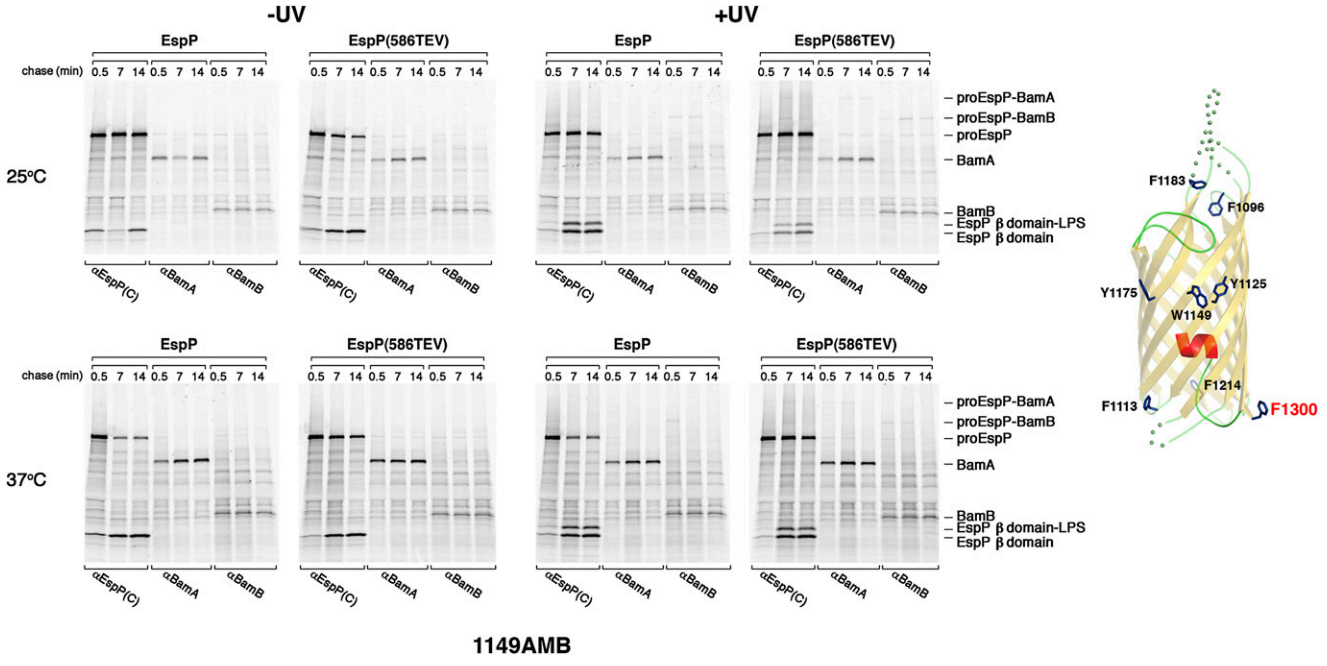

Fig. S7. Crosslinking of EspP β domain residues 1149 to Bam complex subunits and LPS. AD202 cells were transformed with pDULEBpa and a derivative of pRI22 or pRI23 harboring an amber codon at residue 1149. Cells were subjected to pulse-chase labeling at 25 °C or 37 °C after the addition of 200 μM IPTG, and samples were processed as described in the legend to Fig. S2. The crystal structure of the β domain and the location of residue 1149 are shown on the right.

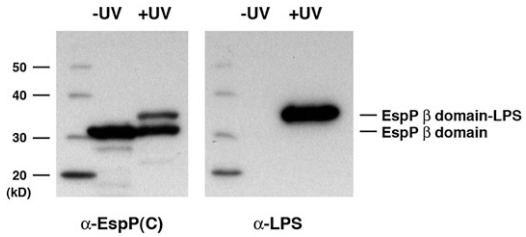

Fig. S8. Detection of an ~33-kDa EspP β domain crosslinking adduct with anti-LPS. AD202 cells were transformed with pDULEBpa and pJH110, a plasmid that encodes a C-terminally His-tagged version of EspPΔ1(1149AMB). The His-tagged EspP β domain and crosslinking adducts were purified from UV-irradiated and untreated cells and subjected to Western blot analysis using antisera generated against an EspP C-terminal peptide and LPS.

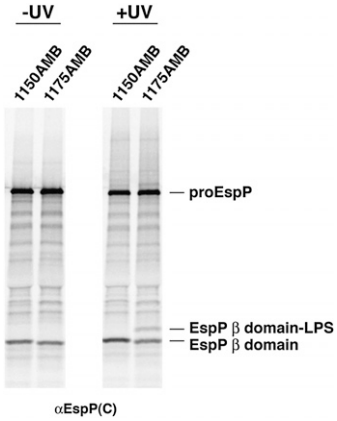

Fig. S9. EspP β domain residue 1150 is not crosslinked to LPS. AD202 cells were transformed with pDULEBpa and a derivative of pRI22 harboring an amber codon at residue 1150 or 1175. Cells were pulse labeled and subjected to a 1-min chase at 37 °C after the addition of 200 μM IPTG. Half of each sample was UV irradiated, and equal portions were used for immunoprecipitations with an anti-EspP C-terminal antiserum.

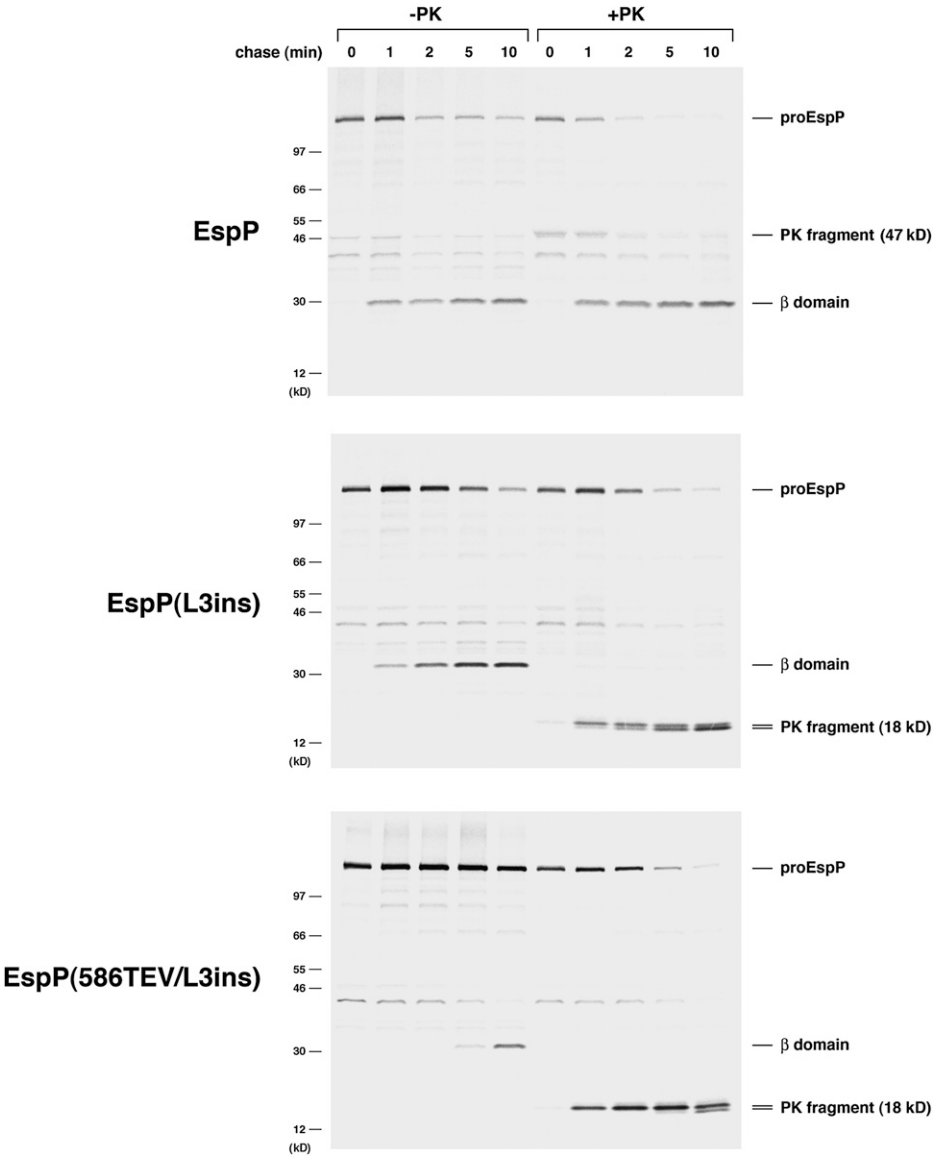

Fig. S10. The extracellular loops of the EspP β domain penetrate the outer membrane at an early stage of assembly. AD202 cells transformed with pRLS5 (P_{trc}-*espP*), pPT17 [P_{trc}-*espP(L3ins)*], or pPT18 [P_{trc}-*espP(586TEV/L3ins)*] were subjected to pulse-chase labeling at 37 °C after the addition of 10 μM IPTG, and half of each sample was treated with PK. Equal portions of each sample were used for immunoprecipitations with an anti-EspP C-terminal antiserum. Although the appearance of an ∼18-kDa PK fragment seemed to parallel the initiation of passenger domain secretion, a slightly faster migrating fragment appeared much later in parallel with passenger domain cleavage. The slightly smaller fragment presumably resulted either from a change in the accessibility of loop 3 after the release of the passenger domain or from a conformational change in the β domain immediately before or after passenger domain cleavage.