Expanded View Figures

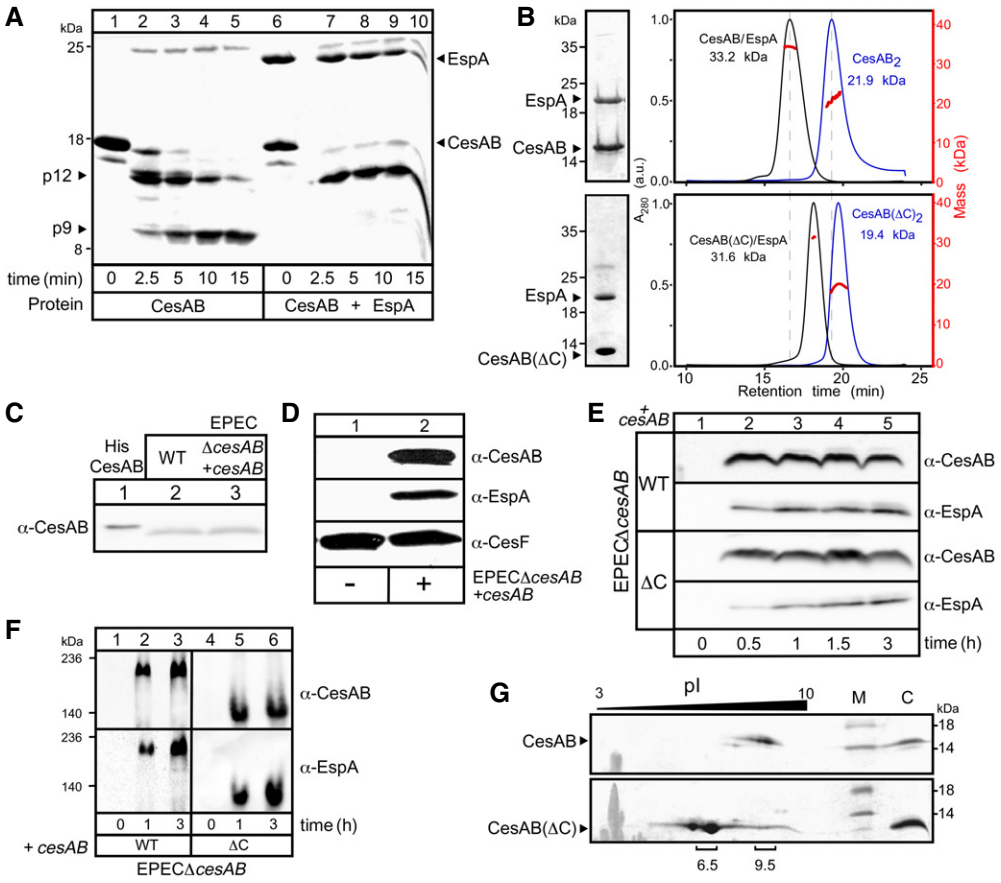

Figure EV1. The CesAB N-core is essential for EspA stability (related to Fig 1).

A Limited trypsinolysis of His-CesAB, alone (lanes 1–5) or EspA-bound (lanes 6–10). Peptides were analyzed on 5.5 M urea-15% SDS–PAGE and Coomassie Blue-stained. Tryptic peptides p12 and p9 were N-terminally sequenced. CesAB alone was rapidly cleaved, and p12 was formed. Further cleavage of p12 generated p9. This last cleavage did not occur when CesAB was bound to EspA. A representative experiment is shown. $n = 3$.

B Co-purified His-CesAB/EspA (top) or His-CesAB(ΔC)/EspA (bottom) complexes, analyzed by 15% SDS–PAGE (left) and GPC-MALS (right; 50 μM; Buffer A). UV traces and masses are shown. Like wt CesAB, CesAB(ΔC) can form stable homodimers as well as heterodimers with EspA (compare blue and black lines on top and bottom panels). A representative experiment is shown. $n = 5$.

C Plasmid-borne *cesAB* was expressed to similar levels with those of a chromosomal copy. The *in vivo* expression of *cesAB* from an EPECΔ*cesAB* strain complemented *in trans* with a pASK-IBA7plus vector carrying *cesAB* (lane 3), following addition of AHT (OD_{600} = 0.3; 37°C; 2.5 ng/ml AHT; 3 h), was compared to that of a wild-type EPEC strain (lane 2). Equal amount of cells were analyzed on 15% SDS–PAGE and immunostained using α-CesAB. Purified His-CesAB (2 ng; lane 1) served as a molecular weight/protein amount marker. A representative experiment is shown. $n = 3$.

D CesAB is essential for the intracellular stabilization of EspA. EPECΔ*cesAB* was complemented with either an empty pASK-IBA7plus vector (left) or one carrying *cesAB* (right) (OD_{600} = 0.3; 37°C; 2.5 ng/ml AHT; 3 h). Equal amount of cells were analyzed on 15% SDS–PAGE and immunostained with the indicated polyclonal antisera. A representative experiment is shown. When CesAB was not present, EspA was not detected; once CesAB synthesis was restored, both proteins were detected (top and middle panels). CesF, a T3S chaperone whose stability was not affected by CesAB (Elliott *et al*, 2002), served as an internal control (bottom panel). $n = 3$.

E CesAB C-tail is not essential for the stabilization of EspA *in vivo*. EPECΔ*cesAB* was complemented with a pASK-IBA7plus vector carrying either *cesAB* (top panel) or *cesAB(ΔC)* (bottom) (OD_{600} = 0.3; 37°C; 2.5 ng/ml AHT). At the indicated time post-induction, equal amount of cells were analyzed by 15% SDS–PAGE and immunostained with the indicated polyclonal antisera. A representative experiment is shown. $n = 3$.

F CesAB/EspA (left) and CesAB(ΔC)/EspA (right) complexes formed *in vivo*. At the indicated time post-induction, equal amount of cytosolic proteins from EPECΔ*cesAB* cells carrying either *cesAB* or *cesAB(ΔC)* plasmids were analyzed on 7% Native-PAGE and immunostained with the indicated antisera. The difference in migration of the two complexes is probably due to the difference in the pI of CesAB and CesAB(ΔC) (see panel G). A representative experiment is shown. $n = 3$.

G The C-tail of CesAB is positively charged. Thus, it shifts the pI of the protein to high values. CesAB (top) and CesAB(ΔC) (bottom) were separated (100 μg in rehydration buffer) according to their native pI in the 1st dimension (IPGphor strips; GE; 13 cm; pH range 3–10; 50 μA; 18,000 Vh) and their denatured mass in the 2nd dimension (15% SDS–PAGE). Proteins were visualized by Coomassie Blue staining. A representative gel is shown. Molecular weight markers (M) and purified proteins (C), run in the 2nd dimension, are indicated. $n = 3$.

Source data are available online for this figure.

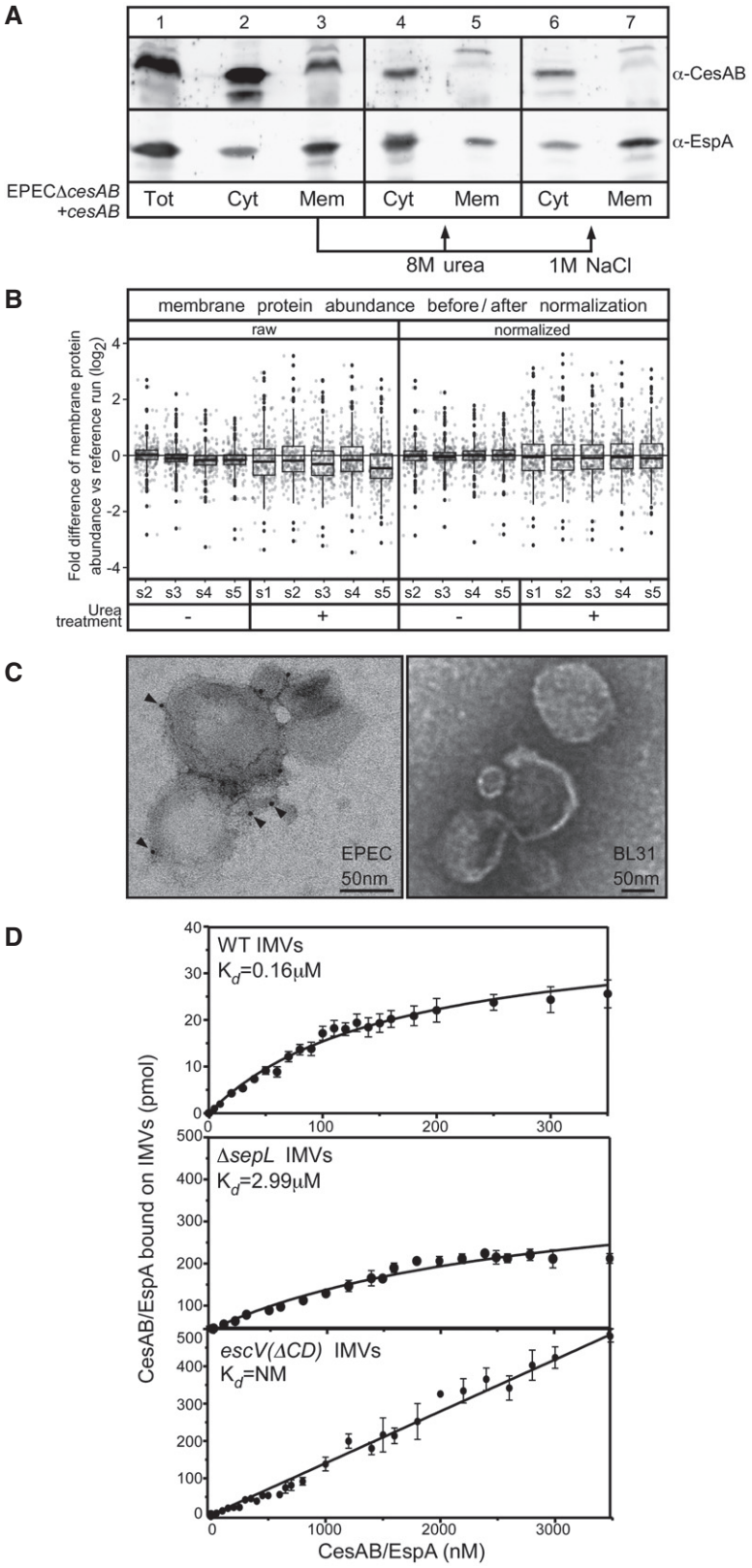

Figure EV2. CesAB/EspA membrane localization *in vivo* and IMVs characterization (related to Figs 1 and 2).

A Sub-cellular localization of CesAB and EspA. EPECΔ*cesAB* cells complemented with a plasmid carrying *cesAB* (OD_{600} = 0.3; 37°C; 2.5 ng/ml AHT; 3 h) were lysed and ultracentrifuged (300,000 *g*, 30 min, 4°C). A representative experiment is shown. CesAB and EspA were detected in both the cytosolic (Cyt) and membrane fraction (Mem). The majority of the membrane-localized CesAB could be extracted by washing the membrane fraction with 8 M urea or high salt (as indicated), implying electrostatic interactions with either the membrane lipids or a protein component. On the contrary, the majority of membrane-localized EspA could not be extracted by salt treatment, implying tight membrane association. n = 3.

B Quality control for the normalization of the different MS runs of IMVs used in this study. The normalization of the different runs was based on the peptide intensity of the integral membrane proteins, since these were not affected by the urea treatment, as described (Callister *et al*, 2006). A box plot, representing a single IMV run versus the reference run, with or without urea treatment (as indicated), is plotted before (raw) and after normalization (normalized). Clearly, following normalization the difference of mean protein abundance (i.e., the middle of the box plot) of integral membrane proteins was corrected (i.e., log of fold difference between runs is now close to zero). s1–s5: repeats of IMVs preparation from EPEC. Dots represent outliers (see Appendix); square box represents the value-range between 1st and 3rd quartile; lines above and below boxes represent range from 1st quartile to the maximum value and from the 3rd quartile to the minimum value, respectively.

C Localization of EscV on IMVs, using electron microscopy. IMVs, derived either from EPEC (left) or BL31 (right) *Escherichia coli* strain, were probed with polyclonal antibodies against the cytoplasmic domain of the membrane-embedded EscV protein, using goat anti-rabbit secondary antibodies attached to 5-nm nanogold particles and were negatively stained. A representative picture is shown. Gold particles, detected on the periphery of EPEC IMVs (left; arrowheads), indicate the presence of T3S injectisomes in the membrane vesicles, containing EscV with its C-domain exposed and accessible. Gold particles were not detected on BL31-derived IMVs (right). n = 3.

D Determination of equilibrium dissociation constants (K_ds) of CesAB/EspA for wt (top panel), Δ*sepL* (middle panel), and *escV*(Δ*CD*) (bottom panel) EPEC IMVs. Data, analyzed by nonlinear regression, represent average values (for 20–23 concentration points); error bars: standard mean error (SEM), as in Fig 2E. *escV*(Δ*CD*) IMVs exhibited linear, non-saturable binding, that did not converge to a K_d. n = 12–14.

Source data are available online for this figure.

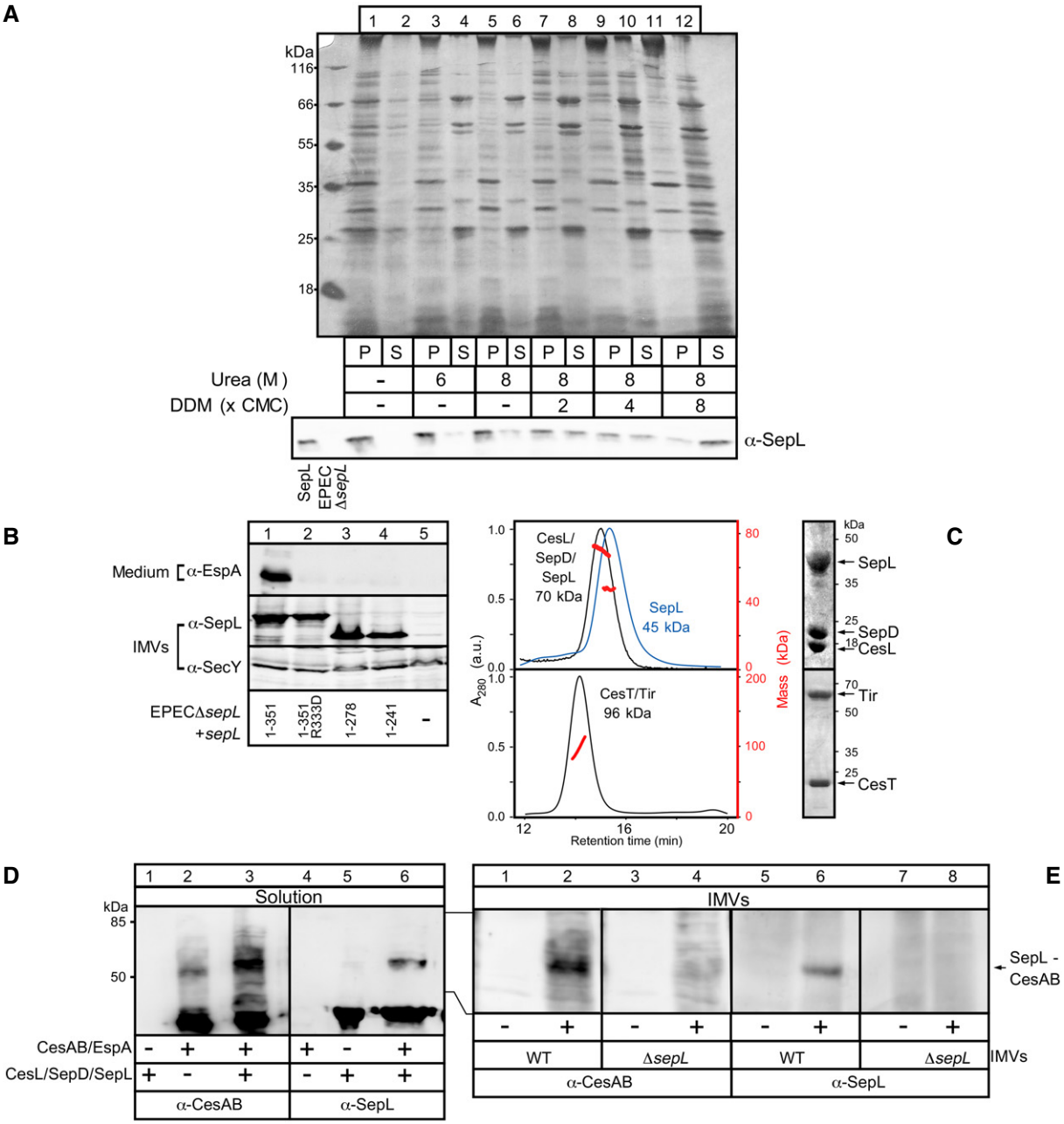

Figure EV3.

Figure EV3. SepL receptor function determination (related to Figs 3–5).

A The association of SepL with the membrane is tight. EPEC IMVs (40 μg of total membrane protein/sample) were incubated with the indicated urea or/and DDM concentration (30 min; on ice). Following ultracentrifugation (100,000 *g*, 30 min, 4°C; Optima Max-XP, TLA100 rotor; Beckman Coulter), samples were analyzed on 15% SDS–PAGE and either silver- (top) or immunostained with α-SepL (bottom). A representative experiment is shown. S = supernatant, P = pellet; $n = 4$.

B EspA secretion of (top), and SepL localization on IMVs prepared from (middle), EPEC Δ*sepL* cells that were complemented with the indicated plasmid-borne *sepL* derivatives (lanes 1–4) or an empty vector (lane 5) (as in Appendix Fig S1A and C). 1–351 is the full-length, wild-type SepL. Immunostaining SecY (bottom), an inner membrane protein unrelated to T3SS, served as loading control. Wild-type (lane 1) and R333D (lane 2) SepL and its C-terminal truncation mutants (lanes 3 and 4) were localized on the IMVs; gel migration differences are due to mass differences. However, SepL mutants did not restore EspA secretion. A representative experiment is shown. $n = 3$.

C The SepL alone or the co-purified His-CesL/SepD/SepL (top, blue, and black line, respectively) and His-CesT/Tir (bottom) complexes were analyzed by 15% SDS–PAGE (right) or GPC-MALS (left), as in Fig EV1B. The mass determined for His-CesL/SepD/SepL indicated a stoichiometric ratio of 1:1:1 (top) while that for His-CesT/Tir indicated a ratio of 2:1 (bottom), in agreement with previous results (Thomas *et al*, 2005; Younis *et al*, 2010).

D CesAB/EspA interacted with SepL in solution. His-CesAB/EspA and His-CesL/SepD/SepL protein complexes (10 μM each) were mixed (100-μl reactions) and cross-linked using a bifunctional chemical cross-linker (SDA-NHS-diazirine; see Appendix Supplementary Materials and Methods). Protein samples were analyzed on 15% SDS–PAGE and immunostained using α-CesAB and α-SepL antisera. When the two complexes were mixed, a new protein species of ~60 kDa appeared after cross-linking, containing both CesAB and SepL (lanes 3 and 6), indicating a cross-linked product between CesAB/SepL (theoretical mass 64 kDa). This band was not present when CesAB/EspA or CesL/SepD/SepL were mixed alone with the cross-linker (lanes 1–2, and 4–5). A representative experiment is shown. $n = 4$.

E CesAB/EspA interacted with membrane-bound SepL on IMVs. His-CesAB/EspA (10 μM) was mixed (100 μl reactions) with IMVs (1 μg/μl total membrane protein) prepared from either wt or Δ*sepL* EPEC strains and cross-linked using a bifunctional chemical cross-linker (SDA-NHS-diazirine; see Appendix Supplementary Materials and Methods). Protein samples were analyzed on 15% SDS–PAGE and immunostained using α-CesAB and α-SepL. When wt IMVs were mixed with CesAB/EspA, a protein band of ~60 kDa appeared after cross-linking which contained both CesAB and SepL (lanes 2 and 6), indicating a cross-linked product between CesAB/SepL (theoretical mass 64 kDa). This band was not present when wt (lanes 1 and 5) or Δ*sepL* (lanes 3 and 7) IMVs alone were mixed with the cross-linker, or when CesAB/EspA was mixed and cross-linked with Δ*sepL* IMVs (lanes 4 and 8). A representative experiment is shown. $n = 4$.

Source data are available online for this figure.

Figure EV4. SepL and EscV C-domain peptide array analysis (related to Figs 3 and 4).

A SepL immobilized peptide arrays (13-mers, 10 residues overlap) were probed with the indicated purified protein ligands (200 nM; 25 ml Buffer C; 25°C; 1 h): His-MBP-EscV C-domain (CD), His-MBP, His-CesAB/EspA, His-CesAB(ΔC)/EspA, His-CesAB(DRE), and His-CesAB, respectively, as described (Karamanou *et al*, 2008). Following washes, proteins that were bound to the array were electro-transferred onto nitrocellulose membranes and immunostained with α-CesAB or α-MBP antibodies. Representative experiments are shown; $n = 4$–6.

B The intensity of signals from experiments like those presented in (A) was quantified using Image Quant software (GE), as described (Karamanou *et al*, 2008). Mean values were plotted using GraphPad Prism; signals below 1.2 (dashed line) were considered as background; $n = 4$; bar graphs represent mean values; error bars standard deviation (SD).

C EscV C-domain immobilized peptide arrays (13-mers; 10 residues overlap) were probed with the purified protein ligands (200 nM; 25 ml Buffer C; 25°C; 1 h): His-MBP-SepL and His-MBP. Experiments were performed as in (A) and immunostained with α-MBP antibodies. Representative experiments are shown; $n = 4$.

D The intensity of signals from experiments like those presented in (C) was quantified as described in (B); $n = 4$; bar graphs represent mean values; error bars standard deviation (SD).

E SepL sequence (1–351), CesAB, and EscV binding sites (a–e) identified by the peptide arrays are indicated (color code unique for each used ligand; as indicated). Residues mutated in this study (red) and their substitutions (red, below) are indicated.

F EscV C-domain sequence (residues 334–675) and domains (D1–4; Abrusci *et al*, 2013; Saijo-Hamano *et al*, 2010). SepL-binding sites (α–γ) identified by peptide arrays are indicated (yellow). Residues mutated in this study (red) and their substitutions (red, below) are indicated.

Source data are available online for this figure.

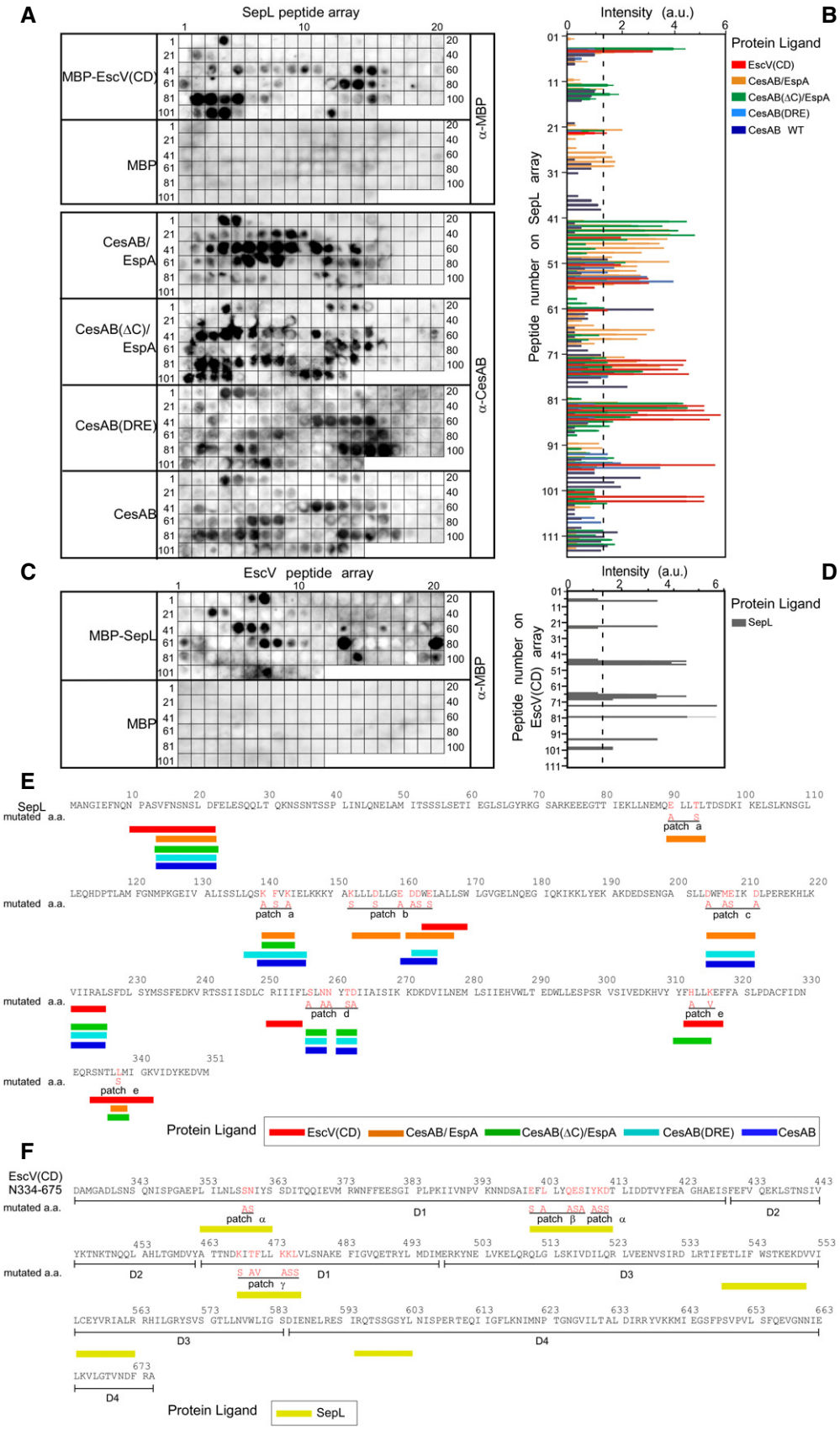

Figure EV4.

Figure EV5. The CesAB C-tail acts as an activator for EspA secretion (related to Fig 6).

A EPEC*ΔcesAB* cells transformed with a pASK-IBA7plus vector carrying *cesAB* and *ΔcesABΔespA* cells transformed with a pASK-IBA7plus vector carrying *cesAB(ΔC)/espA* were grown (OD_{600} = 0.3; 37°C), and plasmid gene expression was induced (2.5 ng/ml or 100 ng/ml AHT as indicated; 3 h). Cells were separated from the spent growth medium by centrifugation (50 ml; 5,000 *g*, 20 min, 4°C) and resuspended in 50 mM Tris pH 8, to reach the same OD_{600}. The indicated relative amount of cells was loaded on 15% SDS–PAGE and probed with α-EspA and α-CesAB. EPEC*ΔcesABΔespA* cells transformed with *cesAB(ΔC)/espA* plasmid (lane 2; 100 ng/ml AHT) synthesized ~10 times more CesAB(ΔC) and EspA compared to EPEC*ΔcesAB* cells transformed with a *cesAB* plasmid (lane 1; 2.5 ng/ml AHT). A representative experiment is shown. *n* = 3.

B CesAB and EspA signals from (A) were quantified using Image Quant (GE). Expression levels of *espA* (lane 1, top) and *cesAB* (bottom) using 2.5 ng/ml AHT were considered as 1; all other values (lane 2) were expressed as folds over this (*y*-axis). *n* = 3; bar graphs represent mean values; error bars standard deviation (SD).

C Secreted polypeptides from the spent growth medium from (A) and (D) were treated and analyzed as described in Appendix Fig S1A. Proteins were immunostained with α-EspA. EspA was not secreted in the absence of either SepL (lanes 2 and 3) or the CesAB C-tail (lanes 4 and 5), even though in both cases CesAB and EspA were over-expressed (see A + B and D + E, respectively). A representative experiment is shown. *n* = 3.

D EPEC*ΔcesAB* transformed with a pASK-IBA7plus vector carrying *cesAB* and EPEC*ΔsepL* transformed with a pASK-IBA7plus vector carrying *cesAB/espA* were grown (OD_{600} = 0.3; 37°C), and plasmid gene expression was induced (2.5 ng/ml or 100 ng/ml AHT as indicated; 3 h). Cells were separated from the spent growth medium by centrifugation (50 ml; 5,000 *g*, 20 min, 4°C) and resuspended in 50 mM Tris pH 8, to reach the same OD_{600}. The indicated relative amounts of cells were loaded on 15% SDS–PAGE and probed with α-EspA and α-CesAB. CesAB and EspA were synthesized at least 10 times more in EPEC*ΔsepL* cells carrying *cesAB/espA* plasmid (lane 2; 100 ng/ml AHT) compared to EPEC*ΔcesAB* complemented with a *cesAB* plasmid (lane 1; 2.5 ng/ml AHT). A representative experiment is shown. *n* = 3.

E CesAB and EspA signals from (D) were quantified using Image Quant (GE). Expression levels of *espA* (lane 1, top) and *cesAB* (bottom) using 2.5 ng/ml AHT were considered as 1; all other values (lane 2) were expressed as folds over this (*y*-axis). *n* = 3; bar graphs represent mean values; error bars standard deviation (SD).

F The C-tail of CesAB is essential for EspA secretion. EPEC*ΔcesAB* cells, transformed with a pASK-IBA7plus vector carrying *cesAB* (lanes 1–4), or *cesAB(ΔC)* (lanes 5–8), or *cesAB(6A)* (lanes 9–12) were grown, as in Fig EV1D. At the indicated time post-induction, 50 ml culture was centrifuged, to separate cells from the spent growth medium (5,000 *g*, 20 min, 4°C). EspA secretion was monitored in the spent growth medium, as in Appendix Fig S1A (top panel). CesAB expression was monitored in total cells, using α-CesAB (bottom). A representative experiment is shown. *n* = 4.

G Secretion of Tir from EPEC*ΔescVΔsepL* strain transformed with a pASK-IBA7plus vector empty or carrying the indicated *escV* derivatives, analyzed as in Appendix Fig S1A using α-Tir; *n* = 3.

H Secretome analysis of the indicated EPEC strains (5 h post-inoculation; as in Appendix Fig S1A), visualized by Coomassie Blue- (top) or immunostaining (as indicated); *n* = 3.

I–M Infection of HeLa cells by EPEC wt or EPEC*ΔcesAB* cells transformed with a pASK-IBA7plus vector empty or carrying the indicated *cesAB* derivatives, as described in Appendix Fig S2B (without induction of plasmid gene expression). n = nucleus; white arrowheads indicate bacterial colonies (in blue) and actin-pedestals (in green). *n* = 4.

N Infection of HeLa cells by EPEC*ΔsepL* cells transformed with a pASK-IBA7plus vector carrying *cesAB/espA*, as described in Appendix Fig S2B. Plasmid gene expression was induced with 100 ng/ml AHT prior to the incubation with HeLa cells to ensure *cesAB/espA* overexpression (as in A). As anticipated, HeLa cells were not infected due to lack of EspA secretion. *n* = 4.

Source data are available online for this figure.

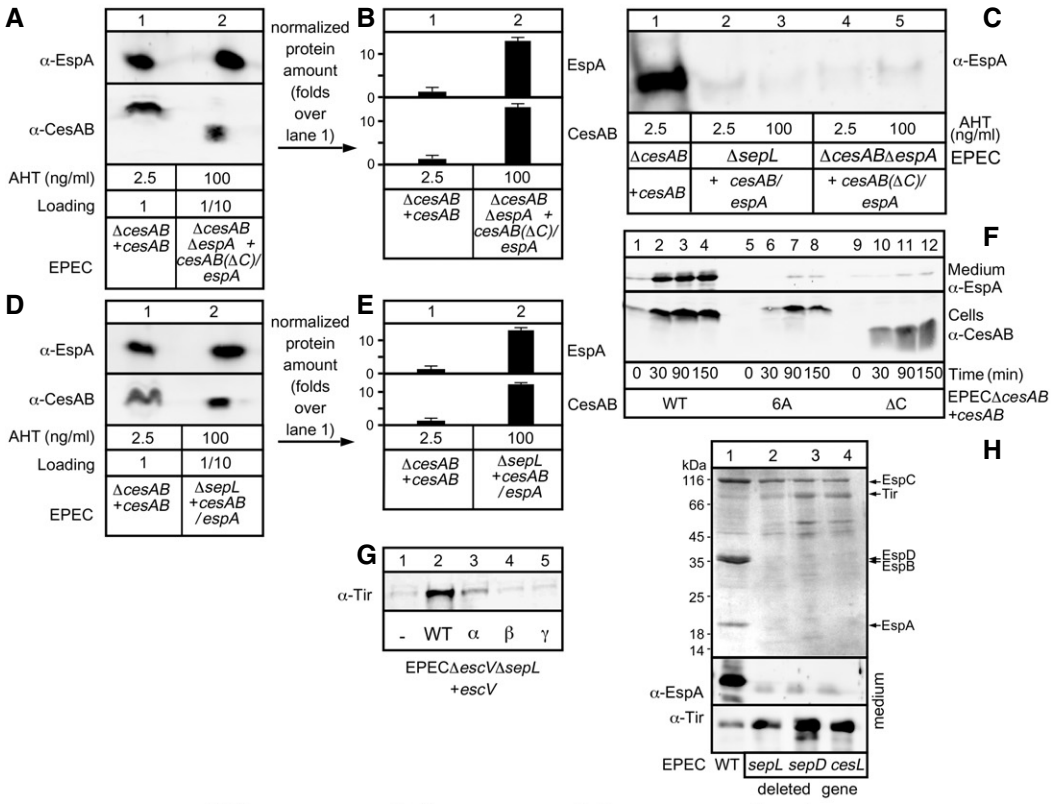

Figure EV5.