Supplemental Information

An affinity switch controls hierarchical Type III protein targeting and secretion

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Abbreviations
EPEC: Enteropathogenic *E. coli*
T3S: Type 3 Secretion
T3SS: Type 3 Secretion System
AHT: Anhydrotetracycline
IMVs: Inverted Membrane Vesicles
HDX: Hydrogen Deuterium Exchange
MS: Mass Spectrometry
MALS: Multi Angle Light Scattering
DMEM: Dulbecco's Modified Eagle Medium
TCA: Trichloroaetic Acid
DDM: *n*-Dodecyl-β-D-maltopyranoside

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Supplementary Figures:

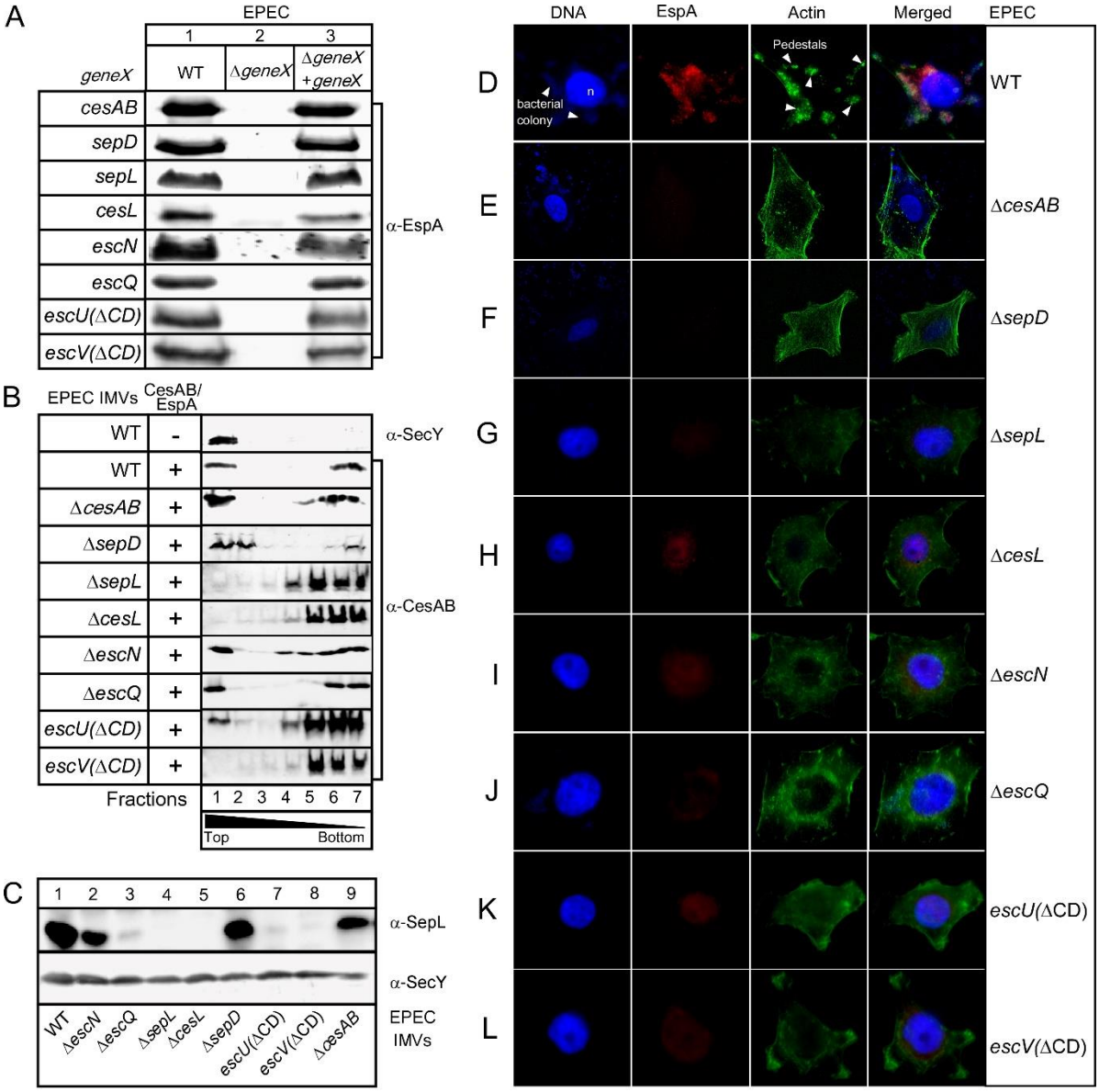

Figure S1: T3SS elements required for CesAB/EspA membrane targeting (Related to Figure 3 and 6)

A. The indicated EPEC gene knock out-strains (Δ*geneX*; lane 2) were tested for EspA secretion, under the same conditions with wild type EPEC (lane 1). 5 hours post-inoculation cells were separated from the spent growth medium (50 ml; 20 min; 3,000 x g; 4 ^{o}C) which was then TCA precipitated (20% w/v). TCA pellets were resuspended in volume adjusted according to OD_{600} and proteins were analyzed on 15% SDS-PAGE. EspA secretion was monitored by immunostaining. Representative experiments are shown. None of these knock out-strains could secrete EspA (lane 2; Δ*geneX*). In all cases, EspA secretion was restored by complementing the deletion strain with a plasmid carrying the respected gene (lane 3; Δ*geneX* + *geneX*). *n=3.*

B. The ability of CesAB/EspA to bind to urea treated IMVs, prepared from the indicated EPEC knock out-strains, was examined using flotation assays (as in Fig. 2D). Representative experiments are shown. When the *sepL* "gatekeeper" or the cytoplasmic domain of EscV were deleted, the migration of CesAB/EspA to the top fractions was compromised. In the absence of CesL, a SepL chaperone (Younis et al, 2010), SepL was not be detected on the IMVs (see panel C) hence, the inability of CesAB/EspA to bind on them. *n=6-9.*

C. SepL content on urea treated IMVs, prepared from wild type or the indicated EPEC deletion strain (20 μgr total membrane protein) were analyzed on 15% SDS-PAGE and immunostained with α-SepL. A representative experiment is shown. The presence of SepL on IMVs was highly correlated with the ability of CesAB/EspA to bind to them. The very low amounts of SepL that were detected in EPECΔ*escQ* (lane 3) and *escU*(Δ*CD*) (lane 7) were sufficient for CesAB/EspA binding on IMVs, suggesting that there is no linear correlation between the two. Immunostaining of the membrane protein SecY (bottom panel) served as an unrelated loading control against which SepL values were adjusted before plotting them in Fig. 3D. *n=3.*

D-L: The indicated EPEC gene knock out-strains were tested for their ability to infect HeLa cells under the same conditions with wild type EPEC. Nucleus and bacterial DNA are indicated in blue (stained with DAPI; TO-PRO®-3; Thermo Fisher Scientific); secreted EspA in red (stained with Cy3 α-rabbit; Jacksons ImmunoResearch Europe Ltd); actin in green (stained with Cy2-Phaloidin; Thermo Fisher Scientific). A merged picture is shown on the right. Arrowheads indicate bacterial colonies and actin pedestals. For each EPEC knock-out strain a representative picture is shown. Unlike wild type EPEC, none of the indicated knock-out strains was able to infect HeLa cells. *n=4*

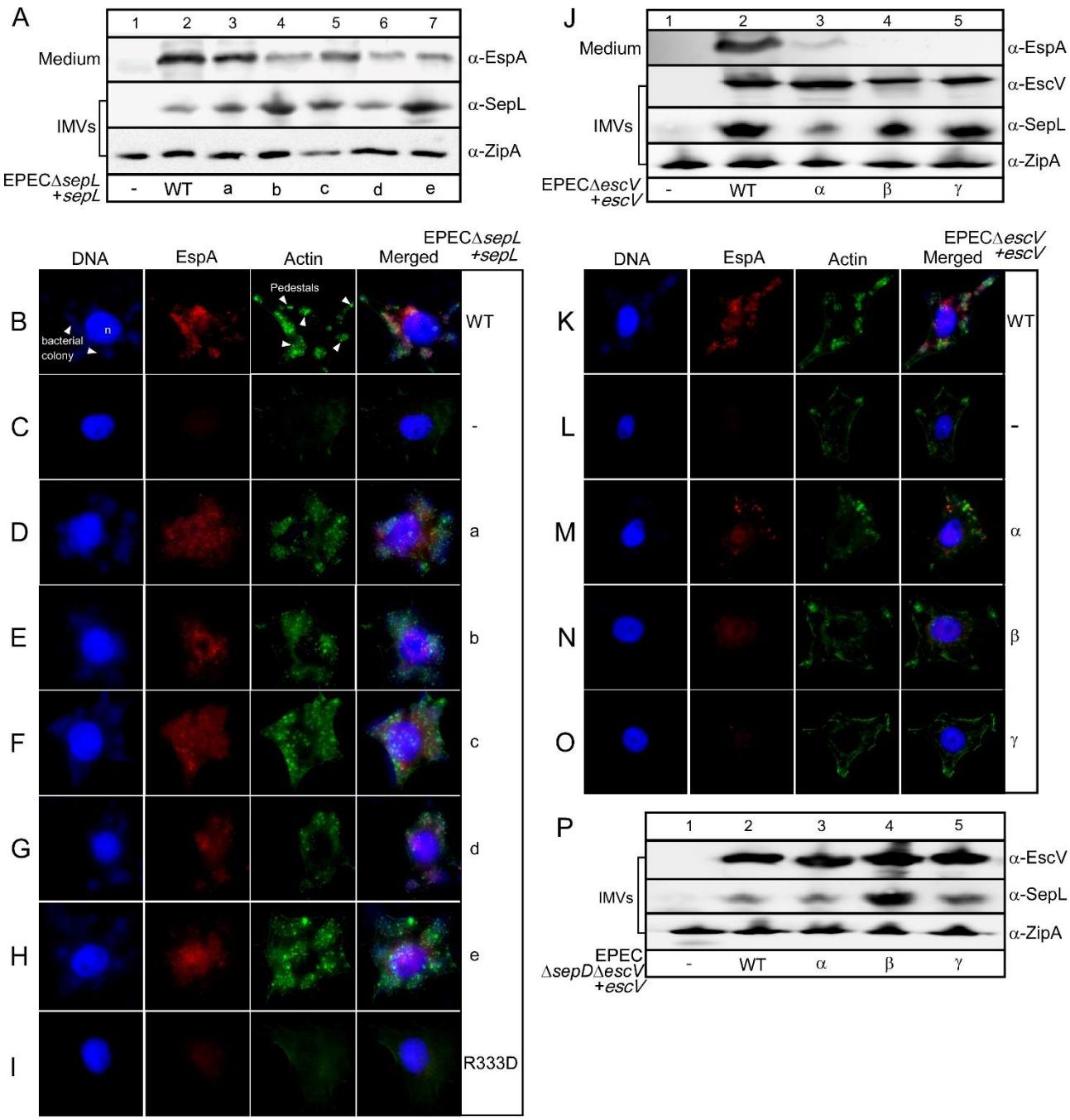

Figure S2: *in vivo* characterization of SepL and EscV mutants (Related to Figures 4 and 6)
A. EspA secretion (top; as in Fig. S3A), *sepL* membrane localization (IMVs; middle; as in Fig. S3C) in EPEC*ΔsepL* cells carrying a pASK-IBA7plus vector empty (lane 1), or with *sepL* (lane 2), or *sepL* mutant derivatives (lanes 3-7). Signals were quantified using Image J software (Schneider et al, 2012). Immunostaining of ZipA (bottom) served as an unrelated loading control against which SepL values were adjusted before plotting them in Fig. 4C. A representative experiment is shown. *n=4*
B-I. Infection of HeLa cells by EPEC*ΔsepL* cells carrying a pASK-IBA7plus vector empty, or with the indicated *sepL* derivatives (without induction of plasmid gene expression). SepL R333D derivative was used as a negative control, as it has been proposed to affect translocator/chaperone binding (Botteaux et al, 2009; Burkinshaw et al, 2015). Nucleus and bacterial DNA are indicated in blue (stained with DAPI; TO-PRO®-3; Thermo Phisher Scientific); secreted EspA in red (stained with Cy3 α-rabbit; Jacksons Immuno-Research lab); actin in green (stained with Cy2-Phaloidin; Thermo Phisher Scientific). A merged picture is shown on the right. Arrowheads indicate bacterial colonies and actin pedestals. Representative pictures are shown. *n=4*.

J. EspA secretion (top panel; as in Fig. S3A), EscV membrane localization (IMVs; second panel; as in S3C after immunostaining with α-EscV CD) and SepL membrane localization (IMVs; third panel; as in Fig. S3C) in EPEC*ΔescV* carrying a pASK-IBA7plus vector empty (lane 1), or with *escV* (lane 2), or with *escV* mutant derivatives (lanes 3-5). *escV* mutants restored EscV localization on IMVs but either could not or could barely secrete EspA. SepL localization on those IMVs was also slightly affected. Immunostaining of ZipA (bottom panel) served as an unrelated loading control against which SepL values were adjusted before plotting them in Fig. 4F. A representative experiment is shown. *n=4*

K-O. Infection of HeLa cells by EPEC*ΔescV* cells carrying a pASK-IBA7plus vector empty or with the indicated *escV* derivatives, as in panel B. *n=3*

P. EscV (IMVs; top panel; as in panel J) and SepL membrane localization (IMVs; middle panel; as in Fig. S3C) in EPEC*ΔescVΔsepD* carrying a pASK-IBA7plus vector empty (lane 1), or with *escV* (lane 2), or with *escV* mutant derivatives (lanes 3-5). *escV* mutants restored EscV localization on IMVs. SepL localization on those IMVs was slightly affected. Immunostaining of ZipA (bottom panel) served as an unrelated loading control against which SepL values were adjusted before plotting them in Fig. 4H. A representative experiment is shown. *n=4*

Supplementary tables:

Table S1: Hydrogen/Deuterium exchange profile of CesAB (Related to Figure 1). The deuterium (D) uptake, absolute values and relative to the complete deuteration control, is given for all identified peptides of the CesAB sequence (98% sequence coverage) for CesAB, CesAB/EspA and CesAB(DRE), as indicated. The overlapping peptides indicated by hashtag (#) were used for the D uptake determination of the sequence residues 59-62, for higher resolution localization of D uptake at this region. D uptake of peptides with mutated sequences (residues in red) were not compared (-).

Identified CesAB peptides			**Absolute D uptake**				**% D uptake (relative to complete deuteration)**		
Start	**End**	**Sequence**	**Complete deuteration**	**CesAB**	**CesAB/ EspA**	**CesAB (DRE)**	**CesAB**	**CesAB/ EspA**	**CesAB (DRE)**
3	23	IVSQTRNKELLDKKIRSEIEA	12.204945	5.972235	2.889644	-	48.9%	23.7%	-
23	30	AIKKIIAE	4.691717	0.45995	0.34798	0.190344	9.8%	7.4%	4.1%
23	31	AIKKIIAEF	5.54809	0.546953	0.414805	0.186649	9.9%	7.5%	3.4%
24	30	IKKIIAE	3.808228	0.353473	0.26307	0.16863	9.3%	6.9%	4.4%
24	31	IKKIIAEF	4.121063	0.472674	0.375524	0.239781	11.5%	9.1%	5.8%
31	40	FDVVKESVNE	5.805299	2.258135	0.840623	0.648278	38.9%	14.5%	11.2%
31	41	FDVVKESVNEL	7.208374	2.639588	0.839591	0.725584	36.6%	11.6%	10.1%
31	58	FDVVKESVNELSEKAKTDPQAAEKLNKL	20.711527	7.266918	2.411325	2.429544	35.1%	11.6%	11.7%
32	40	DVVKESVNE	4.998914	2.102245	0.615076	0.593526	42.1%	12.3%	11.9%
32	58	DVVKESVNELSEKAKTDPQAAEKLNKL	19.99834	7.144325	2.239756	2.408962	35.7%	11.2%	12.0%
32	61	DVVKESVNELSEKAKTDPQAAEKLNKLIEG	22.482801	7.965473	3.292445	2.738683	35.4%	14.6%	12.2%
32	75	DVVKESVNELSEKAKTDPQAAEKLNKLIEGYTYGEERK LYDSAL	34.398828	13.800839	3.705901	5.308861	40.1%	10.8%	15.4%
41	58	LSEKAKTDPQAAEKLNKL	10.676177	3.535546	1.355372	1.415413	33.1%	12.7%	13.3%
41	61	LSEKAKTDPQAAEKLNKLIEG	12.891826	4.148516	1.744197	1.587595	32.2%	13.5%	12.3%
41	75	LSEKAKTDPQAAEKLNKLIEGYTYGEERKLYDSAL	24.287387	9.908216	2.87125	4.040528	40.8%	11.8%	16.6%
42	58	SEKAKTDPQAAEKLNKL	10.144713	3.141387	1.048611	1.274797	31.0%	10.3%	12.6%
56	63	NKLIEGYT	1.344411	0.720293	1.02296	0.910831	53.6%	76.1%	67.7%
#59	75	IEGYTYGEERKLYDSAL	10.2792	5.153114	1.24488	2.329313	50.1%	12.1%	22.7%
#62	75	YTYGEERKLYDSAL	8.322433	3.907156	1.011225	2.061401	46.9%	12.2%	24.8%
68	75	RKLYDSAL	3.826801	1.544169	0.411798	0.833902	40.4%	10.8%	21.8%
76	83	SKIEKLIE	4.757447	0.606036	0.325324	0.218148	12.7%	6.8%	4.6%
82	107	IETLSPARSKSQSTMNQRNRNNRKIV	14.805574	12.86523	12.098505	11.930598	86.9%	81.7%	80.6%

Table S2: T3SS proteins identified by LC-MS/MS (Related to Fig. 2). T3SS proteins present on isolated WT EPEC IMVs detected by LC-MS/MS in various surface proteolysis experiments. 33 LEE-encoded proteins and 5 non-LEE effectors have been detected with medium or high confidence scores (see Supplementary Methods). For each protein, the number of IMV preparations out of the total in which it was detected is indicated (as a percentage; green: 70-100%; orange; 35-70%; red < 35%). The number of peptides used for the comparison in Fig. 2C is shown. Non-detected proteins are marked with (-).

Structural sub-assembly	Subunit function/role	T3SS common nomenclature	EPEC protein nomenclature	Uniprot Accession number	Confidence of identification	Total number of peptides detected	% of IMV preparations in which this protein was detected	No. peptides used for Urea vs Non-urea IMVs comparison
Translocators	Translocator	SctB	EspB	Q05129	High	33		17
	Translocator	SctE	EspD	B7UM93	High	22		13
	Translocator	SctA	EspA	B7UM94	High	10		10
Needle	Needle component	SctF	EscF	B7UM90	High	4		1
OM ring	Secretin	SctC	EscC	B7UMB3	High	13		13
OM-IM connector	Inner rod	SctI	EscI	B7UMB0	-	-		-
IM ring	Lipoprotein ring component	SctJ	EscJ	B7UMB1	High	7		7
	Major IM ring component	SctD	EscD	B7UM96	High	22		17
Export apparatus	Translocase channel	SctR	EscR	B7UMC1	-	-		-
	Translocase channel	SctS	EscS	B7UMC0	-	-		-
	Translocase channel	SctT	EscT	B7UMB9	-	-		-
	Minor component, external channel	SctU	EscU	B7UMB8	High	7		1
	Major component, external channel	SctV	EscV	B7UMA7	High	21		18
Cytoplasmic ring	Component of 6-pod assembly	SctQ	EscQ	B7UMA3	Medium	1		
	Connector of 6-pod with EscD	SctK	EscK	B7UMC3	High	11		9

ATPase complex	External Stator connecting ATPase and Cytoplasmic ring	SctL	EscL	B7UMC2	High	6		
	Hexameric ring-structure ATPase	SctN	EscN	B7UMA6	High	4		2
	Central stalk, inserting in ATPase ring	SctO	EscO	B7UMA5	-	-		-
Assembly regulators	Molecular ruler regulating needle/filament length	SctP	EscP	B7UMA4	High	5		5
Gatekeeper	Gatekeeper/ Affinity switch	SctW	SepL	B7UM95	High	14		10
	Subunit 1 of SctW chaperone heterodimer		SepD	B7UMB2	High	1		1
	Subunit 2 of SctW chaperone heterodimer		CesL	B7UMA8	High	3		4
Chaperones	For early substrates (EscF)		EscG	B7UM89	High			
	For early substrates (EscF)		EscE	B7UMC5	-	-		-
	For middle substrates (translocators)		CesAB	B7UMC4	High	4		3
	For middle substrates (translocators)		CesD	B7UMB4	High	7		4
	For middle substrates (translocators)		CesD2	B7UM91	High	9		9
	For late substrates (effectors)		CesT	P21244	High	7		6
	For late substrates (effectors)		CesF	B7UMA1	High	6		6

Transcription regulators	Global regulators		Ler	B7UMC6	High	1		1
	negative regulators		grIR	B7UMB6	Medium	2		
	Positive regulators		grIA	B7UMB5	High	2		2
	Histone-like nucleoid-like structuring protein		H-NS	B7UQC9	-	-		-
Muramidase			etgA	B7UMB7	-	-		-
Attachment receptor			Intimin	P19809	High	53		43
Secreted effector (LEE encoded)			Tir	B7UM99	High	30		21
			Map	B7UMA0	High	11		8
			EspZ	B7UMA9	High	2		-
			EspF	B7UM88	High	9		9
			EspG	B7UMC8	High	16		12
			EspJ	B7UMB1	High	6		-
			EspH	B7UMA2	-			-
Secreted effector (non-LEE encoded)			EspL	B7UI20	High	5		-
			NleA	B7UR60	High	2		-
			NleB2	B7UI21	High	6		-
			NleE1	B7UI22	High	3		-
			NleI	B7UNX2	High	1		-

Supplementary Materials and methods

Buffers

Buffer A	50mM Tris/HCl pH 8.0; 50mM NaCl
Buffer B	50mM Tris/HCl pH 8.0; 50mM NaCl; 50% Glycerol
Buffer C	50mM Tris/HCl pH 8.0; 50mM KCl; 5mM $MgCl_2$
Buffer D	50mM HEPES pH:7.5; 50mM KCl; 5mM $MgCl_2$; 5% glycerol
Buffer E	50mM HEPES-KOH pH 6.6; 50mM KCl
Buffer F	50mM Tris/HCl pH 8.0; 20% Glycerol
1X PBS	137mM $NaCl_2$; 2.7mM KCl; 4.3mM Na_2HPO_4; 1.4mM KH_2PO_4
Rehydration buffer	8M urea; 2% Triton; 2% IPG Buffer; 40mM DTT; 1% Bromophenol Blue

Antisera

Rabbit polyclonal antibodies against the indicated purified proteins, or protein domains, were raised by Davids Biotechnologie, Germany. T3SS antibodies were further purified by 9 cycles of negative immuno-absorption, using membranes isolated from *EPEC* strains that lacked the gene of interest, i.e. for α-CesAB, membranes isolated from EPECΔ*cesAB* cells were used.

Protein-antigen	Animal Source	Reference or commercial source
CesAB	Rabbit	(Chen et al, 2011; Creasey et al, 2003)
EspA	Rabbit	(Chen et al, 2011; Creasey et al, 2003)
SepL	Rabbit	This study
EscV Cytoplasmic domain	Rabbit	This study
CesF	Rabbit	This study
Tir	Rabbit	This study
MBP	Rabbit	This study
SecY	Rabbit	(Karamanou et al, 2008)
SecA	Rabbit	(Karamanou et al, 2008)
SecB	Rabbit	This study
OmpA	Rabbit	This study
PhoA	Rabbit	(Gouridis et al, 2009)
ZipA	Rabbit	This study
α-Rabbit	Goat	Jackson ImmunoResearch Europe Ltd
α-Mouse	Goat	Jackson ImmunoResearch Europe Ltd
α-His	Mouse	SEROTEC

α-Rabbit 5nm GOLD	Goat	British Biocell International (Cardiff, UK)

Dyes and labeled secondary antibodies

Dyes/labelled secondary antibodies	Commercial source
Cy™ 3-Affinipure α-Rabbit	Jacksons Immuno-Research Lab
Cy2 Oregon Green®4889 Phalloidin	Thermo Fisher Scientific
TO-PRO®-3	Thermo Fisher Scientific
DAPI	Thermo Fisher Scientific

Vectors

Vector name	Antibiotic resistance	Origin	Reference
pASKIBA7 plus	Ampicillin	pBR322	IBA life sciences (http://www.iba-lifesciences.com/isotope/2/2-1406-000-DS-2-1406-000-pASK-IBA7plus.pdf) (Guzman et al, 1995)
pET Duet 1	Ampicillin	pBR322	Novagen
pKD4	Kanamycin	R6Kγ	(Datsenko & Wanner, 2000)
pKD3	Chloramphenicol	R6Kγ	(Datsenko & Wanner, 2000)
pKD46	Ampicillin	R101 w/repA101ts	(Datsenko & Wanner, 2000)
pET 16B	Ampicillin	pBR322	Novagen
pET 22B	Ampicillin	pBR322	Novagen
pMAL-c2X	Ampicillin	pBR322	New England Biolabs
pET16B His-MBP-TEV	Ampicillin	pBR322	This study

Genetic constructs

Genes were cloned in the indicated plasmid vectors using a combination of restriction sites (as indicated). Gene-mutations were introduced by following the Quick Change Site-Directed Mutagenesis protocol (Stratagene-Agilent); templates and primers were as indicated. Restriction enzymes and T4 DNA Ligase were either from Minotech (Greece), or Promega, or NEB. For PCR mutagenesis PFU Ultra Polymerase (Stratagene) was used; for gene amplification either Expand High fidelity Polymerase (Roche) or DNA *Taq* polymerase (Thermo scientific). *Dpn*I was used to cleave the maternal methylated DNA (NEB). All PCR-generated plasmids were sequenced (Macrogen Europe). Plasmids were stored in DH5α cells.

Gene	Uniprot KB accession	Plasmid name	Vector	Cloning/PCR strategy or source
Chaperones and exported proteins				
cesAB	B7UMC4	pIMBB585	pETDuet-1	The *cesAB* gene, was amplified from EPEC strain E2348/69 using primers X345 and X346, digested with BamHI-HindIII and cloned in pETDuet-1.
cesAB and *espA*	B7UMC4 and B7UM94	pIMBB648	pETDuet-1	The *espA* gene was amplified from EPEC strain E2348/69 using primers X375 and X376, digested with MunI-KpnI and cloned in pIMBB585.
cesAB(ΔC) and *espA*	B7UMC4 and B7UM94	pIMBB803	pETDuet-1	The *cesAB* (N1-86) fragment was amplified from EPEC strain E2348/69 using primers X345 and X431, digested with BamHI-HindIII and cloned in pIMBB648 instead of *cesAB* gene.
cesAB and *espA*	B7UMC4 and B7UM94	pIMBB1289	pASK-IBA7plus	The *espA* gene was amplified from pIMBB648 using primers X1018 and X1019, digested with HindIII-HindIII and cloned in pIMBB806. Primer X1018 inserts an RBS.
cesAB(ΔC) and *espA*	B7UMC4 and B7UM94	pLMB1792	pASK-IBA7plus	The *espA* gene was digested from pIMBB1289 using HindIII-HindIII and cloned in pIMBB807
cesAB(ΔC)	B7UMC4	pIMBB801	pETDuet-1	The *cesAB* (N1-86) fragment was amplified from EPEC strain E2348/69 using primers X345 and X431, digested with BamHI-HindIII and cloned in pETDuet-1.
cesAB	B7UMC4	pIMBB806	pASK-IBA7plus	The *cesAB* gene was amplified from pIMBB585 using primers X453 and X346, digested with NheI-HindIII and cloned in pASK-IBA7plus.
cesAB(ΔC)	B7UMC4	pIMBB807	pASK-IBA7plus	The *cesAB*(N1-86) fragment was amplified from pIMBB585 using primers X453 and X431, digested with NheI-HindIII and cloned in pASK-IBA7plus

cesAB(6A)	B7UMC4	pIMBB1413	pASK-IBA7plus	The 6 mutations (R89A, K91A, R99A, R101A, R104A, K105A) in pIMBB806 were introduced sequentially using primer pairs X1221-X1222, X1223-X1224 and X1225-X1226.
cesAB(DRE)	B7UMC4	pIMBB1287	pETDuet-1	The mutations *D14L, R18D* and *E20L* were introduced in pIMBB585 using primer pairs X954-X955, X958-X959 and X1000-X1001.
cesAB(DRE)	B7UMC4	pIMBB1294	pASK-IBA7plus	The *cesAB(D14L/R18D/E20L)* gene was digested from pIMBB1287 using BamHI-HindIII and cloned in pASKIBA7plus vector.
cesAB(DRE/ΔC)	B7UMC4	pLMB0006	pETDuet-1	The *cesAB(D14L/R18D/E20L* (N1-86) was amplified from pIMBB1287 using primers X1442 and X431, digested with NcoI-HindIII and inserted in pETDuet-1.
cesT	P21244	pIMBB1157	pETDuet-1	The *cesT* gene was amplified from EPEC strain E2348/69 using primers X865 and X866, digested with BamHI-HindIII and cloned in pETDuet-1.
cesT and tir	P21244 and B7UM99	pIMBB1158	pETDuet-1	The *tir* gene was amplified from EPEC strain E2348/69 using primers X867 and X868, digested with MfeI-KpnI and cloned in pIMBB1157.
SepL-related constructs				
sepL	B7UM95	pIMBB1189	pET 16B	The *sepL* gene was amplified from EPEC strain E2348/69 using primers X980 and X981, digested with NdeI-XhoI and cloned in pET 16B.
sepD	B7UMB2	pIMBB1188	pET 16B	The *sepD* gene was amplified from EPEC strain E2348/69 using primers X978 and X979, digested with NdeI-XhoI and cloned in pET 16B.
cesL	B7UMA8	pIMBB1549	pETDuet-1	The *cesL* gene was amplified from EPEC strain E2348/69 using primers X1730 and X1731 digested with BamHI-SalI and cloned in pETDuet-1.
cesL	B7UMA8	pLMB1821	pASK-IBA7plus	The *cesL* gene was amplified from EPEC strain E2348/69 using primers X2068 and X1731 digested with NheI-SalI and cloned in pASK-IBA7plus.
cesL and *sepD*	B7UMA8 and B7UMB2	pIMBB1547	pETDuet-1	The *sepD* gene was amplified from EPEC strain E2348/69 using primers X1732 and X1733, digested with SalI-HindIII and cloned in pIMBB1549.
cesL, sepD and *sepL*	B7UMA8, B7UMB2 and B7UM95	pIMBB1548	pETDuet-1	The *sepL* gene was digested from pIMBB1189 using NdeI-XhoI and cloned in pIMBB1547.

His-*sepL*	B7UM95	pIMBB1305	pASK-IBA7plus	The *sepL* gene was digested from pIMBB1189 using XbaI-XhoI and cloned in pASKIBA7plus.
His-*sepD*	B7UMB2	pIMBB1304	pASK-IBA7plus	The *sepD* gene was digested from pIMBB1188 using XbaI-XhoI and cloned in pASKIBA7plus.
sepL(R333D)	B7UM95	pIMBB1543	pASK-IBA7plus	The mutation R333D was introduced in pIMBB1305 using primers X1735 and X1736.
sepL(N1-241)	B7UM95	pIMBB1541	pET 16B	The *sepL* fragment was amplified from EPEC strain E2348/69 using primers X980 and X1737, digested with NdeI-XhoI and cloned in pET 16B.
sepL N1-278	B7UM95	pIMBB1542	pET 16B	The *sepL* fragment was amplified from EPEC strain E2348/69 using primers X980 and X1738, digested with NdeI-XhoI and cloned in pET 16B.
sepL N124-351	B7UM95	pIMBB1540	pET 16B	The *sepL* fragment was amplified from EPEC strain E2348/69 using primers X1734 and X981, digested with NdeI-XhoI and cloned in pET 16B.
His-*sepL N1-241*	B7UM95	pLMB0090	pASK-IBA7plus	The *sepL* fragment was digested from pIMBB1541 using XbaI-XhoI and cloned in pASKIBA7plus.
His-*sepL N1-278*	B7UM95	pLMB0089	pASK-IBA7plus	The *sepL* fragment was digested from pIMBB1542 using XbaI-XhoI and cloned in pASK-IBA7plus.
His-*sepL N124-351*	B7UM95	pLMB0091	pASK-IBA7plus	The *sepL* fragment was digested from pIMBB1540 using XbaI-XhoI and cloned in pASK-IBA7plus.
sepL patch a	B7UM95	pLMB1755	pASK-IBA7plus	The synthetic gene fragment from pUC47 *sepL Patch a* was digested with XbaI-HindIII and cloned in pIMBB1305.
sepL patch b	B7UM95	pLMB1756	pASK-IBA7plus	The synthetic gene fragment from pUC47 *sepL Patch b* was digested with XbaI-HindIII and cloned in pIMBB1305.
sepL patch c	B7UM95	pLMB1757	pASK-IBA7plus	The synthetic gene fragment from pUC47 *sepL Patch c* was digested with HindIII-XhoI and cloned in pIMBB1305.
sepL patch d	B7UM95	pLMB1758	pASK-IBA7plus	The synthetic gene fragment from pUC47 *sepL Patch d* was digested with HindIII-XhoI and cloned in pIMBB1305.
sepL patch e	B7UM95	pLMB1759	pASK-IBA7plus	The synthetic gene fragment from pUC47 *sepL Patch e* was digested with HindIII-XhoI and cloned in pIMBB1305.
Major export apparatus component constructs				

escV	B7UMA7	pLMB0088	pASK-IBA7plus	The *escV* gene was amplified from EPEC strain E2348/69 using primers X1814 and X1710, digested with NheI –XhoI and cloned in pASK-IBA7plus.
escV patch α	B7UMA7	pLMB1762	pASK-IBA7plus	The synthetic gene fragment from pUC47 *escV Patch α* was isolated by NheI-HindIII digestion and inserted in pLMB0088.
escV patch β	B7UMA7	pLMB1763	pASK-IBA7plus	The synthetic gene fragment from pUC47 *escV β* was digested with NheI-HindIII and inserted in pLMB0088.
escV patch γ	B7UMA7	pLMB1764	pASK-IBA7plus	The synthetic gene fragment from pUC47 *escV γ* was digested with HindIII-XhoI and inserted in pLMB0088.
Constructs for protein purification and *in vivo* complementation				
escV(Δ21-509)	B7UMA7	pIMBB1459	pETDuet-1	The N1-20 and the N510-676 domains of EscV were amplified from EPEC strain E2348/69 using the primer pairs X1278-X1279 and X1280-X1281.The N1-20 product was digested with BamHI-PstI and the N510-676 by PstI-HindIII and were cloned sequentially in pETDuet-1.
espA	B7UM94	pIMBB485	pET 16B	The *espA* gene was amplified from EPEC strain E2348/69 using primers X258 and X259, digested with NdeI-BamHI and cloned in pET 16B.
cesF	B7UMA1	pIMBB664	pETDuet-1	The *cesF* gene was amplified from EPEC strain E2348/69 using primers X379 and X380, digested with BamHI-SalI and cloned in pETDuet-1.
Tir	B7UM99	pIMBB1448	pETDuet-1	The *tir* gene was amplified from EPEC strain E2348/69 using primers X1265 and X897, digested with BamHI-HindIII and cloned in pETDuet-1.
His-*mbp-TEV*	P0AEX9	pIMBB491	pET 16B	The *mbp* gene was amplified from pMAL-c2X using primers X239 and X240, digested with BamHI-HindIII and cloned in pETDuet-1.
His-*mbp-TEV-sepL*	B7UM95	pIMBB1191	pET 16B His MBP TEV	The *sepL* gene was amplified from EPEC strain E2348/69 using primers X980 and X981, digested with NdeI-XhoI and cloned in pET16B-MBP-TEV.
*escV(*N334-675)	B7UMA7	pLMB1676	pET 16B	The *escV* gene was amplified from EPEC strain E2348/69 using primers X1850 and X1710, digested with NdeI-XhoI and cloned in pET 16B.
His-*mbp-TEV-escVCD*	B7UMA7	pLMB1643	pET 16B - MBP-TEV	The *escV* C-terminal domain (N334-675) fragment was digested with NdeI- XhoI from pLMB1676 and inserted in pIMBB1191.
His-*secA*	P10408	pIMBB7	pET 16B	(Sianidis et al, 2001)
prophoA- His	P00634	pIMBB882	pET 22b	(Gouridis et al, 2009)

escN	B7UMA6	pIMBB1179	pASK-IBA7plus	The *escN* gene was amplified from EPEC strain E2348/69 using primers X698 and X253, digested with NheI-BamHI and cloned in pASK-IBA7plus
escQ	B7UMA3	pLMB0065	pASK-IBA7plus	The *escQ* gene was amplified from EPEC strain E2348/69 using primers X1665 and X1444, digested with NheI-HindIII and cloned in pASK-IBA7plus
escU	B7UMB8	pLMB0064	pASK-IBA7plus	The *escU* gene was amplified from EPEC strain E2348/69 using primers X1526 and X1527, digested with NheI-BamHI and cloned in pASK-IBA7plus
secB	P0AG86	pIMBB490	pET 16B	The *secB* gene was amplified from *E. coli* DH5a strain using primers X264 and X265, digested with NdeI-BamHI and cloned in pET 16B
ompA	P0A910	pIMBB454	pET22B	The *ompA* gene was amplified from *E. coli* DH5a strain using primers X191 and X193, digested with NdeI-EcoRI and cloned in pET 22B
zipA	P77173	pIMBB1476	pET22B	The *zipA* gene was amplified from *E. coli* DH5a strain using primers X1408 and X1409, digested with NdeI-XhoI and cloned in pET 22B
Constructs used for ^{35}S-labelling				
MM His-*cesAB*	B7UMC4	pLMB0003	pETDuet-1	The *cesAB* gene was amplified from pIMBB585 using primers X1442 and X346, digested with NcoI-HindIII and cloned in pETDuet-1.
MM His-*cesAB(ΔC)*	B7UMC4	pLMB0004	pETDuet-1	The *cesAB* (N1-86) gene was amplified from pIMBB585 using primers X1442 and X431, digested with NcoI-HindIII and cloned in pETDuet-1.
MM His-*cesAB(DRE)*	B7UMC4	pLMB0005	pETDuet-1	The gene was amplified from pIMBB1287 using primers X1442 and X346, digested with NcoI-HindIII and cloned in pETDuet-1.

Bacterial strains

To generate the deletion strains, the indicated gene was replaced by a gene encoding for the indicated antibiotic (Datsenko & Wanner, 2000)

Bacterial strain E. *coli*	**Description (gene deleted)**	**Reference/**
DH5a		Invitrogen
BL21 (DE3)		(Studier et al, 1990)
BL21.19 (DE3)		(Mitchell & Oliver, 1993)
BL31(DE3)		(Chatzi et al, 2017)
EPEC wt	*Escherichia coli* O127:H6 (strain E2348/69)	(Levine et al, 1978)

EPEC Δ*escN*	Δ*escN*:: *nptII*(Kan^R)	(Iguchi et al, 2009)
EPEC Δ*cesAB*	Δ*cesAB*:: *nptII*(Kan^R)	(Iguchi et al, 2009)
EPEC Δ*sepL*	Δ*sepL*:: *nptII*(Kan^R)	(Munera et al, 2010)
EPEC Δ*sepD*	Δ*sepD*:: *nptII*(Kan^R)	(Iguchi et al, 2009)
EPEC Δ*cesAB*/Δ*espA*	Δ*cesAB*:: *cat*(Cam^R), Δ*espA*:: *nptII*(Kan^R)	(Chen et al, 2011)
EPEC Δ*escQ*	Δ*escQ*:: *nptII*(Kan^R)	This study
EPEC Δ*escV*	Δ*escV*:: *nptII*(Kan^R)	This study
EPEC *escU*ΔCytoplasmic domain	*escU*Δ264-345:: *nptII*(Kan^R)	This study
EPEC *escV*ΔCytoplasmic domain	*escV*Δ334-675:: *nptII*(Kan^R)	This study
E2348/69 Δ*cesL*	Δ*cesL*:: *nptII*(Kan^R)	This study
EPEC Δ*escV*Δ*sepL*	Δ*escV*:: *nptII*(Kan^R), Δ*sepL*:: *cat*(Cam^R)	This study
EPECΔ*escV*Δ*sepD*	Δ*escV*:: *nptII*(Kan^R), Δ*sepD*:: *cat*(CamR)	This study

List of primers

Primers used for gene cloning and mutagenesis

Primer Name	Forward/ Reverse	Gene or/and Mutation Inserted	Restriction Site	Sequence (5'-3') (Mutated codons are bold, restriction sites underlined)
X191	F	*ompA*	NdeI	GGAATTCCATATGAAAAAGACAGCTATCGC
X193	R	*ompA*	EcoRI	CGGAATTCTTAAGCCTGCGGCTGAGTTAC
X239	F	*Mbp*	AseI	CCGGATTAATATGAAAACTGAAGAAGGTAAACTGG
X240	R	*mbp* Inserts Tev cleavage site	NdeI	CCCTTAAGCATATGGGCGCCCTGAAAATAAAGATTCTCAGTGGCTTCATCGACAGTCTGACG
X253	R	*escN*	BamHI	GCGGGATCCTCAGGCAACCACTTTGAATAGG
X258	F	*espA*	NdeI	GGGAATTCCATATGGATACATCAACTACAGCA
X259	R	*espA*	BamHI	GCGGGATCCTTATTTACCAAGGGATATTCC
X264	F	*secB*	NdeI	GGGAATTCCATATGTCAGAACAAAACAACACTGAAATG
X265	R	*secB*	BamHI	GCGGGATCCTTAGGCATCCTGATGTTCTTCAGTACC
X345	F	*cesAB*	BamHI	CGCGGATCCGATGAGTATTGTGAGCCAAACAAG
X346	R	*cesAB*	HindIII	CCCAAGCTTTCATACTATTTTTCTATTATTTCTATTCCG
X375	F	*espA*	MfeI	GGCCAATTGGGATACATCAACTACAGCATCAGTTG

X376	R	*espA*	KpnI	GGGGTACCTTATTTACCAAGGGATATTCCTG
X379	F	*cesF*	BamHI	GCGGGATCCGAATGAACAATTTTGCAAAGATC
X380	R	*cesF*	SalI	GGCGTCGACTCAAAGTGAAAGTAGTTTTATTATTTC
X431	R	*cesAB N1–86*	HindIII	CCCAAGCTTTCAACTCAGTGTCTCTATTAGTTTTTC
X453	F	*cesAB*	NheI	GCGCCCTAGCTAGCATGAGTATTGTGAGCCAAACAAG
X698	F	*escN*	NheI	GCGCCCTAGCTAGCATGATTTCAGAGCATGATTCTG
X865	F	*cesT*	BamHI	CGCGGATCCGATGTCATCAAGATCTGAACTT
X866	R	*cesT*	HindIII	CCCAAGCTTTTATCTTCCGGCGTAATAATG
X867	F	*Tir*	MfeI	GGCCAATTG*G*CCTATTGGTAACCTTGGT
X868	R	*Tir*	KpnI	GGGGGTACCTTAAACGAAACGTACTGGTCC
X897	R	*Tir*	HindIII	CCCAAGCTTTTAAACGAAACGTACTGGTCCCGG
X954	F	*cesAB/D14L*		AAAGAACTATTA**CTG**AAAAAAATAAGATCAGAAATTGAGGCG
X955	R	*cesAB/D14L*		CGCCTCAATTTCTGATCTTATTTTTTT**CAG**TAATAGTTCTTT
X958	F	*cesAB/D14L/R18D*		AAAGAACTATTA**CTG**AAAAAAATA**GAC**TCAGAAATTGAGGCG
X959	R	*cesAB/D14L/R18D*		CGCCTCAATTTCTGA**GTC**TATTTTTTT**CAG**TAATAGTTCTTT
X978	F	*sepD*	NdeI	GGGAATTCCATATGAACAATAATAATGGCATAGCA
X979	R	*sepD*	XhoI	GACCCGCTCGAGTTACACAATTCGTCCTATATC
X980	F	*sepL*	NdeI	GGGAATTCCATATGGCTAATGGTATTGAATTTAAT
X981	R	*sepL*	XhoI	GACCCGCTCGAGTTACATAACATCCTCCTTATAATC
X1000	F	*cesAB/E20L*		CTGAAAAAAATACTGTCA**TTA**ATTGAGGCGATAAAAAAA
X1001	R	*cesAB/E20L*		TTTTTTTATCGCCTCAAT**TAA**TGACAGTATTTTTTTCAG
X1018	F	petDuet 1 RBS	HindIII	CCCAAGCTTTTAAGTATAAGAAGGAGATATACATATGGC
X1019	R	*espA*	HindIII	CCCAAGCTTTTATTTACCAAGGGATATTCCTGAAATAGT
X1221	F	*cesAB/R89A/K91A*		GAGACACTGAGTCCAGCA**GCA**TCT**GCA**AGCCAATCAACAATGAAT
X1222	R	*cesAB/R89A/K91A*		ATTCATTGTTGATTGGCT**TGC**AGA**TGC**TGCTGGACTCAGTGTCTC
X1223	F	*cesAB(R89A/K91A)/ R99A/R101A*		CAATCAACAATGAATCAA**GCG**AAT**GCA**AATAATAGAAAAATAGTA
X1224	R	*cesAB(R89A/K91A)/ R99A/R101A*		TACTATTTTTCTATTATT**TGC**ATT**CGC**TTGATTCATTGTTGATTG
X1225	F	*cesAB(R89A/K91A/ R99A/R101A)/ R104A/K105A*		CAAGCGAATGCAAATAAT**GCAGCA**ATAGTATGAAAGCTTGACCTG
X1226	R	*cesAB(R89A/K91A/ R99A/R101A) /R104A/K105A*		CAGGTCAAGCTTTCATACTAT**TGCTGC**ATTATTTGCATTCGCTTG
X1265	F	*Tir*	BamHI	CGCGGATCCGATGCCTATTGGTAACCTTGGTAATAATGTA
X1278	F	*escV*	BamHI	CGCGGATCC*G*ATGAATAAACTCTTAAATATATTTAAAAAAGCA
X1279	R	*escV N1-20*	PstI	AAAACTGCAGAGCCAGAATAAGATCGTGATATGA
X1280	F	*escV N510-675*	PstI	AAAACTGCAGCGCCAGCTTGGTTTGAGCAAA
X1281	R	*escV N510-675*	HindIII	CCCAAGCTTTCATGCTCTGAAATCATTTACCGT

X1408	F	*zipA*	NheI	GGGAATTCCATATGATGCAGGATTTGCGTCTGATATTA
X1409	R	*zipA*	XhoI	GACCCGCTCGAGGGCGTTGGCGTCTTTGACTTCGCG
X1442	F	*cesAB*	NcoI	CATGCCATGGGCATGATGAGCAGCCATCACCATCATCAC
X1444	R	*escQ*	HindIII	CGCAAGCTTTTAATCACATACTACGCTAATAGT
X1526	F	*escU*	NheI	GCGCCCTAGCTAGC-GAAAAAACAGAAAAGCCCACAC
X1527	R	*escU*	BamHI	CGCGGATCCTTAATAATCAAGGTCTATCGCAATAC
X1665	F	*escQ*	NheI	GCGCCCTAGCTAGC-ATGAAGCCATTGAGTTCACAAT
X1710	R	*escV*	XhoI	GGCCTCGAGTCATGCTCTGAAATCATTTAC
X1730	F	*cesL*	BamHI	CGCGGATCCGATGAATCTTTTAGTTAAAAGAAATG
X1731	R	*cesL*	SalI	ACGCGTCGACTTATGATGTCATCCTGCGAACG
X1732	F	*sepD*	SalI	ACGCGTCGACTTTAAGAAGGAGATATAC*C*ATGAACAATAATAATGGCATAG
X1733	R	*sepD*	HindIII	CGCAAGCTTTTACACAATTCGTCCTATATCAG
X1734	F	*sepL N124-351*	NdeI	GGGAATTCCATATGATGCCAAAGGGGGAAATTGTTG
X1735	F	*sepL/R333D*		ATTGATAACGAGCAG**GAC**AGTAATACATTATTA
X1736	R	*sepL/R333D*		TAATAATGTATTACT**GTC**CTGCTCGTTATCAAT
X1737	R	*sepL N1-241*	XhoI	GACCCGCTCGAGTCATCATCTTACCTTGTCTTCAAAAG
X1738	R	*sepL N1-278*	XhoI	GACCCGCTCGAGTCATCATAAAATGACATCTTTATC
X1814	F	*escV*	NheI	GCGCCCTAGCTAGCATGAATAAACTCTTAAATATATTTAAAAAAGCA
X1850	F	*escV N334-675*	NdeI	GGAATTCCATATGGATGCTATGGGAGCTGATTT
X2068	F	*cesL*	NheI	GCGCCCTAGCTAGCATGAATCTTTTAGTTAAAAGAAATG

Primers used for gene replacement.

Primers X1697, X1700, X1702 and X2067 were designed internally to the deleted gene to avoid polar effects due to the deletion of sequences in overlapping neighbor genes.

Primer Name	Forward/ Reverse	Gene or fragment deleted	Resistance introduced	Sequence (5'-3')
X1494	F	*escV*	KanR	ACTTTTCAACAGCATGTGCAGATTATTGAGCGCGTTCGCAGGATGACATCTAGGCTGGAGCTGCTTC
X1697	F	*escQ*	Chlo^R	CGATTTTTATTTGCCGTTGTTGCCAGTTATCGGAACCGGTCGCCTCTATACATATGAATATCCTCCTTAG
X1698	R	*escQ*	Chlo^R	CCTGATAATGATGACGACATCATACACCTCCCTATATAACGCATTCCTGATAGGCTGGAGCTGCTTC
X1700	R	*escU*	Kan^R	CAAGGTCTATCGCAATACGAATCAATTGTGCCACAGGTTCAAAAAAGTCTCATATGAATATCCTCCTTAG
X1701	F	*escV*	Kan^R	TCTTAAATATATTTAAAAAAGCAGAGTCATATCACGATCTTATTCTGGCTTAGGCTGGAGCTGCTTC
X1702	R	*escV*	Kan^R	GTTCGATATTATTCCCAACCTCCTGAAAAGAGAGCACGGGGACTGACGGGCATATGAATATCCTCCTTAG
X1721	F	*escUΔ263-345*	Kan^R	GAAGTTTGGCGAATAACATCAAAAAATCAACCGTTATTGTTAAAAACCCGTGATAATAGGCTGGAGCTGCTTC
X1722	F	*escVΔ333-675*	Kan^R	TAGCCTGGAAATTACAGAAGAAAAGAACATTTGGAGCTGGCAATAATAAGTGATAATAGGCTGGAGCTGCTTC
X2066	F	*cesL*	Kan^R	GCTTTTTCAAGCAATAATATTCACACGTATACTTACTTCAGAGCCTGCAGTAGGCTGGAGCTGCTTC
X2067	R	*cesL*	Kan^R	ACATCGATATTGGTTAACAAACAACTTAAGCACAATTTATTATCGTAGACCATATGAATATCCTCCTTAG
X2081	F	*sepL*	Chlo^R	ACAGTACTATTTAATGGAATATTCATAATTAATGATTACGTGAGTTTCCACATATGAATATCCTCCTTAG
X2082	R	*sepL*	Chlo^R	ACCTCTTCATAATCTTTCTTAGCATGACAAAAACTATAAAAAAAACAATATAGGCTGGAGCTGCTTC

Synthetic genes

To introduce the following multiple mutations (red in the sequence), the indicated *sepL* or *escV* DNA fragments were synthesized by GenScript® , delivered as pUC47 clones and subcloned in pASK IBA7plus vector (IBA life sciences).

Gene/mutant	Mutation introduced	Amino acid sequence
sepL		
Patch a	E90S/ T93A/ K140A/ F141S/ K143A	MANGIEFNQNPASVFNSNSLDFELESQQLTQKNSSNTSSPLINLQNELAMITSSSLSETIEGLSLGYRKGSARKEEEGTT IEKLLNEMQSLLALTDSDKIKELSLKNSGLLEQHDPTLAMFGNMPKGEIVALISSLLQSASVAIELKKKYAKLLLDLLGE DDWELALLSWLGVGELNQEGIQKIK
Patch b	K152S/ D156S/ E160A/ D161A/ D162S/ E164S	MANGIEFNQNPASVFNSNSLDFELESQQLTQKNSSNTSSPLINLQNELAMITSSSLSETIEGLSLGYRKGSARKEEEGTT IEKLLNEMQELLTLTDSDKIKELSLKNSGLLEQHDPTLAMFGNMPKGEIVALISSLLQSKFVKIELKKKYASLLLSLLGA ASWSLALLSWLGVGELNQEGIQKIK
Patch c	D204A/ M207A/ E208S/ D211A	YEKAKDEDSENGASLLAWFASIKALPEREKHLKVIIRALSFDLSYMSSFEDKVRTSSIISDLCRIIIFLSLNNYTDIIAI SIKKDKDVILNEMLSIIEHVWLTEDWLLESPSRVSIVEDKHVYYFHLLKEFFASLPDACFIDNEQRSNTLLMIGKVIDYK EDVM*
Patch d	S257A/ N259A/ N260A T262S/ D263A	YEKAKDEDSENGASLLDWFMEIKDLPEREKHLKVIIRALSFDLSYMSSFEDKVRTSSIISDLCRIIIFLALAAYSAIIAI SIKKDKDVILNEMLSIIEHVWLTEDWLLESPSRVSIVEDKHVYYFHLLKEFFASLPDACFIDNEQRSNTLLMIGKVIDYK EDVM*
Patch e	H313A/ K316V/ L338S	YEKAKDEDSENGASLLDWFMEIKDLPEREKHLKVIIRALSFDLSYMSSFEDKVRTSSIISDLCRIIIFLSLNNYTDIIAI SIKKDKDVILNEMLSIIEHVWLTEDWLLESPSRVSIVEDKHVYYFALLVEFFASLPDACFIDNEQRSNTLSMIGKVIDYK EDVM*
escV		
Patch α	S359A/ N360S/ Y410A/ K411S/ D412S	MNKLLNIFKKAESYHDLILALFFFMAVMMMIIPLPTVVVDIIIAINISTALLLLMLSIYIKNPLELTSFPTILLITTLMR LSLSVSTTRLILLHHDAGDIIYSFGNFVVGGNIVVGLVIFTIITIVQFMVITKGAERVAEVSARFSLDGMPGKQMSIDGD MRAGVIDPLEAKVLRSRVQKESQFYGSMDGAMKFVKGDAIAGIIIVLVNLFGGVLIGMWQFDMPFSEALSLFSVLSVGDA LVAQIPALIISVTAGVVVTRVPGESEKEENLAGDIVQQVSVNSRPFLISAALMLVMAIIPGFPTLVFLFLAVCLLGIAWK LQKKRTFGAGNNKDAMGADLSNSQNISPGAEPLILNLSASIYSSDITQQIEVMRWNFFEESGIPLPKIIVNPVKNNDSAI EFLLYQESIASSTLIDDTVYFEAGHAEISFEFVQE
Patch β	E401S/ L403A/ Q406A/ E407S/ S408A	MNKLLNIFKKAESYHDLILALFFFMAVMMMIIPLPTVVVDIIIAINISTALLLLMLSIYIKNPLELTSFPTILLITTLMR LSLSVSTTRLILLHHDAGDIIYSFGNFVVGGNIVVGLVIFTIITIVQFMVITKGAERVAEVSARFSLDGMPGKQMSIDGD MRAGVIDPLEAKVLRSRVQKESQFYGSMDGAMKFVKGDAIAGIIIVLVNLFGGVLIGMWQFDMPFSEALSLFSVLSVGDA LVAQIPALIISVTAGVVVTRVPGESEKEENLAGDIVQQVSVNSRPFLISAALMLVMAIIPGFPTLVFLFLAVCLLGIAWK LQKKRTFGAGNNKDAMGADLSNSQNISPGAEPLILNLSSNIYSSDITQQIEVMRWNFFEESGIPLPKIIVNPVKNNDSAI SFALYASAIYKDTLIDDTVYFEAGHAEISFEFVQE
Patch γ	K468S/ T470A/ F471V/ K474A/ K475S/ L478S	STNSIVYKTNKTNQQLAHLTGMDVYATTNDSIAVLLASLVSSNAKEFIGVQETRYLMDIMERKYNELVKELQRQLGLSKI VDILQRLVEENVSIRDLRTIFETLIFWSTKEKDVVILCEYVRIALRRHILGRYSVSGTLLNVWLIGSDIENELRESIRQT SSGSYLNISPERTEQIIGFLKNIMNPTGNGVILTALDIRRYVKKMIEGSFPSVPVLSFQEVGNNIELKVLGTVNDFRA*

Immobilized peptide arrays

The following 13 mer-, overlapping (by 10 residues) peptides were synthesized and immobilized on cellulose membranes by PepSpot Peptides, JPT Peptide Technologies, Germany.

SepL											
No	Sequence	No	Sequence	No	Sequence	No	Sequence	No	Sequence	No	Sequence
1	MANGIEFNQNPAS	20	ETIEGLSLGYRKG	39	DPTLAMFGNMPKG	58	GVGELNQEGIQKI	77	DLSYMSSFEDKVR	96	HVWLTEDWLLESP
2	GIEFNQNPASVFN	21	EGLSLGYRKGSAR	40	LAMFGNMPKGEIV	59	ELNQEGIQKIKKL	78	YMSSFEDKVRTSS	97	LTEDWLLESPSRV
3	FNQNPASVFNSNS	22	SLGYRKGSARKEE	41	FGNMPKGEIVALI	60	QEGIQKIKKLYEK	79	SFEDKVRTSSIIS	98	DWLLESPSRVSIV
4	NPASVFNSNSLDF	23	YRKGSARKEEEGT	42	MPKGEIVALISSL	61	IQKIKKLYEKAKD	80	DKVRTSSIISDLC	99	LESPSRVSIVEDK
5	SVFNSNSLDFELE	24	GSARKEEEGTTIE	43	GEIVALISSLLQS	62	IKKLYEKAKDEDS	81	RTSSIISDLCRII	100	PSRVSIVEDKHVY
6	NSNSLDFELESQQ	25	RKEEEGTTIEKLL	44	VALISSLLQSKFV	63	LYEKAKDEDSENG	82	SIISDLCRIIIFL	101	VSIVEDKHVYYFH
7	SLDFELESQQLTQ	26	EEGTTIEKLLNEM	45	ISSLLQSKFVKIE	64	KAKDEDSENGASL	83	SDLCRIIIFLSLN	102	VEDKHVYYFHLLK
8	FELESQQLTQKNS	27	TTIEKLLNEMQEL	46	LLQSKFVKIELKK	65	DEDSENGASLLDW	84	CRIIIFLSLNNYT	103	KHVYYFHLLKEFF
9	ESQQLTQKNSSNT	28	EKLLNEMQELLTL	47	SKFVKIELKKKYA	66	SENGASLLDWFME	85	IIFLSLNNYTDII	104	YYFHLLKEFFASL
10	QLTQKNSSNTSSP	29	LNEMQELLTLTDS	48	VKIELKKKYAKLL	67	GASLLDWFMEIKD	86	LSLNNYTDIIAIS	105	HLLKEFFASLPDA
11	QKNSSNTSSPLIN	30	MQELLTLTDSDKI	49	ELKKKYAKLLLDL	68	LLDWFMEIKDLPE	87	NNYTDIIAISIKK	106	KEFFASLPDACFI
12	SSNTSSPLINLQN	31	LLTLTDSDKIKEL	50	KKYAKLLLDLLGE	69	WFMEIKDLPEREK	88	TDIIAISIKKDKD	107	FASLPDACFIDNE
13	TSSPLINLQNELA	32	LTDSDKIKELSLK	51	AKLLLDLLGEDDW	70	EIKDLPEREKHLK	89	IAISIKKDKDVIL	108	LPDACFIDNEQRS
14	PLINLQNELAMIT	33	SDKIKELSLKNSG	52	LLDLLGEDDWELA	71	DLPEREKHLKVII	90	SIKKDKDVILNEM	109	ACFIDNEQRSNTL
15	NLQNELAMITSSS	34	IKELSLKNSGLLE	53	LLGEDDWELALLS	72	EREKHLKVIIRAL	91	KDKDVILNEMLSI	110	IDNEQRSNTLLMI
16	NELAMITSSSLSE	35	LSLKNSGLLEQHD	54	EDDWELALLSWLG	73	KHLKVIIRALSFD	92	DVILNEMLSIIEH	111	EQRSNTLLMIGKV
17	AMITSSSLSETIE	36	KNSGLLEQHDPTL	55	WELALLSWLGVGE	74	KVIIRALSFDLSY	93	LNEMLSIIEHVWL	112	SNTLLMIGKVIDY
18	TSSSLSETIEGLS	37	GLLEQHDPTLAMF	56	ALLSWLGVGELNQ	75	IRALSFDLSYMSS	94	MLSIIEHVWLTED	113	LLMIGKVIDYKED
19	SLSETIEGLSLGY	38	EQHDPTLAMFGNM	57	SWLGVGELNQEGI	76	LSFDLSYMSSFED	95	IIEHVWLTEDWLL	114	MIGKVIDYKEDVM
EscV CD											
No.	Sequence	No.	Sequence	No.	Sequence	No.	Sequence	No.	Sequence	No.	Sequence
1	DAMGADLSNSQNI	20	NPVKNNDSAIEFL	39	KTNQQLAHLTGMD	58	VKELQRQLGLSKI	77	LRRHILGRYSVSG	96	NIMNPTGNGVILT
2	GADLSNSQNISPG	21	KNNDSAIEFLLYQ	40	QQLAHLTGMDVYA	59	LQRQLGLSKIVDI	78	HILGRYSVSGTLL	97	NPTGNGVILTALD
3	LSNSQNISPGAEP	22	DSAIEFLLYQESI	41	AHLTGMDVYATTN	60	QLGLSKIVDILQR	79	GRYSVSGTLLNVW	98	GNGVILTALDIRR

4	SQNISPGAEPLIL	23	IEFLLYQESIYKD	42	TGMDVYATTNDKI	61	LSKIVDILQRLVE	80	SVSGTLLNVWLIG	99	VILTALDIRRYVK
5	ISPGAEPLILNLS	24	LLYQESIYKDTLI	43	DVYATTNDKITFL	62	IVDILQRLVEENV	81	GTLLNVWLIGSDI	100	TALDIRRYVKKMI
6	GAEPLILNLSSNI	25	QESIYKDTLIDDT	44	ATTNDKITFLLKK	63	ILQRLVEENVSIR	82	LNVWLIGSDIENE	101	DIRRYVKKMIEGS
7	PLILNLSSNIYSS	26	IYKDTLIDDTVYF	45	NDKITFLLKKLVL	64	RLVEENVSIRDLR	83	WLIGSDIENELRE	102	RYVKKMIEGSFPS
8	LNLSSNIYSSDIT	27	DTLIDDTVYFEAG	46	ITFLLKKLVLSNA	65	EENVSIRDLRTIF	84	GSDIENELRESIR	103	KKMIEGSFPSVPV
9	SSNIYSSDITQQI	28	IDDTVYFEAGHAE	47	LLKKLVLSNAKEF	66	VSIRDLRTIFETL	85	IENELRESIRQTS	104	IEGSFPSVPVLSF
10	IYSSDITQQIEVM	29	TVYFEAGHAEISF	48	KLVLSNAKEFIGV	67	RDLRTIFETLIFW	86	ELRESIRQTSSGS	105	SFPSVPVLSFQEV
11	SDITQQIEVMRWN	30	FEAGHAEISFEFV	49	LSNAKEFIGVQET	68	RTIFETLIFWSTK	87	ESIRQTSSGSYLN	106	SVPVLSFQEVGNN
12	TQQIEVMRWNFFE	31	GHAEISFEFVQEK	50	AKEFIGVQETRYL	69	FETLIFWSTKEKD	88	RQTSSGSYLNISP	107	VLSFQEVGNNIEL
13	IEVMRWNFFEESG	32	EISFEFVQEKLST	51	FIGVQETRYLMDI	70	LIFWSTKEKDVVI	89	SSGSYLNISPERT	108	FQEVGNNIELKVL
14	MRWNFFEESGIPL	33	FEFVQEKLSTNSI	52	VQETRYLMDIMER	71	WSTKEKDVVILCE	90	SYLNISPERTEQI	109	VGNNIELKVLGTV
15	NFFEESGIPLPKI	34	VQEKLSTNSIVYK	53	TRYLMDIMERKYN	72	KEKDVVILCEYVR	91	NISPERTEQIIGF	110	NIELKVLGTVNDF
16	EESGIPLPKIIVN	35	KLSTNSIVYKTNK	54	LMDIMERKYNELV	73	DVVILCEYVRIAL	92	PERTEQIIGFLKN	111	ELKVLGTVNDFRA
17	GIPLPKIIVNPVK	36	TNSIVYKTNKTNQ	55	IMERKYNELVKEL	74	ILCEYVRIALRRH	93	TEQIIGFLKNIMN		
18	LPKIIVNPVKNND	37	IVYKTNKTNQQLA	56	RKYNELVKELQRQ	75	EYVRIALRRHILG	94	IIGFLKNIMNPTG		
19	IIVNPVKNNDSAI	38	KTNKTNQQLAHLT	57	NELVKELQRQLGL	76	RIALRRHILGRYS	95	FLKNIMNPTGNGV		

Preparation of Inverted inner membrane vesicles (IMVs)

EPEC cells, grown overnight (200 mL LB; 37 ^{o}C) were used to inoculate 15 L LB (37 ^{o}C; 5 h). Cells were harvested (5,000 x g; 20 min; 4 ^{o}C; Avanti J-26S XPI, Beckman; JLA 8.1000 rotor), resuspended in Buffer F and lysed using a French press (8,000 psi; 5-6 times). Unbroken cells were removed (3,000 x g; 10 min; Sigma 3-16KL; rotor 11180) before the supernatant was ultra-centrifuged (100,000 x g; 90 min; 4 ^{o}C; fixed angle 45Ti rotor; Optima XPN-80, Beckman). The membrane pellet was Dounce-homogenized in Buffer F, loaded (2.5 mL) on top of a 5-step sucrose gradient (1.9; 1.7; 1.5; 1.3; 1.1 M sucrose in 50 mM Tris pH: 8.0; 6 mL / layer) and centrifuged (100,000 x g; 16 h; 4 ^{o}C; swinging bucket SW32 Ti rotor, Optima XPN-80, Beckman) (equilibrium centrifugation). Inverted Inner Membrane Vesicles (IMVs) were collected from gradient fractions 2-3, resuspended in Buffer F and re-centrifuged (100,000 x g; 90 min; 4 ^{o}C; fixed angle 45Ti rotor; Optima XPN-80, Beckman). The membrane pellet was homogenized in 6 M Urea-Buffer C (35 min; ice), loaded on top of an equal volume of 0.2 M Sucrose-Buffer C and centrifuged (100,000 x g; 90 min; 4 ^{o}C; swinging bucket SW32 Ti rotor, Optima XPN-80, Beckman). Finally, IMVs were collected (Chang et al, 1978; Cunningham et al, 1989; Lill et al, 1989; Lill et al, 1990; Rhoads et al, 1984), homogenized in Buffer C, passed through an Avestin LiposoFast-Basic system (100 nm pore size filter; 15-21 times) and stored in aliquots at -80 ^{o}C.

***In vitro* protein labeling with [^{35}S]-methionine**

For protein labeling *in vitro* we used the Easy Tag^{TM} L-[^{35}S]-methionine (1 mC, Perkin Elmer) and the TNT® Quick coupled Transcription/Translation systems (Promega), according to the manufacturer's instructions and as described (Gouridis et al, 2010; Gouridis et al, 2013). At the end of the labeling process, a buffer exchange step was included, using hand-made columns (1 ml syringes; G-50 resin; equilibrated with Buffer A). Proteins were aliquoted and stored in Buffer B (maximum 3-5 days; -20 ^{o}C).

Affinity measurements/ Determination of equilibrium dissociation constants (K_ds)

Proteins, stored in Buffer B, were serially diluted in Buffer C (20 concentration points well distributed within the 0.01-5 μM range were used) and added to reactions (final volume 20 μl in Buffer C) containing IMVs (20 μg total membrane protein/reaction), as described (Gouridis et al, 2009; Gouridis et al, 2010; Gouridis et al, 2013). The [^{35}S]-labelled protein, diluted (4 times), was added to all the reactions, as a tracer (1 μl / reaction). Samples were incubated (20 min; ice), overlaid on an equal volume of BSA/sucrose cushion (0.2 M Sucrose; 1 mg/ml BSA in Buffer C) and centrifuged (300,000 x g; 20 min; 4°C; rotor TL-100; Optima Max-XP, Beckman-Coulter). The pellet (containing IMVs and IMVs-bound proteins) was resuspended in 300 μl buffer C by using a waterbath sonicator and then proteins were immobilized on a nitrocellulose membrane using a vacuum manifold (Bio-Dot apparatus; Bio-Rad). Binding of [^{35}S]-labelled proteins on IMVs was visualized by using a high resolution phosphor storage screen (GE Healthcare) on a Typhoon FLA 9500 system (GE Healthcare). For signal - quantification we used the Image Quant software (GE Healthcare). Data were analyzed by non-linear regression fit for one binding site, using Prism 5 (GraphPad). For the determination of one K_d, 9-14 repetitions were performed, each repeat using 20-25 concentration points.

CesAB/EspA *in vivo* and *in vitro* cross-linking

Purified His CesAB/EspA (50 μM; Buffer D) was incubated with succinimidyl 4,4-azipentanoate (SDA-NHS-Diazerine; 240 μM; 2 h; dark; 4 °C) and quenched (10 mM Tris/HCl pH: 8; 30 min; ice). The labeled complex was diluted (to 10 μM in Buffer C), mixed with either His-CesL/SepD/SepL complex (10 μM in Buffer C) or IMVs (100 μg total membrane protein),

incubated (100 μl reactions; 5 min; 4 ^{o}C) and UV irradiated (10 cm dist. from UV source; BLX-365 UV; Vilber Lourmat; 15min; 4 °C). IMV-containing samples were ultra-centrifuged (250,000 x g; 15 min; 4 ^{o}C) and resuspended in 100 μl Buffer C. Cross-linked polypeptides were analyzed on 12% SDS-PAGE, electrotransferred and immunostained. The synthesis of SDA-NHS-Diazerine will be detailed elsewhere.

IMVs immunostaining coupled with electron microscopy

IMVs (10 μg total membrane protein / 50 μl; Buffer C) were sonicated (3 x 15 sec; bath sonicator; 4 ^{o}C), applied (5 μl) on a glow-discharged 400 mesh carbon and formvar coated copper grids (TedPella; Redding CA, USA). After 5min (RT), grids were washed (1 x PBS) incubated with α-EscVC-domain antibodies (1:500; 1 h; RT) and then with 2nd^{ary} α-rabbit coupled to 5 nm GOLD particles (1:500; 1 h; RT). Grids were further incubated with 1% Uranyl Acetate in Milli Q water (1 min; RT), blotted, dried and observed (JEM1400; 80 kV; JEOL USA, Inc; equipped with Olympus SIS 11Mpxl; VIB/KUL imaging facility).

Hydrogen-deuterium exchange (HDX) mass spectrometry (MS)

Local HDX-MS was performed as described (Tsirigotaki et al, 2017) with slight modifications. Proteins (Buffer B) were dialyzed (16 h; Buffer E; 4 ^{o}C) and concentrated (144 μM). 1 μl of protein sample was diluted with 49 μl deuterated buffer E [lyophilized; freshly dissolved in D_2O (99.9% atom D, Euriso-top); pD 6.6] on ice, resulting in 98% v/v D content. pD refers to the corrected value for the isotope effect. At 10 sec, the exchange reaction was quenched by acidification (pD 2.5) and freezing in liquid nitrogen. The quenching solution contained urea (2 M), formic acid (Ultra-pure, Merck) and protease XIII (1.3 mg/ml) from *Aspergillus saitoi* (Sigma). In-solution digestion was allowed (2 min; ice) prior to freezing. 48 pmol CesAB from the quenched reaction (0.96 μM) were injected into a nanoACQUITY UPLC/ HDX (Waters) coupled with a Synapt G2 (Waters) mass spectrometer. Data acquisition was performed using the MassLynX interface (version 4.1 SCN870, Waters) and data analysis using the ProteinLynX Global Server (PLGS v3.01, Waters) and DynamX software (v3.0, Waters).

Surface proteolysis of IMVs

IMVs (10 μg total membrane protein) were resuspended [50 mM Ammonium Bicarbonate Solution (ABS)] using 3 x 15sec pulses in a bath sonicator. For the reduction and alkylation of cysteines, samples were first incubated with 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (30 min; 22 ^{o}C) and then with 10 mM Iodoacetamide (IAA) (30 min; 22 ^{o}C; dark). Protein samples were incubated with Trypsin (0.1 μg; Trypsin Gold, Promega; 15 h; 37 ^{o}C; shaking) acidified (Trifluoroacetic acid; TFA; pH <2), lyophilized (Speedvac; Savant) and desalted using custom made C18 column-tips (Rappsilber et al, 2007; Tsolis & Economou, 2017a)**.**

LC-MS/MS analysis

Lyophilized samples were dissolved in aqueous solution [0.1% v/v formic acid (FA); 5% v/v Acetonitrile (ACN)] prior to their analysis on a nano-Reverse Phase LC coupled to a Q Exactive™ Hybrid Quadrupole Orbitrap mass spectrometer, through a nano-electrospray ion source (Thermo Scientific). Peptides were first separated using a Dionex UltiMate 3000 UHPLC system on an EasySpray C18 column (Thermo Scientific; OD 360 μm; ID 50 μm; 15 cm length; C18; 2 μm) at of 300 nLmin^{-1}. The LC mobile phase consisted of two buffer solutions; Buffer 1 (0.1% v/v FA) and Buffer 2 (0.08% v/v FA; 80% v/v ACN). A 60 min multistep Buffer 1:Buffer 2 gradient was used, as follows (% are shown in parentheses below): 0–3 min (96:4); 3–15 min (90:10); 15–35 min (65:35); 35–40 min (35:65); 40-41 min (5:95);

41-50 min (5:95); 50-51 min (95:5); 51-60 min (95:5). The separated peptides were analyzed in the Orbitrap QE operated in positive ion mode (nanospray 1.5 kV; source temperature 250 °C). The instrument was operated in data-dependent acquisition mode: survey MS scan resolution 70,000; full width half maximum (FWH M) for the mass range of *m/z* 400-1600 for precursor ions; MS/MS scans of the top 10 most intense peaks with +2, +3 and +4 charged ions above a threshold ion count of 16,000 at 35,000 resolution. MS/MS was performed using: normalized collision energy (NCE; 25%); isolation window 3.0 *m/z*; apex trigger 5-15 sec; dynamic exclusion 10 sec. Data were acquired with Xcalibur 2.2 software (Thermo Scientific).

MS data analysis

Raw MS files were analyzed by MaxQuant v1.5.3.30 (Cox & Mann, 2008). MS/MS spectra were searched by the Andromeda search engine against the Uniprot EPEC E2348/69 sequence (taxon id: 574521; 4,595 proteins; February 2016) and common contaminants as described (Cox et al, 2011), using a peptide and protein false discovery identification rate (FDR) of 1%. Confidence of T3SS protein identification was derived from the maximum peptide identification score and minimum posterior error probability (PEP) of the Andromeda search engine output (identification confidence: "High" = Score > 75 and PEP < 0.0001; "Medium" = Score > 40 and PEP < 0.01). Protease specificity was set to trypsin, allowing for a maximum of two missed cleavages. Dynamic (methionine oxidation and N-terminal acetylation) and fixed (S-Carbamidomethylation of cysteinyl residues) modifications were selected. Precursor and MS/MS mass tolerance was set to 10 ppm for the parent ion mass and 20 ppm for the fragment ion mass. Peptide features were aligned between different runs by matching the retention time and feature masses ("match between runs" feature; match time window 0.7 min; mass alignment window 10 min). Peptide intensities across the MS runs were normalized based on the abundance of membrane proteins. EPEC non-T3S inner membrane proteins were selected based on the degree of homology with the *E. coli* K12 proteins (STEPdb; (Orfanoudaki & Economou, 2014). One run of the dataset was selected as reference; a normalization factor was calculated by subtracting the mean of log peptide intensities of each MS-run from the reference one (Callister et al, 2006). Log-peptide intensities for all the identified peptides in the dataset were corrected based on the calculated normalization factors. For comparison of the protein abundance across the runs we used the sum of normalized peptide intensities. To identify proteins with differential abundance between EPEC IMVs and Urea treated IMVs we used a combination of two-side t-test and fold difference of mean abundance. Sum of peptide intensities (TIC: total ion current) were $\log_2$ transformed and tested for significance using a two-side t-test, without assumption of equal variance. Missing values were omitted. Calculated p-values were adjusted for multiple hypothesis testing error using the "*Benjamini-Hochberg"* (Benjamini & Hochberg, 1995) method, as previously described (Tsolis et al, 2016; Tsolis & Economou, 2017b). Proteins identified in at least three repeats in each group were compared. Fold difference was calculated by dividing the mean raw TIC from untreated IMVs over the urea treated ones. Proteins identified in at least three repeats in each of the comparing groups were considered significant if the adjusted p-value was < 0.05 and the fold difference was > 2x (or $\log_2$ fold difference > 1). EscF, Ler and EscN that were identified in 5 repeats of untreated IMVs and in 2 or less of urea-treated ones were considered significant in the untreated condition, assuming they were no longer detected, due to low abundance. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al, 2016) partner repository with the dataset identifier PXD007087.

Western Blot analysis

Protein samples were analyzed in SDS-PAGE gels and transferred onto nitrocellulose membrane (PROTRAN) using Semi-Dry Transfer protocol (30min; RT; 20V; 300mA)

following manufacturer's instruction (BIORAD). Images were acquired using Las 4000 (GE Healthcare) and Image Quant Las^{TM} 4000, version 1.2 software. For image acquisition the manufacturer's setting were used (Resolution/ Sensitivity: standard mode; Exposure time 10-16 min; Image dimensions 210 x 140 mm; Image resolution 176 dpi)

Analysis of protein structures

Protein structures were downloaded from the Protein Data Bank (http://www.rcsb.org/; PDB codes are indicated) and visualized using Swiss PDB viewer and The PyMOL Molecular Graphics System (Version 0.99, Schrödinger, LLC). FASTA protein sequences were downloaded from Uniprot (http://www.uniprot.org/; STEPdb; (Orfanoudaki & Economou, 2014). SepL and EscV CD sequence alignment and conservation was performed using the "ConSurf Database software" (http://bental.tau.ac.il/new_ConSurfDB/). The EscV CD model was made using SWISS-MODEL SERVER (http://swissmodel.expasy.org) and Shigella *flexneri* MxiA (4A5P) based on their homology. Disorder prediction was performed on the IUPred web server (http://iupred.enzim.hu/; (Dosztanyi et al, 2005).

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