

# **Supplementary Figure 1 | Role of EtpA and flagella in ETEC adherence. a**, Affinity-purified antibodies against flagellin (H48) inhibit ETEC H10407 (O78:H11) adherence to intestinal epithelial cells. (nrs = normal preimmune rabbit sera). **b**,. Antibody<sup>\*</sup> directed at serotype-specific regions of flagellin has no effect on adherence. As shown at left, crude antisera vs full-length [1-498] H48 flagellin, as well as affinity-

purified antisera against H48<sub>[1-498]</sub> inhibited adherence of H10407; whereas antibodies recognizing either of the serotype-specific regions (H48<sub>[173-399]</sub>, H11<sub>[174-385]</sub>) had no effect. Inset: Western blot to verify that antibody affinity-purified against serotype-specific region of H48 (H48<sub>[173-399]</sub>), retains ability to recognize this antigen, but not the polyhistidine-tagged H11 recombinant, H11<sub>[174-385]</sub>. Control lane at right contains same antigen (rFliC-H11<sub>[174-385]</sub>-6His) probed with anti-6His antibody. **c**, Anti-EtpA antisera<sup>\*</sup> inhibits adherence of multiple motile EtpA-producing ETEC strains H10407 (CFAI, O78:H11), DS61-1 (CFAII, O6:H16), and Tx-1 (CFAI, O78:H12). Relative amounts of EtpA produced by the respective strains are indicated in the anti-EtpA immunoblot (inset). [densitometry measurements by ImageJ: H10407=61445, DS61-1=5957, Tx-1=33863]. Flagellin (FliC) is shown here as a loading control. **d**, addition of exogenous rEtpA to *etpA* mutant restores adherence in dose-dependent fashion.

\*Neither antibody against EtpA or flagellin in these experiments could be shown to inhibit motility (in soft agar assays following mixing of bacteria with antibody); likewise we could not demonstrate that under the conditions used here that the antibodies led to clumping of the bacteria or that they affected growth of the organisms, suggesting that the effect on adherence is specific.



**Supplementary figure 2 | EtpA interacts with intestinal surface molecules. a**, Recombinant EtpA bound to the surface of Caco-2 intestinal epithelial cells following incubation of cells with rEtpA purified from supernatants of EtpA-expressing Top10(pJY019) as shown by confocal immunofluorescence imaging. EtpA-negative controls (row 2) demonstrate labelling of cells with plasma membrane marker (CellMask, Invitrogen), but not EtpA (primary and secondary antibody concentrations are the same as in row 1). **b**, EtpA binds to murine intestinal mucosa. Frozen sections from mouse intestine incubated with biotinylated EtpA followed by detection with streptavidin-coated quantum dots (Qdot525, green). Cell membranes labelled with CellMask plasma membrane marker (red), and nuclei with DAPI (blue). (In control, row 2, red filter has been removed to demonstrate amount of bleed-through red fluorescence).



#### b. Search Result for MALDI-TOF data

Rank	Probability	Est. Z Score	Protein ID	%Cov.	pl	kDa
1	1.0	2.40	<u>giļ41651 embļCAA35488.1  <b>hsg48</b> (<i>E. coli</i>)</u>	42	4.5	51.29

	Measured Mass (Da)	Computed Mass (Da)	Error (ppm)	Residues	Peptide sequence
1	1035.511	1035.559	-47	167-176	TLGLDGFSVK
2	1190.589	1190.589	0	21-31	NQSALSSSIER
3	1419.754	1419.735	13	413-425	ALDDAIASVDKFR
4	1439.815	1439.809	4	471-484	AQIIQQAGNSVLAK
5	1560.807	1560.825	-12	126-140	VSGQTQFNGVNVLAK
6	1670.757	1670.745	7	456-470	IQDADYATEVSNMSK
7	1686.759	1686.740	11	456-470	IQDADYATEVSNM*SK
8	1741.900	1741.920	-11	146-161	IQVGANDNQTITIDLK
9	1755.846	1755.852	-3	343-360	TITYTDSSGAASSPTAVK
10	2137.971	2138.012	-19	322-342	DTNGNLYAADVNETTGAVSVK
11	2248.092	2248.092	0	435-455	LDSAVTNLNNTTTNLSEAQSR
12	2302.179	2302.191	-5	120-140	LDEIDRVSGQTQFNGVNVLAK
13	2337.137	2337.144	-3	177-199	NNDTVTTSAPVTAFGATTTNNIK
14	2628.268	2628.273	-2	67-91	NANDGISVAQTTEGALSEINNNLQR

#### Mass and Sequence Detail for the 14 matched peptides to hsg48.

\*mass difference (approximately 16 Da) between peptides 6 and 7 is likely due to oxidized methionine residue at position 468 in peptide 7).

## Supplementary Figure 3 | co-purification of EtpA and flagellins

**a**, Co-purification via gel filtration column chromatography of EtpA (arrowhead) and second ( $\approx$ 50 kDa) protein (arrow) from supernatant of recombinant *E. coli* (H48) expressing the ETEC etpBAC TPS locus. Numbers above gel represent fractions collected at flow rate of 1 ml/min (therefore correspond to timeline at bottom of the chromatogram). Downward arrow indicates point of elution for standard (thyroglobulin, 670 kDa) under same conditions. **b**, MALDI-TOF identification of the  $\approx$  50 kD co-purifying protein as flagellin serotype H48 (accession number <u>CAA35488</u>) **c**, immunoblots of gel filtration-purified H10407 supernatants showing co-purification of EtpA with FliC (H11).



# Supplementary figure 4 | EtpA binds to conserved regions of flagellin at flagellar tips when the FliD cap protein is absent.

**a**, Molecular pull down assays with EtpA and FliD protein demonstrate no apparent interaction between EtpA and FliD . last lane in each immunoblot contains 10% of the protein used in the protein interaction studies as a positive control. **b**, FliD inhibits binding of EtpA to FliC-coated beads in molecular pull-down assays. **c-i**, Co-labelling experiments using primary antibodies against EtpA and FliD or conserved regions of FliC and FliD. **c-e**, EtpA (labelled with anti-mouse 20 nm gold conjugate (arrow) or FliD (labelled with 10 nm anti-rabbit conjugate, arrowheads) identified at the tips of flagella. Out of 42 intact flagella examined, 11 (26%) were labelled with EtpA alone, while 15 (36%) labelled with FliD alone. **f-g**, Labelling of FliD (10 nm anti-mouse gold conjugate), or **h-i**, conserved regions of FliC (1-173) (5 nm anti-rabbit gold conjugate). **j-k**, rEtpA added exogenously to the *etpA* mutant localizes to the tips of flagella. [final EtpA concentrations are ≈ 3 µg/ml in **j**, and 15 µg/ml in **k**]. (Here anti-myc primary antibody was used to recognize the tag on the end of rEtpA).



## Supplementary figure 5 | EtpA does not compromise flagellar length.

Examination of WT and *etpA* mutant strains revealed no appreciable difference in motility or length of the flagella, indicating that EtpA normally attaches to the tips in a way that does not interfere with flagellar maturation. Sizes were determined by measuring flagella [in transmission electron microscopy images] of either the H10407 parent ETEC strain or the *etpA* mutant grown in liquid culture and fixed on grids.





	а. С	340	16 2 - B	360		380	9445 B	400	
EtpA [H10407]	GMEMNTVENV	SGVVEASGMH	R- ODGNIVLD	G- GDSGVVHL	SGTLOADNA-	- SGOGGKVVV	QGKNILLDKG	SNITATGGOG	357
EtpA [E24377A]	DMEMNTVENV	SGMMEAGGME	B- OD GNINLD	G- GDSGVVHL	SGTLOADNA-	- SGOGGKVVV	OGKNILLDKG	SSITATGGOG	357
AAZ57198	ESGVGVNLGN	LYAREGDILL	S- SSGKLVLK	NSLAGGNTTM	TGT - DVSLSG	DNKAGGNLSV	TGTTGLTLNO	SHEVTOKNEV	339
AAN78830	ESGVGVNLGN	LYARDGDITL	D-ASGRETVN	NSLATGANTA	KGQ- GVTLTG	DHKAGGNLSV	SSRRDIVLSN	GTENSDKDES	339
YP_543880	ESGVGVNLGN		N - SAGKLVLK	NSLAGGNTTV	TGT - NMSL SG	DNKAGGNL SV	TGTTGLTLNO	SREVTOKNEV	339
YP_001402134	DMLMDTVIDN	TGILQAKGLS	A- KNGALYLD	G- GGEGVVSQ	MGT DWNNQ-	- OGRGGRAVV	EGKRIYLNKN	SNIEAOGSAG	362
NP_253231	DSLEKTVVNN	QGTLEABTLB	S- AEGRIVLD	G- GEOGTVRV	AGKODASAL-	GGGNGGLVLN	QGANVEI ORT	AQVDTHADOG	343
AAG07929	DSELKTVVNN	OGTLEARTLE	S- AEGRINLD	G- GEOGTVEN	AGKODASA -	GGGNGGLVLN	OGANVE OBT	AQVDTHADOG	343
NP_252771	NALENTVVNS	QGALEARSLE	G- KNGRIVLD	G- GPDGKVMV	GGALSANALN	GPGHGGTVEV	BGQAVEVALG	TOVNTLASNG	348
ZP_01363794	DSELKTVVNN	QGTLEABTLB	S- AEGRIVLD	G- GEQGTVRV	AGKODASAI -	GGGNGGLVLN	QGANVEIORT	AQVOTHADOG	343
ZP_01367514	NALENTVVNT	QGALEARSLE	G- HNGRINLD	G- GPDGKVMV	GGALSANALN	GPGHGGTVEV	RGQAVEVALG	TOWNTLASNG	348
ZP_01295999 ZP_01296804	TTVLGSVVNN	QGALEARSLE	G- OSGKIILD	G- G- SGKVLV	AGALSANALN	EPGHGGTVEM	KAAEVEVNLA	TOVNTLASNG	342
ZP_1297404	DSELKTVVSN	QGVIEAKTLQ	N-RDGRIVLD	A - GN - GTLOV	AGRODASAS-	GOGNGGVVEN	RGAKVEVHOY	AKVOTRSKOG	342
ZP_00138031	DSLLKTVVNN	OGTLEARTLE	S- AEGRIVLD	G- GEOGTVRV	AGKODASA -	GGGNGGLVLN	QGANVE LORT	AQVDTHADOG	343
ZP_00965590	DSELKTVVNN	OGTLEARTLE	S- AEGRIVLD	G- GEOGTVRV	AGKODASAL-	GGGNGGLVLN	QGANVELORT	AQVETHADOG	343
ZP_00967564	NALLNTVVNS	QGA <b>IE</b> A <b>RSL</b> A	G-KNGRIVLD	G-GSDGKVMV	GGALSANALK	GPGHGGTVEV	BGQAVEVALG	TOVNTLASNG	348
ZP_00971001	DSLEKTVVNN	OGTLEARTLE	S- AEGRIVLD	G- GKOGTVRV	AGKODASA -	GGGNGGLVLN	OGANVE I OBT	AQVDTHADOG	343
ZP_00977642	GNLLGAVINN	SGTIEAKGLA	S- BGGRITLD	G-GTVKV	AGKLDASAAE	AGSPAGTVVT	RGERVDVAHD	VOVDTRA - GN	339
ZP_00983072	DAAISSVVNN	TGVIEARSLO	S- KNGRIVLD	NDAANGNTTV	AGTENVSSA-	- DGVGGNVVI	EGKTIAID-N	AAVNADGALG	337
YP_773181	GDLLGAMMNN	TGTEAKGEN	S- RSGKITLE	G-DLWKM	AGKLDASGOE	ADASGGMMTT	BGERNEMAND	MONDTHAPGG	340
Consensus	DSLLKIVVNN	QGATEARXLR	S-XNGRTVLD	G-GXXGTVXV	AGKLDASAL -	GXGNGGTVEV	RGANVEVALG	AQVDILASQG	
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	BILLETVVNN	QUATCARALR	S & UKIVLU		AUREDASAR	GELEUUFVEV	RUSAVEVSES	TOVELEADER	
		420 1		440 1		460		480 I	
EtpA [H10407]	G- GEVYVGGG	WQGKDSNIR-	NADKVVMQG-	GARIDVSATO	OGNGGTAVLW	SDSYTNEH-G	Q- ISAKGGET	GGNGG- BVET	431
ZP 00725946	G- GEVYVGGG	WOGKOSNIH-		GARIDISATO		SDSYTNEH-G	Q- SAKGGET	GGNGG- QMET	431
AAZ57198	LSSSGQIVQ-	NGGELT -	AGONAMESAO	HLNQ- TSGTV	NAAENVTLTT	TD - DTTLK - G	BSWAGKTLTM	- SSGS- LNNG	409
AAN78830	TAGGRITO-		AGROVILAAK	NITODIASO	NAABDIVTVA	SD - TETTO- G	<b>QITAGONETA</b>	- SATT- TOD	410
YP_543880 YP_672417	LSSSGOIVO-	NGGELT-	AGONAMESAO	HLNQ- TSGTV	NAAENWTETT	TN-DTTLK-G	RSTAGKTLTV	- SSGS- LNNG	409
YP_001402134	G- GTVLVGGG	WOGKONOIR-	NATAVVMDK-	GSNIDVSASB	NGPGGTAVEW	SEDYTGEH - G	N-IRARGGPO	SGDGG- BVET	436
NP_253231	ATGTWRILS-	HEVSVAA	MGQANAAGD -	GSGOVHVA	OGPAGANASD	SNGVTIVQQQ	PAVOLAAGAN	- GTSAVQSQS	415
AAG07469	LNGTWK AA-	DKIDV	RPSAVSDGV-	TVHADTLSEN	LASTNIELVS	TKGDLDLD - G	S- WNWASGNE	LGLGS- AADL	418
NP_252771	LNGTWKLAA-	· · · <b>DKIDV</b> · ·	RPSAVSDGV-	TVHADTESAN	LASTNIELVS	TKGDLDLD-G	S- VNWASGNR	LGLGS- AADL	418
ZP_01363794 ZP_01367514	ATGTWRIES-	HEVSVAA	MGQANAAGD -	GSGOVHVA	OGPAGANASD ASTNIEUS	SNGVT VQQQ	PANDLAAGAN S. USWASGNE	- GTSAVQSQS	415
ZP_01295999	OT GTWK LAA -	NNLEVAS	SVL RDAATL -	KASTLADN	LETTSIELAS	TOGOLKVD - A	P-LSWNSGNK	LGLSAERGNV	413
ZP_01296804	NNGIWKISA-	DKNDV	HRTALASGG-	TVHVDTLSBN	LATTNIELNS	TKGDLNLN-G	P- VAWASGNE	LALNS-AGDL	412
ZP_1297404 ZP_00138031	ATGTWRIIS-	HEVSVAA	MGOANAAGO -	GSGOVHVA	OGPAGANASD		PAVDLAAGAN	- GTSAVOSOS	413
ZP_00137527	LNGTWKIAA-	DKIDV	RPSAVSDGV-	THADTLSEN	LASTNIELVS	TKGDLDLD - G	S- VSWASGNE	LGLGS- AADL	418
ZP_00965590	ATGTWRILS-	HEVSVAA	MGQANAAGD -	GSGOVHVA	OGPAGANASD	SNGVTIVQQQ	PAVDLAAGAN	- GTSAVOSOS	415
ZP_00967564 ZP_00971001	ATGTWEILS-		HPSANSUGM-	- GSGOVHVA			S- WSWASGNH	- GTSAVOSOS	418
ZP_00973255	LNGTWK AA-	DKIDX	RPSAVSDGV-	TVHADTLSEN	LASTNIELVS	TKGDLDLD - G	S- VNWASGNE	LGLGS - AADL	418
ZP_00977642	AAGKWTIEA-	· · · ANAGY · ·	NG- TDAAGR-	SIDADTESEN	GTTNVELAN	TOGDL TVG-G	P- VSWT SDNA	LTLTSRKGNV	409
ZP_00983072 YP_773181	BTGTWKIEA-		NGNSSESGS	ALGADILSA		TKGNLTVD - G	S- VNWASDNT	TITSOHGOV	411
Consensus	LTGTWKIAA-	DKVDVA-	NGQAVAAGD-	XADTLSRN	LGPTNIELVS	TKGDXXXD-G	P-VSWASGNX	LGLGS-XADL	1127151
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**Supplementary Figure 6 | EtpA and other putative TpsA adhesins maintain a high degree of similarity in their amino terminal ends.** Shown is a CLUSTAL alignment of TpsA molecules (lacking C-terminal cysteines) with homology to EtpA as determined by BLASTP searches. Primary amino acid sequences were downloaded from GenBank using accession numbers at left. The 1<sup>st</sup> 500 amino acid residues of each sequence was then aligned using CLUSTAL algorithm within CLC Protein Workbench using BLOSUM scoring matrix and open gap and gap extension penalties of 1.