Supplementary Table 1 | Bacterial strains and plasmids

stain or plasmid	relevant genotype or description	reference(s)
strain		
H10407	ETEC serotype O78:H11; CFAI LT^+/ ST^+, $EtpA^+$	30,31
Tx-1	ETEC serotype O78:H12; CFAI, ST^+, $EtpA^+$	31
DS61-1	ETEC serotype O6:H16; CFAII, LT/ST, $EtpA^+$	31
DS220-4	ETEC serotype O11:H33, CFAII, $EtpA^-$	31
E24377A	ETEC serotype O139:H28; CSI/CS3 LT+/ ST+, EtpA+	32,33
E. coli Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
E. coli LMG194	F- ΔlacX74 galE thi rpsL ΔphoA (Pvu II) Δara714 leu::Tn10 (TetR)	Invitrogen
jf1289	isogenic *etpA* mutant	31
jf1412	*fliC* mutant; non-motile; Km^R	this study
jf721	*fliD*::NKBOR, Km^R; non-motile	34
MG1655	descendent of prototype *E. coli* K-12 strain, OR:H48:K-	35
jf1668	etpA mutant containing selectable Cm^R cassette generated by introduction of 8590 bp *Xho*I/*Hind*III pJMF1019 fragment. *etpA* deletion verified by PCR [primers jf092605.3/jf122205.1] to distinguish *etpA*::Cm^R (3044 bp) from wild type *etpA* (1692 bp).	36
jf876	ΔlacZYA derivative of H10407, Km^R	34
jf945	ΔlacZYA derivative of H10407	34
plasmid		
pJY019	*etpBAC* expression plasmid	31
pJL016	*etpBA* cloned into pBAD/*Myc*-His A*, native etpA stop codon	36
pJL017	*etpBA* cloned into pBAD/*Myc*-His A*, with etpA in-frame with myc and 6His coding regions.	36
pJL030	*etpC* gene cloned into pACYC184, Cm^R	this study
pTrcHis B	expression plasmid for polyhistidine fusions, Amp^R	Invitrogen
pBADmycHisA	Arabinose-inducible expression plasmid, Amp^R	Invitrogen
pFLAG-CTC	expression plasmid vector Amp^R	Sigma
pJY044	$FliC_{MG1655}$ (serotype H48)/pFLAG-CTC expression plasmid. *fliC* cloned using primers jf011706.1 and jf011706.2	this study
pJMF1019	pJL017-GPS4 linker scanning insertion (Cm^R) @ *etpA* nucleotide position 328	this study
pJMF1078	pJL017 linker scanning insertion @ *etpA* 2636 (leaving in-frame scar after *Pme*I digestion/religation)	this study
pJMF1087	pJL017 linker scanning insertion @ *etpA* 1242 (leaving in-frame scar after *Pme*I digestion/religation)	this study
pKR001b	full-length FliC (H48) (AA residues M_1-G_{498}) from MG1655 as 6His fusion in pTrcHis B	this study
pKR005b	residues S_{174}- G_{498} $FliC_{MG1655}$ in pTrcHis B	this study
pKR007b	residues M_1-F_{173} $FliC_{MG1655}$ in pTrcHis B	this study
pKR008	full length H11 flagellin amplified from H10407 using primers jf053006.3 and jf053006.4 and cloned into *Bgl*II, *Sna*BI sites of pBAD/*Myc*-His C	this study
pKR009	residues M_1-F_{173} $FliC_{H10407}$ (H11) amplified from H10407 using primers jf053006.3 and jf053006.5 and cloned into *Bgl*II, *Sna*BI sites of pBAD/*Myc*-His C	this study

pKR011	residues N_{174}-L_{385} $FliC_{H10407}$ (H11) amplified from H10407 using primers jf053006.6 and jf053006.7 and cloned into *Bgl*II, *Sna*BI sites of pBAD/*Myc*-His C	this study
pFliD-FLAG-CTC	fliD gene from MG1655, amplified using primers jf011706.3 / jf100307.5 and cloned into HindIII/BglII sites of pFLAG-CTC in-frame with FLAG tag.	this study
pGPS4	Tn7-based linker-scanning mutagenesis donor, Cm^R	NEB
pGPS5	Tn7-based linker-scanning mutagenesis donor, Km^R	NEB

*vector *PmeI* site was subsequently removed using site-directed mutagenesis (QuikChange II, Stratagene) with primers jf071706.1 and jf071706.2

Supplementary Table 2. Primers used in these studies

designation	sequence (5'-3')
jf121905.1	GGAAACCCAATACGTAATCAACGACTTGCAATATAGGATAACGAATCGTGTAGG CTGGAGCTGCTTC
jf121905.2	TGCCAACACGGAGTTACCGGCCTGCTGGATGATCTGCGCTTTCGACATATGAAT ATCCTCCTTA
jf011706.1	AATAATAAGCTT**ATG**GCACAAGTCATTAATACC
jf011706.2	AATAATAGATCT**TTA**ACCCTGCAGCAGAGA
jf031505.1	AATAATCTCGAGAATGGTGGTGAAATTCATG
jf110705.2	AATAATAAGCTTTTGCCAGTACACCTCACT
jf071706.1	CATCATCATCATTGAGTTCAAACGGTCTCCAGCTTGG
jf071706.2	CCAAGCTGGAGACCGTTTGAACTCAATGATGATGATG
primer N	ACTTTATTGTCATAGTTTAGATCTATTTTG
primer S	ATAATCCTTAAAAACTCCATTTCCACCCCT
jf092605.3	CAGATTGTGGCAGGTTCA
jf122205.1	CTAAAACAGAATCCCGCTATC
jf030106.1	AATAATAGATCTATGGCACAAGTCATTAATACC
jf030106.2	AATAATAAGCTTTACCCTGCAGCAGAGACAGAAC
jf050206.1	AATAATAGATCTAGCGTTAAAAATAACGATACA
jf050206.2	AATAATAAGCTTTAAAACCATCAAGGCCAAGAGT
jf011706.3	AATAATAAGCTTATGGCAAGTATTTCATCG
jf100307.5	AATAATAGATCTCTTGGAATTACTGTTGTT
jf053006.3	AATAATAGATCTATGGCACAAGTCATTAAT
jf053006.4	AATAATTACGTAACGCAGCAGAGACAGTAC
jf053006.5	AATAATTACGTAAAAACCGTCCAGGCCGAG
jf053006.6	AATAATAGATCTAATATCGATGGCGCGCAG
jf053006.7	AATAATTACGTACAGAATCGGGCTACCACC

jf121905.1/.2: underlined regions represent H1/H2 homology tails[13] for recombination with *fliC* region
AAGCTT *Hind*III site
AGATCT BglII site
CTCGAG *Xho*I site
TACGTA *Sna*BI site
jf071706.1: underlined base represents (T>C) mutation to remove the *Pme*I site (GTTTAAAC).
jf071706.2: reverse complement of jf071706.1

Supplementary methods

Adherence assays

Bacteria grown overnight in Luria broth from stocks maintained at -80°C were diluted (1:100) into fresh media and incubated at 37°C, 225 rpm to mid-logarithmic growth phase. Bacteria were then added to target epithelial cells at a multiplicity of infection (MOI) of approximately 10:1. After 1 hour incubation at 37°C, 5% CO_2, nonadherent bacteria were removed by repeated washing with RPMI, and cell-associated organisms were recovered by lysis of the epithelial cells in 0.1% Triton-X-100 as previously described [31].

Construction of *fliC* isogenic deletion mutant

To construct a deletion in *fliC*, a Kanamycin cassette was amplified from pKD4 using primers jf121905.1 and jf121905.2, where underlined sequences represent upstream and downstream regions flanking *fliC,* respectively in the *E. coli* K-12 (MG1655) sequence [37]. This amplicon was then introduced into H10407(pKD46) as previously described [31,38] to replace the *fliC* gene. Kanamycin-resistant colonies were then screened for motility on soft agar (1% tryptone, 0.7% NaCl, 0.35%. agar).

To complement the resulting strain, jf1412, we constructed a flagellin expression plasmid encoding *fliC* from MG1655 (serotype H48). Briefly primers jf011706.1 and jf011706.2 were used to amplify *fliC* $_{H48}$ from MG1655 genomic DNA (Underlined regions of primers represent *Hind*III and *Bgl*II sites, and bold residues indicate *fliC* start and stop codons, respectively). The resulting amplicon was cloned into pFLAG-CTC yielding pJY044).

linker scanning mutagenesis of *etpA*

To construct a system that would enable efficient screening of *etpA* mutants, we first amplified both the *etpB* and *etpA* genes using primers jf031505.1 and jf110705.2. The resulting amplicon was digested with *Xho*I and *Hind*III and cloned into the corresponding sites of pBAD/*Myc*-His A placing *etpA* in-frame with the myc epitope and polyhistidine-encoding regions of the vector. Site-directed mutagenesis (QuikChange II, Stratagene) using primers jf071706.1 and jf071706.2 was performed to remove a *Pme*I site of the vector. The resulting plasmid pJL017, was then used as the target for *in vitro* transposase (TnsABC)-mediated Tn7-based transprimer linker scanning mutagenesis (LSM) using either pGPS4 or pGPS5 (New England Biolabs) as the transprimer donors. Recombinant plasmids generated by LSM were then digested with *Xho*I/*Hind*III to identify insertions within *etpA,* followed by sequencing using transprimer-specific primers N and S to identify the precise location of the mutation within *etpA*. Plasmids containing mapped, non-redundant insertions were then digested with *Pme*I and religated to remove the majority of the transprimer sequence yielding *etpA* mutants with 15 bp scar sequences each containing a unique *Pme*I site. These plasmids were then introduced into *E. coli* Top10 along with pJL030 containing the *etpC* gene (required to promote optimal secretion of EtpA) cloned on a compatible vector plasmid (pACYC184). Following induction with arabinose (0.0002%) the resulting Amp^R/Cm^R colonies screened for EtpA secretion by immunoblotting TCA-precipitated supernatants. Plasmids encoding EtpA mutants that were effectively secreted were then introduced into the isogenic *etpA* mutant jf1668. After confirming EtpA secretion by immunoblotting these constructs were employed in subsequent adherence and protein interaction studies.

Construction of an isogenic etpA mutant for use in intestinal competition experiments

To create a strain with a selectable marker for competition assays, we first used plasmid pJMF1019 resulting from linker scanning mutagenesis of pJL017 with GPS4. This plasmid contains a transposon insertion encoding a chloramphenicol resistance (Cm^R) cassette at *etpA* nucleotide position 328. A 8590 bp XhoI/HindIII restriction fragment from pJMF1019 containing this mutagenized etpA locus was then introduced into H10407 by lambda red-mediated allelic exchange as previously described [13] to generate the etpA-negative, Cm^R mutant (jf1668) used for the competition experiments. Verification of the *etpA* deletion was performed by PCR using primers jf092605.3 and jf122205.1 to distinguish the mutant *etpA*::Cm^R sequence (3064 bp) from the wild type *etpA* sequence (1692 bp). Competent jf1668 cells were then used as the recipients for complementing plasmids derived from pJL017 as outlined above. Following induction with 0.0002% arabinose, production of rEtpA (or mutant versions of this protein) was confirmed by immunoblotting TCA-precipitated culture supernatants [31].

Interaction of EtpA with host cell surface proteins

Binding of EtpA to the surface of intestinal epithelial cells.

To examine potential binding of EtpA to intestinal epithelial cells, rEtpA was added to HCT-8 or Caco-2 cells grown on glass coverslips at a final concentration of 12.5 µg/ml in PBS. After incubation with the cells at 4°C for one hour, cells were washed, and fixed with ice cold methanol. Bound EtpA was detected by immunofluorescence using rabbit polyclonal anti-EtpA primary antibodies[3] (1:250), and secondary goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes) (1:500). During subsequent washes with PBS, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was added at a final concentration of 300 nM, and plasma membranes were stained with CellMask (Deep

Red, Molecular Probes). Confocal Imunofluorescence images were acquired on a BioRad MRC1024 imaging system equipped with a krypton/argon laser. Tiff files were saved and pseudocolor images were then created in ImageJ (v1.41a) (http://rsb.info.nih.gov/ij/) without adjustment to contrast or brightness of the original images by merging red and green channel data.

Purified recombinant EtpA was biotinylated by incubation with 2mM biotin LC hydrazide (Pierce) for 4 hours at 4°C. Labeled protein was then dialyzed overnight in PBS at 4°C to remove excess biotin. Target epithelial cell monolayers were fixed with 3% paraformaldehyde, then washed with PBS, and blocked with a solution of 1% BSA in PBS for 1 hour. Binding of EtpA was first investigated by adding biotinylated protein at final concentrations of 0.05, 0.1, and 0.2 μM, and incubating at 37°C for 1 hour. After washing with PBS, bound label was detected by using streptavidin-HRP (1:10000) and tetramethylbenzidine/H_2O_2 (TMB) peroxidase substrate (Kierkegaard & Perry Laboratories). Reactions were stopped after significant color development by the addition of 1M H2SO4, and the OD_{405} was determined spectrophotometrically. Similar experiments were then conducted using labeled EtpA at a final concentration of 0.05 μM combined with increasing amounts of unlabeled EtpA.

To visualize EtpA binding to intestine, frozen sections of mouse ileum were blocked, incubated with biotinylated rEtpA, washed, and bound rEtpA was detected with streptavidin-coated quantum dots (Qdot525 ITK SA). Both CellMask membrane stain and DAPI were incorporated into final washes. Mucin was identified in intestinal sections using primary rabbit polyclonal antibodies against mucin 2 (Santa Cruz Biotechnology, H-300; sc-15334) at a dilution of 1:500, followed by detection with goat-anti-rabbit AlexaFluor 594-labelled antibody (Molecular Probes).

Expression and purification of recombinant EtpA

LMG194(pJY019) [31] expressing the EtpBAC two-partner secretion system was grown overnight in Luria broth containing ampicillin (100 µg/ml) at 37°, 225 rpm. Overnight cultures were diluted 1:100 in 400 ml of fresh media, grown to OD_{600} of ≈ 0.5, and induced by the addition of arabinose (0.0001 mM final concentration) for 4 hours. Supernatant was sterile-filtered through a 0.22 µm filter (GP Express plus, Millipore), then concentrated (≈150X) via filtration through a 30,000 MWCO filter (YM30, Millipore). The retentate was desalted using buffer exchange with 50 mM sodium phosphate, 150 mM NaCl, pH 7.2 and concentrated further to a final volume of ≈ 1 ml (≈400 X) following centrifugation in a 100,000 MWCO filter (YM-100 Centriplus, Millipore), then loaded onto a Sephacryl S-300 high resolution gel filtration column. Elution of protein (flow rate of 0.5 ml/minute) was monitored by absorbance at 280 nm and the profile recorded using LP Data View software (BioRad). 1 ml fractions were then collected (BioFrac, BioRad) and stored at 4°C prior to analysis by SDS-PAGE and immunoblotting. Expression of flagellin-free EtpA was accomplished by performing similar strategy using *E. coli* Top10, which does not make native flagellin [39].

Immunoblotting and antibodies

To detect EtpA, immunoblotting was performed using highly cross-absorbed anti-EtpA[31] rabbit polyclonal rabbit antisera at a dilution of 1:5000, and goat anti-rabbit immunoglobulin G(Fc)-horseradish peroxidase (1:60,000; Pierce). Rabbit polyclonal antisera raised against *E. coli* (MG1655) flagella [40] were used (1:10,000) to detect flagellin molecules. Affinity purification of these antibodies was performed by absorption against recombinant full-length flagellin (H48, MG1655) immobilized on nitrocellulose followed by elution of FliC-specific antibody in 100 mM glycine, pH 2.5 and subsequent neutralization with 1M Tris, pH 8.0 as previously described [41]. Additional rabbit

polyclonal anti-flagellin specific antisera were subsequently generated against the recombinant full-length polyhistidine-tagged (H48) flagellin as well as the peptide encompassing the 1st 173 amino acids of this molecule, followed by affinity purification as outlined above. Control immunoblot demonstrated specificity of anti-EtpA, anti-flagellin (FliC), and anti-FliD antibodies for their respective antigens.

Polyclonal antibodies against full-length FliD-FLAG (MG1655) were obtained by intramuscular immunization of rabbits with suspension of the protein in Alum, and following intranasal immunization of mice with IVX908-FliD-FLAG suspensions. Antibodies against the FLAG epitope tag (anti-FLAG M2 mouse monoclonal) were obtained from Sigma.

All blocking and incubation steps were performed at room temperature in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 and 5% milk. Detection was carried out with a luminal-based chemiluminescent substrate (SuperSignal; Pierce). To examine relative amounts of EtpA produced by ETEC strains, 1.2 ml of overnight cultures in Luria broth was spun at 5,000 rpm for 3 minutes. 1 ml of culture supernatant was then concentrated to a final volume of 50 µl using (10 kDa MWCO) centrifugal concentration devices (Millipore), after which 20 µl was analyzed by immunoblotting as above. ImageJ (v1.41a, Max OS X, http://rsb.info.nih.gov/ij/) was used in subsequent densitometry measurements of scanned immunoblot films.

Identification by Mass Spectrometry

The protein bands of interest were excised from Coomassie-stained polyacrylamide, destained, and placed in 25 mM ammonium bicarbonate and allowed to expand. Following digestion of the excised band with sequencing-grade trypsin (Promega, Madison, WI) for 16 hours at 37°C, the resulting peptides were extracted, and the sample was subjected to analysis by mass spectrometry[42]. MALDI-ToF mass spectra

were recorded on a Bruker Ultraflex MALDI-TOF/TOF reflecting time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). Matrix-related ions and trypsin autolysis products were used for internal spectra calibration. ProFound software[43] was used to search a current non-redundant database downloaded from NCBI.

Cloning, expression and purification of recombinant flagellin molecules

To clone the full-length *fliC* (H48) gene, primers jf030106.1 and jf030106.2 were used to amplify *fliC* from MG1655 genomic DNA. The product was then digested with *Bgl*II and *Hind*III (underlined) and ligated into the corresponding sites on pTrcHisB (Invitrogen) in-frame with the polyhistidine encoding region to produce pKR001b. Primers jf050206.1 and jf030106.2 were used to amplify and clone the region encoding amino acids 174-498 [$FliC_{174-498}$] to produce pKR005b. Finally, the region encoding residues 1-173 of FliC was amplified using primers jf030106.1 and jf050206.2 and cloned into pTrcHisB to produce pKR007b. These resulting plasmids, were then introduced into *E. coli* Top10 (Invitrogen), which does not produce detectable amounts of native flagellin [39], and expression of the corresponding recombinant flagellin molecules was induced with 0.2 mM arabinose. Recombinant flagellin molecules with myc and polyhistidine tags at their carboxy terminal ends were purified from clarified bacterial lysates using nickel-affinity chromatography (HisTrap, Pharmacia).

To construct a FliD expression plasmid, the *fliD* gene from MG1655 was amplified from genomic DNA using primers jf011706.3 / jf100307.5 and cloned into HindIII/BglII sites of pFLAG-CTC in-frame with the FLAG tag. Recombinant FLAG epitope-tagged FliD was expressed in *E. coli* Top10 and purified from bacterial culture lysates (B-PER, Pierce) using anti-FLAG M2 affinity gel (Sigma).

In vitro protein-protein interaction studies

Membrane-based protein interaction studies

For these preliminary studies of protein-protein interaction, varying amounts of target (bait) proteins were dotted and absorbed onto the surface of nitrocellulose filter strips. Prey proteins were biotinylated with 2 mM biotin-LC-hydrazide (Pierce) for 1 hr at 4°C in a total volume of 0.5 ml of PBS. Unconjugated biotin was removed by overnight dialysis against PBS. After blocking nitrocellulose strips with 3% non-fat dry milk in PBS for 30 min at room temperature, biotinylated prey proteins (10 µg/ml) in PBS were added to the strips and incubated for 1 hour at room temperature. Strips were washed 5 times with PBS to remove traces of unbound prey protein. Bound biotinylated prey proteins were detected with streptavidin-HRP (1:20,000; Sigma) using chemiluminescent substrate (Supersignal, Pierce).

Co-immunoprecipitation

Anti-EtpA IgG polyclonal antibodies were first purified from highly cross-absorbed anti-EtpA antisera[31] by protein G affinity column chromatography (HiTrap ProteinG, Amersham Biosciences). Preimmune sera from the same rabbit were treated in an identical fashion. The resulting antibodies (200 µg) were then coupled to solid support gel matrix (AminoLink Plus, Seize, Pierce) using a sodium cyanoborohydride (50 mM) in phosphate buffered saline, pH 7.0. After quenching the reaction with 1M TrisHCl, and washing with 1M NaCl, resin containing anti-EtpA antibodies was equilibrated in binding buffer (140 mM NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate, 10 mM KCl, pH 7.4). Concentrated supernatants from ETEC strains were dialyzed against binding buffer, concentrated approximately 5 fold to a final volume of 0.4 ml, then incubated overnight at 4°C with matrix containing either pre-immune IgG or anti-EtpA

(IgG) antibodies. The matrix was then washed 3x with buffer (25 mM Tris, 150 mM NaCl, pH 7.2) Bound proteins were eluted (ImmunoPure, IgG Elution Buffer, pH 2.8, Pierce), separated by SDS-PAGE, and transferred to nitrocellulose for subsequent immunoblotting.

Molecular pull-down assays

Polyhistidine-tagged flagellin molecules prepared by metal affinity chromatography, and EtpA purified by S-300 molecular sieve chromatography, were added together in solution in a molar ratio of 1:1 (approximately 150 pmol each) in PBS, pH 7.4 to a final volume of 3 ml. After incubation for 1 hour at 4°C polyhistidine tagged proteins and interacting EtpA were pulled down by the addition of 100 µl of Talon (Co^{2+}) metal affinity bead suspension in PBS. Beads were then washed three times to remove unbound proteins. After incubation for an additional hour at 4°C, and washing with PBS, bound proteins were released from Talon beads by incubation in SDS-PAGE sample buffer and used for immunoblotting. FLAG pull-down experiments were performed in a similar fashion, using anti-FLAG (M2) affinity gel (Sigma).

Protein sequence analysis (*in silico*)

To identify potential domains within EtpA, the amino sequence from residues 73-1787 was analyzed using algorithms located on the PredictProtein server. Molecules identified by PSI-BLAST [44] as having regions of homology to EtpA were then used in sequence alignments with CLUSTALW[45] http://www.ebi.ac.uk/clustalw/ or MUSCLE[46] http://www.drive5.com/muscle/ algorithms provided via CLCbio Workbench (v4.01). Regions of homology between EtpA and flagellin were then superimposed on 3D structure of flagellin[47] using Protein Workshop[48] (v1.35 http://www.pdb.org/robohelp/viewers/proteinworkshop.htm)

Transmission electron microscopy

For *in situ* immunogold labeling of EtpA we followed a protocol similar to that previously described by Jin et al[49]. Briefly, 10 µl suspensions of live bacteria were applied to the surface of UV-sterilized Formvar/carbon-coated nickel grids (FCF300-Ni, Electron Microscopy Sciences, Hatfield, PA). Bacteria were grown on the surface of grids in a humidified chamber at 37°C for approximately 4 hours followed by immediate fixation by 2% formaldehyde/ 0.5% glutaraldehyde solution in 50 mM sodium cacodylate, pH 7.2. Immunogold detection was then carried out using highly cross-absorbed anti-EtpA polyclonal rabbit antisera[31] (1:50) or affinity purified anti-flagellin antibodies followed by anti-rabbit IgG gold (10 nm) conjugate (Sigma). Negative staining was then carried out in 1 % phosphotungstic acid, pH 6.5.

Scanning Electron Microscopy (SEM)

Non-confluent Caco-2 or HCT-8 intestinal cells were used to seed coverslips pre-treated with poly-l-lysine, and grown for 24-48 hours in 5% CO_2 atmosphere. ETEC H10407 was grown overnight in Luria broth, and diluted 1:100 into fresh media. After growing for approximately 6 h, 100 µl of bacterial culture was added to individual wells of a 6-well tissue culture plate containing target intestinal cells on coverslips. After 15 minutes of incubation at 37°C, 5% CO_2, media was replaced with 0.1% glutaraldehyde in PBS and incubated at room temperature for 15 minutes. Coverslips were washed 3x with PBS followed by subsequent fixation with 3% glutaraldehyde in PBS. coverslips were then fixed with 2.5% Glutaldehyde in 0.1M Cacodylate buffer, pH=7.35 for at least 2 hours, rinsed (3x5 min) in 0.1M Cacodylate Buffer, pH=7.35, post fixed with 2% Osmium Tetroxide pH= 7.35 (Electron Microscopy Sciences), and rinsed again with 0.1M Cacodylate Buffer, followed by rinsing with deionized water. Samples were subjected to *en bloc* staining with 2% aqueous Uranyl Acetate for 2 hrs (Electron Microscopy Sciences), rinsed (3X5 min) with deionized water, dehydrated through graded series of

Ethyl Alcohol (10% through Absolute) dried via critical point dryer (Tousimis Sandai 790) in which absolute ethyl alcohol was replaced with liquid carbon dioxide. After venting samples were mounted onto SEM stubs and sputter-coated (Electron Microscopy Sciences EMS 550) with gold-palladium. SEM analysis was done with a Philips Environmental Scanning Electron Microscope (FEIC Philips XL30 ESEM).

Measurement of flagellar length

Dilutions of bacteria grown in liquid (Luria broth) cultures were fixed onto grids for transmission electron microscopy as above. Images obtained from multiple independent grids were saved as TIFF files. After these images were imported into ImageJ, flagellar length was determined using the measure and label plugin (http://rsb.info.nih.gov/ij/plugins/measure-label.html). Tabulated data for each flagellum was exported to Prism v5.0a.

Intestinal colonization

The previously described model of murine intestinal colonization [50] was used in competition experiments between etpA mutant jf1668 complemented with EtpA expression plasmid pJL017, or the same mutant complemented with pJMF1087 which expresses EtpA that has lost the ability to bind to FliC following introduction of a linker mutation. Briefly, 10 mice were pre-treated with streptomycin for 48 hours prior to challenge to facilitate colonization with ETEC. Approximately 12 hours prior to challenge mice were fasted and placed on water alone and then were given cimetidine 2 hours before inoculation. Each mouse then received 1 $x10^4$ cfu of both jf1668(pJL017) and 1 $x10^4$ cfu of jf1668(pJMF1087) together in a final volume of 0.4 ml by gavage. Immediately following challenge, the water supply for the mice was changed to include ampicillin (50 µg/ml) and arabinose (0.0002%) to maintain plasmid selection and to induce production of rEtpA, respectively. 24 hours after challenge mice were sacrificed and intestinal lysates were prepared by incubation in saponin. Lysates diluted in PBS

were then plated onto Luria agar plates containing chloramphenicol, 20 µg/ml and ampicillin, 100 µg/ml. Following overnight incubation at 37°C, colony PCR was performed using primers jf122205.1 and jf092605.3 to generate a ≈1690 bp amplicon that was subsequently digested with *Pme*I to distinguish the WT amplicon generated from pJL017 from the mutant amplicon containing a *Pme*I site.

To examine the role of flagella in intestinal colonization with ETEC, 10 mice were challenged simultaneously by gavage with approximately 1 x 10^4 cfu of strain jf876 containing a Km^R cassette in the *lacZYA* locus, and an equal number of jf1412 (containing an identical Km^R cassette in the *fliC* gene) in a final volume of 0.4 ml. After 24 hours, dilution of intestinal lysates[50] were plated onto Luria agar plates containing Km [25 µg/ml] and the ß-galactosidase indicator, Xgal (5-Bromo-4Chloro-3-Indolyl-ß-D-galactopyranoside)[51] permitting visual identification of the Δ*fliC* strain (blue) relative to the WT (white). The competitive index (CI) for each colonized mouse was then calculated as follows: CI=[(mutant (blue)/wild type (white))$^{\text{output cfu}}$/(mutant/wild type) $^{\text{input CFU}}$] where the input fraction was determined directly by colony counting (cfu) prior to preparation of the inoculum.

Immunization of mice with recombinant flagellin and subsequent ETEC challenge

After obtaining preimmune sera, groups of 10 mice were immunized with either 7.5 µg of IVX908 (Protillin, ID Biomedical), a mucosal adjuvant based on *Neisseria* outer membrane proteins non-covalently complexed to LPS [52], or with IVX908 (7.5 µg) rFliC (H48), (30 µg) in a total volume of 20 µl (10 µl/nostril) on days 0, 25, 48, and 66. Mice in both groups were then challenged 2 weeks after the last immunization with 1 x 10^4 cfu of jf876, the *lacZYA*::Km^R version of ETEC H10407 (flagellar serotype H11). 24 hours following challenge, mice were sacrificed and intestinal lysates diluted in PBS were

plated onto Luria agar plates containing Km [25 μg/ml] to assess colonization. IVX908 was kindly supplied by the laboratory of Dr. James Dale.

Assessment of murine immune responses following vaccination with recombinant flagellin

Following completion of the immunization schedule outlined above sera from mice were tested for reactivity to full-length, serotype specific and conserved regions of both $FliC_{H48}$ and $FliC_{H11}$. Briefly, the respective recombinant polyhistidine-tagged proteins were diluted to a final concentration of 4 μg/ml in 0.1 M $NaHCO_3$ buffer, pH 8.6. Wells of ELISA plates were coated overnight at 4°C, washed with TBS (Tris buffered saline) containing 0.05% Tween-20, and blocked for 1 h at 37°C with 1% BSA in TBS-T (Blocker, Pierce). Dilutions of immune and preimmune mouse sera were prepared in 1% BSA in TBS-T. After incubation for 1 hour at 37°C, plates were washed with TBS-T, and secondary Goat anti-mouse (IgA,IgM,IgG) secondary antibody added at a final concentration of 1:10,000. After incubation for 1 hour at 37°C, plates were washed and developed with TMB Peroxidase Substrate [3,3',5,5'-Tetramethylbenzidine]. Absorbance measurements were determined kinetically[53] at a wavelength of 620 nm. Data was acquired using a Molecular Devices Spectramax 340PC microplate reader and recorded and analyzed using SoftMax Pro software v5.0.1. Absorbance values were recorded at 30 second intervals and the Vmax expressed as milli-units/min.

[30.] Evans, D. J., Jr. and Evans, D. G., Three characteristics associated with enterotoxigenic Escherichia coli isolated from man, *Infect Immun* **8** (3), 322 (1973).
[31.] Fleckenstein, J. M., Roy, K., Fischer, J. F. et al., Identification of a two-partner secretion locus of enterotoxigenic Escherichia coli, *Infect Immun* **74** (4), 2245 (2006).
[32.] Rasko, D., Roskovitz, M., Brinkley, C. et al., Escherichia coli E24377A plasmid pETEC_80, complete sequence, Available at http://www.ebi.ac.uk/cgi-bin/dbfetch?db=embl&style=html&id=CP000795, (2007).
[33.] Levine, M. M., Ristaino, P., Marley, G. et al., Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic Escherichia coli: morphology, purification, and immune responses in humans, *Infect Immun* **44** (2), 409 (1984).
[34.] Dorsey, F. C., Fischer, J. F., and Fleckenstein, J. M., Directed delivery of heat-labile enterotoxin by enterotoxigenic Escherichia coli, *Cellular Microbiology* **8** (9), 1516 (2006).
[35.] Bachmann, B., *Derivations and Genotypes of some mutant derivatives of Escherichia coli K-12*, 2nd ed. (ASM Press, Washington, D.C., 1996).
[36.] Roy, K., Hamilton, D., Allen, K. P. et al., The EtpA exoprotein of enterotoxigenic Escherichia coli promotes intestinal colonization and is a protective antigen in an experimental model of murine infection, *Infect Immun* **76** (5), 2106 (2008).
[37.] Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A. et al., The complete genome sequence of Escherichia coli K-12, *Science* **277** (5331), 1453 (1997).
[38.] Datsenko, K. A. and Wanner, B. L., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, *Proc Natl Acad Sci U S A* **97** (12), 6640 (2000).
[39.] Hayashi, F., Smith, K. D., Ozinsky, A. et al., The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5, *Nature* **410** (6832), 1099 (2001).
[40.] Majander, K., Anton, L., Antikainen, J. et al., Extracellular secretion of polypeptides using a modified Escherichia coli flagellar secretion apparatus, *Nat Biotechnol* **23** (4), 475 (2005).
[41.] Harlow, E., Lane, D., and Harlow, E., *Using antibodies : a laboratory manual*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999).
[42.] Cummings, E. D., Brown, J. M., Sarva, S. T. et al., High-throughput proteomics processing of proteins in polyacrylamide in a multiwell format, *Journal of proteome research* **6** (4), 1603 (2007).
[43.] Zhang, W. and Chait, B. T., ProFound: an expert system for protein identification using mass spectrometric peptide mapping information, *Anal Chem* **72** (11), 2482 (2000).
[44.] Altschul, S. F., Madden, T. L., Schaffer, A. A. et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res* **25** (17), 3389 (1997).
[45.] Chenna, R., Sugawara, H., Koike, T. et al., Multiple sequence alignment with the Clustal series of programs, *Nucleic Acids Res* **31** (13), 3497 (2003).
[46.] Edgar, R. C., MUSCLE: multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Res* **32** (5), 1792 (2004).
[47.] Yonekura, K., Maki-Yonekura, S., and Namba, K., Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy, *Nature* **424** (6949), 643 (2003).
[48.] Moreland, J. L., Gramada, A., Buzko, O. V. et al., The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications, *BMC Bioinformatics* **6**, 21 (2005).
[49.] Jin, Q. and He, S. Y., Role of the Hrp pilus in type III protein secretion in Pseudomonas syringae, *Science* **294** (5551), 2556 (2001).
[50.] Allen, K. P., Randolph, M. M., and Fleckenstein, J. M., Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic Escherichia coli strains, *Infect Immun* **74** (2), 869 (2006).
[51.] Maloy, S. R., Stewart, V. J., and Taylor, R. K., in *Genetic analysis of pathogenic bacteria: a laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), Vol. 1, pp. 177.

[52.] Chabot, S., Brewer, A., Lowell, G. et al., A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice, *Vaccine* **23** (11), 1374 (2005).
[53.] Tsang, V. C., Wilson, B. C., and Maddison, S. E., Kinetic studies of a quantitative single-tube enzyme-linked immunosorbent assay, *Clinical chemistry* **26** (9), 1255 (1980).