Directed delivery of heat-labile enterotoxin by enterotoxigenic Escherichia coli

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Summary
Enterotoxigenic Escherichia coli (ETEC), leading causes of diarrhoeal morbidity and mortality in developing countries, are heterogenous pathogens that elaborate heat-labile (LT) and/or heat-stable (ST) enterotoxins which elicit watery, cholera-like diarrhoea. The molecular events permitting efficient delivery of LT remain undefined. Here, we characterize the role of host–pathogen interaction as it relates to the delivery of LT by ETEC. Separation of bacteria from both T2SS and toxin are distributed at one pole of the ETEC (H10407) prototype. Although LT secretion via the type II secretion system (T2SS) was responsive to a variety of environmental factors, neither toxin release nor delivery depended on transcriptional activation of genes encoding LT or the T2SS. Fusions of green fluorescent protein to GspM (a component of the T2SS system for LT) and to LT demonstrated that the T2SS system (T2SS) requires a functional multimeric type II secretion system. Once secreted, both CT and LT via their B subunits bind preferentially to GM-1 gangliosides (Angstrom et al., 1994) on the eukaryotic cell surface. Following internalization of the toxin, the enzymatically active A subunit is allosterically activated by ADP ribosylation factors (ARFs) (Tsai et al., 1987; Lee et al., 1991). The active A1 portion of the LT catalyses the ADP ribosylation of the heterotrimeric GTPase Gsα, consequently activating adenylate cyclase (Moss and Richardson, 1978). Resulting increases in cAMP lead to activation of cAMP-dependent protein kinase A (PKA), which in turn phosphorylates multiple serine residues within the R-domain of CFTR (Cheng et al., 1991) allowing ATP hydrolysis and gating of the chloride channel (Sheppard and Welsh, 1999).

Introduction
Enterotoxigenic Escherichia coli (ETEC) are the leading cause of traveller’s diarrhoea, and account for hundreds of thousands of deaths among children in developing countries annually. These infections are caused by a genetically and phenotypically diverse group of organisms that produce either heat-labile (LT) and/or heat-stable (ST) enterotoxins.

The heat-labile toxin and the closely related cholera toxin (CT) have been the subject of intensive investigation. The toxins are assembled from a single A subunit and five B subunits into heterohexameric holotoxin in the periplasm (Hirst et al., 1984; Yamamoto et al., 1984; Streatfield et al., 1992; Overbye et al., 1993). Secretion through the outer membranes of Vibrio cholerae (Overbye et al., 1993; Sandkvist et al., 1997) and ETEC (Tauschek et al., 2002) requires a functional multimeric type II secretion system. Once secreted, both CT and LT via their B subunits bind preferentially to GM-1 gangliosides (Angstrom et al., 1994) on the eukaryotic cell surface. Following internalization of the toxin, the enzymatically active A subunit is allosterically activated by ADP ribosylation factors (ARFs) (Tsai et al., 1987; Lee et al., 1991). The active A1 portion of the LT catalyses the ADP ribosylation of the heterotrimeric GTPase Gsα, consequently activating adenylate cyclase (Moss and Richardson, 1978). Resulting increases in cAMP lead to activation of cAMP-dependent protein kinase A (PKA), which in turn phosphorylates multiple serine residues within the R-domain of CFTR (Cheng et al., 1991) allowing ATP hydrolysis and gating of the chloride channel (Sheppard and Welsh, 1999).

Unfortunately, these significant advances in elucidating the biology of LT and CT have not yet led to an effective vaccination strategy. While LT possesses potent mucosal adjuvant activity, anti-LT immunity or immunization with the related CT B subunit surprisingly affords only modest protection against LT + ETEC (Clemens et al., 1988; Steinsland et al., 2003). Sero-epidemiological investigations have demonstrated that the presence of anti-LT antibody does not correlate with protection against subsequent LT + ETEC infections (Clemens et al., 1990).

The reasons for the disparity between the potent immunogenicity of LT and its relative ineffectiveness as a protective antigen are not clear. Genetically detoxified variants of LT have been used to generate rabbit and mouse polyclonal antibodies against the A subunit which neutralize the activity of LT on Y1 adrenal cells in vitro (Piazza et al., 1994). However, earlier investigations using recombinant E. coli strains expressing LT demonstrated...
that toxin delivery was most efficient when bacteria were attached to cells (Zafriri et al., 1987) and that while anti-LT antibodies were capable of neutralizing exogenously added toxin, they were ineffective in counteracting LT produced by adherent bacteria (Ofek et al., 1990). One explanation for these observations might be that ETEC are capable of delivering LT in a manner that circumvents antibody-mediated neutralization. Data presented here support the hypothesis that ETEC deliver their toxin payload to target epithelial cells in a highly directed fashion whereby LT is protected from detection by neutralizing antibody.

Results

Close interaction with target epithelial cells is required for efficient toxin delivery

Previous studies by Ofek et al. (1990) suggested that anti-LT antibodies were able to neutralize free LT, but these same antibodies were ineffective in preventing delivery of LT by intact bacteria. To further investigate mechanisms by which ETEC might deliver LT and avoid antibody-mediated neutralization, we first examined the contribution of ETEC–host cell contact to toxin delivery. Physical separation of bacteria from target epithelial cells was first achieved by growing HCT-8 monolayers in plastic wells beneath 0.4 µM filters, followed by introduction of ETEC to the upper compartment above the filter. Under these conditions, there was minimal activation of cAMP in the target monolayers (Fig. 1A, I), whereas introduction of wild-type (WT) bacteria to the apical surface of cells grown on the filter led to the anticipated increases in cAMP (Fig. 1A, II). To our surprise, bacteria introduced to the lower compartment beneath the basal surface of the filter lead to similar increases in cAMP (Fig. 1A, III). We speculated that although the bacteria would be incapable of traversing the filter pores, the target epithelial cells might be able to send projections through these spaces to the basal surface of the filter where they could then be engaged by the bacteria. Indeed, scanning electron microscopy (SEM) of the basal filter surface following infection with H10407 revealed numerous cellular projections through the filter pores to which multiple WT organisms were attached (Fig. 1B). Under these conditions, we observed an average of 21.9 of these cellular projections bearing one or more attached bacteria, per 100 µm², while there were relatively few bacteria (< 1 per 100 µm²) that were simply attached to the underside of the filter. These results suggested that bacteria might need to swim to the eukaryotic cell surface in order to effectively deliver LT. To test this hypothesis, we employed strain jf721, an immotile mutant (Fig. 1C) of H10407 bearing a TnKKBOR (Ros-signol et al., 2001) insertion in fliD, which encodes the flagellar cap protein required for appropriate flagellar assembly. Although the ΔfliD mutant secreted LT nearly as well as the WT (Fig. 1D), cAMP responses from HCT-8 monolayers exposed to this mutant were greatly diminished, even when the bacteria added to the target epithelial cells on the same side of the filter. Together, these results suggest that the bacteria must be in close proximity to target epithelial cells in order to effectively deliver LT.

ETEC secrete and deliver preformed heat-labile toxin

As shown in Fig. 2A, the amount of detectable LT in culture supernatants was highly responsive to environmental
conditions. Virtually no toxin was detected in culture supernatants of WT ETEC grown in Luria broth (LB) at pH 7.0, while significant LT was identified in cultures grown in CAYE or LB at higher pH (data not shown). As predicted, a mutant strain (jf1123) bearing an isogenic deletion in \textit{gspE}, which encodes a putative cytoplasmic ATPase regulating the T2SS, secreted very little toxin under either of these conditions. In separate experiments, complementation of jf1123 with pFLAG-CTC-gspE restored production of toxin to near WT levels (\(P = 0.14\)) confirming that secretion through the T2SS is responsive to environmental influences.

Because our experiments also suggested that physical interaction of ETEC with epithelial cells is important for toxin delivery, we questioned whether toxin production or the secretion apparatus itself might be transcriptionally upregulated either in response to growth conditions or in response to pathogen–host cell interaction as has been demonstrated for other virulence factors (Pettersson et al., 1996; Zhang and Normark, 1996). To explore this possibility, we utilized both transcriptional fusions and real-time reverse transcription polymerase chain reaction (RT-PCR). However, as shown in Fig. 2B–E, our data suggested that the apparatus as well as the heat-labile toxin is constitutively expressed under these conditions, and that neither toxin secretion nor synthesis is coupled to increased transcription in response to either environmental stimuli or cell contact. These findings suggest that one or more post-transcriptional events are likely involved in directing the release of preformed toxin and its delivery to host cells.

**Polarization of the secretion apparatus by ETEC**

One mechanism by which ETEC might avoid neutralization of the heat-labile toxin is by limiting secretion to a focus near the point of cell contact effectively sequestering toxin away from neutralizing antibodies. Indeed, immunofluorescence images obtained using intestinal epithelial cell monolayers infection with jf478 (expressing the FLAG epitope-tagged version of LT) demonstrated toxin in close proximity to sites of bacterial attachment (Fig. 3). We questioned whether ETEC have the capacity to deliver LT in a vectored fashion by polarizing toxin secretion similar to \textit{V. cholerae} (Scott et al., 2001). To study this question, we chose to examine the subcellular localization of GspM, as the related protein (EspM) in \textit{V. cholerae} is a cytoplasmic membrane component of the type II secretion system for CT (Sandkvist et al., 1999) which localizes to one pole of the organism. For these experiments, a recombinant plasmid (pCD004) bearing a gfpmut2–gspM fusion was introduced and expressed in a gspM isogenic deletion mutant (jf1124). Complementation of jf1124 with pCD004 significantly restored the secretion of LT (Table 1) by the mutant to levels approximating the WT (\(P = 0.0005\) for comparison of mutant versus complemented strain) sug-
gesting that the GspM–green fluorescent protein (GFP) chimeric molecule remains functional. As shown in Fig. 4A, GspM–GFP fusion protein localized to one pole of the bacteria, and as shown in Fig. 4B localization occurred largely at the ‘old’ pole. Interestingly, when eltA/B was replaced with a plasmid expressing the chimeric GFP-LT localization to one pole was also noted, whereas expression of GFP alone resulted in a diffuse fluorescence pattern (insert in Fig. 4C). We were unable to demonstrate accumulation of GFP in the periplasm by immunoblotting of periplasmic extracts using anti-GFP antisera suggesting that this fusion molecule is not efficiently transported across the inner membrane.

Live cell imaging of bacteria expressing the GFP–GspM fusion (jf1128) was also consistent with the hypothesis that these organisms may deliver their toxin payload in highly vectored fashion. In these studies, organisms could be seen to approach and attach to epithelial targets, frequently with their fluorescent pole facing the target epithelial cell (shown in Fig. 5 and in Movie S1 in Supplementary material). Interestingly, we noted that multiple organisms were attracted repeatedly to specific regions of the cell surface, and that most of the bacteria remained attached only transiently. These findings might explain why in static images of fixed cells we saw toxin deposition both where bacteria were clustered as in Fig. 3 and at sites where we could not identify any attached organisms.

Table 1. Complementation of gspM mutant with gfpmut2-gspM expression plasmid restores LT secretion.

| Strain designation | Description | Ganglioside ELISA (mean OD_{650} ± SD) | Probability* |
|--------------------|-------------|---------------------------------------|--------------|
| jf394              | WT H10407   | 0.84 ± 0.14                           | –            |
| jf1124             | Isogenic gspM mutant | 0.07 ± 0.01                          | 0.006        |
| jf1128             | jf1124(pCD004)* | 0.70 ± 0.03                           | ns           |

a. Student’s t-test (one-tailed, paired comparisons with WT values)
b. Isogenic gspM deletion strain complemented with gfpmut2-gspM expression plasmid.

Table 1. Complementation of gspM mutant with gfpmut2-gspM expression plasmid restores LT secretion.

For these experiments overnight cultures were diluted in CAYE and grown at 37°C, 225 r.p.m. for approximately 4 h to the same OD_{500} LT in culture supernatant was quantified by mixed-ganglioside ELISA. Values represent the means from three separate determinations on the same culture supernatant.
Taken together, these results suggest that ETEC assemble both the toxin and its cognate secretion apparatus at one pole of the bacterium and that these organisms orchestrate assembly and secretion of toxin in a highly polarized fashion. Importantly, the studies included here provide a compelling argument that these organisms should no longer be depicted as unsophisticated in their mechanism of toxin delivery.

**Discussion**

While much is known about the biological effects of the heat-labile toxin, and homologous CT, the precise molecular details involved in toxin delivery are still being elucidated. Identification of the T2SS involved in secretion of LT (Tauschek *et al.*, 2002) provided opportunities to further examine its role in delivery of this toxin to intestinal...
epithelial cells. The studies reported herein suggest that transport of LT to epithelial cells is distinctly orchestrated by ETEC such that both the toxin and its cognate secretion apparatus assemble at one pole of the organism, and that effective toxin delivery occurs in a process that requires intimate interaction of the bacterium with the host cell.

The processes by which bacteria distribute proteins to particular locations within the cell are still being elucidated. However, a number of bacterial proteins have been shown to localize to discrete locations within the respective organism (Shapiro et al., 2002). Demonstrations here that both the secretion apparatus and the effector heat-labile toxin localize to one end of the bacterium are reminiscent of the directed secretion of protein by *V. cholerae* (Scott et al., 2001), and like *Vibrio*, ETEC appear to assemble their homologous T2SS predominately at the old pole.

A variety of different models have been proposed to explain targeting of proteins within the bacterial cell (Pugsley and Buddelmeijer, 2004), and the molecular requirements for protein polarity in prokaryotic organisms are being defined. Fascinating studies with *Caulobacter crescentus* demonstrate that the actin-like MreB protein forms highly organized arrays to largely determine polarity in these asymmetric model organisms (Gitai et al., 2004). It is interesting to note that MreB also regulates cell shape (Wachi et al., 1989) as well as chromosome segregation (Kruse et al., 2003) in *E. coli*, suggesting that mechanisms which determine protein sorting in bacteria are highly conserved. Indeed, proteins which are normally polarized in their respective organisms, such as IcsA in *Shigella* (Goldberg et al., 1993) and GspM in *V. cholerae* (Scott et al., 2001), appear in multiple locations in spherical *E. coli* lacking MreB (Nilson et al., 2005).

Regardless of the precise mechanism(s) underlying the polarization of LT and its cognate secretion apparatus, localization of the toxin to one pole of the organism could have important physiological consequences. Conceivably, the ability to focus toxin secretion to one pole of the bacterium as it encounters the host epithelium might permit ETEC to direct the delivery of LT in such a way as to escape antibody-mediated neutralization as was suggested in earlier studies by Ofek et al. (1990). Furthermore, our live cell imaging studies suggest that ETEC orchestration of toxin delivery may extend beyond polarization of the toxin and its cognate secretion apparatus, as these organisms appear to target specific regions of the host cell. Certainly, additional studies will be required to elucidate whether this occurs in response to bacterial and/or host cell factors.

Recent demonstrations that mutants defective in production or secretion of LT are markedly deficient in colonization of the small intestine suggest that this toxin is not simply present to promote dissemination of the organism back into the environment by causing diarrhoea, but that it plays a far more complex role than was previously appreciated (Berberov et al., 2004; Allen et al., 2006). This may relate to the fact that LT has multiple effects on target host cells in addition to its known role of promoting secretion of fluid into the small intestine. Increased intracellular cAMP concentrations enhance interaction of the cyclic nucleotide with the regulatory subunit of PKA promoting translocation of the PKA catalytic subunit to the nucleus where it phosphorylates transcription factors such as CREB (cAMP response element-binding protein) that bind to cAMP response elements (CRE) in the promoters of a host of cAMP-responsive genes (Lalli and Sassone-Corsi, 1994; Mayr and Montminy, 2001). In effect, ETEC may be able to co-ordinate secretion at the cell surface where it is quickly internalized escaping neutralization by mucosal antibodies, thereby preserving its ability to serve one or more effector molecule functions.

While our studies support a model in which LT is delivered in a vectored fashion, the precise molecular events surrounding toxin delivery are yet to be determined. In these studies we demonstrate that although LT secretion is highly responsive to environmental signals, release of toxin from the periplasm is not coupled to transcriptional activation of the T2SS. Furthermore, transcription of the genes for LT was not enhanced by these same environmental signals, suggesting that delivery of toxin involves post-transcriptional events that co-ordinate the release of preformed enterotoxin from the periplasmic space of ETEC. These findings are in fact consistent with current models of type II secretion indicating that cytoplasmic protein E (e.g. EpsE in *V. cholerae*) (Sandkvist et al., 1995) may govern secretion through its autoprophosphorylation and interactions with other elements of the secretion apparatus resulting in energy transduction (Camberg and Sandkvist, 2005) that promotes final assembly of a pilus-like structure (Sandkvist, 2001) and secretion of the toxin.

Another aspect of toxin delivery studied here, flagellar locomotion, has been shown to be essential for virulence in a diverse group of pathogens (Giron et al., 2002; Dons et al., 2004; Stecher et al., 2004) including *V. cholerae* (Richardson, 1991; Gardel and Mekalanos, 1996). Our data suggest that flagellar-based motility is required for efficient delivery of LT. However, flagella have recently been shown to play a number of other roles in addition to motility including a direct role in adherence (Giron et al., 2002), secretion of virulence proteins (Stecher et al., 2004) and induction of innate immunity (Steiner et al., 2000; Ogushi et al., 2001). More specifically, flagellar cap proteins (FlID) have been shown to directly mediate adhesion to mucin (Arora et al., 1998) or to epithelial cells (Tasteyre et al., 2001).
Interestingly, recent studies demonstrate that much of the toxin secreted by ETEC remains associated with vesicles that bud from the surface of these organisms (Horstman and Kuehn, 2000). Elegant *in vitro* experiments also showed that LT binds to the surface of these vesicles via LPS in a process that is dependent on secretion to the surface of the organism via type II secretion (Horstman and Kuehn, 2002) GM-1 ganglioside, a major cellular receptor for LT, is largely confined to lipid rafts, and as expected, ETEC-derived vesicles were largely internalized via raft-dependent endocytosis (Kesty *et al*., 2004). Although it seems clear that much of the LT secreted by ETEC when grown to high density in liquid culture remains associated with vesicles, and that these vesicles can in turn effectively deliver the toxin to target epithelial cells *in vitro*, their actual role during the course of infection with intact organisms has not been established. However, these studies, and those included herein, further allude to a previously unappreciated level of complexity for organisms that were once thought to simply release LT via lysis in the small intestine, and they raise a number of important questions relevant to the pathogenesis of ETEC.

**Experimental procedures**

**Measurement of heat-labile toxin in culture supernatants**

Strains were grown overnight at 37°C from -80°C glycerol stocks in LB, then 1:100 dilutions were subcultured in either LB or Casamino Acids-Yeast extract medium (Mundell *et al*., 1976) [CAYE: 2.0% Casamino Acids, 0.15% yeast extract, 0.25% NaCl, 0.871% K2HPO4, 0.25% glucose, and 0.1% (v/v) trace salts solution consisting of 5% MgSO4, 0.5% MnCl2, 0.5% FeCl3] and grown at 37°C, 225 r.p.m. Measurement of LT was performed using mixed-ganglioside ELISA as previously described (Clements, 1990).

**Transcription assays**

**Reporter fusions**. To gauge the transcriptional activity of *eltA*, the gene for the heat-labile toxin A subunit, we constructed an operon fusion with a promoterless chloramphenicol acetyltransferase (cat) gene. Primers Agel.pKK232-8.f (5′-GGACCGGT GGACCCCCGGCAATAA-3′) and pKK232-8.r (5′-GGACCGGT GCCTTTAAAGGGCACCAATA-3′) were used to amplify *cat* from pKK232-8 (Brosius, 1984). This ampiclon containing translational stop codons in all three reading frames upstream from the initiation codon of the *cat* gene was cloned into an Agel site within the *eltA* gene on pLT005.1 which bears the entire *eltA*::*opern* and flanking sequence cloned from ETEC H10407 (Table 2). The resulting plasmid, pLT006, was digested with SphI/NheI yielding a 2603 bp fragment containing the *eltA::cat* fusion which was then cloned into pST76K-Sac to construct pLT007. This suicide plasmid was then used to replace *eltA* with the single-copy *eltA::cat* fusion by double homologous recombination (Fleckenstein *et al*., 2000). To determine chloramphenicol acetyl transferase activity, bacteria were collected by centrifugation, sonicated, and CAT was measured in sonicates using a commercially available ELISA (5 Prime >3 Prime).

To facilitate the use of β-galactosidase as a transcriptional reporter, we first constructed a *lac* deletion in H10407, employing methodology identical to that described for *E. coli* K-12 (Datsenko and Wanner, 2000). First primers jf052202.1 (5′-CAGCGTTGGCCTCCTCAGGCCGTTG-3′) and jf052202.2 (5′-GGAAATCCGCAAGAAGCAGCCGCACGCAAGT-3′) were used to amplify an antibiotic resistance cassette from pKD13 (Datsenko and Wanner, 2000), which was then introduced into H10407(pKD46). After selection for kanamycin-resistant (Km³), ampicillin-sensitive (Ap³), lac- colonies on plates containing Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) the antibiotic resistance marker was eliminated by introduction of FLP recombinase helper plasmid, pCP20. After confirmation of the *lacZA* deletion by PCR using primers jf052202.3 (5′-CAGCGTTGGCCTCCTCAGGCCGTTG-3′) and jf052202.4 (5′-CGAAATCCGCAAGAAGCAGCCGCACGCAAGT-3′), the resulting Km³, Ap³, lac- strain, jf945, was then used as the host for subsequent reporter fusions.

To identify potential promoter sequences for the locus encoding the T2SS of H10407 responsible for the export of LT, we employed the prokaryotic Neural Network Promoter Prediction (NNPP2.2) algorithm (Reese, 2001) (http://www.fruitfly.org/seq_tools/promoter.html). Primers jf070202.1 (5′-CCGGAATT CCTGAAAAACACCAGAACCT-3′) and jf070202.2 (5′-CCGGGATCCTGCTTGATG-3′) were used to amplify an 880 bp segment which encompasses a putative promoter region extending from −865 to +15 relative to the gspD start codon. This ampiclon was then digested with EcoRI and BamHI and cloned into pRS551 to construct a transcriptional fusion with *lacZA* (Simons *et al*., 1987). The resulting plasmid pWY068 was then used to construct single-copy fusions in jf945 using λRS45 as previously described (Simons *et al*., 1987; Hand and Silhavy, 2000). After selection on minimal lactose plates, strains bearing potential fusions were examined by PCR to identify single-copy λ integrants (Powell *et al*., 1994), and β-galactosidase activity was confirmed by O-nitrophenyl-β-D-galacto-pyranoside (ONPG) assays (Miller, 1992). All subsequent β-galactosidase measurements were carried out using 4-methyl-umbelliferyl-β-D-galacto-pyranoside (MUG) (Miller, 1992; Kliarsfeld *et al*., 1994). Strain reports grown to mid-logarithmic phase were used to infect triplicate wells containing either HCT-8 cells (at a multiplicity of infection of 100:1) or tissue culture media alone in black-walled (Costar 3603) plates. After 4 h, the bacteria were removed from the wells and supernatants from triplicate wells were pooled into tubes on ice. Monoayers were washed with RPMI, then subjected to lysis in MUG assay buffer containing 0.1% Triton X-100, 20 mM NaCl, 1 mM MgCl2 and 0.01% BSA in phosphate-buffered saline (PBS), pH 7.8. MUG was added to the buffer immediately prior to assay from 500-fold concentrated stock in DMSO such that the final concentration was 0.2 mg ml⁻¹. The number of organisms present in the different fractions was determined by dilution of aliquots in 0.9% NaCl and plating onto L-agar. Tubes containing bacteria from HCT-8 supernatants and media fractions were centrifuged, the supernatant was removed, and replaced with an equal volume of MUG assay buffer. The resulting suspensions were added to triplicate wells of the 96-well black plate
Table 2. Bacterial strains and plasmids.

| Strain or plasmid | Relevant genotype or description | Reference(s) |
|-------------------|----------------------------------|--------------|
| **Strain**        |                                  |              |
| H10407            | ETEC serotype O78:H11, LT, ST    | Evans et al. (1977) |
| jf478             | H10407 derivative bearing FLAG-eltA epoq-tagged LT | This study |
| J570              | H10407 derivative with deletion in eltA | This study |
| J532              | H10407 derivative with single-copy eltA::cat transcriptional fusion | This study |
| J945              | lacZYA derivative of H10407 | This study |
| jf721             | H10407 derivative, R::NKBOR, KmR, non-motile | This study |
| jf1123            | Isogenic gspE deletion mutant | This study |
| jf1124            | Isogenic gspM deletion mutant | This study |
| jf1128            | Strain 1124 bearing plasmid pCD004, CmR | This study |
| **Plasmids**      |                                  |              |
| pLT005            | 1695 bp eltA/B amplicon cloned in pT7Blue-3 | This study |
| pKK232-8          | Promoterless cat plasmid | Brosius (1984) |
| pRS551            | lacZYA transcriptional fusion vector, AmpR, KmR | Simons et al. (1987) |
| pLT006            | Insertion of cat from pKK232-8 into Agel site of eltA on pLT005 | This study |
| pLT007            | 2603 bp Sphl/NheI insert from pLT006 containing eltA::cat fusion cloned into pST76K-Sac | This study |
| pGAP18            | Expression of amino-terminal gfpmut2 fusions | Pogliano et al. (2001) |
| pST76K-Sac        | Temperature-sensitive (Ts) sacB suicide plasmid, KmR | Posfai et al. (1997) |
| pT7Blue-3         | PCR cloning vector, AmpR, KmR | Novagen |
| pUC19             | 2686 bp cloning vector, AmpR | Norrander et al. (1983) |
| pFLAG-CTC         | 5336 bp expression plasmid, AmpR | Sigma |
| pLTFO05           | 1744 bp FLAG-eltA-eltB amplicon cloned in pT7Blue-3 | This study |
| pLTFO06           | 1793 bp Sphl/SalH fragment from pLTFO05 cloned into pUC19 | This study |
| pLTFO07           | 1817 bp KpnI/Sphl fragment from pLTFO06 in pST76K-Sac | This study |
| pJFF016           | for signal peptide encoding sequence (2RS) cloned into pT7Blue-3 | This study; Masip et al. (2004) |
| pJFF028           | 140 bp HindIII/Xmal insert from pJFF016 cloned into pFLAG-CTC | This study |
| pJFF032           | Fragment encoding 2RS-GFP-LT Chimera cloned on pFLAG-CTC | This study |
| pCP20             | FLP recombinase helper plasmid, AmpR, Ts | Datsenko and Wanner (2000) |
| pKD46             | λ Red recombinase plasmid, AmpR, Ts | Datsenko and Wanner (2000) |
| pWY068            | 880 bp gspD promoter region cloned into pRS551 | This study |
| pCD001            | 2933 bp amplicon with 1434 bp in-frame gspE deletion in pT7Blue-3 | This study |
| pCD002            | 3047 bp KpnI/Sacl insert from pCD001 cloned into pST76K-Sac | This study |
| pFLAG-CTC-gspE    | gspE cloned as 1497 bp HindIII/KpnI fragment in frame with the FLAG epitope | This study |
| pCD004            | gspM-gfpmut2 fusion on pGAP18, CmR | This study |

**Amplification:** containing the HCT-8 lysates. β-Galactosidase activity in lysates was determined using a Fluoromax plate reader (Bio-Rad, Hercules, CA) at excitation and emission wavelengths of 355 and 460 nm, respectively, and expressed as units of clot/h.</p>

**Real-time RT-PCR.** Bacterial RNA was extracted using guanidine isothiocyanate (GTC)-containing buffers and eluted from silica gel-based membranes according to the manufacturer’s protocol (Qiagen, RNeasy protocol for isolation of total RNA from bacteria). Recovered RNA was then treated with DNase and reverse-transcribed using reverse transcriptase, DNase-treated DNA polymerase (TaqGold), Taqman probes (Applied Biosystems), and oligonucleotide primers. Primers used in these assays were as follows: gspG (forward) 5’-GCCAAGCTTGACTGAGC-3’ (reverse) 5’-GGCTTTTTG CAGATCCGCTTT -3’ (TaqMan Probe) TGGTGGCGCATAA; gspC (forward) 5’-GCTGGAAAAGACGACGAT-3’ (reverse) 5’-CCAGCATG-GCGTGGATCA-3’ (TaqMan Probe) 5’-GGCCGATCCAT CAA-3’; eltB (forward) 5’-CAGGGCAAAAGAGAAATGGTTATCA-3’ (reverse) 5’-CTTGTGGTGAGATATTTCCATTC-3’ (TaqMan Probe) 5’-CTCGCCAGTGAATC-3’.

**Construction of mutant strains, and FLAG-LT, and GFP gene fusions**

In order to construct an epitope-tagged version of LT, we first designed sense oligonucleotide Lat1.FLAG.01 (5’-GACTACAGGAGGCAGCAGTACAGGAGACCAATTGACG-3’) and antisense primer Lat1.FLAG.02 (5’-CTTTGATC TCGTCTTGGTAGCTCATTTGCATTATGCGGCA-3’) that incorporates the FLAG octapeptide (DYKDDDDK) coding sequence (underlined) in frame with the region of eltA encoding the amino-terminus of the A subunit. In the first round of PCR using H10407 plasmid DNA as template, primer 5’-long, conserved (Schlor et al., 2000) (5’-GGCCAGGTGGTAGGAGACGCGAGAGGATGA-3’) was combined with Lat1.FLAG.02 and in a separate reaction, primer BamH.LT(h).001 (5’-CCGGCAACGGAAGGAGAAGGATTCATAC-3’) was combined with Lat1.FLAG.01. Next, the products of the first two reactions were combined in a single PCR

Amplification. 

**Amplicon, KmR, CmR, ampicillin, kanamycin and chloramphenicol resistance, respectively; Ts, temperature-sensitive.**
using 5’LT-consensus and BamHI-LT(ht).002 primers to yield a 1744 bp amplicon, which was cloned into pT7Blue-3 to yield pLTFO05. This plasmid was then sequenced using the R-20 vector primer (5’-CAGGATCTGACATGATTAGCT-3’) confirming the construction of the FLAG--eltA gene fusion. In the resulting construct, the sequence corresponding to the FLAG epitope-tag begins one codon beyond that encoding the signal peptide for the A subunit. Next, a 1793 bp SphI/Sall fragment from pLTFO05 encoding the FLAG--LT fusion, LTB, and flanking sequence was cloned into corresponding sites of pUC19 (Norlander et al., 1983). The resulting plasmid, pLTFO06, was used to express recombinant epitope-tagged holotoxin in E. coli DH5α. Following induction of DH5α(pLTFO06) with IPTG, periplasmic lysates were prepared as previously described (Fleckenstein et al., 2000). Total protein from these periplasmic extracts was then tested by gangioside-capture ELISA to demonstrate binding to GM-1 ganglioside, and reactivity with both anti-LTB rabbit polyclonal antibodies and anti-FLAG (M2) monoclonal antibodies, suggesting the assembly of epitope-tagged holotoxin. Finally, an 1817 bp KpnI/Sall fragment from pLTFO06 was cloned into the pST76Ksac suicide vector (Posfai et al., 1997) to yield pLTFO07 which was introduced into H10407 to replace the native eltA allele by double homologous recombination as previously described (Fleckenstein et al., 2000) yielding strain j1123. To complement this mutant, gspE was amplified with primers Jf080503.1 (5’-AACGTTTGTGCTTGTAAGCAG-3’) and Jf080503.2 (5’-GGTACCCGCTCCATGTTACCCG-3’) and cloned as a 1497 bp HindIII/KpnI fragment in-frame with the FLAG epitope sequence on pFLAG-CTC to yield pFLAG-CTC,gspE. The resulting plasmid was then introduced into strain j1123.

Strain Jf721 employed in these studies is the product of random mutagenesis of H11047 with the pGBKKBOR plasposon previously described by Rossignol et al. (2001). Jf721 is one of several Km5, non-motile colonies obtained after growth of H11047(pGBKKBOR) on plates containing Km at 42°C. Genomic DNA isolated from this strain was digested with BglII, religated and used to transform DH5αpir. Sequencing of plasmid DNA from the resulting recombinant revealed a single insertion in filD encoding the flagellar cap protein.

**GFP fusions**

In construction of an amino-terminal fusion of GFP to GspM the gspM gene was first amplified using primers Jf073003.1 (5’-GTCGAATCCGGGTAAATTT-3’) and Jf073003.2 (5’-AAAGCTTTACCCCGTGGAAAATCT-3’), cloned into pT7Blue-3, and excised with Sall and HindIII for cloning into the corresponding sites of pGAP18 (Pogliano et al., 2001). The resulting plasmid, pCD004, was then introduced into strain j1124 yielding j1128.

To promote correct folding of GFP in amino-terminal fusions GFP to the A subunit of the heat-labile toxin, recombinant molecules were directed across the cytoplasmic membrane of the bacteria via the Twin arginine transport (Tat) system (Berks et al., 2000; Thomas et al., 2001). The twin arginine signal peptide encoding region (2RSP) of forA (Masip et al., 2004) was first amplified from MG1655 genomic DNA using primers Jf022604.3 (5’-AAGCTTTATGAAATACACTCTGTTA-3’) and Jf022604.4 (5’-CCCGGCGGCGCGCTGGCCGCG-3’) and cloned into pT7Blue-3 yielding pJF016. The 140 bp HindIII and XmaI insert product was then directly cloned into pFLAG-CTC, resulting in pJFF028. Next, gfpmut2 was amplified from pGAP18 using primers Jf022604.1 (5’-CCCGGAGTTAAAGAGGAAAGA-3’) and Jf022604.2 (5’-GGTACCCCGTGGATGTACTC-3’), and digested with XmaI and KpnI, for directional cloning in frame with the 2RSP in pJF028, yielding pJFF029. Primers Jf022504.1 (5’-GGTACCAAATCGCAGAATCT-3’ and Jf022504.2 (5’-GGTACCATCTTTTACTGACTT-3’) were then used to amplify eltA and eltB encoding the heat-labile toxin subunits from H11047 genomic DNA. The resulting 1105 bp amplicon was digested with KpnI/Sall and cloned in-frame with gfpmut2 on pJFF029 to yield pJFF032, an expression plasmid encoding the 2RS-GFP-LT chimera.

**Toxin delivery studies**

To determine whether contact with epithelial cells is required for effective delivery of LT, we employed 10 µm thick polyester mem-
Immunofluorescence studies

Following bacterial interaction with target host cells grown on glass coverslips or on polyester membranes, samples were washed extensively with RPMI, then fixed with 3.7% paraformaldehyde in PBS, and washed with 1% glycine in PBS to quench excess aldehyde groups. After washing with PBS and blocking with 1% BSA (1x Pierce Blocker in PBS) for 1 h at room temperature, rabbit polyclonal anti-O78 antiserum (Pennsylvania State University Gastroenteric Disease Center Wiley Laboratory, http://ecoli.cas.psu.edu/) and monoclonal antibodies (M2) directed against the FLAG epitope tag (Sigma) were added at concentrations of 1:5000 and 1:2000, respectively, and incubated for 1 h. Secondary antibodies, goat anti-rabbit (IgG) labelled with AlexaFluor594 (Molecular Probes) and goat anti-mouse IgG (FITC) (Sigma) were employed at concentrations of 1:2000 and 1:200 respectively. To stain nucleic acid, coverslips were incubated with PBS containing 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) at a final concentration of 1:2000 for 5 min, followed by washing in PBS. Initial imaging was performed using epifluorescence on a Zeiss Axiopt microscope. Subsequent immunofluorescence microscopy was performed using a Zeiss Axioplan instrument and deconvolution of images was accomplished with AxiocVision software. Individual colour images were acquired as ZVI (Zeiss) or TIFF files, and assembled as composite colour images in Photoshop (v. 7.0 Adobe) followed by editing to adjust contrast and brightness of the combined image.

Live cell imaging

To obtain real-time images of bacteria expressing GFP-labelled proteins interacting with host cells, either HCT-8 or CaCo-2 cells were grown overnight at low density on glass bottom culture dishes (MatTek, Ashland, MA). Strain jf1128 was grown overnight in 2 ml of LB containing chloramphenicol (25 µg ml⁻¹) at 37°C, then diluted 1 : 100 into fresh media, grown to mid-logarithmic phase and induced for 1 h by the addition arabinitol (0.2%, final concentration). Bacteria were then added to dishes and images were immediately acquired on a Nikon Eclipse TE2000-E inverted microscope fitted with an environmental chamber maintained at 37°C, 5% CO₂.

Scanning electron microscopy

Samples for SEM were prepared by fixation in 2.5% glutaraldehyde in 0.05 M cacodylate for 1 h, followed by washing in 0.18 M sodium cacodylate buffer. Samples were then treated with 1% osmium tetroxide in 0.1% sodium cacodylate buffer for 1 h, and washed again in 0.18 M cacodylate buffer. Samples were then dehydrated in increasing concentrations of ethanol ranging from 35% to 100%.

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Supplementary material

The following supplementary material is available for this article online:

Movie S1. Real time images acquired using HCT-8 target epithelial cells and strain jfl128. Image acquisition was initiated immediately following the addition of bacteria. Movie demonstrates bacteria repeatedly targeting specific regions of the target cell, often with the polarized T2SS facing the point of host cell contact.

This material is available as part of the online article from http://www.blackwell-synergy.com