Adhesin Degradation Accelerates Delivery of Heat-labile Toxin by Enterotoxigenic Escherichia coli*

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Many enteric pathogens, including enterotoxigenic Escherichia coli (ETEC), produce one or more serine proteases that are secreted via the autotransporter (or type V) bacterial secretion pathway. These molecules have collectively been referred to as SPATE proteins (serine protease autotransporter of the Enterobacteriaceae). EatA, an autotransporter previously identified in ETEC, possesses a functional serine protease motif within its secreted amino-terminal passenger domain. Although this protein is expressed by many ETEC strains and is highly immunogenic, its precise function is unknown. Here, we demonstrate that EatA degrades a recently characterized adhesin, EtpA, resulting in modulation of bacterial adhesion and accelerated delivery of the heat-labile toxin, a principal ETEC virulence determinant. Antibodies raised against the passenger domain of EatA impair ETEC delivery of labile toxin to epithelial cells suggesting that EatA may be an effective target for vaccine development.

Recent studies suggest that EatA, a SPATE protein previously described in ETEC (Fig. 1a) (3), is immunologically recognized following both experimental murine and natural human infections with ETEC (13). In vivo expression of EatA and the recent identification of eatA genes in most of the recently sequenced ETEC strains (14–16), including the prototype H10407 strain in which it was originally discovered (3), suggest that it likely plays an important role in virulence of this pathovar. Similar to SepA, its close Shigella homologue (17), EatA has been associated with accelerated virulence in a rabbit ileal loop model (3). However, the precise functions of EatA as well as SepA remain unknown.

To date, the majority of ETEC virulence studies have focused specifically on the role of plasmid-encoded fimbrial colonization factors, or the established enterotoxins. However, more recent data suggest that many elements of ETEC virulence, specifically processes pertaining to bacterial adhesion and intestinal colonization, are actually quite complex (2, 18) and likely involve multiple factors. These include integral outer membrane proteins (19), the TibA autotransporter protein (20), the secreted EtpA adhesin molecule (5, 21), and flagella (21), as well as the heat-labile toxin (22).

Here, we further examine the contribution of eatA to virulence and demonstrate that it plays a significant but unanticipated role in modulating adherence of ETEC by degrading the EtpA adhesin. Importantly, the presence of EatA was shown to accelerate delivery of the heat-labile toxin to target epithelial cells.

**EXPERIMENTAL PROCEDURES**

* Bacterial Strains and Plasmids—A complete list of bacterial strains and plasmids used in these experiments is provided in Table 1. LMG194Δflic was constructed as described previ-
ous (21) using λ red recombination (23) to interrupt the flagellin (flIC) gene. Briefly, a kanamycin resistance cassette was amplified from pKD4 using primers jf121905.1 (5′-gagctgcttc-3′) and jf121905.2 (3′-ccaatacgtaatcaacgacttgcaatataggataacgaatcGTGTAGGCTG-3′) and ligated into the BamHI site of pKD4. The resulting plasmid was then transformed into the electroporated E. coli Mid pSB001 was then isolated and used to transform the plasmid into LMG194 carrying the eatAΔ/flIC mutant jf904 to ampicillin and chloramphenicol resistance. 6,7 The PCR product was introduced into LMG194 carrying the eatAΔ/flIC mutant jf904 to ampicillin and chloramphenicol resistance. The resulting plasmid was then religated on itself and re-introduced into Plasmids pBAD-TOPO/lacZ/V5-His: pBAD-TOPO vector containing lacZ control insert pSB001: 7064-bp BamHI subclone from pBAD-TOPO/lacZ/V5-His pSP014: 4186-bp EatA amplicon cloned into pBAD-TOPO pSP019: pSP014 altered by SDM for His-134→Arg-134 substitution of EatA pljL017: eatenA cloned on pBAD-myc His plasmid with eatenA in-frame with C-terminal myc-His6 tags pljL030: eatenA on pACYC184

**TABLE 1**

| Strains    | Relevant genotype or description                                                                 | Ref./source |
|------------|--------------------------------------------------------------------------------------------------|-------------|
| H10407     | ETEC serotype O78:H11, LT+, ST+, eatA+, etpBAC+                                                   | 3, 5, 39     |
| jf721      | Deletion of eatA encoding a subunit of LT                                                        | 24          |
| jf946      | Derivative of H10407 lacZ::KmR                                                                  | 24          |
| jf904      | H10407 eatA:CamR mutant                                                                         | 3           |
| LMG194     | F− ΔlacX74 galE thi rpsL AphoA (ProVII) Δara-714 leu::Tn10                                        | Invitrogen  |
| LMG194Δ/flIC| LMG194 with flIC::KmR                                                                          | This study  |
| TOP10      | F− mcrΔ (mcr−frdRMS-mcrBC) F800lac ΔM15 ΔlacX74 recA1 araD139 Δ (ara leu) 7697 galU galK rpsL (StrR) | Invitrogen  |
| jf1443     | ΔeatA (pljL001)                                                                                  | This study  |
| jf1446     | ΔeatA (pBADmynCHisA)                                                                             | This study  |

| Plasmids   | Relevant genotype or description                                                                 | Ref./source |
|------------|--------------------------------------------------------------------------------------------------|-------------|
| pBAD-TOPO/lacZ/V5-His | pBAD-TOPO vector containing lacZ control insert                                                | 3; Invitrogen |
| pSB001     | 7064-bp BamHI Δlac subclone from pBAD-TOPO/lacZ/V5-His                                         | This study  |
| pSP014     | 4185-bp EatA amplicon cloned into pBAD-TOPO                                                      | 3           |
| pSP019     | pSP014 altered by SDM for His-134→Arg-134 substitution of EatA                                   | 3           |
| jfL017     | eatenA cloned on pBAD-myc His plasmid with eatenA in-frame with C-terminal myc-His6 tags       | 21, 26      |
| jfL030     | eatenA cloned on pACYC184                                                                         | 21, 26      |

**In Vitro Bacterial Adherence Assays**—Adherence assays for these experiments were performed as described previously (21). Briefly, bacteria were grown overnight from frozen glycerol stocks in 2 ml of Luria broth containing antibiotics where appropriate at 37 °C, 225 rpm. The following morning strains were diluted 1:100 in fresh media and grown to mid-logarithmic growth phase (~90 min of incubation at 37 °C). For strains complemented with arabinose-inducible plasmids, arabinose was added to a final concentration of 0.02% for the last 30 min of incubation. Bacteria were then added (at a multiplicity of infection (bacteria/target epithelial cell) of 100:1) to CaCo-2 cell monolayers seeded the previous evening in 96-well tissue culture plates. After 1 h, 37 °C, 5% CO2, monolayers were washed with RPMI 1640 medium and lysed in 0.01% Triton X-100 to release both adherent and intracellular bacteria. Dilutions were then plated onto Luria agar plates and incubated overnight at 37 °C. To identify intracellular bacteria, monolayers were washed at 3 h following infection and the media replaced with fresh pre-warmed media containing gentamicin (100 µg/ml) and incubated for 1 additional hour. After washing to remove remaining antibiotics, monolayers were lysed with Triton X-100 to release intracellular organisms and plated as above.

**Immunofluorescence Imaging**—To examine the pattern of bacterial attachment to the cell surface, organisms were added to Caco-2 epithelial cells grown on sterile glass coverslips pre-treated with poly-1-lysine and incubated in 5% CO2 atmosphere at 37 °C for 1–2 h. After washing three times with tissue culture media, cells were fixed with paraformaldehyde, washed with PBS, and blocked with PBS containing 1% BSA. Anti-O78 rabbit polyclonal antibody followed by AlexaFluor-488-labeled anti-rabbit antibody were used to identify the attached organisms, and cells were stained with both diamidino-2-phenylindole (DAPI) and membrane stain (CellMask, Red, Invitrogen). After acquisition of images on a Zeiss LSM510 confocal microscope, the number of individual bacteria adherent to cells was examined by importing TIFF image files into ImageJ (version 1.44) and tracking the number of adherent bacteria/cell using the cell counter plugin module (Image > plugins > analyze > cell counter).

**ETEC Intestinal Colonization Studies**—jf946, a derivative of ETEC H10407 containing a kanamycin resistance marker in the lacZYA locus (24), and the jf904 eatA mutant (3) were tested in the streptomycin–treated murine colonization model as described previously (25). Briefly, mice (CD-1, Charles River) were treated with streptomycin in drinking water (5 g/liter) for 48 h prior to inoculation, which was changed to antibiotic–free sterile water the evening before challenge. Cimetidine (50 mg/kg) was administered intraperitoneally 1.5 h prior to gavage challenge with bacteria in a final volume of 400 µl. Mice were euthanized 24 h later, and bacterial colonization was assessed by plating saponin (5% solution in PBS) lysates of small intestinal segments onto Luria agar plates containing kanamycin (Km, 25 µg/ml) or chloramphenicol (Cm, 25 µg/ml). To assess the number of ETEC organisms shed in stool, fecal pellets were collected from individual mice, resuspended in PBS, and dilutions plated onto replicate Luria agar plates.

Competition studies were carried out as described previously (21), with co-administration of 1 × 109 of both the jf946 and...
Affinity Purification of Recombinant Proteins—Production and purification of recombinant Myc-polyhistidine-tagged EtpA glycoprotein (rEtpA-myc-His) was carried out as described previously in detail (26). Briefly, E. coli Top10 carrying both pJL017 and pJL030 was induced with arabinose and the secreted rEtpA-myc-His protein recovered from concentrated supernatants by batch metal affinity chromatography (Talon, Clontech).

To produce recombinant EatA passenger protein (rEatAp), the arabinose-inducible EatA expression plasmid pSP014 (3) was first introduced into LMG194Δflic, selecting for ampicillin- and kanamycin-resistant transformants. LMG194Δflic(pSP014) was then grown in 250 ml of Luria broth containing kanamycin (25 μg/ml) and ampicillin (100 μg/ml) to an A600 of ~0.5 then induced for 2 h with arabinose at a final concentration 0.02%. Culture supernatants containing rEatAp were then concentrated ~50-fold by ultrafiltration using a 100-kDa MWCO filter. After buffer exchange using column loading buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.2) and further concentration to a final volume of 0.5 ml, rEatAp was purified to homogeneity by gel filtration chromatography on a Sephacryl S-300 high resolution column (26). Mutant rEatp(H134R) was prepared in the same fashion after introduction of pSP019 into LMG194Δflic.

Separation and Detection of Proteins in Culture Supernatants—After growth of bacteria in Luria broth for the indicated time, cultures were centrifuged at 10,000 × g for 20 min to pellet bacteria, and supernatants of either H10407 or the jf904 cultures were centrifuged at 10,000 × g for 30 min at room temperature. Next, the membrane was incubated with polyclonal rabbit antisera raised against rEatA passenger domain (r6H-EatA(88–581)) (3) diluted 1:10 in TBS-T for 2 h. Antibodies were then eluted with 100 mM glycine, pH 3.0, neutralized with 1 M Tris-HCl, pH 8.8, dialyzed against PBS, concentrated, and then sterile-filtered.

Far Western Analysis—To detect interaction of EatA with EtpA, 5 μg of recombinant EtpA.myc.His was electrophoretically transferred onto nitrocellulose and incubated with an equimolar amount of mutant protease activity-deficient EatA (H134R) for 2 h at room temperature. Membrane was washed and blocked in TBS, 3% milk for 30 min and probed with polyclonal affinity-purified primary antibody raised against r6H-EatA(88–581) (3) followed by detection with HRP-labeled anti-rabbit antibody (1:5000) and chemiluminescent substrate.

EtpA Degradation Studies—Varying amounts of rEatAp or rEatAp(H134R) were mixed with 1 μg of rEatA-myc-His and incubated at 37 °C. To inhibit protease activity, 4-amidinophenylmethanesulfonyl fluoride hydrochloride was added at a final concentration of 25 μM. The contents of the reaction was mixed with loading dye (six times) and separated by SDS-PAGE prior to immunoblotting for EtpA.

Heat-labile Toxin Delivery Studies—To examine the efficiency of LT delivery to target host cells, bacteria from overnight cultures of the wild type H10407 or the ΔeatA strain were added to Caco-2 epithelial cell monolayers. Following incubation of the bacteria with monolayers for 1–3 h, cells were lysed, and intracellular concentration of cAMP was determined by competitive enzyme immunoassay (GE Healthcare).

LT Production—To measure production of LT by wild type and eatA mutant, bacteria were grown at 37 °C, 225 rpm in 2 ml of casamino acids/yeast extract medium (CAYE, 2% 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) (28) Supernatants were then tested in mixed ganglioside ELISAs as described previously (29).

Transcriptional Analysis by Real Time PCR—To assess specific gene transcripts, total RNA was first isolated from bacteria (RNeasy, Qiagen) and quantified spectrophotometrically. RNA was freed of contaminating DNA by repeated DNase treatments. Total RNA from bacteria was converted to cDNA by use of reverse transcriptase and random hexamers. Gene expression was quantified by use of an ABI-PRISM 7900HT sequence detection system with gene-specific primers in PCR buffer containing SYBR Green. Gene-specific transcripts were normalized to the housekeeping gene arcA.

Processing of LT by EatA Protease—To examine the activity of EatA in cAMP assays, the purified recombinant passenger domain of EatA (rEatAp) was added exogenously to Caco-2 target epithelial monolayers with or without purified LT holotoxin. After 3 h, cells were lysed, and cAMP concentrations were determined as above. To examine proteolytic cleavage of
EatA Modulates ETEC Virulence

FIGURE 1. EatA modulates epithelial cell adhesion. a, schematic of EatA protein structure showing from left to right the signal peptide (black), the passenger domain (open) with the site of serine protease catalytic triad (His-134, Asp-162, and Ser-267), and the β-barrel transport domain (gray). b, Caco-2 cell adhesion assays showing adhesion by ETEC wild type strain H10407 (wt) or the eatA mutant (jf904) complemented with empty vector plasmid (pSBO01), plasmids expressing either rEatA (pSP014), or mutant protein bearing a mutation in the putative serine protease motif (pSP019). Shown below each strain are immunoblots of corresponding TCA-precipitated culture supernatants demonstrating production of EatA protein. c, addition of exogenous recombinant EatA passenger domain (rEatAp), but not the mutant protein rEatAp(H134R) restores adhesion to wild type levels (α = no protein added). d, antibodies against the EatA passenger domain alter adhesion of H10407 to target cells. Shown are total cell-associated bacteria shown in presence (+) or absence (−) of affinity-purified antibody (α) directed against the EatA passenger. * denote p values determined by two-tailed t test (unpaired); *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

RESULTS

EatA Modulates Adherence of ETEC to Target Epithelial Cells in a Process Dependent on Its Serine Protease Activity—To date, most studies of ETEC adherence to the intestinal epithelium have focused specifically on the role of plasmid-encoded fimbrial colonization factors. However, more recent data suggest that the process of ETEC adhesion is complex and likely involves multiple virulence factors, including the secreted adhesin molecule, EtpA (5), flagella (21), as well as the heat-labile toxin (22). Each of these factors has been shown to promote adhesion of ETEC to epithelial cells and to promote intestinal colonization. Curiously, by contrast, we observed that a mutant strain deficient in the production of another putative virulence protein, the EatA autotransporter (diagramed in Fig. 1a), appeared more adherent than wild type bacteria using in vitro intestinal epithelial cell monolayer adhesion assays (Fig. 1b), suggesting that this molecule might negatively modulate interactions between ETEC and the host cell. This inhibitory effect appeared to require protease activity of the EatA passenger domain as complementation of the mutant with a plasmid expressing wild type recombinant protein restored adhesion to levels similar to those observed with the parent strain, whereas complementation with a plasmid-expressing protein bearing a H134R mutation in the EatA serine protease catalytic domain had no effect (Fig. 1b). Similarly, we were able to restore adherence of the eatA mutant to WT levels by addition of exogenous recombinant EatA passenger protein (rEatAp), although this was not true for the mutant rEatAp(H134R) molecule (Fig. 1c). Interestingly, antibodies raised against the passenger domain also enhanced adherence of the WT strain but had no appreciable effect on the mutant (Fig. 1d), further suggesting that EatA normally modulates the interaction of ETEC with target host cells.

EatA Negatively Modulates Intestinal Colonization—Because molecules influencing bacterial adherence in vitro also tend to participate in intestinal colonization, an essential virulence trait for enteric pathogens, we examined the relative ability of WT and eatA mutant ETEC strains to colonize small intestine. The results of these studies paralleled those obtained in vitro, with the eatA mutant exhibiting enhanced intestinal colonization at either 24 or 72 h following infection (Fig. 2, a and b). Similarly, the eatA mutant was shed with greater abundance in stool (Fig. 2, d and e). However, the eatA mutant did not out-compete the wild type ETEC strain when these organisms were introduced simultaneously (Fig. 2c), perhaps suggesting that levels of EatA secreted in vivo are sufficient to reverse the hyper-colonization phenotype exhibited by the mutant when introduced alone.

EatA Binds and Proteolytically Degrades the EtpA Adhesin—We next questioned whether EatA might normally suppress bacterial adhesion by targeting one or more bacterial adhesins for proteolytic degradation. Because the passenger domain of EatA is released from the bacteria and freely secreted into the surrounding media (3), we first examined culture supernatants from WT bacteria or the eatA mutant. As predicted, these demonstrated that the EatA passenger was absent from the ∆eatA mutant (jf904) strain. However, we also noted an abundance of EtpA glycoprotein in culture supernatants of the jf904 strain relative to the WT (Fig. 3a). Immunoblots of culture supernatants demonstrated significant accumulation of EtpA in the eatA mutant cultures, whereas complementation of the eatA gene in trans on pSP014 yielded EtpA near or below WT levels (Fig. 3b). In contrast, the eatA mutant complemented with plasmid pSP019-expressing EatA with a mutation (H134R) in the predicted serine protease site exhibited the same phenotype as the uncomplemented mutant with accumulation of EtpA in the media. These differences did not appear to result from appreciable differences in growth of eatA+ and eatA− strains (Fig. 3c) or from altered transcription of the etpBAC locus responsible for the production and secretion of EtpA. Transcript levels for all three genes in the etpBAC locus (etpB, etpA, and etpC) were not appreciably different in the WT strain relative to those in the eatA mutant suggesting that the accumulation of EtpA in the mutant is not due enhanced transcription of the EtpBAC
operon. Likewise, we did not see appreciable alteration in other known adhesin genes, including those for the plasmid-encoded colonization factors or the chromosomally encoded type 1 fimbrial antigens (Table 2). Therefore, we next examined whether EatA might interact with and/or degrade EtpA. Incubation of purified rEatAp(H134R) with rEtpA.myc.His immobilized on nitrocellulose suggested that these proteins might at least transiently interact (Fig. 3d).

**FIGURE 2. EatA regulates colonization of murine small intestine.** Intestinal colonization at 24 h (a) or 72 h (b) following infection. c, competition assay between jf876 (lacZ::KmR) and jf904 (eatA::CmR). The competitive index = ([mutant (CmR)/wild type (KmR)]output cfu/[mutant/wild type] input cfu). Fecal shedding of organisms at 48 h (d), and 72 h (e) following infection. a, b, d, and e involved administration of $1.5 \times 10^6$ cfu of either jf876 and H10407 derivative (lacZ::KmR) or strain jf904 (eatA::CmR). Competition assay in c involved co-administration of $\approx 1 \times 10^4$ cfu of both strains.

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Therefore, we next examined whether EatA might interact with and/or degrade EtpA. Incubation of purified rEatAp(H134R) with rEtpA.myc.His immobilized on nitrocellulose suggested that these proteins might at least transiently interact (Fig. 3d). Incubation of rEtpA with varying amounts of rEatA passenger domain resulted in rapid degradation of the EtpA glycoprotein that was at least partially inhibited by the addition of the serine protease inhibitor, 4-amidinophenylmethanesulfonyl fluoride hydrochloride (Fig. 3e). In addition, affinity-purified polyclonal antibodies directed against the EatA passenger domain similarly inhibited degradation of the EtpA adhesin (Fig. 3f). As predicted, mutant EatAp containing a mutation in the predicted serine protease active site (H134R) was markedly attenuated in its ability to degrade EtpA (Fig. 3, g and h). Together, these results suggest that EatA modulates EtpA-dependent bacterial adhesion and intestinal colonization by targeting this novel adhesin for destruction.

**EatA Accelerates Delivery of Heat-labile Toxin**—In general, adhesion by bacteria is thought to be an important prerequisite for delivery of bacterial effector molecules, including exotoxins such as LT. However, for some pathogens (30), including some pathogenic E. coli (18, 31), the ability to negatively modify adhesion events also appears to be an important virulence trait. However, little is known regarding how modulation of adhesion will affect toxin delivery.

Whereas the eatA mutant and WT strains produced and secreted similar amounts of LT (Fig. 4a), and neither the transcript levels for toxin genes nor those encoding the type II secretion apparatus for LT were demonstrably different between WT and the eatA mutant (Table 2), the eatA mutant was curiously retarded...
in its ability to stimulate cAMP production in target epithelial cells. Complementation of serine protease activity provided by EatA restored cyclic nucleotide activation to near WT levels (Fig. 4b).

Although the eatA mutant was able to stimulate cAMP production relative to the LT (eltA deletion) mutant, this plateaued early after infection of the epithelial cells (Fig. 4c).

**Table 2**

| Functional group       | Gene | Forward primer                                      | Reverse primer                                      | Fold-change*  |
|------------------------|------|----------------------------------------------------|----------------------------------------------------|---------------|
| Type 1 fimbriae        | fimA | 5′-gccgcaaccaggctgttcttgcgcgtttg-3′               | 5′-aaccggcttgcgctgctgtgttc-3′                       | 0.76 ± 0.83   |
|                        | fimH | 5′-gcgggtgcgtttggtgcgtgcttgcgctgctgtgttc-3′       | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.63 ± 1.56   |
| EtpBAC TPS             | etpB | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.52 ± 1.09   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |
|                        | etpA | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.62 ± 0.71   |
|                        | etpC | 5′-gcgggtgcgtttggtgcgtgctgctgctgtgttc-3′          | 5′-gccgcaaccaggctgttcttgcgcgtttgttc-3′             | 1.98 ± 1.92   |

* Fold-change in transcript for WT relative to eatA mutant; each value represents average of ≥4 biological replicates ± S.D.
EatA Modulates ETEC Virulence

Because ETEC-infected patients, as well as mice challenged with these organisms, mount robust immune responses to the EatA passenger domain, we examined the effect of affinity-purified polyclonal antibody directed against this region of the autotransporter (EatAp) on toxin delivery (Fig. 4d). Importantly, addition of these antibodies significantly inhibited cAMP production in target Caco-2 epithelial cells, suggesting that neutralization of EatA could limit the ability of these organisms to effectively deliver active toxin. Together, these results suggest that EatA modulates bacterial adhesion in a way that favors efficient delivery of LT to epithelial cells and that targeting EatA in vaccines could present a novel strategy to prevent diarrheal illness.

Conceivably, EatA could act directly on LT or indirectly on the host cell to promote intoxication, or alternatively, EatA might directly stimulate cAMP production. Notably, however, the addition of exogenous rEatAp to Caco-2 cells was not sufficient to stimulate the production of cAMP, nor did addition of LT together with rEatAp enhance toxin activity (Fig. 5a). Interestingly, both cholera toxin and the similar E. coli heat-labile toxin require proteolytic cleavage or “nicking” of a peptide loop joining the major (A1 and A2) domains of their respective A subunits for complete activation (32). Because Vibrio cholerae strains secrete a protease capable of proteolytic nicking of cholera toxin (33), we also examined whether EatA might serve a similar function in processing LT. Although incubation with trypsin resolved the A subunit into its respective A1 and A2 domains (Fig. 5b), the native A subunit remained intact following addition of EatA. Collectively, these data suggest that increased cAMP levels observed when intestinal cells are infected with the wild type pathogen result from enhanced toxin delivery by the bacteria and not from accelerated toxin processing.

eatA Mutants Form Large Sessile Colonies—Theoretically, more adherent bacteria should be more effective in delivery of the toxin to host cells. To investigate the apparent discord between the adhesion and toxin delivery phenotypes exhibited by the eatA mutant, we also examined the pattern of ETEC adherence to target epithelial cells. We noted that the wild type strain typically adhered as single organisms or groups of a few organisms (Fig. 6, a–d), and the mutant occasionally appeared in the form of large clusters with many organisms (Fig. 6, e–j). Nevertheless, we also found that for the eatA mutant, there were also significantly more individual bacteria attached per cell (ΔeatA = 0.62 ± 0.41 bacteria/cell versus WT = 0.29 ± 0.09 bacteria/cell; p = 0.0007 by Mann-Whitney comparison) suggesting that these mutants exhibit true hyper-adherence relative to the parent strain.

DISCUSSION

Bacterial adhesion to target host cells is thought to be an essential prerequisite for efficient delivery of toxin by entero- toxigenic E. coli (2, 34, 35). Indeed, earlier studies demonstrated that intimate bacteria-host cell contact is an absolute requirement for ETEC to activate cAMP production in target intestinal epithelial cells (24).

This study is therefore seemingly at odds with this paradigm in that a mutant that is hyper-adherent is surprisingly less efficient in delivering LT. However, the investigations here suggest that the manner in which bacteria adhere may be a more relevant determinant of effective toxin delivery than the total number of bacteria that stick to a cell.

Interestingly, earlier investigations, which included real time analysis of wild type ETEC interactions with host cells, demonstrated that the majority of adhesion events are quite transient lasting only seconds to minutes (24). Theoretically, strains that adhere too avidly and remain stuck to a single epithelial cell may...
be incapable of regenerating toxin payload that is then delivered to other target cells during subsequent adhesion events.

Although the biochemical actions and cellular processing of heat-labile toxin have been thoroughly investigated, there is surprisingly scant data regarding the role of bacterial surface structures in facilitating toxin delivery to target host cells. Likewise, although the ETEC type II secretion (T2SS) apparatus for heat-labile toxin is known (36) and is clearly required to stimulate cAMP in intestinal cells (24), the proximate triggers involved in the secretion of LT from the periplasmic space of ETEC so that it can be efficiently delivered to ganglioside receptors at the cell surface are still very poorly understood. This study adds further evidence that this process is one that is elegantly orchestrated by the bacteria.

It is becoming increasingly apparent that toxin delivery and colonization of the intestine involve the coordinate action of multiple genes in addition to the well studied fimbrial colonization factors and the toxins themselves. Indeed, the recent sequencing of multiple ETEC strains suggests that both EatA autotransporter and the EtpA two-partner secretion adhesin protein are highly conserved but specific to the ETEC pathovar among *E. coli* strains (37), further suggesting that these genes likely play important roles in virulence.

Interestingly, proteolytic degradation of an extracellular two-partner secretion adhesin, similar to EtpA, has previously been shown to facilitate colonization of *Bordetella pertussis*. Here, the *B. pertussis* SphB1 protease releases the filamentous hemagglutinin adhesin from the surface of these pathogens, presumably promoting colonization of other sites in the mucosa (30).

By contrast EatA appears to limit intestinal colonization of ETEC by degradation of EtpA. Ultimately, diarrheal pathogens face a hostile environment in the intestinal lumen where they must compete with normal flora and escape both innate and adaptive host responses. Limiting colonization could be one mechanism of avoiding host defenses. Adopting a "hit-and-run" strategy that allows the organism to deliver its toxin payload while escaping the ultimate consequences of unrestricted attachment could be to the advantage of the pathogen.

The production of a virulence factor that limits damage in its host is also not without precedent. The present data imply that EatA fits an emerging paradigm for bacterial virulence proteins that may serve in part to limit damage to the host (38). As earlier experiments demonstrated that EatA could contribute directly to pathogenesis and accelerate fluid secretion into the intestinal lumen (3), it may serve a bi-functional role in acting both as a direct effector molecule and as an anti-colonization factor that modulates damage to the host.

The ETEC H10407 strain, in which both the *eatA* autotransporter gene (3) and the *etpBAC* two-partner secretion locus (5) were originally identified, has been the subject of considerable basic and clinical investigation since its early isolation in 1971 as one of the first strains of enterotoxin-producing *E. coli* from an adult with severe cholera-like diarrheal illness in Bangladesh (39, 40). H10407, which makes CFA/I fimbrial colonization factors and both heat-stable and LT, is also associated with severe illness in human volunteer challenge studies (41). In these studies, when compared with ETEC strain B7A that possesses CS6 fimbria, and also makes both toxins (ST+/LT+), H10407 caused nearly twice the volume of diarrhea even at a lower inoculum. Intriguingly, B7A does not make either the EtpA adhesin or the EatA protease studied here, raising the possibility that severe clinical illness may require a full complement of virulence genes that extends beyond fimbrial colonization factors and toxins.

Of note, early enthusiasm for fimbrial colonization factors as vaccine targets in part stemmed from experiments with a derivative of H10407 (H10407-P), which through serial laboratory cultivation had lost its largest (94.8 kb) virulence plasmid (42). H10407-P lacks the ability to produce CFA/I and is dramatically attenuated in human volunteers (43). Interestingly, later studies showed that both the *eatA* gene (3) and the *etpBAC* locus (5) are also encoded on this same plasmid, findings supported by the recent publication of the H10407 genome (15).

It is important to note that although we demonstrate here that EtpA is one substrate for EatA, there may be additional and as yet unidentified bacterial as well as eukaryotic targets for this protease. Indeed, SpeB, a cysteine protease produced by streptococci, degrades multiple bacterial effectors (44, 45) and targets host defense molecules, including immunoglobulins (12), and cytokines for degradation. Likewise, the presence of a close ETEC homologue of EatA (SepA) in *Shigella*, which does not make EtpA, would also suggest that EatA and similar molecules could serve a number of important virulence functions. Further studies will clearly be needed to assess the complete role of EatA in virulence of ETEC.

Nevertheless, the findings included here suggest that the molecular dynamics of ETEC intestinal colonization, effective toxin delivery, and ensuing diarrheal illness are complex events offering new opportunities to develop intervention strategies. These include the possibility that EatA and other novel virulence molecules could be effective vaccine targets. Additional study of the relative contributions of these and other virulence genes to the overall fitness of ETEC strains as human pathogens could ultimately prove to be important to the rational selection of antigens for incorporation into vaccines designed to prevent severe illness among children in developing countries where ETEC remains among the leading causes of death because of diarrheal disease.

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