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Molecular Determinants of Enterotoxigenic *Escherichia coli* Heat-Stable Toxin Secretion and Delivery

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC), a heterogeneous diarrheal pathovar defined by production of heat-labile (LT) and/or heat-stable (ST) toxins, causes substantial morbidity among young children in the developing world. Studies demonstrating a major burden of ST-producing ETEC have focused interest on ST toxoids for ETEC vaccines. We examined fundamental aspects of ST biology using ETEC strain H10407, which carries *estH* and *estP* genes encoding STh and STp, respectively, in addition to *eltAB* genes responsible for LT. Here, we found that deletion of *estH* significantly diminished cyclic GMP (cGMP) activation in target epithelia, while deletion of *estP* had a surprisingly modest impact, and a dual *estH estP* mutant was not appreciably different from the *estH* mutant. However, we noted that either STh or STp recombinant peptides stimulated cGMP production and that the loss of *estP* was compensated by enhanced *estH* transcription. We also found that the TolC efflux protein was essential for toxin secretion and delivery, providing a potential avenue for efflux inhibitors in treatment of acute diarrheal illness. In addition, we demonstrated that the EtpA adhesin is required for optimal delivery of ST and that antibodies against either the adhesin or STh significantly impaired toxin delivery and cGMP activation in target T84 cells. Finally, we used FLAG epitope fusions to demonstrate that the STh propeptide sequence is secreted by ETEC, potentially providing additional epitopes for antibody neutralization. These studies collectively extend our understanding of ETEC pathogenesis and potentially inform additional avenues to mitigate disease by these common diarrheal pathogens.

KEYWORDS diarrhea, enterotoxigenic, heat-stable toxin, pathogenesis, toxoids, vaccines

Diarrheal illnesses in low-income countries continue to cause substantial morbidity and remain one of the leading causes of death in young children under the age of 5 years in developing countries. Among the bacterial causes of diarrheal illness, enterotoxigenic *Escherichia coli* strains (ETEC) are commonly linked to more-severe forms of illness in young children (1). These organisms are perennially the most common cause of diarrhea in those who travel to areas of endemicity where sanitation is poor (2, 3); however, they have been identified repeatedly as the etiology of diarrheal outbreaks and sporadic cases of illness in industrialized countries, including the United States (4–8).

Acute clinical presentations of ETEC infection may range from mild self-limited illness to severe choleralike diarrhea (9–11). In addition, ETEC and other diarrheal pathogens have been linked to pernicious sequelae, including malnutrition, growth stunting, and impaired cognitive development (12). Presently, there are no vaccines to protect against these common infections. ETEC strains are a genetically (13) and serotypically (14) diverse pathovar of *E. coli* defined by the production of heat-labile (LT)

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and/or heat-stable (ST) enterotoxins that activate production of host cyclic nucleotides to alter intestinal salt and water transport that culminate in net fluid losses and secretory diarrhea.

Heat-stable toxins are synthesized as 72-amino-acid proteins consisting of a signal peptide, a propeptide, and a carboxy-terminal region of 18 to 19 amino acids, which forms the mature active enterotoxin (15). Two enterotoxins cause disease in humans: STp (ST1a), 18 amino acids, and STh (ST1b), 19 amino acids. Both of the mature toxins contain a total of six cysteine residues that form three intramolecular disulfide bonds (16). Their overall structure is shared with two similar mammalian peptides, guanylin and uroguanylin. Each of the bacterial and mammalian peptides binds to guanylate cyclase C (17, 18), leading to increases in intracellular cyclic GMP (cGMP) (19). Increases in cGMP result in activation of protein kinases that phosphorylate and activate the cystic fibrosis transmembrane regulatory (CFTR) channel and inhibit sodium-hydrogen ion exchange via sodium/hydrogen exchanger 3 (NHE3) (20). These effects lead to a net loss of salt and water into the intestinal lumen with ensuing watery diarrhea.

Bacteria producing any of the toxins LT, STh, or STp have been linked to diarrheal illness in humans (21–24), and recent studies suggest that ST-producing ETEC is commonly represented among the pathogens that cause severe diarrheal illness among young children in low-income countries, leading to substantial interest in the development of a vaccine that incorporates ST toxoids (25).

Enterotoxigenic *E. coli* strain H10407, originally isolated from a case of severe choleralike diarrheal illness in Bangladesh, is to date the most extensively characterized isolate of this pathovar. Interestingly, this isolate encodes all three canonical enterotoxins associated with ETEC diarrheal illness in humans (26, 27), with the gene for STh on the largest (94,797-bp) virulence plasmid p948 (NCBI GenBank accession number [NC_017724.1](#)) and the genes for both LT and STp on a 66,681-bp plasmid, p666 (NCBI GenBank accession number [NC_017722.1](#)) (28). In addition, H10407 encodes a copy of the ST-like EAST1 peptide (29) on the large p948 plasmid (30). H10407 is frequently used as the challenge strain in controlled human infection models to test candidate vaccines. Therefore, we set out to examine the relative contribution of STh and STp to the accumulation of cGMP in host epithelia by H10407 and the ability of anti-ST and antiadhesin antibodies to mitigate effective toxin delivery by the bacteria.

RESULTS

Contributions of STh, STp, and EAST1 to activation of cGMP in target epithelial cells. Understanding the individual contributions of ST and ST-like molecules of ETEC is relevant to development and testing of toxin neutralization strategies. H10407 encodes three peptides with the potential to activate cGMP in target intestinal epithelial cells: STh, STp, and EAST1, a heat-stable toxin originally identified in enteroaggregative *E. coli* (29). STh is encoded by the *estH* gene on the large, 94,797-bp plasmid p948 (NCBI GenBank accession [NC_017724.1](#)). The *astA* gene, which encodes the EAST1 peptide (30), is embedded within an *IS1414* insertion sequence (31) and is also located on the p948 plasmid immediately downstream from the *etpBAC* adhesin locus (28, 32). STp is encoded by the *estP* gene on a second, 66-kb virulence plasmid (NCBI GenBank accession number [FN649417.1](#)) in H10407.

We found that deletion of *estH* resulted in appreciable decreases in cGMP production at or near background levels of cGMP production by uninfected cells and that complementation of *estH* in *trans* restored activation of this cyclic nucleotide (Fig. 1A). In contrast, the *estP* mutant was not appreciably different from the wild-type strain, and the introduction of the *estP* mutation to the *estH* strain did not yield further measurable decreases in cGMP production by target epithelial cells. Similar results were obtained at various multiplicities of infection (MOIs) (Fig. 1B), suggesting that this was not related to a spurious inoculum effect. In addition, infection of small intestinal enteroids demonstrated that while deletion of *estH* alone resulted in a substantial decrease in the production of cGMP in target epithelia to background levels, deletion of *estP* had no demonstrable effect on cyclic nucleotide production (Fig. 1C). However, we found that

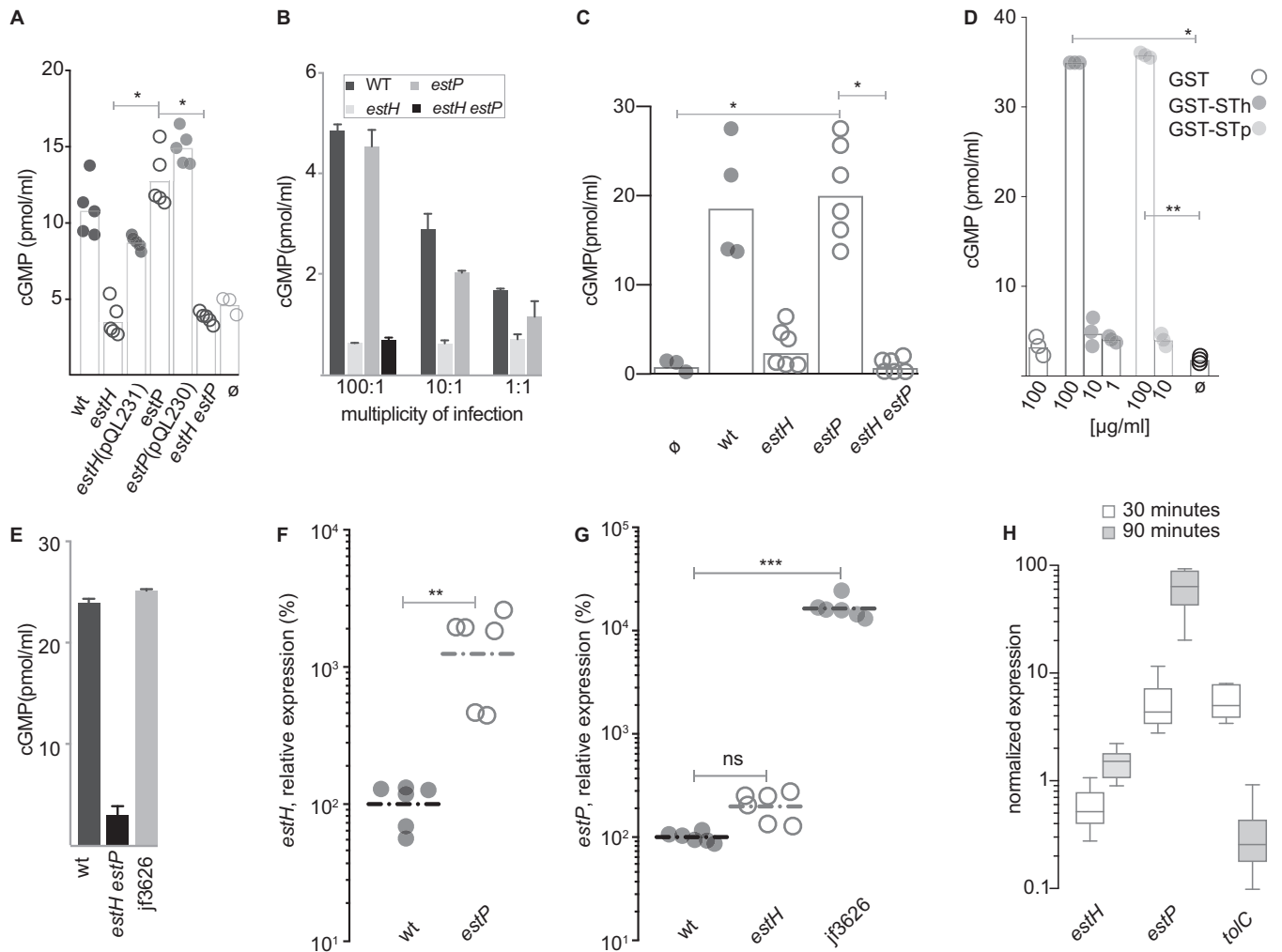


FIG 1 Relative contributions of STh and STp to cGMP production in target epithelia. (A) cGMP production in T84 target epithelial cells following infection with wild-type *estH*, the complemented *estH* mutant *estH*(pQL231), the *estP* mutant, the complemented *estP* mutant *estP*(pQL230), and the *estH estP* dual deletion mutant. \emptyset , uninfected cells. Data represent the combined results of two experimental replicates with each data point representing a technical replicate. (B) cGMP production in T84 cells at various multiplicities of infection with H10407 (wt), the *estH* or *estP* mutant, or the *estH estP* dual deletion mutant ($n = 3$ technical replicates; shown as mean values \pm standard errors of the means [SEM]). (C) cGMP production in small intestinal enteroids following infection with the wild type (wt), the *estH* or *estP* mutant, or the *estH estP* dual deletion mutant. (D) cGMP production in T84 cells following addition of purified GST, GST-STh, or GST-STp fusions. Numbers on the x axis represent final concentrations of protein in micrograms per milliliter. \emptyset , untreated cells. Data include three technical replicates where geometric means are represented as bars. In panels A, C, and D, bars represent geometric means, with statistical comparisons by analysis of variance (ANOVA) (Kruskal-Wallis); *, $P < 0.05$; **, $P < 0.01$. (E) Complementation of the *estH estP* mutant with *estP* in *trans* (jf3626) restores wild-type levels of toxin activity following infection of T84 cells (bars represent results for 3 technical replicates \pm SEM). (F) Transcription of *estH* gene in wild-type and *estP* mutant bacteria. Individual symbols represent 6 technical replicates combined from two independent experiments. Horizontal lines represent geometric means of expression relative to the wild type (H10407); **, $P = 0.002$ by Mann-Whitney (two-tailed) nonparametric comparison. (G) Transcription of *estP* gene in *estH* mutant bacteria relative to the wild type. The *estP*-complemented *estH estP* mutant, jf3626, is shown as a positive transcription control (***, $P < 0.0002$ by ANOVA, Kruskal-Wallis testing). (H) Transcription of genes encoding ST toxins STh (*estH*), STp (*estP*), and TolC. Data are normalized relative to the *arcA* housekeeping gene and represent the ratio of transcripts attached to planktonic bacteria at 30 and 90 min after infection of monolayers. Whisker plots represent the range of data obtained over six replicates from two independent experiments. Horizontal lines represent mean values. Data for each group represent mean of 3 replicates \pm SEM.

in distinct contrast to an A14Q mutant STh peptide (25), the target T84 cells did respond to glutathione S transferase (GST) fusions to either STh or STp (Fig. 1D) and that *estP* complementation of the *estH estP* dual deletion mutant in *trans* was sufficient to completely restore activation of cGMP to wild-type levels (Fig. 1E), clearly suggesting that enterocytes have the capacity to respond to either peptide. These results suggested that deletion of *estP* results in a compensatory increase in *estH* transcription and/or secretion of the encoded peptide. Interestingly, we found that relative to what was seen in the wild-type strain, *estH* transcription was significantly increased in the *estP* mutant (Fig. 1F), while transcription of *estP* in the *estH* mutant was not substantially

increased (Fig. 1G). Collectively, these results suggest that both ST peptides likely act in conjunction with heat-labile toxin to cause the diarrheal illness associated with the H10407 strain, but the molecular details governing transcriptional regulation of toxin production have not been completely elucidated.

Because we previously demonstrated that a number of virulence factors are transcriptionally modulated by ETEC pathogen-host cell interaction (33) we also examined transcription of both ST toxin genes and *tolC*, which encodes the putative export channel for ST. Interestingly, we found that in contrast to *estH*, *estP* transcription was significantly influenced by bacterial cell contact, with transcription of the gene encoding STp enhanced by bacterial adhesion, as was the *tolC* gene (Fig. 1H). These data suggest that similar to LT, the secretion and delivery of one or both of the STh and STp heat-stable toxins are likely highly orchestrated by contact with the intestinal epithelia.

In addition to STh and STp, earlier studies demonstrated that ETEC strains, including H10407 (31), also encode EAST, a heat-stable toxin originally identified in enteroaggregative *E. coli* (29, 31). Although the structures of the mature EAST and STh peptides are similar, there is only 37% identity (7/19 amino acids) between the two molecules (29), a finding that could impact the rational design of toxoids if EAST contributes to ETEC diarrheal illness. However, induction of cGMP in monolayers infected with a strain carrying an isogenic mutation of the *astA* gene, encoding EAST on the large p948 virulence plasmid of H10407 (3.8 ± 0.42 pmol/ml), was not significantly different from that of the wild-type parent (4.0 ± 0.35 pmol/ml), while deletion of both *estP* and *estH* resulted in complete abrogation of cGMP production (0.52 ± 0.06 pmol/ml), findings that suggest that EAST may not contribute significantly to diarrheal illness caused by this particular ETEC isolate.

ToIC is required for effective ETEC secretion of STh and STp. The precise mechanism for secretion of heat-stable toxins from ETEC strains associated with disease in humans is presently uncertain. Prior studies of heat-stable toxin investigated the secretion of STb (ST-II) (34) or STp (STIa) (35) from laboratory strains of *E. coli* containing recombinant expression plasmids. While both studies suggested that the outer membrane protein TolC is involved in secreting these toxins from the recombinant *E. coli* background, there are conflicting data regarding the involvement of the STb (STII) toxin in human disease (36, 37), and unlike the STh and STp toxins, STb does not bind to guanylate cyclase C. Similarly, to our knowledge, there has been no verification of the role of *tolC* in mediating the secretion of either of the ST1 toxins (STh and STp) from strains of ETEC isolated from humans. Therefore, to verify the importance of TolC in secretion of both STh and STp from ETEC strains that cause human illness, we constructed an isogenic *tolC* mutant in the ETEC H10407 strain and tested the ability of the mutant bacteria to deliver ST to target epithelial cells.

We found that mutants lacking *tolC* were devoid of detectable toxin in culture supernatants (Fig. 2A) and were markedly deficient in their ability to deliver ST toxins to epithelial monolayers, as we observed only background levels of cGMP production following infection with the *tolC* mutant strain, and complementation with *tolC* in *trans* restored the ability of the bacteria to provoke a cGMP response in targeted cells (Fig. 2B).

Optimal delivery of ST requires the EtpA adhesin. We have previously demonstrated that intimate interaction of ETEC with intestinal epithelial cells is essential for efficient delivery of heat-labile toxin to intestinal epithelial cells (38). Moreover, delivery of LT requires the concerted action of several ETEC adhesins with different receptor specificities (39, 40, 52) including EtpA, a plasmid-encoded secreted adhesin (32) that serves as a molecular bridge between bacteria and intestinal epithelial cells (42). EtpA is expressed by a diverse population of ETEC isolates (43), is recognized during the course of infection (44), and is a protective antigen in preclinical models of ETEC infection (40, 45, 46).

Because we are exploring the role of EtpA as a protective immunogen (47) and ST-producing ETEC strains have predominated in recent epidemiologic studies (1, 48), we set out to examine the dependence of ST delivery on EtpA-mediated bacterial

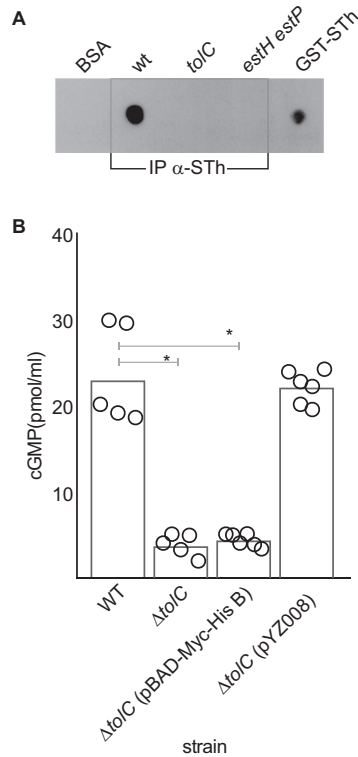


FIG 2 Role of *toIC* in ST secretion and delivery to target epithelial cells. (A) Immunoblot detection of ST toxins in culture supernatants of the wild-type H10407 strain, *toIC* mutant, or *estH estP* dual deletion mutant following immunoprecipitation of culture supernatants with affinity-purified anti-STh antibodies (IP α -STh). BSA and GST-STh fusion are shown as negative and positive controls, respectively. (B) cGMP production by T84 target epithelial cells following infection with wild-type (wt) ETEC strain H10407 (STh, STp), the *toIC* mutant, the vector-complemented mutant (pBAD-Myc-His B) or the *toIC*-complemented mutant (pZY008). Data shown represent the combined results of two experimental replicates. Individual data points represent technical replicates, and bars indicate the geometric mean values, obtained in the presence (+) of phosphodiesterase inhibitors (PDE-I). Statistical comparisons by ANOVA (Kruskal-Wallis): *, $P < 0.05$.

adhesion by comparing cGMP activation of target intestinal epithelial cells infected with wild-type ETEC to that in cells infected with an isogenic *etpA* mutant. These studies demonstrated that cGMP activation in target epithelia by wild-type bacteria was significantly accelerated relative to that seen with the *etpA* mutant (Fig. 3), suggesting that efficient delivery of these small peptides also requires effective bacterium-host interactions.

Similarly, we found that antibodies directed against either the EtpA adhesin molecule or STh significantly inhibited the delivery of heat-stable toxins to target cells (Fig. 4). Although we were not able to demonstrate that the combination of these antibodies was synergistic, these data provide additional support to the concept that EtpA could be useful as a target to engender coverage against a wide variety of ETEC isolates that produce heat-stable and/or heat-labile enterotoxins (43, 49).

Delivery and secretion of epitope-tagged STh. Understanding the nature of ST secretion and its delivery to epithelial receptors could be relevant to an informed development of ST toxoid molecules. STh is synthesized as a 72-amino-acid molecule that includes a 19-amino-acid signal peptide, followed by a 34-amino-acid propeptide and a mature ST molecule of 19 amino acids. Although most attempts to develop ST toxoids have targeted the mature peptides (16, 25), there are earlier but conflicting data regarding the precise form of ST that is secreted into the extracellular milieu (15, 50), with some data suggesting that the propeptide may be exported with subsequent processing to the mature peptide outside the bacteria (51). To investigate the potential secretion of the propeptide, we engineered 3 \times FLAG-epitope fusions to the amino-

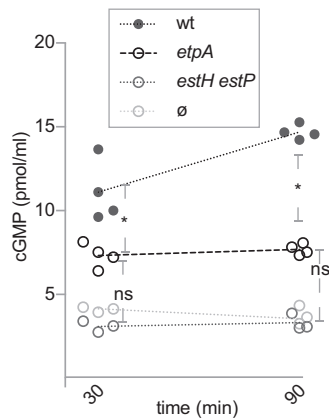


FIG 3 The EtpA adhesin is required for optimal delivery of heat-stable toxins. Shown are comparisons of cGMP activation in target T84 intestinal epithelial cells following infection with wild-type ETEC H10407 or the *etpA* mutant ($n = 4$ replicates), or the *estH estP* dual deletion mutant and uninfected monolayers ($n = 3$). Dashed lines in each group connect geometric mean values obtained at 30 and 60 min following the addition of bacteria. *, $P < 0.05$; obtained by Mann-Whitney (two-tailed) comparisons.

terminal end of the propeptide region (FLAG³-pro-STh) of STh and compared the export and delivery of the resulting peptides to those of carboxy-terminal fusions to the mature peptide (pro-STh-FLAG³) (Fig. 5).

We were able to recover either the amino-terminal or carboxy-terminal FLAG-tagged molecules from culture supernatants of *estH estP* mutant ETEC transformed with the pSTh-FLAG³ or pFLAG³-STh plasmids, respectively (Fig. 5a). Both molecules appeared to yield functional ST mature peptides, as either plasmid was sufficient to complement the ability of the *estH estP* mutant to activate cGMP in target epithelial cells (Fig. 5b). Likewise, we were able to demonstrate binding of FLAG-epitope-tagged molecules to target epithelial cells following infection with either of the complemented strains (Fig. 5c and d). Collectively, these data appear to reaffirm earlier observations of STh (51), suggesting that both the mature form (19 amino acids) of the peptide and the extended pro-STh (53 amino acids) may be found outside the bacteria.

DISCUSSION

Because of the significant global burden of diarrheal illness caused by ETEC, these pathogens have been a target for vaccine development since they were first identified

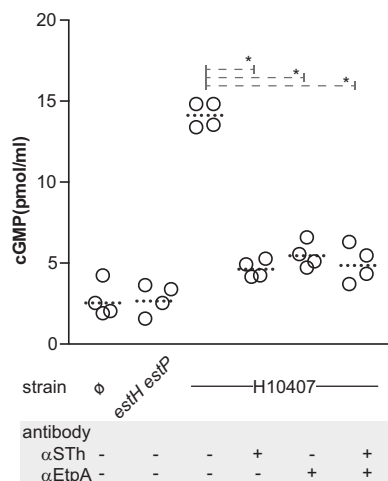


FIG 4 Antitoxin and antiadhesin antibodies inhibit heat-stable toxin delivery. Shown are monolayers infected with the dual heat-stable toxin mutant (*estH estP*), wild-type H10407, or uninfected control monolayers (ø). Dotted horizontal lines for each group represent geometric means. *, $P = 0.028$ by Mann Whitney two-tailed nonparametric comparisons. Both anti-STh and anti-EtpA antibodies were affinity purified and cross adsorbed and used at final dilution of 1:25.

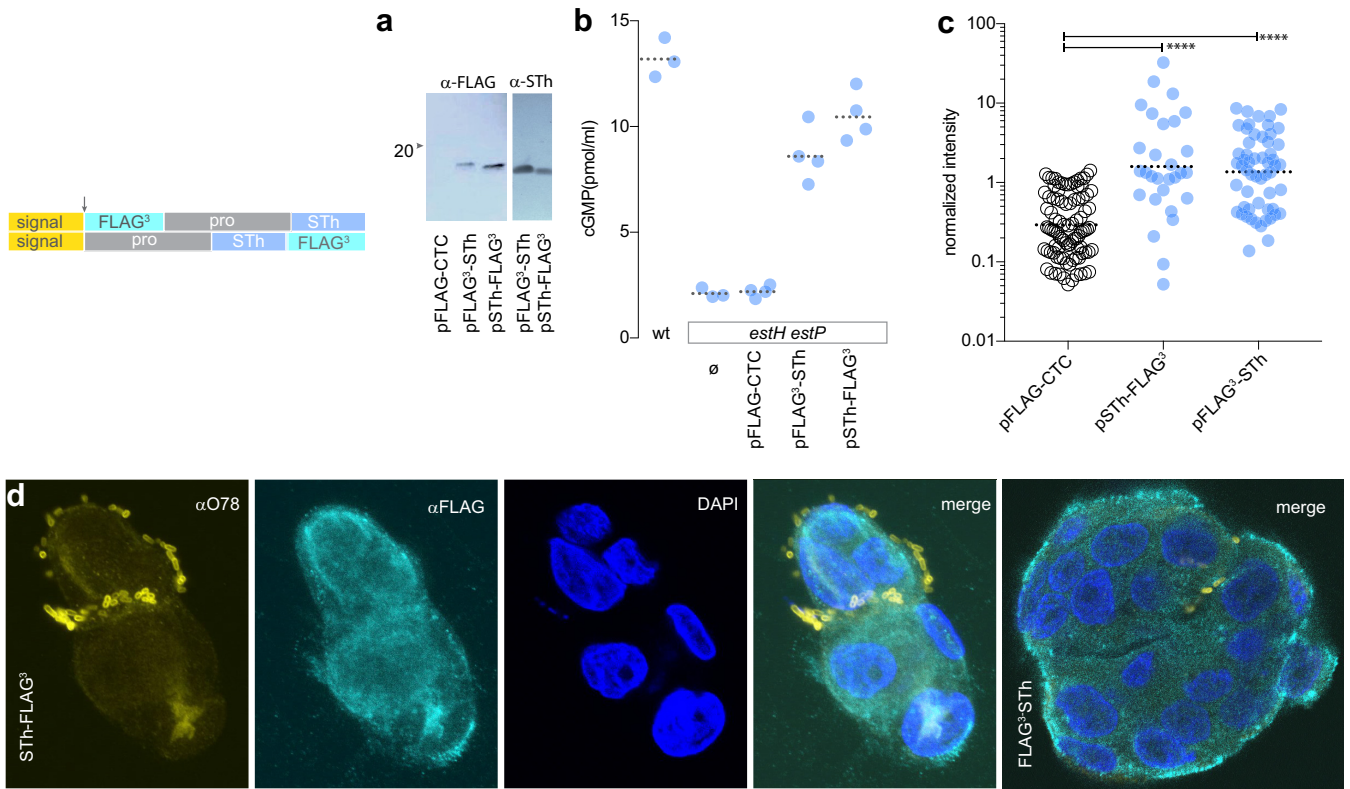


FIG 5 Secretion and delivery of FLAG-epitope-tagged STh. The schematic (top left) depicts the location of the region encoding the 3×FLAG peptide relative to the *estH* gene. The arrow shows the location of the predicted signal peptide cleavage site which is retained in both constructs. (a) Anti-FLAG and anti-STh immunoblots of FLAG³-STh and STh-FLAG³ peptides recovered by anti-FLAG immunoprecipitation from culture supernatants of the ETEC *estH estP* mutant bearing pFLAG³-STh and pSTh-FLAG³. (b) Complementation of the *estP estH* mutant strain with pSTh-FLAG³ or pFLAG³-STh restores toxicity upon infection of T84 cells. (c) Quantitation of FLAG³-tagged ST delivered to epithelial cells by *estH estP* bacteria complemented with pSTh-FLAG³ or pFLAG³-STh. Values represent fluorescence intensity per field. ****, $P < 0.0001$ by ANOVA (Kruskal-Wallis test). (d) Confocal immunofluorescence images of bacteria expressing pST-FLAG³ (anti-O78, yellow) attached to T84 cells (nuclei, blue) and the distribution of FLAG epitope-tagged toxin (cyan).

as a cause of severe diarrheal illness more than 4 decades ago. Currently, there is no vaccine available that affords broad-based protection against ETEC, in part due to the substantial antigenic diversity within the pathovar and limited mechanistic insight into immunologic correlates of the protection that appears to follow early infections among young children in areas of endemicity. Moreover, many features of the molecular pathogenesis of these common pathogens have not been explored in sufficient detail to inform vaccine development.

Because ST-producing ETEC isolates comprise a large proportion of strains associated with symptomatic diarrheal illness (1), we investigated the molecular contributions of the known heat-stable toxins, their proposed secretion apparatus, and bacterial adhesion to toxin delivery. The present studies suggest that efficient delivery of ST toxins is a complex process that requires the ability to effectively engage host cells and produce at least transient adhesion afforded by EtpA and other adhesins (52). Moreover, as antibodies directed at either the EtpA adhesin or ST impaired toxin delivery, our studies provide further support for development of a vaccine platform that combines ST toxoid (25) and antiadhesin approaches. However, further studies will be needed to define an optimal vaccination approach capable of generating sufficient antibodies to neutralize binding of the small ST peptides to cognate receptors in the small intestine.

Our data provide an important validation of the potential biologic importance of both STh and STp toxins. While recent large-scale epidemiologic data have highlighted the importance of ETEC strains producing STh as a cause of moderate to severe diarrhea in young children (1), subsequent reevaluation of these data has suggested that STp-producing strains also contribute significantly to the overall burden of illness

associated with ETEC (48). Indeed, earlier clinical studies demonstrated that while there were regional differences in the distribution of STp and STh among ETEC isolates, both peptides were commonly associated with diarrheal illness, and the clinical presentations attributed to ETEC producing either ST peptide were indistinguishable (24). In keeping with these observations, we found no difference in the ability of STh or STp peptides to elicit cGMP production (19) in target epithelia. Although deletion of the gene encoding STh resulted in lower cGMP production in infected monolayers than when the *estP* gene was deleted from the ETEC H10407 strain, we discovered a compensatory increase in transcription of *estH* in the *estP* mutant. Collectively, therefore, our results suggest that both peptides likely contribute to diarrheal illness caused by H10407. Indeed, deletion of the *tolC* gene, which encodes the membrane transporter for both peptides, resulted in complete abrogation of cGMP activation in target epithelial cells, similar to deletion of both the *estH* and *estP* toxin genes from H10407.

Earlier studies also noted that many ETEC strains, including H10407, bear copies of an insertion sequence (31) that encompasses the *astA* gene encoding EAST1 (30), a cGMP-activating peptide structurally similar to ST1 that was originally identified as a heat-stable enterotoxin in enteroaggregative *E. coli* (29). However, we saw similar increases in cGMP production by target epithelial cells following infection with the *astA* mutant and wild-type ETEC. We cannot rule out the possibility of EAST1 expression from additional copies of *astA* not residing on the large, p948 virulence plasmid or that EAST1 is not optimally expressed *in vitro*. However, the complete absence of a cGMP response in cells infected with the STh/STp deletion mutant might alternatively suggest that EAST1 does not contribute to ETEC virulence and that further toxoid vaccine development can simply focus on engendering neutralizing antibody responses to the established ST and LT enterotoxins.

The reaffirmation of TolC as a key mechanism for export of ST1 toxins could be relevant to the management of diarrheal illness. The rapid emergence of multidrug resistance in the *Enterobacteriaceae* that is in part dependent on drug efflux through TolC has stimulated interest in efflux inhibitors to enhance the potency of available antimicrobial agents (53–55). Theoretically, these inhibitors could offer novel therapeutic agents for treatment of ETEC.

Our studies also revisit the concept that the larger propeptide form of STh may be exported by the bacteria, and the data presented here are consistent with prior observations suggesting that some processing of the propeptide occurs outside the bacteria (15, 51). Further study will be needed, however, to determine whether the propeptide sequence contributes to immunologic recognition of ST following infection and whether these larger molecules might be exploited in the development of improved toxoids to neutralize ST.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used are listed in Table 1. For general purposes, ETEC bacteria were grown in lysogeny broth at 37°C overnight from frozen glycerol stocks. To optimize synthesis and secretion of ST toxin, bacteria were grown in Casamino Acids-yeast extract-sucrose medium (CAYE-ST) (2% Casamino Acids, 0.6% yeast extract, 43 mM NaCl, 38 mM K₂HPO₄, 203 mM MgSO₄, 25 mM MnCl₂, 18 mM FeCl₃, 2% sucrose) at 37°C overnight from frozen glycerol stocks.

Construction of mutants lacking production and secretion of heat-stable toxins. Lambda red recombinase-mediated recombination (56) was used to disrupt genes encoding heat-stable toxins in H10407. In brief, we used the primers jf070212.1 and jf070212.2 to amplify the kanamycin (Km) cassette from pKD13 with a 36-bp overhang sequence from the *estH* gene and primers jf070212.3 and jf070212.4 to amplify the kanamycin cassette from pKD13 with a 36-bp overhang sequence from the *estP* gene. The PCR product was electroporated into competent H10407 containing helper plasmid pKD46 and selected on LB plates with 50 μg/ml kanamycin. Kanamycin-resistant (Km^r) colonies were screened by PCR with primers jf071612.1 and jf071612.2 for the *estH* locus and with primers jf071612.3 and k2 for *estP*. The deletion mutants were further confirmed by toxin multiplex PCR as previously described (57).

To construct complemented strains, we amplified the *estP* gene with primers jf030316.3 and jf060614.1 for cloning into the XhoI site of pBAD/mycHisA, yielding pQL239; *estH* was amplified with primers jf072414.5 and jf072414.6 and cloned into the EcoRV site of pACYC184 to produce pSMD001. We also cloned *estH* and *estP* genes individually and together in pETDuet-1 at NcoI and EcoRI sites and at BglII and XhoI sites with primer pairs jf060614.2/jf060614.3 and jf060614.4/jf060614.5, respectively. This resulted in plasmids pQL230 (*estP*), pQL231(*estH*), and pQL238 (*estP estH*) (Table 1). After confirmation by

TABLE 1 Bacterial strains and plasmids used in this study^d

Strain or plasmid	Description	Source or reference(s)
Strains		
H10407	Wild-type ETEC strain O78:H11; CFA/1; LT, STh, STp; EtpA	28, 32, 64
jf2656	H10407 derivative with isogenic deletion of <i>estP</i>	This study
jf2649	H10407 derivative with isogenic deletion of <i>estH</i>	This study
jf2847	H10407 derivative with isogenic deletion of <i>estP</i> and <i>estH</i>	This study
jf3038	Deletion of <i>astA</i> on the large p948 virulence plasmid of H10407; Km ^r	This study
jf3076	jf2656 complemented with pQL230	This study
jf3081	jf2649 complemented with pQL231	This study
JW5503-1	<i>E. coli</i> K-12 in-frame Δ <i>tolC732::Km^r</i>	58
jf4652	H010407 derivative with <i>tolC::Km^r</i> mutation	This study
jf4709	jf4652 with pBAD/mycHisB	This study
jf4712	jf4652 complemented with pYZ008	This study
jf4644	jf2847 with pFLAG-CTC, Cm ^r Km ^r Amp ^r	This study
jf4648	jf2847 with pFLAG-STh, Cm ^r Km ^r Amp ^r	This study
jf4651	jf2847 with pSTh-FLAG, Cm ^r Km ^r Amp ^r	This study
jf3626	jf2847 with pQL239, Cm ^r Km ^r Amp ^r	This study
AAEC191A	Afimbriate <i>E. coli</i> K-12 derivative	61
Plasmids		
pBAD/mycHisA	pBR322ori; PBAD; Amp ^r ; <i>araC</i> ; C-terminal <i>myc</i> epitope tag; arabinose-inducible expression plasmid	Invitrogen
pBAD/mycHisB	pBR322ori; PBAD; Amp ^r ; <i>araC</i> ; C-terminal <i>myc</i> epitope tag; arabinose-inducible expression plasmid	Invitrogen
pKD13	oriRSKγ Tn5 neomycin phosphotransferase (Km ^r), FRT, β-lactamase (Amp ^r)	56
pKD46	Lambda red recombinase helper plasmid	56
pQL239	<i>estP</i> cloned into XhoI site of pBAD/myc-HisA in frame with myc-His tags	This study
pACYC184	p15Aori, Cm ^r Tc ^r	65
pSMD001	<i>estH</i> cloned into EcoRV site of pACYC184	This study
pGPS4	oriRSKγ Cm ^r Tc ^r	NEB
pETDuet-1	pBR322ori; <i>lacI</i> ; Amp ^r ; IPTG-inducible expression plasmid	Novagen
pQL230	<i>estP</i> cloned into BglII and XhoI sites of pETDeut-1	This study
pQL231	<i>estH</i> cloned into NcoI and EcoRI sites of pETDeut-1	This study
pQL238	<i>estP</i> cloned into BglII and XhoI sites and <i>estH</i> cloned into NcoI and EcoRI sites of pETDeut-1	This study
pGEX-4T1	<i>lacI^a</i> , Amp ^r ; expression plasmid for N-terminal GST fusions	GE Healthcare Life Sciences
pCW002 ^a	5,047-bp GST-STh expression plasmid, Amp ^r	This study
pCW003 ^b	<i>estP</i> cloned into BamHI and EcoRI sites of pGEX	This study
pYZ011 ^c	5,047-bp GST-mSTh expression plasmid for mSTh (A14Q), Amp ^r	This study, 25
pYZ008	H10407 <i>tolC</i> cloned into pBAD/mycHisB	This study
pFLAG-CTC	5.3-kb plasmid, expressing C-terminal FLAG fusion proteins, Amp ^r	Sigma
pFLAG ³ -STh	5,609-bp Flag-pro-STh expression plasmid, Amp ^r	This study
pSTh-FLAG ³	5,612-bp pro-STh-Flag expression plasmid, Amp ^r	This study

^aAddgene accession number 90225 (<https://www.addgene.org/90225/>).

^bAddgene accession number 90226 (<https://www.addgene.org/90226/>).

^cAddgene accession number 110570 (<https://www.addgene.org/110570/>).

^dAbbreviations: Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; NEB, New England Biolabs; FRT, FLP recombination target.

sequencing, the respective plasmids were transformed into the deletion strains, and complementation was confirmed by PCR.

To generate an ETEC *tolC* deletion mutant, primers jf110716.44/45 were first used to amplify a Δ*tolC732::Km^r* fragment from JQ55031 (58). Next, the regions flanking *tolC* in the H10407 genome were amplified with primer pairs jf110716.38/jf110716.43 and jf110716.46/jf110716.39 to amplify 1,027 bp of upstream sequence and 985 bp of downstream sequence, respectively. The three resulting fragments were then fused by PCR with primer pairs jf110716.38/jf110716.39, using high-fidelity polymerase Phusion (Thermo Fisher Scientific), with denaturing for 2 min at 98°C, followed by 30 cycles of 10 s at 98°C, 30 s at 65°C, and 1.5 min at 72°C and a final extension for 10 min at 72°C. The resulting 3,325-bp amplicon was then introduced into H10407(pKD46) as described above. Kanamycin-resistant, ampicillin-sensitive colonies were then screened by colony PCR using primer pair jf112016.50/51 flanking the entire amplicon for a 4,269-bp product and primers jf101716.21/22 specific to *tolC* gene (603-bp product). Primers k2/jf112016.51 (2,024-bp product) were then used to confirm the *tolC* gene deletion and the Km^r cassette integration in the H10407 genome. To complement the *tolC* mutant, the *tolC* gene was amplified from H10407 genomic DNA using primers jf120716.59/jf120716.60, and the plasmid vector backbone was amplified from plasmid pBAD/myc-His B using primers jf120716.52/jf120716.53. The recombinant pYZ008 complementation plasmid was assembled using an adaptation of circular polymerase extension cloning (CPEC) (59) (30 s of denaturation at 98°C, followed by 20 cycles of 10 s at 98°C, 30 s at 55°C, and 1.5 min at 72°C and a final extension for 10 min at 72°C). Following sequence verification, pYZ008 or the

pBAD/myc-His B vector control plasmids were then used to transform the $\Delta tolC$ mutant. Complementation was confirmed by PCR with primers jf101716.21/22.

FLAG-STh fusions. FLAG epitope fusions were constructed to introduce the 3 \times FLAG sequence between the signal peptide of *estH* and the beginning of the propeptide-encoding region (on plasmid pFLAG³-STh) or at the 3' end of *estH* (on pSTh-FLAG³). The 3 \times FLAG fragment was first constructed by annealing complementary synthetic oligonucleotides jf092616.9 encoding positive-strand bases 1 to 43 and jf092616.10 representing negative-strand bases 66 to 19 of a 66-bp sequence encoding the 3 \times FLAG peptide (DYKDHGDYKDHIDYKDDDDK).

To generate plasmid pFLAG³-STh, in which the 3 \times FLAG-encoding sequence was inserted between the STh signal peptide and the STh propeptide, primers jf101916.33/jf092616.4 were first used to amplify the nucleotides (1 to 63) of *estH* encoding the signal peptide. Next, primers jf092616.1/jf092616.2 were used to amplify the 3 \times FLAG fragment from the above-described synthetic oligonucleotide 3 \times FLAG template flanked by nucleotide extensions representing nucleotides 44 to 60 and 64 to 99 of *estH*, while primers jf092616.3/jf101916.34 were used to amplify the 3' end of *estH* from nucleotide 64 to the native stop codon. The three fragments were fused in a single PCR using primers jf101916.33/jf101916.34. Next, the vector backbone was amplified using primers jf101916.31/jf101916.32 from pFLAG-CTC (Sigma), followed by the final assembly of pFLAG³-STh by CPEC. Similarly, to make plasmid pSTh-FLAG³, primers jf092616.5/jf101916.35 were used to amplify 3 \times FLAG with a 5' nucleotide extension representing nucleotides 197 to 216 of *estH*, while primers jf101916.33/jf092616.8 were used to amplify the *estH* gene with a 5' nucleotide extension corresponding to pFLAG-CTC. The resulting amplicons were then fused in a single PCR using primers jf101916.33/jf101916.35 and assembled with the pFLAG-CTC backbone by CPEC.

Cloning, expression, and purification of recombinant ST proteins peptides. To construct a GST-STh or mutant GST-mSTh (A14Q) expression plasmid, synthetic oligonucleotides that encompassed the region of the *estH* gene corresponding to the mature peptide minus the native start codon, preceded by an in-frame flexible linker sequence (60), were synthesized (IDT, Coralville, IA). The forward sequence (jf042715.1 for STh and jf042517.1 for mSTh), preceded by a BamHI overhang sequence (GATCC), and the reverse sequence (jf042715.2 for STh and jf042517.2 for mSTh), preceded by an EcoRI overhang sequence (AATTC), were mixed in a 1:1 molar ratio, heated to 95°C for 5 min, and cooled to room temperature. The annealed double-stranded 91-bp DNA fragments were then cloned directly into the pGEX-4T1 vector digested with BamHI and EcoRI, yielding plasmids pCW002 and pYZ011, respectively. A similar strategy was to construct a GST-STp expression plasmid using forward oligonucleotide jf031915.5 and the reverse sequence jf031915.6, resulting in plasmid pCW003. *E. coli* TOP10(pCW002), TOP10(pCW003), and TOP10(pYZ011) were then used to express recombinant GST-STh, GST-STp, and GST-mSTh, and the resulting fusion proteins were purified by affinity chromatography. In brief, the bacterial cultures were grown in Luria broth at 37°C to an optical density at 600 nm (OD₆₀₀) between 0.6 and 0.7 and induced with 1 mM (final concentration) IPTG (isopropyl- β -D-thiogalactopyranoside) for 1 to 3 h. The cell pellets were resuspended in 30 ml cold phosphate-buffered saline (PBS) containing 5 mM dithiothreitol (DTT), 1 protease inhibitor tablet (Roche), and 0.1 mg/ml lysozyme. Following sonication, supernatants were clarified by centrifugation at 12,000 rpm for 20 min at 4°C, followed by passage through a 0.45- μ m filter. Filtered supernatants were then loaded onto columns (glutathione Sepharose High Performance; GE Healthcare) preequilibrated with PBS, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.3]). After washing with 20 column volumes of PBS containing 1 mM DTT, GST fusion proteins were eluted in fresh buffer containing 100 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione and dialyzed against PBS.

To liberate native STh or mutant STh (mSTh) from its GST fusion partner, GST-fusion protein was dialyzed in 50 mM NH₄HCO₃ (pH 8.5) containing 1 unit of thrombin (Sigma) per mg GST-fusion protein at 4°C overnight, centrifuged at 4,000 \times g for 20 min with a 10-kDa-molecular-weight cutoff (MWCO) centrifugal filter to collect the filtrate, and dried by vacuum centrifugation. Peptide concentrations were then determined by measurement of the molar extinction coefficient at 280 nm (Take3; BioTek).

Transcriptional analysis of *estH*, *estP*, and *tolC* genes. To assess transcription of toxin genes in mutant strains relative to wild-type H10407, the bacteria were grown overnight in 2-ml cultures of lysogeny broth at 37°C and 225 rpm and then diluted 1:100 into fresh medium and grown for an additional 3 h. To assess modulation of transcription upon cell contact, confluent T84 cells were infected with early-log-phase H10407 bacteria ($\sim 10^9$ CFU) for 30 or 90 min. Total RNA was isolated from adherent and planktonic (nonadherent) bacterial fractions using an RNAspin Mini isolation kit (25050070; GE Life Sciences) and treated with DNase I (18068015; Thermo Fisher). PCR for the *arcA* housekeeping gene was used to confirm the removal of DNA. Total RNA was reverse transcribed (SuperScript VILO cDNA synthesis kit, 11754250; Thermo Fisher). RNA transcripts were quantified by real-time (RT) PCR (Fast SYBR green master mix, number 4385612 from Thermo Fisher; ViiA 7 real-time PCR system from Applied Biosystems). Primers specific to the *arcA*, *estH*, *estP*, and *tolC* genes are listed in Table 2. All transcripts were normalized to *arcA* and are presented as a ratio of transcripts in adherent bacteria relative to transcripts in planktonic bacteria.

Production and purification of anti-ST polyclonal antibody. To generate rabbit polyclonal antibody that recognizes STh, New Zealand White rabbits were immunized (Rockland) with recombinant GST-STh. We used lyophilized *E. coli* AAEC191A (61) and an immobilized *E. coli* lysate column (44938; Pierce) to adsorb *E. coli*-reactive antibodies, followed by protein G column purification (HiTrap, GE17-0404-01). IgG GST-STh antibodies were affinity purified using GST-STh immobilized on nitrocellulose as previously described, and anti-GST-reactive antibodies were removed by cross-adsorption against GST coupled to glutathione agarose resin (G-250-100; Gold Biotechnology, St. Louis, MO).

TABLE 2 Primers used in this study

Primer	Sequence (5'–3')	Description
jf070212.1	ATGAAAAAATCAATATTATTTATTTTCTTCTGTAGTGTAGGCTGGAGCTGCTTCG	Forward primer for amplifying <i>estH</i> flanking from pKD13
jf070212.2	TTAATAGCACCCGGTACAAGCAGGATTACAACACAATTCCGGGGATCCGTCGACC	Reverse primer for amplifying <i>estH</i> flanking from pKD13
jf070212.3	ATGAAAAAGCTAATGTTGGCAATTTTATTTCTGTAGTGTAGGCTGGAGCTGCTTC	Forward primer for amplifying <i>estP</i> flanking from pKD13
jf070212.4	TTAATAACATCCAGCACAGGCAGGATTACAACAAAGATTCCGGGGATCCGTCGACC	Reverse primer for amplifying <i>estP</i> flanking from pKD13
jf071612.1	GGCGCACACAAATATAAAG	368-bp amplicon in H10407- <i>estH</i> downstream primer
jf071612.2	AGCGGAGAGTATAGTATGA	<i>estH</i> upstream
jf071612.3	AAAACCAGATAGCCAGAC	168 bp upstream from <i>estP</i> on H10407 plasmid p666
k2	CGG TGC CCT GAA TGA ACT GC	Primer binding Km ^r cassette for confirming <i>estP</i> and <i>toIC</i> deletion
jf072312.1	ATGAAAAAATCAATATTATTTATTTTCTTCTGTACCCTGTTATCCCTAGATT	Forward primer for amplifying <i>estH</i> from pGPS4
jf072312.2	TTAATAGCACCCGGTACAAGCAGGATTACAACACAATAACGGTCTAAGGTAGC	Reverse primer for amplifying <i>estH</i> from pGPS4
jf072412.5	GGCCTCTTGCGGGATATCTAAATGAAAAAATCAATATTA	pACYC184_ECORV_TAA_STh forward primer for in-fusion cloning
jf072412.6	GTCGGAATGGACGATATCTTAATAGCACCCGGTACA	pACYC184_ECORV_TAA_STh reverse primer for in-fusion cloning
jf060614.2	AGGAGATATACCATGGATGAAAAAATCAATATTA	Forward primer for STh in NcoI and EcoRI sites on pETDuet-1 vector
jf060614.3	CGCCGAGCTCGAATCTTAATAGCACCCGGTACA	Reverse primer for STh in NcoI and EcoRI sites on pETDuet-1 vector
jf060614.4	TACATATGGCAGATCTATGAAAAAGCTAATGTTG	Forward primer for STp in BglII and XhoI sites on pETDuet-1 vector
jf060614.5	CTTTACCAGACTCGAGTTAATAACATCCAGCACA	Reverse primer for STp in BglII and XhoI sites on pETDuet-1 vector
jf042715.1	GATCCGATCCCCGGGTACCGAGCTCGAATAGTAGCAATTACTGCTGTGAATTGTG TTGTAATCCTGCTTGTACCGGGTCTATTGAG	5'-Mature <i>estH</i> gene with BamHI, linker, gene, stop codon
jf042715.2	AATTCTCAATAGCACCCGGTACAAGCAGGATTACAACACAATTCACAGCAGTAA TTGCTACTATTCGAGCTCGGTACCCGGGGATCG	3'-Mature <i>estH</i> gene with linker, gene, stop codon, EcoRI
jf042517.1	GATCCGATCCCCGGGTACCGAGCTCGAATAGTAGCAATTACTGCTGTGAATTGTG TTGTAATCCTcaatGTACCGGGTCTATTGAG	5'-Mutant <i>estH</i> gene with BamHI, linker, gene, stop codon
jf042517.2	AATTCTCAATAGCACCCGGTACAAttgAGGATTACAACACAATTCACAGCAGTAA TTGCTACTATTCGAGCTCGGTACCCGGGGATCG	3'-Mutant <i>estH</i> gene with linker, gene, stop codon, EcoRI
jf031915.5	GATCCGATCCCCGGGTACCGAGCTCGAACACATTTACTGCTGTGAATCTTGT GTAATCCTGCCTGTGCTGGATGTTATTGAG	5'-Mature <i>estP</i> gene with BamHI, linker, gene
jf031915.6	AATTCTCAATAACATCCAGCACAGGCAGGATTACAACAAAGTTCACAGCAGTAA AATGTGTTTCGAGCTCGGTACCCGGGGATCG	3'-Mature <i>estP</i> gene with linker, gene, EcoRI
jf021915.1	CGCTTACAGACAAGCTGTG	Reverse sequencing primer that binds 70 bp downstream of the pGEX cloning site
jf021915.2	CCAGCAAGTATATAGCATGG	Forward sequencing primer that binds 117 bp upstream of pGEX cloning site
jf110716.38	CGGGCGCAGTCTGTTCTATTG	Forward primer for amplifying 1,027-bp left-flank segment of H10407 <i>toIC</i>
jf110716.43	TCATTTGCATTCCTTGTGGTGAAGCAGATTTAGCGC	Reverse primer for amplifying 1,027-bp left-flank segment of H10407 <i>toIC</i>
jf110716.44	AAAGGGTTATGTGTAGGCTGGAGCTGCTTCG	Reverse primer for amplifying Km ^r cassette from strain JQ5503-1
jf110716.45	CTTACCACAAGGAATGCAATGATTCCGGGGATCC	Forward primer for amplifying Km ^r cassette from strain JQ5503-1
jf110716.39	CTTTCAACCTGGGCGAGGG	Reverse primer for amplifying 985-bp right-flank segment of H10407 <i>toIC</i>
jf110716.46	CCTACACATAACCTTTCCGTAAGTATGACGACGACGGGGCTTCGG	Forward primer for amplifying 985-bp right-flank segment of H10407 <i>toIC</i>
jf101716.21	CGATCGTGATGCTGCCTTTG	603-bp amplicon in H10407- <i>toIC</i> forward primer
jf101716.22	AGCGACAGGTTGCGTTTTTC	603-bp amplicon in H10407- <i>toIC</i> downstream primer

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5'–3') ^a	Description
jf112016.50	ATTGCCATTGCTCACCAATAAAC	Forward primer binding 2 kb upstream of H10407 <i>tolC</i>
jf112016.51	CTTGCACTGTTAAACTGGTCG	Reverse primer binding 2 kb downstream of H10407 <i>tolC</i>
jf120716.52	GAACAAAACATCATCTCAGAAGAGGATCTGAATAGCG	Forward primer to amplify 4,036 bp of pBAD/myc-His B
jf120716.53	GGTAATTCCTCTGTAGCCCAAAAAACGG	Reverse primer to amplify 4,036 bp of pBAD/myc-His B
jf120716.59	GGTAACAGGAGGAATTAACCATGCAAAATGAAGAAATTGCTCCCCATTCT	Forward primer H10407 <i>tolC</i> gene
jf120716.60	CTCTCTGAGATGAGTTTTTGTCTCAGTTACGGAAAGGGTATGACCGTTACT	Reverse primer H10407 <i>tolC</i> gene
jf092616.1	TTTCACCTTTCGCTCAGGATTACAAAGACCAC	Forward bp 44–60 of <i>estH</i> -5'3×FLAG
jf092616.2	GAAGACCCTGCTGGTTAGCCTTGTCATCGTC	Reverse bp 83–64 of <i>estH</i> -3'3×FLAG
jf092616.3	GCTAAACCAGCAGGGTCTTCAAAAAGAAAAATTACA	Forward primer bp 64–99 of <i>estH</i>
jf092616.4	ATCCTGAGCGAAAGGTGAAAAAGATAATACAGAAAGA	Reverse primer bp 63–27 of <i>estH</i>
jf092616.5	CTGCTTGATACCGGGTGCTATGATTACAAAGAC	Forward primer bp 197–216 of <i>estH</i> -5'3×FLAG
jf092616.8	ATAGCACCCGGTACAAGCAGGATTACA	Reverse primer representing bp 216–190 of <i>estH</i>
jf092616.9	GATTACAAAGACCACGATGGTGACTATAAAGACCATGATATCG	Base pairs 1–43 and 66 through 19 of the 3×FLAG-encoding sequence
jf092616.10	CTTGTCATCGTCGCTTTTATAATCGATATCATGGCTTTTATAGTCACC	For amplifying pFLAG-CTC vector backbone
jf101916.31	CAGATCTGGTACCCGGGAATTCT	
jf101916.32	TGAAGATCGATCTCTCGATCGAGTGA	
jf101916.33	ATCCCGGGTACCAGATCTGATGAAAAATCAATATTATTTTCTTCTGTATT	Forward primer beginning with pflag-CTC-sequence followed by <i>estH</i> sequence bp 1–38
jf101916.34	GATCGAGATCGATCTTCATTAATAGCACCCGGTACAAGCAGG	Reverse primer beginning with pflag-CTC sequence followed by 3' <i>estH</i> native reverse sequence (bp 219–196)
jf101916.35	GATCGAGATCGATCTTCATTAATAGCACCCGGTACAAGCAGG	Reverse primer beginning with pflag-CTC-sequence followed by bases 69–34 of the 3×FLAG sequence
jf092313.5	TCTTCCCTCTTTAGTCAG	<i>estP</i> RT-PCR forward primer
jf092313.6	ACAGGCAGGATTACAACAAAG	<i>estP</i> RT-PCR reverse primer
jf092313.7	TACAAGCAGGATTACAACAC	<i>estH</i> RT-PCR forward primer
jf092313.8	AGTGTCTCTGAAAGCATG	<i>estH</i> RT-PCR reverse primer
jf092210.1	ATCAATCTGCCGGTAAGAACGGT	<i>arcA</i> RT-PCR forward primer
jf092210.2	TCCAGATCACCGCAGAAGCGATAA	<i>arcA</i> RT-PCR reverse primer
jf030316.3	TGGATGCGAGCTCGAGCATGAAAAAGCTAATGTTG	<i>estP</i> forward primer for pBAD/mycHisA-STp cloning
jf060614.1	AGCTGCAGATCTCGAGTTAATAACATCCAGCACA	<i>estP</i> reverse primer for pBAD/mycHisA-STp cloning

^aSee "Description" column for explanation of underlined bases.

Anti-STp antibody was purified from rabbit antisera (provided by Weiping Zhang, Kansas State University) by cross-adsorption against an immobilized *E. coli* lysate column, which was followed by affinity purification against recombinant GST-STp immobilized on nitrocellulose membranes for affinity purification.

Immunoprecipitation and detection of heat-stable toxins in culture supernatants. Purified anti-STh polyclonal IgG was immobilized with AminoLink Plus coupling resin (Pierce Direct IP kit, 26148; Thermo Scientific). Clarified culture supernatants of overnight cultures of ETEC were mixed with protease inhibitor cocktail (88666; Thermo Fisher) and filtered through a 0.45- μ m filter. Sixty milliliters of supernatant was then filtered through a 10-kDa-molecular-weight-cutoff membrane (Amicon). Filtrates were then dried by vacuum centrifugation and dialyzed in PBS against a 1-kDa-cutoff membrane (Float-A-Lyzer G2, MWCO 0.5 to 1 kDa; Spectrum Labs). Immunoprecipitations were conducted by incubation of filtrates with anti-STh-immobilized resin for 2 h at room temperature followed by elution with 4.5% acetic acid. Eluates were dried by vacuum centrifugation and reconstituted in PBS. Immunoprecipitated samples or purified protein/peptide as controls were applied directly to 0.22- μ m-pore-size polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and detected by anti-STh and/or anti-STp primary antibody and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody using Clarity Western ECL substrate (Bio-Rad).

FLAG-STh fusion peptides were prepared from strain jf2847 carrying pFLAG³-STh, pSTh-FLAG³, or the blank vector pFLAG-CTC. Briefly, following overnight growth at 37°C in lysogeny broth containing ampicillin (100 μ g/ml), cultures were diluted 1:100 in fresh medium, grown to an OD₆₀₀ of ~0.2, and then induced with 1 mM IPTG for 7 h. Forty milliliters of clarified supernatant was mixed with 200 μ l of anti-FLAG (M2) affinity gel (A2220; Sigma), and then the mixture was incubated overnight at 4°C with

agitation. After washing with Tris-buffered saline (TBS) buffer, bound proteins were eluted with 0.1 M glycine-HCl, pH 3.5, separated by SDS-PAGE, transferred to nitrocellulose for subsequent immunoblotting, and detected by anti-FLAG antibody (F1804; Sigma).

Confocal immunofluorescence imaging and quantification. To examine the delivery of FLAG-tagged STh toxin to intestinal epithelial cells, *estP estH* mutant strains with or without plasmids encoding FLAG-tagged STh were grown overnight and diluted 1:50 in CAYE-ST medium, grown to an OD₆₀₀ of ~0.2, and then induced with 1 mM IPTG for 2 h. The bacteria were added to T84 cells at a multiplicity of infection (MOI) of ~1:50, maintaining the induction with 1 mM IPTG. After infection with the bacteria, T84 cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1.5% bovine serum albumin (BSA)-PBS at 37°C for 1 h. Anti-O78 rabbit polyclonal antiserum (Penn State University) diluted 1:300 in PBS with 0.02% Tween 20 (PBST) and 1.5% BSA was used to identify H10407 and monoclonal anti-FLAG M2 antibody (number F1804; Sigma) diluted 1:500 in PBST with 1.5% BSA to detect FLAG-tagged STh. Following incubation overnight at 4°C, slides were washed 3 times with PBS and incubated for 1 h at 37°C with goat-anti-rabbit IgG (H&L) Alexa Fluor 488 conjugate (A11070; Thermo Fisher) and goat-anti-mouse IgG (H&L) conjugated to Alexa Fluor 594 (A11032; Thermo Fisher) at a dilution of 1:500 in PBST containing 1.5% BSA. After washing, DAPI (4',6-diamidino-2-phenylindole) was added at 1:6,000. Confocal microscopy images were acquired using a Nikon C2+ confocal microscope system. To quantitate binding of FLAG-epitope-tagged STh molecules, fluorescence detection was normalized to the DAPI signal using NIS-Elements DUO software (v4.4).

In vitro assessment of toxin delivery. Cultures of bacteria were grown overnight in lysogeny broth from frozen glycerol stocks in CAYE-ST medium. Phosphodiesterase inhibitors (PDE-I) vardenafil hydrochloride trihydrate (number Y0001647), rolipram (number R6520), and cilostazol (number C0737) (all from Sigma-Aldrich) were each added to target intestinal epithelial cells at a final concentration of 16.7 or 50 μM and incubated with cells for 1.5 h (T84 cells) or 2 h (enteroids). Bacteria or toxin was added to T84 monolayers seeded into 96-well plates, and the treatment was continued for the indicated duration. After washing in PBS, cyclic GMP (cGMP) levels were determined by enzyme immunoassay (EIA; K020-H1; Arbor Assays) using the acetylated protocol as directed by the manufacturer.

To examine the capacity of antibodies to neutralize ST delivery, antibody against STh and/or EtpA was added directly to T84 cell monolayers at the indicated dilution at the time of infection. After 1.5 h, cells were washed by PBS and lysed, and cGMP assays were performed as described above.

Growth and differentiation of T84 cells and small intestinal enteroids. T84 intestinal epithelial cells (ATCC CCL-248) were cultured in 96-well plates for 24 to 48 h at 37°C, 5% CO₂ incubator, to >90% confluence in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM)/F12 medium supplemented with L-glutamine (2.5 mM) and fetal bovine serum (FBS; 5% [vol/vol]). Enteroids were grown as previously described in detail (62, 63). Briefly, cells obtained from the Washington University Digestive Diseases Research Core Center BioSpecimens Core were propagated in Matrigel (BD Biosciences, San Jose, CA) in 24-well plates at 37°C with a 1:1 mixture of L-WRN (Wnt3a, R-spondin, and Noggin) conditioned medium (CM) and primary culture medium (Advanced DEM/F12; Invitrogen) supplemented with 20% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 μM Y-27632 (ROCK inhibitor; Tocris Bioscience, R and D Systems, Minneapolis, MN), and 10 μM SB 431542 (TGFBR1 inhibitor; Tocris Bioscience, R and D Systems). Cells were then added to Transwell filters (Corning) and incubated in differentiation medium (1:20 mixture of L-WRN CM and primary culture medium) lacking SB 431542 and were grown 2 to 3 days until confluence. Prior to addition of bacteria, cells were switched to differentiation medium lacking antibiotics.

Accession number(s). Plasmids pCW002, pCW003, and pYZ011 have been deposited in Addgene (<https://www.addgene.org/>) under accession numbers 90225, 90226, and 110570, respectively.

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