Bacterial Surface Association of Heat-labile Enterotoxin through Lipopolysaccharide after Secretion via the General Secretory Pathway*

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Heat-labile enterotoxin (LT) is an important virulence factor expressed by enterotoxigenic Escherichia coli. The route of LT secretion through the outer membrane and the cellular and extracellular localization of secreted LT were examined. Using a fluorescently labeled receptor, LT was found to be specifically secreted onto the surface of wild type enterotoxigenic Escherichia coli. The main terminal branch of the general secretory pathway (GSP) was necessary and sufficient to localize LT to the bacterial surface in a K-12 strain. LT is a heteromeric toxin, and we determined that its cell surface localization was mediated by the its B subunit independent of an intact G_{M1} gangioside binding site and that LT binds lipopolysaccharide and G_{M1} concurrently. The majority of LT secreted into the culture supernatant by the GSP in E. coli associated with vesicles. Only a mutation in hns, not overexpression of the GSP or LT, caused an increase in vesicle yield, supporting a specific vesicle formation machinery regulated by the nucleoid-associated protein HNS. We propose a model in which LT is secreted by the GSP across the outer membrane, secreted LT binds lipopolysaccharide via a G_{M1}-independent binding region on its B subunit, and LT on the surface of released outer membrane vesicles interacts with host cell receptors, leading to intoxication. These data explain a novel mechanism of vesicle-mediated receptor-dependent delivery of a bacterial toxin into a host cell.

Enterotoxigenic Escherichia coli (ETEC)\(^1\) is an important pathogen responsible for traveler’s diarrhea and >700,000 childhood deaths annually because of diarrhea in third world countries (1–4). ETEC produces two toxins implicated in the etiology of diarrhea, heat stable toxin and heat-labile entero-toxin (LT) (1, 5). LT, which is encoded on a relatively uncharacterized 60-kb virulence plasmid (1), is one of a group of AB\(_5\) toxins (6, 7) that also includes Shiga toxin, pertussis toxin, and cholera toxin (CT). These heteromeric toxins consist of a catalytic A subunit (LTA) and a pentamer of receptor-binding B subunits (LTB) (8, 9). In addition to structural homology, LT shares 80% sequence homology with the Vibrio cholerae toxin CT (10, 11). The ring-shaped B pentamer of both LT and CT mediates binding to the host epithelial receptor G_{M1} (12–14). LT is more promiscuous than CT in that it can also bind other receptors containing a terminal galactose (13). After binding, the receptor/toxin complex is internalized, and LTA is trafficked to the cytosol (13, 15, 16). Upon entry into the cytosol, the catalytic subunit constitutively activates adenylate cyclase, resulting in water and electrolyte efflux from the host cells (17). One major difference between the CT and LT lies in the fact that although CT is secreted from the cell, LT reportedly remains periplasmic (5, 18–20). Some studies have also found LT to be associated with membranes extracellularly (3, 21, 22). Despite the equivalent activity that CT and LT exhibit in bioassays, disease caused by ETEC is much less severe than that caused by V. cholerae (2). This finding suggests that the difference between V. cholerae and ETEC virulence may depend on the efficiency of toxin secretion and the delivery mechanism (1).

The main terminal branch of the general secretory pathway (GSP) is a well conserved set of proteins encoded by 13–15 genes clustered in an operon that allow secretion through the outer membrane (23–25). CT secretion in V. cholerae progresses through the three steps of the type II secretion pathway: translocation across the inner membrane, folding in the periplasm, and secretion through the outer membrane (23–31). Extracellular CT secretion occurs through the GSP (32). An examination of many Gram-negative bacteria including those from genera Pseudomonas, Klebsiella, Erwinia, and Vibrio has shown that homologs of this pathway (Xcp, Pul, Out, and Eps, respectively) are responsible for the secretion of a large host of soluble extracellular proteases and toxins across the outer membrane (24).

When introduced on an exogenous plasmid, LT is secreted solubly from V. cholerae in the same manner as CT (18, 29, 30), whereas K-12 *E. coli* transformed with a CT-expressing plasmid does not secrete CT (30, 33). These results suggest that *E. coli* does not possess or express the secretion apparatus present in *V. cholerae*. However, these experiments have not been conducted in ETEC where a secretion system may be active. Enterotoxigenic and K-12 *E. coli* contain a gsp gene cluster homologous to the eps genes encoding the secretion machinery for CT, but these genes are not expressed by K-12 strains under laboratory conditions (34, 35). Lack of expression of these genes may be attributed to the transcription factor HNS, which has been shown to negatively regulate the GSP (36) as well as other virulent factors including LT (37). Transformation of hns-deficient *E. coli* K-12 with a plasmid encoding

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\footnotesize{1} The abbreviations used are: ETEC, enterotoxigenic *E. coli*; G_{M1}, Gal_{l,2}Gal_{l,3}Gal_{l,4}Ac_{p,1}–4(NEuAc)_{2,3}Gal_{l,2}4Glc-ceramide; LT, heat-labile enterotoxin; CT, cholera toxin; LTA and LTB, LT subunits A and B; GSP, general secretory pathway; CFU, colony forming units; RFU, relative fluorescence units; LPS, lipopolysaccharide; DOC, deoxycholate; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

\footnotesize{2} A. L. Horstman and M. J. Kuehn, unpublished data.
the full gsp operon along with one of its substrates, chitinase, is sufficient to cause the secretion of chitinase (36). In ETEC, the deletion of leOA causes a buildup of LT in the periplasm, a decrease of LT in the supernatant, and a decrease of toxicity in vivo (38). The molecular role that the leOA gene product plays in LT secretion remains uncharacterized.

Although not secreted from ETEC in a soluble form, LT has been observed to be associated with LPS in a particulate fraction of the culture supernatant (3, 39). These observations were been observed to be associated with LPS in a particulate fraction. LT secretion remains uncharacterized.

Previously, we have shown that active LT is enriched in and present on the surface of the ETEC vesicle (21). Upon further examination of the data presented by Kolling and Matthews (46), we propose that in addition to its internal localization, Shiga toxin is similarly localized to the surface of vesicles derived from enterohemorrhagic E. coli. In addition, the toxins VacA and leukotoxin have been found to be displayed on the surface of vesicles derived from H. pylori and A. actinomycetemcomitans, respectively (53, 54). This activity suggests that membrane vesicles may be natural vehicles for intercellular transport of virulence factors to host cells during a bacterial infection.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Bacterial strains and plasmids used in this investigation are listed in Table I. Strains were grown in LB (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) and maintained on LB agar. Transformations were performed using a modified CaCl2 protocol (55). Antibiotics were added as required at the following concentrations: 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 35 µg/ml chloramphenicol. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (1.0 mM) was used to induce the expression of LTB constructs. Y1 adenoidal cells (ATCC number CCL-79) were maintained in F12K media supplemented with 2.5% fetal calf serum and 12% horse serum as per ATCC instruction. Unless specified, reagents were purchased from Fisher.

**Construction of pPLT**—A 2.0-kb fragment containing eltAB including its promoter (37) was generated by PCR from the template plasmid pWL520 using primers PoL (5′-AACGACGACGTAC-3′) and Kpn (5′-TTTTTGTGCCTAGTTTTTCTACCTGATGCGGC-3′). The fragment was cut with PoL and Kpn and cloned into similarly digested pUC19 (New England Biolabs), resulting in pPLT.

**BODIPY-G_Mi Labeling**—Cells were grown to mid-log phase (A600 ∼0.3) and serially plated for colony forming units (CFU). 30 ml of cells were centrifuged at 6000 × g for 10 min, washed once in ice-cold HEPS (50 mM, pH 6.8), and resuspended in 1.0 ml of HEPS. Cells (100 µl) were mixed with 100 µl of 300 nM BODIPY-G_Mi ( Molecular Probes) in methanol or 100 µl of methanol, and then mixtures were incubated on ice for 30 min. Cells were pelleted and washed three times in ice-cold HEPS. Cells were resuspended in 200 µl of HEPS, and 100 µl was applied to duplicate 96-well microtiter plates. Fluorescence was measured on a FLUOstar Galaxy fluorescence scanner and Microplate Reader (BMG Labtechnologies) and normalized to cell number (RFU/CFU).

**Bacterial Immunoprecipitation Assay—Strains** strains of E. coli, Shigella flexneri, Pseudomonas aeruginosa, Borrelia burgdorferi, Actinobacillus actinomycetemcomitans, and Helicobacter pylori interact with bacteria and mammalian cells via an adherence and/or fusion mechanism (39, 49–51). This activity suggests that membrane vesicles may be natural vehicles for intercellular transport of virulence factors to host cells during a bacterial infection.

**RESULTS**

**Cell Surface Detection of LT**—LT is present on the surface of vesicles produced by growing ETEC (21). Because vesicles are

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expected to form by pinching off of the outer membrane, we reasoned that components bound to the vesicle surface are probably also bound to the cell surface. To determine whether LT is bound to the surface of growing ETEC, we developed a detection assay using BODIPY-GM1, a fluorescently labeled membrane-impermeable receptor for LT. BODIPY-GM1 was incubated with early to mid-log phase ETEC, the cells were washed, and cell-associated fluorescence was measured. BODIPY-GM1 labeling of E9034A and E9034P, a virulence plasmid-cured derivative of E9034A that lacks LT, was compared (Fig. 1A). In contrast to E9034A, very little fluorescence was associated with E9034P. We also tested an isogenic pair of strains, H10407, a wild type ETEC, and ΔleOA, a mutant that accumulates LT in the periplasm and prevents LT secretion into the supernatant (38). Any LT in the ΔleOA supernatant would be a result of cell lysis and would be available for “nonspecific” cell association. BODIPY-GM1 associated with ΔleOA cells 7 times less than with H10407 cells (Fig. 1A). These results showed that Gm1-accessible LT is bound to the surface of wild type ETEC and that the exterior localization of LT did not result from cell lysis.

**LT Secretion via the GSP—**Because LT and CT secretion depended on the native gsp gene cluster in V. cholerae (18, 32), we wanted to investigate whether the GSP secretion machinery is responsible for LT secretion across the outer membrane and, consequently, the association of LT to the cell surface in E. coli. Wild type ETEC strains are not ideal for the dissection of this secretory pathway, because ETEC may possess multiple gsp loci in its chromosome and in its multiple uncharacterized virulence plasmids and the gsp and LT operons are repressed by HNS (36, 37). MC4100, a K-12 strain, has successfully been used to study GSP-dependent secretion of another substrate, chitinase (36). Although K-12 E. coli contain gsp genes, their level of expression appeared to be insufficient for the secretion of chitinase. The presence of a low copy number gsp-carrying plasmid (pCHAP4278) coupled with derepression of gsp genes in an hns-deficient background strain (designated here as Δhns) was found to be necessary for chitinase secretion. Therefore, we used MC4100 and Δhns as “clean” background strains in which to study LT secretion via the GSP. We transformed Δhns with a plasmid encoding either a functional GSP (pCHAP4278) to create Δhns/GSP or the gsp gene cluster with a deletion in gspD and gspE (pCHAP4280) to create Δhns/ΔGSP (Table I). Strains Δhns, Δhns/GSP, and Δhns/ΔGSP were transformed with pPLT, a plasmid encoding LT under the control of its native promoter to create Δhns/LT, Δhns/GSP/LT, and Δhns/ΔGSP/LT, respectively. LT production was equivalent for all strains carrying pPLT as measured by Y1 cell toxicity of total cell lysates (data not shown). The addition of the plasmid-expressed GSP moderately slowed the growth rate of Δhns, and this was compounded by the addition of plasmid-expressed LT (data not shown). To confirm that the slowed growth rate was not a result of cell lysis, we examined the membrane integrity of each of our strain constructs using two established assays, DOC resistance, and periplasmic RNase leakage (56, 57). None of the strains exhibited periplasmic leakage; however, ΔleOA was completely unable to grow on DOC, and several of the GSP- and LT-expressing strains were moderately sensitive to DOC (Table II). Therefore, GSP and LT expression increase detergent sensitivity of the outer membrane but do not compromise the outer membrane barrier.

The presence of LT on the surface of the gsp and LT isogenic strains was investigated using the BODIPY-GM1 binding assay. Strains GSP, Δhns, Δhns/GSP, and Δhns/ΔGSP did not bind BODIPY-GM1 (data not shown). Externally bound LT was detected on Δhns/GSP/LT, which contains both GSP and LT plasmids (Fig. 1B). Strain GSP/LT, which has a functional HNS and contains both GSP and LT plasmids bound 5-fold less BODIPY-GM1 than Δhns/GSP/LT, probably because of repression of both GSP and LT expression. Expression of chromosomal GSP (Δhns/LT) or of the mutant GSP (Δhns/ΔGSP/LT) resulted in a 346- and 30-fold respective reduction in the amount of LT secreted to the surface (Fig. 1B). Therefore, BODIPY-GM1 labeling of the LT-expressing strains depended on functional GSP expression, strengthening the argument.
that cell lysis does not cause LT association with the outer membrane. Together, these results show that LT is actively secreted through the GSP and remains associated with the surface of bacteria after secretion.

**LTB Mediates Binding to Bacterial Surface**—As LT is composed of A and B subunits, it was necessary to determine which subunit was responsible for cell surface localization. Expression of only the LTB pentamer in the Δhns/GSP strain (Δhns/GSP/LTB) resulted in cell surface localization of LTB as detected by BODIPY-GM1 (Fig. 1B), indicating that the A subunit was unnecessary for LT binding to the outer membrane. As expected, BODIPY-GM1 did not bind Δhns/GSP/LTB cells that express LTB with a point mutation that is defective in G

| Strains | Genotype | Relevant characteristics | CFS | Ref. |
|---------|----------|--------------------------|-----|------|
| E9034A  | Wild-type ETEC (LT<sup>+</sup>) | + | 73 |
| E9034P  | ETEC cured of virulence plasmid (LT<sup>−</sup>) | − | 73 |
| H10407ΔleuA | Wild-type ETEC (ATCC 35401) (LT<sup>−</sup>) | + | 38 |
| MC4100 ΔleuA | H10407 mutant defective in LT secretion (LT<sup>−</sup>) | − | 38 |
| GSP | MC4100 | K-12 E. coli | − | 36 |
| GSP/LT | MC4100/pCHAP4278 | K-12 expressing GSP | − | This work |
| Δhns | MC4100 hns1001::kan | + | This work |
| Δhns/LT | MC4100 hns1001::kan/pPLT | + | This work |
| Δhns/GSP | MC4100 hns1001::kan/pCHAP4278 | − | | |
| Δhns/GSP/LT | MC4100 hns1001::kan/pCHAP4278/pPLT | − | | |
| ΔhnsΔGSP | MC4100 hns1001::kan/Δgsp4280 | + | | |
| ΔhnsΔGSP/LT | MC4100 hns1001::kan/Δgsp4280/pPLT | + | | |
| Δhns/GSP/LTB | MC4100 hns1001::kan/pCHAP4278/pMMB68 | − | | |
| Δhns/GSP/LTB<sub>eltBG33D</sub> | MC4100 hns1001::kan/pCHAP4278/pTRH64 | − | | |

| Plasmids | pWD600 | eltAB and −4 kb of uncharacterized downstream DNA, Tet<sup>R</sup> |
|----------|--------|--------------------------------------------------|
| LT      | pUC19  | Coding vector, Amp<sup>R</sup> |
| GSP     | pPLT   | eltAB under control of native promoter, cloned from pWD6000 into pUC19, Amp<sup>R</sup> |
| ΔGSP    | pCHAP4280 | gspAB and gspC-R under control of native promoter, Cm<sup>R</sup> |
| LTB     | pMMB68 | eltB under control of IPTG-inducible promoter |
| LTB<sub>eltBG33D</sub> | pTRH64 | eltB<sub>eltBG33D</sub> under control of IPTG-inducible promoter |

| TABLE II | Strains and plasmids used in this study |
|----------|----------------------------------------|

| Name/alias | Genotype | Relevant characteristics | CFS | Ref. |
|------------|----------|--------------------------|-----|------|
| E9034A     | Wild-type ETEC (LT<sup>+</sup>) | + | 73 |
| E9034P     | ETEC cured of virulence plasmid (LT<sup>−</sup>) | − | 73 |
| H10407ΔleuA | Wild-type ETEC (ATCC 35401) (LT<sup>−</sup>) | + | 38 |
| MC4100 ΔleuA | H10407 mutant defective in LT secretion (LT<sup>−</sup>) | − | 38 |
| GSP | MC4100 | K-12 E. coli | − | 36 |
| GSP/LT | MC4100/pCHAP4278 | K-12 expressing GSP | − | This work |
| Δhns | MC4100 hns1001::kan | + | This work |
| Δhns/LT | MC4100 hns1001::kan/pPLT | + | This work |
| Δhns/GSP | MC4100 hns1001::kan/pCHAP4278 | − | | |
| Δhns/GSP/LT | MC4100 hns1001::kan/pCHAP4278/pPLT | − | | |
| ΔhnsΔGSP | MC4100 hns1001::kan/Δgsp4280 | + | | |
| ΔhnsΔGSP/LT | MC4100 hns1001::kan/Δgsp4280/pPLT | + | | |
| Δhns/GSP/LTB | MC4100 hns1001::kan/pCHAP4278/pMMB68 | − | | |
| Δhns/GSP/LTB<sub>eltBG33D</sub> | MC4100 hns1001::kan/pCHAP4278/pTRH64 | − | | |

| Strains | Genotype | Relevant characteristics | CFS | Ref. |
|---------|----------|--------------------------|-----|------|
| E9034A  | Wild-type ETEC (LT<sup>+</sup>) | + | 73 |
| E9034P  | ETEC cured of virulence plasmid (LT<sup>−</sup>) | − | 73 |
| H10407ΔleuA | Wild-type ETEC (ATCC 35401) (LT<sup>−</sup>) | + | 38 |
| MC4100 ΔleuA | H10407 mutant defective in LT secretion (LT<sup>−</sup>) | − | 38 |
| GSP | MC4100 | K-12 E. coli | − | 36 |
| GSP/LT | MC4100/pCHAP4278/pPLT | K-12 expressing GSP and LT | − | This work |
| Δhns | MC4100 hns1001::kan | K-12 with Kan<sup>R</sup> cassette insertion into hns, Kan<sup>R</sup> | − | 36 |
| Δhns/LT | MC4100 hns1001::kan/pPLT | K-12 with Kan<sup>R</sup> cassette insertion into hns, expressing LT, Kan<sup>R</sup>, Amp<sup>R</sup> | − | This work |
| Δhns/GSP | MC4100 hns1001::kan/pCHAP4278 | K-12 with Kan<sup>R</sup> cassette insertion into hns, expressing wt GSP, Kan<sup>R</sup>, Cm<sup>R</sup>, Amp<sup>R</sup> | − | This work |
| Δhns/GSP/LT | MC4100 hns1001::kan/pCHAP4278/pPLT | K-12 with Kan<sup>R</sup> cassette insertion into hns, expressing mutant GSP and LT, Kan<sup>R</sup>, Cm<sup>R</sup>, Amp<sup>R</sup> | − | This work |
| Δhns/GSP/LTB | MC4100 hns1001::kan/pCHAP4278/pMMB68 | K-12 with Kan<sup>R</sup> cassette insertion into hns, expressing wt GSP and wt LTB, Kan<sup>R</sup>, Cm<sup>R</sup>, Amp<sup>R</sup> | − | This work |
| Δhns/GSP/LTB<sub>eltBG33D</sub> | MC4100 hns1001::kan/pCHAP4278/pTRH64 | K-12 with Kan<sup>R</sup> cassette insertion into hns, expressing wt GSP and G<sub>M1</sub> binding mutant LTB<sub>eltBG33D</sub>, Kan<sup>R</sup>, Cm<sup>R</sup>, Amp<sup>R</sup> | − | This work |

**TABLE II**

| Strain | Growth on DOC plate<sup>a</sup> | RNase<sup>b</sup> |
|--------|-------------------------------|----------------|
| H10407 | ++                            | −              |
| ΔleuA  | ++                            | −              |
| MC4100 | +                             | −              |
| Δhns   | +                             | −              |
| Δhns/LT| +                             | −              |
| GSP    | +                             | −              |
| GSP/LT | +                             | −              |
| Δhns/GSP | +                   | −              |
| Δhns/GSP/LT | +             | −              |
| Δhns/ΔGSP | +               | −              |
| Δhns/ΔGSP/LT | +           | −              |

<sup>a</sup> +++, thick growth; +, growth; −, no growth.

<sup>b</sup> −, no detectable RNase activity in area surrounding bacterial growth.

that cell lysis does not cause LT association with the outer membrane. Together, these results show that LT is actively secreted through the GSP and remains associated with the surface of bacteria after secretion.
We tested whether LPS could block the toxicity of LT in the same manner as G\textsubscript{M\textsubscript{1}} or whether that association could enhance LT toxicity. LT toxicity is commonly assayed using Y1 adrenal mouse cells (58), and we determined that LPS and G\textsubscript{M\textsubscript{1}} alone (up to 6.0 nmol) were nontoxic to Y1 cells (data not shown). Y1 cells were treated with 6 nmol, 600 pmol, and 60 pmol of LT to elicit varying degrees of toxic response (Fig. 3, black, gray, and hatched bars). When LT was preincubated with a 10-fold excess of G\textsubscript{M\textsubscript{1}}, the toxicity of LT was blocked (Fig. 3, last group). Preincubation of LT with up to a 1000-fold molar excess of LPS, however, had no effect on the toxicity of LT (Fig. 3, LPS groups). Thus, although soluble LPS can block the binding of LT to the surface of bacteria (Fig. 2B), LPS does not inhibit or enhance the interaction of LT with its host cell surface receptor G\textsubscript{M\textsubscript{1}}.

**LT Secreted by the GSP Is Vesicle-associated**—Previous work has demonstrated that LT secreted by ETEC is associated with vesicles (21, 40, 41). To investigate whether this association of LT with vesicles was simply because of a lack of GSP expression, we wanted to determine whether LT secreted by the GSP is soluble or associated with vesicles. We first examined the toxicity of unfraccionated cell-free supernatants from the strains constructed in this work using the Y1 adrenal cell assay. The toxicity of the MC4100 supernatants clearly depended on the expression of LT and the presence of a functional plasmid-expressed GSP (Table I). To differentiate whether this toxicity was soluble or associated with vesicles, we fractionated the cell-free supernatant of strain \textDelta hns/GSP/LT. Y1 cell toxicity was used to assay soluble LT and cell-free supernatants before and after filtration (Fig. 4A). After filtration of a pure soluble LT sample, all of the activity was detected in the 100-kDa filtrate, whereas purified vesicles did not enter the filtrate (data not shown). Before filtration, the average LT activity in the total cell-free supernatant of the LT- and GSP-expressing constructs had altered outer membrane vesicle production. Because vesicle production may be linked to protein secretory pathways, we also examined whether the strains containing various secretion constructs had altered outer membrane vesicle production. When total vesicles produced per CFU were compared, we determined that the deletion of \textit{hns} caused a 3-fold increase in vesiculation (Fig. 4B). The addition of plasmid-born GSP, mutant GSP, and/or LT had no effect on the total vesicle production compared with the \textDelta hns parent strain. Therefore, the vesicle production level in \textit{E. coli} is not dramatically affected by...
We considered that the GSP might be responsible for the production of LT, as E. coli has been detected in ETEC and other pathogenic strains. The GSP-dependent secretion of LT is HNS-regulated.

Toxocity

\[ y = 0.4082 \ln(x) + 2.94 \]

A. toxicity of total and size-fractionated (100-kDa filtrate) cell-free supernatants from cultures of Δhns/ GSP/LT (n = 16) (black bars) and Δhns/GSP (n = 8) (gray bars). Error bars = mean ± S.E. Inset, standard curve of LT toxicity on Y1 cells. Error bars = mean ± S.E.; n ≥ 6. B. vesicle yields per CFU of indicated strains compared with Δhns. Error bars = mean ± S.E.; n = 6.

Bacterial proteins have been shown to bind to lipids; therefore, we tested common membrane phospholipids and found that they did not inhibit LT binding. The association of LT with LPS, protein, and possibly minor amounts of phospholipid.

**DISCUSSION**

This work describes the first comprehensive study of the bacterial secretion mechanism of heat-labile enterotoxin in *E. coli*. The mode by which LT is released by ETEC to interact with host cells has long been a paradox. Previously, it was observed that LT remained in the periplasmic space and therefore was unavailable to intoxicate cells. However, LT had also been detected extracellularly and associated with LPS and, more specifically, with vesicles. Given the role of the GSP in the secretion of LT and the fact that the gsp operon has been detected in ETEC and other pathogenic *E. coli* (34, 65), we considered that the GSP might be responsible for the secretion of LT to the cell surface.

In previous work, we discovered that active LT is present on the surface of ETEC vesicles and that ETEC vesicles could bind immobilized receptor (21). In this study, we developed the BODIPY-GM1 assay to detect externally bound LT, and we found that LT was associated specifically with the surface of ETEC cells, which are the source of the outer membrane vesicles. LT could become associated with the cell surface through two possible mechanisms: nonspecific association resulting from cell lysis or a specific transport step across the outer membrane. Three lines of evidence demonstrated that cell lysis was not the means by which LT became bound to the cell surface: the lack of binding of BODIPY-GM1 to ΔLeoA cells, GSP-dependent BODIPY-GM1 labeling of cells, and the lack of periplasmic leakage from our strains. Thus, surface-localized LT specifically depended on the co-expression of LT and the functional plasmid-encoded GSP. These data indicate that the GSP is necessary and sufficient for the secretion of LT, LT remains associated with the surface of the cell after secretion, and LT is capable of simultaneously associating with the outer membrane and the G3M1 receptor.

Because LT localized to the outside of cells, either protein or lipid components of the outer membrane were mediating toxin binding. The outer leaflet of the outer membrane is composed of LPS, protein, and possibly minor amounts of phospholipid. Bacterial proteins have been shown to bind to lipids (66); therefore, we tested common membrane phospholipids and found that they did not inhibit LT binding. The association of LT with the bacterial surface was resistant to protease and inhabitable with soluble LPS, indicating that LT binds to LPS in the outer membrane. We reasoned that because LT binds G3M1 by a terminal galactose and agarose beads through this same sugar (61), LT might be binding LPS on the surface of *E. coli* through a galactose in the O-antigen. Therefore, we were surprised to discover that LT bound to the surface of E9034P, which has a polymannose O-antigen (67), K-12 (expressing the GSP), which does not express O-antigen, and Ra LPS. In addition, none of the simple sugars such as mannose, glucose, galactose, or lactose inhibited LT binding. Therefore, LT may recognize sugar complexes in LPS in the O-antigen (if it is present) or in the core.

The fact that BODIPY-G3M1 bound cells in an LTB-dependent manner demonstrates that LTB can simultaneously bind LPS and G3M1 receptor. LTBG3M1 was found to be associated with cells to the same extent as wild type LTB; therefore, the intact G3M1 binding site is not required for the LTB-LPS interaction. The presence of independent G3M1 and LPS binding sites on LTB is supported by our findings that G3M1, not LPS, was able to block the toxicity of LT, and conversely, LPS but not G3M1 inhibited LT binding to bacteria. Based on these data, one LTB subunit potentially could bind both LPS and G3M1 simultaneously via distinct sites. However, LPS and G3M1 are sterically large molecules; thus, we propose that concurrent LT binding to LPS and G3M1 occurs by LPS engaging one or more LTB subunits of the pentamer and G3M1 engaging one or more of the remaining LTB subunits.
We have proposed that vesicles are one means by which active LT can be delivered to eukaryotic cells, a conclusion that is supported by previous observations (3, 22, 39). However, we considered that vesicles may not be the only form of transport and that in in vitro culture conditions, a soluble LT secretion pathway (e.g. the GSP) was either not expressed or not functional. To address whether LT would be secreted solubly if the GSP pathway was available, we assayed the culture supernatant of E. coli expressing LT and the GSP in the derepressing hns background strain. LT from the supernatant of Δhns/GSP/LT was retained on a 100-kDa filter, indicating that LT secreted by the GSP remains associated with vesicles rather than being liberated solubly into the medium. Thus, although CT is secreted in a soluble form by the GSP, LT secreted by the GSP is exclusively associated with outer membrane vesicles. The difference between the extracellular localization of these two similar toxins may be attributed to the fact that CT is less promiscuous in that it can only bind monosialo-GM1 (12) or that the LPS core structures of E. coli and V. cholerae differ (68, 69).

Very little is currently known regarding the regulation of vesicle production by pathogenic bacteria. We investigated whether HNS, LT, or GSP expression affected vesicle production, because it was possible that an increase in vesicle cargo could induce vesicle formation. Our data indicate that only HNS expression affected the amount of vesicles produced. The hns-dependent increase in vesicle yield could be either a primary or secondary effect. HNS could regulate a specific machinery that mediates the formation of vesicles. Thus, in an hns mutant, a negative regulator of vesicle production could have been lost, resulting in an increase in vesicle production. Alternatively, the difference between the extracellular localization of these two similar toxins may be attributed to the fact that CT is less promiscuous in that it can only bind monosialo-GM1 (12) or that the LPS core structures of E. coli and V. cholerae differ (68, 69).

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