Significance of Enterotoxigenic *Escherichia coli* (ETEC) Heat-Labile Toxin (LT) Enzymatic Subunit Epitopes in LT Enterotoxicity and Immunogenicity

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) strains producing heat-labile toxin (LT) and/or heat-stable toxin (STa) are a top cause of children's diarrhea and travelers' diarrhea. Holotoxin-structured GM_1-binding LT is a strong immunogen and an effective adjuvant, and can serve a carrier or a platform for multivalent vaccine development. However, the significance of peptide domains or epitopes of LT particularly enzymatic LT_A subunit in association with LT enterotoxicity and immunogenicity has not been characterized. In this study, we identified B-cell epitopes *in silico* from LT_A subunit and examined epitopes for immunogenicity and association with LT enterotoxicity. Epitopes identified from LT_A subunit were individually fused to a modified chicken ovalbumin carrier protein, and each epitope-ovalbumin fusion was used to immunize mice. Data showed all 11 LT_A epitopes were immunogenic; epitope 7 ([105]SPHPYEQEVSA[115]) induced greater titers of anti-LT antibodies which neutralized LT enterotoxicity more effectively. To examine these epitopes for the significance in LT enterotoxicity, we constructed LT mutants by substituting each of 10 epitopes at the toxic A1 domain of LT_A subunit with a foreign epitope and examined LT mutants for enterotoxicity and GM_1-binding activity. Data showed that LT mutants exhibited no enterotoxicity but retained GM_1-binding activity. The results from this study indicated that while not all immunodominant LT_A epitopes were neutralizing, LT mutants with an individual epitope substituted lost enterotoxicity but retained GM_1-binding activity. These results provided additional information to understand LT immunogenicity and enterotoxicity and suggested the potential application of LT platform for multivalent vaccines against ETEC diarrhea and other diseases.

IMPORTANCE No vaccine is licensed for enterotoxigenic *Escherichia coli* (ETEC) strains, which remain a leading cause of diarrhea in children from developing countries and international travelers. GM_1-binding heat-labile toxin (LT) which is a key virulence factor of ETEC diarrhea is a strong vaccine antigen and a self-adjuvant. LT can also serve a backbone or platform for MEFA (multiepitope fusion antigen), a newly developed structural vaccinology technology, to present heterogeneous epitopes (by replacing LT epitopes) and to mimic epitope antigenicity for development of broadly protective vaccines. Data from this study identified neutralizing LT epitopes and demonstrated that substitution of LT epitopes eliminated LT enterotoxicity without altering GM_1-binding activity, suggesting LT is potentially a versatile MEFA platform to present heterogeneous epitopes for multivalent vaccines against ETEC and other pathogens.

KEYWORDS ETEC (enterotoxigenic *Escherichia coli*), LT (heat-labile toxin), epitope, vaccine platform

Enterotoxigenic *Escherichia coli* (ETEC) strains continue to be a leading cause of children's diarrhea and travelers' diarrhea (1–4). Currently, there is no vaccine licensed for ETEC diarrhea (4–7). ETEC bacteria produce colonization factor antigen (CFA) or coli surface antigen (CS) adhesins and enterotoxins. CFA and CS adhesins facilitate ETEC bacteria to attach to host cell receptors and to colonize small intestines; enterotoxins, including heat-labile toxin (LT) and heat-stable type 1b toxin (STa), elevate intracellular cyclic AMP or cGMP levels in host epithelial cells to cause water and fluid hypersecretion and watery diarrhea (8–10).

LT (LT-I) produced by ETEC is a typical AB_5 holotoxin and binds GM_1 receptor (9, 11–13). The enzymatic A subunit (LT_A) consists of an A1 domain and an A2 domain. The A1 peptide is ADP-ribosylating; the A2 peptide noncovalently associates the A subunit to the GM_1-binding B subunit (LT_B) pentamer to form the AB_5 holotoxin structure. LT, detoxified LT mutants, and the nontoxic B subunit are strong immunogens to induce anti-LT antibodies; thus, they are often targeted as antigens for ETEC vaccine development (14–16). Interestingly, LT and LT mutants are also effective mucosal and parenteral adjuvants (17–23). In addition, LT, LT_B, and derivatives can serve carrier proteins to facilitate or to up-immunoregulate immunogenicity of carrying proteins and peptides (24–30).

Recently, MEFA (multiepitope fusion antigen) technology has been developed to design structure-based multivalent vaccines (31–35). Aided with protein computational modeling and molecular dynamics stimulation (35), this MEFA technology applies a special backbone protein to present multiple foreign epitopes and to mimic epitope native antigenicity, thus having a single protein to induce protective antibodies against multiple heterogeneous virulence factors. The strongly immunogenic, GM_1-binding, and self-adjuvant LT or LT mutants become an ideal backbone or platform for the MEFA technology. By applying a nontoxic and GM_1-binding LT backbone to present a STa toxoid (19 amino acids in length) and epitopes of the most important CFA adhesins (i.e., the other ETEC virulence determinants), ideally through substitution of LT_A subunit epitopes, we can develop a broadly protective mucosal vaccine against ETEC diarrhea.

However, before LT is applied as a MEFA backbone or platform for ETEC vaccine development, epitopes of LT particularly the enzymatic LT_A subunit domain A1 (LT_{A1}) need to be characterized for the significance associated with LT immunogenicity and enterotoxicity. While antigenic elements from the toxic LT_A subunit need to be included in an ETEC vaccine to induce antibodies neutralizing LT enterotoxicity, a nontoxic GM_1-binding LT is preferred as a safe antigen to induce systemic and mucosal immunity against ETEC diarrhea. If this safe GM_1-binding LT backbone can also induce neutralizing antibodies against LT enterotoxicity, it becomes ideal for ETEC mucosal vaccine development. In this study, we designed experiments to identify immunodominant and neutralizing B-cell epitopes from LT_A subunit and to investigate the significance of each epitope in LT enterotoxicity and GM_1-binding activity. The results from this study should improve our understanding of LT immunogenicity and enterotoxicity and provide useful information to assess the application of LT as a versatile MEFA backbone or platform for multivalent mucosal vaccine development.

RESULTS

Eleven continuous B-cell epitopes were *in silico* identified from LT_A subunit. Eleven continuous B-cell epitopes were identified from LT_A subunit, ranging from 10 to 13 amino acid residues in length (Table 1). Ten epitopes (e2 to e11) were identified from the enzymatic LT A1 domain (indicated in green in Fig. 1), and one epitope (e1) was located at the LT A2 domain (indicated in gray in Fig. 1). Computation modeling using Phyre2 (36) showed all epitopes were surface exposed (Fig. 1A and D). All predicted epitopes were located at α-helix or β-sheet of protein secondary structure (Fig. 1B and C). This LT_A protein model was deposited under PDB accession no. 1LTA at the protein database.

Epitope-ovalbumin fusion proteins reacted with anti-LT antibodies. Each LT_A epitope was genetically fused to a modified chicken ovalbumin gene (37), by replacing

TABLE 1 LT$_A$ subunit continuous B-cell epitopes were identified from epitope prediction programs[a]

Epitope	aa sequence	Position (aa)	Length (aa)	CT counterpart epitope sequence
e1	TITGDTCNEETQ	193–204	12	SSMSTCDEKTQ
e2	NGDKLYRADSR	1–11	11	NDDKLYRADSR
e3	DSRPPDEIKRSGG	9–21	13	DSRPPDEIKQSGG
e4	RGHNEYFDRGTQ	25–36	12	RGQSEYFDRGTQ
e5	YDHARGTQTG	42–51	10	YDHARGTQTG
e6	RYDDGYVSTS	54–63	10	RHDDGYVSTS
e7	SPHPYEQEVSA	105–115	11	SPHPDEQEVSA
e8	HRNREYRDRY	140–149	10	HRNRGYRDRY
e9	APAEDGYRLAG	156–166	11	APAADGYGLAG
e10	AGFPPDHQAWREE	165–177	13	AGFPPEHRAWREE
e11	HHAPQGCGNSSRT	181–193	13	HHAPPGCGNAPRS

[a]Epitope amino acid sequences, positions, and lengths are indicated. The epitope prediction programs used are described elsewhere (44, 45). CT counterpart epitopes are also included; heterogeneous residues are indicated by underlining. The sequences of LT and CT were published previously by Holmes et al. (52). aa, amino acids.

an ovalbumin epitope, for epitope-ovalbumin fusions (Fig. 2A). DNA sequencing confirmed that LT$_A$ epitopes were correctly inserted into the modified chicken ovalbumin gene. Computational modeling indicated each LT$_A$ epitope was surface exposed, and epitope replacement did not appear to alter the ovalbumin protein overall structure or protein antigenic propensity. All recombinant epitope-ovalbumin fusion proteins expressed in pET28a and *E. coli* BL21(DE3) were 6×His tag-less, at the expected size of 34 kDa (Fig. 2B).

All 11 epitope-ovalbumin fusion proteins reacted with anti-LT antiserum. Western blot using anti-LT antiserum as the primary antibody showed each epitope-ovalbumin fusion was recognized (Fig. 2C). When they were immobilized on wells of enzyme-linked immunosorbent assay (ELISA) plates, epitope-ovalbumin fusion proteins reacted with anti-LT antiserum at a similar level, indicated by similar optical density (OD) readings. Interestingly, anti-CT (cholera toxin) antiserum reacted with epitope-ovalbumin fusions differently, likely because of the heterogeneity of LT epitopes and CT counterpart epitopes (Table 1). Western blotting showed anti-CT reacted strongly with e3-ovalbumin and e4-ovalbumin fusion proteins (fusion proteins carrying the LT$_A$ epitope 3 or 4) and moderately with e7-ovalbumin fusion, but weakly reacted with the rest of the epitope fusions. Anti-CT antiserum reacted with all epitope-ovalbumin fusion proteins immobilized in wells of ELISA plates, with greater reactivity with e3-ovalbumin and e4-ovalbumin fusions.
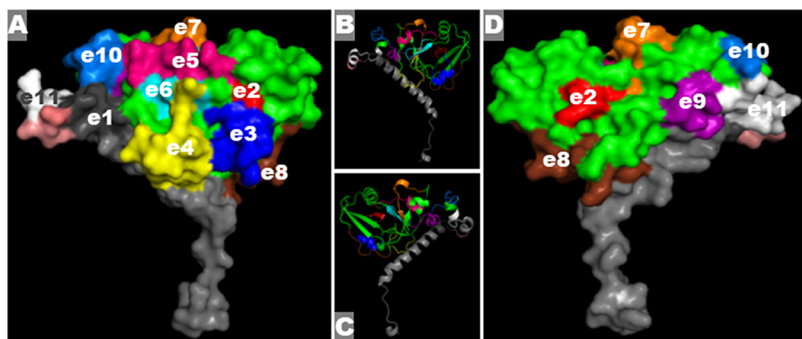

FIG 1 *In silico* epitope identification and protein computational modeling to show epitopes of the A subunit of LT (LT$_A$). (A and D) The front (A) and back (D) of the LT$_A$ protein model show the LT$_A$ subunit structure and 11 LT$_A$ epitopes (in different colors). Green, LT$_{A1}$ domain backbone; gray, LT$_{A2}$ domain. (B and C) Secondary structure of LT$_A$ subunit, with identified epitopes in the same colors used in panels A and D.
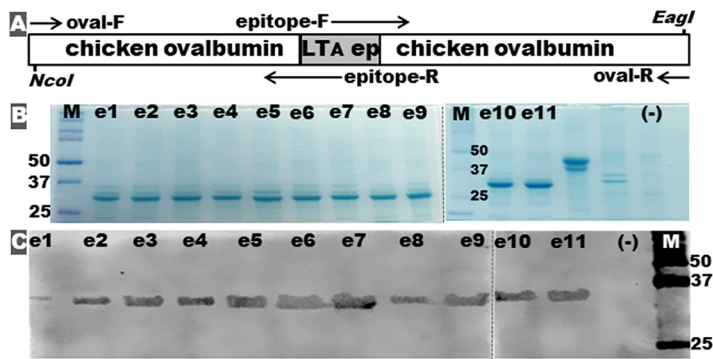
FIG 2 Construction and detection of epitope-ovalbumin fusion proteins. (A) Illustration of the genetic structure of an epitope-ovalbumin fusion gene, with PCR primers used to insert each LT_A epitope into the intron-truncating chicken ovalbumin gene. (B) SDS-PAGE Coomassie blue staining shows each extracted epitope-ovalbumin fusion protein (e1 to e11). (C) Western blot with anti-LT antiserum to characterize each epitope-ovalbumin fusion protein (e1 to e11). Note that e1 to e11 represent epitope-ovalbumin fusion proteins carrying the LT_A subunit epitope e1, e2, e3, e4, e5, e6, e7, e8, e9, e10, or e11. "(−)" refers to proteins of host strain *E. coli* BL21-CodonPlus (DE3). M, molecular marker (Bio-Rad Precision Plus Protein Dual Color Standards), with the 25-, 37-, and 50-kDa bands marked.

Mice immunized with epitope-ovalbumin fusion proteins developed anti-LT antibodies. Mice subcutaneously immunized with each epitope-ovalbumin fusion protein developed antibody response to LT (Fig. 3A). The anti-LT IgG titers (in $\log_{10}$) in the groups immunized with the fusion proteins carrying epitope e1, e2, e3, e4, e5, e6, e7, e8, e9, e10, or e11 were 1.78 ± 0.04, 1.77 ± 0.07, 2.58 ± 0.45, 1.67 ± 0.11, 1.75 ± 0.08, 2.08 ± 0.60, 3.42 ± 0.20, 2.13 ± 0.56, 2.24 ± 0.62, 2.04 ± 0.44, or 2.30 ± 0.76, respectively. The group immunized with the fusion carrying epitope 7 (e7) developed serum anti-LT IgG titers significantly greater than the other immunization groups ($P \leq 0.01$). Serum samples from the mice immunized with ovalbumin protein or phosphate-buffered saline (PBS) had no anti-LT antibody response detected.
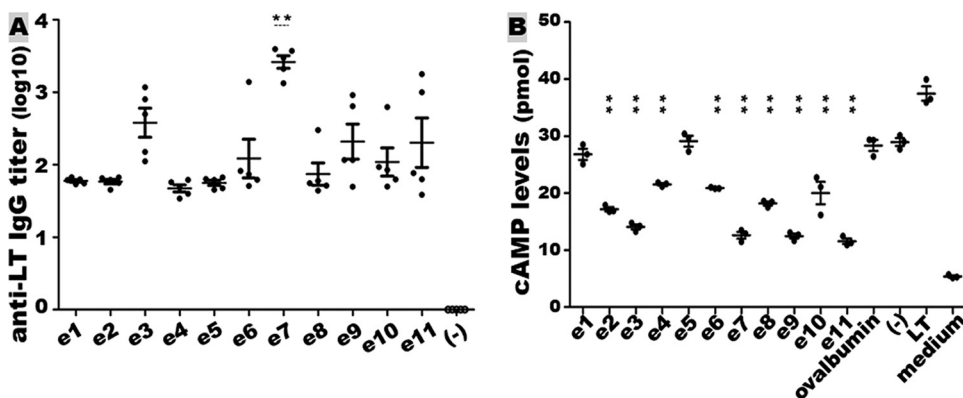

FIG 3 Mouse serum anti-LT IgG antibody titration and neutralization. (A) Anti-LT IgG titers ($\log_{10}$) from the group subcutaneously immunized with the epitope-ovalbumin fusion protein (e1 to e11) or the control group immunized with protein carrier ovalbumin (−). Bars indicate the mean titer in each group. e1 to e11 represent the groups of mice immunized with fusion proteins carrying different LT_{A1} epitopes. Each solid dot refers to an IgG titer from each mouse immunized with the epitope-ovalbumin fusion protein, and empty cycles for the titer of the control mice. **, $P < 0.01$ compared to all other immunized groups. (B) Mouse serum antibody *in vitro* neutralization activity against LT enterotoxicity. Mouse serum samples from the group immunized with each epitope-ovalbumin fusion protein (e1 to e11) and from two control groups immunized with ovalbumin protein (ovalbumin) or PBS (−) mixed with 20 ng of LT were incubated with T-84 cells. T-84 cell intracellular cAMP levels were measured with an EIA cAMP kit (Enzo Life Science). Portions (20 ng) of LT directly were added to T-84 cells to show enterotoxicity in the stimulation of cAMP levels in T-84 cells. Inclusion of T-84 cells in cell culture medium (without LT or serum) was to show baseline cAMP levels. "**" (in the vertical scale) indicates $P < 0.01$, when the cAMP levels in T-84 cells incubated with LT exposed to the immunized mouse sera were compared to the cAMP levels in T-84 cells incubated with LT exposed to the serum of the group immunized with ovalbumin or PBS.
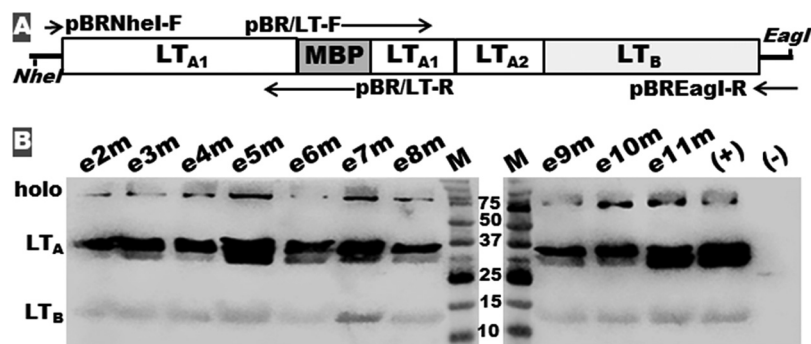
FIG 4 Illustration of the construction and detection of LT mutants e2m to e11m and LT with LT$_{A1}$ epitope e2, e3, e4, e5, e6, e7, e8, e9, e10, or e11 substituted by the MBP epitope KDAQTNSSS. (A) LT mutant genetic structure and chimeric gene construction. Primers pBR/LT-F and pBR/LT-R were specific for the substitution of each LT$_{A1}$ epitope with the MBP epitope. (B) Western blot with anti-LT antiserum to detect LT$_B$ subunit, mutated LT$_A$ subunit, and AB$_5$ holotoxin-structured LT mutants. Lanes: (+), LT from recombinant LT strain 8460; (−), proteins from host strain *E. coli* DH5α. M, molecular marker, in kilodaltons.

Mouse serum samples from the groups immunized with epitope-ovalbumin fusions except the fusions carrying e1 (e1 located at the nonenzymatic LT A2 domain) or e5 significantly prevented LT from stimulating intracellular cyclic AMP (cAMP) in T-84 cells (Fig. 3B), indicating antibody neutralization activities against LT enterotoxicity. The cAMP levels in T-84 cells incubated with 20 ng of LT exposed to the serum sample of mice immunized with e1-ovalbumin or e5-ovalbumin fusion were 27.6 ± 1.7 or 28.5 ± 2.2 (pmol/ml), respectively. These cAMP levels were not significantly different compared to the levels in T-84 cells incubated with LT exposed to the sera of mice immunized with PBS (29.8 ± 1.2 pmol/ml; $P = 0.10$, $P = 0.58$) or ovalbumin protein (29.3 ± 1.1 pmol/ml; $P = 0.17$, $P = 0.73$). The cAMP levels in T-84 cells incubated with LT exposed to the sera of mice immunized with e2-ovalbumin (17.3 ± 1.7 pmol/ml), e3-ovalbumin (13.7 ± 1.1 pmol/ml), e4-ovalbumin (21.7 ± 0.7 pmol/ml), e6-ovalbumin (20.9 ± 1.4 pmol/ml), e7-ovalbumin (12.2 ± 0.9 pmol/ml), e8-ovalbumin (18 ± 0.7 pmol/ml), e9-ovalbumin (12.2 ± 0.7 pmol/ml), e10-ovalbumin (21.9 ± 1.1 pmol/ml), or e11-ovalbumin (11.1 ± 0.7 pmol/ml) were significantly lower than the cAMP in the cells treated with LT exposed to the serum from mice immunized with PBS ($P < 0.01$) or ovalbumin ($P < 0.01$).

LT mutants with each LT$_{A1}$ epitope substituted by a foreign epitope retained AB$_5$ holotoxin structure and GM$_1$-binding ability. LT mutants were produced by replacing nucleotides coding each LT$_{A1}$ epitope of native *eltAB* genes with nucleotides coding maltose-binding protein (MBP) epitope KDAQTNSSS (Fig. 4A). All 10 LT mutants formed AB$_5$ holotoxin structure. Western blotting with anti-LT antiserum detected LT$_B$ subunit, LT$_A$ subunit, and holotoxin mutant proteins from each mutant strain and control CT, but not host strain DH5α (Fig. 4B).

Holotoxin-structured LT mutants were secreted across outer membrane. LT mutant proteins recovered from bacterial culture filtrates bound GM$_1$ (Fig. 5A). GM$_1$ ELISA using antibodies specific to LT$_A$ subunit detected holotoxin-structured GM$_1$-binding LT proteins from each mutant strain. The ELISA OD values were similar to the OD detected from the culture filtrate of LT recombinant strain 8460. GM$_1$-binding activity was not detected from proteins of host strain DH5α.

LT mutants with an LT$_{A1}$ epitope substituted showed no LT enterotoxicity. LT showed no enterotoxicity *in vitro* after each individual LT$_{A1}$ epitope substituted with the MBP epitope. T-84 cells incubated with overnight growth filtrates of each LT mutant strain showed no elevation of intracellular cAMP levels (Fig. 5B). The overall cAMP levels in T-84 cells treated with 10 LT mutant filtrates were 6.2 ± 2.2 pmol/ml, which were not different than the baseline cAMP levels in cells treated with PBS, which were 5.6 ± 0.1 pmol/ml ($P = 0.10$). In contrast, a significant elevation of cAMP levels was detected in
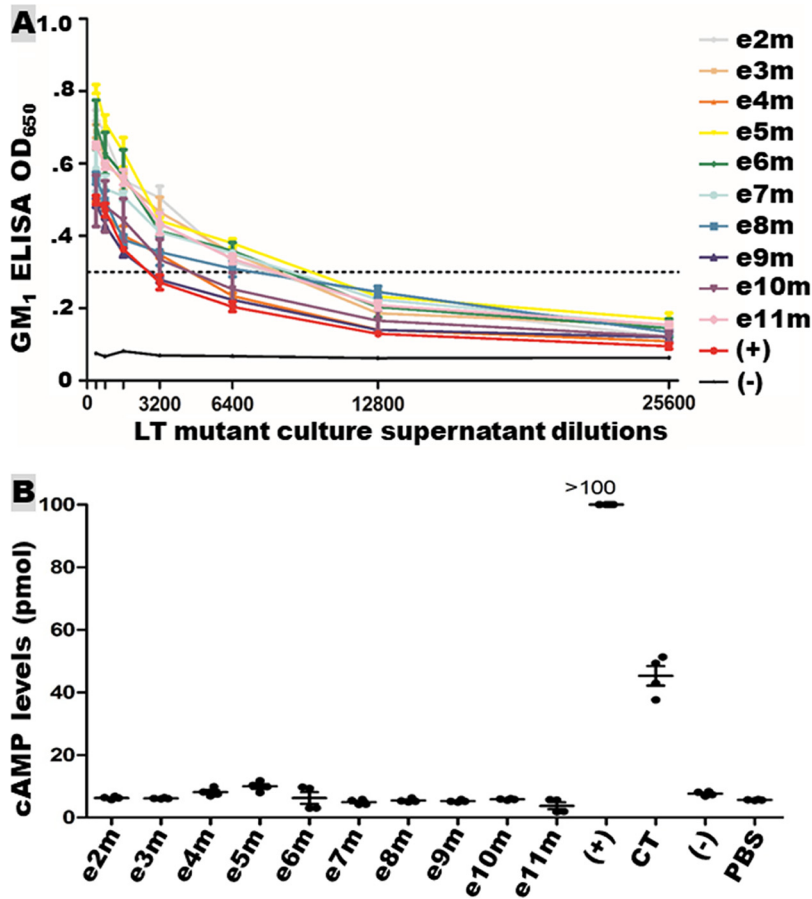
FIG 5 Holotoxin-structured LT mutant protein secretion and enterotoxicity. (A) GM_1 ELISA to show LT mutant protein across outer membrane secretion and GM_1-binding activity. Overnight culture filtrates of LT mutants e2m to e11m (LT toxin with epitope e2, e3, e4, e5, e6, e7, e8, e9, e10 or e11 substituted with MBP epitope KDAQTNSSS), positive strain 8460, and negative-control strain *E. coli* DH5α were used in GM_1 ELISA. Mouse antiserum specific to LT_A detected only the LT_A peptide linked to LT_B pentamer (the A subunit of AB_5 LT mutants). (B) LT mutant protein enterotoxicity detected in T-84 cells using an EIA cAMP kit (Enzo Life Sciences). Overnight growth filtrates (75 μl) from each LT mutant (across outer membrane secreted LT mutant proteins), positive-control strain 8460, or negative-control strain *E. coli* DH5α were added to T-84 cells for stimulation of intracellular cAMP levels (pmol). Portions (10 ng) of CT and PBS were added to T-84 cells to show CT enterotoxicity in elevating cAMP and baseline cAMP levels, respectively.

T-84 cells incubated with 10 ng of CT ($P < 0.001$) or the filtrates of LT-producing *E. coli* strain 8460 ($P < 0.001$) (Fig. 5B).

DISCUSSION

Epitopes from LT_A subunit particularly the A1 domain were investigated for the significance in LT immunogenicity and enterotoxicity in this study. While LT A2 peptide associates LT_A subunit to the LT_B pentamer to form LT AB_5 holotoxin and LT_B subunits (pentamer) bind GM_1 receptor, LT A1 peptide is the enzymatic domain responsible for LT enterotoxicity. Consequently, while antibodies specific to LT A2 peptide and LT_B subunit may disrupt the formation of LT holotoxin or block LT binding to host receptor GM_1, it is antibodies derived from the A1 peptide that are expected to directly neutralize LT enterotoxicity. Data from the present study showed that while all *in silico*-identified epitopes from the LT A1 peptide (when inserted in chicken ovalbumin protein) reacted with anti-LT antiserum and induced anti-LT antibodies in subcutaneously immunized mice, epitopes 3, 7, 9, and 11 induced greater anti-LT IgG antibody responses. Among them, epitope 7 was significantly effective at inducing anti-LT IgG antibody. *In vitro* antibody neutralization study has indicated that while antibodies

derived from all epitopes except e1 and e5 showed neutralizing activity, antibodies derived from epitopes 3, 7, 9, and 11 were more effective in neutralizing LT enterotoxicity. Antibodies derived from epitope 1 (e1) did not show any neutralizing activity against LT enterotoxicity, confirming that A2 peptide plays no role in LT enzymatic activity and that antibodies derived from A2 do not neutralize LT enterotoxicity. It remained unclear why antibodies induced by immunodominant epitope 5 showed no neutralizing activity. This may suggest that immunodominant epitopes are not necessarily neutralizing epitopes. It needs to be pointed out that the present study used the same volume of mouse serum from each group in an LT enterotoxicity neutralization assay; thus, the greater neutralization activity against LT from antibodies derived from fusions with 3, 7, 9, and 11 epitopes may result from higher anti-LT antibody titers. Future studies using standardized anti-LT antibodies or the same amount of mouse serum anti-LT antibodies (e.g., μg) from each group or *in vivo* studies may measure better anti-LT neutralizing activities.

Results from this study suggested that LT, if serving as a carrier protein, can have any of the LT A1 epitopes potentially substituted with foreign epitopes. However, if LT is also used as an antigen for ETEC vaccine development, epitopes 7, 9, 11, and/or 3 need to be retained to induce LT$_{A1}$-specific antibodies to neutralize LT enterotoxicity. Data from this study showed that a replacement of any one of the LT$_{A1}$ epitopes with a foreign epitope resulted in a complete loss of LT enterotoxicity *in vitro*. It was reported that substitution of a single amino acid residue at certain regions of the LT A1 peptide can significantly affect LT enterotoxicity (38). Indeed, LT mutated at R7K, V53D(E), S61F, S63K, A72R, V97K(Y), Y104K(D/S), E110D, E112K, S114K(E), or R192G showed abolition or reduction of toxicity (38–42). Replacing an epitope (10 to 13 amino acid residues) can alter LT$_A$ subunit binding to NAD, thus abolishing LT ribosylating and enterotoxic activities, particularly for epitopes 2, 6, 7, and 11 that are at or near the NAD binding site. Future *in vivo* studies will help to confirm or better characterize the enterotoxicity of these LT epitope mutants.

Data also indicated that LT mutants, after having an individual epitope substituted with a foreign epitope, still formed an AB$_5$ holotoxin structure. It was reported that certain individual amino acid residues have a significant effect on LT holotoxin structure formation or stability (38, 43). While LT mutants R7K, V53D, S63K, V97K, and Y104K formed a stable holotoxin structure, with only a reduction in holotoxin yield (43), mutants with a mutation at other residues, for example, L41F, A45Y or A45E, V60G, and H70P, showed a poor assembly of AB$_5$ structure (38). Data from the present study showed that all epitope mutants formed an AB$_5$ holotoxin structure. However, this study did not quantitatively measure mutant AB$_5$ holotoxin stability. Western blotting examining periplasm proteins suggested variations in the yield of LT$_A$ subunit and holotoxin structured LT mutant proteins (Fig. 4B). GM$_1$ ELISA to analyze proteins across the outer membrane indicated that all epitope mutants secreted AB$_5$ LT proteins at a similar level.

GM$_1$ ELISA to examine LT proteins across the outer membrane suggested that LT with an individual epitope substituted remained bound to the GM$_1$ receptor. Since the *eltA* gene coding the LT$_A$ subunit and the *eltB* gene coding the LT$_B$ subunit have individual promoters and ribosome-binding sites, the two *elt* genes transcribe and translate independently. In addition, the B subunits assembled into the LT$_B$ pentamer and noncovalently associated with the LT$_A$ subunit to form LT holotoxin in bacterial periplasm. Without altering the *eltB* gene structure, all LT epitope mutants were expected to produce LT$_B$ subunits and LT$_B$ pentamer, just like native LT. GM$_1$ ELISA using antibodies specific to the LT$_A$ subunit in the present study detected only the LT proteins which were assembled in the AB$_5$ structure, secreted across the outer membrane, and bound to the GM$_1$ receptor.

A nontoxic, GM$_1$-binding LT molecule secreted across the outer membrane can be an ideal protein carrier and an antigen delivery vehicle for epitopes from heterogeneous virulence factors to target host mucosal immunity. Such a molecule can serve a versatile MEFA (multiepitope fusion antigen) platform for the development of multi-

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
BL21-CodonPlus (DE3)	F$^-$ ompT hsdS(r$_B^-$ m$_B^-$) gal dcm	Agilent Technologies
9511	3×STa-chicken ovalbumin fusion + pET28α in DH5α Kan$^+$	37
9529	e1-ovalbumin + pET28α in BL21 Kan$^+$	This study
9546	e2-ovalbumin + pET28α in BL21 Kan$^+$	This study
9550	e3-ovalbumin + pET28α in BL21 Kan$^+$	This study
9547	e4-ovalbumin + pET28α in BL21 Kan$^+$	This study
9551	e5-ovalbumin + pET28α in BL21 Kan$^+$	This study
9552	e6-ovalbumin + pET28α in BL21 Kan$^+$	This study
9548	e7-ovalbumin + pET28α in BL21 Kan$^+$	This study
9543	e8-ovalbumin + pET28α in BL21 Kan$^+$	This study
9553	e9-ovalbumin + pET28α in BL21 Kan$^+$	This study
9544	e10-ovalbumin + pET28α in BL21 Kan$^+$	This study
9549	e11-ovalbumin + pET28α in BL21 Kan$^+$	This study
8460	eltAB genes + pBR322 in TOP10, LT recombinant strain	25
9646	LT mutant with e2 substituted with MBP epitope, in DH5α Amp$^+$	This study
9647	LT mutant with e3 substituted with MBP epitope, in DH5α Amp$^+$	This study
9648	LT mutant with e4 substituted with MBP epitope, in DH5α Amp$^+$	This study
9649	LT mutant with e5 substituted with MBP epitope, in DH5α Amp$^+$	This study
9650	LT mutant with e6 substituted with MBP epitope, in DH5α Amp$^+$	This study
9651	LT mutant with e7 substituted with MBP epitope, in DH5α Amp$^+$	This study
9652	LT mutant with e8 substituted with MBP epitope, in DH5α Amp$^+$	This study
9653	LT mutant with e9 substituted with MBP epitope, in DH5α Amp$^+$	This study
9654	LT mutant with e10 substituted with MBP epitope, in DH5α Amp$^+$	This study
9655	LT mutant with e11 substituted with MBP epitope, in DH5α Amp$^+$	This study
Plasmids		
pBR322		Promega
pMAL-c5X		NEB
pET28a		Novagen

valent mucosal vaccines against enteric or other pathogens. MEFA is a structure-based vaccine technology to carry and to present multiple heterogeneous epitopes to induce broad antibody responses. Currently, this MEFA technology is mainly applied to produce chimeric proteins for development of multivalent parenteral vaccines. With outer membrane secretion and GM$_1$-binding ability, this LT-platform MEFA can be used for mucosal vaccine development in general. The LT epitope mutants examined in this study had a single epitope substituted with a foreign epitope; future studies using multiple epitopes simultaneously replaced by different foreign epitopes are needed to confirm a stable AB$_5$ structure and GM$_1$-binding activities. MEFA technology using protein modeling and molecule dynamics simulation can optimize epitope substitution for stable protein structure (35). Indeed, a tripartite LT mutant, which had LT segments replaced by porcine ETEC adhesin K88 and F18 subunit domains, not only formed the AB$_5$ holotoxin structure and bound GM$_1$ but also induced antigen-specific protective mucosal immunity (29). A recent study showed that replacing LT$_{A1}$ epitopes 2, 4, 5, 6, 8, 9, and 10 with seven epitopes from pig ETEC fimbrial adhesins K88 and F18, STb, Stx2e, and STa toxoid (with epitope 3 and 7 as LT antigens to induce antibodies neutralizing LT enterotoxicity) did not affect holotoxin formation, protein secretion, or binding to GM$_1$ (data not shown). Replacing these LT$_{A1}$ epitopes with epitopes of human ETEC CFA adhesins (CFA/I, CS1-CS6) and an STa toxoid, we can develop a multivalent ETEC vaccine to target small intestinal mucosal immunity. Similarly, by replacing LT A1 epitopes with neutralizing epitopes from the virulence factors of other enteric pathogens, effective multivalent vaccines can be developed against different enteric diseases.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 2. Recombinant strain 9511 which carries intron-truncated chicken ovalbumin gene (37) was used as the template to construct epitope-ovalbumin fusion genes. Vector pET28a (Novagen, Madison, WI) was used to clone epitope-ovalbumin fusion genes; E. coli strain BL21-CodonPlus (DE3) (Agilent Tech-

TABLE 3 PCR primers used to construct epitope-ovalbumin fusions in this study

Primer	DNA sequence (5′–3′)[a]
oval-F	CATGCCATGGGCGGACCCGGACCTGGTA
oval-R	TATCGGCCGTTAGGTCCGGGTCCTTCGT
LT-e1F	ggtgatacttgtaatgaggagacccagGATGAAGATACCCAGGCGATGCCGTTTCGC
LT-e1R	ctcattacaagtatcacctgtaattgtTTTAAACGCTTTTTCCCACAGGCCTTTAAA
LT-e2F	ggcgacaaattataccgtgctgactctagaGATGAAGATACCCAGGCGATG
LT-e2R	acggtataatttgtcgccatttgcataTTTAAACGCTTTTTCCCACAG
LT-e3F	agatgaaataaaacgttccgggaggtGATGAAGATACCCAGGCGATG
LT-e3R	gaacgttttatttcatctgggggtctagagtcTTTAAACGCTTTTTCCCACAG
LT-e4F	aatgagtacttcgatagaggaactcaaGATGAAGATACCCAGGCGATG
LT-e4R	tctatcgaagtactcattatgccctctTTTAAACGCTTTTTCCCACAG
LT-e5F	agaggaacacaaaccggcGATGAAGATACCCAGGCGATG
LT-e5R	gccggtttgtgttcctctcgcgtgatcataTTTAAACGCTTTTTCCCACAG
LT-e6F	gatgacggatatgtttccacttctGATGAAGATACCCAGGCGATG
LT-e6R	ggaaacatatccgtcatcatatctTTTAAACGCTTTTTCCCACAG
LT-e7F	ccatatgaacaggaggtttctgcgGATGAAGATACCCAGGCGATG
LT-e7R	aacctcctgttcatatgggtgagggctTTTAAACGCTTTTTCCCACAG
LT-e8F	aacagggaatatagagaccggtatGATGAAGATACCCAGGCGATG
LT-e8R	gtctctatattccctgttacgatgTTTAAACGCTTTTTCCCACAG
LT-e9F	gcagaggatggttacagattagcaggtGATGAAGATACCCAGGCGATG
LT-e9R	tctgtaaccatcctctgccggagcTTTAAACGCTTTTTCCCACAG
LT-e10F	accggatcaccaagcttggagagaagaaGATGAAGATACCCAGGCGATG
LT-e10R	caagcttggtgatccggtgggaaacctgcTTTAAACGCTTTTTCCCACAG
LT-e11F	ccacaaggttgtggaaattcatcaagaacaGATGAAGATACCCAGGCGATG
LT-e11R	atttccacaaccttgtggtgcatgatgTTTAAACGCTTTTTCCCACAG

[a]Underlined nucleotides indicate NcoI and EagI restriction sites; nucleotides in lowercase are from each LT~A~ epitope.

nologies, Santa Clara, CA) was used to express recombinant epitope-ovalbumin fusion proteins. Recombinant *E. coli* strains were cultured in Luria broth (LB) medium supplemented with kanamycin (30 μg/ml).

LT mutants were constructed by using LT recombinant strain 8460 (25), which had the native *eltAB* genes cloned in pBR322 (Promega, Madison, WI) to express holotoxin-structured GM$_1$-binding LT toxin, as the template. Mutant LT genes with nucleotides coding each LT$_{A1}$ epitope substituted with MBP epitope KDAQTNSSS were cloned in pBR322 vector and expressed in *E. coli* strain DH5α (Promega).

LT$_A$ epitope *in silico* identification and protein computational modeling. LT$_A$ subunit B-cell continuous epitopes were predicted using B-cell epitope prediction software (44, 45). Peptides that have the highest antigenicity scores were identified as epitopes. Protein modeling was carried out by using Phyre2 online server (36) based on amino acid sequences; the secondary structure stability of epitope-ovalbumin fusion proteins was assessed using CHARMM (46), as previously described (35).

Epitope-ovalbumin fusion gene construction. Nucleotide fragments coding each *in silico* identified LT$_A$ epitope were genetically fused to a modified chicken ovalbumin gene which had introns truncated (37), by using splicing overlap extension (SOE) PCR and specific primers (Table 3). Two PCR products amplified with the primers oval-F/epitope-R and epitope-F/oval-R (primers epitope-F and epitope-R were specific to each LT$_A$ epitope) were overlapped. Each overlapped fragment was digested with restriction enzymes EagI and NcoI (New England BioLabs, Ipswich, MA). Digested epitope-ovalbumin products were ligated into expression vector pET28α and expressed as tag-less proteins in *E. coli* strain BL21-CodonPlus (DE3).

Epitope-ovalbumin fusion protein expression and characterization. Epitope-ovalbumin fusion proteins that carried each LT$_A$ epitope (e1 to e11) were expressed and purified as described previously (33, 34, 37). Briefly, a single colony from each epitope-ovalbumin fusion recombinant strain was cultured in 5 ml of LB medium supplemented with kanamycin (30 μg/ml) at 37°C overnight. Next, 2 ml of overnight bacterial culture was added to 200 ml of 2× yeast extract tryptone (Fisher Scientific, Waltham, MA) medium supplemented with kanamycin (30 μg/ml) and cultured at 37°C. After the culture OD reached 0.5 to 0.7, bacteria were induced with 30 μM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma, St. Louis, MO) and cultured for four more hours. Bacteria pellets collected from centrifugation (12,000 rpm for 15 min) were suspended in 10 ml of bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, Rochester, NY) to collect inclusion body proteins according to the manufacturer's protocol.

Extracted inclusion body proteins after three to five washes with PBS were solubilized and refolded by following a protein refolding protocol (Novagen). Briefly, each extracted epitope-ovalbumin fusion protein was mixed with 1× IB solubilization buffer {50 mM CAPS [3-(cyclohexylamino)propanesulfonic acid] [pH 11.0]} supplemented with 0.3% *N*-lauroylsarcosine and 1 mM dithiothreitol (DTT), followed by incubation at room temperature for 40 min. The suspension was centrifuged at 12,000 rpm for 20 min at room temperature to collect solubilized proteins (in supernatant). Proteins in supernatant were transferred to molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA), refolded using dialysis buffer (20 mM Tris-HCl [pH 8.5]) supplemented with 0.1 mM DTT at 4°C for 3 to 4 h, dialyzed in dialysis buffer without DTT with two to three buffer changes, and collected and stored at −80°C.

Portions (10 μg) of each refolded epitope-ovalbumin fusion protein were examined by using sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. Electrophoresed proteins were visualized with Coomassie blue straining and characterized in a Western blot using anti-LT mouse serum or anti-CT rabbit polyclonal antiserum (Sigma) and IRDye-labeled goat anti-mouse or anti-rabbit IgG secondary antibodies (1:10,000 dilution; LI-COR, Lincoln, NE), as described previously (31, 47, 48).

The immunodominance of each recombinant epitope-ovalbumin fusion protein was examined by ELISA with anti-LT or anti-CT antiserum. Each fusion protein (500 ng per well) was used to coat wells of Immulon 2HB plates (Thermo Fisher Scientific) at 37°C for 1 h, followed by 4°C overnight. Wells were incubated with 10% skim milk (in PBS) at 37°C for 1 h, followed by three washes with PBS–0.05% Tween 20 (PBST). Wells were incubated with 2-fold-diluted anti-LT mouse serum or anti-CT rabbit serum (1:200 to 1:12,800) at 37°C for 1 h. After three washes with PBST, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG (Sigma; 1:3,000) was added to each well. Wells were incubated at 37°C for 1 h, washed with PBST, and combined with 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) Microwell peroxidase substrate (KPL, Gaithersburg, MD) at room temperature for 30 min. The OD_{650} was recorded to measure the epitope reactivity with anti-LT or anti-CT antiserum.

Epitope-ovalbumin fusion protein mouse immunization. Eight-week-old female BALB/c mice (Charles River Laboratories International, Inc., Wilmington, MA), five per group, were subcutaneously immunized with each epitope-ovalbumin fusion protein. Portions (40 μg) of fusion protein (in 25 μl of PBS) mixed with 25 μl of Montanide ISA 51 VG (Seppic, Fairfield, NJ) were injected into each mouse. Two control groups were included: one immunized with the modified chicken ovalbumin protein (40 μg in 25 μl) and ISA adjuvant (25 μl) and the other immunized with sterile PBS (50 μl). Immunized mice received two boosters at the same dose of the primary in an interval of 2 weeks. Mice were euthanized 2 weeks after the second booster. The mouse immunization study complied with the Animal Welfare Act by following the 1996 National Research Council guidance and was approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC 3800).

Epitope-ovalbumin fusion-induced anti-LT IgG antibody titration. The anti-LT IgG antibody response was examined from each mouse serum sample as described previously (32, 47, 48). Briefly, LT (a gift from John Clements, Tulane University; 200 ng per well) was coated in wells of Immulon 2HB 96-well plates (Thermo Fisher Scientific). Wells washed with PBST were incubated with 10% skim milk at 37°C for 1 h to block uncoated sites, followed by three washes with PBST and incubation with 2-fold-diluted mouse serum samples (1:400 to 1:25,600). Wells were washed with PBST, incubated with HRP-conjugated goat anti-mouse IgG secondary antibodies (1:3,000; Sigma), and then washed with PBST and incubated with TMB Microwell peroxidase substrate (KPL). OD_{650} readings were used to calculate anti-LT antibody titers, as previously described (32, 33, 47–49).

Epitope-ovalbumin fusion-induced anti-LT antibody neutralization assay. Serum samples pooled from five mice in each group were used for an antibody neutralization assay with T-84 cells (American Type Culture Collection [ATCC], Fairfax, VA) and an EIA cAMP kit (Enzo Life Sciences, Inc., Farmingdale, NY) as described previously (24, 25, 32, 47). Briefly, 30 μl of serum sample pooled from each immunization group or the control group (in duplicate) was incubated with 20 ng of LT for 30 min at room temperature. Each serum-toxin mixture was transferred to T-84 cells (10^5 cells per well) cultured in Dulbecco's modified Eagle medium-F12 (DMEM-F12) medium (ATCC) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) in a 24-well microplate (Corning, Inc., Corning, NY). After incubation for 3 h in a CO_2 incubator, the T-84 cells were lysed. Collected cell lysates were measured for intracellular cAMP levels (pmol or pmol/ml) with an EIA cAMP kit according to the manufacturer's protocol (Enzo Life Sciences). LT alone (without serum) was used as a positive control to show the LT enterotoxicity in stimulation of cAMP levels in T-84 cells, and culture medium only (without toxin or serum) was used as a control to show the T-84 cell baseline intracellular cAMP levels.

LT mutant construction and expression. Native *eltAB* genes cloned in pBR322 from recombinant strain 8460 (Table 2) and specifically designed primers (Table 4) were used in PCRs to replace each LT_{A1} epitope with MBP epitope KDAQTNSSS for LT mutants. SOE PCR to overlap two fragments, one amplified with the primers pBRNheI-F and pBR/LT-R and the other amplified with the primers pBR/LT-F and pBREagI-R, generated mutated LT genes (Fig. 4). Primers pBR/LT-R and pBR/LT-F were specific for the replacement of each epitope (Table 4). Overlapped PCR products were digested with NheI and EagI restriction enzymes; digested products were ligated into pBR322 with T4 DNA ligase (New England BioLabs).

The expression of each mutant LT was verified by SDS-PAGE Western blotting with anti-LT antiserum. Overnight growth culture pellets from each LT mutant strain, positive strain 9460 and negative-control strain DH5α, were used to extract total proteins with B-PER. Portions (20 μl) of the total proteins from each strain were used for SDS-PAGE. Recombinant LT strain 8460 and cholera toxin (CT; Sigma) were included as positive controls. Anti-LT serum (1:3,000; a gift from John Clements at Tulane University) was used as the primary antibody, and IRDye-labeled goat anti-mouse IgG (1:10,000; LI-COR) was used as the secondary antibody.

LT mutant secretion and GM_1-binding activity in GM_1 ELISA. GM_1 ELISA was used to examine LT mutant protein secretion and GM_1-binding activity, as described previously (50, 51). Since GM_1 (Sigma) was used as the ELISA coating antigen and antibodies specific to LT_A subunit were used as the primary antibodies, only the A subunit of AB_5 LT mutants (which bind GM_1) could be detected. Briefly, 100 μl of monosialo ganglioside GM_1 (4 μg/ml) was used to coat each well of 96-well Maxisorb plates (Nunc, Roskilde, Denmark) overnight at 4°C, followed by incubation with PBST–10% skim milk to block the uncoated sites. After three washes with PBST, wells were incubated with 100 μl of overnight growth filtrates from each LT mutant strain, positive control 8460, or negative-control strain DH5α. After

TABLE 4 PCR primers used to mutate *eltAB* genes by replacing nucleotides coding each LT$_{A1}$ epitope with maltose-binding protein epitope KDAQTNSSS in SOE PCRs

Primer	DNA sequence (5′–3′)^a
pBRNheI-F	TGCGGTAGTTTATCACAG
pBREagI-R	GTCCCTGATGGTCGTCATCT
pBR/LTe2-F	ACGCGCAGACTAATTCGAGCTCGagaccccccagatgaaata
pBR/LTe2-R	TCGAATTAGTCTGCGCGTCTTTatataatggcgatgctaa
pBR/LTe3-F	ACGCGCAGACTAATTCGAGCTCGggtcttatgcccagaggg
pBR/LTe3-R	TCGAATTAGTCTGCGCGTCTTTgtcagcacggtataattt
pBR/LTe4-F	CGCGCAGACTAATTCGAGCTCGcaaatgaatattaatctt
pBR/LTe4-R	CTCGAATTAGTCTGCGCGTCTTTtctgggcataagacctcc
pBR/LTe5-F	ACGCGCAGACTAATTCGAGCTCGggctttgtcagatatgat
pBR/LTe5-R	TCGAATTAGTCTGCGCGTCTTTataaagattaatattcat
pBR/LTe6-F	CGCGCAGACTAATTCGAGCTCGtctcttagtttgagaagt
pBR/LTe6-R	CTCGAATTAGTCTGCGCGTCTTTtctgacaaagccggtttg
pBR/LTe7-F	CGCGCAGACTAATTCGAGCTCGgcgttaggtggaatacca
pBR/LTe7-R	CTCGAATTAGTCTGCGCGTCTTTgctgtatacgcctaatac
pBR/LTe8-F	CGCGCAGACTAATTCGAGCTCGtattacagaaatctgaat
pBR/LTe8-R	CTCGAATTAGTCTGCGCGTCTTTatgtaatcgttcatcaat
pBR/LTe9-F	ACGCGCAGACTAATTCGAGCTCGggtttcccaccggatcac
pBR/LTe9-R	TCGAATTAGTCTGCGCGTCTTTagctatattcagatttct
pBR/LTe10-F	CGCGCAGACTAATTCGAGCTCGgaaccctggattcatcat
pBR/LTe10-R	CTCGAATTAGTCTGCGCGTCTTTtgctaatctgtaaccatc
pBR/LTe11-F	CGCGCAGACTAATTCGAGCTCGacaattacaggtgatact
pBR/LTe11-R	CTCGAATTAGTCTGCGCGTCTTTatgaatccagggttcttc

^{a}Nucleotides in uppercase from the pBT/LT forward or reverse primers are from the MBP epitope; nucleotides in lowercase are from the *eltA* gene (flanking each LT$_{A1}$ epitope).

incubation at 37°C for 1 h, the wells were incubated with 2-fold-diluted anti-LT$_A$ mouse polyclonal antibodies at 37°C for 1 h. After washes with PBST, each well was incubated with 100 μl of HRP-conjugated goat anti-mouse IgG (1:3,000 dilution; Sigma). Wells were washed with PBST and PBS and incubated with TMB Microwell peroxidase substrate to measure the OD$_{650}$ readings.

LT mutant enterotoxicity assay. The enterotoxicity of each LT mutant was examined using a cAMP EIA kit (Enzo Life Sciences) and T-84 cells as described above. Seventy-five microliter overnight growth filtrates from each LT mutant strain, positive strain 8460, or negative strain DH5α (in duplicate) were added to T-84 cells (10^5) in 24-well plates. Ten nanograms of CT (in 75 μl of PBS) and 75 μl of PBS were included in the assay as a positive control and a negative control, respectively. After 3 h of incubation in a CO_2 incubator, the T-84 cells were lysed with 200 μl (per well) of 0.1 M HCl. Cell lysates were measured for intracellular cAMP levels as described above.

Statistical analysis. Data were analyzed using Prism 5 software (GraphPad, La Jolla, CA). The results are presented as means and standard deviations. Differences between compared groups were calculated with one-way analysis of variance with a confidence interval of 95%. Calculated P values of <0.05 were considered significantly different between groups.

Accession number(s). The LT$_A$ protein model was deposited in the Protein Data Bank under PDB accession no. 1LTA.

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