

## Specificity of the Type II Secretion Systems of Enterotoxigenic *Escherichia coli* and *Vibrio cholerae* for Heat-Labile Enterotoxin and Cholera Toxin<sup>∇</sup>

Benjamin Mudrak<sup>1</sup> and Meta J. Kuehn<sup>1,2\*</sup>

Departments of Molecular Genetics and Microbiology<sup>1</sup> and Biochemistry,<sup>2</sup> Duke University Medical Center, Durham, North Carolina 27710

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**The Gram-negative type II secretion (T2S) system is a multiprotein complex mediating the release of virulence factors from a number of pathogens. While an understanding of the function of T2S components is emerging, little is known about what identifies substrates for export. To investigate T2S substrate recognition, we compared mutations affecting the secretion of two highly homologous substrates: heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* (ETEC) and cholera toxin (CT) from *Vibrio cholerae*. Each toxin consists of one enzymatic A subunit and a ring of five B subunits mediating the toxin's secretion. Here, we report two mutations in LT's B subunit (LTB) that reduce its secretion from ETEC without global effects on the toxin. The Q3K mutation reduced levels of secreted LT by half, and as with CT (T. D. Connell, D. J. Metzger, M. Wang, M. G. Jobling, and R. K. Holmes, *Infect. Immun.* 63:4091–4098, 1995), the E11K mutation impaired LT secretion. Results *in vitro* and *in vivo* show that these mutants are not degraded more readily than wild-type LT. The Q3K mutation did not significantly affect CT B subunit (CTB) secretion from *V. cholerae*, and the E11A mutation altered LT and CTB secretion to various extents, indicating that these toxins are identified as secretion substrates in different ways. The levels of mutant LTB expressed in *V. cholerae* were low or undetectable, but each CTB mutant expressed and secreted at wild-type levels in ETEC. Therefore, ETEC's T2S system seems to accommodate mutations in CTB that impair the secretion of LTB. Our results highlight the exquisitely fine-tuned relationship between T2S substrates and their coordinate secretion machineries in different bacterial species.**

Gram-negative bacteria have evolved a number of methods to secrete proteins into the extracellular milieu, with at least six specific secretion systems currently described (14, 30). Type II secretion (T2S), or the main terminal branch of the general secretory pathway, is a feature of a number of proteobacteria and has been shown to be required for pathogenesis and maintenance of environmental niches in a large number of species (5). The T2S system is a multiprotein complex of 12 to 15 components that spans the inner and outer membranes, allowing for the controlled release of certain folded proteins that have been directed to the periplasm through the Sec or Tat machinery (21). Aside from providing a means of exporting freely released virulence factors from plant, animal, and human pathogens (5), the T2S system has been shown to export surface-associated virulence factors (18), fimbrial components (46), outer membrane cytochromes (36), and a surfactant required for sliding motility in *Legionella pneumophila* (39), among other substrates.

While an increasing number of studies have focused on understanding the structure and function of the components of the T2S system itself, little is known about what identifies a periplasmic protein as a substrate for secretion (21, 32). Because proteins secreted from the same bacterial species need

not share any obvious structural homology, it is not even clear how much of a T2S substrate interacts with the secretion machinery (32). Analysis of two similar substrates that can each be secreted by the T2S systems of two distinct species would provide information about species-specific identification of T2S substrates and, by extension, the nature of the “secretion motif” identifying those substrates. Heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* (ETEC) and cholera toxin (CT) from *Vibrio cholerae* represent one such pair of substrates.

ETEC and *V. cholerae* are enteric pathogens causing significant morbidity and mortality worldwide (33). The causative agents of traveler's diarrhea and cholera, respectively, these two pathogens share a number of similarities, including the nature of their disease symptoms (38). Each pathogen secretes an AB<sub>5</sub> toxin important for colonization and the induction of water and electrolyte efflux from intestinal epithelial cells (1, 29). These toxins, LT and CT, are both encoded by two-gene operons. After *sec*-dependent transport to the periplasm, holotoxin formation occurs spontaneously (13), with one catalytic A subunit (LTA or CTA) assembling with five B subunits (LTB or CTB), which are responsible for the binding properties of the toxins. Export of fully folded and assembled LT or CT is then accomplished by the T2S system (34, 40). In ETEC, this system is encoded by *gspC* to *-M* (40), while in *V. cholerae*, these genes are found in the *eps* operon (34).

LT and CT are very similar in structure, sharing approximately 80% sequence homology and 83% identity in the mature B subunit (16, 24). ETEC is thought to have acquired the

\* Corresponding author. Mailing address: Department of Biochemistry, Duke University Medical Center, Box 3711, Durham, NC 27710. Phone: (919) 684-2545. Fax: (919) 684-8885. E-mail: mkuehn@biochem.duke.edu.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Relevant characteristics	Reference or source
<b>Strains</b>			
MK233	MT616/pRK2013	Helper strain for triparental mating; Cm <sup>r</sup>	9
jf570	H10407 $\Delta$ eltA	ETEC strain with polar insertion in <i>eltA</i> (LT deficient)	8
MK1052	H10407 $\Delta$ eltA/pILT	jf570 carrying inducible LT plasmid; Amp <sup>r</sup>	26
MK1053	H10407 $\Delta$ eltA/pTrec99A	jf570 carrying empty vector; Amp <sup>r</sup>	This work
MK1202	H10407 $\Delta$ eltA/pILT[L25E]	As MK1052, with inducible L25E LT mutant; Amp <sup>r</sup>	This work
MK1201	H10407 $\Delta$ eltA/pILT[Q3K]	As MK1202, with LT[Q3K]; Amp <sup>r</sup>	26
MK1239	H10407 $\Delta$ eltA/pILT[Q3A]	As MK1201, with LT[Q3A]; Amp <sup>r</sup>	This work
MK1231	H10407 $\Delta$ eltA/pILT[E11K]	As MK1201, with LT[E11K]; Amp <sup>r</sup>	This work
MK1240	H10407 $\Delta$ eltA/pILT[E11A]	As MK1201, with LT[E11A]; Amp <sup>r</sup>	This work
MK741	DH5 $\alpha$ <i>degP</i> ::Tn5/pDsbA/pILT	<i>E. coli</i> K-12 <i>degP</i> knockout, carrying a plasmid copy of <i>dsbA</i> and an inducible LT plasmid; Kan <sup>r</sup> Cm <sup>r</sup> Amp <sup>r</sup>	26
MK1101	DH5 $\alpha$ <i>degP</i> ::Tn5/pDsbA/pILTB	As MK741, with pILTB; Kan <sup>r</sup> Cm <sup>r</sup> Amp <sup>r</sup>	This work
P4	P27459 $\Delta$ ctxAB	CT-deficient El Tor <i>V. cholerae</i> strain; Str <sup>r</sup> Kan <sup>r</sup>	10, 11
MK1123	P4/pTrec99A	P4 carrying empty vector; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1225	P4/pICTB	P4 carrying inducible CTB plasmid; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1236	P4/pICTB[Q3K]	As MK1225, with inducible Q3K CTB mutant; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1232	P4/pICTB[E11K]	As MK1225, with CTB[E11K]; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1233	P4/pICTB[E11A]	As MK1225, with CTB[E11A]; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1242	DH5 $\alpha$ <i>degP</i> ::Tn5/pDsbA/pICTB	As MK741, with pICTB; Kan <sup>r</sup> Cm <sup>r</sup> Amp <sup>r</sup>	This work
MK1223	H10407 $\Delta$ eltA/pICTB	jf570 with inducible CTB plasmid; Amp <sup>r</sup>	This work
MK1224	H10407 $\Delta$ eltA/pICTB[Q3K]	As MK1223, with inducible Q3K CTB mutant; Amp <sup>r</sup>	This work
MK1234	H10407 $\Delta$ eltA/pICTB[E11K]	As MK1224, with CTB[E11K]; Amp <sup>r</sup>	This work
MK1235	H10407 $\Delta$ eltA/pICTB[E11A]	As MK1224, with CTB[E11A]; Amp <sup>r</sup>	This work
MK1122	P4/pILTB	P4 carrying inducible LTB plasmid; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1237	P4/pILTB[Q3K]	As MK1122, with inducible Q3K LTB mutant; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1243	P4/pILTB[Q3A]	As MK1122, with LTB[Q3A]; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1244	P4/pILTB[E11K]	As MK1122, with LTB[E11K]; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
MK1238	P4/pILTB[L25E]	As MK1122, with LTB[L25E]; Str <sup>r</sup> Kan <sup>r</sup> Amp <sup>r</sup>	This work
<b>Plasmids</b>			
pILT		IPTG-inducible LT holotoxin; Amp <sup>r</sup>	26
pILTB		IPTG-inducible LTB pentamer; Amp <sup>r</sup>	This work
pICTB		IPTG-inducible CTB pentamer; Amp <sup>r</sup>	This work

genes for CT through horizontal transfer, with the toxins evolving over time to possess slight differences (45). As such, these toxins share the same primary host receptor, the monosialoganglioside G<sub>M1</sub>, and catalyze the same ADP-ribosylation reaction within host cells (38). However, LT is able to bind other host sphingolipids in addition to G<sub>M1</sub> and to interact with sugar residues from the A-type blood antigen, which CT cannot bind (16, 41). Both LT and CT are able to associate with sugar residues in lipopolysaccharide (LPS) on the surface of *E. coli* cells (17). Binding to each of these substrates can be impaired by point mutation (26, 43).

In this study, we report point mutations impairing the release of LT from ETEC and CT from *V. cholerae*. We analyzed the specificity of the defects in substrate recognition by comparing the effects of substituting charged and neutral residues in key regions of LTB and CTB. To confirm that the identified mutations resulted specifically in a secretion defect, we tested the effect of the mutations on (i) ligand binding by each toxin, (ii) toxin stability, and (iii) formation of secretion-competent B-subunit pentamers. By introducing comparable mutations into both toxins, including one previously reported to impair the secretion of CT (6), and exchanging toxin substrates between the two species, we have revealed species-dependent differences in T2S substrate recognition. Although wild-type LT and CT can be heterologously expressed and secreted from *V. cholerae* and ETEC, respectively, the substrate residues

identified by the secretion machinery in each species are distinct. Together, our results demonstrate that highly homologous T2S substrates are recognized in different ways when secreted by two distinct systems.

#### MATERIALS AND METHODS

**Strains and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. Strains were grown at 37°C in LB or CFA (1% Casamino Acids, 0.15% yeast extract, 0.005% MgSO<sub>4</sub>, 0.005% MnCl<sub>2</sub>) and maintained on LB plates (LB with 15 g/liter agar; Genesee Scientific). When appropriate, antibiotics (Sigma) were added at the following concentrations: 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, and 50 µg/ml kanamycin. *E. coli* transformations were carried out using a CaCl<sub>2</sub> protocol as described previously (18). Plasmids were conjugated into *V. cholerae* by triparental mating as described previously (12), using DH5 $\alpha$  (Invitrogen) containing the appropriate plasmid as the donor strain and MT616 carrying pRK2013 as the helper strain. Selection of transconjugants was carried out on LB agar plates supplemented with kanamycin and ampicillin. Similar levels of expression of wild-type and mutant LT, LTB, or CTB constructs were detected after induction with a final concentration of 200 µM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG; Sigma), except for the CTB[Q3K] construct, which required induction with 50 µM IPTG. Y1 adrenal cells (ATCC CCL-79) were maintained in Kaighn's medium supplemented with 10% fetal calf serum (Sigma). Unless specified, reagents were purchased from VWR.

**Plasmid construction.** An IPTG-inducible LTB plasmid (pILTB) was generated by cloning the coding sequence of *eltB* from pILT (26). The gene was amplified by PCR using primers *eltb*-bamh1 and *eltAB*reverse, which include restriction sites for BamHI and PstI, respectively (Table 2). PCR products were digested with BamHI and PstI (New England Biolabs) and then ligated into a similarly digested pTrec99A vector to form pILTB.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3') <sup>a</sup>
eltb-bamh1	CGGGATCCCGGAATTCGGGATGAATTATGAA TAAAG
eltABreverse	AACCTGCAGAACCAATGCATTGGCTAGTTTCCA TACTGATTG
ltbL25Esense	CGATAAATGACAAGATAGAATCATATACGGA ATCG
ltbL25Eantisense	CGATTCCGTATATGATTTCTATCTTGTCAATT ATCG
ltbQ3Ksense	CACACGGAGCTCCTAAATCTATTACAGAAC TATG
ltbQ3Kantisense	CATAGTTCTGTAATAGATTTAGGAGCTCCG TGTG
ltbQ3Asense	CACACGGAGCTCCTGCATCTATTACAGAAC TATG
ltbQ3Aantisense	CATAGTTCTGTAATAGATGCAGGAGCTCCG TGTG
ltbE11Ksense	CAGAACTATGTTGCAAAATATCACAAACACAC
ltbE11Kantisense	GTGTGTTGTGATATTTGCAACATAGTTCTG
ltbE11Asense	CAGAACTATGTTGCGGCATATCACAAACACAC
ltbE11Aantisense	GTGTGTTGTGATATGCGGAACATAGTTCTG
CTB_Q3K_sense	GGCGCATGCAACCCCTAAAAATATTACTGA TTTG
CTB_Q3K_antisense	CAAATCAGTAATATTTTATAGGGGTTGCATG CGCC
CTBE11Ksense	CTGATTTGTGTGCAAAATACCACAACACAC
CTBE11Kantisense	GTGTGTTGTGTTATTTGACACAATCAG
CTB_E11A_sense	CTGATTTGTGTGAGCATACCACAACACAC
CTB_E11A_anti	GTGTGTTGTGTTATGCTGCACACAATCAG

<sup>a</sup> Restriction sites are italic; mutagenic codons are bold.

An IPTG-inducible CTB plasmid based on the pTrec99A vector (pICTB) was generated by cloning the *ctxB* gene from pLMPI1 (20). After restriction with SacI and KpnI (New England Biolabs), the digested DNA fragment containing *ctxB* was gel purified using the QIAquick gel extraction kit (Qiagen) and ligated into similarly digested pTrec99A (GE Healthcare) to form pICTB.

**Mutagenesis.** Site-directed mutagenesis was performed on pILT, pILTB, and pICTB using the QuikChange kit (Qiagen), according to the manufacturer's instructions. Primers used are listed in Table 2. All mutations were verified by sequencing at the Duke University DNA Analysis Facility or Eton Biosciences (Durham, NC) (data not shown). Mutants are named by combining the original amino acid residue, its position in the primary sequence of LTB or CTB after cleavage of the signal sequence, and the new substituted amino acid.

**Cell fractionation.** Strains were diluted 1:50 from overnight cultures, grown for 3 to 4 h at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of ~0.8, and induced 3 h (for CTB constructs) or overnight (for LT or LTB constructs). For some experiments, one Mini Complete protease inhibitor pellet (Roche) was dissolved per 10 ml of culture at the time of induction. Following induction and growth, a sample of total culture was precipitated with 20% trichloroacetic acid (TCA). Each strain was serially plated for CFU, and the remaining cells were pelleted at 10,000 × g for 1 min. Supernatant was collected, and periplasm was isolated by polymyxin B treatment essentially as described previously (19). Both supernatant and periplasmic fractions were passed through Durapore microcentrifuge filter devices (Millipore; 0.45 μm for ETEC fractions, 0.22 μm for *V. cholerae* fractions). For some experiments, a sample of cell-free supernatant was also precipitated with 20% TCA. Periplasmic alkaline phosphatase activity was measured using the SensoLyte kit (AnaSpec) according to the manufacturer's instructions.

**Immunoblotting.** To assess the expression of wild-type and mutant constructs, CFU-matched total culture samples were TCA precipitated, boiled in sample buffer, applied to 15% SDS-polyacrylamide gels, and immunoblotted with anti-CT antibody (1:15,000; Sigma), which is cross-reactive with LT, as described previously (19).

**Toxin purification.** Wild-type and mutant LT, LTB, and CTB were purified from strains MK741, MK1101, and MK1242 and derivatives thereof expressing the appropriate mutant constructs as described previously (7, 26). Briefly, periplasm was isolated using osmotic shock after growth in CFA media with ampicillin, and toxins or pentamers were purified by binding to immobilized D-galactose beads (Pierce). For the LT E11K and E11A mutants, 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) was added during purification at a final concentration of 1 mM to prevent degradation. Toxins and pentamers were assessed for purity by SDS-PAGE and protein staining with Ruby Red (Molecular Probes).

**ELISAs.** Enzyme-linked immunosorbent assays (ELISAs) for binding to the monosialoganglioside G<sub>M1</sub> (Sigma) and blood group A terminal trisaccharide (GalNAcα3[Fucα2]Gal) conjugated to bovine serum albumin (V-Labs) were performed as previously described (26). Purified toxin or pentamer was tested at a final concentration of 1 nM, and a different batch was used for each ELISA. Cell-free supernatant was diluted 1:3 (for ETEC expressing LT or LT mutants) or 1:20 (for ETEC or *V. cholerae* expressing CTB or CTB mutants); periplasm samples were diluted 1:20 after adjustment for levels of alkaline phosphatase activity.

**Toxin stability assay.** Purified toxin or pentamer was added at a final concentration of 50 nM to 1.5 ml of cell-free supernatant from a log-phase culture of toxin-deficient ETEC (MK1053) or *V. cholerae* (MK1123), each carrying the empty pTrec99A vector. One 0.5-ml aliquot was immediately frozen, one incubated at 37°C for 1 h (for LT and LT mutants) or 2 h (for CTB and CTB mutants), and one incubated at 25°C for 4 days. Levels of assembled toxin or pentamer remaining after the incubation were compared by G<sub>M1</sub> ELISA, with data normalized to the levels detected in the aliquot that had immediately been frozen. Each experiment was performed with a separate batch of purified pentamer and a fresh preparation of cell-free supernatant.

**Pronase sensitivity assay.** Purified wild-type or mutant LT or CTB (500 ng) was incubated with water or Pronase (Fluka) at the indicated final concentration and incubated for 1 h at 37°C. Afterward, samples were boiled for 10 min and subjected to SDS-PAGE, followed by staining with Ruby Red. Densitometry of the band corresponding to LTB or CTB was performed in the linear range using ImageJ software (NIH). Data are normalized to the intensity of the untreated LTB or CTB band.

**Toxicity assay.** A bioassay on Y1 cells was performed in duplicate as described previously (19), with at least two independent trials for each mutant toxin.

**In vitro pentamer reassembly assay.** Wild-type and mutant LTB pentamers were assessed for reassembly *in vitro* essentially as previously described (31). Briefly, purified wild-type or mutant LTB (5 μg) was treated with 0.1 M HCl for 1 min in a final volume of 20 μl to induce dissociation into monomers and then neutralized with 80 μl of McIlvaine's buffer (31). After incubation for the indicated times at 4°C, samples were diluted 1:50 into phosphate-buffered saline (pH 7.4) to halt reassembly and immediately frozen. Pentamer reassembly was assessed by a G<sub>M1</sub> binding ELISA. Each trial was carried out with a separate batch of pentamers.

**Cell surface binding assay.** Assessment of the binding of purified toxins to DH5α cells was performed using an immunoblot-based assay as previously described (26). Independent assays were carried out with fresh batches of purified toxin.

**Growth of *V. cholerae* on lipid agar and skim milk agar.** Overnight cultures of MK1225, MK1232, and MK1233 were matched for optical density at 600 nm, diluted 1:1,000, and plated on lipid agar (22) containing ampicillin and 200 μM IPTG.

After induction for 3 h as described above, 100 μl of 10<sup>-7</sup> dilutions of MK1225, MK1232, and MK1233 were plated on LB-skim milk agar (42) containing ampicillin and 200 μM IPTG. All plates were incubated for 36 h at 37°C.

**Statistical analysis.** Results were analyzed by Student's *t* test; two-tailed *P* values less than 0.05 were considered statistically significant. Error bars in the figures represent standard errors of the mean (SEM).

## RESULTS

**Mutations affecting the secretion of LT from ETEC.** In a previous study, we identified two mutations in LTB (L25E and Q3K) that independently reduced bacterial cell surface binding of the toxin (26). Upon further examination, when mutants LT[L25E] and LT[Q3K] were expressed in an LT-deficient ETEC strain (jf570), they were detected at wild-type levels in total culture samples but showed reduced levels in the cell-free supernatant (Fig. 1A and B). Consistent with a secretion defect, LT[Q3K] was found at increased levels in the periplasm (Fig. 1B). In contrast, periplasmic LT[L25E] was found at a level close to that of the wild type, suggesting that it did not accumulate in a presecreted form.

Since levels of the toxins in the supernatants were measured by a G<sub>M1</sub> binding ELISA, we examined whether these mutations affected the toxin's ability to bind G<sub>M1</sub> (Table 3). Purified

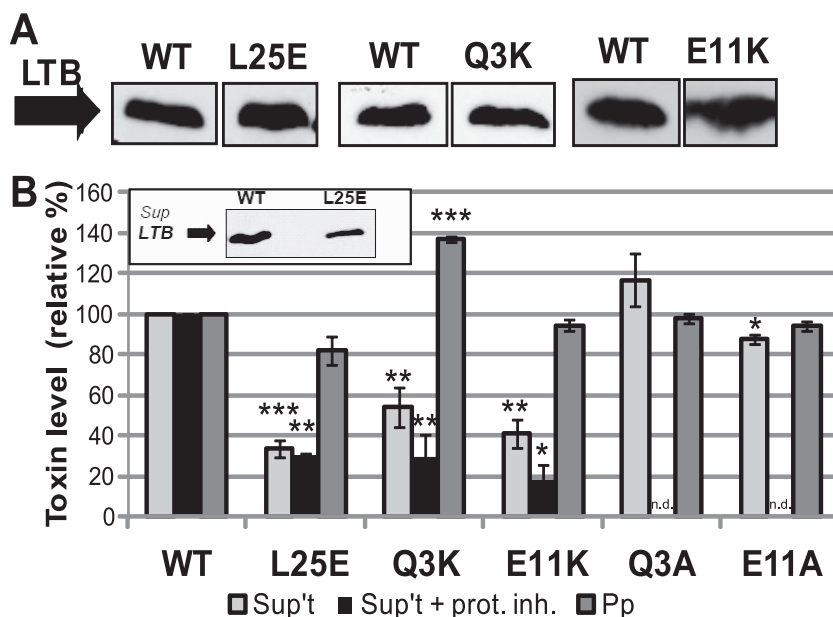


FIG. 1. Three mutations impair the secretion of LT from ETEC. (A) Representative immunoblots of TCA-precipitated total culture samples, adjusted for CFU, showing induced expression of wild-type LT (WT), LT[L25E], LT[Q3K], and LT[E11K] in strain jf570. Blots were probed with cross-reactive anti-CT antibody. (B) Strains expressing wild-type (WT) and the indicated mutant toxins were fractionated to isolate cell-free supernatant (Sup't) and periplasm (Pp). Each fraction was tested for toxin levels by G<sub>M1</sub> ELISA, with wild-type levels set to 100%. Supernatant levels were normalized to CFU, and periplasm levels were normalized to alkaline phosphatase activity. \*, *P* < 0.01; \*\*, *P* < 0.005; \*\*\*, *P* < 10<sup>-6</sup> compared to wild-type (*n* ≥ 3); n.d., not determined. For some experiments, cultures were grown in the presence of a protease inhibitor cocktail (+ prot. inh.) (*n* ≥ 2). Inset: representative immunoblot showing levels of wild-type LT (WT) and LT[L25E] secreted from jf570. Cell-free supernatant fractions were precipitated with TCA, and samples were adjusted for CFU. Blots were probed with cross-reactive anti-CT antibody.

LT[Q3K] showed wild-type levels of G<sub>M1</sub> binding. LT[L25E] bound G<sub>M1</sub> approximately 30% less efficiently than wild-type LT, which, although significant, could not be fully responsible for the observed 65% defect in secretion. To verify the apparent decrease in secretion of LT[L25E] with a G<sub>M1</sub>-independent assay, we immunoblotted cell-free supernatant. The results were consistent with a secretion defect for LT[L25E] (Fig. 1B, inset).

The substitution of lysine for glutamine in LT[Q3K] results in a relatively drastic amino acid change, and therefore, we wondered whether the observed secretion deficiency was due to the addition of a positive charge at that site in the protein.

We made other mutations of Gln-3 to explore the impact of the character of this residue on the secretion phenotype. LT[Q3L] was undetectable when expressed in jf570 (26); however, LT[Q3A] was stable when expressed in ETEC and could be studied (data not shown). LT[Q3A] was detected in the cell-free supernatant at levels slightly higher than those of the wild type, but the difference was not statistically significant, and periplasmic levels of the mutant were equivalent to those of wild-type LT (Fig. 1B). These results suggest that the presence of a charged amino acid at residue 3 impedes the secretion of LTb.

TABLE 3. Binding properties of LT and CTB mutants

Mutant	G <sub>M1</sub> binding <sup>a</sup>		Blood sugar binding <sup>c</sup>		<i>E. coli</i> surface binding <sup>d</sup>	
	%	<i>P</i> value <sup>b</sup>	%	<i>P</i> value	%	<i>P</i> value
LT[L25E]	<b>69.3 ± 5.3<sup>e</sup></b>	<0.005	<b>64.2 ± 7.2</b>	<0.01	<b>35.2 ± 10.9</b>	<0.005
LT[Q3K] <sup>f</sup>	96.0 ± 4.6	0.43	<b>0.67 ± 0.43</b>	<0.005	<b>33.8 ± 8.8</b>	<0.005
LT[Q3A]	95.5 ± 14.4	0.77	<b>4.0 ± 4.0</b>	2.3 × 10 <sup>-6</sup>	<b>36.5 ± 4.7</b>	<0.0005
LT[E11K]	90.9 ± 4.6	0.08	109.6 ± 17.8	0.65	<b>19.6 ± 9.3</b>	<0.001
LT[E11A]	81.5 ± 6.5	0.07				
CTB[Q3K]	<b>93.1 ± 1.9</b>	<0.05				
CTB[E11K]	94.7 ± 2.5	0.08				
CTB[E11A]	<b>106.7 ± 1.8</b>	<0.05				

<sup>a</sup> Purified toxin binding to G<sub>M1</sub> was determined by ELISA; values are expressed as mean percentages of wild-type LT or CTB binding ± SEM (*n* ≥ 3).  
<sup>b</sup> *P* values are for comparison to wild-type binding as determined by Student's *t* test.  
<sup>c</sup> Purified toxin binding to blood group A trisaccharide conjugated to BSA was determined by ELISA; values are expressed as mean percentages of wild-type binding ± SEM (*n* ≥ 2).  
<sup>d</sup> Purified toxin binding to the surface of DH5α *E. coli* cells was determined by a previously described immunoblot-based binding assay (26); values are expressed as mean percentages of wild-type LT binding ± SEM (*n* ≥ 3).  
<sup>e</sup> Significant differences from wild-type (*P* < 0.05) are in bold.  
<sup>f</sup> Data for this mutant were reported previously (26).



**LT secretion mutants show wild-type stability and pentamer assembly.** Degradation could have been responsible for the decreased detection of mutant LT in the cell-free supernatant, and therefore, we investigated the stability of the mutant toxins in that milieu using several methods. The addition of protease inhibitors to the culture media during growth did not change the observed secretion defect of LT[L25E] or LT[Q3K] (Fig. 1B). We also performed a stability assay similar to one described in a study of secretion-impaired mutants of *Pseudomonas aeruginosa* exotoxin A (23) and observed that both mutants were stable over time when purified and incubated with sterile-filtered supernatant from a log-phase ETEC culture (data not shown).

To further assess the stability of these mutants, they were tested for sensitivity to pronase, a protease previously reported to degrade LT (19). Wild-type and mutant LT were equally sensitive to the enzyme, with degradation products visible beginning at a pronase concentration of 50  $\mu\text{g/ml}$  and much of the full-length monomer broken down at a concentration of 500  $\mu\text{g/ml}$  (Fig. 2A and B). Thus, proteolytic degradation was not responsible for reduced detection of the LT[L25E] or LT[Q3K] mutant in the ETEC supernatant.

The first 10 amino acids of LTB have previously been reported to be involved in pentamer assembly, and pentamer assembly is critical for secretion (4, 15). Therefore, we determined whether the effect of the Q3K or L25E mutation on LT secretion was due to a decreased efficiency of pentamer assembly. LT's toxic effects require the interaction of LTA with the LTB pentamer, and thus, we used a toxicity assay to determine whether LTA associates with mutant LTB in a manner equivalent to that of wild-type LT. The toxicity of purified mutant LT was measured using an *in vitro* cell culture assay that is based on a morphological change in Y1 adrenal cells induced by LTA. Both mutants were as toxic as wild-type LT (8 ng of each elicited maximal rounding of  $4 \times 10^5$  cells), suggesting that periplasmic holotoxin formation was not affected by the L25E or Q3K mutation. Furthermore, each mutant behaved similarly to or better than wild-type LT in an established *in vitro* cell-free assay measuring pentamer reassembly after dissociation at extremely low pH (Fig. 2C) (31). Together, these assays indicated that the observed secretion defects for LT[L25E] and LT[Q3K] were not due to defects in their stability or assembly.

Despite the fact that the secretion-defective mutant toxins were wild type in their stability and assembly, we were interested in determining if the mutant toxins displayed more subtle differences in structure. We reasoned that such differences could be detected by measuring the ability of the mutants to bind each of the three major ligands of LT. Therefore, purified mutant toxins were assessed for binding to blood group A sugars and the surface of *E. coli*, in addition to  $G_{M1}$ , and compared to wild-type LT (Table 3). LT[Q3A] showed wild-type binding to  $G_{M1}$  but impaired binding to blood antigen and *E. coli*, consistent with the previously reported phenotypes of LT[Q3K] (26) (Table 3).

LT[L25E] was found to be impaired for binding to all substrates of LTB tested (Table 3). Therefore, although it is stable and assembles into a holotoxin-like wild-type toxin, LT[L25E] appears to have an external interaction surface that does not permit normal substrate recognition. Because of the potential

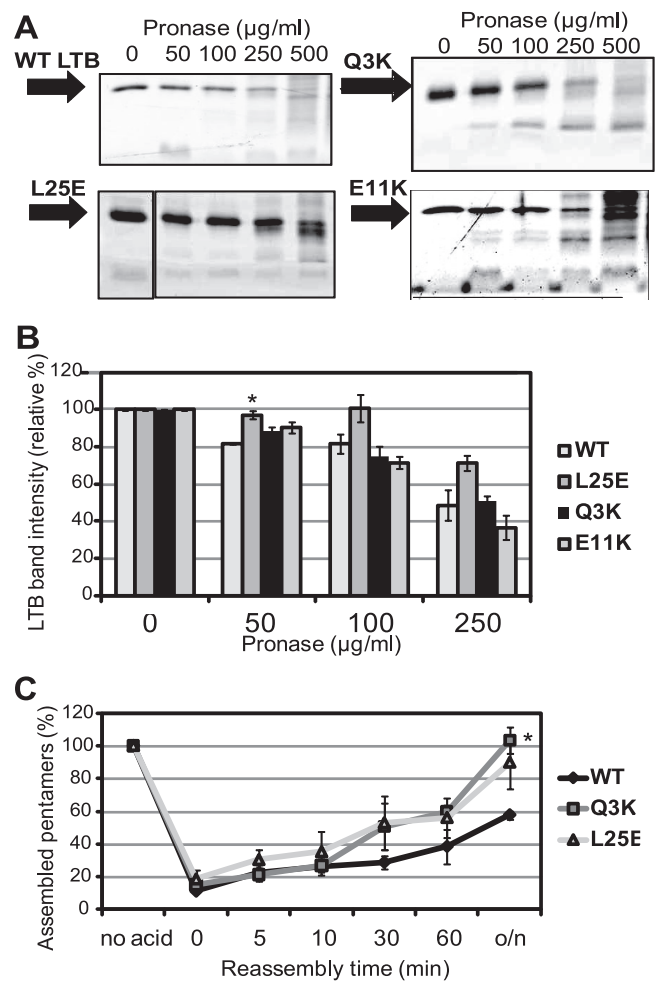


FIG. 2. LT secretion mutants show WT protease sensitivity and pentamer formation. (A) Representative protein gels showing degradation of WT or the indicated mutant toxin. Purified toxin (500 ng) was incubated with pronase at the indicated final concentration for 1 h at 37°C. Following the incubation, samples were boiled and separated by 15% SDS-PAGE, and the gel was stained with Ruby Red. (B) Densitometric measurements of the intensities of the bands corresponding to LTB on the protein gels described in the legend for panel A. The intensity of the band corresponding to untreated WT LTB or the indicated mutant was set to 100%. \*,  $P < 0.05$  compared to WT at the same concentration ( $n \geq 2$ ). (C) Purified WT LTB and the indicated mutants were dissociated into monomers by treatment with acid, and then neutralized and allowed to reassemble into pentamers for the indicated number of minutes or overnight (o/n). Pentamer reformation was assessed by  $G_{M1}$  ELISA, with values normalized to levels of each pentamer without acid treatment (no acid). \*,  $P < 0.01$ , compared to WT at the same time point ( $n \geq 3$ ).

for more global defects that might confound our results, L25E was not further considered in this study.

**The E11K mutation, originally characterized with CT, also impairs the secretion of LT from ETEC.** An E11K mutation in the B subunit of CT reduces its secretion from *V. cholerae* to approximately 60% of wild-type levels (6). Because LT and CT are highly similar secretion substrates, we wondered whether this mutation would affect the secretion of LT from ETEC, and so we introduced the E11K mutation into LTB. Indeed, the levels of LT[E11K] found in ETEC supernatant were substan-

tially reduced, despite a wild-type level of expression (Fig. 1A and B). Periplasmic levels of LT[E11K] were nearly wild type, as was the case for LT[L25E] (Fig. 1B).  $G_{MI}$  binding of purified LT[E11K] was slightly reduced, although not significantly (Table 3). Like the Q3K and L25E mutants, LT[E11K] was stable when incubated in ETEC culture supernatant (data not shown). Additionally, supernatant levels of LT[E11K] did not increase upon addition of protease inhibitor to the growth medium (Fig. 1B). LT[E11K] also behaved similarly to wild-type LT in the pronase sensitivity assay (Fig. 2A and B). While purified LT[E11K] is as toxic as wild-type LT, indicating proper holotoxin formation *in vivo*, the mutation appears to impede the reformation of LTB pentamers *in vitro* after dissociation with acid (data not shown).

To explore the characteristics of residue 11 that are critical for secretion, we tested a more neutral mutation, LT[E11A]. Detected levels of LT[E11A] in the supernatant were decreased only approximately 12% compared to wild-type LT (Fig. 1B), but this value may be the result of its slightly decreased ability to bind  $G_{MI}$  (Table 3). Periplasmic levels of LT[E11A] were normal (Fig. 1B). These data suggest that introduction of a charge at residue 11 is critical for LT[E11K]'s observed secretion defect in ETEC.

Consistent with its location outside the blood antigen binding pocket, LT[E11K] was fully capable of binding blood group A trisaccharide, but intriguingly, this mutant showed a strong impairment in binding to the surface of *E. coli* K-12 cells (Table 3). As such, Glu-11 represents a residue that is located outside the peripheral blood sugar binding pocket but is nevertheless critical for binding to LPS on the surface of *E. coli*.

**The Q3K mutation does not alter the secretion of CTB from *V. cholerae*.** Because the E11K mutation affected LT's secretion from ETEC similarly to its impairment of CT secretion from *V. cholerae*, we were curious whether the Q3K mutation that affected LT secretion from ETEC would also affect CTB secretion from *V. cholerae*. To that end, an inducible plasmid expressing wild-type or mutant CTB carrying the Q3K mutation (CTB[Q3K]) was introduced into the CT-deficient *V. cholerae* strain P4 (10). B-subunit pentamers, without the catalytically active A subunit, were used for reasons of biosafety. Interestingly, a fourfold lower concentration of IPTG was needed to induce CTB[Q3K] to levels matching those of induced wild-type CTB (Fig. 3A).

Unlike LT[Q3K] in ETEC, CTB[Q3K] levels were only slightly reduced in the supernatant of *V. cholerae* P4 (83.3%), and the difference was not significant (Fig. 3B). The  $G_{MI}$  binding levels of purified CTB[Q3K] were slightly reduced compared to wild-type CTB (Table 3); therefore, the level of secreted CTB[Q3K] may be even closer to that of wild-type CTB than our assay indicates. Periplasmic levels of CTB[Q3K] were close to those of wild-type CTB (Fig. 3B). These results suggest a difference in substrate selectivity between the T2S systems of ETEC and *V. cholerae*.

An inducible plasmid expressing CTB[E11K] was also cloned and utilized for comparison studies of our assay system. CTB[E11K] expressed at levels similar to those of wild-type CTB (Fig. 3A), and we could recapitulate its secretion impairment, albeit to an even greater extent than was previously reported ( $20.9 \pm 1.7\%$  of that of the wild type, Fig. 3B). However, in contrast to the near-wild-type secretion phenotype of LT[E11A] from ETEC, alteration of Glu-11 in CTB to

alanine (CTB[E11A]) resulted in significantly reduced CTB levels in the cell-free supernatant, despite wild-type levels of expression (Fig. 3A and B). Although addition of protease inhibitor to the growth medium raised the levels of CTB[E11K] detected, proteolytic degradation cannot account for the full secretion defect (Fig. 3B). The presence of protease inhibitor did not alter the amount of CTB[E11A] detected in the supernatant (Fig. 3B). Like the LT mutants incubated in ETEC supernatant, these CTB mutants were not degraded in *V. cholerae* culture supernatant in our stability assay (data not shown). Moreover, purified wild-type and mutant CTB pentamers behaved similarly in the pronase sensitivity assay (Fig. 3C and D), with CTB[E11A] showing a trend toward increased stability. Despite their secretion defect, periplasmic forms of CTB[E11K] and CTB[E11A] did not accumulate to a level higher than that of wild-type CTB (Fig. 3B).

**The *V. cholerae* T2S system is not blocked by secretion-impaired CTB mutants.** We wondered if nonsecreted CTB mutants would block the T2S system, preventing the export of other T2S substrates. In *V. cholerae*, the T2S system secretes a lipase required for acquisition of olive oil as a carbon source, and degradation of casein occurs in a T2S-dependent manner (35, 37). To test for mutant-dependent blockage of the T2S system, we examined whether *V. cholerae* strains expressing wild-type CTB, CTB[E11K], and CTB[E11A] were able to grow on lipid agar containing olive oil as the sole carbon source and found that their growth was not inhibited (data not shown). Furthermore, these strains were tested for generating zones of clearance on LB-skim milk plates. All strains formed clear haloes of similar sizes (Fig. 3E). Taken together, these results suggest that the T2S system is not completely blocked by mutant CTB, although our data do not rule out the possibility of partial blockage. ETEC did not grow on lipid agar or create a zone of clearance on skim milk plates (data not shown).

**CTB mutants are secreted efficiently from ETEC.** In order to further investigate the common factors in the recognition of T2S substrates, we measured the secretion of CTB[Q3K], CTB[E11K], and CTB[E11A] through a heterologous T2S system in ETEC. In all cases, the mutant toxins expressed at wild-type levels (Fig. 4A). A slight but insignificant impairment in secretion was seen for CTB[Q3K], and this could be partly explained by the mutant's reduced  $G_{MI}$  binding (Fig. 4B; Table 3). Surprisingly, however, whereas CTB[E11K] and CTB[E11A] had strong defects in secretion from *V. cholerae*, these CTB mutants had no significant reduction in secretion from ETEC (Fig. 4B). The modest impairment seen for CTB[E11K] (Fig. 4B) was again accompanied by a slight reduction in  $G_{MI}$  binding of the purified pentamer (Table 3) and could be responsible for the apparent reduction in secreted levels. Intriguingly, the E11A mutation generated a  $G_{MI}$  binding capacity slightly higher than that of wild-type CTB (Table 3), and it is likely that this increased binding is reflected in the slightly elevated levels of CTB[E11A] detected in the supernatant (Fig. 4B). In all cases, periplasmic pentamer levels were close to that of wild-type CTB (Fig. 4B). These data demonstrate that recognition of CT as a secretion substrate occurs differently in the ETEC and *V. cholerae* T2S systems.

**LTB mutants are unstable in *V. cholerae*.** In order to complete our cross-species analysis of the effects of the T2S substrate mutations, wild-type and mutant LT B subunits were expressed in *V. cholerae* strain P4. Again, LTA-deficient constructs were tested

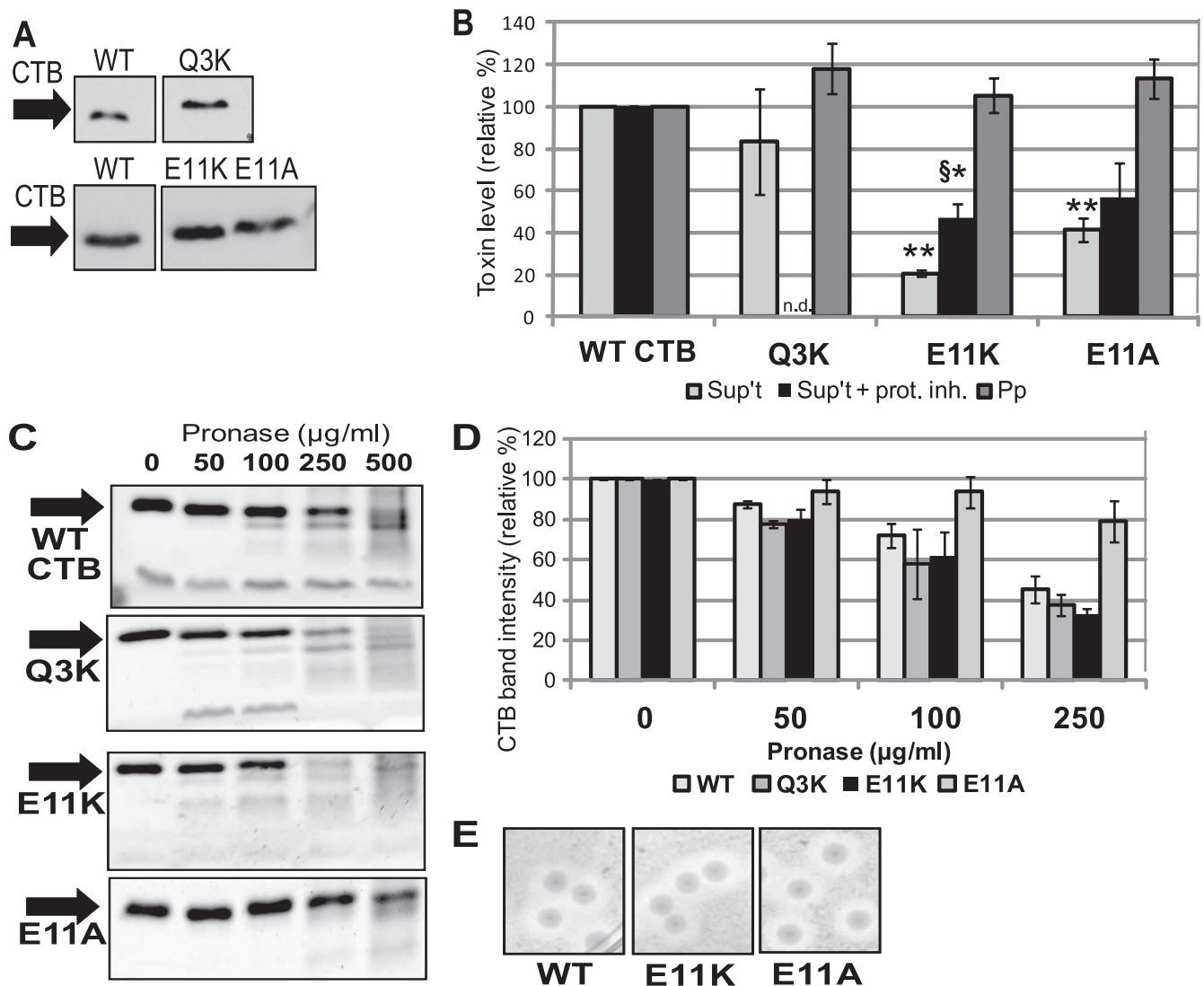


FIG. 3. Mutation of Glu-11 impairs CTB secretion from *V. cholerae*. (A) Representative immunoblot of TCA-precipitated total culture samples, adjusted for CFU, showing induced expression of WT CTB, CTB[Q3K], CTB[E11K], and CTB[E11A] in strain P4. Blots were probed with anti-CTB antibody. (B) Strains expressing WT CTB and the indicated CTB mutants were fractionated to isolate cell-free supernatant (Sup't) and periplasm (Pp). Each fraction was tested for pentamer levels by  $G_{M1}$  ELISA, with WT levels set to 100%. Supernatant levels were normalized to CFU, and periplasm levels were normalized to alkaline phosphatase activity. \*,  $P < 0.05$ ; \*\*,  $P < 0.0005$  compared to wild-type ( $n \geq 3$ ). For some experiments, cultures were grown in the presence of a protease inhibitor cocktail (+ prot. inh.). §,  $P < 0.05$  compared to E11K supernatant levels without protease inhibitor ( $n \geq 2$ ). (C) Representative protein gels showing degradation of WT CTB or the indicated mutant pentamer. Purified pentamer (500 ng) was incubated with pronase at the indicated final concentration for 1 h at 37°C. Following the incubation, samples were boiled and separated by 15% SDS-PAGE, and the gel was stained with Ruby Red. (D) Densitometric measurements of the intensities of the CTB bands on the protein gels described in the legend for panel C. The intensity of the band corresponding to untreated WT or the indicated mutant CTB was set to 100% ( $n \geq 2$ ). (E) Zones of clearance formed by *V. cholerae* on skim milk agar. Strains expressing WT CTB and the indicated mutant pentamers were plated on skim milk agar and incubated for 36 h at 37°C.

for reasons of safety. Although wild-type LTB was readily produced in *V. cholerae*, the Q3K and E11K LTB mutants were detected in small amounts, if at all (Fig. 5), and thus, we were unable to measure the effect of Q3K or E11K on LTB secretion from *V. cholerae*. LTB[L25E] showed significant levels of expressed protein, indicating that some LTB mutations are tolerated by *V. cholerae* (Fig. 5), and LTB[L25E] in *V. cholerae* supernatant was readily detectable by immunoblotting (data not shown). However, the expression of LTB[L25E] was not uniform enough to allow for quantification of its secreted levels. Taken

together, our results provide evidence that, despite a high degree of conservation between the substrates, LTB and CTB are recognized and dealt with in different ways during secretion from ETEC and *V. cholerae* (Table 4).

## DISCUSSION

We have identified three point mutations of LTB (L25E, Q3K, and E11K) that impair LT secretion from ETEC. These mutants behaved like wild-type LT in assays measuring stabil-

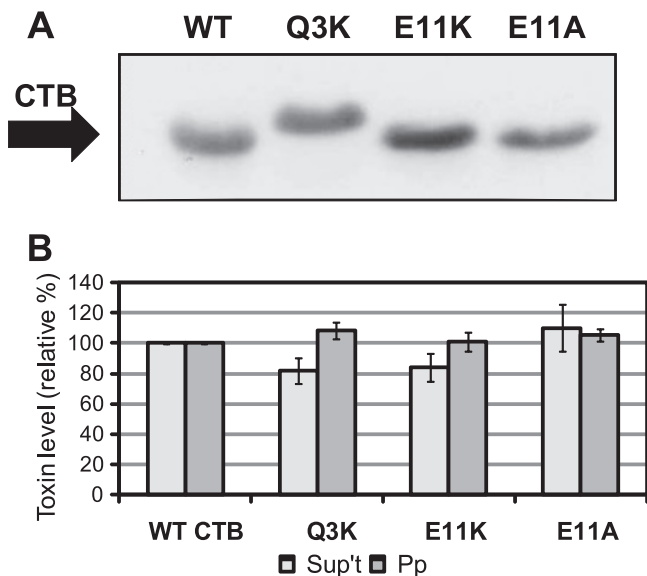


FIG. 4. Secretion of CTB mutants is not impaired in ETEC. (A) Representative immunoblot of TCA-precipitated total culture samples, adjusted for CFU, showing induced expression of WT CTB, CTB[Q3K], CTB[E11K], and CTB[E11A] in strain jf570. The blot was probed with anti-CT antibody. (B) Strains expressing WT CTB and the indicated mutant pentamers were fractionated to isolate cell-free supernatant (Sup't) and periplasm (Pp). Each fraction was tested for pentamer levels by G<sub>M1</sub> ELISA, with WT levels set to 100%. Supernatant levels were normalized to CFU (n ≥ 3), and periplasm levels were normalized to alkaline phosphatase activity (n ≥ 2).

ity (Fig. 2 and data not shown), indicating that the reduced levels found in ETEC cell-free supernatants are due to an effect on secretion and not increased rates of degradation. In contrast to mutants with a substituted lysine residue, LT[Q3A] and LT[E11A] were secreted at essentially wild-type levels from ETEC. Our results are in line with previous research demonstrating that alteration of a single amino acid can impair or block the secretion of other T2S substrates, including CT from *V. cholerae*, aerolysin from *Aeromonas hydrophila*, and Cel5 endogluconase from *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (3, 6, 44).

The similar natures of LT and CT led us to explore whether mutations affecting the secretion of one substrate might affect the secretion of the other. The ability of *V. cholerae* to secrete LT has been recognized for years (27), and our group has

TABLE 4. Summary of the effects of the mutations investigated in this study<sup>a</sup>

Mutation <sup>b</sup>	Secretion from ETEC		Secretion from <i>V. cholerae</i>	
	LT	CTB	CTB	LTB
Q3K	Reduced	Wild type	Wild type	Not detected <sup>d</sup>
Q3A	Wild type	— <sup>c</sup>	—	Not detected
E11K	Reduced	Wild type	Reduced	Little detected
E11A	Near wild type	Wild type	Reduced	—
L25E	Reduced	—	—	Wild type <sup>e</sup>

<sup>a</sup> Data shown characterize levels in cell-free supernatant, as measured by G<sub>M1</sub> ELISA.

<sup>b</sup> All mutations are in the B subunit; the LT holotoxin was expressed in ETEC.

<sup>c</sup> —, not tested.

<sup>d</sup> No mutant LTB could be detected in total culture samples by immunoblotting.

<sup>e</sup> Secreted LTB[L25E] is readily detectable in the cell-free supernatant by immunoblotting.

reported that *E. coli* K-12 carrying a plasmid-borne T2S operon is able to secrete CTB (17). In this study, we have now shown that the native secretion apparatus of ETEC is also able to secrete CTB. Whereas the E11K mutation impairs the secretion of both toxins from their native bacterial species, other mutations affecting the secretion of each toxin are not identical. The Q3K mutation prevents LT secretion and causes LT to back up in the periplasm of ETEC, but the same mutation has no significant effect on the secretion of CT from *V. cholerae*. Furthermore, while the E11A mutation does not strongly impair the secretion of LT from ETEC, CTB[E11A] behaves like the poorly secreted CTB[E11K] when expressed in *V. cholerae*. These results demonstrate that LT and CT are identified as secretion substrates based on distinct sets of residues and/or that mutations of substrates are tolerated to different extents by the T2S systems of ETEC and *V. cholerae*.

Our results investigating the effects of expressing CTB[E11K] or CTB[E11A] on the secretion of other T2S substrates from *V. cholerae* suggest that recognition of the toxin is impaired by these mutations, not that the mutations cause the toxin to remain bound to the secretion machinery, blocking the export of other proteins. However, these assays were not highly quantitative, and therefore, partial blockage may not have been detected. Further studies with a quantitative system will be needed to address this question. As ETEC could not be used in these assays, it is not yet clear whether LT mutants are impaired at the point of being recognized or transiting the outer membrane.

Thus far, there is very little knowledge of how T2S substrates are recognized by the complex T2S apparatus, and an obvious amino acid sequence motif in T2S substrates is lacking. A study of PehA polygalacturonase secretion has provided some of the only direct evidence in support of a hypothetical three-dimensional recognition motif. In that study, one mutation in PehA that reduced the levels detected in *Erwinia carotovora* supernatant was determined to be internal to folded protein and thus to be responsible for impairing the secretion-competent conformation of the substrate (28). However, the stability of the mutant seemed to be reduced. Our data with the L25E mutation provide more definitive evidence for a three-dimensional “structural motif” in T2S substrates. In fact, secretion of the CT/LT B-subunit pentamer may require the simultaneous

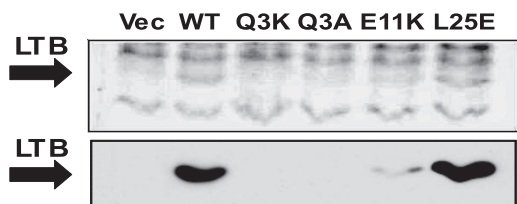


FIG. 5. LTB mutants express poorly in *V. cholerae*. Representative Ruby Red protein stain (top) and immunoblot (bottom) of TCA-precipitated total culture samples, adjusted for CFU, showing induced expression of a vector control (Vec), WT LTB, LTB[Q3K], LTB[Q3A], LTB[E11K], and LTB[L25E] in strain P4. The blot was probed with cross-reactive anti-CT antibody.



recognition of multiple subunits in three-dimensional space. The binding of LT to G<sub>M1</sub>, blood group sugars, and the surface of *E. coli* occurs at the interface between two adjacent B subunits (16, 25, 26). It is particularly notable that the L25E mutation affects the binding of the toxin to all three substrates, each of which binds regions of the B pentamer that do not include the Leu-25 residue (16, 25, 26). Because LT[L25E] did not show any differences from wild-type LT in terms of protease sensitivity or pentamer reassembly, we propose that altered spacing of the monomeric B subunits within the pentamer underlies these results. Therefore, this is strong evidence that the portion of LTB that is recognized by the secretion apparatus spans two adjacent subunits and that L25E alters the three-dimensional organization of the residues in this region.

The determination that CTB[E11K] and CTB[E11A] have two different secretion phenotypes in *V. cholerae* and ETEC allows for future research to determine the portion(s) of the T2S system involved in the recognition of CTB for secretion in these two pathogens. The GspC/EpsC and GspD/EpsD proteins are good candidates for the portions of the T2S apparatus that confer substrate selectivity. Elegant research carried out with two *Erwinia* species determined that the N-terminal portion of OutD (the GspD homolog in *Erwinia*) and a PDZ domain in OutC (GspC) were involved in species-specific substrate recognition (2). Unfortunately, we were unable to assess such roles in ETEC or *V. cholerae* T2S components because we were not able to establish functional secretion of CTB or LTB in a *gspD* knockout strain of ETEC complemented with *epsD* from *V. cholerae*, even in the presence of *epsC* (our unpublished observations). These results are in line with a previous study reporting that the entire *V. cholerae* Eps system was not functional when expressed in *E. coli* (34).

Our results reveal the exquisitely controlled coordination between the T2S machinery and its substrates. Despite the high degree of homology between LTB and CTB, we have discovered that mutations affect their secretion differently in distinct T2S systems, indicating that each substrate has evolved closely with its T2S apparatus to enable its secretion. A number of previous studies have attempted to identify the domain (or domains) present in T2S substrates that mark them for secretion. Our data identifying distinct mutations that affect the secretion of highly similar substrates like LT and CT raise the possibility that it will not be possible to find a common recognition motif among T2S substrates. However, the use of these CT and LT mutants could help elucidate the portions of the T2S complex that are involved in substrate recognition.

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