Secretion and Lipopolysaccharide Binding of Heat-Labile Enterotoxin

by

Benjamin Mudrak

Department of Molecular Genetics and Microbiology
Duke University

Date:_____

Approved:

_____

Dr. Meta J. Kuehn, Supervisor

_____

Dr. Raphael Valdivia

_____

Dr. Hiroaki Matsunami

_____

Dr. Alejandro Aballay

_____

Dr. Joseph St. Geme III

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2010

ABSTRACT

Secretion and Lipopolysaccharide Binding of Heat-Labile Enterotoxin

by

Benjamin Mudrak

Department of Molecular Genetics and Microbiology
Duke University

Date:_____
Approved:

_____
Dr. Meta J. Kuehn, Supervisor

_____
Dr. Raphael Valdivia

_____
Dr. Hiroaki Matsunami

_____
Dr. Alejandro Aballay

_____
Dr. Joseph St. Geme III

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2010

Abstract

       Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of morbidity and

mortality worldwide. The causative agent of traveler's diarrhea, ETEC is often

associated with cholera-like disease, especially in developing countries. One major

virulence factor released by ETEC is the heat-labile enterotoxin LT, which upsets the

balance of electrolytes in the intestine. LT is highly similar to cholera toxin (CT)

produced by *Vibrio cholerae*, both in structure and function. The toxin consists of a

single catalytically active A subunit and a ring of five B subunits mediating its binding

and secretion. Previous work from our lab has shown that, after export by the type II

secretion (T2S) system, LT associates with lipopolysaccharide (LPS) on the bacterial

surface. However, little is known about what identifies LT as a T2S substrate, and the

portion of the toxin that mediates LPS binding has not previously been defined. Site-

directed mutagenesis of residues in a peripheral sugar binding pocket of the toxin was

performed, revealing mutations that affect its binding to LPS, as determined by an *in

vitro* cell surface binding assay. One binding mutant, which is expressed and secreted at

wild-type levels from ETEC, holds particular promise for further studies of the role of the

LT-LPS interaction. Interestingly, some mutations made affected the secretion of the

toxin as detected by ganglioside-binding ELISAs of cell-free supernatant, and several

mutations affected both secretion and LPS binding. These mutations identify residues of

the toxin that are involved in its secretion and association with LPS. In addition, we

introduced mutations affecting the secretion of LT into CT, due to the high similarity

between the two toxins. While one mutation affects the secretion of each, other

mutations affect one toxin but not the other. These results demonstrate that LT and CT

are recognized in different ways during T2S. Combined with an analysis of the effects of

secretion mutations on the stability of the toxin, the results described here highlight the

delicate balance between structure and function of the LT B subunit.

Dedication

To my mentor, Meta, whose optimism about my project has consistently carried me forward.

To my labmates (past and present), whose certified zaniness has made it fun to come to work each and every day. There is no lab like the Kuehn lab.

To my parents, who have always been great supporters of my educational career and who have taken a genuine interest in a protein they'd never heard of from a strain of bacteria they'd never heard of.

To my beautiful wife, Sarah, who is my constant inspiration, best friend, and colleague on the journey to a Ph.D. You know what this journey has been like, and it has meant all the world to me to share it with you.

Contents

List of Tables

List of Figures

1. Introduction

Humans encounter countless bacterial species on a daily basis. While most of these organisms are harmless, many represent a threat to the health of their host. In order to combat these invisible foes, nature has provided several highly regulated immune systems, ranging from the barrier function of the skin to the production of antibodies. Still, bacterial pathogens have evolved along with these protective systems, finding ways to survive and multiply in a human host. Understanding how bacterial pathogens cause disease and evade killing by the immune system is critical for the advancement of disease treatment and prevention.

From a young age, the human gut is colonized by upward of one hundred trillion commensal bacteria, a number that dwarfs the number of cells in the average human body (Steinhoff 2005). Despite this immense throng of living organisms, numerous species of pathogenic bacteria have evolved to colonize the human intestine (which contains approximately 300 m^2 of available surface area), outcompeting the more friendly species and generating a range of unpleasant symptoms (Muller et al. 2005). Some gut pathogens make space for themselves by entering intestinal epithelial cells or by squeezing between them to access the membranes below (Madigan and Brock 2009). Others are thought to bury themselves deep within the crypts present between intestinal villi, a niche not normally occupied by commensal flora (Muller et al. 2005). Some pathogens cause severe diseases requiring medical attention (like *Vibrio cholerae*, discussed below), while others generate self-limiting symptoms. Altogether, intestinal pathogens account for two billion cases of diarrhea per year (WHO 2009), causing a significant burden on health

care institutions worldwide. A better understanding of the virulence mechanisms of intestinal pathogens will lead to better treatment options and preventative measures.

1.1 Enterotoxigenic Escherichia coli

One common form of intestinal disease is termed traveler's diarrhea. In general, this malady is self-limiting in a healthy adult, although antibiotics are prescribed in some cases (Coster et al. 2007). While a large number of pathogens can cause traveler's diarrhea, the most frequent etiologic agent is enterotoxigenic *Escherichia coli*, also called ETEC (CDC 2006). ETEC is endemic in many developing countries, most notably Mexico and Bangladesh, where it is frequently encountered by tourists, members of the military, or other visitors (Coster et al. 2007). Because of poor surveillance, mortality due to ETEC is difficult to estimate, but there are believed to be at least 400,000 ETEC-related deaths in children under the age of 5 each year, with countless others likely classified simply as death due to diarrhea (Qadri et al. 2005). Human ETEC strains are closely related to ETEC isolates obtained from pigs suffering from diarrhea, sharing a number of pathogenic features and virulence factors. However, human and porcine ETEC strains show strong host preferences due to the expression of fimbriae with distinct tropisms (Fleckenstein et al. 2009).

A large number of disease-causing ETEC strains have been isolated, with over 70 O-antigen serogroups having been identified, along with approximately 20 variants of adhesive fimbriae and a handful of enterotoxins (Sanchez and Holmgren 2005). Extrachromosomal plasmids carrying virulence determinants are present in the vast majority of ETEC strains, providing the bacteria with the genes to produce toxins and fimbriae as well as the potential to mobilize the plasmid, creating new enterotoxigenic

strains (Johnson and Nolan 2009). Attempts to generate an effective vaccine against ETEC, particularly for young children, have mostly met with failure due to the highly variable nature of the antigens present in the pathogen (Svennerholm and Tobias 2008).

Virulence factors carried by ETEC. One of the most striking features of ETEC cells is the presence of numerous lengthy fimbriae. These fimbriae, encoded on a virulence plasmid, are termed colonization factor antigens (CFAs) and aid in binding to the host intestinal epithelium. CFAs are grouped into broad classes, with significant variability (Blanco et al. 1991); the prototype ETEC strain H10407, used predominantly in these studies and many others, expresses CFA/I (Roy et al. 2009). Still other strains of ETEC express a type IV pilus called longus, which is closely related to the toxin coregulated pilus of *V. cholerae* (Gomez-Duarte et al. 1999). ETEC also expresses peritrichous flagella, which have been implicated in binding to host intestinal cells (Roy et al. 2009). Flagella have been proposed to play the role of a primary adhesin, anchoring passing ETEC cells to the surface of epithelial cells. As the flagella break down, secondary (but possibly higher-affinity) associations between the host and surface-borne pili and adhesins can then occur. Among these secondary adhesins are TibA (a glycosylated autotransporter) and EtpA (a flagellin-binding protein secreted as part of a two-partner secretion system) (see Table 1).

In addition to these surface structures, several secreted proteins have been reported to influence ETEC pathogenesis. Historically, ETEC strains were classified based on their expression of a heat-labile enterotoxin (LT, described in detail in section 1.3), a heat-stable enterotoxin (ST), or both. STs are a family of small peptide hormone mimics, while LT is closely related to cholera toxin (CT) from *V. cholerae*. These

3

enterotoxins serve to disrupt the balance of electrolytes in the intestine, causing the

diarrhea associated with ETEC infection. Table 1 summarizes several of ETEC's

important virulence factors.

Table 1. Selected virulence factors of enterotoxigenic *E. coli* strain H10407

Virulence factor	MW (kDa)	Relevant characteristics	Reference
LT	87.5	Increases cAMP levels in host cells	(Moss and Richardson 1978)
ST	2.2	Hormone mimic; increases cGMP levels in host cells	(Thompson and Giannella 1985)
Flagella (FliC)	51.3	Important for motility and host cell adherence	(Roy et al. 2009)
CFA/I	15	Glycosphingolipid-binding fimbriae	(Jansson et al. 2006)
Tia	25	Outer membrane adhesin	(Fleckenstein et al. 1996)
TibA	104	Glycosylated autotransporter adhesin	(Lindenthal and Elsinghorst 2001)
EatA	148	Serine protease autotransporter	(Patel et al. 2004)
EtpA	177	Two-partner adhesin; also binds FliC	(Roy et al. 2009)

1.2 **Vibrio cholerae**

Another important intestinal pathogen, *V. cholerae*, is the causative agent of

cholera, a disease causing profuse watery diarrhea and death if oral rehydration therapy is

not available (Sanchez and Holmgren 2005). *V. cholerae* is a gram-negative bacillus

with a characteristic curved morphology. This pathogen has been responsible for a

number of pandemic outbreaks of cholera throughout history, and contains a wide variety

of toxins and virulence determinants in its arsenal (Childers and Klose 2007). Strains of

V. cholerae are divided into two biotypes: classical, which was responsible for historical

outbreaks of cholera, and El Tor, the causative agent of the current pandemic (Peterson

2002). Although they share a majority of their virulence determinants, there are several

differences between these two types of *V. cholerae*. For one, classical strains are more

permissive in their expression of CT-encoding genes; the conditions required for CT

expression in El Tor strains are much more stringent (Sanchez et al. 2004).

1.2.1 Cholera toxin

Like LT in ETEC, the primary virulence factor recognized in *V. cholerae*

(especially from a historical perspective) is CT. CT is a heterohexameric toxin,

consisting of one enzymatic A subunit (CTA) and five B subunits (CTB) mediating

binding and secretion. Despite the nearly identical sequences and true molecular weights

of LT and CT, small electrophoretic differences have been noted for decades, with the

LTB pentamer appearing larger than the CTB pentamer (Clements and Finkelstein 1979).

The enzymatic activity of CT is identical to that of LT, and binding properties are largely

shared between the two toxins (Spangler 1992). The toxins share approximately 81%

sequence similarity as a whole, while the mature B subunits are 83% identical at the

amino acid level (Dallas and Falkow 1980; Holmner et al. 2007). An alignment of the

residues in LTB and classical CTB (which has been used extensively in prior research

studies) is shown in Figure 1. Because of their striking similarity and the limited number

of *E. coli* strains expressing LT, the genes encoding LT are understood to have originated

through horizontal transfer from *V. cholerae* (Yamamoto et al. 1987). Altogether, the

relatedness of LT and CT allows for direct comparisons to be made, and inferences can

be drawn based on differences in their binding properties and other characteristics. All

mutations described in this thesis are in the B subunit of LT or CT.

```
       1                  10                *  20                     30
LTB:  A P Q S I T E L C S E Y H N T Q I Y T I N D K I L S Y T E S
CTB:  T P Q N I T D L C A E Y H N T Q I H T L N D K I F S Y T E S

      31                  40                *  50                     60
LTB:  M A G K R E M V I I T F K S G A T F Q V E V P G S Q H I D S
CTB:  L A G K R E M A I I T F K N G A T F Q V E V P G S Q H I D S

      61                  70                   80                     90
LTB:  Q K K A I E R M K D T L R I T Y L T E T K I D K L C V W N N
CTB:  Q K K A I E R M K D T L R I A Y L T E A K V E K L C V W N N

      91                 100
LTB:  K T P N S I A A I S M E N
CTB:  K T P H A I A A I S M A N
```

Figure 1: Alignment of the amino acid residues of mature LTB and CTB

The amino acid sequences of mature LTB (PDB no. P13811) and classical CTB (PDB no. 3CHBH) are shown, with numbering starting at the first residue after the signal sequence is cleaved. Differences, 17 in all, are boxed. The asterisks mark Tyr-18 and Thr-47, which were deemed critical for blood sugar binding by LT according to examination of crystal structure data (see section 1.3.3 and (Holmner et al. 2007)). The Tyr-18 residue in LTB is also present in CTB from El Tor *V. cholerae* strains, but Thr-47 has been substituted with Ile (not shown).

1.3 Heat-labile enterotoxin

LT was originally named to distinguish it from ST, the heat-stable toxin, although neither of the toxins had been extensively characterized. Incubation of LT at 65-70°C for ten minutes is sufficient to destroy its activity (Gill et al. 1981), whereas boiling does not inactivate ST. As described above, LT is a multimeric AB_5 toxin, composed of a single A subunit (LTA) associated with a ring of five B subunits (LTB) responsible for the toxin's binding properties and secretion (Hardy et al. 1988) (Figure 2). Heat treatment of the toxin breaks down the pentameric ring of B subunits into monomers, releasing LTA. While catalytic activity is maintained by free LTA, the LTB ring is required for entry into

cells of the intestinal epithelium, and disruption of the holotoxin thereby prevents

intoxication of host cells (Spangler 1992).
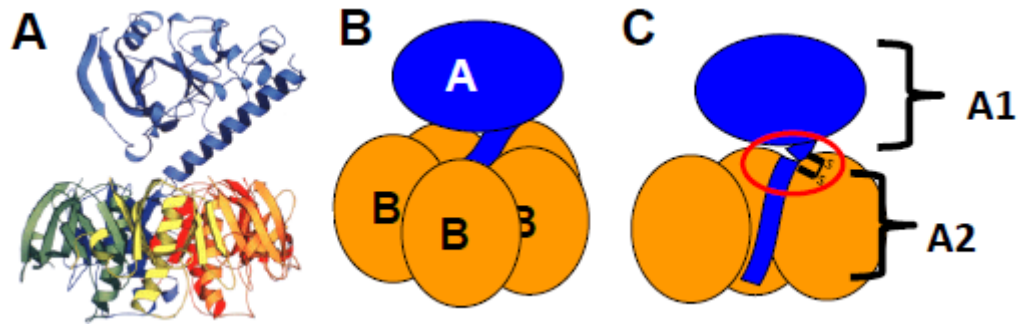
Figure 2: Structure and subunit organization of heat-labile enterotoxin.

The crystal structure of heat-labile enterotoxin **(A)**, adapted from (Sixma et al. 1993), and a schematic of its subunit organization **(B)** are shown. One A subunit is associated with a ring of five B subunits. **(C)** shows a cutaway view of the central core of the holotoxin, with the location of the A subunit's proteolytic processing ("nicking") and its disulfide bond circled. After nicking and reduction of this disulfide bond, the catalytically active A1 fragment is released from the helical A2 fragment.

Catalytic activity. Further studies of LT revealed more about the nature of its

enzymatic activity, which is identical to that of CT. LTA catalyzes the ADP-ribosylation

of the host Gsα protein (Moss and Richardson 1978). Gs is a trimeric G-protein capable

of stimulating the activity of adenylate cyclase to produce greater levels of cyclic AMP

(cAMP) in the cell. ADP-ribosylation of the α subunit greatly reduces its GTPase

activity, leaving it in an activated state, wherein it leaves the apical membrane and

becomes free to interact with basolateral adenylate cyclase molecules (Popoff 1998).

Increased levels of cAMP within the cell lead to the opening of various ligand-gated

membrane channels and the efflux of water and electrolytes into the lumen of the

intestine (Spangler 1992).

During activation, LTA is proteolytically cleaved, releasing a small C-terminal fragment termed A2; the remaining A1 polypeptide is responsible for the observed toxic effects (Popoff 1998) (Figure 2C). This cleavage event, termed "nicking," is not required for the toxic effects of LT, but mutants that are unable to be nicked demonstrate a markedly delayed effect in cell culture (Grant et al. 1994). Trypsin is able to cleave LTA into A1 and A2 *in vitro*, but the identity of the protease(s) involved in this activation step *in vivo* are not known (Clements and Finkelstein 1979). In addition to this cleavage event, a disulfide bond linking A1 and A2 is also reduced after entry into the host cell in order to completely separate the two fragments (Lencer et al. 1999). This disulfide bond is not essential to holotoxin formation, but mutation of the cysteine residues involved makes LTA more sensitive to trypsinolysis and generates a significant lag in cAMP production in cultured intestinal cells compared to wild-type toxin (Okamoto et al. 1998).

Holotoxin formation. As noted above, LTA requires assistance in entering the host cell. This task is handled by a ring of five molecules of LTB, a protein of approximately 11 kDa molecular weight. After transcription, both LTA and LTB are directed to the periplasm by means of a *sec*-dependent signal sequence. LTB also forms an internal disulfide bond, explaining why formation of B-subunit pentamers is dependent on the presence of the periplasmic disulfide oxidoreductase DsbA (Yu et al. 1992; Hardy and Hedges 1996). Spontaneous assembly of AB_5 holotoxin occurs in the periplasm, with the long helical tail of LTA (the A2 peptide) facilitating the oligomerization of five LTB subunits around it (Hofstra and Witholt 1985). Studies

using *V. cholerae*[1] showed that the toxin assembles quite rapidly, while secretion takes

slightly longer (Hirst and Holmgren 1987). LTB pentamers can form in the absence of

LTA, but the kinetics are slower (Hardy et al. 1988).

Host receptor binding and internalization. Once the holotoxin is assembled, it

is secreted from ETEC by the type II secretion system (see section 1.4.3 below). After

secretion, LT is able to cause its toxic effects. In the case of intestinal epithelial cells, the

primary target of LT, the host receptor is understood to be the monosialoganglioside G_{M1}

(Spangler 1992). This molecule is commonly present in lipid rafts on the eukaryotic

membrane and is also the receptor for CT (Popoff 1998). While CT will not bind to

gangliosides other than G_{M1}, LT will also bind some related molecules (Teneberg et al.

1994). However, it is not clear if this additional binding capacity alters its effects *in vivo*.

After internalization mediated by G_{M1} at the apical surface, LTA undergoes retrograde

transport through the Golgi body to the endoplasmic reticulum, where it is ultimately

released into the cytosol to reach target Gsα molecules (Lencer et al. 1999).

The internalization of LT by intestinal cells is critical for proper colonization of

the mouse intestine by ETEC (Allen et al. 2006). Elaboration of LT is also critical for

porcine ETEC to cause disease in gnotobiotic piglets (Berberov et al. 2004). Similar

results were found with CT holotoxin and not with CTB alone (Pierce et al. 1985).

Studies using cultured intestinal cells *in vitro* likewise determined that the activity of

LTA enhances ETEC's association with the epithelium, although a smaller secondary

---

[1] Initially, LT was thought to remain periplasmic in all forms of *E. coli* (even ETEC), but this conclusion was made after expressing LT in lab strains lacking an active type II secretion system. However, due to this confusion, many early studies of LT's oligomerization and secretion were carried out in *V. cholerae*.

role for LTB was indicated (Johnson et al. 2009). This LT-dependent binding enhancement was observed whether intestinal cells were preincubated with the toxin or the toxin was added along with ETEC cells.

Association with outer membrane vesicles. It is important to note that the majority of secreted LT is not freely soluble but, rather, is present in association with outer membrane vesicles (OMVs) released by *E. coli* (Horstman and Kuehn 2002). OMVs are spherical proteoliposomes derived from the outer membrane, which also carry periplasmic components internally (Kuehn and Kesty 2005). ETEC produces a large quantity of OMVs, making this association useful for the dissemination of active toxin (Wai et al. 1995). Based on the existence of a protease-sensitive population of LT and the ability of antibodies recognizing LT to bind to intact vesicles, LT was determined to be bound to the surface of these OMVs as well as carried within their lumens (Horstman and Kuehn 2000). This existence of OMVs coated with LT and carrying more toxin molecules as cargo stands in stark contrast to the secretion of CT from *V. cholerae*, because CT is unable to bind the surface of *V. cholerae* and thus remains soluble (Horstman et al. 2004).

Further research showed that purified lipopolysaccharide (LPS) was able to inhibit the binding of LT to the surface of toxin-deficient ETEC cells, and pretreatment of these cells with protease did not reduce LT binding (Horstman and Kuehn 2002). Together, these results provided evidence that LT directly binds *E. coli* LPS.

As yet, it is unclear what advantages may be provided by the secretion of LT in a vesicle-associated state. The association with LPS may serve to titrate the levels of toxin in the host or, conversely, to deliver toxin-rich bacterial "bombs" to the host in the form

of OMVs (Kuehn and Kesty 2005). Association of LT with LPS may serve an

immunomodulatory function, either by altering the trafficking of LPS and/or LT or

through some other means of regulating host responses. For instance, it has been shown

that ETEC strains expressing LT induce less interleukin-8 production in cultured

intestinal cells than ETEC strains without LT, although the mechanism behind this

observation is unknown (Huang et al. 2004). The ability of LT to remain associated with

the surface of ETEC but still bind G_{M1} (see below) may also indicate a role for LT as an

intimate adhesin molecule. However, CT does not bind the surface of *V. cholerae* but is

nevertheless required for colonization of the intestine. Combined with the *in vitro*

binding results with ETEC described above, it seems more likely that LT's activity, not

its binding properties, is critical for early stages of infection. However, a possible role as

an adhesin has not yet been ruled out.

1.3.1 LT and lipopolysaccharide

The outer leaflet of the outer membrane of gram-negative bacteria is composed

overwhelmingly of LPS. LPS contains a characteristic lipid moiety, Lipid A, covalently

linked to an oligosaccharide chain (Raetz 1993).   In most bacteria, including *E. coli*, this

sugar chain can be divided further into an inner core of approximately five sugars, an

outer core of four to six addition sugars, and in many cases, a series of oligosaccharide

repeats known as the O antigen (Raetz 1993) (Figure 3). The innermost sugar in the

molecule, 3-deoxy-D-manno-octulosonic acid (Kdo) is an atypical seven-carbon sugar,

found only in bacteria, that is linked directly to the glucosamine moiety of Lipid A (Esko

et al. 2009). K-12 laboratory strains of *E. coli* (such as DH5α) do not possess an O

antigen, while ETEC strains and a great number of other pathogens do. Figure 3, a

general schematic of LPS, includes the core sugar residues found in DH5α *E. coli*.

O antigen repeat

Heptose
Glucose
Galactose
} Outer core

Heptose
} Inner core

Kdo
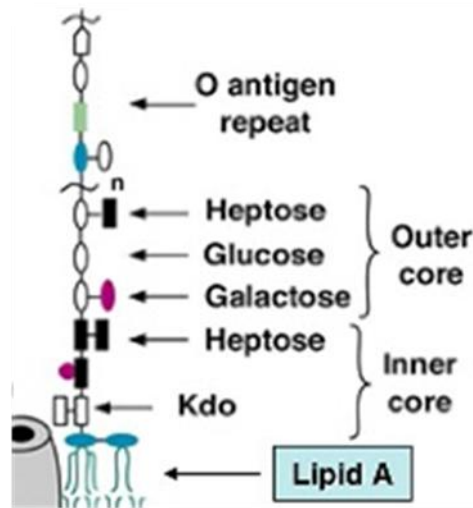
Lipid A

Figure 3: The structure of *E. coli* lipopolysaccharide.

The general organization of an LPS molecule is shown (adapted from (Raetz and
Whitfield 2002)). The lipid anchor of LPS, Lipid A, is found in the outer leaflet of the
outer membrane. It is linked to a chain of sugar residues, beginning with the inner and
outer core, followed in many cases by an oligosaccharide repeat termed the O antigen.
The core sugars shown are present in K-12 *E. coli* such as DH5α.

Binding inhibition studies using truncated forms of LPS, including the minimal

structure Kdo2-Lipid A, led to interesting results. While wild-type LPS with a complete

core could inhibit the binding of LT to *E. coli* cells carrying wild-type or truncated LPS,

Kdo2-Lipid A was only able to inhibit the binding of LT to cells with equally truncated

LPS (Horstman et al. 2004). That is, while binding to Kdo is possible, LT displayed a

greater affinity for LPS with a full oligosaccharide core. The O antigen of LPS appeared

to play no role in this binding event (Horstman et al. 2004). As LT did not bind

efficiently to cells expressing a Kdo kinase, it was concluded that phosphorylation of Kdo

residues blocks the interaction between LT and LPS. Consistent with this observation,

binding of LT to the surface of *V. cholerae* (in which Kdo is phosphorylated) was not

supported (Cox et al. 1996; Horstman et al. 2004).

Other experiments showed that Lipid A could not inhibit the binding of LT to *E.*

coli cells, implicating the sugar residues of LPS as the binding substrate of the toxin

(Horstman and Kuehn 2002). Moreover, soluble G_{M1} did not inhibit bacterial cell-surface

binding, and LT was shown to mediate the internalization of OMVs by eukaryotic cells in

culture in a manner dependent on G_{M1} (Horstman and Kuehn 2002; Kesty et al. 2004).

Therefore, the region of LTB responsible for binding G_{M1} did not seem to be involved in

LPS binding. However, the nature of LTB's LPS binding pocket, and the relative

importance of Kdo and other LPS sugars in the LT-LPS association, remained unknown.

1.3.2 LT and blood group antigens

Recent work by Holmner et al. defined another binding substrate for LT, the sugar

residues of the A-type blood antigen (2007). Prior studies of the binding of LT to

intestinal brush borders indicated that a population of non-G_{M1} receptors existed and that

blood antigens, specifically of the A and B type, may constitute these additional targets of

LT binding (Barra et al. 1992). Further experiments using polarized, differentiated

intestinal cells in culture lent evidence that the blood group A antigen is a functional

receptor for LT (Galvan et al. 2006). While a significant association has been found

between blood type and the severity of cholera symptoms (Harris et al. 2005), no clear

association has been noted for ETEC infection.

Co-crystallography of the blood sugar pentasaccharide GalNAcα3(Fucα2)Galβ4

(Fucα3)Glcβ with LTB revealed a binding site on the periphery of the pentameric ring

that did not overlap with the G_{M1} binding site (Holmner et al. 2007) (Figure 4). The

binding appeared to be critically dependent on residues Tyr-18 and Thr-47 in LTB

(marked with asterisks in Figure 1). Tyr-18 is altered to His in classical CTB, and Thr-47

is substituted with Ile in El Tor strains of *V. cholerae* (Holmner et al. 2007). The lack of

proper side chain interactions with the sugar residues is likely to explain the strongly

reduced binding of CTB to blood antigens compared to LTB (Balanzino et al. 1999).
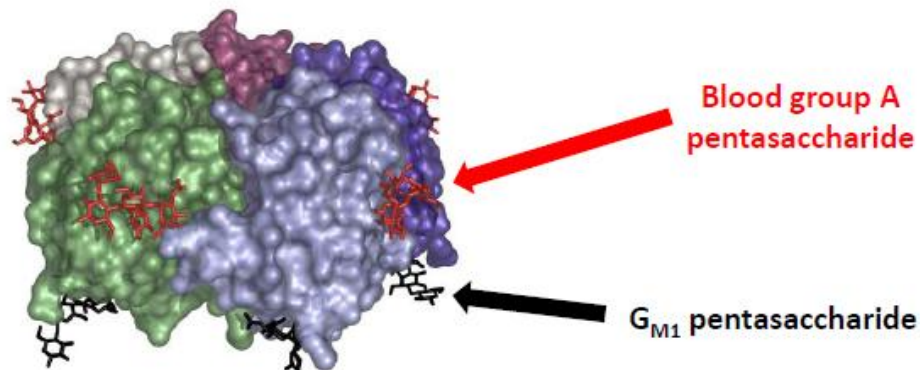
Figure 4: Location of blood sugar and G_{M1} binding pockets on LTB

Space-filling model of the LTB pentamer, with the locations of binding to blood group A
pentasaccharide and G_{M1} pentasaccharide indicated (adapted from (Holmner et al. 2007)).

The relevance of blood sugar binding *in vivo* is complicated by the fact that while

ABH blood group determinants are found in large quantities on the surface of intestinal

cells, some people possess a "secretor" phenotype, presenting blood antigens in their

mucosal secretions (Finne et al. 1989; D'Adamo and Kelly 2001). For these patients, the

blood antigens may actually serve as decoy binding substrates for LT. More importantly,

the association between LT and blood antigens was examined almost exclusively with

soluble LT. The contribution of blood sugar binding in the context of LT's ability to bind

LPS is not clear. Given the presence of five binding sites per holotoxin, binding to both

substrates is not implausible, but future studies involving LPS and blood sugars (perhaps

with vesicle-bound LT) would better mimic a physiologically relevant scenario. All of these binding events require the secretion of LT from ETEC as a first step, and this process will now be discussed, with a focus on the utility of LT and CT as ideal substrates to study the specificity of secretion machinery.

1.4 Bacterial protein secretion

Bacteria possess a wide variety of systems for the export of proteins to the extracellular milieu. For gram-positive bacteria, proteins destined for a role outside the cell are secreted from the cytosol predominantly through Sec machinery (although other pathways exist), after which the proteins can be released or bound to the external cell wall by the activity of sortase enzymes (Marraffini et al. 2006). The Sec machinery, conserved in gram-negative bacteria for transport of polypeptides across the inner membrane, consists of several integral cytoplasmic membrane proteins and an associated ATPase (Veenendaal et al. 2004). Gram-negative bacteria like *E. coli* and *V. cholerae*, due to the existence of a second membrane, have also evolved slightly more complex pathways for secretion to the extracellular milieu.

Direct secretion of cytoplasmic proteins. Some gram-negative secretion pathways, including the type I and type IV secretion systems, are capable of translocating bacterial effector proteins directly from the cytosol to beyond the outer membrane. The type III secretion system (sometimes termed the "injectosome") allows for the introduction of bacterial proteins into the cytosol of a nearby eukaryotic cell, crossing not one but three membranes in the process (Deane et al. 2009). The recently described type VI secretion system shares some structural similarity with the cell-puncturing apparatus of the T4 bacteriophage and may also translocate effectors directly into the host cell

(Pukatzki et al. 2009). However, many questions remain about this new secretion system.

　　　　　Secretion systems with periplasmic intermediates. While the previously mentioned secretion pathways export cytosolic effectors, other systems exist for the secretion of periplasmic proteins. The type II secretion system, which is responsible for the transport of LT and CT to the extracellular space, requires the coordinated efforts of a 12- to 15-subunit complex. This system will be discussed in detail below. However, for some substrates, no multisubunit complex is required. A number of proteins, termed autotransporters, are able to effect their own export from the cell in a process sometimes called type V secretion. Exemplified by IgA protease from *Neisseria gonorrhoeae*, these proteins contain a leader sequence directing them to the periplasm, where they fold and insert into the outer membrane, translocating the "passenger" domain to the extracellular milieu (Pohlner et al. 1987).

　　　　　A second form of type V secretion involves splitting the passenger and integral membrane domains into two separate proteins, frequently located in an operon, which each enter the periplasm in a *sec*-dependent manner before the passenger is secreted through a pore formed by the membrane protein (Mazar and Cotter 2007). In all cases, only specific substrates are secreted, and the identity of these effectors varies in different species. As described above, OMVs can also serve as a method for gram-negative bacteria to release proteins into the extracellular environment, albeit with less selectivity. Together, these systems (and a few others not mentioned here) provide gram-negative bacteria the means to interact with and alter their environment.

16

1.4.1 Type II secretion machinery

The type II secretion (T2S) system is a multiprotein complex spanning the inner and outer membranes of gram-negative bacteria. As of a recent review, genes for T2S had been found in four of the five classes of proteobacteria, although they are most prevalent in γ-proteobacteria, such as *E. coli* and *Vibrio* (Cianciotto 2005). Thus, the system as a whole appears to have arisen in a common bacterial precursor, with even distantly related pathogens such as *Chlamydia* possessing a partial T2S operon. In many cases, T2S is associated with virulence, but some bacteria carrying a T2S system are not pathogens. For instance, K-12 strains of *E. coli* carry a silenced copy of the T2S operon; when levels of the nucleoid-associated protein H-NS are reduced, this system is expressed and secretes an endogenous endochitinase (Francetic et al. 2000).

Outer membrane channel. Early studies of the T2S system were carried out in *E. coli* by expressing genes from *Klebsiella oxytoca* that were responsible for the export of a starch-degrading enzyme, pullulanase (Russel 1998). While the sequence of these genes initially provided little insight into the mechanism by which they enabled pullulanase to escape the cell, one gene, *pulD*, was noted to be similar to a protein encoded by a filamentous phage (Russel 1998). After several years of study, this gene was found to encode an outer membrane "secretin," which formed the channel through which T2S substrates are understood to be released. Secretin proteins form highly stable ring-like oligomers of 12 to 14 identical proteins surrounding a central pore of about 100 Å (Johnson et al. 2006). As can be presumed from its subcellular localization and

multimeric assembly, the secretin protein (GspD[2]) is an essential part of all T2S systems

identified to date, with all reported partial T2S operons containing a gene for this type of

protein (Cianciotto 2005). Elegant work using fluorescently labeled chromosomal T2S

constructs also indicates that GspD may influence the localization and assembly of other

subunits of the T2S apparatus (Lybarger et al. 2009). PulD, the GspD homolog in *K.*

oxytoca, requires the presence of a small outer membrane-anchored lipoprotein, PulS;

without this "pilotin," PulD inserts into the inner membrane, disrupting membrane

potential (Guilvout et al. 2006). While other similar proteins exist, this pilotin is not part

of all T2S systems. It is unclear why some GspD homologs require additional factors for

proper localization while others seemingly do not.

      Inner membrane components. In addition to the outer membrane pore, the T2S

apparatus contains an extensive collection of inner membrane proteins. Perhaps initially

surprising, these components are now understood to provide an energy source for the

export of periplasmic substrates across the outer membrane. The most obvious source of

energy for T2S comes from the cytoplasmic GspE protein, which is recognized to contain

ATPase activity, carrying canonical Walker A boxes for nucleotide binding (Johnson et

al. 2006). GspE has been found to associate with two integral inner membrane T2S

components, GspL and GspM, with the former understood to promote the association of

GspE with the inner membrane and, possibly, to influence its oligomerization state and

---

[2] Here, Gsp refers to the "general secretory pathway," as the T2S system was once termed the main
terminal branch of this pathway. While the genes for T2S components are called *gsp* in some bacterial
species (including *E. coli*), more often than not they are referred to by a unique nomenclature. Fortunately,
the final letter in the gene name (e.g., 'D') is shared in the vast majority of cases, facilitating comparison
between distinct species. However, when a protein component (or gene) is referred to in this text in general
terms, the format 'GspX' (*gspX*) will be used.

activity (Camberg et al. 2007). Similarly, EpsL and EpsM (as the proteins are called in

V. cholerae) have been shown to interact, protecting each other from proteolytic

degradation and forming higher-order complexes (Johnson et al. 2007). Less is known

about GspF, a third inner membrane protein, although OutF (from *Erwinia*) and $XcpS_F$[3]

(from *Pseudomonas aeruginosa*) have been shown biochemically to interact with their

corresponding GspE and GspL homologs (Johnson et al. 2006). A schematic showing

core T2S components and their reported interactions is shown in Figure 5.
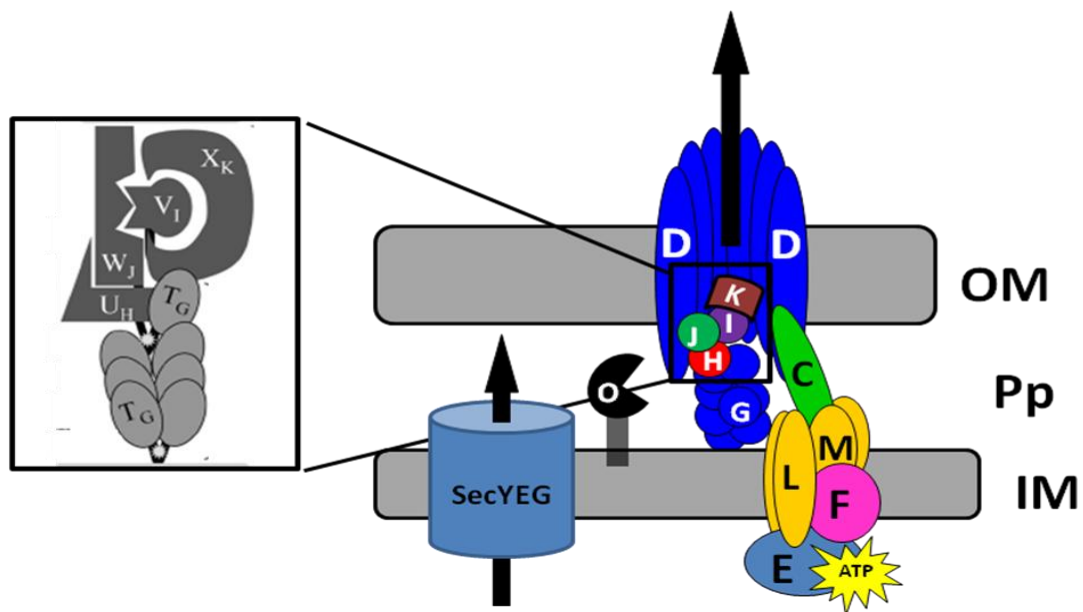
Figure 5: Components of the type II secretion system.

Common components of the bacterial type II secretion system are shown, along with their
localization to the inner membrane (IM), periplasm (Pp), or outer membrane (OM).
GspO represents the inner membrane prepilin peptidase enzyme. Inset: a closer view of
the putative interactions among the minor pseudopilins ($XcpU_H$-X_K) from *P. aeruginosa*
(adapted from (Douzi et al. 2009)).

---

[3] Following the convention of recent publications, in the few cases where a homolog from a completely
different naming system is mentioned, the final letter of the name of the corresponding Gsp system
homolog will be included as a subscript to limit confusion (in this case, XcpS is a homolog of GspF).

The pseudopilus. The so-called "pseudopilins" make up an intriguing set of

components of the secretion apparatus. These five proteins, GspG-K, are processed by a

prepilin peptidase in a manner similar to the way type IV pilin subunits reach their

mature forms through cleavage of an N-terminal sequence and subsequent methylation

(Russel 1998). This similarity is not accidental, as the type IV pilus biogenesis system

shares many features with T2S and is likely to represent an evolutionary linked pathway

(Stathopoulos et al. 2000). In fact, while the T2S system often involves a dedicated

prepilin peptidase (GspO), one protein in *P. aeruginosa* (PilD) is required for both T2S

and the formation of type IV pili (Strom et al. 1991). *V. cholerae* further exploits the

similarity between these two systems by secreting a pilus-independent soluble

colonization factor, TcpF, through the type IV pilus machinery (Kirn et al. 2003).

GspG is considered the major pseudopilin because of its abundance and its ability

to form a visible pilus-like structure (the hyperpseudopilus) when overexpressed, a

characteristic that is not shared by any other pseudopilin (Durand et al. 2005). While

type IV pilins contain disulfide bridges linking two domains to promote stability of the

nascent pilus, calcium ions stabilize GspG subunits in the T2S system (Korotkov et al.

2009). Explanations for the role of the pseudopilus largely fall into two categories:

pushing substrates through the pore formed by GspD or gating the GspD pore, preventing

the unregulated export of non-substrate periplasmic proteins.

The roles of the so-called minor pseudopilins (GspH-K) are less clear, although

several solved structures lend some indication of their function. The crystal structure of a

ternary complex containing GspI, GspJ, and GspK from ETEC indicates that no new

pseudopilin subunits could be added on top of GspK; that is, GspIJK may form a "cap" to

the pseudopilus (Korotkov and Hol 2008). This conclusion is consistent with the fact that

$XcpX_K$ destabilizes overexpressed $XcpT_G$, perhaps by capping hyperpseudopili as they

form (Durand et al. 2005). Recent evidence, also using the T2S proteins from *P.*

aeruginosa, indicates that $XcpV_I$ forms a quaternary complex with the H, J, and K

pseudopilins, perhaps serving as a nucleator for the complex (Douzi et al. 2009). Figure

5 includes a representation of the pseudopilin complex as it is understood to form.

GspC. Of the proteins making up the T2S system, GspC is the hardest to classify.

An integral inner membrane protein, it nonetheless has been reported to interact with

GspD at the outer membrane, perhaps forming a homotrimer in a GspD-dependent

manner (Possot et al. 1999). GspC and GspD homologs are sometimes found as a small

operon, separate from the rest of the T2S genes (Korotkov et al. 2006), and some species

such as *P. aeruginosa* even contain a second *gspCD* operon (in this case, *xphA-xqhA*) that

can stand in for the primary pair of T2S genes (Michel et al. 2007). In the inner

membrane, GspC has been shown to co-purify with GspL and GspM, interacting through

a region of its periplasmic domain near the inner membrane-spanning helix (Gerard-

Vincent et al. 2002). Another study in *Xanthomonas campestris* revealed an interaction

between $XpsN_C$ and the major pseudopilin, XpsG (Lee et al. 2005). Taken together,

these studies indicate that GspC may serve to span the two membranes of gram-negative

bacteria, possibly transferring the energy generated by ATP hydrolysis at the inner

membrane to the outer membrane to enable the export of substrates.

It is also worth noting that GspC has been repeatedly implicated in substrate

selectivity by the T2S machinery. Work done in *P. aeruginosa* and *P. alcaligenes* and

bacteria once classified as *Erwinia* spp. has indicated that portions of GspC are

responsible for species-specific identification of T2S substrates (Bouley et al. 2001; Gerard-Vincent et al. 2002). While entire T2S systems are not frequently suitable for expression in a heterologous system (the Pul complex from *K. oxytoca* functioning in *E. coli* is the exception rather than the rule), individual components can often be swapped between two bacterial species within the same genus. However, GspC proteins do not seem to be exchangeable, even between highly related species (Lindeberg et al. 1996). These observations, coupled with the fact that *gspC* is one of the least conserved T2S genes (Cianciotto 2005), make it tempting to speculate that GspC makes direct contact with T2S substrates.

1.4.2 Type II secretion substrates

As mentioned above, the earliest studies of T2S focused on the pullulanase enzyme (PulA) from *K. oxytoca*. PulA was found to be acylated and form a disulfide bond before secretion, and while acylation was not required for secretion, it enhanced the efficiency (Kornacker et al. 1991; Francetic and Pugsley 2005). These observations lent some of the earliest evidence for the idea that T2S substrates were being secreted in a mature form and not folding outside the cell. A number of periplasmic intermediates were uncovered for other substrates, including CT and exotoxin A from *P. aeruginosa* (Hirst and Holmgren 1987; Lu et al. 1993). The pore-forming toxin aerolysin from *Aeromonas hydrophila* must dimerize in the periplasm before secretion, and other substrates require the formation of disulfide bonds (reviewed in (Russel 1998)). Additionally, reasonable quantities of active substrates can be purified from the periplasm, especially when T2S is disrupted, demonstrating that folding occurs before export.

T2S substrates vary quite widely. Table 2 includes a sampling of T2S substrates, along with the species that secretes them and other notable characteristics. Understandably, given the great variation in these proteins, few substrates can be interchanged among bacterial species. In some cases, similar substrates from two closely related bacterial species that cannot be exchanged and still be secreted. *Erwinia chrysanthemi* (now *Dickeya dadantii*) and *Erwinia carotavora* (now *Pectobacterium carotovorum*) each carry a cellulase enzyme but can only secrete the native version (Lee and Schneewind 2001). Equally confusing, completely unrelated proteins are sometimes able to be secreted by a single system. As mentioned above, *Aeromonas* secretes aerolysin, but it also secretes alkaline phosphatase from *E. coli* if heterologously expressed (Wong and Buckley 1993). The common thread among these substrates is their use of the Sec machinery (or in rare cases, the alternative Tat pathway) to reach the periplasm before subsequent T2S-dependent export (Voulhoux et al. 2001). More importantly, no obvious sequence-based or structural motif is identifiable in all T2S substrates, not even in those secreted by the same species of bacteria (Bouley et al. 2001).

Table 2: Selected type II secretion substrates

Substrate	MW (kDa)	Species	Relevant Characteristics	Reference
LT	87.5	ETEC	AB_5 heterohexamer assembles and forms disulfide bonds before secretion	(Tauschek et al. 2002)
Pullulanase	116	*Klebsiella oxytoca*	N-terminus is acylated before secretion	(Kornacker et al. 1991)
Aerolysin	68	*Aeromonas* spp.	Two proproteins form a dimer before secretion	(Wong and Buckley 1991)
Exotoxin A	66	*Pseudomonas aeruginosa*	Mutations near N-terminus impair secretion	(McVay and Hamood 1995)
Cel5 endoglucanase	42	*Pectobacterium carotovorum*	Carbohydrate binding domain may also be involved in promoting secretion	(Chapon et al. 2000)
MtrC	75	*Shewanella oneidensis*	Integral outer membrane heme-containing c-cytochrome	(Shi et al. 2008)
PehA polygalacturonase	72	*Dickeya dadantii*	Mutations throughout the protein alter secretion properties	(Palomaki et al. 2002)
DraD	16	Uropathogenic *E. coli*	Tip protein of Dr surface fimbriae	(Zalewska-Piatek et al. 2008)

1.4.3 Type II secretion in *V. cholerae* and ETEC

As with CT and LT, the T2S system of *V. cholerae* was identified first and remains better studied. This system is encoded by the genes *epsC-M*, with VcpD serving as the prepilin peptidase protein and processing type IV pilins in El Tor strains (Sandkvist et al. 1997; Marsh and Taylor 1998). Early studies of mutations impairing T2S in *V. cholerae* described altered outer membrane profiles, implicating the Eps system in the process of outer membrane biogenesis (Sandkvist et al. 1997). Further research into the link between the Eps proteins and the outer membrane determined that *V. cholerae* T2S mutants were more sensitive to membrane-perturbing agents (detergents, bile salts, and polymyxin B) and displayed some leakage of periplasmic contents (Sikora et al. 2007).

In ETEC, the genes encoding the T2S system are called *gspC-M* and share a high degree of homology with the *eps* operon in *V. cholerae* (Tauschek et al. 2002). However, they are found as part of a larger operon (*yghJ-pppA-yghG-gspCDEFGHIJKLM*) that is regulated by H-NS in a temperature-dependent manner (Yang et al. 2007). The protein PppA can act as a prepilin peptidase (Francetic et al. 1998), but it has not yet been conclusively shown to process GspG in ETEC. A Δ*gspD* mutant in H10407 seems to leak a small amount of LT out of the periplasm, but did not show any obvious growth defect compared to the parental strain (Tauschek et al. 2002). It is not clear what role, if any, T2S may have in outer membrane biogenesis in ETEC.

Other studies determined that the secretion of LT from ETEC was dependent on the activity of a protein called LeoA, which was later found to be a GTPase (Fleckenstein et al. 1996; Brown and Hardwidge 2007). As only 3% of strains tested in a study of

numerous ETEC isolates carried the *leoA* gene, and Δ*leoA* mutants demonstrate

pleiotropic effects, the role of LeoA in the secretion of LT is certainly not a universal one

(Turner et al. 2006; Brown and Hardwidge 2007).

Most relevant to these studies, *V. cholerae* is able to secrete LT when expressed

heterologously (Neill et al. 1983). Our group has also shown that a lab strain of *E. coli*

carrying a plasmid copy of the *gsp* operon (which is normally silenced in such a strain)

and a deletion in *hns* causing upregulated transcription of the operon can secrete CT

(Horstman et al. 2004). These results are not so surprising, given the high degree of

identity between LTB and CTB. However, they do allow for further characterization of

how these two toxins are recognized as T2S substrates, because many residues are

directly shared by LT and CT, and therefore, mutations made in each toxin can be

directly compared. To date, one mutation (at a residue shared by LTB and CTB)

affecting the secretion of CT had been reported (Connell et al. 1995). This mutant was

reported to bind G_{M1}, but further characterization of its stability was not carried out.

Altogether, these T2S systems and substrates are seemingly as closely related as can be

found in separate bacterial genera. Studies of their specificity would provide great

insight into the complexity of the process of recognizing T2S substrates.

1.5 Significance

ETEC is a tough and prevalent pathogen. Its primary virulence factor, LT, has

been studied for decades, but much remains to be elucidated regarding its course of

action *in vivo*. The work of this thesis is dedicated to a further understanding of this

toxin, largely through studies of point mutations in LTB. Experiments described in

Chapter 2 have revealed the location of the binding pocket responsible for LT's association with LPS. This pocket overlaps with a set of residues involved in binding to blood sugars, but is not identical to the peripheral pocket identified by Holmner et al. (2007). Furthermore, one mutation significantly reducing LT's association with LPS, T47A, was shown to achieve its effects without any other detrimental effects on the normal properties of the toxin, including its expression in ETEC. This mutant also demonstrated impaired binding to the surface of ETEC, a physiologically relevant scenario. The results of assays measuring the surface binding of LT mutants to *E. coli* strains expressing truncated forms of LPS implicate the outer core as the preferred binding site of LT, although binding to Kdo was supported, as in previous studies (Horstman et al. 2004).

The discovery of two mutations that also impaired the export of LT from ETEC allowed for comparison of the secretion of LT and CT, a closely related T2S substrate with a previously reported mutation reducing its secretion efficiency (Connell et al. 1995). As summarized in Chapter 3, experiments analyzing the secretion of mutant LT and CT from both ETEC and *V. cholerae* provide evidence that these two highly similar substrates are recognized in different ways when being secreted by each pathogen. Interestingly, as described in Chapter 4, many of these secretion-impaired mutants demonstrated unexpected stability defects when expressed in *V. cholerae*. For both CT and LT, several mutations caused strain-specific stability phenotypes when expressed in two *V. cholerae* isolates. In addition, expression of some mutants impaired the growth of *V. cholerae*, although they had no detrimental effect on ETEC.

As summarized in Chapter 5, the experiments reported herein highlight the finely tuned nature of LT, defining mutations that alter several of its binding properties and shedding light onto the extent of the changes made by introducing mutations into its B subunit. With the identification of certain mutants, further research can be performed regarding the identity of the T2S components responsible for substrate selectivity in ETEC and *V. cholerae* as well as the role of LT's interaction with LPS and blood sugars in ETEC pathogenesis *in vivo*.

2. Residues of heat-labile enterotoxin involved in bacterial cell surface binding

2.1 Summary

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of traveler's diarrhea worldwide. One major virulence factor released by this pathogen is the heat-labile enterotoxin, LT, which upsets the balance of electrolytes in the intestine. After export from ETEC, LT binds to lipopolysaccharide (LPS) on the bacterial surface. Although the residues responsible for LT's binding to its host receptor are known, the portion of the toxin which mediates LPS binding has not been defined previously. Here, we describe mutations in LT that impair the binding of the toxin to the surface of *E. coli* without altering holotoxin assembly. One mutation in particular, T47A, nearly abrogates surface binding without adversely affecting expression or secretion in ETEC. Interestingly, T47A is able to bind mutant *E. coli* expressing highly truncated forms of LPS, indicating that LT binding to wild-type LPS may be primarily due to association with one or more outer core sugars. In further support of this hypothesis, the E11A mutant is able to bind full LPS cores but cannot bind to truncated forms lacking the outer core. Consequently, we have identified a region of LT, distinct from the pocket involved in eukaryotic receptor binding, that is responsible for binding to the cell surface of *E. coli*.

2.2 Introduction

ETEC, the most common etiologic agent behind traveler's diarrhea, is also a significant cause of mortality worldwide (Turner et al. 2006). Many strains of ETEC elaborate a virulence factor called heat-labile enterotoxin or LT (Sjoling et al. 2006). LT is an AB_5 toxin, consisting of a single A subunit, LTA, and a ring of five B subunits, LTB

(Sixma et al. 1991). LTB mediates the toxin's binding properties, and LTA ADP-ribosylates host Gsα, increasing levels of cAMP and causing the efflux of electrolytes and water into the intestinal lumen (Moss and Richardson 1978; Spangler 1992). Each subunit of LT is translated separately from a bicistronic message and then transported to the periplasm, where holotoxin assembly occurs spontaneously (Hofstra and Witholt 1985). Subsequent export into the extracellular milieu is carried out by the type II secretion system (Russel 1998; Tauschek et al. 2002).

LT binds eukaryotic cells via an interaction between LTB and host gangliosides, primarily the monosialoganglioside G_{M1} (Spangler 1992). The binding site for G_{M1}, situated at the interface of two B subunits, has been identified by crystallography (Merritt et al. 1994). G_{M1} binding can be strongly impaired by a point mutation in LTB that converts Gly-33 to an aspartic acid residue (Tsuji et al. 1985). LT is highly homologous to cholera toxin (CT), both in sequence and structure (Dallas and Falkow 1980; Spangler 1992), contributing to ETEC's potentially cholera-like symptoms (Vicente et al. 2005).

Previous work in our lab has demonstrated that LT possesses an additional binding capacity beyond its affinity for host glycolipids: the ability to associate with LPS on the surface of *E. coli* (Horstman and Kuehn 2002). LPS, the major component of the outer leaflet of the gram-negative outer membrane, consists of a characteristic lipid moiety, Lipid A, covalently linked to a chain of sugar residues (Raetz 1993). In bacteria like *E. coli*, this sugar chain can be further divided into an inner core oligosaccharide of around five sugars, an outer core of four to six additional sugars, and in some cases a series of oligosaccharide repeats known as the O antigen. Lipid A itself cannot inhibit binding of soluble LT to cells containing full-length or truncated LPS, indicating that the

30

LT-LPS interaction involves sugar residues on the surface of *E. coli* (Horstman et al. 2004). The addition of the inner core sugar 3-deoxy-D-manno-octulosonic acid (Kdo) is the minimal Lipid A modification required for LT binding (although longer oligosaccharide chains are preferred), and expression of a kinase that phosphorylates Kdo impairs binding by LT (Horstman et al. 2004). Competitive binding assays and microscopy with fluorescently-labeled ETEC vesicles show that binding to G_{M1} and LPS can occur at the same time, revealing that the binding sites are distinct (Horstman and Kuehn 2002; Kesty et al. 2004). In contrast to LT's ability to bind to the surface of ETEC, CT (or LT, when expressed heterologously) cannot bind *Vibrio* cells, presumably because due to differences in LPS structure, including the presence of phosphorylated Kdo in *Vibrio* spp. (Cox et al. 1996).

As a result of the LT-LPS surface interaction, over 95% of secreted LT is found associated with *E. coli* outer membrane vesicles (OMVs), rather than being secreted solubly (Horstman and Kuehn 2002). OMVs are spherical structures of 50-200 nm diameter that are derived from outer membrane but also enclose periplasmic components (Kuehn and Kesty 2005). As such, active LT is found both on the surface of an OMV and within its lumen (Horstman and Kuehn 2000). ETEC releases a large number of OMVs (Wai et al. 1995), and these vesicles may serve as vehicles for the delivery of LT to host cells.

Recent work by Holmner et al. has uncovered a third binding substrate for LT: human blood group A antigen (2007). This interaction was noted previously as a novel binding characteristic of artificially constructed CT/LT hybrid molecules, but now has been shown to occur with wild-type LT as well (Holmner et al. 2004; Holmner et al.

31

2007). LTB binding to sugar residues in the receptor molecule occurs at a site that is separate from the G_{M1}-binding pocket, in the same region our lab previously proposed to be involved in LPS binding (Horstman et al. 2004; Holmner et al. 2007). While the severity of cholera disease symptoms has been linked to blood type (Harris et al. 2005), the effects of blood type on ETEC infection are less clear. However, it has been demonstrated that LT can use the A-type antigen as a functional receptor in cultured human intestinal cells (Galvan et al. 2004; Galvan et al. 2006), and one recent cohort study found an increased prevalence of ETEC-based diarrhea among children with A or AB blood type (Qadri et al. 2007).

We set out to generate a mutation in LT that reduces its LPS binding without adversely affecting its expression, secretion, or toxicity. In this work, we present the discovery of point mutations in LTB that impair its interactions with the bacterial surface. Examination of these mutations reveals an LPS binding pocket which shares residues with the blood sugar pocket. Studies of LTB mutants binding to bacteria with truncated LPS provide better understanding of the roles that inner and outer core sugars play in toxin binding, and expression, secretion, and toxicity studies demonstrate which mutant is a particularly good candidate for future research. These binding mutants may lead to further discovery of the role that LPS binding plays in the pathogenesis associated with ETEC infection.

Table 3: Strains and plasmids used in Chapter 2.

Strain	Genotype	Relevant characteristics
jf570[1]	H10407Δ*eltA*	ETEC with polar insertion in *eltA* (LT-deficient)
MK1052	H10407Δ*eltA*/ pILT	jf570 carrying inducible LT plasmid; Amp^R
MK1200	H10407Δ*eltA*/ pILT[T47A]	jf570 carrying inducible T47A mutant; Amp^R
MK741	DH5α *degP*::Tn5/ pDsbA/ pILT	*E. coli* K-12, *degP* knockout, carrying a plasmid copy of *dsbA* and an inducible LT plasmid; Kan^R, Cm^R, Amp^R
CWG311	F470 *waaV*::aacC1	R1 core, lacks O-antigen; Gm^R
CWG309	F470 *waaT*::aacC1	R1 core, two terminal Gal residues removed; Gm^R
CWG303	F470 *waaG*::aacC1	R1 core, entire outer core removed; Gm^R
WBB01	JC7236 Δ*waaCF*::tet6	Expresses only Kdo$_2$-Lipid A; Tet^R

Plasmid	Relevant characteristics
pILT	IPTG-inducible LT holotoxin; Amp^R
pDsbA	*dsbA* ligated into pACYC184; Cm^R, Tet^R

2.3 Results

Mutagenesis and assessment of proper assembly of mutant toxins. Given the recent identification of a peripheral sugar binding pocket on LTB by co-crystallization (Holmner et al. 2007), we investigated whether or not this region also plays a role in LPS binding. In that study, residues Gln-3, Tyr-18, Ala-46, and Thr-47 were shown to interact directly with blood antigen sugars (Holmner et al. 2007). Mutations were designed to introduce a highly dissimilar amino acid residue in place of the native residue[2], and the residues were mutagenized in a plasmid containing an inducible LT construct (pILT), generating the following mutants: Q3K, Q3L, Y18A, A46D, and T47A.

The stability of LTB is sensitive to mutation (Alone et al. 2007), so in order to verify that each was folded and assembled, mutant toxins were subjected to multiple

---

[1] The origin of strains not generated in this study can be found in section 2.5.

[2] Detailed methods for the entire thesis are found in Chapter 6.

assays to assess holotoxin formation following purification from strain MK741 (Table 3)

or a derivative thereof carrying the appropriate mutant LT construct. First, wild-type and

mutant toxins were immunoblotted using an anti-LTA monoclonal antibody. All toxins

were detected equally (Figure 6A). The purification process is based on galactose

binding by LTB pentamers, so detection of equal amounts of LTA in purified samples is

a positive indication of holotoxin assembly. Levels of purified LTB were also equal

when compared by Ruby Red staining of SDS-PAGE gels or immunoblotting with cross-

reactive anti-CT antibody (Figure 6A and data not shown).
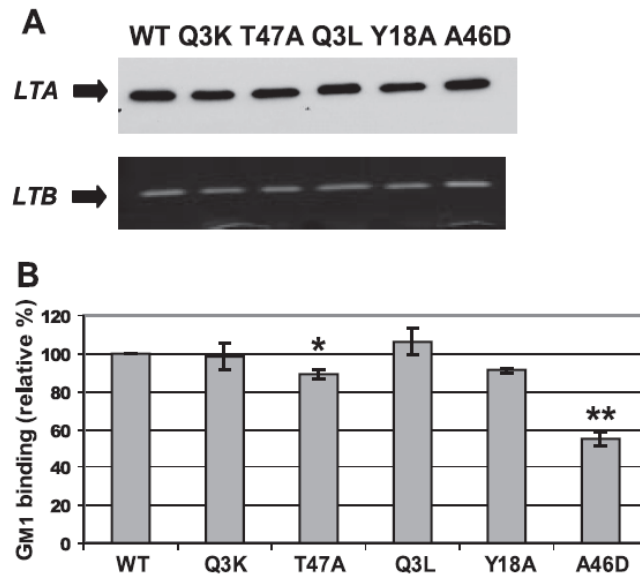
Figure 6: Mutant LTBs assemble into holotoxins that bind G_{M1}.

(A) Representative immunoblot (top) and Ruby Red protein stain (bottom) of 400 ng each of purified wild-type (WT) and the indicated mutant toxins. The blot was probed with anti-LTA antibody. **(B)** Relative G_{M1} binding of purified toxins, as measured in quadruplicate by ELISA. Wild-type binding was set to 100%. *, $p<0.05$; **, $p<0.005$ compared to wild-type ($n\geq2$).

This same panel of purified toxins was also tested for the ability to bind the

eukaryotic receptor G_{M1} by ELISA. Since G_{M1} binding involves residues in adjacent B-

subunits, this assay serves as an indication of wild-type folding and pentamer assembly. Q3K, Q3L, and Y18A bound G_{M1} at wild-type levels, while T47A did show a slight, but statistically significant, reduction in binding to approximately 89% of wild-type (n=4, Figure 6B). A46D demonstrated a stronger impairment of G_{M1} binding, to approximately 55% of wild-type (n=3, Figure 6B).

Finally, each mutant was tested for toxicity using a previously described *in vitro* cell culture bioassay based on a morphological change in Y1 adrenal cells (Donta et al. 1974). At least two separate batches of each mutant exhibited toxic effects comparable to wild-type LT (that is, 4-8 ng of each elicited maximal rounding of 4×10^5 Y1 cells). Taken together, our results indicate that these mutations do not appreciably alter the proper folding of LT, although A46D does somewhat impair host receptor binding.

Mutations in LT's blood sugar binding pocket also impair bacterial surface binding. In order to biochemically validate the role of Gln-3, Tyr-18, Ala-46, and Thr-47 in blood sugar binding, toxins containing point mutations at those residues were tested for binding to blood group A trisaccharide. Each mutation drastically impaired the toxin's ability to bind A trisaccharide conjugated to bovine serum albumin (BSA), as determined by ELISA (Figure 7). For Q3K, Q3L, and Y18A, the level of absorbance detected in some trials was equivalent to the blank, indicating a complete loss of binding. We have therefore demonstrated that four residues identified by co-crystallography (Gln-3, Tyr-18, Ala-46, and Thr-47) are indeed required for LT to associate with blood sugars.
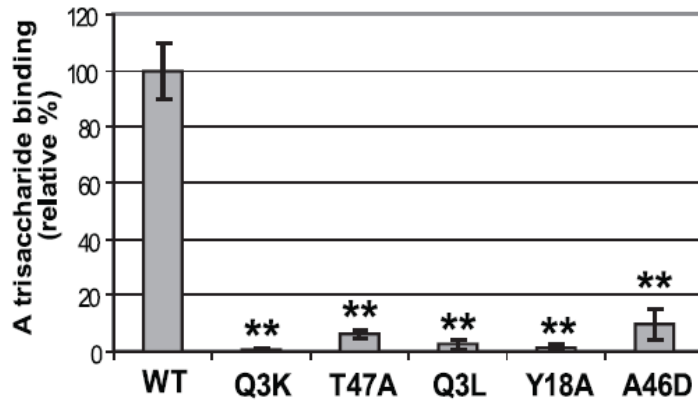
Figure 7: Alteration of putative sugar binding residues in LTB abolishes toxin binding to blood antigen.

Relative binding of wild-type (WT) and the indicated mutant toxins to blood group A antigen terminal trisaccharide (GalNAcα3[Fucα2]Gal) conjugated to BSA. Wild-type toxin binding was set to 100%. Each ELISA was carried out in quadruplicate, and at least two different batches of purified toxin were tested. **, $p<0.005$ compared to wild-type ($n \geq 3$).

To examine whether any of these blood sugar binding mutants were impaired in terms of LPS binding, purified mutant toxins were screened using a bacterial cell surface binding assay. After incubation and washing, we assessed the level of each toxin associated with *E. coli* by immunoblotting (Figure 8A). Initial screening of cell surface binding was carried out using DH5α cells due to the defined oligosaccharide core structure and consistent length of its LPS (Figure 8B), and because previous work from our lab has demonstrated no essential involvement of O antigen in the LT-LPS interaction (Horstman et al. 2004). Densitometric analysis of multiple immunoblots revealed that mutants Q3K, T47A, Q3L, and A46D possess a clearly reduced capacity to bind bacterial cells *in vitro* (Figure 8C). Of these, T47A and A46D showed the strongest impairment, with T47A nearly abolishing surface binding. Mutant Y18A bound the cell surface at wild-type levels, in contrast to its lack of binding to blood group A

36

trisaccharide. For each mutant, unbound toxin was detectable in the supernatant after pelleting cells incubated with the toxin, indicating that the mutants tested were not degraded during the assay (Figure 8D).
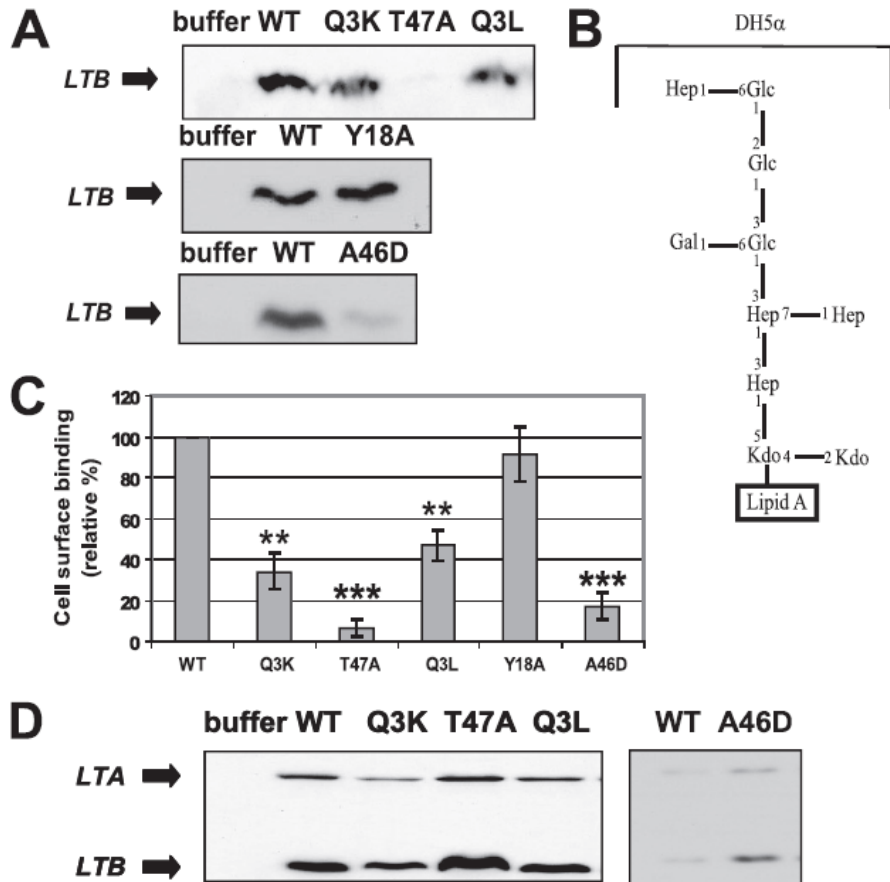
Figure 8: Mutations in LT's blood antigen binding pocket impair the association of the toxin with the *E. coli* cell surface.

(A) Representative immunoblots of DH5α cells after incubation with buffer, wild-type LT (WT), or the indicated mutant toxin. **(B)** Structure of the K-12 oligosaccharide core carried by DH5α (adapted from (Frirdich et al. 2003)). Hep, heptose; Glc, glucose; Gal, galactose. **(C)** Relative binding levels of wild-type and mutant toxins to DH5α cells. Binding of wild-type LT was set to 100%. Each experiment was carried out with a fresh batch of purified toxin. **, $p<0.005$; ***, $p<0.0005$ compared to wild-type ($n=3$). **(D)** Immunoblots of unbound toxin in the supernatant fraction from the incubations described in (A). Blots were probed with cross-reactive anti-CT antibody.

Surface binding of exogenously added wild-type and T47A mutant LT was also compared using fluorescently labeled G_{M1} to detect bound toxin. After incubation of DH5α cells with wild-type LT or T47A, cell-associated fluorescence was quantitated using a 96-well plate reader. T47A was detected at a significantly reduced level after subtraction of the fluorescence from non-specific binding to cells incubated with buffer alone (Figure 9). Measurements using fluorescent G_{M1} did not show as strong an impairment for T47A as our immunoblot-based assay and could not distinguish intermediate levels of impairment (e.g., that of Q3K). This discrepancy likely exists because fluorescent G_{M1} had a relatively high background of non-specific binding (with a value near T47A's before correction) and a tendency to aggregate, as visible by fluorescence microscopy (data not shown). Nevertheless, this independent assay supports our assertion that T47A's binding to the bacterial surface is impaired.
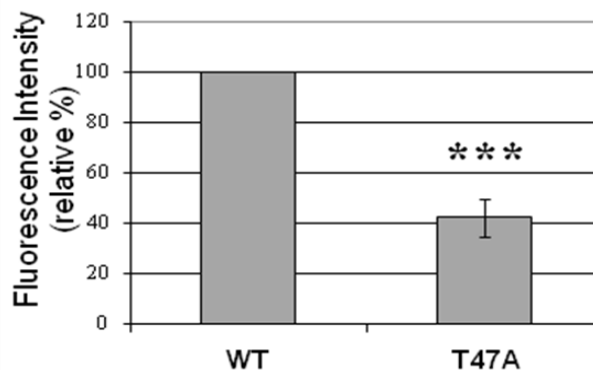
Figure 9: The surface binding impairment of T47A is detectable using a fluorescence-based binding assay

DH5α cells were incubated with wild-type LT (WT) or T47A prior to incubation with fluorescently labeled G_{M1}. The fluorescence associated with cells after washing was measured in 96-well plates with a fluorometer, with background from buffer-treated cells subtracted. ***, $p < 0.0005$ ($n=4$).

Since similar residues appeared to be responsible for binding to both blood sugars and the bacterial surface, we expected that blood sugars could inhibit bacterial surface binding. Consistent with this hypothesis, pre-incubation of LT with an excess of A trisaccharide conjugated to BSA, but not BSA alone, abolished subsequent binding to the bacterial surface (Figure 10A). In contrast, pre-incubation of LT with A trisaccharide conjugated to BSA or BSA alone had no significant effect on G_{M1} binding (Figure 10B). Therefore, the blood sugar and bacterial surface binding sites of LT overlap, but are not identical, and both sites are distinct from the G_{M1} binding pocket.
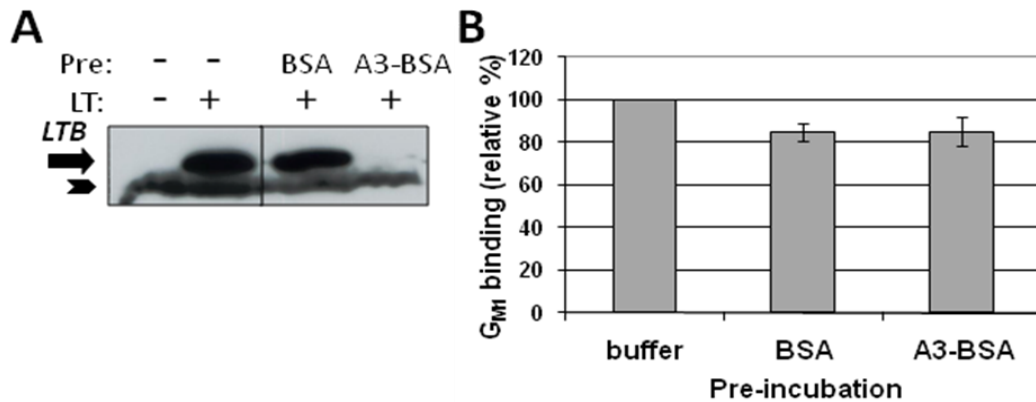
Figure 10: Blood group A trisaccharide inhibits the binding of LT to the surface of *E. coli* but not to G_{M1}

(A) Representative immunoblot of LT bound to DH5α cells (*n=3*). Toxin was preincubated (Pre) with buffer (-) or a 20-fold molar excess of BSA or blood group A trisaccharide conjugated to BSA (A3-BSA) for 1 h at 37°C. The blot was probed with cross-reactive anti-CT antibody. The arrowhead indicates a non-specific band detected. (B) Toxin was pre-incubated under the conditions described in (A) and subsequently tested for G_{M1} binding by ELISA. Binding of buffer-treated LT was set to 100% (*n=2*).

The E11K mutation also impairs the surface binding of LT. Mutant toxin bearing the E11K mutation was generated as part of a study on the secretion of LT (see Chapter 3). Due to the proximity of Glu-11 to the binding pocket described above, this

mutant was also tested for its ability to bind the surface of *E. coli*. Interestingly, the mutant was strongly impaired (Figure 11). However, substitution of Glu-11 with alanine (E11A), a less drastic change, did not impair surface binding. Both mutants were able to bind G_{M1} at near wild-type levels, indicating proper folding and assembly of the pentamers (Figure 11B). Consistent with the location of Glu-11 outside the identified blood sugar binding pocket, the E11K mutation did not reduce binding to blood group A trisaccharide (Figure 11B). Additionally, each mutant was as toxic as wild-type LT in our cell culture assay (data not shown), demonstrating that holotoxin formation occurs normally for E11K and E11A. Together, these results show that at least one residue outside the previously identified peripheral binding pocket in LTB is also involved in LPS binding.
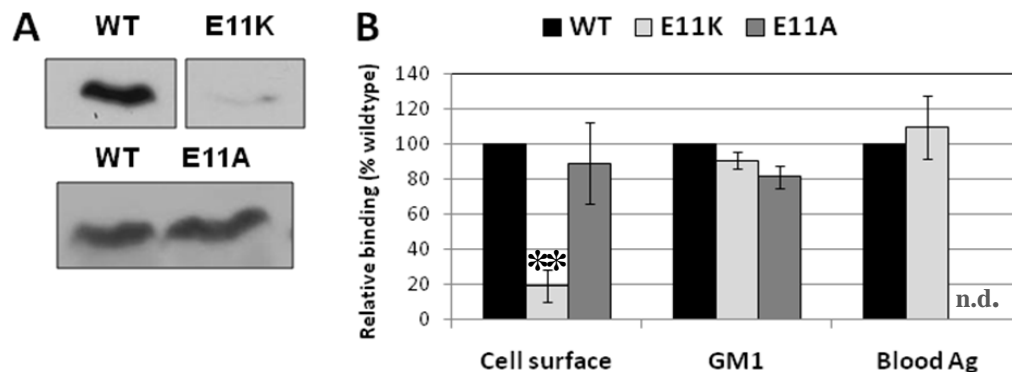
Figure 11: Substitution of Glu-11 with lysine, but not alanine, impairs the surface binding of LT.

(**A**) Representative immunoblots of DH5α cells after incubation with purified wild-type (WT) LT or the indicated mutant toxin. (**B**) Relative levels of binding of wild-type (WT) and mutant toxin to the *E. coli* cell surface, G_{M1}, and blood group A terminal trisaccharide (Blood Ag). Binding to the cell surface was measured by the immunoblot-based assay described above, and binding to G_{M1} and blood sugars was measured by ELISA. **, $p<0.001$ compared to wild-type; n.d., not determined ($n \geq 3$ for all experiments except binding to blood sugars, $n=2$).

It should be noted that we generated mutations in 10 other residues on the periphery of LTB, including many in an area that we had previously speculated may play a role in LPS binding (Horstman et al. 2004). However, these additional mutants exhibited either poor expression in ETEC or had no effect on surface binding in preliminary experiments and were therefore set aside (data not shown). See Chapter 5 for a complete list of LTB mutations generated in these studies and their effects on the stability of the toxin.

Binding of mutant toxins to bacteria expressing truncated forms of LPS. Previous work from our lab has shown that LT requires only the innermost LPS core sugar, Kdo, for binding, although it binds better to LPS with a full oligosaccharide core (Horstman et al. 2004). In order to determine if the mutations described above affected binding to Kdo or other core sugars, we repeated our cell surface binding assay with strains containing truncated forms of LPS from an F470 background. CWG311 features a full-length R1 LPS core, which is similar to the K-12 *E. coli* core found in DH5α, and a disruption in *waaV* to prevent the ligation of O antigen (Heinrichs et al. 1998). CWG309 and CWG303 feature other mutations in this background, truncating LPS to various extents (Figure 12A). Specifically, CWG309 (Δ*waaT*) lacks two terminal galactose residues from the R1 core, and CWG303 (Δ*waaG*) lacks two additional residues, both glucoses, leaving only three heptose and two Kdo residues (Heinrichs et al. 1998; Frirdich et al. 2003). WBB01 is derived from a different strain background and carries deletions in both of *E. coli*'s heptosyltransferases (Brabetz et al. 2000), preventing the addition of sugars beyond the two innermost Kdo residues. As a result, WBB01 expresses Kdo$_2$-Lipid A, the minimal viable LPS structure in *E. coli* and the portion conserved

across all *E. coli* oligosaccharide core types (Raetz 1993; Amor et al. 2000).   Since

changes in LPS structure affect outer membrane protein and lipid composition (Nikaido

2003), comparisons between wild-type and mutant toxin binding could be made for each

strain, but not between strains.   Indeed, some membrane instability has been reported

previously for CWG303, in particular (Yethon et al. 2000).
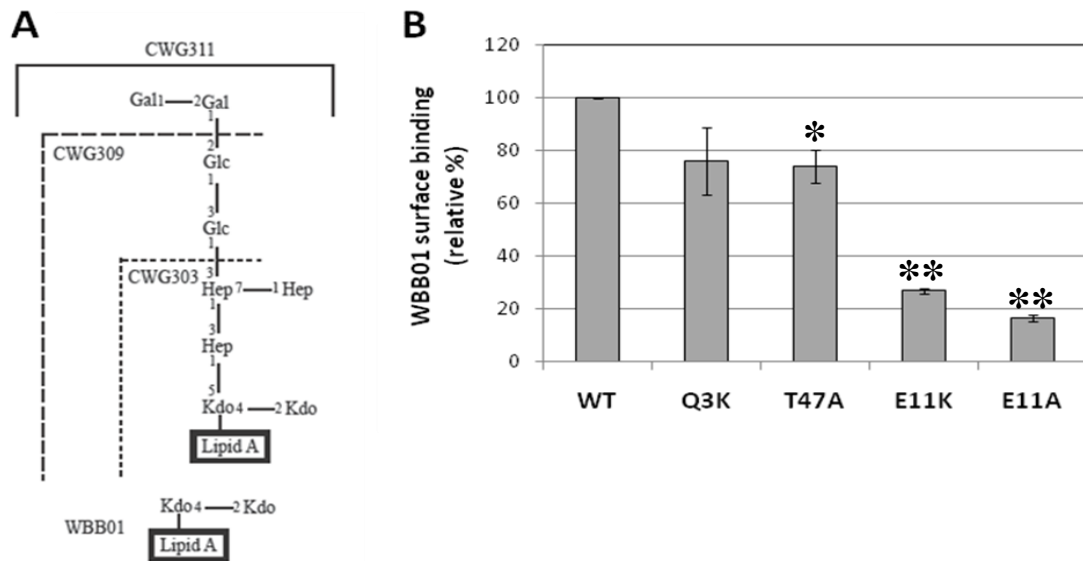
Figure 12: Kdo binding phenotypes of LT mutants

(A) The structure of the R1 LPS oligosaccharide core carried by CWG311, with
truncations present in strains CWG309 and CWG303 indicated (adapted from (Frirdich et
al. 2003)). The LPS structure of WBB01 is also depicted. **(B)** Relative binding of wild-
type (WT) LT and the indicated mutants to the surface of WBB01 cells. Each experiment
was carried out with a fresh batch of purified toxin. *, $p<0.05$; **, $p<0.0005$ compared to
wild-type ($n\geq2$).

Mutants were first tested for binding to cells bearing only Kdo, previously defined

as the determinant of LT binding to LPS (Horstman et al. 2004). The Q3K mutation,

which caused a moderate impairment of DH5α surface binding, did not strongly affect

Kdo binding (Figure 12B). Surprisingly, two mutations that demonstrated strong effects

on binding to full-core LPS showed differing Kdo binding phenotypes. T47A demonstrated considerable binding to WBB01 (although slightly less than wild-type), while E11K was strongly impaired for binding to Kdo (Figure 12B). Pretreatment of WBB01 cells with Pronase did not affect the levels of bound T47A (data not shown). Surprisingly, E11A (which was able to bind to DH5α cells at near wild-type levels; see Figure 11) was highly impaired for Kdo binding (Figure 12B). Thus, mutations that affect Kdo binding do not necessarily impair binding to a full-length core, and mutants that are unable to bind full-length LPS may be able to bind well to Kdo.

LT mutants were further assessed for binding to *E. coli* strains bearing truncated forms of LPS (Figure 12A). T47A showed a strong impairment in binding to the full R1 oligosaccharide core and the same core lacking its two terminal galactose sugars (Table 4). However, the mutation did not seem to affect binding to CWG303 at all, similar to the results with strain WBB01 (Table 4 and Figure 12B). Therefore, the T47A mutant retains the ability to bind Kdo, but is impaired for binding to fuller core structures.

The Q3K mutation was impaired for binding to full-length LPS (either the K-12 or R1 core), but was able to bind strain CWG309, which only differs from the parental strain CWG311 by the loss of two galactose sugars (Table 4). In fact, Q3K bound to the CWG309 and CWG303 LPS truncation strains at levels slightly higher than wild-type LT (Table 4). The Q3A substitution mutant was able to bind to a full-length R1 core (strain CWG311), despite its impairment in binding to the K-12 core of DH5α, and also bound to WBB01 cells expressing only Kdo sugars (Table 4).

The A46D and E11K mutants demonstrated similar, significant binding deficiencies to all strains tested (Table 4). E11A bound to CWG311 at a diminished level

43

Table 4: Binding of purified mutant toxins to *E. coli* cells carrying LPS of varying lengths.

Purified mutant toxin was incubated with *E. coli* strains carrying full-core LPS (DH5α and CWG311) or strains bearing truncated forms of LPS (see Figure 12A), and binding was measured with the immunoblot-based assay described above. All binding values are expressed as percentages of wild-type LT binding. *p*-values represent differences between mutant and wild-type binding according to Student's *t*-test (significant reductions in binding are in bold; $n \geq 2$).

Mutant	DH5α	*p*-value	CWG311	*p*-value	CWG309	*p*-value	CWG303	*p*-value	WBB01	*p*-value
Q3K	**33.8±8.8%**	**<0.005**	**33.8±6.0%**	**<0.01**	106.6±0.7%	<0.05	126.8±5.1%	<0.05	76.1±12.6%	0.20
Q3A	**36.5±4.7%**	**<0.0005**	74.5±9.8%	0.06	--	--	--	--	134.0±26.7%	0.33
E11K	**19.6±9.3%**	**<0.001**	**5.9±2.8%**	**<0.001**	**21.6±8.4%**	**<0.01**	**22.7±7.2%**	**<0.005**	**26.9±1.1%**	**<0.0005**
E11A	89.3±23.4%	0.67	**59.6±13.7%**	**<0.05**	--	--	--	--	**16.4±1.2%**	**<0.0005**
A46D	**16.8±6.3%**	**<0.0005**	**56.5±4.4%**	**<0.001**	**58.9±1.1%**	**<0.001**	**56.5±4.6%**	**<0.001**	**52.5±8.5%**	**<0.05**
T47A	**6.4±3.9%**	**<0.0001**	**5.8±0.2%**	**<0.0001**	**10.5±0.8%**	**<0.0001**	82.5±8.7%	0.11	**74.1±6.3%**	**<0.05**
	Full K-12 core		Full R1 core		R1 core lacking two galactose sugars		CWG309 lacking two glucose sugars		Kdo_2-Lipid A only	

compared to wild-type LT but was able to bind the R1 core of that strain to a much greater extent than E11K, in line with the phenotypes of these two mutants in binding the full core of DH5α (Figure 11 and Table 4). The Y18A mutation, which did not affect DH5α binding at all (Figure 8B), was also tested for binding to CWG311 (carrying the full R1 core) and WBB01. Preliminary results show that Y18A bound these two strains like wild-type LT, indicating that Tyr-18 plays no role in surface binding to any form of LPS (data not shown).

Surface binding of T47A in ETEC. Since T47A was a fully-folded mutant toxin deficient in binding to wild-type strains of *E. coli*, further studies focused on this mutation. The surface features of ETEC and laboratory *E. coli* strains differ in many ways, so we next tested a more biologically relevant scenario: the binding of T47A to ETEC's surface. An LT-deficient ETEC strain, jf570 (H10407Δ*eltA*), was used. This strain contains a polar insertion in *eltA*, the first gene in the operon encoding LT, thus abolishing production of the toxin (Dorsey et al. 2006). T47A exhibited a nearly complete lack of binding to jf570 cells using our surface-binding assay (Figure 13A and B). Once again, the lack of binding was not due to degradation, since T47A was detected in the unbound fraction (Figure 13C).
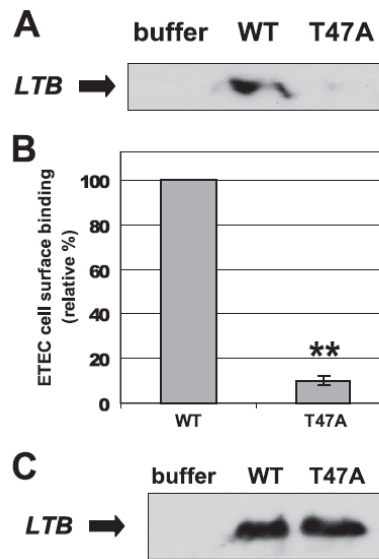
Figure 13: The T47A mutant does not bind the surface of ETEC.

(**A**) Representative immunoblot of jf570 ETEC cells after incubation with buffer, wild-type LT (WT), or T47A mutant toxin. (**B**) Relative binding of wild-type and T47A toxins to ETEC cells are shown, with binding of wild-type toxin set to 100%. Each experiment was carried out using a fresh batch of purified toxin. **, $p<0.001$ ($n=2$). (**C**) Representative immunoblot of unbound toxin from the supernatant of the incubations described in (A). Blots were probed with cross-reactive anti-CT antibody.

Characterization of T47A expression and secretion in ETEC. In order to assess the behavior of the T47A mutant when expressed *in vivo*, an inducible LT plasmid with the T47A mutation was transformed into jf570. After induction overnight, toxin expression levels were determined by immunoblotting total culture samples which had been concentrated by TCA precipitation. Levels of T47A matched those of wild-type LT (Figure 14A).

To examine whether the T47A mutation affected the secretion of LT by ETEC, cultures of strains carrying wild-type LT and T47A were induced and fractionated. Levels of toxin in the supernatant were measured by G_{M1}-binding ELISA, with data

normalized to CFUs. T47A was found in the supernatant at levels slightly higher than wild-type (Figure 14B). A corresponding decrease in levels of T47A in the periplasm was noted as well (Figure 14B). Thus, the numerous properties of LT, including expression, holotoxin assembly, secretion, and host receptor binding, are not strongly affected by the T47A mutation, only the toxin's ability to bind the bacterial surface. E11K, while also a surface binding mutant with good expression in ETEC, was found to have a secretion defect, rendering it less useful for future studies of the role of surface binding in ETEC pathogenesis (see Chapter 3).
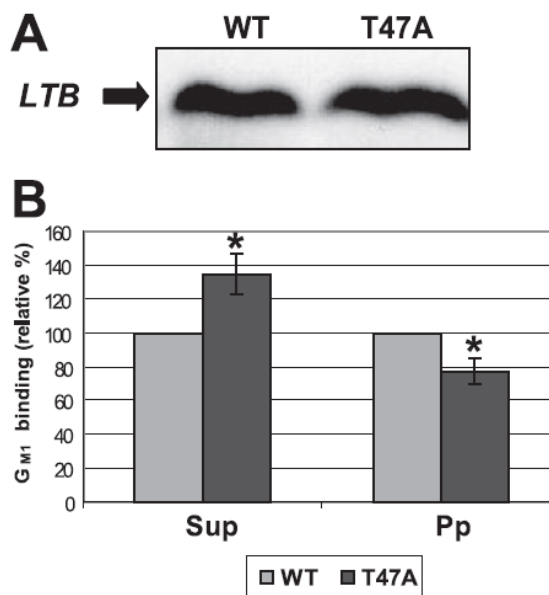
Figure 14: The T47A mutant is expressed at wild-type levels in ETEC but demonstrates altered localization in culture fractions.

(A) Representative immunoblot of TCA-precipitated total culture samples, adjusted for CFU, demonstrating induced expression levels of wild-type (WT) and T47A mutant in strain jf570. The blot was probed with cross-reactive anti-CT antibody. **(B)** MK1052 (WT) and MK1200 (T47A) were fractionated to isolate cell-free supernatant (Sup) and periplasm (Pp). Each fraction was tested for toxin levels by G_{M1} ELISA. Supernatant levels were normalized to CFU, and periplasm levels were normalized to alkaline phosphatase activity. *, $p<0.05$ ($n=4$).

LT binds to liposomes containing LPS and a mixture of LPS and another bacterial lipid *in vitro*. All of the present studies involved testing the binding of LT to the surface of *E. coli*, which due to several prior experiments [3] was understood to be based on LPS (Horstman and Kuehn 2002; Horstman et al. 2004). In order to provide evidence for the direct binding of LT to LPS, liposomes containing LPS were generated and used as the binding substrate in an ELISA. Purified LT was able to bind to 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes containing Ra *E. coli* LPS (Figure 15A). Ra LPS features a full oligosaccharide core but no O antigen, similar to the LPS expressed by DH5α. Background binding to liposomes composed only of DOPC did occur, but to a lower extent (Figure 15A). In our assay system, purified LPS needed to be reconstituted into liposomes with other lipids before use in the ELISA. When LPS was simply resuspended and adsorbed to the 96-well plate, no LT binding could be detected (data not shown).

While LPS alone did not support LT binding by ELISA, purified K-12 Ra LPS mixed with phosphatidylglycerol (PG) was suitable for use as an ELISA substrate. Using an ELISA protocol described previously to study the LPS binding of an RTX toxin from *Actinobacillus pleuropneumoniae* (Ramjeet et al. 2008), wild-type LT was able to bind to LPS mixed with PG after adsorption of the mixture to the wells of a microtiter plate (Figure 15B). The T47A mutant was impaired for binding to LPS:PG, binding

---

[3] Prior results indicating a direct interaction between LT and LPS include: the ability of soluble LPS to compete with LT binding to the *E. coli* surface (as long as the soluble LPS used contains a core structure equal in length or longer than that of the cells in the assay); the undiminished binding of LT to the *E. coli* cell surface after protease treatment; and the reduced surface binding of LT after the expression of an LPS-modifying enzyme (Kdo kinase).

approximately 20% as efficiently as wild-type. In each case, background binding to empty wells was detected, but did not fully explain the signal detected in wells with LPS (Figure 15B). These studies are preliminary, requiring further optimization and repetition, but they nevertheless indicate that LT can interact with purified LPS *in vitro*.
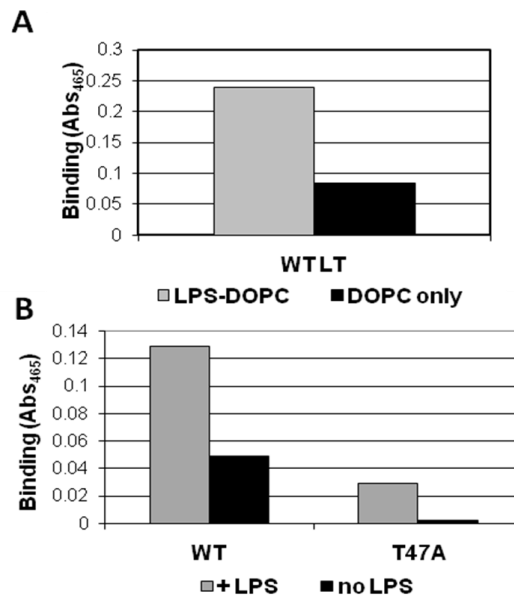
Figure 15: LT binds to purified LPS *in vitro*.

(A) Binding of wild-type (WT) LT (100 ng/μl) to liposomes composed of Ra *E. coli* LPS mixed with DOPC at a 1:10 ratio (LPS-DOPC) and liposomes of DOPC alone. **(B)** Binding of wild-type LT (WT) or T47A (15 ng/μl) to a 1:3 mixture of Ra *E. coli* LPS and phosphatidylglycerol adsorbed to a 96-well plate (+ LPS) or empty wells treated with buffer alone (no LPS). ELISA samples were run in quadruplicate.

2.4 Discussion

Previous work in our lab defined an interaction between LT produced by ETEC and the sugar moieties in LPS on the bacterial surface that was not dependent on the G_{M1}-binding site of LTB (Horstman et al. 2004). A recent report revealed a second sugar-binding pocket at the interface of two B subunits on the periphery of LT (Holmner et al.

2007), leading us to investigate whether that region may also be responsible for binding to LPS. Mutation of this binding pocket confirmed that the area defined by Ala-46, Thr-47, and Gln-3 of adjacent B subunits is necessary for binding to blood group A antigen and is also critical for binding the bacterial cell surface. Both blood antigens and LPS contain complex polysaccharides. With five binding sites per holotoxin, one at each interface between adjacent B subunits, LT may interact with one or more LPS molecules at various places along the sugar chain. With that in mind, it is notable that the mutation of multiple residues was not required in order to disrupt blood group sugar or LPS binding. Similarly, a single amino acid substitution is sufficient to disrupt oligomerization of B subunits (Iida et al. 1989) and to abolish binding to the eukaryotic receptor G_{M1} (Tsuji et al. 1985). Thus, single point mutations can impair each of LT's binding properties, highlighting the close relationship between structure and function in the toxin (see Chapter 5).

Our data demonstrate overlapping, yet distinct LT-LPS and LT-blood sugar binding interfaces, characterized by differences in binding properties of Gln-3, Glu-11, and Tyr-18 mutants. Whereas the T47A mutation strongly impaired binding to both blood sugars and bacteria with full LPS cores, the Q3K mutation totally abolished blood sugar binding but only reduced surface binding by 66% (Figure 7 and Figure 8). The E11K mutation had no effect on blood sugar binding but dropped surface binding by over 80% (Figure 11). It is likely that the residues in the binding pocket vary in their contribution to each intermolecular interaction and that each substrate can accommodate different alterations to the residues in the binding pocket. This point is further supported

50

by the Y18A mutation. While Tyr-18 plays a critical role in blood sugar binding, it is dispensable for binding the bacterial surface (Figure 7 and Figure 8C). Furthermore, like Y18A, CT does not contain a tyrosine residue at that position (see Figure 1 in Chapter 1), yet is still able to bind the surface of *E. coli* (Horstman et al. 2004).

Our analysis of the T47A and E11A mutations supports our previous observation that LT's association with LPS is based on binding to multiple core sugar residues in LPS, although binding to Kdo_2-Lipid A is possible. T47A is unable to bind *E. coli* cells featuring a full core, but is able to bind to Kdo when made available by truncation of surface LPS molecules (Figure 8 and Figure 12). Additionally, while much less T47A associated with liposomes composed of purified full-length LPS than wild-type LT, both toxins bound at a similar, low level to Kdo_2-Lipid A liposomes (D. Rodriguez and M.J. Kuehn, unpublished observations). E11A, in contrast, binds significantly to *E. coli* cells with a full core, but cannot bind to Kdo on the surface of WBB01 (Figure 11 and Figure 12). Therefore, it appears that the primary binding target of wild-type LT, one that is critically dependent on Thr-47, is a sugar residue other than Kdo. Only for truncated LPS does the alternative substrate, Kdo, become a significant factor, presumably due to the contribution of other residues in the area, including Glu-11. These results are in line with previous observations that wild-type LT is competed off the surface of cells expressing Kdo_2-Lipid A by incubation with full-length LPS, but not vice versa (Horstman et al. 2004).

The binding phenotypes of the Gln-3 substitution mutants reveal a possible role for the galactose residues present in LPS as substrates for the sugar binding pocket of LT.

The Q3K mutant is impaired for binding to full-core LPS but can bind CWG309, which lacks only two galactose residues in comparison with its parental strain (Figure 12A and Table 4). Interestingly, in the crystal structure of blood group A antigen bound to LT, Gln-3 makes significant contact with the terminal sugar, an N-acetylgalactosamine residue (Holmner et al. 2007). However, in the blood group B antigen, which LT is also able to bind, a galactose sugar is found at that position. Thus, the Q3K mutation, with its relatively drastic change in charge, may interfere with binding to galactose in LPS as it interferes with binding to the galactose residue in blood sugars (Figure 7). Alteration of Gln-3 to alanine (Q3A) still seems to allow binding to CWG311, with two terminal galactose sugars, but impairs binding to DH5α, which features only one free galactose located in the middle of the outer core (compare Figure 8B with Figure 12A). Thr-47 also makes significant contacts with the terminal N-acetylgalactosamine (or galactose) residue in blood sugars, potentially explaining the reduced binding of T47A (Holmner et al. 2007). Without absolute binding values for DH5α and CWG311, it is not clear whether LT associates more strongly with CWG311 due to the location of its free galactose residues. It is also worth noting that no galactose residues are present in LPS from the two most common O serogroups of *V. cholerae* (Cox et al. 1996).

In contrast to the binding profile of most mutants, which showed variable binding to LPS of different lengths, A46D and E11K showed significant, consistent deficiencies in cell surface association, regardless of the length of LPS (Table 4). For A46D, this result is most likely due to the nature of the amino acid substitution. Introduction of a large, charged aspartate residue in place of the small non-polar alanine may have resulted

in an overall destabilization of the binding pocket. As such, the mutant can bind to the same substrates as wild-type LT, but with a lower affinity in general. In fact, the impact of this mutation may extend beyond the local region, as A46D also demonstrated a similar decrease in G_{M1} binding. Since the binding pockets for the cell surface and G_{M1} are both at the interface of two adjacent subunits, it is possible that the introduction of a bulky aspartate residue alters the spacing of the pentameric LTB ring, thus impairing all binding properties of the toxin. Interestingly, there was a noticeable difference between the levels of A46D binding to two strains featuring full LPS cores: DH5α and CWG311 (Table 4). This disparity may be the result of the slight differences in core residue linkages found between the two strains, with A46D less able to accommodate binding to DH5α's K-12 core.

E11K's general surface binding impairment likely results from one of two possible scenarios. First, binding to both outer core sugars and Kdo may involve Glu-11, and therefore, binding to full-length LPS and truncated LPS is impaired in an E11K mutant. The E11A mutation slightly reduces binding to outer core sugars, but the introduced alanine residues may not actively disrupt that binding event to the same extent as a lysine residue. In this scenario, however, Glu-11 is critical for Kdo binding, and thus, either substitution (E11K or E11A) abolishes Kdo binding. Alternatively, the E11K mutation may exert a general destabilizing effect on the LPS binding pocket. Being a more drastic substitution, it abrogates binding of the pocket to any sugar residue, while the E11A mutation only destabilizes the pocket enough to impair Kdo binding. The wild-type blood sugar-binding capacity of the E11K mutant favors the first scenario. As LPS

and blood antigen binding events involve many of the same residues, this result implies that connections between sugar moieties and Thr-47 and Gln-3 can occur in a normal manner even with the E11K mutation.

Toxin harboring a T47A mutation was the focus of further studies in ETEC since this mutation does not appear to adversely affect any properties of the toxin besides binding to the surface of *E. coli* and blood group antigen. There was a small reduction in G_{M1} binding capacity, but this result may reflect a small contribution by the blood sugar pocket to G_{M1} binding. This speculation is based on the fact that blood group A antigen and G_{M1} each feature a galactose residue that is involved significantly in binding to LT (Merritt et al. 1994; Holmner et al. 2007) and that G33D, the G_{M1}-binding mutant of LTB, retains some ability to bind immobilized galactose (Guidry et al. 1997). Thus, for T47A, the reduced ability of the peripheral pocket to bind galactose may also slightly reduce binding to G_{M1}.

Our assessment of surface binding to ETEC reveals that the impairment detected for T47A is not limited to laboratory strains (Figure 13). Further characterization of T47A's behavior *in vivo* revealed comparable expression of wild-type LT and T47A in ETEC. Unlike T47A, most of the blood sugar binding mutants described above and many other LTB point mutants we generated displayed poor stability when expressed in ETEC (see Chapter 5). While E11K was detectable at levels comparable to wild-type when expressed in ETEC, this mutant demonstrated impaired secretion (see Chapter 3). Therefore, our focus shifted to T47A as a candidate for future research.

When examining secreted and cellular ratios of T47A, we noted slightly increased levels of secreted toxin and slightly decreased levels of periplasmic toxin (Figure 14). The difference between levels of wild-type LT and T47A detected during fractionation may ultimately be due to T47A's deficiency in cell surface binding. That is, when cells expressing equivalent amounts of wild-type and T47A toxin are initially pelleted from culture, they will pull down less T47A than wild-type. As a result, the level of T47A toxin remaining in the cell-free supernatant will be relatively higher. It is also likely that material bound to the cell surface is released during periplasm preparation, so that cells expressing T47A, which binds poorly to the bacterial surface, would release less toxin into the soluble periplasm fraction than cells expressing wild-type LT. Thus, the behavior of toxins expressed in ETEC reinforces the surface binding data based on exogenously added toxin. However, our data do not rule out the possibility that T47A is secreted more efficiently than wild-type LT.

As yet, it is unclear what role the LPS binding properties of LT play during infection. As described in Chapter 1, association of the toxin with LPS on the surface of cells and OMVs may serve to titrate the amount of LT received by host cells. Along these lines, our preliminary data show increased toxicity of supernatant from ETEC expressing T47A compared to wild-type LT, despite equal amounts of toxin present (data not shown). Thus, more of the T47A mutant is available in a soluble form due to its impaired binding to LPS, possibly reaching more target cells. However, the difference is not drastic, and therefore, enough host cells are likely to receive active toxin either solubly or in association with OMVs. In contrast, there may be advantages to

disseminating LT through OMVs. It is possible that an OMV is trafficked differently after internalization when associated with LT (compared to an LT-free OMV), altering host responses while still achieving the ultimate goal of chloride ion and water efflux afforded by the activity of LT. Future studies of the host response to wild-type ETEC supernatant and supernatant from a strain expressing T47A will be needed to address this possibility.

It is worth noting that a recent report has argued that there is no direct binding between LT and LPS (Jansson et al. 2009), although the authors offer no explanation for the data reported here and in previous studies by our lab regarding the surface binding of LT. In their study, all conclusions were based on LPS either bound to thin-layer chromatography plates or evaporated onto 96-well plates. Like those authors, we have not been able to bind LT to LPS that has simply been adsorbed to the bottom of a microtiter well (data not shown). However, when LPS is reconstituted into liposomes, LT binding can be readily detected (Figure 15 and data not shown). Along these lines, differences in LT binding to the ganglioside G_{M2} were previously noted depending on whether the substrate was adsorbed to microtiter plates or present in liposomes (Fukuta et al. 1988; MacKenzie et al. 1997). Mixing LPS with PG as the substrate for LT also made binding possible in an ELISA system (Figure 15). We hypothesize that LPS on a silica plate or microtiter well may not present its sugar residues freely enough to allow for LT to bind. The binding between LT and blood sugars is not a high-affinity event; it is likely that binding to LPS is also of lower affinity than LT binding to G_{M1}, for example (Holmner et al. 2007). Therefore, the presence of LPS molecules in varying orientations

may not make enough sugar available to detect LT binding. The addition of PG into the ELISA system would then provide sufficient spacing for LT to interact freely with the outer core oligosaccharide. While these results provide more direct evidence that LPS in a more native orientation is a suitable binding substrate for LT, liposome binding experiments will need to be repeated before final conclusions can be drawn.

Conclusions. LT is able to mediate the internalization of intact OMVs into host cells (Kesty et al., 2004), and expression of the toxin provides an advantage during initial colonization of the small intestine (Allen et al. 2006). The T47A mutation generates a bacterial surface-binding LT mutant that is free from defects in expression, assembly, secretion, and holotoxin activity. T47A creates a CT-like scenario in which secreted LT is not membrane-bound, allowing for future research to address the role of the LT-LPS interaction in ETEC pathogenesis. The mutations described above also highlight the portion of the LTB molecule responsible for this interaction: a peripheral sugar-binding pocket distinct from (but overlapping with) the toxin's blood sugar binding site. Furthermore, we provide evidence that binding to outer core sugar(s) is critical for LT to bind full-length LPS, while binding to Kdo is possible when LPS is truncated.

2.5 Acknowledgments

58

3. Specificity of the type II secretion systems of enterotoxigenic *Escherichia coli* and *Vibrio cholerae* for heat-labile enterotoxin and cholera toxin

3.1 Summary

The gram-negative type II secretion (T2S) system is a multiprotein complex mediating the coordinated release of virulence factors from a number of pathogenic species. 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 of these toxins 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 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 (Connell et al. 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 CTB secretion from *V. cholerae*, and the E11A mutation altered LT and CTB secretion to varying extents, indicating that these toxins are identified as secretion substrates in different ways. 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. These results highlight

the exquisitely fine-tuned relationship between T2S substrates and their coordinate

secretion machineries in different bacterial species.

3.2 Introduction

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 (Henderson et al. 2000; Pukatzki et al. 2009). 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 (Cianciotto 2005). The T2S system is

a multiprotein complex of 12-15 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 (Johnson et al. 2006). Aside from

providing a means of exporting freely released virulence factors from plant, animal, and

human pathogens (Cianciotto 2005), the T2S system has been shown to export surface-

associated virulence factors (Horstman and Kuehn 2002), fimbrial components

(Zalewska-Piatek et al. 2008), outer membrane cytochromes (Shi et al. 2008), and a

surfactant required for sliding motility in *Legionella pneumophila* (Stewart et al. 2009),

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 (Russel 1998; Johnson et

al. 2006). 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 (Russel 1998). 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. LT from ETEC and CT from *V. cholerae*

represent one such pair of substrates.

ETEC and *V. cholerae* are enteric pathogens causing significant morbidity and

mortality worldwide (Sanchez and Holmgren 2005). 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 (Spangler 1992). Each pathogen secretes

an AB_5 toxin important for colonization and the induction of water and electrolyte efflux

from intestinal epithelial cells (Pierce et al. 1985; Allen et al. 2006). These toxins, LT and

CT, are both encoded by two-gene operons. After *sec*-dependent transport to the

periplasm, holotoxin formation occurs spontaneously (Hardy et al. 1988), 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 and their secretion. Export of

fully folded and assembled LT or CT is then accomplished by the T2S system, described

in detail in section 1.4 (Sandkvist et al. 1997; Tauschek et al. 2002). In ETEC, this

system is encoded by *gspC-M* (Tauschek et al. 2002), while in *V. cholerae*, these genes

are found in the *eps* operon (Sandkvist et al. 1997). Multiple studies have implicated the

Eps system in maintenance of proper outer membrane integrity (Sandkvist et al. 1997;

Sikora et al. 2007), but no role outside of secretion has yet been identified for the Gsp complex in ETEC.

LT and CT are very similar in structure, sharing approximately 80% sequence homology and 83% identity in the mature B subunit (Mekalanos 1983; Holmner et al. 2007). ETEC is thought to have acquired the genes for CT through horizontal transfer, with the toxins evolving over time to possess slight differences (Yamamoto et al. 1987). As such, these toxins share the same primary host receptor, the monosialoganglioside G_{M1}, and catalyze the same ADP-ribosylation reaction within host cells (Spangler 1992). However, LT is able to bind other host sphingolipids in addition to G_{M1} and to interact with sugar residues from the A type blood antigen, which CT cannot bind (Teneberg et al. 1994; Holmner et al. 2007). Both LT and CT are able to associate with sugar residues in lipopolysaccharide (LPS) on the surface of *E. coli* cells (Horstman et al. 2004). Binding to each of these substrates can be impaired by point mutation (Tsuji et al. 1985) (see Chapter 2).

In this chapter, 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 (Connell et al. 1995), 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.

Table 5: Strains and plasmids used in Chapter 3.

Strain	Genotype	Relevant characteristics
MK233[1]	MT616/ pRK2013	Helper strain for tri-parental mating; Cm^R
jf570	H10407Δ*eltA*	ETEC strain with polar insertion in *eltA* (LT-deficient)
MK1052	H10407Δ*eltA*/ pILT	jf570 carrying inducible LT plasmid; Amp^R
MK1053	H10407 Δ*eltA*/ pTrc99A	jf570 carrying empty vector; Amp^R
MK1202	H10407Δ*eltA*/ pILT[L25E]	As MK1052, with inducible L25E LT mutant; Amp^R
MK1201	H10407Δ*eltA*/ pILT[Q3K]	As MK1202, with LT[Q3K]; Amp^R
MK1239	H10407Δ*eltA*/ pILT[Q3A]	As MK1202, with LT[Q3A]; Amp^R
MK1231	H10407Δ*eltA*/ pILT[E11K]	As MK1202, with LT[E11K]; Amp^R
MK1240	H10407 Δ*eltA*/ pILT[E11A]	As MK1202, with LT[E11A]; Amp^R
MK741	DH5α *degP*::Tn5/ pDsbA/ pILT	*E. coli* K-12 *degP* knockout, carrying a plasmid copy of *dsbA* and an inducible LT plasmid; Kan^R Cm^R Amp^R
MK1101	DH5α *degP*::Tn5/ pDsbA/ pILTB	As MK741, with pILTB; Kan^R Cm^R Amp^R
MK1196	MC4100 *hns*::kan1001/ pCHAP4278/pILT	*hns* mutant carrying K-12 T2S operon and inducible LT; Kan^R Cm^R Amp^R
MK1198	MC4100 *hns*::kan1001/ pCHAP4278/pILT[Q3K]	As MK1196, with inducible Q3K LT mutant; Kan^R Cm^R Amp^R
MK1199	MC4100 *hns*::kan1001/ pCHAP4278/pILT[L25E]	As MK1198, with LT[L25E]; Kan^R Cm^R Amp^R
MK1245	MC4100 *hns*::kan1001/ pCHAP4278/pILT[E11K]	As MK1198, with LT[E11K]; Kan^R Cm^R Amp^R
P4	P27459 Δ*ctxAB*	CT-deficient El Tor *V. cholerae* strain; Str^R Kan^R
MK1123	P4/ pTrc99A	P4 carrying empty vector; Str^R Kan^R Amp^R
MK1225	P4/ pICTB	P4 carrying inducible CTB plasmid; Str^R Kan^R Amp^R
MK1236	P4/ pICTB[Q3K]	As MK1225, with inducible Q3K CTB mutant; Str^R Kan^R Amp^R
MK1232	P4/ pICTB[E11K]	As MK1236, with CTB[E11K]; Str^R Kan^R Amp^R

---

[1] The origins of strains and plasmids not generated in these studies are included in section 3.5.

MK1233	P4/ pICTB[E11A]	As MK1236, with CTB[E11A]; Str^R Kan^R Amp^R	
MK1242	DH5α *degP*::Tn5/ pDsbA/pICTB	As MK741, with pICTB; Kan^R Cm^R Amp^R	
MK1223	H10407Δ*eltA*/ pICTB	jf570 with inducible CTB plasmid; Amp^R	
MK1224	H10407Δ*eltA*/ pICTB[Q3K]	As MK1223, with inducible Q3K CTB mutant; Amp^R	
MK1234	H10407Δ*eltA*/ pICTB[E11K]	As MK1224, with CTB[E11K]; Amp^R	
MK1235	H10407Δ*eltA*/ pICTB[E11A]	As MK1224, with CTB[E11A]; Amp^R	
MK1122	P4/ pILTB	P4 carrying inducible LTB plasmid; Str^R Kan^R Amp^R	
MK1237	P4/ pILTB[Q3K]	As MK1122, with inducible Q3K LTB mutant; Str^R Kan^R Amp^R	
MK1243	P4/ pILTB[Q3A]	As MK1243, with LTB[Q3A]; Str^R Kan^R Amp^R	
MK1244	P4/ pILTB[E11K]	As MK1243, with LTB[E11K]; Str^R Kan^R Amp^R	
MK1238	P4/ pILTB[L25E]	As MK1243, with LTB[L25E]; Str^R Kan^R Amp^R	
MK378	H10407	Parent strain of MK379	
MK379	H10407Δ*gspD*	T2S pore protein knockout; Kan^R	
MK1230	H10407Δ*gspD*/pILT	T2S mutant with inducible LT plasmid; Kan^R Amp^R	

Plasmid	Relevant characteristics	
pILT	IPTG-inducible LT holotoxin	Amp^R
pILTB	IPTG-inducible LTB pentamer	Amp^R
pICTB	IPTG-inducible CTB pentamer	Amp^R
pEpsCD	Carries *epsCD* from *V. cholerae* T2S operon	Cm^R
pCHAP4278	Carries *gspC-O* from K-12 *E. coli* T2S operon	Cm^R

3.3 Results

Mutations affecting the secretion of LT from ETEC. In the course of our
screen for bacterial surface binding mutants (described in Chapter 2), we identified two
mutations in LTB (L25E and Q3K) that independently reduced secretion of the toxin.
When mutants LT[L25E] and LT[Q3K] were expressed in an LT-deficient ETEC strain
(jf570, Table 5), they were detected at wild-type levels in total culture samples but
showed reduced levels in the cell-free supernatant (Figure 16). Consistent with a
secretion defect, LT[Q3K] was found at increased levels in the periplasm (Figure 16B).
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 pre-secreted form. Preliminary experiments

with an LT[Q3K,L25E] double mutant detected secreted levels similar to that of

LT[L25E], with periplasmic levels near wild-type (data not shown).
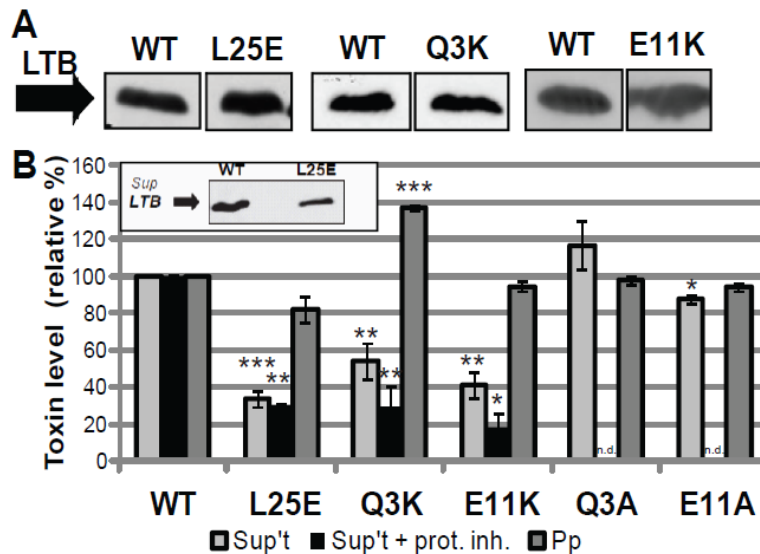
Figure 16: Three mutations impair the secretion of LT from ETEC.

(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. **(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_{M1} ELISA, with wild-type levels set to 100%. Supernatant levels were normalized to CFUs, and periplasm levels were normalized to alkaline phosphatase activity. *, $p<0.01$; **, $p<0.005$; ***, $p<10^{-6}$ compared to wild-type ($n\geq3$); n.d., not determined. For some experiments, cultures were grown in the presence of a protease inhibitor cocktail (+ prot. inh.) ($n\geq2$). **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 CFUs. All blots were probed with cross-reactive anti-CT antibody.

Since levels of the toxins in the supernatants were measured by G_{M1} binding

ELISA, we examined whether these mutations affected the toxin's ability to bind G_{M1}

(Table 6). Purified LT[Q3K] showed wild-type levels of G_{M1} binding. LT[L25E] bound

G_{M1} 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_{M1}-independent assay, we immunoblotted cell-free supernatant. The results were consistent with a secretion defect for LT[L25E] (Figure 16B, inset).

Table 6: Binding properties of LT and CTB mutants

Purified mutant toxin or pentamer was tested for binding to G_{M1} and blood sugars by ELISA and to the surface of *E. coli* with an immunoblot-based assay (see Chapter 2). All binding values are expressed as percentages of wild-type LT or CTB binding ± SEM. *p*-values represent differences between mutant and wild-type binding according to Student's *t*-test, with significant differences in bold ($n \geq 3$ for G_{M1} and surface binding, $n \geq 2$ for blood sugar binding).

Mutant	G_{M1} binding	*p*-value	Blood sugar binding	*p*-value	*E. coli* surface binding	*p*-value
LT[L25E]	**69.3±5.3%**	<0.005	**64.2±7.2%**	<0.01	**35.2±10.9%**	<0.005
LT[Q3K]	96.0±4.6%	0.43	**0.67±0.43%**	<0.005	**33.8±8.8%**	<0.005
LT[Q3A]	95.5±14.4%	0.77	**4.0±4.0%**	2.3×10^{-6}	**36.5±4.7%**	<0.0005
LT[E11K]	90.9±4.6%	0.08	109.6±17.8%	0.65	**19.6±9.3%**	<0.001
LT[E11A]	81.5±6.5%	0.07	-	-	89.3±23.4%	0.67
CTB[Q3K]	**93.1±1.9%**	<0.05	**12.4±12.4%**	<0.05	96.9±19.9%	0.89
CTB[E11K]	94.7±2.5%	0.08	-	-	**16.2±5.6%**	<0.005
CTB[E11A]	**106.7±1.8%**	<0.05	-	-	-	-

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, 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 wild-

type, but the difference was not statistically significant, and levels of LT[Q3A] in the periplasm were equivalent to those of wild-type (Figure 16B). These results suggest that the presence of a charged amino acid at residue 3 impedes the secretion of LTB.

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 two 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] (Figure 16B). We also performed a stability assay similar to one described in a study of secretion-impaired mutants of *Pseudomonas aeruginosa* exotoxin A (McVay and Hamood 1995), observing that both purified mutants were stable over time when incubated in 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 (Horstman and Kuehn 2000). Wild-type and mutant LT were equally sensitive to the enzyme, with degradation products visible beginning at a Pronase concentration of 50 μg/ml, and much of the full-length monomer broken down at a concentration of 500 μg/ml (Figure 17A,B). Thus, proteolytic degradation was not responsible for reduced detection of the LT[L25E] or LT[Q3K] mutant in ETEC supernatant.
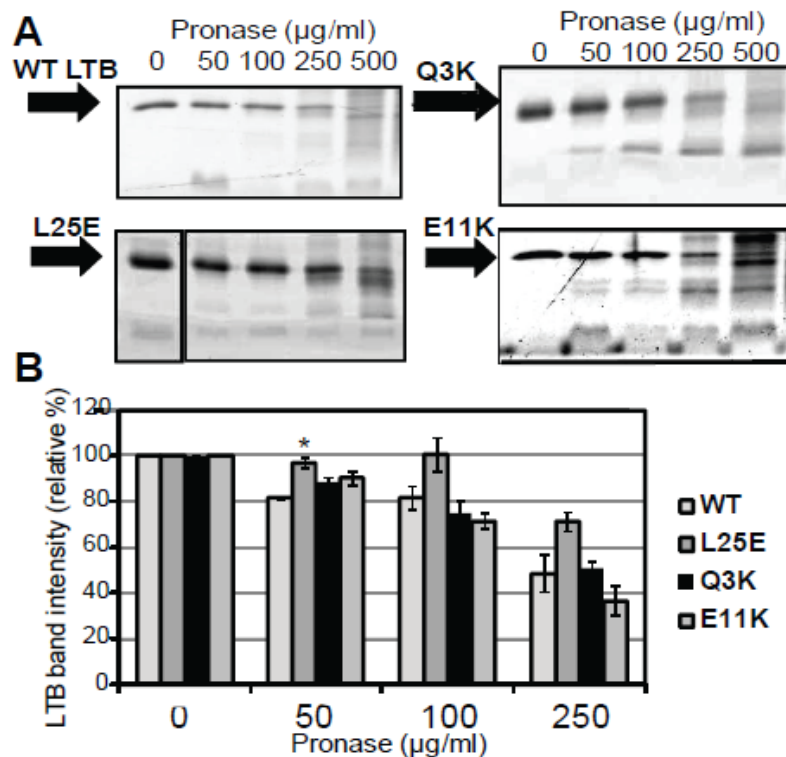
Figure 17: LT secretion mutants show wild-type protease sensitivity

(A) Representative protein gels showing degradation of wild-type (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 stained with Ruby Red. **(B)** Densitometric measurements of the intensities of the bands corresponding to LTB on the protein gels described in (A). The intensity of the band corresponding to untreated wild-type LTB (WT) or the indicated mutant was set to 100%. *, $p < 0.05$ compared to wild-type at the same concentration ($n \geq 2$).

The first ten amino acids of LTB have previously been reported to be involved in

pentamer assembly, and pentamer assembly is critical for secretion (Hirst et al. 1984;

Chung et al. 2006). Therefore, we determined whether the effect of the Q3K or L25E

mutation on LT secretion was due to a decreased efficiency of pentamer assembly.

Purified LT[Q3K] and LT[L25E] were detected by a monoclonal antibody against LTA,

indicating complete holotoxin formation (data not shown). Furthermore, LT's effects

require LTA, and thus we used a toxicity assay to determine whether LTA was able to

associate with mutant LTB in a manner equivalent to 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 (Donta et al. 1974). At least

two separate batches of each purified mutant were as toxic as wild-type LT (8 ng of each

elicited maximal rounding of 4×10^5 Y1 cells), suggesting that periplasmic holotoxin

formation was not affected by the L25E or Q3K mutation. Each mutant also behaved

similar to or better than wild-type LT in an established *in vitro* cell-free assay measuring

pentamer reassembly after dissociation at extremely low pH (Figure 18) (Ruddock et al.

1996). 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.
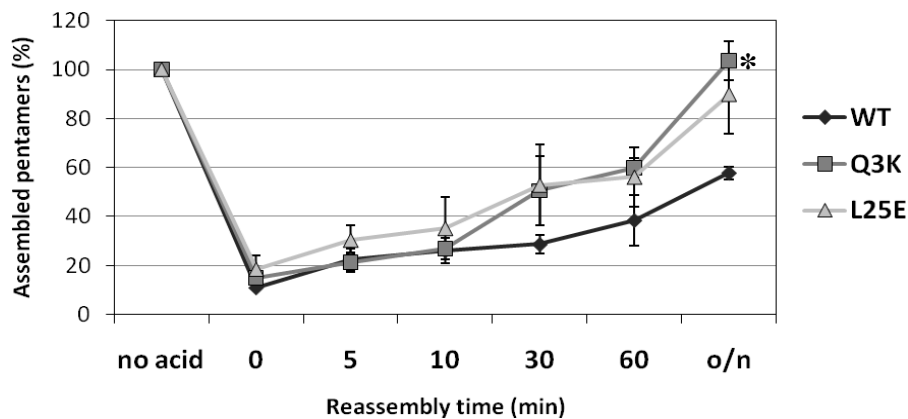
Figure 18: LT secretion mutants show wild-type pentamer formation

Purified wild-type LTB (WT) and the indicated mutants were dissociated into monomers
by treatment with acid, 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 wild-type (WT) at the same time point ($n \geq 3$).

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

reflected by the ability of the mutants to bind each of LT's three major ligands.

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 6).

LT[Q3A], while not impaired for secretion, showed wild-type binding to G_{M1}, but

impaired binding to blood antigen and *E. coli* LPS, consistent with the phenotypes of

LT[Q3K], as described in Chapter 2 (Table 6).

LT[L25E] was found to be impaired for binding to all substrates of LTB tested

(Table 6). Therefore, although it is stable and assembles into a holotoxin like wild-type

LT, LT[L25E] appears to have an external interaction surface that does not permit normal

substrate recognition. Because of the potential for more global defects that might

confound our results, L25E was not further considered in this study.

The E11K mutation, originally characterized in 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 (Connell et al.

1995). 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 substantially reduced, despite a wild-type level of expression (Figure 16).

Periplasmic levels of LT[E11K] were nearly wild-type, as was the case for LT[L25E]

(Figure 16B). G_{M1} binding of purified LT[E11K] was slightly reduced, although not significantly (Table 6).

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 the addition of protease inhibitor to the growth medium, actually reducing slightly instead (p=0.10; Figure 16B). LT[E11K] also behaved similarly to wild-type LT in the Pronase sensitivity assay (Figure 17). While purified LT[E11K] is as toxic as wild-type LT and carries LTA, 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). Other mutations have been shown to prevent reassembly after acid treatment (Cheesman et al. 2004); it is conceivable that Glu-11 plays some role in the reformation process or that an as yet uncharacterized chaperone aids in the oligomerization of this mutant *in vivo*.

To explore the characteristics of residue 11 that are critical for secretion, we tested a more neutral mutation, LT[E11A]. Levels of LT[E11A] detected in the supernatant were only decreased approximately 12% compared to wild-type LT (Figure 16B), but this value may be the result of its slightly decreased ability to bind G_{M1} (Table 6). Periplasmic levels of LT[E11A] were normal (Figure 16B). These data suggest that introduction of a charge at residue 11 is critical for LT[E11K]'s observed secretion defect in ETEC. As discussed in Chapter 2, LT[E11K] was impaired for LPS binding, while LT[E11A] bound DH5α cells at levels equivalent to wild-type LT (Table 6). However, LT[E11K] demonstrated wild-type blood sugar binding, indicating overall stability of the

mutant. Therefore, the E11K mutation appears to have specific effects on secretion and

LPS binding (in contrast to the poor binding of LT[L25E] to all substrates).

The E11K mutation does not affect the secretion of LT through the

endogenous type II system of K-12 *E. coli*. In order to discern whether the secretion-

impairing effects of L25E, Q3K, and E11K were ETEC-specific, we expressed wild-type

LT and these mutants in a lab strain of *E. coli* carrying a plasmid-borne copy of *gspC-O*[2].

Each of the mutants expressed at normal levels (data not shown). LT[L25E] and

LT[Q3K] were found in reduced levels in the supernatant, indicating impaired secretion

(Figure 19). These results were in accordance with the secretion of these mutants from

ETEC (Figure 16B). However, despite the significant effect of the E11K mutation on the

export of LT from ETEC, secreted LT[E11K] was detected at levels equivalent to wild-

type LT in cultures of K-12 *E. coli* (Figure 19).

---

[2] In this case, an extra open reading frame, *gspO*, is found just downstream of *gspM*. The function of this
gene is not known; it does not encode the prepilin peptidase, which is found elsewhere in the *E. coli*
genome.
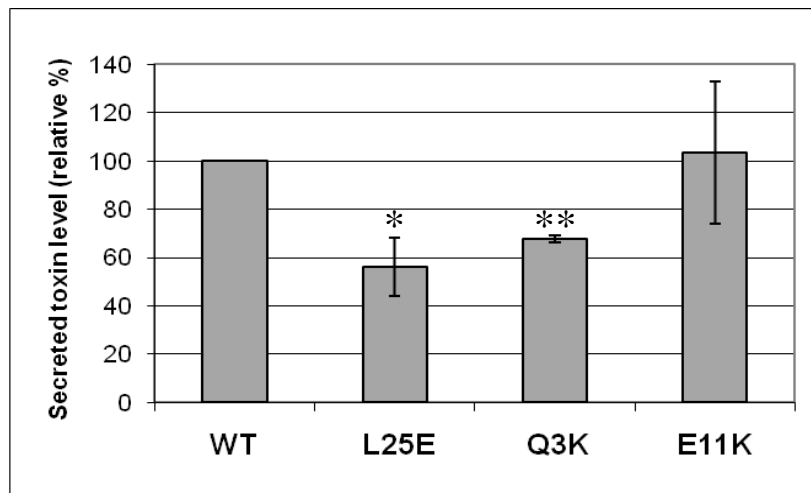
Figure 19: Secretion of LT mutants from K-12 *E. coli*.

Wild-type (WT) and the indicated mutant toxins were expressed in MC4100 *hns*::kan1001/pCHAP4278, a strain of *E. coli* carrying a de-repressed plasmid copy of the endogenous T2S system. Levels of toxin present in the cell-free supernatant were measured by G_{M1} ELISA and adjusted for OD_{600}. Secreted levels of WT LT were set to 100%. *, $p<0.05$; **, $p<0.005$ compared to wild-type ($n\geq2$).

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 by triparental mating as described (Goldberg and Ohman 1984; Fullner et al. 2001). B-subunit pentamers, without the catalytically active A subunit, were used for reasons of biosafety. Interestingly, a fourfold lower concentration of IPTG (50 μM vs. 200 μM) was needed to induce CTB[Q3K] to levels matching induced wild-type CTB (Figure 20A).
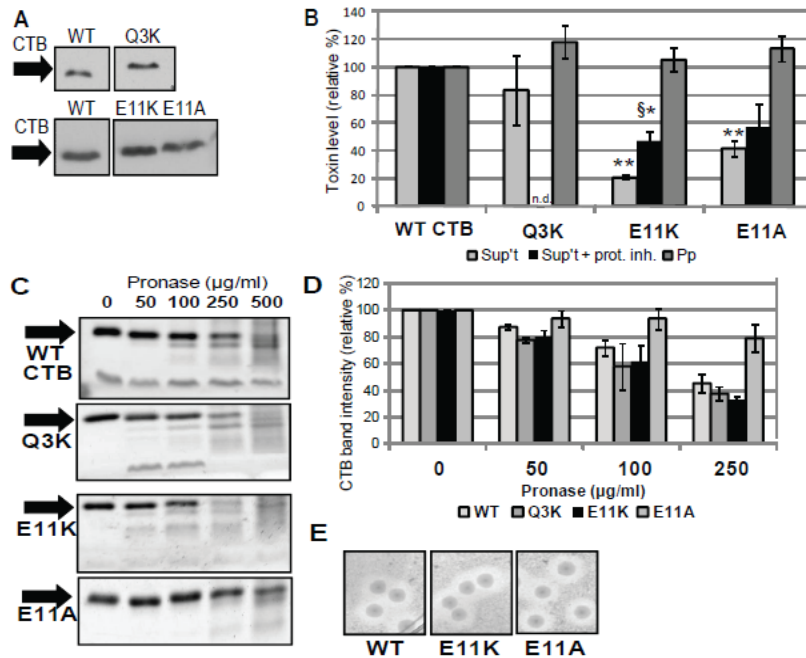
Figure 20: Mutation of Glu-11 impairs CTB secretion from *V. cholerae*

(A) Representative immunoblot of TCA-precipitated total culture samples, adjusted for CFUs, showing induced expression of wild-type CTB (WT), CTB[Q3K], CTB[E11K], and CTB[E11A] in strain P4. Blots were probed with anti-CT antibody. **(B)** Strains expressing wild-type (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 wild-type levels set to 100%. Supernatant levels were normalized to CFUs, and periplasm levels were normalized to alkaline phosphatase activity. *, $p<0.05$; **, $p<0.0005$ compared to wild-type ($n\geq3$). 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 ($n\geq2$); n.d., not determined. **(C)** Representative protein gels showing degradation of wild-type (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 stained with Ruby Red. **(D)** Densitometric measurements of the intensities of the CTB bands on the protein gels described in (C). The intensity of the band corresponding to untreated wild-type (WT) or the indicated mutant CTB was set to 100% ($n\geq2$). **(E)** Zones of clearance formed by *V. cholerae* on skim milk agar. Strains expressing wild-type CTB (WT) and the indicated mutant pentamers were plated on skim milk agar and incubated for 36 h at 37°C.

Unlike LT[Q3K] in ETEC, CTB[Q3K] levels were only slightly reduced in the supernatant of P4 *V. cholerae*, and the difference was not significant (Figure 20B). The G_{M1} binding levels of purified CTB[Q3K] were slightly reduced compared to wild-type CTB (Table 6); 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 (Figure 20B). These results suggest a difference in substrate selectivity by the T2S systems of ETEC and *V. cholerae*.

We also tested CTB[Q3K] for its ability to bind blood sugars and DH5α cells to determine whether the mutation had the same effects on CTB's binding properties that it did on those of LTB. While CT is known to bind to blood sugars with a much lower affinity than LT (Holmner et al. 2007), we were still able to detect some binding in our ELISA, although at a 10- to 20-fold reduction compared with LT binding (data not shown). The CTB[Q3K] mutant, remarkably, showed an even stronger impairment than wild-type CTB, with one trial demonstrating no binding at all above background (Table 6). Thus, the residual binding of CT to blood sugars, which is likely to be drastically reduced compared to LT due to the absence of a tyrosine at residue 18 in CT (see Chapters 1 and 2), may still occur through the residues identified in the blood sugar binding pocket of LT. Surprisingly, CTB[Q3K] displayed no defect in LPS binding compared to wild-type CTB (Table 6), which can bind well to the surface of *E. coli* (Horstman et al. 2004). Therefore, the architecture of the peripheral sugar binding pocket in CTB seems to be slightly different than that of the pocket in LTB, with the Q3K mutation lacking any effect on the LPS binding of the former.

An inducible plasmid expressing CTB[E11K] was also cloned and utilized for comparison studies in our assay system. CTB[E11K] expressed at levels similar to wild-type CTB (Figure 20A), and we could recapitulate its reported secretion impairment, albeit to an even greater extent (20.9±1.7% of wild-type, Figure 20B). However, in contrast to the near wild-type secretion phenotype of LT[E11A] by 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 (Figure 20A,B).

Although the addition of protease inhibitor[3] to the growth medium raised the levels of CTB[E11K] detected (Figure 20B), proteolytic degradation cannot account for the full secretion defect. The presence of protease inhibitor did not alter the amount of CTB[E11A] detected in the supernatant (Figure 20B). 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 (Figure 20C,D), with CTB[E11A] showing a trend toward increased stability compared to wild-type CTB, although not a statistically significant one (p=0.11 for 250 µg/ml). Despite their secretion defect, periplasmic forms of CTB[E11K], and CTB[E11A] did not accumulate to a higher level than wild-type CTB (Figure 20B).

The *V. cholerae* T2S system is not blocked by secretion-impaired CTB mutants. We wondered if non-secreted CTB mutants would block the T2S system,

[3] The protease inhibitor dropped levels of protease activity in *V. cholerae* supernatants to 22% of that of uninhibited supernatant on average (*n*=3), as determined by a colorimetric assay measuring cleavage of the protease substrate azocasein.

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 (Scott et al. 2001; Sikora et al. 2007). 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

(Figure 20E). 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 strain jf570 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 (Figure

21A). A slight but insignificant impairment in secretion was seen for CTB[Q3K], and this

could be partly explained by the mutant's reduced G_{M1} binding (Figure 21B, Table 6).

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 (Figure 21B). The modest impairment seen for CTB[E11K] (Figure 21B)

was again accompanied by a slight reduction in G_{M1} binding of the purified pentamer

(Table 6) and could be responsible for the apparent reduction in secreted levels.

CTB[E11K] did demonstrate an LPS binding impairment compared to wild-type CTB

(Table 6), and therefore, the secreted levels detected may be slightly increased due to the

presence of toxin that does not pellet with whole cells (see section 2.4). However, this

effect was slight for LT[T47A], a mutant with a stronger deficiency in surface binding,

and is unlikely to explain the drastic change in secretion efficiency of CTB[E11K] in *V.*

cholerae and ETEC.
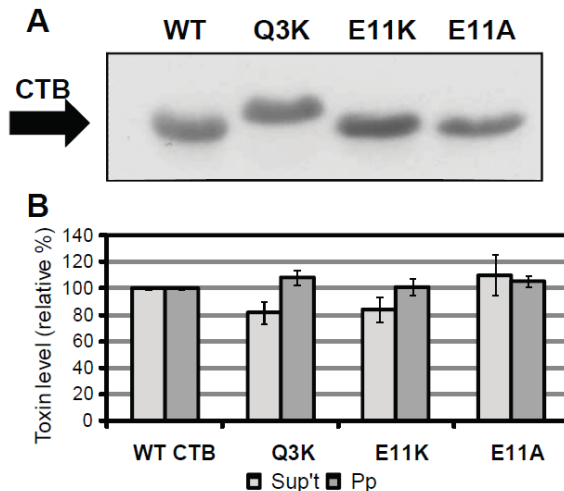
Figure 21: Secretion of CTB mutants is not impaired in ETEC.

(A) Representative immunoblot of TCA-precipitated total culture samples, adjusted for
CFUs, showing induced expression of wild-type CTB (WT), CTB[Q3K], CTB[E11K],
and CTB[E11A] in strain jf570. The blot was probed with anti-CT antibody. **(B)** Strains
expressing wild-type (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_{M1} ELISA, with wild-type levels set to 100%. Supernatant levels
were normalized to CFUs ($n \geq 3$), and periplasm levels were normalized to alkaline
phosphatase activity ($n \geq 2$).

Intriguingly, the E11A mutation generated a G_{M1} binding capacity slightly higher

than that of wild-type CTB (Table 6), and it is likely that this increased binding is

reflected in the slightly elevated levels of CTB[E11A] detected in the supernatant (Figure 21B). In all cases, periplasmic pentamer levels were close to that of wild-type CTB (Figure 21B). These data demonstrate that the 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 for reasons of biosafety. Although wild-type LTB was readily produced in *V. cholerae*, the Q3K and E11K LTB mutants were detected in small amounts, if at all (Figure 22A), and thus we were unable to measure the effect of Q3K or E11K on LTB secretion from *V. cholerae*. See Chapter 4 for a more thorough investigation of the stability of LTB and CTB constructs in ETEC and *V. cholerae*.
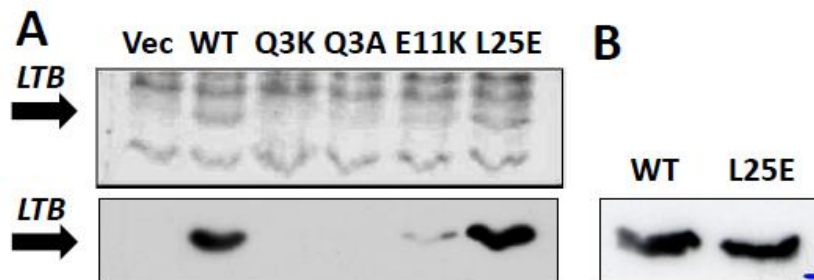
Figure 22: LTB mutants are unstable in *V. cholerae*

(A) Representative Ruby Red protein stain (top panel) and immunoblot (bottom panel) of TCA-precipitated total culture samples, adjusted for CFUs, showing induced expression of a vector control (Vec), wild-type LTB (WT), LTB[Q3K], LTB[Q3A], LTB[E11K], and LTB[L25E] in strain P4. **(B)** Representative immunoblot of TCA-precipitated cell-free supernatant from equivalent cultures of strain P4 expressing WT or L25E mutant LTB. The blots were probed with cross-reactive anti-CT antibody.

LTB[L25E] showed significant levels of expressed protein, indicating that some

LTB mutations are tolerated by *V. cholerae* (Figure 22A), and LTB[L25E] was readily

detectable in *V. cholerae* supernatant by immunoblotting (Figure 22B). 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* (see Table 7).

Table 7: Summary of the effects of the mutations investigated in Chapter 3.

| Mutation | Secretion from ETEC | | Secretion from *V. cholerae* | |
	LT	CTB	CTB	LTB
Q3K	**reduced**	wild-type	wild-type	*LTB not detected*
Q3A	wild-type	--	--	*LTB not detected*
E11K	**reduced**	wild-type	**reduced**	*little LTB detected*
E11A	near wild-type	wild-type	**reduced**	--
L25E	**reduced**	--	--	wild-type[4]

Additional phenotypes of a type II secretion-deficient strain of ETEC. In

their study of *V. cholerae* mutants bearing individual deletions of multiple T2S

component genes, Sikora et al. (2007) uncovered a number of other phenotypes in

addition to the lack of secretion of T2S substrates. Earlier, *V. cholerae* T2S mutants were

reported to have altered outer membrane protein profiles, indicating a possible role for

the Eps machinery in outer membrane biogenesis (Sandkvist et al. 1997). While a

preliminary characterization of H10407 ETEC with a non-polar deletion in *gspD* was

performed in the study identifying ETEC's T2S operon (Tauschek et al. 2002), it was

---

[4] Secreted LTB was readily detectable in cell-free supernatant by immunoblot (see Figure 22B).

unclear if this mutant would demonstrate additional phenotypes along the lines of those observed in *V. cholerae epsD* mutants.

V. cholerae eps mutants are reported to grow more slowly, to leak more periplasmic components, to show increased sensitivity to polymyxin B, and to have altered outer membrane protein profiles compared to a wild-type parental strain (Sandkvist et al. 1997; Sikora et al. 2007). We could not detect any growth difference between H10407 ETEC and an isogenic $\Delta gspD$ mutant (Figure 23A), which is consistent with results from the study in which the mutant was created (Tauschek et al. 2002). That study also reported some leakage of periplasmic LT into the supernatant, which we were able to detect by G_{M1} ELISA for H10407$\Delta gspD$ transformed with pILT (data not shown). Furthermore, outer membrane vesicle (OMV)-free supernatant from H10407$\Delta gspD$/pILT demonstrated toxic effects in the Y1 cell culture assay (data not shown), indicating the presence of soluble secreted toxin. While addition of 5% sucrose to the growth medium reduced the leakiness of a *V. cholerae eps* mutant, it had no effect on the LT released by H10407$\Delta gspD$/pILT as determined by G_{M1} ELISA of cell-free supernatants (data not shown).
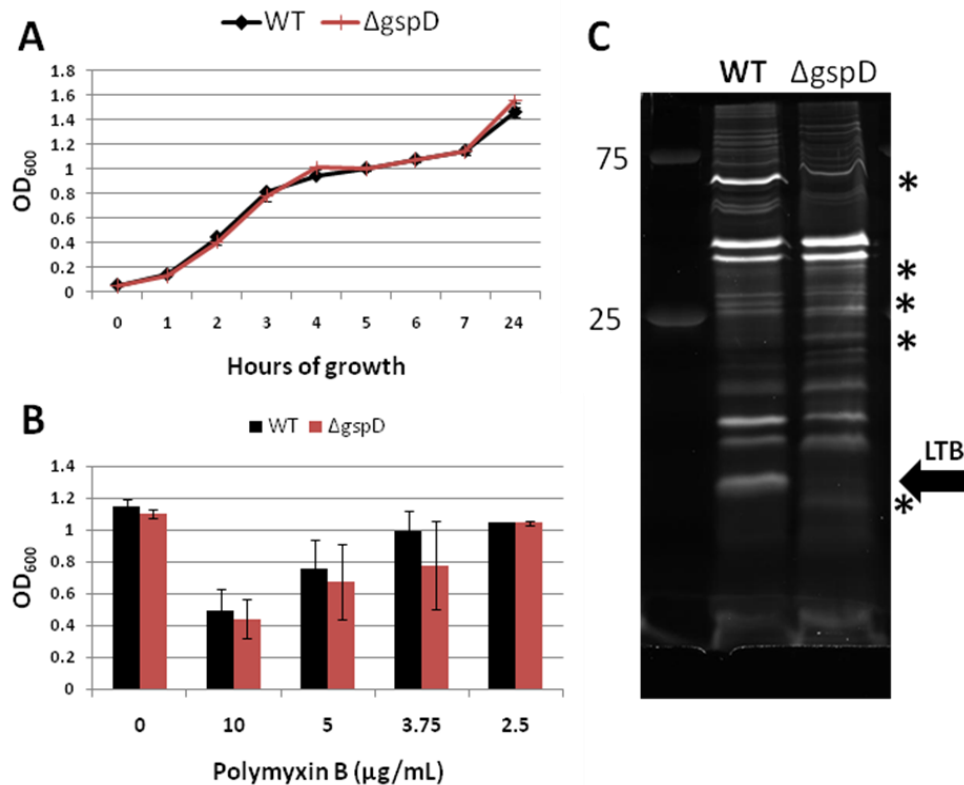
Figure 23: An ETEC type II secretion mutant demonstrates an altered outer membrane vesicle profile but wild-type growth and polymyxin B resistance

(A) Wild-type (WT) H10407 ETEC (MK378) and an isogenic Δ*gspD* mutant (MK379) were diluted 1:80 from overnight cultures, grown at 37°C for the indicated amount of time, and assayed for OD_{600} (*n*=2). **(B)** WT and Δ*gspD* H10407 strains were diluted 1:50 from overnight cultures, grown 2 h at 37°C, and treated with the indicated final concentration of polymyxin B. After an additional 2 h of growth at 37°C, the OD_{600} of each culture was measured (*n*≥2). **(C)** OMVs (1 μg) purified from WT and Δ*gspD* H10407 strains carrying pILT (MK1052 and MK1230, respectively) were run on a 15% SDS gel and stained with Ruby Red. The band corresponding to LTB is indicated with an arrow, and asterisks mark the locations of several noticeable differences between the two protein profiles. Two prominent marker bands are labeled with their corresponding molecular weight (kDa).

H10407Δ*gspD* was also tested for sensitivity to the outer membrane-disrupting antimicrobial peptide polymyxin B. Over a range of polymyxin B concentrations, growth inhibition of the mutant matched that of wild-type H10407 (Figure 23B). Finally,

comparison of the OMV protein profiles of H10407/pILT and H10407Δ*gspD*/pILT

revealed some differences between the two strains (Figure 23C). Some bands present in

the parental strain are missing in the mutant, and several bands change in relative

intensity (marked with asterisks). Interestingly, while both strains carried pILT, the band

corresponding to LTB is strongly enriched in OMVs from the wild-type strain, perhaps

due to leakage of periplasmic LT from the T2S mutant (Figure 23C, arrow). Differences

in the total culture protein profiles of these two strains were also visible but were not as

marked (data not shown).

Together, these data indicate that lack of a functional T2S system alters the outer

membrane profile in ETEC and allows some leakage of periplasmic components but does

not increase sensitivity to polymyxin B or impair growth. That is, the T2S system in

ETEC seems to play a role beyond secretion of substrates, but disruption of the system

does not cause as drastic an effect as disruption of the Eps system does in *V. cholerae*.

Complementation of the Δ*gspD* mutant to restore the secretion of LT was not possible

with a plasmid carrying *epsCD* from *V. cholerae* or *gspC-O* from K-12 *E. coli*, indicating

lack of interchangeability among the components of these T2S systems. While

expression of LT was detectable in H10407Δ*gspD* carrying these plasmids, only a wild-

type, T2S-proficient ETEC strain was able to export detectable levels of LT (Figure 24).
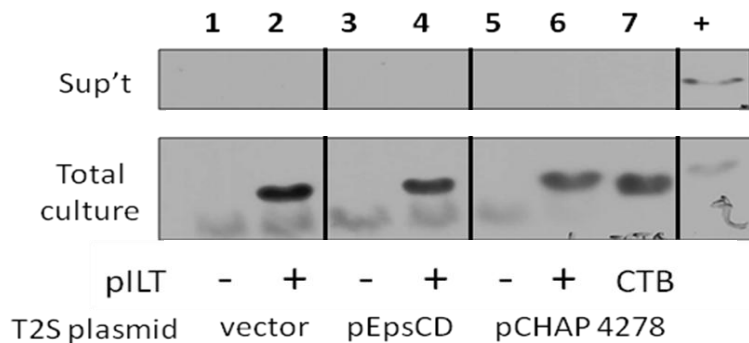
Figure 24: T2S genes from *V. cholerae* and K-12 *E. coli* do not complement an ETEC *gspD* mutant.

H10407Δ*gspD* carrying empty pTrc99A vector (-), pILT (+), or pICTB (CTB) were transformed with plasmid copies of heterologous T2S genes, induced for toxin expression, and fractionated. Cell-free supernatant and total culture samples were immunoblotted for the presence of LT or CTB. Lanes 1 and 2 show strains with no complementation (empty pACYC184 vector), lanes 3 and 4 show strains with *epsCD* from *V. cholerae* (pEpsCD), and lanes 5-7 show strains with *gspC-O* from K-12 *E. coli* (pCHAP4278). The lane marked '+' is a positive control of H10407 with a wild-type T2S system carrying pILT, which was induced and fractionated identically to the mutant strains. Immunoblots were probed with cross-reactive anti-CT antibody.

3.4 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 stability (Figure 17 and data not shown), indicating that the reduced levels found in ETEC cell-free supernatants are due to effects on secretion and not increased rates of degradation. In contrast to mutants with substituted lysine residues, LT[Q3A] and LT[E11A] were secreted from ETEC at wild-type levels. Our results are in line with previous research demonstrating that a single point mutation 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) (Wong and Buckley 1991; Connell et al. 1995; Chapon et al. 2000).

The similar nature 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 (Neill et al. 1983), and our group has reported that K-12 *E. coli* carrying a plasmid-borne T2S operon is able to secrete CTB (Horstman et al. 2004). 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*. Differences in substrate recognition are not limited to the T2S systems of ETEC and *V. cholerae*. Our results using a copy of the K-12 *gsp* operon indicate that the secretion of LT may be dependent on different residues in ETEC and lab strains of *E. coli* (see Figure 16 and Figure 19).

An investigation of the effects of expressing CTB[E11K] or CTB[E11A] on the secretion of other T2S substrates from *V. cholerae* suggested 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 (Palomaki et al. 2002). 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 recognition of multiple subunits in three-dimensional space. The binding of LT to G_{M1}, blood group sugars, and the surface of *E. coli* occurs at the interface between two adjacent B subunits (Merritt et al. 1994; Holmner et al. 2007). 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 (Merritt et al. 1994; Holmner et al. 2007) (Chapter 2).

86

Because LT[L25E] behaved like wild-type LT in terms of protease sensitivity and 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 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 closely related bacteria (formerly considered to be two *Erwinia* species) determined that the N-terminal portion of OutD (the GspD homolog in these species) and a PDZ domain in OutC (GspC) were involved in specific substrate recognition (Bouley et al. 2001).

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* (Figure 24). 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* (Sandkvist et al. 1997). Intriguingly, the T2S operon from K-12 *E. coli* also did not complement H10407Δ*gspD* (Figure 24). While ETEC's T2S operon is related to the T2S operon endogenous to lab strains of *E. coli* according to sequence analysis, there is no

more similarity between the operons in ETEC and K-12 *E. coli* than between those in

ETEC and *V. cholerae* (Tauschek et al. 2002). Therefore, the lack of complementation

from either plasmid is not necessarily surprising.

One limitation to this complementation analysis is that expression from the

plasmid-borne T2S constructs was not controlled; *epsCD* and *gspC-O* were simply placed

on a low-copy vector (pACYC184). While pCHAP4278 (carrying the K-12 T2S operon)

has been previously demonstrated to be functional (Horstman and Kuehn 2002),

expression of *epsCD* from pEpsCD was not verified. However, a previous study found

expression of the genes for CT inserted at the same restriction sites within the

pACYC184 vector (Gennaro et al. 1982).

Conclusions. Our results reveal extensive 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(s) 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.

3.5 Acknowledgments

4. Overexpression of wild-type and mutant LT and CTB constructs results in strain-specific toxic effects

4.1 Summary

The overexpression of a protein of interest is often required for the study of its function. However, there may be unintended consequences of the production of disproportionate levels of any protein, especially one not native to the species being used for overexpression. Indeed, there have been many studies attempting to overexpress heat-labile enterotoxin (LT) and cholera toxin (CT), two ADP-ribosylating toxins from enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae*, respectively. While toxic effects due to such overexpression have been reported on occasion, these proteins can generally be produced in large quantities in either *E. coli* or *V. cholerae*. Here, we report both strain- and species-specific effects of overexpressing B subunits from LT and CT with mutations in the mature protein sequence. Some mutations rendered LTB or CTB unstable, as determined by detection of protein levels by immunoblot. Interestingly, certain mutants impaired the growth of the strains of *V. cholerae* producing them, often with concomitant loss of the plasmid carrying the mutant gene. However, no correlation was found between the instability of the mutant in question and plasmid loss or effects on growth. As the mutations described here were introduced near the N-terminus of the mature protein, it is possible that they affect *sec*-dependent transport of the nascent peptide to the periplasm. Together, our results indicate that the N-terminal portions of mature LTB and CTB are sensitive to mutations that reduce the stability of the proteins and/or produce toxic effects in strain- and species-specific manners.

4.2 Introduction

LT and CT are virulence factors important for host colonization by ETEC and *V. cholerae*, respectively (Pierce et al. 1985; Allen et al. 2006). The generation of active and host-accessible toxin involves a number of steps between transcription and type II secretion. The genes for LT (*eltAB*) and CT (*ctxAB*) are each found as two-gene operons. For LT, these genes are found on an extrachromosomal virulence plasmid, whereas the genes for CT are integrated into the *V. cholerae* genome due to the action of a filamentous phage (Peterson 2002; Fleckenstein et al. 2009). The operons are transcribed as a bicistronic message (four nucleotides of the *eltA* reading frame overlap with the start of the *eltB* gene), and each toxin subunit is translated separately (Yamamoto et al. 1982). Each subunit has an N-terminal signal sequence directing it to the periplasm through the Sec complex. Mature A and B subunits fold in the periplasm, with the helical A2 peptide tail helping to nucleate the formation of a pentameric ring of B subunits (Hofstra and Witholt 1985). Fully assembled holotoxin is then exported from ETEC or *V. cholerae* via the type II secretion system (Sandkvist et al. 1997; Tauschek et al. 2002).

LT comes in two varieties: LT-I, which has been the focus of this thesis, and LT-II, a structurally similar but highly divergent toxin found in some strains of *E. coli* (Popoff 1998). The A subunit of LT-II catalyzes an ADP-ribosylation reaction like that catalyzed by LT-IA (Pickett et al. 1989). As described in previous chapters, the B subunit of LT-I is homologous to CT, while the B subunit of LT-II shows no significant similarity to CTB or LT-IB (Pickett et al. 1989). In contrast to the existence of multiple varieties of LT, only one major type of CT exists.

While ETEC strains fall into many serogroups and categories based on the specific toxins and pili that they produce, *V. cholerae* strains are divided into two major classes: the classical biotype and the El Tor biotype. These are often distinguished biochemically by the hemolytic activity present in El Tor strains (Peterson 2002; Olivier et al. 2007). Classical strains are thought to be responsible for the sixth cholera pandemic around the turn of the twentieth century, while the El Tor biotype is responsible for the current pandemic (Cvjetanovic and Barua 1972). The biotypes are very closely related, but the gene content of certain pathogenicity islands differs, as does the transcriptional regulation of key virulence factors, including CT, which both biotypes produce (Murley et al. 2000; Karaolis et al. 2001). Slight variations in the protein sequence of the CTB subunit have been noted (Holmner et al. 2007), but the signal sequences are identical in most sequenced *V. cholerae* isolates of both biotypes (Goel et al. 2008).

Given the importance of CT and LT in *V. cholerae* and ETEC infection and their extensive use as adjuvants, numerous efforts have been made to overexpress both toxins, for use in research and vaccine production. In one of the most extensive studies of a plasmid-based system for the overexpression of CT, the native *sec* signal sequence of CTB was determined to be a barrier for efficient production of the toxin in *E. coli*, with cell death observed after expression of the construct (Jobling et al. 1997). When the signal sequence from the B subunit of LT-II was used instead, CT could be produced at a high yield (Jobling et al. 1997). For this reason, the inducible CTB plasmid described in Chapter 3 (pICTB) includes the LT-IIB signal sequence. Overexpression of LT has also been reported. The yield of periplasmic toxin in *E. coli* was found to be enhanced by

knocking out the protease/chaperone DegP and providing extra copies of the gene

encoding DsbA, a chaperone involved in disulfide bond formation (Wulfing and

Rappuoli 1997). In this thesis, the inducible LT and LTB constructs used (pILT and

pILTB) contain ETEC's native signal sequences for LTA and/or LTB.

Many of the experiments described in Chapters 2 and 3 involved the

overexpression of LT or CTB from IPTG-inducible plasmids. While overexpression of

LT was achievable in ETEC, LTB mutants (specifically those identified as secretion

mutants; see Chapter 3) were not stable in *V. cholerae* strain P4 (an El Tor isolate). In

this chapter, we report that the induction of mutant LTB expression led to growth defects

in P4, with loss of the inducible plasmid carrying the mutant gene, even in the presence

of selective antibiotics. These growth phenotypes were strain-specific, as most LTB

mutants could be induced and detected in *V. cholerae* strain O395N1 (a classical strain).

Furthermore, overexpression of either wild-type or mutant CTB led to growth

impairment and plasmid loss in strain P4 but not in strain O395N1. Thus, the

overexpression of LTB or CTB mutants that are defective in secretion (or, in this case,

even wild-type CTB) may also produce growth defects in some strains of *V. cholerae*, at

times accompanied by the loss of the mutant plasmid construct. Use of the native signal

sequence from *ctxB* did not allow for the overproduction of CTB in either *V. cholerae*

strain but also did not generate a mutant growth phenotype. In summary, the potential for

overexpressing LTB mutants or CTB constructs is highly strain-specific, with mutations

affecting the stability of the proteins and generating expression-associated toxic effects in

varying ways in different strains.

**Table 8: Strains and plasmids used in Chapter 4.**

Strain	Genotype	Relevant characteristics
P4[1]	P27459Δ*ctxAB*	CT-deficient El Tor *V. cholerae* isolate
O395N1	O395Δ*ctxA*	CTA-deficient classical *V. cholerae* strain
jf570	H10407Δ*eltA*	ETEC strain with polar insertion in *eltA* (LT-deficient)
MK1123	P4/ pTrc99A	P4 carrying empty vector; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1225	P4/ pICTB	P4 carrying inducible CTB plasmid; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1236	P4/ pICTB[Q3K]	As MK1225, with inducible Q3K CTB mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1232	P4/ pICTB[E11K]	As MK1225, with CTB[E11K]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1233	P4/ pICTB[E11A]	As MK1225, with CTB[E11A]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1053	H10407Δ*eltA*/ pTrc99A	jf570 carrying empty vector; Amp^R
MK1223	H10407Δ*eltA*/ pICTB	jf570 with inducible CTB plasmid; Amp^R
MK1224	H10407Δ*eltA*/ pICTB[Q3K]	As MK1223, with inducible Q3K CTB mutant; Amp^R
MK1234	H10407Δ*eltA*/ pICTB[E11K]	As MK1224, with CTB[E11K]; Amp^R
MK1235	H10407Δ*eltA*/ pICTB[E11A]	As MK1224, with CTB[E11A]; Amp^R
MK1122	P4/ pILTB	P4 carrying inducible LTB plasmid; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1237	P4/ pILTB[Q3K]	As MK1122, with inducible Q3K LTB mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1243	P4/ pILTB[Q3A]	As MK1122, with LTB[Q3A]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1244	P4/ pILTB[E11K]	As MK1122, with LTB[E11K]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1238	P4/ pILTB[L25E]	As MK1122, with LTB[L25E]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
MK1105	H10407Δ*eltA*/ pILTB	jf570 carrying inducible LTB plasmid; Amp^R
	H10407Δ*eltA*/ pILTB[Q3K]	As MK1105, with LTB[Q3K]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	H10407Δ*eltA*/ pILTB[Q3A]	As MK1105, with LTB[Q3A]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	H10407Δ*eltA*/ pILTB[E11K]	As MK1105, with LTB[E11K]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	H10407Δ*eltA*/ pILTB[L25E]	As MK1105, with LTB[L25E]; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pTrc99A	O395N1 carrying empty vector; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pICTB	O395N1 with inducible CTB plasmid; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pICTB[Q3K]	O395N1 with inducible CTB[Q3K] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pICTB[E11K]	O395N1 with inducible CTB[E11K] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pICTB[E11A]	O395N1 with inducible CTB[E11A] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pILTB	O395N1 with inducible LTB plasmid; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pILTB[Q3K]	O395N1 with inducible LTB[Q3K] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pILTB[Q3A]	O395N1 with inducible LTB[Q3A] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pILTB[E11K]	O395N1 with inducible LTB[E11K] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pICTB[L25E]	O395N1 with inducible LTB[L25E] mutant; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	P4/ pnCTB	P4 with native CTB construct $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	O395N1/ pnCTB	O395N1 with native CTB construct; $\text{Str}^R \text{Kan}^R \text{Amp}^R$
	DH5α/ pnCTB	K-12 *E. coli* carrying native CTB construct; $\text{Str}^R \text{Kan}^R \text{Amp}^R$

Plasmid	Relevant characteristics	
pILT	IPTG-inducible LT holotoxin	Amp^R
pILTB	IPTG-inducible LTB pentamer	Amp^R
pICTB	IPTG-inducible CTB pentamer	Amp^R
pnCTB	IPTG-inducible CTB with native signal sequence	Amp^R

---

[1] The sources of strains not generated in these studies can be found in section 4.5.

4.3 Results

Overexpression of CTB is burdensome for *V. cholerae* strain P4. In the
course of studying the secretion of LTB, we noticed instability of the Q3K, Q3A, and
E11K mutant constructs in the El Tor *V. cholerae* strain P4 (see Table 8 and Figure 22 in
Chapter 3). This instability was not initially surprising, given the instability of several
LTB mutants in *E. coli* (see Chapter 5) and the existence of numerous proteases in *V.*
cholerae with access to the periplasm (Vaitkevicius et al. 2006). In contrast, wild-type
CTB and a similar set of CTB mutants (Q3K, E11K, and E11A) could be overexpressed
to equal levels in P4 (see Figure 20 in Chapter 3).

Closer inspection of the behavior of *V. cholerae* strain P4 revealed that the
expression of wild-type or mutant CTB under the conditions of our secretion studies
seemed to reduce the numbers of viable cells up to ten-fold when compared with P4
carrying only empty vector (Figure 25A). This stark reduction was especially surprising
given the overall similarity of the OD_{600} readings for these cultures (data not shown). In
contrast to these results, the overexpression of CTB secretion mutants did not reduce the
number of viable ETEC cells in our study conditions (Figure 25B), with each ETEC
strain reaching approximately $3\text{-}10\text{x}10^8$ CFU/ml.
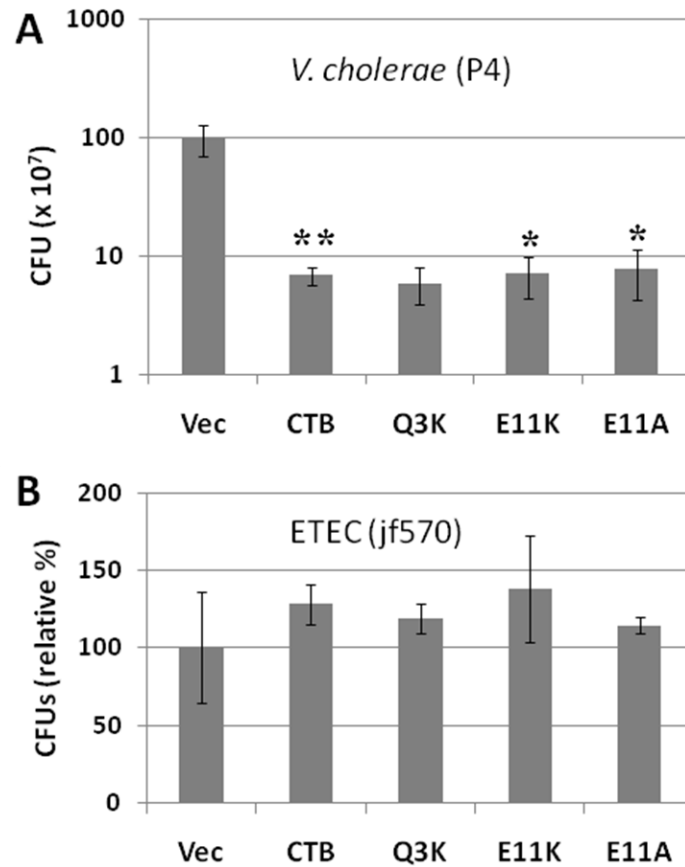
Figure 25: Expression of wild-type or mutant CTB reduces ampicillin-resistant CFU counts in *V. cholerae* strain P4 but not in ETEC.

(A) *V. cholerae* strain P4 carrying empty vector (Vec), wild-type CTB (CTB) or the indicated CTB mutant was diluted 1:50 from an overnight culture, grown for 4 h at 37°C in media containing ampicillin, and then induced for 3 h with 200 μM IPTG (the conditions used to study CTB secretion in Chapter 3). After induction, all strains were plated on media containing ampicillin for CFU determination. Absolute CFU values are plotted on a log scale. *, $p < 0.05$; **, $p < 0.005$ compared to vector ($n \geq 4$). (B) ETEC strain jf570 was grown, induced, and plated as described in (A). Due to some day-to-day variation in the magnitude of the CFU counts for all strains, the values for strains expressing each toxin construct are expressed as relative percentages of the number of CFUs for jf570 carrying empty vector for that trial ($n \geq 3$).

To further characterize the growth of P4 expressing wild-type and mutant CTB,

growth curves were performed for these strains. After dilution from an overnight culture,

P4 carrying empty vector, wild-type CTB, or mutant CTB was induced immediately with 200 μM IPTG and allowed to grow at 37°C. The resulting OD_{600} values at various timepoints can be seen in Figure 26A. Very slight, but reproducible, growth impairments were detected for wild-type CTB and the E11K and E11A mutants.

When a stronger selective antibiotic, carbenicillin, was used for determining the growth curves of P4 expressing CTB constructs, a more pronounced difference between P4 with empty vector and strains carrying pICTB could be observed (Figure 26B). A stronger selective pressure (in the form of a more potent antibiotic) impaired growth more greatly, presumably because *V. cholerae* cells lacking the plasmid were able to survive more easily in ampicillin than in carbenicillin by sharing resistance products.

As the CFU data gathered in the secretion studies (Figure 25) were based on growth on plates with ampicillin, cells which had lost the inducible CTB plasmid (and the ampicillin/carbenicillin resistance gene) for any reason would not have been reflected in the final count. Thus, further experiments were carried out to determine the number of cells still containing wild-type or mutant pICTB. In these experiments, cultures of P4 *V. cholerae* were induced to express wild-type or mutant CTB and then plated on media selecting for any *V. cholerae* cell (the P4 strain is streptomycin resistant) or cells specifically carrying the inducible plasmid. The results showed that while very few

Figure 26: Induction of wild-type and mutant CTB constructs impairs the growth of P4 *V. cholerae* with concomitant plasmid loss

(A) *V. cholerae* strain P4 carrying empty vector (Vec), wild-type CTB construct (CTB), or the indicated mutant construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG and grown in media containing ampicillin at 37°C. OD_{600} readings were taken at the indicated timepoints. **(B)** The same strains were grown and measured as described in part (A), with carbenicill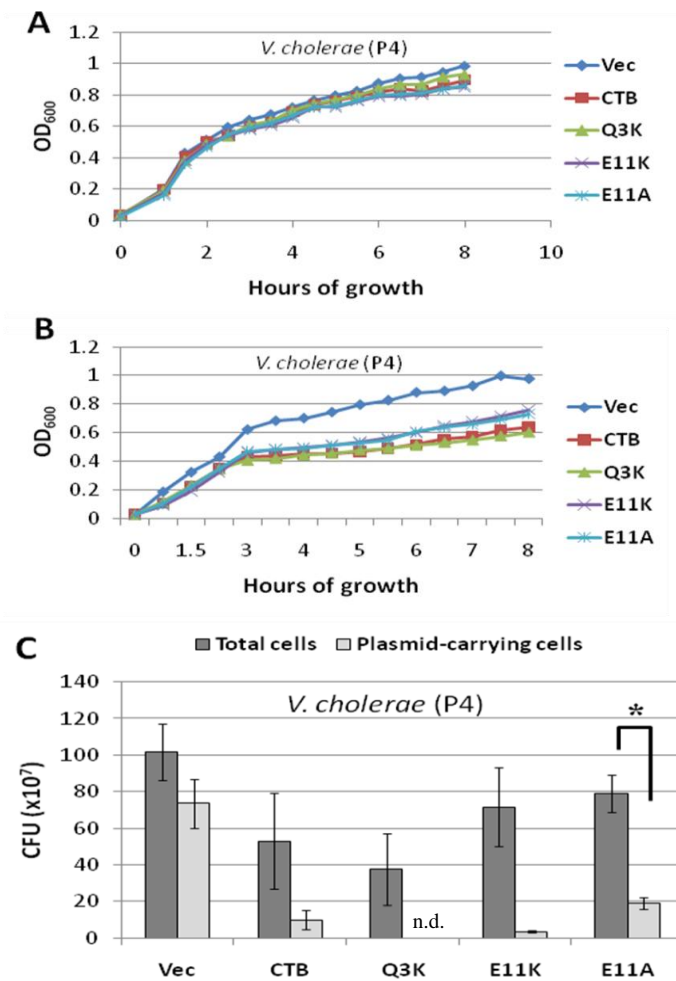in used as the selective antibiotic. **(C)** *V. cholerae* strain P4 carrying empty vector (Vec), wild-type CTB construct (CTB), or the indicated mutant construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG and grown in media containing carbenicillin at 37°C. After eight hours of growth, each strain was serially diluted and plated on media containing streptomycin (selecting for all P4 cells) and ampicillin (selecting for plasmid-carrying cells) for determination of CFUs. Cultures expressing Q3K generated no viable ampicillin-resistant cells at the dilutions tested. ***** $p<0.05$ ($n\geq2$); n.d., none detected.

98

ampicillin-resistant (i.e., plasmid-carrying) colonies were recovered, a significant amount of streptomycin-resistant P4 colonies were formed from each culture (Figure 26C). Thus, induction of wild-type CTB or the Q3K, E11K, or E11A mutant results in loss of the inducible CTB plasmid, even in the presence of selective antibiotics. Together, these results indicate that the burden of overexpressing CTB in P4 leads to plasmid loss. Indeed, it is advantageous for these strains to attempt survival in selective media without a resistance gene rather than to continue to overexpress CTB. In contrast, wild-type or mutant pICTB was carried by essentially 100% of ETEC cells after a three-hour induction (data not shown).

Overexpression of LTB mutants also results in growth defects in P4. To further characterize the capacity of *V. cholerae* to tolerate the overexpression of B-subunit pentamers, P4 strains expressing LTB and LTB mutants were also tested for growth phenotypes and loss of plasmid-based antibiotic resistance. As shown in Figure 27A, overexpression of wild-type LTB had no effect on the growth of P4, while the growth of strains carrying mutant LTB constructs (Q3K, Q3A, and E11K) was distinctly impaired. Likewise, strains carrying mutant LTB lost plasmid-based resistance over the course of the eight-hour growth curve (Figure 27B). Therefore, despite a lack of detectable expression (see Figure 22 in Chapter 3), these mutant forms of LTB nevertheless impair the growth of *V. cholerae* strain P4, and strains carrying mutant LTB constructs appear to lose the plasmids even when grown in selective media.
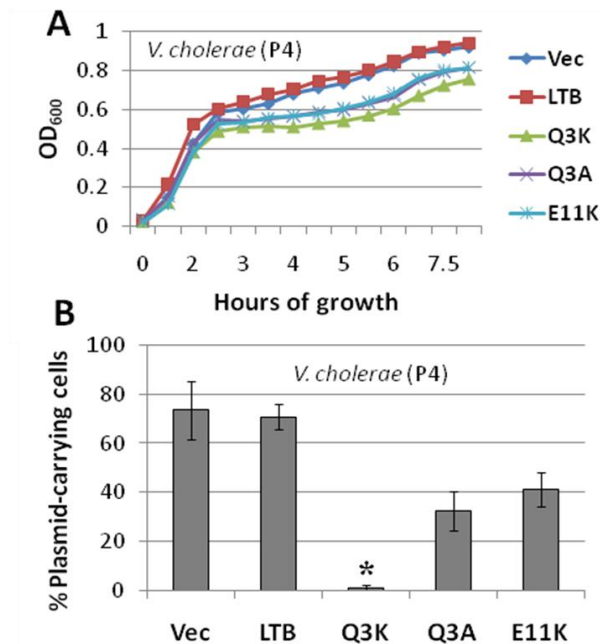
Figure 27: Mutant LTB constructs impair the growth of P4 *V. cholerae* and are lost during induction.

(A) Representative growth curve of *V. cholerae* strain P4 carrying empty vector (Vec), wild-type LTB construct (CTB), or the indicated mutant construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG, and grown in media containing carbenicillin at 37°C. OD_{600} readings were taken at the indicated timepoints. **(B)** Strains were induced and grown for 8 h as described in (A) and plated on media containing streptomycin (selecting for all P4 cells) and ampicillin (selecting for plasmid-carrying cells). The number of ampicillin-resistant colonies was divided by the number of streptomycin-resistant colonies to calculate the percentage of plasmid-carrying cells. * $p<0.005$ compared to LTB and $p<0.05$ compared to vector and E11K ($n \geq 2$).

To further investigate the instability of LTB mutants in strain P4, we attempted to rescue protein production by addition of Mg^{2+} to the medium and expression of LTA along with the LTB mutants. In a study involving numerous LTB mutants (including one with Gln-3 deleted), the addition of 100 mM $MgCl_2$ to the growth medium was reported to increase the stability of these mutants in a cloning strain of *E. coli* (Alone et al. 2007). The authors attributed this effect to impairment of cellular proteases. However, the

presence of 100 mM MgCl$_2$ in the growth medium did not increase the stability of

LTB[Q3K] or LTB[E11K] in *V. cholerae* strain P4 (data not shown). Likewise, the

expression of the Q3K, Q3A, and E11K mutants as holotoxins (i.e., with *eltA* included on

the plasmid construct) did not produce detectable amounts of these mutants (data not

shown). In fact, wild-type LT holotoxin was not detectable in P4 after overnight

induction (data not shown). These results indicate that the inclusion of LTA does not

stabilize mutant pentamers and may instead target overproduced LTB for degradation.

Interestingly, LTB with the L25E mutation could be induced significantly in *V.

cholerae* strain P4 and was readily detectable in the supernatant (see Figure 22 in Chapter

3). This mutation impairs the binding and secretion of LT, possibly altering the spacing

of B subunits (see section 3.4). Expression of this mutant did not alter the growth of P4

or induce plasmid loss (data not shown), establishing that some mutations in LTB are

tolerated by *V. cholerae*.

Mutant LTB constructs are not toxic to ETEC. To discern whether the effects

of overexpressing LTB were inherent to the construct, we transformed the inducible LTB

plasmid and derived mutant constructs into ETEC strain jf570. The OD$_{600}$ values of

cultures grown in ampicillin (data not shown) or carbenicillin (Figure 28) revealed no

impairment of ETEC strains expressing mutant LTB compared to strains carrying wild-

type LTB or vector. At the same concentrations of antibiotics and IPTG, induction of

these constructs generated clearly discernible impairment of the growth of *V. cholerae*

strain P4 (Figure 26B).
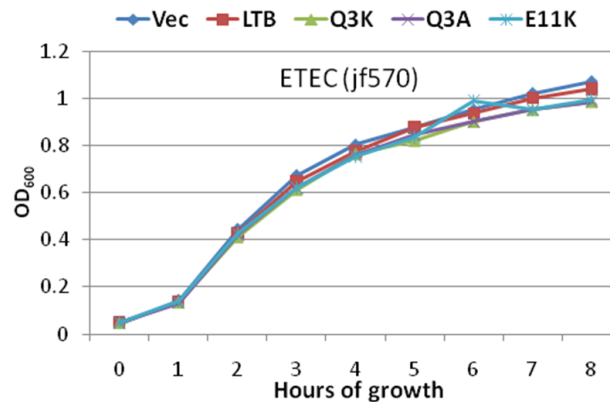
Figure 28: Induction of wild-type or mutant LTB does not impair the growth of ETEC.

Representative growth curve of ETEC strain jf570 carrying empty vector (Vec), wild-type LTB construct (LTB) or the indicated mutant LTB construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG, and grown in media containing carbenicillin at 37°C. OD_{600} readings were taken at the indicated time points.

Analysis of CTB and LTB expression in a second *V. cholerae* strain. To

determine whether the toxicity and instability of mutant constructs was specific to strain

P4, the same panel of mutant plasmids, along with wild-type pICTB and pILTB, were

transformed into the classical *V. cholerae* strain O395N1. This strain carries a deletion in

ctxA (encoding CTA) but still carries the *ctxB* gene. However, the background levels of

CTB produced by O395N1 are undetectable by immunoblotting (see 'Vec' in Figure

29A). Wild-type CTB and CTB[Q3K] were readily detectable in O395N1 total culture

fractions, while CTB[E11K] and CTB[E11A] were detected in slight amounts, if at all

(Figure 29A and data not shown). The instability of these two secretion mutants in

O395N1 stands in contrast to the wild-type levels of induced expression detected in P4

(see Figure 20 in Chapter 3). However, induction of wild-type or mutant CTB had no

strong effect on the growth of O395N1 in carbenicillin (Figure 29B), although O395N1

102

expressing E11A may be slightly impaired. Preliminary results show that the plasmid

constructs are not lost during induction (data not shown).
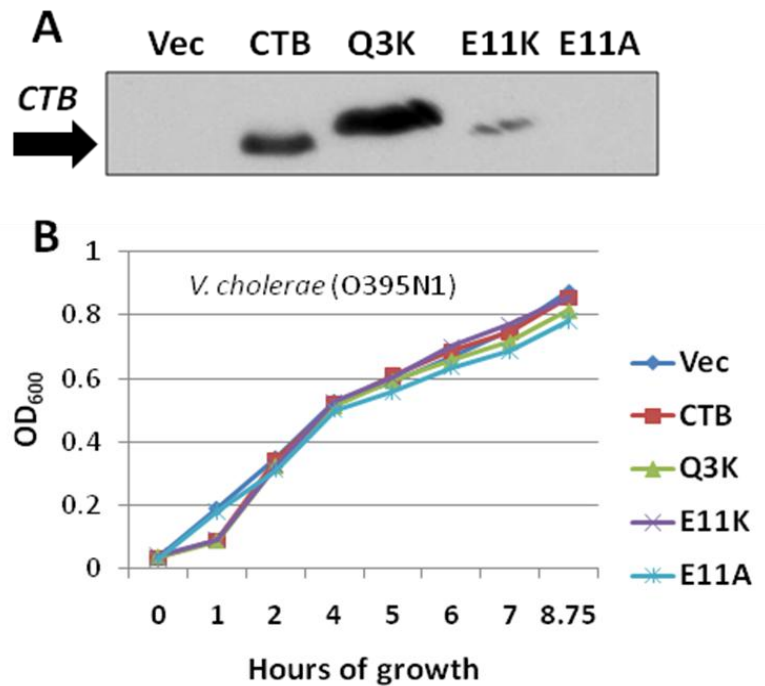
Figure 29: Certain CTB mutants are unstable in a classical strain of *V. cholerae* but do not cause growth impairment.

(A) Representative immunoblot of *V. cholerae* strain O359N1 carrying empty vector (Vec), wild-type CTB construct (CTB), or the indicated mutant construct was diluted 1:50 from an overnight culture, grown 4 h at 37°C, and induced for 3 h. After induction, total culture samples were TCA-precipitated, normalized to the OD_{600} values of the original cultures, and subjected to immunoblotting. The blot was probed with anti-CT antibody. **(B)** Representative growth curve of O359N1 carrying empty vector (Vec), wild-type CTB construct (CTB), or the indicated mutant construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG, and grown in media containing carbenicillin at 37°C. OD_{600} readings were taken at the indicated timepoints.

Interestingly, whereas most LTB mutants were unstable in *V. cholerae* strain P4,

only LTB[E11K] showed low levels of detectable protein in strain O395N1 (Figure 30A).

LTB[Q3K], LTB[Q3A], and LTB[L25E] were detected at levels similar to wild-type

103
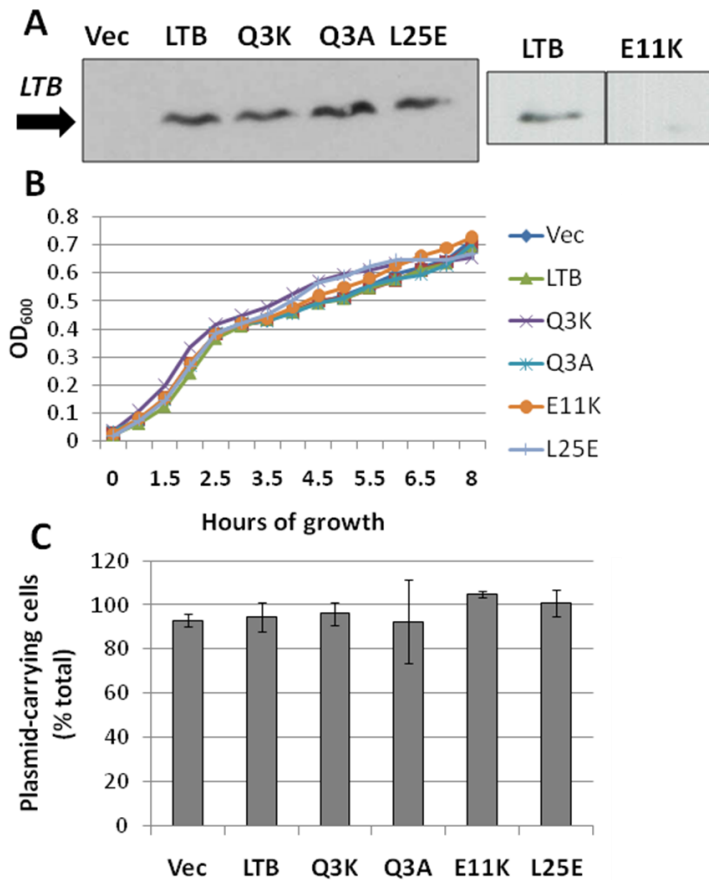
Figure 30: Mutant LTB constructs do not impair the growth of the classical *V. cholerae* strain O395N1.

(A) Representative immunoblot of *V. cholerae* strain O359N1 carrying empty vector (Vec), wild-type LTB construct (LTB), or the indicated mutant construct was diluted 1:50 from an overnight culture, grown 4 h at 37°C, and induced for 3 h. After induction, total culture samples were TCA-precipitated, normalized to the OD_{600} values of the original cultures, and subjected to immunoblotting. Blots were probed with anti-CT antibody. **(B)** Representative growth curve of O359N1 carrying empty vector (Vec), wild-type LTB construct (LTB), or the indicated mutant construct was diluted 1:80 from an overnight culture, induced with 200 μM IPTG, and grown in media containing carbenicillin at 37°C. OD_{600} readings were taken at the indicated timepoints. **(C)** Strains were induced and grown for 8 h as described in (A) and plated on media containing streptomycin (selecting for all O395N1 cells) and ampicillin (selecting for plasmid-carrying cells). The number of ampicillin-resistant colonies was divided by the number of streptomycin-resistant colonies to calculate the percentage of plasmid-carrying cells. Similar absolute numbers of CFUs were found for each strain (*n*=2).

(Figure 30A). In all cases, O395N1 overexpressing wild-type or mutant LTB grew similarly to O395N1 containing empty vector (Figure 30B), and no plasmid loss was detected after eight hours of induction (Figure 30C). Therefore, while some mutant constructs are unstable in O395N1, the specific mutations that impair toxin stability are different from those impairing stability in strain P4. In contrast to the growth defects and plasmid loss noted for P4, no construct altered the growth of O395N1.

CTB with its native signal sequence cannot be overexpressed in the *V. cholerae* strains tested, but induction of the native construct displayed no toxic effects. All of the studies involving the expression and secretion of CTB had made use of pICTB, a construct in which the mature sequence of *ctxB* was fused to the signal sequence from the B subunit of the LT-II toxin, because this sequence had been shown to allow for greater overexpression of CTB in the periplasm (Jobling et al. 1997). Therefore, we sought to determine whether expressing CTB with its native signal sequence would alleviate the toxic effects observed when pICTB was used for pentamer overexpression in strain P4. To that end, the entire *ctxB* gene was cloned from O395N1 genomic DNA and ligated into pTrc99A, the same vector used to create pICTB. This new construct was designated pnCTB for the presence of the native signal sequence.

When pnCTB was conjugated into P4 and IPTG was added to induce the expression of CTB, no pentamer could be detected (Figure 31A). The same was true for strain O395N1, the original source of the cloned *ctxB* gene; no detectable CTB was found after induction of pnCTB, although CTB was readily produced and secreted using the old

construct, pICTB (Figure 31B). Perhaps unsurprisingly, pnCTB did not impair the

growth of either strain and was not lost during eight hours of induction (data not shown).
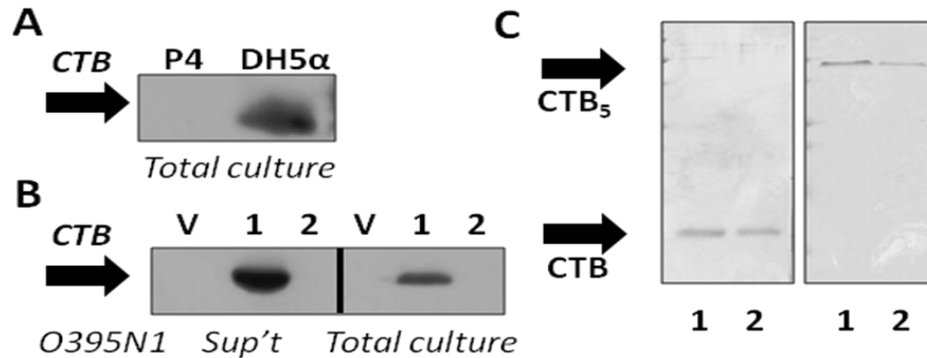
Figure 31: CTB with its native signal sequence can be produced in *E. coli* but cannot be overexpressed in *V. cholerae*.

(A) Representative immunoblot of TCA-precipitated total culture samples from *V. cholerae* strain P4 and *E. coli* strain DH5α carrying pnCTB after induction with 200 μM IPTG for 3 h. **(B)** Representative immunoblot of TCA-precipitated supernatant (Sup't) and total culture and samples from *V. cholerae* strain O395N1 carrying empty vector (V), pICTB (1), or pnCTB (2) after induction with 200 μM IPTG for 3 h. Blots were probed with anti-CT antibody. **(C)** 500 ng of CTB purified from DH5α carrying either pICTB (1) or pnCTB (2) was analyzed by 15% SDS-PAGE and Coomassie staining. Samples were boiled before loading (left panel) or run without boiling (right panel).

On the other hand, induction of pnCTB in DH5α *E. coli* led to detectable levels of

CTB protein (Figure 31A). Furthermore, pentameric CTB could be purified from the

periplasm of DH5α, indicating that there was no inherent problem with the construct

(Figure 31C). Although the original report generating a *ctxB* gene fusion with the LT-IIB

signal sequence described toxicity when expressing *ctxB* with its native signal sequence

in *E. coli* (Jobling et al. 1997), induction of pnCTB had no effect on the growth of DH5α

(data not shown). Therefore, while pentameric CTB could be overexpressed in *E. coli*

using its native signal sequence, it was not detectable under the same conditions in either

of the *V. cholerae* strains tested.

4.4 Discussion

The results presented here show that several B-subunit mutations altering the

secretion of LT also affect the stability of the toxin when overexpressed in *V. cholerae*.

Indeed, beyond reducing the yield of expressed protein, several of these mutant

constructs lead to slower growth in *V. cholerae* strain P4. Furthermore, the constructs are

lost during induction, indicating that overexpression of mutant LTB is burdensome to P4.

Similar phenotypes were seen when both wild-type and mutant CTB were overexpressed

in this strain. Experiments involving the overexpression of the same LTB and CTB

constructs in ETEC and a second *V. cholerae* strain (O395N1) demonstrated that the

constructs were not inherently toxic and that stability of each mutant was strain-

dependent. No correlation between the stability of a construct and growth impairment or

plasmid loss due to its induction could be observed.

As yet, it is unclear why strain O395N1 is able to tolerate the overproduction of

CTB and LTB without apparent growth defects, while strain P4 demonstrates slower

growth and plasmid loss after induction of any CTB construct or certain LTB constructs.

One major difference between these two strains is their biotypes. As mentioned in

section 4.2, *V. cholerae* strains fall into two categories, classical strains (like O395N1)

and El Tor strains (like P4). A number of differences exist between these biotypes

regarding transcriptional and translational regulation and the specific array of virulence

factors produced by each. It is possible that overproduction of B-subunit pentamers is intrinsically easier in classical strains than in El Tor strains. However, the overexpression of both wild-type CTB and a G_{M1}-binding CTB mutant has been reported previously in El Tor strains (Sanchez and Holmgren 1989; Silva et al. 1998). Strain P4 may simply be inefficient at overexpressing CTB compared to many other *V. cholerae* strains, including other El tor isolates.

It is also worth noting that O395N1 still contains a working copy of *ctxB*, although production of the protein is not induced under the conditions used in these studies. Nevertheless, IPTG induction of the plasmid construct may somehow positively regulate transcription of the native *ctxB* gene, allowing for production of a significant amount of CTB. Testing the expression of our CTB and LTB constructs in a larger panel of *V. cholerae* strains (some containing intact *ctxB* genes and some without) will be required to address these possibilities.

It is also unclear why one particular mutant can be stably overexpressed in one strain or species but not in another. For example, LTB[Q3K] is stable when expressed in ETEC and O395N1, but its expression cannot be detected in P4. Strain-specific differences in available proteases could be responsible for these results, or altered folding of mutant B subunits compared to wild-type may impair growth of the strains overexpressing them. Indeed, a poorly folding mutant of LTB has been reported to cause toxic effects when overexpressed in *E. coli* (Wulfing and Rappuoli 1997). However, the mutants described in this chapter assemble into pentamers and bind the host receptor G_{M1} (data not shown; see Chapter 3). In fact, the one mutant that may be folded improperly

(LTB[L25E]; see Table 6 in Chapter 3) does not cause toxic effects when overexpressed in ETEC or *V. cholerae*. Therefore, we favor other possible explanations.

Alterations of a nucleotide sequence that introduce rare codons can lead to ribosome stalling in *E. coli*, resulting in SsrA-dependent degradation of the mRNA and nascent peptide (Withey and Friedman 2003). Indeed, we have noted codon-specific effects on the stability of LT in ETEC strain jf570. Introduction of a rare codon for arginine (AGA) in place of the codon for Lys-84 caused levels of expressed LTB to become undetectable, but when a more commonly used codon (CGT) was introduced instead, wild-type levels of LTB were detected (data not shown). Because the primary protein sequence is identical in both cases, an effect on translation or mRNA stability most likely explains these results. It is possible that the nucleotide changes involved in making the point mutations described above cause similar mRNA-level effects, even though each mutation was designed with an optimal codon (Neidhardt 1996).

Alternatively, the secretion of mutant B subunit through the Sec machinery may be altered. Although the residues of the signal sequence are directly involved in associations with Sec proteins during transit across the inner membrane, mutations near the N-terminus of the mature protein have also been shown to affect *sec*-dependent transport, causing up to 50-fold reduction in transport to the periplasm (Li et al. 1988). Specifically, mutations that alter the net charge of the first 18 amino acids (like the Q3K mutation) have been reported to be the most detrimental, and similar charge-altering mutations located further in the mature sequence do not show any effect (Kajava et al. 2000). This may explain the detrimental effects of LTB[Q3K] and the completely wild-

type behavior of LTB[L25E] in terms of expression and toxicity. It is possible that strain-specific differences in the efficiency of Sec complex formation or in its activity cause differences in the levels of detectable protein.

If the mutations studied in this chapter do significantly delay *sec*-dependent secretion of an overexpressed construct, it is easy to imagine the growth rate of the strain suffering as a result. That is, if numerous LTB peptides are clogging available Sec complexes due to overexpression, the cell may not be able to transport essential proteins to the periplasm or outer membrane. As a consequence, the growth rate would slow down, and there would be selective pressure to lose the plasmid during cell division events. One additional piece of evidence supports this hypothesis. CTB[Q3K] can be overexpressed in ETEC if the strain enters mid-log phase before induction of the plasmid construct (see Figure 25B and Figure 21 in Chapter 3). However, induction of this construct immediately following the dilution of an overnight culture leads to drastic growth impairment, with no increase in OD_{600} seen over an eight-hour window (data not shown). Therefore, ETEC is equipped to handle overexpression of this mutant, but only after entry into mid-log phase. Similar results are seen with LT[Q3A] (data not shown).

As stated above, the inducible CTB construct used in these studies (pICTB) includes the *sec* signal sequence from LT-IIB. Because of the potential for this non-native signal sequence to be responsible for the failure to overexpress stable CTB in strain P4, a second inducible construct containing the native *ctxB* signal sequence (pnCTB) was created. However, CTB could not be overexpressed in either *V. cholerae* strain, even though O395N1 genomic DNA was used to generate the construct. Thus,

there may be a maximal capacity for normal production of CTB in *V. cholerae*, one that can be exceeded by the use of a non-native signal sequence. Interestingly, DH5α *E. coli* could produce high quantities of CTB using the native signal sequence. This suggests that impaired efficiency of the Sec machinery in *V. cholerae* may underlie these results. Alternatively, there may be an increased number of other *sec*-dependent polypeptides in *V. cholerae* as compared to the number in DH5α *E. coli*. Thus, many nascent CTB peptides may be unable to reach the periplasm in *V. cholerae* and be degraded instead, leaving only an undetectable amount.

Conclusions. These experiments highlight the range of phenotypes that can be generated by altering the mature protein sequence of LTB or CTB. As reported in previous chapters, the Q3K mutation alters the blood sugar binding, cell surface binding, and secretion of LTB. In this chapter, we show that the same mutation can lead to drastic instability of the mutant protein in specific strains, along with impairing growth. Similar results can be seen with other mutants, although growth of a second *V. cholerae* strain was not impaired by any mutant tested. Further research will need to be done to determine whether these mutations alter transport through the Sec machinery or render the protein more susceptible to strain-specific proteases.

4.5 Acknowledgements

5. Concluding remarks

5.1 LTB: building community, one binding partner at a time

The B subunit of heat-labile enterotoxin is remarkable for its efficient use of a small number of residues for a relatively large number of purposes. Each B subunit consists of 103 amino acid residues after cleavage of the signal peptide during *sec*-dependent transport into the periplasm. Of those residues, a considerable number become buried in its various β-sheets and small contingent of α-helices (Sixma et al. 1993). With the residues that remain available, an LTB molecule binds the A2 helical peptide tail from the active subunit and forms a pentameric ring (see Figure 2 in Chapter 1), contacting one additional B subunit on each side. At this point, the holotoxin, localized in the periplasm of *E. coli*, is toxic and fully assembled but is still separated from its target molecules by two membranes.

After formation of the holotoxin, an even smaller surface area is exposed for additional contact with other molecules. In order to be secreted from ETEC, LT must be recognized by one or more components of the type II secretion system (as yet, the identity of these components remains unknown). While it has been established that the LTB pentamer (and not LTA) is involved in secretion, it is unclear how much of the B subunit is recognized by the secretion apparatus. The results reported here indicate that the recognition motif may span adjacent B subunits, but individual point mutations can significantly impair toxin export (see Chapter 3).

After release from ETEC, work from our lab has shown that LT associates with LPS on the ETEC surface and on outer membrane vesicles released by the bacteria. Work from this thesis shows that such an interaction occurs through a peripheral sugar binding pocket on LTB. This pocket was first identified as the area on LT responsible for binding to blood antigen sugar residues. Results described in Chapter 2 show that the set of residues involved in LPS binding overlaps significantly with the set of residues required for blood sugar binding. However, these two pockets are not identical, with certain residues required for only one of the two binding events.

A small amount of available surface-exposed area on LTB remains after taking its interactions with other B subunits, LTA, and LPS or blood sugars into account. This portion of the protein is found at the bottom of the pentameric ring, opposite the location of the A subunit. Far from being an extraneous collection of amino acids or merely playing a structural role, this portion of LTB is required for binding to the host receptor, G_{M1}, and several related gangliosides. Like the association of LT with blood sugar or LPS, G_{M1} binding involves residues from adjacent subunits. Thus, LTB uses a vast majority of its available surface amino acids for one or more binding events.

5.2 Residues in perfect balance

As a consequence of its structure/function economy, alteration of the residues in LTB often leads to profound instability. In general, mutations causing complete instability are not reported, but some analysis of the stability of LTB has been described. In a recent study involving the deletion of individual amino acids forming a short α-helix at the N-terminus of mature LTB, many such mutations were reported to render the

protein unstable, even in lab strains of *E. coli* (Alone et al. 2007). In that study, four of

six amino acid deletion mutants were not detectable when expressed from a plasmid.

However, while LTB lacking all six of the amino acids in question (i.e., the entire α-

helix) was not detectable by immunoblotting, transcript for the mutant was produced, and

the mutant could be translated *in vitro* (Alone et al. 2007). Thus, many alterations in the

primary structure of LTB seem to lead to degradation of the protein after its production,

likely as a consequence of major perturbations in folding.

We have also observed profound instability for a similar proportion of LTB

mutants generated in the course of these studies (Table 9). While some point mutations

Table 9: Expression levels of LTB mutants in *E. coli*

LTB mutants were expressed in *E. coli* strain jf570 and/or MC4100 from an inducible plasmid, and expression level was examined by immunoblot analysis of CFU-matched total culture samples using a cross-reactive anti-CT antibody. Mutations causing a reduction in protein stability (17 out of 30; 57%) are shaded in gray.

Mutation	Detectable expression in *E. coli*	Mutation	Detectable expression in *E. coli*
ΔQ3	None	A46D	Reduced
Q3A	Wild-type	A46S	Reduced
Q3K	Wild-type	T47A	Wild-type
Q3L	Poor	T47F	Reduced
T6K	Poor	T47K	Reduced
E11A	Wild-type	F48Y	None
E11K	Wild-type	Q49L	Wild-type
Y18A	Poor	T80A	Wild-type
K23A	Only LTA detected	D83L	Reduced
L25E	Wild-type	W88F	Wild-type
L25I	Poor	W88L	Poor
G33D	Wild-type	N89K	Wild-type
F42E	None	N94L	Wild-type
F42Y	None	S100K	Poor
K43R	Wild-type	S100L	Poor

introduced relatively drastic changes in amino acid character and could be expected to disrupt the folding of LTB, several such drastic mutations had no appreciable effect on the protein's stability.   For example, alteration of Leu-25 to a glutamic acid residue did not reduce expression, while introduction of a closely related isoleucine caused no toxin to be detected (Table 9). A similar effect was seen with Thr-47 (for which only introduction of alanine was tolerated) and Gln-3 (for which substitution with alanine or lysine was tolerated, but not the introduction of leucine or deletion of the amino acid).

Many of these mutations likely brought about degradation of the nascent LTB protein (in some cases, faint bands and/or degradation products were visible after immunoblotting; data not shown). However, we were rarely able to detect the production of LTA, which we would not expect to be altered. It is therefore possible that some mutations altered the stability of the *eltAB* message. As LTA and LTB are transcribed together, disrupting the translation of the mRNA would have profound effects on the production of both proteins. An alternative explanation is that free LTA is not degraded especially quickly, but if LTA has associated with mutant LTB that is destined for degradation, it will be degraded as well. In the case of expressing the K23A LTB mutant, where LTA was readily detectable in the periplasm but LTB was only faintly detected in total culture samples (Table 9 and data not shown), it is possible that the mutation prevents the association of LTA with LTB. Given the extremely rapid association of newly synthesized wild-type LTB and LTA (Hofstra and Witholt 1985), even highly unstable LTB mutants may be able to associate with free LTA, bringing about its degradation as well.

115

Of course, LTB is not unique in the unpredictability of the effects that mutations will have on its stability. For example, similar differences in protein stability based on the introduction of different amino acids at the same position have been reported in studies of the type II secretion substrate PehA polygalacturonase (Palomaki and Saarilahti 1995). However, the high sensitivity of LTB to alteration of its protein sequence requires that the possible effects on its stability to be taken into account when mutations are generated.

5.3 Implications for research with LT

Mutagenesis of LTB reveals an important point about small proteins: a single point mutation rarely generates its effect in a vacuum. When considering stability (in ETEC or *V. cholerae*), secretion, and binding (to LPS, blood sugars, and G_{M1}), the mutations we extensively analyzed in these studies never exhibited effects on only one property. As shown in Table 10, none of the mutations studied thoroughly in this thesis had only one discernible effect. E11A came the closest, by ablating Kdo binding along with only a slight decrease in secretion (to approximately 90% of the secreted levels reached by wild-type LT). On the other end of the spectrum, LT seems crippled by the A46D mutation, which reduced stability *in vivo*, secretion, and binding in each assay (Table 10).

To date, the relevance of LPS and blood sugar binding during infection *in vivo* has not been characterized. It is unclear whether blood sugars can act as a viable alternative receptor alongside G_{M1} and whether LT might be trafficked differently after

Table 10: Effects of selected mutations from these studies.

Mutations tested for their effects on at least five properties of wild-type LT are listed below. Assays for which each mutant demonstrated a significant reduction from wild-type levels of stability, secretion, or substrate binding are shaded in gray. Lighter gray boxes indicate slight (<10%) but statistically significant deviations from wild-type; n.d. = not determined.

Mutation	Stability in ETEC	Secretion from ETEC	DH5α surface binding	Kdo binding	Blood sugar binding	G_{M1} binding
Q3A			▓		▓	
Q3K		▓	▓		▓	
Q3L	▓	*n.d.*	▓		▓	
E11A		░		▓	*n.d.*	
E11K		▓	▓	▓		
Y18A	▓	*n.d.*			▓	
L25E		▓	▓	*n.d.*	▓	▓
A46D	▓	▓	▓	▓	▓	▓
T47A			▓		▓	░

internalization through blood sugars. Moreover, work done with blood sugar binding in cell culture or *in vivo* has exclusively used soluble LT, while the majority of secreted LT is understood to be associated with LPS on the surface of outer membrane vesicles. As the LPS and blood sugar binding pockets overlap significantly (see Chapter 2), there is no reason to assume that soluble LT and vesicle-bound LT will behave identically *in vivo* with regard to blood sugar binding. Due to the presence of five binding pockets per holotoxin, LPS binding and blood sugar binding may occur simultaneously in the context of vesicle-bound LT, but this possibility has not yet been investigated. Mutations described in this thesis impairing only blood sugar binding (Y18A), only LPS binding (E11K), or both (T47A) may be useful for future research into the relative contributions of these binding events.

In the meantime, studies of the association between blood type and ETEC disease should consider the possible complications of LPS binding. Furthermore, comparisons of soluble LT and vesicle-associated LT may need to account for the role of blood sugar binding in the context of actual infection. Thus, due to the wide variety of binding events and the high possibility of structural alteration of LT as a consequence of mutation, careful characterization of all LT mutants generated is advisable.

6. Materials and Methods

Unless specified, all reagents were purchased from VWR.

6.1 Bacterial strains and media

Strains used in these studies are listed in each chapter. Bacterial strains were maintained in LB Miller broth (25 g/l) or on LB-agar plates (LB with 15 g/l agar; ISC) unless otherwise indicated. When appropriate, the following antibiotics (Sigma) were added: ampicillin (100 µg/ml), carbenicillin (100 µg/ml), chloramphenicol (25 µg/ml), gentamicin (30 µg/ml), kanamycin (25 µg/ml), and streptomycin (100 µg/ml). Strains were stored at -80°C in 20% glycerol.

6.1.1 Media

LB Miller broth

10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl

SOC medium

20 g/l Bacto-tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 20 mM glucose (pH 7.0)

CFA medium

1% casamino acids, 0.15% yeast extract, 0.005% MgSO$_4$, 0.005% MnCl$_2$

Lipid agar

50 mM MOPS, 40 mM K$_2$HPO$_4$, 25 mM NaH$_2$PO$_4$, 7.5 mM NH$_3$SO$_4$, 0.4 mM MgSO$_4$, 0.5% olive oil (v/v) (Kagami et al., 1998)

6.1.2 Bacterial transformations

Transformations of K-12 *Escherichia coli* were carried out using a $CaCl_2$ protocol as described (Sambrook 1989). For each transformation, 2 ml of a log-phase culture was spun down (10,000 x g, 1 min). Cells were resuspended in 1 ml of ice-cold 50 mM $CaCl_2$ and incubated for 30 min at 4°C. Afterward, cells were resuspended in 50 µl of ice-cold 50 mM $CaCl_2$, and DNA (~200 ng) was added. The mixture was incubated for 30 min at 4°C, then heat shocked at 42°C for 45 sec. After recovery on ice for 2 min, 950 µl of SOC media was added. Cells recovered for 1 h at 37°C and were plated on selective media overnight.

For transformations involving enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae* strain O395N1, a modified protocol using electroporation was used. For each transformation, 3 ml of a log-phase culture was spun down (10,000 x g, 1 min). Cells were resuspended in 1.5 ml of ice-cold 50 mM $CaCl_2$ and incubated for 30 min at 4°C. Afterward, cells were washed twice with 750 µl of ice-cold 10% glycerol and once with 375 µl of ice-cold 10% glycerol. Finally, cells were resuspended in 100 µl of ice-cold 10% glycerol. DNA was added (~ 400 ng), and the mixture was placed in a 0.2-mm-wide electroporation cuvette (Genesee). Electroporation was carried out at 2.5 V, with 25 µF capacitance and 200 Ω resistance, using a Gene Pulser (Bio-Rad). After electroporation, 1 ml of SOC medium was added, and the cells were allowed to recover for 1 h at 37°C before plating on selective media overnight.

6.1.3 Triparental mating

Plasmids were conjugated into *V. cholerae* strain P4 using triparental mating essentially as described (Goldberg and Ohman 1984). DH5α cells carrying the plasmid of interest were used as the donor strain, and MT616 carrying pRK2013 served as the helper strain. Briefly, 0.5 ml each of the donor, helper, and recipient strains were combined in a 1.5-ml Eppendorf tube and centrifuged (10,000 x g, 1 min). Cells were washed once with 0.5 ml sterile saline (0.85% NaCl), then resuspended in 50 µl sterile saline and spotted onto an LB plate. After incubation for 6 h at 30°C, the resulting bacterial growth was scraped up with a sterile applicator and suspended in 1 ml of sterile saline. Various volumes of suspended cells were plated on LB with kanamycin and ampicillin to select for transconjugants.

6.2 Plasmid construction

Plasmids used are listed in each chapter. All restriction enzymes were purchased from NEB, and restriction digestions were carried out using standard procedures (Sambrook 1989). Vector DNA was incubated with calf intestinal phosphatase (NEB) for 1 h at 37°C to prevent re-ligation. After gel purification of vector and insert with the QiaQuick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions, ligation reactions were carried out at 4°C for at least one hour, using 5 µl of 2X ligation buffer from the PCR Cloning Kit (QIAGEN) and a total of 5 µl of insert and vector in various combinations. PCR reactions were carried out using the FailSafe PCR system (Epicentre), according to the manufacturer's instructions. Where necessary, genomic

DNA was extracted from 2 ml of overnight culture using the MasterPure DNA

Purification Kit (Epicentre), according to the manufacturer's instructions.

pILT. An IPTG-inducible LT holotoxin construct was generated by cloning the

coding sequence of *eltAB* from pPLT (Horstman and Kuehn 2002) using primers

eltABforward and eltABreverse (Table 11). These primers carry restriction sites for

Table 11: Primers used in these studies.

Nucleotide sequences of primers used for cloning and mutagenesis are listed, with restriction sites in italics and mutagenic codons in bold.

Name	Sequence (5'→ 3')
eltABforward	CG*GGATCC*CGCCTCGATGAAAAATATAAC
eltABreverse	AA*CTGCAG*AACCAATGCATTGGCTAGTTTCCATACTGATTG
dsbABamHIfwd	CG*GGATCC*CGGAAGAATTTAGCGAGTTC
DsbA-R(XbaI)	GC*TCTAGA*GCTCCCTGATAAAACAGCAA
eltb-bamh1	CG*GGATCC*CGGAATTCGGGATGAATTATGAATAAAG
epsC01	C*GGATCC*GAATTTAAACAACTTCCTC
epsD01	A*TCTAGA*TCGGTCATTGCTTGGCTTC
ctbF	GA*GGATCC*CGTAAGGATGAATTATGATTAAATTAAAAT
ctbR2	AA*CTGCAG*AGACTATCTATTGGCTAGTTTTCCATACTGATTG
ltbY18Asense	CACAACACACAAATA**GCC**ACGATAAATGACAAG
ltbY18Aantisense	CTTGTCATTTATCGT**GGC**TATTTGTGTGTTGTG
ltbQ3Lsense	CACACGGAGCTCCT**CTG**TCTATTACAGAACTATG
ltbQ3Lantisense	CATAGTTCTGTAATAGA**CAG**AGGAGCTCCGTGTG
ltbQ3Ksense	CACACGGAGCTCCT**AAA**TCTATTACAGAACTATG
ltbQ3Kantisense	CATAGTTCTGTAATAGA**TTT**AGGAGCTCCGTGTG
ltbT47Asense	GAGCGGCGCA**GCA**TTTCAGGTCGAAGTCCC
ltbT47Aantisense	GGGACTTCGACCTGAAA**TGC**TGCGCCGCTC
ltbA46Dsense	CATTACATTTAAGAGCGGC**GAC**ACATTTCAGGTCG
ltbA46Dantisense	CGACCTGAAATGT**GTC**GCCGCTCTTAAATGTAATG
ltbL25Esense	CGATAAATGACAAGATA**GAA**TCATATACGGAATCG
ltbL25Eantisense	CGATTCCGTATATGA**TTC**TATCTTGTCATTTATCG
ltbQ3Asense	CACACGGAGCTCCT**GCA**TCTATTACAGAACTATG
ltbQ3Aantisense	CATAGTTCTGTAATAGA**TGC**AGGAGCTCCGTGTG
ltbE11Ksense	CAGAACTATGTTCG**AAA**TATCACAACACAC
ltbE11Kantisense	GTGTGTTGTGATA**TTT**CGAACATAGTTCTG
ltbE11Asense	CAGAACTATGTTCG**GCA**TATCACAACACAC
ltbE11Aantisense	GTGTGTTGTGATA**TGC**CGAACATAGTTCTG
CTB_Q3K_sense	GGCGCATGCAACCCCT**AAA**AATATTACTGATTTG
CTB_Q3K_antisense	CAAATCAGTAATATT**TTT**AGGGGTTGCATGCGCC
CTBE11Ksense	CTGATTTGTGTGCA**AAA**TACCACAACACAC
CTBE11Kantisense	GTGTGTTGTGGTA**TTT**TGCACACAATCAG
CTB_E11A_sense	CTGATTTGTGTGCA**GCA**TACCACAACACAC
CTB_E11A_anti	GTGTGTTGTGGTA**TGC**TGCACACAAATCAG

BamHI and PstI, respectively. Gel purified PCR products were digested with BamHI and

PstI and then ligated into similarly digested pTrc99A (GE Healthcare) to form pILT.

pDsbA. A low-copy plasmid carrying the *dsbA* gene was generated by

amplification of *dsbA* from DH5α genomic DNA using primers dsbABamHIfwd and

DsbA-R(XbaI). These primers include restriction sites for BamHI and XbaI,

respectively. Gel-purified PCR products were digested with BamHI and XbaI and then

ligated into similarly digested pACYC184 vector to form pDsbA.

pILTB. An IPTG-inducible LTB plasmid was generated by cloning the coding

sequence of *eltB* from pILT. The gene was amplified by PCR using primers eltb-bamh1

and eltABreverse, which include restriction sites for BamHI and PstI, respectively. Gel-

purified PCR products were digested with BamHI and PstI and then ligated into similarly

digested pTrc99A vector to form pILTB.

pICTB. An IPTG-inducible CTB plasmid was generated by cloning the *ctxB*

gene (with the signal sequence from LT-IIB in place of the native sequence) from pLMP1

(Jobling et al. 1997). After digestion with SacI and KpnI, the digested fragment

containing *ctxB* was gel purified and ligated into similarly digested pTrc99A to form

pICTB.

pEpsCD. A low-copy plasmid carrying *epsCD* was generated by cloning the

coding sequence of *epsCD*, from the genomic DNA of O395N1 *V. cholerae*. After

amplification of *epsCD* using primers epsC01 and epsD01 (Lybarger et al. 2009), which

contain restriction sites for BamHI and XbaI, respectively, the PCR product was digested

with BamHI and XbaI and then ligated into similarly digested pACYC184 vector to form pEpsCD.

pnCTB. An inducible plasmid carrying *ctxB* with its native Sec signal sequence was generated by amplification of the gene from *V. cholerae* strain O395N1 genomic DNA using primers ctbF and ctbR2, which contain restriction sites for BamHI and PstI, respectively. After digestion with BamHI and PstI, gel-purified PCR product was ligated into similarly digested pTrc99A to form pnCTB.

6.3 Mutagenesis

Site-directed mutagenesis was performed on pILT, pILTB, and pICTB using the QuikChange kit (QIAGEN), according to the manufacturer's instructions. Primers used for mutagenesis are listed in Table 11. All mutations were verified by sequencing at the Duke University DNA Analysis Facility or Eton Biosciences (Durham, NC). Each mutant is 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.

Random mutagenesis was carried out on pILT using XL-1 Red cells (Stratagene), which possess a mutator phenotype, according to the manufacturer's instructions. LT mutants were screened for binding to G_{M1} using a 96-well ELISA (see below), and clones generating toxin with wild-type G_{M1} binding were sequenced to identify any introduced mutation(s) in *eltB*.

6.4 Toxin purification

Toxin was prepared from DH5α *degP*::Tn5 carrying pDsbA and pILT, pILTB, pICTB, or a mutant construct. Cells were diluted 1:50 from an overnight culture into CFA medium containing ampicillin and grown to an OD600 of approximately 0.8, when they were induced with 200 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sigma).

The next day, periplasm was released by osmotic shock essentially as described (Copeland et al. 1982). Six milliliters of 1 M Tris (pH 7.2) with 1 M NaCl was added to each 200 ml of cells, which were then pelleted (3,500 g, 10 min) and resuspended in 2 ml of 25% (v/v) LB in 1 M Tris with 1 M NaCl. After a brief spin (12,000 g, 1.5 min), cells were resuspended in 750 μl of 30 mM Tris with 2% EDTA. Then, 750 μl of 30 mM Tris with 2% EDTA and 40% sucrose was added, and the tubes were inverted several times. After a 15-min incubation at 25°C, cells were spun again (12,000 g, 1.5 min) and resuspended in 1 ml of ice-cold dH20, with considerable mixing. To each 1 ml sample, 10 μl of 10 mM MgCl2 was added, and the samples were incubated on ice for 5 min. Following the incubation, spheroplasts were pelleted (12,000 g, 3 min at 4°C), and the supernatant, representing the periplasmic fraction, was collected.

Each 1-ml sample of periplasm was incubated at 4°C overnight with 100 μl of 20% immobilized D-galactose beads (Pierce) in TEN buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 200 mM NaCl). For LT mutants E11K and E11A, phenylmethylsulfonyl fluoride (Sigma) or 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) were added at a final concentration of 1 mM during purification to prevent degradation. Beads were pelleted at

125

700 x g for 3 min, washed with TEN, and then resuspended in 1 ml 0.3 M galactose in TEN. Finally, beads were pelleted at 4,000 x g for 5 min, and soluble toxin was retained.

Purified toxin was concentrated in 10-kDa cutoff Ultra4 centrifugal filters (Amicon) and tested for protein concentration using the Coomassie Plus Better Bradford Reagent (Pierce), with bovine serum albumin (BSA) as a standard, according to the manufacturer's instructions. Purified toxin was assessed for purity by SDS-PAGE and protein staining with Ruby Red (Molecular Probes).

6.5 Tissue culture and toxicity bioassay

Y1 mouse adrenal cells (ATCC# CCL-79) were maintained in Kaighn's medium supplemented with 10% fetal bovine serum (Sigma) and penicillin, streptomycin, and fungizone (Sigma). Media was changed every two days (20 ml per 75 cm^2 flask), with cells split 1:5 each week.

A toxicity bioassay based on a morphological change in Y1 mouse adrenal cells was performed essentially as described previously (Donta et al. 1974; Horstman and Kuehn 2002). Y1 cells were washed once with phosphate-buffered saline (PBS) without Mg^{++} and Ca^{++} and then trypsinized for 5-10 min at 37°C. Afterward, cells were seeded into a 24-well plate (4 x 10^5 cells in 400 µl media per well) and left to adhere for 4 h at 37°C. Then, each well received 360 µl of fresh media, and samples were added in duplicate to two wells (in a volume of 40 µl/well). Wells were scored blindly for rounding 18 h later, receiving a score of 1 (<25% rounding), 2 (25-50% rounding), 3 (50-75% rounding), or 4 (>75% rounding).

126

6.7 SDS-PAGE and immunoblotting

6.7.1 Protein staining

Protein samples run on 15% polyacrylamide gels were stained with Ruby Red (Molecular Probes) according to the manufacturer's instructions. Ruby fix/wash solution (10% methanol, 7% acetic acid [v/v]) was used to fix the gel and to wash it after staining.

6.7.2 Immunoblotting

Immunoblotting was performed on protein samples run on 15% polyacrylamide gels essentially as described (Sambrook 1989; Horstman and Kuehn 2002). Proteins were transferred to polyvinyldifluoride membranes, which had been activated in methanol for 1 min, at approximately 10 V for 30 min. Membranes were blocked with Blotto (Tris-buffered saline with 0.1% Tween-20 [v/v], 30% skim milk [w/v], and 0.01% NaN_3) for 1 h at 25°C, then incubated with primary antibody diluted in Blotto overnight at 4°C. Anti-CT antibody (Sigma) was used at 1:15,000, and anti-LTA monoclonal antibody (Abcam) was used at 1:30,000. The next day, membranes were washed 3 times with TBS-T (Tris-buffered saline with 0.1% Tween-20) for 15 minutes each and then incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma), diluted in TBS-T, for 1 h at 25°C. Goat anti-mouse secondary was used at 1:40,000, and goat anti-rabbit secondary was used at 1:20,000. Following six 5-min washes with TBS-T, the membranes were incubated with SuperSignal ECL chemiluminescence reagent (Pierce) for 5 min and exposed to film for various lengths of time.

6.7.3 Densitometry

For some experiments, jpeg files of Ruby Red-stained SDS-PAGE gels or scanned immunoblot films were subjected to densitometry using ImageJ software (NIH Image). For each image, the file was converted to 8-bit grayscale, and background was subtracted using a rolling ball radius of 50. Afterward, a box was drawn around the band of interest, and the integrated density of that band was measured. All densitometry is reported as a percentage of wild-type band intensity, from a band on the same gel or blot. In each case, densitometry was performed in the linear range, at a short exposure time before the bands had become saturated.

6.8 Enzyme-linked immunosorbent assays (ELISAs)

G_{M1} **ELISA.** Purified G_{M1} (1 µg/ml in PBS, pH 7.2; Sigma) was added to 96-well medium-binding EIA/RIA plates (Corning) and adsorbed at least 1 h at 25°C. All other incubations were at 25°C for 1 h with volumes of 100 µl/well unless otherwise noted. After washing with PBS, 360 µl/well of Buffer A (20 mM Tris [pH 7.4], 0.15 M NaCl, 0.1% BSA, 5 mM $CaCl_2$) was added to block. After one wash with PBS, samples (diluted in Buffer A) were added. Purified toxins were tested at a concentration of 1 nM (corresponding to ~10 ng toxin per well); cell-free supernatant was diluted as indicated, and periplasm was diluted 1:20 after adjustment for alkaline phosphatase activity. Following three washes, the plate was incubated with rabbit anti-CT primary antibody (Sigma) at a 1:10,000 dilution in PBS. The plate was then washed three times with PBST (PBS with 0.05% Tween-20) and incubated with secondary (1:15,000 anti-rabbit IgG conjugated to horseradish peroxidase, Sigma). After three final PBST washes, OptEIA

substrate (BD) was added for 5 min. Stop solution (2N H_2SO_4) was added, and A_{410} was measured using a FluoSTAR Optima (BMG Labtech). Background absorbance from blank wells was subtracted from all other values.

Blood sugar ELISA. Blood group A terminal trisaccharide (GalNAcα3[Fucα2]Gal) conjugated to BSA (5 µg/ml in 0.1 M $NaHCO_3$, V-labs) was adsorbed to 96-well Polysorp plates (Nunc) at 25°C for at least 1 h. All other incubations were at 25°C for 1 h with volumes of 100 µl/well unless otherwise noted. After three washes with wash buffer (20 mM Tris, 140 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween-20 [v/v], pH 7.4), 360 µl/well of blocking/binding buffer (20 mM Tris, 140 mM NaCl, 5 mM CaCl2, 0.1% BSA [w/v], pH 7.4) was added. Wells were washed three times, and samples added. All samples consisted of purified toxin or pentamer at a final concentration of 1 nM in binding buffer. The remaining steps were the same as the G_{M1} ELISA, but wash buffer was used for all washes and antibodies were diluted in binding buffer. Blocking/binding buffer and wash buffer were made as previously described (Crouch et al. 2005).

LPS-DOPC liposome ELISA. Liposomes containing Ra LPS (Sigma) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) at a 1:10 ratio and liposomes containing DOPC alone (provided by D. Rodriguez) were adsorbed to Polysorp 96-well plates at 1 mg/well (Nunc) overnight at 37°C. After two washes with PBS, wells were blocked with 360 µl of Buffer A for 1 h at 25°C. Wells were then washed twice with PBS, and samples diluted in Buffer A were added for an incubation of 2 h. Purified toxins were tested at 100 nM. Subsequent antibody incubation steps and washes were carried out as

with the G_{M1} ELISA, except that one-step ABTS solution (Pierce) was used to visualize the bound toxin.

LPS-phosphatidylglycerol ELISA. Purified LPS was tested as an ELISA substrate following a previously published protocol, with slight modifications (Ramjeet et al. 2008). Ra LPS (Sigma) mixed at a 1:3 molar ratio with phosphatidylglycerol (Avanti) was adsorbed to Maxisorp 96-well plates (Nunc) overnight at 4°C, with 20 μg of LPS per well. All other steps were carried out at room temperature (25°C). Following three washes with PBST, wells were blocked with PBS + 2% BSA (w/v) for 1 h. Purified toxin was added at a final concentration of 15 ng/μl, diluted in the same buffer, and incubated for 1 h. Cross-reactive rabbit anti-CT antibody (at a 1:1000 dilution in PBS) was incubated for 1 h, HRP-conjugated anti-rabbit secondary was added for 1 h, and OptEIA substrate was added and allowed to develop for 5 min, with three PBST washes in between each step.

6.9 Cell surface binding assays

Immunoblot-based binding assay. Two milliliters of cells in mid-log phase (OD_{600} ~ 0.5-0.7) were pelleted and resuspended in ice-cold HEPES (50 mM, pH 6.8). Purified toxin was added to a final concentration of 500 nM (for binding to ETEC cells) or 100 nM (all other strains), with TEN buffer added to a final volume of 0.2 ml, and the mixture was incubated with shaking at 4°C for 1 h. Cells were pelleted, and supernatants containing unbound toxins were collected. Cell pellets were washed once, then resuspended in 0.2 ml HEPES. Resuspended cells were concentrated by precipitation with 20% trichloroacetic acid (TCA). Fractions containing unbound toxin were also

130

concentrated with TCA after the addition of 100 µg of BSA as a carrier. Equal volumes of precipitate resuspended in sample buffer were loaded on a 15% polyacrylamide gel and analyzed by immunoblotting with cross-reactive anti-CT as described above. The amount of toxin associated with the cells in each sample was quantified by densitometry of the LTB bands. Each assay was performed using a separate batch of purified toxin. For some experiments, Pronase (Fluka) was added to resuspended WBB01 cells at a final concentration of 0.1 mg/ml, and the mixture was incubated for 15 min at 37°C as previously described (Horstman and Kuehn 2002). Cells were washed three times with HEPES before the addition of toxin as described above.

For some experiments, toxin was pre-incubated for 1 h at 25°C with TEN buffer, BSA, or blood group A trisaccharide conjugated to BSA at 20-fold molar excess before addition to DH5α cells as described above.

Fluorescence-based binding assay. DH5α cells were incubated with buffer, wild-type LT, or T47A, washed, and resuspended identically to the binding assay described above. Then, 100 µl of 1 µM BODIPY-G_{M1} (Molecular Probes) diluted in methanol was added to each sample, and the mixtures were incubated for 30 min at 4°C. Following three washes with 50 mM HEPES with 0.1% Triton X-100 (pH 6.8), pellets were resuspended in 210 µl of HEPES and 100 µl was applied to each of two wells in a black polystyrene 96-well plate (Corning). Fluorescence was measured using a FluoSTAR Optima (excitation: 485 nm, emission: 520 nm). Values for cells incubated with buffer alone were subtracted from the other samples as background fluorescence and non-specific binding.

6.10 Cell fractionation

Strains were diluted 1:50 from overnight cultures, grown for 3-4 h at 37°C to an OD$_{600}$ 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% TCA for at least 1 h at 4°C and pelleted at 16,000 x g for 10 min at 4°C. Protein pellets were washed with acetone and resuspended in 1X sample buffer (50% 1 M Tris [pH 8.8], 25% 5X Laemmli buffer, 25% dH$_2$O). Each strain was serially plated for colony forming units, and the remaining cells were pelleted at 10,000 x g for 1 min. Supernatant was collected, and periplasm was isolated by polymyxin B treatment essentially as described (Horstman and Kuehn 2000). 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.

6.11 Alkaline phosphatase assay

An assay for alkaline phosphatase activity was performed using the SensoLyte alkaline phosphatase kit (AnaSpec), according to the manufacturer's instructions. For each periplasm sample, 50 μl was applied to each of two wells, undiluted. Samples were allowed to develop for 2 h at 25°C, and then A_{420} values were measured in a FluoSTAR Optima plate reader.

6.12 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 toxin-deficient strain of ETEC (MK1053) or *V. cholerae* (MK1123), which each carry empty pTrc99A 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_{M1} ELISA, with data normalized to the levels detected in the aliquot that was immediately frozen. At least two experiments were performed with separate batches of purified pentamer and fresh preparations of cell-free supernatant.

6.13 Protease 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, and data are normalized to the intensity of the untreated LTB or CTB band. At least two separate batches of purified toxin were tested.

6.14 In vitro *pentamer reassembly assay*

Wild-type and mutant LTB pentamers were assessed for reassembly *in vitro* essentially as previously described (Ruddock et al. 1996). 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, then neutralized with 80 µl of McIlvaine's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.0). 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 G_{M1} binding ELISA. Each trial was carried out with a separate batch of pentamers.

6.15 Growth curves

Cells were diluted 1:80 from an overnight culture into 4 ml of media containing appropriate antibiotics. Cultures were induced with 200 µM IPTG immediately after dilution (unless otherwise stated) and grown at 37°C. OD_{600} measurements were taken each hour with a Spectronic 200+ spectrophotometer (Thermo Scientific).

6.16 Determination of plasmid loss

Cells were grown as described for the growth curve. After 8 h of incubation, cultures were serially diluted and plated both on selective media, to determine the number of cells still carrying the plasmid in question (in all cases, this was LB with ampicillin), and media allowing for quantification of all cells present in the culture (LB for ETEC, LB with streptomycin for both *V. cholerae* strains). The number of CFUs on the ampicillin-containing plates was divided by the total number of CFUs to yield the percentage of plasmid-carrying cells.

6.17 Statistical analysis

Results were analyzed for significance using the unpaired Student's *t*-test for equal variance. Values of $p<0.05$ were considered significant. Mean values are reported, and error bars represent standard error of the mean.

References

Allen, K. P., M. M. Randolph and J. M. Fleckenstein (2006). "Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic Escherichia coli strains." <u>Infect Immun</u> 74(2): 869-75.

Alone, P. V., G. Malik, A. Krishnan and L. C. Garg (2007). "Deletion mutations in N-terminal alpha1 helix render heat labile enterotoxin B subunit susceptible to degradation." <u>Proc Natl Acad Sci U S A</u> 104(41): 16056-61.

Amor, K., D. E. Heinrichs, E. Frirdich, K. Ziebell, R. P. Johnson and C. Whitfield (2000). "Distribution of core oligosaccharide types in lipopolysaccharides from Escherichia coli." <u>Infect Immun</u> 68(3): 1116-24.

Balanzino, L. E., J. L. Barra, E. M. Galvan, G. A. Roth and C. G. Monferran (1999). "Interaction of cholera toxin and Escherichia coli heat-labile enterotoxin with glycoconjugates from rabbit intestinal brush border membranes: relationship with ABH blood group determinants." <u>Mol Cell Biochem</u> 194(1-2): 53-62.

Barra, J. L., C. G. Monferran, L. E. Balanzino and F. A. Cumar (1992). "Escherichia coli heat-labile enterotoxin preferentially interacts with blood group A-active glycolipids from pig intestinal mucosa and A- and B-active glycolipids from human red cells compared to H-active glycolipids." <u>Mol Cell Biochem</u> 115(1): 63-70.

Berberov, E. M., Y. Zhou, D. H. Francis, M. A. Scott, S. D. Kachman and R. A. Moxley (2004). "Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic Escherichia coli that produces multiple enterotoxins." <u>Infect Immun</u> 72(7): 3914-24.

Blanco, J., M. Blanco, J. I. Garabal and E. A. Gonzalez (1991). "Enterotoxins, colonization factors and serotypes of enterotoxigenic Escherichia coli from humans and animals." <u>Microbiologia</u> 7(2): 57-73.

Bouley, J., G. Condemine and V. E. Shevchik (2001). "The PDZ domain of OutC and the N-terminal region of OutD determine the secretion specificity of the type II out pathway of Erwinia chrysanthemi." <u>J Mol Biol</u> 308(2): 205-19.

Brabetz, W., S. Muller-Loennies and H. Brade (2000). "3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo) transferase (WaaA) and kdo kinase (KdkA) of Haemophilus influenzae are both required to complement a waaA knockout mutation of Escherichia coli." <u>J Biol Chem</u> 275(45): 34954-62.

Brown, E. A. and P. R. Hardwidge (2007). "Biochemical characterization of the enterotoxigenic Escherichia coli LeoA protein." <u>Microbiology</u> 153(Pt 11): 3776-84.

Camberg, J. L., T. L. Johnson, M. Patrick, J. Abendroth, W. G. Hol and M. Sandkvist (2007). "Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids." <u>EMBO J</u> 26(1): 19-27.

Chapon, V., H. D. Simpson, X. Morelli, E. Brun and F. Barras (2000). "Alteration of a single tryptophan residue of the cellulose-binding domain blocks secretion of the Erwinia chrysanthemi Cel5 cellulase (ex-EGZ) via the type II system." <u>J Mol Biol</u> 303(2): 117-23.

Cheesman, C., R. B. Freedman and L. W. Ruddock (2004). "The disassembly and reassembly of mutants of Escherichia coli heat-labile enterotoxin: replacement of proline 93 does not abolish the reassembly-competent and reassembly-incompetent states." <u>Biochemistry</u> 43(6): 1618-25.

Childers, B. M. and K. E. Klose (2007). "Regulation of virulence in Vibrio cholerae: the ToxR regulon." Future Microbiol 2: 335-44.

Chung, W. Y., R. Carter, T. Hardy, M. Sack, T. R. Hirst and R. F. James (2006). "Inhibition of Escherichia coli heat-labile enterotoxin B subunit pentamer (EtxB5) assembly in vitro using monoclonal antibodies." J Biol Chem 281(51): 39465-70.

Cianciotto, N. P. (2005). "Type II secretion: a protein secretion system for all seasons." Trends Microbiol 13(12): 581-8.

Clements, J. D. and R. A. Finkelstein (1979). "Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from Escherichia coli cultures." Infect Immun 24(3): 760-9.

Connell, T. D., D. J. Metzger, M. Wang, M. G. Jobling and R. K. Holmes (1995). "Initial studies of the structural signal for extracellular transport of cholera toxin and other proteins recognized by Vibrio cholerae." Infect Immun 63(10): 4091-8.

Copeland, B. R., R. J. Richter and C. E. Furlong (1982). "Renaturation and identification of periplasmic proteins in two-dimensional gels of Escherichia coli." J Biol Chem 257(24): 15065-71.

Coster, T. S., M. K. Wolf, E. R. Hall, F. J. Cassels, D. N. Taylor, C. T. Liu, F. C. Trespalacios, A. DeLorimier, D. R. Angleberger and C. E. McQueen (2007). "Immune response, ciprofloxacin activity, and gender differences after human experimental challenge by two strains of enterotoxigenic Escherichia coli." Infect Immun 75(1): 252-9.

Cox, A. D., J. R. Brisson, V. Varma and M. B. Perry (1996). "Structural analysis of the lipopolysaccharide from Vibrio cholerae O139." Carbohydr Res 290(1): 43-58.

Crouch, E., Y. Tu, D. Briner, B. McDonald, K. Smith, U. Holmskov and K. Hartshorn (2005). "Ligand specificity of human surfactant protein D: expression of a mutant trimeric collectin that shows enhanced interactions with influenza A virus." J Biol Chem 280(17): 17046-56.

Cvjetanovic, B. and D. Barua (1972). "The seventh pandemic of cholera." Nature 239(5368): 137-8.

D'Adamo, P. J. and G. S. Kelly (2001). "Metabolic and immunologic consequences of ABH secretor and Lewis subtype status." Altern Med Rev 6(4): 390-405.

Dallas, W. S. and S. Falkow (1980). "Amino acid sequence homology between cholera toxin and Escherichia coli heat-labile toxin." Nature 288(5790): 499-501.

Deane, J. E., P. Abrusci, S. Johnson and S. M. Lea (2009). "Timing is everything: the regulation of type III secretion." Cell Mol Life Sci.

Donta, S. T., H. W. Moon and S. C. Whipp (1974). "Detection of heat-labile Escherichia coli enterotoxin with the use of adrenal cells in tissue culture." Science 183(4122): 334-6.

Dorsey, F. C., J. F. Fischer and J. M. Fleckenstein (2006). "Directed delivery of heat-labile enterotoxin by enterotoxigenic Escherichia coli." Cell Microbiol 8(9): 1516-27.

Douzi, B., E. Durand, C. Bernard, S. Alphonse, C. Cambillau, A. Filloux, M. Tegoni and R. Voulhoux (2009). "The XcpV/GspI pseudopilin has a central role in the assembly of a quaternary complex within the T2SS pseudopilus." J Biol Chem 284(50): 34580-9.

Durand, E., G. Michel, R. Voulhoux, J. Kurner, A. Bernadac and A. Filloux (2005). "XcpX controls biogenesis of the Pseudomonas aeruginosa XcpT-containing pseudopilus." J Biol Chem 280(36): 31378-89.

Esko, J. D., T. L. Doering and C. R. Raetz, Eds. (2009). Essentials of Glycobiology. Cold Spring Harbor, NY, Cold Spring Harbor Press.

Finan, T. M., B. Kunkel, G. F. De Vos and E. R. Signer (1986). "Second symbiotic megaplasmid in Rhizobium meliloti carrying exopolysaccharide and thiamine synthesis genes." J Bacteriol 167(1): 66-72.

Finne, J., M. E. Breimer, G. C. Hansson, K. A. Karlsson, H. Leffler, J. F. Vliegenthart and H. van Halbeek (1989). "Novel polyfucosylated N-linked glycopeptides with blood group A, H, X, and Y determinants from human small intestinal epithelial cells." J Biol Chem 264(10): 5720-35.

Fleckenstein, J. M., P. R. Hardwidge, G. P. Munson, D. A. Rasko, H. Sommerfelt and H. Steinsland (2009). "Molecular Mechanisms of Enterotoxigenic Escherichia coli Infection." Microbes Infect.

Fleckenstein, J. M., D. J. Kopecko, R. L. Warren and E. A. Elsinghorst (1996). "Molecular characterization of the tia invasion locus from enterotoxigenic Escherichia coli." Infect Immun 64(6): 2256-65.

Francetic, O., D. Belin, C. Badaut and A. P. Pugsley (2000). "Expression of the endogenous type II secretion pathway in Escherichia coli leads to chitinase secretion." EMBO J 19(24): 6697-703.

Francetic, O., S. Lory and A. P. Pugsley (1998). "A second prepilin peptidase gene in Escherichia coli K-12." Mol Microbiol 27(4): 763-75.

Francetic, O. and A. P. Pugsley (2005). "Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from Klebsiella oxytoca." J Bacteriol 187(20): 7045-55.

Frirdich, E., B. Lindner, O. Holst and C. Whitfield (2003). "Overexpression of the waaZ gene leads to modification of the structure of the inner core region of Escherichia coli lipopolysaccharide, truncation of the outer core, and reduction of the amount of O polysaccharide on the cell surface." J Bacteriol 185(5): 1659-71.

Fukuta, S., J. L. Magnani, E. M. Twiddy, R. K. Holmes and V. Ginsburg (1988). "Comparison of the carbohydrate-binding specificities of cholera toxin and Escherichia coli heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb." Infect Immun 56(7): 1748-53.

Fullner, K. J., W. I. Lencer and J. J. Mekalanos (2001). "Vibrio cholerae-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin." Infect Immun 69(10): 6310-7.

Galvan, E. M., C. D. Diema, G. A. Roth and C. G. Monferran (2004). "Ability of blood group A-active glycosphingolipids to act as Escherichia coli heat-labile enterotoxin receptors in HT-29 cells." J Infect Dis 189(9): 1556-64.

Galvan, E. M., G. A. Roth and C. G. Monferran (2006). "Functional interaction of Escherichia coli heat-labile enterotoxin with blood group A-active glycoconjugates from differentiated HT29 cells." FEBS J 273(15): 3444-53.

Gennaro, M. L., P. J. Greenaway and D. A. Broadbent (1982). "The expression of biologically active cholera toxin in Escherichia coli." Nucleic Acids Res 10(16): 4883-90.

Gerard-Vincent, M., V. Robert, G. Ball, S. Bleves, G. P. Michel, A. Lazdunski and A. Filloux (2002). "Identification of XcpP domains that confer functionality and specificity to the Pseudomonas aeruginosa type II secretion apparatus." Mol Microbiol 44(6): 1651-65.

Gill, D. M., J. D. Clements, D. C. Robertson and R. A. Finkelstein (1981). "Subunit number and arrangement in Escherichia coli heat-labile enterotoxin." Infect Immun 33(3): 677-82.

Goel, A. K., M. Jain, P. Kumar, S. Bhadauria, D. V. Kmboj and L. Singh (2008). "A new variant of Vibrio cholerae O1 El Tor causing cholera in India." J Infect 57(3): 280-1.

Goldberg, J. B. and D. E. Ohman (1984). "Cloning and expression in Pseudomonas aeruginosa of a gene involved in the production of alginate." J Bacteriol 158(3): 1115-21.

Gomez-Duarte, O. G., A. Ruiz-Tagle, D. C. Gomez, G. I. Viboud, K. G. Jarvis, J. B. Kaper and J. A. Giron (1999). "Identification of lngA, the structural gene of longus type IV pilus of enterotoxigenic Escherichia coli." Microbiology 145 (Pt 7): 1809-16.

Grant, C. C., R. J. Messer and W. Cieplak, Jr. (1994). "Role of trypsin-like cleavage at arginine 192 in the enzymatic and cytotonic activities of Escherichia coli heat-labile enterotoxin." Infect Immun 62(10): 4270-8.

Guidry, J. J., L. Cardenas, E. Cheng and J. D. Clements (1997). "Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of Escherichia coli heat-labile enterotoxin." Infect Immun 65(12): 4943-50.

Guilvout, I., M. Chami, A. Engel, A. P. Pugsley and N. Bayan (2006). "Bacterial outer membrane secretin PulD assembles and inserts into the inner membrane in the absence of its pilotin." EMBO J 25(22): 5241-9.

Hardy, S. J. and P. A. Hedges (1996). "Reduced B subunit of heat-labile enterotoxin associates with membranes in vivo." Eur J Biochem 236(2): 412-8.

Hardy, S. J., J. Holmgren, S. Johansson, J. Sanchez and T. R. Hirst (1988). "Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins in vivo and in vitro." Proc Natl Acad Sci U S A 85(19): 7109-13.

Harris, J. B., A. I. Khan, R. C. LaRocque, D. J. Dorer, F. Chowdhury, A. S. Faruque, D. A. Sack, E. T. Ryan, F. Qadri and S. B. Calderwood (2005). "Blood group, immunity, and risk of infection with Vibrio cholerae in an area of endemicity." Infect Immun 73(11): 7422-7.

Heinrichs, D. E., J. A. Yethon, P. A. Amor and C. Whitfield (1998). "The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of Escherichia coli. The R1 core-specific beta-glucosyltransferase provides a novel attachment site for O-polysaccharides." J Biol Chem 273(45): 29497-505.

Henderson, I. R., J. P. Nataro, J. B. Kaper, T. F. Meyer, S. K. Farrand, D. L. Burns, B. B. Finlay and J. W. St Geme, 3rd (2000). "Renaming protein secretion in the gram-negative bacteria." Trends Microbiol 8(8): 352.

Hirst, T. R. and J. Holmgren (1987). "Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from Vibrio cholerae." Proc Natl Acad Sci U S A 84(21): 7418-22.

Hirst, T. R. and J. Holmgren (1987). "Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from Vibrio cholerae." J Bacteriol 169(3): 1037-45.

Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. Hardy and J. Holmgren (1984). "Mechanism of toxin secretion by Vibrio cholerae investigated in strains harboring plasmids that encode heat-labile enterotoxins of Escherichia coli." Proc Natl Acad Sci U S A 81(24): 7752-6.

Hofstra, H. and B. Witholt (1985). "Heat-labile enterotoxin in Escherichia coli. Kinetics of association of subunits into periplasmic holotoxin." J Biol Chem 260(29): 16037-44.

Holmner, A., G. Askarieh, M. Okvist and U. Krengel (2007). "Blood group antigen recognition by Escherichia coli heat-labile enterotoxin." J Mol Biol 371(3): 754-64.

Holmner, A., M. Lebens, S. Teneberg, J. Angstrom, M. Okvist and U. Krengel (2004). "Novel binding site identified in a hybrid between cholera toxin and heat-labile enterotoxin: 1.9 A crystal structure reveals the details." Structure 12(9): 1655-67.

Horstman, A. L., S. J. Bauman and M. J. Kuehn (2004). "Lipopolysaccharide 3-deoxy-D-manno-octulosonic acid (Kdo) core determines bacterial association of secreted toxins." J Biol Chem 279(9): 8070-5.

Horstman, A. L. and M. J. Kuehn (2000). "Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles." J Biol Chem 275(17): 12489-96.

Horstman, A. L. and M. J. Kuehn (2002). "Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway." J Biol Chem 277(36): 32538-45.

Huang, D. B., H. L. DuPont, Z. D. Jiang, L. Carlin and P. C. Okhuysen (2004). "Interleukin-8 response in an intestinal HCT-8 cell line infected with enteroaggregative and enterotoxigenic Escherichia coli." Clin Diagn Lab Immunol 11(3): 548-51.

Iida, T., T. Tsuji, T. Honda, T. Miwatani, S. Wakabayashi, K. Wada and H. Matsubara (1989). "A single amino acid substitution in B subunit of Escherichia coli enterotoxin affects its oligomer formation." J Biol Chem 264(24): 14065-70.

Jansson, L., J. Angstrom, M. Lebens and S. Teneberg (2009). "No direct binding of the heat-labile enterotoxin of Escherichia coli to E. coli lipopolysaccharides." Glycoconj J.

Jansson, L., J. Tobias, M. Lebens, A. M. Svennerholm and S. Teneberg (2006). "The major subunit, CfaB, of colonization factor antigen i from enterotoxigenic Escherichia coli is a glycosphingolipid binding protein." Infect Immun 74(6): 3488-97.

Jobling, M. G., L. M. Palmer, J. L. Erbe and R. K. Holmes (1997). "Construction and characterization of versatile cloning vectors for efficient delivery of native foreign proteins to the periplasm of Escherichia coli." Plasmid 38(3): 158-73.

Johnson, A. M., R. S. Kaushik, D. H. Francis, J. M. Fleckenstein and P. R. Hardwidge (2009). "Heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells." J Bacteriol 191(1): 178-86.

Johnson, T. J. and L. K. Nolan (2009). "Pathogenomics of the virulence plasmids of Escherichia coli." Microbiol Mol Biol Rev 73(4): 750-74.

Johnson, T. L., J. Abendroth, W. G. Hol and M. Sandkvist (2006). "Type II secretion: from structure to function." FEMS Microbiol Lett 255(2): 175-86.

Johnson, T. L., M. E. Scott and M. Sandkvist (2007). "Mapping critical interactive sites within the periplasmic domain of the Vibrio cholerae type II secretion protein EpsM." J Bacteriol 189(24): 9082-9.

Kajava, A. V., S. N. Zolov, A. E. Kalinin and M. A. Nesmeyanova (2000). "The net charge of the first 18 residues of the mature sequence affects protein translocation across the cytoplasmic membrane of gram-negative bacteria." J Bacteriol 182(8): 2163-9.

Karaolis, D. K., R. Lan, J. B. Kaper and P. R. Reeves (2001). "Comparison of Vibrio cholerae pathogenicity islands in sixth and seventh pandemic strains." Infect Immun 69(3): 1947-52.

Kesty, N. C., K. M. Mason, M. Reedy, S. E. Miller and M. J. Kuehn (2004). "Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells." EMBO J 23(23): 4538-49.

Kirn, T. J., N. Bose and R. K. Taylor (2003). "Secretion of a soluble colonization factor by the TCP type 4 pilus biogenesis pathway in Vibrio cholerae." Mol Microbiol 49(1): 81-92.

Kornacker, M. G., D. Faucher and A. P. Pugsley (1991). "Outer membrane translocation of the extracellular enzyme pullulanase in Escherichia coli K12 does not require a fatty acylated N-terminal cysteine." J Biol Chem 266(21): 13842-8.

Korotkov, K. V., M. D. Gray, A. Kreger, S. Turley, M. Sandkvist and W. G. Hol (2009). "Calcium is essential for the major pseudopilin in the type 2 secretion system." J Biol Chem 284(38): 25466-70.

Korotkov, K. V. and W. G. Hol (2008). "Structure of the GspK-GspI-GspJ complex from the enterotoxigenic Escherichia coli type 2 secretion system." Nat Struct Mol Biol 15(5): 462-8.

Korotkov, K. V., B. Krumm, M. Bagdasarian and W. G. Hol (2006). "Structural and functional studies of EpsC, a crucial component of the type 2 secretion system from Vibrio cholerae." J Mol Biol 363(2): 311-21.

Kuehn, M. J. and N. C. Kesty (2005). "Bacterial outer membrane vesicles and the host-pathogen interaction." Genes Dev 19(22): 2645-55.

Lee, M. S., L. Y. Chen, W. M. Leu, R. J. Shiau and N. T. Hu (2005). "Associations of the major pseudopilin XpsG with XpsN (GspC) and secretin XpsD of Xanthomonas campestris pv. campestris type II secretion apparatus revealed by cross-linking analysis." J Biol Chem 280(6): 4585-91.

Lee, V. T. and O. Schneewind (2001). "Protein secretion and the pathogenesis of bacterial infections." Genes Dev 15(14): 1725-52.

Lencer, W. I., T. R. Hirst and R. K. Holmes (1999). "Membrane traffic and the cellular uptake of cholera toxin." Biochim Biophys Acta 1450(3): 177-90.

Li, P., J. Beckwith and H. Inouye (1988). "Alteration of the amino terminus of the mature sequence of a periplasmic protein can severely affect protein export in Escherichia coli." Proc Natl Acad Sci U S A 85(20): 7685-9.

Lindeberg, M., G. P. Salmond and A. Collmer (1996). "Complementation of deletion mutations in a cloned functional cluster of Erwinia chrysanthemi out genes with Erwinia carotovora out homologues reveals OutC and OutD as candidate gatekeepers of species-specific secretion of proteins via the type II pathway." Mol Microbiol 20(1): 175-90.

Lindenthal, C. and E. A. Elsinghorst (2001). "Enterotoxigenic Escherichia coli TibA glycoprotein adheres to human intestine epithelial cells." Infect Immun 69(1): 52-7.

Lu, H. M., S. Mizushima and S. Lory (1993). "A periplasmic intermediate in the extracellular secretion pathway of Pseudomonas aeruginosa exotoxin A." J Bacteriol 175(22): 7463-7.

Lybarger, S. R., T. L. Johnson, M. D. Gray, A. E. Sikora and M. Sandkvist (2009). "Docking and assembly of the type II secretion complex of Vibrio cholerae." J Bacteriol 191(9): 3149-61.

MacKenzie, C. R., T. Hirama, K. K. Lee, E. Altman and N. M. Young (1997). "Quantitative analysis of bacterial toxin affinity and specificity for glycolipid receptors by surface plasmon resonance." J Biol Chem 272(9): 5533-8.

Madigan, M. T. and T. D. Brock (2009). Brock biology of microorganisms. San Francisco, CA, Pearson/Benjamin Cummings.

Marraffini, L. A., A. C. Dedent and O. Schneewind (2006). "Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria." <u>Microbiol Mol Biol Rev</u> 70(1): 192-221.

Marsh, J. W. and R. K. Taylor (1998). "Identification of the Vibrio cholerae type 4 prepilin peptidase required for cholera toxin secretion and pilus formation." <u>Mol Microbiol</u> 29(6): 1481-92.

Mazar, J. and P. A. Cotter (2007). "New insight into the molecular mechanisms of two-partner secretion." <u>Trends Microbiol</u> 15(11): 508-15.

McBroom, A. J., A. P. Johnson, S. Vemulapalli and M. J. Kuehn (2006). "Outer membrane vesicle production by Escherichia coli is independent of membrane instability." <u>J Bacteriol</u> 188(15): 5385-92.

McVay, C. S. and A. N. Hamood (1995). "Toxin A secretion in Pseudomonas aeruginosa: the role of the first 30 amino acids of the mature toxin." <u>Mol Gen Genet</u> 249(5): 515-25.

Mekalanos, J. J. (1983). "Duplication and amplification of toxin genes in Vibrio cholerae." <u>Cell</u> 35(1): 253-63.

Merritt, E. A., T. K. Sixma, K. H. Kalk, B. A. van Zanten and W. G. Hol (1994). "Galactose-binding site in Escherichia coli heat-labile enterotoxin (LT) and cholera toxin (CT)." <u>Mol Microbiol</u> 13(4): 745-53.

Michel, G. P., E. Durand and A. Filloux (2007). "XphA/XqhA, a novel GspCD subunit for type II secretion in Pseudomonas aeruginosa." <u>J Bacteriol</u> 189(10): 3776-83.

Moss, J. and S. H. Richardson (1978). "Activation of adenylate cyclase by heat-labile Escherichia coli enterotoxin. Evidence for ADP-ribosyltransferase activity similar to that of choleragen." <u>J Clin Invest</u> 62(2): 281-5.

Muller, C. A., I. B. Autenrieth and A. Peschel (2005). "Innate defenses of the intestinal epithelial barrier." <u>Cell Mol Life Sci</u> 62(12): 1297-307.

Murley, Y. M., J. Behari, R. Griffin and S. B. Calderwood (2000). "Classical and El Tor biotypes of Vibrio cholerae differ in timing of transcription of tcpPH during growth in inducing conditions." <u>Infect Immun</u> 68(5): 3010-4.

Neidhardt, F. C., Ed. (1996). <u>Escherichia coli and Salmonella</u>. Washington, D.C., ASM Press.

Neill, R. J., B. E. Ivins and R. K. Holmes (1983). "Synthesis and secretion of the plasmid-coded heat-labile enterotoxin of Escherichia coli in Vibrio cholerae." <u>Science</u> 221(4607): 289-91.

Nikaido, H. (2003). "Molecular basis of bacterial outer membrane permeability revisited." <u>Microbiol Mol Biol Rev</u> 67(4): 593-656.

Okamoto, K., T. Nomura, Y. Fujii and H. Yamanaka (1998). "Contribution of the disulfide bond of the A subunit to the action of Escherichia coli heat-labile enterotoxin." <u>J Bacteriol</u> 180(6): 1368-74.

Olivier, V., G. K. Haines, 3rd, Y. Tan and K. J. Satchell (2007). "Hemolysin and the multifunctional autoprocessing RTX toxin are virulence factors during intestinal infection of mice with Vibrio cholerae El Tor O1 strains." <u>Infect Immun</u> 75(10): 5035-42.

Palomaki, T., R. Pickersgill, R. Riekki, M. Romantschuk and H. T. Saarilahti (2002). "A putative three-dimensional targeting motif of polygalacturonase (PehA), a protein secreted through the type II (GSP) pathway in Erwinia carotovora." <u>Mol Microbiol</u> 43(3): 585-96.

Palomaki, T. and H. T. Saarilahti (1995). "The extreme C-terminus is required for secretion of both the native polygalacturonase (PehA) and PehA-Bla hybrid proteins in Erwinia carotovora subsp. carotovora." <u>Mol Microbiol</u> 17(3): 449-59.

Patel, S. K., J. Dotson, K. P. Allen and J. M. Fleckenstein (2004). "Identification and molecular characterization of EatA, an autotransporter protein of enterotoxigenic Escherichia coli." Infect Immun 72(3): 1786-94.

Peterson, K. M. (2002). "Expression of Vibrio cholerae virulence genes in response to environmental signals." Curr Issues Intest Microbiol 3(2): 29-38.

Pickett, C. L., E. M. Twiddy, C. Coker and R. K. Holmes (1989). "Cloning, nucleotide sequence, and hybridization studies of the type IIb heat-labile enterotoxin gene of Escherichia coli." J Bacteriol 171(9): 4945-52.

Pierce, N. F., J. B. Kaper, J. J. Mekalanos and W. C. Cray, Jr. (1985). "Role of cholera toxin in enteric colonization by Vibrio cholerae O1 in rabbits." Infect Immun 50(3): 813-6.

Pohlner, J., R. Halter, K. Beyreuther and T. F. Meyer (1987). "Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease." Nature 325(6103): 458-62.

Popoff, M. R. (1998). "Interactions between bacterial toxins and intestinal cells." Toxicon 36(4): 665-85.

Possot, O. M., M. Gerard-Vincent and A. P. Pugsley (1999). "Membrane association and multimerization of secreton component pulC." J Bacteriol 181(13): 4004-11.

Pukatzki, S., S. B. McAuley and S. T. Miyata (2009). "The type VI secretion system: translocation of effectors and effector-domains." Curr Opin Microbiol 12(1): 11-7.

Qadri, F., A. Saha, T. Ahmed, A. Al Tarique, Y. A. Begum and A. M. Svennerholm (2007). "Disease burden due to enterotoxigenic Escherichia coli in the first 2 years of life in an urban community in Bangladesh." Infect Immun 75(8): 3961-8.

Qadri, F., A. M. Svennerholm, A. S. Faruque and R. B. Sack (2005). "Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention." Clin Microbiol Rev 18(3): 465-83.

Raetz, C. R. (1993). "Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction." J Bacteriol 175(18): 5745-53.

Raetz, C. R. and C. Whitfield (2002). "Lipopolysaccharide endotoxins." Annu Rev Biochem 71: 635-700.

Ramjeet, M., A. D. Cox, M. A. Hancock, M. Mourez, J. Labrie, M. Gottschalk and M. Jacques (2008). "Mutation in the LPS outer core biosynthesis gene, galU, affects LPS interaction with the RTX toxins ApxI and ApxII and cytolytic activity of Actinobacillus pleuropneumoniae serotype 1." Mol Microbiol 70(1): 221-35.

Roy, K., G. M. Hilliard, D. J. Hamilton, J. Luo, M. M. Ostmann and J. M. Fleckenstein (2009). "Enterotoxigenic Escherichia coli EtpA mediates adhesion between flagella and host cells." Nature 457(7229): 594-8.

Ruddock, L. W., J. J. Coen, C. Cheesman, R. B. Freedman and T. R. Hirst (1996). "Assembly of the B subunit pentamer of Escherichia coli heat-labile enterotoxin. Kinetics and molecular basis of rate-limiting steps in vitro." J Biol Chem 271(32): 19118-23.

Russel, M. (1998). "Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems." J Mol Biol 279(3): 485-99.

Sambrook, J., E.F. Fritsch, and T. Maniatis (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Press.

Sanchez, J. and J. Holmgren (1989). "Recombinant system for overexpression of cholera toxin B subunit in Vibrio cholerae as a basis for vaccine development." Proc Natl Acad Sci U S A 86(2): 481-5.

Sanchez, J. and J. Holmgren (2005). "Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea." Curr Opin Immunol 17(4): 388-98.

Sanchez, J., G. Medina, T. Buhse, J. Holmgren and G. Soberon-Chavez (2004). "Expression of cholera toxin under non-AKI conditions in Vibrio cholerae El Tor induced by increasing the exposed surface of cultures." J Bacteriol 186(5): 1355-61.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey and V. J. DiRita (1997). "General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae." J Bacteriol 179(22): 6994-7003.

Scott, M. E., Z. Y. Dossani and M. Sandkvist (2001). "Directed polar secretion of protease from single cells of Vibrio cholerae via the type II secretion pathway." Proc Natl Acad Sci U S A 98(24): 13978-83.

Shi, L., S. Deng, M. J. Marshall, Z. Wang, D. W. Kennedy, A. C. Dohnalkova, H. M. Mottaz, E. A. Hill, Y. A. Gorby, A. S. Beliaev, D. J. Richardson, J. M. Zachara and J. K. Fredrickson (2008). "Direct involvement of type II secretion system in extracellular translocation of Shewanella oneidensis outer membrane cytochromes MtrC and OmcA." J Bacteriol 190(15): 5512-6.

Sikora, A. E., S. R. Lybarger and M. Sandkvist (2007). "Compromised outer membrane integrity in Vibrio cholerae Type II secretion mutants." J Bacteriol 189(23): 8484-95.

Silva, A., R. Fando and J. A. Benitez (1998). "Overexpression of a mutant B subunit in toxigenic Vibrio cholerae diminishes production of active cholera toxin in vivo." Curr Microbiol 37(4): 231-5.

Sixma, T. K., K. H. Kalk, B. A. van Zanten, Z. Dauter, J. Kingma, B. Witholt and W. G. Hol (1993). "Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin." J Mol Biol 230(3): 890-918.

Sixma, T. K., S. E. Pronk, K. H. Kalk, E. S. Wartna, B. A. van Zanten, B. Witholt and W. G. Hol (1991). "Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli." Nature 351(6325): 371-7.

Sjoling, A., F. Qadri, M. Nicklasson, Y. A. Begum, G. Wiklund and A. M. Svennerholm (2006). "In vivo expression of the heat stable (estA) and heat labile (eltB) toxin genes of enterotoxigenic Escherichia coli (ETEC)." Microbes Infect 8(12-13): 2797-802.

Spangler, B. D. (1992). "Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin." Microbiol Rev 56(4): 622-47.

Stathopoulos, C., D. R. Hendrixson, D. G. Thanassi, S. J. Hultgren, J. W. St Geme, 3rd and R. Curtiss, 3rd (2000). "Secretion of virulence determinants by the general secretory pathway in gram-negative pathogens: an evolving story." Microbes Infect 2(9): 1061-72.

Steinhoff, U. (2005). "Who controls the crowd? New findings and old questions about the intestinal microflora." Immunol Lett 99(1): 12-6.

Stewart, C. R., O. Rossier and N. P. Cianciotto (2009). "Surface translocation by Legionella pneumophila: a form of sliding motility that is dependent upon type II protein secretion." J Bacteriol 191(5): 1537-46.

Strom, M. S., D. Nunn and S. Lory (1991). "Multiple roles of the pilus biogenesis protein pilD: involvement of pilD in excretion of enzymes from Pseudomonas aeruginosa." J Bacteriol 173(3): 1175-80.

Svennerholm, A. M. and J. Tobias (2008). "Vaccines against enterotoxigenic Escherichia coli." Expert Rev Vaccines 7(6): 795-804.

Tauschek, M., R. J. Gorrell, R. A. Strugnell and R. M. Robins-Browne (2002). "Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of Escherichia coli." Proc Natl Acad Sci U S A 99(10): 7066-71.

Teneberg, S., T. R. Hirst, J. Angstrom and K. A. Karlsson (1994). "Comparison of the glycolipid-binding specificities of cholera toxin and porcine Escherichia coli heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine." Glycoconj J 11(6): 533-40.

Thompson, M. R. and R. A. Giannella (1985). "Revised amino acid sequence for a heat-stable enterotoxin produced by an Escherichia coli strain (18D) that is pathogenic for humans." Infect Immun 47(3): 834-6.

Tsuji, T., T. Honda, T. Miwatani, S. Wakabayashi and H. Matsubara (1985). "Analysis of receptor-binding site in Escherichia coli enterotoxin." J Biol Chem 260(14): 8552-8.

Turner, S. M., R. R. Chaudhuri, Z. D. Jiang, H. DuPont, C. Gyles, C. W. Penn, M. J. Pallen and I. R. Henderson (2006). "Phylogenetic comparisons reveal multiple acquisitions of the toxin genes by enterotoxigenic Escherichia coli strains of different evolutionary lineages." J Clin Microbiol 44(12): 4528-36.

Turner, S. M., A. Scott-Tucker, L. M. Cooper and I. R. Henderson (2006). "Weapons of mass destruction: virulence factors of the global killer enterotoxigenic Escherichia coli." FEMS Microbiol Lett 263(1): 10-20.

Vaitkevicius, K., B. Lindmark, G. Ou, T. Song, C. Toma, M. Iwanaga, J. Zhu, A. Andersson, M. L. Hammarstrom, S. Tuck and S. N. Wai (2006). "A Vibrio cholerae protease needed for killing of Caenorhabditis elegans has a role in protection from natural predator grazing." Proc Natl Acad Sci U S A 103(24): 9280-5.

Veenendaal, A. K., C. van der Does and A. J. Driessen (2004). "The protein-conducting channel SecYEG." Biochim Biophys Acta 1694(1-3): 81-95.

Vicente, A. C., L. F. Teixeira, L. Iniguez-Rojas, M. G. Luna, L. Silva, J. R. Andrade and B. E. Guth (2005). "Outbreaks of cholera-like diarrhoea caused by enterotoxigenic Escherichia coli in the Brazilian Amazon Rainforest." Trans R Soc Trop Med Hyg 99(9): 669-74.

Voulhoux, R., G. Ball, B. Ize, M. L. Vasil, A. Lazdunski, L. F. Wu and A. Filloux (2001). "Involvement of the twin-arginine translocation system in protein secretion via the type II pathway." EMBO J 20(23): 6735-41.

Wai, S. N., A. Takade and K. Amako (1995). "The release of outer membrane vesicles from the strains of enterotoxigenic Escherichia coli." Microbiol Immunol 39(7): 451-6.

WHO. (2009). "WHO fact sheet." from http://www.who.int/mediacentre/factsheets/fs330/en/index.html.

Withey, J. H. and D. I. Friedman (2003). "A salvage pathway for protein structures: tmRNA and trans-translation." Annu Rev Microbiol 57: 101-23.

Wong, K. R. and J. T. Buckley (1991). "Site-directed mutagenesis of a single tryptophan near the middle of the channel-forming toxin aerolysin inhibits its transfer across the outer membrane of Aeromonas salmonicida." <u>J Biol Chem</u> 266(22): 14451-6.

Wong, K. R. and J. T. Buckley (1993). "Aeromonas spp. can secrete Escherichia coli alkaline phosphatase into the culture supernatant, and its release requires a functional general secretion pathway." <u>Mol Microbiol</u> 9(5): 955-63.

Wulfing, C. and R. Rappuoli (1997). "Efficient production of heat-labile enterotoxin mutant proteins by overexpression of dsbA in a degP-deficient Escherichia coli strain." <u>Arch Microbiol</u> 167(5): 280-3.

Yamamoto, T., T. Gojobori and T. Yokota (1987). "Evolutionary origin of pathogenic determinants in enterotoxigenic Escherichia coli and Vibrio cholerae O1." <u>J Bacteriol</u> 169(3): 1352-7.

Yamamoto, T., T. Tamura, T. Yokota and T. Takano (1982). "Overlapping genes in the heat-labile enterotoxin operon originating from Escherichia coli human strain." <u>Mol Gen Genet</u> 188(2): 356-9.

Yang, J., D. L. Baldi, M. Tauschek, R. A. Strugnell and R. M. Robins-Browne (2007). "Transcriptional regulation of the yghJ-pppA-yghG-gspCDEFGHIJKLM cluster, encoding the type II secretion pathway in enterotoxigenic Escherichia coli." <u>J Bacteriol</u> 189(1): 142-50.

Yethon, J. A., E. Vinogradov, M. B. Perry and C. Whitfield (2000). "Mutation of the lipopolysaccharide core glycosyltransferase encoded by waaG destabilizes the outer membrane of Escherichia coli by interfering with core phosphorylation." <u>J Bacteriol</u> 182(19): 5620-3.

Yu, J., H. Webb and T. R. Hirst (1992). "A homologue of the Escherichia coli DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in Vibrio cholerae." <u>Mol Microbiol</u> 6(14): 1949-58.

Zalewska-Piatek, B., K. Bury, R. Piatek, P. Bruzdziak and J. Kur (2008). "Type II secretory pathway for surface secretion of DraD invasin from the uropathogenic Escherichia coli Dr+ strain." <u>J Bacteriol</u> 190(14): 5044-56.

Biography

Benjamin Mudrak was born April 24, 1982 in Alexandria, VA. He was raised in Fairfax County, a suburb of Washington, D.C. He graduated from Thomas Jefferson High School for Science and Technology in May, 2000 and set off for the University of Virginia that fall. An Echols scholar at UVA, Ben graduated with distinction, receiving a B.S. degree in Biology and a minor in Religious Studies. He entered graduate school at Duke University through the Cell and Molecular Biology certificate program and was awarded a James B. Duke Fellowship. He decided to join the Kuehn lab in April of 2005, affiliating with the department of Molecular Genetics and Microbiology. In May of 2009, Ben married a fellow Duke graduate student, Sarah Wiley, becoming the legal guardian of two awesome cats in the process. Ben has presented his research at the American Society for Microbiology General Meeting, the U.S.-Japan 42nd Panel Meeting on Cholera and Other Bacterial Enteric Infections, and Mid-Atlantic Microbial Pathogenesis Meetings. In March of 2010, he plans to defend his thesis, preparing to graduate alongside his wife in May.

Publications

Mudrak B., Rodriguez, D.L., and M.J. Kuehn. 2009. Residues of heat-labile enterotoxin involved in bacterial cell surface binding. *Journal of Bacteriology*. 191(9): 2917-25.

Mudrak B. and M.J. Kuehn. 2010. Specificity of the type II secretion systems of enterotoxigenic *Escherichia coli* and *Vibrio cholerae* for heat-labile enterotoxin and cholera toxin. *Journal of Bacteriology*. 192(7): 1902-1911.

Mudrak B. and M.J. Kuehn. Heat-labile enterotoxin: Beyond G_{M1} binding. *Toxins*. Review. In preparation.