IDENTIFICATION OF POTENTIAL TONB-INTERACTIVE SITES IN THE PERIPLASMIC DOMAIN OF THE EXBD PROTEIN

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ABSTRACT

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The TonB energy transduction complex (TonB, ExbB, and ExbD) couples the proton motive force (PMF) to acquire and transport iron in gram-negative bacteria. ExbD is needed to gather energy from the PMF and facilitates conformational changes in the TonB C-terminus. The goal of this study was to determine possible interaction sites between ExbD and TonB. Site-directed mutagenesis was performed on possible interaction sites to create three constructs that selected specific *E. coli exbD* codons and were replaced with *Y. enterocolitica exbD* codons. The codons were chosen by identifying residues that diverged significantly in the ExbD protein compared to homologues in the family *Enterobacteriaceae*. Structural predictions were generated to compare the periplasmic space of ExbD of the homologues. The solvent accessibility for *E. coli* ExbD was generated from the structural predictions with surface residues as possible contact sites. The function of each construct was assessed by a spot titer assay of sensitivity to four group B colicins: M, B, Ia, and D. My results showed that two of these codon changes did not significantly affect the ability of ExbD to support TonB-dependent uptake of colicins, while a third codon change reduced sensitivity to one of the four colicins tested.

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CHAPTER I. INTRODUCTION

The three domains of life are Bacteria, Eukarya, and Archaea (Woese et al., 1990; Doolittle, 1999). The Domain of Bacteria is generally considered as two polyphyletic subgroups on the basis of their cell wall architecture; gram-positive bacteria and gram-negative bacteria. Initially distinguished on the basis of the staining technique of Christian Gram (Gram, 1884); the cell wall of the latter group is a dual membrane system, interfacing with the external environment through an outer membrane (OM), separated from the cytoplasmic membrane (CM) by an aqueous compartment termed the periplasmic space.

The OM acts as a defense by providing a selective permeability barrier for the gram-negative bacteria against harmful compounds such as antibiotics, enzymes, and bile salts (Nikaido & Vaara, 1985; Guest & Raivio, 2016). The components of the outer membrane consist of phospholipids, lipid-anchored polysaccharides, and proteins. Phospholipids are fatty acids with a phosphate group. The fatty acids, the tail region of the phospholipids, are hydrophobic while the phosphate group, the head region of the phospholipids, is hydrophilic. Because they have both hydrophobic and hydrophilic regions, they are amphipathic. Due to the negative charge from the phosphate group, it causes the head of the phospholipid to be polar. The phospholipids can be found in the outer leaflet, although, they are primarily on the inner leaflet of the OM forming a single layer with the head group towards the periplasmic space (White, 2000; Nikaido, 2003; Delcour, 2009).

Lipid-anchored polysaccharides are located exclusively on the outer leaflet of the OM (Kamio & Nikaido, 1976; Simpson et al., 2015). In the model organism *Escherichia coli*, the predominant lipid-anchored polysaccharide is lipopolysaccharide (LPS). There are three distinct

regions in an LPS molecule: the O-antigen, core, and lipid A. The O-antigen is highly diverse among bacterial species and even within strains of species. The O-antigen is a polysaccharide and can differ structurally by chain length, composition, and/or position. Since the O-antigen is on the outer most layer of the outer leaflet of the OM, it influences the surface properties of the bacteria, including the barrier and adhesion to biotic surfaces, abiotic surfaces, and to other cells. The core region is a short chain of oligosaccharides and is attached directly to lipid A and to the O-antigen. The structure of the core region is highly diverse, containing two KDO (3-deoxy-D-*manno*-oct-2-ulosonic acid) residues at the inner end, which is then extended with three variously modified heptose residues in most species. The heptose residues can be phosphorylated, have phosphoethanolamine residues, or have phyrophosphoethanolamine residues. The outer core region is made of hexose residues with the O-antigen attached to the last hexose. The core region provides a barrier to hydrophobic antibiotics and other substances (Delcour, 2009). Lipid A is the hydrophobic anchor region of LPS that forms an asymmetrical bilayer with the phospholipids. It is composed of glucosamine moieties with six saturated fatty acids attached. Most wild-type strains of *Enterobacteriaceae* synthesize a complete core region and the O-antigen. However, the *E. coli* K-12 strain produces a rough LPS which lacks the O-antigen and has a shorter core region (Stevenson et al., 1994). As such, the outer membrane of *E. coli* K-12 does not confer the same degree of barrier function as found in environmental isolates with wild-type cell walls. (White, 2000; Raetz & Whitfield, 2002; Nikaido, 2003; Ruiz, Kahne, & Silhavy, 2006; Delcour, 2009)

Proteins are an important part to the OM. There are two main groups of proteins in the OM. The first are lipoproteins which are small proteins (4-45 kDa) but exist in large quantities (~$7x10^5$ per cell; Nikaido, 1985). Lipoproteins can be anchored to the periplasmic side of either

the CM or the OM, with most of the lipoproteins in *E. coli* localized in the OM (Tokuda & Matsuyama, 2004). The murein lipoprotein (Braun's lipoprotein, Lpp) is anchored to the OM while about one-third is covalently linked to the peptidoglycan layer (Vollmer & Bertsche, 2008). The N-terminus residue in the lipoprotein is modified to a glycerylcysteine with three fatty acids attached (Hantke & Braun, 1973). Lipoproteins have a variety of functions including biogenesis and maintenance of cell surface structures, transport of substrates, drug efflux, and host-pathogen interactions (Babu et al., 2006; Tokuda et al., 2007; Okuda & Tokuda, 2011).

The other main group of proteins are β-barrel channels. These proteins allow for transport and signal transduction (Galdiero et al., 2007; Fairman et al., 2011). β-barrel proteins can vary in the number of strands they have, ranging from 8 to 24, but normally have an even number of strands (Schulz, 2002; Fairman et al., 2011). Some of the β-barrel proteins are nonspecific diffusion channels, or porins, while others are highly specific transporter channels (which will be discussed later). The nonspecific porins form an aqueous channel allowing diffusion of small molecules (< 600 daltons) (Galdiero et al., 2007). Porins can be found in all gram-negative bacteria. There are three general diffusion porins in *E. coli* K-12; OmpF, OmpC, and PhoE. These porins form trimeric 16-stranded β-barrels across the membrane forming an aqueous channel allowing passive diffusion of hydrophilic solutes. Other porins have channels with structures that are selective for certain molecules while excluding others; allowing for facilitated diffusion of specific molecules. For example, the LamB porin of *E. coli* has a structure that allows for diffusion of maltodextrin polymers of a size that would prevent passive diffusion through other porins. (Nikaido, 1985; White, 2000; Shultz, 2002; Nikaido, 2003; Delcour, 2009; Fairman et al., 2011)

The periplasmic region is composed of oligosaccharides, proteins, and peptidoglycans. It is a gel-like consistency (Hobot et al., 1984) and is generally estimated to be 13 to 25 nm wide (Leduc et al., 1989; Graham et al., 1991). The width variation may be due to expansion and contraction because of osmotic shifts or differences in strains or growth conditions (Oliver, 1996). During growth in low osmolarity media, many gram-negative bacteria have been shown to synthesize oligosaccharides (Miller et al., 1986) indicating that the synthesis of the oligosaccharides are osmoregulated (Bohin, 2000). There are many different proteins in the periplasm with different functions including transport (e.g. solute binding proteins), folding (e.g. molecular chaperone protein Skp and peptidyl-prolyl isomerases), hydrolytic enzymes (e.g. alkaline phosphatase), detoxifying enzymes (e.g. β-lactamase which degrades penicillin), and enzymes that promote the biogenesis of the cell envelope (e.g. lipoprotein carrier protein p20) (Oliver, 1996; White, 2000; Betton, 2007; White, 2007).

The peptidoglycan layer lies in the periplasmic space and is connected to the OM layer by lipoproteins. The peptidoglycan layer is what determines the strength, rigidity, and shape of the cell wall. Peptidoglycan is made of carbohydrate polymers cross-linked by short peptide bridges. The polymers are comprised of alternating N-acetylglucosamine and N-acetylmuramic acid attached by β-1,4 linkages. The average chain length of the polymer strands across gram-negative bacteria is between 20 and 40 disaccharide units (Glauner, 1988; Quintela et al., 1995). The peptide can vary across bacterial species, however the amino acids that make up the peptides in most gram-negative bacteria are normally L-alanine, D-glutamate, a diamino acid (normally *meso*-A_2pm), and D-alanine (Vollmer et al., 2008). In *E. coli*, the peptidoglycan layer has been shown to be 6.35 +/- 0.53 nm thick (Matias et al., 2003). Damage to the peptidoglycan layer, either during biosynthesis (by mutation or antibiotics) or degradation (by lysozymes), will cause

cells to be more susceptible to cell lysis (Vollmer & Bertsche, 2008; Vollmer et al., 2008; Silhavy et al., 2010).

The CM is a protein-rich phospholipid bilayer. The phospholipids are composed of a polar phosphate-containing head group and a hydrophobic tail region consisting of fatty acid chains. In aqueous environments the phospholipids assemble into a bilayer, with the tail region forming a hydrophobic core while the head groups face outward forming a hydrophilic surface on both sides of the bilayer. The phosphate group can form noncovalent interactions with cations, water, and polar groups on proteins. Water, gases, small hydrophobic molecules, and lipid-soluble molecules are able to freely diffuse across the CM. Efficient passage of other molecules across the membrane requires proteins that either allow for diffusion or mediate active transport processes (Kadner, 1996; von Heijne, 2006). These are integral proteins that are embedded into the CM. Peripheral proteins associate with the surfaces of the CM either by electrostatic interactions with the polar head groups and with embedded proteins, or, in the case of amphitropic proteins, by hydrophobic interactions with the hydrophobic core of the bilayer. (Singer, 1972; White, 2000)

TonB-Dependent Ligands

The TonB system provides a mechanism for the active transport of a variety of ligands across the OM of gram-negative bacteria including: iron-complexed siderophores, vitamin B_{12}, nickel chelates, and carbohydrates (Schauer et al., 2008; Noinaj et al., 2010). A more extensive list is provided in Table 1. In *E. coli*, the TonB system is primarily used for iron transport. While a few organisms have been found not to need iron, like *Lactobacillus plantarum* (because

it is able to grow in milk which is a highly iron-restricted medium) and *Borrelia burgdorferi* (in order to help it evade its host's attempt to starve pathogens of iron) (Archibald, 1983; Posey & Gherardini, 2000; Aguirre et al., 2013), iron is an essential nutrient for most bacterial species. Iron is used in redox reactions including in electron-transport chains, metabolism and metabolic products, and RNA and DNA synthesis. Under physiological conditions at a neutral pH and in the presence of oxygen, iron is oxidized to an insoluble ferric form of Fe^{3+}. Bacteria have developed different mechanisms to gather iron from its environment (Andrews et al., 2003). Bacteria mainly use siderophores to obtain iron; however, some organisms are able to gather iron without the use of siderophores. Other methods of gathering iron include: The ABC transport system (such as Sfu from *Serratia marcescens* (Angerer et al., 1990)), the ferrous iron transport (Feo) system while in anaerobic conditions (Hantke, 1987; Kammler et al., 1993), organisms utilizing its host proteins (such as *Neisseria* spp. which use the proteins transferrin (Tf), lactoferrin (Lf), hemoglobin (Hb), and haptoglobin-hemoglobin as sources of iron (Andrews et al., 2003; Leon-Sicairos et al., 2015)), and obligate parasites, such as *Francisella*, that live within the intracellular vesicles that its host cell uses in iron uptake (Leon-Sicairos et al., 2015).

Siderophores are iron chelating compounds that have a high affinity for Fe^{3+} and have a low molecular mass (600-1000 DA). There are two major groups of siderophores based on their structure: hydroxamates and catecholates. Hydroxamates are hydroxamic acids with the functional group RC(O)N(OH)R'. Catecholates have catechol rings that help form a hexadentate complex with the iron. A hexadentate complex is when the iron binds to six places on the catechol rings. In *E. coli* K-12, the ferric uptake regulator (Fur) regulon includes the genes for biosynthesis and transport of the catechol siderophore enterobactin and the uptake of a variety of hydroxamate siderophores (Panina et al., 2001; Ollinger et al., 2006). Although iron is essential

for most bacteria, excess iron is toxic for the cell (Halliwell & Gutteridge, 1984) because during the Fenton reaction, ferrous iron reacts with peroxide, producing hydroxyl radicals, which cause cell damage (Carpenter & Payne, 2014). *Enterobacteriaceae* are able to control iron homeostasis by the ferric uptake regulator (Fur) protein (Stojiljkovic et al., 1994; Carpenter & Payne, 2014). Regulation of iron homeostasis is based on iron availability with Fur acting as a positive repressor. In other words, during high iron availability, Fur represses the transcription of iron-regulated genes (Hantke K, 1981). Since siderophores are expensive to bacteria and iron is too important for its acquisition to be left to simple diffusion, most bacteria use specific receptors to bind the iron siderophores and provide for their active transport. (Postle, 1990; Ferguson & Deisenhofer, 2002; Faraldo-Gomez & Sansom, 2003; Wandersman & Delepelaire, 2004)

Table 1: List of substrates and their distribution across phyla. The taxonomic groups abbreviations are: Alt, Altermonadales; CFB, *Chlorobium/Bacteroides* group; Pse, Pseudomonadales; Vib, Vibrionales; Xan, Xanthomonadales, α, α-proteobacteria; β, β-proteobacteria; ε, ε-proteobacteria. Adapted from Schauer et al., 2008.

Substrates	**Phylogenetic Distribution**	**Reference/Source**
Maltodextrin	*Caulobacter crescentus*	Neugebauer et al., 2005
Nickel	*Helicobacter pyloi* (ε), *Bradyrhizobium japonicum* (α), β *(Dechloromonas aromatic, Rubrivivax gelatinosus)*	Schauer et al., 2007; Rodionov et al., 2006
Sucrose	*Xan* (*Xcc*), Alt (*Shewanella* spp.)	Blanvillain et al., 2007
Cobalt	*Novosphingomonas aromaticivorans* (α), *D. aromatic* (β)	Rodionov et al., 2006
Thiamin	Alt (*Shewanella, Colwellia* spp.), Xan, CFB (*Bacteroides fragilis*), α (*Gluconobacter oxydans*), β (*Burkholderia cepacia*)	Gelfand & Rodionov, 2007; Rodionov et al., 2002
Chito-oligosaccharides	Alt (*Shewanella, Colwellia* spp.), Xan, some α (*C. crescentus*)	Yang et al., 2006
Cobalamin (vitamin B_{12})	*Escherichia coli*, Vib (*Vibrio cholera*), Pse (*Pseudomonas aeruginosa*), Alt (*Shewanella* spp.), Xan, α (*C. crescentus, Rhodobacter sphaeroides, Rhodopseudomonas palustris*), β (*Ralstonia solanacearum, Burkholderia pseudomallei*)	Rodionov et al., 2003
Copper	Pse (*Pseudomomas stutzer, Pseudomonas putida, Pseudomonas aeruginosa*)	Lee et al., 1991; Mokhele et al., 1987; Wunsch et al., 2003

TonB-Dependent Transporters

TonB-dependent transporters (TBDT) are proteins in the OM of bacteria that use the cytoplasmic protein complex TonB-ExbB-ExbD to transport ferric siderophore complexes across the OM. The mechanism by which siderophores are transported once they bind to the TBDTs is not well understood. The model organism *Escherichia coli* K-12 has seven TBDTs: BtuB (for vitamin B_{12}), FepA, FhuA, FecA, FhuE, Cir, and Fiu that are for Fe^{3+}siderophores (Nikaido, 2003; Noinaj et al., 2010). TBDTs all have a similar structure of a C-terminus 22-stranded antiparallel β-barrel domain that is inserted in the OM and provides a central aqueous channel, closed on the periplasmic face by a ~150 residue N-terminus globular domain that acts as a hatch to the barrel (Schulz, 2002; Nikaido, 2003; Chimento et al., 2005; Noinaj et al., 2010).

The β-barrel is normally around 70Å in height and 35-47Å in diameter. The β-strands form a 45° angle to the axis of the barrel while forming a right handed twist. Also, the β-strands that form the transmembrane part of the barrel extend past the leaflets of the OM. The β-strands are connected by 11 long loops on the external membrane side and 10 short turns on the periplasmic side. Ligand-binding sites for specific ferric siderophore complexes reside on the external surface on both the β-barrel and the globular domain. (Ferguson & Deisenhofer, 2002; Nikaido, 2003)

The structure of the globular domain is a four stranded β-sheet with several short α-helices mixed between. The globular domain forms a hatch, blocking passage through the barrel (Ferguson & Deisenhofer, 2002; Nikaido, 2003). This involves the positioning of a short extreme N-terminal motif termed the TonB box that is conserved across all TBDT and involved in the recognition of the transporter by the CM protein TonB (Larsen et al., 1997; Cadieux &

Kadner, 1999). Once the ligands bind, there is a conformational change in the globular domain. In the TBDT FhuA, an alpha-helix (the switch helix) is unwound after ligand binding (Ferguson et al., 1998; Locher et al., 1998). This conformational change is believed to alter the accessibility of the TonB box. Altogether, these conformational changes signal ligand occupancy to TonB (Ferguson et al., 1998; Locher et al., 1998; Kodding et al., 2005).

Bacteriophage and colicins are able to hijack the TBDTs to enter the cells and are TonB-dependent. Bacteriophages are viruses that are able to infect and multiply within their host (Ackermann, 2011). The bacteriophages T1 and φ80 are both able to exploit the TonB-dependent transport system for irreversible adsorption into the cell (Hancock & Braun, 1976). Colicins are proteins that are produced by and are lethal for some strains of *E. coli* (reviewed in: Cascales et al., 2007). Colicins use OM receptor proteins to gain entry into the bacteria, where they are able to release their toxins. There are two groups of colicins and they use different machineries to gain access to the cell: Group A colicins use the Tol system (Davies & Reeves, 1975a) while group B colicins use the TonB system (Davies & Reeves, 1975b). These proteins have a molecular mass ranging from 40 to 80 kDa. All colicins consist of three domains: an N-terminal domain that is used for translocating through the membrane, a central domain that is used for receptor binding, and a C-terminal domain that carries the toxic cargo (Ohno-Iwashita & Imahori, 1980; Benedetti et al., 1991). Just like TBDT, group B colicins have the TonB box as well (Schramm et al., 1987) and are unable to translocate across the OM if it is deleted (Mende & Braun, 1990). Since colicins can parasitize the OM transport proteins and kill the bacteria, spot titer assays are a way to measure the activity of the TonB system (Larsen et al., 2003; Devanathan & Postle, 2007; Jakes & Cramer, 2012).

TonB Energy Transduction Complex

For active transport to occur, energy must be delivered to the OM. There are several multi-protein systems that are able to harness the energy from the CM. The Tol system, which includes the proteins TolA, TolQ, and TolR, are required for the integrity of the OM (Lazzaroni et al., 1999). The Mot system, which includes the proteins MotA and MotB, are required for the flagellar motor (Stolz & Berg, 1991). A third multi-protein system is the TonB system.

There are three proteins in the TonB energy transduction complex: TonB, ExbB, and ExbD (Figure 1) with a per-cell ratio of 1:7:2, respectively (Higgs et al., 2002). The three proteins span the CM and cross into the periplasm. The TonB complex is needed to couple the proton motive force (PMF) from the CM and deliver the energy to the TBDT for active transport (Bradbeer, 1993; Noinaj et al., 2010; Bulathsinghala et al., 2013). In order for TonB to make a conformational change in the periplasm, ExbB, ExbD, and TonB all need to be functional and PMF present (Larsen et al., 1999; Ollis et al., 2012). All three proteins are needed for different aspects to harness the proton motive force.

The TonB protein of *E. coli* is 239 amino acids long and has three domains (Hannavy et al., 1990; Roof et al., 1991). The N-terminus domain (residues 1-32) consists of two sections: a short cytoplasmic domain (residues 1-11) and a transmembrane domain (TMD) which acts as a signal-anchor (residues 12-32). A histidine residue (His20) in the TMD is critical for TonB function and has been found to be the only residue in TonB TMD whose replacement by alanine will result in a loss of function (Larsen et al., 2007). Swayne and Postle substituted every other amino acid with His20 (H20X) and found H20N was the only substitution that supported full

activity (2011). They determined His20 is important for its structure and not its ability to be protonated. (Larsen et al., 2007; Postle et al., 2010; Swayne & Postle, 2011; Ollis et al., 2012)

The intermediate domain (residues 33-149) is in the periplasm and has a Pro-Glu and Pro-Lys alternating repeats between residues 66-100. This proline-rich region is non-essential for TonB function in laboratory settings, but allows TonB to extend to the OM across a range of osmotic environments (Larsen et al., 1993). The C-terminus domain (residues 150-239) directly interacts with the TBDTs and is believed to deliver the energy from the PMF for active transport. Residues 158 to 162 is the region known to interact with TonB boxes (Gudmundsdottir et al., 1989; Schoffler & Braun, 1989; Vakharia-Rao et al., 2007; Postle et al., 2010). Seven residues have been found to be functionally important in the C-terminus domain. The residues include G186 and six aromatic residues (Y163, F180, F202, W213, Y215, and F230). When two of the seven residues are replaced with alanine, it renders TonB inactivate, indicating no single residue is essential. This suggests at some point the seven residues interact with one another (Ghosh & Postle, 2004; Postle, et al., 2010; Ollis & Postle, 2012).

Structures have been solved for the crystallized TonB C-terminus domain (Chang et al., 2001) and an NMR study (Peacock et al., 2005), as well as for the TonB C-terminus co-crystallized with the TBDTs BtuB and FhuA (Pawelek et al., 2006; Shultis et al., 2006). These structures suggest a configuration of TonB with one to two alpha-helicies and three to four beta sheets. Postle et al. found that the dimeric crystal structures did not represent the TonB C-terminus configuration *in vivo* (2010), which is significant because TonB has been found to function as a dimer *in vivo* (Sauter et al., 2003; Gresock et al., 2015). It is known that the *tonB* gene is needed for active transport (Hancock & Braun, 1976; Postle, 1990); however, the exact

mechanism remains unknown. TonB is able to interact and make contact with TBDTs *in vivo* (Larsen et al., 1997) and assumed to transmit the energy from the PMF for active transport, but there is no study supporting this hypothesis. When the TonB C-terminus interacts with TBDTs it has been indicated to act as a monomer (Freed et al., 2013; Gresock et al., 2015); however, two alternative models have recently been proposed (Celia et al., 2016; Klebba, 2016).

The ExbB protein of *E. coli* is 244 amino acids long and has three transmembrane domains (predicted as residues: 16-39, 128-155, and 162-194). Most of ExbB is in the CM while the N-terminus and the loop from TMD 2 to 3 are displayed in the periplasm. The C-terminus and the loop from TMD 1 to 2 are displayed in the cytoplasm (Kampfenkel & Braun, 1993). The function of ExbB is thought to be a scaffold and a signal transducer between the cytoplasm and the periplasm (Larsen et al., 1999; Baker & Postle, 2013). A new model (which will be discussed below) was proposed that ExbB forms a pentamer (Celia et al., 2016).

The ExbD protein of *E. coli* is 141 amino acids long and has a structure similar to TonB. ExbD has three domains: the N-terminus (residues 1-22) which is in the cytoplasm, the TMD (residues 23-42) which is predicted to be helical in structure, and the C-terminus (residues 43-141) which is displayed in the periplasm (Kampfenkel & Braun, 1992). The TMD is the most conserved topological domain and is conserved across the ExbD homologs TolR and MotB (Cascales et al., 2001). A solved solution structure of the periplasmic domain of *E. coli* ExbD (residues 44-141) using nuclear magnetic resonance (NMR) spectroscopy suggested three well-defined regions (Garcia-Herrero et al., 2007). An N-terminus domain (residues 44-63) and a C-terminus domain (residues 134-141), which are both flexible in structure. The third region is a folded region consisting of two α-helices and five β-strands (residues 64-133). However, *in vivo*

evidence suggests that this region is needed to be flexible in order for conformational changes to occur for homodimerization and interaction with TonB, leaving the NMR structure to be questioned (Ollis & Postle, 2011; Ollis & Postle, 2012; Sverzhinsky et al., 2015). In the present working model, ExbD gathers energy from the PMF and facilitates conformational changes in the TonB C-terminus (Brinkman & Larsen, 2008; Ollis & Postle, 2012; Ollis et al., 2012).

Studies have shown two amino acids that are important for ExbD to function. In the TMD, there is an essential aspartate that is conserved across all species of ExbD and its homologs of TolR and MotB (Braun et al., 1996). The aspartate residue (D25) is the only charged amino acid in the TMD, which means it can be protonated by the PMF (Swayne & Postle, 2011). This residue has also been shown to be necessary for ExbD-TonB periplasmic interactions (Ollis et al., 2009; Ollis & Postle, 2011). The second important amino acid is a leucine at position 132 in the tail of the C-terminus. If L132 is substituted, it will inactivate ExbD (Braun et al., 1996; Ollis et al., 2009). L132 helps in the assembly of TonB and ExbD when the PMF is independent in stage II of TonB energization (Ollis & Postle, 2012), which will be discussed below.

A region of 30 residues (residues 92 to 121) has been shown to be important for ExbD protein-protein interactions. Deletions within this region prevented the formation of formaldehyde cross-linked complexes of ExbD homodimers, ExbD-TonB heterodimers, and ExbD-ExbB heterodimers (Ollis et al., 2012). Within this region of the ExbD C-terminus, specific residues have been found to interact with the C-terminus of TonB, suggesting that ExbD helps position TonB for the correct conformation to interact with the TBDT. (Ollis & Postle, 2011; Ollis & Postle, 2012)

The current working model of TonB energization can be broken down into three stages. In stage I, ExbD and TonB do not interact with each other. During this stage, TonB is sensitive to proteinase K and it is unable to formaldehyde cross-link with ExbD. Substitutions at ExbD L132 and TonB H20 leaves TonB stalled at this stage. In stage II, ExbD and TonB can assemble and form a heterodimer and does not require the PMF. This conformation leaves TonB resistant to proteinase K and is also unable to formaldehyde cross-link with ExbD. In both stage II and III ExbB is required and acts as a scaffold. Moving from stage II, which was PMF-independent, to stage III requires the PMF. The PMF allows a conformational change within the periplasmic domains of both ExbD and TonB so they are now able to be cross-linked with formaldehyde. This rearrangement leaves TonB sensitive to proteinase K. (Ollis et al., 2012; Gresock et al., 2015) However, there is still a lot unknown about the energy transduction cycle of TonB. This includes the oligomeric state of TonB in all three stages along with the role of homodimerization of the TonB C-terminus and where it fits in. TonB and ExbD are known to form a heterodimer, yet it is still unknown if the proteins are configured in a monomer, homodimer, heterodimer, etc during the entirety of the energy transduction cycle. Most importantly, how this cycle becomes re-energized still needs to be answered.

A current working model incorporates the cellular ratios of individual components (Higgs et al., 2002), the role of which has not been previously accounted for (Gresock et al., 2015). Gresock et al. propose a model in which during stage I, TonB and ExbD are both in a homodimer form ($TonB_2$ and $ExbD_2$) that form independent complexes with an ExbB tetramer ($ExbB_4$). During stages II and III, TonB and ExbD form a dimer of heterodimers with each other. In stage IV, the TonB C-terminus interacts with a TBDT only in monomeric form and TonB H20 is required for rehomodimerization of TonB. Gresock et al. speculate that after stage III, the

energy of $ExbD_2$-$ExbB_4$ is depleted and needs to be restored. They suggest that there is a separate pool of $ExbD_2$-$ExbB_4$ that exists that replenishes $ExbD_2$-$ExbB_4$ for stage I. In this model, only 2 ExbB are unaccounted for. Gresock et al. explain this deficit by suggesting pools of free $ExbB_2$, which are stable, that are precursors to the ExbB tetramers.

The Klebba laboratory has recently proposed a different model of TonB action; the rotational surveillance and energy transfer (ROSET) model (Klebba, 2016). The ROSET model suggests the dimerized C-terminus domain of TonB (which has a LysM motif (Kaserer et al., 2008)) binds to the peptidoglycan layer. This affinity for the peptidoglycan and TBDTs allows the dimerized C-terminus domain of TonB to survey the underside of the OM for occupied TBDTs (where monomeric TonB C-terminus interacts with the TonB box of the TBDT). This model also incorporates previous research that showed rotational motion of the TonB N-terminus domain (Jordan et al., 2013). Jordan et al. created a GFP-TonB fusion protein which showed the reorientation of the light from the GFP-TonB protein, suggesting that TonB has a rotational movement which they found was powered by the electrochemical gradient from ExbB and ExbD. Klebba suggests this rotational movement allows lateral movement of the TonB-ExbB-ExbD complex through the CM. The rotation of TonB also provides the force that causes a conformational change to the globular domain in the TBDTs which provides transport of the ligand.

A new model has just been proposed of the TonB energy transduction complex. In this model, the stoichiometry of the complex consists of a pentamer of ExbB, a dimer of ExbD, and at least one TonB (Celia et al., 2016). In this study, they used X-ray crystallography, electron microscopy, double electron-electron resonance (DEER) spectroscopy, and crosslinking to

determine that the quaternary structure of ExbB is a pentamer with the five transmembrane domains forming a transmembrane pore with the transmembrane helix of ExbD inside. A cross-linking analysis and DEER spectroscopy analysis was performed and determined ExbD is a dimer. They confirmed the stoichiometry results of ExbB and ExbD with and without the presence of TonB. To accommodate for the two ExbD proteins in their model, they propose that the first copy of ExbD has its transmembrane helix inside the transmembrane pore of the ExbB pentamer, while the second copy of ExbD is located outside the ExbB pentamer. They ran electrophysiology studies and found that the Ton subcomplex (ExbB-ExbD) forms channels that are pH-sensitive and cation-selective with the periplasmic domain of ExbD serving as a cation-selective filter with D25 of ExbD serving as an important factor towards the ion selectivity. Based on their findings, they suggest two models for how the PMF energy is harnessed. Their first model is the 'electrostatic piston' model. In this model, the ExbD transmembrane helix that is inside the transmembrane pore of ExbB moves up and down within the pore. Their second model is the 'rotational' model. In this model, the ExbD transmembrane helix that is inside the transmembrane pore of ExbB rotates creating rotational motion.

Hypothesis

Previous research by Kate Butler took *exbD* homologues of *Yersinia enterocolitica*, *Serratia liquefaciens*, *Proteus mirabilis*, and *Vibrio parahaemolyticus* and examined their ability to complement an *E. coli ΔexbD* strain (Butler, 2013). Phenotypic characterization of these clones found that *Y. enterocolitica* is less efficient than *E. coli*, *S. liquefaciens*, and *P. mirabilis*

at supporting TonB function, while the *V. parahaemolyticus* ExbD did not demonstrably support TonB function.

It is known that ExbD makes contact with TonB causing a conformational change in the TonB periplasmic domain most likely occurring with multiple contact sites. Evolutionary changes caused surface interactions between species to diverge, which I believe is the case with *Y. enterocolitica*. I hypothesize there is an essential binding region that is different in the ExbD C-terminus of *Y. enterocolitica* that is causing it to engage TonB less efficiently. This study examined this hypothesis by identifying residues that diverged in the ExbD protein in the family *Enterobacteriaceae* that possibly are involved in the interaction between ExbD and TonB. This strategy will allow us to investigate differences between surface contact sites.

Specific Aims

Specific Aim 1: To perform site-directed mutagenesis to make three constructs replacing specific *E. coli exbD* codons with *Y. enterocolitica* codons. These constructs will then be used to complement a Δ*exbD* strain.

Specific Aim 2: To build 3D models of the predicted structures by using SWISS-MODEL.

Specific Aim 3: To evaluate the constructs for their efficiency of ExbD by performing sensitivity assays against group B colicins.

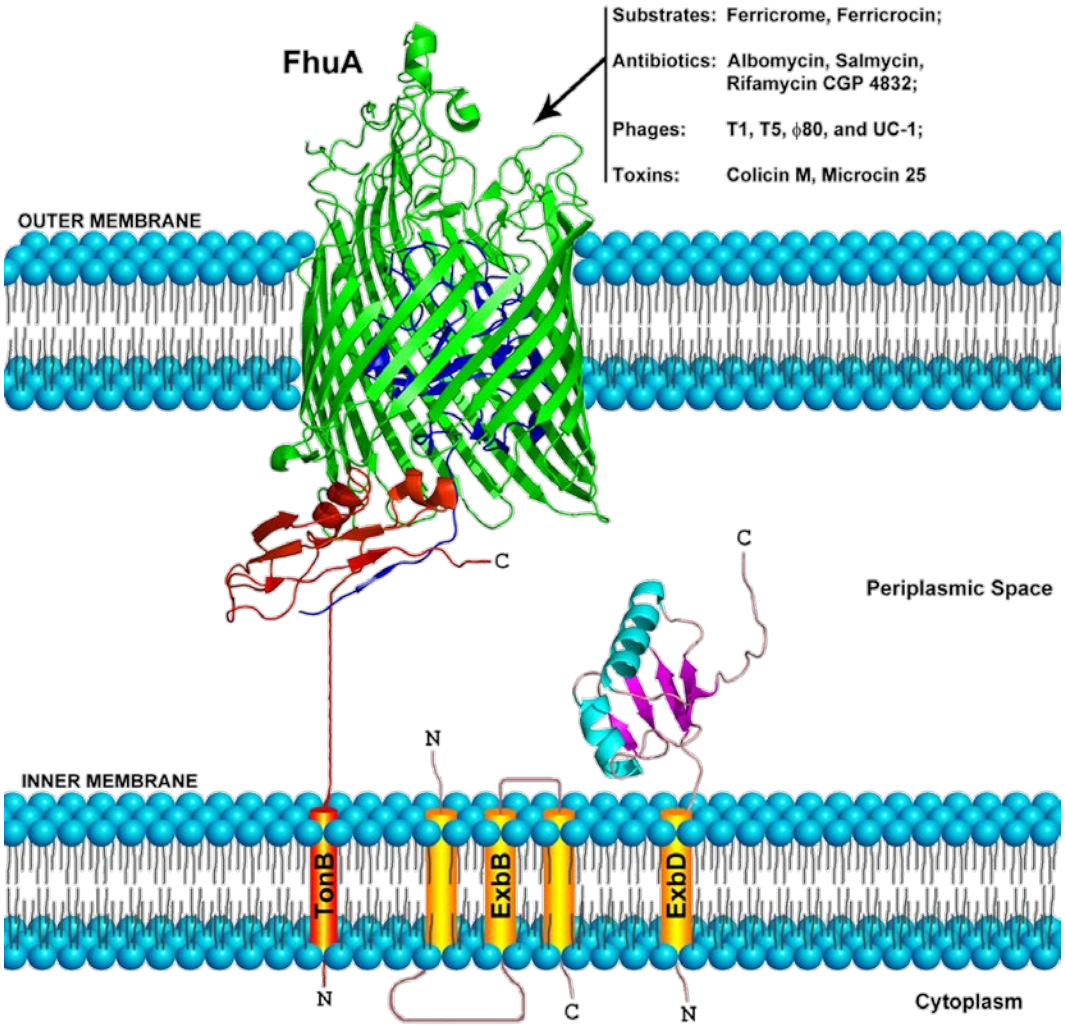


Figure 1: The TonB transduction complex. The components of the TonB system include the CM-anchored proteins TonB, ExbB, and ExbD, coupled with a TBDT (in this case the ferrichrome transporter FhuA protein is depicted). The system provides energy to allow transport of certain substrates and other molecules across the OM. The ribbon structures represent the solved crystal structure of FhuA and TonB (Pawelek et al., 2006) and the solved NMR structure of the C-terminus of ExbD (Garcia-Herrero et al., 2007). Adapted from Ivanov, 2012.

CHAPTER II. MATERIALS AND METHODS

Media

Bacterial strains were cultured in Luria-Bertani (LB) broth and on LB agar plates (Miller, 1972). Both liquid cultures and agar plates were supplemented with 100 μg ml^{-1} ampicillin where required. Colicin sensitivity assays were performed on tryptone (T)-plates overlaid with cells suspended in T-top agar (Miller, 1972) supplemented with 100 μg ml^{-1} ampicillin and 0.001% w/v $_L$-arabinose. Cultures were grown at 37°C.

Bacterial Strains

The bacterial strains used in this study are listed in Table 2. All experiments were performed using isogenic derivatives of the K12 *E. coli* strain W3110 (Hill and Harnish, 1981). RA1035 is a derivative of W3110 with a deletion of the homologues *tolQ* and *tolR* genes (Brinkman and Larsen, 2008), and served as the wild-type strain. RA1045 is a derivative of RA1035 with a deletion of the coding region of the *exbD* gene (Brinkman and Larsen, 2008). The NEB 5-alpha strain is a cloning strain optimized for recovery of recombinant plasmids and purchased in a chemically competent form (New England Biolabs, Ipswich, MA).

Table 2: Strains used in this study.

Strain	Genotype/Phenotype	Reference/Source
W3110	K12 *E. coli rrnD rrnE inversion*	Hill and Harnish, 1981
RA1035	W3110 *ΔtolQR*	Brinkman and Larsen, 2008
RA1045	W3110 *ΔtolQR,* Δ*exbD*	Brinkman and Larsen, 2008
NEB 5-alpha	Chemically competent *E. coli*	New England Biolabs, Inc.

Plasmids

The plasmids used in this study are listed in Table 3. The plasmid pBAD24 was used as the cloning vehicle for the plasmids constructed in this study. pBAD24 provides ampicillin resistance and is arabinose-regulated (Guzman et al., 1995). pKP393 is a pBAD24 derivative that encodes an *exbD* gene of *E. coli* (Brinkman & Larsen, 2008). The plasmid pRA055 is a pBAD24 derivative that encodes an *exbD* gene of *Y. enterocolitica* (Butler, 2013). Three new pBAD24 derivatives with site specific mutations were constructed for this study.

To construct the plasmids, an overnight culture of pKP393 was grown in LB broth supplemented with 100 μg ml^{-1} ampicillin at 37°C. Plasmid DNA was extracted using the Plasmid Purification Protocol procedure (Qiagen, Hilden, Germany). The resulting plasmid was used as the DNA template for an extra-long polymerase chain reaction (XL PCR), to mutate and amplify the site specific mutations in the *exbD E. coli* gene. The primers used for each construct (listed in Table 4) were used to mutate specific codons in the *E. coli* strain to encode amino acids found in *Y. enterocolitica*. Each primer contained complementary base pairs (about 15-20) to the template DNA, the mutational base pairs, and a restriction enzyme site to confirm the mutation

occurred. Primers oRA0701 and oRA0702 were used to replace the motif asparagine, aspartic acid, proline, valine, threonine, aspartic acid at site 78-83 with aspartic acid, glutamine, glutamine, valine, aspartic acid, arginine. Primers oRA0697 and oRA0698 were used to replace an alanine with a lysine at site 92. Primers oRA0699 and oRA0700 were used to replace an alanine with a serine at site 125.

Each XL PCR reaction had a total volume of 50 µl, with 1.5 µl dNTP mix at 300 µM, 1 µl each of forward and reverse primers at 0.5 µM, 1 µl of template DNA pKP393 at 1 ng/µl, 2 µl of Taq polymerase at 5 units/50 µl reaction, 10 µl of 5X buffer, and 33.5 µl of water. No $MgSO_4$ was added to the reaction. Each XL PCR reaction ran for a total of 35 cycles in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.), with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 65°C for 5 minutes through the first 34 cycles, and 10 minutes for the final cycle. The resultant products were evaluated by resolving samples on 1% agarose gels to confirm the production of amplimers of predicted size.

The resultant amplimers were double digested with the restriction enzyme DpnI to degrade methylated DNA to remove the starting template, and the specific restriction enzyme that was built into the primers of each construct. The digestions were performed in a total volume of 50 µl, with 2 µl DNA, 5 µl 10X reaction buffer, 1 µl DpnI, 2 µl of the other restriction enzyme, and 40 µl ddH_2O. Restriction digests were performed for 90 minutes at 37°C. Following digestion, samples were purified using a PCR product purification kit (Qiagen, Hilden, Germany). The samples were then treated with DNA ligase and transformed into NEB 5-alpha chemically competent *E. coli* cells from NEB, following the product protocol. To select for transformants, 100 µl aliquots were spread onto LBamp100 plates and were incubated

overnight at 37°C. Colonies were picked and grown overnight in 5 ml of LB broth at 37˚C with shaking, with plasmid then recovered by standard alkaline lysis method (Sambrook et al., 1989). Plasmids were then screened by restriction analysis to identify predicted restriction sites. Once screened, selected individual plasmids were purified using PCR product purification kit (Qiagen, Hilden, Germany) and verified by Sanger sequencing contracted to the DNA Sequencing Facility at University of Chicago Comprehensive Cancer Research.

Table 3: Plasmids used in this study.

Plasmid	**Genotype/Phenotype**	**Reference/Source**
pBAD24	*araBAD* promoter, AraC, Amp^r	Guzman et al., 1995
pKP393	pBAD24 encoding *E. coli* ExbD	Brinkman and Larsen, 2008
pRA055	pBAD24 encoding *Y. enterocolitica* ExbD	Butler, 2013
pRA062	pBAD24 encoding *E. coli* ExbD with mutation (N78D, D79Q, P80Q, T82D, D83R)	Present study
pRA063	pBAD24 encoding *E. coli* ExbD with mutation (A92K)	Present study
pRA064	pBAD24 encoding *E. coli* ExbD with mutation (A125S)	Present study

Table 4: Primers used in this study.

Primer #	Description	Sequence (5' to 3')
oRA0701	*E. coli exbD* forward mutagenic 78-83	CCCACGTGGACCGTGAAACAATGATTACGGCGTTG
oRA0702	*E. coli exbD* reverse mutagenic 78-83	CCCAGCTGCTGGTCACCGATAAACATCGAGTTGTCTGC
oRA0697	*E. coli exbD* forward mutagenic 92	CCGTTAACCGAAGGCAAGAAAGACACCACC
oRA0698	*E. coli exbD* reverse mutagenic 92	CCGTTAACTTATTCAACGCCGTGATCATTG
oRA0699	*E. coli exbD* forward mutagenic 125	Phos – GGATACCTGAAGATAGGTCTGGTCGG
oRA0700	*E. coli exbD* reverse mutagenic 125	Phos – GGACTGATGCAGCGTATCCATTACC

Transformation

During the initial cloning of the *exbD* mutants, the plasmids were recovered by transformation of ligated DNA into high efficiency competent NEB 5-alpha cells, following the manufacturer's instructions (New England Biolabs, Ipswich, MA). For the spot titer assays, the plasmids were moved to the bacterial strains RA1045 and RA1035. RA1045 and RA1035 were rendered competent for transformation using the TSS protocol (Chung et al., 1989). Briefly, fresh overnight cultures of RA1045 and RA1035 were subcultured at a ratio of 1:100 in 5 ml of

fresh LB and grown to A_{550} = 0.4 (as determined in a Spectronic 20 spectrophotometer with a path length of 1.5 cm). Cultures were divided into 1.0 ml aliquots and incubated on ice for 5 minutes, then centrifuged at 14,000 xg for 5 min at 4°C. The supernatant was removed and the cells were suspended in 100 µl 1X TSS (LB broth supplemented with 10% (w/v) polyethylene glycol (PEG 8000), 5% (v/v) dimethyl sulfoxide (DMSO), and 50 nM $MgCl_2$, pH 6.5). Two microliters of plasmid was added to the cells. The mixture was incubated on ice for 30 min, followed by 2 min at 37°C, followed by another 2 min on ice. Once done incubating, 500 µl LB was added and incubated for 40 min at 37°C. After incubation, 100 µl of the cells were plated onto LB plates supplemented with ampicillin and were incubated overnight at 37°C. The following day, the transformats were streaked for isolation on LB plates supplemented with ampicillin. Phenotypes were determined by spot titer assays.

Colicin Preparations

All colicin tests used working stocks of bacterial lysates from cells induced to express specific colicin genes. Colicin B was prepared by Kate Butler (Butler, 2013) from *E. coli* W3110 transformed with the plasmid pES3 (kindly provided by V. Braun), which bears the gene that encodes colicin B (Pressler et al., 1986). Laboratory stocks of colicins D, Ia, and M were produced by Ray Larsen from *E. coli* K12 strains carrying the plasmid pT04, encoding Colicin M (kindly provided by V. Braun) and natural plasmids encoding colicins D and Ia (kindly provided by A. Pugsley). The purity and specificity of each preparation was confirmed by testing against a set of W3110 derivatives bearing single deletions of the genes encoding the specific TBDT used as a receptor for each of the colicins (Larsen, data not shown).

Spot Titer Assay

Overnight cultures were grown in LB broth supplemented with 100 μg ml^{-1} ampicillin at 37°C. The overnight cultures were subcultured 1:100 in 5 ml LB supplemented with 100 μg ml^{-1} ampicillin and 0.001% w/v L-arabinose and grown to A_{550} = 0.4 (as measured in a Spectronic 20 spectrophotometer with a path length of 1.5 cm). Aliquots of 100 μl of each culture were added to 3 ml of molten (50°C) T-top agar, also supplemented with 100 μg ml^{-1} ampicillin and 0.001% w/v L-arabinose. This solution was mixed and poured onto T-plates. Once solidified, 5 μl spots of serial five-fold dilutions of each colicin, out to 5^{-9}, were spotted onto the plates. All samples were done in triplicate and incubated at 37°C overnight. The plates were scored the next day, with the highest dilution that produced a zone of clearing recorded.

CHAPTER III. RESULTS

Alignment Comparison

Comparison of the protein sequence alignment of ExbD homologues from the family *Enterobacteriaceae* is shown in Figure 2. In the transmembrane domain, the sequence is conserved across all four homologues. A sequence analyses compared the C-terminus to predict amino acid divergence between the homologues. Highlighted in red, navy blue, and purple are the sites chosen to perform the site-directed mutagenesis between *E. coli* and *Y. enterocolitica*. Table 5 compares the amino acid properties between the sites chosen for site-directed mutagenesis between *E. coli* and *Y. enterocolitica*. Figure 3 shows the crystal and cartoon structure of the C-terminus of *E. coli* ExbD.

The first construct, pRA062, consisted of five amino acids mutated and portions of the mutation were within the third beta strand. N78D was the first mutation and is part of the turn between β2 and β3. The mutation changed the charge of the side chain from polar to a negative charge. The van der Waals volumes of both amino acids are relatively the same; however, asparagine has an amide group and aspartate has a carboxylic acid group with the OH deprotonated. The second mutation was D79Q and was within the third beta strand. The mutation changed the charge from a negative charge to polar. Glutamine is a slightly larger amino acid (van der Waals volume of 114 $Å^3$) and contains an amide group compared to aspartate (van der Waals volume of 91 $Å^3$) which contains a carboxylic acid. The third mutation was P80Q, and was also within the third beta strand. The mutation changed the charge of the side chain from non-polar to polar and also increased the van der Waals volume from 90 $Å^3$ to 114 $Å^3$. This mutation would cause an extreme difference in the side chain's flexibility due to

proline being very restrictive in how it is able to fold since it is in a locked, cyclic structure, compared to glutamine which is very flexible. The next amino acid in the sequence is a valine, which is conserved across *E. coli* and *Y. enterocolitica* and is the last amino acid in the third beta strand. The fourth mutation was T82D which is in-between the β3 strand and the α1 helix. The mutation changed the charge from polar to a negative charge. Threonine contains a hydroxyl group and has a van der Waals volume of 93 $Å^3$, while aspartate contains a carboxylic acid and has a van der Waals volume of 91 $Å^3$. The last mutation for this construct was D83R which was also in-between the β3 strand and the α1 helix. This mutation caused an extreme difference in the charge, van der Waals volume, and length of the side chain. The mutation changed the charge from a negative charge to a positive charge which would affect binding to this site. It also changed the size difference between the amino acids with aspartate having a van der Waals volume of 91 $Å^3$ and a smaller side group compared to arginine which has a van der Waals volume of 148 $Å^3$ and contains a very long side group.

Using the amino acid explorer common substitutions from NCBI, the substitutions for each mutation was ranked by how often they substitute for an amino acid using data from the BLOSUM62 matrix. If the mutation has a positive score it means it substitutes frequently for that amino acid in homologous proteins while a negative score means it substitutes rarely. The mutation N78D had a positive score and the mutation D79Q had a neutral score, suggesting that these mutations would probably not cause a big affect. However, the mutations P80Q, T82D, and D83R, all had a negative score suggesting these mutations could cause a disruption in the folding pattern of ExbD causing it to engage TonB less efficiently. No previous research has found any evidence of this section of residues being functionally important, however, with three

of the five mutations with negative scores in the BLOSUM62 matrix, these residues would be a good section to consider.

The second construct, pRA063, was the mutation A92K and it was at the end of the first alpha helix. The mutation changed the charge from non-polar to a positive charge. The amino acids differ greatly in size with alanine having an extremely small side group and a van der Waals volume of 67 $Å^3$ whereas lysine has an extremely long side group with an amine group attached to it and a van der Waals volume of 135 $Å^3$. Before the mutation, the alpha-helix would fold with the alanine facing the interior of the protein because it is hydrophobic. However, with the mutation, the lysine would probably cause the protein to fold slightly different with the lysine facing the exterior of the protein because it is hydrophilic with a positive charge. Using the amino acid explorer common substitutions from NCBI, this mutation had a negative score suggesting it is not a good substitution for alanine and might cause ExbD to fold improperly.

ExbD A92C has been shown to be a site of ExbD homodimeric and heterodimeric interaction with TonB A150C *in vivo* (Ollis et al., 2009). ExbD A92C was only able to form the heterodimeric complex with TonB A150C when the TMDs of both proteins were functional. However, ExbD A92C was able to form homodimers even without a functional TMD (Ollis et al., 2009). This indicates that ExbD A92 might be an import residue for ExbD function.

The third construct, pRA064, was the mutation A125S and it is right outside the second alpha helix. The mutation changed the charge from non-polar to polar and also increased the van der Waals volume slightly from 67 $Å^3$ to 73 $Å^3$. The side chains are about the same size except serine has a hydroxyl group. *Y. enterocolitica* is the only homologue out of the family *Enterobacteriaceae* that does not have an alanine at position 125 which is why this site was

chosen. However, the amino acid explorer common substitutions from NCBI showed that this mutation had a positive score predicting that this mutation would probably not cause an effect.

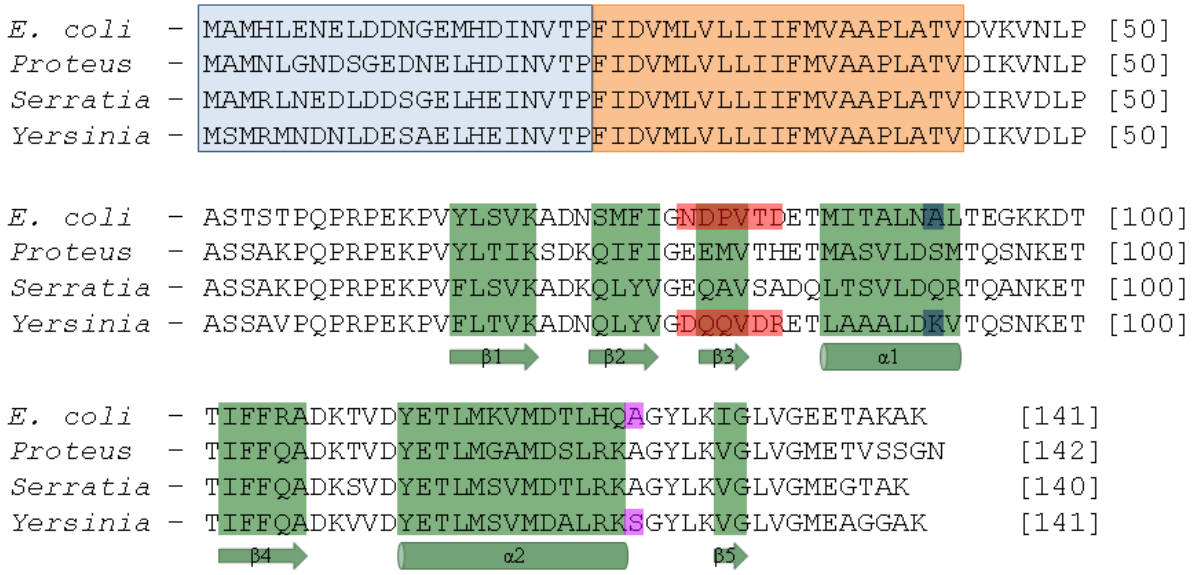
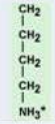


Figure 2: Sequence alignment of the predicted ExbD protein. The homologues that were used are: *Escherichia coli* W3110, *Proteus mirabilis* strain HI4320, *Serratia liquefaciens* ATCC 27592, and *Yersinia enterocolitica* subsp. *Enterocolitica* 8081. Highlighted in the light blue box is the N-terminus domain and highlighted in the orange box is the transmembrane domain. Highlighted in green are the secondary structures of the protein with the cylinders representing the alpha-helices and the arrows representing the beta-sheets. The red box highlights the amino acid change in construct pRA062. The navy blue box highlights the amino acid change in construct pRA063. The purple box highlights the amino acid change in construct pRA064.

Table 5: Amino acid properties between the sites chosen for site-directed mutagenesis between *E. coli* and *Y. enterocolitica* for each construct. In the center of the table is the location of the residue that was mutated. The amino acids for *E. coli* (before the mutation) are on the left and the amino acids for *Y. enterocolitica* (after the mutation) are on the right.

	E. coli					*Y. enterocolitica*			
Plasmid	**R-group**	**van der Waals vol (Å³)**	**Charge**	**Amino Acid**	**Location**	**Amino Acid**	**Charge**	**van der Waals vol (Å³)**	**R-group**
pRA062		96	Polar	Asparagine	78	Aspartic Acid	Negatively Charged	91	
		91	Negatively Charged	Aspartic Acid	79	Glutamine	Polar	114	
		90	Non-polar	Proline	80	Glutamine	Polar	114	
		93	Polar	Threonine	82	Aspartic Acid	Negatively Charged	91	
		91	Negatively Charged	Aspartic Acid	83	Arginine	Positively Charged	148	
pRA063	CH_3	67	Non-polar	Alanine	92	Lysine	Positively Charged	135	
pRA064	CH_3	67	Non-polar	Alanine	125	Serine	Polar	73	

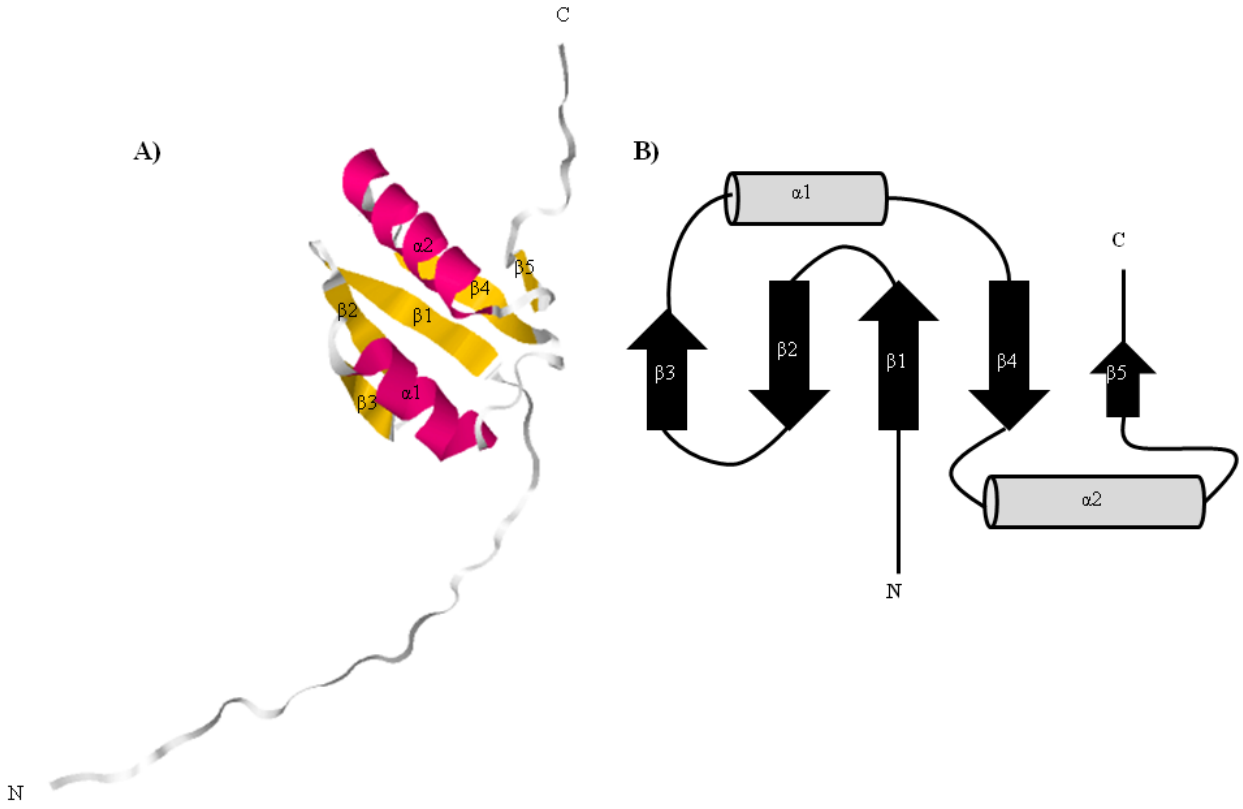
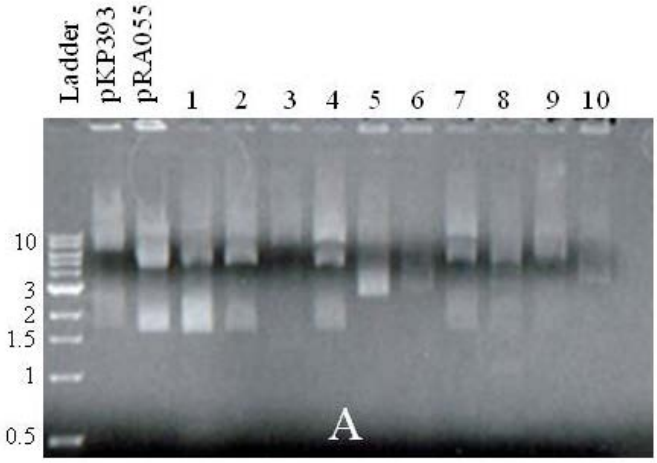
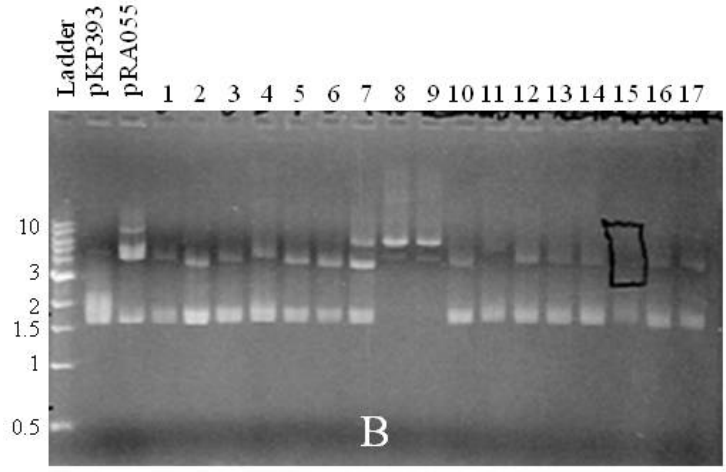


Figure 3: Structure of *E. coli* ExbD C-terminus domain. A) The solved crystal structure of the *E. coli* ExbD C-terminus (Source: Protein Data Bank code 2PFU). The figure is modified with labels of the N- and C- termini, the alpha helices, and the beta sheets. B) Cartoon ribbon depiction of the secondary structure of the *E. coli* ExbD C-terminus. Labeled are: the N-and C-termini, the alpha helices (which are shown as cylinders), and the beta sheets (which are shown as arrows).

Construct Formation

A PCR-based strategy was used to create three mutant clones with site specific mutations. PCR primers were designed to make specific codon mutations in the *exbD E. coli* gene to encode amino acids found in *Y. enterocolitica*. pKP393 was purified, diluted, and used as the DNA template for an extra-long polymerase chain reaction (XL PCR). The resultant DNA was purified following the PCR Purification Spin Protocol (Qiagen, Hilden, Germany). Each construct was analyzed using restriction enzyme recognition sites that were built into each primer set. The samples were treated with DNA ligase and transformed into NEB 5-alpha cells. The colonies were then screened using an Alkaline lysis: Mini-preparation of plasmid DNA procedure as described by Sambrook et al. followed by restriction mapping (1989) (Figure 4). For ExbD(N78D, D79Q, P80Q, T82D, D83R), sixty-three colonies were screened, three showed the predicted pattern of having the insert and were selected for sequence analysis; however, the results of the sequence data showed all three samples had a frameshift at the mutation site, suggesting that the primers were the cause of the frameshift. With the new designed primers, ten colonies were screened and four showed the predicted pattern of having the insert and were selected for sequence analysis. The results of the sequence data revealed one sample showed the predicted sequence. For ExbD(A92K), thirty colonies were screened and three showed the predicted pattern of having the insert and were selected for sequence analysis. The results of the sequence data revealed all three samples showed the predicted sequence. For ExbD(A125S), sixty-three colonies were screened and six showed the predicted pattern of having the insert and were selected for sequence analysis. The results of the sequence data revealed two samples showed the predicted sequence. Figure 5 shows the resulting sequences of the constructs after the mutations.


Ladder
pKP393
pRA055
1 2 3 4 5 6 7 8 9 10
10
3
2
1.5
1
0.5
A


Ladder
pKP393
pRA055
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
10
3
2
1.5
1
0.5
B

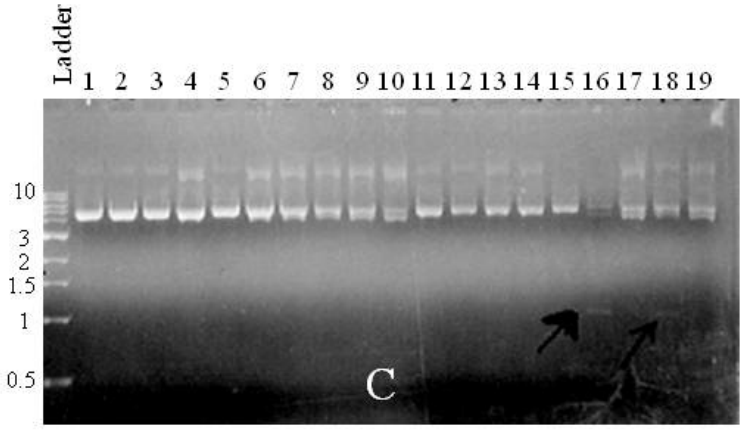

Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
10
3
2
1.5
1
0.5
C

Figure 4: Restriction mapping of each construct. Representative 1.0% (w/v) agarose gels showing the restriction mapping of A) ExbD(N78D, D79Q, P80Q, T82D, D83R), B) ExbD(A92K), and C) ExbD(A125S). A 1 kb ladder was used as a size standard with the lengths indicated in kb on the left of each gel. The two controls, pKP393 (*E. coli* ExbD) and pRA055 (*Y. enterocolitica* ExbD) are next to the 1 kb ladder in A) and B). If there was an insert in ExbD(N78D, D79Q, P80Q, T82D, D83R), the fragments would show the band pattern of pRA055. Lanes 1, 2, 4, and 7 showed the predicted pattern. If there was an insert in ExbD(A92K), the fragments would show the pattern of three bands at 1.6 kb. Lanes 1, 4, and 7 showed the predicted pattern. If there was an insert in ExbD(A125S), the fragments would show the pattern of one band at 4.2 kb and one band at 0.8 kb. Lanes 16 and 18 showed the predicted pattern.

A)

E. coli (pKP393)

226 ATC GGT AAC GAT CCG GTC ACC GAT GAA ACA ATG 258
76 I G N D P V T D E T M 86

pRA062

226 ATC GGT GAT CAG CAG GTT GAC CGT GAA ACA ATG 258
76 I G D Q Q V D R E T M 86

Y. enterocolitica (pRA055)

226 GTT GGC GAT CAG CAG GTT GAC CGT GAG ACA CTG 258
76 V G D Q Q V D R E T L 86

B)

E. coli (pKP393)

262 ACG GCG TTG AAT GCG TTA ACC GAA GGC 288
88 T A L N A L T E G 96

pRA063

262 ACG GCG TTG AAT AAA TTA ACC GAA GGC 288
88 T A L N K L T E G 96

Y. enterocolitica (pRA055)

262 GCG GCT CTG GAT AAA GTG ACC CAA TCT 288
88 A A L D K V T Q S 96

C)

E. coli (pKP393)

361 ACG CTG CAT CAG GCG GGT TAC CTG AAG 387
121 T L H Q A G Y L K 129

pRA064

361 ACG CTG CAT CAG TCG GGT TAC CTG AAG 387
121 T L H Q S G Y L K 129

Y. enterocolitica (pRA055)

361 GCA TTG CGT AAA TCG GGT TAT CTC AAG 387
121 A L R K S G Y L K 129

Figure 5: Predicted nucleotide and amino acid sequences of the constructs. The original cloned *E. coli* (blue) and *Y. enterocolitica* (red) *exbD* gene and gene products are displayed with the corresponding codons of the recombinant constructs encoded by plasmids A) pRA062, B) pRA063, and C) pRA064.

Structural Predictions

To compare the periplasmic space of ExbD of different homologues, modeling structures were generated using the program SWISS-MODEL (Figure 6). The program identifies templates based on Blast and HHblits and the quality of the template is estimated. The models are then generated off of a selected template. The template that was used to generate the models was the NMR structure (2PFU) of *E. coli* ExbD.

The results of the structural predictions show that between the homologues, the structures are very similar. However, the model of *Y. enterocolitica* has a lower quality model in a few areas. For the construct ExbD(N78D, D79Q, P80Q, T82D, D83R), *Y. enterocolitica* has a very low quality score for the model. For the construct ExbD(A92K), *Y. enterocolitica* has a medium score between a low and high quality model. For the construct ExbD(A125S), *Y. enterocolitica* has a very high quality score for the model. These scores indicate that the mutations would probably not cause a significant structural difference. A disadvantage of using a program to determine structural models is it only estimates where the new side chain will be from the template backbone. Also, the program is only a prediction of structural orientation; it cannot show the protein's *in vivo* conformation or the protein's conformation when it is interacting with other proteins.

The solvent accessibility (SOA) for *E. coli* ExbD (Table 6) was generated from the SWISS-MODEL structural prediction. Solvent accessibility is whether or not a residue is near the surface of a protein, exposing it to a solvent (Goldman et al., 1998). If a residue is near the surface, then it could be a possible contact site. For the construct ExbD(N78D, D79Q, P80Q, T82D, D83R), only the first position that was mutated had a high solvent accessibility. This indicates that the overall mutation probably did not have an effect on surface contact sites. Position 92 has a high solvent accessibility which indicates for ExbD(A92K) the mutation could affect surface contact sites. Position 125 had a very low solvent accessibility which indicates for ExbD(A125S) the mutation probably would not affect surface contact sites.

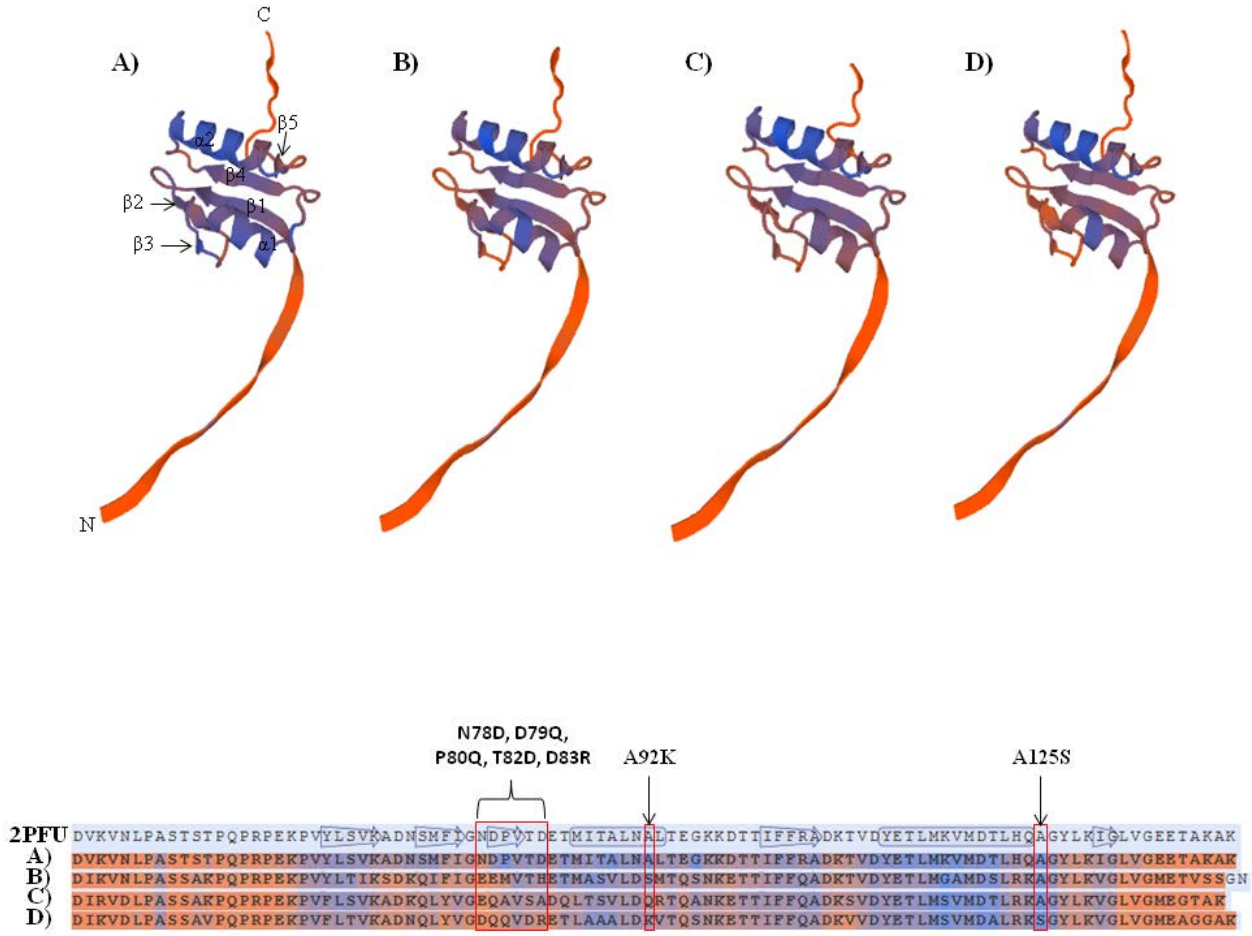


Figure 6: Structural predictions of the periplasmic space of ExbD. Modeling structure (SWISS-MODEL) from the NMR structure (2PFU) of ExbD of the periplasmic space (residues 44-141). Shown are the homologues of A) *Escherichia coli* W3110, B) *Proteus mirabilis* strain HI4320, C) *Serratia liquefaciens* ATCC 27592, and D) *Yersinia enterocolitica* subsp. *Enterocolitica* 8081. Figure (A) is modified with the labels of the N- and C- termini, the alpha helices, and the beta sheets, with figures (B), (C), and (D) in the same orientation. Underneath the 3D structures is the 2D alignment of the predicted models. The mutation sites for each construct are boxed off in the alignment. Colors, for both the 3D structures and the 2D

alignment, are based off the QMean4 score with red indicating a low quality model and blue indicating a high quality model.

Table 6: Solvent accessibility of *E. coli* ExbD. The solvent accessibility (SOA) was generated from the SWISS-MODEL structural prediction. Highlighted in red are the sites that were mutated in each construct. Higher numbers means higher solvent accessibility and lower numbers means lower solvent accessibility.

pRA062		pRA063		pRA064	
AA	**SOA**	**AA**	**SOA**	**AA**	**SOA**
I76	0.09	T88	0.56	T121	0.21
G77	0.38	A89	0.09	L122	0.06
N78	0.76	L90	0.01	H123	0.38
D79	0.21	N91	0.33	Q124	0.63
P80	0.38	A92	0.71	A125	0.12
V81	0.13	L93	0.40	G126	0.15
T82	0.42	T94	0.08	Y127	0.01
D83	0.18	E95	0.56	L128	0.74
E84	0.57	G96	0.16	K129	0.36
T85	0.30				
M86	0.02				

Functional Analysis: Spot Titer Assay

The function of each construct was assessed by a spot titer assay of sensitivity to four group B colicins, M, B, Ia, and D, which require the TonB system to enter and kill the bacteria (Figure 7 and Table 7). The positive control allowed import of all the colicins tested and the negative control did not show sensitivity to any of the colicins. Between the two controls, pKP393 (*E. coli* ExbD) and pRA055 (*Y. enterocolitica* ExbD) there was a difference between colicins B and D with pRA055 being more sensitive. If the mutations affected the function of *E. coli* ExbD, then the results of the constructs would be similar to pRA055 (*Y. enterocolitica* control).

Comparing the results of the spot titer assay from the first construct, ExbD(N78D, D79Q, P80Q, T82D, D83R), there was no difference in sensitivity to colicin M and colicin Ia between all of the controls and this construct. For colicins B and D, this construct was at equal levels of sensitivity with pKP393 (*E. coli* ExbD control) and the positive control and was sensitive to a higher dilution than pRA055 (*Y. enterocolitica* ExbD control). Comparing the amino acid properties of the sites chosen indicate the mutations would have caused *E. coli* ExbD to be less efficient like the *Y. enterocolitica* ExbD control. However, the functional assay results suggest that the mutations made to this construct did not have an effect on the function of ExbD.

Comparing the results of the spot titer assay from the second construct, ExbD(A92K), there was no difference in sensitivity to colicin M and colicin Ia between all of the controls and this construct. ExbD(A92K) was sensitive to a higher dilution than pRA055 (*Y. enterocolitica* ExbD control) to colicin B and colicin D. This construct was at equal levels of sensitivity with pKP393 (*E. coli* ExbD control) for both colicin B and colicin D and the positive control for

colicin B. However, ExbD(A92K) was sensitive to a lower dilution than the positive control for colicin D. Comparing the amino acid properties of the site chosen indicates the mutation could have had an effect on the alpha-helix folding, interfering with ExbD function. However, the functional assay results suggest that the mutation did not have a significant effect on the function of ExbD.

Comparing the results of the spot titer assay from the third construct, ExbD(A125S), there was no difference in sensitivity to colicin M and colicin Ia between all of the controls and this construct. This construct was at equal levels of sensitivity with pKP393 (*E. coli* ExbD control) and the positive control for both colicin B and colicin D. This construct was sensitive to a higher dilution than pRA055 (*Y. enterocolitica* ExbD control) to colicin B and colicin D. With this site having an alanine across all the other homologues except *Y. enterocolitica*, this residue was believed to be an important site potentially causing *Y. enterocolitica* ExbD to engage TonB less efficiently. However, the functional assay results suggest that this mutation did not have an effect on the function of ExbD.

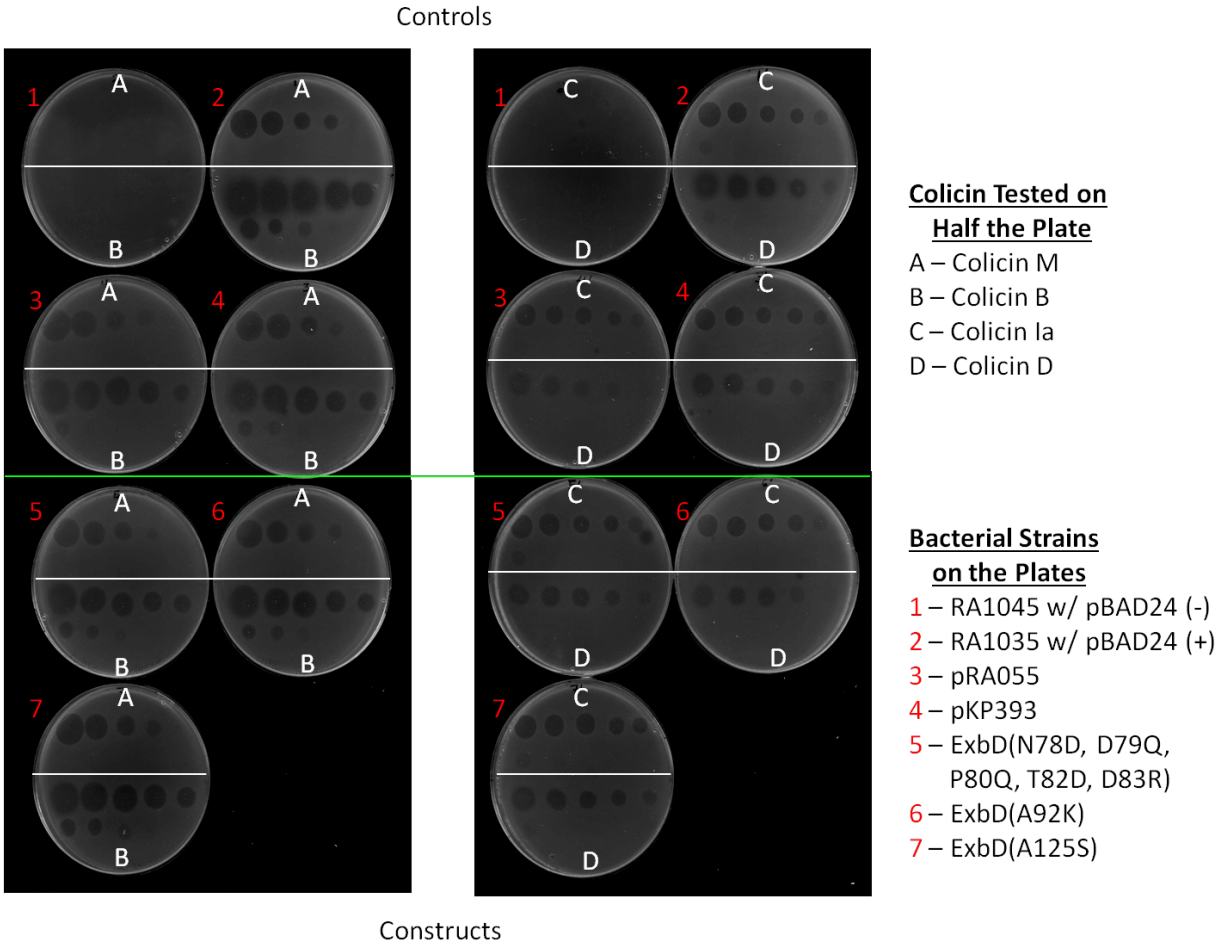


Figure 7: Comparison of constructs in this study to controls in the spot titer assay. The most representative plate used in the spot titer assay showing the zone of clearances. The red numbers are the bacterial strains that are on the plates. The white letters are the colicin that was used in the test. The circles inside the plates are the zones of clearance (which indicates the absence of cell growth in the presence of the test agent).

Table 7: Colicin spot titer assay results. The number recorded for each of three sets is the highest five-fold dilution with a zone of clearing with R indicating resistance.

	Colicin M	Colicin B	Colicin Ia	Colicin D
- Control	R, R, R	R, R, R	R, R, R	R, R, R
+ Control	-3, -3, -3	-7, -8, -8	-5, -5, -5	-5, -5, -5
pKP393 (*E. coli* ExbD)	-3, -3, -3	-7, -7, -7	-5, -5, -5	-4, -4, -5
pRA055 (*Y. ent* ExbD)	-3, -3, -3	-6, -6, -6	-5, -5, -5	-3, -3, -3
ExbD (N78D, D79Q, P80Q, T82D, D83R)	-3, -3, -3	-7, -7, -7	-5, -5, -5	-4, -5, -5
ExbD (A92K)	-3, -3, -3	-7, -7, -7	-5, -5, -5	-4, -4, -4
ExbD (A125S)	-3, -3, -3	-7, -7, -8	-5, -5, -5	-5, -5, -5

CHAPTER IV. DISCUSSION

ExbD is important in the TonB energy transduction complex. It is needed to gather energy from the PMF and facilitates conformational changes in the TonB C-terminus (Brinkman & Larsen, 2008; Ollis & Postle, 2012; Ollis et al., 2012). In this study, site-directed mutagenesis was performed on possible interaction sites to create three constructs that selected specific *E. coli exbD* codons and were replaced with *Y. enterocolitica exbD* codons. The codons were chosen by identifying residues that diverged significantly in the ExbD protein compared to homologues in the family *Enterobacteriaceae*. The goal was to determine possible interaction sites between ExbD and TonB. My results showed that two of these codon changes did not significantly affect the ability of ExbD to support TonB-dependent uptake of colicins, while a third codon change reduced sensitivity to one of the four colicins tested.

My results show that the mutation for construct ExbD(A92K) possibly had an effect on the efficiency of ExbD at supporting TonB function. ExbD(A92K) was selected because it diverged from the homologues, which was predicted to possibly cause an effect on the ability of ExbD to support TonB. Both the amino acid explorer common substitutions and the structural prediction suggested the mutation could cause a disruption in the folding pattern of ExbD causing it to engage TonB less efficiently. The solvent accessibility data shows the residue is near the surface, indicating the mutation could potentially affect surface contact sites, disrupting the efficiency to engage with TonB. The construct was tested using a functional analysis and confirmed that the mutation potentially did affect surface contact sites because ExbD(A92K) was sensitive to a lower dilution than the positive control for colicin D.

Residue 92 of ExbD is within a 30 residue region (residues 92 to 121) that has been shown to be important for ExbD protein-protein interactions (Ollis et al., 2012). Specifically, A92 has been show to be a site of *in vivo* ExbD homodimeric and heterodimeric interaction with TonB (Ollis et al., 2009). ExbD homodimers were able to form with an inactive TMD but the heterodimeric complex with TonB was unable to form unless both TMDs of both proteins were functional (Ollis et al., 2009). Ollis and Postle have suggested that multiple interfaces are involved in both homodimerization of ExbD and heterodimeric interactions with TonB (2011, 2012). Since this site mutation is suggested to be part of the multiple interfaces for interactions, then the mutation A92K in pRA063 could have hindered the homodimerization of ExbD or the heterodimeric interactions with TonB.

Since the mutation changed it to a *Y. enterocolitica* residue, I propose that ExbD(A92K) was able to still form ExbD homodimers. Since the two ExbD proteins would have the same mutated residue, they should be able to recognize it as a homodimerization site. However, ExbD(A92K) would not be as efficient at forming heterodimeric interactions with TonB because it would be a *Y. enterocolitica* residue in one of the ExbD recognition sites with an *E. coli* TonB recognition site. But because ExbD and TonB interact with multiple interfaces, the heterodimer was still able to form but just less efficiently. This would agree with a previous suggestion that ExbD homodimer formation has less stringent structural requirements than initial heterodimer formation (Ollis et al., 2012). If this proposition is true, regarding the TonB energization model (Ollis et al., 2012; Gresock et al., 2015), ExbD(A92K) would cause a slight disruption in stage II which is when ExbD and TonB form a heterodimer.

Ollis and Postle found ExbD A92C contacted two regions of TonB (2012). These regions were on each side of the area of TonB (residues 159-164) that makes contact with the TonB box of TBDTs BtuB (Cadieux et al., 2000) and FecA (Ogierman & Braun, 2003). This suggests that ExbD helps position TonB into a conformation that will interact with the TonB box of TBDTs. If ExbD(A92K) does not efficiently interact at those regions around TonB, then regarding the TonB energization model (Ollis et al., 2012; Gresock et al., 2015), ExbD(A92K) might cause a disruption in stage IV which is when TonB C-terminus interacts with a TBDT. If direct contact between ExbD and TonB is applied to the ROSET model (Klebba, 2016) then ExbD(A92K) could disrupt the rotational movement efficiency of TonB, which was generated by ExbB and ExbD. This would disrupt the proposed lateral movement of the TonB-ExbB-ExbD complex through the CM and the force causing a conformational change in the globular domain in the TBDT.

Celia et al. proposed that the ExbD periplasmic domain performs as a cation-selective filter (2016). Since the mutation of ExbD(A92K) is in the periplasmic domain, it could interfere with the cation-selective filtering. If ExbD(A92K) interferes with the cation-selective filtering, then it could disrupt the proposed 'electrostatic piston' and the 'rotational' models of how the TonB energy transduction complex harnesses the PMF to produce energy (Celia et al., 2016).

A previous study by Koebnik et al. took a *Y. enterocolitica tonB* gene and complemented it with an *E. coli tonB* mutant (1992). In this study they found that the *Y. enterocolitica* TonB was unable to function as well as the *E. coli* TonB. The authors believed the reason for this was due to the TonB boxes of the TBDTs and colicins diverged from the TonB box consensus sequence. To expand on their findings, comparing my results to theirs, I believe that residues

that diverged in the TonB protein were unable to recognize the *E. coli* ExbD surface contact sites or the *E. coli* TonB boxes. This would disrupt stage II and stage IV in the TonB energization model preventing the efficiency of the system.

My results show that the mutations for construct ExbD(N78D, D79Q, P80Q, T82D, D83R) and ExbD(A125S) did not have a considerable effect on the ability of ExbD to support TonB activity as measured by colicin sensitivity. ExbD(N78D, D79Q, P80Q, T82D, D83R) was selected because it diverged from the homologues, which was predicted to possibly cause an effect on the ability of ExbD to support TonB. The amino acid explorer common substitutions suggested three of the five sites (P80Q, T82D, and D83R) could cause a disruption in the folding pattern of ExbD with the structural prediction also suggesting the mutations could disrupt the folding pattern. However, the solvent accessibility data showed only one of the mutated residues is near the surface, indicating the mutation region would probably not disrupt surface contact sites. The construct was tested using a functional analysis which confirmed with the models that the mutation did not have an effect on the ability of ExbD to support TonB activity.

ExbD(A125S) was selected because *Y. enterocolitica* is the only homologue out of the family *Enterobacteriaceae* that does not have an alanine at position 125. However, the amino acid explorer common substitutions suggested this mutation would not cause an effect on ExbD. Both the structural predictions and the solvent accessibility data supported this theory suggesting it would not disrupt the folding pattern and that also the residue is buried. This indicates the mutation would probably not disrupt surface contact sites. The construct was tested using a functional analysis which confirmed with the models that the mutation did not have an effect on the ability of ExbD to support TonB activity.

The results of this study did not specifically determine contact sites between ExbD and TonB. However, this study did gather useful knowledge about species divergence in *Enterobacteriaceae*. The results show that even though the folding structure was not inhibited by the mutagenesis, it still led to some minute surface changes that could affect binding contact. These results show that the minute changes can affect the ability of ExbD to function efficiently.

Prospectus

This study provided evidence for specific residues of ExbD that diverged from the family *Enterobacteriaceae* and showed they did not significantly affect the efficiency of ExbD to support TonB function. However, the mutations of the divergent residues did show how they could disrupt surface contact sites. This study also offered a different approach at determining important residues. The first study for future research is to make the reciprocal alteration of this study: i.e., to make three constructs replacing specific *Y. enterocolitica exbD* codons with *E. coli* codons then complementing them with a Δ*exbD* strain. This would provide more evidence if these sites are important for ExbD to function efficiently. Another future study should include identification and evaluation of residues that diverge in the TonB protein across the family *Enterobacteriaceae* and create constructs with those specific mutations to determine their efficiency at contacting ExbD and TBDTs.

REFERENCES

Ackermann HW. 2011. Bacteriophage taxonomy. Microbiology Australia. **32**:90-94.

Aguirre JD, Clark HM, Mcllvin M, Vazquez C, Palmere SL, Grab DJ, Seshu J, Hart PJ, Saito M, Culotta AC. 2013. A manganese-rich environment supports superoxide dismutase activity in a Lyme disease pathogen, *Borrelia burgdorferi*. J. Biol. Chem. **288**:8468-8478.

Andrews SC, Robinson AK, Rodriguez-Quinones F. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27**:215-237.

Angerer A, Gaisser S, Braun V. 1990. Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic binding-protein-dependent iron transport mechanism. J. Bacteriol. **172**:572-578.

Archibald F. 1983. *Lactobacillus plantarum*, an organism not requiring iron. FEMS Microbiol. Lett. **19**:29-32.

Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: A web-based environment for protein structure homology modeling. Bioinformatics. **22**:195-201.

Babu MM, Priya ML, Selvan AT, Madera M, Gough J, Aravind L, Sankaran K. 2006. A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. J. Bacteriol. **188**:2761-2773.

Baker K, Postle K. 2013. Mutations in *Escherichia coli* ExbB transmembrane domains identify scaffolding and signal transduction functions and exclude participation in a proton pathway. J. Bacteriol. **195**:2898-2911.

Benedetti H, Frenette M, Baty D, Knibiehler M, Pattus F, Lazdunski C. 1991. Individual domains of colicins confer specificity in colicin uptake, in pore-properties and in immunity requirement. J. Mol. Biol. **217**:429-439.

Benkert P, Biasini M, Schwede T. 2011. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics. **27**:343-350.

Betton JM. 2007. Periplasmic chaperones and peptidyl-prolyl isomerases. In: *The periplasm* (Ehrmann M. ed). Washington, D.C.:ASM Press.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T. 2014. SWISS-MODEL: Modeling protein tertiary and quaternary structure using evolutionary information. Nucl. Acids Res. doi: 10.1093/nar/gku340.

Blanvillain S, Meyer D, Boulanger A, Lautier M, Guynet C, Denance N, Vasse J, Lauber E, Arlat M. 2007. Plant carbohydrate scavenging through TonB-dependent receptors: A feature shared by phytopathogenic and aquatic bacteria. PLoS one. **2**, e224.

Bohin JP. 2000. Osmoregulated periplasmic glucans in Proteobacteria. FEMS Lett. **186**:11.

Bradbeer C. 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J. Bacteriol. **175**:3146-3150.

Braun V, Gaisser S, Herrmann C, Kampfenkel K, Killmann H, Traub I. 1996. Energy-coupled transport across the outer membrane of *Escherichia coli*: ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. J. Bacteriol. **178**:2836-2845.

Brinkman KK, Larsen RA. 2008. Interactions of the energy transducer TonB with noncognate energy-harvesting complexes. J. Bacteriol. **190**:421-427.

Bulathsinghala CM, Jana B, Baker KR, Postle K. 2013. ExbB cytoplasmic loop deletions cause immediate, proton motive force-independent growth arrest. J. Bacteriol. **195**:4580-4591.

Butler K. 2013. Functional complementation of ExbD *E. coli* by homologous ExbD genes. MS Thesis, Bowling Green State University, Bowling Green, Ohio.

Cadieux N, Bradbeer C, Kadner RJ. 2000. Sequence changes in the ton box region of BtuB affect its transport activities and interaction with TonB protein. J. Bacteriol. **182**:5954-5961.

Cadieux N, Kadner RJ. 1999. Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. Proc. Natl. Acad. Sci. USA. **96**:10673-10678.

Carpenter C, Payne SM. 2014. Regulation of iron transport systems in *Enterobacteriaceae* in response to oxygen and iron availability. J. Inorg. Biochem. **133**:110-117.

Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, Riley M, Slatin S, Cavard D. 2007. Colicin Biology. Microbiol. Mol. Biol. Rev. **71**:158-229.

Cascales E, Lloubes R, Sturgis JN. 2001. The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB. Mol. Microbiol. **42**:795-807.

Chang C, Mooser A, Pluckthun A, Wlodawer A. 2001. Crystal structure of the dimeric C-terminal domain of TonB reveals a novel fold. J. Biol. Chem. **276**:27535-27540.

Chimento DP, Kadner RJ, Wiener MC. 2005. Comparative structural analysis of TonB-dependent outer membrane transporters: Implications for the transport cycle. Proteins: Structure, Function, and Bioinformatics. **59**:240-251.

Chung CT, Niemela SL, Miller RH. 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86**:2172-2175.

Davies JK, Reeves P. 1975a. Genetics of resistance to colicins in *Escherichia coli* K-12: Cross-resistance among colicins of group A. J. Bacteriol. **123**:102-117.

Davies JK, Reeves P. 1975b. Genetics of resistance to colicins in *Escherichia coli* K-12: Cross-resistance among colicins of group B. J. Bacteriol. **123**:96-101.

Delcour AH. 2009. Outer membrane permeability and antibiotic resistance. Biochim. Biophys. Acta. **1794**:808-816.

Devanathan S, Postle K. 2007. Studies on colicin B translocation: FepA is gated by TonB. Mol. Microbiol. **65**:441-453.

Doolittle WF. 1999. Phylogenetic classification and the universal tree. Science. **284**:2124-2128.

Fairman JW, Noinaj N, Buchanan SK. 2011. The structural biology of β-barrel membrane proteins: a summary of recent reports. Curr. Opin. Struct. Biol. **21**:523-531.

Faraldo-Gomez JD, Sansom MS. 2003. Acquisition of siderophores in gram-negative bacteria. Nature Rev. Mol. Cell Biol. **4**:105-116.

Ferguson AD, Deisenhofer J. 2002. TonB-dependent receptors - Structural perspectives. Biochim. Biophys. Acta. **1565**:318-332.

Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W. 1998. Siderophore-mediated iron transport: Crystal structure of FhuA with bound lipopolysaccharide. Science. **282**:2215-2220.

Freed DM, Lukasik SM, Sikora A, Mokdad A, Cafiso DS. 2013. Monomeric TonB and the Ton box are required for the formation of a high-affinity transporter-TonB complex. Biochemistry. **52**:2638-2648.

Galdiero S, Galdiero M, Pedone C. 2007. β-barrel membrane bacterial proteins: Structure, function, assembly, and interaction with lipids. Curr. Protein Pept. Sci. **8**:63-82.

Garcia-Herrero A, Peacock RS, Howard SP, Vogel HJ. 2007. The solution structure of the periplasmic domain of the TonB system ExbD protein reveals an unexpected structural homology with siderophore-binding proteins. Mol. Microbiol. **66**:872-889.

Gelfand MS, Rodionov DA. 2007. Comparative genomics and functional annotation of bacterial transporters. Phys. Life Rev. **5**:22-49.

Ghosh J, Postle K. 2004. Evidence for dynamic clustering of carboxy-terminal aromatic amino acids in TonB-dependent energy transduction. Mol. Microbiol. **51**:203-213.

Glauner B. 1988. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal. Biochem. **172**:451-464.

Goldman N, Thorne JL, Jones DT. 1998. Assessing the impact of secondary structure and solvent accessibility of protein evolution. Genetics. **149**:445-458.

Graham LL, Harris R, Villiger W, Beveridge TJ. 1991. Freeze-substitution of gram-negative eubacteria: General cell morphology and envelope profiles. J. Bacteriol. **173**:1623-1633.

Gram HCJ. 1884. Ueber die isolirte Färbung der Schizomyceten: in Schnitt-und Trockenpräparaten. Fortschr Med. **2**:185-189.

Gresock MG, Kastead KA, Postle K. 2015. From homodimer to heterodimer and back: Elucidating the TonB energy transduction cycle. J. Bacteriol. **197**:3433-3445.

Gudmundsdottir A, Bell PE, Lundrigan MD, Bradbeer C, Kadner RJ. 1989. Point mutations in a conserved region (TonB box) of *Escherichia coli* outer membrane protein BtuB affect vitamin B12 transport. J. Bacteriol. **171**:6526-6533.

Guest RL, Raivio TL. 2016. Role of the gram-negative envelope stress response in the presence of antimicrobial agents. Trends Microbiol. **24**:377-390.

Guzman L, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177**:4121-4130.

Halliwell B, Gutteridge MC. 1984. Oxygen toxicity, oxygen radicals, transition metals and decease. J. Biochem. **219**:1-14.

Hancock RE, Braun V. 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophage T1 and φ80 to *Escherichia coli*. J. Bacteriol. **125**:409-415.

Hannavy K, Barr GC, Dorman CJ, Adamson J, Mazengera LR, Gallagher MP, Evans JS, Levine BA, Trayer IP, Higgins CF. 1990. TonB protein of *Salmonella typhimurium*. A model for signal transduction between membranes. J. Mol. Biol. **216**:897-910.

Hantke K. 1981. Regulation of ferric iron transport in *Escherichia coli* K-12: Isolation of a constitutive mutant. Mol. Gen. Genet. **182**:288-292.

Hantke K. 1987. Ferrous iron transport mutants in *Escherichia coli* K-12. FEMS Microbiol. Lett. **44**:53-57.

Hantke K, Braun V. 1973. Covalent binding of lipid to protein. Eur. J. Biochem. **34**:284-296.

Higgs PI, Larsen RA, Postle K. 2002. Quantification of known components of the *Escherichia coli* TonB energy transduction system: TonB, ExbB, ExbD, and FepA. Mol. Microbiol. **44**:271-281.

Hobot JA, Carlemalm E, Villiger W, Kellenberger E. 1984. Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. **160**:143-152.

Ivanov, YV. 2012. The roles of moron genes in the *Escherichia coli* enterobacteria phage phi-80. Ph. D. Dissertation, Bowling Green State University, Bowling Green, Ohio.

Jakes KS, Cramer WA. 2012. Border crossings: Colicins and transporters. Annu. Rev. Genet. **46**:209-231.

Jordan LD, Zhou Y, Smallwood CR, Lill Y, Ritchie K, Yip WT, Newton SM, Klebba PE. 2013. Energy-dependent motion of TonB in the gram-negative bacterial inner membrane. Proc. Natl. Acad. Sci. USA. **110**:11553-11558.

Kadner RJ. 1996. Cytoplasmic membrane. In: *Escherichia coli and Salmonella, cellular and molecular biology* (2nd ed). Washington, D.C.:ASM Press.

Kamio Y, Nikaido H. 1976. Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase C and cyanogens bromide activated dextran in the external medium. Biochemistry. **15**:2561-2570.

Kammler M, Schon C, Hantke K. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. **175**:6212-6219.

Kampfenkel K, Braun V. 1992. Membrane topology of the *Escherichia coli* ExbD protein. J. Bacteriol. **174**:5485-5487.

Kampfenkel K, Braun V. 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **268**:6050-6057.

Kaserer WA, Jiang X, Xiao Q, Scott DC, Bauler M, Copeland D, Newton SM, Klebba PE. 2008. Insight from TonB hybrid proteins into the mechanism of iron transport through the outer membrane. J. Bacteriol. **190**:4001-4016.

Klebba PE. 2016. ROSET model of TonB action in gram-negative bacterial iron acquisition. J. Bacteriol. **198**:1013-1021.

Kodding J, Killig F, Polzer P, Howard SP, Diederichs K, Welte W. 2005. Crystal structure of a 92-residuce C-terminal fragment of TonB from *Escherichia coli* reveals significant conformational changes compared to structures of smaller TonB fragments. J. Biol. Chem. **280**:3022-3028.

Koebnik R, Bäumler AJ, Heesemann J, Braun V, Hantke K. 1993. The TonB protein of Yersinia enterocolitica and its interactions with TonB-box proteins. Mol. Gen. Genet. MGG. **237**:152-160.

Larsen RA, Chen G, Postle K. 2003. Performance of standard phenotypic assays for TonB activity, as evaluated by varying the level of functional, wild-type TonB. J. Bacteriol. **185**:4699-4706.

Larsen RA, Deckert GE, Kastead KA, Devanathan S, Keller KL, Postle K. 2007. His20 provides the sole functionally significant side chain in the essential TonB transmembrane domain. J. Bacteriol. **189**:2825-2833.

Larsen RA, Foster-Hartnett D, McIntosh MA, Postle K. 1997. Regions of *Escherichia coli* TonB and FepA proteins essential for in vivo physical interactions. J. Bacteriol. **179**:3213-3221.

Larsen RA, Thomas MG, Postle K. 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol. Microbiol. **31**:1809-1824.

Larsen RA, Wood GE, Postle K. 1993. The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein. Mol. Microbiol. **10**:943-953.

Lazzaroni JC, Germon P, Ray M-C, Vianney A. 1999. The Tol proteins of *Escherichia coli* and their involvement in the uptake of biomolecules and outer membrane stability. FEMS Microbiol. Lett. **177**:191-197.

Leduc M, Frehel C, Siegel E, Van Heijenoort J. 1989. Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli*. J. Gen. Microbiol. **135**:1243-1254.

Lee HS, Abdelal AH, Clark MA, Ingraham JL. 1991. Molecular characterization of nosA, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to make copper-containing N2O reductase. J. Bacteriol. **173**:5406-5413.

Leon-Sicairos N, Reyes-Cortes, R, Guadron-Llanos AM, Maduena-Molina J, Leon-Sicairos C, Canizalez-Roman A. 2015. Strategies of intracellular pathogens for obtaining iron from the environment. BioMed Res. Int. **215**: doi:10.1155/2015/476534.

Locher KP, Rees B, Koebnik R, Mitschler A, Moulinier L, Rosenbusch JP, Moras D. 1998. Transmembrane signaling across the ligand-gated FhuA receptor: Crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell. **95**:771-778.

Matias VR, Al-Amoudi A, Dubochet J, Beveridge TJ. 2003. Cyro-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. J. Bacteriol. **185**:6112-6118.

Mende J, Braun V. 1990. Import-defective colicin B derivatives mutated in the TonB box. Mol. Microbiol. **4**:1523-1533.

Miller JH. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Miller KJ, Kennedy EP, Reinhold VN. 1986. Osmotic adaptation by gram-negative bacteria: Possible role for periplasmic oligosaccharides. Science. **231**:48-51.

Mokhele K, Tang YJ, Clark MA, Ingraham JL. 1987. A *Pseudomonas stutzeri* outer membrane protein inserts copper into N2O reductase. J. Bacteriol. **169**:5721-5726.

Neugebauer H, Herrmann C, Kammer W, Schwarz G, Nordheim A, Braun V. 2005. ExbBD-dependent transport of maltodextrins through the novel MalA protein across the outer membrane of *Caulobacter crescentus*. J. Bacteriol. **187**:8300-8311.

Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **67**:593-656.

Nikaido V. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1-32.

Noinaj N, Guillier M, Barnard TJ, Buchanan SK. 2010. TonB-dependent transporters: Regulation, structure, and function. Annu. Rev. Microbiol. **64**:43-60.

Ogierman M, Braun V. 2003. Interactions between the outer membrane ferric citrate transporter FecA and TonB: Studies of the FecA TonB box. J. Bacteriol. **185**:1870-1885.

Ohno-Iwashita Y, Imahori K. 1980. Assignment of the functional loci in colicin E2 and E3 molecules by characterization of their proteolytic fragments. Biochemistry. **19**:652-659.

Okuda S, Tokuda H. 2011. Lipoprotein sorting in bacteria. Annu. Rev. Microbiol. **65**:239-259.

Oliver DB. Periplasm. In: *Escherichia coli* and *Salmonella*, cellular and molecular biology. (2nd ed). Washington DC: ASM Press.

Ollinger J, Song K, Antelmann H, Hecker M, Helmann JD. 2006. Role of the Fur regulon in iron transport in *Bacillus subtilis*. J. Bacteriol. **188**:3664-3673.

Ollis AA, Kumar A, Postle K. 2012. The ExbD periplasmic domain contains distinct functional regions for two stages in TonB energization. J. Bacteriol. **194**:3069-3077.

Ollis AA, Manning M, Held KG, Postle K. 2009. Cytoplasmic membrane protonmotive force energizes periplasmic interactions between ExbD and TonB. Mol. Microbiol. **73**:466-481.

Ollis AA, Postle K. 2011. The same periplasmic ExbD residues mediate *in vivo* interactions between ExbD homodimers and ExbD-TonB heterodimers. J. Bacteriol. **193**:6852-6863.

Ollis AA, Postle K. 2012. Identification of functionally important TonB-exbD periplasmic domain interactions *in vivo*. J. Bacteriol. **194**:3078-3087.

Panina, EM, Mironov AA, Gelfand MS. 2001. Comparative analysis of FUR regulons in gamma-proteobacteria. Nucleic Acids Res. **29**:5195-5206.

Pawelek PD, Croteau N, Ng-Thow-Hing C, Khursigara CM, Moiseeva N, Allaire M, Coulton JW. 2006. Structure of TonB in complex with FhuA, *E. coli* outer membrane receptor. Science. **312**:1399-1402.

Peacock RS, Weljie AM, Howard SP, Price FD, Vogel HJ. 2005. The solution structure of the C-terminal domain of TonB and interaction studies with TonB box peptides. J. Mol. Biol. **345**:1185-1197.

Posey JE, Gherardini FC. 2000. Lack of a role for iron in the Lyme disease pathogen. Science. **288**:1651-1653.

Postle K. 1990. Aerobic regulation of the *Escherichia coli tonB* gene by changes in iron availability and the *fur* locus. J. Bacteriol. **172**:2287-2293.

Postle K, Kastead KA, Gresock MG, Ghosh J, Swayne CD. 2010. The TonB dimeric crystal structures do not exist *in vivo*. mBio. **1**:1-7.

Pressler U, Braun V, Wittmann-Liebold B, Benz R. 1986. Structural and functional properties of colicin B. J. Biol. Chem. **261**:2654-2659.

Quintela JC, Caparros M, De Pedro MA. 1995. Variability of peptidoglycan structural parameters in gram-negative bacteria. FEMS Microbiol. Lett. **125**:95-100.

Raetz CR, Whitfield C. 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. **71**:635-700.

Rodionov DA, Hebbeln P, Gelfand MS, Eitinger T. 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: Evidence for a novel group of ATP-binding cassette transporters. J. Bacteriol. **188**:317-327.

Rodionov DA, Vitreschak AG, Mironov AA, Gelfand MS. 2003. Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J. Biol. Chem. **278**:41148-41159.

Rodionov DA, Vitreschak AG, Nironov AA, Gelfand MS. 2002. Comparative genomics of thiamin biosynthesis in prokaryotes. New genes and regulatory mechanisms. J. Biol. Chem. **277**:48949-48959.

Roof SK, Allard JD, Bertrand KP, Postle K. 1991. Analysis of *Escherichia coli* TonB membrane topology by use of PhoA fusions. J. Bacteriol. **173**:5554-5557.

Ruiz N, Kahne D, Silhavy TJ. 2006. Advances in understanding bacterial outer-membrane biogenesis. Nat. Rev. Microbiol. **4**:57-66.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sauter A, Howard SP, Braun V. 2003. In vivo evidence for TonB dimerization. J. Bacteriol. **185**:5747-5754.

Schauer K, Gouget B, Carriere M, Labigne A, de Reuse H. 2007. Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery. Mol. Microbiol. **63**:1054-1068.

Schauer K, Rodionov DA, de Reuse H. 2008. New substrates for TonB-dependent transport: Do we only see the 'tip of the iceberg'?. Trends Biochem. Sci. **33**:330-338.

Schoffler H, Braun V. 1989. Transport across the outer membrane of Escherichia coli K-12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217**:378-383.

Schramm E, Mende J, Braun V, Kamp RM. 1987. Nucleotide sequence of the colicin B activity gene *cba*: Consensus pentapeptide among TonB-dependent colicins and receptors. J. Bacteriol. **169**:3350-3357.

Schulz GE. 2002. The structure of bacterial outer membrane proteins. Biochim. Biophys. Acta. **1565**:308-317.

Shultis DD, Purdy MD, Banchs CN, Wiener MC. 2006. Outer membrane active transport: Structure of the BtuB:TonB complex. Science. **312**:1396-1399.

Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb. Perspect. Biol. **2**:a000414.

Simpson BW, May JM, Sherman DJ, Kahne D, Ruiz N. 2015. Lipopolysaccharide transport to the cell surface: Biosynthesis and extraction from the inner membrane. Phil. Trans. R. Soc. B. doi:10.1098/rstb.2015.0029.

Singer N. 1972. The fluid mosaic model of the structure of cell membranes. Science. **175**:720-731.

Stevenson G, Neal B, Liu D, Hobbs M, Packer NH, Batley M, Redmond JW, Lindquist L, Reeves P. 1994. Structure of the O-antigen of *Escherichia coli* K-12 and the sequence of its rfb gene cluster. J. Bacteriol. **176**:4144-4156.

Stojiljkovic I, Baumler AJ, Hantke K. 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **236**:531-545.

Stolz B, Berg HC. 1991. Evidence for interactions between MotA and MotB, torque-generating elements of the flagellar motor of *Escherichia coli*. J. Bacteriol. **173**:7033-7037.

Sverzhinsky A, Chung JW, Deme JC, Fabre L, Levey KT, Plesa M, Carter DM, Lypaczewski P, Coulton JW. 2015. Membrane protein complex $ExbB_4$-$ExbD_1$-$TonB_1$ from *Escherichia coli* demonstrates conformational plasticity. J. Bacteriol. **197**:1873-1885.

Swayne C, Postle K. 2011. Taking the *Escherichia coli* TonB transmembrane domain "offline"? Nonprotonatable Asn substitutes fully for TonB His20. J. Bacteriol. **193**:3693-3701.

Tokuda H, Matsuyama SI. 2004. Sorting of lipoproteins to the outer membrane in *E. coli*. Biochim. Biophys. Acta. **1693**:5-13.

Tokuda H, Matsuyama SI, Tanaka-Masuda K. 2007. Structure, function, and transport of lipoproteins in *Escherichia coli*. In: *The periplasm* (Ehrmann M. ed). Washington, D.C.:ASM Press.

Vakharia-Rao H, Kastead KA, Savenkova MI, Bulathsinghala CM, Postle K. 2007. Deletion and substitution analysis of the *Escherichia coli* TonB Q160 region. J. Bacteriol. **189**:4662-4670.

Vollmer W, Bertsche U. 2008. Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. Biochim. Biophys. Acta. **1778**:1714-1734.

Vollmer W, Blanot D, de Pedro MA. 2008. Peptidoglycan structure and architecture. FEMS Microbiol. Rev. **32**:149-167.

von Heijne G. 2006. Membrane-protein topology. Nature Rev. Mol. Cell Biol. **7**:909-918.

Wandersman C, Delepelaire P. 2004. Bacterial iron sources: From siderophores to hemophores. Annu. Rev. Microbiol. **58**:611-647.

White D. 2000. *The Physiology and Biochemistry of Porkaryotes* (2nd ed.). New York: Oxford University Press.

White D. 2007. The Physiology and Biochemistry of Prokaryotes (3ed ed.) New York: Oxford University Press.

Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. **87**:4576-4579.

Wunsch P, Herb M, Wieland H, Schiek UM, Zumft WG. 2003. Requirements for CuA and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier *Pseudomonas putida*. J. Bacteriol. **185**:887-896.

Yang C, Rodionov DA, Li X, Laikova ON, Gelfand MS, Zagnitko OP, Romine MF, Obraztsova AY, Nealson KH, Osterman AL. 2006. Comparative genomics and experimental characterization of N-acetylglucosamine utilization pathway of *Shewanella oneidensis*. J. Biol. Chem. **281**:29872-29885.

Yoneyama H, Nakae T. 1996. Protein C (OprC) of the outer membrane of *Pseudomons aeruginosa* is a copper-regulated channel protein. Microbiology. **142**:2137-2144.