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Identification of a TolA Protein Binding Site for Bacterial Toxins

Monica Ferrante
monicaf@bgsu.edu

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Monica Ferrante

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Dr. Raymond Larsen, Department of Biological Sciences, Advisor
Dr. Neocles Leontis, Department of Chemistry, Advisor

Abstract

Group A colicins are proteinaceous bacteriocins encoded by plasmids that exploit the cellular envelope protein TolA to translocate the cell wall barrier and cellular envelope of the bacterium *Escherichia coli*. These colicins offer protocols for studying certain protein-protein interactions involved in such membrane transport functions. Previous experimentations suggest the carboxyl-terminal domain of TolA protein contains specific amino acid binding regions required for the translocation of group A colicins into *E. coli*. The amino acid sequence of this domain varies between *E. coli* and other gram-negative bacterial species. It has been suggested that this diversity could be utilized to identify specific TolA residues involved in the binding of colicins. Additionally, other *Enterobacteriaceae* species' TolA genes were cloned into a plasmid that is regulated by the monosaccharide sugar, arabinose. This plasmid was transformed into an *E. coli* strain that lacked a functional *tolA* gene. The plasmid was expressed in this strain. Homologous complementation of this phenotype lacking *tolA* expression has been demonstrated through resistance to the bile acid deoxycholate. The expression of this complemented phenotype through cloned *ΔtolA* genes demonstrates expression and translocation of TolA protein through the outer membrane into the periplasmic space. Bacterial strains possessing these constructs and functional TolA were tested for sensitivity against two group a colicins: A and N. Two such species, *Chronobacter muytjensii* and *Yersinia enterocolitica* complements the phenotype lacking functional TolA with their own encoded TolA proteins. These functional proteins grant sensitivity to various group A colicins, apart from colicin N. The TolA proteins of *C. muytjensii*, *E. coli, Y. enterocolitica* and the corresponding genetic sequences can be contrasted to highlight site of differing residues. These sites of genetic sequence divergence in *E. coli*, *C. muytjensii*, and *Y. enterocolitica* can be altered through mutagenesis to identify sequence domains required for colicin N binding to TolA, and thus import into the corresponding bacterium.

Introduction

Escherichia coli is one of the most prominently studied and thoroughly understood gram-negative bacteria. As a member of the vast *Enterobacteriaceae* family, *E. coli* appear bacillus-shaped and metabolically function as facultative anaerobes. As such, *E. coli* primarily survive the synthesis of ATP through aerobic respiration when oxygen is available, but may compensate for a lack of oxygen by fermenting available sugars to be used in glycolysis. Like other family members, *E. coli* is able to reduce nitrate to nitrite and exercise motility through the usage of flagella. One of the most prominent roles of *E. coli* is its vast types of symbiotic relationship with various higher-level organisms, such as humans, via the occupation of their digestive systems. While some *E. coli* strains have been accepted as commensals that prohibit other pathogenic bacteria from colonizing and help facilitate digestion, other strains have proven harmful and infections in various clinical settings. It is this significance of these strains that casts *E. coli* and other members of the gram-negative *Enterobacteriaceae* family, such as the *Enterobacter* genus, into the radars of researchers.

As is characteristic of the majority of gram-negative bacteria, *E. coli* possess a bi-layered membrane. The first layer consists of the outer membrane (OM) that exists as the most external membrane of the cell, acts as a barrier between cell contents and the environment, and facilitates a point of contact for the extracellular space. The second layer is the cytoplasmic membrane (CM) that acts as an internal membrane to contain the cell contents and separate them from the OM. An aqueous area separates these two membranes using a thin layer of peptidoglycan. This construction is unique to gram-negative bacteria as gram-positive bacteria do not possess an OM and compensate for this absence with a broad peptidoglycan layer. The two membranes differ in their structure to facilitate their unique functions. The OM is constituted by glycolipids, such as lipopolysaccharides (LPS) (Silhavy *et al*., 2010). This composition prevents membrane-damaging substances, such as antibiotics or detergents, from entering the cell (Nikaido, 2003). The CM is composed of a standard phospholipid bilayer and membranous

proteins. The CM is capable of synthesizing energy that facilitates the translocation of molecules through the cellular envelope via an ion-electrochemical gradient (Bauman, 2004).

Although the OM provides gram-negative bacteria with opportunities for survival not feasible for gram-positive bacteria, limitations do exist. One of the primary issues is the OM's inability to create its own energy. This restriction forces the OM, that requires energy for some transport of molecules through the membranes, to turn to the CM and the energy from the proton gradient of the proton motive force (Postle and Kadner, 2003). However, not all molecules require energy to be translocated. For example, hydrophilic molecules are able to enter the cell through porins that consist of beta barrel proteins. These molecules must be approximately 600 Daltons or less in order to diffuse. Other porins, such as outer membrane protein F (OmpF) and OmpC, exist to facilitate passive diffusion. Of course, not all molecules fall within 600 Daltons thus must be transported across the *E. coli* OM using pores capable of active transport. Various protein-systems have evolved to address the need for the OM's energy demand for active transport. Such protein systems occupy the periplasmic space to facilitate an interface between the OM and CM and share the CM's energy with the OM.

One such system that exists with various gram-negative bacteria is the Tol system. Although the physiological function of this system has not been fully determined, its function can be modeled after the TonB system which has been more thoroughly researched. Much of the Tol system has been predicted from the perspective of the TonB system. Both protein systems consist of three proteins fixated to the CM. ExbB, ExbD, and TonB exist in the TonB system and are paralogous to the TolQ, TolR, and TolA proteins of the Tol protein system (Postle and Larsen, 2007). ExbB consists of three segments that span the CM and an N-terminus in the periplasmic space that binds to the CM (Kampfenkel and Braun, 1993a). ExbB forms a complex with ExbD that utilizes the proton motive force. The TonB protein is also consists of three components. The N-terminus domain utilizes the ExbB/ExbD complex to allow TonB to interact with the CM (Krewulak and Vogel, 2011, Larsen and Postle, 2001, Lazar, 2014). The second domain fills the space between the OM and CM and consists of repeats of proline patterns (Brewer et al., 1990). The

final domain, the C-terminus, is ultimately the main point of focus of this project. It is the primary effector domain that interacts with OM transporter proteins (Ferguson and Deisenhofer, 2002; Lazar, 2014). The TonB system utilizes its three proteins to import vitamin B_{12} and facilitate iron transfer through OM active transport (Gresock *et al.* 2011). This active transport is enabled through energy created in the CM by the proton motive force (Postle and Larsen, 2007; Lazar, 2014).

One physiological function shared among the TonB and Tol system is their facilitation of colicin translocation into the OM and through the CM. Group B colicins have been able to parasitize the TonB system and gain cell entry. Complementarily, group A colicins behave similarly with the Tol system. However, resistance to these bacteriocins have been obtained through mutations in these genes. The entry of colicins into bacteria like *E. coli* have proven worthy of study due to their unique mechanism of entering the cell. Rather than being required to stimulate OM receptors to enter the cell, some mutated strains permit colicins to enter cells without having colicins bind to such receptors. Strains permitting this receptor-independent translocation of colicins are referred to as *tol* strains (Webster, 1991). Figure 1 illustrates both the Tol and TonB systems to offer a side-by-side comparison

The Tol Protein System

As previously stated, much of the understanding that has been gathered about the Tol system has been acquired through experiments that predicted its behavior paralleled that of the TonB system. Associations between the Tol system and OM stability have been established through various experiments. Such studies have exposed cells with mutated Tol systems to pharmaceuticals and detergents and transmembrane protein leakage or cell lysis due to a collapsing OM integrity (Lazzaroni *et al.*,1999; Lazar, 2014). As such, dependence of OM stability has been linked to the necessity for a functional Tol system. Although such relationships have been supported between these two entities through such experimentation, it has been suggested that perhaps another protein or protein system supports the action of the Tol system and thus is equally necessary for OM integrity. Pal is one such protein that is believed to act as an interface between the CM and OM while forming an energy-consuming complex with the Tol

system (Gerding *et al*., 2007; Lazar, 2014). This complex has recently been identified as a facilitator of chemotaxis as it may aid in the maintenance of chemoreceptor polarity (Santos *et al.*, 2014; Lazar, 2014).

The Tol proteins are encoded by two operons. The first operon contains the *orf1*, *tolQ*, *tolR*, and *tolA* genes whereas the second operon encodes the *tolB*, *pal*, and *orf2* genes. Two promoters exist in this gene cluster. The first promoter, P_1, is believed to promote the transcription of all seven genes mentioned. However, the second promoter, P_B, only promotes the expression of the final three genes mentioned (Vianney, et al., 1996; Lazar, 2014). Further research has found dependence of *tolR* expression on the among of *tolQ* transcribed (1996). A similar dependence was found between the Ton genes *exbD* and *exbB* (Ahmer *et al*., 1995). This relationship that exists in the two paralogous protein systems possibly exists to ensure both proteins required to form the ExbB/D or TolQ/R complexes are present (Lazar, 2014). *Orf1* and *orf2* are contemporarily known as *ybgC* and *ybgF*, respectively. These genes have not been demonstrated to be necessary for a functional Tol system (Lazdunski *et al.*, 1998). However, it is known that ybgC transcription and translation yields an acyl-coenzyme A thioesterase and ybgF encodes a periplasmic protein that may interact with the TolA protein (Zhuang et al., 2002; Sturgis, 2001).

The five remaining Tol proteins mentioned are far better understood than YbgC and YbgF. As previously mentioned, the TolQ and TolR CM proteins are paralogous to TonB's ExbB and ExbD proteins. It has been demonstrated that TolQ shares 26.3% genetic sequence identity with ExbB, but the 79.1% of amino acids can be substituted by other amino acids and conserve its function. A similar relationship exists between TolR and ExbD with 25% genetic sequence identity being shared (Eick-Heklmerch and Braun, 1989; Lazar, 2014). The ExbB/D and TolQ/R complexes and their relationship to the CM are also comparable. TolQ is an integrated CM protein similar to ExbB while TolR is a transmembranous protein similar to ExbD (Kampfenkel and Braun, 1993). The TolQ/R complex has been shown to restore partial function to TonB systems lacking ExbB and ExbD (Braun et al., 1996; Brinkman and Larsen, 2008; Lazar, 2014).

TolB and Pal proteins do not possess and paralogous relationships to proteins within the TonB system. TolB is a periplasmic protein that exists on the peripheral side of the OM and is constituted of an N-terminal domain and a C-terminal domain (Bouveret *et a*l., 1995). These two proteins interact in such a way that may support OM integrity (Ray *et al*., 2000). Pal is the only protein of the Tol system that directly contacts the OM (Lazar, 2014). This contact is facilitated by the N-terminal of Pal while the C-terminal is fixated to the thin peptidoglycan layer that exists in the periplasmic space (Lazzaroni and Poratlier, 1992).

The central protein of this project, the TolA protein of the Tol system, is paralogous to the TonB protein of the TonB system (Levengood and Webster, 1989). Both proteins are associated with the CM and consist of three domains, each isolated from the others through glycine strings. The first domain of TolA anchors the protein to the CM and interacts with TolQ and TolR (Journet *et al*., 1999). It is believed that the creation of the electrochemical gradient and proton motive force (pmf) of the CM are aided by a complex formed between TolQ, A and R (Germon et al., 2001). Domain I is highly conserved in all *E. coli* strains (Lazar, 2014). The second domain of TolA is separated contains high concentrations of alanine, lysine, and glutamic acids (Lazar, 2014). This domain is believed to provide a tangible link between the OM and CM through the periplasm.

The third domain of TolA is the most pertinent TolA domain to this project. Domain III is believed to form complexes with various porins such as OM proteins C and F that other domains are unable to facilitate (Derousiche *et al*., 1996). The genetic sequence of Domain III is also highly conserved among all *E. coli* strains. The C-terminal of TolA interacts with TolB to facilitate interactions between the other protein components of the CM and OM as well as between Pal and the OM (Walburger *et al*., 2002). The TolA-Pal interactions are powered by the pmf (Cascales *et al.,* 2002). It is ultimately the C-terminal of this domain that facilitates colicin import via interactions with the colicin N-terminal.

Both colicins and bacteriophage are able to parasitize the Tol system to enter target cells. A protein synthesized by viral Ff bacteriophage genes has been shown to interact with Domain III of TolA

to initiate infection (Riechmann and Holliger, 1997). Similar interactions have been shown between the TonB system and T1 phages (Hantke and Braun, 1978). However, in terms of colicins, only certain classifications of colicins can exploit either protein system. Group A colicins require the Tol system for translocation across the cellular envelope, but group B colicins utilize the TonB system (Davies and Reeves, 1975a, b).

Colicin Bacteriocins

Some *Escherichia coli* strains are capable of synthesizing and secreting toxins that eliminate bacteria with close genetic similarity. These toxins, more specifically known as colicins, are encoded by plasmids and may be an offensive mechanism to eliminate nearby bacterial competition. Various colicins have been identified from other bacterial species (Lazar, 2014). Colicins have been classified as either group A or B, depending on which protein system they parasitize to enter the cell (Davies and Reeves, 1975a, b). In addition to plasmids encoding the colicin itself, a protein capable of preventing the colicin-synthesizing cell from being harmed by the colicin is also encoded (Jacob *et al*., 1952). This protective protein is known as the immunity protein and is encoded by the same plasmid that encodes the colicin (Jacob *et al*., 1952). This supports the notion of colicins being a competitive survival advantage since progeny must receive the plasmid in order to confer colicin immunity and retain offensive colicin production.

All colicins are composed of three domains: The N-terminal domain, the central domain, and the C-terminal domain. Each domain possesses its own unique function. Colicin translocation is initiated by the central domain binding to the appropriate receptor on the OM of the target cell (Brunden *et al*., 1984; Lazar, 2014). The stimulated receptor depends on which colicin is attempting import into the cell. Table I details which proteins and receptors are required for translocation of which colicins. Once the colicin's central domain has bound to the OM receptor, the N-terminal aids in translocation of the colicin to the CM and its passing through the OM and periplasm (Benedetii *et al*., 1991). For group A colicins,

translocation cannot occur without TolA, thus provides the basis for this project (Benedetii *et al*., 1991). The C-terminal domain is ultimately responsible for cytotoxicity (Lazar, 2014).

Colicins can exercise cytotoxicity through several techniques that are specific to different targets. One such mechanism is through compromising the synthesis of peptidoglycan. The group B colicin, colicin M, accomplishes this through preventing peptidoglycan subunits from polymerization (Ghachi *et al*., 2006). Other colicins, such as the group A E2 and E3 colicins, function by acting as endonucleases and inhibiting protein synthesis by degrading DNA or RNA (Housden *et al*., 2005). Another common mechanism for practicing colicin cytotoxicity is the perforation the CM, ultimately creating pores (Hilsenbeck *et al*., 2004). These pores alter CM homeostasis and disturb the cell ion gradients that allow the cell to utilize the proton motive force in a phenomenon known as uncoupling (Hilsenbeck *et al*., 2004).

The Relationship Between the Tol Protein System and Colicin Translocation

Before group A colicins can completely enter the target cell, they must pass through the OM into the periplasmic space. In this area between the two membranes, the colicin will encounter the Tol proteins. In order for the group A colicins to enter the periplasmic space, they must first stimulate the OM proteins and receptor proteins, then be transported through the OM by OmpF or TolC porins (Houdsen et al., 2005). Colicin N is an exception to this process as it only requires OmpF to enter the periplasmic space and forgoes the need for receptor proteins or other OM proteins (Baboolal *et al.*, 2008). Upon colicin binding to an OM receptor, an unfolding of the colicin protein occurs to facilitate its translocation through the OM (Baboolal *et al.*, 2008). The N-terminus of the colicin further progresses translocation through its communication with TolB as it passes through an OmpF porin (Loftus et al., 2006). The region of the colicin's N-terminal responsible for binding TolB is referred to as the "TolB box" (Lazar, 2014).

Once the N-terminal of the colicin has been bound to TolB, the colicin binds TolA at a site distinct from that of TolB. The colicin binding site that interacts with this third domain of colicin A, known as the "TolA box" is believed to be located between residues 52-97 of colicin N (Li *et al.*, 2012). Once the colicin is bound to TolA, it releases TolB then binds to TolR. The same residues used to bind TolB are used to bind TolR, thus TolB must be released before TolR can be bound by the colicin (Journet *et al*., 2011). The exact interactions that exist between TolQ and colicins are largely undefined to date.

The mechanism by which energy is supplied to proteins within the Tol system during colicin import seems to vary depending on the colicin in question. Like the TonB system, it the Tol system may uses an electrochemical ion gradient within the CM to power colicin translocation (Braun et al., 1980). However, colicin A, a group A colicin, has proven to successfully translocate without the use of such a gradient. Colicin A utilizes voltage-dependent potassium efflux channels to contact the CM (Bourdineaud *et al*., 1990). A lag time exists between colicin A contacting the CM and efflux which suggests that the uptake of colicin A is energy independent (Lazar, 2014). It is possible that other group A colicins may obtain an interface with the CM in similar energy-independent ways after initiating import through the Tol system.

This project aims to identify specific sites in the domain III of the TolA protein that facilitate colicin N binding and interactions. Such ideas expand upon the previous works of Weitzel and Larsen (2008), Zook (2012), and Lazar (2014) and adopt a similar approach that focuses on understanding TolA/colicin N interactions from an evolutionary perspective. Once it was determined that *Yersinia enterocolitica* TolA could function within the *ΔtolA* 1038 *E. coli* strain in terms of conferring colicin A sensitivity and OM integrity, additional colicins were tested (Weitzel and Larsen, 2008). Eventually, it was determined that sensitivity to colicin N was not conferred in this system due to the lack of the appropriate colicin binding region domain III of the *E. coli* TolA C-terminal (2008). This binding domain was posited to be in the final 85 residues of domain III of the *E. coli* C-terminal TolA. These results are cohesive with the recent identification of a residue segment of domain III (residues 44-66) that has proven

to be crucial for colicin N activity and mimic the residue of G3P bacteriophage (*Gokce et al*., 2000; Lazar, 2014).

This research was further expanded to include *Chronobacter* species into the *ΔtolA* 1038 bacteria (Zook, 2012; Lazar, 2014). Such experimentation determined that the *C. muytjensii* strain was able to facilitate OM integrity in the 1038 strain just as Weitzel and Larsen had done with *Y. enterocolitica* TolA. in the *ΔtolA* 1038 *E. coli* strain. Such results warranted further study using this evolutionary-derived strategy to examine genetically divergent *tolA* genes in different species and restore group A colicin sensitivities (Lazar, 2014). Such previous studies revealed only partial sensitivity to colicin N in *C. muytjensii* and *Y. enterocolitica* thus questions remained regarding role of the C-terminus of domain III TolA in group A colicin binding.

Hypothesis and Research Questions

This purpose of this project is to expand upon these related studies in terms of identifying specific colicin N binding sites within TolA residues that are required for colicin N translocation (Lazar, 2014). Sensitivity assays of colicins A and N were executed through spot titers (Larsen *et al.,* 2001). The wild-type *E. coli, ΔtolA E .coli*, and *C. muytjensii* clones from Zook were researched in this study. The *Y. enterocolitica* clones from Weitzel and Larsen were used as well. Previous work by Gertz (2016) provided data and models for *C. muytjensii* mutants that lacked a terminal Q on the C-terminus. Ultimately, this project's aim is to identify a colicin N binding site within domain III of TolA from *E. coli*, *C. muytjensii*, and *Y. enterocolitica* using site-directed mutagenesis of this region and spot titer assays to measure colicin sensitivity.

Materials and Methods

Media

Bacterial strains were cultured in Luria-Bertani (LB) broth and on Luria-Bertani agar (LBA) plates (Lazar, 2014). Colicin sensitivity spot titers were executed by mixing bacterial strains in liquid cultures with liquid tryptone agar (0.75% w/v agar; "T-top"). This mixture was spread onto tryptone agar plates (1.5% w/v agar; "T-plates"; Lazar, 2014). Both broth and solid media were laced with 100 μg mL^{-1} ampicillin and L-arabinose when specified.

Bacterial Strains

Each bacterial strain presented in this project is summarized in Table II of the appendix. The wild-type strain utilized was the *Escherichia coli* (*E. coli*) K12 strain W3110 (Hill and Harnish, 1981). The *ΔtolA* strain used, RA1038, was a W3110 derivative engineered by Weitzel and Larsen (2008). The bacterial strains used to produce the colicin stock solutions were derived from *E. coli* K12 strains BZB2191 and BZB2123 for colicins A and N respectively. These strains carry the plasmid that encode for these colicin. These cells were provided by Dr. Anthony Pugsley (Institut Pasteur; Paris, France). Another K12 *E. coli* derived strain, NEB5-α, was used to observe the recombinant plasmids constructed for this project. These cells were acquired from New England Biolabs (Ipswich, MA). Plasmids containing functional *tolA* genes from *Chronobacter muytjensii* (*C. muytjensii*) and *Yersinia enterocolitica* (*Y. enterocolitica*) also used in this project. Both strains were originally obtained from the American Type Culture Collection as ATCC51329 and ATCC23715 respectively.

Colicin Stock Solutions

The colicin stock solutions of colicins A and N were previously synthesized by Lazar (2014). These were obtained from bacterial strains that contained the appropriate plasmid to encode for each colicin respectively. The *E. coli* K12 strains BZB2101 and BZB2123 contained the plasmids that encoded for colicins A and N and respectively (Lazar 2014). These strains were provided by Dr. Anthony Pugsley (Institut Pasteur; Paris, France). The *E. coli* K12 strains BZB2101 and BZB2123 were cultured overnight in 5mL LB. The following day, 2.5mL aliquots of each respective overnight culture was added to 50 mL

of LB, respectively. This mixture was grown at 37 °C with shaking until absorbance at 550 nanometer wavelength was equal to 1.0. Absorbance was measured in a Spectronic 20 spectrophotometer with a path length of 1.5 cm. Once an absorbance of 1.0 was achieved, 25 µl of mitomycin C (1mg/ml) was added to cause colicin production. The cells were incubated at 37 °C for four more hours, then harvested with a 10 minutes centrifugation at 6,000 rpm at 4°C. The supernatant was then discarded from each sample and the pellets were suspended in 5mL of 1X M9 salts. The resuspensions were transferred to a new tube. The cells were lysed using sonication and then filtered through a sterile 0.45 µm filter. The product was stored at -20°C. Serial five-fold dilutions were prepared using LB as the solvent.

Plasmid Constructs

Table III itemizes each of the plasmids utilized and researched in this project. In order to identify homologous complementation or DNA sequence overlap among the various TolA proteins studied within *E. coli*, plasmids containing these genes had to be made (Lazar 2014). The plasmid pBAD24, whose expression is regulated by the monosaccharide arabinose, facilitated this matter (Guzman *et al*., 1995; Lazar, 2014). This plasmid encodes ampicillin resistance to aid in mutant screening. Transforming this cell into the 1038 Δ*tolA* to act as negative control during spot titer phenotyping. A positive control, previously constructed by Weitzel and Larsen (2008), was employed. This plasmid, known as pRA004, encoded the *E. coli* TolA protein.

The pBAD24-derivative encoding *C. muytjensii* strain *tol* gene, pRA043, used was previously constructed by Zook (2012). This construct was made using a slightly more complex technique and was based off of the sequence for *Klebsiella pneumoniae* (subspecies *pneumoniae*, strain MGH7857, Genebank accession NC_009648.1). Primers were designed to amplify the homologous initial codons of the *tolA* gene between this strain and *E. coli* (Lazar, 2014; Zook, 2012). These initial sequence portions were used as little similarity existed between the sequences upstream from the *tolA* gene. The utilized bases of both *K. pneumoniae* and the W3110 *E. coli* strain were 883,749-883,763. These bases were

added to the three-prime end of an EcoRI site to facilitate cloning (Lazar, 2014; Zook 2012). This forward primer, ORA0296, created by Zook (2012) can be seen in Table III. The reverse primer was constructed to complement a portion of the sequence between *tolA* and *tolB* of *K. pneumoniae* (Lazar, 2014; Zook, 2012). The bases 835,211-835,159 were added to the three-prime end at a KpnI site to facilitate cloning. This primer was designated ORA0270. PCR reactions were run using these primers and the DNA templates to clone the constructed mutants. The sequences of the genes of interest were confirmed with dideoxy sequencing (Lazar, 2014; Zook, 2012).

Each primer was approximately 30 base pairs long that complemented the template DNA. These primers also contained the intended mutation to remove the glutamine (Q) from the extreme carboxy terminus of the *C. muytjensii* strain and add the glutamine to the extreme carboxy terminus of the *E. coli* of the *tolA* gene (Gertz, 2016). This essentially caused one strain's mutated extreme carboxy terminus to appear as the C-terminus of the wild-type strain. pRA0703 and pRA0704 were the forward and reverse primers used to do so in *E. coli*, respectively, and pRA0705 and pRA0706 were used to mutate *C. muytjensii* (Gertz, 2016). The mutation to create pRA076 was executed at residue 421 (the final amino acid codon) to add a terminal glutamine. The deletion of the terminal glutamine to create pRA067 was done at residues 1267-1269 of the amino acid sequence (the final codon).

The *Y. enterocolitica* primers were created through similar means, however, the focus was on a more upstream portion of the C-terminus. The lysine encoded by bases 895-897 differed from the alanine and serine found at that site in *E. coli* and *C. muytjensii*, respectively. Due to the different biochemical natures and behaviors of lysine and the other two amino acids, it was posited that this site could confer colicin N sensitivity to *Y. enterocolitica* if mutated to possess the alanine as *E. coli* does. 5' phosphorylated primers ORA0732 and ORA0733 were constructed to induce this mutation through site-directed mutagenesis.

PCR and Mutating the tolA Gene in its C-Termini

Primers are designed in the lab and ordered from the Invitrogen Company. Two sterile PCR tubes were obtained. One tube will serve as a negative control for each respective reaction and will be labeled "EC (-)" or "CM (-)" and will designate their mutation as appropriate. The experimental tube was labeled "EC (+)" or "CM (+)" and designated the mutation appropriately. The experimental tube reactions consisted of 35 μL of deionized water, 1 microliter of the appropriate plasmid (pORA004, pORA028, pORA043), 10 μL of 10x Reaction Buffer, 1 μL of each appropriate forward and reverse primer 1μl of deoxynucleotides/dNTP's, and 1μL of *Taq* DNA polymerase. The negative control tubes will contain each of these contents, but with an extra 1 μL of deionized water and the omission of any plasmid to use as template DNA. One tube contained 2 μL of $MgCl_2$ and another contained 1 μL $MgCl_2$ to replace the same volume of water and facilitated PCR better. The tubes will be run on a previously constructed PCR setting in the thermocycler called "KGXL". These samples will be stored in the freezer. Each PCR was run for a total of 35 cycles, with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 6 minutes through the first 34 cycles, and 12 minutes for the final.

Gel Electrophoresis

A sample of the PCR product was resolved on a 1% agarose gel at 110v with a 1 kb ladder side standard, to confirm the product contained an amplification of the entire plasmid. A 1% agarose gel was made using 0.5x Tris-EDTA buffer. Approximately 0.8 gram of agarose was measured out and mixed with 80 mL of 0.5x Tris-EDTA buffer. The mixture was weighed, then heated at 30 second intervals in the microwave until the solution appeared clear and the agarose was fully dissolved. The mixture was weighed between intervals and any lost mass was replaced by the addition of deionized water. The sample was equilibrated to ~50 °C for 10 minutes. 4 μL of ethidium bromide was added to the agarose. This mixture was poured into an assembled gel box and allowed to harden for 15-20 minutes. Sterile Eppendorf tubes were appropriately labeled for each sample being run on the gel. 5 μL of deionized water and 3 μL of loading dye were added to each tube. 5 μL of the appropriate purified PCR sample was added to the corresponding tube. Each sample mixture was injected into its own well within the gel. The anode

of the electric power supply was attached to the electrode closest to the wells and the cathode to the farthest electrode from the wells. The voltage was set at 110V and the gel was run for approximately 1 hour. DNA movement was observed by placing the gel above ultraviolet light. Protective eye-ware is worn during viewing. Samples that moved farthest from the anode were smaller than those that traveled less distance.

Qiagen Purification

All samples were purified using this kit. Sterile columns were obtained and appropriately labeled for both the negative control and experimental tube. 250 μL of the provided PB buffer was added to the PCR sample. The mixture was placed in its respective column, which was then placed in its respective collection tube. The sample was centrifuged at 14,000 revolutions per minute (rpm) for 1 minute. The flow through was discarded. 750 μL of the PE buffer was added to each sample's column. The tubes were centrifuged again at the same speed for the same time period. The flow through was discarded. The tubes were centrifuged at 14,000 rpm for 30 seconds. The collection tubes were discarded. The columns were placed in sterile, appropriately labeled Eppendorf tubes. 30-50 μL of 10 mM Tris elution buffer was added to each sample. The sample was allowed to rest for 2 minutes. The samples were then centrifuged at 14,000 rpm for one minutes. The column was discarded and the final purified sample remained in the Eppendorf tube. These samples were stored in the freezer.

DpnI Site Digestion and Ligation

The PCR product was digested with the DpnI restriction enzyme to remove methylated DNA and the initial template DNA from pRA004, pRA028, and pRA043. 2 μL of each sample's purified PCR product was added to its own sterile Eppendorf tube. 2 μL of Buffer #4 was added to each tube along with 14 μL of deionized water, 1 μL of the DpnI enzyme. The mixtures were incubated 37 °C for one hour.

The purified, digested PCR product were ligated to re-circularize the products into a plasmid. To perform the ligation, tubes were prepared and appropriately labeled for each species. 8 μL of dionized

water, 10 µL of Quick Ligase Buffer, 1 µL of the appropriate digested DNA, and 1 µL of Quick ligase were added to each tube. Negative controls were made for each of these tubes. They lack DNA but compensate with an additional microliter of water. All tubes were incubated at room temperature for 30 minutes.

Transformation of Each Plasmid into Competent 2987α Cells

Once cells were ligated, the circularized products were transformed into DH5-α chemically competent *E. coli* cells from New England Biolabs per product protocol (Lazar, 2014). 20 µL of frozen C2987α cells were deposited in sterile Eppendorf tubes. They were labeled appropriately for each strain that will be transformed (oRA 004, 028, and 043). 2 µL of the digested/ligated DNA from above were added to each tube as appropriate. The tubes were incubated on ice for 30 minutes, then heat shocked for two minutes at 37 °C. The tubes were put back on ice for two minutes and mixed with 500 µL of LB. They were then incubated at 37 °C for one hour. 100 µL of each was plated on its own LB-Amp plated at incubated overnight at 37 °C. Growth was evaluated and selection for transformants on each plate was conducted the following day.

Alkaline Lysis for Plasmid Purification

Overnight cultures of each plasmid strain in the 2987α cells were made and appropriately labeled. The media used will be LB-Amp (2 mL). The samples were incubated at 37 °C. Then, 1.5 mL of each oFvernight culture was placed in its appropriate sterile Eppendorf tube and labeled. The tubes were centrifuged for 5 minutes at 14,000 rpm. The supernatant was aspirated off of each sample. The pellet was mixed and resuspended in 100 µL of Solution I (11.7 mL of 20% 50mM glucose, 2.5 mL of 1.0 M Tris (pH 8.0), 2.0 mL of 0.5 M DTA (pH 8.0), and 84 mL of deionized water). 200 µL of Solution II was added (8.8 mL of deionized water, 200 µL of 10N NaOH, and 1.0 ml of 10% SDS). The solution was gently mixed, then stored on ice for 5 minutes. 150 µL of Solution III (50 ml of 5M potassium acetate, 11.5 mL acetic acid, and 28.4 mL deionized water) was then added to each tube and mixed.

The tubes were incubated on ice for 5 minutes, then centrifuged for 5 minutes at 14,000 rpm. The supernatant was poured into fresh, appropriately labeled tubes. 200 µl of buffer-saturated phenol and 200 µL of 1:24 chloroform:isoamyl alcohol was added to each tube, then mixed with a Vortex. The tubes were centrifuged for 2 minutes at 14,000 rpm. The top layer was collected and deposited into a new, sterile, and appropriately labeled tube. 1 ml of 100% ethanol was added to each tube and mixed. The mixture rested at room temperature for 2 minutes. The tubes were then centrifuged for 5 minutes at 14,000 rpm and the supernatant was aspirated afterwards. Each tube was rinsed with 1 ml of 70% ethanol, spun for 1 minute at 14,000 rpm, and the ethanol was removed. The tubes were air dried for 30 minutes. The pellet was resuspended in 20-50 µL of deionized water and stored in the freezer

Transformation into 1038 TolA$^-$ Cells

The plasmids from overnight cultures were transformed into the *ΔtolA* strain RA1038 using the protocol demonstrated by Chung *et al.* (1989). Overnight cultures were made in 5 mL of LB broth culture of the 1038 cells. These cells were diluted 1:100 by placing 50 µL of the overnight cultures in 5 mL of LB broth. One test tube was allotted for each plasmid (pRA004, pRA028, pRA043) respectively. These cultures were incubated at 37 °C and shaken until their absorbance measurement reads 0.5 on the spectrometer. These cultures were divided as 1 mL aliquots in Eppendorf tubes and chilled on ice for 5 minutes. The tubes were be spun for ten minutes at 14,000 rpm. The supernatant was removed with a pipet tip/aspiration system. The pellet was suspended in chilled 1X TSS buffer (containing 1.0g of Tryptone, 0.5g of yeast extract, 1.0g of NaCl, 10g of polyethylene glycol, 4 mL of DMSO, 100 mL deionized water, and 5 ml of $MgSO_4$. The solution had a 6.21 pH. It was filtered using a 0.45 micrometer syringe filter). 1 µL of the appropriate plasmid was placed in the correct tube and incubated on ice for half an hour. The tubes were then heat shocked for two minutes at 37 °C. 500 µL of LB broth was added. The tubes were incubated at 37 °C for one hour. 100 µL of each sample were plated on its own LB-Ampicillin (LB-Amp) plates. They were incubated overnight at 37 °C. Negative controls were made for each sample in the same way, but they lacked any plasmid. Growth of the plates was evaluated to identify 1038

transformants. These cells were inoculated into 5 mL LB supplemented with ampicillin to prepare for spot titer phenotypes on T-plates.

Genetic Sequencing

DNA sequences of plasmids that behaved with the appropriate phenotype were ordered from the University of Chicago Comprehensive Cancer Center. These sequences can be found in the Appendix. The DNA samples of each plasmid were prepared for sequencing then sent off. Once the sequences were returned, they were analyzed to ensure the appropriate mutation is in each sequence. Further screening for mutants was done by creating overnight cultures of each strain, plating them on LB-Amp, and evaluating their growth.

T-Top Plates

Overnight cultures of the species plated above were made in 5 mL of LB-Amp. 5 μL of 100 mM arabinose was diluted in 5 mL of LB-Amp broth. 25 μL of each culture was deposited in 5 ml of LB-Amp with and without L-arabinose, respectively. Therefore, two tubes existed for each sample and only one of those possessed L-arabinose. Pre-made T-top solution was warmed until liquid. 50 μL of 10X ampicillin and 20μL Ca^{2+} in the form of 500 mM $CaCl_2$ were added to 20 ml of T-top agar. 100 μL of each sample was mixed in a separate tube with 3 mL of T-top broth. This was poured on T-plates until evenly spread and cooled until polymerization.

Colicin Sensitivity Spot Titers

To test transformed colonies for susceptibilities to colicins A and N, spot titers were performed. The T-top plates made previously were used for this experiment. The plates were divided into half, with the top half labeled as “colicin A” and the bottom half as “colicin N”. 9 sterile Eppendorf tubes were obtained for each colicin and labeled a concentration from 10^0 to 10^{-8}. Each colicin was respectively diluted 1:10 from 10^0 to 10^{-9}. 1 μL of each sample was spotted onto the appropriate plate. This was done in such a manner that when viewed from the agarose side of the plate, the front row read 10^{-1} to 10^{-4} and

the bottom row read 10^{-5} to 10^{-9}. These plates were incubated for 24 hours at 37 °C then evaluated for zones of inhibition. Any zone of inhibition is indicative of a functioning Tol system and TolA protein that has been successfully parasitized by the colicin.

Triplicate

The spot titers were performed in triplicate as described above to ensure accurate and reproducible results. Images of these results were captured and can be seen below.

Results

The spot titer results of pRA067 and pRA077 are illustrated below in Figures 2 and 3 for colicins A and N respectively. Images captured of this experiment's plates can be seen in Figure 4. These results are also tabulated in Table IV. The pRA004 (*E. coli* wild-type) positive control experienced sensitivity to both colicins A and N through the 5^{-9} dilution. The pRA043 (*C. muytjensii* wild-type) experienced sensitivity to colicin A to the same degree, but only experienced sensitivity to colicin N through the 5^{-4} dilution. The mutants behaved in such a way that mimicked the sensitivity of the wild-type species its mutated extreme C-terminus was constructed to mirror. The mutated *E. coli* strain (pRA076) that was designed to possess a terminal glutamine (Q) and appear as the extreme C-terminus of *C. muytjensii* experienced the same degree of sensitivity to colicin A as the previously mentioned strains. However, it exhibited less sensitivity to colicin N than wild-type *E. coli* (pRA004) and its sensitivity to colicin mirrored that of wild-type *C. muytjensii* (pRA043). The mutated *C. muytjensii* strain (pRA067) that had its extreme C-terminus Q deleted to appear as the extreme C-terminus of wild-type *E. coli* exhibited sensitivity to colicin A through 5^{-9} like all other strains. However, a greater degree of sensitivity to colicin N was observed when compared to wild-type *C. muytjensii*.

Figure 5 displays the spot titer results obtained from pRA077 in 1038 *ΔtolA* cells. Sensitivity to colicin A extended from 5^{-1} to 5^{-9} dilutions. However, sensitivity to colicin N only extended from 5^{-1} to 5^{-4}

dilutions as was characteristic of the wild-type strain, pRA028 (Gertz, 2016). This suggest that the binding domain mutated may not prove significant to domain III of TolA binding to colicin N.

Conclusion

Overall, the expected results were successfully obtained from project for *E. coli* and *C. mutjensii*. By mutating the glutamine in the extreme carboxy-terminus of the TolA protein in *E. coli* and *C. muytjensii*, colicin N sensitivities were altered. By switching the extreme carboxy termini of the two species where the glutamine through the presence or absence of a glutamine, the sensitivity of colicin N was altered. The results obtained support the belief that the presence of a terminal glutamine on the extreme carboxy terminus of the TolA protein substantially alters the translocation across the cell envelope and the import of colicin N in *E. coli* and *C. muytjensii*. The results obtained suggested the extreme C-termini of these species prove crucial in facilitating group A colicin translocation, most notably of colicins A and N for these two species. Additionally, this project has identified this TolA region as a necessary binding site for colicin N translocation, and potentially other Group A colicins. However, results were less favorable for pRA077. Although this mutant possessed the intended mutation, it exhibited similar colicin N sensitivity to the wildtype pRA028. This suggests that this exact region is not crucial for colicin N binding to domain III of TolA. Future experimentation in which the extreme-terminal glutamine of this strain is removed may affect colicin N sensitivity as seen in the previous strains.

Future experimental prospects consist of testing colicin A and N sensitivities of species with various degrees of genetic similarity to the TolA of the species in this project. Mutations can be made in these species to determine if any other regions of TolA alter colicin N import into these species. One such intended project is introducing the same mutation induced in pRA077 in a previously constructed *Y. enterocolitica* that lacked a terminal glutamine (Gertz, 2016). This new strain would possess two mutations such that the extreme C-terminus sequence would be identical to *E. coli* and the upstream mutation would possess an alanine as *E. coli* does. Mutating *Y. enterocolitica* to possess a sequence more

similar to *E. coli* is desirable as wild-type *E. coli* proves more sensitive to colicin N than wild-type *C. muytjensii*.

Appendix

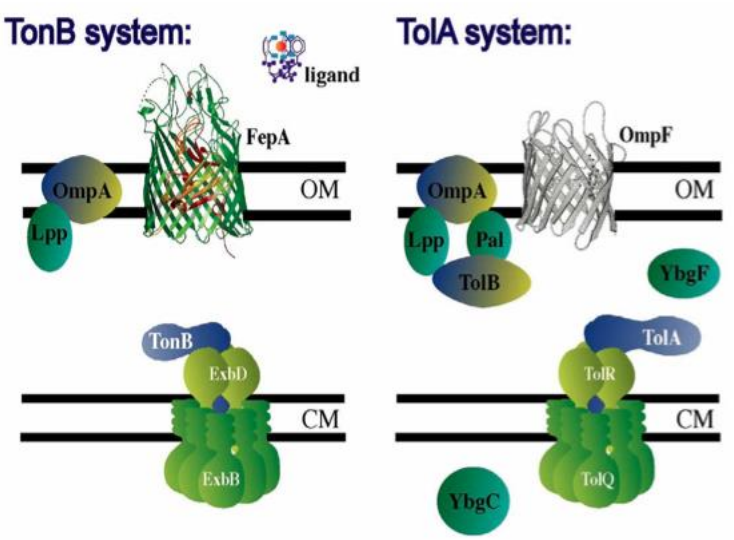

Figure 1. The TonB and Tol protein systems (adapted from Lazar, 2014). ExbB, ExbD, and TonB of the TonB system appear to be analogous to TolQ, TolR, and TolA proteins of the Tol system, respectively. These proteins interact with Lpp and OmpA in the TonB system whereas the Tol system requires these in addition to TolB and Pal. The TonB system aids in transporting iron and vitamin B_{12} while the Tol system supports OM integrity. Both systems can be used by phage or colicins to enter the cell. Both require outer membrane proteins like FepA and OmpF to facilitate such translocations.

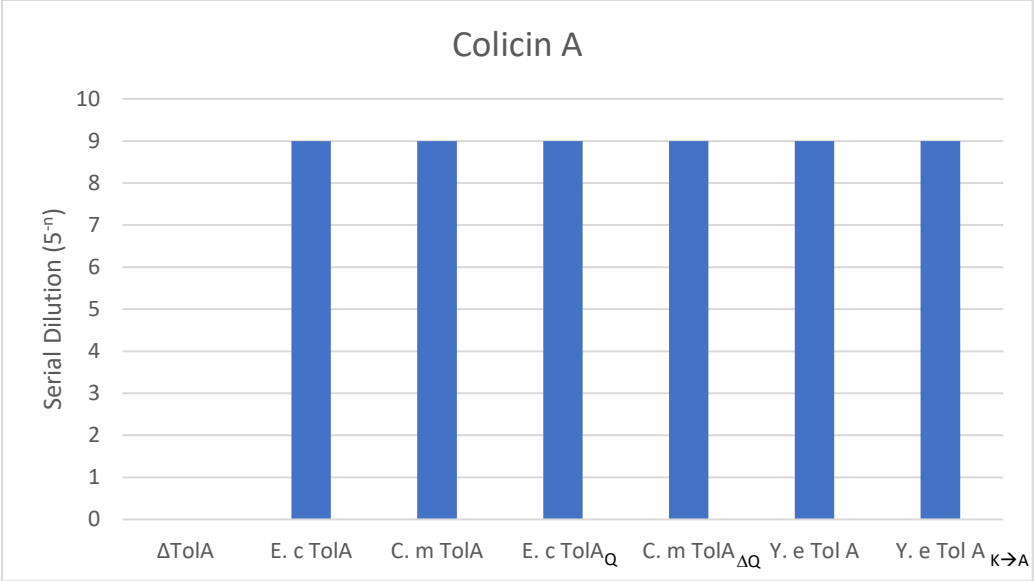

Figure 2. Colicin A sensitivities experienced by each sample. Colicin A is used as a positive control as all samples demonstrate equivalent sensitivity.

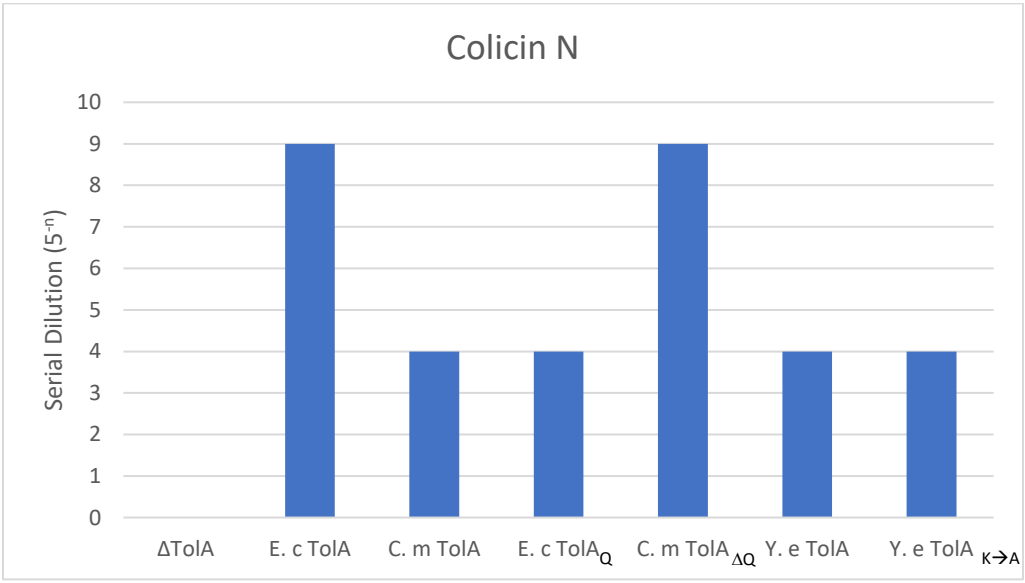

Figure 3. Colicin N sensitivities experienced by each sample. Samples with an *E. coli*-like C-terminus experienced more sensitivity than those with a *C. muytjensii*-like C-terminus.

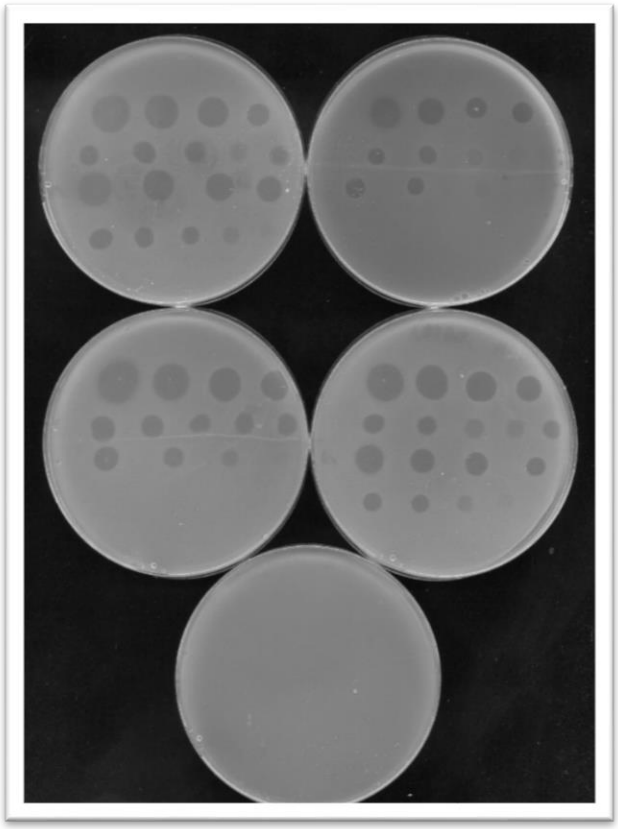

Figure 4. The spot titer plate results observed of pRA067 and pRA076 plasmids in 1038 *ΔtolA* cells. pRA004 and pRA076 can be seen in the first row from left to right. The second row consists of pRA043 and pRA067 from left to right. The final plate consists of pBAD24 to function as a negative control. The top half of the plate contains the colicin A spot titers and the bottom half of the plate contains the colicin N spot titers.

Figure 5. The spot titer plate results observed of pRA077 in 1038 *ΔtolA* cells. pRA077 behaved similarly to wild-type *Y. eneterocolitica* in regard to colicin N sensitivity although the mutation was present. c

E.coli (pRA004) KNNGASGADINNYAGQIKSAIESKFYDASSY**A**GKTCTLRIKLAPDGM

Y. ent (pRA028) KKSGASAGDISGYLGQLTAAIQSKFYDADLY**K**GRTCNLRIKLAPDGL

C. muy (pRA03) KNNGASGAEINGYASQIKAAIESKFYDASSY**S**GKTCTLRIKLAPDGL

E.coli LLDIKPEGGDPALCQAALAAAKLALIPKPPSQAVYEVFKNAPLDFKP**-**

Y. ent LIDVKQEGGDPALCQAAIAAAKLAKIPKPPSQDVYEVFKNAPLVFKP**Q**

C. muy LLDIQSEGGDPALCQAAISAARQAKIPKPPSQAVYEVFKNAPLLFKP**Q**

Figure 6. Comparison of the domain III amino acid sequences of TolA protein of the three wild-type strains used in this study. Bolded letters indicate where mutations were made as described previously.

Table I. List of Group A colicins and their receptors, translocation mechanisms and cytotoxicity. Adapted from Cascales *et al.* (2007) and Lazar (2014).

Colicin	**Primary receptor (OM)**	**Translocater/ Secondary Receptor**	**Mechanism of Translocation**	**Cytotoxicity**
A	BtuB	OmpF	TolA,B,Q,R	Pore-forming
N	LPS	OmpF	TolA,Q,R	Pore-forming

Table II. Summary of bacterial strains used. Adapted from Lazar (2014)

Strain	**Relevant genotype/phenotype**	**Source/Reference**
W3110	Wild type *E. coli*	Hill and Harnish, 1981
NEB5-α	Chemically competent *E. coli*	New England Biolabs
RA1038	W3110 (*ΔtolA)*	Weitzel and Larsen, 2008
ATCC51329	*Cronobacter muytjensii*	American Type Culture Collection
ATCC23715	*Y. enterocolitica*	American Type Culture Collection
BZB2101	Carries a low-copy plasmid that encodes colicin A	Anthony Pugsley, Institut Pasteur
BZB2123	Carries a low-copy plasmid that encodes colicin N	Anthony Pugsley, Institut Pasteur

Table III. Summary of plasmids used.

Plasmid	**Releveant Genotype/Phenotype**	**Source or Reference**
pBAD24	*araBAD* promoter, AraC, amp^r	Guzman *et al.*, 1995
pRA004	pBAD24 encoding *E. coli* TolA	Weitzel and Larsen, 2008
pRA028	pBAD24 encoding *Y. enterocolitica* TolA	Weitzel and Larsen, 2008
pRA043	pBAD24 encoding *C. muytjensii* TolA	Zook, 2012
pRA067	pBAD24 encoding *C. muytjensii* TolA with mutations	Gertz, 2016
pRA076	pBAD24 encoding *E. coli* TolA with mutations	Present Study
pRA077	pBAD24 encoding *E. coli* TolA with mutations	Present Study

Table III. Primers constructed and utilized in this project

Primer #	**Description**	**Sequence**
Amplification primers	**Bacterial Species**	**Primer Sequence**
ORA0269	*Klebsiella pneumoniae* (MGH78578, NC_009648.1) bases 883749-883763	5'-CCGAATTCACGATGTCAAAGGCAACCG
ORA0270	*Klebsiella pneumoniae* (MGH78578, NC_009648.1) bases 835211-883763	5'-GCTGGTACCCTACTACTCGTAATGCCTGC
Mutational primers		
ORA0703	*E. coli tolA* forward, mutagenic	5'-GGCAGTATACGAAGTGTTCAAAAACGCGCCATTGCTGTTTAAACCGCAGTAATCTAGAGTCGACCTGCAGGC
ORA0704	*E. coli tolA* reverse, mutagenic	5'-GGCGCGTTTTTGAACACTTCGTATACTGCCTGGTATACTGCCTGGCTTGGTGGTTTCGGGATCTTCGCAAGTTTAGCTGCTGCC
ORA0705	*C. muytjensii* forward, mutagenic	5'-GGCTGTATACGAAGTCTTTAAAAACGCATTGGATTTTAAACCGTGATTACCGGTAACAAAACCGCC
ORA0706	*C. muytjensii* reverse, mutagenic	5-CGCGTTTTTAAAGACTTCGTATACAGCCTGGCTTGGCGGTTTAGGAATCTTAGCCTGTCGGGCGGCGG

ORA0732	*Y. enterocolitica* forward, mutagenic	5'-P-CCGCACCTGTAATCTGC
ORA0733	*Y. enterocolitica* reverse, mutagenic	5-P-CCGGCGTAAAGATCAGC

Table IV. Sensitivity to group A colicins. Scores in the table indicate the highest fivefold dilution of the indicated colicin resulting in zones of inhibition. A score of 9 would indicate that the cells are sensitive the given colicin through the 5^{-8} dilution.

TolA phenotype	**Colicin A**	**Colicin N**
ΔTolA	0	0
E. coli (pRA004)	9	9
C. muytjensii (pRA043)	9	4
E. coli$_{Q}$ (pRA076)	9	4
C. muytjensii$_{\Delta Q}$ (pRA067)	9	9
Y. enterocolitica (pRA028)	9	4
Y. enterocolitica (pRA077)	9	4

TolA Amino Acid Sequences of Wild-Type Species

Escherichia coli

Domain I:

1- MSKATEQNDKLKRAIIISAVLHVILFAALIWSSFDENIEASAGGGGG -47

Domain II:

48- SSIDAVMVDSGAVVEQYKRMQSQESSAKRSDEQRKMKEQQAAEELREKQAAEQ

ERLKQLEKERLAAQEQKKQAEEAAKQAELKQKQAEEAAAKAAADAKAKAEADAKAA

EEAAKKAAADAKKKAEAEAAKAAAEAQKKAEAAAAALKKKAEAAEAAAAEARKKA

ATEAAEKAKAEAEKKAAAEKAAADKKAAAEKAAADKKAAEKAAAEKAAADKKAAA

EKAAADKKAAAAKAAAEKAAAAKAAAEADDIFGELSSGKNAPKT -310

Domain III:

311-GGGAKGNNASPAGSGNTKNNGASGADINNYAGQIKSAIESKFYDASSYAGKTCTLR

IKLAPDGMLLDIKPEGGDPALCQAALAAAKLAKIPKPPSQAVYEVFKNAPLDFKP-421

Chronobacter muytjensii

MAKATEQNDKLKRAIIISVVLHLLLIALLIWSSFDEHINEDAAGGGGGSAIDAVMVDPGAVVQQY
NRQQQQQASAKQAAEQREKQAQQQAEELREKQAAEQE

RLKKLEQERLEAQEKAQQQAEQQKQAEEAAKRAQEQQKQAEEAAAKAAADAKAKADAQ
AKAAEEAAKKAAADAQKKAQEEAAKAAAAAKKAQEEAEKKAAADAAKKAQQDAEKKAA
AEAAKKAAAEKAAAEKAAAAEKAAAEKKAAAAKAAAEKAAAEKAAADKKAAAEKAAAKKA
AAAKAAAEKAAAAEGVDDLLGDLSSGKNAPKTGGGAKGSNAAPAGSGNSKNNGASGAEINGY
ASQIKAAIESKFYDASSYSGKTCTLRIKLAPDGLLLDIQSEGGDPALCQAAISAARQAKIPKPPSQA
VYEVFKNAPLLFKPQ

Yersinia enterocolitica (Based off *Yersinia pestis*; Lazar, 2014; Zook, 2012).

MWKSSVGKATEQNDKLNRAVIVSVVLHIILIALLIWGSLTQTTE
MGGGGAGGEVIDAVMVDPGAVTEQYNRQQQQQTDAKRAEQQRQKKAEQQAEELQQKQA
AEQQRLKELEKERLQAQEDARQAAEEQKKQAAEQQKQAAEQQKAAAAAAAKAKEEQKQ
AEAAAAQAKAEADKLVKAQAEAQKKAEAEAKKEAAVAAAAKKQAEADAKKAAADAKKAAA
AEAKKKAAAEAAAATDVDDLFGGLANSKNAPKSGSGAGAAAAGKGGGKKSGASAGDISGYLG
QVTAAIQSKFYDADLYKGRTCNLRIKLAPDGLLIDVKQEGGDPALCQAAIAAAKLAKIPKPPSQD
VYEVFKNAPLVFKPQ

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