The Significance of the N-terminal Region of TolQ in Maintaining Tol-associated Energy-dependent Functions and Cell Division in *Escherichia coli*

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ABSTRACT

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The composition of the Tol protein system is conserved throughout the Gram negative bacteria and functions to couple cytoplasmic membrane-derived energy to outer membrane targets in K-12 strains of Escherichia coli. Outer membrane integrity is reliant upon the Tol system and the Tol proteins are parasitized by a number of bacteriophage and bacterial toxins termed group A colicins. The broad host range bacteriophage P1 utilizes the Tol system of E. coli, specifically the TolQ protein, not for adsorption or translocation, but rather at some unknown point during the phage particle maturation process. The addition of a T7 tag to the N-terminus of TolQ in Escherichia coli renders cells unable to support the infectious phage life cycle. The T7 tag addition also inhibits TolQ participation in processes necessary for maintenance of outer membrane integrity and its ability to participate in crosstalk with the paralogous TonB system. The T7 tag addition to the N-terminus of TolQ however, does not inhibit TolQ protein synthesis or the ability of the protein to serve its role in the translocation of group A colicins. These findings indicate an important role for the N-terminal region of TolQ in energy-dependent processes, one that is inhibited by the addition of the T7 tag. Additionally, a role for the Tol system proteins during the cell division process has been suggested. The T7 tag addition to TolQ does not lead to a chaining or filamentous growth pattern in E. coli cells. However, the overexpression of native TolQ does result in this morphological abnormality. This finding clearly supports past data indicating a role of TolQ during the cell division process, a role that is interrupted by the T7 tag addition to TolQ.
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Chapter One

Introduction

*Escherichia coli* has long served as the archetypical bacterial model system for laboratory studies of the unique qualities associated with Gram negative bacteria. Gram negative bacteria are characterized by the presence of an inner cytoplasmic membrane (CM) and a distinct outer membrane (OM), separated by a thin layer of peptidoglycan. The peptidoglycan layer is located within an aqueous compartment found between the CM and the OM and known as the periplasmic space (Oliver, 1996). The CM is a phospholipid bilayer with the characteristic ability to establish and maintain an ion electrochemical gradient (Kadner, 1996). Conversely the OM, with its outer leaflet composed of lipopolysaccharide, lacks the ability to self-energize and is dependent upon energy harnessed at the CM to drive its energy-dependent processes (Postle and Kadner, 2003). Protein complexes associated with the OM, CM, and periplasmic space mediate the energization of the OM.

One such system, the TonB system, energizes the transport of vital nutrients across the diffusion barrier of the outer membrane. This well-studied system minimally consists of the energy-transducing protein TonB and the energy-harvesting pair, ExbB and ExbD. Together, this protein complex mediates the import of iron siderophores and vitamin B$_{12}$ across the outer membrane and into the periplasmic space in *E. coli* (Held and Postle, 2002). The TonB system is also parasitized by group B colicins and bacteriophages T1 and φ80 for cell entry. Import of each of these extracellular materials has been shown to be energy-dependent (Braun and Herrmann, 1993). Anchored in the CM, TonB appears to span the periplasmic space to interact with OM receptors such as
FepA and BtuB (reviewed in Postle and Kadner, 2003), aiding the transport of their ligands across the OM. This interaction of TonB with OM transporter is dependent upon a proton motive force (PMF) (Bradbeer, 1993). ExbB and ExbD, also anchored in the CM, harvest CM-derived energy and in turn, energize TonB. The coupling of TonB to the CM proton gradient drives active transport at the OM (Larsen, et al., 1999).

The Tol system was first identified through mutational analysis (reviewed in Lazzaroni et al., 2002). Mutations in the Tol protein system render cells unable to perform specific functions associated with the OM. Early studies revealed that mutations in the Tol system conferred tolerance to group A colicins and filamentous bacteriophages M13, f1, fd, and Ike, whose entry into cells require passage across the diffusion barrier of the OM (reviewed in Cascales et al., 2007). Other studies have provided evidence that mutations in the Tol system also disrupt OM integrity. Tol mutants exhibit membrane blebbing, leak periplasmic contents into the medium, and are hypersensitive to bile salts, detergents, and some antibiotics (Lazzaroni et al., 1989). Additionally, Tol mutants experience disruptions in cell division under certain conditions (Gerding et al., 2007).

Characterization of the tol-pal gene cluster has revealed it to be the product of seven genes, five of which encode proteins with relatively well established roles. The tol-pal gene cluster contains two promoters, $P_1$ and $P_B$. $ybgC$, $tolQ$, $tolR$, $tolA$, $tolB$, $pal$, and $ybgF$ are transcribed from $P_1$, while the internal promoter $P_B$ synthesizes a second transcript composed of $tolB$, $pal$, and $ybgF$ (Vianney et al., 1996). The TolQ and TolR proteins, anchored in the CM, form a heteromultimeric complex and harvest the energy of the CM proton gradient to energize TolA, the energy transducing protein of the Tol system. The TolQ protein, with its 19 residue N-terminal domain located in the periplasm
and its C-terminal domain in the cytoplasm (Vianney et al., 1994), possesses three transmembrane domains, a small periplasmic loop, and two relatively large portions inside the cytoplasm (Kampfenkel and Braun, 1993). TolR possesses a single transmembrane domain present near its N-terminus (Muller et al., 1993). TolA is anchored by one transmembrane domain near its N-terminus and extends into the periplasm (Levengood et al., 1991) to interact with the OM associated protein TolB and the lipid-anchored OM protein Pal (Vianney et al., 1996). The Tol system, mediated through Pal, interacts with two non-tol-encoded proteins, OmpA and the major lipoprotein Lpp. Two additional genes, ybgC and ybgF, encode proteins whose contributions to Tol system remain unclear (Cascales et al., 2002).

TolQ and TolR share sequence similarities with the ExbB and ExbD proteins, through which energy of the CM proton gradient is coupled via TonB protein to OM proteins that participate in a number of energy-dependent processes including iron siderophore transport via FepA and cobalamin transport via BtuB (Postle and Larsen, 2007) (Figure 1). Nucleotide sequence homology of exbB with tolQ and exbD with tolR were found to be 59.2 and 49.7% respectively. More importantly, amino acid sequence comparisons show that ExbB and TolQ share 26.3% identical amino acids and 79.1% of the non-identical amino acids are conservative replacements. Similarly, the amino acid sequences between ExbD and TolR are 25% identical and 70% conserved (Eick-Helmerich and Braun, 1989). Membrane topologies for TolQ and TolR are very similar to those of ExbB and ExbD, respectively, with the highest amino acid sequence homology in the transmembrane regions (Kampfenkel and Braun, 1993). TolA and TonB are homologous only in their N-terminal transmembrane domains (Germon et al., 2001).
Biochemical and genetic analyses indicate that the components of the Tol system and the TonB system function largely through interactions involving their transmembrane domains (Larsen et al., 2007, Lazdunski et al., 1995, Lazzaroni et al., 1995). Although TolQ/TolR and ExbB/ExbD support distinct processes at the OM, the two complexes can inefficiently substitute for one another through a process that is referred to as crosstalk. However, crosstalk does not occur between TolA and TonB, reflective of their specialized functions in the cell (Braun and Hermann, 1993). From a historical standpoint, it is likely that the Tol system and the TonB system are paralogues, having originated from a common transport system in E. coli (Eick-Helmerich and Braun, 1989).

While the true function of the Tol system is unknown, it is clear that many cellular processes are influenced by this collection of proteins. Much evidence exists for a central role for Tol proteins in biomolecule uptake and OM stability (reviewed in Lazzaroni et al., 1999). Filamentous bacteriophage and Group A colicins both parasitize the Tol system to gain entry into the cell (reviewed in Cascales et al., 2007). P1 bacteriophages utilize TolQ (Sun and Webster, 1987) presumably during virion maturation and/or release (Smerk, MS Thesis, 2007).
Figure 1. Components of the TonB and Tol systems of *Escherichia coli*.

The topology and partitioning of the TonB system and representative proteins with which it interacts are depicted at the figure left. TonB associates with the CM through a heteromultimeric complex of ExbB and ExbD and services the TonB-dependent OM transporters, represented here by the ferric enterobactin transporter FepA. TonB also interacts with OM proteins Lpp and OmpA.

The topology and partitioning of proteins encoded by the *tol* operon, and proteins with which Tol proteins are known to interact are depicted at the right of the figure. A heteromultimeric complex of TolQ and TolR, along with TolA, associates with the cytoplasmic membrane (CM) as depicted. Outer membrane (OM) associated *tol* gene products are TolB and Pal. Other proteins known to interact with Tol system components include Lpp, OmpA, and the porin OmpF. The roles of the two other *tol*-encoded proteins YbgC and YbgF are unknown.
The first reference to an infectious disease of bacteria came in a paper written in 1915 by Fredrick W. Twort when he described a plaque as a “glassy transformation.” Félix d’Herelle named the “ultraviruses” that invaded and multiplied within bacteria bacteriophages (Waldor et al., 2005). For many years thereafter, most study of bacteriophages was limited to their therapeutic use as a treatment for bacterial infectious disease, rather than inquiries into their biological nature. Research on phage therapy continued for two decades in a limited fashion, largely overshadowed by the discovery and successful use of antibiotics. In the 1930’s and 1940’s, Emory Ellis and Max Delbrück conducted studies that led to the unraveling of the mystery of bacteriophages. From studies of viral life cycles (Ellis and Delbrück, 1938) to describing the nature of genetic material (Hershey and Chase, 1952) and its tendency toward mutation (Luria and Delbrück, 1943), phage biology became a central component in the development of modern molecular biology (Waldor et al., 2005). Since the 1960’s, advances in biochemistry and molecular biology have allowed scientists to not only elucidate the biology of phages but to also successfully incorporate them into numerous areas of research. A small number of bacteriophage are routinely used in the laboratory today as model phages, similar to the use of *Escherichia coli* and *Arabidopsis thaliana* in bacterial and plant studies, respectively. Early studies of the virulent *E. coli* bacteriophage T4 led to advancements in genetics, providing a means to study mutation, genetic recombination and gene regulation (Benzer, 1955, Benzer, 1959). Kuhn, *et al.* (1987) also used T4 as a model to describe the steps of viral-self assembly. The temperate *E. coli* phage lambda has served as a valuable research tool as well, providing insight into the genetic regulation that governs the lytic and lysogenic phage life cycles (Ptashne *et al.*, 1980).
The temperate bacteriophage P1 infects *E. coli* as well as a number of other enteric bacteria. The discovery of P1 in 1951 (Bertani, 1951) brought unique features not found in T4 or lambda into the laboratory. Unlike the virulent *E. coli* phage T4, which is only capable of undergoing the lytic life cycle and the temperate *E. coli* phage lambda, which exists largely in a lysogenic state after integration into the host chromosome, the P1 prophage exists as an autonomously replicating plasmid of low copy number within the bacteria cell. While lambda is used for specialized transduction in the lab, P1 is an effective vector for the process of generalized transduction, the non-specific movement of genes from a donor cell to a recipient cell, usually of the same bacterial species, by means of phage progeny. Recently sequenced, the 93,601bp genome of P1 contains 117 identified genes, 49 for which homologues have not been identified (Lobocka, *et al.*, 2004). Of the 45 operons in the P1 genome, four are directly responsible for determining the pathway that the phage will employ, lytic or lysogenic, and four control plasmid maintenance. Most of the remaining operons are necessary for lytic development. Phage DNA is injected into the host cell, entering through an as yet unknown pore in the inner membrane, circularizes by recombination, and either follows a lytic life cycle or is maintained as a single copy lysogen in the form of a stable plasmid. Both environmental and genetic factors determine the life cycle path followed, largely regulated by the well characterized tripartite immunity system of *immC, immI, and immT* (Heinrich, *et al.*, 1995). P1 replicates by a process during which it can package an estimated 10% more DNA than comprises its genome, twice as much as can lambda (Yarmolinsky and Sternberg, 1988). P1 phage head packaging is error-prone and phage DNA packaging is of low fidelity. Host cell DNA is often packaged in P1 progeny instead of viral DNA,
making P1 an effective mediator of generalized transduction (Lennox, 1955).

Additionally, restriction-modification genes found within the P1 genome are routinely used in modern genetic engineering techniques (Lobocka, et al., 2004). Studies of P1 have also led to technical and informational advances in the areas of DNA recombination, site-specific recombination, and plasmid partition, incompatibility, and addiction systems (Lehnherr, 2006).

The P1 virion is composed of an icosahedral head attached to a tail with 6 tail fibers bearing regions responsible for host specificity (Lobocka, et al., 2004). In 1969, the receptor for P1 adsorption was identified as the terminal glucose moiety in the core region of the lipopolysaccharide (LPS) molecule in the OM of Gram negative bacteria (Franklin, 1969). Upon interaction of P1 tail fibers with this receptor, the phage tail contracts and pushes the tail tube through the baseplate. The tail tube then punctures the outer cell membrane and likely also pushes through the cell wall (Dreiseikelmann, 1994). The P1 genome is then injected into the periplasm and enters the cell through an as yet unidentified mechanism. Subsequent lysogeny is regulated by a number of mediators (reviewed in Lehnherr, 2006), and under conditions unfavorable for the lysogenic growth cycle, a lytic life cycle is initiated, the plasmid genome is replicated and phage-specific promoters initiate transcription of late genes whose products are required for phage progeny components, lysis control and finally, lysis (Lehnherr, 2006). P1 DNA packaging occurs by a processive headful mechanism, by which concatemeric units of viral DNA are cleaved at methylated phage packaging sites (pac) and fed unidirectionally into empty proheads until the head is full. This process results in terminally redundant
viral molecules that can become circular by homologous recombination upon injection into the host cell (Sternberg and Coulby, 1990).

The use of the terminal glucose residue of the core region of LPS as its receptor confers a broad host range on P1, and although a relatively large group of bacterial species can become infected by P1, only a subset of these species are capable of producing infectious, viable progeny. The process of P1 virion assembly has not yet been fully described, but Walker and Anderson (1970) observed three head sizes representing infectious phage particles with large heads (P1B), and non-viable progeny with small heads (P1S) and minute heads (P1M). Further characterizations of the phage particles have shown that many non-viable phage progeny are packaged with empty heads and revealed numerous genes responsible for P1 virion head size (Walker and Walker, 1983). Upon assembly of infectious phage particles, the timing of cell lysis is regulated by the protein products of three late genes; an endolysin that degrades the cell wall, a holin that punctures the cytoplasmic membrane, allowing the endolysin to reach the cell wall, and an antiholin protein that acts as an antagonist to the holin to ensure lysis does not occur prior to phage progeny assembly (Schmidt, et al., 1996). As new phage particles accumulate in the cytoplasm of host cells, the endolysin functions to cleave peptidoglycan, weakening the host cell wall. Increased pressure within the cell causes the cell to lyse, releasing 100-200 phage particles (Lehnerr, 2006).

With the sequencing of the P1 genome recently completed, characterization of the genetic control mechanisms governing the P1 bacteriophage life cycle is well underway (Lobocka et al., 2004). A number of genes involved in the maintenance of either the lytic or lysogenic life cycle of P1 as well as numerous genes involved in plasmid genome
replication, virus particle construction and assembly, and host cell lysis have already been characterized through mutational analyses (Lobocka et al., 2004). However, physical events surrounding virus assembly and maturation have been much less described. As observed with many other well characterized bacteriophages, it is likely that P1 virus progeny assemble in association with the inner host membrane (Lehn herr, 2006).

Numerous studies have focused on phage particle entry into the host cell and it has been clearly demonstrated that the Tol proteins are required for filamentous phage infection (Sun and Webster, 1987). However, much less is known about the mechanisms required for phage assembly and lysis from the host cell. In one such study demonstrating that the TolQ protein (previously referred to as fii) is a product of the tol-pal gene cluster and is required for filamentous phage infection, it was noted that some tolQ mutants do not plaque P1 (Sun and Webster, 1986). Further analyses of various Tol system mutants were examined and it was concluded that TolQ is the only Tol system protein essential to the production of viable, infectious P1 phage progeny. Interestingly, mutations in tolQ that disrupt TolQ energy-harvesting capabilities do not necessarily inhibit P1 maturation (Smerk, 2007). These observations suggest that TolQ mutational analyses should not be assessed in the same manner that typical Tol-associated functions are. Additionally, the ability to support P1 maturation can be used to assess TolQ function independent of functionality of other Tol proteins.

Specific Aims:

Previous studies have provided evidence that P1 takes advantage of the proteins of the Tol system to assemble and subsequently release viable and infectious phage progeny. Further investigations have indicated that of the complete energy-transducing Tol system,
only TolQ is specifically required for this process (Sun and Webster, 1986). This study was aimed at the mutational analysis of TolQ, with the specific objective of identifying the particular region or domain of the TolQ protein that is required for the maturation of viable P1 progeny.

To accomplish this objective, the following specific aims were developed:

1. Construct a TolQ protein that contains a T7 tag at the N-terminus, and then subject the gene encoding the fusion protein to mutagenesis. The T7 tag will allow protein expression levels to be determined via Western blotting during analyses.

2. Through mutational analysis identify the region of the TolQ protein essential for P1 maturation. The screening of mutagenized E. coli impaired in P1 maturation will reveal the specific structural features of TolQ involved in this process.

The background, methods, results, and conclusions related to this objective are thoroughly described in Chapter Two. Implications of findings related to this objective are further investigated in Chapters Three and Four.
Chapter Two

Generation of N-terminal T7-tagged TolQ and P1 Sensitivity Assays

Introduction

While conducting studies that concluded with the identification and characterization of the TolQ protein, Sun and Webster (1986) noted that various Tol system mutants do not plaque P1. They further identified a role for TolQ in filamentous bacteriophage infection, but the role of TolQ in the P1 life cycle was largely overlooked by many. Later, during analyses of numerous Tol system mutants, it was confirmed that only the TolQ protein is necessary for the production of viable P1 progeny (Smerk, 2007). In order to investigate the role of TolQ in the P1 life cycle, a plasmid encoding TolQ and TolR (pRA003) was genetically modified to so that TolQ possessed a tag that would allow for detection of protein levels within cells by Western analysis. Specifically, 11 residues from an unrelated T7 phage protein for which specific antibodies are available were fused to the N-terminus of TolQ. This construct was designed to be under control of the arabinose promoter and included TolR to conserve the stoichiometry of the energy-harvesting pair. Once constructed, the plasmid encoding T7/TolQ/TolR was transformed into a tolQ mutant and subjected to P1 growth assays to ensure its ability to complement the mutant strain. Additionally, sequencing and restriction analyses were used to confirm the construct.
Materials and Methods

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are summarized in Table 1. The wild type strain W3110 is described by Hill and Harnish (1981). All of the mutant strains are W3110 derivatives, made in the Larsen lab. Plasmids are constructed from the pBAD24 and pACYC184 vectors and are to be described elsewhere.

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<td>pBAD24 encoding T7-tagged TolQ</td>
<td>Larsen Lab, unpublished</td>
</tr>
<tr>
<td>pRA030</td>
<td>pACYC184 encoding TolQ/TolR</td>
<td>This study</td>
</tr>
<tr>
<td>pRA031</td>
<td>pBAD24 encoding TolQ</td>
<td>This study</td>
</tr>
<tr>
<td>pRA032</td>
<td>pBAD24 encoding T7-tagged TolQ and TolR</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 1.** Descriptions of strains and plasmids used in this study, including genotypes and phenotypes relative to wild type strain.
**Media.** Bacterial strains were maintained on Luria-Bertani (LB) plates or grown in LB broth and shaken at 37°C (Miller, 1972). Broth was supplemented with either 100µg mL\(^{-1}\) ampicillin or 34µg mL\(^{-1}\) chloramphenicol as necessary. P1 maturation assays were performed in LB supplemented with 5mM CaCl\(_2\) and 0.2% glucose to promote phage binding, 100µg mL\(^{-1}\) ampicillin, and 0.001% w/v L-arabinose. Deoxycholate sensitivity assays were performed in LB supplemented with 100µg mL\(^{-1}\) ampicillin and 0.001% w/v L-arabinose. Colicin A, colicin B, and φ80 spot titer sensitivity assays were performed on tryptone (T)-plates overlaid with cells suspended in T-top (Miller, 1972) supplemented with 100µg mL\(^{-1}\) ampicillin, and 0.001% w/v L-arabinose. Cells for filamentation assays were grown in LB with 100µg mL\(^{-1}\) ampicillin and 0.1%, 0.01%, 0.001%, 0.0001%, or 0.00001% w/v L-arabinose.

**P1 bacteriophage preparation.** P1\(^\text{kc}_{\text{vir}}\) (P1) was obtained from the American Type Culture Collection. This strain is a non-lysogenic P1 derivative that possesses a mutation that prevents the phage from entering the lysogenic life cycle. This P1 variant remains a lytic phage during infection cycles. P1 preparations were made from lab stock cultures using methods for the collection of phage lysate by P1 generalized transduction as described by Miller (1972). Overnight cultures of W3110 were subcultured 1:200 in LB supplemented with 0.2% glucose and 5mM CaCl\(_2\) and grown at 37°C with shaking. After 60 minutes, 100µL of ten-fold dilutions (10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\)) of P1 phage preparations were added to 5mL W3110 subcultures. An initial A\(_{550}\) reading was taken on a Spec20 spectrophotometer with a 1.5cm pathlength at the time of P1 addition and at each 45 minute interval thereafter. The culture that grew to the highest A\(_{550}\) before clearing, as indicated by a decline in absorbance, was chosen for P1 preparation. To prepare P1 stock
cultures, 50µL chloroform was added to the cell culture and the tube was vortexed to lyse remaining cells, releasing P1. The culture was then centrifuged at top speed for 5 minutes to remove cell debris. The supernatant containing the P1 preparation was harvested by pipette. An additional 50µL chloroform was added to the supernatant, the prep was vortexed and finally stored at 4°C.

**Colicin B preparations.** Colicin B dilutions were prepared from lab stock (Larsen *et al.*, 2003). Ten-fold dilutions were prepared in tryptone (T) broth for colicin B sensitivity assays.

**Colicin A preparations.** Colicin A preparations were harvested from *E. coli* strain BZB2101 (provided by Anthony Pugsley, Institut Pasteur). An overnight culture of cells was subcultured 1:20 in 50mL LB and grown at 37°C with shaking. At $A_{550}=1$, 50µL mitomycin C (1mg mL$^{-1}$) was added to flasks, and cultures were incubated for 4 additional hours at 37°C with shaking. Cells were then transferred to centrifuge tubes and spun for 10 minutes at 5000 rpm at 4°C. Supernatant was removed and discarded, and cells were resuspended in 10mL 1X M9 salts and transferred to a clean tube. Cells were then lysed by passage through a French press at approximately 20,000 psi. The sample was passed through press a second time to lyse a maximum number of cells, collected, and stored at -20°C. Five-fold dilutions of colicin A were prepared in T broth for colicin A sensitivity assays.

**φ80 preparations.** φ80 stocks, received from the Postle laboratory (Department of Molecular Biology, The Pennsylvania State University, College Park, PA) were diluted ten-fold in T broth for φ80 sensitivity assays.
**pRA032 construction.** pBAD24 derivatives pRA003 and pRA019 carrying tolQ/tolR genes and T7 tag/tolQ, respectively, were each digested with BssHII for 1 hour at 50°C and run on 1% agarose gel electrophoresis to separate digestion fragments. The T7/tolQ fragment (753nt) from pRA019 and the pBAD24 vector/tolR fragment (4971nt) from pRA003 were cut from the agarose gel, and DNA was recovered from gel slices using the QIAGEN® Sciences (Germantown, MD 20874, USA) MinElute Gel Extraction Kit. The vector/tolR fragment was dephosphorylated before DNA ligation. Ligation of the T7/tolQ fragment into the Vector/tolR fragment was carried out at a ratio of 3 insert to 1 vector using T4 DNA ligase from Invitrogen® (Carlsbad, CA 92008, USA) according to product protocols (Figure 2).

**Transformation.** At conclusion of ligation period, an appropriate amount of ligation mixture (3µL) was transformed into DH 5-alpha chemically competent E. coli cells from NEB® (Ipswich, MA 01938, USA) according to product protocol. 100µl aliquots of transformed cells were plated on LB plates with an ampicillin concentration of 100µg mL⁻¹ for selection of transformants. Transformants were grown overnight in 5ml LB 100µg mL⁻¹ ampicillin and subcultured 1:100 in LB 100µg mL⁻¹ ampicillin. Plasmid DNA was collected from DH 5-alpha cells using Alkaline lysis: Mini-preparation of plasmid DNA procedure (Sambrook et al., 2001). pRA032 construction was confirmed by sequence analysis and restriction mapping. The pRA032 plasmid was transformed into RA1035 (ΔtolQ/tolR) as previously described (Chung et al., 1989).
Figure 2. Simplified plasmid map of the pRA032 (also designated T7QR) construct generated within the pBAD24 vector. The vector contains two origins of replication, an ampicillin resistance gene (amp<sup>R</sup>), and the araC gene encoding a positive regulatory protein required for L-arabinose utilization.

Amino acid sequence of the T7-Tol Q fusion protein and the Tol R protein. The purple residues indicate the T7 sequence. The light blue indicates the Tol Q and the dark blue indicates the Tol R. The black residues indicate amino acids introduced as a result of the design of the construct.

```
MASMTGGQQMGRGEFVTDMTNILDIFKLASKLIMLILIGFISASW
AIIIQRTILNAAREAEEAFEDFWSGIELSRLYQESQGKRDNLTGSEQIFY
SGFKEFVRLHRANSHAPEAVVEGASRAMRISMNRELENLETIPFLGTGSI
SPYIGLFVTWGMHAFIALGAVKQATLQMVAPIAEALIATAIGLFAAPA
VMAYNRLNQRVKNLELYDNFMEETAILHRQAFTVSESNG...MARARGRGR
RDLKSEINIVPLLVDVLLLIFMATAPIITQSVEVDLPDATESQAVSSNDN
PPVIVEVGIGQYTVVVEKDRLELPPEQVVAEVSSRFKANPTVFLIGGAK
DVPYDEIIKALNNLHSAVGKSVGLMTQPI<sup>389</sup>
```
**P1 Growth Curves.** To determine function of the T7-tagged TolQ protein, the ability of ΔtolQ/tolR cells (RA1035) carrying the T7/tolQ/tolQ plasmid (pRA032) to support P1 maturation was assessed through use of a modified P1 generalized transduction procedure (Miller, 1972). Overnight LB cultures were subcultured 1:100 in LB supplemented with 100µg mL⁻¹ ampicillin, 5mM CaCl₂, 0.2% glucose, and 0.001% w/v L-arabinose. Initial A₅₅₀ readings were taken with a Spec20 spectrophotometer with a light path length of 1.5cm. Cultures were shaken at 37°C for 60 minutes before the addition of 100µL of an appropriate ten-fold dilution of P1 phage stock. A₅₅₀ readings were taken at the point of P1 addition and at subsequent 45 minute intervals. The ability to support P1 maturation was determined based on whether absorbance readings increased continuously and leveled off, indicating culture saturation or whether absorbance readings rose, then dropped sharply, indicating culture lysis. Lysed cultures support P1 maturation, and therefore, indicate functional Tol Q. P1 growth curves were performed in triplicate.

**Results**

The ability of the T7-tagged TolQ protein to support P1 maturation was assessed through analyses of growth profiles of tolQ/tolR mutants (RA1035) carrying plasmid encoded T7-TolQ fusion protein and TolR (pRA032). This assay was performed to confirm the construction of the pRA032 plasmid, and it was fully expected that the plasmid encoded T7/TolQ/TolR would rescue tolQ/tolR mutants (RA1035) in the same way that plasmid encoded TolQ/TolR (pRA003) does. W3110/pBAD24 represents the wild type ability to support P1 maturation. RA1035/pBAD24 represents the tolQ/tolR mutant phenotype, depicting the inability to support P1 maturation and a continuous P1 infectious life cycle.
As seen in Figure 3, while RA1035/pRA003 performs similarly to wild type *E. coli*, RA1035/pRA032 is essentially phenotypically identical to the *tolQ/tolR* mutant in its inability to maintain P1 infection. Both W3110/pBAD24 and RA1035/pRA003 cultures underwent significant lysis, first evident after the 120 minute time point, as evidenced by a decline in absorbance. RA1035/pBAD24 and RA1035/pRA032 cell cultures continued to grow exponentially throughout the experimental period, even after the addition of P1.

**Figure 3. P1 Growth Curves.** Growth curves of W3110/pBAD24, RA1035/pBAD24, RA1035/pRA003, and RA1035/pRA032 cells incubated with a $10^{-3}$ dilution of P1. Cell density is depicted on the y-axis as measured by light absorbance at 550nm ($A_{550}$) over time (x-axis). Cultures were inoculated with P1 at t=0 and absorbance was measured spectrophotometrically at 60 minute intervals for 4 hours. Only W3110/pBAD24 and RA1035/pRA003 demonstrated overall lysis, while RA1035 with pBAD24 and pRA032 continued to grow exponentially. Assays were performed in triplicate. The data shown here from one replicate represents the pattern observed in all three assays.
Discussion

Previous studies that indicated that TolQ is an essential component in the P1 maturation process (Smerk, 2007, Sun and Webster, 1986) led me to question which specific region of TolQ was required for this process to occur. Although it has been shown that tolQ mutants do not release infectious P1 viral particles, non-infectious viral progeny are released (Smerk, 2007). It is unknown at which point during viral replication the infectious life cycle of P1 is interrupted in tolQ mutants. Because a large portion of the TolQ protein is located within the cytoplasm, there are numerous regions of the TolQ protein that could serve this unknown role in P1 progeny maturation. Therefore, in preparation of the native tolQ gene for mutational analysis, I added the T7 bacteriophage encoded T7 phage gene 10 protein, a commonly used epitope tag. This T7 tag was added to the N-terminus of TolQ to allow for protein level detection during analyses. Once the T7 tag was added to TolQ in the pRA032 plasmid, a P1 growth assay was performed to confirm plasmid construction. The P1 growth assays indicated that while plasmid encoded TolQ/TolR (pRA003) in tolQ/tolR (RA1035) mutants supported P1 infectious progeny maturation, the plasmid encoded fusion T7-TolQ and TolR (pRA032) did not. While sequencing and restriction analysis confirmed pRA032 plasmid construction, this fusion protein was unable to perform its undefined role in the P1 replication cycle.

While my original goal in adding the T7 tag to the N-terminus of TolQ was to make the protein detectable by Western analysis and subject the construct to mutagenesis, I recognized that the addition of the T7 tag to the N-terminus of TolQ and the subsequent loss of TolQ function in P1 maturation may have created a TolQ mutant that behaved just as I had aimed to find through mutagenesis. Specifically, the addition of the T7 tag to the
N-terminus of TolQ abolished the ability of the TolQ protein to support P1 lytic progression following initial release.

Because this interesting TolQ “mutant” had arisen as a consequence of being fused with the T7 epitope tag, I chose to further characterize the T7-TolQ fusion product using standard assays that are commonly employed to assess Tol system function. This further characterization of pRA032 is the subject of Chapter 3 and my revised objective.

**Revised Objective:** The addition of the T7 tag to the N-terminus of TolQ affects the ability of cells to support P1 phage maturation. I will further characterize the ability of the T7-TolQ fusion protein to support other Tol-associated processes.

To accomplish this objective, I developed the following specific aim:

Through the use of phenotypic assays designed to assess Tol system function, construct a phenotypic profile for the T7-TolQ fusion protein. The phenotypic assays will include DOC sensitivity, colicin sensitivity and φ80 susceptibility.
Chapter Three

Analysis of Impact of T7 Addition on Tol-associated Function

Introduction

As *E. coli* live in the harsh environment of the digestive tract, they are continuously exposed to a variety of potential toxins. One potent antimicrobial agent is bile produced by the liver. Therefore, *E. coli*, as well as a number of other Gram negative enteric bacteria possess numerous mechanisms and employ various strategies to protect themselves from the effects of exposure to bile salts (Begley *et al*., 2005). The maintenance of an intact outer membrane is vital to the survival of enteric bacteria. *E. coli* with compromised outer membranes are susceptible to the destabilizing and deleterious effects of exposure to bile salts. Deoxycholate (DOC) is a bile salt that is commonly used in the laboratory to assess the outer membrane integrity of many Gram negative bacteria (Zwaig and Luria, 1967). As previously noted, the true physiological function of the Tol system remains to be described. However, it is clear that Tol system mutants suffer from a compromised outer membrane and are hypersensitive to numerous chemical and biological agents, including DOC (Lazzaroni *et al*., 1999). A rather straightforward analysis of Tol system function includes growth of Tol mutants at concentrations of DOC that are not lethal to wild type cells. Because outer membrane integrity is dependent upon a complete and functioning Tol system, cells with lethal Tol mutations will not be able to grow at DOC levels that are non-lethal to wild type cells.

Some strains of *E. coli* produce protein toxins, bacteriocins that are lethal to related strains, presumably in defense of a particular ecological niche. These bacteriocins are called colicins when they are produced by *E. coli*. Colicin-producing strains possess
an immunity mechanism that protects them from the effects of the bacteriocin. The ability to produce colicins is encoded on extrachromosomal genetic elements, plasmids that are referred to as colicinigenic factors. These plasmids are transferred through conjugation and are either found as small, multicopy plasmids within the cell or as large, monocopy plasmids that also encode additional genes advantageous for the bacterium (Cascales, et al., 2007). Colicins are composed of three globular protein domains. The central domain recognizes and binds to the receptor on a sensitive cell. The N-terminal domain mediates the translocation of the colicin into the sensitive cell and the C-terminal domain is responsible for the lethal action of the colicin on the cell (Braun, et al., 1994).

Colicin action on the sensitive cell can come in the form of nucleases that degrade RNA or DNA, or they can physically create pores in the target cell membrane. Secondary effects include nutrient starvation, as colicins often utilize iron and vitamin B receptors (Cascales, et al., 2007).

Colicins are classified into two groups based on the receptors they use to enter sensitive cells. Group A colicins use the Tol system to reach their target and include colicins A, E1-E9, K, L, N, S4, U, and Y. Group B colicins utilize the TonB system for translocation and include colicins B, D, H, Ia, M, 5, and 10. Group A colicins are encoded on small plasmids and are released into the medium, while group B colicins are encoded on large plasmids and are not secreted (Cascales, et al., 2007).

Numerous studies have been undertaken to determine the energy requirements for colicin entry into the cell. It has been concluded from results of these studies that while group B colicins require the energy provided by the ExbB/ExbD/TonB complex to cross the cell wall, group A colicins do not require Tol-derived energy at any time during
translocation, although they require the structural presence of various combinations of Tol proteins (Cascales et al., 2007).

Because the mode of colicin action, receptors, and translocation systems of sensitive cells have been well-characterized (reviewed in Cascales et al., 2007), colicins are often used as tools in the laboratory to assess the activity or presence of the Tol and the TonB system proteins. Particularly in mutational analyses, a loss of sensitivity to colicins can serve as a clear indicator of loss of function in one or all of the components of either system.

Bacteriophage φ80 is similarly used in the laboratory for mutational analyses. φ80 requires an intact and energized TonB protein for host cell infection (Hancock and Braun, 1976). It has been determined that φ80 assays serve as sensitive measures of TonB system activity, likely because only one phage is needed to kill a single cell (Larsen et al., 2003). The sensitivity of this assay also allows for its use in the analysis of Tol system function in TonB system mutants, as any sensitivity to φ80 in these mutants can be attributed to crosstalk from the Tol system. In our studies, φ80 sensitivity assays are used to determine the ability of TolQ and TolR to engage in crosstalk with TonB in ΔexbB/exbD strains.

In order to construct a phenotypic profile for the T7-tagged TolQ fusion protein, I subjected *E. coli* mutants carrying the pRA032 plasmid (T7/tolQ/tolR) to the aforementioned assays and analyzed the results of these assays, both separately and collectively. Because studies have demonstrated the ability of ExbB and ExbD to substitute in function for TolQ and TolR (Braun and Hermann, 1993), pRA032 was transformed into RA1033, a *tolQ* mutant that is also lacking the genes encoding ExbB
and ExbD. In this mutant strain, all pRA032 phenotypes could be unequivocally attributed to Tol function alone, as no crosstalk between the TonB system ExbB and ExbD and the Tol system would occur.

Materials and Methods

**Western analysis of pRA032.** Before performing phenotypic assays for the T7-tagged TolQ fusion protein, Western analysis was used to confirm expression of the plasmid-encoded genes. Cells were grown in LB supplemented with 100µg mL⁻¹ ampicillin at 0.01% L-arabinose to an A₅₅₀ of 0.4 before trichloroacetic acid (TCA) was added to a final concentration of 10%. Cultures were vortexed and set on ice for 15 minutes. Cultures were then centrifuged at 4°C for 5 minutes, aspirated, and the pellet was washed with 1mL 100mM Tris-HCl (pH 7.9). Wash was immediately aspirated and the pellet was centrifuged for 5 minutes at 4°C. The remaining supernatant was aspirated, the pellet was resuspended in 2µL 1M Tris-HCl and 25µL 2X Laemmli sample buffer, and tubes were boiled for 5 minutes (Abelson et al., 1990). Samples were subjected to electrophoresis on SDS 11% polyacrylamide gels. Resolved proteins were electrotransferred to Immobilon P® (Millipore Corp. Bedford, MA 01730, USA) membrane and immunoblot analyses performed using Novagen T7 Tag® (Madison, WI 53719, USA) anti-mouse Immunoglobulin HRP Conjugate and enhanced chemiluminescence (ECL), as previously described (Larsen et al., 1993, Higgs, et al., 1998).

**Deoxycholate sensitivity assay.** Deoxycholate (DOC) sensitivity assays were performed to determine the ability of T7-TolQ to participate in maintenance of outer membrane integrity. Overnight cultures were subcultured 1:100 in LB with 100µg mL⁻¹
ampicillin at 0.001% L-arabinose for 60 minutes before the addition of DOC at 0.25%. A₅₅₀ readings were taken on the Spec20 spectrophotometer with a path length of 1.5cm at 60 minute intervals for 240 minutes. Outer membrane integrity was determined based on whether absorbance readings increased throughout the assay or decreased after the addition of DOC, indicative of cell lysis. Cells with compromised outer membranes lyse upon the addition of DOC. DOC sensitivity assays were performed in triplicate.

**ColicinA, colicin B, and φ80 spot titers.** Overnight cells were subcultured 1:100 in LB supplemented with 100µg mL⁻¹ ampicillin at 0.001% L-arabinose to an A₅₅₀ of 0.4, as determined with the Spec20 spectrophotometer. One hundred microliters cell cultures was suspended in 3ml molten T(tryptone)-top agar containing 100µg mL⁻¹ ampicillin and a 0.001% L-arabinose concentration and overlaid onto room temperature T plates. Serial five-fold dilutions of crude colicin A and colicin B preps and serial ten-fold dilutions of φ80 phage prep were applied to plate surfaces as 5µL aliquots and plates were incubated overnight at 37°C. Results were recorded as the highest dilution at which clearing of the bacterial lawn was observed. Colicin A, colicin B, and φ80 spot titers were performed in triplicate.

**Results**

**Western analysis of T7-TolQ.** The Western blot results verified that T7-tagged TolQ was expressed at detectable levels in our system (Figure 4). Lane 10 contained a T7 positive control with an apparent molecular mass of 31.1 kDa. Exposure to the T7 antibody revealed a strong band at the correct position when compared to the molecular mass standard run in lane 1. A stained membrane was used to determine molecular masses on the developed blot. Lanes 8 and 9 contained protein preps of
RA1033/pRA032 cells grown at 0.01% \(L\)-arabinose and 0.1% \(L\)-arabinose respectively. Significantly, a clear band was present in each of these lanes at around 25kDa. The predicted mass of the TolQ protein is 25.6 kDa, suggesting a T7-tagged TolQ protein was present at detectable levels in these cells. Also, the band seemed to intensify in the protein sample from the higher \(L\)-arabinose concentration. Interestingly, a clear band was also present at a much higher molecular mass in all protein preparations regardless of \(L\)-arabinose concentration at approximately 78 kDa. This band was even present in the samples grown with no \(L\)-arabinose present, but there was no band in the cells containing the pRA003 plasmid (no T7 tag). At this time it is unclear what this band represents but it is likely a readthrough product of the plasmid. It does not however, negate the conclusion that T7-tagged TolQ was being synthesized at the \(L\)-arabinose concentrations used for the subsequent analyses.

![Western analysis of T7-TolQ fusion protein](image)

**Figure 4. Western analysis of T7-TolQ fusion protein.** Lane 1 was loaded with 5\(\mu\)L of a protein size standard. Lanes 2 and 3 contained protein samples from RA1033 cells carrying the pRA003 plasmid encoding TolQ and Tol R at 0 and 0.1% \(L\)-arabinose concentrations. Lanes 4 through 9 contained protein samples from RA1033 cells carrying the pRA032 plasmid encoding T7-TolQ and TolR expressed at 0, 0.00001, 0.0001, 0.001, 0.01, and 0.1% \(L\)-arabinose concentrations, respectively. Protein extracts were loaded by volume (20\(\mu\)L). Lane 10 contained 5\(\mu\)L of the T7 positive control. A band at the appropriate size of a T7-TolQ fusion protein was detectable in lanes 8 and 9.
**DOC sensitivity assay.** As expected, wild type cells (W3110) with a control plasmid (pBAD24) showed no sensitivity to the deoxycholate treatment. The selected DOC concentrations were not sufficient to markedly impact growth or viability of cells with an uncompromised outer membrane. On the other hand, the RA1033 ($\Delta$tolQ/$\Delta$exbB/$\Delta$exbD) mutants demonstrated a clear susceptibility to the deoxycholate at these levels. The lack of energy transduction normally supplied from TolQ and TolR, and the absence of any cross-talk capacity from components from the Ton system (ExbB and ExbD), clearly influenced the cell’s ability to sustain the integrity of the outer membrane. These same cells supplied with functional TolQ and TolR from the pRA003 plasmid exhibited resistance to deoxycholate comparable to wild type performance. This signified that the re-establishment of energy transduction returns OM integrity maintenance. However, cells expressing the TolQ protein with the T7 tag on the N terminus were just as susceptible to deoxycholate as mutant cells without TolQ present at all. Functional TolR was present in both systems, so it appeared that it was solely the modified TolQ responsible for the distinction observed. This also indicated that the modified TolQ was unable to establish the energy transduction capacity necessary to allow these cells to be insensitive to DOC treatment (Figure 5).
Figure 5. **0.25% DOC Growth Curve.** Growth curves of W3110/pBAD24, RA1033/pBAD24, RA1033/pRA003, and RA1033/pRA032 cells incubated in LB at 0.25% deoxycholate (DOC). Cell density is depicted on the y-axis as measured by light absorbance at 550nm ($A_{550}$) over time (x-axis). DOC was added at t=0 and absorbance was measured spectrophotometrically at 60 minute intervals for 4 hours. Complete lysis was observed in RA1033/pBAD24 and RA1033/pRA032 cells. Assays were performed in triplicate. The data shown here from one replicate represents the pattern observed in all three assays.

**Colicin A, colicin B, and φ80 spot titers.** Examination of the colicin assay data revealed two important factors regarding the impact of the T7 tag addition to TolQ. Colicin A sensitivity assays showed that mutants lacking the TolQ protein (RA1033) were resistant to lysis by this colicin, whereas the wild type cells (W3110) that possess endogenous TolQ could be parasitized by Colicin A up to a dilution factor of $5^{-6}$. Mutant cells with the pRA003 plasmid supplying functionally competent TolQ and TolR displayed sensitivity to Colicin A comparable to wild type cells, with clearing zones.
being obvious up to a $5^7$ dilution factor. Importantly, the T7/TolQ/TolR (pRA031) plasmid also revealed sensitivity to Colicin A out to at least $5^{-5}$ dilutions of the same crude preparation. This was significant because it demonstrated that the TolQ with the T7 tag addition must have been produced and inserted into the cell membrane in order for the assay to display any ability of the colicin to enter and kill the cells. Therefore any observed results that showed a lack of function would not be attributable to a lack of protein production or membrane insertion. Secondly, it showed that the modified TolQ was able to support some processes that were associated with endogenous TolQ, but not all. For example, membrane integrity remained compromised in cells expressing only T7-TolQ, but these cells were still susceptible to Colicin A. It has been determined previously that parasitism by colicin A likely does not require energy transduction by the Tol system, but rather simply a structurally competent protein (Cascales et al., 2007). Therefore, the T7 tag may not radically modify the overall structure of the TolQ protein, but may instead specifically interfere with its capability to harvest and transfer energy (Figure 3).

Unlike Colicin A, Colicin B has been clearly demonstrated to require TonB system-associated energy to parasitize E. coli cells (Jiang et al., 1997). Colicin B sensitivity in these cells was also examined to assess the ability of TolQ and TolR to supply energy via cross-talk, as the mutant cells also lack the ExbB and ExbD proteins that energize the TonB protein, and whether or not the T7 tag addition modifies the capacity to energize TonB. As expected, RA1033 mutants showed no sensitivity to colicin B treatment at any level. In comparison, wild type cells showed sensitivity to colicin B treatments up to a $5^{-8}$ dilution. This sensitivity was equaled by RA1033 cells
when TolQ and TolR were plasmid expressed (pRA003). However, RA1033 cells carrying pRA032 (T7/tolQ/tolR) were resistant to Colicin B treatment at all concentrations. Therefore, it was apparent that the T7-modified TolQ protein could not participate in crosstalk with the TonB system in the same manner as the unmodified TolQ can (Figure 6). Similarly, φ80 spot plates showed clearings to $10^{-7}$ dilution in wild type and RA1033 cells expressing plasmid encoded TolR and TolQ (pRA003), while tolQ/exbB/exbD mutants (RA1033) and RA1033 expressing the T7TolQ fusion protein showed no sensitivity at all to φ80 (data not shown).

![Figure 6. Colicin Spot Assays.](image)

Figure 6. Colicin Spot Assays. Colicin A and colicin B spot plate assays on W3110/pBAD24, RA1033/pBAD24, RA1033/pRA003, and RA1033/pRA032. Spots were placed in decreasing colicin concentration from right to left, top to bottom. Sensitivity of each strain to colicins was scored as the highest dilution that a clearing in the lawn could be observed. Complete resistance to both colicin treatments was observed in RA1033/pBAD24 cells, and to colicin B in RA1033/pRA032 cells. Assays were performed in triplicate. The data shown here from one replicate represents sensitivity levels observed in all three assays.
Discussion

The role of the Tol system in the P1 bacteriophage infection cycle was confirmed early during studies of the gene cluster (Sun and Webster, 1986). During these studies, it was noted that certain TolQ mutants do not plaque P1. This essential role for TolQ in the P1 infection cycle was confirmed through mutational analyses more recently (Smerk, 2007). My first specific research aim was to add an antibody-detectable epitope tag to the N-terminus of TolQ and subject the resulting gene product to mutagenesis. I would then screen mutants for loss of function in P1 infectivity. Although the correct TolQ sequence in the construct was confirmed through sequence analysis, the addition of the T7 tag to the N-terminus of TolQ appeared to render the protein unable to participate in the P1 maturation process. Because the construct contained the wild-type TolQ sequence, this fusion protein was an interesting candidate for Tol system analysis. Specifically, I was interested if this fusion protein made of the short, 11 amino acid tag fused to the TolQ protein N-terminus was able to support any other Tol-system functions.

I first confirmed that the construct was being transcribed and translated by performing Western analysis on cells lacking a chromosomal TolQ gene (RA1033) but carrying the plasmid-encoded T7/tolQ/tolR genes (pRA032). Once I had determined that the T7-tagged TolQ protein was being made in these cells (Figure 4), I set forth to characterize the phenotype of this fusion protein with regards to well known Tol system functions within the cell. I first characterized the ability of the T7-TolQ fusion protein to perform in the maintenance of outer membrane integrity by conducting deoxycholate sensitivity assays (Zwaig and Luria, 1967). I then assessed its participation in the uptake of group A colicins, which has been shown to require the Tol system (Davies and Reeves, 1975b),
and its ability to substitute for ExbB and ExbD in the energization of TonB through crosstalk between the Tol and TonB systems (Braun and Hermann, 1993). Crosstalk capability was assessed through the use of colicin B (Davies and Reeves, 1975a) and φ80 spot titer assays (Larsen et al., 2003). Finally, I considered the results of all assays collectively to construct a phenotypic profile for the T7-TolQ fusion protein.

The DOC sensitivity assay clearly demonstrated that the T7-TolQ fusion protein was unable to substitute for the wild type TolQ in its role in maintenance of the outer membrane. While RA1033 (ΔtolQ/ΔexbB/ΔexbD) cells expressing tolQ/tolR were able to grow at DOC concentrations of 0.25% as wild type cells (W3110) could, RA1033 cells expressing the T7/tolQ/tolR construct (pRA032) were sensitive to this concentration of DOC. Growth curves showed that these cells grew similarly to the tolQ/exbB/exbD mutants (RA1033) carrying no plasmid-encoded Tol or TonB system genes (Figure 5).

Colicin A spot titer assays were performed on ΔtolQ/ΔexbB/ΔexbD cells (RA1033) expressing the plasmid-encoded T7-TolQ fusion protein to evaluate the ability of the T7-TolQ fusion protein to partake in the Tol-associated uptake of Group A colicins. Results of these assays indicate that tolQ mutants (RA1033) exhibit no sensitivity to group A colicins, as seen by the lack of any clearing in bacterial lawns. Interestingly, mutants expressing the T7-TolQ fusion protein were sensitive to colicin A, showing only about a ten-fold reduction in sensitivity when compared to wild type cells and RA1033 cells expressing a plasmid-encoded TolQ/TolR construct (pRA003) (Figure 6). Several other group A colicins, including E1, K and N showed sensitivities similar to those observed with colicin A (data not shown). These results indicated that the T7-TolQ
fusion protein sufficiently substituted for wild type TolQ in the process of Tol-dependent colicin A uptake.

Colicin B spot plate assays were carried out to determine whether or not the T7-TolQ fusion protein was able to participate in crosstalk with the TonB system, specifically as a substitute for ExbB in exbB/exbD mutants (RA1033). The results of these assays clearly indicated that while wild type W3110 and mutant (RA1033) cells expressing plasmid-encoded tolQ/tolR (pRA003) were equally sensitive to colicin B, both RA1033 (ΔtolQ/ΔexbB/ΔexbD) and RA1033 expressing the T7-TolQ fusion protein (pRA032) exhibited no sensitivity to colicin B, as evidenced by the lack of any clearing in bacterial lawns (Figure 6). All four strains performed essentially identically in φ80 spot plate assays (data not shown). These results indicated that the T7-TolQ fusion protein does not participate in crosstalk between the Tol and TonB systems.

Taken together, results of Western analysis, DOC sensitivity assays, and colicin A, colicin B, and φ80 spot plate assays present an interesting picture of the phenotypic capabilities of the T7-TolQ fusion protein (Table 2). Collectively, results of these assays reveal that while the T7-TolQ fusion protein represents a loss of function mutant in some Tol-associated processes, the protein functions similar to wild type TolQ in others. Specifically, Western analysis confirmed the translation of this protein and sequence analysis confirmed the correct amino acid sequence of the product. Additionally, colicin A sensitivity spot plate assays clearly illustrate that the T7-TolQ fusion protein performs in the uptake of this bacteriocin, a Tol-associated process (Davies and Reeves, 1975b).
### Table 2. Phenotypic profile for T7-TolQ.

1Strain and Plasmid descriptions: Wild type *E. coli* is W3110 (Hill and Harnish, 1981). RA1033 is W3110/ΔtolQΔexbBΔexbD (Brinkman and Larsen, 2008). pRA003 supplies a functional tolQ/tolR sequence inserted into the plasmid vector pBAD24. pRA032, also constructed from pBAD24, contains the gene sequence for the T7 tag, tolQ, and tolR (This study, unpublished). Expression of tolQ/tolR and T7/tolQ/tolR is under the control of the L-arabinose promoter.

2S, Sensitive: Cells are sensitive to colicins A and B and bacteriophage φ80, support P1 maturation, and are not hypersensitive to 0.25% deoxycholate.

2R, Resistant: Cells are resistant to colicins A and B and bacteriophage φ80, do not support P1 maturation, and are hypersensitive to 0.25% deoxycholate.

“+” denotes the functional, wild type phenotype

“−” denotes the non-functional, mutant phenotype


5 Hancock and Braun, 1976.

6 Energy requirement unknown.

<table>
<thead>
<tr>
<th>Genotype¹ Phenotype²</th>
<th>Col A (No energy required)³</th>
<th>Col B (Energy required)⁴</th>
<th>φ80 (Energy required)⁵</th>
<th>P1 (Energy Required?)⁶</th>
<th>DOC (Energy required?)⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type (W3110)</td>
<td>+(S)</td>
<td>+(S)</td>
<td>+(S)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TolQ/ExbB/ExbD Mutant (RA1033)</td>
<td>-(R)</td>
<td>-(R)</td>
<td>-(R)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TolQ/ExbB/ExbD Mutant+TolQ/TolR (RA1033/pRA003)</td>
<td>+(S)</td>
<td>+(S)</td>
<td>+(S)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TolQ/ExbB/ExbD Mutant+T7/TolQ/TolR (RA1033/pRA032)</td>
<td>+(S)</td>
<td>-(R)</td>
<td>-(R)</td>
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</tr>
</tbody>
</table>

Considering the ways in which these assays are traditionally used to assess Tol system function, these results may initially appear to be somewhat inconsistent and confusing. However, when they are considered from the perspective of energy-requirement, there appears to be a pattern. While the import of Group A colicins appears
to be an energy-independent process (Bourdineaud, *et al.*, 1990, Lazdunski *et al.*, 1998, reviewed in Cascales. *et al.*, 2007), an energized TonB system is required for the import of group B colicins and bacteriophage φ80 (Braun *et al.*, 1980, Hancock and Braun, 1976, reviewed in Cascales *et al.*, 2007). It is likely that most or all Tol-associated processes that maintain OM integrity are energy-dependent processes (Nikaido, 2003). The energy requirements for P1 maturation and/or release have not yet been characterized.

Mutational analyses in the N-terminal transmembrane domain of TolA illustrated that TolQ interacts with the N-terminus of TolA through its first transmembrane domain (Germon, *et al.*, 1998). Similarly, mutational analyses of the TolR transmembrane domain revealed that its interaction with TolQ occurs in the third transmembrane domain of TolQ (Journet, *et al.*, 1999). These interactions are assumed to occur and be necessary for the TolQ/TolR pair in the harvesting of energy and the subsequent transfer of this energy to TolA during energy-dependent Tol system processes. These processes are numerous and include the entry of filamentous phage DNA (Sun and Webster, 1987), nutrient import and molecule exclusion (Lazzaroni, *et al.*, 1999), and the transport of newly synthesized OM components across the periplasm (Lloubès, *et al.*, 2001). Upon examination of the phenotypic profile of the T7-TolQ fusion protein (Figure 4), the pattern that emerges is one of a division between energy-dependent and energy-independent Tol functions. Because TolQ and TolR can together substitute for ExbB and ExbD, which have themselves been clearly demonstrated to couple the TonB protein to the electrochemical gradient of the CM (Larsen *et al.*, 1999, Larsen and Postle, 2001), it is presumed that TolQ-TolR complexes similarly harvest CM-derived energy. Indeed,
much as ExbB/ExbD mediates energy-dependent conformational changes in TonB (Larsen et al., 1999), TolQ/TolR has been shown to support energy-dependent conformational changes in TolA (Germon, et al., 2001).

From these studies of the T7-TolQ fusion protein, I conclude that the addition of the 11 amino acid T7 epitope tag to the N-terminus of TolQ impairs its ability to substitute for wild type TolQ in the P1 maturation process and in its role in OM maintenance. T7-TolQ cannot participate in crosstalk with the TonB system, as evidenced by resistance to colicin B and φ80 in ΔexbB/ΔexbD cells expressing the T7-TolQ fusion product. I also conclude that this fusion protein is expressed in my system and can substitute efficiently for wild type TolQ in the uptake of group A colicins. In this study, the phenotypic assays I chose consisted of multiple energy-dependent Tol-associated processes and one energy-independent Tol-associates process. Collective analysis of the assay results suggest that the addition of the N-terminal T7 tag to TolQ allows for the protein to be translated and inserted into the cytoplasmic membrane but inhibits its energy-harvesting capabilities. These results are not surprising, as interaction with TolA occurs via the TolQ first transmembrane domain, located only 19 amino acids from the N-terminus. It is not unreasonable to speculate that such an N-terminal modification would not prevent the interaction between the transmembrane domains of these proteins, but may instead impair energy-dependent conformational shape changes that occur during energy transfer. Whether the T7 tag addition to the N-terminus of TolQ affects its insertion in the CM or the stability of the protein or it alters TolQ function in some other way remains to be studied. However, based on the assays performed for
which energy requirements have been described, the T7 tag addition appears the render TolQ unable to participate in energy-dependent processes.

While it is a reasonable assumption that OM maintenance consists of numerous energy-dependent processes, the energy requirement for the maturation of P1 phage particles has not yet been described. It is tempting to speculate from this study that P1 maturation requires an energized Tol system, but more data is needed to confirm this conclusion. One way that this data could be collected would be to demonstrate directly that the P1 infection cycle is dependent exclusively on Tol-transduced energy. Because the true physiological role of the Tol system has yet to be determined, this type of analysis is not a simple matter. Another more realistic aim would be to subject the T7-TolQ fusion protein to further assays designed to test Tol-associated functions. Ideally, the energy-requirement for these processes would be clearly defined, and the data collected could be added to the existing phenotypic profile of the fusion protein, providing a larger and more comprehensive picture of its phenotypic capabilities.
Chapter Four

Cell Division Phenotypes Associated with Expression Levels of TolQ and T7-TolQ

Introduction

Unlike with the TonB system, which as been clearly demonstrated to have a specific, energy-dependent role in active transport across the outer membrane (Braun, et al., 1980), the true physiological role of the Tol proteins remains to be defined. It has been demonstrated that numerous cellular processes in Gram negative bacteria are either directly or indirectly affected by the presence and/or absence of Tol proteins. Mutational analyses have led to the identification of numerous phenotypes that consistently appear in cells with impaired Tol function (reviewed in Lazzaroni et al., 2002). Mutations in the Tol system confer tolerance to group A colicins and filamentous bacteriophage (reviewed in Cascales et al., 2007). Tol mutations also disrupt OM integrity, resulting in membrane blebbing, leaking of periplasmic contents into the medium, and hypersensitivity to bile salts, detergents, and some antibiotics (Lazzaroni et al. 1989). While maintenance of OM integrity is the most widely accepted assigned role for the Tol system, evidence indicates that this system also has either a direct or peripheral role in the cell division process. Meury and Devilliers (1999) reported that E. coli tolA mutants display impaired cell division patterns at high and low osmolarities. This distinct morphological phenotype consisting of filamented or chaining cells has also been observed in tol-pal mutants of Vibrio cholerae (Heilpern and Waldor, 2000), Pseudomonas putida (Llamas et al., 2000) and Erwinia chrysanthemi (Dubuisson et al., 2005). Considering the unique phenotypes observed upon expression of the T7-tagged TolQ protein, with the loss of
some Tol-associated processes and the retention of others, the next question to be asked was what, if any, morphological effect would expression of the T7-tagged TolQ protein have on cell division in *E. coli* under conditions of high and low osmolarities as well as under normal growth conditions.

More recently, using GFP-tagged Tol components, Gerding *et al.* (2007) provided evidence that all five Tol proteins localize to constriction sites during cell division and that Tol mutants experience delayed OM invagination and contain outer membrane blebs at constriction sites and cell poles. Results of this study led to a proposed mechanism that includes Tol system components as mediators of OM invagination during cell division. The septal ring (SR) is a complex of proteins that collectively participate in bacterial cytokinesis, the last of which to join the ring being FtsN. The model proposed by Gerding *et al.* (2007) asserts that active FtsN at the SR recruits the Tol system components to cell constriction sites as invagination of the cytoplasmic membrane (CM) and peptidoglycan (PG) layer occur. At the SR, energized TolA reaches through newly synthesized PG to interact with Pal, tethering the OM to the CM and serving to both spatially and temporally coordinate invagination of the OM, PG, and the CM (Gerding *et al.*, 2007).

This model suggests a requirement for functional TolQ and TolR to energize TolA in the tethering process. Therefore, we decided to investigate whether or not *tolQ* mutants display any type of observable cell division phenotype and if so, whether the mutants can be rescued with plasmid-expressed TolQ. More specifically however, we were interested in determining if the T7-tagged TolQ fusion protein would be unable to mimic the unaltered TolQ protein in rescuing this phenotype. Since the model suggests
the role of the Tol system in cell division is dependent upon an energized TolA, and TolA is energized primarily by the TolQ/TolR protein complex, such an observation would fit nicely with the rest of the accumulated data suggesting that the addition of an N-terminal T7 tag disrupts the energy-dependent function of TolQ. It was expected that if the model proposed by Gerding et al. (2007) held correct, cells expressing the T7-TolQ fusion protein would exhibit phenotypes associated with the inability of the cell division process to complete successfully. Specifically, it was expected that if TolQ participates in the energization of TolA, which in its energized state tethers the OM to invagination of the CM and PG during cell division through interaction with Pal, cell chaining and/or filamentation of cells expressing the T7-TolQ fusion protein would result.

**Methods (a)**

**pRA030 construction.** pBAD 24 derivative pRA003 carrying tolQ/tolR genes and pACYC184 vector were digested with SphI and ClaI for 60 minutes at 37°C before enzymes were heat inactivated at 65°C for 20 minutes. NEB® (Ipswich, MA 01938, USA) Antarctic phosphatase was used to dephosphorylate the vector at 37°C for 15 minutes. The phosphatase was heat inactivated at 60°C for 15 minutes before mixture was ligated at room temperature for 60 minutes with T4 DNA ligase from Invitrogen® (Carlsbad, CA 92008, USA). At conclusion of the ligation period, an appropriate amount of ligation mixture (5µL) was transformed into DH 5-alpha chemically competent *E. coli* cells from NEB® (Ipswich, MA 01938, USA) according to product protocol. Aliquots of transformed cells (100µL) were plated on LB plates with an ampicillin concentration of 100µg mL⁻¹ for selection of transformants. Transformants were grown overnight in 5ml LB 100µg mL⁻¹ ampicillin and subcultured 1:100 in LB 100µg mL⁻¹ ampicillin. Plasmid
was collected from DH 5-alpha cells using Alkaline lysis: Mini-preparation of plasmid DNA procedure (Sambrook et al., 2001).

**pRA031 construction.** pBAD24 derivative pRA003 carrying *tolQ/tolR* genes was digested with NcoI and XbaI for 90 minutes at 37°C before enzymes were heat inactivated for 20 minutes at 65°C. The digested plasmid was then Klenow filled at 37°C for 30 minutes. T4 DNA ligase from Invitrogen® (Carlsbad, CA 92008, USA) was used to ligate the plasmid. At the conclusion of ligation period, an appropriate amount of ligation mixture (5µL) was transformed into DH 5-alpha chemically competent *E. coli* cells from NEB® (Ipswich, MA 01938, USA) according to product protocol. One hundred microliter aliquots of transformed cells were plated on LB plates with an ampicillin concentration of 100µg mL⁻¹ for selection of transformants. Transformants were grown overnight in 5ml LB 100µg mL⁻¹ ampicillin and subcultured 1:100 in LB 100µg mL⁻¹ ampicillin. Plasmid was collected from DH 5-alpha cells using Alkaline lysis: Mini-preparation of plasmid DNA procedure (Sambrook et al., 2001).

**RA1033 mutant analysis.** In order to test the hypothesis that cells expressing the T7-TolQ fusion protein would grow as filaments, the RA1033 (*ΔtolQ/ΔexbB/ΔexbD*) strain of *E. coli* was chosen to carry the plasmid encoding the T7-tagged TolQ protein. RA1033 was chosen because it is a TolQ mutant that is also lacking the genes encoding ExbB and ExbD, which can inefficiently energize Tol A through crosstalk (Braun and Hermann, 1993). Overnight LB 100µg mL⁻¹ Amp cultures of RA1033/pRA032, RA1033/pRA003, and RA1033/pBAD24 were subcultured 1:200 in LB with 100µg mL⁻¹ ampicillin supplemented with 0.01%. Cells were grown for 24 hours at 37°C with shaking, at which time smears were made of each culture. Smears were heat fixed to the
slides and stained using safranin for 60 sec. Slides were then viewed using a compound light microscope and photographed. Visual abnormalities in morphology were noted.

Figure 7. Overexpression of TolQ/TolR and T7/TolQ/TolR in mutants. RA1033 (ΔtolQ/ΔexbB/ΔexbD) cells expressing pBAD24 (no Tol or TonB components), pRA003 (tolQ/tolR), and pRA032 (T7/tolQ/tolR) grown in 0.01% L-arabinose for 24 hours, stained with safranin, and viewed at 1000x. The RA1033/pRA003 cells demonstrated a septate filamentation phenotype.

Results (a)

Curiously, while RA1033 mutants lacking the ability to energize TolA displayed no readily observable division phenotype after 24 hours of growth in L-arabinose-supplemented media, the expression of wild type TolQ in these E. coli cells resulted in a filamentous phenotype (Figure 7). Cells were clearly elongated in a polar fashion to a greater extent than either cells of wild type or the RA1033 mutant. This might be
expected in a cell lacking the ability to secure the OM to the invaginating PG and CM at septal ring positions, creating a contiguous outer membrane envelop around several otherwise divided cells. Most interestingly, expression of the T7-tagged TolQ fusion protein (pRA032) did not generate a filamentous phenotype in these cells. These findings suggest that whatever impact the expressed TolQ has in the cell division process, the T7-tagged TolQ is unable to replicate.

Methods (b)

Analysis of wild type cells. Even in cells completely lacking endogenous TolQ, 0.01% L-arabinose was sufficient to supply protein beyond wild type levels, as seen by Western blot analysis (Chapter 3, Figure 4). Therefore, we surmised that the division phenotype we were observing may have actually been the result of a TolQ overexpression condition rather than a mutant phenotype. To examine this possibility, wild type E. coli (W3110) expressing either TolQ (pRA003 and pRA030) or T7-tagged TolQ (pRA032) at 0.1% L-arabinose induction levels to over-express the Tol proteins in these cells were examined. The cells were treated, stained, and viewed in the same manner as described for the RA1033 transformants.
Figure 8. Overexpression of TolQ/TolR and T7/TolQ/TolR in wild type W3110 cells. W3110 expressing pBAD24 (no Tol components expressed), pRA003 (tolQ/tolR), and pRA032 (T7/tolQ/tolR) cells grown in 0.1% L-arabinose for 24 hours stained with safranin and viewed at 1000x. Only W3110/pRA003 cells demonstrated a septate filamentation phenotype.

Results (b)

As previously seen with TolQ (pRA003) and T7-tagged TolQ (pRA032) expression in RA1033 (ΔtolQ/ΔexbB/ΔexbD), the overexpression of both TolQ and T7-tagged TolQ in W3110 wild type cells showed that overexpression of TolQ in wild type *E. coli* cells causes a filamentous phenotype, while the overexpression of T7-tagged TolQ does not (Figure 8). I will refer to this morphological phenotype as septate filamentation, as cells appear to have divided with regard to the CM and PG layers, while the OM does
not appear to have divided. Individual cells appeared to be chained and enclosed in a continuous OM sheath. The results were mimicked with the overexpression of TolQ and TolR in the pACYC184 vector (pRA030) under chloramphenicol selection (data not shown).

Methods (c)

*TolQ over a range of expression levels.* In order to correlate TolQ expression levels with the degree of filamentation and eliminate other variables that may also affect cell division, W3110/pRA031, wild type *E. coli* carrying the plasmid-encoded TolQ protein was grown in LB ampicillin (100µg mL⁻¹) with levels of L-arabinose at 0, 0.1, 0.01, 0.001, and 0.0001%. The cultures were treated, stained, and viewed in the same manner as described for RA1033 transformants.

Results (c)

Upon viewing wild type cells (W3110) over-expressing pRA031 (*tolQ*), it was observed that the degree of septate filamentation correlated directly with L-arabinose concentration levels, with the most elongated cells seen at 0.1% L-arabinose and nearly wild type cell morphology seen at 0.0001% L-arabinose (Figure 9).
Figure 9. TolQ expression levels in W3110 wild type cells. Wild type cells (W3110) expressing *tolQ* (pRA031) grown in increasing concentrations of L-arabinose (10-fold increases from 0% to 0.1%) for 24 hours, stained with safranin, and viewed at 1000x. There is an apparent direct correlation between TolQ expression levels and degree of cell filamentation.
Methods (d)

**TolQ and T7-TolQ overexpression in the absence of plasmid-encoded TolR.**

Since the T7 tag again appeared to disrupt the overexpression phenotype noted in these cells, it was tempting to speculate that TolQ was solely responsible for this filamentous appearance. pRA032 also overexpresses TolR. To further demonstrate that the overexpression of TolQ alone lead to a filamentous phenotype, and that the presence of TolR on the expression vector had no impact, wild type cells (W3110) carrying either pRA019 (T7/tolQ) or pRA031 (tolQ) were grown at 0.1% L-arabinose, treated, stained, and viewed in the same manner as described for the RA1033 transformants.

![Image of cells expressing TolQ](image.png)

**Figure 10. Overexpression of T7-TolQ and TolQ in W3110 wild type cells.** W3110 cells expressing T7-tolQ (pRA019) and tolQ (pRA031) grown in 0.1% L-arabinose for 24 hours, stained with safranin, and viewed at 1000x. Only W3110/pRA031 cells demonstrated a septate filamentation phenotype.

Results (d)

Overexpression of pRA031 encoding TolQ with the TolR protein chromosomally encoded and pRA019 encoding T7-TolQ, also with TolR chromosomally encoded provided the same results as the overexpression of pRA003 (tolQ/tolR) and pRA032
(T7/tolQ/tolR) (Figure 10). The overexpression of wild type TolQ resulted in septate filamentation and the overexpression of the T-7 TolQ fusion protein did not.

Methods (e)

**Overexpression of TolA, TolQ, and TolR.** Wild type cells (W3110) carrying plasmids encoding TolA (pRA004), TolR (pRA002) or TolQ (pRA031) were grown for 24 hours at 0.01% $L$-arabinose, stained, and viewed for filamentation.

![Image of W3110/pRA004 cells expressing tolA (pRA004), tolR (pRA002), and tolQ (pRA031) grown in 0.1% $L$-arabinose for 24 hours, stained with safranin, and viewed at 1000x. Only W3110/pRA031 cells demonstrated a septate filamentation phenotype.](image-url)

**Figure 11. Overexpression of TolA, TolR, and TolQ.** W3110 cells expressing tolA (pRA004), tolR (pRA002), and tolQ (pRA031) grown in 0.1% $L$-arabinose for 24 hours, stained with safranin, and viewed at 1000x. Only W3110/pRA031 cells demonstrated a septate filamentation phenotype.
Results (e)

As predicted, the overexpression of the wild type TolR and wild type TolA did not cause cell filamentation, while only overexpression of wild type TolQ did (Figure 11).

Discussion

Analysis of the cell division phenotypes presents a complex picture of the role of the Tol system in this process. In this study, E. coli mutants lacking one or more Tol proteins were never observed to display a filamentous division phenotype, even when grown under conditions of high and low osmolarities (data not shown), as reported by Meury and Devilliers (1999). On the contrary, if a trend were to be discerned, the cells of such mutants appeared on the whole shorter with a slightly irregular morphology compared to W3110 wild type cells. However, a clear division impairment was evident in RA1033 cells transformed with the pRA003 construct containing tolQ and tolR when grown at 0.01% L-arabinose levels.

Based on initial observations, it did not appear that filamentation was caused by deficiency of a functional TolQ protein, but instead was the reverse. The sensitivity of the L-arabinose inducible promoter in the pRA003 construct is such that even in cells completely lacking endogenous TolQ protein, induction at 0.01% L-arabinose would have expressed the TolQ and TolR proteins at higher than normal levels in these cells. Therefore the observed phenotype was initially attributed to an overexpression effect.

To support the conclusion that overexpression of TolQ lead to a disruption in normal cell division, W3110 cells carrying functional TolQ and T7-tagged TolQ were grown in 0.1% L-arabinose. As expected, wild type cells over-expressing functional
TolQ and TolR showed an even more pronounced septate filamentation phenotype than that observed in the RA1033 mutants with the same construct. Significantly, at no time did a comparable division phenotype appear in cells over-expressing the T7-tagged TolQ protein. Therefore, this once again indicated that the addition of the T7 tag on the N-terminus of this protein interfered with its normal activity.

In order to specifically identify that it was overexpression of the TolQ protein that caused this cell division defect, an additional construct was generated with just the tolQ gene under L-arabinose induction (pRA031), without the accompanying tolR gene. This construct was transformed into W3110 cells and then these cells were grown under increasing ten-fold concentrations of L-arabinose (ranging from no L-arabinose to 0.1%) for 24 hours and then stained. Such a series was intended to demonstrate two things: that the filamentation phenotype was directly affected by TolQ levels, and that overexpression of TolQ alone was sufficient to result in filamentous cells. As the data demonstrated, the degree of filamentation correlated directly with the concentration of L-arabinose. As L-arabinose induction levels increased, the extent of filamentation intensified, culminating with 0.1% levels resulting in almost universally filamented cells. A T7-TolQ construct (pRA019) behaved similarly to the pRA032 plasmid, resulting in no significant filamentation under overexpression conditions. Examination of overexpression with TolR and TolA also helped support the notion that this was strictly a TolQ-related phenomenon. Neither produced filamentous cells when grown at 0.1% L-arabinose concentrations.

All of these results pose a puzzling question. What role does TolQ have in bacterial cell division? A more immediate question may be what is the nature of the
interaction of Tol system components with each other and other division factors during septal ring formation? It is possible that as part of its role in cell division TolQ is required to complex with a number of necessary factors, and that overexpression of TolQ may serve to “tie up” one or more of these factors in areas away from the target septal rings, preventing them from participating in this process to any large extent. This model appears specific for TolQ interactions, as overexpression of its energy-harvesting counterpart TolR had no significant filamentation phenotype. It also appears that the N-terminus may be a significant region in this interaction, since the T7-tag modification prevents filamentation. These observations do not necessarily discount the model proposed by Gerding et al. (2007) suggesting the Tol proteins act as an OM tether during cell division but instead suggest that the mechanism may be more complex than initially assessed. However, this is still merely speculation, and it is hoped that further investigation into these overexpression phenotypes may help elucidate the specific role TolQ has in cell division.
Chapter Five

Summary and Proposed Future Directions

The Tol system has been implicated in numerous cellular processes. However, its precise physiological role in both laboratory and natural environments remains unclear. The N-terminally modified TolQ presents a novel combination of phenotypic effects that demonstrates a separation of Tol characteristics. Interestingly, it appears to distinguish between known Tol-associated energy-dependent functions and those that may only require a structurally intact Tol system. Specifically, it indicates significance for the N-terminal periplasmic region or the first transmembrane domain of TolQ in participating in these functions. Previous mutants have revealed the transmembrane domains of TolQ through which interactions with other Tol proteins occur (Lazzaroni et al., 1995). The interaction of TolQ with TolA takes place via the N-terminal, first transmembrane domain of TolQ. The primary goal of this project was to modify the TolQ protein by the addition of an antibody detectable tag, subject the fusion protein to mutagenesis, and characterize specific TolQ mutants, namely those that had lost the ability to support P1 maturation. It was quickly determined that the addition of the T7 tag to the amino terminus of TolQ provided just this type of “mutant.” This finding required the objectives of this study to be updated. The amended aim was to assess the impact of modifying the N-terminal region of TolQ as it relates to the various functions associated with the Tol system and TolQ alone. It was clear from preliminary studies that the N-terminal T7 modification of Tol Q impacted only a subset of known Tol-associated functions. In particular, susceptibility to Group A colicins was maintained. However, other Tol-associated functions appeared to be disrupted by the modification. The
modification rendered cells unable to support P1 maturation and compromised outer membrane integrity as assayed through DOC treatment. Additionally, it diminished the cross-talk capabilities of the Tol system, as demonstrated by resistance to colicin B and bacteriophage φ80. These results suggested that the various Tol system functions can be separated, perhaps on the basis of the requirement of Tol-associated energy. The exact nature of how such an alteration can accomplish this is unclear. It is possible that modifying the N-terminus may impact the ability of TolQ to interact with other components of the Tol system.

Through the course of these investigations, the majority of the assays were conducted looking at both TolQ and TolR, expressed together to preserve stoichiometric levels. However, it appears that the modification to the TolQ protein alone was responsible for the observed phenotypes. This was supported by examining the pRA031 construct in the cell division studies, which carries tolQ without accompanying tolR. In order to clearly demonstrate that the TolQ phenotype is independent of TolR co-expression, we intend to examine the previously described TolQ functions using TolQ alone (pRA031), and T7-tagged TolQ alone (pRA019) in our assays.

Although the results presented here suggest the N-terminal region of TolQ is significant for its ability to transfer energy to TolA, the exact nature of this capacity remains unclear. How the N-terminus participates in this process and what key features are necessary for energy transfer are still questions that remain unanswered. Therefore, the N-terminal region of TolQ continues to be a region of the protein that requires further analysis. Thorough characterization of the TolQ amino terminus will require employing various methods to modify this portion of the protein and examine the impact it has on
energy transfer. One strategy will be to use hydroxylamine mutagenesis to generate point mutations in the N-terminus. This protocol produces G:C to A:T transitions that cannot be reverted. The resulting selection and screens will hopefully provide mutants in the N-terminal region of TolQ that display phenotypes comparable to those observed with the T7-tag that can then be characterized. Another method will be to generate different constructs with various other additions to the N-terminus other than the T7-tag. This will help in understanding if various residues or the overall size of the region is significant in TolQ’s ability to transfer energy to TolA. Also, in order to rule out the possibility that the T7 tag itself somehow interferes with this process, a construct will be generated with the T7-tag added to the C-terminus instead. This will also hopefully help us accomplish the original goal of generating a functional TolQ protein that can be traced via Western blotting in the course of further studies.

Recent investigations have also provided support for the hypothesis that the N-terminus may be an important component in energy transfer. These studies involve a construct that has an exbB gene similar to wild type except for the addition of only two amino acids at the N-terminus. The exbB gene is the parologue to tolQ in the TonB system and carries out a very similar function of the harvest and transfer of CM-derived energy. Preliminary studies with this modified ExbB protein reveal a reduced capacity for colicin B and Φ80 sensitivity and iron transport (Teleha, Borisov, and Larsen, unpublished observations). Considering the similar nature of the roles of these two proteins, this is consistent with the notion that the N-terminus is significant for TolQ energy transfer as well.
Another question that remains to be answered is whether or not the phenotypes associated with the T7-tag addition represent a dominant situation. In order to test this, the phenotypic assays used to investigate the T7-tag impact in the Tol system mutants can be carried out in wild type cells. The phenotypes that develop will provide insight into the dominant nature of the TolQ modification.

The cell division data present a tempting picture to assign a defined role to the Tol system. The overexpression of wild type TolQ results in septate filamentation of *E. coli* cells, while the overexpression of the T7-tagged TolQ fusion protein fails to generate a similar phenotype. Yet, the nature of the mechanism is still poorly understood. We hope to clarify that role with further analyses. First, various other mutants in TolQ will be analyzed, including some of the ones we will generate in our other studies. Importantly, it is predicted that while TolQ is involved in regulating proper cell division, there are likely myriad other factors it would be necessary to associate with during this process. Therefore, we hope to identify these partners of TolQ using a yeast two-hybrid system. In this procedure, libraries are screened looking for proteins that may interact with a known protein, in this case TolQ. When TolQ binds to this protein, the interaction brings together two subunits of a transcription factor that promotes the expression of a selection gene. Therefore, surviving yeast cells should contain plasmid constructs expressing proteins that interact with TolQ. A better understanding of the associations of TolQ with other partners may help elucidate the overall process of cell division in Gram negative bacteria.

Careful examination of the cell division images suggests that TolQ overexpression does not impact septal ring formation, and that the filaments generated
are actually separated cells sharing a continuous outer membrane envelope. However, the limits of light microscopy do not provide confirmation that this is true for all filaments formed, or identification of any other anomalies that may be present at the septal ring or in the outer membrane itself. Therefore, electron microscopy may be used to better observe this phenomenon.

Electron microscopy will also be useful in further investigating the nature of the relationship between P1 phage and the Tol system. Since P1 can infect Tol mutants, but cannot subsequently reinfect new cells, progeny virion assembly and maturation are likely impaired. Observation of this process and P1 interactions with cellular components using electron microscopy will likely shed light on what effect Tol mutants have on P1 lytic cycles.

As an additional assay to test the role of TolQ in cell division of Gram negative bacteria, we also intend to over-express the protein in other bacterial systems. It has already been shown that the tol-pal gene cluster is well conserved across the Gram negative bacteria. In particular, the tolQ and tolR genes seem most conserved within the eubacteria and are always found present together. These homologues often are found in multiple copies and are often found outside the context of the tol-pal gene cluster on host genomes. Even an archean has been identified with tolQ and tolR homologues. This widespread homology indicates both a long history and an important cellular function for this protein system (Sturgis, 2001). Additionally, numerous studies have located products of the tol-pal gene cluster to contact regions between the inner and outer membranes. The conserved nature of this system should allow us to over-express the E. coli TolQ protein in other bacteria and potentially observe the same filamentous
phenotype. Such an observation would help support the role of this protein as a cell
division factor across Gram negative species.
References


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