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The Functionality of the TonB System Across the Transduction Pathway

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HONORS PROJECT

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Abstract:

The transport process at the outer membrane of gram negative bacteria, *Escherichia coli*, is influenced by the TonB protein. The TonB protein energizes transport through specific outer membrane proteins that are involved in iron uptake. It does this by using the energy that is collected from the ion electrochemical gradient of the cytoplasmic membrane, by complexes of ExbB and ExbD proteins. The functions of this system involve a set of poorly understood interactions between TonB, ExbB, ExbD, and the outer membrane transporters (Postle and Larsen 2007, Weiner 2005). Modifying the amino terminus of ExbB produces a drastic effect that is seen during energy transduction for transport functions. This proves the importance of the amino terminus.

Introduction:

In *E.coli*, 239 amino acids comprise the TonB protein, with the amino-terminal residues being 2-65 (Postle and Good 1983). This terminal region contains a hydrophobic signal sequence necessary for export of elements to the cytoplasmic membrane, where the TonB is located in a disproportional amount in the periplasmic space (Hannavy et al. 1990; Roof et al. 1991). The TonB protein provides the energy to pump specific ligands across the outer membrane, which is basically unenergized, through transducing the potential energy of the cytoplasmic membrane proton gradient to TonB-gated transporters (Moeck and Coulton 1998). The cytoplasmic membrane is a typical phospholipid bilayer that creates and collects energy from ion gradients for conversion to use elsewhere in the cell. This vital protein, once it has energized the membrane, is essential for the harvesting capacity of cobalamin and iron- sequestering molecules from the external environment. TonB provides stored potential energy to the outer membrane by
shuttling between the cytoplasmic and outer membrane (Larsen et al. 2003). However, this is not a simple action performed by TonB; ExbB and ExbD proteins interact at the cytoplasmic membrane and at the outer membrane, associations with TonB-gated transporters and several other proteins occur (Larsen et al. 2003). The amino-terminal region is responsible for anchoring TonB to the cytoplasmic membrane (Postle and Skare 1988); thus it is evident that TonB is directly impacted between itself and the energy source when the amino terminus is mutated (Karsson et al. 1993; Jaskula et a. 1994). Since TonB is anchored by its amino terminus in a cytoplasmic membrane complex with ExbB and ExbD proteins (Braun et al. 1996; Jaskula et al. 1994; Higgs et al. 2002; Higgs et al. 1998), it is clear that a mutation in this area of the protein may have drastic effects on structure and function. This system is designated by its prominence to iron acquisition, allowing iron transport experiments to be valuable in determining the mutated terminus effects on energy transduction (Postle and Larsen 2007).
**Figure 1.** TonB-dependent outer membrane receptor, FhuA. This receptor occurs as a beta-barrel (shown in green), which can be opened and closed by the N-terminal plug domain, or “cork” (shown in dark blue) to control the transport of substrates. The TonB-ExbB-ExbD-complex transmits the pmf of the CM to FhuA receptor to initiate transport. Cylinders, within the CM, indicate trans-membrane domains. Capital letters “C” and “N” correspond to the C- and N-termini, respectively. The N termini are the location of concern. Ribbons represent the solved crystal structure of FhuA with TonB (Pawelek et al., 2006) and liquid-crystal NMR structure of C-terminal portion of ExbD protein (Garcia-Herrero et al., 2007), visualized by Executable build of The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.
**Methods:**

**Media:** Bacteria was grown and maintained on Luria-Bertani (LB) agar plates as described (Larsen, Chen, Postle 2003). When necessary, Ampicillin was added in various concentrations.

**Colicin and phage assays:** Spot titer assays were done using tenfold serial dilutions of bacteriophage φ80 and fivefold serial dilutions of both colicin B and Ia. Cells were grown overnight in LB Amp. LB Amp plates were used for the plating of the cells, along with molten T-top agar at 60 Celsius containing various concentrations of Arabinose being .01%, .001%, and .005%. Each vile contained 100 µL of desired cells; one containing wild-type, one containing the plasmid, and one without plasmid. 3 mL of molten T-top was transported to vile containing the cells, placed on vortex, and this suspension was poured over room temperature LB plates. These plates sat undisturbed for 5 minutes. Following this, 5µ aliquots of colicin were dropped and incubated upright at 37 Celsius overnight. All experiments were completed in triplicate.

**Western Blotting:** SDS was used to denature and separate the cell components by size, as previously discussed (Laemmli, 1970). The blotting procedure was modified from prior research (Skare, 1993). 20 minute polymerization for the 11% resolving gel and 40 minute polymerization for the stacking gel were required. The 10 µL samples ran for 45 minutes at 150 Volts. For the transfer to membrane, the system was run for 10V overnight. The primary solution contained 25mL blocking with 10 or 25 µL antibody anti-ExbB. These concentrations were 1:2500 or 1:1000, respectively. The primary solution was saved after the addition of 200µL sodium azide. The secondary solution contained 25mL blocking with 5µL anti-Rabbit, resulting in a 1:5000 concentration. ECL Western Kit #4 was used to allow the film to fluoresce. During
visualization, the film spent 3 minutes in developer, rinsed, 3 minutes in fixer, rinsed, and hung to dry.

**Iron $^{55}$[Fe] Ferrichrome Transport:** Transport assays were performed. Cells were grown overnight in 5 mL LB Amp. 100µL of this solution was then sub cultured in 20 mL T-Broth containing 20µL .001% Amp using sidearm flasks. Varying amounts of Arabinose was added based on prior research of expression levels. These cells were grown to $A_{550}=0.4$ and then placed on ice. Cells were transferred to labeled, disposable plastic tubes and spun for 10 minutes at 10 rpm. 5 mL of M-9 was added after the supernatant was removed. This new solution was placed on the vortex. Lab coat, gloves, and goggles were necessary after this point. The Ferrichrome mixture contained 46 µL 72.7 MM Ferrichrome, 21 µL 10 MM HCl. Following the mixture of these two elements, 10 µL of 1 MM $^{55}$Fe was added and placed in a 37 Celcius bath for 15 minutes. The first sample to be tested was brought to 30 Celcius in a shaking water bath. 15 µL of $^{55}$Fe was added to this first sample. After adding a small amount of LiCl to wet the textured filter, 250 µL of sample was added, rinsed three times with 0.1 M LiCl, and set out to dry. This process was repeated at 4, 7, and 10 minutes. At 4 minutes, the next sample was added to the water bath to equilibrate. This process was repeated until complete. The amount of $^{55}$Fe that was taken up by the cell was determined through counting done by the scintillation machine.

**Results:**

**Assays:** The ability of an ExbB protein encoded by an $exbB$ gene was modified to include two additional amino acids (valine and proline) to the beginning of the protein. This mutant ExbB, while still active, was less efficient than the corresponding wild-type ExbB control protein. These results were found by doing spot titer experiments in triplicate with the Col $I_A$
and Col B protein. These proteins require a functional TonB system to enter and kill the cell. The amount of Col I\textsubscript{A} required to kill cells expressing the mutant ExbB was about 5 times more than needed to kill the cells expressing the wild-type ExbB – meaning that the mutant did not transport the toxin as well as the wild-type. The Col I\textsubscript{A} and Col B experiments showed that the wild-type was more active than the mutant. Concentration of .01% Arabinose was the most successful in highlighting this difference. However, both were more active with Col I\textsubscript{A}. These results have been reproduced numerous times and thus advocate that the amino terminus of ExbB is somehow important to the function of the TonB system.
Figure 2. Spot Titer’s in Triplicate of Colicin and Phage Assays using various concentrations of arabinose for induction. The Y-axis represents the number of confluent plaques seen after incubation period. The blue series represents the resistant ExbB cells containing the vector. The
red series represents the protein ExbB with modified amino terminus. The green series represents the wild-type ExbB cell. Various concentrations of arabinose are represented in the x-axis. The ExbB cells with the vector are resistant, as it should be. For each experiment, it is clear that the mutant ExbB cell was less efficient than the wild-type ExbB, which was very active. All experiments had a higher number of plaques using Col Ia. Col B at .01% Arabinose produced the greatest difference between the wild-type and mutated ExbB, thus why we chose this concentration and prep to further analyze the effects of the mutant.

**Western Blotting:** The induction needed to behave as wild-type includes 20 microliters of .001% arabinose added to RA1017/pkp390 and 5 microliters of .001% arabinose added to RA1017/pkp660 matched the wild-type W3110/pBAD 24.

**Iron Transport:** Transport experiments were done to alongside of the Collicin experiments because while colicin assays are more sensitive, they are less precise. Transport is not as sensitive, but more precise. From analyzing the graph, it is clear that the R values are taken as accepted, as they are fairly close to 1. The negative R value for the cell with the vector is common and actually desirable. The wild-type ExbB was 0.933 and the mutated ExbB was 0.906. These numbers show a pretty precise measurement taken during iron transport and one that we will take to be true.
Figure 3. Results of iron transport. The Western Blotting result can be seen in the upper left portion of the graph, taken from the developed film. The purpose of this graph is to analyze the R value for each ExbB cell. The y-axis represents the counts per minute, as calculated by the

Slopes:
- Wild type cells: 208.8 cpm/min (R = 0.887)
- Δ exbB cells w/ wildtype exbB: 92.8 cpm/min (R = 0.933)
- Δ exbB cells w/ mutant exbB: 61.3 cpm/min (R = 0.906)
- Δ exbB cells w/ vector: -11.0 cpm/min (R = 0.485)
scintillation counter. The x-axis represents the time point at which the cells were harvested during the transport experiment. The R value represents the “fitness” of the linear graph, meaning how accurate are the points corresponding to the slope. This measured on a scale from 0-1.

**Figure 4.** Bar Graph Representing the Calculated R Values. The y-axis represents the R value from 0-1. The blue bar represents the wild-type cell. The orange bar represents the cell containing the wild-type ExbB protein. The purple bar represents our cell with the mutated amino terminus. The red bar represents the ExbB cell with the vector.
Discussion:

From the research conducted, it is concluded that the extreme terminus of ExbB in the TonB protein is important to the overall functioning of the cell. The impact of function is not completely understood at this time. Future mutagenesis experiments might prove that it is either less stable due to the mutation, or that it doesn’t engage the TonB system as well to harvest energy. I documented that this was true, but now experiments as to why this occurs will be for future study. Future study might also determine whether the mutation is dominant or recessive, which is unknown at this point in time. I proved that the mutated amino-terminal region makes the TonB system less active than its wild-type counterpart. This leads us to believe that his mutation inhibits function by resulting in a protein less stable or a process that is less efficient.
References:


\textit{J. Bacteriol.} 179: 3213-3221


