TonB Not Directly Related to Efflux of Antibiotics in E. coli

Amber Gombash
agombas@bgsu.edu

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TonB not directly related to efflux of antibiotics in *Escherichia coli*

Amber Gombash

HONORS PROJECT

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[Signature]

Dr. Ray Larsen, Biological Sciences

[Signature]

Dr. Roudabeh Jamasbi, Public and Allied Health
Fig. 1. Overnight cultures (top) and pour plates
Abstract:

Studies in *Pseudomonas aeruginosa* have suggested that the TonB energy transduction system directly contributes to efflux-mediated antibiotic resistance, ostensibly by energizing one or more efflux systems. We have found Δ*tonB* strains of *Escherichia coli* to similarly be more sensitive to certain antibiotics relative to wild-type strains. To test the hypothesis that this enhanced sensitivity involved the energization of efflux systems, sensitivity patterns for a variety of antibiotics were evaluated using a set of strains differentially lacking genes encoding the Acr efflux system, the universal outer membrane efflux portal TolC, and TonB. No correlation was evident between the resistance phenotypes of TonB system mutants and efflux mutants. Addition comparisons using Tol system components excluded the possibility that the increased sensitivity of TonB strains involved disruption of the barrier function of the outer membrane. Further comparisons, using strains in which iron transport was altered and cells were grown under iron limiting conditions, suggest that enhanced sensitivity to select antibiotics is simply another aspect of the pleomorphic *tonB* phenotype attributable to iron starvation. Understanding the TonB system is important for public health.

Introduction:

Gram-negative bacteria have an inner (cytoplasmic) and outer membrane with a periplasmic space between. The outer membrane protects bacteria from environmental stressors but also limits the ability of nutrients to enter the cell [1][2][3][4]. There are three ways to cross the outer membrane: diffusion through non-specific protein porins, diffusion through stereospecific protein porins, and active transport with mediation from high-affinity outer membrane receptor proteins [2]. Molecules less than 600 Da, such as amino acids, sugars, and short peptides or oligosaccharides, can diffuse through porin channels [1][2]. Vitamin B12 and iron-siderophore complexes are essential to bacteria but too large to pass through the porins. In order to uptake these valuable substances, specific high-affinity active transporters are needed. The TonB system transports energy from the cytoplasmic membrane (generated through an action potential) to the outer membrane [3]. This energy transduction is executed through an unknown mechanism.
The TonB energy transduction system allows transporters to deliver B12 and iron-siderophore complexes into the periplasmic space. Additionally, the TonB system contributes to the pathogenesis by Gram-negative bacteria [3]. Ferric hydroxide complexes, the form that iron takes in the presence of oxygen, are insoluble. To reconcile this, *E. coli* secretes siderophores (Greek: “iron bearer”) that have an extremely high affinity for iron. TonB then allows the iron-siderophore complexes to transverse the outer membrane. Iron is an important prosthetic group and cofactor for Gram-negative bacteria [4].

There are three components to antibiotic resistance in Gram-negative bacteria: degradation by bacteria-secreted enzymes, outer-membrane blockage, and a pump that removes the antibiotic as it enters—efflux. This paper focuses on efflux. It has been suggested that the TonB system drives efflux [5][6].

**Materials and Methods:**

To study TonB, Gram-negative bacteria *Escherichia coli* were used. *E. coli* is the most commonly used bacteria in the laboratory setting. However, “the *P. aeruginosa* TonB gene complemented TonB mutations in both *Escherichia coli* and *Pseudomonas putida* WCS358, highlighting the interchangeability of action in different organisms” [5]. Since different Gram-negative bacteria have similar TonB systems, this research has wider implications than solely the model organism *E. coli*.

This research on antibiotic resistance was conducted over the past two years in Dr. Ray Larsen’s microbiology laboratory. Nine strains of *Escherichia coli* that have single stain deletions on a W3110 background were used: W3110- wildtype, RA1079 (ΔfepA), RA1054 (ΔtolC), RA1055 (ΔacrA), RA1056 (ΔacrB), RA1038 (ΔtolA), KP1052 (Δfur⁻), KP1270 (ΔaroB⁻), KP1344 (ΔTonB⁻)—the parenthetical information being the single-gene deletions. FepA, fur⁻, and TonB⁻ genes are needed for iron uptake; the tolA gene is needed for outer membrane integrity; the acrA, acrB, and tolC genes are needed for efflux; the aroB⁻ gene insertion brings too much iron into the cell.
Basic laboratory procedures in bacteriology were used. The nine strains of *Escherichia coli* were cultured on agar plates under refrigeration. The wild type *E. coli*, W3110, was the control.

A single bacterial colony of each strain was put in broth (five milliliters of lysogeny broth in separate large test tubes); these cultures were incubated with shaking overnight. The next day, one hundred microliters of each broth culture was pipetted into separate small test tubes. Three milliliters of hot T-top agar was pipetted into each small test tube (cells suspended in T-top) and immediately poured onto T-top agar plates, one plate per strain. Three pre-prepared antibiotic discs (same antibiotic, i.e. in triplicate) were placed on each plate. Plates were put in the incubator overnight at 37°C. Zones of inhibition were measured in the morning, in millimeters.

![Antibiotics](image)

**Fig. 2.** Example of zones of inhibition, measured in millimeters [7]

This procedure was repeated for each of the following antibiotics: rifampicin, vancomycin, erythromycin, and ceftazidime. Also, ten microliters of a 50:50 EDTA/SDS detergent mixture was tested against the strains in triplicate by pipetting 10μL onto blank discs.
Results:

*Results of Preliminary Studies* - This research builds on laboratory research previously conducted by Dr. Ray Larsen.

TonB mutants were less susceptible (smaller kill zone) than efflux mutants to the antibiotic erythromycin. Also, TonB mutants were less susceptible than efflux mutants to deoxycholate. However, TonB mutants were more susceptible than efflux mutants to rifampicin.

Fig. 4. TonB⁻ strains (yellow) do not correlate with the efflux strains (red)
The integrity of the outer membrane in the TonB strains is intact based on the amount of outer membrane protein observed in TonB strains, which was a similar amount compared to the wildtype.

**Fig. 5.** Immunoblotting of SDS-PAGE resolved samples; used to determine that TonB had not shed its outer membrane.

**Results of this project**

The aroB mutant, which cannot produce a siderophore and consequently has less iron uptake similar to the TonB mutant, had the same susceptibility as the TonB mutant for erythromycin, rifampicin, and vancomycin and similar susceptibility for ceftazidime and EDTA/SDS.

The efflux mutants were more susceptible to erythromycin than the TonB mutant. All strains were fairly susceptible to rifampicin, with the mutants being slightly more so than the wildtype. The iron and the efflux strains were resistant to vancomycin, while the barrier mutant was susceptible. The efflux mutants were more susceptible to EDTA/SDS than the iron and barrier mutants and the wildtype. The barrier mutant and the mutant that takes in excess iron (fur-) were susceptible to ceftazidime; the other iron mutants and the wildtype were slightly more susceptible than the efflux mutants.
Fig. 6. TonB⁻ strain (yellow) does not correlate with the efflux strains (red); rather, it is similar to strains with varied iron intake (orange).

Fig. 7. Complete set of tests on nine strains of *Escherichia coli*.
Conclusion:

Efflux mechanisms are important for erythromycin resistance. Efflux mechanism and barrier functions are both important for rifampicin resistance. Efflux is most likely not important for vancomycin resistance.

Loss of the integrity of the outer membrane (barrier) is more important for the susceptibility of *E. coli* to vancomycin.

While it has been suggested that TonB drives efflux [5][6], research does not confirm this: the TonB\(^{-}\) mutant was unrelated to the acrA, acrB, and tolC mutants (efflux mutants) for the tested antibiotics. For example: with erythromycin, the TonB mutant strain was very resistant while each of the efflux mutants was not.

It is already understood that TonB is important for iron transport. Iron is growth-limiting, generally speaking, and important for bacterial virulence [8]. The results suggest that TonB mutants are more susceptible to certain antibiotics because they are iron limited, not because TonB plays a specific role in antibiotic efflux in *E. coli*.

Discussion:

*Public Health & Antibiotic Resistance*- The majority of this research focused on microscopic mechanisms of antibiotic resistance. Understanding how microorganisms resist antibiotics is important to public health. The implications of understanding the TonB system may be far reaching. “When more is understood about the mechanism of TonB function, it may be possible
to design therapeutics that attack TonB directly” [4]. Knowing the specific role of TonB in *E. coli* and other gram-negative bacteria is vital. Gram-negative bacteria are especially threatening because they are becoming increasingly more resistant to common drugs, and there are not a lot of new drugs in production to treat these infections [9][10]. As microbiologists Ratledge and Dover assert: “The only reason for studying a pathogen is to learn how to kill it” [11].

Bacteria are becoming increasingly resilient to previously effective treatments; this is termed antibiotic resistance (ABR). The CDC has set threat levels for bacteria [12]. Vancomycin-resistant Staphylococcus aureus, erythromycin-resistant group A Streptococcus, and clindamycin-resistant group B Streptococcus are all considered “concerning threats,” meaning these bacteria should be monitored, but there may be other therapeutic options. Multidrug-resistant Acinetobacter, drug resistant Campylobacter, extended spectrum Enterobacteriaceae (ESBL), vancomycin-resistant Enterococcus (VRE), multidrug-resistant Pseudomonas aeruginosa, drug-resistant non-typhoidal Salmonella, drug-resistant Salmonella serotype typhi, drug-resistant Shigella, methicillin-resistant Staphylococcus aureus (MRSA), drug-resistant Streptococcus pneumoniae, and drug-resistant tuberculosis are all considered “serious threats.” Serious threats of ABR may have some therapeutic options but require monitoring and prevention. Most concerning are the “urgent threat” that require the public attention and limited transmission because of little or no other treatment options. One urgent threat is Clostridium difficile (CDIFF), which causes life-threatening diarrhea and is commonly a nosocomial infection. It causes 250,000 infection, 14,000 deaths, and over a billion dollars in medical care per year. Another urgent threat is carbapenem-resistant Enterobacteriaceae (CRE), which causes a bloodstream infection and is often a nosocomial infection. There are 9,000 drug-resistant infections (7,900 Klebsiella spp.; 1,400 E. coli) and 600 deaths per year. The last urgent threat listed by the CDC is drug-resistant Neisseria gonorrhoeae, an STD. Of the 820,000 gonococcal infections per year, 246,000 are drug-resistant infections (188,600 tetracycline resistant, 11,480 less susceptible to cefixime, 3,280 less susceptible to ceftriaxone, and 2,400 less susceptible to azithromycin).

Bacteria are becoming more resistant to previously effective treatments for a number of reasons [12]. First and foremost, use of antibiotics, in general, promotes resistance of
antibiotics. In a bacterial infection of millions of bacteria, few may have evolved to resist the antibiotic; they survive the treatment, and proliferate. Furthermore, these bacteria may conjugate with other bacteria, transferring antibiotic resistance. Since ABR is a natural process of bacterial evolution, it can be slowed but not stopped. Misuse/overuse of antibiotics also promotes ABR. This includes use of antibiotics unnecessarily for viral infections. Additionally, stopping the use of a course of antibiotics when symptoms improve contributes to ABR; taking the full course of an antibiotic prescription to prevent reinfection by the “fittest” bacteria that survived. Also, antibiotics are routinely given to livestock for nontherapeutic use (up to 70% of all antibiotics sold in the U.S.); if these animals acquire ABR, improper handling or processing of food could spread the resistant bacteria to humans [13].

ABR poses a significant threat to public health. The CDC estimates that more than two million people in the United States are infected with bacteria resistant to antibiotics each year, directly resulting in the death of at least 23,000 people [12]. ABR poses a significant economic burden, as well. Direct healthcare costs of ABR is estimated at $20 billion and over $35 billion in lost wages, extended stays in hospitals, and premature deaths.

Unfortunately, our economic model encourages misuse and overuse of antibiotics because drug sales are tied to reimbursement. Since 1987, no new antibiotic classes with novel mechanisms of action have been discovered [14]. Of the few antibiotics in the Research & Development (R&D) pipeline targeted at gram-negative bacteria, none have a novel mechanism of action [15].

There are a number of things we can do to combat ABR. The CDC recommends four actions for this fight: preventing infection (safe food handling, hand washing, immunizations, etc.) and the spread of resistance (using antibiotics only when needed and as directed), tracking (for further strategy development on prevention), improving prescribing/stewardship of antibiotics (stop inappropriate and unnecessary use), and developing new antibiotics and diagnostic testing (new drugs to replace the ones that have stopped working) [12]. One of the barriers to the R&D of new antibiotics is a lack of incentive for pharmaceutical companies. Currently, the R&D of antibiotics requires less clinical trial development time and carries additional five years of market exclusivity than other drugs [16]. However, antibiotics are
administered over a short span of time and do not carry the financial incentive of lifelong use as drugs that treat chronic conditions.

Antibiotics are three times more likely to be withdrawn from the market than non-antibiotics [16]. We do not just need new antibiotics; we need new, better antibiotics.

Further Research- A follow up research question may be: does varying the amount of iron in the medium change the susceptibility of \textit{E. coli} to antibiotics? To test this, enriched medium (with additional iron) and minimal medium (with less or no iron) could be used. The \textit{aroB} and \textit{TonB} mutants are expected to grow poorly on minimal media and be more susceptible to antibiotics.
TonB does not directly contribute to efflux-mediated antibiotic resistance in Escherichia coli

Amber Gombash and R.A. Larsen
Department of Biology, Bowling Green State University

Abstract

TonB does not directly contribute to efflux-mediated antibiotic resistance in Escherichia coli. The resistance of tonB mutants to deoxicholate suggested that TonB does not play a major role in the barrier function of the OM. Conversely, tonB mutants showed enhanced sensitivity to thiophen-2-carboxylic acid (TPCA). These results suggest that TonB may be involved in the regulation of outer membrane protein expression. The loss of function phenotypes observed in tonB mutants were consistent with the hypothesis that TonB is required for the proper assembly of the outer membrane proteins.

Barrier function of OM

Fig. 2
- Wild type
- TonB
- Efflux

Role of iron homologs

Fig. 3
- Achromobacter luxi
- Efflux

Results & Conclusions

- Efflux mechanisms are important for resistance.
- TonB status of the cell does not affect resistance.
- Conversely, iron excess does not confer resistance.
- Both iron and TonB are essential for resistance.
- Absence of iron increases sensitivity to antibiotics.
- Absence of TonB increases sensitivity to antibiotics.
- Efflux is required for resistance.

Uniform deletion strains

The loss of function phenotypes observed in tonB mutants were consistent with the hypothesis that TonB is required for the proper assembly of the outer membrane proteins. These results strongly suggest that iron is required for the expression of critical outer membrane proteins.

References

[Provide references here]
Fig. 10. (above) flyer for Pittsburgh Bacterial Meeting

Fig. 11. (below) Presenting research at the poster session at the Pittsburgh Bacterial Meeting

Sources:


