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Development of Nucleic Acid Aptamers to Inhibit Bacterial Efflux Pumps

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Abstract

Multidrug resistance in bacteria, defined as the ability of a bacterial strain to resist the killing effects of more than one antibiotic, represents a major threat to global healthcare. Every year in the United States, two million people are infected with a multidrug resistant strain of bacteria. According to the Center for Disease Control (CDC), out of those two million people, about 35,000 will die from their infection. Thus, these multidrug resistant diseases are considered by the CDC to be the most dangerous diseases in the world. While multidrug resistance can occur through several different mechanisms, a major contributor to multidrug resistance are the bacterial efflux pumps. Efflux pumps are transporters that reside in the membrane of a bacterial cell, and they function by pumping out toxic organic compounds, including antibiotics, from the cell. These efflux pumps often lack specificity for the compounds that they can expel from the cell which means that a single type of efflux pump can confer resistance to many types of antibiotics all at once. When bacterial cells produce high levels of these efflux pumps in their membranes, it can give rise to a multidrug resistance characteristic. I intend to inhibit the efflux pump using single-stranded DNA aptamers. These aptamers should either clog the pump opening and/or bind to the Tol C region of the pump, making it inactive. This would allow antibiotics to once again be effective and work to their full potential.

Acknowledgements

I would like to thank Dr. Matthew Lopper for his support throughout my research project and the many hours of planning and problem shooting. I would like to give thanks to the University of Dayton Chemistry Department for the support of my research. I also want to thank the University of Dayton Honors program and Dean's Summer Fellowship program for funding my project. Thank you to everyone who has made this project possible.

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Societal Importance

Multi-drug resistant bacterial infections represent a significant threat to our public healthcare system. According to the CDC, about 2.8 million people in the United States get an antibiotic resistant infection each year. Over 35,000 people will die per year from an infection that will not respond to antibiotics.[1] The rise of antibiotic-resistant strains of bacteria can be attributed to several causes, such as the overuse of antibiotics in livestock and the prescription of antibiotics to treat illnesses that cannot be treated with antibiotics (this includes most viral infections). Patient compliance is also a problem. Some individuals who are prescribed antibiotics do not finish the entire antibiotic regimen or hold back some of their prescribed antibiotics and take them at a later occasion when they are sick but have not received a proper medical diagnosis.[2] When this occurs, it allows for the most resistant bacteria to persist. After many decades of these practices, we are now faced with greater numbers of antibiotic-resistant strains of bacteria that our existing antibiotics cannot kill, and this has led to higher mortality rates from infections.

Multidrug Resistance

While there are several ways that bacteria can become multidrug resistant, one common mechanism is through the use of bacterial efflux pumps. The efflux pump is a transporter that is located in the membrane of the bacterial cell and it gives a bacterium the ability to export toxins from the cell. While efflux pumps are common to many types of bacteria, they tend to be found at higher levels in the membranes of multidrug resistant strains.[3] Bacterial efflux pumps tend to be nonspecific with respect to the compounds that they can export which means that bacteria that overexpress them can resist the killing

effects of many different types of antibiotics. The ESKAPE pathogens in particular are known to utilize their efflux pumps to become multidrug resistant.[4] The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*) cause over two million illnesses and kill about 23,000 people a year. According to the CDC, these pathogens are the leading cause of multidrug resistant infections occurring in the world.[5]

Bacterial Efflux Pump

There are many different families of the efflux pump that bacteria can utilize to become antibiotic resistant. The different families are ATP-binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), and resistance-nodulation-cell division (RND) families. Each family differs in structure, placement, and mechanism for how they pump out substrates (Figure 1).

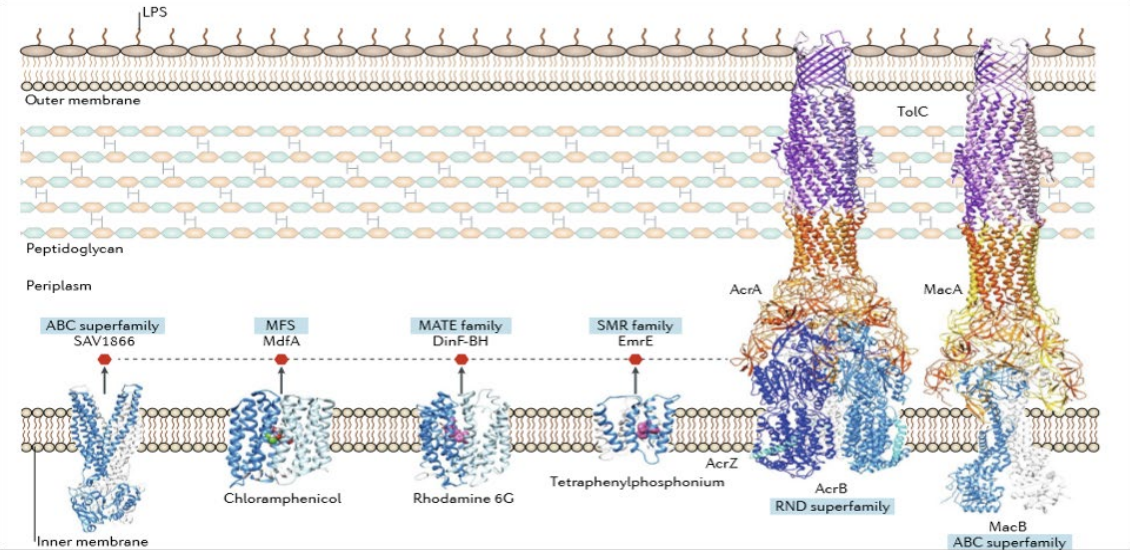

Figure 1. Bacterial efflux pump families. Each family of efflux differs with structure, mechanism, and placement within the cell membrane.[6]

The ABC transporter directly uses ATP as energy to function, while the other families use electrochemical gradients as their energy source. The structure of the ABC transporter has transmembrane domains (TMDs) that have substrate binding sites and nucleotide-binding domains (NBDs) that the ATP will bind to and be hydrolyzed. The MFS transporter is the most diverse family of transporters. They consist of uniporters, symporters, and antiporters. The structure of the transporter includes two domains. To pump substrates out, they must go through two conformational switches from inward-open to outward-open. The MATE transporter structure has amino-terminal and carboxy-terminal domains related by pseudo-twofold symmetry that form a V-shaped central cavity open to the extracellular space. The mechanism uses hydrogen ions and sodium gradients to efflux out substrates. The SMR transporters are small and have four transmembrane helices that function as dimers. During the transport process, the pump has two conformations to efflux substrates. The conformations are the inward-facing and outward-facing states.[6] The RND efflux pump goes through three different conformations to bind substrates and efflux them. The AcrAB-TolC and MexAB-OprM are multidrug efflux pumps that belonging to the RND superfamily and are the two most common types of pumps.[7]

The RND AcrAB-TolC efflux pump is composed of three different components. It is made up of an inner membrane transporter (AcrB), an adaptor protein (AcrA), and an outer membrane channel (TolC). AcrA is found within the periplasm of the cell and it holds AcrB and TolC together. AcrB is found within the inner membrane of the cell. TolC is

found within the outer membrane. Once the protein components are assembled, they make a transporter system that spans from the cytoplasm to the outside of the cell (Figure 2).[6]

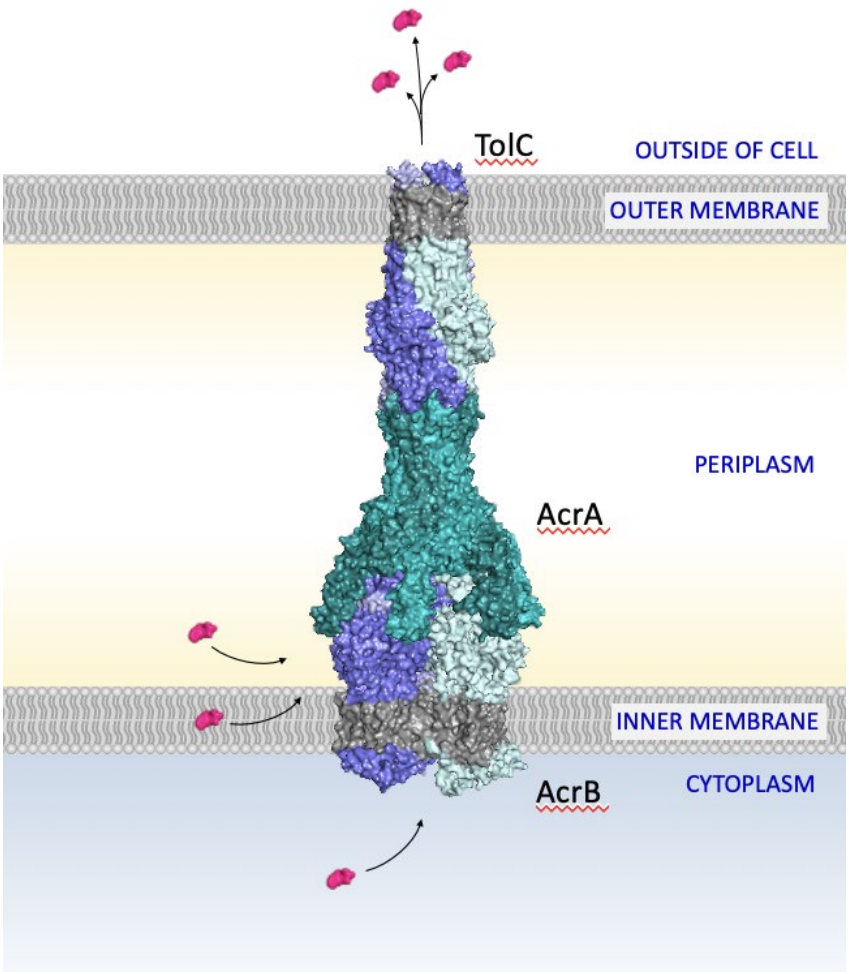

Figure 2. RND bacterial efflux pump showing how AcrA, AcrB, and TolC interact with one another in the intact efflux pump. Molecules can be pumped out of the cell through this efflux pump either from the cytoplasm or from the periplasm. Structural model generated from PDB ID 5O66.

The AcrB portion of the efflux pump is the active portion of the efflux pump. AcrB is a trimer of 1,049 amino acids. It is made up of an extra-membrane headpiece and a transmembrane region. The three transmembrane domain is arranged like a ring with a

central hole. The hole crosses the membrane and connects to the bottom of the headpiece. When looking at AcrB from a side view, the head piece and the transmembrane portion are visible (Figure 3).[8] This is the portion of the efflux pump that is thought to be responsible for expelling antibiotics from the cell.

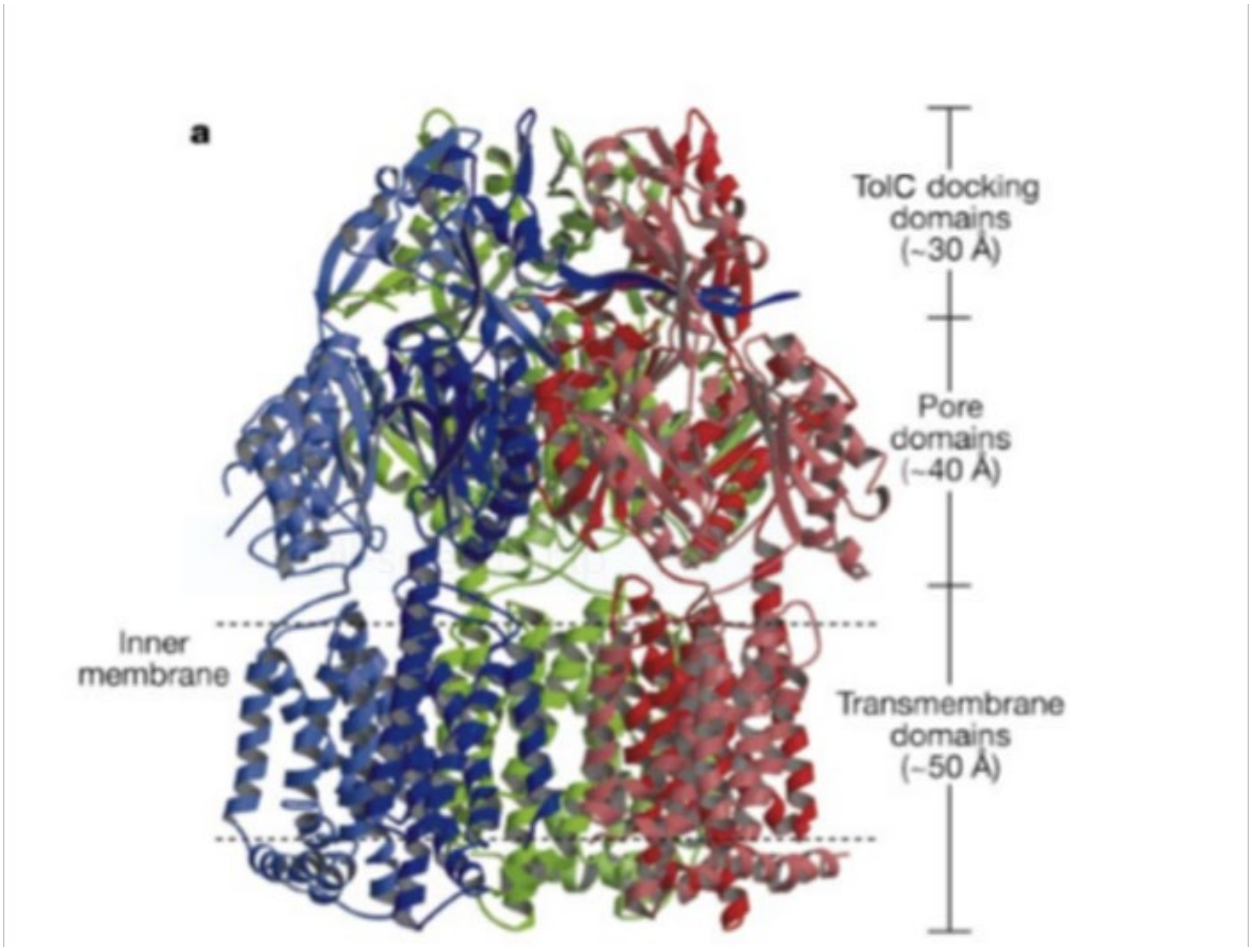

Figure 3. Side view of the AcrB portion of the pump. The funnel portion is seen at the top of the pump.[8]

The AcrB region is what allows for the large variety of substrates to be expelled. AcrB has three different conformations which allows for the expelling of different antibiotics. The first step is known as the access state and the vestibule opens to the periplasm. This is where substrates can enter the vestibule, but the binding site is too small for any substrate to bind to it. In the binding state, the vestibule remains open, and the binding site expands enough for the substrate to bind to it. The substrates will bind to multiple locations in the aromatic pocket inside the vestibule. The exit from the binding site is blocked off so no substrate can get out. During the extrusion state the vestibule is closed and the exit finally opens. The substrate is then pushed into the funnel portion due to the binding site shrinking and expelling the substrate out into TolC (Figure 4). These processes are fueled by the proton motive force.[9]

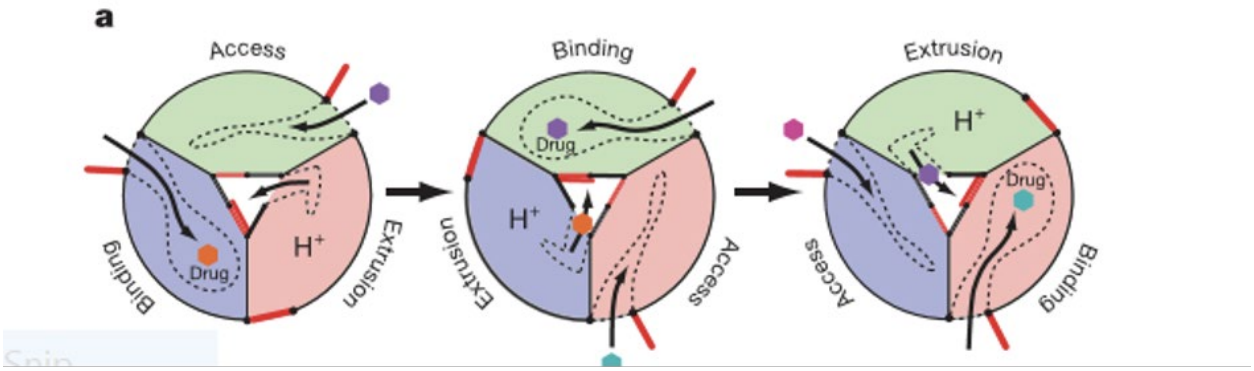

Figure 4. Birds-eye view of the AcrB mechanism. The funnel is shown where the antibiotic will be pushed through. After exiting the funnel, the antibiotic would leave the cell through the TolC channel.[9]

The TolC portion of the efflux pump is a trimer with 428 amino acids. The appearance of the trimer looks like a hollow cylinder (Figure 5). The pump is split into β-

domain, an α- helical domain, and a mixed α/β-domain. The top portion of the structure is viewed to be open, and the bottom portion of the structure is tapered closed.[10]

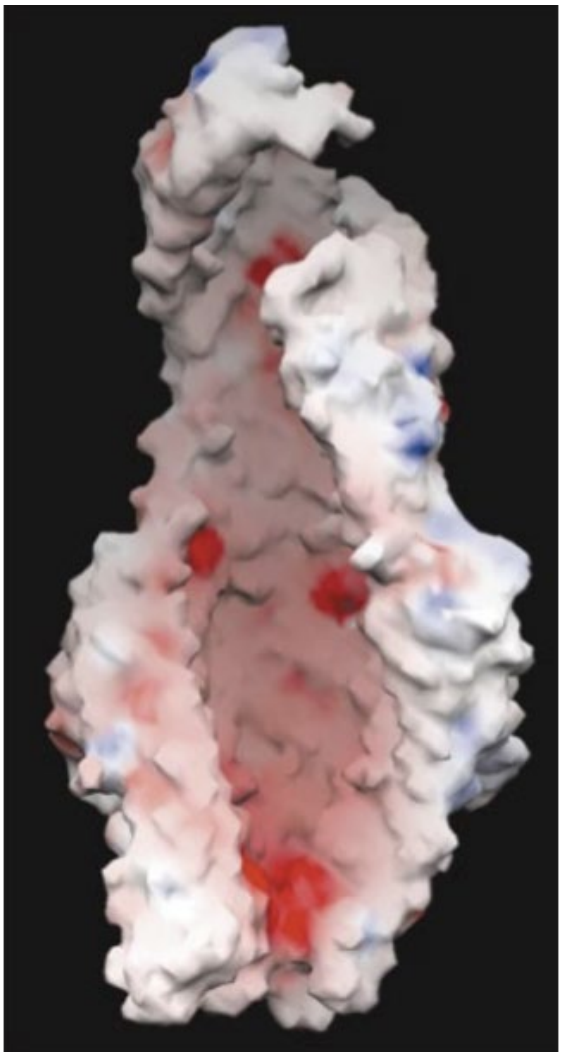

Figure 5. Cross-section of the interior of TolC. It is a hollow cylinder-like structure acting as a channel.[10]

TolC has an important role in the export of substrates. During transport it is open so substrates can fit through the channel. The opening mechanism includes the inner pair of coils rotating around the pair next to it to dilate the entrance. The structural repeat of the TolC portion allows for the channel to open. Figure 6 shows the different conformations of TolC from a bird's eye view. The mechanism of the TolC tunnel was proposed to facilitate the first passage of substrates across two membranes.[10] The assumption was made that the substrates follow their concentration gradient to the outside of the cell. The closing of the pump also ensures there is only one way out for the substrates to go.

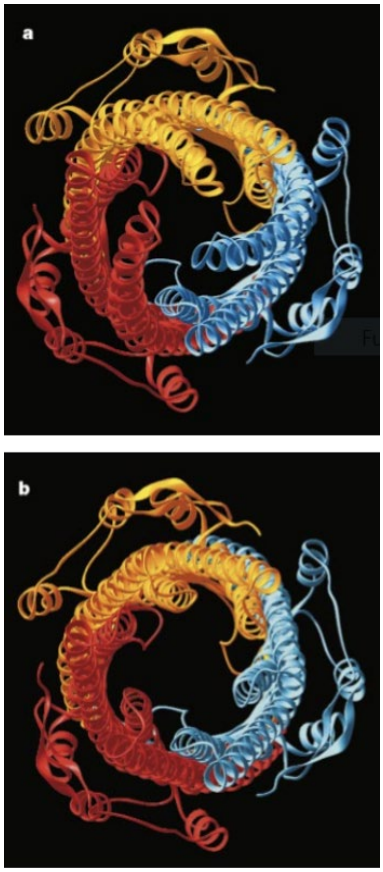

Figure 6. Open and closed conformations of TolC. A) The closed conformation. B) The open conformation.[10]

With the mechanisms of AcrB and TolC put together it allows for the substrate to be effectively pumped from the bacterial cell. The figure below shows the efflux pump assembled with the functional mechanisms to efflux out substrates. The overall pathway of the substrates is to bind to AcrB and be actively pumped into TolC. Once in TolC the substrates can then follow their concentration gradient outside the cell (Figure 7).

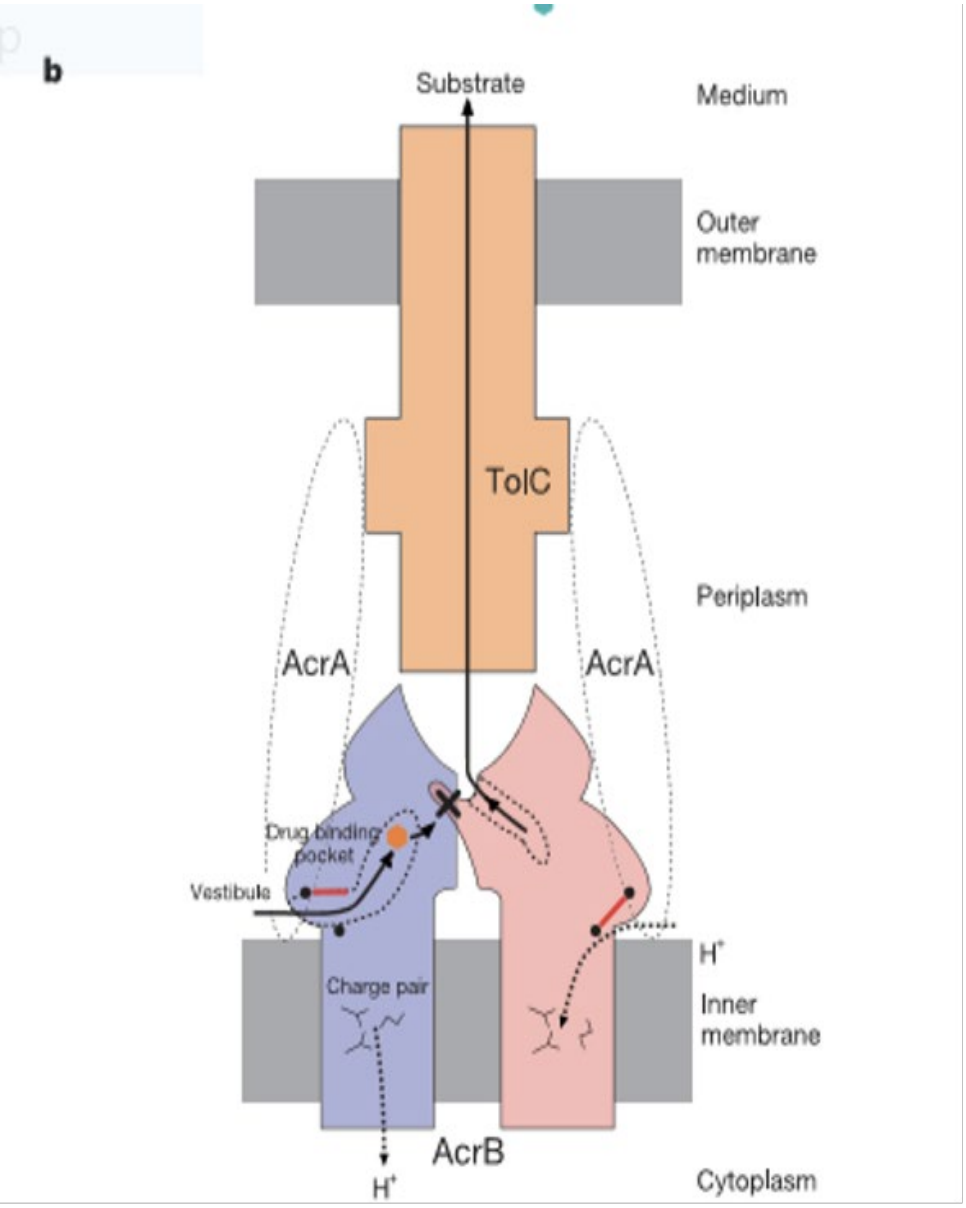

Figure 7. The mechanism of the bacterial efflux pump.[9]

Given the importance of bacterial efflux pumps in conferring multi-drug resistance upon bacteria that overexpress them, many researchers have sought to identify inhibitors of efflux pumps to counter multidrug resistance.[11] Many researchers have investigated inhibiting the bacterial efflux pump. Some compounds that have been evaluated are both plant extracts and polyclonal antibodies.[12,13,14] There has been evidence that there are compounds that have an inhibitory effect on this pump.

Objective and Related Work

The goal of my research is to develop nucleic acid aptamers that will bind to and inhibit bacterial efflux pumps that, when functional, give bacterial cells the ability to survive in the presence of antibiotics. By blocking the activity of bacterial efflux pumps, these nucleic acid aptamers will prevent bacterial cells from developing resistance to the antibiotics that we use to treat infections.

Questions to be addressed:

1. Can we develop single-stranded DNA aptamers that will bind to the outer opening of the TolC component of bacterial efflux pumps?
2. Will single-stranded DNA aptamers that bind to the efflux pump block the export of antibiotics through the pump and will this increase the susceptibility of the bacteria to antibiotics?

Given the role that this efflux pump plays in exporting antibiotics from the cell, we intend to develop single-stranded DNAs that will bind to the outer opening of the pump and block its function. There is precedence in the literature that this approach can work. Researchers have found that antibodies that bind specifically to the TolC portion of the

pump can sensitize the cells to antibiotics that the cells otherwise were able to tolerate.[14] This research shows that macromolecules that bind to TolC can influence bacterial susceptibility to antibiotics. I am hopeful that single-stranded DNA aptamers that bind to TolC will have the same effect as the TolC antibodies, but with the added advantage that single-stranded DNA aptamers are cheap and easy to synthesize in large quantities while antibodies are not.

Methods and Expectations

I will develop single-stranded DNA aptamers that can bind to the bacterial efflux pump through a process known as cell-SELEX. Cell-SELEX is a procedure for selecting molecules that have desired properties from a large and diverse set of starting molecules. The desired molecules are then amplified and passed through more selection processes to result in a final population of molecules that in this case will have a strong binding affinity for the outer portion of the efflux pump in the live cells.

My Cell-SELEX procedure will use live *E. coli* cells that overexpress the AcrA-AcrB-TolC efflux pump. A mixed population of aptamers with many different DNA sequences (~10^{15} different sequences) will be added to the cells and incubated for 30 minutes. This will give a chance for any aptamers that happen to have a structure that lends itself to binding to TolC to do so. Following the incubation period, the cells will be collected and the solution that contains the non-bound aptamers will be discarded. The cells will be heated to release the bound aptamers. The collected aptamers will then be incubated with cells that do not express the TolC component of efflux pump. This step represents a negative selection step, and it will allow me to select for more specific binding between

the aptamers and TolC. Aptamers that pass these selection steps will be passed on through additional rounds of Cell-SELEX (Figure 8) to gradually select for the strongest binders.

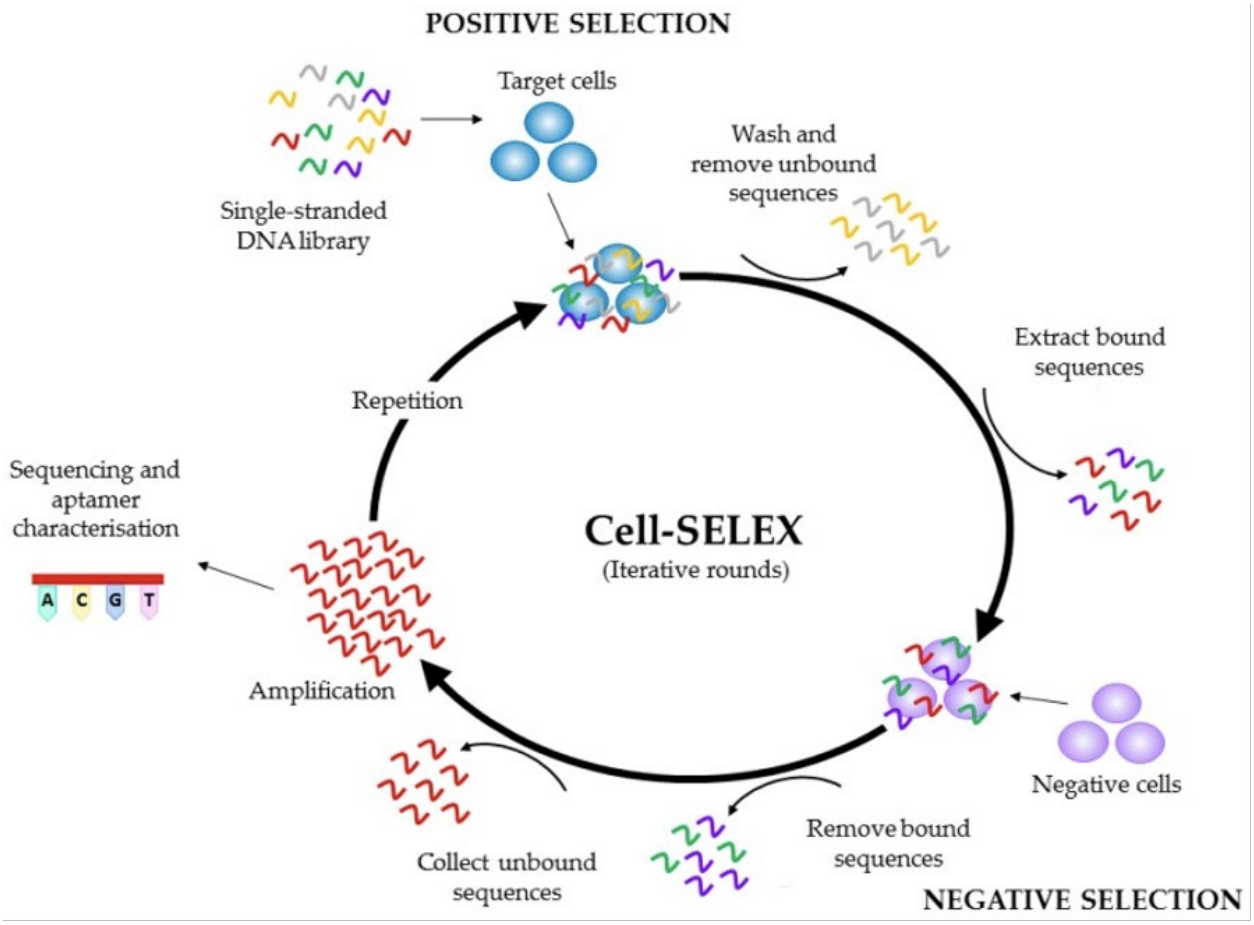

Figure 8. Cell-SELEX procedure.[15] Multiple rounds of Cell-SELEX will be performed.

After many rounds of Cell-SELEX has been performed, I will use growth curves to examine the effects that the aptamer pools have on antibiotic susceptibility in live cells. For each pool of aptamers, I will measure cell growth in the presence and absence of antibiotics and aptamers and compare them with the growth characteristics of cells grown with neither antibiotics nor aptamers present. This will allow me to determine if the aptamers have an inhibiting effect on the efflux pump. By comparing the different growth

curves to the control groups, it will allow me to measure the efflux pump inhibitory activity of the aptamers (Figure 9).

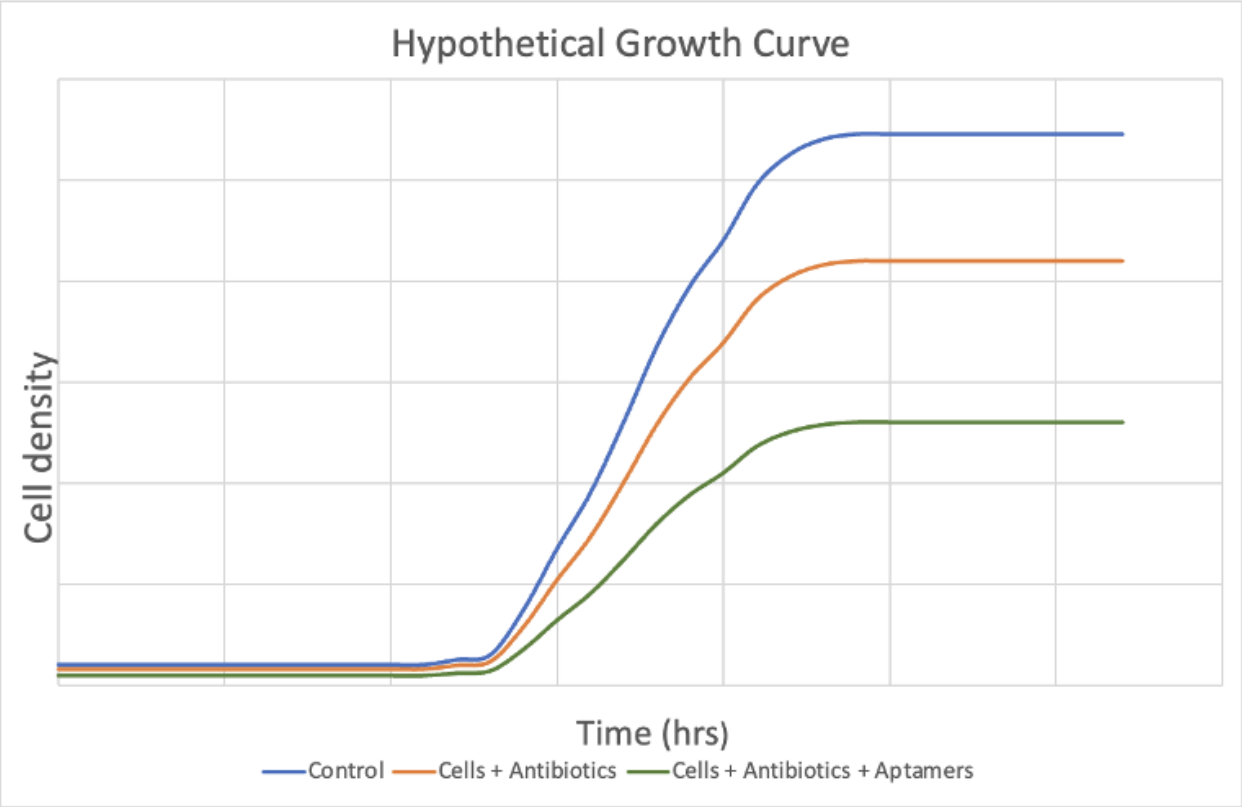

Figure 9. Hypothetical growth curve comparing cells grown with only aptamers, cells grown with just antibiotics, and cells grown with antibiotics and aptamers. Here, the presence of the aptamers enhances the killing effects of the antibiotics.

Once the pools of aptamers have been identified for having activity, I will determine which aptamer pool has the most activity and further my investigation on that pool. The aptamers will be cloned using standard molecular cloning techniques and their specific nucleotide sequences will be determined by conducting DNA sequencing. Once I have identified the specific nucleotide sequence of the active aptamers, I will purchase

synthetic versions of the aptamers from commercial sources that specialize in custom DNA synthesis. The synthetic aptamers will be tested for their potency against a broad panel of antibiotics and in several strains of bacteria including both Gram-positive and Gram-negative strains. This will allow me to determine the species specificity of the aptamers. I hypothesize that aptamers that have been selected in cell-SELEX using *E. coli* will show greatest activity against *E. coli* and related bacteria and that they will be less active against evolutionarily distant bacterial strains.

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