Honors Research (Bio 4950H and 4952H) Application

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Title of Project: Phage Peptidoglycan Hydrolase (PPH) as an antagonist of gram negative bacteria.

Background Information on Project: Bacteriophages are viruses that infect bacteria and replicate within them, typically causing lysis. The canonical model of bacteriophage lysis includes three proteins, endolysins, holins, and spanins. Endolysins degrade peptidoglycan, the bacterial cell wall located in the periplasm. Holins are proteins that create micron scale holes in the inner membrane of the bacterial cells, allowing endolysins access into the periplasm. Spanins complete the lysis of cells by fusing the outer and inner membranes. While this is the most common and best understood model of bacteriophage lysis, other models exist, including the single-target and SAR endolysin models (1). Phage peptidoglycan hydrolase (PPH) is an endolysin that was isolated from phages Atu_ph02 and Atu_ph03, which infect *Agrobacterium tumefaciens.* PPH does not contain all of the features common among previously described phage endolysins. First, PPH has a transmembrane domain on its C-terminus, which is extremely atypical. Second, the genomes of these phages do not contain obvious holin or spanin proteins suggesting that PPH may function independently. Indeed, expression of PPH alone in *Agrobacterium tumefaciens* without any accessory proteins is sufficient to cause cell lysis. There is a highly conserved glutamic acid residue in the putative peptidoglycan hydrolase domain of the endolysin protein, suggesting that PPH may have a role in breaking the bonds between sugars, thereby degrading peptidoglycan (2). By mutating this highly conserved residue we hoped to diminish the effects of PPH on gram negative bacteria. When tested in *A. tumefaciens,* cells expressing PPH with an E32A mutation do in fact grow better and exhibit delayed lysis. Because of the efficient lysis caused by PPH, these phages may have potential to be utilized as enzybiotics. When I started in the lab during the summer of 2017, my goal was to determine if PPH can trigger lysis of other gram negative bacteria as it does on *A. tumefaciens,* and to characterize its peptidoglycan degrading activity. The project began with an examination of how PPH affects *Escherichia coli.* In order to determine the viability of cells when PPH is expressed, I performed multiple spotting assays as well as growth curves. I found that *E. coli* cells do not grow efficiently when PPH is expressed. When performing microscopy on these cells, they exhibit a failure to divide, causing filamentation followed by spheroplasting and finally cell lysis. These spheroplasts and the transition of rod-shaped cells to spheroplasts are extremely similar to the analysis of lambda endolysin activity without the use of spanin proteins (3). These cells have an intact outer membrane but compromised peptidoglycan. When examining my spheroplasts, they do not stay intact as has previously been described (3), but instead lyse after a variable period of time.

Experimental Approaches:
I have two main goals moving forward with this year. My first goal is to further characterize the properties of the spheroplasts. I want to see if the cells still contain peptidoglycan as well as how they respond to permeabilization with different concentrations of EDTA. In order to determine if the cells still contain peptidoglycan, I will use fluorescent-D amino acid (FDAA) staining, using a previously established protocol (4). FDAA stains target D-amino acids that make up the peptidoglycan cell walls of bacteria. If there is no FDAA staining within these spheroplasts, there is no intact peptidoglycan in the cells, as is to be expected. If there is FDAA staining or there is only staining in a specific region, we can gain more knowledge about these cells and the effect of PPH. I would like to stain cells prior to induction with PPH, in order to visualize the transition from rod-shaped cell to spheroplasts in terms of the cell wall. This experiment would give me information about the cell wall before, during, and after the transition. Next, I want to determine how spheroplasts respond when EDTA is utilized to create holes in the outer membrane. We hypothesize that if the outer membrane is permeablized, the transition from rod-shaped cell to spheroplast will be bypassed and the cells will lyse immediately. I will be utilizing time-lapse microscopy, spotting assays, and growth curves in order to determine what occurs when different concentrations of EDTA are applied to the cells. I will conduct these experiments on cells expressing wildtype and mutant forms of PPH to better understand the contribution of the E32 residue during peptidoglycan hydrolysis.

My second goal is to understand the function of the atypical transmembrane domain that occurs on the C-terminus of the PPH endolysin. Canonical endolysins do not have a transmembrane domain and SAR-endolysins may have one, but it occurs on their N-terminus. The Brown lab has constructed a variant of PPH without the transmembrane domain, and I hope to perform several experiments such as spotting assays, microscopy and growth curves in different conditions to determine the function of this domain in PPH function in *E. coli*. For example, if the transmembrane domain functions to deliver PPH across the inner membrane, then mutants lacking the domain should not cause cell lysis. In addition to these goals, my lab has prepared additional mutants of PPH, so that I can try to continue to define roles for different parts of the protein.

Overall, by completing these two goals, I hope to gain a better understanding of how PPH works, and how it can be optimized to better kill gram negative cells. Lastly, if time allows, I will determine the effect of PPH expression in other gram negative bacteria, such as *Caulobacter crescentus*.

Predicted Outcomes: When looking at FDAA staining, I would expect to see no peptidoglycan in the spheroplasts. In accordance with Joel Berry's proposal of the structure of spheroplasts, as the cells are transitioning from rod-shaped cells to spheroplasts, there should only be peptidoglycan on one side of the cell as the endolysin moves through the cell slowly degrading the peptidoglycan from one end (3). Thus, it will be interesting to observe what happens to FDAA labeled peptidoglycan during the transition from rod-shaped cells to spheroplasts and I

expect to observe that the peptidoglycan is being degraded directionally within the cells. I expect that a combined treatment of EDTA during PPH expression will cause the cells to transition directly from rod-shaped to lysis, skipping the spheroplast stage. They should not be able to maintain a spheroplast shape with only an intact inner membrane. When considering the variant of PPH that does not contain a transmembrane domain, I predict that the cells will be able to grow similarly to wild type, as the endolysin will not be able to make its way into the periplasm without the transmembrane domain or a holin protein to create holes in the membrane.

Throughout the course of this year, I also expect to gain a lot from this project. I hope to improve my microscopy skills, as well as gain knowledge of different staining techniques that I had not previously encountered. Through the process of the honors research program, I hope to become better at science communication and gain many skills that can help me in my graduate school endeavors.

Overall Significance: When considering PPH as an effective antagonist of gram negative bacteria, it is important to note that antibiotic resistance is becoming of increasing concern to our current world. By paying close attention to these bacteriophages and attempting to characterize them, we are able to see a possible solution to this problem. Phages have evolved to kill their hosts so it is reasonable to expect that phage biology contains an untapped reservoir of potential antimicrobial strategies that could be exploited for use during the emerging antibiotic resistance crisis. There is so much room for discovery in the world of bacteriophages, so many have yet to be characterized, and there are many phages that have yet to be discovered. PPH in particular is an extremely unique endolysin and may have even more exciting potential as an antimicrobial peptide.

Cited Literature:

1. Young, Ryland. “Phage Lysis: Three Steps, Three Choices, One Outcome.” *Journal of Microbiology,* vol. 52, no, 3, 2014, pp. 243-258.

2. Rodrigues-Rubioa, Lorena, et al. “DUF3380 Domain from a Salmonella Phage Endolysin Shows Potent N-Acetylmuramidase Activity.” *Applied and Environmental Microbiology,* vol. 82, no. 16, Aug. 2016, pp. 4875-4981.

3. Berry, Joel, et al. “The Spanin Complex is Essential for Lambda Lysis.” *Journal of Bacteriology,* vol. 914, no. 20, Oct. 2012, pp. 5667-5674.

4. Kuru, Erkin, et al. “In Situ Probing of Newly Synthesized Peptidoglycan in Live Bacteria with Fluorescent D-Amino Acids.” *PMC,* vol. 124, no. 50, Oct. 2012, pp. 12687-12691