category: microbiology

Research Plan

Sarah Keane

Proposed Title: **The Effects of Membrane Stress and Defects in Lipoprotein Maturation of *Acinetobacter Baylyi* ΔInt**

3A. Rationale:

Throughout the past decade, frequency of antibiotic resistance in the United States has increased drastically. According to the Centers for Disease Control and Prevention, "Each year in the U.S., at least 2 million people get an antibiotic-resistant infection, and at least 23,000 people die. Fighting this threat is a public health priority that requires a collaborative global approach across sectors."(cdc.gov, 2018). Antibiotic resistance is caused by bacteria in a population that have mutations that allow them to thrive even in the presence of a drug. Drug resistant bacteria continue to grow and infect more humans because they cannot be killed by the original drug of choice. This vicious cycle of antibiotic resistance can lead to outbreaks of diseases and infections. *Acinetobacter baumannii* is a gram-negative pathogen with a high rate of bacterial resistance formation. Lipoprotein pathways assist in the viability of A.baumannii. These protein trafficking pathways can be examined through the use of *Acinetobacter baylyi,* cousin like structure of *A.baumannii* (consisting of many similar traits). *A.baylyi* is safe to use in the laboratory as it is a harmless soil microbe. Acinetobacter baylyi ADP1 has been used in molecular biology studies that address a broad range of questions. Varying from traditional genetics to general morphology. A.baylyi has an efficient system for natural transformation and chromosomal incorporation of exogenous DNA. Studies that have used this microbe focus on a wide array of problems, including gene duplication and amplification, horizontal gene transfer, bioreporters, and metabolic reconstruction. (Elliott & Neidle, 2011).

Directing the flow of protein traffic is an important job for all cellular organisms. In gram-negative bacteria, this traffic includes lipoproteins. Lipoproteins are synthesized as precursors in the cytoplasm and receive their acyl modifications as they are exported across the inner membrane. After this processing, they are sorted by the Lipoprotein outer membrane localization (Lol) transport system. The third tail of a lipoprotein enables it to be identified as a lipoprotein, and then it is assigned to a location in the cell membrane (Lovullo, et al. 2015). The third acyl chain is added by the enzyme lnt, which until recently was thought to be essential in all Gram-negatives. It has been shown that Acinetobacter species can tolerate a complete loss-of-function mutation in lnt. Absence of a fully functional Lnt impairs modification of lipoproteins, increases outer membrane permeability and susceptibility to antibiotics, and alters normal cellular morphology (Gwin, et al. 2018). In my research I will be using a bacteria strain that lacks the Lnt gene.

By deleting the Lnt gene from *Acinetobacter baylyi*, the bacteria will be placed under a great amount of stress. The stress that the bacteria will be put under will play a role in increasing the rate of suppressor mutations to occur. A suppressor mutation is a second mutation that masks the phenotypic effects of an earlier mutation, in this situation a suppressor mutation accounts for the absence of the Lnt gene. If I can identify antibiotic resistant mutants of *Acinetobacter baylyi Δlnt*, this will help us better understand the protein trafficking pathways of *Acinetobacter baylyi*. Finding a mutant will advance the research of the lipoprotein systems in A.baylyi because phenotypic analysis can point researchers inti the direction of what pathway is actually being used.

In this experiment *Acinetobacter baylyi* Δlnt will serve as a model organism for *Acinetobacter baumannii*, a deadly pathogen that is commonly found in intensive care units. I

aim to understand the nature of drug resistant *Acinetobacter baylyi Δlnt* bacteria by attempting to analyze how the suppressor mutant is still thriving without the function of Lnt, potentially uncovering the alternate protein trafficking pathway that it is using. The results of this experiment may include a real-world application as if the lipoprotein systems of A. *baumannii* are understood, then an anti-microbial can be created to fight this deadly pathogen.

3B. Hypothesis(es), Research Question(s), Engineering Goal(s), and Expected Outcomes:

Hypothesis: If antibiotics are used to attack the cellular membrane of *Acinetobacter baylyi*, then those bacteria that both originally lack the lnt gene and are also placed under antibiotic stress may form a novel mutation that allows them to survive under this stress. If these suppressors are morphologically analyzed, we can determine if this mutation is a novel mutation, helping us further understand the lipoprotein systems in *Acinetobacter baylyi/baumannii.*

Research Question: What are the Effects of Membrane Stress and Defects in Lipoprotein Maturation of *Acinetobacter Baylyi* Δlnt

Expected outcome: By using a suppressor mutant of *A. baylyi*, my goal is to make the lipoprotein trafficking paradigm in *A.baylyi* clearer.

3C. Procedures

Preparations:

- All strains of *A. baylyi* and *A. baylyi* Δlnt will be first streaked out on LB agar plates to isolate single colonies
- All strains will be grown in LB (Lennox, Carolina Biological); at 30°C overnight

- To determine colony morphology, overnight cultures of each strain (WT and Δlnt) will be serially diluted and then aliquots will be spread on the surface of a series of different agar plates

Plating:

- Dilutions will be plated on Bacitracin plates at 1400, 1200, 1100, 950, 850, 750, 500, and 250 µg/ml grown in LB at 30°C overnight
- Dilutions will be plated on MacConkey Agar plates and were grown at 37°C and 42°C
- Dilutions will be plated on Vancomycin plates at 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml grown in LB at 30°C overnight
- The plates will be incubated overnight and photographed the next morning.

Potential Outcomes That Will Determine the Next Step of Experimentation:

- If the wild type strain grows well on the Bacitracin plates, but there is little to no growth for the Δlnt plates then I will not pursue Bacitracin as my antibiotic of choice. If there is growth, I will isolate any suppressors that emerge and complete further morphological analysis on them.
- If there is no emergence of suppressors on the MacConkey plates I will not continue to use MacConkey plates as my isolation method of suppressor mutants.
- If Δlnt Vancomycin resistant suppressors are found and isolated on Vancomycin 50 plates, I will complete further research on these potential suppressors.
- If WT Vancomycin resistant suppressors were found and isolated on Vancomycin 100 plates, I will complete further morphological testing on these wild type suppressors.

Suppressor Analysis:

Kirby Bauer Disk Assay:

- Kirby Bauer Disk Assays will then be completed with any found suppressors of WT and Δlnt
- Plates will be incubated overnight at 30°C
- The next morning, the zones of inhibition on these plates will be measured and analyzed
- Use of a microscope to measure and analyze the size of each suppressor cell
- Use of a spectrophotometer to measure and analyze the optical density of each suppressor

EOP Assay:

- Two 96 well plates will be used to dilute each of the 12 strains of bacteria from 10^0 to 10^-5, creating a ten-fold dilution moving from left to right.
- 2 plates will be used for each type of agar because all 12 strains could not fir onto 1 plate.
- A replicator will be sterilized, flamed and dipped from the 96 well plate onto each agar plate.

Growth Curve Assay:

- Liquid cultures of the 12 strains will be measured will be made 18-24 hours prior to experimentation.

- The optical density of each liquid culture will be measured in a Biophotometer using a 1ml cuvette with 800ul of LB and 200ul of LC.
- The spectrophotometer will then be used to measure the optical density of each strain of bacteria every 20 minutes over an 18-hour period.

Risk and Safety: Hazardous Chemicals

A. The bacteria *A. baylyi* and *A. baylyi* ΔInt will be the only living organisms used in the experiment and is not associated with human disease. It is classified as Biosafety 1 based on the U.S. Public Health Service Guidelines.

B. During the course of experimentation I will use appropriate safety equipment (goggles, gloves, lab coats, etc.) at all times while working in the lab.

C. Appropriate safety instructions will be followed to work safely in the laboratory.

D. Students will be trained in microbiology lab practices under supervision.

E. All living cultures/worksurfaces will be decontaminated with bleach.

F. Chemicals and biohazardous materials will be properly disposed in designated waste containers in the lab. The waste will be picked up by the Environmental Health and Safety Office of by Hofstra University.

G. Material Safety Data Sheets (MSDS) forms for all chemicals(ethanol).will be available and used during experimentation.

H. The only potentially hazardous chemical that will be used is 70% ethanol for cleaning workspaces.

References

"Antibiotic / Antimicrobial Resistance." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 10 Sept. 2018. https://www.cdc.gov/drugresistance/index.html.

Botos I, Noinaj N, Buchanan SK.Insertion of proteins and lipopoly-saccharide into the bacterial outer membrane.Philos Trans R SocLond B *Biol Sci*2017;372:20160224.

Clardy, J., Fischbach, M. A., & Currie, C. R. (2009). The natural history of antibiotics. *Current Biology*, *19*(11). doi: 10.1016/j.cub.2009.04.001

Chen, T.-L., Siu, L.-K., Lee, Y.-T., Chen, C.-P., Huang, L.-Y., Wu, R. C.-C., … Fung, C.-P. (2008). Acinetobacter baylyi as a Pathogen for Opportunistic Infection. *Journal of Clinical Microbiology*, 46(9), 2938–2944. doi: 10.1128/jcm.00232-08

Cuff, L. E., Elliott, K. T., Seaton, S. C., Ishaq, M. K., Laniohan, N. S., Karls, A. C., & Neidle, E. L. (2012). Analysis of IS1236-Mediated Gene Amplification Events in Acinetobacter baylyi ADP1. *Journal of Bacteriology*, 194(16), 4395–4405. doi: 10.1128/jb.00783-12

Elliott, K. T., & Neidle, E. L. (2011). Acinetobacter baylyi ADP1: Transforming the choice of model organism. *IUBMB Life*, 63(12), 1075–1080. doi: 10.1002/iub.530

Galoustian, G., Science X. "Antibiotic Resistance Surges in Dolphins, Mirroring Humans." Phys.org, Phys.org, 15 Sept. 2019, https://phys.org/news/2019-09-antibiotic-resistance-surges-dolphins-mirroring.html.

Glew, R. H., R. C. Moellering, Jr., and L. J. Kunz. 1977. Infections with *Acinetobacter calcoaceticus* (*Herellea vaginicola*): clinical and laboratory studies. *Medicine* (Baltimore) 56:79-97.

Grabowicz, M., & Silhavy, T. J. (2017). Redefining the essential trafficking pathway for outer membrane lipoproteins. *Proceedings of the National Academy of Sciences*, *114*(18), 4769–4774. doi: 10.1073/pnas.1702248114

Grabowicz M, Silhavy TJ. Envelope stress responses: an interconnected safety net. Trends *Biochem Sci* 2017;42:232–242.

Gwin, C. M., Prakash, N., Belisario, J. C., Haider, L., Rosen, M. L., Martinez, L. R., & Rigel, N. W. (2018). The apolipoprotein N-acyl transferase Lnt is dispensable for growth in Acinetobacter species. *Microbiology*, *164*(12), 1547–1556. doi: 10.1099/mic.0.000726

Harding, C. M., Hennon, S. W., & Feldman, M. F. (2017). Uncovering the mechanisms of Acinetobacter baumannii virulence. *Nature Reviews Microbiology*, *16*(2), 91–102. doi: 10.1038/nrmicro.2017.148

Konovalova A, Mitchell AM, Silhavy TJ.A lipoprotein/b-barrelcomplex monitors lipopolysaccharide integrity transducing infor-mation across the outer membrane

Linares, J.F., Gustafsson, I., Baquero, F., and Martinez, J.L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc. Natl. Acad. Sci.* USA 103, 19484–19489

Lovullo, E. D., Wright, L. F., Isabella, V., Huntley, J. F., & Pavelka, M. S. (2015). Revisiting the Gram-Negative Lipoprotein Paradigm. *Journal of Bacteriology*, 197(10), 1705–1715. doi: 10.1128/jb.02414-14

MacLean, R. C., & Millan, A. S. (2019, September 13). The evolution of antibiotic resistance. *Science*, 365(6458), 1082–1083. doi: 10.1126/science.aax3879

Midolo, P. D., Turnidge, J., Lambert, J. R., & Bell, J. M. (1995). Validation of a modified Kirby-Bauer disk diffusion method for metronidazole susceptibility testing of Helicobacter

pylori. *Diagnostic Microbiology and Infectious Disease*, 21(3), 135–140. doi: 10.1016/0732-8893(95)00066-j

Morán-Barrio, J., Cameranesi, M. M., Relling, V., Limansky, A. S., Brambilla, L., & Viale, A. M. (2017). The Acinetobacter Outer Membrane Contains Multiple Specific Channels for Carbapenem β-Lactams as Revealed by Kinetic Characterization Analyses of Imipenem Permeation into Acinetobacter baylyi Cells. *Antimicrobial Agents and Chemotherapy*, *61*(3). doi: 10.1128/aac.01737-16

Nhu, N. T. K., Riordan, D. W., Nhu, T. D. H., Thanh, D. P., Thwaites, G., Lan, N. P. H., … Stabler, R. A. (2016). The induction and identification of novel Colistin resistance mutations in Acinetobacter baumannii and their implications. *Scientific Reports*, 6(1). doi: 10.1038/srep28291

Okuda S, Sherman DJ, Silhavy TJ, Ruiz N, Kahne D.Lipopolysac-charide transport and assembly at the outer membrane: the PEZmodel.Nat Rev Microbiol 2016;14:337–345.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). Acinetobacter baumannii: Emergence of a Successful Pathogen. *Clinical Microbiology Reviews*, 21(3), 538–582. doi: 10.1128/cmr.00058-07

Pourhajibagher M, Hashemi FB, Pourakbari B, Aziemzadeh M, Bahador A. Antimicrobial Resistance of Acinetobacter baumannii to Imipenem in Iran: A Systematic Review and Meta-Analysis. *Open Microbiol J.* 2016;10:32–42. Published 2016 Mar 29. doi:10.2174/1874285801610010032

Selman A. Waksman and H. Christine Reilly. *Industrial & Engineering Chemistry Analytical Edition* 1945 17 (9), 556-558. DOI: 10.1021/i560145a007