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Synergistic Effects of Rhamnolipids and Antibiotics Against Bacteria

Abigail Mottahedeh[1,2], Ella Shusterman[1,2], Meredith McCarthy[1] and Dr. Lisa Runco[2]

[1]North Shore Hebrew Academy High School Great Neck, New York
[2]Cold Spring Harbor DNA Learning Center Lake Success, New York

ABSTRACT

Antibiotics are used to combat bacterial infections by slowing down and preventing the proliferation of bacteria. Antibiotic resistance is a threat to human health, attributed to its overuse and misuse. Altering the membrane permeability to induce antibiotic uptake may be an effective strategy used against both Gram-positive and Gram-negative infectious bacteria. Rhamnolipids (RLs) are biosurfactants produced by *Pseudomonas aeruginosa.* RLs surface-active properties operate by creating holes in bacterial cell membranes, increasing target cell permeability; allowing antibiotics to penetrate the cell. Rhamnolipids enhance the effect of antibiotics by targeting the intracellular machinery of bacteria. This project tested the susceptibility of bacteria when exposed to antibiotics with and without the addition of RLs, to quantitatively determine if RLs increase antibiotic potency. By analyzing the zones of inhibition data, the results demonstrated that RLs potentiated the antibiotics. Notably, kanamycin coupled with RLs had the most effect inhibiting bacterial growth. To further assess rhamnolipid biosynthesis, a BLAST search was performed exclusively on two genes, *rhlA* and *rhlB.* These genes code for the production of two proteins necessary for rhamnolipids. The search indicated a 48% correlation with putative proteins found in *Burkholderia pseudomallei.* Therefore, based on the experimental results and the BLAST analysis, further research should be conducted to explore the possible role of using rhamnolipids as antibiotic enhancers. Specifically, future experiments could focus on isolating the putative proteins of *B.pseudomallei* to genetically modify *E.coli*. Furthermore, isolated studies analyzing the genes of proteins to determine their role in the pathogenicity of *Burkholderia* species.

Introduction

Antibiotics are powerful and effective treatments that have been approved in the medical profession to combat bacterial infections. Specifically, antibiotics are capable of decelerating, and ultimately preventing, the growth and reproduction of bacterial species. There are circumstances when harmful bacteria which are causing an infection are present in an excessive amount, preventing the white blood cells in our immune system to eliminate them, the use of antibiotics becomes absolutely necessary (Leekha et al., 2011).

Antibiotics contain structures that target both Gram-positive bacteria and Gram-negative bacteria. In contrast to each other, Gram-positive bacteria have a thick peptidoglycan layer without an outer lipid membrane, while Gram-negative bacteria have a thin peptidoglycan layer with an outer lipid membrane. Antibiotics target specific machinery of bacteria and use mechanisms to reduce cell proliferation, such as inhibiting cell wall, protein, and cell membrane synthesis and function (Kiss et al., 2017). However, due to the overconsumption and misuse of antibiotics, antibiotic resistant bacteria is becoming an increasing threat to human health as the potency of certain antibiotics is diminishing over time (Allison et al., 2011). According to the Centers for Disease Control and Prevention (CDC), of the estimated 154 million prescriptions for antibiotics written during each calendar year, approximately one-third to one-half of the antibiotic use in humans is unnecessary or inappropriate (Radlinski et al., 2019). Antibiotics are deemed ineffective

in terminating infections because the bacteria causing the infection learns to adapt and change to the mechanisms of antibiotics (Allison et al., 2011).

Altering membrane permeability to induce antibiotic uptake is an effective technique against both Gram-positive and Gram-negative bacteria that can cause infections and diseases (Wood et al., 2018). Rhamnolipids (RLs) are biosurfactants naturally produced by the bacteria, *Pseudomonas aeruginosa.* There is ample documentation supported with laboratory evidence demonstrating that RLs cause a change to the permeability of cell membranes. On a genetic level, the biosynthesis of mono-RL in *P. Aerugnosia* occurs through two sequential steps. During the first step, RhlA, which is encoded by the rhlA gene, includes the synthesis of the fatty acid dimer moiety of RLs to form three hydroxy fatty acid precursors. The next step, the membrane bound RhlB rhamnosyltransferase, which is encoded by the rhlB gene, uses dTDP-L-rhamnose and an HAA molecule as precursors, yielding mono-RLs (Delcour, 2009).

The function of RLs is to serve as a virulence factor, that assists *P. aeruginosa* by disrupting the hosts' epithelia to promote the paracellular invasion of rhamnolipid-deficient *P.aeruginosa* (Pearson et al., 1997). Furthermore, RLs have the ability to promote the uptake in biodegradation of poorly soluble substrates, they possess antimicrobial activities, they are involved in surface motility and play a role in bacterial biofilm development (Radlinski et al., 2019). In addition, RLs possess unique structures that contain surface active properties which are strategically optimized in diverse contexts; such as therapeutics, cosmetics, and use in agriculture. For example, RLs exhibit antimicrobial activities and surface active properties against several microbes, with low host toxicity, illustrating the possible applications in pharmaceuticals and therapeutics (Qingxin, 2017).

In a study conducted by Lauren C. Radlinski, at University of North Carolina, RLs were shown to potentiate aminoglycoside activity against the bacteria *S.aureus*. Aminoglycosides are a particular group of antimicrobial agents that require proton motor force (PMF) for bacterial internalization. In this experiment, the RLs took on the role of a PMF (Krause et al., 2016). Therefore, the RLs that were used, possessed the ability to induce distinct modifications to the *S.aureus* membrane, which promoted the uptake of tobramycin, an antibiotic (Radlinski et al., 2019).

Methods

As a result of the surmounting lack of effectiveness associated with the use of antibiotics, paralleled with a resurgence to study infection surveillance and control practices; renewed efforts in vaccination; and increased attention to deficiencies in sanitation has transpired (Dusane et al., 2010). The objective of this study is to identify a novel method of administering antibiotics, while reducing the possibility of antibiotic resistance. The experimental hypothesis states, the synergistic effect of coupled rhamnolipids with antibiotics will be greater, when compared to rhamnolipids or antibiotics independently, in susceptibility tests. The null hypothesis, that rhamnolipids and antibiotics will not affect the potency of antibiotics against bacteria used in this study.

The experimental design and laboratory techniques utilized for this experiment were vital to the success of capturing the effectiveness of RLs when combined with the antibiotics. To measure the susceptibility of the RLs, the zones of inhibition, specifically the circular areas surrounding the location of the antibiotic disc, in which the bacteria colonies did not grow, were measured and calculated. The bacteria selected for this study were *Escherichia coli, Bacillus megaterium, Staphylococcus epidermidis,* and *Micrococcus luteus*.

With precision, an aseptic technique was used to promote bacteria growth of isolated colonies while grown on luria broth (LB) agar plates. First, the agar plates were incubated overnight at 37 degrees Celsius, and the isolated colonies were selected from the bacteria plate and inoculated 10mL of liquid LB in a test tube. Afterwards, the bacterial isolates grew overnight in an incubator that was in a constant vibration motion, at 37 degrees Celsius.

This experiment contained six types of plates prepared for each species. The first plate exhibits pure bacterial growth. The following plate that was prepared contained the individual bacteria species in combination with 30 micrograms per milliliter of suspended RL 90% solution. These first two plates served as a reference to compare bacterial growth without exposure to antibiotics.

The succeeding two plates demonstrated the effects solely of the antibiotics against the individual bacteria species, which included both Gram-positive and Gram-negative bacteria species. The antimicrobial susceptibility discs investigated in this experiment individually contained the following dosage; 10 mcg of ampicillin, 30 mcg of chloramphenicol, 15 mcg of erythromycin, 30 mcg of kanamycin, 10 mcg of penicillin, and 30 mcg of tetracycline. The experiment was purposefully designed to include a diverse range of bacteria species and a variety of antibiotics to carefully assess the effect of RLs.

In preparation for the final two plates, the 90% rhamnolipid suspension was diluted and spread as a thin film onto the LB agar plates and placed at room temperature to dry. Next, the bacteria was applied onto labeled plates from the liquid culture. The final two plates tested the antimicrobial susceptibility discs with the added presence of rhamnolipids against the individual Gram-positive or Gram-negative bacteria species.

In summary, the bacterial growth, or lack of growth, was observed and analyzed as a result from being exposed to different living conditions. These conditions included, bacteria with the RLs, bacteria with an antibiotic susceptibility disc, and bacteria with an antibiotic susceptibility disc combined with RLs. Following an overnight incubation at 37 degrees Celsius, zones of inhibition for each agar plate were measured in mm, and recorded for analysis.

Results

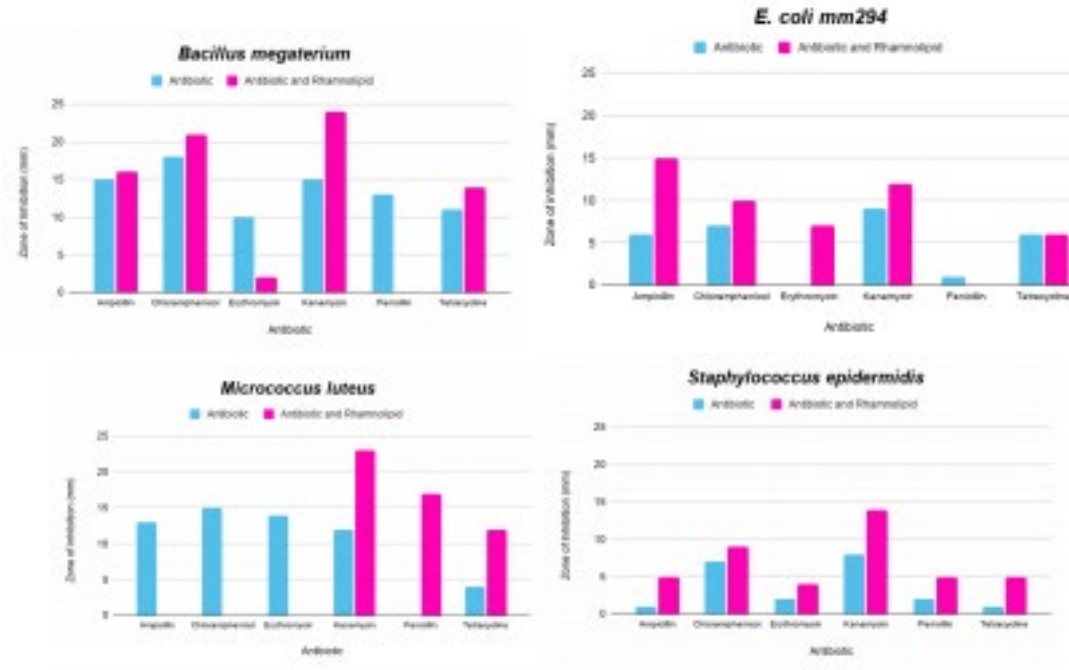


Figure 1: The zones of inhibition for each agar plate were measured (mm) and recorded for analysis. The results differentiate between the effects of the bacteria species and the antibiotic versus the bacteria species and the antibiotic coupled with the RLs. (A)*B.megaterium* (B)*E.coli mm294* (C)*S.epidermidis* (D)*M.luteus*

The results of the experiment support the experimental hypothesis. After analysis of the collected data, the evidence supports that the RLs potentiated the tested antibiotics. This claim is supported by the zone of inhibition calculations. Specifically, demonstrated by the analysis of *E.coli,* the RLs increased the zone of inhibition of ampicillin, chloramphenicol, erythromycin, kanamycin, and tetracycline. However, for *E. coli,* the RLs were ineffective against penicillin. Specifically, demonstrated by the analysis of *Bacillus megaterium*, the RLs increased the zone of inhibition of ampicillin, chloramphenicol, kanamycin, and tetracycline. However, they were ineffective for erythromycin and penicillin. Specifically, demonstrated by the analysis of *Staphylococcus epidermidis,* RLs increased the zone of inhibition

of all tested antibiotics. Therefore, RLs worked the most effectively against thisbacteria when coupled with the antibiotics. Finally, demonstrated by the analysis of *Micrococcus luteus,* the RL's were effective and increased the zones of inhibition for kanamycin, penicillin, and tetracycline. However, analysis of the effectiveness of RLs coupled with ampicillin, chloramphenicol, and erythromycin are undeterminable against *Micrococcus luteus*. A technical error occurred on the plate which yielded results that could not be interpreted, for the components of the plate were jumbled in the incubator.

Conclusion

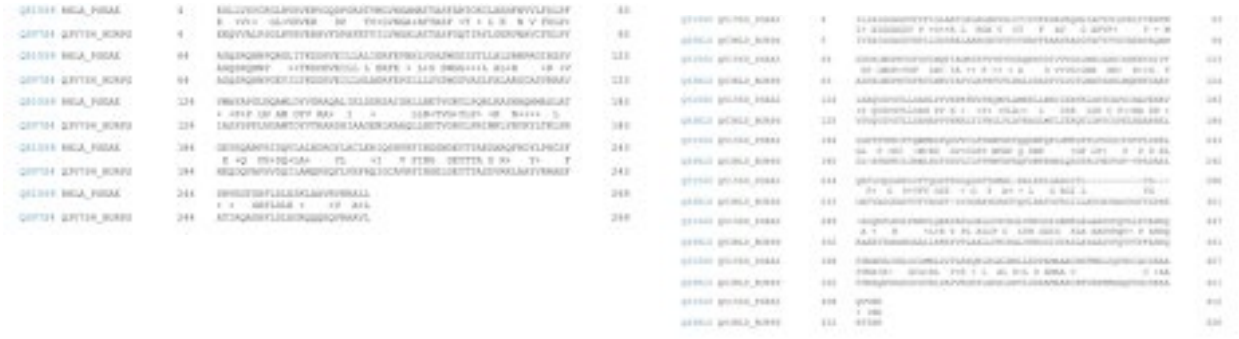
Predominantly, kanamycin had the greatest negative effect on the different bacteria species in the presence of RLs. A paired samples t-test was conducted. This provides statistical evidence that a significant difference does exist between the antibiotic use when coupled with kanamycin against bacterial growth. with a df=3, which yielded a value of p=0.026, with a=0.05. On a separate note, kanamycin is an aminoglycoside, unlike the other antibiotics used in our experiment. Since this specific antibiotic group requires a PMF to stimulate bacterial uptake, their efficacy as therapeutics targeting bacteria is limited. However, the RL's in this case induce PMF and the independent aminoglycoside uptake is used to restore sensitivity to otherwise tolerant bacteria. This makes kanamycin a highly effective antibiotic when combined with rhamnolipids (Krause et al., 2016).

In addition, the results show that RLs had the greatest effect on the permeabilization of *E.coli.* This is particularly interesting because *E. coli* was the only bacterium tested that is Gram negative, which are defined by the thinner peptidoglycan layers found in their cell walls when compared to those of Gram-positive bacteria. The RLs contained the ability to penetrate and create pores in their cell walls because they enabled the membrane to disintegrate.

Blast

The unique qualities of *Pseudomonas aeruginosa,* the bacteria selected for RL synthesis, have influenced extensive research with the purpose of finding different strains that contain the same gene sequence. A BLAST search against RLs enabled the comparison between different species.To further assess the innate function of RLs, a BLAST search of *rhlA* and *rhlB*, which are the genes that code for the enzymes required for rhamnolipid biosynthesis, resulted a strong correlation, measuring a 48.5% comparison, with putative proteins found in *Burkholderia pseudomallei*. Notably, *B. pseudomallei* is another example of a Gram-negative. This bacteria bacteria strain is bipolar, aerobic, motile rod-shaped that infects humans and animals which causes the disease, melioidosis. Both *B. pseudomallei* and *P. aeruginosa* target cells found in the respiratory system.

A BLAST search query results for *P. aeruginosa* RhlB was performed. The match yielded results that 47.3% are similar to *B. pseudomallei.* A practicing guideline states that two sequences are homologous if they are more than 30% identical over their entire lengths (Zulianello et al., 2006). The BLAST results determined a match between species *Pseudomonas* for RhlA and RhlB and for putative protein expressed by species *Burkholderia.* Based upon the results from this experiment and the BLAST analysis, isolated studies of the putative genes and their ability to synthesize and express RLs, or a version of RLs, that may serve a function in the pathogenicity of *Burkholderia* species would be beneficial. In *Pseudomonas*, RLs serve as a virulence factor, disturbing host epithelia, promoting bacterial invasion. An example of future research given this data, an experimental design to include cloning putative genes from *Burkholderia* pathogenic strain into *E. coli* to express the genes in *E. coli* to test for expression and synthesis of RLs would allow for a great extension which would yield supporting data.



(A) (B)

Figure 2: (A) BLAST search query results for *Pseudomonas aeruginosa* RhlA. (B) BLAST search query results for *Pseudomona aeruginosa* RhlB.

Discussion

The RL combination represents a new approach for resolving chronic or relapsing infection and slowing the spread of resistance. The RLs can be used in medication or antibiotics, as they are currently approved to be used in food products, cosmetics and pharmaceuticals by the US Environmental Protection Agency. Altering membrane permeability to induce antibiotic uptake is an extremely effective strategy against both Gram-positive and Gram-negative bacteria that can cause skin infections and other harmful diseases such as meningitis and pneumonia (Wood et al., 2018). These diseases can both be caused by bacterial infections that lead to inflammation of organs, meningitis being the brain and pneumonia being the lungs.

Therefore, when these conditions are caused by a bacterial cell, introducing RLs coupled with antibiotics could potentially increase a more efficient breakdown of the bacterial cells. The RLs create pores in the cell membrane, making it more permeable for antibiotics to penetrate the cell, allowing them to destroy particular cell machinery, ultimately causing bacterial cell death. For example, meningitis in newborns is often caused by *E. Coli* bacteria (CDC, 2019). Based upon our research, there is evidence that the antibiotics' efficacy against *E. coli* will increase when combined with RLs, therefore newborns with bacterial meningitis could be cured in a faster and more efficient way.

There are numerous future studies that can be performed to improve the assessment of the effectiveness of RLs. The testing of different antibiotics, different strains of bacteria, and the different RL concentrations. All of these changes in materials will have a different effect on the susceptibility of RLs.

Specifically, RLs can be used as a potential biocontrol agent for crop culture protection. The possible future research could demonstrate the interaction of natural RLs with plant and fungal membrane models at a molecular scale. RLs have been approved for use in biopesticide formulations. The research could demonstrate the activity of RLs against both fungal and insect pathogens. Additionally, RLs can be used as a supplement utilized in the composting of green waste to generate fertilizers.

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