RAMAPO COLLEGE OF NEW JERSEY

Investigating the *Biofilm* Formation of the Bacteria *Pseudomonas aeruginosa* in a Polymicrobial Setting

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INTRODUCTION

Bacteria rarely exist as single plankotic forms. Several microbial species exist as polymicrobial biofilm communities co-operating the survival of each other. Biofilms are a hallmark feature of various pathogenic and opportunistic pathogenic bacteria that allow them to communicate with each other both *intra-species* and *inter-species* via quorum sensing. We are interested in exploring the quorum sensing phenotypes of the opportunistic bacteria *Pseudomonas aeruginosa* in a polymicrobial setting. *P. aeruginosa* is an opportunistic bacterium that often results in serious infections in health-care settings, especially in immunocompromised patients. This nosocomial opportunistic pathogen produces many virulence factors which are under the regulation of the quorum sensing gene circuit. Two of the very important virulence phenotypes that we are interested in are: production of the blue-green pigment *pyocyanin* and ability to form *biofilms*.

Our previous work suggested that there is a difference in the pigment production when *Pseudomonas* is co-cultured with other gram positive and gram-negative bacteria. As a continuation of this work, we are currently looking at another quorum sensing phenomenon which is the *biofilm* formation of the bacteria in a polymicrobial setting. We are interested in the polymicrobial biofilm co-culture of *Pseudomonas aeruginosa* with other nosocomial bacterial strains *Klebsiella pneumoniae (KP), Escherichia coli (EC), Staphylococcus aureus (SA) and Enterococcus faecalis (EF).*

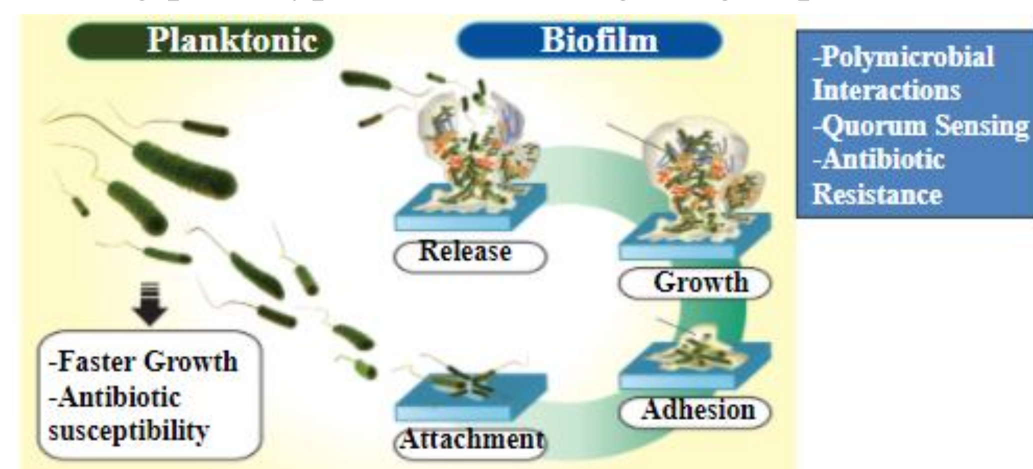
Our preliminary results indicate that the polymicrobial biofilms are not only more complex and stronger than the single species biofilms, but also show greater resistance to the antibiotic treatment *in-vitro*. Our studies are aimed at a long-term goal of developing anti-biofilm agents targeting polymicrobial communities rather than the plankotic bacteria. Future experimental efforts will be focused on identifying differential expression of the quorum-sensing genes of *Pseudomonas aeruginosa* in a co-culture model. The research will be an important contribution to the evolving field of anti-microbial peptides targeting bacterial biofilms and bacterial quorum sensing.

Polymicrobial Biofilms

Biofilms are complex, thriving microbial communities attached to biotic and abiotic surfaces via extracellular matrix made of polysaccharides, proteins and DNA. Bacteria living in biofilm are physiologically different from their planktonic counterparts in many ways. The planktonic form is usually characterized by mobility, faster growth and better susceptibility to antibiotics. Biofilm growth can be seen as an adaptation to a low-nutrient, stressful environment. The Figure below shows various stages of biofilm formation in bacteria.

The first step of biofilm formation is the reversible attachment to a surface through interactions between the bacterial cell wall and the substrate. The contact triggers the release of extracellular matrix components and the colonies grow creating a differential micro-environment to support intra-species and inter-species communication. The biofilm matures and becomes resistant to the antibiotics which are otherwise effective on various planktonic bacteria.

Standard antibiotic susceptibility testing is done on microbes that are grown planktonically whereas the clinical infections are from polymicrobial biofilms. Therefore, it is possible that interactions between microbes could influence the success of antimicrobial treatment. The current study is aimed at understanding the role of *Pseudomonas aeruginosa* quorum sensing phenotypes in modulating the group behaviors in a mixed microbial community.



-Polymicrobial Interactions
-Quorum Sensing
-Antibiotic Resistance

1. Optimization of Biofilm Assay

→ Overnight bacterial cultures of *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Staphylococcus aureus* and *Klebsiella pneumoniae* were added to the sterile Tryptic Soy Broth (TSB) media in 12-well plates and were incubated at 37 ºC for 24-48 hours

→ The supernatant was then discarded, and the adhered cells were rinsed three times with distilled water, and the wells were patted dry on a paper towel

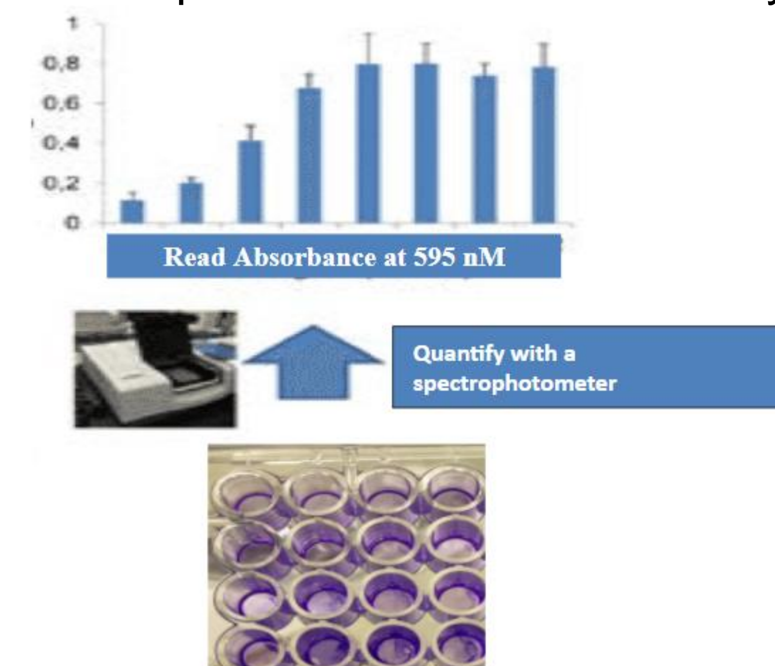
→ 0.1% Crystal Violet (CV) solution was added to each well to stain the adhered biomass and the plate was incubated for 30 min at room temperature

→ The CV dye was discarded, and the wells were again rinsed three times with distilled water and patted dry.

→ 70% ethanol was then added to each well to release the bound CV dye from the biofilm, and the biofilm was quantified by reading the absorbance at 595 nm

→ Experiments were performed in triplicates to ensure reproducibility

Schematic Representation of Biofilm Assay:



2. Quantification of *Pseudomonas* Biofilms

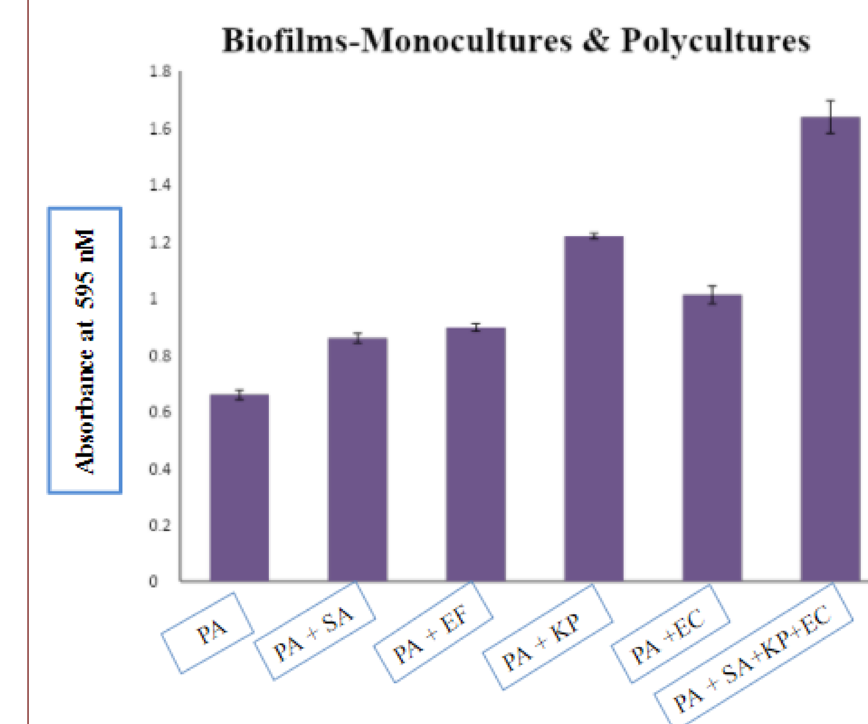
→ Overnight culture of *Pseudomonas* was seeded on 12-well plates and incubated at 37 ºC for 24 hours

→ Planktonic cells were washed using distilled water and the each well was inoculated with another strain of then bacteria to induce mixed biofilms

→ Monoculture control wells of *Pseudomonas* were given a fresh media change and the plate was again incubated at 37 ºC for 24 hours

→ Biofilms were quantified using the experimental procedure outlined previously

Results: The microbial environment around Pseudomonas modulates the extent of biofilm formation



3. Antibiotic Effect

→ Monoculture biofilm of *Pseudomonas* was established by adding actively growing overnight culture of the bacteria into the 12 well plates

→ After 24 hours, planktonic cells were removed, and fresh Tryptic Soy Broth media was added to each well.

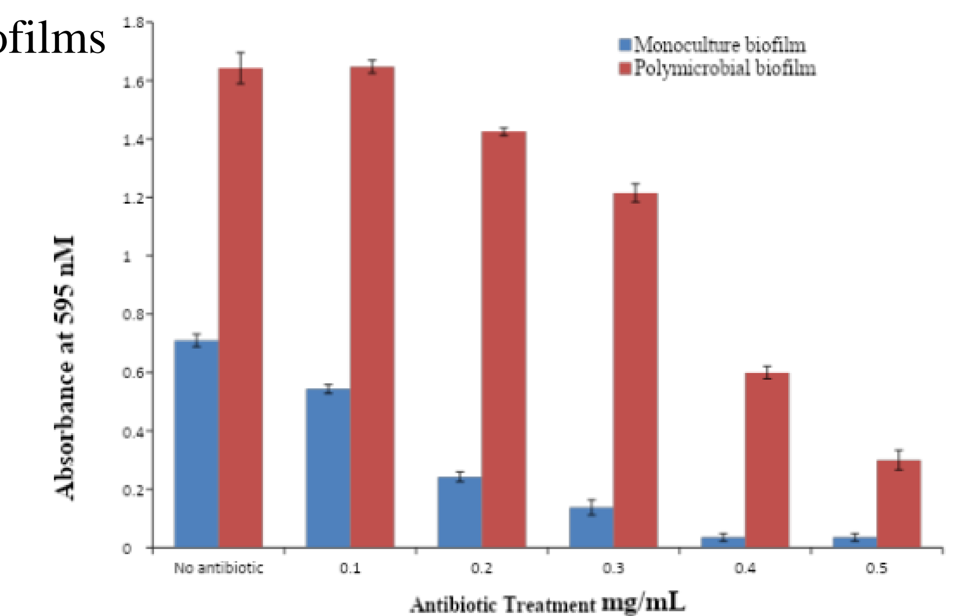
→ Overnight cultures of *Staph aureus, E.coli, Enterococcus faecalis, & Klebsiella pneumoniae* were added to the respective wells to allow the polymicrobial biofilm attachment

→ Additionally, each well was supplemented with the antibiotic gentamicin at various concentrations of 0.1 mg/mL to 0.5 mg/mL which also includes the known MIC value for planktonic *Pseudomonas aeruginosa*

→ The 12-well plates were incubated at 37 ºC for 24 hours

→ Monoculture biofilms and polymicrobial biofilms were quantified using the Crystal Violet Dye absorbance method as mentioned previously

Results: Polymicrobial biofilms are more resistant to the antibiotic treatment than the monoculture biofilms



CONCLUSION

Our results indicate that the biofilm formed by the bacteria *Pseudomonas aeruginosa* in a polymicrobial setting is stronger and has greater resistance to antibiotic treatment than the monoculture biofilm. Our previous results suggested that the virulence factor pyocyanin production by *P. aeruginosa* is highly modulated in a polymicrobial setting . In the current study we observe that the similar regulation is also seen for the biofilm formation of the bacteria in the presence of various other species of bacteria (EC, KP, SA and EF which are also nosocomial bacteria). We hypothesize that there could be a strong inter-connection between the two quorum-sensing phenotypes pyocyanin production and biofilm formation in a polymicrobial setting. To address this, we are currently working on mimicking the polymicrobial environment by working with cell-free supernatant of *Pseudomonas* and other bacteria and assessing their ability to inhibit the biofilm formation. Soon we also would work on generating knock-out/mutant strains of *Pseudomonas* for the pyocyanin gene to establish its role on the polymicrobial-*Pseudomonas* biofilm formation. Eventually the goal is to identify novel drug targets that could serve as quorum quenchers or quorum-sensing inhibitors. This approach has a promising potential for the future in minimizing and delaying the problem of antibiotic resistance.

Future Work

→To test the susceptibility/resistance of the polymicrobial biofilms to various antibiotics at their planktonic MIC values

→To treat the mono-culture biofilms of *Pseudomonas* and other nosocomial bacteria with the cell-free supernatants of each bacterial strain

→ To generate the *Pseudomonas* mutant or knock-out strains and identify the role of Pyocyanin in the formation of polymicrobial biofilms

References & Acknowledgements

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