Characterizing phenotypes in *Pseudomonas aeruginosa* mutants under different oxygen conditions.

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Abstract

Biofilms are ubiquitous in the natural world, inhabiting living and nonliving surfaces. They play a role in environmental cleanup, crop disease, and a wide variety of bodily infections. Bacteria that form biofilms are especially difficult to eradicate due to their sticky quality and increased antibiotic resistance. Biofilm communities also support bacteria resistance to environmental stress. *Psuedomonas aeruginosa*, an opportunistic bacteria, frequently infects cystic fibrosis patients and forms biofilms in the lungs. Here we show that *Psuedomonas aeruginosa* biofilm formation and colony morphology is influenced by the intracellular redox state. The amount of oxygen availability dictates the degree of oxidative stress experienced by the bacteria. The higher the oxygen level, the greater the oxidative stress. The results show that in situations of oxidative stress, the formation of biofilms can support survival.

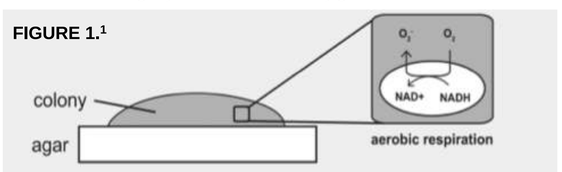
Introduction

Multicellularity offers benefits that are not available to single-celled organisms such as specialization in function and division of labor. This provides them with greater chances of survival during times of limited resources. The heterogeneity of biofilms supports bacterial survival under stressful conditions[1]. The diversity observed in *Pseudomonas aeruginosa* biofilms contributes to its antibiotic resistance. Previous research has shown that phenazines, redox-active molecules, can stimulate anaerobic killing in *P. aeruginosa*[2]. To study this mechanism, mutant strains resistant to anaerobic killing were created. One of these mutants, Candidate #2, is the focus of our study. Candidate #2 contains a putative mutation in ferredoxin reductase and is unable to perform the following reaction:

$$Fd_{ox} + 2H_2 + NAD^+ \rightleftharpoons Fd_{red}^{2-} + NADH + 3H^+$$

Without ferredoxin reductase, the cell may be skewed to a more oxidized state, causing the intracellular redox state of the cell to change. When a cell is more oxidized, it becomes highly sensitive to oxygen.

FIGURE 1.[1]



Candidate #2's sensitivity to oxygen makes it a good model to observe variations in colony and biofilm phenotypes.

Project Goals

Purpose 1: To observe Candidate #2's altered colony and biofilm phenotypes due to changes in intracellular redox state under aerobic and anaerobic conditions.

Purpose 2: To observe Candidate #2's growth and biofilm formation under different oxygen tension.

Experimental Methods

Congo Red Assay

To address Purpose 1, we used a Congo Red assay to test for exo-polysaccharides. Exo-polysaccharides protect bacteria against external stresses and play a pivotal role in biofilm formation

We used 20 ml plates of 1% Agar, 1% tryptone, and 40µg/ml Congo Red. Plates dried for 24 hours before bacterial inoculation. 10µl of each *P. aeruginosa* strain (Δphz and Candidate #2) were spotted in the center of the plates. Plates incubated at 25°C for up to 5 days. On the last day, they were scanned for phenotype analysis.

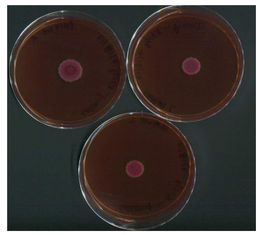


FIGURE 2. Congo Red plates after 5 days of incubation

Crystal Violet Assay

To address Purpose 2, we used a Crystal Violet Assay to test for biofilm formation. Cultures of *P. aeruginosa* were grown overnight (12-16 hours) and then 10^7 cells, as determined by an optical density of 0.01, were put into 10ml of MOPS or MOPS with KNO_3. 200µl of each cell solution was added to 3 wells (biological triplicates) in 96-well plates. Plates are incubated at 25°C for 3 days at various oxygen tensions: 21%, 15%, 5%, 1%, <1%, and 0%.

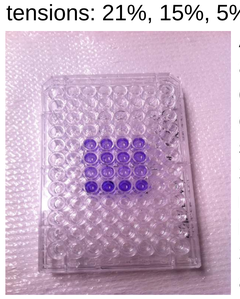


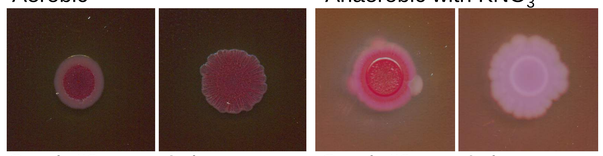
FIGURE 3. Microtiter plate with CV stain

After incubation, plates are read by a microtiter plate reader at 500nm to determine planktonic growth. Then cells are shaken out. The plate is submerged in water and shaken out 3-4 times then allowed to dry for 10 minutes. 200µl of 1% Crystal Violet is added to each well and stained for 30 minutes. Plates are then shaken and rinsed another 3-4 times and air-dried for 10 minutes. 200µl of 95% ethanol is added to each well. A second microtiter reading is done at 595nm quantify biofilm formation data.

Results and Discussion

Congo Red Assay Results

Aerobic | Anaerobic with KNO_3



Cand. #2 | Δphz | Cand. #2 | Δphz

In aerobic conditions, Candidate #2 is smaller and deeper in pink color. The deeper pink is indicative of more biofilm formation. Δphz is able to use oxygen so it is spread out and wrinkled to maximize surface exposure to oxygen.

In anaerobic conditions, Candidate #2 is significantly more pink than Δphz. Colony is rough and highly clustered. Δphz is light pink and does now grow biofilm well in anaerobic conditions. Candidate #2 has higher biofilm formation in aerobic and anaerobic conditions.

Crystal Violet Assay Results

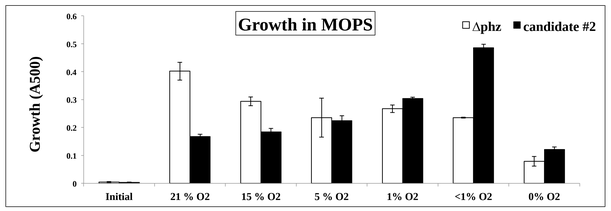


FIGURE 4. Growth of Δphz decreases as oxygen availability decreases in the absence of nitrate. Growth of Candidate #2 increases as oxygen levels decrease because oxygen is toxic to Candidate #2.

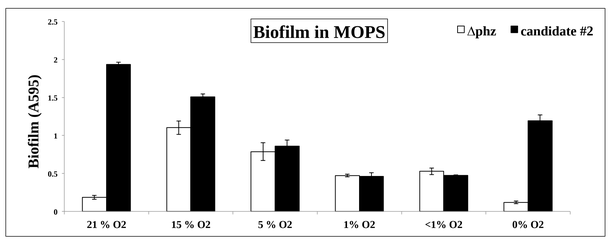


FIGURE 5. As oxygen levels decrease, Δphz is undergoing stress and starts to form biofilms. Meanwhile, Candidate #2 is alleviated from oxidative stress and biofilm levels decrease. At 0% oxygen, both strains are under intense stress but Candidate can form biofilm to survive, Δphz cannot.

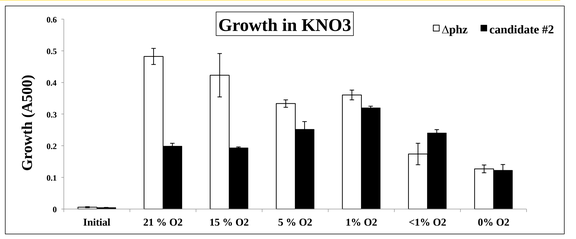


FIGURE 6. Growth of Δphz decreases as it experiences lower oxygen levels. Growth of Candidate #2 is stunted after 1% oxygen because nitrate, an alternative electron acceptor, is toxic to it.

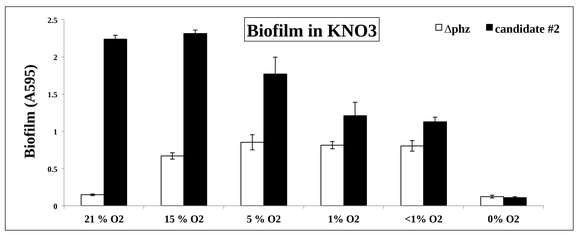


FIGURE 7. Candidate #2 forms better biofilms than Δphz in all oxygen conditions. Δphz is able to sustain biofilm growth because of the addition of nitrate.

Conclusion

The putative ferredoxin reductase mutation in Candidate #2 has been shown to have an effect on *Pseudomonas aeruginosa*'s intracellular redox state and therefore influence biofilm formation and colony morphology. Candidate #2 forms dark, small colonies on Congo Red agar, indicating biofilm formation. With a more oxidized redox state, its growth defect may be due to oxygen or nitrate toxicity. Oxygen tension contributes to oxidative stress, possibly leading to more biofilm formation in Candidate #2.

For future studies, it will be interesting to explore what factors help the bacteria modulate their intracellular redox state allowing them to affect their colony morphology and biofilm formation.

Acknowledgements

1. Wang Lab, Department of Civil and Environmental Engineering, Northwestern University
2. Chicago Botanic Gardens NSF REU

References

1. Dietrich (2013) *Journal of Bacteriology*. Volume 195 1371-81
2. Lee Y, Wang Y. (2013) *In preparation*