

Stay or Go: A Study on Oxygen Tension on the Biofilm Formation of Cystic Fibrosis Bacteria

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By Brandon O. Blackwell and Marilu E. Santos, Ph.D.

Biology

May 2015

Brandon Blackwell

Name

Honors College Scholar

05/15/2015

Date

Ms Santos

Name

Faculty Mentor

05/15/2015

Date

Mark Milewicz

Mark Milewicz, Ph.D.

Dean, Esther G. Maynor Honors College

5/20/15

Date

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## ABSTRACT

### STAY OR GO: A STUDY ON OXYGEN TENSION ON THE BIOFILM FORMATION OF CYSTIC FIBROSIS BACTERIA

Brandon O. Blackwell and Marilu E. Santos, Ph.D.  
Bachelor of Science in Biology – Biomedical Emphasis  
University of North Carolina Pembroke  
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*Pseudomonas aeruginosa* is an opportunistic pathogen commonly associated with mucus-involved disease processes such as cystic fibrosis. In the setting of cystic fibrosis where the airway is obstructed by mucus, the availability of oxygen is greatly reduced (Eschbach, 2004). The ability of *Pseudomonas aeruginosa* to form biofilms greatly contributes to its survival and virulence in the bronchial airways (Haley, 2012). This study investigated in vitro the effect of reduced oxygen tension on the biofilm formation of *Pseudomonas aeruginosa* and on the biofilm formation of a “mock community” of bacterial species that are known to thrive with *Pseudomonas* biofilms in cystic fibrosis patients. It was predicted that with oxidative tension, *Pseudomonas* sp. and the mock community would form biofilms.

Using BD™ *Pseudomonas* Isolation Agar, an overnight culture of *Pseudomonas aeruginosa* ATCC 27853 was grown and spot inoculated on solid, semi-solid, and liquid culture media. *Klebsiella pneumoniae* ATCC 700603 and *Streptococcus aureus* ATCC 17503 were grown separately and together with *P. aeruginosa* in a “mock” community for 1 to 10 days at the human body temperature of 37°C and the ambient temperature of 25°C. The biofilm colony morphology on solid media was characterized, the depth of growth was measured from semi solid media and the optical density readings were obtained in liquid media. Results showed that biofilms grew larger and deeper when incubated at 37°C suggesting that the human body temperature favors biofilm formation. Oxidative tension favored the “go” or dispersal of biofilm cells both from single bacteria and “mock” community resulting to remarkably giant colonies. This study provided evidence of biofilm formation and a better understanding of biofilm behavior during oxidative tension.

## Background

Some bacterial species have the ability to form biofilm communities to aid in increasing protection and virulence. A biofilm is defined as a microbial community encased in an extracellular polymeric substance (Wei, 2013). The extracellular polymeric substance secreted by biofilms and increased cell density are factors contributing to the virulence of the community, protecting the bacteria involved from an immune response and antibiotics. This extracellular polymeric substance matrix serves as the foundation for biofilms, contributing to various processes of the cell including cell-to-cell interactions and attachment of the biofilm (Harmsen, 2010). A species of bacteria commonly associated with biofilms is the opportunistic pathogen, *Pseudomonas aeruginosa* (Eschbach, 2004). Bacterial infections associated with *P. aeruginosa* include otitis media, heart valve endocarditis, and persistent lung infections in cystic fibrosis patients (Haley, 2012). In cystic fibrosis, thick mucus is present in the airway which restricts oxygen flow into the lungs. In mucus involved infections, *Pseudomonas aeruginosa* is not the only culprit involved; studies have shown other species of bacterial are commonly associated with *P. aeruginosa* infections such as *Klebsiella pneumoniae* and *Streptococcus aureus* (Willner, 2012). Regulation of surface behaviors dictates whether or not *Pseudomonas aeruginosa* will swarm or form a biofilm. By forming a biofilm, *P. aeruginosa* is said to exhibit a “stay” behavior and by swarming, the bacteria exhibits a “go” behavior (O’Toole, 2008).

## Materials and Methods

The culture media used to grow the bacteria used in this experiment was BD™ *Pseudomonas* Isolation agar. Overnight cultures of *Pseudomonas aeruginosa* ATCC 2785, *Klebsiella pneumonia* ATCC 700603, and *Streptococcus aureus* ATCC 17503 were grown as both individual cultures and together as a “mock” community. The resulting overnight cultures were used for the spot and stab inoculation of culture media. Solid culture media refers to using 13.6 milligram of agar per 1 liter of purified water, semi-solid culture media refers to using 7.0 milligrams of agar per 1 liter of purified water, and liquid culture media refers to using no agar in the media. The overnight cultures of *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, and “mock” community were spot inoculated on solid and semi-solid plates, and solid, semi-solid, and liquid stabs. Spot inoculation was performed in triplicate, while stab inoculation was performed in duplicate. Each condition was incubated at two different temperatures, the ambient temperature of 25°C and the human body temperature of 37°C and observed at both five and eight days after inoculation. The morphological features of the resulting biofilms growing on the spot inoculated culture media were observed. The depth of penetration within the solid and semi-solid stab culture media was measured from the surface. Optical density measurements at 600 nm were taken for the liquid culture media.

## Results and Discussion

As demonstrated in figure 1, biofilms were seen to grow at both 25°C and 37°C for *P. aeruginosa*, *K. pneumoniae*, and for the “mock” community while no biofilm growth was present for *S. aureus* at either temperature. The comparison between biofilm formation versus colony formation can be seen in figures 2 and 3. In figure 2, *K. pneumoniae* is seen to form a large biofilm, where as in figure 3, *S. aureus* is seen to form uniform colonies. Biofilms were observed to grow larger when incubated at 37°C than when incubated at 25°C, indicating that conditions within the human body favor the biofilm formation in these bacteria. On both solid and semi-solid plates, *S. aureus* did not thrive when incubated at 25°C. The “mock” community shown in the left upper quadrant of figure 1 demonstrates characteristics of both *P. aeruginosa* (left lower quadrant) and *K. pneumoniae* (right upper quadrant). The green pigment that is characteristic of *P. aeruginosa* is present throughout the “mock” community, with a pale, irregular border consistent with the biofilm of *K. pneumoniae*. Under 40x magnification using a light microscope, branching and dispersal patterns were observed within the bacterial biofilms as seen in figure 4.

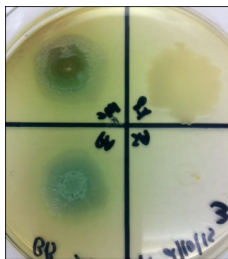


Figure 1. Whole plate sample containing *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and “mock” community.



Figure 2. *K. pneumoniae* biofilm showing macroscopic morphological characteristics.



Figure 3. *S. aureus* did not form biofilms at 37°C.

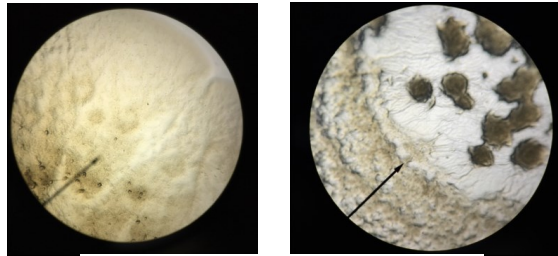


Figure 4. Biofilm branching patterns observed via light microscopy at 40X magnification.

In both the solid and semi-solid agar stabs, biofilms were seen to penetrate within the culture media deeper than bacteria was initially inoculated, with biofilms reaching depths of 29 mm below the surface of the culture media. Figure 5 shows the penetration of biofilms at 37°C for the stab inoculated semi-solid culture media. As the biofilms reach further depths within the culture media, less oxygen is available for the bacteria to utilize. Because biofilms were seen to penetrate deep below the surface, this behavior shows the anaerobic characteristics of the biofilms. In cystic fibrosis, possessing anaerobic characteristic is essential for survival of *P. aeruginosa*. The mucus in which *P. aeruginosa* is harbored restricts air flow to the lungs, providing an anaerobic environment for the biofilm to develop. This anaerobic environment is favorable to producing alginate, a polysaccharide that is found in the extracellular polymeric substance responsible for contributing to the virulence of *P. aeruginosa* biofilms. In vitro, when compared to aerobic conditions, alginate is found in higher concentrations under anaerobic conditions (Worlitzsch, 2002).

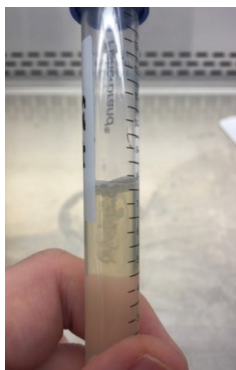


Figure 5. Solid agar stab of “mock” community incubated at 37°C.

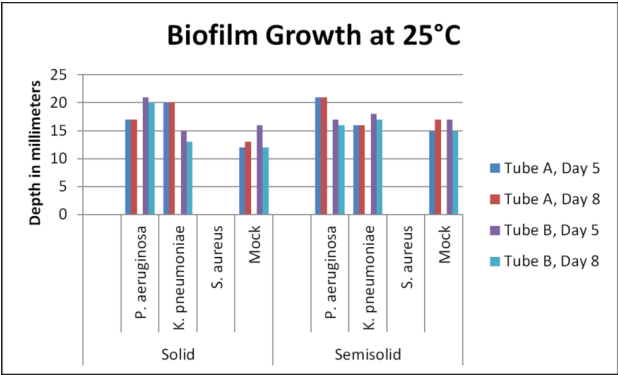


Figure 6. Biofilm growth within stab cultures at 25°C

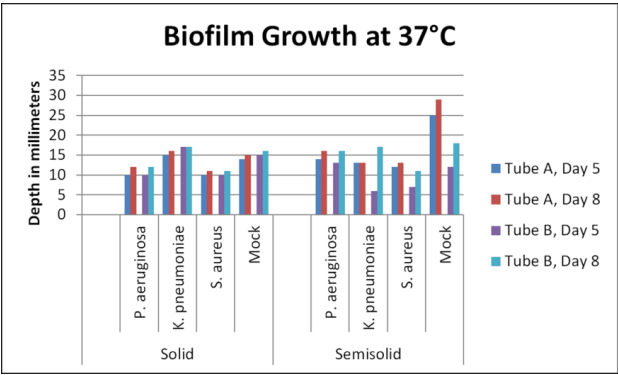


Figure 7. Biofilm growth within stab cultures at 37°C

The graphs above show the penetration of biofilms for both solid and semi-solid culture media at 25°C and 37°C. At the human body temperature of 37°C, there is a higher degree of biofilm penetration in the semisolid culture media. From the conditions presented in this study, this scenario is the best representative of physiological conditions. The most virulent result was from a multi-bacterial biofilm in a mucus-like substrate at human physiologic temperature.

	Day 5		Day 8	
25°C	Tube A	Tube B	Tube A	Tube B
<i>P. aeruginosa</i>	0.218	0.207	0.330	0.478
<i>K. pneumoniae</i>	0.600	0.644	0.645	0.528
<i>S. aureus</i>	0.013	0.051	0.360	0.285
Mock community	0.829	0.553	0.775	0.481

Table 1. Optical density readings for liquid culture media at 25°C at 600 nm.

	Day 5		Day 8	
37°C	Tube A	Tube B	Tube A	Tube B
<i>P. aeruginosa</i>	0.368	0.206	0.390	0.429
<i>K. pneumoniae</i>	1.023	1.051	1.086	1.353
<i>S. aureus</i>	0.017	0.186	0.506	0.353
Mock community	0.926	1.161	1.196	1.339

Table 2. Optical density readings for liquid culture media incubated at 37°C at 600 nm.

Tables 1 and 2 above show the optical density readings at 600 nm for bacteria grown in liquid culture media at both 25°C and 37°C. The readings show a general trend of bacterial growth between the two data collection days. The optical density data does not provide any proof

as to whether or not biofilms are being formed, but it does however show that the bacteria inoculated inside of the culture media are successfully growing.

Cystic fibrosis is an autosomal recessive disorder caused by the mutation in the cystic fibrosis transmembrane regulator gene (CFTR). Dysfunction of this gene is brought about by the deletion of the amino acid phenylalanine. The CFTR gene which is usually involved in chloride movement across lung epithelial cells malfunctions, causing a sticky, salty secretion to be introduced into the lungs (Yang, 2015). The resulting secretion contributes to the ability of bacteria such as *P. aeruginosa* to enact surface behaviors and readily form biofilms. Knowing that the bacteria involved in cystic fibrosis favor the “stay” behavior under bodily conditions, reducing the ability of the bacteria to form biofilms is essential in treating or preventing cystic fibrosis. By correcting these dysfunctional genes, the cellular processing and chloride gating defects within lung epithelial cells would no longer produce this secretion that is advantageous to biofilm forming bacteria.

From the data collected, it can be concluded that the bacteria studied, prefer to establish biofilm communities at 37C, which answers the “stay” or “go” question. The formation of biofilm communities demonstrates a “stay” behavior. For *Staphylococcus aureus* at 25°C, a “go” behavior was observed. At this temperature, *S. aureus* did not form a biofilm. *S. aureus* did not form a biofilm on its own and it is unknown if it is a part of the “mock” community. It has been shown that in chronic infections such as cystic fibrosis, small colony variants of *S. aureus* are able to thrive with support from products produced by *P. aeruginosa* (Goss, 2011).

There is much more to be learned from further advancement of this study. The “mock” community could be Gram stained to identify its bacterial composition. The spatial location of

the different bacterial species within the biofilm could shed light on how to better approach the treatment of these biofilms. Since *S. aureus* was not able to form biofilms on its own, or grow at 25°C, Gram staining would allow visualization to ascertain if *S. aureus* was present as a participant in the “mock” community biofilm. Furthering the understanding of biofilms could lead to developing treatments for diseases such as cystic fibrosis that are contributed to by biofilm forming bacteria. Understanding that *P. aeruginosa* biofilms do not need oxygen in order to flourish and determining optimal survival conditions can help with the future treatment of these biofilms.

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