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Spatial Distribution of Respiratory Metabolisms in Lab-Grown and *in vivo* *Pseudomonas aeruginosa* Biofilms.

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There has been a surge in studies characterizing the human microbiome and its link to human health and disease [1]. Bacterial communities that infect the lung of cystic fibrosis patients have been an area of particular emphasis [2], yet there has been little progress made in the development of effective treatments for chronic infections of the CF airways. A lack of successful therapies can be partially attributed to the void in our understanding of the metabolic processes utilized by CF pathogens *in vivo,* how these processes are spatially distributed throughout the lung cavity, and how they relate to disease progression. Moreover, we know very little of how laboratory observations of *Pseudomonas aeruginosa* biofilm physiology correlate with metabolisms and other cellular processes carried out within the host. This is made more complicated by recent conflicting reports of low oxygen measurements in airway mucus [3] and convincing evidence of aerobic respiration as measured by *in vivo* gene expression data [4]. Therefore, unique approaches are needed to study the respiratory pathways (oxygen and/or nitrate respiration) of *Pseudomonas aeruginosa* while growing in biofilms under both controlled laboratory conditions, and *in vivo,* in order to better understand how this bacterium generates energy and thrives within the lungs of CF patients over the course of infection.

In this study, we utilized two fluorescent microscopy approaches to characterize the spatial expression of two terminal oxidases (CyoA, a low affinity oxidase; and Cco2, a high affinity oxidase) and a regulator of nitrate reductase (NarX) in a simulated stratified CF lung environment. This growth chamber (Figure 1) [5] consisted of an artificial sputum medium supplemented with a nitrate source (100 mM KNO_3) at one end and an oxygen-permeable zone at the other (Figure 1A), creating three distinct strata that are thought to be present in the airways – an oxygen-rich zone, an anoxic nitrate-rich zone, and a central region containing neither oxidant. We then used an anaerobic variant of green fluorescent protein to monitor the expression of each protein (CyoA, Cco2, NarX) within each growth environment. As predicted, CyoA, which is highly expressed in the presence of oxygen [6], showed the highest level of GFP fluorescence in the upper 0.5 mm of the growth chamber (where oxygen was abundant). Conversely, Cco2, was more highly expressed throughout the anoxic region of the entire growth chamber. NarX was also expressed throughout the growth chamber, though its highest expression was restricted to the anoxic region nearest the nitrate source. We then used a novel *in situ* gene expression detection approach known as hybridization chain reaction (HCR; 7) to corroborate GFP fluorescence with gene expression levels. This method, which is based on a chain of recognition and hybridization events between fluorescently labeled nucleic acid hairpins, can detect specific mRNA sequences at high spatial resolution. Using probes specific for *cyoA, cco2,* and *narX,* we were able to visualize expression patterns that supported our GFP analysis. Most notable was the restricted expression of *cyoA* in the upper ~300 μm of the growth chamber (Figure 1B). These results suggest that use of gene-specific fluorophores can be used *in vivo* to better understand which respiratory metabolisms are employed by *P. aeruginosa* during infection.

Based on the success of our *in vitro* observations, these methods are now being applied to *Pseudomonas aeruginosa* biofilms in more complex environments that are clinically relevant – sputum from cystic

fibrosis patients, explanted lungs from late-stage CF patients, and infected paranasal sinus tissue in CF patients suffering from chronic rhinosinusitis. Preliminary data generated by targeting *P. aeruginosa* metabolisms *in vivo* has shown that expression *cyoA* is abundant in all three host environments, suggesting that *P. aeruginosa* is respiring aerobically throughout the respiratory tract at multiple stages of infection. However, that *narX* is also detected (albeit at lower expression levels), indicates that the upper and lower respiratory tract contains a heterogeneous growth environment that supports the versatile metabolisms of *P. aeruginosa.* Further development and application of fluorescent microscopic approaches towards the *in vivo* physiology of host-associated microbial communities will not only help us better understand the infectious disease process, but potentially identify novel strategies for infection control.

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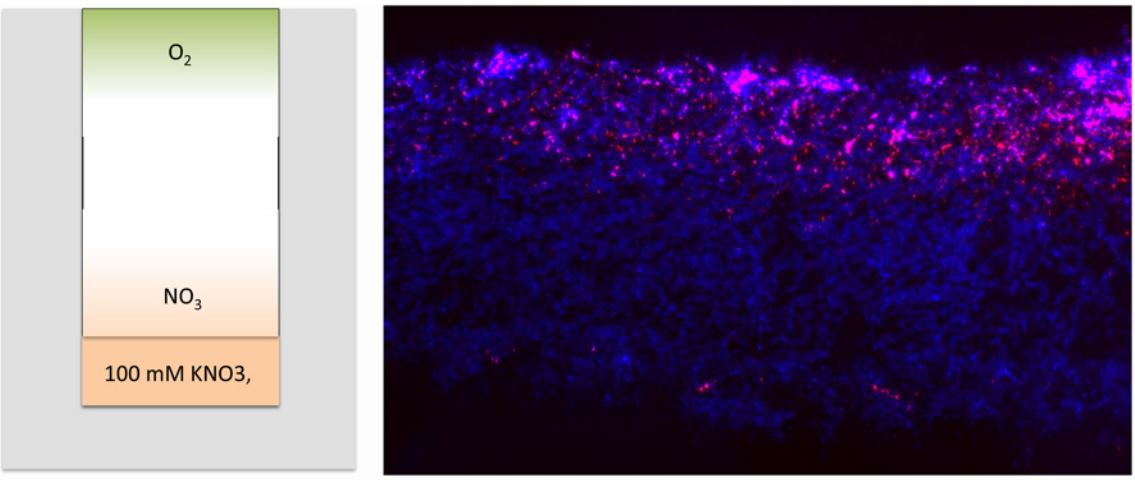

Figure 1. A) Simulated stratified CF lung chamber supplemented with 100 KNO_3 and O2, generating discrete growth environments for *P. aeruginosa.* (B) Using gene-specific fluorescent probes targeting *cyoA* (red), a terminal oxidase expressed under high oxygen concentrations, is restricted to the upper layer of biofilm growth (blue = DAPI) in the simulated CF lung environment.

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