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A messenger molecule governs interdependency in an evolved *Pseudomonas aeruginosa* biofilm community

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by

Thomas MacElliott Johnson

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A messenger molecule governs interdependency
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Senior Honors Thesis

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Abstract:

Biofilm populations are known to harbor great diversity, but the importance of this diversity is not fully understood. A likely contributor to this variation is the second messenger molecule cyclic-di-GMP: low levels associate with a planktonic lifestyle while high levels favor biofilm formation. In ongoing studies of an evolving biofilm population of *Pseudomonas aeruginosa* (PA), we observed extensive ecological diversification that may relate to this lifestyle switch. In PA, the gene *bifA* encodes a phosphodiesterase that is known to degrade cyclic-di-GMP and reduce biofilm. This gene was cloned onto a plasmid under control of an inducible promoter and the plasmid was added to each of seven different members of a diverse biofilm community. We also constructed communities lacking a single member. The fitness of each biofilm community, with a single member expressing *bifA*, lacking a member, or unaltered, was then quantified. Reductions in community fitness were observed when a single genotype expressed *bifA* and hence experienced low levels of cyclic-di-GMP. This result demonstrates that this messenger molecule regulates the biofilm lifestyle at the population level as well as the individual level. Subtraction of individual community members produced varied effects on fitness that associated with their ecological role. Together these results suggest that the synergy of diverse biofilms depends on a balance of genotypes varying in their regulation of molecules that define their niches.

Introduction:

The opportunistic pathogen *Pseudomonas aeruginosa* is one of the principal model organisms in the study of biofilm formation. *P. aeruginosa* growth and development in surface associated biofilm communities is found in a multitude of environments and is known to cause chronic infections in the lungs of cystic fibrosis patients. The mechanism of transition from being a free swimming planktonic cell to a biofilm community is complex and involves a distinct change in gene expression within the microbial cell. *P. aeruginosa* biofilm formation is believed to occur through a series of four subsequent steps: (i) surface association of planktonic cells via reversible attachment, (ii) irreversible attachment of cells to surface (iii) aggregation of surface-attached cells into microcolonies, (iv) encasement of microcolonies in newly synthesized exopolysaccharides. (Klausen et al., 2003; Caiazza and O'Toole, 2004; Kuchma et al., 2007)

The biofilm's dynamic structure is composed of layers of microniches that allow for great biodiversity to develop (Costerton et al., 1994). Environmental conditions and metabolic activities differ throughout the biofilm, which produces gradients in nutrient, waste and signaling molecule concentrations (Stewart and Franklin, 2008). Heterogeneous in composition, a mature biofilm consists of an assortment of cells displaying differential gene expression patterns and physiological activities according to spatial distribution within the biofilm (Costerton et al., 1994; Stewart and Franklin, 2008). The large number of local selection pressures within biofilms force cells to adapt and inhabit the microniche that is most favorable for them. Because there are so many microniches available during biofilm development, the biofilm community exhibits great biodiversity and variants that would be out competed to extinction in a planktonic community are allowed to persist in the biofilm (Nguyen and Singh, 2006; Poltak and Cooper, 2011). As conditions change new microniches arise, which further increases the biodiversity

present. The net effect is an overall biofilm population that is better equipped for survival, as seen in clinical environments where biofilm growth of pathogenic bacteria have increased antimicrobial tolerance and cause chronic infections that are nearly impossible to eradicate (Nguyen and Singh, 2006; Smith et al., 2006).

The effect of biodiversity can lead to increased biofilm productivity of the community by two mechanisms: selection and complementarity (Loreau, 2000; Loreau and Hector, 2001). The selection effect refers to process of dominant species over-yielding in the community because of extreme trait values (Loreau, 2000; Loreau and Hector, 2001). Whereas, the complementarity effect refers to the enhancement of production by the community because of permanent association (for e.g. resource partitioning) between community members (Loreau, 2000; Loreau and Hector, 2001; Langenheder et al., 2010).

Discovered in 1987, the second messenger molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is now considered a key player in the control the transition from living as a planktonic cell to the biofilm lifestyle ubiquitous for all bacteria (Morgan et al., 2006; Ryan et al., 2006; Dow et al., 2007; Hengge, 2009). Generally, when present in high concentrations c-di-GMP acts to stimulates the synthesis of adhesion molecules and the exopolysaccharide matrix of biofilms while also inhibiting motility associated mechanisms such as flagellar expression and flagellar motor function (Morgan et al., 2006; Dow et al., 2007; Hengge, 2009). At low c-di-GMP concentrations the motile planktonic lifestyle is promoted along with the synthesis of virulence factors (Ryan et al., 2006; Hengge, 2009).

Cellular c-di-GMP concentrations are antagonistically controlled via synthesis by diguanylate cyclases (DGCs) containing the conserved GGDEF domain in their active site and degradation catalyzed by specific phosphodiesterases (PDEs) with conserved EAL or HD-GYP

domains (Morgan et al., 2006; Dow et al., 2007; Kuchma et al., 2007; Hengge, 2009). There is an abundance of GGDEF, EAL and HD-GYP proteins in the bacteria; in *P. aeruginosa* alone there is known to be at least 40 of these proteins (Ryan et al., 2006). Sequence analysis has shown that many of these proteins also contain signal input domains, which respond to environmental conditions (Morgan et al., 2006; Ryan et al., 2006; Dow et al., 2007). After recognizing an environmental cue, the production or degradation of c-di-GMP will act to regulate bacterial physiology or gene expression (Ryan et al., 2006; Hengge, 2009). If a signal input domain is not present then a sensor protein associated with the DGC or PDE may act to recognize environmental cues and modulates enzyme activity (Morgan et al., 2006; Thormann et al., 2006).

One PDE of interest in *P. aeruginosa* is BifA (PA4367), which has been identified as a participant in the regulation of biofilm formation and motility (Kuchma et al., 2007; Merighi and Lory, 2010). BifA is a dual GGDEF/EAL domain-containing protein, yet it has an inactivated GGDEF domain and only PDE enzyme activity (Kuchma et al., 2007). Dual GGDEF/EAL domain proteins are not uncommon and phenotype evaluations show that usually only one domain is catalytically active, yet both domains are important for proper functioning as the inactive domain can act as an allosteric effector site (Schmidt et al., 2005; Weber et al., 2006; Kuchma et al., 2007; Hengge, 2009).

Localized to inner membrane, BifA functions together with the DGC SadC to modulate the concentration of c-di-GMP in the cell, which inversely regulates biofilm formation and swarming motility (Kuchma et al., 2007; Merritt et al., 2007). Phenotypic analysis indicates that at least one other DGC supplies c-di-GMP into the same concentration pool that BifA moderates (Kuchma et al., 2007; Merritt et al., 2007). The c-di-GMP signal is predicted to be transferred by the protein SadB to the Pel machinery, which in turn produces components of the

exopolysaccharide matrix of the biofilm (Caiazza and O'Toole, 2004; Kuchma et al., 2007; Merritt et al., 2007).

This investigation analyses how an experimentally evolved *P. aeruginosa* biofilm population is affected as a whole by changes in c-di-GMP concentration within single members of the community. Our strategy was to quantify population fitness of the wildtype evolved community as well as constructed communities where a particular member of the diverse population was substituted with its *bifA* complemented equal or where the member was subtracted entirely from the population. We also measured if a change in the diversity of the biofilm community occurred because of the decreased c-di-GMP levels expressed in a single community member. We found that community fitness decreased when a member of the community was expressing decreased levels of c-di-GMP or was subtracted entirely from the population. Further, we found that the level of biodiversity decreased as the population dynamics were altered when a member of the community was expressing decreased levels of c-di-GMP. These results demonstrate that the messenger molecule c-di-GMP regulates the biofilm lifestyle at the population level as well as the individual level. Furthermore, the synergy of diverse biofilms is dependent on a balance of genotypes varying in their regulation of molecules that define their niches.

Methods and Materials:

Experimental evolution:

Three replicate ancestral populations of *P. aeruginosa* PA14 were grown on 7mm polystyrene beads suspended in 5 mL of M63 media in a test tube for ninety days or ~550 generations.

Populations were selected for reversible surface attachment by daily transfer of the bead to a new test tube where cells were required to adhere to a new bead in order to persist. Three replicate planktonic lines were also passaged as a control.

***P. aeruginosa* *bifA* complementation:**

i) Preparation of electrocompetent cells. *Escherichia coli* SM10 λ pir cells were grown overnight in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 28°C with shaking. Fresh LB medium (50 ml) was inoculated with the 1.5 ml of overnight culture and incubated at 28°C with shaking until the optical density reached 0.600. The cells were then chilled and centrifuged at 4000 x g for 10 minutes at 4°C. The pellet was washed once with 200 ml of ice cold water and then again with 100 ml. The pellet was then resuspended in 10 ml of ice cold 10% glycerol, centrifuged again, and resuspended in 800 μ l of ice cold 10% glycerol for storage at -80°C.

ii) Electroporation. Arabinose-inducible, N-terminal His-tagged *bifA*-containing pMQ80 plasmid (2 μ g) was added to 100 μ l of the electrocompetent cells in a chilled electroporation cuvette and electroporated at 18-kV/cm using an Eppendorf 2510 electroporator. Immediately after electroporation, 800 μ l of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂-6H₂O, 10 mM MgSO₄-7H₂O, 20 mM glucose) was added to the cells and incubated for 60 minutes at 37°C with shaking. The cells were then spread plated onto LB agar (1.3% agar) containing 10 μ g/ml of gentamicin and incubated at 37°C overnight.

iii) SM10 bi-parental matings. Overnight cultures of SM10 cells containing the pMQ80-His-*bifA*⁺ plasmid were grown with 10 µg/ml of gentamicin. Overnight cultures of the *P. aeruginosa* B1-90d community members were also grown. The SM10 cells were centrifuged for 5 minutes at 4000 x g, then resuspended in 1ml of LB medium. To the center of an LB agar plate 50 µl of SM10 was mixed with 100 µl of a B1-90d community member and incubated for two hours at 37°C. To the plate 1 ml of phosphate buffered saline (PBS) was added and washed over the plate ten times. LB agar plates supplemented with 80 µg/ml of gentamicin and 20 µg/ml nalidixic acid were then inoculated with 100 µl of the PBS solution and incubated at 37°C for 24 hours.

Biodiversity assays and Fitness assays:

Monocultures of each B1-90d community member were grown overnight in 1 ml Tsoy and 4 ml M63 media with a polystyrene bead. For fitness assays, *P. aeruginosa* wildtype strain PA14 was also grown over night and then equal amounts of competitors were added to 5 ml of M63 media with a new polystyrene bead and CFU/mL was determined for each competitor at the time of inoculation and after 24 hours off of the bead by plating onto indicator plates (1% tryptone supplemented with coomassie blue and congo red), allowing variation in colony morphology to be more apparent due to dye uptake. Communities were reconstructed by adding them together in their natural relative frequency with substituted complements or subtracted members. All beads were aseptically removed and sonicated in 1.5 mL PBS to remove all biofilm cells before passage or plating. For expression of *bifA*, arabinose was added to the media at a final concentration of 0.5%. In fitness assays the relative fitness was calculated as the ratio of the $\ln(\Delta\text{CFU/mL})$ over a 24 hour time period. In biodiversity assays, population dynamics were calculated as the difference between the observed yield of a mixed community and its expected yield (based on the monoculture yields of the component species).

Results:

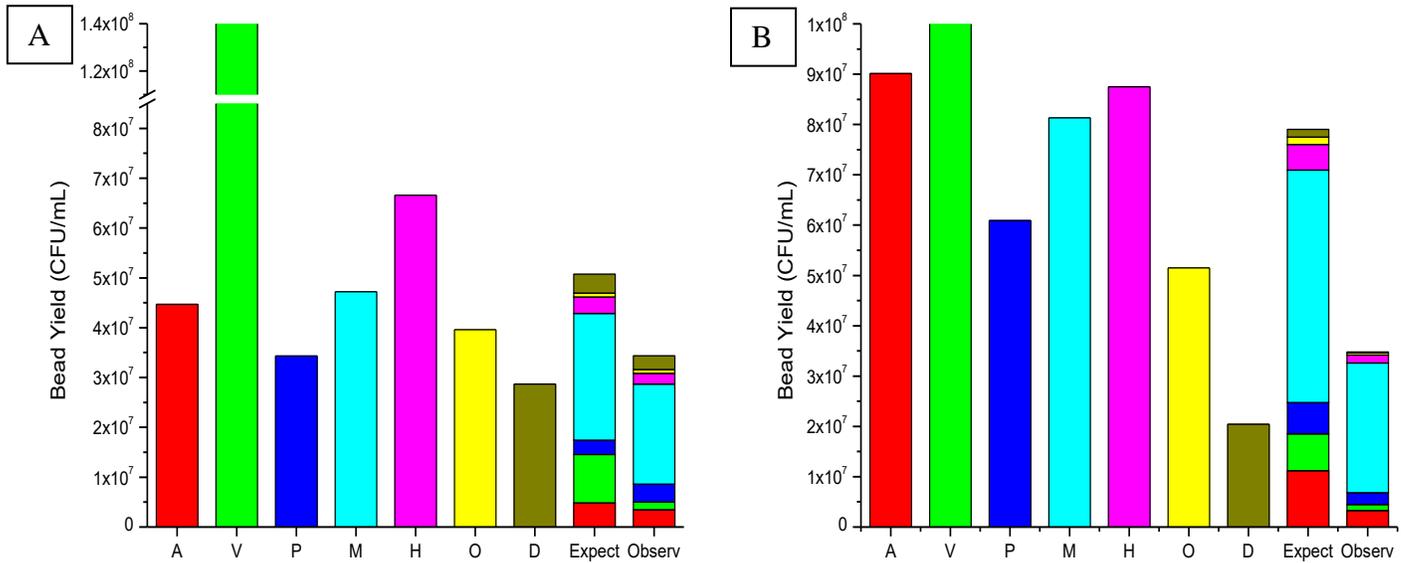


Figure 1: Productivity of the seven community members isolated from evolved biofilm B1-90d population grown in monoculture and in mixed community. Productivity in monoculture was used to predict the expected mixture yield assuming no interaction between types (Loreau and Hector, 2001). Observed productivity is the total yield of the mixed community. A. Natural community and B. Community with M + *bifA*.

Community member monoculture yield is not synonymous with yield in mixture.

We compared the productivity of the B1-90d biofilm community mixtures with the viable cell counts of their constituent morphotypes grown in monoculture (Figure 1). The cell yields for both the wildtype mixed community and the M + *bifA* mixed community were lower than many of their constituents in monoculture and significantly lower than the expected mixture yields. For each morphotype, the yield in monoculture was found to be much greater than the morphotypes relative yield in mixture. The observed yields for the natural community and the M + *bifA* community were found to be very similar, yet the proportions of each member in the communities differed. Between the two communities the M type remained dominant in mixture, even when expressing decreased levels of c-di-GMP. The M + *bifA* community shows a

dramatic decrease in the D variant, which was almost lost completely from the mixed community. Compared to the wildtype B1-90d community, in the M + *bifA* community the proportion of variant A decreased 6.9%, V decreased 22 %, P decreased 36%, M increased 27%, H decreased 27%, O decreased 45% and D decreased 95%. This indicates that the decreased levels of c-di-GMP expressed in variant M caused the population dynamics to change as each community member other than M had decreased abundance in the community.

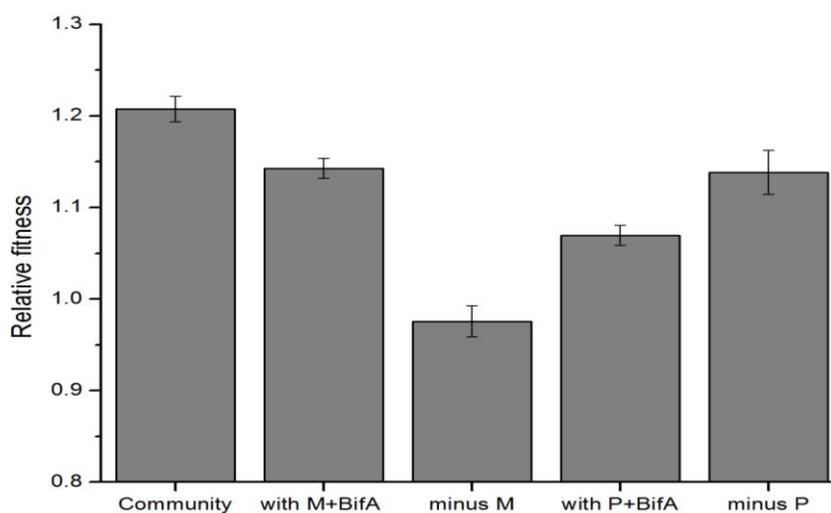


Figure 2: Community relative fitness values for the wildtype evolved B1-90d community, M + *bifA* substitute, minus M, P + *bifA*, and minus P populations. Relative fitness is the colonization efficiency of the total communities relative to the PA14 ancestor.

Community fitness declines when a member expresses decreased levels of cyclic-di-GMP or is subtracted entirely.

The relative fitness of five different community populations was determined and compared to determine the effect that certain manipulations to the population had (Figure 2). The wildtype B1-90d evolved community had the highest relative fitness (1.20), while each of the

manipulated communities was found to have decreased relative fitness's. When the predominate member of the natural community, M (representing ~50% of the population), was substituted with its bifA complement, the community fitness decreased to 1.14, and when M was subtracted completely from the community the community fitness was measured to be 0.96. When the community member, P (representing ~8% of the population), was substituted with its bifA complement, the community fitness was 1.07, and when P was subtracted entirely the community fitness was 1.14.

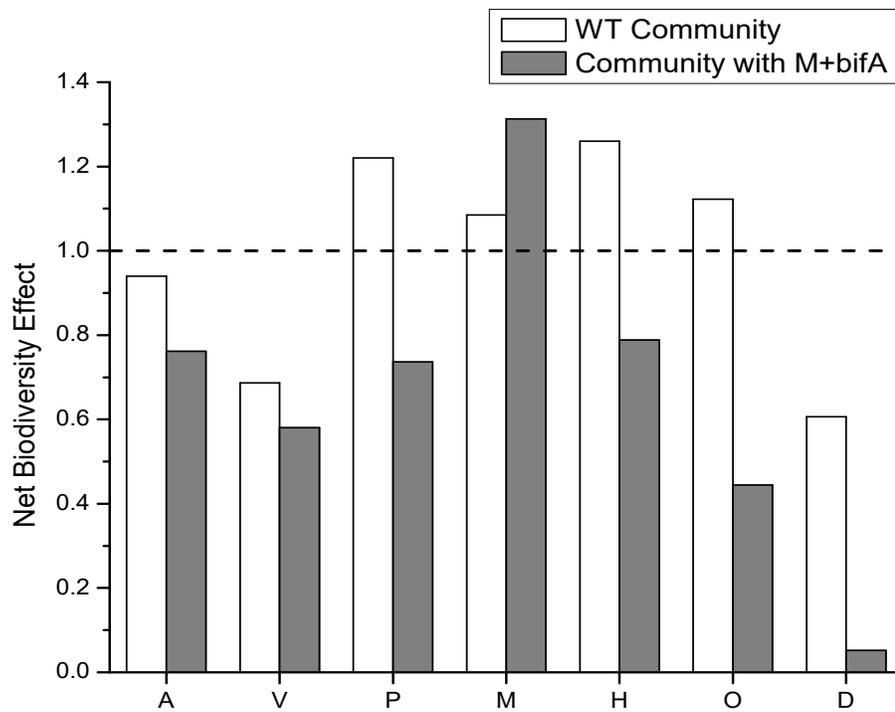


Figure 3: Net biodiversity effects (NBEs) for each community member. NBE is determined as the relative observed yield divided by the expected yield in mixture. NBE values less than one indicate lower than expected observed yield, while values greater than one indicate higher than expected observed yield.

Cyclic-Di-GMP alteration in one community member negatively impacts the dynamics of the entire population.

As noted above the proportional abundance of each of the community members was altered when the M variant was substituted with its *bifA* complemented self. Variant M had an increase in dominance while the six other members had decreases in their relative abundances. The net biodiversity effect (NBE) for each community member was also calculated (Figure 3) for both the wildtype B1-90d and M + *bifA* populations. NBE values less than one indicate lower than expected observed yield, while values greater than one indicate higher than expected observed yield. Variant A had a NBE in the wildtype community of 0.94 and a NBE in of 0.76 in the M + *bifA* community, which is a 19% decrease. This indicates that A experienced a slight decreased observed yield in the wildtype community and a more pronounced decrease in the M + *bifA* community. Variant V had a NBE in the wildtype community of 0.69 and a NBE in of 0.58 in the M + *bifA* community, which is a 16% decrease. This indicates that V experienced a decreased observed yield in the wildtype community, which was worsened in the M + *bifA* community. Variant P had a NBE in the wildtype community of 1.22 and a NBE in of 0.74 in the M + *bifA* community, which is a 40% decrease. This indicates that P experienced a higher than expected observed yield in the wildtype community, but had a decreased observed yield in the M + *bifA* community. Variant M had a NBE in the wildtype community of 1.08 and a NBE in of 1.31 in the M + *bifA* community, which is a 21% increase. This indicates that M experienced a slight increased observed yield in the wildtype community, which increased more in the M + *bifA* community. Variant H had a NBE in the wildtype community of 1.26 and a NBE in of 0.79 in the M + *bifA* community, which is a 37% decrease. This indicates that H experienced a higher than expected observed yield in the wildtype community, but had a decreased observed yield in

the M + *bifA* community. Variant O had a NBE in the wildtype community of 1.12 and a NBE in of 0.44 in the M + *bifA* community, which is a 60% decrease. This indicates that O experienced a slight higher than expected observed yield in the wildtype community, but had a significant decreased observed yield in the M + *bifA* community. Variant D had a NBE in the wildtype community of 0.61 and a NBE in of 0.05 in the M + *bifA* community, which is a 91% decrease. This indicates that V experienced a decreased observed yield in the wildtype community and then was almost completely lost in the M + *bifA* community. These results show that the six community members other than variant M experience much lower yields in the M + *bifA* community than what is expected based on their individual monoculture yields.

Conclusions:

Biofilms are ubiquitous structures found in a multitude of natural, industrial and clinical environments. Strong selective pressures produced a diverse biofilm community consisting of distinct morphological and genetic differences. The diversity is harbored in biofilms thanks to its complex structure and the microniche environments within. Interactions between variant members of a biofilm community heighten the stability provided by the biofilm lifestyle. When this diversity is altered the community as a whole is affected, presumably until selection forces cause the biofilm community to adapt to restore their diversity.

Before we can understand the significance of biodiversity present in a community, we must analyze the two components of diversity. These components are species richness (the number of species per unit area) and species equitability (the abundance of individual species in the community) (Polley et al., 2007). Putting these components together allows us to answer the question of whether or not the production of a community has a direct positive relationship with community biodiversity (Mouquet et al., 2002). Furthermore, we can determine if the

biodiversity is under the influence of complementarity or selection effects. Where the complementarity effect refers to enhanced production due to associations, and the selection effect reflects population dominance by a variant carrying extreme traits (Loreau and Hector, 2001). In our evolved biofilm populations we are dealing with only one species (*P. aeruginosa*) and therefore we are analyzing community member richness and equitability. Biofilm community member was deemed genetically different from each other by the observation of distinct phenotypic and colony morphology differences.

We used the NBE values to determine if complementarity or selection effects were occurring in the two biofilm communities analyzed. The selection effect is a covariance measurement between monoculture yield and change in relative yield in the community (Loreau and Hector, 2001). A selection effect may be evident if the NBE of one or more community member is significantly different from the NBE of the other members. This would occur if one member with dominated the mixed community and had high monoculture yields. A complementarity effect occurs if community member yields in mixture are on average higher than expected, relative to their weighted monoculture yields (Loreau and Hector, 2001). Positive complementarity effects would be evident if the average of each NBE value for all community members was greater than one.

The NBE values from the wildtype evolved B1-90d biofilm community indicate that there are no complementarity effects occurring. The average of the NBE values for each of the seven members of this community is 0.99, which according to Loreau and Hector (2001) would indicate that there are no complementarity effects because the value is so close to 1. Analyzing the individual NBE values for each community member separately shows that some variants are being influenced by positive complementarity effects (variants P, H and O); while others are

reacting to negative complementarity effects (variants V and D). These complementarity effects offset in the community as a whole, which causes the average NBE to be approximately equal to 1. It is not surprising to see offsetting complementarity in a biofilm community because of its inherent structure where microniche differentiation and facilitation mechanisms are known to dominate. There is no indication that selection effects are occurring as there is no dominant community member present. A selection effect would be more evident in the early stages of evolution for the population or if a major environmental change was to occur, which would introduce new microniches and selection pressures.

The NBE values from the M + *bifA* biofilm community indicates that there are negative complementarity and selection effects occurring. The average of the NBE values for each of the seven members of the community is 0.67, which indicates negative complementarity effects are occurring as the value is much less than 1 (Loreau and Hector, 2001). The NBE values for each individual member of this community indicate that the six variants other than M have an observed yield significantly lower than the expected yield in mixture. Furthermore, these six variants display a significant decrease in abundance compared to the wildtype B1-90d community. There are indications that positive selection effects are occurring as well because the community is now dominated by the M variant that increased its abundance in the population 16% and now represents 74% of the population. However, it may be the decrease of the other six members of the community that is more significant. Their total decrease could be the result of negative selection, as negative selection effects can occur in communities where a larger proportion of the population is unfit for the environment (Loreau and Hector, 2001).

Relative fitness measurements for the biofilm communities in this investigation demonstrate that decreasing the concentration of c-di-GMP within one member of a diverse community affects the entire population's fitness. The M + *bifA* and P + *bifA* biofilm communities had decreased population fitness relative to their ancestor PA14. The P + *bifA* community had a relative fitness value that was found to be lower than a constructed community where the P variant was absent entirely. This indicates that it is worse for the biofilm community to have a community member expressing lower levels of c-di-GMP, which would promote the planktonic state, then to subtract that member entirely. However, when relative fitness of a community constructed with the M variant subtracted entirely was measured, the resulting population fitness was found to be significantly lowered than the M + *bifA* community. This result is most likely observed because the M variant is the most prevalent member of the biofilm community and it plays an important role in maintaining the stability of the biofilm community.

C-di-GMP is known to play a role in modulating a cell's transition from the motile planktonic state to biofilm formation (Morgan et al., 2006; Ryan et al., 2006; Dow et al., 2007; Hengge, 2009). Evidence provided here indicates that c-di-GMP may function in regulation of biofilm formation at the population level, not just the individual level. We have shown that interfering with the c-di-GMP levels can lower the strength of the community and population dynamics by decreasing biodiversity. The ability to destabilize a biofilm population by introducing a bacterial strain that over expresses PDEs for c-di-GMP degradation could possibly be a future method of treating biofilm infections. These studies contribute to our understanding of biofilm diversity in general, which may help explain the observed dynamics of *P. aeruginosa* biofilm populations found in lung infections of cystic fibrosis patients.

Future Directions:

Expansion of this investigation is necessary to understand the mechanisms behind the destabilization of a biofilm community by cells expressing decreased levels of c-di-GMP. Future studies include confocal imaging of biofilms produced by the wildtype community along with the altered populations, which would help demonstrate effects of altered c-di-GMP levels on biofilm structure. We also plan to repeat all experiments by supplementing the *bifA* gene with the pseudomonas *sadC* gene which acts oppositely to *bifA* by increasing the production of c-di-GMP.

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