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Jennifer E. Brophy April 11, 2016

Population dynamics and recombination of *Streptococcus pneumoniae* strains in nasopharyngeal biofilms

by

Jennifer Brophy

Jorge Vidal
Adviser

Department of Biology

Jorge Vidal

Adviser

Bruce Levin

Committee Member

Rustom Antia

Committee Member

2016

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Jennifer Brophy

Jorge Vidal

Adviser

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Invasive pneumococcal disease (IPD), caused by *Streptococcus pneumoniae*, is a leading cause of death among children worldwide. Asymptomatic nasopharyngeal colonization by *S. pneumoniae*, a prerequisite for IPD, is estimated to occur in up to 90% of healthy children. Recent studies have shown that ~50% of colonized children carry more than one strain (co-colonization). Colonization is mediated by pneumococcal biofilms, which form on the nasopharyngeal epithelium. Nasopharyngeal biofilms may contribute to the emergence of antibiotic-resistant strains and new pneumococcal serotypes, thought to occur by genetic recombination in the human nasopharynx. The aims of this thesis were to study the population dynamics of two pneumococcal strains and to begin exploring pneumococcal nasopharyngeal recombination. We first examined two *S. pneumoniae* strains to determine whether some serotypes colonize the nasopharynx more effectively, i.e., produce more biofilms, and release more planktonic (i.e., invasive) pneumococci on human nasopharyngeal cells. Genome-sequenced reference strains, serotype 19F strain GA13499 and serotype 2 strain D39, were grown separately or together in bioreactors containing human nasopharyngeal cells for 24 h. GA13499 encodes resistance to trimethoprim whereas D39 carries resistance to tetracycline in a plasmid encoding the *tet*M gene. Therefore, blood agar plates with the appropriate antibiotics were used to compare viable biofilm cells and planktonic bacteria grown separately or together. Our results demonstrated that serotype 19F strain GA13499 produced more biofilms, and released more planktonic bacteria, than strain D39, either when grown alone or together with D39. The addition of the other strain did not significantly affect biofilm counts or counts of planktonic bacteria of either strain. We recovered recombinant pneumococci encoding resistance to both antibiotics. While we have reasons to believe they are 19F that acquired the *tet*M-encoding plasmid from strain D39, the direction of recombination and the mechanism governing the recombination direction needs to be further investigated. Should these recombinant pneumococci be 19F, the biofilm recombination frequency was 2.35×10^{-4} and the planktonic recombination frequency was 1.02×10^{-3}.

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Abstract

Invasive pneumococcal disease (IPD), caused by *Streptococcus pneumoniae*, is a leading cause of death among children worldwide. Asymptomatic nasopharyngeal colonization by *S. pneumoniae*, a prerequisite for IPD, is estimated to occur in up to 90% of healthy children. Recent studies have shown that ~50% of colonized children carry more than one strain (co-colonization). Colonization is mediated by pneumococcal biofilms, which form on the nasopharyngeal epithelium. Nasopharyngeal biofilms may contribute to the emergence of antibiotic-resistant strains and new pneumococcal serotypes, thought to occur by genetic recombination in the human nasopharynx. The aims of this thesis were to study the population dynamics of two pneumococcal strains and to begin exploring pneumococcal nasopharyngeal recombination. We first examined two *S. pneumoniae* strains to determine whether some serotypes colonize the nasopharynx more effectively, i.e., produce more biofilms, and release more planktonic (i.e., invasive) pneumococci on human nasopharyngeal cells. Genome-sequenced reference strains, serotype 19F strain GA13499 and serotype 2 strain D39, were grown separately or together in bioreactors containing human nasopharyngeal cells for 24 h. GA13499 encodes resistance to trimethoprim whereas D39 carries resistance to tetracycline in a plasmid encoding the *tet*M gene. Therefore, blood agar plates with the appropriate antibiotics were used to compare viable biofilm cells and planktonic bacteria grown separately or together. Our results demonstrated that serotype 19F strain GA13499 produced more biofilms, and released more planktonic bacteria, than strain D39, either when grown alone or together with D39. The addition of the other strain did not significantly affect biofilm counts or counts of

planktonic bacteria of either strain. We recovered recombinant pneumococci encoding resistance to both antibiotics. While we have a reason to believe they are 19F that acquired the *tet*M-encoding plasmid from strain D39, the direction of recombination and the mechanism governing the recombination direction needs to be further investigated. Should these recombinant pneumococci be 19F, the biofilm recombination frequency was 2.35×10^{-4} and the planktonic recombination frequency was 1.02×10^{-3}.

Introduction

Streptococcus pneumoniae is a gram-positive bacterial pathogen responsible for numerous invasive diseases in humans. The pathogen enters the body through the inhalation of airborne droplets and subsequently colonizes the nasopharynx (de Beer, Stoodley et al. 1997, Cox and Link-Gelles 2014) (Musher 1992). This colonization is asymptomatic and is common in children worldwide (Shak, Vidal et al. 2013). Bacterial persistence is mediated by pneumococcal biofilms, which form on the nasopharyngeal epithelium. Recent studies have shown that pneumococcal biofilms can contain multiple serotypes (i.e., strains) of *S. pneumoniae* (Sakai, Telekar et al. 2013). From nasopharyngeal biofilms, planktonic *S. pneumoniae* bacteria can migrate to infect other parts of the body resulting in invasive pneumococcal disease (Randle, Ninis et al. 2011).

Invasive pneumococcal disease (IPD), including pneumococcal pneumonia, pneumococcal meningitis, and bacteremia, is a leading cause of death among children

worldwide. IPD is estimated to cause between 700,000 and 1 million deaths per year among children younger than age 5 (O'Brien, Wolfson et al. 2009). In the United States, both the rate of IPD and the likelihood of fatality due to IPD are highest among children younger than age 2 and adults aged 65 or older (Cox and Link-Gelles 2014). Following the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in 2000, there was a dramatic decline in IPD among children ages 5 and younger. Reduction in the seven serotypes protected by PCV7 (4, 6B, 9V, 14, 18C, 19F, and 23F) accounted for most of this decline. The PCV13 vaccine was introduced in 2010 in the USA, and protects against the seven PCV7 serotypes as well as six additional serotypes (1, 3, 5, 6A, 7F, 19A) (Cox and Link-Gelles 2014). IPD due to these six serotypes has since decreased (Moore, Link-Gelles et al. 2015).

While PCV7 and PCV13 caused a large decline in overall IPD rates, *S. pneumoniae* colonization rates among adults and children worldwide remain high. Up to 90% of healthy children and ~15% of adults are estimated to be carriers of *S. pneumoniae* (Blanchette-Cain, Hinojosa et al. 2013). In addition, serotype replacement, or an increase in the prevalence of non-PCV serotypes, has occurred since vaccine introduction. The carriage of non-vaccine serotypes has risen greatly since the introduction of PCV7, and IPD due to these serotypes has also increased in many populations (Weinberger, Malley et al. 2011). In examining these challenges, it is necessary to consider the complex interactions that occur between multiple pneumococcal strains during co-colonization in the same host. These interactions, which take place in nasopharyngeal biofilms, can lead to the increased growth or inhibition of certain strains as well as homologous recombination (Shak, Vidal et al. 2013).

Biofilms are microbial communities that are encased in an extracellular matrix and adhere to a surface. Though the specific role of biofilm formation in *S. pneumoniae* colonization is not entirely clear, recent studies have suggested that the formation of biofilms provides many benefits to *S. pneumoniae*. Critical to colonization success, the bacteria housed in biofilms tend to be less susceptible to host immune defenses than their planktonic counterparts (Fux, Costerton et al. 2005). Vuong et al. demonstrated with *Staphylococcus epidermis* mutants that polysaccharide intercellular adhesion (PIA), a major component of the staphylococcal biofilm matrix, protects biofilm bacteria from phagocytosis by host immune cells (Vuong, Voyich et al. 2004). Further studies have shown that secreted antibodies bind to heterogeneous biofilm matrices and cannot achieve penetrance (de Beer, Stoodley et al. 1997).

Bacteria in biofilms also tend to be less susceptible to antibiotics compared to planktonic bacteria. This appears to be in part due to non-inherited resistance (Levin and Rozen 2006). Multiple hypotheses attempt to explain non-inherited antibiotic resistance in bacteria, but two stand out as the most well-supported (Anderl, Zahller et al. 2003). First of all, it has been suggested that the bacteria within biofilms are physically less accessible to antibiotics that spread via diffusion, and thus cannot be targeted by these drugs. Studies done with *Klebsiella pneumoniae* biofilms found that the inability of ampicillin to effectively penetrate the biofilm structure of wild type populations led to increased resistance (Anderl, Franklin et al. 2000). Chao et al. similarly demonstrated that *S. pneumoniae* biofilm populations were far more resistant to both penicillin and gentamicin than planktonic populations were due to reduced drug penetrance (Chao, Marks et al. 2014). The second hypothesis suggests that some biofilm bacteria enter a

stationary phase in which they are phenotypically inactivated or slow-growing, and thus lack the processes that many antibiotics target. Further studies done on *K. pneumoniae* demonstrated that some biofilm bacteria enter a stationary phase when nutrients such as oxygen and glucose are lacking in availability. These bacterial biofilms allowed the penetrance of ciprofloxacin but were not killed, suggesting that other mechanisms characteristic of the stationary phase contributed to the resistance (Anderl, Zahller et al. 2003).

Recent studies have also revealed the occurrence of increased genetic exchange within *S. pneumoniae* biofilms. *S. pneumoniae* housed in biofilms upregulate genes involved in competence and horizontal genetic transfer (Chao, Marks et al. 2014). Chao et al. suggested that bacteria in nasopharyngeal colonies are responsible for the majority of horizontal genetic transfer between *S. pneumoniae* strains, with invasive strains less involved in this exchange (Chao, Marks et al. 2014). Thus, antibiotic resistance appears to be transmitted primarily through interactions that happen within nasopharyngeal biofilms. Furthermore, genetic exchange appears to be enhanced by the presence of multiple strains of *S. pneumoniae* in the nasopharynx (Chao, Marks et al. 2014). The multiplicity of these strains potentially provides the genetic diversity needed for horizontal transfer and evolution within the host. This could be a contributing factor to the large portion of pneumococcal strains that have obtained resistance to one or more antibiotics (Weinberger, Malley et al. 2011, Cox and Link-Gelles 2014). Both vertical and horizontal gene transfer, coupled with antibiotic misuse, have been implicated in the increasing numbers of single- and multi-drug resistant *S. pneumoniae* strains (Croucher, Harris et al. 2011).

Because these complex interactions take place within the nasopharyngeal environment, efforts have been made to better understand *S. pneumoniae* biofilm formation *in vivo*. Using bioreactor technology, colonization in the delicate environment of the human nasopharyngeal epithelium can be imitated. The bioreactor contains a surface of human respiratory epithelial cells (i.e., lung, nasal, or pharyngeal cells) and a chamber through which media is constantly flowing. *S. pneumoniae* strains are inoculated into the chamber and form biofilms on the human cell surface. Planktonic cells disperse into the supernatant filling the chamber. The bioreactor's *in vivo* mimicking efficacy has been demonstrated through increased biomass grown on the epithelial cells in a bioreactor compared to an abiotic surface (Vidal, Howery et al. 2013). The bioreactor technology also allows biofilm cells to stay alive for 24 h, whereas biofilm cells grown in microtiter plates undergo autolysis within 24 h. Thus, the mimicking efficacy of this technology allows live bacterial cells to be recovered at the end of 24 h experiments (Vidal, Howery et al. 2013).

Because the presence of multiple strains of *S. pneumoniae* has several implications including the potential exchange of antibiotic resistance determinants and persistence within a host, the population dynamics of co-colonization within *S. pneumoniae* biofilms requires further investigation. Therefore, our research aim was to elucidate the population dynamics between two strains of *S. pneumoniae*. Given the limited time to conduct our research we decided to study only a vaccine type and a non-vaccine type. The two strains that we chose to experiment with are serotype 19F strain GA13499 and serotype 2 strain D39. 19F is a vaccine serotype targeted by both PCV7 and PCV13. Prior to the introduction of PCV7, it was one of the five most prominent

serotypes worldwide, and was identified as a type particularly likely to become resistant to antibiotics (Hausdorff, Feikin et al. 2005). 19F's carriage and IPD prevalence have decreased since the introduction of PCV7, but the carriage of non-vaccine serotypes, such as serotype 2, has increased in turn (Browall, Backhaus et al. 2014). Recent studies have suggested that non-vaccine serotypes have a reduced invasive potential, contributing to lower rates of IPD even when carriage rates are increased (Browall, Backhaus et al. 2014). Pneumococcal strains were inoculated in a bioreactor with cultures of human pharyngeal cells. We studied the effects of the co-colonization of serotype 19F and serotype D39 on the biomass of individual strains; populations of planktonic (i.e., invasive) bacteria leaving the biofilm structure were also studied. Our experimental design also allowed us to monitor recombination resulting in antibiotic resistance. I hypothesized that serotype 19F would produce greater biofilm and planktonic populations than serotype 2 due to similar characteristics observed with other vaccine types. I also hypothesized that the addition of the other strain would allow both strains to grow to greater concentrations, perhaps sharing genetic material that aids both strains in colonization and the generation of planktonic cells.

Experimental design

Strains used and main characteristics. Our first research aim was to determine whether some *S. pneumoniae* serotypes are more successful (e.g., fitness advantage) than others in colonizing human nasopharyngeal cells. To investigate this question, two *S. pneumoniae* strains belonging

to different serotypes were studied. The strains we selected for these experiments were: serotype 19F strain GA13499 and serotype 2 strain D39. Serotype 19F GA13499 is a clinical isolate from the Atlanta, GA metropolitan area. It is a vaccine serotype. The strain is resistant to trimethoprim, encoding the resistance in the chromosome (hereafter called GA13499cTrim); its genome sequence is available. Serotype 2 strain D39 is a reference genome sequenced strain for which a series of transformation/recombination-defective mutants or variants with different antibiotic resistances are available in our laboratory at the Rollins School of Public Health. We chose to experiment with a strain of D39 that has been previously published to express resistance to tetracycline by encoding the *tet*M gene in a plasmid (SPJV01 hereafter called D39pTet). Strain D39, which was used in Griffith's experiments discovering the transformation ability of bacteria, is known to be transformable whereas transformation of GA13499cTrim has not been achieved using the traditional transformation protocol (Griffith 1928).

Preliminary experiments to confirm strain phenotypes. Strains were inoculated onto blood agar plates (BAP) and incubated for 16 hours in a 37°C CO_2 incubator. With these fresh cultures I inoculated a series of BAPs containing increasing concentrations of either tetracycline or trimethoprim with the strains mentioned above. These studies confirmed that GA13499cTrim is trimethoprim-resistant and grows on BAP containing 5 μg/ml trimethoprim whereas the strain was sensitive to 1 μg/ml of tetracycline. As expected, D39pTet was tetracycline-resistant (1 μg/ml) while neither GA13499cTrim nor D39pTet grew on BAP containing 5 μg/ml of trimethoprim and 1 μg/ml of tetracycline.

Preparation of inocula for experiments. Bacteria were inoculated in Todd-Hewitt broth containing 0.5% (weight/volume) yeast extract (THY) and were incubated at 37°C in a CO_2 incubator until the suspensions had optical densities between 0.2 and 0.3. Serial dilution techniques were used to count *S. pneumoniae* cells (cfu/ml) in these suspensions. Aliquots of these bacteria were added with 10% glycerol (v/v) and these inocula were stored at -80°C until used.

Preparation of cell cultures. Human pharyngeal Detroit 562 cells (ATCC CCL-198) were cultured in EMEM (Eagle's minimum essential medium; Lonza, Walkersville, MD) supplemented with non-heat-inactivated 10% fetal bovine serum (FBS) (Atlanta biologicals), 1% nonessential amino acids (Sigma), 1% glutamine (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml), and the pH was buffered with HEPES (10 mM) (Gibco) (Vidal et al. 2013). Faidad Khan, a PhD candidate from Pakistan, assisted in the preparation of cell cultures and running of the bioreactors.

Bioreactor experiments. The interactions between GA13499cTrim and D39pTet were investigated using three bioreactors which were inoculated with similar inoculum (~$2.0x10^7$ cfu/bioreactor) from each strain. Specifically, one bioreactor was inoculated with GA13499cTrim, whereas a second bioreactor was inoculated with D39pTet. The third bioreactor was inoculated with both GA13499cTrim and D39pTet. The inoculated bioreactors were allowed to sit at 37°C for 1 hour under static conditions. After this time, a peristaltic pump provided a constant flow of 0.2 ml/min of cell culture medium containing no antibiotics. Further incubation occurred for 23 h.

After this 24 h period, biofilms from each bioreactor were dissolved in 1 ml PBS. Serial dilution and plating techniques were used to determine final bacterial counts of both biofilm bacteria and planktonic cultures. GA13499cTrim biofilm solution was placed on BAP containing 5 μg/ml trimethoprim to recover 19F cells. This solution was also placed on BAP containing 1 μg/ml tetracycline and double-antibiotic plates with both 5 μg/ml trimethoprim and 1 μg/ml tetracycline to verify no spontaneous mutations conferring GA13499cTrim resistance to tetracycline occurred. Similarly, D39pTet biofilm solution was placed on BAP containing 1 μg/ml tetracycline to recover D39 cells. This solution was also placed on BAP containing 5 μg/ml trimethoprim and double-antibiotic plates with both 5 μg/ml trimethoprim and 1 μg/ml tetracycline. GA13499cTrim/D39pTet biofilm bacterial suspension was inoculated on BAP containing individual antibiotics. The GA13499cTrim/D39pTet biofilm solution was then concentrated ~9-fold and the resulting concentrate was placed on double-antibiotic BAP containing both 5 μg/ml trimethoprim and 1 μg/ml tetracycline. The plates were placed in a CO_2 incubator at 37°C for 16 hours before cfu counts were obtained. This experimental setup allowed us to quantify the biofilm growth of serotype 19F and serotype 2 both separately and together. The concentrates grown on double-antibiotic plates allowed us to check for recombination resulting in double-antibiotic-resistant biofilm *S. pneumoniae*.

Secondly, the proposed research investigated whether co-colonization in nasopharyngeal biofilms influenced the generation of planktonic cells in different *S. pneumoniae* serotypes. After the 24 h incubation period, the supernatant contained in the three bioreactors was collected. The *S. pneumoniae* concentrations of these supernatant solutions were determined using serial

dilution and the same plating as described for biofilms above. Concentrated supernatant solutions were made and plated on double-antibiotic BAPs. The plates were placed in a CO_2 incubator at 37°C for 16 hours before cfu counts are obtained. Thus, we were able to quantify the planktonic cells of serotypes 19F and 2 grown separately and in the same bioreactor, and were able to check for the occurrence of recombination resulting in double-antibiotic resistant planktonic *S. pneumoniae*.

All experiments were repeated 4 times to ensure that the results were reproducible. Concentrations of each strain grown alone were be compared to the concentrations of each strain grown together with the other serotype. The concentrations of the two strains grown together in the bioreactor were compared to determine competitiveness. These comparisons were analyzed using two-tailed t-tests, with significance denoted as $P<0.05$. Comparisons with sample populations in which sufficient normality was not achieved were analyzed using two-tailed Mann-Whitney U tests, with significance at $P<0.05$.

Results

Five replications of the experiment were completed and the three types of antibiotic plates recovered populations of interest. The limit of detection on these plates was 50 cfu/ml. Figures 1, 3, 4, and 5 show mean cfu/ml concentrations recovered. The median concentrations are shown within the bars, so that a sense of variability in the sample populations can be obtained.

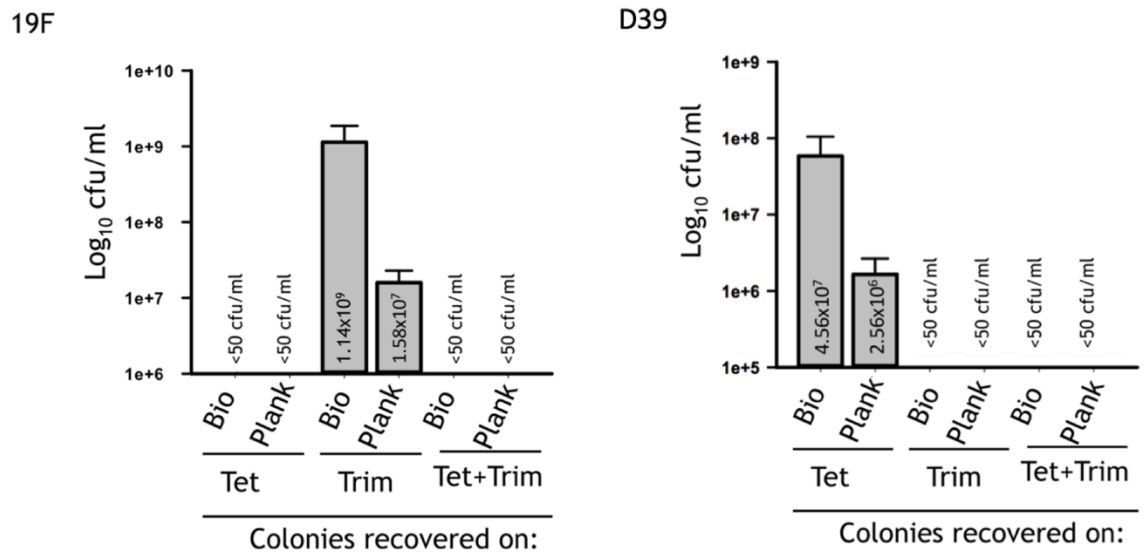

Figure 1. Concentrations (cfu/ml) of *Streptococcus pneumoniae* biofilm (Bio) and planktonic (Plank) populations recovered 1 µg/ml tetracycline, 5 µg/ml trimethoprim, and 1 µg/ml tetracycline + 5 µg/ml trimethoprim plates. Populations were allowed to incubate for 24 h in Bioreactors 1 and 2, which contained the single strains 19F GA13499cTrim (left) and D39pTet (right) grown alone.

Figure 1 shows the concentrations of colonies recovered on tetracycline, trimethoprim, and double-antibiotic plates from Bioreactors 1 and 2, which contained 19F GA13499cTrim alone or D39pTet alone. The trimethoprim plates allowed both the biofilm and planktonic populations of 19F to be recovered and quantified. As expected, the biofilm population was much greater than the planktonic population. The tetracycline and double-antibiotic plates served as negative controls for 19F, and recovered no colonies. This suggests that spontaneous mutation resulting in double-antibiotic resistant *S. pneumoniae* GA13499cTrim did not occur. The tetracycline plates allowed both the biofilm and planktonic populations of D39pTet to be recovered and quantified. The biofilm population of D39pTet was similarly much greater than the planktonic population, but this difference was less pronounced than with 19F GA13499cTrim. The trimethoprim and double-antibiotic plates served as negative controls for D39pTet, and recovered no spontaneous mutants. Figure 2 is an image of the different types of plates included in the experiment.

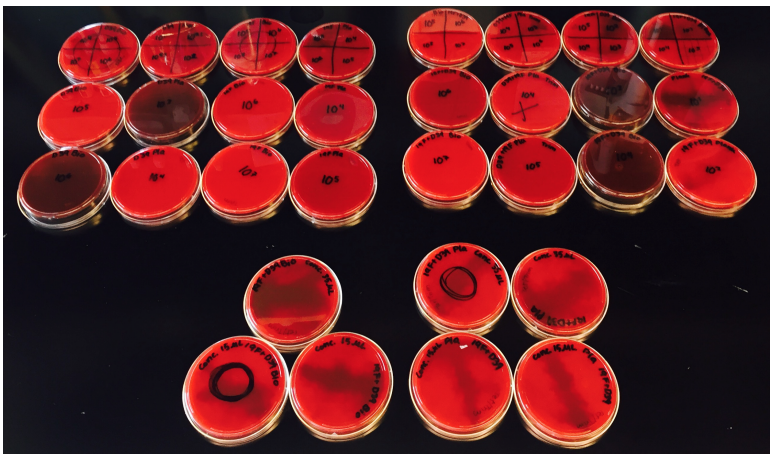

Figure 2. BAP with *S. pneumoniae* colonies resulting from serial dilutions. The top left section of plates contains either tetracycline or trimethoprim along with either 19F GA13499cTrim or D39pTet grown individually in the bioreactor. The top right section of plates contains either tetracycline or trimethoprim along with either 19F GA13499cTrim or D39pTet grown together in the bioreactor. The bottom section of plates contains both antibiotics, and the growth observed on these plates is resistant to both tetracycline and trimethoprim. These *S. pneumoniae* cells have undergone recombination.

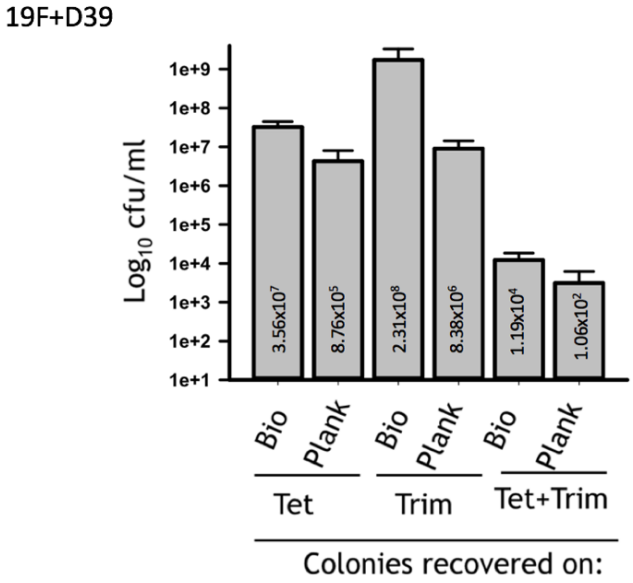

Figure 3. Bacterial counts (cfu/ml) of *Streptococcus pneumoniae* biofilm and planktonic populations recovered on 1 µg/ml tetracycline, 5 µg/ml trimethoprim, and 1 µg/ml tetracycline + 5 µg/ml trimethoprim plates. Populations were allowed to incubate for 24 h in Bioreactor 3, which contained strains 19F GA13499CTrim and D39pTet inoculated and grown together.

Figure 3 shows concentrations of colonies recovered on tetracycline, trimethoprim, and double-antibiotic plates from Bioreactor 3, which contained both 19F GA13499cTrim and D39pTet. We isolated D39 biofilm and planktonic populations on tetracycline plates, while we

recovered 19F biofilm and planktonic populations on trimethoprim plates. On BAP with both antibiotics, trimethoprim and tetracycline, we were able to isolate recombinant bacteria from both populations, biofilms and planktonic bacteria. We hypothesize that recombination took place in biofilms and that the presence of recombinant bacteria in planktonic cultures was due to the active release of recombinant bacteria from biofilms. Given parents of recombinant bacteria have the antibiotic resistant determinant encoded in the chromosome and in a plasmid, GA13499cTrim and D39pTet respectively, we hypothesize that the direction of recombination allowed GA13499cTrim take up the plasmid from D39pTet and become resistant to tetracycline. Should these recombinant pneumococci be 19F, the biofilm recombination frequency was $2.35x10^{-4}$ and the planktonic recombination frequency was $1.02x10^{-3}$. These recombination frequencies were calculated first by dividing the number of recombinant pneumococci obtained in an experiment by the number of 19F bacteria (biofilm or planktonic) recovered from the bioreactor containing both strains. These values were then averaged among the experiments for both biofilm and planktonic populations.

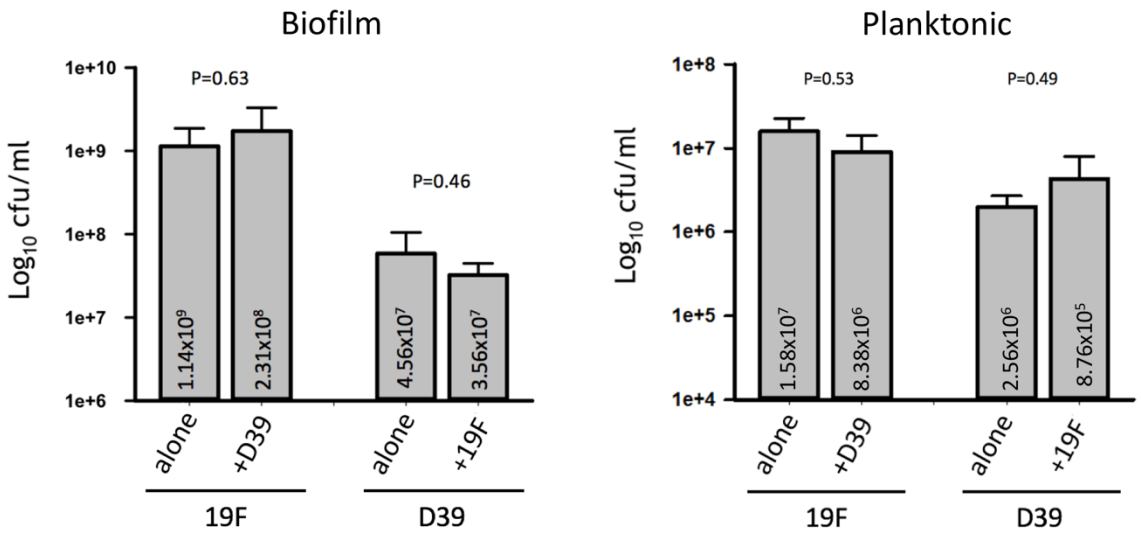

Figure 4. Comparison of bacterial counts (cfu/ml) of *Streptococcus pneumoniae* biofilm and planktonic populations grown alone and coupled with the other strain after 24 h in bioreactor technology.

A comparison of both biofilm populations and planktonic populations grown alone compared to those grown with the other strain can be seen in Figure 4. 19F GA13499cTrim biofilm populations showed no significant difference in concentration when grown alone or with D39pTet (P=0.63). D39pTet biofilm populations similarly underwent no significant changes in concentration with the addition of 19F GA13499cTrim (P=0.46). These results were also seen in planktonic populations. There was no significant difference between the concentration of the 19F GA13499cTrim planktonic population grown alone and the concentration of the 19F GA13499cTrim planktonic population grown with D39pTet (P=0.53). Lastly, the addition of 19F GA13499cTrim did not significantly alter D39pTet planktonic population concentration (P=0.49).

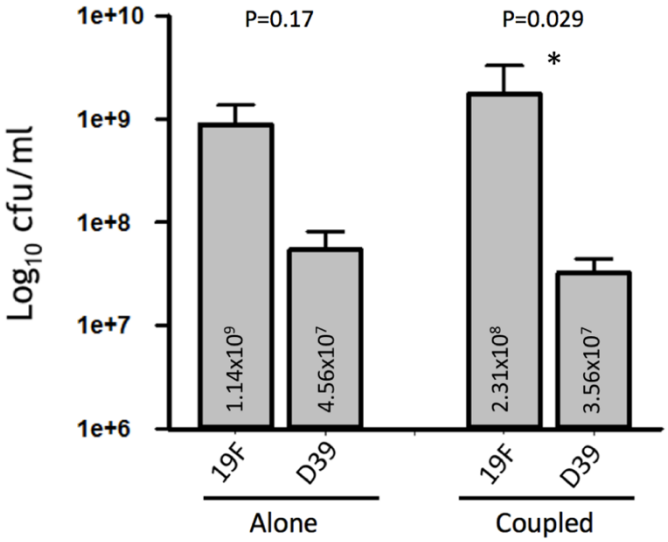

Figure 5. Comparison of bacterial counts (cfu/ml) of *Streptococcus pneumoniae* 19F GA13499cTrim and D39pTet biofilm populations grown alone or coupled with the other strain after 24 h in the bioreactor.

One can see in Figure 5 that 19F GA13499cTrim appears to be a superior colonizer on nasopharyngeal cells when compared to D39pTet. Although a tendency of GA13499cTrim to form more biofilms than D39pTet is observed, our results do not show a significant difference between the population concentrations of 19F and D39 grown alone (P=0.17). This was perhaps due to considerable variations within some experiments. However, a statistically significant difference was observed between biofilm counts of 19F and D39 when they were grown together (P=0.029). This indicates that 19F GA13499cTrim is able to colonize better in the presence of D39pTet than alone, and possesses better colonizing abilities than D39pTet when coupled with this strain.

Discussion

Four major conclusions can be discerned from the results of our experiments. Both strains produced far greater concentrations of biofilm cells compared to planktonic cells. The addition of the other strain did not significantly change the biofilm or planktonic populations of either strain, indicating strains can cohabit the same niche, at least for 24 h. Strain 19F GA13499cTrim appears to be a better colonizer than D39pTet, especially when the two strains are infecting the same microenvironment. These *in vitro* observations correlate with epidemiological studies showing strains belonging to serotype 19F (vaccine serotype) are more prevalent in the nasopharynx and disease cases than strain of serotype 2 (non-vaccine). Lastly, recombination

occurred between the two strains resulting in populations that are resistant to both trimethoprim and tetracycline.

The finding of greater concentrations of biofilm populations compared to planktonic populations in all three bioreactors is consistent with current knowledge about *S. pneumoniae* virulence. Bacteria persist in biofilms under optimal conditions, typically leaving only when they are threatened or outside signals trigger a transition to the planktonic phenotype (Chao, Marks et al. 2014). Because the bioreactors are able to mimic optimal conditions similar to those in the nasopharynx, cells are able to persist in the biofilms in greater numbers. We were surprised by our finding that the addition of the other strain did not significantly alter biofilm or planktonic populations for either 19F GA13499cTrim or D39pTet. This contrasted with my hypothesis that both biofilm and planktonic populations would be enhanced from shared genetic material increasing the fitness of both strains. Perhaps these strains establish population sizes that are based on strain-specific characteristics, and are thus not affected by the presence of the other strain. Our results suggest that there is little strain competition in this pair. Our experiments found that 19F GA13499cTrim produces significantly greater biofilms when coupled with D39pTet, but not when it is grown alone. This suggests that the addition of D39 helps 19F to become a better colonizer. This could perhaps be explained by the transfer of genetic material from D39pTet to 19F GA13499cTrim, increasing the fitness of the latter strain. Our findings underscore the importance of the PCV7 and PCV13 vaccines, both of which protect against serotype 19F. If the uptake of the vaccine is increased in risk populations, 19F will continue to decrease in prevalence worldwide.

Our experiments recovered biofilm and planktonic populations that were able to growth on plates containing both trimethoprim and tetracycline. This means that genetic transfer occurred resulting in double-antibiotic resistant populations. Though the identities of this particular population (i.e., recombinant) were not determined, the following possibilities exist. Recombinant bacteria could be entirely D39pTet, entirely 19F, a mixture of some proportion of the two strains, or a new serotype altogether. Based on previous experiments in the laboratory, we have reasons to believe that recombinant bacteria are mostly, if not entirely, serotype 19F. These recombinant bacteria likely received the plasmid encoding tetracycline resistance from D39pTet, and thus are resistant to both trimethoprim and tetracycline. This characterization of the recombinant populations is supported by knowledge of serotype 19F's increased ability to resist antibiotics (Hausdorff, Feikin et al. 2005). It is also supported by studies showing that strains with greater colonization success tend to have increased competence and by our observations than 19F GA13499cTrim produces more biofilms (increasing the chance of acquiring DNA from D39pTet if this DNA is available within the biofilm population) (Chao, Marks et al. 2014).

If the recombinant pneumococci are indeed 19F, the recombination frequencies that we observed were 2.35×10^{-4} for the biofilm population and 1.02×10^{-3} for the planktonic population. The biofilm recombination frequency that we obtained is relatively similar to the frequency obtained recently by Marks et al. They found a recombination frequency between of D39 *S. pneumoniae* in nasopharyngeal biofilms to be around 4.4×10^{-4} (Marks, Reddinger et al. 2012). This same study reported a planktonic recombination frequency significantly lower than ours:

10^{-9}. However, this is most likely due to differences in experimental design, as this study did not use bioreactor technology to obtain planktonic counts and instead relied upon previous research on recombination rates during sepsis (infection) (Marks, Reddinger et al. 2012).

While our experiments showed that the coupling of serotype 19F GA13499cTrim and serotype 2 D39pTet did not significantly alter population dynamics in most respects, they did show that coupling can cause the colonization success of one strain to increase. Furthermore, they demonstrated that *S. pneumoniae* in biofilms transfer genetic material resulting in recombinant biofilm and planktonic cells. These genetic transfers created a population with increased fitness that was resistant to both trimethoprim and tetracycline. Studies such as these can be used to examine the population dynamics between different strains of *S. pneumoniae* in order to better understand the effects co-carriage *in vivo*. Similar research can be done to identify strains that colonize particularly well, such as 19F in our case. Experiments such as these have important implications for the development of new pneumococcal conjugate vaccines, helping researchers to target the serotypes that have the greatest colonizing abilities, those that generate the most planktonic cells, and those that most frequently undergo recombination to become antibiotic-resistant.

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