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Rebekah Kunkel Date

The role of the LuxS quorum-sensing system in *Streptococcus pneumoniae* biofilms

By

Rebekah Kunkel
Master of Science

Graduate Division of Biological and Biomedical Science
Population Biology, Ecology, and Evolution

_____

Keith Klugman
Advisor

_____

Jorge Vidal
Advisor

_____

Karen Conneely
Committee Member

_____

William Shafer
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

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Rebekah Kunkel
B.S., University of Illinois, 2005
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Advisors: Keith Klugman, MD, PhD
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A thesis submitted to the Faculty of the
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Abstract

The role of the LuxS quorum-sensing system in *Streptococcus pneumoniae* biofilms
By Rebekah Kunkel

The ability of *Streptococcus pneumoniae* to colonize the nasopharynx is an important risk factor for pneumococcal disease and carriage. Biofilm production has been associated with the persistence of other respiratory pathogens and *S. pneumoniae* is able to form biofilm structures within the nasopharynx. Previous studies have demonstrated that the LuxS quorum-sensing system plays a role in the ability of *S. pneumoniae* to persist within the nasopharynx of experimentally infected mice. This quorum-sensing system has also been shown to play a role in biofilm production among certain species of streptococci.

This thesis project investigated the role of the *lux*S gene in biofilm formation in *S. pneumoniae* strain D39. Insertional inactivation was used to construct an isogenic mutant of *lux*S. The *lux*S mutant genotype was confirmed using PCR and sequencing. Complemented strain was prepared by cloning the *lux*S wild type gene in a Gram positive replicative plasmid. Quantitative differences in biofilm production by wild type, mutant, and complemented strains were assessed. The D39 *lux*S mutant produced ~80% less biofilm biomass than the wild type. Complementation restored biofilm biomass to wild type levels. A series of experiments demonstrated that, once produced, biofilm growth is in part due to expansion and reproduction of the sessile cells, rather than recruitment of planktonic cells. These new insights into the genetics of biofilm growth may allow researchers to better control pneumococcal infections and prevent disease.

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Table of Contents

List of Tables and Figures

Chapter 1: *Streptococcus pneumoniae* and biofilms

Streptococcus pneumoniae and disease

Streptococcus pneumoniae can cause severe infections, such as otitis media, septicemia, meningitis, and pneumonia (Musher 1992). The role of *S. pneumoniae* in pneumonia is of particular concern, since pneumonia is one of the leading causes of under-five mortality, responsible for approximately 20% of deaths, and the majority of these deaths can be attributed to *S. pneumoniae* infection (World Health Organization, 2009). One of the Millennium Development Goals is to reduce under-five mortality by two-thirds between 1990 and 2015; control of pneumonia, and thus control of *S. pneumoniae*, is an important aspect of obtaining this goal (United Nations Children's Fund/World Health Organization, 2006).

S. pneumoniae can be found widely in all human populations, being of particular concern for individuals with weakened immune systems, such as the elderly and others suffering from immunodeficiencies. For example, invasive pneumococcal disease is a common early stage infection among HIV-infected patients (Hart, Beeching et al. 2000; Karstaedt, Khoosal et al. 2001). With the spread of HIV and increases in the number of immunosuppressed individuals, understanding the pathogenesis of *S. pneumoniae* is becoming increasingly important to develop appropriate prevention, control, and treatment strategies.

S. pneumoniae is transmitted from person-to-person through direct contact with respiratory secretions. Oftentimes, exposure to the pathogen only results in transient

colonization of the nasopharynx, but the pneumococcus is capable of causing disease in a variety of bodily locations, including the sinuses, ear canals, lungs and meninges. *S. pneumoniae* infections can be severe, resulting in conditions such as arthritis, endocarditis, meningitis, abscesses, sinusitis, and fasciitis. The diversity of these infection locations indicates that *S. pneumoniae* possesses the ability to express a wide variety of phenotypic characteristics that allow it to invade and thrive in many different environments (Oggioni, Trappetti et al. 2006).

S. pneumoniae strains are highly diverse; there are over 90 different serotypes and genomic differences between strains can be as high as 10% (Hoskins, Alborn et al. 2001; Tettelin, Nelson et al. 2001; Shen, Gladitz et al. 2006). Recombination events occur frequently, often affecting major antigens (Croucher, Harris et al. 2011). Phenotypic differences include variation in capsule production and types of virulence factors. This variety means researchers must study many strains in order to obtain a complete understanding of *S. pneumoniae* behavior.

Carriage and persistence

The microflora within the nasopharynx are constantly changing over the course of a person's lifespan, with frequent acquisition and elimination of bacterial species (Faden, Harabuchi et al. 1994; Harabuchi, Faden et al. 1994). *S. pneumoniae* is one of the most frequent colonizers and most children are colonized at least once within the first few months of life (Hendley, Sande et al. 1975; Gray, Turner et al. 1982; Koornhof, Wasas et

al. 1992; Musher 1992; Reichler, Allphin et al. 1992). Colonization with *S. pneumoniae* can lead to disease or can be asymptomatic; when colonization is asymptomatic, it is known as carriage or persistence. Length of carriage varies and pneumococcal strains may be carried for only a few days or up to several months (Ortqvist, Hedlund et al. 2005). Carriage plays an important role in the bacterium's survival. During carriage, *S. pneumoniae* is provided with a stable environment where it is able to avoid destruction by the host's defense mechanisms; the nasopharynx also provides a location for the bacterium to be readily transmitted to new hosts (Hava, LeMieux et al. 2003; Hall-Stoodley and Stoodley 2009; Moscoso, Garcia et al. 2009).

Complete elimination of colonizing *S. pneumoniae* strains from the nasopharynx is difficult, as *S. pneumoniae* often remains in the nasopharynx following antibiotic treatment. Consequently, nasopharyngeal carriage of *S. pneumoniae* is an important risk factor for recurrent otitis media infections (Leibovitz, Greenberg et al. 2003; Libson, Dagan et al. 2005). Despite these important clinical ramifications of *S. pneumoniae* colonization, our understanding of the molecular mechanisms involved in colonization and persistence remains limited.

Molecular studies have identified several virulence factors that may play a role in *S. pneumoniae* colonization. Some of these factors protect the pneumococcus from the immune defense mechanisms. For example, PspA, the pneumococcal surface protein, protects *S. pneumoniae* from the bactericidal protease activity of apolactoferrin and the capsule inhibits opsonization (Shaper, Hollingshead et al. 2004; Hyams, Camberlein et al. 2010). The PsrP protein and the RlrA pilus are adhesion proteins which help the bacterium attach to epithelial cells (Barocchi, Ries et al. 2006; Sanchez, Shivshankar et

al. 2010). Finally, the neuraminidases, NanA and NanB, have been shown to be vital for both colonization and persistence within the nasopharynx, as well as for survival at non-mucosal sites (Manco, Hernon et al. 2006).

Studies have found evidence that the *lux*S quorum-sensing system plays a role in the virulence and persistence of *S. pneumoniae*. In mice, a *lux*S mutant was found to be less virulent than the wild type strain (Stroeher, Paton et al. 2003). Additionally, following challenge with a mixture of wild type and mutant bacteria, only wild type bacteria were found colonizing the blood and lungs of mice (Stroeher, Paton et al. 2003). A later study found that a *lux*S mutant had a reduced ability to persist within the nasopharynx, compared to the wild type bacteria (Joyce, Kawale et al. 2004).

Biofilms

Effective colonization of a host requires that the bacterium have mechanisms to remain within its ideal environment, out - compete other microorganisms in that niche, and evade the host immune system. Several pathogens, including respiratory pathogens, have been shown to use biofilms as a method of persistence (Hall-Stoodley, Hu et al. 2006; Sloan, Love et al. 2007; Armbruster, Hong et al. 2009; Sekhar, Kumar et al. 2009). Biofilms are complex communities of microorganisms growing on a solid substrate within an extracellular polymeric matrix. The extracellular polymeric matrix is composed of extracellular DNA (eDNA), polysaccharides, and other biopolymers excreted by the microorganisms; this matrix helps to stabilize the microbial aggregates on the solid

surface, as well as to each other. The matrix provide a stable environment, protecting the community from a variety of threats, including physical forces, changes in pH, desiccation, heat shock, and chemical agents, such as such as detergents and antibiotics (Hall-Stoodley and Stoodley 2009; Moscoso, Garcia et al. 2009). Biofilms can be composed of a single bacterial species or multiple bacterial species, and may even contain multiple types of microbes, such as algae, protozoa, and fungi (Davey and O'Toole G 2000).

Three distinct stages have been identified in *S. pneumoniae* biofilm development (Allegrucci, Hu et al. 2006). During the first stage, individual planktonic cells begin to adhere to the surface substrate. This is followed by bacterial aggregation and the formation of microcolonies. Finally, the biofilm structure grows and matures. During this final stage, the biofilm reaches its maximum thickness and cellular metabolism slows. The bacterial cells in each stage of development are physiologically distinct from cells in the other stages of development, but within mature biofilms, cells exhibiting characteristics of all stages of development can be found.

All biofilms, even those composed of a single species, are highly diverse, with structural heterogeneity and variations in gene expression and protein synthesis. Gradients in nutrient availability and signaling factors play a role in producing this heterogeneity (Mah and O'Toole 2001). Surface cells remain metabolically active; they continue to divide and increase biofilm thickness. In contrast, cells located in the deeper layers of the biofilm may be in a dormant state and will not become activated unless the outer layers of the biofilm are destroyed or the biofilm disperses (Wolcott, Rhoads et al. 2010).

In the clinical realm, biofilm growth in the human body is generally detrimental. Over 60% of human bacterial infections are can be attributed to biofilm growth, including native valve endocarditis, burn wound infections, chronic otitis media, and infections in patients affected by cystic fibrosis (Sauer 2003; Hall-Stoodley, Costerton et al. 2004; Moscoso, Garcia et al. 2009). Biofilms are of particular concern in for hospital infection control, as bacteria will often adhere to implanted medical devices, such as catheters and prosthetics, causing serious internal infections (Donlan 2001). These infections, oftentimes occurring in immunocompromised individuals, can result severe outcomes, including additional surgeries, amputation to remove diseased tissue, and even death.

S. pneumoniae strains are known to form biofilms *in vivo* (Moscoso, Garcia et al. 2009). In a recent study, Sanchez *et al* (2010) demonstrated that *S. pneumoniae* TIGR4 was able to form biofilms in the nasopharynx of experimentally infected mice (Sanchez, Shivshankar et al. 2010). Studies in chinchillas found that pneumococcal biofilm formation was enhanced in the presence of *H. influenzae* (Weimer, Armbruster et al. 2010). More importantly, biopsies collected from children with recurrent otitis media demonstrated the presence of *S. pneumoniae* biofilms on adenoid and mucosal cells in 92% of specimens (Hall-Stoodley, Hu et al. 2006).

Several molecular factors have a demonstrated role in *S. pneumoniae* biofilm formation. For example, mutation in genes encoding the LytA, LytB, or LytC amidases resulted in a decreased ability of *S. pneumoniae* to form biofilms (Moscoso, Garcia et al. 2006). A mutation in the *psp*A gene, which encodes the surface protein PspA, also diminished biofilm production (Moscoso, Garcia et al. 2006). PsrP, one of the adhesion proteins associated with virulence, has been found to be associated with biofilm

production in animal models (Sanchez, Shivshankar et al. 2010). Several other adhesin proteins, including CbpA and PcpA, have also been shown to play a role in proper biofilm formation and maturation (Moscoso, Garcia et al. 2006).

Quorum-sensing

Behavioral coordination of multiple organisms for activities such as biofilm formation requires environmental signaling and detection. One of these mechanisms is known as quorum-sensing (Costerton, Stewart et al. 1999). Bacteria utilize quorum-sensing to determine surrounding bacterial densities through the release of hormone-like signaling molecules [known as autoinducers (AI)]; these molecules allow bacterial populations to communicate their presence and coordinate group behavior (Petersen, Ahmed et al. 2006). When the AI reach a critical threshold level, bacterial genes are activated or deactivated, regulating physiologic processes and resulting in coordinated behavior among the organisms (Moscoso, Garcia et al. 2009).

One of the major classes of autoinducers is the N-acyl-L-homoserine lactones (AHLs), which are produced by Gram-negative bacteria (Vendeville, Winzer et al. 2005). Gram-positive bacteria use post-translationally modified peptides known as autoinducer peptides (AIPs) (Sturme, Kleerebezem et al. 2002). One important autoinducer is AI-2, which is used by both Gram negative and Gram positive bacteria (Vendeville, Winzer et al. 2005). Quorum-sensing systems improve bacterial access to nutrients and environmental niches and also enhance production of virulence factors and other defense

capabilities against the host and other microorganisms (Miller and Bassler 2001; Sauer 2003).

Several studies have linked quorum-sensing systems with biofilm formation. In *P. aeruginosa*, mutants of the *las*R/*las*I quorum-sensing system produced early-stage biofilms that never fully matured; supplementation of the media with the *las*I gene product was sufficient to restore wild type biofilm production (Davies, Parsek et al. 1998; Surette, Miller et al. 1999). Inactivation of the *cep* quorum-sensing system in *Burkholderia cepacia* caused defects in biofilm production (Huber, Riedel et al. 2001). Despite these initial findings, the role of quorum-sensing in biofilm production remains unclear. For example, in *Staphylococcus aureus*, intraspecific quorum-sensing was found to serve as a negative regulator of biofilm formation and several microarray studies in *E. coli* and *P. aeruginosa* failed to find evidence for an increase in the expression of quorum-sensing genes during biofilm growth (Vuong, Saenz et al. 2000; Whiteley, Bangera et al. 2001; Schembri, Kjaergaard et al. 2003).

LuxS

The *lux*S gene plays an important role in quorum-sensing for many bacterial species. LuxS, the enzyme encoded by *lux*S, is involved in the catabolism of the AI-2 (Surette, Miller et al. 1999). AI-2 can be detected from the environment by sensory proteins, leading to activation of signaling cascades that alter gene production (Leibovitz, Greenberg et al. 2003). The *lux*S quorum-sensing system was first identified in *Vibrio*

harveyi, but it has since been found in other bacterial species. Initial studies in *V. harveyi* found that the production of AI-2, regulated by *lux*S, controlled bioluminescence in *V. harveyi* (Musher 1992; Harabuchi, Faden et al. 1994; Surette, Miller et al. 1999; Chen, Schauder et al. 2002).

Bioinformatic analyses have found *lux*S homologues in many bacterial species, including both Gram-negative and Gram-positive bacteria (Federle and Bassler 2003; Vendeville, Winzer et al. 2005). A *lux*S gene, capable of producing AI-2, has been identified in *S. pneumoniae* strains (Stroeher, Paton et al. 2003; Joyce, Kawale et al. 2004). The universality of these homologues suggests that AI-2 may serve as method for cross-species communication, a theory that is supported by the fact that the AI-2 signal does not appear to be species-specific (Surette, Miller et al. 1999; Miller and Bassler 2001; Winans and Bassler 2002; Xavier and Bassler 2003; Vendeville, Winzer et al. 2005). AI-2 molecules from other bacterial species are capable of inducing bioluminescence in *V. harveyi* and culture media from other *lux*S-encoding species can complement a *V. harveyi lux*S mutant (Surette and Bassler 1998; Petersen, Ahmed et al. 2006).

The role of the *lux*S-controlled quorum-sensing system on biofilm formation is highly variable among species and remains unclear. The *lux*S gene in *S. gordonii* was essential to form biofilms with strains of *P. gingivalis* lacking *lux*S (Sanchez, Shivshankar et al. 2010). Studies in *S. mutans* and *S. anginosus* found that a mutation in the *lux*S gene resulted in defective biofilm production (Wen and Burne 2002; Petersen, Pecharki et al. 2004; Wen and Burne 2004; Yoshida, Ansai et al. 2005; Petersen, Ahmed et al. 2006). In a chinchilla model, *lux*S was necessary for biofilm formation and

persistence in *H. influenzae* (Armbruster, Hong et al. 2009). A *Klebsiella pneumoniae* *lux*S mutant had reduced capacity to form microcolonies during the early stages of biofilm development (Balestrino, Haagensen et al. 2005). Viable cell counts decreased in biofilms formed by a *lux*S mutant of *S. intermedius* compared to the wild type (Ahmed, Petersen et al. 2009). In contrast to these findings, wild type strains of *Helicobacter pylori* and *Staphylococcus epidermidis* are unable to produce biofilms and a *lux*S mutation results in biofilm formation, suggesting that *lux*S may repress biofilm formation in these species (Cole, Harwood et al. 2004; Xu, Li et al. 2006). The variation in these findings demonstrates more studies are needed to fully understand the role of *lux*S on biofilm formation.

Remaining scientific questions

Despite the clinical importance of biofilms, research on this stage of bacterial life remains limited and many unanswered questions still remain. This thesis project focused on two important areas of *S. pneumoniae* biofilm research: 1) based on the role that *lux*S has been shown to play in nasopharyngeal persistence and in biofilm production in other bacterial species, we sought to understand the role of the *lux*S gene on biofilm formation in *S. pneumoniae* strain D39; and 2) we were interested in further understanding early biofilm growth and development, specifically, investigating the role of biofilm cells in the growth and formation of early biofilms. The next two chapters focus on these experiments and our findings.

Chapter 2: Construction and characterization of a *lux*S isogenic mutant

Introduction

Despite the well-known importance of nasopharyngeal carriage on the pathogenesis and transmission of *S. pneumoniae*, little research has focused on identifying genetic factors responsible for the continued persistence of this bacterium in the nasopharynx. Biofilm structures have been shown to play a role in the persistence of other pathogens, suggesting they may also be important for the persistence of *S. pneumoniae* within the nasopharynx (Sloan, Love et al. 2007; Armbruster, Hong et al. 2009; Sekhar, Kumar et al. 2009). The *lux*S gene has also been shown to play a role in the virulence and persistence of *S. pneumoniae* (Stroeher, Paton et al. 2003; Joyce, Kawale et al. 2004). In addition, research studies in other *Streptococci* species have identified *lux*S as an important gene in biofilm development (Merritt, Qi et al. 2003; Wen and Burne 2004; Yoshida, Ansai et al. 2005; Petersen, Ahmed et al. 2006).

Given these findings, we sought to investigate the role of *lux*S in *S. pneumoniae* biofilm production. During this study, we constructed a *lux*S isogenic mutant by inactivating the *lux*S gene in *S. pneumoniae* strain D39, complemented this mutation in trans, and tested its ability to biofilms. We then compared biofilm production in the wild type, mutant, and complemented strains to understand the role of *lux*S in *S. pneumoniae* biofilm formation.

Methods

Bacterial strain and growth conditions

S. pneumoniae strains were grown in tryptic soy agar (TSA) supplemented with 5% sheep's blood (BAP). Strains were grown at 37°C, 5% CO_2. Bacterial stocks were stored at -80°C in 2% milk. For mutant selection, antibiotics were used at the following concentrations: erythromycin (0.2 ug/ml) and tetracycline (0.1 ug/ml). *Escherichia coli* cells were grown in Luria-Bertani medium containing 0.5ug/ml of erythromycin with aeration at 37°C.

Preparation of the luxS-ery-luxS mutator cassette

To inactivate the *lux*S gene in strain D39, a DNA cassette containing the *erm*B gene (which confers resistance to erythromycin) flanked by 5' and 3' regions of *lux*S was prepared. Briefly, the *erm*B gene was PCR amplified using primers 57L and 58R (Table 1). and cloned into pCR2.1 TOPO. Then, 5' (~210 bp) or 3' (~160bp) sequences of luxS were PCR amplified using primers LuxS5-L and Lux5-R or Lux3-L and LuxS3-R, respectively. Those PCR fragments were purified using Qiaquick gel extraction (Qiagen) and digested with *Kpn*I and *Bam*HI (5' *lux*S fragment) or *Xho*I and *Xba*I (3' *lux*S fragment). Digested fragments were again purified and ligated, using T4 DNA ligase (Promega), upstream or downstream respectively of pCR2.1 TOPO encoding the *erm*B to create plasmid pLuxS-ery-LuxS. The whole cassette *lux*S-ery-*lux*S (~1.1 kb) was PCR amplified and then purified. PCR conditions were as follows: 95°C for 5 min, 30 cycles

of 95°C for 15 sec, 60°C for 30 sec, 68°C for 2 min, and 68°C for 10 min. Purity of the *lux*S-ery-*lux*S cassette was checked by 1% gel electrophoresis.

Preparation of S. pneumoniae D39 competent cells

S. pneumoniae D39 cells were grown overnight on TSA plates supplemented with 5% sheep's blood (BAP) at 37°C, 5% CO_2. 5 ml of complete transformation media (CTM) [10 ml THY + 500 ul of 0.1M $CaCl_2$ + 100 mg BSA, filter sterilized (Havarstein, Coomaraswamy et al. 1995)] was inoculated with the wild type strain and grown at 37°C, 5% CO_2 until the culture reached an optical density of OD600=0.6. A 1:200 dilution of the culture was made in fresh CTM media and incubated at 37°C, 5% CO_2 until the culture reached an optical density of OD_{630}=0.05. Cells were frozen immediately in liquid N_2 in 300 ul aliquots containing 10% glycerol and stored at -80°C until use.

Transformation of competent cells

Purified *lux*S-ery-*lux*S cassette DNA (approximately 1 ug) was added to 20 ul of competent cells, along with 10 ul of competence-stimulating peptide (CSP) to a final concentration of 5 ng/ul, and 180 ul of CTM media. The transformation mixture was incubated in a 37°C water bath for 2 hours, then plated onto BAP containing 0.2 ug/ml of erythromycin and incubated at 37°C and 5% CO_2 for 48 hours. Isolated colonies were streaked on TSA plates containing erythromycin and incubated at 37°C, 5% CO_2 overnight. Potential mutants were screened and confirmed by PCR using primers 57L & 58R (Table 1). PCR conditions were as follows: 95°C for 5 min, followed by the 30

cycles at 95°C for 15 sec., 55°C for 30 sec., 68°C for 1 min, and finally 68°C for 10 min. Fragments were visualized by running a 1% agarose gel.

Sequencing

To further confirm the mutation in the obtained erythromycin resistant strains, genomic DNA was purified using the QIAamp DNA minikit (QIAGEN) following the manufacturer's instructions. DNA was amplified by PCR using primers 15L & 6R. PCR conditions were as follows: 95°C for 5 min, followed by the 30 cycles at 95°C for 15 sec., 55°C for 30 sec., 68°C for 1 min, and finally 68°C for 10 min. The PCR product was purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions, eluted in water and quantified using a spectrophotometer. The sequence was cloned into the pCR2.1-TOPO cloning vector (Invitrogen) and sequenced using the M13 TOPO reverse primer by Beckman Coulter Genomics.

Biofilm timecourse assay

An overnight BAP culture was used to prepare a cell suspension in THY broth (OD600=0.05) and grown at 37°C, 5% CO_2 for two hours until the culture reached an OD600=0.2. An aliquot ($7x10^5$ CFU/ml) of the bacterial culture was added to THY in 96-well flat-bottom polystyrene plates in triplicate. Plates were incubated for 24 hrs at 37°C, 5% CO_2. Biofilms were washed two times with PBS, then fixed with 2% paraformaldehyde for 15 minutes. After paraformaldehyde removal, biofilms were washed twice with PBS, then incubated in 0.5% Triton X-100 for 5 minutes. Biofilms were washed 2 times with PBS, then blocked with 2% bovine serum albumin (BSA).

Biofilms were stained for 1 hour with an anti-*S. pneumoniae* antibody (~40 ug/ml) coupled to fluorescein isothiocyanate [(FITC) ViroStat, Portland, ME)]. Biofilm biomass was quantified by measuring FITC-fluorescence readings using a VICTOR X3 Multilabel Plate Reader (Perkin-Elmer).

Results

Preliminary studies found that *S. pneumoniae* D39 did not encode the *erm*B gene or other genes known to confer resistance to erythromycin (data not shown). As mentioned, the *lux*S-ery-*lux*S cassette was transformed into *S. pneumoniae* strain D39 and the resulting erythromycin resistant strains were screened by PCR. Results from the colony PCR in Figure 2 show our mutant screening. Two sets of primers were used; one set of primers (15L & 6R) amplified the upstream region along with the *lux*S fragment, while the second set of primers (15L & 39L) served as a control reaction to ensure proper orientation of our gene cassette. Two potential mutant strains were identified and the mutant from colony #3, which we named SPJV05, was chosen for further testing.

SPJV05 was complemented using a pReg696 plasmid encoding the wild type *lux*S gene to create SPJV06. The wild type, mutant, and complemented strain were further tested with PCR using both the *lux*S-specific primers (5L & 6R) as well as the primers specific for the upstream region (15L & 6R). The PCR results in Figure 3 show that in both PCR reactions, the amplified fragment from the mutant strain was approximately 800 bp larger than the wild type strain, demonstrating that an insertional event had

occurred. PCR results from the complemented strain shows that the complemented strain contained both the wild type *lux*S (Figure 3B) and mutated *lux*S genes (Figure 3C), which is expected since pReg696 is a replicative plasmid.

To further confirm the mutation, DNA extracted from SPJV05 was used as a template and the PCR product was cloned into the pCR2.1-TOPO vector immediately downstream of the M13 reverse primer. After sequencing, bioinformatic analysis confirmed that the inserted fragment was the erythromycin resistance gene (Figure 4). Together, the PCR and sequencing results confirmed that we prepared a *lux*S isogenic mutant of *S. pneumoniae* strain D39.

To assess the role of the *lux*S gene in *S. pneumoniae* biofilms, we performed a 24-hour biofilm formation assay experiment (Figure 5), using the SPJV05 mutant created in our laboratory along with EJ3, a *lux*S deletion mutant kindly provided by Dr. Elizabeth Joyce from the Department of Microbiology and Immunology, Stanford University School of Medicine. EJ3 was used to create two additional strains—SPJV04, which was complemented with the pReg696-*lux*S plasmid, and SPJV02, which contained an empty pReg696 vector. Biofilm formation by the wild type strain was set as 100% biofilms. Both mutant strains produced about 20% of the wild type biofilm biomass, a difference that was statistically significant. Both complemented strains restored biofilm biomass back up to the levels seen in the wild type strain. Insertion of the empty vector into the mutant strain did not result in an increase in biofilm biomass.

To begin to understand how LuxS controls biofilm formation, a time-course study was conducted that evaluated early biofilm production. After 6 hours of growth, the wild type strain had produced ~45% of 24 hr biofilm biomass; after 8 hours of growth, biofilm

biomass had nearly doubled, reaching ~80% (Figure 6A). In contrast, SPJV05 only produced 15% of biofilm biomass at 8 hours. Microscopic examination of early pneumococcal biofilm structures after 6 hours of growth are shown in Figures 6B and 6C. Wild type biofilms were fairly uniform, with consistent coverage over the attachment surface. In contrast, *lux*S mutant biofilms were sparsely distributed with many visible gaps in the biofilm matrix. Since no growth deficiencies were seen in the mutant strain during the experimental runs, the differences in biofilm morphology can be attributed to the absence of the *lux*S quorum-sensing gene.

Table 1: Primers used in this study

Primer	Sequence
5L	ACATCATCTCCAATTATGATATTC
6R	GACATCTTCCCAAGTAGTAGTTTC
15L	TATGTCCAATATGTACCACGAC
39L	TTGGTACCGAACTTGACCACACCATTGTC
57L	AAAAATTTGTAATTAAGAAGGAGT
58R	CCAAATTTACAAAAGCGACTCA
Lux5-L	TTGGTACCGAACTTGACCACACCATTGTC
Lux5-R	TTGGATCCATGGTGAACAGTCAATCATGC
Lux3-L	TTCTCGAGACGTCACACCAGTGCTAAAAT
Lux3-R	TTTCTAGAATGAGTCTTGCCCATTCTTTA

Figure 1: Insertional inactivation of the *lux*S gene using a cassette containing an erythromycin resistance marker. Schematic representation of the insertional event. Disruption of the *lux*S wild type gene was achieved through integration of a *lux*S-ery-*lux*S DNA cassette carrying a sequence homologous to a *lux*S internal fragment.

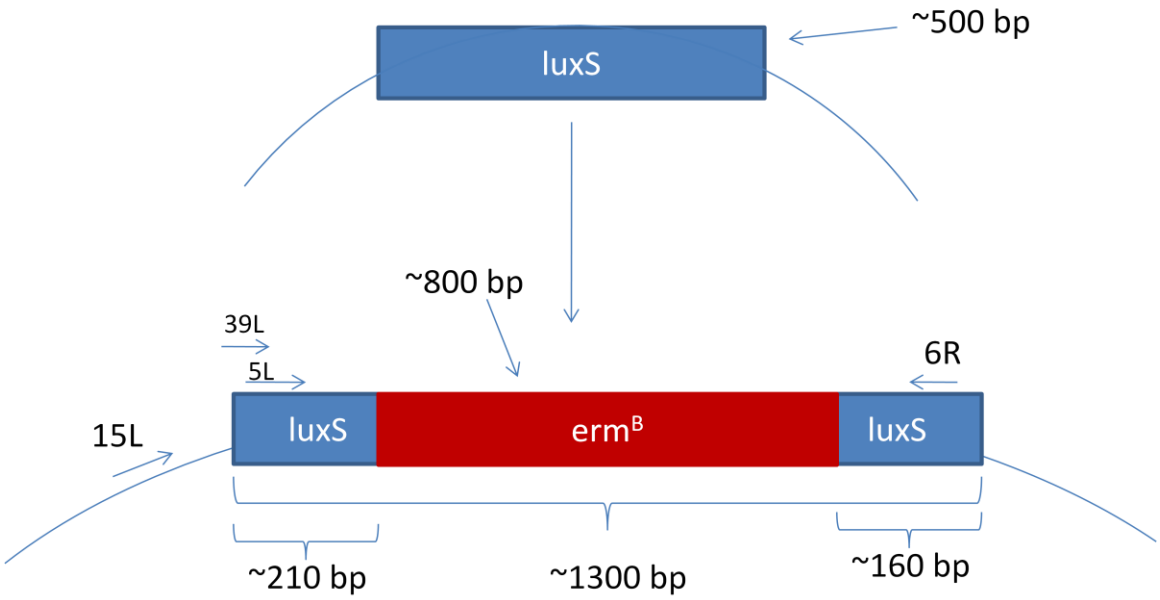


Figure 2: Colony PCR of potential *S. pneumoniae lux*S mutants. The PCR products were amplified using the following primers: lanes 1-4, 15L & 6R; lanes 5-8, 15L & 39L. Lanes: 1 & 5, colony #1; 2 & 6, colony #2; 3 & 7, colony #3; 4 & 8, colony #4.

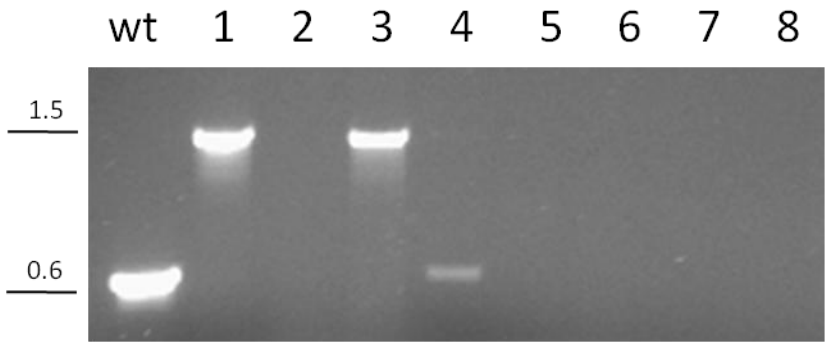
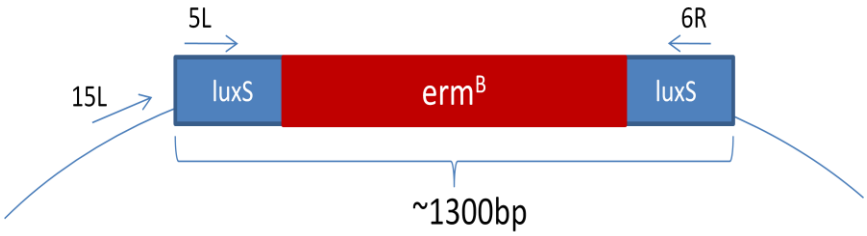
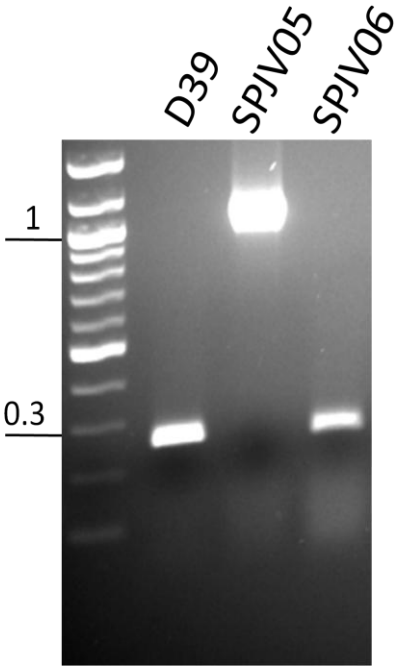


Figure 3: PCR Confirmation of the *lux*S mutant. D39 refers to the wild type. SPJV05 is the *lux*S mutant. SPJV06 is the *lux*S mutant complemented with pReg696-luxS (A) Primer locations. (B) PCR products using primers 5L & 6R (C) PCR products using primers 15L & 6R. The marker used is a 100-bp DNA ladder.

A)



B)



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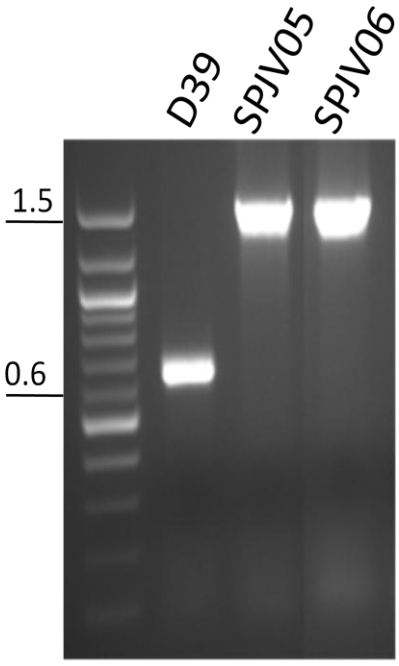


Figure 4: Sequencing results. Green region corresponds to sequence homology with the TOPO cloning vector. The blue region corresponds to the *lux*S gene. The pink region corresponds to the erythromycin resistance gene.

Figure 5: Biofilm formation by *S. pneumoniae* strain D39 is regulated by the *lux*S gene. Strains were inoculated in 96-well plates and incubated for 24 hrs at 37°C in a 5% CO_2 atmosphere. Biofilms were stained with an anti-*S. pneumoniae* antibody coupled to fluorescein and quantified using a fluorometer. Biofilm biomass of wild type strain D39 was set to 100% to calculate the biomass of the other strains. Error bars represent the strandard error of the mean. Asterisks (*) indicate statistical significance ($p<0.05$) calculated using a non-parametric Student *t*-test in comparison to wild type D39.

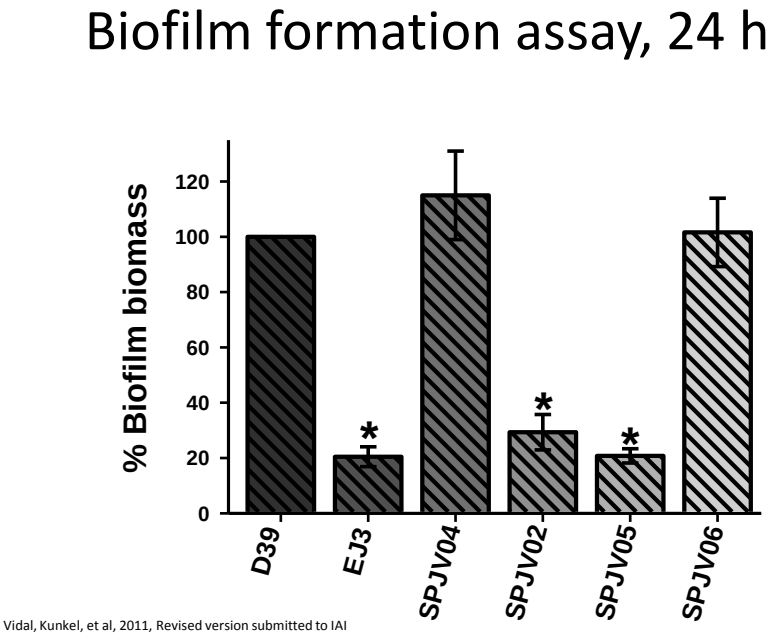
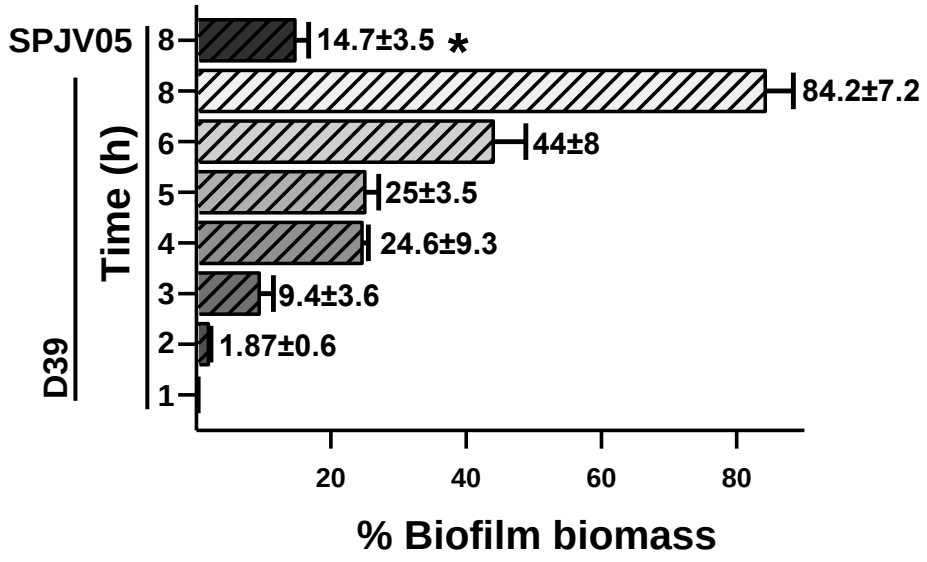
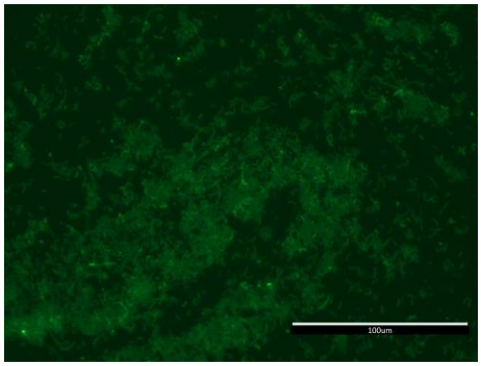


Figure 6: Percentage of 24 hr biofilm biomass formed by *S. pneumoniae* D39 and SPJV05 *lux*S mutant. Strain D39 or SPJV05, was inoculated in 96-well plates and incubated for 1-8 or 24 h at 37°C in a 5% CO2 atmosphere. (A) Biofilms were stained with an anti-*S. pneumoniae* antibody coupled to fluorescein and quantified using a fluorometer. Biofilm biomass of wt strain D39 24 h post-inoculation was set to 100% and all others calculated. Error bars represent the standard error of the mean calculated using data from three independent experiments. Asterisk (*) indicates statistical significance ($p \leq 0.05$), calculated using non-parametric Student *t*-test, in comparison with wt D39 8 h post-inoculation. Biofilms formed by the (B) wild type *S. pneumoniae* D39 after 6 h; and (C) SPJV05 *lux*S mutant *S. pneumoniae* after 6 h.

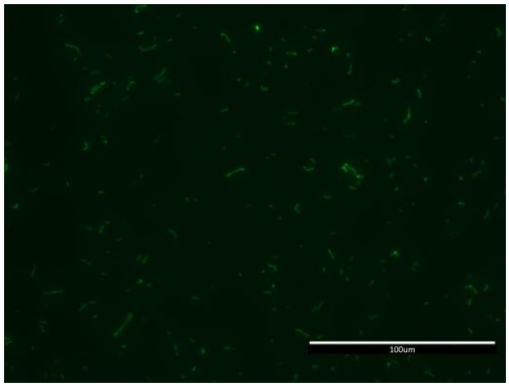
A)



B)



C)



Discussion and significance

In this study, we successfully produced a *S. pneumoniae lux*S isogenic mutant to demonstrate that the *lux*S gene has a significant effect on the ability of the D39 strain of *S. pneumoniae* to form early biofilms. Inactivation of *lux*S was achieved through disruption of the *lux*S wild type gene through integration of cassette DNA carrying a sequence homologous to a *lux*S internal fragment. Insertional inactivation allowed us to target the mutation directly to the *lux*S gene, keeping the upstream region intact so as to not affect the expression of other genes in the process. This helped to confirm that our observed phenotype was due to the inactivation of *lux*S gene and not the result of polar effects.

We used *S. pneumoniae* strain D39 for this study rather than some of the other commonly studied strains, such as R6 and TIGR4, due to our interest in early biofilm formation. D39 produces more robust biofilm biomass, in microtiter plates, than the TIGR4 and R6 strains, making it a better candidate for studies focused on early biofilm formation (Vidal et. al., submitted).

The data in this study demonstrate that *lux*S plays a role in early biofilm formation, but the mechanism by which *lux*S affects biofilm production remains unknown. The *lux*S quorum-sensing system is involved in the regulation of many proteins and it not clear which of these proteins may be causing our observed biofilm phenotype. One possibility is that the *lux*S mutation causes an inability of the bacterium to adhere to the surface substrate, as has been seen in studies of *E. coli* (Genevaux, Bauda et al. 1999). This was seen in a recent study in *Streptococcus suis*, which found a

decrease in the expression of genes involved in adhesion in *lux*S mutants compared to wild type bacteria (Wang, Zhang et al. 2011). The effect of a *lux*S mutation on adherence in *S. pneumoniae* is an important topic for future research.

Another hypothesis is that a delay in DNA competence (the ability to release and take up DNA) is causing the altered biofilm phenotype (Oggioni, Trappetti et al. 2006; Trappetti, Gualdi et al. 2011). Previous research studies have shown that the competence stimulating peptide signaling factor involved in natural cellular competence may play a role in biofilm production (Petersen, Pecharki et al. 2004; Suntharalingam and Cvitkovitch 2005). In addition, it was recently shown that a *lux*S mutation in the RX strain of *S. pneumoniae* (a derivative of D39), causes a delay in cellular competence (Kovacs, Halfmann et al. 2006; Romao, Memmi et al. 2006). Due to the similarities between D39 and RX, it is likely that the *lux*S mutation would also cause a delay in the cellular competence of D39, as well. If the defect in biofilm production in the *lux*S mutant is due to a delay in competence, then biofilm production should be delayed, but not fully prevented. Due to our focus on early biofilm growth, we do not know if our mutant would have formed mature biofilms over a longer incubation time. This could be investigated by using exogenous competence stimulating peptide to induce early biofilm formation in the mutant strain. Future studies in our laboratory will further investigate the relationship between DNA competence and its impact on biofilm formation.

This study demonstrates the importance of *lux*S during *in vitro* biofilm formation in an *in vitro* setting, but does not confirm that a *lux*S mutation would have the same impact on biofilm production in the natural environment of the nasopharynx. In microtiter plates, limited nutrient and signal exchange may prevent proper biofilm

development, making it difficult to study biofilm formation beyond the very early stages (Ghigo 2003). A previous *in vivo* study on mice found that the *lux*S quorum-sensing system was involved in nasopharyngeal persistence, supporting our findings (Joyce, Kawale et al. 2004). These types of *in vivo* studies, while more difficult to execute, will become necessary to fully understand the role of quorum-sensing in colonization and persistence of *S. pneumoniae* within the nasopharynx. *In vitro* studies remain important, however, providing a solid basis for understanding the methods by which *lux*S may control biofilm production in its natural environment.

It is unlikely that *lux*S controlled quorum-sensing system is the only signaling network necessary for early biofilm formation. As mentioned earlier, the competence quorum-sensing system is known to play a role in *S. pneumoniae* biofilm production (Oggioni, Trappetti et al. 2006; Trappetti, Gualdi et al. 2011). *S. pneumoniae* lives in a competitive multi-species environment and its ability to compete with other species could play an important role the survival of the bacterium. Using a ubiquitous signaling molecule, such as AI-2, which allows the bacterium to communicate with other species, may help *S. pneumoniae* better establish its own ecological niche, but other *S. pneumoniae*-specific biofilm regulons may be necessary for *S. pneumoniae* to outcompete these other species.

Chapter 3: Early biofilm growth and development

Background and importance

Historically, the majority of bacterial studies have focused on studying free-living bacteria in liquid media, known as planktonic cells, with very little emphasis on the behavior and characteristics of cells growing in biofilms. This is an important research gap to fill, as over 60% of all human bacterial infections can be attributed to biofilm growth (Sauer 2003; Hall-Stoodley, Costerton et al. 2004; Moscoso, Garcia et al. 2009). It also known that the behavior of biofilm cells differs greatly from the behavior of planktonic cells, emphasizing the importance of expanding the research arena to include studies on both stages of bacterial life (Davey and O'Toole G 2000).

The switch from planktonic to biofilm growth is highly regulated and it is suspected that initial biofilm formation is triggered by the release of quorum-sensing molecules by planktonic cells. Once the initial attachment to the surface has begun, new microorganisms begin to attach to the extracellular matrix, providing more surface area for further colonization. Previous studies in our laboratory have found that there is a substantial increase in biofilm biomass between 6 hrs and 8 hrs of incubation (Figure 7). This increase could be attributed to cellular division of the cells within the biofilm or recruitment of planktonic cells from the surrounding liquid substrate. These preliminary studies sought to provide us with an initial understanding of the methods behind early biofilm expansion and growth in *S. pneumoniae*.

Methods

Transformation with the pMV158-GFP plasmid

To more easily visualize biofilm growth over time, we transformed our *S. pneumoniae* strains with a plasmid [pMV158-GFP (Nieto and Espinosa 2003)] containing a green fluorescent gene encoded downstream of a maltose-inducible promoter. Purified pMV158-GFP was added to 20 ul of wild type *S. pneumoniae* competent cells, along with 1 ul of competence-stimulating peptide (CSP) to a final concentration of 0.5 ng/ul, and 180 ul of complete transformation media [10ml THY + 500 ul of 0.1M $CaCl_2$ + 100mg BSA, filter sterilized (Havarstein, Coomaraswamy et al. 1995)]. The transformation mixture was incubated in a 37°C water bath for 2 hours, then plated onto 5% blood-agar plates containing 0.1 ug/ml of tetracycline and incubated at 37°C and 5% CO_2 for 48 hours. Isolated colonies were streaked on TSA plates containing tetracycline and incubated at 37°C, 5% CO_2 overnight.

Studies of biofilm growth

Bacterial cells were resuspended in PBS on microscope slides and visualized using fluorescence microscopy to ensure expression of the green fluorescent protein. Cells were grown in 96-well flat-bottom polystyrene plates. Plates were incubated at 37°C in a 5% CO_2-enriched atmosphere. After a 6 hour incubation, supernatant was removed. To remove any remaining planktonic cells, wells were washed twice with THY and biofilms were freshly incubated with 150 μl of THY + 2% maltose (Time 0).

Quantification of biomass by fluorescence or by viable cell counts

These biofilms (Time 0) were incubated 1, 2, 3, or 4 hours and biofilm biomass was quantified by measuring fluorescence readings using a VICTOR X3 Multilabel Plate Reader (Perkin-Elmer). Alternatively, biofilm cells were resuspended by scraping the bottom and lateral walls of the wells with a pipet tip and flushing was used to break up cellular aggregates. CFU counts were performed on the resuspended biofilm structures by plating an aliquot of the serial dilutions onto blood agar plates. Plates were incubated at 37°C, 5% CO_2 and colony counts were obtained following 48 hours of incubation.

Results

To assess the ability of biofilm cells to grow following initial biofilm structure development, planktonic cells were removed and biofilm growth was measured hourly for an additional 4hrs to monitor changes in biomass. This allowed us to examine changes in biofilm biomass during the 6-8 hr time point following initial biofilm inoculation. We observed an increase in biofilm biomass at all time points. With biofilm biomass at time 0 (6 hr biofilms immediately following removal of planktonic cells) set at 100%, biofilm biomass doubles within two additional hours of growth (Figure 8). At times 3 and 4, biofilm biomass is significantly larger than that seen at time 0, with biofilm biomasses of ~324% and ~482% respectively ($p<0.05$, Student *t*-test). The increase in biofilm biomass at all time points following removal of planktonic cells

demonstrates that biofilm cells could play a role in the growth and expansion of biofilm structures.

To further confirm, by a direct method, that the biofilms were growing, a series of experiments were conducted to quantify the biofilm colony forming units per milliliter (CFU/ml). A representative sample of the average colony forming units over time is presented in Table 2. An average of $6x10^8$ CFU/ml were seen at time 0, which increased to $2.3x10^{10}$ CFU/ml at time 3, confirming the findings from the fluorescent biofilm assay and providing further evidence for the growth of biofilm cells. In addition, the viable cell counts at time 3 for biofilms grown in the presence of planktonic cells and biofilms without planktonic cells, were $1.3x10^{10}$ CFU/ml and $2.3x10^{10}$ CFU/ml, respectively. This suggests that biofilm cells, once they have produced ~50% of the biofilm biomass, are capable of reaching the same biomass density that can be produced by both planktonic and biofilm cells.

Figure 7: *S. pneumoniae* biofilm growth following removal of planktonic cells after 6 hrs of incubation. Time = hours since planktonic cell removal. Biomass at Time 0 is set at 100%. Asterisk (*) indicates statistical significance ($p<0.05$) and was calculated using the non-parametric t-test.

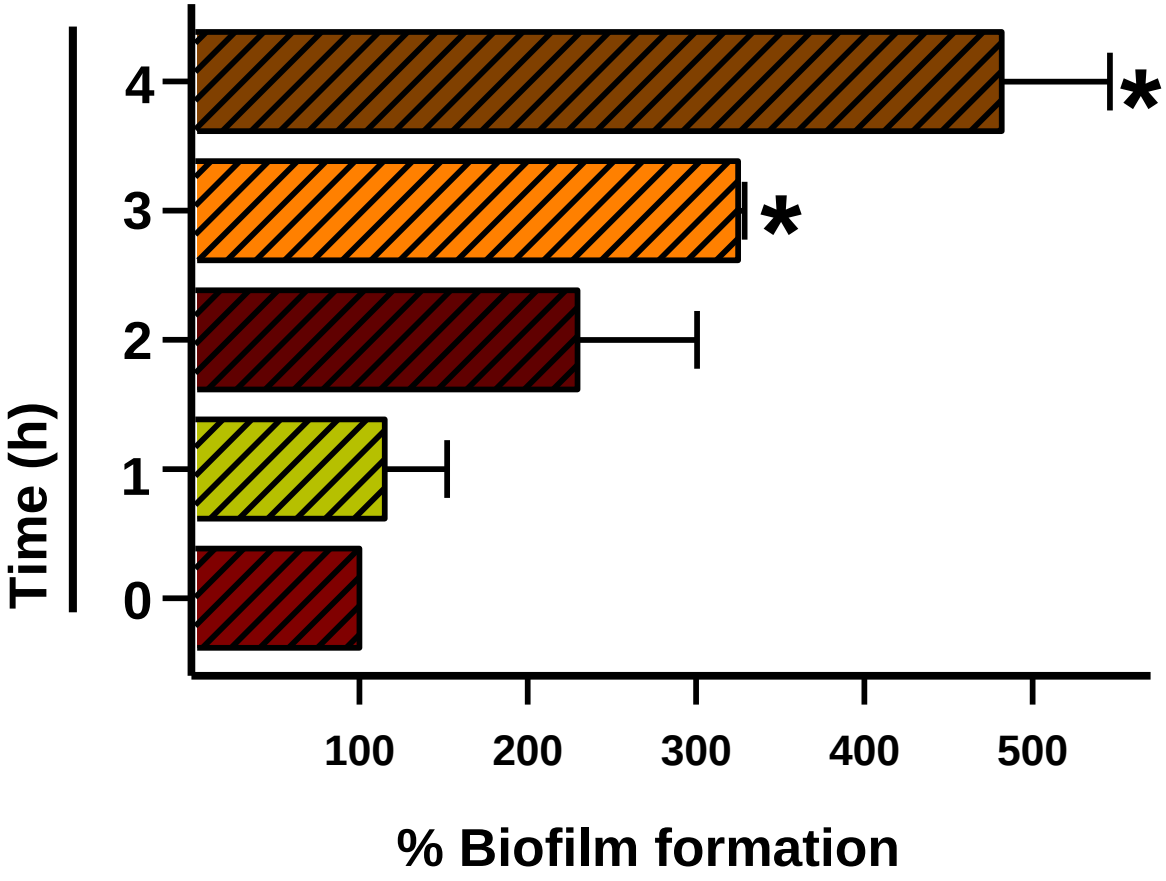


Table 2: Colony forming units/ml (CFU/ml) counts for 6 hr biofilms (Time 0) following removal of planktonic cells

Time	**Total growth time**	**Average (CFU/ml)**
0	6hr	6×10^8
1	7hr	4.1×10^9
2	8hr	5.3×10^9
3	9hr	2.3×10^{10}
3	9hr (w/planktonic cells)	1.3×10^{10}

Discussion and significance

This preliminary study provides some baseline information on the mechanism of early biofilm growth. From other work done in our laboratory, we knew that biofilm biomass doubles during the 6 to 8 hour timeframe. In addition, pneumococci undergo autolysis following prolonged incubations (Oggioni, Trappetti et al. 2006). This meant that 6hr biofilms provide an ideal timepoint to investigate whether an increase in biomass was due to replication of the biofilm cells or due to recruitment of planktonic cells. Evidence from this study demonstrates that biofilm growth during early biofilm development can occur in the absence of planktonic cells. Both of our methods, fluorescence readings and viable cell counts, demonstrated that biofilm cells were replicating in the absence of planktonic cells.

For this study, we used green fluorescent protein (GFP) production to quantify biofilm biomass. This method allowed us to quickly visualize biofilm structures at any point in time without having to utilize staining techniques which may disrupt the fragile biofilm structures. Although this technique is convenient, it may not be suitable for biofilm studies over longer periods of time. During the later stages of biofilm growth, biofilm cells, particularly those located within the inner layers, decrease their metabolic rates. As metabolic rates decrease, so does the production of cellular proteins, including GFP.

For the viable cell counts, we used a pipet tip to manually resuspend biofilm structures. Although this method may seem too variable, we found that it was a reliable

and convenient method, providing us with similar bacterial counts to those obtained with sonication (data not shown).

This study does not eliminate the possibility that planktonic cells could contribute to biofilm growth and expansion in natural environments. It is known, however, that planktonic cells will undergo autolysis after prolonged incubation periods; this means we would expect the contribution of planktonic cells to diminish over time (Oggioni, Trappetti et al. 2006). Whether they do not contribute at all remains unknown; the biofilms in this study are exposed to particularly favorable conditions, which could cause them to grow more robustly than they would in natural environments. After six hours of growth, they are provided with fresh media, which provides them with new nutrients and removes waste products. In addition, the removal of planktonic cells means there are fewer competitors for the available nutrients. These altered conditions could cause biofilm cells to increase their metabolism and replicate more rapidly.

It is generally believed that all biofilm cells are in a dormant state. This study, however, demonstrates that some biofilm cells maintain sufficient metabolic activity to allow for replication. In fact, evidence suggests that biofilm cells are far from inactive; while some genes are down-regulated, others are up-regulated. Proteomic studies have shown that when cells attach to the surface substrate and begin to express the biofilm phenotype, the expression of many genes is altered throughout all stages of biofilm development (Prigent-Combaret, Vidal et al. 1999; Allegrucci, Hu et al. 2006). Recent studies from our laboratory have shown that the transcription of *lyt*A, *psp*A, *nan*A, and *ply* were all increased in biofilm cells compared to planktonic cells, making these genes important candidates for further study (Vidal et. al., unpublished).

In conclusion, this study has provided us with preliminary evidence that pneumococcal biofilms can grow and expand. These results have important clinical and treatment implications. Since biofilm cells are capable of causing biofilm growth and expansion, it is vital that treatments be targeted against biofilm cells to successfully eliminate an infection. These cells are more resistant to standard therapies, so clinicians must be aware of the need for altered treatment regimens. Continuing studies in our laboratory will investigate this phenomenon more thoroughly.

Chapter 4: Conclusion and future directions

This investigation into the role of the *lux*S quorum-sensing system in *S. pneumoniae* biofilms has provided us with some new information regarding biofilm growth and development. We have shown that the *lux*S gene is required for early biofilm formation. We have also demonstrated that biofilm growth can occur in the absence of planktonic cells due to replication of the cells within the biofilm. Both of these findings open the door to other areas of biofilm research that require further study and investigation. Several of these studies are discussed in more detail in this chapter.

Relationship between *lux*S and DNA competence

Future studies are planned in our laboratory to investigate the relationship between DNA competence and biofilm formation. Previous studies have found that biofilm production is induced by the presence of the competence stimulating peptide (CSP) in other *Streptococci* species (Loo, Corliss et al. 2000; Li, Tang et al. 2002; Cvitkovitch, Li et al. 2003; Petersen, Pecharki et al. 2004; Qi, Kreth et al. 2005). More recently, it was has been shown that biofilm production in *S. pneumoniae* is controlled by the competence quorum-sensing system, which is regulated by CSP (Oggioni, Trappetti et al. 2006). Addition of exogenous CSP increased biofilm biomass in D39 wild type, but not in mutants of the *com*D gene, which encodes the CSP receptor (Oggioni, Trappetti et al. 2006). In addition, *com*C mutants were unable to form biofilms, but the addition of

CSP allowed the mutants to produce biofilms at wild type levels. The relationship between the competence quorum-sensing system and LuxS remains unknown and provides a rich area for further investigation.

Biofilm formation and protein expression

Expression of *S. pneumoniae* virulence factors and the physiologic state of the bacteria are affected by the type of infection and the location of the infection in the body (Polissi, Pontiggia et al. 1998; Hava, LeMieux et al. 2003; LeMessurier, Ogunniyi et al. 2006). Planktonic bacteria are able to more efficiently induce septic infections, while biofilm bacteria were more successful at causing tissue infections, such as pneumonia (Oggioni, Trappetti et al. 2006). Although biofilm formation is not considered a virulence factor in the traditional sense, it plays an important role in the colonization ability of the pathogen, suggesting that we would also observe different expression patterns for proteins involved in biofilm formation during *in vivo* situations.

Follow up studies in our laboratory investigated differences in transcription levels between the wild type and *lux*S mutant strains. These studies demonstrated that the *lux*S quorum-sensing system was involved in the transcription of *lyt*A and *ply* mRNA, suggesting genes could play a role in biofilm formation. On the other hand, levels of *lyt*A, *psp*A, *nan*A, and *ply* were all increased in biofilm cells compared to planktonic cells.

In light of our finding that bacterial cells in the biofilm are able to replicate, it would be interesting to study the regulation of genes involved in growth and replication.

Not only would these studies help us to better understand the effects of the biofilm state on gene expression, but it may also help us to understand what genes play a role in the switch from colonizer to pathogenic invader.

Applications in Population Biology, Ecology, and Evolution

Many of the research questions in biofilm development are particularly relevant for researchers in the area Population Biology, Ecology, and Evolution and inclusion of these non-traditional researchers may help to expand upon current theories and understanding. Mathematical spatial analyses can be used to study the biogeography of microbial communities and density dependence models can be used to further understand the relationship between quorum-sensing and biofilm formation (Frederick, Kuttler et al. 2011). Due to the highly coordinated behavior of bacteria within these communities, biofilms could represent the link between unicellular and multicellular life. Biofilm structures would have provided the bacteria with protection from the difficult conditions on early earth, facilitating interactions between individual cells and promoting the development of signaling pathways (Hall-Stoodley, Costerton et al. 2004). Further investigation into this possibility by evolutionary biologists could provide enlightenment for important evolutionary questions.

Based on ecological theory, the heterogeneity seen in the bacterial biofilms should provide a selective advantage for the community. Bacteria within a biofilm are able to cooperate, with certain strains providing necessary metabolites for other strains (Hammer

and Bassler 2003; Mai-Prochnow, Evans et al. 2004). This leads to mutual dependence between species, causing a bacterial symbiosis and providing a unique system in which to study the role of phenotypic variation in species-species interactions. Further investigation into the physical and biochemical interactions within these communities will enhance current research in microbial ecology.

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