1-1-2014

The Effects of Temperate Phage on Streptococcus Pneumoniae During Colonization

Hilary Kay DeBardeleben
University of Pennsylvania, hdebardeleben@gmail.com

The Effects of Temperate Phage on Streptococcus Pneumoniae During Colonization

Abstract
The first step in the pathogenesis of bacterial pathogens is colonization of the host. During colonization, bacteria encounter host defenses, other commensal flora and selective pressures from lytic and temperate bacteriophage. Phages and bacteria have a long history of co-evolution, which has led to bacterial resistance mechanisms and phage counter-resistance mechanisms. One mechanism that phage have adapted is to provide a fitness advantage to the host during colonization and disease, thereby promoting survival of both bacteria and phages. Herein I examine how temperate phages affect streptococcal fitness while colonizing the host, specifically looking at the interactions between Streptococcus pneumoniae, phage element Spn1 and colonization of a murine nasopharynx.

Temperate phages have been identified in the genomes of up to 70% of clinical isolates of Streptococcus pneumoniae. How these phages affect the bacterial host during colonization has not been previously demonstrated. To ask this question, we used a clinical isolate, which carries a novel prophage Spn1. Spn1 was detected as integrated and episomal forms both in vitro and in vivo. Surprisingly, Spn1 also expresses both lytic and lysogenic genes during normal growth conditions. Since Spn1 could not be spontaneously cured, a clean deletion was made to create the Spn1- strain. We used a competitive colonization assay in a murine model to test the fitness of the Spn1+ vs. the Spn1- strain. The Spn1- strain outcompeted the Spn1+ strain seventy-fold after seven days of colonization. To determine if Spn1 is causing a fitness defect by a trans-acting factor, we made an Spn1+ mutant that does not become an episome or express any phage genes. This mutant competed equally with the Spn1- strain, indicating that the fitness defect required expression of phage genes. Further experiments interrogating autolysis, chain length and resistance to lysis by penicillin indicated differences in the cell wall physiology associated with the presence of Spn1. This change in cell wall physiology may be responsible for the fitness defect of Spn1+ strain during colonization. This study provides new insight into how bacteria and prophages can interact and how these interactions may impact the relationship between bacteria and the host.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Jeffrey N. Weiser

Keywords
bacteriophage, colonization, evolution, streptococcus pneumoniae

THE EFFECTS OF TEMPERATE PHAGE ON *STREPTOCOCCUS PNEUMONIAE* DURING COLONIZATION

Hilary K DeBardeleben

A DISSERTATION
in
Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

2014

Supervisor of Dissertation

*Signature*_____
Jeffrey N. Weiser, M.D.
Professor of Microbiology and Pediatrics

Graduate Group Chairperson

*Signature*_____
Daniel S. Kessler, Ph.D.
Associate Professor of Cell and Developmental Biology

Dissertation Committee

Frederick D. Bushman, Ph.D. (Chair)	Professor of Microbiology
Mark Goulian, Ph.D	Professor of Biology
Mechtild Pohlschröder, Ph.D	Associate Professor of Biology
Sunny Shin, Ph.D.	Assistant Professor of Microbiology

Acknowledgements

I would first like to acknowledge my research mentor, Jeff. I couldn't have asked for a better advisor. I make sure to tell all the rotating students how happy we are in the Weiser Lab and that you really can't go wrong in choosing Jeff as your PI. The members of the Weiser lab also contributed positively to my PhD experience and I'm very glad to call them my friends. Science is so much easier when the people around you share in your joys and frustrations. I would particularly like to acknowledge Aoife, my baymate, animal shelter co-volunteer, choir buddy and friend for putting up with all my pestering and for generally making life in the lab more fun. I hope to be able to continue my M4M collaborations with the Weiser lab for many years.

My friends in the graduate group have also improved my PhD experience. Long lunches, beach trips, ultimate frisbee, happy hours and game nights make life much more enjoyable. These friends truly understand the ups and downs of science and I appreciate them always being there to commiserate or celebrate.

I would also like to acknowledge my family, particularly my parents. I have never doubted how proud they are of me and I truly appreciate their support. I would also thank my second family for their enthusiasm at every step of this process; I could not have married into a better clan. That brings me to the last most important

member of my family, my husband. There were more than a few times when I would come home from the lab frustrated, angry or just sad. He was and is a steady presence, helping me get through hard times just by believing in me.

ABSTRACT

THE EFFECTS OF TEMPERATE PHAGE ON *STREPTOCOCCUS PNEUMONIAE* DURING COLONIZATION

Hilary K. DeBardeleben

Jeffrey N. Weiser

The first step in the pathogenesis of bacterial pathogens is colonization of the host. During colonization, bacteria encounter host defenses, other commensal flora and selective pressures from lytic and temperate bacteriophage. Phages and bacteria have a long history of co-evolution, which has led to bacterial resistance mechanisms and phage counter-resistance mechanisms. One mechanism that phage have adapted is to provide a fitness advantage to the host during colonization and disease, thereby promoting survival of both bacteria and phages. Herein I examine how temperate phages affect streptococcal fitness while colonizing the host, specifically looking at the interactions between *Streptococcus pneumoniae*, phage element Spn1 and colonization of a murine nasopharynx.

Temperate phages have been identified in the genomes of up to 70% of clinical isolates of *Streptococcus pneumoniae*. How these phages affect the bacterial host during colonization has not been previously demonstrated. To ask this question, we used a clinical isolate, which carries a novel prophage Spn1. Spn1 was detected as

integrated and episomal forms both *in vitro* and *in vivo.* Surprisingly, Spn1 also expresses both lytic and lysogenic genes during normal growth conditions. Since Spn1 could not be spontaneously cured, a clean deletion was made to create the Spn1- strain. We used a competitive colonization assay in a murine model to test the fitness of the Spn1+ vs. the Spn1- strain. The Spn1- strain outcompeted the Spn1+ strain seventy-fold after seven days of colonization. To determine if Spn1 is causing a fitness defect by a trans-acting factor, we made an Spn1+ mutant that does not become an episome or express any phage genes. This mutant competed equally with the Spn1- strain, indicating that the fitness defect required expression of phage genes. Further experiments interrogating autolysis, chain length and resistance to lysis by penicillin indicated differences in the cell wall physiology associated with the presence of Spn1. This change in cell wall physiology may be responsible for the fitness defect of Spn1+ strain during colonization. This study provides new insight into how bacteria and prophages can interact and how these interactions may impact the relationship between bacteria and the host.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

History and Background

Classification

Streptococcus pneumoniae, the pneumococcus, is a gram-positive organism and member of the lactic acid bacteria. Its closest relatives are *S. mitis* and *S. oralis*, commensal organisms that occupy the same niche, the human nasopharynx (145). Further classification of the pneumococcus relies on the immunogenic properties of its capsule (15). The capsule is a thick polysaccharide layer, covalently linked to the cell wall. There are over ninety known serotypes, which have been given number/letter designations. Capsule type does not necessarily indicate genotype since pneumococci are known to switch capsular types through natural transformation. Therefore, there is also a separate classification system that relies on multi-locus sequence typing. However, the capsule type is easier to determine and therefore is more frequently used as a method of classifying pneumococci.

History

The pneumococcus was first identified in 1880 by Sternberg who isolated it from septic rabbits that had been injected with his saliva and separately by Pasteur from the saliva of an infant. The connections between the pneumococcus and various disease states such as

pneumonia and otitis media were made within ten years of its discovery. Initial observations also noted the presence of capsule, the pneumococcal virulence factor that would eventually be used to produce a vaccine. (6)

Over the years the pneumococcus has served as a model bacterium for many important discoveries in cell and molecular biology. Following its discovery a scientist named Hans Christian Gram, working under Carl Friedlander to identify organisms responsible for causing pneumonia, developed a test to differentiate bacteria in histological samples. It was this test that first differentiated pneumonia caused by the pneumococcus vs. *Klebsiella pneumoniae* and ultimately was used to categorize almost all bacterial species into two groups, Gram-positive and Gram-negative. The Gram stain is a method so simple and effective that it is still used extensively today. (6)

The pneumococcal capsule and its role in virulence also led to the discovery of transformation and the biological properties of DNA. Bacteriologist Fred Griffith first noted the smooth vs. rough colony morphology indicative of the presence of a capsule and further determined that lack of a capsule meant a significant decrease in virulence of the pneumococcus. He infected mice with the avirulent rough colonies mixed with heat killed smooth colonies and recovered smooth colonies from the mice who succumbed to infection. He called this process transformation since the living bacteria were transformed from rough to smooth colony morphology (61). Oswald Avery expanded on this study and showed only DNA from the smooth colonies was required for

transformation (8). This established DNA as the biological molecule responsible for heredity and created the field of modern molecular biology.

Research on the pneumococcus has also led to historic findings in the field of immunology. Early on in pneumococcal research, it was established that capsule was immunogenic. Neufeld based his quellung reaction on this property of the capsule and began the identification of serotypes (112). At the time it was thought that only proteins could be immunogenic and that the capsule must be similar to a protein or associated with a protein. This notion was finally overturned when biochemical studies on the capsule conclusively showed the pneumococcal capsule is a polysaccharide (7). Furthermore, the protective effects of passive immunization with anti-sera, which we now know are largely due to antibodies to the capsule, were first demonstrated in a pneumococcal infection model (6).

Antibiotic resistance

Treatment of the pneumococcus is largely dependent on beta-lactam antibiotics. After the discovery of penicillin, the potential for the pneumococcus to develop resistance to antibiotics was very quickly appreciated (53). However, the appearance of clinical isolates resistant to penicillin did not occur until 1965 and resistance to beta-lactams rose in prevalence throughout the 1970s (5). By the late 90's antibiotic resistance was rampant amongst pneumococci with many strains resistant to multiple drugs. Introduction of the

conjugate vaccine reduced beta-lactam resistance among invasive isolates by 57% due to the fact that the serotypes included in the vaccine were also the most likely to be penicillin resistant. However, this study also observed an increase in non-susceptible isolates amongst non-vaccine strains, particularly 19A (84).

The rise in antibiotic resistance amongst pneumococci has led to an interest in developing phage lysins as antimicrobial agents. Phage lysins are proteins produced by phage in order to lyse the cell at the end of the phage life cycle. Pneumoccocal phage lysin Cp1-l has been shown to reduce the incidence of disease progression from colonization to otitis media when applied intranasally in a mouse model (102). Most phage lysins are specific to the host bacterium from which the phage was isolated. The strength and limitation of phage lysins as antimicrobial agents lie somewhat paradoxically in their specificity. While ideally an antimicrobial would be specific enough to eliminate only the pathogen responsible for disease, the lack of rapid and sensitive diagnostic tools to identify disease-causing organisms severely limits that approach. (118)

Vaccines

Pneumococcal capsule is the major antigen and antibodies against the capsule are protective against pneumococcal colonization and disease. Several vaccines based on the capsule are in use today. The 23-valent polysaccharide vaccine is recommended for use in the elderly and others at risk for pneumococcal disease. More recently the 7-valent

conjugated vaccine, PCV7, was introduced into the vaccine program for children. This vaccine has been successful at eliminating pneumococcal colonization and disease not only in the children vaccinated but also in the elderly population, which indicates that the vaccine also provides herd immunity (66, 164). Each of these vaccines is only effective against the capsule types that they target. Other capsule types are increasing in prevalence after the introduction of the conjugate vaccine, a phenomenon referred to as "serotype replacement" (110). With over 90 known capsule types of *S. pneumoniae*, and the high cost associated with adding new capsule types to the vaccine, new broader vaccine targets are needed.

Pneumococcal Colonization

Colonization is a precursor to disease

The pneumococcus is responsible for roughly one million deaths worldwide annually. Disease outcomes resulting from pneumococcal infection include pneumonia, otitis media, septicemia and meningitis. Disease largely affects young children and the elderly. The first step in the pathogenesis of all pneumococcal disease is colonization of the human nasophayrnx. Colonization is asymptomatic. When the pneumococcus leaves this niche and enters normally sterile body sites such as the lung, middle ear or bloodstream, pneumococcal disease occurs. However, disease is thought to be an evolutionary dead end for the bacterium since transmission does not occur from these sites (20).

Progression to disease is more likely in the context of co-infection or immune suppression. The most common co-infection leading to pneumococcal pneumonia is with the influenza virus. In a chinchilla model of otitis media, co-infection with *S. pneumoniae* and influenza A virus significantly increased likelihood of disease compared to *S. pneumoniae* or influenza A virus alone (58). Experimental infection of adult volunteers with influenza A virus also showed an increased likelihood of acquiring nasopharyngeal carriage of *S. pneumoniae* (154). The mechanism by which the influenza virus increases susceptibility to the pneumococcus is not completely understood.

Epidemiology

Pneumococcal colonization is most prevalent in children. By the age of one year, nearly 100% of children have tested positive for pneumococcal carriage (4). There is a drop off in carriage rates after four years of age and further decreases during adulthood (147). Though colonization is cleared within a few months, children are often serially colonized by different serotypes. Repeated colonization may lead to a build-up of immunity to non-capsule antigens, which leads to the decrease in susceptibility during adulthood. However, adults older than sixty-five have a resurgence in susceptibility to pneumococcal disease, possibly due to declining immune function (147). Colonization in children is the main reservoir for disease and transmission (162). Reduction in rates of colonization amongst children has led to a reduction in pneumococcal disease in the entire population,

especially the elderly (66).

Animal models of colonization

Several animal models are actively used to study different bacterial and host factors involved in pneumococcal colonization. The most common model animal used is mice. In this model, bacteria are inoculated into unanaesthetized adult mice. Colonization persists for approximately twenty days depending on the strain of pneumococcus used. Mice are particularly easy to work with and a common model for all biomedical research. Therefore, investigation of host factors using genetic approaches is much easier in a mouse model than perhaps any other mammalian model system. However, not all strains of pneumococci can colonize mice. Also adult mice do not transmit the bacteria even when co-housed making studies on transmission limited. Other models are not as tractable as mice but have other benefits. Chinchilla models are used to study progression from colonization to otitis media due to the anatomy of their ears (58). Initial colonization studies were done in infant rats which can be colonized by a greater variety of pneumococcal strains (161). A ferret model was developed to study transmission (103) but was quickly replaced by an infant mouse model (49). Lastly, human carriage models are being developed to study colonization since carriage is a relatively safe, asymptomatic state (62). This model could verify discoveries previously found in small animal models.

Dynamics of pneumococcal colonization

Though the carriage state is largely considered to be asymptomatic, in both human and mouse models of colonization pneumococcal carriage is protective against secondary infection (20). This indicates an active host response to the pneumococcus during colonization which must be countered in order for the pneumococcus to persist.

The first obstacle encountered by the pneumococcus upon inoculation into a host is mucocilliary-mediated clearance. The pneumococcus overcomes this host defense by expressing a negatively charged capsule, which repels the negatively charged sialic acid residues on mucus. The capsule allows the pneumococcus to avoid agglutination in the mucus layer and move to the epithelial surface (111). Upon reaching the epithelial surface the pneumococcus must adhere to cells in order to persist. Adherence factors CbpA and PsaA are both involved in adherence to epithelial cells binding to human immunoglobulin receptor PlgL and E-cadherins respectively (65). The glycosidases NanA, BgaC and SpuA have also been shown to improve adherence likely by removing sugars that otherwise mask receptors on the epithelial cell surface (83).

Within 1-3 days of colonization there is an influx of neutrophils into the nasopharynx, however this influx is not sufficient to clear the pneumococcus. Instead pneumococcal density declines only after macrophages are recruited to the site of colonization at 7-10 days post inoculation (42). Pneumococci employ several factors to avoid

opsonphagocytosis by neutrophils. The main component in avoiding complement deposition is capsule. However, exoglycosidases NanA, StrH and BgdA have also been shown to inhibit complement deposition (37). Mice lacking T-Cells and B-Cells (SCID mice) and mice lacking MHCII complex, which have significantly reduced numbers of CD4 T-Cells, are impaired in clearance of pneumococcal colonization indicating that clearance is also dependent on CD4 T-cells (150).

The development of antibody during primary colonization is not important for bacterial clearance (100). However, antibody is important for blocking secondary infection. In a human challenge study protection from colonization correlated with serum antibody to the protein PspA, however this protein is antigenically variable (101). Additionally, antibody generated against an unencapsulated strain was able to protect against all serotypes of the pneumococcus in a mouse model of colonization, though the combiation of antigens responsible for this effect is unknown (125).

Intraspecies competition

Several epidemiological studies have shown simultaneous colonization by multiple serotypes of *S. pneumoniae*. The likelihood of this happening is increased in young children and in areas endemic for pneumococcal disease (25). Furthermore, the emergence of serotype replacement strains is indicative of the possibility that vaccine strains had previously been able to outcompete the non-vaccine strains. Therefore, by

eliminating the vaccine strains the vaccine has expanded the niche for non-vaccine strains. This hypothesis has led to inquiries about intra-species competition among pneumococci. Initial studies show that colonization by one pneumococcal strain can inhibit another isogenic strain from establishing colonization (91). Further studies into the mechanism of competition amongst pneumococci led to the discovery of bacteriocins.

Bacteriocins are small proteins, similar to anti-microbial peptides found in eukaryotes, that are used to compete with similar bacterial species. The Blp locus of *Streptococcus pneumoniae* encodes a regulatory region, a bacteriocin and an immunity locus (44). Two strains of capsule types 6A and 19A were able to inhibit growth of their isogenic Blp locus mutants and the growth of a separate strain, Tigr4, *in vitro*. The 19A strain was also able to inhibit colonization of its isogenic Blp mutant and Tigr4 in a mouse model. Bacteriocin loci of four other strains were tested and found to have activity against not only *S. pneumoniae* but also against other members of the genus Streptococcus and two non-streptococcal species, *Micrococcus luteus* and *Lactococcus lactis* (93). The importance of co-colonization and competition amongst pneumococci and with other colonizing bacteria is highlighted by the conservation of bacteriocins across many pneumococcal strains (44).

Interspecies competition

The human nasopharynx is colonized by many species of bacteria and constitutes a

complex ecology (89). Many pathogens of note, such as *Staphylococcus aureus*, *Haemophilus influenza* and *Neisseria meningitidis,* directly compete with the pneumococcus for space and resources within this niche. The production of hydrogen peroxide by *S. pneumoniae* inhibits growth of many co-colonizing species (119). However for some species it has been shown that a much more complex interaction is occurring.

In large scale epidemiological studies, it has been noted that colonization by *S. pneumoniae* is protective against colonization by *S. aureus* While *S. aureus* is affected by hydrogen peroxide produced by *S. pneumoniae in vitro*, direct competition via this mechanism has not been shown *in vivo*. Furthermore, in hosts where the immune system is compromised, no inverse correlation is seen between these two species. Cross-reactive antibodies generated against *S. pneumoniae* are protective against colonization by *S. aureus* in a mouse model. This may indicate that competition between the pneumococcus and *S. aureus* is immune mediated (90).

Another common pathogen, *Haemophilus influenzae* competes with the pneumococcus for space and resources within the human nasopharynx. Co-colonization with the pneumococcus and *H. influenzae* leads to rapid clearance of the pneumococcal strain that is dependent on opsonophagocytic killing (95). Co-colonization of either airway epithelial cells or a mouse model of colonization leads to synergistic increase in the production of cytokines and influx of neutrophils (123). This host influx is not sufficient

to clear *H. influenzae* but rather eliminates competing flora, such as the pneumococcus. Pneumococci with capsule types that increase resistance to opsonophagocytosis, and therefore more virulent strains of pneumococci, are more likely to survive this increased host influx (94). Thus it is likely that competition with *H. influenzae* has driven the evolution of the pneumococcus towards virulence.

Pneumococcal Physiology

Colony morphology

Streptococcus pneumoniae naturally produces a large amount of hydrogen peroxide, which can be lethal to the bacterium. Therefore it is traditionally grown on agar containing sheep's blood as a source of catalase, which catalyzes the reaction converting hydrogen peroxide to water and oxygen. Sheep's blood agar is opaque, making observations on colony morphology difficult. However, addition of purified catalase to tryptic soy agar (TSA) allowed the bacteria to be grown on a clear medium. This enabled researchers to make observations on the variations in colony morphology of the pneumococcus. (161)

When grown on TSA, *Streptococcus pneumoniae* exhibits two distinct colony morphologies noted as transparent or opaque (Figure 2A). This phenotype is phase variable meaning there is likely random switching between the two morphologies.

However, the genetic basis for the phase variability, which resembles a bistable switch, is unknown. Biochemical analysis of opaque vs. transparent strains indicated differences in amount of capsule and teichoic acids which may contribute to the visible phenotype (82). However, it should be noted that when capsule is genetically removed, variations in colony morphology are still visible meaning the basis for opacity includes other factors and may be multi-factorial.

Colony morphology also plays a role in pneumococcal colonization and invasive disease. Transparent variants colonize the nasopharynx of infant rats at higher densities than the opaque variants. Furthermore, in one strain in this study, phase-switching was observed. In this strain an opaque variant was inoculated but a transparent variant was recovered indicating that the transparent morphology was selected for during colonization (161). In contrast, opaque variants are more virulent during invasive disease than transparent variants (82). This was determined using an intraperitoneal infection model in adult mice where injection with opaque strains resulted in 100% mortality and injection with transparent strains resulted in near 100% survival out to twenty days. In the few mice that did succumb to infection after injection with the transparent strain, it was noted that the bacteria recovered from the spleen had also phase-switched and became opaque variants. This indicates a strong selection for opaque variants during invasive disease.

Cell morphology

The pneumococcus was initially described as a diplococcus due to its cell morphology. Later the organism was reassigned to the genus *Streptococcus* after scientists determined that there is variation in chain length and because of additional phenotypic data. The mismatched timing of cell division and cell separation results in chain formation. Two proteins LytA and LytB are known to be involved in cell separation and division. LytA is a cell wall amidase and mutations in *lytA* have multiple phenotypic effects including the formation of long chains (Figure 2B) (130). LytB is responsible for separation following cell divison and mutants in *lytB* form very long chains with a characteristic tethering visible between cells (Figure 2C) (45). Chain length has been found to be important during colonization due to increased adherence to the epithelium by longer chained pneumococci (126). Conversely, short chained pneumococci are better adapted for invasive disease because their lower surface area per particle allows them to better evade complement deposition on the bacterial surface (38).

Cell wall structure and synthesis

The pneumococcal cell wall is synthesized at two points along the bacterial surface for elongation and division. These two steps are inversely regulated to maintain the ovoid shape of the cell. The two groups of genes responsible for cell wall synthesis, one for division and one for elongation, are homologous to those found in rod-shaped bacteria. The three major components of the cell wall peptidoglycan, wall teichoic acid and capsule are all synthesized at the mid-point in the cell. Peptidoglycan precursors are

synthesized in the cytosol and linked via a phosphate bond to the cell membrane. Each precursor is theoretically flipped to the cell surface where it is added to the already synthesized peptidoglycan. Also at this point, penicillin-binding proteins begin cross-linking the peptide bridges forming the lattice structure of peptidoglycan. Teichoic acids and capsule are synthesized in the cytosol and exported by separate machinery from the peptidoglycan. At the same zone of new growth they are inserted and covalently linked to the peptidoglycan. A complex network of signaling pathways tightly regulates synthesis of these three components. (98)

Autolytic growth and LytA

The pneumococcus is naturally autolytic, meaning in stationary phase the bacteria release a lytic amidase resulting in lysis of the entire bacterial culture. The amidase responsible for this action is LytA (130). LytA is a choline binding protein, which is associated with the cell wall due to choline containing teichoic acids (81). LytA has no known secretion signal. It was proposed that LytA is transported to the cell wall by binding teichoic acids while they are inside the cell during cell wall synthesis (56). LytA is specifically associated with the nascent peptidoglycan likely because of its role in cell division and separation (104). Crystal structures of LytA revealed a rigid choline binding domain and a more flexible amidase domain, which has specific substrate requirements indicating that regulation of LytA may be at the substrate level (105). LytA is activated and will lyse the bacterial cell when membrane integrity is lost causing a change in the proton gradient

(56). LytA has been found to be important in pneumococcal virulence. After intranasal inoculation, mutants in *lytA* had lower bacterial titers than the wild type in the nasopharynx at 24 hours, in the lungs and blood at 48 hours and were unable to reach the CSF after 96 hours. This study also found defects in the virulence of *lytA* mutants when inoculated directly into the lungs, intraperitoneally or intravenously (116). The mechanism for LytA's role during pneumococcal pathogenesis is unclear.

The importance of the cell wall in vivo

As a Gram positive pathogen the pneumococcal cell wall is surface exposed and therefore able to directly interact with the mammalian host. Many important virulence factors are choline binding proteins. These proteins bind to the choline found on cell wall teichoic (also known as the c-polysaccharide) and lipoteichoic acids. Some examples include LytA, CbpA, which is known to improve bacterial adherence to the epethelium and bind IgG receptors, and PspA which can prevent complement deposition on the bacterial surface (81). Phosphorylcholine in the cell wall can directly interfere with host responses by binding rPAF (the receptor for Platelet Activating Factor) and activating host signaling which can lead to downstream effects on the immune response (35). Another important virulence factor, capsule, is covalently linked to peptidoglycan, the main component of the cell wall.

The cell wall is directly exposed to host defenses, which include enzymes that can break

down peptidoglycan. The most prevalent peptidoglycan-degrading enzyme on the mucosal surface is lysozyme. Many successful pathogens, including *Streptococcus pneumoniae,* alter their cell wall in order to resist lysozyme (43). Furthermore, after they have been broken down by lysozyme, the host can detect components of the bacterial cell wall through pattern recognition receptors such as Nod2 and TLR2 (120, 142). This detection generally leads to activation of host immunity and bacterial clearance.

Pneumococcal Phages

Temperate phages

There are two types of phages, temperate and virulent. Both types will adsorb to the bacterial surface and inject phage DNA into the bacterial cell. A temperate phage, depending on the conditions within the cell, will enter either a lytic or lysogenic pathway. A virulent phage can only enter the lytic pathway. Phages that follow the lytic pathway usually hijack the bacterial machinery to transcribe and translate genes for DNA replication, capsids, tail and lysis of the bacterial cell. New phages are assembled and escape the bacterial cell after lysis has occurred. A temperate phage that follows the lysogenic pathway will not replicate but integrate into the bacterial genome at a specific site where it is called a prophage. It remains in this state as the bacteria grow and divide and under certain conditions in the cell, usually in times of bacterial stress, the prophage will excise and enter the lytic pathway.

The regulation of temperate phages has been most thoroughly studied in lambda phages, which infect the species *E. coli*. Lambda phage genomes are organized by function with the lysogeny genes all encoded in the same direction on one end and the genes for the lytic pathway encoded in the other direction away from the lysogeny cluster. A regulatory region between these two clusters contains three operator sites called O_R1, O_R2 and O_R3. The lysogeny cluster encodes a C1repressor, which maintains the integrated state of the prophage. It binds to the first operator site to repress the lytic genes. Binding to the second site promotes transcription of itself. Binding to the third site represses its own transcription. When the bacterial cell experiences stress it upregulates proteases. These proteases cleave the C1repressor. Once enough of the repressor has been cleaved to prevent binding to the O_R1 site the expression of lytic genes is no longer repressed. The first lytic gene is *cro*, another regulatory protein. Cro further promotes expression of the lytic genes by binding the second operator site. This system of regulation creates a rapid switch from the lysogenic to lytic pathway. (121)

Co-evolution

In an effort to resist predation by phage, bacteria have evolved numerous mechanisms that target phage at all points the phage cycle. For example bacteria have evolved restriction modification (RM) systems to distinguish self DNA from non-self DNA. An RM system involves a DNA methylase and a restriction enzyme that is inhibited by

methylation. This allows unmethylated DNA, such as an invading phage genome, to be cut by the restriction enzyme while methylated bacterial DNA is unaffected. Bacteria have also evolved a CRISPR-Cas system to achieve a similar end, the digestion of invading phage DNA. CRISPRs encode short sequences of previously encountered foreign DNA called a spacer. When the same DNA sequence is encountered the spacer binds to the foreign DNA and the Cas family of proteins recognizes this interaction and cuts up the invading DNA. Bacteria will also alter or cover outer surface proteins with other proteins or sugars, which are used by phages as receptors for adsorption. Phages have co-evolved to counteract all of these mechanisms. Phages have been shown to encode their own methylases to counteract restriction enzymes. Point mutations in DNA sequences targeted by CRISPRs are enough to evade Cas systems. Lastly, phages evolve to bind to new receptors and digest sugars to reach receptors on the bacterial surface for adsorption. This evolutionary arms race is a classic example of predator prey relationship and is indicative of the enormous selective pressure applied by phages on bacteria in the environment. (85)

The pneumococcus has two restriction modification systems, DpnI and DpnII. These systems are mutually exclusive because DpnI restricts only methylated DNA while DpnII restricts unmethylated DNA. This allows survival of phage attack in a mixed population. Phages produced in a DpnII containing bacterium will be restricted when infecting a DpnI containing bacterium and vice versa. However, restriction of foreign DNA presents a problem for the pneumococcus during natural transformation, a process that improves

survival of the population by creating diversity (137). The pneumococcus overcomes this by have enzymes specific to cleaving double stranded DNA, which is how phage DNA is injected into a cell. Also when competence is induced, the DpnII system upregulates a methylase that will methylate single stranded DNA as it enters the cell, thus protecting foreign DNA taking up during transformation. (80)

The pneumococcal capsule may be another phage resistance mechanism since capsule prevents phage infection in a laboratory setting (17). There is very little known about the specific receptors phage use to adsorb to the pneumococcus, however, any receptor would likely be covered by the capsule. In natural settings phages may be able to overcome the presence of the capsule or there may be a population of unencapsulated pneumococci that serve as a reservoir for phage.

Lastly, CRISPR-Cas systems have not been identified in any sequenced pneumococcal strains. CRISPR-Cas systems have been artificially introduced to pneumococci and can inhibit uptake of foreign DNA including natural transformation (19). It is likely that because of the inhibition of natural transformation, which is advantageous to the bacterial population, CRISPR-Cas systems have been selected against in the pneumococcus.

Prevalence

Prophages have been identified in up to 70% of clinical isolates of *Streptococcus*

pneumoniae. Initial studies identified the phage lysin by homology to the bacterial gene *lytA* (122). In this study southern blots with probes for *lytA* resulted in multiple bands indicating multiple copies of *lytA*. It was further determined that isolates containing multiple bands were reactive to chemical induction of prophages by mitomycin C and supernatants of these isolates contained phage particles detected in electron micrographs. Later studies were more specific in the identification of prophages by using a PCR based system that detected multiple phage genes (128). This system was less likely to find phage remnants and they found that about 50% of the clinical isolates they tested harbored phages. The prophages were clustered based on sequence similarity into three distinct groups simply numbered 1, 2 and 3. Group 1 was the most common type of prophage found followed by group 2 and then 3. There were no significant correlations between the phage groups and origin or serotype of the bacterial isolates. Each of these studies only identified phages with known, albeit common, phage genes. Later studies were less biased by using whole genome sequencing. One study in particular focused on a single clone of *S. pneumoniae,* PMEN-1 (33). Though they were not specifically looking for phage, they found prophages from all three previously identified groups in up to 30% of the isolates sequenced. They also identified a new group of phages (Group 2b) that integrate into the ComE locus and inhibit competence in *S. pneumoniae*. This inhibition of competence led to a halt in the evolution of the organism not seen in strains that did not harbor group 2b prophages. This is an example of how phage might negatively impact host fitness.

The PMEN-1 study determined that the accessory genome, genes that are most variable between isolates, largely consisted of prophages indicating phages are a source of diversity for the pneumococcus (33). In other streptococci, such as *S. pyogenes,* phage are the main source of diversity and carry many virulence factors and toxins. Therefore, the phage profile often determines the virulence of the organism (106). This does not hold with *S. pneumoniae*. No known virulent toxins are encoded on pneumococcal phages (127). Also the phages of *S. pyogenes* have been shown to lysogenize other streptococcal species such as *S. suis* and *S. equi* thus bringing new virulence factors to emerging pathogens (73, 144). However, no pneumococcal phages are thought to have originated in any other species.

Phenotypic effects of pneumococcal phages

Pneumococcal phages can affect biofilm formation, colony morphology and adherence. Extracellular DNA is an important part of the extracellular matrix in pneumococcal biofilms (63). By lysing a sub-population of bacteria, prophages contribute to the presence of extracellular DNA (30). Consequently, pneumococcal lysogens form thicker biofilms. The presence of pneumococcal phage MM1 caused a transparent colony morphology, which has preciously been associated with improved fitness during colonization (92). Furthermore, MM1 lysogens had improved adherence to A549 cells in the Tigr4 background independent of colony morphology. The mechanism for this increased adherence is unknown but this study did not address the possibility of increased

biofilm formation affecting adherence. Both of these phenotypic effects could improve bacterial fitness during colonization.

Regulation of integration and excision

The attachment site and integration of pneumococcal phages has only been studied in one phage, MM1. MM1 has a 15bp attachment site located, when the phage is in a circular form, between the lysin and the integrase in a non-coding region. The phage attachment site is an exact match to the bacterial attachment site located within the coding region in the 3' end of the coding region of a conserved hypothetical protein (60). A non-replicating plasmid encoding just the phage integrase (and no other phage genes) can integrate into the bacterial genome using this attachment site. Regulation of the integrase and other lysogeny genes likely depends on two promoter regions located between the lytic and lysogeny clusters. C1Repressor can bind to and repress gene expression at both of these promoters, which indicates a "genetic switch" similar to what is seen in lambdoid phages (115).

Pneumococcal phage genomes

There are several studies comparing temperate phages within streptococcal species including *S. suis*, *S. thermophilus* and *S. pneumoniae*. Temperate phage genomes from all three species were organized into functional clusters (lysogeny, replication,

packaging, morphology and lysis). These findings support the modular evolution theory, that phages have gene modules that recombine to create new phages. However, in the phages of *S. pneumoniae* recombination was also seen at the single gene level. All temperate phages of the pneumococcus in this study carried a LytA homologue in the lysis cluster, however virulent phages have a different lysin. There was a high level of conservation amongst these phages in the integrase gene as well. Within the other functional clusters there is more diversity particularly within the morphology cluster. Based on the nucleotide similarity, the ten phages were grouped into three families. (127)

Putative virulence factors were only seen in two of the ten phages. Phage φSpn_6 encoded a putative toxin/antitoxin system homologous to the MazEF system in *E. coli*. This system promotes maintenance of the phage by producing a stable toxin and an unstable antitoxin. When the phage is lost the unstable antitoxin is degraded and the stable toxin remains killing the bacteria (68). In E. coli the MazEF system is also important for bacterial stress responses. PblA and PblB found in *S. mitis* phage SM1 have been shown to bind to the surface of the bacterium and improve bacterial adherence to platelets (14). Phage φSpn_11 encoded a tail and tape measure protein similar to PblA and PblB. The rest of the phages in this study, with the exception of the MM1 family, also encoded a protein similar to PblB. The role of the toxin system or PblA/B *in vivo* has not been tested experimentally. (127)

Pneumococcal phage lysins

Almost all known temperate phages of the pneumococcus contain similar phage lysins. These phage lysins are homologous to and functionally redundant with the bacterial protein LytA. Sequence homology of LytA and phage lysins was first noted in a study looking at the presence of LytA in clinical isolates. In this study, Southern blots to detect *lytA* showed two bands indicating two *lytA* genes. Further work determined that this second band indicated the presence of a homologous phage lysin (122). Recombination occurs frequently between phage lysins and LytA contributing to the diversity and evolution of these genes (109, 163). Chimeric enzymes mixing the choline binding and amidase domains of these two proteins are functional even when lacking a high degree of sequence similarity (50). Phage lysin Svl and LytA are functionally redundant during the last part of the phage cycle, phage progeny release (57). Phage lysins are usually secreted through holins, small proteins that aggregate to form pores in the membranes of cells. Pneumococcal phages encode 1-3 holins upstream of the lysin (127). However, pneumococcal phage lysins and LytA are likely secreted by a different mechanism, by binding choline-containing teichoic acids as the cell wall is synthesized (56). Both proteins require holin dependent permeabilization of the membrane for activation rather than for secretion.

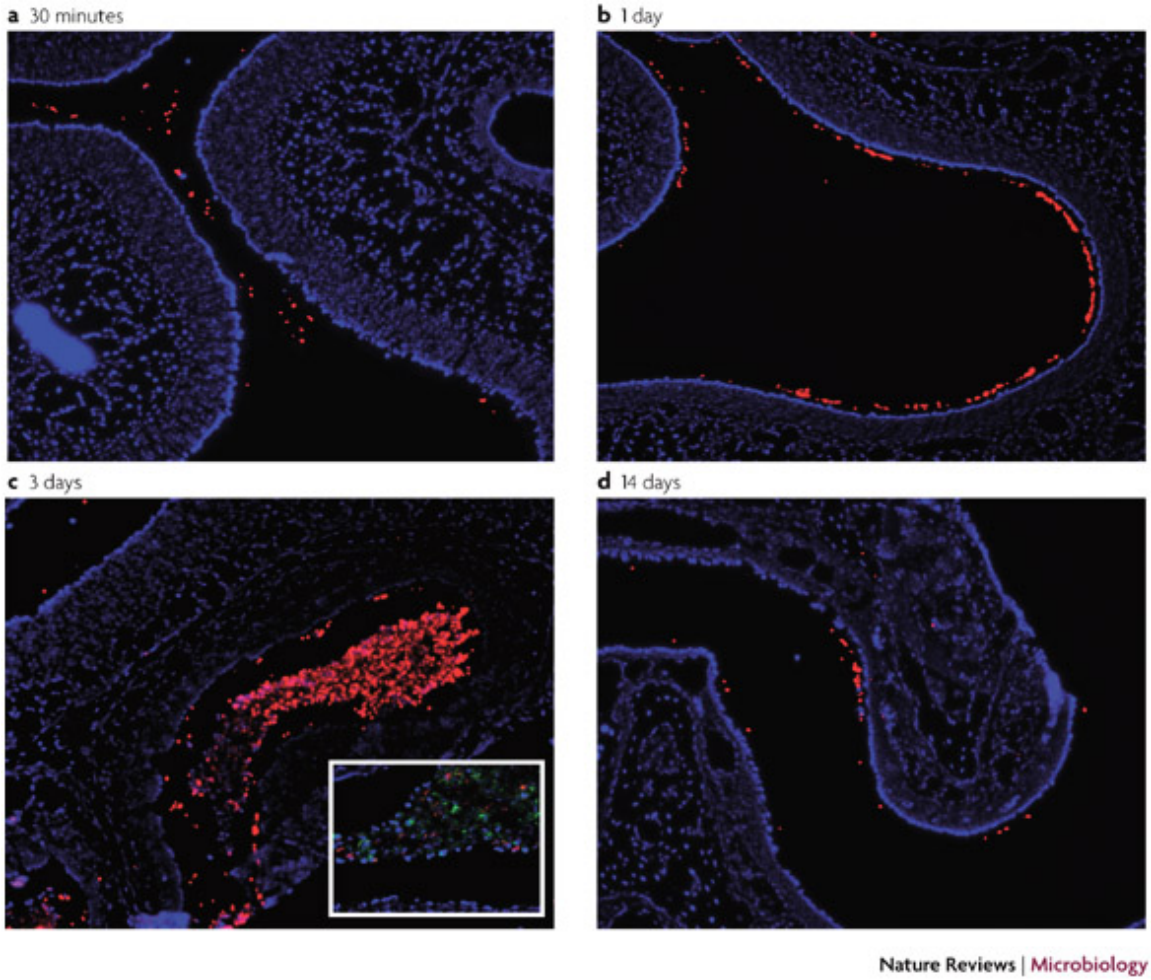


Figure 1. Tissue sections of pneumococcal colonization from 30m to 14 days (a-b). Pneumococci are stained in red using anti-capsular immunoflouresence. Epithelial cells are stained in blue using DAPI. Neutrophils are stained in green using anti-Ly6-G immunoflourescence. Adapted from Kadioglu *et al* 2008. Images courtesy of Aoife Roche

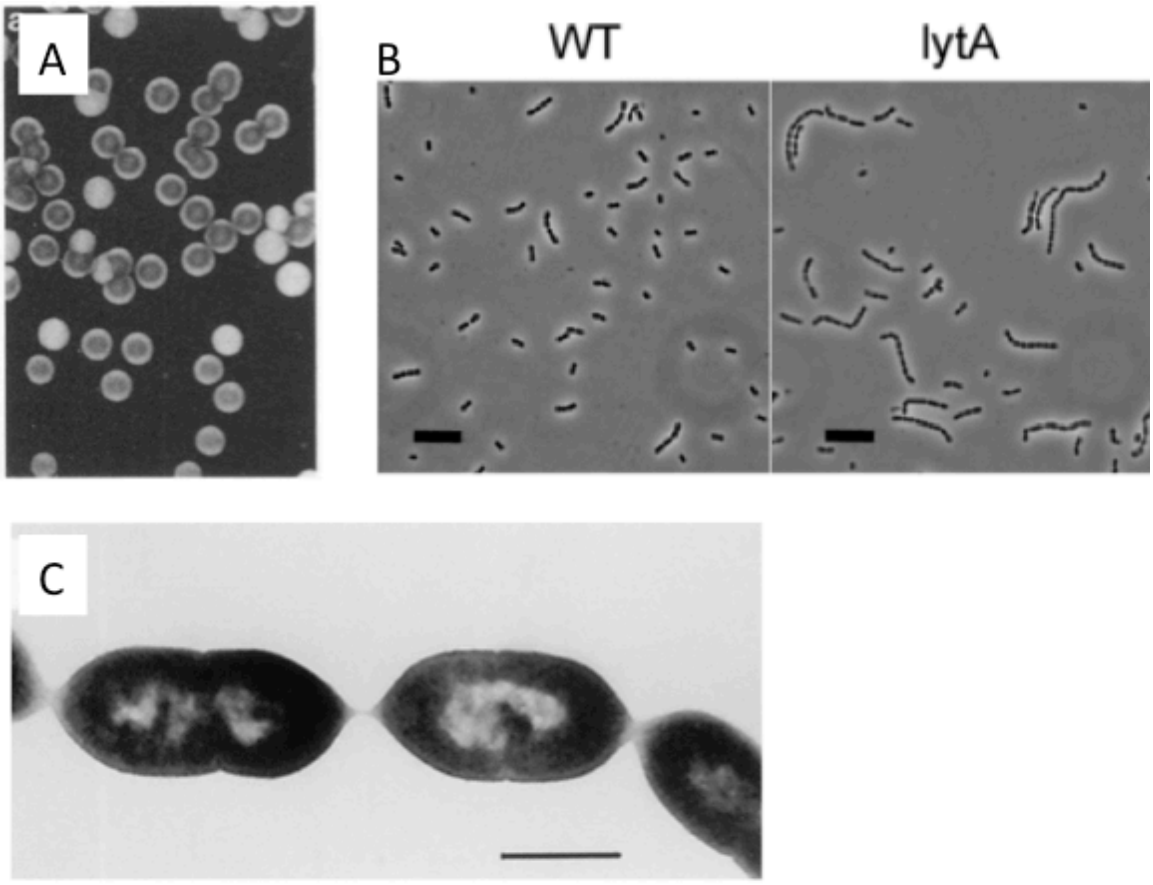


Figure 2. Colony and cell morphology of *Streptococcus pneumoniae* **A.** Opaque and transparent colonies adapted from Weiser et al. 1994 **B**. Brightfield microscopy of pneumococcal strain Tigr4, wild type and Δ*lytA* mutant adapted from Dalia et al. 2010. **C.** Cell morphology of the Δ*lytB* mutant adapted from De Las Rivas et al. 2002

Dissertation Goals

The pneumococcus is a leading cause of bacterial pneumonia resulting in roughly one million deaths worldwide each year. The first step in the pathogenesis of pneumococcal disease is colonization. Blocking colonization prevents pneumococcal disease, therefore it is important to understand the bacterial and host factors involved in this step of pathogenesis. One ecological pressure important for all bacteria is that of bacteriophage. A high percentage of clinical isolates of pneumococci carry prophages. How these phages affect the pneumococcus during colonization is unclear. It is the goal of this dissertation to determine how phages might affect the pneumococcus during colonization.

The first aim of this dissertation is to examine the effects of phage on related streptococcal species. In Chapter two I will discuss the diverse mechanisms by which phage can affect streptococci while colonizing or infecting a mammalian host. Though there are many known phage encoded toxins within streptococcal species, particularly for *S. pyogenes*, we will also discuss the full range of phage associated fitness advantages. These include phage promotion of diversity, phage mediated competition amongst different species or strains of streptococci and phage associated adherence factors such as directly encoded adhesins or assistance in biofilm formation. This variety of mechanisms sheds light on how phage can affect all pathogens including *S. pneumoniae*, the focus of this dissertaion.

The second aim of this dissertation is to characterize the novel phage element Spn1 and its effects on the pneumococcal isolate P1121. In chapter three I will examine the molecular biology and activity of Spn1. I will then assess how Spn1 affects P1121 during colonization in a mouse model. In an effort to determine a mechanism by which Spn1 is having an effect on fitness *in vivo* I will discriminate cis vs. trans acting effects of Spn1. I will also test for other physiological changes associated with the presence of Spn1.

This work will provide new insight into interactions between phages and the pneumococcus. Furthermore, it will elucidate factors that are important for the bacteria during colonization of a host.

Chapter 2

Effects of bacteriophage on interactions between bacteria and their mammalian hosts: Lessons from streptococci

Hilary DeBardeleben[1] and Jeffrey N. Weiser[1,2]

Departments of Microbiology[1] and Pediatrics[2], University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

This review is submitted to *Infection and Immunity*

Abstract

Bacteria and phage have a long history of co-evolution involving resistance and counter-resistance mechanisms. One strategy that phage have adopted is to improve bacterial fitness thereby rendering themselves advantageous to the survival of their bacterial host. Phages that have adopted this strategy are abundant in many clinically and agriculturally important species that reside primarily within a mammalian host. We have chosen to take an in depth look at the genus *Streptococcus*, for which the effect of phage has been intensely studied, to explore the multiple ways that phage can affect their bacterial hosts during colonization and disease. Streptococcal phages have been shown, for example, to impact diversity and adaptation, antibiotic resistance, adherence, and virulence of their bacterial host. Streptococcal phages also affect their bacterial hosts' ability to compete *in vivo* with other members of the same species or same genus. These mechanisms provide insight into the role of bacteriophage in the microbial ecology of bacterial pathogens and should be relevant to other bacterial species.

Introduction

An important and often overlooked factor in bacterial fitness and adaptation is the role of bacteriophage. Bacteria and phages have co-evolved in a never-ending arms race of resistance and counter-resistance mechanisms. In many instances, phages have evolved to be useful to the bacterial host. In this way, a bacterial host that carries a prophage is more fit and, therefore, both the phage the bacteria carrying it are more likely to survive. Bacteria that colonize and infect mammalian hosts are no exception. A well known example of phage contributing to microbial pathogenesis is the phage-encoded cholera toxin of *Vibrio cholerae*, which induces diarrhea, thereby enhancing bacterial transmission (155). In other examples, such as Shiga toxin expressed by enterohemorrhagic *Escherichia coli* and diphtheria toxin expressed by *Corynebacterium diptheriae*, phage-encoded toxins appear to contribute to bacterial survival by disrupting the function of mammalian cells involved in defense (54, 114). Phages also affect their bacterial hosts in many other ways. Phages can serve as gene shuttles providing access to a "pool of diversity" for bacteria inhabiting environments containing other members of the same or related species. These beneficial effects of phage need to be balanced with their potential adverse effects, such as bacterial lysis. Phage-mediated lysis, however, may benefit one strain through niche clearance if it acts on competing susceptible members of the same species or other constituents of the microflora. While the phages of many important bacterial species have been investigated in detail in regards to their structure, invasion, gene regulation, and other characteristics, here we will review

specifically those studies that examined the impact of phages on bacterial species during mammalian colonization or the pathogenesis of disease.

Phages that impact their bacterial hosts in a positive way are temperate. Temperate phages can enter either the lysogenic or lytic pathway after invasion of the bacterium. The lytic pathway involves the normal production of phage DNA and proteins to make new phage and subsequently lyse the cell. The lysogenic pathway leads to phage integration into the bacterial genome. In this stage, the integrated phage is called a prophage while the bacterial host is called a lysogen. Prophages usually express a set of genes to maintain lysogeny, while the rest of the genes are repressed. However, some phages have been found to express additional genes that affect the biology of their host bacteria. Classically, when the bacterium experiences stress, the SOS response leads to an increase in production of bacterial proteases. The proteases cleave the C1 repressor, which maintains the lysogenic state of the phage. Without the C1 repressor the prophage is reactivated. During reactivation, the prophage is excised from the genome and enters the lytic cycle. Temperate phages can impact host fitness at many stages of the phage lifecycle.

We have chosen to limit our discussion to phages of the clinically and agriculturally important genus of *Streptococcus*, and a few closely related relatives, in order to discuss in more detail the diverse mechanisms by which phage impact bacterial-mammalian host interactions (summarized in Figure 1). The bacterial species that

make up this genus, despite being closely related, are found in diverse environments and perform a large set of functions. Many streptococcal species are human pathogens with a clear impact on human health, including *S. pyogenes*, and *S. pneumoniae* as well as the closely related genus *Enterococcus,* which includes *E. faecalis* and *E. faecium*. Animal pathogens, such as *S. equi* sp. and *S. suis,* have a significant impact on the farm animal industry. Others species largely colonize humans and animals and only occasionally cause disease, including *S. mitis*, *S. oralis* and *S. mutans*. Still others, such as *S. thermophilus*, are used extensively in industrial processes, such as cheese production. With so many species in this genus that affect human welfare in a number of ways, it is important to study the relationships between the streptococci and factors that impact their fitness.

New virulent and temperate phages are continually being discovered for streptococcal and enterococcal species. Dozens of these phage genomes have been sequenced and characterized. The frequency of lysogenized isolates is high amongst many streptococcal species. For *S. pneumoniae*, it has been estimated up to 70% of clinical isolates are lysogenized(122). *S. pyogenes* is almost always lysogenized or poly-lysogenized, with studies showing that the most virulent strains have acquired multiple phages over the years(135, 139, 141). Other streptococcal species are less well studied epidemiologically but are likely to have similar rates of lysogeny. Morphologically, the majority of phages that infect this genus are tailed, siphoviridae, podoviridae and myoviridae(2). A few non-tailed phages have been

recently identified but these are not as common (99). The phage genomes found in streptococci (and most bacteria) are organized into functional modules. These modules usually include groups of genes for lysis, replication, structural components and lysogeny. In streptotcocci, there are often groups of genes with higher GC content than the rest of the prophage in the region between the lysin and the attachment site of the phage, indicating a genomic rearrangement (151). In some species, these genes encode virulence factors, which may indicate that other genes of unknown function in this region also aid in bacterial adaptation to its environment. In *S. thermophilus,* these genes may be transcribed directly from the prophage without phage activation.

Traditional views of phage-bacterial interactions surmise that a particular phage will be specific to one host species or strain of bacteria. However, it is becoming increasingly evident that promiscuous phages, phages that can infect a broad spectrum of hosts, not only exist but are a huge source of genetic diversity in bacteria (31). Evidence of promiscuity in Streptococcal phages has been around for over 30 years. Phage host promiscuity has not only been seen in large sequencing studies but also demonstrated experimentally(157). Promiscuous phages can act as agents of horizontal gene transfer creating a large shared gene pool amongst susceptible streptococci. This also allows for transfer of phage-encoded factors that aid in colonization or disease across species. The phenotypic effects of different phage on their streptococcal host species are summarized in Table 1.

Phage-mediated horizontal gene transfer

In order to adapt to a changing host environment, it is beneficial for bacteria to have access to a pool of genetic material to promote diversity. The genetic determinants of host specificity in streptococci, for example, can be very small and often come down to just a small subset of genes which can easily be attributed to horizontal gene transfer or lysogenic conversion (73). As is the case for other bacterial genera, members of *Streptococcus* have many phages that contribute to diversity via transduction of virulence genes and antibiotic resistance cassettes both within and across bacterial species (47). Evidence of phage-mediated horizontal gene transfer and lysogenic conversion within and between species has been largely indirect, consisting of sequencing and analysis of homologous phages within their bacterial hosts and experimental demonstration of cross-species infective phages.

The genomic and experimental evidence of phage-generated diversity within streptococcal species has been well studied and reviewed (33, 55, 143, 152). Phages that infect *S. pyogenes*, for instance, contribute to the diversification of the species by transducing the genes for toxins, such as superantigens, and virulence factors, such as hyaluronidases, which we will cover later in this review. (78, 167) It has also been shown that for *S. pneumoniae* serotype 1, variations in virulence in a mouse model of infection can be attributed to a few highly variable genomic regions, one of which is a prophage (67). Phages of *S. pneumoniae* also harbor a homologue of the

bacterial virulence factor LytA, indicating genomic rearrangements between the prophage and bacterial genome (109). In each of these examples, phage is contributing to the species diversity, which allows the bacterial hosts to better adapt to changing environments.

There is also considerable evidence of homologous phages infecting different species of streptococci, for both species residing in the same niche and across niches. In geographic areas where multiple streptococcal species are endemic, phages originating in *S. pyogenes* not only provide variation within this species but also seem to have crossed over into commensal species, such as *S. dysgalacatiae* subsp. *equisimilus*, a group C *Streptococcus*. (39, 40) Genomic studies of *S. pyogenes* suggested over 90 phage-associated horizontal gene transfer events originating from several zoonotic species, including *S. equi*, *S. dysgalactiae* and *S. canis*. (88) *S. equi* was observed to have the greatest number of genetic exchange events with *S. pyogenes*, despite the fact that *S. equi* is an obligate horse pathogen while *S. pyogenes* is uniquely adapted to human hosts. Three phage encoded superantigens that define the emergence of a particularly virulent strain of *S. pyogenes* (M3) were also identified in phages of *S. equi* that predate the emergence of M3 (73). Conversely, the toxins SpeH and SpeI were recently acquired in *S. equi* and likely originated from *S. pyogenes*. Both examples indicate a common phage pool leading shared virulence factors between the two species. In another study, phages of each of the species *S. pyogenes*, *Lactococcus lactis* and *S. thermophilus* have been sequenced and show a

close phylogenetic relationship implicating a potential shared pool of cross-reactive phages infecting both human pathogens and species relevant in the food industry that are not found in humans (46). Although these studies document homologous phages among multiple species from different environments, they do not show direct evidence of cross infectivity *in vivo*. However, these observations have broad implications for how the bacteria that humans consume and acquire through contact with animals may affect the human microbiota.

Phages of streptococci have been implicated in the transfer of antibiotic resistance within and between species. Antibiotic resistance, particularly in prominent streptococcal species that infect humans, including *S. pyogenes* and *S. pneumoniae*, has become a serious and increasing public health issue (3, 149). Phages have been shown to be capable of transducing genes encoding resistance to commonly prescribed antibiotics, including tetracycline, chloramphenicol, macrolides, lincomycin and clindamycin, *in vitro* between non-clinical strains of *S. pyogenes* (148). Additionally, prophages have been detected in clinical isolates of *S. pyogenes* resistant to erythromycin (75). By inducing these phages and infecting other strains of *S. pyogenes,* it was determined that these prophages carry the erythromycin resistance gene. In fact, this was the first example of a phage in a clinical isolate found to carry an antibiotic resistance element. Erythromycin and other macrolide resistance are due to an efflux pump encoded by two genes, *mefA* and *msrD*. One of these genes, *mefA*, and the gene responsible for tetracycline resistance, *tetO*, were

identified and characterized on the *S. pyogenes* phage Φm46.1 (48). Tetracycline resistance, encoded by the gene *tetW*, has also been reported on *S. suis* phage φSsUD.1. *In vitro* φSsUD.1 demonstrates cross infectivity with *S. pyogenes* indicating the possibility that it is capable of transducing antibiotic resistance between bacterial species inhabiting different hosts (117).

Although much of the best evidence for horizontal gene transfer amongst streptococci comes from metagenomic sequencing studies, there have been experimental demonstrations of cross reactive phage between group A and group C streptococci (157). In this study, phages that were induced and propagated on group A streptococci were able to plaque with consistently high titer on several group C streptococcal indicator strains. Conversely, phages induced from group C streptococci also formed plaques on group A streptococcal indicator strains, though to a lesser degree. Lastly, movement by transduction of streptomycin and bacitracin resistance markers has been observed by these same cross-reactive phages. A more recent study also showed that phage 149, isolated from an M2 isolate of *S. pyogenes,* was able to infect and lysogenize many M-types of *S. pyogenes* as well as the species *S. equi* (153). These two studies provide the most direct evidence of phage-mediated gene transfer between different species of streptococci and highlight the potential of phages to be a mode of transfer for the substantial genetic diversity found in numerous streptococcal and related species.

Phage-encoded virulence factors

The contribution of bacteriophage to bacterial fitness *in vivo* is largely dependent on the expression of phage encoded virulence factors. These virulence factors are well known to account for much of the strain to strain variation in pathogenicity, particularly in group A streptococci but also in other streptococcal species, and may be a determining factor in clinical outcomes (33, 55, 152). Phage encoded virulence factors include toxins that directly cause damage to the host (e.g., superantigens), enzymes acting on mammalian cell processes, (e.g., phospholipases), or immune evading factors (e.g., DNases). The general topic of phage encoded exotoxins has been reviewed in detail elsewhere (32). Examples of each of these categories of phage-encoded virulence factors are provided below.

Superantigens cause an influx and non-clonal activation of T-cells leading to a cytokine storm and are responsible for much of the pathogenicity of group A streptococci (10). These virulence factors consist of streptococcal pyrogenic exotoxins, SpeA, C and H to M, which are exclusively found on phage genomes (138). Other superantigens such as mitogenic factors 3 and 4 (mf3, mf4) and Streptococcal Super Antigen (SSA) have been found on prophages in the particularly virulent M3 clone of *S. pyogenes* and have been determined to enhance virulence in a mouse model (16). Phages have been largely responsible for dissemination of these superantigens to other species of streptococci, including *S dysgalactiae*, *S. canis* and *S. equi* (73, 79, 158)

Phosphlipases, common component of snake venoms, break down fatty acids to release arachidonic acid, thus causing host tissue damage. A recently emerged M3 strain of *S. pyogenes* was found to have acquired a phospholipase homologue, SlaA, by acquisition of a prophage (135). It was further determined that SlaA was induced by contact with human saliva. Mutants lacking SlaA had decreased adherence to a human cell line and decreased virulence in a mouse model. SlaA and another putative phospholipase, SlaB, were also identified in infectious equine isolates of *S. equi* and *S. zooepidemicus* (73, 124). In this case, SlaA was encoded on an active prophage while SlaB was found within a prophage remnant in all strains. Some of these strains predate the emergence of the expression of SlaA by *S. pyogenes,* indicating the M3 clone may have acquired SlaA from phages of *S.equi* or *S. zooepidemicus*. (73)

Other relevant virulence determinants include the DNases, Spd-1, Sda1 and SdaD (23, 141). The most common of these DNases, Sda1, was shown to be important for virulence in a mouse cutaneous lesion model (28). This was attributed to degradation of the DNA scaffold of Neutrophil Extracellular Traps (NETS), which demonstrate bactericidal activity against strains of group A streptococci that do not express Sda-1. Sda-1 was also found on phages in bovine mastitis isolates of *S. dysgalactiae* subsp *dysgalactiae* (124) and on a phage in an invasive human isolate of *S. dysgalactiae* subsp *equisimilus* (158). In both instances, the phages were

thought to have originated in group A streptococci.

Lastly, there are some phage-encoded virulence factors that have yet to be ascribed to a specific mechanism. For example, in a zebra fish model of infection, lysogenized *S. suis* was more virulent than the wild-type strain (144). However, sequencing of the phage responsible, SMP, revealed no known toxin or virulence factor (96). Another potential virulence factor with an incompletely understood mechanism is the phage-encoded LytA homologue, usually annotated as the phage lysin, which is present in almost all known phages of *S. pneumoniae* (127). Chromasomally-encoded LytA is a cell surface anchored amidase responsible for autolysis and affects virulence in several murine models of pathogenesis (18, 72, 116). The mechanism of LytA's involvement *in vivo* is unclear but it has been speculated that LytA's contribution to shedding of the cell wall may be responsible. The role of the homologous phage lysin *in vivo* is unknown. It is likely that this phage lysin was acquired from the bacterial genome based on its high GC content and ability to recombine with the bacterial LytA (109). The phage lysin is also functionally redundant with LytA during the final stage of the phage lytic cycle, phage progeny release (57). Each of these examples indicate that further work in determining mechanisms for the potential phage-encoded virulence factors of many streptococcal phages is needed.

Effects of phage on colonization and adherence

A key aspect of streptococcal colonization and disease is the ability to adhere to host tissues, which allows the bacterium to establish its niche in the mammalian host. Streptococcal phages have been shown to improve bacterial adherence to host cells in a number of systems, including *S. mitis* and *S. pneumoniae*. Additionally, for *S pneumoniae*, phages have been shown to enhance biofilm formation, which is thought to prolong bacterial survival in the host. In *S. pyogenes*, phage may also increase host tissue invasion. In each of these examples, phages increase bacterial persistence at the site of infection promoting both phage and bacterial survival within the mammalian host.

The most well studied phage-encoded adherence factors are the platelet binding factors A and B, encoded by *S. mitis* phage, SM1 (13). These proteins (PblA/B) are homologous to, and are thought to function primarily as, phage tail structural components. As such, PblA/B are expressed only during phage induction (14). However, these proteins have also been shown to increase bacterial adherence to platelets, which could be important in colonization of inflamed tissues and facilitate the establishment of infected thrombotic vegetations in streptococcal endocarditis. During the normal phage lifecycle, PblA/B are released when holins permeabilize the cell membrane for lysis (107). Once released from the bacterium, the choline binding motif on these proteins will bind to choline residues on the cell wall and are displayed on the bacterial cell surface. At this point, PblA/B is able to bind to sialic acid motifs on platelet gangliosides (108). Similarly, the phage lysin of SM1 has also

been shown to be a choline binding protein that can adhere to fibrinogen domains on the surface of platelets (133). Thus, by bridging bacterial and host cells, PblA/B and lysin are thought to improve bacterial adherence. It is assumed in this model that only a portion of the population of bacteria is lysed by the phage, thus improving adherence in the remaining population. The presence of PblA/B and lysin and subsequent increased adherence to platelets has been correlated with enhanced virulence of *S. mitis* in a rat endocarditis model.

For the airway pathogen *S. pneumoniae*, lysogeny by the phage MM1 increases adherence to A549 respiratory tract epithelial cells in culture (92). The MM1 prophage is also correlated with a shift in bacterial phenotype, which is detected as a more transparent colony morphology. The transparent phenotype has been previously shown to express less capsular polysaccharide per cell and, thereby, allow for increased display of adhesins on the cell surface, enabling stronger binding to host cells (82, 161). Phase variants with transparent colony morphology have previously been shown to colonize the mucosal surface more efficiently than more opaque forms. The mechanism for the effect of MM1 phage on colony phenotype and corresponding increased adherence in cell culture is unknown. Although many types of phage lysogenizing *S. pneumoniae* have been described, the *in vivo* relevance of phage for persistence and for colonization by this species has not yet been addressed (92).

Biofilms are often associated with adherence to surfaces in many species of bacteria. For example, in the pneumococcus, biofilms have been shown to occur in otitis media models (166). Pneumococcal biofilms, which are thought to increase bacterial persistence, require DNA as a component of the extracellular matrix. By lysing a small portion of the bacterial population, phages provide a source of exogenous DNA, which serves to augment biofilm thickness (30). Phage-enhanced biofilm formation has been demonstrated with the temperate phage SV-1 but could be applicable to all temperate phages of the pneumococcus and potentially other bacteria. By enhancing biofilm formation, SV-1 could be improving bacterial persistence and survival in the middle ear during otitis media infections. However, a direct role for phage-mediated biofilm formation in virulence in an otitis media model has not yet been demonstrated.

Phages infecting *S. pyogenes* have been shown to encode hyaluronan lyases (76). There is a significant amount of hyaluronan in the extracellular matrix of the human epithelial surface colonized by the organism. There is a correlation between bacteria that can produce hyaluronidases and disease (77). Breakdown of the hyaluronan allows the bacteria to invade the epithelium and access additional tissues for binding. It is likely that *S. pyogenes* phage, such as H4489, encode these enzymes primarily to break down the hyaluronan which is also found in the capsule of *S. pyogenes* in order to gain access to the bacterial cell for infection. However, antibodies to the phage encoded hyaluronan lyase have been found following *S.*

pyogenes infection, indicating that these enzymes are being released *in vivo* (64). It is plausible, therefore, that the hyaluronan lyase also assists with breakdown of the host hyaluronan, although there has been no direct demonstration of this possibility.

Phage-mediated competition

An evolving story is that of the involvement of phage in competition between bacterial species. When colonizing a niche, microbes must compete for limited space and resources. Bacteria use various means to achieve a competitive advantage over microbes of the same and different species. One mechanism, recently shown in studies performed with *E. coli* and *E. faecalis*, is that phage can be used as a lytic weapon against phage-sensitive competing bacteria (24, 52). Streptococcal phage that cross-infect between species could be mediators of this competition by the same mechanism described. The high prevalence of co-colonization by multiple strains or species in the oropharynx and incidences of cross-reactive phages indicate this could also occur among streptococci. There is also evidence of a role for prophages and phage lysins in competition between streptococci and other bacterial genera.

The direct effect of phage-mediated competition occurs between a lysogenic population and a competitor(s) that is susceptible to phage lysis. A portion of lysogenic populations of bacteria will spontaneously lyse, thus releasing phages at a

low level, while the rest of the lysogenic population is immune to these phages due to phage immunity islands encoded by the prophage itself. However, released phages can infect and, by undergoing the lytic pathway, kill competing bacterial strains that are not lysogens. Temperate phages used in this strategy are more successful when they have an intrinsically low rate of entering the lysogenic pathway. This was first modeled mathematically and then demonstrated in *E. coli* using an *in vitro* system (24). Both the model and the *in vitro* data showed phage-mediated predation to be an effective strategy for competing bacteria. More recently, phage-mediated competition was shown to occur *in vivo* using *E. faecalis* , a close relative of the streptococci, in a mouse gastritis model. In this study *E. faecalis* strains carrying the prophage competed more effectively against susceptible, non-lysogenic *E. faecalis* strains. When the competing strains were both lysogens the competitive advantage of the first strain was diminished due to resistance to phage infection in both strains. Competition was also less effective if the prophage on the first strain was rendered inactive. The authors hypothesized that the prophage of the first strain was lysing and killing the second strain of *E. faecalis* (52). There are many examples of simultaneous colonization or infection by multiple strains of the same streptococcal species (26) and different streptococcal species (89) occupying the same niche. As noted above, there are phage that cross infect between streptococcal species (129, 157). Although there should be ample opportunity for streptococcal phage to confer a competitive advantage by this mechanism, phage-mediated competition among streptococci has not yet been demonstrated.

Lastly, phage may also be involved in competition between streptococci and other bacterial species. Studies have shown that certain phage lysins have broad antibacterial activity and can affect multiple strains across several genera of bacteria including most streptococci, staphylococci, listeria and enterococcus (59). This was shown by the addition of purified phage lysin, PlySs2 from an *S. suis* phage, to *in vitro* cultures. Intraperitoneal injections of PlySs2 were also able to clear infection in 92% of mice in a bacteremic model of co-infection by *S. aureus* and *S.pyogenes*. No activity was seen against gram-negative bacteria. Purified phage lysins are not a good model of how phage lysins might act during the natural phage cycle. Further work is required to determine if these lysins can affect multiple species when contained within a phage. An alternative mechanism of competition is to activate prophages of other species by inducing bacterial hosts' stress responses. This was demonstrated in competition between *S. pneumoniae* and *Staphylococcus aureus*, two species previously demonstrated to have an inversely correlated presence during natural colonization (21). In this example, the production of high levels of hydrogen peroxide by the metabolism of *S. pneumoniae* did not directly damage *S. aureus* via oxidation but rather induced an SOS response in *S. aureus* that lead to a loss of the repression of prophages harbored in its genome, thus activating the lytic cycle of the phage (132). *In vitro* this activation leads to lysis of *S. aureus* – an effect that could presumably increase niche space for *S. pneumoniae*. However, this *S. pneumoniae*-induced effect of phage activation in *S. aureus* lysogens has not

been demonstrated *in vivo*.

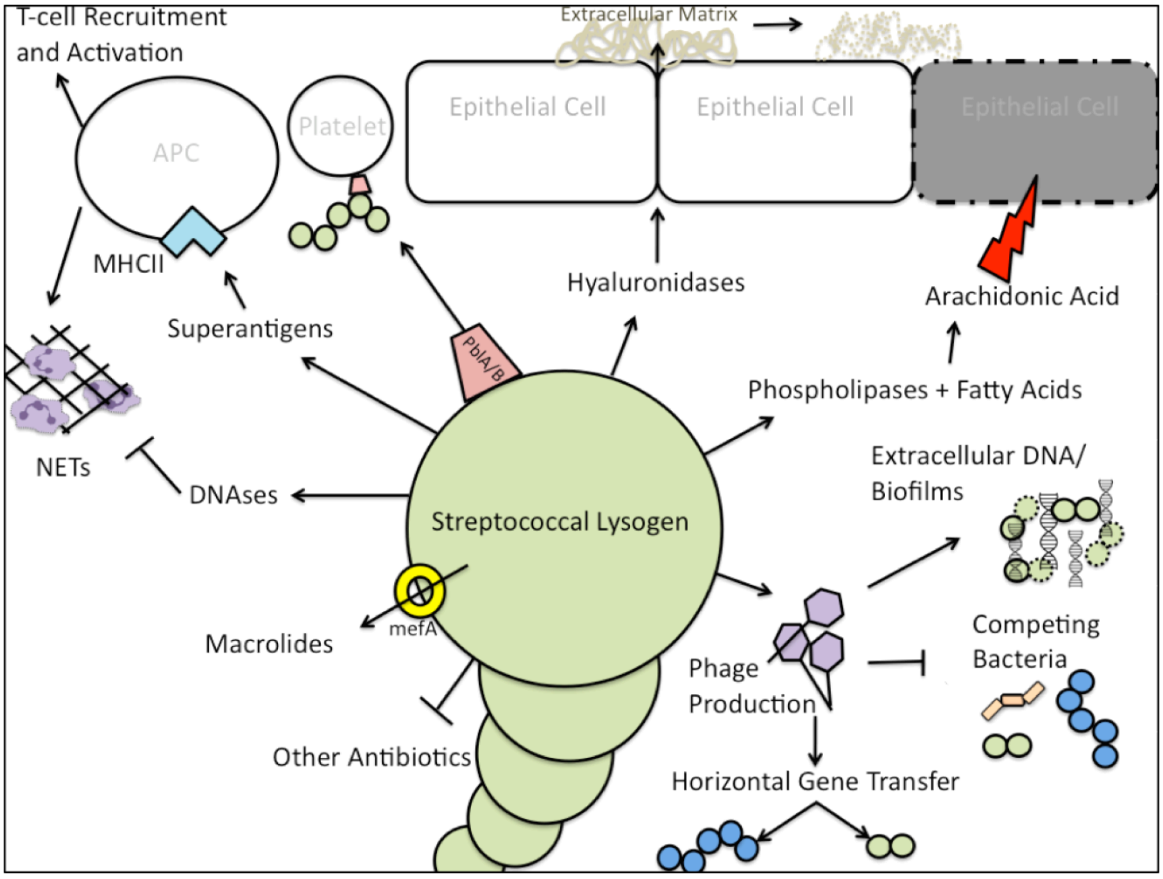
Conclusion

Bacteriophages are parasites of the microbial world, only able to live and replicate within a host bacterium. Though little work has been done to characterize the detrimental effects of prophage carriage on the bacterial host *in vivo*, the possible negative effects of temperate phages include low levels of lysis within the population, protein and DNA synthesis burden and interruption of neighboring bacterial genes. Theoretically, these negative effects on bacterial physiology must be overcome if the phage is to maintain a relationship with its host bacterium. By providing a benefit to the host bacterium in its natural environment, a phage can overcome any fitness defect it may innately cause. The genetic mobility of phages allows these fitness and virulence factors to move across species, creating a reservoir of genetic diversity to possibly create new pathogens, similar to what has been shown in marine phages (31). We have highlighted a few strategies employed by the phages of streptococci that improve bacterial fitness within mammalian hosts. The implications of phage encoded fitness advantages include changes in bacterial virulence, adaptation and host specificity and are relevant in clinical, veterinary and agricultural settings.

Table 1. The effects of temperate phage on the fitness of Streptococci

Phenotypic effect	Species	Phage	Mechanism	Ref
Adherence	*S. mitis*	SM1	Platelet binding proteins A/B	(14)
	S. pneumoniae	MM1	Unknown, possibly increased adherence due to transparent morphology	(92)
	S. pneumoniae	SV1	Biofilm formation due to excess extracellular DNA	(30)
	S. pyogenes	All	Hyaluronidases breakdown the extracellular matrix allowing bacterial access to epithelium	(12)
Immune Evasion	*S. pyogenes, S. agalactaiae, S. equi*	Many	Spe family and mitogenic factors recruit and activate T cells	(138)
	S. Pyogenes	Many	DNAses break down Neutrophil Extracellular Traps	(28)
Host Tissue Damage	*S. pyogenes, S. dysgalactaiae, S. equi, S zooepidemicus*	Φseq2	Phospholipases (SlaA, SlaB) lead to arachidonic acid from breakdown of fatty tissue which damages host cells	(73, 124, 135)
Antibiotic Resistance	*S. pyogenes*	Fm46.1	mefA, tetO	(48)
	S. suis	φSsUD.1	tetW	(117)
Competition	*S. pneumoniae*	CP-1 and others	Phage lysins delivered exogenously can lyse and kill competing bacteria	(59)
	S. aureus	80α, Φ11	Hydrogen peroxide from *S.pneumoniae* activates prophages in S. aureus	(132)
	E. faecalis	ΦV1, ΦV7	bacteria compete better when lysogenized against unlysogenized competititors	(52)
Increased Virulence	*S. pneumoniae*	All	Phages harbor LytA homologue, bacterial LytA is important in pathogenesis	(109, 116)
	S. suis	SMP	Lysogenized strains show increased mortality in zebrafish model	(144)

Figure 1.


T-cell Recruitment and Activation
Extracellular Matrix
APC
Platelet
Epithelial Cell
Epithelial Cell
Epithelial Cell
MHCII
Superantigens
PblA/B
Hyaluronidases
Arachidonic Acid
Phospholipases + Fatty Acids
NETs
DNAses
Streptococcal Lysogen
Extracellular DNA/ Biofilms
mefA
Macrolides
Competing Bacteria
Phage Production
Other Antibiotics
Horizontal Gene Transfer

Chapter 3

Tolerance of a phage element by *Streptococccus pneumoniae* leads to a fitness defect during colonization

Hilary DeBardeleben[1] , Elena S. Lysenko[1], Ankur B. Dalia[3], and Jeffrey N. Weiser[1,2]

Departments of Microbiology[1] and Pediatrics[2], University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Department of Molecular Biology and Microbiology[3], Tufts University School of Medicine, Boston, Massachusetts 02111

This work is submitted to *The Journal of Bacteriology*

Abstract

The pathogenesis of the disease caused by *Streptococcus pneumoniae* begins with colonization of the upper respiratory tract. Temperate phages have been identified in the genomes of up to 70% of clinical isolates. How these phages affect the bacterial host during colonization is unknown. Herein, we examined a clinical isolate that carries a novel prophage element, designated Spn1, which was detected in both integrated and episomal forms. Surprisingly, both lytic and lysogenic Spn1 genes were expressed under routine growth conditions. Using a mouse model of asymptomatic colonization, we demonstrate that the Spn1- strain outcompeted the Spn1+ strain >70-fold. To determine if Spn1 causes a fitness defect through a *trans*-acting factor, we constructed an Spn1+ mutant that does not become an episome or express phage genes. This mutant competed equally with the Spn1- strain, indicating that expression of phage genes or phage lytic activity is required to confer this fitness defect. *In vitro*, we demonstrate that the presence of Spn1 correlated with a defect in LytA-mediated autolysis. Furthermore, the Spn1+ strain displayed increased chain length and resistance to lysis by penicillin compared to the Spn1- strain, indicating that Spn1 may alter the cell wall physiology of its host strain. We posit that these changes in cell wall physiology allow for tolerance of phage gene products and may be responsible for the relative defect of the Spn1+ strain during colonization. This study provides new insight into how bacteria and prophages interact and affect bacterial fitness *in vivo.*

Introduction

Streptococcus pneumoniae, or the pneumococcus, is a gram-positive opportunistic pathogen responsible for ~1 million deaths worldwide each year (168). Although a leading source of infection, the pneumococcus is most commonly found in a commensal relationship with its human host (20). Pneumococcal colonization is characterized by sequential and overlapping episodes that each last for days to months. Colonizing pneumococci provide the reservoir of organisms causing disease and are likely the only source of bacteria for host-to-host transmission (162). Therefore, colonizing pneumococci are likely to be the focus of selective pressure and adaptation for the organism. During colonization, pneumococci encounter varied challenges, one of which is predation by bacteriophages. Up to 70% of clinical isolates of *S. pneumoniae* harbor genetic elements resembling prophages based on the presence of the phage-encoded lysin, which is a homologue of the chromosomally-encoded cell wall amidase, LytA (122). How these prophage elements affect the physiology of their pneumococcal host during colonization has not been fully explored.

The predator-prey dynamic between phages and the bacteria they infect has been an important factor driving bacterial evolution (160). This arms race has resulted in the evolution of phages that promote the fitness of their bacterial host, including its survival within a mammalian host and ability to cause disease (27). For example,

prophages may encode toxins or other virulence factors that increase the ability of their bacterial host to persist during infection. Phages may also impact genetic diversity within a species through lysogenic conversion or horizontal gene transfer, which facilitates bacterial adaptation to the host environment (e.g. antibiotic resistance)(148).

In contrast to the positive effects of phages on bacterial pathogenesis, few studies have assessed the negative effect of phages on bacterial survival during infection. Aspects of the temperate phage lifecycle that could theoretically have a negative impact are phage lysis, increased burden of DNA and protein synthesis, or *cis*-acting effects on genes flanking the phage attachment site. The variety of phage resistance mechanisms employed by bacteria indicates that negative effects of carrying prophage may be a significant factor in bacterial fitness (85).

In the case of the pneumococcus, prophage SV-1 has been shown to enhance biofilm formation by increasing amounts of extracellular DNA(30). Similarly, pneumococcal prophage MM1 has been shown to increase bacterial adherence to human epithelial cells in culture (92). However, the contribution of these or other pneumococcal phages to positive or negative effects on bacterial fitness *in vivo* has not been demonstrated.

Using a newly identified prophage, Spn1, found in a highly successful pneumococcal

lineage, and a well-established mouse model (165), we examined how the prophage effects *S. pneumonia*e during colonization. The bacterial isolate harboring Spn1 was obtained from a human challenge study and shown to be fit for colonizing the natural host for up to 122 days (101) and the murine nasopharynx for 21 days (165). Here, we find that the Spn1 element was stably present and transcriptionally active in this isolate. We show that the presence of Spn1 correlates with a negative impact on bacterial fitness during colonization. We also provide evidence to show that Spn1 is associated with changes in the cell wall physiology of its host. We posit that this change is a mechanism for tolerance of phage products, which may be the underlying cause of this fitness defect.

Materials and Methods

Bacterial Growth Conditions

Unless noted otherwise, bacteria were grown in C+Y medium pH ~6.8 (86) without shaking in sealed tubes at 37°C. Growth was monitored via absorbance readings at 620nm. Bacteria were plated on blood agar plates or tryptic soy medium (TS) + Catalase (4741 U/plate) (Worthington Chemicals). C+Y was supplemented with streptomycin (200μg/mL), kanamycin (500μg/mL), erythromycin (1μg/mL), neomycin (5μg/mL), or spectinomycin (200μg/mL) where indicated.

Sequencing of Spn1

P1121, a bacterial isolate from a human challenge study (Table 1) was sequenced on an Illumina HiSeq and de novo assembled using CLC's Main Workbench (CLC bio). Two assembled contigs containing prophage coding regions were identified by NCBI's BLAST program. The gap between the two contigs was sequenced using Sanger sequencing technology. Once the sequence was complete the genome was submitted to RAST for annotation (9).

RNA isolation and qRT-PCR

RNA was isolated using the TRIzol® reagent and protocol (Lifetechnologies) with modifications. Bacteria were harvested at mid-log phase and a 2:1 volume of RNAprotect™ Bacteria Reagent (Qiagen) was added to the culture. Bacteria were then resuspended in TRIzol®, frozen and then disrupted by bead beating for 5min. RNA was isolated by Phenol/chloroform extraction as written in the TRIzol® protocol. After re-suspension of the RNA in RNase free water, samples were treated with Dnase(Qiagen) for 30min at 37°C. DNase was inactivated at 65°C for 10min before RNA was then used to make cDNA. RT-PCR was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with the Power SYBR Green PCR Mastermix (Applied Biosystems). mRNA levels were quantified using ΔCT values and compared to an internal control, *gyrA*. Primers are listed in Table 2.

DNAse Protection Assay

Supernatants from cultures treated or untreated with 5μg/mL mitomycin C (Sigma-Aldrich) were filtered through a .22μm syringe filter. Samples were divided and treated or not with DNAse (Qiagen) and incubated for 30min at 37°C. DNAse was then inactivated at 65°C for 10min. DNA was quantified via quantitative PCR using the Power SYBR Green PCR Mastermix (Applied Biosystems).

Generation of Mutants

Bacterial mutants were created in the P1121 background by first amplifying regions upstream and downstream of the gene of interest via PCR. For each mutant, a set of primers 1-6 was designed. Primers amplifying the flanking regions are designated 1&2 (upstream) and 5&6 (downstream). Antibiotic cassettes were amplified using primers 3&4. PCR was carried out using standard techniques with either Taq DNA Polymerase (Invitrogen) and related reagents for non-cloning related PCR assays or Platinum Pfx Polymerase (Invitrogen) and related reagents for PCR related to cloning. Primers used are listed in Table 2. Primers 2-5 were tagged with an overlapping sequence. A DNA fragment was then created using overlap-extension PCR (70) with the amplified regions flanking the antibiotic resistance cassette of choice. Antibiotic resistance markers used were erythromycin resistance, Em^r (pCR2.1-TOPO plasmid with Em^r insertion from pMU1328(1)), kanamycin resistance, Kn^r (Janus Cassette (140)), and spectinomycin resistance, Sp^r (pCR2.1-TOPO with Sp^r insertion from pBI143 (aad9, GenBank accession number U30830)(136)). This PCR fragment was transformed directly into bacteria using a

transformation protocol as previously described(74). Mutants with an unmarked deletion were made via the Janus Cassette as previously described (140). Primers and antibiotic cassettes used for each mutant strain are listed in Table 2. Constructs were confirmed by sequencing across junctions.

Mitomycin C Assays

Bacteria were grown to mid-log phase then diluted to an OD_{620} of 0.1 in C+Y with or without 0.5μg/mL of mitomycin C (Sigma-Aldrich). Growth at 37°C without shaking was recorded as the OD_{620} over 4 hours after treatment. Samples were collected for RNA isolation two hours after addition of mitomycin C. Control strains TIGR4 (MM1-) and TIGR4M (MM1+) were obtained from Dr. V. Fischetti, Rockefeller University (Table 1).

Mouse Model of Colonization

Bacteria were grown to mid-log phase (OD_{620} 0.5) in C+Y medium. 1mL of culture was pelleted and resuspended in 100μL of PBS. For competitive assays bacteria were combined in a 1:1 ratio after re-suspension. 10μL of resuspended bacteria were dropped onto the nostrils of non-anaesthetized wild-type female C57BL/6 mice, 6-8 weeks of age. Inoculum counts were ~$1x10^6$ CFU/ml. For competitive assays, at least 50 colonies of the inoculum were plated to determine streptomycin sensitivity or resistance and thereby the input ratio. Seven days after inoculation, nasal lavages were taken as previously described (100) and plated on TS with

neomycin (5μg/mL) to determine colony forming units. For competitive assays, at least 50 colonies from each mouse were tested for streptomycin resistance or sensitivity to determine an output ratio. Competitive indices were calculated by (output ratio)/(input ratio). Statistics were calculated using a one sample T-test on log values of all competitive indices and compared to a hypothetical value of 0 which would indicate no competitive advantage or disadvantage.

In vitro Competitive Growth Assay

Bacterial strains were grown up to OD_{620} 0.5 in C+Y then diluted to OD_{620} 0.1. 1mL of each strain and 2mL of fresh culture for a total of 4ml were combined to start the assay. Cultures were incubated, without shaking at 37°C and plated for bacterial counts at 0 hours and 5 hours on selective media for an input and output ratio respectively to calculate the competitive index.

Growth and Autolysis Assays

Bacteria were diluted from mid-log phase culture to a low starting OD_{620} (~.05) and grown in a 96-well plate in an incubating plate reader set at 37°C in 180μl C+Y medium with 10μl of catalase (30,000U/mL)(Worthington Chemicals) for 24 hours. Penicillin sensitivity was determined across a range of concentration from 10-50ng/ml (50ng/mL exceeded the MIC for both strains, defined as total inhibition of growth) under these conditions. Absorbance at 600nm was measured every 15 minutes with 5 seconds of shaking on a low setting before every reading. Co-

cultures were comprised of 100μl fresh medium (Brain Heart Infusion(BHI)) and 50μl of each of the strains grown to mid log phase and diluted to a low OD_{620} (~.05) in BHI.

Western Blot

Whole cell lysates were created by resuspending bacteria (harvested at mid-log phase) in loading dye (30 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol and 2% β-mercaptoethanol) and incubated at 95° for 5min. Lysates were loaded onto a 10% glycine gel (Mini-Protean TGX Gel, BioRad) and run under standard conditions. Protein was transferred to a PVDF membrane via a semi-dry transfer. After transfer, the membrane was allowed to dry, blocked with 2% BSA solution in phosphate buffered saline, and incubated with 1:100 dilution of anti-LytA Rabbit IgG (obtained from Dr. R. Lopez, Biological Research Center, Madrid, Spain) or 1:40 dilution of anti-pneumolysin Mouse IgG (Novacastra Laboratories Ltd., Buffalo Grove, IL) for two hours. This was followed by incubation with an appropriate secondary antibody conjugated to alkaline phosphatase (anti-Rabbit IgG or anti-Mouse IgG respectively, diluted 1:5000 (Sigma)). Proteins were visualized with a 5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium reaction.

Microscopy

Bacteria were grown to mid-log phase in C+Y medium. Samples were blinded before images at 400x magnification were taken on a Nikon E600 brightfield microscope.

ImageJ analysis was performed using the "threshold" function followed by the "analyze particles" function to quantify particle size. For transmission electron microscopy, samples were grown to mid-log phase then pelleted. Pelleted bacteria were high pressure frozen, freeze substituted into acetone with 2% OsO4 & 0.1% UA, embedded in EPON resin, cut into 70 nm sections, and imaged on JEOL 1010 microscope at X mag on AMT 1K x 1K camera

Results

Identification of Phage Element Spn1

Phage element Spn1 was first identified in a human colonization isolate of *Streptococcus pneumoniae* via PCR using the prophage typing system described by Romero *et al* (128). Subsequent sequencing of the entire locus (43kb) revealed a novel phage element most closely related to phage040922 (GenBank accession no. FR671406.1). However, the integrase (*int*) and lysin (*mml*) of Spn1 shared 99% and 95% identity, respectively, to the previously characterized pneumococcal phage MM1 (GenBank accession no. AJ302074.2). The sequence of phage element Spn1 was annotated using RAST, which predicted 63 open reading frames. Similar to lambda phage, the genome was organized into lytic (lysis, structural and replication genes) and lysogenic (Figure 1A) functional clusters. The Spn1 phage element integrates between two convergently transcribed genes annotated as SP_1563 (a

pyridine-nucleotide-disulpho-oxidoreductase) and SP_1564 (conserved hypothetical protein) in the TIGR4 genome (GenBank accession no. AE005672.3). The attachment site is likely to be the same attachment site for MM1, a 15 base pair sequence located at the 3' end of the coding sequence for SP_1564(Figure 1B)(60). Integration of Spn1 does not interrupt the coding region or promoter sequence of either flanking gene. Both flanking genes are highly conserved amongst published *S. pneumoniae* strains.

Spn1 activity

We designed PCR primers to amplify across the attachment site of both the prophage and the bacteria to determine if Spn1 was capable of excising (Table 2). Both sets of primers amplified a product in non-induced genomic DNA samples indicating a background level of spontaneous excision and circularization of Spn1 from the P1121 genome (Figure 1C). Using qRT-PCR on bacterial RNA isolated from non-induced log-phase cultures, we determined that Spn1 expresses its lysogenic genes as expected but also expresses lytic genes, including those encoding its lysin (Mml) and holins (Figure 1D). Despite the expression of lytic genes, we were unable to identify plaques on any indicator strain using a plaque assay that was previously described for *S. pneumoniae*(57). Additionally, there was no evidence of phage particles using a DNAse protection assay or in transmission electron micrographs of strain P1121. A selectable marker was inserted within Spn1 to determine whether

the prophage could be cured as indicated by loss of the marker during passage. No Spn1- revertants were identified during *in vitro* growth (spontaneous cure rate <.002 per generation). Spn1 was then removed from strain P1121 using Janus cassette technology to generate an unmarked, in-frame deletion, Spn1- (140).

Spn1 induction by mitomycin C

The P1121 lysogen was treated with the DNA crosslinking agent mitomycin C (5μg/mL) to trigger the SOS response and activate the Spn1 prophage. Mitomycin C treatment of strain TIGR4 with or without phage MM1 served as controls. There was inhibition of growth correlating with the presence of phage MM1, but no effect of mitomycin C on the growth of strain P1121 carrying Spn1 as compared to the isogenic Spn1- construct (Fig. 2A). To confirm that the mitomycin C treatment was activating the Spn1 prophage under these conditions, phage gene expression was assessed. Gene expression was greatly increased in the presence of mitomycin C for three lytic genes and one lysogenic gene across Spn1 (Figure 2B). Thus, Spn1 is transcriptionally activated by mitomycin C, but not able to lyse its bacterial host.

Spn1 causes a defect in fitness in vivo

To determine the effect of Spn1 on its bacterial host *in vivo* we used a murine nasopharyngeal colonization model. When tested individually, the Spn1+ and Spn1-

strains each colonized mice at similar densities at seven days post-inoculation (Figure 3A). To compare their relative fitness, we competed Spn1+ and Spn1- strains 1:1 in the same model of colonization. At seven days post-inoculation, the Spn1- strain significantly outcompeted the Spn1+ strain (competitive index (CI) >70, Figure 3B). Competitive growth *in vitro* did not correlate with competitive effect *in vivo*, indicating that Spn1 confers a fitness disadvantage that is specific to the *in vivo* environment (Fig. 3B). In order to differentiate strains post-colonization, one strain was marked with an *rpsL* mutation conferring resistance to streptomycin. Data shown is a combination of experiments with either the Spn1+ or Spn1- strain having been marked to ensure that the streptomycin resistance phenotype was not the cause of the fitness effect *in vitro* or *in vivo*.

A trans-acting factor from Spn1 affects in vivo fitness

To assess whether the effect of Spn1 on *in vivo* fitness was caused by *cis*-acting effects on the flanking genes, we generated mutants in *SP_1563* and *SP_1564* in strain P1121 and tested them in the colonization model. The loss of *SP_1563* resulted in a defect in colonization (Fig. 4A). To determine if Spn1 was affecting transcription of the flanking genes, we compared mRNA levels of Spn1+ and Spn1- strain using qRT-PCR. Expression of *SP_1563* and *SP_1564* were unaffected by the presence of Spn1. Therefore, an effect of Spn1 on either flanking gene was unlikely to be the cause of the fitness defect *in vivo* (Figure 4B).

We attempted to reinsert the Spn1 prophage in the Spn1- strain but were unable to identify transformants acquiring this large genetic element (43kb). By placing selectable markers within both the *int* and *mml* genes at opposite poles of the prophage, it was possible to obtain transformants of the Spn1- strain in which the entire locus was restored. However, interruption of both the *int* and *mml* genes resulted in a strain in which Spn1 no longer excised spontaneously (Fig. 1C) or transcribed genes across the entire prophage (Fig. 4C). Theoretically, this mutant should maintain any *cis*-acting effects on the flanking genes but lack *trans*-acting effects due to phage gene expression. The competitive index of the Spn1+Δ*intΔmml* strain vs. the Spn1- strain was not significantly different from 1, indicating that the two strains compete equally (Fig. 4D). This result suggests a *trans*-acting factor requiring phage gene expression is required for the effect of Spn1on *in vivo* fitness. This result also eliminated the possibility that the burden of carrying an additional ~40 kilobases of DNA was responsible for our observations on fitness during colonization.

Three candidate genes were identified as potential trans-acting factors in the fitness effect of Spn1. First, the phage lysin, Mml, was interrupted to create a Spn1+Δ*mml* strain. The competitive index of Spn1- vs. Spn1+Δ*mml* was significantly greater than 1 indicating that the Spn1 mediated fitness effect was not due to Mml (Figure 4E). The second candidate gene PblB, a large phage tail protein, was removed to create

the Spn1+Δ*pblB* strain. Competition between Spn1+ and Spn1+Δ*pblB* resulted in a competitive index not significantly different from 1 indicating no competitive advantage for either strain (Figure 4E). Lastly, the integrase gene (*int*), a DNA binding protein that mediates integration of the phage (115), was interrupted to create Spn1+Δ*int*. The competitive index of Spn1- vs. Spn1+Δ*int* is significantly greater than 1 indicating that Spn1- has the fitness advantage. This leads us to conclude that Mml, PblB or integrase alone does not confer the fitness defect to the Spn1+ strain *in vivo*.

The presence of Spn1 correlates with resistance to autolysis

The phage lysin, Mml and the major autolysin of *S. pneumoniae*, LytA, are homologous and thought to be functionally redundant cell wall amidases (57). In order to determine if autolysis is affected by the presence of Spn1, we measured bacterial growth in nutrient broth medium over a period of 24 hours. Autolysis was delayed in the Spn1+ strain by ~5 hours (Figure 5A). As a control, *lytA* was interrupted to ensure autolysis was LytA dependent in both the Spn1+ and Spn1- strain. No autolysis was observed in the absence of LytA. In agreement with *in vitro* competitions (Figure 3B), the Spn1+ strain outgrew the Spn1- strain, however, this effect was reversed in the absence of LytA (Figure 5A). The presence or absence of Mml had no effect on growth or autolysis (data not shown). We checked levels of LytA expression by Western blot and detected no difference in expression of LytA

between Spn1+ and Spn1- strains (Figure 5B).

To determine if Spn1 affected LytA activity, we complemented LytA activity by comparing growth of LytA+ and LytA- bacteria singly and in a co-culture. If active, the LytA secreted from the LytA+ strain should be able to lyse the LytA- strain, resulting in a growth curve similar to that of a LytA+ strain grown in isolation. To determine if Spn1 conferred resistance to LytA, we measured the growth of a co-culture of LytA+ and LytA- strains in both Spn1+ and Spn1- backgrounds. As expected, the growth pattern mimicked that of a LytA+ strain grown in isolation, indicating the LytA is active and able to lyse the co-cultured strain in both the Spn1+ and the Spn1- backgrounds (Figure 5C). This was confirmed by plating bacteria and colony counts at 0, 5 and 8 hours (data not shown). To test if LytA's target, the cell wall, is altered in the Spn1+ strain, we co-cultured the Spn1+ and Spn1- strains where both strains contained LytA. The growth curve mimicked the curve of Spn1+ strains grown in isolation (Figure 5C). This leads us to conclude that Spn1 confers resistance to LytA-mediated autolysis.

LytA has previously been associated with virulence(116). To determine if the delay in autolysis was the cause of the fitness defect *in vivo*, we competed Spn1+Δ*lytA* and Spn1-Δ*lytA* strains 1:1 in the murine model of colonization. The competitive advantage of the Spn1- strain was *lytA*-independent (Figure 3C). Thus, the altered cell wall physiology conferred by Spn1 may result in a fitness defect *in vivo*

independent of its effects on promoting resistance to LytA-mediated autolysis.

Spn1 affects chain length

LytA is also involved in cleavage of the cell wall to allow for cell separation during division. Deletion of *lytA* leads to the formation of long chains (130). Additionally, chain length has been shown to have an important role during both colonization and invasive disease (38, 126). Using brightfield microscopy we observed that the mean chain length of the Spn1- strain was greater than the Spn1+ strain (Fig 6A&B). The role of the phage encoded homologue Mml in septation and chain length has not been elucidated. Surprisingly deletion of *lytA* or *mml* did not lead to long chains in Spn1+ bacteria. However, Spn1+Δ*lytAΔmml* bacteria did form long chains – a result consistent with the functional redundancy of these amidases. Even when the effects of Mml are accounted for by comparing the Spn1- strain to the Spn1+Δ*mml* strain, the Spn1- strain grows as longer chains than the Spn1+ strain. The observations on chaining provided further evidence that the presence of Spn1 affects cell wall physiology.

To determine if the difference in chain length was causing the fitness defect *in vivo*, we assessed competitive colonization of the Spn1-Δ*lytA* strain and the Spn1+Δ*lytAΔmml* strain, which both grow as long chains. Despite controlling for chain length, the *in vivo* fitness defect of the Spn1+ background remained (Figure

3C).

Spn1 affects penicillin-mediated lysis

Penicillin acts by inhibiting penicillin-binding proteins, which are required for cross linking of the peptidoglycan backbone to prevent osmotic lysis (159). Strain P1121 is considered sensitive to penicillin (minimum inhibitory concentration (MIC) 50ng/mL). However, to further demonstrate that Spn1 altered the pneumococcal cell wall, we examined whether there were small differences in penicillin sensitivity conferred by the Spn1 prophage. The presence of Spn1 resulted in a five-fold higher MIC to penicillin. This effect was also demonstrated by differences in growth of Spn1+ and Spn1- strains at concentrations of penicillin near the MIC (Fig. 6C). This difference in growth in the presence of penicillin was independent of LytA (Data not shown).

Discussion

Our findings demonstrate that expression of the phage-like genetic element Spn1 affects cell wall physiology and nasopharyngeal colonization of *S. pneumoniae*. Several aspects of this study merit further comment. Excision and integration of the Spn1 element occurs at the same site as the related phage MM1 (60). Through PCR analysis it appears that a circularized form of the prophage occurs during normal bacterial growth, although we were unable to detect packaged phage by

transmission electron microscopy or DNAse protection assays. We were also unable to demonstrate phage infectivity by plaque assays (even after induction by mitomycin C), transduction, or by detection of lysogenic conversion after co-culture, *in vitro* or *in vivo*. We were also unable to infect TIGR4 or R36A strains with Spn1. It remains possible that under different growth conditions or in its natural environment, Spn1 forms infectious phage particles. Spn1 could have become defective after infecting this strain or it could have been obtained through transformation rather than infection. It was unexpected that the lytic genes of Spn1 were expressed during normal growth yet we did not detect increased bacterial lysis compared to an isogenic strain lacking Spn1. The circularized form may be a precursor to Spn1 gene expression (and allow for amplification of gene expression), since mutations at both ends of the element in Spn1+Δ*int*Δ*mml* prevented both circularization and expression of lytic and lysogenic genes. The inability of Spn1 to silence expression of lytic genes, which has not been observed in wild-type lambdoid phages, appears to have required adaptation by the host bacterium (121).

The tolerance of prophage gene expression, even when further induced with mitomycin C, suggests that the phage lysins, which usually target the cell wall, are ineffective against strain P1121. It is possible that the lysins are inactive or only a small portion of the population is being activated by mitomycin C. However, our observations suggest that strain P1121 modifies its cell wall so that it is less susceptible to lysis. Several lines of evidence point to an alteration in cell wall

physiology when this strain carries Spn1. The Spn1+ strain has a delayed autolysis phenotype, not due to any alteration in amounts or activity of the major amidase responsible for autolysis, LytA. This suggests that LytA's target, the cell wall, is more resistant to its enzymatic activity. Moreover, the Spn1+ strain showed an increased resistance to penicillin. We did not detect an elevated amount of peptidoglycan when purified from the Spn1+ strain or a thicker cell wall in electron micrographs of the Spn1+ strain (data not shown). The effect of penicillin suggests that alteration of the cell wall could be due to increased cross-linking, since this is the step inhibited by the drug. Increased integrity through more extensive cross-linking within the cell wall could also explain the increased resistance to LytA and delayed autolysis – phenotypes previously linked to altered penicillin resistance (104, 113). Finally, alterations in cell wall physiology may be seen as differences in cell division/separation resulting in abnormal chain formation. The presence of Spn1 correlated with decreased average chain length.

Studies on LytA have shown that *lytA* mutants do not autolyse and display increased chain length (130). However, in this study, these effects of LytA were unpaired. The Spn1+ strain demonstrated delayed autolysis indicating resistance to LytA, yet shorter chains, which was previously thought to be associated with an increase in susceptibility to LytA. This apparent discrepancy could be indicative of the secretion and localization of LytA on the bacterial cell surface. A recent study on Mml and LytA showed that phosphorylcholine is required for secretion of both of

these lysins. The authors of that study proposed that LytA binds the phosphorylcholine as it is added to the cell wall during division (56). This is supported by another study demonstrating that LytA is localized to the nascent peptidoglycan (104). This suggests that the change in the cell wall that leads to resistance to LytA in the Spn1+ strain might occur during maturation of the peptidoglycan, leaving the nascent peptidoglcyan formed during division susceptible to LytA-mediated cleavage, resulting in reduced chain length.

The precise nature of the alteration in the pneumococcal cell wall required for tolerance of Spn1 is the subject of further investigation. Factors regulating cell wall synthesis, quantity of peptidoglycan per cell and level of cross-linking are incompletely understood. These differences could be the direct action of an unknown protein encoded on the prophage. However, it is possible that the over-production of prophage products, including the lysin and holins, leads to a general stress response in the host bacterium. Stress responses in bacteria often induce changes in the cell wall. For example in *Staphylococcus aureus* there is evidence of cell wall thickening in the presence of vancomycin (34). Also the CiaRH two-component system in *S. pneumoniae* is thought to maintain cell wall integrity in the presence of various stressors (97).

The presence of Spn1 was associated with reduced fitness during competitive colonization – an effect that may be related to the changes in its cell wall. There are

several phenotypes associated with the presence of Spn1 that may affect fitness during colonization. Most notably chain length increases adherence to epithelial cells (126), and the presence and action of LytA is important during pathogenesis, although the exact mechanism is unclear (116). However, when we eliminate differences in chain length and the effects of LytA, but maintain the presence of Spn1, we still observe a fitness defect in the Spn1+ strain during competitive colonization. This implies that the inherent differences in the cell wall, not these subsequent phenotypes, are responsible for the fitness defect *in vivo.* Theoretically, alteration of the cell wall in response to the prophage could be a metabolic burden on the Spn1+ strain, which gives the Spn1- strain the edge in competitive colonization. The *in vitro* competition data supports this concept since the competitive effect is lost in a nutrient rich environment. Additionally, the cell wall anchors many factors linked to fitness for colonization, which could also be playing a role (81).

The competitive fitness defect and alteration of the cell wall are both dependent on prophage gene expression. This indicates that a *trans*-acting factor encoded on the prophage could be responsible for these phenotypes. We eliminated the potential contribution of three candidate trans-acting factors, Mml, PblB and Integrase. Mml has previously been shown to be redundant with LytA in phage progeny release indicating it contributes to bacterial lysis (57). Integrase is a DNA binding protein involved in the excision and integration of the prophage (115). PblB was originally

annotated on phages of *Streptococcus mitis* and is thought to mediate adherence to platelets (14). We saw no differences in adherence of the Spn1+ and Spn1- bacteria to A549 human epithelial cells in culture (data not shown). While we eliminated several potential *trans*-acting factors, there are ~63 open reading frames annotated on Spn1, many of which have an unknown function and could be affecting bacterial fitness. The increased protein-synthesis burden of carrying Spn1 could also account for the effect on fitness *in vivo.*

The question remains as to why the bacterium does not spontaneously cure Spn1 if it is associated with a fitness cost. *S. pneumoniae* is highly proficient at recombination and it should readily eliminate any disadvantageous genetic elements, yet Spn1 remains stably integrated (71). Moreover, in a study of human experimental pneumococcal colonization, Spn1 was present in the initial clinical isolate used to inoculate several volunteers, and also in all the isolates retrieved from participants up to 122 days later (101)(data not shown). Although this lineage was successful at colonization in this study, it may not be adept at competing with other pneumococci. Colonization by multiple strains of pneumococci commonly occurs in children (25). Prophages are known to protect against super-infection by other related phages. It is possible that selective pressure from other phages during natural colonization is sufficient to maintain this element.

In conclusion, we have determined that a novel prophage, Spn1, is associated with

altering the bacterial cell wall and a fitness defect during competitive colonization. This type of interaction between a prophage and its bacterial host, where the bacteria tolerate the phage despite its negative effects, has, to our knowledge, not been described previously.

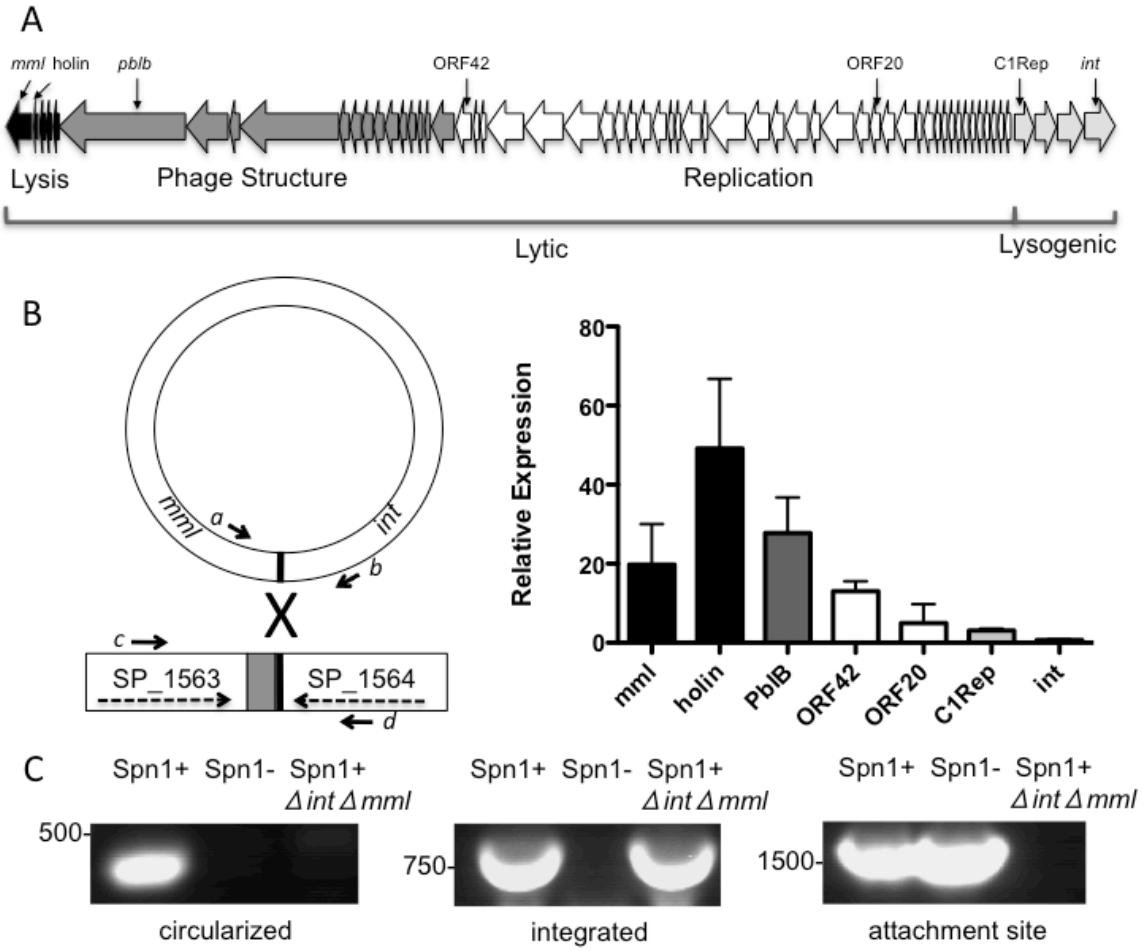


Figure 1. Genetic map and activity of Spn1 **A.** The strain P1121, including the genetic element Spn1, was sequenced on an Illumina Hi-Seq and annotated using RAST. Sixty-three open reading frames were identified and are indicated as block arrows showing the direction of transcription. Genes were arranged in lytic or lysogenic clusters by putative function in; lysis (black), structural genes (dark gray), replication genes (white) and lysogeny (light gray). **B**. Schematic of Spn1 (circle) integration between genes *SP_1563* and *SP_1564* (dashed arrows show direction of transcription). The attachment site, 15bp at the 3' end of the coding region of *SP_1564* is indicated by a black bar. The light gray bar indicates a noncoding intergenic region. Small arrows represent the location of primers. **C**. PCR products showing the presence of a circularized element (primers *a* and *b*), integration at the attachment site (primers *a* and *d*), or the attachment site lacking Spn1(primers *c* and *d*) in the indicated strains. Size markers are indicated in base pairs. **D**. Levels of mRNA relative to the housekeeping gene *gyrA* as determined by qRT-PCR for genes across Spn1. The position of specific genes is designated in **A** and shaded to match the functional cluster in the genetic map. Data are shown as Mean ±SEM, *P<0.05, ns, non-significant by Student t-test.

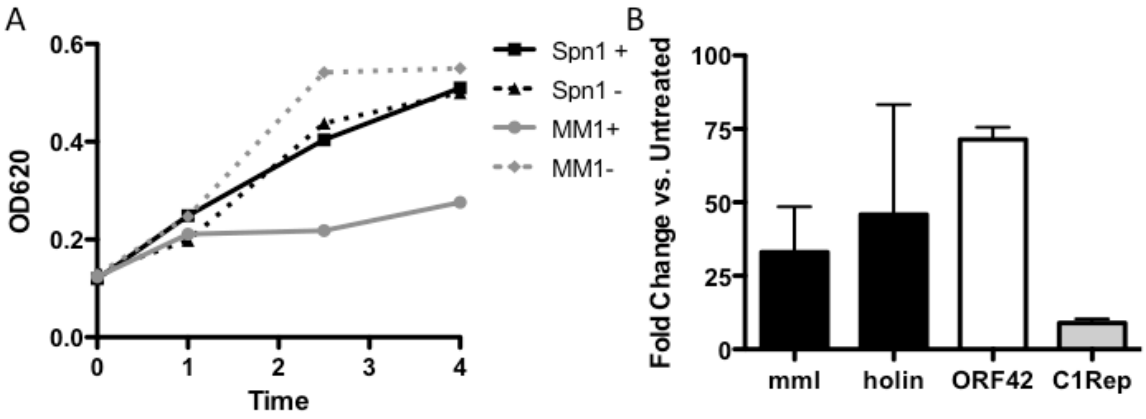


Figure 2. Activation of Spn1 by mitomycin C. **A.** Growth of strain P1121 with or without Spn1 and strain TIGR4 with and without MM1 in C+Y medium. Mitomycin C (5µg/mL) was added at time 0. **B.** Relative transcript abundances as determined by qRT-PCR for the Spn1 gene indicated relative to internal control *gyrA* is shown relative to no mitomycin C treatment. Data are shown as Mean ±SEM.

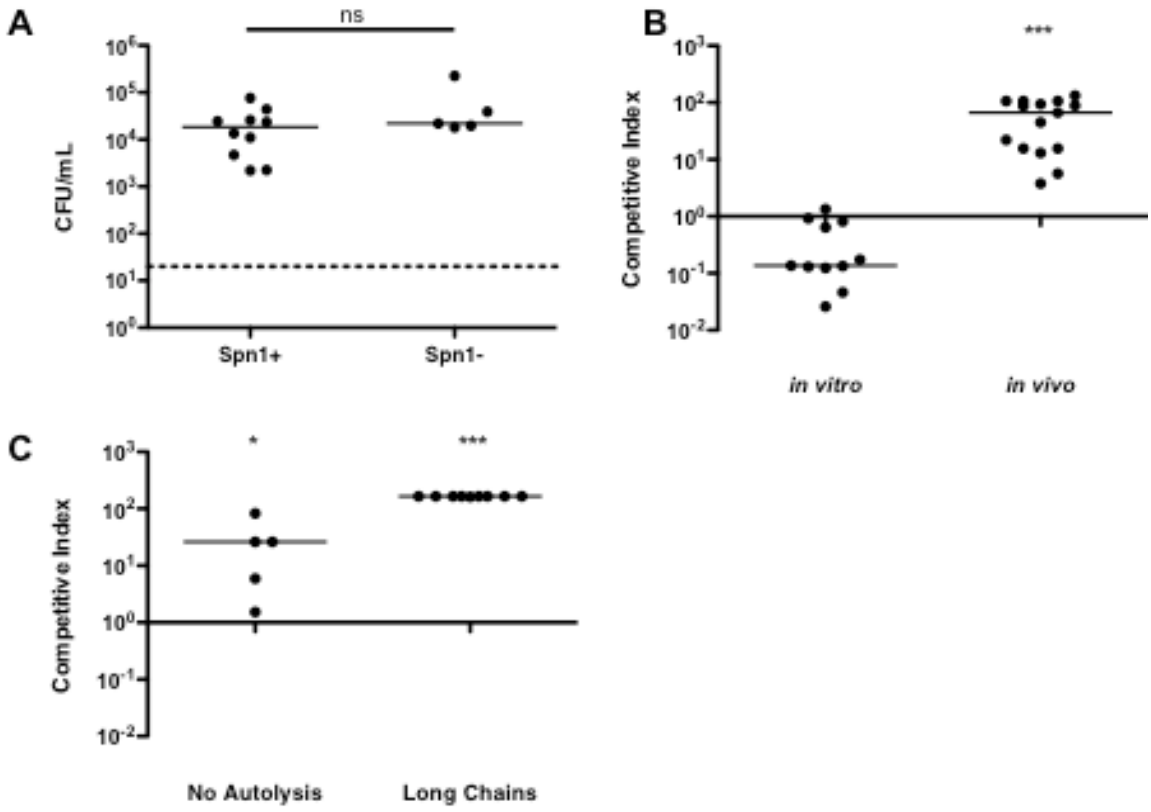


Figure 3. Spn1 in colonization. **A.** Density of Spn1+ and Spn1- in nasal lavages obtained 7 days after inoculation. Dashed line indicates limit of detection. Comparisons between strains were made using the Mann Whitney test. **B**. Competitive indexes (CI=output ratio/input ratio) of Spn1-/Spn1+ following growth for 5 hrs in C+Y medium (*in vitro*) or 7 days of colonization (*in vivo*). **C.** Competitive indexes for Spn1-Δ*lytA*/Spn1+Δ*lytA* and Spn1-Δ*lytA*/Spn1+Δ*lytA*Δ*Mml* to determine the competitive advantage without autolysis and with equivalent chain length, respectively. Each symbol is from a single mouse or culture. CI is based on patching ≥50 colonies on selective medium to distinguish strains. One sample t-test was used to determine if CIs were significantly different from a hypothetical value of 1, which would indicate no competitive advantage or disadvantage. *P<0.05, ***P<0.001. ns, non-significant.

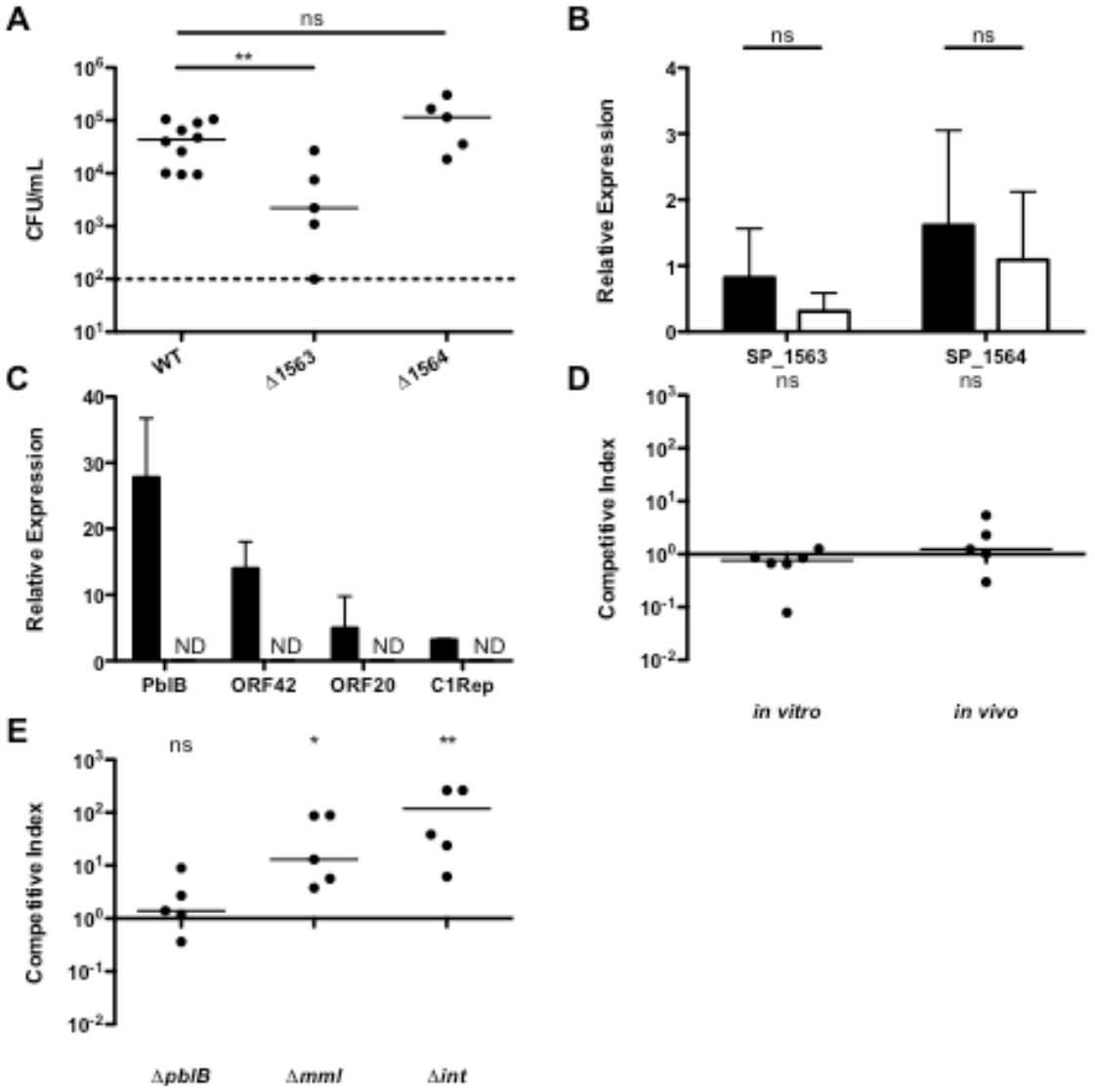


Figure 4. Spn1 gene expression is required for the fitness defect *in vivo* **A.** Density of Spn1+(WT), Spn1+Δ*SP_1563* and Spn1+Δ*SP_1564* in nasal lavages obtained 7 days after inoculation. Comparisons between mutant and WT strains were made by Mann Whitney test. **B.** Levels of mRNA relative to the internal control *gyrA* as determined by qRT-PCR of genes *SP_1563* and *SP_1564* in the presence (black bars) or absence (white bars) of Spn1. Data are shown as Mean ±SEM. **C**. Levels of mRNA compared to internal control *gyrA* as determined by qRT-PCR for sample phage genes in either the Spn1+ strain or the Spn1+Δ*int*Δ*Mml* strain. Expression was undetected (ND) in the Spn1+Δ*int*Δ*mml* strain. Data are shown as Mean ±SEM. **D.** Competitive indexes of Spn1-/ Spn1+Δ*int*Δ*mml* following growth for 5 hours in C+Y medium or 7 days of colonization. **E.** Competitive indexes of Spn1+Δ*pblB*/Spn1+, Spn1-/Spn1+Δ*mml* and Spn1-/Spn1+Δ*int* following 7 days of colonization. One sample t-test was used to determine significant difference from a hypothetical value of 1, which would indicate no competitive advantage. *P<0.05, **P<0.01, ns, non-significant.

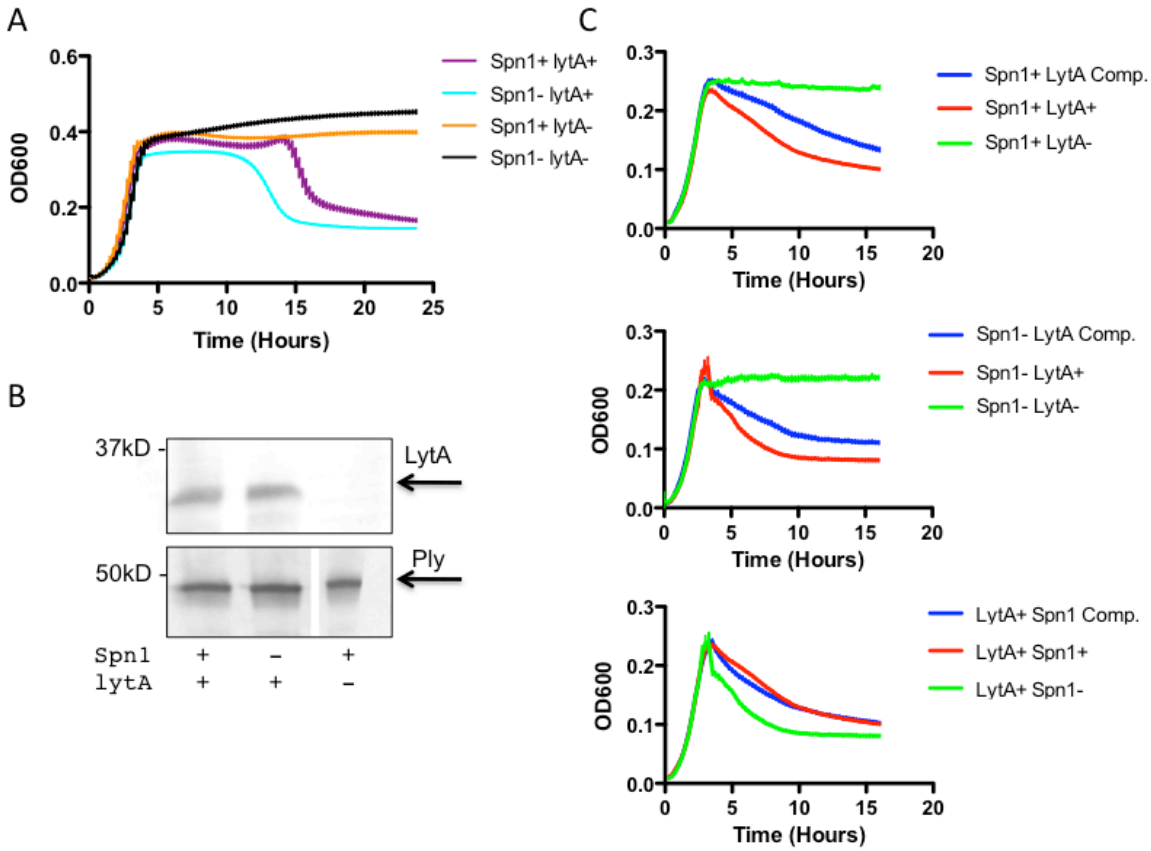


Figure 5. Spn1 has an effect on LytA-mediated autolysis. **A**. Growth and autolysis of strain P1121 (Spn1+) and designated mutants in C+Y broth medium over 24 hours at 37°C. Data are shown as Mean ±SEM. **B.** Protein levels of LytA as determined by Western blot on whole cell lysates of Spn1+, Spn1- and Spn1+Δ*lytA* strains with anti-LytA antibody. Pneumolysin was used as a loading control**. C.** Growth and autolysis of strain P1121 and designated mutants with and without LytA or Spn1 complemented by co-culture in BHI for 16 hours at 37°C. For each graph shown, red and green lines are strains grown in isolation. Blue line is the co-culture of those two strains. Data are shown as Mean ±SEM.

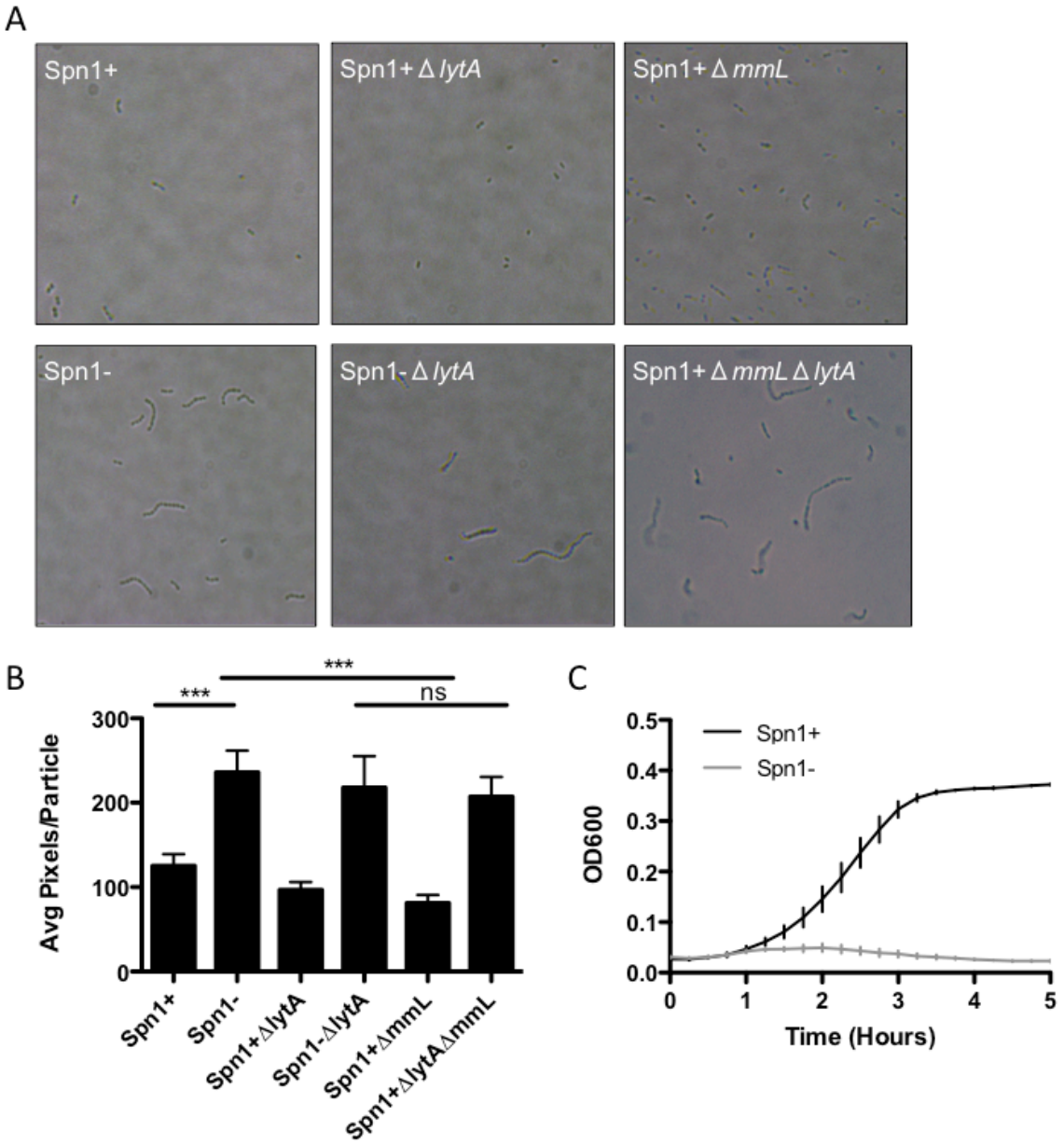


Figure 6. Spn1 has an effect on the cell wall. **A.** Images taken of strain P1121 and mutant indicated in mid-log phase culture using a brightfield microscope. **B.** Average pixels per particle as determined by ImageJ analysis to quantify chain length. Student T test was used to compare groups. ***P<0.001. ns, non-significant. Data are shown as Mean ±SEM. **C.** Growth of Spn1+ and Spn1- strains in 30ng/mL of penicillin in TS over 5 hours at 37°C. Data are shown as Mean ±SEM.

Table 1. Bacterial Strains

Strain No.	*Designation*	*Background*	*Description*[a]	*Source*
P1121	Spn1+		Human challenge study isolate	(101)
P1397	Spn1+ Sm^r	P1121	Sm^r spontaneous mutation	This study
P2197	MM1-	TIGR4		(92)
P2198	MM1+	TIGR4		(92)
P2282	Spn1+Δ*lytA*	P1121	*lytA*::Sp^r	This study
P2308	Spn1+Δ*mml*	P1121	*mml*::Km^r	This study
P2345	Spn1+Δ*SP_1563*	P1121	*sp_1563*::Sp^r	This study
P2348	Spn1+Δ*SP_1564*	P1121	*sp_1564*::Em^r	This study
P2352	Spn1- Sm^r	P1397	unmarked in-frame Spn1 deletion, Sm^r	This study
P2373	Spn1+Δ*pblB*	P1121	*pblB*::Em^r	This study
P2379	Spn1-Δ*lytA*	P2385	*lytA*::Sp^r transformed with P2282	This study
P2380	Spn1+Δ*mml*Δ*lytA*	P2308	*mml*::Km^r *lytA*:Sp^r transformed with P2282	This study
P2385	Spn1-	P2352	transformed with P1121 to remove Sm^r	This study
P2386	Spn1+Δ*int*Δ*mml*	P2385	*mml*:: Km^r *int*::Em^r transformed with P2387	This study
P2387	Spn1+Δ*int*Δ*mml*	P2308	*mml*::Km^r *int*::Em^r transformed with 2388	This study
P2388	Spn1+Δ*int*	P1121	*int*::Em^r	This study

a Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Km^r, kanamycin resistant; Em^r, erythromycin resistant

Table 2. Primers for mutant construction and qRT-PCR

Primer	*Gene*	*Direction*	*Sequence*	*Source*
AttF	*SP_1563*	Forward	TGTGGGTGGTGGTCCTGTCG	This study
AttR	*SP_1564*	Reverse	ATGCCAAACTGGCCCGTCAC	This study
EGP4	*int*	Forward	GAAGATAGGAGGATAAACTGG	(60)
EGP9	*mml*	Reverse	AACTGCAGAAATTGTTCTTTCACCGCAGG	(60)
Spn1-2	*mml*	Reverse	CATTATCCATTAAAAATCAAACAACTGCAGAAATTGTTCTTTCACCGCAGG	This study
Spn1-3	*Janus*	Forward	CCTGCGGTGAAAGAACAATTTCTGCAGTTGTTTGATTTTTAATGGATAATG	This study
Spn1-4	*Janus*	Reverse	CCAGTTTATCCTCCTATCTTCCTTTCCTTATGCTTTTGGAC	This study
Spn1-5	*int*	Forward	GTCCAAAAGCATAAGGAAAGGAAGATAGGAGGATAAACTGG	This study
1563-1	*SP_1561*	Forward	TATAGCCGCCCGGTGTCTGG	This study
1563-2	*SP_1563*	Reverse	CACTTTATTAATTTGTTCGTATGTATTCAGTTTCTCCTTTGTTTTTTCTAGTCAGTTTAT	This study
1563-3	*SpecR*	Forward	ATAAACTGACTAGAAAAAACAAAGGAGAAACTGAATACATACGAACAAATTAATAAAGTG	This study
1563-4	*SpecR*	Reverse	CGAATCCTATGTGACTCGTGGTTCTTTTTTCCCGAGCTCGAATTGACGCGGATCC	This study
1563-5	*SP_1563*	Forward	GGATCCGCGTCAATTCGAGCTCGGGAAAAAAGAACCACGAGTCACATAGGATTCG	This study
1563-6	*mml*	Reverse	CGCTGCAACAGGCTGGCAGA	This study
1564-1	*int*	Forward	TGGAGAAGAAAACTCCCCAGGCA	This study
1564-2	*SP_1564*	Reverse	GGTGCAAGTCAGCACGAACGATATAAAGCAACCCCTTGAATTATCAA	This study
1564-3	*ermR*	Forward	ATAATTCAAGGGGTTGCTTTATATCGTTCGTGCTGACTTGCACC	This study
1564-4	*ermR*	Reverse	GATGCGCATTATACAGGTGAAAAATGAGTAACGTGTAACTTTCCAAAT	This study
1564-5	*SP_1564*	Forward	ATTTGGAAAGTTACACGTTACTCATTTTTTCACCTGTATAATGCGCATC	This study
1564-6	*SP_1565*	Reverse	GGCCAGCCGTCGAGTAGTGC	This study
mml-1	*SP_1563*	Forward	GCAGCCTTTTATGCCCACCTACGCC	This study
mml-2	*mml*	Reverse	CTCTGGAATAGGCATAGACACTATCCACCGCAGGCTCAGGCTTGCGG	This study
mml-3	*kanR*	Forward	CCGCAAGCCTGAGCCCTGCGGTGGATAGTGTCTATGCCTATTCCAGAG	This study
mml-4	*kanR*	Reverse	GACGCATGGAAAGGACGATAGGGACACGTTTTTGTGGTGAGAAAC	This study
mml-5	*mml*	Forward	GTTTCTTCACCACAAAAACGTGTCCCTATCGTCCTTTCCATGCGTC	This study
mml-6	*pblB*	Reverse	CTGAGCATAAGGAATAGGAGGTGTG	This study
int-1	*int*	Forward	ATGTGGATGGAAGAACTTTCCAAC	This study
int-2	*int*	Reverse	GGTGCAAGTCAGCACGAAACAAGTGCTTTTATTTCTTGCATGGT	This study
int-3	*ermR*	Forward	ACCATGCAAGAAATAAAAGCACTTGTTTCGTGCTGACTTGCACC	This study
int-4	*ermR*	Reverse	GTACTTTTGGTGATATTCTCGACGATTAAGAGTAACGTGTAACTTTCCAAAT	This study
int-5	*int*	Forward	ATTTGGAAAGTTACACGTTACTCTTAATCGTCGAGAATATCACCAAAAGTAC	This study
int-6	*int*	Reverse	AGTATCTAATTTATTGACCAGTTTCTCCTCC	This study
PblB-1	*ORF61*	Forward	GAAGCTGGTAAAGAGGCGGT	This study
PblB-2	*pblB*	Reverse	ATGGTGCAAGTCAGCACGAATCCTTGGCAAACATGGCTCT	This study
PblB-3	*ermR*	Forward	AGAGCCATGTTTGCCAAGGATTCGTGCTGACTTGCACCAT	This study
PblB-4	*ermR*	Reverse	ATGGTTTCAGCCTCTTGAGCTAGTAACGTGTAACTTTCCAA	This study
PblB-5	*pblB*	Forward	TTGGAAAGTTACACGTTACTAGCTCAAGAGGCTGAAACCAT	This study
PblB-6	*ORF55*	Reverse	GTCAGGATTGCCCTCTGCAT	This study
LytA-1	*dinF*	Forward	TTGGCTAGTTCGACAGATGGTTAC	This study
LytA-2	*lytA*	Reverse	CACTTTATTAATTTGTTCGTATGTATTCTTCTACTCCTTATCAATTAAAACAACTCA	This study

LytA-3	*specR*	Forward	TGAGTTGTTTTAATTGATAAGGAGTAGAAGAATACATACGA ACAAATTAATAAAGTG	This study
LytA-4	*specR*	Reverse	ATGCGCTGTTCTGATTTGAAAGACATTCCCCCGAGCTCGA ATTGACGCGGATCC	This study
LytA-5	*lytA*	Forward	GGATCCGCGTCAATTCGAGCTCGGGGGAATGTCTTTCAAA TCAGAACAGCGCAT	This study
LytA-6	*SP_1935*	Reverse	TGAGTTCTATTGGCATTTTCTCTG	This study
RTmmlF	*mml*	Forward	CCACTCAACAGGCAACCGTA	This study
RTmmlR	*mml*	Reverse	CTTCCGTTGTTCACAGGACC	This study
RTholinF	*holin*	Forward	TTGCGTAAAGGCGGAGAGAA	This study
RTholinR	*holin*	Reverse	AGCTACTTGCTCAACGGCAT	This study
RTPblBF	*pblB*	Forward	TATCAAGGCCGAATCGGTGG	This study
RTPblBR	*pblB*	Reverse	GGCACCATTCCCCATACCAA	This study
RTORF42F	*ORF42*	Forward	GGACGCAGACTACAGCAAGT	This study
RTORF42R	*ORF42*	Reverse	CATATCGGCAGGCACGTACT	This study
RTORF20F	*ORF20*	Forward	AAGAGTCAAATCGGTGGCGT	This study
RTORF20R	*ORF20*	Reverse	GCTCCTTTGTCAGCAATCGAC	This study
RTC1RepF	*C1Rep*	Forward	TCGCACAGATAATCCTACCATCGCT	This study
RTC1RepR	*C1Rep*	Reverse	TGAAGCGCATAGCAGTAGAGGCA	This study
RTintF	*int*	Forward	CGACTTAGCGAACGTTTCCAGCCA	This study
RTintR	*int*	Reverse	ACGTGGGGGTCGTGGTGTCC	This study
RT1563F	*SP_1563*	Forward	CGACCTCAACCGTCACAAGA	This study
RT1563R	*SP_1563*	Reverse	TAGCAGCAGTCACCGATAGC	This study
RT1564F	*SP_1564*	Forward	AGGTCGTTACGGGTTTCTCG	This study
RT1564R	*SP_1564*	Reverse	ACCTATCTCCAGCGAGCAGA	This study
RTgyrAF	*gyrA*	Forward	GCCCTTTGGCAGTCCGACCA	This study
RTgyrAR	*gyrA*	Reverse	ACGTGGGGGTCGTGGTGTCC	This study

Acknowledgements

This work was supported by grants from The National Institutes of Health to J.N.W. (R01 AI078538, T32 A1060516), The Morphology Core of the Center for Molecular Studies in Digestive and Liver Diseases (NIH P30 DK050306) and the Electron Microscopy Resource Laboratory.

Chapter 4

Discussion and Conclusions

Discussion

Spn1 gene expression

Via qRT-PCR we were able to detect the expression of phage genes in the pneumococcus under normal growth conditions and an increase in phage gene expression with the addition of mitomycin C. There are two possible explanations for the data we have seen regarding phage gene transcription. One explanation is the entire population of bacteria is constitutively expressing all phage genes at a low level all the time and addition of mitomycin C leads to an increase in gene expression across Spn1. The other possibility is that a subset of the population of P1121 is spontaneously entering the lytic cycle. This would mean that part of the population is expressing lysogenic genes, while the rest of the population is expressing lytic genes. When mitomycin C is added we could be seeing a shift in the ratios of these two populations. Bacteria commonly have mechanisms, such as phase variation, to create subsets of populations expressing different genes (69). This heterogeneity allows the population as a whole to adapt to changing environments. The qRT-PCR assay cannot distinguish between these two possibilities. In either case the expression of lysins, holins and other phage genes, whether it be from within each bacterium or from a neighboring bacterium, appears to be having an effect on the overall physiology and fitness of strain P1121.

Spn1 infectivity

Though we were able to detect phage excision and gene expression, we could not detect phage particles or infectivity despite extensive attempts to do so. Four techniques were attempted, plaque assays, selection after lysogenic conversion, DNase protection assays and electron microscopy.

Plaque assays using *Streptococcus pneumoniae* are known to be difficult and successful plaque assays are usually performed under non-physiologic condition. For example phages will not infect encapsulated pneumococci in a laboratory setting (17). Typically the only indicator strain used for plaque assays is R36A an unencapsulated lab strain. The correct growth conditions for top agar is also difficult due to *S. pneumoniae*'s oxygen requirements and hydrogen peroxide production. Typically S*. pneumoniae* will only form micro-colonies within the top agar instead of the lawn needed for the spread of phage infection to create a plaque. Lastly the phages themselves seem to be very unstable, lasting only about 24 hours after being harvested. Clearly the high incidence of prophages amongst pneumococcal isolates indicates that phages are successfully infecting the bacteria in their natural environment.

To more closely mimic the infection that might occur in nature, and in order to be able to detect low infection rates, we looked for lysogenic conversion of the Spn1-

strain using a phage marked with a kanamycin resistance cassette in the gene *mml*. It had previously been shown that Mml and LytA were redundant during lysis after phage infection so replacing this gene should have no effect on Spn1's ability to infect other bacteria (57). We used the streptomycin resistance marker to differentiate the originally Spn1+ and Spn1- strains. After co-colonizing mice with Spn1+Δ*mml*(kan^r) and Spn1-(str^r), lavages were plated using a double selection, TS with kanamycin and streptomycin. No colonies grew indicating that no Spn1- bacteria had acquired the phage during co-colonization. We repeated this experiment after co-culture in several types of media and buffers and also using antibiotic markers in different phage genes but never detected lysogenic conversion of the Spn1- strain.

It is possible that Spn1 is not infectious but can still make phage particles. In electron micrographs of P1121 from uninduced cultures, we did not see phage particles (Figure 1A). However, with unknown phage morphology, it might be difficult to discern a phage particle from other extracellular structures. Currently no antibodies against any Spn1 encoded protein have been made making further identification using immunogold labeling impossible. To further determine the presence of phage capsids we used a DNase protection assay. In this assay we induced potential phage particles using mitomycin C. We then treated with DNase. If phage capsids are present they will protect phage DNA from the DNase but not bacterial DNA. After DNase treatment we quantified phage DNA using qPCR but

could not reliably detect protection of phage DNA from DNase. Often times phage capsids are not stable. There is the possibility that variations in the conditions of both EM experiments and the DNase protection assays altered stability of the capsid making it difficult for us to detect.

Spn1 stability

Bacteria are rapidly evolving organisms and *S. pneumoniae* is particularly adept at DNA recombination. Therefore it seems unlikely that the pneumococcus would retain a genetic element that confers a fitness defect. Yet phage Spn1 is highly stable, remaining integrated in the bacterial genome throughout an experimental human colonization period of 122 days (101). A simple explanation for this is that Spn1 provides a fitness advantage in some setting other than colonization of a murine nasopharynx.

It is possible that Spn1 provides a fitness advantage during invasive disease. The environment in the bloodstream or in sterile sites such as the lung or middle ear have different selective pressures and require a separate set of pneumococcal genes for survival. We were unable to test the effect of Spn1 on the fitness of P1121 in these environments because this strain of bacteria is avirulent in the mouse model. However, it is unlikely that this is the reason for Spn1's stable presence. Disease states are an evolutionary dead end for the bacteria with no transmission occurring

from any of its disease sites. Therefore, any factor that improves bacterial fitness only in a disease state is unlikely to be maintained.

A key selective pressure in all bacterial pathogens is the ability to be transmitted from one host to another. The pneumococcus is likely transmitted from its carrier state. Co-housed adult mice do no transmit the pneumococcus. However a transmission model was developed using infant mice co-infected with influenza virus (49). This model is very dependent on litter size and therefore highly variable. Recent work in the lab also shows a possible bottleneck, making competitive experiments difficult to analyze. Therefore this model would not be useful in testing the effects of Spn1 on transmission. Another aspect of transmission is survival on fomites such as plastic or other surfaces. The pneumococcus can survive desiccation and persist for up to three weeks on plastic petri dishes (156). We repeated these experiments and did not see improved survival in the Spn1+ strain (data not shown). However, it remains possible that Spn1 improves transmission in some other way.

Another possibility is that the phage has evolved mechanisms to maintain itself. In several species of bacteria, abortive infection systems (ABi) have been characterized as a phage resistance mechanism. These systems detect phage infection and induce an apoptosis like pathway, which limits the spread of phage infection (22). Subsequently, phages have evolved to encode ABi resistance proteins. To our

knowledge, no ABi system has been identified in the pneumococcus. However, Spn1 encodes a putative abortive infection bacteriophage resistance protein in its lysogeny module. Theoretically this factor could be preventing the bacteria from detecting phage infection and inducing apoptosis-like pathway. However, it is unlikely that this highly adaptive species could not evolve to counter resist this mechanism.

The most likely reason for the stability of Spn1 is a difference between the natural human host and the murine model. There are certain host defenses that differ between human and murine immune systems. Also the microbiota of the human nasopharynx is very different from that of a mouse. As the natural host, a human nasopharynx is more likely to carry other strains of pneumococci and pneumococcal phages. One possibility is that P1121 can use Spn1 in bacterial competition similar to the mechanism described in *E. faecalis* where phages released from a lysogen infect and kill competing strains of the same species (52). Another likely possibility is that Spn1 protects the bacteria from super-infection by other phages. The C1repressors of a prophage can bind to invading phages of the same family to prevent superinfection. The pressures from phage in a human nasopharynx might be enough to maintain the presence of Spn1 despite its associated fitness defect. We were unable to test this hypothesis without a working assay to detect phage infection. Lastly the human host from which strain P1121 or its precursor was isolated from could have been exposed to penicillin. Perhaps the small difference in

resistance to penicillin-mediated lysis is enough to maintain the presence of Spn1.

Cell wall in vivo

We have shown that the change in the cell wall associated with Spn1 might be the cause of the fitness defect *in vivo*. There are several potential mechanisms behind this effect. The change in the cell wall might affect metabolic burden or affect the many cell wall bound virulence factors such as choline binding proteins, c-polysaccharide and capsule.

The simplest explanation for a physiological change in the cell wall affecting bacterial fitness *in vivo* is a change in metabolic burden. This mechanism seems likely due to the finding that the fitness defect is ameliorated during growth in nutrient rich broth. Experiments in minimal media to test this possibility were grossly affected by the streptomycin resistance marker and therefore did not provide useful data. Metabolic burden would theoretically also be affected by the burden of translating phage proteins. We found that the Spn1+ strain had a higher protein content, specific to the supernatant, which could indicate the burden of phage proteins (Figure 2A). However, the relative protein content did not change in the presence of mitomycin C, which correlated with the increased gene expression (Figure 2B and Chapter 3 Figure 2B). It is therefore likely that both the metabolic burden of both phage protein synthesis and the compensatory change in the cell

wall are playing a role *in vivo*. However, there are multiple other potential effects that a change in the cell wall could have on bacterial fitness.

Many of the cell wall associated proteins in the pneumococcus, including LytA, bind to the choline containing teichoic acids that form part of the lattice of the cell wall. These proteins are involved in many aspects of colonization and virulence of the pneumococcus (81). The phenotypic effects related to LytA could be due to a change in localization due to altered accessibility of the choline binding domains of these proteins to the teichoic acids in the cell wall. We attempted to test this hypothesis by washing Spn1+ and Spn1- strains with 2% choline, which removes all choline binding proteins from the surface of the pneumococcus, and then performing a western blot for both LytA and the surface binding protein involved in adherence, PspA, which would indicate the quantity of choline binding proteins on the surface. We did not see a difference in the amount of LytA or PspA bound to the surface, however this assay would not indicate differences in localization of choline binding proteins on the cell wall.

Possible alternative structures of the cell wall

Though we have established several phenotypic differences associated with Spn1 that are indicative of a change in the cell wall, we have not determined exactly what this change might be. There are many possible changes including thickening of the

cell wall, increased crosslinking, changes in amount of teichoic acids and modifications to the glycan backbone.

Thickening of the cell wall in response to environmental stress has been seen in the *S. aureus* response to vancomycin (34). In this study, simple electron micrographs were sufficient to see the change in the thickness of the cell wall. In our study, no difference could be seen in micrographs comparing Spn1+ and Spn1- strains and measurements using ImageJ analysis showed no difference (Figure 1A&B). It is possible that a smaller change, not visible by this technique is occurring.

Penicillin and LytA both act (directly or indirectly) on the peptide crosslinks of the peptidoglycan. There is a large variation in pneumococcal muropeptides, the unit of the cell wall consisting of a peptide chain linked to a unit of the glycan backbone. Isolated muropeptides can vary in length of the peptide chain (three four or five peptides), the amount of crosslinks between peptide chains (monomeric, dimeric or trimeric) and branching (containing either a Ser-Ala, Ala-Ala branch or no branch at all) (29). The regulation responsible for these differences in the pneumococcus is unknown. Changes in the composition of peptide chains within the peptidoglycan could be conferring the resistance to LytA and penicillin mediated lysis phenotypes in the Spn1+ strain.

The phenotypes related to LytA and Mml are indicative of the change in the cell wall

being related to maturation of the peptidoglycan. LytA mutants typically have the dual phenotype of lack of autolysis and long chain formation (130). In the Spn1+ strain we see delayed autolysis, indicating resistance to LytA but shorter chain formation indicating an increase in sensitivity to LytA. At the septum, where LytA and LytB are involved in septation, there is either very little cross-linking, or a specific type of cross-linking making it easier for these enzymes to act at this site. As the peptidoglycan matures increased cross-linking is observed (87). This may mean that increased crosslinking during peptidoglycan maturation leads to the seemingly contrary LytA phenotypes in autolysis and chain length. These two phenotypes could also indicate the localization of Mml. Since it is involved in chain formation, but not autolysis, Mml is likely localized to the septum. It is known that during exponential growth LytA is localized to the nascent peptidoglycan (104). Also, LytA and the phage lysin of phage SV-1 are likely co-secreted in a choline dependent, holin independent manner (56). Both of these studies support the idea that Mml is localized at the septum.

The pneumococcus modifies its cell wall in order to resist host defenses such as lysozyme. Specifically, the enzyme PgdA deacetylates the Gluc-nac residue on the glycan backbone of the peptidoglycan to resist the muramidase activity of lysozyme (41). Comparison of Spn1+ and Spn1- strains showed no LytA independent difference in resistance to lysozyme (data not shown). It is unlikely that there is a change in the glycan backbone of the cell wall since the phenotypes observed of the

Spn1+ strain in autolysis and pencillin resistance are related to the peptide cross-links. However, the importance of changes to the glycan backbone during colonization has laid the groundwork for the importance of the structural components of the cell wall as a target for host defenses *in vivo*.

Lastly, the cell wall change associated with Spn1 could be within the teichoic (WTA) and lipoteichoic (LTA) acids. WTA is made up of repeating units and can differ in length. Also the amount of phosphoryl choline bound to each of these units is variable. These two aspects of the WTA are thought to be the same in LTA (29). Length and amount of choline would likely alter accessibility of WTA and LTA to choline binding proteins, which, as I have already mentioned, are frequently important for pneumococcal virulence. Specifically, the availability of WTA and LTA to LytA for binding to the cell surface could affect the phenotypes of autolysis and chain length.

Mechanism of Spn1 effect on cell wall

Though we can detect phenotypic differences associated with the presence of Spn1 we have not determined a mechanism by which Spn1 may be affecting these changes. It is possible that an unknown gene on Spn1 is directly involved in altering the cell wall. However, it is also possible that expression of holins and lysins has led to a general stress response by the bacteria. Three stress response systems in

Streptococcus pneumoniae, the Clpl heat shock protein and the CiaRH and WalRK two component systems, have been shown to lead to changes in bacterial physiology including the structure of the cell wall.

The heat shock protein ClpL affects the pneumococcal cell wall and penicillin resistance (146). In response to heat shock, a commonly used stress inducer, ClpL upregulates transcription of the *pbp2X* gene and directly interacts with Pbp2X helping it translocate across the membrane. Penicillin binding proteins are largely responsible for crosslinking the peptide bridges of peptidoglycan. In this study they also saw a ClpL dependent thickening of the cell wall, which may indicate that it is not involved in the Spn1 associated effects.

The two component WalRK system, responds to stress and regulates several genes that modify the peptidoglycan in *Streptococcus pneumoniae*. The WalRK system has been shown to respond to oxidative stress and mutations in this pathway lead to resistance to vancomycin and daptomycin (51). The regulon of WalRK includes the essential PcsB protein, which works in conjunction with $FtsX_{Spn}$ a protein involved in cell division (134). WalRK also regulates several non-essential peptidoglycan hydrolases and carboxy-peptidases, including LytB, that can alter cell morphology (11). The role for WalR and WalK *in vivo* is strain dependent with some studies showing virulence defects in WalR or WalK mutants and others reporting no difference between WalR and WalK mutants and wild-type strains (51).

The CiaR and CiaH two component system also alters the cell wall in response to stress. Activation of the CiaRH system leads to resistance to many cell wall inhibiting antibiotics such as cycloserine and bacitracin (97). Disruption of the CiaRH system leads also leads to early autolysis and loss of competence (36). Furthermore, CiaRH mutants have defects during colonization (131). Based on the similarity between phenotypes elicited by Spn1 and CiaRH mutants it is possible that this system is involved in the effects we have shown.

A likely stress inducer produced by phage would be holins. Holins are small proteins that oligomerize to form a pore in the membrane. In lambda phages, this pore allows the phage to time the release of phage lysins, however in pneumococcal phages it seems to trigger a loss of membrane integrity and proton gradient leading to activation of lysins (56). We tested the fitness of a mutant lacking the entire lysis cluster, Mml and four putative holins. This mutant had similar penicillin resistance to the WT Spn1+ strain (Figure 3A). Also, this mutant did not outcompete the wild type Spn1+ strain *in vivo* (Figure 3B). Therefore we concluded that the holins alone or in combination with Mml are not the trans-acting factor involved in the Spn1 associated fitness defect and are not triggering a bacterial stress response.

Future Directions

There are several questions brought up in the previous discussion that merit further experimentation.

I discussed the possibility that phage stability is indicative of a difference in the model host versus the natural host. To test this we could repeat the experimental human carriage studies to test competitive fitness of the Spn1+ and Spn1- strains. Furthermore, we could look at samples from epidemiology studies on co-colonization to see if the presence of Spn1 correlates with a lack of co-colonizing strains. Lytic phage stocks of CP-1 and DP-1 are available commercially and could be used to check for phage resistance in the Spn1+ strain. Though it is unlikely that Spn1 confers a resistance to these phages because they are not of the same family, they are amongst the most common virulent phages found.

Another future direction is to uncover the mechanism by which Spn1 confers a change in the cell wall and therefore lead to a defect during colonization. One possible technique to pursue this question is using RNAseq to determine differences in bacterial gene transcription between Spn1+ and Spn1- strains. We could then look more specifically at the genes involved in the pneumococcal stress response, such as the genes regulated by the CiaRH system or WalRK system and *clpL* using

qRT-PCR. To further determine the regulation by these two compnent systems we could use western blots to determine the phosphorylation status of the signaling component of each of these two component systems. We could also look downstream of these regulatory genes at transcript levels of PBPs and see if changes in PBP transcription are dependent on the CiaR/H system, WalRK system or ClpL.

To test the effects of metabolic burden we could remove large, non-specific sections of phage genes. If fitness correlates roughly with numbers of genes removed, but not any one specific gene, it could be simply an effect of protein synthesis burden. This method could also potentially identify a specific phage gene involved in the fitness defect and/or the change in the cell wall.

The best way to determine the exact change in the cell wall is by isolating the different components of the peptidoglycan and using biochemical analyses to determine variations in branching or crosslinking between the Spn1+ and Spn1- strain. After purification of the peptidolglycan, treatment with LytA will separate glycan strands from peptides. Treatment with cellosyl will cleave the peptidoglycan into muropeptides. Analysis of glycan strands by HPLC will indicate length of glycan strands, which is indicative of their acetylation. Analysis of muropeptides by HPLC and mass spectrometry will indicate glycan modifications, cross-linking and length of peptide chain. WTA can be isolated from cell wall treated with both cellosyl and LytA by size exclusion chromatography. Mass spec analysis of the WTA will indicate

number of repeats and amount of phosphocholine within a chain. Once the exact change in the cell wall is determined it may be easier to determine the mechanism by which Spn1 confers this change. (29)

Lastly, it would be interesting to determine if the relationship between Spn1 and P1121 is unique or if it is found in other pneumococcal strains. We could first look at similar phages such as phage 040922 and the bacterial isolate it was found in to see if phage gene expression is similar to what we see in Spn1. We could then follow up with assays to assess fitness and physiology in this pair. We could also design a PCR based prophage typing assay specific to Spn1-like phages and use it to test large strain collections of the pneumococcus.

Conclusions

Although many phages confer a fitness advantage to their bacterial host during colonization and disease, the phage element Spn1 does the opposite during colonization in a murine model. This phage element is novel and has a unique symbioses with its bacterial host, the pneumococcal strain P1121. Spn1 expresses both lytic and lysogenic genes within a normal growing population. This is different from the carefully controlled gene expression seen in wild-type lambda phages. For some unknown reason, the bacteria is tolerating the presence of Spn1 rather than eliminating it. However, this tolerance has led to physiological changes in the bacterium that may result in decreased fitness in vivo.

The Spn1+ and Spn1- strains both colonize the murine nasopharynx adequately. However, when assessed in a competitive assay, the Spn1- strain outcompeted the Spn1+ strain. This fitness advantage was dependent on phage gene expression. Other phenotypes associated with the presence of Spn1 all indicate a change in the cell wall. Spn1 confers a resistance to LytA mediated autolysis and to lysis by the action of penicillin. Both of these phenotypes reflect possible changes in the peptide crossbridges within peptidoglycan. Spn1 also affects bacterial chain formation. The phenotypes of chain length and the protein LytA have both been found to be important during colonization. We separated those phenotypes from the genotypic presence of Spn1. Neither chain length nor LytA were important for the fitness

defect associated with Spn1 during colonization. This leads us to conclude that the inherent change in the cell wall, associated with Spn1, possibly in order to tolerate phage products, results in a competitive fitness defect during colonization.

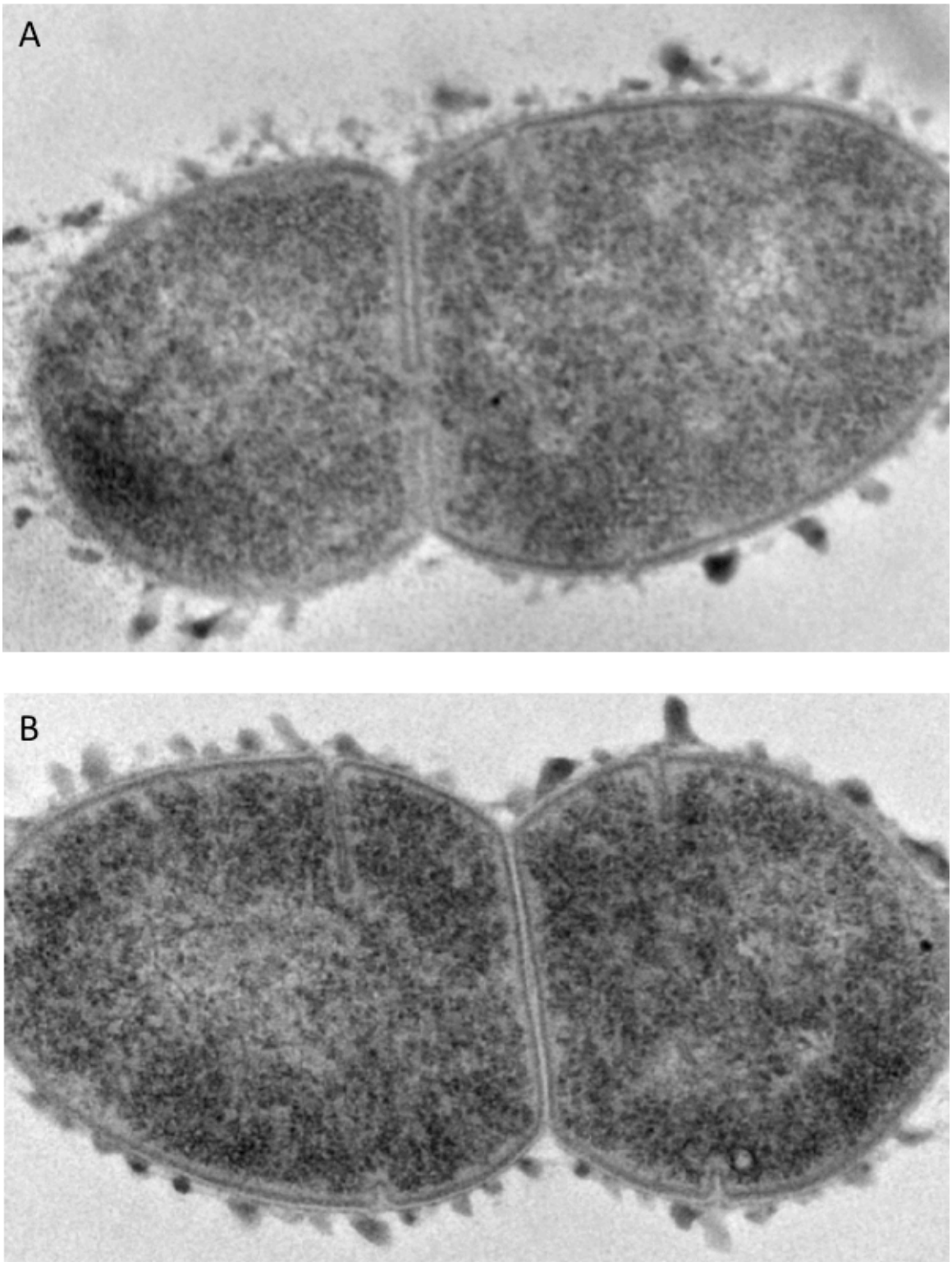


Figure 1. Electron Micrographs of **A.** Spn1+ and **B.** Spn1- strains

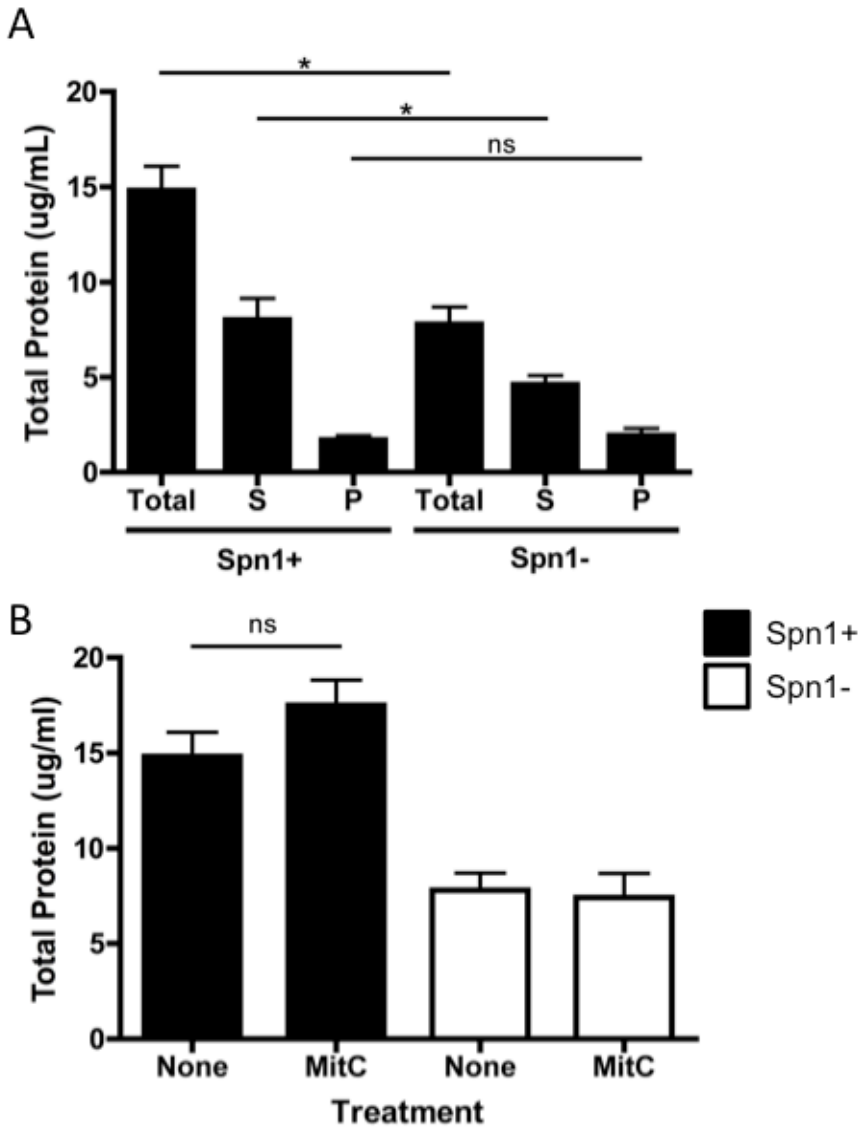


Figure 2. Relative protein content associated with Spn1 **A.** Total content as determined by BCA assay normalized by OD after mild sonication to break up chains. Supernatant (S) and pellet (P) assayed after 1 hour of ultracentrifugation **B.** Total protein content assayed and normalized the same as in A with and without mitomycin C treatment. "ns" not significant, *P<.05 by Student T-Test

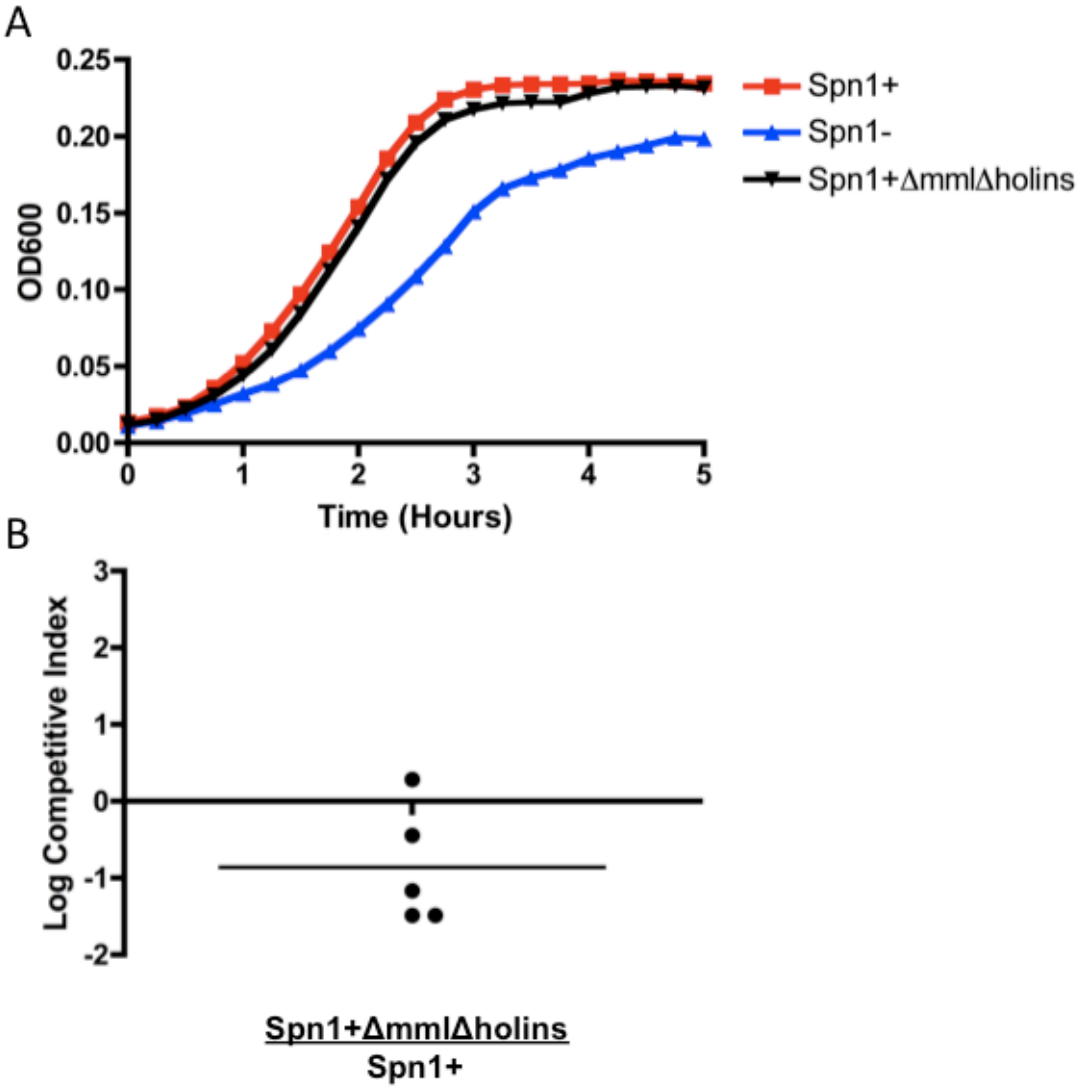


Figure 3. The effect of holins on pneumococcal fitness **A.** Growth as determined by OD in Tryptic Soy broth with 10ng/ml of penicillin over 5 hours **B.** Competition between the Spn1+*ΔmmlΔholins* and the Spn1+ strains during seven days of colonization

Bibliography

1. **Achen MG, Davidson BE, Hillier AJ**. 1986. Construction of plasmid vectors for the detection of streptococcal promoters. Gene **45**:45–9.
2. **Ackermann H-W**. 2009. Phage classification and characterization. Methods Mol. Biol. **501**:127–40.
3. **Albrich WC, Monnet DL, Harbarth S**. 2004. Antibiotic selection pressure and resistance in Streptococcus pneumoniae and Streptococcus pyogenes. Emerg. Infect. Dis. **10**:514–517.
4. **Aniansson G, Alm B, Andersson B, Larsson P, Nylen O, Peterson H, Rigner P, Svanborg M, Svanborg C**. 1992. Nasopharyngeal colonization during the first year of life. J. Infect. Dis. **165**:S38–S42.
5. **Appelbaum P**. 1992. Antimicrobial resistance in Streptococcus pneumoniae - An overview. Clin. Infect. Dis. **15**:77–83.
6. **Austrian R**. 1981. Pneumococcus - The 1st One Hundred Years. Rev. Infect. Dis. **3**:183–189.
7. **Avery OT, Goebel WF**. 1933. Chemoimmunological studies on the soluble specific substance of the pneumococcus: I. The isolation and properties of the acetyl polysaccharide of Pneumococcus Type I. J. Exp. Med. **58**:731–55.
8. **Avery OT, Macleod CM, McCarty M**. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus Type III. J. Exp. Med. **79**:137–58.
9. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O**. 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9**:75.
10. **Bannan J, Visvanathan K, Zabriskie JB**. 1999. Structure and function of streptococcal and staphylococcal superantigens in septic shock. Infect. Dis. Clin. North Am. **13**:387–396.
11. **Barendt SM, Sham L-T, Winkler ME**. 2011. Characterization of mutants deficient in the L,D-carboxypeptidase (DacB) and WalRK (VicRK) regulon, involved in peptidoglycan maturation of Streptococcus pneumoniae serotype 2 strain D39. J. Bacteriol. **193**:2290–300.
12. **Benchetrit L, Gray E, Wannamaker L**. 1977. Hyaluronidase activity of bacteriophages of group A streptococci. Infect. Immun. **15**:527–532.
13. **Bensing BA, Rubens CE, Sullam PM**. 2001. Genetic loci of Streptococcus mitis that mediate binding to human platelets. Infect. Immun. **69**:1373–80.
14. **Bensing BA, Siboo IR, Sullam PM**. 2001. Proteins PblA and PblB of Streptococcus mitis, which promote binding to human platelets, are encoded within a lysogenic bacteriophage. Infect. Immun. **69**:6186–92.
15. **Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kaltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG**. 2006. Genetic Analysis of the Capsular Biosynthetic Locus from All 90 Pneumococcal Serotypes. PLoS Genet **2**:e31.
16. **Beres SB, Sylva GL, Barbian KD, Lei B, Hoff JS, Mammarella ND, Liu M-Y, Smoot JC, Porcella SF, Parkins LD, Campbell DS, Smith TM, McCormick JK, Leung DYM, Schlievert PM, Musser JM**. 2002. Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc. Natl. Acad. Sci. U. S. A. **99**:10078–83.
17. **Bernheimer HP, Tiraby J-G**. 1976. Inhibition of phage infection by pneumococcus capsule. Virology **73**:308–309.
18. **Berry AM, Paton JC**. 2000. Additive Attenuation of Virulence of Streptococcus pneumoniae by Mutation of the Genes Encoding Pneumolysin and Other Putative Pneumococcal Virulence Proteins. Infect. Immun. **68**:133–140.
19. **Bikard D, Hatoum-Aslan A, Mucida D, Marraffini LA**. 2012. CRISPR interference can prevent natural transformation and virulence acquisition during in vivo bacterial infection. Cell Host Microbe **12**:177–86.

20. **Bogaert D, De Groot R, Hermans PWM**. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect. Dis. **4**:144–54.
21. **Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rümke HC, Verbrugh HA, Hermans PWM**. 2004. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. Lancet **363**:1871–1872.
22. **Bouchard JD, Dion E, Bissonnette F, Moineau S**. 2002. Characterization of the Two-Component Abortive Phage Infection Mechanism AbiT from Lactococcus lactis. J. Bacteriol. **184**:6325–6332.
23. **Broudy TB, Pancholi V, Fischetti VA**. 2002. The in vitro interaction of Streptococcus pyogenes with human pharyngeal cells induces a phage-encoded extracellular DNase. Infect. Immun. **70**:2805–11.
24. **Brown SP, Le Chat L, De Paepe M, Taddei F**. 2006. Ecology of microbial invasions: amplification allows virus carriers to invade more rapidly when rare. Curr. Biol. CB **16**:2048–2052.
25. **Brugger SD, Frey P, Aebi S, Hinds J, Mühlemann K**. 2010. Multiple colonization with S. pneumoniae before and after introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine. PLoS One **5**:e11638.
26. **Brugger SD, Hathaway LJ, Mühlemann K**. 2009. Detection of Streptococcus pneumoniae strain cocolonization in the nasopharynx. J. Clin. Microbiol. **47**:1750–1756.
27. **Brüssow H, Canchaya C, Hardt W-D**. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol. Mol. Biol. Rev. **68**:560–602, table of contents.
28. **Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, Feramisco J, Nizet V**. 2006. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr. Biol. **16**:396–400.
29. **Bui NK, Eberhardt A, Vollmer D, Kern T, Bougault C, Tomasz A, Simorre J-P, Vollmer W**. 2012. Isolation and analysis of cell wall components from Streptococcus pneumoniae. Anal. Biochem. **421**:657–66.
30. **Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M**. 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae. PLoS One **5**:e15678.
31. **Casas V, Magbanua J, Sobrepeña G, Kelley ST, Maloy SR**. 2010. Reservoir of bacterial exotoxin genes in the environment. Int. J. Microbiol. **2010**:754368.
32. **Casas V, Maloy S**. 2011. Role of bacteriophage-encoded exotoxins in the evolution of bacterial pathogens. Future Microbiol. **6**:1461–73.
33. **Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD**. 2011. Rapid pneumococcal evolution in response to clinical interventions. Science **331**:430–4.
34. **Cui L, Ma X, Sato K, Okuma K, Tenover FC, Mamizuka EM, Gemmell CG, Kim M-N, Ploy M-C, El-Solh N, Ferraz V, Hiramatsu K**. 2003. Cell wall thickening is a common feature of vancomycin resistance in Staphylococcus aureus. J. Clin. Microbiol. **41**:5–14.
35. **Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI**. 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature **377**:435–8.
36. **Dagkessamanskaia A, Moscoso M, Hénard V, Guiral S, Overweg K, Reuter M, Martin B, Wells J, Claverys J-P**. 2004. Interconnection of competence, stress and CiaR regulons in Streptococcus pneumoniae: competence triggers stationary phase autolysis of ciaR mutant cells. Mol. Microbiol. **51**:1071–86.
37. **Dalia AB, Standish AJ, Weiser JN**. 2010. Three surface exoglycosidases from Streptococcus pneumoniae, NanA, BgaA, and StrH, promote resistance to opsonophagocytic killing by human neutrophils. Infect. Immun. **78**:2108–16.
38. **Dalia AB, Weiser JN**. 2011. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. Cell Host Microbe **10**:486–96.
39. **Davies MR, McMillan DJ, Van Domselaar GH, Jones MK, Sriprakash KS**. 2007. Phage 3396 from a Streptococcus dysgalactiae subsp. equisimilis pathovar may have its origins in streptococcus

pyogenes. J. Bacteriol. **189**:2646–52.
40. **Davies MR, Tran TN, McMillan DJ, Gardiner DL, Currie BJ, Sriprakash KS**. 2005. Inter-species genetic movement may blur the epidemiology of streptococcal diseases in endemic regions. Microbes Infect. **7**:1128–38.
41. **Davis KM, Akinbi HT, Standish AJ, Weiser JN**. 2008. Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by Streptococcus pneumoniae. PLoS Pathog. **4**:e1000241.
42. **Davis KM, Nakamura S, Weiser JN**. 2011. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of S. pneumoniae colonization in mice. J. Clin. Invest. **121**:3666–76.
43. **Davis KM, Weiser JN**. 2011. Modifications to the peptidoglycan backbone help bacteria to establish infection. Infect. Immun. **79**:562–70.
44. **Dawid S, Roche AM, Weiser JN**. 2007. The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo. Infect. Immun. **75**:443–451.
45. **De Las Rivas B, García JL, López R, García P**. 2002. Purification and polar localization of pneumococcal LytB, a putative endo-beta-N-acetylglucosaminidase: the chain-dispersing murein hydrolase. J. Bacteriol. **184**:4988–5000.
46. **Desiere F, McShan WM, van Sinderen D, Ferretti JJ, Brüssow H**. 2001. Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic Streptococci: evolutionary implications for prophage-host interactions. Virology **288**:325–41.
47. **Desiere F, Lucchini S, Canchaya C, Ventura M, Brüssow H**. 2002. Comparative genomics of phages and prophages in lactic acid bacteria. Antonie Van Leeuwenhoek **82**:73–91.
48. **Di Luca MC, D'Ercole S, Petrelli D, Prenna M, Ripa S, Vitali LA**. 2010. Lysogenic transfer of mef(A) and tet(O) genes carried by Phim46.1 among group A streptococci. Antimicrob. Agents Chemother. **54**:4464–6.
49. **Diavatopoulos DA, Short KR, Price JT, Wilksch JJ, Brown LE, Briles DE, Strugnell RA, Wijburg OL**. 2010. Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. FASEB J. **24**:1789–98.
50. **Diaz E, Lopez R, Garcia J**. 1991. Chimeric pneumococcal cell-wall lytic enzymes reveal important phytsiological and evolutionary traits. J. Biol. Chem. **266**:5464–5471.
51. **Dubrac S, Bisicchia P, Devine KM, Msadek T**. 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol. Microbiol. **70**:1307–22.
52. **Duerkop B a, Clements C V, Rollins D, Rodrigues JLM, Hooper L V**. 2012. A composite bacteriophage alters colonization by an intestinal commensal bacterium. Proc. Natl. Acad. Sci. U. S. A. **109**:17621–6.
53. **Eriksen KR**. 1945. Studies on induced resistance to penicillin in a pneumococcus type I. Acta Pathol. Microbiol. Scand. **22**:398–405.
54. **Freeman VJ**. 1951. Studies on the virulence of bacteriophage-infected strains of Corynebacterium diphtheriae. J. Bacteriol. **61**:675–88.
55. **Friães A, Lopes JP, Melo-Cristino J, Ramirez M**. 2013. Changes in Streptococcus pyogenes causing invasive disease in Portugal: Evidence for superantigen gene loss and acquisition. Int. J. Med. Microbiol.
56. **Frias MJ, Melo-Cristino J, Ramirez M**. 2013. Export of the pneumococcal phage SV1 lysin requires choline-containing teichoic acids and is holin-independent. Mol. Microbiol. **87**:430–45.
57. **Frias MJ, Melo-Cristino J, Ramirez M**. 2009. The autolysin LytA contributes to efficient bacteriophage progeny release in Streptococcus pneumoniae. J. Bacteriol. **191**:5428–5440.
58. **Giebink GS, Berzins IK, Marker SC, Schiffman G**. 1980. Experimental otitis media after nasal inoculation of Streptococcus pneumoniae and influenza A virus in chinchillas. Infect. Immun. **30**:445–50.
59. **Gilmer DB, Schmitz JE, Euler CW, Fischetti VA**. 2013. Novel bacteriophage lysin with broad lytic activity protects against mixed infection by Streptococcus pyogenes and methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. **57**:2743–50.
60. **Gindreau E, López R, García P**. 2000. MM1, a temperate bacteriophage of the type 23F

Spanish/USA multiresistant epidemic clone of Streptococcus pneumoniae: structural analysis of the site-specific integration system. J. Virol. **74**:7803–7813.
61. **Griffith F**. 1928. The Significance of Pneumococcal Types. J. Hyg. (Lond). **27**:113–59.
62. **Gritzfeld JF, Wright AD, Collins AM, Pennington SH, Wright AKA, Kadioglu A, Ferreira DM, Gordon SB**. 2013. Experimental human pneumococcal carriage. J. Vis. Exp.
63. **Hall-Stoodley L, Nistico L, Sambanthamoorthy K, Dice B, Nguyen D, Mershon WJ, Johnson C, Hu FZ, Stoodley P, Ehrlich GD, Post JC**. 2008. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. BMC Microbiol. **8**:173.
64. **Halperin SA, Ferrieri P, Gray ED, Kaplan EL, Wannamaker LW**. 1987. Antibody Response to Bacteriophage Hyaluronidase in Acute Glomerulonephritis after Group A Streptococcal Infection. J. Infect. Dis. **155**:253–261.
65. **Hammerschmidt S**. 2006. Adherence molecules of pathogenic pneumococci. Curr. Opin. Microbiol. **9**:12–20.
66. **Hammitt LL, Bruden DL, Butler JC, Baggett HC, Hurlburt DA, Reasonover A, Hennessy TW**. 2006. Indirect effect of conjugate vaccine on adult carriage of Streptococcus pneumoniae: an explanation of trends in invasive pneumococcal disease. J. Infect. Dis. **193**:1487–94.
67. **Harvey RM, Stroeher UH, Ogunniyi AD, Smith-Vaughan HC, Leach AJ, Paton JC**. 2011. A Variable Region within the Genome of Streptococcus pneumoniae Contributes to Strain-Strain Variation in Virulence. PLoS One **6**.
68. **Hazan R, Engelberg-Kulka H**. 2004. Escherichia coli mazEF-mediated cell death as a defense mechanism that inhibits the spread of phage P1. Mol. Genet. Genomics **272**:227–34.
69. **Henderson IR, Owen P, Nataro JP**. 1999. Molecular switches - the ON and OFF of bacterial phase variation. Mol. Microbiol. **33**:919–932.
70. **Higuchi R, Krummel B, Saiki RK**. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16**:7351–67.
71. **Hiller NL, Ahmed A, Powell E, Martin DP, Eutsey R, Earl J, Janto B, Boissy RJ, Hogg J, Barbadora K, Sampath R, Lonergan S, Post JC, Hu FZ, Ehrlich GD**. 2010. Generation of genic diversity among Streptococcus pneumoniae strains via horizontal gene transfer during a chronic polyclonal pediatric infection. PLoS Pathog. **6**:e1001108.
72. **Hirst RA, Gosai B, Rutman A, Guerin CJ, Nicotera P, Andrew PW, O'Callaghan C**. 2008. Streptococcus pneumoniae deficient in pneumolysin or autolysin has reduced virulence in meningitis. J. Infect. Dis. **197**:744–51.
73. **Holden MTG, Heather Z, Paillot R, Steward KF, Webb K, Ainslie F, Jourdan T, Bason NC, Holroyd NE, Mungall K, Quail MA, Sanders M, Simmonds M, Willey D, Brooks K, Aanensen DM, Spratt BG, Jolley KA, Maiden MCJ, Kehoe M, Chanter N, Bentley SD, Robinson C, Maskell DJ, Parkhill J, Waller AS**. 2009. Genomic evidence for the evolution of Streptococcus equi: host restriction, increased virulence, and genetic exchange with human pathogens. PLoS Pathog. **5**:e1000346.
74. **Hsieh Y-C, Wang J-T, Lee W-S, Hsueh P-R, Shao P-L, Chang L-Y, Lu C-Y, Lee C-Y, Huang F-Y, Huang L-M**. 2006. Serotype competence and penicillin resistance in Streptococcus pneumoniae. Emerg. Infect. Dis. **12**:1709–14.
75. **Hyder SL, Streitfeld MM**. 1978. Transfer of Erythromycin Resistance from Clinically Isolated Lysogenic Strains of Streptococcus pyogenes via Their Endogenous Phage. J. Infect. Dis. **138**:281–286.
76. **Hynes WL, Hancock LE, Ferretti JJ**. 1995. Analysis of a 2nd Bacteriophage Hyaluronidase Gene from Streptococcus pyogenes - Evidence for a 3rd Hyaluronidase Involved in Extracellular Enzymatic Activity. Infect. Immun. **63**:3015–3020.
77. **Hynes WL, Walton SL**. 2000. Hyaluronidases of Gram-positive bacteria. FEMS Microbiol. Lett. **183**:201–207.
78. **Ikebe T**. 2002. Dissemination of the Phage-Associated Novel Superantigen Gene speL in Recent Invasive and Noninvasive Streptococcus pyogenes M3/T3 Isolates in Japan. Infect. Immun. **70**:3227–3233.

79. **Ingrey KT, Ren J, Prescott JF**. 2003. A fluoroquinolone induces a novel mitogen-encoding bacteriophage in Streptococcus canis. Infect. Immun. **71**:3028–33.
80. **Johnston C, Polard P, Claverys J-P**. 2013. The DpnI/DpnII pneumococcal system, defense against foreign attack without compromising genetic exchange. Mob. Genet. Elements **3**:e25582.
81. **Kadioglu A, Weiser JN, Paton JC, Andrew PW**. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat. Rev. Microbiol. **6**:288–301.
82. **Kim JO, Weiser JN**. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae. J. Infect. Dis. **177**:368 –377.
83. **King SJ**. 2010. Pneumococcal modification of host sugars: a major contributor to colonization of the human airway? Mol. Oral Microbiol. **25**:15–24.
84. **Kyaw MH, Lynfield R, Schaffner W, Craig AS, Hadler J, Reingold A, Thomas AR, Harrison LH, Bennett NM, Farley MM, Facklam RR, Jorgensen JH, Besser J, Zell ER, Schuchat A, Whitney CG**. 2006. Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant Streptococcus pneumoniae. N. Engl. J. Med. **354**:1455–63.
85. **Labrie SJ, Samson JE, Moineau S**. 2010. Bacteriophage resistance mechanisms. Nat. Rev. Microbiol. **8**:317–327.
86. **Lacks S, Hotchkiss RD**. 1960. A study of the genetic material determining an enzyme in Pneumococcus. Biochim. Biophys. Acta **39**:508–18.
87. **Laitinen H, Tomasz A**. 1990. Changes in composition of peptidoglycan during maturation of the cell wall in pneumococci. J. Bacteriol. **172**:5961–7.
88. **Lefébure T, Richards VP, Lang P, Pavinski-Bitar P, Stanhope MJ**. 2012. Gene repertoire evolution of Streptococcus pyogenes inferred from phylogenomic analysis with Streptococcus canis and Streptococcus dysgalactiae. PLoS One **7**:e37607.
89. **Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch S V, Kolter R**. 2010. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. MBio **1**.
90. **Lijek RS, Weiser JN**. 2012. Co-infection subverts mucosal immunity in the upper respiratory tract. Curr. Opin. Immunol. **24**:417–23.
91. **Lipsitch M, Dykes J., Johnson S., Ades E., King J, Briles D., Carlone G.** 2000. Competition among Streptococcus pneumoniae for intranasal colonization in a mouse model. Vaccine **18**:2895–2901.
92. **Loeffler JM, Fischetti VA**. 2006. Lysogeny of Streptococcus pneumoniae with MM1 phage: improved adherence and other phenotypic changes. Infect. Immun. **74**:4486–4495.
93. **Lux T, Nuhn M, Hakenbeck R, Reichmann P**. 2007. Diversity of bacteriocins and activity spectrum in Streptococcus pneumoniae. J. Bacteriol. **189**:7741–51.
94. **Lysenko ES, Lijek RS, Brown SP, Weiser JN**. 2010. Within-host competition drives selection for the capsule virulence determinant of Streptococcus pneumoniae. Curr. Biol. CB **20**:1222–1226.
95. **Lysenko ES, Ratner AJ, Nelson AL, Weiser JN**. 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog. **1**:e1.
96. **Ma YL, Lu CP**. 2008. Isolation and identification of a bacteriophage capable of infecting Streptococcus suis type 2 strains. Vet. Microbiol. **132**:340–7.
97. **Mascher T, Heintz M, Zähner D, Merai M, Hakenbeck R**. 2006. The CiaRH system of Streptococcus pneumoniae prevents lysis during stress induced by treatment with cell wall inhibitors and by mutations in pbp2x involved in beta-lactam resistance. J. Bacteriol. **188**:1959–68.
98. **Massidda O, Nováková L, Vollmer W**. 2013. From models to pathogens: how much have we learned about Streptococcus pneumoniae cell division? Environ. Microbiol. **15**:3133–57.
99. **Mazaheri Nezhad Fard R, Barton MD, Heuzenroeder MW**. 2010. Novel Bacteriophages in Enterococcus spp. Curr. Microbiol. **60**:400–6.
100. **McCool TL, Weiser JN**. 2004. Limited role of antibody in clearance of Streptococcus pneumoniae in a murine model of colonization. Infect. Immun. **72**:5807–13.
101. **McCool TL, Cate TR, Moy G, Weiser JN**. 2002. The Immune Response to Pneumococcal Proteins during Experimental Human Carriage. J. Exp. Med. **195**:359–365.
102. **McCullers JA, Karlström A, Iverson AR, Loeffler JM, Fischetti VA**. 2007. Novel strategy to prevent otitis media caused by colonizing Streptococcus pneumoniae. PLoS Pathog. **3**:e28.

103. **McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B**. 2010. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J. Infect. Dis. **202**:1287–95.
104. **Mellroth P, Daniels R, Eberhardt A, Rönnlund D, Blom H, Widengren J, Normark S, Henriques-Normark B**. 2012. LytA, major autolysin of Streptococcus pneumoniae, requires access to nascent peptidoglycan. J. Biol. Chem. **287**:11018–29.
105. **Mellroth P, Sandalova T, Kikhney A, Vilaplana F, Hesek D, Lee M, Mobashery S, Normark S, Svergun D, Henriques-Normark B, Achour A**. 2014. Structural and functional insights into peptidoglycan access for the lytic amidase LytA of Streptococcus pneumoniae. MBio **5**:e01120–13.
106. **Minami M, Oshima Y, Kuriyama M, Hasegawa T**. 2012. Prevalence of bacteriophage-associated super-antigen genes in Streptococcus pyogenes isolated from pediatric patients in Japan. Int. J. Infect. Dis. **16**:e203–e204.
107. **Mitchell J, Siboo IR, Takamatsu D, Chambers HF, Sullam PM**. 2007. Mechanism of cell surface expression of the Streptococcus mitis platelet binding proteins PblA and PblB. Mol. Microbiol. **64**:844–57.
108. **Mitchell J, Sullam PM**. 2009. Streptococcus mitis phage-encoded adhesins mediate attachment to {alpha}2-8-linked sialic acid residues on platelet membrane gangliosides. Infect. Immun. **77**:3485–90.
109. **Morales M, García P, de la Campa AG, Liñares J, Ardanuy C, García E**. 2010. Evidence of localized prophage-host recombination in the lytA gene, encoding the major pneumococcal autolysin. J. Bacteriol. **192**:2624–32.
110. **Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R**. 2008. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin. Infect. Dis. **46**:174–82.
111. **Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN**. 2007. Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance. Infect. Immun. **75**:83–90.
112. **Neufeld F**. 1902. Über die Agglutination der Pneumokokken und über die Theorieen der Agglutination. Z Hyg Infekt. **40**:54–72.
113. **Novak R, Braun JS, Charpentier E, Tuomanen E**. 1998. Penicillin tolerance genes of Streptococcus pneumoniae: the ABC-type manganese permease complex Psa. Mol. Microbiol. **29**:1285–96.
114. **O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB**. 1984. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science (80-.). **226**:694–696.
115. **Obregón V, García P, López R, García JL**. 2003. Molecular and biochemical analysis of the system regulating the lytic/lysogenic cycle in the pneumococcal temperate phage MM1. FEMS Microbiol. Lett. **222**:193–197.
116. **Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI**. 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J. Infect. Dis. **190**:1661–9.
117. **Palmieri C, Varaldo PE, Facinelli B**. 2011. Streptococcus suis, an Emerging Drug-Resistant Animal and Human Pathogen. Front. Microbiol. **2**:235.
118. **Pastagia M, Schuch R, Fischetti VA, Huang DB**. 2013. Lysins: the arrival of pathogen-directed anti-infectives. J. Med. Microbiol. **62**:1506–16.
119. **Pericone CD, Overweg K, Hermans PW, Weiser JN**. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect. Immun. **68**:3990–7.
120. **Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE**. 2014. NOD proteins: regulators of inflammation in health and disease. Nat. Rev. Immunol. **14**:9–23.
121. **Ptashne M**. 2004. A genetic switch: phage lambda revisited. CSHL Press.
122. **Ramirez M, Severina E, Tomasz A**. 1999. A high incidence of prophage carriage among natural isolates of Streptococcus pneumoniae. J. Bacteriol. **181**:3618–3625.
123. **Ratner AJ, Lysenko ES, Paul MN, Weiser JN**. 2005. Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. Proc. Natl. Acad. Sci. U. S. A. **102**:3429–34.

124. **Rato MG, Nerlich A, Bergmann R, Bexiga R, Nunes SF, Vilela CL, Santos-Sanches I, Chhatwal GS**. 2011. Virulence gene pool detected in bovine group C Streptococcus dysgalactiae subsp. dysgalactiae isolates by use of a group A S. pyogenes virulence microarray. J. Clin. Microbiol. **49**:2470–9.
125. **Roche AM, King SJ, Weiser JN**. 2007. Live attenuated Streptococcus pneumoniae strains induce serotype-independent mucosal and systemic protection in mice. Infect. Immun. **75**:2469–75.
126. **Rodriguez JL, Dalia AB, Weiser JN**. 2012. Increased chain length promotes pneumococcal adherence and colonization. Infect. Immun. **80**:3454–9.
127. **Romero P, Croucher NJ, Hiller NL, Hu FZ, Ehrlich GD, Bentley SD, García E, Mitchell TJ**. 2009. Comparative genomic analysis of ten Streptococcus pneumoniae temperate bacteriophages. J. Bacteriol. **191**:4854–4862.
128. **Romero P, García E, Mitchell TJ**. 2009. Development of a prophage typing system and analysis of prophage carriage in Streptococcus pneumoniae. Appl. Environ. Microbiol. **75**:1642–1649.
129. **Ronda C, García JL LR**. 1989. Infection of Streptococcus oralis NCTC 11427 by pneumococcal phages. FEMS Microbiol. Lett. **53**:187–92.
130. **Sanchez-Puelles JM, Ronda C, Garcia JL, Garcia P, Lopez R, Garcia E**. 1986. Searching for autolysin functions. Characterization of a pneumococcal mutant deleted in the lytA gene. Eur. J. Biochem. **158**:289–93.
131. **Sebert ME, Palmer LM, Rosenberg M, Weiser JN**. 2002. Microarray-based identification of htrA, a Streptococcus pneumoniae gene that is regulated by the CiaRH two-component system and contributes to nasopharyngeal colonization. Infect. Immun. **70**:4059–67.
132. **Selva L, Viana D, Regev-Yochay G, Trzcinski K, Corpa JM, Lasa I, Novick RP, Penadés JR**. 2009. Killing niche competitors by remote-control bacteriophage induction. Proc. Natl. Acad. Sci. U. S. A. **106**:1234–8.
133. **Seo, Ho Seong , Yan Q. Xiong2, Jennifer Mitchell3, Ravin Seepersaud1, Arnold S. Bayer2 PMS**. 2010. Bacteriophage lysin mediates the binding of streptococcus mitis to human platelets through interaction with fibrinogen. PLoS Pathog. **6**:e1001047.
134. **Sham L-T, Barendt SM, Kopecky KE, Winkler ME**. 2011. Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsXSpn cell division protein in Streptococcus pneumoniae D39. Proc. Natl. Acad. Sci. U. S. A. **108**:E1061–9.
135. **Sitkiewicz I, Nagiec MJ, Sumby P, Butler SD, Cywes-Bentley C, Musser JM**. 2006. Emergence of a bacterial clone with enhanced virulence by acquisition of a phage encoding a secreted phospholipase A2. Proc. Natl. Acad. Sci. U. S. A. **103**:16009–14.
136. **Smith CJ, Rollins LA, Parker AC**. 1995. Nucleotide sequence determination and genetic analysis of the Bacteroides plasmid, pBI143. Plasmid **34**:211–22.
137. **Smith JM, Dowson CG, Spratt BG**. 1991. Localized sex in bacteria. Nature **349**:29–31.
138. **Spaulding AR, Salgado-Pabón W, Kohler PL, Horswill AR, Leung DYM, Schlievert PM**. 2013. Staphylococcal and streptococcal superantigen exotoxins. Clin. Microbiol. Rev. **26**:422–47.
139. **Sumby P, Porcella SF, Madrigal AG, Barbian KD, Virtaneva K, Ricklefs SM, Sturdevant DE, Graham MR, Vuopio-Varkila J, Hoe NP, Musser JM**. 2005. Evolutionary origin and emergence of a highly successful clone of serotype M1 group a Streptococcus involved multiple horizontal gene transfer events. J. Infect. Dis. **192**:771–82.
140. **Sung CK, Li H, Claverys JP, Morrison DA**. 2001. An rpsL cassette, janus, for gene replacement through negative selection in Streptococcus pneumoniae. Appl. Environ. Microbiol. **67**:5190–6.
141. **Suvorov AN, Polyakova EM, McShan WM, Ferretti JJ**. 2009. Bacteriophage content of M49 strains of Streptococcus pyogenes. FEMS Microbiol. Lett. **294**:9–15.
142. **Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S**. 1999. Differential Roles of TLR2 and TLR4 in Recognition of Gram-Negative and Gram-Positive Bacterial Cell Wall Components. Immunity **11**:443–451.
143. **Tang F, Bossers A, Harders F, Lu C, Smith H**. 2013. Comparative genomic analysis of twelve Streptococcus suis (pro)phages. Genomics **101**:336–44.
144. **Tang F, Zhang W, Lu C**. 2013. Lysogenic Streptococcus suis isolate SS2-4 containing prophage SMP showed increased mortality in zebra fish compared to the wild-type isolate. PLoS One **8**:e54227.

145. **Tapp J**. 2003. Phylogenetic relationships and genotyping of the genus Streptococcus by sequence determination of the RNase P RNA gene, rnpB. Int. J. Syst. Evol. Microbiol. **53**:1861–1871.
146. **Tran TD-H, Kwon H-Y, Kim E-H, Kim K-W, Briles DE, Pyo S, Rhee D-K**. 2011. Decrease in penicillin susceptibility due to heat shock protein ClpL in Streptococcus pneumoniae. Antimicrob. Agents Chemother. **55**:2714–28.
147. **Tuomanen EI**. 2004. The pneumococcus. ASM Press.
148. **Ubukata K, Konno M, Fujii R**. 1975. Transduction of drug resistance to tetracycline, chloramphenicol, macrolides, lincomycin and clindamycin with phages induced from Streptococcus pyogenes. J. Antibiot. (Tokyo). **28**:681–8.
149. **Urbásková P, Zemlicková H**. 2011. Antibiotic resistance of Streptococcus pneumoniae and Streptococcus pyogenes in 1996-2009. Epidemiol. Mikrobiol. Imunol. Cas. Spol. pro Epidemiol. a Mikrobiol. Ces. Lek. Spol. JE Purkyne **60**:10–20.
150. **Van Rossum AMC, Lysenko ES, Weiser JN**. 2005. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. Infect. Immun. **73**:7718–26.
151. **Ventura M, Bruttin A, Canchaya C, Brüssow H**. 2002. Transcription Analysis of Streptococcus thermophilus Phages in the Lysogenic State. Virology **302**:21–32.
152. **Vlaminckx BJM, Schuren FHJ, Montijn RC, Caspers MPM, Beitsma MM, Wannet WJB, Schouls LM, Verhoef J, Jansen WTM**. 2007. Dynamics in prophage content of invasive and noninvasive M1 and M28 Streptococcus pyogenes isolates in The Netherlands from 1959 to 1996. Infect. Immun. **75**:3673–9.
153. **Vojtek I, Pirzada ZA, Henriques-Normark B, Mastny M, Janapatla RP, Charpentier E**. 2008. Lysogenic transfer of group A Streptococcus superantigen gene among Streptococci. J. Infect. Dis. **197**:225–34.
154. **Wadowsky RM, Mietzner SM, Skoner DP, Doyle WJ, Fireman P**. 1995. Effect of experimental influenza A virus infection on isolation of Streptococcus pneumoniae and other aerobic bacteria from the oropharynges of allergic and nonallergic adult subjects. Infect. Immun. **63**:1153–7.
155. **Waldor MK, Mekalanos JJ**. 1996. Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin. Science (80-.). **272**:1910–1914.
156. **Walsh RL, Camilli A**. 2011. Streptococcus pneumoniae is desiccation tolerant and infectious upon rehydration. MBio **2**:e00092–11.
157. **Wannamaker LW**. 1973. Intergroup phage reactions and transduction between group C and group A streptococci. J. Exp. Med. **137**:1338–1353.
158. **Watanabe S, Kirikae T, Miyoshi-Akiyama T**. 2013. Complete genome sequence of Streptococcus dysgalactiae subsp. equisimilis 167 carrying Lancefield group C antigen, and comparative genomics of S. dysgalactiae subsp. equisimilis strains. Genome Biol. Evol. **5**:1644–1651.
159. **Waxman DJ, Strominger JL**. 1983. Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. Annu. Rev. Biochem. **52**:825–69.
160. **Weinbauer MG, Rassoulzadegan F**. 2003. Are viruses driving microbial diversification and diversity? Environ. Microbiol. **6**:1–11.
161. **Weiser JN, Austrian R, Sreenivasan PK, Masure HR**. 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect. Immun. **62**:2582–2589.
162. **Weiser JN**. 2010. The pneumococcus: why a commensal misbehaves. J. Mol. Med. (Berl). **88**:97–102.
163. **Whatmore AM, Dowson CG**. 1999. The autolysin-encoding gene (lytA) of Streptococcus pneumoniae displays restricted allelic variation despite localized recombination events with genes of pneumococcal bacteriophage encoding cell wall lytic enzymes. Infect. Immun. **67**:4551–6.
164. **Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, Reingold A, Cieslak PR, Pilishvili T, Jackson D, Facklam RR, Jorgensen JH, Schuchat A**. 2003. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N. Engl. J. Med. **348**:1737–46.
165. **Wu HY, Virolainen A, Mathews B, King J, Russell MW, Briles DE**. 1997. Establishment of a

Streptococcus pneumoniae nasopharyngeal colonization model in adult mice. Microb. Pathog. **23**:127–137.

166. **Yadav MK, Chae S-W, Song J-J**. 2012. In VitroStreptococcus pneumoniae Biofilm Formation and In Vivo Middle Ear Mucosal Biofilm in a Rat Model of Acute Otitis Induced by S. pneumoniae. Clin. Exp. Otorhinolaryngol. **5**:139–44.
167. **Yu C-E, Ferretti JJ**. 1991. Molecular characterization of new group A streptococcal bacteriophages containing the gene for streptococcal erythrogenic toxin A (speA). MGG Mol. Gen. Genet. **231**:161–168.
168. WHO | Estimated Hib and pneumococcal deaths for children under 5 years of age.