INTERACTIONS BETWEEN INFLUENZA A VIRUS AND STREPTOCOCCUS PNEUMONIAE IN NASAL COLONIZATION AND MIDDLE EAR INFECTION

BY

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<td>Acute otitis media</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BgaA</td>
<td>β-galactosidase A</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BSL-2</td>
<td>Biosafety level 2</td>
</tr>
<tr>
<td>BV</td>
<td>Blood vessel</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
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<tr>
<td>C3b</td>
<td>Complement 3b</td>
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<td>CbpA/PspC</td>
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</tr>
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<td>CD4</td>
<td>Cluster of differentiation 4</td>
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<td>Cluster of differentiation 8</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>ΔNanA</td>
<td>NanA-deficient</td>
</tr>
<tr>
<td></td>
<td><em>S. pneumoniae</em></td>
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<tr>
<td>DANA</td>
<td>N-acetyl-2,3-dehydro-2-deoxy neuraminic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco PBS</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEC</td>
<td>External ear canal</td>
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</table>
Eno  α-Enolase
ET  Eustachian tube
g  Gravity
GFP  Green fluorescent protein
H₂O₂  Hydrogen peroxide
H₂SO₄  Sulfuric acid
H&E  Hematoxylin and eosin
HA  Influenza hemagglutinin
HCl  Hydrochloric acid
*H. influenzae*  *Haemophilus influenzae*
hsdS  Type I restriction modification system, S subunit gene
IAV  Influenza A virus
IFN  Interferon
IgA  Immunoglobulin A
IgG  Immunoglobulin G
IL-1  Interleukin 1
IL-6  Interleukin 6
IL-8  Interleukin 8
IL-17  Interleukin 17
LytA  Autolysin A
M  Molar
mM  Millimolar
M2  Influenza matrix protein 2
MARCO  Macrophage receptor with
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<td><em>Moraxella catarrhalis</em></td>
</tr>
<tr>
<td>MCB</td>
<td>Mucociliary border</td>
</tr>
<tr>
<td>MCP 1</td>
<td>Monocyte chemoattract protein 1</td>
</tr>
<tr>
<td>ME</td>
<td>Middle ear</td>
</tr>
<tr>
<td>MEC</td>
<td>Middle ear cavity</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<td>mL</td>
<td>Milliliter</td>
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<td>MUAN</td>
<td>2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid</td>
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<td>n</td>
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</tr>
<tr>
<td>NA</td>
<td>Influenza neuraminidase</td>
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<td>NanA</td>
<td>Neuraminidase A</td>
</tr>
<tr>
<td>NanB</td>
<td>Neuraminidase B</td>
</tr>
<tr>
<td>NanC</td>
<td>Neuraminidase C</td>
</tr>
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<td>NFκB</td>
<td>Nuclear factor κB</td>
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<td>NK cells</td>
<td>Natural killer cells</td>
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<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>NOD-1</td>
<td>Nucleotide-binding oligomerization domain-containing protein 1</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature media</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Otitis media</td>
</tr>
<tr>
<td>OME</td>
<td>Otitis media with effusion</td>
</tr>
<tr>
<td>Acronym</td>
<td>Standardized Name</td>
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<td>---------</td>
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<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PavA</td>
<td>Pneumococcal adherence and virulence factor A</td>
</tr>
<tr>
<td>PB1-F2</td>
<td>Influenza accessory protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PCV</td>
<td>Pneumococcal conjugate vaccine</td>
</tr>
<tr>
<td>plgR</td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>PsaA</td>
<td>Pneumococcal surface adhesin A</td>
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<tr>
<td>PspA</td>
<td>Pneumococcal surface protein A</td>
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<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<td>RIG-1</td>
<td>Retinoic acid inducible gene 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RSV</td>
<td>Respiratory syncitial virus</td>
</tr>
<tr>
<td>SatABC</td>
<td>Sialic acid transporter</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Spp.</td>
<td>Multiple species in same genus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SpxB</td>
<td>Pneumococcal pyruvate oxidase</td>
</tr>
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<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
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<tr>
<td>S. pneumoniae, pneumococcus</td>
<td><em>Streptococcus pneumoniae</em></td>
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<tr>
<td>StrH</td>
<td>β-N-acetylhexosaminidase</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infectious dose 50%</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TH1 cell</td>
<td>T helper 1 cell</td>
</tr>
<tr>
<td>TH17 cell</td>
<td>T helper 17 cell</td>
</tr>
<tr>
<td>THY&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Todd-Hewitt yeast broth supplemented</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TM</td>
<td>Tympanic membrane</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>URTI</td>
<td>Upper respiratory tract infection</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UspA1</td>
<td><em>M. catarrhalis</em> adhesin</td>
</tr>
<tr>
<td>Vol</td>
<td>Volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>wt</td>
<td>Weight</td>
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YSK

Sialic acid-free media
ABSTRACT

*Streptococcus pneumoniae* (pneumococcus) is a tremendously variable pathogen. Reflecting this, it is both a nearly ubiquitous nasopharyngeal colonizer and also a leading cause of otitis media, one of the most common diseases of childhood. Despite this, the factors underlying its transition from colonizer to otopathogen are incompletely understood and are of great interest. Epidemiologically, coinfection with influenza A virus is strongly linked to the incidence of otitis media in children and experimental models have identified numerous mechanisms by which viral infection predisposes to increased pneumococcal infection. Unclear, however, are the roles of specific pneumococcal factors in this coinfection process. This study was undertaken to examine the largely unexplored importance of two such factors of *S. pneumoniae* in coinfection with influenza A virus, namely phase variation and the pneumococcal neuraminidase NanA, using a novel animal model of nasal colonization and middle ear infection.

Following the widespread implementation of the pneumococcal conjugate vaccines, the incidence of invasive pneumococcal disease in children continues to decrease. As a result, the burden of *S. pneumoniae*-influenza virus coinfection has begun to shift somewhat from lethal, invasive presentations to more common, less invasive disease processes such as otitis media. As such, there is a need for readily accessible and relevant animal models. In this study, we utilize a mouse model wherein mice are infected intranasally with influenza A
virus followed four days later by intranasal infection with a colonizing strain of S. 
*pneumoniae* that, when inoculated alone, does not disseminate to cause lethal, 
systemic disease in mice. This model establishes nasal colonization to at least 
21 days in the absence of bacteremia. Preceding viral infection, however, 
induces substantial inflammatory changes to the nasal epithelium resulting in a 9-
fold increase in the magnitude of pneumococcal colonization. This increased 
colonization following influenza infection is correlated with a 37-fold increase in 
middle ear infection as well as histologic findings consistent with the disease 
process of acute otitis media.

Using this model, we next investigated the effect of antecedent influenza 
 virus infection on specific pneumococcal sub-populations inherent within strains 
of *S. pneumoniae*. As a method of adaptation to different host environments, *S. 
pneumoniae* can phase-vary via a stochastic process between a transparent 
phase traditionally associated with nasal colonization and an opaque phase 
associated with invasive disease. These phases have been proposed to 
contribute to the transition from colonization to disease; however, their interaction 
with influenza A virus coinfection is unclear. Using pneumococcal phase variants 
enriched for either the opaque or transparent phases, we observed that the 
opaque phase exhibited impaired nasal colonization relative to the transparent in 
the absence of viral infection. *In vitro*, the opaque phase also demonstrated 
diminished biofilm formation and adherence to epithelial cells. However, 
coinfection with influenza virus ameliorated this colonization defect *in vivo*. 
Further, viral coinfection ultimately induced a similar magnitude of middle ear
infection by both phase variants. These data indicate that despite inherent differences in colonization, the influenza A virus exacerbation of experimental middle ear infection is independent of the pneumococcal phase; providing new insights into the synergistic link between pneumococcus and influenza in the context of otitis media.

*S. pneumoniae* expresses NanA, a neuraminidase that catalyzes the cleavage of terminal sialic acids from host glycoconjugates and is involved in nasal colonization and, potentially, pneumococcal disease. Influenza A virus also expresses a neuraminidase which can synergistically enhance lethal pneumococcal pulmonary infection in mice. The specific role of pneumococcal NanA in this interaction, however, is not known. Using our mouse coinfection model, we demonstrate that NanA-deficient pneumococci exhibit both impaired nasal colonization and middle ear infection. Coinfection with neuraminidase-expressing influenza A virus potentiates both nasal colonization and middle ear infection but not to the same magnitude of wild-type, suggesting a potentially unexplored role of NanA in this process. Using *in vitro* models, we show that while NanA contributes to epithelial adherence and biofilm viability, its effect on the latter is actually independent of its enzymatic sialidase activity, suggesting an additional function for this protein. These data indicate that NanA contributes both enzymatically and non-enzymatically to pneumococcal pathogenesis and that it is not a redundant bystander during coinfection with influenza A virus. Rather, its expression is required for the full synergism between these two pathogens. Based on these studies, we envision a model in which NanA is
involved in the pneumococcal-influenza A virus interaction both via its overlapping sialidase activity with the viral neuraminidase but also by a previously unappreciated, non-enzymatic role involving biofilm formation; the precise nature of which is in need of further study.
INTRODUCTION

The importance of bacterial-viral coinfections in life-threatening diseases has been recognized for two centuries (5) and their significant role in non-lethal illnesses is being rapidly appreciated by the scientific community. The synergism of two such pathogens, *Streptococcus pneumoniae* (pneumococcus) and influenza A virus (IAV), represents a particularly great public health burden. The significance of this coinfection is clearly illustrated by the 1918 Spanish Flu pandemic, where a majority of the 50-100 million deaths were in fact due to secondary bacterial infections (6), and also remains evident in the present day. Indeed, influenza and pneumonia remain the leading cause of infectious-related mortality in the United States (US) while a 2009 study estimated that invasive pneumococcal disease globally continues to account for up to 11% of all deaths in children under the age of five (7, 8).

Due to this prevalence, significant attention has successfully been paid to the interaction of IAV and *S. pneumoniae* in the context of severe disease. This has led to it being one of the most well-studied aspects of any bacterial viral coinfection (5). However, this attention on but one facet of this coinfection process has come at a cost: namely that much less is known regarding the interactions between these two pathogens that contribute to exceedingly common yet less invasive conditions such as nasopharyngeal colonization and otitis media (OM).
**Nasopharyngeal Colonization**

The initiation of nearly all respiratory tract infections and disease processes begins with nasal colonization (9), defined generally as the acquisition and resultant carriage of microbes in the nasopharynx. This environment is populated by a diverse polymicrobial microbiota that is generally composed of numerous commensal species that play important roles in immune system stimulation and maturation, mucosal structure, and competition with other pathogenic genera (10-12). Often included in this flora, however, are several potential, or opportunistic, pathogens including *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *S. pneumoniae* (10, 13-21). Most instances of colonization by these opportunistic pathogens are asymptomatic and only a small subset will result in clinical disease (22-24). However, the sheer prevalence of their colonization rates, depicted in Table I and reviewed elsewhere (10, 24), ensures that these bacteria are among the leading causes of mucosal diseases such as otitis media, sinusitis, and pneumonia (1, 25-28). In general, each episode of colonization can be classified into three inter-related categories: acquisition, carriage, and clearance.

**Acquisition**

While not all exposures will result in nasal colonization, the acquisition of nasal colonizing commensals begins at birth and continues dynamically throughout life (10). Colonization by opportunistic pathogens can similarly occur as early as the first hours of life and greater than 50% of children will be
Table I. Reported nasopharyngeal carriage rates from a selection of studies published during the period of 2013-2015.
<table>
<thead>
<tr>
<th></th>
<th>Nasopharyngeal Carriage Rates %†</th>
<th>References</th>
</tr>
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<tr>
<td><em>S. pneumoniae</em></td>
<td>27-63</td>
<td>(29-35)</td>
</tr>
<tr>
<td><em>H. influenzae</em> ‡</td>
<td>25-36</td>
<td>(29, 32, 33, 36)</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>24-58</td>
<td>(32, 36)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>13-56</td>
<td>(29, 33, 37-39)</td>
</tr>
</tbody>
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† Includes all age ranges and vaccination statuses. ‡ Includes encapsulated and non-typeable strains.
colonized by these pathogens at least once by one year of age (17, 19, 40, 41). Numerous factors predispose to colonization and include environmental factors such as crowding, smoke exposure, low socio-economic status, and even pacifier use (17, 24, 42-44). In addition, there is a peak in colonization by these opportunistic pathogens in the winter months, coinciding with a similar peak in viral upper respiratory tract infections (URTIs) (45, 46). Specific host contributions similarly play an important role in predisposing to the acquisition of colonization. Indeed, young age is a particularly strong risk factor for the rapid and frequent acquisition of potential colonizing bacteria (24, 41, 45, 47, 48). This is in part attributable to environmental exposures such as day care but also to poorly developed and immature immunity to these pathogens (49-51). Reflecting this, opportunistic pathogen-specific mucosal IgA antibodies, which are critical for preventing bacterial adherence to the respiratory mucosa (50, 52), are undetectable prior to initial colonization (53-55). Beyond simple naivety, the immune response in young children also tends towards hypo-responsiveness and, particularly in infants, a T_H2-skew; factors that impair bacterial clearance (56-60). Entwined with this susceptibility to acquisition is the inability to mount a sufficient T cell-independent immune response to polysaccharide antigens, such as the capsules of H. influenzae and S. pneumoniae. Based on these factors, despite exposure to opportunistic pathogens early in life, serum pathogen-specific IgG and IgA do not begin to rise until approximately two years of age (53).
As introduced above, an additional critical component in the acquisition of colonizing opportunistic pathogens are viral URTIs. These are significantly more common in children, who face an average of 5-6 such clinical infections each year (45, 61, 62). The incidence, prevalence, and density of nasal colonization by opportunistic pathogens such as *S. pneumoniae* are increased during and/or following viral URTI (19, 63-65). Further, the symptomology of acute viral URTI, notably rhinitis, is correlated strongly with the acquisition of opportunistic pathogens in children and has been shown in mice to increase transmission (33, 66). Specifically, positive associations have been observed either *in vivo* or *in vitro* between at least one of the opportunistic bacterial colonizers described above and viruses including influenza virus, parainfluenza virus, human rhinovirus, human metapneumovirus, respiratory syncitial virus (RSV), and coronavirus (67). Interestingly, even the live-attenuated temperature-sensitive influenza vaccine was recently shown in mice to increase the colonization density of both *S. pneumoniae* and *S. aureus* (68), though whether a similar effect occurs in humans is not yet known.

Bacterial interactions in the nasopharynx, both antagonistic and synergistic, further contribute to the population of colonizing bacteria. Antagonistic competition between commensal viridans streptococci (non-pneumococcal α-hemolytic streptococci) and *S. pneumoniae, H. influenzae, M. catarrhalis*, and even *S. pyogenes* has been observed in clinical isolates from children (69). Similarly, commensal bacteria including *Corynebacterium* spp., *Dolosigranulum* spp., and *Lactococcus* spp. appear to be protective for
colonization by opportunistic pathogens (70, 71). Indeed, a lack of diversity in the nasal microbiota, as has been observed following viral infections (64), increases the risk of opportunistic pathogen acquisition (71). In addition, disruption of this flora by antibiotic treatment can predispose to acquisition of opportunistic pathogens such as *S. pneumoniae* (72). These pathogens also interact with each other, as colonization by *S. pneumoniae* can inhibit acquisition of *S. aureus*, purportedly in a H$_2$O$_2$-dependent manner via the pneumococcal pyruvate oxidase SpxB (24, 29, 73). Conversely, there is a generally positive correlation between colonization by *S. pneumoniae* and the acquisition of *H. influenzae* and *M. catarrhalis* (1, 65, 74).

**Carriage**

Following acquisition, opportunistic pathogens such as *S. pneumoniae* generally colonize the nasopharynx for approximately 1-3 months in children and shorter durations in adults (10, 18, 40, 75). Colonization of *S. pneumoniae* tends to peak at around 2-3 years of age in developed countries before gradually declining in both incidence and bacterial density through adolescence and into adulthood (24, 63, 76, 77). Interestingly, a recent study of children in India noted an earlier peak at 6-7 months, indicating that numerous factors including geography are contributing to this process (45). Paradoxically, despite increased rates of disease and mortality from *S. pneumoniae* in the elderly, nasal colonization rates remain relatively low in this population (78-80).
Simultaneous carriage of *S. pneumoniae, H. influenzae*, and *M. catarrhalis* can be quite common, particularly in children, during viral URTIs, or during episodes of acute OM (65, 81). The formation of mixed-species biofilms as well as inter-species quorum sensing has been shown to contribute to this process (82-84). The population of particular bacterial species colonizing the nasopharynx at any one time has previously been presumed to be comprised of specific clones of each species. Recently, however, the co-colonization of multiple distinct genetic lineages of the same bacterial species has also begun to be appreciated. This is most evident in *S. pneumoniae* where multiple serotypes can be isolated from the nasopharynx during asymptomatic carriage (85). As with multi-species colonization, this is more common in children and is, intriguingly, associated with increased nasal bacterial density (86). Further, co-colonization enhances the diversity of carried serotypes by enabling even poorly colonizing serotypes to more readily persist in the nasopharynx (87).

Opportunistic pathogens colonizing the nasopharynx utilize multiple mechanisms to persist in this site. Reaching and subsequently adhering to the mucosal epithelial surface is a key first step. *H. influenzae* utilizes fimbriae and outer membrane proteins (OMP) P2 and P5 to adhere to glycoconjugates and mucins, respectively (88, 89). Similarly, *M. catarrhalis* utilizes the OMP UspA1 to adhere to epithelial cells via several moieties including fibronectin (90). Once attached to the epithelial surface, opportunistic pathogens such as *S. pneumoniae* and *S. aureus* can then invade airway mucosal cells as a means of persistence and immune evasion (91, 92). Other immune evasion mechanisms
also involve epigenetic regulation mediating, for example, the variable decoration of lipooligosaccharide with phosphorylcholine in *H. influenzae* which inhibit the binding of antibodies (93). Biofilms are also a critical component of carriage by these opportunistic pathogens and will be discussed in more detail in a later section.

**Clearance of Colonization**

While colonization rates decrease with increasing age, the mechanisms mediating this observation and indeed the mechanisms affecting clearance in general are still not fully understood. Extracellular pathogens such as *S. pneumoniae* are typically cleared most efficiently via antibody-mediated complement deposition and opsonophagocytosis (24, 94), though the importance of this mechanism in the nasopharynx is unclear. As episodes of nasal colonization decrease as age, and thus pathogen exposure, increases, the development of pathogen-specific antibodies is likely intimately involved in pneumococcal clearance. Nasal colonization typically results in both local IgA and systemic IgG production to various proteins of *S. pneumoniae, H. influenzae*, and *M. catarrhalis* (54, 95, 96). Of these, IgA appears to be the most critical as increased amounts of IgA at the mucosal surface are associated with improved clearance of colonization (52, 54). Despite a critical role in preventing invasive disease by *S. pneumoniae*, as will be discussed later, the importance of antibodies against pneumococcal capsular polysaccharide in the context of nasal
colonization is, somewhat surprisingly, a contested subject. On one side, vaccination with pneumococcal capsular polysaccharide does indeed reduce colonization rates of that serotype. Conversely, while some studies have documented increases in capsule-specific antibodies following colonization, others have not (96-99). Further, these antibodies did not correlate with protection from colonization in a model of human experimental carriage (100). Finally, while epidemiological analyses have observed serotype-specific protection from colonization, implying a role for capsule-specific antibodies, this was only in a subset of serotypes. These same studies also described epidemiologic evidence of serotype-independent protection (101, 102), indicating that capsular polysaccharide-independent mechanisms also contribute to clearance. Taken together, these results suggest that antibodies to both protein and capsular antigens are involved in the clearance of colonization, though other factors likely contribute as well to serotype-independent clearance.

Illustrating that additional factors are involved, the clearance of *H. influenzae* requires both OMP-specific antibodies and innate signalling via TLR stimulation and NOD-1 (103, 104). Further, clearance of *S. pneumoniae* from the nasopharynx has in fact been observed to be independent of antibody production in one study (60). Rather, clearance relied on serotype-independent CD4+ T\textsubscript{H}1 and T\textsubscript{H}17 cells, innate recognition of pneumolysin via TLR2 and TLR4, and phagocytosis by both macrophages and neutrophils (60, 105, 106). Reflecting this, immunization of mice with pneumococcal proteins screened for their ability to stimulate T\textsubscript{H}17 cells significantly improved protection from colonization (107).
While reconciling differences in these studies can be difficult, when taken in sum, clearance of nasal colonization by opportunistic pathogens appears to be a complex and multi-factorial process that relies on all arms of the immune system.

In most cases, nasal colonization is ultimately cleared without any evidence of clinical disease or symptoms. However, colonization is also the necessary, although not sufficient, step in the initiation of numerous disease processes. The precise mechanisms that mediate this transition are still not fully understood but appear to center on alterations to the nasopharyngeal microenvironment. One of the most significant of these are viral URTIs that contribute to the preferential outgrowth of opportunistic pathogens in the nasopharyngeal flora (61, 67, 108), a process termed dysbiosis. In this context, nasal colonization can result in protean disease presentations including otitis media, sinusitis, pneumonia, and meningitis, among others.

**Otitis Media**

Otitis media is an exceptionally common disease that can affect as many as 80% of children at least once and has a global incidence rate of nearly 11% (109, 110). It is also the leading cause of pediatric sick office visits and antibiotic prescriptions resulting in direct healthcare costs estimated at US $3 billion each year in the US alone (111-113). While a majority of cases are acute in nature and self-limiting, they can persist and/or become recurrent; potentially leading to hearing loss and speech or learning delays (112, 114).
Rather than a singular diagnosis, OM is in fact a clinical spectrum of disease processes. At its core, it is essentially inflammation of the middle ear space with resultant effusion, typically stemming from infection. Acute otitis media (AOM) is the most readily identifiable presentation and classically involves an earache in a young child. Despite the seeming simplicity of this diagnosis, indiscriminate antibiotic prescriptions over the past decades have led to more stringent diagnostic guidelines that now require otoscopic evidence of effusion and inflammation along with acute inflammatory symptoms (112). Specifically, the most recent guidelines now require either severe bulging of the tympanic membrane (TM) with or without otorrhea or mild TM bulging with associated pain and/or inflammation for the diagnosis of AOM (115). Distinguishing AOM from the separate but related disease process of otitis media with effusion (OME) can be quite difficult clinically. OME is essentially a diagnosis of exclusion from AOM and is defined as the presence of middle ear effusion, indicating localized inflammation, but in the absence of acute inflammatory signs or symptoms such as an erythematous tympanic membrane or otalgia (116). While OME may precede, follow, or occur independently of an episode of AOM, distinguishing between the two is important for appropriate treatment strategies (117), as will be discussed later.

Given the widespread significance of this disease process, the causative agents of OM are of intense interest. These otopathogens reach the middle ear space via ascension of the eustachian tube connecting the posterior nasopharynx to the middle ear. As such, nasal colonizing opportunistic
pathogens including *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* are the most commonly isolated bacterial causes of OM (27), with specific rates varying depending on numerous factors including the pneumococcal conjugate vaccine (PCV), as depicted in Table II. This vaccine, which will be discussed in more detail in an ensuing section, contains components of the capsular polysaccharide of 7-13 serotypes and was introduced in its first iteration as the PCV-7 in the US in 2000 for use in children. In general, isolation of *S. pneumoniae* has mildly declined since that time while the detection of *H. influenzae* has resultantly increased (4), though these findings vary from study to study and are reflected in the wide range of acute OM rates depicted in Table II. In addition to vaccines, the development of more sensitive diagnostic assays has further altered the microbiology of OM, or at least our appreciation of it. Indeed, while OM was previously thought to be a predominantly bacterial disease, it is now estimated that as many as 66% of AOM cases contain both bacteria and viruses (1), as depicted in Figure 1. As with nasal colonization, specific bacterial-viral interactions are associated with the development of AOM more than others (118). In addition to viral-bacterial coinfections, more recent work also suggests that OM caused solely by viral infections may be more common than previously thought (81, 119).

Several risk factors have been identified in the pathogenesis of OM including, unsurprisingly, concurrent viral URTI. Illustrating this, one study observed that greater than 60% of symptomatic viral URTIs, particularly RSV and influenza virus, were associated with the development of OM, often within the
Table II. Reported etiology of AOM in children aged 5 months to 12 years old from 3 published studies in the United States from 1995-2006. Table adapted from Coker et al. (4, 120-122)
<table>
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<th></th>
<th>Pre-PCV7</th>
<th>Post-PCV7</th>
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<tr>
<td><em>S. pneumoniae</em></td>
<td>44-54</td>
<td>31-44</td>
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<tr>
<td><em>H. influenzae</em></td>
<td>18-43</td>
<td>24-57</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>4-9</td>
<td>1-11</td>
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Figure 1. Etiology of otopathogens in middle ear effusions of AOM detected by molecular analysis. Graph adapted from Ruohola et al. (1)
17% Bacteria + Viruses
65% Bacteria Alone
27% Virus Alone
4% No Detected Pathogen
4%
first five days (61). Viral infection predisposes to OM via several mechanisms including mucosal inflammation, hypercytokinemia, neutrophil impairment, and increased bacterial adherence (123-126). In particular, the viral impairment of normal eustachian tube (ET) function appears critical in this process. Physiologically, the ET ventilates the middle ear space and enables pressure equilibration. Viral-induced disruption of ET ciliary function, sloughing of cellular debris, and edema can induce ET obstruction (127, 128). This obstruction can then lead to the development of negative middle ear pressure which facilitates effusion. Indeed, experimental IAV infection of adult volunteers was shown to induce negative middle ear pressure in nearly 60% of participants (129).

In addition to viral URTI, age is also a key component in the pathogenesis of OM. Prevalence tends to peak between 6-36 months of age, coinciding with peak nasal colonization of otopathogens, and declines thereafter, particularly at 6 years old (61, 130). This association with young age is partly mediated by an immature immune system, as described above for nasal colonization, but also by anatomy. The eustachian tube in children is both more horizontal and shorter, allowing easier access for nasopharyngeal secretions to reach the middle ear. Similarly, the tensor veli palatini muscle is underdeveloped in young children, revealing a more patent ET orifice. Unsurprisingly, being colonized with potential otopathogens such as *S. pneumoniae* is also associated with an increased incidence of OM (118). Other risk factors are also involved and include male sex, socioeconomic status, allergies, immunodeficiencies such as asplenia, and
environmental exposures including tobacco smoke, crowding, and daycare (117, 131).

As in the nasopharynx, the precise mechanisms mediating the clearance of OM by the immune system are still not fully understood. Both protection from and clearance of OM are mediated, at least in part, by the adaptive immune response and pathogen-specific antibodies. Indeed, antibodies in the middle ear effusion are positively correlated with clearance (132). As such, it is unsurprising that the antibody response and the pathogen-specific memory B cell population following OM has been shown to be reduced in otitis prone children (133, 134). The antibodies themselves are likely spilling over from the neighboring adenoids, nasal-associated lymphoid tissue, and the bloodstream (135) as T cells, specifically CD4$^+$ T$_H$ cells, and not plasma cells, are the dominant lymphocyte present in middle ear effusions (136). In general, however, immune cells are not present at high levels in healthy middle ear tissue and instead migrate in following stimulation (137).

The innate immune response during OM also plays a critical role in initially controlling the infection and bridging to the adaptive immune response. Epithelial cells of the middle ear mucosa express numerous pattern-recognition receptors (PRRs) and TLRs and produce pro-inflammatory cytokines including IL-1β and anti-microbial peptides such as β-defensins following pathogen stimulation (138). The infiltration of macrophages into the middle ear during OM further contributes to pathogen clearance, though this effect may be species- and, for S. pneumoniae, serotype-dependent (139). Though most innate and adaptive
immune cells migrate to the middle ear, even the healthy mucosa contains Langerhans cells that have been hypothesized to play a key role in ultimately mediating the resolution of OM by linking the innate and adaptive immune responses (140, 141). Somewhat paradoxically, however, certain components of this immune response have actually been shown to promote OM. Illustrating this, though neutrophils are a major component of the immune response to OM and purulent effusions, both *S. pneumoniae* and *H. influenzae* have been shown to survive and even proliferate within neutrophil extracellular traps (NETs) (142, 143).

Via a combination of the mechanisms described above, a majority of OM episodes resolve spontaneously. Despite this, antibiotics are still prescribed in as many as 80% of cases (144). However, with complications arising from OM continuing to decrease in developed countries (112), newer treatment guidelines have attempted to limit antibiotic usage as much as is feasible. As such, “watchful waiting” with adjunctive analgesic therapy is now recommended in all cases of non-severe AOM in children older than 24 months (117). If antibiotics are warranted, β-lactams antibiotics including high-dose amoxicillin with or without clavulanate and cefdinir are generally first-line therapeutics (115). Recurrent or recalcitrant OM can occur in upwards of 25% of children and often requires the placement of tympanostomy tubes to address ET dysfunction (145).
**Streptococcus pneumoniae**

*S. pneumoniae* is a Gram-positive, α-hemolytic encapsulated diplococcus that is a widespread colonizer of the human nasopharynx, generally in the absence of clinical disease. However, when the nasopharyngeal microenvironment is altered such as by concomitant viral URTI, pneumococci can readily disseminate to cause disease. Indeed, *S. pneumoniae* remains one of the leading bacterial causes of diseases ranging from otitis media and sinusitis to pneumonia, meningitis, and even a newly-appreciated role in the initiation of cardiac micro-lesions (7, 146, 147). The anti-phagocytic polysaccharide capsule encasing *S. pneumoniae* is a primary virulence determinant contributing to these disease processes and is used as a means of typing this pathogen. Currently, over 90 pneumococcal serotypes have been identified by Quellung reaction, though more likely exist (148). Interestingly, epidemiological studies suggest that serotype can predict disease presentation. Reflecting this, the invasiveness of a specific serotype is generally inversely correlated with its colonization prevalence (149). For example, serotypes 1, 5, and 7 cause a high proportion of invasive pneumococcal disease in unvaccinated populations but are infrequently carried; the inverse is the case for serotypes such as 6B, 19F, and 23F (24, 149, 150).

Taking advantage of this clustering of a relatively small number of serotypes causing a disproportionate amount of disease in children, the seven-valent PCV was introduced containing capsular components from the seven most invasive serotypes in the Western hemisphere conjugated to a protein immunogen (151). This conjugation allows children less than two years of age to
mount a T cell-dependent immune response to the carbohydrate capsular antigen. At the time of its introduction, serotypes covered by PCV-7 accounted for greater than 80\% of invasive pneumococcal disease and over 85\% of antibiotic resistant strains (152, 153). Since its implementation in 2000 and the introduction of the 13-valent PCV in 2010, these vaccines have had a profound impact in preventing vaccine-type invasive pneumococcal disease (154, 155). Their effects on nasal colonization and otitis media, however, have been much more muted. Indeed, while carriage of vaccine-type serotypes has decreased, the overall prevalence of \textit{S. pneumoniae} colonization is largely unchanged due to the phenomenon of serotype replacement (102). The effect of vaccination on pneumococcal OM is even less clear with some studies reporting a mild to moderate effect and others reporting near elimination of vaccine-type OM and replacement by \textit{H. influenzae} (156-158). These differences may be attributable to the time at which each study was performed following implementation of the vaccine, as pneumococcal OM rates appear to decrease immediately following vaccine introduction before rebounding in 3-5 years (159). In general, the all-cause reduction in OM since PCV introduction is approximately 6-9\% (160). While PCV implementation has had only modest effects on rates of pneumococcal colonization and OM, it has succeeded in significantly decreasing antibiotic resistance as well as complications including recurrent OM, tympanostomy tube placement, and mastoiditis (117, 154, 161). In sum, a shift in developed countries is occurring towards serotypes and strains associated more with nasal colonization and uncomplicated mucosal diseases such as OM.
The interaction of these less virulent pneumococcal strains in the context of OM with common coinfecting viruses such as influenza A virus is in significant need of further study and is addressed in this project.

The human nasopharynx is the primary ecological niche of *S. pneumoniae* and as such it is well-adapted to establish colonization at this site. To reach the epithelial surface, the negatively charged capsule helps to prevent ensnarement and aggregation of *S. pneumoniae* in the similarly-charged host mucins distributed throughout the nasopharynx (162). Once at the mucosal surface, at least three exoglycosidases, NanA, BgaA, and StrH, are involved in sequentially cleaving terminal carbohydrate moieties such as sialic acid from host-associated glycoconjugates to expose potential adherence sites (163). The products released via this enzymatic activity can also be used as a carbon source by the bacteria via the SatABC sialic acid transport and metabolism locus (164). In addition to cryptic receptors revealed by enzymatic activity, pneumococci can also adhere to numerous sites throughout the upper airway epithelial surface via a plethora of cell-surface adhesins. These include CbpA/PspC binding to the laminin receptor and the plgR (165, 166), PsaA binding to E-cadherin (167), and the phosphorylcholine residues of teichoic and lipoteichoic acid on the pneumococcal surface binding to the receptor for platelet activating factor (PAF) (168). Adherence of *S. pneumoniae* to plgR and the PAF receptor has further been shown to mediate translocation of the bacteria across the cellular surface (169, 170). A subset of adhesins including PavA and Eno can then bind to extracellular matrix components beneath the epithelial barrier (171, 172).
Once adherent to the mucosal surface, \textit{S. pneumoniae} can then begin to form biofilms. Biofilms are generally defined as adherent, highly-organized multicellular microbial communities encased in an extracellular matrix (173). In pneumococci, this matrix is comprised \textit{in vivo} of extracellular DNA from both the bacteria and the host, proteins, and polysaccharides (174, 175), although the specific composition is still unclear. While the precise definition of biofilms continues to be debated, increasing evidence indicates that these microbial communities are the “preferred state of bacterial growth in nature” (176). As such, they have been observed clinically in the adenoids of children as well as in the nasopharynges of mice (177, 178). Further, there are several distinct differences when bacteria are in a biofilm versus a planktonic state. Biofilm-associated bacteria possess a distinct transcriptome with reduced capsular expression and metabolic activity and increased natural competence for genetic exchange (87, 175, 179, 180). Biofilms additionally provide physical protection from clearance by host immune mediators (181, 182). Interestingly, pneumococci in the biofilm state are also generally less invasive than their planktonic counterparts (183). This is mediated in part as they are both more adherent via up-regulation of factors such as CbpA and also elicit a dampened inflammatory response from macrophages via down-regulation of virulence factors such as pneumolysin (184). A multitude of other pneumococcal virulence factors are involved in the formation of biofilms including SpxB, CbpA, and LytA (179, 183-186). Of note, the pneumococcal neuraminidase NanA in particular is up-regulated during biofilm growth and has been implicated in the formation of
biofilms *in vitro* during co-culture with epithelial cells (184, 187). The specific role of NanA in pneumococcal biofilms, however, and whether this interaction with epithelial cells is even required, is in need of further study and is addressed in the project discussed herein.

In addition to contributing to nasal colonization, biofilms have similarly been implicated in pneumococcal disease processes including OM. Indeed, these structures have been observed in the middle ears of experimentally-infected chinchillas and in the middle ear mucosa of children with recurrent OM (82, 174). Biofilm bacteria, for multiple reasons including reduced metabolic activity, are significantly more resistant to antibiotics than their planktonic counterparts (84, 179, 188). The high rates of antibiotic failure and recurrent OM in children have been attributed in part to these microbial biofilms in the middle ear.

While biofilms enable *S. pneumoniae* to persist in both the nasopharynx and middle ear, the factors enabling this pathogen to transition from biofilms in the former site to the latter are of great interest. Recent work has identified one such factor, influenza virus, contributing to this transition. Indeed, infection with IAV, both *in vitro* and *in vivo*, was shown to induce dissemination of pneumococci from the biofilm state (189). Specifically, the viral induction of febrile temperature as well as increased concentrations of extracellular ATP, norepinephrine, and glucose was observed to mediate this transition (189).
Somewhat paradoxically, transmission of *S. pneumoniae* from host to host occurs almost exclusively during nasal colonization and not during disease (190). As such, its propensity to induce disease stands in stark contrast to the clear selective pressure for colonization. As a result, it has been hypothesized that the same factors that enable pneumococci to colonize the nasopharynx can also enable it to cause disease when this colonization is either disrupted or the host environment is altered (23). Illustrating this, pneumococci that were induced to disseminate from the biofilm state by viral infection were associated with increased expression above that of both biofilm and planktonic bacteria of key virulence factors including pneumolysin, PspA, CbpA, and NanA (180). These factors, which contribute to persistence during colonization, also have important roles in immune evasion and induction of inflammation, particularly once pneumococci have disseminated from the nasopharynx. Pneumolysin is a cytolysin with additional protean functions including inhibition of ciliary activity, inhibition of respiratory burst in phagocytes, and stimulation of CD4+ T cell activation and inflammatory cytokine secretion (191). Interestingly, pneumolysin is also required in the initiation of an appropriate anti-pneumococcal immune response (192). PspA possesses several immune evasion properties including inhibition of C3b complement deposition and lactoferrin bactericidal activity (193). Reflecting its critical role in virulence, antibodies to this protein are protective for colonization (100). CbpA similarly inhibits complement deposition but via a separate mechanism involving the recruitment of Factor H (194). Finally, the
neuraminidase NanA also contributes to immune evasion by desialylating immune factors including lactoferrin, IgA2, and secretory component (195).

*Streptococcus pneumoniae* also adapts between nasal colonization and disease via phase variation. This is a method of bacterial adaptation and immune evasion that involves a stochastic shift in phenotypic profiles that is reversible, heritable, and occurs at rates more frequently than from mutation alone (196). *S. pneumoniae* phase-varies between a transparent phase typically associated in animal models with nasal colonization and an opaque phase typically associated with disease (197). The two phases are identified by their colony morphology when viewed on a translucent agar surface under oblique transmitted light. Although shifts in colony appearance were noted earlier, the phases were first isolated and characterized in 1994 by Weiser et al. (197). Since then, these phases have been shown to be relevant clinically as well. Indeed, studies have identified that the transparent phase is found more frequently in the nasal washes of children while the opaque is more predominant in the bloodstream and in middle ear effusions (198, 199).

Several features of the transparent phase make it ideally suited for nasal colonization and are summarized in Table III. Capsular polysaccharide is down-regulated which serves to enhance epithelial adherence, as a thick capsule can mask many of the pneumococcal cell-surface adhesins and its negative charge
Table III. Features of the transparent and opaque phases in *S. pneumoniae*. 
<table>
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<th></th>
<th>Transparent</th>
<th>Opaque</th>
<th>Reference</th>
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<tr>
<td>Nasal colonization</td>
<td>↑</td>
<td>↓</td>
<td>(197)</td>
</tr>
<tr>
<td>Virulence in intra-peritoneal infection</td>
<td>↓</td>
<td>↑</td>
<td>(200)</td>
</tr>
<tr>
<td>Site of isolation</td>
<td>Nasal wash</td>
<td>Bloodstream</td>
<td>(198, 199, 201)</td>
</tr>
<tr>
<td>Transformability</td>
<td>↑</td>
<td>↓</td>
<td>(202)</td>
</tr>
<tr>
<td>Capsule amount</td>
<td>↓</td>
<td>↑</td>
<td>(199, 200, 203)</td>
</tr>
<tr>
<td>Complement deposition</td>
<td>↑</td>
<td>↓</td>
<td>(204)</td>
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<tr>
<td>Susceptibility to opsonophagocytosis</td>
<td>↑</td>
<td>↓</td>
<td>(203)</td>
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<tr>
<td>Phosphorylcholine</td>
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<td>↓</td>
<td>(200)</td>
</tr>
<tr>
<td>Pneumolysin</td>
<td>↔</td>
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<td>(200)</td>
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<tr>
<td>NanA</td>
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<td>↓</td>
<td>(195)</td>
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<td>CbpA</td>
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<tr>
<td>PspA</td>
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<td>(200, 205)</td>
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can repel from the similarly-charged epithelial surface (200, 205). In addition, phosphorylcholine residues on pneumococcal cell wall-associated teichoic and lipoteichoic acid are more abundant in this phase and contribute to adherence via the PAF receptor (168). Conversely, the increased expression of phosphorylcholine and reduced expression of capsule render the transparent phase more susceptible to host clearance mechanisms, particularly in sites more immunologically privileged than the nasopharynx. Specifically, C3b deposition, complement-mediated killing, and susceptibility to opsonophagocytosis are all greater in the transparent phase, mediated by increased activation of the alternative pathway of complement (203, 204). Further, expression of the neuraminidase NanA, which contributes in multiple manners to nasal colonization (206), is significantly increased in the transparent phase relative to the opaque (195). Finally, recent work has also demonstrated that biofilm formation, a hallmark of colonization, appears to select for the transparent phase, although more work is needed as this finding has not been universal (184, 207).

The opaque phase of *S. pneumoniae* is identified more frequently during disease states rather than from nasal washes in both clinical studies and animal models (197, 198, 201, 205, 208, 209). Much of the increased virulence of this phase has been attributed to its increased expression of capsule (205). Indeed, one study identified that this specifically mediated the enhanced resistance of the opaque phase to opsonophagocytosis (203). Beyond capsule, this phase also exhibits increased expression of PspA which, as described previously, has roles in immune evasion and virulence (193, 200, 210). Somewhat surprisingly,
however, the pro-inflammatory virulence factor pneumolysin was shown to be expressed similarly between the two phases (200). Microarray analysis of the opaque phase has also identified significantly reduced expression of genes involved in bacteriocin production/immunity and carbohydrate uptake and metabolism (195). Interestingly, virulent pneumococci dispersed from the biofilm state by influenza virus were recently shown to actually have the opposite expression profile with increased expression of genes associated with sugar metabolism and bacteriocins (180). These dispersed populations have previously been shown to be predominantly in the opaque phase (189), illustrating the still not fully understood balance between these two phases in \textit{S. pneumoniae}. Given these intriguing prior findings, the project presented herein was designed to determine whether factors such as IAV coinfection alter the traditional paradigm regarding the opaque and transparent phases. Specifically, the potential ability of IAV coinfection to differentially affect nasal colonization and middle ear infection by both pneumococcal phases was investigated.

Phase variation is widespread among many upper respiratory tract pathogens in addition to \textit{S. pneumoniae} including \textit{H. influenzae}, \textit{Neisseria} spp., and non-pneumococcal streptococci. In nearly all cases, its mechanism is largely understood and is often mediated by either slipped-strand DNA mispairing or epigenetic effects such as DNA methylation (196). These switches occur randomly at the molecular and cellular level but are ultimately preferentially selected for based on environment conditions. Despite over two decades of study, the genetic mechanism underpinning phase variation in \textit{S. pneumoniae},
however, has been difficult to elucidate. Early work suggested that the box A and C regulatory element may mediate this shift between phases (211). Located downstream of these elements was a sequence which is involved in phase switch frequency in certain but not all serotypes. This region, however, was ultimately not required for phase variation (211). In addition, the specific features of *S. pneumoniae* that ultimately contribute to visible colony opacity are similarly unknown. This has, in group A streptococci, been traced to chain length; however, this was not observed in the pneumococcal phases (197). Electron micrographs have indicated evidence of increased autolysis by the transparent phase. While the autolysin LytA is similarly up-regulated in this phase, a ΔLytA mutant still exhibited phase variation (205). In addition, as capsule production appears to be a key difference between the two phases, it has been hypothesized that the amount of capsular polysaccharide may impact colony opacity. As acapsular pneumococci similarly exhibit two colony morphologies (168), however, this mechanism is not likely. Taken together, these results suggest that phase variation in *S. pneumoniae* is a complex process involving differential regulation and expression of numerous genes rather than a single virulence factor.

**Influenza A Virus**

Influenza viruses are single-stranded, negative sense RNA viruses belonging to the family *Orthomyxoviridae*. The influenza viral genome contains
eight segments encoding as many as 12 polypeptides. Based on differences in the encoded proteins, influenza viruses are classified into three types. Of these, influenza A and B viruses cause the vast majority of disease in humans and are responsible for annual epidemics. IAV is further subdivided into subtypes based on antigenic differences in the surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins, as will be discussed in more detail later. Currently, H1N1 and H3N2 subtypes of IAV are circulating in the population. While the primary reservoir of influenza viruses is avian, it is a major human pathogen responsible for a staggering 3-5 million severe infections each year (212). Indeed, influenza viral infections are estimated to occur in 10-20% of the world’s population (213). Approximating the societal impact of this infection, a 2007 report estimated that influenza each year in the US alone was responsible for 3.1 million hospitalized days and US $10.4 billion in direct medical costs (214).

As with *S. pneumoniae*, the pathogenesis of influenza viral infection begins at the mucosal surface. The three surface expressed viral glycoproteins, NA, HA, and M2, play critical roles at this stage and in the influenza viral replication cycle. The neuraminidase (NA) cleaves sialic acid residues from host mucins, enabling the virus particle to reach the epithelial surface without becoming entrapped and cleared (215). Once at the epithelium, the viral hemagglutinin (HA) binds to $\alpha2,3$- and $\alpha2,6$-linked sialic acid on the cell surface and mediates fusion with the host cellular membrane and endocytosis into the cell (216). As the endosome acidifies, the matrix protein ion channel M2 enables acidification of the virion core, which then releases the viral nucleic acid and
allows it to reach the nucleus for viral replication (217). Newly synthesized viral RNA and proteins then reassemble at the host cell surface before budding as a new virion. The NA is then involved in its primary role of cleaving sialic acid at the host cell surface, thereby preventing the HA from inducing aggregation of budding virions and inhibiting dissemination (218). This somewhat antagonistic interplay between the functions of the HA and NA is critical in the pathogenesis of influenza virus and has implications for the management of infection (216), as will be discussed later.

Much of the pathology resulting from influenza viruses stem from the pro-inflammatory environment present during and following infection. Indeed, an important component of the 1918 influenza pandemic that claimed over 50 million lives has been traced to a unique variant of the viral accessory protein PB1-F2 that was shown to be hyper-inflammatory (6, 219, 220). Influenza infections induce this inflammatory environment by two main mechanisms. The first is direct via the cytolytic destruction of host cells during viral replication. This is mediated both non-specifically by virion budding and specifically by the pro-apoptotic activity of viral proteins such as PB1-F2 and the inhibition of anti-apoptotic activity by the M2 protein (221, 222). The second mechanism of inflammation is inter-related and is driven by the immune response itself attempting to clear the viral infection. Necrosis and apoptosis of respiratory mucosal cells triggers an inflammatory cascade with the secretion of pro-inflammatory cytokines including IL-1, IL-6, and tumor necrosis factor-α (TNF-α) and chemokines including monocyte chemoattract protein-1 (MCP-1) and IL-8.
These result in the influx of neutrophils, macrophages, and T cells which can then themselves secrete additional cytokines (223); ultimately contributing to tissue damage and resulting in hypercytokinemia in some cases (224). Further, the induction of type I interferons (IFN-α and IFN-β), while critical for viral clearance, have also been implicated in host epithelial cell damage (225). Thus, controlling the extent of this pro-inflammatory environment via modulatory cytokines such as IL-10 is also important in the response to influenza infection. Reflecting this, IL-10 was shown to be required for viral clearance and prevention of mortality in animal models (226). While the immunopathology of influenza infection undoubtedly contributes to human disease, disrupting this cascade with potential therapeutics is difficult as these same inflammatory mediators are required for the control of viral infection (227).

Regarding the course and clearance of influenza infection, viral load will generally peak in the first 2-4 days, followed quickly by a peak in the inflammatory cytokines and IFNs described above via PRRs including TLRs and RIG-1 inducing NFκB transcriptional activity (228). The Type I IFN response specifically is critical in controlling early viral infection by inducing transcription of interferon-stimulated genes and induction of an antiviral state (228, 229). Reflecting this significance, influenza virus has multiple mechanisms encoded in its limited genome devoted to disrupting this response (230, 231). Additionally, type I IFNs and other cytokines secreted from epithelial cells and macrophages also serve a key role in activating dendritic cells and bridging to the adaptive immune response (125). Ultimately, influenza viral infections are generally
cleared by a combination of innate effector cells including natural kills (NK) cells, macrophages, and neutrophils. Later in the course of infection, virus-specific CD4$^+$ and CD8$^+$ T cells further contribute to viral clearance via production of IFN-$\gamma$ and cytolytic effector function, respectively (229). Virus-specific antibodies directed against the viral HA and NA can then be detected in serum and mediate future protection (232).

This protection from future influenza infections, however, is generally short-lived. Due to the immense selective pressure of HA- and NA-specific antibodies, these viral proteins are highly antigenically variable. This is mediated by the lack of proof-reading activity and the error-prone nature of the influenza RNA polymerase generating a constant pool of potentially beneficial mutations in these proteins (233, 234). When combined with the selective host environment, this results in antigenic drift of influenza A viruses and can lead to annual epidemics if neutralizing immunity is lost. In addition, as the viral genome is segmented, genetic reassortment can occur whereby influenza A viruses acquire a novel HA or NA to which widespread immunity is not present. These antigenic shifts may lead to pandemics, as has occurred in 1918, 1957, 1968, 1977, and 2009 (235).

To attempt to account for this extensive antigenic variation and the resultant short-lived immunity, influenza vaccines in both an inactivated form administered intramuscularly and a live-attenuated form administered intranasally are formulated each year. These vaccines contain a representative H1N1, H3N2, and influenza B virus that are predicted by the WHO to be circulating in a
given region that year. While each vaccine exhibits seasonal variability in effectiveness, particularly the 2014-2015 northern hemisphere formulation (236), they have been remarkably effective at preventing influenza morbidity and mortality, particularly in the elderly and in children (237, 238). With regards to their effectiveness at preventing influenza complications such as OM, however, the results are more mixed. Indeed, while two studies have reported significant reductions in all-cause OM (239, 240), two others either observed reductions in only OME (241) or reported no preventative effect at all (242).

As influenza vaccine prophylaxis provides incomplete protection, antiviral therapeutics represent the second line of defense against infection. Of the only two classes of antivirals currently licensed for use, resistance against the viral M2 protein inhibitors (e.g. Amantadine and Rimantadine) has made these medications nearly unusable in the US, with greater than a 90% resistance rate (243, 244). The second class of antivirals, the inhibitors of the viral neuraminidase (e.g. Oseltamivir and Zanamivir), act by impairing the release of progeny virions from host cells thereby decreasing viral infectivity (245). These medications, if given in the first 48 hours, are currently first-line therapeutics for prophylaxis and are recommended in patients with confirmed or suspected influenza infection with comorbidities or requiring hospitalization (246). Resistance against this class is significantly lower as the inhibitors act at the highly-conserved active site of the viral NA. Indeed, resistance rates have been reported at 0.3% in adults and 4% in children (247). These medications have proven effective at ameliorating influenza infection (248), and have also shown
promising, albeit limited, efficacy at reducing influenza-associated complications in a limited number of trials (249, 250).

While influenza infections themselves are significant, much of the associated morbidity and mortality is attributable to these downstream complications, notably from bacterial coinfections. Viral infection alone is generally not associated with severe mortality, with the current exception of H5N1 avian influenza circulating in southeast China (251). In combination with bacterial coinfection, however, influenza is the 8th leading cause of death in the US and is estimated to account for 250,000-500,000 deaths annually worldwide (8, 212). *S. pneumoniae* is a leading co-pathogen of influenza infections; however, at least for children in the developed and vaccinated world, the trends of pneumococcal disease have shifted dramatically in the past 15 years. As described previously, the incidence of invasive pneumococcal disease has declined significantly since the introduction of the PCVs in 2000 (152, 252, 253). Despite this, the synergistic interaction between *S. pneumoniae* and influenza virus has not gone away. Rather, it appears that the context has shifted somewhat away from lethal disease and more towards localized diseases such as OM. Indeed, even in the vaccine era, as many as 33% to 66% of influenza viral infections in young children are still associated with an ensuing case of AOM, often caused by a bacterial coinfection (61). Thus, a greater understanding of how IAV ultimately contributes to the development of OM by colonizing strains of *S. pneumoniae* in the vaccine era is needed and is a major aim of the project presented herein.
Interactions between *S. pneumoniae* and Influenza A virus

Many virulence features of both *S. pneumoniae* and IAV have additional and/or synergistic roles when in coinfection. For both pathogens, the mucosal surface is a key area of interaction. As such, the traditional paradigm, put forth nearly 100 years prior, holds that preceding IAV infection pathologically damages the epithelial surface, enabling bacterial attachment and outgrowth (254). As discussed above, many influenza infections are indeed cytotoxic to respiratory epithelial cells and expose the underlying basement membrane in the process, to which *S. pneumoniae* can strongly adhere via components such as fibronectin (255, 256). IAV infection has also been shown to decrease mucociliary velocity *ex vivo*, thereby contributing to increased pneumococcal adherence (257). Interestingly, however, coinfection with influenza viruses that induce little to no pathologic damage can also promote pneumococcal pulmonary infection (5, 258), suggesting that factors beyond indiscriminate mucosal damage and dysfunction are involved.

The viral alteration of specific host receptors represents another mechanism by which pneumococcal mucosal infection is enhanced. This is mediated, in large part, by the proinflammatory cytokine environment induced by IAV infection. Specifically, cytokines such as IL-1β up-regulate expression of the PAF receptor on epithelial cells to which *S. pneumoniae* can adhere via its surface-associated phosphorylcholine (168). Illustrating this, PAF receptor-deficient mice coinfectected with IAV have been shown to be more resistant to post-influenza pneumococcal pulmonary infection than their receptor-expressing
counterparts (259). This observation has been challenged elsewhere (260), however, suggesting that this relationship may be more intricate than currently appreciated. In addition, mucin production is regulated in part by NFκB and as such is similarly increased by a pro-inflammatory milieu similar to that observed during IAV infection (261). Recently, pneumococci have been shown to utilize this increased mucin as both a carbon source and for adherence (262). As an additional specific mechanism, the IAV NA can activate transforming growth factor-β (TGF-β) from its latent form and this activation correlates with the enzymatic activity of the viral NA (263). This increase in TGF-β has been shown to mediate deposition of fibronectin, integrins, and collagen to which pathogens such as *S. pneumoniae* and *S. pyogenes* can adhere (255, 264). Finally, the enzymatic activity of the IAV NA can also contribute directly by revealing cryptic receptors for pneumococcal adherence, as will be discussed more in an ensuing section.

Another key component influencing pneumococcal-IAV interactions is the alteration of the immune response to these pathogens. While neutrophils are a predominant component of the inflammatory response to both *S. pneumoniae* and IAV, prior influenza infection impairs neutrophil activity and coinfection enhances neutrophil apoptosis (124, 265). This is mediated, at least in part, by the excess of type I IFNs induced by IAV infection impairing the appropriate production of neutrophil chemokines in response to *S. pneumoniae* (266). Type I IFNs also suppress the production of IL-17 by γδ T cells which is important for pneumococcal clearance (267). Similarly, though somewhat paradoxically, the
overwhelming increases in IFN-γ observed in experimental models of IAV infection can also impair the macrophage response to *S. pneumoniae* via both the down-regulation of the scavenger receptor MARCO and by depleting this cell population in the airway (268, 269). In addition, as IAV begins to be cleared, an anti-inflammatory state mediated in large part by IL-10 begins to arise that ultimately impairs the capability of immune cells to respond adequately to an ensuing pneumococcal challenge (270, 271). Finally, though less well-explored, the interaction between these two pathogens is mutually synergistic and *S. pneumoniae* coinfection also contributes to IAV infection. Reflecting this, IAV titers have generally been observed to increase in animal coinfection models following bacterial inoculation (272, 273). The mechanisms driving this interaction are yet unclear but likely reflect alterations in the anti-viral adaptive immune response induced by *S. pneumoniae* infection (274).

**Pneumococcal Phase Variation**

Despite the confirmed presence of distinct phases of *S. pneumoniae* in the nasopharynges of children (198), the interaction of these phases during polymicrobial infections has been largely unexplored. Coinfection of *S. pneumoniae* with non-typeable *H. influenzae* has been shown to select for the transparent phase (275), though whether this is a unique interaction or indirectly from the increased biofilm formation observed when these two pathogens are co-cultured is not known. Additionally, an interaction with influenza A virus has been observed where IAV infection specifically induces dissemination of opaque phase pneumococci from the biofilm state (189). Further work is needed to investigate
the relationship between the pneumococcal phases and IAV, particularly in the in vivo environment. As such, this project aimed to first investigate how these phases may differentially contribute to nasal colonization and next to elucidate whether the synergistic pneumococcal-influenzal interaction extends to both the opaque and transparent phases. Ultimately, this project was designed to assess whether IAV coinfection disrupted the traditional dichotomous paradigm of colonization versus disease between the pneumococcal phases, as has been observed in a prior clinical study (201).

Neuraminidases

A particularly interesting area of potential interaction between influenza A virus and S. pneumoniae involves the neuraminidases that are expressed by both pathogens. Neuraminidases are exoglycosidases containing a sialidase catalytic domain that removes terminal sialic acid residues from host glycoconjugates. Specifically, the influenza neuraminidase NA is a tetramer surface glycoprotein which, in the presence of Ca\(^{2+}\), catalyzes the cleavage of α2,3- and α2,6-linked sialic acids, the latter of which are most significant for human infection (276). As discussed previously, the enzymatic activity of NA plays important roles in facilitating both virion release from host cells and preventing viral aggregation in host mucins at the mucosal surface (215, 276). Interestingly, despite viral replication and infectivity in vitro relying on these functions of NA (277), IAV clinical isolates in the past 10 years have been identified with minimal to no neuraminidase activity (278, 279), indicating a clear potential mechanism of resistance to viral neuraminidase inhibitors. Efforts to
account for this partial dispensability of IAV NA activity have focused on the antagonistic yet functional balance between HA binding to and NA cleaving sialic acid (216). Indeed, in influenza strains with impaired NA function, mutations have been observed near the sialic acid binding pocket of HA, decreasing its binding affinity and thus decreasing its reliance on NA activity (280). A second potential hypothesis, however, which has received considerably less attention, involves the contributions of the polymicrobial in vivo environment. Two predominant members of the oropharyngeal flora that, like S. pneumoniae, also express a neuraminidase, Streptococcus oralis and Streptococcus mitis, were just recently shown to be capable of rescuing influenza virus replication from inhibition by the viral NA inhibitor zanamivir in vitro (281). A similar effect has been observed in vitro with S. pneumoniae as well (282), suggesting a similar activity of the bacterial and viral proteins.

Neuraminidases from both S. pneumoniae and influenza virus are very distinct based on DNA sequence (~15% homology); however, they share a similar catalytic domain topology as well as conserved key catalytic residues (3, 283, 284). Bacterial neuraminidases appear to diverge from influenza NA in the addition of several non-catalytic repeating segments and, in the case of bacteria such as Vibrio cholerae, the addition of lectin-like carbohydrate binding motifs (285). These domains have been shown to significantly increase the catalytic efficiency of bacterial neuraminidases (286).

S. pneumoniae expresses at least three neuraminidases, NanA, -B, and –C. All three contain an N-terminal signal sequence for export, though NanA also
has a C-terminal surface-anchoring motif (3, 287). Similarly, they all possess, by sequence homology, an N-terminal lectin-like carbohydrate binding domain in addition to the C-terminal catalytic domain (3), as depicted in Figure 2. Of the three, NanA is the most highly conserved and is expressed in all strains, while NanB and NanC are expressed in 96% and 51% of studied strains, respectively (288). NanA is transcribed approximately 10-fold greater and its activity is roughly 100-fold higher than NanB at relevant physiologic pH (289, 290). As discussed previously, NanA is canonically involved in vivo in bacterial adherence via desialylation of host cell-associated glycoconjugates. More recently, NanA has also been shown to be involved in immune evasion, biofilm formation with epithelial cells, and metabolism via utilization of sialic acid as a carbon source (195, 291-293). The precise roles of NanB and NanC in vivo are less clear, though various studies have suggested a role in pulmonary infection (290) and invasion of the cerebrospinal fluid (288), respectively.

Due to the apparent similarities in the enzymatic activity of the influenza and pneumococcal neuraminidases, this particular interaction has been theorized to contribute to viral-bacterial synergism. There are several pieces of evidence to support this hypothesis. First, in a mouse model of pulmonary coinfection, the IAV NA specifically was observed to exacerbate pneumococcal infection and mortality (294). Recapitulating this result in vitro, pneumococcal adherence was increased to IAV-infected epithelial cells and this effect could be reversed by treatment with a viral neuraminidase inhibitor in a time frame where its effects on
Figure 2. Representative domain map of *S. pneumoniae* NanA. Adapted from Uchiyama et al and Xu et al (2, 3).
viral replication can be excluded (294, 295). Providing further evidence of this synergistic interaction, maximal pneumococcal adherence to IAV-infected epithelial cells \textit{in vitro} and pulmonary infection \textit{in vivo} correlated with the neuraminidase activity of a panel of recombinant influenza A viruses (296). The mechanisms underlying these observations have traditionally centered on the synergistic exposure of pneumococcal binding sites. Indeed, lectin labelling of chinchilla eustachian tube epithelial cells has been shown to be maximally altered during coinfection with both pathogens (208). Newer studies have further contributed that IAV may also enhance pneumococcal adherence by directly activating TGF-β tissue remodeling and that the sialic acid released by the viral NA can independently promote pneumococcal proliferation (5, 262, 297).

These studies have led to the hypothesis that influenza virus neuraminidase inhibitors may have the additional effect of preventing sequelae such as coinfection with \textit{S. pneumoniae}. This effect would, theoretically, be present even if the medications were given outside of the first 48 hours following influenza infection, greatly increasing their clinical utility. One study in coinfected mice did indeed observe that oseltamivir treatment, even when delayed, reduced pneumococcal pulmonary infection independently of its effect on viral replication (294). Clinically, efficacy trials of viral neuraminidase inhibitors have generally not been designed to assess complications such as bacterial coinfections. However, a recent meta-analysis estimated that acute OM was approximately half as likely to develop in patients with confirmed influenza virus infection treated with neuraminidase inhibitors (298), though more randomized clinical trials are
sorely needed. Conversely, infection by a clinical IAV variant possessing reduced neuraminidase activity was not observed to alter colonization by *S. pneumoniae* in humans (299). In addition, the ability of viral neuraminidase inhibitors to interact with pneumococcal neuraminidases *in vivo* is not clear. Though both oseltamivir and zanamivir have been shown to bind pneumococcal NanA, their inhibitory effect relative to the influenza NA is only moderate for the former and nearly undetectable for the latter (300, 301). Complicating our understanding of this interaction further, the contributions of pneumococcal NanA during IAV coinfection, though likely important, have been almost entirely unexplored. Taken together, the contributions of the influenza virus NA to *S. pneumoniae* infection are well-documented, yet further work is needed regarding its pneumococcal counterpart NanA. Thus, this project aimed to investigate the specific role of NanA in pneumococcal-influenzal synergism *in vivo*.

**Statement of Research Purpose**

The research presented herein is centered on the interactions between influenza A virus and *S. pneumoniae* in a mouse model. First, we investigated the ability of a preceding viral infection to induce a prototypical colonizing, less virulent pneumococcal strain to colonize the nasopharynx and infect the middle ear, as occurs most frequently in children. Next we investigated whether this influenza viral synergism extended to the intra-strain phenotypic alterations between the opaque and transparent phases inherent within pneumococci.
Finally, we investigated the specific role of the major pneumococcal neuraminidase NanA in the IAV-induced exacerbation of nasal colonization and middle ear infection as well as a potential, extra-enzymatic role for this protein with significant implications for future therapeutic usage and targeting.
Chapter I:

Influenza A Virus Pneumococcal Nasal Colonization and Middle Ear Infection
Independently of Phase Variation


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INTRODUCTION

Otitis media (OM) is among the most common diseases of childhood, affecting most children at least once before the age of three and accounting for billions of dollars in healthcare expenditures annually in the US alone (111, 302). *Streptococcus pneumoniae* (pneumococcus) is the most commonly isolated pathogen of OM even following the advent of widespread vaccination with the pneumococcal conjugate vaccine (303). Pneumococci can also asymptotically colonize the nasopharynx (22, 50, 304, 305). There remains much to be learned regarding the factors contributing to the transition from carriage to OM disease.

Epidemiologic evidence strongly indicates a role for upper respiratory tract viruses, particularly influenza A virus (IAV), in the dissemination of *S. pneumoniae* from its nasopharyngeal niche to the middle ear (61, 81, 126, 305, 306). Indeed, experimental IAV infection of human volunteers was shown to increase both nasal pneumococcal burden and incidence of OM (307, 308). This synergistic interaction has also been observed across different pneumococcal strains and serotypes in ferrets (309) and, extensively, in chinchillas (310, 311). The development of mouse models, though often requiring either the use of artificial or invasive procedures (312, 313) or infant mice (66, 314, 315), has further significantly contributed to and widened the field. Largely stemming from these models, the viral factors which predispose to OM are well-studied and include eustachian tube dysfunction, ciliary dysmotility, mucosal inflammation, and neutrophil impairment (81, 126, 128, 223, 316). How this viral predisposition
affects specific pneumococcal subpopulations during middle ear infection, however, is less well understood.

*S. pneumoniae* is a tremendously variable pathogen that is well adapted to persist within the human nasopharyngeal microbiota. In addition to including numerous capsular types, pneumococcal populations also undergo intra-strain phase variation in which bacteria spontaneously and reversibly shift between an opaque and transparent colony types (197). Present data indicate that pneumococci exist in humans as a heterogeneous combination of these phases during both colonization and disease (198). In prior studies, the transparent phase was shown to be more efficient in nasopharyngeal colonization in vivo (197) along with increased adherence to activated lung epithelial cells in vitro (317) and an increase in transparent phase pneumococci within biofilm communities (184). Biofilms are surface-attached bacterial communities that are encased in an extracellular matrix and display an altered metabolic phenotype (173). These structures in particular have previously been linked both clinically and experimentally to nasal colonization and OM (174, 178, 185). The opaque phase, in contrast, is associated with invasive disease (197), with increased resistance to opsonophagocytosis and host clearance (203), likely mediated through increased capsular expression (200).

We hypothesized that coinfection with IAV may alter pneumococcal colonization and, possibly, alter the dynamics of middle ear infection by the two phases, disrupting this traditional paradigm regarding the transparent and opaque phases. Previous work in a chinchilla model has indicated that, at later
time points, opaque phase variants predominated in both the nasopharynx and middle ear following IAV infection (318). As the opaque phase is not traditionally associated with colonization in animal models (197) or in humans (198), this current study was designed to further investigate the interaction of IAV and the two phases of *S. pneumoniae* in a distinct animal model and using a well-studied colonizing pneumococcal strain.

To investigate our hypothesis, we first aimed to develop a readily accessible adult mouse model of nasal colonization and middle ear infection that mimicked the natural course of infection. As such, we utilized an intranasal inoculation of a colonizing pneumococcal strain (EF3030) that has been shown to colonize the mouse nasopharynx in the absence of lethal, systemic disease (319, 320). We found that preceding IAV infection enhanced nasal colonization by *S. pneumoniae* and this was correlated with a significant increase in middle ear infection. We then employed this model to investigate whether viral coinfection altered the pathogenesis of middle ear infection by the two pneumococcal phases. Our results demonstrated inherent differences in bacterial adherence to nasopharyngeal epithelial cells and biofilm viability in vitro as well as nasal colonization in vivo between the two phases. Despite this, IAV coinfection ultimately enabled both phases to colonize the nasopharynx and infect the middle ear similarly. This provided evidence that the pathogenesis of pneumococcal colonization and middle ear infection during IAV coinfection was phase-independent.
MATERIALS AND METHODS

**Infectious agents and growth conditions.** *S. pneumoniae* EF3030 (serotype 19F) is a nasopharyngeal isolate that has previously been shown to colonize the mouse nasopharynx in the absence of lethal, systemic disease (319, 320). Pneumococci were cultured on trypticase soy agar (Beckton-Dickinson) with 5% sheep blood (Hemostat) and 4 µg/ml gentamicin (Sigma). For freezer stocks, *S. pneumoniae* were grown in brain-heart infusion (BHI) broth (Beckton-Dickinson) supplemented with 10% heat-inactivated horse serum and 10% catalase (2,500 U/ml, Worthington) until mid-late logarithmic phase (OD$_{600\text{ nm}}$ 0.6-0.85), diluted 1:1 in 50% glycerol, and frozen at -80°C. Influenza A/PR/8/34-GFP (H1N1) used in this study was generously provided by Adolfo García-Sastre (321). Viral stocks were prepared in embryonated eggs and titered via determination of the median tissue culture infectious dose (TCID$_{50}$) in Madin-Darby canine kidney cells.

**Phase determination and variant isolation.** Colony phenotype was determined under oblique light, as described previously (91, 197). Phase variants of the parent EF3030 strain were isolated prior to each experiment and were confirmed to contain >90% of colonies in the same phase of at least 100 counted colonies. These variants retained the ability to shift between the transparent and opaque phases. The percent opacity of each inoculum was confirmed prior to each experiment.
**Coinfection model.** Female, 6 week old BALB/c mice (Jackson Laboratory) were housed in a BSL-2 facility. All mouse infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee. Mice were anesthetized with Avertin (20 mg/ml 2,2,2-Tribromoethanol, Acros Organics) intraperitoneally and infected intranasally with $3 \times 10^3$ TCID$_{50}$ IAV diluted in 20 µl sterile phosphate-buffered saline (PBS) on ice or an equal volume of vehicle control. Four days later, mice were again anesthetized and inoculated intranasally with $5 \times 10^6$ CFU *S. pneumoniae* in 20 µl BHI on ice or vehicle control. Bacterial density was confirmed by serial dilution and plate count. The phase composition of each inoculum was also confirmed. Mice were weighed and assessed for disease at least twice daily. While minimal and moderate signs of disease were apparent in mice inoculated with EF3030 alone and IAV alone, respectively, a small subset of coinfected mice (<10%) exhibited moribund disease. These mice were euthanized on that day. At days 2 and 4 post-bacterial infection, mice were euthanized and the nasopharynx and bilateral bullae were aseptically excised and homogenized (PowerGen 100, Fisher Scientific) in sterile PBS. Aliquots of these homogenates were serially diluted and plated for bacterial quantification and phase determination following 20 hour incubation at 37°C and 5% CO$_2$.

**qRT-PCR.** Tissue homogenates were snap frozen and stored at -80°C. Aliquots were then freeze-thawed three times and RNA was isolated with TRIzol reagent (Ambion) and reverse transcribed to cDNA using random primers (Invitrogen), deoxynucleoside triphosphates (Promega), and Superscript III reverse
transcriptase (Invitrogen) supplemented with RNasin (Promega) following each manufacturer's specifications. Influenza RNA was then detected by real-time quantitative PCR using TaqMan Universal PCR Master Mix (Applied Biosystems) and IAV primers (322) with a 7500 Real Time PCR System (Applied Biosystems), essentially as described previously (323).

**Histopathology.** Excised nasopharynges and bullae from pre-selected mice were fixed in 4% paraformaldehyde, decalcified, embedded in O.C.T. Solution (Sakura Finetek), and sequentially frozen by placing at room temperature for 3 hours, -20°C for 12 hours, and -80°C for 24 hours or until ready for use. Samples were cryosectioned into approximately 5 µm slices, stained with hematoxylin and eosin, mounted with Permount (Electron Microscopy Sciences), and examined microscopically.

**Adherence Assay.** The human nasopharyngeal carcinoma cell line Detroit 562 (ATCC CCL-138) was maintained in Eagle’s Minimal Essential Media with L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cells were grown in T75 flasks (Corning) at 37°C and 5% CO₂. Adherence assays were performed essentially as described previously (184, 324). Approximately 2x10⁵ cells/well were seeded onto 24-well flat-bottom, tissue culture-treated plates (Costar) and allowed to grow to confluence (~4x10⁵ cells/well). When cells were ready to infect, bacteria were grown in BHI broth supplemented with 2,500 U/mL catalase to mid-logarithmic phase (OD₆₀₀nm = 0.45-0.65). Cell monolayers were washed three times in warmed Dulbecco’s PBS (DPBS, Lonza) and overlaid with 1 ml/well serum- and
antibiotic-free media containing 1x10^7 CFU of *S. pneumoniae* opacity variants. The bacterial density and phase composition of each inoculum was determined. To initiate bacterial-epithelial cell interactions, the plates were centrifuged at 1300 x g for 5 minutes and then incubated at 37°C and 5% CO₂ for 1 hour. Following incubation, the monolayer was washed three times with DPBS to remove non-adherent bacteria. Epithelial cells and adherent bacteria were then dissociated from the plate by treatment with 0.25% Trypsin-EDTA (Gibco) for 10 minutes. The number of adherent bacteria was determined by serial dilution and plate count. Pneumococcal adhesion was expressed as percent adherence relative to the inoculum. The experiment contained 3 biological replicates per opacity variant and included 6 variants of each phase.

**Static Biofilm Assay.** This assay was performed essentially as described previously (84). Briefly, *S. pneumoniae* opacity variants were inoculated into Todd-Hewitt broth (Beckton-Dickinson) with 0.5% yeast extract (Difco) and supplemented with 10% heat-inactivated horse serum and 2,500 U/ml catalase and were seeded onto 24-well flat-bottom plates (Costar) at 5x10^5 CFU/well. The bacterial density and phase composition of each inoculum was confirmed prior to each experiment. After incubation at 37°C and 5% CO₂ for 4 or 24 hours, the media was removed and the adherent biofilms were resuspended in sterile PBS by scraping and vigorous pipetting. The biofilm viability, defined as the total recovered numbers of surface-attached *S. pneumoniae*, and phase composition were then determined via plate count and phase determination, respectively.
Each experiment contained 3 biological replicates and was repeated at least three times.

**Statistical analysis.** Pairwise comparisons of nasal colonization, middle ear infection, and phase composition were analyzed by Mann-Whitney $U$ test. The percent adherence of pneumococcal phase variants to epithelial cells and in vitro biofilm viability was analyzed by Student’s $t$ test. Correlation between nasal colonization density and middle ear bacterial burden was assessed by Spearman’s rank correlation test. Where noted in the text, fold-change was calculated using the geometric mean, except for biofilm viability which was calculated using the arithmetic mean. Bacterial counts below the limit of detection were considered uninfected. For statistical analysis and graphing, these points were plotted at the limit of detection. A $p$-value $<0.05$ was considered to be significant. Statistical analyses were performed using GraphPad Prism, version 5.01 (GraphPad Software).
RESULTS

**Nasal colonization is increased by prior influenza virus infection.** Mice were infected intranasally with IAV followed four days later by *S. pneumoniae* to investigate the effect of antecedent viral infection on pneumococcal colonization. The mouse nasopharynx was permissive to IAV infection as influenza viral RNA was still detectable in 75-89% of mice by day 8 post-viral infection (**Table IV**). Further, all mice challenged with *S. pneumoniae* exhibited nasal colonization at both days 2 and 4 post-bacterial infection (**Figure 3A**). This colonization was observed to persist for at least 20 days (not shown). In mice coinfected with both IAV and *S. pneumoniae*, the magnitude of pneumococcal colonization was significantly increased by ~8-fold at both days 2 and 4 (*p* = 0.0003 and *p* = 0.0043, respectively; **Figure 3A**). Bacterial coinfection, alternatively, did not have a significant effect on the proportion of IAV-infected nasopharynges (*p* >0.05, Fisher’s exact test; **Table IV**).

**Influenza virus enhances pneumococcal middle ear infection.** In children, the magnitude of nasal colonization is linked to the incidence of OM (325). We next aimed to determine whether the increase in nasal pneumococcal colonization following IAV infection was similarly associated with middle ear infection. IAV coinfection significantly increased the middle ear pneumococcal burden by nearly 40-fold at both days 2 and 4 post-bacterial infection (*p* <0.0001 and *p* = 0.025, respectively; **Figure 3B**). Prior viral infection also altered the incidence of middle ear bacterial infection as 100% of coinfected mice possessed detectable numbers of bacteria in the normally sterile middle ear space of at least
Table IV. Presence of IAV RNA in the nasopharynx and middle ear. The percentage of tissues that contained detectable IAV RNA by qRT-PCR at the indicated time points is shown. Data are pooled from two replicate experiments.
<table>
<thead>
<tr>
<th>Tissue Type and Inoculum</th>
<th>% Tissue Containing IAV RNA (Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Nasopharynx</strong></td>
<td></td>
</tr>
<tr>
<td>IAV</td>
<td>100.0 (0.0)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAV + <em>S. pneumoniae</em></td>
<td>76.9 (28.4)</td>
</tr>
<tr>
<td><strong>Middle Ear</strong></td>
<td></td>
</tr>
<tr>
<td>IAV</td>
<td>27.8 (13.8)</td>
</tr>
<tr>
<td>IAV + <em>S. pneumoniae</em></td>
<td>15.4 (10.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Days post-bacterial infection, corresponding to day 6 and day 8 post-viral infection, respectively.

<sup>b</sup> Data was evaluated via Fisher's exact test and no significant differences between IAV- and co-infected tissue were present ($p > 0.05$).
Figure 3. Influenza A virus exacerbates nasal colonization and middle ear infection by *S. pneumoniae*. Mice were infected intranasally with influenza A virus (IAV) PR8-GFP followed four days later with *S. pneumoniae* (Sp) EF3030. 

**A**, Magnitude of nasal colonization and **B**, middle ear infection by *S. pneumoniae* in the tissue homogenates from days 2 and 4 post-bacterial infection (corresponding to days 6 and 8 post-viral infection). Each data point represents a single nasopharynx or ear and the data are pooled from two replicate experiments. The short, solid lines denote the geometric mean and the dotted line represents the limit of detection of the assay. Statistical analysis was performed using two-tailed Mann-Whitney *U* test and is denoted by * *p* <0.05, ** *p* <0.01, or *** *p* <0.001. 

**C**, Correlation between nasal colonization density of *S. pneumoniae* and the middle ear pneumococcal burden in coinfected mice. Each ear is correlated to its respective nasopharynx. The data contain both time points and are pooled from two replicate experiments. Statistical analysis was performed using Spearman’s rank correlation test and is denoted by Spearman’s rank correlation coefficient (r) and *p*-value (*p*). 

**D**, Middle ear (ME) pneumococcal burden at day 2 post-bacterial infection recovered from mice infected with *S. pneumoniae* alone (black bar), coinfected with IAV and possessing detectable viral RNA in the ME (white bar), or coinfected with IAV but containing no detectable viral RNA in the ME (hatched bar) from a separate experiment. Each bar represents the geometric mean +/- 95% confidence intervals. The presence of IAV RNA was detected by qRT-PCR and no IAV RNA was detectable in the ME by day 4 post-bacterial infection (day 8 post-viral infection). Data are pooled from two replicate experiments. Statistical analysis was performed using two-tailed Mann-Whitney *U* test and is denoted by ** *p* <0.01 or *** *p* <0.001.
one ear as compared to 75% of singly-infected mice ($p = 0.01$; Fisher’s exact test). These coinfected mice were also more prone to develop bilateral middle ear infection (88% vs 35%, $p = 0.0004$; Fisher’s exact test, **Supplemental Figure 3**), a frequently-used clinical criterion for the initiation of antimicrobial therapy (326). Further, the magnitude of middle ear infection was found to be directly correlated to the density of nasal pneumococcal colonization but only during coinfection with IAV (**Figure 3C**), echoing clinical data (325).

Using the presence of IAV RNA in the middle ear as a surrogate marker for viral infection at that site, coinfection with pneumococci had no effect on the proportion of ears infected with IAV ($p > 0.05$, Fisher’s exact test; **Table IV**). The middle ear pneumococcal burden, however, was significantly elevated in coinfected mice even in those ears without detectable IAV RNA ($p<0.01$; **Figure 3D**). Further, the pneumococcal burden in the middle ears that did contain IAV RNA at the designated time points was significantly greater than ears from both singly-infected mice and ears from coinfected mice that did not contain viral RNA ($p<0.01$, **Figure 3D**).

**Influenza virus induces increased nasal epithelial inflammation.** IAV-induced inflammation in the middle ear has previously been shown to propagate pneumococcal OM (315). To determine whether IAV induced a similar effect in the nasopharynx, nasal sections from both singly- and coinfected mice were compared using histopathologic staining and microscopic analyses. The nasal epithelium from mock infected mice was intact and contained a thick mucociliary border (**Figure 4**, column 1). During colonization with *S. pneumoniae*, an
Figure 4. **Influenza virus induces nasal epithelial changes.** Representative histological images of the lateral wall of the nasal cavity from mice (n ≥ 4) infected intranasally with influenza A virus (IAV) followed four days later by *S. pneumoniae* from both days 2 and 4 post-bacterial infection (corresponding to days 6 and 8 post-viral infection). Representative images from mock-, *S. pneumoniae* alone-, and IAV alone-infected mice are also shown. Tissues were stained with hematoxylin and eosin and examined microscopically at magnification x40. The scale bar denotes 20 µm. Insets were examined at magnification x60 and the respective scale bars denote 10 µm. Relevant structures are labelled including the mucociliary border (MCB). Filled arrowheads mark areas of ciliary denudation, open arrowheads denote areas of epithelial disruption.
inflammatory infiltrate was present within the nasal meatuses consisting primarily of cells morphologically resembling neutrophils (Figure 4, column 2), as has been noted previously (23). Despite this infiltrate, the nasal epithelium was largely unchanged from mock-infected mice with the notable exceptions of isolated areas of ciliary denudation and areas of increased mucus production (Figure 4, column 2). In contrast, IAV infection, both in the presence and absence of *S. pneumoniae*, was associated with substantial damage to the nasal epithelium including widespread ciliary denudation, epithelial disruption, areas of microvascular hemorrhage and exposure of the lamina propria (Figure 4, columns 3-4). Epithelial changes appeared similar between IAV-only and coinfectected mice. A predominantly neutrophilic infiltrate was again present within the lumen of the nasal meatuses of both IAV-only (Figure 4, column 3) and coinfectected mice (Figure 4, column 4). Unlike mice colonized with pneumococci alone, this infiltrate was also epithelial-associated (Figure 4, columns 3-4). These data indicate that IAV alters the nasopharyngeal epithelium, suggesting a potential mechanism by which nasal colonization is enhanced following viral infection.

**Middle ear inflammation is increased in coinfectected mice.** As pneumococci were detected in the middle ears of both singly- and coinfectected mice, middle ear sections were evaluated histopathologically to determine whether the increased bacterial burden was associated with increased inflammation or signs of disease. No evidence of inflammation or infiltrate was observed in mock-infected mice (Figure 5, column 1). A minimal inflammatory
Figure 5. Middle ear inflammation and infiltrate is increased during coinfection. Representative histological images of the middle ear (n ≥ 8) from mice infected intranasally with influenza A virus (IAV) followed four days later by *S. pneumoniae* from both days 2 and 4 post-bacterial infection (corresponding to days 6 and 8 post-viral infection). Representative images from mock-, *S. pneumoniae* alone-, and IAV alone-infected mice are also shown. Tissues were stained with hematoxylin and eosin. The samples were examined microscopically at magnification x4 and x40 and the scale bars denote 100 µm and 20 µm, respectively. Insets were examined at magnification x60 and the respective scale bars denote 10 µm. Relevant structures are labelled including the middle ear cavity (MEC), tympanic membrane (TM), external ear canal (EEC), and blood vessels (BV).
infiltrate was occasionally present in mice infected with *S. pneumoniae* alone. Of note, one ear was observed to contain a large inflammatory infiltrate within the lumen. In most cases, however, the tissues appeared similar to mock-infected mice (Figure 5, column 2), as has been described previously (314). The heterogeneity of these findings is likely attributable to the high variability of bacterial burden that we detected in the ears of singly-infected mice. While most values were clustered around ~10^2-10^3 CFU/ml, the range extended to as high as ~10^6 CFU/ml (Fig. 1B). As middle ear inflammatory infiltrates have been shown to correlate with bacterial burden (327), it is likely that the disparate finding that we observed in one ear was due to an abnormally high magnitude of middle ear infection in that animal.

In mice infected with IAV alone, an inflammatory infiltrate was also largely absent. However, there was evidence of mucosal inflammation with increased edema, epithelial thickening, and vasodilation (Figure 5, column 3). These findings were more prominent in coinfected mice (Figure 5, column 4). Further, an inflammatory infiltrate consisting primarily of neutrophils was readily apparent in the middle ear lumens of coinfected mice (Figure 5, column 4). Taken together, these results indicate that the increased magnitude of middle ear bacterial infection following IAV was associated with increased evidence of both mucosal inflammation and inflammatory infiltration. As these are two histological findings linked to the clinical diagnosis of OM (328), these data are suggestive that middle ear disease was likewise increased during bacterial-viral coinfection in these mice.
During coinfection, the transparent phase is found more frequently in the nasopharynx, the opaque in the middle ear. Pneumococci exhibit phase variation between a transparent phase associated more with nasal colonization and an opaque phase associated more with disease (197). To determine whether this association was also present during bacterial-viral coinfection, the phase composition of colonies isolated from mice inoculated with IAV and S. pneumoniae was assessed. The pneumococcal strain used in this study, EF3030, was comprised of a heterogeneous combination of both phases, as occurs naturally in humans (198). This strain was confirmed to contain approximately 73% opaque phase colonies at the time of inoculation. The propensity of the transparent and opaque phases to be isolated from the nasopharynx and ear, respectively, persisted in vivo during coinfection with IAV. By day 4, colonies isolated from the nasopharynx were more transparent while those from the middle ear were more opaque ($p = 0.037$; Table V), reflecting a similar study in children with acute OM (198). Of note, a similar comparison could not be made in singly-infected mice as insufficient bacterial counts to evaluate phase composition were consistently isolated from the middle ears of these mice.

Transparent phase pneumococci are more adherent to nasopharyngeal epithelial cells. Since transparent phase pneumococci were isolated more frequently from the nasopharynges of both singly- and coinfectected mice, we hypothesized that this may be mediated by increased epithelial adherence. To test this, phase variants of EF3030 were generated and were confirmed to
Table V. Percent opacity of colonies isolated from IAV- and co-infected mice. The mean percentage of opaque phase colonies isolated from either the nasopharynx or middle ear of singly- or co-infected mice at the indicated time points from two pooled experiments.
**% Opaque Phase Colonies (Standard Error)**

<table>
<thead>
<tr>
<th>Inoculum (% opaque phase colonies in inoculum)</th>
<th>Day 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nasopharynx</td>
<td>Middle Ear</td>
<td>Nasopharynx</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF3030 (73.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae-only</td>
<td>69.5 (3.4)</td>
<td>-</td>
<td>64.8 (5.0)</td>
</tr>
<tr>
<td>IAV + S. pneumoniae</td>
<td>64.1 (4.0)</td>
<td>72.2 (5.5)</td>
<td>62.0 (5.8)</td>
</tr>
<tr>
<td>Opaque Variant (99.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae-only</td>
<td>99.5 (0.2)</td>
<td>-</td>
<td>98.4 (0.6)</td>
</tr>
<tr>
<td>IAV + S. pneumoniae</td>
<td>96.1 (1.4)</td>
<td>98.1 (0.8)</td>
<td>99.8 (0.1)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Transparent Variant (5.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae-only</td>
<td>9.1 (2.0)</td>
<td>-</td>
<td>6.8 (2.3)</td>
</tr>
<tr>
<td>IAV + S. pneumoniae</td>
<td>7.6 (1.2)</td>
<td>22.8 (4.5)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>9.6 (1.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated as: (Number of opaque phase colonies) / (Total number of colonies) x 100.

<sup>b</sup> Days post-bacterial infection, corresponding to day 6 and day 8 post-viral infection, respectively.

<sup>c</sup> Insufficient CFUs were isolated from the middle ears of singly-infected mice to assess the percentage of opaque colonies.

* <sup>p</sup> < .05, ** <sup>p</sup> < 0.01 compared to the respective nasopharynx by the Mann-Whitney U-test. † <sup>p</sup> < .05 compared to respective S. pneumoniae-only inoculated mice by the Mann-Whitney U-test.
contain >90% of colonies in the same phase. Transparent phase pneumococci of this strain demonstrated significantly increased adherence to Detroit 562 cells, a human nasopharyngeal carcinoma cell line, than their opaque counterparts (41.1% vs 25.4%, respectively; \( p = 0.002 \); Figure 6A). Of note, Cundell et al. previously reported increased adherence by the transparent phase to activated but not resting A549 immortalized alveolar epithelial cells (317). As invasive blood isolates were employed in the prior study, this discrepancy is likely attributable to differences in the pneumococcal strains studied.

**Biofilm viability is enhanced in transparent phase pneumococci.** To further explore potential mechanisms to explain why the transparent phase was isolated more frequently from the nasopharynx in this model, we investigated biofilm viability in vitro. Biofilms are critical for nasal colonization and persistence in both animal models and children (178, 329). Further, bacterial adherence is the essential first step in biofilm formation. As such, we hypothesized that a transparent phase variant of EF3030 would also exhibit enhanced biofilm viability along with increased adherence. In an in vitro biofilm model, we found that biofilm viability, defined as the numbers of surface-attached pneumococci, was significantly increased in the transparent variant by 3.5-fold at 4 hours and 3-fold at 24 hours (\( p = 0.0002 \) and \( p = 0.0002 \), respectively; Figure 6B). In the time frame studied, no significant phase shifting of the phase variants during biofilm formation was observed (not shown). These results indicated that biofilm formation not only selects for the transparent phase from a mixed-phase inoculum, as has been noted previously (184), but that transparent phase
Figure 6. The transparent phase displays enhanced epithelial adherence and biofilm viability in vitro.  

A. Adherence of *S. pneumoniae* opacity variants to Detroit 562 nasopharyngeal epithelial cells after 60 minutes, expressed as the percentage of adherent bacteria relative to the inoculum. Bars represent the mean ± SEM. The experiment contained 3 biological replicates per opacity variant and 6 variants of each phase. Statistical analysis was performed using two-tailed Student’s *t* test and denoted by ** *p* <0.01.  

B. Viability of pneumococcal opacity variants from 4- and 24 hour static biofilms. Bars represent the mean ± SEM of 3 replicate experiments. Statistical analysis was performed using two-tailed Student’s *t* test and denoted by ** *p* <0.01 and *** *p* <0.001.
pneumococci themselves demonstrate increased in vitro biofilm viability independent of phase shifting.

**Coinfection with influenza virus ablates the nasal colonization defect of opaque phase pneumococci.** The opaque phase is noted for both its increased invasiveness but also its impaired ability to colonize the nasopharynx (197). This phenotype is likely linked, in part, to the reduced adherence and biofilm viability in this phase that we observed in vitro. Theoretically, this impairment in nasal colonization ability could serve as a natural check by limiting the number of the opaque phase in the nasopharynx, thereby inhibiting pneumococci from too rapidly killing its host. As such, we aimed to determine whether the IAV enhancement of pneumococcal colonization in Figure 3A extended to opaque phase pneumococci, potentially disrupting a delicate balance. To investigate this, phase variants that were confirmed prior to each experiment to contain >90% of colonies in the same phase were inoculated to mice either alone or four days following IAV infection. The variants used in this study are not “locked” thereby allowing us to assess whether the viral coinfection altered phase shifting in vivo. Confirming prior studies (197), the opaque phase variant when infected alone exhibited impaired colonization relative to the transparent variant, although this was only statistically significant at day 2 ($p = 0.005$; Figure 7A). Prior viral infection, however, increased the nasal colonization of the opaque variant by 16-fold and the transparent variant by 11-fold. As a result, IAV coinfection ultimately removed the colonization defect of the opaque variant such that the nasal pneumococcal burden between the variants was indistinguishable at both days
**Figure 7. Coinfection with influenza virus alters nasal colonization and middle ear infection by both the opaque and transparent phases.** Mice that had been either mock-infected or infected intranasally with influenza A virus (IAV) four days prior were infected with either the opaque or transparent phase variant of *S. pneumoniae* EF3030. **A**, Magnitude of nasal colonization and **B**, middle ear infection by *S. pneumoniae* in the tissue homogenates from days 2 and 4 post-bacterial infection (corresponding to days 6 and 8 post-viral infection). Each data point denotes a single nasopharynx or ear and the data are pooled from two replicate experiments. The short, solid lines indicate the geometric mean and the dotted line represents the limit of detection of the assay. Statistical analysis was performed using two-tailed Mann-Whitney U test and denoted by * p <0.05, ** p <0.01, and *** p <0.001. **C and D**, Correlations between nasal colonization density of *S. pneumoniae* and the middle ear pneumococcal titers in mice coinfect ed with IAV and either the opaque variant (C) or the transparent variant (D). Each ear is correlated to its respective nasopharynx. The data contain both time points and are pooled from two replicate experiments. Statistical analysis was performed using Spearman’s rank correlation test and is denoted by Spearman’s rank correlation coefficient (r) and p-value (p).
and 2 and 4 \( (p = 0.81 \text{ and } p = 0.38, \text{ respectively}; \text{Figure 7A} \). Of note, IAV coinfection did not alter the pneumococcal phase composition in the nasopharynx or middle ears relative to singly-infected mice with the exception of a small but statistically significant increase in the proportion of opaque colonies in the nares of mice coinfected with IAV and the opaque variant at day 2 \( (p = 0.0129; \text{Table V} \). Taken together, these results demonstrate that IAV coinfection overcomes inherent colonization deficits of the opaque phase including reduced adherence and biofilm viability, ultimately enabling it to colonize the nasopharynx commensurate with its transparent counterpart.

**Influenza virus exacerbates middle ear infection by both pneumococcal phases.** We next sought to determine whether the increased nasal colonization by both phase variants following IAV infection altered the magnitude of middle ear infection. As the opaque phase has been shown to be isolated more frequently from the ears of children with OM (198), we were initially surprised to discover in singly-infected mice that the number of pneumococci in the middle ears was similar between both phase variants (Figure 7B). This finding likely stems from the colonizing strain used in this study which has been shown to inherently disseminate poorly from the nasopharynx in the absence of external stimuli (320). During coinfection with IAV, middle ear infection by both the opaque and transparent variants was significantly increased relative to singly-infected mice. Interestingly, however, bacterial titers were similar between the phase variants (Figure 7B). This similar magnitude of pneumococcal middle ear infection following IAV was not solely explained by phase shifting in vivo.
Despite similar titers, colonies isolated from the ears of mice infected with the opaque variant remained 98.1-100% opaque while those from mice infected with the transparent variant were 22.8-26.4% opaque (Table V).

Since we observed a correlation between nasal colonization density and middle ear infection in the parent EF3030 strain (Figure 3C), we hypothesized that a similar mechanism would account for the equal magnitudes of middle ear infection by both phase variants following coinfection. To investigate this, the correlation between the nasopharyngeal bacterial density and the middle ear pneumococcal burden in each mouse was again assessed. In the absence of IAV, there was no significant correlation by either phase variant (p >0.05; Spearman’s rank correlation test; not shown). Following IAV infection, there was indeed a highly significant correlation between nasal colonization and the resultant magnitude of middle ear infection with the opaque phase variant (p <0.001; Spearman’s rank correlation test; Figure 7C). Interestingly, no such relationship was observed during IAV coinfection with the transparent variant (Figure 7D). This indicated that the increase in the magnitude of middle ear infection by the transparent variant was not simply due to greater nasal colonization density following IAV coinfection and that multiple factors are likely involved. Taken together, however, these findings establish that the influenza exacerbation of pneumococcal middle ear infection was phase-independent. Further work is needed to investigate the mechanisms by which IAV enhances nasopharyngeal dissemination by both phases.
DISCUSSION

The importance of IAV in the induction of pneumococcal dissemination from the nasopharynx has been well-studied. However, pneumococci are known to persist in the human nasopharynx as a mixture of two phases that differ in amounts of capsular polysaccharide and exposure of bacterial surface moieties (198, 200, 203). How these specifically interact with influenza virus in the context of middle ear infection is less appreciated.

In this study, we first established a murine model wherein a pre-existing influenza A virus infection enhanced the nasopharyngeal colonization by a pneumococcal strain that naturally contains both phases. Prior work has shown that IAV can also enhance nasal colonization when given after pneumococcal inoculation (66). Together, these findings suggest that this synergism is likely occurring quite frequently as these pathogens are serially acquired and cleared. Indeed, children experience a median of 7 carriage episodes before the age of 2 and face an average of 6 respiratory viral infections each year (61, 330). The increased nasal colonization observed in our model following IAV infection correlated with an increased magnitude of middle ear pneumococcal infection, mirroring clinical data from children (15, 325). Of note, this finding regarding middle ear bacterial burden contradicted those of Short et al. who noted that the H1 hemagglutinin of IAV-PR8/34 (H1N1) was unable to induce pneumococcal OM in infant C57BL/6 mice (315). This disparity is likely attributable to differences in both the age and strain of mice, particularly as the content of sialic acid, the hemagglutinin ligand, varies among mouse species (331). However,
our findings indicate that pneumococcal middle ear infection can also be exacerbated in an adult murine model by H1N1 influenza, the most prevalent circulating sub-type in the US (332).

Using this model, we discovered the presence of viral RNA in the middle ears of a subset of both IAV and coinfected mice. As qRT-PCR is not a direct measure of viral infectivity, it is possible that this simply represents contaminating viral RNA from the nasopharynx. This is a difficult question as there are contrasting opinions in the field regarding this subject (expertly reviewed elsewhere (333)). However, in both children and animal models, viral infection of the middle ear has been linked to inflammation (315, 333). In this study, we detected IAV RNA and evidence of mucosal inflammation as late as six days post-viral infection in mice infected with IAV alone. Thus, it appears likely that in this model the presence of viral nucleic acid represents an infection. Supporting this hypothesis, we found that pneumococcal middle ear burden was greatest in IAV-infected ears. Clinically, this direct synergism in IAV-infected ears may account for the increased rates of antibiotic failure in OM cases where both bacteria and viruses are detected in the ear (334).

The mechanisms underpinning this IAV-pneumococcal synergism remain to be fully elucidated. From our findings, it seems apparent that the viral enhancement of bacterial colonization is a critical component. As adherence is required for pneumococci to form the surface-attached biofilms that are hallmarks of colonization (174, 178), the nasal epithelial changes we observed following IAV infection likely contribute to this process. In addition, IAV is known to expose
specific pneumococcal binding sites through its neuraminidase activity (294) as well as the induction of epithelial platelet activating factor expression (317). Recent work by Marks et al. suggests that in addition to increasing nasal colonization, influenza A virus also induces pneumococcal dissemination from the nasopharynx via changes to the host microenvironment including hyperthermia and nutrient availability (189). Once translocated from the nasopharynx, the virally-induced mucosal inflammation we observed in the middle ears of IAV-infected mice (and exacerbated by bacterial coinfection) can then itself contribute to the establishment of pneumococcal middle ear infection (315).

In light of these findings, an important question remains: is this IAV predisposition to enhanced middle ear infection by *S. pneumoniae* a uniform process that universally enables increased outgrowth and dissemination? The work of McCullers et al. began to answer this question and found that the IAV modulation of pneumococcal disease was bacterial strain-dependent (309). Tong et al. further explored whether the intra-strain opaque and transparent phases are differentially affected by preceding IAV infection in a chinchilla model of OM (318). The authors’ conclusions in that study complement our findings with one key distinction. Tong et al. similarly observed an increase in both nasal colonization and middle ear infection by both phase variants following IAV infection, ultimately to similar levels of bacterial burden at early time points. In contrast to our findings, however, Tong et al. did not observe a difference in inherent nasal colonization between the phase variants in the absence of viral
infection (318). Thus, our findings further contribute that IAV infection is sufficient to alter the nasopharyngeal microenvironment such that the opaque phase is capable of colonizing similar to its transparent counterpart. This has significant implications as an invasive serotype, such as serotype 1, which is almost always found in invasive disease and rarely in nasal carriage, has been shown to be significantly more associated with the opaque phase (201). The discrepancy in the observed colonization by the opaque phase between this study and that by Tong et al. is likely accounted for by differences in the serotypes studied and in the animal models employed. Indeed, the distinction in virulence between the two phases has previously been shown to differ between animal species (197, 200).

The ablation of the colonizing defect of the opaque phase by IAV occurred despite the impaired epithelial adherence and biofilm viability that we observed in vitro. Previously, pneumococcal adherence to lower respiratory tract epithelial cells has been shown to increase following influenza A viral infection (295). Additionally, both phase variants have been shown to adhere via the receptor for platelet activating factor, though to a greater extent by the transparent (317). As this specific receptor has been shown to be up-regulated by rhinovirus infection (335), we hypothesize that influenza viral infection may have a similar effect, thereby enabling nasal colonization to increase by both phases. Further, the expression and activity of the pneumococcal neuraminidase, critical for carriage, is diminished in the opaque phase (195, 206). As the IAV neuraminidase has been shown to synergistically interact with pneumococci (294), it is conceivable
that the viral neuraminidase could complement the opaque phase. This area is in need of further study.

The opaque phase has been shown to more efficiently infect and persist in invasive sites such as the lungs and bloodstream in animal models (197, 205). While not generally considered an invasive site, we observed that middle ear infection was similar between the two phase variants following IAV coinfection. It is important to note, however, that studies characterizing the two phases were initially performed using more invasive strains derived from blood isolates (197, 200). As such, the distinction between phases may be more subtle in a colonizing strain such as EF3030 (197, 200, 319, 320). Reflecting this possibility, we did not observe an increase in the incidence or magnitude of middle ear infection by the opaque phase variant in the absence of IAV. While similar numbers of the opaque and transparent variants were found in the middle ear during IAV coinfection, pneumococci within this site were still more frequently in the opaque phase relative to the nasopharynx. This study could not distinguish whether this represented, in the case of the transparent variant, preferential expansion of the small subpopulation of opaque phase bacteria in the inoculum or phase shifting in vivo. As pneumococci phase vary at frequencies ranging from $10^{-3}$ to $10^{-7}$ per generation, it is likely that both are occurring and contributing to the similar magnitude of middle ear infection by both phase variants.

Despite a similar magnitude of infection, the IAV exacerbation of pneumococcal middle ear infection by both phases appears to occur, in part, via
divergent mechanisms. In the opaque phase, the enhanced middle ear infection is directly linked to the increased nasal colonization density induced by viral coinfection. This was reflected in our correlation data and is in agreement with the recent findings by Marks et al. that opaque phase pneumococci disperse more readily from a biofilm state (189). In children, the incidence of OM is linked to nasal colonization density. Our findings thus further suggest that the opaque phase is primarily responsible for this clinically-observed phenotype (325). As this correlation is absent during IAV coinfection with the transparent variant, additional factors beyond nasal colonization density are likely involved. Indeed, once the preceding viral infection enables transparent phase pneumococci to ascend the Eustachian tube, the increased adherence and biofilm viability that we observed in vitro can then enable this phase to increasingly infect the middle ear. Supporting this hypothesis, transparent phase pneumococci have been shown to bind more readily to specific N-acetylglucosamine residues in the Eustachian tube revealed by IAV (208).

In summary, these results indicate that coinfection with influenza A virus increases both nasal and middle ear epithelial inflammation and that this is associated with significantly enhanced nasal colonization and middle ear infection by *S. pneumoniae*. Further, despite inherent differences in in vitro biofilm viability and adherence and in vivo colonization, this effect is independent of the predominant pneumococcal phase.
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CHAPTER II:

Pneumococcal neuraminidase A (NanA) is involved in biofilm formation and synergistic interaction with influenza A virus in nasal colonization and middle ear infection


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INTRODUCTION

*Streptococcus pneumoniae* (pneumococcus) is a widespread colonizer of the human nasopharynx, found in as many as 85% of children under the age of four (304). While this colonization is typically asymptomatic and self-limiting, it is also the requisite precursor for most if not all pneumococcal diseases (24, 336). Otitis media (OM) specifically is frequently caused by *S. pneumoniae* and is one of the most significant public health burdens with as many as 700 million cases worldwide each year, disproportionately affecting preschool-aged children (109, 159). Coinfection with respiratory tract viruses, particularly influenza A virus (IAV), is epidemiologically and experimentally linked to the transition of *S. pneumoniae* from asymptomatic colonizer to otopathogen (61, 66, 81, 274, 308, 309, 315, 337). The role of specific virulence factors in this viral-bacterial interaction, however, remains to be fully explored.

Neuraminidases are widely expressed as virulence factors by a number of bacteria and viruses, including both *S. pneumoniae* and IAV. These proteins possess sialidase activity to catalyze the cleavage of terminal sialic acid residues from glycoconjugates. The influenza neuraminidase has a well-established role in the pathogenesis of IAV infection and inhibitors to this target are currently first-line therapeutics in the United States (338). In addition, IAV neuraminidase activity has been shown to interact synergistically with *S. pneumoniae*. Indeed, pneumococcal adherence in vitro and pulmonary infection in vivo is increased during coinfection with IAV (262, 294, 339). This effect is, at least in part, reliant on the activity of the viral neuraminidase as IAV neuraminidase inhibitors have
shown protection against pneumococcal pneumonia in mice independent of their effect on viral replication (294). Providing an intriguing clinical correlation, the IAV N2 neuraminidase has been shown to have increased enzymatic activity relative to the N1; potentially contributing to the increased incidence of secondary pneumococcal pneumonia in H3N2 as compared to H1N1 epidemics (296, 340).

*S. pneumoniae* expresses as many as three neuraminidases, of which NanA is the most highly conserved, expressed, and active at physiologic pH (195, 289, 290, 341, 342). The modification of host glycoconjugates by NanA plays a multifactorial role in pneumococcal pathogenesis. Most well-known, the sialidase activity of NanA exposes desialylated residues on host glycans and mucins to which *S. pneumoniae* can adhere (293, 343). In addition, NanA desialylates host immune factors thereby potentially promoting persistence (195, 344). Further, the sialic acid released by neuraminidase activity can act as a carbon source as well as a diffusible signal for enhanced pneumococcal proliferation both in vivo and in vitro (291, 297). Interestingly, recent evidence has also indicated that neuraminidase activity may impact biofilm formation. Indeed, reductions in biofilm biomass in vitro have been observed in neuraminidase-deficient and neuraminidase inhibitor-treated *S. pneumoniae* as well as in *Pseudomonas aeruginosa* and *Tannerella forsythia* (187, 345-347). These findings are generally supported by animal models where NanA-deficient pneumococci are impaired in nasal colonization, otitis media, pneumonia, and bacteremia (206, 290). There are, however, conflicting studies (195, 348),
suggesting a complex role for NanA in pneumococcal pathogenesis that is still being appreciated.

Taken together, it is evident that IAV neuraminidase activity significantly contributes to pneumococcal colonization and disease. Left unclear, however, is the role of the pneumococcal neuraminidase NanA in this synergistic interaction, particularly as it relates to nasal colonization and middle ear infection. To assess this, we utilized a mutant strain of *S. pneumoniae* that does not express NanA in our previously-established mouse model of IAV coinfection (337). Our studies identified that prior IAV infection potentiates but cannot completely restore nasal colonization and middle ear infection by NanA-deficient pneumococci, suggesting that the activity of the viral neuraminidase was insufficient to fully complement the absent expression of NanA. Additionally, we demonstrated in vitro that pneumococcal adherence but, interestingly, not biofilm viability can be reduced by a neuraminidase inhibitor. Taken together, these results imply a potential intrinsic role for NanA distinct from its enzymatic activity in pneumococcal pathogenesis.
MATERIALS AND METHODS

Infectious agents, growth conditions, and materials. S. pneumoniae EF3030, a serotype 19F nasopharyngeal isolate was used in all studies. This strain has been shown to colonize the mouse nasopharynx in the absence of lethal systemic, disease (319, 320). Phase variants of the parent EF3030 strain containing >90% of colonies in the same phase were isolated as described previously (337). ΔNanA EF3030 used in this study was generously provided by David E. Briles and is described elsewhere (349). The IAV strain used in this study, Influenza A/PR/8/34-GFP (H1N1), was provided by Adolfo García-Sastre (321). Bacterial and viral strains were propagated and maintained as described previously (337). Except where otherwise indicated, all materials were obtained from Sigma-Aldrich.

Coinfection model. All animal experiments were approved by the Wake Forest University Health Science Institutional Animal Care and Use Committee. Coinfection experiments were performed as described previously (337). Briefly, female, 6-8 week old Balb/c mice (Jackson Laboratory) were inoculated intranasally with $3 \times 10^3$ TCID$_{50}$ of IAV or vehicle control; followed four days later by intranasal inoculation of $5 \times 10^8$ CFU S. pneumoniae or vehicle control. Nasal and middle ear bacterial burdens were enumerated by tissue homogenization, serial dilution, and plating at days 2 and 4 post-bacterial infection.

Neuraminidase Activity. Neuraminidase activity was assessed using the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid
MUAN (0.35% wt./vol.) was resuspended in 0.25M sodium acetate buffer (pH 7), aliquoted, and stored at -20°C. For the assay, *S. pneumoniae* was grown in brain heart infusion broth (Beckton-Dickinson) supplemented with 10% heat-inactivated horse serum and 10% catalase (2,500 U/mL, Worthington) until mid-logarithmic phase. *S. pneumoniae* was then centrifuged at 3600 x *g* at 4°C, resuspended in 0.1 M Tris-HCl (pH 8), freeze-thawed three times in liquid nitrogen, and lysates were stored at -80°C. To measure neuraminidase activity, 10 µL of lysate was mixed with 10 µL MUAN and incubated for 2 hours at 37°C. The reaction was stopped by the addition of 50mM sodium carbonate (pH 9.6). Fluorescence was detected using a POLARstar Omega plate reader (BMG Labtech) with an excitation wavelength of 355 nm and emission wavelength of 460 nm. The background mean fluorescence intensity of Tris-HCl vehicle incubated with MUAN was subtracted from each sample reading. Each reaction was measured in triplicate and the experiment was repeated twice. Where indicated in the text, the competitive neuraminidase inhibitor N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), a sialic acid transition state analog, was added to the bacterial lysates at varying concentrations and incubated at 37°C for 15 minutes prior to incubation with MUAN.

**Adherence Assay.** The adherence of EF3030 and its ΔNanA derivative to Detroit 562 cells (ATCC CCL-138), a human nasopharyngeal epithelial carcinoma cell line, was assessed after 60 minutes of adherence, as described previously (337). Where indicated, vehicle control or the neuraminidase inhibitor
DANA was added at 300 µg/ml prior to incubation of the bacteria with epithelial cells. Each experiment contained at least three biological replicates and was repeated six times.

**Biofilm Assay.** The biofilm assay was performed in Todd-Hewitt broth supplemented with 0.5% yeast extract, 10% heat-inactivated horse serum, and 2,500 U/ml catalase (THY*), essentially as described previously (337). Where indicated, pneumococci were mixed with 300 µg/ml DANA prior to inoculation into the biofilm wells. Biofilm assays were also performed using sialic acid-free media (YSK) (297). Each experiment contained at least three biological replicates and was repeated a minimum of two times.

**Sialic Acid Assay.** Sialic acid content was determined by a modified thiobarbituric acid assay (350-352). In this assay, 200 µl of sample was oxidized by incubation with 100 µl 0.2M sodium periodate/9M H₂SO₄ for 30 minutes at 37°C. Oxidation was stopped by addition of 1 ml sodium arsenite (10% wt./vol.) in 0.5M sodium sulfate/0.05M H₂SO₄. The chromophore was then developed by addition of 3 ml 2-thiobarbituric acid (0.6% wt./vol.) in 0.5M sodium sulfate and boiling for 15 minutes. The solution was cooled, mixed 1:1 in acid butanol (5% vol./vol.), and the OD₅₄₉ and OD₅₃₂ of the butanol fraction were determined. The sialic acid content (µmoles), corrected for the presence of 2-deoxyribose, was calculated by the equation: [(0.021 x OD₅₄₉ – 0.0078 x OD₅₃₂) x Total Reaction Volume] (352). Each sample was measured in triplicate and the experiment was repeated three times.
**Statistical Analysis.** Nasal colonization and middle ear bacterial infection were compared pairwise by a Mann-Whitney *U* test. Samples with bacterial counts below the limit of detection were considered uninfected. These samples were plotted at the limit of detection for graphing and statistical analysis. The percent adherence of pneumococci to epithelial cells relative to the inoculum was compared using a one-way analysis of variance with a Bonferroni posttest. Biofilm viability was analyzed by Student’s *t* test, with the exception of DANA-treated biofilms, which were normalized to untreated wells and analyzed by a one-way analysis of variance with Dunnett posttest. Where indicated in the text, fold-change refers to the geometric mean. *P* < 0.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism, version 5.01 (GraphPad Software).
RESULTS

Nasal colonization is impaired in NanA-deficient pneumococci and is partially restored by influenza coinfection. To assess the role of the pneumococcal neuraminidase NanA in nasal colonization during coinfection with influenza virus, mice were infected intranasally with IAV followed four days later by wild-type or NanA-deficient S. pneumoniae. The ΔNanA mutant was confirmed to grow similarly to wild-type in planktonic culture, to possess minimal neuraminidase enzymatic activity, and was confirmed to not express NanA when assessed by Western blot (data not shown). As predicted from prior studies (206, 290), the magnitude of nasal colonization by the ΔNanA mutant was significantly reduced at both days 2 and 4 post-bacterial infection relative to wild-type ($P < 0.0001$). Coinfection with IAV significantly increased the nasal colonization density of both wild-type and ΔNanA pneumococci ($P < 0.0001$) (Figure 8A). This, however, was not sufficient to enable the ΔNanA mutant to colonize the nasopharynx to the same magnitude as the wild-type during coinfection with IAV. Indeed, during viral coinfection, the nasal pneumococcal burden of the ΔNanA mutant was approximately 5-fold lower than the wild-type at both time points studied (Figure 8A). Taken together, these findings suggest that expression of both the viral and pneumococcal neuraminidase is required for complete synergistic activity between the two pathogens in the nasopharynx.

Influenza A virus potentiates middle ear infection by NanA-deficient pneumococci but not to wild-type levels. Preceding IAV infection has
Figure 8. NanA is involved in nasal colonization and middle ear infection by *S. pneumoniae* in the presence and absence of influenza A virus coinfection. Mice were infected intranasally with influenza A virus (IAV) PR8-GFP followed four days later by intranasal inoculation with either *S. pneumoniae* EF3030 wild-type or EF3030ΔNanA. Data represent the total pneumococcal burden in tissue homogenates from the nasopharynx (A) and middle ear (B) from days 2 and 4 post-bacterial infection, corresponding to days 6 and 8 post-viral infection. Each data point denotes a single nasopharynx or middle ear. The solid horizontal bars indicate the geometric mean and the dotted line denotes the limit of detection of the assay. Data are pooled from two replicate experiments. Statistical analysis was determined using a two-tailed Mann-Whitney *U* test (**, *P* <0.01; ***, *P* <0.001).
previously been shown to increase the magnitude of middle ear infection by S. pneumoniae EF3030 (315, 337). To determine whether pneumococcal NanA was required in this interaction or if its absence could be fully complemented by coinfection with neuraminidase-expressing IAV, the middle ear bacterial burden in singly- and co-infected mice was enumerated. Middle ear infection by ΔNanA pneumococci was significantly reduced relative to wild-type at day 4 ($P < 0.0001$) (Figure 8B), illustrating an important role for NanA in this process even in the absence of influenza virus. Coinfection with IAV significantly increased middle ear infection by both wild-type and NanA-deficient pneumococci at days 2 and 4 post-bacterial infection ($P = 0.0031$ and $P < 0.0001$ for wild-type and $P = 0.0012$ and $P < 0.0001$ for ΔNanA, respectively) (Figure 8B). Despite this, middle ear infection by the ΔNanA mutant was still significantly less than wild-type following coinfection with IAV ($P < 0.0001$). Taken together, these data indicate that NanA has an intrinsic role in pneumococcal middle ear infection which cannot be fully complemented by coinfection with neuraminidase-expressing IAV.

**NanA enzymatic activity is important for pneumococcal adherence to epithelial cells.** As IAV coinfection could not fully restore colonization and middle ear infection by NanA-deficient pneumococci in vivo, we postulated that NanA may be involved independent of its enzymatic activity. This would account for the inability of IAV coinfection to fully complement the absence of NanA expression. To begin investigating this, we assessed pneumococcal adherence in vitro. NanA expression is up-regulated by exposure to epithelial cells and
adherence to these cells is a critical and requisite step in pneumococcal colonization (191, 353, 354). We isolated the role of NanA sialidase activity in this process using N-acetyl-2,3-dehydro-2-deoxy neuraminic acid (DANA), a competitive transition-state analog of sialic acid that acts as a potent neuraminidase inhibitor. Utilizing the fluorogenic sialic acid analog 2’-(4-methylumbelliferyl)-α-D-N-acetyleneuraminic acid (MUAN) as an indicator of neuraminidase activity, we determined that maximal inhibition occurred at approximately 300 µg/ml DANA (Figure 9A), comparable with prior findings (187). Of note, DANA also inhibited the residual neuraminidase activity of the ΔNanA mutant, likely attributable to inhibition of NanB as well (3). Epithelial adherence to Detroit 562 cells, a human nasopharyngeal carcinoma cell line, was significantly impaired in the ΔNanA mutant. A similar magnitude of impairment could be induced in the wild-type strain treated with 300 µg/ml DANA (Figure 9B). These data indicate that NanA, specifically its enzymatic activity, is important in pneumococcal adherence. As both IAV and S. pneumoniae express neuraminidases with seemingly overlapping activity (282, 294), these findings suggest a potential mechanism whereby IAV coinfection contributes to enhance nasal colonization by NanA-deficient pneumococci.

**NanA is involved in pneumococcal biofilm formation independent of its enzymatic activity.** To continue investigating how NanA specifically may be impacting pneumococcal pathogenesis, we next assessed its effect on biofilm formation in vitro. Biofilms are surface-attached microbial communities encased in an extracellular matrix that possess well-described roles in both nasal
Figure 9. The sialidase activity of NanA is inhibited by DANA and contributes to epithelial adherence in vitro. (A) Inhibition of NanA sialidase activity in lysates of wild-type and ΔNanA S. pneumoniae following 15 minute incubation with N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) at varying concentrations. The assay was performed using the fluorogenic sialic acid analog 2'-((4-methylumbelliferyl)-α-D-N-acetylneuraminic acid. Each data point represents the mean +/- SEM. Each sample was measured in triplicate and the experiment was performed twice. (B) Adherence of wild-type and ΔNanA pneumococci incubated with DANA (300 μg/mL) or vehicle control after 60 minutes, expressed as the percent adherence relative to the inoculum. Each experiment contained at least three biological replicates and was repeated six times. Statistical analysis was determined by a one-way analysis of variance with a Bonferroni posttest (*, P <0.05; ***, P <0.0001).
colonization and middle ear infection (174, 178). NanA has previously been shown to be involved in pneumococcal biofilm formation in association with human epithelial cells (187). We hypothesized that NanA may also be involved in pneumococcal biofilms independent of its sialidase function on host epithelial cells, as has been observed in biofilms of *Pseudomonas aeruginosa* where the neuraminidase contributes to inter-bacterial aggregation (346).

Utilizing a previously established model of in vitro biofilm viability on an abiotic polystyrene surface (84, 337), we found that biofilm viability by NanA-deficient pneumococci was significantly reduced by 3.25-fold relative to wild-type (*P* < 0.0001) (Figure 10A). While epithelial cells and their sialylated glycoconjugates were not present in this model, the growth media itself (THY+) was found by thiobarbituric acid assay to contain numerous sialylated residues (Figure 10B). To determine whether this biofilm defect persisted in the absence of both epithelial cells and sialylated moieties, we performed a similar experiment in growth media (YSK) confirmed to be sialic acid-free (Figure 10B) (297). Interestingly, even in the absence of detectable sialic acid, NanA-deficient pneumococci similarly exhibited a significant 3.25-fold reduction in biofilm viability (*P* = 0.0003) (Figure 10C). Further, a biofilm defect could not be induced in wild-type pneumococci grown in sialic acid-replete media and treated with a neuraminidase inhibitor at concentrations sufficient to inhibit enzymatic activity (Figure 10D), mirroring a recent study of *S. pneumoniae* clinical isolates (300).
Figure 10. NanA contributes to in vitro biofilm viability independently of its sialidase activity. (A) Viability of S. pneumoniae wild-type and ΔNanA from 4-hour static biofilms grown in THY+ media. (B) Sialic acid content of THY+ media and YSK media as measured by a modified thiobarbituric acid assay, as described in Methods and Materials. Bars represent the mean +/- SEM. Each measurement was made in triplicate and the experiment was repeated three times. (C) Viability of wild-type and ΔNanA pneumococci from 4-hour static biofilms grown in sialic acid-free YSK media. (D) Static biofilm viability of wild-type and ΔNanA pneumococci in the presence of increasing concentrations of DANA. Biofilm viability was normalized to and expressed as a percentage relative to the untreated control. Statistical analysis was performed using a one-way analysis of variance with Dunnet posttest (*, \( P < 0.05 \)). Each experiment contained at least three biological replicates and was repeated at least twice. (E) Viability of S. pneumoniae opaque and transparent phase variants (confirmed to contain >90% of colonies in the same phase) from 4-hour static biofilms grown in sialic acid-replete THY+ media. For all biofilm assays, bars denote the mean +/- SEM of pooled experiments. Statistical analysis was performed using two-tailed Student’s \( t \) test (**, \( P < 0.01 \); ***, \( P < 0.001 \)).
To further confirm these findings, we also examined the biofilm viability of transparent and opaque phase variants of \textit{S. pneumoniae}; sub-populations typically associated with nasal colonization and disease, respectively (197).

Specifically, the transparent phase has previously been shown to exhibit increased biofilm viability (337). Independently, NanA expression and activity have also been shown to be significantly up-regulated in the transparent phase (195). To determine if the enzymatic activity of NanA contributed to the observed difference in biofilm viability between the two phases, biofilms of phase variants of EF3030 confirmed to contain >90\% of colonies in the same phase were assessed in sialic acid-free media (YSK). In this model, biofilm viability by the transparent variant remained significantly increased relative to the opaque variant even in the absence of sialic acid (\textbf{Figure 10E}). Taken together, these findings suggest that the role of NanA in pneumococcal biofilm formation by EF3030 is independent of its enzymatic sialidase activity. Further work is needed to determine the specific non-enzymatic contributions of NanA to this process and whether this finding contributes to the incomplete ability of IAV coinfection to fully restore nasal colonization and middle ear infection by NanA-deficient pneumococci to wild-type levels.
DISCUSSION

Coinfection of *S. pneumoniae* and IAV is linked both experimentally and epidemiologically with the development of OM (61, 81, 355). The activity of the viral neuraminidase altering the respiratory and eustachian tube epithelium has long been proposed as a mechanism by which preceding IAV infection potentiates pneumococci in the initiation of this disease (208, 356). The contributions of the bacterial neuraminidase to this process, however, are less well appreciated. In this study, we examined the role of and potential mechanisms whereby the primary pneumococcal neuraminidase, NanA, interacts with IAV during the exacerbation of nasal colonization and middle ear infection.

Extensive prior data illustrates that *S. pneumoniae* utilizes the sialidase activity of NanA to strip away sialic acid moieties, thereby enhancing adherence to host cells (293, 343). Reflecting this, we observed in this study that a *S. pneumoniae* strain deficient in the expression of NanA was impaired in nasal colonization and middle ear infection in vivo, as has been similarly observed in outbred mice and chinchillas (206, 290), respectively. Of note, however, these findings are not universal in all animal models (163, 195, 357) and may reflect differences in pneumococcal serotypes or model species. Indeed, sialic acid content can vary extensively between animal models (331). More generally, this discordance in the literature reflects increasing appreciation for the numerous roles of NanA in pneumococcal pathogenesis. As such, the significant reduction in middle ear infection by the ΔNanA mutant even during single infection is likely indicative of the roles of the multiple important roles of this virulence factor. Of
particular import in this model, NanA-deficient pneumococci are impaired in both modifying the receptor complement of the eustachian tube as well as in desialylating and thereby altering the function of host immune factors (195, 358). Illustrating this clinically, neuraminidase activity is frequently detected in the middle ear effusions of children with pneumococcal OM (359).

In addition to its canonical role in enhancing pneumococcal adherence during coinfection, the contributions of the IAV neuraminidase has recently been expanded to include the released sialic acid acting as a signalling molecule to induce pneumococcal outgrowth both in vitro and in vivo (262, 297). In the study presented here, we further contribute that the pneumococcal neuraminidase NanA itself is not a redundant bystander in this process, but rather is required for the full synergistic interaction between these two pathogens. Indeed, we show that while coinfection with IAV increases both nasal colonization and middle ear infection by NanA-deficient pneumococci, it is not to wild-type levels. Interestingly, this contrasts somewhat with the findings of King et al. who observed that NanA was not involved in the outgrowth of pneumococci in the lungs during IAV coinfection (357). This difference is likely tissue niche-specific, particularly as NanA has been shown to be expressed between 15- and 22-fold higher in the nasopharynx than in the lungs (353).

The relative inability of IAV coinfection to fully restore the nasal colonization and middle ear infection of the ΔNanA mutant may stem from one or both of two potentialities. First, this may result from an incomplete ability of IAV neuraminidase activity to fully complement the absent enzymatic activity of
NanA. Reflecting this possibility, the desialylation of airway epithelial cells appears to be maximal during coinfection (262, 358). Conversely, however, IAV preinfection of A549 epithelial cells was previously shown to be sufficient to restore wild-type adherence in a ΔNanA mutant (356). A similar result was achieved by pre-treatment of epithelial cells with exogenous neuraminidase (163). As an alternative hypothesis, NanA may be involved in pneumococcal pathogenesis in a manner that would not be supplemented by the activity of the IAV neuraminidase. Supporting this hypothesis, Uchiyama et al. reported a critical, non-enzymatic role for NanA in brain endothelial cell invasion. The researchers identified, in addition to the C-terminal catalytic domain, an N-terminal lectin-like adhesion domain that was required for the full virulence of the bacteria (2). Interestingly, another pneumococcal exoglycosidase, the β-galactosidase BgaA, has also been shown to participate in cellular adherence independent of its enzymatic activity (360).

Adherence to epithelial cells represents a critical first step in pneumococcal pathogenesis (354). The sialidase activity of NanA has been shown to extensively modify the carbohydrate moieties of chinchilla tracheal and eustachian tube epithelial cells and most, though not all, studies have reported reduced adherence by NanA-deficient pneumococci (163, 195, 208, 293, 345, 358). Utilizing a neuraminidase inhibitor, we demonstrated that the sialidase activity of NanA was required for wild-type adherence. As the IAV neuraminidase exhibits a similar enzymatic function (282), these findings correlate well with prior data in which an IAV neuraminidase inhibitor was
observed to reduce pneumococcal adherence to IAV-infected airway epithelial cells (294).

While we confirmed that the role of NanA in the in vitro epithelial adherence of *S. pneumoniae* EF3030 was dependent on its enzymatic activity, whether a similar role exists during biofilm formation, a downstream step in pneumococcal colonization and disease, remained unclear. Parker et al. have reported the inhibition of pneumococcal biofilm formation in vitro during coculture with epithelial cells following treatment with several neuraminidase inhibitors (187). As the sialic acid released from host cellular glycoconjugates can also independently stimulate biofilm growth (297), the specific role of NanA in this process is still difficult to identify. Neuraminidases of both *P. aeruginosa* and *Porphyromonas gingivalis* have been implicated in biofilm formation independently of epithelial cells (346, 361). In the present study, we similarly identified a role for the NanA neuraminidase in pneumococcal biofilms. Interestingly, however, this could not be inhibited by treatment with a neuraminidase inhibitor or by growth in sialic acid-free media, indicating a non-enzymatic role for NanA in this process. Lending support to this hypothesis, Brittan et al. recently noted that biofilm formation by *Lactococcus lactis* was enhanced by in situ complementation with pneumococcal NanA and that this effect that was not reversible by treatment with neuraminidase inhibitors (345).

Based on these findings, we propose a revised model of *S. pneumoniae*-influenza A virus coinfection wherein the pneumococcal neuraminidase NanA is potentially both complemented by the viral neuraminidase and also
independently involved in contributing to nasal colonization and middle ear infection. Thus, we hypothesize that the synergistic activity of the IAV NA contributes to bacterial adherence in vivo, as suggested previously (294). Ultimately, however, we hypothesize that NanA-deficient pneumococci are less able to colonize the nasopharynx and infect the middle ear in vivo even during coinfection with neuraminidase-expressing IAV due to the non-enzymatic contributions of NanA to biofilm formation. In sum, these findings establish an important role for the pneumococcal NanA during coinfection with IAV and further illustrate the complex interactions occurring between even analogous virulence factors of these two pathogens in the context of nasal colonization and middle ear infection.
CONCLUSIONS

Remarkably, the significance of the polymicrobial interactions between *Streptococcus pneumoniae* and influenza virus was identified as early as 1803 (5). This relationship was first noted due to the increased mortality from bacterial pneumonia during influenza epidemics. It was observations such as these that led the French physician Luis Cruveilhier to remark in 1919, “If la grippe [the flu] condemns, the superinfection executes” (362). Much has changed, though, in the past century. Indeed, following the widespread implementation of both seasonal influenza vaccines and the pneumococcal conjugate vaccines, the incidence of invasive pneumococcal disease has declined significantly, particularly in children (152, 252, 253). We would argue, however, that the synergistic interaction between these two pathogens has not receded into history. Rather, that the context of this interaction has shifted somewhat in the developed world, from invasive to localized disease processes such as otitis media. Indeed, as many as 60% of IAV infections in young children are still associated with a resultant OM diagnosis (61). As such, the goal of this research was to investigate the interaction of IAV and a weakly virulent, colonizing pneumococcal strain in the context of nasal colonization and middle ear infection. Specifically, the roles of phase variation in *S. pneumoniae* and the pneumococcal neuraminidase NanA during IAV coinfection were examined.
Coinfection Model

*S. pneumoniae* strain EF3030 readily colonizes the murine nasopharynx with only minimal to moderate ascension to the middle ear (*Figure 3*). Despite this, preceding IAV infection significantly increases the magnitude of pneumococcal nasal colonization and this correlates directly with a significant increase in the magnitude of middle ear infection (*Figure 3*). Additionally, we observed that transparent phase pneumococci were consistently isolated more frequently from the nasopharynx while the opaque phase was isolated more frequently from the middle ear (*Table V*). In short, the transparent phase has been shown to express less capsule and to be more susceptible to host clearance while the opaque phase expresses more capsule and is more resistant (61, 197, 199, 203). This could thus begin to explain how this colonizing strain of *S. pneumoniae* is able to more readily persist in the middle ear during IAV coinfection, as the pneumococcal population at this site gradually shifts more to the opaque phase.

There are several factors that are hypothesized to contribute to the increase in nasal colonization density when *S. pneumoniae* is inoculated during an ongoing IAV infection. Most evident initially are the inflammatory changes observed throughout the nasopharyngeal epithelium in coinfected mice (*Figure 4*), likely contributing to bacterial adherence. In addition, these epithelial alterations were also associated with a predominantly neutrophilic infiltrate. In
addition to their traditional role as phagocytes, neutrophils have been identified in the past decade to also mediate pathogen clearance via the formation of NETs. These are made via the extrusion of neutrophil extracellular DNA that is studded with antimicrobial components including neutrophil elastase and histones to trap and kill bacteria (363). Pneumococci, however, are resistant to NET-mediated killing in part as they produce both an elastase inhibitor and an endonuclease to degrade extracellular DNA (364, 365). Further, *S. pneumoniae* was recently shown to not only persist within middle ear NETs during coinfection with IAV, but these NETs were actually required for maximal infection (143). Supporting this finding, we similarly observed a large neutrophilic infiltrate in the middle ears of coinfected mice ([Figure 5](#)). Interestingly, we also observed structures resembling NETs in the nasopharynges of coinfectected mice by hematoxylin & eosin (H&E) staining ([Supplemental Figure 1](#)). While this needs to be confirmed by fluorescent microscopy, as is the gold-standard, this observation offers an additional potential mechanism by which nasal colonization is increased following IAV infection. It remains unclear why these NET-like structures were only observed in the nasopharynx when *S. pneumoniae* is coinfectected with IAV. Intriguingly though, in the middle ear, IAV was recently shown to independently induce NET formation via a nonspecific antibody-mediated process (143).
Implications of Coinfection Model

As the burden of disease caused by *S. pneumoniae* has shifted in children over the past two decades from invasive to more common, localized infections such as OM (366), there is a need for relevant animal models. The studies presented herein provide a novel, translational, and accessible model to investigate the interactions between influenza virus and colonizing strains of *S. pneumoniae* in the context of middle ear infection. Of particular import beyond the pathogens studied, this model was designed to mimic as closely as possible the natural disease course observed in children. Thus, it utilizes young adult rather than immunocompromized infant mice and also a simple intranasal route of infection rather than invasive or artificial mechanisms to induce bacterial ascension to the middle ear (312, 313, 315).

The results from this coinfection model exhibit several interesting parallels to components of the disease process of otitis media in children. While simply being colonized with potential otopathogens is a risk factor for OM (118), the density of nasal colonization is further correlated with the development of OM for *S. pneumoniae, H. influenzae*, and *M. catarrhalis* (325). We similarly observed a correlation with *S. pneumoniae*, however, only during coinfection with IAV (Figure 4). This indicated that IAV contributes to middle ear infection by both increasing nasal pneumococcal density and by predisposing to enhanced bacterial ascension. As an additional risk factor, a recent clinical study observed that nasal colonization of non-pneumococcal α-hemolytic streptococci was decreased in children with viral URTI and acute OM (367). These bacteria are
also detected at lower levels in otitis-prone children but can inhibit the growth of *S. pneumoniae in vitro* (368, 369). Thus, an intriguing possibility is that influenza viral coinfection may both increase pneumococcal outgrowth in the nasopharynx while simultaneously decreasing the numbers of inhibitory microorganisms. The animal model presented in this study is well-positioned to assess this potentiality *in vivo*. Of note, the use of nasal sprays containing non-pneumococcal α-hemolytic streptococci as OM prophylaxis in clinical studies have provided promising, albeit mixed, results (370, 371).

An additional clinical link observed in this coinfection model was the increased incidence of bilateral middle ear infection in coinfected mice *(Supplemental Figure 2)*. As clinicians continue to grapple with the difficulty of antibiotic stewardship, the presence of bilateral OM has been suggested as an indicator for initiating antimicrobial therapy. Specifically, current guidelines call for antibiotics in children between 6 and 24 months of age with bilateral OM even with mild symptoms (115). There are currently no similar recommendations for antiviral therapy. Interestingly though, vaccination of children with recurrent OM with a virosomal influenza vaccine significantly decreased the number of days with bilateral middle ear effusion in this cohort (372). As our data suggest that bilateral middle ear infection is associated with viral coinfection, influenza virus vaccination in children with recurrent bilateral OM may be especially beneficial, in addition to traditional antibacterial therapy.

Otitis media is, by its definition, a disease of inflammation. Reflecting this, histologic hallmarks of this disease process in its acute form include infiltration of
inflammatory cells into the middle ear lumen, mucosal edema, vasodilation, and fibrin deposition (328). Middle ear sections from coinfected mice in this study contained these histologic findings (Figure 5), confirming that a disease process resembling acute OM was in fact occurring in these mice. Interestingly, though quantifiable pneumococci were detected in the middle ears of a majority of singly-infected mice (Figure 4), evidence of inflammation was not (Figure 6). As non-proteinaceous fluid is generally not visible on an H&E stain, the presence of a middle ear effusion could not be ruled out in these mice; however, it is clear that acute OM was not present histologically. These observations alluded to a critical question currently being debated clinically: namely, whether bacteria in the middle ear represent an infection and, relatedly, whether the middle ear is in fact a sterile site. The sterility of this site in healthy patients has been held as dogma for decades and was further supported by a recent study that detected no bacteria by culture or PCR in healthy patients undergoing cochlear implantation (373). Remarkably, however, another recent study observed, by electron microscopy, bacteria and even bacterial biofilms in over 65% of healthy patients undergoing a similar procedure (374). S. pneumoniae is typically found in the middle ear in a biofilm state and biofilm-derived bacteria are less inflammatory (174, 183). Further, as middle ear inflammatory cytokines in children are correlated with bacterial load (375), it is plausible that a low number of pneumococci in the biofilm state may be present in the middle ear without inducing an acute inflammatory disease process, as was observed in singly-infected mice in this study. Finally, these findings also warn of the pitfalls of
assuming in animal models that the simple presence of bacteria in a presumably sterile site is indicative of a disease process occurring.

**Pneumococcal Phases During Coinfection**

Utilizing phase variants of *S. pneumoniae*, which are essentially enriched sub-populations containing nearly all bacteria in the opaque or transparent phases, we confirmed in single infection that the opaque phase exhibited a colonization defect *in vivo* (Figure 7 and Supplemental Figure 3). We hypothesized that this likely stemmed from a decrease in both epithelial adherence and biofilm viability by the opaque phase relative to the transparent, as we observed *in vitro* (Figure 6). Further, this difference in biofilm viability persisted and was in fact even more apparent when the assay was performed at 34°C rather than 37°C (Supplemental Figure 4), approximating the ambient temperature of the posterior nasopharynx (376).

Prior IAV infection, however, ultimately overcame these inherent differences in adherence and biofilm formation and enabled both phases to colonize the nasopharynx and infect the middle ear to a similar extent (Figure 7). We hypothesized that phase shifting *in vivo* may contribute to these findings, enabling the classically less invasive transparent phase to persist in the immunologically privileged site of the middle ear. Supporting this hypothesis, in mice inoculated with the transparent variant, there was a significant increase in the proportion of opaque colonies in the middle ear relative to the nasopharynx.
(Table V). However, the proportion of opaque phase colonies in the middle ears of these mice was still only ~25-30\%, indicating that other mechanisms must be involved. Uncovering these additional mechanisms is an active area of research. As discussed in the conclusions of Chapter I, we propose that this may be mediated by many of the same features that enable the transparent phase to readily colonize the nasopharynx. One such feature specifically is biofilm formation, as these are routinely detected in the middle ear during pneumococcal OM (174, 275). This hypothesis is congruent with our correlation data indicating that the transparent phase during IAV coinfection could infect and persist in the middle ear irrespective of the density of nasal colonization (Figure 7). This suggested that a smaller number of transparent phase bacteria disseminating from the nasopharynx to the middle ear could ultimately induce a similar magnitude of middle ear infection as the opaque phase. Further work is needed to determine whether it is the initiation of middle ear infection or resistance to clearance or both that is most altered by IAV coinfection and enables a similar magnitude of middle ear infection by both phases.

**Implications of the Pneumococcal Phases During Coinfection**

Rather than a simple bacteriologic phenomenon, phase variation within pneumococci is also relevant clinically and has been observed in essentially all studied lab strains as well as in clinical isolates from both children and adults (195, 198, 201). Thus, the results presented herein have important implications
regarding the pathogenesis, prevention, and treatment of pneumococcal diseases such as OM.

Current pneumococcal vaccines (PCVs) in children engender protection against type-specific capsular polysaccharide. However, with >90 pneumococcal serotypes, incomplete vaccine protection against non-vaccine serotypes illustrates the significant need for vaccines directed against pneumococcal proteins (377, 378). While these are largely conserved between serotypes, the expression of many, by their nature as surface-exposed proteins, have been shown to phase vary (205). Specifically, a popular vaccine target has been the choline-binding protein PspA (379, 380). As an added benefit, PspA is the rare pneumococcal choline-binding protein whose expression is significantly increased in the more virulent opaque phase and down-regulated in the transparent phase (200). However, our finding that IAV coinfection enables the transparent phase, with its reduced PspA expression, to infect the middle ear similar to the opaque suggests there may be an added benefit to targeting a non-phase variable protein such as pneumolysin.

Antibiotic failure rates in OM can be quite high, though they are decreasing in the PCV era (381). As such, interest in prophylactic alternatives is high. Antibiotic prophylaxis has been attempted previously using low-dose therapy over a long period of time (382). The moderate effectiveness of this approach ultimately did not outweigh the negative sequelae of allergic reactions, gastrointestinal disturbance, and further contributing to antibiotic resistance and, as such, this approach is no longer recommended (115). The observed
effectiveness of this regimen was attributed to disrupting the positive relationship between nasal colonization density of otopathogens such as *S. pneumoniae* and the risk of OM (325). We observed a similar correlation in our model but only when the opaque phase variant was coinfe cted with IAV (*Figure 7*). If these results recapitulate what is occurring in children, it suggests that targeted prophylactic antibiotics to debulk nasal pneumococcal density during influenza season may be beneficial. Prior findings may actually enable this strategy to be further refined. Indeed, the predominant phase of pneumococcal isolates has been shown, in some cases, to be independently associated with serotype (201). Specifically, serotypes 1, 4, and 23F were shown to be associated with the opaque phase regardless of the site of isolation (201). Thus, a particularly intriguing prophylactic approach could involve specifically targeting children with recurrent OM harboring one of these serotypes in their nasopharynx above a set culture or PCR detection threshold during the winter months. While more work is needed to determine if similar correlations are present when pneumococcal colonization precedes IAV infection, the potential to reduce even a fraction of OM cases could have a significant impact.

The traditional paradigm regarding the pneumococcal phases *in vivo* has typically held that *S. pneumoniae* entering the nasopharynx at acquisition are comprised of a mixture of both phases which then shift towards the transparent phase for colonization or to the opaque if the host microenvironment has been altered in some way (197, 199, 202, 203, 205, 209). Our findings, and those of others, however, posit that these two phases are more nuanced than would first
appear. Indeed, our findings suggest that coinfection with a respiratory virus as common as IAV renders the predominant phase of pneumococci at acquisition to be essentially irrelevant in the resultant development of middle ear infection. Providing further complexity to this paradigm, some serotypes of *S. pneumoniae* isolated from invasive sites have been shown to be more associated with the transparent phase (201). When taken together with the results of Briles et al. who identified contributions of both phases to nasal colonization (91), it becomes apparent that the role of these pneumococcal phases is not nearly as dichotomous as previously thought.

Finally beginning to unravel these complex interactions, the molecular mechanism underlying phase variation in *S. pneumoniae* was just recently discovered, over two decades after its first characterization (197). The mechanism itself is mediated by a previously uncharacterized Type I restriction modification system that preferentially methylates the lagging strand of pneumococcal DNA throughout the genome, causing epigenetic effects (383). Random genetic rearrangements of the *hsd*S gene, the specificity sub-unit of the methylation apparatus, alter its recognition sequence and thus induce different methylation patterns throughout the *S. pneumoniae* genome. The rearrangements were ultimately found to generate six specific gene expression profiles, correlating with six distinct epigenetic phases in strain D39: SpnIIIA through SpnIIIF (383). The possibility exists that the methylation patterns of other strains may vary between or even within serotypes. These six epigenetic phases contributed to the previously described phenotypic opacity phases.
(opaque and transparent), although incompletely (383). Indeed, most of the newly identified epigenetic phases contained a mixture of opaque and transparent phase colonies. A similar Type III restriction modification system mediating phase variation has been described previously in Gram-negative pathogens including *Neisseria* spp. and *Haemophilus* spp. (384).

While more work is needed regarding this exciting new discovery, it is interesting to note that two of the newly discovered epigenetic phases, SpnIIIA and SpnIIIB, are strikingly similar to the opaque and transparent colony morphology phases, respectively. Specifically, the SpnIIIA phase possesses increased capsule, increased persistence in the bloodstream, and reduced nasal colonization in a mouse model while the SpnIIIB phase was the inverse (383). This correlated with the opaque and transparent phases being predominant in SpnIIIA and SpnIIIB, respectively, though this was not absolute (383). While related, the opacity phases appear to be somewhat distinct from the newly-discovered genetic phases. Indeed, the SpnIIIE phase was comprised of an identical proportion of opaque phase colonies as SpnIIIA but the two exhibited vastly different phenotypes *in vivo* (383). These findings, while novel, also confirmed a hypothesis we had posited that even within the opaque and transparent phases, pneumococci could further adapt to persist in different host environments. Illustrating this, the opaque phase up-regulated capsule even further when it is exposed to an anaerobic environment (199). Taken together, these new observations provide additional means to explain how both the
opaque and transparent phases could ultimately infect the middle ear similarly during IAV coinfection.

**Pneumococcal Neuraminidase A During IAV Coinfection**

In these studies, we demonstrate, using a NanA-deficient mutant, that the primary pneumococcal neuraminidase NanA is involved both *in vitro* in epithelial adherence (Figure 9) and biofilm viability (Figure 10) and also *in vivo* during single infection in nasal colonization and middle ear infection (Figure 8). Contrary to our original hypothesis as well as unpublished work by Jonathan McCullers using a highly virulent pneumococcal strain (356), coinfection with IAV increased but did not fully restore nasal colonization and middle ear infection by the ΔNanA mutant to wild-type levels (Figure 8). This indicated that the similar enzymatic activity of the IAV NA was not sufficient to fully replace the absent expression of NanA in *S. pneumoniae*.

Based on these findings, we hypothesized that NanA was potentially involved intrinsically in pneumococcal pathogenesis independent of its enzymatic activity and thus not fully complemented by viral coinfection. This hypothesis was based on the knowledge that the neuraminidases of other bacteria such as *Vibrio cholerae* have been shown to contain a second, non-catalytic, lectin-like carbohydrate-binding domain (285). A putative lectin-like domain in the NanA of *S. pneumoniae* was recently identified by Uchiyama et al. and implicated in brain endothelial cell invasion, independent of the enzymatic activity of NanA. Indeed,
the carbohydrate-binding domain was absolutely required for cellular invasion and this could not be inhibited by treatment with neuraminidase active site inhibitors (2). We identified that a similar domain is also present in strain EF3030 (data not shown), as determined by nucleotide sequence homology to strain D39 (2). In testing our hypothesis in vitro, we observed that bacterial adherence to epithelial cells did require the sialidase activity of NanA while, interestingly, biofilm viability did not (Figures 9 and 10). Based on these findings, we proposed a model wherein pneumococcal NanA participates in biofilm formation independently of its enzymatic activity, potentially via its N-terminal lectin-like domain. Thus, the IAV NA during coinfection could supplement the enzymatic role of NanA in regards to adherence, as has been observed previously in vitro (5). Impairments in biofilm formation, however, would ultimately prevent NanA-deficient pneumococci from colonizing the nasopharynx and infecting the middle ear at wild-type levels, even during IAV coinfection.

**Implications of Pneumococcal NanA During IAV Coinfection**

Elucidating the mechanistic underpinnings of the synergistic interactions between *S. pneumoniae* and influenza A virus are critical in the identification of potential therapeutics. Indeed, the influenza virus neuraminidase inhibitors, which are now so widespread, represent one of the great victories of rational drug design. More significantly, these therapeutics have effectively contributed to reduce severe influenza virus infections (385). A growing number of studies
have also indicated that these medications may also decrease the incidence of secondary complications such as bacterial coinfections and OM (298, 386). In the case of pneumococcal coinfections, this effect appears to be mediated by a reduced ability of the viral neuraminidase to alter the host microenvironment to the benefit of the bacteria (262, 294). In addition, as some viral neuraminidase inhibitors exhibit moderate \textit{in vitro} activity against NanA as well, therapeutics such as these have also been proposed to directly inhibit pneumococcal growth and infection (187). Our findings suggest that current neuraminidase inhibitors that target the catalytic domain, even experimental therapeutics targeted against the pneumococcal neuraminidase such as XX1 (187), would only partially inhibit the functions of NanA in pneumococcal pathogenesis, potentially altering therapeutic efficacy.

Relatedly, the studies presented herein further identify NanA specifically as a virulence factor that directly contributes to and is required for full synergism between pneumococci and IAV; highlighting an attractive therapeutic target to disrupt this interaction. Our findings, along with those of other recent studies (2, 387), further suggest that the design of future drugs targeting this protein should potentially be expanded beyond inhibitors of the catalytic domain. Supporting this hypothesis, sequence analysis of the non-catalytic domain demonstrates that these sites have long been targets of the host immune response in clearing pneumococcal infection (342); though the resultant variability of these domains could be a confounding factor. An intriguing approach would be to test the ability of a monoclonal antibody against the N-terminal lectin-like domain of NanA to
inhibit biofilm formation. Of potential note, a novel neuraminidase inhibitor was recently identified to inhibit pneumococcal biofilm formation in vitro via its interaction with NanA at a site that, while currently unknown, is distinct from that of the active site (300). Small molecule screening of potential therapeutics targeting this lectin-like domain would also be beneficial.

While largely studied as a virulence factor (2, 290, 294, 387), the NanA genetic locus is also involved in pneumococcal carbohydrate metabolism and it and its associated operon are specifically up-regulated in the presence of sialic acid and its derivative N-acetylmannosamine (388). Conversely, this locus is down-regulated in the presence of simple sugars such as glucose (388). Marks et al. recently demonstrated that extracellular glucose is increased following IAV infection and that it serves as a signal for pneumococcal dissemination from the nasopharynx via a yet to be discovered mechanism (189). An interesting possibility from these data then is that NanA expression may be down-regulated during IAV coinfection, while pneumococci, in essence, co-opt the enzymatic activity of the IAV neuraminidase. This potentiality could have ramifications regarding the use of NanA as a protein-based pneumococcal vaccine target, which has shown promise in animal models (389, 390). Our findings however, suggest that NanA continues to be expressed even in the glucose-rich host environment previously associated with acute viral infection (189) as its expression is required for the full virulence of S. pneumoniae even during IAV coinfection.
Future Directions

The findings presented herein provide novel insights into the interactions occurring between *S. pneumoniae* and influenza A virus during nasal colonization and middle ear infection. Specifically that when IAV precedes pneumococcal acquisition, both pneumococcal phases are equally capable of causing middle ear infection and that the bacterial neuraminidase NanA contributes significantly to this coinfection process, potentially via a currently unappreciated mechanism. Much more work is needed, however, to further understand the intricacies of this complex yet widespread coinfection process. As such, we propose that there are three important questions to address in future work: (1) What is the effect of the order of pathogen introduction in this coinfection process? (2) What specific features of both phases of *S. pneumoniae* enable it to colonize the nasopharynx and infect the middle ear during IAV coinfection? (3) What is the non-enzymatic contribution of NanA to pneumococcal pathogenesis?

Episodes of pneumococcal colonization cycle frequently in children, with a median duration of carriage of 30-90 days for infants (330). As such, there is significant clinical relevance in investigating the effects of coinfection when pneumococci are acquired in the context of a pre-existing viral infection, as was modeled in this study. However, an equally relevant clinical scenario is when a child asymptptomatically colonized with a carriage strain of *S. pneumoniae* is then coinfected with a respiratory virus such as IAV. As such, reversing the order of pathogen introduction in our coinfection model may yield interesting and even
contradictory results. In the field as a whole, few studies have investigated this order of pathogen acquisition specifically; of those that have, the results have been disparate. Indeed, one study identified synergistic exacerbation of pneumococcal infection in infant mice (315) while another study in adult mice observed protection (391). In general, however, experimental evidence appears to suggest that pneumococcal colonization prior to acquisition of IAV provides some protection from lethal pulmonary infection (100, 392), though, interestingly, at the expense of long-term antibody-mediated antiviral immunity (393). The effect of this order of pathogen introduction in a non-lethal disease process such as OM, however, is largely unexplored and in need of further study.

A second direction of investigation, which is already ongoing, is investigating the epigenetic phases present in *S. pneumoniae* strain EF3030 which could be the same or different from the six identified previously in the more virulent D39 strain (383). Using an RT-PCR approach, we anticipate being able to estimate the relative proportion of each epigenetic phase in pneumococci isolated from both the nasopharynx and middle ear in our *in vivo* coinfection model. This data can be used to assess whether epigenetic phase shifts occur within the opacity phases (i.e., two transparent phase colonies in distinct epigenetic phases). Next, we will continue our work to, for the first time, generate phase-locked mutants of each of the six newly-discovered genetic phases in strain EF3030. These locked phase variants represent an exceptionally powerful tool to study how pneumococci adapt to the host environments of the nasopharynx and, following IAV coinfection, the middle ear.
The first step in this investigation will involve characterizing the phenotypes of these phases by qRT-PCR and Western blot to assess for relative expression of virulence factors such as capsule, NanA, PspA, CbpA, LytA, and pneumolysin. These investigations will also need to be extended to the opaque and transparent phases (if present) of each of the epigenetic phases. Following this, functional assays involving in vitro assessments of epithelial adherence, biofilm formation, resistance to opsonophagocytosis, and neuraminidase activity will further delineate the relative roles of these epigenetic phases. Employing these variants in our in vivo coinfection model would then enable an identification of which phases contribute to the synergistic coinfection with IAV. Ultimately, the results obtained from the above described in vitro and in vivo experiments will be used to delete key virulence factors such as NanA in these phase-locked pneumococcal strains. This will contribute to reveal which specific components of each phase are indeed responsible for their distinct phenotypes and which are required for the synergistic interaction with IAV, thereby identifying potential therapeutic targets.

Studies such as these will begin to reveal precisely how antecedent IAV infection enabled similar nasal colonization and middle ear infection by the colonial morphology opaque and transparent phase variants in this study. Finally, an additional interesting avenue of study will be determining how exactly the opacity phases are related with the newly-discovered epigenetic phases.

The next step in investigating the full role of NanA in this coinfection process necessitates isolating the specific non-enzymatic contribution that we
observed affecting biofilm viability in vitro. As such, we are actively using inverse PCR for site-directed mutagenesis to generate mutants of EF3030 that express modified NanA proteins that lack either the C-terminal catalytic domain or the putative N-terminal lectin-like carbohydrate-binding domain. Utilizing these mutants in this coinfection model will determine whether NanA possesses a previously unappreciated role in pneumococcal colonization and middle ear infection that works in conjunction with its previously described synergistic sialidase activity with the IAV neuraminidase. Further, we will also determine the specific contribution of IAV neuraminidase activity to pneumococcal infection in this coinfection model. This will be investigated via the treatment of mice intra-nasally with either exogenous neuraminidase or an inflammatory TLR agonist instead of IAV prior to inoculation of either wild-type or NanA-deficient S. pneumoniae.

In sum, these future directions will contribute to further advance our understanding of this complex yet critically important bacterial-viral coinfection.
APPENDIX
Supplemental Figure 1. Representative H&E-stained images of the nasopharynx of coinfected mice displaying NET-like structures. The nasopharynges of coinfected mice were aseptically excised, fixed overnight in 4% paraformaldehyde, decalcified, embedded in OCT, frozen at -80°C, cryo-sectioned into 5 μm slices, stained with H&E, and mounted with Permount. Slides were viewed and photographed at x40 magnification. Cellular infiltrate in the nasal lumen are comprised of cells morphologically resembling neutrophils.
Supplemental Figure 2. Percent mice with bilateral middle ear infection are increased during coinfection. Mice were infected as described in the text. Data represent both days 2 and 4 post-bacterial infection and represent the mean +/- SEM. Statistical significance determined by Fisher’s exact test, * indicates $P < 0.05$. 
Percent Mice with Bilateral Middle Ear Infection

% Mice

S. pneumoniae
S. pneumoniae + IAV

*
Supplemental Figure 3. Nasal colonization and clearance of opaque and transparent phase variants of *S. pneumoniae* EF3030. Mice were inoculated intranasally with $5 \times 10^6$ CFU of either the opaque or transparent phase variants. These variants were isolated by serial passage and were confirmed prior to each experiment to contain >90% of colonies in the same phase. At various times post-infection, the mice were euthanized and the nasal colonization density was enumerated. Data represent the geometric mean +/- 95% confidence intervals. The dashed line represents the limit of detection of the assay.
Supplemental Figure 4. *In vitro* biofilm viability is increased in the transparent phase variant at 34°C. 1x10^7 CFU of the opaque or transparent phase variant in 1 mL Todd-Hewitt both with 0.5% yeast extract and supplemented with 10% heat-inactivated horse serum and 2,500 U/mL catalase was inoculated into a 24-well plate. The phase composition of each variant was confirmed prior to each experiment. At 4- and 24-hours, the media was removed and adherent bacteria were lifted from the surface of the well by scraping and vigorous pipetting and plated. Each bar represents the mean +/- SEM. Significance was determined by Student’s *t* test. *** indicates *P* < 0.001.
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**ORAL PRESENTATIONS:**

January, 2015:  “Influenza A virus alters pneumococcal nasal colonization and middle ear infection independently of phase variation.” Mid-Atlantic Microbial Pathogenesis Meeting; Wintergreen, VA.

October, 2014:  “Interactions between influenza and pneumococcus in nasal colonization and middle ear infection.” Departmental Seminar; Winston-Salem, NC.

October, 2014:  “Is it otitis or is it otitis?” Departmental Fall Symposium; Winston-Salem, NC.

May, 2014:  “Influenza A virus alters pneumococcal colonization and middle ear infection by *S. pneumoniae*.” University of Alabama at Birmingham Microbial Pathogenesis Meeting; Birmingham, AL.

February, 2014:  “Influenza A virus alters colonization and middle ear infection by *Streptococcus pneumoniae*.” Departmental Seminar; Winston-Salem, NC.
October, 2013: “The interaction of influenza A virus and pneumococcal phase variation in acute otitis media.” Departmental Fall Symposium, Winston-Salem, NC.

POSTER PRESENTATIONS:

May, 2014: “The interaction of influenza A virus and pneumococcal phase variation in otitis media.” 114th American Society for Microbiology General Meeting; Boston, MA


February, 2013: “Influenza A virus modulates pneumococcal otitis media.” Mid-Atlantic Microbial Pathogenesis Meeting; Wintergreen, VA.


PROFESSIONAL MEMBERSHIPS:

American Society for Microbiology
American Medical Association

PUBLICATIONS


