HAEMOPHILUS INFLUENZAE AND MORAXELLA CATARRHALIS INTRA- AND INTERSPECIES QUORUM SIGNALING

BY

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<td><em>A. actinomycetemcomitans</em></td>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
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<td>luxS</td>
<td>gene encoding the AI-2 synthase</td>
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<td>AI-2</td>
<td>autoinducer-2</td>
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<td>DPD</td>
<td>(S)-4,5-dihydroxy-2,3-pentanedione, AI-2 precursor</td>
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<td>Lsr</td>
<td>LuxS regulated</td>
<td></td>
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<tr>
<td>AHL/HSL</td>
<td>acyl homoserine lactones, autoinducer-1</td>
<td></td>
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<tr>
<td>AIP</td>
<td>autoinducer peptide</td>
<td></td>
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<tr>
<td>PCho</td>
<td>phosphorylcholine</td>
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<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances, exopolysaccharide</td>
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<tr>
<td>OM</td>
<td>otitis media</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
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<tr>
<td>OapA</td>
<td><em>H. influenzae</em> opacity-associated protein A</td>
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<td>HMW1/HMW2</td>
<td><em>H. influenzae</em> high molecular weight adhesins</td>
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<td>Hia</td>
<td><em>H. influenzae</em> adhesin</td>
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<td><em>H. influenzae</em> adhesin</td>
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<td>P2</td>
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<td><em>H. influenzae</em> outer membrane protein 5</td>
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P6  H. influenzae outer membrane protein 6
OMP 26  H. influenzae outer membrane protein 26
UspA1/UspA2/UspA2H  M. catarrhalis ubiquitous surface proteins
Hag/MID  M. catarrhalis hemagglutinin and adhesin
McaP  M. catarrhalis adherence protein
OMP CD  M. catarrhalis outer membrane protein CD
OMP E  M. catarrhalis outer membrane protein E
FHA  filamentous hemagglutinin adhesin
MhaC/MhaB1/MhaB2  M. catarrhalis FHA-like proteins
Kdo  keto-3-deoxy-d-manno-octulosonic acid
CopB  M. catarrhalis outer membrane protein B2, iron acquisition protein
C3  complement component 3
CEACAM1  carcinoembryonic antigen family of cell adhesin molecules
PAF  platelet-activating factor
TNF-α  tumor necrosis factor alpha
TLR  Toll-like receptor
LEE  locus of enterocyte effacement
SAM  S-adenosylmethionine
SAH  S-adenosylhomocysteine
SRH  S-ribosylhomocysteine
h  hour
min  minute(s)
<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micron</td>
</tr>
<tr>
<td>CFU, cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>sBHI</td>
<td>supplemented brain heart infusion</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>MIC</td>
<td>minimal inhibitory concentration</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>TS</td>
<td>trimethoprim-sulfamethoxazole</td>
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ABSTRACT

Otitis media (OM) is among the most common childhood diseases worldwide. OM infections often persist for long periods of time and can be highly resistant to antibiotic treatment due in part to persistence of bacteria within biofilms. The majority of OM infections also involve coinfection by multiple species. For instance, the leading causative agents *Haemophilus influenzae* and *Moraxella catarrhalis* are more frequently found to coexist than to be isolated individually. Polymicrobial infection and biofilm formation may have a dramatic impact on the severity and treatability of OM. Thus, there is a pressing need to investigate polymicrobial infection and to identify factors that contribute to the establishment of these infections. One factor that may contribute to the establishment of polymicrobial infection is bacterial communication via quorum signaling. Autoinducer-2 (AI-2) is termed an interspecies signal as the genetic determinant for AI-2 production (*luxS*) is highly conserved and many species possess the machinery to respond to AI-2. *H. influenzae* has a *luxS* homolog whereas *M. catarrhalis* does not but may still sense and respond to AI-2. Therefore, the focus of this project was to investigate the impact of AI-2 intra- and interspecies bacterial communication and *H. influenzae* and *M. catarrhalis* polymicrobial infection on biofilm formation, antibiotic resistance, and persistence in experimental OM.

For *H. influenzae*, a *luxS* mutant formed biofilms with decreased biomass and biofilm-associated lipoooligosaccharide (LOS) glycoforms, and had a persistence defect during late stages of infection in the chinchilla model of OM. Internalization of and response to AI-2 was found to require NTHI_0632. Studies utilizing an NTHI_0632
mutant revealed accumulation of AI-2 in culture supernatants and altered LOS composition, biofilm formation, and persistence similar to that observed for a \textit{luxS} mutant. Modulation of \textit{luxS} expression was also found to be critical for nontypeable \textit{H. influenzae} biofilm development and dispersal as interruption of \textit{luxS} expression resulted in rapid dispersal of established biofilms while continuous expression prevented dispersal. Taken together, these data indicate that AI-2 represents a genuine signaling molecule in \textit{H. influenzae} and support a model in which AI-2 signaling promotes biofilm maturation by modulating LOS composition, followed by decreased signaling to promote dispersal. Interfering with AI-2 signaling may therefore limit \textit{H. influenzae} biofilm formation during infection and potentially force dispersal of already-established biofilms.

We next investigated \textit{H. influenzae} and \textit{M. catarrhalis} polymicrobial biofilm formation and interspecies bacterial communication. \textit{H. influenzae} and \textit{M. catarrhalis} formed polymicrobial biofilms in vitro with enhanced resistance to antibiotics, and production of AI-2 by \textit{H. influenzae} contributed to the enhanced resistance. Incubation with synthetic AI-2 was sufficient to promote increased biofilm formation by \textit{M. catarrhalis} and antibiotic resistance. \textit{H. influenzae} and \textit{M. catarrhalis} polymicrobial infection also promoted \textit{M. catarrhalis} persistence in a \textit{luxS}-dependent manner. We thus conclude that \textit{H. influenzae} promotes \textit{M. catarrhalis} persistence and antibiotic resistance within polymicrobial biofilms via AI-2 interspecies signaling. Disruption of AI-2 signaling during \textit{H. influenzae} and \textit{M. catarrhalis} polymicrobial infection may therefore disrupt polymicrobial biofilm formation and persistent infection. Moreover, successful vaccination against \textit{H. influenzae} may also limit \textit{M. catarrhalis} infectivity by removing a reservoir of the AI-2 signal that promotes \textit{M. catarrhalis} persistence.
INTRODUCTION

Nasopharyngeal Colonization

Immediately after birth, the skin and mucosal surfaces of a normal, healthy neonate are rapidly colonized by commensal bacteria (1). Colonization of the upper airway establishes a major reservoir of bacterial species, particularly in the nasopharynx (1, 2). The normal flora of the nasopharynx is established during the first year of life and includes relatively non-pathogenic bacteria such as commensal Neisseria species and diphtheroids as well as potential pathogens such as Haemophilus influenzae, Moraxella catarrhalis, and Streptococcus pneumoniae (Table 1) (3-11). Colonization by one or more pathogenic species can occur as early as one month of age, and approximately sixty-eight percent of children are colonized by M. catarrhalis, S. pneumoniae, and/or H. influenzae within the first six months of life (4).

The outcome of colonization by H. influenzae follows three general patterns: the initial colonizing strain may be rapidly eliminated from the nasopharynx, the initial colonizing strain may be carried for a prolonged period of time (generally up to five months), or the nasopharynx may be colonized sequentially by multiple different strains of H. influenzae (12). Colonization by M. catarrhalis tends to occur slightly earlier than colonization by H. influenzae but generally follows the same three patterns, with some children rapidly clearing the initial strain, some exhibiting prolonged carriage of a single strain, and some carry and clear up to five different strains of M. catarrhalis (13).
Table 1. Examples of non-pathogenic and potentially pathogenic bacteria that frequently inhabit the nasopharynx.
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<th><strong>Potentially Pathogenic Bacteria</strong></th>
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<td><em>Haemophilus influenzae</em></td>
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<td><em>Prevotella melaninogenica</em></td>
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<td><em>Neisseria perflava/sicca</em></td>
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<td><em>Neisseria flavescens</em></td>
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</tr>
<tr>
<td>Diphtheroids</td>
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</tbody>
</table>
Repeated colonization by the same bacterial strain is more common with *M. catarrhalis* than *H. influenzae* (12, 13).

Nasopharyngeal colonization is a dynamic process, and the composition of the nasopharyngeal flora is in constant flux (1, 2). This continual turnover is due in part to the generation of an immune response against colonizing bacteria. Maternal antibody can passively protect newborns from infection by potential pathogens even though the level of antibody steadily declines during the first six months of life. However, approximately fifty percent of infants have maternal strain-specific antibody at the time of initial colonization by *H. influenzae*, indicating that passive antibody protection does not prevent nasopharyngeal colonization (14-16). Production of immunoglobulin in healthy infants begins at approximately two to three months of age, albeit at a much slower rate than antibody production occurs in adults. IgG serum levels begin to rise at approximately two years of age, and IgM and IgA appear over the first three years of life (16). This is important as the dominant serum antibody against *H. influenzae* and *M. catarrhalis* is IgG, although the level of IgM antibody against *M. catarrhalis* is comparable to IgG for the first two years of life (16, 17).

In contrast to serum immunoglobulin, children do not possess mucosal antibodies against the potentially pathogenic nasopharyngeal flora prior to colonization (16). The mucosal antibody response is predominantly IgA, and the magnitude of this response influences which of the three general colonization patterns will occur (16). Colonization by *H. influenzae* promotes production of IgA and secretory IgA, particularly against the outer membrane protein P6. This antibody response is associated with elimination of the initial colonizer and generally protects against subsequent colonization by the same strain.
of *H. influenzae* (14, 18). P6 is also highly conserved among *H. influenzae* strains and thus may protect against colonization by different strains, whereas an antibody response generated against a strain specific outer membrane protein, such as P2, would not be cross-protective (16). Upon exposure to *M. catarrhalis*, serum IgM and IgG specific for the surface protein UspA are present at low levels during the first two years of life followed by an increase in IgG levels, and antibody specific for the Hag protein can also be detected (16, 17, 19). The mucosal response to *M. catarrhalis* includes IgA specific for UspA and the Hag protein (16, 17, 19). Again, an antibody response generated against conserved surface proteins may provide cross-protection against different strains of *M. catarrhalis* while antibodies generated to phase variable proteins or strain-specific proteins would provide only limited protection.

While many studies have been conducted to investigate nasopharyngeal colonization, there is a large amount of variability in the reported rates of carriage between studies conducted in different geographical areas. This suggests that, in addition to differences in methodology between studies and individual variation, there are social and environmental factors that impact the dynamics of nasopharyngeal colonization (2). Even in countries with similar environments, such as Italy and Portugal, colonization studies with healthy pre-school children show a large degree of variation, and differences in bacterial carriage have even been found between nearby day-care centers (2, 20). Factors known to influence bacterial colonization include an individual’s genetic background and local host immune response, socio-economic factors, and environmental factors. Low socio-economic status, housing and general living conditions, limited access to health care, poor hygiene, and sleeping in the prone position increase the
frequency of colonization (2, 21-24). Having a large family size and high number of siblings and attending day-care increases exposure to other children and frequency of colonization (2, 21). Carriage rates vary depending on time of year, and allergies and viral infection can increase the frequency of colonization by different pathogenic bacteria (2, 25). Exposure to cigarette smoke also impacts the nasopharyngeal flora of both the smoking adult and children exposed to second-hand smoke (23, 26). In general, any factor that causes damage to the mucosal surfaces of the nasopharynx can promote bacterial attachment or impair the host immune response, thus impacting clearance of bacteria and increasing colonization (4, 23, 25).

Additionally, the composition of the nasopharyngeal flora impacts colonization by new species as competition between bacterial populations for the same niche can prevent colonization by potentially pathogenic bacteria (7, 8, 26, 27). For instance, viridans group streptococci and Prevotella melaninogenica can interfere with the growth of potential pathogens and antagonize colonization by these species (2, 5). Bacterial interference has mostly been studied in the context of viridans streptococci and Group A β-hemolytic streptococci, but α-hemolytic streptococci have also been shown to antagonize H. influenzae, M. catarrhalis, and S. pneumoniae (2, 28). Competitive interactions also exist between the potentially pathogenic species that colonize the nasopharynx. Colonization by H. influenzae is negatively associated with colonization by S. pneumoniae, while colonization by both H. influenzae and M. catarrhalis results in a positive association with S. pneumoniae colonization (29). Colonization by Staphylococcus aureus reduces the odds of S. pneumoniae colonization by approximately forty percent, and also reduces the odds of H. influenzae colonization (29).
Nasopharyngeal colonization by *H. influenzae* is negatively associated with colonization by *M. catarrhalis*, but colonization by both *H. influenzae* and *S. pneumoniae* results in a positive association with colonization by *M. catarrhalis* (29). Antibiotic treatment further influences the composition of the nasopharyngeal normal flora as many of the commensal species that interfere with colonization by pathogens are more susceptible to antimicrobial agents, particularly amoxicillin (5). Thus, antibiotic treatment may deplete the less-pathogenic species within the nasopharynx, consequently allowing for colonization by pathogens.

While most if not all humans harbor potential pathogens within the nasopharynx, these organisms are generally carried asymptptomatically and only a very small percentage of colonized individuals develop clinical infection (2). The development of an immune response against colonizing species generally protects against the development of clinical infection with colonizing species. However, factors such as genetic/immunologic predisposition to infection and abnormalities in host defense, viral infection or allergies, and anatomical defects can establish a permissive environment for the egress of opportunistic pathogens from the nasopharynx (30, 31). Egress from the nasopharynx introduces opportunistic pathogens to new sites throughout the respiratory tract, where they can colonize and ultimately establish opportunistic infections including sinusitis, pneumoniae, chronic obstructive pulmonary disease (COPD) exacerbations, and otitis media (OM) (14, 32-35).
Otitis Media

Otitis media (OM) refers to inflammatory middle ear disease and it represents one of the most common childhood infections (36-39). It is estimated that approximately 85% of children experience at least one episode of OM by three years of age (40). Based on the high incidence of OM and a high rate of spontaneous recovery, OM is thought to be a natural and inevitable part of childhood, like the common cold (41). However, proper management of OM is critical as untreated infections can lead to severe complications including mastoiditis, meningitis, hearing loss, delayed communicative development, behavioral changes, and impaired psychosocial development (40, 42, 43). OM represents a significant economic burden worldwide and the direct cost is estimated at $3-5 billion annually in the US. However, the true impact of OM is drastically underestimated as direct cost estimates do not account for lost wages for caregivers or the burden of OM complications (40, 41, 44).

OM is the result of the dynamic interplay between microbial load and the immune response, and OM pathogenesis is multifactorial (Figure 1) (41). Environmental factors such as number of siblings, attending group day care, and time of year influence bacterial load, while factors such as age, genetic predisposition, and atopy impact the immune response to opportunistic pathogens (41). Children who are prone to OM tend to exhibit frequent nasopharyngeal colonization by potential OM pathogens and repeated colonization by the same strain or serotype, indicating failure to develop a broadly-protective immune response, and antibodies against capsular polysaccharides are generally not produced until approximately two or three years of age (16, 45). In
Figure 1. Important factors involved in the pathogenesis of otitis media (41).
addition, the complement system in children is only approximately 50% of adult levels, and children’s monocytes do not increase production of C3 in response to LPS stimulation. Children also produce fewer neutrophils in response to infection than adults, and their neutrophils do not adhere as well to endothelium or migrate as well as adult neutrophils (45). These deficiencies of the childhood immune response may influence the balance between bacterial load and host response in favor of the opportunistic pathogen.

The Eustachian tube also plays a major role in development of OM as bacteria migrate from the nasopharynx to the middle ear via this organ, which consists of a lumen with mucosa, cartilage, surrounding soft tissue, and peritubal muscles (46). In relation to the middle ear, the Eustachian tube functions to regulate pressure by equilibrating gas pressure in the middle ear with atmospheric pressure. This is critical because hearing is optimal when gas pressure within the middle ear is similar to the air pressure of the outer ear canal, so factors that alter the balance of pressure impact hearing. The Eustachian tube is normally closed, but it opens when swallowing to allow for gas exchange that equilibrates pressure. Interestingly, the Eustachian tubes of children cannot equilibrate pressure as well as an adult Eustachian tube (46). This is due in part to differences in the anatomy of the Eustachian tube between children and adults, as the Eustachian tubes of children are shorter, more horizontal, and less rigid (Figure 2) (47, 48). The Eustachian tube also protects the middle ear from nasopharyngeal secretions, and drains middle ear secretions to the nasopharynx via a “pumping action” as it closes (46). Thus, factors that impact the anatomy of the Eustachian tube or the mucociliary system can also influence development of OM by impairing the balance of pressure or clearance of middle ear secretions.
secretions (41, 46). Furthermore, viral infection and bacterial toxins can impair mucociliary function, and viral infection or allergies can cause inflammatory swelling of the Eustachian tube mucosa and allow for aspiration of nasopharyngeal secretions to the middle ear (46).

OM is generally divided into two main categories for clinical diagnosis: acute OM and OM with effusion (40, 49). In this context, acute OM refers to the presence of middle-ear effusion (MEE) and symptoms of middle ear inflammation, such as otalgia, otorrhoea, fever, or irritability. OM with effusion refers to the presence of MEE without any signs or symptoms of an acute infection (41). Despite these two main classifications, OM actually encompasses a continuum of presentations ranging from a single acute OM episode to chronic or recurrent infections that may or may not involve the presence of MEE (Figure 3) (40, 49-51). Between fifty and eighty-five percent of children experience at least one episode of acute OM by three years of age (41, 52). Acute OM can have several outcomes, including relief of symptoms and resolution of MEE, relief of symptoms but persistence of MEE for weeks or months, development of suppurative complications, perforation of the tympanic membrane and otorhea, relief of symptoms of the acute infection followed by relapse of symptoms, or recurrent episodes of acute OM with or without persistent MEE (42). Recurrent OM is defined as having three or more new episodes of acute OM within a six-month period or four infections in the span of one year. Approximately 14-22% of children experience recurrent OM before one year of age, and 60% of children who had at least one episode of acute OM by six months of age experience recurrence (53). It is also estimated that approximately 20% of children develop chronic OM or OM with effusion (40, 41).
Figure 3. Classification of otitis media (49).
Are there symptoms / signs of inflammatory middle ear disease?

**Otitis Media**

How long has the disease been present?

Less than 8 weeks → **Acute otitis media**

More than 8 weeks → **Chronic otitis media**

Is there middle ear fluid? (Impaired mobility / Opacity / Air-fluid / Flat Tympanogram)

Yes → Perforation with otorrhoea?

    Yes → Chronic suppurative otitis media

    No → **Acute suppurative otitis media**

Is TM red / bulging?

    Yes → **Acute non-suppurative otitis media**

    No → **Otitis media with effusion**

Is the perforation central or marginal?

    (R) → Central

    (R) → Marginal

**Tubotympanic disease**

**Atticoantral disease**

Is there squamous epithelium within the middle ear?

→ **Cholesteatoma**
While viruses such as respiratory syncytial virus and rhinovirus can cause OM, the pathogens responsible for the majority of OM cases are *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*. These three pathogens are more frequently found in the nasopharynx compared to other potential OM pathogens, and early colonization and frequency of colonization are associated with both an early first episode of acute OM and the frequency of OM (4, 14, 16). The predominant acute OM pathogen is *S. pneumoniae*, which accounts for approximately 35% of episodes, while *H. influenzae* and *M. catarrhalis* cause approximately 20% and 15% of cases, respectively (16). Patients with OM with effusion and chronic OM have increased nasopharyngeal carriage and colonization density of *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* compared to patients experiencing acute OM, and the hierarchy shifts to *H. influenzae* and *M. catarrhalis* replacing *S. pneumoniae* as the predominant causative agents (54). *H. influenzae* can be detected by PCR in 52-55% of cases of chronic OM with effusion, *M. catarrhalis* is detected in 16-46% of cases, and *S. pneumoniae* can be detected in 8-30% of cases (55, 56).

OM is the leading reason for pediatric office visits and new antibiotic prescription to children (57). Recommended treatment of OM varies depending on symptoms, severity, and recurrence of the infection. Acute OM and OM with effusion may be handled through watchful waiting, prescription of antibiotics, or use of oral steroids. Chronic and recurrent cases of OM sometimes warrant further medical interventions such as myringotomy for ventilation, placement of tympanostomy tubes, or adenoidectomy. The aim of medical intervention is to improve hearing, reduce middle ear effusion fluid,
and/or reduce the incidence of acute OM episodes, particularly when antibiotic treatment is ineffective (41).

The rate of antibiotic prescription for children less than 5 years of age experiencing OM is as high as 80% (58). Regular and high-dose amoxicillin are the most common prescriptions for OM (59). However, resistance to beta-lactam antibiotics is staggeringly high for *H. influenzae*, as approximately 60% of strains produce beta-lactamase and numerous beta-lactamase negative, ampicillin resistant (BLNAR) strains exist that are resistant through mutations in the penicillin binding proteins (60-62). Beta-lactamase production is also essentially ubiquitous in *M. catarrhalis* clinical isolates (61).

Other antibiotics prescribed for treatment of OM include cefdinir, cefaclor, azithromycin, trimethoprim, and amoxicillin/clavulanate. For recurrent or nonresponsive OM, high dose amoxicillin, amoxicillin/clavulanate, or ceftriaxone are recommended (63). However, a recent study found that approximately 13% of all acute OM episodes result in treatment failure or recurrence, regardless of the treatment method (59). Failure of bacteriological eradication rates are particularly striking for nonsusceptible pneumococci, for which cefaclor, azithromycin, trimethoprim, and cefdinir are only as efficacious as placebo (53). For *H. influenzae*, azithromycin and trimethoprim have a high rate of failure, and cefaclor is only as efficacious as placebo (53, 64). Other studies have shown that antibiotic treatment provides only minimal and transient benefit, particularly for patients experiencing OM with effusion or chronic infection (65). The high rate of treatment failure, recurrence, and chronic infection suggest that OM pathogens have evolved numerous strategies to evade the host immune response and resist the action of antimicrobial agents. Thus, researching mechanisms behind evasion
of the immune response and antibiotic resistance not only broadens knowledge of bacterial pathogens and disease, but may also guide development of new therapeutics or treatment and prevention strategies for infection.

**Bacterial Biofilms**

Since the first published observations of bacteria preferentially growing attached to a surface, the concept of biofilms has expanded our understanding of bacterial persistence mechanisms. Bacterial biofilms are generally described as surface-adherent communities of bacteria encased in a matrix of extracellular polymeric substances (EPS), although the exact composition of this matrix as well as the structure of the biofilm varies between species. The vast majority of bacteria are known to exist in biofilm communities, both in nature as well as in human and animal hosts, and the clinical significance of biofilms is a vast area of study (66-73).

While the composition and architecture of biofilms differ between species, biofilm development can be generally divided into five stages: initial attachment to a surface, production of EPS, early development of the biofilm architecture, maturation, and dispersion of single cells from the biofilm (Figure 4) (74). Attachment to a surface can be mediated by flagella and type IV pili, fimbria, extracellular DNA, and polysaccharides (75). Once the initial bacteria are attached to a surface, microcolonies are formed and cell aggregation begins to establish the biofilm. This occurs through surface motility of the attached bacteria, binary division of attached cells to form clusters, and recruitment of planktonic bacteria to the developing biofilm (74, 76, 77). Following
Figure 4. **Biofilm development (74).** Diagram showing the development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered “irreversible” attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The bottom panels (a-e) show each of the five stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum (74).
the initial stages of attachment and microcolony formation, a switch occurs from irreversible attachment to permanent attachment to the substratum. This irreversible attachment is associated with a switch from flagella-based motility to type IV pili-based twitching motility and interactions between the attached bacteria involving adhesins (74).

Production of EPS provides structural support for the growing biofilm as the EPS matrix contributes to attachment, cell-to-cell interconnection, and interactions between subpopulations within the biofilm, as well as playing a role in tolerance to antimicrobials and the host immune response (75). The slime-like EPS is a biosynthetic polymer frequently composed of polysaccharides, proteins, nucleic acids, and phospholipids, although the exact composition varies between species (74). As the biofilm matures, a complex architecture is established with channels, pores, and redistribution of the bacteria away from the substratum. The cell clusters grow larger, and metabolic activity within the center of the clusters diminishes due to limited nutrient availability (74, 78). In addition to altered metabolism, the expression of genes involved in phospholipid and lipopolysaccharide-biosynthesis, membrane transport and secretion, and stress response pathways are drastically different in mature biofilms compared to planktonic bacteria.

Dispersal or detachment of cells from a biofilm is a natural and advantageous process through which bacteria egress from a mature biofilm to establish new bacterial communities, particularly when facing unfavorable conditions (79, 80). Many factors have been implicated in regulating the dispersal process for different bacterial species, including nutrient availability, carbon, oxygen, and calcium concentrations, iron availability, glucose depletion, protease activity, nitric oxide exposure, intracellular cyclic di-GMP levels, and various cell-cell signaling mechanisms (81-90). Once
initiated, the dispersal process involves a reduction in bacterial adhesiveness and modulation or breakdown of the EPS matrix through factors such as proteases or nucleases. The detaching bacteria are released back into a planktonic mode of growth to seed and colonize new sites, thus completing the biofilm developmental cycle (74).

An important aspect of the biofilm mode of growth is the inherent resistance to antimicrobial agents provided by the biofilm structure. Biofilm-associated bacteria can withstand antibiotic concentrations up to 1,500 times greater than bacteria in liquid broth cultures (67, 91). This is considered to be an inherent resistance phenotype because mechanical disruption of the biofilm community generally restores susceptibility to antimicrobial agents (91). Factors such as the thickness of the biofilm and EPS composition promote the inherent resistance of biofilms by binding antimicrobial agents or delaying penetration through the biofilm, and the altered metabolic state and stress response of biofilm-associated bacteria limit the efficacy of antibiotics that target cell wall synthesis or require actively replicating bacteria (92-95). Interestingly, the organisms responsible for chronic infections are generally sensitive to antibiotics when cultured in the lab, but antibiotic treatment fails to resolve the clinical infection (96). This discrepancy can be explained by the establishment of bacterial biofilms during chronic infection.

It is hypothesized that biofilm formation is involved in over 60% of all microbial infections, particularly chronic infection (91). In contrast to acute disease, chronic infections tend to be much less aggressive, are frequently culture negative, and persist for months or years with alternating periods of acute exacerbation and quiescence. During such infections, bacteria exist in both a biofilm state and as individual planktonic cells
that trigger overt symptoms of infection. The planktonic population is rapidly cleared by the host immune response or antibiotic treatment while the biofilm population continues to exist under the radar, thus resulting in periodic acute exacerbation and culture negative infection (96). Biofilm formation on medical devices such as prosthetic heart valves, orthopedic implants, venous and urinary catheters, contact lenses, intrauterine devices, and dental unit water lines can contribute to the development and persistence of chronic infections. The existence of bacterial biofilms on patient tissue has also been described for numerous chronic infections, including native valve endocarditis, chronic bacterial prostatitis, cystic fibrosis, osteomyelitis, rhinosinusitis, and periodontitis (97-100).

In the context of OM, chronic infections persist for long periods of time and are generally not well-resolved by antibiotic treatment or host immunity. Chronic OM effusion samples are also frequently culture-negative (50, 51, 101). This suggests that the causative agents are sequestered within the middle ear space or surface-adherent, as would be expected for bacteria existing as a biofilm community, and that the planktonic population that triggers overt symptoms of infection is most likely rapidly killed by the host immune response or antibiotic treatment. Evidence supporting the existence of bacterial biofilms in OM includes detection of bacterial products in culture-negative effusion fluids, indicating that bacteria are present and metabolically active even if they cannot be recovered by traditional culture methods (102-104). Biofilms have also been directly observed in patient tissue (105) and in the chinchilla experimental model of OM (106-109). Thus, chronic OM is recognized as involving bacterial persistence within biofilm communities (67, 72, 110).
Polymicrobial Infection

In the context of human infection, both acute and chronic diseases can be caused by combinations of viruses, bacteria, fungi, and parasites (Table 2) (111-115). Polymicrobial interactions during infection can be divided into four general categories: synergistic infections, infections predisposing to polymicrobial disease, additive infections, and microbial interference. During synergistic infection, one microorganism generates a niche that is favorable for colonization and/or infection by another microorganism. This is best exemplified by the metabolic interdependence of periodontal pathogens (111, 116, 117). Certain initial colonizers like *Streptococcus gordonii* can grow as a solitary and independent resident on the tooth with other species, while *Streptococcus oralis* and *Actinomyces naeslundii* fail to colonize independently but form a mutualistic relationship that enables both species to grow and form a biofilm (116). A classic example of infection predisposing polymicrobial disease is the relationship between respiratory tract viral infection and the development of bacterial respiratory tract infection or OM (111). In additive polymicrobial infection, combinations of two or more generally non-pathogenic microorganisms lead to conditions such as bacteremia, abdominal abscess or secondary periontitis, lung abscess, odontogenic infection, brain abscess, mastoiditis, liver infections, and soft tissue infection or fasciitis (111). Additive polymicrobial interactions can contribute to whooping cough, in which *Bordetella pertussis* uses filamentous hemaglutinin (FHA) and pertussis toxin (TOX) to adhere to human respiratory cilia. These two proteins are secreted during infection and can bind to the surface of other resident species, such as *H. influenzae*, *S. aureus*, and *S. pneumoniae*. 
Table 2. Polymicrobial human infections. Combinations of microorganisms in human polymicrobial disease (111).
<table>
<thead>
<tr>
<th>Causal agents</th>
<th>Disease</th>
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<tbody>
<tr>
<td><strong>Synergistic polymicrobial infections</strong></td>
<td></td>
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<tr>
<td>Human metapneumovirus with coronavirus or respiratory syncytial virus</td>
<td>SARS, bronchiolitis</td>
</tr>
<tr>
<td>Measles and <em>Mycobacterium tuberculosis</em> and <em>Staphylococcus aureus</em></td>
<td>Measles</td>
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<tr>
<td>Epstein-Barr virus and retrovirus</td>
<td>Multiple sclerosis</td>
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<tr>
<td>HTLV-I, HTLV-II, and/or HIV-1, HIV-2</td>
<td>AIDS</td>
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<tr>
<td>HTLV-I and HTLV-II</td>
<td>Respiratory and urinary tract infections</td>
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<tr>
<td>HIV and M <em>tuberculosis</em></td>
<td>AIDS</td>
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<tr>
<td>HBV or HCV and HIV-1</td>
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<tr>
<td>HIV and enteric viruses, <em>Acinetobacter radioreisent</em>, <em>M</em> <em>tuberculosis</em>,</td>
<td>AIDS</td>
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<tr>
<td><em>Ehrlichia chaffeensis</em>, <em>Candida albicans</em>, <em>Histoplasma capsulatum</em>,</td>
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<tr>
<td><em>Cryptosporidium parvum</em>, <em>Trichomonas vaginalis</em>, and others</td>
<td></td>
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<tr>
<td>Lyme disease with babesiosis or ehrlichiosis</td>
<td>Lyme disease</td>
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<tr>
<td><em>Stenotrophomonas maltophilia</em> and <em>Aspergillus fumigatus</em></td>
<td>Corneal infection</td>
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<tr>
<td><strong>Infections predisposing to polymicrobial disease</strong></td>
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<tr>
<td>Influenza viruses, parainfluenza viruses, respiratory syncytial viruses,</td>
<td>Respiratory disease</td>
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<tr>
<td>adenoviruses, measles viruses, rhinoviruses, and coronaviruses with</td>
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<tr>
<td><em>Streptococcus pneumoniae</em>, <em>Strep pyogenes</em>, <em>Haemophilus influenzae</em>,</td>
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<tr>
<td><em>Staph aureus</em>, <em>Neisseria meningitidis</em>, <em>M tuberculosis</em>, or <em>Bordetella</em></td>
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<tr>
<td>pertussis</td>
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<tr>
<td>Coronavirus and <em>Escherichia coli</em></td>
<td>SARS</td>
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<tr>
<td>Respiratory tract viruses and bacterial infections</td>
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<tr>
<td>Varicella-zoster virus and <em>Strep pyogenes</em></td>
<td>Otitis media</td>
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<tr>
<td><strong>Additive polymicrobial infections</strong></td>
<td></td>
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<tr>
<td>Aerobic and anaerobic gram-positive and gram-negative bacteria and</td>
<td>Periodontal disease</td>
</tr>
<tr>
<td><em>Candida</em> spp</td>
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<tr>
<td><em>Prevotella</em>-like bacteria</td>
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<td><em>B pertussis</em>, <em>Strep pneumoniae</em>, <em>Staph aureus</em>, <em>H influenzae</em></td>
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<td><em>Nocardia asteroides</em> and <em>Cryptococcus neoformans</em></td>
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<td>Herpes zoster and tuberculosis</td>
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<td>Pseudomonas aeruginosa, <em>S maltophilia</em>, <em>Prevotella oris</em>, <em>Fusobacterium</em></td>
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<tr>
<td>gonidofirmans, <em>Bacteroides fragilis</em>, <em>Leptotrichia</em>-like spp, <em>Abiotrophia</em></td>
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<tr>
<td><em>defecta</em>, <em>Citrobacter murliniae</em>, <em>Lautropia mirabilis</em>, and <em>Sarcina</em></td>
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<tr>
<td><em>ventriculi</em></td>
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<tr>
<td>Aerobic and anaerobic gram-positive and gram-negative bacteria</td>
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<tr>
<td>HBV, HCV, and HDV</td>
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<tr>
<td>HCV and HIV</td>
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<tr>
<td>Norwalk-like virus and <em>Aeromonas sobria</em> or <em>E coli</em></td>
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<tr>
<td><em>Schistosoma haematobium</em> and <em>S mansoni</em></td>
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<tr>
<td>Combinations of <em>Corynebacterium urealyticum</em>, <em>Gardnerella vaginitis</em>,</td>
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<tr>
<td><em>Anaerococcus lactoalyticus</em>, <em>Bact vulgatus</em>, <em>Dialister inusis</em>, <em>Fusobacterium</em></td>
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<tr>
<td>nucleatum*, <em>Lactobacillus iners</em>, <em>Leptotrichia ammionii</em>, <em>P buccalis</em>,</td>
<td></td>
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<tr>
<td><em>P rominicola</em>, <em>Rahnella aquatilis</em>, and <em>Strep intermedius</em></td>
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<tr>
<td><em>Staphylococcus</em> ssp, <em>Streptococcus</em> ssp, and HACEK group</td>
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<tr>
<td><strong>Microbial interference</strong></td>
<td></td>
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<tr>
<td>Flavivirus and HIV</td>
<td>AIDS</td>
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<tr>
<td><em>Strep pneumoniae</em> and <em>Staph aureus</em></td>
<td>Staph-aureus-related disease</td>
</tr>
</tbody>
</table>
This confers the ability to adhere to the cilia of the respiratory tract and provides an anchor to the respiratory epithelium that is not normally present for these species, potentially contributing to the development of secondary pneumonias during whooping cough (111, 118). Microbial interference is discussed above in the context of nasopharyngeal colonization.

As many acute and chronic infections are polymicrobial in nature, and chronic infections frequently involve biofilm formation, it stands to reason that polymicrobial biofilms may play a central role in chronic infection and disease progression. Chronic wound infections tend to be polymicrobial and involve biofilm formation, although a recent study showed that biofilms in chronic wound infections are generally composed of single-species aggregates (98). However, oral biofilms can contain over fifty species of bacteria, and the accumulation of these biofilm communities leads to development of dental carries and periodontitis, providing a direct link between polymicrobial biofilms and infection (119, 120).

A single tooth and the surrounding tissue can harbor a community of several hundred species, making dental plaque and the oral microbial community one of the best models for studying interspecies interactions, polymicrobial biofilms, and polymicrobial infection (121-124). The development of multispecies oral biofilms is impacted by bacterial adhesion and host receptor interactions, as well as bacterial cell-to-cell interactions. Bacteria first must colonize the enamel surfaces via adhesion to the salivary pellicle (119). This is primarily carried out by oral streptococci, which then recruit other bacteria to the growing biofilm. Coaggregation of oral streptococci with other oral microbes depends on cell-surface adhesins and receptors. Through these interactions,
primary colonizers such as *Streptococcus sanguinis* and *Streptococcus gordonii* recruit *Actinomyces oris*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, and *Candida albicans* to the biofilm (119, 125, 126). Further coadhesion of bacteria from the saliva leads to the development of complex microenvironments within oral biofilms (119, 124, 127).

The complex interactions between species within oral biofilms influence both development and composition of the biofilm (121). For instance, while saliva is the main source of nutrients for supragingival dental plaque, very few oral bacteria can utilize saliva as a sole nutrient source. The presence of multiple species within the biofilm allows for a mutualistic interaction in which dental plaque as a whole can actually degrade host mucins and other salivary components to survive in this environment (119, 128, 129). Metabolic products of the organisms within a multispecies biofilm can also influence biofilm composition. For example, lactic acid produced by *Streptococcus mutans* can be metabolized by *Veillonella* species and *Streptococcus oligofermentans* but limits the presence of *Streptococcus sanguinis* in dental plaque (121, 130, 131). *S. oligofermentans* converts lactic acid into hydrogen peroxide, which is highly toxic to *S. mutans*, shifting the population towards the decreased presence of *S. mutans* of *P. gingivalis* (121, 132). *Fusobacterium nucleatum* and *Prevotella intermedia* can impact biofilm composition by altering the pH of the biofilm environment (121). These species can tolerate low pH, and produce ammonia and organic acids when using glutamic and aspartic acids from the crevicular fluid, which contributes to establishing a more neutral pH in the biofilm and allows for survival of acid-sensitive species, such as *P. gingivalis* (121, 133).
Many oral bacteria also produce bacteriocins that can kill other species within dental plaque (119, 121). For instance, *S. mutans* produces at least five different bacteriocins, some of which can inhibit the growth of *S. sanguinis*, and the bacteriocins produced by *S. pyogenes* and *S. salivarius* are structurally similar and can interfere with two-component signaling by the other species (121, 134). Thus, the presence of a single highly pathogenic organism within the complex dental plaque community does not solely determine the properties of the biofilm or infection (121). Instead, the complex interactions between organisms within a multispecies biofilm determine biofilm composition and ability to cause disease. The same may also be true for other polymicrobial infections involving multispecies biofilms.

As with most upper airway infections, epidemiological data indicate that the majority of chronic OM infections are polymicrobial (55, 111). For example, *H. influenzae* and *M. catarrhalis* are frequently present together in patient samples from chronic and recurrent OM (55, 104, 135). Interestingly, a recent study found *M. catarrhalis* and to be more frequently isolated from polymicrobial OM infections than as the single causative agent (136). This suggests that the presence of other bacterial pathogens impacts persistence of *M. catarrhalis* or the ability of this species to cause disease. *H. influenzae* and *M. catarrhalis* are also the leading causes of chronic OM, suggesting that biofilm formation by these species as well as polymicrobial interactions may contribute to disease. Polymicrobial biofilms containing combinations of *H. influenzae, M. catarrhalis, S. pneumoniae*, and *S. aureus* have been directly observed on adenoid tissue from children with recurrent OM (137), and experimental data supports the formation of *H. influenzae* and *S. pneumoniae* polymicrobial biofilms during OM.
(138). However, further research is necessary to investigate polymicrobial biofilm formation during infection by *H. influenzae* and *M. catarrhalis*.

An important factor to consider with polymicrobial infection is the impact on treatment efficacy. According to the long-standing concept of indirect pathogenicity, bacterial disease and/or response to treatment can be influenced by bacteria sharing the same environment (139, 140). It is well-documented in the literature that the presence of multiple bacterial species during infection can significantly impact treatment efficacy. For example, *M. catarrhalis* is thought to confer passive antibiotic resistance upon other pathogens via secretion of beta-lactamase (139-145). This is of particular interest for respiratory tract infections and OM, where other opportunistic pathogens such as *S. pneumoniae* are still relatively sensitive to beta-lactam antibiotics in the absence of a beta-lactamase producing coinfection partner, and *M. catarrhalis* is frequently found as part of a polymicrobial infection. Polymicrobial biofilm formation during infection may further contribute to antibiotic treatment failure due to specific features of the biofilm, transfer of resistance determinants between species, or secretion of beta-lactamase providing passive protection to susceptible organisms (92). However, the overall impact of polymicrobial infection on bacterial persistence, virulence, or response to treatment is not presently clear, particularly for *H. influenzae* and *M. catarrhalis* coinfection.

*Haemophilus influenzae*

*Haemophilus influenzae* is a Gram-negative coccobacillus that commonly inhabits the nasopharynx and upper airways of children and healthy adults (146, 147). *H.
*H. influenzae* can grow aerobically or as a facultative anaerobe, and the species is defined by a nutritional requirement for β-nicotinamide adenine dinucleotide and heme (148, 149). As a human-adapted pathogen, *H. influenzae* is not a natural colonizer or pathogen in most animal models, which may complicate interpretation of animal studies on pathogenesis and vaccination (32, 150). However, several animal models exist for the study of *H. influenzae* infection and pathogenesis, including the use of rats, mice, and chinchillas (32).

*H. influenzae* strains can be broadly divided into two main categories, encapsulated strains and nontypeable *H. influenzae* (NTHI). Encapsulated strains of *H. influenzae* are further divided into serotypes a through f based on differences in their polysaccharide capsular material (148, 151, 152). These encapsulated strains, particularly serotype b (Hib), are the causative agents of invasive childhood disease such as meningitis, pneumonia, epiglottitis, septicemia, cellulitis, osteomyelitis, and septic arthritis (153). However, the prevalence of these infections has been significantly reduced by a vaccine to the capsular polysaccharide of serotype b (154). In contrast, NTHI strains lack the genetic material to produce a polysaccharide capsule and mainly cause opportunistic infections of the airway mucosa, including bronchitis, sinusitis, and OM (32, 33, 146, 155).

Approximately 45% of healthy children are colonized by NTHI during the first two years of life (156), and NTHI colonizes the nasopharyngeal region of up to 80% of the population as a whole (148). The colonization process begins with attachment of NTHI to mucosal or epithelial surfaces through the interaction of bacterial attachment factors and host receptors. NTHI strains preferentially adhere to mucus, non-ciliated
cells, and damaged epithelium (51). The high rate of colonization by NTHI is due in part to the ability of this species to use numerous mechanisms for attachment coupled with antigenic variation (32). The fimbriae encoded by the hif locus mediate adherence of serotype b strains to all cell types of the respiratory tract and are thought to be an important factor in the early stages of colonization, but not all NTHI strains possess this locus (146, 157). Some NTHI strains express phase-variable pili that mediate agglutination of human erythrocytes and promote adherence to human oropharyngeal epithelial cells, human nasopharyngeal tissue, and mucin (51, 158-160). For these strains, pilin-mediated binding appears to require sialic acid-containing lactosylceramide structures present on oropharyngeal epithelial cells and abundant in the upper respiratory tract (32, 161).

Many strains of NTHI possess the phase-variable high molecular weight adhesins HMW1 and HMW2, which bind a wide variety of mammalian cells by interacting with structures containing glycoproteins related to heparan sulfate (32, 162, 163). HMW1 specifically interacts with glycoproteins containing N-linked oligosaccharide chains with sialic acid in an α-2,3 configuration, but the specificity of HWM2 is not currently known (164). Together, HMW1 and HMW2 mediate attachment to human conjunctival cells, but individually the binding specificity of HMW1 is for oropharyngeal cells while HMW2 has greater affinity for genital tract epithelial cells (32). Approximately 25% of NTHI strains lack HMW1/HMW2, but these strains generally express a different high molecular weight protein named Hia that can mediate adhesion to Chang epithelial cells (164-166). Studies of mutant strains lacking HMW1, HMW2, and Hia led to the identification of the Hap adhesion that mediates adherence to human epithelial cells.
through interactions with fibronectin, laminin, and type IV collagen (164, 167, 168). In addition to a role in adhesion, the Hap protein also mediates bacterial aggregation and plays a role in microcolony formation by NTHI (169). The NTHI outer membrane proteins P2 and P5 promote adherence to mucin-coated nasopharyngeal epithelial cells by binding the mucin oligosaccharide moieties that contain sialic acid (32, 170). The OapA surface-associated lipoprotein also plays a role in adherence and colonization by NTHI as this protein is required for efficient nasopharyngeal colonization in infant rats, and inactivation of OapA limits attachment to Chang conjunctival cells (51, 171, 172).

Another bacterial factor that promotes NTHI adherence and colonization is lipooligosaccharide (LOS). NTHI LOS contains non-repeating oligosaccharides consisting of glucose, galactose, N-acetylglucosamine, phosphorylcholine, and N-acetyl-neuraminic acid rather than repeating polymeric O-side chains (51, 173). LOS composition and structure can impact the ability of NTHI to colonize the nasopharynx. For instance, polystyrene beads coated with purified NTHI LOS can adhere to human bronchial epithelial cells (174), and expression of a higher-molecular-weight LOS by NTHI enhances adhesion to rat nasopharyngeal cells (175). One way that LOS promotes adherence is through the addition of phosphorylcholine (PCho). PCho is a host structure and a component of platelet-activating factor (PAF) that NTHI adds to its LOS by scavenging choline from host cells (176). Adherent bacteria expressing PCho co-localize with host cell PAF receptor, and this interaction influences invasion of human bronchial epithelial cells by NTHI (174).

Following initial attachment, NTHI must either evade the host immune response to exist as a commensal or take advantage of its surroundings to cause disease. Similar to
the multifaceted approach used for adhesion, NTHI has many ways to evade or take advantage of host defenses. IgA is the predominant immunoglobulin at mucosal surfaces, and IgA1 accounts for over 90% of the IgA present within the respiratory tract (177). At least two different types of IgA1 proteases have been identified in *H. influenzae* clinical isolates, and almost all strains express one of these proteases (164, 178). Both types of IgA1 protease cleave the heavy chain in the hinge region resulting in a Fab fragment that can still bind antigen and an Fc fragment that lacks biological function, but they differ in the proline residue at which they cleave (32). There are over 30 antigenic types of IgA1 proteases derived from *H. influenzae* strains, and NTHI strains can change their IgA1 protease to cleave at a different site and alter antigenic properties (32, 179).

NTHI may also evade host defenses by entering into host cells or invading the subepithelial space (32, 164). NTHI has been observed between epithelial cells of the bronchi and bronchioles in tissue sections from patients with bronchitis and in adenoid tissue (180-182). Further investigation of adenoid tissue from children with otitis media showed that NTHI was present within macrophage-like cells, where they survived gentamicin treatment and appeared to be dividing (182). *H. influenzae* strains lacking capsule have been observed to rapidly associate with monolayers of human umbilical vein endothelial cells and are internalized into membrane-bound vacuoles in which the bacteria can persist (183). Several lines of evidence suggest that by adhering to epithelial cells with multiple ligands, NTHI actually stimulates a proinflammatory response that promotes engulfment by the epithelial cells (146, 184, 185). NTHI strains have been shown to adhere to and invade Chang epithelial cells (186), and the interaction between PCho moieties on the LOS with the PAF receptor is one factor that mediates adherence to
and invasion of bronchial cells by NTHI (174). Importantly, NTHI can also dampen the induced proinflammatory response after colonization or invasion have occurred (146, 187-189).

Another mechanism of immune evasion by NTHI involves phase variation to reversibly alter expression of surface structures that are immunogenic or stimulate the host antibody response (164). The *hmw1A* and *hmw2A* genes that encode HMW1 and HMW2 include seventeen 7-bp direct repeats in the promoter region, and slipped-strand mispairing in these repeat regions alters the total number of repeats in the region, which in turn alters protein expression. Specifically, increasing the number of repeats results in decreased levels of mRNA and decreased protein expression, enabling NTHI to spontaneously vary between expression states of the HMW adhesins (190). Phase variation of pili can also provide a significant survival advantage as antibody directed against pili enhances complement-mediated clearance (164, 191). The *hifA* and *hifB* promoters share a common RNA polymerase binding site, and the region of promoter overlap contains tandem TA dinucleotide repeats. The number of tandem repeats influences the spacing between the -10 and -35 regions of the promoter and therefore influences gene expression, so variation in the number of repeats allows for shifts between expression of both genes and full piliation or failure to express either gene, resulting in loss of piliation (164, 192). The heme-binding proteins of *H. influenzae*, such as *hgpA*, are also subject to phase-variable expression due to the presence of CCAA repeats within their 5’ coding region (164, 193).

The composition of NTHI LOS is also highly variable and NTHI strains express a diverse population of LOS forms, with one individual strain capable of producing up to
25 different forms (164, 174, 194, 195). The NTHI specific subset of LOS glycoforms expressed by NTHI can impact adherence and invasion of host cells, as discussed above (175, 196). These modifications are also thought to aid in masking the bacteria from host immune responses and thus may contribute to evasion of immune clearance (197). LOS biosynthesis involves a number of genes and enzymatic steps, and several of the LOS biosynthetic genes contain long stretches of tandem 4-bp repeats within the 5’ coding region (164). Slipped-strand mispairing within these regions causes spontaneous variation by generating reversible translational frame shifts that can alter the N terminus of the protein or eliminate protein expression altogether (164, 198). Tetrameric repeat regions are present in oafA, lex2A, lic1A, lic2A, lic3A, lic3A2, lgtC, lav, and NTHI_1769. The protein encoded by oafA is an O-antigen LOS acetylase linked to serum resistance (199), lex2A and lic2A are involved in expression of digalactoside moieties of LOS, and the Galα1-4Galα moiety added as a result of lic2A expression aids in protection from antibody-mediated killing (200-203). The lic1A gene is part of the lic1 operon which contains four genes necessary for choline uptake, phosphorylation, and transfer onto the LOS (204, 205), and lic3A encodes a sialyltransferase (206). lgtC codes for a glycosyltransferase linked to serum resistance (207, 208), and NTHI_1769 encodes a hypothetical glycosyltransferase (209). Thus, phase variation of these LOS biosynthetic genes can impact the host response to NTHI, attachment to and invasion of host cells, and the ability of NTHI to resist host factors.

NTHI surface proteins also undergo antigenic drift, which is an irreversible process that causes antigenic variation by substitution, deletion, or addition to amino acids in the immunodominant regions of a surface protein that prevent recognition by
previously generated host antibodies (164). For NTHI, this has been best studied in the P2 and P5 outer-membrane proteins. The P2 protein is highly variable in size and amino acid sequence between strains of NTHI, and variation of the P2 sequence occurs within a clonal population during persistent infection (164, 210, 211). Frequent nucleotide changes in a P2 surface-exposed loop have been observed in a rabbit model that allow for survival and persistence of bacteria even though bactericidal antibodies are produced against P2 (212). The size of the P5 protein also varies over time during infection, suggesting that this protein is an important target of the immune response and also under considerable selective pressure to acquire point mutations and deletions in putative surface-exposed loops (164, 213). The P1 protein can also change during persistent infection to evade host bactericidal antibody (32, 214).

Biofilm formation is another mechanism used by NTHI that provides protection from the host immune response and innate clearance mechanisms of the airway. Many studies support the formation of NTHI biofilms during infection, particularly in the context of OM (55, 104, 215-218). Specifically, NTHI bacterial products have been detected in culture-negative effusion fluids (102-104), and biofilms containing NTHI have been directly observed both in patient tissue (105) and in the chinchilla experimental model for OM (106-109). The NTHI extracellular matrix has been visualized, but the exact composition of the exopolysaccharide is not yet known (146, 219). However, the presence of sialic acid (N-acetyleneuraminic acid or Neu5Ac), double-stranded DNA, type IV pilin protein, and the outer-membrane proteins Hap, HMW1, and HMW2 have been detected in the extracellular matrix of various NTHI isolates (109, 146, 219). Additionally, a study of NTHI clinical isolates found that pili
expression is important in biofilm formation, and the outer-membrane proteins P2, P5, and P6 are expressed during biofilm growth (218).

LOS composition has also been shown to be an important factor in the establishment of NTHI biofilms and persistence in the biofilm mode of growth (107, 108, 220, 221). Prior work in our lab and others has shown that sialylated LOS forms predominate in vivo, where they promote resistance to complement-mediated killing and formation of biofilm communities (109, 220, 222-224). NTHI requires the putative undecaprenyl-phosphate α-N-acetyl-glucosaminyltransferase encoded by wecA to produce a full biofilm, and mutation of siaA that encodes a sialyltransferase or siaB that encodes a CMP-Neu5Ac synthetase also reduce biofilm production (220, 223). Similarly, LOS glycoforms containing PCho are enriched for in persistent NTHI populations in vivo and promote biofilm maturation and NTHI persistence in the airway (107, 108, 200, 221, 225-227). Mutants lacking the PCho transferase encoded by licD form biofilms with decreased biofilm density and thickness, while a strain constructed to be locked constitutively into the PCho+ phase produces increased amounts of extracellular material and biofilms with increased density and thickness compared to the parental strain (108).

Due to the high rate of antibiotic treatment failure for NTHI disease, particularly biofilm-associated infections like OM, attention has been focused on vaccination to prevent *H. influenzae* colonization and infection and therefore reduce the need for antibiotics (228). As discussed above, the prevalence of infection due to encapsulated *H. influenzae* type b has been significantly reduced by a vaccine against the type b capsular polysaccharide, but vaccination against NTHI infection remains an area of active
NTHI protein D is a highly conserved lipoprotein that appears to be present in all *H. influenzae* strains that is expressed on the cell surface, exhibits limited antigenic drift, and plays a critical role in the pathogenesis of NTHI infection, making it an ideal target for NTHI vaccination (228-233). In addition, antibodies generated against protein D have shown protective efficacy in animal models of NTHI infection (228, 234, 235). Protein D was included as a carrier protein in the 11Pn-PD pneumococcal vaccine in part to provide protection against NTHI infection (228). In the Pneumococcal Otitis Efficacy Trial (POET), 11Pn-PD was found to have a similar vaccine efficacy against pneumococcal serotypes covered by the vaccine as the 7vCRM and 7v-PncOPMc vaccines (52.6%), and approximately 32% efficacy against NTHI (228, 236). The PHiD-CV vaccine against NTHI and *S. pneumoniae* also contains pneumococcal capsular polysaccharide serotypes conjugated to protein D, and provides a similar level of protection against NTHI infection as the 11Pn-PD vaccine (228). NTHI outer-membrane proteins P6 and OMP26 are also being investigated as potential vaccine candidates (146). P6 is a surface-exposed and immunogenic protein that is highly conserved in both encapsulated and NTHI strain (146, 237-240), and OMP26 can elicit clearance of homologous and heterologous NTHI strains in vivo (146, 241).

*Moraxella catarrhalis*

*Moraxella catarrhalis* is a Gram-negative, aerobic diplococcus and common commensal of the upper respiratory tract that has been known by a variety of names, including *Micrococcus* (*Mikrokokkus*) *catarrhalis*, *Neisseria catarrhalis*, and
Branhamella catarrhalis (242, 243). Due to difficulties in distinguishing M. catarrhalis from commensal Neisseria species such as Neisseria cinerea, the species was considered a harmless commensal organism and was classified as Neisseria catarrhalis based on colony morphology and other phenotypic characteristics (243, 244). Based on this classification, clinical laboratories did not test for M. catarrhalis in samples from patients with respiratory infections through most of the 1900s (243). However, DNA hybridization studies conducted in 1970 revealed that little homology existed between M. catarrhalis and Neisseria species, resulting in reclassification of the species to Branhamella catarrhalis (243). Further DNA-DNA and rRNA-DNA hybridization studies and 16S rDNA sequence comparisons showed that the species was actually more closely related to Moraxella species, resulting in further reclassification to Moraxella catarrhalis (243, 244). Controversy over the nomenclature still exists, but the classification as Moraxella catarrhalis is generally used in current literature (243).

M. catarrhalis was cataloged as a respiratory pathogen in the early 1900s before the species was classified as a harmless commensal (243, 245). Recent studies have shown that between 28 and 100% of children are colonized by M. catarrhalis during the first year of life, and they may be colonized by and clear up to four different strains of M. catarrhalis by two years of age (156, 243). Even though M. catarrhalis is clearly capable of colonizing humans without causing infection, during the past 40 years it has become increasingly clear that M. catarrhalis is an important respiratory pathogen and can cause infections in both healthy children and immunocompromised individuals (242). M. catarrhalis is a causative agent of respiratory infections such as bronchitis, laryngitis, pneumonia, sinusitis, COPD exacerbations, and is one of the three leading causative
agents of OM (242, 243). Severe infections caused by *M. catarrhalis* are rare, but there have been reports of septic arthritis, bacteremia, cellulitis, osteomyelitis, endocarditis, and pericarditis caused by *M. catarrhalis* (243, 246-253).

While the mechanisms of *M. catarrhalis* colonization and pathogenicity are increasingly active areas of research, very few studies have been successfully conducted in vivo under conditions that mimic human infection. Studying the underlying mechanisms of *M. catarrhalis* pathogenesis during infection is complicated by the fact that this species is a human-adapted pathogen that does not normally cause infection in animals and tends to be rapidly cleared from most animal models (243, 254). Despite this difficulty, animal models such as the mouse model of pulmonary clearance, severe combined immunodeficient (SCID) mice, and the chinchilla model of OM have been utilized to investigate vaccine candidates and the impact of mutations on pathogenesis (243). In the context of OM research and the chinchilla model, *M. catarrhalis* fails to persist in middle-ear effusion fluid and does not establish a productive infection or the majority of the symptoms observed during human OM infection (243, 255). Thus, there is a pressing need to establish a better model that allows for prolonged persistence of *M. catarrhalis* and more closely mimics the course of human infection.

To date, many studies have focused on the ability of *M. catarrhalis* strains and isogenic mutants to adhere to epithelial cells. Similar to what has been observed for *H. influenzae*, *M. catarrhalis* utilizes a number of adhesins for attachment and colonization. The most extensively studied *M. catarrhalis* outer-membrane proteins are the ubiquitous surface proteins (Usps), which include UspA1, UspA2, and the hybrid UspA2H protein that consists of a UspA1-like N terminus domain and a UspA2-like C terminus domain.
The UspAs are trimeric autotransporters that mediate their own insertion into the outer membrane (258). UspA1 has been shown to mediate binding to Chang conjunctival epithelial cells, HEp-2 laryngeal epithelial cells, A549 alveolar epithelial cells, and to fibronectin and laminin of the extracellular matrix (258-262). Binding of UspA1 to host cells is due to its interaction with CEACAM1, which is expressed on a variety of epithelial cells, endothelial cells, and leukocytes (258, 263). UspA2 also binds to laminin and fibronectin, but does not interact with CEACAM molecules (258, 261, 262). UspA2H also appears to mediate binding to Chang cells and may bind fibronectin (258, 259).

The Hag protein (also referred to as MID) is another autotransporter protein that plays a role in adhesion. Hag mediates agglutination of human erythrocytes and attachment to A549 cells, human middle ear epithelial cells, NCIH292 lung epithelial cells, and Chang cells (258, 264-266). In addition, studies of mutants lacking uspA1 and hag led to identification of another autotransporter adhesion molecule, named McaP (258, 267). McaP is a highly conserved protein that appears to mediate adhesion to Chang cells, A549 cells, and polarized human bronchial cells (258, 268). Other M. catarrhalis proteins that play a role in adhesion include OMP CD, which is important for adhesion to middle ear mucin and A549 cells, and the Mha proteins MhaC, MhaB1, and MhaB2 that appear to be required for binding to HEp-2 cells (258, 269-273). Pili have also been observed for some M. catarrhalis strains, and type IV pili appear to contribute to airway colonization and adherence to Chang cells (274).

M. catarrhalis LOS also plays a role in adhesion to host cells. The LOS of M. catarrhalis does not have O-side chains, and M. catarrhalis strains can be typed based on
the branched oligosaccharide chains of their LOS into three major serotypes, designated A, B, and C (275, 276). Studies of an \(lpxA\) mutant lacking a UDP-N-acetylglucosamine acyltransferase involved in lipid A biosynthesis revealed that this gene is required for LOS biosynthesis and that loss of LOS diminishes adhesion to Chang cells, A549 cells, and HeLa cervical cells (277, 278). Loss of the Kdo transferase encoded by \(kdtA\) results in an LOS structure with just lipid A and no core oligosaccharides that is impaired in adhesion to Chang and HeLa cells but can still attach to A549 cells (258, 279). Mutation of \(lgt3\) and loss of the encoded LOS glycosyltransferase also impacts LOS structure by truncating the core oligosaccharide, resulting in reduced adhesion to Chang and HeLa cells (280).

Similar to NTHI, adhesion of \(M. catarrhalis\) to host cells induces an immune response that \(M. catarrhalis\) must evade to successfully colonize and persist. One way that \(M. catarrhalis\) evades the host immune response is through invasion of host cells (258). \(M. catarrhalis\) has been shown to actively invade bronchial epithelial cells and A549 cells to reside within vacuolar structures, and can also invade Chang cells (278, 281). The ability of \(M. catarrhalis\) to invade adenoid and tonsil tissues has also been demonstrated in vivo (282). Furthermore, invasion by \(M. catarrhalis\) appears to require UspA1, LOS, and binding to CEACAM1 (278, 283).

\(M. catarrhalis\) utilizes several other strategies to evade the host immune response, including evasion of complement, inhibition of TLR2 signaling, and phase variation (256, 258). The Usp proteins can interfere with the classical complement pathway by binding to the C4 binding protein, and they interfere with the alternative pathway by neutralizing C3 (284, 285). Binding of UspA2 to vitronectin can also inhibit formation of the
membrane attack complex (286). The outer-membrane proteins OMP CD, OMP E, and CopB also appear to be important for *M. catarrhalis* serum resistance (258, 272, 287, 288). In addition, LOS composition impacts serum resistance as mutations that impact lipid A synthesis, Kdo synthesis, and core oligosaccharide structure all increase sensitivity to the bactericidal activity of human serum (258, 277, 279, 280). The interaction between UspA1 and CEACAM1 can actually inhibit the TLR2-mediated proinflammatory response as CEACAM1 co-localizes with TLR2 on the epithelial surface, and binding of UspA1 to CEACAM1 results in recruitment of SHP-1 that inhibits phosphorylation PI3K, thus inhibiting activation of the PI3K-Akt-NF-κB pathway that is necessary for the TLR2 proinflammatory response (256, 258, 289). The Hag/MID protein also contributes to evasion of the immune response by inducing non-specific IgM production from B-cells without T-cell help, potentially serving to redirect the adaptive immune response (256, 290). Finally, UspA1 and Hag/MID expression are subject to phase variation via slipped-strand mispairing. UspA1 phase variation occurs at the level of transcription due to poly(G) repeats upstream of the *uspA1* open reading frame, while Hag/MID is subject to translational phase variation due to poly(G) repeats at the 5’ end within the open reading frame (265, 291, 292).

*M. catarrhalis* strains can also exist in biofilm communities, and biofilms containing this species have been detected in middle ear mucosa specimens (105, 256). In experiments investigating stationary biofilm formation with crystal violet-based assays, UspA1, UspA2H, and Hag/MID were determined to be involved in biofilm formation by *M. catarrhalis* (256, 293, 294). Specifically, *uspA1* mutants exhibited a 50% decrease in biofilm production compared to the parental strain (295). A recent study
also demonstrated biofilm formation by *M. catarrhalis* under continuous media-flow conditions, and showed that expression of type IV pili enhances biofilm formation under these conditions (274). However, further study is necessary to identify other factors involved in biofilm formation and development, and to determine the importance of biofilm formation to *M. catarrhalis* persistence and virulence in vivo, particularly during conditions that more closely mimic human infection.

Currently there are no vaccines available to protect against *M. catarrhalis* infection, and several challenges exist to the development of such a vaccine including development of a better animal model that simulates human respiratory tract infection by this species (296). The ideal *M. catarrhalis* vaccine antigen would be present on the surface of all strains, conserved among strains, expressed during infection of a human host, immunogenic in infants, and capable of inducing a protective immune response (296). Adhesins and other potential vaccine antigens currently under investigation include Hag/MID, the Mch proteins, a metallopeptidase-like adhesion named McmA, the Usp proteins, McaP, OMP E and OMP CD, type IV pili, LOS, and several other surface-expressed proteins (296-300).

**Quorum Signaling**

As bacteria generally exist in large communities, cell-to-cell communication within the population is an advantageous method for coordinating gene expression and population behavior as a whole, thus allowing bacterial communities to behave like multicellular organisms. Communication between bacteria can be mediated by signals
produced at specific stages of growth, such as indole produced by *Escherichia coli* in stationary phase, or by signals related to population density that are utilized in a concentration-dependent manner (301-303). Quorum sensing refers to the later mechanism of bacterial cell-to-cell communication, wherein changes in population phenotypes are mediated by the accumulation of signaling mediators. These signaling mediators are produced and released by bacteria during growth and accumulate as the population density increases until a threshold stimulatory concentration is reached that initiates signal transduction (301, 304-310).

Quorum sensing signal mediators are more akin to pheromones than hormones, as they are secreted outside of the producing cell to facilitate communication between individual bacteria (311). The signaling mediators are also frequently referred to as “autoinducers” as they tend to regulate and promote their own synthesis as a downstream effect of signaling. Examples of quorum signaling networks include acyl homoserine lactones (AHL) in Gram-negative bacteria, species-specific peptide autoinducers in Gram-positive bacteria, and autoinducer-2 (AI-2) in both Gram-positive and Gram-negative bacteria (312). A particular AHL can only be detected by the species that produces it, so AHL signaling as well as the peptide autoinducers are generally considered to be intraspecies communication. AI-2, on the other hand, is a ribose-derived signaling mediator that is commonly referred to as an interspecies signal as many bacterial species produce and respond to a similar collection of molecules (301, 310). An individual species can produce multiple types of signal mediators, and the response pathways to the signals tend to be interconnected and integrated with the organism’s ability to sense environmental factors beyond cell density, such as temperature, pH,
osmolarity, oxidative stress, and nutrient availability, thus adding layers of complexity to the study of quorum signaling and regulation of gene expression (311).

The autoinducer peptide (AIP) signaling system exclusive to Gram-positive bacteria utilizes polypeptides as signaling molecules that function as autoinducers for the producing strain and inhibitors of other organisms (313). In *S. aureus*, the AIP is encoded by the *agrD* gene. Translated AgrD is targeted to the cell membrane for processing and release into the extracellular environment. The AgrD signal receptor, AgrC, has a histidine kinase domain that phosphorylates the response regulator AgrA. Phosphorylated AgrA binds direct repeats in the promoter region of a select set of genes to activate transcription (313). The inhibitory function of an AIP depends on the interaction between the AIP and the signal receptor AgrC, wherein the cyclic AIP structure is required for binding to AgrC, but the N-terminal tail of the AIP mediates activation of AgrC (313). Thus, AIPs from other species bind AgrC but do not activate signaling and can interfere with binding and signaling by the endogenous AIP (314).

AIP signaling in *S. aureus* initiates transcription of several exoproteins, including alpha-, beta-, and delta-hemolysins, enterotoxin B, serine proteases, and a toxic shock syndrome protein, while repressing transcription of cell surface proteins involved in adhesion, such as protein A and fibronectin-binding protein (311, 313). The Agr system is an important component of pathogenesis, as *S. aureus* strains lacking this system are less virulent in animal models of osteomyelitis (313, 315). AIP signaling polypeptides have been characterized in several Gram-positive species, including *Staphylococcus epidermidis*, *Staphylococcus intermedia*, *Enterococcus faecalis*, *Lactobacillus plantarum*, and *Listeria monocytogenes* (313).
The AHL signals exclusive to Gram-negative bacteria are also referred to as autoinducer 1 (AI-1) or the LuxI/LuxR system (313). In this system, LuxI is the AHL synthase and LuxR is the transcription factor that regulates gene expression in response to AHL signaling. AHLs are generated by transferring a fatty acid chain from an acylated carrier protein to S-adenosylmethionine (SAM), which is carried out by LuxI (313). The structure of the AHLs produced by each Gram-negative species depends on the acyl carrier protein used by LuxI, resulting in signaling molecules that are genus or species specific and have acyl chain lengths between C₄ and C₁₈ (312, 313). Species that produce AHLs tend to produce multiple AHLs that vary in the length of the acyl chain, saturation, and oxidation (311). Once produced, AHLs diffuse out of the bacterial cell to accumulate in the extracellular environment as the population density increases. At high population densities, the local environmental concentration of AHL is high enough to allow for diffusion back into bacterial cells, where the AHL binds to cytoplasmic LuxR (311, 313).

The AHL system was first described in *Vibrio fischeri*, a bioluminescent marine bacterium that colonizes the light organs of marine organisms such as the bobtail squid (312). Within the light organ, bacteria reach a high population density and utilize LuxR/AHL to regulate expression of the luciferase operon (*luxICDABE*) and bioluminescence (312). The LuxR/AHL complex also increases expression of *luxI*, resulting in an autoinduction positive feedback loop that floods the extracellular environment with more AHL signal to aid in rapid coordination of gene expression for the entire population in response to high cell density (312). Homologs of the LuxI/LuxR system have been identified in numerous Gram-negative species, including *Pseudomonas*.
aeruginosa, Agrobacterium tumefaciens, Pantoea stweartii, Rhodopseudomonas palustris, Acidithiobacillus ferroxidans, Yersinia pseudotuberculosis, Yersinia enterocolitica, Burkholderia pseudomallei, Burkholderia cepacia, Rhizobium leguminosarum and Escherichia coli (301, 311-313). The LuxI/LuxR quorum signaling system controls a wide range of bacterial functions in different species, including regulation of virulence determinants such as elastase and proteases, siderophore production, cell division, polysaccharide synthesis, adhesion, and motility (301, 316, 317).

In contrast to the peptide autoinducers and AHL signaling, the AI-2 signaling system is unique because it is present in many Gram-positive and Gram-negative bacteria, and the AI-2 signaling molecules are actually generated as a byproduct of bacterial metabolism. The genetic determinant of AI-2 production is luxS, which is part of the activated methyl cycle (Figure 5) (308). The generation of AI-2 starts with transfer of a methyl group from S-adenosylmethionine (SAM) to various substrates (such as DNA, RNA, and proteins), resulting in S-adenosylhomocysteine (SAH). SAH is toxic to cells and must be rapidly converted to homocysteine via the action of an SAH hydrolase, or to S-ribosylhomocysteine (SRH) by cleavage of adenine. LuxS hydrolyzes SRH to form homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). Homocysteine feeds back into the activated methyl cycle, while DPD spontaneously interconverts between cyclic furanone structures in the presence of water (312, 313, 318). This collection of structures is referred to as AI-2, although bacteria sense and respond to specific discrete structures within the collection. Vibrio harveyi and Vibrio cholerae sense and respond to (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (or S-THMF borate), which
Figure 5. Production of AI-2 and known structures (319). The activated methyl cycle (AMC) is responsible for the generation of S-adenosylmethionine (SAM) and the recycling of methionine by detoxification of S-adenosylhomocysteine (SAH). LuxS acts on S-ribosylhomocysteine (SRH) to recycle homocysteine. The AI-2 precursor 4,5-dihydroxy-2,3-pentadione (DPD) is formed as a byproduct of this reaction, and forms the molecules collectively referred to as AI-2 (319).
Activated Methyl Cycle

Al-2 Structures
is generated in the marine environment when DPD cyclizes and reacts with borate (312, 320). Other species such as *Salmonella typhimurium* and *Escherichia coli* recognize an arrangement of DPD lacking borate, \((2R, 4S)-2\text{-methyl}-2,3,4\text{-tetrahydroxytetrahydrofuran (or } R\text{-THMF})\) (312, 321).

Investigating the role of a quorum signaling system in virulence generally involves mutation of the autoinducer synthase. Homologs of *luxS* have been identified in numerous bacterial species, including *H. influenzae* (322), and inactivation of *luxS* impacts factors such as production of toxins, motility, and virulence in animal models of infection for many pathogens (311, 323-329). However, as AI-2 is produced as a byproduct of metabolism and LuxS plays an integral role in the activated methyl cycle, the results of *luxS* mutation studies must be carefully scrutinized to determine the role of AI-2 quorum signaling versus potential metabolic defects. AI-2 has been shown to act as an autoinducing quorum signal in *V. harveyi* and *V. cholerae* (312), but other studies suggest a purely metabolic role for *luxS* in many species (319, 329-337).

In addition to the role of *luxS* in metabolism and AI-2 quorum signaling, mutation of *luxS* also resulted in the discovery of another quorum signaling molecule referred to as AI-3 (338). Infection by enterohemorrhagic *E. coli* (EHEC) results in the formation of lesions on the intestinal epithelial cells, termed attaching and effacing (AE) lesions, and the genes involved in formation of AE lesions are contained on a pathogenicity island known as the locus of enterocyte effacement (LEE) (339). Expression of the LEE gene is controlled in part by quorum signaling, as supernatants taken from a *luxS* mutant that do not contain AI-2 fail to activate transcription of the LEE genes, while supernatant from the parental strain and AI-2 produced by intestinal flora activate transcription of *LEE1*.
However, the luxS mutant was still able to produce AE lesions on cultured HeLa epithelial cells, which led to the discovery that norepinephrine and epinephrine contained in the cell culture media were activating transcription of LEE1 in the luxS mutant. Furthermore, studies using Sep Pack columns revealed that bacterial supernatant fractions containing AI-2 failed to activate transcription of LEE1, while a column fraction containing catecholamine-like compounds was able to activate transcription of LEE1 (338). The enzymatic activity of LuxS does not directly produce AI-3, but mutation of luxS impacts AI-3 synthesis due to the impact of this mutation on metabolism (340). Mutation of luxS limits the ability of bacteria to convert SRH to homocysteine, which is needed for de novo synthesis of methionine and therefore production of SAM, an important methyl donor in bacteria (341). In E.coli, homocysteine can also be produced by a pathway utilizing oxaloacetate, glutamate, and the AspC and TyrB transaminases to produce aspartate, which can then be used to generate homoserine and homocysteine (Figure 6). Interestingly, the addition of aspartate to the growth medium of a luxS mutant restores AI-3 production, as does expression of an SAH hydrolase, suggesting that mutation of luxS impacts AI-3 production by altering cellular metabolism to exclusively use the oxaloacetate pathway for production of homocysteine (340). Although the structure of AI-3 has yet to be determined, the role of AI-3 signaling in pathogenesis and the potential for inter-kingdom hormone signaling between bacteria and their host are active areas of ongoing investigation (331, 338, 342-347).

Due to the pleiotropic effects of a luxS mutation, the only way to distinguish between the role of LuxS in metabolism, the effect of luxS mutation of production of AI-3, the potential use of AI-2 as a nutrient, and the role of AI-2 as an autoinducing quorum
Figure 6. Pathways for homocysteine production (340).
*luxS* mutant can only synthesize homocysteine using oxaloacetate

**Pathway 1**

- Methionine $\xrightarrow{\text{MetK}}$ S-Adenosylmethionine (SAM)
- S-Adenosylhomocysteine $\xrightarrow{\text{Pfs}}$ S-Ribosylhomocysteine
- **Homocysteine**
- AI-2 precursor DPD

**Pathway 2**

- Oxaloacetate $+\text{L-Glutamate} \xrightarrow{\text{AspC}}$ L-Aspartate
- L-Aspartate $\xrightarrow{\text{TyrB}}$ L-aspartyl-4-phosphate
- L-aspartate-semialdehyde
- Homoserine $\xrightarrow{}$ O-succinyl-L-homoserine
- Cystathionine
signaling molecule is to determine if a particular species of bacteria can sense and respond to AI-2 and to elucidate the signaling pathway. In *V. harveyi*, sensing of AI-2 occurs through a two-component system involving LuxP and LuxQ (348), where LuxP is a periplasmic protein that binds AI-2 and LuxQ is a histidine kinase that senses and propagates the signal (349). At low cell density, the local concentration of AI-2 is too low to initiate signaling and the two-component system favors kinase activity. Conserved histidine residues of LuxQ are phosphorylated by ATP, followed by consecutive transfer of the phosphate group to a conserved aspartate residue in the LuxQ C-terminus receiver domain, to the phosphotransfer protein LuxU, and finally to the LuxO response regulator (312). Phosphorylated LuxO activates transcription of the Qrr1-5 small regulatory RNAs, which destabilize the mRNA encoding LuxR, the master transcriptional regulator for quorum-sensing (350). Thus, at low cell density, no LuxR protein is produced. When population density is high enough that the local concentration of AI-2 reaches a certain threshold level, LuxQ phosphatase activity predominates ultimately resulting in dephosphorylated LuxO. Under these conditions, Qrr1-5 are not transcribed so the mRNA encoding LuxR is stabilized and protein is produced. LuxR regulates the luciferase operon and at least 50 other genes, including the *V. harveyi* type III secretion system (312, 351, 352). It is important to note that while the above describes signaling in response to AI-2, *V. harveyi* utilizes multiple quorum signals and LuxU is the only phosphotransfer protein, so integration of the quorum signals used by this species involves convergence of the pathways at phosphorylation of LuxU (312).

Notably, the LuxP/LuxQ system has only been identified in *Vibrio* species to date and a different mechanism exists for responding to AI-2 in *Salmonella typhimurium*,
*Escherichia coli*, *Sinorhizobium meliloti*, and *Aggregatibacter actinomycetemcomitans*. These organisms internalize AI-2 via the Lsr (LuxSr-regulated) system, an ABC transporter with homology to the ribose ABC transporter (Figure 7) (313, 353-355). Rather than a two-component signal cascade, the Lsr system involves binding of AI-2 by the periplasmic protein LsrB followed by transport through a heterodimeric membrane channel comprised of LsrC and LsrD driven by the ATPase LsrA (354, 356, 357). Once internalized, AI-2 is phosphorylated by LsrK. Phosphorylation of AI-2 causes the signaling molecule to be retained in the cytoplasm where it appears to interact with LsrR, a DNA-binding protein that represses transcription of *lsrABCD* in the absence of AI-2 (354, 356, 358). In addition to LsrB, the ribose binding protein RbsB of *A. actinomycetemcomitans* has also been shown to interact with AI-2 (355, 359). Binding of AI-2 by ribose-binding proteins is plausible as AI-2 is a ribose-derivative via the activated methyl cycle. Thus, ribose transport systems may also participate in the internalization of or response to AI-2.

In addition to the role of bacterial communication in regulation of virulence factor expression, the different types of quorum signaling appear to be important factors in the establishment and regulation of bacterial biofilm communities (74, 122, 360). Quorum signaling can impact numerous aspects of the biofilm mode of growth, such as regulation of adhesin expression and attachment, production of EPS, biofilm maturation, and dispersal. For the Gram-positive bacterium *Staphylococcus aureus*, *agr*-mediated quorum signaling is repressed during initial biofilm formation, but induction of the *agr*-quorum signaling system is required for biofilm dispersal (82). Biofilm development by *Pseudomonas aeruginosa* is a complex and dynamic process regulated by several
Figure 7. Diagram of the Lsr AI-2 transport system (356).
different two-component sensor systems and quorum signaling systems. AHL is required for normal biofilm development by *P. aeruginosa* (74, 360), and recent evidence suggests that signaling through two-component systems can promote biofilm dispersal by decreasing production of one of the other *P. aeruginosa* quorum signaling mediators (361).

Mutation of *luxS* has been shown to impact biofilm development for many species, including *Aggregatibacter actinomycetemcomitans*, *Actinobacillus pleuropneumoniae*, *Aeromonas hydrophila*, *Lactobacillus rhamnosus*, *Streptococcus mutans*, *Staphylococcus epidermidis*, and *Escherichia coli* (308, 326, 332, 337, 357, 362-364). For most species, LuxS and AI-2 signaling are thought to promote biofilm formation and development. For instance, mutation of *luxS* in *Aggregatibacter actinomycetemcomitans* results in biofilms with decreased total biomass and biofilm depth compared to the wild-type strain, and both genetic complementation and the addition of exogenous AI-2 restore the biofilm defect. In addition, mutation of the AI-2 binding proteins of this species similarly impact biofilm development, showing that internalization of and response to AI-2 are required for normal biofilm development (357). AI-2 signaling is also thought to promote biofilm formation for *E. coli* as inactivation of the AI-2 kinase LsrK or the transcriptional regulator LsrR result in decreased average thickness and biomass (362). Alternatively, AI-2 signaling in *Vibrio cholerae* decreases expression of factors involved in adhesion, cholera toxin production, and biofilm formation and induces expression of the Hap protease, thus promoting biofilm detachment rather than initial attachment and maturation (365).
As the AI-2 signal is produced by a wide variety of bacteria and can possibly be detected by most species, the use of AI-2 for interspecies quorum signaling may be one method for coordinating behavior within multispecies biofilms (365-367). While little is known about interspecies communication in biofilms, studies of dental plaque organisms support the interspecies signaling capacity of AI-2. For instance, a *S. mutans luxS* mutant grown in a two-compartment system can be complemented by *S. gordonii, S. sobrinus, S. anginosus, P. gingivalis,* and *A. actinomycetemcomitans* secreted products (363). However, a *P. gingivalis luxS* mutant cannot complement the *S. mutans luxS* biofilm defects, suggesting that AI-2 is responsible for restoring the wild-type phenotype (363).

Two-species biofilm studies of *A. naeslundii* and *S. oralis* showed that mutualistic biofilm growth with saliva as a nutrient source requires AI-2 production by *S. oralis* (368). Biofilms formed by wild-type *A. naeslundii* and a *S. oralis luxS* mutant were sparse and the total biomass of each species within the biofilm was approximately 10-fold lower than biofilms formed with wild-type *S. oralis*. A similar role for AI-2 signaling has been explored for *S. gordonii* and *P. gingivalis*, in which mutation of *luxS* in both species abolishes biofilm formation on polystyrene. Complementation of the mutation in *S. gordonii* restores production of AI-2 by this species, which in turn results in full restoration of dual-species biofilm development (369). AI-2 signaling may be particularly important for incorporation of *P. gingivalis* into early dental biofilms, as early biofilm formation is dominated by Gram-positive species but both Gram-positive and Gram-negative species can produce and respond to the AI-2 signal (121).

Another interesting aspect of AI-2 signaling that adds intriguing support to the potential role of this molecule in interspecies signaling is the finding that several species
of bacteria that do not produce AI-2 can internalize and respond to exogenous AI-2 provided by other species (353, 370). For instance, *P. aeruginosa* lacks a *luxS* homolog yet uses the concentration of exogenous AI-2 as a means of sensing the population density of other species within its environment (370). *Sinorhizobium meliloti*, a soil bacterium also known to lack a *luxS* homolog, can internalize exogenous AI-2 produced by other species through an Lsr transporter homologous to the system described in *S. typhimurium* and *E. coli* (353). Homologs of AI-2 transport and/or sensory mechanisms have been identified in many other bacterial species that lack *luxS*, although the majority have yet to be functionally defined (371).

In the context of OM, bacterial communication has the potential to impact virulence, biofilm formation, chronic infection, and polymicrobial interactions between the leading OM pathogens. The role of AI-2 signaling is of particular interest due to its potential role in polymicrobial interactions. *H. influenzae*, *S. pneumoniae*, *S. aureus*, and *S. pyogenes* all have *luxS* homologs and thus produce the AI-2 signal, although it has yet to be determined if AI-2 is being used specifically as a quorum signaling molecule in these species (323, 325, 372, 373). *M. catarrhalis* does not possess a *luxS* homolog but may still sense or respond to the AI-2 signal. Additionally, a recent study investigating *M. catarrhalis* and *H. influenzae* colonization of the nasopharynx found that the high frequency of co-colonization is not associated with any specific genotypic clones of either species, suggesting that a ubiquitous system, such as quorum signaling, facilitates the high frequency of co-colonization (135). Thus, interspecies signaling via AI-2 has the potential to play a prominent role in the interaction between *H. influenzae* and *M. catarrhalis* during biofilm formation and the establishment of polymicrobial OM.
Statement of Research Purpose

The research presented herein focuses on AI-2 intra- and interspecies bacterial communication during *H. influenzae* and *M. catarrhalis* infection. As *H. influenzae* produces AI-2 and *M. catarrhalis* does not, we first sought to investigate the role of AI-2 quorum signaling in *H. influenzae* biofilm formation and persistence, particularly in the context of otitis media. We next sought to determine the mechanism used by *H. influenzae* to internalize and respond to AI-2, and to elucidate the role AI-2 in biofilm formation verses dispersal. Following this work, we transitioned into polymicrobial studies to determine if *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms, and to investigate the impact of polymicrobial biofilm formation and interspecies bacterial communication on bacterial persistence, antibiotic resistance, and disease progression.
CHAPTER I:

LuxS Promotes Biofilm Maturation and Persistence of Nontypeable *Haemophilus influenzae* In Vivo via Modulation of Lipooligosaccharides on the Bacterial Surface


The following manuscript was published in *Infection and Immunity*, volume 77, issue number 9, pages 4081-4091, 2009 and is reprinted with permission. Stylistic variations are due to the requirement of the journal. Chelsie E. Armbruster performed the experiments and prepared the manuscript. Dr. W. Edward Swords acted in an advisory and editorial capacity. Copyright © American Society for Microbiology, 2009.
INTRODUCTION

*Haemophilus influenzae* is a gram-negative bacterium that frequently inhabits the nasopharynx and upper airways of children and health adults (147). Nontypeable *H. influenzae* (NTHI) strains lacking capsular polysaccharides are the strains carried most frequently and can cause opportunistic infections of the airway mucosa that include bronchitis, sinusitis, and otitis media (OM) (146). OM is among the most common pediatric infections and affects the majority of all children by the age of 3 years (36). NTHI is one of the leading causes of OM (38, 374), including chronic and recurrent OM infections that are generally not resolved by antibiotic treatment or host immunity (50). The chronic or recurrent presentations of OM have long been thought of as involving bacterial persistence within a biofilm (67). Biofilms have been directly observed both in the chinchilla experimental model for OM (106-109, 226) and in middle-ear tissues from patients with OM (105).

Most factors associated with NTHI disease promote evasion of host clearance and persistence in vivo (146). These factors include specific subtypes of the lipooligosaccharides (LOS) on the bacterial surface (375, 376), many of which contain structures found on host cells and are thought to mask the organism from host immune defenses (377). Previous work by our group and other groups has elucidated that role of the LOS modifications in NTHI persistence during biofilm growth (107-109, 220). Specifically, LOS forms decorated with sialic acid and phosphorylcholine predominate in vivo (200, 221, 222, 225) and are known to promote biofilm formation and/or maturation and persistence in vivo (107-109, 220).
Quorum sensing refers to density-dependent changes in the phenotype(s) of bacterial populations that occur due to accumulation of a soluble signaling mediator (301). For example, various homoserine lactone derivatives mediate quorum signaling for some gram-negative bacteria, whereas gram-positive species typically produce peptide signaling molecules (310). Autoinducer 2 (AI-2) is a furanosyl borate diester molecule that was first identified as a quorum signal molecule in *Vibrio harveyi* (378). AI-2 is produced by many different bacterial species and thus has been referred to as an interspecies signaling system (310). The genetic determinant of AI-2 production is *luxS*, which is part of the methionine-homocysteine cycle (308). Homologs of *luxS* have been identified in numerous bacterial species, including *H. influenzae* (322), and have been implicated in biofilm formation, maturation, or dispersal in several different species (308). For NTHI, *luxS* mutants are known to have increased virulence in the chinchilla infection model for OM (323). In this study, an isogenic *luxS* mutant was generated using the well-characterized NTHI 86-028NP strain background, and the effect of AI-2 on NTHI biofilm formation, maturation, and persistence in vivo was assessed.
MATERIALS AND METHODS

Bacterial strains and culture conditions. A complete list of bacterial strains and plasmids (with descriptions and references) used in this study is provided in Table 3. All NTHI strains were cultivated in brain heart infusion (BHI) medium (Difco) supplemented with hemin (ICN Biochemicals) and NAD (Sigma); this medium is referred to below as supplemented BHI (sBHI) medium.

Generation of NTHI 86-028NP luxS. A ~2.4-kb DNA fragment containing the luxS open reading frame (NTHI 0621) was amplified from NTHI 2019 genomic DNA using primers LuxS Forward and LuxS Reverse (Table 4), and cloned into pCRBlunt (InVitrogen) according to the manufacturer’s instructions to generate pCR-luxS. This amplicon was excised using EcoRI and cloned into EcoRI-digested pUC19 to generate pUCluxS. Plasmid pUCluxS was then digested with BsrGI, a unique site within luxS, and a kanamycin-resistance cassette excised from pUC4K (379, 380) was ligated to the blunted ends using T4 DNA ligase. The resulting construct was transformed into chemically-competent E. coli to yield plasmid pUCluxS::Kn. The expected sequence of pUCluxS::Kn was confirmed by sequence analysis, then the construct was linearized by digestion with SfoI and introduced into NTHI 86-028NP via natural transformation as described previously (107, 108). Transformants were plated onto sBHI agar containing 2% Fildes’ reagent (Difco) and 30 µg/ml ribostamycin (Sigma), and incubated for 48 h at 37°C. Transformants carrying the expected construct were verified by PCR using LuxS verification primers (Table 4), and by loss of detectable AI-2 production as compared with the parental strain in the Vibrio harveyi bioassay (see Fig. S1 in the appendix).
Table 3. Bacterial strains and plasmids.
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<th>Strain or Plasmid</th>
<th>Description</th>
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Table 4. Primers used in this study.
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<td>5'-FAM-CTCATGGACAAAGCCATTG-3'</td>
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<tr>
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<td>5'-CCGGAAAAACATCAATCTTTAGG-3'</td>
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<tr>
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<td>5'-GCTAAAAATACTTACGCTAAATAAG-3'</td>
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<tr>
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<td>FamOafA F</td>
<td>5'-FAM-TACCGGAATATATGCAATATCATATT-3'</td>
</tr>
<tr>
<td>OafA R</td>
<td>5'-TCTCTCTCAACCTAAAGTTTCT-3'</td>
</tr>
<tr>
<td>1769 F</td>
<td>5'-GTGTGCTGAGATGTTTATAATC-3'</td>
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<tr>
<td>Fam1769 R</td>
<td>5'-FAM-CCAAAGATAATTTCTGACCAG-3'</td>
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<tr>
<td>FamLav F</td>
<td>5'-FAM-CAGTTTTATACGTTACACCG-3'</td>
</tr>
<tr>
<td>Lav R</td>
<td>5'-GTGATATCAACACAGCAAAGG-3'</td>
</tr>
<tr>
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</tr>
<tr>
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<td>5'-GAATGGTCAAAACCCCAG-3'</td>
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</tr>
<tr>
<td>Hmw2A R</td>
<td>5'-ACAACAATTACACAGCTTCTTG-3'</td>
</tr>
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</table>

*aFAM, 6-carboxyfluorescein
NTHI 86-028NP and NTHI 86-028NP luxS constitutively expressing the green fluorescence protein (gfp) were generated essentially as described previously for strain 2019 (108).

**Generation of NTHI 86-028NP luxS IRA::luxS.** Plasmid pUCluxS was digested with SfoI and HincII. The 1600 bp fragment containing luxS was gel purified and ligated into pIRA::Cm which had previously been digested with SfoI and dephosphorylated. This plasmid is a vector containing a 1.6-kb intergenic, noncoding region between nucleotides 794506 and 796038 of the *H. influenzae* Rd genome and a chloramphenicol resistance cassette (382). The ligation product was transformed into chemically-competent *E. coli* to yield plasmid pIRA::luxS. pIRA::luxS was confirmed by restriction digest with BsrGI and EcoRI as well as PCR with 2LuxS verification primers (Table 4), then linearized by digestion with NotI and introduced into NTHI 86-028NP luxS via natural transformation. Transformants were plated onto sBHI agar containing 2% Fildes’ reagent (Difco) and 1.5 µg/ml chloramphenicol (Sigma) and incubated as above. Transformants carrying the expected construct were verified by PCR with the 2LuxS verification primers to contain both wild type luxS and luxS::Kn, and AI-2 production was confirmed as above.

**Generation of NTHI 86-028NP licON.** NTHI 86-028NP licON is a mutant harboring an in-frame deletion of the CAAT repeat region in licA, and was generated as previously described for 2019 licON (108).

**Assessment of phase-variable NTHI genes.** Determination of on/off state of phase-variable genes was performed as previously described (207). Individual colonies of each
NTHI strain were harvested from plate cultures suspended in 0.1 ml of 10% suspension of Chelex (BioRad). Genomic DNA was prepared by vortexing suspensions for 15 sec, boiling for 10 min, chilling on ice for 2 min, and centrifugation at 8000 x g for 2 min. Supernatants (0.05 ml) were transferred to fresh tubes for PCR amplification using primers directed at sequences flanking repeat regions within phase-variable genes (Table 2). Unmodified primers were purchased from Operon Biotechnologies (Huntsville, AL). Primers tagged with 6-carboxyfluorescein were purchased from Integrated DNA Technologies (Coralville, IA). PCR was performed in a final volume of 0.025 ml containing GoTaq Flexi Buffer, 0.005 ml of template, 5 pmol each of F/R primer, 2 mM MgCl$_2$, 10 mM deoxynucleotide triphosphates, 1.5 U GoTaq DNA Polymerase (Promega). Amplification procedures were as follows: 94˚C for 2 min; 30 cycles of 94˚C for 1 min, 54.9˚C for 1 min, and 68˚C for 1 min, followed by a final extension for 7 min at 68˚C. Amplicons were submitted to the DNA Sequencing Facility of the WFUHS Biomolecular Resource Laboratory for sizing by means of GeneMapper (Applied Biosystems, Inc., Foster City, CA) analysis; amplicons were diluted 1/1500 to 1/3000, depending on the concentration of representative samples on agarose gels. The number of repeats for each region was estimated based on fragment size, and used in accordance with the previously defined genomic sequence of each region to define “on” or “off” status of each phase-variable region.

**Continuous flow system.** In vitro biofilm cultures were performed using a continuous-flow system as previously described (108). NTHI 86-028NP and NTHI 86-028NP luxS expressing gfp were cultured overnight in sBHI broth and diluted to OD$_{600}$ of 0.15 (~10$^8$...
CFU/ml) in sBHI broth, and 10 ml of this suspension was then injected into the port of a commercial microscopy flow-cell (Stovall) and incubated for 3 h at 37°C without medium flow to permit bacterial surface attachment. Continuous flow of sBHI media was then initiated at a rate of ~60 ml per hour and maintained for 72 h with interruptions at 24 h and 48 h for imaging by confocal laser scanning microscopy (CLSM). Z-series images of biofilms in the in vitro flow chambers were collected using a Zeiss LSM510 CLSM microscope at 24, 48, and 72 hours post-inoculation. Eight image stacks, each representing a different field of view, were compiled for each strain at each timepoint. These images were obtained at consistent positions (~5 mm) relative to the inlet. The Z-slice images were exported into MATLAB (version 5.1) and COMSTAT analysis was performed using the Image Processing Toolbox as previously described (383).

**Scanning electron microscopy.** In vitro biofilm samples were fixed within the flow chamber for 60 min with 2.5% glutaraldehyde in PBS and then rinsed twice. Samples were then dehydrated, fixed, and prepared for SEM analysis as previously described (220). Biofilm samples were mounted onto stubs and sputter coated with palladium, then viewed with a Philips SEM-515 scanning electron microscope.

**Dual-well complementation assay.** A two-compartment system was established essentially as described previously (363). Lab-TekII 2-chamber #1.5 German coverglass slides (Nunc) were sterilely coated with 0.700 ml poly-L-lysine (Sigma) for 15 min. Overnight plate cultures of NTHI 86-028NP gfp and NTHI 86-028NP luxS gfp were suspended in sBHI and diluted to an OD₆₀₀ of 0.150. Each chamber slide was inoculated
with 1.5 ml of the strain whose biofilm structure parameters were being tested. A 10 mm tissue culture insert with a 0.2 µm Anapore membrane (Nunc) was then aseptically transferred into the chamber and inoculated with and 0.5 ml of NTHI 86-028NP gfp or NTHI 86-028NP luxS gfp. Cultures were incubated for 12 h at 37˚C and 5% CO₂ under stationary conditions. Tissue culture inserts and remaining liquid were carefully removed, and Z-series images of biofilms on the surface of the chamber slide were collected using a Nikon Eclipse C1 CLSM microscope. Ten image stacks, each representing a different field of view, were compiled for each culture condition. The Z-slice images were exported into MATLAB (version 5.1) for COMSTAT analysis.

**Colony immunoblots.** PCho expression was assessed essentially as described previously (174, 384). NTHI colonies from plates with 10-500 colonies were lifted onto nitrocellulose membranes and air-dried, then washed (2 washes, 10 min/wash) in TSBB buffer (10 mM Tris, 0.5 M NaCl, 0.5% Tween, pH = 8.0). Membranes were incubated in anti-phosphorylcholine monoclonal antibody HAS (Statens Serum Institut) in TSBB overnight, washed in fresh TSBB (5 washes, 10 min/wash), and incubated overnight in goat anti-mouse IgM alkaline phosphatase conjugate in TSBB, washed in TSBB, and incubated for 10 min in alkaline phosphatase (AP) buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5). PCho⁺ colonies were visualized using 5-bromo-4-chloro-3-indolyl phosphate (Sigma, 0.16 mg/ml) and nitroblue tetrazolium (Sigma, 0.33 mg/ml) in AP buffer. Colonies were classified as having high or low reactivity with the antibody based on intensity of staining.
**Whole-bacteria ELISA.** ELISAs were performed on intact bacterial cells as described previously (382). NTHI strains were harvested from overnight plate cultures into sterile PBS, pelleted, and washed twice in PBS. Bacteria were then diluted in distilled H₂O to an OD₆₀₀ of ~0.15, and 0.1 ml was then dispensed into each well of a MaxiSorp-coated 96-well microtiter plate (Nunc) and dried uncovered at 37°C for 24 h or until all liquid had evaporated. For detection of PCho, dried plates were washed once and blocked for 30 min in TSBB. Wells were incubated with 0.1 ml mouse IgM HAS antibody diluted 1:2500 in TSBB overnight at room temperature, washed once, and incubated for 1 h in the dark with goat-anti-mouse IgM-horseradish peroxidase (HRP) conjugate diluted 1:2500 in TSBB. Wells were then washed once, and incubated in the dark for approximately 20 min with 0.1 ml of TMB substrate reagent (OptEIA, Becton-Dickinson). The reaction was stopped with 0.05 ml of 2 N H₂SO₄, and OD₄₅₀ measured using a plate reader (Labsystems Multiskan Plus). For lectin reactivity, wells containing dried bacteria were washed and blocked as above, then treated with either 0.1 ml neuraminidase buffer alone (0.15 M NaCl, 4 mM CaCl₂, pH 5.5) or 80 mU/well of *Clostridium perfringens* neuraminidase (Sigma) overnight at 37°C in a humidified chamber. Wells were washed and blocked with 1 x TSBB for 30 min and incubated for 4 h in the dark with 0.1 ml HRP-conjugated *Limax flavus* (LFA) diluted 1:750 in LFA buffer + Tween (0.05 M Tris, 0.3 M NaCl, 0.5% Tween, pH = 7.5). Wells were then washed in LFA buffer without added Tween and developed as above.

**Lipooligosaccharide purification.** LOS was isolated from NTHI strains using a modified proteinase K procedure (382). Briefly, NTHI cells were harvested from
overnight plate cultures, diluted in sterile PBS, pelleted, and lysed in 2.0% sodium dodecyl sulfate, 10 mM EDTA, 0.06 M Tris (pH 6.8). Lysates were boiled for 5 m and treated overnight with proteinase K (Sigma). LOS was then precipitated with sodium acetate-ethanol, centrifuged, washed with 70% ethanol, and resuspended in sterile distilled H₂O.

**Western immunoblotting.** Purified LOS samples were analyzed by SDS-PAGE (385, 386). Following overnight transfer to a nitrocellulose membrane, quantities of LOS remaining on the gel were compared by silver stain (387). Membranes were washed twice in 1x TSBB (5x TSBB, pH 8.0: 50 mM Tris, 2.5 M NaCl, 2.5% Tween). Membranes were then incubated overnight at room temperature with mouse anti-\(P\)Cho IgM Has antibody (Statens Serum Institut). Membranes were washed 5 times in TSBB and incubated for 2 h with goat-anti-mouse IgM-HRP (Sigma), washed 1 time in TSBB, washed twice with distilled H₂O, and washed 5 times with PBS prior to developing with Pierce western blotting substrate.

**Chinchilla infection studies.** Bacterial persistence and biofilm formation in the middle ear chamber were assessed as described previously (107, 108). Chinchillas (500-800 g body weight) were purchased from Rauscher's chinchilla ranch (Larue, OH) and allowed to acclimate to the vivarium for >7 d prior to infection. No animals showed visible signs of illness by otoscopy prior to infection. The animals were anesthetized with isofluorane and infected via transbullar injection with \(~10^3\) CFU of NTHI 86-028NP or NTHI 86-028NP \(luxS\); all inocula were confirmed by plate-counting. The degree of inflammation
was assessed at 48 h intervals by otoscopic examination using a qualitative scoring system of 1 to 4, with 4 being the most severe (107). Criteria assessed included erythema, tympanic membrane discoloration and opacity, vessel dilation, and fluid accumulation. At 7 d, 14 d, and 21 d post-infection animals (3/group) were euthanized and middle-ear chambers were aseptically opened. Fluids within the middle ear were recovered and middle ear lavage was performed using 1.0 ml sterile PBS. Viable numbers of bacteria within middle ear fluid were determined by plate counting the combined retrieved fluids. Right bullae were excised and homogenized in 10 ml sterile PBS, then plated to determine CFU of tissue-associated bacteria (107).

**Microscopic analysis of infected tissues.** At each timepoint, the left bullae were excised and preserved in chilled 4% paraformaldehyde for 24 h then transferred to 70% ethanol. Following fixation, bullae were submerged in Decalcifier II solution (Surgipath) for approximately 6 h, trimmed and embedded in paraffin, and sectioned (4-6 μm). Individual sections were mounted onto microscope slides, treated with xylene to remove paraffin, and stained with hematoxylin and eosin according to standard methods that we have described previously (227, 388). The stained sections were distributed into blinded sets and examined by light microscopy by a trained pathologist (N.K.).

**Statistics.** Significance determined by nonparametric *t* tests and two-way ANOVA with post-hoc tests of significance. Analyses were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA).
RESULTS

Construction and confirmation of NTHI 86-028NP luxS. A null allele of luxS was constructed in which a kanamycin resistance cassette was cloned in the coding sequence (see Materials and Methods). This allele was introduced into NTHI 86-028NP by natural transformation and confirmed by PCR analysis (data not shown) and the V. harveyi bioluminescence assay (see Fig. S1 in the appendix). As expected, culture supernatants from NTHI 86-028NP luxS elicited approximately sevenfold less luminescence from the AI-2 sensor strain BB170 than supernatants from the parental NTHI 86-028NP strain. The luxS mutation was complemented genetically by insertion of the wild-type luxS allele into the IRA site of NTHI 86-028NP luxS (see Materials and Methods). Culture supernatants from NTHI 86-028NP luxS IRA::luxS elicited approximately nine fold-greater luminescence from the AI-2 sensor strain than culture supernatants from NTHI 86-028NP luxS, indicating that there was successful restoration of AI-2 production. No differences in growth rates in sBHI broth were observed (data not shown).

Assessment of phase-variable loci. NTHI populations undergo phase variation via slipped-strand mispairing within repeat regions in a number of open reading frames, including genes involved in the biosynthesis and assembly of LOS components and surface proteins (389). The tetrameric repeat regions of nine phase-variable genes of NTHI 86-028NP and NTHI 86-028NP luxS were amplified using PCR and analyzed by the GeneMapper procedure, which allows single-nucleotide resolution of DNA fragments and thus can be used to assess the number of repeats within a region and estimate the
“on” or “off” status (207, 390). The only notable difference in the phase status of NTHI 86-028NP and NTHI 86-028NP luxS was a difference in oafA, which encodes an acetyltransferase that can impact bacterial resistance to serum killing by modification of the LOS core region (199). The majority of NTHI 86-028NP colonies had the “off” configuration, whereas all of the NTHI 86-028NP luxS colonies tested were phase “on” (Table 5). All seven other LOS biosynthetic genes were found to be in the “on” configuration for the majority of colonies tested for both strains. The status of lav was determined to be “off” for the majority of colonies tested for both strains.

In addition to the phase-variable genes containing internal tetrameric repeats, the heptameric repeat regions in the promoter for the open reading frames encoding the bacterial adhesins Hmw1A and Hmw2A were also assessed. For these genes, the number of repeats correlates with the level of protein expression rather than phase status (190). All colonies tested contained comparable numbers of repeats, and in all cases the total number of repeats was within a range which indicates optimal adhesion expression (190).

**Comparison of biofilm structure and density.** NTHI 86-028NP and NTHI 86-028NP luxS constitutively expressing gfp were used to ask how luxS impacts biofilm structure and density (Fig. 8). NTHI 86-028NP luxS produced a visible biofilm in a continuous-flow chamber and remained attached at all time points up to 72 hours post-inoculation. SEM performed on mature biofilms formed by both NTHI 86-028NP and NTHI 86-028NP luxS revealed no qualitative differences (Fig. 8A to D), similar to previous results for different *H. influenzae* isolates (323).
Table 5. Analysis of tetrameric repeat regions.
<table>
<thead>
<tr>
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<th>NTHI 86-028NP</th>
<th>NTHI 86-028NP luxS</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Number of colonies</td>
</tr>
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</tr>
<tr>
<td></td>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
</tbody>
</table>

*NTHI 1769 encodes a hypothetical glycosyltransferase.
Figure 8. Microscopic analysis of in vitro biofilm formation in flow chambers. NTHI strains expressing gfp were cultured in flow cells under continuous-media-flow conditions for 72 h. (A to D) SEM of biofilms formed by NTHI 86-028NP and NTHI 86-028NP luxS at magnifications of x5,000 (A and B) and x10,000 (C and D). (E) CLSM vertical z-series images of biofilms formed by NTHI 86-028NP and NTHI 86-028NP luxS at 24 h, 48 h, and 72 h. (F to I) Biofilm measurements for NTHI 86-028NP (open bars) and NTHI 86-028NP luxS (gray bars) obtained using COMSTAT, including measurements of total biomass (F), average thickness (G), roughness (H), and surface/volume ratio (I). Significance was determined by a two-way ANOVA with a post hoc test of significance (**P ≤ 0.01, *P ≤ 0.05). The error bars indicate standard errors of the means.
However, CLSM analyses of biofilms formed by NTHI 86-0-28NP and NTHI 86-028NP luxS in continuous-flow systems revealed significant structural differences, including reductions in biofilm thickness and overall density for NTHI 86-028NP luxS at 24 h and 48 h post-inoculation (Fig. 8E). To quantify the differences between NTHI 86-028NP and NTHI 86-028NP luxS biofilms, the CLSM images were processed using the COMSTAT software package, as we have done previously for other NTHI mutants (108). Biofilms were analyzed to determine the total biomass (Fig. 8F), average thickness (Fig. 8G), roughness (Fig. 8H), and surface/volume ratio (Fig. 8I). The values for these parameters were significantly less for biofilms formed by NTHI 86-028NP luxS than for biofilms formed by NTHI 86-028NP at both the 24-h and 48-h time points. By 72 h, all of the parameters were comparable for the two strains. Based on these results, we concluded that luxS significantly promotes maturation of NTHI 86-028NP biofilms, as manifested by increased thickness and density for the parental strain.

As the LuxS protein is an integral part of the methionine-homocysteine cycle, it is imperative to determine if the observed difference in biomass was caused by lack of soluble quorum-sensing molecules, such as AI-2, or by a metabolic defect that was strictly due to mutation of luxS. To distinguish between these possibilities, a dual-well assay was used to complement NTHI 86-028NP luxS with soluble products from wild-type strain NTHI 86-028NP (Fig. 9). The biofilms formed by NTHI 86-028NP luxS alone in this system again had decreased biomass (Fig. 9B) and average thickness (Fig. 9C) compared to those formed by the wild-type strain. Importantly, soluble products from NTHI 86-028NP fully restored the ability of NTHI 86-028NP luxS to form biofilms whose biomasses and average thicknesses were equal to those of biofilms formed by
Figure 9. Complementation of luxS with exogenous AI-2. NTHI strains expressing gfp were separated by 0.2 µm Anapore membranes and cultured in two-well chamber slides under stationary conditions for 12 h. Biofilm measurements of total biomass (A) and average thickness (B) were obtained from CLSM vertical z-series images using COMSTAT. Significance was determined by a nonparametric t test (***P≤0.001, *P≤0.05). The error bars indicate standard errors of the means.
We therefore concluded that the decrease in the density of the NTHI 86-028NP luxS biofilms was directly due to the soluble signaling products of luxS and not to a metabolic effect.

LOS composition. The decreased biomass observed for an NTHI 86-028NP luxS biofilm was similar to previously observed phenotypes for mutations affecting LOS composition. Specifically, our previous work has shown that sialylation promotes biofilm formation (220), whereas PCho affects later stages of biofilm maturation (107, 108). Therefore, the surface accessible sialic acid and PCho contents of NTHI 86-028NP and NTHI 86-028NP luxS LOS populations were compared (Fig. 10). Whole-bacteria ELISAs were performed using L. flavus lectin to detect sialic acid and using anti-PCho monoclonal antibody to detect phosphorylcholine. Strains NTHI 86-028NP, NTHI 86-028NP luxS, NTHI 86-028NP luxS IRA::luxS, NTHI 86-028NP licD, and NTHI 86-028NP licON were first analyzed to determine the surface sialic acid content (Fig. 10A). As a control for background reactivity, the results were compared to the reactivity of samples treated with neuraminidase to remove sialic acid residues (see Materials and Methods). The resulting data show that the luxS mutant had accessible levels of sialic acid similar to those of NTHI 86-028NP. These data are consistent with the in vitro biofilm data, as a reduction in the surface sialic acid content of LOS would most likely have resulted in a more pronounced qualitative difference in biofilm appearance as determined by SEM (220).

The surface-accessible PCho of the same five strains was also analyzed (Fig. 10B). In five independent experiments, NTHI 86-028NP luxS had significantly lower
Figure 10. Analysis of LOS composition. Modified whole-bacterium ELISA using treatment with 80 mU neuraminidase as a negative control for reactivity with *L. flavus* lectin for detection of surface sialic acid (A) (n=2) and ELISA using anti-\textit{P}Cho monoclonal for detection of surface phosphorylcholine (B) (n=5). Significance was determined by a nonparametric \( t \) test (***\( P \leq 0.001 \), **\( P \leq 0.01 \)). The error bars represent standard errors of the means. (C) Western blot of purified LOS for detection of total \( \textit{P}Cho \) from NTHI 86-028NP (lane 1), NTHI 86-028NP \textit{luxS IRA:luxS} (lane 2), NTHI 86-028NP \textit{luxS} (lane 3), NTHI 86-028NP \textit{licD} (lane 4), and NTHI 86-028NP \textit{lic}\textsuperscript{ON} (lane 5).
A

B

C

91
$P$Cho levels than NTHI 86-028NP, yet it had higher levels than NTHI 86-028NP $licD$, which was used as a negative control. Importantly, genetic complementation of the $luxS$ mutation was able to partially restore the decrease in surface $P$Cho levels. Based on these data, we concluded that NTHI 86-028NP $luxS$ has a lower level of surface-exposed $P$Cho than NTHI 86-028NP.

In order to determine if the decrease in surface-accessible $P$Cho was due to an overall decrease in $P$Cho expression, LOS was purified from the NTHI strains described above and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (Fig. 10C). The lanes were loaded with similar amounts of LOS, as determined by silver staining (data not shown). As shown in Fig. 10C, lane 3, the purified LOS of NTHI 86-028NP $luxS$ contained only minimal levels of $P$Cho, indicating that the observed decrease in the level of surface-accessible LOS was due to an overall decrease in $P$Cho expression rather than to masking of the moieties by other components of the LOS. Importantly, the purified LOS of NTHI 86-028NP $luxS$ IRA::luxS had an increased level of $P$Cho compared to the purified LOS of NTHI 86-028NP $luxS$. These data indicate that mutation of $luxS$ in NTHI alters the total $P$Cho content of LOS.

The reactivities of individual NTHI colonies with anti-$P$Cho monoclonal antibody were also assessed by colony immunoblotting to determine the proportion of colonies with reduced levels of surface $P$Cho (Table 6). The vast majority of the colonies of wild-type strain NTHI 86-028NP consistently showed high reactivity for $P$Cho, while colonies of NTHI 86-028NP $luxS$ generally showed low reactivity. Genetic complementation of the $luxS$ mutation resulted in an increase in the percentage of colonies which reacted with anti-$P$Cho monoclonal antibody. NTHI 86-028NP $lic^{ON}$ was used as a positive control.
Table 6. Surface expression of PCho as assessed by colony immunoblotting.
<table>
<thead>
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<th>Strain</th>
<th>Colony reactivity</th>
<th>Reactive colonies/total</th>
<th>Percent Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTHI 86-028NP</td>
<td></td>
<td>998/1004</td>
<td>99.4</td>
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<tr>
<td>NTHI 86-028NP luxS</td>
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<td>84/1314</td>
<td>6.4</td>
</tr>
<tr>
<td>NTHI 86-028NP luxS IRA::luxS</td>
<td></td>
<td>19/70</td>
<td>27.1</td>
</tr>
<tr>
<td>NTHI 86-028NP licD</td>
<td></td>
<td>0/46</td>
<td>0.0</td>
</tr>
<tr>
<td>NTHI 86-028NP lic&lt;sup&gt;ON&lt;/sup&gt;</td>
<td></td>
<td>24/24</td>
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</tbody>
</table>
for highly reactive colonies, while NTHI 86-028NP licD was used as a negative control. These data confirm that mutation of luxS results in colonies that have an overall decrease in PCho moieties.

**Persistence and biofilm formation in the chinchilla model for OM.** To investigate the role of LuxS in establishment of OM, chinchillas were inoculated with $\sim 10^3$ CFU/ear of NTHI 86-028NP or NTHI 86-028NP luxS, and the bacterial counts and the presence of a biofilm within the middle ear were assessed at 7, 14, and 21 days post-infection (Fig. 11). At day 7, four of six ears infected with NTHI 86-028NP and three of six ears infected with NTHI 86-028NP luxS contained biofilms (Fig. 11B and E). By 14 days post-infection, three of six ears infected with NTHI 86-028NP and two of six ears infected with NTHI 86-028NP luxS contained biofilms, indicating that NTHI 86-028NP luxS was not deficient in the ability to form biofilms in vivo and that it could persist until 14 days post-infection in these structures (Fig. 11C and F). Interestingly, the biofilms formed by NTHI 86-028NP luxS in the chinchilla middle ear appeared to be more diffuse than those formed by NTHI 86-028NP. This was particularly evident at the 14-day time point (Fig. 11F), and may reflect the structural differences observed in the continuous-flow system and dual-well assay.

Importantly, by 21 days post-infection only one of six ears infected with NTHI 86-028NP luxS contained a biofilm, while all of the ears infected with NTHI 86-028NP contained biofilms. It is also interesting that the one remaining NTHI 86-028NP luxS biofilm (Fig. 11H) was much smaller and more diffuse than the NTHI 86-028NP biofilms at this time point (Fig. 11D), possibly indicating that the infection in this ear was
Figure 11. LuxS promotes biofilm formation and persistence in the chinchilla model of OM. (A) Middle ear of an uninfected control animal at 21 days post-infection. (B to H) Biofilms in the middle ear visualized at 7 days (B and E), 14 days (C and F), and 21 days (D, G, and H) post-infection. Panels B, C, and D show biofilms from animals infected with NTHI 86-028NP, and panels E, F, G, and H show biofilms from animals infected with NTHI 86-028NP luxS. Panel G is representative of five of six ears infected with NTHI 86-028NP luxS at 21 days post-infection, while panel H shows the only ear containing biofilm at this time point. (I and J) Bacterial counts for middle-ear effusion and homogenized bullae after infection with NTHI 86-028NP (filled symbols) or NTHI 86-028NP luxS (open symbols). The limit of detection was 30 CFU/ear. Statistical significance was determined by two-way ANOVA with a post-hoc test of significance. The horizontal bars indicate the median values.
resolving. The remaining ears infected with NTHI 86-028NP luxS appeared to have cleared the infection at day 21, with no biofilm and little or no effusion (Fig. 11G).

Counts of viable bacteria in middle-ear effusions (Fig. 11I) and bullar homogenates (Fig. 11J) were also determined at 7, 14, and 21 days post-infection. Comparable numbers of planktonic (effusion) and surface-adherent (homogenate) populations were observed at 7 and 14 days. The data indicated that NTHI 86-028NP luxS had a significant persistence defect as compared to NTHI 86-028NP at 21 days, as shown by the finding that only two of six ears infected with the luxS mutant had viable effusion counts above the limit of detection. This persistence defect was not as pronounced in the surface-adherent population, but a decrease in viable counts was still observed.

**Inflammatory response to NTHI 86-028NP luxS.** Clinical assessments of infected animals were performed at 48-h intervals throughout the course of the infection studies (see Materials and Methods). On average, animals infected with NTHI 86-028NP luxS tended to have higher otoscopy scores than animals infected with NTHI 86-028NP during the early periods post-infection, which was followed by a steady decrease in scores at later time points (data not shown).

To obtain an estimation of the inflammatory response in the middle ear, the left bullus from each animal was excised and processed for hematoxylin and eosin staining at all timepoints (Fig. 12). Bullar sections from control animals showed no signs of middle-ear inflammation, only smooth bone with very few neutrophils and little evidence of tissue damage (Fig. 12A). Bullae from most infected animals had extensive
Figure 12. Hematoxylin and eosin staining of bullar sections. (A) PBS control (magnification, x20) showing smooth bone surface with few neutrophils and no significant evidence of tissue injury. (B) Focal osteomyelitis (arrow) 14 days after inoculation with NTHI 86-028NP. The marrow space has been effaced by intense inflammation, as indicated by comparison to the normal bone marrow on the left. Magnification, x2. (Inset) Higher magnification (x40) of the exudate. (C to E) Stained sections from animals at 7, 14, and 21 days after infection with NTHI 86-028NP. Magnification, x20. (F to H) Sections from animals at 7, 14 and 21 days after infection with NTHI 86-028NP luxS. Magnification, x20. The arrows indicate periosteal hyperplasia.
fibrinosuppurative inflammation of the middle ear characterized by abundant infiltration of neutrophils (many degenerative), epithelial necrosis, fibrin, and in some cases hemorrhage. One animal inoculated with NTHI 86-028NP developed osteomyelitis (Fig. 12B).

Images of bullae from animals infected with NTHI 86-028NP at day 7 post-infection reflected more acute inflammation along with the presence of protein-rich edema fluid, fibrin, neutrophils, and hemorrhaging (Fig. 12C). At later time points more prolonged injury was shown by reactive hyperplasia of the periosteum with the production of new bone (Fig. 12D and E). At 21 days, exudates were rich in degenerate neutrophils and partially filled the middle-ear chamber. The inflammation observed in bullar sections from animals infected with NTHI 86-028NP luxS similarly reflected the time course of infection, much like that observed for NTHI 86-028NP (Fig. 12F to H). However, due to the great variation in scores for each group no quantitative differences in the magnitude of inflammation could be identified.
DISCUSSION

Biofilms are surface-attached microbial communities that promote resistance to environmental clearance. In the context of infectious disease, biofilms are important determinants of bacterial persistence in long-term infections, such as chronic and recurrent OM. The importance of quorum signaling in the formation and maturation of biofilms has long been appreciated (308). For some species, quorum signaling promotes biofilm development and maturation (360), whereas in other species the converse is true and quorum signals mediate biofilm dispersal (82). In this study, we used a genetic approach to ask how mutation of luxS impacts the biofilm formation by and persistence of NTHI. The results clearly show that in luxS mutants there is significantly decreased production of PCho+ LOS glycoforms. NTHI 86-028NP luxS produces biofilms whose density and thickness are significantly decreased and has a corresponding persistence defect in experimental otitis media.

The results of the phase status analyses clearly exclude the possibility of an unlinked phase variation event contributing to or conferring the observed changes in biofilm and persistence. In particular, it is notable that the phase status of the lic1 operon, which determines addition of PCho to a specific subpopulation of LOS forms (384), is comparable between NTHI 86-028NP and NTHI 86-028NP luxS. Other potential reasons for changes in PCho on the bacterial surface and within total LOS could be related to changes in the oligosaccharide acceptor populations, which have been associated with other regulatory systems. Nonetheless, the current data are consistent
with luxS-mediated control of phosphorylcholine-positive LOS forms on the bacterial surface conferring a biofilm-related persistence phenotype in NTHI populations.

Mutation of luxS has the potential to have metabolic effects in addition to causing potential changes in quorum signaling (373), and in some situations these effects rather than production and subsequent sensing of AI-2 are thought to be responsible for changes in biofilm and/or virulence properties (319, 330-336). We have clearly shown by use of a dual-well assay that soluble products from wild-type strain NTHI 86-028NP fully complement the biofilm density defects of NTHI 86-028NP luxS, thus demonstrating that in NTHI the role of luxS in biofilm development is most likely a role in production of soluble quorum-signaling molecules rather than a role in metabolism. Additionally, our data do not support the conclusion that mutation of luxS impairs growth of NTHI 86-028NP, as no growth defect was noted in vitro and the persistence defect observed in the chinchilla infection studies was apparent only at the later stages of infection. These results are more consistent with a defect in biofilm formation or maturation than with a strictly metabolic deficit.

In some circumstances production of an as-yet-unidentified autoinducer-3 (AI-3) may also be impacted by mutation of luxS, thus conferring changes in quorum signaling by a separate pathway (331). On this note, the genomic sequence of NTHI 86-028NP has homologs of the genes encoding the QseB/QseC two-component regulators that are the apparent sensors for AI-3 (209, 391). The data presented here support the conclusion that luxS activity, either via direct AI-2 quorum signaling or via an independent (AI-3?) quorum signal pathway, can affect the shift in LOS glycoform populations that occurs in NTHI biofilm communities. Because the sensory network for AI-3 is also affected by
host hormone levels (331), it is also possible that host factors produced during the course of disease could impact quorum-signaling networks during NTHI infection. It is, however, important to recognize that the biofilm density and persistence phenotypes reported here are strikingly similar to results of infection studies with PCho⁺ mutant strains (107, 108). Thus, based on these data, we conclude that luxS impacts changes in LOS glycoform populations that are essential for full biofilm maturation, with resulting effects on NTHI persistence during experimental OM. These findings are also consistent with previous findings for luxS mutants with other NTHI strain backgrounds, which showed that more fulminant OM disease with severe inflammation and higher planktonic bacterial counts were observed at an early time point (323). Like data obtained in previous studies by our group and others (107-109), these data clearly show that stable biofilm communities promote long-term NTHI persistence in vivo and are necessary for establishment of the chronic, lower-grade inflammation that is the hallmark of chronic and recurrent OM.

Otitis media frequently presents as a polymicrobial infection, including both viral and bacterial coinfections and concurrent infection with multiple bacterial strains or species (29, 105). Recent epidemiologic studies have clearly established that coinfections with specific organisms commonly associated with OM can apparently affect long-term bacterial persistence in either a positive or negative sense, depending on the specific combination(s) of organisms involved (29). Because the AI-2 signaling pathway is widely conserved among bacterial species, understanding how different species impact one another in the contexts of opportunistic airway infections and in the context of biofilm formation and maturation is an important topic for further study.
CHAPTER II:

NTHI_0632 mediates quorum signal uptake in nontypeable *Haemophilus influenzae* strain 86-028NP


The following manuscript has been submitted to *Molecular Microbiology*. Stylistic variations are due to the requirement of the journal. Chelsie E. Armbruster performed the experiments and prepared the manuscript. Dr. W. Edward Swords acted in an advisory and editorial capacity.
INTRODUCTION

*Haemophilus influenzae* is a common airway commensal that inhabits the nasopharynx and upper airways of children and healthy adults (146, 147). Nontypeable *H. influenzae* strains lack capsular polysaccharides and mainly cause opportunistic infections of the airway mucosa, such as bronchitis, sinusitis, and otitis media (OM) (50, 146). OM is among the most common childhood infections (36, 37) and the leading reason for pediatric office visits and new antibiotic prescription to children (57). While capsular polysaccharide vaccines have reduced the prevalence of infection caused by encapsulated strains of *H. influenzae* and other otitis media pathogens such as *Streptococcus pneumoniae*, nontypeable strains remain a leading cause of both the acute and the chronic and recurrent presentations of OM (50).

OM infections often persist for long periods of time and can be highly resistant to immune clearance and antibiotic treatment. The highly resistant nature of these infections has long been thought to occur through persistence of bacteria within biofilm communities (67, 72, 110). Clinical evidence of bacterial biofilms includes the direct observation of biofilms in patient samples (105, 112, 137, 392) and in the chinchilla experimental model for OM (106, 393). As biofilms provide protection from the immune response and antibiotic treatment, understanding the mechanisms involved in their formation during chronic infection may provide new targets for disruption and treatment of biofilm-related infections (394).

Biofilm formation for many bacterial species is controlled in part through cell density-dependent quorum signaling networks, wherein changes in bacterial population
phenotypes are mediated by accumulation of a soluble signaling mediator (301, 308-310). Such signaling networks include autoinducer-2 (AI-2), a ribose derivative produced by both Gram-negative and Gram-positive bacteria that is conserved among numerous bacterial species and thus referred to as an inter-species signal (301, 310). Mutation of the genetic determinant of AI-2 production (luxS) was previously shown to impact biofilm thickness/maturation and persistence of nontypeable H. influenzae strain 86-028NP in an experimental animal model of OM (395). The focus of this study is to understand how strain 86-028NP internalizes and responds to AI-2.

In Vibrio harveyi, sensing of AI-2 occurs through a two-component system involving LuxP and LuxQ (348). LuxP is similar to periplasmic ribose binding proteins and binds AI-2, while LuxQ contains sensor kinase and response regulator domains to propagate the signal (349). Salmonella typhimurium, Escherichia coli, Sinorhizobium meliloti, and Aggregatibacter actinomycetemcomitans utilize the Lsr (LuxS regulated) ABC transporter, an AI-2 transport system with homology to the ribose ABC transporter (321). Rather than the two-component signal cascade described in V. harveyi, the Lsr system mediates AI-2 uptake via binding by LsrB and transport through a heterodimeric membrane channel (353, 356, 357, 396). In addition to LsrB, it was determined in A. actinomycetemcomitans that the ribose binding protein RbsB also binds AI-2 (355, 359).

Nontypeable H. influenzae strain 86-028NP does not possess a homolog of LuxPQ transport system. However, the 86-028NP genome contains an open reading frame annotated as a ribose transport system that includes a binding protein encoded by NTHI_0632 (209). NTHI_0632 has 40.3 percent identity to E. coli LsrB, and 87 percent identity to A. actinomycetemcomitans RbsB that was found to bind AI-2. Based on this
observation and the homology between the Lsr AI-2 transport system and ribose transporters, it was hypothesized that 86-028NP may utilize the protein encoded by NTHI_0632 for internalization of and response to AI-2. An NTHI_0632 mutant was therefore generated in 86-028NP to determine the impact of this mutation on production and internalization of AI-2, biofilm formation, and the ability of nontypeable *H. influenzae* to establish a chronic infection in the chinchilla model of OM.
EXPERIMENTAL PROCEDURES

**Bacterial strains and culture conditions.** All strains of nontypeable *H. influenzae* were cultivated in brain heart infusion (BHI) medium (Difco) supplemented with 10 µg/mL hemin (ICN Biochemicals) and 10 µg/mL NAD (Sigma); this medium is referred to below as supplemented BHI (sBHI). Nontypeable *H. influenzae* strain 86-028NP is a nasopharyngeal isolate from a child with chronic otitis media (381) for which the genomic sequence has been determined (209). 86-028NP *luxS::Kn* contains a kanamycin resistance cassette disrupting the autoinducer-2 synthase (NTHI_0621) (395). 86-028NP *licD::Kn* contains a kanamycin resistance cassette disruption the phosphorylcholine transferase (NTHI_1594) (226).

**Generation of strain 86-028NP 0632::Cm.** A 2313 bp DNA fragment containing the NTHI_0632 open reading frame was amplified from 86-028NP genomic DNA using primers GCA ATC GCC GCT TCA ATG G (forward) and CTA CCG CTA CCC CCG TCA GG (reverse). The amplicon was ligated into pCR2.1 following InVitrogen protocol to generate pCR0632. Plasmid pCR0632 was digested with *SspI*, a unique site within NTHI_0632, for ligation with a chloramphenicol resistance cassette. The resulting construct was transformed into chemically-competent *E. coli* to generate pCR0632::Cm. The expected sequence was confirmed by PCR with primers GGT TTG GCT GTT TCT GGC TCT GC (forward) and CGA TAG TTG CTG CCA TTT TGC CAC (reverse), and by sequence analysis. The plasmid DNA was linearized with *SfoI* and introduced into 86-028NP via natural transformation as described previously (107, 108). Transformants
were plated onto sBHI agar containing 2% Fildes’ reagent (Difco) and 1.5 µg/ml chloramphenicol (Sigma), and incubated for 48 h at 37˚ C. Transformants carrying the expected construct were verified by PCR.

**Generation of strain 86-028NP 0632::Cm luxS::Kn.** A double mutant strain was generated by disruption luxS (NTHI _0621) in 86-028NP 0632::Cm. The previously published plasmid pUCluxS::Kn (395) was linearized with SfoI and introduced into 86-028NP 0632::Cm via natural transformation. Transformants were plated onto sBHI agar containing 2% Fildes’ reagent (Difco) and 30 µg/ml ribostamycin (Sigma), and verified by PCR to contain both luxS::Kn and 0632::Cm.

**Autoinducer-2 production and depletion.** Nontypeable *H. influenzae* strain 86-028NP and 86-028NP 0632::Cm from overnight plate cultures were diluted in sBHI to an OD_{600} of approximately 0.100 and grown to stationary phase. Samples were taken at hourly intervals to assess production of AI-2 using the *Vibrio harveyi* bioluminescence assay. Luminescence produced by *V. harveyi* BB170 (348) following 3 h incubation with supernatant samples was determined in a Turner Designs TD-20/20 luminometer for 10 s. Data are reported as relative light units [counts per 10 s]. For AI-2 uptake studies, sBHI was supplemented with 0.2 µM (S)-4,5-Dihydroxy-2,3-pentanedione (DPD, Omm Scientific) when indicated, inoculated with ~10^8 cfu of *H. influenzae*, and incubated at 37˚C and 150 rpm. Samples were taken at hourly intervals, centrifuged, filter-sterilized and stored at -20˚C for bioluminescence. This concentration of DPD was chosen as it elicits approximately equivalent luminescence from *V. harveyi* as nontypeable *H.*
*influenzae* strain 86-028NP late exponential phase culture supernatant (OD$_{600}$ of approximately 0.750). For inhibition studies, 86-028NP *luxS*::Kn cultures were supplemented with ribose (Sigma), xylose (Sigma), or sucrose (Sigma) to a final concentration of 0.1, 1.0, or 10 mM as indicated.

**Real time RT-PCR.** 86-028NP and 86-028NP *luxS*::Kn were grown in sBHI to stationary phase, and 0.5 ml samples were taken at the indicated optical densities for isolation of RNA as per RNeasy Mini kit instructions (Qiagen). On-column DNase treatment of samples was performed using the RNase-Free DNase set (Qiagen), and RT-PCR master mixes were prepared using the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). A complete list of primers and probes is provided in Table 7. All primers were purchased from Eurofins, and Fam/Tamra probes were purchased from Sigma. An ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used under the following conditions: 1 cycle of 48˚C for 30 minutes, 1 cycle of 95˚C for 10 minutes, and 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute.

**Continuous flow system.** Nontypeable *H. influenzae* biofilms were established in a continuous-flow system as previously described (108). 86-028NP, 86-028NP *luxS*::Kn, and 86-028NP 0632::Cm were diluted to ~10$^8$ cfu/ml in sBHI broth, injected into the port of a commercial microscopy flow-cell (Stovall), and incubated for 3 h at 37˚C without medium flow to permit bacterial surface attachment. Continuous flow of sBHI media was initiated at a rate of ~60 ml per hour and maintained for 24 h.
Table 7. Real time PCR primers.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA Forward</td>
<td>5’-GCATTACCTGACGTTCGAGATG-3’</td>
</tr>
<tr>
<td>gyrA Reverse</td>
<td>5’-CCTTCGCGATCCATTGAGAA-3’</td>
</tr>
<tr>
<td>gyrA Probe</td>
<td>5’-6TTTTAAAAACCAGTTTCCACGCGCGTAC0-3’</td>
</tr>
<tr>
<td>luxS Forward</td>
<td>5’-TGCGGAAGCTATACGGAACA-3’</td>
</tr>
<tr>
<td>luxS Reverse</td>
<td>5’-CCTATACCGCGTGCGATAACA-3’</td>
</tr>
<tr>
<td>luxS Probe</td>
<td>5’-6TCCCTTAGAAGATGACACGAAATTGCCAA0-3’</td>
</tr>
<tr>
<td>0632 Forward</td>
<td>5’-TGATGCTCATAAAATTCAATGTGCTT-3’</td>
</tr>
<tr>
<td>0632 Reverse</td>
<td>5’- TTTCCGTTACATTCAAACCTTTTG-3’</td>
</tr>
<tr>
<td>0632 Probe</td>
<td>5’-6CCAGTCAGCCAGCAGATTGGATCGA0-3’</td>
</tr>
</tbody>
</table>
Scanning electron microscopy (SEM). In vitro biofilm samples were fixed within one half of the flow chamber for 60 min with 2.5% glutaraldehyde in PBS and then rinsed twice. Samples were then dehydrated, fixed, and prepared for SEM analysis as previously described (220). Biofilm samples were mounted onto stubs and sputter coated with palladium, then viewed with a Philips SEM-515 scanning electron microscope.

Confocal Laser Scanning Microscopy (CLSM). In vitro biofilm samples in the top half of the flow chamber were washed once with PBS and stained with a LIVE/DEAD BacLight viability kit (Invitrogen). Z-series images of biofilms were collected using a Zeiss LSM510 CLSM microscope. Eight image stacks, each representing a different field of view, were compiled for each strain. The Z-series images were visualized using the Zeiss LSM Image Browser software and exported into MATLAB (version 5.1) for COMSTAT analysis as previously described (383).

Whole-bacteria ELISA. ELISAs were performed on intact bacterial cells as described previously (382, 395). Bacteria were diluted in distilled H$_2$O to an OD$_{600}$ of 0.150 (~10$^8$ cfu/ml), and 0.1 ml was dispensed into each well of a MaxiSorp-coated 96-well microtiter plate (Nunc) and dried overnight uncovered at 37°C for 24 h. Plates were washed once and blocked for 30 min in TSBB buffer (10 mM Tris, 0.5 M NaCl, 0.5% Tween, pH = 8.0). Wells were incubated with 0.1 ml anti-phosphorylcholine monoclonal antibody HAS (Statens Serum Institut) overnight at room temperature, washed once, and incubated for 1 h in the dark with goat-anti-mouse IgM-horseradish peroxidase (HRP) conjugate. Wells were then washed once and incubated in the dark for approximately 20
min with 0.1 ml of TMB substrate reagent (OptEIA, Becton-Dickinson). The reaction was stopped with 0.05 ml of 2 N H$_2$SO$_4$, and OD$_{450}$ measured using a plate reader (Labsystems Multiskan Plus).

Assessment of phase-variable genes. Determination of ON/OFF state of phase-variable genes was performed as previously described (207, 395). Individual colonies of each nontypeable H. influenzae strain were harvested from plate cultures suspended in 0.1 ml of 10% suspension of Chelex (BioRad). Genomic DNA was prepared by vortexing suspensions for 15 sec, boiling for 10 min, chilling on ice for 2 min, and centrifugation at 8000 x g for 2 min. Supernatants (0.05 ml) were transferred to fresh tubes for PCR amplification and sizing by means of GeneMapper (Applied Biosystems, Inc., Foster City, CA) analysis as previously described (395). Numbers of repeats for each region were estimated based on fragment size, and used in accordance with the previously defined genomic sequence of each region to define “on” or “off” status of each phase-variable region.

Chinchilla infection studies. Bacterial persistence and biofilm formation in the middle ear chamber were assessed as described previously (107, 108, 395). Chinchillas were purchased from Rauscher’s chinchilla ranch (Larue, OH) and allowed to acclimate to the vivarium for >7 d prior to infection. No animals showed visible signs of illness prior to infection. The animals were anesthetized with isofluorane and infected via transbullar injection with ~10$^3$ CFU of 86-028NP, 86-028NP luxS::Kn, or 86-028NP 0632::Cm. All inocula were confirmed by plate-counts. The degree of inflammation was assessed at 48
h intervals by digital otoscopic examination using a qualitative scoring system of 1 to 4, with 4 being the most severe (107). Animals were euthanized at 7, 14, 21, and 28 days post-infection and middle-ear chambers were aseptically opened. Fluids within the middle ear were recovered and middle ear lavage was performed using 1.0 ml sterile PBS, and both bullae were excised and homogenized in 10 ml sterile PBS (107). Total cfu/ear were determined by combining bacterial counts obtained for homogenized bullae and middle-ear effusion fluid with lavage.

**Statistics.** Significance determined by nonparametric t test, unpaired t test, or two-way ANOVA with post-hoc tests of significance as indicated. All P values are two-tailed at a 95% confidence interval. Analyses were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA).
RESULTS

Construction and confirmation of 86-028NP 0632::Cm. A null allele of NTHI_0632 was constructed by insertion of a chloramphenicol resistance cassette into the coding sequence (see Experimental Procedures). This allele was introduced into nontypeable H. influenzae strain 86-028NP by natural transformation and verified by PCR (data not shown). No differences in growth rate were observed for 86-028NP 0632::Cm compared to the parental strain in rich broth culture. 86-028NP and 86-028NP 0632::Cm also grew similarly in minimal media with glucose as a carbon source, and neither strain was able to utilize ribose as a sole carbon source (data not shown). Notably, samples taken at hourly intervals during growth in rich media and assessed for AI-2 using the Vibrio harveyi bioluminescence assay revealed a significant accumulation of AI-2 in 86-028NP 0632::Cm supernatants during late-exponential and early-stationary phase (Figure 13). In contrast, the level of AI-2 in supernatant harvested from cultures of the parental strain decreased at these time points, suggesting that strain 86-028NP begins to internalize AI-2 in an NTHI_0632-dependent manner as it approaches stationary phase.

NTHI_0632 mediates AI-2 uptake in nontypeable H. influenzae strain 86-028NP. To further investigate the role of NTHI_0632 in depletion or internalization of AI-2, 86-028NP luxS::Kn was utilized to study the kinetics of AI-2 depletion during growth, independent of AI-2 production. Broth cultures were inoculated with 86-028NP luxS::Kn and supplemented with the synthetic AI-2 precursor dihydroxypentanedione (DPD) as indicated, and samples were taken to assess the amount of DPD remaining in the cultures
Figure 13. AI-2 accumulates in late-exponential phase cultures of 86-028NP 0632::Cm. Nontypeable H. influenzae strain 86-028NP and an NTHI_0632 mutant were cultured in sBHI to stationary phase. Supernatant samples were taken at hourly intervals for determination of AI-2 production by Vibrio harveyi bioluminescence. Dashed line indicates background luminescence elicited by a negative control. Significance was determined by a two-way ANOVA with a post-hoc test. *P<0.05. Error bars indicate mean and standard deviation.
over time (Figure 14A). Over the course of 7 hours, 86-028NP luxS::Kn depleted the majority of DPD present in the culture. While some loss of signal was observed for an uninoculated control, the level of depletion observed for 86-028NP luxS::Kn was significantly greater. As expected, 86-028NP luxS::Kn did not produce detectable AI-2 during the time course. Notably, mutation of NTHI_0632 on an 86-028NP luxS::Kn background almost completely abrogated depletion of DPD by this strain (Figure 14A). In five replicate experiments, 86-028NP luxS::Kn depleted 60% of DPD from culture supernatants on average, while the double mutant only depleted 15% of total DPD signal (Figure 14B). In addition, DPD levels were unaffected by cell-free culture supernatants from 86-028NP luxS::Kn and 86-028NP 0632::Cm luxS::Kn, indicating that DPD is not being degraded by factors present in the culture supernatants (data not shown).

Because NTHI_0632 has homology with the ribose transporter RbsB, we next tested the impact of ribose on DPD uptake. *H. influenzae* 86-028NP luxS::Kn was cultured in media supplemented with 0.2 µM DPD alone or with increasing concentrations of ribose, and samples were taken for assessment of DPD depletion (Table 8). The addition of 0.1 mM ribose had no effect on DPD depletion, while ribose in excess of 1 mM inhibited depletion of DPD. Notably, this inhibition of depletion only occurred when ribose was present in 1000-fold molar excess, suggesting a higher affinity for AI-2 than for ribose. Incubation with millimolar concentrations of another pentose sugar (xylose) also limited DPD depletion, while the addition of sucrose had no impact (Table 8). Based on these results, we conclude that in nontypeable *H. influenzae* strain 86-028NP, NTHI_0632 encodes an AI-2 binding protein with low affinity for pentose sugars.
Figure 14. Mutation of NTHI_0632 limits AI-2 depletion. 86-028NP luxS::Kn and 86-028NP 0632::Cm luxS::Kn were cultured in sBHI media alone or sBHI supplemented with 0.2 µM of the chemically synthesized AI-2 precursor (S)-4,5-Dihydroxy-2,3-pentanedione (DPD). Samples were taken at hourly intervals to assess depletion of AI-2 by *V. harveyi* bioluminescence. An uninoculated control sample of sBHI supplemented with DPD was included as a negative control for depletion. (A) A representative graph showing kinetics of AI-2 depletion by 86-028NP luxS::Kn and 86-028NP 0632::Cm luxS::Kn. (B) Combined results of five experiments showing the percent of AI-2 depleted by 86-028NP luxS::Kn versus 86-028NP 0632::Cm luxS::Kn as the cultures reached late-exponential phase (OD$_{600}$ of approximately 0.750), normalized to uninoculated control samples. Error bars indicate mean and standard deviation.
Table 8. Inhibition of DPD depletion.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent DPD Depletion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Standard Deviation</th>
<th>P Value&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>No Treatment</td>
<td>58.66</td>
<td>10.91</td>
<td>-</td>
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<tr>
<td>Ribose</td>
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<tr>
<td>0.1 mM</td>
<td>42.68</td>
<td>7.92</td>
<td>0.07</td>
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<td>1.0 mM</td>
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<td>Xylose</td>
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<td>0.1 mM</td>
<td>60.46</td>
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<td>16.83</td>
<td>&lt;<strong>0.01</strong></td>
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<td>Sucrose</td>
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<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>60.50</td>
<td>1.13</td>
<td>0.19</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>61.20</td>
<td>10.89</td>
<td>0.17</td>
</tr>
<tr>
<td>10 mM</td>
<td>58.10</td>
<td>2.26</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent DPD depleted from culture at an OD<sub>600</sub> of approximately 0.800 compared to an uninoculated control.

<sup>b</sup>P values determined by unpaired t test of percent DPD depletion with the indicated treatment compared to an untreated control.
**NTHI_0632 transcript levels correlate with AI-2 production.** For some bacterial species, expression of the AI-2 transport system is induced by the presence of AI-2 (354). It was therefore hypothesized that if the protein encoded by NTHI_0632 functions as an AI-2 binding protein, transcript levels may increase during late-exponential phase when bacteria reach peak AI-2 production. 86-028NP and 86-028NP luxS::Kn were therefore cultured in rich media to stationary phase, and samples were taken at various time points during growth to assess transcript levels for luxS and NTHI_0632 by real time RT-PCR (Figure 15). For 86-028NP, luxS transcript levels increased by approximately two-fold during late-exponential phase (Figure 15A), and NTHI_0632 transcript levels increased by approximately five-fold during late-exponential to early-stationary phase (Figure 15B). Notably, both luxS and NTHI_0632 transcript levels remained low in the luxS mutant (Figure 15A and B). As 86-028NP luxS::Kn was shown to deplete AI-2 from culture in Figure 14, low level expression of NTHI_0632 must still be sufficient to mediate AI-2 uptake, whereas increased expression of NTHI_0632 may be optimal for internalization of the high concentration of AI-2 encountered during early-stationary phase. Additionally, incubation with exogenous AI-2 was sufficient to promote increased NTHI_0632 transcript levels in 86-028NP luxS::Kn cultures (data not shown). From these data, we conclude that transcription of NTHI_0632, and luxS to a lesser extent, increases in response to AI-2 signaling.

**NTHI_0632 promotes biofilm maturation.** Previous studies with the 86-028NP luxS mutant showed that loss of AI-2 quorum signaling altered overall biofilm development and maturation, resulting in biofilms with reduced biomass and average thickness (395).
Figure 15. Increased \textit{luxS} and \textit{NTHI_0632} transcript levels correlate with AI-2 production. Nontypeable \textit{H. influenzae} 86-028NP and 86-028NP \textit{luxS::Kn} were cultured in sBHI media to stationary phase. Samples were taken during lag phase (OD\textsubscript{600} 0.200), exponential phase (OD\textsubscript{600} 0.500), late-exponential phase (OD\textsubscript{600} 0.750), and early stationary phase (OD\textsubscript{600} 0.950) for isolation of RNA and real time RT-PCR analysis of \textit{luxS} (A) and \textit{NTHI_0632} (B) transcript levels. Values represent the ratio of \textit{luxS} or \textit{NTHI_0632} to \textit{gyrA} transcript. Error bars indicate mean and standard deviation.
As NTHI_0632 is necessary for depletion of DPD, we hypothesized that mutation of NTHI_0632, and the concomitant loss of AI-2 internalization would diminish biofilm maturation, as we have previously observed for luxS mutants. To test this hypothesis, nontypeable H. influenzae strain 86-028NP, 86-028NP luxS::Kn, and 86-028NP 0632::Cm were cultured in a continuous flow system to establish biofilms (Figure 16). Both mutants formed visible biofilms in the continuous flow system. Scanning electron microscopy (SEM) analysis of 86-028NP, 86-028NP luxS::Kn, and 86-028NP 0632::Cm biofilms at 24 h post-inoculation showed no qualitative differences between strains at any magnification (Figure 16A-F), as previously reported for 86-028NP luxS::Kn and other H. influenzae isolates (323, 395).

Further analysis of biofilms visualized by Live/Dead staining revealed differences in biofilm thickness by confocal laser scanning microscopy (CLSM) for 86-028NP luxS::Kn and 86-028NP 0632::Cm compared to the parental strain (Figure 17). Representative three-dimensional images of biofilms formed by 86-028NP, 86-028NP luxS::Kn, and 86-028NP 0632::Cm demonstrate the reduced biofilm biomass for the quorum signaling mutant strains (Figure 17A-C). Similar to our previous work with 86-028NP luxS::Kn, both the luxS mutant and 86-028NP 0632::Cm formed biofilms with significantly decreased biomass (Figure 17D), surface to biovolume ratio (Figure 17E), average thickness (Figure 17F), and maximum thickness (Figure 17G) compared to the parental strain. From these studies, we conclude that mutation of NTHI_0632 and the resulting loss of AI-2 internalization impacts biofilm formation in a similar manner as loss of AI-2 production.
Figure 16. 86-028NP 0632::Cm forms a biofilm under continuous-flow conditions.

Nontypeable *H. influenzae* 86-028NP, 86-028NP *luxS::Kn*, and 86-028NP 0632::Cm were cultured under continuous-media-flow conditions for 24 h. SEM of biofilms formed by 86-028NP (A and B), 86-028NP *luxS::Kn* (C and D), and 86-028NP 0632::Cm (E and F) at magnifications of x500 (A, C, and E) and x1,000 (B, D, and F).
Figure 17. Mutation of NTHI_0632 results in a similar biofilm maturation defect as mutation of luxS. Biofilms formed by 86-028NP, 86-028NP luxS::Kn, and 86-028NP 0632::Cm under continuous flow conditions were visualized with a live/dead stain by CLSM. Z-series images were used to create representative volume views of biofilms formed by 86-028NP (A), 86-028NP luxS::Kn (B), and 86-028NP 0632::Cm (C). Z-series images were also exported to COMSTAT to obtain biofilm measurements, including total biomass (D), surface to biovolume ratio (E), average biofilm thickness (F), and maximum biofilm thickness (G). Statistical significance determined by unpaired \( t \) test, error bars indicate standard error of the mean.
To further investigate the impact of AI-2 quorum signaling on biofilm formation, nontypeable *H. influenzae* strain 86-028NP, 86-028NP *luxS::Kn*, and 86-028NP *0632::Cm* stationary biofilms were formed in the presence or absence of DPD, and biofilms were analyzed for changes in total biomass (Figure 18). In media lacking DPD, 86-028NP formed thick biofilms while 86-028NP *luxS::Kn* and 86-028NP *0632::Cm* biofilms had significantly decreased total biomass, as previously observed. The addition of 0.2 µM DPD to 86-028NP did not significantly alter biofilm formation or total biomass. As this concentration of DPD mimics the amount produced by 86-028NP during late-exponential phase, this suggests that there is most likely a threshold concentration of AI-2 required by nontypeable *H. influenzae* to initiate biofilm formation/development, and increasing the concentration of AI-2 beyond this threshold has no further additive effect on total biomass of the biofilm. For 86-028NP *luxS::Kn*, the addition of DPD resulted in a dramatic increase in biofilm thickness, fully restoring biomass to the level observed for the parental strain. Notably, 86-028NP *0632::Cm* did not respond to DPD, as indicated by biofilm biomass remaining decreased under both treatment conditions. Taken together, these results provide concrete evidence that the biofilm defects observed for 86-028NP *luxS::Kn* are the direct result of loss of AI-2 signaling rather than a metabolic defect due to mutation of *luxS*, and that NTHI_0632 is required by nontypeable *H. influenzae* strain 86-028NP to respond to AI-2.

The decreased biofilm thickness and total biomass observed for 86-028NP *luxS::Kn* were previously attributed in part to alterations in lipooligosaccharide (LOS) moieties (395). Specifically, 86-028NP *luxS::Kn* had decreased phosphorylcholine (*PCho*), an LOS modification that correlates with biofilm maturation and persistence in
Figure 18. Nontypeable *H. influenzae* 86-028NP requires NTHI_0632 to respond to exogenous AI-2. 86-028NP, 86-028NP luxS::Kn, and 86-028NP 0632::Cm were cultured in sBHI media alone (white bars) or sBHI supplemented with 0.2 µM DPD (gray bars) and allowed to establish stationary biofilms for 12 h. Biofilms were visualized by live/dead staining and CLSM. Vertical z-series images were exported to COMSTAT for analysis of total biofilm biomass. Statistical significance determined by unpaired t test. *P*<0.05 compared to 86-028NP in sBHI. **P**<0.01 compared to 86-028NP luxS::Kn in sBHI. Error bars indicate standard error of the mean.
vivo (107, 108). The level of surface-accessible \( P\text{Cho} \) on 86-028NP 0632::Cm was assessed by whole-bacterium ELISA for comparison to 86-028NP, 86-028NP \( luxS::\text{Kn} \), and 86-028NP \( licD::\text{Kn} \), a mutant lacking the \( P\text{Cho} \) transferase that is thus unable to decorate LOS with \( P\text{Cho} \) (Figure 19). As previously reported, 86-028NP \( luxS::\text{Kn} \) had significantly reduced surface-accessible \( P\text{Cho} \) compared to the parental strain. 86-028NP 0632::Cm also had reduced \( P\text{Cho} \) as compared to the parental strain. Notably, the amount of surface-accessible \( P\text{Cho} \) detected on 86-028NP 0632::Cm was almost identical to the level detected on 86-028NP \( luxS::\text{Kn} \). 86-028NP \( licD::\text{Kn} \) had negligible surface-accessible \( P\text{Cho} \), as expected.

The biosynthetic genes involved in \( P\text{Cho} \) decoration of LOS, as well as other LOS moieties, are subject to phase variation via slip-strand mispairing within repeat regions (389). Therefore, the phase status of seven LOS biosynthetic genes was assessed for 86-028NP 0632::Cm for comparison to the parental strain (see Experimental Procedures). No differences were observed for the phase status of genes involved in \( P\text{Cho} \) decoration of LOS (Table 9). Additionally, 86-028NP 0632::Cm phase status only differed from that of 86-028NP for \( oafA \), which was generally in the “ON” phase for 86-028NP 0632::Cm colonies and in the “OFF” phase within most 86-028NP colonies. Interestingly, altered phase status of \( oafA \) was also observed for 86-028NP \( luxS::\text{Kn} \) (395). The results of the phase status assessment clearly show that the reduction in surface-accessible \( P\text{Cho} \) observed for 86-028NP 0632::Cm was not due to phase variation of the LOS biosynthetic genes necessary for the addition of \( P\text{Cho} \) to LOS.
Figure 19. Mutation of NTHI_0632 reduces surface-accessible PCho. A modified whole-bacterium ELISA was used to detect surface phosphorylcholine using an anti-PCho monoclonal antibody. Significance determined by unpaired t test, error bars indicate standard error of the mean.
Surface PCho level (OD$_{590}$)
Table 9. Analysis of tetrameric repeat regions.
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</tr>
<tr>
<td>lex2A</td>
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<td>6</td>
</tr>
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<td>lic1A</td>
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<td>lav</td>
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<td>0</td>
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<table>
<thead>
<tr>
<th>Gene</th>
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<th>OFF</th>
</tr>
</thead>
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<tr>
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<td>86-028NP</td>
<td></td>
</tr>
<tr>
<td>0632::Cm</td>
<td>0632::Cm</td>
<td></td>
</tr>
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</table>

^1769 refers to NTHI_1769, a hypothetical glycosyltransferase
**Mutation of NTHI_0632 limits persistence in the chinchilla model of OM.** Our previous study of 86-028NP luxS::Kn showed that production of AI-2 was a critical factor in the ability of nontypeable *H. influenzae* to establish a persistent infection in the chinchilla model of otitis media (395). Based on the similarities between 86-028NP luxS::Kn and 86-028NP 0632::Cm biofilm formation and PCho expression, it was hypothesized that mutation of NTHI_0632 and the resulting defect in AI-2 internalization would result in a similar persistence defect in the chinchilla model of otitis media. To test this hypothesis, chinchillas were inoculated via transbullar injection of approximately $10^3$ cfu/ear of 86-028NP, 86-028NP luxS::Kn, or 86-028NP 0632::Cm, and bacterial load was determined at 7, 14, 21, and 28 days post-infection. Comparable numbers of each strain were recovered at 7 and 14 days post-infection (Figure 20). By 21 days post-infection, a significant persistence defect was observed for the quorum signaling mutants with 12.50% of ears (2 of 16) infected with 86-028NP luxS::Kn and 31.25% of ears (5 of 16) infected with 86-028NP 0632::Cm completely cleared of infection. A similar trend was observed at 28 days post-infection, with complete clearance of bacteria occurring in 33.33% ears (2 of 6) infected with 86-028NP luxS::Kn and 50.00% of ears (4 of 8) infected with 86-028NP 0632::Cm. Notably, no clearance was observed for ears infected with 86-028NP at any time post-infection. We thus conclude that NTHI_0632 encodes a protein critical for AI-2 quorum signaling in strain 86-028NP, and that the AI-2 signaling system promotes the establishment of biofilms and chronic infection by nontypeable *H. influenzae*.
Figure 20. Mutation of NTHI_0632 limits bacterial persistence in vivo. Chinchillas were infected via transbullar inoculation with ~10^3 cfu of nontypeable *H. influenzae* 86-028NP, 86-028NP *luxS::Kn*, or 86-028NP *0632::Cm*. Total bacterial load per ear was determined by serial dilution and plating of effusion fluid, middle ear lavage, and bullar homogenate at 7, 14, 21, and 28 days post-infection. Data points represent total bacterial load for individual ears, and data shown are the combined results for three independent studies. The dashed line indicates limit of detection. Error bars represent the geometric mean and 95% confidence intervals.
DISCUSSION

It has long been recognized that bacteria can persist within biofilm communities during chronic disease, and establishment of these bacterial communities affords protection from both immune clearance and antibiotic treatment (91, 92, 95, 96). Due to the high rate of antibiotic resistance for chronic biofilm diseases such as OM, research focused on identifying factors which contribute to the establishment of bacterial biofilms and persistence will further our understanding of biofilm-associated disease and may aid in the development of new therapeutics. One factor which contributes to biofilmformation, maturation, and dispersal for numerous species is bacterial cell-to-cell communication via quorum signals (308, 360). Our previous work established that nontypeable H. influenzae 86-028NP produces AI-2 and requires luxS to form mature biofilms and establish a chronic infection (395), but it was unclear as to how 86-028NP was responding to this signaling mediator. In this study, we used a genetic approach to identify the factor encoded by NTHI_0632 as being required for uptake of and response to AI-2 by nontypeable H. influenzae strain 86-028NP, and further addressed the role of AI-2 quorum signaling in biofilm development and persistence. The results of this study clearly show that sensing of AI-2 promotes the establishment of mature nontypeable H. influenzae biofilm communities in vitro and during experimental OM.

While previous work from our laboratory reported a prominent role for luxS in biofilm maturation and persistence, the product of luxS is a component of the activated methyl cycle and therefore plays a role in bacterial metabolism. Thus, for some species, the effects of a luxS mutation on biofilm development and virulence can be attributed to a
metabolic defect rather than interference with production and sensing of AI-2 (319, 330, 333, 334). However, through the use of the AI-2 precursor DPD in the present study, we have clearly shown that the biofilm defects of a luxS mutant can be fully complemented by the addition of quorum signal. The results of this study also prove that mutation of a gene involved in the response to AI-2 in a strain background with luxS intact still results in defects in biofilm maturation, indicating that this phenotype in 86-028NP is due to loss of AI-2 quorum signaling rather than any potential metabolic impact of the luxS mutation. The NTHI_0632 mutation also resulted in decreased phosphorylcholine levels, as was previously observed for mutation of luxS, indicating that AI-2 signaling regulates the formation of mature nontypeable H. influenzae biofilm at least in part through modulation of LOS composition (395). Additionally, the results of the phase status analyses in concert with our previous study of the luxS mutation clearly exclude the possibility of an unlinked phase variation event resulting in the observed changes in biofilm maturation.

In S. typhimurium and E. coli, transcription of the lsr operon is regulated by AI-2 and thus decreased in a luxS mutant (354, 356). Therefore, the data reported in the present study showing reduced NTHI_0632 transcript levels in 86-028NP luxS::Kn and upregulation of transcript in the parental strain during peak AI-2 production and upon the addition of AI-2 to 86-028NP luxS::Kn cultures are consistent with NTHI_0632 encoding an AI-2 regulated protein. Based on these data, NTHI_0632 clearly represents a luxS-regulated (Lsr) gene, even though the protein encoded by NTHI_0632 only shares 40.3% identity to the E. coli LsrB. We thus propose that NTHI_0632 may be part of an Lsr transport system that is functionally homologous to the transport systems of E. coli and S.
typhimurium. The minimal impact of pentose sugars on depletion of DPD (unless in excess of 1000 times the DPD concentration) also supports the conclusion that, for 86-028NP, NTHI_0632 most likely encodes an AI-2 binding protein with limited affinity for pentose sugars. It is intriguing to speculate that the rest of the putative ribose transport operon of nontypeable H. influenzae strain 86-028NP may be similarly regulated by AI-2 and function as part of a high-affinity AI-2 transporter, and possibly as a low-affinity pentose sugar transporter. Additionally, the downstream phosphorylation and detection of AI-2 and the signaling induced in response to detection of AI-2 remain to be investigated in nontypeable H. influenzae.

In the context of experimental OM, mutation of either luxS or NTHI_0632 results in a similar persistence defect, indicating that both production of AI-2 and the ability to sense and respond to the AI-2 signal contribute to establishment of a chronic infection. This finding also proves that the persistence defect previously observed for 86-028NP luxS::Kn was not due to a metabolic defect or to alterations in other possible quorum signaling networks, such as AI-3, which may be impacted by a luxS mutation (331). However, it still remains a possibility that host factors produced during the course of infection could impact nontypeable H. influenzae signaling networks and contribute to biofilm formation and persistence. While a clear trend towards complete clearance was observed for 86-028NP luxS::Kn and 86-028NP 0632::Cm compared to the parental strain, it is notable that some of the animals infected with the quorum signaling mutants still carried a bacterial load similar to that observed for the parental strain at 28 days post-infection. The data thus support a prominent role for AI-2 quorum signaling in concert with other as yet undetermined factors that promote the establishment of a chronic,
biofilm-associated disease. It is also possible that a mutant deficient in both the ability to produce and respond to AI-2 may have a more severe persistence defect beyond what was observed for either quorum signaling mutant strain.

Nontypeable *H. influenzae* remains a leading cause of OM and other upper respiratory tract infections, despite promising vaccine candidates. Our data indicate that AI-2 quorum signaling is utilized by strain 86-028NP to promote biofilm formation and establishment of chronic, resistant infections. Furthermore, our previous work suggests AI-2 produced by nontypeable *H. influenzae* impacts *Moraxella catarrhalis* biofilm formation, antibiotic resistance, and persistence in the chinchilla model of OM (145). Therefore, therapeutics aimed at interfering with AI-2 binding and signaling may prevent biofilm development or disrupt already established biofilms to augment current OM treatments. For nontypeable *H. influenzae* strain 86-028NP, the protein encoded by NTHI_0632 appears to be a promising target for interference with AI-2 signaling as mutation of this gene clearly has a significant biological impact on AI-2 uptake, biofilm development, and persistence during experimental OM. However, it should be noted that there is considerable genomic heterogeneity among *H. influenzae* strains, and the possibility remains of other means for quorum signal uptake in other strain backgrounds. Further investigation into transport and sensing of AI-2 in *H. influenzae* will allow for identification of virulence factors controlled by AI-2 signaling, and may reveal new target candidates for disruption of quorum signaling as a therapy for treatment of nontypeable *H. influenzae* infections.
CHAPTER III:

LuxS Expression Promotes Nontypeable *Haemophilus influenzae* biofilm development and prevents dispersal

Chelsie E. Armbruster, Gayle C. Foster, Brian S. Learman, Bing Pang, and W. Edward Swords

The following manuscript has been submitted to *mBio*. Stylistic variations are due to the requirement of the journal. Chelsie E. Armbruster performed the experiments and prepared the manuscript. Dr. W. Edward Swords acted in an advisory and editorial capacity.
INTRODUCTION

*Haemophilus influenzae* is a Gram-negative bacterium and common airway commensal that inhabits the nasopharynx and upper airways of children and healthy adults (11, 146, 397). Encapsulated strains of *H. influenzae* cause invasive disease such as meningitis, but the prevalence of these infections has been significantly reduced through widespread use of a conjugate vaccine against serotype b (154, 398). Nontypeable *H. influenzae* strains lack capsular polysaccharides and mainly cause opportunistic infections of the airway mucosa, such as bronchitis, sinusitis, and otitis media (OM) (146). Nontypeable *H. influenzae* infections frequently persist for long periods of time and can be highly resistant to immune clearance and antibiotic treatment at least in part due to persistence of bacteria within biofilm communities (50, 67, 72, 110). Bacterial biofilms have been directly observed in animal models of nontypeable *H. influenzae* infection (106, 109, 392) and in patient samples (105, 112, 137, 392). Therefore, understanding the mechanisms involved in biofilm formation during infection may provide new targets for disruption and treatment of biofilm-related infections.

Biofilm development for many bacterial species is controlled in part through cell density-dependent quorum signaling networks, wherein changes in bacterial population phenotypes are mediated by accumulation of a soluble signaling mediator (301, 308-310). Such signaling networks include autoinducer-2 (AI-2), a ribose-derivative produced by both Gram-negative and Gram positive bacteria and often referred to as an inter-species signal (301, 310). In some systems quorum signaling promotes biofilm development and maturation, while in others the primary role of quorum signaling in biofilm development
is to promote the release of bacteria from the biofilm through a process known as dispersal (74, 82, 360, 361).

For nontypeable \textit{H. influenzae} strain 86-028NP, the genetic determinant of AI-2 production (\textit{luxS}) was previously shown to be required for biofilm maturation and persistence in an experimental animal model of OM (395). However, for a more rigorous analysis of the role of \textit{luxS} expression in biofilm formation, maturation, and dispersal, we generated a strain of nontypeable \textit{H. influenzae} 86-028NP in which \textit{luxS} expression is induced in the presence of xylose. The results of this study argue that LuxS and AI-2 quorum signaling not only promote biofilm thickness and maturation but act to prevent or delay biofilm dispersal, and support a model wherein overall \textit{luxS} expression and AI-2 quorum signaling peak as the biofilm starts to mature and decrease as the biofilm approaches dispersal.
MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Nontypeable *Haemophilus influenzae* was cultivated in brain heart infusion (BHI) medium (Difco) supplemented with hemin (ICN Biochemicals) and NAD (Sigma); this medium is referred to below as supplemented BHI (sBHI). *H. influenzae* strain 86-028NP is a nasopharyngeal isolate from a child with chronic otitis media (381). 86-028NP *luxS* contains a kanamycin resistance cassette disrupting the Autoinducer-2 synthase (NTHI0621), and 86-028NP *luxS gfp* is an AI-2 synthase mutant expressing the green fluorescence protein (395).

**Generation of WES204 and WES204 gfp.** A xylose-inducible *luxS* strain of *H. influenzae* 86-028NP was constructed in a similar manner as described by Wong et. al. for *H. influenzae* Rd (399). A complete list of primers is provided in Table 10.

*Generation of a plasmid containing the xyl region of 86-028NP.* Primers 1 and 2 were used to PCR amplify a 1379 bp region of 86-028NP genomic DNA containing *aspC2* and a partial *xylA* fragment containing the *xylA* promoter. Primers 3 and 4 were used to PCR amplify another *xylA* fragment starting at nucleotide 805 and part of *xylB*. Using primers 2 and 4, the above fragments were joined to create a segment containing *aspC2*, the *xylA* promoter and a deletion of *xylA* nucleotides 4-804, and a partial *xylB* fragment. This product was ligated into pCR2.1 following InVitrogen protocol to create pCR.xyl containing engineered *XbaI* and *KpnI* sites, and clones were verified by sequencing with primer 5 and *KpnI* restriction digest (*KpnI* cuts pCR.xyl twice to yield two bands of approximately 3892 bp and 2852 bp).
Table 10. Primers used in this study.
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<td>2</td>
<td>5'-GCTCTAGATTTAAATCTCCCTATTGTTTAATAAAAACCC-3’</td>
</tr>
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<td>3</td>
<td>5’-CCATATGGGAAGAGCGCTCTTCCATATGGGATCCAAGC-TAACCACGCAGCCTTG-3’</td>
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<td>4</td>
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</table>
Preparation of the xyl cloning vector. Vector pUC19 was prepared by SapI digestion and T4 DNA polymerase fill-in, followed by ligation to an annealed XbaI linker. The ligation product was transformed into chemically competent Escherichia coli to generate pUCXbaI, and confirmed by XbaI digest (XbaI cuts twice to yield two bands of approximately 2423 bp and 273 bp). pUCXbaI was then digested with KpnI, gel-purified, digested with XbaI, dephosphorylated, and run on an agarose gel. The larger band generated by the restriction digests was excised from the gel using a Qiagen Gel Extraction kit.

pCRxyl was digested with XbaI and KpnI, the fragment containing xyl was ligated into the prepared pUCXbaI vector, and transformed into chemically competent E. coli to create pUCxyl. pUCxyl was then linearized with BamHI, dephosphorylated, and ligated to a gentamycin resistance cassette (excised from pGmΩ with BamHI) to generate pUCxyl-Gm (approximately 7100 bp). Roche’s Expand Long Template PCR kit was used with primers 7 and 8 to introduce an XhoI site in pUCxyl-Gm. Amplification procedures were as follows: 58°C anneal temperature for the first 10 cycles, then 60°C anneal temperature for 25 cycles. The resulting amplicon was purified using a Qiagen PCR Purification Kit, digested with XhoI, incubated with T4 DNA ligase to join the overhanging ends, and transformed into chemically competent E. coli to create pUCxylGM. Transformants were verified by BsrBI digest to yield three bands (3546 bp, 1801 bp, and 1454 bp). pUCxylGM was then digested with SapI, dephosphorylated, and the larger band was excised from an agarose gel using a Qiagen Gel Purification kit.
**Generation of luxS flanked by SapI sites.** Primers 9 and 10 were used to PCR amplify luxS lacking its start site from pUCluxS (395). The resulting product was amplified with primers 11 and 12 to engineer SapI sites on both ends of the luxS gene. These sites allow for precise insertion of luxS into pUCxyl/GM under control of the xylA promoter. The amplicon was ligated into pCR2.1 and transformed into chemically competent *E. coli* to create pCRluxSSapI. Transformants were sequenced using primers M13F and M13R, and verified by EcoRI and BsrGI restriction digest. pCRluxSSapI was then digested with SapI and the 500 bp band was excised from an agarose gel using a Qiagen Gel Extraction kit, and ligated into SapI-digested pUCxyl/GM. The ligation product was transformed into chemically competent *E. coli* to generate pUCxyl/GMluxS. Transformants were verified using primers 5 and 6.

**Generation of pUCxylSPluxS.** A spectinomycin resistance cassette (400) was prepared by BamHI digest and excised from an agarose gel using a Qiagen Gel Extraction kit. pUCxyl/GMluxS was digested with BamHI, dephosphorylated, and run on an agarose gel. The larger band (lacking the Gm resistance cassette) was excised using a Qiagen Gel Extraction kit, ligated with the spectinomycin cassette, and transformed into chemically competent *E. coli* to generate pUCxyl/SPluxS. Transformants were verified by KpnI restriction digest to yield two bands (2935 bp and 1974 bp).

**Transformation into 86-028NP luxS and 86-028NP luxS gfp.** pUCxylSPluxS was linearized by SfoI and introduced into both nontypeable *H. influenzae* strain 86-028NP luxS and 86-028NP luxS gfp via natural transformation as described previously (107,
156. Transformants were plated onto sBHI agar containing 2% Fildes’ reagent (Difco) and 30 µg/ml spectinomycin, and incubated for 24 h at 37° C. Transformants carrying the expected inducible luxS construct under control of the xylA promoter were verified by PCR using primers 5 and 13.

Autoinducer-2 production. H. influenzae strains 86-028NP and WES204 from overnight plate cultures were diluted in sBHI or sBHI supplemented with 5 mM xylose (Sigma) to an OD_{600} of approximately 0.150 and grown to stationary phase. Samples were taken during growth to assess production of AI-2 using the Vibrio harveyi bioluminescence assay. Luminescence produced by V. harveyi BB170 (348) following 3 h incubation with supernatant samples was determined in a Turner Designs TD-20/20 luminometer for 10 s. Data are reported as relative light units [counts per 10 s].

Whole-bacterium ELISA. ELISAs were performed on intact bacterial cells as described previously (382, 395). Bacteria were diluted in distilled H_{2}O to an OD_{600} of ~0.15, and 0.1 ml was dispensed into each well of a MaxiSorp-coated 96-well microtiter plate (Nunc) and dried overnight uncovered at 37°C for 24 h. Plates were washed once and blocked for 30 min in TSBB buffer (10 mM Tris, 0.5 M NaCl, 0.5% Tween, pH = 8.0). Wells were incubated with 0.1 ml anti-phosphorylcholine monoclonal antibody HAS (Statens Serum Institut) overnight at room temperature, washed once, and incubated for 1 h in the dark with goat-anti-mouse IgM-horseradish peroxidase (HRP) conjugate. Wells were then washed once and incubated in the dark for approximately 20 min with 0.1 ml of TMB substrate reagent (OptEIA, Becton-Dickinson). The reaction was stopped with
0.05 ml of 2 N H$_2$SO$_4$, and OD$_{450}$ measured using a plate reader (Labsystems Multiskan Plus).

**Colony immunoblots.** PCho expression was assessed as described previously (174, 384). Bacterial colonies were lifted from sBHI plates onto nitrocellulose membranes and air-dried, then washed (2 washes, 10 min/wash) in TSBB buffer (10 mM Tris, 0.5 M NaCl, 0.5% Tween, pH = 8.0). Membranes were incubated in anti-phosphorylcholine monoclonal antibody HAS (Statens Serum Institut) in TSBB overnight, washed in fresh TSBB (5 washes, 10 min/wash), and incubated overnight in goat anti-mouse IgM alkaline phosphatase conjugate in TSBB, washed in TSBB, and incubated for 10 min in alkaline phosphatase (AP) buffer (100 mM NaCl, 5 mM MgCl$_2$, 100 mM Tris, pH 9.5). PCho$^+$ colonies were visualized using 5-bromo-4-chloro-3-indolyl phosphate (Sigma, 0.16 mg/ml) and nitroblue tetrazolium (Sigma, 0.33 mg/ml) in AP buffer. Colonies were classified as having high or low reactivity with the antibody based on intensity of staining.

**Confocal Laser Scanning Microscopy (CLSM).** Stationary biofilms were established in Tab-TekII coverglass slides (Nunc). Each chamber was inoculated with ~10$^8$ cfu/ml of *H. influenzae* 86-028NP, 86-028NP luxS, or WES204 in sBHI or sBHI + 5mM xylose and incubated for 12, 24, or 48 h at 37˚C and 5% CO$_2$. For induction experiments, supernatants were carefully removed and replaced with sBHI or sBHI + 5mM xylose as indicated. For visualization, biofilms were washed once with PBS and stained with a LIVE/DEAD BacLight viability kit (Invitrogen). Z-series images of biofilms were
collected using either a Zeiss LSM510 CLSM microscope or a Nikon Eclipse C1 CLSM microscope. Six image stacks, each representing a different field of view, were compiled for each strain. The Z-series images were visualized using the Nikon Elements software and exported into MATLAB (version 5.1) for COMSTAT analysis as previously described (383).

**Continuous flow system.** *H. influenzae* biofilms were established in a continuous-flow system as previously described (108). Strains 86-028NP *gfp*, 86-028NP *luxS* *gfp*, and WES204 *gfp* were diluted to ~10^8 CFU/ml in sBHI broth, injected into the port of a commercial microscopy flow-cell (Stovall), and incubated for 3 h at 37˚C without medium flow to permit bacterial surface attachment. Continuous flow of sBHI media was initiated at a rate of ~60 ml per hour and maintained for 48 h. For induction of WES204, a 3-way stopcock luer lock was utilized to switch media flow from flasks containing sBHI to sBHI supplemented with 5 mM xylose, or vice versa, after biofilms had established for 24 hours. Confocal microscopy of biofilms was performed at 24, 27, and 48 h. For the 96 h biofilm experiment, strain WES204 *gfp* was cultured under continuous flow conditions in sBHI supplemented with 5 mM xylose for 96 h with confocal microscopy at 24 h intervals.

**Scanning electron microscopy (SEM).** Biofilm samples from the 96 h continuous flow experiment were washed once with PBS and fixed within one half of the flow chamber for 60 min with 2.5% glutaraldehyde. Samples were then dehydrated, fixed, and prepared for SEM analysis as previously described (220). Biofilm samples were
mounted onto stubs and sputter coated with palladium, then viewed with a Philips SEM-515 scanning electron microscope.

**Real time RT-PCR.** 86-028NP biofilms were established in triplicate in a 24-well tissue culture plate for 6, 12, 24, 48, or 72 h at 37°C and 5% CO₂. At each timepoint, supernatants were carefully removed, biofilms were suspended in RNA Protect and centrifuged for isolation of RNA from cell pellets as per RNeasy Mini kit instructions (Qiagen). On-column DNase treatment of samples was performed using the RNase-Free DNase set (Qiagen), and RT-PCR master mixes were prepared using the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). Primers for luxS were purchased from Eurofins, and a Fam/Tamra probe was purchased from Sigma (Table 10). An ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used under the following conditions: 1 cycle of 48°C for 30 minutes, 1 cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

**Statistics.** Significance determined by nonparametric *t* test, paired or unpaired *t* tests, or two-way ANOVA with post-hoc tests of significance. All *P* values are two-tailed at a 95% confidence interval. Analyses were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA).
RESULTS

Generation and confirmation of WES204. It has previously been shown that a xylose-inducible expression system can be utilized in *H. influenzae* to achieve tight regulation of a gene of interest (399). Therefore, an inducible *luxS* strain of nontypeable *H. influenzae* 86-028NP was generated by placing *luxS* under control of the *xylA* promoter, which is activated by the presence of xylose. To accomplish this, a plasmid was constructed containing a deletion of ~800 bp of *xylA* and a fusion of *luxS* to the *xylA* promoter, with flanking regions to allow for homologous recombination into the *xyl* region of the *H. influenzae* genome (see Materials and Methods, Figure 21A). The inducible construct was introduced into a *luxS* null strain of *H. influenzae* to prevent endogenous *luxS* expression, ensuring that AI-2 can only be produced in the presence of xylose. The resulting inducible *luxS* strain of nontypeable *H. influenzae* 86-028NP was named WES204.

As *luxS* is the genetic determinant of AI-2 production, induction of *luxS* by xylose should result in production of AI-2. To test AI-2 production, WES204 was cultured in media containing varying concentrations of xylose, and supernatant samples were taken to assess total AI-2 production over time compared to the parental strain 86-028NP (Figure 21B). Incubation with 5 mM xylose resulted in similar levels of AI-2 as produced by 86-028NP. This was particularly striking when the cultures reached an OD$_{600}$ of approximately 0.800, the stage of growth that corresponds with peak AI-2 production. Importantly, no AI-2 was detected in cultures of WES204 in the absence of
Figure 21. Generation of an inducible *luxS* strain and confirmation of induction.

(A) Schematic showing generation of the inducible-*luxS* strain (WES204), depicting *luxS* under control of the *xylA* promoter and flanking regions used for homologous recombination into the *xyl* region of the 86-028NP *luxS* chromosome. (B) *H. influenzae* strain WES204 was cultured in sBHI supplemented with increasing concentrations of D-xylose. Supernatant samples were taken at three times during growth for *V. harveyi* bioluminescence to compare AI-2 production under the inducing conditions to 86-028NP. Error bars represent mean and standard deviation.
xylose, and WES204 did not exhibit any growth defects in minimal medium or rich media in the presence or absence of xylose (data not shown).

**Surface-accessible phosphorylcholine is modulated by luxS.** Previous work from our laboratory showed that luxS and AI-2 quorum signaling impact lipooligosaccharide composition (395). Specifically, the luxS mutant expressed less surface-accessible phosphorylcholine (PCho) than the parental strain. To further verify nontypeable *H. influenzae* strain WES204, colony immunoblotting and whole-bacterium ELISA were used to determine the impact of luxS induction on PCho expression (Figure 22). By colony immunoblotting, WES204 colonies lifted from sBHI plates showed a similar percentage of PCho+ colonies as 86-028NP luxS, and both had fewer PCho+ colonies than the parental strain (Figure 22A). WES204 colonies lifted from sBHI plates supplemented with 5 mM xylose had a higher percentage of PCho+ colonies than colonies taken from plain sBHI plates, and a similar percentage of PCho+ colonies as the parental strain. The same trend was observed with whole-bacterium ELISA, where 86-028NP luxS and un-induced WES204 had decreased surface accessible PCho compared to 86-028NP, and WES204 from plates supplemented with xylose had similar levels of PCho as 86-028NP (Figure 22B). Taken together, these data indicate that induction of luxS with 5 mM xylose restores surface PCho to a similar level as observed for the parental strain.

**Induction of luxS expression promotes stationary biofilm development.** Using the inducible luxS system, the impact of luxS expression on biofilm development can be directly tested by modulating the timing of expression and observing the resulting
Figure 22. Induction of *luxS* expression increases phosphorylcholine expression. *H. influenzae* strains 86-028NP, 86-028NP *luxS*, and WES204 were cultured overnight on sBHI agar with or without 5 mM xylose. (A) Bacterial colonies were lifted onto nitrocellulose membranes and incubated with anti-PCho monoclonal antibody for determination of the percentage of colonies positive for PCho expression. (B) Whole-bacteria ELISA using anti-PCho monoclonal antibody for detection of surface-accessible PCho. Error bars represent mean and standard deviation. ***P<0.0001
changes in biofilm structural parameters. Stationary biofilms were established by nontypeable *H. influenzae* strains 86-028NP, 86-028NP *luxS*, and WES204 for 12 hours in media with or without 5 mM xylose. Biofilms were then visualized by live/dead staining and confocal microscopy (Figure 23A-D). As anticipated, WES204 biofilms established under inducing conditions were similar to those formed by 86-028NP (Figure 23A-B), while WES204 biofilms in the absence of xylose were comparable to those formed by the *luxS* mutant (Figure 23C-D). The presence of xylose did not significantly alter biofilm formation by 86-028NP or 86-028NP *luxS* (data not shown). COMSTAT analysis of the biofilm samples further confirmed that induction of *luxS* by xylose significantly increased the average thickness (Figure 23E) and total biomass (Figure 23F) of WES204 biofilms to a similar level as observed for the parental strain. Thus, *luxS* expression is necessary for full biofilm formation by nontypeable *H. influenzae* strain 86-028NP and induction of *luxS* results in the formation of biofilms that resemble those formed by the parental strain.

The inducible system can also be used to determine the temporal requirement for *luxS* expression during biofilm development. To investigate the impact of inducing *luxS* expression after initial biofilm formation, stationary biofilms were established in media with or without xylose for 21 hours. At this time, supernatants were carefully removed and replaced with fresh media with or without xylose as indicated, and biofilms were incubated for an additional three hours prior to visualization by live/dead staining and confocal microscopy. As above, WES204 biofilms established with xylose for 24 hours were similar to those formed by 86-028NP and had increased average thickness (Figure 23G) and total biomass (Figure 23H) compared to un-induced WES204 biofilms.
Figure 23. Induction of luxS expression promotes biofilm development in a stationary system. Stationary biofilms were established by H. influenzae strains 86-028NP, 86-028NP luxS, and WES204 in sBHI with or without 5 mM xylose as indicated, and stained with a BacLight live/dead stain for confocal microscopy. Vertical z-series images were compiled to generate representative volume views of 12 h biofilms formed by 86-028NP (A), WES204 induced by xylose for 12 h (B), 86-028NP luxS (C), and un-induced WES204 (D). Z-series images of biofilms formed for 12 h (E and F), 24 h (G and H), or 48 h (I and J) were exported into Matlab for COMSTAT analysis of biofilm average thickness (E, G, I) and total biomass (F, H, J). Error bars represent mean and standard deviation.
Notably, induction of *luxS* for three hours after the initial stages of biofilm development was sufficient to increase both biofilm thickness and biomass, suggesting that AI-2 quorum signaling can promote biofilm thickness and maturation in a relatively rapid manner, regardless of the stage of biofilm development. Similar results were obtained with biofilms established for 48 hours, where induction of *luxS* three hours prior to visualization by confocal microscopy was again sufficient to drive increased biofilm thickness (Figure 23I) and total biomass (Figure 23J).

**Modulation of *luxS* expression alters both biofilm development and dispersal.** The previous experiments have established a role for *luxS* expression in promoting biofilm development and thickness in a static culture, regardless of the timing of *luxS* induction. However, as it can be technically challenging to study biofilm dispersal in stationary culture, the role of *luxS* and AI-2 quorum signaling in biofilm dispersal remain unclear. In order to address biofilm dispersal and further verify our stationary biofilm findings, biofilms were established by gfp-expressing WES204 under continuous media flow conditions (Figure 24). Biofilms were imaged by confocal microscopy at 24 hours and, using 3-way stopcock luer locks, the media source to each chamber was either held constant or switched from media alone to media containing xylose (or vice versa) as indicated. Biofilms were imaged again three hours after the media switch and at 48 hours to observe the impact of *luxS* induction and interruption of *luxS* expression on biofilm development. As expected, WES204 established and maintained in media alone had significantly decreased total biomass (Figure 24A) and average thickness (Figure 24B) compared to biofilms established and maintained in media with xylose. Additionally, the
Figure 24. Modulation of *luxS* expression alters biofilm development and dispersal.

Biofilms were established by *gfp*-WES204 under continuous media flow conditions in sBHI or sBHI supplemented with 5 mM xylose as indicated and imaged by confocal microscopy at 24, 27, and 48 h post-inoculation for COMSTAT analysis of total biofilm biomass (A) and average thickness (B). At 24 h post-inoculation, the media source was switched when indicated from sBHI to sBHI supplemented with xylose or vice versa and maintained for the remainder of the experiment. Error bars represent mean and standard deviation.
same trend observed in stationary culture was observed for WES204 biofilms established in media alone and switched to media with xylose, where induction of luxS promoted increased total biomass and average biofilm thickness. Interestingly, biofilms established in media containing xylose and switched to media alone exhibited a dramatic decrease in total biomass and decreased average thickness three hours after the interruption of luxS expression produced by the media switch. These biofilms remained at decreased biomass and thickness, similar to the levels observed for un-induced WES204, for the duration of the experiment following the switch to media without xylose.

**Continuous luxS expression prevents biofilm dispersal.** The results of the media switch experiment indicate that luxS expression not only influences biofilm development but also appears to provide an anti-dispersal signal, as interruption of luxS expression resulted in rapid dispersal of WES204 biofilms. If LuxS and AI-2 quorum signaling act to prevent or postpone biofilm dispersal, it is possible that production of quorum signal decreases as the biofilm matures and approaches dispersal. To test this hypothesis, stationary biofilms of nontypeable *H. influenzae* strain 86-0-28NP were established for 6, 12, 24, 48, or 72 hours, and RNA was isolated for real time RT-PCR analysis of luxS transcript levels (Figure 25). Interestingly, luxS transcript levels were highest at six hours and decreased steadily as the biofilms aged. By 72 hours post-inoculation, a time point thought to correspond with nontypeable *H. influenzae* biofilm dispersal, luxS transcript levels were approximately half the level observed for gyrA transcript.

The decreased transcription of luxS as biofilms mature and approach dispersal supports a model in which LuxS and AI-2 quorum signaling act early on during
Figure 25. Transcription of *luxS* decreases as biofilms approach dispersal. *H. influenzae* 86-028NP stationary biofilms were established in sBHI for 6, 12, 24, 48, and 72 h for isolation of RNA and measurement of LuxS transcripts by real time RT-PCR. Data are expressed as the ratio of LuxS to GyrA. Error bars represent mean and standard deviation.
biofilm formation to promote development towards a mature biofilm, followed by a decrease in AI-2 signaling to allow for dispersal. If this model is correct, continuous induction of luxS should actually prevent biofilm dispersal. To test this hypothesis, biofilms were established by gfp-expressing *H. influenzae* strains 86-028NP and WES204 under continuous media flow conditions for 96 hours with confocal microscopy every 24 hours (Figure 26A). 86-028NP biofilms were cultured in media alone, while WES204 biofilms were cultured with xylose for the duration of the experiment to maintain continuous induction of luxS expression. As expected based on the previous experiments, biofilms formed by 86-028NP and induced WES204 had comparable average thickness at both 24 and 48 hours, again confirming that induction with 5 mM xylose drives biofilm development in a similar manner as the parental strain. As the biofilms progressed to 72 and 96 hours, 86-028NP biofilms began to disperse as evidenced by a significant decrease in average thickness, while fully induced WES204 biofilms remained at approximately the same thickness with only a modest decrease between 72 and 96 hours (Figure 26A). SEM images of the biofilms at 96 hours post-inoculation revealed the extent of biofilm dispersal for *H. influenzae* strain 86-028NP, compared to the full biofilm structure present for induced WES204 at this time point (Figure 26B). 86-028NP mostly dispersed from the surface of the flow chamber, as evidenced by the presence of only a few large clusters of bacteria. In contrast, the biofilm formed by WES204 in media with xylose still covered the entire surface of the flow chamber with the full, thick biofilm structure characteristic of nontypeable *H. influenzae* visible at higher magnification. We thus conclude that luxS expression both promotes biofilm development and acts to prevent or delay dispersal.
Figure 26. Continuous luxS expression prevents biofilm dispersal. Biofilms were established by *H. influenzae* gfp-expressing strains 86-028NP and WES204 for 96 h under continuous media flow, with confocal microscopy every 24 h for COMSTAT analysis of average biofilm thickness (A). 86-028NP biofilms were cultured in sBHI, and WES204 was cultured in sBHI supplemented with 5 mM xylose. Error bars represent mean and standard deviation. **P<0.01, ***P<0.001. (B) Biofilms were processed for SEM at 96 h post-inoculation.
A

Average Thickness (microns)

86-028NP  
WES 204

24 h  48 h  72 h  96 h

**  ***

B

86-028NP 96 h  Induced luxS 96 h

0.1 mm  0.1 mm

0.01 mm  0.01 mm
DISCUSSION

Previous work from our laboratory and others established a prominent role for LuxS and AI-2 quorum signaling in nontypeable *H. influenzae* pathogenesis (323, 395). Specifically, we found that loss of AI-2 quorum signaling alters biofilm development and maturation and ultimately results in a persistence defect during chronic infection (395). In this study, we directly tested the role of *luxS* expression in biofilm development, maturation, and dispersal using an inducible *luxS* strain of nontypeable *H. influenzae*. The results of this study indicate that *luxS* expression and AI-2 quorum signaling not only promote biofilm thickness and maturation, but also act to prevent or delay biofilm dispersal during the development and maturation process.

AI-2 signaling is a tightly regulated process in which *luxS* expression and subsequent production of AI-2 peak as bacteria reach a specific growth stage and population density, followed by internalization of and response to the AI-2 signal. Gene regulation induced by AI-2 signaling generally includes upregulation of *luxS* expression which further increases AI-2 production. For nontypeable *H. influenzae*, increased *luxS* transcription correlates with peak AI-2 production (C. E. Armbruster et al., manuscript submitted). As auto-regulation of AI-2 production/*luxS* expression would most likely require the native *luxS* promoter, it is possible that having *luxS* under control of the *xylA* promoter may alter the kinetics and regulation of AI-2 production. However, based on the bioluminescence studies it is clear that induction of *luxS* with 5 mM xylose resulted in production of a similar amount of AI-2 as observed for the parental strain, particularly at the stage of growth that correlates with peak AI-2 production. Induction of *luxS* was also
clearly sufficient to promote phosphorylcholine expression and normal biofilm development, indicating that if regulation of AI-2 production was slightly altered by placing luxS under control of the xylA promoter, it did not have a noticeable impact on biofilm-related phenotypes.

In the context of the nontypeable H. influenzae biofilm developmental cycle, luxS expression is clearly involved in regulation of both biofilm maturation and the dispersal processes, as interruption of luxS induction caused rapid biofilm dispersal while continuous induction completely inhibited dispersal. Many factors have been implicated in signaling biofilm development and dispersal for other bacterial species, including availability of carbon and oxygen, calcium concentration, iron availability, glucose depletion, protease activity, nitric oxide exposure, intracellular cyclic di-GMP levels, and various cell-cell signaling mechanisms (81, 82, 84-90). Quorum signaling systems are also frequently interconnected with global regulators and pathways for sensing physiological and environmental signals that can work in concert to promote biofilm formation or dispersal (88, 401-404). It has yet to be determined exactly how luxS expression and AI-2 quorum signaling regulate biofilm formation and dispersal in nontypeable H. influenzae, but the AI-2 signaling pathway may integrate with pathways for sensing and responding to the concentration of iron, oxygen, and/or various nutrient sources.

The rapid biofilm dispersal observed upon interruption of luxS expression supports a role for AI-2 quorum signaling in regulation of biofilm development and dispersal at least in part by inhibiting expression or activation of factors involved in dispersal. Biofilm dispersal generally involves a reduction in bacterial adhesiveness and
modulation or breakdown of the EPS matrix through factors such as proteases or nucleases to release bacteria back into a planktonic mode of growth (74). Thus, one possibility is that AI-2 quorum signaling promotes the expression of factors involved in adherence to a substrate or aggregation of the bacteria, and decreased AI-2 signaling reduces expression of these factors. Several factors involved in adhesion and aggregation of *H. influenzae* may fit with AI-2 regulation of dispersal in this manner (51). Expression of *luxS* also clearly plays a role in modulation of lipoooligosaccharide phosphorylcholine content, which impacts adhesion and biofilm maturation (395). Therefore, AI-2 signaling may impact biofilm development and dispersal through temporal regulation of lipoooligosaccharide content in a manner that is independent of phase variation at the lic locus. In addition, based on the rapid changes in biofilm structural parameters in response to modulation of *luxS* expression, it is likely that AI-2 signaling also regulates the expression or activity of an enzyme involved in degradation of structural components of the biofilm.

Based on our findings, inhibition of AI-2 quorum signaling may represent an effective strategy for limiting single species and possibly polymicrobial biofilm formation or inducing dispersal of established biofilms (145, 394). However, dispersal is a natural and advantageous process through which bacteria are released from a biofilm to establish new bacterial communities, particularly when facing unfavorable conditions (79, 80). In the context of biofilm-associated infection, one theory is that opportunistic pathogens such as nontypeable *H. influenzae* form biofilms during infection to limit the immune response and overall inflammation and establish persistent communities that more closely resemble a carriage state or colonization than active infection. These
biofilm communities are thus resistant to host immune clearance, as well as being more resistant to antibiotic treatment. Designing therapeutics that inhibit quorum signaling to force biofilm dispersal for this type of infection could increase efficacy of antibiotic treatment by breaking up the highly resistant biofilm structures and allowing the immune system and antimicrobial compounds access to the bacteria. Alternatively, forcing biofilm dispersal could result in severe systemic disease as the bacteria leave a localized site of infection, potentially spreading to the blood stream and throughout the host. In this context, biofilm formation has been proposed as a mechanism by which pathogens accumulate a sufficient quantity of bacteria to cause severe infection upon dispersal (67, 405, 406). Therefore, further study is necessary to investigate the specifics of *H. influenzae* biofilm dispersal in vitro and during experimental infection to determine the impact of inducing dispersal on both the severity and treatability of nontypeable *H. influenzae* infections.
CHAPTER IV:

Indirect Pathogenicity of Haemophilus influenzae and Moraxella catarrhalis in Polymicrobial Otitis Media Occurs via Interspecies Quorum Signaling

INTRODUCTION

Otitis media (OM) is one of the most common childhood infections (36-38) and is the leading reason for pediatric office visits and new antibiotic prescription to children (57). OM infections often persist for long periods of time, and are frequently recalcitrant to antibiotic treatment (50, 51). Due to the highly resistant nature of chronic and recurrent OM, these infections have long been thought of as involving bacterial persistence within a biofilm (67, 72, 110). Clinical evidence of bacterial biofilms includes direct observation of biofilms in patient tissue (105) and in the chinchilla experimental model for OM (106-109, 393). Persistence of bacteria within a biofilm community can also greatly increase resistance to antibiotics (216, 407) through numerous mechanisms including phenotypic heterogeneity and slower growth of bacteria within the biofilm, delayed antibiotic penetration through matrix material/exopolysaccharide, and the presence of persister cells (92-95).

As with most upper airway infections, epidemiological data indicate that the majority of chronic OM infections are polymicrobial in nature (55). For example, *Haemophilus influenzae* and *Moraxella catarrhalis* are frequently present together in patient samples from chronic and recurrent OM (55, 104). Interestingly, a recent study found *M. catarrhalis* to be more frequently isolated from polymicrobial OM infections than as the single causative agent of OM (136). This suggests that the presence of other bacterial pathogens may impact persistence of *M. catarrhalis* or the severity of disease caused by this species. Additionally, *M. catarrhalis* is thought to confer passive antibiotic resistance upon other OM pathogens via secretion of beta-lactamase (139-144).
However, the impact of polymicrobial infection on bacterial persistence, virulence, or response to treatment is not presently clear.

Interbacterial communication via quorum signaling is one factor which may impact establishment of chronic polymicrobial infection as quorum signaling is known to influence biofilm development for many species (308, 360). Autoinducer-2 (AI-2) is commonly referred to as an interspecies signal, as the genetic determinant for AI-2 production (luxS) is conserved among numerous bacterial species (301, 310, 322). AI-2 is known to influence biofilm for many species, including *H. influenzae* (395), and in some instances AI-2 can impact the development of polymicrobial biofilms (366, 367). In this study, we addressed the hypothesis that polymicrobial infection impacts biofilm development and resistance during OM disease. The results clearly show that *H. influenzae* promotes *M. catarrhalis* persistence by means of interspecies quorum signals that increase the resistance of *M. catarrhalis* in biofilm.
MATERIALS AND METHODS

Bacterial strains and culture conditions. A complete list of bacterial strains used in this study is provided in Table 1. *M. catarrhalis* strains were cultivated in brain heart infusion (BHI) medium (Difco) and *H. influenzae* strains were cultivated in BHI supplemented with hemin (ICN Biochemicals) and NAD (Sigma); this medium is referred to below as supplemented BHI (sBHI). For experiments using trimethoprim-sulfamethoxazole, *H. influenzae* and *M. catarrhalis* were cultured in Morse’s defined medium (408) supplemented with hemin and NAD. *H. influenzae siaB* was constructed essentially as described previously for 2019 *siaB* (409) and confirmed by immunoblot for decreased reactivity with *Limax flavus* (LFA) lectin (EY Laboratories).

SEM. Stationary in vitro biofilm cultures were grown in Tab-TekII coverglass slides (Nunc). Each chamber was inoculated with \(~10^8\) cfu/ml of either *H. influenzae*, *M. catarrhalis*, or a 1:1 mixture of both species and incubated for 48 h at 37°C and 5% CO₂. Biofilm samples were fixed for 30 min with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) and rinsed once. Samples were then dehydrated, fixed, and prepared for scanning electron microscopy (SEM) analysis as previously described (220). Biofilm samples were mounted onto stubs, sputter coated with palladium, and then viewed with a Philips SEM-515 scanning electron microscope.

Confocal Laser Scanning Microscopy (CLSM). In vitro biofilm cultures were grown using a continuous-flow system as previously described (108). *H. influenzae* and *M. catarrhalis* were cultured overnight in sBHI broth and diluted to \(~10^8\) cfu/ml. Chamber
Table 11. Bacterial strains.
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<td><em>H. influenzae</em> 86-028NP</td>
<td>Nasopharyngeal isolate from child with OM</td>
<td>(381)</td>
</tr>
<tr>
<td><em>H. influenzae</em> licD</td>
<td><em>H. influenzae</em> 86-028NP NTHI1594 mutant</td>
<td>(226)</td>
</tr>
<tr>
<td><em>H. influenzae</em> licON</td>
<td><em>H. influenzae</em> 86-028NP constitutive <em>PCho</em>⁺</td>
<td>(108)</td>
</tr>
<tr>
<td><em>H. influenzae</em> luxS</td>
<td><em>H. influenzae</em> 86-028NP NTHI 0621 mutant</td>
<td>(395)</td>
</tr>
<tr>
<td><em>H. influenzae</em> siaB</td>
<td><em>H. influenzae</em> 86-028NP NTHI 1891 mutant</td>
<td>This study</td>
</tr>
<tr>
<td><em>H. influenzae</em> Rd</td>
<td><em>H. influenzae</em> RD KW20</td>
<td>(410)</td>
</tr>
<tr>
<td><em>M. catarrhalis</em> 7169</td>
<td><em>Moraxella catarrhalis</em> serotype B strain</td>
<td>(411)</td>
</tr>
</tbody>
</table>
slides were inoculated with each strain alone or a 1:1 mixture of both species and incubated for 24, 48, and 72 h at 37°C and 5% CO₂. At each timepoint, biofilms were fixed and stained with rabbit polyclonal anti-\textit{H. influenzae} sera (382) and/or monoclonal antibody 4G5 (412). Mab 4G5 was generously provided by Anthony Campagnari. All secondary antibodies were purchased from Jackson Laboratories. Biofilms were visualized using a Zeiss LSM510 CLSM microscope and the Zeiss LSM Image Browser software.

\textbf{Antibiotics.} Antibiotics used were ampicillin (Sigma), clavulanate (Sigma), clarithromycin (Abbott Laboratories), trimethoprim (Sigma), and sulfamethoxazole (Sigma). Trimethoprim-sulfamethoxazole experiments were conducted using a 1:5 ratio. Concentrations listed refer to trimethoprim.

\textbf{Biofilm antibiotic protection studies.} Bacteria were grown overnight on sBHI or BHI plates, then suspended in sBHI and diluted to \textasciitilde10⁸ cfu/ml. A 24-well microtiter plate was inoculated with a single species suspension diluted 1:1 with PBS, or a 1:1 mixture of both bacterial suspensions. Cultures were incubated at 37°C and 5% CO₂ for either 4 h or 24 h to establish biofilms. Supernatants were then carefully removed and replaced with either fresh sBHI or sBHI with antibiotic, and cultures were returned to 37°C and 5% CO₂ for 24 h. Following incubation, supernatants were removed and biofilms were resuspended in PBS for serial dilution and plating to enumerate viable bacteria. Polymicrobial biofilms were plated onto both BHI and sBHI containing 2 µg/ml clarithromycin to distinguish between \textit{M. catarrhalis} and \textit{H. influenzae} respectively.
**M. catarrhalis AI-2 studies.** All studies were conducted using 0.2 μM dihydroxypentanedione (DPD, Omm Scientific). This concentration of DPD was chosen to simulate the amount of AI-2 produced by *H. influenzae* as it elicits approximately equivalent luminescence from *Vibrio harveyi* as *H. influenzae* late exponential phase culture supernatant. For AI-2 depletion studies, BHI or sBHI were supplemented with DPD when indicated, inoculated with ~10⁸ cfu of *M. catarrhalis* or *H. influenzae luxS*, and incubated at 37°C and 150 rpm for 6 h. Samples were taken at 0.25, 0.5, 0.75, 1, 2, 3, and 6 h, centrifuged, filter-sterilized and stored at -20°C for bioluminescence. Luminescence produced by *Vibrio harveyi* BB170 (348) following 3 h incubation with supernatant samples was determined in a Turner Designs TD-20/20 luminometer for 10 s. Data are reported as relative light units [counts per 10 s]. Tetracycline studies were conducted by incubating *M. catarrhalis* with 10 μg/ml tetracycline (Sigma). *M. catarrhalis* cultures were incubated with tetracycline during overnight growth in a broth culture to completely inhibit bacterial growth/viability, as well as at the start of the 7 h AI-2 depletion study to monitor the role of protein synthesis in AI-2 depletion. For crystal violet staining of *M. catarrhalis* biofilms, wells of a 24-well tissue culture plate containing sBHI or sBHI supplemented with 0.2 μM DPD were inoculated with ~10⁸ cfu of *M. catarrhalis* and incubated at 37°C and 5% CO₂. Supernatants were carefully removed at each timepoint, biofilms were washed 1x with H₂O, stained with 0.1% crystal violet for 30 min, washed 2x with H₂O, and solubilized in ethanol for 10 minutes prior to measuring optical density at 600 nm. For CLSM and SEM, *M. catarrhalis* biofilms were established in sBHI or sBHI supplemented with 0.2 μM DPD for 24 h. Biofilms were
then prepared for SEM as above, or washed once with PBS and stained with a LIVE/DEAD BacLight viability kit (Invitrogen) prior to imaging by CLSM as above.

**Chinchilla infection studies.** Bacterial persistence and biofilm formation in the middle ear chamber were assessed as described previously (107, 108). Chinchillas were purchased from Rauscher’s chinchilla ranch (Larue, OH) and allowed to acclimate to the vivarium for >7 d prior to infection. No animals showed visible signs of illness by otoscopy prior to infection. The animals were anesthetized with isofluorane and infected via transbullar injection with \( \sim 10^3 \) cfu of *H. influenzae* or *H. influenzae luxS*, \( \sim 10^4 \) cfu of *M. catarrhalis*, or a 1:1 mixture of both species. All inocula were confirmed by plate-count. At 7 d or 14 d post-infection, animals (4/group) were euthanized and middle-ear chambers were aseptically opened. Fluids effusion fluids were recovered and middle ear lavage was performed using 1.0 ml sterile PBS. Viable bacteria were enumerated by plate counting the combined retrieved fluids. Fluids from animals which received polymicrobial inocula were plated onto both sBHI containing 2 \( \mu \)g/ml clarithromycin and BHI lacking NAD and hemin. Bullae were excised and homogenized in 10 ml sterile PBS, then plated to determine cfu of tissue-associated bacteria (107).

**Statistics.** Significance determined by nonparametric *t* test, unpaired *t* test with Welsh’s correction, or two-way ANOVA with post-hoc tests of significance. All *P* values are two-tailed at a 95% confidence interval. Analyses were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA).
RESULTS

*H. influenzae* and *M. catarrhalis* form polymicrobial biofilms in vitro. Based on clinical evidence for the coexistence of *H. influenzae* and *M. catarrhalis* in OM cases, it was hypothesized that these bacterial species would coexist in culture and in vitro biofilms. Static biofilms of *H. influenzae*, *M. catarrhalis*, or a mixture of both species were established in microscopy chamber slides and the surface attached bacterial communities were examined by scanning electron microscopy (SEM, Figure 27A) and confocal laser scanning microscopy (Figure 27B) at varying times during biofilm development. As previously observed, *H. influenzae* formed matrix-encased biofilm communities on the chamber slide surface (108, 220, 395). In contrast, *M. catarrhalis* formed smaller surface-attached clusters. In coculture, *H. influenzae* and *M. catarrhalis* formed polymicrobial biofilms with both species incorporated into the biofilm structure as indicated by the presence of larger *M. catarrhalis* diplococci interspersed with the smaller *H. influenzae* coccobacilli (Figure 27A). Immunostaining and confocal laser scanning microscopy showed that *M. catarrhalis* communities were present in discrete regions within the *H. influenzae* biofilm structure (Figure 27B). Based on these results, we conclude that *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms.

Polymicrobial biofilms provide passive antibiotic resistance. Beta-lactam antibiotics are commonly prescribed to children with OM (63). *M. catarrhalis* strains are nearly universally resistant to these antibiotics via secretion of beta-lactamase, and passive resistance during coinfection with *M. catarrhalis* has been postulated as a mechanism for
Figure 27. *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms in vitro.

Stationary biofilms were established in chamber slides for visualization of bacteria by SEM and confocal laser scanning microscopy (CLSM). (A) Samples of *H. influenzae* and *M. catarrhalis* single species or polymicrobial biofilms were taken at 48 h and prepared for SEM. Images shown are at three different levels of magnification. (B) CLSM was performed on 24, 48, and 72 h biofilms following staining for *H. influenzae* (red) and *M. catarrhalis* (green).
antibiotic resistance of many airway pathogens. Therefore, we asked if *M. catarrhalis* could provide passive protection for a beta-lactam sensitive *H. influenzae* strain within a polymicrobial biofilm. Biofilms were established for 24 h and treated with ampicillin or a combination of ampicillin and the beta-lactamase inhibitor clavulanate (Figure 28). As anticipated, biofilms formed by *H. influenzae* were susceptible to ampicillin treatment and *M. catarrhalis* biofilms were resistant. Polymicrobial biofilm formation increased recovery of viable *H. influenzae*, indicating that *M. catarrhalis* provided protection against ampicillin treatment. The addition of clavulanate abolished the increased recovery of *H. influenzae*, indicating that protection was due to beta-lactamase produced by *M. catarrhalis*. As anticipated, clavulanate also reduced recovery of viable *M. catarrhalis* from single species biofilms. Of note, formation of a polymicrobial biofilm with *H. influenzae* increased recovery of *M. catarrhalis* even in the presence of clavulanate, suggesting that inherent properties of the polymicrobial biofilm provide antibiotic protection in addition to secreted beta-lactamase.

A combination of trimethoprim-sulfamethoxazole was next used to test the hypothesis that polymicrobial biofilms provide passive protection independent of diffusible resistance determinants. *H. influenzae* biofilms were more susceptible to this antibiotic combination while *M. catarrhalis* biofilms were more resistant (data not shown). Polymicrobial biofilms formed by *H. influenzae* and *M. catarrhalis* afforded protection to *H. influenzae* from trimethoprim-sulfamethoxazole as indicated by increased recovery of viable *H. influenzae* from these biofilms (Figure 29A). Control experiments indicated that bacteria recovered from polymicrobial biofilms retained similar broth culture susceptibility characteristics as the inocula (data not shown). These
Figure 28. **Beta-lactam protection in a polymicrobial biofilm.** Stationary biofilms of *H. influenzae* Rd and/or *M. catarrhalis* were established in chamber slides for 24 hours and treated with 100 µg/ml ampicillin or ampicillin with 25 µg/ml clavulanate for an additional 24 h. Biofilms were resuspended in sterile PBS, serially diluted, and plated on sBHI + clarithromycin and BHI plates for enumeration of viable *H. influenzae* Rd and *M. catarrhalis* respectively. Data are represented as mean ± SEM. *P*<0.05 compared to *H. influenzae* + ampicillin. **P*<0.05 compared to polymicrobial *H. influenzae* + ampicillin. ***P*<0.05 compared to *M. catarrhalis* + ampicillin.
Figure 29. Polymicrobial biofilm formation protects *H. influenzae* and *M. catarrhalis* from antibiotic treatment. Single species or polymicrobial stationary biofilms were established for 4 h and treated with (A) 60 µg/ml trimethoprim-sulfamethoxazole (TS) or (B) 6 µg/ml clarithromycin for 24 h. Biofilms were resuspended in sterile PBS, serially diluted, and plated on sBHI + clarithromycin or BHI for enumeration of viable *H. influenzae* and *M. catarrhalis* respectively. Data are represented as the mean for three combined experiments, two replicates per experiment. Error bars represent SEM. *P<0.05.
data show that antibiotic protection can be provided by the polymicrobial biofilm independent of genetic changes or transfer of resistance determinants between species.

In order to determine the contribution of *H. influenzae* biofilm to antibiotic protection within a polymicrobial biofilm, the macrolide clarithromycin was used at a concentration that eradicates *M. catarrhalis* within biofilm (Figure 29B). Polymicrobial biofilms formed by *M. catarrhalis* and *H. influenzae* protected *M. catarrhalis* from clarithromycin treatment, as indicated by a significant increase in recovered viable *M. catarrhalis*. Importantly, the amount of protection afforded to *M. catarrhalis* by biofilms containing *H. influenzae* mutants with biofilm defects (siaB, licD, luxS) (109, 220, 226, 382, 395) was significantly diminished as compared with the parental strain (Figure 29B). Conversely, protection of *M. catarrhalis* was increased in biofilms formed with *H. influenzae lic\textsuperscript{ON},* a mutant strain which forms thicker biofilms (108). Control experiments showed that bacteria recovered from all polymicrobial biofilms retained broth susceptibility characteristics similar to the inocula, indicating that the increased antibiotic resistance observed in polymicrobial biofilms was not due to genetic changes or transfer of resistance determinants between species (data not shown). Based on these data, we conclude that the maturation state and/or overall biomass of the polymicrobial biofilm play integral roles in the antibiotic protection provided by *H. influenzae* biofilms.

**Autoinducer-2 (dihydroxypentanedione) promotes *M. catarrhalis* biofilm thickness and antibiotic resistance.** The decreased clarithromycin protection of *M. catarrhalis* by *H. influenzae luxS* could be due to differences in biofilm thickness or the maturation state of the *luxS* mutant, similar to the mechanism for decreased protection by *H. influenzae*
licD. However, another possibility is that *M. catarrhalis* responds to AI-2 produced by *H. influenzae*, and the decrease in protection observed with *H. influenzae luxS* would thus be due to loss of the AI-2 quorum signal. *M. catarrhalis* is not known to possess a *luxS* homolog and did not produce detectable AI-2 during growth in a broth culture as measured by a *Vibrio harveyi* bioluminescence assay (Figure 30A). However, recent evidence suggests that bacterial species which do not make AI-2 may still sense and respond to the AI-2 signal (353, 370). To test this hypothesis, *M. catarrhalis* was cultured in broth supplemented with chemically synthesized AI-2 precursor dihydroxypentanedione (DPD), and samples were taken to determine the level of DPD remaining in the culture over time (Figure 30A). *M. catarrhalis* depleted DPD over the course of 6 hours, which indicates uptake and/or degradation of DPD, while an uninoculated control showed minimal decrease in the AI-2 signal by 6 h. Notably, the amount of DPD depleted by *M. catarrhalis* was similar to the amount depleted by *H. influenzae luxS*. To determine if depletion of AI-2 requires live bacteria and/or active protein synthesis, *M. catarrhalis* cultures were incubated with tetracycline overnight prior to the addition of DPD, or incubated with tetracycline concurrent with the addition of DPD. Samples were taken over the course of 7 h for comparison of DPD depletion to untreated *M. catarrhalis* (Figure 30B). Both tetracycline treatments completely inhibited depletion of DPD by *M. catarrhalis*, suggesting that depletion is an active process which requires protein synthesis. Additionally, incubation of *M. catarrhalis* culture supernatant with DPD did not result in depletion (data not shown). Taken together, these data indicate that *M. catarrhalis* is most likely depleting AI-2 by means of an uptake system rather than passive binding or external degradation of this signaling molecule.
Figure 30. AI-2 promotes *M. catarrhalis* biofilm development and antibiotic resistance. (A) *M. catarrhalis* was cultured in BHI media or BHI supplemented with 0.2 μM synthetic AI-2 (DPD) to determine AI-2 production and depletion as measured by *Vibrio harveyi* bioluminescence. *H. influenzae luxS* was cultured in sBHI supplemented with DPD to measure depletion. An uninoculated control of BHI media with DPD shows the minimal degradation of the AI-2 signal during 6 h of incubation at 37°C. (B) Depletion of DPD by *M. catarrhalis* following incubation with 10 μg/ml tetracycline was measured by bioluminescence over a period of 7 h. (C) *M. catarrhalis* biofilms were established in the presence or absence of DPD and stained with crystal violet for determination of biofilm biomass at 4, 6, 12, 24, and 48 h. Data represent the mean of three combined experiments, three replicate wells per experiment. Error bars represent SEM. (D-E) *M. catarrhalis* 24 h biofilms were established in the presence (E) or absence (D) of DPD and stained with a viability kit for CLSM visualization of surface coverage and biofilm thickness. (F-G) Z-series images from D and E were compressed to show total viable and non-viable staining of biofilms established in the presence (G) or absence (F) of DPD. (H-I) SEM images of *M. catarrhalis* 24 h biofilms established with (I) or without (H) DPD. (J) *M. catarrhalis* biofilms were established for 4 h in the presence or absence of DPD, then treated with 6 μg/ml clarithromycin for 24 h and plated for enumeration of viable bacteria. Data represent the mean of three replicates ± SEM. *P<0.05, **P<0.01, ***P<0.001.
To assess the impact of exogenous AI-2 on *M. catarrhalis* biofilm formation, stationary *M. catarrhalis* biofilms were established in the presence or absence of DPD and stained with crystal violet at 4, 6, 12, 24, and 48 hours (Figure 30C). Incubation with DPD resulted in an overall increase in *M. catarrhalis* biofilm biomass that was particularly evident at 24 and 48 hours. Viability staining and CLSM of *M. catarrhalis* biofilms confirmed the increased *M. catarrhalis* biofilm density in the presence of DPD, and further demonstrated an increase in bacterial viability within biofilm. *M. catarrhalis* biofilms established in media lacking DPD formed small clusters with mostly non-viable staining (Figure 30D) while biofilms established in the presence of DPD were thicker on average and showed an increased number of viable bacteria within the larger biomasses (Figure 30E). Compressed z-series images confirmed the increased viable staining present in the larger biomasses of DPD treated *M. catarrhalis* (Figure 30G) compared to untreated *M. catarrhalis* biofilms (Figure 30F). SEM of *M. catarrhalis* biofilms similarly demonstrated the impact of DPD on biofilm development, with treatment resulting in increased formation of bacterial clusters compared to *M. catarrhalis* biofilms established in media lacking DPD (Figure 30H-I).

Based on the imaging results, we hypothesized that DPD could increase resistance of *M. catarrhalis* to antibiotic treatment. To test this hypothesis, *M. catarrhalis* biofilms were established in the presence or absence of DPD for 4 h and treated with clarithromycin. Treatment with DPD did not significantly alter the overall recovery of viable *M. catarrhalis* from control wells (Figure 30J). However, *M. catarrhalis* biofilms established in the presence of DPD were inherently more resistant to clarithromycin, as indicated by increased recovery of viable bacteria following incubation with antibiotic.
Similar results were obtained using trimethoprim-sulfamethoxazole (data not shown). Taken together, these studies indicate that while *M. catarrhalis* 7169 does not produce AI-2, this strain does respond to the interspecies quorum signaling molecule by producing biofilms with increased biomass and resistance to antibiotic treatment.

**Interspecies quorum signaling during polymicrobial infection promotes persistence of *M. catarrhalis*.** The in vitro studies of polymicrobial biofilms support a prominent role for interspecies quorum signaling in development of *M. catarrhalis* biofilms with increased resistance phenotypes. As *M. catarrhalis* is frequently isolated from polymicrobial OM infections, we hypothesized that *M. catarrhalis* could utilize AI-2 produced by *H. influenzae* or other OM pathogens to persist in vivo. Therefore, infection studies were performed using the chinchilla model of OM to test this hypothesis. As previously observed, high numbers of *H. influenzae* and *H. influenzae luxS* were detected in middle ear effusion fluid (Figure 31A) and bullar homogenate (Figure 31B) at both 7 d and 14 d post-infection for single species and polymicrobial infection groups. Counts of *M. catarrhalis* within middle-ear effusion fluids were at or below the level of detection at both timepoints (Figure 31A), regardless of the type of infection. Animals infected with *M. catarrhalis* alone had bacterial load within the bullar homogenates at both 7 and 14 d post-infection that was consistent with the initial inocula. However, in the coinfected animals, significantly greater numbers of *M. catarrhalis* were observed in bullar homogenates 14 d post-infection (Figure 31B). Based on these results, we conclude that *M. catarrhalis* survives exclusively within surface-attached communities within the chinchilla middle-ear chamber, and that coinfection with *H. influenzae* provides a
Figure 31. Polymicrobial infection augments *M. catarrhalis* persistence in vivo.

Chinchillas were infected with $10^3$ cfu of *H. influenzae* or *H. influenzae luxS*, $10^4$ cfu of *M. catarrhalis*, or a mixture of both species. (A) Middle ear effusion fluids were removed for enumeration of viable *H. influenzae* and *M. catarrhalis* by plating on sBHI + clarithromycin or BHI, respectively. (B) Bullae were removed at each timepoint and homogenized for enumeration of viable *H. influenzae* and *M. catarrhalis* as above. Data represent the mean for four experiments ± SEM. *P<0.005 compared to cfu from *M. catarrhalis* single species bullar homogenate.
permissive environment in which *M. catarrhalis* can proliferate. We next asked if the increase in *M. catarrhalis* numbers during coinfection with *H. influenzae* were dependent on interspecies quorum signaling by performing similar coinfection studies using *H. influenzae luxS*. Notably, no increase in *M. catarrhalis* counts was observed during coinfection with *H. influenzae luxS* at either timepoint. Taken in concert with the experiments showing increased *M. catarrhalis* biofilm density and resistance following treatment with synthetic AI-2, these experiments show that *M. catarrhalis* can utilize exogenous AI-2 provided by *H. influenzae* to establish a persistent infection.
DISCUSSION

According to the long-standing concept of indirect pathogenicity, bacterial disease and/or response to treatment is subject to influence by other bacteria sharing the same environment (139, 140). In this study, *H. influenzae* was shown to promote persistence and antibiotic resistance of *M. catarrhalis* via protection within the biofilm structure in response to interspecies quorum signaling. The data presented in this study therefore provide concrete validation of the concept of indirect pathogenicity and provide a mechanism to support how this can occur during polymicrobial otitis media infections.

In the context of infectious disease, biofilm formation has long been considered to be an important determinant of bacterial persistence. It has also long been appreciated that growth within a biofilm increases resistance to antibiotics through multiple mechanisms that include delayed antibiotic penetration of the biofilm and changes in the metabolic state of bacteria within biofilms (92-95). In this study, both in vitro and in vivo models were used to ask how *H. influenzae* and *M. catarrhalis* polymicrobial biofilm formation impacts antibiotic resistance and bacterial persistence. While *M. catarrhalis* can be considered an indirect pathogen through production of beta-lactamase, the results of this study clearly prove that both *M. catarrhalis* and *H. influenzae* can provide antibiotic protection to other pathogens within a polymicrobial biofilm in a manner independent of diffusible resistance determinants. Additionally, the abrogation of antibiotic protection observed for polymicrobial biofilms formed with *H. influenzae* biofilm mutants demonstrates a role for biofilm maturation/total biomass in antibiotic
protection. This study and others support the hypothesis that biofilm can provide a barrier that protects susceptible organisms contained within it.

In addition to the impact of biofilm biomass on antibiotic susceptibility, this study solidifies the role of interspecies communication during establishment of polymicrobial biofilms. Mutation of the luxS gene, the genetic determinant of AI-2 production, alters H. influenzae biofilm maturation and density as well as bacterial persistence, indicating that AI-2 plays a critical role in development and maturation of H. influenzae biofilms (395). While M. catarrhalis does not produce AI-2, the results clearly demonstrate the critical role of interspecies quorum signaling via AI-2 in establishment of persistent polymicrobial biofilms containing this species and indicate the presence of an AI-2 transport system in M. catarrhalis. The main AI-2 transport system that has been described outside of the Vibrio species is the Lsr ABC transporter. The Lsr system, identified in Salmonella typhimurium, Escherichia coli, Sinorhizobium meliloti, and Aggregatibacter actinomycetemcomitans, has homology to the ribose ABC transporter and involves binding of AI-2 by LsrB and transport through a heterodimeric membrane channel (353, 356, 357, 396). In addition to LsrB, it was determined in A. actinomycetemcomitans that the ribose binding protein RbsB can also bind AI-2 (355, 359). The mechanism for sensing of DPD/AI-2 by M. catarrhalis has yet to be defined, but as the presence of this signaling molecule clearly alters M. catarrhalis biofilm development, this will be an important topic for future studies. Additionally, the increased antibiotic resistance following treatment with DPD also provides further support for quorum signaling contributing to establishment of a diffusion barrier to delay or limit antibiotic penetration of the biofilm.
The augmented persistence observed during infection by *M. catarrhalis* and the parental strain of *H. influenzae* but not with *H. influenzae luxS* further demonstrates the role of interspecies quorum signaling in establishment of polymicrobial OM. One possible explanation for the increase in *M. catarrhalis* persistence is that *M. catarrhalis* becomes incorporated into the biofilm scaffold provided by *H. influenzae*, and this incorporation protects *M. catarrhalis* from host factors thus allowing for increased persistence. As *H. influenzae luxS* has a persistence defect in the chinchilla model of OM, it is possible that the luxS mutant does not provide sufficient biofilm structure for protection of *M. catarrhalis*, or alternatively that other factors lacking in *H. influenzae luxS* are required for enhancement of *M. catarrhalis* persistence. However, the results demonstrating depletion of synthetic AI-2 by *M. catarrhalis*, as well as the increased *M. catarrhalis* biofilm density and resistance elicited by synthetic AI-2, argue that AI-2 quorum signaling from *H. influenzae* promotes *M. catarrhalis* persistence in polymicrobial biofilm. Therefore, we conclude that the production of AI-2 by *H. influenzae* promotes *M. catarrhalis* resistance within biofilm, and thereby promotes *M. catarrhalis* persistence within the middle-ear chamber. It is notable that prior studies involving infection of rodents with *M. catarrhalis* have historically failed to mimic the chronic and recurrent infections that are typical of human patients with opportunistic airway infections, but rather are typically transient in nature (255). The results presented here may indicate that this difference in bacterial persistence occurs as consequence of the absence of an “infection partner” such as *H. influenzae* to provide AI-2, as opposed to any species differences.
As we observed that both *M. catarrhalis* and *H. influenzae* can deplete AI-2/DPD yet only one of these species produces the signal, there could be competition between *M. catarrhalis* and *H. influenzae* for available AI-2 during coinfection. Based on the AI-2 requirement for *H. influenzae* to establish a chronic infection, any competition with *M. catarrhalis* would most likely have a negative impact on *H. influenzae* persistence. However, similar numbers of viable bacteria were recovered from coinfectected animals as those which received *H. influenzae* alone, indicating that AI-2 uptake by *M. catarrhalis* did not interfere with *H. influenzae* biofilm formation or persistence. Additionally, the presence of *M. catarrhalis* during stationary biofilm formation was not observed to negatively impact *H. influenzae* biofilm formation or antibiotic resistance. Taken together, these observations support a model wherein *H. influenzae* requires only a certain threshold level of AI-2, but may produce AI-2 in excess of the threshold concentration needed to promote biofilm development. In this model, AI-2 depleted from the biofilm environment by *M. catarrhalis* would not have a detrimental impact on *H. influenzae* biofilm development. Another possible explanation is that *M. catarrhalis* may only require a minimal concentration of AI-2 to alter biofilm development. This would be advantageous, as *M. catarrhalis* could utilize any AI-2 producing species as an “infection partner” to promote its own persistence without directly competing for the AI-2 signal. Further research will be necessary to determine the minimal AI-2 concentration required by both *M. catarrhalis* and *H. influenzae* to promote biofilm development.

The data presented in this study and others provide substantial evidence for the influence of polymicrobial infection on severity of disease and the outcome of antibiotic treatment, particularly for chronic infections involving persistence of bacteria within
biofilms. Notably, the results of this study imply that vaccination against upper airway pathogens such as the unencapsulated strains of *H. influenzae* may have a greater impact than expected. For instance, successful vaccination against *H. influenzae* may also disrupt establishment of disease by *M. catarrhalis*. Further research is necessary to elucidate the interactions between all three of the leading causative agents of OM and the impact of other polymicrobial upper airway infections on resistance to relevant antibiotics. Knowledge of the bacterial species present during highly recalcitrant infections may provide insight into which course of antibiotic treatment would be most effective. Additionally, AI-2 may represent an ideal target for disruption of numerous chronic and/or recurrent infections.
CONCLUSIONS

Despite widespread vaccination efforts, OM remains a significant health problem worldwide and the rate of treatment failure continues to escalate (53, 58, 59, 63, 64). One factor that may contribute to the high rate of treatment failure is the formation of biofilm communities during infection, as biofilm-associated bacteria can withstand antibiotic concentrations up to 1,500 times greater than bacteria in liquid broth culture (67, 91). Biofilms have been directly observed in patient samples and in the chinchilla experimental model for OM, indicating that biofilm formation is an important aspect of OM disease (105, 106, 112, 137, 392, 393). Therefore, understanding mechanisms involved in biofilm formation may provide new targets for disruption and treatment of biofilm-related infection.

Biofilm formation for many species is controlled in part through bacterial cell-to-cell communication, or quorum signaling, wherein changes in bacterial population phenotypes are mediated by accumulation of a soluble signaling mediator (301, 308-310). Such signaling networks include autoinducer-2 (AI-2), a ribose derivative produced by both Gram-negative and Gram-positive bacteria. AI-2 is commonly referred to as an interspecies signal as many bacteria possess the machinery to sense and respond to AI-2 (301, 310, 365, 371). Interspecies quorum signaling via AI-2 has therefore been proposed as one method used by bacteria to coordinate behavior within multispecies biofilms (365-367).

Polymicrobial biofilm formation may also have a profound effect on the course, severity, and treatability of OM. Recent epidemiological studies have established that the
majority of infections are polymicrobial in nature, including OM (55, 91, 111). However, the vast majority of pathogenesis studies are conducted using single species cultures of bacteria and fail to account for the impact of polymicrobial infection. There is a pressing need to better understand the mechanisms underlying the establishment of polymicrobial infection, and to investigate the impact of multiple species on the severity and treatability of OM disease. *H. influenzae* and *M. catarrhalis* are more frequently isolated from polymicrobial infection than individually, and these species are the leading causes of the chronic and recurrent presentations of OM that involve biofilm formation (55, 104, 135, 136). Thus, the focus of this body of work was to investigate coinfection by *H. influenzae* and *M. catarrhalis* and the impact of polymicrobial biofilm formation on persistence and antibiotic resistance, with an emphasis on the role of intra- and interspecies bacterial communication via AI-2 quorum signaling.

**Autoinducer 2 Quorum Signaling in Haemophilus influenzae**

Nontypeable *H. influenzae* strain 86-028NP was isolated from a child with chronic OM, and previous studies of this isolate have shown that 86-028NP readily forms biofilms in vitro and in the chinchilla model of OM (107, 226, 381). A nontypeable *H. influenzae luxS* mutant deficient in the ability to produce AI-2 quorum signal was still able to form biofilms, but with reduced total biomass and average thickness compared to the parental strain (Figure 8). Notably, the biofilm defect could be complemented by soluble products from the parental strain (Figure 9). The defect in biofilm development was found to relate to decreased LOS PCho content (Figure 10 and Table 6) that was not
linked to phase variation of LOS biosynthetic genes (Table 5). Similar to H. influenzae, biofilm formation by the opportunistic pathogen Klebsiella pneumoniae has been shown to involve both production of lipopolysaccharide (LPS) and AI-2 quorum signaling (413, 414). Inactivation of luxS or genes involved in AI-2 transport in K. pneumoniae alters the expression of LPS-related genes (413, 414), indicating that AI-2 quorum signaling can impact polysaccharide production and possibly LOS or LPS composition in other species in addition to nontypeable H. influenzae.

One factor involved in PCho expression that may be regulated in part by AI-2 quorum signaling is the transcriptional regulator CsrA. In H. influenzae, PCho expression varies based on the level of aeration in the culture, and differential expression of PCho expression requires csrA (415). A csrA mutant strain of H. influenzae has increased PCho expression under aerobic conditions, and decreased expression of the galU gene that is required for synthesis of the LOS outer core (415). As CsrA is a pleiotropic regulator of glycogen biosynthesis and degradation and gluconeogenesis in E. coli, regulation of PCho expression by CsrA may involve sensing of environmental conditions and coordinate regulation of genes involved in metabolism and LOS biosynthesis (415-419). Furthermore, CsrA activity is modulated by small RNAs in E. coli (416, 420, 421), and sRNA expression is regulated by quorum signaling in many bacterial species (350, 362, 422, 423). Therefore, AI-2 signaling in H. influenzae may modulate LOS composition and PCho content by directly or indirectly regulating CsrA activity.

Nontypeable H. influenzae 86-028NP luxS also had a significant persistence defect, as the majority of chinchillas infected with the luxS mutant appeared to have
cleared this strain from the middle ear chamber and exhibited decreased numbers of surface-adherent bacteria (Figure 11). The persistence defect may also be related to the decreased LOS PCho content observed for the luxS mutant. The loss of PCho incorporation into LOS observed for a licD mutant is associated with increased pattern recognition and induction of innate immune responses, such as production of nitric oxide and TNF-α by immortalized mouse macrophages (226). Loss of PCho expression is also associated with decreased binding of H. influenzae to the human bronchial epithelial cells via the PAF receptor, by extension decreasing the ability of H. influenzae to subjugate host cell signaling (174, 188). Furthermore, loss of PCho expression is also associated with rapid clearance from a mouse pulmonary-infection model, induction of a greater inflammatory response early during infection in the chinchilla model of OM, and a defect in the formation of visible biomasses within the chinchilla middle ear chamber (107, 108, 227). Loss of PCho expression was not associated with a persistence defect in the chinchilla model at 7 or 14 days post-infection, but the impact of this mutation on persistence during a longer course of infection has not been addressed. Thus, decreased PCho expression by the luxS mutant may be responsible for the persistence defect observed for this strain.

In support of this hypothesis, another study of H. influenzae luxS mutants found that infection resulted in increased bacterial load and a greater inflammatory response 10 days post-infection compared to the parental strain (323). Our study did not include a 10-day timepoint and no statistically significant increase in bacterial load was observed for the luxS mutant at 7 or 14 days post-infection. However, otoscopy scores for animals infected with the luxS mutant tended to be higher between 7 and 14 days post-infection.
than the scores for animals infected with the parental strain, indicating that NTHI 86-028NP luxS may have induced a greater inflammatory response at early stages of infection. This finding would be consistent with the induction of a greater inflammatory response by mutants lacking PCho. If mutation of luxS increases bacterial load around 10 days post-infection, loss of Ai-2 signaling in H. influenzae may also induce increased inflammation due to the inability of this strain to sense population density, resulting in overgrowth of the population and increased stimulation of the host immune response. Infection with the luxS mutant could thus be self-limiting due to inability to maintain a stable biofilm community under the radar of immune detection, and subsequent induction of an increased inflammatory response through several mechanisms pertaining to the mutation. However, it is important to note that while otoscopy scores appear to support increased inflammation during infection with the luxS mutant, this hypothesis is not supported by histological analysis of bullar sections (Figure 12).

Notably, the biomasses present in the middle ear chambers of animals infected with the luxS mutant at 7 and 14 days post-infection were visibly different than those formed during infection with the parental strain (Figure 11). While NTHI 86-028NP generally formed cohesive masses within the middle ear space, mutation of luxS resulted in the formation of biomasses that were more diffuse in appearance and were difficult to separate from the effusion fluid. This may reflect the differences in biofilm maturation observed for NTHI 86-028NP luxS compared to the parental strain. For instance, the diffuse nature of the biomasses formed by the luxS mutant could be due in part to decreased PCho as this particular LOS modification plays a role in adhesion (174). Expression of other cellular adhesins important for bacterial cell-to-cell interactions may
also be decreased in the absence of AI-2 signaling, reducing the ability of the bacteria to form a cohesive biomass during infection.

Another possible explanation for the differences between the biomasses formed by the luxS mutant versus the parental strain relates to differences in the composition of the biomass. For instance, the biomasses formed during infection with the luxS mutant may incorporate more host material than those formed during infection by the parental strain. Previous work from our laboratory demonstrated that nontypeable H. influenzae induces formation of neutrophil extracellular traps (NETs), and that components of these structures such as neutrophils elastase and histone protein are incorporated into the biomasses that form during experimental OM (424). Sections of the biomasses formed during infection with the luxS mutant revealed increased staining for neutrophil elastase as well as co-localization of bacteria with elastase, and biomasses taken from animals 14 days post-infection contained fewer bacteria and a greater proportion of dead cells stained with propidium iodide compared to biomasses from animals infected with the parental strain (Supplemental Figure 2).

These results support the hypothesis that mutation of luxS alters the host response to infection by increasing induction of NET formation. Both the parental strain and NTHI 86-028NP luxS can induce NET formation in vitro, and NETs induced by the luxS mutant appear to cover a greater surface area than those formed by the parental strain (Supplemental Figure 3), but no quantitative analyses of NET size or number of viewing fields positive for NET formation have been performed. Nontypeable H. influenzae is normally resistant to the bactericidal activity of NETs (424, 425), but the effects of the luxS mutation may impact susceptibility to this form of neutrophil killing. Susceptibility
of the luxS mutant to NET-mediated killing has not been directly assessed, but prior work from our laboratory demonstrated that factors related to biofilm formation, such as sialylation of LOS, also promote survival in NETs (424). Therefore, the impact of luxS mutation on LOS PCho content or other factors may be responsible for the persistence defect due to increased susceptibility to NET-mediated killing rather than induction of a more potent immune response.

An important consideration for investigating AI-2 quorum signaling through the study of a luxS mutant strain is to determine the impact of the mutation on metabolism, production of AI-3, the potential use of AI-2 as a metabolite, and the role of AI-2 as genuine quorum signaling molecule and autoinducer. The best way to distinguish between these possibilities is to determine if a particular species can respond to the AI-2 signal, and to elucidate the signaling pathway. For nontypeable H. influenzae strain 86-028NP, the protein encoded by NTHI_0632 appears to mediate AI-2 transport. Mutation of NTHI_0632 led to the accumulation of AI-2 in culture supernatants during a stage of growth when the parental strain decreased AI-2 expression and/or internalized extracellular AI-2 (Figure 13), and limited the ability of NTHI 86-028NP to deplete exogenous AI-2 (Figure 14). If AI-2 were to be utilized as a nutrient source by H. influenzae, mutants deficient in the ability to produce or internalize AI-2 would most likely exhibit growth defects. However, no such defects were observed in rich media or minimal media for strains lacking luxS, NTHI_0632, or both gene-products. Moreover, increased transcription of NTHI_0632 correlated with peak AI-2 production and peak expression of luxS, and transcription was also induced by the addition of exogenous AI-2 (Figure 15 and data not shown), consistent with this gene representing part of a luxS-
regulated AI-2 transporter (354, 356). Taken together, these results indicate that rather than utilizing AI-2 as a nutrient source, *H. influenzae* utilizes AI-2 as a genuine signaling molecule that regulates expression of its own synthase and transport apparatus.

NTHI_0632 was also required by nontypeable *H. influenzae* strain 86-028NP for a biological response to AI-2. Strain 86-028NP 0632::Cm formed biofilms with reduced total biomass and average thickness, had decreased LOS PCho content, and had a persistence defect in the chinchilla model of OM, similar to a *luxS* mutant (Figure 17-20). Notably, complementation with exogenous AI-2 restored biofilm development for the *luxS* mutant but had no impact on biofilm development for the NTHI_0632 mutant (Figure 18). As *luxS* expression and AI-2 production are intact in the NTHI_0632 mutant, these findings are consistent with NTHI_0632 encoding a factor involved in AI-2 transport and exclude the possibility that the phenotype observed for the *luxS* mutant was due to the potential impact of this mutation on metabolism or imbalance of the activated methyl cycle. The impact of a *luxS* mutation on production of AI-3 also relates to the metabolic imbalance that can result from lack of LuxS enzymatic activity (340), so the results of the NTHI_0632 studies also exclude the possibility that the observed defects in biofilm development and persistence are due to loss of AI-3 production and signaling.

Despite these findings, the two-component system implicated in sensing of AI-3 and/or host norepinephrine and epinephrine may still be involved in sensing of AI-2 or host products by *H. influenzae*. In enterohemorrhagic *E. coli*, a two-component system composed of the sensor kinase QseC and the response regulator QseB mediates sensing and interpretation of AI-3, host epinephrine, and norepinephrine (426). The QseBC signaling cascade regulates genes responsible for biosynthesis of flagella and motility,
iron-uptake systems, and adhesin expression, and sensing of AI-3 is linked to the formation of attaching and effacing lesions (338, 342, 347, 426). In *S. typhimurium*, sensing of epinephrine and norepinephrine impacts regulation of genes that encode virulence factors, colonization of the swine GI tract, reactivation of subacute infection, and fecal excretion of bacteria, although the exact role of the QseBC signaling cascade in this species is an ongoing area of investigation (344, 346, 427-431).

Notably, expression of QseBC in *A. actinomycetemcomitans* is increased by incubation with AI-2, and increased expression requires the AI-2 receptors LsrB and RbsB (432). A *qseC* mutant of *A. actinomycetemcomitans* had similar defects in biofilm growth and development of periodontitis as a *luxS* mutant, suggesting that the QseBC two-component system may link detection of AI-2 and regulation of factors involved in biofilm development and virulence in *A. actinomycetemcomitans* (432). The genome of nontypeable *H. influenzae* strain 86-028NP includes homologs of the genes encoding QseB and QseC (209, 426), but the functionality of these homologs has yet to be characterized in *H. influenzae*. Therefore, it is possible that *H. influenzae* utilizes the QseBC system to sense host epinephrine and norepinephrine, or as part of the AI-2 sensory and response pathway.

The *luxS* and NTHI_0632 studies clearly demonstrate a role for AI-2 signaling in *H. influenzae* biofilm development and persistence in an experimental model of OM. However, quorum signaling systems have also been implicated in regulation of biofilm dispersal for many species (82, 87, 88, 365). To directly address the role of *luxS* expression and AI-2 signaling in *H. influenzae* biofilm dispersal, an inducible strain of NTHI 86-028NP was generated in which *luxS* expression is induced upon addition of
xylose to the culture media (Figure 21). Using this strain, it was determined that expression of luxS and subsequent production of AI-2 not only act to promote biofilm formation but actually prevent dispersal. Induction of luxS expression resulted in the formation of biofilms with similar total biomass and average thickness as those produced by the parental strain, and biofilms formed in the absence of xylose were similar to those formed by a luxS mutant as expected (Figure 23 and 24). Notably, disruption of luxS expression after biofilms had been established for 24 hours resulted in rapid biofilm dispersal (Figure 24), and continuous induction of luxS expression for 96 hours completely prevented biofilm dispersal (Figure 26). Transcription of luxS was also found to be highest during early stages of biofilm development, followed by a steady decrease in luxS transcript levels as biofilms approached dispersal (Figure 25).

The results of the inducible-luxS study in concert with the rest of the H. influenzae AI-2 studies support a model in which luxS expression and subsequent AI-2 signaling promote biofilm development and maturation, at least in part by modulation of LOS composition, followed by decreased signaling as biofilms approach dispersal (Supplemental Figure 4). Based on this model, the decreased biofilm biomass and average thickness observed for the luxS mutant may be due to the continual expression of factors involved in biofilm dispersal in the absence of the developmental signal provided by AI-2. The proposed role of AI-2 signaling in H. influenzae would be advantageous for an opportunistic pathogen adopting the biofilm mode of growth during infection. Once the bacteria reach a potential site of infection, they would benefit from sensing population density to gauge when best to devote resources to production of EPS and the building of a biofilm. The biofilm population would also benefit from having a way to rapidly escape
from the biofilm structure and localized site of infection in response to unfavorable environmental conditions.

Many factors have been implicated in the dispersal process for different bacterial species, including availability of carbon and oxygen, calcium concentrations, iron availability, glucose depletion, protease activity, nitric oxide exposure, intracellular cyclic di-GMP levels, and various cell-cell signaling mechanisms (81-90). Quorum signaling systems are also frequently interconnected with other global regulators and pathways for sensing physiological and environmental signals that can work in concert to promote biofilm formation or dispersal (88, 401-404). For instance, biofilm formation by *Serratia* species requires production and sensing of homoserine lactone and expression of quorum-signaling regulated genes involved in production of adhesins and factors necessary for activating cell aggregation (404, 433), but nutrient cues can circumvent the impact of quorum signaling on biofilm formation and motility (88, 404, 434). *Serratia liquefaciens* is unable to swarm in minimal media supplemented with glucose, even in the presence of exogenously added quorum signal, yet a quorum signaling mutant can form biofilms similar to the wild-type strain when cultured in LB medium (434). By extension, regulation of the AI-2 signaling pathway in *H. influenzae* may involve signaling systems that sense environmental conditions and the QseBC system potentially sensing host hormone signals. Based on our proposed model, integration of these signals would repress *luxS* expression and production of AI-2 and activate factors necessary for rapid dispersal of the biofilm when the bacteria encounter unfavorable environmental conditions.
In the context of nuclease or protease production to initiate biofilm dispersal, the *H. influenzae* Hap protein is one intriguing candidate for regulation of dispersal by AI-2 signaling as this autotransporter protein functions as both an adhesin and a protease (435). The protease domain of one Hap molecule can cleave neighboring molecules, and inhibition of Hap autoproteolysis increases adherence and bacterial aggregation (169, 435). Autoproteolysis also appears to be dependent on Hap expression and the density of Hap precursor present on the bacterial cell. The expression level of Hap can either promote a high-level of adherence and aggregation and limited autoproteolysis, or increase autoproteolysis with a low or moderate level of adherence (435, 436). Therefore, it is possible that AI-2 signaling negatively regulates Hap expression, and a decrease in AI-2 signaling increases Hap expression to promote the switch to autoproteolysis, which would then promote rapid biofilm dispersal by severing cell-to-cell tethers and decreasing adhesion to the substrate. However, further research is necessary to identify the biofilm components and pathways through which quorum signaling regulates dispersal.

**Implications of *Haemophilus influenzae* AI-2 Studies**

While several promising vaccine candidates are being explored for nontypeable *H. influenzae*, several challenges yet remain to vaccination such as the considerable genomic heterogeneity among *H. influenzae* strains (228, 437). Due to potential challenges with vaccination and increasing rates of antibiotic treatment failure, there remains a need for alternative and effective OM treatments. Ideally, new therapeutic
treatments would be designed to exert minimal selective pressure to limit the development of bacterial resistance. For nontypeable *H. influenzae*, the ideal therapeutic would also take into account biofilm formation and the heterogeneity of strains, and be effective for treating a wide range of infections caused by *H. influenzae* including OM, sinusitis, bronchitis, and COPD exacerbations. Quorum signaling has been suggested to be an ideal target for the design of novel antibacterial agents as quorum signaling systems control virulence, pathogenic traits, and biofilm development for many species of bacteria (438, 439). The use of quorum signaling inhibitor compounds can also render biofilms more susceptible to antimicrobial treatments (438, 440-442), indicating that the combined use of signaling inhibitors and antibiotics may be effective for treatment of biofilm-related infections such as OM.

Recent efforts aimed at interfering with bacterial communication via quorum signaling have mostly focused on inhibiting AHL and peptide autoinducer signaling (443, 444). However, our results suggest that therapeutics aimed at disrupting AI-2 quorum signaling may prevent establishment of *H. influenzae* biofilms or disrupt already-established biofilms. As NTHI_0632 appears to mediate AI-2 binding and transport in nontypeable *H. influenzae* strain 86-028NP, AI-2 analogues could be used to interfere with recognition of and response to the AI-2 signal. One complication to this strategy is that many of the current AI-2 nucleoside analogues such as adenosine derivatives only antagonize the *V. harveyi* LuxPQ sensor system (445). Other AI-2 analogs have been shown to antagonize signaling in *S. typhimurium* and are nontoxic toward mammalian cells, but signaling in *V. harveyi* could still be activated by exogenous AI-2 in the presence of the analog (446). Another complication in targeting AI-2 transport or
sensory systems is the duration of inhibition. For alkyl-DPDs, inhibition of *V. harveyi* signaling lasted less than 2 hours (447), which may limit their therapeutic potential.

Another consideration in the design of AI-2 inhibitors for treatment of nontypeable *H. influenzae* infection is the finding that disruption of *luxS* expression results in rapid dispersal of already-established biofilms. Dispersal is generally considered to be an advantageous process through which bacteria egress from a biofilm to establish new bacterial communities, particularly when faced with unfavorable conditions (79, 80). The forced release of bacteria from a biofilm community back into a planktonic state would most likely result in a bacterial population that would now be susceptible to antimicrobial agents, making the combined use of quorum signaling inhibitors and antibiotics an effective treatment for biofilm-related infection. However, biofilm formation and subsequent dispersal have been proposed as a mechanism used by pathogenic bacteria to accumulate a sufficient number of bacteria to cause severe infection upon dispersal (67, 405, 406). Thus, the use of therapeutics that inhibit quorum signaling and induce biofilm dispersal could result in systemic disease by forcing a large population of bacteria to rapidly leave a localized site of infection, potentially spreading to the blood stream and throughout the host. Further research will be necessary to determine the impact of *H. influenzae* biofilm dispersal on antibiotic susceptibility versus the development of systemic disease.
Haemophilus influenzae and Moraxella catarrhalis Indirect Pathogenicity

As with most upper airway infections, epidemiological data indicate that the majority of chronic OM infections are polymicrobial (55, 111). H. influenzae and M. catarrhalis are frequently present together in patient samples from chronic and recurrent OM (55, 104, 135), and M. catarrhalis is more frequently isolated from polymicrobial OM infections than as the single causative agent (136). M. catarrhalis is the third leading cause of acute OM and the second leading cause of chronic and recurrent OM (16, 55, 56). In contrast to human patient data, infection with pure cultures of M. catarrhalis in animal models, such as the chinchilla model of OM, results in only transient infection and rapid clearance (Figure 31) (243, 254). This discrepancy could indicate that M. catarrhalis is highly adapted to human hosts and therefore unable to cause infection in animal models. Another possibility is that the environment encountered by M. catarrhalis during single species infection in an animal model is significantly different than the environment encountered during infection of a human host, and lacking factors that M. catarrhalis requires to establish a persistent and productive infection. Our results favor the later hypothesis, and indicate that M. catarrhalis utilizes AI-2 produced by H. influenzae to promote biofilm formation and persistence.

In the chinchilla model of OM, M. catarrhalis was undetectable in middle ear effusion fluid at 7 and 14 days post-infection, and the surface-adherent bacteria present in the bullar homogenate failed to increase bacterial load above the number of colony forming units present in the initial inocula (Figure 31). Animals infected with M.
*catarrhalis* also generally did not exhibit the formation of visible biomasses within the middle ear chamber. However, coinfection with nontypeable *H. influenzae* increased persistence of *M. catarrhalis* (Figure 31) and resulted in formation of biomasses within the middle ear space, indicating that *M. catarrhalis* benefits from the presence of other species during infection and can establish a persistent infection in the chinchilla model of OM with help. Notably, coinfection with a luxS mutant of *H. influenzae* did not provide sufficient help to *M. catarrhalis* to promote increased persistence, indicating that AI-2 produced by *H. influenzae* was responsible for the increased persistence. Even though *M. catarrhalis* lacks a luxS homolog, further investigation revealed that *M. catarrhalis* utilized AI-2 and the AI-2 precursor DPD to augment biofilm development and enhance resistance to antibiotics in the biofilm mode of growth (Figure 30).

These results thus support a role for interspecies AI-2 signaling during polymicrobial infection, and indicate that *M. catarrhalis* may need an infection partner in order to persist in animal infection models. Coinfection studies in the chinchilla model of OM may be a more accurate representation of human infection in this context. Our results also shed new light on the finding that *M. catarrhalis* is more frequently isolated from polymicrobial OM than as the single causative agent (136). Acute OM caused by *M. catarrhalis* generally results in a less severe infection associated with a lower chance of developing OM complications such as mastoiditis compared to infection caused by *H. influenzae, S. pneumoniae*, or *S. pyogenes* (136, 448, 449). This is strikingly similar to the transient infection caused by *M. catarrhalis* that has been observed in animal models of single-species infection. Therefore, in a human host *M. catarrhalis* may utilize AI-2 produced by other species and/or the biofilm scaffold provided by these species to
promote its own persistence, while causing only transient disease in the absence of an infection partner. Additionally, a recent study that examined nasopharyngeal colonization by *M. catarrhalis* and *H. influenzae* found a high occurrence of co-colonization that was not associated with any specific genotypic clones of either species, suggesting that a ubiquitous system such as interspecies quorum signaling may be responsible for the high frequency of co-colonization by these species (135).

The exact mechanism used by *M. catarrhalis* to sense and respond to exogenous AI-2 is currently unknown. A sequenced genome of *M. catarrhalis* strain 7169 is not yet available, but a full genome sequence of strain RH4 was published in 2010 that should provide targets for investigating potential AI-2 uptake systems and response pathways present in *M. catarrhalis* (450). Currently, no genes present in the *M. catarrhalis* strain RH4 genome have been annotated as homologs of the Lsr transport system, the ribose transport system, or the QseBC two-component system that may play a role in response to AI-2. However, a LuxR-type regulator is encoded by MCR_1062 (450), suggesting that *M. catarrhalis* strain RH4 may also respond to AI-2 and further indicating that an AI-2 sensory mechanism most likely exists in *M. catarrhalis* that has yet to be identified.

In an effort to identify proteins differentially expressed in *M. catarrhalis* biofilms established in the presence of DPD, we generated samples for 2D gel analysis. A total of eight polypeptide spots were identified as changing under the treatment conditions; three increased when biofilms were established with DPD and five decreased (Supplemental Table 1). Of the eight polypeptide spots, one that increased 1.9 fold in the presence of DPD was identified as the Hag/MID protein of *M. catarrhalis*. Hag is a trimeric autotransporter protein known to be involved in adhesion of *M. catarrhalis* and biofilm
formation (258, 264-266, 293), and thus would likely be involved in the increased clumping and biofilm formation observed when *M. catarrhalis* is incubated with DPD (Figure 30). Preliminary studies of *M. catarrhalis* strain O35E and an isogenic *hag* mutant revealed that Hag is required for enhanced antibiotic resistance in response to DPD treatment (Supplemental Figure 5). It is therefore likely that AI-2 signaling modulates Hag expression, although this has yet to be directly addressed. Further research is necessary to determine exactly how Hag is regulated in this context, to elucidate the role of Hag in biofilm development in the presence of AI-2, and to identify the other factors regulated by AI-2 signaling in *M. catarrhalis*.

In addition to altering bacterial persistence and disease severity, the presence of multiple bacterial species during infection can significantly impact treatment efficacy (139, 140). For example, the vast majority of *M. catarrhalis* clinical isolates produce beta-lactamase that is thought to confer passive antibiotic resistance upon other pathogens (139-145). This is of particular interest for respiratory tract infections and OM, where other opportunistic pathogens such as *S. pneumoniae* are relatively sensitive to beta-lactam antibiotics in the absence of a beta-lactamase producing coinfection partner. The formation of biofilms during infection can also significantly impact treatment efficacy as these structures are inherently more resistant to antimicrobials (67, 91). Biofilm formation during polymicrobial infection may result in incorporation of multiple bacterial species into biofilm communities (138), and thus has the potential to further enhance the antibiotic resistant nature of the biofilm.

Our results clearly show that *H. influenzae* and *M. catarrhalis* readily form polymicrobial biofilms when cultured together in vitro (Figure 27). Both *H. influenzae*
and *M. catarrhalis* were detected in biomasses removed from the middle-ear chamber of chinchillas during polymicrobial infection, indicating that these species also coexist in biofilm structures during experiment OM (Supplemental Figure 6). The impact of polymicrobial biofilm formation on antibiotic susceptibility was assessed in vitro, and both *H. influenzae* and *M. catarrhalis* were able to confer resistance to susceptible strains independent of the production of secreted resistance factors such as beta-lactamase (Figure 28 and 29). Through the use of isogenic *H. influenzae* mutants, the enhanced antibiotic resistance provided by polymicrobial biofilm formation was found to relate to total biofilm biomass, LOS composition, and interspecies AI-2 signaling (Figure 29). However, features of the *M. catarrhalis* biofilm important for enhanced antibiotic resistance and protection of *H. influenzae* have yet to be identified beyond the role of Hag in promoting resistance to clarithromycin (Supplemental Figure 5).

Several inherent features of a biofilm can enhance antibiotic resistance over planktonic growth. Factors such as the thickness of the biofilm and EPS composition promote resistance by binding antimicrobial agents or delaying penetration through the biofilm, and the altered metabolic state and stress response of biofilm-associated bacteria limit the efficacy of antibiotics that target cell wall synthesis or require actively replicating bacteria (92-95). Biofilms also provide an ideal environment for the transfer of resistance determinants that can be further enhanced by the presence of multiple strains and species within the biofilm (451-455). Based on our antibiotic protection studies and the use of isogenic *H. influenzae* mutants that did not enhance antibiotic resistance, our results support the conclusion that biofilm thickness and/or EPS composition limit access of the antibiotic to bacteria within the biofilm. Following each
antibiotic protection study, bacteria were isolated from the polymicrobial biofilms, suspended in liquid growth media, and subjected to a standard MIC test which revealed that the bacteria regained susceptibility to antibiotics once removed from the polymicrobial biofilms (data not shown). Thus, the enhanced resistance observed for polymicrobial biofilms was not due to mutation or transfer of resistance determinants.

While several studies have demonstrated the ability of *M. catarrhalis* to confer passive protection through production of beta-lactamase as discussed above, our results clearly show that the incorporation of multiple species into a biofilm enhances the inherent antimicrobial resistance of the biofilm independent of secreted factors such as beta-lactamase or acquisition of resistance genes. In addition, these results demonstrate that both *M. catarrhalis* and *H. influenzae* contribute important factors to the polymicrobial biofilm that provide protection from a variety of antimicrobial agents. Furthermore, our results indicate a central role for interspecies AI-2 signaling in polymicrobial infection, polymicrobial biofilm formation, and antibiotic resistance.

**Implications of Polymicrobial Studies**

The most commonly prescribed antibiotic for treatment of OM is the beta-lactam amoxicillin (59, 63). However, the rate of antibiotic treatment failure continues to increase for amoxicillin as well as other commonly prescribed antibiotics for treatment of airway infections and OM (50, 53, 59, 64, 456). Treatment efficacy is clearly influenced by the formation of biofilm communities during infection, and work from our laboratory has demonstrated that the presence of multiple bacterial species significantly impacts
antibiotic resistance in an experimental model of OM (457). The results of this study also clearly demonstrate that coinfection with *H. influenzae* and *M. catarrhalis* promotes enhanced resistance to antibiotics in vitro, indicating that polymicrobial infections caused by these species may contribute to the high rate of treatment failure. As the majority of OM infections are now thought to be polymicrobial and polymicrobial infection and biofilm formation clearly alter antibiotic susceptibility, there is a pressing need to utilize combinations of antibiotics that are effective against all of the leading OM pathogens or to design new therapeutics to replace or augment current OM treatments. The results of our *H. influenzae* and *M. catarrhalis* coinfection studies also support the use of quorum signaling inhibitors to interfere with bacterial communication and thus potentially disrupt the polymicrobial biofilm communities that develop during infection.

Due to the diversity of autoinducer molecules and the complicated interactions of these signaling molecules between different species, targeting bacterial communication as a therapeutic treatment for infections poses many challenges. However, the interspecies signaling capacity of AI-2 and conservation of *luxS* in numerous bacterial species makes this system an attractive target for interference with quorum signaling, particularly in polymicrobial biofilms (394). In the context of OM, AI-2 quorum signaling clearly influences biofilm formation and disease severity for *H. influenzae* and *M. catarrhalis*. *S. pneumoniae* is also capable of producing AI-2, and mutation of *luxS* reduces virulence and persistence in a murine model of nasopharyngeal carriage (324, 325). As all three of the leading OM pathogens appear to utilize the AI-2 signal, one target for blocking bacterial interspecies communication would be to interfere with recognition of AI-2. Through NCBI genome searches, it is clear that different strains of nontypeable *H.*
*H. influenzae* have homologs of the Lsr transport system and/or NTHI_0632. While *M. catarrhalis* utilizes the AI-2 signal, the sensory system or AI-2 transport of this species is not yet known. The AI-2 transport system of *S. pneumoniae* is also not currently known. Thus, targeting AI-2 signaling by interfering with signal binding would only be effective for *H. influenzae* at present, and is addressed above.

Another target for disrupting bacterial communication would be to inhibit AI-2 production by targeting LuxS. This strategy would not directly impact *M. catarrhalis* as this species does not possess a luxS homolog or produce AI-2, but it should impact *H. influenzae* and *S. pneumoniae* and indirectly limit the ability of *M. catarrhalis* to establish persistent infection by decreasing the reservoir of AI-2. This strategy may also disrupt metabolism in *H. influenzae* and *S. pneumoniae* in addition to inhibiting quorum signaling due to the role of LuxS in the activated methyl cycle (329). Research focusing on the mechanism of action of LuxS has identified key amino acid residues and catalytic intermediates which could be suitable targets for inhibiting the enzymatic reaction that generates AI-2 (458). LuxS substrate analogues of s-ribosyl homocysteine (SRH) act as time-dependent inhibitors of AI-2 production in *V. harveyi*, *Bacillus subtilis*, and *E. coli*. It therefore may be possible to generate modified SRH analogues which permanently inhibit LuxS and AI-2 production.

Another strategy to block bacterial communication is through external quenching of AI-2. This could be accomplished by degradation or cleavage of AI-2, use of AI-2 binding proteins, or alteration of AI-2 to limit sensing of the signal. One example of AI-2 quenching in the literature is the use of the *E. coli* AI-2 kinase LsrK for in vitro phosphorylation of AI-2 (459). The addition of LsrK and ATP to bacterial cultures can
generate phospho-AI-2 that is not transported into bacterial cells and is degraded over time without stimulating quorum signaling pathways. Such strategies may prove the most effective against interspecies communication between OM pathogens as LsrK can phosphorylate DPD, the precursor of all discrete structures of AI-2, thus targeting bacterial communication regardless of the specific AI-2 structure or transport/sensor mechanisms present during the infection.

Based on our results and current literature, interference with AI-2 quorum signaling would most likely have a beneficial effect on patients experiencing polymicrobial OM by limiting biofilm formation or promoting biofilm dispersal to increase susceptibility to antimicrobials and the host immune response. However, as mentioned above for nontypeable H. influenzae, the rapid biofilm dispersal that would most likely result from interference with AI-2 signaling may increase systemic disease by flooding the middle ear space and potentially the bloodstream with a large number of bacteria as they egress from the biofilm and the localized site of infection. It is also important to consider the implications of AI-2 inhibition for all OM pathogens, not just H. influenzae and M. catarrhalis. Mutation of luxS in S. aureus actually promotes production of capsular polysaccharide and increases survival compared to the parental strain when cultured with human blood and monocytes (460). For S. pyogenes, mutation of luxS was recently found to increase acid tolerance and intracellular survival in epithelial cells and macrophages (461). Therefore, targeting AI-2 signaling during infections involving these species may actually increase disease severity. However, as mutation of luxS can impact factors independent of AI-2 quorum signaling it is possible that some of the phenotypes reported in these studies are not the direct result of AI-2
signaling. If this is the case, external binding, modification, or degradation of AI-2 would not have a negative impact the severity of disease caused by these species, but inhibition of LuxS/AI-2 production should not be considered for such infections.

Another consideration in treating polymicrobial OM by interfering with AI-2 signaling concerns the potential impact of such therapeutics on normal flora. It has previously been shown that avirulent oropharyngeal flora in cystic fibrosis patients as well as intestinal microflora from healthy individuals produce AI-2 (338, 370). There is limited information concerning the role of AI-2 quorum signaling and normal flora. However, the possibility exists that AI-2 signaling may be critical for the establishment or maintenance of normal flora and defense against pathogens. Thus, it would be ideal to engineer an AI-2 inhibitor that remains localized to the site of infection to avoid potentially disrupting signaling by intestinal or other microflora.

Despite the challenges in manipulating bacterial communication, AI-2 remains an intriguing target for disruption of chronic, recurrent, and polymicrobial infections. The evidence suggests that therapeutics targeting AI-2 may not be universal treatments for OM as each strategy has limitations, and not all OM pathogens utilize LuxS and AI-2 in the same manner. Additionally, interfering with AI-2 signaling may not completely inhibit biofilm formation or disrupt established biofilms as numerous other factors are involved in biofilm development. However, limiting bacterial communication may be sufficient to knock down key species and make biofilms more susceptible to immune clearance or antibiotics. In the case of *H. influenzae* and *M. catarrhalis* coinfection, where almost all *M. catarrhalis* strains provide passive protection from beta-lactam
antibiotics, limiting AI-2 should decrease the bacterial load of *M. catarrhalis* and consequently may decrease antibiotic treatment failure for OM.

In addition to the implications for AI-2 inhibition as a therapeutic target, the results of our polymicrobial studies further suggest that successful vaccination against *H. influenzae* may decrease the incidence of disease due to *M. catarrhalis* by removing a reservoir of the AI-2 signal that promotes *M. catarrhalis* biofilm formation and antibiotic resistance. *H. influenzae* and *M. catarrhalis* also provide antibiotic protection against trimethoprim-sulfamethoxazole and clarithromycin in polymicrobial biofilms, and both species have been shown to protect *S. pneumoniae* from beta-lactam antibiotics such as amoxicillin (144, 457). Thus, successful vaccination against *H. influenzae* and/or *M. catarrhalis* would most likely have a significant impact on treatment efficacy in a polymicrobial setting in addition to limiting the incidence of disease caused by these species.

As nontypeable *H. influenzae* and *M. catarrhalis* are common components of the nasopharyngeal flora, the impact of vaccination against these species on disease incidence and severity must be considered in the context of the other pathogenic bacteria that frequently colonize the nasopharynx and cause opportunistic infection. Preventing nasopharyngeal colonization by *H. influenzae* and/or *M. catarrhalis* may significantly impact the overall composition of the nasopharyngeal flora. For instance, nasopharyngeal colonization by *S. pneumoniae* was found to decrease following introduction of the pneumococcal vaccine, and resulted in a corresponding increase in colonization by *H. influenzae* and *S. aureus* (462-464). Colonization of the nasopharynx by *H. influenzae* is negatively associated with colonization by *M. catarrhalis*, *S.
pneumoniae, and S. aureus, suggesting that if vaccination against H. influenzae limits nasopharyngeal colonization by this species, it may also increase colonization by the other leading OM pathogens (29). Co-colonization by H. influenzae and M. catarrhalis is positively associated with colonization by S. pneumoniae (29), suggesting that vaccination strategies or AI-2 inhibition aimed at limiting colonization of both H. influenzae and M. catarrhalis may also limit nasopharyngeal colonization by S. pneumoniae. However, colonization by S. pneumoniae decreases the odds of S. aureus colonization by up to 40% (29), indicating that removal of both H. influenzae and M. catarrhalis from the nasopharynx may further increase colonization by S. aureus or other species. Thus, treatment or prevention strategies that reduce nasopharyngeal colonization by nontypeable H. influenzae and M. catarrhalis may alter the composition of the nasopharyngeal flora and promote colonization by other pathogenic organisms such as S. pneumoniae, S. aureus, and possibly S. pyogenes.

Shifts in nasopharyngeal colonization due to vaccination are important to consider as the composition of the nasopharyngeal flora may influence development of opportunistic infections, similar to how the composition of the intestinal flora can influence colonization by pathogens and development of disease (465-469). Nontypeable H. influenzae and M. catarrhalis generally cause less severe infection than the other OM pathogens (136, 448, 449, 470, 471). Therefore, vaccination against these species and increased nasopharyngeal colonization by S. pneumoniae, S. aureus, and/or S. pyogenes may result in an overall increase in severe OM infections and OM complications. S. pneumoniae infection is also associated with a high rate of systemic disease and mortality in the chinchilla model of OM that can be tempered by coinfection with nontypeable H.
influenzae, further indicating that while vaccination against nontypeable *H. influenzae* may decrease antibiotic treatment failure it also has the potential to increase the incidence of severe infection and/or development of systemic disease (138, 457).

Clearly, complex interactions exist between potential pathogens within the nasopharynx and during polymicrobial infection, and factors that impact colonization or persistence of one species can have a dramatic impact on the persistence and antibiotic susceptibility of other species as well as on disease progression. The role of interspecies bacterial communication during polymicrobial infection is just beginning to be critically investigated, and there are many mechanisms that bacteria may use to sense and interact with each other and their human and animal hosts that have yet to be studied in this setting. Thus, further research is necessary to determine how multiple species interact during polymicrobial infection as well as the impact of vaccination on the composition of nasopharyngeal flora and the incidence and severity of disease. Further investigation of polymicrobial interactions will also provide a more complete understanding of the role of AI-2 interspecies communication in polymicrobial infection, and will be necessary to fully understand the impact of AI-2 inhibition on antibiotic susceptibility, biofilm formation and dispersal during infection, and disease severity.

**Future Directions**

The research presented herein provides insight into production and uptake of AI-2 by nontypeable *H. influenzae* and the role of this signaling molecule in biofilm development and persistence, as well as *H. influenzae* and *M. catarrhalis* polymicrobial
interactions and the role of interspecies bacterial communication in biofilm formation, antibiotic resistance, and infection. However, many questions remain to be addressed. For *H. influenzae*, the complete AI-2 signaling pathway has yet to be elucidated. As NTHI_0632 is part of an operon that encodes an ABC transporter, analysis of the other genes included in this operon would provide a better understanding of the mechanism used by nontypeable *H. influenzae* to internalize AI-2. Additionally, if AI-2 initiates a phosphorylation cascade in *H. influenzae*, identification of an AI-2 kinase may allow for further elucidation of the AI-2 signaling cascade. If the signaling pathway in *H. influenzae* is similar to *S. typhimurium* or *E. coli*, studies utilizing phosphorylated AI-2 may provide further insight into transcription factors and genes that are regulated by AI-2 signaling. Knowledge of the AI-2 signaling pathway in *H. influenzae* will be useful to identify compounds that inhibit AI-2 signaling in this species.

The induction of *H. influenzae* biofilm dispersal by shutoff of luxS expression is another interesting area that requires further study. Once AI-2 signaling inhibitors have been identified and characterized for nontypeable *H. influenzae*, these compounds can be utilized to determine if inhibition of AI-2 signaling forces biofilm dispersal in a similar manner as disruption of luxS expression. The chinchilla model of OM could be used to follow up on the impact of biofilm dispersal on development of systemic infection versus restoring susceptibility to antibiotic treatment. While it would be technically challenging to precisely control luxS expression in vivo using the inducible system, AI-2 inhibitors that force biofilm dispersal could be used following establishment of chronic infection. The chinchilla model of OM could also be used in this manner to determine if AI-2
inhibitors increase susceptibility to antibiotic treatment in vivo, and to assess the impact of biofilm dispersal on systemic infection.

The results of the *H. influenzae* and *M. catarrhalis* studies clearly show that *M. catarrhalis* benefits from polymicrobial biofilm formation and coinfection, and that *M. catarrhalis* utilizes AI-2 produced by *H. influenzae*. However, the interaction of *M. catarrhalis* with the other leading OM pathogens has yet to be investigated. Further research is necessary to determine if *M. catarrhalis* and *S. pneumoniae* coinfection also augments biofilm formation, antibiotic resistance, or bacterial persistence in a similar manner as coinfection with *H. influenzae*. The ability of *M. catarrhalis* to respond to AI-2 produced by other OM pathogens such as *S. pneumoniae*, *S. aureus*, and *S. pyogenes* also needs to be determined to elaborate on the role of interspecies bacterial communication in polymicrobial interactions. If *M. catarrhalis* can respond to AI-2 produced by these species or other common inhabitants of the nasopharynx, AI-2 signaling may play a role in nasopharyngeal colonization by *M. catarrhalis* or egress from the nasopharynx to the middle ear or lungs. The AI-2 signaling pathway of *M. catarrhalis* and factors regulated by AI-2 in this species also remain to be investigated.

In addition to the role of AI-2 signaling in *M. catarrhalis* biofilm formation and pathogenesis, the role of AI-2 signaling versus mutation of *luxS* has yet to be fully addressed for many of the species that commonly inhabit the nasopharynx and cause OM. Before AI-2 inhibitors can be considered for treatment of OM, the impact of AI-2 signaling and inhibition on other OM pathogens and nasopharyngeal composition must be determined. In addition to in vitro testing, compounds that inhibit AI-2 signaling should also be studied in experimental models of polymicrobial infection. Further
investigation into the dynamics of polymicrobial infection with all three of the leading OM pathogens will also be necessary to determine the impact of each additional species on biofilm formation and composition, bacterial persistence, and antibiotic susceptibility.

Another line of study that warrants further investigation is the impact of polymicrobial infection on the host immune response. While *H. influenzae* clearly promotes persistence of *M. catarrhalis* and can limit systemic disease due to *S. pneumoniae*, the role of the immune system in this interaction is not yet clear. It is possible that *H. influenzae* somehow promotes host tolerance of other OM pathogens. Another possibility is that infection with *H. influenzae* induces host factors that somehow promote the biofilm mode of growth for *M. catarrhalis* and *S. pneumoniae*, thus resulting in their incorporation into biofilms and limiting systemic disease while simultaneously promoting persistence in the localized site of infection. As components of neutrophil extracellular traps are present in the biomasses that form during experimental OM, it would be interesting to determine the impact of incubation with different combinations of bacteria on NET formation or NET-induced killing as well as the relative proportion of NET components present in the middle ear space during infection. The composition of the nasopharyngeal flora may also influence the immune response developed against OM pathogens or influence egress from the nasopharynx and development of opportunistic infection. Thus, the impact of vaccination and AI-2 inhibition on the host immune response also needs to be addressed.

The results of the polymicrobial studies clearly show that the presence of multiple species during infection and incorporation of multiple species into biofilm communities can significantly impact treatment efficacy. Incorporation of additional species such as *S.
*pneumoniae* into the biofilm communities that form during infection may further enhance resistance or provide protection against a broader range of antimicrobial agents. Thus, antibiotic susceptibility testing for the treatment of OM needs to account for biofilm formation and polymicrobial infection rather than solely measuring minimal inhibitory concentrations for pure broth cultures of a single species. The chinchilla model of OM can also be utilized to investigate the efficacy of new antimicrobial agents against polymicrobial infection and biofilm-related disease.
Supplemental Figure 1. Bioassay for AI-2 production. *V. harveyi* BB170 was cultured in AB media and AI-2 activity was assessed. Broth cultures of NTHI strains were grown to late log phase or early stationary phase (OD<sub>600</sub> of ~1.0). Cultures were centrifuged, and the supernatants were collected, filter-sterilized, and stored at -20°C. *V. harveyi* AI-2 sensor strain BB170 was harvested from overnight plate cultures and suspended in 10 ml AB media supplemented with iron. The bacterial suspension was vortexed and diluted to an OD<sub>600</sub> = 0.7 in 20 ml of AB media supplemented with iron. The sensor culture was incubated at 30°C in a shaking incubator at 100 rpm to OD<sub>600</sub> ~1.0, then diluted 1:5000 in AB media without iron and dispensed in 0.9 ml aliquots into a 24-well culture dish. Thawed NTHI supernatants (0.1 ml) were added to the wells (3 replicates) and incubated at 30°C and 100 rpm for 3 h, and luminescence was measured using a Turner Designs TD-20/20 luminometer.
Supplemental Figure 2. Infection with NTHI 86-028NP luxS impacts biomass composition in vivo. CLSM of biomasses removed from chinchilla middle ear chambers at 7 days (A,B) and 14 days (C-F) post-infection. Panels A, C, and E show biomasses excised from animals infected with NTHI 86-028NP and panels B, D, and F show biomasses excised from animals infected with NTHI 86-028NP luxS. Panels A, B, C, and D were stained with rabbit polyclonal anti-NTHI sera (red) and a monoclonal antibody to neutrophil elastase (green). Panels E and F were stained with propidium iodide (red) and rabbit polyclonal anti-NTHI sera (green).
Supplemental Figure 3. NTHI 86-028NP and NTHI 86-028NP luxS induce NET formation. Representative images of NETs formed by human neutrophils following incubation with $10^4$ cfu of NTHI 86-028NP or NTHI 86-028NP luxS for 5 hours. NETs were stained with propidium iodide (red) and anti-histone antibody (green).
Supplemental Figure 4. AI-2 quorum signaling in NTHI biofilm formation and dispersal. Diagram of the five general stages of biofilm development indicating our current understanding of AI-2 quorum signaling during biofilm maturation and dispersal. AI-2 signaling appears to be most important during microcolony formation and biofilm maturation and decreases as biofilms approach dispersal, most likely resulting in de-repression of proteases or nucleases that induce dispersal. AI-2 is produced by luxS and internalized by a transport system including NTHI_0632. Genes of the LuxS regulated (Isr) transport system of E. coli are shown in parallel to an NTHI operon annotated as a ribose transporter that includes genes predicted to encode two members of a heterodimeric membrane channel, an ATP-binding protein, NTHI_0632, a sugar kinase, and an operon regulator. AI-2 signaling upregulates expression of luxS and NTHI_0632, and promotes PCho expression which is required for full maturation of NTHI biofilms. Image adapted from (472), (74), and (356).
Al-2 signaling begins
PCho expression increases

AI-2 signaling decreases
Decreased luxS expression

De-repression of proteases/nucleases?

Adhesins

1 2 3 4 5

NTHI_0632

LuxS

AI-2

Upregulates luxS
Upregulates NTHI_0632
Increases PCho expression

E. coli

H. influenzae

Al-2 binding
Membrane channel
ATP binding
ATP
ADP

???

Al-2

LsrR

lsrK

lsrA

lsrC

lsrD

lsrE

lsrF

lsrG

lsrH

NTHI_0632
Supplemental Table 1. 2D gel analysis of *M. catarrhalis* biofilms established in the presence of absence of DPD. *M. catarrhalis* biofilms were established for 24 hours in BHI or BHI supplemented with 0.2 µM DPD. Supernatants were removed and biofilms were suspended in PBS and centrifuged, and cell pellets were sent to Kendrick Laboratories for 2D gel analysis. Reference spot number, pI, and MW are given for changing polypeptide spots. Also shown are fold increases or decreases (difference) of the polypeptides for DPD treated versus Untreated. The differences are calculated from spot percentages (individual spot density divided by total density of all measured spots). Polypeptide spots increased in DPD treated vs Untreated by a fold increase of > 1.7 and p value < 0.05 are highlighted in blue, while spots decreased in DPD treated vs Untreated with a fold decrease of ≤ -1.7 and p value < 0.05 are highlighted in red. A total of 584 spots were analyzed. Spot #2 was identified as the Hag autotransporter protein through MASCOT searches conducted by Jeremiah Davie of Dr. Anthony Campagnari’s lab at the University of Buffalo.
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Supplemental Figure 5. *M. catarrhalis* 035E requires Hag for increased resistance to clarithromycin in response to DPD. Biofilms were established for 4 h by *M. catarrhalis* strain O35E or a hag mutant in BHI alone or BHI supplemented with 0.2 µM DPD, followed by 24 h incubation in media alone (white bars) or media with 6 µg/ml clarithromycin (gray bars). Biofilms were suspended in sterile PBS, serially diluted, and plated onto BHI for enumeration of viable *M. catarrhalis*. Error bars represent the mean and standard deviation for triplicate wells.
Supplemental Figure 6. *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms during experimental OM. Biomasses were removed from the middle-ear chambers of animals co-infected with wild type *H. influenzae* and *M. catarrhalis* at 7 (A) and 14 (B) days post infection and stained for *H. influenzae* (red) and *M. catarrhalis* (green). Images were obtained by confocal laser scanning microscopy (CLSM).
Permission to reprint the article entitled “LuxS Promotes Biofilm Maturation and Persistence of Nontypeable Haemophilus influenzae In Vivo via Modulation of Lipooligosaccharides on the Bacterial Surface.”
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Letter indicating permission to reprint sections of the editorial entitled “Interspecies bacterial communication as a target for therapy in otitis media.”
Dear Chelsie,

In this instance we will be happy for you use sections of the editorial at no charge.

Sam Cavana

Reprint Sales Manager

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-----Original Message-----
From: Chelsie Armbruster [mailto:carmbrus@wfubmc.edu]
Sent: 07 March 2011 05:36
To: Sam Cavana
Subject: Author request for permission

To whom it may concern,

I am an author of an editorial for Expert Review of Anti-infective Therapy entitled "Interspecies bacterial communication as a target for therapy in otitis media," and I would like to request permission to include sections of the editorial in my graduate thesis for the Wake Forest University School of Medicine.

Thank you,

Chelsie E. Armbruster
carmbrus@wfubmc.edu
REFERENCE LIST


286


Carriage of *Haemophilus influenzae* among Brazilian children attending day care centers in the era of widespread Hib vaccination. *Vaccine* 29:1438-1442.


CIRRICULUM VITAE

EDUCATION

2007-present      Wake Forest University School of Medicine
                  Ph.D. Program in Microbiology and Immunology
                  “Haemophilus influenzae and Moraxella catarrhalis intra- and
                  interspecies quorum signaling”
                  Advisor: Dr. W. Edward Swords

2003-2007         Rochester Institute of Technology
                  B.S. Honors degree in Biotechnology
                  Psychology Concentration
                  Advisors: Dr. Jean A. Douthwright and Dr. Irene M. Evans

PUBLICATIONS


**PRESENTATIONS**

2011  NTHI_0632 Mediates AI-2 Uptake in Nontypeable Haemophilus influenzae strain 86-028NP. Poster presentation at the Mid-Atlantic Microbial Pathogenesis meeting (Wintergreen, VA).

2010  Bacterial Eavesdropping and Indirect Pathogenicity during Polymicrobial Otitis Media. Poster presentation at the Fifth Annual NIH National Graduate Student Research Festival (Bethesda, MD).

2010  Haemophilus influenzae and Moraxella catarrhalis Polymicrobial Biofilms Promote Antibiotic Resistance and Bacterial Persistence. Poster presentation, 110th American Society for Microbiology General Meeting (San Diego, CA).
2010 Nontypeable *Haemophilus influenzae* Strain 86-028NP Utilizes the Ribose-binding Protein RbsB to Respond to Autoinducer-2. Poster presentation, 110th American Society for Microbiology General Meeting (San Diego, CA).

2009 Polymicrobial biofilms promote enhanced antibiotic resistance for *Haemophilus influenzae* and *Moraxella catarrhalis*. Poster presentation, 5th American Society for Microbiology Conference on Biofilms (Cancun, Mexico).


2005 Construction and Transformation of a Recombinant Plasmid Containing the Genes for Production of β-galactosidase, the Green Fluorescent Protein, and Ampicillin Resistance. Poster presentation, 105th American Society for Microbiology General Meeting (Atlanta, GA).


**HONORS/AWARDS**

2011 Awarded a Student Travel Grant to present at the 2011 Mid-Atlantic Microbial Pathogenesis Meeting

2010 Selected to attend the Fifth Annual NIH National Graduate Student Research Festival

2010 Appointed to the Wake Forest University Department of Microbiology and Immunology NIH-funded Predoctoral Training Program in Immunology and Pathogenesis (T32 AI007401)

2009 Awarded a CAP Student Travel Grant to present at the 109th American Society for Microbiology General Meeting

2009 Runner up cash award for best poster presentation in the Basic Sciences category at the 9th Annual Wake Forest Graduate Student Research Day
2007 Awarded a Graduate School Fellowship for Wake Forest University for 2007 through 2012
2007 Graduated from RIT with Full Honors and from the Honors Program (GPA 3.96)
2006 Recipient of the RIT Outstanding Undergraduate Award
2005 Induction into Golden Key International Honors Society
2004 Awarded a certificate of achievement for presentation at RIT’s Undergraduate Research Symposium
2004 Awarded a scholarship through the RIT Honors Program for 2004 through 2007
2004 Accepted to the RIT Honors Program
2003 Awarded a Presidential Scholarship to attend RIT for 2003 through 2007

TEACHING/ADMINISTRATIVE RELATED EXPERIENCE

2010 **Student Lecturer**, Advanced Topics in Microbiology and Immunology, Wake Forest University.
Chose two journal articles and a review article concerning *Mycobacterium tuberculosis* latency for discussion during an Advanced Topics session. Guided student presentations on *M. tuberculosis* and latency, and led discussion of the journal articles and review article.
Dr. W. Edward Swords and Dr. Sean Reid, Department of Microbiology and Immunology

2010 **Student Host for Invited Seminar Speaker**, Wake Forest University.
Selected and invited Dr. Dennis Metzger to be a student-hosted speaker for the Microbiology and Immunology seminar series. Scheduled and hosted the seminar, assisted with travel arrangements for Dr. Metzger, and organized seminar and meetings with graduate students and post-doctoral fellows.

2010 **Student Lecturer**, Advanced Topics in Microbiology and Immunology, Wake Forest University.
Chose discussion paper, precursor paper, and review article for presentation and discussion during an Advanced Topics session. Led student discussion of quorum signaling and catecholamine signaling in *Salmonella typhimurium* and EHEC.
Dr. Jason Grason and Dr. Sean Reid, Department of Microbiology and Immunology

2006 **Teaching Assistant**, Advanced Immunology Laboratory, RIT, 03/2006 - 06/2006.
Prepared reagents, helped run experiments, and assisted students with laboratory technique and troubleshooting.
Dr. David A. Lawlor, Department of Biology.
2005  **Teaching Assistant**, Molecular Biology Laboratory, RIT, 03/2005 - 06/2005. Optimized laboratory protocols, prepared reagents, and implemented a new laboratory protocol generated during independent research. Graded assignments and assisted students with laboratory technique. Dr. Irene M. Evans and Dr. Jean A Douthwright, Department of Biology.