INTERACTIONS BETWEEN *STREPTOCOCCUS PNEUMONIAE* AND *MORAXELLA CATARRHALIS* IN POLYMICROBIAL COMMUNITIES AFFECT ANTIMICROBIAL RESISTANCE AND SURVIVAL IN VIVO

by

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<td>autoinducer 2</td>
</tr>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CbpA</td>
<td>choline binding protein A</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>ChoP</td>
<td>phosphorylcholine</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal scanning laser microscopy</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSP</td>
<td>competence simulating peptide</td>
</tr>
<tr>
<td>DPD</td>
<td>4,5-dihydroxy 2,3-pentanedione</td>
</tr>
<tr>
<td>ENT</td>
<td>ear, nose, and throat</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
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<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
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<tr>
<td>H. influenzae, NTHi</td>
<td>(nontypeable) <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Hib</td>
<td><em>Haemophilus influenzae</em> b</td>
</tr>
<tr>
<td>Hyl</td>
<td>hyaluronidase</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgD</td>
<td>immunoglobulin D</td>
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<tr>
<td>LOD</td>
<td>limit of detection</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>-------------</td>
<td>-------------------------------------------------</td>
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<tr>
<td>Isr</td>
<td>luxS-regulated</td>
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<tr>
<td>LuxS, luxS</td>
<td>homocysteine lyase</td>
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<tr>
<td>M. catarrhalis</td>
<td>Moraxella (Branhamella) catarrhalis</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
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<tr>
<td>µg</td>
<td>microgram(s)</td>
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<td>ml</td>
<td>milliliter(s)</td>
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<td>microliter(s)</td>
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<td>µm</td>
<td>micrometer(s)</td>
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<tr>
<td>ng</td>
<td>nanogram(s)</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>OM</td>
<td>otitis media</td>
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<tr>
<td>OME</td>
<td>otitis media with effusion</td>
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<tr>
<td>OMP</td>
<td>outer membrane protein</td>
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<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCV13</td>
<td>pneumococcal conjugate vaccine 13 (Prevnar)</td>
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<tr>
<td>PgdA</td>
<td>peptidoglycan deacetylase</td>
</tr>
<tr>
<td>Ply</td>
<td>pneumolysin</td>
</tr>
<tr>
<td>PPSV23</td>
<td>pneumococcal polysaccharide vaccine 23 (Pneumovax)</td>
</tr>
<tr>
<td>PspA</td>
<td>pneumococcal surface protein A</td>
</tr>
<tr>
<td>RLU/s</td>
<td>relative light units per second</td>
</tr>
<tr>
<td>rPAF</td>
<td>platelet activating factor receptor</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td><em>S. aureus</em></td>
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<tr>
<td><em>S. pneumoniae</em>, pneumococcus</td>
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<tr>
<td>SPRE</td>
<td></td>
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<tr>
<td>sRNA</td>
<td></td>
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<tr>
<td>THY</td>
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<td>TSB</td>
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<td>US</td>
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<td><em>V. harveyi</em></td>
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ABSTRACT

Otitis media is an extremely common pediatric disease affecting millions of children each year worldwide. The three leading pathogens associated with this disease are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. The initial step in the development of otitis media is nasopharyngeal colonization. The next step involves ascension through the Eustachian tube to access the middle ear space. For persistence within their host, it has been hypothesized that these bacteria are able to form biofilms on the mucosal epithelial cell surface. Recent studies have shown that these bacteria not only form biofilms in the middle ears of patients and experimentally infected animals with otitis media, but these surface-attached communities may contain multiple species of bacteria. Within a polymicrobial biofilm, the bacteria are able to share certain resources, including metabolic byproducts, virulence factors, and signaling molecules. Prior studies in the lab have shown that beta-lactamase production and quorum sensing play important roles in polymicrobial biofilms with *S. pneumoniae* and *H. influenzae*, and *M. catarrhalis* and *H. influenzae*, respectively. In these studies, the importance of both beta-lactamase and interspecies quorum signal in polymicrobial biofilms with *S. pneumoniae* and *M. catarrhalis* was assessed using in vitro and in vivo models.

Beta-lactamases are enzymes that inactivate the functional portion of beta-lactam antibiotics. Species of both Gram positive and Gram negative bacteria can produce a beta-lactamase and the spread of beta-lactamase genes is an increasing problem worldwide. A major concern with beta-lactamase producing bacteria is the effect they may have on co-residing species: the thought being that beta-lactamase production by an adjacent organism will affect the susceptibility of bacteria for which antimicrobial
therapy has been employed to treat. Virtually all strains of *M. catarrhalis* isolated today test positive for beta-lactamase activity. Additionally, this organism has been found in polymicrobial infections with other otopathogens, including *S. pneumoniae*. It has been hypothesized that beta-lactamase producing strains of *M. catarrhalis* and *H. influenzae* may in part account for the high treatment failure of *S. pneumoniae* in middle ear infections. Prior studies have shown that beta-lactamase produced by *H. influenzae* can provide passive resistance to pneumococcus in vitro and in vivo. Similarly, other studies suggested that *M. catarrhalis* may provide passive resistance to pneumococcus. To further investigate this possibility, an in vitro biofilm assay was used to determine if beta-lactamase produced by *M. catarrhalis* could passively protect *S. pneumoniae* from beta-lactam killing when both species are in a polymicrobial biofilm. Single species pneumococcal biofilms were readily killed by beta-lactam treatment. However, in polymicrobial biofilms with *M. catarrhalis*, pneumococcus was protected from killing by the beta-lactamase. Furthermore, pneumococcus was not protected from beta-lactam killing in polymicrobial biofilms with an isogenic beta-lactamase deficient mutant. Therefore, we concluded that beta-lactamase production by *M. catarrhalis* provides passive protection to pneumococcus in polymicrobial biofilms. These studies further support the hypothesis that beta-lactamase producing bacteria within the same microbial community as a beta-lactam susceptible species could provide passive resistance to beta-lactam antibiotics.

Quorum sensing is a process that involves the production of small, diffusible molecules that mediate bacterial cell-to-cell communication such that, at a target density, the bacteria can synchronize changes in gene expression optimal for residence within the
bacterial community. The interspecies quorum signal, autoinducer 2 (AI-2), is produced by many species of bacteria and provides a “universal” signal that allows multiple species of bacteria to communicate. Prior studies have found that this molecule is produced by both \textit{H. influenzae} and \textit{S. pneumoniae}, but not \textit{M. catarrhalis}. Despite its inability to produce AI-2, \textit{M. catarrhalis} can detect and respond to AI-2 produced by \textit{H. influenzae}, resulting in enhanced biofilm formation, resistance to macrolide antibiotics, and persistence within a chronic otitis media model. Therefore, in vitro antimicrobial protection assays and rodent nasopharyngeal colonization models were used to determine whether AI-2 produced by pneumococcus could protect \textit{M. catarrhalis} in a similar fashion. An isogenic AI-2 deficient mutant was constructed in a non-invasive pneumococcal strain; this mutant did not have any defects in growth, biofilm formation, antibiotic resistance, or survival in vivo. When tested in an in vitro polymicrobial biofilm assay, passive protection of \textit{M. catarrhalis} from macrolide antibiotic killing was found to be AI-2 independent. However, in vivo, production of AI-2 by pneumococcus enhanced \textit{M. catarrhalis} nasopharyngeal colonization when in a co-infection. Furthermore, quorum signal production by pneumococcus affected ascension of one or both of these pathogens into the middle ear during co-infection. These data demonstrate the importance of multispecies biofilm formation in antibiotic resistance and sheds new light on the role of interspecies communication during bacterial co-colonization and middle ear infection.
INTRODUCTION

Nasopharyngeal Colonization to Disease

Colonization of the nasopharynx begins immediately after birth. During this time, the nasopharynx is colonized by avirulent commensal organisms as well as potential pathogens [1, 2]. Among the resident flora, the otopathogens *Streptococcus pneumoniae* (pneumococcus), *Moraxella catarrhalis*, and *Haemophilus influenzae* also asymptotically colonize young children early in life [3-7]. Carriage rates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* tend to be especially high in children within the few years of life [4-6, 8, 9]. Although these organisms typically colonize without causing any clinical symptoms, alterations in the nasopharyngeal environment can lead to dissemination to adjacent sites and sometimes, especially with *S. pneumoniae* the blood, stream, causing opportunistic infections. In addition, nasopharyngeal secretions containing potential pathogens can mediate the spread of these organisms from one individual to another [1, 10]. Therefore, nasopharyngeal colonization is important for both disease development and the spread of pathogens [2].

Initiation and duration of nasopharyngeal colonization is influenced by both bacterial and host factors. For long-term survival in the nasopharynx, the bacteria must overcome innate host defenses, adhere to the epithelial cell surface, and establish a surface-attached community. To initiate adherence to epithelial cells, the bacteria must first get past the mucus barrier. The nasopharynx is a mucosal site. Therefore, the epithelial layer is protected by a thick mucus barrier that can trap inhaled microbes and prevent attachment. Additionally, the beating of cilia on the epithelial cells in the upper
respiratory tract provides constant removal of mucus-ensnared bacteria. The mucus layer also contains antimicrobial peptides, including lysozyme, beta-defensins, lactoferrin, and secretory immunoglobulin that can bind to bacteria and facilitate clearance [11, 12]. Therefore, to gain access to the epithelial layer below, potential pathogens have developed mechanisms to evade entrapment and killing within this mucosal barrier. For example, most pneumococcal strains produce a negatively charged polysaccharide capsule which repels the mucopolysaccharides in mucus, preventing entrapment [12]. Also, the production of a peptidoglycan deacetylase, PgdA, prevents lysozyme binding to peptidoglycan [13]. Both pneumococcus and NTHi produce an IgA protease that specifically targets human IgA1, which encompasses over 90% of the secretory IgA in the upper respiratory tract, preventing binding and antibody-mediated clearance of these bacteria [14].

Once through the mucus layer, the bacteria can adhere to epithelial cells through binding of bacterial surface molecules to host cell surface components [1, 15], or through binding to extracellular matrix materials [12, 16]. The specifics of these interactions will be discussed in detail later. In general, adhesion involves binding of surface-exposed molecules expressed on the bacterial and host cell surface, or through binding to components of the extracellular matrix within the basement membrane [1, 12, 16]. Expression of type IV pili or fimbriae can also mediate bacterial attachment [16, 17].

Finally, the attached bacteria grow and form surface attached communities, or biofilms, that can persist within the nasopharynx for extended periods of time. Figure 1 illustrates the events involved in biofilm formation [18]. Biofilm formation is initiated by the adherence of bacteria to a biotic or abiotic surface (Figure 1). Adherence in the
nasopharynx involves association of bacterial surface molecules with host epithelial cells, as previously mentioned. In the second stage, the bacteria begin to produce an extracellular polymeric substance (Figure 1). In pneumococcus, the extracellular polymeric substance has been shown to contain polysaccharides, proteins, and/or nucleic acids [18-20]. The extracellular polymeric substance encases the bacteria and allows for a stronger association with the surface to which they are attached. The third and fourth stages involve maturation and the development of biofilm architecture (Figure 1). Typically, mature biofilms are three-dimensional in nature and contain water channels, which allows for perfusion of nutrients and oxygen deep within the biofilm [18]. The fifth stage involves dispersal of the biofilm, or of a subset of bacteria from the biofilm, and repopulation of these organisms at new sites (Figure 1). Biofilm dispersal can be an active process, mediated by the bacteria themselves, or passive, with environmental factors such as shear forces playing a role. For active dispersal, secretion of enzymes that degrade extracellular matrix components have been described. A surface protein releasing enzyme (SPRE) has been identified in \textit{S. mutans}. Similar enzymes were found in other species of streptococci, including one strain of pneumococcus [21]. In the context of nasopharyngeal colonization, dispersal is a possible mechanism for the spread of colonized bacteria. The dispersed bacteria can either or be carried to a new potential host through exposure to contaminated nasal secretions, or disseminate and colonize new sites within the same host, possibly causing disease.

Colonization and persistence is not only mediated by bacterial factors. There are host factors that can also influence the duration of carriage, the most significant of which is age. Studies have shown that the carriage rate of potential pathogens is greatly reduced
Figure 1: Schematic of the events involved in biofilm formation [18]. (1) Attachment of bacteria to a surface. (2) The attached bacteria begin to grow on the surface and produce an extracellular matrix. (3) The biofilm begins to mature and early biofilm architecture is developing. (4) Establishment of a mature biofilm. (5) Dispersal of the biofilm, or of a subset of bacteria from the biofilm, and recolonization of a new site.
with increasing age [1, 22]. The enhanced prevalence of otopathogen colonization in young children is a consequence of immune immaturity. Due to the state of their immune systems, children do not produce effective response to certain bacterial antigens, including polysaccharides, which make them more prone to colonization and infection. However, breast milk contains some immune factors, including secretory immunoglobulins, which help protect infants from infections, including otitis media [3, 4, 23]. To this end, studies have shown that breastfeeding may affect nasopharyngeal colonization with potential pathogens. However there are also some studies that suggest otherwise [4, 24]. Furthermore, young children are routinely vaccinated to protect against *H. influenzae* b (Hib) and *S. pneumoniae* colonization and subsequent infection. While the Hib vaccine is very effective against typeable *H. influenzae*, it does not provide protection against nontypeable *H. influenzae* (NTHi). On the other hand, the formulation of the pneumococcal vaccine that is given to children (PCV13) provides protection against the 13 serotypes that were most commonly associated with disease, most notably invasive disease. While vaccination has significantly reduced colonization with serotypes represented in the vaccine, the prevalence of other serotypes not included in the vaccine has improved [25].

The development of the nasopharyngeal normal flora is also highly influenced by the environment. Some of environmental factors known to affect colonization are listed in Table I. There is also a wealth of data that indicates the existing normal flora can influence the ability of an otopathogen to colonize the nasopharynx [26-32]. In addition, antibiotic use can have a significant effect on the balance of normal flora species and in turn affect colonization, carriage, and infection with potential pathogens [33].
Typically, colonizing potential pathogens persist within the nasopharynx without causing disease [1]. However, disruptions in the environment of the nasopharynx can allow these organisms to disseminate to adjacent sites, including the lungs, middle ear space, and nasal sinuses, and cause disease. In severe cases, some species can even access the blood stream and cause life-threatening systemic infections. Studies have shown that prior viral upper respiratory tract infection can cause inflammation and damage to the epithelial cell layer, which can enhance the likelihood of potential pathogens causing disease [2, 30, 32, 34-36]. In addition, inherited predisposing conditions, like cystic fibrosis (CF), can enhance the propensity of potential pathogens to cause disease. CF is a genetic disorder that most critically affects the lungs. A mutation in the CFTR gene causes a defect in chloride and sodium transport, which in turn leads to dehydration of airway mucus, reduced mucociliary clearance, mucus build-up, and chronic infections [37]. As a result, CF patients develop lung infections caused by airway opportunists, including Pseudomonas aeruginosa, S. aureus, H. influenzae, Stenotrophomonas maltophilia, Achromobacter xylosoxidans, and Burkholderia species [38]. The exact mechanisms that allow potential pathogens to switch from colonization to disease are not well understood. However, some of these organisms carry a set of virulence factors that could play a role in this critical switch. The virulence factors employed by S. pneumoniae and M. catarrhalis will be reviewed later.

**Otitis Media**

Otitis media (OM) is a common pediatric disease world-wide, affecting most children within the first three years of life [39]. It is the most common ailment for which children visit the doctor, antibiotics are prescribed, and parents miss days from work to
Table I: Examples of environmental factors that can influence nasopharyngeal colonization.

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>References</th>
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<tbody>
<tr>
<td>Season</td>
<td>[40]</td>
</tr>
<tr>
<td>Socio-economic status</td>
<td>[41]</td>
</tr>
<tr>
<td>Geographical location</td>
<td>[42]</td>
</tr>
<tr>
<td>Exposure to smokers</td>
<td>[4]</td>
</tr>
<tr>
<td>Daycare attendance</td>
<td>[43]</td>
</tr>
<tr>
<td>Allergies</td>
<td>[44]</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>[45]</td>
</tr>
<tr>
<td>Family size</td>
<td>[46]</td>
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</tbody>
</table>
care for a sick child in the US [47]. In addition to the high rate of morbidity, it is also a significant economic burden, costing >$3.5 billion dollars per year in the US alone [48, 49]. The majority of OM cases are acute in nature. However, complications due to recurrent/chronic OM can cause irreparable damage to the ears, sometimes with effects lasting into adulthood [50, 51]. Some of these complications include hearing loss, balance and motor problems, and speech and learning delays [52].

Age is the most important risk factor associated with this disease [53]. The age-related incidence of OM seems to peak between the ages of 6 and 18 months. After this point, the incidence of OM tends to decline with increasing age [54]. Young children are at the highest risk for developing OM due to their immature immune systems and ear, nose, and throat (ENT) anatomy. As discussed previously, young children do not make adequate responses to certain bacterial antigens. This inadequacy not only affects the rate of colonization, it also has an affect on the propensity of potential pathogens to cause infection [4]. In addition, the anatomy of the ENT in young children makes them prone to developing this disease. As shown in Figure 2 (left), children have a short, horizontally-oriented Eustachian tube. In this position, it is more difficult to keep the Eustachian tube clear of secretions from both the nasopharynx and the inner ear, which allows pathogens easier access to the middle ear space. As children get older, the craniofacial features elongate and the Eustachian tube becomes longer and more vertical (right). Additionally, environmental factors, many of which are listed in Table I, anatomic abnormalities affecting the ENT, such as cleft lip and palate, immunodeficiencies, or genetic conditions, such as down syndrome, can also affect disease development [47].
Figure 2: Schematic of Eustachian tube anatomy in infants (left) and adults (right) [55]. The Eustachian tube of a young child is shorter and more horizontal than the Eustachian tube of an adult. The adult Eustachian tube tends to be longer and more vertically oriented. Secretions from the nasopharynx can more easily contaminate the Eustachian tube in young children. It is for this reason that children are prone to developing OM.
Ascension of otopathogens from the nasopharynx into the middle ear typically requires an initiating event, i.e. upper respiratory tract infection, allergies, etc., that causes inflammation and in the upper respiratory tract and obstruction of the isthmus, which is the narrowest portion of the Eustachian tube [53]. Also, dysfunction of the Eustachian tube compromises the natural flow of secretions out of the Eustachian tube and away from the middle ear. This blockage causes negative pressure to build up in the behind the tympanic membrane and otopathogens in nasopharyngeal secretions are then introduced into the middle ear by aspiration through the Eustachian tube [53]. The bacteria can then colonize the middle ear mucosa and cause middle ear inflammation and effusion.

OM encompasses a broad spectrum of disease presentations. However it can be separated into two basic classifications: acute otitis media (AOM), middle ear inflammation caused by infection, and otitis media with effusion (OME), the presence of middle ear effusion that is not the result of an infection [56]. AOM presents with a rapid onset of symptoms and is diagnosed by the presence of acute inflammation in the middle ear, i.e. bulging of the tympanic membrane, and the presence of middle ear effusion. The child may also present other signs that indicate AOM, including otalgia, irritability, excessive crying, otorrhea, and/or fever [56]. OME on the other hand, is typically caused by Eustachian tube dysfunction, which causes build-up of middle ear secretions and middle ear effusion. With OME, there is usually no inflammation or bulging of the tympanic membrane.

The three most frequently isolated pathogens from patients with OM are *S. pneumoniae*, NTHi, and *M. catarrhalis*. The prevalence of each species in middle ear
fluids from patients with AOM may vary depending on the region in which the study was conducted. Table II lists the results from a study conducted in Rochester, NY by Casey et al post-PCV7 introduction [53, 57]. In general, pneumococcus is the most commonly isolated otopathogen, followed by NTHi, then M. catarrhalis. However, some studies have found as much as a 40% reduction in the number of S. pneumoniae isolates found in middle ear fluids after PCV7 introduction, with a marked increase in the number of AOM cases caused by Gram-negative otopathogens, most notably NTHi [58, 59].

Studies have shown that the majority of the time, AOM will spontaneously resolve on its own in otherwise healthy children over 2 years of age [56, 60]. However, in cases where age and illness severity is a factor, antimicrobials are prescribed to treat these infections [56]. If antimicrobial intervention is necessary, amoxicillin (80-90 mg/kg per day) is the first line of antibiotics prescribed, unless there is a penicillin allergy. If initial antimicrobial treatment fails, amoxicillin-clavulanate (90 mg/kg/day amoxicillin and 6.4 mg/kg/day clavulanate) is the second line of treatment. For the management of recalcitrant AOM, surgical intervention may be employed, depending on severity and frequency of disease. Insertion of tympanostomy tubes has been shown to reduce the number of recurrent AOM episodes in patients with recalcitrant AOM [61]. Recalcitrant AOM occurs at an alarmingly high rate, with 20% of children being affected within their first years of life [62, 63]. Recent studies in children with chronic OM have shown that these otopathogens are able to form biofilms in the middle ears [64]. Biofilm bacteria can be up to 1,000 times more resistant to antimicrobial therapy than planktonic bacteria [65, 66]. In addition, some strains of NTHi and virtually all strains of
Table II: **Prevalence of otopathogens in middle ear aspirates.** Table adapted from Casey et al [53, 57]

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Percent of aspirates *</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>31.7 (66)</td>
</tr>
<tr>
<td>nontypeable <em>Haemophilus influenzae</em></td>
<td>28.4 (59)</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>13.9 (29)</td>
</tr>
</tbody>
</table>

*The number of positive aspirates is represented in parentheses
*M. catarrhalis* produce a beta-lactamase; these bacteria are especially resistant to beta-lactam antibiotics [67-69]. Considering each of these points, the high rate of antimicrobial treatment failure may be explained by the resilient nature of biofilm bacteria and beta-lactamase-producing strains. Nonetheless, amoxicillin continues to be the first line of treatment because it is cost-effective, it tastes better, and the side effects are minimal in comparison to other antibiotics used to treat this disease [47].

**Streptococcus pneumoniae**

*Streptococcus pneumoniae*, or pneumococcus, is a Gram-positive organism that colonizes the human nasopharynx and typically resides there as a commensal species. However, under conditions where the host is immunosuppressed or immunocompromised, it can cause opportunistic infections of the upper and lower respiratory tract, as well as severe, systemic diseases. The World Health Organization estimates that 1.6 million people die each year from invasive pneumococcal disease [70]. In patient samples, pneumococcus is identified by both culture- and biochemical-based tests (Figure 3). Typeable pneumococcus is alpha-hemolytic on a blood agar plate, optochin-sensitive, and bile soluble. For serotyping, the standard method used is the quellung reaction, in which serotype specific anti-capsular antibodies are mixed with the bacteria on a slide then visualized by light microscopy. If the capsule “swells,” this indicates a positive reaction to the antibody. Some non-culture-based techniques have been developed within the last decade, and these have also been used for identification of pneumococcus. PCR-based practices target areas of the genome where the sequence is unique to pneumococcus, including 16S-rRNA, *lytA*, *ply*, and *rpoA* [71-75].
The virulence factors employed by pneumococcus play important roles in colonization and disease. Most pneumococcal strains produce a polysaccharide capsule, which prevents entrapment in mucus and effective opsonophagocytosis [12, 76, 77]. Furthermore, studies using isogenic capsule-deficient mutants demonstrate that capsule production is necessary for effective colonization in these strains [78]. Capsule production is phase variable; studies suggest low capsule production is permissible to colonization, allowing appropriate exposure of adhesion molecules, while high capsule production is associated with disease, enhancing resistance to serum-mediated killing and opsonophagocytosis [79-81]. For adherence, expression of surface-associated exoglycosidases NanA, BgaA, and StrH remove terminal sugars on glycoconjugates and allows for exposure of host cell receptors [12, 82]. Also, expression of phosphorylcholine (ChoP) aids in bacterial adherence through binding to the platelet activating factor receptor (rPAF) on epithelial cells [12]. Pneumococcus encodes 10-15 choline binding proteins that also aid in adherence [83]. Choline binding protein A (CbpA) is non-covalently bound to ChoP. It binds to the human secretory component found on secretory IgA and the polymeric immunoglobulin receptor [84, 85]. Binding to the human secretory component not only aids in adherence, it also allows pneumococcus to translocate across the epithelial barrier and gain access to the connective tissue beneath, also known as the basement membrane [12]. Once in the basement membrane, the hyaluronidase (Hyl) can facilitate spread through the hyaluronan-rich extracellular matrix within the basement membrane [86]. Bacterial adherence within this area is facilitated through binding of PavA and Eno, expressed on the bacterial cell surface, to fibronectin and plasminogen, respectively [83, 87].
Figure 3: Standard clinical laboratory practices for positively identifying pneumococcus in a patient sample [75]. Alpha-hemolytic colonies that are optochin-sensitive can then be serotyped using the Quellung reaction. If positive, the colonies can be positively identified as typeable pneumococci.
Expression of pneumococcal virulence factors also plays an important role in invasion and disease. As previously discussed, the capsule is important for resistance to complement deposition and opsonophagocytosis, which aids in invasion and disease. Loss of capsule expression in encapsulated strains results in attenuated virulence in animal models of invasive pneumococcal disease [88-90]. Pneumolysin (Ply) is a cytolytic toxin and a TLR4 agonist that plays an important role in disease [91, 92]. Production of this toxin has been associated with enhanced bacterial survival in the upper and lower respiratory tract, spread of bacteria from the lungs to the blood stream, and death due to bacteremia in animal models of invasive pneumococcal disease [93-96]. For Ply release, LytA production is stimulated through the competence stimulating peptide quorum sensing system. This enzyme digests the cell wall to release cytoplasmic Ply into the extracellular environment [97]. To aid in serum resistance, pneumococcal surface protein A (PspA) prevents binding of C3 on the pneumococcal surface [12]. Several metal-ion-binding lipoproteins have been shown to be essential for pneumococcal disease. PsaA is a divalent metal-binding lipoprotein that is part of an ABC transporter system with specificity for manganese and iron. When psaA is deleted, virulence is abolished in murine models of pneumonia, bacteremia, and colonization [12, 98-100]. Similarly, PiaA and PiuA are part of an ABC transporter system specific for iron uptake; a mutation in either piaA or piuA reduced bacterial virulence in pneumonia and bacteremia models, but a double-mutant resulted in much greater attenuation [101, 102].

For the treatment of pneumococcal diseases, beta-lactam antibiotics are typically employed because most strains of pneumococcus are susceptible. However, the rate of antibiotic resistance in pneumococci is increasing at an alarming pace. Currently, the
percentage of antibiotic resistant strains isolated is around 30% in the US. However, the percentage of penicillin-resistant strains can be as high as 80% in some countries [103]. The mechanism of beta-lactam antibiotic resistance usually involves mutation of penicillin-binding proteins (PBP) so that beta-lactams can no longer bind and inhibit cell wall biosynthesis [104]. Pneumococci do not produce beta-lactamases [103], so the addition of clavulanate will not make resistant strains more susceptible to beta-lactam treatment.

As the rate of antibiotic resistance continues to increase in pneumococcal stains, preventing colonization through vaccination may hinder the spread of these strains and prevent other strains from developing resistance. There are two formulations of pneumococcal vaccine available: the polysaccharide vaccine (PPSV23) and the conjugate vaccine (PCV13). These vaccines are typically given to high risk individuals. PPSV23 is given to the elderly (65+) and patients between the ages of 2 - 65 years old that are immunocompromised, for instance AIDS patients, immunosuppressed, or have a congenital defect [105, 106]. This formulation of the vaccine includes polysaccharide antigens from 23 of the most commonly isolated serotypes in disease. The pneumococcal conjugate vaccine, PCV13, is typically given to young children < 2 years of age; it includes polysaccharide antigens from 13 of the most common disease-causing serotypes conjugated to a diptheria toxoid [107]. Through vaccination against pneumococcus, the main goal is to prevent nasopharyngeal colonization by these disease-prone serotypes, which is the initial step necessary for development of disease. In children, vaccination has been very successful in preventing disease caused by the included serotypes. However, vaccination does not protect against serotypes not included in the vaccine.
Studies have shown that these serotypes have increased in prevalence in both colonization and disease [108-110]. To account for this short-coming of the current formulations, research on other possible vaccine candidates, including some of the virulence factors previously mentioned, with a broader serotype coverage is now the main focus in pneumococcal vaccine research.

**Moraxella catarrhalis**

*Moraxella (Branhamella) catarrhalis* is a Gram-negative, unencapsulated diplococcus that, like pneumococcus, typically resides in the human nasopharynx as a commensal species. In fact, in 1921, Gordon described *M. catarrhalis* a “saprophyte of negligible virulence found in the throat of heathy adults” [111]. Today, *M. catarrhalis* is a known pathogen that is typically associated with localized disease, but is not commonly a cause of severe invasive disease. In chronic obstructive pulmonary disease (COPD) patients, *M. catarrhalis* is the second most common cause of exacerbations, causing 2-4 million episodes per year in the US [16, 112, 113]. Carriage of *M. catarrhalis* is extremely common in young children; two-thirds of all children are colonized within the first year of life [7]. In adults, the carriage rate is much lower, with only 3-5% of adults being colonized [16, 112]. For identification in a clinical laboratory, *M. catarrhalis* can be grown on a chocolate or blood agar plate for 16-48 hours in 5-10% CO₂ at 35-37°C. Colonies are smooth, round, uniform, opaque, non-haemolytic, and can be easily be pushed along the surface of the agar like a “hockey puck [111].” *M. catarrhalis* can further be identified by microscopy, i.e. Gram stain, and biochemical tests; it is oxidase, tributyrin, and DNase positive [114].
The virulence factors employed by *M. catarrhalis* are not as well characterized as the virulence factors in pneumococcus. The lack of good animal models for *M. catarrhalis* diseases makes characterization difficult. In *M. catarrhalis*, adhesion to epithelial cells is a multifactorial event that involves (1) initial, long distance attachment by type IV pili, then (2) outer membrane proteins (OMPs) for close-up attachment [16].

UspAs are multifunctional, phase-variable proteins that play an important role in attachment. UspA1 binds human CEACAM1 and UspA2 binds components of the extracellular matrix, including fibronectin, laminin, and vitronectin. The surface molecule that UspA2H binds is not known, but it has been shown to mediate attachment in Chang cells [115-120]. Like UspAs, Hag/MID is also phase-variable and multifunctional. It plays an important in attachment and has been shown to bind IgD, agglutinate human erythrocytes, and adhere to several cell lines, including human middle ear epithelial cells [121-124]. Other OMPs shown to be involved in adhesion to epithelial cells are McaP, OMP CD, and the Mha proteins [125-127]. The role of LOS in *M. catarrhalis* adhesion is still debatable. Studies using LOS-defective mutants have shown reduced adhesion to various epithelial cell lines in vitro [128, 129]. However, it is not clear whether it plays a direct role in adhesion. One hypothesis is that LOS maintains membrane integrity, and, without it, the expression of important adhesion molecule on the bacterial surface may be altered [16, 129].

The ability to resist clearance is another important factor in *M. catarrhalis* colonization and disease. Studies have shown that *M. catarrhalis* can invade tissues to hide from host immune factors and antibiotics [16]. As mentioned previously, UspA1 binding to CEACAM1 is important for adherence, however, this interaction may also
play a role in tissue invasion [115]. Furthermore, Hag/MID has been shown to induce activation in human B cells in vitro. It has been hypothesized that this interaction may play a role in survival of *M. catarrhalis* within the subepithelial layer of adenoid and tonsilar tissue [130, 131]. The innate immune response is the first line of defense against pathogens. Studies have shown that *M. catarrhalis* can resist complement-mediated killing through the expression of UspA2, OMP CD, OMP E, CopB, and LOS [125, 132-135]. Another important persistence factor is biofilm formation. This is a poorly understood aspect of *M. catarrhalis* pathogenesis. However, studies have suggested that expression of CopB, OMP J, Hag, type IV pili, UspAs and OMP G1b may play a role in biofilm formation [136-140].

The nasopharynx is a nutrient-limiting environment. This is an innate defense mechanism so that potential pathogens cannot colonize and/or cause disease. However, *M. catarrhalis* has devised several mechanisms to acquire nutrients in limiting conditions so that it can colonize this site. For example, iron is a key nutrient that is not readily available in the upper respiratory tract. However, *M. catarrhalis* can steal the iron from host iron-binding proteins, including lactoferrin, transferrin, and hemoglobin. Lactoferrin and transferrin are present in mucus, and OMPs LbpA/B and TbpA/B, respectively, can utilize the iron bound to these molecules [141, 142]. CopB has been shown to utilize iron from both lactoferrin and transferrin [143]. Additionally, *M. catarrhalis* expresses HumA which can steal the iron from metalloproteins containing heme. Furthermore, MhuA has been shown to utilize the iron in hemoglobin as a sole iron source in limiting conditions [144, 145]. For uptake of essential nutrients, especially under nutrient-
limiting conditions, the M35 porin has been shown to be essential in a murine nasopharyngeal colonization model [146].

Virtually all strains of *M. catarrhalis* isolated today produce a beta-lactamase [67]. This beta-lactamase has been shown to be highly active against amoxicillin, which is the first line of antibiotics used to treat OM. It is hypothesized that beta-lactamase production by *M. catarrhalis* and *H. influenzae* may account for the high treatment failure seen in OM cases. The use of clavulanate will inhibit most of these beta-lactamases and render these organisms susceptible to amoxicillin treatment [147]. However, clavulanate is not administered until the initial treatment has failed, which adds to the high financial burden and substantial amount of antibiotics given to treat this disease. As a prophylactic measure, the development of a *M. catarrhalis* vaccine may provide some relief from the morbidity and financial burden caused this pathogen.

**Polymicrobial Biofilms in Otitis Media**

Based on conventional detection methods, OM was believed to mostly be caused by a single pathogen. However, more recent epidemiological data and studies in children with recurrent/chronic OM have shown that a significant number of these cases are actually polymicrobial infections. Advances in technology, namely PCR-based assays, have played a key role in detection of multiple species in chronic OM samples. In addition, confocal laser scanning microscopy (CLSM) on tissue samples from chronic OM patients has shown that these bacteria are able to form biofilms in the middle ear [64, 148]. This finding is important because biofilms have been shown to be significantly more resistant to antibiotic treatment, which could provide one possible reason why there
is such high treatment failure with OM [149]. Also, since conventional detection methods often miss the detection of multiple species, treatment of these infections may not be as effective, especially if beta-lactamase producing organisms are present [150-152]. This proposed protection of typically susceptible bacteria by beta-lactamase-producing bacteria is one example of indirect pathogenicity, where the susceptibility or virulence of one organism is affected by another. There have been other proposed mechanisms of indirect pathogenicity that may affect the survival of otopathogens during OM. For example, production of interspecies quorum signal may mediate passive resistance when multiple pathogens are in a polymicrobial community. Evidence of this has been shown in prior work in the lab [153]. The formation of polymicrobial communities by otopathogens may provide prolonged survival and enhance resistance. Characterizing the methods of indirect pathogenicity utilized by these organisms may aid in the development of more effective treatments for this disease.

**Beta-lactamase Production**

In 1940, Abraham and Chain discovered an enzyme with “penicillin-destroying activity [154].” Soon after, similar enzymes were found to have activity against cephalosporins (cephalosporinases) and some having activity against both types of beta-lactams [155]. These enzymes were found to hydrolyze the beta-lactam bond of this class of antibiotics; therefore they were re-termed beta-lactamases. Classification of beta-lactamases is extremely difficult due to the wide diversity of these enzymes. However, Bush *et al* proposed a classification scheme based on the function and molecular structure (Table III) [156]. In general, there are 4 basic molecular classifications (A-D), which are based on nucleic and amino acid sequence similarity.
Based on the molecular classification, the enzymes can be further broken-down into 4 functional groups (1-4). For example, Group 1 consists of clavulanate-sensitive enzymes, with cephalosporinase activity, that belong to molecular Group C.

Beta-lactamase production can be found in species of both Gram-positive and Gram-negative bacteria, as well as mycobacteria [155]. However, beta-lactamase production is generally found in species of Gram-negative organisms, including the otopathogens *M. catarrhalis* and NTHi. The beta-lactamase produced by *M. catarrhalis* is encoded by the *bro* gene. There are two isoforms: BRO-1 and BRO-2. However, beta-lactamase producing strains only produce one of either isoform [157-159]. BRO-1 is the heavier of the two isoforms, and is most abundant among *M. catarrhalis* strains. BRO-2 is less abundant and its substrate metabolism level is slightly lower than BRO-1 [159]. The acquisition and spread of BRO seemed to occur rapidly, with the first ampicillin-resistant strains of *M. catarrhalis* being described over 30 years ago [160, 161]. Moreover, the origin of this beta-lactamase is still unknown. It has been hypothesized that *M. catarrhalis* aquired this enzyme from a Gram-positive organism [162]. Evidence to support this hypothesis is based on molecular and structural similarities of this enzyme to beta-lactamases typically found in Gram-positive organisms. For example, the enzyme is membrane-bound which is a form found in Gram-positive organisms. Also, the GC content is relatively higher than genes flanking *bro*; the GC content of *bro* is ~60% while the GC content of flanking genes is ~30% [162]. Moreover, the high GC content more closely resembles the GC content found in Gram-positive organisms. Finally, studies have shown that BRO is sensitive to clavulanate so *M. catarrhalis* strains expressing this enzyme are easily killed by amoxicillin/clavulanate [163].
Table III: Classification scheme proposed by Bush, Jacoby, and Mederios for beta-lactamases. Group designation is based on functionality and class is based on molecular similarity. Table adapted from Bush et al [156]. * Not determined

<table>
<thead>
<tr>
<th>Group</th>
<th>Molecular class</th>
<th>Preferred substrates</th>
<th>Inhibited by clavulanate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>Cephalosporins</td>
<td>-</td>
</tr>
<tr>
<td>2a</td>
<td>A</td>
<td>Penicillins</td>
<td>+</td>
</tr>
<tr>
<td>2b</td>
<td>A</td>
<td>Penicillins, Cephalosporins</td>
<td>+</td>
</tr>
<tr>
<td>2be</td>
<td>A</td>
<td>Penicillins, narrow-spectrum and extended spectrum cephalosporins, monobactams</td>
<td>+</td>
</tr>
<tr>
<td>2br</td>
<td>A</td>
<td>Penicillins</td>
<td>±</td>
</tr>
<tr>
<td>2c</td>
<td>A</td>
<td>Penicillins, carbenicillin</td>
<td>+</td>
</tr>
<tr>
<td>2d</td>
<td>D</td>
<td>Penicillins, cloxacillin</td>
<td>±</td>
</tr>
<tr>
<td>2e</td>
<td>A</td>
<td>Cephalosporins</td>
<td>+</td>
</tr>
<tr>
<td>2f</td>
<td>A</td>
<td>Penicillins, cephalosporins, carbenamens</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>Most beta-lactams, including carbenams</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>ND*</td>
<td>Penicillins</td>
<td>-</td>
</tr>
</tbody>
</table>
It has been hypothesized that beta-lactamase producing bacteria may provide protection to typically susceptible species of bacteria from beta-lactam treatment. Weimer, et al conducted in vitro and in vivo studies that support this hypothesis, demonstrating that the beta-lactamase produced by NTHi can protect pneumococcus from amoxicillin treatment. A few studies have suggested that the beta-lactamase produced by *M. catarrhalis* can protect pneumococcus from amoxicillin treatment in vitro. However, it is not known whether this protection is mediated by the beta-lactamase alone or if there could be other factors involved.

**Quorum Sensing**

Quorum sensing is a means of density-dependent cell-to-cell communication utilized by bacteria. It involves the synthesis of small, diffusible molecules that, when detected, results in changes in gene expression. There are two types of quorum sensing systems. Intraspecies systems are very specific to a particular species of bacteria, and only this species is able to produce, detect and respond to the molecule(s). Interspecies systems involve the synthesis of a molecule that multiple species of bacteria can recognize and respond to. For example, pneumococcus possesses both an intra- and interspecies quorum sensing system. The competence stimulating peptide (CSP) system is an intraspecies system that involves the synthesis of small peptide pheromones. Response to these signals results in upregulation of factors involved in transformation, biofilm formation, and virulence [97, 164-166]. Pneumococcus is also able to produce the interspecies quorum signal autoinducer-2 (AI-2).
The AI-2 quorum sensing system was first discovered and described in *Vibrio* species. Since then, the machinery to produce this signal has been found in over half of the bacterial genomes published on the NCBI database [167, 168]. AI-2 production has been described in both Gram-positive and Gram-negative species of bacteria; the genetic determinant for its production is *luxS*. The enzyme produced by this gene, homocysteine lyase (*LuxS*), acts in concert with Pfs to cleave S-adenosylhomocysteine into homocysteine in a two step process. First, Pfs cleaves S-adenosylhomocysteine into S-ribosylhomocysteine and adenine. Then, LuxS cleaves S-ribosylhomocysteine into homocysteine and the biproduct 4,5-dihydroxy 2,3-pentanedione (DPD), which spontaneously cyclizes in solution into AI-2 [169]. On the other hand, bacteria that do not encode Pfs and LuxS, and therefore do not produce AI-2, instead encode a single enzyme, SAH hydrolase, which cleaves S-adenosylhomocysteine into homocysteine and adenosine in one step [169].

There are several well described systems for uptake and response to AI-2. In *Escherichia coli* and *Salmonella typhimurium*, AI-2 regulates the *luxS* regulated (*lsr*) genes, which encode the machinery necessary for AI-2 uptake and processing [170, 171]. In *V. harveyi*, AI-2 uptake and response is mediated by a set of genes responsible for response to another signal HAI-1, which is the primary quorum sensing pathway that regulates bioluminescence [167] Both of these signaling networks have cross-talk and results in expression or repression of LuxR. As illustrated in Figure 4, the membrane-bound receptor complex (LuxPQ) contains the AI-2 binding protein, LuxP, and the sensor kinase LuxQ, which can switch from kinase to phosphatase activity in response to the level of signal available in the environment. At low levels of signal, LuxQ
Figure 4: The AI-2 signaling network in *V. harveyi* [167]. When no AI-2 is available, LuxQ autophosphorylates, which initiates a phosphorylation cascade. This results in the repression of the regulator LuxR. When AI-2 is detected through binding of LuxP, LuxQ switches to phosphatase activity and drains phosphate from the system. This results in derepression of LuxR and the upregulation of LuxR-regulated genes.
autophosphorylates and stimulates a phosphorylation cascade, which leads to the expression of genes that encode a set of 5 small regulatory RNAs (sRNAs) that block translation of *luxR*. When the level of signal is high, LuxQ switches to phosphatase activity and drains phosphate from the system which represses expression of the sRNAs and derepresses the expression of LuxR. This protein is responsible for activating genes responsible for bioluminescence as well as 100 other genes. A LuxR family transcriptional regulator has been identified in *M. catarrhalis* [172]. Interestingly, data has shown that while *M. catarrhalis* cannot produce AI-2, it is able to take up and respond to exogenous AI-2 [153]

**Statement of Research Purpose**

The research presented herein focuses on the importance of beta-lactamase and interspecies quorum sensing in polymicrobial biofilms with *S. pneumoniae* and *M. catarrhalis*. First, we wanted to determine if beta-lactamase production by *M. catarrhalis* will protect *S. pneumoniae* from beta-lactam killing. For these studies, in vitro polymicrobial biofilms were treated with amoxicillin, and the survival of pneumococcus was assessed. Then, we wanted to assess the role(s) of interspecies quorum signal in antibiotic resistance, and survival and persistence of *M. catarrhalis* and pneumococcus in polymicrobial infections. For these studies, in vitro polymicrobial biofilms were treated with azithromycin and the survival of *M. catarrhalis* was assessed. Also, co-infections in two rodent models of nasopharyngeal colonization with subsequent AOM were used to assess survival and pathogenesis of both pneumococcus and *M. catarrhalis*. 
Chapter I:

Residence of *Streptococcus pneumoniae* and *Moraxella catarrhalis* within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence *in vivo*

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ABSTRACT

Otitis media is an extremely common pediatric ailment caused by opportunists that reside within the nasopharynx. Inflammation within the upper airway can promote ascension of these opportunists into the middle ear chamber. Otitis media can be chronic/recurrent in nature, and a wealth of data indicates that in these cases the bacteria persist within biofilms. Epidemiological data demonstrates most cases of otitis media are polymicrobial, which may have significant impact on antibiotic resistance. In this study, we used *in vitro* biofilm assays and rodent infection models to examine the impact of polymicrobial infection with *Moraxella catarrhalis* and *Streptococcus pneumoniae* (pneumococcus) on biofilm resistance to antibiotic treatment and persistence *in vivo*. Consistent with prior work, *M. catarrhalis* conferred beta-lactamase dependent passive protection from beta-lactam killing to pneumococci within polymicrobial biofilms. Moreover, pneumococci increased resistance of *M. catarrhalis* to macrolide killing in polymicrobial biofilms. However, pneumococci increased colonization *in vivo* by *M. catarrhalis* in a quorum signal-dependent manner. We also found that co-infection with *M. catarrhalis* affects middle ear ascension of pneumococci in both mice and chinchillas. Therefore, we conclude that residence of *M. catarrhalis* and pneumococci within the same biofilm community significantly impacts resistance to antibiotic treatment and bacterial persistence *in vivo*.
INTRODUCTION

Otitis media (OM) is a significant public health problem worldwide, affecting the majority of all children at least once by three years of age [173]. OM is typically caused by colonization of the middle ear space by bacterial opportunists that normally reside within the nasopharyngeal microbiota. These infections can be chronic and/or recurrent in nature, and a wealth of data indicates that the bacterial populations persist within biofilm communities [64, 174-176]. Recent epidemiology data also clearly demonstrate that most cases of OM involve simultaneous infection with multiple agents [177-180], and our recent work shows that otopathogens can coexist within biofilm communities [181-183]. Such polymicrobial infections can have a profound impact on the progression, severity, and response of infections to treatment. It is therefore of great importance to understand how different bacterial species interact during OM infections.

In particular, Moraxella catarrhalis has long been thought to be of importance in the context of polymicrobial infections due to the expression of beta-lactamase by virtually all clinical isolates [67]. It is for this reason that M. catarrhalis is frequently implicated as a cause of high treatment failures with beta-lactam antibiotics against pathogens that are otherwise susceptible. The general hypothesis is that the production of beta-lactamase affords passive protection [184, 185].

In addition, many species of bacteria can produce and/or respond to small, diffusible molecules in a process termed quorum sensing. It has been hypothesized that production of interspecies quorum signal, auto-inducer 2 (AI-2), could have an effect on persistence and/or virulence of multiple species of bacteria residing within a polymicrobial community. AI-2 is produced as a bi-product of the activated methyl cycle
where LuxS cleaves S-ribosylhomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which spontaneously cyclizes in solution into AI-2. First described in *Vibrio* species [186-188], AI-2 production has been demonstrated in species of both gram-positive and gram-negative bacteria [169], including *Streptococcus pneumoniae* (pneumococcus). While *M. catarrhalis* cannot produce its own AI-2, our recent work highlights the importance of interspecies quorum signaling to the persistence of *M. catarrhalis* bacteria in vivo, with other otopathogens potentially augmenting biofilm formation and persistence by *M. catarrhalis* through production of AI-2 [181]. The objective of this study was to define interactions of *M. catarrhalis* and *S. pneumoniae* within polymicrobial biofilms, and their implications for resistance of bacteria within biofilm to antibiotic treatment or host clearance.
MATERIALS AND METHODS

Bacterial strains and growth conditions

A list of all bacterial strains, plasmids, and primers is provided in Table IV. \textit{S. pneumoniae} EF3030 is a serotype 19F strain which typically establishes nasopharyngeal carriage or localized airway infection in murine models \cite{88, 189}. Pneumococci were grown on trypticase soy agar (BD) supplemented with 5\% defibrinated sheep blood (Hemostat) and 4 µg ml\(^{-1}\) gentamicin. For freezer stocks, \textit{S. pneumoniae} was grown in Todd Hewitt broth with 0.5\% yeast extract (THY) additionally supplemented with 10\% horse serum and ~2,500 U/ml of catalase to late logarithmic phase (OD\(_{600}\) 0.850 – 1.000), then diluted 1:1 in 50\% glycerol and frozen at -80°C.

A DNA fragment containing the \textit{luxS} open reading frame was amplified by PCR using \textit{S. pneumoniae} genomic DNA using primers (SpluxF and SpluxR), and cloned using the TOPO-TA Cloning kit (Invitrogen). Presence of inserts within clones was verified via PCR with primers (SpLuxverF and SpLuxverR) and by DNA sequencing. A null allele of \textit{luxS} was generated by ligation of a spectinomycin-resistance marker into an \textit{AleI} restriction site within the coding sequence. The resulting plasmid (pLuxS::Sp) was used for natural transformation of \textit{S. pneumoniae} EF3030 using established methods \cite{190}; transformants were plated onto blood agar containing spectinomycin (100 µg ml\(^{-1}\)).

\textit{Moraxella catarrhalis} strain O35E is a well characterized laboratory strain \cite{191}, and a beta-lactamase deficient mutant in this background (hereafter referred to as 035E \textit{bro}’) has been recently described \cite{192}. \textit{M. catarrhalis} strains (O35E and O35E \textit{bro}’) were grown on brain heart infusion (BHI) agar containing vancomycin (3 µg ml\(^{-1}\)).
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For *in vitro* biofilm assays, bacteria were grown in either THY broth supplemented with 10% horse serum and ~2,500 U ml\(^{-1}\) of catalase (hereby referred to as supplemented THY) or trypticase soy broth (TSB) supplemented with ~2,500 U ml\(^{-1}\) of catalase (hereby referred to as supplemented TSB). In each assay, *M. catarrhalis* was seeded 3 logs higher than pneumococcus in single species and polymicrobial biofilms for equivalent survival of both species at time of harvest in polymicrobial biofilms.

**Antibiotic protection assays**

Antibiotic protection assays were performed essentially as described previously \[153, 183\]. *S. pneumoniae* EF3030 and/or *M. catarrhalis* O35E, or isogenic mutants as indicated in the text, were seeded into 24 well flat-bottom plates (Costar) using inocula of \(10^5\) and \(10^8\) colony-forming units (CFU) ml\(^{-1}\), respectively, in supplemented THY. After incubation (4 hours at 37°C), azithromycin (6 µg ml\(^{-1}\)) or amoxicillin (1 µg ml\(^{-1}\)) was added as indicated in the text, concentrations of both antibiotics were chosen based on minimal inhibitory concentrations for the strains used in this study; buffer was added to negative control wells. After incubation (16 hours at 37°C) the biofilms were scraped from the surface, resuspended in phosphate-buffered saline (PBS; pH = 7.2) and serial dilutions were prepared and analyzed by plating on appropriate media to define viable counts of each species (blood agar plates supplemented with gentamicin to select for pneumococcus and BHI plates supplemented with vancomycin to select for *M. catarrhalis*).

**Confocal laser scanning microscopy (CLSM)**
CLSM was performed as previously described with some modifications [153]. *S. pneumoniae* EF3030 and/or *M. catarrhalis* O35E were seeded in 4 well Permanox chamber slides (Thermo Scientific) as previously stated and grown for 24 hours at 37°C in supplemented TSB. The biofilms were fixed (0.3% paraformaldehyde), frozen in embedding medium (Tissue-Tek), and cryosectioned laterally (~5 µm per section). Immunofluorescent staining was performed using rabbit polyclonal antiserum against pneumococcal surface protein A (PspA) and monoclonal antibody Mab 3F5-5E5 which recognizes a conserved *M. catarrhalis* surface epitope [145] along with appropriate fluorescent secondary antibody conjugates (*S. pneumoniae*: Alexa Fluor 488 donkey anti-rabbit IgG, *M. catarrhalis*: Texas Red goat anti-mouse IgM) (Molecular Probes). Microscopy was performed using a Nikon Eclipse confocal laser scanning microscope. Images were analyzed using the COMSTAT program within MatLab 7.0.4 software.

**Mouse infections**

BALB/c mice (9 week old females, 5 per group) were serially infected for 3 days with $10^7$ CFU of *S. pneumoniae* EF3030, its *luxS* mutant, or *M. catarrhalis* O35E via intranasal inoculation either alone or both species of bacteria in a co-infection. At 3, 7, and 10 days post-infection (after the third inoculation), the bullae and nasopharynx were harvested from the mice, homogenized in PBS, serially diluted, and plated onto appropriate media to determine viability of either species of bacteria at each site. All mouse infection experiments were performed according to protocols approved by the Wake Forest Animal Care and Use Committee.

**Chinchilla infections**
Adult chinchillas (each weighing 400 – 600 g; 8 per group) were intranasally inoculated with either *S. pneumoniae* EF3030 (10^5 CFU), its luxS mutant (10^5 CFU), *M. catarrhalis* O35E (10^8 CFU), or both species of bacteria simultaneously. Chinchillas were given a higher inoculum of *M. catarrhalis* to improve its survival in this particular animal model. Animals were monitored daily for clinical signs of infection and examined by otoscopy at 48 hour intervals. At 2 and 7 days post-infection, the bullae and nasopharyngeal epithelia were collected and homogenized in PBS. Samples were then serially diluted and plated onto appropriate media to obtain viable counts. All chinchilla infections were performed according to protocols approved by the Wake Forest Animal Care and Use Committee.

**Statistics**

Statistical analyses were performed using GraphPad Prism 5 software. *In vitro* data was analyzed using the Mann-Whitney *U* test for significance. A one-way ANOVA with Newman-Keuls post-test was used to determine statistical significance for *in vivo* bacterial counts. Incidence of OM was also assessed using a chi-square test; counts on or above the limit of detection (LOD) were considered infected while counts below the LOD were considered uninfected.
RESULTS

**Beta-lactamase mediates passive protection of pneumococci by M. catarrhalis.**

To determine whether the production of a beta-lactamase by *M. catarrhalis* provides protection of *S. pneumoniae* from beta-lactam antibiotic killing in polymicrobial biofilms, amoxicillin was added to static biofilms as previously described in the methods section. *S. pneumoniae* within monospecies biofilms was readily killed by amoxicillin (Fig. 5). However, growth of pneumococci with *M. catarrhalis* in polymicrobial biofilms completely abolished amoxicillin-mediated killing. Additional studies with the beta-lactamase deficient *M. catarrhalis* bro’ mutant showed that this protective effect was dependent upon beta-lactamase (Fig. 5). Based on these data we conclude that beta-lactamase production by *M. catarrhalis* provides passive protection to *S. pneumoniae* in polymicrobial biofilms from beta-lactam antibiotic killing.

**S. pneumoniae passively protects M. catarrhalis from azithromycin.**

A luxS mutant was created in *S. pneumoniae* strain EF3030 as described in the methods, and the absence of detectable AI-2 quorum signal was confirmed (data not shown). Previous studies have shown that nontypeable *Haemophilus influenzae* stimulates formation of antibiotic-tolerant biofilms by *M. catarrhalis*, via interspecies quorum signaling [193]. To determine if *S. pneumoniae* promoted antibiotic resistance within *M. catarrhalis* biofilms in a similar fashion, biofilms containing *S. pneumoniae* and/or *M. catarrhalis* were tested for resistance to azithromycin. In the absence of a co-infecting species, *M. catarrhalis* within biofilm showed some resistance to azithromycin (Fig. 6). However, culture of *M. catarrhalis* within polymicrobial biofilm with *S. pneumoniae* enhanced resistance of *M. catarrhalis*
Figure 5: *M. catarrhalis* protects *S. pneumoniae* from beta-lactam killing. *S. pneumoniae* EF3030 and *M. catarrhalis* O35E were seeded in a 24 well plate alone or together (at a ratio of 1:1,000) as described in the methods section. After 4 hours at 37°C, 1 μg/ml of amoxicillin or buffer was added to each well. Biofilms were resuspended, serially diluted, and plated at 16 hrs post-antibiotic treatment to determine viability. Results depict pneumococcal counts from 3 independent experiments.
to azithromycin. Surprisingly, polymicrobial biofilms with the luxS mutant conferred equivalent protection to *M. catarrhalis* as when it is in a polymicrobial biofilm with the parental strain (Fig. 6). This was in contrast to the previous work with nontypeable *H. influenzae*, this passive protection was unaltered by abolition of quorum signal production (Fig. 6). Based on these data we conclude that *S. pneumoniae* provides passive protection to *M. catarrhalis* from azithromycin killing by an AI-2 independent mechanism.

*S. pneumoniae and M. catarrhalis form polymicrobial biofilms in vitro.*

As the additional resistance was independent of quorum signal, we hypothesized that the polymicrobial biofilms may have increased density. This was addressed using confocal microscopy; polymicrobial biofilms grown in static conditions were cryosectioned and differentially stained to identify both species (Fig. 7 A-E). Cross-sections of the polymicrobial biofilms show that both species of bacteria were able to homogenously grow in dense clusters within the biofilm. Images of these cross-sections were then analyzed using the COMSTAT program and the biomass of *M. catarrhalis* was determined in each condition based on staining specifically identifying this species of bacteria (represented in red). Interestingly, while the biomass of *M. catarrhalis* increased in polymicrobial biofilms with either *S. pneumoniae* or its luxS' mutant (Fig. 7 F), viable counts were comparable between monospecies and polymicrobial biofilms (data not shown). Based on these data we conclude that the biomass of *M. catarrhalis* increases in polymicrobial biofilms with *S. pneumoniae* independent of AI-2 production.
Figure 6: *S. pneumoniae* protects *M. catarrhalis* from macrolide killing. *S. pneumoniae* EF3030 and *M. catarrhalis* O35E were seeded in a 24 well plate alone or together as described in the methods section. After 4 hours at 37°C, 6 µg/ml of azithromycin or buffer was added to each well. Biofilms were resuspended, serially diluted, and plated at 16 hrs post-antibiotic treatment to determine viability. Images depict *M. catarrhalis* counts from 5 independent experiments. *** denotes a *P* value < 0.001.
Figure 7: *M. catarrhalis* forms polymicrobial biofilms with *S. pneumoniae* in vitro. *S. pneumoniae* EF3030 (green) and *M. catarrhalis* O35E (red) were seeded in 4 well Permanox chamber slides alone or together at 1:1,000 (as described in the methods section) and grown for 24 hours at 37°C. Biofilms were then fixed, frozen within OCT medium and cryosectioned (~5 µm/slice) and placed on slides. Bacteria were visualized using antibodies specific for pneumococi (rabbit anti-PspA) and Moraxella (monoclonal antibody 4G5), along with relevant fluorescent secondary antibody conjugates (Molecular Probes). Representative images of *S. pneumoniae* (A.), its luxS mutant (B.), *M. catarrhalis* (C.), polymicrobial biofilms with *S. pneumoniae* and *M. catarrhalis* (D.), and polymicrobial biofilms with *S. pneumoniae* luxS and *M. catarrhalis* (E.) were taken using CLSM. Images (n=5 frames per group) were analyzed by COMSTAT to determine biomass of *M. catarrhalis* (F.) alone or in polymicrobial biofilms. Scale bar = 10 µm.
Quorum signal (AI-2) production promotes nasopharyngeal colonization and affects middle ear ascension in polymicrobial infections

Previous studies have shown that production of the interspecies quorum signal, AI-2, by nontypeable *H. influenzae* improved *M. catarrhalis* survival and persistence in the middle ears of experimentally infected chinchillas [193]. To assess the role of AI-2 in co-infections with *S. pneumoniae* both a murine and chinchilla model was used. In mice, nasopharyngeal colonization was established after 3 serial inoculations of bacteria (Fig. 8A). At all time points, *S. pneumoniae* was not affected by the presence or absence of *M. catarrhalis* (Fig. 8A). However, at 3 days post-infection, the numbers of colonizing *M. catarrhalis* increased by a log when it was in a co-infection with *S. pneumoniae* (Fig. 8B). Moreover, in co-infections with the luxS\(^{-}\) mutant, the numbers of colonizing *M. catarrhalis* was equivalent to when it was alone. At later times post-infection, *M. catarrhalis* was quickly cleared from the nasopharynx. From these data, we conclude that during co-infection the production of AI-2 by *S. pneumoniae* increased colonization of *M. catarrhalis* in the nasopharynx.

Although *M. catarrhalis* was not recovered from any middle ear samples (data not shown), co-infection with *M. catarrhalis* did have an effect on the presence of *S. pneumoniae* in the middle ear. At day 3 post-infection, the CFU counts of *S. pneumoniae* were significantly reduced when it was in a co-infection with *M. catarrhalis* compared to when it was in a single infection (Fig. 8 C). Furthermore, this effect was not seen in co-infections with the luxS\(^{-}\) mutant. However at later times post-infection (days 7 and 10), clearance of *M. catarrhalis* from the nasopharynx coincided with improved recovery and prolonged survival of *S. pneumoniae* in the middle ear (Fig. 8B and C). Additionally,
Figure 8: Quorum sensing promotes nasopharyngeal colonization of *M. catarrhalis* and affects ascension of pneumococci during polymicrobial infection in mice. 9 week old female BALB/c mice were serially infected for 3 days with *S. pneumoniae* EF3030, its luxS mutant, and *M. catarrhalis* O35E intranasally either alone or together as described in the methods section. At 3, 7, and 10 days post-infection, the superior middle-ear bullae and nasopharynx were harvested from the mice, homogenized, and serially diluted and plated to determine viability of *S. pneumoniae* in the nasopharynx (A.), *M. catarrhalis* in the nasopharynx (B.), and *S. pneumoniae* in the middle ear (C.). ● represents *S. pneumoniae*, ■ represents *S. pneumoniae* luxS alone,▲ represents *M. catarrhalis* alone, ○ represents polymicrobial infections with *S. pneumoniae* and *M. catarrhalis*, and □ represents polymicrobial infections with *S. pneumoniae* luxS and *M. catarrhalis*. * denotes a P value between 0.01 and 0.05. n = 5 animals per group.
this effect was not seen in co-infections with the luxS mutant, as the counts of pneumococcus in the middle ears of these animals peaked at day 3, similar to the single infections. All together, these data show that production of AI-2 by S. pneumoniae enhances colonization of M. catarrhalis in the nasopharynx. In turn, this alters the disease progression of AOM by delaying ascension of S. pneumoniae into the middle ear.

In addition, a chinchilla intranasal infection was performed as described in the methods. In the nasopharynx, S. pneumoniae colonization was increased during a co-infection with M. catarrhalis independent of quorum signal production (Fig. 9 A). There was a statistically significant increase in the bacterial load of pneumococcus when it was in a co-infection. Also, there was a log increase in the bacterial load of the luxS mutant when it was in a co-infection. Although not statistically significant, there was an increase in both the number of colonized nasopharynxes (alone: 50%; co-infection: 100%) and bacterial load of M. catarrhalis in co-infected animals. Furthermore, during co-colonization with the luxS mutant, the number of animals colonized with M. catarrhalis dropped to almost equivalent levels as the single-infected animals (~63%) (Fig. 9 B). At day 7 post-infection, M. catarrhalis and S. pneumoniae counts decreased in both single and co-infected animals, which may be due to host clearance of the bacteria from this site.

Both S. pneumoniae and M. catarrhalis was able to ascend into the middle ear during single and co-infection (Fig. 9 C-D). While there was no difference in the bacterial load in the middle ears of single and co-infected animals, the incidence of OM increased during co-infection (percent of infected ears during single infection – 50% and co-infection – 78%). Also, during a co-infection, the bacterial load and number of
Figure 9: Quorum sensing promotes nasopharyngeal colonization and ascension of both *S. pneumoniae* and *M. catarrhalis* during polymicrobial infections in chinchillas. Adult chinchillas were intranasally inoculated with *S. pneumoniae* EF3030, its luxS- mutant, and *M. catarrhalis* O35E either alone or together in a co-infection as described in the methods section. Nasopharyngeal epithelia and bullae were harvested at days 2 and 7 post-infection. Each sample was homogenized, serially diluted, and plated to determine the viability of *S. pneumoniae* in the nasopharynx (A.), *M. catarrhalis* in the nasopharynx (B.), *S. pneumoniae* in the middle ear (C.), and *M. catarrhalis* in the middle ear (D.). ● represents *S. pneumoniae* alone, ■ represents *S. pneumoniae* luxS- alone, ▲ represents *M. catarrhalis* alone, ○ represents polymicrobial infections with *S. pneumoniae* and *M. catarrhalis*, and □ represents polymicrobial infections with *S. pneumoniae* luxS- and *M. catarrhalis*. * denotes a P value between 0.01 and 0.05. n = 8 animals per group.
colonized ears with *S. pneumoniae* was significantly higher during co-infections with the parental strain of pneumococcus as opposed to co-infection with the *luxS* mutant at 2 days post-infection. Similarly, the bacterial load of *M. catarrhalis* in the middle ears of co-infected animals with the parental strain of pneumococcus was significantly higher at day 2 post-infection than in co-infections with the *luxS* mutant. At day 7, both the number of infected ears and the bacterial load decreased, which may be due to host clearance of the bacteria. The results from the middle ear suggest that during co-infections, AI-2 production promotes ascension of both species of bacteria in the middle ear. Taken together, the results from both animal models suggest that quorum signaling plays an important role in nasopharyngeal colonization and middle ear ascension during co-infections with *S. pneumoniae* and *M. catarrhalis*. 
DISCUSSION

It has long been understood that the outcome, severity, and success of treatment of bacterial infection can be profoundly influenced by other microbes within the microbiota or in co-infection [194, 195]. Our previous work has clearly demonstrated that polymicrobial infection significantly influences persistence of otopathogens, at least in part, by affecting biofilm formation, with related impact on bacterial resistance to host clearance and antibiotics [181-183]. For pneumococcal infections, the incidence of antibiotic treatment failure dramatically exceeds the occurrence of antibiotic resistant pneumococcal strains [196]. In the case of beta-lactam resistance, this has often led to speculation that co-infection with bacteria expressing beta-lactamase might confer passive protection [150, 197]. In keeping with this hypothesis, experimental evidence has indicated that *M. catarrhalis* can confer such passive protection within biofilms [181, 184, 185, 198]. The unique beta-lactamase produced by *M. catarrhalis* is encoded by the *bro* gene, which produces either one of two isoforms, BRO-1 or BRO-2 [157-159]. The heavier isoform, BRO-1, is the most commonly found isoform among *M. catarrhalis* strains. It also differs from BRO-2 in the amounts that are produced and *in vitro* rate of substrate metabolism [159]. The work presented in this study clearly demonstrates that *M. catarrhalis* can afford passive protection from beta-lactam killing upon pneumococci residing within the same biofilm. Importantly, our experiments conclusively point to beta-lactamase production as the sole determinant of this protection, as no passive resistance was observed with an isogenic *M. catarrhalis bro* mutant lacking beta-lactamase activity.
Our previous work showed that *M. catarrhalis* uses quorum signal eavesdropping to enhance biofilm formation and, in turn, improve antibiotic resistance [193]. However in this study, we found that polymicrobial biofilms with *S. pneumoniae* enhanced antimicrobial resistance despite the production of a quorum signal. To further investigate this improved resistance we found that there was an AI-2-independent increase in the biomass of *M. catarrhalis*. In addition, there seemed to be a change in the overall structure of the polymicrobial biofilms. The bacteria formed dense clusters surrounded by an extensive amount of open space, which could be due to water channel formation or extracellular matrix material which was not accounted for in this study. The significance of these findings is important, especially given recent epidemiological evidence demonstrating the increased occurrence of *M. catarrhalis* in conjunction with other bacterial species as opposed to alone [177]. These results demonstrate the resilient nature of polymicrobial biofilms and suggest other microbe-microbe interactions not characterized in this study may play a role in antimicrobial resistance.

Mice and chinchillas were infected via the intranasal route to assess how colonization of both *M. catarrhalis* and *S. pneumoniae* affect nasopharyngeal colonization and persistence, ascension of the Eustachian tube, and development of OM. Both murine and chinchilla models have been used to study colonization of the nasopharynx and middle ears by otopathogens [172, 199, 200]. Each model offers different advantages for studying OM [201], which provides a stronger argument for the trends observed herein. In both of these models, the results suggest a role for quorum sensing in nasopharyngeal colonization as well as middle ear ascension and colonization during co-infections. These results are quite convincing, especially considering the stark
differences between these two models. Not only are these two different species of animals, but the infection schemes were different as well. Additionally, the increased bacterial load and incidence of OM in co-infected chinchillas was an interesting outcome, which seems to closely model what has been seen in young children [202]. This is tantalizing evidence that suggests communication between these two species via the interspecies quorum signal AI-2 could mediate the increased incidence of OM in children. luxS is widely expressed by a number of species of bacteria, including S. pneumoniae [203]. To date, there have not been any reports of S. pneumoniae strains that do not contain this gene. Therefore, it is possible that targeting AI-2 production could mitigate the incidence of OM in children that are colonized with these two species of bacteria.

In conclusion, these studies show that M. catarrhalis and S. pneumoniae can form polymicrobial communities which, under antibiotic and environmental pressure, can render either bacterium more resistant to clearance. It is important to understand the impact of polymicrobial communities on otopathogens and other nasopharyngeal normal flora to develop better strategies for preventing and treating OM.
ACKNOWLEDGEMENTS

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CONCLUSIONS

The use of PCR-based detection techniques and experimental evidence of polymicrobial biofilm formation by otopathogens has changed the way OM is viewed and may possibly change how this disease is treated in the future. In recent years, more studies have focused on the implications of these polymicrobial communities in pathogenesis, and prevention and treatment of this disease. The general hypothesis is that different species of bacteria form multispecies communities to enhance their own survivability and persistence through indirect pathogenicity. In support of this hypothesis, previous studies in the lab have shown that beta-lactamase production and quorum sensing can affect resistance to antibiotics and persistence in the middle ears of experimentally infected chinchillas when either pneumococcus and NTHi, or M. catarrhalis and NTHi are in a polymicrobial infection [153, 183, 199]. However, interactions between pneumococcus and M. catarrhalis within polymicrobial biofilms have yet to be characterized. The purpose of this research is to determine the role(s) of beta-lactamase production and quorum sensing in polymicrobial infections with pneumococcus and M. catarrhalis.

Beta-lactamase production in polymicrobial biofilms

In 1986, Simon and Sukai suggested that beta-lactamase producing bacteria may provide passive protection to typically susceptible species of bacteria. They found that beta-lactamase producing S. aureus isolates from patients who failed penicillin treatment protected hemolytic streptococci isolates in culture [204, 205]. Similarly, it has been hypothesized that beta-lactamase producing otopathogens may provide passive protection
to pneumococcus, which may explain the high rate of treatment failure seen in OM [150, 206-208]. Brook and Gober found that after amoxicillin treatment only 64% of patients were considered clinically cured, while 92% of patients treated with amoxicillin-clavulanate were cured [209]. Furthermore, previous studies in the lab have shown that beta-lactamase production by NTHi can provide passive protection to pneumococcus in vitro and in vivo, although some protection was afforded by the polymicrobial biofilm in vivo [183].

In these studies, polymicrobial biofilms with pneumococcus and *M. catarrhalis* were seeded in vitro and treated with amoxicillin. Even in biofilm, pneumococcus is readily susceptible to amoxicillin treatment (Figure 5). However, the results showed passive protection of pneumococcus from amoxicillin treatment when it was in a polymicrobial biofilm with *M. catarrhalis*. Additionally, pneumococcus was not protected from amoxicillin treatment in polymicrobial biofilms with an isogenic beta-lactamase-deficient mutant (Figure 5). While these studies implicate the beta-lactamase as the sole mediator of protection in vitro, the mechanism by which the beta-lactamase provides protection to pneumococcus was not evaluated. More specifically, these studies do not address whether secretion of the beta-lactamase is important in conferring this protection in polymicrobial biofilms. Unlike the secreted beta-lactamase produced by NTHi, studies by Eliasson, *et al* suggested that BRO beta-lactamase is membrane-bound, which would suggest that close-proximity would be required to provide passive protection [157]. However, more recent studies by Schaar, *et al* suggest that this beta-lactamase can be carried on outer membrane vesicles and can act in *trans* to protect pneumococcus and susceptible species of NTHi from beta-lactam antibiotic killing [198].
It is possible that BRO can protect pneumococcus when it is in close-proximity, i.e. in a polymicrobial community, or when it is at a more distal site if enough BRO-containing outer membrane vesicles are produced. Thus, proximity may be more important in vivo for *M. catarrhalis* to confer passive protection of pneumococcus from beta-lactam killing.

**Implications of beta-lactamase protection results**

In comparison to NTHi, beta-lactamase expression is especially wide-spread among *M. catarrhalis*. The findings in these studies show that beta-lactamase production by *M. catarrhalis* can affect the susceptibility of pneumococcus to amoxicillin treatment. Furthermore, it is important to note that this beta-lactamase has been shown to be inhibited by clavulanate [163]. Therefore, the addition of clavulanate to amoxicillin should render organisms protected by production of BRO beta-lactamase more susceptible to antimicrobial therapy; although the beta-lactamase deficient mutant did show some resistance to amoxicillin treatment in biofilm (data not shown). Without beta-lactamase to protect it, these results showed that pneumococcus was susceptible to amoxicillin treatment, even in biofilm, in vitro (Figure 5). While the biofilm may still provide some protection in vivo, these results provide promising insight into the treatment of polymicrobial infections in OM and highlight the importance of adding a beta-lactamase inhibitor when *M. catarrhalis* is one of the co-colonizing organisms. Considering the high rate of initial treatment failure and rate of recalcitrant AOM, a more individualized treatment approach based on organisms found in the middle ears of OM patients may provide a more promising future in the treatment of this disease [210].
A *luxS* mutation in *S. pneumoniae* EF3030

A mutation in the *luxS* gene of *S. pneumoniae* strain EF3030 was created as previously mentioned; this mutant was designated EF3030 *luxS*. Deficiency in AI-2 production of EF3030 *luxS* was confirmed using the *V. harveyi* bioluminescence assay as described by Bassler, *et al* [211] (Supplemental Figure 1). Previously described *luxS*-deficient mutants in pneumococcus were shown to have defects in biofilm formation, and survival and persistance in an animal model of colonization and/or disease [166, 203, 212-214]. To characterize potential deficiencies in this mutant, in vitro biofilm assays and murine infection studies were performed to determine if EF3030 *luxS* had defects in biofilm formation, antibiotic resistance, and/or survival and persistance within an animal model of nasopharyngeal colonization and subsequent AOM. As shown in Supplemental Figures 2-4, this mutant did not have defects in any of the phenotypic characteristics tested.

Unlike previously described *luxS*-deficient mutants, the mutant described herein was created in a nasopharyngeal isolate that has been used to study localized infection, and is quickly cleared from the bloodstream of rodents inoculated intravenously [88]. The *luxS*-deficient mutants with defects in biofilm development and persistance were created in strains of pneumococcus that were isolated during severe infections and are typically used to study invasive pneumococcal disease [203, 212, 214]. These obvious differences in the strain background could explain why EF3030 *luxS* did not present the defects that others have described previously. Further studies to assess more subtle differences between the parental and mutant strains must be performed to determine if the
Quorum sensing in vitro

Production of interspecies quorum signal (AI-2) by pneumococcus does not have the same effect on *M. catarrhalis* in polymicrobial biofilms as was previously seen with NTHi [153]. These studies show that while *M. catarrhalis* is protected from macrolide killing in polymicrobial biofilms with pneumococcus, this protection was not conferred by production of AI-2, as *M. catarrhalis* survival was equivalent in polymicrobial biofilms with the *luxS* deficient mutant. Therefore, the enhanced resistance provided to *M. catarrhalis* was attributed to the biofilm rather than AI-2 production. This hypothesis was further supported by results of COMSTAT analysis on CLSM images of single and polymicrobial biofilms (Figure 7). The biomass of *M. catarrhalis* was enhanced in polymicrobial biofilms, regardless of AI-2 production. In studies conducted by Armbruster, et al, enhanced biofilm biomass as a consequence of AI-2 uptake and response was implicated as the mechanism of antimicrobial resistance for *M. catarrhalis* [153]. It is possible that in polymicrobial biofilms with pneumococcus the increase in *M. catarrhalis* biomass could provide enough protection to see enhanced resistance despite AI-2 production. Further experiments to assess the contribution of biofilm to enhanced resistance should be performed to further support this hypothesis.

Quorum sensing in vivo

For in vivo studies, two animal models for nasopharyngeal colonization and subsequent AOM were used: a murine and chinchilla model. In both cases, animals
were inoculated intranasally with one or both species of bacteria. The results of these studies suggest a role for quorum sensing in nasopharyngeal colonization, ascension, and OM during polymicrobial infections with pneumococcus and *M. catarrhalis*.

In mice, concurrent inoculation enhances colonization of *M. catarrhalis* in the nasopharynx. This effect was dependent upon the production of AI-2 by pneumococcus (Figure 8). With the enhanced colonization of *M. catarrhalis* in the nasopharynx and the production of quorum signal, pneumococcus was retained at this site and it did not ascend into the middle ear. However, once *M. catarrhalis* is cleared from the nasopharynx, pneumococcus was able to ascend and colonize the middle ear until it was cleared from this site by the host. In all, interspecies quorum signal production promoted higher loads of *M. catarrhalis* in the nasopharynx, which in turn delayed disease progression of AOM caused by pneumococcus. A summary of the findings from this model is illustrated in Supplemental Figure 5.

In chinchillas, concurrent intranasal inoculation promoted the colonization of both pneumococcus and *M. catarrhalis* (Figure 9). For pneumococcus, enhanced colonization was achieved during polymicrobial infection regardless of AI-2 production. However, similar to murine infection, enhanced colonization of *M. catarrhalis* was consistently observed with production of AI-2. Furthermore, polymicrobial infection with the *luxS*-deficient mutant resulted in a bimodal distribution, with some animals retaining very high numbers of *M. catarrhalis* while others had low numbers (Figure 9). This result was very interesting and suggests that other bacterial or host factors may be playing a role in co-colonization. Considering chinchillas are outbred animals with very limited reagents available for immunologic studies, determining how host factors affect co-colonization
may not be feasible at this time. Exploring other microbial interactions is a more viable option that may shed more light on the cause of this bimodal response. Ascension and middle ear colonization was achieved by both species of bacteria (Figure 9). In chinchillas, production of interspecies quorum signal promoted ascension into the middle ear during polymicrobial infection. In all, production of quorum signal promotes colonization of *M. catarrhalis* in the nasopharynx, which in turn promotes colonization of pneumococcus. Furthermore, quorum signaling enhances ascension and middle ear colonization of both species of bacteria during polymicrobial infection. A summary of the findings from this model is illustrated in Supplemental Figure 6.

**Implications of interspecies quorum sensing in protection and persistence**

The role of interspecies quorum signal in pathogenesis has been a more recent area of focus. Its role as a mediator of indirect pathogenesis is even more important given the wide-spread nature of this quorum sensing system. It is important to understand how both signal producers and responders are affected in the presence/absence of AI-2. Communication through this interspecies network allows bacteria to detect the presence of other species and sense the population density of AI-2 producing bacteria based on the level of signal in the environment. The advantage of being an AI-2 producer, like pneumococcus, allows for genetic manipulation of quorum signaling eavesdroppers, like *M. catarrhalis*. The global changes in gene expression occurring in *M. catarrhalis* single species communities resulted in an increase in biofilm biomass and antimicrobial resistance [153]. Although this effect was not observed in polymicrobial biofilms with pneumococcus in vitro (Figure 7), the role of interspecies quorum sensing in vivo seems to support the previous studies, with enhanced *M.*
*catarrhalis* nasopharyngeal and middle ear colonization in the rodent models (Figures 8-9).

The ability to enhance the biomass of a co-colonizing species could provide enhanced protection for AI-2 producing bacteria under host and/or antimicrobial stress. In addition, it is possible that certain virulence factors could provide a survival advantage to co-residing species. Along those lines, the studies herein suggest that production of the BRO beta-lactamase by *M. catarrhalis* could provide passive protection to pneumococcus if they reside together in a polymicrobial community. The ability to enhance the colonization of this beta-lactamase producing bacteria may provide protection to pneumococcus when beta-lactam antibiotics are in the environment. Thus pneumococcus could utilize the production of interspecies quorum signal to promote the growth, survival, and/or persistence of *M. catarrhalis* and in turn enhance its own survival under antibiotic stress. Other virulence factors such as nutrient acquisition proteins or proteins required for exposure of cell surface molecules may also provide a survival advantage to AI-2 producing bacteria in a polymicrobial environment. In future studies, it would be interesting to determine which genes are affected by response to AI-2 in *M. catarrhalis*, especially in a polymicrobial community.

The role of interspecies quorum signal in the formation of dental plaque has been more thoroughly investigated, and may provide an example for how polymicrobial communities in the ENT behave in response to this molecule. In oral biofilms, production of AI-2 has been shown to mediate both interspecies competition as well as cooperativity. For example, it is hypothesized that commensal organisms produce and respond to picomolar amounts of AI-2, while periodontal pathogens produce and respond
to much higher amounts of signal [215, 216]. Furthermore, high amounts of AI-2 inhibit the growth of the commensal bacteria. Therefore, when pathogenic oral bacteria begin to grow on the preformed commensal biofilms, they produce such high levels of AI-2 that the commensal bacteria cease to grow. Eventually, the biofilm is overtaken by the pathogenic bacteria, which forms the tooth-decaying plaque [215]. The high amount of AI-2 produced by the pathogenic species provides a mechanism to outcompete the commensal oral bacteria so that they can survive and persist within the oral biofilm. In addition, cooperativity between pathogenic species has been attributed to production of AI-2. Spatial arrangement of organisms within periodontal biofilms is an important aspect of plaque formation, and it is orchestrated via production of AI-2. Initial attachment of periodontopathogenic species is mediated by “bridging” bacterial species which connect the early colonizing bacteria to the late, generally more pathogenic colonizers. *Fusobacterium nucleatum* is a very important bridging species. Studies have shown that production of AI-2 by this organism can enhance or mitigate biofilm formation and coaggregation of early and late colonizers in polymicrobial communities [215, 217-219]. The results of the studies presented herein and from others in the lab suggest that otopathogens are able to use AI-2 production to mediate interspecies cooperativity amongst one another. However nothing is known about how AI-2 production affects other commensal species in the nasopharynx or whether under certain conditions AI-2 production can mediate interspecies competition between these bacteria. Therefore, determining the level of AI-2 otopathogens respond to and how AI-2 produced by these organisms affects biofilm formation in other co-residing species will be important to better understand the role of this molecule in survival and pathogenesis.
Future Directions

Like with NTHi, protection of pneumococcus was conferred in polymicrobial biofilms in vitro, and this protection was mediated by production of beta-lactamase by *M. catarrhalis*. However, Weimer *et al.* found that in a chinchilla chronic otitis model, both the beta-lactamase produced by NTHi and the polymicrobial biofilm could protect pneumococcus from beta-lactam killing [183]. Therefore, beta-lactam protection of pneumococcus by *M. catarrhalis* should be assessed in vivo to determine if the polymicrobial biofilm within an animal would be more resilient than the polymicrobial biofilm in vitro and therefore would also provide some level of protection to pneumococcus. However, using the chronic otitis model in chinchillas may not provide the best assessment for antimicrobial protection since EF3030 causes very severe invasive disease in the majority of animals when inoculated via transbulla route (data not shown). Thus, the bacteria would have to be inoculated intranasally in either mice or chinchillas and the antibiotic would have to be administered by another route (orally, subcutaneously, intravenously, etc.) so that the antibiotic would reach the nasopharynx and middle ear at clinically relevant levels. These experiments would determine whether the beta-lactamase is sufficient enough to be the sole mediator of protection for pneumococcus in polymicrobial infections with *M. catarrhalis* or if the biofilm provides added protection to pneumococcus in vivo.

The results from both rodent models suggest that AI-2 production plays an important role in both colonization and bacterial persistence in simultaneous co-infections with pneumococcus and *M. catarrhalis*. However, the importance of interspecies quorum signal production in colonization and persistence was not assessed
when one species is already established and then the other is introduced. For these experiments, the mouse model may be the best system to test this in. *M. catarrhalis* seems to colonize the nasopharynx of mice more readily and survive longer when they are not in a co-infection (Figures 8-9). In addition to further exploring the importance of AI-2 in subsequent colonization, further analyses of the expression of genes affected by quorum sensing in both pneumococcal strain EF3030 and *M. catarrhalis* O35E should be assessed. For these studies, a more global approach may be the most informative. A microarray analysis of each strain in the presence and absence of AI-2 should be performed. Because EF3030 produces AI-2 at high levels during growth, the isogenic *luxS*-deficient mutant should be used for the microarray analysis. Once the genes affected by AI-2 uptake/response are identified, the importance of these genes in interspecies interactions via quorum sensing can be assessed. Furthermore, future studies exploring other interactions that are not mediated by interspecies quorum sensing should be performed. A study by Marks et al. showed that genetic exchange between pneumococci is enhanced during nasopharyngeal colonization and biofilm formation on an epithelial cell surface [220]. It would be interesting to explore the possibility of genetic exchange between these two naturally competent species of bacteria, especially since *M. catarrhalis* seems to be receptive to acquiring some Gram-positive genes (see beta-lactamase discussion in the Introduction).
Characterization of a luxS mutation in S. pneumoniae strain EF3030

Rationale: Previous studies have shown that mutating or deleting the luxS mutation in S. pneumoniae causes defects in biofilm formation and survival and persistence in rodent models of nasopharyngeal colonization or invasive disease [166, 203, 212-214]. For our studies, S. pneumoniae strain EF3030 was chosen because it has been previously shown to establish localized infection or carriage and does not typically cause sepsis [88, 189].

Methods

Generating EF3030 luxS mutant

Detailed methods for generating the luxS mutant in EF3030 are outlined in the Materials and Methods section in Chapter I. In short, a spectinomycin cassette was inserted in the middle of the luxS gene, thereby disrupting production of LuxS in this strain. Absence of AI-2 production was confirmed in this mutant strain by treating a reporter strain of V. harveyi, which will bioluminesce in response to AI-2 only, with cell-free culture supernatants as previously described [211]. In these experiments, the supernatants were obtained from bacteria grown in biofilms rather than broth culture.

Biofilm growth curves

Biofilms of EF3030 and EF3030 luxS mutant were seeded in 24 well plates (Costar) and grown in BHI broth (Difco) supplemented with 10% horse serum and ~2,500 U/ml of catalase at 37°C. At various times post-inoculation, biofilms were harvested and viability was determined by serially diluting and plating onto blood agar plates supplemented with 4 µg/ml gentamicin.

COMSTAT analysis of continuous flow biofilms

Single chamber flow cells (Stovall) were inoculated with 10^5 CFU/ml of EF3030 or EF3030 luxS mutant in THY broth. The biofilms were grown for 24 hours at 37°C. The biofilms were then stained using the
Live/Dead staining kit (Molecular Probes) and visualized on a Nikon Confocal microscope. For each biofilm, Z-stack series were generated at six different locations on the biofilm. These images were analyzed via COMSTAT program in MatLab 7.0.4 software.

**Antibiotic resistance assay**  EF3030 and EF3030 luxS were inoculated in BHI broth supplemented with 10% horse serum and ~2,500 U/ml of catalase and allowed to grow for 4 hours at 37°C in 0.5% CO₂. Azithromycin was then added to each biofilm at various concentrations; mock treated wells were given buffer. The biofilms were allowed to grow for an additional 24 hours before the media was removed and the biofilms were resuspended in 1 ml of PBS, serially diluted and plated on blood agar plates supplemented with 4 µg/ml gentamicin to determine viability.

**Mouse infections**  6 week old BALB/c mice (n=5 mice per group) were intranasally inoculated with 10⁷ CFU of EF3030 or EF3030 luxS for three consecutive days in 50 µl of freezing medium (THY supplemented with ~2,500 U/ml catalase and 25% glycerol). After the third inoculation, the bullae and nasopharynx was harvested at days 3 and 7 post-infection. Each sample was homogenized in 1.5 ml PBS and serially diluted and plated on blood agar plates supplemented with 4 µg/ml gentamicin to determine bacterial viability.

**Results:**  The luxS⁻ mutant in EF3030 did not have any defects in growth, biofilm formation, resistance to antibiotics, or survival and persistence in vivo.
Supplemental Figure 1: Confirmation of AI-2 deficiency in EF3030 luxS−. EF3030 (WT) and EF3030 luxS− was seeded into 24 well plates at 10^5 CFU/ml in THY supplemented with ~2,500 U/ml of catalase and 10% horse serum. At various times post-inoculation, 1 ml of spent media was carefully removed from the well and the bacteria were spun down at 13000 rpm for 5 min. The levels of AI-2 in the supernatant were indirectly measured using the V. harveyi reporter strain BB170; the level of fluorescence (measured in RLU/s) produced by this strain corresponds to the level of AI-2 in the supernatant [211]. DPD in growth media control contains 200 ng of synthetic DPD.
Supplemental Figure 2: EF3030 luxS\(^-\) has no defect in biofilm formation in continuous flow conditions. EF3030 (WT) and EF3030 luxS\(^-\) were seeded in single-chamber flow cells (Stovall) at 10\(^5\) CFU/ml. Biofilms were grown for 24 hours under continuous flow conditions, with a media flow rate of 1 ml/min. Afterwards, the biofilms were stained (Live/Dead® cell viability kit – Life Technologies) and imaged by CSLM. The biomass (A.), average thickness (B.), surface to biovolume ratio (C.), and maximum thickness (D.) were determined using COMSTAT analysis. A students’ \(t\) test was performed to determine statistical significance.
Supplemental Figure 3: EF3030 luxS− biofilms do not have a defect in resistance to antibiotics. EF3030 (WT) and EF3030 luxS− was seeded into 24 well plates at $10^5$ CFU/ml in THY supplemented with ~2,500 U/ml of catalase and 10% horse serum. After 4 hours, azithromycin (or buffer for the negative control) was added to the biofilms; then 24 hours later, the biofilms were resuspended in PBS, serially diluted, and plated to determine biofilm viability.
Supplemental Figure 4: EF3030 luxS− does not have a survival defect in vivo. Mice were intranasally inoculated with $10^7$ CFU of EF3030 or EF3030 luxS− for three consecutive days. After which the nasopharynx (A.) and bullae (B.) were homogenized in PBS, serially diluted, and plated to determine viability. ● represent EF3030 and ■ represent EF3030 luxS−. n=5 mice per group.
Supplemental Figure 5: Summary of murine model results. During concurrent colonization production of AI-2 by pneumococcus promotes colonization of *M. catarrhalis* in the nasopharynx and pneumococcus is retained within this site. Eventually, pneumococcus outcompetes *M. catarrhalis* in the nasopharynx. This allows pneumococcus to disseminate, ascend the Eustachian tube, and colonize the middle ear.
Supplemental Figure 6: Summary of chinchilla model results. During concurrent colonization in the nasopharynx, the presence of *M. catarrhalis* promotes colonization of pneumococcus at this site. In turn, the production of AI-2 by pneumococcus promotes colonization of *M. catarrhalis*. Both species of bacteria are able to colonize the middle ear. However, the production of quorum signal by pneumococcus promotes colonization of both species of bacteria in the middle ear.
Quantitation of extracellular matrix material

**Rationale:** One possible explanation for the increase in *M. catarrhalis* biomass when it was in a polymicrobial biofilm is that there was an increase in the production of extracellular matrix material. To test this hypothesis, the amount of extracellular DNA, protein, and carbohydrate was determined.

**Methods:** Single species or polymicrobial biofilms of pneumococcus (EF3030), the *luxS* mutant, and *M. catarrhalis* (O35E) were seeded in 6 well plates (Costar) and allowed to grow for 24 hours at 37°C with 0.5% CO₂. The spent media was removed and the biofilm was resuspended in PBS, serially diluted, and plated on selective agar plates to determine viability (blood agar supplemented with 4 µg/ml of gentamicin to select for pneumococcus; BHI agar supplemented with 3 µg/ml of vancomycin to select for *M. catarrhalis*). The bacteria were spun down at 5,000 x g for 15 min and the supernatant was removed and saved to quantitate extracellular matrix material. Extracellular DNA, protein, and carbohydrate were quantitated using previously described methods [213, 221-223]. In general, extracellular DNA and carbohydrate were purified using phenol:chloroform:isoamyl alcohol (23:23:1; pH 7.3); the extracellular carbohydrates were dialyzed in deionized water. Standard curves of glucose and bovine serum albumin were generated to quantitate extracellular carbohydrate and protein, respectively.

**Results:** There was no difference in extracellular protein and carbohydrate between single species and polymicrobial biofilms. However, there was a drastic difference in extracellular DNA content. Single species pneumococcal biofilms contained high levels of extracellular DNA while *M. catarrhalis* biofilms contained very low levels.
Surprisingly, the polymicrobial biofilms contained amounts of extracellular DNA comparable to *M. catarrhalis* single species levels.

**Conclusions:** The amount of extracellular DNA within the polymicrobial biofilms were lower than what was expected. The amount of pneumococci in the polymicrobial biofilms was comparable to the single species biofilms. Therefore, we expected comparable amounts of extracellular DNA to pneumococcal single species biofilms. It is known that most strains of *M. catarrhalis* produce a DNase so supernatants from *M. catarrhalis* biofilms were collected and pneumococcal biofilms were treated with the supernatants to test for DNase activity. Even after 24 hours, the amount of extracellular DNA in the treated pneumococcal biofilms was the same as the amount of extracellular DNA isolated from untreated controls (data not shown). It is possible that *M. catarrhalis* may have some effect on autolysis in pneumococcus, which is a mechanism used by this species of bacteria to release DNA into the extracellular environment. It is also possible that there may be other interactions between these two species of bacteria that have yet to be described. Additional studies should be done to further investigate these findings.
Supplemental Table I: The composition of the extracellular matrix is altered in polymicrobial biofilms

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<td>2 (1)</td>
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<td><em>M. catarrhalis</em></td>
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<td>3 (1)</td>
<td>28 (8)</td>
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The average quantities (expressed in µg for eCarbohydrate and eProtein; eDNA is expressed in ng µl⁻¹) of each component are represented on the first line, with standard error of the mean (SEM) in parentheses. Geometric mean of total the CFU counts are represented in bold, with SEM in parentheses. eDNA and eCarbohydrate: n=3 replicates; eProtein: n=4 replicates
REFERENCE LIST


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**ANTONIA C. PEREZ**  
**CURRICULUM VITAE**

**EDUCATION**

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**EXPERIENCE**

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**MEMBERSHIPS**

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**ORAL PRESENTATIONS**

A murine model for pneumococcal otitis media. Wake Forest University Department of Microbiology and Immunology Fall Symposium, 2010.

Effects of quorum signaling on biofilm formation and persistence *in vivo* in *Streptococcus pneumoniae*. Wake Forest University Department of Microbiology and Immunology Fall Symposium, 2011.

Interactions between *Moraxella catarrhalis* and *Streptococcus pneumoniae*: to compete or coexist? Mid-Atlantic Microbial Pathogenesis Meeting, 2013.

Microbial interactions between upper airway opportunists affect bacterial resistance, and survival and persistence *in vivo*. North Carolina American Society for Microbiology Annual Branch Meeting, 2013.
AWARDS

Travel Grant to the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA 2011.

Department of Microbiology and Immunology Training Grant, Wake Forest University, 2011-2013.

Travel Grant to the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA 2013.

PUBLICATIONS


