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

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Otitis media is often linked to the presence of polymicrobial biofilms, and the interactions of the different organisms are likely to play an important role in disease progression and treatment outcome. In this study, Perez *et al.* demonstrate that *Streptococcus pneumoniae* and *Moraxella catarrhalis* form multispecies biofilms *in vitro* and *in vivo* and can protect each other from the activity of antibiotics. These findings shed new light on failure of antibiotic treatment and may open the door to better strategies for preventing and treating otitis media.

Keywords

otitis; persistence; biofilm; antibiotic.

Abstract

Otitis media (OM) 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. OM can be chronic/recurrent in nature, and a wealth of data indicates that in these cases, the bacteria persist within biofilms. Epidemiological data demonstrate that most cases of OM 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 3 years of age (Klein, 2000). 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 (Post, 2001; Ehrlich *et al.*, 2002; Hall-Stoodley *et al.*, 2006; Swords, 2012). Recent epidemiology data also clearly demonstrate that most cases of OM involve simultaneous infection with multiple agents (Chonmaitree *et al.*, 2008; Pettigrew *et al.*, 2008; Revai *et al.*, 2008; Holder *et al.*, 2012), and our recent work shows that otopathogens can coexist within biofilm communities (Armbruster *et al.*, 2010; Weimer *et al.*, 2010, 2011). 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 (Bernhard *et al.*, 2012). 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 (Budhani & Struthers, 1997, 1998).

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 by-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 (Kuo et al., 1994; Gilson et al., 1995; Surette et al., 1999), AI-2 production has been demonstrated in species of both Gram-positive and Gram-negative bacteria (Xavier & Bassler, 2003), 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 (Armbruster et al., 2010). 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 1. *Streptococcus pneumoniae* EF3030 is a serotype 19F strain which typically establishes nasopharyngeal carriage or localized airway infection in murine models (Bries et al., 1992; Palaniappan et al., 2005). Pneumococci were grown on trypticase soy agar (BD) supplemented with 5% defibrinated sheep blood (Hemostat) and 4 μg mL⁻¹ gentamicin. For freezer stocks, *S. pneumoniae* was grown in Todd Hewitt broth with 0.5% yeast extract (THY) additionally supplemented with 10% horse serum and c. 2500 U mL⁻¹ of catalase (blood agar) to late logarithmic phase (OD₆₀₀ nm 0.850–1.000), then diluted 1 : 1 in 50% glycerol, and frozen at −80 °C.

A DNA fragment containing the luxS open reading frame was amplified by PCR using *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 luxS was generated by ligation of a spectinomycin resistance marker into an *Ale* restriction site within the coding sequence. The resulting plasmid (pLuxS::Sp) was used for natural transformation of *S. pneumoniae* EF3030 using established methods (Yother et al., 1986); transformants were plated onto blood agar containing spectinomycin (100 μg mL⁻¹).

*Moraxella catarrhalis* strain O35E is a well-characterized laboratory strain (Unhanand et al., 1992), and an isogenic beta-lactamase-deficient mutant in this background (hereafter referred to as O35E bro⁻) has been recently described (Balder et al., 2013). *Moraxella catarrhalis* strains (O35E and O35E bro⁻) were grown on brain heart infusion (BHI) agar containing vancomycin (3 μg mL⁻¹).

For *in vitro* biofilm assays, bacteria were grown in either THY broth supplemented with c. 2500 U mL⁻¹ of catalase (hereby referred to as supplemented THY) or trypticase soy broth (TSB) supplemented with c. 2500 U mL⁻¹ of catalase (hereby referred to as supplemented TSB). In each assay, *M. catarrhalis* was seeded 3 logs higher than pneumococci in single species and polymicrobial biofilms for equivalent survival of both species at the time of harvest in polymicrobial biofilms.

**Antibiotic protection assays**

Antibiotic protection assays were performed essentially as described previously (Armbruster et al., 2010; Weimer et al., 2011). *Streptococcus 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 10⁸ and 10⁵ colony-forming units (CFU) mL⁻¹, respectively, in supplemented THY. After incubation (4 h at 37 °C with 5% CO₂), azithromycin (6 μg mL⁻¹) or amoxicillin (1 μg mL⁻¹) was added as indicated in the text. Concentrations of both antibiotics were included to ensure that the *M. catarrhalis* strains were fully susceptible to each antibiotic and were used as a control for antibiotic protection. After incubation for 4 h, bacteria were killed with 1% sodium lauryl sulfate and CFU/mL of *S. pneumoniae* were determined using a plate count method on blood agar plates (BD). Each experiment was performed in triplicate and repeated at least twice.

**Table 1** Bacterial strains, plasmids, and primers

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<tr>
<th>Species</th>
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**Acknowledgments**

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA (AI078394) and by the Academy of Finland (310980). The authors appreciate technical assistance from Dr. W. Haapala, University of Oulu, Finland. The manuscript was improved by Dr. K. Skouby, University of Aarhus, Denmark. The authors declare no conflicts of interest.

**Conflict of interest**

None declared.

**Funding**

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA (AI078394) and by the Academy of Finland (310980).
were chosen based on minimal inhibitory concentrations for the strains used in this study; buffer was added to negative control wells. After incubation (16 h at 37 °C), the biofilms were scraped from the surface and 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 scanning laser microscopy (CLSM)**

CLSM was performed as previously described with some modifications (Armbruster et al., 2010). *Streptococcus pneumoniae* EF3030 and/or *M. catarrhalis* O35E were seeded in 4-well Permanox chamber slides (Thermo Scientific) as previously stated and grown for 24 h at 37 °C in supplemented TSB. The biofilms were fixed (0.3% paraformaldehyde), frozen in embedding medium (Tissue-Tek), and cryosectioned laterally (c. 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 specific epitope (*M. catarrhalis* surface epitope (Furano et al., 2005) 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 in combination with 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 (10^6 CFU), its luxS mutant (10^6 CFU), *M. catarrhalis* O35E (10^6 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-h 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 were 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 described in the methods section. *Streptococcus pneumoniae* within monospecies biofilms was readily killed by amoxicillin (Fig. 1). However, growth of pneumococci with *M. catarrhalis* in polymicrobial biofilms completely abolished amoxicillin-mediated killing. Additional studies within the beta-lactamase-deficient *M. catarrhalis bro^-^ mutant showed that this protective effect was dependent upon beta-lactamase (Fig. 1). 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.

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

A luxS mutant was created in *S. pneumoniae* strain EF3030 as described in the methods section, and the absence of detectable AI-2 quorum signal was confirmed (data not shown). Previous studies have shown that nontypeable *Haemophilus influenzae* stimulates the formation of antibiotic-tolerant biofilms by *M. catarrhalis*, via interspecies quorum signaling (Armbruster et al., 2010). To determine whether *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. 2). However, culture of *M. catarrhalis* within polymicrobial biofilm with *S. pneumoniae* enhanced resistance of *M. catarrhalis* 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. 2). This was in contrast to the previous work with nontypeable
H. influenzae; this passive protection was unaltered by abolition of quorum signal production (Fig. 2). Based on these data, we conclude that S. pneumoniae provides passive protection to M. catarrhalis from azithromycin killing by an AI-2-independent mechanism.

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

As the additional resistance to antibiotic treatment 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. 3a–e). Cross-sections of the polymicrobial biofilms showed 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. 3f), 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.

**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, Al-2, by nontypeable H. influenzae improved M. catarrhalis survival and persistence in the middle ears of experimentally infected chinchillas (Armbruster et al., 2010). To assess the role of Al-2 in co-infections with S. pneumoniae, both murine and chinchilla models were used. In mice, nasopharyngeal colonization was established after three serial inoculations of bacteria (Fig. 4a). At all time points, S. pneumoniae was not affected by the presence or absence of M. catarrhalis (Fig. 4a). 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. 4b). Moreover, in co-infections with the luxS mutant, the numbers of colonizing M. catarrhalis were 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 Al-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 with when it was in a single infection (Fig. 4c). 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

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**Fig. 1** Moraxella catarrhalis protects Streptococcus pneumoniae from beta-lactam killing. Streptococcus pneumoniae EF3030 and M. catarrhalis O35E were seeded in a 24-well plate alone or together (at a ratio of 1 : 1000) as described in the methods section. After 4 h at 37 °C, 1 μg mL⁻¹ of amoxicillin or buffer was added to each well. Biofilms were resuspended, serially diluted, and plated at 16 h post-antibiotic treatment to determine viability. Results represent pneumococcal counts from three independent experiments.

**Fig. 2** Streptococcus pneumoniae protects Moraxella catarrhalis from macrolide killing. Streptococcus pneumoniae EF3030 and M. catarrhalis O35E were seeded in a 24-well plate alone or together as described in the methods section. After 4 h at 37 °C, 6 μg mL⁻¹ of azithromycin or buffer was added to each well. Biofilms were resuspended, serially diluted, and plated at 16 h post-antibiotic treatment to determine viability. Results represent M. catarrhalis counts from five independent experiments. ***denotes a P < 0.001.
survival of *S. pneumoniae* in the middle ear (Fig. 4b and c). Additionally, 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 OM 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. 5a). There was a statistically significant increase in the

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**Fig. 3** *Moraxella catarrhalis* forms polymicrobial biofilms with *Streptococcus pneumoniae* in vitro. *Streptococcus pneumoniae* EF3030 (green) and *M. catarrhalis* O35E (red) were seeded in 4-well Permanox chamber slides alone or together at 1 : 1000 (as described in the methods section) and grown for 24 h at 37 °C. Biofilms were then fixed, frozen within OCT medium, and cryosectioned (c. 5 μm/slice) and placed on slides. Bacteria were visualized using antibodies specific for pneumococci (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.
Fig. 4 Quorum sensing promotes nasopharyngeal colonization of Moraxella catarrhalis and affects ascension of pneumococci during polymicrobial infection in mice. Nine-week-old female BALB/c mice were serially infected for 3 days with Streptococcus 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.

Discussion

It has long been understood that the outcome, severity, and success of treatment for bacterial infection can be profoundly influenced by other microorganisms within the microbiota or in co-infection (Maddocks & May, 1969; Maddocks, 1980). 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 (Armbruster et al., 2010; Weimer et al., 2010, 2011). For pneumococcal infections, the incidence of antibiotic treatment failure dramatically exceeds the occurrence of antibiotic-resistant pneumococcal strains (Harrison et al., 2009). 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 (Kaieda et al., 2005; Brook, 2009). In keeping with this hypothesis, experimental evidence has indicated that M. catarrhalis can confer such passive protection within biofilms (Budhani & Struthers, 1997, 1998; Armbruster et al., 2010; Schaar et al., 2011). 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 (Wallace et al., 1989; Eliasson et al., 1992; Bootsma et al., 1996). 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 (Wallace et al., 1989). 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* revertant 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 (Armbruster et al., 2010). 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

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**Fig. 5** Quorum sensing promotes nasopharyngeal colonization and ascension of both *Streptococcus pneumoniae* and *Moraxella catarrhalis* during polymicrobial infections in chinchillas. Adult chinchillas were intranasally inoculated with *S. pneumoniae* EF3030, its *luxS* revertant 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* revertant alone,▲ represents *M. catarrhalis* alone, ○ represents polymicrobial infections with *S. pneumoniae* and *M. catarrhalis*, and □ represents polymicrobial infections with *S. pneumoniae* *luxS* revertant and *M. catarrhalis*. * denotes a *P* value between 0.01 and 0.05. *n* = 8 animals per group.
evidence demonstrating the increased occurrence of *M. catarrhalis* in conjunction with other bacterial species as opposed to alone (Petitgrew et al., 2008). These results demonstrate that 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 (Krishnamurthy et al., 2009; Weimer et al., 2010; Hoopman et al., 2012). Each model offers different advantages for studying OM (Chiavolini et al., 2008), 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 (Ruohola et al., 2013). 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* (Stroehler et al., 2003). 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

The authors thank Anthony Campagnari and David Briles for providing antibodies and Eric R. Lafontaine for providing *M. catarrhalis* strain O35E bro+ strain. This work was supported by grants from NIH/NIDCD (DC007444, DC10051, and DC12205) awarded to W.E.S. A.C.P. and K.A.M. were also supported by NIH training grant (T32AI007401).

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