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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*.

Keywords

Otitis; persistence; biofilm; antibiotic

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 (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, Weimer, *et al.*,

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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, Budhani & Struthers, 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 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 (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. *S. pneumoniae* EF3030 is a serotype 19F strain which typically establishes nasopharyngeal carriage or localized airway infection in murine models (Briles, *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 ~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 *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*I 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 a beta-lactamase deficient mutant in this background (hereafter referred to as 035E *bro*$^{-}$) has been recently described (Balder, *et al.*, 2013). *M. 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 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 (Armbruster, *et al.*,2010; Weimer, *et al*., 2011). *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 phospate-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). *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 (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 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. 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.

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 (Armbruster, *et al.*, 2010). 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. 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 confered 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.

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. 3 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. 3 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.

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 (Armbruster, *et al.*, 2010). 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. 4 A). At all time points, *S. pneumoniae* was not affected by the presence or absence of *M. catarrhalis* (Fig. 4 A). 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. 4 B). Moreover, in co-infections with the *luxS*$^{-}$ mutant, the numbers of colonizing *M. catarrhalis* was equivalent to when it is 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. 4 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. 4 B 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 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. 5 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. 5 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. 5 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 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 (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, Weimer, *et al.*, 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, Budhani & Struthers, 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*$^{-}$ 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 (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 evidence demonstrating the increased occurrence of *M. catarrhalis* in conjunction with other bacterial species as opposed to alone (Pettigrew, *et al.*, 2008). 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 (Krishnamurthy, *et al.*, 2009, Hoopman, *et al.*, 2012, Weimer, *et al.*, 2010). 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* (Stroeher, *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.

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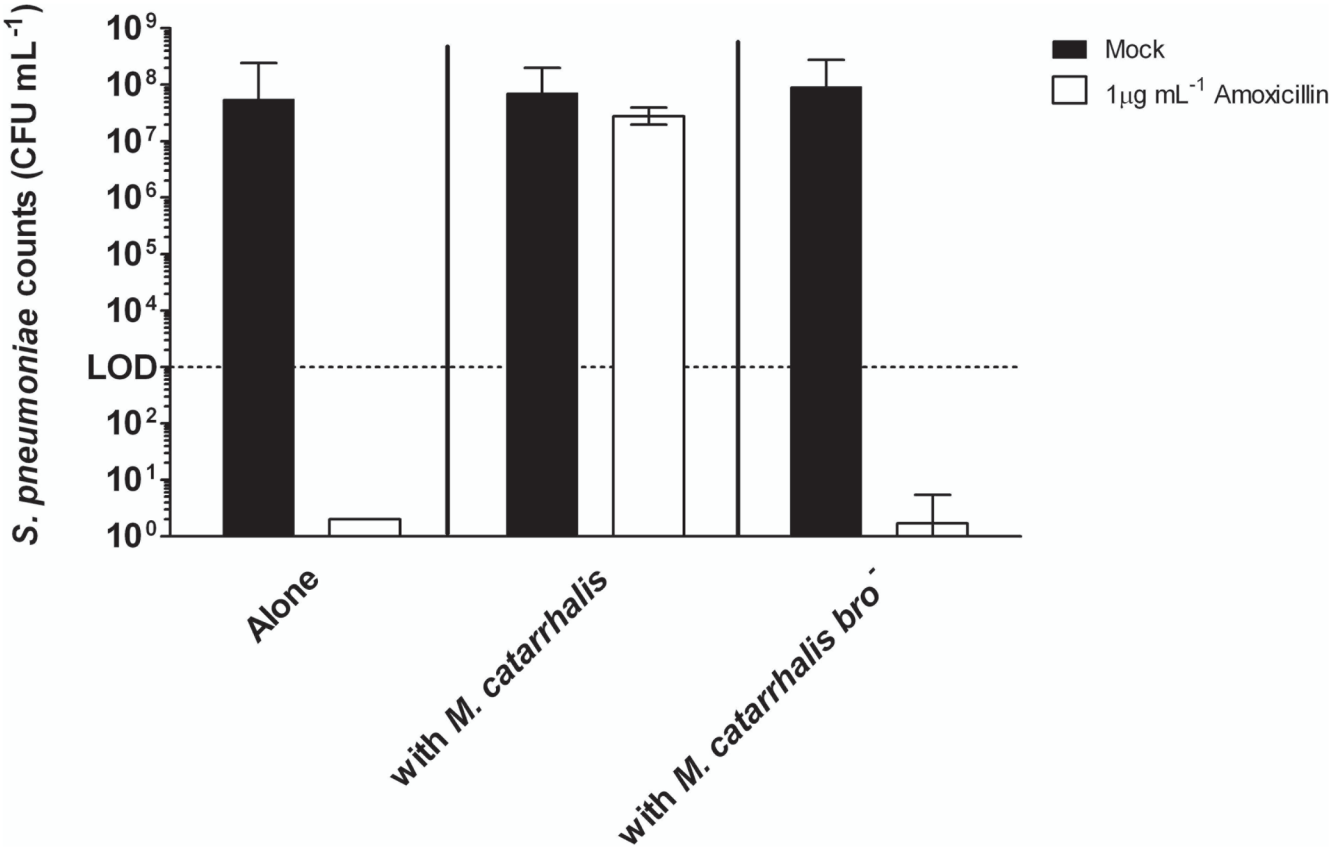

Figure 1. *M. catarrhalis* protects *S. pneumoniae* from beta-lactam killing

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.

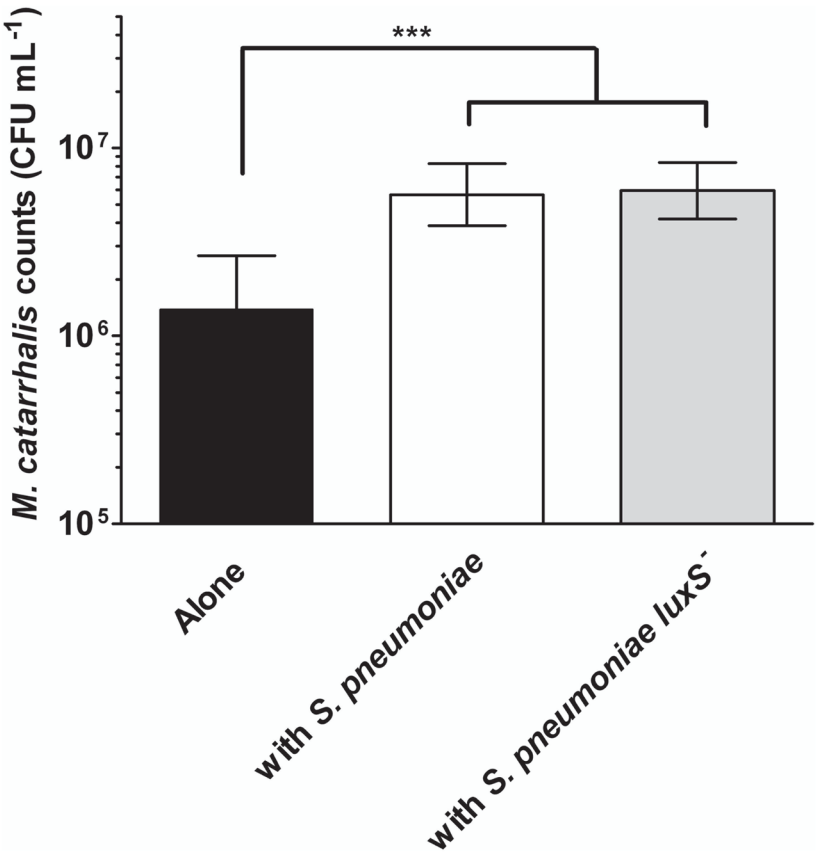

Figure 2. *S. pneumoniae* protects *M. catarrhalis* from macrolide killing

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.

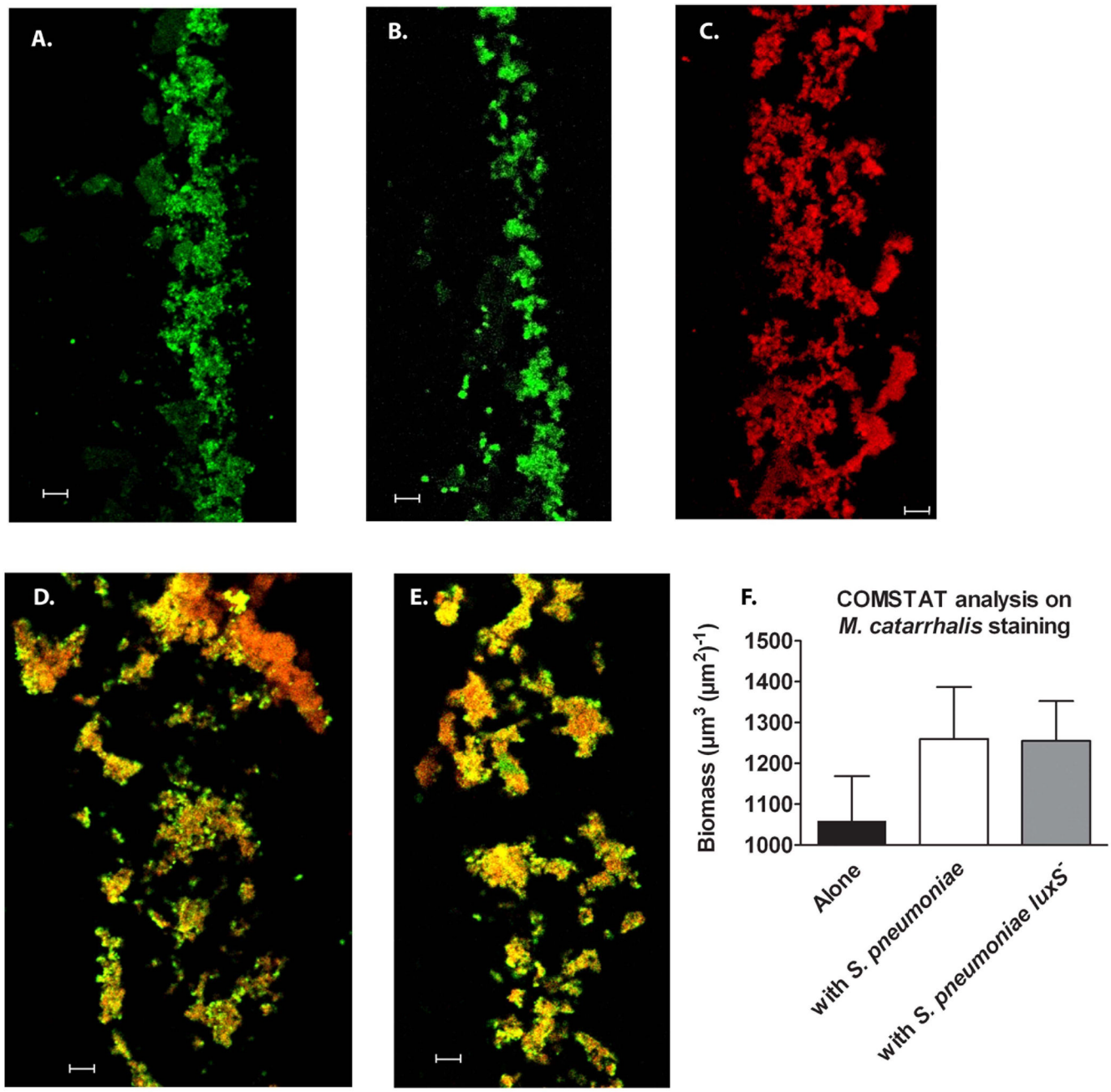

Figure 3. *M. catarrhalis* forms polymicrobial biofilms with *S. pneumoniae in vitro*

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 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.

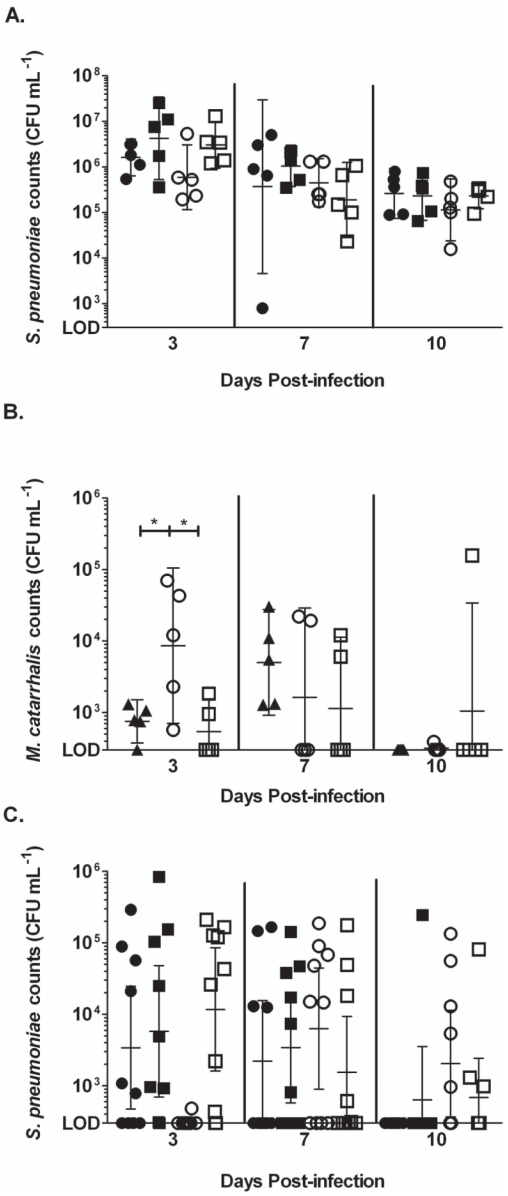

Figure 4. Quorum sensing promotes nasopharyngeal colonization of *M. catarrhalis* and affects ascension of pneumococci during polymicrobial infection in mice

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

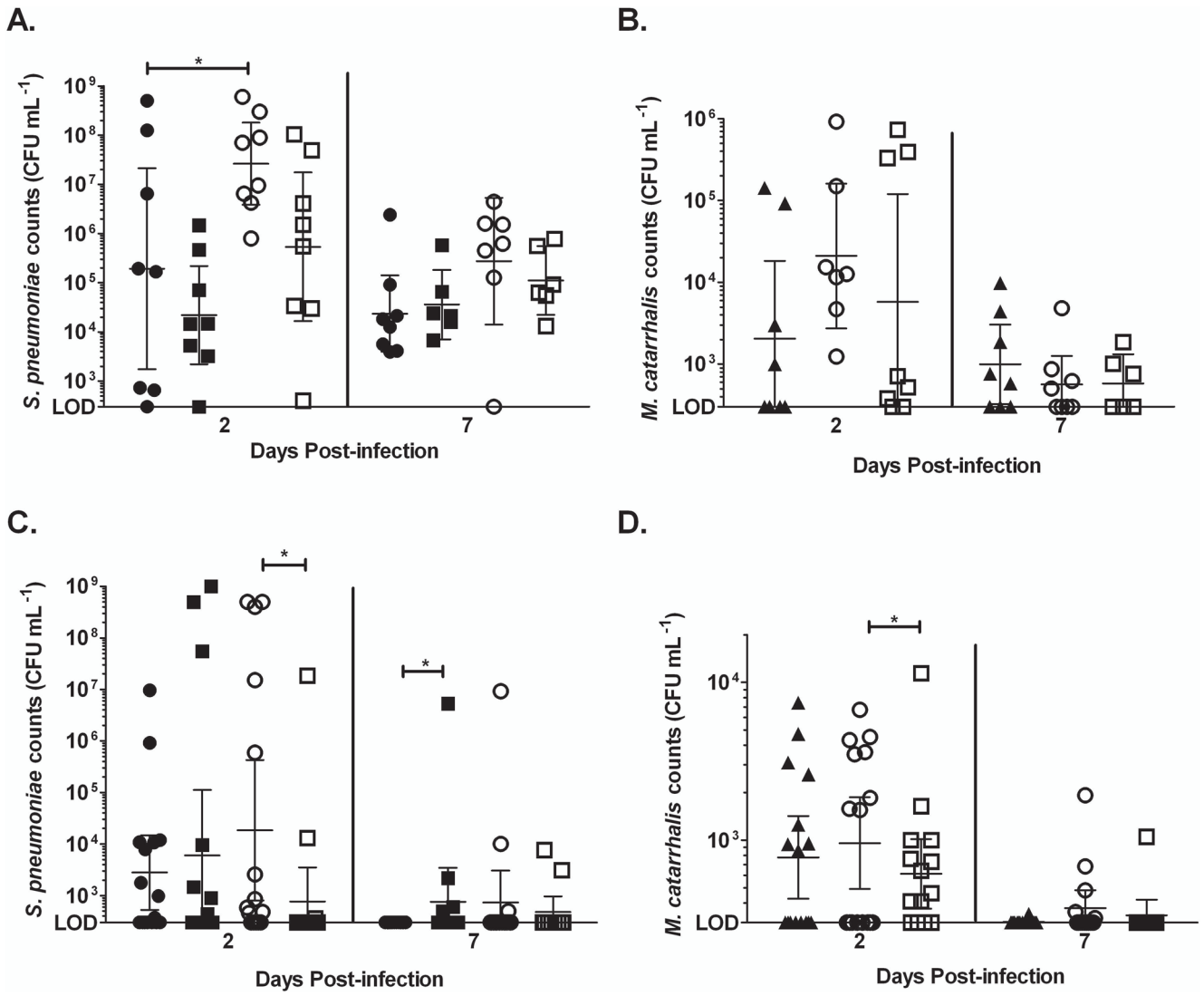

Figure 5. Quorum sensing promotes nasopharyngeal colonization and ascension of both *S. pneumoniae* and *M. catarrhalis* during polymicrobial infections in chinchillas

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

Table 1

Bacterial strains, plasmids, and primers

Bacterial strains

Species	Strain name	Reference
S. pneumoniae	EF3030	Briles *et al.*, 1992
	EF3030 *luxS*$^{-}$	this study
M. catarrhalis	O35E	Unhanand, *et al.*, 1992
	O35E *bro*$^{-}$	Balder, *et al.*, 2013

Plasmids

Name	Reference
pCR2.1	Invitrogen®
pSpR	Whitby *et al.*, 1998
pCRSpLuxSSp	this study

Primers

Name	Sequence	Reference
Splux		this study
Forward	5′-CGTGTTCGTCGCAATCCATACTC-3′	
Reverse	5′-ACAAGAAATCATCCGCCGTTACTA-3′	
Spluxver		this study
Forward	5′-GACGTTCAAAGGCATCATCTG-3′	
Reverse	5′-CCTACTGCCGGCCTTCACACTATC-3′	
M13		Invitrogen ®
Forward (−20)	5′-GTAAAACGACGGCCAGT-3′	
Reverse	5′-CAGGAAACAGCTATGAC-3′	