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

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**ABSTRACT** Otitis media (OM) is among the leading diseases of childhood and is caused by opportunists that reside within the nasopharynx, such as *Haemophilus influenzae* and *Moraxella catarrhalis*. As with most airway infections, it is now clear that OM infections involve multiple organisms. This study addresses the hypothesis that polymicrobial infection alters the course, severity, and/or treatability of OM disease. The results clearly show that coinfection with *H. influenzae* and *M. catarrhalis* promotes the increased resistance of biofilms to antibiotics and host clearance. Using *H. influenzae* mutants with known biofilm defects, these phenotypes were shown to relate to biofilm maturation and autoinducer-2 (AI-2) quorum signaling. In support of the latter mechanism, chemically synthesized AI-2 (dihydroxypentanedione [DPD]) promoted increased *M. catarrhalis* biofilm formation and resistance to antibiotics. In the chinchilla infection model of OM, polymicrobial infection promoted *M. catarrhalis* persistence beyond the levels seen in animals infected with *M. catarrhalis* alone. Notably, no such enhancement of *M. catarrhalis* persistence was observed in animals infected with *M. catarrhalis* and a quorum signaling-deficient *H. influenzae luxS* mutant strain. We thus conclude that *H. influenzae* promotes *M. catarrhalis* persistence within polymicrobial biofilms via interspecies quorum signaling. AI-2 may therefore represent an ideal target for disruption of chronic polymicrobial infections. Moreover, these results strongly imply that successful vaccination against the unencapsulated *H. influenzae* strains that cause airway infections may also significantly impact chronic *M. catarrhalis* disease by removing a reservoir of the AI-2 signal that promotes *M. catarrhalis* persistence within biofilm.

**IMPORTANCE** Otitis media (OM) is one of the most common childhood infections and is a leading reason for antibiotic prescriptions to children. Chronic and recurrent OM involves persistence of bacteria within biofilm communities, a state in which they are highly resistant to immune clearance and antibiotic treatment. While it is clear that most of these infections involve multiple species, the vast majority of knowledge about OM infections has been derived from work involving single bacterial species. There is a pressing need for better understanding of the impact of polymicrobial infection on the course, severity, and treatability of OM disease. In this study, we show that communication between bacterial species promotes bacterial persistence and resistance to antibiotics, which are important considerations in the diagnosis, prevention, and treatment of OM. Moreover, the results of this study indicate that successful preventive measures against *H. influenzae* could reduce the levels of disease caused by *M. catarrhalis*.
frequently isolated from polymicrobial OM infections than from single-species OM infections (24). This suggests that the presence of other bacterial pathogens may impact the persistence of *M. catarrhalis* or the severity of disease caused by this species. Additionally, *M. catarrhalis* is thought to confer passive antibiotic resistance upon other OM pathogens via secretion of beta-lactamase (25–30). However, the impact of polymicrobial infection on bacterial persistence, virulence, or response to treatment is not presently clear.

Interbacterial communication via quorum signaling is one factor which may impact the establishment of chronic polymicrobial infection, as quorum signaling is known to influence biofilm development for many species (31, 32). Autoinducer-2 (AI-2) is commonly referred to as an interspecies signal, as the genetic determinant for AI-2 production (*luxS*) is conserved among numerous bacterial species (33–35). AI-2 is known to influence biofilms for many species, including *H. influenzae* (36), and in some instances, AI-2 can impact the development of polymicrobial biofilms (37, 38). In this study, we addressed the hypothesis that polymicrobial infection impacts biofilm development and resistance during OM disease. The results clearly show that *H. influenzae* promotes *M. catarrhalis* persistence by means of interspecies quorum signals that increase the resistance of *M. catarrhalis* in biofilm.

**RESULTS**

*H. influenzae* and *M. catarrhalis* form polymicrobial biofilms in vitro. Based on clinical evidence for the coexistence of *H. influenzae* and *M. catarrhalis* in OM cases, it was hypothesized that these bacterial species would coexist in culture and *in vitro* biofilms. Static biofilms of *H. influenzae*, *M. catarrhalis*, or a mixture of both species were established in microscopy chamber slides, and the surface-attached bacterial communities were examined by scanning electron microscopy (SEM) (Fig. 1A) and confocal laser scanning microscopy (CLSM) (Fig. 1B) at various times during biofilm development. As previously observed, *H. influenzae* formed matrix-encased biofilm communities on the chamber slide surfaces (13, 36, 39). In contrast, *M. catarrhalis* formed smaller surface-attached clusters. In coculture, *H. influenzae* and *M. catarrhalis* formed polymicrobial biofilms with both species incorporated into the biofilm structure, as indicated by the presence of larger *M. catarrhalis* diplococci interspersed with the smaller *H. influenzae* coccobacilli (Fig. 1A). Immunostaining and confocal laser scanning microscopy showed that *M. catarrhalis* communities were present in discrete regions within the *H. influenzae* biofilm structure (Fig. 1B). Based on these results, we concluded that *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms.
Polymicrobial biofilms provide passive antibiotic resistance.

Beta-lactam antibiotics are commonly prescribed to children with OM (40). M. catarrhalis strains are nearly universally resistant to these antibiotics via secretion of beta-lactamase, and passive resistance during coinfection with M. catarrhalis has been postulated as a mechanism for antibiotic resistance of many airway pathogens. Therefore, we asked if M. catarrhalis could provide passive protection for a beta-lactam-sensitive H. influenzae strain within a polymicrobial biofilm. Biofilms were established for 24 h and treated with ampicillin or a combination of ampicillin and the beta-lactamase inhibitor clavulanate (Fig. 2). As anticipated, biofilms formed by H. influenzae were susceptible to ampicillin treatment, and M. catarrhalis biofilms were resistant. Polymicrobial biofilm formation increased the recovery of viable H. influenzae, indicating that M. catarrhalis provided protection against ampicillin treatment. The addition of clavulanate abolished the increased recovery of H. influenzae, indicating that protection was due to beta-lactamase produced by M. catarrhalis. As anticipated, clavulanate also reduced the recovery of viable M. catarrhalis from single-species biofilms. Of note, formation of a polymicrobial biofilm with H. influenzae increased the recovery of M. catarrhalis, even in the presence of clavulanate, suggesting that inherent properties of the polymicrobial biofilm provide antibiotic protection in addition to secreted beta-lactamase.

A combination of trimethoprim-sulfamethoxazole was next used to test the hypothesis that polymicrobial biofilms provide passive protection independent of diffusible resistance determinants. H. influenzae biofilms were more susceptible to this antibiotic combination, while M. catarrhalis biofilms were more resistant (data not shown). Polymicrobial biofilms formed by H. influenzae and M. catarrhalis afforded protection to H. influenzae from trimethoprim-sulfamethoxazole, as indicated by the increased recovery of viable H. influenzae from these biofilms (Fig. 3A). Control experiments indicated that bacteria recovered from polymicrobial biofilms retained broth culture susceptibility characteristics similar to those of the inocula (data not shown). These data show that antibiotic protection can be provided by the polymicrobial biofilm independently of genetic changes or transfer of resistance determinants between species.

In order to determine the contribution of H. influenzae biofilm to antibiotic protection within a polymicrobial biofilm, the macrolide clarithromycin was used at a concentration that eradicates M. catarrhalis within a biofilm (Fig. 3B). Polymicrobial biofilms formed by M. catarrhalis and H. influenzae protected M. catarrhalis from clarithromycin treatment, as indicated by a significant increase in recovered viable M. catarrhalis bacteria. Importantly, the amount of protection afforded to M. catarrhalis by biofilms containing H. influenzae mutants with biofilm defects (siaB, licD, and luxS) (14, 36, 39, 41, 42) was significantly diminished compared with that of the parental strain (Fig. 3B). Conversely, pro-
tation of M. catarrhalis was increased in biofilms formed with H. influenzae licON, a mutant which forms thicker biofilms (13). Control experiments showed that bacteria recovered from all polymicrobial biofilms retained broth susceptibility characteristics similar to those of the inocula, indicating that the increased antibiotic resistance observed in polymicrobial biofilms was not due to genetic changes or transfer of resistance determinants between species (data not shown). Based on these data, we concluded that the maturation state and/or overall biomass of the polymicrobial biofilm play integral roles in the antibiotic protection provided by H. influenzae biofilms.

Autoinducer-2 (dihydroxypentanedione) promotes M. catarrhalis biofilm thickness and antibiotic resistance. The decreased clarithromycin protection of M. catarrhalis by H. influenzae luxS could be due to differences in biofilm thickness or the maturation state of the luxS mutant, similar to the mechanism for decreased protection by H. influenzae licD. However, another possibility is that M. catarrhalis responds to AI-2 produced by H. influenzae, and the decrease in protection observed with H. influenzae luxS would thus be due to the loss of the AI-2 quorum signal. M. catarrhalis is not known to possess a luxS homolog and did not produce detectable AI-2 during growth in a broth culture, as measured by a Vibrio harveyi bioluminescence assay (Fig. 4A). However, recent evidence suggests that bacterial species which do not make AI-2 may still sense and respond to the AI-2 signal (43, 44). To test this hypothesis, M. catarrhalis was cultured in broth supplemented with the chemically synthesized AI-2 precursor dihydroxypentanedione (DPD), and samples were taken to determine the level of DPD remaining in the culture over time (Fig. 4A). M. catarrhalis depleted DPD over the course of 6 h, which indicates uptake and/or degradation of DPD, while an uninoculated control showed a minimal decrease in the AI-2 signal after 6 h. Notably, the amount of DPD depleted by M. catarrhalis was similar to the amount depleted by H. influenzae luxS. To determine if depletion of AI-2 requires live bacteria and/or active protein synthesis, M. catarrhalis cultures were incubated with tetracycline overnight prior to the addition of DPD or incubated with tetracycline concurrent with the addition of DPD. Samples were taken over the course of 7 h for comparison of M. catarrhalis DPD depletion to that of untreated M. catarrhalis (Fig. 4B). Both of the tetracycline treatments completely inhibited depletion of DPD by M. catarrhalis, suggesting that depletion is an active process which requires protein synthesis. Additionally, incubation of M. catarrhalis culture supernatant with DPD did not result in depletion (data not shown). Taken together, these data indicate that M. catarrhalis is most likely depleting AI-2 by means of an uptake system rather than passive binding or external degradation of this signaling molecule.

To assess the impact of exogenous AI-2 on M. catarrhalis biofilm formation, stationary M. catarrhalis biofilms were established in the presence or absence of DPD and stained with crystal violet at 4, 6, 12, 24, and 48 h (Fig. 4C). Incubation with DPD resulted in an overall increase in M. catarrhalis biofilm biomass that was particularly evident at 24 and 48 h. Viability staining and confocal laser scanning microscopy (CLSM) of M. catarrhalis biofilms confirmed the increased M. catarrhalis biofilm density in the presence of DPD and further demonstrated an increase in bacterial viability within biofilm. M. catarrhalis biofilms established in media lacking DPD formed small clusters with mostly nonviable staining (Fig. 4D), while biofilms established in the presence of DPD were thicker on average and showed an increased number of viable bacteria within the larger biomasses (Fig. 4E). Compressed z-series images confirmed the increased viable staining present in the larger biomasses of DPD-treated M. catarrhalis (Fig. 4G) compared to those of untreated M. catarrhalis biofilms (Fig. 4F). SEM of M. catarrhalis biofilms similarly demonstrated the impact of DPD on biofilm development, with treatment resulting in increased formation of bacterial clusters compared to those formed in M. catarrhalis biofilms established in media lacking DPD (Fig. 4H and I).

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

Interspecies quorum signaling during polymicrobial infection promotes persistence of M. catarrhalis. The in vitro studies of polymicrobial biofilms support a prominent role for interspecies quorum signaling in the development of M. catarrhalis biofilms with increased resistance phenotypes. As M. catarrhalis is frequently isolated from polymicrobial OM infections, we hypothesized that M. catarrhalis could utilize AI-2 produced by H. influenzae or other OM pathogens to persist in vivo. Therefore, infection studies were performed using the chinchilla model of OM to test this hypothesis. As previously observed, high numbers of H. influenzae and H. influenzae luxS bacteria were detected in middle ear effusion fluid (Fig. 5A) and bullar homogenate (Fig. 5B) samples at both 7 days and 14 days postinfection for single-species and polymicrobial infection groups. Counts of M. catarrhalis within middle ear effusion fluid samples were at or below the level of detection at both time points (Fig. 5A), regardless of the type of infection. Animals infected with M. catarrhalis alone had bacterial loads within the bullar homogenates at both 7 and 14 days postinfection that were consistent with the initial inocula. However, in the coinfected animals, significantly higher numbers of M. catarrhalis bacteria in bullar homogenate samples at 14 days postinfection were observed (Fig. 5B). Based on these results, we conclude that M. catarrhalis survives exclusively in surface-attached communities within the chinchilla middle ear chamber and that coinfection with H. influenzae provides a permissive environment in which M. catarrhalis can proliferate. We next asked if the increase in M. catarrhalis bacterium numbers during coinfection with H. influenzae were dependent on interspecies quorum signaling by performing similar coinfection studies using H. influenzae luxS. Notably, no increase in M. catarrhalis bacterium counts was observed during coinfection with H. influenzae luxS at either time point. Taken in concert with the experiments showing increased M. catarrhalis biofilm density and resistance following treatment with synthetic AI-2, these experiments...
show that *M. catarrhalis* can utilize exogenous AI-2 provided by *H. influenzae* to establish a persistent infection.

**DISCUSSION**

According to the long-standing concept of indirect pathogenicity, bacterial disease and/or response to treatment is subject to influence by other bacteria sharing the same environment (28, 29). In this study, *H. influenzae* was shown to promote persistence and antibiotic resistance of *M. catarrhalis* via protection within the biofilm structure in response to interspecies quorum signaling. The data presented in this study, therefore, provide concrete validation of the concept of indirect pathogenicity and provide a mechanism to support how this can occur during polymicrobial otitis media infections.
In the context of infectious disease, biofilm formation has long been considered to be an important determinant of bacterial persistence. It has also long been appreciated that growth within a biofilm increases resistance to antibiotics through multiple mechanisms, which include delayed antibiotic penetration of the biofilm and changes in the metabolic state of bacteria within biofilms (18–21). In this study, both in vitro and in vivo models were used to ask how *H. influenzae* and *M. catarrhalis* polymicrobial biofilm formation impacts antibiotic resistance and bacterial persistence. While *M. catarrhalis* can be considered an indirect pathogen through the production of beta-lactamase, the results of this study clearly prove that both *M. catarrhalis* and *H. influenzae* can provide antibiotic protection to other pathogens within a polymicrobial biofilm in a manner independent of diffusible resistance determinants. Additionally, the abrogation of antibiotic protection observed for polymicrobial biofilms formed with *H. influenzae* biofilm mutants demonstrates a role for biofilm maturation/total biomass in antibiotic protection. This study and others support the hypothesis that biofilm can provide a barrier that protects susceptible organisms contained within.

In addition to the impact of biofilm biomass on antibiotic susceptibility, this study solidifies the role of interspecies communication during the establishment of polymicrobial biofilms. Mutation of the *luxS* gene, the genetic determinant of AI-2 production, alters *H. influenzae* biofilm maturation and density as well as bacterial persistence, indicating that AI-2 plays a critical role in the development and maturation of *H. influenzae* biofilms (36). While *M. catarrhalis* does not produce AI-2, the results clearly demonstrate the critical role of interspecies quorum signaling via AI-2 in the establishment of persistent polymicrobial biofilms containing this species and indicate the presence of an AI-2 transport system in *M. catarrhalis*. The main AI-2 transport system that is required for the enhancement of *M. catarrhalis* persistence within biofilm and thereby promotes antibiotic resistance following treatment with DPD also provides further support that quorum signaling contributes to the establishment of a diffusion barrier to delay or limit antibiotic penetration of the biofilm.

The augmented persistence observed during infection by *M. catarrhalis* and the parental strain of *H. influenzae* but not by *H. influenzae luxS* further demonstrates the role of interspecies quorum signaling in the establishment of polymicrobial OM. One possible explanation for the increase in *M. catarrhalis* persistence is that *M. catarrhalis* becomes incorporated into the biofilm scaffold provided by *H. influenzae*, and this incorporation protects *M. catarrhalis* from host factors, thus allowing for increased persistence. As *H. influenzae luxS* has a persistence defect in the chinchilla model of OM, it is possible that the luxS mutant does not provide sufficient biofilm structure for protection of *M. catarrhalis* or, alternatively, that other factors lacking in *H. influenzae luxS* are required for the enhancement of *M. catarrhalis* persistence. However, the results demonstrating depletion of synthetic AI-2 by *M. catarrhalis*, as well as the increased *M. catarrhalis* biofilm density and resistance elicited by synthetic AI-2, argue that AI-2 quorum signaling from *H. influenzae* promotes *M. catarrhalis* persistence in polymicrobial biofilm. Therefore, we conclude that the production of AI-2 by *H. influenzae* promotes *M. catarrhalis* resistance within biofilm and thereby promotes *M. catarrhalis* persistence within the middle ear chamber. It is notable that prior studies involving infection of rodents with *M. catarrhalis* have historically failed to mimic the chronic and recurrent infections that are typical of human patients with opportunistic airway in-

**FIG 5** Polymicrobial infection augments *M. catarrhalis* persistence in vivo. Chinchillas were infected with 10^7 CFU of *H. influenzae* or *H. influenzae luxS*, 10^4 CFU of *M. catarrhalis*, or a mixture of both species. (A) Middle ear effusion fluids were removed for enumeration of viable *H. influenzae* and *M. catarrhalis* bacteria by plating on sBHI medium plus clarithromycin or BHI medium, respectively. (B) Bullae were removed at each time point and homogenized for enumeration of viable *H. influenzae* and *M. catarrhalis* bacteria, as described above. Data represent the mean results from four experiments ± SEM. *** P < 0.005 compared to the number of CFU from *M. catarrhalis* single-species bullar homogenate.
fections; instead, rodent infections are typically transient in nature (50). The results presented here may indicate that this difference in bacterial persistence occurs as a consequence of the absence of an “infection partner,” such as H. influenzae, to provide AI-2, as opposed to any species differences.

As we observed that both M. catarrhalis and H. influenzae can deplete AI-2/DDP, yet only one of these species produces the signal, there could be competition between M. catarrhalis and H. influenzae for available AI-2 during coinfection. Based on the AI-2 requirement for H. influenzae to establish a chronic infection, any competition with M. catarrhalis would most likely have a negative impact on H. influenzae persistence. However, the numbers of viable bacteria recovered from coinfected animals were similar to the numbers recovered from those which received H. influenzae alone, indicating that AI-2 uptake by M. catarrhalis did not interfere with H. influenzae biofilm formation or persistence. Additionally, the presence of M. catarrhalis during stationary biofilm formation was not observed to negatively impact H. influenzae biofilm formation or antibiotic resistance. Taken together, these observations support a model wherein H. influenzae requires only a certain threshold level of AI-2 but may produce AI-2 in excess of the threshold concentration needed to promote biofilm development. In this model, AI-2 depleted from the biofilm environment by M. catarrhalis would not have a detrimental impact on H. influenzae biofilm development. Another possible explanation is that M. catarrhalis may require only a minimal concentration of AI-2 to alter biofilm development. This would be advantageous, as M. catarrhalis could utilize any AI-2-producing species as an “infection partner” to promote its own persistence without directly competing for the AI-2 signal. Further research will be necessary to determine the minimal AI-2 concentration required by both M. catarrhalis and H. influenzae to promote biofilm development.

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

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** A complete list of bacterial strains used in this study is provided in Table 1. M. catarrhalis strains were cultivated in brain heart infusion (BHI) medium (Difco), and H. influenzae strains were cultivated in BHI medium supplemented with hemin (ICN Biochemicals) and NAD (Sigma); this medium is referred to herein as supplemented BHI (sBHI). For experiments using trimethoprim-sulfamethoxazole, H. influenzae and M. catarrhalis were cultured in Morose’s defined medium (51) supplemented with hemin and NAD. H. influenzae siaB was constructed essentially as described previously for strain 2019 siaB (52) and confirmed by immunoblotting to have decreased activity with Limax flavus (LFA) lectin (EY Laboratories).

**SEM.** Stationary in vitro biofilm cultures were grown in Lab-Tek II cover glass slides (Nunc). Each chamber was inoculated with ~10^8 CFU/ml of H. influenzae, M. catarrhalis, or a 1:1 dilution mixture of both species and incubated for 48 h at 37°C and 5% CO2. Biofilm samples were fixed for 30 min with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) and rinsed once. Samples were then dehydrated, fixed, and prepared for scanning electron microscopy (SEM) analysis as previously described (39). Biofilm samples were mounted onto stubs, sputter coated with palladium, and then viewed with a Philips SEM-515 scanning electron microscope.

**CLSM.** In vitro biofilm cultures were grown using a continuous flow system as previously described (13). H. influenzae and M. catarrhalis were cultured overnight in sBHI broth and diluted to ~10^9 CFU/ml. Chamber slides were inoculated with each strain alone or a 1:1 dilution mixture of both species and incubated for 24, 48, and 72 h at 37°C and 5% CO2. At each time point, biofilms were fixed and stained with rabbit polyclonal anti-H. influenzae sera (41) and/or monoclonal antibody 4G5 (53). MAb 4G5 was generously provided by Anthony Campagnari. All secondary antibodies were purchased from Jackson Laboratories. Biofilms were visualized using a Zeiss LSM 510 CLSM and Zeiss LSM Image Browser software.

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

**Biofilm antibiotic protection studies.** Bacteria were grown overnight on sBHI or BHI medium plates, suspended in sBHI medium, and diluted to ~10^6 CFU/ml. A 24-well microtiter plate was inoculated with a single-species suspension diluted 1:1 with PBS or a 1:1 dilution mixture of both bacterial suspensions. Cultures were incubated at 37°C and 5% CO2, for either 4 h or 24 h to establish biofilms. Supernatants were then carefully removed and replaced with either fresh sBHI or sBHI medium with the antibiotic, and cultures were returned to 37°C and 5% CO2, for 24 h. Following incubation, supernatants were removed, and biofilms were resuspended in PBS for serial dilution and plating to enumerate viable bacteria. Polymicrobial biofilms were plated onto both BHI medium and sBHI medium containing 2 μg/ml clarithromycin to distinguish between M. catarrhalis and H. influenzae, respectively.

**M. catarrhalis AI-2 studies.** All studies were conducted using 0.2 μM dihydroyxypentanediol (DPP; Omm Scientific). This concentration of DPP was chosen to simulate the amount of AI-2 produced by H. influenzae, as it elicits luminescence from Vibrio harveyi that is approximately equal to that elicited by H. influenzae late-exponential-phase culture supernatant. For AI-2 depletion studies, BHI or sBHI medium was supplemented with DPP when indicated, inoculated with ~10^6 CFU of M. catarrhalis or H. influenzae luxS, and incubated at 37°C and 150 rpm for 6 h. Samples were taken at 0.25, 0.5, 0.75, 1, 2, 3, and 6 h, centrifuged, filter sterilized, and stored at ~20°C for bioluminescence. Luminescence produced by Vibrio harveyi BB170 (54) following a 3-h incubation with su-

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**TABLE 1 Bacterial strains**

<table>
<thead>
<tr>
<th>Strain/mutant</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>H. influenzae</td>
<td>Nasopharyngeal isolate from child with OM</td>
<td>55</td>
</tr>
<tr>
<td>licD mutant</td>
<td>86-028NP NTHI 1594 mutant</td>
<td>42</td>
</tr>
<tr>
<td>licON mutant</td>
<td>86-028NP constitutive PCho</td>
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<td>luxS mutant</td>
<td>86-028NP NTHI 0621 mutant</td>
<td>36</td>
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<tr>
<td>siaB mutant</td>
<td>86-028NP NTHI 1891 mutant</td>
<td>This study</td>
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<tr>
<td>M. catarrhalis 7169</td>
<td>Serotype B strain</td>
<td>57</td>
</tr>
</tbody>
</table>

^a PCho^*+, phosphorylcholine positive.
permanant samples was determined in a Turner Designs TD-20/20 lumi-
nometer for 10 s. Data are reported as relative light units (counts per 10 s).
Tetracycline studies were conducted by incubating M. catarrhalis with 10 μg/ml tetracycline (Sigma). M. catarrhalis cultures were incubated with tetracycline during overnight growth in a broth culture to completely inhibit bacterial growth/viability as well as at the start of the 7-h AI-2 depletion study to monitor the role of protein synthesis in AI-2 depletion.

Inhibit bacterial growth/viability as well as at the start of the 7-h AI-2 with tetracycline during overnight growth in a broth culture to completely

10

M. catarrhalis were inoculated with ~108 CFU of M. catarrhalis and incubated at 37°C and 5% CO2. Supernatants were carefully re-
depth study to monitor the role of protein synthesis in AI-2 depletion.


Jurcisek, J. A., L. Greiner, H. Watanabe, A. Zaleski, M. A. Apicella, and L. O. Bakaletz. 2005. Role of saltic acid and complex carbohydrate bio-


Chinchilla infection studies. Bacterial persistence and biofilm forma-
tion in the middle ear chamber were assessed as described previously (12, 13). Chinchillas were purchased from Rauscher’s Chinchilla Ranch (La-
Rue, OH) and allowed to acclimate to the vivarium for >7 days prior to infection. No animals showed visible signs of illness by otoscopy prior to infection. The animals were anesthetized with isoflurane and infected via transbular injection with ~107 CFU of Haemophilus influenzae or H. influenzae luxS−, ~105 CFU of M. catarrhalis, or a 1:1 mixture of both species. All inocula were confirmed by plate counting. At 7 days or 14 days postinfection, animals (four per group) were euthanized, and middle ear chambers were aseptically opened. Effusion fluid samples were recovered, and middle ear lave was performed using 1.0 ml sterile PBS. Viable bacteria were enu-
merated by plate counting the combined retrieved fluids. Fluid samples obtained from animals which received polymicrobial inocula were plated onto both sBHI medium containing 2 μg/ml clarithromycin and BHI medium lacking NAD and hemin. Bacterial colonies were excised and homogenized in 10 ml sterile PBS and then plated to determine the number of CFU of sBHI medium processed as described above or washed once with PBS and stained with a Live/Dead BacLight viability kit (Invitrogen) prior to imaging by CLSM, as described above.

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

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REFERENCES
14. Jefferson, K. K., D. A. Goldmann, and G. B. Pier. 2005. Use of confocal microscopy to microscopically analyze the rate of vancomycin penetration through Staph-
genomic role of beta-lactamase-producing moraxellae by use of a


