

# 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*.

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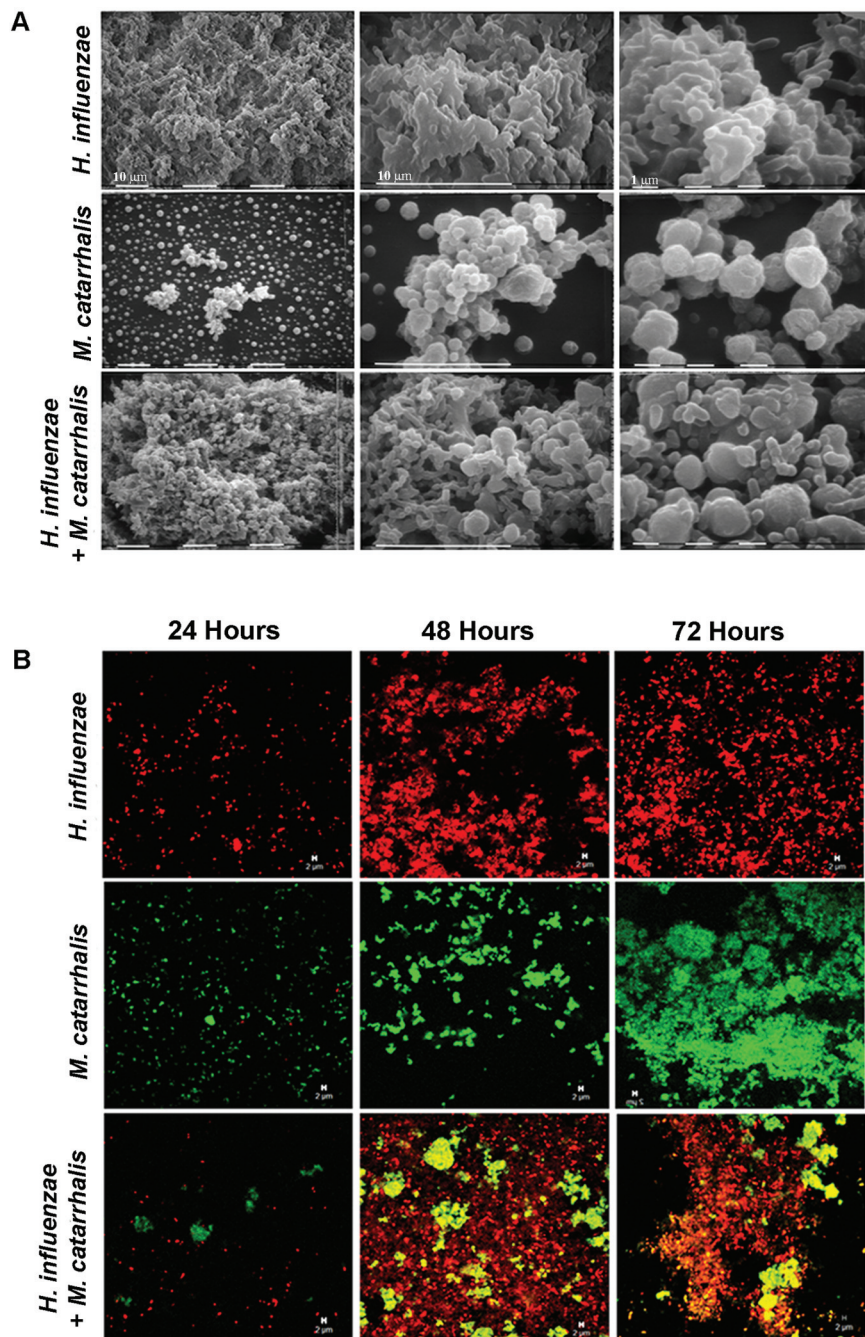
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Otitis media (OM) is one of the most common childhood infections (1–3) and is the leading reason for pediatric office visits and new antibiotic prescriptions to children (4). OM infections often persist for long periods of time and are frequently recalcitrant to antibiotic treatment (5, 6). Due to the highly resistant nature of chronic and recurrent OM, these infections have long been thought of as involving bacterial persistence within a biofilm (7–9). Clinical evidence of bacterial biofilms includes direct observation of biofilms in patient tissue samples (10) and in the chinchilla experimental model of OM (11–15). Persistence of bacteria within a biofilm community can also greatly increase re-

sistance to antibiotics (16, 17) through numerous mechanisms, including phenotypic heterogeneity and slower growth of bacteria within the biofilm, delayed antibiotic penetration through matrix material/exopolysaccharide, and the presence of persister cells (18–21).

As with most upper airway infections, epidemiological data indicate that the majority of chronic OM infections are polymicrobial in nature (22). For example, *Haemophilus influenzae* and *Moraxella catarrhalis* are frequently present together in samples obtained from patients with chronic and recurrent OM (22, 23). Interestingly, a recent study found *M. catarrhalis* to be more



**FIG 1** *H. influenzae* and *M. catarrhalis* form polymicrobial biofilms *in vitro*. Stationary biofilms were established in chamber slides for visualization of bacteria by SEM and confocal laser scanning microscopy (CLSM). (A) Samples of *H. influenzae* and *M. catarrhalis* single-species or polymicrobial biofilms were taken at 48 h and prepared for SEM. Images shown are at three different levels of magnification. (B) CLSM was performed on 24-, 48-, and 72-h biofilms following staining of *H. influenzae* (red) and *M. catarrhalis* (green).

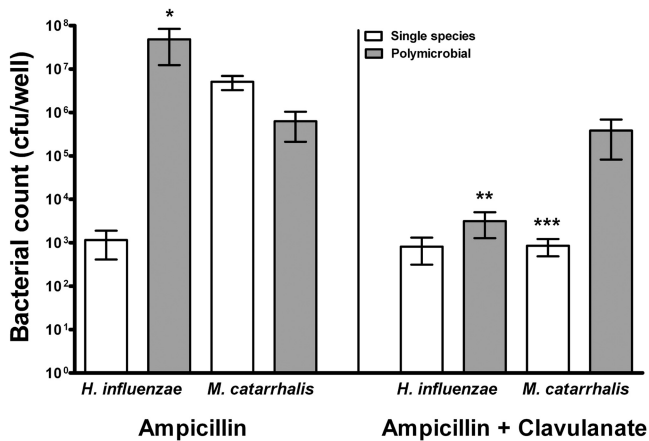
frequently isolated from polymicrobial OM infections than from single-species OM infections (24). This suggests that the presence of other bacterial pathogens may impact the persistence of *M. catarrhalis* or the severity of disease caused by this species. Additionally, *M. catarrhalis* is thought to 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.

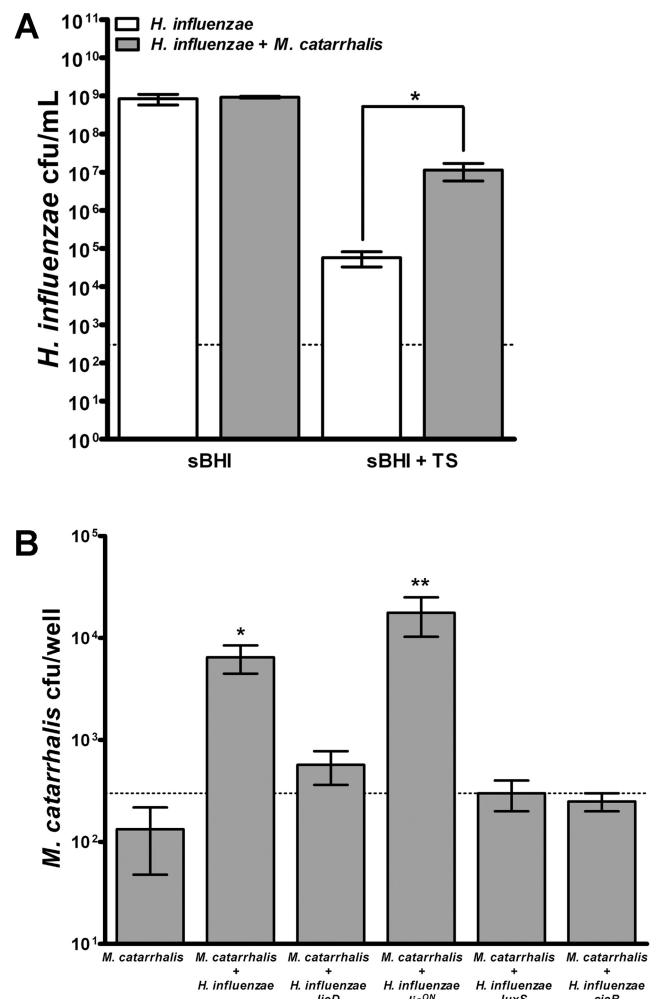


**FIG 2** Beta-lactam protection in a polymicrobial biofilm. Stationary biofilms of *H. influenzae* Rd and/or *M. catarrhalis* were established on chamber slides for 24 h and treated with 100  $\mu$ g/ml ampicillin or ampicillin with 25  $\mu$ g/ml clavulanate for an additional 24 h. Biofilms were resuspended in sterile PBS, serially diluted, and plated on sBHI medium plus clarithromycin and BHI medium plates for enumeration of viable *H. influenzae* Rd and *M. catarrhalis* bacteria, respectively. Data are represented as means  $\pm$  SEM. \*,  $P < 0.05$  compared to *H. influenzae* plus ampicillin; \*\*,  $P < 0.05$  compared to polymicrobial *H. influenzae* plus ampicillin; \*\*\*,  $P < 0.05$  compared to *M. catarrhalis* plus ampicillin.

#### 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



**FIG 3** Polymicrobial biofilm formation protects *H. influenzae* and *M. catarrhalis* from antibiotic treatment. Single-species or polymicrobial stationary biofilms were established for 4 h and treated with 60  $\mu$ g/ml trimethoprim-sulfamethoxazole (TS) (A) or 6  $\mu$ g/ml clarithromycin (B) for 24 h. Biofilms were resuspended in sterile PBS, serially diluted, and plated on sBHI medium plus clarithromycin or BHI medium for enumeration of viable *H. influenzae* and *M. catarrhalis* bacteria, respectively. Data are represented as the mean results from three combined experiments, with two replicates per experiment. Error bars represent SEM. \*,  $P < 0.05$ .

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-



tection of *M. catarrhalis* was increased in biofilms formed with *H. influenzae lic<sup>ON</sup>*, 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.

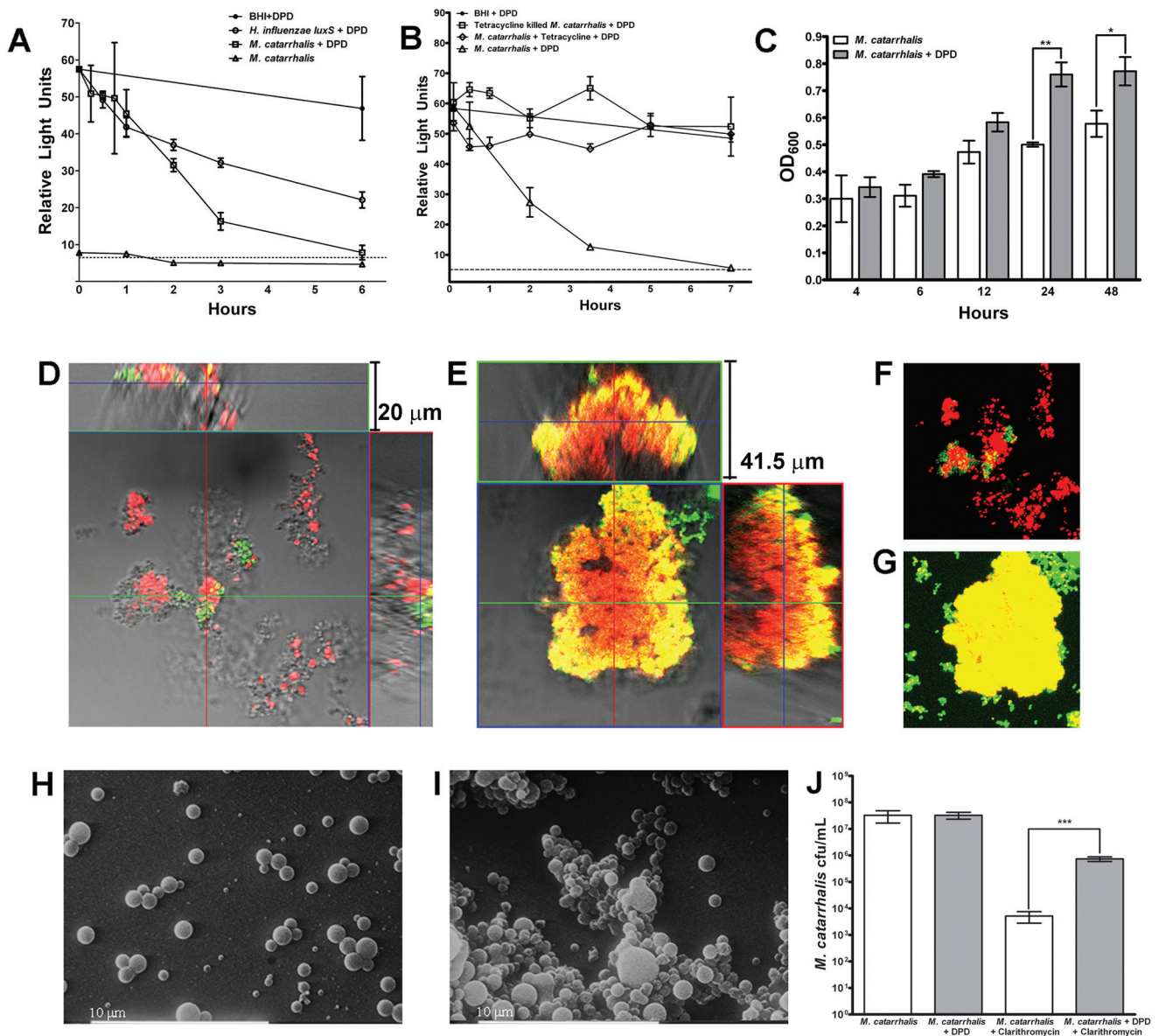
**Autoinducer-2 (dihydroxypentanedione) promotes *M. catarrhalis* biofilm thickness and antibiotic resistance.** The decreased clarithromycin protection of *M. catarrhalis* by *H. influenzae luxS* could be due to differences in biofilm thickness or the maturation state of the *luxS* mutant, similar to 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 coinfecting 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



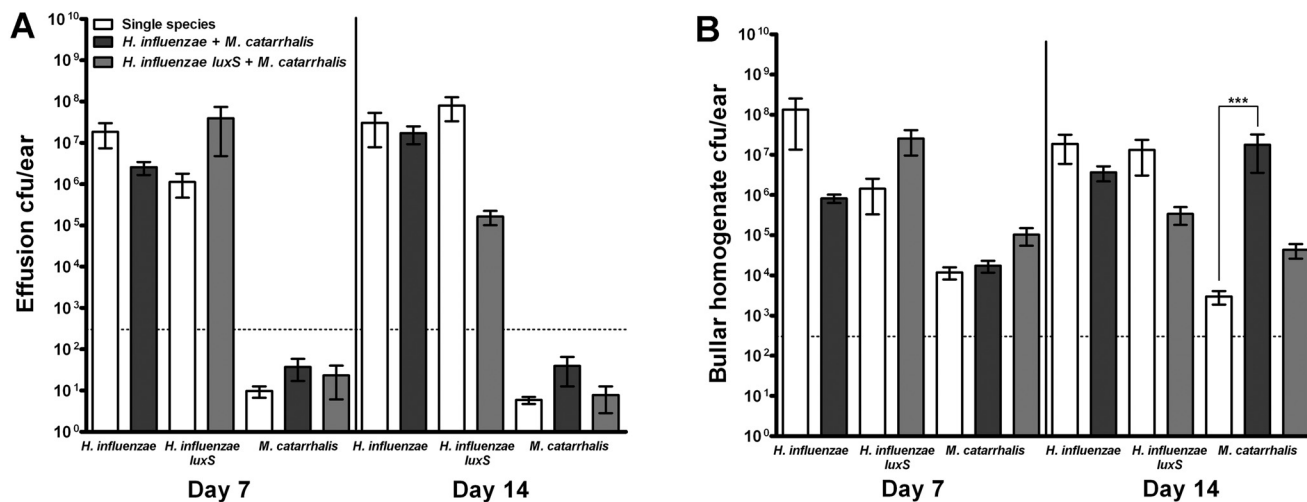
**FIG 4** AI-2 promotes *M. catarrhalis* biofilm development and antibiotic resistance. (A) *M. catarrhalis* was cultured in BHI medium or BHI medium supplemented with 0.2  $\mu\text{M}$  synthetic AI-2 (DPD) to determine AI-2 production and depletion, as measured by *Vibrio harveyi* bioluminescence. *H. influenzae luxS* was cultured in sBHI medium supplemented with DPD to measure depletion. An uninoculated control of BHI medium with DPD shows the minimal degradation of the AI-2 signal during 6 h of incubation at 37°C. (B) Depletion of DPD by *M. catarrhalis* biofilms were established for 24 h following incubation with 10  $\mu\text{g}/\text{ml}$  tetracycline was measured by bioluminescence over a period of 7 h. (C) *M. catarrhalis* biofilms were established in the presence or absence of DPD and stained with crystal violet for determination of biofilm biomass at 4, 6, 12, 24, and 48 h. Data represent the mean results from three combined experiments, with three replicate wells per experiment. Error bars represent SEM. (D and E) *M. catarrhalis* biofilms were established for 24 h in the presence (E) or absence (D) of DPD and stained with a viability kit for CLSM visualization of surface coverage and biofilm thickness. (F and G) Z-series images from panels D and E were compressed to show total viable and nonviable staining of biofilms established in the presence (G) or absence (F) of DPD. (H and I) SEM images of 24-h *M. catarrhalis* biofilms established with (I) or without (H) DPD. (J) *M. catarrhalis* biofilms were established for 4 h in the presence or absence of DPD and then treated with 6  $\mu\text{g}/\text{ml}$  clarithromycin for 24 h and plated for enumeration of viable bacteria. Data represent the means from three replicates  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

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.



**FIG 5** Polymicrobial infection augments *M. catarrhalis* persistence *in vivo*. Chinchillas were infected with  $10^3$  CFU of *H. influenzae* or *H. influenzae luxS*,  $10^4$  CFU of *M. catarrhalis*, or a mixture of both species. (A) Middle ear effusion fluids were removed for enumeration of viable *H. influenzae* and *M. catarrhalis* 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  $\pm$  SEM. \*\*\*,  $P < 0.005$  compared to the number of CFU from *M. catarrhalis* single-species bullar homogenate.

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 has been described outside *Vibrio* species is the Lsr ABC transporter. The Lsr system, identified in *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, *Sinorhizobium meliloti*, and *Aggregatibacter actinomycetemcomitans*, has homology to the ribose

ABC transporter and involves binding of AI-2 by LsrB and transport through a heterodimeric membrane channel (44–47). It was determined in *A. actinomycetemcomitans* that the ribose binding protein RbsB, in addition to LsrB, can bind AI-2 (48, 49). The mechanism for the sensing of DPD/AI-2 by *M. catarrhalis* has yet to be defined, but as the presence of this signaling molecule clearly alters *M. catarrhalis* biofilm development, this will be an important topic for future studies. Additionally, the increased antibiotic resistance following treatment with DPD also provides further support 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-



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/DPD, yet only one of these species produces the signal, there could be competition between *M. catarrhalis* and *H. influenzae* for available AI-2 during coinfection. Based on the AI-2 requirement for *H. influenzae* to establish a chronic infection, any competition with *M. catarrhalis* would most likely have a negative impact on *H. influenzae* persistence. However, the numbers of viable bacteria recovered from coinfecting 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 Morse’s defined medium (51) supplemented with hemin and NAD. *H. influenzae* *siaB* was constructed essentially as described previously for strain

TABLE 1 Bacterial strains

Strain/mutant	Description	Reference
<i>H. influenzae</i>		
86-028NP	Nasopharyngeal isolate from child with OM	55
<i>licD</i> mutant	86-028NP NTHI 1594 mutant	42
<i>lic<sup>ON</sup></i> mutant	86-028NP constitutive PCho <sup>+</sup>	13
<i>luxS</i> mutant	86-028NP NTHI 0621 mutant	36
<i>siaB</i> mutant	86-028NP NTHI 1891 mutant	This study
Rd	Rd KW20	56
<i>M. catarrhalis</i> 7169	Serotype B strain	57

<sup>a</sup> PCho<sup>+</sup>, phosphorylcholine positive.

2019 *siaB* (52) and confirmed by immunoblotting to have decreased reactivity 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<sup>8</sup> 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% CO<sub>2</sub>. 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<sup>8</sup> 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% CO<sub>2</sub>. 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<sup>8</sup> 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% CO<sub>2</sub> 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% CO<sub>2</sub> 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 dihydroxypentanedione (DPD; Omm Scientific). This concentration of DPD 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 DPD when indicated, inoculated with ~10<sup>8</sup> 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-

pernatant samples was determined in a Turner Designs TD-20/20 luminometer for 10 s. Data are reported as relative light units (counts per 10 s). Tetracycline studies were conducted by incubating *M. catarrhalis* with 10  $\mu\text{g}/\text{ml}$  tetracycline (Sigma). *M. catarrhalis* cultures were incubated with tetracycline during overnight growth in a broth culture to completely inhibit bacterial growth/viability as well as at the start of the 7-h AI-2 depletion study to monitor the role of protein synthesis in AI-2 depletion. For crystal violet staining of *M. catarrhalis* biofilms, wells of a 24-well tissue culture plate containing sBHI medium or sBHI medium supplemented with 0.2  $\mu\text{M}$  DPD were inoculated with  $\sim 10^8$  CFU of *M. catarrhalis* and incubated at 37°C and 5%  $\text{CO}_2$ . Supernatants were carefully removed at each time point, and biofilms were washed one time with  $\text{H}_2\text{O}$ , stained with 0.1% crystal violet for 30 min, washed two times with  $\text{H}_2\text{O}$ , and solubilized in ethanol for 10 min prior to measurement of the optical density at 600 nm. For CLSM and SEM, *M. catarrhalis* biofilms were established in sBHI medium or sBHI medium supplemented with 0.2  $\mu\text{M}$  DPD for 24 h. Biofilms were then prepared for SEM 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.

**Chinchilla infection studies.** Bacterial persistence and biofilm formation in the middle ear chamber were assessed as described previously (12, 13). Chinchillas were purchased from Rauscher's Chinchilla Ranch (LaRue, 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 transbullar injection with  $\sim 10^3$  CFU of *H. influenzae* or *H. influenzae luxS*,  $\sim 10^4$  CFU of *M. catarrhalis*, or a 1:1 mixture of both species. All inocula were confirmed by plate 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 lavage was performed using 1.0 ml sterile PBS. Viable bacteria were enumerated by plate counting the combined retrieved fluids. Fluid samples obtained from animals which received polymicrobial inocula were plated onto both sBHI medium containing 2  $\mu\text{g}/\text{ml}$  clarithromycin and BHI medium lacking NAD and hemin. Bullae were excised and homogenized in 10 ml sterile PBS and then plated to determine the number of CFU of tissue-associated bacteria (12).

**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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