



Streptococcus pneumoniae Modulates *Staphylococcus aureus* Biofilm Dispersion and the Transition from Colonization to Invasive Disease

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ABSTRACT *Streptococcus pneumoniae* and *Staphylococcus aureus* are ubiquitous upper respiratory opportunistic pathogens. Individually, these Gram-positive microbes are two of the most common causative agents of secondary bacterial pneumonia following influenza A virus infection, and they constitute a significant source of morbidity and mortality. Since the introduction of the pneumococcal conjugate vaccine, rates of cocolonization with both of these bacterial species have increased, despite the traditional view that they are antagonistic and mutually exclusive. The interactions between *S. pneumoniae* and *S. aureus* in the context of colonization and the transition to invasive disease have not been characterized. In this report, we show that *S. pneumoniae* and *S. aureus* form stable dual-species biofilms on epithelial cells *in vitro*. When these biofilms are exposed to physiological changes associated with viral infection, *S. pneumoniae* disperses from the biofilm, whereas *S. aureus* dispersal is inhibited. These findings were supported by results of an *in vivo* study in which we used a novel mouse cocolonization model. In these experiments, mice cocolonized in the nares with both bacterial species were subsequently infected with influenza A virus. The coinfecting mice almost exclusively developed pneumococcal pneumonia. These results indicate that despite our previous report that *S. aureus* disseminates into the lungs of mice stably colonized with these bacteria following influenza A virus infection, cocolonization with *S. pneumoniae* *in vitro* and *in vivo* inhibits *S. aureus* dispersal and transition to disease. This study provides novel insight into both the interactions between *S. pneumoniae* and *S. aureus* during carriage and the transition from colonization to secondary bacterial pneumonia.

IMPORTANCE In this study, we demonstrate that *Streptococcus pneumoniae* can modulate the pathogenic potential of *Staphylococcus aureus* in a model of secondary bacterial pneumonia. We report that host physiological signals related to viral infection cease to elicit a dispersal response from *S. aureus* while in a dual-species setting with *S. pneumoniae*, in direct contrast to results of previous studies with each species individually. This study underscores the importance of studying polymicrobial communities and their implications in disease states.

KEYWORDS Polymicrobial infections, secondary bacterial pneumonia, *Staphylococcus aureus*, *Streptococcus pneumoniae*

Streptococcus pneumoniae (the pneumococcus), a Gram-positive diplococcus, is one of the most common opportunistic pathogens in the upper respiratory tract. By the age of 2 years, nearly 95% of children are colonized in the nasopharynx, in the form of a bacterial biofilm, by one of the greater than 90 serotypes of *S. pneumoniae*. Biofilm formation is a crucial step in pathogenesis, as biofilms promote bacterial persistence, competence, immune evasion, and resistance to antibiotics, all while serving as reser-

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voirs for local and invasive disease (1–7). The pneumococcus, which predominantly causes upper respiratory tract infections, is the primary etiologic agent of both otitis media and secondary bacterial pneumonia (5, 8). Pneumococcal pneumonia following influenza A virus (IAV) infection poses a serious health threat to both young and elderly people. Recent studies have shown that host physiological changes in response to IAV infection, such as increased temperature, as well as the release of norepinephrine, glucose, and ATP, cause the pneumococcus to disperse from asymptotically colonizing biofilms (9, 10). The bacteria, once dispersed from the biofilm in response to these stimuli, cause invasive disease, as they express an altered transcriptome and hypervirulent phenotype compared to the biofilm-associated bacteria (11–13).

Another common upper respiratory tract opportunistic pathogen is *Staphylococcus aureus*. *S. aureus* colonizes the anterior nares and nasopharynx of 30 to 80% of individuals, often in biofilms, which serve as a reservoir for local and invasive disease (14–17). In addition to many other diseases, *S. aureus* is a common cause of secondary bacterial pneumonia following IAV infection during seasonal influenza outbreaks and especially during pandemics (14, 18–25). Secondary staphylococcal pneumonia is severe, resulting in extensive damage to the respiratory tract, including necrosis, as well as dissemination into the bloodstream; it has been linked to mortality in previously healthy children with no underlying complications (21, 25–28). Recent studies from our laboratory have demonstrated that physiological changes linked to IAV infection induce *S. aureus* to disperse from biofilms *in vitro* and to disperse and disseminate from asymptotically colonized murine nasal tissues into the lungs, where they cause pronounced staphylococcal secondary pneumonia (29).

The human nasopharynx is a very diverse physiological niche, and the microbiome has become much more complex since the introduction of the pneumococcal conjugate vaccine (PCV) (15, 30–32). While this vaccine has been very effective against relevant invasive pneumococcal serotypes, non-PCV serotype replacement has occurred and may be a contributing factor for the rise of *S. aureus* colonization of the nasopharynx, otitis media, and staphylococcal secondary pneumonia (22, 23, 25, 33, 34). It is possible that the change in colonizing pneumococcal serotypes in the nasopharynx has altered the competitive environment of this niche, facilitating the emergence of *S. aureus*. Previous *in vitro* studies have suggested that these species are antagonistic, whereby the pneumococcus uses hydrogen peroxide production to kill *S. aureus* (35). In contrast, subsequent models of cocolonization with both species have shown that this antagonistic relationship does not occur in animal models (36, 37). Moreover, this observation has been supported by recent epidemiological studies reporting an increased cocolonization rate of the human nasopharynx, as both species were detected in 10 to 24% of patients sampled (38–40).

The complex nature of the interaction between *S. aureus* and *S. pneumoniae* in the nasopharynx and the clinical increase in cocolonization warrants further analyses. No studies to date have examined the dynamics of dual-species cocolonization or the transition from colonization to secondary bacterial pneumonia. Therefore, we have developed an *in vitro* and *in vivo* system to investigate the effects on dual-species biofilms of host physiological changes related to IAV infection. Our results suggest that while stable dual-species biofilms can be formed and stable cocolonization of the nasal tissue can be established, the presence of *S. pneumoniae* inhibits staphylococcal dispersal and dissemination to invasive disease while remaining unaffected itself.

RESULTS

***S. aureus* and *S. pneumoniae* form robust dual-species biofilms *in vitro* on fixed H292 cells.** Previous studies have shown that both *S. aureus* strain UAMS-1 and *S. pneumoniae* strain EF3030 form robust single-species biofilms in our *in vitro* model system, and these closely mimic *in vivo* biofilms (29, 41). Given the rise in cocolonization rates with these species since introduction of the PCV, we performed studies to determine if these two species form biofilms on fixed human H292 cells *in vitro*. The data in Fig. 1 indicate that, following coculture using this *in vitro* system, high levels of

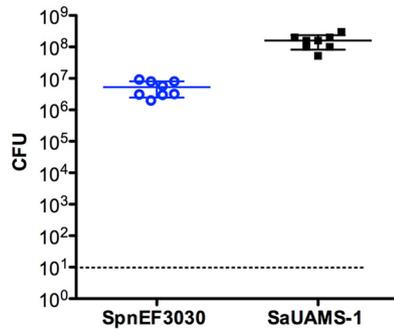


FIG 1 *S. pneumoniae* EF3030 and *S. aureus* UAMS-1 form dual-species biofilms *in vitro*. Biofilm-associated EF3030 and UAMS-1 were enumerated from cocultured biofilms after 48 h ($n = 8$). Both species of bacteria were recovered from each well. The dashed line indicates the limit of detection. Solid bars are means \pm standard deviations.

both UAMS-1 and EF3030 were recovered from harvested biofilms (approximately 10^8 and 10^7 CFU per well, respectively). These quantitative data suggest that in a dual-species model, *S. aureus* UAMS-1 and *S. pneumoniae* EF3030 form stable biofilms for 48 h under conditions that more closely mimic the microenvironment *in vivo*. A representative dual-species biofilm was visualized via confocal laser scanning microscopy (CLSM). In Fig. 2, both bacterial strains are shown colocalized in the mixed-species biofilm, with towers of UAMS-1 visible over a mat-like distribution of EF3030.

The *S. aureus* response to host physiological changes induced by viral infection is modulated in the presence of *S. pneumoniae in vitro*. As mentioned above, IAV infection induces a wide range of host physiological responses. We have previously shown that both *S. pneumoniae* and *S. aureus* in single-species studies disperse from the biofilm in response to physiological changes in the host that are associated with IAV infection *in vitro* and disseminate to the lungs *in vivo* (9, 29). In these previous studies, the greatest effect by a single factor for both organisms was seen upon exposure to

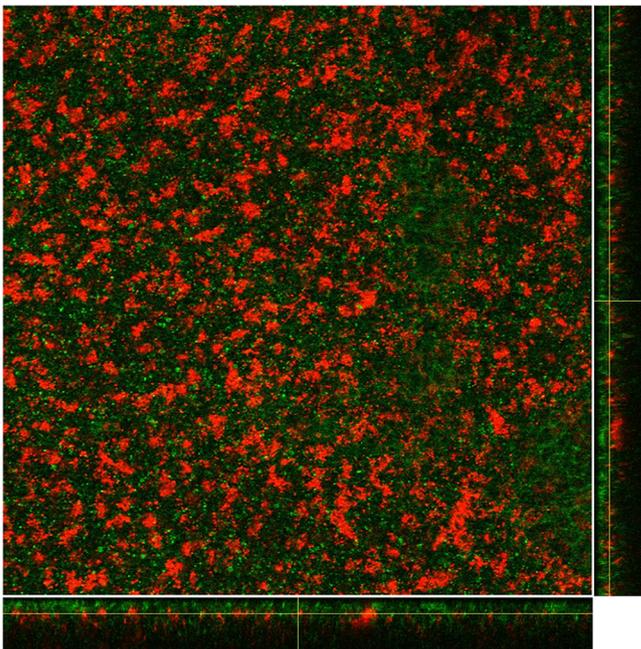


FIG 2 CLSM images of dual-species biofilms formed by *S. pneumoniae* EF3030-GFP (green) and *S. aureus* UAMS1-dsRED (red) following 48 h of coculture. The image shows the average intensity projections through the confocal image stack, with the maximum-intensity x - z and y - z projections shown along the bottom and side of the image; a representative image of a single slice from the central region of the biofilm is shown. Images were produced using ImageJ.

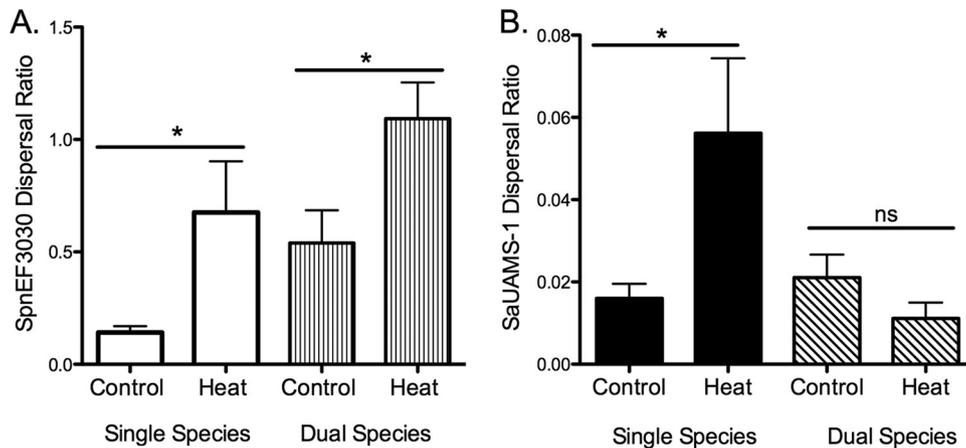


FIG 3 *In vitro* dispersal of 48 h *S. pneumoniae* EF3030 (A) and *S. aureus* UAMS-1 (B) single-species and dual-species biofilms following 4 h of heat treatment (38.5°C). Dispersal values are presented as the ratio of supernatant to biofilm from a minimum of four independent experiments (means \pm standard errors of the means). Statistical analysis of the effect heat dispersal compared to controls was performed with the unpaired Student *t* test. *, $P < 0.05$.

febrile-range temperatures. We repeated this experiment to determine if this same physiological change induced dispersal in a dual-species biofilm. Figure 3A shows that *S. pneumoniae* EF3030 responded to heat stimuli by dispersing from the biofilm, as previously reported (9). However, in contrast to our recent data (29), *S. aureus* UAMS-1 did not disperse from the dual-species biofilm in response to this stimulus, as shown in Fig. 3B. Moreover, staphylococcal dispersal inhibition was also observed when *S. pneumoniae* EF3030 was cocultured with *S. aureus* NRS123, and also coculture of *S. aureus* NRS123 in the presence of *S. pneumoniae* D39 (data not shown). These data demonstrate stable dual-species biofilm formation and dispersal inhibition are not strain specific. Taken together, these *in vitro* studies suggest that pneumococci may modulate the response of staphylococci to physiological changes associated with IAV infection.

***Streptococcus mitis* does not inhibit *S. aureus* dispersal.** We next determined if *S. aureus* dispersal could be inhibited by another streptococcal species. *S. mitis* was selected for these experiments, as this organism is a closely genetically related oropharyngeal commensal that shares physiological and molecular traits with *S. pneumoniae*, in addition to colonizing the same human niche. We grew dual-species biofilms of *S. aureus* UAMS-1 and *S. mitis* strain NS51, an ATCC type strain shown in previous studies to form biofilms (42). Figure 4 shows that *S. aureus* UAMS-1 dispersal was unaffected by the presence of *S. mitis*, suggesting that the previously noted inhibition may be pneumococcus specific.

***S. aureus* and *S. pneumoniae* asymptotically cocolonize the murine nasopharynx.** A majority of murine secondary bacterial pneumonia studies consist of an aspiration of IAV followed 3 to 7 days later with a second aspiration of a high titer of bacteria. As the bacteria are introduced directly into the lungs, these experimental *in vivo* systems bypass the initial colonization of the nasal tissues, which is likely important for infection. Based on our *in vitro* biofilm results, we performed studies using our recently described murine nasal colonization model to determine if *S. aureus* UAMS-1 and *S. pneumoniae* EF3030 form dual-species biofilms *in vivo*. The data in Fig. 5 show that nasal tissues harvested from the inoculated mice were cocolonized with both UAMS-1 and EF3030, suggesting that these two species can concurrently and stably colonize the murine nasopharynx for 48 h. Moreover, it is important to note that bacteria remained in the nasopharynx in the majority of these animals, and the few mice from which bacteria were recovered from the lungs did not exhibit any detectable symptoms of pneumonia.

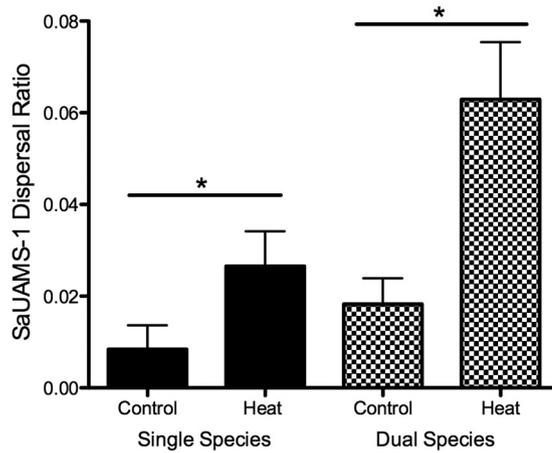


FIG 4 *In vitro* dispersal of *S. aureus* UAMS-1 from 48-h single-species biofilms compared to dual-species biofilms with *S. mitis* N555 following 4 h of heat treatment (38.5°C). Dispersal values are presented as the ratio of supernatant to biofilm from a minimum of four independent experiments (mean result ± standard deviation). Statistical analysis on heat dispersal versus that in controls was performed with the unpaired Student *t* test. *, *P* < 0.05.

IAV coinfection in asymptotically cocolonized mice primarily leads to secondary pneumococcal pneumonia. Based on the *in vitro* dispersal data, we performed the following studies to determine if *S. pneumoniae* also modulated *S. aureus* dispersal *in vivo*. Mice were cocolonized with *S. aureus* UAMS-1 and *S. pneumoniae* EF3030 for 48 h and then infected with IAV strain PR8 via aspiration. At 48 h post-virus infection, animals were sacrificed and harvested nasal and lung tissues were evaluated for bacterial burden by CFU counts. Figure 6 shows that 96 h after initial bacterial inoculations, comparable bacterial burdens of *S. aureus* and *S. pneumoniae* were detected in murine nasal tissues for both the control cohort and the cohort that received both bacteria and virus. Only mice that received both virus and bacteria, however, developed pronounced secondary bacterial pneumonia, displaying piloerection, lethargy, and increased body temperature. Moreover, these data indicate that the majority of these mice (62.5%) developed secondary pneumococcal pneumonia, while only two mice had staphylococci in the lungs (12.5%). In addition, none of the animals had both bacterial species recovered from the lungs; in all cases, the pneumonia was

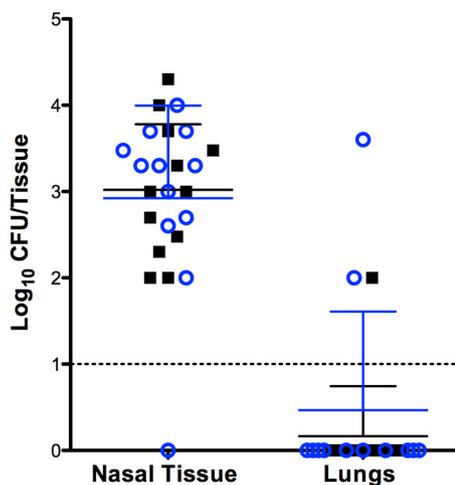


FIG 5 Dual-species colonization of the murine nasal mucosa. Samples were harvested 48 h after colonization; each point presents the CFU recovered from individual mice (*n* = 12). Blue circles represent *S. pneumoniae* EF3030, and the black squares represent *S. aureus* UAMS-1. The dashed line indicates the limit of detection. Bars indicate standard deviations.

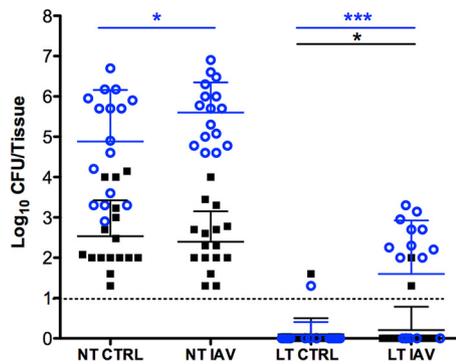


FIG 6 Dissemination following IAV coinfection. Forty-eight hours post-bacterial colonization, IAV cohorts received intranasal virus inoculations while control cohorts (CTRL) received only saline, and the infection was allowed to progress an additional 48 h prior to harvest. Each point represents the CFU recovered from nasal tissues (NT) and lung tissues (LT) harvested from individual mice ($n = 16$) 96 h after cocolonization with *S. pneumoniae* EF3030 (blue circles) and *S. aureus* UAMS-1 (black squares). The dashed line indicates the limit of CFU detection. Solid bars indicate the standard deviations. Statistical analysis was performed with the nonparametric Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$.

monomicrobial. These results suggest the effect on dispersal that *S. pneumoniae* exerts on *S. aureus in vitro* may also be occurring *in vivo*, thereby inhibiting *S. aureus* from disseminating to the lungs when cocolonizing pneumococci are present in the upper respiratory tract.

DISCUSSION

While IAV causes significant amounts of tissue damage and inflammation, the development of secondary bacterial pneumonia is one of the most serious complications associated with infection. It is a primary cause of both morbidity and mortality during seasonal influenza epidemics and during influenza pandemics (22, 23, 43–45). The two main etiologic agents of secondary bacterial pneumonia are the upper respiratory pathogens *S. pneumoniae* and *S. aureus*. It appears that biofilms colonizing the upper respiratory tract are the most likely reservoir for invasive disease caused by both of these species. Interestingly, epidemiological studies have shown an increase in the incidence of cocolonization in the nasopharynx by both *S. pneumoniae* and *S. aureus* even though these species are traditionally viewed as mutually exclusive and antagonistic species (35, 38–40).

Despite this increase in cocolonization of the nasopharynx, there are essentially no case reports of dual-species secondary bacterial pneumonia. This suggests that although *S. pneumoniae* and *S. aureus* can coexist on the mucosal surface of the upper respiratory tract and both species are able to cause secondary bacterial pneumonia, the virulence capacity of *S. aureus* appears to be diminished in the presence of the pneumococcus. Despite this intriguing observation, few studies have been performed to investigate these complex bacterial interactions in the context of secondary bacterial pneumonia. Furthermore, little is known about the events involved in the transition from colonization to invasive disease or about the effects of a polymicrobial environment on this transition.

We began to address this gap in knowledge by investigating the transition of *S. pneumoniae* and *S. aureus* from colonization to invasive disease following IAV infection in a dual-species model. We previously showed that, individually, both bacterial species will respond to physiological changes induced by IAV infection by dispersing from the biofilm and disseminating from the nasal tissue to the lungs (9, 29). Using a novel *in vitro* dual-species biofilm model, we were able to demonstrate that *S. pneumoniae* and *S. aureus* form robust dual-species biofilms on prefixed upper respiratory epithelial cells that remain stable for up to 48 h. Biofilm formation at 34°C with the addition of an epithelial cell substratum more closely mimics the ecologic niche of the upper respiratory tract (41). These findings suggest that the previously

reported antagonistic relationship between these species may be the result of various environmental factors, including growth conditions, which could affect the ability of these two bacterial species to coexist.

Using this physiologically relevant model, we were able to investigate the effects of host changes in response to IAV infection on dual-species biofilms. Our results demonstrated that in a dual-species biofilm, *S. pneumoniae* responds to these stimuli by dispersing from the biofilm, consistent with previous reports using single-species biofilms (9). However, *S. aureus* did not disperse from the dual-species biofilm, which is in direct contrast to our recent report showing that *S. aureus* disperses from a single-species biofilm in response to the same stimuli (29). Importantly, *S. aureus* dispersal was not inhibited in the presence of *S. mitis*, an oropharyngeal colonizer that is closely related to *S. pneumoniae*; these findings support the specificity of the observed inhibition. To our knowledge, these are the first results suggesting that *S. pneumoniae* modulates the pathogenesis of *S. aureus*, although the mechanism responsible for this observation remains currently undefined and warrants additional studies.

Our *in vitro* dual-species biofilm results were confirmed *in vivo*. Using an intranasal colonization model, we demonstrated that mice could be simultaneously colonized with both *S. pneumoniae* and *S. aureus* for up to 96 h. These results extend two previous studies reporting dual colonization in a neonatal mouse and rat nasal model for 72 h (36, 37). We investigated the effect of concomitant IAV infection on cocolonized mice. Previously published models of secondary bacterial pneumonia have overlooked the natural route of infection, bypassing the transition from colonization to invasive disease. Our novel secondary pneumonia model allows us to investigate this critical step in pathogenesis, and our *in vivo* coinfection experiments confirmed the *in vitro* dispersal results. When cocolonized mice receive IAV, the majority of these mice develop pneumococcal pneumonia within 48 h of viral infection. The finding that few of these mice developed staphylococcal pneumonia suggested that the inhibition of biofilm dispersal that we observed *in vitro* is a phenomenon that also occurs *in vivo*. Furthermore, these data show that the nasal tissues of the mice remain stably colonized with both species for up to 96 h. These are the first studies to effectively demonstrate that colonization with these two species of bacteria alters the pathogenesis of one species in a host environment. It is important to note that in all mice that developed pneumonia, the bacteria harvested from the infected lung tissue were exclusively pneumococci or staphylococci, but never both. The disseminated lung infections were strictly monomicrobial, even though the nasal tissues indicated stable cocolonization by both species. The finding that none of the cocolonized IAV-infected mice developed a dual-species bacterial pneumonia is consistent with most clinical case reports of secondary bacterial pneumonia (46).

There are numerous potential explanations for these results. A recent report showed that the transcriptional profile of *S. pneumoniae* shifts in response to dispersal from a stable biofilm, with an upregulation of pneumococcal virulence factors and bacteriocins (11). It is possible that this increase in bacteriocin production by dispersing streptococci leads to the killing of *S. aureus* as it also disperses from the biofilm, a common method used to eliminate competition (47–50). Another explanation is that *S. pneumoniae* biofilm structural components trap *S. aureus* in the extracellular matrix, preventing dispersal. Alterations in the regulation of known *S. aureus* biofilm dispersal mechanisms, including exoenzymes and surfactants, such as proteases, nucleases, and phenol-soluble modulins, via cocolonization with *S. pneumoniae* could negatively affect *S. aureus* dispersal from established biofilms (recently reviewed in references 51 and 52). Alternatively, the presence of the pneumococcus may induce *S. aureus* to increase expression of adhesins and/or other biofilm-associated factors, thus promoting a more cohesive structure that stunts dispersal. These possibilities are merely speculative, and detailed mechanistic and transcriptomic studies designed to delineate these complex bacterial interactions are warranted prior to making any definitive conclusions.

This study is the first to demonstrate that the presence of *S. pneumoniae* alters the pathogenesis of *S. aureus*. These findings may provide insight into the rise of *S. aureus* secondary bacterial pneumonia since the introduction of the PCV. While global use of PCV has greatly reduced carriage of vaccine serotype pneumococci and invasive disease caused by these strains, colonization by non-PCV serotype replacement strains continues to occur, and broad PCV implementation has clearly changed the microbiome of the nasopharynx overall. More studies are needed to better understand the complex interactions that exist between various bacterial species that share common microenvironments and the contributions of interspecies competition to the dynamics of the microbiome of a given ecologic niche. Our data provide the foundation for future experiments designed to delineate the mechanisms used by *S. pneumoniae* and *S. aureus* during cocolonization of the human upper respiratory tract and define their contributions to the subsequent development of invasive disease.

MATERIALS AND METHODS

Reagents. Bacterial and cell culture materials were from VWR (Radnor, PA) and Invitrogen (Carlsbad, CA). Chemically defined pneumococcal growth medium (CDM) was formulated as previously described (53). Sheep blood was purchased from Biolink, Liverpool, NY.

Eukaryotic cell lines and bacterial and viral strains. NCI-H292 bronchial epithelial cells were grown and prepared as previously described (54). This study used *Streptococcus pneumoniae* strains EF3030, a serotype 19F otitis media isolate (55), and D39, a classical serotype 2 Avery strain (56). *Staphylococcus aureus* strain UAMS-1 (ATCC 49230) (57) was a generous gift from Steve Gill (University of Rochester, Rochester, NY), and methicillin-resistant strain NRS123 was from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (58). *Streptococcus mitis* strain NS51 was obtained from ATCC (ATCC 49456) (42). Chromosomally encoded fluorescent variants for confocal microscopy were generated in *S. pneumoniae* EF3030 by natural transformation with the *hlpA-gfp_Camr* construct from strain JW500, as described elsewhere (59) (strain JW500 was a generous gift from Jan-Willem Veening, University of Lausanne, Lausanne, Switzerland), creating strain EF3030-GFP. UAMS-1-dsRED, was constructed via chromosomal integration using pRFP-F, as described previously (60), and was a generous gift from Steve Gill (University of Rochester, Rochester, NY). Bacteria were grown in CDM at 37°C unless otherwise noted. Viral stocks of influenza A virus strain A/PR/8/34 were a mouse-adapted H1N1 strain (with titers determined), generously provided by Paul Knight (University at Buffalo, Buffalo, NY) and used for the viral coinfection studies (61).

Static biofilms on fixed epithelial substratum. Static pneumococcal biofilms were grown as previously described (41). *S. aureus* biofilms were grown using the same model with slight modifications. Briefly, *S. aureus* was grown in CDM at 37°C to mid-log phase. Bacteria were diluted to a final inoculum of 2×10^7 CFU in a 500- μ l volume of CDM and seeded onto a fixed H292 cell substratum with 500 μ l of additional CDM. Biofilms were placed at 34°C for growth, with medium changed every 12 h for 48 h. Dual-species biofilms were grown using the same model with additional modifications. In brief, streptococci were grown in CDM to mid-log phase, diluted in 500 μ l of fresh medium to a final inoculum of 5×10^6 CFU, and seeded onto fixed H292 epithelial cells in an additional 500 μ l of fresh medium. Biofilms were placed at 34°C for 24 h. Five hundred microliters of CDM was removed, and 2×10^7 CFU of *S. aureus* grown as mentioned above were added gently to the preformed streptococcal biofilms and then placed at 34°C for an additional 24 h. A minimum of three independent assays with four replicates each were performed.

Biofilm dispersal. Static monomicrobial or dual-species biofilms were prepared as outlined above. Biofilms were dispersed as previously described (9). Briefly, biofilms were shifted to a febrile-range temperature of 38.5°C for 4 h. Supernatants from each biofilm were removed, sonicated, and plated onto blood agar plates for bacterial quantification. Remaining biofilm bacteria were suspended in phosphate-buffered saline, sonicated, and plated onto blood agar plates for bacterial quantification. Values reported are the calculated ratios of supernatant to biofilm CFU, a measure of bacterial dispersal, as previously reported (29).

Confocal microscopy. In order to identify and localize individual bacteria in the dual-species biofilms, cocolonized biofilms were prepared on a fixed epithelial substratum as described above, except we used glass-bottom 24-well plates (MatTek Corp., Ashland, MA) and chromosomally encoded fluorescent constructs (EF3030-GFP [green] and UAMS-1-dsRED [red]). Image stacks were acquired by CLSM at 630 \times magnification on a Zeiss Axiovert 200 M inverted microscope with an attached Zeiss LSM 510 Meta NLO imaging system. Images were produced from the raw LSM files by using the freely available ImageJ software analysis program.

Mouse nasopharyngeal colonization and influenza virus infection model. Nasopharyngeal single- and dual-species colonization experiments were performed as previously described, with limited modifications (62). Briefly, nonanesthetized 5-week-old BALB/cByJ mice were intranasally inoculated with either 2×10^8 CFU of *S. aureus* UAMS-1 or 5×10^6 CFU of *S. pneumoniae* EF3030 in a 20- μ l volume by pipetting the inoculum into the nares. For dual-species colonization experiments, nonanesthetized 5-week-old BALB/cByJ mice were intranasally inoculated with both UAMS-1 and EF3030 in a 20- μ l

volume as described above with 30 min in between inoculations. For influenza virus coinfection experiments, mice colonized for a period of 48 h were anesthetized with isoflurane and 100 PFU of IAV in 20 μ l was pipetted into the nares. At 48 h post-viral inoculation (96 h post-bacterial colonization), nasopharyngeal tissue and lungs were collected, homogenized, and plated onto blood agar plates to assess bacterial burdens.

Statistical analyses. Statistical significance was determined using the Mann-Whitney U test or Student's *t* test, as indicated, and Prism 5 from GraphPad Software, Inc. (La Jolla, CA). A *P* value of <0.05 was considered significant.

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