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Influence of bacterial interactions on pneumococcal colonization of the nasopharynx

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Abstract

Streptococcus pneumoniae (the pneumococcus) is a common commensal inhabitant of the nasopharynx and a frequent etiologic agent in serious diseases such as pneumonia, otitis media, bacteremia, and meningitis. Multiple pneumococcal strains can colonize the nasopharynx, which is also home to many other bacterial species. Intraspecies and interspecies interactions influence pneumococcal carriage in important ways. Co-colonization by two or more pneumococcal strains has implications for vaccine serotype replacement, carriage detection, and pneumonia diagnostics. Interactions between the pneumococcus and other bacterial species alter carriage prevalence, modulate virulence, and affect biofilm formation. By examining these interactions, this review highlights how the bacterial ecosystem of the nasopharynx changes the nature and course of pneumococcal carriage.

Keywords

Streptococcus pneumoniae; the pneumococcus; co-colonization; carriage; nasopharynx; interaction

***Streptococcus pneumoniae*: a commensal and a pathogen**

The Gram-positive bacterium *Streptococcus pneumoniae* (the pneumococcus) is a common asymptomatic resident of the human nasopharynx [1] and is also a primary etiologic agent in pneumonia, otitis media, meningitis, and sepsis. Pneumococcal pneumonia is the leading cause of childhood death in developing countries [2] and imposes a significant burden of morbidity, mortality, and healthcare costs in developed countries such as the USA [3]. Colonization of the nasopharynx is a necessary – but not sufficient – step along the path to pneumococcal disease [4,5]. On entering the nasopharynx, and during its residence there, the pneumococcus shares this anatomical and physiological niche with an array of other viral and bacterial inhabitants. Although there have been recent reviews of host–pneumococcal [6,7] and viral–pneumococcal interactions [8], there has been less written on the direct bacterial–bacterial interactions that influence pneumococcal carriage. This review highlights recent advances in our understanding of the bacterial interactions that alter the nature of pneumococcal carriage and its potential to progress to pneumococcal disease.

The nasopharynx lies at a vital crossroads connecting the nose, ears, mouth, and lower respiratory tract (Figure 1). Directly posterior to the nasal cavity and superior to the oropharynx, the nasopharynx is also close to the paranasal sinuses and directly connected to the middle ear by the Eustachian tubes. A dynamic environment with a constant flow of air, blood, and mucus, the nasopharynx modulates incoming air quality and temperature for the host, while also providing a home to millions of microbes. Colonization by the pneumococcus may occur at any time during a person's life, but is most common during infancy, with acquisition even documented in the first day of life [9]. The prevalence of pneumococcal carriage increases in the first few years of life, peaking at approximately 50% to >70% in hosts 2–3 years of age [4,10] and decreasing thereafter until stabilizing at 5–10% in hosts over 10 years of age in developed countries [4,11,12] but as high as 25–60% in some developing countries [13,14]. The median duration of carriage is estimated to be 31 days in adults and 60.5 days in children, with length of carriage dependent on capsular serotype and previous immunologic exposure, as well as the host's age and immunocompetence [15,16]. However, a recent study using intensive sampling of African children found a shorter duration of pediatric carriage: an overall mean of 31.3 days, with serotype-specific means ranging from 6.7 to 50 days [17]. Similar studies in adults may also reveal that the duration of carriage is shorter than previously deduced from studies using monthly assessments.

Once carriage is established in the nasopharynx, the pneumococcus can migrate through the Eustachian tubes to cause otitis media [18], descend the respiratory tract to cause pneumonia [19], or invade the bloodstream through the respiratory epithelium to cause bacteremia or meningitis (Figure 1) [20]. The success of the pneumococcus as both a commensal and a pathogen can be attributed to a repertoire of cellular components that are useful during both carriage and disease [21]. Ultimately, regulation of these dual-use cellular components and changing conditions in the nasopharynx mediate if and when the pneumococcus progresses from a commensal resident to a pathogen. In this review, we argue that the abilities of the pneumococcus to persist and harm are influenced by within-species and cross-species population dynamics.

Effects of co-colonization on serotype distribution, genetic exchange, and density

Pneumococcal strains interact directly when they co-colonize a single host, an occurrence more prevalent than previously believed. Co-colonization is clinically and epidemiologically important because pneumococcal strains interfere with or aid in subsequent colonization by additional strains [22–24], and the pneumococcus is highly prone to homologous recombination and transformation of exogenous DNA [24–28]. In fact, it has recently been demonstrated that strains with poor colonization efficiency are better at colonizing a mouse model when a second strain with high colonization efficiency is already present [24]. Furthermore, this phenomenon appears to occur without genetic exchange, possibly through biofilm formation [24].

Recent work has begun to explore specific molecular mechanisms of interaction, including bacteriocin production and competence-induced fratricide in inter-strain dynamics. The *blp* locus in the pneumococcal genome encodes an antimicrobial bacteriocin peptide and corresponding immunity peptide. A strain harboring the *blp* locus has a competitive advantage in a mouse colonization model [22], and the importance of this locus in the wild is supported by a comparative genomic study that demonstrated extensive variation at the *blp* locus, indicative of diversifying selection [23]. Autolysis is another important mediator of inter-strain dynamics; long recognized for its ability to rapidly release the toxin pneumolysin, autolysis may actually be a form of competence-induced fratricide regulated

by the Com quorum-sensing (QS) system [29]. In addition to upregulating factors necessary for uptake and transformation of DNA, the Com system activates the two-peptide bacteriocin CibAB and its immunity factor CibC; these bacteriocins target non-competent pneumococci that do not produce CibC, causing LytA-, LytC-, and CbpD-mediated lysis and DNA release from sister cells [30,31]. The role of bacteriocins and competence-induced fratricide *in vivo* is not yet clear, but it has become increasingly important to understand the mechanisms of strain interaction because it is now apparent that co-colonization is far more common than previously believed.

The original study of co-colonization by Gundel and Okura used a technique involving serial intraperitoneal saliva injections into mice to detect multiple serotypes [32]. The cost of this animal work led to a standard assay with streaking out of enriched nasopharyngeal specimens and serotyping of a handful of colonies. However, this approach yielded low rates of co-colonization; only 1.3–1.9% of samples from pediatric populations in South Africa were found to have more than one serotype using this laborious and costly method [33]. Testing of enriched cultures directly with the capsular reaction test using pooled and type-specific serum proved more sensitive, detecting co-colonization in 9.9% of pediatric Danish carriers [34]. However, a more cost-effective approach using PCR amplification of a noncoding region adjacent to the *ply* gene, followed by restriction fragment length polymorphism (RFLP) analysis yielded similar rates of co-colonization, 7.9% and 9.5% of pediatric carriers in two studies [35,36]. More recently, studies have demonstrated the utility of sweep techniques in which nasopharyngeal specimens are plated onto blood agar plates and incubated before all growth is swept up and assayed by multiplex PCR or latex agglutination [37]. A study of indigenous children in Venezuela using the sweep approach followed by multiplex PCR revealed a co-colonization rate of 20% [38]. In a careful comparison of the sweep technique and a microarray-based approach against traditional WHO culture methods, sweep–agglutination and microarray resulted in co-colonization rates of 43.2% and 48.8%, respectively, compared to 11.2% by the WHO-recommended culture [39]. Clearly, multiplex PCR, sweep, and microarray techniques demonstrate that co-colonization is more common than previously believed, and our ability to accurately detect co-colonization will only improve with the refinement of new techniques such as metagenomic sequencing and sequence-based serotype identification [40].

New studies are beginning to examine the theoretical and practical aspects of co-colonization on serotype-specific interactions and the implications for pneumococcal vaccine development and deployment. Current formulations of the pneumococcal conjugate vaccine (PCV) protect against 7, 10, or 13 capsular vaccine types (VTs), but have no direct effect on the more than 80 non-vaccine types (NVTs). Introduction of PCV unmask the presence of NVTs that may have been at lower density or under less scrutiny during pre-PCV epidemiological studies [41]. In addition, NVTs are likely to gain a competitive advantage, both within hosts and within populations, when strains capable of competition are removed by vaccination [42,43]. PCV introduction has also precipitated a shift towards more NVT-caused invasive pneumococcal disease (IPD) [43]. In general, even with the increase in NVT-caused IPD, the rates of pneumococcal disease are still far below the pre-PCV era [44]. However, in certain populations, namely in Native Alaskan children and adults, IPD has actually increased [45], and in the USA, NVT 19A emerged prior to the introduction of PCV13 as a more virulent and drug-resistant cause of IPD [46,47]. This increase in IPD due to serotype 19A is likely to be because of its increased carriage prevalence in a population immunized with PCV7.

Co-colonization is also important because the pneumococcus has a significant propensity for genetic exchange through transformation and homologous recombination. A longitudinal study of chronic otitis media demonstrated extensive genomic rearrangements *in vivo* [26],

and animal models of pneumococcal disease have demonstrated that even minor genetic differences can significantly alter virulence [23]. A multi-locus sequence typing (MLST) study of pediatric strains in Gambia found extensive recombination and serotype switching among carriage strains; the authors suggested this finding may be due to high rates of co-colonization in this population [48]. Furthermore, a study of 240 full genome sequences from the PMEN1 lineage revealed more than 700 recombination events and 10 capsule-switching events [28], suggesting extensive diversity within a relatively contemporary highly related clone and thus a high frequency of recombination events. More studies using whole-genome sequencing of isolates obtained sequentially from carriers are needed to pinpoint the frequency of these recombination and capsule-switching events, especially in relation to the changing flora of the nasopharynx post-vaccination.

It has recently been suggested that co-colonization with multiple strains of *S. pneumoniae* increases the density of nasopharyngeal carriage [36]. Brugger and colleagues found that subjects co-colonized with two or more strains had higher carriage density than subjects colonized with one strain [36]. Lower density carriage is less likely to be detected by culture methods than by real-time quantitative PCR (qPCR) [49,50], so the findings of previous, culture-based studies may be distorted by the relative frequency or rarity of co-colonization. Nasopharyngeal pneumococcal density is also showing promise as a diagnostic tool for pneumonia [51,52]. If co-colonization is correlated with nasopharyngeal pneumococcal density, then this factor will need to be controlled for when exploring density-based pneumonia diagnostics.

Interactions between nasopharyngeal bacterial species

The nasopharyngeal microbial community is established in the first year after birth and varies throughout a person's lifetime [53]. The five most common bacterial families in the nasopharynx are Moraxellaceae, Streptococcaceae, Corynebacteriaceae, Pasteurellaceae (including the genus *Haemophilus*), and Staphylococcaceae (Table 1) [10,54–56]. The first microbiome studies to analyze 16S rRNA sequences have revealed high inter-individual variability in nasopharyngeal communities, as well as possible correlations with season [55,56]. In addition to bacteria, viruses and the host immune system are also major determinants of nasopharyngeal dynamics. The synergy between viral co-infections and bacterial pneumonia has been an ongoing area of research [8,57] and a recent review by Lijek and Weiser examines the evidence that mucosal immunity mediates interactions between *S. pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* [7]. Here we consider how direct bacterial–bacterial interactions may impact clinically important nasopharyngeal dynamics.

With the widespread deployment of PCV and a seemingly concurrent rise in community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) infections, there has been speculation as to whether these two trends may be related [54,58,59]. Epidemiological studies have indeed revealed an inverse correlation between carriage of *S. pneumoniae* and carriage of *S. aureus* [50,54,60,61]. The presence of the pneumococcus in the nasopharynx appears to inhibit *S. aureus* colonization. *In vitro* studies demonstrated that hydrogen peroxide (H₂O₂) produced by the enzyme pyruvate oxidase (SpxB) of *S. pneumoniae* kills *S. aureus* strains. In addition, targeted mutagenesis of staphylococcal catalase results in decreased survival of *S. aureus* in the presence of the pneumococcus [62]. The mechanistic explanation is that without catalase to degrade hydrogen peroxide, the staphylococcal SOS response is triggered, leading to bacteriophage-induced lysis [63,64]. However, the *in vivo* relevance of hydrogen peroxide-mediated killing is in question because a neonatal rat study using an SpxB-deficient *S. pneumoniae* strain or a catalase-deficient *S. aureus* strain demonstrated no significant difference in co-colonization densities [65]. The finding that

carriage of *S. aureus* was less likely in individuals carrying piliated strains of *S. pneumoniae* suggests that the pneumococcal pilus may somehow reduce the likelihood of staphylococcal colonization or sustained carriage [66].

Regardless of mechanism, for PCV to contribute to the rise in MRSA there would need to be an inverse correlation specifically between VT the pneumococcus and MRSA. Cross-sectional studies have demonstrated an inverse association between carriage of *S. aureus* and vaccine-type *S. pneumoniae* [54,60]. However, interventional studies that assessed bacterial carriage before and after PCV introduction are divided. Some studies found that carriage prevalence of *S. aureus* remained steady [67–69] whereas others found increased prevalence of *S. aureus* following PCV [61,70,71]. Importantly, although some have searched for it [58], no study has demonstrated an association between pneumococcal carriage and MRSA carriage. Given that the inverse correlation between *S. aureus* and *S. pneumoniae* is absent in immunodeficient HIV-positive populations [61,72], an immune-mediated mechanism may be responsible for this relationship. Thus, although there does appear to be a link between carriage of *S. aureus* and carriage of VT *S. pneumoniae*, there is not sufficient evidence to assert that the increase in MRSA is a result of PCV rollout and no increase in community-acquired MRSA has been seen in the years following PCV rollout in Europe [73].

Pneumococcal carriage in the nasopharynx has also been associated with carriage of two other major nasopharynx-based pathogens, *H. influenzae* and *Moraxella catarrhalis*. Although there are exceptions, most studies find a positive association in humans between the presence in the nasopharynx of *S. pneumoniae* and *H. influenzae* and between *S. pneumoniae* and *M. catarrhalis* [74–76]. The epidemiologic association between *S. pneumoniae* and *H. influenzae* was recently extended by the observation that their carriage densities are also positively correlated [50]. Animal studies concur with the human epidemiology, with a neonatal rat study demonstrating that resident *S. pneumoniae* facilitated colonization of *H. influenzae* [77]. In contrast to epidemiologic data showing a positive correlation between the pneumococcus and *H. influenzae*, *in vitro* studies by Pericone and colleagues demonstrated that *S. pneumoniae* inhibits the growth of *H. influenzae*, *M. catarrhalis*, and *Neisseria meningitidis* via hydrogen peroxide production [78]. This apparent paradox suggests that cultivation of the two pathogens together *in vitro* does not accurately reflect their physiological interactions *in vivo*. In a longitudinal study of PCV introduction in the Netherlands, researchers found that whereas overall pneumococcal prevalence remained steady (with VTs decreasing and NVTs increasing), *H. influenzae* prevalence increased [71], suggesting that if there is an association between colonization with *S. pneumoniae* and *H. influenzae*, the relationship may be serotype-specific. Ongoing and future human studies of pneumococcal colonization [79] have the potential to help us better understand the relationship between the pneumococcal colonization event and the nasopharyngeal carriage of other bacterial species.

Although the mechanisms behind inter-species interactions are still being investigated, it is likely that bacteriocins play a role. It has been shown that *S. pneumoniae* bacteriocins inhibit the growth of other streptococcal species [80], and bacteriocins produced by *Streptococcus mitis* and *Streptococcus salivarius* inhibit the growth of the pneumococcus [81]. In addition to growth inhibition via secreted signals, there is also a growing body of evidence that cross-species pheromones alter bacterial gene expression. Recent studies have shown that cross-species signals can modify the expression of pneumococcal virulence genes. In contrast to the findings of Pericone *et al.* [78], Cope and colleagues demonstrated that co-culture of clinical strains of *S. pneumoniae* with *H. influenzae* does not change planktonic growth rates [82]. However, co-culture of these two strains did upregulate expression of *SpxB* and downregulate expression of *ply*, the gene that encodes pneumolysin, and *pavA*, the gene that

encodes pneumococcal adherence and virulence factor A [82]. These results suggest that bacteria sharing the nasopharynx have the potential to alter one another's gene expression. The mechanisms of interspecies gene regulation are just beginning to be explored, but the well-studied QS system LuxS/autoinducer-2 (AI-2) may be partly responsible for interspecies communication. AI-2, synthesized by the enzyme LuxS, is a molecule secreted and recognized by many bacterial species and has the potential to alter growth and virulence [83]. As reviewed in the next section, pneumococcal LuxS/AI-2 plays an important role in biofilm formation.

Pneumococcal biofilms in the nasopharynx

There is mounting evidence that the nasopharynx hosts biofilms created by many bacterial species, including the pneumococcus [84,85], *S. aureus*, *Staphylococcus epidermidis* [86], *N. meningitidis* [87], and *H. influenzae* [88]. Pneumococcal biofilms form in stages, starting with attachment to the substratum mediated by pneumococcal adhesion factors such as CbpA, PcpA, and PspA [85,89], and followed by bacterial aggregation and matrix maturation [90,91]. A mature matrix is mainly composed of extracellular DNA (eDNA), proteins, and polysaccharides [91,92] linking pneumococcal cells and attaching them to host cells in a tight mesh (Figure 2). Recent studies suggest that biofilm formation, cellular autolysis, and genetic competence are all intertwined and regulated by the Com and LuxS/AI-2 QS systems.

The Com system is well known for its ability to control the genetic competence of the pneumococcus and mediate killing of non-competent sister cells [30]. Competence-stimulating peptide (CSP), the signal secreted by the Com QS system, is strain-specific and regulates autolysis and genetic competence, which allows for release and uptake of DNA from other streptococci [93]. Recent studies have shown that gene transference among streptococci is more efficient in early biofilm structures [24,94], and LytC, a lysozyme implicated in ComC-mediated lysis of non-competent neighbors, is a common component of DNA-protein complexes in pneumococcal biofilms [92]. Two recent studies have also demonstrated that strains deficient in LuxS are unable to form biofilms at early time points and have decreased ability for eDNA release and competence [90,95]. How these two QS systems work together in biofilm formation is unknown, but perhaps LuxS/AI-2 is necessary for attachment of early biofilms and pneumococcal biofilm maturation can only be completed with activation of the Com system and release of the eDNA required for matrix development.

The pneumococcus is also capable of participating in multi-species biofilms. Co-incubation of *H. influenzae* and *S. pneumoniae* increases the biomass of pneumococcal biofilms [96]. In addition, these interspecies biofilms are able to confer protection against antibiotics to strains of susceptible *S. pneumoniae* through passive transference of β -lactamase produced by resistant *H. influenzae* [97]. Bio-film formation in *S. pneumoniae* and *H. influenzae* is in part regulated by the LuxS/AI-2 QS mechanism [90,95,98]. Accordingly, animal studies also demonstrate that the LuxS/AI-2 system regulates persistence of the pneumococcus in the mouse nasopharynx [99] and persistence of *H. influenzae* biofilms in the ear epithelium of chinchillas [98].

AI-2 is produced by most bacterial pathogens and normal flora strains, so it is quite possible that the pneumococcus secretes AI-2 in the nasopharynx to modify the behaviors of other bacterial species or that the pneumococcus responds to AI-2 secreted by other colonizers. In support of this theory, exogenous addition of purified AI-2 significantly increased the biofilm biomass of *S. pneumoniae* strain D39 [90]. Furthermore, *M. catarrhalis*, which does not possess a luxS homolog, produced more robust biofilms in response to AI-2 secreted by

H. influenzae strains [100]. A number of other streptococcal species that colonize the pharynx, such as *S. intermedius*, *S. oralis*, *S. gordonii*, and *S. mutans*, utilize LuxS/AI-2 to control biofilms [83]. Whether AI-2 is used for competition or cooperation among species in the pharynx requires further investigation.

Concluding remarks

Our understanding of how microbes and humans live together in health and disease is rapidly evolving. The pneumococcus is a clinically important and biologically fascinating organism that we can better understand by observing its interactions with the human host and with other microbes. Additional pneumococcal strains and other bacterial species play key roles in pneumococcal colonization. Metagenomic sequencing of all DNA in the nasopharynx will not only allow us to take a better census of the microbes and viruses, but will also help us to understand the full genetic and transcriptional activity of these populations [101]. Furthermore, experimental human studies of pneumococcal colonization of the nasopharynx have the potential to distinguish cause and effect when a correlation or change in bacterial composition is observed [79,102]. Ultimately, the challenge will be to identify biological and clinical relevance when examining large complex networks of intraspecies and interspecies interactions inside the human host.

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Figure 1.

Diagram of the nasopharynx and pathways to pneumococcal diseases. The nasopharynx lies directly posterior to the nasal cavity and superior to the oropharynx. The pneumococcus can spread from its home in the nasopharynx through the Eustachian tube to cause otitis media, to the sinus cavities to cause sinusitis, through the larynx to the lower respiratory tract to cause pneumonia, and then in some cases, bacteremia. Image adapted from an illustration by Patrick J. Lynch distributed under Creative Commons Attribution 2.5 License.

Figure 2.

Streptococcus pneumoniae forms multicellular structures *in vitro* on top of human lung cells.

(a) Scanning electron micrograph of pneumococcal cells in 8-h biofilms grown on top of human lung epithelial cells show bacterial cells connected by an extracellular matrix. (b) Confocal images of GFP-expressing *S. pneumoniae* biofilms demonstrate cells co-localizing with sialic acid residues (stained red) present on human lung epithelial cells (nuclei stained blue). The top image is an optical *xy* section and the bottom is an *xz* section.

Table 1

Average frequency of 16S rRNA sequences present in nasal swabs of children

Operational taxonomic unit (OTU) ^a	Frequency (%) ^b
Unclassified Moraxellaceae	19.0
<i>Streptococcus</i>	17.9
<i>Corynebacterium</i>	7.0
<i>Moraxella</i>	6.5
<i>Haemophilus</i>	4.7
Unclassified Pasteurellaceae	4.1
<i>Staphylococcus</i>	3.8
<i>Acinetobacter</i>	3.4
<i>Dolosigranulum</i>	3.2
<i>Propionibacterium</i>	3.1
Unclassified Proteobacteria	2.6
<i>Lactococcus</i>	2.6

^a Adapted with permission from Laufer *et al.* [55].

^b Average frequency, among 108 children, of 16S rRNA sequences in nasal swab samples. Only OTUs with an average frequency >2% are listed.