MINIREVIEW

Haemophilus influenzae and Streptococcus pneumoniae: living together in a biofilm

Alexandra Tikhomirova & Stephen P. Kidd

Research Centre for Infectious Disease, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, SA, Australia

Bacterial opportunists including Haemophilus influenzae and Streptococcus pneumoniae frequently reside in the nasopharynx of healthy children from where it can migrate to cause severe infections involving lungs, middle ear and the brain. It is now clear that biofilms promote bacterial persistence during many of these infections. This MiniReview nicely summarizes a large amount of work on Haemophilus and pneumococcus in biofilms alone and in co-culture.

Keywords
multispecies biofilms; nontypeable Haemophilus influenzae; otitis media; Streptococcus pneumoniae; inter-species interactions; bacterial pathogenesis.

Abstract

Streptococcus pneumoniae and Haemophilus influenzae are both commensals of the human nasopharynx with an ability to migrate to other niches within the human body to cause various diseases of the upper respiratory tract such as pneumonia, otitis media and bronchitis. They have long been detected together in a multispecies biofilm in infected tissue. However, an understanding of their interplay is a recent field of study, and while over recent years, there has been research that has identified many specific elements important in these biofilms, to date, it remains questionable whether the relationship between H. influenzae and S. pneumoniae is competitive or cooperative. Additionally, the factors that govern the nature of the interspecies interaction are still undefined. This review aims to collate the information that has emerged on the cocolonization and co-infection by S. pneumoniae and nontypeable H. influenzae (NTHi) and their formation of a multispecies biofilm.

Introduction

Streptococcus pneumoniae and Haemophilus influenzae are commensal bacteria that inhabit the nasopharynx of healthy humans, and asymptomatic nasopharyngeal carriage in healthy children has been documented to range up to 80% for H. influenzae (LaCross et al., 2013) and at least 20% for S. pneumoniae (Bogaert et al., 2004). However, both bacterial species have the capacity to migrate from this anatomical niche and, in doing so, to cause various diseases. This includes transit to the bronchi to cause bronchitis (Bresser et al., 2000; Priftis et al., 2013), to the lungs to cause pneumonia (Berk et al., 1982; Weiss et al., 2004), to the middle ear to cause otitis media (OM; Hall-Stoodley et al., 2006), to the blood to cause septicemia (Goetzheuer et al., 2000) and across the blood-brain barrier to cause meningitis (Goetzheuer et al., 2000). For H. influenzae, a vaccine exists against the encapsulated type b of H. influenzae, which was the predominant cause of invasive diseases such as meningitis and septicaemia (Adam et al., 2010). Since the introduction of this vaccine, there has been a decline in invasive diseases caused by the vaccine type (Adams et al., 1993; Peltola, 2000; Leung et al., 2012; Georges et al., 2013). However, the other serotypes of encapsulated H. influenzae (a, c, d, e and f; Waggoner-Fountain et al., 1995; Urwin et al., 1996; Murphy et al., 1999; Adderson et al., 2001) as well as the multitude of nontypeable unencapsulated strains (also known as the nontypeable Haemophilus influenzae or NTHi; Falla et al., 1993; Murphy et al., 1999) still pose a threat, particularly for the development of diseases of the respiratory tract such as OM (Ribeiro et al., 2003). Importantly, a majority of strains colonizing the paediatric nasopharynx consist of nontypeable strains, carriage itself posing a high risk of development of respiratory tract infection due to these NTHi (Spinola et al., 1986).
There also exists a range of vaccines against some *S. pneumoniae* serotypes. The polysaccharide vaccine consists of purified polysaccharides from 23 serotypes of *S. pneumoniae* (specifically, 1, 2, 3, 4, 5B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F; Nuorti & Whitney, 2010) and does offer protection from pneumococcal bacteraemia and pneumonia in adults (Shapiro et al., 1998; Cornu et al., 2001; Melegaro & Edmunds, 2004). However, it is less immunogenic than the conjugate 7- or 13-valent vaccines (Ahman et al., 1998).

In the conjugate vaccines, the polysaccharides are conjugated to diphtheria toxin, which is highly immunogenic and generates a more robust immune response (Ahman et al., 1998). Importantly, however, while both vaccines offer protection from carriage and invasive disease caused by the vaccine serotypes (Fenoll et al., 2009; Isaacman et al., 2010; Ho et al., 2011; Egere et al., 2012; Alonso et al., 2013), over 90 serotypes of *S. pneumoniae* (van der Linden et al., 2013) have been described, and the majority of these are not included in the polysaccharide or conjugate vaccines. Hence, both *H. influenzae* and *S. pneumoniae* still pose a threat to human health due to colonization and disease is still a possible outcome of infection by nonvaccine serotypes. Currently, the specific molecular factors and signals that cause the transit from a commensal colonization of the nasopharynx to an invasion of sterile sites of the respiratory tract remain incompletely defined, although host factors such as the anatomy of the eustachian tubes (Stenström et al., 1991; Bylander-Groth & Stenström, 1998), age (Teele et al., 1989) and immune competence (Engel et al., 2001; Faden, 2001) seem to play a role. Importantly, to cause invasive disease of the respiratory tract, these bacteria must be able to adapt to the specific conditions of the microniche they transit to in the lung, middle ear or cerebrospinal fluid. This includes adaptation to the niche-specific pH, nutrient availability and antimicrobial compounds.

In addition, both bacterial species must have the capability to persist within the site after initial colonization. This includes expression of appropriate adhesins and the ability to evade the host immune response (Fig. 1). It is known that for both bacterial species, a key feature of their colonization, survival and persistence, as well as the disease outcome when they become established in a particular niche, is their ability to form a biofilm.

**Coexistence within the multispecies biofilm as a mode of bacterial persistence**

In recent years, it has been proposed that the biofilm lifestyle of both *S. pneumoniae* and NTHi is responsible for the persistence of these bacteria in the chronic forms of OM (Hall-Stoodley et al., 2006) and chronic rhinosinusitis (Cope et al., 2011). The monospecies biofilm formation of both *S. pneumoniae* and *H. influenzae* has been described in both *in vitro* and *in vivo* settings (Reid et al., 2009; Weimer et al., 2010; Cope et al., 2011; Carruthers et al., 2012). More importantly, however, fluorescence *in situ* hybridization (FISH) techniques have shown that both *S. pneumoniae* and *H. influenzae* are present in middle ear tissues excised from chronic OM patients (Hall-Stoodley et al., 2006). The persistence of these species within a biofilm provides a vast array of phenotypes that allow for both bacterial adaptation to a host niche and for the persistence of these species in a niche for a prolonged period. Specifically, residence within a biofilm allows for global changes in gene and protein expression profiles, which has many effects on cell physiology including an increased resistance to antimicrobial agents, as well as expression of cell surface appendages such as the type IV pili, lipopolysaccharide or lipooligosaccharide and the release of biomolecules such as extracellular DNA (eDNA), which all promote adhesion and cohesion properties of biofilm cells, thereby increasing persistence (Table 1). However, although *H. influenzae* and *S. pneumoniae* are known and coexist within a biofilm in many chronic diseases of the respiratory tract, the nature of the interplay between these pathogens and the effect of co-infection on disease outcome has not yet been extensively studied. There are, however, significant factors that seem to have been determined to function in this interplay.

**The NTHi and *S. pneumoniae* multispecies biofilms: the basis for the chronicity of OM**

OM is an inflammatory disease of the middle ear, affecting over 70% of children before 3 years of age (Teele et al., 1989; Berman, 1995). OM poses a serious risk of progression to systemic disease (Klein, 2000) as well as impaired hearing (Hunter et al., 1996), which in children can progress to speech delay (Shriberg et al., 2000) and impaired cognitive development (Williams & Jacobs, 2009). The major aetiological agents of OM are the bacterial pathogens *S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* (Hall-Stoodley et al., 2006). While OM is commonly treated with antibiotics (Dowell et al., 1999) or tympanostomy tube placement (Gebhart, 1981), chronic (COM) or recurring (ROM) forms of OM are often unresponsive to these treatments (Williams et al., 1993; Ojano-Dirain & Antonelli, 2012).

The opportunistic pathogens *S. pneumoniae* and *H. influenzae* (NTHi in most cases) are significant contributors to the pathogenesis of acute OM (AOM). Recently, FISH techniques demonstrated the presence of biofilms harbouring both NTHi and *S. pneumoniae* on middle ear membrane tissue from COM patients (Hall-Stoodley et al., 2006; Thornton et al., 2011). A biofilm is a bacterial lifestyle in which the cells reside adhered to a substratum and to each other and are encased in a self-produced extracellular polymeric substance matrix (EPS matrix; Sutherland, 2001; Flemming & Wingender, 2010; Hoa et al., 2010). An important feature of bacterial biofilms is their persistent nature and their insensitivity to immune mediators and clinically used antimicrobial agents (Domenech et al., 2013a, b). These features can be explained both by the physiology of the biofilm-resident cells themselves and by the physical properties of the EPS matrix components. The presence of an EPS matrix allows for biofilm persistence by physically...
limiting the diffusion of antimicrobial compounds into the biofilm (Høiby et al., 2010), thus allowing for the increased persistence of biofilm-resident cells. Additionally, the biofilm-resident cells themselves have been shown to undergo changes in gene expression profiles upon transition from planktonic to biofilm state (Prigent-Combaret et al., 1999; Becker et al., 2001; Sauer & Camper, 2001; Sauer et al., 2002). The switch in gene expression that defines biofilm-resident cells seems to have global effects on cellular functions. This includes changes not only in the surface components expressed, but also in the metabolic and biosynthetic pathways for maintaining intracellular conditions such as pH and redox balance (Schembri et al., 2003). Often, these global physiological changes include a physiological state with an increased recalcitrance to antimicrobial agents (Mah & O’Toole, 2001), which contributes to an increased persisitence. The biofilm nature of COM explains the difficulty in treatment of COM with antibiotics (Williams et al., 1993; Segal et al., 2005; Rovers et al., 2006), as biofilm communities display increased recalcitrance to antibiotics and explains the resistance to tympanostomy tube placement (Post, 2001), as both pathogens are able to re-establish the biofilm on the tympanostomy tube.

The *H. influenzae* and *S. pneumoniae* Biofilm Lifestyle

Residence within a biofilm for both *H. influenzae* and *S. pneumoniae* involves global changes in gene expression and physiology to enable them to adapt to the environmental conditions.
Table 1: The systems with an increased expression in the biofilm state compared to the planktonic state for *Haemophilus influenzae* and *Streptococcus pneumoniae* monospecies biofilms and in the coculture biofilm where increased expression compares the expression of genes in the coculture biofilm compared to the monospecies biofilms.

<table>
<thead>
<tr>
<th>Factors upregulated</th>
<th>Genes involved</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors upregulated in the <em>H. influenzae</em> monospecies biofilm compared to the planktonic state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IV pilus</td>
<td>pilA-D comABCDEF</td>
<td>Adhesion to host cells, twitching motility, co-localization with eDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Jurcisek &amp; Bakaletz, 2007)</td>
</tr>
<tr>
<td>eDNA</td>
<td>Unknown; possibly involvement of</td>
<td>Maintenance of biofilm structure and stability</td>
</tr>
<tr>
<td>qurorn-sensing luxS for release</td>
<td></td>
<td>(Jurcisek &amp; Bakaletz, 2007)</td>
</tr>
<tr>
<td>Sialylated lipooligosaccharide</td>
<td>lic3A, siaA, lsgB (Jones et al., 2002)</td>
<td>Adhesion to host cells, maintenance of biofilm architecture</td>
</tr>
<tr>
<td>Phosphorylcholine decorated lipooligosaccharide</td>
<td>licABCD (Weiser et al., 1997)</td>
<td>Reduced induction of host innate immune response</td>
</tr>
<tr>
<td>Outer membrane protein P6</td>
<td>omp p6 (Chang et al., 2011)</td>
<td>Adhesion (Gallagher et al., 2006)</td>
</tr>
<tr>
<td>Hap</td>
<td>hap (Fink &amp; St. Geme, 2003)</td>
<td>Adhesion (Webster et al., 2006)</td>
</tr>
<tr>
<td>HMW1</td>
<td>hmw1A (Barenkamp &amp; St Geme, 1994)</td>
<td>Adhesion (Webster et al., 2006)</td>
</tr>
<tr>
<td>HMW2</td>
<td>hmw2A (Barenkamp &amp; St Geme, 1994)</td>
<td>Adhesion (Webster et al., 2006)</td>
</tr>
<tr>
<td>IgA1 protease</td>
<td>Iga (Poulsen et al., 1992)</td>
<td>Proteinolysis of secretory forms of IgA1 antibodies, involvement in microcolony formation (Webster et al., 2006)</td>
</tr>
<tr>
<td>Factors upregulated in the <em>S. pneumoniae</em> monospecies biofilm compared to the planktonic state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eDNA</td>
<td>LuxS-mediated ltaA autolysis</td>
<td>Adhesion, cohesion and biofilm structure</td>
</tr>
<tr>
<td></td>
<td>implicated in eDNA release</td>
<td>(Moscoco et al., 2006; Hall-Stoodley et al., 2008)</td>
</tr>
<tr>
<td>CbpA</td>
<td>cbpA (Sanchez et al., 2011)</td>
<td>Adhesion to laminin receptor</td>
</tr>
<tr>
<td>PsrP</td>
<td>psrP (Sanchez et al., 2011)</td>
<td>Host cell and intraspecies adhesion (Sanchez et al., 2011)</td>
</tr>
<tr>
<td>ChoP</td>
<td>licD2 (Zhang et al., 1999)</td>
<td>Adhesion to host PAFR</td>
</tr>
<tr>
<td>Factors upregulated in the <em>H. influenzae</em>/<em>S. pneumoniae</em> multispecies biofilm compared to the monospecies biofilms</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em> type IV pilus</td>
<td>pilABCD comABCDEF</td>
<td>Possibly adhesion, cohesion and biofilm structure</td>
</tr>
<tr>
<td></td>
<td>(Bakaletz et al., 2005; Carruthers et al., 2012)</td>
<td>(Cope et al., 2011)</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> H2O2 production</td>
<td>spxB (Spellerberg et al., 1996)</td>
<td>Possibly to compete with <em>H. influenzae</em> for nutrients or space or to mediate <em>H. influenzae</em> lysis and eDNA release (Cope et al., 2011)</td>
</tr>
</tbody>
</table>

Conditions and persist in the host. However, while it is a multispecies biofilm that contributes to the pathogenesis of chronic OM, to date, the biofilms of both species have been studied primarily in monoculture.

It is known that in a biofilm, one major phenotypic cellular changes is that of a production of an EPS matrix (Branda *et al.*, 2005; Flemming & Wingender, 2010). The composition of the matrix differs between bacterial species, but generally includes lipids, proteins and nucleic acids (Flemming & Wingender, 2010). The EPS matrix has been shown to reduce the permeability of the biofilm matrix to antimicrobial compounds, as well as contributing to the adhesion and cohesion of the cells to the host cells and to each other (Branda *et al.*, 2005; Flemming & Wingender, 2010). Various adhesion factors have been shown to be upregulated in the *H. influenzae* and *S. pneumoniae* biofilms (Table 1). In this context, there may be features of the host tissue, the middle ear mucosa for instance, that permit adhesion and biofilm (in particular, multispecies biofilm) or there may be features of the bacterial survival strategies that mean the properties and stresses of a specific niche act to signal for the bacterial cells to switch to a biofilm (and in particular, a multispecies biofilm). In addition, the biofilm mode of life involves global changes in the expression of genes that are not involved in the EPS matrix and rather may be involved in the response to low nutrient levels, which may vary throughout the biofilm, as well as oxidative stresses or local pH stress (Schembri *et al.*, 2003; Beenken *et al.*, 2004).

Importantly, changes in the expression of genes and their protein products have been observed from the planktonic to the biofilm state for both *H. influenzae* and *S. pneumoniae*. The *S. pneumoniae* biofilm cells have been shown to have an increased adhesion by up to 12× compared with their planktonic counterparts (Sanchez *et al.*, 2011). This was due to a reduced expression of capsular polysaccharide which exposed bacterial cell surface proteins and an increased expression of phosphorylcholine which is known to bind to the host receptor PAfR and increased expression...
of protein CbpA which binds to Laminin receptor on host cells and PsrP which binds keratin 10 (Sanchez et al., 2011). eDNA has also been shown to form a part of the S. pneumoniae EPS matrix (Moscoso & Claverys, 2004; Moscoso et al., 2006; Hall-Stoodley et al., 2008). Additionally, it was recently shown that eDNA–protein complexes play a role in the EPS matrix. Thus, the lysozyme LytC protein was shown to bind to the eDNA in S. pneumoniae biofilms (Domenech et al., 2013a, b). A novel polysaccharide component to the S. pneumoniae EPS matrix which was distinct from the capsule has also been identified (Domenech et al., 2013a, b). A clear part of the switch in lifestyle is that an upregulation of the gene expression of some stress response genes was observed in the biofilm of S. pneumoniae compared with the planktonic state. Specifically, there was an upregulation in the expression of stress-related chaperons and proteases including GroEL, class I heat shock proteins, the universal stress family proteins, thioredoxin, 2 Clp proteases and 3 transposases, as well as 18 hypothetical proteins (Sanchez et al., 2011). Hence, it appears that stress response plays a role in the maintenance of S. pneumoniae in the biofilm state; the profile of transcriptionally active genes defines that lifestyle and this certainly indicates that in a biofilm lifestyle, the cell is predisposed to a stress response state.

Webster et al. (2006) showed an involvement of the lipoooligosaccharide of H. influenzae in the monospecies biofilm as well as the proteins Hap, HMW1 and HMW2. The secreted IgA protease was also associated with the EPS matrix; however, it was primarily localized at the outer regions of the matrix, corresponding to a role in protection from the host immune response via degradation of the secretory forms of IgA1 (Webster et al., 2006). A study by Gallaher et al. (2006) investigated numerous proteins associated with the H. influenzae EPS matrix and found the involvement of outer membrane proteins 5 and 6 (OMP5 and OMP6), as well as various proteins implicated in biofilms of other bacteria such as GroEL, KasA/FabB, UspA and peroxiredoxin; however, their precise function in the H. influenzae EPS matrix have not been thoroughly investigated. In addition, both eDNA and the type IV pilus (Tfp) have recently been identified as aspects of the H. influenzae EPS matrix and were shown to contribute to the stability and structure of the biofilm (Jurcisek & Bakaletz, 2007).

While genes expressed in the biofilm for each species allow adaptation to and persistence within the host environment, little is known about the genes expressed in a multispecies biofilm. In the case of a multispecies biofilm, adhesion genes in the EPS matrix would also be required to facilitate cell–cell cohesion between the two different species that possess different cell surface appendages and proteins. In addition, the architecture of the multispecies biofilm would need to facilitate the spatial localization of cells that would allow for the survival of both species. This would involve complex sublocalizations depending on nutrient and oxygen requirements, as well as protection from external stresses and intrabiofilm stresses such as the waste of ‘self-species’ cells and the waste or toxic by-products of the other competing species. An overview of the sequence of events proposed to occur in multispecies biofilm development is given in Fig. 1. Currently, little is known about the genes expressed in the multispecies biofilm; however, in part, the genes expressed would rely on the nature of the interaction between H. influenzae and S. pneumoniae (Fig. 1). To date, despite several studies in this regard, it remains unclear as to whether this interaction is synergistic, antagonistic or both. It is worthy at this juncture to collate the data from what is known and analysing the supposed conflicts.

Mutual protection and stability within the multispecies biofilm?

The colocalization of NTHi and S. pneumoniae has suggested a synergistic interaction between the two organisms. Indeed, some studies have demonstrated that the coexistence in a multispecies biofilm provides advantages to one or both of these species. One advantage is the passive protection from antibiotic therapy seen in the multispecies biofilm. Weimer et al. (2011) showed that the β-lactamase-producing NTH strain 86-028NP was able to protect both planktonic and biofilm-resident S. pneumoniae from amoxicillin treatment in the chinchilla middle ear. This finding demonstrates the importance of sharing a diverse gene bank within the multispecies community of the respiratory tract where gene expression has repercussions not only for one bacterial species, but the whole microbiome of the resident environment. However, this study demonstrated that β-lactamase production by H. influenzae was not the only method of S. pneumoniae protection against amoxicillin. In fact, the β-lactamase-deficient strain 86-028NP bla was able to protect biofilm S. pneumoniae from amoxicillin. In addition, the coculture 86-028NP bla strain could be isolated from bullar homogenate biofilms, whereas the monoculture inoculated 86-028NP bla was unable to survive on its own. Hence, while β-lactamase production played a role in interspecies synergy, the formation of a multispecies biofilm by even β-lactamase-deficient strain was shown to be beneficial in protection from antibiotics for both species. While the reason for such protection was not further analysed, most likely the protection was due to the formation of an extensive matrix of EPS in the formation of a multispecies biofilm; the EPS matrix is known to limit the physical diffusion of antimicrobials into close proximity with the biofilm-resident cells.

A recent study by Cope et al. (2011) also demonstrated synergy between H. influenzae and S. pneumoniae. Specifically, both species reached higher cell densities in coculture compared with monoculture, and in polymicrobial biofilms, they were shown to modulate the expression of each other’s virulence genes, resulting in persistent biofilm (Cope et al., 2011). Specifically, it was shown that in a coculture biofilm setting, physical contact of H. influenzae with S. pneumoniae resulted in an upregulation of pilA expression by the H. influenzae (Table 1). The pilA of H. influenzae is known to encode the major subunit of the Tfp (Bakaletz et al., 2005), an important appendage in adhesion and biofilm stability of the monospecies biofilm. Hence, this indicates that in coculture, genes may be
expressed that facilitates biofilm formation, and which perhaps may benefit both species in the biofilm.

Overall, a stable and robust biofilm would be beneficial to both species due to the possibility of passive protection from antimicrobial substances and due to an extensive EPS matrix to promote adhesion to host surfaces and protection from antimicrobial substances. However, a multispecies biofilm would also have the greater benefit in the wider diversity of the biofilm genome, where the genetic reservoir would harbour genes from not only different strains, but also different species. It has been shown that in vivo S. pneumoniae can uptake genes from lysed cells within the biofilm and that the competence for genetic uptake is 10^7-fold higher in the biofilm state than in the planktonic state (Marks et al., 2012). Thus, the potential for uptake of genes by different species within the biofilm would be high and would allow for an increased interspecies genetic transfer and diversity, which could impact both treatment outcomes and progression of the disease.

**Biocide production, an antagonistic interaction or for the greater common good?**

In contrast to the aforementioned interactions, there have been some studies that have demonstrated not synergistic, but competitive interactions with contradictory competition outcomes. Specifically, the H_2O_2 generated by S. pneumoniae was toxic to NTHi (Pericone et al., 2000). However, it is worth noting that Cope et al. (2011) showed that the spaB of S. pneumoniae responsible for H_2O_2 production was upregulated in the coculture S. pneumoniae – H. influenzae biofilm (Table 1). Hence, it could be that the toxic H_2O_2 production plays a competitive role in the multispecies biofilm or, in contrast, that H_2O_2 production plays a more complex role. Specifically, H_2O_2 could locally reduce *H. influenzae* viable cells, which could result in cell death and subsequent release of nutrients and other cellular components such as genomic DNA. Indeed, such a release of nutrients could serve to benefit both species within the biofilm, providing that a subset of viable *H. influenzae* cells was still present. Additionally, released DNA could serve multiple roles in a multispecies biofilm; if taken up by competent cells in the biofilm, it could increase genetic diversity of both species, it could be utilized as a nutrient or contribute to the EPS matrix increasing biofilm adhesion of cohesion.

**Competition for nutrients, attachment space and optimal conditions**

As both species possess individual genomes and utilize unique metabolic pathways, it is likely that as well as the metabolic production of H_2O_2 by S. pneumoniae, there exists a myriad of biological factors that may impact the growth of one of the species. These factors include metabolic by-products produced by one of the species, which can be beneficial or detrimental to the other species. The metabolic by-products generated are in turn dependent on the environmental conditions, nutrient availability and physical conditions of the environment.

Recently, it has been observed that in a batch coculture model of S. pneumoniae and H. influenzae growth, S. pneumoniae was able to outcompete *H. influenzae* (A. Tikhomirova & S.P. Kidd, unpublished observation, 2012). This effect was not dependent on the strains used and was not a result of H_2O_2-mediated competition as an spaB isogenic mutant of S. pneumoniae also inhibited *H. influenzae* planktonic and biofilm growth. Importantly, this growth inhibition effect was time dependent, where *H. influenzae* cell death occurred only after the entering of both species into stationary phase. This growth inhibition effect appeared to be a result of a pH change occurring in the S. pneumoniae growth after reaching stationary phase (A. Tikhomirova & S.P. Kidd, unpublished data, 2013). Hence, it appears that by modifying the pH of the immediate environment during certain growth stages, S. pneumoniae can diminish the viability of *H. influenzae*. Hence, *H. influenzae* may be more adapted for survival and potentially biofilm formation in an environment where pH is elevated. This is supported by the finding that the type IV pili important for *H. influenzae* biofilm formation were expressed in a chemically defined medium at pH 8.5–9.0, but not 7.2 (Bakalatz et al., 2005). The pH of the middle ear environment has not been extensively studied; however, it has been shown that the pH of middle ear effusions of OM patients with chronic secretory forms of OM extends to a basic pH that ranges from pH 7–9 (Nuuinen et al., 1993; Wezyk & Makowski, 2000). If *S. pneumoniae* lowers the pH during a multispecies infection, *H. influenzae* must have mechanisms to regulate the pH to maintain a neutral or basic pH of the environment. One pH regulatory mechanism in *H. influenzae* is urease. The *H. influenzae* urease is a nickel-dependent enzyme, which is involved in catalysing the hydrolysis of urea to ammonia and carbon dioxide (Mobley et al., 1995). In other bacteria, the urease functions to raise the pH of the environment, allowing bacterial survival at low pH environments (Ferrero & Lee, 1991; Stingl et al., 2002). In a chinchilla model of OM, it has been demonstrated that the ureH, a homologue of a gene requires for expression of an active urease in *Helicobacter pylori*, was upregulated in the chinchilla middle ear (Mason et al., 2003). Additionally, in human chronic obstructive pulmonary disease, antibodies against *H. influenzae* urease increased with exacerbations of the disease, indicating urease expression in chronic diseases of the respiratory tract (Murphy & Brauer, 2011). The ure operon was also found to be more prevalent in *H. influenzae* isolates from OM or COPD than from isolates obtained from throats of healthy individuals (Zhang et al., 2013). It would be likely that if *S. pneumoniae* growth dramatically reduces the pH of the environment (A. Tikhomirova & S.P. Kidd, unpublished data), then for a coculture growth within the middle ear, acidification of the environment would be prevented or shifted to a more alkalizing state that would facilitate survival, type IV pili expression and a multispecies biofilm formation. Hence, in chronic forms of OM that involve multispecies infections, *H. influenzae* may require urease or other systems to
maintain the pH at a neutral or slightly basic state, which is consistent with the clinical observations for the presence of urease.

**Exploiting the host immune system to outcompete a competitor**

In addition to H$_2$O$_2$-mediated competition of *S. pneumoniae* with *H. influenzae*, there have been reports of immune-mediated competition between these two species. Shakhnovich et al. (2002) have shown that *S. pneumoniae* produces an neuraminidase capable of desialylating the sialic acid – decorated lipooligosaccharide structures of *H. influenzae*. It has been hypothesized that in vivo, such desialylation could lead to a reduction in *H. influenzae* viability due to the decrease in sialic acid association with complement factor H and, thus, an increase in complement-mediated removal of *H. influenzae* from the host. It is important also to note that sialic acid was shown to be central in *H. influenzae* biofilm formation (Swords et al., 2004) and that mutants deficient in siaB produced only asiaylated lipooligosaccharide and formed reduced biofilm in a continuous flow system, and this leads to a reduced persistence and colonization in the gerbil OM model (Swords et al., 2004). In the chinchilla OM model, NTHi mutants deficient in sialyltransferases siaA and lsgB also displayed an atypical biofilm phenotype where, while a biofilm was formed, it contained mostly nonviable bacteria (Jurcisek et al., 2005).

However, the opposite – that is, the immune-mediated competition of *H. influenzae* with *S. pneumoniae* – has also been shown. Lysenko et al. (2005) showed that NTHi was dominant in a mouse model through its stimulation of opsonophagocytic killing of *S. pneumoniae*. In other animal studies, NTHi had an improved colonization of the rat nasopharynx when *S. pneumoniae* was previously established, seemingly through mediating immune competition with *S. pneumoniae* (Margolis et al., 2010). Importantly, the immune-mediated competition of *H. influenzae* with *S. pneumoniae* was specific to particular *S. pneumoniae* strains (Margolis et al., 2010). However, the factors that govern these strain-specific interactions remain unclear. It had been suggested that because NTHi directed the opsonophagocytosis response against *S. pneumoniae*, encapsulated *S. pneumoniae* strains would prevail in a coculture setting, and the capsular type and thickness would determine the survival of *S. pneumoniae* in this setting (Lysenko et al., 2010). It is well known that *S. pneumoniae* has a phase-variable capsular expression that is linked to colony morphology determined by the opaque or transparent colony phenotype (Kim & Weiser, 1998; Kim et al., 1999). Specifically, *S. pneumoniae* is able to switch from expression of high levels of capsular polysaccharide, which is determined by the opaque colony phenotype, to lower levels of capsular polysaccharide expression and the transparent morphology (Kim & Weiser, 1998; Kim et al., 1999). It could be suggested by the mathematical model of Lysenko et al. (2010) that in a co-culture setting, opaque strains expressing higher levels of capsular polysaccharide would predominate. However, this theory is contradicted by several findings. Firstly, capsule expression was shown to be reduced in monospecies *S. pneumoniae* biofilm models (Hall-Stoodley et al., 2008), and reduced capsular expression mutants displayed augmented biofilm formation (Muñoz-Elias et al., 2008; Qin et al., 2013). Likewise, expression of a capsule by capsular *S. pneumoniae* strains has been associated with a reduced biofilm formation as compared to nontypeable strains (Moscoso et al., 2006). This suggests that capsular expression may not be favoured in the biofilm lifestyle of *S. pneumoniae*. Most importantly, however, in a chinchilla OM co-infection model, there was shown to be an increased proportion of translucent *S. pneumoniae* types within the multispecies biofilm (Weimer et al., 2010).

Hence, the indication is that there is selection for strains that have a capacity for survival in coculture and in the multispecies biofilm, mediated through yet to be identified determinants that are not necessarily related to capsular type or capsular expression.

**The EPS matrix and interspecies interactions**

While specific factors that impact the interspecies interactions have not yet been identified, it is likely that synergistic relationships may be established by strains that would benefit the mutual biofilm. It is known that the EPS matrix plays a vital role in biofilm stability, protection against antimicrobial compounds and adhesion to host cells – factors that would benefit both species in a host environment. Therefore, it could be suggested that strains that would benefit the mutual biofilm could be those that are able to establish an extensive EPS matrix structure or strains where the EPS matrix components could synergistically interact with the EPS matrix components of the other species. To date, some studies support this hypothesis. Cope et al. (2011) showed that the NTHi pilA, which encodes the Tfp, was upregulated in *S. pneumoniae*/NTHi multispecies biofilm. Previously, it was shown that in the NTHi monospecies biofilm, the type IV pili increased biofilm stability (Jurcisek et al., 2007) and mediated twitching motility (Bakaletz et al., 2005). NTHi type IV pili also bind DNA (Aas et al., 2002) and were colocalized with eDNA (Jurcisek & Bakaletz, 2007) in a monospecies biofilm formed in the chinchilla middle ear. The type IV pilA DNA-binding role is key to mature NTHi biofilm formation as a complex mature biofilm structure could be formed via twitching motility-directed migration of individual cells along eDNA (Allesen-Holm et al., 2006). It is important that both *S. pneumoniae* (Moscoso et al., 2006) and NTHi (Jurcisek & Bakaletz, 2007) are known to release eDNA into their own EPS matrix but the role and interactions of type IV pilA and eDNA in the multispecies biofilm remain unclear. The evidence suggests that they are important and may function in strain-specific selection.

**Molecular factors of the communication, inter-/intraspecies interplay**

To initiate and maintain formation of a biofilm, resident cells must be able to regulate expression of appropriate genes that
may facilitate biofilm formation or promote survival in the presence of strain-specific selection forces. The environmental basis for bacteria switching into their biofilm state is complex and the factors responsible for this are not fully understood, although the physical, chemical and biological conditions of the immediate environment such as nutrient starvation, pH stress and oxygen levels likely play a role in inducing biofilm formation. Recently, it has been shown that a knockout in the *H. influenzae* nickel uptake system *nik-KLMOO-nimR* displayed an increased biofilm formation, which was attributed to a change in the surface properties of the cell (Ng & Kidd, 2013). Specifically, the mutant cells were more negatively charged and hydrophobic compared with the wild type, which stimulated cell–cell aggregation and biofilm formation. Hence, it is likely that for *H. influenzae*, the environmental conditions that determine a requirement for nickel and the local concentration of nickel can modulate biofilm formation propensity (Ng & Kidd, 2013).

It is also known that other environmental factors including volatile pollutants can also stimulate biofilm formation, and it has recently been shown that cigarette smoke condensate augmented biofilm formation of *S. pneumoniae* (Mutenne et al., 2013). It is important that for many bacterial species, cell density-dependent quorum-sensing signals are vital in the formation of monospecies biofilms (Merritt et al., 2003; Yoshida et al., 2005). For both *H. influenzae* and *S. pneumoniae* monospecies biofilms, quorum sensing appears to be an important initiation factor.

In many bacterial species, the quorum-sensing signal autoinducer-2 (AI-2) encoded by the *luxS* gene, is vital for inducing biofilm formation (Merritt et al., 2003; Yoshida et al., 2005; González Barrios et al., 2006). AI-2 is produced as a by-product in the homocysteine synthesis pathway, where S-ribosylhomocysteine is converted to homocysteine via the action of the *luxS*-encoded S-ribosylhomocysteine lyase (Walters et al., 2006). The by-product of this reaction is 4,5-dihydroxy-2,3-pentadione (DPD); however, as this compound is highly unstable, it is able to spontaneously cyclize, forming several furanone ring structures, including AI-2 (Walters et al., 2006). The action of AI-2 involves a density-dependent sensing by a cell surface uptake system or a two-component regulatory system. In *H. influenzae*, the AI-2 uptake receptor was found to be RbsB (Armbruster et al., 2011). However, it is also important to note that in enterrohaemorrhagic *Escherichia coli* (EHEC), an alternate QS system involving the autoinducer AI-3 has also been shown to be affected by *luxS* (Walters et al., 2006), and interestingly, in *H. influenzae*, the QseB/QseC 2 component regulatory system was found to be a homologue of the EHEC QseB/QseC AI-3 sensing system (Ünal et al., 2012). Hence, in the majority of studies investigating AI-2-mediated quorum sensing via analysis of *luxS* knockouts, the phenotype of both AI-2 and AI-3 mutant strains could be mistaken for a purely AI-2 lacking phenotype. Importantly, in *S. pneumoniae*, while *luxS* has been shown to be important for biofilm formation, a homologue of the AI-2 receptor or the AI-3 receptor has not yet been determined, which may be due to sequence diversity of the receptor (Rezzonico & Duffy, 2008). While for both species, the precise downstream signalling events for AI-2 or AI-3 sensing are not yet known, certainly the ultimate result is the regulation of quorum-sensing-dependent genes. Both *H. influenzae* and *S. pneumoniae* encode *luxS*, which was shown to be important for formation of a monospecies biofilm in both species (Moscoso et al., 2006; Vidal et al., 2011, 2013). In *H. influenzae*, *luxS* was shown to modify the lipooligosaccharide moieties on the bacterial surface, which was thought to account for the reduction in biofilm formation in a *luxS* mutant (Armbruster et al., 2009). In *S. pneumoniae*, *luxS* controls transcription of *lytA*, a autolysin implicated in biofilm formation (Vidal et al., 2011), and possibly involved in lysis-induced eDNA release into the EPS matrix (Romao et al., 2006), as well as early and late competence genes of the *com* competence quorum-sensing system (Trappetti et al., 2011; Vidal et al., 2011), which are implicated in competence and competence-dependent fratricide and associated DNA release (Steinmoen et al., 2002; Vidal et al., 2013). Hence, AI-2-mediated quorum sensing may be involved in initiating biofilm formation by establishing the EPS matrix in *S. pneumoniae* strains capable of eDNA release, possibly through coregulation of the *com* genes.

In the context of a multispecies biofilm, it is likely that the presence of the other bacterial species is also sensed, inducing formation of a multispecies biofilm. Currently, a specific sensing pathway whereby one species is able to sense the presence of another, especially in the context of *H. influenzae* and *S. pneumoniae*, has not been established. Importantly, although both species encode *luxS*, AI-2 production may be sensed by strains that may not produce AI-2, as has been observed with *M. catarrhalis* in the *M. catarrhalis/H. influenzae* multispecies biofilm (Armbruster et al., 2010). It could be that each bacterial species is also able to sense the AI-2 produced by the other species as well as the self-produced AI-2 and that coordinated production of AI-2 by both species is responsible for a quicker switch to the biofilm lifestyle or the more efficient formation of a robust biofilm.

Importantly, the expression of *luxS* itself may be dependent on more global environmental signals. For example, it has been shown in *S. pneumoniae* that extracellular iron Fe(III) stimulated *luxS* expression, and, in turn, increased the biofilm formation (Trappetti et al., 2011). It is possible that not only extracellular iron, but also environmental factors signalling of the presence of the other species may be sensed resulting in *luxS* expression, more efficient interspecies sensing and subsequent biofilm formation.

**Haemophilus influenzae** and **S. pneumoniae** working together to avoid the host immune response

In the presence of external signals, which are currently unclear, *H. influenzae* and *S. pneumoniae* are able to sense each other’s presence in the niche and initiate polymicrobial biofilm formation. This would involve expression of appropriate adhesins for such a multispecies biofilm. It is known that in the multispecies biofilm, residents are offered protection from host immune mediators and
antimicrobial agents due to reduced permeability into the biofilm. However, such anti-immunity biofilm properties are sometimes more diverse and may depend on the nature of the EPS matrix components. For instance, it has recently been shown that in the NTHi biofilm, eDNA as an anionic molecule, can bind to the cationic β-defensin three antimicrobial peptide, thus deactivating it (Jones et al., 2013). It is important that if both H. influenzae and S. pneumoniae are together able to release higher levels of eDNA into the biofilm matrix, the antimicrobial action of the eDNA could be augmented allowing for a longer persistence of the biofilm. Another mechanism of biofilm-related host defence resistance is neutrophil extracellular trap (NET) utilization. It is known that host neutrophils produce NETs that are comprised of double-stranded DNA, histones and elastase (Yousefi et al., 2009). These structures have microbicidal activity due to entrapping bacterial cells within the DNA matrix and killing them by granular components (Brinkmann et al., 2004). However, it has been shown that NTHi biofilms can exist in a viable state within the NET structures, and such survival has been correlated to a higher bacterial load during experimentally induced OM (Hong et al., 2009). Hence, it does appear host NETs, derived as an immune response to infection, are actually utilized by bacterial communities to maintain persistence. It has also been shown that the NTHi cells are able to induce the formation of NETs, possibly due to signalling of NTHi lipoooligosaccharide through the TLR4 (Juneau et al., 2011). It has been shown that the S. pneumoniae protein α-enolase also stimulates the formation of NETs (Mori et al., 2012). While this study showed that the α-enolase actually increased the bactericidal function of NETs, this was shown in human blood, where bacteria are normally present in the planktonic form. Hence, in an environment where biofilm formation is favoured, such as the middle ear, the α-enolase activity may promote persistence rather than bacterial clearance. Hence, bacterial infection can promote NET formation, which under certain conditions may augment persistence. While the function of a multispecies infection in NET formation and function has not been investigated, it is likely that in the context of biofilm formation in the middle ear, H. influenzae and S. pneumoniae can exploit the host immune response to facilitate a more protected biofilm lifestyle.

**Conclusion**

In disease situations, H. influenzae and S. pneumoniae form a multispecies biofilm; however, the nature of this interaction is only starting to be understood. The review of the literature collates research from different perspectives and shows that the current research suggesting the relationship between these pathogens may be strain specific and may depend on multiple and possibly strain-specific factors (Fig. 1). Synergistic interactions may be enhanced in strains with an ability to generate a structured EPS matrix. Evidence is emerging that type IV pili associations with eDNA function in the EPS matrix and could lead to strain selection within the multispecies biofilm. In the future, transcriptomic and proteomic analyses of the multispecies biofilms may help to establish the stress response and EPS-component genes that are involved in generating and maintaining the multispecies H. influenzae/S. pneumoniae biofilm. It is also important to note that the effects of strain or serotype replacement of either S. pneumoniae or H. influenzae as a result of vaccine use and novel vaccine development on multispecies interactions remain unknown. Such alterations in microbial ecology of the nasopharynx could potentially be either detrimental or beneficial for interspecies interactions and could thus either reduce or augment the risk of COM. Such a risk would need to be noted when introducing new vaccines directed against the common otopathogens H. influenzae and S. pneumoniae. Understanding the interactions between these species will enable selection of drugs that may inhibit these interactions and thus their capacity to form a multispecies biofilm as a means of survival. The upregulation of pilA in the biofilm, for instance, suggests the possible involvement of the type IV pili in biofilm formation. Administering a Tfp blocker may reduce the multispecies biofilm formation. Means of dispersing the biofilm structure in COM patients by targeting the structures that allow its formation may assist in the administration of other therapeutic agents such as antibiotics, which may otherwise not be able to permeate into the biofilm. In addition, if S. pneumoniae pH-mediated competition is important in the multispecies biofilm clinically, then administering a urease inhibitor may reduce the ability of H. influenzae to survive in the multispecies community and contribute to the multispecies biofilm formation. Further studies will likely expose new targets for drug design that may be involved in the multispecies biofilm formation. In addition, it is important to note the strain variation present among the H. influenzae and S. pneumoniae isolates, which may determine the differences in their interspecies interactions and also in the molecular factors utilized to facilitate these interactions. So, while new molecular targets for COM therapy will be identified, future therapy should focus on diagnosing the disease based on the specific isolates involved and administering therapeutic agents based on the classification of the strains involved. Another aspect that is open for investigation in OM therapy of the future should be the prognosis of OM development; specifically, any strain-specific therapy that is utilized should optimally involve screening of nasopharyngeal communities to analyse the probability of strain replacement and the development of subsequent OM cases. This will allow the optimal selection of therapy on an individual basis.

**Acknowledgements**

We acknowledge the support of the Research Centre for Infectious Diseases.

**References**


Nuutinen J, Torkkeli T & Penttilä et al. (2000) Pathogens and Disease a decade after the advent of conjugates.


