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Non-typeable *Haemophilus influenzae* chronic colonisation in Chronic Obstructive Pulmonary Disease (COPD)

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Key words: *Haemophilus influenzae*, biofilm, COPD, chronic colonisation

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Abstract

*Haemophilus influenzae* is the most common cause of bacterial infection in the lungs of chronic obstructive pulmonary disease (COPD) patients and can lead to episodes of acute exacerbation resulting in increased hospitalisation and mortality. Although *H. influenzae* has developed multiple mechanisms to prolong its colonisation in the lower airways of COPD patients, a key reason for this persistence is the ability of *H. influenzae* to adhere to host epithelial cells in order to form biofilms. The formation of biofilms is associated with changes in bacterial behaviour such as reduced cellular metabolism and production of an obstructive extracellular matrix (ECM). Herein we discuss the mechanisms by which *H. influenzae* adheres to host airway epithelial cells to induce the formation of biofilms, the role these biofilms play in the pathogenesis of COPD and the mechanisms by which these cellular aggregates promote persistence in the lungs through immune system evasion and antibiotic tolerance.
Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive but preventable condition associated with loss of lung function because of exposure to noxious particles or gases (Vogelmeier et al., 2017). It is typically characterised by airway obstruction due to small airways disease and parenchymal destruction in varying proportions (Rabe and Watz, 2017).

An increase in disease severity is linked to a greater frequency of events associated with a sudden decline in lung function, known as an acute exacerbation of COPD (AECOPD) (Vogelmeier et al., 2017). These heterogeneous events, caused by interactions between pathogens, the lung environment and the associated host response, lead to an increase in airway inflammation and a worsening of clinical symptoms (Sapey and Stockley, 2006; Sethi and Murphy, 2008). Furthermore, disrupted innate lung defences contribute to an increased frequency of airway infections making the patient more susceptible to AECOPD (Sethi, 2010). Knowledge of the nature of the inflammatory changes within the airways during exacerbations is however, limited due to the risks involved in carrying out bronchial biopsies in patients with moderate or severe COPD (Hogg, 2004).

Viruses are detected in approximately 50% of COPD exacerbations (Seemungal et al., 2001; Rohde et al., 2003), but an association between exacerbations and bacterial infections is more difficult to assess. This is because, in addition to acute bacterial infections driving the acquisition of AECOPD, the airways of COPD patients are intermittently colonised by pathogenic bacteria, with bacteria present at both stable and periods of exacerbation. Molecular science techniques such as strain typing allows specific bacterial strains to be isolated from patients and provided evidence that the acquisition of new bacterial strains is a key trigger for an exacerbation (Veeramachaneni and Sethi, 2006). The associated, inappropriately elevated, inflammatory response (Sethi and Murphy, 2008) is then driven by the higher levels of immune cells present in COPD airways such as macrophages,
neutrophils, eosinophils and dendritic cells (Barnes, 2014; Di Stefano et al., 1994; Finney et al., 2014; Van Pottelberge et al., 2010).

Of all the bacterial strains capable of colonising the respiratory tract of COPD patients, *H. influenzae* is arguably the most clinically relevant. Non-Typeable *H. influenzae* (NTHi) is frequently isolated from COPD patients (Sethi et al., 2002) and is associated with 30% of acute exacerbations (Sethi, 2010). Additionally, NTHi is detected in ~30% of COPD patients during stable disease over extended periods of time (Marin et al., 2010; Patel et al., 2002; Wilkinson et al., 2003). This indicates that NTHi has a number of mechanisms utilised in the airways that surmount innate and adaptive host defences as well as antibiotic treatments. This review will summarise the current understanding of *H. influenzae* biofilm formation and other mechanisms employed to enable persistence within the COPD lung environment.

*Haemophilus influenzae*

*H. influenzae* is a human-restricted, Gram-negative coccobacillus that exists as a commensal organism within the nasopharyngeal flora of most humans (Erwin and Smith, 2007). When growing aerobically, *H. influenzae* requires the presence of hemin and nicotinamide adenine dinucleotide (NAD; factors X and V, respectively) (Artman et al., 1983). Strains of *H. influenzae* are classified based on the presence, or absence of a chemically distinct polysaccharide capsule, which are divided into six serotypes (a to f) (Falla et al., 1994). Strains lacking a polysaccharide capsule are designated as non-typeable *H. influenzae* (NTHi).

As well as being a major cause of AECOPD, *H. influenzae* has been implicated in other conditions such as the inflammatory disease of the middle ear, otitis media (OM) and pneumonia (Gilsdorf et al., 2004). *H. influenzae* normally spreads from person to person by inhalation of respiratory droplets or through direct contact of respiratory secretions (King,
812012). *H. influenzae* serotype b (Hib) strains cause invasive infections in infants and children 
but since the development of the Hib conjugate vaccine, infection by *H. influenzae* is 
predominately due to NTHi (Van Eldere et al., 2014; Zarei et al., 2016). 
NTHi strains commonly colonize the upper respiratory tract and cause mucosal infections in 
children and adults (Agrawal and Murphy, 2011).

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**Initial adherence of NTHi to the COPD airway**

The first step in the timeline of bacterial infection is the initial adhesion to the host however, 
as a respiratory pathogen, NTHi must first overcome the mucociliary escalator before 
attachment to the airway epithelium can occur (Ganesan et al., 2013). NTHi possess a 
number of cell surface proteins (summarised in Figure 1) that facilitate adhesion to and 
subsequent invasion of the respiratory mucosa, enabling escape from host immune defences. 
The type IV pili (T4P), regulated by the products of the *pilA* and *comE* genes, are composed 
of helically arranged pilin subunits that form filamentous polymer assemblies. Their presence 
on the surface of a number of Gram-negative bacteria facilitates uptake of DNA across the 
bacterial cell membrane, mediates adherence to mammalian epithelial cells and promotes 
genetic adaptability which also contributes to biofilm formation (Jurcisek et al., 2007). *H. 
influenzae* fimbriae bind to mucins (Kubiet et al., 2000) with the T4P associated with high 
levels of adherence to the Intracellular Adhesion Molecule-1 (ICAM-1) (Novotny and 
Bakaletz, 2016). ICAM-1 is an adhesion molecule which is part of the immunoglobulin 
superfamily. It facilitates reversible adhesion and signal transduction between cells but is also 
exploited by a number of bacterial and viral pathogens such as NTHi and rhinovirus to 
facilitate their own adhesion and uptake. ICAM-1 is present on the surface of a number of 
different cell types including airway epithelial cells at a basal level, but is increased following 
exposure to cigarette smoke (Scott and Palmer, 2003) and in the airway epithelial cells of
smokers and COPD patients (Shukla et al., 2017). Exposure to NTHi can increase the expression of its receptor in airway epithelial cells through upregulation of ICAM-1 (Avadhanula et al., 2006). The presence of further attachment points on the surface of airway epithelial cells then act as a positive feedback loop for attachment and infection by NTHi. The P1 fimbriae bind to the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) cell surface glycoprotein present on the surface of epithelial cells (Tchoupa et al., 2015) whereas the P5 fimbriae facilitates cellular adhesion via mucins, CEACAM-1 and ICAM-1 (Avadhanula et al., 2006).

The exposed extracellular matrix (ECM) present within the COPD airway environment also facilitates attachment of NTHi by *Haemophilus* Adhesion Protein (HAP) (Fink et al., 2002). HAP is a ubiquitously expressed adhesion protein that binds to basement membrane components such as fibronectin, laminin and collagen IV (De Chiara et al., 2014; Sekiguchi and Yamada, 2018). HAP, however, is not required for biofilm formation (Hendrixson and St Geme, 1998). Additionally, the P4 fimbriae adheres to fibronectin, laminin and vitronectin, and, as with P1, P2 and P5, is present in biofilms (Murphy and Kirkham, 2002; Wu et al., 2014a). Protein E of NTHi is involved in adhesion and activation of epithelial cells (Singh et al., 2010), interacts with laminin and vitronectin present within the ECM (Hallstrom et al., 2011) and upregulates IL-8 and ICAM-1 expression in airway epithelial cells (Singh et al., 2013). Protein E is also involved in evading host defences by binding to the proenzyme plasminogen causing its catalytic conversion to plasmin (Osman et al., 2018), a serine protease which, in addition to its primary role in fibrinolysis, has been shown to inhibit the complement pathway (Godier and Hunt, 2013).

Additionally, the NTHi high molecular weight (Hmw) adhesins Hmw1 and Hmw2 are key glycoproteins that display high levels of homology and are also key proteins in the process of binding to respiratory epithelial cells (Geme III and Yeo, 2009; Rempe et al., 2016).
Expression of both Hmw1 and Hmw2 are associated with invasive and virulent strains of *H. influenzae* (Vuong et al., 2013). In addition to Hmw proteins, *H. influenzae* possesses proteins that mediate adhesion to epithelial cells. Using dual RNA-sequencing, Baddal et al. (2015) identified the upregulation of multiple virulence factors, which included the gene encoding *Haemophilus influenzae* adhesion (Hia), another cell surface adhesion molecule found to mediate adherence to airway epithelial cells (Spahich and St Geme, 2011).

The adhesion molecules described above, highlight adaptations that allow HTHi to adhere to airway epithelial and exploit the damaged, chronically inflamed environment present in the airways of COPD patients. Although adhesion to the airways facilitates infection, it is also the critical first step in the formation of a biofilm (Rabin et al., 2015).

**NTHi biofilms and bacterial persistence**

A widely utilised mechanism of bacterial persistence within the respiratory tract is the formation of biofilms (Murphy and Kirkham, 2002; Murphy et al., 2004; Murphy et al., 2005). Biofilms are described as a community of cells encased in a self-produced ECM, adhered to a biotic or abiotic surface (López et al., 2010). In the airways, biofilms may act as reservoirs of bacteria, which can trigger the reoccurrence of lower respiratory infections and contribute to frequent exacerbator phenotypes in COPD (Sethi, 2010). They are characterised by changes in cell behaviour, such as differential gene expression and ECM production which, in turn, can lead to increased virulence, antimicrobial tolerance and decreased susceptibility to killing from immune cells (Singh et al., 2017). Biofilms also differ significantly in structure within the lungs in that, instead of forming the ‘traditional’ mushroom-like structure, they take on a small aggregate phenotype (Darch et al., 2018). These aggregates contain between $10^1$ and $10^4$ cells whilst retaining biofilm-associated characteristics such as increased antimicrobial tolerance.
Using a multi-omics approach, Harrison et al. (2019) characterised the transcriptome, metabolome and proteome of *H. influenzae* to identify the driving forces behind its biofilm formation. Significant increases in amino acids and enzymes involved in central metabolic processes such as serine and glycine were identified, along with increases in adhesin production in combination with enriched DNA metabolism pathways. Another interesting observation from the same study was a significant increase in the expression of an uncharacterised ATP-binding cassette (ABC) periplasmic protein. Many members of this protein family provide bacteria with multi-drug resistance (Wilkens, 2015) therefore, the increased expression of these pathways have been hypothesised to increase *H. influenzae* survival *in vivo* through contribution to horizontal gene transfer throughout the biofilms.

As previously discussed, adhesion proteins of *H. influenzae* play a key role in the attachment of the bacteria to epithelial cells. Indeed, it appears that *H. influenzae* increases adhesin protein production in response to contact with human respiratory epithelia as evident in co-cultures where the expression of T4P is significantly increased within 30 minutes following exposure (Mokrzan et al., 2019). In agreement with these findings, (Baddal, 2020) showed that *H. influenzae* adhesion to Calu-3 cells increased in a time dependant manner, up to 72 hours, at which point the colonising bacterial cells were also found to induce epithelial cell apoptosis.

In 2013, Bjarnsholt and colleagues compared bacterial biofilms formed *in vivo* to those grown *in vitro*. Biofilms communities have been studied *in vitro* for decades but it is extremely difficult to properly mimic *in vivo* conditions such as host immune response and nutrient availability (Bjarnsholt et al., 2013). Biofilms formed by *H. influenzae* *in vivo* can therefore, differ greatly from those formed *in vitro* (Brown et al., 2019b). From analysing images of *in vivo* *H. influenzae* biofilms formed on the middle ear of chinchillas, Brown et al. (2019b) developed a reproducible, *in silico*, agent-based model to simulate biofilm formation.
Results of this model showed that *H. influenzae* biofilms *in vivo* are formed following a similar spatial distribution pattern to its *in vitro* counterparts. However, *in vivo* biofilms were more than 10-fold smaller, likely as a result of host factors clearing planktonic and poorly integrated biofilm cells. These data highlight the importance of closely replicating host environments when studying biofilms in disease pathogenesis.

In many species of bacteria, proper biofilm development is dependent on the release and uptake of density-dependant small molecules, known as quorum sensing (Miller and Bassler, 2001). In biofilms, quorum sensing pathways can regulate development, whereas others can induce or promote biofilm dispersal. One such quorum sensing network is the auto-inducer 2 (AI-2) pathway which is controlled by the *luxS* operon. Utilising a *H. influenzae* mutant, where the *luxS* operon was controlled by the *xylA* promoter (which is activated in the presence of xylose), Pang et al. (2018) showed that *luxS* operon activation and interruption influenced biofilm maturation and dispersal, respectively. Induction of *luxS* synthesis also resulted in increased transcription of a glycosyltransferase enzyme. In other bacterial biofilms formed by streptococcal species, glycosyltransferases are key enzymes in the production of biofilm ECM which also aid the integration of opportunistic fungal pathogens such as *Candida albicans* (Souza et al., 2020).

The ECM of *H. influenzae* biofilms plays an integral role in resisting the effects of the host immune system by providing physical protection against phagocytosis and neutrophil extracellular traps (NETs) (Juneau et al., 2011; Langereis and Hermans, 2013). In a study by Murphy and colleagues the formation of biofilms by clinical isolates of NTHi was found to be dependent on the production of a 30-kDa protein identified as peroxiredoxin–glutaredoxin (pdgX). PdgX levels were significantly increased in NTHi biofilms compared to planktonic growth and 44% of study participants displayed considerably higher levels of antibodies to pdgX (Murphy et al., 2005). As well as being crucial for effective biofilm production, pdgX
provides protection against oxidative stress induced by neutrophils invading NETs (Juneau et al., 2015).

Additional key components of *H. influenzae* biofilms which have multiple crucial roles are extracellular DNA (eDNA) and DNABII proteins (Devaraj et al., 2018; Marti et al., 2017). DNABII proteins and eDNA are released into the surrounding area, with *H. influenzae* Δ*comE* strains failing to secrete both proteins. *H. influenzae* T4P is also expressed through the pore formed by the *comE* product (Das et al., 2017; Jurcisek et al., 2017), suggesting this pilus acts as a type 4 secretion system that is utilised to ‘inject’ eDNA and DNABII proteins into the biofilm matrix. In addition to stimulating biofilm formation, eDNA binds to human β-defensin-3, reducing its antimicrobial properties (Jones et al., 2013).

As well as the underlying transcriptional changes that accompany biofilm development, production of a robust ECM and immunoglobulin A (IgA) proteases are key to the immunoresistance and persistence shown by *H. influenzae*. IgA proteases produced within a biofilm can integrate into the ECM but are primarily found on the matrix surface where they thwart the host immune response by cleaving human IgA, a critical first line immunoglobulin localised in mucus membranes. The *H. influenzae* genome codes for two types of IgA proteases, *igaA* and *igaB*. All strains are able to produce *igaA* and 40% of strains have *igaB* (Murphy et al., 2015). There are two variants of each protease, with varying specificities of the hinge region found in human IgA. These proteases are variably expressed *in vivo* but play an important role in bacterial survival in respiratory epithelial cells (Murphy et al., 2017). Although this is yet to be confirmed, *igaB* appears to play a larger role in bacterial survival than *igaA*.

Despite a high level of heterogeneity between biofilms produced by clinical isolates of *H. influenzae* (Puig et al., 2014), biofilm size does not influence antimicrobial tolerance. Reimche et al. (2016) tested the effect of 4 antibiotics (amoxicillin, azithromycin,
clarithromycin and ceftriaxone) on pre-formed *H. influenzae* biofilms and found no correlation between biofilms of differing biomass and/ or thickness and their ability to survive treatment.

Antibiotics are a commonly used tool in the treatments of AECOPD, however, sub-inhibitory concentrations of β-lactams have been shown to induce *H. influenzae* biofilm formation through the formation of tightly packed biofilms of increased overall biomass, albeit containing a reduced number of viable bacteria (Wu et al., 2014b). The semi-dormant state of *H. influenzae* biofilms display reduced metabolism and protein synthesis while still maintaining the ability to modulate responses to oxidative stress (Post et al., 2014). This altered cell activity exacerbates the additional challenge of limited drug diffusion through biofilm ECM components (Singh et al., 2017). Decreased protein synthesis also minimises the effects of antibiotics that operate as protein synthesis inhibitors, such as macrolides (Kanoh and Rubin, 2010).

In addition to β-lactams, cigarette smoke extract (CSE) and electronic cigarette vapour extract (ECVE) have been shown to significantly influence *H. influenzae* biofilm pathogenicity (Gilpin et al., 2019). Both CSE and ECVE significantly increased *H. influenzae* pathogenicity in the *Galleria mellonella* infection model while also causing a significant increase in interleukin-8 (IL-8) and tumour necrosis factor α (TNF-α). Although both extracts exhibited a stimulatory effect on the biofilm production of a number of isolates such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the effect on *H. influenzae* biofilm formation was not significant.

Heteroresistance is a phenomenon frequently observed in pathogenic bacteria where subgroups of bacteria possess a lower susceptibility to an antibiotic treatment than the general population (Nicoloff et al., 2019). Planktonic *H. influenzae* have been shown to display a heteroresistant phenotype against imipenem (Cherkaoui et al., 2017). It can be expected that
This tolerance would be strengthened within a biofilm community and likely attributable to the differential behaviour of biofilm integrated cells (Flemming et al., 2016). For example, fluctuating gene expression and cell metabolism can lead to the development of heteroresistant and persisting cell phenotypes.

**Polymicrobial biofilms**

Biofilms are rarely composed of a single species and the clinical significance of these polymicrobial biofilms is becoming increasingly recognised. The first reports of the polymicrobial nature of biofilms can be dated back to over three centuries ago where Antoni van Leeuwenhoek drew illustrations representing *Streptococcus* chains and *Fusobacteria* which he had observed in dental plaque (Brown et al., 2019a). To date, dental plaque remains one of the most studied and best characterised models of polymicrobial biofilms. 16S RNA sequencing has shown that the oral microbiome is similar to the lung (Bassis et al., 2015), indicating a relationship between oral and lung microbiomes. Salivary flow and microaspiration have been identified as major causes or bacterial migration from the oral cavity to the lungs resulting in oral pathogens such as *Porphyromonas* and *Prevotella* entering the lower respiratory tract (Huffnagle et al., 2017). This migration creates an opportunity for *Porphyromonas* gingivalis, a prolific oral pathogen, to interact with other bacteria such as *Streptococcus*, one of the most abundant genera in COPD lungs (Millares et al., 2019; Sinha et al., 2018; Zakharkina et al., 2013); an interaction that is known to be synergistic, promoting biofilm formation (Simionato et al., 2006). Within a polymicrobial biofilm organisms are in constant communication with one another either through direct contact or via the release and uptake of quorum sensing molecules (Peters et al., 2012).

As the lungs of COPD patients are home to a plethora of microorganisms (Garcia-Nuñez et al., 2015; Wang et al., 2016; Wang et al., 2017) multiple opportunities exist for pathogens such as *H. influenzae* to interact with other bacteria and fungi. Limited attention has,
however, been given to characterising the interactions between respiratory pathogens. A number of studies have focused on the relationship between *H. influenzae* and *S. pneumoniae* and their impact on OM which show a competitive link between the organisms. For example, a study by Bair and Campagnari (2020) reports *S. pneumoniae* to cause a rapid decrease in viable *H. influenzae* cells in dual-species biofilms after 24 h, which supports findings by Thornton et al. (2011) who failed to observe both species together in the same specimen from children with OM.

In acute OM cases, when isolated in combination with *S. pneumoniae*, *H. influenzae* was significantly more likely to be able to produce biofilms (Vermee et al., 2019). These two pathogens have previously been reported to modulate the expression of each other’s virulence genes with transcription of *H. influenzae* T4P significantly increased in dual-species biofilms (Cope et al., 2011). Using a chinchilla infection model of OM, a polymicrobial *in vitro* biofilm of both *H. influenzae* and *S. pneumoniae* was able to persist in the middle ear due to *H. influenzae* providing *S. pneumoniae* with protection against amoxicillin treatment (Murrah et al., 2015). While previously, *H. influenzae* 86-028NP, a β-lactamase producing strain, was found to protect both planktonic and biofilm *S. pneumoniae* cells from amoxicillin (Weimer et al., 2011). β-lactamase production is however, undoubtedly not the sole mechanism for antibiotic protection. Although the mechanisms behind this have yet to be explored, it could be hypothesised that the combined, increased production of bacterial ECM prevents diffusion of antimicrobials, therefore protecting both organisms.

**Additional mechanisms of persistence**

**Epithelial cell invasion**

As discussed previously, NTHi utilises a range of adhesin proteins to adhere to the human respiratory tract. If the host fails to clear the invading bacteria, NTHi cells can then follow
one of two paths that ultimately result in chronic colonisation. The first path leads to the bacterium being engulfed by host respiratory epithelial cells. Secondly, the bacteria may either remain on the epithelial cell surface or will have been bound by other host factors such as fibronectin potentiating the development of a biofilm.

*H. influenzae* is widely regarded as an extracellular pathogen and has shown the ability to persist in the intercellular space between epithelial cells thanks to the adhesion and invasion protein Hmw1 (Mell et al., 2016). The ability of *H. influenzae* to cause the downregulation of protocadherin 8 and cadherin 6, which are important in epithelial cell-cell interactions, and claudin 3 and claudin 8, which play a role in tight junction formation, highlight other potential mechanisms that may explain the ability of *H. influenzae* to invade intracellular space (Baddal et al. (2015).

In addition to this, evidence also exists showing *H. influenzae* is able to invade epithelial cells and persist. Ahearn et al. (2019) identified an open reading frame (ORF) in *H. influenzae* as a key virulence factor involved in cell invasion. Although the product of this ORF is yet to be fully characterised, the ability of *H. influenzae* cells lacking ORF NTHI1441 to invade bronchial epithelial cells was significantly reduced, even though cellular attachment was observed.

*H. influenzae* also utilises the outer membrane protein P1 (OmpP1), a receptor for bactericidal long chain fatty acids (LCFA), to invade epithelial cells, via the CEACAM-1 receptor (Moleres et al., 2018) with *H. influenzae fadL* (which codes for OmpP1) null mutants much less effective. Computational analyses showed host factors pressure loss of function mutations in *fadL*, which ultimately reduces *H. influenzae* invasion potential but increases tolerance to bactericidal LCFA which may benefit long term persistence.

Genetic adaptations
Mechanisms such as antigenic variation, epigenetic variation and phase variation are also exploited by *H. influenzae* to increase fitness and prolong persistence within the host (Ahearn et al., 2019). In addition, where encapsulated *H. influenzae* rely on their capsule to evade the immune system, NTHi strains have been forced to develop alternative mechanisms. A number of genes have been identified which are involved in protection against complement mediated killing (Nakamura et al., 2011). The majority of these genes are related to lipooligosaccharide (LOS) synthesis. *H. influenzae* LOS structure is controlled by the expression of a number of genes such as *lic1A, lic2A, lic3A, lic3B* and *lex2A* (Phillips et al., 2019). LOS gene expression is regulated via phase variation, which allows for *H. influenzae* to quickly adapt in response to the external environment and therefore increasing bacterial fitness while inhabiting the human respiratory tract. Expression of adhesion genes such as *hmw* and *hia* are also controlled through phase variation (Atack et al., 2015b; Giufrè et al., 2008). For example, passage of *H. influenzae* cells through a chinchilla model of infection selected for cells that highly expressed the Hia protein proving that *H. influenzae* rapidly responds to its environment.

Despite playing an integral role in colonisation and persistence, surface adhesins such as Hmw1 and Hmw2 elicit a strong host immune response (Winter and Barenkamp, 2014) but this response can be moderated by *H. influenzae* using phase variation. Davis et al. (2014) reported that *H. influenzae* is able to repress the expression of *hmw* genes thanks to the presence of 7bp tandem repeats located within the promoter region. This same study showed that the protein production decreases as the number of tandem repeats increase.

Epigenetic variation in *H. influenzae* is dependent on *modA* expression. *H. influenzae* possess over 20 variations of *modA* alleles (*modA1 – modA22*). *ModA* genes encode a DNA-methyltransferase, which is prone to random on/off switching (Atack et al., 2015a). Isolates from patients with COPD possessed higher amounts of variation in the expressed *modA*
alleles highlighting the importance of epigenetic variation in providing *H. influenzae* with the necessary advantages to allow survival within the human respiratory tract. Specifically, Δ*modA10 H. influenzae* showed increased attachment to and invasion of human bronchiole epithelial cells (VanWagoner et al., 2016). When ‘switched on’ *modA2* increases bacterial susceptibility to oxidative stress and neutrophil mediated killing (Brockman et al., 2017). Cells expressing *modA2* form biofilms with significantly greater biomass compared to when this gene is ‘switched off’. Despite greater biomass, the ECM of these biofilms was deficient in eDNA (Brockman et al., 2018). It can be deduced that in contrast to *modA10*, *modA2* is detrimental to the survival of *H. influenzae*.

*H. influenzae* also exhibits heterogeneity between strains in the presentation of surface antigens, the differential expression of which results in strain specific immune responses (De Chiara et al., 2014; Sethi et al., 2004). Contrary to phase variation, antigenic variation in proteins such as P1, P2 and P5 occur as a result of insertion and deletion mutations which have been reviewed extensively elsewhere (Gilsdorf et al., 2004).

**Future directions**

Most of our understanding of *H. influenzae* biofilms and persistence during infection still comes from OM, highlighting a need to address the current gaps in our knowledge of COPD pathology. Future work in addressing the role of *H. influenzae* biofilms in COPD should focus on developing definitive criteria for the diagnosis of biofilms in the respiratory tract and methods that can be used to confirm their presence. For example, a promising new technique of real-time molecular imaging could prove useful in the identification of *H. influenzae* biofilms. This method uses the environmentally sensitive fluorophore, 7-nitrobenz-2-oxa-1,3-diazole conjugated to a polymyxin which fluoresces upon contact with the lipid A component of Gram-negative bacteria (Akram et al., 2018). More recent studies have shown that similar smart probes can be used to label cell metabolites (Benson et al.,
Combining these two methods may provide a crucial opportunity to develop a method of selectively labelling molecules associated with *H. influenzae* biofilms such as pgdX, which if successful, could allow for the rapid diagnosis of *H. influenzae* biofilms in the COPD lung or indeed other conditions. Importantly, recent studies have begun to unravel the complex network of inter-kingdom interactions between bacteria and fungi in lung diseases such as cystic fibrosis and pneumonia. Despite the mycobiome of the COPD lung being previously defined (Su et al., 2015) the interactions between fungi and bacteria in COPD have yet to be studied in detail. Fungi such as *C. albicans* and *Aspergillus fumigatus* are known to regularly interact with bacteria causing more serious infection (Kean et al., 2017; Reece et al., 2017). Future studies should aim to confirm if there is indeed co-association of fungi and bacteria in the COPD lung and should investigate if these interactions play a role in the pathogenesis and progression of COPD.

**Conclusions**

As a pathogen with a major impact on the lives of COPD patients due to chronic colonisation, it is important to understand the underlying mechanisms of persistence employed by *H. influenzae*. One of the many methods of persistence utilised by *H. influenzae* is the formation of biofilms. The development of these complex microbial communities provides numerous advantages over the host immune system and present multiple challenges for effective antimicrobial therapies. Despite current advances in the field of *H. influenzae* biofilms, some aspects of this process particularly around the perceived synergy of polymicrobial biofilms remain enigmatic. Future work should aim to build upon our understanding of *H. influenzae* biofilms within COPD airways which will be aided by the development of more refined and accurate diagnostic...
tools for *in situ* biofilm identification. The rapid and continuous advancement of sequencing tools, should also be exploited to further characterise the molecular mechanisms behind *H. influenzae* biofilm formation. This would enable the identification of key metabolites and proteins within biofilm communities that could be utilised as novel targets for new antimicrobial therapies. The ability to prevent *H. influenzae* biofilm development or the provision of a rapid diagnosis and effective treatment options would be an extremely beneficial step to reduce AECOPD and help stall the devastating progression of COPD.
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**Figure 1**: Summary of the roles of adhesion molecules present in strains of NTHi. Panel A shows the interactions of P1 and P5 adhesins as well as the type 4 pili with cell surface glycoproteins CEACAM-1 and ICAM-1 to facilitate adherence to the surface of airway epithelial cells. Panel B highlights the roles of the P4 adhesin, PE and HAP in adhering to components of the basement membrane present in the airways. Panel C shows the adhesion of HiF and the P5 adhesin to mucins present in the mucus secreted in the airway as well as the enzymatic conversion of plasminogen to plasmin by PE to disrupt the complement cascade. This figure was created using BioRender.

**Figure 2.** *Haemophilus influenzae* biofilms interact with the host and antibiotics. *H. influenzae* biofilms utilise a number of mechanisms to persist in the host. Production of a robust ECM aids in the resistance to neutrophils and NETS (1) and IgA proteases on the matrix surface cleaves IgA (2). The thick ECM also prevents phagocytosis by blocking macrophage access to biofilm cells (3), pairing this with lowered cell metabolism and protein synthesis the activity of antibiotics (4) on biofilm cells is minimal. Another important mechanism of resistance is eDNA that is incorporated into the biofilm matrix which binds β-defensin (5), reducing its antimicrobial properties. Finally, likely as a stress response, β-lactam antibiotics induce biofilm formation in *H. influenzae* (6). Flat headed arrows indicate an inhibitory effect whereas pointed arrows indicate a stimulatory effect.
A. NTHi – Epithelial cell interactions

B. NTHi – ECM component interactions

C. NTHi – Immune component interactions

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