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Interactions and Mechanisms of Respiratory Tract Biofilms Involving *Streptococcus Pneumoniae* and Nontypeable *Haemophilus Influenzae*

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Abstract

The pathology associated with human respiratory tract bacterial agents that exist as opportunistic commensals in the nasopharynx cause infections. This is particularly true for the middle ear disease otitis media (OM) and exacerbations of chronic obstructive pulmonary disease (COPD). *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are a commonly recurrent combination and the formation of bacterial biofilms by these pathogens in the bronchial airway or middle ear contributes significantly to the chronic nature of these diseases. While *S. pneumoniae* and NTHi have been extensively studied in mono-culture, our knowledge about how they exist together, either in their free-living (planktonic) form or as a biofilm, or indeed the implication of co-infection is still limited. Several key elements are believed to contribute or are induced: (1) a set of sugar metabolic pathways; (2) surface structures in *S. pneumoniae* and NTHi when they are able to co-exist equally; (3) epithelial cell contact that dramatically increases the rate of biofilm formation; (4) chemical modifications of NTHi surface structures involved in host cell interactions; and (5) transcription factors that regulate particular surface molecules and the switch to a biofilm state. There appears to be multiple mechanisms involved and that these are active under specific conditions.

Keywords: biofilm metabolism, multispecies biofilm, *Streptococcus pneumoniae*, *Haemophilus influenzae*, metal ions in biofilm
1. Introduction

Human respiratory tract bacterial infections, like otitis media (OM) and exacerbations of chronic obstructive pulmonary disease (COPD), are caused by bacterial agents that exist as opportunistic commensals in the nasopharynx. *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are a commonly recurrent combination. The formation of bacterial biofilms in the bronchial airway or middle ear contributes significantly to the chronic nature of these diseases. Biofilms are very difficult to remove by either the host’s natural processes or antibiotic therapies, making them an important element within the vicious cycle of the infection exacerbations.

*S. pneumoniae* and NTHi are extensively studied individually, yet our knowledge about how they exist together, either in their free-living (planktonic) form or as a biofilm, or indeed the implication of co-infection is still limited in contrast to a single species. Studies have shown that in mono- and co-culture planktonic states and in biofilm development several key elements contribute or are induced. These include: (1) a set of sugar metabolic pathways employed especially by *S. pneumoniae* in co-culture when it dominates; (2) surface structures in *S. pneumoniae* and NTHi when they are able to co-exist equally; (3) epithelial cell contact that dramatically increases metabolic process associated with biofilm formation; (4) chemical modifications of NTHi surface structures that have a direct role in the interaction with host epithelial cells; and (5) certain transcription factors that have an integral role in the regulation of particular surface molecules and the switch to a biofilm state. There appears to be multiple mechanisms involved and that these are active under specific conditions.

2. Co-existence within the multispecies biofilm as a mode of bacterial resistance and persistence

In most environmental situations that bacteria exist, they are within communities and in a biofilm. By definition therefore, in nature these are multi-species biofilms. It is surprising, therefore, that the vast amount of knowledge that exists on bacterial biofilm formation and function is from mono-species studies. How the individual species function within an environment, the physical and chemical nature of their biofilm and its eventual impact on the environment (this is particularly true of bacterial persistence within an anatomical niche) will be different when as a mono-culture compared to multi-species culture. *H. influenzae* is a commensal bacterial species that inhabits the nasopharynx of healthy humans, and it is accepted knowledge that its asymptomatic nasopharyngeal carriage is in the range of up to 80% [1]. However, *H. influenzae* is not the only species to colonise the nasopharyngeal niche; the other bacterial species within this niche include *S. pneumoniae*, *Staphylococcus aureus*, and *Moraxella catarrhalis*. Most commonly the species known to co-colonise the nasopharyngeal niche with *H. influenzae* is the Gram-positive species *S. pneumoniae*. Asymptomatic nasopharyngeal carriage for *S. pneumoniae* has been documented to be at least 20% [2].

Both *H. influenzae* and *S. pneumoniae* are able to transit from this site of their commensal lifestyle to other anatomical niches and thereby cause various diseases. This includes the subsequent
infection of the bronchi to cause bronchitis [3, 4], the lungs to cause pneumonia [5], the middle ear to cause OM [6], the blood to cause sepsis, and across the blood–brain barrier to cause meningitis [7]. An increasing number of clinical, diagnostic or epidemiological studies with a focus either on bacterial carriage or the microbiota within an infection have co-located *S. pneumoniae* and *H. influenzae* together [8]. Further to this, in many diseases there are other bacteria present – as mentioned, in the middle ear of OM patients there is *S. aureus* and *M. catarrhalis*, but then in different parts of the respiratory tract, these and other microorganisms are known to co-exist (whole genome sequencing of the bacterial population in patients with cystic fibrosis has shown the presence of a diverse range of bacteria including *S. pneumoniae* and NTHi but also *Pseudomonas aeruginosa*) [9]. In the case of OM, to some degree, there is evidence that at least infection with *S. pneumoniae* alone represents a different clinical and epidemiological case than compared to *S. pneumoniae* together with NTHi [10]. There seems to be a distinction also based on strains; specifically *S. pneumoniae* serotype variations effecting their colonisation and interaction with NTHi. Also, there are non-encapsulated *S. pneumoniae* strains that obviously have a different molecular pathogenesis but also cause OM, and have been shown to co-exist with NTHi [11].

Upon entry to their new niche *H. influenzae* and *S. pneumoniae* require systems that permit their adaptation to the specific physical and chemical properties that exist in the lung, middle ear, blood or cerebrospinal fluid. These include oxygen levels, pH, nutrient availability, the presence of toxic reactive chemicals (reactive oxygen and nitrogen species), and immune factors such as antimicrobial compounds. Given the likely inhospitable nature of migration from nasopharyngaeal niche, it seems necessary that there are eventual benefits from this switch in lifestyle. However, the specific molecular factors and signals that cause the transit from the commensal colonisation of the nasopharynx to, for instance, an invasion of sterile sites of the respiratory tract is not well known. There are clearly host factors such as the anatomy of the eustachian tubes [12], and then age and immune competence [13]. Within either their original commensal site or the further migrated locations (in particular the middle ear and the lung), it is known that *H. influenzae* and *S. pneumoniae* have an ability to persist for prolonged periods of time. In the first instance this requires the bacterial cells to attach and remain present. This process includes expression of appropriate adhesins and the ability to evade the host immune response. For both these bacterial species, an essential factor in colonisation and then their survival and persistence is their formation of a biofilm. In the case of the lung and middle ear, this is now known to be a multi-species biofilm.

3. The nature of bacterial persistence and resistance within a biofilm

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 (EPS) matrix [14, 15]. An important feature of bacterial biofilms is their persistent nature and their insensitivity to immune mediators and clinically used antimicrobial agents [16]. These features can be explained both by the changed physiology of the biofilm-resident bacterial cells themselves and by the physical properties of the EPS matrix components. The presence of an EPS matrix
provides protection and biofilm persistence by physically limiting the diffusion of antimicrobial compounds into the biofilm [17]. Additionally, within the biofilm the bacteria have altered gene expression profiles as compared to their planktonic state [18–20]. This switch in gene expression has global effects on cellular functions. This includes changes not only in the surface structures that are expressed for adhesion and cell-cell interactions, but also in the metabolic and biosynthetic pathways and the systems for maintaining intracellular conditions such as pH and redox balance [21]. There is a reduced metabolic activity; a reduction in energy production, cell division, protein synthesis, and other molecular pathways. These changes create a cellular state with an increased recalcitrance to a broad range of antimicrobial agents [22], at the very least they have reduced or no targets for many antibiotics (DNA replication, protein synthesis, and cell wall biosynthesis). The resistance provided by a biofilm state to the bacterial cells is against effectors of both the innate and acquired immunity as well as antibiotics. The nature of the biofilm (its chemical composition and physical properties), the process of its initiation and formation, and the eventual maturation (the structure), will impact the function and stability of the biofilm. This will be different for a mono-culture compared to a multi-species biofilm.

In summary, a model for different stages of biofilm development by *H. influenzae/S. pneumoniae* is well demonstrated [23]. Figure 1 shows this model in the development of the mixed-species biofilm. Stage 1 involves adaptation and adhesion, where the bacteria recognise the physical and chemical conditions of their new environment (such as the oxygen level, nutrients, and pH) and indeed the biological conditions (the immune mediators that are present and the host cells and their receptors). Specific bacterial adhesins bind to cognate host cell receptors. These surface exposed adhesins structures are expressed with the particular function for attachment to host cells. For *H. influenzae*, these include the type IV pili, lipooligosaccharide (LOS) decorated to form sialylated LOS or phosphorylcholine LOS (discussed later), outer membrane proteins (OMP P5, P6, Hap, HMW1, HMW2), and extracellular DNA (eDNA). For *S. pneumoniae*, there is also a role for eDNA including a range of surface structures and proteins such as capsule, Pho, CbpA, PsrP. Stage 2 is the recognition and response to the interspecies stresses. This involves the expression of systems designed for survival in the presence of the stresses generated by the other species such as; chemical stresses, pH, and immune-mediated stress. *S. pneumoniae* growth generates acidic by-products that lowers the local pH. It is also well known that *S. pneumoniae* can produce H$_2$O$_2$ either naturally or perhaps by induction, and this has been argued to be a factor in *S. pneumoniae* out-competing *H. influenzae* (although there is evidence that this not the case) [24]. In addition, *S. pneumoniae* produces an extracellular enzyme (NanA) that desialylates the sialic acid decorated *H. influenzae* LOS, thereby reducing its ability for adhesion. At the same time, *H. influenzae* stimulates certain host immune factors that specifically induce opsonophagocytosis of *S. pneumoniae*, although capsule-specific strains will survive this process [25]. These events through Stage 2 will remove the sensitive strains of both species from the niche such that in Stage 3 there is now co-operation between the strains that have survived and is ready to form a multi-species biofilm. This co-operation is complex and still poorly understood but does include quorum sensing (QS; as discussed in the next section of this chapter), co-aggregation and adhesion, formation of an EPS matrix, and subsequently biofilm formation. Stage 4 is the
maturation of the biofilm permitting its persistence and, through an extension of the EPS matrix, the resistance to exogenous antimicrobial compounds. Host neutrophil extracellular traps (NETs) seem to be incorporated specifically into the EPS matrix and further to this, there is non-specific binding of host immune factors to EPS matrix components such as to the eDNA, further protecting the bacteria that exist within the biofilm. The role of eDNA and the association of NETs in the integrity and structure of biofilm is discussed in the next section of this chapter.

**Figure 1.** A model for the development of the *H. influenzae*-*S. pneumoniae* multispecies biofilm. Based on the available literature, there can be identified discrete stages for *H. influenzae* (Hi) and *S. pneumoniae* (Spn) response to the host environment and their survival together and biofilm formation. **Stage 1 – Environmental response and initial adhesion:** the bacteria respond to stresses in the host-pathogen environment by switching from a free-living lifestyle (planktonic) to a biofilm active form (a change in cellular metabolism and surface structures). This includes cell-cell interactions and specifically the binding of bacterial adhesins to host cell receptors. **Stage 2 – Competition and strain selection:** As Spn grows it lowers the local pH and generates hydrogen peroxide, both of which are bactericidal to Hi. The SpnNanA enzyme desialylates the Hi lipooligosaccharide (LOS) reducing its capacity to attach to host cells. The Hi is known to stimulate neutrophils and opsonophagocytosis of Spn. The strains that do survive can then co-operate. **Stage 3 – Multispecies biofilm:** there is signalling between the bacterial cells by quorum sensing (QS) through AI-2/AI-3 such that the bacteria recognise the multispecies environment. There is development of the extracellular polymeric substance (EPS) matrix made up of components from both bacterial species (type IV pili, eDNA, LOS and protein), providing co-operative adhesion and stability of the biofilm structure. **Stage 4 – Biofilm maturation:** the EPS develops providing further protection to the bacterial cells from antibiotics and host phagocytic cells. There is also incorporation of host immune factors, such as NET structures into the EPS.

In very particular disease situations, it is apparent that the biofilm formation is a key virulence factor. For *S. pneumoniae* and the NTHi, they are clearly present together in middle ear tissues of recurrent OM (ROM) and chronic OM (COM) patients [6] and in sputum samples from COPD [26]. The mono-species biofilm formation of both bacterial species has been well described [27, 28], and although both are known to co-exist in planktonic and biofilm states, the understanding of the nature of the interplay between these pathogens and the effect of co-
infection on the disease is only just starting to emerge [23]. The persistence of these species within a biofilm provides a vast array of phenotypes that allow for both the bacterial adaptation to a host anatomical niche and for the persistence of these species within a niche for a prolonged period. The switch to a biofilm state is characterised by global changes to their surface structures, physiology (energy production), metabolic processes, and stress response (discussed in a later section of this chapter).

4. Antibiotic resistance within a biofilm

Within a biofilm, bacteria display added resistance to host defences and antibiotic therapies; biofilms are 1000x more resistant to antibiotics than the planktonic state. An unusual stress response by NTHi that employs nickel [Ni (II)] ion uptake seemingly as a signalling process that links the cell’s stress response to the cell physiology and the composition of its surface structures has also been identified [29].

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 [6]. The bacterial aetiological agents in OM are S. pneumoniae, H. influenzae, and M. catarrhalis [6]. All these bacteria have also been located in tissue samples within a biofilm. While OM is commonly treated with antibiotics [30] or tympanostomy tube placement [31], COM or ROM forms of OM are often unresponsive to these treatments. The antibiotic treatment is complicated by the presence of multiple species of bacteria as well as the biofilm lifestyle of these bacteria. This has been shown in various studies [8, 32]. Firstly by the induction of a polymicrobial biofilm in the presence of each other and then specifically the antibiotic resistance provided to the oto-pathogenic bacteria within a polymicrobial biofilm [32]. This can be in a directed role (through signalling pathways, see later) or passively. In addition to this antibiotic resistance, there is some thought that the tympanostomy tube insertion has little impact on removing the bacteria or even on the biofilms formed; it is believed that it could promote biofilm formation and particularly in a polymicrobial situation [33].

The formation of bacterial biofilm during COM largely explains the difficulty in treating COM with antibiotics, as well as the resistance to tympanostomy tube placement, as both pathogens are able to re-establish the biofilm on the tympanostomy tube. However, while it has been established that both species are capable of forming a multi-species biofilm, the physical or molecular interactions between H. influenzae and S. pneumoniae in vivo or furthermore, when within the biofilm, have not been well defined. Research findings relating to the outcomes of this interaction are conflicting, as recently reviewed [23]. There are studies showing that the interaction between H. influenzae and S. pneumoniae is of a synergistic nature, whereby the formation of a multi-species biofilm would benefit both species, and protect them from host antimicrobials, shear forces, and antimicrobial agents. It was shown that a β-lactamase producing strain of H. influenzae could protect S. pneumoniae from β-lactam treatment [34]. This same study showed that the formation of a multi-species biofilm with a bla\- strain of H. influenzae also had a protective effect on both the biofilm resident S.
pneumoniae and the biofilm resident H. influenzae blacells, which alone were susceptible to beta-lactamase treatment. A synergistic interaction was also shown, where H. influenzae and S. pneumoniae reached higher cell densities in co-culture than in mono-culture, and were able to modulate each other’s gene expression in the biofilm [35]. Associated with this has been the demonstration that H. influenzae inhibits autolysis and fratricide of S. pneumoniae, and thereby H. influenzae improves the biofilm formation by S. pneumoniae, although this effect was only observed at later stages of culturing, and suggested that bacteria in co-culture biofilms may have altered biofilm formation processes, as previous study showed that in mono-cultures of S. pneumoniae autolysis promoted biofilm formation [36].

As is obvious from this array of findings, the nature of the interactions between these species remains unclear. It is likely, that these interactions are dependent on a multitude of specific host, genomic, and environmental factors, and that the discrepancy observed between studies is a result of the variation of one or more of these parameters. In addition, most studies have investigated the role of H. influenzae/S. pneumoniae interactions from the perspective of biofilm formation. Our recent study analysed global gene expression patterns in the H. influenzae/S. pneumoniae co-culture situation. This revealed the potential for either synergistic or antagonistic interactions between H. influenzae and S. pneumoniae, which is largely dependent on the growth dynamics and environmental conditions. We have shown that both species undergo vast changes in their transcriptional profile in response to the growth environment, and further influenced by the presence of the other species, and we thereby proposed that these environmental parameters and transcriptional patterns determine the synergistic or antagonistic nature of the H. influenzae/S. pneumoniae interactions [24]. Indeed under conditions of neutral or lower pH, the presence of different strains of S. pneumoniae induces H. influenzae into a Viable But Non-Culturable (VBNC) state [24]. For other bacterial species, this VBNC state was differentiated from the dead state by several observations; firstly, VBNC cells have an intact membrane, in contrast to dead cells, they are metabolically active and continue respiration, VBNC cells continue gene transcription and mRNA production, and were shown to have continued uptake and incorporation of amino acids into proteins [37, 38]. Given these characteristics, the induction of H. influenzae cells under specific conditions into the VBNC state by S. pneumoniae does not preclude it from a multi-species biofilm. This switch in cell type during co-culture highlights the complex nature of the impact of the bacteria being together than being in mono-culture.

5. Signalling and sensing mechanisms associated with biofilm formation

Most acute respiratory infections are often dominated by one organism, however, chronic bacterial infections mostly encompass mixed species microbial communities. In the natural environment, bacteria mostly coexist or compete with various microbial species, therefore, it is important to understand the impact of co-infections on persistent infections. The nasopharyngeal commensals such as H. influenzae, S. pneumoniae, S. aureus, and M. catarrhalis are linked with many respiratory tract infections, with several virulence factors of these microbes involved and recognised in biofilm formation. This highlights the need to understand the
complex interactions between these microbes and how they influence each other to form biofilms that contribute to persistent and chronic infections.

The matrix of microbial biofilm is usually composed of biopolymers that include polysaccharides, protein, and extracellular DNA (eDNA), referred to as the EPS. It is well established that bacteria use a signalling network for cell-to-cell communication, known as QS, to carry out coordinated activities including migration to a suitable environment, nutrient acquisition, and biofilm formation with the release of various signal molecules or autoinducers (AI) [39]. Such mechanisms have been identified in both S. pneumoniae and NTHi. Although different systems are used by different bacteria, the principles of QS such as: AI signal molecules are often undetected when bacteria are in low density, but commonly detected at high density; availability of receptors for AI are usually cytoplasmic or membrane bound; and their detection is critical for any co-ordinated gene expression and/or repression to be carried out by the bacteria [40, 41]. N-acyl homoserine lactones (AHL’s) are the most studied class of AI signal molecule and are commonly involved in the QS by gram-negative bacteria. The enzymes involved in the synthesis of autoinducer N-3-(oxo-hexanoyl)-homoserine lactone (3OC6HSL) AHL’s are: LuxR and LuxR-type synthases, and substrate S-adenosylmethionine. The AHL’s then traverse across the bacterial membranes through efflux pumps to bind to their respective regulators and initiate their activity. LuxR, a receptor for 3OC6HSL, and a well recognised transcriptional activator of the luciferase luxICDABE operon that activates its expression [42]. In contrast, in gram-positive bacteria, the modified oligopeptides or autoinducing peptides (AIPs) are mediated by specialized transporters that act as autoinducers in the QS systems. The AIP’s bind to the bacterial membrane bound two-component histidine kinase receptors, which further activates the cytoplasmic regulator that transcribes the genes associated with QS [43]. A recent review on AI-2 mediated signalling in bacteria has compiled different functions that are regulated by AI-2 including biofilm formation, antibiotic susceptibility, virulence factor production, motility, in both gram-positive and gram-negative bacteria [44]. Some of the noted examples of AIPs based QS include; ComD/ComE in S. pneumoniae, AgrC/AgrA in S. aureus, and ComA/ComP in Bacillus subtilis [42]. The manipulation of the identified QS systems in both gram-positive and gram-negative bacteria must be addressed further to develop newer biotechnological therapies towards treating chronic and persistent infections.

6. Quorum sensing mechanisms and signalling in H. influenzae biofilm formation

NTHi biofilm formation is well recognised due to bacterial aggregation involving various bacterial components such as lipooligosaccharide, proteins, extracellular DNA (eDNA), and host material derived from inflammation [45]. QS for NTHi was first suggested because of the presence of luxS gene in H. influenzae Rd genome, with luxS gene known to be involved in the production of AI-2 [46]. The most studied, identified QS systems in NTHi are the LuxS/RbsB and QseB/QseC systems.
6.1. LuxS/RbsB system

The role of *luxS* gene in NTHi biofilm formation has been extensively studied in both *in vivo* and *in vitro*, with the mutants lacking this gene forming biofilm, although with decreased biofilm thickness and biomass, that was further shown to be due to decreased phosphorylcholine incorporation into the LOS structure of the NTHi [47–49]. A certain *in vivo* study recently demonstrated the involvement of RbsB protein, a known periplasmic binding protein in mediating the uptake of AI-2 signals in NTHi [50]. Similar to the *luxS* mutants, the *rbsB* mutants also produced biofilms with reduced thickness and biomass, which were reflective of the decreased phosphorylcholine levels in the LOS of NTHi. These observations strongly indicate that QS clearly contributes to the establishment of a chronic infection.

6.2. QseB/QseC system

This two-component signalling system in NTHi was first described in enterohemorrhagic *Escherichia coli* and shown to regulate expression of virulence genes in a QS system independent of AI-2 [51]. A certain study involving the NTHi mutants lacking the *qseC* gene showed decreased biofilm production and was AI-2 independent, indicating that there could be other alternative signalling molecules affecting NTHi biofilm formation [52]. Although much progress has been done in understanding and identifying the QS system involved, not much is known about the nature of the QS signal molecules secreted by NTHi, or how does AI-2 affect the gene expression that could further alter the bacterial phenotype to produce biofilm is yet to be determined.

6.3. Role of extracellular DNA in NTHi biofilms

eDNA has been implicated as a major structural component of NTHi biofilms facilitating survival and replication of NTHi within a biofilm [53]. The association of NTHi pili and eDNA in biofilms, and its involvement in increasing bacterial adherence and biofilm formation is also well recognised [53, 54]. Recently, the protein responsible for providing the stabilisation of eDNA within the NTHi biofilm was identified as DNABII that binds to the eDNA and offers stabilization to the biofilm structure [55]. In addition to the bacterial eDNA, host eDNA also facilitates NTHi biofilm formation. The human neutrophils through making the NETs entrap the pathogens with the help of their genomic DNA [56]. The presence of these NETs had been demonstrated in various studies [53, 55, 57] but their role in pathogenesis is still unclear. A recent review has described the diverse mechanisms by which both gram-positive and gram-negative bacteria release eDNA, how eDNA and extracellular polymer matrix of a biofilm interact with each other, and the chemical behavior of eDNA and these interactions are responsible for the integrity and structure of biofilm development [58]. eDNA is often supplied by both host and a pathogen, and is linked to bacterial biofilms, QS, structural maintenance of biofilm, and offers a protective environment to pathogens residing inside, and further contributes to chronic and persistent infections. This prompts the need for developing therapeutics to target disruption of the extracellular matrix. A recent study has provided with a promising result to show an effective way involving human β-defensin to remove the eDNA
from the extra cellular polymer matrix, alter the NTHi biofilm formation, and effectively kill the NTHi residing within the biofilm [59].

6.4. Quorum sensing mechanisms and signalling in *S. pneumoniae* biofilm formation

In *S. pneumoniae* and in most of the gram-positive bacteria, QS often involves recognition of secreted peptides through the two-component regulatory systems. Over the last 40 years, the main QS systems in *S. pneumoniae* that have been identified and deciphered in detail are: LuxS/Autoinducer 2 (AI-2), the ComABCDE, and the BlpABCSRH systems [60]. Recently, there has been a growing interest in deciphering QS signalling or bacterial cross-talk between different strains of the same species. It has been suggested that bacteria belonging to the same pherotype are able to recognise peptides secreted by the same group but not the ones secreted by the other members. These pherotypes were previously identified for different QS systems including Agr in *S. aureus*, ComCDE in *S. pneumoniae*, ComQXPA in *B. subtilis*, and PapR/PlcR in *B. cereus* [61–63]. A recent study identified a QS mechanism in *Streptococci* genus that belongs to the Rgg family and involves a short hydrophobic peptide (SHP) that acts as a pheromone [64]. The functionality of the SHP/Rgg cell-cell communication mechanism in three different *Streptococci* species was demonstrated and cross-talk between strains was observed. More recently, an *in vitro* study demonstrated the involvement of a secreted peptide pheromone, competence-stimulating peptide (CSP) in influencing and development of *S. pneumoniae* biofilm [65].

In the recent years, an alternative group of QS peptides have been identified which are secreted by bacteria upon interaction with an oligotransporter and a cytoplasmic receptor protein, and initiate the process of QS [66, 67]. One of such peptides is the Phr signalling peptides of the *Bacillus species* that regulate different functions such as; sporulation, genetic competence, virulence gene expression, biofilm formation, and transfer of genetic elements [68]. The role of pneumococcal oligopeptide permease (Opp) (homologous to the phr peptides in the *Bacillus species*) in colonisation and virulence is well known [69, 70]. A recent study has identified TprA/PhrA signalling system to mediate QS in various strains of the pneumococci and its involvement in regulating the QS system in media containing galactose, which is one of the main energy sources required by the pneumococci during nasopharyngeal colonisation [68]. As biofilms are associated with colonisation, further studies are warranted to investigate the involvement of TprA/PhrA signalling system in biofilm formation, if any.

6.5. ComABCDE pathway

ComABCDE pathway is one of the most studied QS system regulated by the CSP, encoded by the *comC* gene and exported by the ATP-dependent ComAB transporter. In this system, the membrane-bound histidine kinase receptor, ComD, recognises the CSP which further leads to autophosphorylation of the histidine kinase, involving a transfer of a phosphate group from ComD to ComE [60]. Although the biological role of the Com QS in colonisation, bacterial carriage, and disease by *S. pneumoniae* is not yet fully known, there are certain studies that has demonstrated genetic transference to be more efficient in competent *Streptococci* biofilm cells.
in comparison with planktonic cells [71], and induction of competence a well recognised link between the switch from planktonic to biofilm form [72]. A certain study demonstrated competent cells releasing pneumolysin from the neighbouring non-competent cells by a cell- lysis mechanism, suggesting an indirect relationship between competence and virulence [73]. Further studies are warranted to understand how this mechanism relates to pneumococcal infections. Various pathogenic bacteria including *Ps. aeruginosa*, *H. influenzae*, *S. pneumoniae* form biofilms on different substrates including tissues and human epithelia. Moreover, *S. pneumoniae* and *H. influenzae* upon interaction with human airway epithelial cells have been shown to produce more biofilm in comparison with no contact of epithelial cells [74, 75]. However, the production, regulation, and the mechanism by which enhanced pneumococcal biofilms are formed upon host-microbial interactions are not fully elucidated. Recently, an *in vitro* study has described a mechanism involved in the regulation of biofilm autolysis, and studies involving mutant strains lacking the *comC* and *luxS* showed that early pneumococcal biofilm on human cells are regulated by both Com and LuxS/AI-2 QS system [76]. In other *Streptococcus* species, such *S. gordonii* and *S. mutans*, there are reports about the involvement of ComCDE QS system in regulating both competence development and biofilm formation [77, 78].

### 6.6. BlpABCSRH pathway

This pathway is also one of the well characterised QS system in *S. pneumoniae*. The pathway consists of a secretion apparatus (BlpAB), a two-component regulatory system (BlpSRH), and an ABC transporter (BlpA) [79]. Being similar to the Com pathway, it is suggested that both pathways could converge at a common site where the response regulators of both pathways bind to the same motif, and activate the transcription of the same target gene [79]. In this QS system, a peptide pheromone encoded by *blpC* gene regulates the production of class II bacteriocins and their immunity proteins [80]. As bacteriocins are known to inhibit growth of competing bacteria, leading to intense microbial competition, it could be important to further elucidate how these complex regulatory networks operate during the course of an infection.

### 6.7. LuxS/AI-2

Autoinducer-2 (AI-2) is one of the most common QS signal in both gram-positive and gram- negative bacteria synthesised by S-ribosyl homocysteine lyase (LuxS) [81]. LuxS converts S- ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which further cyclises to active AI-2 [82]. Although its involvement in the biofilm development and virulence in several bacterial species is widely recognised [83, 84], the regulation and mechanism of LuxS has not been clear until now. It is believed that LuxS-controlled QS system might be only a part of the regulatory network that controls competence and LytA-dependent autolysis. Recently, the role of LuxS in controlling *S. pneumoniae* biofilms was first demonstrated using luxS mutants that failed to form early biofilms [85], and overexpression of *luxS* gene resulted in hyper-biofilm-forming phenotype [36]. Another study involving human respiratory cells showed that both the LuxS/AI-2 and Com QS systems as the main regula-
tors in the process of biofilm production [76]. Another report confirmed the down regulation of pneumolysin in mutants lacking the luxS gene but not in a comC knockout mutant, suggesting that pneumolysin is predominantly regulated by the LuxS/AI-2 system [86]. Future studies involving molecular interactions between Com and LuxS/AI-2 QS systems could provide new research directions in further elucidating gene expressions during early biofilm formation, or how mature biofilms are formed upon activation of either of these systems.

7. Quorum sensing mechanisms and signalling in mixed species biofilm formation

Although AI-2 signalling has been vastly studied under monospecies experimental set-ups, the polymicrobial nature of any microbial biofilm cannot be underestimated. There are certain co-culture studies that have demonstrated how AI-2 signalling by *E. coli* induced production of haemagglutinin protease, which facilitates detachment of *Vibrio cholera* from the intestinal mucosa [87]. This indicates how the presence of *E. coli* influences the dynamics and spread of cholera disease. Similar approaches have also been adopted to investigate interspecies signalling amongst nasopharyngeal microflora such as *M. catarrhalis* and *H. influenzae*, and demonstrated that biofilm formation by *M. catarrhalis* is promoted by the AI-2 signalling by *H. influenzae* [88]. Both *H. influenzae* and *M. catarrhalis* are well recognised causative pathogens of many respiratory infections including OM, and chronic OM is often associated with multi-species biofilm formation and antibiotic resistance [89]. Although *M. catarrhalis* lacks LuxS/AI-2 QS system, there are certain studies that have demonstrated increased biofilm production, antibiotic resistance by *M. catarrhalis* in the presence of *H. influenzae* [88]. Our own studies have found that nasal co-colonisation of *M. catarrhalis*, *H. influenzae*, and *S. pneumoniae* resulted in increased colonisation load and incidence of OM in mice [90], increased bacterial adherence to epithelial cells in vitro. The complex microbe-host interactions during biofilm production in our study suggested the importance of understanding why certain strains and serotypes differentially influence biofilm formation, in which the epithelial cell contact was a key contributor to increased biofilm formation [75]. Figure 2 shows the possible mechanisms that could be involved in a multi-species biofilm. It shows how mixed bacterial species could behave differently and produce more biofilm upon interaction with host epithelial cell contact in comparison with no cell contact on abiotic surfaces. A recent review has collated the extensive work on *H. influenzae* and *S. pneumoniae* multi-species biofilm including; co-existence within the biofilm to reflect their persistence, changes in gene expression and physiology, how they adapt to environmental conditions, and molecular factors involved in bacterial cross-talk [23]. Another study showed how co-existence of *M. catarrhalis* and *S. pneumoniae* within a biofilm confers antibiotic resistance and bacterial persistence, and facilitates increased *M. catarrhalis* biofilm production which is not dependent on AI-2 signalling [32]. These observations highlight the importance of interspecies AI-2 signalling and the resilient nature of multi-species biofilm, and how do they impact on bacterial persistence and virulence.
Figure 2. Possible mechanisms involved in multispecies biofilm formation. Mixed bacterial species behave differently and produce more biofilm upon interaction with host epithelial cell contact in comparison with no cell contact on abiotic surfaces. The figure shows increased biofilm production [more extracellular polymeric substance (EPS) matrix] by *H. influenzae*-*S. pneumoniae* in a multispecies environment upon contact with human epithelial cells (left panel). The right panel shows a decreased production of biofilm when grown on an abiotic surface (tissue-culture polystyrene plate). The potential role of host cell contact, possibility of any altered metabolic changes, or increased quorum sensing (QS)/autoinducer-2 (AI-2) signalling, upon host-cell interactions could hold key to further elucidate the mechanisms involved in increasing multispecies biofilm formation.

8. Future directions in QS signalling research

Despite the progress made in QS system and signalling pathways, there are several challenges ahead to better understand how these networks function. The challenges include; deciphering the messages obtained from the chemical properties, different sensing mechanisms and integration with other QS pathways, environmental factors, and cellular metabolism. A modern approach that involves developing a chemical probe to identify novel AI-2 receptors [91] along with the availability of genetic screening and bioinformatics could be a promising tool to further elucidate the role of different signalling systems in individual organisms. Therapeutic approaches to combat horizontal gene transfer by bacteria, multi-drug resistance, or to target induction of bacterial community behaviours could be helpful in answering control of bacterial communities within multi-species biofilm that presents a major problem in chronic disease including cystic fibrosis or OM. Another approach could be the use
of quorum quenching or cause interference in AI-2 based signalling by developing antagonistic analogue molecules [92]. Recently, nanotechnology has provided some promising results with the manipulation of the AI-2 signalling on certain subpopulation of targeted bacteria [93].

9. The host-pathogen environment and role of metal ions in bacterial cells

The exact nature of the properties of an environment will influence if a resident bacterial species can grow freely in an active, planktonic lifestyle or whether the environmental properties represent non-optimal or stressed conditions and therefore act as a trigger for the bacteria to switch to a biofilm lifestyle. These properties include the chemical and physical properties affecting growth; the pH, oxygen levels, nutrient levels, temperature, osmotic pressure, redox state, water availability, and the presence of toxic compounds such as reactive chemicals. In host-pathogen environment the presence, absence, or changes in the levels of these properties can become a stress for the bacteria. Many host cells either intrinsically or by induction as a response to the bacterial being present, generate toxic levels of reactive oxygen or nitrogen species (ROS and RNS respectively). The immune response from cells such as macrophages (and other cells) stimulates the production of the ROS superoxide and hydrogen peroxide as part of their anti-microbial processes. They are also known to generate nitric oxide (NO) and other RNS as a response to infection. It is appreciated that there are differences in this range of physical and chemical properties between anatomical niches. It has been well established in numerous bacterial species that the presence of hydrogen peroxide and NO stimulate biofilm formation [94, 95]. In the context of bacteria such as S. pneumoniae and NTHi, when acting as a commensal of the nasopharynx and when persisting in niches such as the lung and middle ear, and perhaps more relevant than the short-lived immune generated toxic chemicals (ROS and RNS), are the intrinsic features of the environment (the oxygen levels, the pH nutrients levels, and the availability of essential micro-nutrients such as the metal ions, iron, copper, manganese, and nickel).

It is not understood how measures of variation observed in the secretions taken from the middle ear and lungs of patients relate to conditions that favour biofilm formation and persistence of bacteria. The pH in serous (7.92), mucous (8.55), and serous/mucous (8.33) middle ear fluids varies slightly across a weak alkaline range [96]; sputum from COPD patients was lower in pH from those with more significant disease, and this was shown to be associated with increased cytokine levels [97]. The contribution to defined growth conditions for the co-existence of S. pneumoniae and NTHi have been important determinants for understanding the mechanisms and factors important at different growth phases in planktonic and biofilm states. Studies ultimately found that at a higher pH, NTHi survived in co-culture with S. pneumoniae and as part of the competitive microbial environment in batch cultures, S. pneumoniae drives a decrease in pH that continues below pH 5.75, at which point NTHi is unable to grow. Such in vitro observations do not appear to be a representative of the host environment, therefore studies using flow cell chambers have provided a more realistic model system, showing that even under low pH condition, the NTHi can survive [24].
The environmental concentration of transition metal ions can have significant influence on the survival of the bacteria and its lifestyle. For many metals, even though essentially they can quickly become toxic. Obviously, there is therefore a necessary tight regulation of their homeostasis; under metal starvation, there is an up-regulation of uptake systems, but as the metal concentration exceeds the cellular requirement there is activation of efflux systems [98].

In addition to some metal ions, their toxicity is closely linked to other environmental factors (or stresses) that the cells also required to respond – for instance iron toxicity is associated with oxidative stress (through Fenton Chemistry) and therefore iron homeostasis is regulated in conjunction with oxidative stress responses. Nickel levels and nickel function (such as binding to proteins) is affected by pH. Copper also is linked to pH, as the concentration increases there is an increase in acidity, and for copper there is also a link to Fenton chemistry and oxidative stress. Other metals seem to have an anti-oxidant role for the cell (such as zinc and manganese). The homeostasis of a metal is therefore affected by cellular requirement as well as other environmental factors that are affecting the metal toxicity or function. The influence of metal ions on bacteria within the environment is complex. It is further the subject of the metal bioavailability and cellular requirements. It is known that the correct access to metals influences cell lifestyle – this can be direct (where their role is either as a co-factor for a biomolecule or through directly within transcriptional pathways) or indirectly (acting as signalling system for stresses). As suggested previously, the response of cellular networks to the environmental level of a metal ion, the metabolic and physiological (energy generation) cell systems, and cell surface structures, will vary under different growth and environmental conditions.

Metal ion uptake is known to be important for bacterial survival within the host. Iron uptake systems are proven virulence factors for many pathogens [99, 100] and the control of zinc, copper, and manganese levels within the host environment has been shown to be important for bacterial survival and virulence. There have been different studies associating the niche-specific metal ion concentration and subsequent expression and role of particular surface structures [101]. A key example is the struggle between host and bacteria for iron. Iron is central for many pathways required in growth and survival, indeed for humans as well as for the bacteria, but iron chemistry also links it tightly to oxidative stress. Alternatively, an inability to acquire iron simply in itself can become a stress for the bacteria and induce the bacteria into a biofilm lifestyle. Iron acquisition and homeostasis is considered a virulence factor for many pathogens. In addition to the stress, some iron acquisition pathways have surface structures and of these iron-acquiring systems they have a dual role in adhesion and initiation of biofilm formation. In the context of multi-species biofilms, there is very little known of the role of transition metals. For Pneumococcus alone, iron sensing and iron transport is complicated, overlapping with other transition metal systems. The regulation is predominantly through RitR and transport is via PiaABCD, PitADBC, and PiuCDA [101]. Disruption of these pathways is shown to affect nasopharyngeal carriage and adhesion (indeed using OM model assays; these different reports have been well reviewed) [101]. Iron has been suggested through different studies to act as a signal for numerous processes in Pneumococcus, although the fine balance required for iron levels seems to be highlighted by its positive and negative effects on biofilm formation. Certainly, iron-limited conditions altered the protein expression of a
number of surface structures (such as PsaA) and therefore affected biofilm formation [102]. Other work linked iron levels to LuxS regulatory controlled processes, increasing levels of iron actually enhanced biofilm formation and other processes [36]. For NTHi, there is also a correlation to iron acquisition pathways, iron regulatory pathways, and biofilm formation. Iron uptake is up-regulated as NTHi migrates to the middle ear, a *niche* that it is known to exist within a biofilm. The central iron-responsive transcription factor (ferric uptake regulator, Fur) is required for long term survival of NTHi *in vivo* [103], and by regulating many genes it controls biofilm initiation and maturation. More directly, culturing NTHi sequentially through iron-replete and iron-depleted conditions revealed iron restriction induces biofilm formation [104]. These studies also used an experimental OM model and observed survival in the middle ear and the biofilms formed in the middle ear of bacterial cells taken from iron-rich and iron-depleted cultures. The iron-depleted cultures survived longer and interestingly showed a changed architecture in their biofilm [104]. While this work revealed significant outcomes and raised intriguing questions with regards to the cell biology, it did clearly show that iron levels play an essential role in NTHi biofilm formation in the middle ear.

Other transition metal ions variously have vital role for bacterial survival. Some of these roles are as co-factors for important enzymes and then simply for growth, while other functions includes in stress response. In the case of *S. pneumoniae* these functions for transition metals have been intensively studied and this has been well reviewed [101]. In pneumococcal pathogenesis, transition metal ions such as iron, zinc, copper, and manganese are critical for its survival within a host, although in differing degrees. Although the exact function is not always clear and could be direct or indirect such as; the signalling through global transcriptional pathways or in competition with other metals for specific binding sites in biomolecules [101]. There are a series of surface proteins in *S. pneumoniae* that function in metal ion uptake (or even in efflux) but are concurrently essential in adhesion and at least the first stages of biofilm formation on epithelial cells. These structures include the choline binding protein PcpA, the serine protease PrtA, and the manganese uptake system PsaBCA. PsaR regulates all these and this is at least in response to environmental and cellular manganese levels. While the transport proteins for copper seem to be up-regulated during infection, their role in virulence is not known [105]. Although the copper export proteins (CopA certainly) do seem to be involved at various stages during pathogenesis and certainly are linked to pneumococcal survival in the nasopharynx and lung [106]. Likewise, with other transition metals, there seems to be a central role in survival as perhaps shown in infection and systems animal model studies. These however have not always been directly associated with an exact process within pathogenesis or directly in biofilm formation [101].

The local concentration of zinc has a significant role in the pathogenesis of *S. pneumoniae* and this includes in its biofilm formation within the host [107]. There are a number of zinc uptake and efflux systems that have been studied in pneumococcus (AdcABC and AdcAII regulated by AdcR, Pht proteins and CzcD). Metal limiting conditions can result in a growth limitation. Maintaining cellular zinc (and manganese) levels is important to controlling the redox balance and defending against oxidative stress. AdcAII has been shown itself to have a direct role in pathogenesis; while mutants lacking either *adcA* or *adcAII* increased invasive
infection of human lung epithelial cells, it was $adcAII$ alone that was required for attachment and colonization on the nasopharynx, presumably through biofilm formation [108]. The Pht proteins bind zinc and facilitate zinc uptake, have been shown to be essential in attachment of $S. pneumoniae$ to respiratory epithelial cells [109]. The $pht$ genes along with $adc$ operon are regulated by AdcR in response to zinc but the PsaR regulator is now also known to be zinc-responsive (in addition to manganese). Further to the complex nature of the transcriptional response to environmental metal ion levels, both AdcR and PsaR have been shown to additionally respond to cellular concentrations of nickel. In PsaR, the nickel competes against manganese’s binding and has an opposite effect to manganese for PsaR function on its regulon [110]. AdcR also independently responds to nickel – exogenous nickel levels having a direct role in regulating the Pht proteins and affecting the AdcR control of the $adc$ operon [111]. Nickel has clearly been shown for NTHi to directly have a role in cell’s lifestyle. The maintenance of intracellular nickel has a role in the nature of the cell surface, the surface charge and hydrophobicity, and the outer membrane protein and LOS composition, and this is independent of nickel binding proteins [112]. Further to this, it was shown that this nickel-induced effect on the bacterial cell also translated to a loss in type IV pili-mediated twitching motility in NTHi [29]. The importance of nickel uptake for the growth of NTHi is well known, and when limited, the bacteria makes the switch to a biofilm state [112]. This was correlated to a control of intracellular pH levels. However, it was shown not simply to be pH stress that was influencing NTHi survival or biofilm formation when in co-culture with $S. pneumoniae$, but growth dynamics [24].

The exact nature of the environmental transition metal composition therefore impacts on both NTHi and $S. pneumoniae$ lifestyle and their ability to attach to host cells and initiate biofilm formation. There is little analysis of the consequential impact of metal ions in NTHi/$S. pneumoniae$ co-culture and biofilm formation and much of our discussion has therefore focussed on mono-culture studies.

10. Bacterial metabolic pathways and mechanisms contributing to the biofilm production

Several studies have investigated gene and/or expression to identify the unique metabolic changes associated with transition from planktonic form to biofilm for $S. pneumoniae$. Yadav and co-workers [113] identified the exclusive up-regulation of genes involved in the mevalonate pathway, pyruvate metabolism, carbohydrate metabolism, galactose metabolic process, cell wall biosynthesis, translation, and purine and pyrimidine nucleotide metabolic pathways in biofilm formation, and suggested that these were also important to the growth and survival of bacteria in biofilms. In addition, changes to related genes suggested that the cells in biofilms may be under stress conditions that result in changes in the protein synthesis required to adapt to a new environment. Protein profiles have been compared between log-phase planktonic $S. pneumoniae$ serotype 14 and 1-day and 7-day biofilm cultures using iTRAQ (isobaric tagging for relative and absolute quantification) [114]. This study by Allan and co-workers identified 244 proteins of which >80% were differentially expressed during
biofilm development. Their results indicated that metabolic regulation appears to play a central role in the adaptation from the planktonic to biofilm phenotype. Their study found that 47% of proteins were down-regulated during biofilm development (day 1) and 16% were up-regulated compared to the bacteria in log-phase. As the biofilm matured, approximately 24% of the proteins expressed during biofilm development returned to expression levels similar to the planktonic state and a further 16% were up-regulated. In general, up-regulation was observed with proteins associated with pyruvate, amino acid, and carbohydrate metabolism and there was a down-regulation in glycolysis and some other metabolic proteins. By day 7 of the biofilm, the most noticeable difference was the increase in some proteins that were associated with protein biosynthesis/alteration or degradation and cell division.

Changes in metabolism potentially serve two purposes, firstly changing the bacterial phenotype so as to adapt to the different lifestyle and secondly, the need to utilise alternative metabolic pathways for survival.

The results of these studies suggest that *S. pneumoniae* uses a range of carbohydrates during biofilm formation. The biofilm also has a changing oxygen environment and increases in pyruvate metabolism, particularly lactate dehydrogenase that indicates an adaptation to this. The down-regulation of many virulence proteins generally associated with infection, persistence, and its ability to compete suggest a significant shift in its need to protect and respond to threats from the external environment. This is accompanied by the down-regulation in NADH oxidase that acts as an oxygen sensor and improving glucose catabolism. At the same time, processes important to carbohydrate selection and capsule production were increased, as was pyruvate oxidase, important to *S. pneumoniae* aggregate formation.

Comparison of NTHi biofilm to planktonic form in one study has shown that 127 proteins are significantly differentially expressed [115]. Of particular note was the major down-regulation in proteins involved in purine, pyrimidine, nucleoside, and nucleotide processes; protein synthesis; and energy metabolism. Up-regulation was detected for proteins involved in the cell envelope, DNA metabolism, transcription, and metabolism of phospholipids and fatty acids. Similar to the conclusions drawn from the metabolic changes to *S. pneumoniae*, NTHi appears to enter a state of decreased energy metabolism and protein biosynthesis at the same time adjusting its metabolism to the changes in the aerobic environment and energy derived from carbohydrate metabolism. Another study found that one of the triggers for biofilm formation was exposure to sub-inhibitory concentrations of beta-lactam antibiotics [116]. While very similar gene expression changes were found as reported by Post et al., including an increase in biofilm biomass and decreased protein production, the concomitant up-regulation of the genes involved in glycogen production was proposed to be associated with an ability for the bacteria to be sustained as they become metabolically inactive. This aligns with recent work reported by Kidd where in mixed *S. pneumoniae* and NTHi biofilm, *S. pneumoniae* is able to convert NTHi to a non-culturable state [29].

It appears that similar changes in metabolic processes might occur as bacteria transition from the planktonic state, through early biofilm development to the mature biofilm (Table 1). As yet, very little is known about the metabolic changes that enable mixed biofilm formation, particularly associated with the shift in the processes associated with interspecies competi-
tion and mechanisms of cooperation. Additionally, the role of the human mucosal surface and respiratory tract environment on metabolic changes have not yet been investigated.

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<th>Planktonic</th>
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<th>Mature biofilm</th>
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<tr>
<td>Up-regulation</td>
<td>Cell wall organisation</td>
<td>Some enzymes involved in biosynthesis/alteration or degradation and cell division</td>
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<td>Stress response</td>
<td>Amino acid, pyruvate, pyrimidine processes</td>
<td>Transport</td>
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<td>Virulence</td>
<td>Glycolysis and some other metabolic proteins</td>
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<td>Specific carbohydrate metabolism</td>
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<td>Down-regulation</td>
<td>Translation</td>
<td>Many metabolic processes changed during biofilm development return to normal levels of expression</td>
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Table 1. Summary of key metabolic processes altered during biofilm formation and maturation.

11. Conclusion

The biofilm is the dominant factor in persistence; being recalcitrant to antibiotic and host antimicrobial processes. Understanding the mechanisms that contribute to this persistence will help to design the next generation therapeutics. Many key questions are still unresolved. Identifying the genes involved in enabling bacterial co-existence, particularly in the transition to a biofilm state, may provide new targets for preventing the transition to a state of chronic, persistent colonisation. Understanding the specific cell-to-cell factors affecting the signalling/sensing mechanisms that could alter bacterial cell-surface and the host characteristics that play a role might enable us to identify individuals likely to be susceptible to chronic disease situations. Our knowledge is still limited about the differences in the general charac-
teristics, biofilm architecture, and signalling mechanisms associated with single and co-species biofilms.

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