MINIREVIEW

Novel concepts in nontypeable Haemophilus influenzae biofilm formation

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Abstract

Nontypeable Haemophilus influenzae (NTHi) is a Gram-negative microbe that frequently colonizes the human host without obvious signs of inflammation, but is also a frequent cause of otitis media in children and exacerbations in chronic obstructive pulmonary disease patients. Accumulating data suggest that NTHi can reside in biofilms during both colonization and infection. Recent literature proposes roles for phosphorylcholine, sialic acid, bacterial DNA, but also eukaryotic DNA in the development of NTHi biofilms. However, many questions remain. Until now, there are insufficient data to explain how NTHi forms biofilms. Here, we review the recent advances in NTHi biofilm formation with particular focus on the role that neutrophils may play in this process. We propose that recruitment of neutrophils facilitates NTHi biofilm formation on mucosal sites by the initiation of neutrophil extracellular traps.

Introduction

The formation of biofilms has been described for both nonpathogenic and pathogenic bacteria. Bacterial biofilm formation is a multistep process that starts with adhesion to a surface, after which attached bacteria divide and give rise to a biomass referred to as a biofilm. In general, biofilms are considered advantageous to pathogens, because they protect them from antimicrobial actions of the host immune system and offer resistance to antibiotics. In addition, they are proposed to function as a nutrient source in nutrient-limiting environments (Stewart & Costerton, 2001; Jefferson, 2004).

The definition of a biofilm is still under debate. To date, there is no convincing evidence that nontypeable Haemophilus influenzae (NTHi) produces extracellular polymeric substances. Despite the inability to produce extracellular polymeric substances, NTHi forms a discrete biomass in vivo, consisting of live and dead bacteria as well as host cells, and in this review, we will refer to this as a biofilm.

The latest review that focused on NTHi biofilm formation clearly evaluated literature published until 2008 (Moxon et al., 2008). The authors suggested that NTHi biofilm formation is not a distinct genetically programmed behavioral activity, but rather the aggregation of bacteria and the elaboration of bacterial components [lipooligosaccharide, proteins, and extracellular DNA (eDNA)] and host material generated by inflammation. Recently, Ng & Kidd (2013) showed that metabolic stress in the form of disrupting nickel transport induced NTHi biofilm formation, which might indicate that biofilm formation is partly regulated, at least by the availability of nutrients. Here, we review recent literature and discuss novel insights, in particular the potential role of quorum sensing, lipooligosaccharide modifications, and neutrophils in biofilm formation.
NTHi biofilm formation in health and disease

Many reports are aimed to establish a link between NTHi biofilm formation and its ability to cause disease, such as otitis media (OM). NTHi presenting at inflammatory sites, such as the middle ears of children with OM, is usually terminal for the bacteria as they are normally cleared from the site by host defense mechanisms and therefore are not transmitted to another host. In this regard, the ability of NTHi to form biofilms would not be expected to evolve or be retained within the bacterial population. Therefore, NTHi biofilm formation should have other advantages besides the ability to cause disease. Possibly, biofilm formation is already needed for colonization. For Neisseria meningitidis, it has been postulated that the ability to utilize eDNA for colonization allows isolates to colonize the host for long periods of time (Lappann & Vogel, 2010). These bacterial strains build sticky biofilms that might resist detachment, which prevents transmission (settlers). In contrast, hypervirulent N. meningitidis strains that lack the capacity to utilize eDNA detach more easily and transmit to another host (spreader). Although evidence supporting that NTHi forms biofilms during colonization is very limited, NTHi isolates collected from the nasopharynx of children with OM show variation in biofilm amount formed in vitro (Torretta et al., 2012), which might point toward similar groups for NTHi (settlers and spreaders). In order to address this, studies need to be undertaken wherein the ability to form biofilms must be related to duration of colonization and transmission to another host. Torretta et al. (2012) showed that NTHi strains collected from children with OM formed significantly more biofilms compared with NTHi strains collected from healthy children. However, it is not possible to draw firm conclusions from this study due to very low number of isolates (12 OM strains, 1 healthy control strain), but it at least shows that NTHi collected from the nasopharynx have the ability to form biofilms in vitro.

The ability for NTHi to form a biofilm in vivo during disease was first visualized on tympanostomy tubes collected from children with OM (Post, 2001). These data show that NTHi forms a biofilm on an abiotic surface in the presence of antimicrobial activity of the human immune system. Hall-Stoodley et al. (2006) evaluated biofilms present in middle ear effusions obtained from children undergoing tympanostomy tube placement. In this study, the vast majority of the middle ear effusions contained bacterial biofilms based on morphological criteria and fluorescence in situ hybridization (FISH) analysis. A high prevalence of NTHi, but also Streptococcus pneumoniae or Moraxella catarrhalis, was found in these biofilms, and a growing body of evidence suggests that NTHi resides in multispecies biofilms (Armaribster et al., 2010; Weimer et al., 2010), but this will not be discussed in further detail in this review.

Another location where NTHi is frequently found to cause inflammatory disease is the lungs of patients with chronic obstructive pulmonary disease (COPD). Usually, the lungs of healthy adults are free of bacteria based on available culture methods, whereas NTHi, but also Haemophilus haemolyticus, Haemophilus parainfluenzae, M. catarrhalis, and S. pneumoniae, are frequently found to colonize the lungs of patients with COPD (Sethi et al., 2007). NTHi isolates collected from sputum of patients with COPD were able to form biofilms in vitro (Murphy & Kirkham, 2002). However, to our knowledge, no studies have clearly shown the presence of biofilms in the lungs of patients with COPD. Starner et al. (2006) found structures consistent with NTHi biofilms in bronchial alveolar lavage fluid from asymptomatic patients with cystic fibrosis by electron microscopy. Additional studies must be undertaken to provide evidence that NTHi biofilm formation enables colonization of the inflamed lungs for prolonged times.

Studies on the role of NTHi biofilms in disease in vivo frequently use the chinchilla model for OM. For instance, infections of chinchillas with various mutant bacteria proposed a role for quorum sensing, phosphorylcholine, and sialic acid in biofilm formation (discussed below). However, the route of infection often used in these studies, transtympanic, is not the natural route wherein NTHi causes OM in the human host. Recently, we achieved spontaneous development of OM in the mouse following coinfection with influenza A virus and NTHi in the nose (Langereis et al., 2012). Biofilm formation by NTHi in this model has not been assessed to date.

In vitro biofilm formation

Formations of biofilm in vitro do not represent what happens in vivo, but at least it is a start. Although these models offer conditions that are far from natural for NTHi, they do appear to be a valuable tool to study molecular aspects of initial bacterial attachment and biofilm outgrowth, in greater detail than can be achieved by in vivo infection models.

A static biofilm assay is used in most studies. Although this is a very artificial method, its high-throughput nature can be instrumental in determining potential factors involved in biofilm formation through comparison of biomass formed between wild-type and specific mutant bacteria. In this assay, NTHi is grown on a plastic or glass surface such that attachment and subsequent outgrowth can be assessed (Fig. 1a; O’Toole & Kolter, 1998).
biomass formed is subsequently stained with crystal violet, which stains not only live bacteria, but also lysed bacteria and bacterial products including eDNA. Therefore, this method is suitable to determine the involvement of certain genes or bacterial phenotypes in the ability of NTHi to form biomass over time, but does not necessarily implicate that these genes are needed for biofilm formation in vivo. Therefore, in vivo experiments are essential for determining genes involved in biofilm formation.

Another widely used in vitro method wherein NTHi can form a biofilm is a continuous flow system (Davies et al., 1998). In this assay, bacteria attach to a surface where after a flow is initiated in the chamber (Fig. 1b). The formation of a biofilm can vary dependent on the flow rate in the chamber.

Both of the in vitro methods have advantages and disadvantages. The static biofilm formation is relatively easy and allows rapid screening of large numbers of strains and conditions for biomass formation based on the simple measurement of optical density. One can question whether such measurement can distinguish between true biofilm formation and just simple adherence to plastic. NTHi biofilms formed in a continuous flow chamber displayed water channels after 5 days; similar structures were also identified for biofilms in an experimental chinchilla model for OM in vivo (Greiner et al., 2004; Jurcisek et al., 2005; Jurcisek & Bakaletz, 2007). Therefore, although artificial, the in vitro continuous flow system can grow biofilms with features resembling those of biofilms formed in vivo.

**Quorum sensing**

Quorum sensing has been shown to coordinate activities within bacterial populations, including the formation of biofilms. This process is dependent on the production and sensing of small diffusible molecules that act as signals called autoinducers (AI; Miller & Bassler, 2001). These signaling molecules are chemically diverse and include competence factor peptides, homoserine lactones, and derivatives of the bacterial metabolic byproduct.
dihydroxypentanodione, which is the precursor for AI-2. Quorum sensing appears to have effects on the development of biofilms in many bacterial species, including NTHi (Swords, 2012).

In recent years, various studies have reported quorum sensing in NTHi, including the identification and function of the LuxS/RbsB and QseB/QseC systems (Fig. 2). However, questions about the relationship between quorum sensing and NTHi biofilms remain.

The LuxS/RbsB system
Quorum sensing for NTHi was first suggested due to the presence of the luxS gene in the H. influenzae Rd genome. This gene is responsible for the production of the signaling molecule AI-2 in many bacterial species (Surette et al., 1999). Although the molecular structure of NTHi AI-2 has not yet been determined, the role of the luxS gene has been studied extensively. NTHi mutants lacking the luxS gene retained the ability to form a biofilm in vitro (Daines et al., 2005), although quantitative analysis showed decreased biofilm thickness and biofilm density after 24 and 48 h, but not after 72 h (Armbruster et al., 2009). Additionally, the authors showed decreased phosphorylcholine incorporation into the lipooligosaccharide structure of NTHi. This is of particular interest because the presence of this molecule is linked to the development of NTHi biofilms (discussed below) and could therefore explain the results obtained with the luxS mutant strain. The NTHi luxS mutant also showed decreased survival in a chinchilla model for OM, although this only became significant 21 days postinfection, whereas in vitro results showed an attenuation biofilm formation at 2–3 days (Armbruster et al., 2009, 2011).

As AI-2 is secreted, recipient bacteria should bear a receptor or transport mechanism enabling them to react to the presence of this molecule. Recently, RbsB, a periplasmic binding protein responsible for AI-2 uptake in other bacterial species (Shao et al., 2007), was similarly shown to mediate uptake of AI-2 in NTHi (Armbruster et al., 2011). A mutant lacking the rbsB gene showed no depletion of AI-2 from the medium, and presented decreased biofilm mass in a continuous flow system after 24 h and in a stationary system after 12 h compared with the wild-type strain. Interestingly, the rbsB mutant also showed decreased phosphorylcholine levels in the lipooligosaccharide of NTHi, indicating that quorum sensing might be linked to phosphorylcholine expression, either by decreased incorporation or altered phase variation frequency.

The QseB/QseC system
In addition to the LuxS/RbsB system, the two-component system QseB/QseC, which regulates quorum sensing in other bacteria, for example, Escherichia coli, was recently shown to influence NTHi biofilm formation in vitro (Unal et al., 2012). Deletion of the qseC gene from the NTHi genome did not affect growth or adhesion of the bacterium, but did decrease biofilm formation under static and semi-static (shaking with 200 r.p.m.) conditions after 24 h, which was independent of AI-2 production (Unal et al., 2012). These data indicate that there are alternative signaling molecules besides AI-2 that affect NTHi biofilm formation. In E. coli, QseC was shown to sense human epinephrine or norepinephrine (Clarke et al., 2006), but possibly also a postulated bacterial quorum-sensing molecule, named AI-3 (Hughes et al., 2009). Whether or not this is also the case for NTHi has to be determined.

The field of NTHi quorum sensing has made progress toward understanding the mechanisms involved, but some major challenges remain. For instance, dihydroxypentanodione induces NTHi biofilm maturation in vitro (Armbruster et al., 2011), but the effect on gene expression and bacterial phenotype has to our knowledge not yet been investigated. Also, the nature of the quorum-signaling molecules secreted by NTHi is not known.

It should be mentioned that, given the genomic diversity among NTHi strains, not all sequenced NTHi isolates possess homologues to the essential quorum-sensing genes, luxS and rbsB. Swords (2012) showed that in three of 18 publically accessible NTHi genomic sequences, no predicted sequence for AI-2 transport was present, pointing to some significant diversity in the mechanism...
or the presence of quorum sensing in disease-causing strains. Therefore, a potential shortcoming in these studies is that NTHi quorum-sensing data are obtained with a selected number of NTHi strains and experiments should be reproduced, preferably with a range of NTHi clinical isolates.

Another critical aspect that has to be addressed is the discrepancies between in vitro and in vivo experiments with NTHi. A luxS deletion mutant strain showed decreased biofilm formation after 24–48 h growth in vitro compared with the wild-type strain, whereas biofilms were equivalent after 72-h growth (Armbruster et al., 2009). Contrastingly, in vivo in the chinchilla middle ear, the wild-type and luxS and rbsB mutant strains showed equivalent growth until day 14, where after reduced bacterial counts were observed for the mutants (Armbruster et al., 2009, 2011). These latter data would indicate that quorum sensing does not affect disease onset, but rather influences disease resolution. Taken together, although quorum sensing has been shown to be pivotal for biofilm formation in other bacterial species, its role for NTHi remains ambiguous because strains mutated in luxS, rbsB, and qscC are still able to form significant biofilms.

Biofilm composition

The current data on NTHi biofilm formation do not support the idea that NTHi produce specific extracellular polymeric substances to promote biofilm formation. As an alternative, NTHi appears to use lipooligosaccharide-related material containing specific moieties such as sialic acid and phosphorylcholine. It is also considered that eDNA is needed to form NTHi biofilms, constituting part of the extracellular matrix wherein NTHi can survive and replicate. Jurcisek & Bakaletz (2007) showed the presence of NTHi pili associated with eDNA in biofilms in the middle ears of chinchillas and deletion of individual pili genes decreased bacterial adhesion and biofilm formation in vitro (Carruthers et al., 2012). The role for pili proteins in NTHi biofilm formation has to be replicated in other strains to confirm their role in NTHi biofilm formation. Other proteins including high-molecular-weight adhesins, _Haemophilus_ adhesion and penetration protein (hap), and outer membrane protein P5 or P6 are found in NTHi biofilms (Gallaher et al., 2006; Webster et al., 2006), but they will not be discussed in further detail in this review.

Sialic acid

NTHi is not able to synthesize sialic acid and is therefore dependent on uptake from the host (Apicella, 2012). Incorporation of sialic acid into lipooligosaccharide of NTHi has been shown to promote biofilm formation (Swords et al., 2004), and NTHi mutants defective in sialic acid incorporation exhibited decreased biofilm formation in vitro (Greiner et al., 2004; Swords et al., 2004).

The effect of sialic acid on biofilm formation has also been studied in various in vivo models. Incorporation of sialic acid into NTHi lipooligosaccharide permits persistent colonization in an experimental Mongolian gerbil model for OM, in a chinchilla model for OM, and a rat pulmonary challenge model (Swords et al., 2004; Jurcisek et al., 2005). However, these in vivo data are difficult to interpret because when sialic acid is lacking, this renders NTHi sensitive to complement-mediated killing (Figueira et al., 2007). Therefore, additional studies should be carried out to determine by which mechanism sialic acid promotes biofilm formation.

Phosphorylcholine

Incorporation of phosphorylcholine into NTHi lipooligosaccharide enhances adherence to upper airway epithelial cells (Swords et al., 2000), so it is not unexpected that phosphorylcholine also plays a role in biofilm formation because attachment to a surface is fundamental to this process. In an initial study, NTHi licD mutant, who are defective for incorporation of phosphorylcholine into lipooligosaccharide, retained the ability to form a biofilm in a static biofilm assay in vitro after 8 h (West-Barnette et al., 2006). However, later studies wherein biofilms were visualized after 24- to 72-h growth by confocal microscopy indicated that the presence of phosphorylcholine in lipooligosaccharide increased biofilm mass and thickness (Hong et al., 2007). These data are consistent with phosphorylcholine facilitating biofilm maturation: however, the means by which it realizes this remains unknown.

A role for phosphorylcholine in biofilm maturation was also observed in vivo. An NTHi licD mutant strain showed decreased biofilm formation after 14 days in the chinchilla judged by macroscopic analysis of the middle ears compared with the wild-type strain, whereas a mutant strain that incorporated more phosphorylcholine showed increased biofilm formation (Hong et al., 2007).

A potential shortcoming in these studies is that most experiments are conducted with a limited selection of mainly laboratory strains. Recently, we have determined the presence of phosphorylcholine in lipooligosaccharide and measured the ability to form a biofilm in a static biofilm assay for over 100 clinical NTHi isolates, but we were not able to find any correlation between phosphorylcholine expression measured by flow cytometry and biofilm formation (C. Puig, S. Marti, P.W.M. Hermans, C. Ardanuy, J. Liñares & J.D. Langereis, unpublished results). Additionally, deletion of the licA gene from the genome of a selection of these clinical NTHi isolates, which decreased
or abrogated phosphorylcholine incorporation into lipooligosaccharide, had positive and negative effects on biofilm formation. Therefore, further studies using an extended panel of NTHi isolates should be undertaken to determine the role of phosphorylcholine incorporation into lipooligosaccharide in the initiation and outgrowth of NTHi biofilms in vitro and in vivo.

**Extracellular DNA**

The presence of eDNA in the biofilm matrix plays a major role in both biofilm formation and biofilm stability for many bacterial species, including the respiratory pathogens *Pseudomonas aeruginosa* (Allesen-Holm et al., 2006), *S. pneumoniae* (Moscoso et al., 2006), and NTHi (Jurcisek & Bakaletz, 2007). Jurcisek & Bakaletz (2007) showed the presence of host immune cells, as well as fine strands of eDNA material, in a 4-day-old NTHi biofilm in an experimental chinchilla model for OM. The authors suggested that the eDNA, which came from the bacteria themselves, might play a predominant role in biofilm formation in vivo. Recently, the eDNA-associated protein DNABII has been shown to be important in stabilizing the eDNA within the NTHi biofilm (Goodman et al., 2011). DNABII was bound to eDNA in the biofilm. Interestingly, an antibody raised against this protein prevents biofilm formation, but also decreases the biomass of existing biofilms. Based on these data, it appears that an organized eDNA structure enhance NTHi biofilm formation.

Besides bacterial eDNA, host eDNA is also implicated in facilitating NTHi biofilm formation. Jurcisek & Bakaletz (2007) showed that immune cells, such as neutrophils, are present within NTHi biofilms in vivo. Neutrophils are especially interesting as these cells make neutrophil extracellular traps (NETs), a mechanism by which neutrophils entrap microorganisms by extrusion of their genomic DNA (Brinkmann et al., 2004). Several studies have indicated that these NETs are present in NTHi biofilms (Hong et al., 2009; Goodman et al., 2011; Juneau et al., 2011). Although NETs are supposed to kill bacteria, this is obviously not the case for NTHi. Juneau et al. (2011) and coworkers showed that NTHi induced the formation of NETs, in which NTHi was able to grow and survive, even when additional neutrophils were added. Also, eDNA present in NETs interacts with β-defensin-3 to significantly decrease its antimicrobial activity toward NTHi (Jones et al., 2013). Altogether, the question of whether or not NETs are able to kill entrapped bacteria directly remains controversial (Menegazzi et al., 2012).

This resistance to NET-mediated killing, at least in part, was dependent on the lipooligosaccharide structure because *htrB*, *rfaD*, and *siaB* mutant strains were more susceptible to NET-mediated killing (Hong et al., 2009). This susceptibility to NET-mediated killing could therefore explain why the NTHi *htrB* and *rfaD* mutants were attenuated for survival in the chinchilla model for OM in vivo (DeMaria et al., 1997).

Recently, we found in mice that a prior influenza A virus infection facilitated development of OM by NTHi (Langereis et al., 2012), which was observed for *S. pneumoniae* previously (Short et al., 2011). In these experiments, influenza A virus-induced neutrophil infiltration into the middle ears and *S. pneumoniae* was largely present within these large neutrophil infiltrates (Short et al., 2011). Therefore, we find it likely that this influx of neutrophils to the
middle ears facilitates *S. pneumoniae*, and supposedly also NTHi biofilm formation, although this needs to be confirmed in future studies.

Although evidence for a role for eDNA, either bacterial- or host-derived, in NTHi biofilms is increasing, its role in pathogenesis is unclear. Based on the literature and our own unpublished observations, we propose that the formation of NETs is an essential step in the development and maintenance of the NTHi biofilm.

**Model for NTHi biofilm formation in vivo**

We propose that NTHi biofilms can exist in at least two clinically relevant phenotypes: (1) a ‘classical’ biofilm, wherein bacteria are attached to a surface such as the mucosa of the middle ear, in adenoid tissue, or tympanic tube implants; (2) large biofilm aggregates consisting of host material, especially neutrophils, proteins, and lipooligosaccharide that are not attached to the mucosa or host surfaces per se.

We postulate that NTHi benefits from the recruitment of neutrophils while developing a biofilm (Fig. 3). Upon adhesion to the epithelial surface, NTHi activates pattern recognition factors that result in secretion of neutrophil chemotactic factors, such as IL-8 and leukotriene (LT) B4. Once NETs are induced, these NET structures form a scaffold in which NTHi can reside and form a biofilm. This hypothesis could be tested in a mouse model wherein neutrophil recruitment can be modulated. Also, the essential nature of NET formation could be evaluated by treating infected animals with DNase that can degrade NETs. The true contribution of biofilm/aggregation in NTHi to disease needs to be determined in animal models or by detailed analyses of NTHi obtained from patient materials.

**Concluding remarks**

Although the definition of an NTHi biofilm is still under debate, we are convinced that that NTHi can form large aggregates, containing bacterial and host material that enhance bacterial survival within the human host. Eradication of NTHi, for instance in the middle ears of children with OM or the lungs of patients with COPD, could be achieved by interfering with the ability of NTHi to form these biofilms. However, at present, insufficient knowledge is available to understand the molecular mechanisms underlying NTHi biofilm formation. Unraveling these mechanisms, by for instance the use of genomewide mutagenesis screens as used recently for NTHi by our laboratory (Langereis *et al.*, 2013), would offer the potential to identify novel therapeutic targets for the prevention or treatment of inflammatory diseases caused by NTHi.

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