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Original article

The roles of epithelial cell contact, respiratory bacterial interactions and phosphorylcholine in promoting biofilm formation by *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae*

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Abstract

Streptococcus pneumoniae and nontypeable *Haemophilus influenzae* (NTHi) often share a common *niche* within the nasopharynx, both associated with infections such as bronchitis and otitis media. This study investigated how the association between NTHi and *S. pneumoniae* and the host affects their propensity to form biofilms. We investigated a selection of bacterial strain and serotype combinations on biofilm formation, and the effect of contact with respiratory epithelial cells. Measurement of biofilm showed that co-infection with NTHi and *S. pneumoniae* increased biofilm formation following contact with epithelial cells compared to no contact demonstrating the role of epithelial cells in biofilm formation. Additionally, the influence of phosphorylcholine (ChoP) on biofilm production was investigated using the *licD* mutant strain of NTHi 2019 and found that ChoP had a role in mixed biofilm formation but was not the only requirement. The study highlights the complex interactions between microbes and the host epithelium during biofilm production, suggesting the importance of understanding why certain strains and serotypes differentially influence biofilm formation. A key contributor to increased biofilm formation was the upregulation of biofilm formation by epithelial cell factors.

Keywords: Mixed-species biofilm; Epithelial cells; Differential biofilm formation; Phosphorylcholine

1. Introduction

The human respiratory tract is colonised by multiple species at any given time. Microbe–host interactions often begin at the mucosal surface of the nasopharynx. The epithelial lining provides a *niche* for both disease and non-disease causing bacteria to colonise and/or establish an infection [1]. Respiratory tract infections such as otitis media and exacerbations of chronic obstructive pulmonary disease are frequently polymicrobial in nature and can lead to serious clinical outcomes. Nasopharyngeal commensal microbes such as *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* often share a common colonisation *niche* and are linked with many respiratory tract infections. Bacterial acquisition and carriage rates for these bacteria have been reported in many studies and are well reviewed [2]. The mean age of first acquisition of any of these bacteria is 6 months of age [3] with early nasopharyngeal colonisation with *S. pneumoniae* and NTHi being recognised as a risk factor for the onset of otitis media. For Australian Aboriginal children, the median age of colonisation by these bacteria has been found to be just 10 and 20 days, respectively, in comparison with 209 and 270 days for non-Aboriginal children [4]. A recent review has focussed on bacterial colonisation involving these bacteria within a

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microbial community and how this might influence a persistent disease or infection state [5].

Sometimes, antibiotic treatment does not eradicate bacteria from the nasopharynx and bacterial biofilm formation has been considered as an etiological factor in bacterial persistence [6]. Microbial biofilms are reported as present in periodontal disease, gastrointestinal infections, medical device-related infections (catheters, prosthetic heart valves, and joint prostheses) and so forth [7]. Biofilms are reported as also able to confer antibiotic resistance to other bacteria within a biofilm [8]. Biofilm formation is a continuous process that can result in a multispecies microbial community [9]. Upon maturation, microbial biofilms often release free-floating bacterial cells that are believed to contribute to both persistent and recurrent infections [10,11]. Bacteria within the biofilm display a different phenotype than the planktonic form with the multiple microenvironments developed forming a symbiotic living society that augments nutrient exchange, cross-talk between bacteria, and offers protection from the host defence mechanisms [12].

A role for contact with respiratory epithelial cells during biofilm formation was demonstrated for *S. pneumoniae* [13]. More biofilm was formed following interaction with 1HAEo$^-$ epithelial cells compared to without contact [13], and *S. pneumoniae* taken from biofilms were more adhesive to A549 pneumocytes and Detroit 562 pharyngeal epithelial cells [14]. Additionally, pneumococcal biofilms grown on respiratory epithelial cells are shown to display similar structural and antibiotic resistance pattern to those of biofilms in vivo [15].

The addition of phosphorylcholine (ChoP) to the glycans of pathogens has many potent biological consequences arising from ChoP's multiple interactions with host proteins [16]. The pathway and gene locus for ChoP modification was initially described by Weiser et al. in 1997 [17] for *H. influenzae* and subsequently, homologues for genes in the *lic1* locus were found in *S. pneumoniae* [18], resulting in these two bacteria being important to the investigation of the properties of ChoP-modified glycans. ChoP is a small, zwitterionic molecule and on *H. influenzae* is attached to the lipooligosaccharide [19]. It not only contributes to persistence and colonisation by NTHi through enhancing adherence to host cells, but also facilitates bacterial invasion into airway epithelial cells [20,21]. There are a number of studies that have associated expression of ChoP with the growth and maturation of NTHi biofilms [22,23]. Therefore, in the present study we investigated the influence of ChoP on biofilm production using the mutant strain of NTHi 2019 that lacks ChoP (ChoP$^-$).

Our investigation of microbe–host dynamics involved in airway infection and nasal colonisation in mice [24] showed synergistic increases in adherence/colonisation for combinations of *M. catarrhalis* and *S. pneumoniae*, however, an apparent suppression of this synergy was observed when NTHi was present in the same environment. The results indicated that studies were needed to better understand the behaviour of bacteria in a polymicrobial environment when on the epithelial surface and the impact on biofilm formation. This study has investigated the effect of intra- and inter-species infection, the potential contribution of contact with respiratory epithelial cells and whether bacterial ChoP was important to biofilm formation for NTHi and *S. pneumoniae*. The study has found that co-infection combinations did increase biofilm formation; that a contact interaction with A549 epithelial cells triggered changes in the bacteria resulting in increased biofilm formation; and that ChoP on NTHi plays a role in this response. Differences in specific inter- and intra-species combinations suggest that the association between colonising bacteria and biofilm formation may be linked to the complex interactions between the different bacterial colonisers and the host epithelium.

2. Materials and methods

2.1. Bacterial strains and culture

Bacterial strains of *S. pneumoniae* with capsular types 6B, 14, 19F, 23F, and non-typeable *H. influenzae* 289, 2019, and 86-028NP were recovered from frozen stocks. *S. pneumoniae* were initially grown on blood agar supplemented with 5% defibrinated horse blood, and NTHi on brain-heart infusion agar supplemented with 5% partially lysed defibrinated horse blood, respectively in 5% CO_2 at 37 °C. Bacterial colonies were then grown to mid-log phase using Todd-Hewitt broth (for *S. pneumoniae* strains) or brain-heart infusion broth containing 10 μg/ml of haemin (Sigma Aldrich) and 10 μg/ml of NAD (Sigma Aldrich) (for NTHi strains), hereafter referred to as supplemented BHI (sBHI). In addition, a previously described phosphorylcholine (ChoP$^-$) mutant of NTHi 2019, NTHi 2019 *licD* [20], was propagated in sBHI containing 15 μg/ml of kanamycin. To establish equal multiplicity of infection by these strains, approximately 10^7 CFU/ml bacterial suspensions were prepared in sterile phosphate buffered saline (PBS).

2.2. Biofilm assay

For the biofilm assays, equal volumes of appropriate bacterial strains were grown to mid-log phase using sBHI. Two hundred μl of this suspension was added in duplicate to 96-well flat-bottom tissue culture treated plates and incubated for 0, 24, 48, and 72 h at 37 °C in 5% CO_2. The plates were then washed using sterile nanopure water and adherent biofilm-forming cells were stained with 50 μl of 1% crystal violet for 15 min. The plates were washed again and air-dried followed by addition of 200 μl of 95% ethanol and shaking for 10 min to suspend the bound crystal violet before measuring the optical density at 595 nm. The assays were performed in duplicate and repeated three times.

2.3. Epithelial cell culture

A549 lung epithelial (ATCC, CCL-185) was grown in RPMI 1640 containing 10% heat-inactivated Fetal Calf Serum (Lonza Australia Pty. Ltd.), 100 units of penicillin/ml, 100 μg/ml of streptomycin, and 1% L-glutamine (Invitrogen). The cells were grown to achieve 90–95% confluence in sterile 96 well tissue culture plates at a concentration of approximately