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Intercellular adhesion and biocide resistance in nontypeable *Haemophilus influenzae* biofilms

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ABSTRACT

Respiratory infections caused by nontypeable *Haemophilus influenzae* (NTHi) are a major medical problem. Evidence suggests that the ability to form biofilms on mucosal surfaces may play a role in NTHi pathogenesis. However, the factors that contribute to NTHi biofilm cohesion remain largely unknown. In this study we investigated the biofilm growth and detachment phenotypes of eight NTHi clinical strains *in vitro*. We found that the majority of strains produced biofilms within 6 h when cultured statically in tubes. Biofilm formation was inhibited when culture medium was supplemented with proteinase K or DNase I. Both enzymes also caused significant detachment of pre-formed NTHi biofilms. These findings indicate that both proteinaceous adhesins and extracellular DNA contribute to NTHi biofilm cohesion. Treatment of NTHi biofilms cultured in centrifugal filter devices with DNase I, but not with proteinase K, caused a significant decrease in fluid convection through the biofilms. These results suggest that extracellular DNA is the major volumetric component of the NTHi biofilm matrix. Mechanical or enzymatic disruption of NTHi biofilms cultured in microtiter plates significantly increased their sensitivity to killing by SDS, cetylpyridinium chloride, chlorhexidine gluconate, povidone iodine and sodium hypochlorite. These findings indicate that biocide resistance in NTHi biofilms is mediated to a large part by the cohesive and protective properties of the biofilm matrix. Understanding the mechanisms of biofilm cohesion and biocide resistance in NTHi biofilms may lead to new methods for treating NTHi-associated infections.

1. Introduction

Haemophilus influenzae is a gram-negative bacterium that comprises part of the normal nasopharyngeal flora of most humans [1]. Most strains lack capsular polysaccharides and are referred to as nontypeable *H. influenzae* or NTHi [2]. Some NTHi strains cause otitis media, which is a major medical problem that can lead to childhood hearing loss [3]. NTHi also causes bronchitis, sinusitis and other chronic respiratory infections [3].

Biofilms are communities of bacteria, encased in a self-synthesized extracellular matrix, growing attached to a biotic or abiotic surface [4]. Evidence suggests that the ability to form biofilms may contribute to NTHi pathogenesis [1]. Matrix-encased biofilm-like structures have been observed on middle ear tissue of experimentally infected chinchillas [5], and on middle ear mucosa biopsy specimens obtained from children undergoing tympanostomy tube placement [6]. Structures consistent with a biofilm have also been observed on cultured human airway epithelial cells [7] and on abiotic surfaces *in vitro* [8]. Like most biofilms, NTHi biofilms cultured *in vitro* exhibit increased resistance to killing by antibiotics compared to the resistance exhibited by planktonic cells [9–11]. Biofilm formation may account for the recalcitrance of many chronic NTHi infections to antibiotic therapy [1].

Numerous studies have identified NTHi cellular components, such as pili, surface proteins and lipooligosaccharide, that contribute to mucosal surface attachment and colonization [12,13]. Few studies, however, have identified cellular components that mediate biofilm-specific processes such as intercellular adhesion and biocide resistance. NTHi strains carrying mutations in the pilin genes *hifA* [14] or *pilA* [15] exhibit reduced biofilm formation *in vitro*. Adhesive pili have been shown to mediate biofilm formation in numerous other bacteria [16,17]. However, not all NTHi strains produce pili [18]. Sialylated lipooligosaccharides may also play a cohesive role in NTHi biofilms [19,20]. Several studies have shown that DNA is present in the matrix of NTHi biofilms recovered from the middle ear of the chinchilla [21–23], although no cohesive role for matrix DNA was identified. To date, there is no evidence that the NTHi biofilm matrix contains polysaccharide adhesins, which are common matrix components in most other bacterial biofilms [24].

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The purpose of the present study was to identify extracellular components that mediate NTHi biofilm cohesion, and to further characterize NTHi biofilm resistance. We tested the ability of various matrix-degrading enzymes, detergents, antiseptics and disinfectants to inhibit, detach or kill biofilms produced by eight NTHi clinical strains *in vitro*. Our findings demonstrate that both proteinaceous adhesins and extracellular DNA mediate biofilm cohesion and biocide resistance in NTHi biofilms.

2. Results

2.1. Biofilm formation by NTHi strains

Eight NTHi clinical strains were tested for their ability to form biofilms in polystyrene culture tubes (Fig. 1). After staining the tubes with Crystal Violet, which stains bacterial cells and biofilm matrix components but not polystyrene [25], a visible biofilm was evident on the surface of the tube for most strains (Fig. 1A,B). The amount of biofilm formation varied among the eight strains, but was highly reproducible within each strain. For example, strains NJ9724 and NJ9726 consistently formed weaker biofilms than did the other six strains. Biofilm formation was evident at both the air–liquid interface and at the bottom of the tube. Biofilms were tenaciously attached to the surface of the tube and were resistant to detachment by vortex agitation. The biofilm phenotypes were stable after three passages on agar. Quantitation of Crystal Violet binding revealed that most strains produced a detectable amount of stainable, surface-associated biomass (Fig. 1C).

Quantitation of biofilm formation by CFU enumeration revealed that strong biofilm-forming strains such as NJ9725 produced 10^7–10^8 biofilm cells per tube after 24 h, which constituted approximately 20% of the total CFU per tube. After 48 h, the amount of Crystal Violet staining remained the same, but the total CFU decreased to $<10^2$ per tube.

2.2. Inhibition of NTHi biofilm formation by DNase I and proteinase K

DNase I and proteinase K, when added to the culture medium at the time of inoculation, significantly inhibited biofilm formation by NTHi strain NJ9725 as determined by Crystal Violet staining (Fig. 2A). Both enzymes also inhibited biofilm formation by the other five NTHi strains that exhibited a strong biofilm phenotype (data not shown). When assayed by CFU enumeration, the presence of DNase I in the growth medium resulted in significantly less surface-associated biofilm cells (Fig. 2B). The total CFU per tube values were approximately the same for cultures grown with or without DNase I or proteinase K, indicating that neither enzyme severely affected cell growth or viability. These findings confirm that both proteinaceous adhesins and extracellular DNA contribute to NTHi biofilm cohesion.

2.3. Detachment of NTHi biofilms by DNase I and proteinase K

To further confirm that proteinaceous adhesins and DNA mediate NTHi biofilm cohesion, we treated 24-h-old biofilms for 1 h with 1 mg mL^{-1} proteinase K or DNase I (Fig. 3). Proteinase K caused significant detachment of biofilm biomass in all strains (Fig. 3A). A time-course study indicated that detachment of NJ9725 biofilms by proteinase K was very rapid, with most of the biomass detaching within 15 s (Fig. 3B). In contrast, DNase I caused only partial detachment of biofilms produced by most NTHi strains after 1 h of treatment (Fig. 3C). These findings suggest that proteinaceous adhesins are a major mediator of intercellular cohesion in mature NTHi biofilms. NTHi biofilms were not detached by treatment with the carbohydrate-modifying agent sodium metaperiodate (data not shown), which has been shown to detach biofilms whose cohesion depends on polysaccharide adhesins [26,27].

2.4. Extracellular DNA facilitates fluid convection in NTHi biofilms

To determine whether the presence of proteins and DNA in the NTHi biofilm matrix significantly alters the physical properties of the biofilms, NJ9725 biofilms were cultured for 24 h in centrifugal filter devices in the presence or absence of proteinase K and DNase I, and the devices were then subjected to low-speed centrifugation for increasing amounts of time (Fig. 4). The volume of broth that flowed through the biofilm was <50% of the volume that flowed through control filter devices inoculated with sterile BHI broth, indicating that NTHi biofilms inhibit bulk fluid convection. Fluid convection through NJ9725 biofilms cultured in the presence of

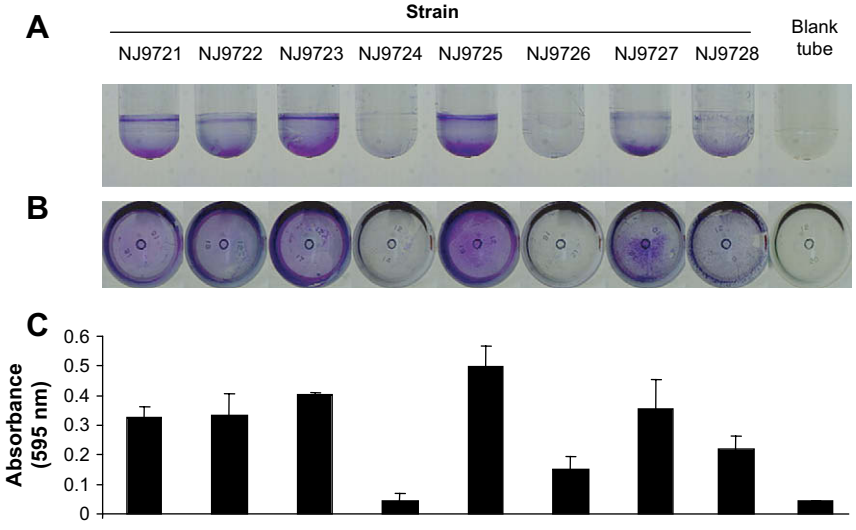

Fig. 1. Test tube biofilm assay. NTHi strains were cultured for 24 h in 17 × 100-mm polystyrene tubes. Tubes were rinsed with water and stained with Crystal Violet. Panels (A) and (B) show photographs of stained tubes taken from the side and bottom, respectively. Also shown is a control tube that was inoculated with sterile BHI broth, cultured and stained with Crystal Violet using the same procedure. In panel (C), tubes were destained with 33% acetic acid and the absorbance of the dye solution (at 595 nm) was measured in a spectrophotometer. Values in panel (C) show mean absorbance and range for duplicate tubes.