BMC Microbiology

Research article

Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili

Timothy F Murphy*[1,2] and Charmaine Kirkham[1,2]

Address: [1]Division of Infectious Diseases, Department of Medicine, and Department of Microbiology, University at Buffalo, State University of New York and [2]Department of Veterans Affairs Western New York Healthcare System (151) 3495 Bailey Avenue, Buffalo, NY 14215, USA

E-mail: Timothy F Murphy* - murphyt@acsu.buffalo.edu; Charmaine Kirkham - camchar@acsu.buffalo.edu

*Corresponding author

Published: 15 April 2002

BMC Microbiology 2002, **2**:7

Received: 18 January 2002

Accepted: 15 April 2002

This article is available from: http://www.biomedcentral.com/1471-2180/2/7

© 2002 Murphy and Kirkham; licensee BioMed Central Ltd. Verbatim copying and redistribution of this article are permitted in any medium for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: Nontypeable *Haemophilus influenzae* is an important cause of otitis media in children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD). Several lines of evidence suggest that the bacterium grows as a biofilm in the human respiratory tract.

Results: Fifteen clinical isolates from middle ear fluid of children with otitis media and 15 isolates from sputum of adults with COPD were studied in an in vitro assay of biofilm formation. Striking variability among isolates was observed in their ability to form biofilms. Analysis of cell envelopes revealed minimal differences in banding patterns in polyacrylamide gels, alteration of expression of an epitope on lipooligosaccharide, and preservation of expression of selected epitopes on outer membrane proteins P2, P5 and P6 in biofilms compared to planktonically grown cells. A pilus-deficient variant showed a marked impairment in biofilm formation compared to its isogenic parent.

Conclusions: Nontypeable *H. influenzae* forms biofilms in vitro. Clinical isolates show substantial variability in their ability to grow as biofilms. Three major outer membrane proteins (P2, P5 and P6) are expressed during growth as a biofilm. Expression of lipooligosaccharide is altered during growth as a biofilm compared to planktonic growth. Pili are important in biofilm formation. As the role of biofilms in human infection becomes better defined, characterization of biofilms may be important in understanding the pathogenesis of infection and immune response to nontypeable *H. influenzae* in children with otitis media and adults with COPD.

Background

Nontypeable *Haemophilus influenzae* is an important human respiratory tract pathogen [1–4]. The bacterium causes acute otitis media as established by pure culture of the organism from middle ear fluid during disease [5–9]. In addition, nontypeable *H. influenzae* has been implicated as a cause of otitis media with effusion, which refers to the presence of fluid in the middle ear in the absence of acute symptoms. Acute otitis media and otitis media with effusion represent an enormous health problem for children worldwide [10–12]. A large amount of effort has been devoted to understanding the pathogenesis of otitis media with a goal toward developing ways to prevent the disease [1,13,14].

Many middle ear fluids from children with otitis media with effusion are sterile by culture. However, assays using the polymerase chain reaction (PCR) have demonstrated that DNA of nontypeable *H. influenzae* is present in a substantial proportion of these middle ear fluids [15–20]. Furthermore, reverse transcriptase PCR-based assays have shown the presence of bacterial mRNA, indicating that bacteria are present in a viable and metabolically active but non-culturable state [21]. These observations suggest that in otitis media with effusion, bacteria are in a physiological state which differs from that of bacteria growing planktonically in a free floating phase. Some authors have proposed that in otitis media with effusion, nontypeable *H. influenzae* grow in the form of a biofilm in the middle ear [15,22,23].

A biofilm is a structured community of bacterial cells enveloped in a self-produced polymeric matrix and adherent to an inert or living surface [22,24]. It is estimated that 99.9% of bacteria in nature are attached to a surface in the form of a biofilm. Over the past decade bacteria in the form of biofilms have been recognized as important causes of a variety of human infections, including infections of prosthetic devices, endocarditis, dental caries, pneumonia in cystic fibrosis, prostatitis and others [22,25–28]. Biofilms are more resistant to immune clearance mechanisms and to antibiotics compared to planktonic bacteria [29,30]. In order to develop strategies to treat and prevent infections caused by bacteria in biofilms, including otitis media with effusion, it will be important to elucidate the characteristics of bacterial pathogens as biofilms.

In addition to being a common cause of otitis media, nontypeable *H. influenzae* colonizes the lower respiratory tract of adults with chronic obstructive pulmonary disease (COPD) and is an important cause of exacerbations of the disease [4,31]. An ongoing prospective study of bacterial infection in COPD at the Buffalo Veterans Affairs Medical Center has revealed interesting features regarding the dynamics of colonization in COPD. While active turnover of new strains of nontypeable *H. influenzae* occurs, some strains have the ability to persist for many months. Monthly sputum cultures reveal intermittent negative cultures in spite of continuous colonization by the same isolate proven by molecular typing, indicating that the organism is present in spite of negative cultures of sputum [32] (and authors unpublished observations). These observations suggest that nontypeable *H. influenzae* grows as a biofilm in the respiratory tract of adults with COPD, reminiscent of *Pseudomonas aeruginosa* and its well established propensity to grow as a biofilm while causing infection in patients with cystic fibrosis [27].

In view of these lines of evidence suggesting that nontypeable *H influenzae* grows as a biofilm during human respiratory tract infection, the goals of the present study are to 1) Develop an assay to study biofilms of nontypeable *H. influenzae* in vitro; 2) Characterize the extent to which isolates from the middle ear fluid of children with otitis media and from the sputum of adults with COPD form biofilms; 3) Begin to elucidate the characteristics of the outer membrane antigens of nontypeable *H. influenzae* during growth as a biofilm; and 4) Preliminarily assess the role of pili in biofilm formation.

Results

Biofilm formation by clinical isolates

Fifteen isolates recovered from the sputum of adults with COPD and 15 isolates recovered from the middle ear fluid of children with otitis media were studied for biofilm formation. Figure 1a and 1b show that striking differences were observed among strains with regard to the ability to form biofilms. Values depicted in the bar graphs represent the mean of three independent experiments performed in quadriplicate. The error bars represent the standard deviation. Although some variability in values was seen, the level of biofilm formation was highly consistent from experiment to experiment. Strains which formed good biofilms (e.g., strains 5P11H1, 6P8H1, 13P26H1, 31P13H1, 56P40H1 and 74P10H1 from Figure 1b) and strains which formed poor biofims (e.g., 5P10H1, 6P5H, 7P49H1, 6P32H1 and 13P33H1) do so consistently. Inspection of Figure 1 reveals that one cannot readily divide strains into those which form biofilms and those which do not. Rather, a broad range of ability to form biofilms was seen among these randomly chosen 30 clinical isolates.

Conditions for biofilm formation

In an effort to identify growth conditions which might result in increased biofilm formation, eight strains which showed a range of ability to form biofilms were chosen for further study. The strains included 4 isolates from middle ear fluid (955, 1826, 1749, 9289) and 4 isolates from the sputum of adults with COPD (74P10H1, 5P11H1, 5P10H1, 14P13H1). The following reagents were added individually to aliquots of media: 2% glucose, 5% glucose, 2% sucrose, 5% sucrose, 0.2 M NaCl, 2% ethanol. A biofilm assay was performed and the results were compared with biofilms in growth media for each of the strains. None of the added reagents caused an increase in biofilm formation for any of the strains.

Biofilm growth curve

To characterize the kinetics of biofilm formation, the level of biofilm formation was measured at selected time points over a 24 hour period for 4 strains. Figure 2 shows that a gradual increase in biofilm is seen over approximately 16 hours. After approximately 16 hours, biofilm formation shows a leveling off and the optical density re-

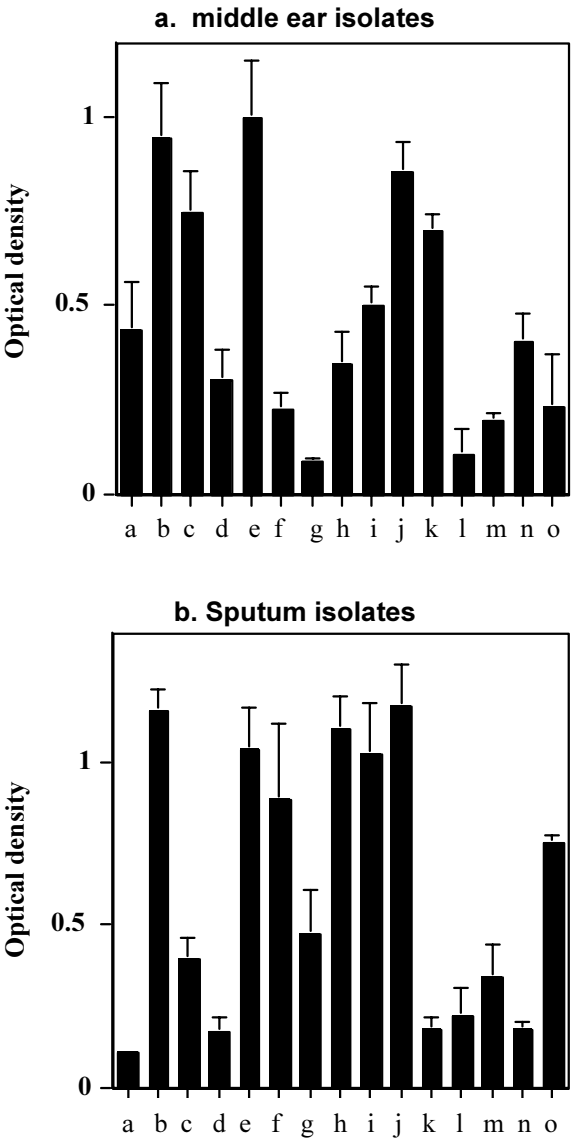

Figure 1
Biofilm formation by clinical isolates of nontypeable *H. influenzae*. Bars represent the means of three independent experiments performed in quadruplicate. Error bars represent standard deviation. Y axis is optical density at 570 nm. la: middle ear isolates: a. 83; b. 955; c. 1128; d. 1749; e. 1826; f. 2536; g. 2846; h. C4556; i. 8131; j. 8183; k. 8981; 1. 9289; m. C1425; n. DL200; o. 4505. 1b: sputum isolates: a. 5P10H1; b. 5P11H1; c. 5P30H; d. 6P5H; e. 6P8H1; f. 13P26H1; g. 14P13H5; h. 31P13H1; i. 56P40Hl; j. 74P10H1; k. 7P49H1; l. 6P32H1; m. 12P37H1; n. 13P33H1; o.18P16H1.

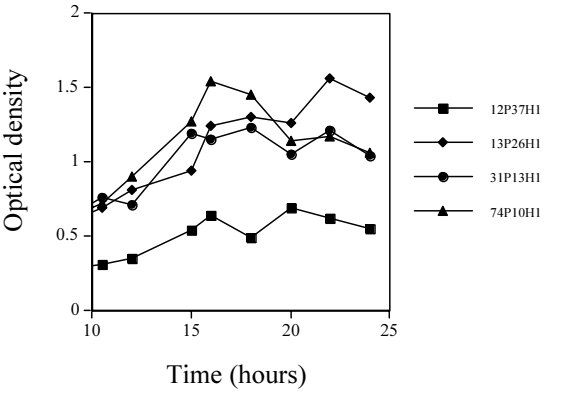

Figure 2
Time course of biofilm formation by four strains of nontypeable *H. influenzae* as noted on the right. X axis: time in hours. Y axis: optical density at 570 nm.

mains relatively stable to 24 hours at an optical density which is characteristic of each of the 4 strains.

Expression of outer membrane antigens during growth as a biofilm

To preliminarily assess the expression of outer membrane antigens by nontypeable *H. influenzae* during growth as biofilms, cell envelopes of strain 74P10H1 grown as a biofilm in a 24 well plate were purified. Simultaneously, cell envelopes of strain 74P10H1 grown on agar plates were purified by the same method. Figure 3 is a Coomassie blue stained gel of cell envelopes of strain 74P10H1 grown on agar medium compared to cell envelopes of the same strain grown as a biofilm. Differences are noted in minor bands and differences in the intensity of bands are noted. The major bands appear similar in the two preparations.

To further assess the expression of epitopes on selected individual outer membrane antigens, purified cell envelopes were assayed in immunoblot assays with well characterized polyclonal and monoclonal antibodies. Polyclonal antibodies to a peptide corresponding to loop 6 of the outer membrane protein P2 [33], which is the only known porin of nontypeable *H. influenzae*, revealed expression of P2 in the bacteria grown as a biofilm and in planktonically grown bacteria (Figure 4). Similarly, the epitopes recognized by monoclonal antibodies to outer membrane protein P5, an OMP A-like protein, and P6, a highly conserved protein in all strains of nontypeable *H. influenzae*, were expressed in the outer membranes of cells grown as biofilms and cells grown planktonically (Figure 4).

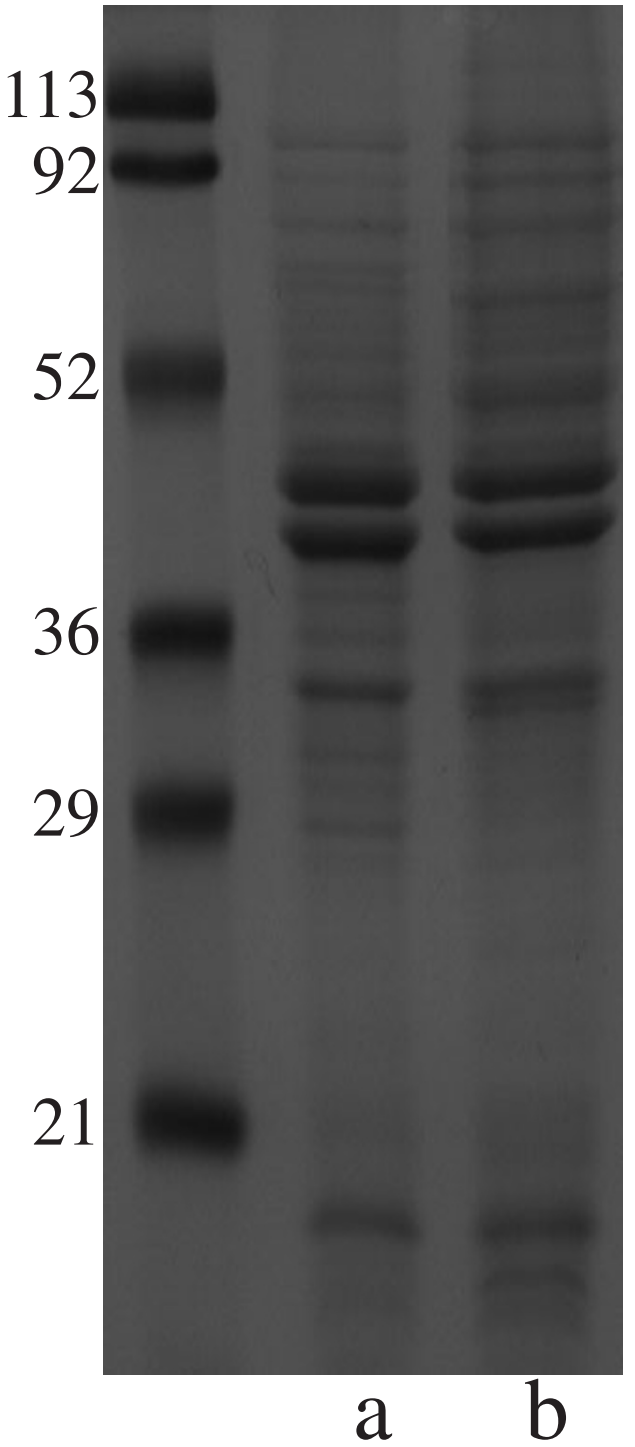

Figure 3
Coomassie blue stained sodium dodecyl sulfate polyacrylamide gel of cell envelopes of nontypeable *H. influenzae* 74P10H1 grown on agar plates (lane a) and as a biofilm (lane b). Molecular mass standards are noted in kDa on the left.

When bacteria were grown on agar plates using the same media as for growth in biofilms (brain heart infusion + hemin + nicotinamide adenine dinucleotide), the expression of a lipooligosaccharide epitope was reduced compared to the level of expression in biofilms (Figure 4). This epitope appears to be expressed at similar levels as in biofilms when the bacteria are grown on chocolate agar and in brain heart infusion broth (data not shown). Bacteria grow more slowly on the brain heart infusion agar compared to chocolate agar. The observation that the expression of epitopes on outer membrane proteins P2, P5 and P6 was similar in biofilms and agar (Figure 4) indicates that the alteration in expression of the epitope on lipooligosaccharide is not explained by slow growth, but represents altered expression of lipooligosacharide under different growth conditions.

Role of pili in biofilm formation

To assess the potential role of pili in biofilm formation, a variant of a strain of nontypeable *H. influenzae* which lacks expression of pili was tested for biofilm formation along with its isogenic parent strain. Strain M37-1 lacks expression of pili as indicated by absence of hemagglutination and absence of a 22 kDa band on SDS-PAGE corresponding to pilin [34]. In an experiment comparing biofilm formation in 4 duplicate wells, strain M37-1, the pilus deficient variant, yielded an average optical density of 0.204 whereas its isogenic parent strain, M37, yielded an average optical density of 0.884. This experiment was repeated multiple times and the pilus deficient variant showed a 3 to 4 fold decrease in biofilm formation compared to its isogenic parent strain in every experiment.

Discussion

Assays of 30 randomly chosen clinical isolates from the middle ear of children with otitis media and from the sputum of adults with COPD show that isolates of nontypeable *H. influenzae* demonstrate striking differences in their propensity to grow as biofilms in vitro. These differences are highly reproducible in that individual isolates show similar levels of biofilm formation on repeated experiments

The assay for biofilm formation used in this study is based on the ability of bacteria to adhere to plastic wells in microtiter plates. This method has been used to study biofilm formation by a variety of other gram-positive and gram-negative bacteria [35–38]. The method has both advantages and disadvantages compared to studying biofilm formation in flow cells, the other widely used method. The microtiter plate assay yields reproducible results and is convenient, allowing one to study large numbers of strains and conditions. Furthermore, the method yields quantitative results, based on measuring the optical density of wells. An important potential limitation of the mi-

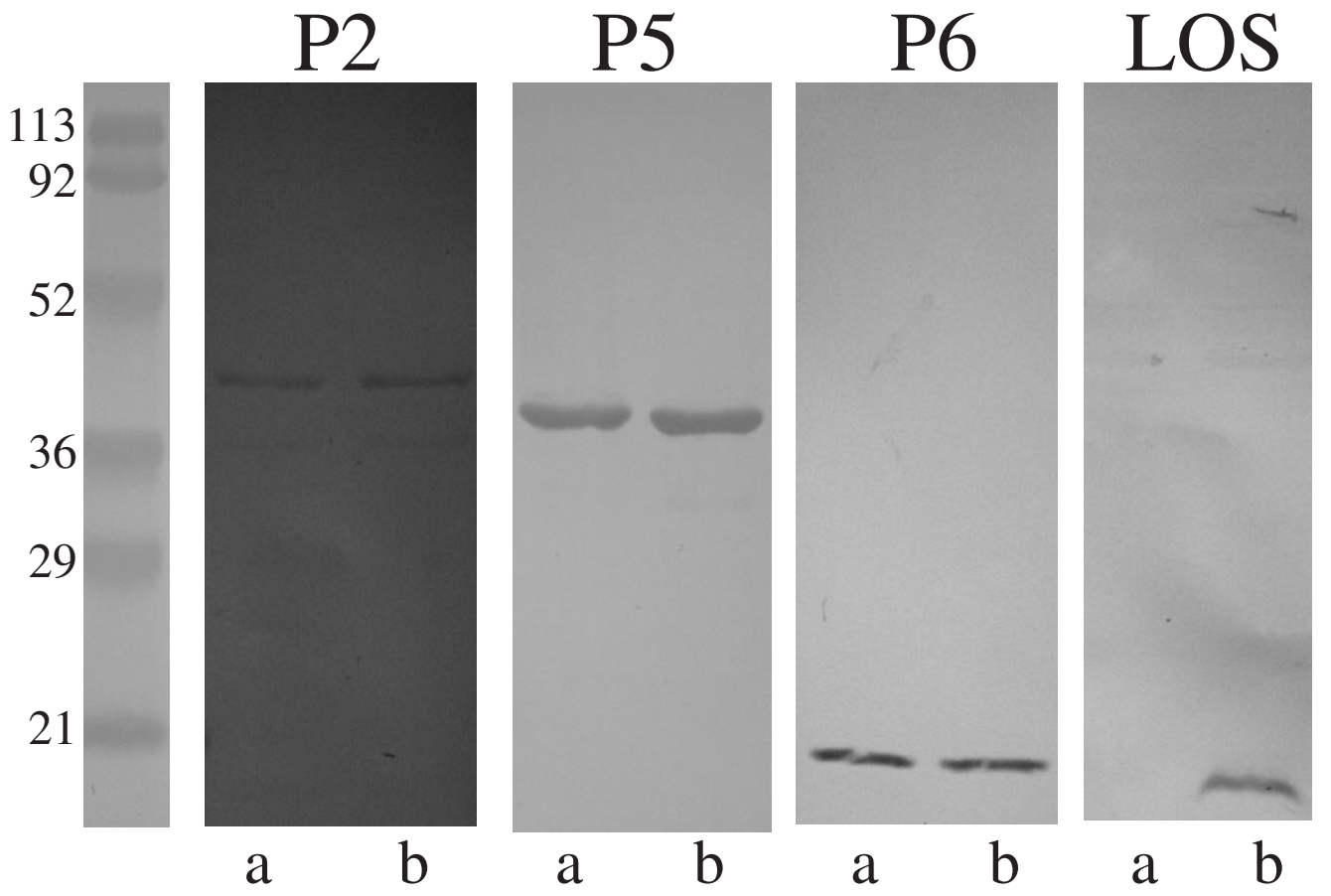

Figure 4
Immunoblot assays of cell envelopes of nontypeable *H. influenzae* 74P10H1 grown on agar plates (lanes a) and as a biofilm (lanes b). Panels were probed with antibodies to outer membrane antigens as follows: P2: rabbit polyclonal antiserum raised to loop 6 of P2; P5: monoclonal antibody 2C7; P6: monoclonal antibody 7F3; LOS (lipooligosaccharide): monoclonal antibody 6E4. Molecular mass standards are noted in kDa on the left.

crotiter plate method is that one might question whether the method is capable of distinguishing true biofilm formation from bacteria simply adhering to plastic wells. Two lines of evidence suggest that the method is measuring biofilm formation. First, Watnick and Kolter [36] showed by confocal laser microscopy that the morphology of biofilms of *Vibrio cholerae* grown on cover slips in static culture is similar to the morphology of biofilms grown in a flow cell. And second, with regard to this study, the protein patterns in SDS polyacrylamide gel electrophoresis and expression of lipooligosaccharide epitopes are different in bacteria recovered from biofilms grown in micotiter wells compared to planktonic cells grown in media. These observations suggest that the assay is detecting bacteria as biofilms rather than simply measuring planktonic bacteria adhering to plastic wells. However, more definitive answers regarding biofilm formation in human infection caused by nontypeable *H. influenzae* awaits detailed studies involving confocal laser microscopy of clinical samples containing the organism.

The importance of nontypeable *H. influenzae* as a human respiratory tract pathogen, has stimulated a large body of research to elucidate the antigenic characteristics of the outer membrane of the organism. This work is important in understanding mechanisms of pathogenesis, characterizing the human immune response to infection and guiding vaccine development. This large body of work has been performed exclusively on bacteria grown planktonically. In view of the mounting evidence that nontypeable *H. influenzae* grows as a biofilm in otitis media with effusion and in the respiratory tract of adults with COPD, it will be important to characterize the bacterial surface antigens of the organism during growth as a biofilm. The present study used well characterized polyclonal and

monoclonal antibodies to begin to address this important question.

P2, the major outer membrane protein in the outer membrane is the only known porin protein of *H. influenzae*. Immunoblot assays with antiserum raised to a conserved surface exposed loop of P2 showed that expression of P2 was similar in amount, molecular mass, and immunoreactivity in bacteria grown planktonically and as a biofilm. This observation is in contrast to expression of porin proteins of *Escherichia coli* and *Pseudomonas aeruginosa* whose expression are altered during growth as a biofilm [38,39].

Similarly, expression of outer membrane protein P5, an OMP A-like protein, and P6, a highly conserved 16 kDa protein, is preserved in cells grown as biofilms based on immunoblot assays with monoclonal antibodies (Figure 4). The expression of P5 and P6 in biofilms is especially important since these proteins are under consideration as vaccine antigens. Finally, alteration of expression of an epitope on lipooligosaccharide suggests that the organism expresses a different lipooligosaccharide molecule during growth as a biofilm. Similarly, expression of lipopolysaccharide of *Pseudomonas aeruginosa* is altered during growth as biofilms [40]. These studies of outer membrane antigen expression are limited by the sensitivity of immunoblot assays. While the assays establish that P2, P5 and P6 and the epitopes recognized by the antibodies are expressed in biofilms and that expression of lipooligosaccharide is altered, one must be cautious in drawing firm conclusions regarding the level of expression and the surface accessibility of the epitopes recognized by the antibodies based on results of immunoblot assays alone because they are not quantitative assays.

An isogenic variant of nontypeable *H. influenzae* deficient in expression in pili showed a marked reduction in the ability to grow as a biofilm. This observation parallels that made in several other bacterial species showing the pili are critical in the early stages of biofilm formation [36,41].

This paper is the first report of formation of biofilms by *H. influenzae*. Work on biofilm formation by nontypeable *H. influenzae* should focus in several areas. It will be important to define more precisely the extent to which the organism grows as biofilms during colonization and infection of the human respiratory tract. The biofilm of many bacterial species is composed of extracellular polysaccharide such as capsule [42,43]. Since nontypeable *H. influenzae* does not express a capsule, it will be interesting to characterize the composition of the biofilm. As the role of biofilms in human infection becomes better defined, elucidating the human immune response to antigens expressed during growth as biofilms may be important in understanding host responses and in devising strategies to prevent infection by nontypeable *H. influenzae*.

Conclusions

Nontypeable *Haemophilus influenzae* forms biofilms in vitro. Clinical isolates show substantial variability in their ability to grow as biofilms. Three major outer membrane proteins, P2, P5 and P6, are expressed during growth as a biofilm. Expression of an epitope on lipooligosaccharide is altered during growth as a biofilm. Pili are important in biofilm formation. As the role of biofilms in human infection becomes better defined, characterization of biofilms may be important in understanding the pathogenesis of infection and immune response to nontypeable *H. influenzae* in children with otitis media and adults with COPD.

Materials and methods

Bacterial strains

A total of 30 isolates of nontypeable *H. influenzae* were studied. Fifteen were isolated from the sputum of 11 adults with COPD as part of a prospective study being conducted at the Buffalo Veterans Affairs Medical Center. The isolates recovered from the same patient were proven to be distinct strains of nontypeable *H. influenzae* based on outer membrane protein patterns [32,44]. Fifteen isolates were recovered from the middle ear fluids obtained by tympanocentesis from children with otitis media. Twelve were from Buffalo, New York; 1 from Columbus, Ohio; 1 from Seattle, Washington; and 1 from Dallas, Texas.

Nontypeable *H. influenzae* strain M37 and an isogenic variant designated M37-1 which is deficient in expression of pili were kindly provided by Robert Munson [34].

Bacteria were grown on chocolate agar or in brain heart infusion broth supplemented with hemin and nicotinamide adenine dinucleotide both at 10 μg/ml. In the experiments to compare cell envelopes of bacteria grown planktonically with those grown as biofilms, bacteria were grown on brain heart infusion agar supplemented as noted above. Media was supplemented with glucose, sucrose, NaCl and ethanol in selected experiments as described in the Results.

Biofilm growth assay

The assay to grow and quantitate nontypeable *H. influenzae* biofilms was essentially the same assay which has been used for several other bacterial species [35–38]. An overnight broth culture was diluted 1:200 in fresh broth and 200 μl was inoculated into the wells of a 96 well Linbro tissue culture plate (ICN Biomedical, Inc., Aurora, Ohio). The plates were incubated at 37°C under 5% CO_2 for 24 hours. Before biofilm quantitation, growth was assessed by measuring the optical density at 490 nm

(OD_{490}) in a BioRad plate reader. To quantitate biofilm formation, 20 µl of Difco crystal violet (Becton Dickinson, Sparks, Maryland) was added to each well and incubated at room temperature for 15 minutes. Wells were washed vigorously with distilled water and the plate was air dried. A volume of 230 µl of 95% ethanol was added to each well and the OD_{570} was measured. All strains were tested in quadruplicate. Each plate included 4 wells which contained sterile broth instead of bacteria but were treated identically otherwise. The OD_{570} was standardized against these wells.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and immunoblot assays

Cell envelopes were subjected to SDS PAGE and Coomassie blue staining using previously described methods [44]. Immunoblot assays were performed as described previously [45]. After incubating with the antibodies, blots were developed with the appropriate murine or rabbit peroxidase conjugated secondary antibody.

Purification of cell envelopes

To study the outer membrane of nontypeable *H. influenzae*, bacteria were grown as a biofilm overnight in a 24 well Linbro tissue culture plate. Broth was aspirated and the wells were washed 3 times with 1 ml of 0.01 M HEPES, pH 7.4. Bacteria in the biofilm were harvested by scraping the wells with the tip of a 1 ml micropipette tip. To prepare cell envelopes, cells were suspended in 0.01 M HEPES, pH 7.4 and sonicated on ice by using a Branson Sonifier (small tip, setting 7) [44]. The suspension was centrifuged at 12,000 x g for 2 minutes at 4°C to remove unbroken cells and debris. The supernatant was recovered and centrifuged at 12,000 x g for 45 minutes. The resulting pellet was suspended in sample buffer and subjected to SDS-PAGE and immunoblot assay.

Monoclonal and polyclonal antibodies

Monoclonal antibody (mab) 7F3 recognizes a surface exposed epitope on outer membrane protein P6 [45,46]. Mab 6E4 (Provided by Alan Lesse) recognizes an epitope on lipooligosaccharide [47]. Mab 2C7 (provided by Alan Lesse) recognizes an epitope on outer membrane protein P5. Rabbit polyclonal antiserum raised to a peptide corresponding to loop 6 of outer membrane protein P2 recognizes P2 of many strains of nontypeable *H. influenzae* was described previously [33].

Acknowledgements

This work was supported by grants AI 19641 and AI 46422 from the National Institutes of Health and by the Department of Veterans Affairs. The authors thank Dr. Sanjay Sethi for performing statistical analysis and Dr. Thomas Hiltke for critically reviewing the manuscript.

References

1. Foxwell AR, Kyd JM, Cripps AW: **Nontypeable *Haemophilus influenzae:* pathogenesis and prevention.** *Microbiol.Mol.Biol.Rev.* 1998, **62:**294-308
2. Rao VK, Krasan GP, Hendrixson DR, Dawid S, St.Geme JW III: **Molecular determinants of the pathogenesis of disease due to non-typable *Haemophilus influenzae*.** *FEMS Microbiol Rev* 1999, **23:**99-129
3. Murphy TF: **Bacterial otitis media: pathogenetic considerations.** *Pediatr Infect Dis J* 2000, **19:**S9-S16
4. Murphy TF: ***Haemophilus influenzae* in chronic bronchitis.** *Semin.Respir.Infect.* 2000, **15:**41-51
5. Klein JO: **Otitis media.** *Clin.Infect.Dis.* 1994, **19:**823-833
6. Ruuskanen O, Heikkinen T: **Otitis media: etiology and diagnosis.** *Pediatr Infect Dis J* 1994, **13:**S23-S26
7. Dagan R, Leibovitz E, Greenberg D, Yagupsky P, Fliss DM, Leiberman A: **Early eradication of pathogens from middle ear fluid during antibiotic treatment of acute otitis media is associated with improved clinical outcome.** *Pediatr Infect Dis J.* 1998, **17:**776-782
8. Kilpi T, Herva E, Kaijalainen T, Syrjanen R, Takala AK: **Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life.** *Pediatr Infect Dis J* 2001, **20:**654-662
9. Gehanno P, Panajotopoulos A, Barry B, Nguyen L, Bingen E, Berche P: **Microbiology of otitis media in the Paris, France, area from 1987 to 1997.** *Pediatr Infect Dis J* 2001, **20:**570-573
10. Kaplan B, Wandstrat TL, Cunningham JR: **Overall cost in the treatment of otitis media.** *Pediatr Infect Dis J* 1997, **16:**S9-S11
11. Stool SE, Field MJ: **The impact of otitis media.** *Pediatr Infect Dis J.* 1989, **8:**S11-S14
12. Faden H: **The microbiologic and immunologic basis for recurrent otitis media in children.** *Eur J Pediatr* 2001, **160:**407-413
13. Poolman JT, Bakaletz L, Cripps A, Denoel PA, Forsgren A, Kyd J, *et al*: **Developing a nontypeable *Haemophilus influenzae* (NTHi) vaccine.** *Vaccine* 2000, **19:**S108-S105
14. Klein JO: **Management of otitis media: 2000 and beyond.** *Pediatr Infect Dis J* 2000, **19:**383-387
15. Dingman JR, Rayner MG, Mishra S, Zhang Y, Ehrlich MD, Post JC, *et al*: **Correlation between presence of viable bacteria and presence of endotoxin in middle-ear effusions.** *J Clin Microbiol* 1998, **36:**3417-3419
16. Ueyama T, Kurono Y, Shirabe K, Takeshita M, Mogi G: **High incidence of *Haemophilus influenzae* in nasopharyngeal secretions and middle ear effusions as detected by PCR.** *J Clin Microbiol* 1995, **33:**1835-1838
17. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ: **Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions.** *J Clin Microbiol* 1997, **35:**2854-2858
18. Hendolin PH, Paulin L, Ylikoski J: **Clinically applicable multiplex PCR for four middle ear pathogens.** *J Clin Microbiol* 2000, **38:**125-132
19. Post JC, Preston RA, Aul JJ, Larkins-Pettigrew M, Rydquist-White J, Anderson KW, *et al*: **Molecular analysis of bacterial pathogens in otitis media with effusion.** *JAMA* 1995, **273:**1598-1604
20. Post JC, Aul JJ, White GJ, Wadowsky RM, Zavoral T, Tabari R, *et al*: **PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media.** *Amer. J. Otolaryngol.* 1996, **17(2):**106-111
21. Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD: **Evidence of bacterial metabolic activity in culture-negative otitis media with effusion.** *JAMA* 1998, **279:**296-299
22. Costerton JW, Stewart PS, Greenberg EP: **Bacterial biofilms: a common cause of persistent infections.** *Science* 1999, **284:**1318-1322
23. Post JC, Ehrlich M, Ehrlich GD: **New paradigms in infectious disease: applications to otitis media.** *Pittsburgh, PA* 2000
24. O'Toole G, Kaplan HB, Kolter R: **Biofilm formation as microbial development.** *Annu Rev Microbiol* 2000, **54:**49-79
25. Davey ME, O'Toole GA: **Microbial biofilms: from ecology to molecular genetics.** *Microbiol Mol Biol Rev* 2000, **64:**847-867
26. Donlan RM: **Biofilm formation: a clinically relevant microbiological process.** *Clin Infect Dis* 2001, **33:**1387-1392
27. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP: **Quorum-sensing signals indicate that cystic fibrosis**

lungs are infected with bacterial biofilms. *Nature* 2000, **407(6805):**762-764
28. Kolenbrander PE: **Oral microbial communities: biofilms, interactions, and genetic systems.** *Annu Rev Microbiol* 2000, **54:**413-437
29. Meluleni GJ, Grout M, Evans DJ, Pier GB: **Mucoid *Pseudomonas aeruginosa* growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients.** *J Immunol* 1995, **155:**2029-2038
30. Evans DJ, Allison DG, Brown MRW, Gilbert P: **Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate.** *J Antimicrob Chemother* 1991, **27:**177-184
31. Sethi S, Murphy TF: **Bacterial infection in chronic obstructive pulmonary disease in 2000. A state of the art review.** *Clin Microbiol Rev* 2001, **14:**336-363
32. Murphy TF, Sethi S, Klingman KL, Brueggemann AB, Doern GV: **Simultaneous respiratory tract colonization by multiple strains of nontypeable *Haemophilus influenzae* in chronic obstructive pulmonary disease: implications for antibiotic therapy.** *J Infect Dis* 1999, **180:**404-409
33. Neary JM, Yi K, Karalus RJ, Murphy TF: **Antibodies to loop 6 of the P2 porin protein of nontypeable *Haemophilus influenzae* are bactericidal against multiple strains.** *Infect Immun* 2001, **69:**773-778
34. Coleman T, Grass S, Munson R Jr: **Molecular cloning, expression, and sequence of the pilin gene from nontypeable *Haemophilus influenzae* M37.** *Infect Immun* 1991, **59:**1716-1722
35. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R: **Genetic approaches to study biofilms.** *Methods in Enzymology* 1999, **310:**91-109
36. Watnick PI, Kolter R: **Steps in the development of a *Vibrio cholerae* El Tor biofilm.** *Mol Microbiol* 1999, **34:**586-595
37. Froeliger EH, Fives-Taylor P: ***Streptococcus parasanguis* fimbria-associated adhesin FapI is required for biofilm formation.** *Infect.Immun.* 2001, **69:**2512-2519
38. O'Toole GA, Kolter R: **Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis.** *Mol Microbiol* 1998, **28:**449-461
39. Prigent-Combaret C, Vidal O, Dorel C, LeJeune P: **Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*.** *J Bacteriol* 1999, **181:**5993-6002
40. Giwercman B, Fomsgaard A, Mansa B, Hoiby N: **Polyacrylamide gel electrophoresis analysis of lipopolysaccharide from *Pseudomonas aeruginosa* growing planktonically and as biofilm.** *FEMS Microbiol Immunol* 1992, **4:**225-230
41. Pratt LA, Kolter R: **Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili.** *Mol Microbiol* 1998, **30:**285-293
42. Sutherland IW: **Biofilm exopolysaccharides: a strong and sticky framework.** *Microbiology* 2001, **147:**3-9
43. Danese PN, Pratt LA, Kolter R: **Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture.** *J Bacteriol* 2000, **182:**3593-3596
44. Murphy TF, Dudas KC, Mylotte JM, Apicella MA: **A subtyping system for nontypable *Haemophilus influenzae* based on outer-membrane proteins.** *J Infect Dis* 1983, **147:**838-846
45. Nelson MB, Munson RS Jr, Apicella MA, Sikkema DJ, Molleston JP, Murphy TF: **Molecular conservation of the P6 outer membrane protein among strains of *Haemophilus influenzae:* analysis of antigenic determinants, gene sequences, and restriction fragment length polymorphisms.** *Infect Immun* 1991, **59:**2658-2663
46. Murphy TF, Nelson MB, Dudas KC, Mylotte JM, Apicella MA: **Identification of a specific epitope of *Haemophilus influenzae* on a 16,600-dalton outer membrane protein.** *J Infect Dis* 1985, **152:**1300-1307
47. Campagnari AA, Spinola SM, Lesse AJ, Abu Kwaik Y, Mandrell RE, Apicella MA: **Lipooligosaccharide epitopes shared among gram-negative non-enteric mucosal pathogens.** *Microb Pathog* 1990, **8:**353-362

Publish with **BioMed** Central and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright

BioMedcentral.com

Submit your manuscript here:
http://www.biomedcentral.com/manuscript/

editorial@biomedcentral.com