Eur Respir J, 1995, 8, 709–714
DOI: 10.1183/09031936.95.08050709
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European Respiratory Journal
ISSN 0903 - 1936

Interaction of fimbriated and nonfimbriated strains of unencapsulated *Haemophilus influenzae* with human respiratory tract mucus *in vitro*

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Interaction of fimbriated and nonfimbriated strains of unencapsulated Haemophilus influenzae *with human respiratory tract mucus* in vitro. *W. Barsum, R. Wilson, R.C. Read, A. Rutman, H.C. Todd, N. Houdret, P. Roussel, P.J. Cole. ©ERS Journals Ltd 1995.*

ABSTRACT: Adherence to mucus may influence bacterial colonization of the respiratory tract. Clinical isolates of nontypable *Haemophilus influenzae* (NTHi) from the respiratory tract are often fimbriated. We wondered whether fimbriated strains have a different adherence from related nonfimbriated strains.

A microtitre plate assay has been developed to study adherence of nontypable *H. influenzae* to mucus. Wells were coated by incubation either with sol phase of sterile mucoid secretions or with purified preparations of mucins. Two laboratory pairs of fimbriated (F+) and nonfimbriated (F-) nontypable *H. influenzae*, and six fresh clinical isolates of fimbriated nontypable *H. influenzae* each with nonfimbriated partners derived by serial passage on agar, were cultured to mid-log phase, washed, and then added to the wells. They were then incubated at 37°C for 30 min before washing to remove unbound bacteria. Adherent bacteria were desorbed by agitation with 0.5% Tween 80 and a viable count performed.

The two fimbriated laboratory strains (n=12 and n=17), and 5 of the 6 fimbriated clinical isolates were more adherent to sol phase than their respective nonfimbriated partners. Two nonfimbriated clinical isolates were more adherent to plastic than their fimbriated partners. A fimbriated laboratory strain was more adherent than its nonfimbriated partner both to a purified preparation of high molecular mass mucin and to the glycopeptide fraction of the same.

We conclude that fimbriated strains of nontypable *H. influenzae* have increased adherence to sol phase of mucus and purified human respiratory tract mucin. The interactions of fimbriae with mucus are likely to be complex, and may involve both nonspecific and specific interactions.

Eur Respir J., 1995, 8, 709–714.

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Keywords: Adherence
bacterial
fimbria
non-typable *Haemophilus influenzae*
mucus

Received: March 10 1993
Accepted after revision August 9 1994

The mucociliary apparatus of the upper and lower respiratory tract plays an important role in preventing paranasal sinus and bronchial infections by conveying inhaled particles, including bacteria, out of the airways to the pharynx, where they are swallowed or expectorated. In situations in which mucociliary clearance is impaired, bacterial adherence to mucus is likely to influence colonization of the respiratory tract.

Haemophilus influenzae is a frequent cause of acute respiratory tract infection in developing countries, and is commonly associated with exacerbations of tobacco smoke-related chronic bronchitis and with chronic bronchial sepsis (including bronchiectasis) in developed countries. FARLEY *et al*. [1] demonstrated that nontypable *H. influenzae* (NTHi) infecting an organ culture of human adenoid tissue adhered avidly to mucus. READ *et al*. [2] recently showed that this also occurred in an organ culture of human nasal turbinate tissue; and that, in this system, mucus rather than the epithelial surface was the initial site of interaction of NTHi with human airway mucosa.

More than 50% of NTHi isolates from sputum or nasopharynx are fimbriated [3, 4]. The role of fimbriae in the pathogenesis of infection by *H. influenzae* is not clear, but they probably act as adhesins to cell surfaces. There have been few studies of the interactions between NTHi and mucus. We have adapted a previously described *in vitro* system [5], in which mucus is bound to microtitre plates, and have investigated the difference in adhesion between closely related fimbriated (F+) and nonfimbriated (F-) strains of NTHi.

Materials and methods

Mucus

Upper respiratory tract mucus was obtained by washing the nose of five normal volunteers after nasal challenge

with saline spray (5–10 ml of saline at 0.1 ml·spray^{-1}). Expectorated tracheobronchial secretions were obtained from one individual with mucus hypersecretion. Saliva or saline was removed by aspiration with a Pasteur pipette. Pooled secretions were collected and shown to be sterile on microbial culture by standard techniques. Sol phase was obtained by high speed centrifugation (27,000×g for 30 min) and aspirated from the gel phase which was discarded. The sol phase was ultrasonicated (75 W) on ice for 3 min, intermittently (30 s interval every min), then stored at -70°C. The composition of the sol phase was investigated by polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis. The polyacrylamide gels were stained by Coomassie blue and by Schiff reagent after periodic oxidation (PAS); the agarose gels were stained by amido black, PAS and toluidine blue.

Purified preparations of high molecular mass tracheobronchial mucin, and mucin glycopeptides obtained after proteolysis, were prepared as follows: sputum was diluted (1:12) with deionized water and stirred overnight, then dialysed and lyophilized. A preparation of high molecular mass mucin was obtained by caesium bromide density gradient centrifugation [6], and was further purified by a second step of caesium bromide density gradient centrifugation. Mucin glycopeptides were obtained by pronase digestion of mucin and separated by gel chromatography on Sepharose 4B [7]. Both mucin preparations were dissolved in phosphate-buffered saline (PBS), at a concentration of 100 µg·ml^{-1}, prior to use in the assay.

Coating of wells

Adherence assays were performed in 96 well microtitre plates (Flow Laboratories, Buckinghamshire, UK). One hundred microlitres of sol phase or mucin preparation was added to wells. The plates were left at 37°C overnight to allow coating of the wells. Wells were washed twice with sterile PBS.

Bacteria

The following pairs of nontypable *H. influenzae* were used:

1) Unencapsulated strain R890 (F+) was derived from unencapsulated strain R906 (F-) by transformation with chromosomal deoxyribonucleic acid (DNA) from a fimbriated type b capsulated strain of *H. influenzae* [8] (gifts from A. Smith, University of Washington, Seattle, USA).

2) VA (F+) is a Dutch clinical isolate and has a relatively stable F+ phenotype. VA (F-) was derived by serial passage of VA (F+) on agar (gifts from L. van Alphen, University of Amsterdam, The Netherlands).

3) Six clinical strains were isolated from sputum of patients with chronic obstructive airways disease during infective exacerbations. All strains were identified as *H. influenzae* by requirements for X and V factors on sucrose plates [9], and demonstrated to be fimbriated by transmission electron microscopy (TEM). Nonfimbriated strains (by TEM) were derived by serial passage on Levinthal agar (six subcultures).

Assay of bacterial adherence to mucus

Bacteria were stored in 10% glycerol broth in liquid nitrogen and plated out overnight on Levinthal agar, as required. Two colonies were touched with a loop, transferred to 20 ml of enriched brain heart infusion broth (Oxoid, Hampshire, UK) (supplemented with haemin and β-nicotinamide adenine dinucleotide, 5 µg·ml^{-1} and 10 µg·ml^{-1}, respectively), and incubated for 4 h (mid-log phase) at 37°C on an orbital shaker (10 cycles·min^{-1}). The bacteria were pelleted by centrifugation at 2,000×g for 15 min at 4°C, washed three times and resuspended in PBS. The viable count of the starting inoculum was quantitated by serial dilutions and culture on Levinthal agar overnight. One hundred microlitres of the bacterial suspension in PBS was added to each well. Experiments were performed in triplicate. The plates were incubated at 37°C for 30 min for most experiments. The wells were then washed twice with 250 µl PBS to remove unbound bacteria. Adherent bacteri were desorbed by agitation with 250 µl 0.5% polyoxyethylenesorbitan (Tween 80) (Sigma) in PBS for 15 min. Fifty microlitres was removed, diluted in 450 µl of broth, then quantitated by overnight inoculation of serial dilutions plated on Levinthal agar.

This experiment protocol was determined by a series of preliminary experiments (data not shown). Siliconization of plastic did not affect nonspecific binding of NTHi and was, therefore, omitted. Mucus coating of wells, as tested by Alcian blue staining, was maximal after overnight incubation. The concentration of 0.5% Tween 80 was chosen as the maximum which did not affect bacterial viability. Eighty to ninety five percent of bacteria adherent to the well were desorbed after 15 min using this concentration of detergent. To determine the influence of time of incubation on the adherence of *H. influenzae* to mucus, incubation time was varied prior to Tween treatment. The adherence of strains R890 and R906 was greatest at 30 min. Sputum sol phase was ultrasonicated, as this was shown to improve binding of bacteria to coated wells.

Transmission electron microscopy (TEM)

The degree of fimbriation of bacteria prior to and after performing the adherence assay was evaluated. Two colonies were picked with a loop and transferred into 1 ml of PBS in an Eppendorf, spun for a few seconds, and then centrifuged at 2000×g for 5 min. The bacteria were washed once in 1 ml of ammonium acetate 0.1 M, then resuspended in the same solution. A droplet of this suspension was placed on a waxy surface and touched with the matt surface of a 400 mesh, carbon-coated copper grid (Agar Scientific, Stansted, UK), which was then allowed to dry in air under a lamp. A droplet of 1% potassium phosphotungstate (BDH, Poole, UK) was placed into the grid and the excess removed with tissue paper.

The grid was dried and examined by TEM. The percentage of fimbriated bacteria in the first 50 bacteria seen was calculated.

Calculation of indices of adherence

The adherence index (AI) was calculated as $(X) \times Z^{-1}$ and the specific adherence index (SAI) as $(X-Y) \times Z^{-1}$ where X=number of bacteria desorbed from mucus coated wells, Y=number of bacteria desorbed from uncoated control wells (*i.e.* adherent to plastic), and Z=number of bacteria in initial inoculum.

Statistical analysis

For each experiment performed in triplicate, the mean adherence index and the mean specific adherence index were obtained. Statistical comparisons were made by Wilcoxon's Rank analysis, using Statmaster programme and Commodore computer. A value of $p<0.05$ was considered significant.

Results

The viable counts (colony forming units (cfu)·ml^{-1} ±standard error) of inocula and degree of fimbriation were: R890 (F+) 1.63±0.47 $\times 10^9$, 94%, and R906 (F-) 1.87±0.66 $\times 10^9$, 8%; VA (F+) 4.76±0.57 $\times 10^8$, 96%, and VA (F-) 5.23±0.64 $\times 10^8$, 8%. There was no change in the degree of fimbriation of bacteria before and after adherence experiments.

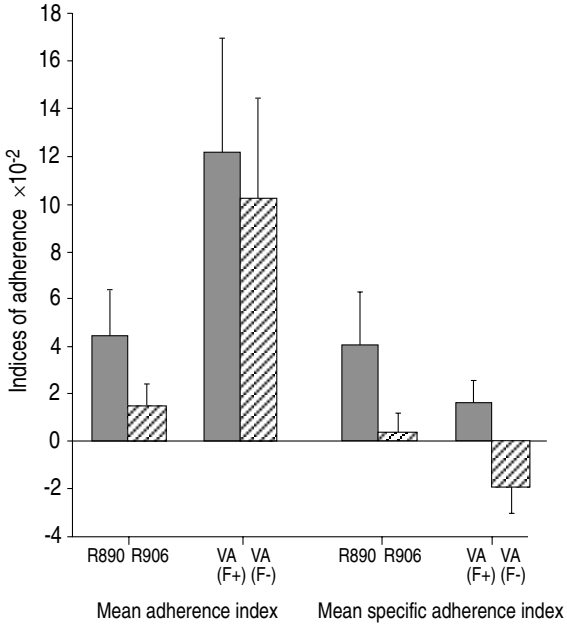

Fig. 1. – Effect of fimbriae on adherence of nontypable *Haemophilus influenzae* to mucus sol phase. The mean adherence index and mean specific adherence index of fimbriated strains R890 (n=12) and VA (F+) (n=17) was greater than nonfimbriated strains R906 ($p<0.05$ and $p<0.02$, respectively) and VA (F-) ($p<0.04$ and $p<0.05$, respectively). Error bars indicate the SEM. ▬: fimbriated; ▭: nonfimbriated.

Effect of fimbriae on the ability of NTHi to adhere to respiratory tract mucus sol phase

The results of the adherence assay of these strains are shown in figure 1. In each case, the fimbriated bacteria were more adherent than the nonfimbriated. In 5 of the 12 experiments (R890/R906), pooled nasal secretions from normal volunteers were used, whilst in the other 7 experiments and in the VA series sol phase was prepared from sputa of one individual with mucus hypersecretion. Whether sol phase was prepared from nasal secretion or sputum, the binding of R890 exceeded R906 (SAI nasal secretion: F+=0.94±0.35 $\times 10^{-2}$, and F-=-1.13±0.654 $\times 10^{-2}$ ($p<0.05$); SAI sputum: F+=6.29±3.83 $\times 10^{-2}$, and F-=1.6±1.583 $\times 10^{-2}$ ($p<0.02$)). There was no significant difference in adherence of R890 and R906 to nasal or sputum sol phase.

The analysis of the sputum sol phase by PAGE and agarose gel electrophoresis showed that it contained mostly proteins and only a little mucin. There were several proteins, mainly at about 55/60 kDa and 15 kDa. Adherence of *H. influenzae* to purified mucin preparations was, therefore, studied. Adherence of R890 (F+) exceeded R906 (F-) to both high molecular weight mucin and mucin glycopeptides, respectively, (fig. 2). R890 (F+) was more adherent to sputum sol phase than to high molecular weight mucin and mucin glycopeptides ($p<0.02$), whereas adherence of F- to all three preparations were similar.

The adherence assay was performed with clinical isolates of NTHi. The viable counts (cfu·ml^{-1}) of inocula and degree of fimbriation of those strains were: R1 (F+) 3.25±0.67 $\times 10^8$, 94%, and R1 (F-) 2.5±0.41 $\times 10^8$, 28%; R2 (F+) 2.4±0.47 $\times 10^8$, 84%, and R2 (F-) 2.71±0.98 $\times 10^8$, 6%; R3 (F+) 3.91±0.88 $\times 10^8$, 84%, and R3 (F-) 5.41±1.2 $\times 10^8$, 24%; R4 (F+) 3.91±0.71 $\times 10^8$, 82%, and R4 (F-) 4±0.68 $\times 10^8$, 4%; R5 (F+) 3.07±1.19 $\times 10^8$, 80%, and R5 (F-) 3.4±0.9 $\times 10^8$, 4%; R6 (F+) 3.3±1.15 $\times 10^8$,

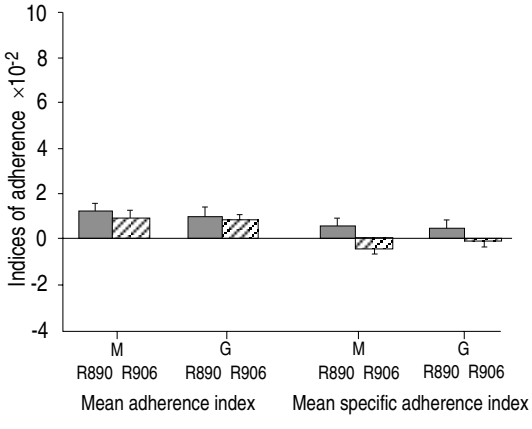

Fig. 2. – Effect of fimbriae on adherence of nontypable *Haemophilus influenzae* to high molecular weight mucin (M) and its constituent glycopeptides (G). The mean specific adherence index of fimbriated strain R890 (n=8) to both high molecular weight mucin ($p<0.02$) and to its constituent glycopeptides ($p<0.04$) was significantly greater than nonfimbriated strain R906, but there was no significant difference between the mean adherence indices.

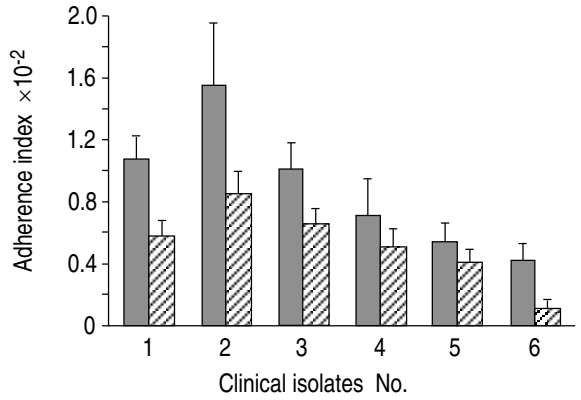

Fig. 3. – Mean adherence index of six clinical isolates of fimbriated (F+) and nonfimbriated (F-) nontypable *Haemophilus influenzae* to sputum sol phase (n=6). For each clinical isolate the adherence index of the fimbriated strains was significantly ($p<0.05$) greater than its nonfimbriated derivative. Errors bars indicate the SEM. ▬: fimbriated; ▭ : nonfimbriated.

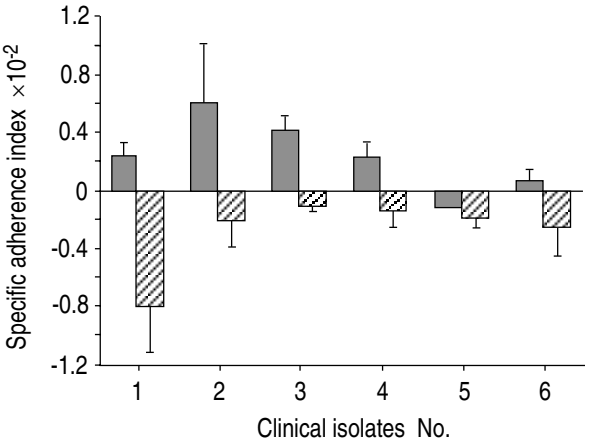

Fig. 4. – Mean specific adherence index of six clinical isolates of fimbriated (F+) and nonfimbriated (F-) nontypable *Haemophilus influenzae* to human sputum sol phase (n=6). For five of the six clinical isolates the mean specific adherence index of the fimbriated stains was significantly ($p<0.03$) greater than its nonfimbriated derivative. Strain No. 5 was not significantly different. Error bars indicate the SEM. ▬: fimbriated; ▭ : nonfimbriated.

46%, and R6 (F-) $2.86\pm1.04 \times 10^8$, 0%. The results of the adherence of these pairs to sputum sol phase are shown in figures 3 and 4. In 5 of the 6 strains, SAI of F+ was significantly greater than F- ($p<0.03$), whilst for all strains AI of F+ was significantly greater than F- ($p<0.05$).

Two of the F-clinical isolates (strains 1 and 6 in fig. 3 and 4) were significantly ($p<0.05$) more adherent to plastic than their F+ partners, but in the other four clinical isolates, and the two laboratory pairs (to both sol phase and purified preparations), there was no significant difference in the adherece of F+/F- strains to plastic.

Discussion

Bacterial adherence to mucosal surfaces is thought to be the primary initiating event in respiratory infections in man [10]. In this study, we have shown that NTHi adhere to sol phase of mucus and purified human respiratory tract (HRT) mucin, and that adherence is increased if the bacteria are fimbriated.

Previous studies have shown that bacteria such as *Pseudomonas aeruginosa* [11–17], *H. influenzae* [18–20], and *Streptococcus pneumoniae* [21, 22] adhere to epithelial cells. However, these studies have usually used dispersed cells obtained from the mouth, nose, or trachea, and have not examined the interaction of bacteria with the intact respiratory mucosal surface. In the respiratory tract, the mucociliary system is an important defence mechanism against inhaled micro-organisms. Micro-organisms are trapped in mucus, which is their first contact with the mucosal surface, and subsequently moved cephalad by ciliary beating. The majority of bacteria infecting the airways are intraluminally associated with secretions, rather than attached to the epithelial surface [23]. Respiratory tract mucus consists of mucin and other biological substances, such as lactoferrin, immunoglobulins, lipids and glycoproteins. Mucin consists of a population of high molecular weight glycoproteins with different peptide cores (apomucins), to which are attached hundreds of carbohydrate side-chains, each containing from 1 to 20 sugars [24]. It has been suggested that specific interactions occur between certain bacteria and mucins [25, 26, 27], which may be similar to the adhesin-receptor interactions that are responsible for bacterial adherence to epithelial cells [10].

NTHi is an important human pathogen both in adults and children, causing a wide range of diseases, including acute and chronic otitis media, sinusitis, pneumonia and exacerbations of chronic bronchitis. The pathogenesis of these diseases involves colonization of the respiratory mucosa. There have been a number of studies that have investigated the interaction of various bacterial species with mucus during respiratory infections.

The capacity of *H. influenzae* to adhere to mucus has been noted previously. FARLEY *et al.* [1] observed that large numbers of *H. influenzae* adhered to mucus of human adenoid organ cultures. READ *et al.* [2] supported this observation in an organ culture model utilizing human nasal turbinate tissue. They demonstrated that NTHi were associated with mucus but not epithelium after 14 h of incubation, and adherence to epithelium only occurred when epithelial damage was present. It has been suggested that binding of NTHi to human tracheobronchial mucin may involve specific interactions [28]. Certain species of bacteria (*P. aeruginosa*, *H. influenzae* and *S. pneumoniae*) that infect the respiratory tract are not only capable of adhering to mucus, but also stimulate its secretion [29].

The majority of fresh isolates of NTHi from the respiratory tract are fimbriated [4]. Fimbriae have been identified as one of the determinants of *H. influenzae* adherence to epithelial cells [20], although other adhesins are also involved [2, 4]. Therefore, study of the role of fimbriae during respiratory infection by *H. influenzae* is warranted, and we have investigated their role in bacterial adherence to mucus.

We have adapted the system of VISHWANATH and RAMPHAL [5] to enable us to measure bacterial interaction with mucus sol phase and purified mucin. This provides an

opportunity to examine difference in adhesion of closely related bacterial strains. It also offers an opportunity, in the future, to explore the nature of adherence, *i.e.* to identify adhesin-receptor interactions if these are present. The weakness of the system was the incomplete coating of the wells by the preparations. This problem was circumvented by measuring bacterial adherence to both coated and uncoated (plastic only) wells, and calculating SAI. In some cases the SAI was negative, indicating that the strains were more adherent to plastic than the partially mucus coated wells. The SAI was frequently negative for non-fimbriated strains, and two F- clinical isolates were more adherent to plastic than their respective F+ partners. The relevance of this observation is uncertain. The surface of non-typable *H. influenzae* is composed of outer membrane proteins, lipooligosaccharide and fimbriae. Differences in adherence to plastic are likely to be due to physical properties of the bacterial surface such as charge, and may be influenced by the relative proportions of lipoligosaccharide and protein present. Fimbriae are filamentous proteins that would not only increase the protein content of the bacterial surface, but also by projecting from the bacterial surface, its spacial organisation.

Fimbriae enhance *H. influenzae* adherence to buccal cells, and it has been suggested that fimbriae may give *H. influenzae* an advantage when colonizing the nasopharynx by increasing adherence [20]. However, it has also been demonstrated in an organ culture of human nasal turbinate tissue [2] that fimbriae neither permit adherence to normal ciliated respiratory epithelium, nor enhance bacterial adherence to areas of epithelial damage. In the present study, we have shown that fimbriae enhance the adherence of NTHi to the sol phase of nasal and tracheobronchial secretions, purified high molecular weight mucin, and its constituent glycopeptides derived by proteolysis. It is possible that fimbriae could confer a colonization advantage by facilitating adherence to mucus, although fimbriae could also disadvantage the bacterium in situations of normal host defence, when mucus is cleared normally. In situations in which mucus might be slow moving or static, fimbriae could confer a colonization advantage. There may be areas in the upper respiratory tract, such as overlying the adenoid and tonsil, where pools of mucus form. In the lower respiratory tract, mucus clearance is impaired in chronic bronchitis [30], bronchiectasis [31], and cystic fibrosis [32, 33]. Fimbriae could also enhance the chance of the bacterium staying within mucus rather than adhering to epithelium, which could select for organisms that lose fimbriae before adhering to damaged epithelium. This concept would be analogous to the loss of fimbriae that has been observed in isolates from the bloodstream [34].

In an attempt to identify the components of mucus involved in adherence, we have analysed sputum sol phase, and also used purified preparations in the adherence assay. We have demonstrated that fimbriae enhance adherence of NTHi to sputum sol phase, but that this preparation contains mostly proteins, and only contains small amounts of mucin. Fimbriae also enhance adherence to purified human tracheobronchial mucin and its constituent glycopeptides, although the adherence indices were smaller than for sol phase.

In summary, we have shown that fimbriated strains of NTHi have increased adherence to sol phase of mucus and purified HRT mucin. The majority of fresh isolates of NTHi from the respiratory tract are fimbriated, and possession of fimbriae may provide the bacteria with a colonization advantage in conditions with impaired mucociliary clearance. The interactions of fimbriae with mucus are likely to be complex, and may involve both nonspecific and specific (adhesin-receptor) interactions.

Future work should aim to characterize the nature of such interactions. The association of large numbers of bacteria with secretions during lower respiratory tract infections [23] emphasizes the importance of improving mucus clearance in the management of these conditions.

Acknowledgements: The authors thank A. Smith (University of Washington, Seattle, USA) for donating strains R890 and R906, L. van Alphen (Dept of Medical Microbiology, University of Amsterdam, The Netherlands) for donating VA strains, P. Webber (Dept of Microbiology, Royal Brompton Hospital, London, UK) for providing the clinical isolates and J. Burditt for manuscript preparation.

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