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Frequency of Fimbriation of Nontypable *Haemophilus influenzae* and Its Ability To Adhere to Chinchilla and Human Respiratory Epithelium

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To date, we have examined nearly 60 clinical isolates of nontypable *Haemophilus influenzae* (26 nasopharyngeal, 33 from middle ear effusions) and have found that 100% were fimbriated. The percentage of cells bearing fimbriae within each isolate varied from less than 10 to 100%, with fimbriae being either peritrichous or bipolar in distribution. Fimbriae were approximately 2.4 to 3.6 nm in width; however, there was a high degree of variability in both length and number of fimbriae per individual bacterial cell among these isolates. All isolates tested adhered to both human oropharyngeal cells and chinchilla tracheal epithelium regardless of the degree to which the particular isolate was fimbriate. The level or degree of fimbriation did not correlate with either site of isolation, biotype, strength of hemagglutination reaction, or type of effusion present in the ear. These appendages appear to be quite different from those described for type b *H. influenzae* in which the ability to adhere and strength of ability to hemagglutinate correlated strongly with degree of fimbriation.

Bacterial fimbriae are defined as nonflagellar proteinaceous surface appendages that do not participate in the transfer of bacterial or viral nucleic acids (19). They are known to be critical to the successful adherence of many pathogens to host cells, which is considered to be the first step in the infectious process (3). Although it has been shown by several investigators (9, 14) that nontypable *Haemophilus influenzae* (NTHI) are generally more adherent than type b strains, to date the possession of fimbriae by nontypable isolates has been infrequently reported (1; N. G. Guerina, S. Langermann, H. W. Clegg, T. W. Kessler, D. A. Goldman, and J. R. Gilsdorf, Pediatr. Res. **16**:242A, 1982).

Because of its importance as a causative agent for both acute and chronic otitis media, we have investigated the frequency of fimbriation for minimally passaged middle ear and nasopharyngeal (NP) isolates of NTHI. In this study, isolates from cases of chronic otitis media with effusion were assessed for the possession of fimbriae after one or four passages on artificial medium, both grossly by an ability to hemagglutinate and electron microscopically by a negative staining technique. In addition, we addressed the issue of whether the extent to which these organisms were fimbriated was influenced by biotype, site of isolation, type of effusion present in the ear, or ability to hemagglutinate and how the level of fimbriation affected ability to adhere to chinchilla ciliated tracheal epithelium or human oropharyngeal cells.

(This study was presented in part at the Fourth International Symposium on Recent Advances in Otitis Media, Bal Harbour, Fla., 1 through 4 June 1987.)

MATERIALS AND METHODS

Specimens. Middle ear effusions (MEEs) and NP samples were obtained from patients undergoing routine myringotomy and tube insertion for chronic otitis media with effusion at the Children's Hospital, Columbus, Ohio.

Culturing and isolation of bacteria. All specimens were held on ice until cultured for bacteria on blood and chocolate agars within 3 h of surgery. Presumptive *H. influenzae* isolates were identified by their typical colonial morphology on chocolate agar and concomitant lack of growth on blood agar. Definitive identification was determined by their growth requirements for NAD and hemin by use of X, V, and X-V factor strips (BBL Microbiology Systems, Cockeysville, Md.) on brain heart infusion agar. Nontypable isolates were defined as those organisms that failed to agglutinate with commercial type-specific rabbit antisera prepared against types a through f (Difco Laboratories, Detroit, Mich.).

Negative staining. A solution of 2% ammonium molybdate–2% ammonium acetate in distilled water was used to negatively stain bacteria. Organisms were taken from an 18- to 24-h chocolate agar plate after one or four passages (for those isolates already maintained in laboratory culture collections) on artificial medium and suspended in a minimal amount of distilled water. Two drops of the bacterial suspension was mixed with an equal amount of stain, and a Formvar-coated copper grid was floated on the surface for 5 min. After staining, grids were blotted, allowed to air dry, and viewed immediately by transmission electron microscopy; 100 to 200 randomly chosen bacterial cells were scanned and rated for the presence or absence of fimbriae.

Hemagglutination. Fresh human erythrocytes (RBCs) were used to assess the ability of these isolates to hemagglutinate. RBCs were collected, washed twice in 0.15 M phosphate-buffered saline (PBS) (pH 7.4), and suspended to 5% (vol/vol) in PBS. The strength of hemagglutination was assessed within 2 days of blood collection by mixing 2 drops of RBCs with several colonies from an 18- to 24-h chocolate agar plate and rocking for 5 min. Each well of the slide was read independently by two observers using a nonhemagglutinating *Staphylococcus epidermidis* as a negative control and a stably fimbriate, strongly hemagglutinating type b *H. influenzae* as a positive control. Each isolate was assessed on two or three separate occasions with different RBC

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preparations. The strength of reaction was rated from 0 to 4^+.

Adherence to chinchilla tracheal epithelium. Adherence to tracheal epithelium was assessed as previously reported (2). Briefly, chinchilla half-tracheas were embedded in a perfusion chamber modeled after the original design of Gabridge and Hoglund (7). Colonies of NTHI from 18- to 24-h chocolate agar plates were gently suspended to approximately 10^8 CFU/ml, and 2 ml was added to the perfusion chambers. The bacterial suspension was rocked back and forth through the tracheal lumen in a CO_2 incubater for 1 h before homogenization of the tracheal tissue and determination of adherent CFU per average tracheal length. A minimum of five replicates was done for each isolate.

Adherence to human oropharyngeal cells. Human oropharyngeal cells were harvested by gentle scraping of the soft palate in front of the uvula and suspension in cold PBS (0.01 M, pH 7.2). Cells were vortexed to separate clumps and collected over a polycarbonate membrane (12-μm pore size; Nuclepore Corp., Pleasanton, Calif.). Cells from several volunteers were pooled and adjusted to a density of 2×10^4 to 8×10^4 cells per ml of PBS. Bacterial suspensions were prepared as described above to a density of 10^8 to 10^9 CFU/ml as confirmed by plate count, and the degree of fimbriation was assessed before each trial by negative staining. Equal amounts of oropharyngeal cells and bacteria were mixed in sterile siliconized glass vials and incubated for 90 min at 37°C in a reciprocal shaking water bath (75 rpm; Fisher Scientific Co., Pittsburgh, Pa.). Controls consisted of equal volumes of oropharyngeal cells and sterile PBS. Cells with adherent bacteria were then collected over a 12-μm polycarbonate membrane and washed free of nonadherent bacteria with 100 ml of sterile PBS. Cells were harvested into a minimal amount of PBS, cytocentrifuged (Shandon Elliott centrifuge) onto a glass slide, fixed with ethanol, and Gram stained. Determination of adherence was performed by direct microscopic count of 25 nonoverlapping oropharyngeal cells per slide, selected randomly and representing the four quadrants of the cytocentrifuged area of the slide. Only gram-negative bacilli resembling *H. influenzae* were counted. A total of 100 oropharyngeal cells were counted for each isolate per trial, and a total of three replicates were done. Only data collected from those trials in which control preparations (two per trial) rated less than 3 of 25 oropharyngeal cells with five or more adherent bacteria per cell were included. All counts were done blindly by the same operator.

Statistical analysis. Determination of the correlation between a high degree of fimbriation and biotype, effusion type, site of isolation, or strength of hemagglutination was done by the Fisher exact test. Comparison of adherence of NP versus MEE isolates to tracheal epithelium was by individual Student t-tests. Differences in ability to adhere to human oropharyngeal cells was determined by analysis of variance and Student-Newman-Keuls multiple comparison procedure. For all analyses, $P \leq 0.05$ was chosen as the level of significance.

RESULTS

Examination of negatively stained bacterial preparations by transmission electron microscopy revealed that 100% of the isolates bore fimbriae. The percentage of cells bearing fimbriae within each isolate varied from less than 10% to 100%. Fimbriae were either peritrichous or bipolar in distribution and were approximately 2.4 to 3.6 nm in width (Fig. 1). Although this study does not attempt to present a detailed morphological analysis of these structures, it was noted that there was a high degree of variability among isolates with regard to both length of fimbriae and number of fimbriae per individual bacterial cell (compare Fig. 1 and 3).

When tested for the ability to hemagglutinate RBCs, isolates varied in the strength of the hemagglutination reaction (Table 1), and no correlation was found between a high degree of fimbriation and strength of the hemagglutination. In addition, highly fimbriated isolates did not correlate with either side of isolation, biotype, or effusion type. These isolates hemagglutinated chinchilla RBCs as well, but as with human RBCs there were no apparent correlations (data not shown).

The relative adherence of 10 isolates from five randomly selected NP-MEE pairs (clinical pairs represent those isolated from the same patient) to chinchilla tracheal epithelium is illustrated in Table 2. An enhanced ability to adhere did not consistently correlate with site of isolation, nor in the example of isolates 297 and 1714 and 1885 MEE and 1885 NP, where the two were very different in degree of fimbriation, was adherence related to the percentage of cells bearing fimbriae. In addition, when comparing two isolates recovered that were both highly fimbriated and gave a 4^+ hemagglutination reaction with one that was minimally fimbriated and gave no or a weak hemagglutination, we found no significant difference in ability to adhere to chinchilla tracheal epithelium (Table 3). When observed by transmission electron microscopy, isolates were found adhering to ciliated epithelial cells only.

Adherence to human oropharyngeal cells by 11 NTHI isolates which were varied in their fimbriate status and site of isolation was also assessed (Fig. 2). Both the average number of adherent bacteria and the percentage of cells with five or more adherent bacteria per cell were greater than those of controls ($P \leq 0.005$) for all isolates tested. All isolates adhered and appeared to do so by interaction of fimbriae with the host cell (Fig. 3). However, there was no significant difference among the isolates which consistently correlated with either site of isolation or extent of fimbriation. Isolate 1128, an unstable strain that was tested in states of both low and high degrees of fimbriation, demonstrated no significant difference in relative ability to adhere in this system.

DISCUSSION

NTHI is the primary pathogen in chronic otitis media and an important pathogen in acute otitis media. It remains the most frequently culturable microorganism from chronic middle ear fluids despite the existence of various treatment regimens. The mechanisms of pathogenesis used by this organism, however, remain essentially unknown. Although it is generally acknowledged that NTHI isolates are more adherent than their type b counterparts (9, 14), the mechanism of this association has not been demonstrated.

One possible mechanism of superior ability to adhere is the possession of bacterial fimbriae, which are gaining recognition as significant surface antigens of bacterial cells. Known to mediate adherence and critical to the virulence of several pathogens, they have the additional value of being useful vaccine components. Preparations of bacterial fimbriae have been successfully used to immunize against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae* (11). Fimbriae have, however, been infrequently reported for NTHI and, when found, have been on only a small percentage of isolates (1; Guerina et al., Pediatr. Res. **16**:242A, 1982). One of the problems that exists when

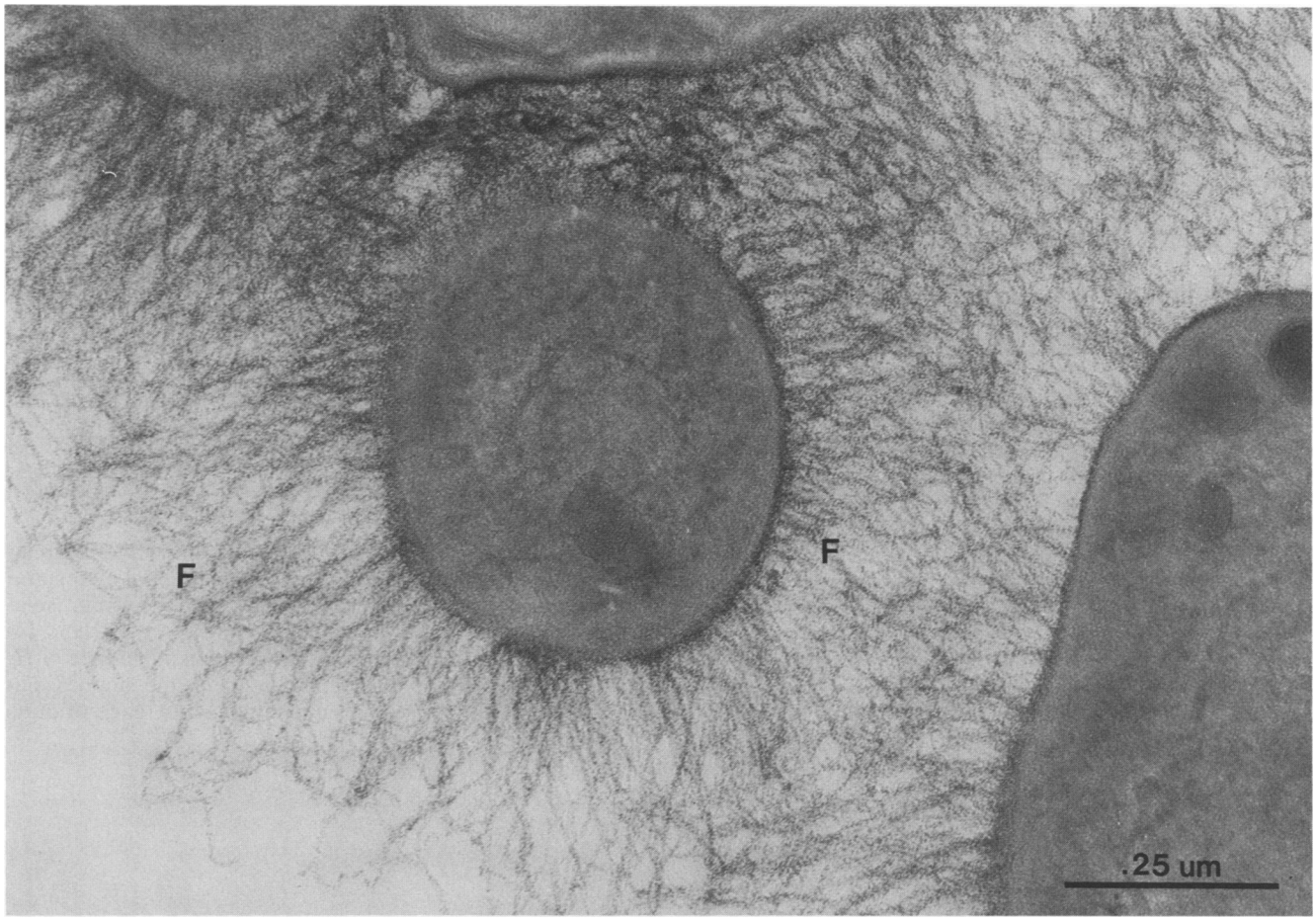

FIG. 1. Transmission electron micrograph of a thin-sectioned fimbriate isolate of NTHI. F, Fimbriae.

attempting to demonstrate fimbriae is the fact that many organisms cease production of these appendages as they are passaged on artificial medium. In addition, they are often fragile, and observation is difficult and tedious when the number of fimbriae per bacterial cell is low. We attempted to demonstrate their presence by observing isolates from the primary isolation plate or within four passages in vitro. Organisms were handled very gently, and a minimum of 100 cells was observed per isolate. As a result we were able to demonstrate the presence of fimbriae on all of our isolates of NTHI. This characteristic was not influenced by site of isolation, type of effusion, or biotype, nor did it correlate directly with strength of hemagglutination. This was not expected, and we attempted to show a connection between hemagglutination and fimbriation by two other methods: the nitrocellulose hemadsorption method of Connor and Loeb (4) and the selective enrichment of Guerina et al. (Pediatr. Res. **16**:242A, 1982) (data not shown). Neither method demonstrated to us a connection between the extent of fimbriation of individual isolates and the ability to bind to erythrocytes, thus confirming the results observed with direct hemagglutination. Nonfimbrial hemagglutinins have been reported for other organisms (5, 6, 16, 18), and it is our belief on the basis of these data that this may be true for this

TABLE 1. Comparison of strength of ability to hemagglutinate with degree of fimbriation for 59 isolates of NTHI

Strength of hemagglutination	No. of isolates with the indicated degree of fimbriation:			
	0–24%	25–49%	50–74%	75–100%
0	13	2	1	0
1^+	15	3	2	4
2^+	7	2	2	3
3^+	1	1	0	1
4^+	0	0	0	2

TABLE 2. Relative adherence of NP-MEE pairs of NTHI isolates to chinchilla tracheal epithelium

Isolate no.	Site of isolation	% of cells bearing fimbriae	Avg no. of adherent CFU/half trachea, 10^6, ± SEM (n = 5)
266	NP	25	1.9 ± 0.96
1590	MEE	<10	1.2 ± 0.29
1848NP	NP	<10	0.29 ± 0.05
1848MEE	MEE	<10	1.7[a] ± 0.59
1885NP	NP	60–75	0.87 ± 0.25
1885MEE	MEE	20	1.9 ± 1.1
214	NP	<10	1.6 ± 0.28
1371	MEE	<10	2.1 ± 1.3
297	NP	10	2.8[a] ± 0.62
1714	MEE	90	1.1 ± 0.12

[a] Significant difference within pair.

TABLE 3. Influence of percent fimbriation and ability to hemagglutinate on adherence

Isolate no.	Site of isolation (effusion type)	% of cells bearing fimbriae	Ability to hemagglutinate	Avg no. of adherent CFU/ half-trachea, $10^5 \pm$ SEM ($n = 5$)
86-042	NP	100	++++	5.8 ± 0.73
1712	MEE (Se[a])	75	++++	5.3 ± 0.51
1657	MEE (Se)	<10	0/+	6.3 ± 0.50

[a] Se, Serous.

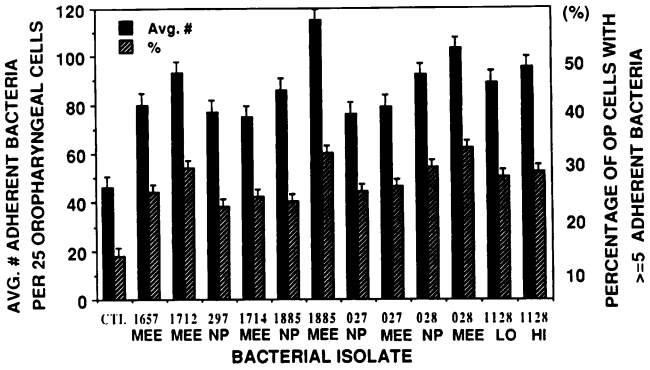

FIG. 2. Adherence of several clinical isolates of NTHI to human oropharyngeal cells. Clinical pairs are 1885 MEE and 1885 NP, 297 NP and 1714 MEE, 027 NP and 027 MEE, and 028 NP and 028 MEE. Degree of fimbriation for those not reported in Tables 2 and 3: 027 NP, 100%; 027 MEE, 75%; 028 NP, 10%; 028 MEE, 75%; 1128 LO, 10%; 1128 HI, approximately 50%.

organism as well. The RBC receptor may be another cell surface molecule or fimbrial type. Investigations are underway to further characterize this interaction.

Since adherence has also been found to correlate with the presence of fimbriae and ability to hemagglutinate (8, 10, 12, 15, 19; Guerina et al., Pediatr. Res. **16**:242A, 1982), we investigated this phenomenon for our clinical isolates of NTHI with both human and chinchilla respiratory epithelial cells. All of our isolates adhered to these cells; however, the relative ability to adhere among the isolates was not found to correlate with either site of isolation or extent of fimbriation. In addition, when comparing the ability of two isolates of very different strengths of ability to hemagglutinate and extent of fimbriation to adhere to chinchilla tracheal epithelium, we saw no significant difference. These data are in part similar to those of others who have found a lack of correlation between adherence of *H. influenzae* to monkey respiratory tissue and capsulation, anatomical site of strain isolation, or biotype (17). In addition, Porras et al. (13, 14) demonstrated a lack of correlation between adherence of *H. influenzae* to human buccal epithelial cells and site of isolation, type of infection, or frequency of occurrence of otitis media.

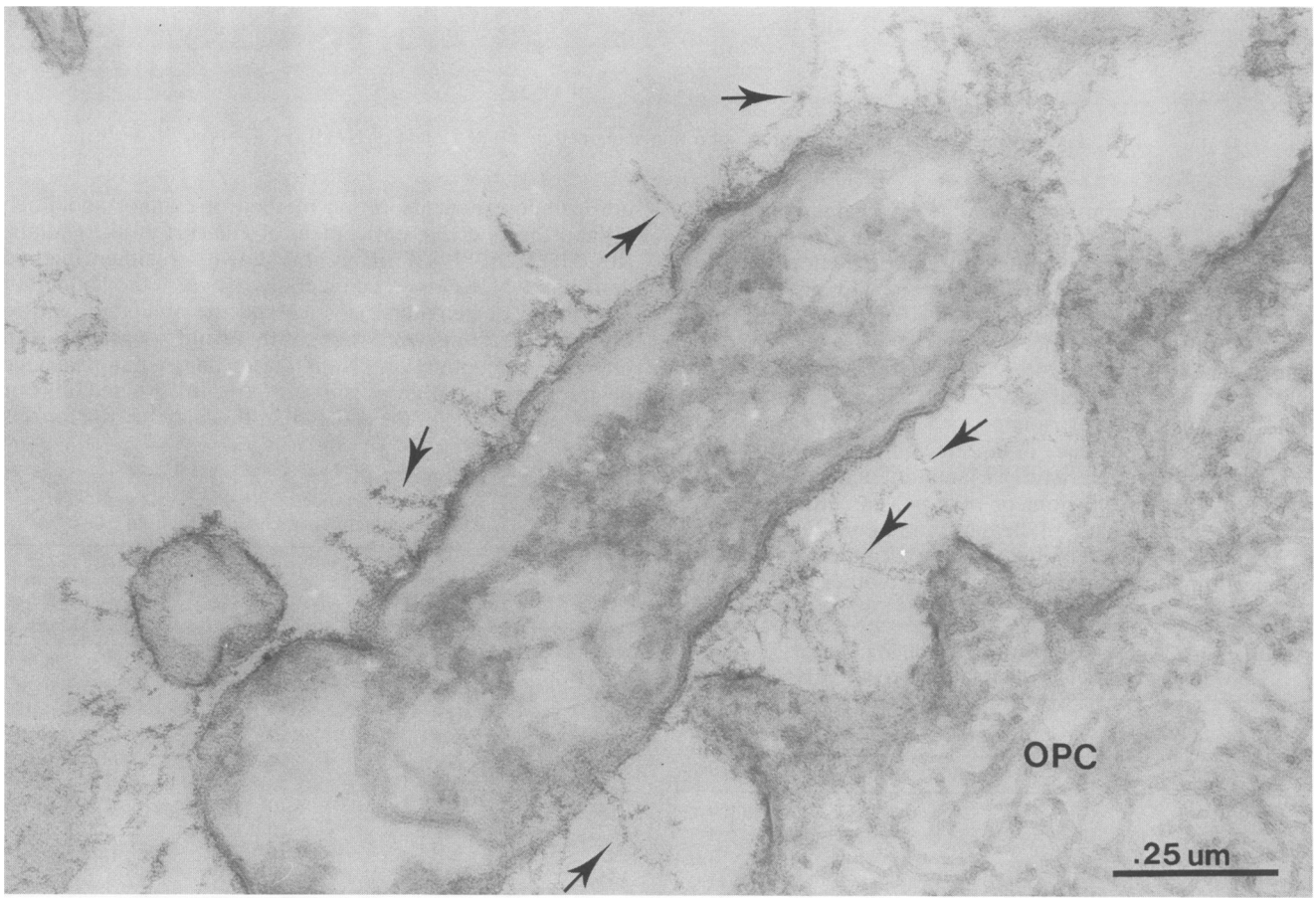

FIG. 3. Interaction of a fimbriated isolate of NTHI with a human oropharyngeal cell (OPC). Arrows indicate individual fimbriae.

Our data have indicated that unlike type b *H. influenzae*, in which the degree of fimbriation has been found to influence adherence as well as ability to hemagglutinate (Guerina et al., Pediatr. Res. **16**:242A, 1982), this does not hold true for NTHI and therefore seems to represent a unique type of fimbriae. The seemingly universal production of fimbriae by these isolates nonetheless indicates the significance of their existence. The possession of fimbriae regardless of the degree seems to provide the mechanism by which the organism interacts with epithelial cells in the oropharynx, after which other pathogenic mechanisms or differences in immune status of the host may determine whether otitis media will occur. An additional aspect of these structures which is potentially of clinical importance is the possibility of their use as a vaccine component. Their outermost surface location, proteinaceous nature, and prevalence among clinical isolates of NTHI make them logical candidates for a vaccine aimed at prevention of adherence.

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