The Presence of Fimbriae (Pili) in Three Species of *Neisseria*

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SUMMARY

Fimbriae (pili) were demonstrated in three of four *Neisseria* species (*Neisseria catarrhalis*, *N. perflava*, *N. subflava*) by electron microscopy. Only one of three *N. catarrhalis* strains examined exhibited these non-flagellar appendages. Haemagglutination occurred with all strains bearing such surface structures, but different species reacted in different ways. *Neisseria catarrhalis* and *N. perflava* agglutinated mouse and rabbit red cells at 0 and 37°; *N. subflava* reacted similarly only with rabbit erythrocytes, and with human O cells at 0°. No reactions were observed with guinea pig, goat or sheep red cells. Prior heating of the organisms destroyed all obvious haemagglutination activity; addition of D-mannose did not inhibit haemagglutination.

INTRODUCTION

Bacterial fimbriae, or pili, are described as filamentous surface appendages having various diameters and lengths. Their presence on bacterial cells can be detected directly by standard electron microscopy procedures, e.g. negative staining and shadow-casting, and indirectly by haemagglutination (HA) tests. Haemagglutination alone, however, is not a reliable indicator of the presence of fimbriae as it can be caused by other means in their absence, i.e. 'non-fimbrial haemagglutination' (Duguid, Smith, Dempster & Edmunds, 1955; Duguid, Anderson & Campbell, 1966). Moreover, certain fimbriated bacteria have been reported not to produce the HA reaction (Duguid & Gillis, 1958; Cruickshank, 1965).

Fimbriae (pili) are differentiated from flagella on the basis of their smaller diameter, the absence of the sinuous shape characteristic of flagella and lack of an association with motility. Early studies of fimbriation (Duguid & Gillies, 1957, 1958; Brinton, 1959; Thornley & Horne, 1962; Brinton, 1965) established these characteristics, and demonstrated the presence of fimbriae in a large number of genera in the Enterobacteriaceae. More recent studies have shown that genera belonging to other families also possess fimbriae. Examples are species of *Pseudomonas* (Fuerst & Hayward, 1969), *Vibrio* (Tweedy, Park & Hodgkiss, 1968) and *Herellea* (Swanson & Goldschneider, 1969). Fimbriae are apparently not limited to Gram-negative bacteria, since they have reported for variants of group A streptococci (Swanson & McCarty, 1969) and numerous strains of *Corynebacterium renale* (Yanagawa & Otsuki, 1970). The findings of the former investigation, however, should not be considered conclusive, because standard procedures for the characterization of fimbriae were not used.

As far as we know, this is the first report of fimbriae (pili) in the genus *Neisseria*.

We were able to study bacterial haemagglutination with the scanning electron microscope, which proved to be of great value in exploring the spatial relationship between fimbriae and erythrocytes.

Throughout this report we use the term 'fimbriae' rather than 'pili' since we contend that the former term not only has priority, but that its meaning has been clearly expressed by Duguid *et al.* (1955). Like Fuerst & Hayward (1969) we agree with the views presented by Duguid (1966) and Duguid & Anderson (1967) in support of the use of the term 'fimbriae'.

METHODS

Organisms. Of the nine bacterial species used, *Neisseria catarrhalis*, *N. perflava*, *Proteus mirabilis*, *Mima polymorpha* and *Vibrio metschnikovii* came from the stock culture collection of the Bacteriology Section, Division of Biological Sciences, University of Southern California, Los Angeles. The remaining four species, *N. catarrhalis* (ATCC 8176, ATCC 8193), *N. flava* (ATCC 14221), and *N. subflava* (ATCC 19243) were from the American Type Culture Collection, Rockville, Maryland. Biochemical tests were made to confirm the identity of all *Neisseria* strains. *Mima polymorpha*, *N. catarrhalis* (ATCC 8193), *P. mirabilis*, and *V. metschnikovii* were used for purposes of comparing haemagglutination reactions of fimbriate bacteria with non-fimbriate ones. Electron microscope examinations of these bacterial species showed fimbriae only on *P. mirabilis*.

Culture methods. All organisms were cultured on tryptic soy agar slants and in tryptic soy broth (Difco Laboratories, Detroit, Michigan) at 28° for 48 h.

Haemagglutination tests. Bacterial haemagglutination, and its sensitivity to mannose, was studied with red blood cells from six animal species: goat, guinea pig, human (O, Rh₀+), two strains of mice (Swiss albino, C₃H/HeJ), rabbit and sheep. Red cells were prepared as described by Cruickshank (1965). Bacteria were grown on solid media for 48 h. at 28°, washed off with sterile saline (0.9%) and centrifuged. Pellets were twice washed with sterile saline, resuspended in saline, and refrigerated until needed.

The haemagglutination procedure was similar to that of Tweedy *et al.* (1968), except that tests were done in duplicate in 10 × 75 mm. glass tubes, and incubated for 1 h., one set at 37° and the other at 4°.

Experiments were made to ascertain the effect of heat on fimbriate bacteria before testing their haemagglutinating activity. The bacteria were harvested, washed once with sterile distilled water and suspended in sterile distilled water. After 1 h. at 85°, the suspension was cooled, centrifuged, and the bacteria suspended in sterile saline. Haemagglutination only of human (O, Rh₀+), and mouse (strain C₃H/HeJ) red cells was looked for in heated and control (unheated) suspensions. Mannose sensitivity tests were done by adding D-mannose (final concn. 0.7%, w/v) to the reaction mixture.

The ability of fimbriated *Neisseria catarrhalis*, *N. perflava*, *N. subflava* and *Proteus mirabilis* to adhere to red cell ghosts was also investigated. Ghosts were prepared by adding 2 ml. whole blood to 10 ml. sterile distilled water, centrifuging, and washing the deposit in sterile physiological saline. The washed ghosts were suspended in 5 ml. saline and used as described for haemagglutination.

Electron microscopy. Bacteria from cultures on solid media were harvested and fixed with 0.25% formaldehyde. With liquid cultures, the bacteria were first centri-

fuged and then treated with 0.25% formaldehyde. Preparations were washed twice with sterile distilled water before examination. Specimens were placed on 1% collo-dion-coated copper grids (200 mesh), and excess fluid removed with filter paper. The grids were allowed to dry and then shadowed with either gold/palladium, or platinum/palladium alloys. Triplicate specimens were always prepared. A RCA EMU-3 F electron microscope (Radio Corporation of America, Camden, New Jersey) operated at an accelerating voltage of 100 kV, and a Philips 300 microscope (Philips Electronic and Pharmaceutical Industries Corporation, Mt Vernon, New York) operated at 80 kV were used.

For negative staining, the bacteria were fixed in formaldehyde suspended in distilled water and mixed with equal volumes of a 1% (w/v) phosphotungstic acid (PTA), pH 6.8. Usually 0.3% (w/v) sucrose was added to the PTA to enhance its spreading and staining effectiveness.

Scanning electron microscopy. Equal volumes of 2% (w/v) glutaraldehyde in phosphate buffer (Cokelet & Meiselman, 1968), pH 7.4, were added to tubes in which haemagglutination was observed. The red cells were collected by centrifugation (2000 rev./min. for 10 min.), washed twice in sterile distilled water and suspended in 2 ml. phosphate buffer. Samples were placed on polished flat surfaces of circular aluminium specimen stubs and mixed with one or two drops of a 1:600 dilution of Photo-Flo 200 solution (Eastman Kodak Company, Rochester, New York) to facilitate spreading. After drying at 60°, the specimens were fastened to a turntable (Cone Shadowing Accessory, Edwards High Vacuum Ltd, Manor Royal, Crawley, Sussex), coated with a thin film of carbon and then shadowed with gold, 200 Å thick. A Cambridge Steroscan electron microscope, Mark II (Cambridge Instrument Co. Ltd, London) was used with an accelerating voltage of 20 kV and a specimen angle of 51°. Photographs were made with a Polaroid sheet film, Type 55 P/N (Polaroid Corporation, Cambridge, Massachusetts).

RESULTS

Electron microscopy

The presence of fimbriae was observed in one of the three strains of *Neisseria catarrhalis*, in *N. perflava* and in *N. subflava* ATCC 19243 (Pl. 1, fig. 1, 2, 3; Pl. 2, fig. 4, 5). Measurements of length were made from prints of negatives of shadowed cells, but only from those structures which were perpendicular to the line of shadowing. Photographs of phosphotungstic acid-stained cells were used in the case of *N. catarrhalis*.

The majority of the organisms in both shadowed and negatively stained preparations were found to bear fimbriae. The number of fimbriae per cell varied considerably; some cells had as few as 25, others had 60 to 100. Determining the actual count was often difficult as many surface structures formed bundles. Furthermore, although the organisms were fixed before examination, whole fimbriae, as well as smaller fragments, apparently became detached since filaments were seen scattered over the background of shadowed specimens. Individual fimbriae exhibited slight curvatures and tapering along their lengths. Autolysed cells retained fimbriae.

The arrangements and dimensions of the fimbriae differed among the *Neisseria* species. Cells of *Neisseria catarrhalis* exhibited a 'peri-fimbriae' pattern (Pl. 1, fig. 1). Although intercellular connexions were observed with all three species of *Neisseria*,

N. catarrhalis showed a far greater number of them (Pl. 1, fig. 2). Based on measurements of length, two fimbriae types were seen which differed in their distribution. Several cells had numerous short filaments emerging from their outer borders, with an occasional long filament extending beyond them. In other organisms the longer type predominated. The diameters of both appendages ranged from 4.0 to 4.5 nm. The lengths of the shorter structures measured 0.5 to 0.73 μm ., while the longer filaments were 12.5 to 14.1 μm . long (Pl. 1, fig. 3). As these cultures were not synchronous, it is possible that both types of appendages were of a similar nature, differing only in their particular stage of development.

With *Neisseria perflava*, the distribution of surface appendages did not vary to the extent described for *N. catarrhalis*. In general, most cells showed both short and long fimbriae. The former exhibited diameters of 2 to 3 nm. and lengths of about 7 μm . The longer filaments differed from those of *N. catarrhalis* and *N. subflava* by showing a branching effect near the distal ends (Pl. 2, fig. 4); this may have been due to an unravelling of fimbriae bundles, or to the effect of drying specimen preparation.

The fimbriae of *Neisseria subflava* appeared to be of two types. The predominant form was a short type, 0.2 to 0.3 μm . long and 6 nm. in diameter, whilst the long type was 2.3 to 3.4 μm . in length and 6 nm. in diameter.

Selected cultural characteristics of Neisseria species bearing fimbriae. The results of a comparative study of the degree of fimbriation exhibited by agar and broth cultures were in keeping with the observations of other investigators (Duguid & Gillies, 1958; Duguid, 1959). We found that organisms grown on agar media showed fewer fimbriae than those from liquid media. Furthermore, bacteria from subsequent agar subcultures showed a decrease in numbers of fimbriae. Preliminary results of studies involving the cultivation of *N. catarrhalis* at 37° in both types of media showed that this temperature also decreased the number of fimbriae. A similar observation was noted by Duguid (1959) with respect to certain strains of *Klebsiella*.

Table 1. *Haemagglutinating activities of live fimbriated Neisseria species and control micro-organisms*

Micro-organism	Fimbriae	Systems*							
		Mouse		Human		Guinea-pig		Rabbit	
		0°	37°	0°	37°	0°	37°	0°	37°
<i>Mima polymorpha</i>	—	—	—	—	—	n.t.	n.t.	n.t.	n.t.
<i>Neisseria catarrhalis</i>	+	+	+	—	—	—	—	+	+
<i>N. catarrhalis</i> ATCC 8193	—	—	—	—	—	n.t.	n.t.	n.t.	n.t.
<i>N. perflava</i>	+	+	+	—	—	—	—	+	+
<i>N. subflava</i> ATCC 19423	+	—	—	+	—	—	—	+	+
<i>Proteus mirabilis</i>	+	—	—	—	—	+	+	—	—
<i>Vibrio metschnikovii</i>	—	(+)	(+)	—	—	+	—	+	—

* + = Agglutination; (+) = weak agglutination; — = no agglutination; n.t. = not tested.

Haemagglutination tests

The reactions produced by preparations containing live *Mima polymorpha*, *Neisseria catarrhalis*, *N. catarrhalis* ATCC 8193, *N. perflava*, *N. subflava* ATCC 19423, *Proteus*

mirabilis and *Vibrio metschnikovii* are shown in Table 1. Because no *Neisseria* species or control organisms agglutinated goat or sheep red cells, these results were not included in the table. No auto-agglutination was observed in the controls, which contained red cells alone and saline.

Both *Neisseria catarrhalis* and *N. perflava* agglutinated only red cells from rabbit and the two mouse strains used. *Neisseria subflava*, on the other hand, agglutinated only rabbit and human red cells. Thus these species can be differentiated according to their haemagglutinating activities. With respect to control organisms, only *Proteus mirabilis* and *Vibrio metschnikovii* produced reactions. *Proteus mirabilis* agglutinated mouse red cells at both 0 and 37°. *Vibrio metschnikovii* reacted strongly with rabbit red cells at 0° but not at 37° and only with mouse cells at 0 and 37°. The latter reaction was characterized by extremely small granular formations.

Although prior heat treatment eliminated all haemagglutinating activity, D-mannose (final concentration 0.7%, w/v) had no effect on any of the fimbriated *Neisseria* species tested.

Scanning electron microscope examination of systems showing haemagglutination revealed the pronounced adhesive property of both bacterial cells and their fimbriae to erythrocytes (Pl. 2, fig. 6). Fimbriae were also observed to adhere to one another. Examinations of specimens by transmission electron microscopy did not present so true a representation of spatial relationships between bacteria, fimbriae and red cells. The means of attachment was not as evident as in the scanning micrographs. To demonstrate the attachment of fimbriae to red cells, agglutinated erythrocytes were lysed *in situ*, fixed overnight in 2% (v/v) glutaraldehyde and negatively stained. Although these preparations showed the adherence of bacteria to red cells, they did not yield any additional information. The reactions of *Proteus mirabilis* were also examined by scanning and by transmission electron microscopy. These organisms also adhered to red cells; however, from scanning electron micrographs it appeared that erythrocytes were stuck to the surfaces of large accumulations of bacteria (Pl. 2, fig. 7). Fimbriae were not obvious in such preparations even though they could be seen in pure specimens of the organism.

Agglutinated ghosts of mouse and human red cells examined by electron microscopy clearly showed the adherence of bacteria to them. However, fimbriae were either not seen or were observed only with great difficulty.

DISCUSSION

Our electron microscope examinations have shown the existence of fimbriae in three *Neisseria* species, which resemble in several respects the surface appendages of other bacteria. These structures of *Neisseria catarrhalis*, *N. perflava* and *N. subflava* exhibited diameters and lengths which, for the most part, were within the range of measurements used by Brinton (1965) and Duguid *et al.* (1966) in their respective characterizations of pili and fimbriae. Specifically, the appendages of *N. catarrhalis* appear to be similar to the type 4 fimbriae, while the longer structures resemble the type 6 fimbriae described by Duguid *et al.* (1966). With respect to the former category, *N. catarrhalis* appendages showed approximately the same diameters, exhibited mannose resistance and were capable of reacting with untreated erythrocytes. Similarity to the type 6 structures was based on the unusual lengths of the fimbriae observed, the

relative low numbers of these appendages and the display of the 'weak' mannose sensitivity (MS) reactions in haemagglutination tests. We interpret the latter property to mean mannose insensitivity. One feature of the type 6 fimbriae does, however, present a problem, since the diameter of the structures observed by Duguid (Duguid *et al.*, 1966) was exceptionally large (about 10 nm.). If each of these fimbriae was in fact composed not of just one fibril but of a bundle of such structures, then it is conceivable that their individual diameters would be the same as those of *N. catarrhalis*. Clarification of this point would be helpful in the characterization of the fimbriae of *N. catarrhalis*. Although values for length were not given by Duguid *et al.* (1966) for type 4 fimbriae, the surface structures of *N. perflava* and *N. subflava* resemble these appendages more than any of the others described by Duguid *et al.* (1966).

Any discussion of the occurrence of fimbriation among different bacterial species, as emphasized by Fuerst & Hayward (1969), requires an interpretation of negative findings. The absence of fimbriae on cells from cultures of *Neisseria catarrhalis* (ATCC 8176, ATCC 8193) and *N. flava* (ATCC 14221) may be attributable to any one, or a combination of the factors listed by Fuerst & Hayward (1969): these include genetic incapability to form fimbriae, inhibitory effects of the cultural conditions used, and the phase of development at the time sampling was made. It would appear that additional studies using a variety of culture conditions and other strains of these bacteria are warranted to confirm our findings.

The *Neisseria* species bearing fimbriae showed haemagglutinating activities which differed in some respects from, and yet in other ways resembled, the reactions described for the fimbriated organisms in the family Enterobacteriaceae. The finding that haemagglutination occurred with washed organisms suggested that the bacteria themselves and/or their fimbriae were responsible for the phenomenon, and not a diffusible haemagglutinin. Confirmation of this point was provided with the aid of the scanning electron microscope. This instrument clearly showed the adherent and cohesive nature of *Neisseria* fimbriae. The latter property was of particular advantage in detecting the surface appendages as they tended to form aggregates or bundles. A similar situation was not observed with the highly fimbriated *Proteus mirabilis*.

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EXPLANATION OF PLATES

The bar marker in all photographs represents 1 μ m.

PLATE 1

- Fig. 1. *Neisseria catarrhalis* stained with phosphotungstic acid. Numerous fimbriae are visible. $\times 41,000$.
- Fig. 2. A gold/palladium shadowed preparation of *N. catarrhalis* from a 48 h. broth culture. Several intercellular connexions are apparent. $\times 45,700$.
- Fig. 3. A platinum/palladium (Pt/Pd) shadowed specimen of *N. catarrhalis* with long fimbriae (arrows). Note the thickened basal portion of the structure, as well as the tapering effect. $\times 10,000$.

PLATE 2

- Fig. 4. *Neisseria perflava*, a Pt/Pd shadowed cell exhibiting branching fimbriae. Both short and long filaments are shown. $\times 32,000$.
- Fig. 5. *N. subflava* ATCC 19243, shadowed with Pt/Pd. The predominance of short fimbriae are evident. One long appendage (arrow) can be seen emerging from the shadow region. $\times 32,000$.
- Fig. 6. A scanning micrograph of the cells of *N. catarrhalis*, and their fimbriae causing the agglutination of mouse red cells. The adhesive nature of the fimbriae (arrows) can be seen. $\times 10,000$.
- Fig. 7. A scanning micrograph of *Proteus mirabilis* agglutinating red blood cells. The adherence of red cells to aggregates of bacteria can be seen. $\times 5000$.