Electron Microscope Observations on the Structure of Fimbriae, with Particular Reference to Klebsiella Strains, by the use of the Negative Staining Technique

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SUMMARY

The method of application of negative staining with potassium phoshotungstate for the electron microscopy of bacterial surface structure is described. The method has been used to study the fimbriae of Klebsiella strains, and has confirmed, qualitatively, the results of other workers who used the shadow-casting technique. The diameters of the two types of fimbriae associated with the MS and MR adhesins were found to be 65-70 Å and about 48 Å, respectively. These figures are about 30% less than earlier estimates. The resistance of both types of fimbriae to prolonged autolysis was shown. Other applications of the negative staining method include the study of bacterial flagellation, and of the structure of cells disintegrated by autolysis or other methods.

INTRODUCTION

Electron microscope studies of bacterial fimbriae have been reported by Houwink (1949), Duguid, Smith, Dempster & Edmunds (1955), Duguid (1959) and by Brinton (1959), who referred to them as 'pili'. All these workers used the shadow-casting technique. The fimbriae have been described as filamentous appendages associated with the cell surface, and differ in their appearance from bacterial flagella when examined in the electron microscope after similar methods of preparation (Houwink & van Iterson, 1950). Duguid's (1959) measurements from shadowed preparations of Klebsiella strains showed them to have a diameter of about 70 Å and to be of variable length. Unlike flagella, they appeared to be much more rigid structurally, with no evidence of their forming patterns with wavelengths or amplitude. He reported the possibility of the presence of two types of fimbriae in the shadowed preparations: one type with a diameter of 100 Å and more rigid than the other type of 70 Å diameter. He suggested that such a difference in the apparent sizes might result from the preparation methods used for electron microscopy.

The shadow-casting method is usually used to increase contrast in the electron microscope specimen so as to permit suitable detail to be observed in a relatively electron transparent object, thus enabling the surface detail of bacteria to be made visible. By this method the dimensions of fine structure are difficult to measure accurately, and resolution is limited by the granulation effects produced by the

evaporation technique. For these reasons it is difficult to interpret electron micro-
graphs revealing structure of a size in the region of 30–40 Å.

The negative staining technique with potassium phosphotungstate has been
applied successfully to the study of several viruses to elucidate their structure at the
molecular level (Brenner & Horne, 1959; Brenner et al. 1959; Horne, Watson
Wildy & Farnham, 1960; Wildy, Russell & Horne, 1960; and others). This same
 technique has been applied to the study of bacteria, and particular attention has
been paid to the structure of the fimbriae of Klebsiella, as this gave good opportunities
for testing the method.

METHODS

The electron microscope specimen support grids were covered with a thin film of
nitrocellulose and stabilized by an additional layer of evaporated carbon (Bradley,
1954). For mounting the bacteria, a solution of 1 % (w/v) phosphotungstic acid was
adjusted to pH 7.2 with KOH. The bacteria, prepared as described below, were
suspended in the phosphotungstate solution to give about $10^8$–$10^9$ bacteria/ml. This
concentration was estimated roughly by making dilutions with a Pasteur pipette,
until the turbidity was barely visible in the capillary portion. A drop of the sus-
pension was then placed on the prepared supports, and the surplus fluid removed
with filter paper, leaving a thin film of liquid covering each grid. The films were
allowed to dry and were then ready for examination in the electron microscope.

Preparation of the bacteria. When grown on agar the bacteria were usually
sufficiently clean to be mixed with the phosphotungstate and mounted directly;
from a broth culture they often required washing once in distilled water. Capsulated
organisms presented some difficulty, as the capsular material often distorted and
broke the supporting film on drying. Also, the large amount of phosphotungstate
absorbed by the capsule tended to obscure structures such as fimbriae which passed
through it. With some Klebsiella strains the capsular material was easily removed by
autolysis for 14 days at room temperature. This treatment had been used on the cell
shown in Fig. 4.

Source of cultures. All strains of Klebsiella were obtained from Dr J. P. Duguid
(University of Edinburgh). Two pseudomonads, one pigmented (MT/F4/2.4) and
one non-pigmented (MT/F5/146) were isolated by one of the authors, the Aerobacter
sp. BE/21, was from Dr B. P. Eddy (Low Temperature Research Station), and
the Proteus vulgaris culture was from the Low Temperature Research Station collec-
tion. Staphylococcus aureus, NCTC 8532, was obtained from the National Collection
of Type Cultures.

RESULTS AND DISCUSSION

Effects of phosphotungstate on bacteria

Immersion in 1 % (w/v) phosphotungstate solution had no obvious effect on the
bacteria. Motile strains remained actively motile and the viability of Escherichia
coli and a Lactobacillus strain was unaltered after 10 min. in the phosphotungstate.

Comparison of electron microscopy with light microscopy

Bacteria dried in phosphotungstate solution on a glass slide can be examined by
phase-contrast microscopy, since the dried phosphotungstate film has a very high
refractive index and hence appears dark in contrast to the light bacteria. Plate 1,
The electron microscopy of fimbriae of Klebsiella

fig. 1, shows two variations of this effect: A, in which only a narrow band of phosphotungstate surrounds the cells, as is usual in the electron microscope preparations; B, where a more extensive film of phosphotungstate covers the whole background. In fig. 1A, two cells appear dark and wider than the others: these are probably filled with phosphotungstate and flattened; in fig. 1B, a cell filled with phosphotungstate shows a light outline.

The width of these cells was compared with that of cells photographed with phase contrast in a wet preparation (Pl. 1, fig. 1C), and the shrinkage in width in the phosphotungstate preparation appeared to be 9%. The Gram-stained preparation (Pl. 1, fig. 1D) showed 25% shrinkage in width compared with C. The distortion resulting from the phosphotungstate method is therefore relatively small.

Appearance in the electron microscope

In negatively stained preparations the phosphotungstate appears dark because of its high electron density in comparison with biological material, and shows a similar picture to that obtained with phase contrast. The cell surface structure in both intact and disrupted bacteria is revealed where the phosphotungstate has penetrated into folds or spaces. The effects of this penetration by the electron dense material are shown in Pl. 1, fig. 2, and at higher magnification in Pl. 3, fig. 9. There is little evidence of collapse of bacteria when they are embedded in sufficient amounts of phosphotungstate to preserve the three dimensional structure. Staphylococcus aureus is shown in Pl. 1, fig. 3, where the dark grooves in the cell surface have presumably been filled with electron-dense material in regions where two cells are in the process of dividing. The depth of focus in the electron microscope is sufficient to show the grooves in both the upper and lower surfaces, and the elliptical shape is evidence of good preservation.

Surface structures such as flagella and fimbriae are outlined by a dark margin. In Pl. 1, fig. 2, an Aerobacter strain is seen with peritrichous flagella, which have remained attached to the cells, extending from the cell surface. When observed in the absence of phosphotungstate in shadowed preparations, these are frequently detached or fragmented.

Fimbriae

Strains of Klebsiella were chosen because their fimbriae have previously been studied in detail by Duguid (1959) who used shadow-casting techniques for electron microscopy. He reported that some strains possessed an adhesin which was mannose-sensitive (MS), other adhesins were mannose-resistant (MR), while still other strains possessed both types of adhesin. Those observed to have the MS adhesin had fimbriae about 100 Å diameter, compared with the 70 Å diameter fimbriae associated with the MR adhesin. Strains with both types of adhesin comprised a mixture of organisms, some with the thicker fimbriae, some with the thinner ones; each organism had fimbriae of one thickness only.

Before studying the structure of fimbriae by negative staining methods, preparations were examined after shadow casting. A selection of the strains Duguid (1959) used was studied by using his methods for growing, fixing the cells in 0.25% (w/v) formaldehyde and washing, before mounting. The fixing procedure is necessary when the organisms are air dried from liquid suspensions ready for shadowing;
without this most of the fimbriae became detached from the cell surface and scattered over the background. Our results obtained from metal-shadowed preparation were in good agreement with those reported by Duguid (1959).

Mounting in phosphotungstate revealed the presence of fimbriae which were basically similar to those in the shadowed preparations, but which appeared to have smaller diameters. Two types were clearly visible, corresponding to the two types of adhesin, thus confirming the previous observations. Plate 2, fig. 4, shows an electron micrograph from a negatively stained preparation of Klebsiella, strain K7, which possesses the MR adhesin. With this strain and with strain K9, capsular material was removed after the growth period of 2 days at 37° by leaving the culture to autolyse for 14 days at 20° before washing and mounting. Measurements showed the fimbriae to have a mean diameter of 48 Å in strain K7, while the fimbriae shown in Pl. 2, fig. 6, from a preparation of Klebsiella NCTC9644 (with MS adhesin), were of 71 Å diameter. Klebsiella, strain A3 MR, possesses both types of adhesin, and thus cells with the thicker fimbriae (Pl. 3, fig. 7) and the thinner type (Pl. 3, fig. 9) were both found. Several of Duguid's strains were examined in the electron microscope and measurements of the fimbriae are given in Table 1.

### Table 1. Measurements of fimbriae of Klebsiella strains

<table>
<thead>
<tr>
<th>Type of adhesin (Duguid 1959)...</th>
<th>NCTC 9644</th>
<th>NCTC 9662</th>
<th>A3MR</th>
<th>K7</th>
<th>K9</th>
</tr>
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<tbody>
<tr>
<td>Diameter of fimbriae (Å)</td>
<td>MS Thck</td>
<td>MR Thck</td>
<td>MS and MR Thck</td>
<td>MR Thck</td>
<td>MR Thck</td>
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<td>67*</td>
<td>43</td>
<td>66</td>
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<tr>
<td>Mean:</td>
<td>71</td>
<td>47</td>
<td>65</td>
<td>47</td>
<td>48</td>
</tr>
</tbody>
</table>

* Each figure is the mean of measurements of 15 fimbriae attached to one cell.

The resistance of fimbriae to autolysis is illustrated in the electron micrograph shown in Pl. 3, fig. 8. The region shows an organism of Klebsiella strain NCTC 9668, which has both types of adhesin, and which was allowed to autolyse in distilled water at 37° for 41 days. The cell wall is recognizable with a large number of intact fimbriae attached to the surface, although the cell contents had almost entirely disappeared. These fimbriae were found to have a mean diameter of 77 Å, while those of another cell in the same autolyse preparation were 50 Å wide. These measurements correspond to those of the thick and thin types of fimbriae in fresh preparations of this organism, and show that both types remained unchanged in size after extensive autolysis.

On a number of electron micrographs showing fimbriae with a mean diameter of 48 Å the appearance suggested a regular periodicity of 45–50 Å along the filament axis. These dimensions were judged from 50 measurements, but as this structure was
The electron microscopy of fimbriae of Klebsiella 55

similar in size to the background structure, accurate estimation was difficult. The periodie structure can be seen in Pl. 2, fig. 5, showing at higher magnification an area of Pl. 2, fig. 4, containing separated fimbriae. It is suggested that the small roughly spherical structures may be subunits arranged in the form of threads, and that they are less tightly packed than the more rigid fimbriae of larger diameter. The number of fimbriae per cell appeared to be about 200–400 for the thicker fimbriae and 400–700 for the thinner type.

Three Klebsiella strains, NCTC 5054, 7761 and 5046 of serotypes, 1, 2 and 3 respectively, found by Duguid to lack fimbriae in shadowed preparations, were examined by negative staining; his results were confirmed.

Other observations by the use of negative staining

This method has been found useful for detecting the type of flagellation in doubtful cases, as the actual point of attachment of flagellum to cell can often be clearly seen (Pl. 4, fig. 10A). Kluyvera spp. which had been previously considered to have polar flagella (Asai, Okumura & Tsunoda, 1957), were found by Eddy (1960) to possess a small number of lateral flagella when examined in phosphotungstate.

Structures which resembled basal granules attached to flagella were visible in some of the electron micrographs. A typical region is shown in Pl. 4, fig. 10B, which shows a pigmented pseudomonad autolysed on the surface of agar for 21 days at 20°C.

The process of autolysis, or, of course, any other form of cell damage, can be followed through different stages. Plate 4, fig. 11A, shows a Proteus vulgaris cell which had collapsed and lost most of its contents, but which retained flagella after 20 days at 20°C. The area in Pl. 4, fig. 11B, shows the same organism after 3 months at 37°C, when the cell walls had disintegrated into rounded fragments. A large number of fimbriae remained, but no flagella were visible in the electron micrographs. A detailed study, by a combination of physical and chemical methods, of the structure of bacterial flagella is in progress and will be described elsewhere.

It is suggested that the method of examining certain surface structures in bacteria by negative staining is less laborious than metal shadowing. The preliminary preparation and treatment of organisms by washing and fixing is simpler. Moreover, from the results obtained and described above, there is some evidence that phosphotungstate has itself some action in preserving structure. The observations reported in this communication about fimbriae are qualitatively the same as those seen in shadowed preparations, but the estimates of size are lower by about 30%.

We are indebted to Dr J. P. Duguid for the cultures of Klebsiella strains and for information about them.

REFERENCES


EXPLANATION OF PLATES

**PLATE 1**

Fig. 1. A pigmented pseudomonad (M1/F4/2.4), grown on agar for 24 hr. at 25°. Light micrographs x 3400. A and B. Bacteria suspended in 1% (w/v) phosphotungstate dried on slide. Phase contrast. C. Aqueous suspension, phase contrast. D. Gram stain.

Figs. 2–11. Bacteria negatively stained by phosphotungstate (Except where mentioned, no previous fixation or washing was used.) Electron micrographs showing cells and appendages surrounded by the electron dense material.

Fig. 2. Aerobacter, strain BE/21, grown on agar for 24 hr. at 37°, showing peritrichous flagella which have remained intact and attached to the organisms.

Fig. 3. *Staphylococcus aureus*, NCTC 8332, grown on agar for 2 days at 30° and washed twice, with the grooves between cells in process of division filled with phosphotungstate.

**PLATE 2**

Figs. 4–9. Klebsiella strains, grown in nutrient broth for 2 days at 37° and washed twice before mounting. Large numbers of fimbriae remained intact and attached to the cells, whether formaldehyde fixation was used (fig. 7) or not (figs. 4–6, 8, 9).

Fig. 4. Klebsiella K7, autolysed in broth for 14 days at 20° to remove capsular material, showing fimbriae of the thinner type.

Fig. 5. A portion of Fig. 4, enlarged, showing the appearance of periodic structure.

Fig. 6. Klebsiella, NCTC 9644, with the thicker type of fimbriae, which appear to have a more rigid structure.

**PLATE 3**

Fig. 7. Klebsiella, A3MR, a cell with the thicker fimbriae. Formaldehyde fixation used.

Fig. 8. Klebsiella NCTC 9606, autolysed in water for 41 days at 37°. A cell with fimbriae of the thicker type.

Fig. 9. Klebsiella, A3MR, a cell with the thinner fimbriae, showing penetration of the phosphotungstate into crevices which appear to lie below the cell surface.

**PLATE 4**

Fig. 10. A. A non-pigmented pseudomonad (MT/F5/146), grown on agar for 24 hr. at 25°, showing a single polar flagellum which appears to emerge from an indentation in the cell surface. B. A pigmented pseudomonad (MT/F4/2.4), grown and autolysed on agar for 21 days at 20°, with 2 polar flagella which appear to be associated with a single structure resembling a basal granule.

Fig. 11. A. *Proteus vulgaris*, grown on agar for 2 days at 37°, autolysed for 20 days at 20°. Although the cell appears to have collapsed and lost most of its contents, numerous peritrichous flagella remain attached. B. *Proteus vulgaris*, grown on agar for 24 hr. at 37°, suspended in water and autolysed for 14 weeks at 37°. No flagella visible, but large numbers of fimbriae remain associated with the cell wall, which has partially disintegrated into rounded fragments.