INFECTION AND IMMUNITY, Oct. 1974, p. 872-876

Vol. 10, No. 4
Printed in U.S.A.

Ultrastructure of Pili and Annular Structures on the Cell Wall Surface of *Neisseria meningitidis*

I. W. DEVOE AND J. E. GILCHRIST

Department of Microbiology, Macdonald Campus of McGill University, Quebec H9X 3M1, Canada

Received for publication 31 July 1974

In a survey of negatively stained preparations of the prototype strains of *Neisseria meningitidis*, pili were detected on three strains. However, these pili were detected on fewer than 5% of cells in populations of these three strains. Those individual cells with pili were seldom observed to contain more than two or three pili per cell. In contrast, nearly all cells of the nonprototype group B strain ATCC 13090 had numerous pili on their surfaces. When viewed in frozen-etched replicas, a few pili were observed lying on the cell surface of this latter strain. An annular structure was also found in frozen-etched replicas. This structure usually consisted of a series of concentric rings that were always found on the flat side of these bean-shaped cells. It is concluded that such structures represent a differentiated portion of the cell wall, which is involved in cross-wall formation during synthesis of the cell septum in dividing cells.

The surface morphology and surface-associated structures of various neisseriae have been the subject of several recent investigations (5, 9, 12, 14). The neisseriae in thin section exhibit a typical gram-negative cell envelope morphology (1, 3, 11), i.e., two trilaminar membrane-like layers separated by a periplasmic zone containing an electron-dense peptidoglycan layer. Surface pili have been reported on the gonococcus (5, 12) and three species of nonpathogenic neisseriae (14). Jephcott et al. (5) mentioned in a presentation of data on the pili of the gonococcus that similar fibrilar structures have been observed on meningococci, although evidence for such structures was not presented. It has been suggested that the presence of pili on the gonococci may be associated with virulence in this organism (12). This proposal is based on the finding that cells from gonococcal colony types one and two were found to be piliated and virulent, whereas those from colony types three and four were neither piliated nor virulent (5, 6, 12). DeVoe and Gilchrist (2) recently reported an additional structural feature on the meningococcal cell wall. A detailed analysis on three prototype strains of meningococcus revealed that relatively large quantities of endotoxin were released into the medium in the form of cell wall blebs that originated at the outer trilaminar layer of the cell. Such blebs were formed and released only during the active stage of growth, whereas these structures were absent on stationary phase cells. The relationship of such a mechanism for endotoxin release to meningococcal pathogenicity is unknown; however, proposals from several sources (4, 7, 8) suggest a prominent role for endotoxin in the pathology of meningococcal disease.

In this report, we present an ultrastructural study on both pili of the meningococcus and a newly discovered structural feature associated with the flat side of the bean-shaped meningococcus.

MATERIALS AND METHODS

Organisms. *Neisseria meningitidis* strains 791 (serogroup A), SD1C (serogroup B), M-60 (serogroup D), X Slaterus (serogroup X), Y Slaterus (serogroup Y), Z Slaterus (serogroup Z), 29E (serogroup 29E), and W-135 (serogroup W-135) were obtained from the Neisseria Repository, Naval Medical Research Unit No. 1, School of Public Health, University of California, Berkeley. Group B strain ATCC 13090 (serogroup B) was obtained from the American Type Culture Collection.

Maintenance of stock cultures. Stock cultures were maintained on Mueller-Hinton agar slants at −80 C. The procedures described by Vedros (Neisseria Repository Bulletin, U.S. Naval Research Unit No. 1, School of Public Health, University of California, Berkeley) were followed for routine examination for strain purity.

Cell growth. Cells from thawed slants were used as the inoculum for Mueller-Hinton agar (Difco) plates which were incubated (24 h) in a candle jar (37 C) at 100% humidity. Cells from the 24-h cultures were used as the inoculum for 10 ml of Mueller-Hinton broth, which was incubated (37 C) with agitation (100 rpm) until cultures were in the mid-log phase of growth. A 0.05-ml portion of this culture was used to inoculate 10 ml of fresh Mueller-Hinton broth. The final culture was incubated as above to the mid-log stage of growth (in most strains approximately 3 h).

Negative staining. Specimens for negative staining were deposited onto Formvar-coated carbon-stabilized copper grids and stained with 0.01% phosphotungstate (pH 7.5).

Freeze etching. This work was carried out by a procedure described previously (13).

Electron microscopy. All electron micrographs were obtained by use of an AEI-EM6B.

RESULTS

During studies on several strains of meningococci, we routinely observed cell-free pili in negatively stained preparations of supernatant fluids (Fig. 1) of three prototype strains (group B strain SD1C, group X, and group Y). Close examination of the individual cells from these cultures revealed that approximately 5% of the cell population possessed pili attached to the cell surface; however, the individual piliated cells only rarely exhibited more than five pili per cell, usually only one or two. The morphology of the pili themselves was identical in all stains; however, in the preparation of the group Y strain, small bundles of intertwined pili were often observed (Fig. 2). These bundles of pili were similar in many respects to those observed in the gonococcus (9).

In contrast to the paucity of pili found in the prototype strains, numerous pili were evident on the surface of over 90% of cells of the group B strain ATCC 13090 (Fig. 3). As in the prototype strains, cell-free pili were also found in the culture supernatant fluids of actively growing cells.

In the case of the gonococcus (6, 10), piliated cells are associated with a distinct colonial morphology. Pili were not observed on all meningococcal cells in either the cultures of the piliated ATCC 13090 strain or those of the piliated prototype strains. An attempt was made to separate the piliated cells from nonpiliated forms, on the basis of colonial morphology, in a manner similar to that employed with gonococcus. Colonial morphologies in all instances were consistently identical. Moreover, negatively stained preparations of cells from selected colonies of the various strains revealed that the proportion of piliated cells in the population was similar to that described above for broth cultures. Therefore, the low frequency of pili in the cell populations of the prototype strains appeared to be characteristic for these cells under the conditions for growth used here.

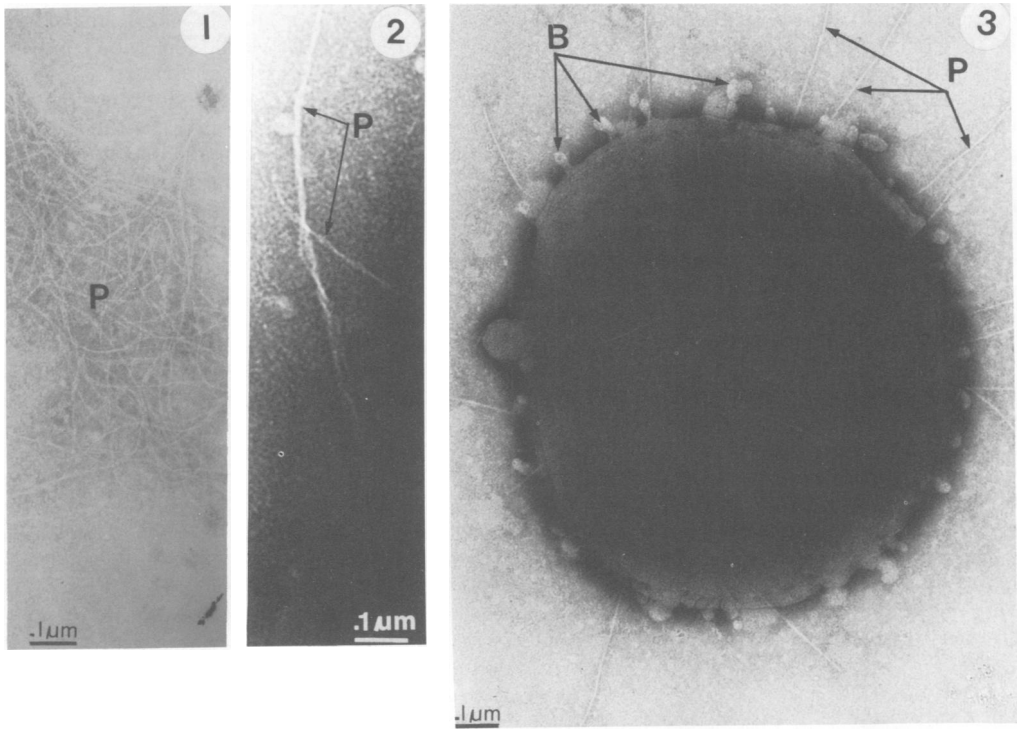

FIG. 1. *Cell-free pili (P) in negative stain from a broth culture of N. meningitidis group X prototype strain ×66,000.*

FIG. 2. *Negative stain preparation of intertwined pili (P) from N. meningitidis group Y. ×75,000.*

FIG. 3. *Negative stain preparation of N. meningitidis group B ATCC 13090. P, Pili; B, bleb-like evagination of outer cell-wall layer. ×60,000.*

Multiple pili on over 90% of cells in the ATCC 13090 strain appeared to be the exception among the number of meningococcal laboratory strains observed to date.

Swanson (9) concluded from freeze-fracture studies on the gonococcus that the extension of most pili out and away from the cell surface in negatively stained preparations was probably an artifact produced by the staining process itself. Abundant pili were observed wrapped around the cell surface in his frozen-etched replicas. In contrast, our frozen-etched replicas of meningococcal strain ATCC 13090 (Fig. 4 and 5) revealed very few pili on the cell surface, suggesting that they were removed during the cleavage stage of specimen preparation. It is, therefore, reasonable to conclude that in the case of this single meningococcal strain the extended pili observed in negatively stained preparations were representative of the state of pili in the culture.

An additional feature noted in our frozen-etched replicas was the distinct morphology associated with the flat side of these bean-shaped cells (Fig. 5 to 8). This side of the cell exhibited a distinct annulus-like structure. As the orientation of these structures appeared to be random with respect to the plane of cleavage, it seems improbable that they were artifacts produced by either the cleavage process or the subsequent etching of the specimen. These annular structures were oriented such that much of the structures was revealed above the frozen milieu (Fig. 5 to 8). Dividing cells are shown in Fig. 6 and 8. In these cells, the remnants of the annular structure was faintly visible on the cell surface. In all dividing cells on which these structures were observed, the structures were in a plane perpendicular to the line of septum formation. A small concave fragment is shown in Fig. 7 that appears to be the remnant of the cell wall from a sister cell removed during cleavage. The fact that this attached fragment is seen near the center of the annular structure is further evidence that these concentric rings are associated with that part of the wall involved in cell division. As these distinct structural features are associated with the flat side of the cell walls, which are in juxtaposition during cell division, it is reasonable to consider such structures may constitute a division scar.

DISCUSSION

The importance of meningococcal surface structural components to meningococcal disease is unknown. However, a role for the endotoxin from the cell surface of this organism in the pathology of meningococcal disease was postulated by early workers in this field (7), and more recently by others (4, 8). DeVoe and Gilchrist (2) recently reported a mechanism whereby rapidly growing meningococci released substantial amounts of endotoxin-containing cell wall without cell lysis. During the active stage of growth, the outer cell wall of these organisms evaginated to form multiple blebs, which were subsequently released into the medium. These surface blebs were also found in abundance on cells of prototype meningococcal strains as well as on the surface of cells from primary throat cultures of known meningococcal carries (DeVoe and Gilchrist, unpublished data).

Surface pili have been reported on the gonococcus (5, 12), meningococcus (5), and three nonpathogenic neisseriae (14). There appears to be a direct correlation in the gonococcus between virulence and the presence of such pili (5, 6, 12). Although pili are numerous on the surface of the piliated gonococci (5, 12), in our experience with laboratory strains of meningococci generally only a small proportion of cells in cultures exhibited pili. In a survey, we found only three prototype strains that possessed detectable pili; however, the proportion of the population of these three strains with surface-attached pili was relatively small, in the range of 5%. In contrast, over 90% of the cells in the populations of the group B strain ATCC 13090 were piliated when grown under identical laboratory conditions to those used for the prototype strains. In a survey of over 30 laboratory strains, we have found only the group B strain ATCC 13090 to be heavily piliated (DeVoe and Gilchrist, unpublished data). Close examination of negatively stained preparations from most strains revealed that generally a very low number of cells in the population possessed these structures, and then one can detect only one or two pili per cell. The presence of abundant pili on the group B ATCC 13090 strain, therefore, appears to be the exception among laboratory strains of meningococci.

An explanation for the general paucity of pili in meningococcal cultures was recently uncovered when fresh throat cultures from known carriers of meningococci were analyzed (DeVoe and Gilchrist, manuscript in preparation). All cells from primary cultures of these carriers were piliated; however, serial subculture of the same strains resulted in the loss of pili from these cells. Therefore, it seems likely that, at least in the carrier state, meningococci do possess pili, but during the conversion of these cells to laboratory strains such structures are lost.

The annular structures reported here on the surface of meningococci in frozen-etched repli-

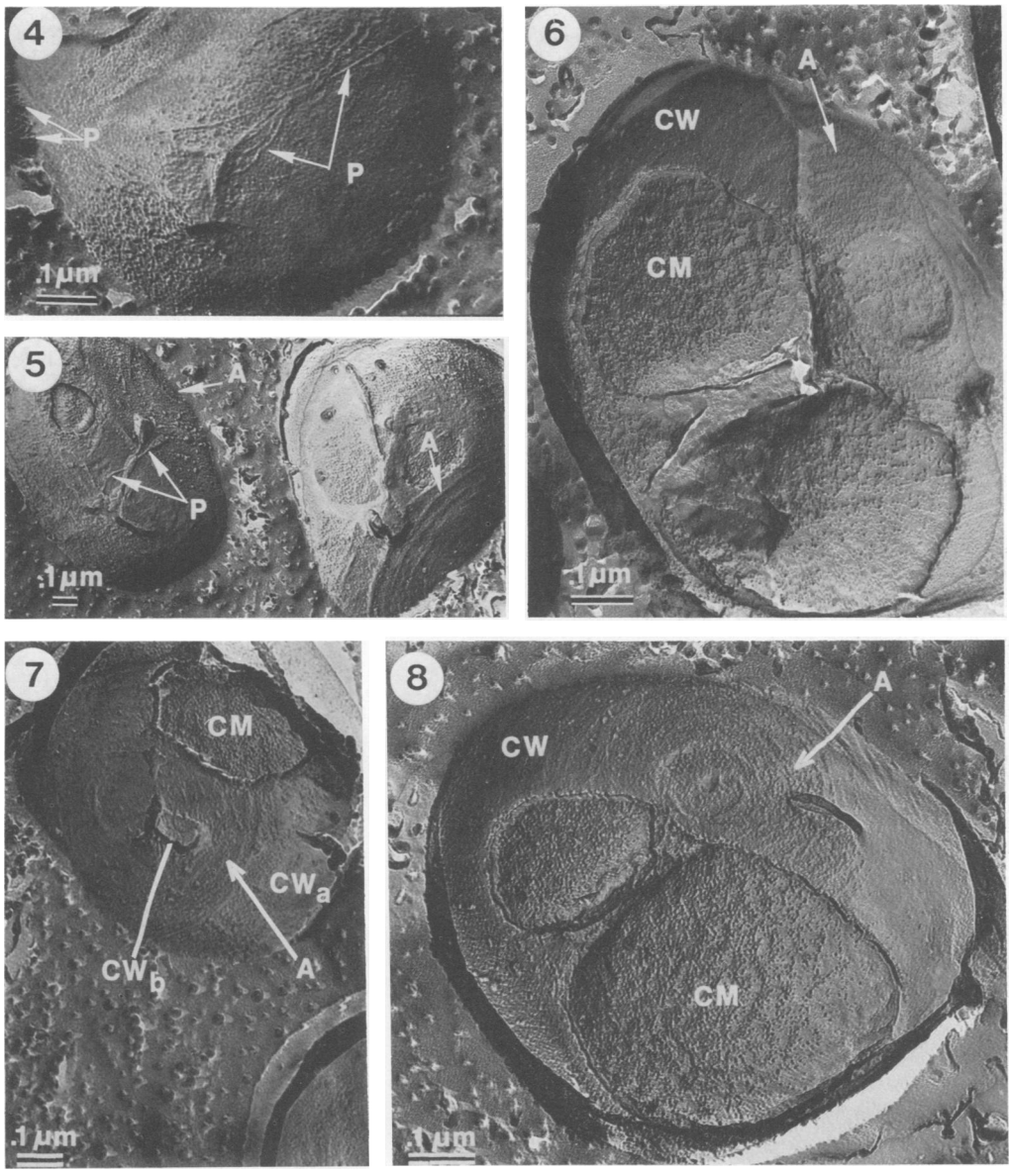

FIG. 4-8. *Frozen-etched replica of cell surface of N. meningitidis group B (ATCC 13090). P, Pili; A, annular structure on flat side of cells; CM, cytoplasmic membrane; CW, cell wall; CWa, cell wall surface of cell shown in plane of cleavage; CWb, cell wall fragment of sister cell which was itself removed during cleavage.*

cas were always found in association with the flat side of the bean-shaped diplococcal cells. This differentiated area of the cell wall is no doubt produced during the synthesis of cross-wall formation during cell division. When observed on the surface of a dividing cell, these annular structures were found to be in a plane perpendicular to the line of new septum formation. We are presently investigating this area of the wall in greater detail.

ACKNOWLEDGMENT

This work was supported by the National Research Council of Canada.

LITERATURE CITED

1. Cesarini, J. P., M. Vandekerkov, R. Faucon, and J. Nicoli. 1967. Ultrastructure de la paroi de *Neisseria meningitidis*. Ann. Inst. Pasteur (Paris) **113**:833-841.
2. DeVoe, I. W., and J. E. Gilchrist. 1973. The release of endotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. J. Exp. Med. **138**:1156-1167.

3. DeVoe, I. W., J. E. Gilchrist, and D. W. Storm. 1973. Ultrastructural studies on the fate of group B meningococci in human peripheral blood leukocytes. Can. J. Microbiol. **19**:1355-1359.
4. Ducker, T. B., and R. L. Simmons. 1968. The pathogenesis of meningitis: systemic effects of meningococcal endotoxin with the cerebrospinal fluid. Arch. Neurol. **18**:123-128.
5. Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. Brief report: *Neisseria gonorrheae*. III. Demonstration of presumed appendages to cells from different colony types. Acta. Pathol. Microbiol. Scand. Sect. B **79**:437-439.
6. Kellogg, D. S., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. L. Pirkle. 1963. *Neisseria gonorrheae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274-1279.
7. Murray, E. D. G. 1929. The Meningococcus. Special Report Series no. 124. Medical Research Council, London, England.
8. Scherp, H. W. 1955. Neisseria and neisserial infections. Ann. Rev. Microbiol. **9**:319-334.
9. Swanson, J. 1972. Studies on gonococcus infection. II. Freeze-fracture studies on gonococci. J. Exp. Med. **136**:1258-1271.
10. Swanson, J. 1973. Studies on gonococcus infection. III. Pili: their role in attachment of gonococci to tissue culture cells. J. Exp. Med. **137**:571-589.
11. Swanson, J., and I. Goldschneider. 1969. The serum bactericidal system: ultrastructural changes in *Neisseria meningitidis* exposed to normal rat serum. J. Exp. Med. **129**:51-80.
12. Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. **134**:886-906.
13. Thompson, J., and I. W. DeVoe. 1972. Physiological and morphological effects of phenethyl alcohol upon a gram-negative marine bacterium. Can. J. Microbiol. **18**:841-852.
14. Wistreich, G. A., and R. F. Baker. 1971. The presence of fimbriae (pili) in three species of Neisseria. J. Gen. Microbiol. **65**:167-173.