CURRENT MICROBIOLOGY, Vol. 8 (1983), pp. 57–61

Current Microbiology

An International Journal

Effects of Culture pH on the Expression of Meningococcal Pili

D. Brener* and I. W. DeVoe**

Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4

Abstract. The effects of pH on piliation of the meningococcus was investigated. Group B *Neisseria meningitidis* (M2092), ATCC 13090, was piliated when grown in Mueller-Hinton Broth at pH 7.2, but pili were not synthesized after cells were transferred to the same medium at pH 6.6 even though cells grew well at the lower pH. Meningococci possessed a sizable reservoir of pilus subunits, as evidenced by the persistent repiliation in the presence of chloramphenicol after repeated mechanical depiliation. The failure of cells to synthesize proteins during such treatment was confirmed by the near absence of ^{3}H-leucine uptake in chloramphenicol-treated cells, whereas those without chloramphenicol incorporated ^{3}H-leucine. Cells from which pili had been mechanically removed could not regenerate pili on their surfaces in the presence of cyanide or at 4°C, even though such cells presumably possessed reservoirs of pilus subunits. Acid-dissociated pili (pH 1.7) spontaneously repolymerized in the presence of KCl and $MgCl_2$ at pH 7.0 after 48 h at 4°C.

Meningococcal pili were first mentioned in the literature by Jephcott et al. [13]. Subsequently, Froholm et al. [12] observed pili on genetic transformation-competent meningococci but not on strains that were genetically incompetent. During a survey of meningococcal prototype strains in negative stain, DeVoe and Gilchrist [6] found pili in very low numbers (1 to 2 per cell) on only 5% of cells in the populations. In contrast, nearly all cells of the nonprototype meningococcus ATCC 13090 exhibited numerous pili on their surface [6]. This same strain was subsequently reported [2] to have two colonial types that were indistinguishable except for their size. Large diameter pili (4.5 nm) were found on cells from large colonies, whereas cells from small colonies exhibited small diameter pili (2.5 nm).

DeVoe and Gilchrist [6] reported that all meningococci from cerebrospinal fluid or the oropharynx were piliated on initial laboratory cultivation, but pili disappeared on subculture. Two strains were exceptions to this general finding and remained piliated for an indefinite number of subcultures [8]. Salit and Morton [20] have more recently reported similar findings. McGee et al. [16] have discovered that the composition of the medium can affect the retention of meningococcal pili on laboratory cultivation.

The control of pili synthesis by nutrients in the medium opens an interesting area for study. Such studies take on added significance in view of the recent reports [11,17] that gonococci are not piliated in urethral exudates or biopsy samples from cervical gonorrhea. In this study we present evidence that pH of the growth medium not only affects the elaboration of pili on the cell surface, but also affects the intracellular pool of pilus subunits in the meningococcus.

Materials and Methods

Organism. *Neisseria meningitidis* group B (strain M2092) was obtained from the American Type Culture Collection (ATCC 13090). Maintenance of stock cultures and routine examinations for purity were described elsewhere [5,6].

Cultural conditions. Frozen working agar-slant cultures (−70°C) were thawed at 37°C and streaked onto Mueller-Hinton (M-H) agar (Difco Laboratories, Detroit, MI) plates that were incubated for 16 h in a candle extinction jar (37°C, 100% relative humidity). In some experiments, the M-H medium was buffered with 25 mM tris (hydroxymethyl)-aminomethane-maleate (Sigma Chemical Co., St. Louis, MO) and titrated with 4 M NaOH to the desired pH value as previously described [1].

Electron microscopy. Negatively stained specimens (0.5–1% phosphotungstic acid, pH 7.4; Fisher Scientific Co., Fairlawn, NJ) were prepared as previously described [6]. Electron micrographs were obtained with a Phillips EM300 electron microscope.

Studies on pili synthesis. Meningococci were grown in M-H broth to absorbance 0.6, 600 nm. Cells were harvested (8,000 × *g*, 10

* Present address: National Research Council of Canada Ottawa, Ontario, Canada K1A 0R6

** To whom reprint requests should be addressed.

0343-8651/83/0008-0057 $01.00

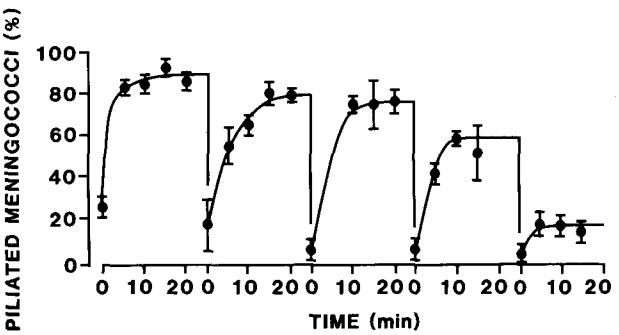

Fig. 1. Effect of sequential removal of pili from chloramphenicol-treated *N. meningitidis* (M2092) grown in M-H broth at pH 7.2. Pili were mechanically removed and cells were further incubated for 25 min intervals in a shaking (100 rpm) water bath at 37°C. Each point represents the mean of 50 well-separated meningococci observed in negatively stained preparations by electron microscopy.

min) and resuspended in fresh M-H broth supplemented with either chloramphenicol (20 μg per ml, final; Fisher Scientific Co.) or KCN (500 μM; prepared fresh). In some experiments cells were rapidly chilled to 4°C. Cells were mechanically depiliated in an Omni—Mixer (Model 17105; DuPont Co., DE), centrifuged (8,000 × *g*, 10 min), and resuspended in fresh M-H broth with chloramphenicol (repeated five times). Fifty cells were examined after each repeat to determine the degree of piliation.

Effect of pH on piliation. A pili preparation was obtained by mechanically depiliating cells as described above, removing cells and large cell debris (12,000 × *g*, 10 min), and collecting pili by high-speed centrifugation (75,000 × *g*, 1 h). Pili were acid-dissociated and subsequently repolymerized as previously described for *Escherichia coli* [3]. Meningococcal pili were reversibly dissociated by subjecting a cell-free pili preparation to a pH of 1.7 (±0.2). Repolymerization of pili was accomplished by dialysis against a solution of 0.2 M KCl, 0.1 M $MgCl_2$, pH 7.0, for 48 h at 4°C.

In some experiments meningococci were cultured in M-H media at either pH 6.6 or 7.2, collected by centrifugation (8,000 × *g*, 10 min), and subsequently resuspended to 2 × 10^8 cells per ml in fresh M-H media at the opposite pH value of pH 7.2 or 6.6, respectively. Cell suspensions were further incubated at 37°C and aliquots were screened for piliated cells.

Results

Before determining the effect of pH on pili expression, it was important to determine whether meningococci maintained intracellular reservoirs of the components required for pili synthesis. Pili were easily removed repeatedly from cells by violent agitation in a laboratory blender. Chloramphenicol was used to prevent protein synthesis, and ^{3}H-leucine incorporation was used to monitor what protein synthesis, if any, did occur. The results of these experiments are shown in Fig. 1. Cells failed

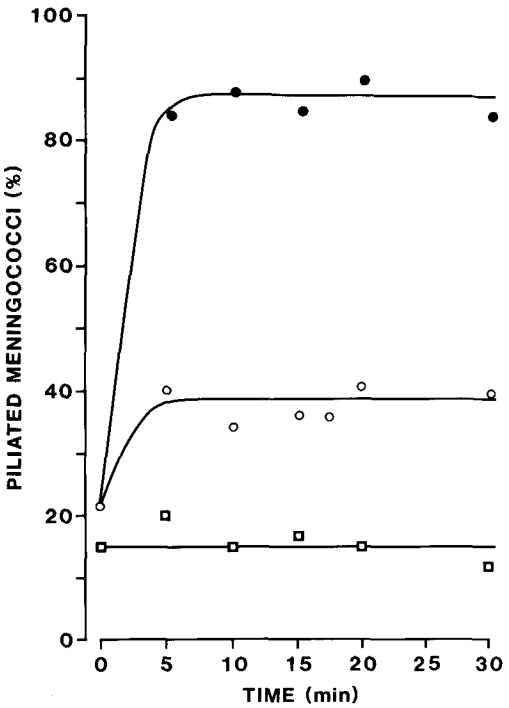

Fig. 2. Effect of cyanide and temperature on piliation of *N. meningitidis* (M2092) grown in M-H broth at pH 7.2. Pili were mechanically removed, cells washed, and then reincubated in M-H broth at 37°C (●); M-H broth + 500 μM KCN at 37°C (○); and M-H broth at 4°C (□).

to synthesize protein in the presence of 20 μg of chloramphenicol per ml, as evidenced by the <5% ^{3}H-leucine incorporation during the 120 min incubation (not shown) when compared to control cells under similar conditions but without chloramphenicol. The reservoir of pili precursors was relatively large as shown by the ability of cells to regenerate these surface structures repeatedly in the absence of *de novo* protein synthesis. Control cells regenerated pili indefinitely after their mechanical removal.

The assembly of pili, or the translocation of subunits from within the cell to the surface, was affected by both a terminal oxidase inhibitor and by low temperature (Fig. 2). As shown above, cells that had been mechanically depiliated regenerated pili rapidly at 37°C in M-H broth at pH 7.2. However, the interruption of the respiratory chain with cyanide or the lowering of the temperature to 4°C prevented repiliation of cells, even though precursor reservoirs were present in the cells. Although spontaneous self-assembly of pili from cell-free precursors can take place at 4°C (see below), such spontaneous extracellular self-assembly of pili can be ruled out (Fig. 2) as a mechanism for the

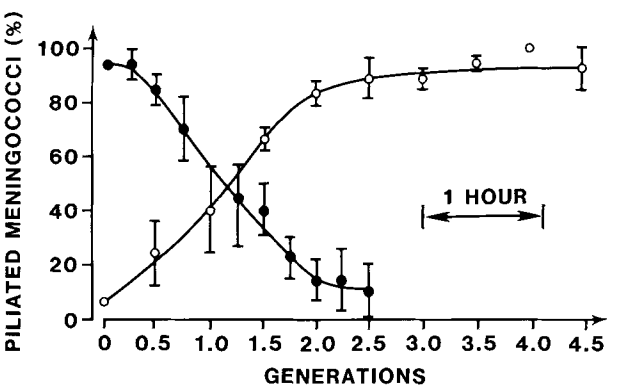

Fig. 3. Effect of culture medium pH on synthesis of pili on *N. meningitidis* (M2092). Cultures were grown in M-H broth at pH 6.6 or 7.2, washed, then resuspended in M-H broth at pH 7.2 (○) or 6.6 (●), respectively.

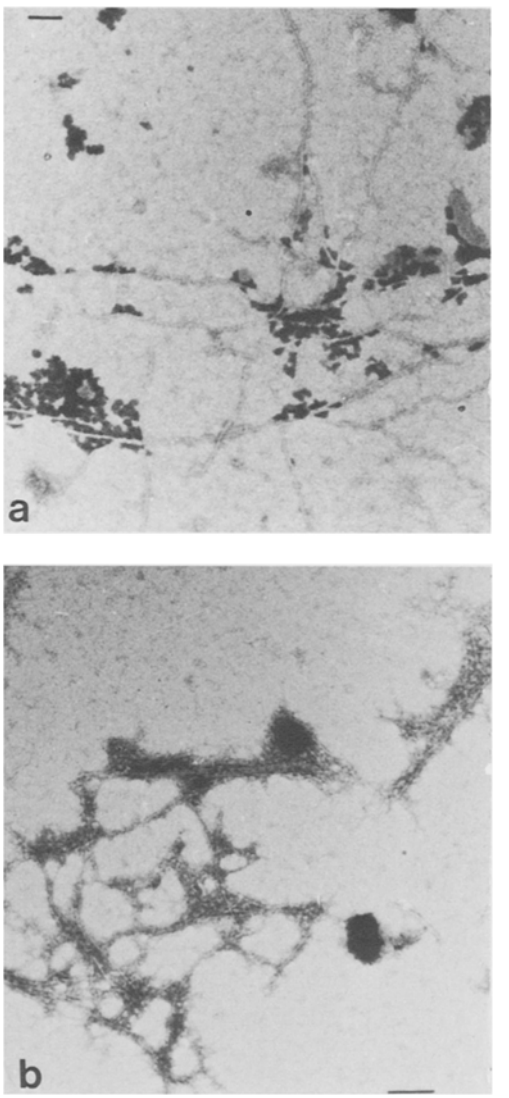

Fig. 4. Negatively stained pili (a), prior to dissociation; and (b), after spontaneous assembly. Bar represents 0.1 μm.

reappearance of pili on meningococci after mechanical depiliation. The implication from the experimental results is that a functioning respiratory chain and a fluid membrane are required for the assembly and/or translocation of pilin subunits in intact cells.

Previously we reported that the pH of growth was an important factor in predetermining the virulence of meningococci [1]. This prompted us to examine the effect of pH on piliation. Piliated meningococci grown at pH 7.2 became nonpiliated when grown in a buffered M-H broth at pH 6.6, the lowest pH at which meningococci will grow [1]. The absence of pili on cell surfaces at pH 6.6 was not due to the simple dissociation of these structures at this pH, since isolated pili remained intact at this pH (data not shown). Therefore, cells grown at pH 6.6 either synthesized pilus subunits but could not assemble them on their surfaces, or they did not produce such subunits at all.

To determine the kinetics of pili appearance or disappearance, piliated cells grown at pH 7.2 were transferred to the same medium but at pH 6.6, whereas those nonpiliated cells from a pH 6.6 culture were transferred to a pH 7.2 medium. The rate of appearance or disappearance of pili on cells is shown in Fig. 3. The rate of loss mirrors that of the appearance of piliated cells. In these experiments, a cell with at least a single pilus was scored as a piliated cell. The loss of pili from cell surfaces required two generations before >90% of cells were nonpiliated. If pili were merely diluted out in the population as a result of cell division, one could expect the number of cells with pili to remain relatively constant for several generations; i.e., the number of pili per cell would decrease by half at each cell division. It is obvious that pili were lost by some other mechanism. Moreover, the appearance of piliated cells is a relatively slow process indicating that in spite of the abrupt pH change, not all cells elaborated pili immediately. There is no lag period for the initiation of cell division when cells are transferred from neutral to the lower pH [1], or vice versa. Therefore, one can conclude that pH affects the regulation of pili synthesis rather than pili assembly, itself.

Pili isolated after mechanical shearing from cells were isolatd by ultracentrifugation and washed free of whole cell debris. Such isolated pili (Fig. 4a) were acid-dissociated by lowering the pH of the suspending fluid to pH 1.7. The evidence for the

dissociation was the complete disappearance of pili from negatively stained preparations. The dissociated pili spontaneously repolymerized during the 48 h dialysis against 0.1 M $MgCl_2$ and 0.2 M KCl at pH 7.0 (4°C), as evidenced by their reappearance in negatively stained preparations (Fig. 4b).

Discussion

It is well established that bacteria may exhibit genetic phase variation in the synthesis of pili as a result of changes in environmental or nutrient conditions [7,9,10,16,18,21]. *E. coli, Klebsiella pneumoniae,* and *Shigella flexneri* shift to a nonpiliated phenotype in response to frequent subculture in stationary broth (nonaerated) or to anaerobiosis [14]. Just as we have found with meningococci, species of *Salmonella,* which are piliated at pH 7.3, generally lose their pili when grown at low pH. When subcultured at neutral pH, pili reappear [9]. Piliated *S. flexneri* convert to nonpiliated forms when grown in a medium containing 1% glucose [19]—a medium in which the pH decreases as growth proceeds.

A morphological feature of primary isolates of meningococci is the presence of surface pili [7]. Such pili are present in primary culture whether cells are taken from the oropharynx or cerebrospinal fluid. This observation suggests that the phenotypic expression of pili may be an important survival determinant in the host. In most meningococcal strains examined, pili are seen on initial isolation but are lost on subsequent culture in the same medium [7,20]. McGee et al. [16] have found that piliation can be retained if initial isolates are grown on rich media, e.g., isovitalex-supplemented chocolate agar.

In this report we have presented evidence for a reservoir of pilus subunits in the meningococcus. Assembly of these subunits into pili was blocked by cyanide, suggesting the requirement for a functional respiratory chain for the complete phenotypic expression of meningococcal pili. Moreover, cells incubated at 4°C did not produce surface pili even though cell-free self-assembly can occur at this temperature. The translocation of the pilus subunits through the cell membranes may be inhibited by the reduction in membrane fluidity one could expect at 4°C.

Cells transferred from neutral to low pH did not lose their pili immediately, which rules out dissociation of the pilus subunits at the lower pH. Moreover, cells grown at the lower pH did not immediately produce surface pili when transferred to the higher pH 7.2, suggesting that a reservoir of pilus subunits was not preformed. Therefore, it seems likely that environment pH somehow regulates the genetic expression of the pilus genes.

A functional role for meningococcal pili has yet to be shown. However, there is indirect evidence that pili may aid in the attachment to host cell surfaces. Salit and Morton [20] and McGee and Stephens [15] have demonstrated the adherence of meningococci to pharyngeal cells but have found they adhere poorly to Hela cells. Moreover, mannose did not inhibit such adherence [4,15]. It is not unreasonable that control of meningococcal pili synthesis through environmental factors may act to the advantage of the organism in some as yet unknown fashion.

Literature Cited

1. **Brener, D., DeVoe, I. W., Holbein, B. E.** 1981. Increased virulence of *Neisseria meningitidis* after *in vitro* iron-limited growth at low pH. Infection and Immunity **33**:59–66.
2. **Brener, D., Gilchrist, J. E., DeVoe, I. W.** 1977. Relationship between colonial variation and pili morphology in a strain of *Neisseria meningitidis*. FEMS Microbiology Letters **2**:157–161.
3. **Brinton, Jr., C. C.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. Transactions of the New York Academy of Sciences **27**:1003–1054.
4. **Craven, D. E., Frasch, C. E.** 1978. Pili and nonpili mediated adherence of *Neisseria meningitidis* and its relationship to invasive disease, pp. 250–252. In: Brooks, G. F., Gotschlich, E. C., Holmes, K. K., Immunobiology of *Neisseria gonorrhoeae*. Washington, DC: American Society for Microbiology.
5. **DeVoe, I. W.** 1976. Egestion of degraded meningococci by polymorphonuclear leukocytes. Journal of Bacteriology **125**:258–266.
6. **DeVoe, I. W., Gilchrist, J. E.** 1974. Ultrastructure of pili and annular structures on the cell wall surface of *Neisseria meningitidis*. Infection and Immunity **10**:872–876.
7. **DeVoe, I. W., Gilchrist, J. E.** 1975. Pili on meningococci from primary cultures of nasopharyngeal carriers and cerebrospinal fluid of patients with acute disease. Journal of Experimental Medicine **141**:297–305.
8. **DeVoe, I. W., Gilchrist, J. E.** 1978. Piliation and colonial morphology among laboratory strains of meningococci. Journal of Clinical Microbiology **7**:379–384.
9. **Duguid, J. P., Anderson, E. S., Campbell, I.** 1966. Fimbriae and adhesive properties in *Salmonellae*. Journal of Pathology and Bacteriology **92**:107–138.
10. **Duguid, J. P., Gillies, R. R.** 1956. Non flagellar filamentous appendates ("Fimbriae") and haemagglutinating activity in dysentery bacilli. Journal of General Microbiology **15**:vi.
11. **Evans, B. A.** 1977. Ultrastructural study of cervical gonorrhea. Journal of Infectious Diseases **136**:248–255.

12. **Froholm, L. O., Jyssum, K., Bøvre, K.** 1973. Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta Pathologica Microbiologica Scandinavia. Section B. **81**:525–537.
13. **Jephcott, A. E., Reyn, A., Birch-Andersen, A.** 1971. *Neisseria gonorrhoeae*. III. Demonstration of presumed appendages to cells from different colony types. Acta Pathologica et Microbiologica Scandinavia. Section B. Supplement. **79**:437–439.
14. **Maccacaro, G. A., Turri, M.** 1959. Effect of temperature and other experimental variables on the course of infectious transmission of surface properties of *Escherichia coli*. Giornole di Microbiologia **3**:84–96.
15. **McGee, Z. A., Stephens, D. S.** 1981. Attachment of *Neisseria meningitidis* to human mucosal surfaces, influence of receptor cell. Journal of Infectious Diseases **143**:525–532.
16. **McGee, Z. A., Street, C. H., Chapell, C. L., Cousar, E. S., Morris, F., Horn, R. G.** 1979. Pili of *Neisseria meningitidis:* Effect of media on maintenance of piliation, characteristics of pili, and colonial morphology. Infection and Immunity **24**:194–201.
17. **Novotony, A. P., Short, J. A., Walker, P. D.** 1975. An electron microscope study of naturally occurring and cultured cells of *Neisseria gonorrhoeae*. Journal of Medical Microbiology **8**:413–427.
18. **Old, D. C., Duguid, J. P.** 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. Journal of Bacteriology **103**:447–453.
19. **Ottow, J. C. G.** 1975. Ecology, physiology and genetics of fimbriae and pili. Annual Review of Microbiology **29**:79–108.
20. **Salit, I. E., Morton, C.** 1981. Adherence of *Neisseria meningitidis* to human epithelial cells. Infection and Immunity **31**:430–435.
21. **Tweedy, J. M., Park, R. W. A., Hodgkiss, W.** 1968. Evidence for the presence of fimbriae (pili) on *Vibrio* species. Journal of General Microbiology **51**:235–244.