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NOTES

Altered Outer Membrane Protein in Different Colonial Types of *Neisseria gonorrhoeae*

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Dark-colored colony types of *Neisseria gonorrhoeae* (T3 and dark variants of T1 and T2) had markedly increased amounts of an approximately 28,000-dalton outer membrane protein, as compared with light-colored colony types (T4 and light variants of T1 and T2). The presence of this protein appeared to be unrelated to piliation. The apparent molecular weight of this protein on sodium dodecyl sulfate-polyacrylamide gels varied, depending on methods used to solubilize envelope proteins. In view of the location of this protein on the outer membrane, this protein could be important to the pathogenicity or antigenicity of the organism as well as to colonial characteristics in vitro.

Morphological variation of *Neisseria gonorrhoeae* colonies was first described by Kellogg et al. (7). Colony types T1 and T2 differ from T3 and T4 in several respects: T1 and T2 colonies are smaller (7), possess surface pili (17), are virulent (7), and are competent in genetic transformation (14). T2 colonies have a more sharply defined border than T1 colonies (7). Both T1 and T2 colonies may exist as light- or dark-colored variants (1, 2). T3 colonies are darker and more granular, and exhibit more cell clumping than T4 colonies. No biochemical differences between colony types, other than piliation, have been described, however. We report here evidence of altered outer membrane proteins in dark-colored variants of both piliated and nonpiliated gonococci, which we discovered serendipitously during study of membrane proteins of antibiotic-resistant strains.

The strains studied were FA19 (15), FA140 (15), FA248 (a *met-1* mutant of F62), and FA19X (local isolate). Cultures of the colony types to be compared were grown on the same day in 1.5-liter amounts of GC base broth (GCBB-DS) (11). The liquid cultures were inoculated with overnight GCBA-DS (11) plate cultures to an initial density of 10 Klett units, and were grown at 37°C in 5% CO_2 to mid-log phase (100 to 120 Klett units) 4 to 5 h later. Generation times were nearly identical for all four colony types under these conditions. Purity of the colony types of the harvested cells was usually 90% or better. The cells were collected by centrifugation at 4°C, washed once with 40 ml of ice-cold minimal medium Davis (Difco), suspended again in 40 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.4 (HEPES buffer), and ruptured by two passages through an ice-cold French pressure cell at 20,000 lb/in^2 before overnight storage at −70°C.

Membranes were prepared from thawed ruptured cells. After incubation for 15 min at 37°C in the presence of 1 mM $MgCl_2$, 40 μg of deoxyribonuclease per ml, and 40 μg of ribonuclease per ml, all subsequent steps were performed at 4°C. Unbroken cells were removed by one 5-min centrifugation at 3,000 × *g*. The clear supernatant was centrifuged for 20 min at 48,000 × *g*, and the opaque pellet was washed one time by suspension and centrifugation at 48,000 × *g* for 1 h in 10 mM HEPES to yield crude outer membrane fractions. The supernatant of the 20-min centrifugation at 48,000 × *g* was centrifuged at 214,000 × *g* for 1.5 h to give a reddish transparent pellet. This was washed one time by recentrifugation in 10 mM HEPES to yield crude cytoplasmic membrane fractions.

The crude membrane fractions were loaded onto separate sucrose gradients as follows: 5 mg of membrane protein, in a total volume of less than 1 ml, was layered onto a 15 to 55% continuous sucrose gradient of 37.5 ml, containing 10 mM HEPES, pH 7.4. Centrifugation was for 16 h at 27,000 rpm in a Spinco SW27 rotor. The crude outer membrane formed a major peak at buoyant density $\rho^\circ = 1.22$ g/cm^3, whereas crude cytoplasmic membrane formed a major peak at buoyant density $\rho^\circ = 1.14$ g/cm^3. Similar gra-

dients containing 5 mM ethylenediaminetetraacetic acid resulted in loss of the cytoplasmic membrane peak at $\rho° = 1.14$ g/cm^3. Approximately 3-ml amounts from each of the $\rho° = 1.14$ and 1.22 g/cm^3 peaks were recovered in drops by use of an Isco density gradient fractionator device. These were diluted 10× with 10 mM HEPES buffer and centrifuged for 2 h at 214,000 × *g*. After resuspension of the pellets in 10 mM HEPES, portions were used to determine protein concentration (8) and succinic dehydrogenase activity (5). The rest was solubilized by treatment with sodium dodecyl sulfate (SDS) and urea and by boiling (method II of Schnaitman [13]), or as described later in this paper.

SDS-polyacrylamide disc gel electrophoresis was carried out essentially as described by Schnaitman (13) unless otherwise noted. Slab gels contained 7.5% polyacrylamide, 0.266% bisacrylamide, 0.5 M urea, 0.1% SDS, 0.1 M NaH_2PO_4 (pH 7.2), prepared with 0.1% *N*,*N*,*N'*,*N''*-tetramethylethylenediamine, 0.1% ammonium persulfate, and 0.1% sodium sulfite. An EC474 standard vertical gel electrophoresis apparatus (E-C Apparatus Corp., St. Petersburg, Fla.) was used. Running times were 30 min at 75 mA and 7 h at 150 mA in a circulating 0.1% SDS-0.1 M NaH_2PO_4 buffer, pH 7.2. Gels were stained with Coomassie brilliant blue and destained as described previously (16).

Results of a typical experiment are shown in Fig. 1. In this experiment, proteins were solubilized in 5 M urea-0.5% SDS, but were boiled only 2 min, according to the method of Pett et al. (10). Crude envelope proteins of strain FA140 showed consistent increases in two bands, designated 16A and 20A, in T3 and dark-colored T1 and T2 colony types, as compared with T4 and light-colored T1 and T2 colony types. No differences in gel patterns between either light-colored T1 or T2 and T4 colonies, or between dark-colored T1 or T2 and T3 colonies were discerned, except for slight increases in a 21,000- to 22,000-molecular-weight band in piliated T1 and T2 colonies. (Other minor differences shown in Fig. 1 were not reproducible.) The T2 colony type in all figures is a dark variant. Similar differences in envelope proteins of isogenic dark- or light-colored colonial variants were noted in each of 12 different membrane preparations from four strains and confirmed by coelectrophoresis of ^{3}H- and ^{14}C-labeled proteins from T3 and T4 colonies on single SDS-10% polyacrylamide gels (data not shown).

The calculated molecular weights of proteins 16A and 20A for strain FA140 were 28,000 and 20,000, respectively. Strain FA248 showed a slightly lower molecular weight for both proteins. The 20,000-molecular weight protein sometimes increased more strikingly in the dark-colored T1 and T2 colonies, and in T3 colonies, than depicted in Fig. 1. The occasional inconsistency in the relative amounts of the 28,000- and 20,000-molecular weight proteins (bands 16A and 20A, Fig. 1) was troublesome. Schnaitman (13) and Inouye and Yee (4)

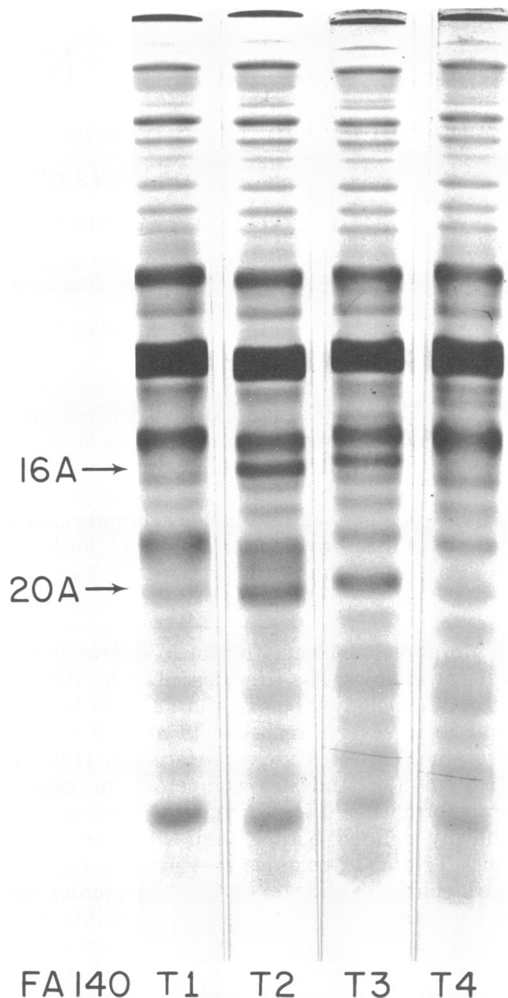

FIG. 1. *Cell envelope proteins of FA140 colony types derived from a single T1 colony in a total of two subcultures. Envelopes were prepared as described in text except that membranes were pelleted from French-pressed cells by centrifugation at 48,000 × g for 1 h instead of 20 min, resulting in increased amounts of cytoplasmic membrane protein. Protein was solubilized in SDS-urea by heating at 100°C for 2 min, and the SDS-10% polyacrylamide gels were prepared and run essentially as described by Pett (10). Amount of protein on each gel was approximately 115 μg.*

showed that certain membrane proteins of *Escherichia coli* migrate either as single or multiple bands on SDS-polyacrylamide gels, depending on the methods used for solubilization. Similar analysis of variables during solubilization of envelope proteins showed that the presence of urea and the extent of heating markedly influenced the relative amounts of the 28,000- and 20,000-molecular-weight proteins (Fig. 2). When envelope proteins were solubilized by boiling for at least 5 min in the presence of 5 M urea, only the 28,000-molecular weight band was observed. Conversely, omission of both urea and boiling during solubilization resulted in predominantly 20,000-molecular weight protein. Solubilization with urea but without boiling, or boiling without urea, resulted in a major band at 28,000 molecular weight and a minor band at 20,000 molecular weight.

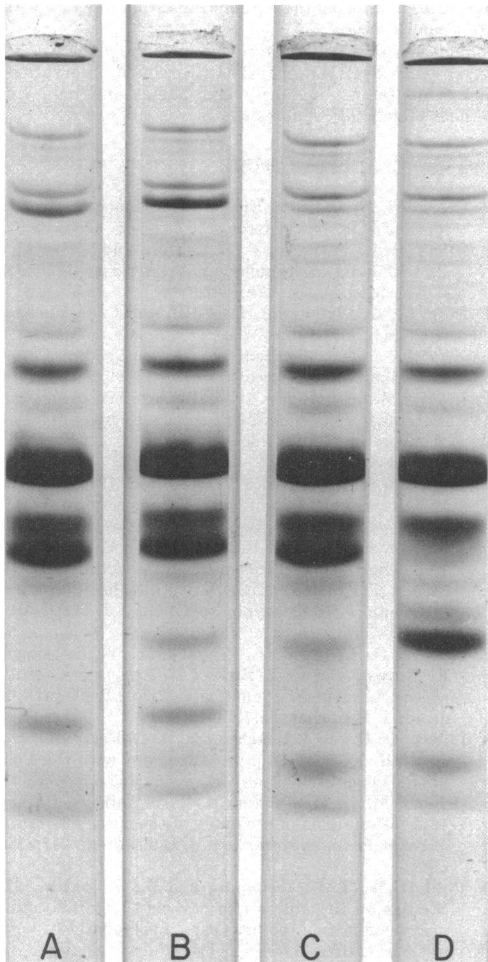

FIG. 2. *Effect of solubilization conditions. Purified outer membrane of FA19 T2 (dark) was solubilized in 0.5% SDS, 1% β-mercaptoethanol, 50 mM* NaH_2PO_4 *(pH 7.2) at a protein concentration of 375 μg/ml under different conditions: (A) 5 min, 100°C with 5 M urea included in the solubilizing solution; (B) 37°C, 1 h with urea; (C) 5 min, 100°C, no urea; (D) 37°C, 1 h, no urea. Gels were SDS-10% polyacrylamide described by Schnaitman (13). Amount of protein on each gel was approximately 15 μg.*

These results suggest that dark-colored colonies may contain markedly increased amounts of only one protein, and that the apparent molecular weight of this protein may differ, depending on conformational changes induced by urea and heating in the presence of SDS. Further experiments are necessary, however, to prove this point. In subsequent experiments, proteins were always solubilized by boiling for at least 5 min in 5 M urea-0.5% SDS, and only the 28,000-molecular weight protein was observed.

We tried to determine whether the 28,000-molecular weight protein in crude envelope preparations of dark-colored colonies was in the cytoplasmic or outer membrane. Many attempts to prepare purified outer membranes by the methods of Johnston and Gotschlich (5), Wolf-Watz et al. (18), and Schnaitman (12) were unsuccessful. However, it was possible to prepare partially purified cytoplasmic and outer membranes by differential centrifugation in HEPES, followed by separation on linear 15 to 55% sucrose gradients. Outer membrane and cytoplasmic membrane preparations had buoyant densities of $\rho^\circ = 1.22$ and 1.14 g/cm^3, respectively, which are typical of purified membranes of the gonococcus (5). Specific activities of the cytoplasmic membrane marker enzyme, succinic dehydrogenase, were sixfold higher in cytoplasmic than in outer membranes. Comparisons of proteins from outer and cytoplasmic membranes on slab gels clearly located the 28,000-molecular weight protein in the outer membrane (Fig. 3).

Digestion of crude envelopes with 2% sodium lauryl sarcosinate (Sarkosyl), which reportedly removes selectively cytoplasmic membrane in *Escherichia coli* (3), appeared effective in removing cytoplasmic protein. Assays for succinic dehydrogenase showed no activity in Sarkosyl-treated membranes, but activity was easily detectable in the supernatant of Sarkosyl-solubilized membranes or in crude membranes assayed in the presence of 2% Sarkosyl. Again the 28,000-molecular weight protein was readily apparent in the detergent-treated membranes (data not shown).

Neither the functional role of this cell surface

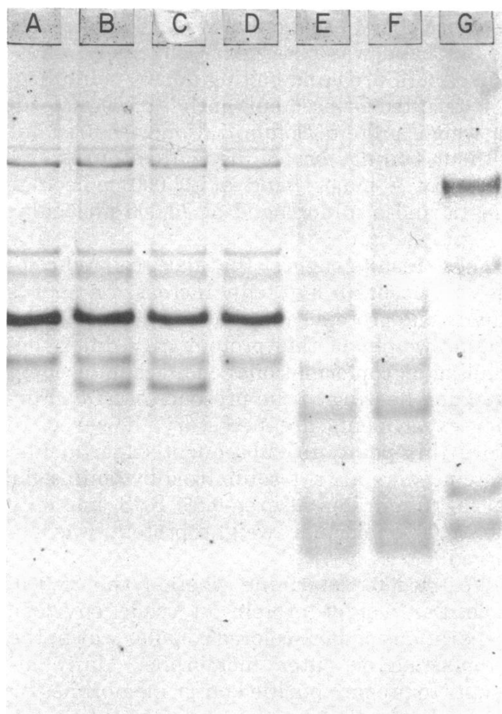

FIG. 3. *Outer membranes (OM) and cytoplasmic membranes (CM) of different colony types of FA140 purified by sucrose density gradient centrifugation. (A) OM, light T1; (B) OM, dark T2; (C) OM, T3; (D) OM, T4; (E) CM, light T1; (F) CM, dark T2; (G) 2 μg each of molecular weight marker proteins bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 45,000), myoglobin (molecular weight 17,800), and cytochrome c (molecular weight 12,400). Preparation and running of the 7.5% polyacrylamide slab gel is described in the text. All membrane proteins were solubilized in the presence of urea and 5 min boiling by Schnaitman's method II (13). Samples represent 15 μg of OM protein and 25 μg of CM protein.*

protein, nor the mechanisms of its apparent relationship with color are known. Since each of four tested strains showed a color-associated protein, our results probably can be extended generally to most other gonococcal isolates. We recently learned that J. Swanson has independently made similar observations (personal communication). These results are at variance, however, with those of Johnston et al., who found no differences in outer membrane proteins of isogenic colonial variants (6).

Although Diena et al. found no differences in virulence of dark- as compared to light-colored piliated gonococci in chicken embryos, and no differences in the response of light and dark gonococci to the bactericidal effect of human serum (2), it is possible that the 28,000-molecular weight outer membrane protein of dark-colored colonial variants may affect the pathogenicity or antigenicity of the organism. Future comparisons between piliated and nonpiliated gonococci should be restricted to similarly colored variants, lest findings attributed to differences in piliation actually result from differences in the 28,000-molecular weight outer membrane protein.

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