COLONY MORPHOLOGY OF PILIATED *NEISSERIA MENINGITIDIS*

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Kellogg (1, 2) was the first to recognize that *Neisseria gonorrhoeae*, when grown on solid agar, grew in distinctive colony types. He was able to show that gonococci growing in colonies, which he designated as types 1 and 2, were able to cause disease, whereas those he described as types 3 and 4 did not (1, 2). The type 1 and 2 colonies were characterized by a perceptible dark ring surrounding each colony. Upon repeated nonselective transfers of these bacteria, the majority of the population would give rise to type 3 and 4 colony phenotypes. It was later found that the cardinal feature of the bacteria that grew in type 1 and 2 colonies was the presence of pili on their surfaces (3, 4). Since this discovery there has been an effort to understand this gonococcal surface structure and the genes that produce it (for reviews, see references 5 and 6). This single property, i.e., the colony morphology, has allowed researchers to quickly evaluate whether the gonococci they were propagating were piliated, and infectious, or not. However, such an association between piliation and colony morphology has not been observed for *N. meningitidis*. This has made the study of the role of pili in meningococcal colonization and invasion more difficult.

In the present study it was found that when meningococci were grown on agar media at 30°C in a candle extinction jar; the bacteria grew in colonies that resembled the colonial morphologies of gonococci. Similar to gonococci (7), the colonial morphologies could be maintained by selective transfer and a population of bacteria showing each of the phenotypes obtained. Each of the meningococcal colonial phenotypes were examined for characteristics of piliation, i.e., the production of pilin by Western blots, using an mAb reactive to all Neisserial pilin proteins (8); competence of the bacteria for transformation (9); the ability of the bacteria to form pellicles in static broth cultures (9); and the presence of surface pili by EM (9). The findings of these studies suggest that the same associations between colony phenotypes and presence of pili exist in meningococci and gonococci; that is, those bacteria forming colonies with a sharp perimeter and a dark ring had all the characteristics of piliated bacteria, while those growing with a diffuse edge did not. Likewise, colony opacity in meningococci is associated with the production of opacity-associated proteins as measured by the reactivity in Western blots to an antibody reactive to class 5 proteins (10), much like that of gonococci displaying the opague colonial phenotype (7).

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Materials and Methods

Organisms. The organisms used were meningococcal strains originally isolated from clinical cases of meningitis. M1080 (a type 1 strain) was kindly provided by Dr. Carl Frasch, Bureau of Biologics, Food and Drug Administration, and 8765 (a type 15 strain) was kindly provided by Dr. John Boslego, Walter Reed Army Institute of Research. Neither strain had undergone lyophilization. Lyophilized strains BNCV and S-946 (both type 2) were received from Dr. Frasch, and MC8532 (type 15) was from Dr. Boslego. The organisms were grown on agar medium, previously described (7), in a candle extinction jar in an incubator maintained at 30°C. Static broth cultures were performed as described by Frøholm et al. (11) in the same medium lacking the agar.

SDS-PAGE and Western Blot. For whole cell lysates, single colonies of a particular phenotype were removed from the agar surface using small strips of filter paper and submerged in 30 μl of water. To this was added 30 μl 2× SDS-PAGE preparation buffer, and the suspension was placed in a microwave oven on high output for 10 s. The SDS-PAGE was a variation of Laemmli's method (12), as described previously (13). Electrophoretic transfer to Immobilon P (Millipore Corp., Bedford, MA) was performed according to the methods of Towbin et al. (14), with the exception that the paper was first wet in methanol. The blots were probed in most cases with two mAbs: MCO2, which was kindly supplied by Dr. John Swanson, Rocky Mountain Laboratory (Hamilton, MT), and is crossreactive with all Neisserial pili (8); and 4B12, which is reactive with gonococcal opacity proteins as well as most class 5 proteins (10), the meningococcal equivalent (15). The Western blots were probed with phosphatase-conjugated reagents (16).

Transformation. An overnight culture of each of the strains, M1080, S-946, 8765, or MC8532, was inoculated to an OD_{600} of 0.05 in 1 ml of broth containing 15 g/liter proteose peptone (no. 3; Difco Laboratories Inc., Detroit, MI), 5 g/liter NaCl, 7.15 g/liter Hepes (Aldrich Chemical Co., Milwaukee, WI), pH 7.2, to which was added $MgCl_2$ to a final concentration of 10 mM, and a solution identical to IsoVitaleX (Baltimore Biological Laboratories, Cockeysville, MD) was added to a final concentration of 1%. The organisms were incubated at 30°C for 30 min with 500 ng to 2 μg of DNA used for transformation. In a series of experiments designed to quantitate the transformation efficiency of the different colonial phenotypes, meningococcal strains treated with 2 μg of gonococcal MS11 chromosomal DNA (containing a gene encoding streptomycin resistance) (17) were assayed as follows. 3–4 ml of medium as above, but containing 0.7% agarose (SeaKem agarose; FMC BioProducts, Rockland, ME) was mixed with the organisms after exposure to the DNA for 30 or 60 min and poured over typing medium agar plates (7). The plates were allowed to set at room temperature and then incubated in CO_2 at 37°C for 5 h. A second layer of soft agar was then added, either with or without streptomycin, allowing a final streptomycin concentration after diffusion throughout the plate of 500 μg/ml (18). Colonies were counted after further overnight incubation in CO_2 at 37°C.

In a second series of experiments a fragment of meningococcal DNA containing the open reading frame of the class 4 gene (*rmpM*) of meningococcal strain BNCV was inserted into *Escherichia coli* plasmid pBR322 and the *rmpM* gene replaced by the *ermC'* gene (19) derived from *Bacillus subtilis* (20). The construct was Hae III methylated, and transformation of meningococcal strains were attempted to produce an *rmpM*$^-$, *ermC*$'^+$ phenotype (19). The strains were grown and incubated with DNA as above, but then 3 ml of fresh broth was added and the mixture incubated with tumbling rotation at 37°C for 5 h. Gradient culture plates were prepared by adding 50 μl of either 1 mg/ml or 10 mg/ml erythromycin to the center of the plates and then distributing this volume over two-thirds of the plate, allowing a diffusion gradient to form. Duplicate samples of 50 μl each of neat, 1/10, and 1/100 dilutions of organisms were then distributed over the entire plates, which were incubated under CO_2 at 37°C overnight. Similarly, a 6.6-kb fragment of gonococcal strain VD302 DNA cut with Cla I (21) containing the gonococcal gene *recA* interrupted by a β-lactamase gene (22) was used to transform meningococcal strain M1080. The transformation was carried out as above, except that the selective gradient was made using 50 μl of 1 mg/ml ampicillin.

Electron Microscopy. Meningococci of a particular colony phenotype were removed from the agar plate using a dacron swab and resuspended in 0.15 M ammonium acetate. Other-

wise, the negative staining with uranyl acetate was performed as previously described for gonococci (23).

Results

Colony Morphology. When several different strains of meningococci were propagated on solid media and either incubated at 37°C in a CO_2 incubator for 8–10 h or at 30°C for 16–24 h, differences in colony morphologies become readily apparent as observed by a dissecting microscope with both oblique light and with the polished mirror substages. The similarity between gonococcal colony phenotypes and the meningococcal colonies, when grown under these conditions, was obvious. As observed by the oblique lighting, one colony type was smaller and had a sharp perimeter with a dark ring, while a second was larger with a more diffuse edge (Fig. 1). Both of these colony types could be maintained by selecting single colonies from each and transferring them to fresh agar media. After 16–24 h at 30°C, the observed colonies of the new plate were homogeneously representative of the parent colony type with a few exceptions. The strains that had been lyophilized showed only the diffuse edge phenotype. The sharp perimeter phenotype with a dark ring was visible on Mueller-Hinton agar after overnight incubation at 30°C, but was difficult to see because of the opacity of the medium. Colonies grown overnight on either Mueller-Hinton agar or typing medium (7) at 37°C failed to show the sharp perimeter phenotype.

When the colonies were observed using the polished substage mirror, two pre-

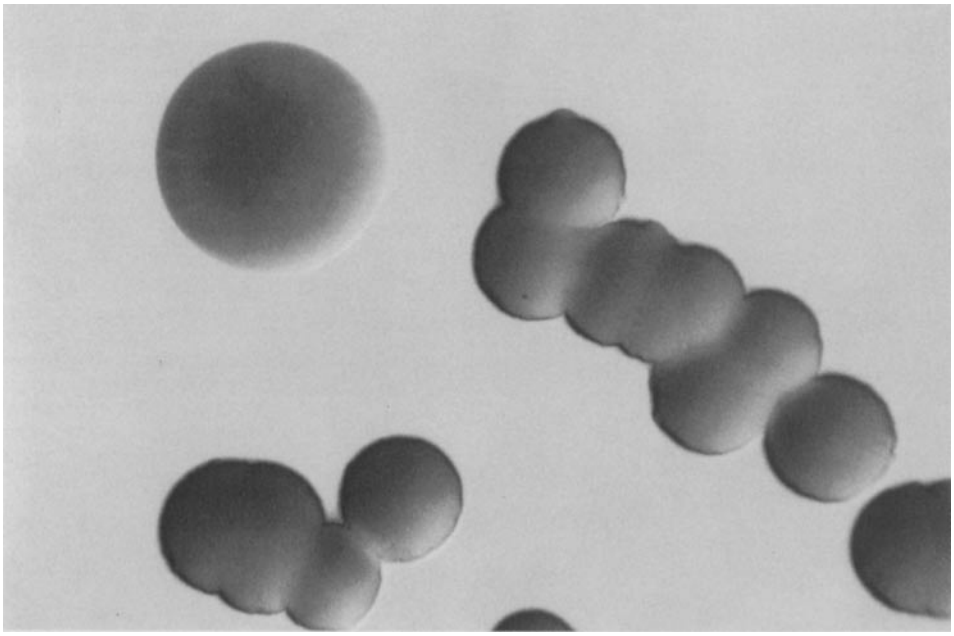

FIGURE 1. Photograph (through a dissecting microscope with a diffusing substage) of colonies on solid agar after a 20-h incubation at 30°C. Note the many sharp-edged colonies, showing a dark peripheral line, and the larger single diffuse-edged colony lacking the dark peripheral line.

dominant colony types could be seen. One was clear and the other was opaque (Fig. 2 *D*). These colony types seemed independent of the colony types observed with the diffusing substage. Both transparent as well as opaque colonies could be seen with sharp edges and others with diffuse edges. Thus, through single colony transfers, four distinct colony phenotypes could be separated: (*a*) sharp-edged transparent; (*b*) sharp-edged opaque; (*c*) diffuse-edged transparent; and (*d*) diffuse-edged opaque. Sharp edge colonies of both the transparent and opaque phenotypes observed with the polished substage mirror showed a sharper peripheral light reflection than those with a diffuse edge (Fig. 2 *B*).

Western Blot Analysis. When whole cell lysates of meningococci from the sharp-edged colonies and the diffuse-edged colonies were subjected to SDS-PAGE, Western blot analysis, and probed with a pilin-specific mAb (MCO2), the antibody reacted with a protein of ~18,000 mol wt in the lysates from sharp-edged colonies. In most cases, no such reactivity was found in the lysates from the diffuse-edged colonies (Fig. 3). The molecular weight of the reactive protein varied somewhat from colony

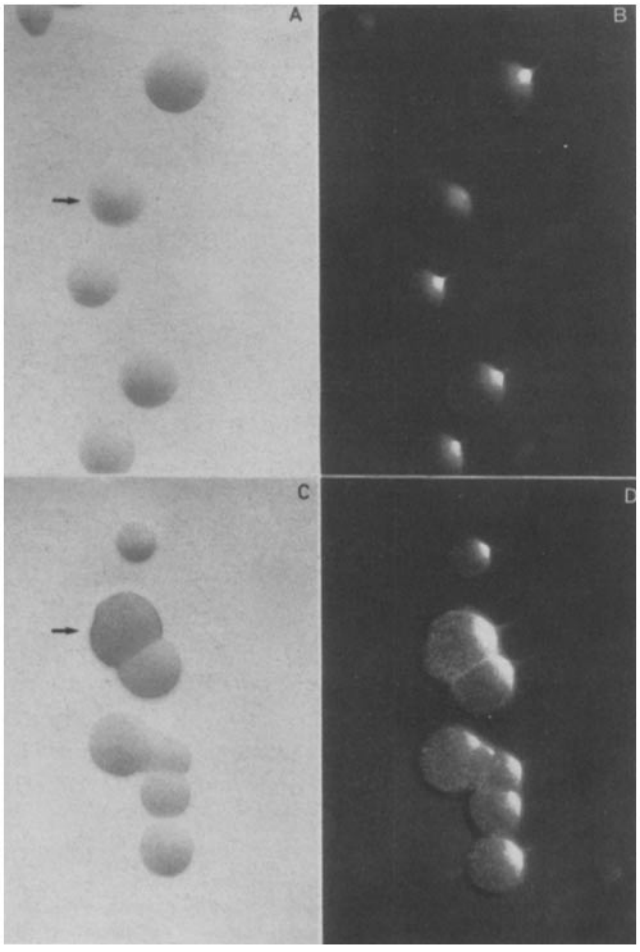

FIGURE 2. Photographs of colonies on solid agar taken through a dissecting microscope. (*A*) A colony (*arrow*) showing a diffuse edge is seen among several colonies with a sharp edge; (*B*) the same group of colonies, but with a polished mirror substage. Note that when the polished mirror is used, there is a sharp point of light at the edge of the colonies with a sharp edge, while this point of light is lacking in the diffuse-edged colony. (*C* and *D*) Opaque colonies are seen in comparison with transparent colonies (*A* and *B*), both with the diffusing substage and with the polished mirror substage.

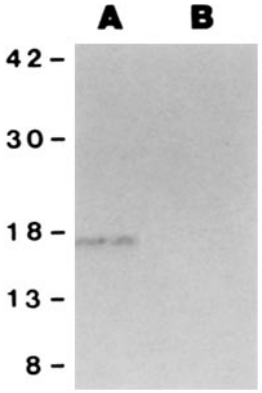

FIGURE 3. A Western blot analysis of whole cell lysates run on SDS-PAGE. In lane *A*, a colony showing a sharp edge morphology was picked and processed as described in Materials and Methods, while in lane *B*, a diffuse edged colony was selected. After electrophoretic transfer, the blot was reacted with the gonococcal pili mAb MCO2 and developed using an alkaline phosphatase anti-mouse IgG conjugate. Note that in the whole cell lysate of the sharp-edged colony, there is a reactive protein that migrates at 18,000 mol wt, while no such reactivity is seen in the lysate from the diffuse-edged colony. Molecular weight standards were OVA (42,000), carbonic anhydrase (30,000), β lactoglobulin (18,000), lysozyme (13,000), and bovine trypsin inhibitor (8,000).

to colony in wild-type strains. However, when probing colonies from the M1080 variant, into which the gonococcal *rec*$^-$ gene had been transformed, the reactive pilin remained the same molecular weight (Fig. 4). This MCO2 reactivity was not influenced by the opacity of the colony. However, the opacity of the colony did influence the reactivity of the other mAb, 4B12. Whole cell lysates from transparent colonies showed a reduced reactivity with the 4B12 antibody while those from opaque colonies had a reactive protein or proteins (Fig. 5). These proteins had a mol wt of ~28,000 and seemed to be in the class 5 family of outer membrane proteins. The 4B12 reactivity, unlike that of the MCO2 antibody, had no correlation with the type of edge of the colony.

Assay for Competence. The quantitative assessment of the transformation efficiency of the sharp and diffuse colonial phenotypes is shown in Table I. Streptomycin resistance was transferred at a higher frequency to both strains when the sharp edge colonial phenotype was used as the recipient strain. In addition, strain M1080 showing the sharp edge phenotype was successfully transformed using the *rmpM*$^-$, *ermC'*, construct (19) to produce a mutant strain resistant to erythromycin and lacking the class 4 protein (19). Repeated attempts to transform strains S-946 and MC8532, both lacking the sharp edge phenotype, were unsuccessful. Chromosomal DNA from the *rmpM*$^-$, *ermC'* M1080 was used to successfully transform both the parent M1080 and the type 15 strain 8765, which also showed the sharp edge phenotype. In addition, the 6.6-kb Cla I fragment of chromosomal DNA from gonococcal strain VD302 with a β-lactamase insertion inactivating the *recA* gene (22) transformed sharp edge phenotype M1080 to a stable piliated phenotype (Fig. 4).

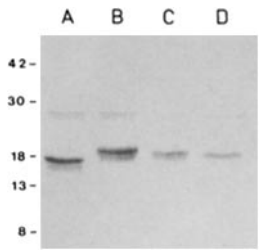

FIGURE 4. A Western blot analysis of whole cell lysates from two sharp-edged colonies from *recA*-competent organisms (lanes *A* and *B*) and two colonies from a *recA*-defective strain (lanes *C* and *D*). The gonococcal pili mAb MCO2 was used to detect the pilin protein. Note that in the competent organisms, the pilin molecular weight is different, even though the two colonies were selected from the same plate, while the *recA*-defective organisms have pilin of the same molecular weight. Molecular weight standards were OVA (42,000), carbonic anhydrase (30,000), β lactoglobulin (18,000), lysozyme (13,000), and bovine trypsin inhibitor (8,000).

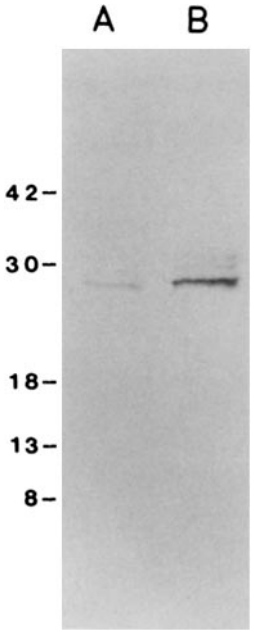

FIGURE 5. A Western blot analysis of whole cell lysates from colonies showing a transparent (lane *A*) and a opaque (lane *B*) colony phenotype. The gonococcal protein II mAb 4B12 was used in this assay. Note that in the lysates from the opaque colony there is a reactive protein at ~28,000 mol wt, with less reactivity seen in the lysates from the transparent colony. Molecular weight standards were OVA (42,000), carbonic anhydrase (30,000), β lactoglobulin (18,000), lysozyme (13,000), and bovine trypsin inhibitor (8,000).

Pellicle Formation. When bacteria from each of the four colony phenotypes were grown in static broth cultures at 37°C in a CO_2 incubator, pellicles on the surface of the broth culture could be observed in those cultures starting with bacteria from sharp-edged colonies. No such pellicles could be seen from cultures from diffuse-edged colonies. When the bacteria from the pellicle were recovered and propagated on agar media, they always grew with a sharp-edged colony phenotype. In fact, in cases where the bacteria were growing in a mixture of colony phenotypes, one could select for organisms that would grow with a sharp colony edge by this pellicle formation process.

Electron Microscopy. By uranyl acetate-negative staining EM, pili could be observed extending from the bacteria that grew with the sharp-edged pattern (Fig. 6). These pili had the characteristics of previously described gonococcal and meningococcal pili (3, 4, 11). No such surface structure was observed on organisms that grew with a diffuse edge.

TABLE I

Transformation to Streptomycin Resistance

Strain	Colonial phenotype	T/E 30 min	T/E 60 min
		× 10^5	
M1080	Sharp	592	5,291
8765	Sharp	628	4,441
M1080	Diffuse	38	768
8765	Diffuse	<1	59

T/E = no. of transformants per ml/no. of viable cells exposed to DNA per ml.

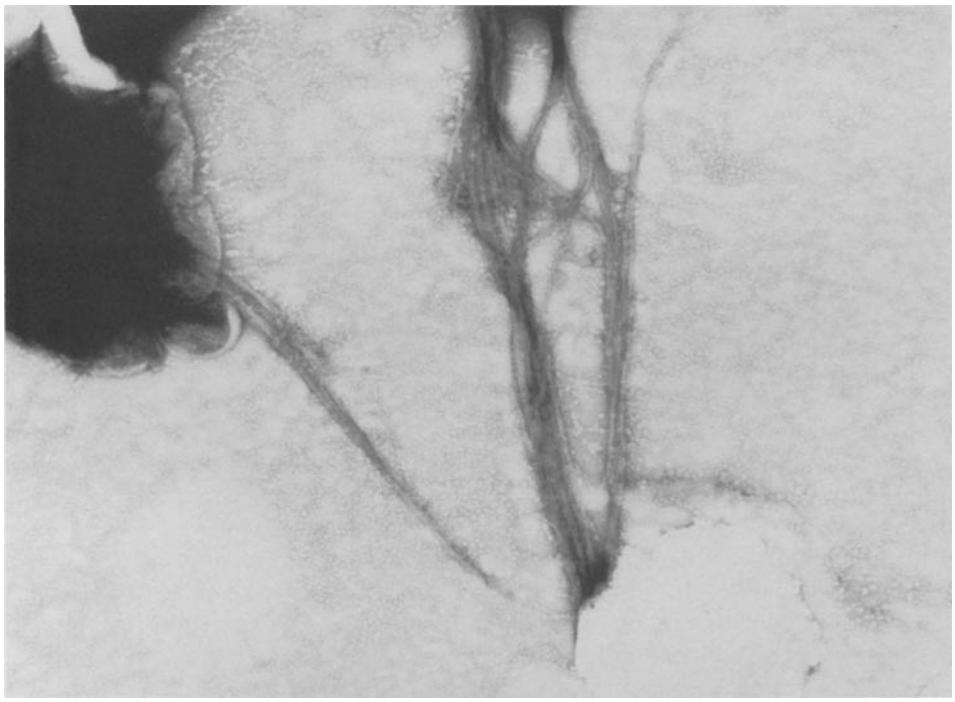

FIGURE 6. An EM of bacteria displaying the sharp-edged colony morphology, which have been negatively stained as described in Materials and Methods. Note that the pili radiating from the organisms are sometimes in large bundles. Bacteria displaying the diffuse-edged morphology lacked these pili.

Discussion

One of the most important characteristics that makes the study of *N. gonorrhoeae* easier is the growth patterns of organisms on agar media. Many of the changes in the outer membrane of the gonococcus are reflected in these growth patterns, and several gonococcal colonial phenotypes are well known. As the molecular biology, genes, and proteins of both the gonococcus and the meningococcus are being defined, it is becoming clear how genetically close these two species are. Many of the outer membrane components are very similar, if not identical (24–32). Also, many of the variations that the gonococcus undergoes have been observed in the meningococcus (5, 6). Thus, it has been hard to understand why meningococci do not display colony phenotypes similar to gonococci. With the exception of the observations made by Stephens and McGee (33), who reported a loose association between meningococci displaying a transparent colony phenotype and the presence of low molecular weight proteins in the outer membranes of these organisms, no association between surface protein expression and colony phenotype has ever been made with meningococci. However, Koomey et al. (22) pointed out that growth on agar media selects for gonococcal organisms that are pilus negative and grow in flat colonies with diffuse edges. Those organisms, which maintain pili on their surface, autoagglutinate and grow in domed colonies with defined edges. Furthermore, he reported that a wild-

type pilus-positive gonococcus would generate pilus-negative variants at a rate of 2.2×10^{-5}/CFU/generation. Since the generation time for meningococci is approximately one-third that of gonococci, a meningococcal agar culture grown at 37°C for 24 h would be comparable with a gonococcal culture grown at 37°C for 72 h. All the gonococcal colony phenotypes, including those that were originally described by Kellogg (1, 2), plus the opacity phenotypes reported by Swanson (34), disappear by 48 h.

Variants where the *recA* gene has been deleted maintain the piliation phenotype longer than *recA*-competent organisms but still loose signs of opacity at the same rate (personal observation). Furthermore, even these *recA*$^-$ variants lose the piliated colonial phenotypic characteristics by 72 h because of the selective pressure of the agar media (22). These data are consistent with our findings that the piliated phenotype and characteristics of opacity were not recognizable in meningococci grown at 37°C overnight. Thus, the observations made by Stephens and McGee (33) cannot be directly compared with those presented here because of the differences in growth temperatures used in the two studies.

The association between piliation and competence in meningococci was first made in 1973 (11). Although these authors established this association of competence with the presence of pili by EM and also the formation of a surface pellicle in stationary broth culture, they were unable to detect differences in colony morphology between the phenotypes on solid agar (11). Meningococci show loss of competence with sequential passage on solid media (35), and a recognizable piliation phenotype is essential to allow selection of competent bacteria for repeated passage. Some isolates of noncompetent meningococci have been shown to revert to competence at a frequency of $\sim 10^{-3}$ (36). Similarly, P$^-$r gonococci have been shown to revert to a P$^+$ phenotype at a frequency of 6.5×10^{-4} (22). The relatively high ratio of transformants to viable cells exposed to the transforming DNA of the diffuse edge phenotypes in the streptomycin transformation in this paper suggests a high rate of revertants, especially in the M1080 strain. The diffuse colonial phenotypes used in that experiment were a first generation subculture from a sharp-edged phenotype culture.

Although competent meningococci have been isolated from lyophilized strains (36), in our experiments, the phenotype is less easily lost from strains frozen at −70°C. The lack of an observable piliated phenotype after overnight incubation at 37°C and the failure of lyophilized strains to retain the piliated phenotype are probable reasons that the gonococcal phenotypes have not previously been shown to be applicable to the meningococcus.

Summary

An association between piliation and colony morphology has not been observed for the meningococcus. We have found that growth of meningococci overnight at 30°C in a candle extinction jar allows observation of distinct colonial phenotypes correlated to the presence or absence of piliation and the expression of opacity-associated proteins. These phenotypes are similar to those observed in gonococci grown overnight at 37°C.

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