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Relationship of Pili to Colonial Morphology Among Pathogenic and Nonpathogenic Species of *Neisseria*

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Growth in colonies with type 1 morphology and the presence of pili are characteristics that have been associated with virulence of gonococci for humans. To determine whether the presence of pili per se might be responsible for colony type 1 morphology, the relationship of pili to colony type was examined in various species of *Neisseria*. Short pili (175 to 210 nm in length) were seen only on nonpathogenic neisseria, whereas long pili (up to 4,300 nm) were seen on organisms of both nonpathogenic and pathogenic species. Although long pili, similar to those found on organisms from high-domed, type 1 colonies of gonococci, were observed on organisms from high-domed, type 1 colonies of nonpathogenic *Neisseria* species, they were also observed on low-convex, type 4 colonies of meningococci and nonpathogenic neisseria. Among meningococci there was no difference in the morphology of colonies consisting of organisms with many long pili and colonies consisting of organisms that completely lacked pili. Thus, there was no consistent relationship of pili to colonial morphology. Unless the pili of *N. gonorrhoeae* are unique among *Neisseria* species in their influence on colonial morphology, it is likely that factors other than pili determine colony type 1 morphology of gonococci. Whether these same factors, either alone or in conjunction with pili, are also responsible for gonococcal virulence warrants further investigation.

The factor or factors that make gonococci virulent for humans are unknown. Kellogg and his colleagues showed that after prolonged passage in vitro, gonococci which grew in colonies with type 1 morphology caused gonorrhea in human volunteers, whereas gonococci from type 4 colonies did not (8, 9). Swanson et al. (17) and Jephcott et al. (7) subsequently demonstrated that gonococci from colonies with type 1 morphology had pili, whereas gonococci from type 4 colonies were not piliated. Although colony type 1 morphology and pili have thus been associated with virulence, it is not clear whether pili are responsible for either colony type 1 morphology or virulence or whether other virulence-associated factors must be considered. In the current study we have examined the relationship of pili to colony type in pathogenic and nonpathogenic species of *Neisseria* to determine whether the presence of pili per se might be responsible for colony type 1 morphology among neisseria.

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MATERIALS AND METHODS

Microorganisms. The microorganisms studied in detail were: (i) *N. gonorrhoeae*, strain 192 A, colony types 1 and 4; (ii) *N. meningitidis* group A, strain 1636; (iii) *N. meningitidis* group B, strain 1643; (iv) *N. pharyngis*, strain NPH 30948; and (v) *N. subflava* ATCC 19243, colony types 1, 2, 3, and 4. These microorganisms were kindly examined in the laboratories of A. P. Johnson, Clinical Research Centre, Harrow, England; A. J. Macara, Central Public Health Laboratory, Colindale, England; and R. E. Weaver, Center for Disease Control, Atlanta, Ga., and their identity was confirmed as indicated above. Strain NPH 30948 was one of a group of four commensal neisseria that were identified as *N. pharyngis;* recently, strains identified as *N. pharyngis* and *N. subflava* have been included in a single group designated *N. subflava* (2, 14). The four colonial variants of *N. subflava* ATCC 19243 were examined in the laboratories in Colindale and Atlanta and were indistinguishable by standard biochemical tests.

Microorganisms were inoculated onto agar medium consisting of GC agar base (Difco) plus 2% (vol/vol) IsoVitaleX (Baltimore Biological Laboratory, Cockeysville, Md.) and incubated at 37°C in 2% (vol/vol) CO_2 in air for 18 to 22 h. Colonial morphology was examined and typical colonies were photo-

graphed, using a Zeiss Universal microscope with camera attachment and angled, transmitted light as described by Kellogg and colleagues (8). The same culture was then used to make negatively stained preparations for study by electron microscopy.

Electron microscopy studies. The tops of colonies on agar plates were scraped with a platinum loop and the organisms were dispersed in a drop of tissue culture fluid, HEPES-MEM (*N*-2-hydroxyethylpiparazine-*N'*-2-ethanesulfonic acid-buffered minimal essential medium), prepared as previously described (10). Two methods were used to negatively stain the organisms. In the first, a Formvar-carbon-coated grid was inverted and floated on the surface of the drop of organism suspension for 1 min. It was blotted from the edge with filter paper and floated for 2 min on the surface of 1 drop of 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The grid was again blotted from the edge with filter paper and floated for a few seconds in each of 2 separate drops of distilled water. After the grid was blotted, it was next floated on 1 drop of 1% sodium phosphotungstate, pH 6.0, for 20 s. It was then blotted thoroughly around the edge and allowed to dry.

The second method, a modification (12) of the method of Valentine et al. (21), was more difficult but demonstrated fine pili better than the first. The suspension of neisseria organisms in HEPES-MEM was introduced between the carbon film and mica substrate with a Pasteur pipette. The film was then floated for 2 min on the surface of a solution of 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, so that the side with adherent organisms was immersed in the solution. The film was then transferred to distilled water for a few seconds and finally to a 0.5% solution of sodium phosphotungstate, pH 6.0, for 20 s. A grid was then applied to the film as described by Parkhouse et al. (12). The grids were examined with a Philips 300 electron microscope at 60 kV.

RESULTS

Colonial morphology and occurrence of pili. The colonial morphology of the various *Neisseria* species, as inferred from the appearance of colonies in incident light, was generally one of two basic sorts: (i) high domed, with *N. gonorrhoeae*, type 1, as the prototype (Fig. 1a), or (ii) low convex, with *N. gonorrhoeae*, type 4, as the prototype (Fig. 1c). Within these groups there were slight variations.

Of the colonies of *N. gonorrhoeae* that were sampled, all of those that had type 1 morphology consisted of organisms that had long pili (Fig. 1b). None of the colonies of *N. gonorrhoeae* that had type 4 morphology contained organisms with pili (Fig. 1d).

Four strains of another pathogenic neisseria, *N. meningitidis*, were examined to determine the morphology of their colonies and the relationship of colonial morphology to the presence of pili. The colonies of all four strains were low convex, like those of *N. gonorrhoeae*, colony type 4 (Fig. 1c), and most were about the same size (0.6 to 1 mm in diameter). Although the morphology of the colonies was identical to that of type 4 gonococci, which lack pili, three of the four strains of meningococci had long pili indistinguishable from those found on colony type 1 gonococci. The three piliated strains of meningococci, one of group A and two of group B, had numerous pili on over 90% of the organisms examined. Representative colonies and organisms with pili are shown in Fig. 1e-h.

Of four freshly isolated commensal neisseria, all of which were identified as *N. pharyngis*, only one strain, NPH 30948, had pili. Nevertheless, the colonies of all four strains had similar morphology (Fig. 2a); they were slightly larger and more convex than type 4 colonies of gonococci. *N. pharyngis*, strain NPH 30948, had both long and short pili present on a majority of the organisms (Fig. 2b). Whereas only two or three long pili were present on about half of the organisms, short pili were present in profusion on most of the organisms.

N. subflava ATCC 19243, on primary culture from the lyophilized vial sent by the American Type Culture Collection, grew in four distinctive colony types that correlated roughly with the colony types of *N. gonorrhoeae* described by Kellogg et al. (8, 9). Colonies that we have designated type 1 were about 0.3 to 0.5 mm in diameter and were high domed with a central peak (Fig. 2c). Colonies designated type 2 were about the same size as type 1, but were darker and slightly wrinkled and had more vertical, refractile margins. Colonies of *N. subflava* designated type 3 (Fig. 2e) were low convex like colony type 3 of *N. gonorrhoeae*, but were much smaller (0.1 to 0.2 mm in diameter). Colonies designated type 4 (Fig. 2g) were about the same size as gonococcal colonies of type 4, but were slightly more domed. After the first two selective passages these colony types remained relatively stable, except that types 1 and 3 occasionally produced type 4 colonies. All four colony types could be repeatedly isolated from the original lyophilized material, which had been frozen after resuspension in heart infusion broth. The proportion of each colony type in a series of 200 colonies counted was: type 1, 4%; type 2, 6%; type 3, 14%; and type 4, 76%. These findings indicate that this strain of *N. subflava*, like strains of *N. gonorrhoeae*, may dissociate into at least four characteristic colony types.

Both long and short pili similar to those described for *N. pharyngis*, strain NPH 30948, were present on organisms from all four colony types of *N. subflava* (Fig. 2d, f, and h). How-

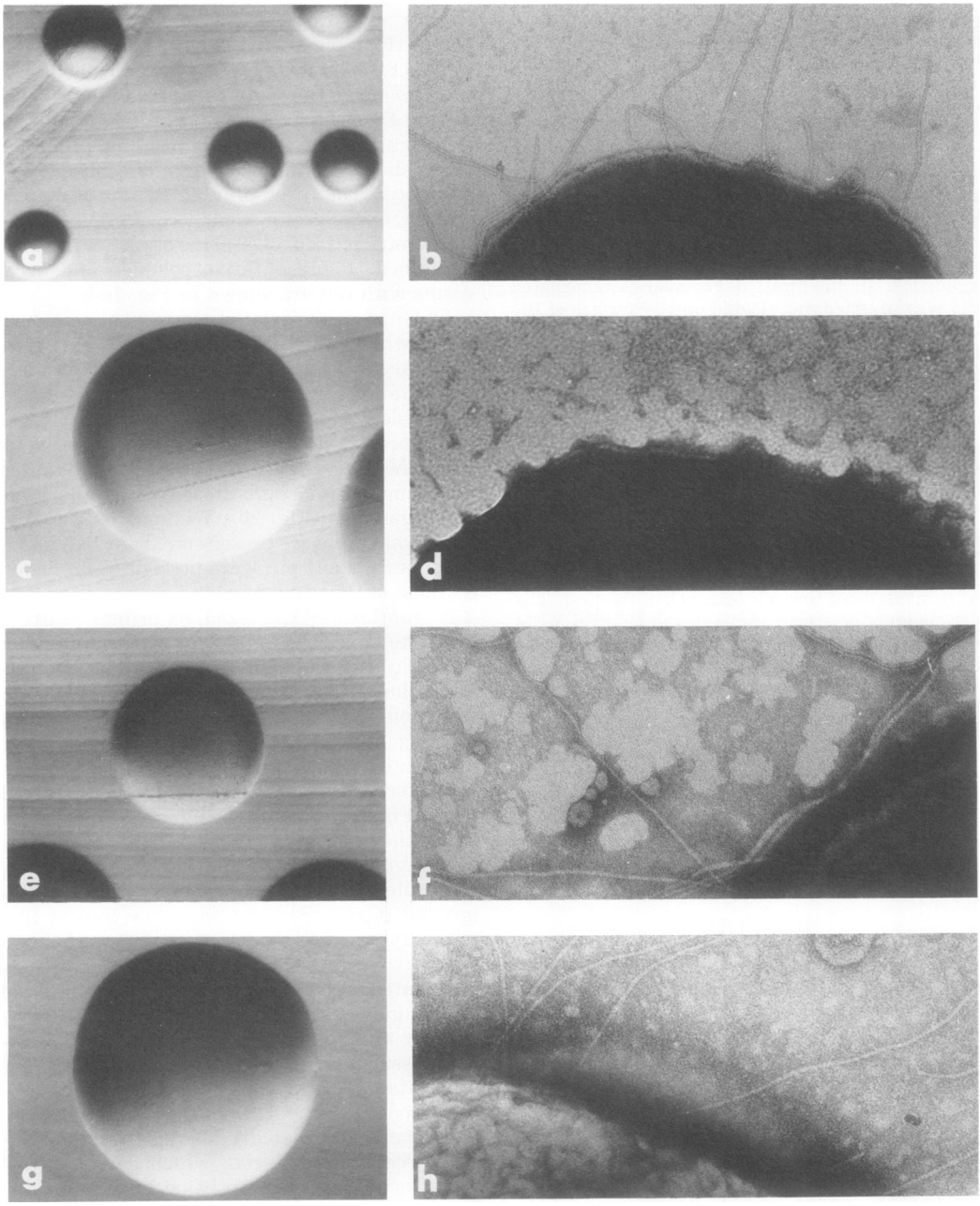

FIG. 1. *Relationship of colony type to presence or absence of pili among species of Neisseria. Colonies photographed after 18 to 22 h of incubation at 37°C (×50); negative stains prepared at the same time show the edge of an organism (phosphotungstic acid, ×110,000). (a, b) N. gonorrhoeae, colony type 1; (c, d) N. gonorrhoeae, colony type 4; (e, f) N. meningitidis, group A; (g, h) N. meningitidis, group B.*

ever, pili of both kinds were observed on only 20 to 40% of the organisms from any given colony type.

Data concerning the relationship of type of pili and density of pili to colonial morphology in various species of *Neisseria* are summarized in Table 1.

Characteristics of pili. The long pili were of

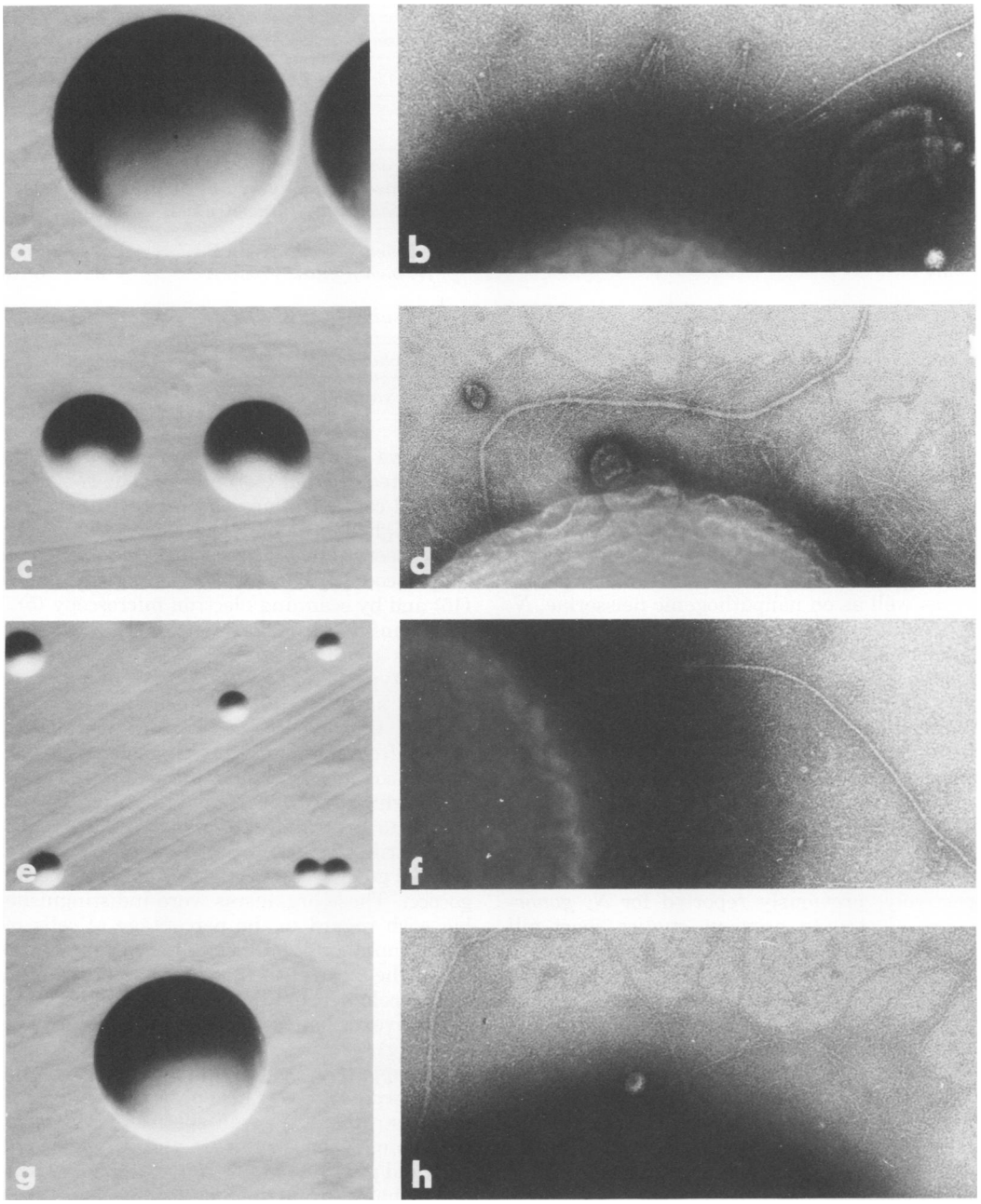

FIG. 2. *Relationship of colony type to presence or absence of pili among species of Neisseria. Colonies photographed after 18 to 22 h of incubation at 37°C (×50); negative stains prepared at the same time show the edge of an organism (phosphotungstic acid, ×110,000). (a, b) N. pharyngis, strain NPH 30948; (c, d) N. subflava ATCC 19243, colony type 1; (e, f) N. subflava ATCC 19243, colony type 3; (g, h) N. subflava ATCC 19243, colony type 4.*

similar dimensions in all *Neisseria* species and strains tested. The average diameter was approximately 7 nm, and the length varied up to 4,300 nm. The exact length of the long pili is uncertain because they may have broken during preparation of the specimen. The short pili were more uniform, with diameters of approximately 4 nm and lengths of 175 to 210 nm.

TABLE 1. *Relationship of colonial morphology to absence of pili or presence of long pili or long and short pili in various species of Neisseria*

Pili	High-domed morphology			Low-convex morphology		
	Strain	Long pili[a]	Short pili	Strain	Long pili	Short pili
No pili				*N. gonorrhoeae* type 4	0	0
				N. meningitidis group B	0	0
				N. pharyngis (three strains)	0	0
Long pili only	*N. gonorrhoeae* type 1	4+	0	*N. meningitidis* group B	4+	0
				N. meningitidis group A	3+	0
Long and short pili	*N. subflava* type 1	1+	4+	*N. subflava* type 3	1+	4+
	N. subflava type 2	1+	4+	*N. subflava* type 4	1+	4+
				N. pharyngis	1+	4+

[a] Increasing density of pili is indicated on a scale of relative values of 0 to 4+.

DISCUSSION

Pili occur on organisms from a variety of species of the genus *Neisseria*. They have been described previously on the pathogenic neisseriae *N. gonorrhoeae* (7, 17) and *N. meningitidis* (3), as well as on nonpathogenic neisseriae, *N. catarrhalis*, *N. perflava*, and *N. subflava* (23). Observations in the current studies confirm previous findings that only long pili occur on piliated organisms of pathogenic species of *Neisseria*, whereas both long and short pili occur together on piliated organisms of nonpathogenic species. The long pili on organisms of both pathogenic and nonpathogenic neisseria had similar morphology; the length and width generally fell within the range of values of 500 to 4,000 nm and 5.5 to 8.5 nm in diameter, respectively, previously reported for *N. gonorrhoeae* (7, 17). In previous studies short pili were found to be 200 to 300 nm long and to have diameters of 6 nm, the same as those of long pili (23). Our observations are similar, except that we found the short pili to have diameters (4 nm) distinctly smaller than those of the long pili (7 nm).

The question of whether colonial morphology among *Neisseria* species is determined by the presence or absence of pili on the organisms is stimulated by the association of colony type 1 and 2 morphology as well as the presence of pili with virulence of *N. gonorrhoeae* for human volunteers (8, 9). Previously, there has been no systematic examination of the relationship of pili to colonial morphology among other *Neisseria* species. DeVoe and Gilchrist (3) reported that colonies yielding piliated meningococci were identical to those yielding nonpiliated meningococci, but the techniques of examination of colonies were not described, so it is uncertain whether differences would have been detected if they had existed. In the current study we used a lighting system that clearly delineated colonial morphology. The two major colonial configurations observed with this system, high domed and low convex, have also been observed in studies of various colony types of gonococci by vertical sectioning of colonies (15) and by scanning electron microscopy (5).

The influence of long pili on colonial morphology could be studied in *N. gonorrhoeae* colony type 1, *N. meningitidis*, *N. pharyngis*, and *N. subflava*, all of which had long pili. Among this group the colonial morphology ranged from high-domed, type 1 colonies to low-convex colonies similar to those of type 4 gonococci, which lack pili. This failure of long pili per se to determine colonial morphology was most striking in the comparison of colonies of piliated gonococci with those of piliated meningococci. These organisms were indistinguishable with regard to the percentage of cells piliated, number of pili per cell, and characteristics of the pili, yet the gonococci produced high-domed colonies and the meningococci produced low-convex colonies (compare Fig. 1a with Fig. 1e and g).

The apparent lack of influence of pili on colonial morphology was also observed among different strains of a given species of *Neisseria*. Strains of *N. meningitidis* and *N. pharyngis* that had pili grew in low-convex colonies indistinguishable from colonies formed by strains that completely lacked pili. Conversely, four different colony types of *N. subflava* ATCC 19243 were formed by piliated organisms that were indistinguishable by electron microscopy studies (Fig. 2c to h). Although it is possible that the pili of gonococci have a property, unique among neisseria, that allows them to determine colonial morphology, this seems unlikely since among gonococci, too, colonies of type 1 and 2 have different morphology, yet both consist of piliated organisms with the

same morphological characteristics (7, 9, 17). These observations suggest that pili either have no influence on or are not the only factors responsible for the morphology of colonies of virulent gonococci.

If some factor other than pili determines type 1 morphology of gonococcal colonies, this same factor, either alone or in conjunction with pili, may also be responsible for the virulence of gonococci from these colonies. There are a number of observations which suggest that factors other than pili play a role in the virulence of gonococci. In the present studies both high-domed, type 1 colonies and possession of pili were found among nonpathogenic as well as pathogenic species of *Neisseria;* thus, neither of these characteristics per se determines the virulence of *Neisseria* species. Indeed, among strains of *N. gonorrhoeae* the relationship of type 1 colonial morphology and the presence of pili to virulence is not as clear as is usually implied. In the original experiments of Kellogg et al. nonpiliated organisms from type 4 colonies, when tested after selective passages 18 and 38 in vitro, were still pathogenic in volunteers, and it was not until 69 or more passages that there was attenuation of virulence (9). It is possible that the type 4 colonies of passages 18 and 38 contained some organisms of colony type 1 or that the type 4 organisms underwent transition to type 1 in vivo. An alternative explanation is that early passage, nonpiliated, type 4 gonococci were capable of attaching to, invading, and damaging the genital mucosa in humans, which they have been demonstrated to do in human fallopian tube organ cultures (20). Thus it would appear that gonococci, like other bacteria, may be able to attach to host cells by means of surface factors other than pili (4, 16, 18, 19). Nonetheless, pili have been postulated to promote virulence by enhancing attachment to host cells (1, 6, 11, 13, 22), and in this respect it is interesting to note the profuse occurrence of long pili on pathogenic neisseria in contrast to the sparse occurrence of long pili on nonpathogenic neisseria. However, even if pili do aid in attachment of gonococci to host cells, attachment alone does not explain the damage to host cells that follows. These considerations suggest not only that factors other than pili are responsible for the virulence-associated morphology of type 1 and 2 gonococcal colonies but also that these or other nonpilar factors play a role in the virulence of gonococci.

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