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Effect of Colony Type and pH on Surface Charge and Hydrophobicity of *Neisseria gonorrhoeae*

KARL-ERIC MAGNUSSON,[1]* ERIK KIHLSTRÖM,[1] LENA NORLANDER,[2] ANDERS NORQVIST,[2] JOHN DAVIES,[2] AND STAFFAN NORMARK[2]

Department of Medical Microbiology, University of Linköping, S-581 85 Linköping, Sweden,[1] and Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden[2]

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The effect of colonial variation and growth at pH 7.2 or pH 6.0 on the surface properties of *Neisseria gonorrhoeae* was assessed by the use of two-phase partitioning and hydrophobic interaction chromatography. Cells grown at pH 7.2 tended to be both hydrophobic and to possess a slight negative charge. Growth at pH 6.0 appeared to decrease hydrophobicity and to increase the negative surface charge. Possession of a series of outer membrane proteins, termed the colony opacity-associated proteins, did not appear to significantly affect charge or hydrophobicity. Piliated cells tended to have a higher negative charge than nonpiliated variants. They also tended to be less hydrophobic at pH 7.2, but became more hydrophobic at pH 6.0. The implications of these findings are discussed.

In contrast to the case with enteric gram-negative bacteria, advances in our knowledge of the role played in gonococcal infection by the envelope structure of *Neisseria gonorrhoeae* has been hampered by a relative lack of knowledge of the physicochemical nature of the gonococcal surface. It has, however, been shown that *N. gonorrhoeae* has an envelope structure which is similar to that of other gram-negative bacteria (14, 31).

It also seems that two different components of the outer membrane may be important in determining virulence. The presence of pili, one of the components, on the cell surface can be easily monitored because piliated cells have a characteristic colonial morphology (11, 15, 16). Most recent isolates are piliated but give rise in vitro to large numbers of nonpiliated variants (16). Experiments in human volunteers and a variety of animal models (5, 15) suggest that these nonpiliated (T3 and T4) variants are relatively avirulent when compared with the piliated (T1 and T2) colonial variants.

In addition, the presence or absence in the outer membrane of a series of proteins, termed the "colony opacity-associated" (COA) proteins (26, 27), seems also to influence the degree of virulence displayed by a particular strain. Recently it has been shown that piliated variants lacking the COA proteins are distinctly more virulent in an animal model than are piliated variants that possess them (24). Piliated variants, whether or not they possess the COA proteins, appear to be more virulent in these models than either of the nonpiliated variants. The presence of these proteins in vitro can also be monitored easily because cells containing the proteins form colonies that are distinctly darker in color, and more opaque, under specific lighting conditions (26, 27). Variants possessing or lacking the COA proteins can give rise to each other at a high frequency in vitro. We were therefore interested in determining the effect of piliation and possession or absence of the COA proteins on the physicochemical nature of the cell surface.

Recent evidence has suggested that the pH of the medium may also affect the composition of the gonococcal surface (7). We have therefore assessed the effect of growth at different pH values on the physicochemical nature of the gonococcal cell surface. We have investigated the effects of these variables on the surface charge and relative hydrophobicity of the gonococcal surface by using a combination of aqueous two-phase partitioning systems (TPP) and hydrophobic interaction chromatography (HIC).

MATERIALS AND METHODS

Gonococcal strains. The strain used in these experiments is 82409/55, which was originally obtained from A. Reyn, Copenhagen, Denmark.

Media and growth conditions. The solid medium used was GC medium base (Difco) supplemented with 1% (vol/vol) Kellogg supplement (20). Plates were incubated at 37°C in 6% CO_2. The liquid medium was identical to the solid medium, except that agar was omitted and 10 mM $NaHCO_3$ was added. In some experiments, the pH of the GC medium was adjusted to pH 6.0 with *N*-2-hydroxyethylpiperazine-*N'*-2-eth-

anesulfonic acid buffer. Growth was followed in a Klett-Summerson photometer (red filter).

Colonial morphology. The presence or absence of pili was scored by colonial morphology and confirmed by scanning electron microscopy (6, 11, 15, 16). Colonial morphology was scored essentially by the methods of Kellogg et al. (16) and Swanson (26, 27). The protein composition of the outer membranes of the variants are in agreement with the assignments of Swanson (26, 27). There was no detectable difference in the outer membrane protein profiles between piliated and nonpiliated variants possessing the same coloration and opacity. T1 and T2 variants are piliated; T3 and T4 variants are nonpiliated. T2 and T3 variants possess the COA proteins, whereas in the T1 and T4 variants they are virtually absent.

Labeling of bacteria. Bacteria were labeled by growth of 25-ml cultures in the presence of 4 μCi of tritiated adenine per ml (23 Ci/mmol) (Radiochemical Centre, Amersham, England). They were harvested at 50 Klett units, washed by suction filtration on a filter, and resuspended in the appropriate buffer for TPP (phosphate-buffered saline, pH 7.3) or HIC [1 M $(NH_4)_2SO_4$–0.01 M phosphate buffer (pH 6.8)].

TPP. (i) Procedure. A two-phase system, basically that of Albertsson (1), was prepared from stock solutions of 20% (wt/wt) polyethylene glycol 6000 (PEG; Carbowax 6000, Union Carbide, New York, N.Y.), 20% (wt/wt) dextran T 500 (Pharmacia Fine Chemicals AB, Uppsala, Sweden), 0.1 M tris(hydroxymethyl)-aminomethane buffer (Tris) (pH 7.0), and distilled water. The basal system contained 4.4% (wt/wt) PEG and 6.2% (wt/wt) dextran in 0.03 M tris(hydroxymethyl)aminomethane buffer. It was allowed to equilibrate at 4°C overnight in a separation funnel. The bottom phase (rich in dextran) and the top phase (rich in PEG) were then collected and stored separately at 4°C. To prepare phase systems for the partitioning studies, 2 ml of the bottom phase and 2 ml of the top phase were pipetted into graduated test tubes. For tests with hydrophobic PEG, 0.2 ml of PEG (20 g/liter) esterified with palmitic acid (PEG-palmitate [P-PEG]) dissolved in phosphate-buffered saline (0.13 mmol of palmitic acid per g of polymer [13]) was added. In the system with charged PEG, 12.5% of the PEG had been exchanged with positively charged PEG, bistrimethylamino $[(CH_3)_3N^+]$-PEG (TMA-PEG) (12), during the preparation of the stock solutions. A 0.1-ml volume of a suspension of bacteria (5×10^8/ml) was added to the graduated tubes with the different phase systems, and the tubes were inverted (20 times) for mixing. They were then kept at 4°C for 30 min for separation of the phases. After reading off the volumes of the bottom phase and the total system, 0.5-ml volumes were withdrawn from the two phases. After mixing with a Vortex homogenizer, 0.5 ml was taken from the remainder (the material adhering to the interface). Quantification of the bacteria was made by beta-scintillation counting. The distribution of bacteria was then calculated from volumes of the phases and radioactivity (concentration of bacteria) in the samples taken.

(ii) Presentation of partition data. The result of the TPP was described by the percentage of material in the top (T) and bottom (B) phase. The rest remains at the interface (I). From these data the percentage of bacteria transferred to T from B upon addition of TMA-PEG or P-PEG was calculated relative to the distribution in the basal system and used as a measure of the negative charge and liability to hydrophobic interaction of the bacteria, respectively. Principally, this index consists of three components—the transfer of material (i) from B to I, (ii) from B to T, and (iii) from I to T. If particles are unaffected by ligand PEG, the index equals zero; it becomes 200 if the entire particle population is completely moved from a position in B in the basal system to T in the system containing ligand PEG. This index was chosen so as to monitor all three of the components above. Any index based solely on the transfer to T ignores component (i), whereas one based on the transfer of particles from B would ignore component (iii).

HIC. The interaction with hydrophobic column material was investigated by adsorption to and elution from Octyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) (10, 23, 25). The HIC procedure was performed essentially as described by the manufacturer of Octyl-Sepharose. However, ordinary Pasteur pipettes were used as columns. The tip of the pipette was filled with glass wool on which was laid a nylon net (70-μm pore size) and about 1 ml of the gel. Thus, about 1 ml of space was left for the eluant. The flow rate (22°C) was about 20 ml/h. The columns were equilibrated with the starting buffer, 1 M $(NH_4)_2SO_4$ in 0.01 M phosphate buffer (pH 6.8), whereupon 0.2 ml of the suspension of bacteria (5×10^8/ml) was added. Elution was made by decreasing stepwise (1-ml proportions) the concentration of $(NH_4)_2SO_4$ to nil and at the same time increasing the concentration of Triton X-100 from 0 to 0.1% (vol/vol). Finally, 95% ethanol was used. All eluants except the latter were 0.01 M with respect to phosphate and had a pH of 6.8.

RESULTS

The surface charge and liability to hydrophobic interaction of the different colonial variants of *N. gonorrhoeae* was studied by TPP. The cells were grown in the normal GC medium at pH 7.2 and pH 6.0 (Table 1). The charge of the bacteria is negative if they are moved towards the top phase by positively charged TMA-PEG; they have a tendency to hydrophobic interaction if they are transferred in the same direction by P-PEG. The liability to hydrophobic interaction was also studied by HIC on Octyl-Sepharose. The two methods were used in parallel, since they might show partly different binding characteristics of the bacteria.

Growth at pH 7.2. The result of the TPP (Table 1) indicates that in the basal system the nonpiliated variants (T3 and T4) accumulate more in T than the piliated variants. The results (Tables 1 and 2) demonstrate that the T1 and T2 variants were both more negatively charged and less liable to hydrophobic interaction than T3 and T4. The latter observation was sup-

TABLE 1. *TPP, in a dextran-PEG system, of the colonial variants of N. gonorrhoeae grown at pH 7.2 and pH 6.0*

pH	TPP system	% Material in top (T) and bottom (B) phase[a]							
		T1[b]		T2		T3		T4	
		T	B	T	B	T	B	T	B
7.2	Basal	14 (1)	86 (1)	10 (1)	74 (9)	20 (1)	57 (1)	25 (0)	54 (10)
	TMA-PEG	5 (0)	49 (3)	21 (1)	37 (5)	16 (2)	44 (5)	24 (1)	38 (6)
	P-PEG	21 (3)	48 (4)	16 (1)	53 (1)	53 (11)	33 (3)	49 (5)	18 (1)
6.0	Basal	23 (3)	68 (4)	30 (1)	66 (1)	34 (1)	63 (1)	44 (1)	56 (1)
	TMA-PEG	56 (2)	44 (2)	55 (1)	43 (1)	39 (3)	51 (3)	60 (1)	40 (1)
	P-PEG	35 (5)	57 (7)	35 (2)	38 (0)	36 (3)	64 (1)	46 (2)	54 (2)

[a] Figures in parentheses represent the range (two experiments performed).
[b] Colony type.

TABLE 2. *Change of TPP of the colonial variants of N. gonorrhoeae grown at pH 7.2 and 6.0 upon addition of TMA-PEG or P-PEG to the dextran-PEG system*[a]

pH	TPP system	Change of TPP[b]			
		T1[c]	T2	T3	T4
7.2	TMA-PEG	28	48	9	15
	P-PEG	45	27	57	60
6.0	TMA-PEG	57	48	27	32
	P-PEG	23	33	1	4

[a] Positive figures indicate either a net negative surface charge or liability to hydrophobic interaction.
[b] Calculated from figures in Table 1 as percentage of increase of material in T plus percentage of decrease of material in B compared to the partition in the basal system.
[c] Colony type.

TABLE 3. *HIC on Octyl-Sepharose of the colonial variants of N. gonorrhoeae grown at pH 7.2 and 6.0*

Eluant	pH	% of bacteria applied to column			
		T1[a]	T2	T3	T4
$(NH_4)_2SO_4$[b]	7.2	8	14	10	14
$(NH_4)_2SO_4$/Triton X-100[c]		25	19	13	15
Remainder[d]		67	67	78	71
$(NH_4)_2SO_4$[b]	6.0	15	39	92	90
$(NH_4)_2SO_4$/Triton X-100[c]		2	1	0	1
Remainder[d]		83	59	8	9

[a] Colony type.
[b] Bacteria were eluted by 1 ml of 1 M $(NH_4)_2SO_4$ followed by 1 ml of 0.5 M $(NH_4)_2SO_4$.
[c] Bacteria were eluted by successive 1-ml volumes of 0.5 M $(NH_4)_2SO_4$/0.01% (vol/vol) Triton X-100, 0.25 M $(NH_4)_2SO_4$/0.02% (vol/vol) Triton X-100, 0.10 M $(NH_4)_2SO_4$/0.05% (vol/vol) Triton X-100, and 0.10% Triton X-100.
[d] Bacteria were either eluted by 3 ml of 95% ethanol or remained on the column.

ported by HIC, which gave a greater remainder of hydrophobic material sticking to the Octyl-Sepharose column in the case of the nonpiliated variants (Table 3).

Growth at pH 6.0. All of the variants, when grown at pH 6.0, once again were transferred towards T by TMA-PEG suggesting a net negative surface charge (Table 1). The relative change of the partition, in contrast to the basal system, was greater than after growth at pH 7.2, implying increased negative charge (Table 2). At the same time, the effect of P-PEG was diminished (Table 2), especially for the nonpiliated T3 and T4. This was supported by the HIC pattern, with retained hydrophobic interaction (i.e., a large remainder) for T1 and T2, but with almost no such interaction for T3 and T4 (i.e., more than 90% of the material came out with the void volume (Table 3).

DISCUSSION

From the results (Tables 1 to 3), it can be inferred that each of the variables shown here (piliation, COA proteins, and pH) may well make independent contributions to the total physicochemical characteristics of the cell surface. Thus a T2 variant grown at pH 7.2 will have a net surface charge that reflects the fact that it is piliated, possesses the COA proteins, and was grown at pH 7.2. Furthermore, in some cases (e.g., T2 variants grown at pH 7.2), our data suggest that the effects of these different components, although independent, are not strictly additive but may be cooperative. Nevertheless, some general conclusions can be drawn. When the distribution in the TMA-PEG system for the cell variants grown at pH 6.0 is compared with the value obtained after normal growth (Tables 1 and 2), it can be inferred that growth at pH 6.0 tends to increase the negative charge of the cell

surface. A similar trend can be seen in the piliated cells, which have a tendency to shift more towards T with TMA-PEG, than the nonpiliated strains. This is in agreement with the data of Heckels et al. (8), who demonstrated that piliated variants were slightly more negatively charged than nonpiliated variants. Possession of the COA proteins has no clear effect on the surface charge, irrespective of piliation and the pH during growth.

Using the same approach, it is possible to make some general estimates of the effect of these variables on surface hydrophobicity. On average the variants grown at pH 7.2 are fairly hydrophobic, but growth at pH 6.0 seems to reduce the liability to hydrophobic interaction. The possession of pili also apparently reduces the tendency to hydrophobic interaction at pH 7.2, whereas the reverse is found after growth at pH 6.0 (Tables 2 and 3). Again, the effect of the COA proteins seems negligible. A few discrepancies are found when comparing the TPP and HIC systems in assessing hydrophobicity. This is not too surprising, since, in TPP, hydrophobicity is essentially assayed in a physiological milieu, whereas with HIC the ionic strength in 1 M $(NH_4)_2SO_4$ is far beyond naturally occurring conditions. For lipopolysaccharide variants of *Escherichia coli* and *Salmonella typhimurium*, TPP differentiated well between mutants with defective core structure, which HIC did not. By contrast, HIC differentiated between mutants with impaired synthesis of the outer core of the lipopolysaccharide (to be published).

It is interesting to look at the TPP results for the effects of piliation on hydrophobicity. At pH 7.2 the piliated strains were less hydrophobic than the nonpiliated, which would suggest that the pili were making the cells more hydrophilic. At pH 6.0 the general liability to hydrophobic interaction was decreased compared to pH 7.2, although the pili now apparently had a hydrophobic effect.

Watt and Ward have reported that purified pili have a high content (about 25%) of hydrophobic amino acids, which might facilitate association with other types of cells (29). More recently, Buchanan et al. (4) have demonstrated that 22 of the first 24 residues in the amino-terminal sequence of purified gonococcal pili are hydrophobic in nature. Buchanan has observed that, at lower pH values, the association of free gonococcal pili with human buccal mucosal cells was increased (2, 3). Independently, the sticking of gonococci to a vaginal cell was shown to increase at pH 4.5 (20). The differences observed in the physicochemical effects of pili during different growth conditions might possibly explain the enhanced role of pili (at lower pH values) as a proadhesion factor in the adhesion of gonococci to different types of mammalian cells (2).

Finally, it should be noted that the effects monitored here may not be the direct results of variations in the pili and COA proteins. It is conceivable that such variations have a secondary effect on the amount and nature of phospholipid and lipopolysaccharide exposed on the bacterial cell surface, which will in turn influence the surface charge and liability to hydrophobic interaction. In this respect, the effects of any variations in the structure of the gonococcal lipopolysaccharide may have a particularly important role in determining the physicochemical nature of the gonococcal surface. It has been shown that T1 variants may contain S type lipopolysaccharide (21), in addition to R type lipopolysaccharide (22, 28), the only type found in T4 variants. Furthermore, T1 variants have a larger amount of acidic glycoses, *N*-acetylneuraminic acid and 2-ketodeoxyoctulosonic acid than T4 variants (30).

It has recently been found that the S type lipopolysaccharide of *S. typhimurium* 395 MS renders the surface hydrophilic and virtually uncharged, in contrast to the R type lipopolysaccharide of *S. typhimurium* 395 MR10 (Rd mutant), which has a surface that is negatively charged and liable to hydrophobic interaction (17, 18). Variations in the lipopolysaccharide might therefore partly explain why the T1 variants are less hydrophobic and more negatively charged than T4 variants after growth at pH 7.2 (Tables 2 and 3). After growth at pH 6, all the variants were more negatively charged and less hydrophobic than at pH 7.2, which might speak for the presence of acidic capsular material of carbohydrate nature. In this respect, it is also interesting to note the results we have recently obtained with the TPP system, when comparing a recent clinical isolate with the laboratory strain employed in these studies (unpublished data). Although the distribution between the different phases was similar, the results (for all colonial variants) demonstrate that the clinical isolate was both slightly more negatively charged and slightly less liable to hydrophobic interaction than the laboratory strain. It is tempting to speculate that these changes reflect the presence of a capsule in the recent isolate (9) or possibly differences in the structure of the lipopolysaccharide (22).

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