Effect of Iron on Surface Charge and Hydrophobicity of *Neisseria gonorrhoeae*

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The effect of iron concentration during growth on the physicochemical surface properties of the colonial variants of *Neisseria gonorrhoeae* has been assessed by aqueous two-phase partitioning in a dextran-polyethyleneglycol system containing positively charged trimethylamino-polyethyleneglycol or hydrophobic polyethyleneglycol-palmitate. The complex effects of iron, in combination with other variables known to affect surface charge and hydrophobicity, have provided some clues as to the properties of the gonococcal surface that are important in promoting virulence.

*Neisseria gonorrhoeae* possesses an outer membrane typical of gram-negative bacteria (9, 25). In the gonococci, however, the composition of this structure is highly variable. Alterations to the amount and diversity of a series of proteins termed the colony opacity-associated (COA) proteins have been noted (21, 22). In addition, variants possessing differing levels of piliation can arise at a high frequency (6, 10, 11). Both pili (2, 10, 11) and the COA proteins (19) seem to be of importance in determining the degree of virulence displayed by a particular variant.

It is also possible to alter the gonococcal outer membrane protein composition in vitro by regulating the amount of iron available to the cells. Iron limitation results in an increased level of a series of high-molecular-weight proteins in the outer membrane (16). Iron has a widely reported but poorly understood role in promoting bacterial infection (24). In the case of the gonococci, high and low iron levels have been shown to significantly enhance and decrease, respectively, the virulence displayed by certain variants (17, 18).

Recently we assessed the effects of different states of piliation, variations in the COA proteins, and the pH of the growth medium on the physicochemical nature of the gonococcal cell surface (12). We were therefore interested in determining whether the level of iron in the medium, which also apparently affects virulence, had a demonstrable effect on the surface charge or hydrophobicity of the gonococcal cell surface, or both.

**MATERIALS AND METHODS**

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

**Media and growth conditions.** The solid medium used was GC medium base (Difco) supplemented with 1% (vol/vol) Kellogg's supplement (14). Plates were incubated at 37°C in 6% CO₂. The liquid medium was identical to the solid medium, except that agar was omitted and 10 mM NaHCO₃ was added. Cultures were grown in flasks, with gentle agitation, at 37°C. Growth was followed in a Klett-Summerson photo-electric colorimeter (red filter).

Iron excess was achieved by adding ferric nitrate to give a final (added ferric nitrate) concentration of 100 μM. Iron limitation was achieved by adding an iron (Fe³⁺) chelator, Desferal (Ciba-Geigy Ltd.) to give a final Desferal concentration of 25 μM (16). In some cases, the pH of the GC medium was adjusted to pH 6.0 with N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid buffer.

**Colonial morphology.** The presence or absence of pili was scored by colonial morphology and confirmed by scanning electron microscopy (4, 9, 11). Colonial morphology was scored essentially by the methods of Kellogg (9) and Swanson (21, 22). The protein composition of the outer membranes of the variants are in agreement with the assignments of Swanson (21, 22). There was no detectable difference between the outer membrane protein profiles of 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 (phosphate-buffered saline, pH 7.3) for two-phase partitioning.

**Two-phase partitioning.** 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 tri(3-mercaptopropyl)trimethoxysilane (SHC), and distilled water. The basal system contained 4.4% (wt/wt) PEG and 6.2% (wt/wt) dextran in 0.03 M tri(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 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 [8]) was added. In the system with charged PEG, 12.5% of the PEG had been exchanged with positively charged PEG, bis-trimethylammonium [CH₃₃N⁺]-PEG (TMA-PEG) (7), during the preparation of the stock solutions. A 0.1-ml volume of a suspension of bacteria (5 × 10⁴/ml, and at least 10⁷ cpm/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 determining the volumes of the bottom phase and the total system, 0.5-ml volumes were withdrawn from the respective 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.

The results have been expressed as the percentage distribution between the top and bottom phases in the different systems. The rest of the material (making a total of 100%) is formed at the interface between top and bottom. To facilitate the interpretation of the data, we calculated the change in distribution, relative to the basal system, when positively charged TMA-PEG or hydrophobic P-PEG are added to the system.

### Table 1. TPP in a dextran-PEG system of the colonial variants of N. gonorrhoeae, grown in GC medium containing different iron concentrations

<table>
<thead>
<tr>
<th>Iron concn</th>
<th>TPP system</th>
<th>T1b</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>B</td>
<td>T</td>
<td>B</td>
</tr>
<tr>
<td>Normal</td>
<td>Basal</td>
<td>19</td>
<td>59</td>
<td>19</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>TMA-PEG</td>
<td>20</td>
<td>42</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>35</td>
<td>37</td>
<td>29</td>
<td>44</td>
</tr>
<tr>
<td>Deficient</td>
<td>Basal</td>
<td>12</td>
<td>56</td>
<td>21</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>TMA-PEG</td>
<td>30</td>
<td>31</td>
<td>48</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>42</td>
<td>29</td>
<td>29</td>
<td>50</td>
</tr>
<tr>
<td>Excess</td>
<td>Basal</td>
<td>25</td>
<td>37</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>TMA-PEG</td>
<td>28</td>
<td>41</td>
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<td>41</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>40</td>
<td>40</td>
<td>38</td>
<td>36</td>
</tr>
</tbody>
</table>

* Figures in parentheses represent the range (two experiments performed).

### RESULTS

**Effect of iron on surface charge.** Previously we have shown that, for gonococci grown in GC medium (which has a pH of 7.2), the possession of pili causes a net increase in the negative charge of the variants (12). The presence or absence of the COA proteins, however, did not appear to have a significant effect on charge, as confirmed by the data (Tables 1 and 2). In addition, however, it can be seen that pregrowth of the bacteria in different iron concentrations has a profound effect on their distribution in the TMA-PEG system. Iron limitation seems to result in an even greater negative surface charge among the piliated strains. Among the nonpiliated variants, however, only the T3 cells show an increased negative charge. It should also be noted that a difference between variants possessing or lacking the COA proteins becomes obvious in iron-limited cells. In both cases the variants possessing the COA proteins (T2 and T3) are more negatively charged than the variants lacking them (T1 and T4).

Iron excess, on the other hand, seems to decrease the negative charge of all variants. The increased negative charge seen in the piliated variants at normal iron concentrations is completely abolished, and they in fact seem to become weakly positively charged.

**Effect of iron on hydrophobicity.** It is known that, for gonococci grown at pH 7.2, the possession of pili has a hydrophilic effect (12). Again the possession or absence of the COA proteins seems to have little effect. Iron also has a clear effect on the hydrophobicity of the gonococcal cell surface (Tables 1 and 2). Iron limitation seems to counteract the effects of piliation (the piliated variants become more hydrophobic), but again a clear difference between the
COA variants becomes apparent. In both cases the variants possessing the COA proteins are distinctly more hydrophilic than the variants lacking them.

Growth in high iron concentrations seems to increase the hydrophilic nature of the piliated variants, but has little effect on the nonpiliated variants. As with cells grown in normal media, the possession of the COA proteins has little effect on the hydrophobicity of cells grown in the presence of an excess of iron.

**Effect of iron at pH 6.0.** It has been known for some time that growth at pH 6.0 has an effect on the nature of the cell surface (5). Recently we have shown that all variants grown at pH 6.0 tend to have a higher negative charge, but, in contrast to the situation at pH 7.2, the possession of pili seems to have a hydrophobic effect at pH 6.0 (12). We were therefore interested in determining the effects of the iron concentration on these variations in the physicochemical nature of the gonococcal cell surface. The data (Tables 3 and 4) show that growth in an iron-deficient medium at pH 6.0 completely abolishes the negative charge of all variants. The piliated variants tend to become less hydrophobic, and the nonpiliated variants become more hydrophobic.

**DISCUSSION**

From the data presented above, it would seem that the level of iron in the growth medium can have a profound effect on the physicochemical nature of the gonococcal surface. The data also clearly suggest that the role of iron in determining virulence may not depend solely on the fact that it is an essential growth factor. This effect is not, perhaps, too surprising, because the proteins that appear in the outer membrane during iron limitation are present in amounts sufficient for them to be classified as major components of the cell surface (16). It should be stressed that the effects demonstrated here may not be direct effects of the iron-induced proteins (or of the pili and COA protein), but could be due to the indirect effects of varying protein concentrations on the amount of phospholipid and lipopolysaccharide exposed at the surface. The iron-induced proteins, which presumably act as receptors for the gonococcal siderophores (iron chelators) (15), are also present, in small quantities, in normally grown cells (15, 16). Only in cells grown in high concentrations of iron can they be regarded as being virtually absent. If the iron effects seen here are directly or indirectly due to the presence or absence of the iron-induced proteins, normally grown cells should be intermediate, in terms of hydrophobicity and charge.

**TABLE 2. Change in distribution of the gonococcal colonial variants, grown in GC medium with different iron concentrations, on the addition of positively charged TMA-PEG or hydrophobic P-PEG to the dextran-PEG system**

<table>
<thead>
<tr>
<th>Colony type</th>
<th>TPP system</th>
<th>Change in distribution of colonial variants*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deficient</td>
</tr>
<tr>
<td>T1</td>
<td>TMA-PEG</td>
<td>43</td>
</tr>
<tr>
<td>T2</td>
<td>TMA-PEG</td>
<td>66</td>
</tr>
<tr>
<td>T3</td>
<td>TMA-PEG</td>
<td>34</td>
</tr>
<tr>
<td>T4</td>
<td>TMA-PEG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>57</td>
</tr>
</tbody>
</table>

* Calculated from the data in Table 1 as the increase in percentage of material in T plus decrease of percentage of material in B, compared to the partition in the basal system. A positive figure therefore indicates either a net negative surface charge or a liability to hydrophobic interaction.

**TABLE 3. TPP of the gonococcal colonial variants grown at pH 6.0 under normal or iron-depleted conditions**

<table>
<thead>
<tr>
<th>Iron concn</th>
<th>TPP system</th>
<th>T1*</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Basal</td>
<td>23</td>
<td>68</td>
<td>30</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>TMA-PEG</td>
<td>56</td>
<td>44</td>
<td>55</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>35</td>
<td>57</td>
<td>35</td>
<td>38</td>
</tr>
<tr>
<td>Deficient</td>
<td>Basal</td>
<td>25</td>
<td>38</td>
<td>22</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>TMA-PEG</td>
<td>27</td>
<td>52</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>P-PEG</td>
<td>42</td>
<td>35</td>
<td>31</td>
<td>27</td>
</tr>
</tbody>
</table>

* Figures in parentheses represent the range (two experiments performed).

**Colony type.**
when compared with iron-starved cells as one extreme and cells grown in high iron concentration as the other, and the data (Table 2) demonstrate that, in most cases, this is so. It is also important to note that we have previously shown that the proteins induced in the different variants of the one strain are identical, both in terms of quantity and variety (16; unpublished data). The same is known to be true for the gonococcal siderophores (18).

From the data in Table 2, it would appear that the presence of iron-induced proteins, by themselves, have little effect on surface charge. This is shown by the data on the T4 variant (which lacks both pili and the COA proteins)—there is little change in charge when comparing the iron-starved T4 variant with the same variant grown in the presence of excess iron. When these proteins are present together with either the pili or the COA proteins, however, they do appear to have an effect. When grown in the presence of excess iron, all of the variants are essentially uncharged. When the piliated T1 variant or the T3 variant (possessing the COA proteins) is iron limited, they gain a substantial negative charge. Thus, although neither the iron-induced proteins, pili, nor the COA proteins have any effect on charge in the absence of the others, when they are present simultaneously their combined effect is to make the cell more negatively charged. From the above it might be assumed that the most negatively charged variant would be one which possesses both pili and the COA proteins (i.e., a T2 variant), as well as the iron-induced proteins, and the data in Table 2 demonstrate that this is, in fact, the case.

Previously we showed that piliated variants grown in normal GC media (pH 7.2) are distinctly more negatively charged than nonpiliated variants (12). From the data in Table 2, it would appear that this observation may be the result of an iron effect. If excess iron is added to the medium during growth, the piliated variants become as virtually uncharged as the nonpiliated variants (they possess a weak positive charge, in fact).

It is known that cells grown at pH 6 become more negatively charged (12). This is particularly so in the case of the piliated variants, where the presence of pili also has the effect of increasing the negative surface charge. The data (Table 4) support these previous observations, but in addition show that a dramatic difference is apparent if cells grown at pH 6.0 are also iron starved. At pH 6.0, in contrast to pH 7.2, the effect of the iron-induced proteins is to make the cells weakly positively charged, irrespective of whether or not the COA proteins or pili are also present.

Previous work showed that piliated variants grown at pH 7.2 are less hydrophobic than nonpiliated variants (12). These studies also showed that the presence of the COA proteins had little effect on hydrophobicity. The data presented in Table 2 support these earlier results, but show that the iron-induced proteins can also have an effect on hydrophobicity. Increases in hydrophobicity are apparent when the T1, T2, and T4 variants are iron limited, but the T3 variants become slightly more hydrophilic. The net result is that all the variants are relatively hydrophobic, but both the piliated (T1) and nonpiliated (T4) variants lacking the COA proteins are distinctly more hydrophilic than the corresponding variants possessing them.

Thus, in the absence of the iron-induced proteins, it might be argued that the COA proteins have little effect on hydrophobicity, and that pili have the effect of making the cells slightly more hydrophilic. In the presence of the iron-induced proteins (which themselves make the cells slightly more hydrophobic), however, pili seem to have little effect, whereas the COA proteins decrease hydrophobicity.

Lowering the pH of normal GC medium to pH 6.0 has the effect of decreasing the hydrophobic nature of all variants (12), especially in the case of the nonpiliated variants, which become much more hydrophilic. The effect of simultaneously
limiting iron is to partially restore the hydrophobic character of the nonpiliated variants (Table 4). At the same time the piliated variants become slightly less hydrophobic.

Thus, the iron-induced proteins appear to be one more example of a variable component of the outer membrane that has an important impact in determining the physicochemical nature of the gonococcal surface. The extremely complex pattern that emerges from these studies may provide some clues as to what physicochemical properties are important in a successful gonococcal infection. The ability to be able to alter the entire nature of the cell surface may be of great importance in giving a variant the ability to adhere to a mucosal cell surface and to simultaneously endow it with properties that make it relatively resistant to phagocytosis. This would be particularly so if the immediate environment of these two very different host cells differed in respect to pH or iron concentration. In this respect, it is interesting to note that mutants of Salmonella typhimurium and Escherichia coli (13, 20, 23; unpublished data) that are resistant to phagocytosis tend to be weakly hydrophobic or hydrophilic and to possess a weak negative surface charge. Increases in hydrophobicity and negative charge can be correlated with increased susceptibility to phagocytosis. The data (Table 2) demonstrate that at pH 7.2 the piliated, virulent variants are more hydrophilic and less charged than the nonpiliated avirulent variants. In addition, it has been known for some time that the piliated variants are relatively resistant to phagocytosis, when compared to nonpiliated variants (3).

Recently it has been reported that lowering the serum iron concentration has the effect of reducing the virulence of the piliated variants in the chicken embryo model (17, 18). The effect of lowering the iron concentration is to make the cells both more hydrophobic and more negatively charged (Table 2). It is therefore tempting to speculate that the reduced virulence is the result of induction of the iron-induced proteins, which increases the negative charge and hydrophobic nature of the gonococcal surface, which in turn might lead to augmented susceptibility to phagocytosis.

The increased hydrophilic character of the piliated variants at pH 7.2 presumably also means that they are less likely to adhere to mucosal surfaces. If, however, the pH of that surface was significantly lower, the gonococcal surface, as the data in Table 4 demonstrate, would become distinctly more hydrophobic. Thus, depending on the nature of the environment, the piliated variants could present a surface that was both resistant to phagocytosis and suited for adherence to mucosal surfaces.

The validity of these speculations, must, however, await the results of further experimentation in this field.

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LITERATURE CITED


