

INTRA-STRAIN HETEROGENEITY OF GONOCOCCAL PILI IS RELATED TO OPACITY COLONY VARIANCE

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Clinical isolates of gonococci, when propagated on agar, grow in colonies having predominantly the characteristic morphology of types 1 and 2 (T1, T2)¹ first described by Kellogg (1), who also reported that organisms expressing these phenotypes were more infectious than the organisms of the less predominant phenotypes 3 or 4 (T3, T4) (1). Later it was observed that organisms of T1 or T2 colonies possess pili, whereas those from the T3 or T4 colonies lack such appendages (2, 3).

There has been considerable interest in the isolation and characterization of gonococcal pili because they mediate bacterial adherence to epithelial cells (4-7) and erythrocytes (8). Gonococcal pili isolated from different gonococcal strains are, however, not identical: inter-strain differences have been found using immunological methods (9, 10) and by comparing subunit molecular weights and amino acid compositions (11, 10).

More recently, it has been observed that gonococci can be classified not only into pilated (P+) and nonpilated phenotypes (P-) but also into opacity variants on the basis of colonial morphology (12). The opacity characteristic is invariably correlated with the presence of one or more additional proteins in the outer membrane (13). Furthermore, opacity is related in an as yet unknown manner to the physiology of the host. Strains of gonococci isolated from infected women at the time of menses give rise primarily to transparent (Tr) colonies, whereas isolates obtained in mid-cycle yield predominantly opaque (Op) colonies (14). The high frequency of secondary complications such as pelvic inflammatory disease observed at or near menses (15) suggests that Tr strains are more invasive. As observed by Salit and Gotschlich (16), organisms classified as P+Tr are more virulent than P+Op bacteria in the chick embryo model. In this model, the state of pilation has been directly related to increased virulence (17, 18). Because functional differences between pili of opacity variants might account for these observations, we systematically compared pili isolated from opacity variants derived from 14 strains of gonococci. We found that in 10 of 14 instances there were appreciable differences in the apparent molecular weight of pilin subunits.

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¹ *Abbreviations used in this paper:* Op, opaque; P+, pilated; P-, nonpilated; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; T1, T2, T3, T4, phenotypes 1, 2, 3, and 4 of gonococci; Tr, transparent; Tris-saline, 0.05 M Tris-HCl buffer, pH 6.8, containing 0.15 M NaCl.

Materials and Methods

Gonococci and Medium. Gonococcal strains F62, 2686, and 33 have been maintained in our laboratory for several years. All other isolates were collected by the Department of Microbiology, Cornell University School of Medicine, New York: these gonococci were received on agar plates (New York City Medium) and were nonselectively removed with cotton-tipped swabs and immediately frozen in Greaves' solution (19) at -70°C . All subsequent propagation was done on solid typing medium described by James and Swanson (14). 100- \times 15-mm disposable Petri dishes were used for typing colonies and 150- \times 15-mm disposable Petri dishes for pilus production. To confirm that the variants selected were *Neisseria gonorrhoeae*, Gram stain, oxidase reaction, and sugar fermentations were performed. The bacteria were selectively passaged every 22–24 h by single-colony transfers and incubated at 36.5°C in candle jars. Opacity variants of each strain were selected and maintained separately for a minimum of 30 passages. Colonies were classified based on their appearance as viewed through a dissecting stereomicroscope as previously described (12). Piliation was confirmed by electron microscopy using a Siemens 101 microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) at 60 kV. Specimens were placed on a 400-mesh Formvar (Monsanto Co., St. Louis, Mo.)-coated and carbon-strengthened copper grid and were stained with 1% wt/vol potassium phosphotungstate, pH 6.3. For immunoelectronmicroscopy, twofold serial dilutions of anti-pilus antiserum were prepared and incubated with purified pili or pilated gonococci. Typical beading of the pili was taken as a positive result.

Purification of Pili. Colonial opacity variants were selected and separated after one passage on typing agar. Because Johnston *et al.* (20) reported that multiple strains could be isolated from a single patient, the possibility that opacity variants might represent different strains of the opposite opacity phenotype had to be eliminated. To accomplish this, strain variants were maintained by single colony transfers for at least 30 passages. Before pilus production, a single colony of the opposite opacity phenotype was selected from a 24-h culture and was propagated separately; e.g., a colony with P+Op morphology was selected from a culture of P+Tr organisms. To observe colonial stability, these variants were passed at least twice and not more than five times before use for pilus isolation. 100 150- \times 15-mm Petri plates containing solid media were each inoculated with 100 μl of a broth suspension of gonococci which had an OD of 0.30 at 540 nm (125- \times 16-mm tube). This suspension was prepared by harvesting the organisms from a large Petri dish with a cotton swab and vortexing to disrupt clumps. The plates were incubated for 24 h in candle jars and before harvest, the colonial characteristics were reassessed. If colony stability was $>90\%$, pili were purified using a modification of the method described by Brinton *et al.* (10) and all steps were done at 4°C . Each plate was flooded with 5 ml of 0.65 M ethanolamine-HCl buffer, pH 10.4. The bacteria were brought into suspension with a bent glass rod, placed into a Sorvall Omnimixer cup (400 ml) (DuPont Instruments-Sorval, DuPont Co., Newton, Conn.) and homogenized for 60 s at 4,000 rpm. Bacteria were removed by centrifugation at 17,000 *g* for 10 min. The bacteria were reextracted with ethanolamine buffer under identical conditions. The supernates were pooled and centrifuged at 48,000 *g* for 60 min. The supernate was dialyzed exhaustively against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (Tris-saline). The aggregated pili were sedimented by centrifugation at 17,000 *g* for 20 min and redissolved in the ethanolamine buffer by end-over-end rotation overnight. The solution was centrifuged at 23,000 *g* for 60 min and the pili reaggregated by dialysis against Tris-saline. This procedure was repeated three times and the purified pili were stored in the ethanolamine buffer. Pilus protein concentration was measured by the Coomassie blue method (21) using bovine serum albumin as a standard. Pilus aggregation was monitored by looking for the presence of streaming birefringence and by dark field microscopy (10).

Polyacrylamide Gel Electrophoresis. Samples were boiled for 2 min in 62.5 mM Tris HCl, pH 6.8, buffer, with 2% wt/vol sodium dodecyl sulfate (SDS) and 1% vol/vol 2-mercaptoethanol. They were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (22) and modified by Ames (23) (separating gel, T = 12.5; stacking gel, T = 5.0; C = 2.6). Gels were run at constant current of 25 mA until the bromophenol blue marker had just migrated off the bottom. The proteins were fixed in the gel with 25% vol/vol isopropyl alcohol and 7% vol/vol acetic acid at 45°C for 1 h. Proteins were stained with 0.25%

wt/vol Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, Mo.) in the fixative solution for 1 h at 45°C. Gels were then destained for 5–7 h in fixative solution. Staining for carbohydrate was done using the periodic acid-Schiff method (24) and also by using Stains All (25), a cationic carbocyanine dye (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.).

Immunological Methods. New Zealand white rabbits were immunized with pilin separated by SDS-PAGE. The unstained protein band, representing pilin, was cut out of polyacrylamide gels by comparing the relative distance of migration of a similar gel stained with Coomassie blue. The gel containing pilin was suspended in phosphate-buffered saline PBS, homogenized by grinding and finally repeatedly passed through a 14- and then 16-gauge needle. Subscapular injections were then given at fortnightly intervals for 12 wk and the rabbits were subsequently bled.

Isoelectric Focusing. Samples (30 µg) for isoelectric focusing were precipitated in 10% trichloroacetic acid, washed with absolute ethanol, then with acetone, and vacuum dried. The pili were then solubilized as recommended by Dr. Gianni Piperno, The Rockefeller University, New York (Personal communication.) as follows: 35 µl of solubilization buffer containing 5% wt/vol SDS, 0.3 M Tris, 0.01% wt/vol EDTA, 1% vol/vol 2-mercaptoethanol, pH 8.8 was added to each sample; after the samples were completely solubilized, 10 µl of concentrated Nonidet P-40 (Particle Data, Inc., Elmhurst, Ill.) was added and mixed thoroughly; finally the sample was diluted to 100 µl with a solution containing 9.5 M urea, 5% vol/vol 2-mercaptoethanol, 2% vol/vol Ampholines 3.5–10 (LKB Instruments, Inc., Rockville, Md.), and 8% wt/vol Nonidet P-40.

The isoelectric focusing was accomplished using polyacrylamide slab gels prepared as described by Ames and Nikaido (26) with the following modifications. The 2% Ampholines consisted of 14 parts 3.5–10, 3 parts 9–11, 1 part 4–6, and 1 part 5–7. The samples were applied at the anodal end of the gel and overlaid with a solution of 5 M urea and 1% Ampholines (4 parts 5–7 and 1 part 3.5–10). The electrophoresis was performed using an LKB 2103 power supply at 25 ma for 1 h and 2.0 W for 12 h. The staining procedure was that recommended in LKB bulletin RB 423.

Isopycnic Centrifugation. Purified pili from both opacity variants were diluted to 1 mg/ml in the ethanolamine buffer. From each preparation, 100 µl was taken and added to 10 ml of CsCl in 0.1 M Tris-HCl, pH 8.9, with a density of 1.29 g/cm³, and refrigerated overnight. One-half of each sample was then mixed to prepare a third sample, and each tube was filled with the CsCl solution. The samples were centrifuged in a Beckman L5-65 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using a Beckman SW 27.1 swinging bucket rotor at 25,000 rpm for 68 h at 4°C. Fractions were collected by puncturing the cellulose nitrate tubes and the protein content monitored by the Coomassie blue method (21). The concentration of CsCl was determined by refractometry.

Results

Colony Variation. Gonococci recently isolated from patients usually expressed predominantly either Op or Tr phenotypes. Some isolates gave rise to only a single opacity variant and were not used in this study. From isolates which did give rise to Tr and Op phenotypes, the variants were separated and their colonial characteristics maintained by single colony transfers. Isogenic variants were derived from these clones by selecting the opposite phenotype of that expressed by the majority of the culture. The use of this method avoided comparison between strains, rather than between variants of a single strain.

Pili Isolation. Crude preparations (first cycle) containing pili and some outer membrane vesicles could easily be prepared by centrifuging the homogenized bacteria in an ethanolamine buffer. These conditions allowed the pili to disaggregate and to remain in the supernate. Pili could then be aggregated at neutral pH, in the presence of salt and collected by centrifugation. It is estimated that in crude pilus preparations

TABLE I
Apparent Molecular Weight of Gonococcal Pili

Strain	Opacity variant	
	Op	Tr
F62	16,900	16,600
33	18,500	18,000
MS11	17,500	17,500*
2686	18,000	18,000*
R1	15,800	15,800*
R2	17,500	17,000
R4	18,200	17,500
R5	18,000	17,000
R10	17,000	16,400
R11	17,000	16,900*
R12	17,500	16,000
R16	17,500	16,200
R17	18,600	16,800
R18	18,600	16,200

* In these strains no difference in mobility of pilin was evident.

(first cycle), approximately one-half the protein was pili and the rest was predominantly outer membrane protein. Analysis of such samples by SDS-PAGE (Fig. 1) indicated that: (a) the major outer membrane protein of a particular strain had an identical subunit molecular weight regardless of opacity phenotype but that the subunit molecular weight differed between different strains; (b) other membrane proteins differed within one strain in accord with the observations of other authors (13), i.e., the Op phenotypes possess one or more strain-specific protein band(s) (23,500–31,000 mol wt) which are diminished or absent in the Tr phenotype; and (c) all strains had a protein band migrating at ~15,000–18,000 dalton (presumptively pili) which was rarely visualized when whole bacteria were subjected to SDS-PAGE.

Pili were purified further by disaggregating them at the high pH of the ethanolamine buffer, at which time impurities were removed by centrifugation. The final product using two or three cycles of reaggregation and disaggregation consisted predominantly of pili. In such preparations, only rare outer membrane vesicles could be seen by electron microscopy and scant amorphous material was seen by darkfield microscopy. When viewed by darkfield microscopy, it seemed that pili derived from Tr colonies aggregated maximally at a lower pH (pH 7.0–7.5) than pili derived from Op colonies (pH 8.0–8.5). However, for the purposes of this study, all pili were aggregated under the same conditions; i.e., by dialysis against Tris-HCl, pH 7.5. When analyzed by SDS-PAGE, the majority of the protein migrated as one band with some minor contaminants (especially the major outer membrane protein) (Fig. 2).

The protein band in the 15,000- to 19,000-mol wt range on SDS-PAGE was proven to be pilin by two methods. For three strains, antibodies were raised in rabbits to bands extracted from polyacrylamide gels. Characteristic antibody coating of pili was visualized by electron microscopy. However, antiserum prepared against pilin from either the Op or Tr bacteria-coated pili from both opacity variants. None of the pilin on SDS-PAGE were stainable by the use of the carbohydrate stains. From each 150-

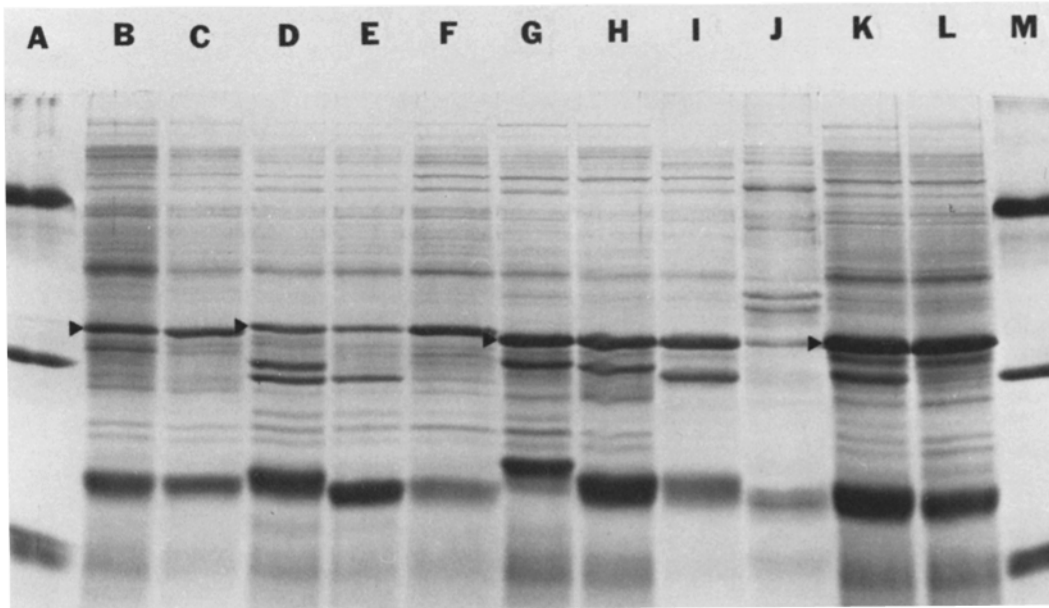


FIG. 1. Crude first cycle pili preparations of isogenic opacity variants of five strains of *N. gonorrhoeae* were boiled in SDS and subjected to SDS-PAGE. The Op and Tr variant of each strain were separated in contiguous lanes with the Op variant on the left. Lanes B and C, R11; Lanes G and H, R17; lanes I and J, R10; lanes K and L, R1. In the case of strain F62, three variants were compared, lane D, F62 Op; lane E, F62 Op/Tr; lane F, F62 Tr. Note that within each strain the major outer membrane protein (indicated in each strain by a triangle) has the same subunit molecular weight, and that this may vary between strains. The Op variants have one or more additional protein bands migrating below the major outer membrane protein. The prominent bands migrating slightly above cytochrome *c* represent pilin. Note the heterogeneity not only between the five strains, but also within strains F62, R17, and R10. Molecular weight standards in lanes A and M are, from top downward: bovine serum albumin, carbonic anhydrase, and cytochrome *c*.

× 10-mm Petri dish, we could obtain ~85 mg of Tr bacteria or 125 mg of Op bacteria (wet weight). The yield of purified pili was ~1–1.5 mg pilus protein for each 10 g of bacteria. Usually, more pili were recovered from the Op bacteria.

Pilus Heterogeneity. In 10 of 14 opacity pairs, the subunit molecular weights of pilins, as determined by SDS-PAGE, differed within a strain (Table I). Most strains had two types of pilin, each with a characteristic mobility. Most often, when this heterogeneity was observed, the pilin from the Op variant had a slower mobility. Isoelectric focusing of pilin from Op and Tr variants of strain R10 indicated that the two populations of pili each gave rise to two bands with differences in isoelectric points (Fig. 3). By two-dimensional electrophoresis (not shown) both bands had a mobility in the second dimension (SDS-PAGE) that was characteristic of pilin. In the case of pili derived from opacity variants of strain MS11, where no difference in pilin mobility on SDS-PAGE was detected, we also saw no difference in the isoelectric focusing pattern. Isopycnic gradient centrifugation in CsCl also demonstrated a difference between purified pili derived from opacity variants. As illustrated in Fig. 4, Tr or Op pili gave rise to a predominant band, i.e., a or b, whereas the mixture yielded both bands. The bands were also different in character with band b appearing to be more flocculent. Thus it appears that pilus expression is related to opacity phenotype.

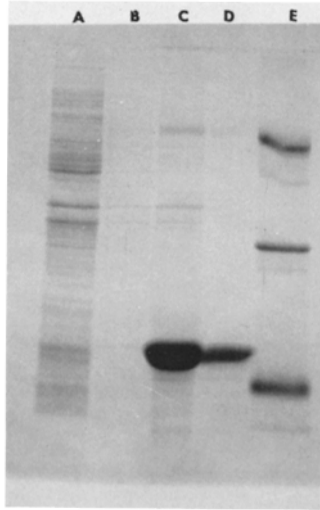


FIG. 2. SDS-PAGE illustrating the purification of pili. Lane A, whole organisms; lane B, supernate from first aggregation at pH 7.5; lane C, 200 μ g of final purified pili; lane D, 75 μ g of final purified pili; and lane E, molecular weight standards: bovine serum albumin, carbonic anhydrase, and cytochrome *c*. Samples were boiled in SDS and subjected to SDS-PAGE. Note that the protein band representing purified pili can scarcely be seen in lysates of whole organisms. Note also that when 200 μ g of purified pili (lane C) is subjected to SDS-PAGE, the majority of the protein migrates as a single band, and that minor contaminating materials cannot be detected when 75 μ g is used (lane D).

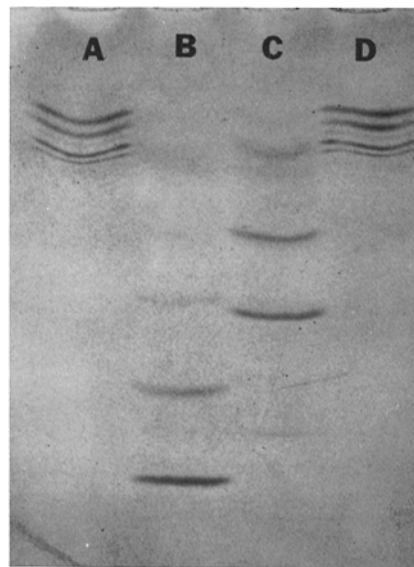


FIG. 3. Isoelectric focussing of pili on a polyacrylamide gel. Lane A and D, beta-lactoglobulin; lane B, pili of R10 Tr; lane C, pili of R10 Op. To determine the pH gradient, an adjacent lane was cut into 5-mm sections, eluted with water and the pH was measured. R10 Tr pili gave rise to two major bands focusing at pH 6.73 and 6.38. Pili from the Op variants also gave rise to two bands which focused at pH 5.89 and 5.40.

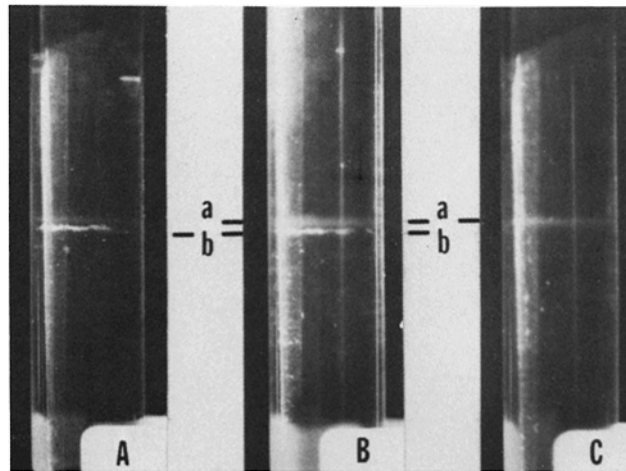


FIG. 4. Isopycnic centrifugation on CsCl gradients of purified pili from opacity variants of strain R10. Tube A, R10 Tr pili; tube C, R10 Op pili; tube B, mixture of R10 Tr and Op pili. The samples were centrifuged at 116,000 g for 68 h at 4°C. Each preparation of pili gave rise primarily to a single band labeled "a" or "b." Centrifugation of the mixture (tube B) yielded a composite pattern. The pili had a density of ~ 1.30 g/cm^3 .

Discussion

When *N. gonorrhoeae* are propagated on a clear medium, a variety of colonial color densities and opacities are seen and these appear to be independent of piliation (12). The relevance of this variability to the pathogenesis of gonorrhea remains unclear, but several interesting findings have emerged when these colonial types have been compared. First, gonococci isolated from infected menstruating females express a Tr phenotype, whereas those bacteria isolated at mid-cycle are mainly of the Op variety (14). Second, the Op colony types are more susceptible to killing by trypsin (12, 14). Third, the outer membranes are not identical in Op and Tr variants derived from a single strain. The Op organisms produce one or more additional protein(s) with apparent molecular weights ranging between 23,500–31,000 which are not found in Tr bacteria (13). Fourth, studies in the chicken embryo model indicate that P+Tr bacteria are more virulent than P+Op organisms of the same strain (16). Because pili are suspected to be a virulence factor in gonococcal infections, it seemed appropriate to purify pili to determine if differences exist between pili obtained from Op and Tr bacteria.

Pili isolated from different strains have been noted to differ chemically (subunit molecular weight, amino acid composition) (11, 10) and immunologically (gel diffusion, enzyme-linked immunosorbent assay [ELISA]) (9, 10). By measuring the apparent molecular weights of pilin derived from different strains, we have confirmed that such inter-strain heterogeneity exists. In addition to such differences between strains, other investigators have implied that there may be more than one pilus type carried by a single strain: Novotny and Turner (27) using morphological criteria found three types of pili in the gonococcus and Robertson et al. (11) noted that pili from a single strain appeared to give rise to two distinct buoyant densities and isoelectric points. These findings were not, however, related by these authors to variability in colony morphology.

We have found that in 10 of 14 strains of gonococci, bacteria from opaque colonies had one pilus type, whereas bacteria from Tr colonies had another. These pili can differ in buoyant density and aggregation behavior in buffers; and their pilins in isoelectric point and mobility on SDS-PAGE. It is not known as yet whether this variability reflects a change in the primary structure or a change in post-translational modification of pilin subunits. There is little doubt that the pilus heterogeneity that we noted was not a result of an inadvertent comparison of bacteria from different strains. Each strain was passed daily from a single colony a minimum of 30 times before the selection of opacity variants, and the pili were isolated from the variants usually within 2 passages and never more than 5 after this separation. In addition the variants always possessed a major outer membrane protein of identical subunit molecular weight (13).

The genetic basis of the phenotypic variation leading to changes in colonial opacity characteristics is not known. Whatever mechanism is proposed, it will need to address the complex nature of the changes observed. Not only does there appear to be a substantial set of distinct Op proteins that can be expressed by different strains of gonococci, but, in addition, a particular strain may elaborate one or more of these, and the concentration found in the outer membrane can be quite variable. The findings reported in this study strongly suggest that the phenotypic expression of pili type is intimately associated with the genetic control of the opacity characteristic. It should be reemphasized that in the chronically infected female, these phenotypic changes occur in association with the menstrual cycle. It is unknown whether some change in the physiology of the reproductive tract induces the phenotypic opacity variation, or whether this change merely reflects a selective advantage of the two forms at different times in the cycle. The drastic changes occurring in the micro-environment of the host at the time of menses may demand a major functional rearrangement of the gonococcal outer membrane. More specifically, if the host's pili receptors are modified, this may necessitate a change in the pilus type expressed if their adhesive function is to be maintained. Whether the observed heterogeneity of pilus expression has an *in vivo* functional basis awaits further characterization of the molecular interaction of Tr and Op pili with host receptors. Thus the observation of the existence of opacity variants seems to lead us to a deeper understanding of the dynamics of the host-parasite relationship.

Summary

We have purified pili from isogenic opacity colony variants that were derived from 14 gonococcal strains. Pili purified from opaque colonies of one strain usually differed from pili purified from transparent colonies of the same strain. In 10 of the 14 strains examined, the apparent subunit molecular weight of pilin isolated from the opaque variants was larger than that seen with pilin obtained from transparent variants. In addition there were demonstrable intra-strain differences in the isoelectric point and buoyant density of pili derived from the opacity variants.

Because gonococci express differing opacity phenotypes during the menstrual cycle, it is possible that the pili of these organisms may also alter *in vivo*.

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