

GONOCOCCAL PILUS SUBUNIT SIZE HETEROGENEITY CORRELATES WITH TRANSITIONS IN COLONY PILIATION PHENOTYPE, NOT WITH CHANGES IN COLONY OPACITY

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Pili are thought to effect or affect attachment of gonococci (Gc)¹ to mucosal cells and thereby mediate or influence important initial interactions between these bacteria and their human host. Previous studies (1, 2) have established structural and antigenic diversity of pili among Gc from different strains. Recently, two groups (3–5) have noted different pilus subunits for Gc variants within individual strains; those differences in pilus subunit size appeared to correlate with differences in the Gc variants' colony opacity/protein II phenotype. In strain P9 variants, four different pilus subunit sizes were found, and each of these pilus types exhibited distinctive adherence avidities in their attachment to various eucaryotic cells in vitro (6). The present study was done to examine the diversity of pilus subunit size forms that exist among intrastain variants of Gc and to reexamine the purported relationship between pilus subunit size and colony opacity/protein II phenotypes.

Materials and Methods

Gc. Gc of strains JS1, JS3, and JS5 (7) as well as R10 and MS11 (3) were examined during this study; most observations were on strain JS3. The colonial piliation phenotype (P⁺, P⁺⁺, or P⁻) was assessed microscopically as described (7); examples of P⁺ and P⁺⁺ are shown in Fig. 1 and will be described later. Protein II/colonial opacity phenotypes were established as noted in an earlier study (8).

Two slightly different media were used during this study. The first was formulated as previously described and contained Thiotone peptone (Baltimore Biological Laboratories, Cockeysville, MD) (7). The second contained meat peptone (40-2304; Baltimore Biological Laboratories) in place of Thiotone peptone, which is no longer available. These media seemed roughly equivalent. Although some strains grew better on one medium than on the other, they all formed colonies for which piliation and opacity phenotypes were readily defined on both media. All organisms were propagated by passage of single selected colonies and were grown for 20–22 h at 36°C in 5% CO₂.

Visualization of Pilus Subunits. Two methods were used for estimating and comparing pilus subunit sizes. In the first, Gc cultures from a single colony passage were swabbed from solid medium if they were acceptably homogeneous (>95% the desired phenotype) and were radioiodinated by the Iodogen method. These radioiodinated Gc were solubilized and their components resolved by sodium dodecyl sulfate-polyacrylamide gel elec-

¹ *Abbreviations used in this paper:* Gc, gonococci; P⁻, nonpiliated; P⁺, piliated; P. I, II, and III, outer membrane proteins I, II, and III; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol.

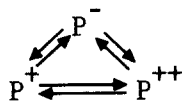
trophoresis (SDS-PAGE) in 15% gels. Autoradiography of the dried slab gel allowed characterization of the protein II constitution of the organisms and permitted visualization of their pilus subunits. The details of iodination, autoradiography, etc., are contained in recent reports from this lab (8, 9).

The second method used single Gc colonies that were selected through a stereo microscope, lifted from the solid medium on a small fragment of filter paper, and immersed in 20 μ l solubilizing solution (containing 4% SDS and 8% 2-mercaptoethanol). After heating in a boiling water bath for 10 min, 10 μ l of each lysate was applied to the slot of an SDS-PAGE slab gel. After electrophoresis, the SDS-PAGE-separated components were electrophoretically transferred to a sheet of nitrocellulose which, after blocking with a solution containing bovine serum albumin, was incubated with rabbit antipilus antiserum diluted 1:500 in the same solution. Details of the immunoblotting procedure are contained in two recent reports (9, 10). The antipilus rabbit serum was obtained from Dr. Gary Schoolnik, Stanford University. It was raised by repeated immunization of a rabbit with pili purified from strain MS11 (transparent colony phenotype) as described by Schoolnik et al. (11).

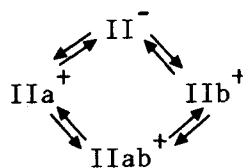
Results

General Comments on Study Design and Results. Previous studies suggested correlations between colony opacity phenotype and pilus subunit size (3, 4), so pilus subunits of the six different protein II/opacity variants in strain JS3 (8) were examined in detail. For each protein II/opacity phenotype (e.g., II⁻, IIa⁺, etc.), two distinct, different small colony forms containing pilated Gc can be found; these are called P⁺ and P⁺⁺ and are shown for strain JS3 opacity variants in Fig. 1. Analogous P⁺ and P⁺⁺ variants have been found in strains JS1, JS5, R10, and MS11. Repeated observations on these strains' opacity and "piliation" variants provided the following conclusions about interrelationships among Gc colonial phenotypes:

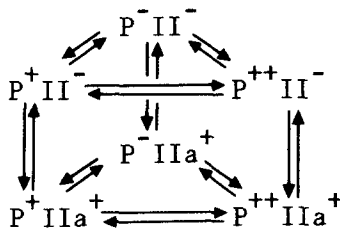
Piliation phenotype transitions



Protein II phenotype transitions



Transitions of both piliation and protein II phenotypes:



These colony phenotype changes were examined to see which, if any, corre-

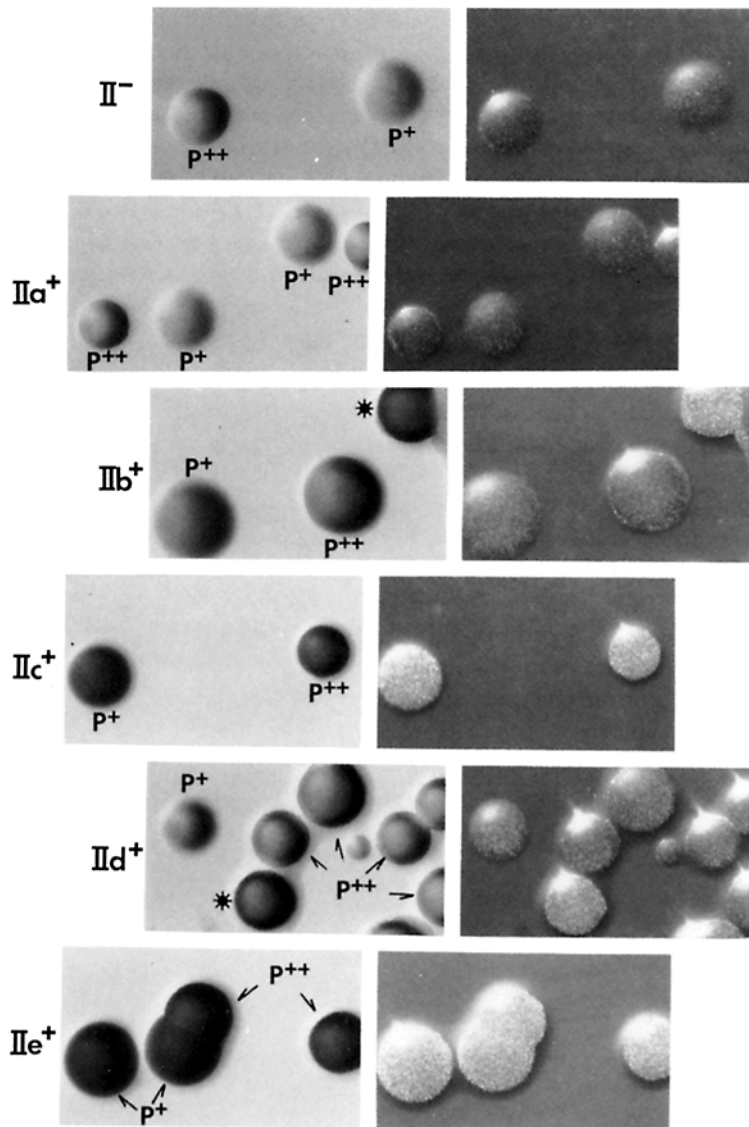


FIGURE 1. P^+ and P^{++} colonial phenotypes in strain JS3 Gc. Six distinct protein II phenotypes are recognized in this strain, as follows: II^- , IIa^+ , IIb^+ , IIc^+ , IIc^+ , IId^+ , and IIe^+ . For each of these protein II phenotypes, a P^{++} colony was selected and passaged; parent-like P^{++} and variant P^+ progeny colonies are seen in these micrographs taken with two lighting arrangements. With a substage diffusing reflector (*left* panel, each pair), colony edges are well-visualized. With a substage polished mirror (*right* panel, each pair), colony opacity is appreciated. Within each protein II phenotype, P^{++} colonies are differentiated from P^+ by virtue of the sharper edges on the former (P^{++}). Little or no difference in colonial opacity is found between II^- and IIa^+ phenotypes. Distinct, enhanced opacity is found for IIb^+ , IIc^+ , IId^+ , and IIe^+ Gc, and each protein II imports a unique degree of opacity as described before (8). The opacity variants (*) shown for IIb^+ and IId^+ phenotypes are $P^{++}IIbx^+$ and $P^{++}IIdx^+$, respectively (IIx = protein II species whose exact identity is not established but whose presence is clear from the enhanced colony opacity; $IIdx^+$ = presence of both IId and IIx). For the protein II/opacity phenotypes associated with marked opacity (such as IIc^+ and IIe^+), P^+ and P^{++} phenotypes may be difficult to distinguish, but their differentiation is possible by noting the P^+ or P^{++} character of II^- variants that arise, since $P^+II^+ \rightarrow P^+II^-$ and $P^{++}II^+ \rightarrow P^{++}II^-$, and $P^{++}II^-$ versus P^+II^- are easily distinguished.

lated with shifts in apparent subunit sizes of Gc pili. In a typical experiment, a single P⁺II⁻ colony was passaged and, from its progeny, Gc with the following colonial phenotypes were selected: P⁺II⁻ (parental phenotype), P⁺⁺II⁻ (change from P⁺ to P⁺⁺), P⁺IIc⁺ (change from II⁻ to IIc⁺), other protein II variants with P⁺ phenotype (such as P⁺II d⁺), and P⁻II⁻ variants. When radioiodination was to be used, the Gc population was expanded by one passage of selected variant colonies and the resultant cultures were used, but the majority of experiments were done with individual parental and variant phenotype colonies whose pili were visualized by immunoblotting. With these general approaches, the parental-type and variant progeny of several hundred Gc colonies of diverse phenotype were examined, and representative findings are presented in Figs. 2-9. The results are described below and show the following: (a) among variants or derivatives of any single strain of Gc, many different pilus subunit sizes are found; (b) change in pilus subunit size accompanies transition in colony piliation phenotype; (c) no change in pilus subunit size accompanies protein II gain or loss (or change in colonial opacity phenotype); (d) many different pilus subunit sizes can occur among "sister" P⁺ colonies arising as variants from a common P⁻ parent colony; and (e) no specific pilus subunit size is characteristic of a particular colony piliation and/or opacity phenotype.

Visualization of Gc Pilus Subunits. Gc pilus subunits are relatively hard to visualize in SDS-PAGE gels either by Coomassie blue staining or by lactoperoxidase-catalyzed radioiodination and autoradiography (J. Swanson, unpublished observations). Pilus subunits can be identified after Iodogen-catalyzed radioiodination of Gc, SDS-PAGE, and autoradiography, as shown in Fig. 2. P⁺ and P⁺⁺ derivatives of each protein II phenotype have bands (18,000-22,000 D range)

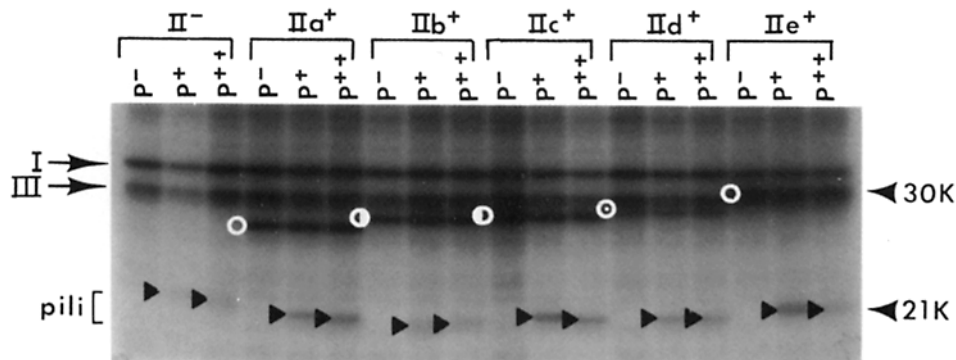


FIGURE 2. Autoradiograph of SDS-PAGE gel of ¹²⁵I-labeled Gc of strain JS3. For each of strain JS3's six P.II phenotypes (II⁻, IIa⁺-IIe⁺), nonpiliated (P⁻) and both piliation forms (P⁺ and P⁺⁺) were radioiodinated, subjected to SDS-PAGE, and visualized by autoradiography. Note that P.II (●, IIa; ○, IIb; ○, IIc; ○, II d; and ○, IIe) have the same apparent molecular weight for P⁻, P⁺, and P⁺⁺ variants of each set. Pilus subunits (▶) are visualized in P⁺ and P⁺⁺ Gc but not in P⁻ preparations. In some P⁺ and P⁺⁺ organisms with the same P.II phenotype (such as P⁺II d⁺ and P⁺⁺II d⁺), pilus subunits seem to have the same size. With other P.II phenotypes (II⁻, IIa⁺, IIb⁺, IIc⁺, and IIe⁺), there are slight differences in the apparent sizes of pilus subunits between P⁺ and P⁺⁺ preparations. The apparent sizes of protein I (I) and protein III (III) subunits are identical for all preparations of this one strain (JS3). (Positions of molecular weight markers in this and subsequent figures are noted as follows: 30 K, carbonic anhydrase; 21 K, soybean trypsin inhibitor; 14.3 K, egg-white lysozyme, which is shown only in Fig. 4-6.)

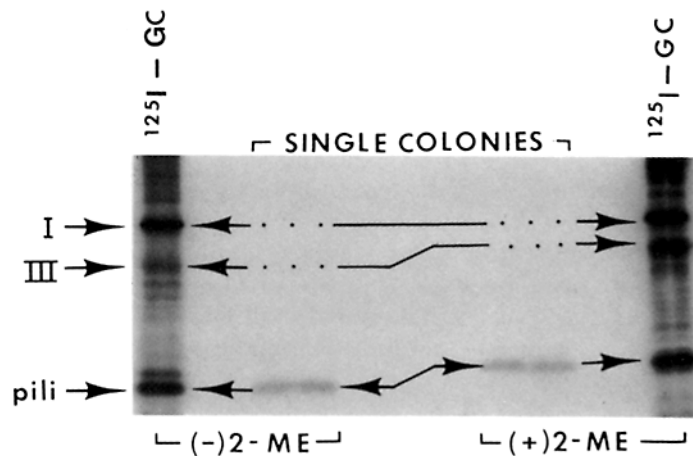


FIGURE 3. Comparison between autoradiography of ^{125}I -labeled Gc and of immunoblotting with antipilus antiserum for visualization of pilus subunits: influence of 2-ME on apparent pilus subunit size. From a single P^+II^- colony's progeny (strain JS3), suspensions of Gc were radioiodinated (^{125}I -Gc) and were solubilized in the presence (+2-ME) and absence (-2-ME) of 2-ME before SDS-PAGE. Single P^+II^- colonies in the progeny population were also selected and subjected to SDS-PAGE followed by immunoblotting with antipilus antiserum. Both protein III (III) and pili exhibit increases in their apparent subunit sizes in the +2-ME versus -2-ME specimens of radioiodinated, autoradiographically visualized organisms and in the immunoblotted single colony preparations; in both kinds of preparations pilus subunits have identical apparent sizes.

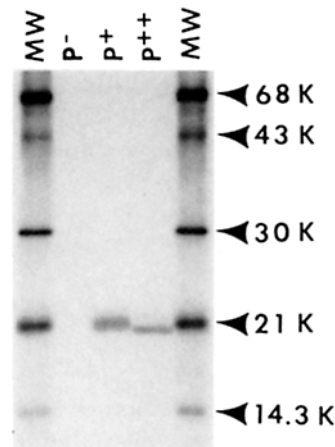


FIGURE 4. Immunoblotting of P^- , P^+ , and P^{++} colonies (strain JS3) with antipilus serum. P^- , P^+ , and P^{++} colonies with protein II^- phenotypes were selected and solubilized as single colonies for SDS-PAGE. With the 1:500 dilution of antipilus rabbit serum used, no bands, except pilus subunits, emit discernible autoradiographic signals. Pilus subunit bands of different apparent size are found in these P^+ versus P^{++} preparations; no pilus subunits are visualized in the P^- Gc.

that are not seen in the P⁻ colony phenotypes. Pilus subunit size differences are found among some of these JS3 variants, but the pilus bands are not very sharp and subtle size differences are probably not distinguishable. Visualization of pilus subunits by immunoblotting with antipilus antiserum plus ¹²⁵I-labeled protein A is more satisfactory, as shown in Fig. 3. These pilus subunits have different electrophoretic mobilities depending on whether 2-mercaptoethanol is present (+2-ME) or absent (-2-ME) during solubilization before SDS-PAGE. Analogous changes in electrophoretic mobilities of pilus subunits in +2-ME versus -2-ME duplicate specimens of individual P⁺ or P⁺⁺ colonies were found for only some strain JS3 variants that were examined (data not shown). Both the +2-ME and -2-ME pilus subunit bands bind antipilus antibodies and give bands with equivalent autoradiographic intensities.

The rabbit antiserum used for all immunoblotting was raised against pili prepared from strain MS11, but it reacted with pili of Gc from strains JS1, JS3, JS5, R10, and MS11. This reaction was not equally intense for all pili in a given strain, as can be seen for strain JS3 in nearly every figure to follow. The antipilus serum was seemingly monospecific for pili at the dilution used (1:500) since it was bound to a visible extent only by pilus subunits, as shown in Fig. 4. At much lower dilution (such as 1:10), reactivities for several outer membrane proteins could be seen with this antiserum and immunoblotting (data not shown). When P⁻, P⁺, and P⁺⁺ Gc are compared, pilus subunits are visualized in P⁺ and P⁺⁺ colonies and are not seen in the P⁻ preparations (Fig. 4). In these preparations, P⁺ organisms have pili of subunit size different from that of P⁺⁺ variants; both P⁺ and P⁺⁺ colonies were selected from among progeny of a single P⁺⁺ colony and both parent and progeny colonies had nonopaque, presumably protein II⁻ phenotypes.

Pilus Subunit Size Heterogeneity Among Intrastrain Variants. Pilus subunit size diversity was regularly seen among colonial phenotype variants of a given strain; examples of the diversity in strain JS3 Gc are shown in Figs. 2, 4-9, and the range of these differences is seen best in Figs. 5 and 6. Technical problems make small differences among pilus subunit sizes difficult to appreciate, and the present methods are not so reproducible as to give a reliable estimate of the total number of subunit size forms that might be found within a single strain. But, even with the crude tools used here, at least a dozen different pilus subunit sizes have been recognized among the variants within strain JS3 as compared by immunoblotting as shown; they ranged from 18,500 to 23,000 D in apparent size.

Lack of Correlation Between Pilus Subunit Size and Colonial Phenotype. No correlation was found between a particular pilus subunit size and a particular colony phenotype, as seen well in Figs. 5 and 6. When multiple P⁺ variants were selected from P⁻ parents of differing protein II phenotypes, several pilus subunit sizes were sometimes found among sister P⁺ colonies of identical phenotype (Fig. 5, lanes 2-5, 6-9, 14-17, 19-21). The same subunit sizes were found for pili among Gc with differing opacity phenotypes (lanes 4, 6, 10-14). This was also demonstrated by selecting identical P⁺⁺II⁻ colonies (Fig. 6, lanes 1-9) from among progeny of parental colonies of several different phenotypes (P⁺⁺IIb⁺, P⁺⁺IIc⁺, P⁺⁺IId⁺, P⁻II⁻, and P⁺II⁻); each parent spawned P⁺⁺II⁻ variants with a pilus subunit size different from those of P⁺⁺II⁻ colonies from other parents.

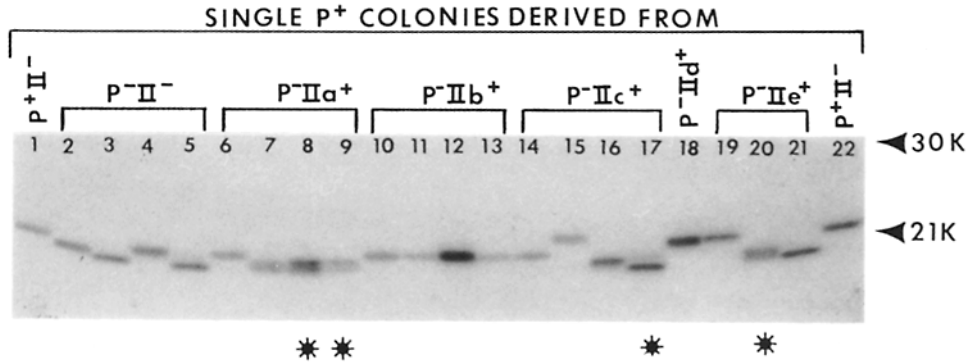


FIGURE 5. Pilus subunit size diversity in P^+ colonies of strain JS3. P^+ colonies with differing P.II phenotypes were selected among progeny of parental colonies whose phenotypes are shown (lanes 2-5 are P^+ progeny of P^-II^- , etc.). Each P^+ colony had the same apparent opacity phenotype (and, presumably, the same P.II constitution) as the P^- parent. All the P^+ colonies of the P^-IIb^+ parent have the same apparent pilus subunit size; but in all other instances, multiple P^+ progeny of each individual P^- colony (P^-II^- , P^-IIa^+ , P^-IIc^+ , P^-IIE^+) differed from one another in pilus subunit size. In several of these single colony preparations (*, lanes 8, 9, 17, and 20), two distinct, different pilus subunits are visualized. Lanes 1 and 22 represent P^+II^- colonies derived from parental colonies of the same P^+II^- phenotype.

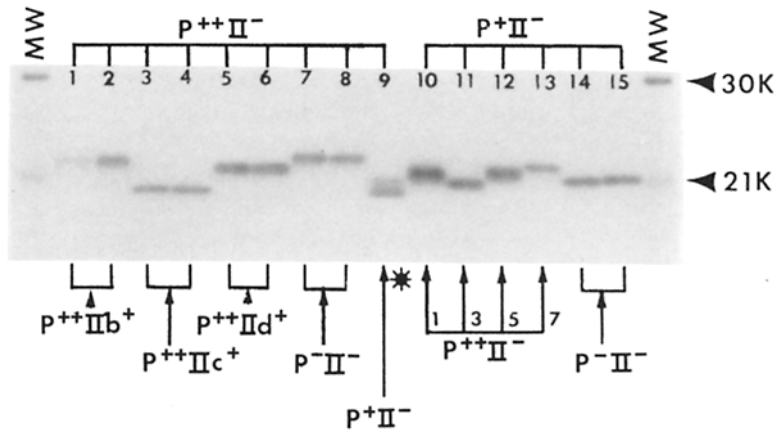


FIGURE 6. Pilus subunit size diversity among strain JS3 Gc of identical colonial phenotypes. This experiment was designed to define whether pilus subunits were the same or different for Gc that have identical colony piliation and opacity phenotypes but are derived from parent colonies each of which has a differing phenotype. $P^{++}II^-$ colonies (lanes 1-9) were selected as variants among the progeny of five colonies of differing phenotypes (shown below figure: $P^{++}IIb^+$, $P^{++}IIc^+$, $P^{++}IId^+$, P^-II^- , and P^+II^-); one or two $P^{++}II^-$ variants were selected from each parent (lanes 1 and 2 from $P^{++}IIb^+$, lane 9 from P^-II^- , etc.). When two variants from the same parent were selected (lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8), both had identical pilus subunit size, but $P^{++}II^-$ colonies from different parents had different pilus subunit sizes. The $P^{++}II^-$ colonies of lanes 1, 3, 5, and 7 were subcultured before being solubilized for SDS-PAGE; in their progeny the next day, variants having P^+II^- phenotypes were selected. These are shown in lanes 10-13; P^+II^- variants from a P^-II^- parent are shown in lanes 14 and 15. Aside from offspring of the same colony (lanes 14 and 15), all these P^+II^- Gc have different pilus subunit sizes except those in lanes 10 and 12, which seem the same.

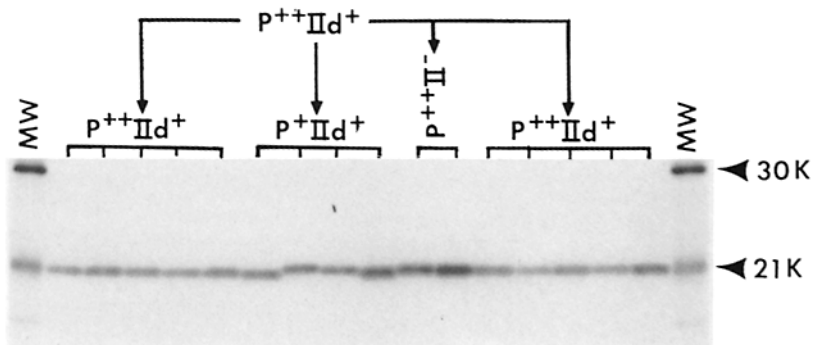


FIGURE 7. Pilus subunit size changes coincident with change in colony piliation phenotype ($P^{++} \rightarrow P^+$) in strain JS3. A single $P^{++}II_d^+$ colony was passaged; the resultant culture contained colonies of parental phenotype ($P^{++}II_d^+$) as well as variants differing either in piliation phenotype ($P^+II_d^+$) or in opacity/protein II phenotype ($P^{++}II^-$). All $P^{++}II_d^+$ colonies have identical pilus subunit sizes; the same subunit size is found in $P^{++}II^-$ variants (after opacity/protein II change). The pilus subunits of $P^+II_d^+$ colonies are slightly different from one another and also from their $P^{++}II_d^+$ sister colonies (parental phenotype); this demonstrates that changes in pilus subunits coincide with $P^{++} \rightarrow P^+$ colony phenotype transitions.

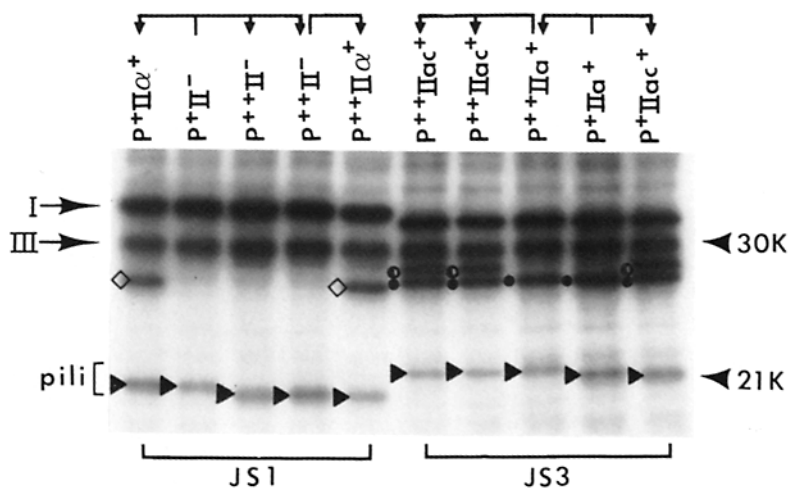


FIGURE 8. Colony opacity and piliation phenotype changes in strains JS1 and JS3 correlated with pilus subunit sizes. Results obtained with two strains examined in similar experiments are presented. A colony (P^+II^- in JS1, $P^+II_{\alpha}^+$ in JS3) was passaged (note arrows above figure) and among its progeny, variant phenotypes were selected. After one passage, the cultures from these variant and parental phenotypes were radioiodinated, subjected to SDS-PAGE, and autoradiographs prepared. For JS1, transition in phenotypes from P^+II^- to $P^+II_{\alpha}^+$ shows no change in pilus subunits; note the acquisition of protein II_{α} (\diamond). Comparison of two different P^+II^- variants and their P^+II^- parents reveals a change (decrease) in pilus subunit size; the subsequent occurrence of a $P^+II_{\alpha}^+$ variant from one of these P^+II^- preparations is not accompanied by a change in pilus subunit size. For strain JS3, the initial colony selected for variant selection was $P^+II_{\alpha}^+$; the variants included $P^{++}II_{\alpha}^+$ with changed piliation phenotype as well as $P^{++}II_{\alpha c}^+$ with changed protein II/opacity phenotype. A slight increase in apparent pilus subunit size is found after change in piliation phenotype, but no change in size is seen with a change in opacity/protein II. Another change in opacity/protein II phenotype is also shown (JS3, $P^+II_{\alpha}^+$ to $P^{++}II_{\alpha c}^+$), and no change in pilus subunit size is present.

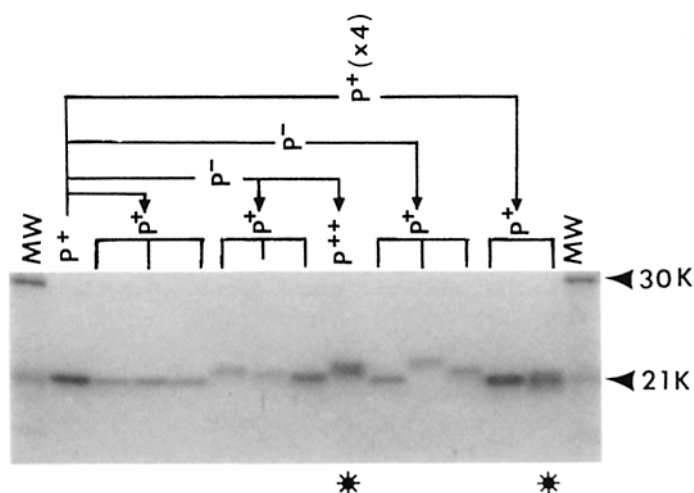


FIGURE 9. Pilus subunits exhibit marked heterogeneity among P^+ progeny of P^- parent colonies (strain JS3). A P^+ colony (this and all others shown are protein II⁻) was chosen and among its progeny both parental-type (P^+) and variant (P^-) colonies were selected. After passage of the P^- colonies once or twice, as shown, P^+ variants and one P^{++} colony were selected. The original parental phenotype was also maintained during four serial passages after which P^+ colonies were again examined. The results seen here include the following: (a) $P^+ \rightarrow P^- \rightarrow P^+$ changes accompany generation of several different pilus subunit sizes, as compared with original P^+ colony; (b) $P^+ \rightarrow P^+$ leads to no change in pilus subunit size; and (c) two colonies (*) have two different size pilus subunits.

Comparable results (also in Fig. 6) were obtained when P^+II^- colonies were derived from five different parent colonies, four of which were $P^{++}II^-$ colonies with seemingly identical colony phenotypes; five different pilus subunits were found in these P^+II^- colonies. These results demonstrate the lack of correlation between Gc pilus subunit size and colony phenotype.

Pilus Subunit Size Changes with Colonial Phenotype Transitions. Transitions in colonial phenotype occur so frequently among most strains of Gc on solid medium that opacity and piliation variants regularly appear among a single colony's progeny in strain JS3. This high frequency permits study of changes in both opacity or piliation phenotype in relationship to pilus subunit size. Numerous observations on strain JS3 (Figs. 6–8), strain JS1 (Fig. 8), and several others (not shown) suggest the following: (a) changes in colonial opacity/protein II phenotype are not accompanied by changes in pilus subunit size (Figs. 7–9), and (b) changes in colonial piliation phenotype are accompanied by changes in pilus subunit size (Figs. 7–9).

The pilus subunit size changes seen coincident with $P^+ \rightleftharpoons P^{++}$ transitions (such as those found in Figs. 7 and 8) are usually small (~ 200 – 500 D differences are typical). Seemingly larger differences in pilus subunit sizes appeared among piliated variants arising as progeny of P^- parent colonies, as previously shown in Fig. 5. This is seen clearly in Fig. 9: in this experiment, the P^+ colonies arising directly from passage of a P^+ parent (one and four times) are compared with P^+ variants arising from a P^- parent colony (derived from original P^+ ancestor colony). P^+ colonies emanating from a P^- parent have a wide variety of pilus

subunit sizes, but P^+ colonies resultant from continual P^+ colony passage are similar or identical in their subunit sizes.

Discussion

Diversity among Gc pili was recognized initially as antigenic heterogeneity of pili from different Gc strains (1). A recent study concluded that "[t]he pili of *all* pathogenic neisseriae (gonococci and meningococci) we have examined to date (about 60) have each had a different (pilus) serotype" (2). The pilus differences among Gc were generally interpreted as being "strain differences"; this view was enlarged by recent demonstrations that several different pilus subunit forms (differing in apparent size by SDS-PAGE, in density, and in isoelectric point) appear among variants in individual strains of Gc (3–5). More recent studies (4, 6) show that not only do the intrastrain variant pili differ in these physical characteristics, but they also exhibit variations in their attachment avidities when incubated with several different kinds of eucaryotic cells.

The present study attempted definition of the extent of pilus subunit size diversity among variants of strain JS3 Gc. Within this strain (and it seems to be typical by virtue of observations on several other, unrelated strains), at least 12 different sizes of pilus subunit were found, but it is clear that this estimate is conservative; the SDS-PAGE method used is relatively insensitive for discriminating subtle differences in pilus subunit sizes, and our initial results with isoelectric focusing provide evidence of additional diversity among pilus subunits of the same apparent sizes (J. Swanson and O. Barrera, unpublished observations).

Changes in pilus subunit size occur coincident with colony "piliation" phenotype changes ($P^+ \rightleftharpoons P^{++}$). Conversely, pilus subunit size usually remains the same in progeny versus parent colonies if there is no change in piliation phenotype, whether or not there is a change in colony opacity/protein II phenotype. Marked diversity in pilus subunit sizes was observed when multiple piliated variants arose from a single P^- parental colony. In one instance, four such P^+ variants each displayed a different pilus subunit size; but in other instances, multiple P^+ variants from a common parental P^- colony all had apparently identical pilus subunits. These differing results may depend on whether the piliated variants arose in the P^- parental colony as daughters of a single P^+ variant before passaging the P^- colony, or whether the P^+ variants arose independently among the separated, already passaged progeny of the P^- colony.

The pilus subunit size diversity that occurred among piliated variants of P^- parent colonies seemed greater than subunit differences found with $P^+ \rightleftharpoons P^{++}$ transitions. As shown in Fig. 5, a large collection of different pilus subunit sizes can be obtained by selecting piliated progeny from P^- colonies that have different opacity/protein II phenotypes, but the observed diversity is not directly related to colony opacity phenotype differences, per se. The seemingly high degree of diversity among pili subunits for P^+ or P^{++} progeny of a P^- colony is curious. Are P^- Gc involved in the pathogenic process whereby Gc establish their initial (or later) contacts with host cells? It might be advantageous if P^- variants were spawned in vivo and if subsequent P^+ (or P^{++}) variant progeny exhibited a broad variety of different pilus forms; this variety in pili might be useful to Gc if only one or a few kinds of pili possess properties that promote Gc adherence to a

certain anatomical niche's cell type, etc.

Antiserum from one rabbit inoculated with strain MS11 pili was used throughout this study; by immunoblotting, this antiserum reacted with at least one band in the 18,000–23,000 D range for all the P⁺ and P⁺⁺ colonies examined. This suggests that the antiserum recognized an antigen common to all Gc pili, but one cannot exclude the possibility that more than a single pilus subunit form was contained in the purified pili used for immunization. Not all the pilus subunit bands gave equivalent intensities by immunoblotting, as can be seen especially well in Fig. 5. The differences in intensities of immunoblotting signals could result from variation either in the number of pili on individual Gc or in the antigenic reactivities of different subunit forms of pili from one variant to the next. In several instances more than one pilus subunit size is found among Gc of individual colonies. It seems most likely that this reflects the level of detection for Gc pilus subunits by immunoblotting (pili on $\sim 1 \times 10^8$ Gc can be visualized by this method; data not shown) plus the high frequency at which variants occur (as judged by the prevalence of variants after passage of single colonies). But we cannot exclude the possibilities that individual pili may be composed of a mixture of different pilus subunit sizes or that different pili, each with a distinct subunit, coexist on individual Gc.

At present, it isn't clear how large a repertoire of different pilus subunit forms a given Gc strain possesses. Nor is it clear how the observed differences in subunit size relate to pilus subunits' chemical structures. Small amounts of both phosphate and polysaccharide are usually found in "purified" pilus preparations (19, 20); if either is an integral part of the pilin subunit molecule, posttranslational modification (by phosphorylation, etc.) might account for the different migration characteristics of different pili. But at this point, we assume that different subunit sizes relate to differing primary amino acid sequences of Gc pili as reflected in others' demonstrations (5) of amino acid composition differences for pili from variants of a given Gc strain. Schoolnik et al. (11) have recently proposed a model for pilin in which each pilin subunit species is composed of an N-terminal constant region and a variable, C-terminal portion. The variations in apparent pilus subunit size may correlate with sequence variability in this C-terminal of pilin molecules.

The frequency of variation in pilus subunit size exceeds that for other Gc surface components that have been studied. Outer membrane protein III (P.III) is present and apparently identical on all Gc examined (9, 14). The monoclonal antibody that recognizes the 2-ME-modifiable P.III of all Gc also binds to 2-ME-modifiable proteins of other neisseriae (J. Swanson, unpublished observations). Lipopolysaccharide (15, 16) and outer membrane protein I (17, 18) each occur in several structural variations among different Gc strains, and changes in each of these constituents probably proceed at a very low frequency compatible with classic mutations. But, for neither lipopolysaccharide nor outer membrane protein I has the extent of diversity or the number of different forms of P.I been established. Changes in outer membrane protein II composition occur at high frequency, and multiple subunit size and antigenic forms of P.II occur within individual strains (7). But pilus subunit size seems to be even more plastic than transitions in Gc's P.II constitution. Although colony to colony and day to day

variations were seen in frequencies of $P^+ \rightleftharpoons P^{++}$ transitions even with a single strain and the same medium, piliation phenotype transitions occur with frequencies roughly in the range of 0.1–5.0% among progeny of single colonies.

The results described here are in apparent conflict with the findings of others. Specifically, our results show there is no correlation between colony opacity/protein II phenotype and pilus subunit size; such correlation was suggested in studies from two groups (3, 4). We suspect that those studies used Gc that were not related as closely in passage history as those used here. As shown in our studies, Gc of any given piliation plus opacity colonial phenotype may have either the same or different pilus subunit size as compared with Gc of any different colonial phenotype in the same strain. The results of Salit et al. (3) also suggest this kind of relationship, in that 9 of the 14 strains they examined showed different pilus subunit sizes between opaque and transparent colony forms, whereas the other 5 strains' opaque versus transparent variants had pilus subunits of the same size.

The lack of correlation between pilus subunit size and colony opacity phenotype also suggests there is no close genetic linkage between pilus subunit size and protein II expression as was suggested in a recent article by Meyer et al. (21). They found differences between P^+ organisms differing in their opacity phenotypes, between P^- Gc of different opacity types, and between P^+ versus P^- Gc of the same opacity phenotype, by Southern hybridization with a plasmid containing molecularly cloned Gc pilus genes. Those differences in hybridization patterns suggest Gc chromosomal DNA rearrangements coincident with a change from " P^+Op " (IIx^+) to " P^+Tr " (II^-), which is interpreted as suggesting that "the switch from the Tr to Op state may involve the organization of the pilus gene as well." That interpretation is not supported by our finding that there is no change in pilus subunit size coincident with either gain or loss of protein II change in colony opacity phenotype. These observations argue against any close genetic linkage between expression of a particular pilus form and of protein II.

Summary

The apparent subunit sizes for pili of gonococci (Gc) have been visualized by using either Iodogen ^{125}I -labeled whole Gc or immunoblotting with antipilus antiserum. These methods permitted definition of pilus subunit sizes for Gc of a given strain that had undergone changes either in piliation phenotype or in colonial opacity/protein II phenotype. The results indicate that pilus subunit size does not change coincident with changes in colony opacity/protein II phenotypes; but change in pilus subunit size is seen after a change in piliation phenotype ($P^+ \rightarrow P^{++}$, and vice versa). Marked diversity in pilus subunit sizes is found for Gc of individual strains when P^+ derivatives of P^- colonies are compared. This diversity extends to pilus subunits of Gc found in single colonies; two distinct pilus forms were demonstrated for Gc residing in several single colonies. These findings show that Gc of a given strain are able to express any of a number of different pilus subunit size forms.

Note added in proof: A recent article reports variation in subunit size of pili on Gc of the same strain isolated from different anatomical sites (cervix versus

urethra) of an individual female; differences in pilus subunits also were found for Gc of the same strain isolated from male versus female sexual partners (Duckworth, M., D. Jackson, K. Zak, and J. E. Heckels. 1983. Structural variations in pili expressed during gonococcal infection. *J. Gen. Microbiol.* 129:1593). Those findings demonstrate that pilus subunit size can differ among variants in a single Gc strain in vivo as well as in vitro.

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