PILUS⁻ GONOCOCCAL VARIANTS

Evidence for Multiple Forms of Piliation Control

By JOHN SWANSON,* SVEN BERGSTRÖM,* OSMAR BARRERA,*
KEN ROBBINS,* AND DAN CORWIN‡

*From the National Institutes of Health, National Institute of Allergy and Infectious Diseases,
*Laboratory of Microbial Structure and Function, and ‡Laboratory of Pathobiology, Rocky
Mountain Laboratories, Hamilton, Montana 59840*

Pili (or fimbriae) occur on a wide variety of gram-negative bacteria (1) and constitute a set of surface appendages with a broad spectrum of functions, biological properties, amino acid sequences, etc. (2). Enterobacteria can simultaneously display multiple pilus forms, such as type 1, F, and K88 (3). In enteropathogenic *Escherichia coli*, K88, K99, CFA I, and CFA II pili (or fimbriae) are thought to affix or anchor organisms to particular gastrointestinal mucosal sites in host-specific fashion (4). *E. coli* Pap pili, which seem involved in adherence of pyelonephritogenic *E. coli* to renal epithelial cells, appear to be pilin subunit polymers that do not have inherent adhesive properties and require an additional gene product for expression of adherence to epithelial cells (5). Adherence of gonococci (Gc)[1] to epithelial cells is greatly enhanced if the organisms are pilus⁺ (6); and enhanced adherence is thought to correlate with and explain the apparent increased virulence of colony types 1 and 2 (pilus⁺) Gc in human male volunteers (7, 8).

Gc express pili in vivo, as seen from the following data: antipilus antibodies appear in sera of patients in response to gonorrheal infections (9); electron microscopy has visualized pili on Gc in clinical specimens (10); and the majority of Gc in clinical cultures have colony morphotypes (type 1 or type 2, equivalent to P⁺ or P⁺⁺, respectively) typical for pilus⁺ gonococci (11). Pilus⁺ phenotype is readily maintained for Gc selectively cultivated in vitro, but pilus⁻ variants (P⁻ morphotype) occur frequently among the P⁺ (or P⁺⁺) colonies that result from passage of a single P⁺ (or P⁺⁺) parent colony. The frequencies at which pilus⁻ variants are spawned by pilus⁺ Gc vary widely among different strains on different batches of growth medium on different days (J. Swanson, unpublished observation).

Little information is available regarding "reversion" of pilus⁻ Gc to pilus⁺ phenotype. An older study (12) found that some pilus⁻ (colony type 4) Gc spawned pilus⁺ variants and others did not, but, in that study, the reverting and

nonreverting phenotypes were from different strains. No information exists regarding "reversion variants" within a single strain.

A recent study (13) used cloned DNA specifying pilin to probe P^+ and P^- Gc genomic DNA by blot hybridization. The authors concluded that genomic rearrangement of DNA containing pilus subunit (pilin) structural genes was related, perhaps causally, to change in the piliation status of Gc. The same study also found rearrangement of DNA containing pilin structural genes when Gc were compared with similar piliation but different colonial opacity/protein II phenotypes. A more recent study (14) by the same group demonstrated deletions in pilin structural genes coincident with rearrangements that accompany pilus$^+$ to pilus$^-$ transitions; with the Gc used, reversion (pilus$^-$ to pilus$^+$) was accompanied by reconstitution of pilin structural genes. Those studies (13, 14) used strain MS11 Gc, which contain two apparently complete, identical pilin structural genes. In contrast, our previous report (15) demonstrated that some, but not all, pilus$^+$ to pilus$^-$ transitions were accompanied by changes in hybridization patterns of ClaI-digested Gc DNA when probed with recombinant plasmid that contained pilin-encoding DNA. We used several strains of Gc, including strain MS11, but our MS11 Gc as well as the other strains studied contained only one copy of the pilin structural gene. We question the proposed causal relationship between pilin structural gene rearrangement and pilus$^+$ to pilus$^-$ (or reverse) phenotype changes of Gc.

In the present study, several pilus$^-$ variants were isolated and examined regarding their (a) scanning electron microscopic appearance, to document their pilus$^-$ state, (b) reversion to pilus$^+$ phenotype, (c) synthesis of pilin subunits (in the absence of complete pilus assembly), (d) synthesis of pilin-specific mRNA, and (e) rearrangement in genomic DNA that contain pilin structural genes. By these measures, three phenotypes were identified among Gc that lack pili. The occurrence and characteristics of these different phenotypes for pilus$^-$ Gc strongly suggest that three distinct mechanisms can "turn-off" Gc pilus production. One mechanism involves pilin gene rearrangement and the resulting pilus$^-$ Gc are "nonreverting" (to pilus$^+$ phenotype). The other two mechanisms evidently operate at posttranscriptional levels and do not involve discernible rearrangement of pilin genes; the resultant pilus$^-$ Gc show high frequency reversion to pilus$^+$ phenotype.

Materials and Methods

Bacteria and Their Cultivation. All Gc were propagated on clear solid medium at 36°C in 5% CO_2 (16). All passages used careful, microscopic selection of single colonies with desired morphotype after 18–20 h growth. In the main, Gc were maintained as P^+ or P^{++} morphotypes with transparent O^- (nonopaque) phenotype through daily passage. Several different strains as well as different "copies" of the same strain (MS11) were examined: strain JS3 (from J. Swanson's lab); strains UU1220 and strain JL43 from Dr. Zell McGee, University of Utah; strain MS11 copies included MS11$_{ms}$ from Dr. Magdalene So, Scripps Clinic and Research Foundation, La Jolla, CA; MS11$_{mk}$ from Dr. Michael Koomey, The Rockefeller University; and MS11$_{zm}$ from Dr. McGee. MS11$_{mk}$ and MS11$_{zm}$ both came from stocks supplied by Dr. Gary Schoolnik, Stanford University, who serially passaged them from the collection of Dr. Emil Gotschlich, The Rockefeller University; Dr. So also received her MS11$_{ms}$ from the Gotschlich collection, where the organisms were retained after their initial isolation at Mount Sinai School of Medicine, New York in 1970.

Recombinant Gc Pilin–encoding Plasmids and Synthetic Oligonucleotides. The recombinant plasmid pVD200, containing a pilin-encoding 4.1 kb ClaI insert of strain MS11$_{mk}$ in pBR325, was originally constructed by Dr. Mike Koomey as described previously (15), as was pVD203, which contains a 1.4 kb SmaI fragment subcloned into pUC8; both pVD200 and pVD203 direct pilin synthesis in *E. coli.* The 1.4 kb insert of pVD203 was isolated by complete XmaI digestion of pVD203, agarose gel electrophoresis, and electroelution. Oligonucleotides corresponding to desired portions of the Gc pilin structural gene were designed according to the gene's DNA sequence published by Meyer et al. (18) (see legend to Fig. 5) and were synthesized on a SAM 1 automated synthesis instrument (Biosearch, Inc., San Rafael, CA). The oligonucleotides were isolated from polyacrylamide gels and were ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) as follows: 10 μM oligonucleotide plus 150 μCi [^{32}P]ATP were added to a reaction mixture containing 5 mM dithiothreitol, 10 mM MgCl$_2$, and 50 mM Tris HCl, pH 7.4. After the addition of 20 U of polynucleotide kinase, the reaction was incubated at 37°C for 30 min, an additional 10 U of kinase was added, and incubation was continued for another 30 min.

DNA Preparation. Individual colonies of desired morphotype were lifted from the solid medium, immersed in 100 μl water, and agitated by vortex mixing. 30-μl portions were applied and spread on each of three plates of medium. After incubation for 18–20 h, the resultant Gc populations were examined microscopically, and, if they were sufficiently homogeneous, the organisms were removed with swabs and suspended in 1.5 ml STE (0.1 M NaCl, 10 mM Tris, pH 7.8, 1 mM EDTA). After centrifugation, the Gc were resuspended in 1.5 ml STE and were either frozen (−70°C) or processed immediately. Briefly, 0.25–0.5 ml portions of the suspension were diluted to 0.5 ml vol, incubated with sodium dodecyl sulfate (SDS) (1% final concentration) and proteinase K (10 μg/ml final concentration) at 68°C for 90 min, cooled in an ice bath, and extracted with an equal volume (500 μl) cold phenol. Phenol extraction, centrifugation (10,000 g, 10 min, microfuge model 11; Beckman Instruments, Inc., Fullerton, CA), and removal of the top, aqueous phase was followed by repetition of the phenol extraction and then two extractions with chloroform containing isobutanol (24:1). DNA was precipitated with ethanol and, after drying and suspension in TE (10 mM Tris, pH 7.8, 1 mM EDTA), digestion was carried out with RNase at 37°C for 1 h. Ethanol precipitation was repeated, as was washing with 70% ethanol and drying in vacuum. The final DNA-containing residue was dissolved in TE and its quantity estimated by agarose gel electrophoresis and ethidium bromide staining. Appropriate aliquots were incubated with ClaI (New England Biolabs) according to the manufacturer's recommendations. The digested DNAs were resolved by agarose gel (0.7–0.9%) electrophoresis in Tris borate (17).

Blot Hybridization (17). Capillary transfer of DNA from agarose gel to nitrocellulose was with 20× SSPE (1× SSPE: 130 mM NaCl, 10 mM NaH$_2$PO$_4$, pH 7.4, 1 mM EDTA) after which the nitrocellulose was baked at 80°C for 2 h in a vacuum oven. Blots to be probed with recombinant plasmid or insert DNA were prehybridized in a mixture containing boiled, sonicated calf thymus DNA (0.1 mg/ml), 0.1% SDS, 1 mM EDTA, 5× Denhardt's solution, and 5× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate) at 37°C for 2–4 h. Hybridization was for 14–16 h at 68°C in the same mixture without calf thymus DNA but with ^{32}P probes labeled by nick translation (Nick Translation Kit; New England Nuclear, Boston, MA). Washing was at 68°C with 1× SCC (30 min) and 0.1× SSC (1 h), each containing 0.1% SDS and 1 mM EDTA. Blots probed with synthetic oligonucleotides were prehybridized at 37°C in a mixture containing herring sperm DNA (0.1 mg/ml), 0.05% SDS, 5× SSC, 0.1 mM EDTA, and 1× Denhardt's. Hybridization with ^{32}P-labeled oligonucleotide probes was at 40°C; washing in 2× SSC containing 0.05% SDS and 1 mM EDTA was at 40°C for 15 min. The synthetic oligonucleotides were ^{32}P labeled with T4 polynucleotide kinase as described above.

RNA Preparation and Northern Blotting (17). Whole Gc were suspended in cold (4°C) guanidine isothiocyanate lysis buffer and total RNA was extracted with hot (65°C) acidic (pH 5.0) phenol. After extraction and ethanol precipitation, the RNA were subjected to electrophoresis in 1.8% agarose containing 20% formaldehyde. This gel was used for

capillary transfer of RNAs to nitrocellulose under conditions identical to those described above for DNA. All glassware used for RNA preparations was baked at 250°C for 24 h and all plasticware was autoclaved. Solutions, except Tris-HCl buffers, were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. Blot hybridization with ^{32}P-labeled DNA derived from pVD203 or with ^{32}P-labeled synthetic oligonucleotides was as described above for genomic DNAs.

Immunoblotting. The presence or absence of pilin was determined by subjecting individual Gc colonies to SDS–polyacrylamide gel electrophoresis (PAGE), electrotransfer to nitrocellulose, incubation with either polyclonal rabbit antipilus serum (courtesy of Dr. Gary Schoolnik, Stanford University) or a monoclonal antibody, MC02, and visualization of pilin antigen–bound antibodies through reaction with ^{125}I–protein A followed by autoradiography. The exact method has been described in detail (16). The MC02 antipilus monoclonal antibody was raised by immunizing BALB/c mice with whole, purified pili from strain MS11 Gc and fusing their spleen cells (harvested 3 d after a "boost" with pili 30 d after primary immunization) with NS1 myeloma cells as described in detail (19). This MC02 monoclonal reacts by immunoblotting with pili from a wide variety of strains and intrastrain colonial variants.

Scanning Electron Microscopy (SEM). For electron microscopy, Gc from a single, selected colony were diluted and spread on a plate of solid medium so that ~200–500 colonies were present after overnight incubation. The population of progeny colonies was carefully scanned with a dissecting microscope so any unwanted variant forms could be removed on filter paper fragments. Several pieces of agar (~3 × 5 mm) containing several colonies were excised, removed from the petri plate, and placed in a small glass dish. Fixation and washing fluids were carefully added to the dish such that the colony-bearing top surface of the agar was not immersed in the liquid. Fixation and washing were accomplished using the following solutions and times: 3% glutaraldehyde in 0.1 M sodium cacodylate (60 min), 0.1% sodium cacodylate (5 min), 2% osmium tetroxide in cacodylate (45 min), sodium cacodylate (5 min), and water (5 min). Afterwards, graded ethanol or acetone solutions (15, 30, 50, 70, 90, 100%) were sequentially added, each for 15 min. The specimens were placed in the critical point drying apparatus in absolute ethanol or acetone for 2–16 h, after which they were subjected to critical point drying and applied to SEM stubs with conductive silver paint. Gold-palladium coating was performed in a Hummer X sputter coater, and the specimens were viewed with a scanning microscope (JSM-35CF; JEOL USA, Peabody, MA), as described elsewhere in detail (20).

Results

Selection of Pilus⁻ Variants. We noted earlier (15) that some P⁻ Gc exhibit blot hybridization patterns (Cla1-digested genomic DNA probed with pilin gene–containing Gc DNA) identical to their pilus⁺ parents, whereas other P⁻ Gc DNAs are different; the differences likely reflect DNA "rearrangement" of pilin-encoding DNA. It seemed possible that P⁻ Gc containing rearranged pilin structural gene DNA might exhibit lower frequencies of reversion to pilus⁺ phenotype than P⁻ Gc whose genomes underwent no pilin gene rearrangement coincident to their loss of pilus⁺ phenotype; if the frequencies of rearranged vs. nonrearranged pilus⁻ Gc were grossly different, they should be distinguishable by microscopic exam. Therefore, six P⁻ colonies that occurred as variant progeny of two sibling P⁺⁺ colonies were selected and serially passaged; at each passage, the occurrence/absence of P⁺ or P⁺⁺ "revertants" among the progeny of the six P⁻ preparations was microscopically assessed. After three successive single P⁻ colony passages, each of the preparations was designated as pilus⁻ reverting (P⁻r) or pilus⁻ nonreverting (P⁻n). Strains MS11$_{mk}$ and JS3 were initially used for selection of these P⁻r and P⁻n phenotypes, which have been stable for 6 mo of
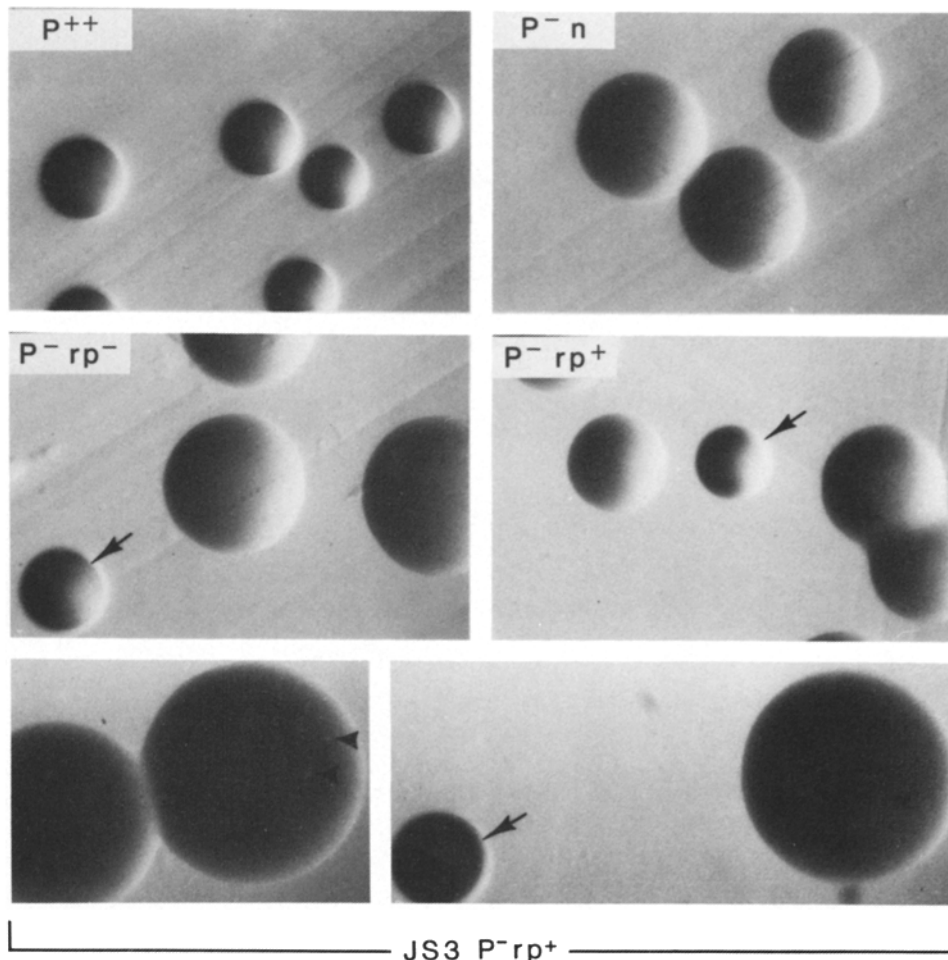
JS3 P⁻rp⁺

FIGURE 1. Gc colonies viewed with a dissecting microscope reveal characteristic small diameters and sharp edges of P^{++} colonies that contrast with the larger diameters and rather indistinct edges of P^- morphotypes. Except for the presence or absence of pilus⁺ revertants (arrows), the colonies of P^-n, P^-rp^-, and P^-rp^+ (see text and Table II for definitions) are practically identical for the transparent (O⁻) colonies of $MS11_{mk}$ shown in the top two rows; the foci described in the text for P^-r colonies are poorly resolved. The bottom row of photomicrographs depicts strain JS3 P^-rp^+, opaque (O⁺) phenotype; a P⁺ revertant is noted (arrow). On the large, moderately opaque P^-rp^+ colonies, small round foci (arrow heads) of lighter color are visible.

daily serial passage; both P^-r and P^-n Gc have been found in three additional strains or strain copies ($MS11_{zm}$, JL43, $MS11_{ms}$; data not shown). Our microscopic method of detecting pilus⁺ revertants is quite crude, and reverting vs. nonreverting characteristics may simply reflect easily distinguished differences in reversion frequencies of these Gc.

Reversion to pilus⁺ phenotype at easily detectable frequencies endows P^-r Gc with colonial morphology subtly different from that for P^-n Gc; P^-r colonies usually have small round foci on their surfaces that are not found on P^-n colonies. Examples of these foci on P^-r colonies are shown in Fig. 1. By light

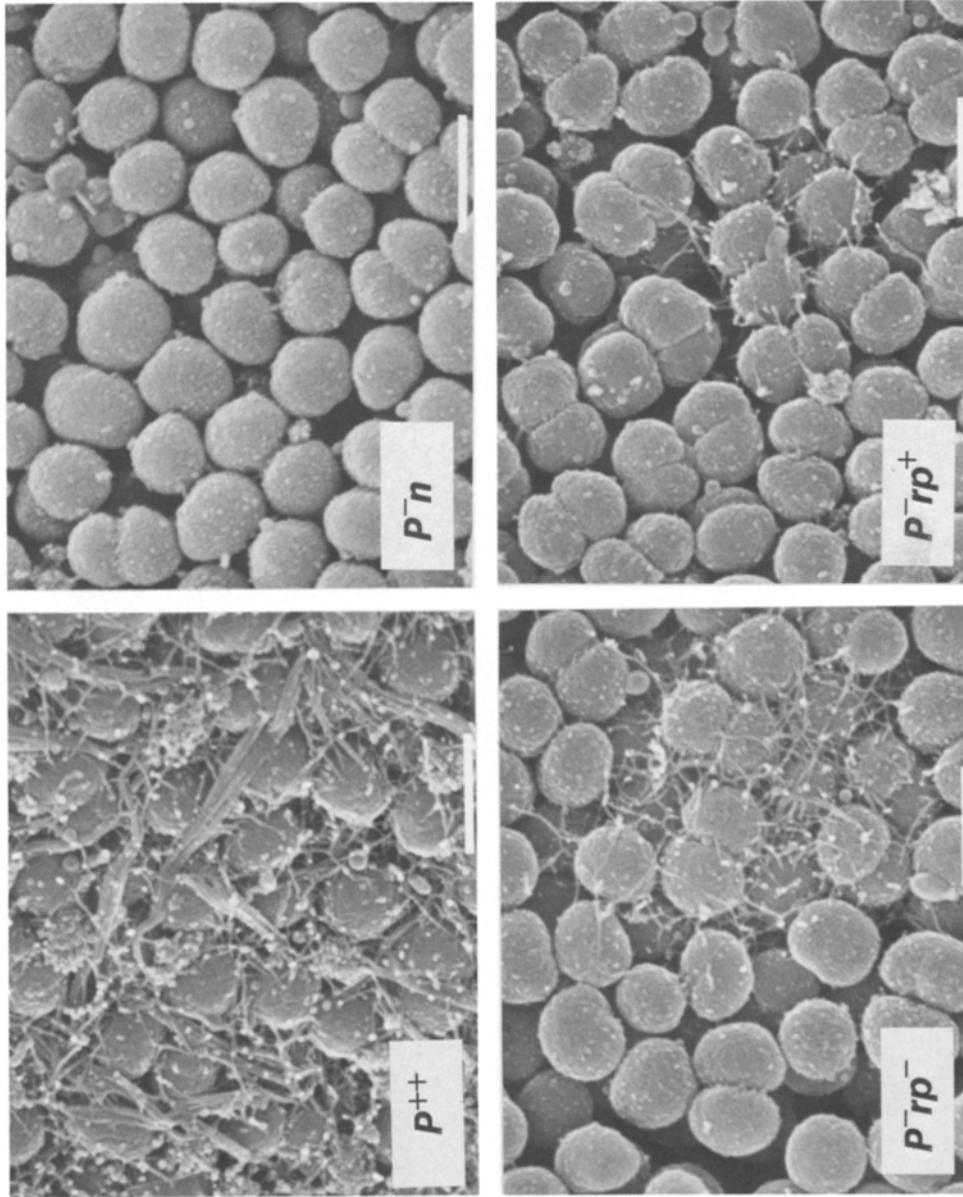FIGURE 2. By SEM, Gc on P⁺⁺ colonies are heavily piliated, with both individual pilus filaments and large bundles of aggregated pili visible. Gc on P⁻n colonies exhibit no pili. In both P⁻rp⁻ and P⁻rp⁺ phenotypes, foci of piliated Gc are found among the majority, pilus⁻ inhabitants of the respective colonies; these represent the foci defined by light microscopy in Fig. 1.
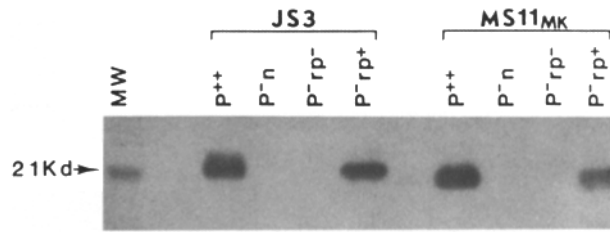
FIGURE 3.   Immunoblotting with antipilus serum reveals M_r 21,000 pilin bands for P^{++} and P^-rp^+ phenotypes but not for either P^-n or P^-rp^- Gc of strains MS11$_{mk}$ and JS3. Note the additional, immunoreactive M_r 16,000 band in the P^-rp^+ Gc of strain MS11$_{mk}$.

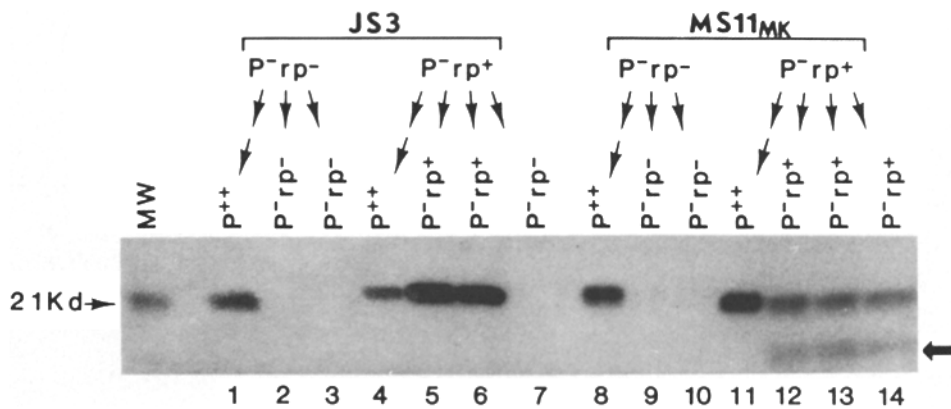

FIGURE 4.   Immunoblotting with a monoclonal antipilus serum to define pilin subunits in P^-rp^- and P^-rp^+ Gc of strains JS3 and MS11$_{mk}$ and their derivatives. One colony of each phenotype in each strain was used for selection of parental phenotype as well as pilus$^+$ revertants (P^{++} morphotype). Note the identity in apparent sizes for pilin subunits strain JS3 P^{++} revertant vs. P^-rp^+ Gc (lanes *4* vs. *5, 6*); note also the transition from P^-rp^+ to P^-rp^- in lane *7*. In strain MS11$_{mk}$, an additional M_r 16,000 band (arrow) is present for P^-rp^+ Gc (lanes *12–14*) and is not present in a P^{++} revertant (lane *11*). This immunoblotting autoradiograph was scanned for semiquantification of pilin signals as shown in Table 1.

microscopy, the foci are best visualized on colonies having modest degrees of opacity. By SEM (Fig. 2) the foci are clearly seen to consist of pilus$^+$ Gc surrounded by the majority occupants of the colony, pilus$^-$ Gc, clearly an expression of their reversion capacities. Similar foci were not present on P$^-$n colonies in strains MS11$_{ms}$ and JS3.

P$^-$rp$^+$ vs. P$^-$rp$^-$ Phenotypes.   Immunoblotting with antipilus serum is sufficiently sensitive to provide clear autoradiographic pilin band signals from individual pilus$^+$ Gc colonies (Figs. 3 and 4). On examining P$^-$ colonies by the same technique, we detected no pilin band signals for P$^-$n phenotypes. Nor were pilin signals visible in several preparations of P$^-$r phenotype; these Gc appear to be pilin$^-$ and are designated P$^-$rp$^-$. Immunoblots of other P$^-$r preparations yielded pilin band signals with identical electrophoretic migration properties and roughly equal autoradiographic intensities as the pilin bands of pilus$^+$ Gc; data presented later suggest that these Gc produce amounts of pilin per bacterial cell equivalent to those of pilus$^+$ Gc. They are denoted P$^-$rp$^+$ since they synthesize pilin but are nonpiliated by SEM (see below). Pilin sizes for P$^-$rp$^+$ Gc resemble those for both
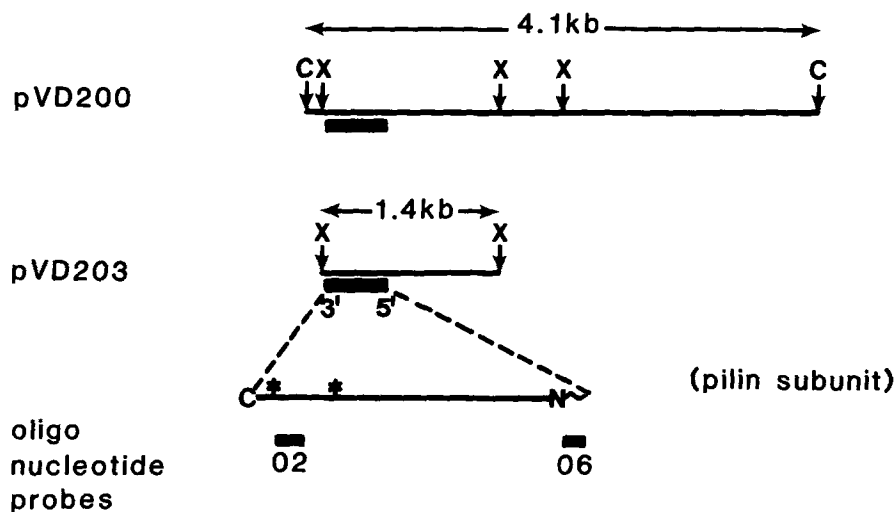
FIGURE 5. Recombinant pilin-encoding plasmids and oligonucleotide probes used for blot hybridizations are depicted. The original cloned DNA is represented by pVD200, in which a 4.1 kb ClaI fragment of Gc DNA was cloned into pBR325; pVD203 is a recombinant subclone whose pilin-encoding, 1.4 kb XmaI fragment is inserted into pUC8. The pilin-encoding portions of these recombinant plasmids are shown by solid boxes. A diagram of the pilin subunit is included to show the pilin-encoding gene regions represented by oligonucleotides O2 and O6 in relation to C- and N-termini and the cysteine residues (*) of the pilin subunit. These oligonucleotide probes have sequences of the complementary, noncoding DNA strand as follows: O2, (3′)TTTCTTTAGCTGTGGTTCGTGGA(5′); O6, (3′)TACTTATGG-GAAGTTTTTCCG(5′).

their pilus⁺ progenitors and their pilus⁺ revertant progeny; in strain MS11, this is ~21,000 M_r. P⁻rp⁺ Gc in both MS11$_{ms}$ and MS11$_{mk}$ exhibited an additional, somewhat smaller (M_r 16,000) band that is clearly immunoreactive with both polyclonal and monoclonal antipilus sera (Fig. 4).

SEM of P⁺⁺, P⁻n, P⁻rp⁻, and P⁻rp⁺ Colonies.   Representative scanning electron micrographs of P⁺⁺, P⁻n, P⁻rp⁻, and P⁻rp⁺ colonies from MS11$_{mk}$ are shown in Fig. 2. In the P⁺⁺ colonies, pili are easily seen as individual filaments and large bundles of laterally aggregated filaments. No pili are observed on Gc comprising P⁻n colonies. In both P⁻r phenotypes, the vast majority of Gc on the colonial surface are pilus⁻; but clusters or foci of pilus⁺ Gc are regularly present on both P⁻rp⁻ and P⁻rp⁺ colonies.

Blot Hybridization of ClaI-digested Genomic DNA from P⁺⁺, P⁻n, P⁻rp⁻, and P⁻rp⁺ Gc.   Blot hybridization of ClaI-digested genomic DNAs revealed no differences among pilus⁺ and reverting pilus⁻ phenotypes (P⁻rp⁻, P⁻rp⁺) for each strain; but each strain's P⁻n phenotype had one rearranged ClaI fragment in its genome. This was seen by using synthetic oligonucleotides O2 and O6 (Fig. 5) as probes in blot hybridization of ClaI-digested Gc genomic DNAs. Strain JS3 showed rearrangement of a 3.6 kb ClaI fragment to 2.6 kb while, in strain MS11$_{mk}$, rearrangement of a 4.1 kb ClaI fragment to 4.9 kb occurred (Fig. 6). The rearranged ClaI fragments contain sequences corresponding to the 3′ portion of the pilin gene, but they lack the pilin gene's 5′ portion, which occurs in a single ClaI fragment of P⁺⁺, P⁻rp⁻, and P⁻rp⁺ Gc DNAs. Several ClaI fragments,
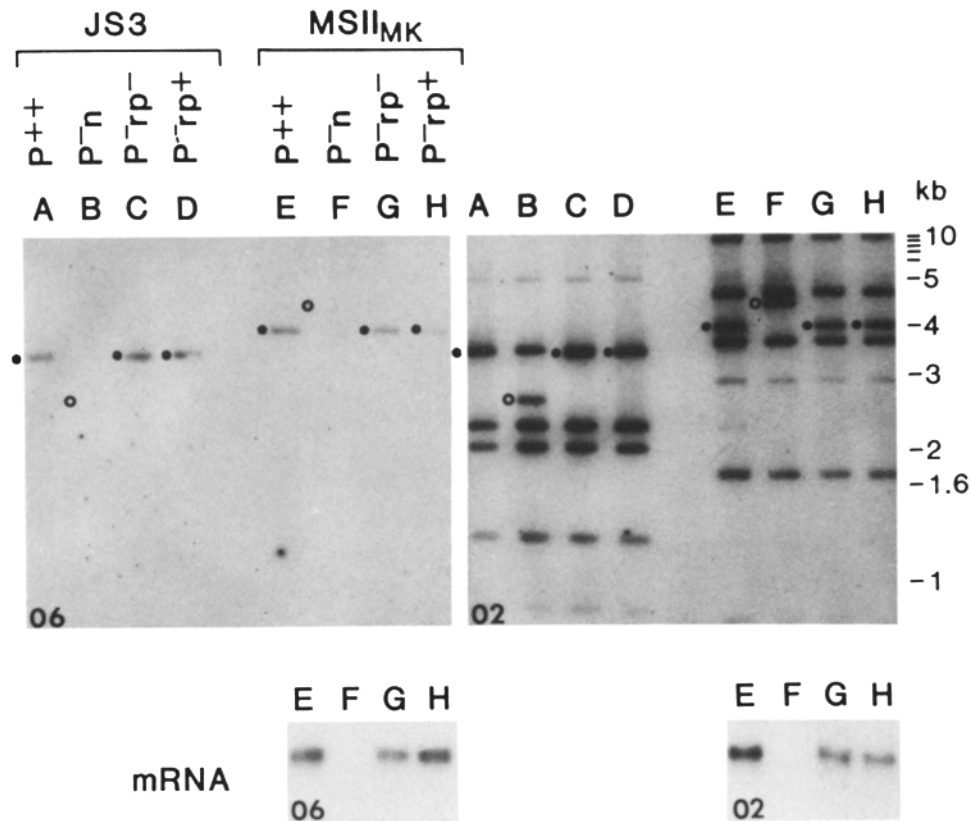
FIGURE 6. Blot hybridizations of strain JS3 and strain MS11$_{mk}$ ClaI-digested DNA (*top*) and strain MS11$_{mk}$ total RNA (*bottom*) after probing with oligonucleotides O6 (5' portion of pilin gene) and O2 (3' portion of pilin gene). With the 5' O6 probe, a single ClaI DNA fragment hybridized in each strain's P^{++}, P^-rp^-, and P^-rp^+ phenotypes; no DNA-DNA hybridization signal was obtained for either P^-n preparation. Multiple ClaI fragments of genomic DNA hybridized with the 3' O2 probe, including those which, on transition from pilus$^+$ to P^-n, underwent rearrangement. Hybridization of a single broad band with identical apparent size (~0.5 kb) occurred in total RNA preparations from P^{++}, P^-rp^-, and P^-rp^+ Gc, with both the 5' O6 and the 3' O2 oligonucleotide probes. Locations of 1 kb ladder DNA molecular weight standards and their sizes are noted on right of figure.

including rearranged forms, hybridize with the pilin gene's 3' probe. All rearranged fragments also contain a 1.9 kb ClaI-XmaI fragment that is a noncoding, flanking region to the pilin gene in the cloned DNA (not shown, see reference 15). Rearranged ClaI fragments of several different sizes all exhibited loss of the pilin gene's 5' portion (two examples shown in Fig. 7). This figure also shows the blot hybridization pattern for Gc from strain MS11$_{ms}$, which can contain two complete pilin-encoding genes as described by Meyer et al. (18).

Pilin-specific mRNA in P^{++}, P^-n, P^-rp^-, *and* P^-rp^+ *Gc.* RNA was extracted from Gc and subjected to agarose gel electrophoresis under denaturing conditions. After transfer to nitrocellulose, the RNAs were hybridized with the pilin-encoding recombinant plasmid pVD203, its 1.4 kb XmaI insert, or with synthetic
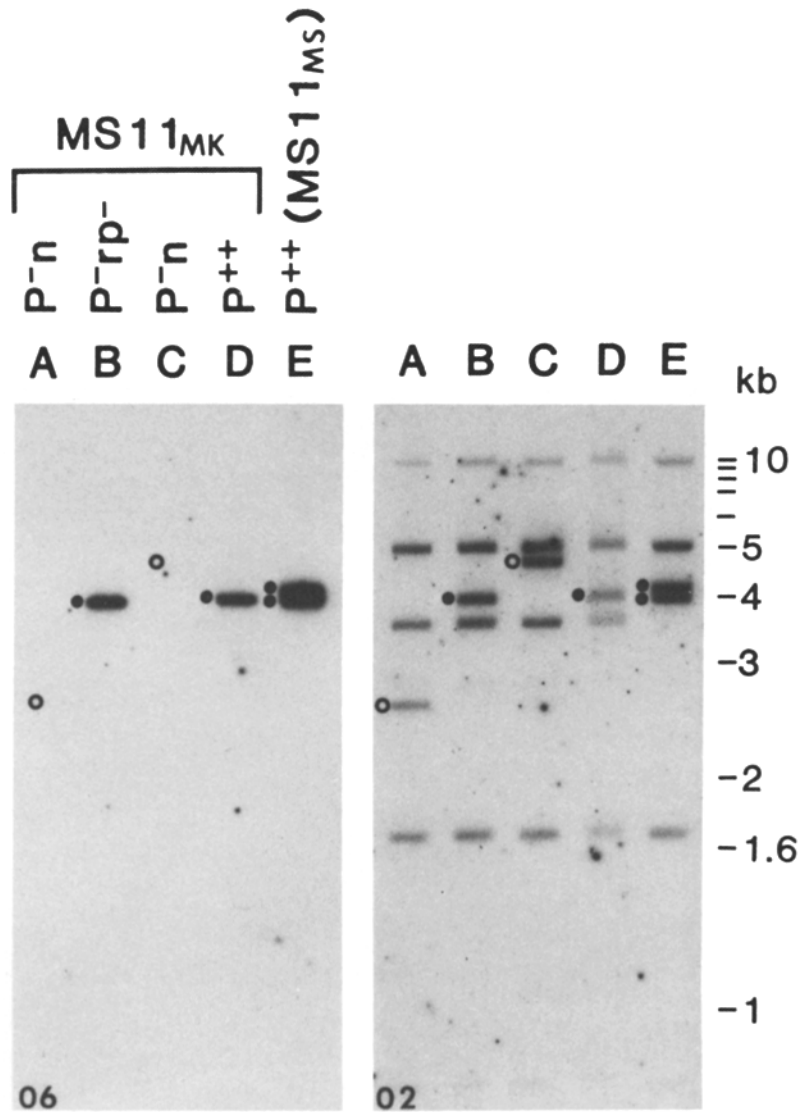
FIGURE 7. Blot hybridization of ClaI-digested DNAs with probes corresponding to the 5' portion of the pilin gene (probe O2) and to a more 3' region of the pilin gene encoding part of the disulfide loop of pilin (probe O6). P^-n, P^-rp^-, P^-n, and P^{++} phenotypes of strain $MS11_{mk}$ are shown in A–D; E contains DNA from P^{++} of strain $MS11_{ms}$. With the 5' probe O2, hybridization is seen for a single 4.1 kb ClaI fragment in P^-rp^- and P^{++} derivatives; no hybridization signal was found for rearranged or other ClaI fragments of P^-n Gc with probe O2. $MS11_{ms}$ Gc contained both 4.1 and 4.3 kb ClaI fragments that hybridized with 5' probe O2. Several ClaI fragments, including the rearranged fragments of P^-n organisms, exhibited hybridization with the 3' O2 probe in each of the different Gc phenotypes. Locations of 1 kb ladder DNA molecular weight standards are noted with their sizes on right of figure.

oligonucleotides constructed according to sequences of different portions of the pilin structural gene. In P^{++}, P^-rp^-, and P^-rp^+ $MS11_{mk}$ RNA preparations, a single broad band of ~0.5 kb hybridized with oligonucleotides O2 and O6 (Fig. 6); no hybridization signal was obtained for P^-n RNAs with any of the probes.
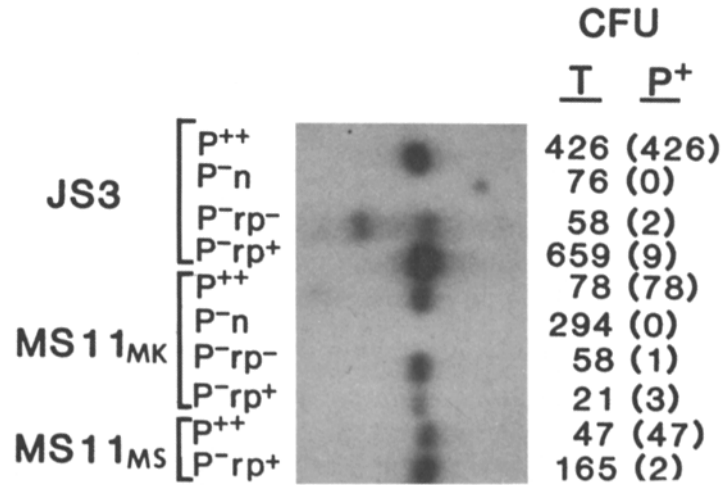
FIGURE 8. Total RNA was extracted from suspensions of Gc of desired phenotypes. Both total and pilus$^+$ CFU were determined for each suspension, and an equivalent amount of RNA extracted from each phenotype was used for gel electrophoresis followed by transfer to nitrocellulose and probing with the 1.4 kb XmaI fragment that contains a complete copy of the cloned Gc pilin gene. As seen by simple examination of the hybridization intensities, the apparent quantity of pilin-specific mRNA correlates with the total CFU rather the the pilus$^+$ CFU in P^{++}, P$^-$rp$^-$, and P$^-$rp$^+$ Gc; no hybridization signal is found with P$^-$n Gc RNA with the pilin gene–containing DNA probe.

To define whether the quantities of pilin-specific mRNA in P$^-$rp$^-$ and P$^-$rp$^+$ Gc reflected only pilus$^+$ variants in these preparations or originated from both pilus$^+$ and pilus$^-$ cells, we extracted RNA from suspensions of Gc and determined total colony-forming units (CFU) and pilus$^+$ CFU. After hybridization with ^{32}P-labeled DNA (1.4 kb XmaI fragment containing a complete pilin gene), the relative intensities of the DNA-mRNA hybridization signals clearly reflected the total number of Gc inhabiting colonies with P$^-$rp$^-$ or P$^-$rp$^+$ phenotypes, not their pilus$^+$ revertants (Fig. 8). In strain JS3, two mRNA-size species showed hybridization with the 1.4 kb DNA probe. The origin and nature of the second, larger (~0.9 kb) mRNA species is under study.

Quantities of Pilin in P^{++}, P$^-$n, P$^-$rp$^-$, and P$^-$rp$^+$ Gc.   Quantification of pilin per Gc was attempted by subjecting individual colonies of Gc to SDS-PAGE followed by immunoblotting with antipilus serum. Before solubilization for electrophoresis, the Gc had been suspended in water and a sample was used for CFU determination and assessment of colonial morphotype. Each autoradiographic pilin band signal was scanned by densitometry and integrated. As shown in Table I, the intensities of pilin signals from P$^-$rp$^+$ appear to correlate with the total CFU, not with only the pilus$^+$ CFU.

A summary of the findings described above for the three pilus$^-$ phenotype variants of Gc is shown in Table II.

Discussion

When Gc with one pilin gene change from pilus$^+$ to pilus$^-$, blot hybridization of their ClaI-digested genomic DNAs revealed rearrangement of pilin-containing

TABLE I

Number of Gc vs. Pilin Immunoblotting

| Lane in Fig. 3B | Strain | Phenotype* | CFU | | Pilin band area‡ |
			Total	P⁺, P⁺⁺	
1	JS3	P⁺⁺ (P⁻rp⁻)	1.9×10^6	1.9×10^6	54
2		P⁻rp⁻	2.5×10^6	1.2×10^4	0
3		P⁻rp⁻	4.1×10^6	3.6×10^4	0
4	JS3	P⁺⁺ (P⁻rp⁺)	2.1×10^6	2.1×10^6	53
5		P⁻rp⁺	8.9×10^6	2.3×10^5	300
6		P⁻rp⁺	6.0×10^6	1.4×10^5	277
7		P⁻rp⁻ (P⁻rp⁺)	2.4×10^6	4.8×10^3	0
8	MS11	P⁺⁺ (P⁻rp⁻)	4.7×10^5	4.7×10^5	122
9		P⁻rp⁻	1.8×10^6	2.4×10^4	0
10		P⁻rp⁻	1.3×10^6	2.4×10^4	0
11	MS11	P⁺⁺ (P⁻rp⁺)	1.3×10^6	1.3×10^6	276
12		P⁻rp⁺	1.9×10^6	9.6×10^4	131, 47 (178)
13		P⁻rp⁺	2.0×10^6	2.4×10^4	124, 63 (207)
14		P⁻rp⁺	1.6×10^6	4.8×10^4	115, 51 (166)

* For Gc with P⁺⁺ phenotype (lanes 1, 4, 8, 11), the phenotypes of their respective P⁻ parent colonies are noted in parentheses.
‡ For lanes 12–14, densitometric values for both 21,000 and 16,000 M_r bands are noted (total in parentheses).

TABLE II

Summary of Characteristics for Pilus⁻ Phenotype Variants

Phenotype	Reversion to pilus⁺	Pili	Pilin	"Pilin" mRNA
P⁻n	−	−	−	−
P⁻rp⁻	+	*	−	+
P⁻rp⁺	+	*	+	+

* Denotes absence of pili by most Gc in colony with foci of pilus⁺ organisms.

DNA in some, but not all of the resultant pilus⁻ variants. Pilus⁻ Gc with a rearranged pilin gene appeared "frozen" in a P⁻n phenotype with no detectable reversion to pilus⁺. Rearranged P⁻n DNAs exhibited partially deleted pilin structural gene sequences; these deletions generated genomes totally devoid of 5' pilin gene sequences. The extent of pilin gene deletion was not defined by the methods used here. Recent observations by Segal et al. (14) show that pilin gene rearrangements involve deletion of variable portions of the structural gene; most deletions include the gene's 5' portion. We previously showed (15) that several forms of Gc pilin gene DNA rearrangement occur to produce ClaI fragments that differ widely in size (2.1, 2.7, 3.0, 4.4, 4.6, and 5.0 kb) (15); each of these rearranged ClaI fragments retains 2.0 kb of DNA sequences, which flanks the pilin gene from pilus⁺ organisms and sequences homologous to central and C-terminal portions of the pilin gene. Several size forms of rearranged ClaI

fragments seen in strain $MS11_{mk}$ recur in organisms of distant in vitro passage relationships. The observed multiplicity of pilin gene rearrangement forms suggests that either (a) a single recombinational mechanism can use any of several nucleotide sequences found in the region of the pilin gene or (b) several different recombinational mechanisms, each with its individual target sequence, operate in Gc. After rearrangement, several copies of the pilin gene's 3' portion remained in the genome; but sequences corresponding to the gene's 5' portion appeared to be lost from the P^-n organism's genome. The absence of a complete pilin gene seems to preclude or greatly diminish reversion to a $pilus^+$ phenotype. No reversion to $pilus^+$ phenotype was seen for several P^-n Gc serially passaged during the 6-mo course of this study.

$Pilus^-$ Gc that show no rearrangement of their single pilin gene DNA compared with their $pilus^+$ parents readily reverted to $pilus^+$ phenotype. Some reverting $pilus^-$ Gc (P^-rp^-) produced neither pili (by SEM) nor pilin subunits (by immunoblotting). However, Gc with P^-rp^- phenotype contained pilin-specific mRNA, indicating that their lacking pilin and pili results from control at unknown, posttranscriptional levels. Whatever the site of control, it is readily reversible and $pilus^+$ revertants are spawned at high frequency. Other reverting $pilus^-$ Gc (P^-rp^+) synthesize pilin subunits (by immunoblotting), but they are not assembled into intact, mature pili (by SEM). The pilin subunits synthesized by P^-rp^+ Gc commonly have sizes identical to that of their $pilus^+$ parents and variant progeny. In strain MS11, P^-rp^+ Gc regularly elaborate two different size products (21 and 16 kd) that react with monoclonal and polyclonal antipilus sera by immunoblotting; their $pilus^+$ revertants produce M_r 21,000 pilin subunits only. The structural basis for a related or identical epitope on proteins of two different sizes is not clear; the M_r 16,000 product may be truncated or defective pilin molecules whose presence could interfere with proper polymerization of native, M_r 21,000 pilin subunits into pili. Rapid reversion to $pilus^+$ occurs among progeny of P^-rp^+ Gc regardless of whether they produce one or two pilin subunit-like products. Because P^-rp^- Gc contain pilin-specific mRNA species that we assume are functional, it appears that an unknown mechanism restricts effective translation of the pilin message, and that reversible activation/deactivation of this restrictive or suppressive activity effects another form of pilus control.

The two strains primarily used here, $MS11_{mk}$ and JS3, display only a single apparent copy of the pilin gene's 5' portion among their $pilus^+$ and reverting $pilus^-$ phenotypes. Meyer et al. (18) found two complete pilin genes in genomic DNA of their strain MS11 (our $MS11_{ms}$); our results agree with theirs for that strain. The recent study by Segal et al. (14) examined DNA rearrangements coincident with changes in piliation status of MS11 Gc. They found that all $pilus^+$ to $pilus^-$ and reverse changes correlate with rearrangement of one, the other, or both pilin genes (14); all of their P^- organisms are described as able to revert regardless of whether one or both pilin genes have undergone rearrangement. The majority of their $pilus^-$ derivatives did not synthesize pilin and might correspond to our P^-rp^- phenotype. A few of their P^- Gc produced low levels of pilin that may be synthesized by $pilus^+$ revertants, and are probably not comparable to our P^-rp^+ that produce pilin in amounts comparable to $pilus^+$ Gc. Our initial studies on strain $MS11_{ms}$ suggest that recombinational events involving

two pilin genes are very difficult to relate to piliation phenotype changes. Obvious rearrangement of one or the other pilin gene may occur without loss of pilus$^+$ character; some MS11$_{ms}$ Gc exhibit apparently intact pilin genes but display a P$^-$n phenotype (Swanson, unpublished observations). It is doubtful whether P$^-$n, P$^-$rp$^-$, and P$^-$rp$^+$ phenotypes would have differentiated in Gc with two pilus genes because of the complexity of their rearrangements. MS11$_{ms}$ displays two complete pilin genes located on different ClaI fragments. Although it is not clear whether these two genes are active and transcribed simultaneously, it seems possible that the control mechanisms exemplified by P$^-$rp$^-$ and P$^-$rp$^+$ Gc could operate on one or both pilin genes to modulate Gc piliation in organisms having two complete pilin structural genes.

P$^-$n Gc that have undergone rearrangement of their pilin-encoding DNA and are unable to revert to pilus$^+$ phenotype should be totally avirulant for humans if piliation is required for the organism's pathogenicity. This question can only be answered by experiments to induce human infections with P$^-$n Gc since it is not clear whether reverting or nonreverting pilus$^-$ Gc were used in two previous human volunteer challenge studies (7, 8). In the first of these classical studies by Kellogg and coworkers (7), colony type 3 Gc caused infection in three of four volunteers, but no infections resulted from intraurethral inoculation with colony type 4 Gc; in the second report (8), colony type 4 but not type 3 organisms were studied and were found to be avirulent. In both trials, colony type 1 or 2 (pilus$^+$) Gc caused typical gonorrheal infections in the male volunteers. Although both colony types 3 and 4 are thought to contain pilus$^-$ Gc, the results cited above (7, 8) leave doubt as to the requirement of pili for Gc virulence. Those results may be explained if their colony type 3 organisms were analogous to a P$^-$r phenotype and displayed virulence because they spawned pilus$^+$ revertants, and if their avirulent type 4 Gc corresponded to a nonreverting P$^-$n phenotype.

If piliation of Gc is required for their virulence, genetic events which produce nonreverting, avirulent, nonpiliated organisms seem, a priori, to confer little or no advantage to the successful pathogenic lives of these obligate human parasites. Genetic mechanisms that permit reversion to pilus$^+$ status and oscillation between pilus$^+$ and pilus$^-$ phenotypes seem more likely to provide Gc with a useful strategy for dealing with host defenses, through varying their surface components and properties. Although piliation is known to endow Gc with enhanced abilities to adhere to epithelial cells, the converse, reduced attachment to mucosal or other cells of the host, may play a role in allowing Gc to leave initial sites of infection and gain access to blood or lymphatic channels. Such a nonadherent, interim state might also enhance Gc transmission from one individual to another, if pilus$^-$ organisms are more easily dislodged from mucosal surfaces of an infected donor during transmission but can subsequently revert and generate pilus$^+$ Gc that more effectively adhere to mucosal sites of the inoculated individual.

Summary

Pilus$^+$ to pilus$^-$ transitions of gonococci (Gc) that involve rearrangement of pilin gene DNA yield the P$^-$n phenotype, which is incapable of reversion (to pilus$^+$). Reversion to pilus$^+$ is found for nonpiliated Gc that have undergone no apparent pilin gene rearrangement. Among the reverting, nonpiliated Gc, two

distinct phenotypes (P⁻rp⁻ and P⁻rp⁺) occur and are differentiated according to their synthesis (or lack) of pilin subunits; both P⁻rp⁻ and P⁻rp⁺ Gc contain pilin-specific mRNA. The occurrence of these different pilus⁻ phenotypes strongly suggests that several mechanisms can account for changes in the piliation status of Gc; one of these involves pilin gene rearrangement but the others apparently operate at posttranscriptional levels. Reverting pilus⁻ Gc may have a pathogenic advantage in being able to reversibly alter their host cell adherence-promoting surface properties through high frequency transitions in piliation status.

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