

GONOCOCCAL PILIN VARIANTS IN EXPERIMENTAL GONORRHEA

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Pili appear to promote the pathogenicity of gonococci (Gc)¹ by mediating their attachment to human hosts' mucosal surfaces (1, 2). Although all Gc pili are assumed to have adherence-promoting properties, they exhibit structural diversity between strains and among variants of a given strain (3–8). This diversity is due to differences in the amino acid sequences of their pilus subunit (pilin) polypeptides. Such pilin sequence diversity is based on the Gc genome's one complete pilin gene and multiple partial pilin genes that participate in homologous recombination. Through nonreciprocal exchange, a partial pilin gene's sequence undergoes insertion into the complete pilin gene; the resulting chimeric complete pilin gene expresses a novel pilin polypeptide. The Gc genome contains over a dozen distinct partial pilin genes as a basic repertoire for synthesis of differing pilin polypeptides (9, 10).

The pathogenic significance of pilus variants generated by the Gc genome's elaborate intergenic recombination system is not clear. Several experimental findings are consistent with the notion that pilus variants play role(s) in the colonization of different mucosal niches by Gc in vivo; such a role would be important mainly to the initial stages of gonorrheal infection. Pilus variation influences adherence properties of Gc; this has been demonstrated with pilus⁺ variants of strain P9 that express different pilin polypeptides and show disparate adherence proclivities to selected eukaryotic cells, in vitro (11). Gc that are isolated from diverse sites in an individual with gonorrhea but represent a single strain express pilin polypeptides of differing electrophoretic mobilities (12).

Pilus variation might also allow Gc to avoid the actions of a human host's antipilus antibodies while they retain an adherence-promoting pilus⁺ status. It is

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¹ *Abbreviations used in this paper:* CNBr, cyanogen bromide; Gc, gonococci; O⁺, opaque colony phenotype; O⁻, nonopaque colony phenotype; P^{+/+}, pilus⁺ colony morphotypes; PII, protein II; pAb, polyclonal antibody.

well established that antipilus antisera raised against pili from a given Gc strain inhibit attachment of the homologous pilus⁺ Gc to tissue culture cells; the same sera are less effective in interrupting attachment of heterologous pilus⁺ Gc (13). Thus, variant pili may exhibit antigenic changes without an attendant loss of adherence-promoting properties. An immune-avoidance role of pilus variation may be relevant to reiterative gonorrhea in a host who possesses antibodies stimulated by Gc pili from preceding infections. This idea is consistent with the changed attachment antigen(s) (presumably pili) found for Gc isolated from individuals' initial infections versus Gc that reinfected the same people 1 mo later and seemed to represent the same strain (14).

Regardless of the exact role(s) that pili play in the genesis of gonorrhea, piliation seems central to the pathogenicity of Gc, and interruption of Gc attachment by antipilus antibodies is one strategy being followed for development of a gonorrhea vaccine (4, 5, 14, 15). But the structural variety observed among Gc pili poses problems for design of such a pilus-based vaccine. One way around this difficulty might be through identification of an oligopeptide that is common to all Gc pilins, is functionally important in mediating Gc adherence, and constitutes a target for antibodies evoked by the oligopeptide vaccine (15–17). These features are described for the oligopeptide comprising pilin amino acids 69–84 (76–91 in our numbering scheme) (18, 19); antibodies raised against this oligopeptide react with intact pili from all strains studied and bind a tryptic fragment of pilin that, in turn, binds to an endometrial carcinoma cell line, in vitro (19). Success of such a vaccine clearly depends on conservation of this oligopeptide in the pilins of both inter- and intrastrain Gc variants.

We have examined the pilins elaborated by Gc during initial phases of experimental gonorrhea in human male subjects. Pilus⁺ Gc were introduced into the urethrae of two subjects who had no history of previous gonorrhea, and the pilins of input Gc were compared with those of Gc reisolated during the resulting incipient infections; input versus reisolated Gc were compared by sequencing their pilin mRNAs and by immunoblotting their pilin polypeptides with selected mAbs. All Gc reisolates expressed pilin polypeptides distinct from the pilin elaborated by input Gc. This report describes their structural and antigenic distinctions and documents changes in Gc reisolates' pilin regions that were previously said to be conserved (19).

Materials and Methods

Gonococci. Strain MS11_{mk} Gc were propagated on a clear solid Gc medium previously described (8). Gc used for urethral challenge were pilus⁺ (P⁺⁺ colony morphotype; reference 8), formed nonopaque (O⁻) colonies, and expressed no outer membrane protein II (PII⁻). Several such P⁺⁺O⁻ colonies were microscopically selected after 20–22 h of growth, were lifted from the clear solid medium with filter paper fragments, were suspended in sterile PBS, and a portion was used to determine total Gc CFUs and to define the presence and number of colonial variants. 1 ml of the suspension (containing ~10⁴ CFU Gc/1 ml for subject A and 10⁵ CFU/1 ml for subject B) was instilled into the urethra of each male subject, who then refrained from urination for 1 h. Subsequent urine specimens were centrifuged to concentrate the Gc and were plated onto Gc medium containing Vancomycin, Colistin, and Nystatin (V-C-N Inhibitor, used as per directions; BBL Microbiology Systems, Cockeysville, MD). Other portions of the centrifuged urine sediment were frozen after mixing with sterile 1% skim milk. In retrospect, this freezing menstruum was unsatisfactory because, unlike Gc swabbed from plates and frozen in skim milk, Gc in urinary sediments were recoverable from frozen specimens only one or two

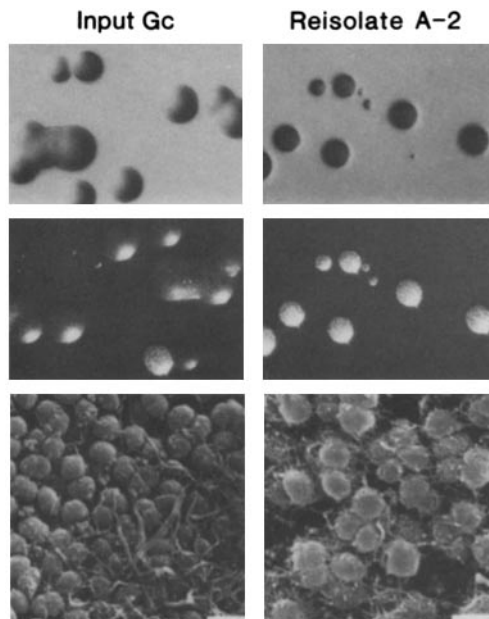


FIGURE 1. Morphologies of input Gc versus reisolate A-2 Gc. A low-power, dissecting microscope shows colonies of input Gc have very regular, distinct edges (P^{++} morphotype; *top*) and are O^{-} (*middle*), except for an occasional O^{+} variant. But in reisolate A-2, the colonies are O^{+} and have irregular edges. Scanning electron micrographs (*bottom*) confirm that both input and reisolate Gc are pilus $^{+}$. The appearances of their pili are quite distinct; pili on input Gc seem prone to lateral association or aggregation, while those on Gc reisolate A-2 do not.

times (thawing and refreezing cycles). Frozen stocks were thawed and plated on Gc medium containing V-C-N Inhibitor; after 18 h of growth, 20 barely visible (with dissecting microscope) colonies were selected, lifted from the agar on filter paper, dispersed in water, plated on Gc medium without inhibitors, and used after 1 d of growth to prepare Gc frozen stocks and whole cell lysates.

Antibodies. Antipilus mAbs were generated by immunization of BALB/c mice with isolated pili from MS11. Spleen cells from immunized mice were fused with NS1 cells (8, 18). These mAbs were assayed for reactivity with cyanogen bromide (CNBr) fragments of pilin and with synthetic oligopeptides, as described by others (18). Polyclonal antiserum was raised in rabbits immunized with whole pilus $^{+}$ Gc (MS11) and was adsorbed several times with formalin-fixed pilus $^{-}$ Gc (P^{-} phenotype; references 20, 21).

Immunoblotting. SDS-PAGE and immunoblotting with mAbs and rabbit antiserum were performed exactly as described in detail elsewhere (8). Use of mAb 9B9 and mAb 7B4 necessitated use of a second antibody (rabbit anti-mouse, Cappel Laboratories, Malvern, PA) before incubation with 125 I-protein A.

DNA and RNA Preparations. Gc grown for 20 h on solid medium were scraped from the plate and were suspended either in 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.8 (for DNA), or in guanidine isothiocyanate lysis buffer (for RNA). Subsequent lysis in SDS, digestion with proteinase K, and phenol extraction in DNA extraction were as described in detail (20), as was the scheme of total RNA extraction (10).

Primer-extension Sequencing of Pilin mRNAs. Oligonucleotides constructed according to the DNA sequence for the cloned pilin gene in pVD203 (21) were used as radiolabeled primer for sequencing pilin mRNA as described elsewhere (10, 20).

Results

Input Gc vs. Reisolated Gc Variants. Input Gc exhibited predominantly $P^{++}O^{-}$ phenotype; 1% were $P^{+}O^{-}$ and 0.3% were $P^{++}O^{+}$. All Gc recovered from both subjects' urine 2–3 d after intraurethral challenge were pilus $^{+}$ formed O^{+} colonies (Fig. 1), and bore one or more outer membrane PII $^{+}$ (data not shown). Colonial morphotypes were difficult to define for the initially reisolated Gc colonies because their top surfaces and edges had a cracked and wrinkled appearance

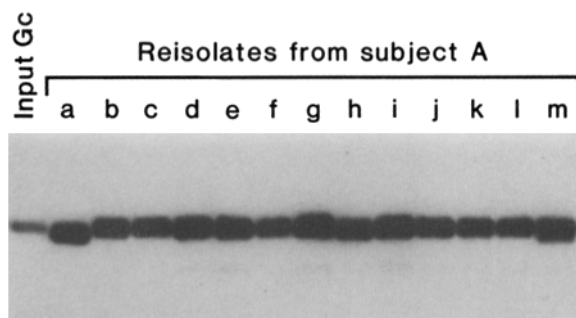


FIGURE 2. Immunoblotting input and reisolated Gc with crossreactive antipilus mAb 02 show relatively little variation in their pilins' electrophoretic mobilities; but slight differences are noted among these 13 colonial reisolates; for example, lanes *a*, *b*, and *m* vs. input Gc. Several Gc depicted here were examined by mRNA sequencing, as follows: (*a*) re-isolate A-1; (*c* and *m*) re-isolate A-2 (see Fig. 3 legend); (*g*) A-4. A-3 was isolated from patient A after he had clinical signs and symptoms of acute urethritis and is not shown in this immunoblot.

due mainly to their $O^+(PII^+)$ status. So, the reisolates' respective colony morphotypes were defined in $O^-(PII^-)$ variants that arose by a single-step $O^+(PII^+) \rightarrow O^-(PII^-)$ change; they were all P^+ or $P^{+/++}$. Regardless of their exact colonial morphotype, scanning electron microscopic examination of colonies in situ after a single passage showed the reisolated Gc were pilus-positive (Fig. 1).

Gc were reisolated from patient A 2 d before he developed any local or systemic symptoms or signs of urethritis. 13 Gc colonies in one of his urine specimens were examined by immunoblotting and had pilins of roughly similar electrophoretic mobilities (Fig. 2). Four of these reisolates were examined by pilin mRNA sequencing; two were identical (Fig. 2, legend). These reisolates provided sequences A-1, A-2, and A-4 noted in Figs. 2–5. Another re-isolate from patient A (A-3) was recovered later, when he had classical symptoms and signs of acute urethritis. Both the original $O^+(PII^+)$ colony forms and their $O^-(PII^-)$ variants were subjected to RNA extraction and pilin mRNA sequencing for three reisolates (A-2, A-3, and A-4); identical pilin mRNA sequences were obtained (data not shown) for all three $O^+(PII^+)$ and $O^-(PII^-)$ pairs.

Pilin mRNA Sequences of Input Gc vs. Reisolated Gc Variants. All the reisolated Gc (A-1, A-2, A-3, and A-4 from patient A; B-1 and B-2 from patient B) had multiple sequence changes in their pilin mRNAs compared with input Gc (Fig. 3). Stretches of nucleotides homologous to partial pilin gene *pilS1* copy 2 (9) resided in mRNAs of three of four Gc reisolates from subject A (A-2, A-3, A-4). In A-2 and A-3, *pilS1* copy 2 sequence occupied 175 nucleotides (522–696), while a shorter 136 nucleotide block (561–696) of the same partial pilin gene appeared in A-4. The *pilS1* copy 2–derived sequence in A-3 and A-4 each contained a 14-nucleotide segment (627–640) homologous to another partial pilin gene, *pilS1* copy 5 (9); as discussed later, this nucleotide stretch also resided in other partial pilin genes. Re-isolate A-1 mRNA contained neither *pilS1* copy 2– nor copy 5–like sequence, but it had a novel block of 136 nucleotides (561–696) that encoded 15 amino acid changes compared with input Gc; the partial pilin gene(s) responsible for the unique nucleotide span in A-1 mRNA has (have) not been characterized as to location and sequence. Pilin mRNAs from A-2, A-3, and A-4 encoded polypeptides of 165 amino acids, while input Gc and re-isolate A-1 mRNAs encoded pilin polypeptides that were 166 amino acids long. Despite these differences, the pilins of all reisolates from subject A had identical sequences in their inter-cysteine regions (amino acids 128–158); most of the sequence

Input Gc (p00203)	(51)	(500)	(600)	(700)	(800)	(900)
reisol A-1	(K) (S) (A) (V) (T) (E) (Y) (Y) (L) (N) (H) (G) (K) (Q) (P) (E) (N) (N) (T) (S) (A) (G) (V) (A) (G) (K) (K) (Y) (V) (K) (E) (E) (V)	AAA TCA GCC GTT ACC GAG TAT TAC CTG AAT CAC GGC AAA TGG CGG GAA AAC AAC ACT TCT GCG GGC GTG GCA TGC	(S) (A) (A)	(P) (P) (S) (D) (I) (K) (G) (K) (K) (Y) (V) (K) (E) (E) (V)	AAA GAG GTT GAA GTT	(E) (S)
pl151 copy 2
reisol A-2
reisol A-3
reisol A-4
reisol B-1
reisol B-2
pl151 copy 5
Input Gc (p00203)	(91)	(100)	(110)	(120)	(700)	(130)
reisol A-1	(K) (N) (G) (V) (V) (T) (A) (T) (M) (L) (S) (S) (G) (V) (N) (N) (E) (I) (K) (G) (K) (K) (L) (S) (L) (S) (K) (N) (K) (N) (P) (C) (G) (Q)	AAA AAC GGC GTC GTT ACC GGC ACA ATG CTT TCA AGC GGC GTA AAC AAT GAA ATC AAA GGC AAA AAA CTC TCC CTG TGG GCC	AGC CGT GAA AAC GGT TGG TTA AAA TGG TTC TGC GGA CAG	(K) (Q) (D)	(K) (E) (N) (G) (S) (S) (V) (K) (N) (K) (N) (P) (C) (G) (Q)	(130)
pl151 copy 2
reisol A-2
reisol A-3
reisol A-4
reisol B-1
reisol B-2
pl151 copy 5
Input Gc (p00203)	(131)	(140)	(150)	(800)	(160)	(167)
reisol A-1	(P) (V) (T) (K) (T) (D) (D) (D) (T) (V) (A) (D) (D) (D) (D) (K) (K) (K) (H) (L) (P) (S) (T) (C) (R) (K) (K) (A) (S) (D) (G) (K) (K) (S) (R)	CCG GTT ACG CGC ACC GAC GAC GAC ACC GTT GCG GAC GGC AAA GAC GGC AAA GAA ATC GAC ACC AAG CAC CTG CGG TCA ACC TGC CGC GAT AAG GCA TCT GAT GCC AAA TGA
pl151 copy 2
reisol A-2
reisol A-3
reisol A-4
reisol B-1
reisol B-2
pl151 copy 5

changes in these pilin polypeptides occurred in their respective amino acid 60–120 regions.

Gc B-1 and B-2 (from patient B) also produced pilin polypeptides with multiple amino acid changes in their 60–120 regions compared with input Gc; B-1 had an additional amino acid 135 change, while the entire amino acid 135–150 region of B-2 was different. In spite of length differences in pilins they encode, B-1 and B-2 mRNAs contained unique blocks of nucleotides that seem to derive from the same uncharacterized partial pilin gene. In B-1, this unique block was 205 nucleotides long (534–738), while in B-2 it extended at least to nucleotide 784, beyond which pilin mRNA sequence was not obtained. Within their unique nucleotide blocks, B-1 and B-2 mRNAs contained *pilS1* copy 5–like sequence in their nucleotide 600–640 regions.

Antigenic Differences of Pilins from Input vs. Reisolated Gc. The amino acid sequence changes of reisolated Gc pilins correlated with their altered antigenicities. This was seen by immunoblotting with selected mAbs that react with pilin of input Gc (Fig. 4) and with an antipilus rabbit serum polyclonal antibody 83 (pAb 83) that recognized pilins from input Gc and all reisolates' pilins. Reisolates A-3 and A-4, in addition to having a pilin of usual size ($M_r \sim 21 \times 10^3$), exhibited another pilin protein of smaller apparent size ($M_r \sim 16\text{--}17 \times 10^3$) reminiscent of P⁻rp⁺ phenotype Gc (20, 21) and some pilus⁺ revertants (22). mAb 1H5 and mAb 7B4 reacted with pilins of input Gc and reisolates A-1, A-2, A-3, A-4, and B-1; only B-2 pilin was not reactive with these two mAbs, whose epitopes are located in CNBr fragment 3 (CNBr-3) (amino acids 100–166). mAbs 9B9 recognized an epitope located in the CNBr fragment 2 (CNBr-2) of input Gc pilin (amino acids 15–99, reference 18); epitopes of mAb 9B9 were absent on pilins of all reisolated Gc. Two other mAbs, whose epitopes are in CNBr-3 of input Gc pilin (mAb 9B12 and mAb 2C7), reacted with pilins from all the reisolates from both subjects (data not shown). These immunoblotting results are

FIGURE 3. Pilin mRNA sequences of input vs. reisolated Gc are shown along with DNA sequences for partial pilin genes *pilS1* copy 2 and *pilS1* copy 5 (19). Reisolates A-1, A-2, and A-4 represent Gc shown in Fig. 2. Re isolate A-3 came from patient A after he had developed gonorrheal urethritis. Reisolates B-1 and B-2 are from subject B. A-1, A-3, and A-4 each represent the identical sequences obtained for O⁺⁺ and O⁻ variants of single reisolates. A-2 represents two independently isolated colonies (*c* and *m* in Fig. 2) that had identical *pilS1* mRNA sequences. The positions of amino pilin amino acids are noted in parentheses. The locations of pilin gene nucleotides are noted as 500, 600, et cetera, with each 20-nucleotide interval noted by a dot. A-1 pilin mRNA contains a block of 136 nucleotides (561–696) that differ in sequence from input Gc mRNA; this block is not homologous to a partial gene of known sequence. In contrast, A-2, A-3, and A-4 mRNAs carry varying length nucleotide stretches attributable to *pilS1* copy 2 (*dotted unshaded areas*). Sequence corresponding exactly to *pilS1* copy 2 occupies positions 522–695 (174 nucleotides long) in A-2; the same length stretch is present in A-3, while A-4 has a shorter 135-nucleotide segment resembling *pilS1* copy 2 (positions 561–695). The portion of *pilS1* copy 2 that is not present in any of the reisolated Gc mRNAs is shown (*solid underlining*). In both A-3 and A-4 the *pilS1* copy 2 stretches contain 14 nucleotide-long islands (627–640) homologous to *pilS1* copy 5 (*dotted shaded areas*). Longer stretches of *pilS1* copy 5–like sequence are found in mRNAs of reisolates B-1 and B-2 (41 nucleotides, 600–640). The remainder of the unique nucleotide blocks in B-1 and B-2 mRNAs (*hatched areas*) do not correspond to any partial pilin gene whose sequence has been determined. In A-3, A-4, B-1, and B-2 the *pilS1* copy 5–like sequences represent a different region of this partial pilin gene than those carried in mRNAs of P⁻rp⁺ phenotype Gc (17, 18, 23); the longest portion of *pilS1* copy 5 we have found among such P⁻rp⁺ Gc is shown (*dashed underlining*, positions 645–779).

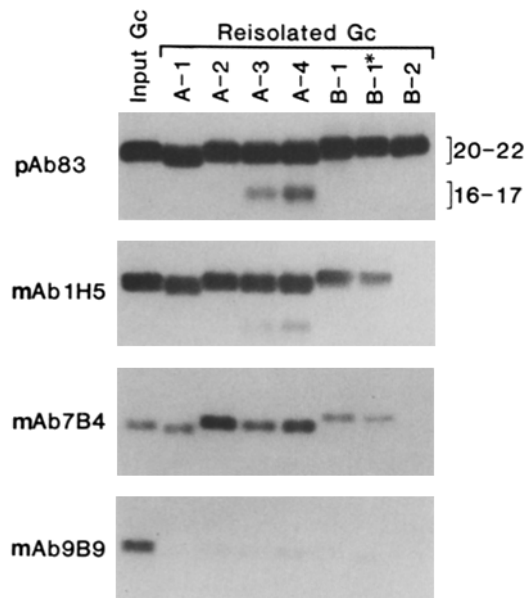


FIGURE 4. Reactivities of pilins from input and reisolated Gc probed with antipilus mAbs in immunoblotting. Whole-cell lysates of input Gc and reisolates A-1, A-2, A-3, A-4, B-1, B-1* (see below), and B-2 were subjected to SDS-PAGE, and transferred to Millipore HAHY membrane. Duplicate membranes were probed with a polyclonal rabbit antipilus serum (pAb 83) or with mAbs 1H5, 7B4, and 9B9. Reisolated B-1* was from patient B's urine, and its mRNA sequence is identical to that of B-1 (data not shown). Epitopes for the mAbs used are tentatively designated as including pilin residues as follows: 1H5, amino acids 136 and 137; 7B4, amino acids 142, 143, and/or 148; 9B9, amino acids 86 and 87. These epitopes' locations were deduced from immunoblotting reactivities for a large series of MS11_{mk} pilin variants whose pilin mRNAs have been sequenced (data not shown). For reisolates A-3 and A-4, pAb 83 and mAb 1H5 (and a broadly crossreactive mAb02, not shown) recognize pilin polypeptides of M_r 20,000–21,000, as well as more rapidly migrating peptides (M_r 16,000–17,000); the latter peptides are typical for P⁺ phenotype Gc but are also present in some pilus⁺ revertants (8, 23). $M_r \times 10^{-3}$ shown at right.

summarized in Fig. 5 along with the amino acid sequence changes in reisolated Gc pilins.

Blot Hybridization Patterns with Pilin Gene Oligonucleotides. Nearly all strains of Gc, including MS11_{mk}, contain only one complete pilin structural gene in their genome. The only known exception is strain MS11_{ms}, which often has two complete pilin genes, has been studied extensively by others (23–25), and is the ancestor of our MS11_{mk} Gc (20). To explore whether the one gene-containing MS11_{mk} input Gc would spawn variants with gross rearrangements in their pilin gene DNA, perhaps even with duplication of their complete pilin gene, genomic DNAs from input and all the reisolated Gc were digested with Cla I and probed with synthetic pilin gene oligonucleotides in blot hybridizations. Results with input Gc and reisolates B-1 and B-2 are shown in Fig. 6. In each Cla I-digested DNA, only one fragment (4.1 kb in input Gc and reisolate B-1; 4.05 kb in reisolate B-2) hybridized with GCO6; this oligonucleotide encodes pilin amino acids 1–7 and its hybridization signal marks each Gc genome's complete pilin gene. All Gc reisolates from patient A exhibited hybridization of 4.1-kb Cla I fragments with GCO6. Blot-hybridization patterns of input versus reisolated organism DNAs were identical when probes GCO2.5 (data not shown) and GCO1.2 (Fig. 6) were used. These oligonucleotides encode the amino acids surrounding cysteine residues 128 and 158, respectively. The hybridization patterns with GCO2.5 and GCO1.2 were as described before for MS11_{mk} Gc (10, 20).

The partial pilin gene origins of the *pilS1* copy 5-like stretch in mRNAs of reisolates A-3, A-4, B-1, and B-2 were explored with oligonucleotide GCO2.9,

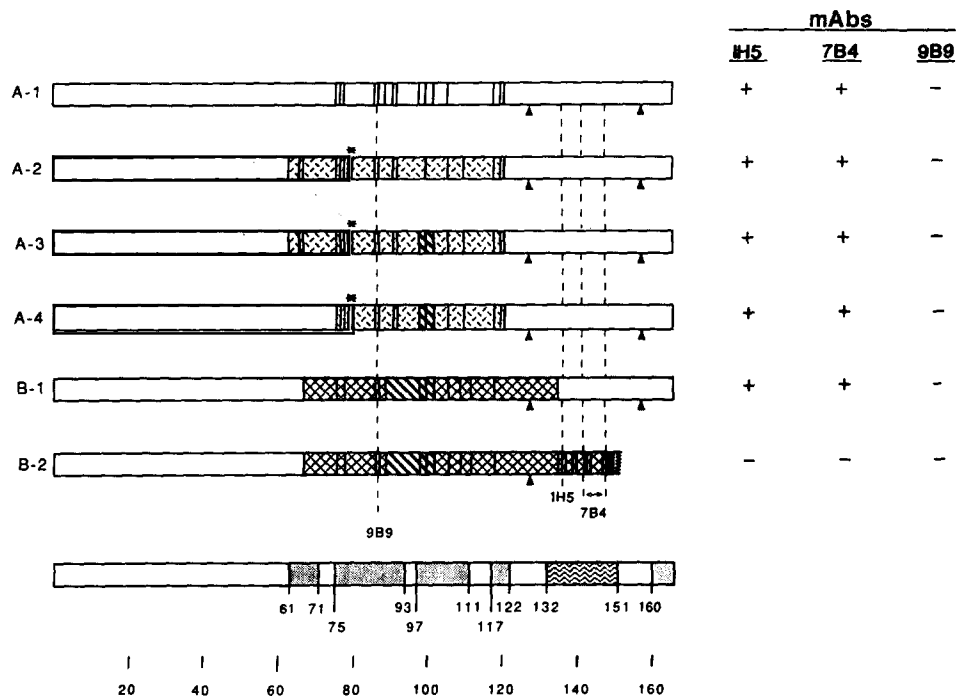


FIGURE 5. Amino acid changes in pilins of reisolated Gc are summarized in comparison with pilin polypeptide of input Gc. The location of each amino acid sequence change encoded by a reisolates' mRNA (A-1, et cetera) is noted by a vertical line at the appropriate residue location (see scale at bottom). Reisolates A-2, A-3, and A-4 have mRNAs that encode pilins 165 amino acids long, compared with 166 for input Gc and reisolates A-1, B-1, and B-2; this is shown by a break and asterisk at position 79 in A-2, A-3, and A-4 as per alignment shown in Fig. 3. Sequences attributed to partial pilin gene *pilS1* copy 2 are noted (□) as are *pilS1* copy 5-like regions (▨). The stretches of unique nucleotides found in mRNAs of reisolated Gc B-1 and B-2 vs. input organism's mRNA are shown (▩). Cysteine residues 128 and 158 are noted (▲). Locations of Gc pilin polypeptides' constant (□), semivariable (▨), and hypervariable (▩) domains are shown in the bottom figure as defined previously (19). Immunoblotting reactivities of these Gc pilins with mAbs 1H5, 7B4, and 9B9 are noted as a plus or minus.

whose sequence is homologous to *pilS1* copy 5 nucleotides 620–640. GCO2.9 hybridized with two *Cla* I fragments (5.0 kb, *pilS1*; 3.8 kb, Δ *pilE2*) in DNAs of input Gc. GCO2.9 reacted with the same two fragments plus another fragment (4.1 kb in B-1, 4.05 kb in B-2 [*pilE1*]) in reisolates B-1 and B-2 DNAs. The hybridization signals of *pilE1* and Δ *pilE2* were equal in B-1 and B-2 DNAs, so each of these fragments contained a single copy of GCO2.9 sequence. *pilS1* yielded a hybridization signal three times more intense than Δ *pilE2* or *pilE1* (by densitometric scanning). This showed that GCO2.9 sequences are distributed in the Gc genome as follows: one copy in the complete pilin gene (*pilE1*) of B-1 and B-2 but not input Gc; one copy in a partial pilin gene contained in the respective Δ *pilE2* loci of both input and reisolated Gc; and three copies of GCO2.9 in *pilS1* of all these Gc. These findings correlate with data of others (9), which show GCO2.9 sequences in three partial pilin genes in *pilS1* (copies 1, 3, and 5), but none of these *pilS1* genes has upstream and downstream sequences

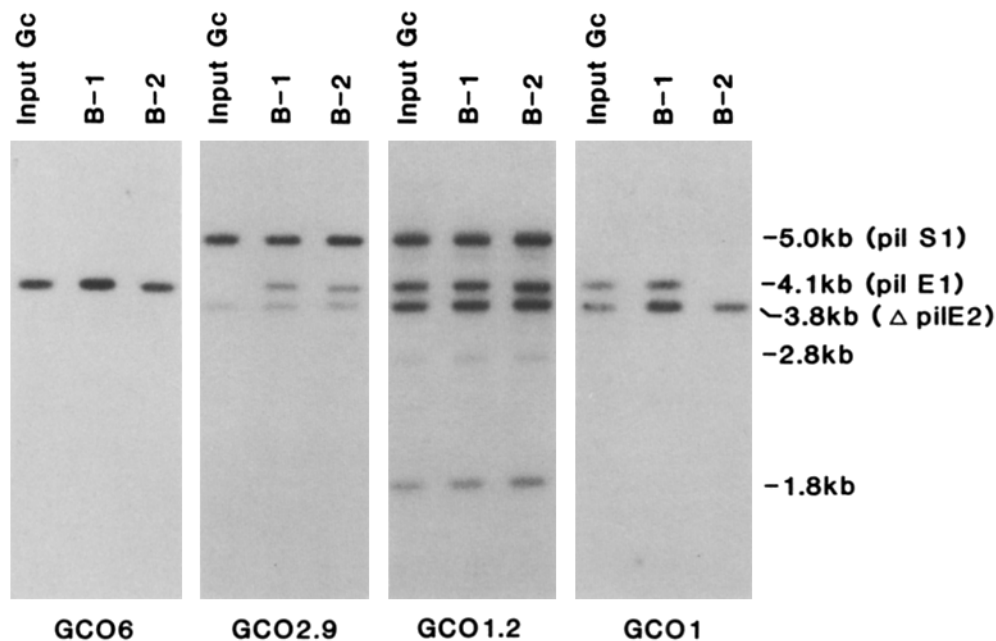


FIGURE 6. Input Gc and two reisolates' DNAs probed in blot hybridization with pilin gene oligonucleotides. Genomic DNAs of input Gc and of Gc reisolates B-1 and B-2 were digested with Cla I and the fragments were resolved by agarose gel electrophoresis. After transfer to a Millipore HAHY membrane, they were hybridized with oligonucleotides GCO6, GCO2.5, GCO1.2, GCO1.05, and GCO1, whose sequences correspond to that of input Gc (10). Blot-hybridization patterns obtained with the three of these common probes, GCO6, GCO1.2, and GCO1, are shown along with that obtained with probe GCO2.9, whose sequence represents nucleotides 620–640 in *pilS1* copy 5 DNA and in pilin mRNAs of reisolates B-1 and B-2.

A single Cla I fragment in each Gc DNA hybridizes with GCO6, which encodes amino acids 1–7 (leader peptide); this Cla I fragment contains each Gc genome's single complete pilin gene. In DNAs of input Gc and most reisolates (A-1, A-2, A-3, A-4, and B-1) the complete gene-containing Cla I fragment is 4.1 kb, but in reisolates B-1 and B-2 it is slightly smaller (~4.05 kb). GCO2.9 hybridizes with 5.0 kb (*pilS1*) and 3.8-kb (Δ *pilE2*) fragments of input and reisolated Gc DNAs; it hybridizes with the complete pilin genes of reisolates B-1 and B-2, but not with that of input Gc. GCO1.2 hybridizes with partial pilin genes in the following Cla I fragments: 5.0, 4.1 (or 4.05), 3.8, 2.8, 1.8, and 0.9 kb. It also hybridizes with the complete pilin gene in input Gc and reisolates B-1 and B-2. GCO1 recognizes partial pilin gene sequences in the 3.8-kb Cla I fragment of input Gc and both reisolates. Only input Gc and reisolates B-1 contain the GCO1 sequence in their respective complete pilin genes; the complete pilin gene of B-2 does not hybridize with GCO1.

(flanking nucleotides 620–640) that correspond to those found in the B-1 and B-2 Gc reisolates' mRNAs.

A complete nucleotide sequence could not be obtained for pilin mRNA of reisolates B-2, as noted above; no extension was seen in RNA sequencing with the primer GCO1, whose sequence (pilin gene nucleotides 844–862) flanks (3') the pilin open reading frame. Blot-hybridization results with GCO1 provided an explanation. The complete expressed pilin gene of B-2 lacked the GCO1 sequence, whereas the complete pilin genes of input Gc and reisolates B-1 hybridized with GCO1 (Fig. 6). Analogous results were obtained with oligonucleotide GCO1.1 (nucleotides 820–836, amino acids 162–166 + TGA stop codon). The complete pilin gene of reisolates B-2 clearly lacked sequences homologous to

GCO1 and GCO1.1, although they have been found in all other pilin mRNAs that we have sequenced (10, 21, 22).

Discussion

Pilus⁺ Gc are adroit at shifting from synthesizing one pilin polypeptide to making another (8). Such changes in pilin structure result when a novel nucleotide stretch, with sequence identical to that of a silent partial pilin gene, is recombined into and expressed by the Gc genome's single complete pilin structural gene (10). The newly constituted chimeric complete pilin gene encodes a novel pilin polypeptide whose synthesis is usually tantamount to the appearance of antigenically unique pili on the pilus⁺ Gc variant's surface. Use of dissimilar partial pilin genes' sequences in different recombination events generates pilins with unique amino acid sequences (10). Antigenically distinct pilins also result when independent events recombine differing length stretches of the same partial pilin gene into the complete pilin gene (22).

All pilus⁺ Gc reisolated from two experimentally infected male subjects expressed pilin mRNAs whose sequences differed from that of input Gc. Most of the independent Gc reisolates from patient A expressed pilin polypeptides that were slightly different from one another, but they differed markedly from pilins of the Gc reisolated from patient B. Partial pilin gene *pilS1* copy 2 sequences resided in mRNAs of most reisolates from patient A; but *pilS1* copy 2 sequence did not appear in mRNAs of Gc reisolated from patient B. Instead, patient B Gc reisolates expressed mRNAs that contained long stretches of sequence from an uncharacterized partial pilin gene X (see below). Short segments of *pilS1* copy 5-like appeared in pilin mRNAs of Gc reisolates from both patients A and B; but their respective reisolates' mRNAs contained slightly different lengths of this sequence. In A-3 and A-4, *pilS1* copy 2 sequences flanked (both 5' and 3') their *pilS1* copy 5-like nucleotide 627-640 segment. In B-1 and B-2, *pilS1* copy 5-like nucleotides 600-640 were flanked by another partial pilin gene's (X) sequence. It seems likely that all these pilin mRNAs (A-3, A-4, B-1, and B-2) reflect two serial gene conversion events. The first event probably recombined a long stretch of *pilS1* copy 2 (or of gene X) into the complete pilin gene to generate a variant like A-1 (or its gene X-containing analogue). The second intragenic recombination event then effected integration of *pilS1* copy 5-like nucleotides into the *pilS1* copy 2 (or gene X) sequence stretches that resided in the complete pilin gene, and this produced new chimeric complete genes, each of which had sequences derived from two different partial genes.

The partial pilin gene origin(s) of *pilS1* copy 5 nucleotides 620-640 sequence (corresponding to oligonucleotide probe GCO2.9) in mRNAs of reisolated pilus⁺ Gc could not be defined. GCO2.9 sequence occurs in three different partial genes in *pilS1* (copies 1, 3, and 5) and in a partial gene within the 3.8-kb Cla I fragment ($\Delta pilE2$). The GCO2.9-like regions in mRNAs from A-3, A-4, B-1, and B-2 were flanked 5' and 3' by sequences that differ from those in *pilS1* copies 1, 3, or 5; the 3.8-kb Cla I fragment's GCO2.9-containing partial gene has unknown sequence. GCO2.9 sequences in A-3 and A-4 mRNAs clearly derived from one of these partial genes since it is flanked by *pilS1* copy 2 sequence in these two reisolates' mRNAs; but the *pilS1* copy 2 partial gene does not contain a nucleotide 620-640 region homologous to GCO2.9 (9). So, it is

not clear whether or not the GCO2.9 sequence found in reisolates' A-3, A-4 vs. B-1, B-2 pilin mRNAs emanated from the same partial pilin gene or not.

Others' studies (9, 26–28) on pilin polypeptides of diverse Gc suggest a model for Gc pilin wherein different oligopeptide regions are conserved, semivariable, or hypervariable as per their comparative amino acid sequences. Gc pilin polypeptides' NH₂-terminal one-third portions represent a lengthy conserved region of identical sequence for all Gc pilins. Several other short, structurally conserved oligopeptide stretches occur in diverse Gc pilins' COOH-terminal two-third portions; these include amino acid regions 71–75, 93–97, 122–132, and 151–160. The latter two conserved oligopeptide stretches include cysteines 128 and 158 that flank the hypervariable pilin domain. Within this hypervariable region, wholesale amino acid differences can occur among pilins of Gc from different strains and from strain MS11_{ms} variants (26) and among the MS11 genome's six silent pilin genes whose sequences are known (9). The hypervariable region of pilin lies within the polypeptide's CNBr-3 fragment (amino acids 100–167); this is thought to be an immunodominant domain whose amino acid sequences engender pilins' strain- and type-specific antigens (13, 15–19, 28). Amino acid changes in this hypervariable domain occurred only in reisolates B-1 and B-2 in the present study; in contrast, pilins from all reisolated Gc displayed multiple amino acid changes in their so-called semivariable regions. These findings demonstrate that type-specific epitopes or antigens cannot be assigned to one or another particular portion of the pilin polypeptide a priori since type-specific epitopes occur in both CNBr-2 and CNBr-3 regions of pilins for strain MS11_{mk} variants. It is not clear what constitutes strain-specific pilin antigens (13); these may represent pilin gene sequences that display preferential use in a given strain. Such preferential use occurs for pVD203-like sequence among pilus⁺ revertants of P⁻rp⁺ phenotype Gc in strain MS11_{mk} (22).

Interest in pilin peptides as potential vaccines has focused attention on a 69–84 oligopeptide (our 76–91) that resides in the largely conserved CNBr-2 fragment (amino acids 15–99); this oligopeptide elicits formation of antibodies that reportedly crossreact with several different pilins and that recognize their antigenic target on intact, polymeric pili from different Gc strains (18, 19). The mAb 9B9 epitope has been mapped to this 69–84 (76–91) oligopeptide (18) whose sequence occurs in pilin mRNA of our input Gc. Rabbit antiserum against this oligopeptide reacted with a tryptic fragment of pilin known to bind human endocervical cells, and it also inhibited adherence of Gc bearing either homologous or heterologous pili to a human endometrial carcinoma cell line (19). Accordingly, the 69–84 (76–91) oligopeptide was suggested as a possible gonorrhea vaccine on the assumption that it would evoke formation of antibodies that would reduce or abrogate attachment of Gc to host mucosal cells and thereby disrupt this phase of Gc pathogenicity. But amino acid changes are found in this oligopeptide region of pilins in all Gc reisolated from the two experimentally infected subjects and the mAb 9B9 epitope was absent from all Gc reisolates' pilins. Changes in this region of reisolates' pilin mRNAs are similar to those found by others among MS11 variants and several clinical isolates (26). These data suggest that a 69–84 (76–91) oligopeptide will not be effective as a gonorrhea vaccine.

The multiple, distinct partial pilin genes in the MS11_{mk} Gc genome provide a

repertoire of sequences that encode novel pilins when their sequences are recombined into the expressed, complete pilin gene (9, 10). Different length stretches from a given region of each partial pilin gene can be integrated into the expression locus by different gene conversion events (22); antigenically variant pilins result. The present findings show another facet of Gc pilin structural diversity; regions of a partial pilin gene sometimes incompletely replace the partial gene-sequence stretch recombined into the complete pilin gene by the preceding gene-conversion event. The resulting chimeric complete pilin gene thereby comes to contain bits and pieces from several different partial pilin genes. These observations suggest that an almost infinite variety of pilin polypeptides can be synthesized by the variants in a given Gc strain.

The size change in the Cla I fragment carrying *pilE1* of reisolate B-2 was the first such gross change we have observed that correlated with a pilin gene-conversion event. This size change correlated with a marked change in sequence for the 3' terminal portion of the B-1 complete pilin gene; it failed to hybridize with oligonucleotides downstream of pilin gene nucleotide 820 (i.e., probes GCO1.1, GCO1.05, and GCO1).

Because the Gc pilin variants in this study arose before a discernible antibody response in the experimentally infected subjects, we surmise that such variants have roles during initial phases of Gc infection in immunologically naive hosts. It is tempting to speculate whether other, different pilin variants would have arisen later in the experimental infections, after the appearance of antipilus antibodies. Would the later variants exhibit striking changes in their intercyysteine regions, as per others' findings (27), in contrast to changes we find in more NH₂-terminal portions of pilus⁺ reisolates' pilin polypeptides? Only Gc challenges of individuals who have previously developed antipilus antibodies or a longer duration study of Gc variants that arise during infection of immunologically naive subjects will answer that question. But it is clear that Gc pilin gene-recombination events produce novel pili whose antigenic differences stem from amino acid changes in several oligopeptide regions of their respective pilin polypeptides.

Summary

When pilus⁺ Gc were introduced into a male subject's urethra, they gave rise to pilus⁺ variants whose pilin mRNAs differed from that of input Gc. The differences stemmed from the Gc genome's single complete pilin gene having undergone gene conversion by different partial pilin genes' sequences and by different length stretches of a single partial pilin gene. In some instances, the variant's pilin mRNA appeared to reflect two independent gene-conversion events that used sequences from two different partial pilin genes. The resulting variants' pilins exhibited antigenic differences compared with the pilin polypeptide of input Gc; these differences were discernible by immunoblotting with mAbs. Amino acid and antigenic changes occurred in a segment of the variants' pilin polypeptides that previously was thought to be conserved or constant in sequence.

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