GENE CONVERSION VARIATIONS GENERATE STRUCTURALLY DISTINCT PILIN POLYPEPTIDES IN *NEISSERIA GONORRHOEAE*

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Reversible transitions in pilus expression by *Neisseria gonorrhoeae* include switches in piliation phase ($pilus^+ \rightleftarrows pilus^-$) and changes in pilin subunit structure ($pilin_a^+ \rightarrow pilin_b^+$) (1–3). Both correlate with nonreciprocal recombination (gene conversion) involving the gonococcus' $(Gc)^1$ complete expressed pilin gene (expression locus) and one partial pilin gene (silent locus). DNA identical in sequence to one of the Gc genome's multiple partial pilin genes becomes incorporated into the complete pilin gene with attendant eviction of the analogous portion of the complete gene's previously expressed version (4). The result is a new chimeric pilin structural gene that encodes a corresponding new pilin polypeptide.

Sequence diversity among the Gc genome's partial pilin gene repertoire is a major reason that a given Gc strain's variants can express antigenically distinct pilin polypeptides. An estimated 12–16 partial genes compose this repertoire in strain $MS11_{mk}$ (4). They each contain sequences analogous to the 3′ half to two-thirds of the complete pilin gene; different partial genes exhibit considerable homology, but each also contains unique sequences (5). When the complete pilin gene of $pilus^+$ Gc undergoes gene conversion by a partial gene's sequence, the resultant Gc synthesize a structurally distinct pilin and usually display $pilus^+$ phenotype; but expression of the sequence from partial pilin gene *pilS1* copy 5 (5) leads to synthesis of an assembly-missense pilin and no pilus formation (P^-rp^+ phenotype) (1, 6). Such P^-rp^+ Gc revert to $pilus^+$ status when their complete pilin gene undergoes a subsequent gene conversion to encode an orthodox pilus-producing pilin polypeptide. The geneses of P^-rp^+ phenotype Gc and their $pilus^+$ revertants are clearly analogous to switches in which $pilus^+$ phenotype is retained but the pilin polypeptide synthesized is changed.

Pilin mRNAs were sequenced for six such $pilus^+$ revertants from a common P^-rp^+ parent to understand the above-described pilin gene conversion events more fully. Four of these revertants' pilin mRNAs contained sequences attributed to the same silent pilin gene. But each of the four revertants' pilin mRNAs

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[1] *Abbreviation used in this paper:* Gc, gonococcus.

contains a different-length stretch of that silent gene's sequence; those differences correlate with expression of antigenically diverse pilin polypeptides by these revertants. Two other revertants' mRNAs bear sequences from another silent locus, are identical to one another, and encode a pilin polypeptide that is immunochemically distinct from pilins of the other pilus$^+$ revertants.

Our results show that structurally and antigenically distinct pilin polypeptides are synthesized by sibling pilus$^+$ revertants of P$^-$rp$^+$ Gc when the parent's complete pilin gene undergoes (*a*) independent gene conversion events by sequences of different partial pilin genes, or (*b*) independent gene conversions by differing-length sequences of the same partial pilin gene.

Materials and Methods

Gonococci (Gc). Strain MS11$_{mk}$ was grown at 37°C on clear medium as described before (1). Gc with P$^-$rp$^+$ phenotype and their pilus$^+$ revertant colonies (with P$^+$ or P^{++} morphotype) were selected with a dissecting microscope, and their piliation (or lack thereof) was confirmed by scanning electron microscopy as described earlier (4). All Gc used were outer membrane protein II$^-$ and exhibited nonopaque (O$^-$) colonial morphology.

Monoclonal Antibodies. mAb 9B9 was obtained from Emil Gotschlich (The Rockefeller University). mAbs 9B9 and 1H5 were generated by immunizing BALB/c mice with purified MS11 pili, while mAbs 02 and 04 were obtained from mice that received whole, pilus$^+$ Gc of strain JS3. Spleen cells of immunized mice were fused with BALB/c NS1/1 cells. Three of these mAbs bound ^{125}I-labeled protein A; the fourth (9B9) required use of rabbit anti–mouse antibody (Cooper Biomedical, Malvern, PA) before incubation with radioiodinated protein A.

Immunoblotting. Suspensions (OD_{550} = 0.6) were made of each Gc phenotype preparation in PBS, and desired amounts were mixed with solubilizing solution (4% SDS plus 2-ME), and then boiled. Separation in 15% acrylamide gels, transfer to nitrocellulose, incubation with monoclonal antibodies, and subsequent steps were exactly as described before (3).

RNA Extraction and Pilin mRNA Sequencing. Gc grown for 18–20 h on solid medium were suspended in cold guanidine isothiocyanate lysis buffer and extracted with hot (60°C) acidic (pH 5.0) phenol. These methods, along with primer extension sequencing of pilin mRNA using pilin-encoding oligonucleotides were done as described previously (6).

Results

Pilin mRNAs of P$^-$rp$^+$ Parent Gc and Their Pilus$^+$ Revertants. P$^-$rp$^+$ phenotype parental Gc (P$^-$rp$^+$, Fig. 1) arose from multiple cycles of P$^-$rp$^+$ pilus$^+$ transitions. From a single P$^-$rp$^+$ colony's progeny, three sibling colonies (*P$^-$rp$^+$, Fig. 1) were selected and were passaged once on solid medium. On the next day, two pilus$^+$ revertants (one each of P^{++} and P$^+$ colony morphotype, as defined by Swanson and Barrera [3]) were selected from each *P$^-$rp$^+$ parental culture; these six pilus$^+$ revertants (A–E) and their immediate parents (*P$^-$rp$^+$) were subjected to RNA extraction and pilin mRNA sequencing after expanding their number by a single passage.

The three *P$^-$rp$^-$ parent Gc mRNAs were identical to one another, are hereafter noted simply as P$^-$rp$^+$ parent, and contain multiple nucleotide differences compared with the cloned pilin gene in recombinant plasmid pVD203 (6): these include a 117 nucleotide block (662–779) in P$^-$rp$^+$ mRNA that derives from partial gene *pilS1* copy 5 (see Fig. 5 in Swanson et al. [4] and Haas and

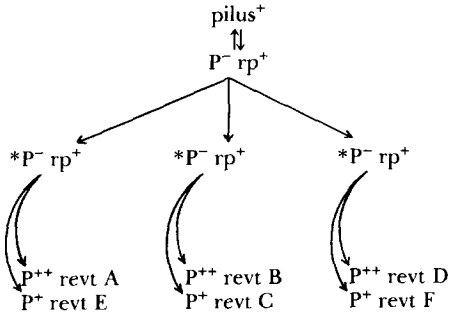

FIGURE 1. $MS11_{mk}$ Gc examined in this study bear the depicted genealogy. Original P^-rp^+ phenotype parent Gc colony was derived from multiple cycles of $pilus^+ \rightleftarrows P^-rp^+$ transitions; among its progeny, three colonies ($*P^-rp^+$) of parental phenotype were selected and passaged once. The next day, two $pilus^+$ revertants (revt) (one each of P^{++} and P^+ morphotype) were selected from each P^-rp^+ phenotype's progeny and were passaged to expand their number. Pilin mRNAs from these six $pilus^+$ revertants (A–E) and their respective P^-rp^+ precursors (noted by asterisk) were examined by primer extension sequencing (Fig. 2); whole-cell lysates of these Gc were subjected to SDS-PAGE followed by immunoblotting with antipilus mAbs as shown in Fig. 3.

Meyer [5]), and additional upstream (516–661 region) single-base changes. These sequence differences aid the definition of partial pilin gene–derived nucleotides, especially if they are pVD203-like, that newly appear in complete pilin genes of $pilus^+$ revertants. As discussed later, a pVD203-like sequence regularly appears among such revertants and seems to reside in several silent loci of the $MS11_{mk}$ genome. The following presentation assumes that a single silent partial pilin gene contributed the pVD203-like sequence for the independent recombination events that generate $pilus^+$ revertants A–D. Each of these revertant's pilin mRNA bears a different length stretch of the pVD203-like sequence; the longest stretch (254 nucleotides, 523–777) occurs in revertant A, and the shortest (38 nucleotides, 741–779) is in revertant D. Pilin mRNAs of revertants B and C contain pVD203-like sequence for 237 nucleotides (540–777) and 91 nucleotides (688–779), respectively (Fig. 2).

A distinctly different partial gene sequence resides in mRNAs of $pilus^+$ revertants E and F; these two revertants' pilin mRNAs have identical sequence changes (compared with P^-rp^+ parent) for a 236-nucleotide block (561–797). Upstream and downstream of this newly inserted block, revertants' E and F pilin mRNAs are identical to their P^-rp^+ parents. These two revertants' pilin mRNAs encode the same pilin polypeptide, 170 amino acids long, compared with pilins of 166 amino acids encoded in the P^-rp^+ parent's and the other four $pilus^+$ revertants' (A–D) mRNAs.

Amino Acid Sequence and Antigenic Differences in $Pilus^+$ Revertants' Pilins. Sequence changes noted above in mRNAs of P^-rp^+ Gc and their $pilus^+$ revertants' result in several amino acid differences in their respective pilin polypeptides, as shown in Fig. 2 and summarized in Fig. 4 in comparison with pVD203. Antigenic differences accompany these amino acid changes, as seen when parental P^-rp^+ Gc and their $pilus^+$ revertants pilins are examined in immunoblotting with mAbs 02, 1H5, and 9B9 (Fig. 3); all these monoclonals react with pVD203-encoded pilin (not shown).

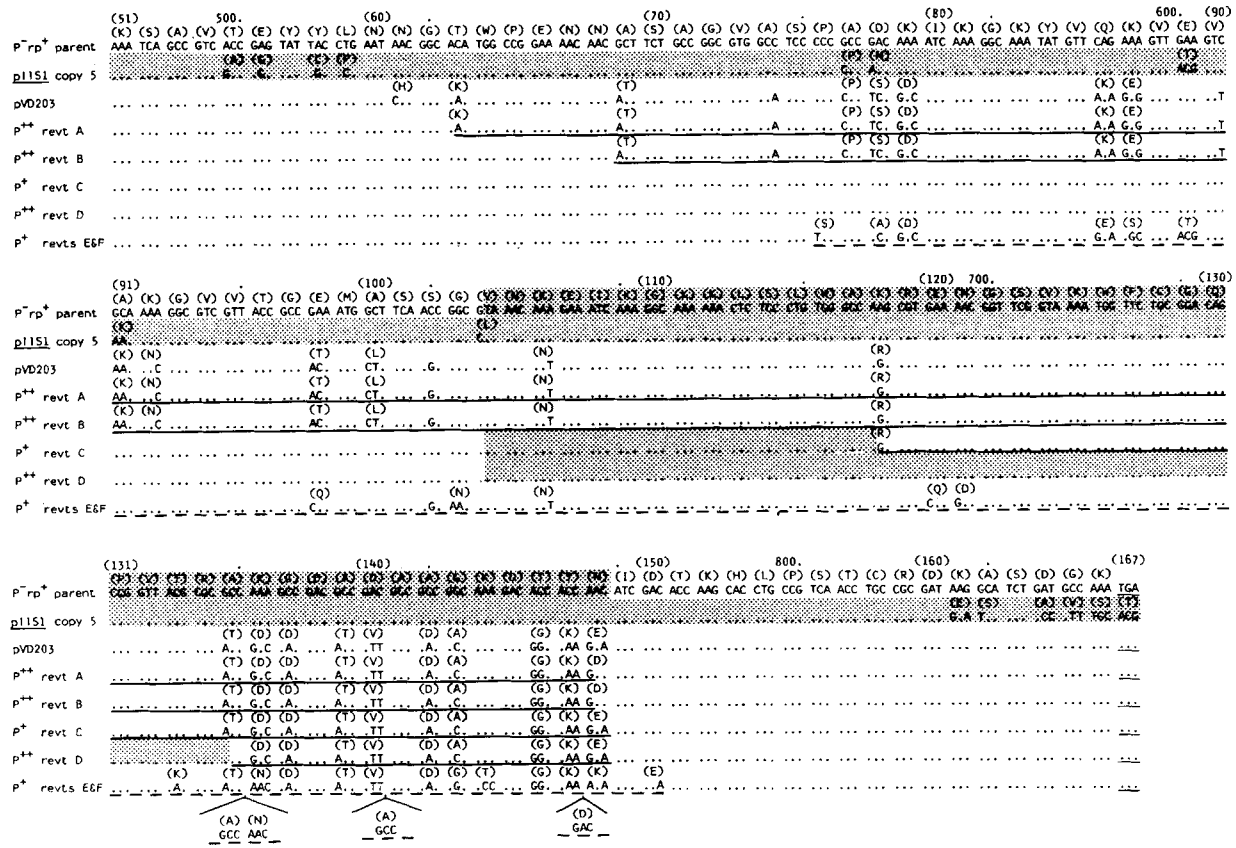

FIGURE 2. Pilin mRNA and deduced amino acid sequences are shown for P⁻rp⁺ Gc and six pilus⁺ revertants (revt) (A–F) along with DNA sequences of the complete pilin gene in pVD203 and the *pilS1* copy 5 partial gene. Only nucleotides encoding amino acid 51 to the TGA termination codon are included; alignments are for maximal homology. Nucleotide positions are noted (500, 600, etc.), as is each 20 nucleotide interval (.); amino acids and their positions are noted in parentheses. Pilin mRNAs from three P⁻rp⁺ phenotype Gc (*P⁻rp⁺ in Fig. 1) were sequenced and gave identical results, and that sequence is noted as P⁻rp⁺ parent. P⁻ rp⁺ parent mRNA contains a 133-nucleotide region (646–779, *shaded region*) homologous to partial pilin gene *pilS1* copy 5. In four pilus⁺ revertants (P⁺⁺, A; P⁺⁺, B; P⁺, C; and P⁺⁺, D), pilin mRNA sequence homologous to pVD203 occurs in varying stretches (*solid line*) as described in the text. Note the *pilS1* copy 5 sequence (*shaded*) in P⁺ revertant C (646–686) and in P⁺⁺ revertant D (646–740) that was not replaced by incoming pVD203-like sequence. Differing downstream (3′) terminations of pVD203-like sequence occur in P⁺⁺ revertants A and B (position 777) versus P⁺ revertant C and P⁺⁺ revertant D (position 779); the nucleotide 777 junction effects an amino acid change not encoded by either the P⁻rp⁺ parent or pVD203 sequences. The intercysteine regions (amino acids 128–158) encoded by pilus⁺ revertant A–D's pilin mRNAs are identical. Newly acquired sequence in pilin mRNAs of pilus⁺ revertants E and F consist of nucleotides 561–797 (*dashed line*). Their mRNAs encode pilins 171 amino acids long compared with 167-amino-acid-long pilins polypeptides of P⁻rp⁺ parent and their sibling revertants, A–D. This is due to the newly inserted sequence of revertants E and F containing four more codons than the analogous regions of P⁻rp⁺, pVD203, and revertants A–D as per the nucleotide alignment shown.

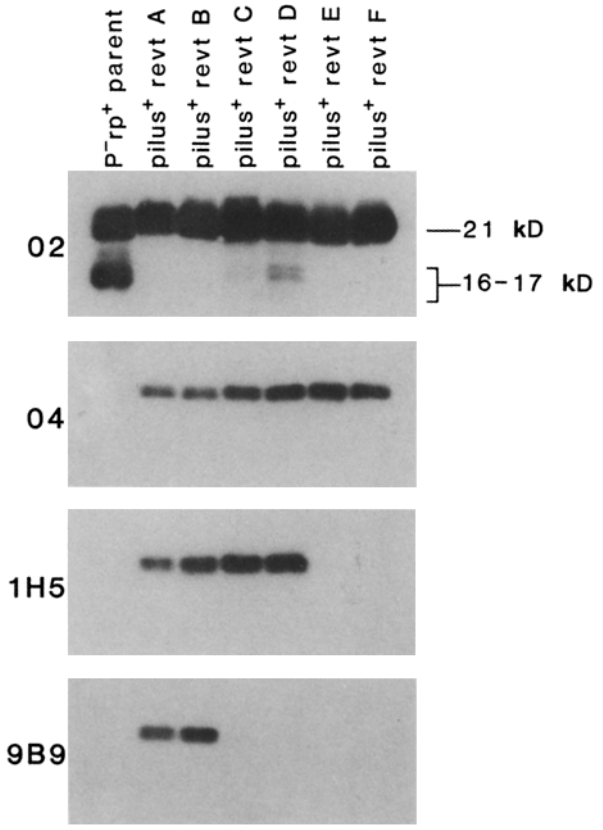

FIGURE 3. Immunoblotting of P^-rp^+ and $pilus^+$ revertant Gc with four mAbs. mAb 02 reacts not only with the M_r ~21,000 pilin polypeptide found in both parental and revertant (revt) Gc, but also with faster-migrating pilin polypeptides (M_r 16,000–17,000) occurring in P^-rp^+ Gc as well as in $pilus^+$ revertants C and D. mAb 02 is broadly crossreactive with pilins from all Gc strains examined. mAb 04 reacts with all six $pilus^+$ revertants but not with the P^-rp^+ parent. mAb 1H5 yields an immunoblotting signal with revertants A–D, but displays no reaction with either P^-rp^+ parent nor $pilus^+$ revertants E and F. mAb 9B9 recognizes an epitope found only on pilins of revertants A and B.

mAb 02 reacts with an undefined epitope that is present on all pilins we have examined to date among diverse Gc strains; this mAb recognizes the pilin proteins (M_r ~21,000) of the P^-rp^+ parental Gc and all $pilus^+$ revertants. mAb 02 also recognizes faster-migrating proteins (M_r 16,000–17,000) that are typical for P^-rp^+ phenotype Gc (1, 4), but which are also seen in $pilus^+$ revertants C and D.

mAb 04 recognizes an epitope that is present on all the $pilus^+$ revertants' pilins but absent from parental P^-rp^+ Gc pilin. Comparison of the mRNA sequences and immunoblotting reactivities with mAb 04 for these and several other variants in $MS11_{mk}$ (not shown) suggest that the mAb 04 epitope involves three amino acids (aspartic acids 136–137 of revertants A–D and 137–138 of revertants E and F; and glycine 146), but the epitope's location cannot be deduced more precisely from present data.

mAb 1H5 recognizes pilins of $pilus^+$ revertants A–D but neither those of the P^-rp^+ parent nor revertants E and F. This mAb (1H5) reacts with a synthetic oligopeptide representing pVD203 pilin amino acids 128–141 (M. Blake, personal communication). In that region, only amino acid position 136 exhibits differences among the $pilus^+$ revertants that correlate with their observed reactivity patterns with mAb 1H5; therefore, amino acid 136 plus adjacent but undefined residues likely make up this mAb's epitope.

mAb 9B9 reacts with two of the $pilus^+$ revertants (A and B), but it does not

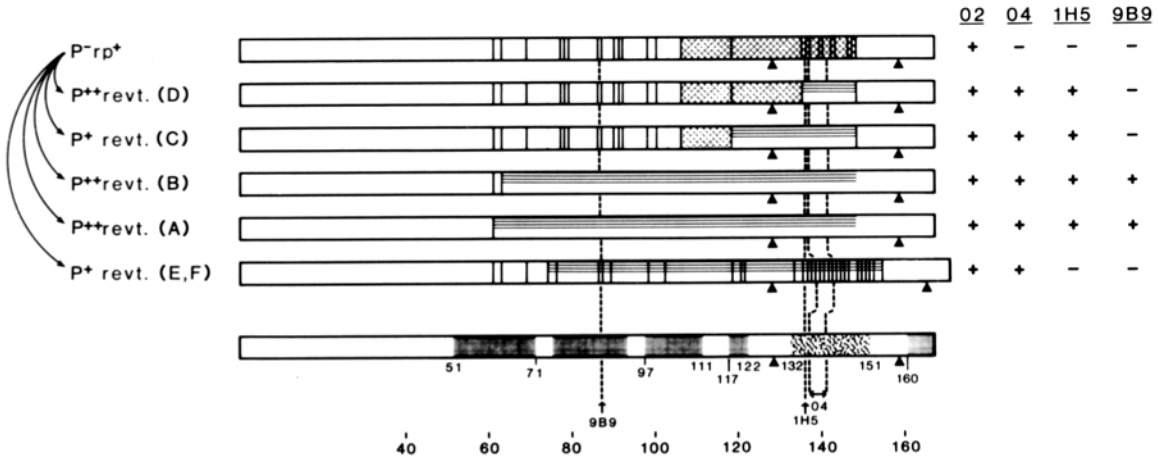

FIGURE 4. Pilin amino acid sequences of P^-rp^+ parent and $pilus^+$ revertant (revt) Gc are summarized by comparison with pVD203 sequence. Differences in each pilin versus that encoded by pVD203 are indicated by vertical lines at appropriate amino acid locations (residue locations as per scale at bottom). The portion of *pilS1* copy 5 found in pilin mRNA of P^-rp^+ Gc is indicated (*crosshatching*) as are remnants of that *pilS1* copy 5 sequence that remain in $pilus^+$ revertants D and C. Cysteine residues are noted (▲) as are the tentatively assigned locations of epitopes for mAbs 9B9, 04, and 1H5. Locations of Gc pilin polypeptides' constant (*open bars*), semivariable (*shaded*), and hypervariable domains (*marbled*) are shown in the bottom pilin polypeptide diagram, as formulated by others (5). Immunoblotting results for each pilin with mAbs 02, 04, 1H5, and 9B9 are shown as plus or minus.

recognize pilins of either the P^-rp^+ parent or the $pilus^+$ revertants C–F. The mAb 9B9 epitope was localized by others (8) to the amino acid 76–91 region (their 69–84 oligopeptide). Our findings also place the mAb 9B9 epitope in this region; and additional data from other $MS11_{mk}$ variants (our unpublished data) suggest that changes in amino acids 86–87 abolish reactivity of this mAb's epitope.

Discussion

Previous studies have demonstrated marked sequence variation of pilin polypeptides synthesized by Gc that represent either inter- or intrastrain variants. Amino acid sequence changes appear in the pilins' hypervariable domains (between cysteine residues 128 and 158) as well as in their semivariable regions (6, 9). Such differences have been attributed to expression of different silent, partial pilin genes' sequences after their incorporation into the expressed complete pilin gene via gene conversion (4, 5). Sequence differences among the Gc genome's repertoire of multiple partial pilin genes clearly account for some of the observed variations in pilin structure; known examples include the *pilS1* copy 5 sequence in pilin mRNA of P^-rp^+ phenotype Gc and of *pilS1* copy 2 sequence in $pilus^+$ Gc reisolated from men whose urethrae were infected with Gc the pilin mRNAs of which have pVD203-like sequence (our unpublished data). These are clear instances where different partial pilin genes' sequences are expressed by Gc and lead to synthesis of structurally and antigenically distinct pilin polypeptides. $Pilus^+$ revertants E and F in the current study contain sequences derived from another, uncharacterized partial gene.

Our present studies point to an additional feature of pilin gene recombination that operates to generate structurally distinct Gc pilin polypeptides, namely, variation in the extent to which a given silent, partial pilin gene's sequence is recombinationally inserted into the Gc genome's expressed pilin gene during independent gene conversion events. In general, each gene conversion event produces a novel complete pilin gene chimera the 5′- and 3′-terminal portions of which are residua of the previously expressed parental pilin gene, and the subterminal 3′ portion of which contains a block of nucleotides with a sequence like that of a silent partial pilin gene. We find different-length stretches of one partial gene's sequence in the chimeric complete pilin genes that are constituted by independent gene conversion events; this implies that pilin gene conversion has no strict requirements regarding lengths of nucleotides undergoing recombinational insertion into the complete pilin gene.

The use of different stretches of a partial gene in different gene conversion events also means that a previously inserted partial pilin gene sequence may not be replaced completely in the complete pilin gene during the next subsequent gene conversion event; so each newly constituted chimeric complete pilin gene may contain sequences representing different partial pilin genes that were involved in several earlier gene conversion events. For example, in the genesis of the parental P^-rp^+ phenotype Gc used here, a 126-nucleotide stretch (653–779) of *pilS1* copy 2 sequence was inserted into the pilin expression locus; that 126-nucleotide-long span in the P^-rp^+ parent's expressed pilin gene is incom-

pletely replaced by new, incoming pVD203-like sequence stretches of 91 and 38 nucleotides in pilus$^+$ revertants C and D, respectively.

Epitopes found in pilins of pilus$^+$ revertant Gc represent admixtures of those encoded by the P$^-$rp$^+$ parent's pilin gene and newly inserted sequences. This admixture varies due to differences in the upstream and downstream junctions between old and new incoming pilin gene sequences. Our present data indicate neither the full range of such variation nor the extent of amino acid differences among the resultant pilin polypeptides, but the multiplicity of sequence differences between P$^-$rp$^+$ versus pVD203 pilin mRNAs predict creation of additional unique chimeric pilin genes among yet-undefined pilus$^+$ revertants through gene conversion of a common P$^-$rp$^+$ parent's complete pilin gene with the same pVD203-like partial pilin gene.

One particular pilin sequence (pVD203-like) occurred in most pilus$^+$ revertants. That this pilin sequence is recurrently and, perhaps, preferentially expressed by pilus$^+$ Gc of strain MS11 is shown best by three groups having found virtually identical, pVD203-like pilin sequences for pilus$^+$ organisms of this strain genealogically separated by multiple in vitro passages in each lab (6, 9, 10). In the present study, the partial pilin gene origin(s) of pVD203-like sequence in our pilus$^+$ revertants is/are not entirely clear. Blot hybridization results suggest that three partial pilin genes have nucleotide 744–767 regions resembling pVD203 in MS11$_{mk}$ (4) and, hence, any or all three might contribute sequence for the gene conversions that generate our pilus$^+$ revertants A–D. If analogous stretches of three different pVD203-like partial pilin genes participated in the gene conversions observed here, the results might resemble products from different gene conversions by different-length blocks of a single partial pilin gene. We think that this is unlikely for two reasons. First, only three partial pilin genes of MS11$_{mk}$ contain GCO (P$^+$ [744–767]) sequence (pVD203-like for nucleotides 744–767); so at least two out of four pilus$^+$ revertants contain sequences from the same partial pilin gene. Second, clear evidence of variable contributions from the well-characterized *pilS1* copy 5 partial gene are found in the pilin mRNAs of different P$^-$rp$^+$ phenotype Gc (compare P$^-$rp$^+$ in Swanson et al. [4] with the P$^-$rp$^+$ parent used here). Variable-length stretches of another partial pilin gene, *pilS1* copy 2 (5), have been found in pilin mRNAs of Gc from experimental human infections after challenge with Gc whose pilins are pVD203-like (our unpublished data). Taken together, these data show that gene conversions events that involve different-length stretches of a single pVD203-like partial pilin gene account for differences observed among our pilus$^+$ revertants A–D from a common P$^-$rp$^+$ parent.

Two partial genes are likely candidates for donation of pVD203-like sequence in the pilin gene conversions we observed here. One is the partial pilin gene lying immediately upstream of the complete pilin gene (in a 0.5 kb Sma I fragment contained in the 4.1 kb Cla I fragment); although this partial gene lies closest to the expression locus, its blot hybridization signal with the pVD203-like GCO (P$^+$ [744–767]) probe suggests incomplete homology, and it lacks both 3′-terminal and flanking sequences of pVD203 (4). The other possible partial pilin gene sequence donor resides in the 3.8 kb Cla I fragment that contains a 5′-deleted form of a previously complete pilin gene (*pilE2*). This is the only partial

pilin gene we can find in the $MS11_{mk}$ genome that contains 3′-terminal and flanking sequences homologous to pVD203, i.e., hybridizes with oligonucleotide probes GCO (P^+ [744–767]) and GC01 (4). Such extensive sequence homology may dictate the preferred use of this partial gene's sequence in gene conversions of P^-rp^+ phenotypes' complete pilin gene to generate $pilus^+$ revertants in strain $MS11_{mk}$.

The above-described findings provide evidence that independently occurring gene conversion events involving any silent pilin gene can generate antigenically distinct pilins through variations in the stretches of silent gene sequence that are recombined into a pilin expression locus. Such variable usage of any given partial pilin gene sequence in gene conversion significantly expands the number of antigenic variant pilins that Gc can synthesize on the basis of a finite pilin gene repertoire. It also suggests lack of specificity in either the length or the sequence of nucleotides that are recombinationally inserted into a complete pilin gene by gene conversion.

Summary

$Pilus^+$ to $pilus^-$ phenotype change occurs in *Neisseria gonorrhoeae* through gene conversion of the gonococcus' complete, expressed pilin gene by nucleotides homologous to the *pilS1* copy 5 partial pilin gene; assembly missense pilin is synthesized but pili are not. Reversion to $pilus^+$ occurs by a subsequent recombinational event that replaces the complete pilin gene's *pilS1* copy 5–like sequence with nucleotides from a different partial gene to effect expression of an orthodox (i.e., pilus producing) pilin. Sibling $pilus^+$ revertants of common parentage can carry different sequences in their expressed pilin genes because they have undergone nonidentical gene conversion events such as (*a*) recombinations with sequences from different partial genes, or (*b*) recombinations with different length nucleotide stretches of the same partial gene; either can yield structurally and antigenically variant pilin polypeptides.

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