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Genetics

Release of soluble pilin antigen coupled with gene conversion in *Neisseria gonorrhoeae*

(antigenic variation/piliation phases/extracellular secretion)

RAINER HAAS, HEINZ SCHWARZ, AND THOMAS F. MEYER

Max-Planck-Institut für Biologie, Infektgenetik, Spemannstrasse 34, D-7400 Tübingen, Federal Republic of Germany

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ABSTRACT **Gene conversion appears to be the frequent mechanism in *Neisseria gonorrhoeae* that leads to an altered expression of pilin, the subunit component of the pili. In this process segments of variable sequence information, the minicassettes, are transferred from silent storage loci into an expression locus. As a putative consequence of the rearrangement in the *pilE* gene, gonococci can enter a different phase of pilin production. Although the removal of a 7-amino acid leader peptide results in the production of typical P^+ pilin used to form pili, the loss of an additional 39 amino acids yields S-pilin, a soluble form of pilin that is efficiently secreted into the extracellular environment. Both pilin types can coexist in an apparently homogeneous culture. P^s cells usually are piliated, although less extensively with regard to the length and the number of the pili when compared with P^+ cells. P^s cells form T3/T4-type colonies also typical of nonpiliated cells (P^-). The observations further suggest that the classical nonsecretory P^- phenotype is not generated as a rule by precise gene conversion but rather by genetic changes that cause the production of an over-length pilin (L-pilin).**

The Gram-negative coccus *Neisseria gonorrhoeae* is a major cause of sexually transmitted disease in humans (for a survey, see ref. 1). This bacterium exhibits a striking capacity to vary the expression of its pilus protein (pilin), the subunit component of the pilus structure conferring adhesion of the bacterium to the host epithelial cells (2, 3). The pilus formation in gonococci can spontaneously turn on and off (commonly referred to as phase variation), and a single cell frequently gives rise to variant offsprings that produce pilin with altered serological and functional properties (antigenic variation) (4, 5). Many *pil* genes are found in the genome of gonococci (6), most of them containing variant sequence information, lacking expression signals and 5′-terminal coding sequences (silent copies, *pilS*) (7, 8). Only one or two *pil* genes have a promoter and complete coding sequences (*pilE*) (7), and these expression genes seem to be constitutively transcribed (9). Silent and expressed *pil* genes each contain six variable gene segments, the minicassettes, that are flanked by strictly conserved sequences (8). The minicassettes are frequently transferred from silent loci into the expression loci by a process typical of gene conversion, programing the expression gene to produce altered pilin (8, 10, 11). In this report we provide evidence for two further phases of pilin production in gonococci, one of which is characterized by the extracellular release of soluble pilin molecules (S-pilin).

MATERIALS AND METHODS

Bacterial Strains and Derivation of Gonococcal Variants. Hybrid plasmids containing gonococcal DNA fragments were transformed into *Escherichia coli* GC1 (6) and subsequently transferred to strain DH1 (12) for large-scale preparation of plasmid DNA. All experiments described are based on *N. gonorrhoeae* strain MS11 originally obtained from E. Gotschlich (Rockefeller University). Single colonies of gonococci were passaged every 18–22 hr on fresh typing agar plates (GC agar base, Baltimore Biological Laboratory) that were supplemented with Isovitale-X and incubated at 37°C and in 5% CO_2 in air. Starting from the original piliated MS11 variant (showing the T2 phenotype) a variety of pilus phase and antigenic variants (T2 and T4) were derived (T1–T4 indicate colony morphology phenotypes). All passages were carefully monitored by using an Olympus binocular microscope operated with transmitted light.

DNA Manipulations. Gonococcal DNA isolation was done as described (13). Restriction enzymes were used according to conditions recommended by the vendors (New England Biolabs, Boehringer Mannheim, and Pharmacia). Expression genes of *pilE1* from various pilus variants were cloned as *Cla* I fragments in plasmid pBA (14). Pilin expression genes were subcloned as *Hpa* I–*Cla* I fragments in pEMBL8 or pEMBL9 (15) and sequenced according to the dideoxynucleotide method (16, 17) using synthetic primers complementary to constant pilus gene sequences (8). DNA filter hybridizations with specific oligonucleotides were performed essentially as described (18).

RNA Preparation and RNA Transfer Blotting. For determination of *pil* mRNA, total RNA was prepared (18) and subjected to electrophoresis on 2% agarose containing 20% formaldehyde. The RNA was blotted onto nitrocellulose and hybridized with the ^{32}P-labeled oligonucleotide complementary to codon positions 1–6 of the pilus gene (RH26) under conditions identical to those described for DNA filter hybridizations (19).

Transmission Electron Microscopy (TEM). Gonococci were adsorbed to pioloform-coated grids, air-dried, and shadowed with carbon/platinum. The samples were viewed in a Philips EM201 at 60 kV.

Quantification of Pilus Antigen. For determination of total pilin synthesis, colonies were collected from agar plates and lysed in sample solution (20). Samples were sonicated, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and immunoblotted. The immunoblots were incubated with rabbit serum raised against a genetically engineered pilin fusion protein, FP.AC7, exhibiting amino acids 21–68 of the conserved/semivariable region of pilin (21), and further developed by using alkaline phosphatase-coupled anti-rabbit secondary antibody (22). For quantification of secretory pilin, gonococci were grown in culture medium to an OD_{595} of 0.8. Cells were collected by centrifugation. The pelleted fraction was resuspended in sample solution and homogenized by sonication. Culture supernatants were precipitated

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Abbreviations: P^+, P^-, P^s, piliation phases of gonococci; T1–T4, colony morphology phenotypes; S-pilin, soluble secretory pilin; L-pilin, over-length pilin; TEM, transmission electron microscopy.

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with 10% trichloroacetic acid on ice, centrifuged, washed with 80% ethanol, and resuspended in sample solution. Aliquots of both samples were separated on 15% NaDod-SO_4/polyacrylamide gels and immunoblotted as above.

Preparation and Sequencing of S-Pilin. Cells of variant B1 were collected from a one-liter culture grown in a Pellicon-filtered (Millipore) medium as described for the isolation of extracellular IgA protease (23). From the supernatant, S-pilin was precipitated with 10% trichloroacetic acid, and the pellet was washed twice in 80% ethanol, dried, and resuspended in sample solution. S-pilin was further purified by a preparative $NaDodSO_4$ gel electrophoresis; the pilin band was excised and isolated by electroelution. Sequencing was done with 50 pmol of S-pilin, using an ABS-A470 protein sequencer.

RESULTS

Isolation and Characterization of Isogenic Pilus Variants. By passing on typing agar and alternate screening for the T2 or T4 colony phenotypes (24), we obtained an inherent series of pilus variants of *N. gonorrhoeae* strain MS11 (6) (Fig. 1). These variant lines were investigated with respect to their morphological, genetic, and biochemical properties. By TEM we observed that the piliation state of the variant cells did not strictly correlate with the colony phenotype: bacterial cells of some colonies with the T4 phenotype—e.g., variants B3, D4, F1, F3, and F7—were heavily piliated, quite like the T2 (P^+) variants A and C (Fig. 2). This was unexpected since hitherto the T4 phenotype was believed to be typical of nonpiliated gonococci (24). Due to these dramatic differences in colony morphology among P^+ variants we happened to isolate variants that could be considered as the result of direct antigenic (P^+ to P^+) switches.

TEM analysis further revealed that other T4 colonies contained cells with low or intermediate piliation, such as the B1, B5, D5, and D3 variants (Figs. 1 and 2). This class of variants produces pilin that appears to be processed into a form of lower molecular weight (for example, variant B1 in Fig. 3*b*; see also Fig. 5*a* for similar variants). No pili could be detected on the surface of variants D1 and F5 (see Fig. 2*f* for variant D1). Thus we isolated in this experiment three classes of isogenic pilus variants (Figs. 1 and 2)—i.e., piliated variants (P^+), nonpiliated variants (P^-), and variants whose pilin was (partially) converted into a smaller form (termed P^s).

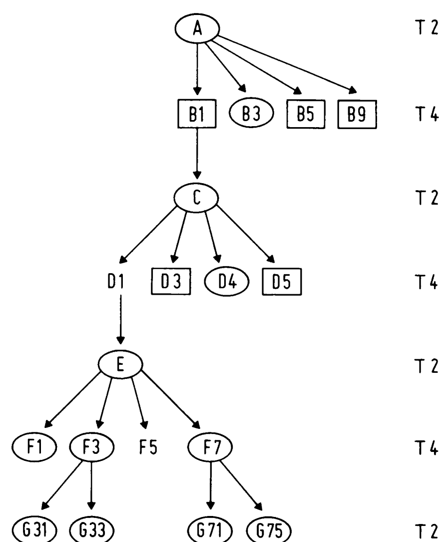

FIG. 1. Derivation scheme of isogenic pilus variants of *N. gonorrhoeae* strain MS11. Variant lines were established by the criteria of their colony appearance (24, 25). Primary progenies (B) were selected from variant A (T2) by screening for T4 colonies and, further, the progenies of those (C) were screened for T2, etc. The isolated variants include three different types of pilus variants: standard piliated cells (P^+, encircled), cells secreting truncated S-pilin (P^s, framed with squares), and nonpiliated cells (P^-, without any frame).

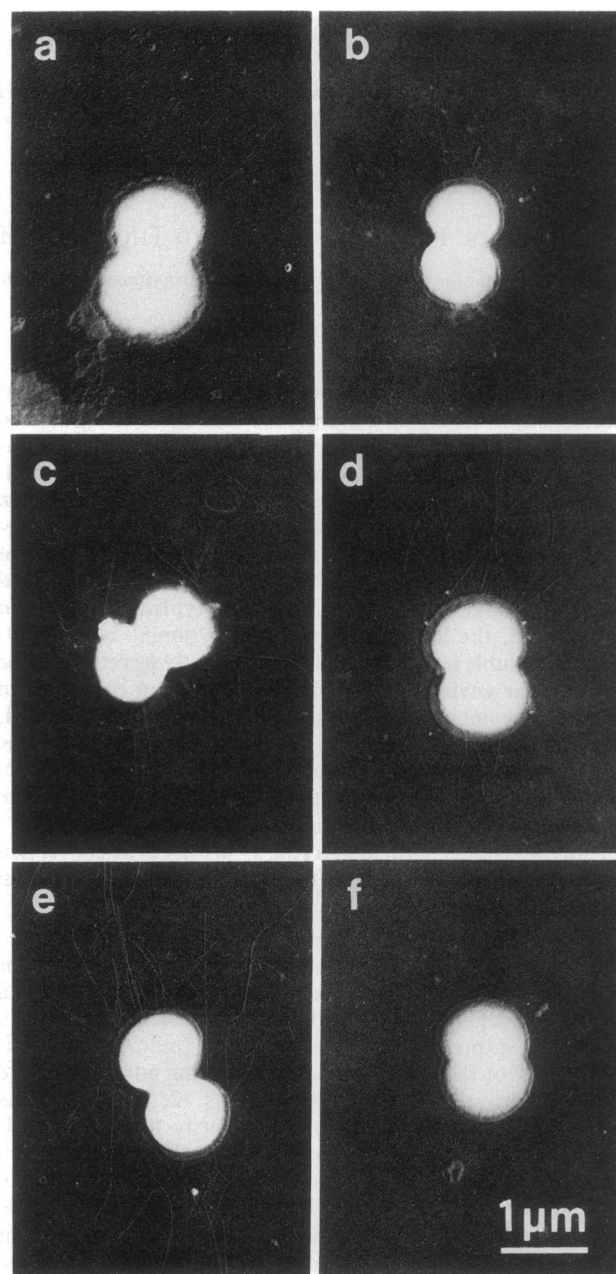

FIG. 2. Piliation of gonococcal cells observed by electron microscopy. Representative examples are shown. (*a*) Variant B1 (P^s). (*b*) Variant B5 (P^s). (*c*) Variant D5 (P^s). (*d*) Variant D3 (P^s). (*e*) Variant C (P^+). (*f*) Variant D1 (P^-).

Transcriptional and Translational Analysis. Genetic analysis of the isogenic pilus variants by DNA filter hybridization showed that, except for the original variant A that carried two identical structural genes, all other variants contained a partially deleted *pilE2* locus (13) (data not shown). Therefore all phenotypic changes in pilus expression had to be accounted for by the remaining functional expression locus *pilE1* (7). The *pilE1* locus did not undergo apparent size changes in any of the variants, except for variants D1 and F5 (P^-). Observations made on the RNA and protein levels were consistent (Fig. 3). The pilin-specific mRNA in variant gonococci was determined by RNA transfer blot analysis using a conserved pilin oligonucleotide probe. Total pilin protein produced in

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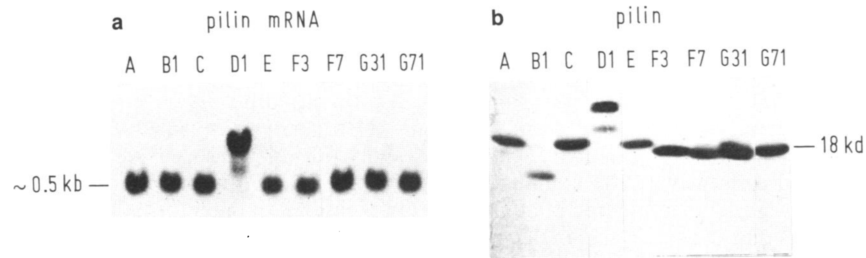

FIG. 3. Production of *pil*-encoded mRNA and pilin in isogenic pilus variants. (*a*) Pilin mRNA in variant gonococci visualized by RNA transfer blot analysis using the labeled oligonucleotide probe RH26, codon positions 1–5 of pilus gene (7). (*b*) Pilus protein visualized by immunoblotting using anti-FP.AC7 rabbit serum. The sizes of mRNAs and pilins are indicated at the margins in kilobases (kb) and kilodaltons (kd), respectively.

the variants was determined by immunoblotting using a cross-reactive antiserum that was raised against a genetically engineered pilin fusion protein containing amino acids 21–68 of the conserved/semivariable region of variant A pilin. Fig. 3 shows that all variant gonococci produced pilus-specific mRNA as well as pilin, thus confirming the constitutive transcription and translation of the *pilE1* locus irrespective of the piliation phase, as observed with other pilin variants (9). The P^- variants D1 and F5 both carried a 500-base-pair extension of yet unknown origin in their *pilE1* expression copy and consequently produced a larger pilin mRNA and an over-length pilin molecule (L-pilin, shown for D1 in Fig. 3 and for F5 in Fig. 5). The L-pilin preparation of variant D1 shows an additional band of lower molecular weight that corresponds in size with a shorter transcript. This band might therefore not be produced by posttranslational degradation. The reversion to P^+ of variants producing L-pilin can be associated with an alteration of the previous minicassettes in the expression gene (unpublished).

Conversion of Minicassettes in the Expression Gene. The expression genes (*pilE1*) of variants B1, E, F3, F7, and G31 were cloned and sequenced. The sequences of variant A loci *pilE1* and *pilS1* were already known (7, 8). A partial sequence was derived from locus *pilS7* (Fig. 4). At first we compared the sequences of variants involved in a direct P^+ to P^+ transition—i.e., the E, F3/7, and G variants (see Fig. 1). F3 and F7 had identical expression genes, indicating that the same antigenic switch had occurred twice, independently. The expression genes of all other variants differed, but alterations were limited to the variable minicassette regions (8) (Fig. 4 *b–d*). By comparing silent and expressed sequences we noted that the *pilE1* loci of variants F3/7 have acquired at least two minicassettes from *pilS1* copy 1—namely, mc2 and mc3 (Fig. 4 *a* and *c*). In conjunction with previous Southern hybridization data (8) the recombination process underlying this sequence acquisition can be interpreted as a precise gene conversion event. Similar gene conversions led to the switches from F to G (Fig. 4 *a* and *d*). Variant G31 has acquired two new minicassettes (mc2 and mc3) from *pilS7* (Fig. 4 *a* and *d*). Further G variants, not shown here, yet have adopted other minicassette combinations in their expression genes. Likewise, the P^s variants, described here as a separate class—for example, variant B1—have a converted expression gene (Fig. 4 *a* and *b*). The

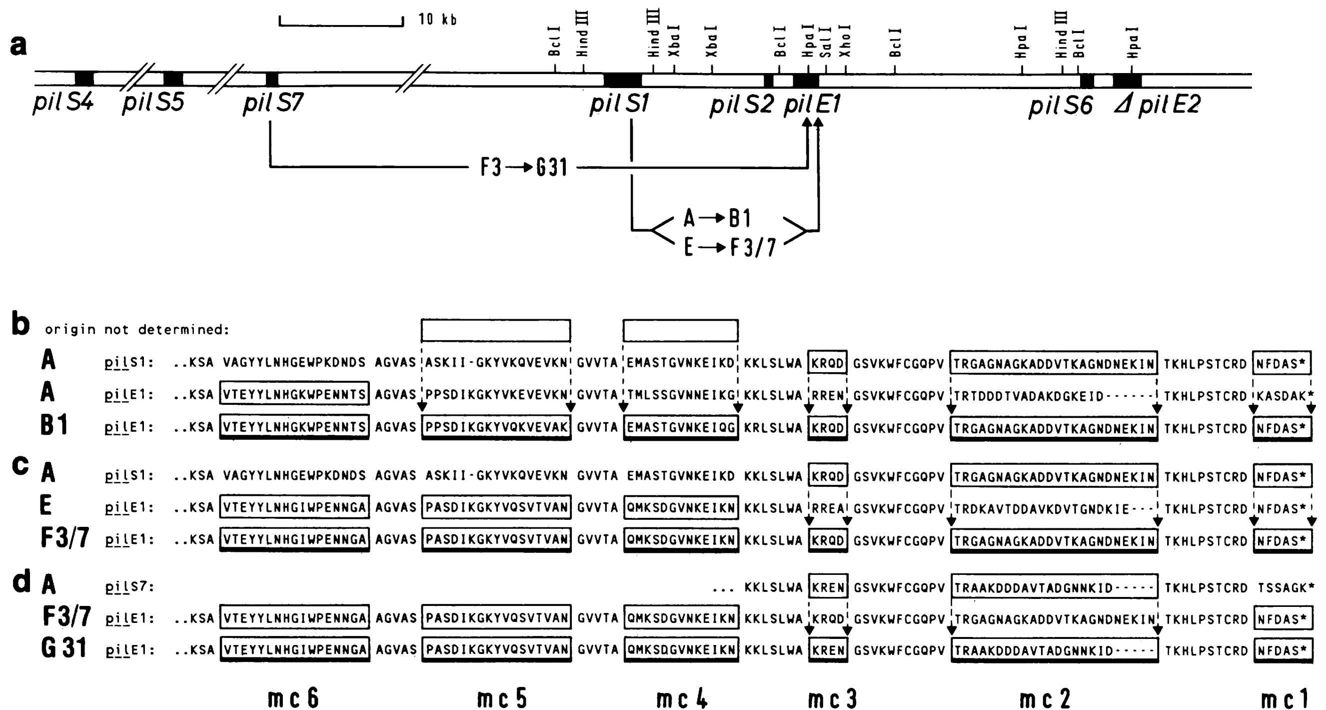

FIG. 4. Minicassette rearrangements in the expression genes of isogenic pilus variants. (*a*) Genomic organization of pilus gene loci, as determined for variant A (ref. 7; nomenclature according to refs. 10 and 26). Arrows indicate the direction of minicassette transfer from silent loci to *pilE1* within distinct variants, as indicated. (*b–d*) Amino acid sequences of various *pilS* and *pilE* gene copies as predicted from the corresponding DNA sequences (this work and refs. 7 and 8). Sequence alterations between the gene copies were restricted to the minicassette regions (mc6 to mc1, sequences framed). Conversions of the expression gene with minicassettes from silent loci are indicated by arrows. The gene loci and their respective pilus variants that were subject to gene conversion are indicated at the margin.

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minicassettes in the *pilE1* gene of this particular variant were donated from *pilS1* and yet another silent locus (Fig. 4*b*).

Production of S-Pilin. Common to the B1 variant and the other P^s variants included in Fig. 1 is the fact that their pilin molecules are degraded to a certain extent into a smaller fragment of distinct size (Figs. 3*b* and 5*a*). Partially truncated pilin has been observed before, however, for nonpiliated gonococci (9). By determining the cellular location of pilin in our isogenic variants we found that the small pilin molecules (S-pilin) produced by P^s variants were efficiently secreted into the extracellular environment (Fig. 5*a* I, 1–6), whereas the large L-pilin of variant F5 was not secreted (Fig. 5*a* II). For variant B1 the amount of truncated pilin found in culture fluids is very high, reaching concentrations of about 2 mg/liter. This variant converts almost all of its pilin into the extracellular S form (Fig. 5*a*). Secretion of S-pilin is highly selective. The only other protein component of the Gram-negative gonococci known to be secreted into the environment is IgA protease (23).

As determined by amino-terminal sequence analysis, S-pilin of variant B1 lacks the conservative region of propilin up to position 39 and begins with an alanine (Fig. 5*c*). This strongly hydrophobic region has been implicated in the polymerization of pilin (27); its loss can therefore explain the solubility of S-pilin. It is unknown if the processing site of all S-pilins is at position 39/40 and, further, if B1 propilin is subjected to two subsequent processing steps, first at the −1/+1 position and then at the 39/40 position. In *E. coli* cells containing a cloned *pilE* gene of P^s variants, truncation to the S form could not be observed (not shown).

TEM analysis revealed that B1 cells exhibit very few and short pili on their cell surfaces (Fig. 2*a*), suggesting that variant B1 pilin in principle can form pili if the pilin is not processed into the S form. This dual capacity of the P^s phase is more pronounced with other variants—e.g., B5, D5, and D3. These variants show an intermediate piliation with respect to the length and number of pili (Fig. 2 *b–d*). The amount of S-pilin is inversely related to the piliation (Fig. 5*a*). There seems to exist a continuous line of stages between the extreme P^s phase cells, which convert almost all of their pilin into the soluble form, and extreme P^+ cells, which produce no detectable quantity of soluble pilin (Fig. 5 *a* and *b*).

DISCUSSION

All phase transitions from P^+ to P^s, or vice versa, as observed here, are associated with a reassortment of minicassettes in the expression gene (Fig. 4). It is therefore tempting to speculate that the alternate processing of propilin might depend upon the intrinsic structural features of propilin that are ultimately determined by the DNA sequence of the pilin structural gene. At this stage, however, we cannot rule out

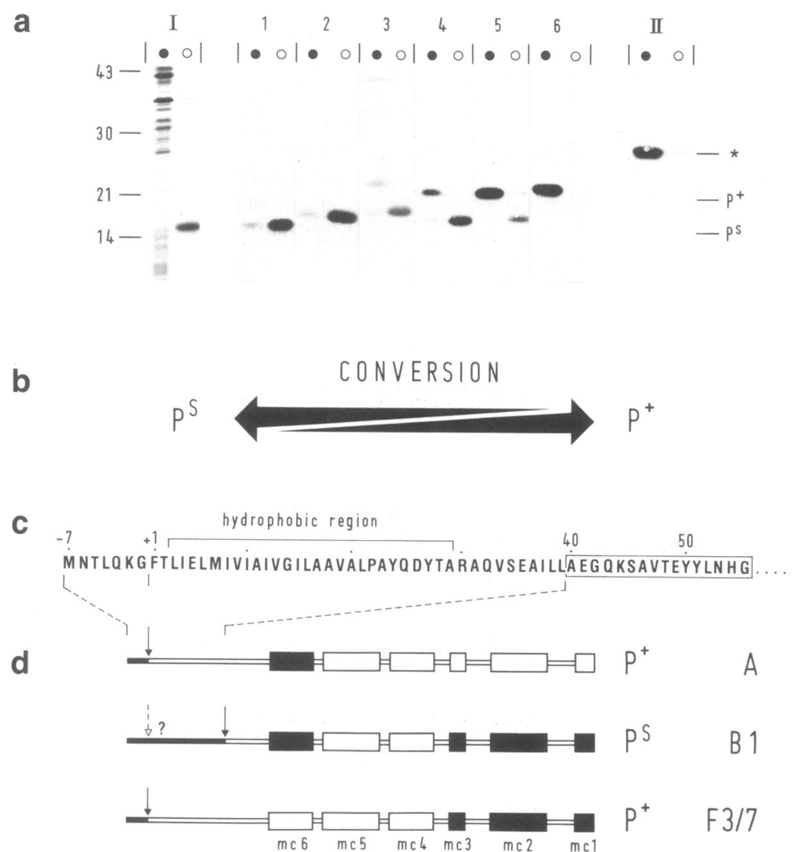

FIG. 5. Localization and characterization of S-pilin. (*a*) Cell pellets including the (insoluble) surface pili (●) and culture supernatants of gonococci (○) were analyzed on a stained protein gel (I) and by immunoblotting with pilin-specific antiserum (1–6, II). Lanes I, Coomassie-stained protein gel of variant B1 fractions. Lanes 1–6, immunoblots of pellets and supernatants of variants B1 (P^s), B5 (P^s), B9 (P^s), D5 (P^s), D3 (P^s), and C (P^+). Lanes II, immunoblot of pellet and supernatant of variant F5 (P^-). The left margin indicates the position of molecular mass markers in kilodaltons; the right margin shows the positions of P^+ and S-pilin as well as the position of the L-pilin of variant F5 (asterisk). (*b*) Schematic drawing suggesting a continuous transition of P^s and P^+ phases, as demonstrated for the piliation stages in *a*, lanes 1–6. Common to these phase transitions is their apparent association with conversion events in the expression gene. (*c*) Amino-terminal sequence of propilin and its processing in gonococci (the single-letter amino acid code is used). Position −1/+1 indicates the processing site for P^+ pilin (7). This site is followed by a hydrophobic region. S-pilin of variant B1 lacks this hydrophobic region and begins with an alanine at position 40, as determined by amino-terminal sequencing (boxed sequence). (*d*) Processing sites used in propilin of variants A, B1, and F3. Homologous minicassettes (■) are distinguished from different minicassettes (□). The immunodominant domain of gonococcal pilin is contained in mc2, whereas mc4 and mc5 include a putative receptor-binding domain (27).

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that S-pilin production is controlled, for example, by an independent protease or that the phenomenon is subject to a more complex mechanism. Likewise for the *E. coli* Pap pili, which in many respects differ from gonococcal pili, it was shown recently that the length and the number of the pili can be influenced by the ratio of different pilus subunit components (28).

The *pilE1* sequences of variants B1 (P^s) and F3 (P^+) only differ in mc4 to mc6 (Figs. 4 *b* and *c* and 5), suggesting that the same type-specific determinant of pilin located in mc2 (29) can be present at the surface pili and the S-pilin. Since pilin is highly immunogenic, the secretion of soluble pilin antigen may exert some deleterious effect on the host immune response, a phenomenon that is well known from other pathogens (30). Although a rationale for the physiological meaning of S-pilin secretion is missing, the phenomenon may have biological impact. The significance of S-pilin *in vivo* is supported by the appearance of truncated pilin molecules (likely to comprise S-pilin) in variants recovered from experimental human infections (26). When variant A pilin (P^+) is compared with variant B1 pilin (P^s), homology in the minicassette mc6 can be seen, whereas minicassettes mc4 and mc5, including a putative receptor-binding domain (27), differ in all three pilin types shown (Fig. 5*d*). A distinct structural signal that triggers the processing of S-pilin therefore cannot be asserted.

The frequent event causing sequence reassortments in the expression gene is reminiscent of gene conversion, in that it reflects an apparently nonreciprocal recombination process. The precise molecular mechanisms involved in this gene conversion, however, are unknown and may involve more complex pathways in gonococci than in spore-forming fungi for which the term "gene conversion" originally was coined. Owing to the unique transformability of piliated gonococci (31), horizontal genetic transfer between two cells may play a role in the pilus variation. To dissect the details of the gene conversion process in gonococci, the construction of transformation- and/or recombination-deficient mutants might be pertinent.

Intragenic conversions as observed in our studies never terminated within a variable minicassette region. This leads us to assume that the conserved sequences, flanking the minicassettes, provide essential homology to confer conversions in a *pil* gene. Indeed, a recent report clearly demonstrated that gene conversion in the *pil* genes is *recA*-dependent and therefore based on homologous recombination (32). Likewise, gene conversion in trypanosomes, effecting the variation of the surface glycoprotein, seems to rely on homologous sequence stretches between the basic copy donor gene and the expression-linked acceptor gene (33).

Our observations suggest that the precise intragenic gene conversion is not an effective mechanism for the generation of nonpiliated, nonsecretory (P^-) phase variants. Previous studies show that complete loss of piliation in the absence of pilin secretion can, however, result as a consequence of expression gene deletions (13, 34) and of the introduction of a translation termination signal in the expression gene (34). Here we introduced a different type of P^- variant (e.g., D1 and F5) that produces an over-length pilin (L-pilin). Such P^- variants, unlike most P^s variants, do not assemble detectable pili on their surfaces. Their large pilin molecules instead remain cell associated. Rearrangements in the expression gene leading to the production of L-pilin occur frequently. Beside the precise gene conversions, that type of rearrangement might thus be important and play a further interesting role in the complex program of pilus phase and antigenic variation in *N. gonorrhoeae*.

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