

PILIATION CHANGES IN TRANSFORMATION-DEFECTIVE GONOCOCCI

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Pili of *Neisseria gonorrhoeae* are virulence-associated organelles (1) whose expression and variation depend on a seemingly simple yet versatile genetic system (2, 3). Its structural elements are an expressed complete pilin gene (*pilE*)¹ and 16 or more partial (5'-deleted), silent pilin genes (*pilS*), whose homologous regions separate stretches of divergent sequence that range from 18 to >70 bp (4) in length. Pilus⁺ (P⁺) cells undergo "antigenic" change when their *pilE* receives a new *pilS* oligonucleotide stretch and comes to encode a novel functional pilin polypeptide (1, 5-8). When a nonsense or missense mutation is created in the newly constituted *pilE*, a "defective" pilin polypeptide is expressed, no pili are formed, and piliation "phase" changes to pilus⁻ (9⁻) (9, 10). Such recombinations usually leave the participating *pilS* unaltered (5, 7), though reciprocal *pilE-pilS* exchanges have been observed in gonococci grown on medium with DNase (11). Missense or nonsense P⁻ variants spawn P⁺ progeny when their *pilE* mutation is "corrected," usually by recombinational replacement of the mutated region with the corresponding portion of yet another *pilS* (7, 9, 10). Thus, pilus phase and antigenic changes reflect the same process (12, 13).

At one extreme, intragenomic *pilS-pilE* recombination would account for the above-described *pilE* sequence changes, but so might transformation by DNA from spontaneously lysed neighbor cells. Either could produce the observed nonreciprocal changes. Initial studies suggested that *pilS-pilE* exchanges proceed by gene conversion-like intra-genomic transfers (2, 5, 7, 9, 10, 14), while several recent reports have suggested that transformation is responsible (11, 15-17). The latter include the observations that DNase reduces the frequency of transitions from P⁺ to P⁻ (11) and from P⁻ to P⁺ (16); they also showed that *pilE* sequences can be transformed into wild-type P⁺ gonococci in vitro (15, 17).

We examined strain FA660, whose DNA uptake deficiency (*dud*) mutation severely impairs its transformability (18), expecting it to display dramatically deranged pilus changes. No detectable amounts of exogenous DNA are taken up by this mutant, whose *recA* function is intact (18). We found that P⁺ *dud* mutants, though virtually nontransformable for streptomycin resistance (*str*^R), exhibited both antigenic

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¹ Abbreviations used in this paper: *dud*, DNA uptake deficiency; P, pilus; *pilE*, complete expressed pilin gene; *pilS*, partial silent pilin gene; *str*^R, streptomycin resistance.

pilus changes and reversible piliation phase transitions. Wild-type strain 228 P⁻ gonococci were also nontransformable to str^R, except as they spawned competent P⁺ revertants. These data argue that *pilE* readily undergoes recombinational modification in the absence of DNA transformation.

Materials and Methods

Gonococci. Wild-type parental strain FA228 and its dud mutant FA660 strain were obtained as lyophilized cultures from Drs. G. Biswas and P. F. Sparling (18) (University of North Carolina, Chapel Hill, NC); both are str^S. The str^R strain MS11 was from our collection; its derivation is described elsewhere (12). All organisms were propagated on clear solid typing medium (19) in 5% CO₂ at 35°C by daily passage of single selected colonies.

SDS-PAGE and Immunoblotting. Gonococci grown on solid medium were suspended in 0.9% NaCl (OD₅₄₀ = 0.3), 1.5-ml portions were centrifuged, and the pellets were resuspended in 80 μl solubilizing solution. After boiling for 5 min, these were diluted 1:10 in solubilizing solution, and 5-μl samples were subjected to separation by SDS-PAGE in 15% gels; the separated components were electrophoretically blotted to membrane (HAHY; Millipore Continental Water Systems, Bedford, MA), and were probed first with the broadly crossreactive antipilin mAb 02, and then with ¹²⁵I-labeled protein A. Details of these techniques have been described before (19).

Transformation. Gonococci grown overnight on plates were suspended in liquid growth medium to OD₅₄₀ = 0.3; to 100-μl portions were added 2 mM CaCl₂ and 20 μg/ml DNA that had been extracted and purified by centrifugation in a cesium chloride gradient from strain MS11. Incubation of recipient gonococci with str^R DNA for 30 min at 37°C was followed by directly plating 20-μl aliquants on clear solid medium containing streptomycin (25 μg/ml); other samples were appropriately diluted and plated on plain medium. P⁺ and P⁻ colonies on plain medium were microscopically differentiated and enumerated the following day; str^R transformants were similarly evaluated, and then again after 2 d incubation. Some transformation assays differed in that the 30-min incubation of gonococci + DNA was followed by 10-fold dilution in liquid growth medium and further incubation (30 or 60 min, 37°C) before plating on both plain and str^R-containing solid media.

Phase Transition Frequencies. Individual P⁺ or P⁻ colonies were microscopically selected, lifted from solid medium on filter paper fragments, suspended in water, and plated on solid medium; the resulting P⁺ and P⁻ colonies were scored the following day.

Blot Hybridizations. Genomic DNAs were prepared from selected gonococci by methods described before (12). After Cla I digestion, they were subjected to agarose gel electrophoresis, blotted to solid matrix (HAHY; Millipore Continental Water Systems) and probed with ³²P-labeled oligonucleotides whose sequences corresponded to selected portions of *pilE* (7). Overnight incubation with radiolabeled oligonucleotide probes was at 37°C. These blots were washed with 1 × SSC for 15 min at 45°C; after autoradiographic exposure, the blots were washed with 1 × SSC at successively higher temperatures (5°C intervals) up to 10°C below the calculated melting temperature for the probe, and then with 0.1 × SSC at the same temperature.

Pilin mRNA Sequencing. RNA was extracted from whole gonococci with hot acidic phenol and precipitated with ethanol. Pilin mRNAs were annealed with custom-designed *pilE* oligonucleotide primers and extended with reverse transcriptase as described previously (9). The mRNA sequences are presented as those of *pilE* DNA.

Results

Pilus Structural/Antigenic Variation in FA660 (dud). 12 colonies were microscopically selected from the almost exclusively P⁺ outgrowth of FA660 (dud) on clear solid medium. They displayed subtle, sometimes questionable differences in edge morphologies. Lack of clear colonial differences among P⁺ variants of FA660 precluded reliable definition of the frequencies at which they arose. Only four different pilins

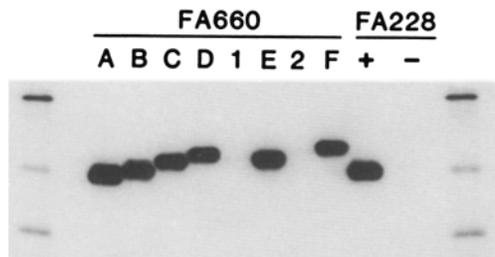


FIGURE 1. Among the 12 P^+ colonies selected from the original outgrowth of FA660, four electrophoretically distinct pilin polypeptides (A, B, C, and D) were defined by immunoblotting. Two P^- variants (1 and 2) produced no detectable pilin and had no pili by EM; each gave rise to a P^+ revertant (E and F) that elaborated a pilin of unique apparent size. One representative P^+ and a P^- variant from parental, wild-type strain FA228 are also shown (+ and -). The whole cell lysates were subjected to PAGE, immunoblotting with a crossreactive antipilin mAb, incubation with ^{125}I protein A, and autoradiography. Molecular mass markers included at each end include proteins of 30, 21, and 14.3 kD (top to bottom).

were electrophoretically differentiated among the 12 variants (Fig. 1); seven had pilins that resembled variant P^+ C, three were like P^+ D, and P^+ A and P^+ B were each unique. Pilin mRNA sequences of the latter three variants carried unique oligonucleotide spans ranging from 68 to >130 nucleotides, compared with the predominant P^+ C (data not shown). Their encoded pilin polypeptides had sequence differences in both the so-called "conserved" and "hypervariable" portions of their respective amino acid 100–160 regions (Fig. 2).

Pilus Phase Variation in FA660 (dud). Two P^- variants (P^- 1 and P^- 2) were identified in the original FA660 outgrowth by their typical large, flat colonies. Neither elaborated detectable pilin (Fig. 1) nor had pili by EM (not shown), but both spawned P^+ revertants at high frequencies (1.66% for P^- 1, 2.6% for P^- 2; Table I). These two P^- variants had distinct pilin mRNA sequences, but both carried the same apparent single base C deletions at nucleotide 326 (GGGCCA to GGGCA), resulting in -1 frame shifts, and downstream nonsense mutations (Fig. 3).

Revertants P^+ E (from P^- 1) and P^+ F (from P^- 2) had new oligonucleotide stretches that spanned and corrected the parental nonsense mutations. P^+ E carried novel sequence for nucleotides 252–337, while P^+ F had new sequence in a more downstream region (310–430); they spawned second generation P^- variants at very disparate frequencies (2.68% for P^+ E, 0.06% for P^+ F).

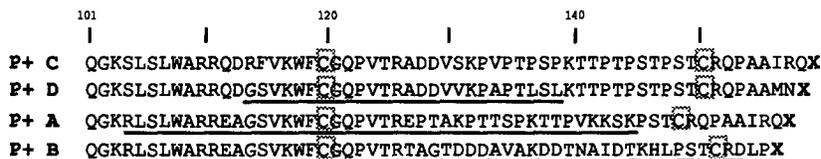


FIGURE 2. The partial amino acid sequences (residues 101 to >150) of FA660 P^+ variants A, B, C, and D (see Fig. 1) were deduced from their pilin mRNA sequences. P^+ A, B, and D each has a unique oligopeptide region (underscored), compared with P^+ C, the predominant form in the FA660 outgrowth; each of these unique portions extends into the "hypervariable" region bounded by cysteine residues (C). The COOH-terminal sequence of P^+ D was not completed, as noted (---), through its translation stop codon. These data have been submitted to the EMBL/GenBank Data Libraries.

TABLE I
FA660 and FA228 Variants Were Assessed for their Transformabilities to str^R

Gonococci	Transformation				Percent change
	P ⁺	P ⁻	str ^R	str ^R /P ⁺	
FA660 P ⁺ A	1.9 × 10 ⁷	2.5 × 10 ⁵	0		[1.32]*
FA660 P ⁺ B	2.1 × 10 ⁷	5.0 × 10 ⁴	0		[0.24]
FA660 P ⁺ C	2.0 × 10 ⁷	1.2 × 10 ⁵	20(1)†	1.0 × 10 ⁻⁶	[0.6]
FA660 P ⁺ D	1.2 × 10 ⁷	1.5 × 10 ⁵	0		[1.25]
FA660 P ⁻ 1	2.0 × 10 ⁵	2.3 × 10 ⁷	0		1.66 ± 0.92 (4)
FA660 P ⁺ E	1.3 × 10 ⁷	2.0 × 10 ⁵	0		2.68 ± 0.74 (9)
FA660 P ⁻ 2	2.4 × 10 ⁵	2.0 × 10 ⁷	0		2.6 ± 0.75 (3)
FA660 P ⁺ F	2.5 × 10 ⁷	1.5 × 10 ⁴	0		0.06 ± 0.04 (6)
FA228 P ⁺	2.5 × 10 ⁷	7.5 × 10 ⁵	1.1 × 10 ⁴ (1,830)	4.4 × 10 ⁻⁴	[3.0]
FA228 P ⁻ x	0	5.5 × 10 ⁷	0		[0]
FA228 P ⁻ y	2.6 × 10 ⁵	4.9 × 10 ⁷	99(16)	3.8 × 10 ⁻⁴	[0.53]

The FA660 (dud) preparations examined included six from the original outgrowth (P⁺ A, B, C, and D; P⁻ 1 and 2) plus P⁺ E and P⁺ F revertants derived from P⁻ 1 and P⁻ 2, respectively. The numbers of P⁺ vs. P⁻ variants in each preparation are shown. Attempts to transform the FA660 variants to str^R often yielded no transformants; a few str^R transformants (one colony observed, calculated total = 20) arose from FA660 P⁺ C in the experiment shown. Identical conditions with FA228 P⁺ yielded many more str^R transformants (1,830 observed; calculated total = 1.1 × 10⁴). Two FA228 P⁻ variants (P⁻ x and P⁻ y) were studied; P⁻ x had a 5' *pilE* deletion and did not revert to P⁺, while P⁻ y had a nonsense *pilE* mutation and regularly generated P⁺ revertants. The number of transformants obtained from P⁻ y (observed = 16, calculated total = 99) and from other P⁻ variants of FA228 (not shown) correlated directly with their respective numbers of P⁺ revertants; that correlation is mirrored in the similar str^R/P⁺ values for FA228 P⁺ vs. P⁻ y.

* Pilus variation frequencies (percent change) were determined specifically for P⁻ 1, P⁺ E, P⁻ 2, and P⁺ F multiple times (number in parentheses) by lifting individual colonies of different serial passages from an agar surface and enumerating the variant vs. parental phenotypes in each. P⁻ 1 and P⁻ 2 spawned P⁺ revertants at similar frequencies (P⁻ 1 = 1.66%; P⁻ 2 = 2.6%), but their revertants generated P⁻ cells at very different frequencies (P⁺ E = 2.68%; P⁺ F = 0.06%).

† The percent change values shown in brackets for all the other variants come from the transformation experiments that are summarized.

Seven second generation P⁻ variants from P⁺ E and P⁺ F all carried identical C deletions and downstream nonsense mutations. P⁻ 3 and P⁻ 5 differed from their P⁺ E parent by only the C deletion (Fig. 3). The five other second generation P⁻ variants contained new pilin mRNA stretches that varied from 13 to 132 nucleotides, bordered (P⁻ 4, and 7) or spanned (P⁻ 6, 8, and 9) the deletion site, and had homologous overlapping regions. These new stretches were composited into the sequence of a hypothetical *pilS* (*pilS_n*) whose recombinational insertion into *pilE* had created the nonsense mutations in the P⁻ variants (Fig. 3). Compared with *pilE* of P⁺ E and P⁺ F, *pilS_n* had several bases changed upstream of the apparent deletion site; downstream, its sequence differed considerably from *pilE* of P⁺ F, but was homologous to that of P⁺ E. (Fig. 3).

The putative *pilS_n* was identified by blot hybridization of FA660 genomic DNA with oligonucleotide probes specific for P⁺ and P⁻ *pilE* sequence (Fig. 4). The P⁺ probe (recognizing GGGCCA) hybridized with *pilE* of P⁺ E variant but not with *pilE* of variant P⁻ 4; it also recognizes seven *pilS* sequences (estimated by densitometric scanning) in both P⁺ and P⁻ variants (Fig. 4 A). The P⁻ probe (recognizing

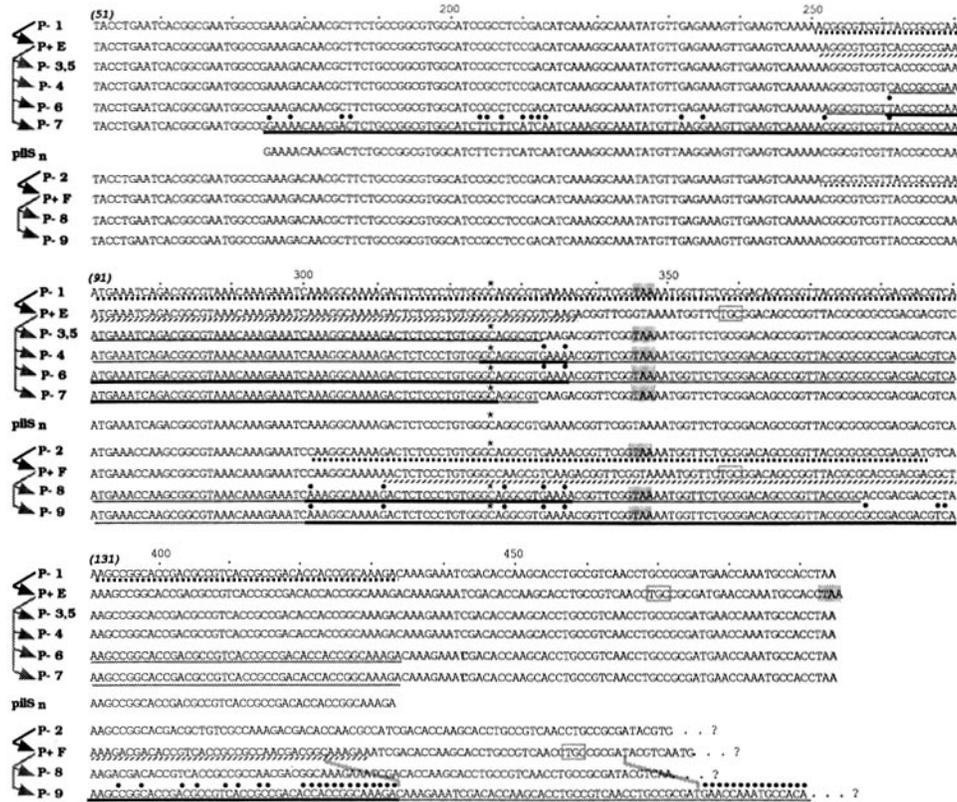


FIGURE 3. Pilin mRNA sequences of FA660 P⁻ 1, P⁻ 2, their respective P⁺ revertants E and F, and seven different second generation P⁻ variants from P⁺ E and F (P⁻ 4, 5, 6, and 7 from P⁺ E; P⁻ 8 and 9 from P⁺ F) are represented as *pilE* DNA sequence. Also shown is the deduced sequence of *pilS_n* that was composited from the new *pilE* oligonucleotide stretches in second generation P⁻ variants. P⁺ E and P⁺ F both carry new oligonucleotide stretches (-----) compared with their respective P⁻ 1 and P⁻ 2 parents; both P⁺ revertants E and F have complete pilin open reading frames that contain codons (TGC) for the two cysteine residues that border the hypervariable portions of all neisserial *pilE*. Each P⁻ variant has an apparent single base (C) deletion (*; nucleotide 326) that creates a downstream premature translation stop codon (TAA). This apparent deletion (GGGCCA- to -GGGCA) is the only difference observed for P⁻ 3, 5 vs. its P⁺ E progenitor. Each second generation P⁻ variant (P⁻ 4, 6, 7, 8, and 9) has a newly inserted oligonucleotide stretch compared with its respective P⁺ E or P⁺ F forerunner; both the minimum (————) and the maximum (-----) lengths of those new oligonucleotide spans, as deduced from base differences of P⁺ vs. *pilS_n*, are shown. The minimum new stretches are composited into a “nonsense *pilS*” (*pilS_n*) sequence. Nucleotide differences of *pilS_n* vs. P⁺ E and P⁺ F, noted with dots above the changed bases in each second generation P⁻ variant sequence, allow definition of their new oligonucleotide stretches. P⁻ 1 and P⁻ 2 also carry sizeable stretches (•••••) homologous to *pilS_n*.

GGGCA) hybridized with one *pilS* (i.e., *pilS_n*) in both P⁻ 4 and P⁺ E, with *pilE* of P⁻ 4, but not with *pilE* of variant P⁺ E (Fig. 4 B). This *pilS_n* was also found in parental, wild-type strain FA228, whose P⁻ variants often carried the same apparent single-base *pilE* deletion present in P⁻ cells of FA660 (data not shown).

Transformabilities of FA660 (dud) and Wild-type FA228. The dud mutant and its

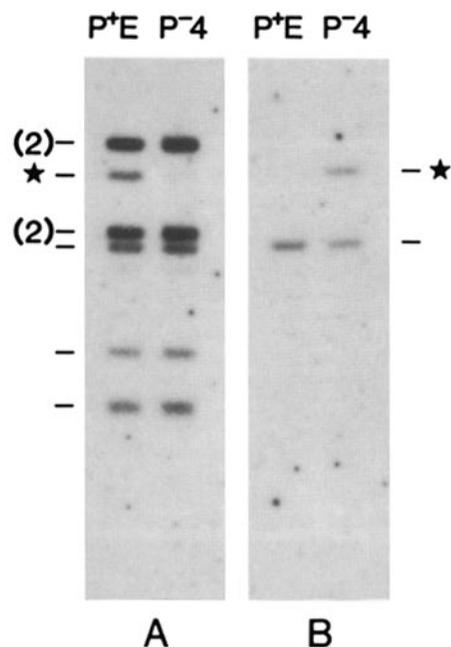


FIGURE 4. Cla I-digested genomic DNAs of P⁺ E and P⁻ 4 were probed in blot hybridization with oligonucleotides, as follows: (A) GCCTGGCCCCA-CAGGGAGA (which recognizes the DNA sequence GGGCCA) and (B) GCCTGCCCCACAGGGAGAG (recognizing GGGCA) (see Fig. 3). Probe A hybridizes to the *pilE* (★) of P⁺ E and to seven *pilS* contained in five Cla I fragments (-), two of which contain two *pilS* each (2-) in P⁺ E and P⁻ 4, as seen by densitometric scanning; probe B hybridizes to only one *pilS*-containing fragment in P⁺ E and P⁻ 4, and to the *pilE* (★) of P⁻ 4.

wild-type parental strain FA228 are both str^S and were assessed for transformation with DNA from str^R strain MS11 (Table I). Multiple attempts to transform the same P⁺ and P⁻ dud mutants that displayed pilus antigenic and phase variation usually yielded no str^R colonies. Identical conditions yielded numerous transformants with P⁺ FA228. Two distinct P⁻ variants of FA228 (P⁻ x and P⁻ y, Table I) were assessed for their transformabilities to str^R. P⁻ x had an extensive 5' *pilE* deletion and did not revert to P⁺ (i.e., P⁻ n phenotype; reference 12), while P⁻ y carried a nonsense *pilE* mutation like that seen with FA660 variants, and regularly gave rise to P⁺ revertants (P⁻ r phenotype). P⁻ x gave rise to no str^R transformants. The str^R transformants from P⁻ y were all P⁺ and, in several experiments, their number correlated with the number of P⁺ revertants that "contaminated" the P⁻ y population. Accordingly, both P⁻ y and the predominantly P⁺ preparations of FA228 exhibited very similar str^R/P⁺ ratios (Table I). Several additional, independently derived P⁻ missense variants of FA228 were similar regarding the numerical correlations between their revertants and str^R transformants (not shown). These data suggest that P⁻ gonococci, per se, are incompetent for transformation; the transformants that arise from populations of predominantly P⁻ cells come from their P⁺ revertants spawned by nonreciprocal intragenomic *pilS*-*pilE* recombination.

Discussion

N. gonorrhoeae displays impressive abilities to alter piliation status and to elaborate structurally variant pili. Reversible pilus phase changes and antigenic variations both arise through sequence changes in *pilE*; the critical difference is whether the altered *pilE* expresses a missense/nonsense pilin or, alternatively, it encodes an orthodox

pilin that can form pili. Which genetic mechanism accounts for such *pilE* modifications is disputed. Initial reports suggested that intragenomic recombinatorial events were responsible, but more recent studies propose transformation as the major cause. The latter opinion is extrapolated from demonstrations that DNase diminishes the frequencies of piliation phase changes (P^+ to P^- , and reverse) and that P^+ gonococci can take up, integrate, and express exogenous *pilE*::CAT fusions that contain the homologous *pilE* sequence.

If transformations were mainly responsible for the *pilE* sequence changes that produce pilin variations, they should be markedly reduced or absent in nontransformable cells. The present report shows that dud mutants display *pilE* sequence changes at frequencies that rival wild-type, competent organisms, in spite of their virtual incompetence for DNA transformation. Antigenic changes and associated *pilE* sequence alterations were identified in P^+ dud gonococci, but we could not define the frequencies at which they occur. P^+ vs. P^- colonies were easily differentiated, and P^+ to P^- transitions in FA660 dud mutants correlated with creation of a nonsense *pilE* sequence whose newly inserted oligonucleotide stretches derived from one particular *pilS*, i.e., *pilS_n*. This *pilS_n* was also present in wild-type parent organisms, arising before generation of the dud mutation. Insertion of *pilS_n* sequence into *pilE* was not accompanied by any definable reciprocal exchange. Reversion (P^- to P^+) correlated with correction of the nonsense *pilE* mutation by a newly incoming sequence to encode an orthodox pilin polypeptide.

Because wild-type competent P^+ gonococci can be transformed by exogenous, homologous *pilE* sequences in *pilE*::CAT fusions, transformation of competent P^+ cells by heterologous *pilE* DNA could be a possible explanation for their pilus changes. Transformation of such competent P^+ cells with missense/nonsense *pilE* might also affect their transition to a revertible P^- phenotype. But neither of these pilus changes has been demonstrated experimentally.

Transformation does not explain P^- to P^+ transitions if P^- gonococci are nontransformable. Early studies found that nonpiliated gonococci were usually totally incompetent, with transformation frequencies of $<10^{-7}$ (20). Whether those P^- cells were capable of reversion to P^+ or were nonreverting P^- variants (P^- n phenotype) is not known. Two recent reports note that P^- n gonococci are incompetent (11, 17). The former concludes that transformation is the major effector of pilin phase changes in revertible P^- variants (11). More recently, P^- n gonococci were reported to be transformed at very low frequencies ($<1.1 \times 10^{-6}$ /CFU) when exposed to large amounts of DNA (100 μ g/ml); P^+ cells transformed at 10,000-fold higher frequencies when incubated with 50–100 times less DNA (1–2 μ g/ml) (21).

Our data indicate that both nonreverting P^- and reverting P^- gonococci are nontransformable. That conclusion rests on (a) the correspondence between reversion and transformation frequencies of wild-type nonsense P^- variants; and (b) the uniform P^+ phenotypes of their *str^R* transformants. Although both wild-type P^- cells and dud mutants are virtually incompetent, they both display pilus changes at high frequencies. These findings indicate that gonococci can utilize transformation-independent means to affect pilus variations. We think that intragenomic recombination is most likely responsible for their observed changes in pilus phenotype and *pilE* sequence.

Summary

Recombination-dependent alterations of their expressed pilin gene (*pilE*) enable gonococci to synthesize a myriad of structurally/antigenically different pili and to reversibly switch their pilus production on and off. These changes have been ascribed both to DNA transformation and to intragenomic recombination between *pilE* and silent pilin genes (*pilS*). We examined the pilus changes in gonococci that are incompetent for transformation because of their DNA uptake deficiency (*dud*) mutation, pilus⁻ (P⁻) phenotype, or both. Though incompetent for DNA transformation, *dud* cells displayed pilus antigenic variation and underwent reversible pilus variations much like their wild-type parent. Wild-type P⁻ with a *pilE* nonsense mutation were also virtually nontransformable, but they reverted to P⁺ at high frequencies. The pilin mRNA sequence changes that accompanied pilus transitions in these nontransformable *dud* and P⁻ gonococci represent insertion of *pilS* stretches into their respective *pilE*, apparently via intragenomic recombination.

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