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Frequency of Pilin Antigenic Variation in *Neisseria gonorrhoeae*

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Variation of the pilus of *Neisseria gonorrhoeae* occurs by the recombination of silent pilin DNA sequences into the pilin expression locus. We have developed a quantitative, competitive reverse transcription-PCR assay which measures the frequency of pilin antigenic variation independently of changes in gonococcal colony morphology and have determined this frequency within a gonococcal population. We have also studied the frequency of antigenic variation during growth and have concluded that growth does not dramatically influence the frequency of pilin antigenic variation, although a reproducible, twofold increase is observed upon the transition into late log/stationary phase.

The gram-negative diplococcus *Neisseria gonorrhoeae* (the gonococcus) is the causative agent of the sexually transmitted disease gonorrhea. Pili, filamentous cell surface structures composed primarily of pilin monomers, mediate the initial attachment of the gonococcus to host mucosal epithelial cells (22) and are essential in the establishment of infection, as nonpiliated gonococci fail to cause disease (9, 10).

Pilin is encoded by *pilE*, the pilin expression gene (12). Gonococcal strain FA1090 contains one *pilE* gene (21), and 19 silent pilin copies distributed among five loci, with one silent copy associated with and located upstream of *pilE* (21). Variation of the gonococcal pilus results from the nonreciprocal transfer of partial pilin sequence information from one silent pilin copy into *pilE* (5, 6, 17). The resultant, altered *pilE* gene sequence may encode either an immunologically distinct pilin monomer which can be assembled into functional pili (antigenic variation) or a pilin monomer which is not produced or is inefficiently assembled, resulting in a switch from a piliated (P^+) to a nonpiliated (P^-) colony phenotype (colony morphology-based phase variation) (1, 6, 23).

Since pilin antigenic variation and changes in gonococcal colony morphology result from similar recombination-mediated processes, estimates of the frequency of antigenic variation have been based on that of colony morphology-based phase variation (5, 23, 24, 27), which has been reported to be about 10^{-4} to 10^{-2} colony morphology variants per total CFUs (1, 16, 27). However, changes in gonococcal colony morphology can also occur through processes distinct from those resulting in antigenic variation, such as the deletion of *pilE* or phase variation of PilC (7, 8, 16). Therefore, equating the frequency of pilin antigenic variation to that of colony morphology-based phase variation is not always appropriate. In order to quantitate *pilS* to *pilE* recombination independently of changes in colony morphology, we have combined a qualitative assay which specifically detects *pilS* to *pilE* recombination events (25), with a competitive reverse transcription-PCR (RT-PCR) strategy. By using this assay, we have determined the frequency of pilin antigenic variation during the growth of a gonococcal population.

Development of a quantitative RT-PCR assay to measure the frequency of pilin antigenic variation. The quantitative RT-PCR assay involves two PCRs which amplify *pilE* (Fig. 1A): a recombination-independent reaction (primers PILRBS and CYS2R), which amplifies all *pilE* template present (25), and a recombination-dependent reaction (primers PILRBS and HV-1), which amplifies only those *pilE* genes into which HV-1 amplifiable silent sequences have recombined (11). To establish the assay, the frequency of pilin recombination in the FA1090 variant 1-81-S2 wild type (WT) (19), which does not initially contain HV-1 target sequences in *pilE* (20), was determined. A constant volume of total RNA isolated from a late-log-phase gonococcal population (target RNA) was mixed with a range of known concentrations of in vitro-derived competitor RNA. The cloned competitor contains PILRBS target sequences at the 5′ end, an extra 100 bp of nonpilin DNA, and both HV-1 and CYS2R target sequences at the 3′ end, downstream of the T7 polymerase promoter in pGEM-3 (pCDS2). This mixture of RNA was then reverse transcribed, and the resulting cDNA was used as template for both the recombination-independent and recombination-dependent PCRs, which were performed in triplicate. By mixing target and competitor RNA prior to the reverse transcription step, the competior template acted as an internal control for the efficiencies of both the reverse transcription reactions and PCRs (15). Target and competitor PCR products were separated by gel electrophoresis (Fig. 1B), and the amount of each product present was measured by densitometry. The fluorescence intensities of the competitor products were then corrected for size, allowing for a direct comparison between competitor and target product molar amounts. The corrected competitor-to-target product fluorescence ratio was plotted as a function of the relative number of competitor RNA molecules present (Fig. 1C). The relative number of competitor RNA molecules at which equal molar amounts of target and competitor products are made reflects the concentration of target *pilE* RNA template present in the original sample (15). This calculation is independent of the level of transcription of total mRNA in the gonococcal cultures, since the amounts of both target RNAs are determined with regard to known concentrations of competitor RNA. The total number of *pilE* template molecules was calculated from the recombination-independent PCRs (primers PILRBS and CYS2R), while the number of *pilE* template molecules containing sequences amplified by HV-1 (due to recombination) was calculated from the recombination-dependent PCRs (primers PILRBS and HV-1) (Fig. 1C). Once the amounts of total and HV-1 amplifiable *pilE* template present were calculated with respect to the same competitor, they were used to determine the proportion of *pilE* genes containing

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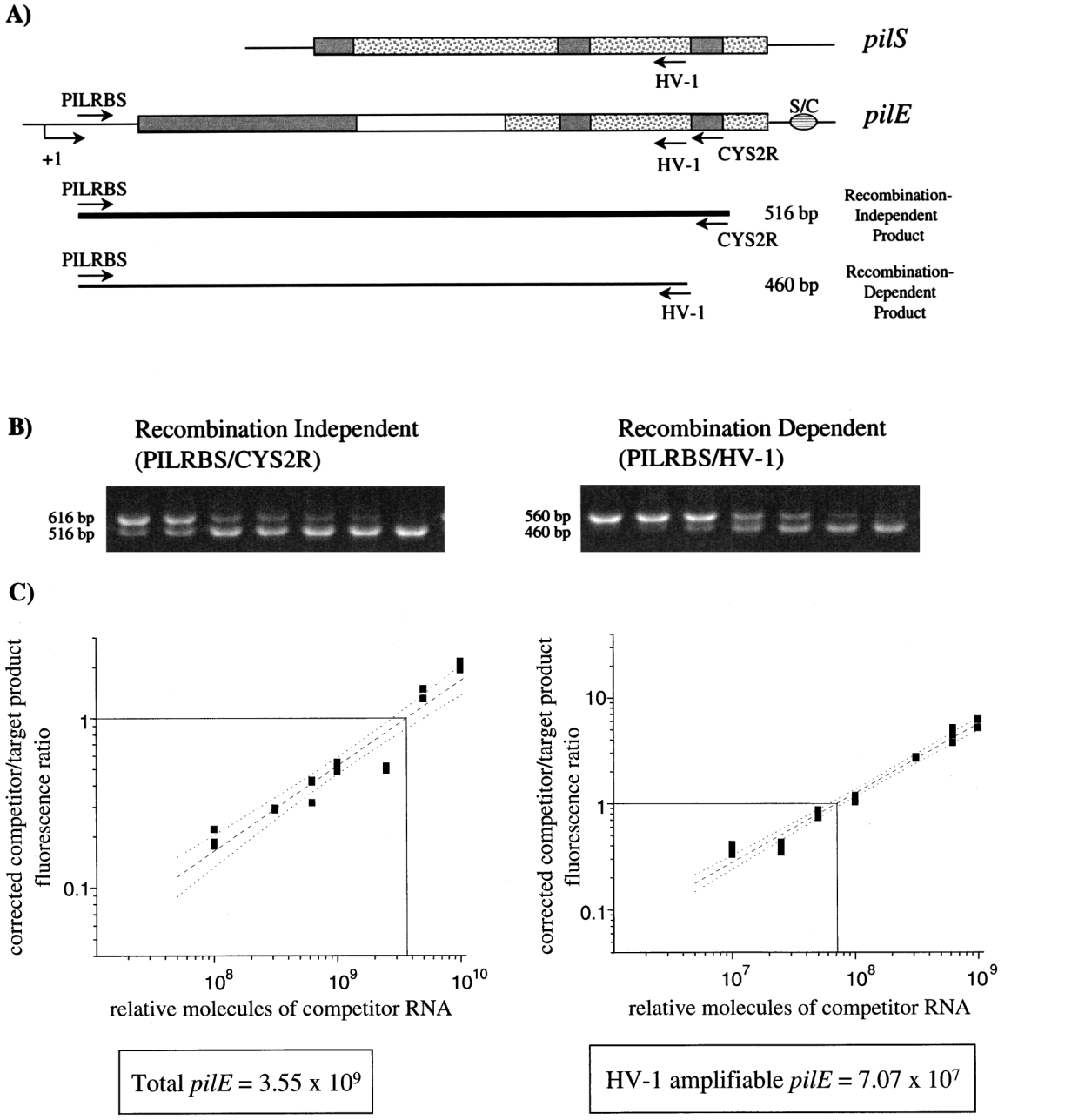

FIG. 1. Quantitation of pilin recombination in variant 1-81-S2 WT at late log phase using competitive RT-PCR. (A) Cartoon of PCR assay detecting *pilS*-to-*pilE* recombination. The major conserved regions of *pilE* and *pilS* are represented by the shaded boxes, and variable sequences are represented by either white or speckled boxes. S/C represents the conserved, 3′ Sma/Cla repeat. The transcription start site of *pilE* is indicated by +1. PILRBS targets the ribosomal binding site of the expression locus and is *pilE* specific (26). CYS2R targets the conserved *cys2* region of all pilin copies (25), while HV-1 targets the HV sequences of *pilS1* copy 4 and *pilS6* copy 1 (19, 20). Recombination-independent primers PILRBS and CYS2R amplify all *pilE* templates present, generating a 516-bp product (thick line). Recombination-dependent primers PILRBS and HV-1 yield a 460-bp product only if HV-1 target sequences have recombined into *pilE* (thin line). (B) Representative agarose gels of the recombination-independent and -dependent PCR products. The products of one recombination-independent PCR and one recombination-dependent PCR are shown. In both gels, the top band is the competitor PCR product, and the bottom band is the target PCR product. (C) Graphs of the PCR data. The *x* axis shows the relative number of competitor RNA molecules added for each cDNA reaction, as determined by spectrophotometry at an optical density of 260 nm. The *y* axis shows the corrected competitor-to-target product fluorescence ratio. A line of least-squares fit (dashed) with 95% confidence level (dotted lines) is shown. The amount of competitor RNA present when equal molar amounts of competitor and target products are made (solid vertical and horizontal lines, respectively) equals the amount of target template present in the original reaction. Total *pilE* is calculated from the recombination-independent reactions, and HV-1 ampifiable *pilE* is calculated from the recombination-dependent reactions.

HV-1 amplifiable sequences within the population. The frequency of HV-1 detection of *pilE* in variant 1-81-S2 WT at late log phase was 1.99×10^{-2} HV-1 amplifiable *pilE* per total *pilE* (HV-1 *pilE*/*pilE*). This is the first quantitative measurement of the frequency of pilin antigenic variation within a gonococcal population and independent of colony morphology-based phase variation.

Contribution of HV-1 amplifiable silent copies to the frequency of antigenic variation. The HV regions of *pilS1* copy 4 and *pilS6* copy 1 in FA1090 contain identical sequences that

TABLE 1. Spectrum of sequences in recombination-dependent PCR product clones

HV sequence	No. of clones (% total)	No. of mismatches to HV-1
pilS1 copy 4 or *pilS6* copy 1	27 (50)	0
pilS1 copy 5 or *pilS2* copy 5	17 (31.5)	2
pilS1 copy 2 or *pilS3* copy 1	5 (9.3)	4
pilS7 copy 1	4 (7.4)	5
pilS1 copy 3	1 (1.8)	3

are specifically targeted by HV-1 (20). However, the HV regions of other silent copies contain sequences similar to HV-1 and therefore may have also been amplified during the recombination-dependent reaction. In order to determine the contribution of the HV-1-specific silent copies to the calculated frequency, recombination-dependent PCR products from the late log phase of preinduced 1-81-S2 *recA6* and of 1-81-S2 *recA6* induced at time zero (t_0) (see below) were cloned and sequenced. None of the 54 clones sequenced contained the original *pilS2* copy 1 sequence in the HV region, confirming that the assay detected the transfer of silent-copy HV sequences into *pilE* (Table 1). Fifty percent of *pilE* PCR products contained HV-1 target-specific *pilS1* copy 4 or *pilS6* copy 1 sequences in the HV region. The other clones sequenced contained HV sequences from a subset of other silent pilin copies (20), each of which contained two to five mismatches to the HV-1 primer. Therefore, although this RT-PCR assay does not detect recombination solely from *pilS1* copy 4 or *pilS6* copy 1 into *pilE*, it accurately and reproducibly quantitates the recombination frequency of HV-1 amplifiable silent pilin sequences into *pilE*.

Effect of growth on the frequency of gonococcal pilin antigenic variation. Since this competitive RT-PCR assay provides a reproducible measurement of the frequency of pilin antigenic variation within a gonococcal population, we used it to study the effect of growth. We used two FA1090 variants which do not initially contain HV-1 amplifiable sequences in *pilE*; 1-81-S2 WT (19) and 1-81-S2 *recA6* (11). Variant 1-81-S2 *recA6* contains the *recA6* allele, in which *lac* regulatory sequences control the transcription of *recA*. In the absence of isopropyl-β-D-thiogalactopyranoside (IPTG), transcription of *recA* is undetectable, and *recA6* variants are deficient in recombination. However, in the presence of IPTG, *recA* is transcribed, and recombination-mediated processes, such as pilin antigenic variation, can occur (18). The use of variant 1-81-S2 *recA6* ensured that the initial *pilE* sequences within the gonococcal population did not contain HV-1 target sequences. Moreover, in the absence of IPTG, recombination-dependent product from the PCR with primers PILRBS and HV-1 was not detected at any point during growth (data not shown).

The frequency of pilin antigenic variation was determined for mid-log, late log, and late log/stationary phases of growth of these two FA1090 variants. Following the induction of *recA* with IPTG at t_0, there was a considerable increase in the frequency by which HV-1 sequences recombined into *pilE* over time in 1-81-S2 *recA6* (Fig. 2). A 3.8-fold increase, from 4.97×10^{-4} to 1.87×10^{-3} HV-1 *pilE*/*pilE*, was observed between mid- and late log phases, followed by a 2-fold increase, from 1.87×10^{-3} to 3.86×10^{-3} HV-1 *pilE*/*pilE*, between late log and late log/stationary phases. Rates of variation, which reflect the number of antigenic variants per generation, were calculated during exponential growth of the gonococcal population and were 2.76×10^{-4} variants per generation (var/gen) from t_0 to mid-log, 4.90×10^{-4} var/gen from mid-log to late log, and 3.36×10^{-4} var/gen from t_0 to late log. The increase in the frequency over time suggested that the number of gonococci within the population containing recombinant, HV-1 amplifiable *pilE* accumulated from the time recombination could oc-

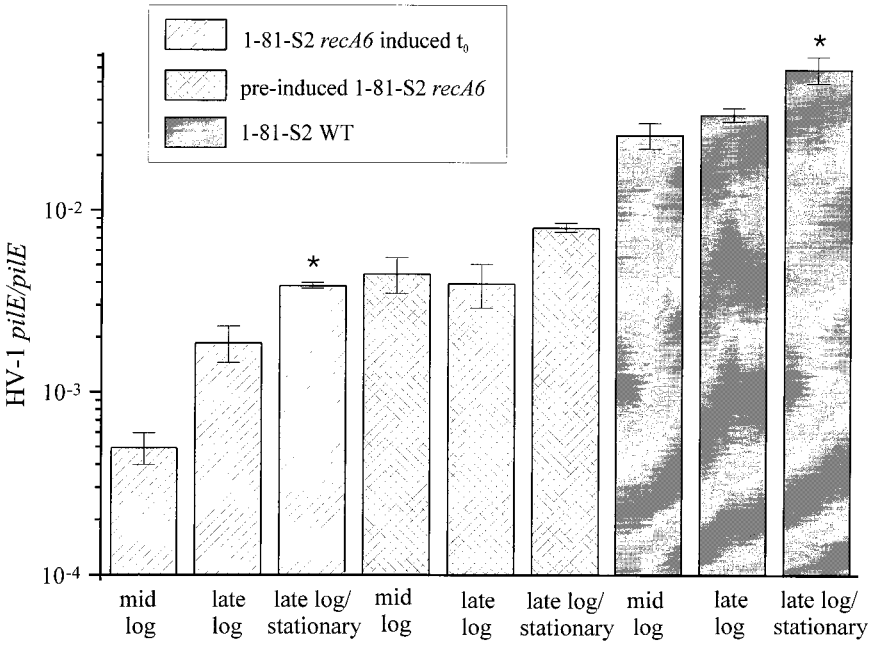

FIG. 2. Average frequency of pilin antigenic variation with respect to growth phase. The frequency of antigenic variation is expressed as HV-1 *pilE*/*pilE* for mid-log, late log, and late log/stationary phases of 1-81-S2 *recA6* induced at t_0, preinduced 1-81-S2 *recA6* and 1-81-S2 WT. Six to 15 individual sets of PCRs were performed for each time point, and the standard errors of the means are shown by the error bars. The asterisk indicates a significant difference relative to the value at late log phase by the Student *t* test with $P < 0.05$.

cur with the induction of *recA* through to late log/stationary phase of growth.

In order to determine whether the level of HV-1 amplifiable *pilE* in variant 1-81-S2 *recA6* reaches an equilibrium within a population undergoing continual pilin recombination, this variant was preinduced with IPTG prior to the growth curve assayed. The frequency of pilin recombination in variant 1-81-S2 *recA6* preinduced with IPTG remained relatively constant throughout early growth and with repeats of growth, with 4.48×10^{-3} HV-1 *pilE*/*pilE* at mid-log and 3.97×10^{-3} HV-1 *pilE*/*pilE* at late log. There was a twofold increase in this frequency upon late log/stationary phase relative to late log from 3.97×10^{-3} to 8.05×10^{-3} HV-1 *pilE*/*pilE* (Fig. 2). The consistency in the frequency over time suggested the presence of an equilibrium subpopulation of HV-1 amplifiable *pilE*-expressing gonococci within the population which was not drastically influenced by growth.

With variant 1-81-S2 WT, the frequency of pilin antigenic variation also remained relatively constant throughout the exponential phase of growth, with 2.59×10^{-2} HV-1 *pilE*/*pilE* at mid-log and 3.35×10^{-2} HV-1 *pilE*/*pilE* at late log (Fig. 2). The rate of variation over this period of growth was 7.92×10^{-3} var/gen. Overall, the frequency by which HV-1 target sequences recombined into *pilE* was approximately 10-fold higher in the 1-81-S2 WT variant than in the 1-81-S2 *recA6* variant, presumably due to the lower levels of *recA* mRNA present in fully induced *recA6* variants compared to those in the WT counterparts (18). The frequency of pilin antigenic variation in the WT variant was consistent both throughout early growth and throughout repeated growth curves assayed. This further suggested the presence of an equilibrium subpopulation of variant gonococci within the population which was not affected by growth. In contrast, there was a significant twofold increase in the frequency of pilin recombination upon late log/stationary phase relative to late log from 3.35×10^{-2} to 5.92×10^{-2} HV-1 *pilE*/*pilE*. This suggested that some event during the transition into the late log/stationary phase of growth may have influenced pilin recombination. Stationary-phase effects have been reported in *N. gonorrhoeae*, such as an enhancement in gonococcal autolysis (2, 3, 13). This increased autolysis, coupled with the natural transformation competence of gonococci, has previously been proposed to affect pilin sequence changes (4, 14). It is possible that an increase in autolysis and the subsequent uptake of released DNA by intact, unlysed gonococci may account for the increase in the frequency of pilin antigenic variation observed upon late log/stationary phase.

Conclusions. This quantitative RT-PCR assay measures pilin antigenic variation independently of changes in gonococcal colony morphology, thus taking into account the fact that pilin antigenic and colony morphology-based phase variation are separable events. Furthermore, this assay can be used to quantitate and to compare the frequency of gonococcal pilin antigenic variation between different variants, growth conditions, or mutants and is adaptable for the detection and quantitation of recombination from other silent pilin copies into *pilE*. Using this quantitative assay to examine pilin antigenic variation frequencies during growth, we can conclude that growth does not dramatically affect the frequency of pilin antigenic variation in a gonococcal population but that a reproducible, twofold increase in the frequency does occur upon transition into the late log/stationary phase of growth.

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