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The frequency and rate of pilin antigenic variation in *Neisseria gonorrhoeae*

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Summary

The pilin antigenic variation (Av) system of *Neisseria gonorrhoeae* (Gc) mediates unidirectional DNA recombination from silent gene copies into the pilin expression locus. A DNA sequencing assay was developed to accurately measure pilin Av in a population of Gc strain FA1090 arising from a defined pilin progenitor under nonselective culture conditions. This assay employs a pilated parental Gc variant with a *recA* allele whose promoter is replaced by *lac*-regulatory elements, allowing for controlled induction of pilin Av. From this assay, the frequency of pilin Av was measured as 0.13 recombination events per colony, with a corresponding rate of pilin Av of 4×10^{-3} events per colony per generation. Most pilin variants retained the parental piliation phenotype, providing the first comprehensive analysis of pilated variants arising from a pilated progenitor. Sequence analysis of pilin variants revealed that a subset of possible recombination events predominated, which differed between pilated and nonpilated progeny. Pilin Av exhibits the highest reported frequency of any pathogenic gene conversion system and can account for the extensive pilin variation detected during human infection.

Keywords

gonorrhea; recombination; pilus

Introduction

Homologous recombination of DNA is a fundamental cellular process involved in replication, DNA repair, and the generation of genetic diversity. Gene conversion, the nonreciprocal incorporation of DNA sequence into a homologous locus, underlies immunoglobulin variation in some mammals (Knight, 1992) and is also used by pathogens such as *Borrelia*, *Campylobacter*, *Plasmodium*, and *Trypanosoma* to antigenically vary virulence-associated surface structures (van der Woude and Baumler, 2004). Antigenic variation (Av) has been implicated in evasion of the host humoral immune response and adaptation to new intracellular niches or to different host species (Liang *et al.*, 2004; Smith *et al.*, 1995). During infection, frequencies of gene conversion range from 10^{-1} to $<10^{-4}$ recombinants per cell, with corresponding rates of 10^{-2} to $<10^{-5}$ recombinants per cell per generation (Horrocks *et al.*, 2004; Keely *et al.*, 2003; Roberts *et al.*, 1992; Roske *et al.*, 2001; Tu *et al.*, 2003; Turner, 1997), but some of these frequencies may reflect selection in the host.

Pilin Av in the human-specific pathogen *Neisseria gonorrhoeae* (Gc) is one of the best-understood systems of gene conversion (Kline *et al.*, 2003). Pilin is the major subunit of the

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neisserial type IV pili, which are present on all clinical isolates of Gc and are essential for establishing urogenital infection (Cohen and Cannon, 1999). Gc carry a pilin expression locus, *pilE*, and multiple storage loci containing unexpressed pilin coding information, collectively termed *pilS*, on their chromosome. During pilin Av, part or all of a *pilS* copy is transferred unidirectionally into *pilE*, giving rise to new pilin variants that differ in their ability to be expressed or assembled into pili. As a result, pilin Av confers a spectrum of pilus-dependent colony morphologies on Gc, from heavily pilated (P+) to nonpilated (P-).

In the Gc strain FA1090, 19 unique *pilS* copies reside in 6 discrete chromosomal loci (Hamrick *et al.*, 2001). All *pilS* copies lack the promoter, ribosomal binding site, and 5' 150 bp found at *pilE* (Haas and Meyer, 1986; Haas *et al.*, 1992; Segal *et al.*, 1986). Based upon sequence divergence in the *pilS* repertoire, *pilS* copies are comprised of a 5' semivariable region, corresponding to the central ~250 bp of *pilE*, and a 3' hypervariable loop and tail (HV_L and HV_T) of ~100 bp in length. Within these variable regions are short (1–34 bp) regions of sequence conserved among all *pilS* copies and *pilE* thought to provide the homology that drives recombination. As with all homologous recombination pathways, RecA function is essential for pilin Av (Kooimey *et al.*, 1987). Pilin Av also requires a RecF-like pathway (RecQ, RecO, RecR, and RecJ), the RecA modulator RecX, the growth regulator RdgC, the Rep helicase and the Holliday junction processing enzymes RecG and RuvB (Kline and Seifert, 2005a; Mehr and Seifert, 1997, 1998; Mehr *et al.*, 2000; Sechman *et al.*, 2005; Skaar *et al.*, 2002; Stohl and Seifert, 2001).

Estimates of the frequency of pilin Av are based upon P-colony appearance or sequence changes in the *pilE* HV_L and range from 10⁻² to 10⁻⁴ variants per colony-forming unit (CFU) (Bergstrom *et al.*, 1986; Kooimey *et al.*, 1987; Rohrer *et al.*, 2005; Segal *et al.*, 1985; Serkin and Seifert, 1998; Wainwright *et al.*, 1994; Zhang *et al.*, 1992). To directly measure pilin Av, we developed a DNA sequencing assay that nonselectively identifies pilin variants arising from a defined *pilE* progenitor. Pilin Av is controlled by the *lac*-inducible regulation of *recA* expression, which allows for the frequency and rate of this process to be accurately measured. We have measured a frequency of pilin Av for FA1090 variant 1-81-S2 of 0.13 recombination events per CFU and a corresponding rate of 4.0×10⁻³ events per CFU per generation, values that are 5–500 times higher than previously reported for this Gc variant. The large number of P+ and P- pilin antigenic variants analyzed allowed for definition of the recombination events underlying pilin Av at a previously unavailable level of detail.

Results

Measuring the frequency and rate of pilin Av with the *pilE* sequencing assay

We developed a novel DNA sequencing-based assay to measure the total amount of pilin Av independent of colony morphology changes in a population of Gc. This assay depends upon three key factors. First, Gc of strain background FA1090 were used, since all *pilS* copies in this strain have been sequenced (Hamrick *et al.*, 2001). Second, the specific FA1090 isolate used harbors the *recA6* allele, in which RecA expression is controlled by *lac*-regulatory elements (Seifert, 1997). In the *recA6* genetic background, RecA-dependent processes like pilin Av are initiated only when Gc are grown in the presence of IPTG (Rohrer *et al.*, 2005; Serkin and Seifert, 1998; Seifert, 1997). The *recA6* isolate encodes the 1-81-S2 pilin variant (Seifert *et al.*, 1994), a highly pilated variant that has been used previously to measure pilin Av (Rohrer *et al.*, 2005; Serkin and Seifert, 1998). The combination of the *recA6* allele with the 1-81-S2 variant sequence ensures that the parental colonies uniformly express one pilin protein. Third, large-scale DNA sequencing allowed the *pilE* genes from a large number of randomly selected progeny to be rapidly examined. In combination, these factors yield the most complete analysis of pilin Av to date.

To perform the assay, 3–7 single colonies of FA1090 1-81-S2 *recA6* Gc are grown in the presence of IPTG to allow pilin Av to occur. These colonies are dispersed and grown on solid medium in the absence of IPTG. At least 48 CFU arising from each parental colony are isolated and serially passaged to ensure the recovery of pure populations of Gc. The *pilE* loci of the progeny are sequenced and compared to both the parental *pilE* allele as well as the *pilS* copies present in the FA1090 genome. These numbers of starting and progeny colonies were determined statistically to yield accurate, reproducible measurements of pilin Av (see Experimental Procedures). The assay was performed with three independent cultures of Gc, generating a total of 497 progeny colonies that were examined for *pilE* sequence changes.

5.2–17.3% of *pilE* clones in the three sequenced populations differed from 1-81-S2, averaging $11.8 \pm 3.5\%$ variation. Strikingly, 0.96% of the *pilE* sequences in each replicate population had undergone two recombination events, comprising 13.3% of all nonparental *pilE* sequences. A recombination event is defined as the incorporation of new *pilS* sequence into *pilE*; when multiple recombination events have occurred, sequences corresponding to different *pilS* copies appear at various sites in *pilE*. Detection of two recombination events in a *pilE* sequence was a reasonable expectation: if 10% of clones exhibit one event, 1% would on average exhibit two. However, multiple recombination events per *pilE* sequence have never before been taken into account when measuring pilin Av. Since the sequencing assay had the unique ability to identify these events, we redefined the frequency of pilin Av as the number of recombination events measured in a population divided by the total number of *pilE* genes sequenced, i.e. the number of CFU. In the 497 P+ CFU examined, the frequency of pilin Av was 0.13 ± 0.04 recombination events per CFU. The DNA sequencing assay is therefore able to detect substantial amounts of pilin Av in a Gc population cultured under nonselective conditions.

Assuming each colony grown in the presence of IPTG arose from one CFU and contained 19 generations of bacteria ($\sim 3 \times 10^5$ CFU per colony), the resulting rate of pilin Av is $\sim 6.8 \times 10^{-3}$ recombination events per CFU per generation. In order to more accurately measure the rate of pilin Av, FA1090 1-81-S2 *recA6* Gc were grown in liquid medium containing IPTG, and the frequency of pilin Av was analyzed at 3 hr intervals up to 12 hr after IPTG induction for three independent cultures. The average generation time of these cultures was 61 min. No antigenic variants were detected in any of the replicate cultures at 3 hr after IPTG induction (a frequency of $< 9.52 \times 10^{-3}$ events per CFU). At 6 hr, the average frequency was 1.05×10^{-2} events per CFU; at 9 hr, 3.52×10^{-2} events per CFU; at 12 hr, 5.13×10^{-2} events per CFU. Using the data from the 6 to 12 hr time points, the rate of pilin Av was calculated to be $4.0 \pm 0.6 \times 10^{-3}$ recombination events per CFU per generation.

The results with FA1090 1-81-S2 *recA6* were validated using Gc with a wild-type *recA* allele, in which pilin Av occurs constitutively. Three experiments were conducted, yielding 180 *pilE* clones. $22.5 \pm 2.7\%$ of progeny CFU encoded nonparental *pilE* sequences, with a corresponding frequency of pilin Av of 0.25 ± 0.01 recombination events per CFU. This frequency is within two fold of that measured in *recA6* Gc. The rate of pilin Av of Gc with a wild-type *recA* allele was estimated by culturing Gc in liquid medium and performing the sequencing assay at 0, 3, 6, 9, and 12 hr after inoculation. Since in this experiment the starting inoculum exhibited a frequency of pilin Av of 0.25 events per CFU, the rate was calculated as the change in frequency at each time point divided by the number of generations and was found to be $1.8 \pm 0.4 \times 10^{-2}$ recombination events per CFU per generation. The frequency and rate calculations with this Gc strain are substantially higher than measured in *recA6* Gc, but these values should only be considered estimates since the starting colonies of FA1090 1-81-S2 Gc may have contained bacteria with nonparental *pilE* sequence. Furthermore, it is likely that each wild-type *recA* colony contains a different percentage of nonparental *pilE* variants, but since these variants arise stochastically, the basal level of variation in a colony cannot be accurately assessed. When the frequency of pilin Av is measured, the variant sequences present at the

start are recorded as if they had arisen during the course of the experiment (although they were already present at the start), thus affecting the calculated rate. In contrast, at the time of *recA* induction the starting population of the *recA6* Gc is uniform in its *pilE* sequence, which then produces a defined rate. These results show that the values calculated with Gc with a wild-type *recA* allele are similar to those measured in the *recA6* strain, and even after taking into account the variability inherent in wild-type colonies, may in fact be higher. We conclude that the *pilE* sequencing assay for Gc reproducibly measures pilin Av, and it reveals that the frequency and rate of this process are significantly higher than previously reported (Rohrer *et al.*, 2005; Serkin and Seifert, 1998).

Nonrandom incorporation of *pilS* sequences in P+*pilE* variants

Prior to this study, large-scale analysis of pilin antigenic variants was limited to Gc exhibiting pilus-dependent colony morphology changes. However, results from the *pilE* sequencing assays clearly demonstrate that a sizable percentage of P+ Gc arising from a P+ progenitor undergo pilin Av. We therefore used the sequence information from these assays to investigate the recombination events arising in P+ variants of FA1090 1-81-S2. 93 nonparental *pilE* sequences were recovered from 1-81-S2 harboring either the *recA6* or the wild-type *recA* allele. Five of the 93 *pilE* clones had undergone two recombination events at *pilE*, generating 98 total recombination events. 92 of the 98 events could be unambiguously assigned to one or two *pilS* copies. The remainder diverged from 1-81-S2 at 1–2 bp and corresponded to sequences found in multiple silent copies. It is likely that these single nucleotide changes are true antigenic variants and not point mutants, since a) the nucleotide changes are in variable regions and are present in several *pilS* copies that have flanking sequence identity to 1-81-S2, and b) no single nucleotide changes were detected elsewhere in *pilE*, including conserved sequences.

The 92 P+ recombination events with identifiable donor silent copies had sequence corresponding to 12 of the 19 *pilS* copies in Gc strain FA1090. Within this subset, certain silent copies predominated (Table 1). The most frequently recovered recombinant exhibited a change in the HV_T region of *pilE* corresponding to the sequence found in both *pilS2c1* and *pilS6c1* (Fig. 1). It cannot be determined whether one or both of these donors contributed to this recombination event, since both share sequence identity with 1-81-S2 *pilE* upstream of the HV_T. However, *pilS2c1* is identical to 1-81-S2 *pilE* for 268 bp upstream of the HV_T (dotted line in 2c1/6c1, Fig. 1), whereas *pilS6c1* only shares 32 bp of sequence with 1-81-S2 *pilE* in this region (solid line). Sequences from *pilS3c1*, *pilS1c1*, and *pilS3c2* also appeared repeatedly. Notably, four recombination events, *pilS2c1/6c1*, *pilS3c1-A* and *-B*, and *pilS3c2-A*, accounted for 57% of the events detected (Fig. 1), and all arose in more than one of the six *pilE* sequencing assays. In total, eight *pilE* variants emerged in more than one of the sequencing assays performed (arrowheads, Fig. 1), indicating that certain recombination events are more frequently recovered than others. The most predominant *pilS* copies appeared at similar levels in both *recA6* and wild-type *recA* genetic backgrounds, indicating there is no mechanistic difference between these two strains (data not shown). The relative frequency of incorporation of *pilS* copies into the *pilE* HV_L differed from full-length *pilE*, but correlated with a previous examination of the repertoire of donor *pilS* sequences in the HV_L arising from the 1-81-S2 variant (Rohrer *et al.*, 2005).

All 98 P+ recombination events were examined for the length and position of variant sequence in *pilE* (Fig. 1). The length of contiguous variant sequence incorporated in *pilE* averaged 55 bp and ranged from 1 bp (A326G) to greater than 200 bp (*pilS3c1* recombinant C, *pilS1c5-B*). Notably, 50% of the recombination events involved incorporation of 15 bp or less of *pilS* sequence (*pilS2c1/6c1*, *pilS3c2-A* and *-E*, *pilS3c3-A*). In contrast, only one recombination event involved incorporation of an entire *pilS* copy (*pilS7c1*). The 98 P+ recombination events occurred throughout *pilE*, with 50% of variants exhibiting changes in the semivariable region.

As previously observed (Hamrick *et al.*, 2001; Howell-Adams and Seifert, 2000; Seifert, 1996), the location of the endpoints of variant information did not support the minicassette model of pilin Av (Haas and Meyer, 1986). Taken together, these results indicate that P+ antigenic variants derived from 1-81-S2 *pilE* are generated from a subset of *pilS* copies that donate short stretches of sequence to *pilE*, and that a small number of pilin variants exhibiting identical recombination events at *pilE* arise repeatedly from this progenitor pilin sequence.

Generation of P- variants by pilin Av

Gc colonies of P- morphology arise from pilin Av, L-pilin production (the incorporation of multiple *pilS* copies in tandem into *pilE*, yielding an aberrantly long, nonfunctional protein), deletion of the *pilE* locus, and phase variation of the structural PilC proteins (Hagblom *et al.*, 1985; Jonsson *et al.*, 1991; Segal *et al.*, 1985; Swanson *et al.*, 1985; Swanson *et al.*, 1986). To explore the relative contribution of these events to P- colony formation, FA1090 1-81-S2 *recA6* was grown on solid medium containing IPTG to induce pilin Av, sectors of colonies exhibiting a less piliated morphology were isolated, and the *pilE* genes were sequenced from progeny retaining the P- morphology. Three replicate experiments were performed with a total of 67 P- CFU analyzed.

The appearance of P- colonies in a population has been used as a surrogate measure of pilin Av, and we observed that 58.6% of the P- clones had undergone pilin Av. The remaining 41.4% of P- colonies exhibited one of three phenotypes: 1) production of L-pilin, determined by the generation of an abnormally long PCR product using *pilE*-specific primers (6.5% of the P- isolates); 2) deletion of the *pilE* locus, assessed by the inability to produce a PCR product with these same *pilE*-specific primers (20.2%); and 3) PilC phase variation, or the retention of parental *pilE* sequence while exhibiting P-morphology (14.7%). The absence of PilC expression was confirmed by Western blot (data not shown). Two of the 67 P- CFU exhibited sequence changes that were detected in the P+ population and were also PilC phase variants (*2c1/6c1* and *1c5*, Fig. 2). These results demonstrate that pilin Av is a common mechanism for generating P- variants, but other mechanisms are of equal prevalence and should be taken into consideration when using phase variation as a surrogate measure of pilin Av.

The 47 *recA6* and 5 wild-type *recA* P- pilin antigenic variants yielded a total of 52 P- variants and 64 recombination events. 61 of the 64 recombination events could be assigned to one or two *pilS* copies (Fig. 2). Compared to the P+ variants, a smaller and different subset of *pilS* copies were represented in the P- variants (Table 1). As with the P+ variants, the most predominant *pilS* copies appeared in each of the replicate P- population at a similarly high frequency (data not shown). These variants fell into two categories. The first class of variants, accounting for 7% of the P- sequences, was predicted to produce full-length pilin protein but produced a P- colony phenotype. These variants are classified as “assembly mutants,” where pilin monomers are not effectively assembled into the growing pilus (Swanson *et al.*, 1986). No pilin protein was detected in these variants by Western blot, but PilC was still expressed (data not shown). This class of variants incorporated *pilS2c1* or *pilS6c1* in the semivariable region of *pilE* (*2c1*- A and -B and *6c1*- A and -B, Fig. 2).

The majority of P- pilin variants also failed to react with pilin-specific antiserum, but these variants were predicted to encode truncated proteins. These P- variants contained sequence encoded in one of 4 *pilS* copies: *pilS2c4*, *pilS3c2*, *pilS6c2*, and *pilS1c3* (Fig. 3). Incorporation of *pilS2c4*, *pilS3c2*, and *pilS6c2* at the 5' end of the *pilE* semivariable region generates a stop codon after residue 94 or 95, and incorporation of *pilS1c3* introduces a stop codon at position 78.3 of the 4 *pilS* copies contain poly(C) tracts that could vary in length as a result of replication errors to restore a P+ phenotype (Koomey *et al.*, 1987). As with P+ variants, certain *pilS* sequences in these P- variants predominated, with *pilS2c4* and *pilS3c2* detected more frequently than the other two (Table 1).

Compared to P+ variants, P- variants on average contained longer tracts of *pilS* sequence which was incorporated closer to the 5' end of the *pilE* semivariable region (Fig. 2). 86% of P-recombination events occurred in the semivariable region of *pilE*, and the average minimum length of *pilS* sequence incorporation in P- variants was 133 bp. Although *pilS* incorporation as short as 1 bp could be detected (C395A, Fig. 2), only 15.7% of variants had incorporated. 15bp or less of *pilS* sequence. In summary, pilin Av accounts for the majority of P- colonies generated after *in vitro* passage of Gc, and these variants arise from recombination with a subset of *pilS* copies that encode truncated or poorly assembled pilin proteins.

Minimal sequence identity is required for pilin Av

RecA-dependent homologous recombination requires sequence identity between donor and recipient DNA of at least 73 bp (Nash, 1996). Each donor *pilS* copy identified in the variant *pilE* population was aligned with 1-81-S2 *pilE* to identify regions of shared sequence (depicted for a subset of *pilS* copies in Figure 1 and Figure 2). For each recombination event where the donor *pilS* copy could be unambiguously identified, the length of identical sequence between the incoming copy and 1-81-S2 *pilE* flanking the recombinant tract was measured. The median amount of shared sequence was 44 bp at the 5' flanking region and 39 bp at the 3' flanking region, and 8 independent recombination events were detected where shared sequence identity was 2–4 bp. No significant difference was noted in the amount of sequence overlap between P+ and P- variants. Minimal regions of shared sequence identity were not necessarily paired with especially extensive sequence identity at the other end of the recombination tract, and the length of sequence overlap was not related to the length of *pilS* sequence appearing in *pilE*. This analysis confirms that minimal sequence identity is needed for *pilE* recombination (Howell-Adams and Seifert, 2000) and further demonstrates that recombination is independent of the length or identity of the *pilS* sequence incorporated, lending further support for the current understanding of pilin Av as a specialized form of homologous recombination.

Discussion

Gonococcal pilin Av is a prototype for prokaryotic gene conversion systems, yet a direct measure of the frequency and rate of this process has never been reported. We developed a *pilE* sequencing assay to address this issue, which revealed that 1 out of 4–8 CFU in a population has undergone recombination at *pilE*. These frequencies are three- to ten-fold higher than measured with a RT-PCR assay examining variants in the HV_L, the most complete assay for pilin Av until this point (Rohrer *et al.*, 2005; Serkin and Seifert, 1998). Strikingly, the maximum frequency of pilin Av may be even higher than reported here, since FA1090 Gc with a different starting *pilE* sequence was found to produce a greater percentage of HV_L variants than 1-81-S2 (Rohrer *et al.*, 2005). To our knowledge, pilin Av exhibits the highest frequency of gene conversion of any pathogenic antigenic variation system examined to this point (van der Woude and Baumler, 2004). One drawback to this assay is its cost, considering that a minimum of 480 PCR products need to be sequenced per time point for a given strain. However, this assay demonstrated that most antigenic variants retain the parental P+ phenotype and provided the first comprehensive analysis of P+ variants arising from a P+ progenitor. It also identified a novel means by which P- variants arise from a P+ progenitor. Finally, by generating 159 nonparental *pilE* sequences encompassing 48 unique pilin variants (Supplemental Fig. 1), the assay produced the most complete picture available about the recombination events of pilin Av.

It is apparent from the P+ and P- variant *pilE* sequences arising from FA1090 1-81-S2 that certain *pilS* copies predominate, even in the absence of any apparent selection pressure. We have calculated that 963 independent antigenic variants could arise following one recombination event with 1-81-S2 *pilE*, if at least 2 bp of sequence identity exists at the end

of a tract of new *pilE* sequence. This is inconsistent with the observation that only 3 recombination events account for 50% of the P+ variant population (*2c1/6c1* and *3c1*- A and -B, Fig. 1). These are not isolated events, since these and other high-frequency recombinants occurred in multiple experiments (arrowheads, Fig. 1). Several explanations for the nonrandom distribution of *pilS* donors were considered, including the chromosomal location of a *pilS* copy or its position in the *pilS* locus, its relative percentage of sequence identity to *pilE*, the presence of pilin-associated repeat elements (Hamrick *et al.*, 2001), and the length of the sequence tracts flanking the *pilE/pilS* recombinants. The antigenic variation data presented in this work does not individually support any of these possibilities. We postulate that either a combination of these factors dictates the prevalence of *pilE* recombination events, or some as-yet unknown sequence element or bacterial factor influences the frequency of *pilS* recombination at the *pilE* locus.

In FA1090 1-81-S2, the predominant mechanism for generating P- variants was recombination with silent copies that shift the *pilE* coding frame and prematurely incorporate a stop codon. These truncations were predicted from the sequence analysis of *pilS* copies by Hamrick *et al.* (Hamrick *et al.*, 2001) but were not previously recovered in P- Gc. It is important to note that these variants differ from S-pilin variants, which encode full-length pilin proteins that undergo posttranslational removal of their N-termini (Kooimey *et al.*, 1991). Although we have no direct evidence that P- variants occur at a measurable frequency during infection, the retention of *pilS* copies encoding truncated pilins in the genome suggests that nonpilated variants may have a role in infection. The first possibility for such a role is that nonpilated Gc have a growth advantage *in vivo* since, *in vitro*, nonpilated Gc have an enhanced metabolic rate compared to an isogenic pilated strain and can outgrow their pilated counterparts when cultured together in liquid medium (H.S. Seifert, unpublished results). The second possibility is that loss of pilation is advantageous during infection. For instance, the reduced adherence capacity of nonpilated bacteria may facilitate Gc movement within a person or transfer between individuals. Furthermore, P- Gc would not be recognized by host pilin-specific antibodies, and in this light a lack of pilation can be considered an extreme form of Av. The third possibility is that nonpilated Gc may alter host cell signaling. In epithelial cells, neisserial pili and their retractile forces induce Ca^{2+} spikes and the activation of protein kinases (Howie *et al.*, 2005; Kallstrom *et al.*, 1998); modulation of these signals by P- Gc could affect, for example, the inflammatory response. If any or all of these possibilities are found to be important for Gc pathogenesis, pilin Av would provide the high-frequency mechanism behind $\text{P+} \leftrightarrow \text{P-}$ variation.

The extent of pilin Av measured by the DNA sequencing assay can be compared to that reported during human infection. The majority of Gc recovered from experimentally infected male volunteers encode nonparental *pilE* sequences after 24 hr of infection, and some *pilE* genes contain more than one recombination tract (Hamrick *et al.*, 2001; Seifert *et al.*, 1994; Swanson *et al.*, 1987). These high levels of pilin variation were thought to be due either to stimulation of the frequency of pilin Av or selection against the parental pilin protein. In contrast, results from the sequencing assay would argue that the frequency of pilin Av *in vitro* is sufficient to account for all pilin variation that occurs during infection. It is not possible to directly compare the rate of pilin Av measured *in vitro* to that occurring *in vivo* because the number of generations of growth in the host cannot be determined: colonization bottlenecks occur early after inoculation that limit the number of bacteria that actually establish infection (Cohen and Cannon, 1999). Assuming all the progeny arising during acute infection were descended from one parental CFU and an *in vitro* rate of pilin Av, after one day approximately 50% of isolates would have undergone a recombination event, which is similar to what has been observed in experimentally infected individuals. Our results also correlate with the observation made in human volunteer studies that a small population of antigenic variants, involving a subset of *pilS* copies, predominate, without needing to invoke selection *in vivo* (Hamrick *et al.*, 2001;

Seifert *et al.*, 1994). However, it is likely that in natural, long-term infections, immune selection is important for driving the appearance of a subpopulation of pilin variants. Since each newly arising pilin variant would give rise to its own unique repertoire of pilin sequences (Rohrer *et al.*, 2005), this ever-expanding pool of variants could facilitate immune evasion as Gc are transmitted within a sexual network and back to previously exposed individuals. We conclude that all observations made about gonococcal pilin Av *in vitro* are sufficient to explain the repertoire of pilin variants arising during infection, underscoring the importance of this process to the persistence and spread of Gc in the human population.

Experimental Procedures

Bacterial strains and growth conditions

1-81-S2 is a P+ variant of Gc strain FA1090 arising after experimental infection of a male volunteer (Seifert *et al.*, 1994). FA1090 1-81-S2 *recA6* contains an isopropyl β-D-thiogalactopyranoside (IPTG)-regulatable Gc *recA* gene, which allows for control of *recA* expression. These strains are phenotypically RecA-when IPTG is absent from the growth medium and phenotypically RecA+ in the presence of IPTG (Seifert, 1997). Gc were grown on GC Medium Base (Difco) plus Kellogg supplements (Kellogg *et al.*, 1963) (GCB) at 37°C in 5% CO₂ for 20 h, with the addition of 1 mM IPTG (Diagnostic Chemicals Limited) when necessary. Colonies were observed in the stereomicroscope to ensure retention of the desired piliation phenotype. Liquid growth of Gc was performed in Gc liquid medium containing Kellogg's supplements and 0.042% Na₂HCO₃ (GCBL) as described (Serkin and Seifert, 1998).

Pilin variation assays

(i). Growth on solid medium—The pilin variation assay was performed as described (Kline and Seifert, 2005b). 3–7 colonies of FA1090 1-81-S2 *recA6* Gc grown on GCB-IPTG were each passaged on GCB. Approximately 48 P+ colonies that arose from each starter colony were passaged two more times to ensure clonal populations, then stored at –80 °C. For Gc with the wild-type *recA* allele, 1 P+ colony of Gc with 1-81-S2 *pilE* sequence was passaged on GCB. 48–96 colony-forming units (CFU) of both P+ and P– colony morphology were collected and stored at –80 °C without further passage. P– colonies comprised ~5% of this population.

To examine P– variants in FA1090 1-81-S2 *recA6*, P– outgrowths arising from P+ colonies on GCB-IPTG were isolated and streak purified for several days on GCB to ensure a homogeneous P– population was obtained. In each experiment, up to 32 independent P– clones were examined.

(ii) Growth in liquid medium—Liquid-grown FA1090 1-81-S2 *recA6* were diluted into GCBL containing 1 mM IPTG at 37 °C. At 3, 6, 9, and 12 h after IPTG induction, an aliquot of the culture was serially diluted and plated. The number of CFU present at each time point was determined, and 96 CFU were collected from each time point. The experiment was performed in triplicate.

(iii) Statistical calculations—To determine the minimum sample size necessary to yield an accurate measurement of pilin Av, we performed a small pilot study with three 1-81-S2 *recA6* starter colonies and 16–24 sequenced progeny from each, which had a standard deviation of 2.8% (data not shown). The formula

$$N=((z*s.d.)/E)^2$$

was then applied, where N = sample size, z = value corresponding to the confidence interval, $s.d$ = standard deviation of the sample, and E = acceptable margin of error for the assay. Using $z = 95\%$ confidence interval and $E \leq 1\%$ margin of error, N was calculated at 48 progeny per starter colony.

***pilE* sequence analysis**—The *pilE* locus was amplified by polymerase chain reaction (PCR) from Gc lysates as described previously (Kline and Seifert, 2005b). Sequencing reactions were carried out with the CEQ Dye Terminator Cycle Sequencing Quick Start Kit using primers specific for *pilE* and were run on a CEQ 2000XL automated sequencer (Beckman Coulter) according to the manufacturer's instructions. Alternatively, purified PCR products were sequenced commercially (SeqWright, Houston, TX).

Calculations for pilin antigenic variation—The *pilE* sequence from each clone was aligned with the parental 1-81-S2 *pilE*, as well as with all *pilS* copies present in the FA1090 genome (Hamrick *et al.*, 2001), using the AlignX program of the Vector NTI software (Informax, Inc.). In all cases, sequence changes observed in the isolates corresponded to one or more *pilS* copies. Nonparental *pilE* sequences were translated using the EditSeq program in the Lasergene software package (DNASTar, Inc.).

Each independent incorporation of *pilS* sequence into a *pilE* clone was designated a recombination event. For FA1090 *recA6* Gc, the frequency of pilin Av was calculated by the following formula: Frequency = Total number of recombination events at *pilE* ÷ Total number of isolates sequenced. For FA1090 1-81-S2 Gc, the frequency of pilin Av was determined separately for P+ and P− CFU, and the overall frequency was calculated by multiplying the frequency for each population by the fraction of colonies with the given colony morphology. Data are presented as the average of the frequencies measured from replicate samples ± SEM. The rate of pilin Av is presented as the average change in frequency divided by the average number of bacterial generations over the course of the experiment ± SEM.

Analysis of PilC and pilin production in P- variants—Lysates of P- variants were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide for PilC, 15% for pilin) and transferred to polyvinylidene difluoride (Millipore). PilC was detected using a polyclonal anti-PilC antiserum (gift of J. Pfeifer and S. Normark) (Nassif *et al.*, 1994) and pilin detected using T36 polyclonal antiserum (gift of M. So) (Forest *et al.*, 1996), with both followed by goat anti-rabbit IgG coupled to horseradish peroxidase (Chemicon). Blots were developed using the ECLPlus chemiluminescence reagent (Amersham).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Recombination events in *P*⁺ variants of *Gc* strain FA1090

pilS copies are abbreviated as in Table 1. The positions in *pilE* of the semivariable (SV), hypervariable loop (HV_L) and tail (HV_T), and conserved *cys1* and *cys2* sequences are indicated. Sequences conserved among all *pilS* copies are gray and divergent regions are white; both are to scale. Recombination events involving the same donor *pilS* copy are grouped together and are presented in decreasing order of frequency of appearance in *pilE*. Hatched boxes indicate recombinant *pilS* sequence in 1-81-S2, and flanking sequence shared by 1-81-S2 and the donor *pilS* copy is indicated by flanking black lines. Six *pilS* copies (boxed) participated in more than one recombination event at *pilE* (labeled A, B, etc.). The alignment of each *pilS* copy with the 1-81-S2 sequence is shown above the corresponding recombination events; regions of each

pilS copy that share identity with 1-81-S2 are indicated by black bars and divergent sequences by white boxes. In recombination events where two *pilS* copies could have served as donor (*2c4/6c1* and *2c1/6c1*), flanking sequence shared with both copies is indicated with a black line; sequence shared with only one copy is indicated by a dotted line. In *pilS2c1/6c1*, the dotted line represents additional homology between *pilS2c1* and 1-81-S2 not present in *pilS6c1*; in *pilS2c4*, the dotted line represents additional homology between *pilS6c1* and 1-81-S2 not present in *pilS2c4*. The number of times each recombination event was recovered is indicated at the right. The eight pilin variants recovered in independent cultures of Gc are indicated by arrowheads. A326G and A326G/A350G are sequences found in multiple *pilS* copies.

Figure 2. Recombination events in P- variants of Gc strain FA1090

Labeling of divergent and conserved regions, and alignments of *pilS* copies with *pilE*, is as in Fig. 1. C395A and A355G/G358A are sequences found in multiple *pilS* copies. Black arrowheads indicate the position of frameshifts that are predicted to cause premature truncation of pilin.

1-81-S2	CGTCACCGAGTATTACCTGAATCACGGCATATGGCCGAAAGACAAC
<i>pilS3c2</i>	-C-----A-----A*-----GA-----C-----
<i>pilS2c4</i>	G--TG--G-----G-----GA-----G-----
<i>pilS6c2</i>	---TG--G-----C-----A-----G-----
<i>pilS1c3</i>	---TG--G-----G-C-----A-----G-A-----
1-81-S2	ACTTCTGCCGGCGTGGCATCC*GCTTCA*ACA**ATCAAAGGCAAA
<i>pilS3c2</i>	GGC-----*-----*-----*-----
<i>pilS2c4</i>	-----*C-CC-*C-GAC-----
<i>pilS6c2</i>	-----*C-CC-T*C-GAC-----
<i>pilS1c3</i>	-----CC-CC-TCC-GAC-----
1-81-S2	TATGTTCAGAAAGTTGAAGTCGAAAAGGCGTCGTTACCGCCCAAA
<i>pilS3c2</i>	-----A-----C-----
<i>pilS2c4</i>	-----A-A-GC---ACG-----C-----G---
<i>pilS6c2</i>	-----A-GC---ACG-----C-----G---
<i>pilS1c3</i>	-----A-----
1-81-S2	TGGCTTCAACCGGCGTAA
<i>pilS3c2</i>	-----
<i>pilS2c4</i>	-----
<i>pilS6c2</i>	--AAA---GA-----

Figure 3. *pilS* copies in strain FA1090 that produce truncated pilin proteins and confer P- colony morphology

Alignments of *pilS* copies with 1-81-S2 *pilE* begin at nucleotide 159 of *pilE*, numbered from the first nucleotide of the ATG start codon. Sequence divergences from 1-81-S2 *pilE* are indicated; identical nucleotide to 1-81-S2, (-); nucleotide missing relative to other *pilS* copies or *pilE*, (*). Frameshifts relative to 1-81-S2 are circled. Each *pilS* sequence terminates with the predicted stop codon (boxed).

Table 1**Distribution of *pilS* copies in variants of FA1090 1-81-S2 Gc**

P+ and P− variants were isolated after passage of FA1090 1-81-S2 and its *recA6* derivative, yielding 92 P+ and 61 P− recombination events where the donor silent copy could be assigned to one or two *pilS* sequences. The percent appearance of each *pilS* copy, or sequence corresponding to two *pilS* copies, was determined. Each *pilS* copy is annotated for its copy number and position in *pilS* as follows: *pilS3c1* = *pilS* locus 3, copy 1.

<i>pilS</i> copy	% appearance in:	
	P+	P−
<i>pilS2c1</i> OR <i>pilS6c1</i>	34.8	6.6
<i>pilS3c1</i>	21.7	1.6
<i>pilS3c2</i>	17.4	36.1
<i>pilS1c1</i>	8.3	--
<i>pilS2c4</i> OR <i>pilS6c1</i>	4.3	--
<i>pilS3c3</i>	3.3	--
<i>pilS1c5</i>	3.3	1.6
<i>pilS2c4</i>	1.1	36.1
<i>pilS1c4</i>	1.1	4.9
<i>pilS7c1</i>	1.1	1.6
<i>pilS6c3</i>	1.1	--
<i>pilS2c5</i>	1.1	--
<i>pilS6c1</i>	1.1	3.3
<i>pilS2c1</i>	--	4.9
<i>pilS6c2</i>	--	1.6
<i>pilS1c3</i>	--	1.6