A real-time semi-quantitative RT–PCR assay demonstrates that the *pilE* sequence dictates the frequency and characteristics of pilin antigenic variation in *Neisseria gonorrhoeae*

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ABSTRACT

A semi-quantitative real-time RT–PCR assay was designed to measure gonococcal pilin antigenic-variation (SQ-PCR Av assay). This assay employs 17 hybridization probe sets that quantitate sub-populations of pilin transcripts carrying different silent pilin copy sequences and one set that detects total *pilE* transcript levels. Mixtures of a DNA standard carrying the silent copy being detected and a clone encoding the starting *pilE* sequence, which is the majority *pilE* template, provided amplification curves that closely matched the experimental data and allowed an analysis of the contribution of different silent pilin copies to variation. The SQ-PCR Av assay was verified using DNA sequence analysis to demonstrate that this methodology allowed an accurate analysis of pilin variation. Both assays showed that with a specific starting *pilE* sequence, only a subset of the silent pilin copies recombine into *pilE* at a detectable level, and that this limited subset was reproducibly detected in replicate cultures. When an isogenic *pilE* sequence variant was examined using both assays, a new subset of silent copy sequences were detected recombining into *pilE* and the overall frequency of variation was increased. Thus, the parental *pilE* sequence influences the frequency of variation and the repertoire of pilin variants produced.

INTRODUCTION

Neisseria gonorrhoeae (also called Gc or the gonococcus) is a Gram-negative diplococcal bacterium and is the causative agent of the human sexually transmitted disease gonorrhea. No vaccines for gonorrhea exist in part because *N.gonorrhoeae* alters some of its surface elements, allowing it to evade the human immune system (1,2). Gonococcal attachment to human epithelial cells is mediated primarily by pili, surface structures composed of multiple units of the protein pilin (3,4). Pili mediate adherence to a number of different eukaryotic cell types, affect interactions with epithelia, endothelia and phago-cytes (5), and are also required for full DNA transformation competence (6–8).

Pilin is encoded by the *pilE* gene present at a single expression locus in the strain FA1090. In addition to *pilE*, FA1090 also contains a total of 18 silent pilin gene copies present in 5 *pilS* loci and 1 silent pilin gene copy present in the *pilE* locus (Figure 1A) (9). These silent copies and *pilE* share a central semivariable (SV) region and 3′ hypervariable (HV) region, but lack the 5′ constant region and promoter found only at *pilE*. The 3′ HV region can be further subdivided into a HV loop (HV$_L$) and a HV tail (HV$_T$). The HV$_L$ is flanked by two conserved regions, *cys1* and *cys2* (Figure 1B) (10).

Pilin antigenic variation (Av) is defined as the high frequency change of amino acid residues in the pilin protein (11,12). During pilin Av, a portion of *pilS* replaces a portion of *pilE*, often producing a new pilin that is efficiently expressed on the cell surface. This RecA-dependent recombination of a *pilS* sequence into *pilE* is unidirectional because the donor *pilS* sequence remains unchanged while the recipient sequence is lost from *pilE* (13,14). Several conserved DNA segments that have been reported to be important for pilin Av include the *cys2* sequence (15), as well as the correct spacing between *cys1* and *cys2* (16), and the 3′-untranscribed sequence of the Sma/Cla repeat (17). Av at *pilE* also requires RecA, and the RecF-like pathway of homologous recombination (18–20), but not the RecBCD pathway (18). The Rep helicase (21) and the RecA modulator RecX (22) are involved in
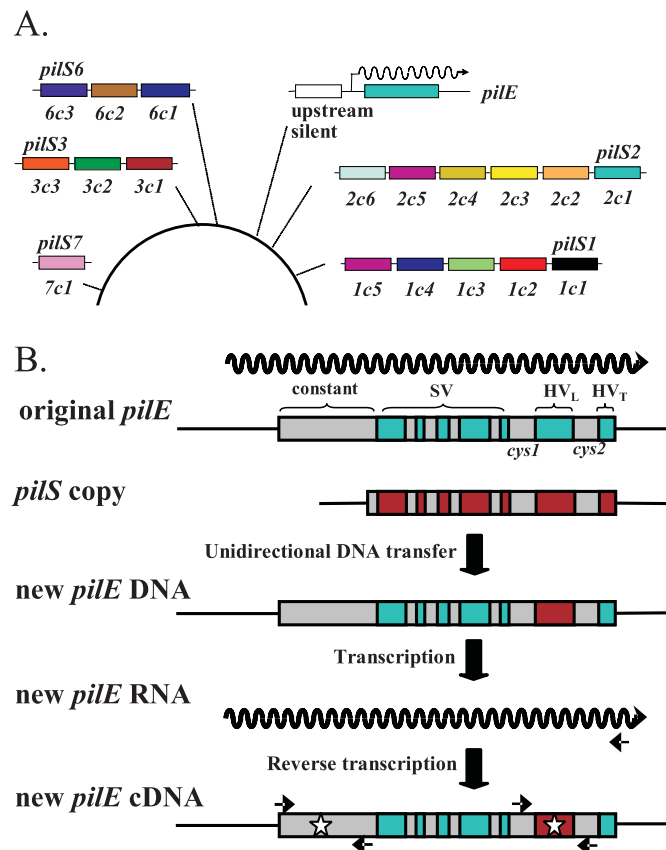
Figure 1. Cartoons showing the pilin loci and the basis for the SQ-PCR assay. (**A**) Pilin loci of gonococcal strain FA1090. The *N.gonorrhoeae* strain FA1090 chromosome contains one pilin expression locus, *pilE*, and five silent pilin loci, *pilS*. The *pilE* locus contains the pilin expression gene *pilE* (light blue rectangle) and the upstream silent pilin copy (white rectangle). The wavy line indicates the *pilE* transcript. Each of the five *pilS* loci contain between one and six silent pilin gene copies (colored rectangles). The silent copies are shown using an abbreviated nomenclature. Silent pilin locus 1 (*pilS1*) gene copy 2 is shown as *1c2*, *pilS3* copy 3 is *3c3*, etc. (**B**) The SQ-PCR Av assay to measure recombination at pilE. Five general portions of the *pilE* gene are shown: the 5′ constant region, a central SV region, the conserved *cys1* and *cys2* regions that flank the HV_L, which is followed by the hypervariable tail (HV_T) (16). The wavy line indicates the transcript encoded by the *pilE* gene and the rectangles indicate coding regions of DNA. Primers are shown as arrows, and the sequence targeted by the pairs of hybridization probes is starred. The 5′ primers are used with the *pilE* constant region hybridization probes, LC-HybCONS 1 and 2, and detect total *pilE* transcript levels. The 3′ primers are used with the 17 pairs of *pilS* HV hybridization probes and detect individual *pilS* to *pilE* recombination at the HV_L of *pilE*.

pilin Av, but the replication restart protein PriA is not involved (23).

Many assays have been developed to analyze *pilS* to *pilE* recombination, but each has its shortcomings (17,24). The most common assay used to measure pilin Av uses the frequency of colonies appearing with a non-piliated colony morphology from a piliated population to estimate the frequency of pilin Av (18,19,22). However, this method underestimates the frequency of Av because not all changes in *pilE* lead to changes in colony morphology. To analyze pilin variation independent of colony morphology changes, Wainwright *et al.* (17) developed an RT–PCR-based assay to measure the transfer of silent copy sequences into *pilE* in a population of bacteria. Briefly, the assay measured the relative quantity of

total and recombinant *pilE* by measuring the presence of new HV_L sequences from either *pilS1* copy 4 or *pilS6* copy 1. Subsequently, Serkin *et al.* (24) improved upon the existing qualitative assay by mixing target and competitor RNA prior to reverse transcription such that competitor template served as an internal control for RT and PCR efficiency. This quantitative, competitive RT–PCR assay was capable of detecting recombination from multiple sources, yet was incomplete in that not all silent pilin copies were detected or could be individually assayed. Thus, in order to detect the contribution of all silent pilin copies individually, a real-time semi-quantitative RT–PCR (SQ-PCR Av) assay was designed to study *pilS* to *pilE* recombination.

This SQ-PCR Av assay was used to demonstrate that with a defined starting *pilE* sequence only a subset of the silent pilin copies are prevalent donors for pilin variation even between replicate cultures. Furthermore, with a different parental *pilE* sequence, a different representation of silent copy sequences was detected recombining into *pilE*, and the overall frequency also changed. Thus, the repertoire of silent copies that donate their sequences to create pilin variants depends on the parental sequence. These data demonstrated that a real-time RT–PCR assay can be used to investigate the mechanisms leading to antigenic variation of pilin in *N.gonorrhoeae* and shows that the starting *pilE* sequence has a major influence on the variants produced.

MATERIALS AND METHODS

Generation of SQ-PCR Av standards

The hypervariable loop (HV_L) regions of all *pilS* copies were previously cloned into the pGEM-3 vector (24). These plasmids served as standards for the SQ-PCR Av assay. Clone E1r was a gift from Terri Hamrick and was used as a standard for assaying total *pilE* transcript levels (9). Clone pGADT7 + GcRecA#9 was used as a standard for the *recA* control (25). All plasmid standards were linearized by digestion with HincII (New England Biolabs). The linearized plasmid standards and Low DNA Mass Ladder (Invitrogen), which has a known concentration, were then run in a 0.8% agarose gel stained with ethidium bromide. Densitometry was performed using ImageQuant (Molecular Dynamics) to quantify the linear plasmid standards. Furthermore, the plasmid standards were normalized using SYBR Green I DNA dye and the LightCycler instrument. Molecules of the gene of interest were calculated as described previously (26), and serial dilutions representing 10^8–10^1 molecules were generated. Pilin competing-template mixed standards were generated by mixing the silent copy standard of interest with the parental copy standard at the total *pilE* transcript level. Non-pilin competing-template mixed standards were produced with *opa* PCR product, *recA* cDNA and *Escherichia coli* chromosome, each at the total *pilE* transcript level.

Bacterial strains and growth conditions

Gonococcal strain FA1090 *recA6* pilin variants 1-81-S2 (27) and 1-81-S2(*3c3*) (this study) were used in all of the experiments. FA1090 *recA6* contains an isopropyl-β-D-thiogalactopyranoside (IPTG)-regulatable gonococcal *recA* allele that produces a phenotypically RecA-deficient strain

without IPTG in the growth medium and a RecA-proficient strain when grown in the presence of 1 mM IPTG that is indistinguishable from wild-type *recA* strain (28). Gonococci were grown on Gc Medium Base (Difco) with Kellogg supplements (GCB) (29) and 1 mM IPTG (Diagnostic Chemicals Ltd) at 37°C in 5% CO_2 for 22, 26 or 30 h.

RNA extraction and reverse transcription

Gonoccoci were swabbed from solid media into ice-cold phosphate-buffered saline (PBS), and Trizol extraction of total RNA was performed according to the supplier's instructions (Invitrogen). DNase treatment was performed twice (24), followed by RNA purification using the RNeasy RNA cleanup protocol (Qiagen) as described previously (24). RNA was run in a 0.8% agarose gel stained with ethidium bromide to partially quantitate and assess the stability of the RNA. 28S ribosomal RNA bands were compared and normalized by performing densitometry with ImageQuant software (Molecular Dynamics). After RNA levels from the experimental samples were normalized, cDNA synthesis was performed as described by Serkin and Seifert (24) using the primer LC-CYS2rev for the 3′ HV region of *pilE*, LC-CONSrev for the 5′ constant region of *pilE* or LC-recArev for *recA* (Supplementary Material). All reverse transcription reactions were performed with a no reverse transcriptase control in order to control for contaminating DNA in the SQ-PCR Av assay. The reverse transcription reaction was purified using the Qiaquick PCR Purification Kit (Qiagen).

Oligonucleotides

All oligonucleotides used are listed in Supplementary Table 1. The forward primer LC-CYS1for and reverse primer LC-CYS2rev hybridize to two previously characterized pilin regions, *cys1* and *cys2* (30), conserved in all *N.gonorrhoeae pilE* and *pilS* copies (9). LC-CYS1for and LC-CYS2rev were used in conjunction with the silent pilin copy probes (Supplementary Material). A second primer set consisting of forward primer LC-CONSfor and LC-CONSrev anneals to the 5′ constant region of the *N.gonorrhoeae pilE* gene and was used in conjunction with the *pilE* 5′ constant region hybridization probes to detect total *pilE* transcript levels. The third primer set consists of forward primer LC-recAfor and reverse primer LC-recArev and anneals to the *N.gonorrhoeae recA* gene. The *recA* primers were used in conjunction with the *recA* hybridization probes to detect *recA* transcript levels, which served as a control for RNA normalization. All primers were reverse phase high-performance liquid chromatography purified (IT Biochem). Primer and probe melting temperatures were calculated using the freeware program TM Utility v.1.3 (Idaho Technology, http://www.idahotech.com/downloads_up/pTmutility_form.htm).

Hybridization probes were designed to specifically target 17 of the 18 silent pilin gene copies (9). An alignment of all *pilS* copies using the software program AlignX (InforMax, Inc.) allowed unique sequences within the HV_L to be identified as potential hybridization probe targets for each silent pilin copy (Supplementary Figure 1). Because the HV_L sequences share sequence similarity (Supplementary Material), probe target sequences for each silent copy were compared against all the other FA1090 silent pilin gene copies (using the BLAST

program) to ensure that the hybridization probe sequences specifically hybridize only to the targeted silent copy. The HV_L sequence of one silent pilin copy, *pilS1* copy 5, is identical to the HV_L sequence of *pilS2* copy 5. Thus, no *pilS1* copy 5 hybridization probes were designed; instead, the pair of *pilS2* copy 5 hybridization probes detects both *pilS1* copy 5 and *pilS2* copy 5. The distance between the donor and acceptor probes in all probe sets was 1 base (31). All donor probes were labeled with fluorescein at the 3′ end, while all acceptor probes were labeled at the 5′ end with LightCycler-Red 640-*N*-hydroxysuccinimide ester. Acceptor probes also had phosphate groups attached to the 3′ end to prevent probe extension (IT Biochem).

Each of the pairs of hybridization probes used the same primer pair for amplification (Figure 1B). Clones carrying the HV_L sequence of each silent copy bordered by the conserved *cys1* and *cys2* priming sites were isolated for use as standards to quantitate the proportion of each silent HV_L sequence that had transferred into the *pilE* of an experimental sample. The purified plasmid standards were gel quantitated as described above, and 10-fold dilutions of these standards were used in each SQ-PCR Av experiment.

The annealing temperature of the hybridization probe pairs were adjusted to yield maximal detection of the targeted silent pilin gene copy sequence while concurrently minimizing undesired hybridization to sequences from the other silent pilin gene copies. Pairs of hybridization probes were deemed optimized when they met two conditions: (i) that all other untargeted silent pilin gene copies were detected at levels <1% of the targeted copy (data not shown) and (ii) that the detection limit was 10^5 copies of linear plasmid mixed standard or less.

Analysis of Southern colony variants and sequencing for the verification of the SQ-PCR Av data

FA1090 1-81-S1 *recA6* was grown at 37°C in CO_2 on GCB agar +/− IPTG for 22 h and was swabbed into ice-cold PBS and plated onto GCB agar. After 22 h, 1055 individual colonies of Gc were selected and frozen in 15 μl of GCBG (GCB with 20% glycerol). The frozen stocks of the individual colonies of Gc were passaged onto GCB agar. Individual colonies from each revived culture were lysed (1% Triton X-100, 2 mM Tris, pH 8, 2 mM EDTA) and the *pilE* gene was PCR amplified using PILRBS (17) and SP3A (32) in a PTC-100 thermocycler (MJ Research). The *pilE* PCR products were electrophoresed on a 0.8% TBE agarose gel, denatured, neutralized and blotted to nylon as described previously (33). The blots were probed with a 3′ end labeled digoxigenin-11-ddUTP (Boehringer Mannheim) oligonucleotide specific to the parental FA1090 1-81-S2 *recA6* HV_L sequence (Supplementary Material, LC-Hyb2c1 1). The blots were probed at 67°C for 2 h and were washed with 0.1× SSC, 0.1% SDS twice at 67°C for 20 min and twice with 0.5× SSC, 0.1% SDS at room temperature. The blots were exposed to Kodak BioMax XAR film. A positive control, *pilE* amplified PCR product of FA1090 1-81-S2 *recA6*, and a negative control, *pilE* amplified PCR product of RM11.2 (*6c1* in the HV_L) were included in each Southern blot. About 3% of the isolates did not hybridize to the *2c1* parental probe in the HV_L. These isolates were sequenced to determine the sequence changes in the *pilE* HV_L.

Furthermore, 5% of randomly selected *2c1* positive clones were sequenced and found to carry the *2c1* HV$_L$ sequence, showing that we were not missing any HV$_L$ variants. For sequencing, the PCR products were treated with shrimp alkaline phosphatase and exonuclease 1 to remove primers and dNTPs. PCR-generated pilin templates (16–33 ng) were used in each sequencing reaction with the PILRBS primer (17) and were analyzed using a Beckman Coulter CEQ 2000XL DNA system as specified by the manufacturer (Beckman).

Sequencing assay of antigenic variation for validation of the SQ-PCR Av assay

A sequencing assay was performed to verify the results of the SQ-PCR Av assay when using the strain FA1090 1-81-S2(*3c3*) *recA6*. This assay was performed as described previously (23). Briefly, FA1090 1-81-S2 *recA6* and variant 1-81-S2 *3c3 recA6* were grown on plates containing IPTG and incubated at 37°C in 5% CO_2 for 20 h. Five starter colonies from each variant were then passaged onto separate GCB plates. A total of 45–50 colonies arising from each starting colony were passaged two more times to ensure clonal populations, and the *pilE* gene of each colony was sequenced as described above.

Real-time RT–PCR and data analysis

Real-time RT–PCR was performed on the LightCycler instrument (Roche Diagnostics). All assays used hybridization probes containing 2 μl of LightCycler DNA Master Hybridization Probes mix (Roche Diagnostics), 3.2 μl of MgCl$_2$ (for a final concentration of 5 mM), 2 μl of each primer (0.5 mM), 2 μl of donor hybridization probe (0.2 mM), 2 μl of acceptor hybridization probe (0.2 mM), 2 μl of template and PCR-grade sterile water for a final volume of 20 μl. PCR amplification for the hybridization probes assays began with initial denaturation (30 s at 95°C) followed by 40 cycles of denaturation (2 s at 95°C), annealing (56–66°C for silent copy probes, 53°C for *pilE* and 60°C for *recA*) and extension (5 s at 72°C). For the optimized annealing temperatures for each pair of hybridization probes see Supplementary Material. Fluorescence in channels F1 and F2 was acquired at the end of every extension step, and the ratio of F2/F1 was analyzed.

Data were exported from LightCycler software and imported into Excel (Microsoft). Standard curves, *P*-values and Pearson Correlation Coefficients '*R*' were generated using the Fit linear function. Pie charts were generated in Microcal Origin (Microcal Software Inc.).

RESULTS

Development of a SQ-PCR Av assay to detect sequence changes in the *pilE* HV$_L$

Many assays have been developed to measure the frequency of pilin Av, but each has limitations (17,20,24). Each previously developed Av assay underestimates the frequency of variation and records only a small proportion of the possible recombination events comprising pilin Av. The specificity supplied by the hybridization step of real-time RT–PCR was used to attempt to detect recombination of all 18 silent gene copies into the *pilE* gene within a large population of bacteria.

A total of 18 pairs of hybridization probes were designed, one probe set reacted specifically with the constant region only found in *pilE* and the other 17 probe sets were designed to measure the level of the 18 silent copy HV$_L$ sequences (two HV$_L$ sequences are identical) in a population of gonococci. To test whether the hybridization probes could detect recombinant *pilE* sequences, *N.gonorrhoeae* strain FA1090 1-81-S2 *recA6* was grown on solid media with or without 1 mM IPTG to control pilin Av. Total RNA was extracted and then reverse transcribed to pilin cDNA for real-time RT–PCR analysis. Pilin cDNA was normalized using the conserved region real-time RT–PCR primers and probes and adjusted to a concentration of ∼1×10^8 molecules per reaction. Once pilin cDNA was normalized, real-time RT–PCR was performed to quantify the parental *2c1* HV$_L$ sequence (Figure 2). Fluorescent acquisition curves of the cDNA from the experimental 22 h pilin cDNA sample of Gc was similar in slope and character to the purified standards during the early amplification cycles, although in the later cycles the slopes diverged between the pilin cDNA and the standards. During the earlier cycles, the experimental cDNA sample was estimated to contain ∼9.7×10^7 molecules carrying the parental *2c1* sequence. This measurement suggested that ∼97% of the *pilE* genes in this population retained the parental *2c1* sequence, while 3% had recombined with silent copies to produce variant *pilE* genes with a changed HV$_L$. To investigate this further, real-time RT–PCR was used to measure the appearance of new HV$_L$ sequences into *pilE* from the other silent copies. However, fluorescent acquisition curves using the HV$_L$ probes all demonstrated a severely depressed slope that could not be compared with or quantified using the purified standard curves (Figure 3A). Some of the silent copy
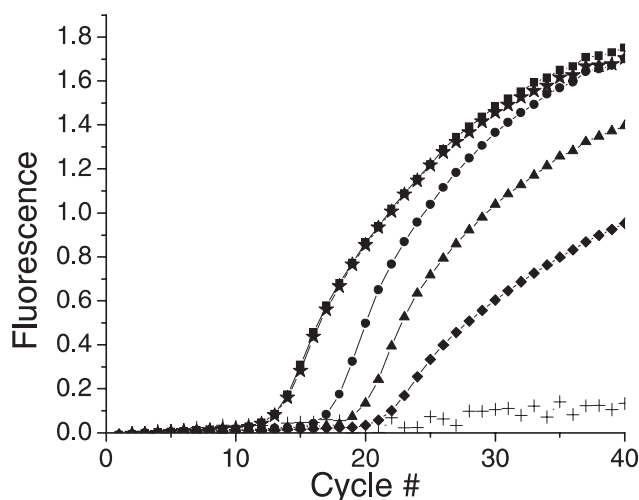

Figure 2. Real-time RT–PCR measurement of total *pilE* transcript levels. The fluorescence acquisition curves are plots of the fluorescence detected versus cycle number. The most concentrated standard (10^8 molecules, squares) begins to rise above background at approximately cycle number 13. The closed symbols represent dilutions of the purified standard (squares, 10^8 molecules; circles, 10^7 molecules; triangles, 10^6 molecules; and diamonds, 10^5 molecules). The cross symbol indicates the no template control, which contained the master mixture and water instead of template. The star indicates the experimental sample of cDNA of Gc grown for 22 h on plates with IPTG to induce RecA expression and antigenic variation and is superimposed on the 10^8 standard curve.
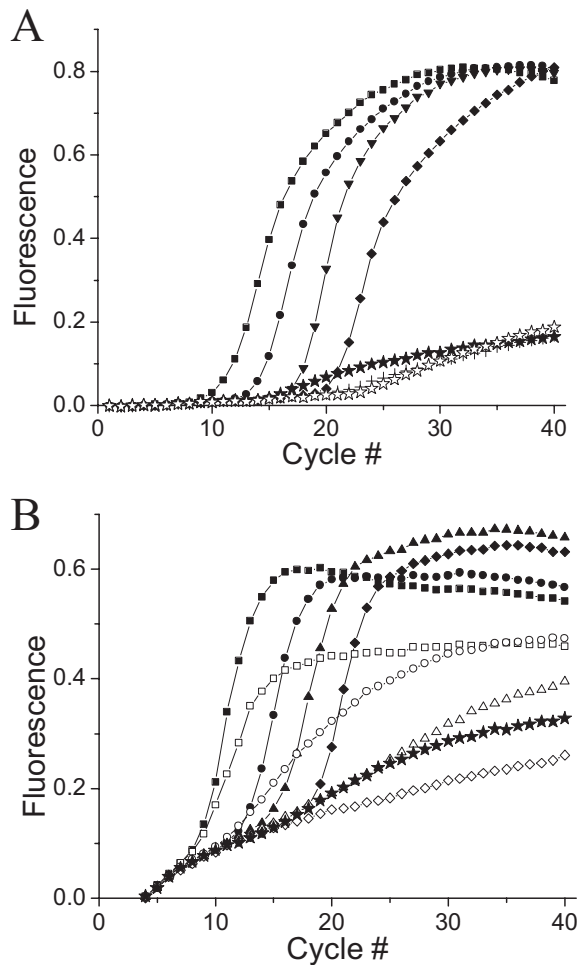
Figure 3. RT–PCR analysis of *3c1* cloned standards and mixed standards for *3c1*. (**A**) Representative fluorescence acquisition curves for *3c1* hybridization probes with dilutions of *3c1* standards (closed squares, 10^8 molecules; circles, 10^7 molecules; triangles, 10^6 molecules; and diamonds, 10^5). This is compared with pilin cDNA detected with *3c1* hybridization probes (star). The opened star is the no RT control for contaminating DNA. The cross symbol is the no template control (background level). (**B**) Fluorescence acquisition curves for *3c1* hybridization probes with dilutions of *3c1* cloned standards (closed squares, 10^8 molecules; circles, 10^7 molecules; triangles, 10^6 molecules; and diamonds, 10^5) and dilutions of *3c1* standards mixed with the 10^8 parental copy standard (*2c1*) (open squares, 10^8 *3c1* molecules; circles, 10^7 *3c1* molecules; triangles, 10^6 *3c1* molecules; and diamonds, 10^5 *3c1* molecules). Total pilin cDNA detected with *3c1* hybridization probes is indicated by the star.

probes produced a signal above background (Figure 3A), while other silent copies were undetectable using these probes (data not shown).

It was unclear whether the frequency of recombination of each silent copy was too low to be quantified by this assay, or whether the complex mixture of cDNAs present in the experimental pilin cDNA presents a problem for the real-time RT–PCR technology. It was probably that there was an excess of either (1) any non-pilin competing template or (2) competing pilin sequences (predominately the parental copy) that interfered with amplification or detection of recombinant *pilE* sequences within the experimental sample. To test whether any non-pilin competing template interfered with

detection, amplification curves of different dilutions of the *pilE* standard in question were mixed with *recA* cDNA, *opa* PCR product, or *E.coli* chromosomal DNA. These real-time RT–PCR amplification curves were similar in slope to the purified standards (data not shown), showing that an excess of non-pilin competing templates does not interfere with the assay. To determine whether the excess of *pilE* molecules carrying the parental HV_L sequence interfered with detection, an excess of purified DNA molecules carrying the parental *2c1* HV_L sequence was mixed with different amounts of purified template carrying the HV_L sequence being detected. The mixed samples produced depressed fluorescence acquisition curves during real-time RT–PCR, which replicated those recorded using pilin cDNA (Figure 3B). These data suggest that the excess of parental *2c1* sequences in the pilin cDNA sample was preventing the lower concentration of recombinant molecules from being quantified using the hybridization probes. Since the mixed standards produced fluoresence acquisition curves with different slopes, depending on the amount of the specific standard present in the mixture, we asked whether these mixed standards could be used to estimate the amount of recombinant HV_L sequence from each silent copy in a complex mixture of *pilE* sequences. All 16 HV_L probes produced depressed amplification curves when the target HV_L sequence was limiting within an excess of parental *pilE* templates, and for each probe set, the florescence acquisition curves became increasingly depressed as the level of the probe reactive sequence was decreased relative to the parental molecules (data not shown). With each probe set, $\sim 10^5$ molecules of the minority sequence were detectable above background within the excess of 10^8 molecules of the *2c1* *pilE* template (data not shown).

These mixed standards were used to approximate the amount of recombinant *pilE* in a population, and the participation of each silent copy to the variation in the HV_L region. Comparing each HV_L probe set against a mixed standard carrying various amounts of each HV_L standard mixed with *2c1* template, six additional silent copy sequences produced detectable amplification curves using *pilE* cDNA, while probes for the remaining 10 silent copy sequences detected nothing above background (data not shown). Pilin cDNA from a culture grown without IPTG, which does not undergo pilin Av, produced undetectable levels of amplification with all of the silent copy probes, showing that the amplification detected was RecA-dependent, as is pilin Av (data not shown). The contribution of all six detectable silent copy sequences to pilin variation was estimated by comparing the amplification of each HV_L subpopulation to the mixed standard (Figure 3B). Surprisingly, the seven detectable HV_L sequences were detected at differing frequencies in *pilE*. The HV_L sequence originating from *pilS2 copy 1* (*3c1*) was estimated to recombine at a frequency of 1.25% of the total *pilE* molecules. The other detectable HV_L sequences were estimated to occur at a frequency of 0.60% for *1c1*, 0.40% for *6c1* and 0.25% for *1c3*, *2c6* and *3c3*. The sum of the recombinant *pilE* molecules along with the parental *pilE* molecules together corresponded to the 10^8 molecules of *pilE* present in each sample. While these results strongly suggested that this assay was accurately describing the recombinant population of *pilE* variants, the validity of these findings was confirmed using a different approach.

Independent validation of the repertoire of silent pilin sequences producing pilin Av

To confirm the results of the SQ-PCR Av assay, individual colonies from the same population examined using the SQ-PCR Av assay were isolated by growing out individual clones from the population on solid medium without IPTG. Clones that had undergone pilin variation in the HV_L region were identified by the absence of hybridization signal to an oligonucleotide specific for the parental *2c1* HV_L sequence (Supplementary Table 2, LC-Hyb2c1 1). This hybridization screen confirmed that 97% of the 1055 colonies assayed retained *2c1* in the HV_L (data not shown). The *pilE* gene was amplified from the 32 colonies that did not hybridize with the *2c1* probe and the *pilE* gene sequenced to determine the silent copies that had donated sequences to alter the HV_L region. All of the *2c1* hybridization negative clones were found to express a new pilin variant with different silent copy sequences carried in the HV_L region (Figure 4). Six non-parental silent gene HV_L sequences were found in *pilE* using the sequencing assay, and this was a similar repertoire of donor silent copies as that determined by the SQ-PCR Av assay (Figure 4). The non-parental silent gene sequences found in *pilE* HV_L often extended into the SV region (data not shown). None of the HV_L sequences that were undetectable in the SQ-PCR assay was found in the sequenced variants (data not shown). While the exact frequency of each silent copy recombining into *pilE* was not identical in the two assays, they were strikingly similar—considering the semi-quantitative estimate of frequency used in the SQ-PCR Av assay and the limited number of colonies assayed by sequencing (Figure 4). From these data, we concluded that the SQ-PCR Av assay for pilin variation can accurately estimate the frequency of variation and reliably detect the silent copies that participate in producing pilin variation. These results show that within this one bacterial culture, only a subset of silent copies contributed to the 3% of variant *pilE* HV_L sequences.

Having verified the findings of SQ-PCR Av assay, the reproducibility of the non-uniform donation of silent copy sequences was assayed in repeat experiments using the same starting pilin variant within replicate bacterial cultures. Two cultures of *N.gonorrhoeae* strain FA1090 1-81-S2 *recA6* were grown with or without IPTG for 22 h in two separate experiments, and two cultures were grown on separate days and each sampled at 22, 26 and 30 h. In each case, growth without IPTG resulted in no detectable recombination in *pilE*. Similar copies were detected in all four IPTG grown cultures at 22 h (Figure 5). The most prevalent gene copy detected was the parental copy, *2c1*. Other detectable gene copies included *1c1*, *2c6*, *3c1*, *3c2*, *3c3* and *6c1*. Essentially the same subset of silent copies recombined into the *pilE* HV_L in replicate cultures, although the exact frequency for each silent copy sequence did vary from culture to culture, and in some cultures a new silent copy was detected (Figure 5). Taken together, these results show that the spectrum of *pilE* HV_L variants produced during pilin Av is not random and that some silent copies are over represented, while others are under represented during pilin variation.
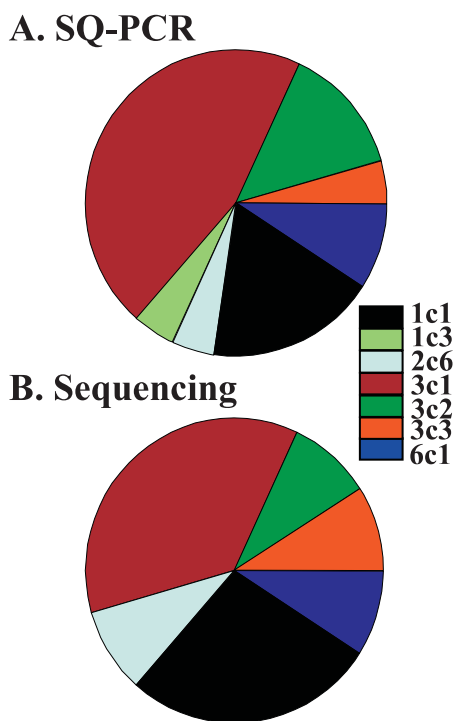
The starting pilin sequence influences the frequency of pilin variation and the spectrum of silent copies acting as donors

There were two main explanations for why a reproducible, nonrandom recombination of the silent copy HV_L sequences into *pilE* was observed. Either the starting *pilE* sequence dictates the repertoire of the donor silent copies used as recombination donors, or the nonrandom use of silent copy donors reflects a mechanistic limit on recombination that is independent of the expressed gene. In order to differentiate between these possibilities, a FA1090 1-81-S2 *recA6* pilin variant containing the *3c3* sequence within the entire *pilE* locus [FA1090 1-81-S2(*3c3*) *recA6*] was recovered from the sequencing assay and used as the parental variant in the SQ-PCR Av assay. The *3c3* variant is 80% identical to the *2c1* variant which is a lower percent identity than a majority of the other silent copy sequences (data not shown).

FA1090 1-81-S2(*3c3*) *recA6* was analyzed using the SQ-PCR Av assay as described above using the mixed standards to estimate the overall frequency of pilin variation and to measure the recombination potential of each silent copy. The *3c3* variant showed a much higher Av frequency than the *2c1* variant, with 12% HV_L variation after 22 h of IPTG induction as compared with 3–5% variation with the *2c1* variant (Figures 5 and 6). Analysis of the contribution of each silent copy to variants derived from the *3c3* variant demonstrated a different repertoire of silent copies than were found during the
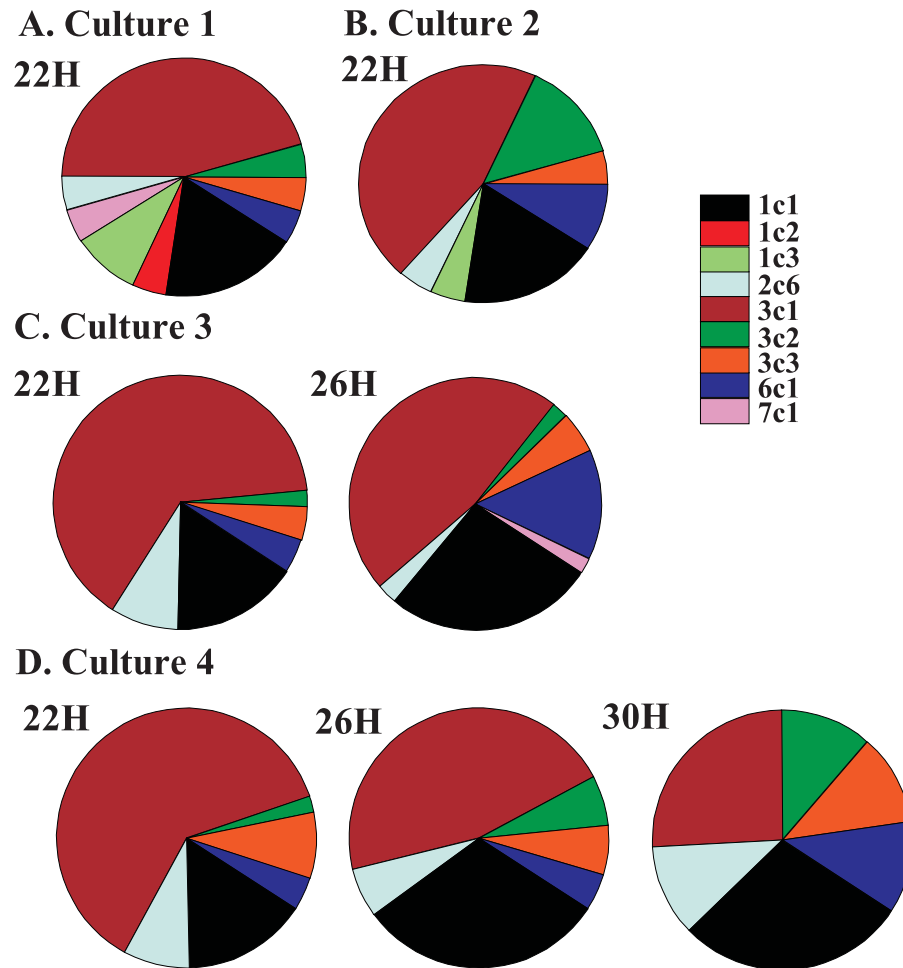
A. SQ-PCR

B. Sequencing

■ 1c1
■ 1c3
■ 2c6
■ 3c1
■ 3c2
■ 3c3
■ 6c1

Figure 4. Analysis of the silent copies recombining into the HV_L of *2c1*. (**A**) Detection of donor silent pilin copies using the SQ-PCR Av assay for *N.gonorrhoeae* strain FA1090 1-81-S2 *recA6* grown for 22 h on solid media in the presence of IPTG. (**B**) Detection of donor silent pilin copies using the *pilE* sequencing assay for *N. gonorrhoeae* strain FA1090 1-81-S2 *recA6* grown for 22 h on solid media in the presence of IPTG. The parental copy sequence, *2c1*, was retained in ∼97% of the *pilE* templates in both assays, and the proportion of silent copy donors to the 3% of variant *pilE* HV_L sequences is shown.

Figure 5. Similar silent copies are detected in separate experiments over time. Detection of newly recombined silent pilin copies using the SQ-PCR Av assay for *N.gonorrhoeae* strain FA1090 1-81-S2 *recA6* with *2c1* as the parental *pilE* sequence grown for 22 (**A–D**), 26 (C and D) and 30 (D) h on solid media in the presence of IPTG in four separate experiments. The parental copy, *2c1*, was retained in ~95% (A and B) and 94% (C and D) of the *pilE* cDNA templates. The graphs represent 5–6% of the *pilE* transcripts that have changed from parental. The legend depicts the silent HV$_L$ sequences detected in *pilE*.

2c1 variation assays. Not only was the repertoire of silent copies arising during Av different between the *2c1* and *3c3* variants, but a broader representation of silent copy donors was found with the *3c3* parental sequence than the *2c1* variant, and the distribution of silent copy donors was more diverse (Figures 5 and 6).

To confirm the results of the SQ-PCR Av assay with the *3c3* variant, the frequency and spectrum of variants produced in the *3c3* variant were analyzed by sequencing the *pilE* gene of random colonies isolated after IPTG induction of RecA. A level of 9% variation in the HV$_L$ was detected by the sequencing assay as opposed to 12% variation measured by the SQ-PCR Av assay. Considering the limitation of both assays, these values are in reasonable agreement. New silent sequences were found in both the SV and HV$_T$ regions, verifying that the SQ-PCR Av assay only records a subset of the total *pilE* variation. The greater repertoire of silent copy donors was also observed in the sequencing assay (data not shown). We conclude that the parental *pilE* sequence influences both the frequency of variation and the diversity of variants produced.

DISCUSSION

A SQ-PCR Av assay was developed to detect all *pilS* to *pilE* recombination at the HV$_L$ region in a population of Gc. The complex nature of the pilin cDNA isolated from Gc prevented efficient real-time RT–PCR analysis even though the primers and probes worked as expected on purified standards. This inefficient real-time RT–PCR was not owing to the use of hybridization probes since we obtained similar results using Taqman probes (data not shown). It has not been investigated whether other detection technologies could provide increased specificity to allow a more quantitative assay using purified standards. It is most likely that even though most of the HV$_L$ sequences are divergent (Supplementary Figure 1), when the parental sequence is in excess it out competes with the real target sequence during the hybridization step and therefore prevents efficient detection.

By using mixed standards, the complex mixture of pilin cDNA templates was mimicked and the frequency with which each detectable silent copy contributed to pilin Av was determined. The accuracy of these estimates relies on comparing the amplification of mixed standards with pilin
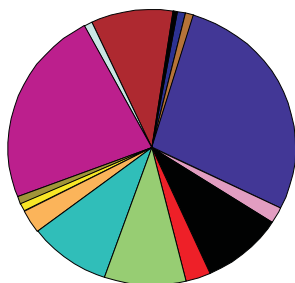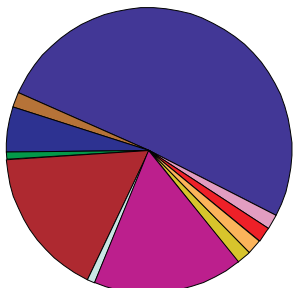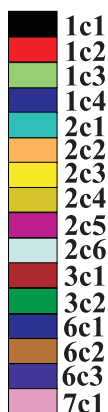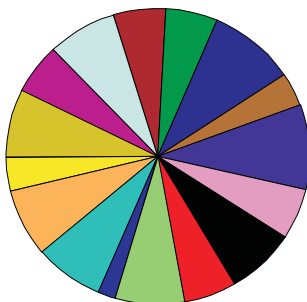
A. Culture 1

B. Culture 2

C. Culture 3

Legend:
- 1c1
- 1c2
- 1c3
- 1c4
- 2c1
- 2c2
- 2c3
- 2c4
- 2c5
- 2c6
- 3c1
- 3c2
- 6c1
- 6c2
- 6c3
- 7c1

Figure 6. A different representation of silent copies is detected with a different parental *pilE* sequence. Detection of newly recombined silent pilin copies using the SQ-PCR Av assay using three replicate cultures (panels **A–C**) of strain FA1090 1-81-S2(*3c3*) S2 *recA6* grown for 22 h on solid media in the presence of IPTG. The parental silent copy was calculated to be 88% of the total number of copies (data not shown). The graphs represent the remaining 12% of the *pilE* transcripts that have changed from parental. The legend depicts the silent HV_L sequences detected in *pilE*.

cDNA samples and can be estimated to be within a 3-fold of the real level and often as close as 2-fold. While frequencies produced are not precise, they allow a good estimate of the relative use of all detected silent copies as donors for pilin Av. The silent copies that were undetected may also recombine into *pilE* and produce variants, just at a level undetectable by either assay. The actual frequency with which these undetected silent copies participate in variation at *pilE* is unknown, but is at least 5-fold lower than the least frequent detected copy, and therefore the variants carrying the undetected copies are truly under represented in this bacterial population. However, even if these under represented copies participate in pilin Av rarely, they could still contribute to bacterial colonization if they are not recognized by an adaptive immune response.

It is not clear why the starting *pilE* sequence influences the frequency at which silent copies recombine into *pilE*. There was no pattern for the donor silent copies recombined more or less frequently in relationship to their position (upstream or downstream) to *pilE* or their position in a silent locus. As a homologous recombination mediated process (34), pilin Av may respond to the ability of the *pilE* recipient and the donor silent copy sequence to allow heteroduplex formation due to sequence similarity. The silent copies that recombined into *pilE* with the *2c1* starting *pilE* variant did show a higher degree of sequence similarity to the *2c1* variant (Clustal W, AlignX, Vector NTI) compared with the silent copies that recombined less frequently. However, these sequence similarity predictions were not supported with the donor silent copy spectrum observed with the *3c3* starting *pilE* variant. Therefore, no rules based on the two examples examined here can be established.

The use of this SQ-PCR Av assay has resulted in several important conclusions about the mechanism of pilin Av. First, the recombination potential of different silent pilin gene copies with *pilE* is not uniform. This observation means that within a limited population of gonococci, immune responses are likely to be raised only to a subset of potential pilin variants. Initially, this would allow the remainder of the pilin variants to contribute to escape of the immune response and be saved for future immune evasion. However, the silent copies that recombine at a very low frequency may become targeted over time as the immune response recognizes and eliminates the more dominant variants. Second, we have clearly demonstrated that the parental *pilE* sequence can influence the frequency and repertoire of pilin variants arising in a population. This observation suggests that particular pilin variants may act in a unique manner during a gonococcal infection, which could explain the various clinical manifestations of the disease in different individuals.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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Conflict of interest statement. None declared.

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