A Double-Strand Break Does Not Promote *Neisseria gonorrhoeae* Pilin Antigenic Variation

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ABSTRACT The major subunit of the type IV pilus (T4p) of *Neisseria gonorrhoeae* undergoes antigenic variation (AV) dependent on a guanine quadruplex (G4) DNA structure located upstream of the pilin gene. Since the presence of G4 DNA induces genome instability in both eukaryotic and prokaryotic chromosomes, we tested whether a double-strand break (DSB) at the site of the *pilE* G4 sequence could substitute for G4-directed pilin AV. The G4 motif was replaced by an I-SceI cut site, and the cut site was also introduced to locations near the origin of replication and the terminus. Expression of the I-SceI endonuclease from an irrelevant chromosomal site confirmed that the endonuclease functions to induce double-strand breaks at all three locations. No antigenic variants were detected when the G4 was replaced with the I-SceI cut site, but there was a growth defect from having a DSB in the chromosome, and suppressor mutations that were mainly deletions of the cut site and/or the entire *pilE* gene accumulated. Thus, the *pilE* G4 does not act to promote pilin AV by generating a DSB but requires either a different type of break, a nick, or more complex interactions with other factors to stimulate this programmed recombination system.

IMPORTANCE *Neisseria gonorrhoeae*, the causative agent of gonorrhea, possesses a DNA recombination system to change one of its surface-exposed antigens. This recombination system, known as antigenic variation, uses an alternate DNA structure to initiate variation. The guanine quadruplex DNA structure is known to cause nicks or breaks in DNA; however, much remains unknown about how this structure functions in cells. We show that inducing a break by different means does not allow antigenic variation, indicating that the DNA structure may have a more complicated role.

KEYWORDS RecA, antigenic variation, double-strand break, guanine quadruplex, homologous recombination, pilus

Programmed recombination is used in many systems to increase diversity, from antibody diversification to *Saccharomyces cerevisiae* mating type switching and antigenic variation in bacterial, viral, and protozoan pathogens (1–4). A DNA double-strand break (DSB) or single-strand nick is required for recombination events to be initiated. For example, *S. cerevisiae* switches between two mating types, **a** and α . Mating type interconversion is initiated by the endonuclease HO, which causes a specific double-strand break that can be repaired with the alternate gene sequence by recombination, leading to a gene conversion event and switching of the mating type (2). *Trypanosoma brucei*, the causative agent of African sleeping sickness, possesses a well-studied antigenic variation (AV) system (5) producing many versions of the variable surface glycoprotein (VSG) for immune avoidance. AV is important for long-term colonization of a host in the face of immune recognition. While many aspects of this programmed recombination process remain unknown, it was reported that a double-strand break could initiate recombination (6).

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Much like *T. brucei*, *Neisseria gonorrhoeae*, the sole causative agent of gonorrhea, also utilizes a gene conversion process known as pilin AV to change the sequence of the pilin major subunit, PilE, and alter the antigenicity or expression of the surface-exposed pilus (7, 8). There are 19 silent donor copies that each lack a promoter, ribosome binding site, and the conserved N-terminal coding sequence. One or more of these copies can recombine into the expression locus. These gene conversion reactions change the coding sequence of *pilE*, but the sequences of the silent copies remain unchanged (9, 10). The factors involved in the RecF-like recombination pathway of *N. gonorrhoeae* (*N. gonorrhoeae* does not encode a RecF ortholog [11]) are RecA, RecOR, and RuvABC and RecG (11–13). Inactivation of each of the *recQ*, *recJ*, and *rep* genes reduces pilin AV frequency but does not alter the types of recombinants produced, suggesting there may be unknown activities redundant to these factors (11, 14–16). Mutation of the Holliday junction-processing proteins RuvABC prevents AV (12, 17), and while it was reported that RecG is also involved in pilin AV, more sensitive assays suggest the helicase does not directly participate in the process (16). However, inactivation of the RuvABC system with a *recG* mutation produces synthetic lethality when pilin AV occurs, suggesting that Holliday junctions are intermediates in the process and that stalled Holliday junctions can be rescued by RecG (17, 18). The involvement of the RecF-like pathway suggests that there may be a gapped intermediate during the pilin AV process (19). The RecBCD holoenzyme repairs double-strand breaks in Gram-negative bacteria and also participates in the repair of DNA damage from nalidixic acid, ultraviolet light, and methylmethane sulfonate damage. While RecBCD has been reported to either enhance or limit pilin AV (20), we have shown that the pathway is not involved in pilin AV (16, 21).

In addition to the *trans*-acting factors involved in pilin AV, there are *cis*-acting factors required for the process. Upstream of the *pilE* gene and promoter, there is a guanine-quartet-forming motif (22). The motif contains four guanine tracts separated by one or two thymines and can fold into a parallel guanine quadruplex (G4) structure. The motif is required for pilin AV, and mutation in any one of the GC base pairs, but not the AT base pairs, interferes with pilin AV (22). RecA was also found to bind to the G4, and RecQ could unwind the G4 (18, 23). In the second G tract of the G4 motif, transcription of a small RNA (sRNA) is initiated (24). Transcription of this noncoding *cis*-acting sRNA is required for pilin AV and is thought to open the duplexed DNA to allow the G4 to form.

G4s are present in many different cell types and have roles in the regulation of different DNA processes, including transcription, DNA replication, translation, telomere processing, and viral packaging (25). G4 structures do not form readily when DNA is in a duplex but can stably form during times of transcription or replication, when DNA is single stranded (26, 27). G4 structures are highly stable once formed under physiological conditions and can hinder the progression of the replication fork if not resolved (28). Replication fork stalling can leave a single-strand gap in the DNA, which can become a DSB (29). In *N. gonorrhoeae*, nicks and DSBs were detected in the region of the G4 and were reduced when the G4 was mutated (22). We hypothesize that the *pilE* G4 causes a DSB by stalling replication to initiate pilin AV.

Similar to the yeast HO endonuclease, I-SceI is a site-specific endonuclease responsible for intron mobility (30, 31). The recognition site of I-SceI is a nonsymmetrical 18-bp sequence not found in the *N. gonorrhoeae* genome. The cut generates a 4-bp 3' overhang (32) (Fig. 1A). The endonuclease was used in *T. brucei* to show that a DSB can allow VSG variation (6). Moreover, RNA-DNA hybrids were shown to form in the VSG region and facilitate recombination, possibly through induction of a DNA break, but the mechanism is not known (33). Therefore, if pilin AV is initiated by a DSB caused by the G4 structure, replacing the G4 sequence with the I-SceI cut site would allow pilin AV recombination. In addition, the effects of the DSB near *pilE* were different than those of inducing DSBs near the origin and terminus of replication, suggesting that the *pilE* region might be more recombinogenic than other portions of the chromosome.

FIG 1 The I-SceI system generates DSBs at the site of *N. gonorrhoeae pilE* G4 and other control loci. (A) Sequences of *pilE* G4 and I-SceI digestion site. (1) *pilE* G4 sequence of *N. gonorrhoeae* strain FA0190. (2) Sequence of I-SceI cut site. The arrows indicate the cut sites of the wild-type I-SceI enzyme. (B) Genomic organization of strain FA0190 showing the *pilE* gene and the locations of I-SceI sites at the NGO1881 locus, near the origin of replication (*ori*), and the NGO0806 locus, near the replication terminus. The locations of I-SceI cut sites are marked by yellow stars. (C) Detection of double-strand breaks in SceI *N. gonorrhoeae* strains. Genomic DNA of SceI *N. gonorrhoeae* strains was isolated from liquid culture that was treated with 8 ng/ml Atc for 60 min. Break site mapping was applied to identify the double-strand breaks. The red boxes indicate the I-SceI cut sites, and the black boxes indicate the linker sequences. The identification of the linker sequence suggests the presence of breaks.

RESULTS

Construction and verification of DSB induction sites in *N. gonorrhoeae*. Since a G4 structure is required to initiate pilin AV (22), we asked whether the G4 structure might act to halt replication at the G4 to produce a recombinogenic DSB. To induce a DSB at the *pilE* G4-forming sequence, the *N. gonorrhoeae pilE* G4 sequence (Fig. 1A, sequence 1) was replaced by the I-SceI cut site (Fig. 1A, sequence 2). The I-SceI endonuclease was cloned under the control of the anhydrotetracycline (Atc)-inducible Tet promoter expressed from an ectopic site on the *N. gonorrhoeae* genome (34). As controls for the effect of DSBs in the *N. gonorrhoeae* chromosome, the I-SceI cut site was also introduced into an intragenic region near the replication origin (NGO1881) and in an intragenic region near the replication terminus (NGO0806) (Fig. 1B).

Production of DSBs in each site was assessed using a primer extension break-site-mapping method (35). DNA was harvested after I-SceI was induced by growth of the strains on medium containing Atc. Primer extension from sites within 100 to 400 bp of the I-SceI sites produced extension products that ended when a break in the template DNA was encountered to produce a blunt end. These breaks were tagged by ligation of an adaptor, and PCR using the primer extension primer and a primer specific for the adaptor produced specific amplicons that were analyzed by Sanger sequencing. Breaks

FIG 2 I-SceI DSBs cause reduced viability. The blue bars represent the survival rates calculated as the number of colonies grown on plates with GCB plus 2 ng/ml Atc divided by the number of colonies grown on GCB plates. The red bars represent the survival rates calculated as the number of colonies grown on plates with GCB with 1 mM IPTG and 2 ng/ml Atc divided by the number of colonies grown on plates with GCB with 1 mM IPTG. *, $P < 0.05$; Student's t test.

at the I-SceI site at each of the three locations were detected with the adaptor sequence occurring at each cut site (Fig. 1C).

DSBs cause *N. gonorrhoeae* growth defects. Serial dilutions of liquid cultures of the strains were spotted on *N. gonorrhoeae* medium base (GCB) (Difco) plates with or without 2 ng/ml Atc. The relative survival was enumerated by calculating the number of CFU on Atc plates divided by the number of CFU on plain GCB plates. An *N. gonorrhoeae* strain with *pilE* G4 replaced by the I-SceI cut site and an Atc-induced I-SceI gene showed a significant reduction of 10^{-5} to 10^{-4} CFU (Fig. 2). This survival frequency rebounded by about 1,000-fold (10^{-2} to 10^{-1} -CFU reductions) when *N. gonorrhoeae* was grown with IPTG (isopropyl- β -D-thiogalactopyranoside) to induce RecA expression, showing that recombination was important to repair the DSB. The survival of the I-SceI-induced NGO0806 and NGO1881 strains was also significantly decreased (10^{-5} - to 10^{-4} -CFU reductions) when a DSB was induced, but there was no effect of RecA induction on survival (Fig. 2). These results confirm that there is efficient cutting of the I-SceI sites in all three strains. It was surprising that RecA induction enhanced the survival of only the G4::Scel strain but not the control strains. As expected, all the strains with only the cut site or only the expressed enzyme displayed survival comparable to that of the parent strain.

An I-SceI-induced DSB at the G4 sequence does not allow pilin AV. We asked whether sublethal amounts of Atc could generate a reduced level of DSBs that might lead to pilin AV. We have developed a simple colony morphology-based assay as a surrogate measure of pilin AV by tracking the emergence of nonpiliated (P^-) blebs on the perimeters of pilated (P^+) colonies over time (a pilus-dependent colony morphology change [PDCMC] assay) (12). Changes in piliation can represent AV rates, and analysis of P^- colonies by sequencing often reveals altered *pilE* sequences. We determined that 0.048 ng/ml Atc allowed a G4::Scel AtcP-I-SceI strain to grow on solid medium (albeit with reduced growth). There was also increased PDCMC, suggesting increased frequencies of P^- variants arising from the P^+ progenitor. During the first 24 h, there was little to no P^- blebbing when DSBs were induced in the G4::Scel AtcP-I-SceI strain with IPTG and Atc, similarly to the same strain without Atc. However, at 48 h, we observed about 3 or 4 P^- blebs per colony when grown on GCB plus IPTG and Atc compared to 1 or 2 P^- blebs per colony for the same strain when grown on

TABLE 1 Analysis of *N. gonorrhoeae* P[−] blebs for *pilE* deletion or *pilE* antigenic variation

Strain from which P [−] blebs were isolated	No. (%) with <i>pilE</i> deletion/truncation	No. (%) with <i>pilE</i> antigenic variation
FA1090 <i>recA6</i>	6/224 (2.7)	9/10 (90)
G4::Scel AtcP-I-Scel	60/80 (75)	0/20 (0)

ITPG plates without Atc (data not shown). PCR and sequence analysis of the *pilE* genes from 80 clones from individual blebs demonstrated that 60 blebs had some or all of the *pilE* gene deleted with loss of the I-Scel cut site, similar to the suppressors detected with lethal amounts of Atc. All of the remaining 20 P[−] cells retained the intact *pilE* gene, but none had any sequence changes in the *pilE* characteristic of pilin AV (Table 1). The P[−] colony morphology was most likely due to PilC phase variation, which results in a similar P[−] colony morphology. In contrast, only 6 out of 224 P[−] blebs derived from the parental strain FA1090 with a normal G4 sequence showed *pilE* deletion, and 9 out of 10 P[−] variants from blebs showed sequence changes in *pilE* characteristic of pilin AV (Table 1). We also tested whether smaller amounts of Atc (0.002 ng/ml, 0.004 ng/ml, 0.008 ng/ml, and 0.02 ng/ml) might produce pilin AV, but no antigenic variants were identified in the induced G4::Scel strain (data not shown).

Suppressor mutations show how *N. gonorrhoeae* recombination differs around the genome. Comparison of the responses of the three strains with I-Scel sites at different loci to I-Scel induction showed that the *pilE* region has unique properties relative to the other two sites. Induction of a DSB in the *N. gonorrhoeae* genome inhibited growth of *N. gonorrhoeae* at 2 ng/ml, but we also observed that a small population of *N. gonorrhoeae* organisms could escape the DSB at the original *pilE* G4 site, and this escape was alleviated by the induction of RecA6. On the other hand, induction of RecA did not increase the number of bacteria that survived when DSBs were induced at NGO0806 or NGO1881.

To understand these differences, 200 suppressors of each strain were identified when the I-Scel enzyme was induced using 2 ng/ml Atc; 90 to 99% of G4::Scel strain suppressors had the P[−] colony morphology, either with or without IPTG induction (Fig. 2). In contrast, only 1 to 2% of NGO1881::Scel and NGO0806::Scel strains had suppressors with a P[−] colony morphology. To understand how suppressors bypassed the I-Scel system and survived I-Scel-induced DSBs, we analyzed the sequence of the I-Scel gene, including the I-Scel promoter region, the I-Scel cut site, and the *pilE* gene.

Sequencing analysis of five G4::Scel P⁺ suppressors identified mutations in the I-Scel promoter and coding region in all five survivors, while P[−] suppressors all (42 out of 42) had *pilE* deletions (Table 2). For the 0806::Scel strain suppressors, 18 out of 18 suppressors had mutations in the I-Scel promoter or coding region. For the 1881::Scel suppressors, 14 out of 16 suppressors had mutations in the I-Scel promoter or coding region and the other two had mutations in the I-Scel cut site.

PCR and sequence analysis of the G4::Scel P[−] suppressors of DSB lethality showed that recombination occurred between different pilin repeat sequences located upstream and downstream of the original *pilE* G4 to remove the I-Scel cut site (Fig. 3). Among 42 P[−] suppressors analyzed, 18 had recombination via repeat I (a 72-bp sequence immediately after the SmaI/Clal site of the upstream silent copy [USS] and *pilE* [36]) that resulted in a complete loss of *pilE* sequence and the I-Scel cut site. Twenty-

TABLE 2 Mapping of I-Scel-induced DSB suppressors

Strain from which suppressors were isolated on GCB + 2 ng/ml Atc + IPTG plates	No. with I-Scel gene/promoter mutation	No. with <i>pilE</i> deletion/truncation	No. with I-Scel digestion site mutation
G4::Scel AtcP-I-Scel, P ⁺	5/5	0	0
G4::Scel AtcP-I-Scel, P [−]	0	42/42	0
0806::Scel AtcP-I-Scel	18/18	0	0
1881::Scel AtcP-I-Scel	14/16	0	2/16

FIG 3 Analysis of P^- suppressors that escaped the I-SceI DSB at the *pilE* G4. (A) Diagrams showing three types of homologous recombination that occurred in G4::SceI AtcP-I-SceI suppressors with P^- colony morphology. The gray arrows indicate the USS sequence. The pink arrows indicate the *pilE* genes. The stars indicate the I-SceI cut site that replaces the original *pilE* G4 site. The orange boxes indicate the repeat I sequence, the green boxes indicate the repeat II sequence, and the blue boxes indicate the repeat III sequence. The dashed lines represent the homologous recombination that is mediated by the repeat sequences. (B) Sequences of repeats I, II, and III.

two P^- suppressors had recombination via repeat II (a 23-bp sequence) and 2 of them via repeat III (a 5-bp sequence). Both repeat II and repeat III were located at the beginning or upstream of the *pilE* and USS sequence and resulted in the loss of the I-SceI cut site, the *pilE* promoter, and part of the *pilE* gene. Taken together, these data confirm that *pilE* AV did not occur in the P^- suppressors when a DSB was induced at the original *pilE* G4 site.

DISCUSSION

We tested the hypothesis that a specific DSB could replace the G4 structure to initiate pilin AV, essentially determining if the only role of the G4 is to induce a DSB. While break site mapping and the phenotypes observed when the I-SceI enzyme was induced demonstrated that DSBs were produced, there was no evidence for pilin AV, even at reduced frequencies. There are a few hypotheses that could account for the lack of pilin AV after an I-SceI-induced DSB was produced. First, it is possible that the type of break does not support critical downstream processing events, since the I-SceI enzyme produces a DSB with a 4-base 3' overhang (Fig. 1). However, even if this were not the preferred form of DSB, there are several exonucleases that could process the 3' ends to allow recombination by other forms of the DSB. We hypothesize that the G4 stalls chromosomal replication to initiate this programmed recombination process. When DNA polymerase encounters an impediment, such as a G4, the replication machinery stops and the replication fork collapses. This collapse would leave a gap of unreplicated DNA. The gap could then be processed either as a gap or, if the intact strand was cleaved, a double-strand break, leading to homologous recombination (28). The dependence of pilin AV on the RecOR recombinase (11) supports the idea of a gap being produced. However, since the G4 structure is a few hundred base pairs from where recombination occurs, the gap would need to be resected into the *pilE* variable regions (22), presumably by the RecJ exonuclease, other exonucleases, or helicases (14). If the 3' overhang could not be properly processed by these enzymes, it would prevent the gap or break from leading to the appropriate recombination reactions. However, a study done in yeast found that a specific quadruplex had an effect on replication stalling only when placed on the leading strand of replication (37). Since the *pilE* G4 is

on the lagging strand of replication, this hypothesis may not be true, unless the G-rich RNA transcript on the other strand forms an RNA-DNA hybrid (R-loop) (38) to stall replication, as proposed (24). Second, it is possible that the G4 structure plays additional roles in the process, such as recruitment of recombination factors, like RecA, or helicases, like RecQ (18, 23). There is some sequence/structural specificity for the *pilE* G4, since different G4-forming sequences at the site of the *pilE* G4 do not allow pilin AV. Also, switching the strand or orientation of the G4 and associated sRNA results in a loss of pilin AV (22, 24). Moreover, adding the *pilE* G4 before a silent pilin copy does not result in the silent copy becoming a recipient for pilin AV (22). All of these data point to a complex and specific role for the G4 that cannot be replaced by an I-SceI DSB. Finally, changing the G4 sequence also changes the sequence of the *cis*-acting G4-associated sRNA. The sRNA is required for AV, but its specific role remains to be elucidated. It is possible that this change can affect the sRNA, because it changes the sequence of the sRNA. It has been hypothesized that the sRNA can form an RNA-DNA hybrid with the C-rich strand of DNA. Changing the sequence of the sRNA would affect the stability of this RNA-DNA hybrid (39).

Although the DSB did not allow pilin AV, it did lead to RecA-dependent recombination, resulting in the deletion of *pilE* and the cut site itself. While deletions were detected in the majority of suppressor mutants, they were not found when a DSB was made at the two other sites in the genome. The appearance of deletions at *pilE* supports the idea that the *pilE* region is a recombination hot spot (40). The number of suppressor mutations was significantly higher when the cut site was near *pilE*, which may be due to an increase in recombinational machinery in the region. There are a lot of repeated sequences in the chromosomal sequences near the *pilE* gene (Fig. 3). Recombination factors may already be recruited to this region to facilitate pilin AV recombination in wild-type cells.

The increased survival of G4 region DSBs is dependent on the expression of RecA, but RecA expression did not increase survival if DSBs were induced at other regions of the genome. There are two pathways involved in homologous recombination, the RecBCD pathway and the RecF-like pathway. In *Escherichia coli*, the RecF pathway generally mediates single-strand gap repair and RecBCD repairs double-strand breaks, but both require RecA. However, at this high level of DSBs, RecA induction does not rescue survival, indicating that the DSBs occur too often to repair or cannot be repaired as readily.

Characterizing suppressor mutations for strains in which a DSB was induced can elucidate how these breaks may or may not be repaired. Deletion of the *pilE* gene and cut site allowed bacteria to escape the I-SceI DSB at the G4 site. However, DSBs at the two other sites in the genome (suppressors mainly) arose from mutations in the endonuclease gene or promoter, even with RecA induction. Deletion of the G4 I-SceI cut site was accomplished using repeats found before and after the cut site in the upstream silent copy and *pilE* to complete homologous recombination. The most common repeat sequence used for recombination was the longest, and the least used was the shortest. The deletion of the cut site and subsequent *pilE* gene most likely contributed to the increased survival of the G4 site DSB. Survival increased with the induction of RecA, which would usually be required for this deletion/recombination event (Fig. 2).

The cut site was not deleted in suppressors from the other two sites in the genome. This may be due to a lack of repeat sequences in the vicinity of the cut sites at NGO0806 and NGO1881 and/or may indicate that *pilE* deletions or recombination happens more readily at the *pilE* gene due to the presence of recombination factors. The most common suppressor mutations when the DSB was located at NGO0806 and NGO1881 were small mutations that may have arisen from errors in replication, not recombination, which happens at a higher frequency (41).

Taking the data together, we can confirm that a DSB does occur in our I-SceI system and cannot substitute for the G4 in pilin AV. DSBs at this location cause a drastic increase in gene deletions. This is not seen at other locations in the genome, near both

the origin and terminus of replication, indicating a difference in the repair mechanisms for this type of damage at the *pilE* locus. We did not test whether DSBs were repaired using the RecBCD or RecF-like pathway, and the differences in the suppressors that arose may indicate the usage of different systems or proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All the strains, plasmids, and oligonucleotides used in this study are listed in Tables S1 and S2 in the supplemental material. *E. coli* TOP10 competent cells (Invitrogen) were grown in Luria-Bertani (LB) broth or solid medium containing 15 g/liter agar at 37°C and used to propagate plasmids. *N. gonorrhoeae* was grown on GCB plus Kellogg supplements I (22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase) and II [1.23 mM Fe(NO₃)₃] at 37°C in 5% CO₂ or in *N. gonorrhoeae* liquid (GCBL) medium (1.5% proteose peptone no. 3 [Difco], 0.4% K₂HPO₄, 0.1% KH₂PO₄, 0.1% NaCl). Liquid-grown gonococcal strains additionally contained Kellogg supplements I and II and 0.042% sodium bicarbonate (42, 43). The *N. gonorrhoeae* strains used in this study were derivatives of FA1090 and are listed in Table S1. All the *N. gonorrhoeae* strains used in the study were constructed with an IPTG-inducible *recA6* allele at the normal *recA* locus that allows the control of *pilE* AV (44). All *N. gonorrhoeae* strains began with the same *pilE* sequence as variant 1-81-S2 (46).

Constructing *N. gonorrhoeae* strains. To replace the *pilE* G4 with the I-SceI cut site, the Scel-G4-P1 and Scel-G4-P2 primers were used to amplify the upstream sequences flanking the G4, and the Scel-G4-P3 and Scel-G4-P4 primers were used to amplify the downstream sequence flanking the G4. Their PCR products were gel purified and added as the template of a stitching by overlap extension (SOE) PCR using Scel-G4-P1 and Scel-G4-P4 as primers. The final PCR product was gel purified and cloned into pGEM3Z via EcoRI and PstI cut sites. The corresponding element was transformed into the *N. gonorrhoeae* chromosome through homologous recombination. *N. gonorrhoeae* strains were selected based on the AV-deficient phenotype as a result of G4 interruption, and the final strains were verified by DNA sequencing.

SOE PCR was used to introduce an I-SceI cut site into the NGO0806 or NGO1881 chromosome intragenic regions. The I-SceI cut site was designed into the P4 and P5 primers, and the 1881-DSB-P1 and 1881-DSB-P2 primers were used to amplify the upstream intragenic region of NGO1881, using *N. gonorrhoeae* genomic DNA as the template. The 1881-DSB-P3 and 1881-DSB-P4 primers were used to amplify the kanamycin (Kan) cassette from pBSL86 (45). The 1881-DSB-P4 and 1881-DSB-P5 primers were used to amplify the downstream intragenic region of NGO1881 using *N. gonorrhoeae* genomic DNA as the template. PCR products from the above-mentioned three PCRs were gel purified and added as the template for the final PCR, using 1881-DSB-P1 and 1881-DSB-P6 as the primers. The final PCR product was gel purified and cloned into pSMART-LC-AMP using Kan for selection. The same approach was used for NGO0806, but with primers 0806-DSB-P1 and 0806-DSB-P2, and 0806-DSB-P3 and 0806-DSB-P4.

pSMART-LC-AMP (Lucigen) carrying I-SceI cut sites in the NGO1881 or NGO0806 intragenic region was transformed into strain FA1090, and the corresponding elements were incorporated into the *N. gonorrhoeae* chromosome through homologous recombination. Kan-resistant *N. gonorrhoeae* strains were selected, and the final strains were verified by DNA sequencing.

Cloning the I-SceI gene into *N. gonorrhoeae*. The I-SceI nuclease was amplified from SMR6276 genomic DNA (a generous gift from Susan Rosenberg, Baylor College of Medicine). Since the I-SceI gene is highly AT rich near the start and stop codons, two rounds of PCR were used to amplify the gene. The Com-I-SceI-short and Com-I-SceI-P2-short primers were used in the first round of PCR, using genomic DNA of SMR6276 as the template. The Com-I-SceI-P1 and Com-I-SceI-P2 primers were used in the second round of PCR, using DNA purified from the first-round PCR product as the template. The final PCR product was gel purified and cloned into the pMR69 plasmid via the KpnI and SpeI sites. pMR69 carrying an Atc-inducible I-SceI gene or K223I-SceI gene was transformed into strain FA1090, and erythromycin (Erm)-resistant strains were selected and verified by DNA sequencing.

Survival assays on solid medium. Strains were grown overnight from freezer stocks on GCB medium plates, resuspended in GCBL, and serially diluted to 10⁻¹ to 10⁻⁶. Ten microliters of each dilution was spotted on plates containing either GCB, GCB plus IPTG, GCB plus Atc, or GCB plus IPTG and Atc and grown for 22 h. Survival was assessed using the number of CFU with Atc treatment divided by the total number of colonies. The role of RecA in the repair of breaks was also assessed using the inducible RecA *recA6* strain (36). The survival of strains with and without RecA expression and breaks was compared to overall growth.

Survival assays with liquid medium growth. Strains were grown overnight from freezer stocks on GCB medium plates. Two plates of confluent colonies were resuspended in 20 ml of GCBL in 50-ml conical tubes. The cultures were grown for 3.5 h, Atc was added at 8 ng/μl, and the cultures were grown for an additional 1.5 h. The cultures were serially diluted, and CFU counts were obtained. One milliliter of the cultures was collected at 60 min and 90 min, and the genomic DNA was extracted using a QiAmp genomic kit for break site mapping.

Break site mapping. DSBs were detected using the primer extension method as described previously (35). Briefly, a double-stranded linker was made with each oligonucleotide concentration at 12.5 μM in 10 mM Tris, pH 7.5, 50 mM NaCl, and 1 mM EDTA. The annealing reaction mixture was heated to 90°C for 5 min, and the heating block was then removed and set on the bench to slowly return to room temperature. Primer extension was then performed using 20 μg of genomic DNA from each sample with 0.66 μM Mapbottom1 primer and 1× Sequenase buffer in 15 μl. The DNA was denatured at 95°C for 3 min, and the primer was annealed at 43°C for 30 min; 1.5 μl of 1:4-diluted Sequenase 2.0 enzyme

(Affymetrix) was added, along with 7.5 μ l of primer extension buffer containing 50 mM MgCl₂, 100 mM dithiothreitol (DTT), and 2 mM deoxynucleoside triphosphate (dNTP). Extension was performed at 45°C for 30 min, and 6 μ l of cold 300 mM Tris-Cl, pH 7.5, was added. Finally, the Sequenase was inactivated at 70°C for 15 min. The linker was then annealed using 100 pmol linker and extension product in T4 ligase buffer at 1 $\times$ and 1 μ l of T4 ligase at 400 U/ μ l at 16°C for 16 h and inactivated at 65°C for 10 min. Two rounds of nested PCR were performed to allow sequencing of the break site products.

Pilin AV assay. Pilin antigenic variation was determined using two different methods.

Pilus-dependent colony phase variation was used on strains that had the same growth rate as the parental strain (12). *N. gonorrhoeae* was grown from a frozen stock overnight. The next day, a single colony was picked and resuspended in 500 μ l GCB and then 1 μ l was diluted in 500 μ l GCB. Thirty microliters of the diluted bacteria was spread onto plates with and without IPTG and/or Atc. The next day, 20 colonies from each condition were selected at 22 h of growth. These colonies were followed at 24, 26, 28, and 30 h for the presence of blebs, which indicated a pilus phase variation event. The phase variation scores were 1 for 1 bleb, 2 for 2 blebs, 3 for 3 blebs, and 4 for 4 or more blebs. This is a surrogate test for pilin AV and has been verified by sequencing methods to confirm that the phase variation rate represents the AV rate.

Pilin antigenic variation was also measured by sequence analysis of P[−] variants. P[−] blebs arising from P⁺ colonies were picked at 48 h of growth on IPTG and 0.048 ng/ml Atc and then plated on GCB plates to select for isolated colonies. A single P[−] colony was isolated and sequenced to determine the *pilE* sequence present with the primers *pilE*-amplify-P1 and *pilE*-amplify-P2.

Characterization of suppressors. Strains with the induced I-SceI endonuclease were grown overnight and collected with a Dacron swab into GCB, and dilutions were spread onto plates containing 2 ng/ml Atc. Colonies carrying suppressor mutations were isolated following growth at 37°C for 26 h. Pilated and nonpilated colonies were identified by colony morphology. Each colony was added to 15 μ l of cell lysis buffer (1% Triton X-100, 10 mM Tris, pH 8, and 2 mM EDTA) to lyse the cells; the reaction mixtures were heated to 94°C for 15 min and 20°C for 5 min, and 2 μ l of this lysate was used as a template for a PCR to amplify the I-SceI cut site, the endonuclease, and the *pilE* gene. To amplify and sequence the I-SceI cut site, G4-Kan-P1 and G4-Kan-P6 were used. The *pilE*-amplify-P1 and *pilE*-amplify-P2 primers were used to amplify the *pilE* gene and identify truncations in *pilE* and the nearby region. To sequence the NGO1881 cut site, primer 1881-DSB-P6 was used. The I-SceI gene and its promoter were amplified and sequenced with pseq-SceI-Forward. The *pilE* gene was amplified and sequenced using primers PilRBS and Sp3A (46).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00256-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

- Maizels N. 2005. Immunoglobulin gene diversification. *Annu Rev Genet* 39:23–46. <https://doi.org/10.1146/annurev.genet.39.073003.110544>.
- Lee CS, Haber JE. 2015. Mating-type gene switching in *Saccharomyces cerevisiae*. *Microbiol Spectr* 3:MDNA3-0013-2014. <https://doi.org/10.1128/microbiolspec.MDNA3-0013-2014>.
- Deutsch KW, Lukehart SA, Stringer JR. 2009. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. *Nat Rev Microbiol* 7:493–503. <https://doi.org/10.1038/nrmicro2145>.
- Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S. 1982. Rapid evolution of RNA genomes. *Science* 215:1577–1585. <https://doi.org/10.1126/science.7041255>.
- McCulloch R, Morrison LJ, Hall J. 2015. DNA recombination strategies during antigenic variation in the African trypanosome. *Microbiol Spectr* 3:MDNA3-0016-2014. <https://doi.org/10.1128/microbiolspec.MDNA3-0016-2014>.
- Boothroyd CE, Dreesen O, Leonova T, Ly KI, Figueiredo LM, Cross GA, Papavasiliou FN. 2009. A yeast-endonuclease-generated DNA break induces antigenic switching in *Trypanosoma brucei*. *Nature* 459:278–281. <https://doi.org/10.1038/nature07982>.
- Hagblom P, Segal E, Billyard E, So M. 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. *Nature* 315:156–158. <https://doi.org/10.1038/315156a0>.
- Criss AK, Kline KA, Seifert HS. 2005. The frequency and rate of pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol Microbiol* 58:510–519. <https://doi.org/10.1111/j.1365-2958.2005.04838.x>.
- Meyer TF, Mlawer N, So M. 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell* 30:45–52. [https://doi.org/10.1016/0092-8674\(82\)90010-1](https://doi.org/10.1016/0092-8674(82)90010-1).
- Hamrick TS, Dempsey JA, Cohen MS, Cannon JG. 2001. Antigenic variation of gonococcal pilin expression in vivo: analysis of the strain FA1090 pilin repertoire and identification of the pilS gene copies recombining with pilE during experimental human infection. *Microbiology* 147:839–849. <https://doi.org/10.1099/00221287-147-4-839>.
- Mehr IJ, Seifert HS. 1998. Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol Microbiol* 30:697–710. <https://doi.org/10.1046/j.1365-2958.1998.01089.x>.
- Sechman EV, Rohrer MS, Seifert HS. 2005. A genetic screen identifies genes and sites involved in pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol Microbiol* 57:468–483. <https://doi.org/10.1111/j.1365-2958.2005.04657.x>.
- Mehr IJ, Seifert HS. 1997. Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol Microbiol* 23:1121–1131. <https://doi.org/10.1046/j.1365-2958.1997.2971660.x>.
- Skaar EP, Lazio MP, Seifert HS. 2002. Roles of the recJ and recN genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. *J Bacteriol* 184:919–927. <https://doi.org/10.1128/jb.184.4.919-927.2002>.
- Kline KA, Seifert HS. 2005. Role of the Rep helicase gene in homologous

- recombination in *Neisseria gonorrhoeae*. *J Bacteriol* 187:2903–2907. <https://doi.org/10.1128/JB.187.8.2903-2907.2005>.
16. Rotman E, Webber DM, Seifert HS. 2016. Analyzing *Neisseria gonorrhoeae* pilin antigenic variation using 454 sequencing technology. *J Bacteriol* <https://doi.org/10.1128/JB.00330-16>.
  17. Sechman EV, Kline KA, Seifert HS. 2006. Loss of both Holliday junction processing pathways is synthetically lethal in the presence of gonococcal pilin antigenic variation. *Mol Microbiol* 61:185–193. <https://doi.org/10.1111/j.1365-2958.2006.05213.x>.
  18. Cahoon LA, Manthei KA, Rotman E, Keck JL, Seifert HS. 2013. *Neisseria gonorrhoeae* RecQ helicase HRDC domains are essential for efficient binding and unwinding of the pilE guanine quartet structure required for pilin antigenic variation. *J Bacteriol* 195:2255–2261. <https://doi.org/10.1128/JB.02217-12>.
  19. Hiom K. 2009. DNA repair: common approaches to fixing double-strand breaks. *Curr Biol* 19:R523–R525. <https://doi.org/10.1016/j.cub.2009.06.009>.
  20. Hill SA, Woodward T, Reger A, Baker R, Dinse T. 2007. Role for the RecBCD recombination pathway for pilE gene variation in repair-proficient *Neisseria gonorrhoeae*. *J Bacteriol* 189:7983–7990. <https://doi.org/10.1128/JB.00980-07>.
  21. Helm RA, Seifert HS. 2009. Pilin antigenic variation occurs independently of the RecBCD pathway in *Neisseria gonorrhoeae*. *J Bacteriol* 191:5613–5621. <https://doi.org/10.1128/JB.00535-09>.
  22. Cahoon LA, Seifert HS. 2009. An alternative DNA structure is necessary for pilin antigenic variation in *Neisseria gonorrhoeae*. *Science* 325:764–767. <https://doi.org/10.1126/science.1175653>.
  23. Kuryavyi V, Cahoon LA, Seifert HS, Patel DJ. 2012. RecA-binding pilE G4 sequence essential for pilin antigenic variation forms monomeric and 5' end-stacked dimeric parallel G-quadruplexes. *Structure* 20:2090–2102. <https://doi.org/10.1016/j.str.2012.09.013>.
  24. Cahoon LA, Seifert HS. 2013. Transcription of a cis-acting, noncoding, small RNA is required for pilin antigenic variation in *Neisseria gonorrhoeae*. *PLoS Pathog* 9:e1003074. <https://doi.org/10.1371/journal.ppat.1003074>.
  25. Seifert HS. 2018. Above and beyond Watson and Crick: guanine quadruplex structures and microbes. *Annu Rev Microbiol* 72:49–69. <https://doi.org/10.1146/annurev-micro-090817-062629>.
  26. Lipps HJ, Rhodes D. 2009. G-quadruplex structures: in vivo evidence and function. *Trends Cell Biol* 19:414–422. <https://doi.org/10.1016/j.tcb.2009.05.002>.
  27. Bochman ML, Paeschke K, Zakian VA. 2012. DNA secondary structures: stability and function of G-quadruplex structures. *Nat Rev Genet* 13:770–780. <https://doi.org/10.1038/nrg3296>.
  28. Mirkin EV, Mirkin SM. 2007. Replication fork stalling at natural impediments. *Microbiol Mol Biol Rev* 71:13–35. <https://doi.org/10.1128/MMBR.00030-06>.
  29. Lambert S, Carr AM. 2013. Replication stress and genome rearrangements: lessons from yeast models. *Curr Opin Genet Dev* 23:132–139. <https://doi.org/10.1016/j.gde.2012.11.009>.
  30. Jacquier A, Dujon B. 1985. An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell* 41:383–394. [https://doi.org/10.1016/S0092-8674\(85\)80011-8](https://doi.org/10.1016/S0092-8674(85)80011-8).
  31. Dujon B. 1989. Group I introns as mobile genetic elements: facts and mechanistic speculations—a review. *Gene* 82:91–114. [https://doi.org/10.1016/0378-1119\(89\)90034-6](https://doi.org/10.1016/0378-1119(89)90034-6).
  32. Plessis A, Perrin A, Haber JE, Dujon B. 1992. Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. *Genetics* 130:451–460.
  33. Briggs E, Crouch K, Lemgruber L, Lapsley C, McCulloch R. 2018. Ribonuclease H1-targeted R-loops in surface antigen gene expression sites can direct trypanosome immune evasion. *PLoS Genet* 14:e1007729. <https://doi.org/10.1371/journal.pgen.1007729>.
  34. Ramsey ME, Hackett KT, Kotha C, Dillard JP. 2012. New complementation constructs for inducible and constitutive gene expression in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Appl Environ Microbiol* 78:3068–3078. <https://doi.org/10.1128/AEM.07871-11>.
  35. Kong Q, Maizels N. 2001. Breaksite batch mapping, a rapid method for assay and identification of DNA breaksites in mammalian cells. *Nucleic Acids Res* 29:E33. <https://doi.org/10.1093/nar/29.6.e33>.
  36. Wainwright LA, Pritchard KH, Seifert HS. 1994. A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. *Mol Microbiol* 13:75–87. <https://doi.org/10.1111/j.1365-2958.1994.tb00403.x>.
  37. Lopes J, Piazza A, Bermejo R, Kriegsmann B, Colosio A, Teulade-Fichou MP, Foiani M, Nicolas A. 2011. G-quadruplex-induced instability during leading-strand replication. *EMBO J* 30:4033–4046. <https://doi.org/10.1038/emboj.2011.316>.
  38. Viguera E, Hernandez P, Krimer DB, Boistov AS, Lurz R, Alonso JC, Schvartzman JB. 1996. The ColE1 unidirectional origin acts as a polar replication fork pausing site. *J Biol Chem* 271:22414–22421. <https://doi.org/10.1074/jbc.271.37.22414>.
  39. Gyi JI, Conn GL, Lane AN, Brown T. 1996. Comparison of the thermodynamic stabilities and solution conformations of DNA-RNA hybrids containing purine-rich and pyrimidine-rich strands with DNA and RNA duplexes. *Biochemistry* 35:12538–12548. <https://doi.org/10.1021/bi960948z>.
  40. Howell-Adams B, Seifert HS. 2000. Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation. *Mol Microbiol* 37:1146–1158. <https://doi.org/10.1046/j.1365-2958.2000.02067.x>.
  41. Barrick JE, Lenski RE. 2013. Genome dynamics during experimental evolution. *Nat Rev Genet* 14:827–839. <https://doi.org/10.1038/nrg3564>.
  42. Kellogg DS, Jr, Peacock WL, Jr, Deacon WE, Brown L, Pirkle DI. 1963. *Neisseria gonorrhoeae*. I. Virulence Genetically linked to clonal variation. *J Bacteriol* 85:1274–1279.
  43. Stohl EA, Criss AK, Seifert HS. 2005. The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol Microbiol* 58:520–532. <https://doi.org/10.1111/j.1365-2958.2005.04839.x>.
  44. Seifert HS. 1997. Insertionally inactivated and inducible recA alleles for use in *Neisseria*. *Gene* 188:215–220. [https://doi.org/10.1016/S0378-1119\(96\)00810-4](https://doi.org/10.1016/S0378-1119(96)00810-4).
  45. Alexeyev MF. 1995. Three kanamycin resistance gene cassettes with different polylinkers. *Biotechniques* 18:52–56.
  46. Seifert HS, Wright CJ, Jerse AE, Cohen MS, Cannon JG. 1994. Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J Clin Invest* 93:2744–2749. <https://doi.org/10.1172/JCI117290>.