

Antigenic Variation in *Neisseria gonorrhoeae* Occurs Independently of RecQ-Mediated Unwinding of the *pilE* G Quadruplex

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ABSTRACT The obligate human pathogen *Neisseria gonorrhoeae* alters its cell surface antigens to evade the immune system in a process known as antigenic variation (AV). During pilin AV, portions of the expressed pilin gene (*pilE*) are replaced with segments of silent pilin genes (*pilS*) through homologous recombination. The *pilE*-*pilS* exchange is initiated by formation of a parallel guanine quadruplex (G4) structure near the *pilE* gene, which recruits the homologous recombination machinery. The RecQ helicase, which has been proposed to aid AV by unwinding the *pilE* G4 structure, is an important component of this machinery. However, RecQ also promotes homologous recombination through G4-independent duplex DNA unwinding, leaving the relative importance of its G4 unwinding activity unclear. Previous investigations revealed a guanine-specific pocket (GSP) on the surface of RecQ that is required for G4, but not duplex, DNA unwinding. To determine whether RecQ-mediated G4 resolution is required for AV, *N. gonorrhoeae* strains that encode a RecQ GSP variant that cannot unwind G4 DNA were created. In contrast to the hypothesis that G4 unwinding by RecQ is important for AV, the RecQ GSP variant *N. gonorrhoeae* strains had normal AV levels. Analysis of a purified RecQ GSP variant confirmed that it retained duplex DNA unwinding activity but had lost its ability to unwind antiparallel G4 DNA. Interestingly, neither the GSP-deficient RecQ variant nor the wild-type RecQ could unwind the parallel *pilE* G4 nor the prototypical *c-myc* G4. Based on these results, we conclude that *N. gonorrhoeae* AV occurs independently of RecQ-mediated *pilE* G4 resolution.

IMPORTANCE The pathogenic bacteria *Neisseria gonorrhoeae* avoids clearance by the immune system through antigenic variation (AV), the process by which immunogenic surface features of the bacteria are exchanged for novel variants. RecQ helicase is critical in AV and its role has been proposed to stem from its ability to unwind a DNA secondary structure known as a guanine quadruplex (G4) that is central to AV. In this work, we demonstrate that the role of RecQ in AV is independent of its ability to resolve G4s and that RecQ is incapable of unwinding the G4 in question. We propose a new model of RecQ's role in AV where the G4 might recruit or orient RecQ to facilitate homologous recombination.

KEYWORDS antigen variation, DNA helicase, G quadruplex, *Neisseria gonorrhoeae*

More than 550,000 Americans are infected annually with *Neisseria gonorrhoeae*, the causative agent of gonorrhea (1). Untreated gonorrhea infections can lead to serious complications, including septic arthritis, pelvic inflammatory disease, and infertility (2). Although gonorrhea can be treated using antibiotics, increasing levels of

Citation Voter AF, Callaghan MM, Tippana R, Myong S, Dillard JP, Keck JL. 2020. Antigenic variation in *Neisseria gonorrhoeae* occurs independently of RecQ-mediated unwinding of the *pilE* G quadruplex. *J Bacteriol* 202:e00607-19. <https://doi.org/10.1128/JB.00607-19>.

Editor Ann M. Stock, Rutgers University-Robert Wood Johnson Medical School

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Received 24 September 2019

Accepted 7 November 2019

Accepted manuscript posted online 18 November 2019

Published 15 January 2020

resistance have the potential to eliminate current therapies available to patients (3). Indeed, strains with resistance to front-line clinical antimicrobial agents have been reported (4, 5). A better understanding of the mechanisms of pathogenesis in *N. gonorrhoeae* is critical for the development of novel therapeutics and treatment strategies required to maintain our ability to treat gonorrhea.

Antigenic variation (AV) is a critical process used by *N. gonorrhoeae* and other pathogens to avoid clearance by the host immune system. During infection, antigens on the surface of the bacterial cells are detected by the host immune system, which directs production of immune cells to clear the infection. However, *N. gonorrhoeae* can evade the immune response by generating new variants of surface antigens. These changes force the immune system to develop new antibodies to combat the infection. An essentially limitless number of variants can be generated through iterations of AV, impairing the development of protective immunity (6, 7).

AV of several surface antigens occurs in *N. gonorrhoeae*, including lipooligosaccharides (8), opacity proteins (9), and the type IV pilus. However, AV is most common in the pilin subunits, indicating its major role in immune system evasion (10). Only a single pilin gene, *pilE*, is actively expressed in *N. gonorrhoeae*, whereas its genome contains 19 silent copies of the pilin gene called *pilS*. Portions of the *pilS* loci replace portions of *pilE* through RecA-mediated homologous recombination during AV (11). While the precise mechanistic steps that drive pilin AV remain unclear, the contributions of several major factors have been characterized (12).

A key early step in *N. gonorrhoeae* pilin AV is formation of a guanine quadruplex (G4) DNA structure (13). G4s are unusual DNA secondary structures that form in guanine-rich nucleic acid sequences through extensive Hoogsteen hydrogen bonding and stacking among the guanine bases. The interactions within G4s form extremely stable structures that can be challenging to unwind. G4s fold in either parallel or antiparallel structures based on the orientation of their phosphodiester backbone. These orientations are typified by the parallel *c-myc* G4 (14) (Fig. 1A, nearly identical to the *N. gonorrhoeae pilE* G4 element) and the antiparallel human telomeric G4 (15) (telo-G4) (Fig. 1B). These two forms are structurally distinct, have differing stabilities, and varied susceptibilities to helicase unwinding. The *pilE* G4-forming sequence is located upstream of the *pilE* gene, and this G4 is known to be essential for AV but not pilin expression (13). Initiation of the AV process occurs when the *pilE* G4-forming sequence is unwound to allow transcription of a small noncoding RNA. Freed from the complementary template strand, the *pilE* G4 sequence folds into a G4 structure (16). Although it has been shown that G4 formation is required for AV and alternate G4-forming sequences fail to initiate AV, the precise role for the G4 has not been defined (13, 16). Because RecQ helicases are known to unwind G4 substrates (17) and $\Delta recQ$ strains have been shown to be partially deficient in AV (12), it has been proposed that unwinding of the *pilE* G4 by the RecQ helicase could be critical to the AV process.

The bacterial RecQ protein is a 3' to 5' DNA helicase (18). The preferred substrate for RecQ is duplex DNA with a 3' single-stranded DNA (ssDNA) element, but bacterial RecQs have also been shown to unwind G4 DNA substrates (17). Bacterial RecQs comprise a helicase motor domain made up of two RecA-like lobes, a structural Zn²⁺-binding domain, a DNA-binding winged helix domain, and a regulatory helicase and RNase D C-terminal (HRDC) domain (Fig. 1C). The *N. gonorrhoeae* RecQ (NgRecQ) is distinct from most other RecQs in that it possesses three HRDCs rather than one. Previous studies have found that truncation of the two C-terminal-most HRDC domains from NgRecQ is sufficient to disrupt AV, and this defect was attributed to a relatively modest decrease in G4 DNA binding and unwinding by the variant (19). However, the truncated NgRecQ also has greatly reduced affinity for duplex and ssDNA and reduced helicase activity on Holliday junction substrates (20). Furthermore, strains with truncated RecQ variants were found to be hypersensitive to UV irradiation, suggesting that the AV deficiency could be the result of general effects on RecQ rather than a G4-specific defect (20). A more precise isolation of the G4 unwinding activity of RecQ is needed to deconvolute the possible roles of RecQ-mediated G4 unwinding in AV.

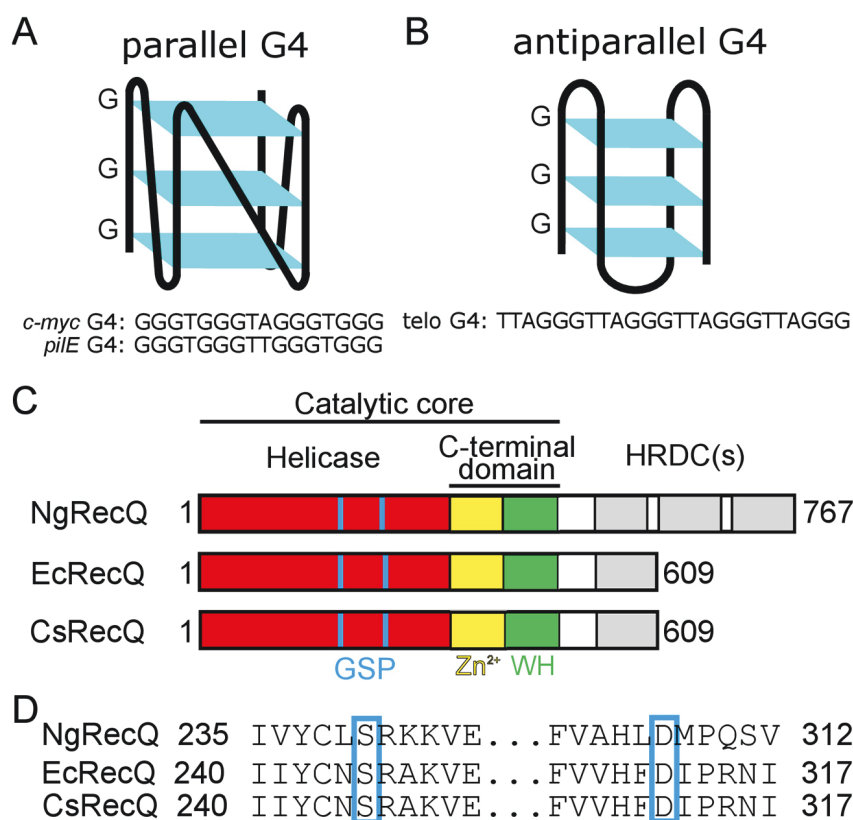


FIG 1 Comparison of the structures of antiparallel and parallel G4s and bacterial RecQ helicases. (A) Model of a parallel G4, such as the *c-myc* and *pilE* G4s used in this study. Each blue structure represents four guanine bases in a quartet structure. G4-forming sequences are shown under each model. (B) Model of an antiparallel G4 typified by the telomeric G4. (C) Comparison of the domain architecture of RecQ helicases from bacterial species. The location of the GSP is denoted by the blue lines. (D) Sequence alignment of GSP between bacterial RecQ helicases. Residues that directly interact with a guanine base within the GSP are boxed.

We recently determined the crystal structure of RecQ from *Cronobacter sakazakii* (CsRecQ) in complex with an unfolded G4. This structure revealed the presence of a guanine-specific pocket (GSP) on the surface of RecQ that was essential for G4 helicase activity but dispensable for unwinding duplex substrates (21). The GSP is conserved in NgRecQ (Fig. 1D), and we reasoned that mutation of the GSP in NgRecQ could be used to determine the role of RecQ-mediated G4 unwinding in AV. We therefore generated *N. gonorrhoeae* strains bearing GSP-defective *recQ*. Surprisingly, these strains underwent AV at the same rate as wild-type cells, which refuted a role for RecQ G4 unwinding in AV. Wild-type and GSP-defective NgRecQ proteins were purified and, although both retained duplex unwinding activity, neither was competent to unwind *pilE* G4 DNA. From these results, we conclude that AV in *N. gonorrhoeae* occurs independently of RecQ-mediated unwinding of the *pilE* G4 and that the role of RecQ is limited to facilitating homologous recombination by RecA.

RESULTS AND DISCUSSION

The GSP of RecQ is dispensable for AV. The specific roles of the RecQ helicase in *N. gonorrhoeae* AV are unclear. Deletion of the *recQ* gene or removal of two HRDC domains from the protein diminish AV (12, 13, 19), but whether this effect is due to a specific loss in G4 unwinding or RecQ activities in homologous recombination has not been defined. To determine the role of RecQ-mediated G4 unwinding on AV, we generated *N. gonorrhoeae* strains bearing NgRecQ variants that we predicted would maintain all enzyme functions except for the ability to unwind G4 DNA. Our previous biochemical analysis of the RecQ proteins from *Escherichia coli* (EcRecQ) and *C. sakazakii*

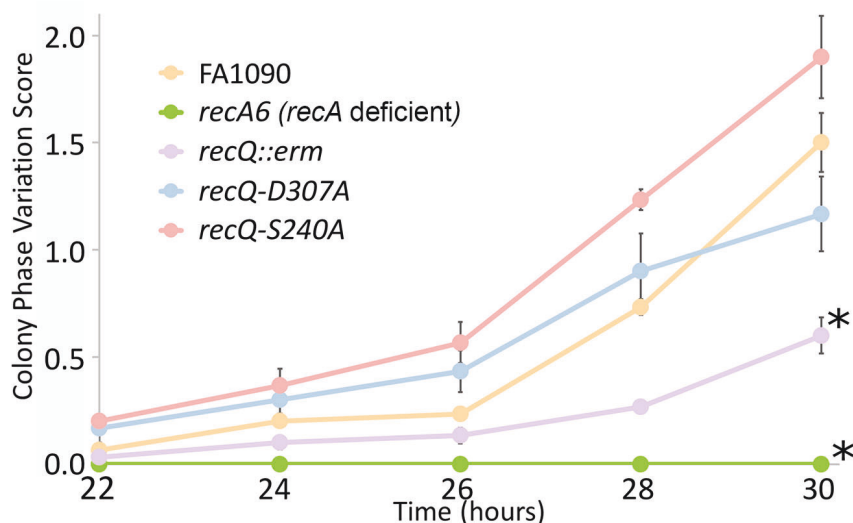


FIG 2 Role of GSP in *N. gonorrhoeae* antigenic variation. Pilin-dependent colony morphology changes in *N. gonorrhoeae* variants are depicted. Each point represents the average colony phase variation score of three biological replicates, with 10 colonies assessed for each replicate. Error bars represent the standard errors of the mean ($n = 10$), and an asterisk indicates a P value of <0.05 , as determined by a two-tailed Student t test relative to the FA1090 strain.

(CsRecQ) demonstrated that disruption of a GSP on the surface of the enzymes resulted in a complete loss of G4 helicase activity, whereas duplex DNA unwinding activity was retained (21). Therefore, we mutated the *recQ* gene in *N. gonorrhoeae* to encode single-site variants containing disabled GSPs (Ser240Ala or Asp307Ala) to distinguish between a G4-specific and G4-independent role for RecQ in the AV process. These strains were compared to *recQ::erm* or *recA6* (RecA deficient) *N. gonorrhoeae* strains that are known, respectively, to impair or eliminate AV (12, 13). We predicted that if RecQ-mediated G4 unwinding was important for AV, then AV in the single-site GSP-deficient strains would be impaired to the same extent as the *recQ::erm* strain. We recapitulated the partial AV defect of the *recQ::erm* strain and complete loss of AV in the uninduced *recA6* strain. However, in contrast to the hypothesis, the strains with GSP-defective RecQ proteins underwent AV at the same rate as wild-type *N. gonorrhoeae* (Fig. 2).

Two possibilities could explain the observed wild-type *N. gonorrhoeae* FA1090 levels of AV in these strains. First, the G4 helicase activity of NgRecQ may be occurring independently of its GSP. This is unlikely since the structural and biochemical studies defining the GSP were conducted using EcRecQ and CsRecQs, which are closely related to NgRecQ (45% identical through the first HRDC domain). In addition, the residues that form the GSP in EcRecQ and CsRecQ are exactly matched in NgRecQ (Fig. 1B and C). Despite this overall similarity, specific features of NgRecQ, especially the presence of two additional HRDC domains, might confer GSP-independent *pilE* G4 unwinding. Alternatively, NgRecQ-mediated unwinding of the *pilE* G4 may be dispensable for AV.

NgRecQ cannot unwind the *pilE* G4 in bulk assays. To distinguish between the possible explanations for AV function with the RecQ GSP-defective variants, we sought to determine whether the G4 helicase activity of NgRecQ occurred independently of its GSP. NgRecQ and the NgRecQ Asp307Ala GSP variant were purified and tested for DNA unwinding *in vitro*. In this experiment, the *pilE* G4 was labeled with 5' 6-carboxyfluorescein (FAM) and a 3' black hole quencher (BHQ). In the folded state, FAM fluorescence was quenched by the nearby BHQ and unwinding was expected to result in an increase in fluorescence intensity. After allowing NgRecQ to bind to the G4, ATP was added, and the fluorescence intensity was measured over time (see Fig. S2A in the supplemental material). Only a modest increase (~3%) in fluorescence intensity was observed after ATP addition for both NgRecQ and the NgRecQ Asp307Ala

variant (Fig. S2B). These results contrast with the high-magnitude, albeit slow increase in fluorescence previously observed during NgRecQ G4 helicase action (19).

The bulk G4 unwinding experiments were conducted with Na^+ as the primary cation, while K^+ is known to stabilize G4s more than Na^+ (22). To measure the potential effect of cation choice on G4 unwinding, we measured the stability of the G4 by performing circular dichroism thermal denaturation assays with the substrate in the presence of either Na^+ or K^+ (Fig. S3). The *pilE* G4 was less stable in NaCl; a T_m of 53°C was observed in the presence of 50 mM NaCl, whereas a T_m in excess of 80°C was observed in the KCl buffer. In the presence of a G4 destabilizing cation such as Na^+ , minor variations in helicase assay setup could result in the appearance of G4 unwinding. However, when the helicase assay experiments were repeated in the presence of KCl, we again failed to observe significant unwinding for either protein (Fig. S2C). Because K^+ is the primary intracellular cation in bacteria, with concentrations greatly exceeding that of Na^+ (23), this result suggested that NgRecQ may not be able to unwind the *pilE* G4 under physiological conditions or even under Na^+ -containing conditions in which the *pilE* G4 structure is relatively less stable.

NgRecQ unwinds antiparallel but not parallel G4s. An established single-molecule helicase assay was used to further assess NgRecQ duplex and G4 DNA unwinding properties. In these experiments, a 5' Cy5-labeled oligonucleotide was tethered to a coverslip and annealed to a test oligonucleotide containing a 5' complementary region (Fig. 3A). The test oligonucleotide contained a 3' Cy3 label, a 3' dT₁₅ region for NgRecQ loading and, if required for the experiment, a G4-forming sequence between the 3' dT₁₅ and the 5' complementary region. Given the 3'-5' polarity of RecQ helicases, the enzyme is expected to bind to the 3' dT₁₅ and translocate toward the 5' end. If the enzyme is competent to unwind the G4 and duplex DNA, the G4-containing test oligonucleotide will be released leading to a loss of Cy3 fluorescence (Fig. 3A). To ensure both NgRecQ proteins were properly folded and active, we first tested the ability of the proteins to unwind a simple duplex substrate that lacked a G4-forming sequence. Both wild-type and the NgRecQ Asp307Ala variant were competent to unwind the duplex substrate, although the Asp307Ala variant had a 2.8-fold-slower unwinding rate than wild-type NgRecQ (Fig. 3B; Table 1).

As was observed for EcRecQ and CsRecQ, NgRecQ was found to robustly unwind a test oligonucleotide containing the antiparallel human telomeric G4 forming sequence [(TTAGGG)₄]. In contrast, antiparallel G4 unwinding was not observed with the NgRecQ Asp307Ala variant, consistent with the essential nature of the GSP for RecQ-mediated G4 helicase activity (Fig. 3C). Because the *pilE* G4 forming sequence adopts a parallel conformation rather than the antiparallel structure of the telomeric G4, we next tested whether NgRecQ was competent to unwind the parallel *pilE* or *c-myc* G4s. (Fig. 1A). Neither the NgRecQ nor the NgRecQ Asp307Ala variant unwound the *pilE* DNA (Fig. 3D). Similar results had previously been obtained with EcRecQ and CsRecQ (21). These results indicate that while the GSP is required for NgRecQ to unwind antiparallel G4 DNA, parallel quadruplexes such as the *c-myc* and the *pilE* G4s are not substrates of the NgRecQ helicase.

RecQ helicases prefer substrates with a 3' ssDNA overhang, which was absent from the substrate in the bulk helicase assay. To explore the requirement of such a tail for G4 unwinding, we repeated the single-molecule fluorescence resonance energy transfer (smFRET) experiments with G4 substrates lacking the dT₁₅ tail. Although NgRecQ was competent to unwind the tailed variant of the telomeric G4, neither NgRecQ variant could unwind the untail telomeric G4 and neither NgRecQ variant was found to unwind the *pilE* G4, regardless of the presence of a 3' tail (Fig. S4; Table 1). These results highlight the critical nature of the 3' tail for RecQ activity and are consistent with our observations from the bulk helicase assay.

Although no unwinding was observed for the parallel G4 substrates, the addition of either NgRecQ or the NgRecQ Asp307Ala variant resulted in an ATP-independent shift to lower FRET states (~ 0.8 to ~ 0.5) (Fig. S5). This shift likely indicates that NgRecQ and

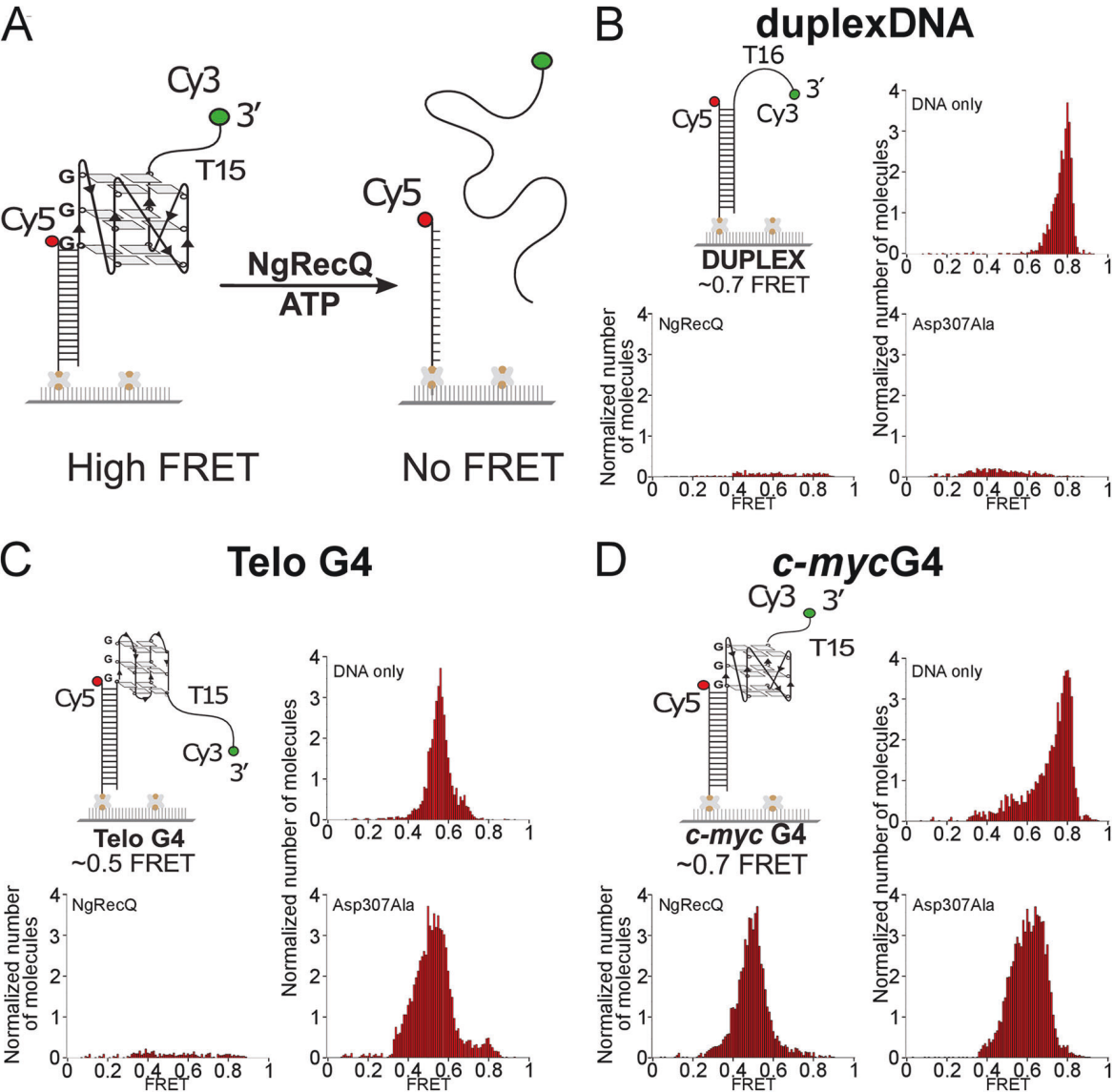


FIG 3 smFRET studies of NgRecQ helicase activity. (A) Scheme depicting the smFRET strategy used to monitor DNA unwinding by NgRecQ. (B) NgRecQ-mediated unwinding of duplex DNA. Histograms of the smFRET signals for the DNA alone (top), or 12-min after the addition of ATP and NgRecQ or NgRecQ Asp307Ala are shown. (C and D) Same as in panel B but for the antiparallel telo-G4 and parallel *pilE* G4 substrates, respectively.

the variant can bind to and possibly alter the structure of the substrate without unwinding. Notably, this was not observed in our prior experiments using the catalytic cores of EcRecQ and CsRecQ (which lacked HRDC domains), so this binding or reorganization may be HRDC dependent. Another possibility is that NgRecQ binds to the 3' ss dT₁₅ tail, stretching the DNA and increasing the distance between the Cy3-Cy5 FRET pair.

TABLE 1 DNA unwinding rates of the NgRecQs^a

RecQ variant	Mean duplex unwinding (s ⁻¹) ± SD	Unwinding rate (s ⁻¹) ± SD			
		telo-G4, tail	telo-G4, no tail	<i>pilE</i> G4, tail	<i>pilE</i> G4, no tail
NgRecQ	0.0223 ± 0.0007	0.0093 ± 0.0005	NU	NU	NU
NgRecQ Asp307Ala	0.0079 ± 0.0011	NU	NU	NU	NU

^aNU, no unwinding.

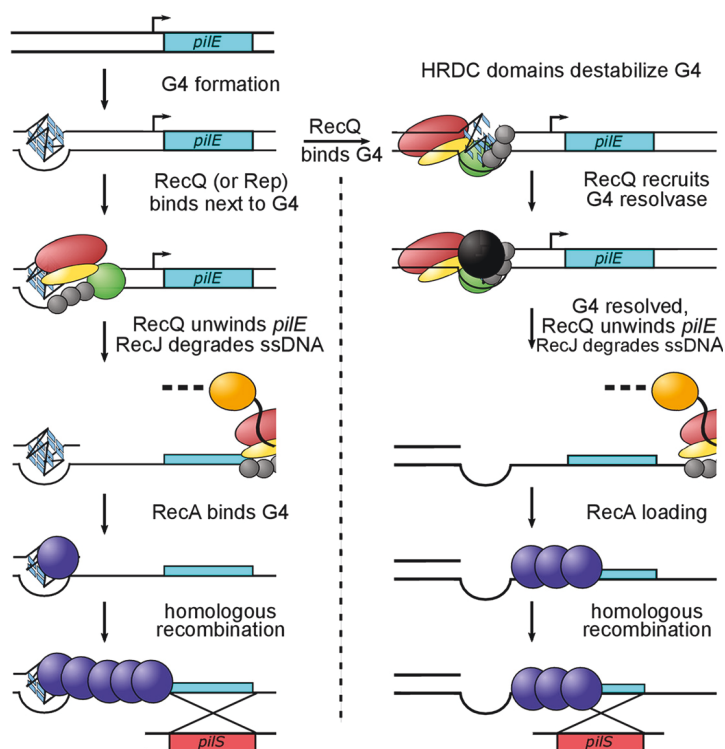


FIG 4 Model of the role of RecQ in *N. gonorrhoeae* AV. After formation of the *pilE* G4, RecQ (domains colored as in Fig. 1) binds to the G4. (Left) The RecQ HRDC domains bind to the G4 or nearby DNA, orienting RecQ to unwind the *pilE* gene. RecJ degrades the unwound ssDNA behind RecQ. RecQ cannot unwind the *pilE* G4, so it remains folded and recruits RecA for strand exchange by homologous recombination. (Right) Alternatively, HRDC domains could destabilize the G4, allowing for unwinding by another G4 resolving helicase. Once the G4 obstruction is removed, RecQ unwinds the *pilE* gene to facilitate RecA loading and homologous recombination.

It has been shown that G4 folding is essential for AV, and there appears to be a requirement for both the compacted structure adopted by the *pilE* G4 and its parallel orientation (13). Indeed, *N. gonorrhoeae* strains in which the *pilE* G4 has been replaced by other G4-forming sequences (including those likely to be unwound by RecQ) cannot undergo AV (13). These findings are consistent with our observations that NgRecQ-mediated unwinding of the *pilE* G4 is not a requirement for AV and that the isolated NgRecQ enzyme is not capable of such unwinding. Despite this, NgRecQ is involved in the AV process (12, 19, 20).

The second and third HRDC domains of NgRecQ are crucial for NgRecQ's role in AV (19, 20) and we propose two possible roles that are consistent with this requirement (Fig. 4). First, the HRDC domains might interact with the G4 to promote homologous recombination without NgRecQ G4 unwinding. In support of this, the shift to a lower FRET state observed in our FRET assays is consistent with binding to the *pilE* G4. Such binding could distort the G4 or adjacent DNA to promote access by another protein, either to unwind the G4 or facilitate RecA-mediated homologous recombination (Fig. 4, right). Similarly, the NgRecQ HRDC domains might bind the *pilE* G4 to orient the helicase in a manner that aids in productive RecA loading. Because deletion of the NgRecQ HRDCs have only a modest impact on either G4 binding or unwinding, the HRDC domains might instead modulate NgRecQ activity. In this scenario, the NgRecQ variant lacking the C-terminal-most HRDC domains may bind to the *pilE* G4 such that the *pilE* G4 or nearby duplex DNA cannot be unwound in preparation for RecA loading. Thus, the HRDCs might serve to recruit NgRecQ to the *pilE* G4 and properly orient the helicase (Fig. 4, left).

A second possibility is that the NgRecQ HRDCs are required for efficient RecA loading via a G4-independent mechanism. To this end, the NgRecQ variant lacking the

C-terminal-most HRDC domains is defective in binding and unwinding some, but not all, DNA substrates (20). In addition, a strain bearing truncated *recQ* was as sensitive to UV-induced DNA damage as a *recQ::erm* knockout (20). Thus, NgRecQ dysregulation resulting solely from the loss of the HRDC domains is sufficient to inhibit NgRecQ-mediated DNA repair. The loss of the HRDC domains very likely impairs NgRecQ-mediated loading of RecA during AV. Further studies are needed to clarify the role of the NgRecQ HRDC domains in AV.

What then is the role of the *pilE* G4 in AV? RecA has a high affinity for the *pilE* G4 and binding to the quadruplex stimulates RecA-mediated strand exchange (24). Furthermore, substitution of the *pilE* G4 with other G4s that RecA cannot bind blocks AV (13). It has been proposed that the critical function of the *pilE* G4 may be to recruit and stimulate RecA (24). Unfortunately, this is a difficult hypothesis to test; RecA-mediated homologous recombination is likely essential for AV independent of its affinity for the *pilE* G4, and there are no known RecA separation-of-function mutants that would allow for isolation of the role of the RecA-G4 interaction. The fate of the G4 remains another outstanding question. Unresolved G4s block replication fork progression and lead to toxic DNA damage. Therefore, the *pilE* G4 is likely resolved by a G4 resolving helicase, such as UvrD (25), DinG (26), or Rep (unpublished observations) prior to replication.

In conclusion, the results presented in this study demonstrate that AV in *Neisseria gonorrhoeae* occurs independently of NgRecQ-mediated unwinding of the *pilE* G4 and that NgRecQ is incapable of unwinding the *pilE* G4. Instead, we propose that the role of NgRecQ in AV is limited to facilitating RecA-mediated homologous recombination. Future experiments will elucidate the structure and role of the RecA G4 interaction in AV and explore how the *pilE* G4 is resolved or tolerated during DNA replication.

MATERIALS AND METHODS

Generation of the GSP variants in NgRecQ. Plasmids pAV305 and pAV306 were each ligated with pIDN1 after HindIII/XhoI digestion (see Table S1 in the supplemental material) (27). Plasmids were transformed into TAM1 *E. coli*, and transformants were screened for plasmids of the expected size. Final constructs pMMC15 (containing the pAV305 NgRecQD307A mutation) and pMMC16 (containing the pAV306 NgRecQS240A mutation) were confirmed by DNA sequencing. *N. gonorrhoeae* strains MMC552 and MMC553 were generated by spot transformation of *N. gonorrhoeae* FA1090 with plasmids pMMC15 and pMMC16, respectively (Table S2). Screening was performed by colony PCR and digestion with either NruI (MMC552) or BssHII (MMC553) (28). The *recQ::ermC* interruption strain MMC536 was generated by spot transforming FA1090 with linear pPK1014 (20). Transformants were selected with 2 μ g/ml erythromycin and confirmed by PCR and sequencing. All strains stocks were confirmed to have identical *pilE* sequences. No differences in solid medium growth rates were observed between the RecQ variant strains (Fig. S1).

Colony phase variation assay. Pilus-dependent colony morphology change assays were performed as described by Sechman et al. (12). Briefly, strains were grown from frozen stocks on GCB agar for 24 h. A single pilated colony was restreaked onto GCB agar and incubated overnight. For each strain, 10 colonies were chosen. At 22, 24, 26, 28, and 30 h, chosen colonies were analyzed using a stereomicroscope and scored by counting the nonpilated outgrowths visible on the colony. Each new outgrowth increases the score by one, until four outgrowths have developed. All colonies with four or more outgrowths receive a score of four. FA1090 *recA6*, wherein *recA* is under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter, was used without induction as a *recA* deficient control (29).

Measurement of growth on solid medium. Growth rate comparisons were performed as previously described (7). Bacteria were struck from frozen glycerol stocks on GCB plates and grown overnight (~16 to 20 h). Three to five pilated colonies were selected and struck for single colonies onto new GCB plates. At 22 and 30 h, four colonies were collected using sterile filter paper disks and suspended in liquid GCBL medium with 0.042% sodium bicarbonate and Kellogg's supplements. Suspensions were dilution plated in duplicate. The mean CFU per colony for each strain at each time point were counted. At each time point, the CFU/colony of all strains were compared by analysis of variance using a significance threshold of $P = 0.05$.

Purification of the NgRecQ variants. BL21-AI *Escherichia coli* cells were transformed with overexpression plasmids encoding N-terminally His-tagged NgRecQ or the NgRecQ Asp307Ala variant. Cells were grown at 37°C to an optical density at 600 nm of 0.6 before protein expression was induced with 0.2% (wt/vol) arabinose and 1 mM IPTG (NgRecQ) or 0.2% (wt/vol) arabinose (NgRecQ Asp307Ala). The cells were grown for a further 4 h at 37°C, harvested by centrifugation, and stored at -80°C. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM 2-mercaptoethanol [BME], 1 mM phenylmethylsulfonyl fluoride, 100 mM dextrose, 15 mM imidazole, 1 Pierce protease inhibitor tablet, 10% [vol/vol] glycerol), lysed by sonication, and clarified by centrifugation. The supernatant was incubated with Ni-nitrilotriacetic acid (Ni-NTA)-agarose resin at 4°C for 1 h and then washed extensively

with lysis buffer. Proteins were eluted from the resin with elution buffer (lysis buffer supplemented with 250 mM imidazole). Eluent was diluted to 50 mM NaCl using dilution buffer (5 mM Tris-HCl [pH 8.0], 1 mM BME, 10% [vol/vol] glycerol) and then loaded onto a HiPrep QFF ion exchange column and eluted with a 0.05 to 1.0 M NaCl gradient. RecQ-containing fractions were identified by SDS-PAGE analysis, concentrated, and further purified with an S-100 size exclusion column before dialysis into storage buffer (20 mM Tris-HCl, 1 M NaCl, 4 mM BME, 40% [vol/vol] glycerol, 1 mM EDTA) and stored at -20°C .

Bulk dually labeled G4 helicase assays. A dually labeled, HPLC purified oligonucleotide with the *pilE* G4 sequence (5' FAM-GGG TGG GT TGG GTG GG-BHQ) was obtained from Integrated DNA Technologies (Coralville, IA). The oligonucleotide was resuspended in water, diluted to 1 μM (molecules) in 20 mM Tris-HCl–100 mM KCl, and then heated to 95°C for 10 min and allowed to slowly cool to room temperature to ensure the G4s were properly folded at the start of the experiment. The oligonucleotide was diluted to a final concentration of 10 nM in a reaction buffer (25 mM Tris-HCl, 0.1 mM dithiothreitol, 3 mM MgCl_2) containing a variable amount of the NgRecQ helicase and either 50 mM NaCl or 100 mM KCl. All measurements were taken using a Photon Technology International, Inc., fluorimeter with a 490-nm excitation, and the emission was measured at 520 nm. After a 10-min incubation, ATP was added to a final concentration of 1 mM, and the emission intensity was recorded. All further intensities were normalized to the first intensity measurement taken after ATP addition.

Circular dichroism. G4-forming oligonucleotides were resuspended in water and then diluted to 5 μM (molecules) in 300 μl of either a KCl (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 3 mM MgCl_2) or an NaCl (25 mM Tris-HCl, 50 mM NaCl, 3 mM MgCl_2) salt buffer. The oligonucleotides were heated to 95°C for 10 min and then allowed to slowly cool to room temperature. Circular dichroic spectra were recorded on an AVIV 420 circular dichroism spectrometer with a step size of 2 nm and a 5-s average. A buffer matched blank lacking DNA was subtracted from each reading. Samples were equilibrated at each temperature for 5 min before data collection. To generate the melting curve, the ellipticity at 260 nm was measured at increasing temperatures for each salt condition. Curve fitting was performed in Prism version 5.0c.

smFRET DNA substrates. Amine-modified ssDNA substrates were purchased from Integrated DNA Technologies. Cy3/Cy5 monofunctional NHS esters were used to label the amine modified ssDNA constructs (GE Healthcare, Princeton, NJ). Amino-modified oligonucleotides (10 nmol in 50 μl of ddH₂O) and 100 nmol of Cy3/Cy5 NHS ester dissolved in 50 μl of 0.1 M NaHCO_3 were combined and incubated with rotation for 4 h in the dark. The labeled oligonucleotides and unreacted dye were separated by P6 columns (Bio-Rad) or ethanol precipitation.

Both G4 and non-G4 substrates consist of a stem of dsDNA with 18 bp and a specific sequence 3' tailed ssDNA (Table S1). A Cy5-Cy3 FRET pair are placed at the junction and the 3' end of the ssDNA, respectively.

T50 (10 mM Tris-HCl [pH 8.0], 50 mM NaCl) buffer was used to anneal the biotinylated and nonbiotinylated oligonucleotides in a 1:1.5 molar ratio to a final concentration of 10 μM duplex. The sample was heated to 95°C for 2 min slow cooled in a thermocycler. The high concentration of annealed DNA was stored at -20°C and was freshly diluted for each measurement to a 10 nM stock concentration in K100 buffer (10 mM Tris-HCl [pH 8.0], 100 mM KCl).

smFRET unwinding assays. All single-molecule unwinding assays were measured by using a custom-built total internal reflection (TIRF) microscope. A 532-nm laser (Coherent) was used to excite the donor dye in the Cy3-Cy5 FRET pair used for the single molecule measurements. The fluorescence emission was separated by a dichroic mirror with a cutoff of 630 nm to split the Cy3 and Cy5 signals, which were then detected on an electron-multiplying charge-coupled device (EMCCD) camera (iXon DU-897ECSO-#BV; Andor Technology). Single-molecule traces from the recorded data were extracted by IDL software. Matlab and Origin software was used to display and analyze the single-molecule traces. All homemade codes are in the smFRET package available at the Center for the Physics of Living Cells (<https://cplc.illinois.edu/software/>; Biophysics Department, the University of Illinois at Urbana-Champaign).

RecQ unwinding experiments were performed in reaction buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 3 mM MgCl_2 , 1 mM ATP) with an oxygen scavenging system containing 0.8% (vol/vol) dextrose, 1 mg/ml glucose oxidase, 0.03 mg/ml catalase 1, and 10 mM Trolox. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Biotinylated FRET DNA (50 to 100 pM) was immobilized on polyethylene glycol-coated quartz surface via biotin-neutravidin linkage for 2 min, and then all unbound DNA was washed away. RecQ and mutant proteins (100 nM) were added at room temperature to initiate unwinding. Then, 10 to 20 short movies (10 s) with an interval of 20 s and separately 3 or 4 long movies (3 min) were taken, monitoring the Cy3 and Cy5 emission intensities over time. These were then analyzed to produce the FRET histograms and trajectories to monitor any unwinding activity. Unwinding rates were calculated as previously reported (21).

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

ACKNOWLEDGMENTS

We thank members of the Keck laboratory for critical reading of the manuscript.

This study was funded by NIH R01 GM098885 to J.L.K. A.F.V. was supported by NIH F30 CA210465 and T32 GM008692.

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