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Neisseria gonorrhoeae RecQ Helicase HRDC Domains Are Essential for Efficient Binding and Unwinding of the *pilE* Guanine Quartet Structure Required for Pilin Antigenic Variation

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The strict human pathogen *Neisseria gonorrhoeae* utilizes homologous recombination to antigenically vary the pilus, thus evading the host immune response. High-frequency gene conversion reactions between many silent pilin loci and the expressed pilin locus (*pilE*) allow for numerous pilus variants per strain to be produced from a single strain. For pilin antigenic variation (Av) to occur, a guanine quartet (G4) structure must form upstream of *pilE*. The RecQ helicase is one of several recombination or repair enzymes required for efficient levels of pilin Av, and RecQ family members have been shown to bind to and unwind G4 structures. Additionally, the vast majority of RecQ helicase family members encode one "helicase and RNase D C-terminal" (HRDC) domain, whereas the *N. gonorrhoeae* RecQ helicase gene encodes three HRDC domains, which are critical for pilin Av. Here, we confirm that deletion of RecQ HRDC domains 2 and 3 causes a decrease in the frequency of pilin Av comparable to that obtained with a functional knockout. We demonstrate that the *N. gonorrhoeae* RecQ helicase can bind and unwind the *pilE* G4 structure. Deletion of the RecQ HRDC domains 2 and 3 resulted in a decrease in G4 structure binding and unwinding. These data suggest that the decrease in pilin Av observed in the RecQ HRDC domain 2 and 3 deletion mutant is a result of the enzyme's inability to efficiently bind and unwind the *pilE* G4 structure.

Neisseria gonorrhoeae is an obligate human pathogen and the causative agent of the sexually transmitted infection gonorrhea. Gonococci generally infect the urogenital tract, and the infection typically presents as urethritis in men and cervicitis in women, but many women can be asymptomatic carriers (1). The *N. gonorrhoeae* type IV pilus is essential for establishing infection (2). Pili assist in epithelial adherence and gonococcal cell aggregation and also mediate twitching motility (3–5). Protective immunity never develops, partially because the bacterium can evade host immune selection by antigenically varying surface antigens, including lipooligosaccharides, the opacity family of outer membrane proteins, and the type IV pilus (6–10).

N. gonorrhoeae possesses one pilin expression locus (*pilE*) and up to 19 silent pilin storage copies, which reside in up to 6 discrete loci (*pilS*) in the genome (11). Pilin antigenic variation (Av) occurs as a result of nonreciprocal DNA recombination between any *pilS* copy and *pilE*, leading to the expression of a new variant (7). *N. gonorrhoeae* pilin Av is a specialized high-frequency recombination system that occurs via a RecF-like pathway of homologous recombination whereby a functional knockout of the *recQ* gene, which encodes the RecQ DNA helicase, results in an Av deficiency (12–15). In addition, pilin Av requires the formation of a *pilE* guanine quartet/quadruplex (G4) structure.

The *pilE* G4-forming sequence was identified using a targeted genetic screen in a DNA region upstream of *pilE*, where insertions were found to block pilin Av but not alter pilin expression (16, 17). In this region, individual mutation of 11 GC base pairs completely blocked pilin Av, and mutation of a 12th GC base pair produced reduced but residual pilin Av frequency (14, 16). Mutation of an adjacent 13th GC base pair did not affect pilin Av, but mutation of this GC base pair in addition to the 12th resulted in a complete loss of function, suggesting that this 13th GC base pair could partially substitute for the mutated 12th GC base pair (14, 16). The organization of these GC base pairs conforms to a G4 motif (16). Biophysical studies showed that this G-rich sequence formed a G4 structure in which mutations that block pilin Av also inhibited structure formation (16). *N. gonorrhoeae* grown on *N*-methyl mesoporphyrin IX, a compound that specifically binds and stabilizes G4 structures but not double- or single-stranded DNA (ssDNA) (18), decreased the frequency of pilin Av (16). Moreover, point mutations in *N. gonorrhoeae* that block pilin Av and G4 structure formation prevented single-stranded nicks from being detected in the G4-forming sequence and complement strand (16).

Pilin Av also requires transcription of a small RNA (sRNA) molecule that initiates within the G4-forming sequence (19). Mutation of the promoter of this sRNA prevents detectable pilin Av from occurring, and replacement of the normal promoter with a phage-specific promoter allows restoration of pilin Av only when the phage polymerase is expressed (19). However, expression of this sRNA from an ectopic locus does not rescue a promoter mutation, showing that this sRNA must be expressed in its normal location to function (19). We postulate that transcription is required to open the DNA duplex, but further roles for this sRNA in the process of pilin Av remain to be determined.

The pathogenic *Neisseria* RecQ helicase is unusual among

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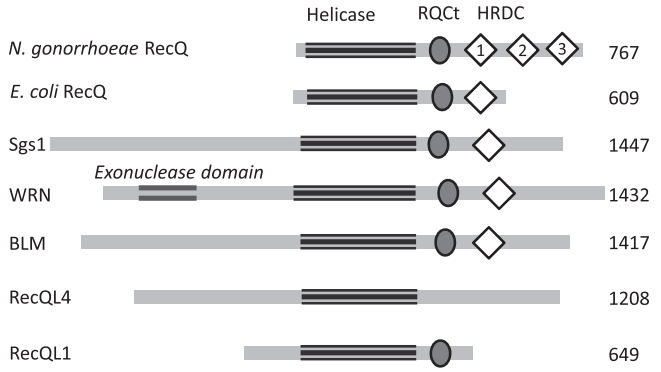

FIG 1 RecQ helicase family members. Schematic representation of the relative location of conserved domains in selected RecQ family members including *N. gonorrhoeae*, *E. coli*, *S. cerevisiae* Sgs1, human WRN, BLM, RecQL4, and RecQL1. If applicable, the exonuclease, helicase, RecQ C-terminal (RQCt), and helicase and RNase D C-terminal (HRDC) domains are designated. HRDC domains 1, 2, and 3 are labeled for *N. gonorrhoeae*. The numbers of amino acid residues in the protein are indicated on the right.

RecQ family members, having three "helicase and RNase D C-terminal" (HRDC) domains (Fig. 1). Only one other organism, *Deinococcus radiodurans*, has been reported to possess this unusual RecQ triple-HRDC domain structure (20), while most RecQs contain one HRDC domain and a few RecQ orthologs have no HRDC repeats (Fig. 1). Previously, it was shown that deletion of the *N. gonorrhoeae* HRDC domains inhibited pilin Av (21). Since RecQ HRDC domains have been shown to provide additional DNA specificity and modulate the affinity of the enzyme for single-stranded, duplex, and Holliday junction DNA structures (20, 22–24) and pilin Av depends on a G4 structure, it was possible that the RecQ HRDC domains might play a role in binding and unwinding the *pilE* G4 structure required for pilin Av (16). Moreover, some RecQ helicase enzymes have been shown to unwind G4 structures (25–28). In addition, only RecQ helicase family members that have an HRDC domain, i.e., *Saccharomyces cerevisiae* Sgs1, human WRN and BLM, and *Escherichia coli* RecQ, have been shown to process G4 structures (25–28), whereas RecQ family members human RecQL1 and RecQL4, which do not have an HRDC domain, do not interact with G4 structures (29, 30) (Fig. 1). Since the *N. gonorrhoeae* RecQ helicase HRDC domains are required for efficient levels of pilin Av, we determined whether these domains facilitate the interaction of RecQ helicase with the *pilE* G4.

MATERIALS AND METHODS

Bacterial growth conditions. *E. coli* One Shot TOP10 competent cells (Invitrogen) were grown in Luria-Bertani (LB) broth or on solid medium containing 15 g/liter agar at 37°C and used to propagate plasmids. *E. coli* strains with plasmids containing kanamycin or erythromycin resistance were selected on media containing 50 or 100 μg/ml of the respective antibiotic. Gonococcal strains were grown on GC Medium Base (Difco) plus Kellogg supplements (GCB) [22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM $Fe(NO_3)_3$; all from Sigma] at 37°C in 5% CO_2; when applicable, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction. Gonococcal transformants were selected on media containing 80 μg/ml kanamycin, 10 μg/ml erythromycin, or 2 μg/ml tetracycline.

Construction of strains. Gonococcal strains VD300, PK133, PK134, PK131, and *recQ*::*ermC* (21) were transformed with chromosomal DNA from a strain containing the *recA6* construct, where *recA* is IPTG inducible and linked to tetracycline resistance (*recA6*) (31). The VD300 *recA6* strain was transformed with a plasmid containing a kanamycin cassette insertion within repeat sequence 1 (RS1) that has no effect on pilin Av (*pilE*::mTn#9) (16), which links a specific *pilE* sequence to kanamycin resistance. Then PK133, PK134, PK131, and *recQ*::*ermC* were transformed with chromosomal DNA from VD300 *pilE*::mTn#9 *recA6* selecting for kanamycin resistance and the presence of specific RecQ mutations, and matching *pilE* genes for all strains were verified by sequence analysis.

Pilin Av assays. Kinetic pilus-dependent colony phase variation assays were performed as previously described (13). The pilin Av sequencing assay (15) was performed by selection and disruption of P+ progenitors in GCB liquid medium (GCBL), which were spread on solid medium with IPTG for RecA induction. After 22 h, the *pilE* genes from 95 piliated colonies from each progenitor were sequenced. The parental strain had 2 progenitors, while the *recQ*::*ermC* mutant and the RecQ mutant with deletion of HRDC 2 and 3 (RecQ HRDC Δ2&3) had 3 progenitors.

Circular dichroism (CD) spectroscopy. CD spectra were recorded at the Northwestern University Keck Biophysics Core Facility on a Jasco-815 CD spectrometer. Spectra were recorded at room temperature in a 1-mm-path-length quartz cuvette as an average of 3 scans over 200 to 400 nm, with a scan rate of 100 nm m^{-1}, a bandwidth of 2.0 nm, and a response time of 2 s. The spectra were normalized by subtraction scan obtained with a buffer solution. Oligonucleotides were measured at a concentration of 2 μM under G4-forming conditions (100 mM KCl and 50 mM Tris [pH 7.5]) or ssDNA conditions (50 mM Tris, pH 7.5).

Fluorescence anisotropy. Protein purification was performed as previously described (21). Concentrated protein stocks of RecQ or RecQ HRDC Δ2&3 mutant in storage buffer were serially diluted in DNA binding buffer (20 mM Tris [pH 8], 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 g/liter bovine serum albumin [BSA], 4% glycerol) and incubated with 10 nM 3′ 6-carboxyfluorescein (FAM)-*pilE* G4 (ssDNA or structured) at room temperature for 30 min. Then, the fluorescence anisotropy for each sample was measured on a Molecular Devices SpectroMax M5 plate reader at 490-nm excitation and 535-nm emission wavelengths. Apparent K_d (dissociation constant), average, and standard error values were calculated using Origin software. Briefly, a sigmoidal fit was found using the equation $y = A_2 + (A_1 - A_2)/[1 + (x/x_0)^p]$, where A_1 is the initial value, A_2 the final value, x_0 is the center, and p is the power.

***pilE* G4 unwinding.** The RecQ or RecQ HRDC Δ2&3 mutant proteins were preincubated with the 5′FAM-3′ Black Hole Quencher (BHQ)-labeled *pilE* G4 structure for 20 min at a 1:1 ratio in unwinding buffer (25 mM Tris [pH 7.5], 50 mM NaCl, 3 mM $MgCl_2$, 0.1 mM dithiothreitol [DTT]). Then, fluorescence intensity was measured over time immediately after the addition of 1 mM ATP. Unwinding efficiency and standard error values were calculated using Origin software, and a sigmoidal fit was determined as described above.

RESULTS

Pilin antigenic variation and the *N. gonorrhoeae* RecQ helicase. During pilin Av, nonpiliated (P−) colonies can arise from piliated (P+) progenitors from a variety of mechanisms such as the introduction of a stop codon or a *pilE* sequence that interferes with pilus assembly (32, 33). Previously, deletion of *N. gonorrhoeae* HRDC domains 2 and 3 or 1 to 3 was shown to decrease the percentage of P− colonies that arose from a P+ progenitor, indicating that these domains are required for pilin Av (21). In contrast, deletion of *N. gonorrhoeae* HRDC domain 3 had no effect on the frequency of pilin Av (21).

Since the starting *pilE* sequence can influence the frequency of pilin Av (34) and there is some variability in measuring P− colonies that arise from P+ progenitors, we constructed the previously reported RecQ HRDC mutant strains (Fig. 2A) but made sure that they all had the same *pilE* sequence, and these assays were per-

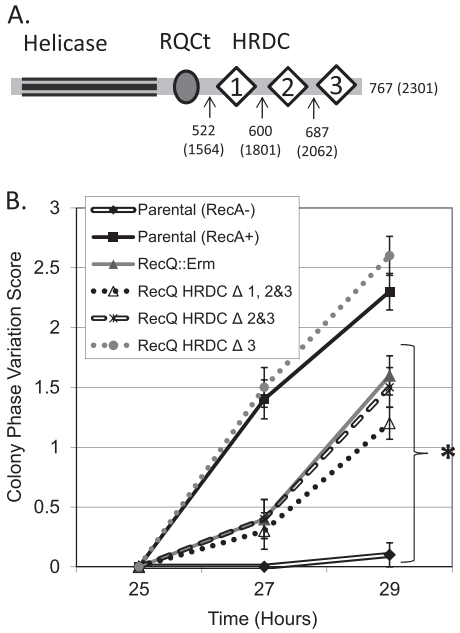

FIG 2 *N. gonorrhoeae* RecQ HRDC domains and pilin antigenic variation. (A) Schematic of the *N. gonorrhoeae* RecQ helicase, in which stop codon insertions are indicated by arrows. Residues are numbered, with the corresponding nucleotide positions in parentheses. (B) Kinetic pilus-dependent colony phase variation assay of the RecQ deletion and HRDC mutants. This standard assay measures the average number of visible pilus-dependent colony morphology changes occurring over time and is a surrogate measure for antigenic variation (Av) (13). RecQ HRDC Δ3 has no pilin Av defect, whereas RecQ HRDC Δ1, 2&3 and HRDC Δ 2&3 show a significant decrease in pilin Av, similar to that of the RecQ null strain. *, $P < 0.05$ as determined by two-tailed Student's *t* test. Error bars represent the standard errors of the means for 10 colonies; shown is a representative of 5 replicates.

formed in a *recA6* genetic background, where *recA* is under the control of IPTG, so that the *pilE* sequence was stable until RecA induction (31, 35). None of the *recQ* mutant strains showed a difference in growth rate, which can influence the frequency of pilin Av (data not shown). The kinetic pilus-dependent colony phase variation assay was used as a surrogate measure for pilin Av in the *recQ* mutants (13) (Fig. 2B). In this assay, we measured P− sectors arising from individual P+ colonies over time after RecA induction. The RecQ HRDC Δ3 mutant had no pilin Av defect, whereas the RecQ HRDC Δ2&3 or RecQ HRDC Δ1, 2&3 mutants both showed an Av defect similar to that seen in the *recQ* null strain (Fig. 2B). These results are consistent with the previously reported phenotypes for these strains (21). Next, we directly determined the frequency of pilin Av by DNA sequencing (15). The *pilE* gene was sequenced from 95 colonies arising from two or three progenitor colonies grown on media containing IPTG. We analyzed two progenitors from the parental strain and three progenitors for the RecQ null and RecQ HRDC Δ2&3 mutant. The frequency of pilin Av for the parental strain was 11.6%, while the RecQ null and the RecQ HRDC Δ2&3 mutants showed decreased frequencies of 0.7% and 2.1%, respectively (Table 1). These results suggest that RecQ HRDC domain 2 or the maintenance of two HRDC domains is required for pilin Av.

***N. gonorrhoeae* RecQ binding to the *pilE* G4 structure and sequence.** G4 structures have been implicated in many biological processes (36–38), and some RecQ helicase family members can process these structures (25–28). Since a RecQ deletion mutant causes an Av deficiency, we tested whether the *N. gonorrhoeae* RecQ helicase could bind the *pilE* G4 sequence or structure by fluorescence anisotropy. First, we determined that the *pilE* G4 3′FAM-labeled oligonucleotide formed the same parallel G4 structure as the unlabeled oligonucleotide (forming conditions) (Fig. 3A). The CD spectra confirm that the *pilE* G4 3′FAM-labeled oligonucleotide forms a parallel G4 structure upon addition of potassium (Fig. 3A), which is the same structure that forms when the oligonucleotide is unlabeled (16). Next, we compared the binding of gonococcal RecQ to the unfolded or folded oligonucleotide (*pilE* G4 structure) by measuring changes in fluorescence anisotropy (Fig. 3B). The apparent K_d of RecQ for the unfolded oligonucleotide was 25.7 ± 5.9 nM, whereas the apparent K_d of RecQ for the formed structure was 55.1 ± 10.5 nM. The apparent K_d of *N. gonorrhoeae* RecQ helicase for the *pilE* G4 structure is comparable to its affinity for duplex DNA, which has an apparent K_d of 58.2 ± 3.9 nM (21). These results confirm that the purified *N. gonorrhoeae* RecQ helicase can bind the G4 structure with relevant affinity to other known substrates.

We then determined that the apparent K_d of the RecQ HRDC Δ2&3 mutant for the single-stranded *pilE* G4 sequence was 46.7 ± 2.5 nM whereas the apparent K_d for the *pilE* G4 structure was

TABLE 1 Frequency of recombination for the parental, RecQ null mutant, and RecQ HRDC Δ2&3 mutant strains

Strain[a]	Progenitor[b]	No. of recombination events[c]/no. of sequenced colonies	Total no. of recombinants/ total no. sequenced	Frequency of recombination (%)
Parental	A	11/95	22/190	11.60
	B	11/95		
RecQ null mutant	A	1/95	2/285	0.70
	B	0/95		
	C	1/95		
RecQ HRDC Δ2&3 mutant	A	0/95	6/285	2.10
	B	5/95		
	C	1/95		

[a] All strains are derivatives of *N. gonorrhoeae* isolate VD300 carrying the IPTG-regulatable *recA* allele.
[b] Single-colony progenitors were grown with IPTG in the medium for 22 h (about 20 generations), the progeny were propagated without IPTG, and the *pilE* genes from the indicated number of progeny colonies were amplified and sequenced.
[c] Number of independent recombination events in the sequenced *pilE* genes. Multiple events in a single *pilE* are recorded as separate events.

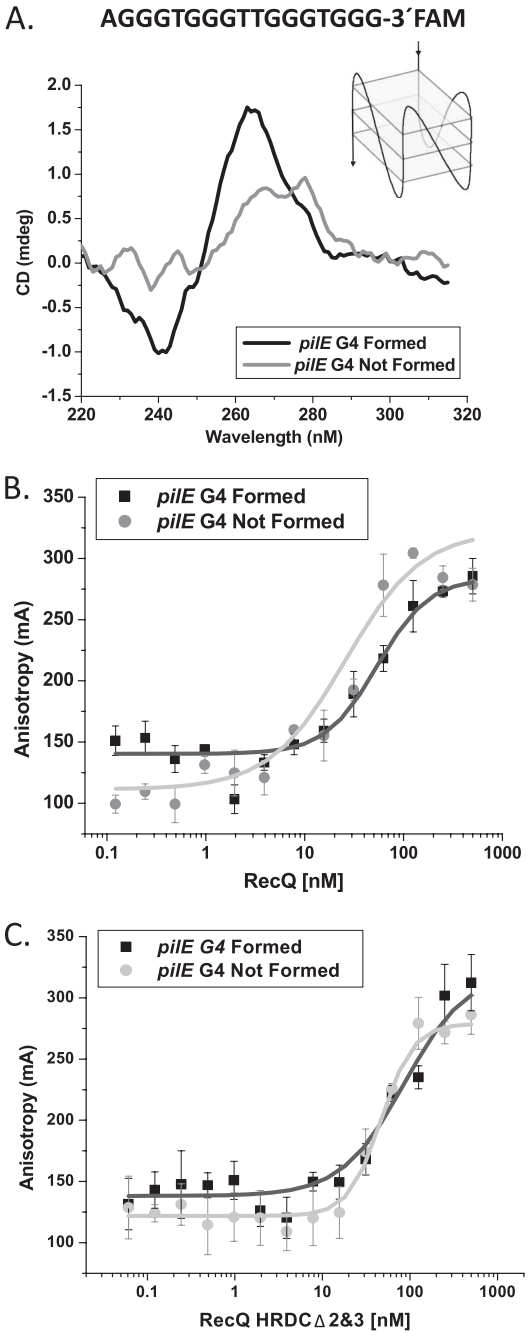

FIG 3 The RecQ HRDCΔ 2&3 mutant is defective for binding the *pilE* G4 structure and sequence. (A) CD spectroscopy of a 3′FAM-labeled *pilE* G4 oligonucleotide under conditions that allow or do not allow formation of the structure. The sequence of the oligonucleotide and a representation of the G4 structure are shown. (B) RecQ binding measured by fluorescence anisotropy of the *pilE* G4 3′FAM-labeled oligonucleotide. The fluorescence anisotropy is plotted against increasing protein concentration per 1 nM DNA; symbols represent RecQ binding to either a formed (black) or unformed (gray) *pilE* G4 3′FAM-labeled oligonucleotide. Error bars represent the standard errors of the means for 3 independent experiments with a total of 6 replicates and for 6 independent experiments with a total of 12 replicates for the formed and unformed oligonucleotides, respectively. (C) RecQ HRDC Δ2&3 binding measured by fluorescence anisotropy of the *pilE* G4 3′FAM-labeled oligonucleotide. The fluorescence anisotropy of RecQ HRDC Δ2&3 is plotted as described above. Error bars represent the standard errors of the means for 3 independent experiments with a total of 6 replicates for the formed and unformed oligonucleotides.

85.6 ± 33.4 nM (Fig. 3C). These results demonstrate that the RecQ HRDC Δ2&3 mutant is approximately 2 times less efficient at binding the *pilE* G4 sequence and structure. Therefore, the *N. gonorrhoeae* RecQ HRDC domains provide some specificity or modulate the affinity of the enzyme for the *pilE* G4 sequence and structure but do not differentially alter DNA recognition.

The *Neisseria gonorrhoeae* RecQ HRDC domains are required for efficient unwinding of the *pilE* guanine G4 structure. Since RecQ family members have been shown to unwind G4 structures (25–28), we tested whether the gonococcal RecQ and HRDC Δ2&3 mutant could also unwind the *pilE* G4 structure. To determine helicase unwinding efficiency, we used a *pilE* G4 oligonucleotide with a 5′FAM and a 3′BHQ (Fig. 4A). Upon G4 structure formation, the FAM and BHQ molecules are in closer proximity, which enables quenching of the fluorophore (Fig. 4B). As the structure is processed or unwound, the increase in fluorescence intensity can be measured over time to calculate the apparent percentage of processed or unwound DNA. The ability of the *pilE* G4 5′FAM-3′BHQ-labeled oligonucleotide to form a parallel G4 structure was confirmed by CD spectroscopy (Fig. 4C) (16). Incubation of the *pilE* G4 5′FAM-3′BHQ-labeled structure with RecQ at a 1:1 ratio without ATP allowed binding, and the change in fluorescence intensity over time after the addition of ATP was recorded as unwinding. It took RecQ 211.4 ± 12.9 s to unwind 50% of the *pilE* G4 5′FAM-3′BHQ-labeled structure (Fig. 4D). Previously, the RecQ HRDC Δ2&3 mutant was reported to have the same unwinding efficiency on duplex DNA as the wild-type enzyme and did not have an altered ATP hydrolysis rate (21). However, the RecQ HRDC Δ2&3 mutant had a defect in unwinding G4 DNA, taking 692.5 ± 45.7 s to unwind 50% of the *pilE* G4 5′FAM-3′BHQ-labeled structure (Fig. 4E). Therefore, the RecQ HRDC Δ2&3 mutant enzyme is 3.5 times less efficient at unwinding the *pilE* G4 than the wild-type enzyme. These data suggest that the phenotype of the RecQ Δ2&3 mutation on pilin Av may be due, at least in part, to its inability to efficiently process the *pilE* G4 structure in the chromosome.

DISCUSSION

The *Neisseria* RecQ helicase is unusual among RecQ family members, having three HRDC domains. Only the RecQ from one other organism, *D. radiodurans*, has a similar triple-HRDC arrangement. There are some parallels that can be drawn between pathogenic *Neisseria* and *D. radiodurans*. The pathogenic *Neisseria* strains exist only in humans, and therefore the *Neisseria* repair capabilities are predicted to be specialized for damage that might occur within the host. For example, the pathogenic *Neisseria* strains possess a diverse range of mechanisms for coping with the reactive oxygen species (ROS) that are encountered during aerobic respiration and interactions with phagocytic cells (39, 40), in addition to evasion of the human immune response by high-frequency recombination at *pilE* (15). *D. radiodurans* is an extremophile that can survive massive levels of DNA-damaging radiation. Both the pathogenic *Neisseria* strains and *D. radiodurans* have a high demand for DNA repair and recombination for survival, and therefore the maintenance of three RecQ HRDC domains suggests that they may utilize similar RecQ-dependent strategies of DNA recombinatorial repair. In addition, these organisms have G4 structures in their genomes (16, 41) suggesting that RecQ may process these structures.

In this work, we confirm that deleting HRDC domains 2 and 3

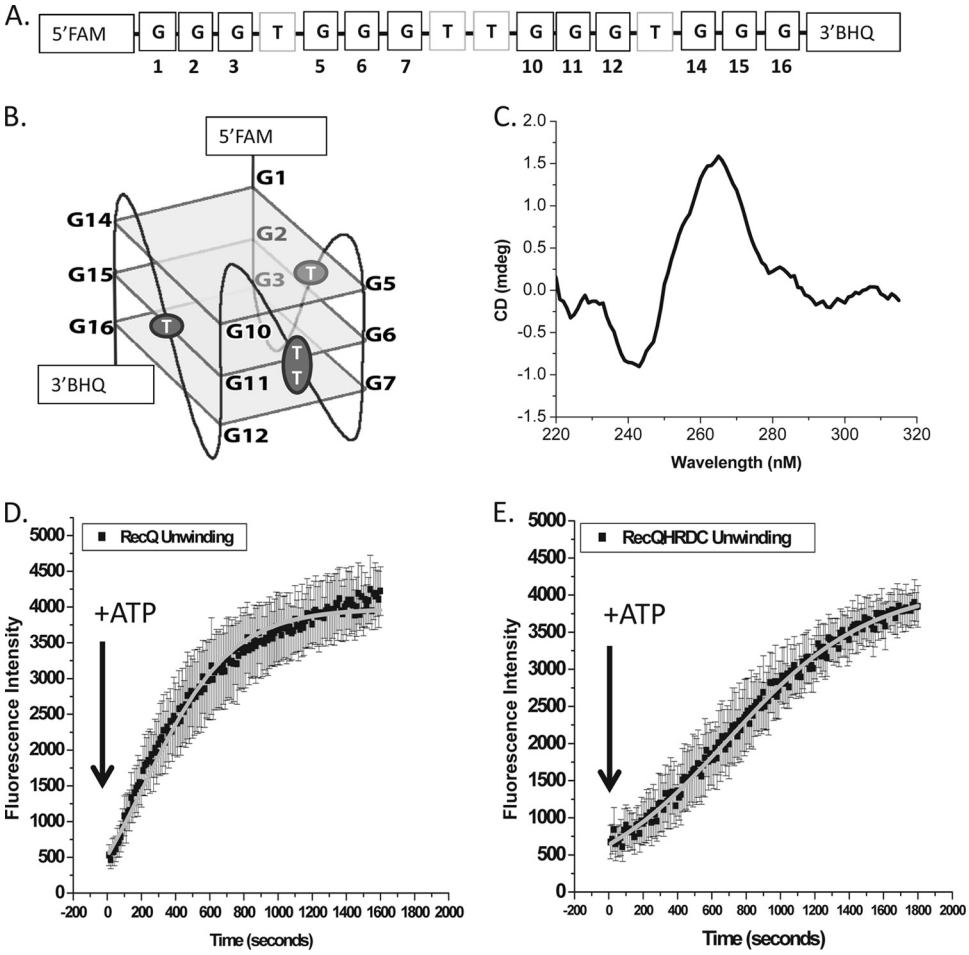

FIG 4 The RecQ HRDC Δ2&3 mutant is defective for unwinding the *pilE* G4 structure. (A) 5′FAM-3′Black Hole Quencher (BHQ)-labeled *pilE* G4 oligonucleotide. (B) *pilE* G4 structured oligonucleotide. The 5′FAM and 3′BHQ labels are indicated, where guanines are numbered and correspond to those shown in panel A. (C) CD spectrum of the 5′FAM-3′BHQ-labeled *pilE* G4 oligonucleotide under conditions that allow G4 formation. (D) RecQ unwinding measured by the change in fluorescence of the 5′FAM-3′BHQ-labeled *pilE* G4 oligonucleotide. Shown is the fluorescence intensity over time of a 1:1 ratio of RecQ to DNA immediately after the addition of ATP. Symbols represent the interaction of RecQ with the 5′FAM-3′BHQ-labeled *pilE* G4 oligonucleotide. Error bars represent the standard errors of the means for 3 independent experiments. (E) RecQ HRDC Δ2&3 unwinding measured by change in fluorescence of the 5′FAM-3′BHQ-labeled *pilE* G4 oligonucleotide. Shown is the fluorescence intensity over time of a 1:1 ratio of RecQ HRDC Δ2&3 to DNA immediately after the addition of ATP. Error bars represent the standard errors of the means for 3 independent experiments.

of *N. gonorrhoeae* RecQ helicase causes a decrease in the frequency of pilin Av that is comparable to that of functional knockout (Table 1). Only RecQ helicase family members that have an HRDC domain can unwind G4 structures (25–28), whereas RecQL1 and RecQL4, which do not have an HRDC domain, do not interact with G4 structures (29, 30). We determined that the *N. gonorrhoeae* RecQ helicase HRDC Δ2&3 mutant is deficient for binding and unwinding the *pilE* G4 structure required for pilin Av (16). Genetic evidence supports a model whereby the initiation of pilin Av occurs as a result of the formation of the *pilE* G4 structure (14, 16), which occurs upstream of RecQ helicase (42), and therefore RecQ may be required to unwind the structure, which is facilitated by its HRDC domains.

The RecQ HRDC Δ2&3 mutant's loss of binding to the *pilE* G4 may be a general loss of binding to all G4 structures. We tested RecQ binding to another G4 sequence found in the gonococcal chromosome that forms a parallel G4 structure *in vitro* and determined that the RecQ HRDC Δ2&3 mutant was 2 times less efficient at binding this structure (data not shown). These results are similar to those observed for the *pilE* G4 structure, which suggests that the RecQ HRDC domains provide some general specificity to G4 structures. Even though deletion of RecQ HRDC 2&3 decreased binding efficiency and unwinding, we did not observe any gonococcal growth defects, suggesting that deletion of the RecQ HRDC domains does not cause pleotropic effects in the cell (21), and the first RecQ HRDC domain may be sufficient to process other RecQ targets or other helicases may compensate for partial RecQ function.

Since the *pilE* G4 structure is located on the lagging stand during DNA replication (14, 16), we hypothesize that the structure stalls replication, causing a nick on the leading strand, which initiates pilin Av. The *pilE* G4 structure then remains on the lagging strand and must be unwound by RecQ helicase lest the structure halt replication, which would be detrimental to the cell. Other helicase families, such as Pif1, FANCJ (nematode DOG-1), and DNA2, have also been shown to unwind G4 structures (43–45). *N.*

gonorrhoeae does not encode a Pif1 helicase. However, the pathogen does encode a DinG helicase that shares a domain with DOG-1. Previously, we created a functional deletion of the *N. gonorrhoeae* DinG helicase, but the mutant did not have a pilin Av defect (data not shown). Although we cannot rule out a role for DinG unwinding G4 structures in the gonococcal cell, the helicase does not function during pilin Av and must not act on the *pilE* G4. *N. gonorrhoeae* also encodes UvrD and Rep helicases, which share a domain with DNA2 helicase. UvrD does not function during pilin Av (46); however, Rep does have a role (47). Further investigation will determine whether Rep helicase unwinds the *pilE* G4 structure. Since pilin Av is not completely blocked upon RecQ deletion, it is possible that there are redundant helicases such as Rep or an as-yet-unidentified helicase that may act at the *pilE* structure.

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