



Analysis of Pilin Antigenic Variation in *Neisseria meningitidis* by Next-Generation Sequencing

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ABSTRACT Many pathogenic microbes evade host immune surveillance by varying the surface antigens, a process termed antigenic variation. While the process of pilin antigenic variation has been extensively studied in the human pathogen *Neisseria gonorrhoeae* (gonococcus [Gc]), relatively few studies of pilin antigenic variation have been conducted with *Neisseria meningitidis* (meningococcus [Mc]). Mc is usually a commensal organism that colonizes the human nasopharynx, but when it translocates to the bloodstream or meninges, it results in the severe and often deadly meningococcal disease. The type IV pili of Mc isolates play a critical role in host surface adherence, and its major pilin component (PilE) can undergo antigenic variation. In this study, Roche 454 pyrosequencing was used to examine the pilin antigenic variation of Mc strain 8013, as well as 8013 *recA*, *recX*, *recQ*, *rep*, and *recJ* mutants, Gc orthologues which have been shown to play a role in pilin antigenic variation. This study confirms that the Mc *recA*, *rep*, and *recJ* genes are essential for pilin antigenic variation. While the Mc *recQ* and *recX* gene products contribute to normal frequencies of antigenic variation, the loss of these factors does not alter the types of pilin variants produced. Overall, this study shows that the mechanisms of pilin antigenic variation are conserved between Gc and Mc.

IMPORTANCE Antigenic variation is a strategy used by many pathogens to escape host immune surveillance and establish persistent infections. This study successfully applies next-generation sequencing to study pilin antigenic variation in the human pathogen *Neisseria meningitidis*. This assay provides an affordable and efficient solution for quantifying antigenic variation frequency in mutant strains and for defining the recombination products of the process. We determined that there is a nonuniformity of silent donor copies used during meningococcus antigenic variation, and by the analysis of selected mutants deficient for specific recombination pathways, we show for the first time that the processes are conserved between *N. meningitidis* and *Neisseria gonorrhoeae*.

KEYWORDS pilus, gene conversion, antigenic variation, 454 sequencing, diversity generation

Many viruses, bacteria, and parasites can alter the expression or composition of their surface-exposed proteins to escape host immune surveillance, a process defined as antigenic variation (1). Antigenic variation is one of the most prevalent strategies that pathogens employ to establish persistent infections in immunocompetent hosts (2). A common mechanism of diversity generation is gene conversion, which involves a unidirectional recombination event that incorporates donor sequences into a homologous locus, while the donor sequence remains unchanged (3).

One of the best-studied antigenic variation systems is the pilin antigenic variation system of the pathogenic *Neisseria*, *Neisseria gonorrhoeae* (gonococcus [Gc]) and *Neisseria meningitidis* (meningococcus [Mc]) (4). Gc is an obligate human pathogen that

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colonizes the urogenital tract and is the sole causative agent of the sexually transmitted infection, gonorrhea. Mc is an obligate human resident that usually colonizes the nasopharynx asymptotically but occasionally causes life-threatening meningitis and sepsis. The emerging antibiotic resistance in Gc and the lethality of disseminated Mc infection make them serious public health threats (5).

Type IV pili (Tfp) are crucial virulence factors for pathogenic *Neisseria* and are important for adhesion, twitching motility, resistance to neutrophil killing, and DNA transformation (3, 6–8). Tfp are filamentous appendages expressed on the bacterial surface that are composed of thousands of copies of PilE subunits encoded by the *pilE* gene. Pilin antigenic variation occurs when variant DNA sequences from a silent *pilS* copy transfer to the expressed *pilE* locus, generating a pilin variant (9, 10). Gc has one expressed *pilE* gene and up to 19 silent copies distributed in four or five silent loci across the chromosome. Most Mc isolates have fewer of these *pilS* copies compared to Gc isolates, usually, four to eight copies contained in one *pilS* locus (11). These *pilS* copies lack a promoter and ribosome binding site, serving as reservoirs for the genetic information used for pilin antigenic variation (4). A subset of Mc isolates do not undergo pilin antigenic variation but instead have *pilE* genes that are more similar to those of commensal *Neisseria* (10, 11) and have altered glycosylation (12).

Many different approaches have been applied to study pilin antigenic variation in Gc isolates. One common approach used to estimate pilin antigenic variation in Gc isolates is the pilus-dependent colony morphology change (PDCMC) assay (13, 14). Some Gc pilin antigenic variation variants produce a nonpiliated (P^-) colony phenotype due to poor expression of the variant or loss of the pilin protein (13, 15). In a Gc pilated (P^+) colony, P^- blebs emerge at the edges of P^+ colonies over time, and their appearance can be used as a reproducible measure of pilin antigenic variation frequency (13). As long as the starting *pilE* sequences and the growth rates between the strains are the same, the frequencies of pilin antigenic variation can be compared between isogenic strains (4). In contrast, PDCMC cannot be used to estimate Mc pilin antigenic variation, as no P^- blebs are observed arising in Mc colonies from pilin antigenic variation for unknown reasons. Traditional DNA sequencing has also been used to quantify the frequency and rate of Gc and Mc pilin antigenic variation (10, 16). Generally, these studies have utilized an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *recA* allele that was engineered at the endogenous *recA* to control when pilin antigenic variation can occur. To analyze pilin variation, individual colonies are grown on solid medium with IPTG to allow for pilin antigenic variation, and individual progeny from those progenitors are subsequently grown on solid medium without IPTG to lock in the resultant changes in the *pilE* genes of individual progeny. Sequencing of *pilE* in progeny colonies provides a measure of the relative level of antigenic variation and the types of recombinants produced (16). The traditional sequencing assay has the advantage of analyzing P^+ or P^- variants, but the assay is labor intensive and expensive and is not sensitive enough to measure pilin variation in mutants with a reduced frequency. Therefore, next-generation sequencing technology (NGS) has been applied to measure the frequency of pilin antigenic variation in populations of Gc and Mc isolates (17, 18).

Gc pilin antigenic variation is mediated via a RecF-like homologous recombination pathway, which can repair single-strand gaps or double-strand breaks (19). Proteins that affect the frequency of Gc pilin antigenic variation include enzymes involved in general DNA recombination or DNA repair pathways. RecA, a recombinase that generally mediates strand exchange, coprotease activity, and G4 structure binding, is required for pilin antigenic variation (20). RecOR is a recombinase that assists in RecA-mediated strand exchange when gapped substrates are used (21) and is also essential for pilin antigenic variation (13, 19). Other factors involved in pilin antigenic variation include RecX, RdgC, and RecG. RecG is a helicase that processes Holliday junctions. RecX has been shown to antagonize RecA and mediates efficient recombination. RdgC has been shown to bind DNA and inhibit RecA strand exchange (13, 14, 19, 22–25). While RuvA, RuvB, and RuvC were reported to be involved in mediating Gc pilin antigenic variation, we have shown that this phenotype was due to the slower

growth of mutants and not a direct reduction in pilin antigenic variation (18). In this study, we tested whether the pathways used by Mc isolates were the same as by Gc by analyzing pilin antigenic variation in Mc strain 8013 *recA*, *recX*, *rep*, *recJ*, and *recQ* loss-of-function mutants.

An analysis of the *pilE* variants produced in Mc8013 showed that mutations that lowered the frequency (*recX* and *recQ*) produced similar patterns of variants to the parental strain. These analyses also show that silent *pilS* copy 3 is the dominant donor sequence and silent copy 5 is used infrequently. This work shows for the first time that Gc and Mc isolates use similar recombination pathways for pilin antigenic variation and demonstrates the utility of NGS to analyze Mc pilin antigenic variation.

RESULTS

Phenotypic analysis of Mc8013 *recA*, *recJ*, *rep*, *recX*, and *recQ* mutants. We picked the serogroup C strain Mc8013 for this study as it has been sequenced, it is relatively easy to transform with Gc DNA, and our group has been vaccinated against serogroup C Mc. To test whether Mc uses similar recombination pathways as Gc for pilin antigenic variation, the orthologues of *recA*, *recJ*, *rep*, *recX*, and *recQ* were identified in Mc8013 via NCBI blast, and the genes with the highest homology were selected for further analysis. Mc8013 genes NMV_0941, NMV_0907, NMV_0300, NMV_1567, and NMV_0939 each share more than 95% protein sequence identity with the corresponding Gc genes and were designated Mc *recA*, *recX*, *recQ*, *recJ*, and *rep*, respectively.

To study the function of Mc *recA*, *recX*, *recQ*, *recJ*, and *rep* genes, loss-of-function mutants were constructed in strain Mc8013 *recA6*. DNA transformation assays using spectinomycin (Spc)-resistant chromosomal DNA revealed that the *recX*, *recJ*, *recQ*, and *rep* mutants exhibited levels of DNA transformation efficiency similar to that of the parental strain, while the *recA* mutant exhibited an approximately 100-fold decrease in DNA transformation efficiency (Fig. 1A). The few CFU detected in the *recA* mutant are likely due to the spontaneous antibiotic resistance, as no DNA control for this mutant showed a similar background level (data not shown). The UV survival assay was performed to measure the DNA repair capabilities of the mutants. The Mc *recA* and *recJ* mutants were the most sensitive to the UV exposure, while the *recQ* mutant had an intermediate phenotype (Fig. 1B). The *recX* and *rep* mutants, on the other hand, exhibited wild-type level sensitivities to UV treatment (Fig. 1B). The nalidixic acid (Nal) survival assay was also performed to measure the sensitivity of the mutants to double-strand breaks induced by the inhibition of gyrase (22). The *recJ*, *rep*, and *recA* mutants showed decreased survival to Nal. The *recX* and *recQ* mutants exhibited slightly reduced survival to Nal exposure that was not statistically significant (Fig. 1C).

Growth of the strains for analysis of pilin antigenic variation. As the growth rates might affect the frequency of pilin antigenic variation, a time course was conducted measuring CFU per colonies of the Mc8013 mutants growing on solid medium. For the Mc8013 parent strain, *recQ*, *recX*, *rep*, and *recJ* mutants, IPTG was added to the solid medium to induce the expression of *recA*. For the *recA*-deficient strain, no IPTG was added to the solid medium (26). Each Mc strain was grown to 1×10^7 CFU/colony on solid medium for the following times: Mc8013 parent strain, 11 h; *recA* mutant, 15 h; *recQ* mutant, 11 h; *recX* mutant, 13 h; *rep* mutant, 15 h; and *recJ* mutant, 13 h. For 1×10^8 CFU/colony, strains were grown for 17 h (Mc8013, *rep*) and 21 h (*recA*, *recQ*, *recX*, and *recJ* mutants).

Measuring the frequency of pilin antigenic variation by next-generation sequencing. Roche 454 pyrosequencing was used to analyze the frequency of *pilE* antigenic variation of strain Mc8013 and the *recJ*, *rep*, *recX*, and *recQ* mutants. The reason that 454 sequencing was chosen over other NGS approaches is that the optimal size range to obtain high-quality reads for 454 sequencing is 200 to 600 bp (it covers the length of the *pilE* PCR product), while the optimal size range for ion torrent sequencing and Illumina sequencing is significantly shorter. Mc8013 has one expressed *pilE* gene and five tandemly arranged variant silent copies upstream of *pilE* (Fig. 2). To amplify the majority of *pilE* variants, the forward PCR primer was designed to bind to

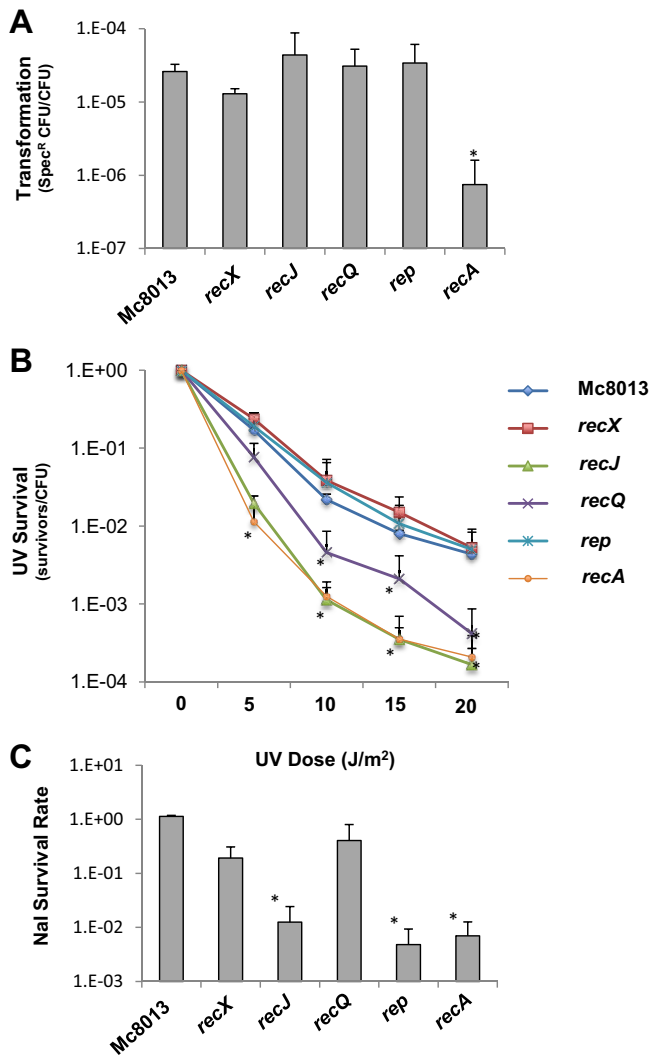


FIG 1 DNA transformation and DNA repair of Mc8013 mutants. (A) DNA transformation efficiency of Mc8013, *recX*, *recJ*, *recQ*, *rep*, and *recA* mutants. (B) UV survival rates of Mc8013, *recX*, *recJ*, *recQ*, *rep*, and *recA* mutants. (C) Nalidixic acid survival rates of Mc8013, *recX*, *recJ*, *recQ*, *rep*, and *recA* mutants. Error bars represent the standard deviations from three independent experiments. *, $P < 0.05$ by the Student *t* test relative to parental Mc8013.

the conserved *pilE* region and the reverse primer was designed to bind to the sequence 33 bp after the stop codon of *pilE* (Fig. 2). Different multiplex identifiers (MIDs) were designed into the forward primers to identify each strain. The resultant PCR products had an appropriate length of 554 bp (including the primers), excluding any changes in length mediated by variation of the donor silent copy.

The 454 sequencing reads were analyzed to determine the pilin antigenic variation frequency for each strain. The sequence reads were first aligned to the *pilE* starting sequence by the software DNASTAR SeqMan_Ngen_12 module, and all *pilE* sequence variants were identified. While the inherent error rate of 454 pyrosequencing resulted in a low level of sequencing errors, including nucleotide mutations, deletions, and additions, *pilE* antigenic variants were identified when the altered sequence matched one or more silent copy sequence. The frequency of pilin antigenic variation was calculated by dividing the number of sequence variants by the total number of *pilE* sequences. The pilin antigenic variation frequency for the Mc8013 parent strain was 2.74% when grown on solid plates for 17 h (1×10^8 CFU per colony) and 1.39% when grown for 11 h (1×10^7 CFU per colony) (Table 1). In both cases, the antigenic variation

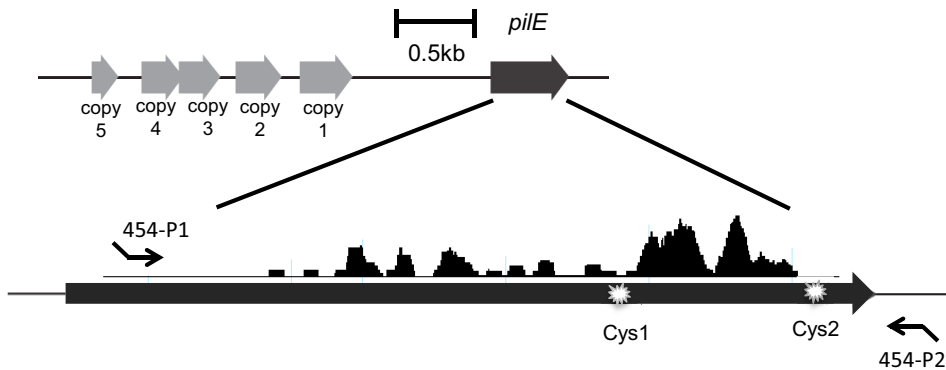


FIG 2 Gene organization of *pilE* and upstream silent copies in Mc8013. The five gray arrows indicate pilin silent copies 1 to 5. The black arrow represents the expressed *pilE* gene in Mc8013. The silent copies and *pilE* share significant DNA homology and their level of discrepancy is indicated by black peaks when aligned (generated by DNASTar SeqMan Pro). Cys1 and Cys2 represent the two conserved cysteine amino acids in the *pilE* gene. The binding sites of the 454 primers are represented by small black arrows, with the 454-P₁ primer binding to the conserved region of *pilE* and the 454-P₂ primer binding to the region 33 bp after the stop codon of *pilE*.

frequency of *recX* mutants was reduced to approximately 20% and the *recQ* mutant to approximately 4% of the parental strain, showing that as in Gc, these genes are not essential for the recombination process but aid in the efficiency. In contrast, the *recA*, *rep*, and *recJ* mutants were totally deficient in pilin antigenic variation (Table 1). These data confirm that the *recX* and *recQ* genes are important for Mc pilin antigenic variation, while *recA*, *rep*, and *recJ* genes are required for Mc pilin antigenic variation, which are similar phenotypes to the same mutants in Gc.

Determination of the donor silent-copy contribution to pilin antigenic variation. During Gc pilin antigenic variation, silent copies at the same or different loci are not equally incorporated into the expressed *pilE* gene (16, 27). We examined whether the use of silent-copy donors in Mc isolates is also nonrandom by analyzing the donor silent-copy profiles in Mc8013 pilin variants. We also tested if RecX or RecQ contributed to silent-copy incorporation in the mutant variants. Among the 132 *pilE* variants that were identified in the Mc8013 parent strain, silent copy 3 was the most frequent donor (63 variants), followed by silent copy 4 (24 variants), silent copy 2 (21 variants), silent copy 1 (14 variants), and silent copy 5 (1 variant) (Fig. 3). Silent copy 3 shares the longest region of continuous homology with the *pilE* sequence (~531 bp), while silent copy 5 is the shortest copy (~210 bp) (Fig. 4). Shared homology may contribute to the selection of silent copies as donors. Eight *pilE* variants were identified with a single nucleotide change that could have originated from different donor silent copies that we presume are minimal antigenic variation events (Fig. 3). One *pilE* variant was identified as a hybrid with donor sequences that originated from both silent copy 3 and copy 4 (Fig. 3). The silent-copy profiles were also analyzed in *recX* and *recQ* mutants, and while the numbers are small, a similar pattern of donor preference was also observed

TABLE 1 Pilin antigenic variation frequencies of Mc8013 strains

Growth (CFU/colony) ^a	Antigenic variation frequency (%) ^b of strain:					
	Mc8013	<i>recA</i>	<i>recX</i>	<i>recQ</i>	<i>rep</i>	<i>recJ</i>
10 ^{7c}	1.39	ND ^d	0.22	0.04	ND	ND
10 ^{8e}	2.74	0.02	0.54	0.12	0.01	0.03

^aStrains were grown on solid medium.

^bAntigenic variation frequency was calculated by the number of *pilE* variant reads divided by the total read number of each strain. All mutants were statistically different from Mc8013 parental strain ($P < 0.05$) using the two-tailed test of population proportion.

^cMc8013 and *recQ* strains were grown for 11 h, *recX* and *recJ* strains were grown for 13 h, and the *recA* and *rep* strains were grown for 15 h.

^dND, no variant reads detected.

^eMc8013 and *rep* strains were grown for 17 h, and *recA*, *recQ*, *recX*, and *recJ* strains were grown for 21 h.

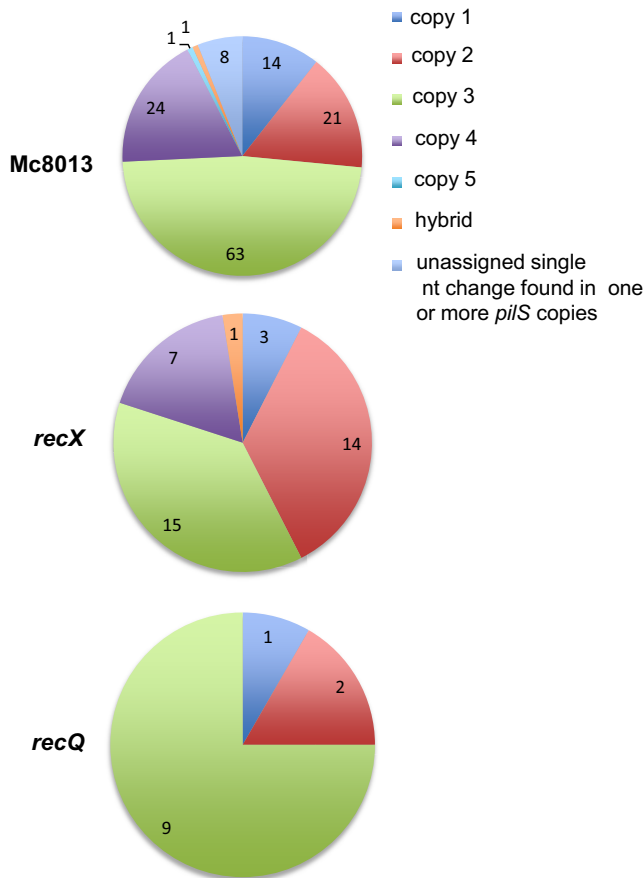


FIG 3 The profile of donor silent copies incorporated into the *pilE* gene. The pie chart represents the numbers of *pilE* variants with donor sequences from a specific silent copy, hybrid silent copies, or an unassigned single nucleotide change. The three pie charts show the donor silent copy profile for Mc8013, the *recX* mutant, and the *recQ* mutant.

in these mutants with reduced frequencies of variation (Fig. 3). These results are consistent with the conclusions that silent-copy incorporation into *pilE* is not random and that RecX and RecQ are not involved in the process of antigenic variation in Mc isolates.

To characterize the products of Mc pilin antigenic variation, each individual *pilE* recombination event from the Mc8013 parental strain was defined (Fig. 4). The number of times each specific recombination event was found and, for each recombination event, the flanking regions shared by *pilE* and the silent copy, as well as the region that recombined, were identified and mapped (Fig. 4). The most frequent antigenic variation event (26% of all variants) was a three-nucleotide (CCA) insertion into *pilE* with the donor sequence originating from silent copy 3 (Fig. 4, silent copy 3). Multiple recombination products were detected that received sequences from each of the five donor loci, showing that even with only five silent loci, the possible pilin diversity generated in Mc isolates is much more than just the number of donor silent copies. Interestingly, none of the recombination events introduced presumptive premature stop codons or a *pilE* truncation, as has been reported previously for Gc isolates (16, 18).

DISCUSSION

Pilin antigenic variation is an important strategy for pathogenic *Neisseria* species to escape host immune surveillance and alter the expression and possibly functions of the type IV pilus. While Gc pilin antigenic variation can be measured using the surrogate assay of colony phase variation (13), this approach is not applicable to Mc, as Mc isolates do not have distinguishable, pilus-dependent colony variation phenotypes.

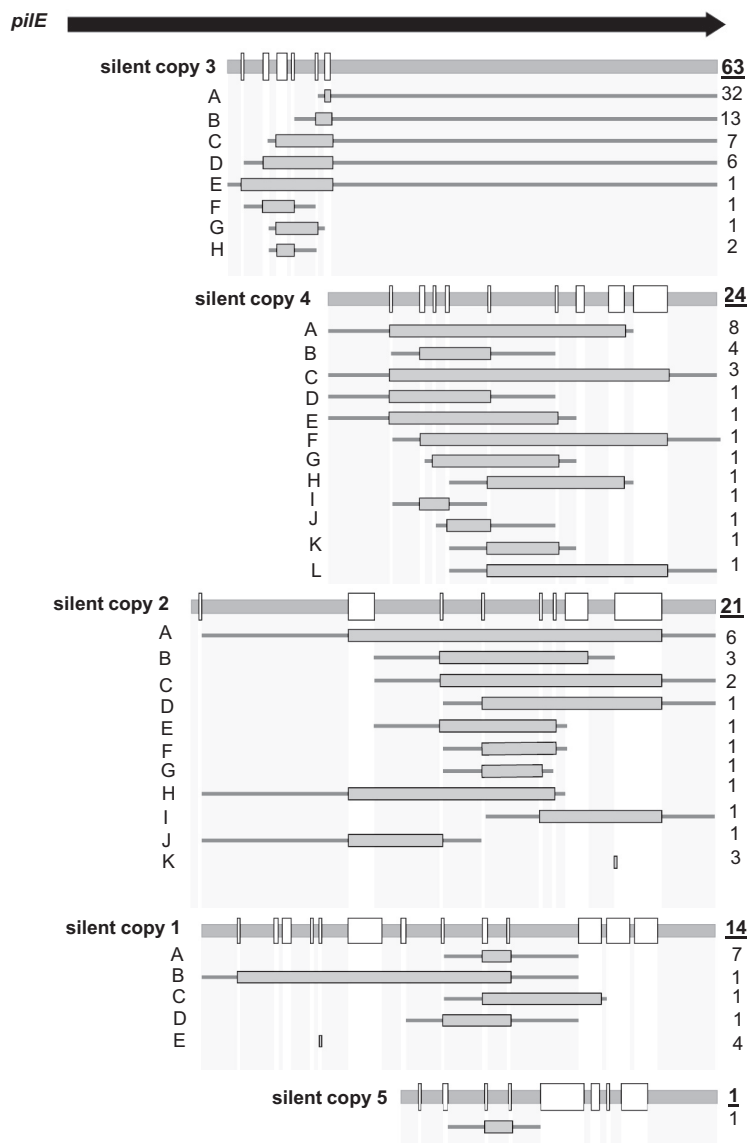


FIG 4 Detailed analysis of *pilE* variants in Mc8013. The *pilE* gene is represented by the arrow at the top. For each silent donor copy, the level of sequence conservation with the starting *pilE* sequence is shown with sequences that are identical with *pilE* by the thick gray horizontal line and sequences that differ between the *pilS* copy and *pilE* represented by white boxes. The frequency of each recombination event is indicated on the right of each cartoon where the underlined number on top indicates the total number of events using the same silent copy donor. For each recombination event, the frequency of individual recombination events is indicated on the right of each cartoon, consisting of a gray box that indicates the extent of the transferred silent copy sequence in *pilE* and thin gray lines that indicate flanking sequences shared by *pilE* and the silent copy for each product.

Traditional Sanger sequencing assays for Gc or Mc pilin antigenic variation involves the amplification of the *pilE* genes from hundreds of individual colonies (10, 28). This assay is labor intensive and not ideal for measuring Mc mutants with decreased pilin antigenic variation frequency. An alternate approach used Illumina sequencing to investigate the pilin antigenic variation in *Neisseria* species (17). However, due to the short reads of the Illumina platform, this approach does not identify individual *pilE* variants but only characterizes the overall population of the recombinants. Roche 454 targeted sequencing of the Mc *pilE* enabled us to identify the *pilE* variants through one strand sequence analysis that covered the entire *pilE* variable region. Moreover, the *pilE* PCR products of different strains were barcoded with unique MIDs and pooled in a

library, thus enabling the measurement of the pilin antigenic variation frequencies of multiple strains at the same time. This procedure enables the detection of Mc *pilE* variation when the frequency is lower than 1%. This study shows it is feasible to measure Mc pilin antigenic variation in a way that determines both the frequency and the characteristics of the recombination reactions. Unfortunately, Roche 454 sequencing technology is no longer being supported, and its availability as a sequencing platform is rapidly disappearing. To continue to use this type of deep sequencing, one would have to use Illumina sequencing, which does not provide individual variants (17), or adopt PacBio or Nanopore sequencing in a way that overcomes the intrinsic error rate of these platforms.

There are many factors that have been shown to be involved in Gc pilin variation, including RecA, RecX, RecJ, RecQ, Rep, RecO, and RecR (reviewed in references 4 and 25). Inactivation of the genes encoding these proteins resulted in different Gc frequencies of pilin antigenic variation. This study showed that the inactivation of *recA* lowered the transformation efficiency, highlighting a role for RecA in DNA uptake and incorporation. Mutations of *recA*, *recJ*, and *recQ* all lowered survival after UV radiation, implicating these genes in pyrimidine dimer repair. RecA, RecJ, and Rep are involved in double-strand break repair. This study confirms that the orthologues of *recA*, *recX*, *recJ*, *recQ*, and *rep* gene products also are involved in Mc pilin antigenic variation, showing that the mechanism is conserved between these related species.

During the course of pilin antigenic variation of *Neisseria* species, it is important to know whether each silent copy has an equal chance to be incorporated into the *pilE* gene. During pilin antigenic variation of Gc isolates, silent copies at the same or different loci are not equally incorporated into the expressed *pilE* gene (16). Our data show that the use of donor silent copies is also nonuniform in Mc strain 8013; in the variants analyzed, silent copy 3 was the dominant donor sequence, while silent copy 5 was represented the least. A further examination of silent copies revealed that silent copy 3 shares the longest homologous region with the parental *pilE* sequence (~531 bp on one side only), and silent copy 5 is the shortest silent copy (~210 bp) (Fig. 4). These factors might explain the frequent use of copy 3 as a donor and the low use of copy 5. However, stretches of sequence homology and the size of the silent copy do not correlate with the relative donor frequency of the other silent copies (Fig. 4). Neither relative homology nor size explains the nonuniform use of Gc silent donor copies (16). Therefore, there may be mechanisms of silent copy usage that pertain to specific silent copies and are not generalizable to other silent copies in Mc isolates. Taken together, our study successfully applied next-generation sequencing to investigate Mc pilin antigenic variation. It confirmed the critical roles of *recA*, *recX*, *recQ*, *recJ*, and *rep* in Mc pilin antigenic variation and revealed the nonuniformity of silent copy incorporation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains, plasmids, and oligonucleotides used in this study are described in Tables 2 and 3. *Escherichia coli* TOP10 competent cells (Invitrogen) were grown in Luria-Bertani (LB) broth or on LB solid medium containing 15 g/liter agar at 37°C and used to propagate plasmids. The Mc strains used in this study were primarily derivatives of Mc strain 8013 (Mc8013) (Table 2). All meningococcal strains used in this study contained the IPTG-inducible *recA6* allele that replaced the original *recA* gene to enable control of *pilE* antigenic variation (26). All Mc strains contained the same starting *pilE* sequence as the published Mc8013 *pilE* gene (identification [ID] [NMV_0019](#)).

Mc strains were grown on GC medium base (Difco) plus Kellogg supplements [22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM Fe(NO₃)₃; all from Sigma] (7) at 37°C with 5% CO₂ for 11 to 21 h. For the transformation assays, Mc strains were grown in Gc liquid medium (GCBL; 1.5% protease peptone no. 3 [Difco], 0.4% K₂HPO₄ [Fisher], 0.1% KH₂PO₄ [Fisher], 0.1% NaCl [Fisher]) with Kellogg supplements and 0.042% sodium bicarbonate (Sigma) at 37°C. Antibiotics were added at the following concentrations for Mc: kanamycin (Kan), 50 µg/ml; erythromycin (Erm), 2 µg/ml; tetracycline (Tet), 0.2 µg/ml; and spectinomycin (Spc), 50 µg/ml. For *E. coli*, the concentrations were Kan, 50 µg/ml; Erm, 250 µg/ml; and Tet, 20 µg/ml. IPTG (1 mM; Diagnostic Chemicals) was added to solid medium when necessary.

Construction of an IPTG-inducible *recA6* strain in Mc strain 8013. The native *recA* gene of Mc8013 was replaced with the Gc FA1090 *recA6* construct by transforming with Gc chromosomal DNA selecting for the Tet marker associated with the *recA6* allele (26). The *recA6* strains are RecA⁺ when IPTG is provided in the medium and are RecA deficient when grown without IPTG (26).

TABLE 2 Strain and plasmid descriptions and sources

Strain or plasmid	Description	Reference or source
<i>N. meningitidis</i> strains		
8013	Mc serogroup C parental strain	33
8013 <i>recA6</i>	Contains an IPTG-inducible <i>recA</i> gene	This study
8013 <i>recQ::erm recA6</i>	The <i>recQ</i> gene is replaced by the <i>erm</i> antibiotic resistant cassette	This study
8013 <i>recJ::kan recA6</i>	The <i>recJ</i> gene is replaced by the <i>kan</i> antibiotic resistant cassette	This study
8013 <i>recX::erm recA6</i>	The <i>recX</i> gene is replaced by the <i>erm</i> antibiotic resistant cassette	This study
8013 <i>rep::kan recA6</i>	The <i>rep</i> gene is replaced by the <i>kan</i> antibiotic resistant cassette	This study
8013 Sp ^r <i>recA6</i>	Spontaneous Spc-resistant mutant, used for DNA transformation assay	This study
Gc FA1090 <i>recQ::erm</i>	The Gc <i>recQ</i> gene is insertionally disrupted by Erm	14
Plasmids		
<i>precXKOerm</i>	Plasmid to knock out <i>recX</i> gene, Erm ^r	31
<i>precJ::Kan</i>	Plasmid to knock out <i>recJ</i> gene, Kan ^r	This study
<i>prep::Kan</i>	Plasmid to knock out <i>rep</i> gene, Kan ^r	This study
<i>precQ::erm</i>	Plasmid to knock out <i>recQ</i> gene, Erm ^r	This study

Insertional inactivation of *recJ*, *rep*, *recX*, and *recQ* in Mc8013 *recA6*. To mutate *recJ* and *rep*, a Kan cassette was used to replace the majority of each gene using the SOEing PCR approach (29). The primers listed in Table 3 and Phusion Hot Start Flex DNA polymerase (NEB) was used for all PCRs in this study; a standard PCR protocol was followed. Two sets of primers were used to amplify the regions adjacent to the genes from the Mc8013 chromosomal DNA template. Complementary primers were used to amplify the kanamycin cassette from plasmid pBSL86 (ATCC). The three PCR products were gel purified and used as the template in another PCR using the two flanking gene-specific primers, resulting in the Kan marker flanked by sequences adjacent to the deleted gene. The correct-sized PCR product was isolated and cloned into a pSMART vector (Lucigen). After confirmation by DNA sequence analysis, each mutation was transformed into Mc8013 *recA6* by liquid transformation as described previously (22), selected on medium containing Kan and verified by colony PCR and sequencing.

To mutate the Mc8013 *recQ* and *recX* genes, flanking primers (Table 3) were used to amplify the mutated gene from Gc FA1090 (*recQ::erm* [30] and *recX::erm* [31]). Each PCR product was cloned into a pSMART vector (Lucigen), and the proper construct was confirmed by DNA sequence analysis and transformed into Mc8013 *recA6* by liquid transformation as described previously (22). The final strain was selected on medium containing Erm and verified by colony PCR and sequencing.

Determination of growth on solid medium. Measurements of growth were performed on solid medium as previously described (22). Briefly, a piece of sterile filter paper was used to pick five colonies after growth on GCB agar containing IPTG for 11 to 21 h, the cells were released from the filter paper into GCBL by vortexing, and serial dilutions were plated to determine CFU/colony.

Liquid transformation assay. Mc transformation efficiency was measured as previously described (32). Briefly, Mc strains were grown for 18 h on GCB plates and resuspended in liquid transformation medium (LTM; 1 mM IPTG, 1.5% protease peptone no. 3 [Difco], 0.1% NaCl, 200 mM HEPES [Sigma], 5 mM MgSO₄, and Kellogg supplements I and II [pH 7.2]) to an optical density at 600 nm of approximately 1.5. Thirty microliters of the cell suspension was added to tubes containing 150 ng Spc^r Mc chromosomal DNA and 200 μl LTM. Following incubation at 37°C for 20 min, transformation solutions were added to prewarmed 2 ml LTM and incubated at 37°C in the presence of 5% CO₂ for 4 h. The mixtures were serially diluted 10-fold in GCBL, and 20-μl serial 10-fold dilutions were spotted on GCB plates in the presence and absence of Spc^r. The transformation efficiencies are reported as antibiotic-resistant CFU divided by total CFU and are the means from three replicates.

UV survival assay. UV survival assays were performed as described previously (22). Briefly, Mc bacterial lawns were grown for 24 h on GCB agar supplemented with IPTG when appropriate, collected with a Dacron swab, and resuspended in 1 ml of GCBL. The cells were serially diluted and spotted onto GCB agar with IPTG when appropriate. Plates were exposed to 0, 5, 10, 15, or 20 mJ/m² UV light (UV Stratalinker 1800; Stratagene) and incubated at 37°C in the presence of 5% CO₂ overnight. The UV survival rate was calculated by dividing the number of CFU in the irradiated sample by the number of CFU in the nonirradiated sample. The assay was performed in duplicates from at least three different experiments.

Nalidixic acid survival assay. Nal survival assays were performed as previously described (28). Bacterial lawns were grown for 18 h on GCB plates, swabbed, and resuspended in 1 ml of liquid medium. Cells were serially diluted and spotted onto GCB agar in the presence or absence of 0.5 μg/ml nalidixic acid (Nal). The survival rate was calculated by dividing the number of CFU surviving Nal treatment by the total number of CFU. The assay was performed in duplicates within at least three independent experiments.

Roche 454 pyrosequencing. Mc strains were grown on fresh GCB plates for 18 h. For each strain, one colony was picked with a sterilized filter disk and dispersed in 500 μl GCBL. A 1-μl culture was diluted in 500 μl GCBL, and 30-, 40-, and 50-μl cultures were spread on fresh GCB plates with or without IPTG. Bacteria were grown to a cell density of 1 × 10⁷ or 1 × 10⁸ CFU per colonies as indicated. Approximately 300 colonies per strain were collected in 1.5-ml GCBL by Dacron swabs. The QIAmp DNA minikit (Qiagen) was used to isolate genomic DNA, according to the manufacturer's instructions. The *pilE* region for each strain was amplified using Phusion Hot Start Flex DNA polymerase (NEB) and 10 ng genomic DNA with 454-P1 primer and 454-P2 primer (Table 3). The primer design was based on 454 sequencing application

TABLE 3 Oligonucleotide sequences and descriptions

Oligonucleotide name	Sequence ^a	Description
<i>ΔrecJ</i> -P1	GGTTCGTCGCTCAATCCTGAA	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecJ</i> -P2	GCAGGTCGACTCTAGAGGATCAACGTGGAACCTTACAGTGC GG GT	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecJ</i> -P3(Kan)	ACCCGCACTGTAAAGTTCCACGTTGATCCTCTAGAGTCGACCTGC	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecJ</i> -P4(Kan)	CGCTTCCCAGTAGTCGATATAAAGTCAGAAGAACTCGTCAAGAAGGCG	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecJ</i> -P5	CGCCTTCTTGACGAGTCTTCTGACTTTATATCGACTACTGGGAAGCG	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecJ</i> -P6	CCGACCACATAAGCCTGAAA	Primer to interrupt Nm8013 <i>recJ</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P1	CCGTGTTCCGGACGGTTATTA	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P2	GCAGGTCGACTCTAGAGGATCGTTTTGATGCCGTCTGAAATC	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P3(Kan)	GATTTTCAGACGGCATCAAACGATCCTCTAGAGTCGACCTGC	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P4(Kan)	CATTGCGGCTCCGGTTAATCTCAGAAGAACTCGTCAAGAAGGCG	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P5	CGCCTTCTTGACGAGTCTTCTGAGATTAACCGGAGCCGCAATG	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>Δrep</i> -P6	CGGCGAACTCTTAAACCATTTT	Primer to interrupt Nm8013 <i>rep</i> by replacing <i>recJ</i> with <i>kan</i>
<i>ΔrecQ</i> -P1	GGGACAATTGGAACGCTTTG	Forward primer to amplify the <i>recQ::erm</i> region from the FA1090 <i>recQ</i> mutant
<i>ΔrecQ</i> -P2	GCAACACCCAATCGTTCAAG	Reverse primer to amplify the <i>recQ::erm</i> region from the FA1090 <i>recQ</i> mutant
<i>ΔrecJ</i> -colony PCR1	ACCCGCACTGTAAAGTTCCACGTT	Forward primer to verify <i>recJ</i> interruption
<i>ΔrecJ</i> -colony PCR2	CGCTTCCCAGTAGTCGATATAAAG	Reverse primer to verify <i>recJ</i> interruption
<i>Δrep</i> -colony PCR1	GATTTTCAGACGGCATCAAAC	Forward primer to verify <i>rep</i> interruption
<i>Δrep</i> -colony PCR2	CATTGCGGCTCCGGTTAATC	Reverse primer to verify <i>rep</i> interruption
454-8013-P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGC GTTCGCCC TTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 parent strain grown on solid medium for 11 h
454-P2	CCTATCCCTGTGTGCCTTGGCAGTCTCAGCGTGAAAATCACTT ACCGC	Reverse primer to amplify the <i>pilE</i> region for 454 sequencing
454- <i>recA</i> -P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTATACATATCGC CCTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recA</i> mutant grown on solid medium for 15 h
454- <i>recX</i> -P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACTCGCCC TTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recX</i> mutant grown on solid medium for 13 h
454- <i>recQ</i> -P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGTGC CCTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recQ</i> mutant grown on solid medium for 11 h
454- <i>recJ</i> -P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCTCGCCC TTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recJ</i> mutant grown on solid medium for 13 h
454- <i>rep</i> -P1	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGAGTATGTCGC CCTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>rep</i> mutant grown on solid medium for 15 h
454-8013-P1-17h	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACGCTCTCGC CCTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 parent strain grown on solid media for 17 h
454- <i>recA</i> -P1-21h	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTCTATCTCGC CCTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recA</i> mutant grown on solid medium for 21 h
454- <i>recX</i> -P1-21h	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCATCTACTCGCC CTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recX</i> mutant grown on solid medium for 21 h
454- <i>recQ</i> -P1-21h	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTGTACTCTCGCCC TTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recQ</i> mutant grown on solid medium for 21 h
454- <i>recJ</i> -P1-21h	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGAGCGTCACTCGCC CTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>recJ</i> mutant grown on solid medium for 21 h
454- <i>rep</i> -P1-17h	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGACTAGCTCGCC CTTCTGCTTATCAA	Forward primer to amplify the <i>pilE</i> region for 454 sequencing, with MID for Nm8013 <i>rep</i> mutant grown on solid medium for 17 h

^aThe MID for each sample is underlined.

brief no. 001-2009. Each 454-P1 primer contains a multiplex identifier (MID) as a unique identifier for samples, and MIDs were designed according to 454 sequencing technical bulletin no. 005-2009. DNA was quantified with a Nanodrop (ND-1000) and by gel densitometry (Bio-Rad Quantity One software). The final genomic DNA concentration for each sample was adjusted to approximately 20 ng/μl, and 2 μl per sample was pooled as a sequencing library. The 454 pyrosequencing was performed by the Microbiome

core facility at the University of North Carolina. The library was sequenced on a Roche 454 Genome Sequencer FLX Titanium instrument using standard GS FLX Titanium XLR70 sequencing reagents and protocols. One-eighth of the plate was used for the sequencing, and 86,765 raw sequence reads were obtained.

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