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Role for the RecBCD Recombination Pathway for *pilE* Gene Variation in Repair-Proficient *Neisseria gonorrhoeae*▽

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The role of the RecBCD recombination pathway in PilE antigenic variation in *Neisseria gonorrhoeae* is contentious and appears to be strain dependent. In this study, *N. gonorrhoeae* strain MS11 *recB* mutants were assessed for recombination/repair. MS11 *recB* mutants were found to be highly susceptible to DNA treatments that caused double-chain breaks and were severely impaired for growth; *recB* growth suppressor mutants arose at high frequencies. When the recombination/repair capacity of strain MS11 was compared to that of strains FA1090 and P9, innate differences were observed between the strains, with FA1090 and P9 *rec*$^+$ bacteria presenting pronounced recombination/repair defects. Consequently, MS11 *recB* mutants present a more robust phenotype than the other strains that were tested. In addition, MS11 *recB* mutants are also shown to be defective for *pilE*/*pilS* recombination. Moreover, *pilE*/*pilS* recombination is shown to proceed with gonococci that carry inverted *pilE* loci. Consequently, a novel RecBCD-mediated double-chain-break repair model for PilE antigenic variation is proposed.

Neisseria gonorrhoeae causes the human mucosal infection gonorrhea. Following sexual transmission of the organism, gonococci bind to columnar epithelial cells utilizing proteins associated with the pilus organelle. The major protein component of the pilus structure is PilE polypeptide. PilE polypeptide undergoes antigenic variation such that the chemical composition of the protein changes and, in doing so, negates an efficacious immune response, thus promoting immune evasion (reviewed in reference 18).

PilE protein is expressed from a single *pilE* gene (1). However, the genome also contains many truncated, variant, nontranscribed copies of *pil* sequence which are located in several different loci (*pilS*) (7). Despite this apparent diversity, the genetic structures of all *pil* genes are very similar, with variable regions being interspersed with conserved DNA segments, with these conserved regions believed to facilitate recombination between *pilE* and a *pilS* gene copy, leading to variant *pilE* alleles (7). Changes in the *pilE* gene sequence occur primarily through RecA-mediated gene conversion events (7, 15, 25, 29) where a portion of the *pilS* gene copy is transferred into the *pilE* gene, with the resident *pilE* sequence being ejected or lost from the chromosome (7, 25). Consequently, this results in the expression of a unique PilE polypeptide.

Homologs of many of the *Escherichia coli* Rec proteins have been identified in *N. gonorrhoeae* (3, 8, 15, 17). Genetic analysis of *pilE* gene variation using *N. gonorrhoeae rec* mutants has proven to be controversial. In *N. gonorrhoeae* strain MS11, inactivation of the *recD* gene was shown to cause hyperrecombination at *pilE*, resulting in progeny with an increased number of nonparental pilus phenotypes (3). Consequently, this led to a proposal that *pilE* gene variation proceeded via a RecBCD-mediated pathway. However, in studies utilizing a different strain (FA1090), insertion mutations in either *recB* or *recC* did not effect gene variation at *pilE* and caused only a modest recombination/repair deficiency (17). Consequently, these observations indicated a significant difference between the proposed behavior of the *N. gonorrhoeae* RecBCD complex and the *E. coli* RecBCD enzyme. To account for these differences, it was proposed that a "RecF-like" pathway accounted for *pilE* gene variation and recombination/repair in strain FA1090, especially as gonococci possess several homologs to genes that are present in the *E. coli* RecF pathway, which include *recO*, *recR*, *recJ*, and *recQ* (8, 17). However, gonococci lack exonuclease I (*sbcB* and *-C*) as well as the *recF* gene, so the relationship of this pathway to the RecBCD pathway is currently unclear. Nonetheless, two studies have demonstrated that this "RecF-like" pathway participates in repairing UV-induced lesions (8, 23).

Although the effects of an insertion mutation in the *recB* gene of *N. gonorrhoeae* strain FA1090 have been documented (17), the effects of a null mutation in strain MS11 have yet to be investigated. The purpose of this study is to determine the effect of *recB* (and *recC*) mutations on recombination/repair in *N. gonorrhoeae* strain MS11. From the data presented, an MS11 *recB* (or *recC*) null mutation severely impedes repair of double-chain breaks caused by nalidixic acid treatment as well as the repair of DNA alkylation lesions caused by methyl methanesulfate (MMS) treatment. In addition to the robust recombination/repair phenotype exhibited by the MS11 *recB* mutants, we present evidence that indicates that *pilE* gene variation proceeds via a RecBCD-mediated pathway in strain MS11. Furthermore, as we are also able to demonstrate that inverting the *pilE* locus does not impede *pilE*/*pilS* recombination, these combined observations allow us to propose a double-chain-break repair model for *pilE* gene variation in strain MS11.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *Neisseria gonorrhoeae* strains MS11 (obtained from John Swanson, Rocky Mountain Laboratories, MT), FA1090

TABLE 1. Bacterial strains utilized in this study

Strain	Description	Source or reference
Escherichia coli		
DH5α	F φ*lac*Z Δ(*lacZYA argF*)U16 *deoR recA1 endA1 hsdR17*(r_κ^- m_κ^+)*phoA supE44* λ^-*thi-1 gyrA96 relA1*	Gibco BRL, Gaithersburg, MD
GY5873	Hfr *argA*::Tn*9 lacMS286* Φ80dII *lacBK1 recB21*	21
Neisseria gonorrhoeae		
MS11 *rec*$^+$	Wild type	J. Swanson
P9 *rec*$^+$	Wild type	J. Saunders
FA1090 *rec*$^+$	Wild type	F. Sparling
MS11 *recB*	*recB*::*ermC*	This study
MS11 *recB opaE*::*recB*$^+$	Wild type *recB* gene placed in *opaE* locus	This study
MS11 *recB sup*	Intragenic *recB* suppressor mutant	This study
FA1090 *recB*	*recB*::*ermC*	This study
P9 *recB*	*recB*::*kan*	This study
MS11 *pro*	ΔProline residue in *recB* active site and *ermC* cassette 3′ of *recB* gene	This study
MS11 *inv*	900-bp segment of the *pilE* locus in an inverse orientation	This study
MS11 *recC*	*recC*::*ermC* (or *kan*)	This study
FA1090 *recC*	*recC*::*ermC* (or *kan*)	This study
P9 *recC*	*recC*::*ermC* (or *kan*)	This study
MS11 Sma::Xho	XhoI linker placed in the SmaI site in the Sma/Cla repeat downstream of *pilE*	9
FA1090 Sma::Xho	XhoI linker placed in the SmaI site in the Sma/Cla repeat downstream of *pilE*	This study

(obtained from Fred Sparling, University of North Carolina), and P9 (obtained from John Saunders, University of Liverpool) were used in this study (Table 1). Strain MS11 has been passaged as a laboratory strain for many years; strains FA1090 and P9 were obtained in the mid 1980s and have undergone limited cultivation. During the course of these studies, gonococci were passaged daily on gonococcal (GC) typing medium (GTM) at 37°C in 5% CO_2 (24). Occasionally, gonococci were resuspended in GC HEPES medium, which is identical in composition to GTM (24) except that the phosphate salts are replaced by 0.2% HEPES, Na^+ salt (Calbiochem, La Jolla, CA), and 0.5% HEPES acid (Calbiochem, La Jolla, CA). Where appropriate, the medium was supplemented with antibiotics: 10 μg/ml of erythromycin or 80 μg/ml of kanamycin (Sigma, St. Louis, MO).

Escherichia coli was grown on Luria-Bertani medium at 37°C, with antibiotics added at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 200 μg/ml; and kanamycin, 40 μg/ml. The *lac* papillation assay was performed as described previously, using *E. coli* strain GY5873 (Table 1) and MacConkey lactose medium (21, 30).

DNA manipulations. All constructs were made in *E. coli* strain DH5α. *N. gonorrhoeae* strain MS11 chromosomal DNA was used as a PCR template to construct the MS11 *recB* mutants by amplifying two segments of the *recB* gene using primers recB4/B5 and recB9/recB10 (Table 2). The PCR products were then sequentially ligated into the pCRII vector (Invitrogen) with an erythromycin or kanamycin gene cassette inserted in the middle. The recombinant plasmid was used to transform *N. gonorrhoeae* to drug resistance to create a *recB* insertional mutant, with the mutation confirmed by PCR in which one of the primers was located within the drug resistance marker (Erm1.5Rev) (Table 2). *N. gonorrhoeae recC* mutants were constructed following PCR amplification of *recC* using primer pair RecC2 and RecC4 (Table 2). The resulting 1,834-bp fragment was cloned into the pCRII vector. The insert was sequenced and an erythromycin (or kanamycin) gene cassette being inserted into a unique HincII site. The recombinant plasmid was then used to transform strain MS11 to drug resistance. The mutation was confirmed by PCR in which one of the primer pairs was located within the drug resistance marker (Erm1.5Rev) (Table 2).

Site-specific mutagenesis (Stratagene) was used to delete the codon that specified the proline residue in the *recB* nuclease active site. A standard PCR was performed using primer pairs RecB12 (upstream primer) and Tracy1 (the deletion primer) (Table 2) with *Pfu Taq* polymerase (*Pfu* Ultra; Stratagene) to amplify the active-site DNA. A second PCR was performed using a primer complementary to the first deletion primer (Tracy2) and the downstream primer RecB6 (Table 2). These PCRs were purified, and a third PCR was run, with these template DNAs serving as primers because they are partially complementary. This final PCR product was cloned, and codon deletions were confirmed by DNA sequencing. A drug resistance marker was then inserted in a unique SalI site that is located downstream of the *recB* transcriptional unit. The codon deletion was crossed into the gonococcal chromosome by transformation. The incorporation of the codon deletion was confirmed by DNA sequencing of selected transformants.

The MS11 *recB* gene was cloned from a pBR322 plasmid library that was created using a Sau3A partial digest of gonococcal chromosomal DNA. Plasmids carrying the gonococcal *recB* gene were identified using *Neisseria*-specific *recB* oligonucleotides (Gc recB1 to -4) (Table 2).

The *pilE* gene was inverted by PCR of specific gene fragments incorporating unique restriction sites, using pVD203 (which carries the *pilE* gene, the downstream Sma/Cla repeat, and approximately 800 bp upstream of the *pilE* start

TABLE 2. Oligonucleotides utilized in this study

Designation	Nucleotide sequence
Cys2R	5′-GCAGGTGACGGCAGGTGC-3′
Erm1.5Rev	5′-TGTTAGCCAAAGCTTCCAAGC-3′
HVPilS(R)	5′-CGTTGTCGTTGCCGGCTTTGGT-3′
InterB6	5′-GCCTGAAGGTAATAGTGCTGGTGC GCGACGGC-3′
PilRBS	5′-GGCTTTCCCCTTTCAATTAGGAG-3′
RecC2	5′-GATTTGTTCCGCAGCGCACAA-3′
RecC4	5′-GGCAACGTATTGATGATTCG-3′
RecB4	5′-CCCAAAGCTTGCACTGCACAAATG GCTGCGCGATCAAATC-3′
RecB5	5′-CTAGTCTAGAGTGCGGGCAGGAA GGTGTCGCCTTCGT-3′
RecB6	5′-CTAGTCTAGAGGTGAGGGTGTGC GTGGTCATA-3′
RecB9	5′-CCCTCGAGGCTCAAGATTTTTGGC GGGAACG-3′
RecB10	5′-GGTCTAGACCCGAAGTCTGCCAGT TTCTGCAATTC-3′
RecB12	5′-AAAAGCTTGGCTGTGCCTGCACGA AATTCTTGAAGA-3′
Tracy1	5′-CGATATGGTCTGCCAAGACGACG GCAATATCTGC-3′
Tracy2	5′-GCAGATATTGCCGTCGTCTTGGCA GACCATATCG-3′
GC recB1	5′-AAACCGCAACCCCGCCGCA-3′
GC recB2	5′-GCGTGGGACGCGCAAGATACC-3′
GC recB3	5′-CGGCGGCGTTGCCAGCGT-3′
GC recB4	5′-GGTGCCGCCGTCCCGAA-3′
probe 245	5′-GCCTTTTTGAAGGGTATTCAT-3′

codon) (1) and pSX2.7 (which carries the *opaE* gene, which resides downstream of *pilE* in the gonococcal chromosome [2]) as templates. The PCR fragments were assembled sequentially through selective restriction digests. The *pilE* inversion included approximately 500 bp upstream of *pilE*, the *pilE* gene itself, and the downstream Sma/Cla repeat. An erythromycin gene cassette was inserted in the SalI site located in the *opaE* gene. The construct was crossed into the gonococcal chromosome by transformation. The structures of the final construct and of putative transformants were confirmed by DNA sequencing and PCR analysis.

Analysis of *N. gonorrhoeae* mutants. (i) Exposure to nalidixic acid. Gonococci were serially diluted in GC HEPES medium (pH 7.4) and were then plated on GTM containing 0.5 μg/ml nalidixic acid and were grown for 2 days. Survival rates were calculated by dividing the total number of colonies growing on the nalidixic acid-containing GTM by the total number of colonies growing on the GTM without antibiotic. The assays were performed concurrently.

(ii) Exposure to MMS. Gonococci were plated on GTM plates to a confluent density, with paper disks saturated with either 0.01%, 0.05%, 0.1%, 0.2%, or 0.3% freshly diluted MMS (Sigma) solution placed on top of the agar, and the plates were incubated overnight. Zones of inhibition identified the growth-inhibiting concentrations of MMS. The assays were performed concurrently.

(iii) Exposure to UV light. Gonococci were serially diluted to the appropriate cell density, plated on solid medium, and exposed to various doses of UV radiation (0, 20, 40, 60, and 80 mJ/cm^2) using a UV Stratalinker 1800. Survival rates were calculated by dividing the total number of visible colonies on an irradiated plate by the total number of visible colonies on the nonirradiated plate. The assays were performed concurrently.

PilE antigenic variation assays. Antigenic variation was determined by two methods. The first method, in which conversion of a *pilE* outside marker is assessed, has been previously described (9). A XhoI linker was inserted into the SmaI site of the Sma/Cla repeat that is located downstream of the *pilE* locus. Chromosomal DNA was purified from an overnight culture, digested with SmaI, and analyzed by Southern analysis using the *pilE*-specific probe 245 (Table 2). Conversion of Sma::Xho to the wild-type Sma configuration requires *pilE*/*pilS* recombination (9). The second method entailed reverse transcription-PCR (RT-PCR) on purified RNA samples, slightly modifying a previously described procedure (27). RT-PCRs were run at 60°C for 30 min using conserved *pilE* primer pairs (PilRBS and Cys2R) under conditions outlined by the manufacturer (Boehringer Mannheim). *Taq* polymerase (2.5 units) was then added, and PCR was performed for 30 cycles. The RT-PCR was then used as a template for a second PCR using *pilE* primers [PilRBS and HVPilS(R)]. A *pilE*/*pilS1* recombination event is required in order to obtain a product from the second PCR. The products were then analyzed by Southern analysis, using the HVPilS(R) primer as a probe.

RNA isolation. RNA was isolated for RT-PCR as described previously (10). The purified RNA was then incubated with 2 units of molecular biology grade DNase (United States Biochemical) for 10 min at 37°C. The DNase was inactivated by boiling it for 5 min.

Southern analysis. Southern blot analyses were performed as described previously, using radiolabeled oligonucleotides as probes (9, 25).

RESULTS

The *N. gonorrhoeae* RecB protein shares some similarity with its *E. coli* counterpart (33% identity and 49% similarity). A canonical ATPase domain (16/22 amino acid identity) is located toward the amino-terminal end of the protein, with the nuclease domain being located toward the carboxy-terminal end. A proline residue is located in the center of the nuclease active site (Fig. 1) (28). Given the disparity that has been observed between various gonococcal strains regarding RecBCD involvement in *pilE* gene variation (3, 17), we explored whether this was due to the presence of the proline residue in the nuclease active site causing a partial *recB* mutant phenotype in strain FA1090, with the defect in strain MS11 being alleviated through suppressor mutations. Site-directed mutagenesis was used to delete the codon that specified the proline residue in the nuclease active site to determine whether this proline residue influenced the functionality of the protein. No differences in the functionality of the proline-minus RecB protein were observed when these mutants were assessed in either strain MS11 or strain FA1090 (the data obtained with strain MS11 are presented in Fig. 2). The functionality of the MS11 *recB* gene was further confirmed by cloning *recB* from an MS11 plasmid library on an approximately 8-kb fragment and then utilizing this clone to complement an *E. coli recB21* mutant in a *lac* papillation assay; extensive *lac* papillation was apparent in *E. coli rec*$^+$ and in the *E. coli recB21* strain carrying the GC *recB* complementation plasmid and was absent in the *E. coli recB21* strain carrying the vector alone when the various bacteria were grown on solid medium (21, 30).

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RECB_NGON   IDMVCQDPDGNICIIDYKSNHLS(14)HYYLQALIYAVAAARY
RECB_NMEN   IDMVCQDPDGNICVIDYKSNHLS(18)HYYLQACIYAVASARY
RECB_MTB    IDVVLRLPGQRYLVVDYKTNHLG(18)DYPLQALLYVVVLHRY
RECB_ECOLI  IDLVFRH-EGRYYLLDYKSNWLG(18)RYDLQYQLYTLALHRY
RECB_HINF   IDLVFRH-NGKYYLVDYKSNFLG(18)HYDWQYLIYTLALHRY
RECB_BBURG  VDLIFKA-NNKIYILDYKTNYLG(18)YYDLQYKIYALGIKKI
RECB_CPNEU  IDLFFEH-EGKYYIIDWKTSFLG(18)KLDYQGRIYVKAVRKF
RECB_BSUB   IDCLYET-EDGLYLLDYKSDRIE(18)RYETQIQLYTKAVEQI
RECB_TPAL   IDLLFLS-NGVWHLVDYKTDYEE(13)RYLPQLQHYARAVQDL
              :* .         ::*:*:.          * . *
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FIG. 1. CLUSTALW alignment of the amino acids that constitute the RecB nuclease active site. Regions of identity are indicated by stars and regions of similarity by dots. The sequences are as follows: *N. gonorrhoeae* strain MS11, GeneID no. 3282190 (NGON); *Neisseria meningitidis* strain FAM18, GeneID no. 4675668 (NMEN); *Mycobacterium tuberculosis* strain CDC151, GeneID no. 925060 (MTB); *Escherichia coli* strain K-12, GeneID no. 947286 (ECOLI); *Haemophilus influenzae* strain RdKW20, GeneID no. 950246 (HINF); *Borrelia burgdorferi* strain B31, GeneID no. 1195485 (BBURG); and *Chlamydophila pneumoniae* strain J138, GeneID no. 919511 (CPNEU). Also included are the homologous AddA proteins previously identified from *Bacillus subtilis* strain 168, GeneID no. 939793 (BSUB), and from *Treponema pallidum* strain Nichols, GeneID no. 2611603 (TPAL) (27).

Analysis of *N. gonorrhoeae* strain MS11 *recB* mutants. *Neisseria gonorrhoeae recB* mutants yielded small colonies on solid growth medium (Fig. 2A). However, larger colonies appeared (with an estimated frequency of 1×10^{-3} per CFU) upon passaging the small *recB* mutants on solid medium containing no antibiotic (Fig. 2A). These suppressor mutants arose through excision of the drug resistance marker and a small segment of the *recB* gene apparently using small direct repeats that flanked the drug resistance cassette in the *recB* gene (data not shown). Besides identification of these intragenic suppressor mutations, wild-type growth could also be restored through extragenic suppressor mutations that abrogated natural competence for DNA transformation, as observed when *pilT* and *comA* mutants were established via DNA transformation of the *recB* mutants. The MS11 *recB* mutants were tested for their abilities to undergo recombinational repair of DNA damage. When double-strand breaks were introduced to the chromosome by exposure of the bacteria to nalidixic acid (6, 16), the decrease in the repair capacity of the small-colony *N. gonorrhoeae recB* mutant was seen to be comparable to that for *N. gonorrhoeae recA* mutants (Fig. 2B). In contrast, wild-type MS11 and the *recB* proline-minus mutant displayed little inhibition of growth, while the intragenic *recB* suppressor mutant showed a modest defect. Complementation of the *recB* mutation with a *recB*$^+$ allele restored wild-type growth and repair capabilities. Comparable observations were made following DNA alkylation by MMS treatment (Fig. 2C). Strain MS11 *N. gonorrhoeae recC* mutants displayed repair phenotypes similar to that of the *recB* mutant following nalidixic acid (data not

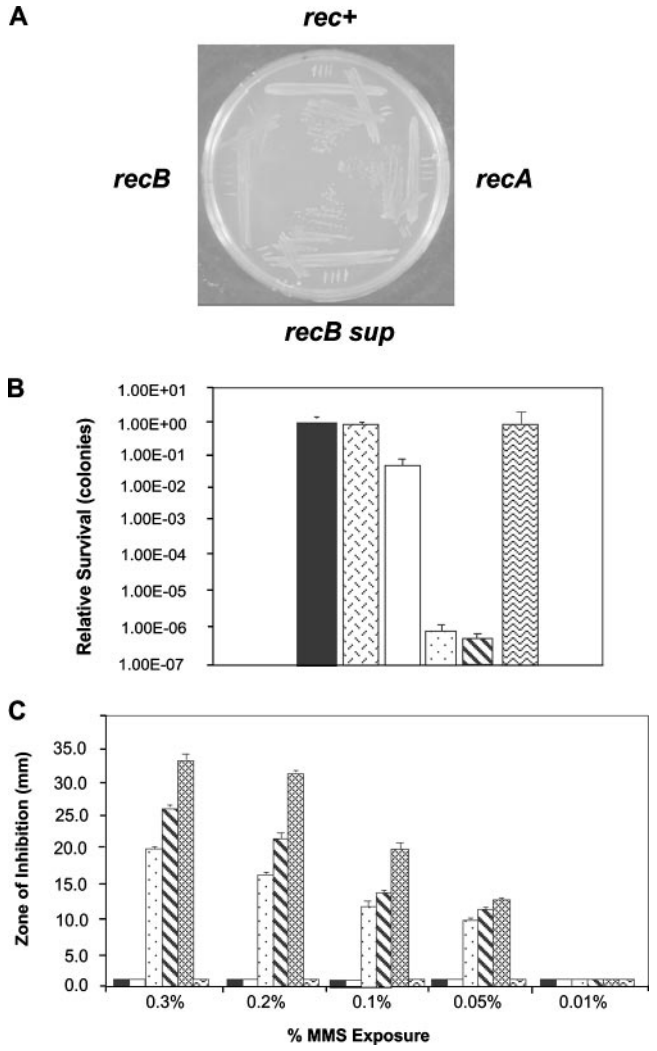

FIG. 2. Analysis of *N. gonorrhoeae* strain MS11 *recB* mutants. (A) Comparison of the growth characteristics of MS11 *rec*$^+$, *recB*, *recA*, and *recB* suppressor mutants. (B) Exposure to nalidixic acid (0.5 μg/ml). Solid bar, *rec*$^+$; hexed bar, proline minus mutant; clear bar, *recB* suppressor; stippled bar, *recB*; diagonally striped bar, *recA*; wavy bar, *recB opaE*::*recB*$^+$. Error bars indicate standard deviations from the mean ($n = 10$). (C) Exposure to MMS. Solid bar, *rec*$^+$; clear bar, *recB* suppressor; stippled bar, *recB*; diagonally striped bar, *recA*; shaded bar, *recC*; wavy bar, *recB opaE*::*recB*$^+$. Data represent two experiments performed in triplicate. Error bars represent standard errors ($n = 6$).

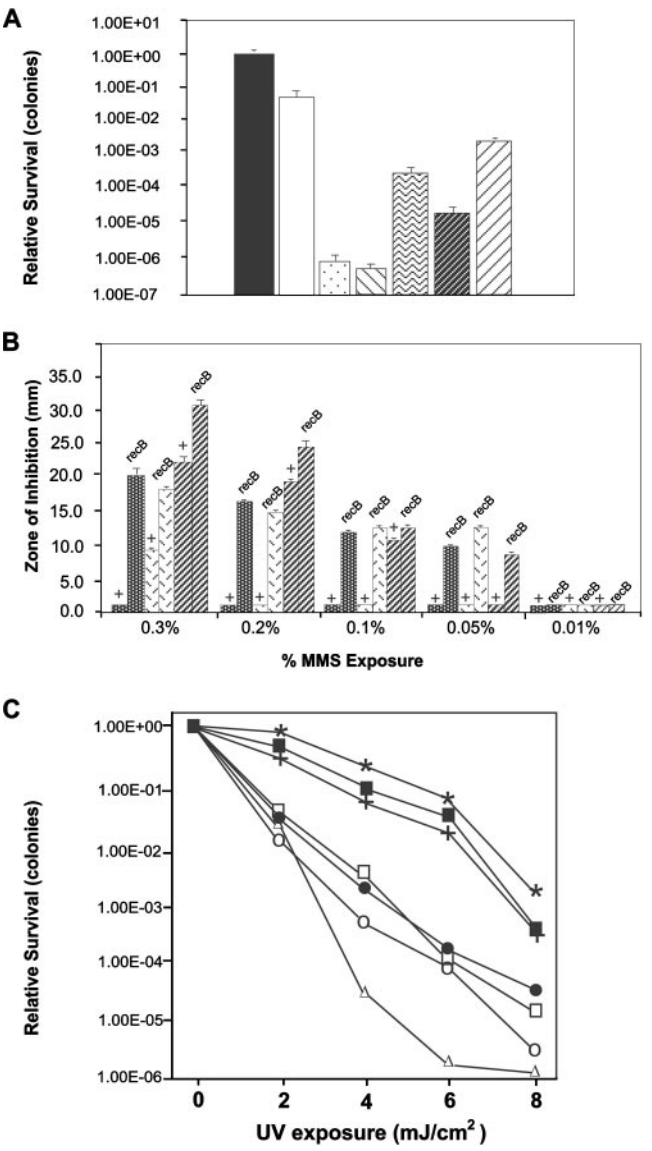

FIG. 3. Comparing the effects of DNA damage-causing reagents between *N. gonorrhoeae* strains MS11, FA1090, and P9. (A) Exposure to nalidixic acid (0.5 μg/ml). Solid bar, MS11 *rec*$^+$; clear bar, MS11 *recB* suppressor; stippled bar, MS11 *recB*; diagonally striped bar, MS11 *recA*; squiggly bar, FA1090 *rec*$^+$; dark diagonal bar, FA1090 *recB*; light diagonal bar, FA1090 $recB_{MS11}$. Strain P9 *rec*$^+$ and *recB* mutants were unable to tolerate nalidixic acid exposure at this concentration. Error bars indicate standard deviations from the mean ($n = 10$). (B) Exposure to MMS. Dark stippled bars, strain MS11; hexed bars, strain FA1090; diagonal bars, strain P9. The + symbol reflects *rec*$^+$ bacteria. Data represent two experiments performed in triplicate. Error bars represent standard errors ($n = 6$). (C) Exposure to UV irradiation. MS 11 *rec*$^+$ (filled squares), FA 1090 *rec*$^+$ (stars), P9 *rec*$^+$ (crosses), MS11 *recB* (open circles), FA 1090 *recB* (closed circles), P9 *recB* (open squares), and MS 11 *recA* (open triangles). Data represent two experiments performed in triplicate. Standard error bars are omitted for clarity.

shown) or MMS (Fig. 2C) treatment. From these data, we conclude that *N. gonorrhoeae* strain MS11 uses RecBCD to repair double chain breaks and alkylated DNA.

The preceding results obtained with strain MS11 differ from those found in a different strain background (FA1090) where only a modest defect in recombinational repair was observed (17). Consequently, we constructed an *N. gonorrhoeae* strain FA1090 *recB* mutant, as well as a *recB* mutant in a European isolate (*N. gonorrhoeae* strain P9), and repeated the assays. As can be seen in Fig. 3A and B, with these other strains, the effect of a *recB* mutation is less severe than that observed with strain MS11, with these data essentially confirming the previously published conclusions for strain FA1090 (14, 17). Comparable results were also found using FA1090 and P9 *recC* mutants (data not shown). However, what became apparent was that the wild-type repair capacities of strains FA1090 and P9 were significantly impaired compared to the wild-type MS11 repair

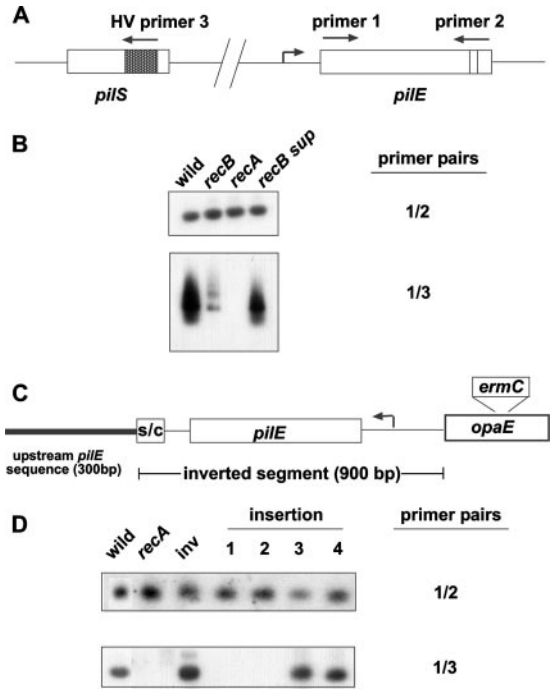

FIG. 4. RT-PCR analysis assessing *pilE*/*pilS* recombination. (A) Schematic showing the relative locations of the oligonucleotide primers used in the assay. (B) Southern hybridizations of RT-PCR products using MS11 RNAs prepared from the wild type, *recB* mutants, *recA* mutants, and the *recB* intragenic growth suppressor. The blots were probed with primer 2 (top) or primer 3 (bottom). (C) Schematic showing the inverted *pilE* chromosomal context (not drawn to scale). S/C represents the Sma/Cla repeat located downstream of *pilE*. (D) Southern hybridizations of RT-PCR products performed on MS11 RNAs prepared from various MS11 strains. The inverted *pilE* locus is designated *inv*. The insertion (no. 1 to 4) mutants carry an erythromycin gene cassette at positions −192 (insertion 1), −221 (insertion 2), −336 (insertion 3), and −743 (insertion 4) relative to the ATG start codon. The blots were probed with primer 2 (top) or primer 3 (bottom).

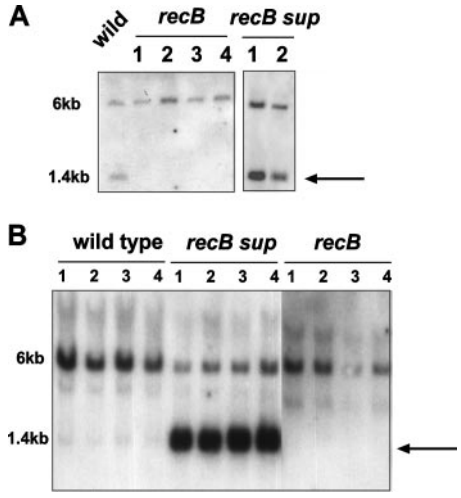

FIG. 5. Coconversion assay assessing *pilE*/*pilS* recombination. (A) XhoI linker DNA is located in the SmaI site in the Sma/Cla repeat downstream of the *pilE* locus. Following restriction of chromosomal DNA with SmaI, the XhoI linker can either be converted back to a wild-type configuration (1.4 kb; arrow) or remain in the mutated state (6 kb) if *pilE*/*pilS* recombination extends beyond the *pilE* locus. (A) Analysis of MS11 *pilE* variant 6 (9) and its *recB* derivatives. (B) Analysis of FA1090 and its *recB* derivatives. *recB sup* are intragenic growth suppressors derived from the cognate *recB* population. Each blot was probed with the *pilE*-specific probe 245. In each panel, lanes 1, 2, 3, and 4 represent independent mutants.

capacity (e.g., repair of nalidixic acid-induced double chain breaks shows a difference of approximately 3 orders of magnitude) (Fig. 3A). Therefore, these observations indicate that for strains FA1090 and P9, wild-type bacteria appear deficient for recombinational repair, with the result that a *recB* mutation has little impact on the repair phenotype. When a recombinant FA1090 strain in which the MS11 *recB* allele replaced the FA1090 *recB* gene was constructed, the repair capability remained that of the FA1090 wild type, indicating that the recombinational repair phenotype presented by the FA1090 wild-type strain probably reflects genetic differences outside the *recB* locus. In contrast to these observations, no difference was observed between the three strains for the repair of UV-induced lesions (*Neisseria* possesses the *uvr* repair pathway); the wild-type strains grouped together, as did the various *recB* mutants (Fig. 3C).

Effect of an *N. gonorrhoeae* strain MS11 *recB* mutation on *pilE* gene variation. Two previously published qualitative assays were used to test whether an MS11 *recB* mutation influenced *pilE* gene variation: (i) an RT-PCR assay in which the selection of primers demands a *pilE*/*pilS* recombination event (Fig. 4A) (27) and (ii) a marker conversion assay that has also been shown to be dependent on *pilE*/*pilS* recombination (Fig. 5) (9). The RT-PCR assessment of the *recB* effect is shown in Fig. 4B. Following standardization of the reaction with respect to the amount of RNA that was used for RT as well as of the amount of the template that was used for the second PCR amplification (Fig. 4B), it is evident that a *recB* mutation reduces the extent of *pilE*/*pilS* recombination compared to that for either the wild type or the intragenic *recB* suppressor strain (Fig. 4B). As would be predicted, no signal was obtained when RNA was isolated and tested from an MS11 *recA* mutant. These observations were then confirmed in the coconversion assay whose results are presented in Fig. 5A. In this assay, a DNA linker is placed downstream of the *pilE* locus. Following *pilE*/*pilS* recombination, the linker can be either converted back to the wild-type configuration or not, due to the presence of homology downstream of *pilE* and the various *pilS* loci. As shown in Fig. 5A, the introduction of the *recB* mutation abrogates conversion of the marker back to the wild-type configuration, whereas with rec^+ bacteria and the intragenic *recB* suppressor, conversion of the marker from the mutated state to the wild-type configuration is apparent. This conversion assay was also used to test *pilE*/*pilS* recombination with strain FA1090. In Fig. 5B, FA1090 rec^+ bacteria show very little conversion of the *pilE* outside the marker, and what little that appears to occur is abrogated in the *recB* mutants. However, when *recB* growth suppressors were isolated, considerable conversion of the outside marker was demonstrable. Coconversion results comparable to those displayed by strain FA1090 were also evident with strain P9 (data not shown).

Effect of inverting the *pilE* locus on *pilE* gene variation. A model that has been proposed for *pilE*/*pilS* recombination involves duplicating the *pilE* locus following a *pilE*/*pilS* recombination event, which then leads to the excision of a *pilE*::*pilS* fusion on a closed-circular piece of DNA. The closed circle then recombines with *pilE*, leading to *pilE* gene variation event (reviewed in references 18 and 26). In this model, in order for the closed circle to be efficiently excised from the chromosome, the duplicated *pilE* genes need to be in direct orientation. Consequently, if this mechanism actually operates in the gonococcus, inverting *pilE* should either totally abrogate or severely curtail *pilE*/*pilS* recombination. However, if RecBCD operates during *pilE* gene variation in strain MS11, this should occur irrespective of *pilE* locus orientation (22). The *pilE* locus was inverted on the MS11 chromosome (Fig. 4C), and the effect of this inversion on *pilE*/*pilS* interactions was assessed by RT-PCR. The blot presented in Fig. 4D shows that an inverted *pilE* locus recombines with *pilS* as efficiently as is observed with the *pilE* locus in direct orientation. The blot also confirms previously published observations (12) that show that large nonhomologous insertions placed immediately upstream of the *pilE* promoter abrogate *pilE*/*pilS* recombination (Fig. 4D, insertions 1 and 2).

DISCUSSION

From the data presented, we conclude that the RecBCD recombination pathway in *N. gonorrhoeae* strain MS11 plays a pronounced role in recombinational repair and *pilE* gene variation. This contrasts with previously reported observations with a different gonococcal strain, where the data indicated that the gonococcal RecBCD recombination pathway was noticeably different from the equivalent pathway in *E. coli* (17). Initially, we examined whether the presence of a proline residue in the RecB nuclease active site may have caused some disruption of RecB functionality, possibly accounting for the previously reported observations. However, we found that the presence or absence of this residue had no effect on enzyme function in the gonococcus. Subsequently, we were able to demonstrate that the MS11 *recB* gene was fully capable of complementing an *E. coli recB21* mutant in a *lac* recombination assay.

MS11 *recB* null mutants were severely deficient in the repair of nalidixic acid-induced chromosomal breaks as well as deficient in the repair of alkylated DNA lesions caused by MMS treatment. Accompanying this repair deficiency was a severe growth defect. Therefore, MS11 *recB* mutants appeared to present a more pronounced phenotype than the corresponding FA1090 *recB* mutants and, as such, behave very much like their *E. coli* counterparts. In addition, MS11 *recB* growth suppressors were readily obtainable, which is commonly seen with *E. coli recB* mutants. Two types of growth suppressors were identified in the MS11 *recB* cultures: (i) intragenic suppressors that arose through the deletion of the antibiotic marker plus a small segment of the *recB* gene and (ii) extragenic suppressors, with two suppressor mutations mapping to genes involved in DNA transformation (*pilT* and *comA*). In the latter suppressors, the growth defect was alleviated. However, the repair defect was retained (data not shown). A possible explanation for the observed discrepancy between the two phenotypes is that the differences reflect ATP usage between the various mutants, as uptake of DNA during DNA transformation requires extensive ATP hydrolysis, as does repair of broken chromosomes. Therefore, by eliminating DNA transformation, higher cellular-ATP levels now become available, leading to an enhanced growth phenotype.

In the three gonococcal strains that were assessed in this study, *recB* mutants for each strain showed diminished repair/recombination capacities compared to the wild type. However, the magnitudes of the defect varied considerably among strains and appeared to reflect the innate repair capacity of the wild type *rec*$^+$ strain, with strain MS11 apparently presenting a full repair capacity. This was evident from the relatively high sensitivities to MMS of P9 *rec*$^+$ and FA1090 *rec*$^+$. Similarly, FA1090 *rec*$^+$ was relatively sensitive to nalidixic acid whereas strain P9 *rec*$^+$ was unable to tolerate these nalidixic acid levels. Consequently, when these innate differences in recombination/repair are taken into account, MS11 *recB* mutants present a more robust phenotype than that observed for the other two strains, which begs the question as to whether wild-type strain FA1090 and wild-type strain P9 are fully functional with respect to recombination/repair. As the functionality of the FA1090 RecBCD protein was not addressed in the previously reported study (17), it is not known whether the observed recombination/repair deficiency of the FA1090 *rec*$^+$ strain is due to a defect in the RecBCD enzyme. However, if the FA1090 RecBCD pathway is defective in wild-type bacteria, this may explain why that particular strain relies on an alternative "RecF-like" pathway for recombination/repair (11). Interestingly, in meningococcal clinical isolates, epidemic *Neisseria meningitidis* clones that present *recB* mutant phenotypes due to small deletions located at the 5′ end of the gene have recently been identified (21). Furthermore, these strains were shown to undergo increases in *pilE* gene variation. Indeed, considerable variation in DNA repair capacity apparently is manifest by clinical meningococcal strains (4, 5). Therefore, the pathogenic *Neisseria* strains may simply utilize whatever active recombination proteins are at their disposal.

The major conclusion of this study is the finding that the RecBCD recombination pathway mediates PilE antigenic variation in *N. gonorrhoeae* strain MS11 in contrast to previously reported observations that proposed that a "RecF-like" pathway mediated *pilE* gene variation (11, 17). From the two assays presented in this paper (as well as a colony morphological analysis not presented), MS11 *recB* mutants appear to be constrained with respect to *pilE*/*pilS* recombination. In the more sensitive RT-PCR assay, the apparent low-level signal obtained from the *recB* RNA sample may reflect suppressor activation of an alternative pathway, such as the "RecF-like" pathway. This then leads to the intriguing possibility that if strain FA1090 is deficient in recombination/repair and does not show decreased levels of PilE antigenic variation in *recB* mutants, then perhaps the identification of a "RecF-like" pathway is due to suppressor activation countering this recombination/repair defect. The blot presented in Fig. 5B supports this contention.

MS11 *recB* mutants show reduced levels of *pilE*/*pilS* recombination, augmenting previous studies that have implicated the RecBCD recombination pathway in *pilE* gene variation. Previously published work has shown that MS11 *recD* mutants presented a hyperrecombination phenotype when *pilE* gene

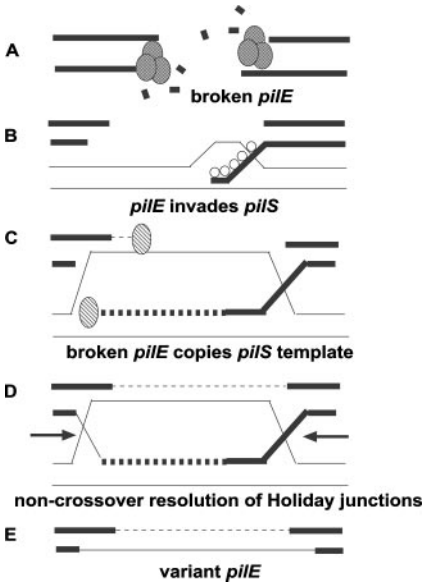

FIG. 6. Double-chain-break repair model for *pilE* gene variation. The model is based on the yeast mating-type switching model in yeast (20). (A) The *pilE* locus is broken, and the ends are acted upon by the RecBCD nuclease yielding 3′ overhangs. (B) The single chain overhangs bind RecA protein, which then seeks homology and invades a homologous *pilS* gene copy. (C) Following invasion of the *pilS* gene copy, the 3′ end is extended by DNA polymerase, using the *pilS* gene copy as a template. (D) Following extension, the Holiday junctions are resolved (arrows). (E) Creation of a variant *pilE* gene consisting of novel *pilS* sequence that was obtained during the DNA polymerase extension.

variation was assessed by a colony morphological assay in conjunction with DNA sequencing (3). In addition, the RecBCD pathway was shown to influence *pilE*/*pilS* templated deletion formation across the *pilE* locus, especially L-pilin-to-pilus+ transitions (9). Given these considerations, coupled with the observation that *pilE*/*pilS* recombination can proceed with an inverted *pilE* locus, a parsimonious double-chain-break repair model that utilizes the RecBCD enzyme can now be proposed for *pilE* gene variation occurring by a gene conversion mechanism in *N. gonorrhoeae* strain MS11 (Fig. 6). In this model, a break occurs at the *pilE* locus, each end of the broken chromosome is resected using the RecBCD enzyme, the single-chain DNAs then search for homology (i.e., *pilS*) using RecA protein, *pilE*/*pilS* pairing occurs, and *pilS* DNA then serves as a template to repair the break at *pilE*, which then leads to *pilE* gene variation and an unchanged *pilS* gene copy. Therefore, we propose that in recombination/repair of proficient gonococci, *pilE* gene variation proceeds via a double-chain-break repair model that utilizes the RecBCD enzyme, whereas in those strains where the RecBCD pathway is compromised, a half-crossing-over model for *pilE* gene variation remains valid (13), as inverting the *pilE* locus should not prevent the formation of the initial recombination intermediate.

The proposed model is very similar to the mating-type switching model for *Saccharomyces cerevisiae* (20). The model proposes that *pilE* is actively broken, which may simply be through the action of cellular nucleases. In the RT-PCR assay, *pilE* promoter insertions (Fig. 4D) that disrupted *pilE*/*pilS* recombination were identified (12). The insertions that disrupted *pilE*/*pilS* recombination lie adjacent to the integration host factor binding site which is located in the *pilE* promoter (10). Consequently, as integration host factor bends the gonococcal promoter (10) and that DNA bending has been implicated in chromosome breaks (19), the effect of the promoter insertions on *pilE*/*pilS* recombination may be due to prevention of *pilE* locus bending, causing *pilE* to remain intact. In the proposed model for *pilE*/*pilS* recombination, no role for potential Chi site activation is stated, as it is unknown whether Chi activates the gonococcal RecBCD enzyme. A BLAST search of the gonococcal chromosome using the Chi sequence (5′-GCTGG TGG-3′) reveals approximately 140 Chi sites occurring on average in 9-kb intervals (the predicted number of Chi sites randomly occurring in the gonococcal genome is approximately 40). This is a smaller number than that observed in *E. coli*, where Chi is found approximately every 3 kb. However, because the gonococcal *recB*, *recC*, and *recD* genes are unlinked on the chromosome, assessment of potential Chi stimulation is difficult to demonstrate experimentally.

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