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AUTHOR: Rachel K. Baker

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ABSTRACT:

The role of the RecBCD recombination pathway in PilE variation in *Neisseria gonorrhoeae* is debatable and dependent on the strain studied. In the MS11 strain, *recB* mutants were assessed for recombination/repair by assessing their ability to repair double-chain breaks. The MS11 *recB* mutants were found to be highly susceptible to these DNA double-chain break treatments and were severely impaired for growth; recB growth suppressor mutants arose at high frequencies. When the recombination/repair capacity of MS11 were compared to other strains (FA1090 and P9), innate difference were observed between each as FA1090 and P9 rec^+ bacteria had pronounced recombination/repair defects. Moreover, MS11 *recB* mutants present a more robust phenotype than the other strains. Additionally, MS11 *recB* mutants are also shown to be defective for *pilE/pilS* recombination. *pilE/pilS* recombination is also shown to proceed with gonococci that carry an inverted *pilE* locus. From this study, an innovative RecBCD-mediated double-chain-break repair model for PilE antigenic variation is proposed.

University Honors Program

Capstone Approval Page

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

Student Name: Rachel Katelyn Baker

Faculty Supervisor: Dr. Stuart Hill/Dr. Bethia King

Faculty Approval Signature: Bethia King

Department of Biological Sciences

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NORTHERN ILLINOIS UNIVERSITY

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

A Thesis Submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With Upper Division Honors

Department of

Biological Sciences

Rachel Katelyn Baker

DeKalb, Illinois

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Abstract

The role of the RecBCD recombination pathway in PilE variation in *Neisseria gonorrhoeae* is debatable and dependent on the strain studied. In the MS11 strain, *recB* mutants were assessed for recombination/repair by assessing their ability to repair double-chain breaks. The MS11 *recB* mutants were found to be highly susceptible to these DNA double-chain break treatments and were severely impaired for growth; recB growth suppressor mutants arose at high frequencies. When the recombination/repair capacity of MS11 were compared to other strains (FA1090 and P9), innate difference were observed between each as FA1090 and P9 *rec*$^+$ bacteria had pronounced recombination/repair defects. Moreover, MS11 *recB* mutants present a more robust phenotype than the other strains. Additionally, MS11 *recB* mutants are also shown to be defective for *pilE/pilS* recombination. *pilE/pilS* recombination is also shown to proceed with gonococci that carry an inverted *pilE* locus. From this study, an innovative RecBCD-mediated double-chain-break repair model for PilE antigenic variation is proposed.

Introduction

Neisseria gonorrhoeae is a gram-negative diplococcus which is an obligate human parasite causing the mucosal disease gonorrhea. Pathogenesis of the organism is initiated via host contact by pili binding host cell receptors. The major protein component of the gonococcal pilus organelle is PilE polypeptide which is encoded by the *pil*E gene. PilE polypeptide has the ability to undergo antigenic variation where the chemical composition of the protein changes and counteracts an efficacious immune response, which will further promote immune evasion (reviewed 18).

In the MS11 *N. gonorrhoeae* strain, there is a single copy of the *pil*E locus (1). However, there are also multiple silent copies of *pil* DNA (designated *pil*S) that are truncated, variant copies of *pil* sequence which are located in several different loci (7). *pil*S lack a promoter and are thus transcriptionally silent. Despite this apparent diversity, the genetic structure of all *pil* genes is very similar with variable regions being interspersed with conserved DNA segments, with these conserved regions believed to facilitate recombination between *pil*E and a *pil*S gene copy leading to variant *pilE* alleles (7). It has been found that most of the changes in *pilE* gene sequence occur predominantly through RecA-mediated gene conversion events (7,15, 25,29) where a segment of the *pilS* gene copy is transferred into the *pilE* gene, with the segments of the original *pilE* sequence being ejected or lost from the chromosome (7, 25). As a result, a unique PilE polypeptide is expressed.

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 caused hyper-recombination at *pilE* resulting in progeny with an increased number of non-parental pilus phenotypes (3). This led to a proposal that *pilE* gene variation proceeded via a RecBCD-mediated pathway. However, in studies using the FA1090 strain, insertion mutations in either *recB* or *recC* did not affect gene variation at *pilE* and only caused a modest recombination/repair deficiency (17). Consequently,

these observations indicated a significant difference between the proposed behavior of the *N. gonorrhoeae* RecBCD complex and *E. coli*'s 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). Gonococci, however, lack exonuclease I (*sbcB/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).

The effects of an insertional mutation in the *recB* gene have previously been detailed in the *N. gonorrhoeae* FA1090 strain (17) yet had not been investigated in the MS11 strain. The effects of *recB* and *recC* mutations on recombination/repair in *N. gonorrhoeae* strain MS11 were thus the purpose of this study. It was determined that the null mutation severely impeded repair of double-chain breaks caused by both nalidixic acid treatment and repair of DNA alkylation lesions caused by MMS (methyl methanesulfonate) treatment. Additionally, evidence suggests that the *pilE* gene variation proceeds via a RecBCD-mediated pathway in strain MS11. Moreover, as we are also able to demonstrate that inverting the *pilE* locus does not hinder *pilE/pilS* recombination, these combined observations allow us to propose a double-chain break repair model for *pilE* gene variation in strain MS11.

Materials and Methods

Table 1. Bacterial strains utilized in this study

Strain	Description	Source
Escherichia coli		
DH5α	F- φ*lac*ZΔ(*lac*ZYA*arg*F) U169*deo*R *rec*A1*end*A1*hsd*R17 (rκ-,mκ+)*pho*A*sup*E44 λ-*thi*-1gyrA96*rel*A1	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.Swanson
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

Table 2. Oligonucleotides used in this study	
Designation	Nucleotide Sequence (5'—3')
Cys2R	GCAGGTGACGGCAGGTGC
Erm1.5Rev	TGTTAGCCAAAGCTTCCAAGC
HVPilS ®	CGTTGTCGTTGCCGGCTTTGGT
InterB6	GCCTGAAGGTAATAGTGCTGGTGCGCGACGGC
PilRBS	GGCTTTCCCCTTTCAATTAGGAG
RecC2	GATTTGTTCCGCAGCGCACAA
RecC4	GGCAACGTATTGATGATTCG
RecB4	CCCAAAGCTTGCACTGCACTGCACAAATGGCTGCGCGATCAAATC
RecB5	CTAGTCTAGAGTGCGGGCAGGAAGGTGTCGCCTTCGT
RecB6	CTAGTCTAGAGGTGAGGGTGTGCGTGGTCATA
RecB9	CCCTCGAGGCTCAAGATTTTTGGCGGGAACG
RecB10	GGTCGAGACCCGAAGTCTGCCAGTTTCTGCAATTC
RecB12	AAAAGCTTGGCTGTGCCTGCACGAAATTCTTGAAGA
Tracy1	CGATATGGTCTGCCAAGACGACGGCAATATCTGC
Tracy2	GCAGATATTGCCGTCGTCTTGGCAGACCATATCG
GC recB1	AAACCGCAACCCCGCCGCA
GC recB2	GCGTGGGACGCGCAAGATACC
GC recB3	CGGCGGCGTTGCCAGCGT
GC recB4	GGTGCCGCCGTCCCGAA
probe 245	GCCTTTTTGAAGGGTATTCAT

Bacterial Strains and Growth Conditions

Neisseria gonorrhoeae strains MS11 (obtained from John Swanson, Rocky Mountain labs, MT), FA1090 (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 lab strain for many years; strains FA1090 and P9 were obtained in the mid 1980's and have undergone limited cultivation. During the course of these investigations, gonococci were passaged daily on GC Typing Medium (GTM) at 37°C in 5% CO_2 (24). Occasionally, gonococci were re-suspended in GC HEPES medium which is identical in composition to GC typing medium (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 kanamycin (Sigma, St. Louis).

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 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 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 pCRII™ vector (Invitrogen) with an erythromycin or kanamycin gene cassette inserted in the middle. The recombinant plasmid was used to transform *N. gonorrhoeae* for drug resistance to create a *recB* insertional mutant, with the mutation confirmed by PCR using primers where one was located within the drug resistance maker (erm 1.5 Rev; Table 2). *N. gonorrhoeae recC* mutants were constructed following PCR amplification of *recC* using primer pair RecC2 and RecC4 (Table 2). The resulting 1834 bp fragment was cloned into the pCRII™ vector. The insert was sequenced and an erythromycin (or kanamycin) gene cassette being inserted into a unique *Hin*cII site. The recombinant plasmid was then used to transform strain MS11 to drug resistance. The mutation was confirmed by PCR using primer pairs where one was located within the drug resistance maker (erm 1.5 Rev; 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 (Stratagene, Pfu Ultra ™) to amplify the active site DNA. A second PCR reaction was performed using a primer complementary to the first deletion primer (*Tracy* 2) and the downstream primer RecB6 (Table 2). These PCR reactions were purified and a third PCR reaction 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 *Sal*I 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 *Sau*3A partial digest of gonococcal chromosomal DNA. Plasmids carrying the gonococcal *recB* gene were identified using Neisseria-specific *recB* oligonucleotides (Gc recB 1-4; 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 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 *Sal*I site located in the *opaE* gene. The construct was crossed into the gonococcal chromosome by transformation. The structure of the final construct and of putative transformants was confirmed by DNA sequencing and PCR analysis.

Analysis of *N. gonorrhoeae* mutants

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 two 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. Assays were performed concurrently.

Exposure to methyl methanesulfate (MMS; Sigma): 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 solution placed on top of the agar and the plates were incubated overnight. Zones of inhibition identified the growth-inhibiting concentrations of MMS. Assays were performed concurrently.

Exposure to UV light: gonococci were serially diluted to the appropriate cell density, plated on solid medium and were exposed to varying doses of UV radiation (0, 20, 40, 60, and 80 mJ/cm^2) using an 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 non-irradiated plate. 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 *Xho*I linker was inserted into the *Sma*I site of the *Sma/Cla* repeat that is located downstream of the *pilE* locus. Chromosomal DNA was purified from an overnight culture, digested with *Sma* I 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 RT-PCR on purified RNA samples, slightly modifying a previously described procedure (27). RT-PCR reactions were run at 60°C for 30 minutes using conserved *pilE* primer pairs (pilRBS and cys2R) under conditions outlined by the manufacturer (Boehringer Mannheim). Two and one half units of *Taq* polymerase were then added and PCR was performed for 30 cycles. The RT-PCR reaction was then used as template for a second PCR reaction using

primers *pilE* (pilRBS) and (HVpilSR). A *pilE/pilS1* recombination event is required in order to obtain a product from the second PCR reaction. The products were then analyzed by Southern analysis using the HVpilSR primer as probe.

RNA Isolation

RNA was isolated for RT-PCR as described (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 for 5 min.

Southern Analysis

Southern blots were performed as described using radiolabelled oligonucleotides as probes (9, 25).

Results

The *N. gonorrhoeae* RecB protein shares some similarity to its *E. coli* counterpart (33% identity and 49% similarity). A canonical ATPase domain (16/22 amino acid identity) is located towards the amino-terminal end of the protein, with the nuclease domain being located towards the carboxy terminal end. A proline residue is located in the center of the nuclease-active site (Fig. 1) (28). Given the inconsistency 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 affected 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 is presented in [Fig. 2]). The functionality of the MS11 *recB* gene was further confirmed by cloning *recB* from an MS11 plasmid library on an approximate 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).

```
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
               :* .          ::*:*:.           * . *
```

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).

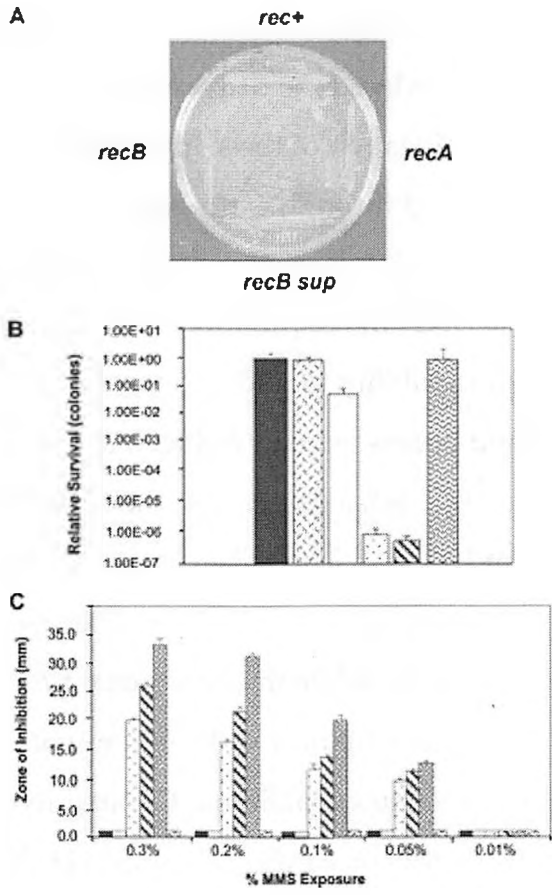

FIG. 2. Analysis of *N. gonorrhoeae* strain MS11 *recB* mutants. (A) Comparison of the growth characteristics of MS11 *rec*$^+$, *recB*, *recA*, and *recB* suppressor mutants. (B) Exposure to nalidixic acid (0.5 μg/ml). Solid bar, *rec*$^+$; hexed bar, proline minus mutant; clear bar, *recB* suppressor; 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).

Analysis of *N. gonorrhoeae* strain MS11 *recB* mutants

Neisseria gonorrhoeae recB mutants generated small colonies on solid growth medium (Fig. 2A). Conversely, larger colonies appeared (with an estimated frequency of 1×10^{-3} per colony forming unit) 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 bordered the drug resistance cassette in the *recB* gene (data not shown). Besides identification of these intra-genic suppressor mutations, wild type growth could also be restored through extra-genic suppressor mutations that nullified 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 capability 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), a decrease in the repair capacity of the small-colony *N. gonorrhoeae recB* mutant was seen to be comparable to that of *N. gonorrhoeae recA* mutants (Fig. 2B). In contrast, wild type MS11 and the *recB* proline minus mutant displayed little inhibition of growth, while the intra-genic *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 observed following DNA alkylation by MMS treatment (Fig. 2C). Strain MS11 *N. gonorrhoeae recC* mutants displayed a repair phenotype similar to that of the *recB* mutant following nalidixic acid (data not shown) and MMS treatment (Fig. 2C). 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 a *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 Figs. 3A and 3B, with these other strains, the effect of a *recB* mutation is less severe than observed with strain MS11 with these data essentially confirming the previously published conclusions for strain FA1090 (14, 17). Comparable observations were also found using FA1090 and P9 *recC* mutants (data not shown). However, what became apparent was that the "wild type" repair capacity for

strains FA1090 and P9 was significantly impaired when compared to the "wild type" MS11 repair capacity (e.g., repair of nalidixic acid-induced double chain breaks shows a difference of approximately three 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 was constructed, in which the MS11 *recB* allele replaced the FA1090 *recB* gene, the repair capability remained that of 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 possess the *uvr* repair pathway); the wild-type strains grouped together, as did the various *recB* mutants (Fig. 3C).

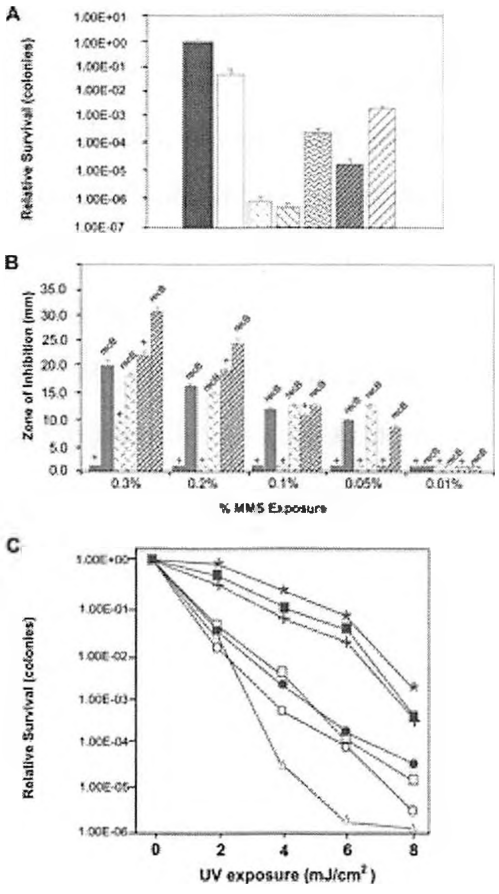

FIG. 3. Comparing the effects of DNA damage-causing reagents between *N. gonorrhoeae* strains MS11, FA1090, and P9. (A) Exposure to nalidixic acid (0.5 μg/ml). Solid bar, MS11 *rec*$^+$; clear bar, MS11 *recB* suppressor; stippled bar, MS11 *recB*; diagonally striped bar, MS11 *recA*; squiggly bar, FA1090 *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.

Effect of an *N. gonorrheae* strain MS11 *recB* mutation on *pilE* gene variation

Two previously published qualitative assays were used to test whether a 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 reverse transcription, as well standardizing the amount of 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 when compared to either "wild type" or to the intra-genic *recB* suppressor strain (Fig. 4B). As would be predicted, no signal was obtained when RNA was isolated and tested from a MS11 *recA* mutant. These observations were then confirmed in the co-conversion assay 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 homology being present 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 intra-genic *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 maker, 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. Comparable co-conversion observations to those displayed by strain FA1090 were also evident with strain P9 (data not shown).

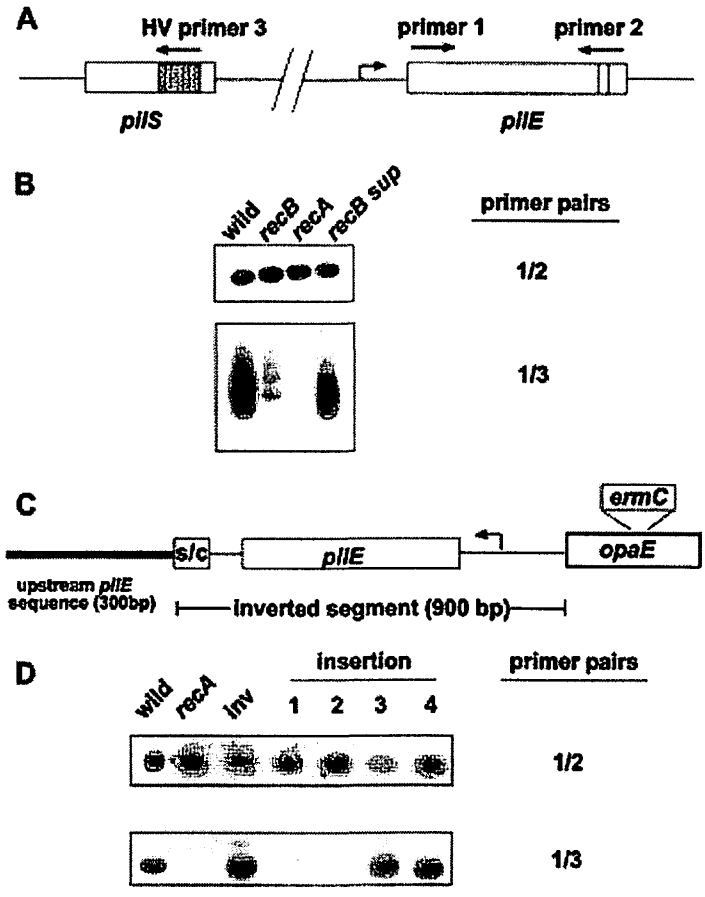

FIG. 4. RT-PCR analysis assessing *pilE/pilS* recombination. (A) Schematic showing the relative locations of the oligonucleotide primers used in the assay. (B) Southern hybridizations of RT-PCR products using MS11 RNAs prepared from the wild type, *recB* mutants, *recA* mutants, and the *recB* intragenic 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).

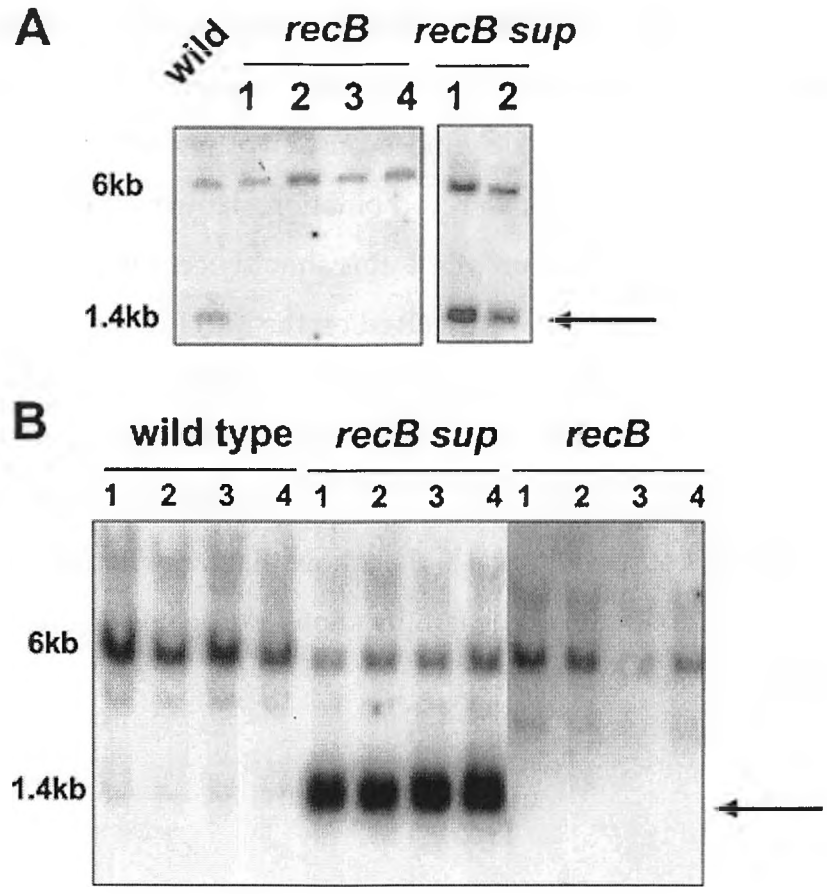

FIG. 5. Coconversion assay assessing *pilE/pilS* recombination. (A) XhoI linker DNA is located in the SmaI site in the Sma/Cla repeat downstream of the *pilE* locus. Following restriction of chromosomal DNA with SmaI, the XhoI linker can either be converted back to a wild-type configuration (1.4 kb; 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.

Effect of inverting the *pilE* locus on *pilE* gene variation

A model has been proposed for *pilE/pilS* recombination that 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 18, 26). In this

model, in order for the closed-circle to efficiently excise from the chromosome, the duplicated *pilE* genes would 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 non-homologous insertions placed immediately upstream of the *pilE* promoter abrogate *pilE/pilS* recombination (Fig. 4D, insertions 1 and 2).

Discussion

Previous studies on the *N. gonorrhoeae* FA1090 strain indicated that the gonococcal RecBCD recombination pathway was distinctly different from the equivalent pathway in *E. coli* (17). However, in the MS11 strain, it has been concluded that the RecBCD recombination pathway plays a prominent role in the recombinational-repair and *pilE* gene variation. One of the steps in examining the RecB gene involved determining whether the presence of a proline residue in the RecB nuclease active site may have caused some disruption of its functionality (e.g., impede nuclease activity), possibly accounting for the previously reported observations. The presence or absence of proline residue was found to have no effect on enzyme function in the gonococcus. Also, 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.

Repair of nalidixic acid-induced chromosomal breaks and alkylated DNA lesions triggered by MMS treatment was found to be severely deficient in MS11 *recB* null

mutants alongside a severe growth defect. For that reason, MS11 *recB* mutants when compared to FA1090 *recB* mutants appear to present a more pronounced phenotype as well as appear to work in a similar manner as their *E. coli* equivalent. Also, MS11 *recB* growth suppressors were readily accessible which is generally seen with *E. coli recB* mutants. In MS11 *recB* cultures, two different types of growth suppressors were found which include intra-genic suppressors and extra-genic suppressors. Intra-genic suppressors arose through the deletion of the antibiotic marker plus a small segment of the *recB* gene. Exra-genic suppressors had two suppressor mutations mapping to genes involved in DNA transformation designated *pilT* and *comA* and the growth defect was lessened. However, the repair defect was retained. One possible explanation for this discrepancy is that various mutants have differences in ATP usage as the uptake of DNA during DNA transformation requires extensive ATP hydrolysis as does repair of broken chromosomes. Thus, by eradicating DNA transformation, higher cellular ATP levels now become available leading to an enhance growth phenotype.

Though *recB* mutants for each of the three strains studied (MS11, P9, FA1090) showed a diminished recombination/repair capacity when compared to wild type, the magnitude of the defect varied considerably among strains and appeared to reflect the innate repair capacity of the wild-type *rec*+ strain. The MS11 strain presented a full repair capacity which was evident from the relatively high sensitivity of P9 *rec*+ and FA1090 *rec*+ to MMS treatment. In the same way, FA1090 *rec*+ was relatively sensitive to nalidixic acid whereas strain P9 *rec*+ was unable to tolerate these nalidixic acid levels. Subsequently, 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. This further leads to the matter as to whether wild-type strain FA1090 and wild-type strain P9 are fully functional with respect to recombination/repair. It is not known whether the observed recombination/repair deficiency of the FA1090 rec+ strain is due to a defect in the RecBCD enzyme as it was not tested in previous reports (17). 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). In comparison, meningonococcal clinical isolates, epidemic *Neisseria meningitides* clones have been recently identified that present a *recB* mutant phenotype due to small deletions located at the 5'end of the gene (21). These strains have been shown to undergo an increase in *pilE* gene variation and indeed considerable variation in DNA repair capacity evident by clinical meningococcal strains (4, 5). It can be concluded from this that pathogenic *Neisseria* basically use whatever active recombination proteins are at their disposal.

The predominant conclusion of this study is discovering that the RecBCD recombination pathway mediates PilE antigenic variation in *N. gonorrhoeae* strain MS11 as compared to a "RecF-like" pathway previously offered to mediate *pilE* gene variation. (11, 17). Two assays performed in this study show that MS11 recB mutants appear to be constricted 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. 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. This is an intriguing possibility which is further supported by the blot found in Fig 5B.

MS11 *recB* mutants show reduced levels of *pilE/pilS* recombination further supporting previous studies implying a RecBCD recombination pathway in *pilE* gene variation. In earlier published work, MS11 *recD* mutants were found to present a hyper-recombination phenotype when *pilE* gene variation was assessed by a colony morphological assay in conjunction with DNA sequencing (3). Also, the RecBCD pathway was shown to influence *pilE/pilS* template deletion formation across the *pilE* locus, particularly L-pilin to pilus+ transitions (9). A parsimonious double-chain break repair model that employs the RecBCD enzyme has been proposed for *pilE* gene

variation occurring by a gene conversion mechanism in *N. gonorrhoeae* strain MS11 from these observations that *pilE/pilS* recombination can progress with an inverted *pilE* locus (Fig 6). The proposed model suggests the following occurrences: a chain break occurs at the *pilE* locus and each end of the broken chromosome is resected using the RecBCD enzyme, the single-chain DNAs then search for homology utilizing *pilS* using RecA protein. Next, *pilE/pilS* pairing occurs after which *pilS* DNA then serves as a template to repair the break at *pilE*. This leads to *pilE* gene variation and an unaffected *pilS* gene copy. Therefore, it has been proposed that in recombination/repair proficient gonococci, *pilE* gene variation proceeds via double-chain break repair model that uses 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.

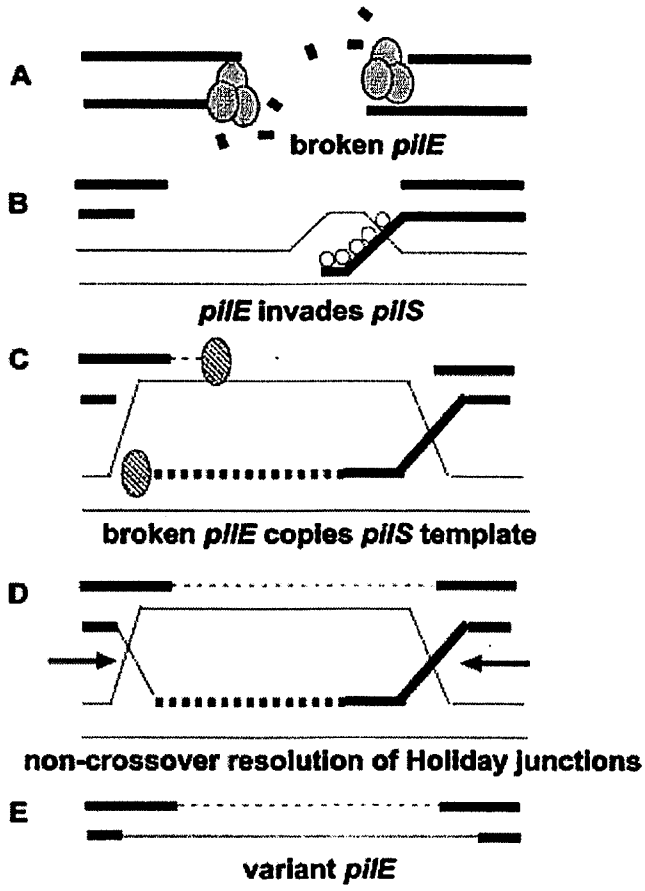

FIG. 6. Double-chain-break repair model for *pilE* gene variation. The model is based on the yeast mating-type switching model in yeast (20). (A) The *pilE* locus is broken, and the ends are acted upon by the RecBCD nuclease yielding 3' overhangs. (B) The single chain overhangs bind RecA protein, which 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.

The proposed model is quite similar to the mating-type switching model for *Saccharomyces cerevisiae* (20). The model proposes that *pilE* is actively broken which could occur through the action of cellular nucleases. In the RT-PCR assay, *pilE* promoter insertions (Fig. 4D) were identified that disrupted *pile/pilS* recombination (12). The insertions which caused disruption in the *pilE/pilS* recombination lie adjacent to the IHF binding site located in the *pilE* promoter (10). As IHF bends the gonococcal promoter

(10) and DNA bending has been linked in chromosome breaks (19), the effect of the promoter insertions on *pilE/pilS* recombination may be due to prevention of the *pilE* locus from bending causing *pilE* to remain intact. In our 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'-GCTGGTGG-3') reveals approximately 140 Chi sites occurring on average in 9 kb intervals though the predicted number of Chi sites randomly occurring in the gonococcal genome is approximately 40. The average of 9 kb intervals is a smaller number than observed in E. coli, where Chi is found approximately every 3 kb. The potential of Chi stimulation is difficult to exhibit experimentally as the gonococcal *recB*, *recC* and *recD* genes are unlinked on the chromosome.

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