NOTES

Role of the Rep Helicase Gene in Homologous Recombination in *Neisseria gonorrhoeae*

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In *Escherichia coli*, the Rep helicase has been implicated in replication fork progression, replication restart, homologous recombination, and DNA repair. We show that a *Neisseria gonorrhoeae* *rep* mutant is deficient in the homologous-recombination-mediated processes of DNA transformation and pilus-based colony variation but not in DNA repair.

Neisseria gonorrhoeae (gonococcus [Gc]) is an obligate human pathogen that possesses a number of mechanisms that have enabled it to persist within human populations, including the homologous recombination-mediated processes of recombinational DNA repair, DNA transformation, and pilin antigenic variation (reviewed in reference 20). One potential participant in these recombination processes is the Rep helicase. *Escherichia coli* *rep* mutants were originally identified by their inability to support phage replication (4, 7). Rep has also been implicated in chromosomal replication (6, 24, 25), DNA repair, and homologous recombination (4, 7, 8) in *E. coli*.

A direct link between replication and recombination has been demonstrated for *E. coli* (21, 36) in a process called replication restart. During this process, replication can be resumed from recombination intermediates formed at stalled replication forks. The *E. coli* replication restart pathway targets branched DNA structures at the stalled replication fork either by a PriA-dependent pathway that utilizes PriB or PriC and DnaT or by a Rep-dependent pathway that uses PriC and possibly DnaT (35). *N. gonorrhoeae* does not possess homologues of PriC or DnaT (20), indicating that the secondary pathway of replication restart may be missing from gonococci.

Construction of an *N. gonorrhoeae* *rep* mutant. In order to study the relationship between replication and recombination in *N. gonorrhoeae*, we identified the gonococcal *rep* homologue for further study. *N. gonorrhoeae* possesses a number of helicases that share homology with *E. coli* Rep helicase, including Rep, UvrD, and RecB. BLAST analyses performed against the *N. gonorrhoeae* strain FA1090 genome database (34) revealed that gonococcal Rep has the highest E value (E-134) and shares the greatest sequence identity (48%) and similarity (65%) with *E. coli* Rep (Fig. 1A). The gonococcal Rep homologue is encoded by a 2,013-bp gene predicted to encode a protein of 671 amino acids. Seven motifs conserved among

ATP-dependent DNA helicases (15) are present in Rep (Fig. 1A). Motifs Ia, III, and V are involved in binding single-strand DNA; motifs I and IV are involved in nucleotide binding; and motifs II and VI are important for helicase function (23). Based on the high degree of sequence similarity and the presence of conserved helicase motifs, we conclude that Gc Rep is the homologue of *E. coli* Rep.

Gonococcal *rep* was cloned and inactivated by inserting a gene encoding tetracycline resistance between codons 303 and 304 (Fig. 1B). To ensure that all phenotypes observed in the *rep* mutant were due to the *rep* mutation, complementation analysis was performed with a functional copy of Gc *rep* elsewhere on the gonococcal chromosome. The close apposition of the upstream *aroD* gene and *rep*, along with the absence of clear promoter sequences directly upstream of *rep*, suggested that *aroD* and *rep* are cotranscribed in an operon. Reverse transcription (RT)-PCR analysis using primer pairs 1 and 3, 4 and 5, and 2 and 5 (Fig. 1B) yield products indicating that these genes are cotranscribed, whereas no product was observed in the no-RT controls (data not shown). Therefore, we placed *rep* under the control of the *lac* promoter in the complement strain to ensure expression.

Replication and growth are not altered in an *N. gonorrhoeae* *rep* mutant. It has been reported that *E. coli* *rep* mutants do not grow as well as corresponding *rep*⁺ strains (8) and that replication forks of *rep* mutants in *E. coli* move more slowly than in wild-type strains (25). We observed that the gonococcal *rep* mutant grew at rates similar to those of the parental and complement strains when numbers of CFU per colony were compared at numerous time points spanning 15 to 40 h of growth (data not shown). In addition, hybridization experiments showed an increase in gene dosage near the origin of replication for *E. coli* *rep* mutant strains compared to that of the parental strain, suggesting that more replicating forks are present in the mutant strain (6, 24). We replicated the gene dosage experiment with *N. gonorrhoeae* by using DNA microarrays. DNA was isolated from the parental and *rep* mutant strains, differentially labeled, and hybridized to a pan-*Neisseria* DNA microarray (J. K. Davies et al., unpublished data). We observed no difference in hybridization intensities and hence

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FIG. 1. *N. gonorrhoeae* *rep* homologue. (A) Amino acid sequence alignment of Rep from *E. coli* (Ec) and Rep from *N. gonorrhoeae* (Gc). Identical residues are boxed in black, and similar residues are boxed in gray. Conserved helicase motifs are underlined and labeled. A conserved DEX(D/H) helicase motif is indicated TXGX, and black circles are over residues of *E. coli* Rep involved in single-stranded-DNA contact (23). (B) Chromosomal map of the region containing the *rep* gene and the complement strain. The *rep* gene (black arrow) was PCR amplified from *N. gonorrhoeae* genomic DNA with primers RepF2 (5'-CGTGGCAAATGCTCAAAAA-3') and RepR1 (5'-GCCTCCAAATTTCCACAGAA-3'), cloned into plasmid pCR2.1-TOPO (Invitrogen), and cut within the predicted Rep open reading frame with MfeI, and the *tetM* gene tagged with the gonococcal uptake sequence required for efficient uptake of DNA (11) was inserted. This plasmid was used to transform *N. gonorrhoeae* (transformants were selected on Gc medium base (GCB) containing 1 mg of tetracycline per liter), creating the gonococcal *rep* mutant strain. The triangle indicates the site and orientation of the inserted *tetM* gene. The *rep* gene was cloned downstream of a dual *tacIAC* promoter (hatched box) and inserted into the neisserial intergenic complementation site (NCIS), a chromosomal site located between the *lctP* and *aspC* genes (27, 28) (linked to a chloramphenicol resistance cassette allowing for selection on GCB containing 2 mg of chloramphenicol per liter) to create the *rep/rep*⁺ complement strain. Gonococcal strains in this study were derivatives of strain FA1090 variant 1-81-S2 (39) containing an IPTG (isopropyl-β-D-thiogalactopyranoside)-regulatable *recA* allele (38) and have matched *pilE* sequences as described previously (41). Large arrows indicate the predicted direction of transcription. The white open reading frame 74 bp downstream of and transcribed in the orientation opposite to that of the *rep* stop codon has homology to DinP (31), which is involved in SOS-induced mutagenesis in *E. coli* (19, 45). The gray open reading frame, whose stop codon is 23 bp upstream of the *rep* ATG, has homology to AroD from *E. coli*. AroD is believed to be involved in the biosynthesis of aromatic amino acids (10). Small arrows (numbered 1 to 5) in *aroD* and *rep* were used for RT-PCR operon analysis.

no difference in gene dosages between the *rep* mutant and the parental strain across the chromosome (data not shown). These data suggest that replication fork progression is unchanged in the gonococcal *rep* mutant compared to that in the parental strain and suggest that gonococcal Rep does not play a role in replication identical to that of the *E. coli* homologue.

A *rep* mutation does not affect DNA repair in *N. gonorrhoeae*.

In *E. coli*, *rep* mutants are deficient in the repair of DNA damage caused by UV light (4, 7) and double-strand (DS) breaks caused by X rays (8). In addition, although *E. coli rep* mutants are not more sensitive to H₂O₂ exposure (18), a recent report showing that *E. coli rep* mutations increase the sensitivity of *recA* mutants to oxidative stress (3) potentially implicates *rep* in the oxidative-damage response. We found that the gonococcal *rep* mutant, the parental strain, and the complement strain displayed equivalent levels of resistance to H₂O₂ (data not shown). Furthermore, the gonococcal *rep* mutant was no more sensitive to UV light or nalidixic acid-induced DS DNA breaks (9, 26) than the parental and complement strains, whereas a *recA*-negative strain was significantly more sensitive to both DNA-damaging agents (data not shown). Colonies arising in the nalidixic acid-induced DS break assay, performed as described in references 40 and 41, resulted from DS break repair and were not simply spontaneous mutations, since colonies were not viable upon passage to nalidixic acid-containing medium (data not shown).

Therefore, in contrast to the DNA repair defect of *E. coli rep* mutants, the gonococcal *rep* mutant did not display increased sensitivity to any DNA-damaging agent tested. It has been proposed that *E. coli rep* mutants experience increased chromosomal breaks due to more frequent replication pauses (29, 44) and that the stalled replication forks trigger the constitutively induced SOS response observed in *E. coli rep* mutants (32, 33, 44). Since gonococci do not possess an SOS response (2), this may explain the lack of a DNA repair phenotype in the *N. gonorrhoeae rep* mutant.

A *rep* mutant has a reduced DNA transformation efficiency.

Gonococci are naturally competent for DNA transformation, the only known means of exchange of chromosomal markers in *Neisseria* species (30), during all stages of growth (1). During transformation, DNA is imported into the bacterial cytosol, where it can be efficiently recombined into the chromosome in a RecA-dependent manner (22).

It has been demonstrated in *E. coli* that *rep* mutants display decreased conjugation frequency when *rep* is absent in the recipient strain (7, 8). We tested whether *rep* might also be involved in the homologous-recombination-mediated process of DNA transformation in *N. gonorrhoeae*. We observed an approximately 10-fold decrease in DNA transformation efficiency in the *rep* mutant strain, similar to the partial transformation defect seen in gonococcal *recBCD* (28) and *recN* (40) recombinase mutants. In contrast, the *recA* mutant strain is completely deficient in DNA transformation (Fig. 2). The transformation efficiency was restored to parental levels in the *rep/rep*⁺ complement strain, indicating that the observed transformation defect in the *rep* mutant was not due to a secondary mutation or to polar effects on another gene. Therefore, we conclude that gonococcal *rep* plays a role in DNA transformation.

FIG. 2. A mutation in *rep* decreases DNA transformation efficiency. Parental strain 1-81-S2 *recA6* containing the IPTG-regulatable *recA* allele (38) is a variant of parental strain FA1090. All strains were grown in 1 mM IPTG for maximal *recA* induction, which yields RecA expression levels similar to those of strains with a wild-type *recA* allele (E. A. Stohl and H. S. Seifert, unpublished data). DNA transformation efficiency was determined as previously described (5). Transformation to nalidixic acid resistance occurred when plasmid DNA containing *gyrB* with a mutation which yields Nal^r was recombined into the gonococcal chromosome. Transformants were selected on GCB plates containing 1.5 mg of nalidixic acid per liter. Error bars represent the standard errors of the means of results of three experiments done in duplicate or triplicate ($n = 6$ to 9). Statistically significant differences ($P < 0.05$) as determined by Student's *t* test are indicated by asterisks for comparison to results with the parental strain and by a pound sign for comparison to the strain carrying the *rep* mutation.

A *rep* mutant is deficient in pilin antigenic variation. Pilin antigenic variation occurs via homologous recombination events between *pilE* and one of multiple unexpressed pilin copies (*pilS*) found at various loci within the chromosome (12, 13, 37). During pilin antigenic variation, a variable portion of a *pilS* copy replaces the corresponding portion of *pilE*, recombining at short regions of identity shared between the silent and expressed genes. The *pilS* copy remains unchanged during this recombination process, and it is hence a gene conversion event. As in DNA transformation, RecA is also essential for pilin antigenic variation (22) and is aided by the RecA modulator RecX (41). In addition, the RecF family members RecO and RecQ (28), RecJ (14, 40), and RecR, along with the branch migration factors RuvA and RecG (36a) are also required for this process. It has been proposed that the two DNA molecules that recombine during antigenic variation may arise just after the genome is replicated (16, 17), suggesting a possible link between antigenic variation and DNA replication. Since the Rep helicase plays a role in replication and recombination in *E. coli* (7, 8), we tested whether the gonococcal *rep* gene is involved in pilin antigenic variation.

Quantification of pilus-based colony morphology changes, reflecting recombination at *pilE*, revealed that inactivation of *rep* leads to a statistically significant approximately twofold reduction in pilus-dependent colony variation. This level of variation was greater than that of the *recA* mutant strain (Fig. 3A). To confirm this observation, a kinetic variation assay that quantifies the number of phase variation events within a colony

FIG. 3. A mutation in *rep* disrupts pilin antigenic variation. Strains are as described in the legend for Fig. 2, and assays were performed as previously described (40). (A) Percent colony phase variation of *N. gonorrhoeae*. Error bars represent the standard errors of the means of results of four experiments performed at least in duplicate ($n = 10$ to 11). (B) Kinetic variation assay, measuring the average number of pilus-dependent colony morphology changes that occur over time. Error bars represent the standard errors of the means of results of three experiments done at least in triplicate ($n = 10$ to 12). Statistically significant differences ($P < 0.05$) as determined by Student's *t* test are indicated by asterisks for comparison to the parental strain and by a pound sign for comparison to the strain carrying the *rep* mutation.

over time was used. Similarly, we observed a statistically significant decrease in the amount of variation in the *rep* mutant, to a level that was less than that of the parental strain but greater than that of the *recA* mutant (Fig. 3B). The *rep/rep*⁺ complement strain showed levels of colony variation that were statistically increased over levels in the *rep* mutant strain and indistinguishable from those in the parental strain. The intermediate effect of *rep* on colony variation is similar to that seen with *recJ* (40) and *recG* and *ruvA* (36a) recombination-deficient strains. Sequence analysis of the *pilE* gene from antigenic variants arising from the *rep* mutant strain revealed a repertoire of *pilE* sequences similar to that of variants arising from the parental strain (data not shown). These data suggest that the antigenic variation defect in the *rep* mutant is not due to a disruption of a major recombination process and subsequent unmasking of a secondary pathway. Instead, the intermediate

defect of antigenic variation suggests that gonococcal *rep* mutations decrease the overall efficiency of pilin antigenic variation. Therefore, we conclude that *rep* is involved in, but not required for, pilin antigenic variation.

One hypothesis explaining the role of Rep in pilin antigenic variation is that DNA structures or protein-bound DNA formed during recombination leading to antigenic variation may lead to replication fork stalling or collapse. If a stalled fork at the recombining *pilE* locus is not restarted, resultant antigenic variants would not be detected. If a subset of replication restart events is Rep dependent, a partial defect in detectable antigenic variation may result, an outcome consistent with our observation. An equally attractive hypothesis explaining the role of *rep* in pilin antigenic variation is that Rep's 3'→5' helicase activity (46) is important for the process, independently of replication restart. Consistent with this hypothesis is the involvement of RecQ, another 3'→5' helicase (42, 43), in pilin antigenic variation. A *recQ* mutant also displays an intermediate variation defect, and it is possible that Rep and RecQ share redundant functions in this process.

Concluding remarks. This work describes the identification and inactivation of the *N. gonorrhoeae* *rep* gene. Several *N. gonorrhoeae* *rep* mutant phenotypes are similar to those of its *E. coli* homologue. The *rep* gene is not essential in *E. coli* (6), nor is it essential in *N. gonorrhoeae*. Both gonococcal *rep* and *E. coli* *rep* are involved in homologous recombination, since *E. coli* *rep* mutants are deficient in conjugational recombination and a Gc *rep* mutant exhibits defects in both DNA transformation and pilin antigenic variation. However, unlike *E. coli* *rep* mutants, gonococcal *rep* mutants do not display defects in DNA replication or DNA repair. In addition, *E. coli* and gonococci differ in the replication restart gene repertoire and the SOS response. Taken together, these findings suggest that the pathways that link replication and recombination delineated in *E. coli* are not identical in the human-specific pathogen *N. gonorrhoeae* and support the notion that this human-restricted pathogen may have more finely tuned systems to survive within their host.

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