

Genetic Characterization of the Nucleotide Excision Repair System of *Neisseria gonorrhoeae*[∇]

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Nucleotide excision repair (NER) is universally used to recognize and remove many types of DNA damage. In eubacteria, the NER system typically consists of UvrA, UvrB, UvrC, the UvrD helicase, DNA polymerase I, and ligase. In addition, when DNA damage blocks transcription, transcription-repair coupling factor (TRCF), the product of the *mfd* gene, recruits the Uvr complex to repair the damage. Previous work using selected mutants and assays have indicated that pathogenic *Neisseria* spp. carry a functional NER system. In order to comprehensively examine the role of NER in *Neisseria gonorrhoeae* DNA recombination and repair processes, the predicted NER genes (*uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd*) were each disrupted by a transposon insertion, and the *uvrB* and *uvrD* mutants were complemented with a copy of each gene in an ectopic locus. Each *uvr* mutant strain was highly sensitive to UV irradiation and also showed sensitivity to hydrogen peroxide killing, confirming that all of the NER genes in *N. gonorrhoeae* are functional. The effect of RecA expression on UV survival was minor in *uvr* mutants but much larger in the *mfd* mutant. All of the NER mutants demonstrated wild-type levels of pilin antigenic variation and DNA transformation. However, the *uvrD* mutant exhibited higher frequencies of PilC-mediated pilus phase variation and spontaneous mutation, a finding consistent with a role for UvrD in mismatch repair. We conclude that NER functions are conserved in *N. gonorrhoeae* and are important for the DNA repair capabilities of this strict human pathogen.

Neisseria gonorrhoeae (the gonococcus [Gc]) is a strict human pathogen and is the sole causative agent of the human sexually transmitted disease gonorrhea. Gc and its closest relatives are only found naturally within the human population with no known animal, insect, or environmental reservoirs. Disease symptoms consistent with gonorrhea have been described throughout history (25), leading to the hypothesis that most of the genus *Neisseria* have evolved within humans for >50,000 years. This adaptation to life within a single organism has produced a streamlined genome and genetic and physiology focused on life within humans. Although these organisms have not been regularly exposed to UV light, extremes of temperature, desiccation, or a wide range of chemicals during their exclusive residence in the human population, the Gc genome encodes a variety of genes involved, or predicted to be involved in DNA repair, including photoreactivation, base excision repair, nucleotide excision repair (NER), recombination repair, and replication restart (10, 18). Among the predicted DNA repair pathways is a full NER pathway.

NER is the most ubiquitous DNA repair pathway and is found in the sequenced genomes from almost every kingdom, genus, and species that has been examined. The core set of genes involved in the *Escherichia coli* NER system are *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* (43). All of these gene products have been purified, and their roles in NER have been defined. The UvrAB complex recognizes and binds to the distortion in the

DNA duplex caused by DNA damage. UvrB recruits UvrC to the lesion, where it acts as a single-stranded DNA endonuclease cleaving the DNA 5' and 3' from the lesion. UvrD (helicase II) then removes the single stranded segment carrying the lesion. DNA polymerase I resynthesizes the DNA copying the undamaged template, and ligase completes the repair (43). The *mfd* gene product TRCF recognizes stalled transcription machinery and targets the NER components to correct lesions that block transcription (36). The NER systems of all organisms are used to recognize and remove chemical lesions in DNA. Although *uvrB* and *uvrC* are induced by the damage-inducible SOS system in *E. coli* and this system is associated with the removal of pyrimidine dimers resulting from UV irradiation, many types of chemical damage can be recognized and repaired by NER, including psoralen monoadducts, cisplatin cross-links, thymine glycol products, O⁶-methyl guanine, and others, as well as abasic sites (43). In a subset of organisms including *E. coli*, a paralog of UvrC has been identified that allows the removal of larger chemical substitutions on DNA, like cholesterol (28).

Although NER has not been extensively studied in the *Neisseria*, several reports have concluded that a functional NER system exists in *Neisseria* spp. In 1984, Campbell and Yasbin demonstrated the excision of nucleotides carrying pyrimidine dimers in UV-irradiated Gc by using an alkaline agarose gel system (7). In 1997, Black et al. demonstrated that the Gc *uvrA* gene could complement an *E. coli* *uvrA* mutant for UV survival (3), and in 1995 the same group demonstrated that a *uvrB* mutant was deficient in UV survival (4). Finally, Davidsen et al. demonstrated that a *Neisseria meningitidis* *uvrA* mutant was also deficient for UV survival but not altered for survival to alkylating agents (11). The involvement of NER in other DNA recombination and repair pathways in pathogenic *Neisseria*,

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such as natural competence, repair of spontaneous mutations, and pilus variations, has not been defined.

In order to fully address the role of NER in Gc, each gene involved in the repair system was cloned and mutated by a transposon insertion and then transformed into laboratory strain FA1090. As expected, NER mutants had substantial sensitivity to UV irradiation, and further studies revealed a complex relationship between the NER system and RecA in repairing UV-mediated DNA damage. NER mutants were also more sensitive to other types of DNA damage but had no defect in the recombination-dependent events of DNA transformation and pilin antigenic variation. Finally, the participation of NER mutants in the control of spontaneous mutation was assayed, and only *uvrD* was found to influence mutation frequencies, presumably due to the role for this helicase in mismatch correction.

MATERIALS AND METHODS

Strains and growth conditions. Gc strain FA1090, pilin variant 1-81-S2, is a pilated (P+) Gc strain carrying a defined *pilE* sequence (35). The FA1090 1-81-S2 *recA6* derivative carries a *recA* gene under the control of *lac* regulatory elements (34). The *recA9* mutant is *recA* deficient due to an insertion of an erythromycin resistance cassette into *recA* (34). Where indicated, spontaneous nonreverting nonpilated (P-) variants of FA1090 1-81-S2 were isolated (Δ *pilE*), and deletion of the *pilE* gene confirmed by PCR. Gc strains were grown on GC Medium Base (Difco) plus select agar (Invitrogen) with Kellogg's supplements (17) and, when appropriate, 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside; Fisher Scientific) was added. Gonococci were grown at 37°C in 5% CO₂ unless otherwise indicated. *E. coli* TOP10 (Invitrogen) were grown on solid Luria-Bertani (LB) agar at 37°C or in LB liquid medium at 37°C with rotation. Antibiotics were used as follows: kanamycin at 50 μ g/ml, chloramphenicol at 1 μ g/ml, and erythromycin at 0.5 μ g/ml for Gc or 250 μ g/ml for *E. coli*.

Identification and cloning of the Gc NER genes. The annotated but unpublished Gc genome (<http://stdgen.northwestern.edu>) was used to identify the gene sequences of *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* in the chromosome of Gc strain FA1090. Primers were designed to amplify an internal region of each gene and PCR was performed by using *Taq* polymerase (Promega). Primers to *uvrB*, *uvrC*, and *uvrD* were designed to incorporate the Gc uptake sequence (GCCGTCT GAA, underlined in the primer sequences below [13]) into PCR-amplified DNA for efficient transformation of Gc. Gc *uvrA* was amplified by using the primers UVRA-1 (CGAAAAATCTCAAAGAAATC) and UVRA-2 (GAGTCCA AGGCGAGTTTG). The primers used to PCR amplify *uvrB* were UVRB-1 (TGCCGCTGAACGAGTATCAACAAATGGTGCTG) and UVRB-2 (GT ACGTCAAACAGGCCGAGC). The primers for *uvrC* were UVRC-1 (TTGC CGTCTGAACGAAGAAGACTACTGCGACAGC) and UVRC-2 (GCAAGG CCGGGCTATTAGGAG). The primers for amplification of *uvrD* were UVRD-1 (TTGCCGCTGAACATGATTGGGTGCTACGCCG) and UVRD-2 (CATGGTG ATGTAGAGGCCGTTTG). For *mfd*, primers were MFD-1 (CGAAGATGCGCTGT TTGCTC) and MFD-2 (GGTCTTCGGTTCTCTCGTAGC). The individual PCR products were cloned into pCR2.1 (Invitrogen) and transformed into TOP10 chemically competent *E. coli* (Invitrogen) according to the manufacturer's instructions. Plasmids were isolated from kanamycin-resistant *E. coli* colonies and a diagnostic enzyme digest was performed with EcoRI to confirm the insert was the correct size. The presence of the correct DNA sequence was confirmed by sequencing with M13 forward and reverse primers flanking the insert.

In vitro transposition and transformation of Gc. *In vitro* transposition of the plasmid clones, pCR2.1::*uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* was performed by using the EZ::Tn5<KAN-2> transposition system (Epicentre Biotechnologies). A 1:1 molar ratio of each plasmid to transposon was used for the *in vitro* transposition reaction (33). The gaps were repaired by using T4 DNA polymerase (New England Bio Labs) and T4 DNA ligase (New England Biolabs). The DNA was introduced into the chromosome of strain FA1090 1-81-S2 *recA6* by spot transformation (14) in the presence of 1 mM IPTG to allow RecA-dependent homologous recombination to take place (34). Kanamycin-resistant Gc transformants were screened by PCR using gene-specific primers to confirm that the transposon had inserted into the appropriate genes. The PCR products were then sequenced by using KAN-2_FP-1 and KAN-2_RP-1 to map the position of the transposon insertion. The primers used read out of the transposon, KAN-

2_FP-1 (ACCTACAACAAAGCTCTCATCAACC) and KAN-2_RP-1 (GCAA TGTAACATCAGAGATTTTGAG). Southern blotting was performed on all of the strains with gene-specific DIG-labeled probes (Roche) according to the manufacturer's instructions to confirm proper insertion into the correct chromosomal locus. Mutations in the NER genes were reintroduced into FA1090 1-81-S2 (wild-type *recA*) by spot transformation with genomic DNA from the *recA6* NER mutants, followed by selection for kanamycin resistance. Retention of the 1-81-S2 *pilE* was confirmed by sequencing with PILRBS and SP3A primers (45).

Complementation of *uvrB* and *uvrD*. *uvrB* and *uvrD* were complemented by using the neisserial intergenic complementation system, where full-length coding sequences of each gene are inserted between the *lctP* and *aspC* genes on the Gc chromosome, a site that has no known effect on Gc recombination or repair processes (26). The primers for complementation of *uvrB* were UVRB_PacI-1 (GCTTAATTAAGCAAACGTATCAGGCAAG) and UVRB_PacI-2 (GCT TAATTAACAAGCTGCGTACATGATTTTC), and the primers used for amplification of *uvrD* were UVRD_PacI-1 (CATTAATTAAGCGCATTCCATTTT CAGAC) and UVRD_PacI-2 (TCTTAATTAAGCCTTGCTCTGCTACG GT). The genes were amplified by using KOD Hot Start DNA polymerase (Novagen) to lessen the likelihood of any mutation occurring during amplification and cloned into PacI-digested plasmid pGCC4. The ligations were transformed into TOP10 chemically competent *E. coli* cells and plated on LB-erythromycin. Erythromycin-resistant colonies were screened for insertion of *uvrB* and *uvrD* using PacI and for correct orientation of the open reading frames using PacI, MluI, and PfiMI. Complementation constructs were introduced into the chromosome of strain FA1090 1-81-S2 by transformation. Proper transformation of all strains was confirmed by PCR and Southern blotting (data not shown), and retention of the 1-81-S2 *pilE* sequence was confirmed by DNA sequence analysis as described above.

Gc phenotypic assays. (i) UV sensitivity assay. Serial dilutions of FA1090 1-81-S2 *recA6* Gc and isogenic NER mutants were plated on GCB agar containing 1 mM IPTG and exposed to UV at fluences of 0 to 80 J/m² by using a Stratallinker 1800 (Stratagene). To test the effect of *recA* on the NER UV phenotype, dilutions of Gc were plated on GCB agar \pm 1 mM IPTG and exposed to UV fluences of 0 to 20 J/m². Survival is expressed relative to unexposed Gc of the same strain. Assays were performed four times.

(ii) H₂O₂ sensitivity assay. Survival of FA1090 Δ *pilE* Gc and isogenic *uvrA*, *uvrB*, and *uvrD* mutants after exposure to 0 to 10 mM H₂O₂ was assessed as previously described (39), except the first plate growth was limited to 10 h. Assays were performed four times.

(iii) Kinetic PDCMC assay. The kinetic assay for pilus-dependent colony morphology changes (PDCMC) was performed as previously described (33). Assays were performed four times per strain.

(iv) PilC expression analysis. FA1090 1-81-S2 *recA6 uvrD* Gc were grown on GCB without IPTG. P- colonies emerging from the culture were propagated, and the resulting lysates were separated on a 10% polyacrylamide gel and transferred to 0.45- μ m-pore-size polyvinylidene difluoride Immobilon-P membrane (Millipore). PilC expression was probed by using a polyclonal antibody (a gift from A. Jonsson), followed by horseradish peroxidase-conjugated anti-rabbit IgG (Chemicon). ECL Plus chemiluminescence (Amersham) was used to develop the blot after transfer. FA1090 Gc that are P- due to an antigenic variation event that gives rise to a truncated *pilE* sequence (8) served as a positive control for PilC expression.

(v) Sequencing assay for pilin Av. The DNA sequencing-based assay for pilin Av was performed for FA1090 1-81-S2 *recA6* and the isogenic *uvrD* mutant as previously described (8). Only P+ colonies arising from the P+ parent were passaged and examined for sequence changes at *pilE*. The assay was performed three times.

(vi) Transformation efficiency assay. Quantitative transformation was performed as previously described (23). FA1090 1-81-S2 *recA6* and isogenic NER mutants were exposed to 1 μ g of chloramphenicol-resistant Gc genomic DNA carrying a 1.1-kb cloned *cat* insertion in the *recX* locus (FA1090 Δ *recX/recX* [41]) for 30 min. The transformation frequency was measured as the number of chloramphenicol-resistant CFU arising after transformation divided by the total CFU present in the transformation mix. The assay was conducted four times.

(vii) Spontaneous mutation assay. FA1090 Δ *pilE* (wild-type *recA*) and isogenic mutants and complements in the NER system were resuspended to a concentration of 10⁷ to 10⁸ CFU/ml in GCB containing 1 mM IPTG for induction of the complement constructs. Gonococci were plated on GCB containing 50 ng of rifampin (Sigma)/ml or 0.8 μ g of nalidixic acid (Sigma)/ml, as well as on GCB to enumerate total CFU. Antibiotic-resistant CFU were enumerated after incubation for ~40 h at 37°C with 5% CO₂ and expressed as a fraction of the CFU

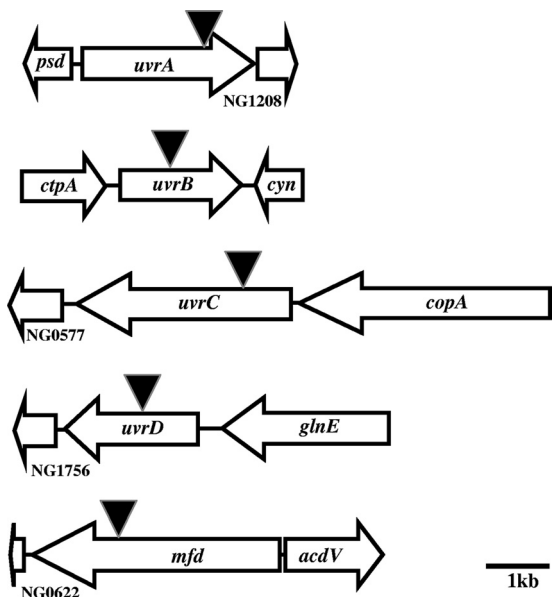


FIG. 1. Location of NER genes in the chromosome of Gc strain FA1090. The locations and orientations of *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* orthologs identified in the chromosome of Gc strain FA1090 are shown along with flanking open reading frames. The figure is drawn to scale. Bar, 1 kb. Gray triangles indicate sites of insertion of the EZ::Tn5<KAN-2> minitransposon to create insertions in each gene.

plated. The median frequency of spontaneous mutation to antibiotic resistance was determined from 10 independent experiments.

RESULTS

Mutation of NER genes affect DNA repair in *N. gonorrhoeae*.

Gene products that mediate the NER and TRCF pathways are conserved throughout evolution and play key roles in the viability of prokaryotes and eukaryotes. Homologs of the *E. coli* *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* genes were identified in the chromosome of Gc strain FA1090 (Fig. 1). Each of these genes is found at discrete sites in the chromosome (Fig. 1). Therefore, Gc appears to encode the full complement of NER and TRCF genes. Each of these genes was cloned, and a mini-Tn5 transposon containing a kanamycin resistance cassette was isolated within the coding region of each gene after *in vitro* transposition (see Materials and Methods). The mutated genes were reintroduced into the pilated Gc strain FA1090, pilin variant 1-81-S2, by DNA transformation, and antibiotic-resistant clones were isolated and verified by PCR and Southern blot analysis (data not shown).

In *E. coli*, the *uvr* gene products protect the organism from various types of DNA damage, including UV irradiation (43). To assess the involvement of the putative NER genes found in the Gc chromosome in DNA repair, we measured the sensitivity of the Gc strains lacking the NER and TRCF genes to DNA damage caused by UV light B (UV) irradiation (Fig. 2). Parental FA1090 Gc exhibited dose-dependent sensitivity to UV irradiation, with a 90% reduction in survival after exposure to 20 J/m² UV and a 3-log reduction in survival after exposure to 80 J/m² UV. As expected, inactivation of the *uvr* genes rendered Gc exquisitely sensitive to UV irradiation. Survival of the *uvr* mutants was reduced by ~4 logs relative to the parental

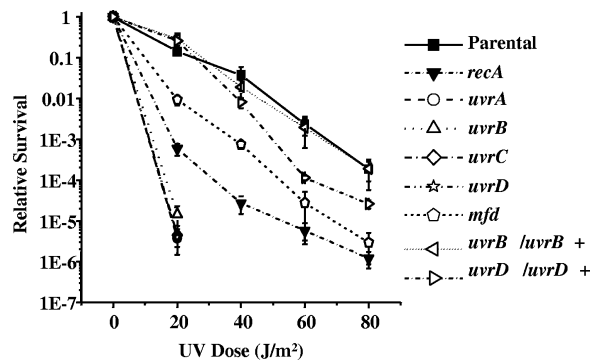


FIG. 2. Sensitivity of Gc NER mutants to UV irradiation. The FA1090 1-81-S2 parental strain and *recA*, *uvrA*, *uvrB*, *uvrC*, *uvrD*, *mfd* isogenic mutants, as well as *uvrB* and *uvrD* complements in which wild-type copies of each gene were inserted elsewhere in the Gc chromosome (*uvrB/uvrB*⁺ and *uvrD/uvrD*⁺), were irradiated with the indicated doses of UV light. Survival was calculated relative to nonirradiated bacteria of the same strain. The curves for the *uvrA*, *uvrB*, *uvrC*, and *uvrD* mutants are nearly identical. Error bars represent the standard deviation of the means of four independent experiments. No CFU were recovered from the *uvrA*, *uvrB*, *uvrC*, and *uvrD* mutants irradiated with >20 J/m² UV. The differences in survival between all NER mutants and the parent at each UV dose were significant at *P* < 0.05 (Student *t* test).

strain after exposure to 20 J/m² UV, a statistically significant decrease (*P* < 0.05 [Student *t* test]), and at higher dosages of UV there was no recovery of *uvr* mutant Gc. The decrease in survival after UV irradiation was much lower than that of the *recA*-null strain, which exhibited a 2- to 3-log decrease in survival relative to the RecA⁺ parent at all UV fluences tested. Complementation of the *uvrB* and *uvrD* mutants, achieved by supplying a wild-type copy of each gene at a second site in the Gc chromosome, restored the survival of the mutants to near-parental levels. In contrast to the *uvr* mutants, the *mfd* mutant was not as sensitive to UV as the other strains. The *mfd* mutant exhibited a 15-fold decrease in survival after exposure to 20 J/m² UV, ~10-fold higher than for the *recA* mutant, but at higher dosages of UV the *mfd* mutant showed sensitivity to UV similar to the *recA* mutant. We conclude that the *uvrABCD* genes are critical to Gc survival after UV irradiation but that the *mfd* gene product plays a minor role in protecting the bacteria from damage caused by UV light. Although complementation was only performed with the *uvrB* and *uvrD* mutants, the expected and identical UV sensitivity phenotypes of the *uvrABCD* mutants strongly suggests that none of these phenotypes are the result of polar effects on downstream genes or second site mutations.

NER is the predominant mechanism for repairing UV damage to DNA in Gc. *recA* plays an important role in recombinational DNA repair in Gc and protects the organism from damage caused by UV light (21). The extreme sensitivity of the Gc NER mutants to UV irradiation led us to examine the effect of *recA* inactivation on this sensitivity phenotype. In order to address this question, the NER genes were inactivated in a strain where the *recA* gene is under the control of *lac* regulatory elements; thus, *recA* expression can be ectopically stimulated or repressed by the presence or absence of IPTG, respectively (34). The resulting Gc strains were subjected to UV

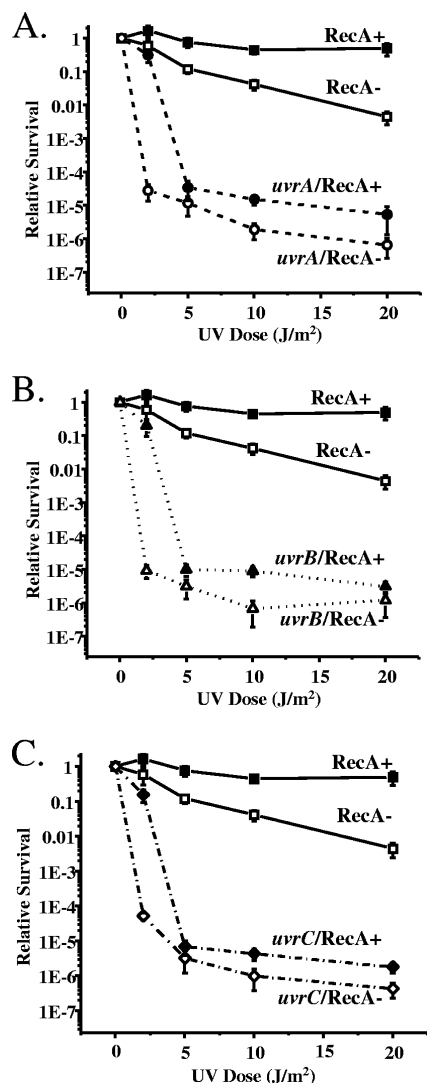


FIG. 3. RecA dependence of the sensitivity of *Gc uvrA*, *uvrB*, and *uvrC* mutants to UV irradiation. *uvrA* (A), *uvrB* (B), and *uvrC* (C) mutants were constructed in the FA1090 *recA6* genetic background, in which *recA* expression is under the control of *lac* regulatory sequences (34). The parental *recA6* strain and isogenic mutants in each of the NER genes were grown in the presence (+) or absence (-) of IPTG to induce or repress RecA expression, respectively. Gonococci were then irradiated with the indicated fluences of UV light, and survival for each strain with or without *recA* expression was calculated as in Fig. 2. Error bars represent the standard deviation of the mean of four independent experiments.

fluences ranging from 0 to 20 J/m² in the presence or absence of *recA* expression, dosages that reduced the survival of the NER mutants compared to the parental strain (Fig. 3 and 4).

We observed a bipartite effect of *recA* on the survival of the *uvrA*, *uvrB*, and *uvrC* mutants after UV irradiation (Fig. 3). At the lowest dose of UV tested (2 J/m²), removal of *recA* expression from these three mutants decreased their survival by ~4 logs. However, at doses of 5 to 20 J/m², no significant difference in survival was observed for the *uvrA*, *uvrB*, and *uvrC* mutants whether or not *recA* was also expressed. These results show that NER is the predominant repair pathway for UV-

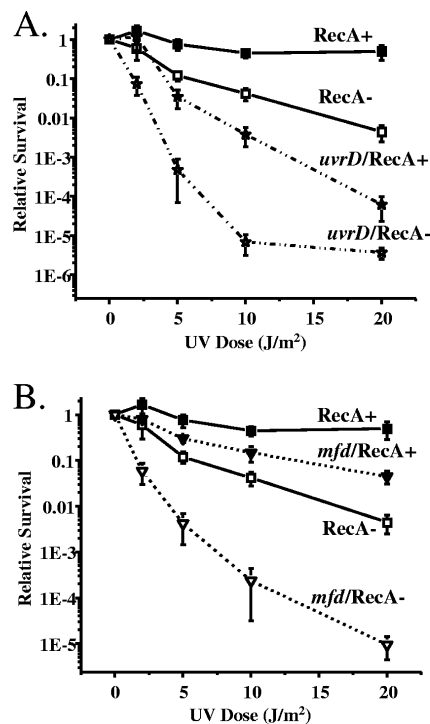


FIG. 4. RecA dependence of the sensitivity of *Gc uvrD* and *mfd* mutants to UV irradiation. *uvrD* (A) and *mfd* (B) mutants were constructed in the FA1090 1-81-S2 *recA6* genetic background, and their survival after exposure to the indicated UV fluences was assessed in the presence (+) or absence (-) of RecA expression as described in Fig. 3. Error bars represent the standard deviation of the mean of six independent experiments.

induced DNA lesions in *Gc* and that under conditions where DNA damage is minimal, RecA can aid in repairing damaged DNA by a pathway independent of the NER gene products.

In contrast to the *uvrABC* mutants, the *uvrD* and *mfd* mutants exhibited a less pronounced response to the lowered dosages of UV irradiation (Fig. 4). In the presence of *recA* expression, the *uvrD* mutant survived significantly better after exposure to 2 to 10 J/m² UV than when *recA* was not induced, but this difference was minimized at 20 J/m² UV (Fig. 4A). Thus, the effect of *uvrD* inactivation on repair of UV-damaged DNA is not as extreme as inactivation of the *uvrABC* genes. At doses of 2 to 20 J/m², inactivation of *mfd* had minimal effects on *Gc* survival after UV irradiation, indicating that TRCF participates in repairing a small subset of all NER lesions in *Gc* (Fig. 4B). The combined effect of inactivation of *mfd* and *recA* on *Gc* survival was significantly greater than inactivating either gene alone. These observations lend further credence to the hypothesis that recombinational repair and nucleotide excision repair independently correct DNA lesions induced by UV irradiation.

NER genes help protect from oxidative DNA damage. As a strict human pathogen primarily infecting the urogenital tract, *Gc* has not been regularly exposed to UV light for tens of thousands of years (25). We therefore reasoned that there must be other types of DNA damage encountered by *Gc* that are repaired by the NER system. One DNA-damaging agent *Gc* is likely to encounter during infection is reactive oxygen

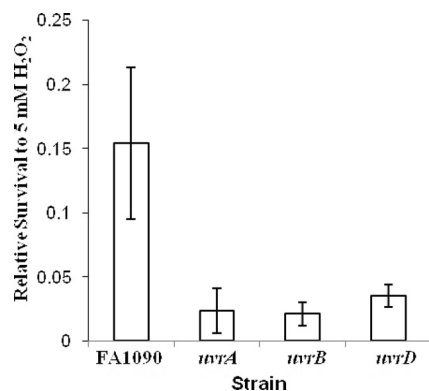


FIG. 5. Sensitivity of Gc NER mutants to oxidative damage. FA1090 Δ *pilE* and isogenic mutants in *uvrA*, *uvrB*, and *uvrD* were exposed to 5 mM H₂O₂ for 15 min. Survival was calculated relative to untreated bacteria of each strain. Experiments were repeated four times.

species (ROS), which can be produced by lactobacilli, neutrophils, and epithelial cells. To test whether one role for the Gc NER system is to protect against ROS-mediated damage, the sensitivity of FA1090 *uvrA*, *uvrB*, and *uvrD* mutants to 0 to 20 mM H₂O₂ was determined (Fig. 5 and data not shown). The NER mutants were more sensitive to 2.5 and 5 mM H₂O₂, exhibiting ~10-fold reduction in survival relative to the FA1090 parent. At higher concentrations of H₂O₂, there was no difference in survival between the NER mutants and the parental strain; surprisingly, the bacteria survived slightly better at higher H₂O₂ concentrations, for reasons that are unclear (data not shown). These results suggest that the NER system plays a role in repairing low levels of oxidative damage to DNA.

NER genes are not required for DNA transformation. Gc are naturally competent for DNA transformation (37), which can occur at frequencies as high as 10^{-1} to 10^{-2} transformants per CFU (5). We examined whether the NER mutants participated in the recombination events underlying DNA transformation by measuring the efficiency with which Gc incorporated a chloramphenicol resistance cassette into their chromosome. Parental Gc exhibited a frequency of transformation to chloramphenicol resistance of $(4.6 \pm 2.0) \times 10^{-3}$ transformants/CFU, which was not significantly different in the NER mutants (data not shown). We conclude that the NER gene products are not important for the natural competence of Gc. Due to the high frequency of pilus phase variation of the *uvrD* mutant this mutant was not tested for transformation competence (see next section).

NER genes do not participate in pilin antigenic variation, but the loss of function of *uvrD* results in a higher frequency of PilC phase variation. Gc possesses a type IV pilus that is important for host infection (27). Changes in piliation state arise rapidly during *in vitro* passage to yield colonies with characteristic morphologies. Piliated Gc give rise to “P+” colonies showing a compact, domed morphology under the stereomicroscope, while underpiliated and nonpiliated Gc yield “P–” colonies with flattened morphology (17, 42). The parental strain used in the present study, FA1090 1-81-S2, exhibits a P+ colony morphology that was retained in the *uvrA*, *uvrB*, *uvrC*, and *mfd* mutants. In contrast, we observed a high fre-

quency of kanamycin-resistant colonies with a P– morphology arising after Gc transformation with the *uvrD* mutant, and this phenotype was retained after backcross (data not shown). We validated this observation by using the PDCMC assay, which quantifies the number of P– outgrowths arising from uniformly P+ colonies over time (33). FA1090 *uvrA*, *uvrB*, *uvrC*, and *mfd* colonies grown on IPTG to induce *recA* expression produced P– outgrowths at the same rate as in the IPTG-induced parental strain, whereas the number of P– outgrowths was consistently higher in the *uvrD* mutant than in any of the other strains tested (Fig. 6A).

PDCMCs can be produced from both antigenic and phase variation events. Changes in the sequence of the *pilE* gene that encodes pilin, the major subunit of the Gc pilus, arise from a DNA recombination-based process called pilin antigenic variation (Av), while the loss of expression of the PilC protein due to slipped-strand mispairing events in the *pilC* promoter prevents presentation of the pilus on the bacterial surface (16). Pilin Av absolutely requires RecA expression, whereas PilC phase variation is RecA independent. To begin to determine whether one or both of these processes were affected in the *uvrD* mutant, we first examined whether PDCMCs occurred in the absence of RecA expression. In the absence of IPTG to induce RecA expression, FA1090 parental colonies did not give rise to any detectable P– outgrowths, in keeping with the observation that the frequency of the RecA-dependent process of pilin Av is >100-fold higher than that of PilC phase variation (unpublished observations). In contrast, P– outgrowths were still detected in the *uvrD* mutant even when *recA* was not expressed (Fig. 6B). This result suggested that the increased PDCMC in the *uvrD* mutant was attributable to increased PilC phase variation. To directly test this possibility, *uvrD* mutant Gc were grown in the absence of IPTG, and lysates from seven P– colonies arising after passage were assayed for PilC expression by immunoblotting. All of the *uvrD* P– colonies had lost PilC expression (Fig. 6C). We conclude that the *uvrD* mutant exhibits a higher frequency of phase variation of PilC, leading to an increase in the emergence of P– colonies. We attribute this increased frequency of phase variation to reports in other bacteria that in addition to NER, UvrD also participates in mismatch repair, which normally corrects slipped-strand mispairing events, as seen in PilC phase variation (24).

Since the possibility remained that the *uvrD* mutant might also exhibit a higher frequency of pilin Av, we directly measured the frequency of recombination at *pilE* using a DNA sequencing-based assay (8). The frequency of *pilE* recombination was calculated as the number of recombination events at *pilE* detected in P+ FA1090 and *uvrD* colonies arising from the parental 1-81-S2 *pilE* variant divided by the total number of *pilE* sequences analyzed. In three independent experiments, the average frequency of pilin Av in the FA1090 parent was 0.11 recombination events per CFU, as previously reported for this pilin variant (8), while the average frequency detected in the *uvrD* mutant was 0.09 events per CFU (Table 1). There were no statistically significant differences in the frequency of pilin Av between parental and *uvrD* mutant Gc in any experiment. We conclude that *uvrD* is dispensable for pilin Av, and effects of *uvrD* inactivation on pilus-dependent colony morphology in Gc are solely due to increased PilC-dependent phase variation.

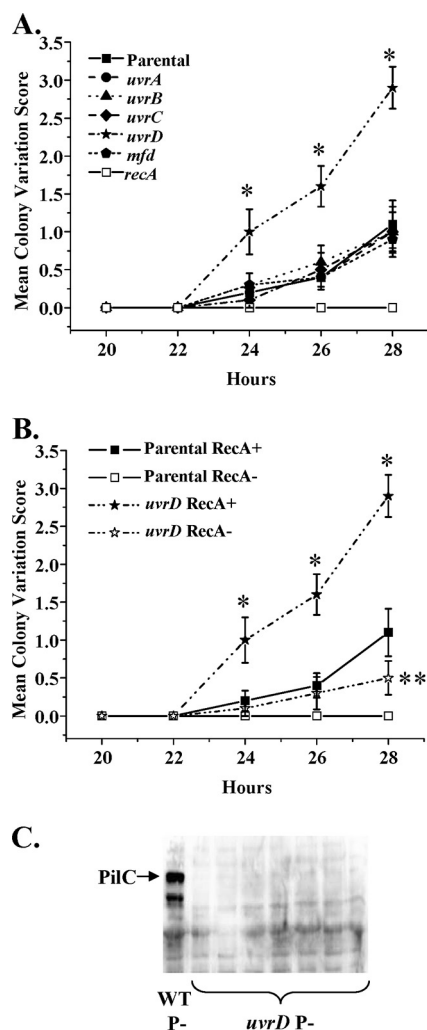


FIG. 6. *uvrD* inactivation leads to a higher frequency of PilC pilus-dependent phase variation events. (A) The parental strain FA1090 *recA6* and isogenic mutants in *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* were passaged on solid medium containing 1 mM IPTG to induce RecA expression. The appearance of P⁺ outgrowths from 10 independent P⁺ colonies was measured from 20 to 28 h as reported by Sechman et al. (33). The mean PDCMC index \pm the standard deviation from 10 independent colonies is reported from four experiments. Asterisks represent significant ($P < 0.025$ [Student two-tailed *t* test]) increases over the parental strain grown on IPTG at the indicated time points. FA1090 *recA6* that were maintained on medium lacking IPTG, which are phenotypically RecA⁻, served as a negative control for PDCMC (*recA*). (B) The parental strain FA1090 *recA6* and the isogenic *uvrD* mutant were passaged on solid medium containing 1 mM IPTG to induce RecA expression (+) or without IPTG to repress RecA expression (-). The mean PDCMC index is reported as in panel A. Asterisks represent significant (*, $P < 0.025$; **, $P < 0.05$ [Student two-tailed *t* test]) increases in PDCMC over the parental strain when maintained under the same growth conditions at the indicated time points. (C) FA1090 *recA6* *uvrD* was grown on medium lacking IPTG, and lysates from seven P⁻ CFU arising after passage were immunoblotted for PilC expression as described in Materials and Methods (lanes 2 to 8). A lysate from FA1090 Gc that is P⁻ due to a pilin antigenic variation event (WT P⁻) served as a positive control for PilC expression (lane 1).

Mutations in *uvrD* result in a statistically higher frequency of spontaneous mutations. Given the effect of a *uvrD* mutant on phase variation events in Gc, we examined whether the *uvrD* or other NER mutants yielded a higher frequency of spontaneous antibiotic resistance mutations. The antibiotics chosen for these experiments were rifampin and nalidixic acid. Rifampin inhibits the initiation of RNA synthesis by binding to the β subunit of RNA polymerase (*rpoB*), while nalidixic acid inhibits the activity of DNA gyrase encoded by *gyrB*. Point mutations in *rpoB* or *gyrB* render the bacteria resistant to rifampin or nalidixic acid, respectively (1). FA1090 Δ *pilE* Gc and each of the NER mutants were plated on solid medium containing either of these antibiotics, and the frequency of mutation was calculated as the number of antibiotic-resistant CFU divided by the total CFU arising on antibiotic-free medium. The median frequency of rifampin resistance in FA1090 Gc was 5×10^{-8} mutants per CFU and for nalidixic acid resistance was 5.6×10^{-6} mutants per CFU (Table 2). Deletion of *uvrA*, *uvrB*, *uvrC*, or *mfd* had no effect on the median frequency with which Rif^r or Nal^r colonies arose (Table 2). However, the *uvrD*-inactivated strain exhibited a 22-fold increase in spontaneous rifampin resistance and a 10-fold increase in spontaneous nalidixic acid resistance (Table 2), increases that were statistically significant relative to the FA1090 parent. Complementation of *uvrD* reduced the frequency of spontaneous mutations below what was observed for the FA1090 parent, which may be attributed to higher levels of UvrD expression driven by *lac* regulatory sequences (Table 2). The differential effect of the *uvrD* and *uvrABC* mutants on spontaneous mutation frequency indicates that NER is not important for correcting mutations that give rise to spontaneous antibiotic resistance and instead implies that the effect of the *uvrD* mutation on this process is due to an additional role in Gc mismatch repair.

DISCUSSION

Using a genetic approach, we have demonstrated that Gc possesses a complete NER pathway that participates in the repair of many types of DNA damage, including damage produced by UV light and hydrogen peroxide treatment. However, the phenotypes of the mutants suggest that the NER system has a more significant role in repairing lesions produced by UV irradiation than the damage produced by hydrogen peroxide treatment. Not surprisingly, the Gc NER system has no role in pilin antigenic variation or DNA transformation, which rely on the Rep and PriA helicases, respectively (19, 20). However, inactivation of *uvrD* demonstrated that the UvrD helicase participates not only in NER but also in mismatch correction, given its effects on PilC phase variation and spontaneous mutation (9a).

TABLE 1. Pilin antigenic variation frequencies

Strain	Avg frequency of pilin Av \pm SEM			Overall frequency
	Expt 1	Expt 2	Expt 3	
FA1090	0.11 \pm 0.02	0.10 \pm 0.03	0.10 \pm 0.02	0.11
<i>uvrD</i> mutant	0.14 \pm 0.06	0.05 \pm 0.03	0.08 \pm 0.02	0.09

TABLE 2. Spontaneous mutation frequencies

Antibiotic resistance and strain	Spontaneous mutation frequency ^a		
	Median	25th/75th percentile	Range (low/high)
Rif^r			
Parental strain	4.99E ⁻⁰⁸	0/1.44E ⁻⁰⁷	0/2.86E ⁻⁰⁶
<i>uvrA</i> mutant	0	0/0	0/2.13E ⁻⁰⁷
<i>uvrB</i> mutant	0	0/0	0/1.67E ⁻⁰⁷
<i>uvrC</i> mutant	1.18E ⁻⁰⁸	0/2.04E ⁻⁰⁷	0/4.00E ⁻⁰⁷
<i>uvrD</i> mutant	1.09E ⁻⁰⁶	3.89E ⁻⁰⁷ /2.19E ⁻⁰⁶	0/1.09E ⁻⁰³
<i>uvrD/uvrD</i> ⁺ mutant	0	0/0	0/6.25E ⁻⁰⁸
<i>mfd</i> mutant	5.30E ⁻⁰⁸	0/1.06E ⁻⁰⁷	0/1.67E ⁻⁰⁶
Nal^r			
Parental strain	5.62E ⁻⁰⁶	9.65E ⁻⁰⁷ /8.61E ⁻⁰⁶	4.21E ⁻⁰⁷ /2.33E ⁻⁰⁵
<i>uvrA</i> mutant	1.28E ⁻⁰⁶	8.11E ⁻⁰⁷ /1.73E ⁻⁰⁵	5.09E ⁻⁰⁷ /5.00E ⁻⁰⁵
<i>uvrB</i> mutant	3.14E ⁻⁰⁶	7.56E ⁻⁰⁷ /9.98E ⁻⁰⁶	3.08E ⁻⁰⁷ /2.10E ⁻⁰⁵
<i>uvrC</i> mutant	1.28E ⁻⁰⁶	3.83E ⁻⁰⁷ /7.24E ⁻⁰⁶	4.55E ⁻⁰⁸ /2.61E ⁻⁰⁵
<i>uvrD</i> mutant	5.63E ⁻⁰⁵	1.35E ⁻⁰⁵ /1.41E ⁻⁰⁴	4.94E ⁻⁰⁶ /5.81E ⁻⁰⁴
<i>uvrD/uvrD</i> ⁺ mutant	4.15E ⁻⁰⁷	1.69E ⁻⁰⁷ /9.51E ⁻⁰⁷	0/7.58E ⁻⁰⁶
<i>mfd</i> mutant	1.92E ⁻⁰⁶	6.07E ⁻⁰⁷ /3.76E ⁻⁰⁶	2.00E ⁻⁰⁷ /1.16E ⁻⁰⁵

^a The frequency of spontaneous mutation of FA1090 $\Delta pilE$ and isogenic NER mutants and complements to Rif^r and Nal^r was calculated from 10 independent experiments. The differences between FA1090 and the *uvrD* mutant are significant ($P \leq 0.005$ [Student *t* test]).

The UvrABC excinuclease from *E. coli* is the prototypical enzyme for NER systems and cooperates with the UvrD helicase and polymerase I to repair a variety of bulky lesions. NER gene expression through the SOS system is well described (6). Although Gc, like many bacteria, operates in the absence of a complete SOS system, the UV sensitivity phenotypes of the *uvrABC* mutants demonstrate that even without a classical SOS system (2), Gc NER is very efficient at repairing bulky DNA lesions such as pyrimidine dimers, but expression of these genes is not upregulated by hydrogen peroxide treatment (39). Not surprisingly, inactivation of the *mfd* gene produces a less severe UV sensitivity phenotype than the *uvrABC* mutants. In *E. coli*, TRCF (the product of the *mfd* gene) has been shown to recruit the UvrAB complex to stalled transcription complexes to first release RNA polymerase and, second, to direct NER to the damaged DNA to initiate repair of the damage (36). The original *E. coli mfd* mutant also showed a mild UV sensitivity phenotype (44) but was originally isolated as a mutant which demonstrated “a mutation frequency decline” when irradiated cells are inhibited for protein synthesis (36). We did not test for a diminished mutation frequency after irradiation of *mfd* Gc since the fastidious nature of this organism makes this analysis difficult to interpret. Since *mfd* only has a role when a pyrimidine dimer blocks transcription and the *uvrABC* gene products are important both when transcription and when replication is inhibited, the relative phenotypes fit the present models.

The effect of recombination repair on the NER mutants was tested by using the IPTG regulatable *recA6* gene, which provides near wild-type levels of RecA when induced and a phenotypically RecA⁻ cell when no IPTG is in the growth medium (34, 40). RecA expression is necessary for both RecBCD and RecF pathways of recombination, which both contribute to UV survival, but without a classical SOS system does not affect gene regulation in response to DNA damage. Thus, the effect of lowering RecA expression in wild-type cells or *uvrABC* mutants has a relatively minor effect on UV survival. This is

similar to the effect reported by Davidsen et al. in a *uvrA recA6* double mutant from the related human pathogen *Neisseria meningitidis* (11). Intriguingly, we observed a large increase in UV sensitivity in the *mfd* mutant in the absence of RecA expression. The exacerbated phenotype in the *mfd recA6* double mutant grown without IPTG suggests that recombinational repair in Gc may be linked to transcriptional stalls, but the mechanism underlying this linkage is unknown at this time.

The UvrD gene product of *E. coli* is a general helicase that participates in both NER and mismatch correction (15, 22). The UV sensitivity profile of the Gc *uvrD* mutant suggests that it contributes to NER in Gc. However, the Gc *uvrD* mutant also showed a RecA-independent increase in pilus-dependent colony phase variation, which was due to an increased frequency of *pilC* phase variation. PilC phase variation occurs by slipped-strand mispairing in a mononucleotide tract in the *pilC* promoter, a mispairing event correctable by the mismatch correction system. We postulate that the increased level of *pilC* phase variation in the *uvrD* mutant is due to the additional participation of the UvrD helicase in mismatch correction, which is further supported by the increased frequency of spontaneous mutation to antibiotic resistance in the *uvrD* mutant but not the *uvrABC* mutants. We did not observe any increased frequency of opaque colonies that result from the slipped-strand mispairing of a pentameric repeat in the *opa* gene signal sequence (29). This is not a surprising finding, since we did not observe increased *opa* gene phase variation in mismatch correction mutants (9a) and since mismatch correction does not influence slipped-strand mispairing of polynucleotide repeats with unit sizes over 4 bp (30).

One unique observation made in the present study is that the *uvr* mutants show sensitivity to oxidative killing by H₂O₂ exposure. Oxidative damage to DNA can be manifested as the presence of 8-oxoguanine, as well as a variety of other modified bases (43). *E. coli uvr* mutants have not been reported to have a strong H₂O₂ sensitivity phenotype, but human cells that are deficient in NER, obtained from patients with xeroderma pig-

mentosum, cannot remove 8-oxo-guanine from DNA (31). Oxidative sensitivity of the Gc NER mutants was only observed at lower levels of H₂O₂ (2.5 to 5 mM) and not at higher H₂O₂ concentrations. These results suggest that at high levels of ROS, either a different type of lesion is formed that cannot be repaired by the NER system, or the lethality is due to damage to molecules in addition to DNA such that NER-mediated repair does not alter the survival of the cells. We favor the latter model due to the fact that the NER system is very effective at repairing high levels of UV-induced damage and that H₂O₂ can damage other molecules, as well as DNA (12).

As an obligate human pathogen whose close relatives are also human specific, most members of the genus *Neisseria* have not been regularly exposed to UV light for tens to hundreds of thousands of years. Examination of the NER system in Gc allows us to explore why continued expression of this repair system has been selected for throughout evolution, even among organisms such as Gc that are not expected to encounter substantial UV-induced DNA damage. The present study conclusively shows that Gc possesses a complete NER system that is functional in its ability to protect the organism from DNA damage, including that caused by UV irradiation, but does not substantially contribute to natural transformation, pilin Av, or repair of spontaneous mutations. Thus, we speculate that Gc encounters other DNA-damaging agents within humans that are repaired, at least in part, by the NER system. Gc are hypothesized to encounter ROS, such as H₂O₂, during human infection, and here we have shown that the NER system in Gc can protect the organism from oxidative damage. One potential source of damage-producing oxidants are neutrophils, which migrate abundantly into the genitourinary tract during acute infection, but results from our lab and others suggest that neutrophils do not produce large quantities of ROS upon infection with Gc, at least when actively growing (9). ROS is also produced by cervical epithelial cells and commensal lactobacilli, and we speculate that the Gc NER system may have been retained to counter these insults (32, 38). In addition, Gc grown with aeration generate ROS, and the NER system may protect Gc from their own by-products of oxidative metabolism. Together, these results indicate that there are broader roles for NER than simply protecting organisms from UV irradiation, underscoring the importance of retaining this ancient, conserved repair system in prokaryotes and eukaryotes alike.

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