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Closely opposed apurinic/apyrimidinic sites are converted to double strand breaks in *Escherichia coli* even in the absence of exonuclease III, endonuclease IV, nucleotide excision repair and AP lyase cleavage

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Abstract

Multiply damaged sites (MDSs) consist of two or more damages within 20 base pairs (bps) and are introduced into DNA by ionizing radiation. Using a plasmid assay, we previously demonstrated that repair in *E. coli* generated a double-strand break (DSB) from two closely opposed uracils when uracil DNA glycosylase initiated repair. To identify the enzymes that converted the resulting apurinic/apyrimidinic (AP) sites to DSBs, repair was examined in bacteria deficient in AP site cleavage. Since exonuclease III (*xth*) and endonuclease IV (*nfo*) mutant bacteria were able to introduce DSBs at the MDSs, we generated unique bacterial mutants deficient in UvrA, Xth and Nfo. However, the additional disruption of nucleotide excision repair (NER) did not prevent DSB formation. *xth nfo nfi* bacteria also converted the MDSs to DSBs, ruling out endonuclease V as the candidate AP endonuclease. By using MDSs containing tetrahydrofuran (an AP site analog), it was determined that even in the absence of Xth, Nfo, NER and AP lyase cleavage, DSBs were formed from closely opposed AP sites. This finding implies that there is an unknown enzyme/repair pathway for MDSs, and multiple underlying repair systems in cells that can process closely opposed DNA damage into lethal lesions following exposure to ionizing radiation.

Keywords

Multiply damaged sites; clustered DNA lesions; ionizing radiation DNA damage; DNA repair; AP endonuclease; AP sites

1. Introduction

Ionizing radiation and chemotherapeutic agents introduce many types of base modifications into DNA, as well as abasic (AP) sites, single strand breaks (SSBs) and double strand breaks (DSBs; 1). However, due to the production of low energy secondary electrons, X- and γ -rays produce clusters of ionizations, causing the production of multiple lesions within 20 bps of DNA (2;3). These multiple lesions are called clustered lesions or multiply damaged sites (MDSs). Although the simplest MDS is a DSB, these complex lesions can contain oxidized purines and pyrimidines, AP sites and SSBs in the same (4;5) or opposing DNA strands (6; 7). MDSs consisting of closely opposed base damages, SSBs or AP sites are of biological relevance as it is possible for base excision repair to convert two opposing damages into two opposing SSB-repair intermediates, and hence a DSB, which is a potentially lethal lesion.

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Repair of defined MDSs using prokaryote and eukaryote proteins or extracts has been studied extensively *in vitro* (for review see 8;9;10). In general, if two lesions are situated in opposite strands and greater than 2 bps apart, the DNA glycosylases and AP endonucleases initiate repair by removing the base damage/ AP site in the MDS and introduce two closely opposed SSBs. We have developed an assay to determine whether MDSs are converted to DSBs in cells (11). The synthetic MDS is ligated into the luciferase coding region in a plasmid (p3'luc) and transformed into bacteria. After allowing time to repair the DNA in the absence of DNA replication, the luciferase activity is measured. A decrease in luciferase activity compared to the undamaged sequence indicates the formation of a DSB. It is also possible to determine the effect of the MDS on plasmid survival by growing the bacteria on solid medium containing carbenicillin. Using this assay we have studied the repair of model MDSs in bacteria where the lesions are situated at specific distances apart in the opposite strands. The loss of luciferase activity and decrease in plasmid survival is lesion dependent as well as dependent upon the distance separating the damages. Two closely opposed 8-oxo-7, 8 dihydroguanines (8-oxodGs) or an 8-oxodG opposite a uracil do not result in the formation of a DSB (12), although a closely opposed 8-oxodG (12;13) or uracil (14) results in an enhanced mutation frequency of 8-oxodG. This suggests that the opposing lesion is inhibiting the repair of the 8-oxodG. However, using our repair assay two uracils situated ≤ 7 bp apart dramatically reduce luciferase activity and result in destruction of the plasmid when repair is initiated by uracil DNA glycosylase in bacteria (11). This indicates that the clustered uracil lesions are converted to DSBs. When the uracils are separated by 13-33 bp luciferase activity is not lost, indicating the MDS can be completely repaired. Our work is therefore in agreement with Dianov et al (15) who showed that, using a plasmid system that allowed replication, two opposing uracils 12 bps apart resulted in an increase in the deletion frequency. Following removal of uracil by uracil DNA glycosylase (Ung), DNA is predominantly cleaved at the 5' side of the AP site forming a SSB by exonuclease III (Xth) or endonuclease IV (Nfo). We previously examined the repair of two uracils situated in opposite DNA strands immediately 5' to each other or separated by 5 bps in bacteria deficient in Xth and Nfo (11). These MDSs reduced luciferase activity, indicating another enzyme or repair system could cleave at the AP sites. AP lyases, associated with certain DNA glycosylase activities, can cleave at the 3' side of the AP site (16), endonuclease V (Nfi) is able to hydrolyze the second phosphodiester bond 3' to an AP site (17), and AP sites can be removed from DNA by nucleotide excision repair (NER, 18;19).

The work described in this paper employs a variety of bacterial strains deficient in DNA repair enzymes and model MDSs, to try to determine which *E.coli* enzymes can cleave at AP sites situated in MDSs. This is the first report to utilize bacteria deficient in UvrA, Xth and Nfo as an informative system for dissecting the roles of repair pathways involved in the processing of MDSs. By using this bacterial mutant deficient in NER and the major AP endonucleases and MDSs containing tetrahydrofuran to eliminate cleavage by AP lyases in the cell, we have shown that there is an alternate repair mechanism or enzyme in *E.coli* that can cleave at AP sites in MDSs to form DSBs. This study demonstrates the detrimental effect of MDSs compared to single lesions. Attempts to repair AP sites even in a small number of MDSs in a cell's genome could have a severe impact on cell survival due to the formation of lethal lesions.

2. Materials and methods

2.1 Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (oligonucleotides) were purchased from Operon Technologies Inc. (Alameda, CA), contained 5' phosphate termini and were purified by polyacrylamide gel electrophoresis. The sequences of the double-stranded oligonucleotides containing MDSs are shown in Table 1. A uracil, thymine or a tetrahydrofuran (known as a d-spacer from Operon Technologies) was placed at X in the sequence (Table 1). An *in vitro* assay did confirm that

the tetrahydrofuran in the oligonucleotide could be cleaved by endonuclease IV, but not by Fpg, even though the Fpg could cleave an AP site generated from a uracil situated in the same DNA sequence. The oligonucleotides used for PCR are as follows:

Primer 1 d(TGGATGGCTACATTCTG)

Primer 2 d(GTCATCGTCGGGAAGACCTGCCACGCC)

2.2 Bacteria

Wild-type *E. coli* (strain BW35- Hfr KL16(PO-45) *thi-1,relA1, spoT1,e14⁻, λ⁻*), *xth⁻* (Δ [*xth-pnca*]90), *nfo⁻* (kanamycin-resistant, *nfo-1::kan*) and *xth⁻nfo⁻* (kanamycin-resistant) bacteria were obtained from Dr. Susan S. Wallace (University of Vermont, Burlington, VT). Tetracycline-resistant uracil DNA glycosylase-deficient (*ung⁻*) bacteria (BD2008, a lambda-minus derivative of BD2007, 20) were obtained from Dr. Bernard Weiss (Emory University, Atlanta, GA). The tetracycline-resistant UvrA-deficient bacteria and the kanamycin and tetracycline-resistant *uvrA⁻xth⁻nfo⁻* were constructed as described below and were handled and grown in dim light. All of these strains were isogenic. *E. coli* deficient in *xth⁻nfo⁻* (RPC501) and *xth⁻nfo⁻nfi⁻* (BW1163) with an AB1157 genetic background (*thr-1, araC14, leuB6*(Am), Δ (*gpt-proA*)62, *lacY1, tsx-33, qsr⁻, glnV44*(AS), *galK2*(Oc), $\lambda⁻, Rac-0, hisG4(Oc), *rfbD1, mgl-51, rpoS396*(Am), *rpsL31*(strR), *kdgK51, xylA5, mtl-1, argE3*(Oc), *thi-1*) were also obtained from Dr. Bernard Weiss (Emory University, Atlanta, GA). These strains are both resistant to kanamycin and BW1163 is also resistant to chloramphenicol. Bacteria were grown in the appropriate antibiotic(s) at 40 µg/ml kanamycin, 10 µg/ml tetracycline and 34 µg/ml chloramphenicol either on solid growth medium or in liquid culture during the preparation of electrocompetent bacteria. Electrocompetent bacteria were generated according to Seidman et al (21), except that the bacteria were harvested at an optical density (600 nm) of 0.2 for the *uvrA⁻xth⁻nfo⁻* bacterial strain.$

2.3 Construction of UvrA-deficient bacterial strains

To generate strains deficient in nucleotide excision repair we obtained the N3055 strain ($\lambda⁻, *IN(rrnD-rrnE)1, uvrA277::Tn10*) from the *E. coli* Genetic Stock Center (Yale University, New Haven CT) and *E. coli* bacteriophage P1 (P1kc) transducing phage (ATCC® 25404-B1) from the ATCC (Manassas, VA). To prepare a P1 lysate carrying the *uvrA277::Tn10* mutation, an overnight culture of N3055 was diluted 100 times into LB, 0.2% glucose and 5 mM CaCl₂. After 60 minutes of growth, $\sim 5 \times 10^8$ P1 phage particles were added and growth continued for 3 hours. After addition of chloroform, the debris was removed by centrifugation and the clear P1 lysate was isolated and vortexed again with chloroform. The P1 lysate was grown on solid medium containing tetracycline to ensure there was no bacterial contamination. To transduce the BW35 strain (or *xth⁻nfo⁻*, KL16 background strain) an overnight culture was resuspended in 10 mM MgSO₄/ 5 mM CaCl₂. After 20 minutes at 37°C, 0.1 ml culture was mixed with the P1 lysate and the cells incubated at 30°C for 30 minutes prior to addition of 0.1 ml 1 M sodium citrate and 1 ml LB. After 30 minutes at 37°C, the bacteria were centrifuged, resuspended in LB and 4 mM sodium citrate and grown on solid medium containing 10 mM sodium citrate and 10 µg/ml tetracycline for BW35, and 40 µg/ml kanamycin and 10 µg/ml tetracycline for the *xth⁻nfo⁻* strain. Colonies were seen after overnight growth for the transduction into BW35, however, approximately six more hours were required to obtain small colonies from the *xth⁻nfo⁻* transduction. Colonies were re-grown on solid medium containing the appropriate antibiotic. To identify colonies that contained the *uvrA* mutation, bacteria were streaked onto solid medium and half of the plate was irradiated with 100 J/m² for *uvrA⁻* or 60 J/m² for *uvrA⁻xth⁻nfo⁻* using 254 nm light from a UV Stratalinker® 2400 (Stratagene®, La Jolla, CA). Bacteria that only grew on the unirradiated side of the plate were considered sensitive to UV light and were tested further using a colony forming assay.$

2.4 Cell survival studies

Sensitivity to UV light was performed using early stationary phase cultures. Bacteria were grown to stationary phase overnight in LB and the appropriate antibiotic. The culture was diluted 25 times into 2×TY medium (22) supplemented with 0.1% glucose and grown at 37°C at 250 rpm for 5 hours. This was diluted 100 times in phosphate buffered saline (PBS, GIBCO™, Invitrogen Corporation, Grand Island, NY) and 5 mls irradiated in a 100 mm Petri dish on ice. The UV light was a 254 nm bulb positioned at a height that resulted in a dose of 0.4 J/m²/s. The cultures were exposed to the UV light for the time required to deliver between 0 and 15 J/m², depending on the bacterial strain. The bacteria were then diluted to obtain colonies after overnight growth on solid medium containing the appropriate antibiotic. The protocol was modified slightly for the *uvrA⁻ xth⁻ nfo⁻* bacterial strain, since it was determined that the bacteria had a slower growth rate and reached stationary phase at a lower optical density than the other mutants and wild-type bacteria. After overnight growth, the culture was diluted only 8 times in 2 × TY medium and 0.1% glucose in order to reach a stationary phase culture after 5 hours. After irradiation and re-plating on solid medium, the colonies were allowed to grow longer (~5 hours) prior to counting. The percent survival was calculated from the number of colonies obtained for irradiated and untreated bacteria.

To exam the sensitivity of the bacteria to tert-butyl hydroperoxide (t-BuO₂H), solid medium (50 ml) was prepared containing a gradient of the chemical. Growth was examined on plates containing different amounts of t-BuO₂H. To prepare the gradient plates, 0-1.5 µl of t-BuO₂H (70 % solution, Sigma-Aldrich, St.Louis, MO) was added to 25 mls of medium (LB, 1.5% agar) and poured into a square plate positioned at an angle and the medium allowed to solidify. The plate was then positioned on a flat surface and a further 25 mls of medium (LB, 1.5% agar) poured on top. Once the medium was solid, single colonies were used to inoculate 8 cm lines of bacteria across the gradient plate. A single colony from each strain was used to establish a line on each plate. The length of the bacterial growth for each strain was measured after an overnight incubation at 37°C.

2.5 Plasmids

pACYC184 (New England Biolabs, Beverly, MA) is a low copy vector with a p15A origin of replication that encodes resistance to chloramphenicol (34 µg/ml) and tetracycline (25-50 µg/ml). p3'luc (11) is a derivative of pBestluc (Promega, Madison, WI). It is a high copy vector with a pUC origin of replication that encodes resistance to ampicillin or carbenicillin (50 µg/ml) and expresses the firefly luciferase open reading frame from a tac promoter.

2.6 DNA repair assay

p3'luc contains unique Pac I and Cla I restriction sites within the 3' end of the firefly luciferase coding region. After the plasmid is linearized with Pac I and Cla I, a 45 bp double-stranded oligonucleotide carrying no damage, a single lesion or a MDS (Table 1) is reinserted into the firefly luciferase open reading frame. Failure to insert the 45 bp oligonucleotide, or breakage or deletion of the DNA at this site results in loss of luciferase activity. Preparation of linearized plasmid and the annealing of complementary oligonucleotides are described in detail in D'souza & Harrison (11). A 30 µl ligation reaction was prepared containing 1.5 µg (~600 fmoles) of linear p3'luc, 1.8 pmoles double-stranded oligonucleotide, 1 mM rATP, 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 1 mM dithiothreitol and 6 units T4 DNA ligase (Stratagene, La Jolla, CA) and incubated overnight at 4°C. Salts were removed from the DNA using the Qiaquick Nucleotide Removal kit (Qiagen Inc., Valencia, CA) and the DNA eluted using 30 µl 10 mM Tris, pH 8.5. Electrocompetent bacteria were co-transformed with 4 µl (~200ng) of the ligation reaction and 0.1-2 ng of pACYC184 at 2.5 kV, 200Ω, 25 µF. Following transformation, the bacteria were incubated at 37°C and 250 rpm for 4 hours in 1.5 ml Luria-Bertani medium (LB) containing 80-100 µM novobiocin (Sigma-Aldrich, St. Louis, MO) and 1 mM IPTG (Sigma-

Aldrich, St. Louis, MO). After 4 hours, the culture was grown on triplicate plates of solid medium to determine the level of transformation of each sample. The medium contained chloramphenicol (34 µg/ml) for the wild-type bacteria, and was also supplemented with tetracycline (10 µg/ml) for *uvrA*⁻ and *ung*⁻, kanamycin (40 µg/ml) for *xth*⁻*nfo*⁻, tetracycline and kanamycin for *uvrA*⁻*xth*⁻*nfo*⁻, or kanamycin and tetracycline for *xth*⁻*nfo*⁻*nfi*⁻ bacteria. Since the *xth*⁻*nfo*⁻*nfi*⁻ are resistant to chloramphenicol and kanamycin, tetracycline was required to select for bacteria carrying pACYC184. To measure luciferase activity, cell-free extracts were prepared from 1 ml of the bacterial culture and 20 µl of extract was mixed with luciferase assay reagent (100 µl; Promega, Madison, WI) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The relative light units/ chloramphenicol resistant colony (or / tetracycline resistant colony for *xth*⁻*nfo*⁻*nfi*⁻ bacteria) were calculated. This activity for each transformation was expressed as a percentage of the activity of the undamaged sequence.

2.7 Plasmid survival assay

Electrocompetent bacteria were co-transformed with 1 µl (~50 ng) of the ligation reaction and 0.1 ng pACYC184 at 2.5 kV, 200Ω, 25 µF. Following transformation, the bacteria were incubated at 37°C and 250 rpm for 4 hours in 1.5 ml LB medium containing 100 µM novobiocin (Sigma-Aldrich, St. Louis, MO) and 1 mM IPTG (Sigma-Aldrich, St. Louis, MO). After 4 hours, the culture was grown overnight on triplicate plates of chloramphenicol (34 µg/ml) or carbenicillin (50 µg/ml)-containing solid medium. The solid medium was also supplemented with kanamycin and/tetracycline depending on the bacterial strain (see above). The number of colonies was counted and a ratio calculated for the number of carbenicillin-resistant (Carb^R)/ the number of chloramphenicol-resistant (Cm^R) colonies. The Carb^R colonies were transferred to a nylon membrane and sprayed with luciferase reagent and the number of colonies that emitted light was determined. Individual Carb^R colonies that did not emit light were used to inoculate a 3 ml LB-carbenicillin culture and plasmid DNA isolated after overnight growth. PCR reactions containing plasmid DNA were performed using 125 nM each of primers 1 and 2 in a 50 µl reaction containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl₂, 200 µM each of dGTP, dCTP, dATP and dTTP, and 2 units Taq DNA polymerase (Promega, Madison, WI). The reactions were performed using a MyCycler™ (BIO-RAD, Hercules, CA) for thirty cycles using an annealing temperature of 50°C. Products were visualized following electrophoresis through a 1.8% agarose gel.

2.8 Statistical analysis

To determine whether the same lesion was processed differently by the different bacteria strains, the data for each type of lesion used in the DNA repair assay was analyzed using the Instat3 program and the unpaired t test. This test was also used to analyze the data from the plasmid survival studies, which compared the Carb^R/Cm^R ratios for ligations transformed into the same bacterial strain.

3. Results

3.1 Generation of nucleotide excision repair-deficient mutants

UvrA is the damage recognition protein of NER. To generate bacteria deficient in NER the *uvrA277::Tn10* mutation was transferred into the KL16 wild-type and *xth*⁻*nfo*⁻ bacteria. The transduction of the *uvrA277::Tn10* into the wild-type KL16 strain was efficient and the colonies grew well. However, extra time (~6 hours) was required to detect small colonies that were kanamycin and tetracycline-resistant following transduction of the *xth*⁻*nfo*⁻ strain. The slower growing colonies that re-grew on antibiotic-containing solid medium were found to be UV sensitive by the initial screen. Previously, Saporito et al (23) were unable to generate a viable triple mutant of *uvrA*, *xth* and *nfo* in the AB1157 background using the *uvrA6* mutation. To confirm that the KL16 triple mutant had altered growth characteristics, we performed growth

studies at 37°C and 250 rpm by monitoring the optical density at 600 nm. We determined that the wild-type, *xth*⁻*nfo*⁻ and *uvrA*⁻ bacteria grew at a similar rate (doubling time of 26-28 minutes), while two of the original *uvrA*⁻*xth*⁻*nfo*⁻ colonies that were expanded to make new strains grew at a slower rate (doubling time 37-38.5 minutes). These times were determined from three experiments for each of the *xth*⁻*nfo*⁻ and *uvrA*⁻*xth*⁻*nfo*⁻ strains and two experiments for each of the wild-type and *uvrA*⁻ strains. To confirm that NER was disrupted in the putative *uvrA*⁻ strains, a colony-forming assay was performed to determine the sensitivity of the bacteria to 254 nm UV light (Figure 1A). Both the *uvrA*⁻ and the *uvrA*⁻*xth*⁻*nfo*⁻ bacteria were very sensitive to UV light and the triple mutant was more sensitive than the single mutant. Previously, Cunningham et al (24) reported a slight sensitization of an AB1157 *xth*⁻*nfo*⁻ strain to UV light, and as can be seen from Figure 1A the KL16 *xth*⁻*nfo*⁻ bacteria were also sensitive to the treatment. To confirm the triple mutant was deficient in *xth* and *nfo*, single colonies of the different strains were used to inoculate individual lines of bacteria across solid medium containing a gradient of t-BuO₂H. Solid medium containing different doses of the chemical were tested. As can be seen from Figure 1B, the sensitivity of the strains to t-BuO₂H was in the order of *uvrA*⁻*xth*⁻*nfo*⁻ > *xth*⁻*nfo*⁻ > *uvrA*⁻ > *xth*⁻ = *nfo*⁻ > wild-type. This is in agreement with Cunningham et al (24) who demonstrated that the *xth*⁻*nfo*⁻ bacteria were more sensitive to t-BuO₂H than each of the single mutants on a gradient plate. We have therefore generated a unique mutant that is deficient in NER and the major *E.coli* AP endonucleases. This bacterial strain will be important for the study of how DNA repair systems manage DNA damage generated by endogenous as well as exogenous reactive oxygen species.

3.2 Repair of two closely opposed uracil lesions

Our previous study (11) demonstrated that two opposing uracils situated ≤ 5 bps apart decreased luciferase activity in bacteria deficient in the two major AP endonucleases, Xth and Nfo. This indicates that enzymes other than Xth and Nfo can convert the MDS to a DSB. To determine if NER was involved in breaking the DNA, we repeated the study using *uvrA*⁻ bacteria (Figure 2A). The results were identical to those of our previous study using wild-type bacteria, i.e. activity was decreased if the lesions were ≤ 7 bps apart, with more severe breakage occurring if the lesions were situated 3' to each other, and the MDSs were repaired if the uracils were 13-33 bp apart. We extended our study of *xth*⁻*nfo*⁻ bacteria to include more MDSs. As can be seen from Figure 2B, the two closely opposed uracils dramatically decreased luciferase activity when they were ≤ 7 bps apart. Unlike the *uvrA*⁻ bacteria, activity did not increase as the lesions moved from immediately 5' to each other to 7 bps apart, and even when the uracils were 13-33 bps apart the activity was ~ 50% of that found with the *uvrA*⁻ mutant. A virtually identical result was found using the triple mutant deficient in UvrA, Xth and Nfo (Figure 2C). We also examined MDSs containing two uracils 0, 7 and 13 bps apart in *xth*⁻*nfo*⁻ and *xth*⁻*nfo*⁻*nfi*⁻ bacteria with an AB1157 genetic background. Again severe decreases in luciferase activity were seen using this *xth*⁻*nfo*⁻ mutant (data not shown). The results using the *xth*⁻*nfo*⁻*nfi*⁻ bacteria (Figure 2D) were very similar to the *xth*⁻*nfo*⁻ mutant, with only small decreases in luciferase activity detected in the triple mutant compared to the double mutant for the same lesions. This work indicates that NER and endonuclease V are not responsible for the cleavage of the MDSs in the absence of Xth and Nfo.

3.3 Repair of a uracil closely opposed to a furan

A furan is an analog of an AP site that cannot be cleaved by β-elimination (25) and so is not a substrate for the AP lyase activity associated with DNA glycosylases such as endonuclease III, endonuclease VIII and formamidopyrimidine DNA glycosylase (Fpg). This lesion can therefore be used to distinguish an AP endonuclease from an AP lyase, since Xth and Nfo can cleave at the 5' side of a furan (25). A MDS consisting of a uracil closely opposed to a furan can therefore not be converted to a DSB by an AP lyase even after Ung has initiated repair.

For this study, the uracil was positioned in the non-transcribed strand and the furan in the transcribed strand. *In vitro* studies (26) have shown that *E. coli* RNA polymerase is blocked not by an AP site, but by the SSB-repair intermediate generated from the AP site. However, as can be seen from Figure 3, a single furan situated in the transcribed strand of p3'luc did not alter luciferase activity in the bacterial strains used in this study.

To form MDSs, the uracil and the furan were positioned in opposite strands and immediately 5' or 3' to each other or separated by 5 or 20 bps. When the assay was performed in bacteria deficient in Ung, all the ligations containing MDSs produced luciferase activity similar to the undamaged sequence (Figure 4A). In the absence of Ung, base excision repair cannot initiate repair at the uracil. Without the removal of the uracil, this lesion cannot result in a SSB-repair intermediate, and hence the MDS cannot be converted to a DSB even if the opposing furan is cleaved. The experiment using *ung*⁻ bacteria therefore demonstrated that all the ligations containing MDSs generated the same amount of circular product as the undamaged sequence. In wild-type bacteria (Figure 4B), MDSs with lesions separated by ≤ 5 bps dramatically decreased luciferase activity compared to the undamaged sequence. When the uracil and furan were situated 20 bps apart, the luciferase activity was only reduced to 81%. However, this was significantly lower than the result for the same lesion in *ung*⁻ bacteria, which suggests that even a uracil and a furan 20 bps apart cannot be completely repaired in the wild-type bacteria. These MDSs with lesions separated by 0-20 bps in *uvrA*⁻ (Figure 4C), *xthnfo*⁻ (Figure 4D), and *uvrA*⁻*xth*⁻*nfo*⁻ (Figure 4E) bacteria produced decreases in activity similar to that seen in the wild-type bacteria. Analysis of MDSs consisting of a uracil and a furan in the AB1157 *xth*⁻*nfo*⁻ (data not shown) and *xth*⁻*nfo*⁻*nfi*⁻ (Figure 4F) mutants also resulted in decreased luciferase expression for all the MDSs. Only small differences were found when the data from the two AB1157 strains were compared. These experiments indicate that in *E. coli* there is an unidentified enzyme or system that can generate a DSB from a MDS containing AP sites when the AP lyases, Xth, Nfo and NER are unable to repair the damage. The work with the *xth*⁻*nfo*⁻*nfi*⁻ mutant suggests that endonuclease V is not the candidate enzyme.

3.4 Repair of two closely opposed furans

Two furans situated 5' to each other in opposite DNA strands and situated 5 bps apart decreased luciferase activity to 1-2% in wild-type and *uvrA*⁻ bacteria (Figure 5A), while mutants defective in Xth and Nfo decreased activity to 24-33% of the undamaged sequence. Therefore even though the alternate repair enzyme/system was able to introduce a DSB at this MDS in the absence of Xth and Nfo, processing of the two furans to a DSB was not as effective as in wild-type cells. Comparison of the *xth*⁻*nfo*⁻ results for two furans 5 bps apart, and a uracil and a furan 5 bps apart (Figure 4D and E) also indicates that the alternate system is more effective at generating a DSB from the MDS consisting of a uracil and a furan. Xth and Nfo are therefore important for the processing of two opposing furans.

When the furans were separated by 20 bps, the activity decreased to only ~70% in the wild-type bacteria (Figure 5B). This same lesion produced similar decreases in activity in the *uvrA*⁻ and *uvrA*⁻*xth*⁻*nfo*⁻ strain. Even though there was a significant difference for two furans separated by 20 bps in the *xth*⁻*nfo*⁻ strain compared to the wild-type, the difference was small (WT = $70 \pm 7\%$ and *xth*⁻*nfo*⁻ = $47 \pm 5\%$ for the average and standard error) and there was not a significant difference between the *xth*⁻*nfo*⁻ and *uvrA*⁻*xth*⁻*nfo*⁻. This suggests that two furans 20 bps apart are repaired to a similar extent in the wild-type and the mutant bacteria.

3.5 Plasmid survival assay

A decrease in luciferase activity indicates that the plasmid was converted to a DSB, and either the linear plasmid was destroyed or it was mis-repaired resulting in a deletion of sequence in the luciferase coding region. To determine whether the plasmid was destroyed, the culture was

grown on carbenicillin-containing solid medium, as well as chloramphenicol-containing solid medium. If the plasmid is destroyed during repair the ratio of the p3'luc (Carb^R)/pACYC184 (Cm^R) will decrease compared to the undamaged sequence, while mis-repair will result in a similar Carb^R/Cm^R ratio with a higher percentage of colonies expressing inactive luciferase. The control ligation, which contained only linear p3'luc, produced only 3-6% of the amount of Carb^R colonies generated from the ligation with the undamaged sequence in all the bacterial strains (Table 2) and 94-100% of these colonies did not express active luciferase. PCR analysis of the plasmid from colonies that did not express active luciferase showed that 85-100% was deleted in the oligonucleotide insert, as previously found (11). A single furan, however, did not reduce the Carb^R/Cm^R in any of the bacterial strains. It was found that Carb^R colonies from ligations that produced a Carb^R/Cm^R ratio similar to the undamaged sequence predominantly expressed active luciferase (> 90%); where as 30-90% of colonies from ligations with a decreased ratio expressed inactive luciferase. Using PCR, it was determined that the plasmid from the majority of these colonies was deleted in the oligonucleotide insert between Pac I and Cla I, as seen in the control ligation. This was therefore likely due to the ligation of linear DNA in the *in vitro* ligation reaction, as previously seen (11;12), and not due to MDS mis-repair.

In *ung*⁻ bacteria, all the MDSs that contained uracil resulted in similar Carb^R/Cm^R ratios as the undamaged sequence, while in wild-type bacteria, MDSs containing uracil separated from the second opposing lesion by ≤ 7 bps caused a dramatic reduction in plasmid survival (3-5%). Therefore the plasmid was only converted to a DSB and destroyed when Ung initiated repair of the uracil(s) in the MDS. Bacteria deficient in Xth and Nfo also destroyed plasmid containing two uracils separated by 7 bps, and a uracil and a furan separated by 5 bps (Table 2). When these lesions were separated by 20 bps the ratio was reduced to ≤ 50% in these bacteria, however it was only a statistically significant reduction in the *uvrA*⁻ *xth*⁻ *nfo*⁻ mutant.

Two furans separated by 5 bps in wild-type and *ung*⁻ bacteria dramatically reduced plasmid survival (~3%), while survival was reduced to only 17-19% in cells deficient in Xth and Nfo (Table 2). A reduced colony-forming ability for a plasmid containing two closely opposed furans was also reported by Takeshita & Eisenberg (27) for wild-type bacteria and it was suggested that the furans blocked DNA replication. However, a single furan in their study and in ours (Table 2) had little effect on the number of colonies obtained from the plasmid, and MDSs consisting of a uracil and a furan in *ung*⁻ bacteria and two furans separated by 20 bps in wild-type bacteria replicated as well as the undamaged sequence (Table 2).

The plasmid survival studies therefore indicate that the reduction in luciferase activity seen with the MDSs in the DNA repair assay was due to the formation of DSBs and destruction of the plasmid. The data also confirms that there is an alternate system generating DSBs in the absence of Xth, Nfo and the AP lyases.

4. Discussion

We have used model MDSs containing two opposing uracils, a uracil and a furan or two opposing furans to try to identify the *E.coli* enzymes that can convert opposing AP sites to DSBs in bacteria. A MDS consisting of two uracils can be converted to two AP sites by Ung. In fact, without the initiation of repair by Ung two closely opposed uracils positioned in p3'luc do not decrease luciferase activity, indicating that a DSB is not formed until the uracil is removed (11). The prime candidates for generating the DSB from opposing uracils ≤ 7 bps apart following Ung (11) are Xth and Nfo, the major *E.coli* AP endonucleases. However, we previously showed that even in the absence of Xth and Nfo, two closely opposed uracils ≤ 5 bps apart resulted in a dramatic decrease in luciferase activity (11). The other proteins in bacteria known to cleave at AP sites are endonuclease V (17), the DNA glycosylases (for review see 16) with associated AP lyase activity (Fpg, endonuclease III and VIII) and NER (18;19).

In order to study the involvement of NER in MDS repair, we generated a mutant deficient in UvrA, Xth and Nfo. Loss of the two major AP endonucleases and NER severely altered cell growth, as well as sensitivity to UV light and an oxidant, t-BuO₂H. A similar deficiency in yeast (*apn1⁻apn2⁻rad1⁻/rad10⁻*) results in lethality probably due to AP lyase conversion of endogenously produced AP sites to SSBs with 3' blocked termini (28). It is possible that the slow growth of the *uvrA⁻xth⁻nfo⁻* mutant was due to similar un-repaired damage, since Xth and Nfo are the major enzymes that remove 3' blocking termini in *E.coli* (29;30) and the 3' to 5' exonuclease activity of DNA polymerase I only removes this damage slowly (31;32).

Using the *uvrA⁻xth⁻nfo⁻* mutant, we have shown that the loss of NER in addition to Xth and Nfo does not prevent DSB formation from MDSs containing two opposing uracils ≤ 7 bps apart. It is also unlikely that endonuclease V generated the DSBs, since luciferase activity was decreased in *xth⁻nfo⁻nfi⁻* bacteria when p3'luc contained two opposing uracils 0-13 bps apart. The remaining candidates are the AP lyases (endonuclease III, endonuclease VIII or Fpg), which cleave at the 3' side of the AP site resulting in 3' phosphate or 3' α , β -unsaturated aldehyde termini that require processing to a 3' hydroxyl prior to complete repair (16). Fpg, endonuclease III and endonuclease VIII can cleave at an AP site closely opposed to an AP site (33) or SSB (34) *in vitro*, although with reduced efficiency, and endonuclease III can convert two closely opposed AP sites in a plasmid to a DSB *in vitro* (35). Cleavage of AP sites by the AP lyases to produce SSBs with blocked 3' termini may explain why MDSs (two uracils separated by 13-33 bps), that were completely repaired in wild-type and *uvrA⁻* bacteria, resulted in reduced luciferase activity (Figure 2B and C) and decreased plasmid survival in bacteria deficient in Xth and Nfo. Even the MDSs with the uracils 0-7 bps apart resulted in significantly lower luciferase activity in the bacteria deficient in Xth and Nfo compared to *uvrA⁻* (Figure 2A) and wild-type bacteria (11).

To determine whether the AP lyases were converting two opposed AP sites to a DSB; substrates were tested that contained a uracil opposite a furan. Due to the lack of a 1'-OH group the furan cannot undergo β -elimination (25), which is the mechanism by which the AP lyases introduce a SSB at the 3' side of the AP site (36). Since the furan is resistant to AP lyase cleavage, it was expected that a uracil opposite a furan would not decrease luciferase activity in the DNA repair assay, or result in decreased plasmid survival bacteria deficient in either Ung or Xth and Nfo. This was indeed found for the *ung⁻* bacteria, however all the *xth⁻nfo⁻* strains tested were able to introduce DSBs at MDSs containing a uracil opposite a furan separated by 5 bps. Therefore inhibiting the AP lyases, and inactivating NER or endonuclease V as well as Xth and Nfo, did not prevent this type of MDS from being converted to a DSB. This is the first work that indicates there is an as yet unknown enzyme or system in *E.coli* that can convert a uracil (processed to an AP site or SSB by base excision repair) opposite a furan to a DSB. This enzyme/ repair pathway does not seem to produce 3' blocked termini as a uracil and a furan separated by 20 bps did not reduce luciferase activity in bacteria deficient in Xth and Nfo compared to wild-type and *uvrA⁻* bacteria, as found for two opposing uracils. It is likely that the AP lyases generate the DSBs at AP sites when Xth and Nfo are absent and that the alternate repair system acts as a second "back-up" repair mechanism. It is not possible to determine from this work the proportion of repair completed by this "back-up" system when Xth, Nfo and AP lyases are able to cleave at the AP sites.

Since this work used a MDS with the furan on the transcribed strand, it was possible that the processing of the furan was related to the strand in which it was situated. Zhou & Doetsch (26) did find that purified *E.coli* RNA polymerase was able to cleave at an AP site in the transcribed strand, although they could not rule out a contaminating enzyme. A MDS was therefore tested in *xth⁻nfo⁻* bacteria that contained a uracil in the transcribed strand 5 bps apart and 5' to a furan in the non-transcribed strand. The luciferase activity (11% of the undamaged sequence, data not shown) was very similar to that of the same MDS with the furan in the

transcribed strand. Therefore cleavage of the furan in the absence of the AP endonucleases was not altered by the strand in which the lesion was positioned.

Comparison of the luciferase activities for the two opposing furans or a uracil opposite a furan 5 bps apart does show that the uracil opposite the furan was cleaved more effectively in the *xth⁻nfo⁻* strain. The uracil opposite the furan can be converted by the AP lyases to a SSB by β -elimination or a nucleotide gap by β and δ -elimination. It is possible that the opposing lesions (two furans or SSB opposite a furan) generate distortion in the DNA that may initiate recombination, but that due to the inhibition of DNA gyrase by novobiocin complete repair cannot occur. Nuclear magnetic resonance studies have demonstrated that two closely opposed furans distort the DNA structure (37) and DNA fragments with a nick or nucleotide gap have increased flexibility and a V-shaped structure (38;39). Previously Kokontis et al (40) proposed that distortion of the DNA structure by two closely opposed benzo[a]pyrene diol epoxide lesions activated recombination resulting in an enhancement of deletion frequency. The recF recombination pathway has also been implicated in cell death and has been proposed to increase DSB formation during thymine-less death (41). MDSs containing closely opposed uracils, AP sites and SSBs may also be generated in the genome during thymine-starvation of bacteria, since a lack of thymine is believed to result in uracil incorporation into DNA (for review see 42). It is beyond the scope of this study to determine whether recF mutants can cleave MDSs containing a furan, although future studies are planned to determine the role of recombination in the repair of MDSs.

Bacteria deficient in Xth and Nfo are known to exist in an SOS-induced state (43). It is therefore possible that the enzyme/system generating the DSBs is a protein that is part of the SOS response. If the SOS-induced state allowed plasmid replication to occur in the *xth⁻nfo⁻* bacteria in the presence of novobiocin, the furan is likely to cause a block to DNA replication (44). Translesion synthesis of a furan is increased by SOS-induction due to the expression of DNA polymerases II, IV and V. However, by-pass is increased by only 3-11 fold to ~13% (44). Replication could therefore result in stalled replication forks and DNA breakage that cannot be repaired by recombination due to the inhibition of DNA gyrase by novobiocin. The collapse of the replication forks could result in DSB formation at the MDSs. Future experiments using RecA mutants will need to be performed to determine whether the mechanism of MDS-breakage is related to the induction of SOS in the *xth⁻nfo⁻* bacteria.

In summary, it is evident from the DNA repair assay and the plasmid survival assay that Xth and Nfo are key enzymes for the complete repair of uracils as well as furans. When these AP endonucleases are not present the data suggests that the AP lyases, and not NER or endonuclease V, cleave at closely opposed AP sites, generating SSBs with 3' blocked termini that are not readily repaired. We do provide evidence for an additional repair enzyme/system which can convert AP sites within MDSs to DSBs. It is unlikely that this repair system predominates in a wild-type cell and we hypothesize that it acts as a "back-up" repair mechanism when the known AP endonucleases and AP lyases are unable to initiate repair of the AP sites. It cannot be ruled out from these studies that this enzyme/system may be part of the SOS response. It is very clear however that MDSs containing AP sites can be converted to potentially lethal lesions by a number of different mechanisms within the cell. Processing of even a small number of these MDSs in the genome can have major consequences on cell survival if complete repair does not occur. Identifying the enzymes that can convert MDSs to DSBs is important. This knowledge will allow us to manipulate the repair systems to promote cell survival or cell death after treatment with DNA damaging agents such as ionizing radiation and bleomycin, which also produces MDSs containing AP sites and SSBs (45). Future work will focus on identifying the "back-up" repair system that exists in *E.coli*.

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Abbreviations

MDS, multiply damaged site
 oligonucleotide, oligodeoxyribonucleotide
 bp, base pairs
 AP, apurinic/apyrimidinic
 SSB, single strand break
 DSB, double strand break
 8oxodG, 8-oxo-7, 8 dihydroguanine
 xth, exonuclease III
 nfo, endonuclease IV
 NER, nucleotide excision repair
 nfi, endonuclease V
 furan, tetrahydrofuran
 UV, ultraviolet
 t-BuO₂H, tert-butyl hydroperoxide
 Carb, carbenicillin
 Cm, chloramphenicol
 BER, base excision repair
 Fpg, formamidopyrimidine DNA glycosylase
 Nt, nucleotide
 Ung, uracil DNA glycosylase

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Figure 1.**Sensitivity of bacterial strains to UV light and tert-butylhydroperoxide**

Bacteria were treated with short wave UV light and plated on solid medium. After overnight growth the colonies were counted to determine the percentage of surviving bacteria. At least three experiments were performed and the average and standard error obtained for each dose are shown graphically (**A**). To determine the sensitivity to t-BuO₂H, gradient plates were prepared containing 0- 987 µg and a line (8 cm long) of bacteria was inoculated for each bacterial strain. After overnight growth the length of the growth was measured. At least six different bacterial streaks for each strain and dose were measured and the average distance of growth and standard error are shown graphically (**B**).

Figure 2.

Repair of MDSs containing two opposing uracils

Ligation reactions containing p3' luc with two closely opposed uracils or no damage (represented by a white bar on the graphs) were co-transformed with pACYC184 into either *uvrA*⁻ (A), *xth*⁻*nfo*⁻ (B), *uvrA*⁻*xth*⁻*nfo*⁻ (C), or *xth*⁻*nfo*⁻*nfi*⁻ (D) bacteria. Luciferase activity was measured after 4 hours and normalized to the number of tetracycline (for *xth*⁻*nfo*⁻*nfi*⁻) or chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity for each sample compared to the undamaged control sequence. At least five transformations were performed for each type of ligation reaction. The average and the standard error are shown graphically. The number of base pairs (bps) separating the two uracils, as well as the orientation (5' or 3') of the uracils with respect to each other, is indicated. * represents a statistical difference ($p < 0.05$) compared to the results obtained with the *uvrA*⁻ bacteria. # represents a statistical difference $p < 0.05$ compared to the *xth*⁻*nfo*⁻ mutant on an AB1157 background.

Figure 3.

A single furan situated in the transcribed strand does not disrupt transcription

Ligation reactions containing p3' luc with no damage or a single furan in the transcribed strand were co-transformed with pACYC184 into either wild-type (WT), *uvrA*⁻, *xth*⁻*nfo*⁻, *uvrA*⁻*xth*⁻*nfo*⁻, or *xth*⁻*nfo*⁻*nfi*⁻ bacteria. Luciferase activity was measured after 4 hours and normalized to the number of tetracycline (for *xth*⁻*nfo*⁻*nfi*⁻) or chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity measured for each sample compared to the undamaged control sequence. At least six transformations were performed. The average and the standard error are shown graphically for the ligation containing a single furan.

Figure 4.

Repair of MDSs containing a uracil closely opposed to a furan

Ligation reactions containing p3' luc with a uracil closely opposed to a furan or no damage (represented by a white bar on the graphs) were co-transformed with pACYC184 into either *ung*⁻ (**A**), wild-type (WT, **B**) *uvrA*⁻ (**C**), *xth*⁻*nfo*⁻ (**D**), *uvrA*⁻*xth*⁻*nfo*⁻ (**E**), or *xth*⁻*nfo*⁻*nfi*⁻ (**F**) bacteria. Luciferase activity was measured after 4 hours and normalized to the number of tetracycline (for *xth*⁻*nfo*⁻*nfi*⁻) or chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity measured for each sample compared to the undamaged control sequence. At least five transformations were performed for each type of ligation reaction. The average and the standard

error are shown graphically. The number of base pairs (bps) separating the two lesions, as well as the orientation (5' or 3') of the uracil and furan with respect to each other, is indicated. * represents a statistical difference ($p < 0.05$) compared to the results obtained with the *ung*⁻ bacteria. # represents a statistical difference $p < 0.05$ compared to the *xth*⁻*nfo*⁻ mutant on an AB1157 background.

Figure 5.

Repair of MDSs containing two closely opposed furans

Ligation reactions containing p3' luc with no damage or two closely opposed furans situated 5' to each other and 5 (**A**) or 20 (**B**) bps apart were co-transformed with pACYC184 into either wild-type (WT), *uvrA*⁻, *xth*⁻*nfo*⁻, or *uvrA*⁻*xth*⁻*nfo*⁻ bacteria. Luciferase activity was measured after 4 hours and normalized to the number of chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity measured for each sample compared to the undamaged control sequence. At least six transformations were performed for each type of ligation reaction, except for the two furans separated by 20 bps in the *uvrA*⁻*xth*⁻*nfo*⁻ bacteria where three transformations were performed.

The average and the standard error are shown graphically. * represents a statistical difference ($p < 0.05$) compared to the results obtained with the wild-type bacteria.

Table 1**Double-stranded oligonucleotides containing a MDS****Position of two damages relative to each other
5' or 3'****bp apart****Sequence**

5'	33	5' TAAATACAAAGGATATCAGGTGGCCCCCGCTGAATXGGAAT 3' 3' TAAXTTATGTTTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	20	5' TAAATACAAAGGATATCAGGTGGCCCCCGCTGAATXGGAAT 3' 3' TAAATTTATGTTTCCTAXAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	18	5' TAAATACAAAGGATATCAGGXGGCCCCCGCTGAATTGGAAT 3' 3' TAAXTTATGTTTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	13	5' TAAATACAAAGGATAXCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAAXTTATGTTTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	7	5' TAAATACAAAGGATATCAGGXGGCCCCCGCTGAATTGGAAT 3' 3' TAAATTTATGTTTCCXATAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	5	5' TAAATACAAAGGATATCAGGXGGCCCCCGCTGAATTGGAAT 3' 3' TAAATTTATGTTTCCTAXAGTCCACCGGGGGCGACTTAACCTTAGC 5'
5'	0	5' TAAATACAAAGGATAXCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAAATTTATGTTTCCTAXAGTCCACCGGGGGCGACTTAACCTTAGC 5'
3'	0	5' TAAATACAAAGGAXATCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAAATTTATGTTTCCTAXAGTCCACCGGGGGCGACTTAACCTTAGC 5'
3'	7	5' TAAAXACAAAGGATATCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAAATTTATGTTTCCXATAGTCCACCGGGGGCGACTTAACCTTAGC 5'

X designates the position of a uracil or a furan in a damage-containing oligonucleotide or a thymine in an undamaged oligonucleotide. Two oligonucleotides were annealed to form a MDS. MDSs examined consisted of either two opposing uracils, a uracil opposite a furan or two opposing furans. The distance (in bps) separating the damages and the orientation (5' or 3') of the damages with respect to each other are shown.

Table 2

Effects of a MDS on plasmid survival

Type of MDS	Bps apart	Wild-type Carb ^R / Cm ^R	<i>ung</i> ⁻ Carb ^R / Cm ^R	<i>xth</i> ⁻ <i>nfo</i> ⁻ Carb ^R / Cm ^R	<i>uvrA</i> ⁻ <i>xth</i> ⁻ <i>nfo</i> ⁻ Carb ^R / Cm ^R
Undamaged	-	13.1±6	17.2±8.5	28.3±18.6	8.4±1.8
One Furan	-	15.9±8.4	18.8±5.4	25.4±6.9	6.3±0.9
Two Uracils	7	1.0±0.62*	17.2±6.5	0.8±0.11*	0.3±0.12*
	20	13.2±7.7	18.8±3.6	9.2±3.7	3.3±1.2*
Uracil and a Furan	5	0.66±0.35*	14.7±4.3	1.6±0.8*	0.95±0.38*
	20	10.3±5.1	15.4±1.7	14.3±4.1	4.1±1.1*
Two Furans	5	0.41±0.23*	0.61±0.42*	4.8±2.6*	1.6±0.8*
	20	9.8±3.7	10.7±2.7	15.8±6.8	6.6±2.5*
No insert	-	0.73±0.48*	0.73±0.54*	1.3±0.6*	0.24±0.06*

Double-stranded oligonucleotides containing no damage, a single furan, or two lesions situated 5' to each other in opposing DNA strands were ligated into p3'luc. At least four transformations were performed for each bacterial strain. After the 4 hour incubation in novobiocin, the culture was grown on carbenicillin or chloramphenicol-containing medium overnight and the colonies counted. A ratio of carbenicillin resistant (Carb^R) to chloramphenicol resistant (Cm^R) colonies was calculated. The average and standard deviation are shown. No insert is the ligation containing only linear DNA.

* represents p < 0.05 compared to the undamaged sequence.