

Cisplatin induces DNA double-strand break formation in *Escherichia coli* *dam* mutants

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Abstract

E. coli dam cells are more susceptible to the cytotoxic action of cisplatin than wildtype. *Dam mutS* or *dam mutL* bacteria, however, are resistant to this agent indicating that active mismatch repair sensitizes *dam* cells to cisplatin toxicity. Genetic data, obtained previously, was consistent with the generation and repair of cisplatin-induced double-strand breaks (DSBs). We measured DSB formation in temperature-sensitive *dam recB* mutants, after exposure to cisplatin, using pulse field gel electrophoresis and observed an increase in linear 100-300 kb DNA fragments corresponding to approximately 15-45 double strand breaks per genome. The formation of these DSBs was temperature and dose-dependent and was decreased in *recBC* bacteria at the permissive temperature or in *dam*⁺ or *mutS* control strains. There was a 3-fold increase in circa 2 mb linear chromosomal fragments in *dam recBC* strains at the non-permissive temperature compared to *recBC* alone. We show that *dam priA* strains are not viable suggesting that DSB formation is dependent on DNA replication restart. The sensitivity of *priA* mutants to cisplatin is also consistent with this conclusion.

INTRODUCTION

Although cisplatin (*cis*-diaminodichloroplatinum(II)) is used for the treatment of a variety of cancers, including testicular cancer for which it is highly effective (1), the molecular mechanism leading to cell death after treatment is not known. Cisplatin reacts with DNA to produce mostly intrastrand crosslinks between adjacent guanines (65% of the total), adjacent guanine and adenines (25%), and guanines separated by a base (1,3-GNG, 5–10%). Interstrand crosslinks comprise about 2% of the total adducts and a small amount of monoadducts is also formed (2;3). The biologically inactive *trans* isomer of cisplatin also produces 1,3-GNG adducts, interstrand crosslinks and monoadducts (4;5), suggesting that cisplatin intrastrand crosslinks between adjacent purines are the biologically important adducts since they efficiently block progression of DNA polymerases *in vitro* and *in vivo* (6). Nucleotide excision repair (NER) removes intrastrand crosslinks but the 1,3-GNG lesions are removed at a rate 50-fold faster than those between adjacent purines (7). The importance of NER is manifested by the increased sensitivity of NER-deficient mutants of *E. coli* (8) and mammalian cells (9-11) to cisplatin. Recombinational repair mechanisms, however, are as important as NER in allowing *E. coli* to survive cisplatin damage (12). Recombination-deficient strains (including *recA*, *recBCD*, *ruvABC*, *priA*) are very sensitive to the cytotoxic action of cisplatin (12-14) and cells deficient in both NER and recombination show greater cisplatin sensitivity than cells lacking one of these repair mechanisms (12). The genetic evidence is consistent with cisplatin damage leading to the formation of double-strand breaks (DSBs) and gaps (12). Cisplatin is also a highly efficient inducer of recombination and the proposed molecular mechanism is through the formation of DSBs (14).

E. coli dam mutants and mammalian cells are highly susceptible to the cytotoxic action of cisplatin and mismatch repair-deficient derivatives are more resistant (15;16). The mismatch repair system in *E. coli* includes the MutS protein which recognizes platinated diguanyl intrastrand crosslinks (17). We recently showed that such GG crosslinks opposite CC bases are recognized by *E. coli* MutS as well as GG crosslinks opposite one or more T bases which could be formed by the action of translesion polymerases (18). After recognition of the lesion by MutS, a ternary complex with MutL and MutH on looped DNA is formed followed by the introduction of a single-strand nick in the newly-synthesized strand. Excision by exonucleases removes the lesion followed by re-synthesis, catalyzed by the replicative polymerase, and ligation by DNA ligase (19). It has recently been proposed that one mechanism of sensitization of *dam* mutants to cisplatin is by interference of recombinational repair by MutS (20).

Recombination is essential for *E. coli dam* mutant survival because mismatch repair-provoked single-strand nicks are converted to double-strand breaks that require recombinational DSB repair (21-23). These breaks can occur anywhere in the chromosome and the frequency with which they form increases the basal level of the SOS response to provide more RecA, RuvA and RuvB proteins (24). The *dam* mutants, therefore, can be viewed as stressed cells in which the recombination system is working at, or near, capacity. We reasoned that if cisplatin does indeed introduce DSBs into DNA, they should be more readily detected in *dam* mutants than wildtype. In this communication, we show that this is the case.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The *E. coli* K-12 strains used in this study were derived from AB1157 (25) and are listed in Table 1. Strains were constructed by P1 *vir* transduction. The phenotype of *mutS*, *mutH* and *mutL* mutations in strains GM56 or GM58 were checked by testing the ability of these strains to grow at 42°C and by the frequency of resistance to rifampicin. Drops of different dilutions of these mutants were placed on L plates and incubated overnight at permissive (30°C) and non-permissive temperature (42°C). GM56 derivatives that acquired *mutS*, *mutL* or *mutH* mutations were able to grow at 42°C. Overnight cultures of these derivatives were plated on L-Rif plates and on L plates. After incubation overnight at 30°C and 42°C, the frequency of mutation was measured by dividing number of rifampicin-resistant mutants per 10⁸ viable cells. Plasmid pMQ148 carries the *dam* gene and produces a two-fold increase in cellular Dam activity (26).

Rich medium was Luria (L) broth which consists of 20 g tryptone, 10 g yeast extract, 1 g NaCl and 4 ml 1 M NaOH/l, supplemented with 2 µg/ml thymine, solidified when required with 16 g agar (Difco). Minimal casamino acids medium was M63 (27), supplemented with 0.4% glucose and 0.2% casamino acids. Minimal salts solution was that described by Davis and Mingioli (28). Antibiotics were used at the following concentrations: ampicillin (Amp), 100 µg/ml; tetracycline (Tet), 15 µg/ml; chloramphenicol (Cam) 30 µg/ml.

Cytotoxicity assay

Overnight cultures in L broth were diluted 100-fold into fresh M63 medium and grown to OD₆₀₀ 0.4-0.5. The cells were centrifuged, resuspended in an equal volume of minimal salts and treated with cisplatin for 60 min. The cisplatin (Sigma) was dissolved in water for 2 h at 37°C before use. The treated cells were diluted and plated on L medium to measure survival.

Preparation of plugs and PFGE migration

We have used a modified procedure of the method described by Seigneur et al (29). Overnight cultures were grown in L medium. The cells were diluted to OD₆₀₀ 0.01 or 0.02 in M63 supplemented with 0.4% glucose, 0.2% casamino acids, thiamine 4 µg/ml, 100 µg/ml deoxyadenosine and 5 µCi/ml of [³H]thymidine (87 Ci/mmol, New England Nuclear). After 3 h of incubation at permissive temperature (30°C) the culture was divided in two when the OD₆₅₀ reached 0.05-0.08. One part was incubated further at 30°C, the other was shifted to 42°C. When they reached OD₆₀₀ 0.4-0.6 (about 3h of incubation) they were centrifuged and resuspended in an equal volume of minimal salts and cisplatin (usually 50 µM) was added and incubated for an additional hour. The cells were centrifuged again and resuspended in M63. Duplicate 1 ml samples of each culture were collected, washed in minimal medium and twice in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4) and finally resuspended in 160 µl of distilled water. The cells were mixed with an equal volume of 2% low melt agarose (in 0.5 × TBE buffer), distributed in 60 µl portions in molds and left for a few minutes at 4°C until set. The plugs (about 5) were incubated overnight at 56°C in 1 ml LE buffer (1% N-laurylsarcosine, 0.5 M EDTA, pH 9.6)

with proteinase K (0.5%) (30). The plugs were then washed three times with TE buffer and stored in TE buffer at 4°C. Portions of each agarose plug were loaded into the wells of 1% agarose gels (Seakem Gold Agarose) with 1% low melt agarose and were subjected to electrophoresis for 24 h at 6 V/cm in $0.5 \times$ TBE, with an initial switching time 60 s and final switching time of 120 s in a CHEF-DR II apparatus. After electrophoresis, the gels were stained with ethidium bromide and photographed. Plugs containing the chromosomes of the yeast *Saccharomyces cerevisiae* were routinely used as molecular weight standards (Bio-Rad). The lanes were cut into 3 mm slices using a razor blade slicer. A total of 35 slices representing 10 cm of gel was usually made to allow the recovery of all fragments between the origin and the location corresponding to 100-200 kb fragments. The agarose slices were melted at 90°C, and diluted 5-fold in 0.1 M HCl at the same temperature. After cooling 0.8 ml portions were added to 5 ml of scintillation fluid (EcoLume, MP Biochemicals) and the radioactivity measured in a Beckman Coulter LS6500 scintillation counter. The fraction of migrating DNA was calculated by dividing the amount of tritium found in a slice by the total amount of tritium found in all the slices including the migration origin.

RESULTS

Survival of *dam recB* strains to cisplatin

RecBC protein has a high affinity for double-stranded DNA ends to initiate processive exonucleolytic degradation (31). To preserve such ends, we have used strain GM56 which contains the temperature-sensitive *recB270* mutation in a *dam-3* genetic background (21). This

strain is viable at 30°C but inviable at 42°C. At the non-permissive temperature, full viability is retained for at least the first hour if the cells are returned to the permissive temperature. We measured the survival of GM56 after a 1 hr exposure to cisplatin at both temperatures. Fig. 1A shows that, as expected, the survival is greater at the permissive temperature than the non-permissive temperature. Derivatives of GM56 (*dam-3 recB270*) were constructed with *mutS* (GM7751) or *mutL* (GM7752) or *mutH* (GM7753) mutations and these no longer showed a temperature-sensitive phenotype. When these mismatch repair-deficient strains were exposed to cisplatin, there was increased survival at 30°C and 42°C compared to GM56 (Fig. 1B) although the strains still showed greater sensitivity at 42°C due to the *recB270* mutation. Similar results were obtained with strain GM58 (*dam-3 recC271*)(data not shown). We also constructed a *dam-3 recB270 recC271* strain (GM7759) but it grew very slowly even at the permissive temperature, suggesting that the growth rate was limited by insufficient RecBC. The poor growth rate of the *recBC* double mutant plus the high probability of selecting derivatives with suppressor mutations that improved growth restricted us to use cells with either the *recB270* or *recC271* alleles for most experiments. The experiments described in this paper were carried out with strains GM56 and GM58 (*dam-3 recC271*) but since the results were identical for both, we report only the data from GM56.

Dose-dependence of DSB formation

To detect the presence of DSBs we have utilized the pulse field gel electrophoresis (PFGE) method described by Seigneur et al (29). This technique allows the separation of linear chromosomes, or fragments of them, which enter the gel from the covalently-closed circular

form which remains in the well. The residual RecBC activity in strain GM56 (*dam-3 recB270*) requires specific conditions for growth in minimal medium and drug exposure. The cells were grown at the permissive temperature (30°C) in log phase with ³H-thymidine and then shifted to the non-permissive temperature (42°C) for 3 hr. The bacteria were harvested and resuspended in minimal medium without glucose or amino acids at 42°C but containing various concentrations of cisplatin for 60 min. The cells were harvested again and resuspended in minimal medium at 42°C and incubated for an additional 60 min at 42°C without cisplatin. The OD₆₀₀ of the culture at this time was about 0.7 and the cells were collected by centrifugation and embedded in agarose, lysed and subjected to PFGE. Fig. 2A shows the resulting ethidium bromide stained gel from cells exposed to 6, 12.5 and 25 uM cisplatin. Most of the cellular DNA remains in the wells of the gel but a conspicuous band is visible migrating at about 2 megabases (mb) in the lane containing untreated cells. A band at this position is barely visible in cells exposed to 6 uM cisplatin, but is absent in cells exposed to higher concentrations. Untreated cells show extensive degradation of chromosomal DNA reflecting that these *dam recB270* bacteria were incubated 2 hr at the non-permissive temperature for growth. With increased cisplatin dosage, the DNA appears as a diffuse band in the range of 150-500 kb.

In the same experiment, a duplicate gel was cut into slices and the radioactivity in each slice was measured (Fig. 2B). In this and subsequent figures, the distributions of radioactivity in slices 4-35 represents linear DNA fragments that have entered the gel while radioactivity in slices 1-3 is the DNA remaining in the well. Only fractions 4-35 are shown in the figures and the data are plotted as percent of total radioactivity in fractions 1-35. The distributions of radioactivity at different doses in Fig. 2B are clearly different from the stained gel in Fig. 2A. The distribution of

DNA fragments from cells exposed to 6 μM is barely above that for the untreated sample but that for cells exposed to 12.5 μM cisplatin is clearly different, with a major peak at slice 16 and an increase in radioactivity in slices 20-32. At 25 μM cisplatin, the distribution of radioactivity is increased in slices 10-35, with a skew towards smaller fragments, peaking at slice 28. In subsequent experiments, we have used 50 μM cisplatin which gave essentially the same distribution as 25 μM , but gave more reproducible results from experiment to experiment. A dose-dependent reduction in the 2 mb fragments migrating in slices 4-6 in Fig. 2B is also evident. We consider the use of radioactive labeling a more accurate and quantitative method than ethidium staining to assess the extent of DNA degradation and have used it throughout this paper. A similar conclusion was reached by Seigneur et al (29). We conclude that cisplatin-induced DSBs follow dose-dependent kinetics.

Time dependence of DSB formation

Strain GM56 was grown as described above and was either exposed to 50 μM cisplatin, or untreated, for 60 min, after which the cells were harvested and lysed (time 0) or incubated at 42°C for 30 or 60 additional min. The results are shown in Fig. 3. At time 0, the radioactivity from untreated cells was found almost exclusively in slices 4-6 corresponding to the 2 mb fragments and similar distributions were obtained with incubation times of 30 and 60 min. Cells exposed to cisplatin, however, showed decreased radioactivity in slices 4-6, relative to the untreated cells, and a corresponding time-dependent increase in fragments peaking at slice 27. The distributions of radioactivity for the untreated and treated cells at time 0 are different. This can be explained by residual DNA replication during cisplatin treatment leading to chromosome

fragmentation. We conclude that cisplatin-induced DSBs are formed in a time and dose-dependent manner.

Genotype dependence of DSB formation

Derivatives of GM56 (*dam recB270*) containing mismatch repair-deficient mutations were constructed to yield GM7751 (*mutS*), GM7752 (*mutL*) and GM7753 (*mutH*). We examined the DNA of these strains after exposure to cisplatin at 42°C by PFGE. The strains accumulated DNA radioactivity in slices 4-6 but not faster-migrating fragments (Fig. 4). This result indicates that mismatch repair in a *dam* genetic background is responsible for the formation of ~300 kb DNA fragments after cisplatin treatment. Exposure of GM56 to cisplatin at 30°C did not produce faster migrating fragments after PFGE indicating that inactivation of RecBC is required to detect DSBs.

In a separate experiment, we introduced a plasmid bearing the *dam*⁺ gene into GM56. As expected, radioactivity was present only in slices 4-6 (corresponding to the 2 mb peak) when this strain was exposed to 50 uM cisplatin (data not shown). We conclude that DSB formation by this method can be detected more readily in *dam* mutant cells with an active mismatch repair system and lacking RecBC.

DSB level in *dam* mutants

In *dam* mutants, mismatch repair-induced single-strand nicks are converted to DSBs (21-23). The profiles of radioactivity for strains not exposed to cisplatin in Fig 4 indicates that there is an increased number of DSBs in the 2 mb peak of mismatch repair-proficient GM56 compared to its *mut* derivatives. This difference represents the endogenous level of mismatch repair-dependent DSBs in *dam* mutants. In order to measure the level of these DSBs, it was necessary to increase the sensitivity of the PFGE assay by using derivatives of strain JJC1263 which has temperature-sensitive mutations in both *recB* and *recC* genes and the results are shown in Table 2. The amount of linear DNA in JJC1263 grown at 30°C and 42°C is about 10% and 20% respectively and these amounts do not change significantly in a *mutS* deletion derivative (GM7757). In contrast, a *dam* deletion derivative of JJC1263 (GM7759) shows a substantially higher level of linear DNA at both 30°C (28%) and 42°C (58%). Almost all the increased radioactivity was associated with the 2 mb peak. This level of linear DNA is higher than that in GM56 (12% at 30°C, 22% at 42°C) and GM58 (18% at 30°C, 33% at 42°C) consistent with the reduced RecBC activity in GM7759. In a *dam mutS* derivative (GM7767) of JJC1263, the amount of linear DNA at the two temperatures is about the same as that for JJC1263 (Table 2). Finally, complementation with a plasmid bearing the *dam*⁺ gene in GM7759 (*dam-16*) reduces the amount of linear DNA to the wildtype level. These results indicate that DSBs in *dam* mutants are due to mismatch repair and that there are about 3-fold more unrepaired DSBs compared to wildtype.

Inviability of *dam priA* mutants

The increased level of DSBs in *dam* mutants can be explained by assuming that replication forks collapse when encountering nicks or gaps created by mismatch repair (23). If this interpretation is correct, then there should be an absolute requirement for reloading the DNA polymerase III holoenzyme complex onto the reconstructed fork after recombinational repair (32). Such loading is catalyzed by the Pri (primosomal) proteins (PriA, PriB, PriC), Rep and DnaT and is initiated by PriA (33). If such a requirement is necessary in *dam* mutants, then a *dam priA* double mutant should not be viable.

There are currently no temperature sensitive alleles of either *dam* or *priA*, so a test of inviability using temperature conditional lethality is not possible. We used a four factor genetic cross with closely linked markers to test if *priA dam* recombinants could be recovered (34). We used a P1*vir* lysate propagated on a *priA2::Kan argE3* donor to transduce to Met⁺, recipients that were *metB1* and *btuB::Tn10*. These markers are closely linked on the *E. coli* chromosome as shown in the upper part of Table 3. Additionally the recipients were either wildtype or *dam-13::Tn9*. The lower part of Table 3, shows that there is a high frequency of co-transduction between *metB* and *priA* (78%) in the wildtype background. In the *dam-13* recipient, however, this figure was 0% suggesting that *priA dam* double mutants are inviable. The lack of Met⁺ Kan^R recombinants cannot be due to a problem with recombination since Met⁺ Tet^S and Met⁺ Arg⁻ recombinants were recovered at equal frequency in wildtype and *dam* recipients.

DISCUSSION

In previous studies on the genetic requirements for recombinational repair of cisplatin damage, it was concluded that gaps and DSBs were the most likely substrates for recombination (12;13). More recently, we investigated the dramatic high-level induction of recombination by cisplatin and concluded, on the basis of the genetic requirement for both RecBCD and RecFOR pathways, that DSBs were the most likely initiating event (14). These findings spurred us to detect DSBs in *E. coli* cells exposed to cisplatin. We have not been able to detect significant cisplatin-induced DSBs at low or moderate doses in wildtype *E. coli* or in those with the *recB270* or *recC271* mutations using PFGE (Fig. 4). The increased susceptibility of *dam* mutants to cisplatin (15) and their absolute requirement for recombination for viability (23) to repair DSBs (22), suggested that if cisplatin does form DSBs at low doses (12.5-50 μ M), we should be able to detect them in *dam recBC* strains and this indeed is the case (Figs. 2, 3, 4). That we had to go to these lengths to demonstrate DSBs, indicates the amazing repair capability of the recombination process in general and of RecBC in particular.

Our results indicate that cisplatin induces 15 to 45 unrepaired DSBs in *dam recB* or *dam recC* strains at a dose of 50 μ M, although this is an underestimate due to the residual RecBC activity that is present in these strains at 42°C. The total number of lesions produced at this dose in the chromosome is about 800 (D. Froim, personal communication) and most of these are removed by NER. If we assume that we have detected half the number of DSBs and that the corrected figure is closer to 80, then mismatch repair processes about 10% of the total lesions in a *dam* mutant

that lead to DSB formation. We caution that the numbers above have been obtained using PFGE and that some other method might give different values.

Before proceeding to cisplatin-induced DSB formation, the mechanism of endogenous DSB formation in *dam* mutants will be considered. Two mechanisms have been proposed: MutH-induced cleavage at the same GATC on both strands of DNA (35) or replication fork collapse at nicks or gaps at sites of mismatch repair (23;36). It is also possible that both mechanisms can operate in the same cell. Both mechanisms require DSB repair proteins but the PriA protein should be required only for the replication fork collapse model. The genetic data shown in Table 3 indicate that cells with $\Delta priA$ and Δdam are inviable. Since the proposed role for the PriA protein is in the loading of the replicase onto a reconstituted fork, this result suggests that replication fork collapse occurs in *dam* mutants. A replication fork collapse model is shown in Fig. 5A as proposed for *dam* mutants by Kuzminov (36). A replication fork in a *dam* mutant approaches a mismatch repair-induced nick or gap and collapses to form a double-stranded end which is a substrate for RecBCD. The nick or gap in the top strand (Fig. 5A, step 2) can be closed by the action of DNA polymerase and/or DNA ligase respectively. RecBCD digests DNA and, after encountering a Chi sequence, promotes RecA strand transfer, followed by RuvAB helicase action and RuvC cleavage to restore the replication fork. PriA indicates the replication restart pathway to reload DNA polymerase III holoenzyme.

At present, we do not know the precise mechanism by which cisplatin-induced DSBs are formed in *dam* mutants. We propose the sequence of events in Fig. 5B as a likely explanation based, in part, on the observation that *E. coli priA* mutants are very sensitive to cisplatin (14). Clearly

mismatch repair instigates DSB formation, as DSBs are not detected in cells defective in this repair process (Fig. 4, Table 2) and the initial event is undoubtedly the recognition of platinated GG crosslinks, opposite CC bases, by MutS (18). After recruitment of MutL and incision by MutH, a gap is formed by exonucleases. In *dam* mutants, unlike wildtype, such gaps can occur on either DNA strand in unreplicated DNA (23). These gaps will lead to replication fork collapse if encountered by DNA polymerase III holoenzyme producing a molecule with a double-stranded end which is a substrate for RecBCD (Fig. 5B). A crucial difference with the replication fork collapse model described in Fig. 5A is that DSB repair cannot use the daughter strand because of the replication-blocking cisplatin lesion. Rather it must first use either homologous DNA from sister molecules (Fig. 5B, step 3) in RecFOR-dependent gap repair (37) or translesion polymerase synthesis (TLS; Fig. 5B, step 8) to close the gap. RecFOR or TLS gap repair must then utilize the RecBCD pathway to reconstitute the replication fork. Under the growth conditions used in this communication, we have shown previously by flow cytometry that there are 4-8 replication origins per cell (38) indicating multiple regions of homology. Steps 3-7 in Fig. 5B diagram how a new replication fork can be established via the RecFOR and RecBCD pathways. We have shown that the MutS (and probably MutL) protein inhibits RecA-mediated strand exchange when one of the substrate molecules contains cisplatin adducts (20). This inhibition of recombinational repair (Fig. 5B, step 4) can contribute to the mismatch repair-dependent sensitization of *dam* mutants to cisplatin. As an alternative to RecFOR gap repair, translesion polymerase synthesis (TLS, Fig. 5B, step 2) could eliminate the gap, and after removal of the adduct by NER, the RecBCD pathway re-establishes the replication fork. A prediction of the model is that *recBCD recFOR* strains deficient in TLS should be very sensitive

to cisplatin toxicity if both pathways can be used independently. We are currently investigating this prediction.

Another outcome not shown in Fig. 5B, is if the strand subjected to RecBCD digestion invades a sister chromosome while the gapped strand is acted upon by the RecFOR pathway to repair it (Fig. 5B, steps 3-5). In this case, however, a new replication fork would be established in the sister chromosome and the chromosome in which replication fork collapse occurred would lose a fork. Either of these outcomes might be detrimental and we have not included them in Fig. 5B.

The involvement of PriA protein, the inhibition of RecA-mediated strand exchange by MutS, the genetic requirements for cisplatin damage repair, and induction of recombination by cisplatin, are all consistent with a crucial role for DNA replication to produce DSBs at cisplatin lesions. We showed in Fig. 3, for the cisplatin-treated zero time sample, that DNA replication could be responsible for chromosome fragmentation. We are currently attempting to obtain more direct evidence for this idea.

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Table 1. *E. coli* K-12 strains used in this study

Strain	Relevant genotype	Reference or Origin
AB1157	<i>thr-1 araC14 leuB6(Am) Δ(gpt-proA)62 lacY1 tsx-33 supE44(AS) galK2(Oc) hisG4(Oc) rfbD1 mgl-51 rpoS396(Am) rpsL31(Str^R) argE3 thi-1</i>	E.A. Adelberg
GM56	<i>dam-3 recB270(Ts) thr-1 araC14 leuB6(Am)Δ (gpt-proA)62 lacY1 tsx-33 supE44(AS) galK2(Oc) hisG4(Oc) rfbD1 mgl-51 rpoS396(Am) rpsL31(Str^R) metB1 thi-1 deoB16 (kdgK51 xylA5 mtl-1)?</i>	(21)
GM58	As GM56 but <i>recC271(Ts) recB⁺</i>	(21)
GM7330	<i>Δ(lacY-lacZ)286 (φ80dIIIΔ lacZ9) ara thi (?)</i>	(14)
GM7547	As GM7330 but <i>sfiA11 btuB319::Tn10 metB1</i>	Lab stock
GM7751	As GM56 but <i>ΔmutS465::Tet</i>	From KM75
GM7752	As GM56 but <i>ΔmutL460::Cam</i>	From KM52
GM7753	As GM56 but <i>ΔmutH461::Cam</i>	From KM55
GM7757	As JJC1263 but <i>ΔmutS465::Tet</i>	Lab stock
GM7759	As JJC1263 but <i>dam-16::Kan</i>	Lab stock
GM7765	As GM7547 but <i>dam-13::Tn9</i>	Lab stock
GM7767	As GM7759 but <i>ΔmutS465::Tet</i>	Lab stock

GM7836	As GM56 but pMQ148 (Dam ⁺)	Lab stock
GM7849	As GM7759 but pMQ148 (Dam ⁺)	Lab stock
JJC1263	As AB1157 but <i>sfiA11 recB270</i> (Ts) <i>recC271</i> (Ts)	B. Michel (39)
KM52	As AB1157 but Δ <i>mutL460</i> ::Cam	K.C Murphy
KM55	As AB1157 but Δ <i>mutH461</i> ::Cam	K.C Murphy (40)
KM75	As AB1157 but Δ <i>mutS465</i> ::Tet	K.C Murphy
SS129	<i>priA2</i> ::Kan Δ (<i>lacY-lacZ</i>)286 (ϕ 80dII Δ <i>lacZ9</i>) <i>sfiB rpsL31 thi-1</i> <i>his-4 argE3</i>	S. Sandler

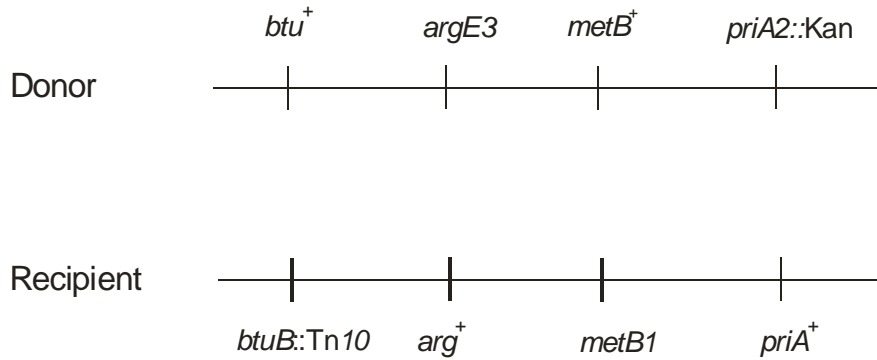
Am = amber, AS = amber suppressor, Oc = ochre, Ts = temperature-sensitive; Str = streptomycin, Tn9 = chloramphenicol resistance. Further information about the strains used in this study can be found at <http://users.umassmed.edu/martin.marinus/dstrains.html>

Table 2. Percent linear DNA in wildtype and *dam* strains

Strain	Genotype	Percent linear DNA	
		30°C	42°C
JJC1263	<i>recB270 recC271</i>	10.6 ± 3.26	20.1 ± 9
GM7757	<i>recB270 recC271 ΔmutS465::Tet</i>	7.7 ± 1.7	22.9 ± 10.25
GM7759	<i>recB270 recC271 dam-16</i>	28.2 ± 2.4	58.5 ± 13.5
GM7767	<i>recB270 recC271 dam-16 ΔmutS465::Tet</i>	9.1 ± 0.19	22.9 ± 4.4
GM7849	<i>recB270 recC271 dam-16/ pMQ148 (Dam⁺)</i>	13.5 ± 4.45	18.1 ± 0.47

The percent linear DNA for each strain was measured as described in the legend to Fig. 2.

Table 3. Frequency of recombinants in transductional crosses with *priA2::Kan* in wildtype and *dam* recipients



Recipient	Percent Met^+ recombinants that were		
	Tet^S	Arg^-	Kan^R
GM7547 (wild)	45	48	78
GM7765 (<i>dam-13</i>)	44	46	0

The arrangement of markers is shown in the upper panel of the Table. Distances are not drawn to scale. A P1 *vir* lysate was prepared on strain SS129 (*priA2::Kan^R*) and used to transduce strains GM7547 (*metB1 btuB3191::Tn10*) and its *dam* derivative GM7765 to Met^+ . One hundred Met^+ transductants from each cross were scored for the unselected markers shown above.

Figure 1. A. Survival of GM56 (*dam recB* (Ts)) after cisplatin treatment at 30 C (filled symbols) and 42°C (open symbols). B. Survival of GM56 derivatives GM7751 (*dam recB*(Ts) *mutS*), GM7752 (*dam recB*(Ts) *mutL*) and GM7753 (*dam recB*(Ts) *mutH*) strains after cisplatin treatment at 30°C (filled symbols) and 42°C (closed symbols). Because the survival values for these strains were identical within experimental limits, only single data points are shown.

Figure 2. Dose-dependent increase in 100-300 kb linear fragments from GM56 (*dam-3 recB270* (Ts)), grown with tritiated thymidine at 30 C, then shifted to 42°C, treated with varying concentrations of cisplatin for 60 min. at 42°C, and incubated for an additional 60 min. at 42°C in the absence of drug. DNA fragments were separated by PFGE and stained with ethidium bromide (A) and radioactivity was measured in slices of a duplicate gel (B). In this and subsequent figures, the distributions of radioactivity in slices 4-35 represents linear DNA fragments that have entered the gel while radioactivity in slices 1-3 is the intact DNA remaining in the well. Only fractions 4-35 are shown in the figures and the data are plotted as percent of total radioactivity in fractions 1-35. The peak of radioactivity at the left of the figure (fractions 4-6) represents the 2 mb DNA fragments.

Figure 3. Time-dependent increase in 100-300 kb linear DNA fragments from GM56 exposed to 50 uM cisplatin. Cells were exposed to cisplatin (open symbols) or not (closed symbols) for 60 min, harvested and resuspended in minimal medium (time 0) and samples removed after 30 and 60 min incubation at 42°C in the absence of drug. For other details, see the legend to Fig. 2.

Figure 4. Formation of linear DNA fragments in GM56 at 42°C after exposure to 50 uM cisplatin (open symbols) or not (closed symbols) in comparison to its *mutS* (GM7751), *mutL* (GM7752) and *mutH* (GM7753) derivatives. For details, see legend to Fig. 2.

Figure 5. Model for DSB formation and its repair. A. A replication fork in a *dam* mutant (not exposed to cisplatin) approaches a mismatch repair-induced nick or gap (1) and collapses (2) to form a double-stranded end which is a substrate for RecBCD (3). The nick or gap in the top strand in (2) can be closed by the action of DNA polymerase and/or DNA ligase respectively. RecBCD digests DNA (3) and, after encountering a Chi sequence, promotes RecA strand transfer (4), followed by RuvAB helicase action and RuvC cleavage (5) to restore the replication fork. PriA indicates the replication restart pathway to reload DNA polymerase III holoenzyme (6). B. A replication fork in a *dam* mutant exposed to cisplatin approaches a mismatch repair-induced nick or gap opposite a cisplatin GG intrastrand crosslink (1) and collapses (2). The gap in the top strand in step (2) cannot be sealed due to the blocking action of the cisplatin adduct on the replicase. RecFOR proteins load RecA at the gap (3) to promote strand invasion of homologous sister chromosomal DNA (4) and subsequent RuvAB helicase action and RuvC cleavage (5) restores the duplex DNA. The lesion is removed by NER (5) allowing RecBCD action (6) to recombine the dislocated double-stranded end molecule from step (2) to restore the replication fork (7) as outlined in Fig. 5A. The figure also shows where MutS can inhibit RecA-mediated strand exchange with platinated substrates. Alternatively, TLS at step (2) could seal the gap (8) allowing NER to remove the lesion and restoring the replication fork (10) by RecBCD action (9) as shown in Fig. 5A.

Fig. 1

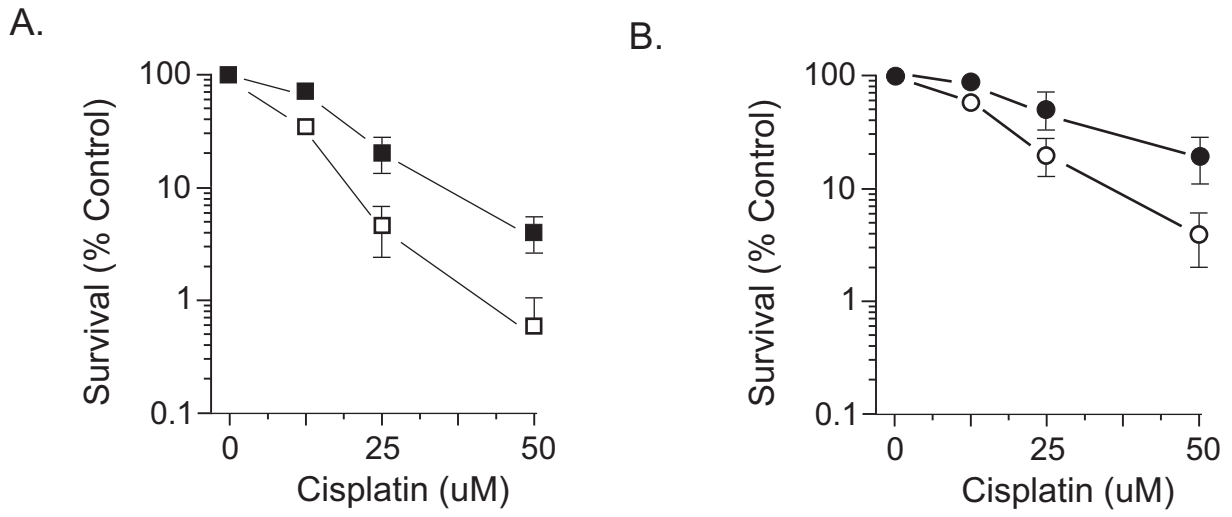


Fig. 2

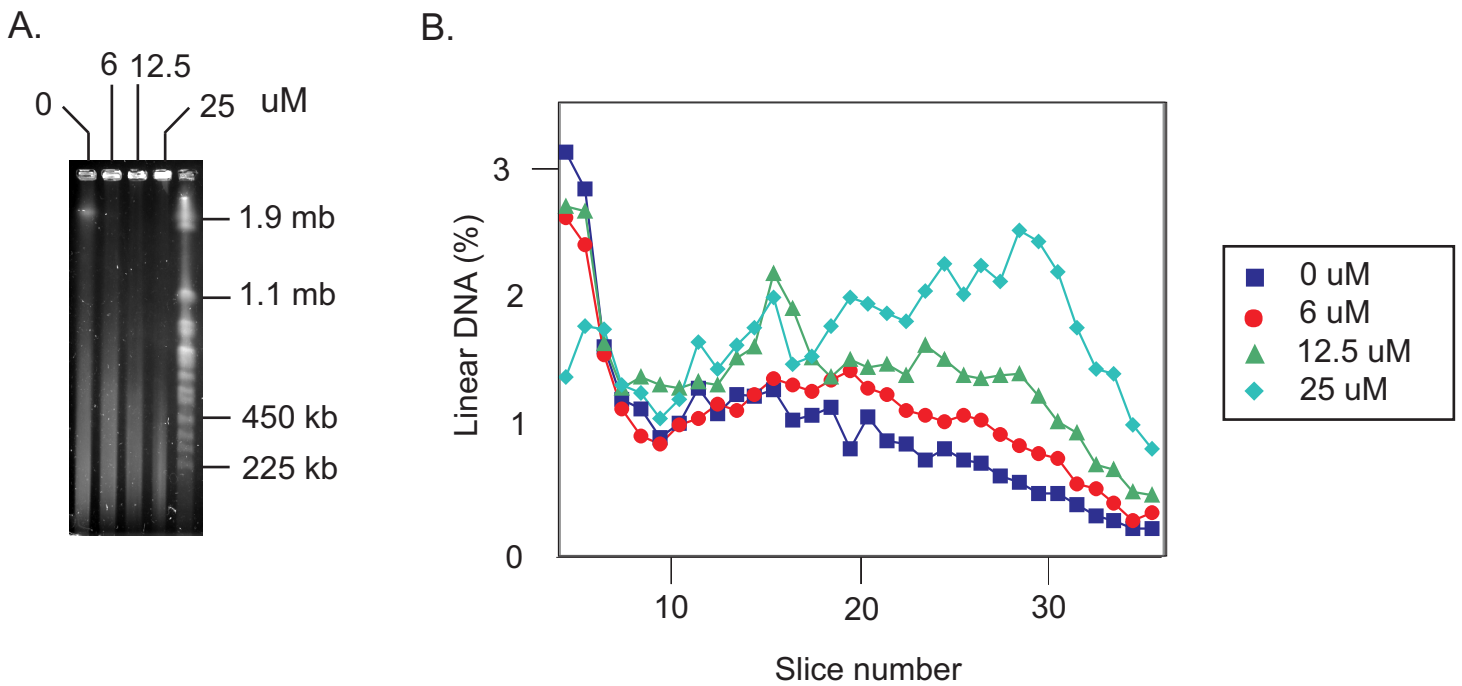


Fig. 5

