

RecN and RecG are Required for Bleomycin Survival

by *Escherichia coli*

Running Title: Genetic Requirements for Bleomycin Survival

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1. Abstract

The sensitivity of a panel of DNA repair-defective bacterial strains to BLM was investigated. *E. coli recA* cells were far more sensitive than were *uvrA*, *dam-3*, and *mutM mutY* strains, underscoring the importance of RecA to survival. Strains *recBCD* and *recN*, which lack proteins required for double strand break (DSB) repair, were extremely sensitive to BLM, while *recF* cells were not. The requirement for DSB-specific enzymes supports the hypothesis that DSBs are the primary cause of bleomycin cytotoxicity. The extreme sensitivity of *recN* cells was comparable to that of *recA*, implying a central role for the RecN protein in BLM lesion repair.

The Holliday junction processing enzymes RecG and RuvC were both required for BLM survival. The *recG ruvC* double mutant was no more sensitive than either mutation alone, suggesting that both enzymes participate in the same pathway. Surprisingly, *ruvAB* cells were no more sensitive than wildtype, implying that RuvC is able to perform its role without RuvAB. This observation contrasts with current models of recombination in which RuvA,B, and C function as a single complex. The most straightforward explanation of these results is that DSB repair involves a structure that serves as a good substrate for RecG, and not RuvAB.

Key Words: recombination, radiomimetic, DNA repair

2. Introduction

The natural product bleomycin (BLM), isolated from *Streptomyces verticilles* [1], is a family of DNA-damaging glycopeptides used as antibiotics and anti-tumor drugs. BLM is an unusual genotoxin in that it targets the furanose rings of DNA rather than the bases or the phosphate linkages of the backbone [2-6]. BLM initiates DNA degradation by generating a free radical in the sugar resulting in two different types of DNA damages, which are dependent on the availability of oxygen [7-12]. At low oxygen tension, oxidized apurinic/aprimidinic (AP) sites are favored [3;5;13;14], while at high oxygen tension strand breaks predominate [2;10;15;16]. Because of these alternative pathways, bleomycin-induced damage is a mixture of strand breaks and abasic sites. *In vitro*, up to one-third of bleomycin-induced DNA lesions (depending on sequence context) are double-stranded [17-20]. These lesions consist of either two chemically identical breaks in opposite strands, or an abasic site with a closely opposed strand break.

The chemistry of DNA cleavage by BLM (reviewed in [7]) correlates well with the physiological impact of this agent *in vivo* [21]. Levin and Demple have demonstrated that BLM generates abasic sites *in vivo* [22]. BLM has also been shown to induce single and DSBs in cells and under physiological conditions and it appears that abasic sites and strand breaks occur in a 1:1 ratio [17]. The double strand lesions are suspected to be the major cause of cell death [23;24].

While mammalian cells are the most medically relevant model of BLM toxicity, the availability of numerous mutants makes bacteria an attractive and useful tool to probe mechanism. BLM sensitivity of *Escherichia coli* is increased by the *lexA* or *recA* mutations [25], demonstrating that the SOS response is an important mechanism cells use

to resist the toxic effects of the drug [26]. Increased sensitivity of *recA* and *recBC* [27] mutants, indicates a role for recombination in BLM survival. This result is consistent with the requirement for recombinational repair as a consequence of gamma and X-irradiation treatments which also directly produce DSBs in DNA [28].

As indicated above, various studies have investigated many genetic requirements for BLM survival. The relative contributions of various DNA repair pathways, however, have not been measured in a quantitative fashion in *E. coli* cells exposed to BLM. In order to probe the genetic aspects of BLM cytotoxicity, we have investigated the BLM sensitivity of a panel of DNA repair mutant strains.

3. Materials and Methods

Bacterial Strains

Bacterial strains used in this study are listed in Table 1.

Cell Growth Medium

LB medium (Bacto tryptone, Bacto yeast Extract, NaCl, pH 7.0) and agar were purchased from Difco. Minimal medium was standard M9. MacConkey agar was purchased from Difco and supplemented with 1% lactose.

Bleomycin Survival Assay

Bleomycin sulfate (Blenoxane) was purchased from Sigma and dissolved in 10 mM HEPES buffer, pH 7.6. The concentration of the stock solution was determined by measurement of OD₂₉₀, using the extinction coefficient 14,000 M⁻¹. The assay for sensitivity of WT and mutant *E. coli* cells to BLM was adapted from a procedure developed by Levin and Demple [22]. Cells were grown at 37°C with aeration to early

logarithmic phase ($OD_{600} = 0.2-0.3$). Aliquots of cells were treated with BLM at the indicated concentrations. These cultures were incubated at 37°C for one hr with aeration. Cells were diluted in minimal medium and plated on LB agar in order to measure colony-forming units (cfu). Surviving fraction was the cfu/mL ratio of drug-treated to control cultures.

Drug-Induced Recombination Assay

Recombinogenesis was assayed by treating GM7330 cells, which carry a non-tandem duplication of partially deleted *lac* operons. Recombination between the two partial alleles is necessary to produce a functional *lacZ* gene [29]. Recombinants are visible as dark red papillants.

GM7330 cells were grown overnight in LB medium and diluted ten-fold in minimal medium. Cells were then plated as a uniform lawn on supplemented MacConkey agar. Indicated concentrations of drug were spotted on sterile disks, which were then placed on top of the bacterial lawn, allowing diffusion of the drug to produce a local concentration gradient. The plates were then incubated for 48 hr at 37°C.

4. Results

Bleomycin survival of recombination-defective *E.coli*

BLM is toxic to wildtype AB1157 (WT) cells, killing 99% of cells at a concentration of about 4.4 μM . Cells defective in RecA were significantly more sensitive to BLM, with 99% cell death occurring at about 0.6 μM (Fig. 1). Cells with mutations in *uvrA*, *dam3*, and *mutM mutY* were slightly more sensitive than WT. These strains all experienced 99% killing at 2-2.5 μM BLM. This difference between these strains and WT was greater than the standard deviation, but the difference was small relative to *recA*.

Cells carrying mutations in the DSB repair genes *recN* and *recBCD* cells were substantially more sensitive than WT (Fig. 2). For both *recN* and *recBCD* strains, 99% of the cells were killed by 0.8-0.9 μM BLM. This difference between WT and mutant cells is comparable to the sensitivity of the *recA* strain. *recF* cells were only slightly more sensitive to BLM than WT.

Cells deficient in the branch migration and resolvase activities RuvC and RecG experienced 99% cell death at 0.9-1.2 μM BLM, a sensitivity similar to that of the *recA* strain (Fig. 3). The *ruvC recG* double mutant did not show additive sensitivity of the parent strains suggesting that the gene products act in the same linear pathway.

Surprisingly, the *ruvAB* strain exhibited essentially the same vulnerability as WT cells.

Recombinogenesis Assay

Many agents that induce strand breaks are recombinogenic. The ability of BLM to induce recombination was examined in GM7330 cells, a strain carrying inverted inactive *lac* operons that could be made functional only through a recombination event.

BLM treatment was compared to cisplatin, which is known to be a potent inducer of recombination [30]. A range of approximately equitoxic doses of each drug was spotted onto small filter disks and placed on top of a bacterial lawn (Fig. 4). The higher doses produce a zone of killing surrounding the disk. Immediately outside the zone of killing, a ring of red papillants indicates induction of recombination. Such recombinants were apparent in the cisplatin treated cells, but not the BLM treated cells.

5. Discussion

Recombination is necessary for repair of BLM lesions

Previous studies have found that *E. coli* cells are rendered vulnerable to BLM by mutations in *recA* [25] and *recBC* [27], suggesting that recombination must be required for BLM tolerance. The ability of BLM to sever DNA strands *in vitro* led to the hypothesis that strand breaks were the cause of BLM toxicity. These results are consistent with the known sensitivity of these strains to gamma and X-irradiation, which produce DSBs [31-33].

In this study, we have built upon the above-mentioned studies by adding detail to the genetic requirements for BLM survival. First, as a baseline, we confirmed the finding of Yamamoto and Hutchinson [25] that *recA* cells are extremely sensitive to BLM. The clear dependence of the cells on the RecA protein for BLM survival implies that BLM produces potentially deadly lesions that require RecA-dependent processing. The nucleotide excision repair system, represented by *uvrA*, does not appear to play a major role, as would be expected if replication-blocking lesions were involved in toxicity. Neither *dam* nor *mutM mutY* mutations had a large impact on BLM survival, indicating that DNA mismatch repair, FAPY glycosylase, and adenine glycosylase were also

unimportant for BLM tolerance. Taken together, these findings support the existing model that strand breaks are responsible for cell death in BLM treated *E. coli*.

The double strand break repair genes *recN* and *recBCD* are required for survival after BLM treatment

BLM produces both single strand breaks, which are repaired by the daughter strand-gap pathway, and double strand breaks, which are repaired by the DSB pathway. To distinguish between single and double strand breaks as mechanisms of toxicity, we compared the effect of specific mutations in genes known to be involved in the DSB and DSG pathways. Absence of the RecF protein had little impact on BLM survival. As daughter-strand gaps typically result from single strand breaks or replication-blocking lesions, this result is consistent with a model in which BLM produces DSBs directly, rather than as a consequence of replication. This view was supported by our finding that double strand breaks decrease, presumably due to repair, rather than increase when cells are permitted to replicate after BLM treatment [34].

In contrast, the *recBCD* and *recN* strains were extremely sensitive to BLM exposure. This finding agrees with the conclusion of Knezevic-Vukcevic and Simic that the RecBCD complex is required for repair of BLM lesions [27]. We note parenthetically that the strain we used has all three genes (*recBCD*) deleted rather than the two point mutations in *recB* and *recC* used previously [27]. The known requirement for these genes in DSB repair [35-39] suggests that the sensitivity of *recN* and *recBCD* strains to BLM is due to their inability to repair DSB lesions. Both strains were essentially as compromised

as *recA* cells (Fig. 2), suggesting that most or all of the RecA-dependent recombination occurring in BLM-exposed cells involves the RecBCD-dependent DSB pathway. The behavior of RecBCD may explain the lack of detectable BLM induced recombination observed in GM7330 cells - the nuclease activity of a RecBCD complex in search of a Chi site may result in loss of sequence information, and ultimately a non-functional gene when recombination is complete.

The biochemical function of the RecN protein has not yet been defined. RecN protein is required for repair of ionizing radiation, but not UV-induced lesions [40] and is therefore suspected to be a DSB repair protein. Notably, the bacterium *Deinococcus radiodurans* is dependent on RecN function for its extreme resistance to radiation [41]. Cells deficient in RecN exhibit greater than normal mutation rates. This effect is dependent on RecA and abrogated by LexA, suggesting that the *recN* mutation causes constitutive SOS activation [42]. RecN is believed to have ATPase activity, but as it has not been purified and assayed for its biochemical properties its role in DSB repair unknown. The extreme sensitivity discovered in this work of *recN* cells to BLM suggests that this protein fulfills as critical a role in DSB repair as does RecA.

Both RecG and RuvC are required for BLM survival but RuvAB is not

Holliday junctions generated during RecA-mediated recombination can be processed by multiple branch migration and resolvase activities, including the RecG and RuvABC complexes. In current models of RuvABC activity, the three proteins assemble on the junction and catalyze branch migration and resolution as a complex [43;44]. Surprisingly, *ruvC* cells were highly sensitive to BLM (Fig. 3), but *ruvAB* cells were not.

The *ruvC recG* double mutant was approximately as sensitive as either single mutant, which is consistent with the two proteins acting in concert or in sequence in a single pathway. Foster *et al.* [45] and Kuzminov [46] have proposed models of RecG-mediated resolution that do not require scission of two strands at the Holliday junction. These models involve a single-strand cut within the D-loop, followed by branch migration towards the nick until the crossover resolves by sliding off the end of the nicked strand. The clear requirement for RuvC seen here suggests that if such a strategy is in use, then the RuvC protein must be performing a novel function. A more straightforward interpretation of these results is that repair of BLM-induced double strand breaks involves branch migration promoted by RecG, and junction resolution by RuvC.

In studies comparing the helicase activities of RuvAB and RecG on forked DNA *in vitro*, the Lloyd group found that RecG was inclined to unwind DNA in the direction that would generate a Holliday junction from a stalled replication fork, while RuvAB is more efficient at unwinding in the opposite direction [47-50]. RuvAB and RecG are thought to have opposite preferences for the direction of branch migration relative to the RecA filament, probably because RecG, and not RuvAB, is able to facilitate branch migration without displacement of RecA [51]. DSB repair requires invasion of an intact homolog by a single-stranded tail (generated by RecBCD), producing a three-armed junction resembling a replication fork. Branch migration must then proceed in the direction of the RecA filament, from the stem of the Y-structure into the two homologous arms (Fig. 5), ultimately generating a four-armed Holliday junction. This process may be similar or essentially identical to the branch migration of forked substrates catalyzed by RecG *in vitro* with high efficiency [49].

The requirement for RecG instead of RuvAB after BLM exposure is in contrast to the repair of lesions caused by cisplatin, which also undergo recombinational repair. Cisplatin produces crosslinks that block replication, leading to degeneration of the replication fork and creation of a daughter-strand gap, which is subjected to DSG repair. Cells lacking RuvAB are exquisitely sensitive to cisplatin, while *recG* cells are less vulnerable, suggesting that RuvAB is the preferred repair activity for cisplatin, and RecG is probably a backup system [30]. RuvAB is known to act at arrested replication forks to prevent the formation of double strand ends [52], which may be its role in cisplatin resistance.

Our initial interest in BLM stemmed from its use in combination cancer chemotherapy with cisplatin. This study demonstrates that the genes required for repair of cisplatin and BLM lesions overlap substantially, but not entirely. The proteins of early recombination, RecA and RecBCD, are involved in tolerance of both drugs. However cisplatin tolerance also depends on RecFOR, due to the occurrence of daughter-strand gaps in cisplatin-treated cells [30]. As discussed above, the post-synaptic activities required are different; cisplatin lesions are processed by RuvABC, while BLM lesions require RecG and RuvC. Although *E. coli* can never fully model the complexity of mammalian cells, these findings suggest that the success of cisplatin-BLM drug regimens may result from the overwhelming of the recombinational repair system. As RecBCD and RuvC are not transcriptionally induced, the cell must rely on constitutive levels of the proteins for processing of both types of lesions, and the amount of RuvC may be inadequate to withstand the dual assault. Another possibility is that the two types of lesions each interfere with repair of the other. The branch migration required to resolve a

daughter-strand gap produced by cisplatin might be stymied by the occurrence of a nearby double-strand break, and vice versa. The need for two different branch migration activities, RecG in one case and RuvAB in the other, might also generate confusion as multiple junctions compete for the attention of these complexes, leading to stalled or inefficient resolution of the recombination intermediates. Finally, the presence of so many junctions at once might create tangles, as the crossed strands are cut and religated. The third drug employed in combination with these two is generally a topoisomerase inhibitor, which would impair the ability of the cell to untangle the resulting knots. This scenario, though speculative, offers an appealing explanation for the power of such triple therapy.

The results of this study support the hypothesis that BLM kills cells by generating double-strand breaks, and that these breaks are repaired by recombination. We conclude that transcriptional induction of *recA* and *recN* is part of the response of cells to DSB formation, and is necessary for recombinational repair of BLM lesions. The proteins RecBCD, RuvC, and RecG are not transcriptionally induced, but are critical for repair, suggesting that their role can be fulfilled by constitutive levels of these proteins. The most surprising of our findings is the apparent requirement for RecG and not RuvAB for resolution of the crossover. The possibility of RecG and RuvC working in close cooperation is an intriguing one and, like the still-undefined function of RecN, will need to be explored *in vitro*.

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Strain	Phenotype	Genotype	Source
AB1157	wildtype	<i>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 glnV44(AS) galK2(Oc) hisG4(Oc) rfbD1 mgl-51 rpoS396(Am) rpsL31(Str^R)kdgK51 xylA5 mtl-1 argE3(Oc) thi-1</i>	E.A. Adelberg
AB2500	uvrA	As AB1157 but <i>uvrA6 deoB16 thyA12</i>	W.D. Rupp
C266	recG	As AB1157 but <i>recG258::Kan</i>	F. Stahl
CS85	ruvC	As AB1157 but <i>ruvC53 eda51::Tn10</i>	R.G. Lloyd
GM113	dam3	<i>dam-3 thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 metB1 rfbD1 mgl-51 rpsL260 kdgK51 mtl-1 thi-1</i>	Lab stock
SP254	recA	As AB1157 but <i>recN262</i>	R.G. Lloyd
GM5560	recN	As AB1157 but <i>recA56 srl300::Tn10</i>	Lab stock
JC9239	recF	As AB1157 but <i>recF143</i>	A.J. Clark
KM21	recBCD	As AB1157 but <i>ΔrecBCD::Kan</i>	K.M. Murphy
N2057	ruvAB	As AB1157 but <i>ruvA60::Tn10</i>	R.G. Lloyd
N3398	recGruvC	As AB1157 but <i>recG258::Kan ruvC53 eda51::Tn10</i>	R.G. Lloyd
JD240	mutMmutY	As AB1157 but <i>mutM::mTn10Kan mutY::mTn10</i>	Lab stock
GM7330	inverted <i>lac</i>	<i>ΔlacMS286φ80dIIIacBK1 ara thi</i>	Lab stock

Table 1 Genotypes of *E. coli* strains All strains are F⁻. Abbreviations used: Am, *amber* mutation; AS, *amber* suppressor; Δ, deletion; Oc, ochre mutation; Str, streptomycin; Kan, kanamycin; Tn5 and Tn10 encode kanamycin and tetracycline resistance respectively; mTn10, miniTn10.

Figure Legends

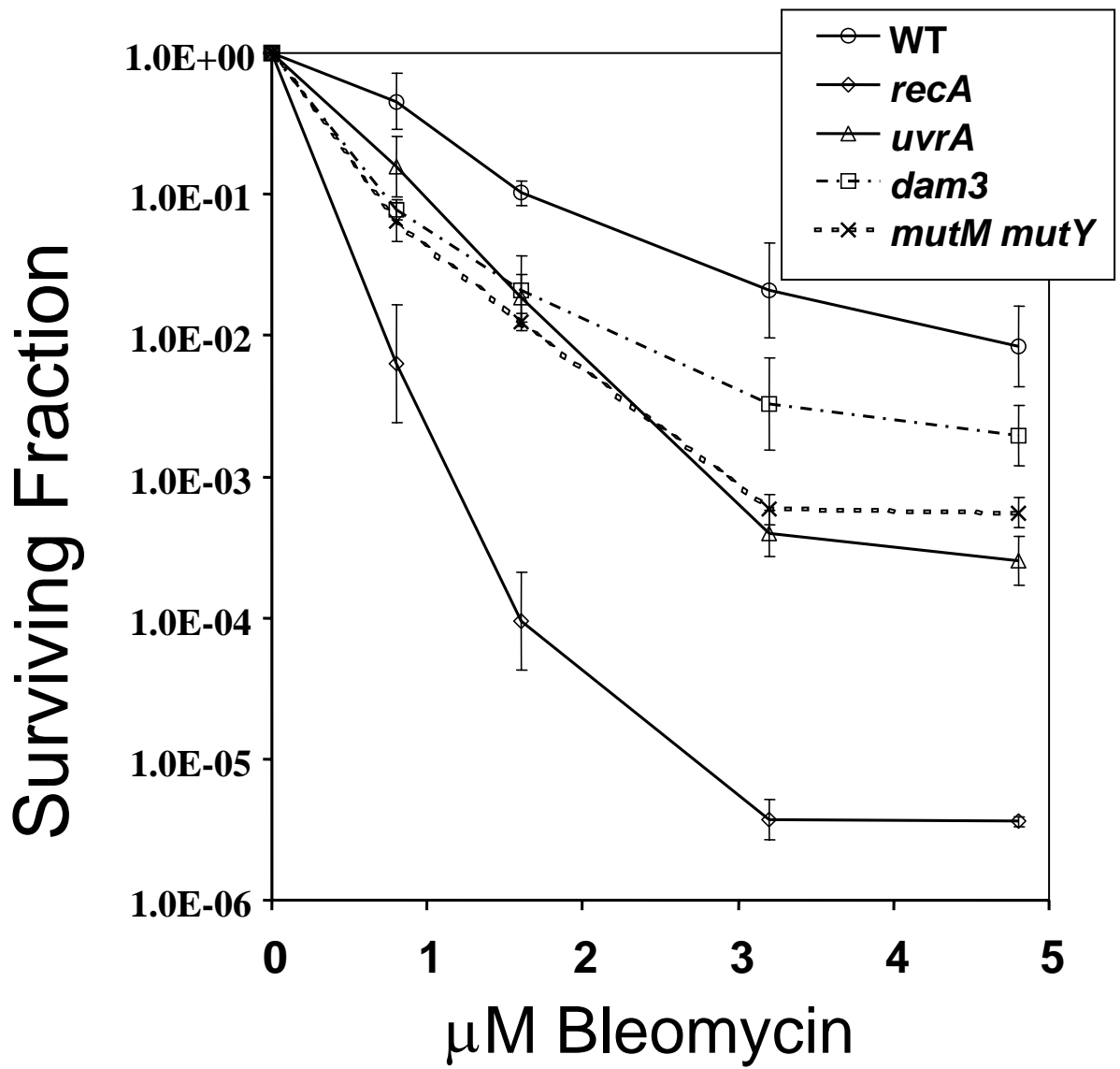
Figure 1 Cell survival after BLM treatment. Cells were treated for one hr with indicated dose of BLM. Surviving Fraction is cfu/mL (treated) / cfu/mL (untreated). WT, wildtype AB1157 cells.

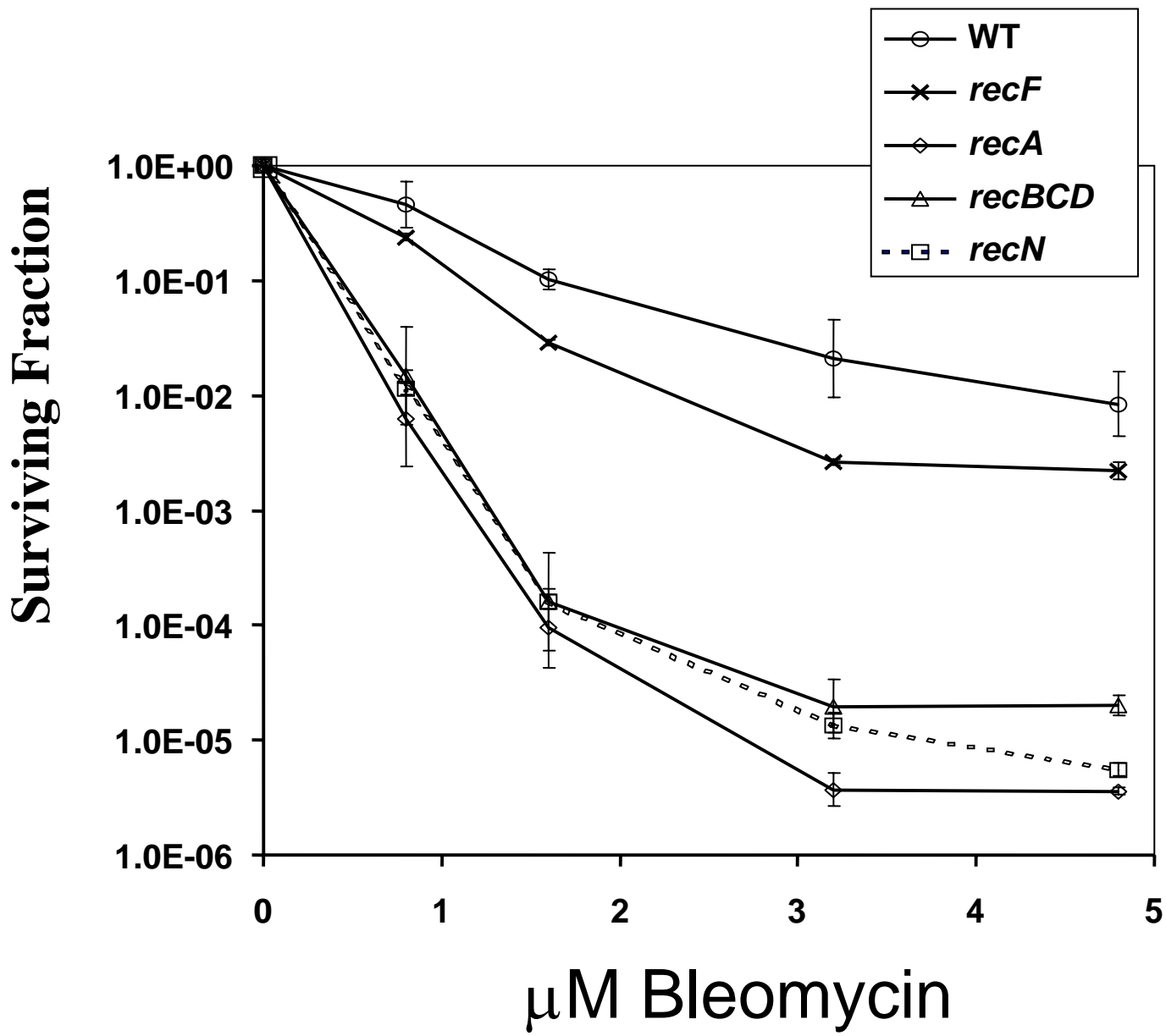
Figure 2 Cell survival after BLM treatment. Cells were treated for one hr with indicated dose of BLM. Surviving Fraction is cfu/mL (treated) / cfu/mL (untreated). WT, wildtype AB1157 cells.

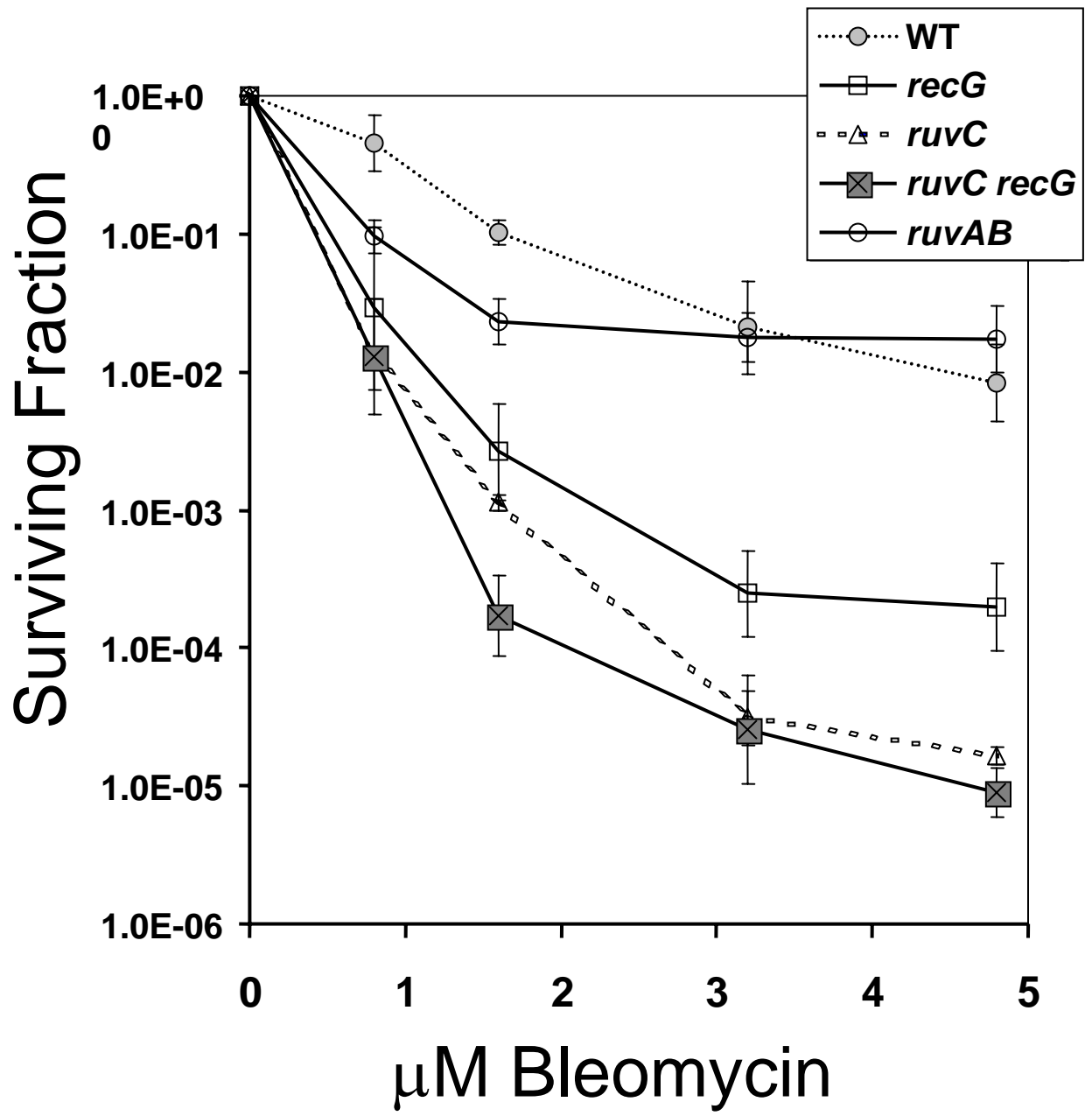
Figure 3 Cell survival after BLM treatment. Cells were treated for one hr with indicated dose of BLM. Surviving Fraction is cfu/mL (treated) / cfu/mL (untreated). WT, wildtype AB1157 cells.

Figure 4 Recombinagenesis Assay. Drug was spotted onto filter disks (dose increases counterclockwise from bottom as indicated). Red papillants represent recombination events.

Figure 5 Proposed schemes for recombinational repair of DSB (a) and DSG (b) lesions.







Cisplatin 120 nmoles 30
60
0

Bleomycin 5 μ moles 1
2
0

