Genetic Requirements for Protection Against Bleomycin Toxicity in *Escherichia coli*

By

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Submitted to the Division of Bioengineering and Environmental Health in Partial Fulfillment of the Requirements for the Degree of Master of Science in Toxicology

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Abstract

Bleomycin is known to cause double strand breaks *in vitro*. Little is known, however, about its mechanism of genotoxicity *in vivo*. One way to probe the mechanism of genotoxicity of a DNA damaging agent *in vivo* is to compare the relative sensitivities of a wild type *Escherichia coli* strain to a panel of isogenic repair deficient mutants. If the pathway defective in the mutant is known (e.g., base excision repair, alkyltransferase repair, nucleotide excision repair, and so on), the sensitivity of the mutant can reveal mechanistic insight into the mode of killing by the DNA damaging agent. In this study, mutants deficient in recombinational repair, specifically *recF, recBCD*, *ruvABC*, *recG* and *recGruvC,* were examined for sensitivity to bleomycin. This sensitivity was tested in both dividing and non-dividing cells in order to analyze the effect of cell division on the cytotoxicity of bleomycin.

When non-dividing cells were treated, the *recBCD* and *recGruvC* mutants, demonstrated high sensitivity to bleomycin. The *recF* mutant, on the other hand, demonstrated no sensitivity. These results were consistent with the conclusion that

bleomycin induces double strand breaks *in vivo* that are repaired by the recombinational repair double strand break pathway. It also suggests that no damage was induced by bleomycin that required repair by the daughter strand gap pathway.

Examining the sensitivity of recombinational repair deficient mutants to bleomycin also gave new insights about the mechanism of recombinational repair. Both *ruvABC* and *recG* gene products resolve Holliday junctions; however, they are thought to work on separate recombinational pathways. In this study, although the *recGruvC* strain was highly sensitive to bleomycin, the *ruvABC* and the individual *recG* and *ruvC* strains were not. This result suggested redundancy in the functions of the RecG and RuvABC proteins.

Dividing cells showed a marked increase in sensitivity to bleomycin as compared to non-dividing cells. In addition, the functional redundancy of *recG* and *ruvABC* mutants was no longer seen. The *ruvABC* strain demonstrated high sensitivity equal to that of the *recGruvC* and *recBCD* strains whereas the *recG* and *ruvC* strains were only slightly sensitive. Under these conditions of increased cytotoxicity, additional functions of the RuvAB enzymes became important for suppression of toxicity. This result suggested a change in the mechanism of bleomycin's genotoxicity.

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Chapter I Introduction and Background

PART A: RESEARCH OBJECTIVES

Bleomycin is a glycopeptide antibiotic isolated from *Streptomyces verticilles* (1). Its ability to kill dividing cells specifically has made this drug important in the treatment of a variety of cancers, including testicular carcinoma, Hodgkin's disease and squamous cell carcinoma of the head and neck (2; 3). Although bleomycin has been used clinically for many years, the exact mechanism of its cytotoxicity, which is believed to be linked with DNA damage, is still poorly understood.

Many studies have focused on the interaction of bleomycin with DNA. They have shown that bleomycin can induce various DNA lesions *in vitro*, namely abasic sites, single strand breaks, and double strand breaks. Little has been done, however, to examine if these damages occur *in vivo*. The primary objective of the present study was to test whether the genotoxicity of bleomycin *in vivo* is due to its ability to induce double strand breaks.

The experimental procedure used to achieve the aforementioned goal was to compare the relative sensitivities of wild type *Escherichia coli* cells and a panel of mutants to bleomycin. The mutants studied were lacking proteins involved in recombinational processing of DNA damage. The results of this analysis revealed mechanistic insight into the genotoxicity of bleomycin. Moreover, beyond the particular context of bleomycin's action *in vivo*, an important offshoot of this work was to provide

generic findings on DNA repair mechanisms that address radical mediated DNA damage *in vivo*.

The remainder of the present chapter is devoted to background (along with a literature review) on both the chemistry of bleomycin *in vivo* (Part B) and the mechanisms of DNA repair (Part C). The rest of this dissertation is organized as follows. Chapter II contains a detailed description of the materials and methodology used for this study. Chapter III presents the results of the various experiments that were conducted. Finally, Chapter IV summarizes the inferences that could be drawn from the experimental data about bleomycin-induced damage and the mechanisms of DNA repair.

PART B: CHEMISTRY OF BLEOMYCIN

The cytotoxicity of bleomycin is thought to be due to its ability to damage DNA (4). Three steps are required for this damage to occur. First, bleomycin must be activated. Second, it must interact with the DNA. Third, the initial damage in DNA leads to degradation. Of these three steps, steps one and three are fairly well understood. However, the mechanism by which bleomycin interacts with DNA (step two) is still unclear. In the following sections I shall present what is known about bleomycin, its structure and the chemistry of its function.

B1. Bleomycin Structure

The structure of bleomycin (BLM), as shown in Diagram 1, is divided up into six functional domains (5):

1. the metal-complexing pyrimidine *beta*-hydroxymidazole moieties;
2. the DNA-binding bithiazole;
3. its terminal amine, which distinguishes various forms of bleomycin (A2 and B2 being the major components of the clinically used formulation, which is called "Bleoxane" (6);
4. the linker region, which attaches the bithiazole to the rest of the molecule and determines the efficiency of double strand break;
5. the disaccharides important for uptake; and
6. the pyrimidinyl propionamide, which effects sequence specificity.

A variety of metal ions (i.e., Fe(II)/Fe(III)), Cu(I)/Cu(II), Co(III), Zn(II), Mn(II) can form complexes with bleomycin to cause DNA damage (7; 8). However, only Fe(II) and Cu(I)/Cu(II) are thought to be important for cytotoxicity (9;10). Demetallo-bleomycin is injected into patients' blood stream where it chelates Cu(II) in blood plasma (9), and in this form enters cells. However, once in the cells, Cu(II) is displaced by Fe(II) (10). It is in this iron bound form that bleomycin is thought to cause DNA degradation.

B2. Bleomycin Activation

Activation of bleomycin, (BLM), requires oxygen and iron and is thought to occur *in vivo* by either of two pathways (see Diagram 2). In the first, oxygen adds to Fe(II)BLM, followed by the addition of one proton and one electron either from another Fe(II)BLM complex (11), or from a thiol (12). Bleomycin can also be activated when peroxide (HOOH) adds to Fe(III)BLM. Fe(III)BLM is a product once activated bleomycin reacts with DNA (13) (see below).

Bleomycin ferric peroxide (HOO-Fe(III)BLM) or "activated bleomycin" is a complex in which one coordination position of Fe(III) is occupied by the peroxide anion (11). This intermediate, with a half-life of 2 min at 4 degrees Celsius is the last detectable intermediate prior to DNA strand scission (11). At this point activated bleomycin (or some transient intermediate form) acquires the ability to abstract hydrogen atoms, which is key to DNA degradation.

It is unclear what exact form of bleomycin actually acts on the DNA. It is possible that the peroxide itself initiates DNA cleavage. However, research by Stubbe *et al.* suggests that the peroxide is actually cleaved via heterolysis of the O-O bond to a high valence iron-oxo species such as O=Fe(V)BLM (8). It has also been argued that this cleavage may be homolytic (resulting in O=Fe(IV)BLM), rather than heterolytic (14) (Diagram 2). However, some evidence, including the lack of production of a peroxide radical (15), has refuted this hypothesis.

Abstraction of a hydrogen returns activated bleomycin to the Fe(III) bleomycin form. This species may be reactivated by addition of hydrogen peroxide (as mentioned

above) or by reduction via some sulfhydryl-induction reduction reaction. Evidence suggests that, *in vivo*, cytochromes (rather than glutathione) may act as this reducing agent for bleomycin (16).

B3. Interaction of Bleomycin with DNA

It is generally accepted that bleomycin initiates DNA degradation by abstracting a hydrogen atom from C-4' of the deoxyribose in DNA (to be described later). However, it remains unclear how this activated bleomycin (HOO-Fe(III)BLM) actually interacts with DNA. One reason for this uncertainty is that determining the structure of iron BLM-DNA via NMR spectroscopy has proven to be very difficult, due in part to the instability of the Fe(III)BLM (17). Much of the work in this field has thus been done on the alternative metallo-BLM, specifically CoBLM. This compound is a less reactive analog of activated BLM, cleaving DNA via the same C-4' hydrogen abstraction mechanism, and with the same sequence context specificity (18; 19). Unlike Fe(III)BLM, Co(III)BLM is exchange-inert and thus stable long enough for investigation by NMR. In addition Co(III) is diamagnetic, which makes it especially amenable to NMR spectroscopy (5).

Using data collected through NMR studies of HOO-CoBLM, Stubbe *et al.* have proposed a well supported model for bleomycin structure and DNA interaction (5) (see Diagram 3). According to this model, the bithiazole tail, inserting from the minor groove, is intercalated between the stacked bases G14 and G15 of DNA. The pyrimidine of bleomycin, important for sequence context specificity, is shown to form hydrogen

bonds with G5 (specifically N3 and the amino group of the pyrimidine with a hydrogen from the 2-amino group and N3 of G5, respectively). In their model, the terminal oxygen of the hydrogen peroxide of HOO-CoBLM is shown to be located 2.5 angstroms from the C-4' hydrogen, facilitating hydrogen atom abstraction form the sugar ring.

B4. DNA Degradation

When bleomycin abstracts a hydrogen atom from the C-4' of the deoxyribose, a free radical is left in its place. At this point two different types of chemistry can occur, resulting in two different types of DNA damage (Diagram 4).

If little oxygen is available (B of Diagram 4), the C-4' radical is believed to be oxidized to a carbonium ion (21). This oxidation could be done by a putative (OH)Fe(IV)=BLM generated by C-4' hydrogen abstraction into OF(III)(21). Reaction of the carbonium ion with water results in a ring that is electron deficient. This sets the system up for the release of the base, resulting in sugar ring opening. The final product is a chemically modified apurinic/apyrimidinic site with a keytone at C-4' and an aldehyde at C-1' (20; 21). The sugar of the AP site most likely exists in equilibrium between ring-closed and ring-open forms.

A very different DNA degradation product will result if oxygen is abundant after hydrogen abstraction. In this second pathway, the oxygen dependent pathway (A of Diagram 4), an oxygen molecule adds to C-4' to form a peroxyl radical species, which decomposes to give a strand break with 5'-phosphate and 3'-phosphoglycolate termini with release of a base-propenal (22). The mechanism by which this chemistry occurs has

been described by McGall *et al.* (Diagram 4) (23). Their work suggests that a rapid C-2' hydrogen removal (step 4 in Diagram 4) induces cleavage of the O-C-1' bond, and thus the opening of the Criegee-rearrangement sugar 4. This step is followed by a slow release of the base propenal which can occur via two pathways (C and D of Diagram 4).

B5. Four Types of Bleomycin-induced DNA Damage

Based upon the discussion above, bleomycin-induced damage is a mixture of strand breaks and abasic sites. These lesions have been shown to form in equal quantities under physiological conditions (24). Cytotoxicity of bleomycin, however, is thought to be due to double strand breaks (25). Between 10 and 33% (depending on sequence context, see below) of bleomycin-induced DNA lesions are bi-stranded (26). These lesions consist of either two chemically identical breaks in opposite strands, or an abasic site with a closely opposed strand break (Two closely opposed abasic sites are never formed; see Diagram 5) (26). The lesions in each strand are formed either directly opposite each other, or with a 1-base stagger.

The choice of sites for initiating damage is based on a highly consistent hierarchy of sequence-dependent selection rules (27). Single strand lesions occur specifically at a 5' G<u>Py</u> 3', where <u>Py</u>= C or T (the underline indicates cleavage site). Breaks occur at 5'-G<u>A</u>-3' and 5' A<u>T</u>-3' with much lower frequency, occurring the least frequently at A<u>T</u>. Following these sequence-dependent selection rules, the "hot spot" for double strand breaks is at 5'-G<u>T</u>AC-3' cleavage (where the frequency of ds:ss lesions is 1:3 vs. the normal 1:10). At this sequence, a highly favorable 5'-G<u>T</u>-3' cleavage site is provided in

each strand. Other sites for bi-stranded damage always consist of a primary site that conforms to the normal GPy sequence-specificity of bleomycin. The secondary site, however, often occurs at sequences that would not be favorable for the formation of a primary lesion (i.e., they do not necessarily follow the context rules described above) (28). The abasic site is always formed at the secondary site, after a strand break event at the first site. The discovery of this pattern of sequence context dependency suggests a model by which one molecule of bleomycin could be generating these double strand breaks. This model will be discussed in the following section.

B6. Mechanism of Double Strand Breaks

Theoretically speaking, there are two general mechanisms by which bleomycin could cause double strand (ds) breaks. The first is that the double strand breaks are formed from independent closely spaced single strand lesions (ss) in opposite strands. This idea was ruled out in 1977 by Povirk *et al.* who demonstrated, using supercoiled ColE1 and T2 DNA, that the ratio of ds to ss breaks was too high for double strand breaks to be caused by random ss breaks (24). Thus, the second possibility, that one single molecule of bleomycin causes breaks in opposite strands, was proposed.

Two observations support the hypothesis that bleomycin is in fact able to attack the secondary site in the opposite strand without dissociating from the DNA. First, a study by Absalon *et al.* of ss/ds-cleavage at a 5'-GTAC/5'-GTAC site revealed that the ratio of the extent of ss to bi-stranded lesions is invariant over at least a 70-fold range in concentration of Fe-BLM and extent in DNA degradation (29). The second rationale is

that according to Burger et al, bi-stranded lesions are formed much faster than bleomycin can be reactivated by sulfhydryls (30). Therefore, in order for one bleomycin molecule to cause bi-stranded lesions without dissociating, it must be rapidly reactivated in situ during the formation of the first strand break. The most accepted model for this reactivation proposes that the original abstracting species of bleomycin is regenerated through a direct reaction between the immediate bleomycin product of the hydrogen abstraction and the 4'-peroxyl radical (31) (see Diagram 6).

One of the most important clues leading to models of the double strand cleavage reaction was the discovery that the two sugars attacked are 15-18 angstroms apart (in B-DNA) (29). This observation suggests that bleomycin must undergo substantial "re-placement" between the first and second cleavage events. A model for this rearrangement proposed by Vanderwall *et al.* (32) suggests that the bithiazole portion of bleomycin remains intercalated between the bases, while the iron chelating complex rotates 180 degrees across the minor grove (see Diagram 7), rotating around the (B-4')-(B-C2) bond (see Diagram 1). These rearrangements place the hydrogen peroxide 3.4 angstroms from the 4' hydrogen of T15, which is the same hydrogen that is experimentally found to be abstracted.

In order to position the metal binding domain in the minor groove of the second cleavage site, the bithiazole ring system is rotated about 117 degrees around an axis perpendicular to the rings while remaining coplanar to the DNA base pairs. These rotations allow the relative orientation of the thiazole rings to the cleavage site to remain the same for both positions. It also positions the bleomycin so that the same hydrogen-bonding interactions occur between the metal binding domain and the G14 on the 5' side

of the second cleavage site. This orientation is consistent with the high rate of double strand cleavage at GTAC sequence, where the preferred GPy is provided at both the initial and secondary cleavage sites.

PART C: BLEOMYCIN DAMAGE *IN VIVO*

Although much is known about the mechanism of action of bleomycin chemistry *in vitro*, little is known about whether this same chemistry occurs *in vivo*. Bleomycin has been shown to induce single and double strand breaks in cells (as well as in isolated DNA) and under physiological conditions it seems that abasic sites and strand breaks occur in a 1:1 ratio (24). According to Povirk, sequence-specificity studies done *in vivo* suggest that these lesions result from drug-DNA interaction rather than, for example, activation of apoptotic endonucleases (28; 33; 34). This is yet to be proven, however, and the *in vivo* action of bleomycin as well as its mechanism of cytotoxicity are still, for the most part, unknown.

One way to study what is occurring *in vivo* is to examine the relative sensitivities of different repair or recombination deficient mutants to bleomycin. *E. coli* is often the organism of choice for these types of studies, because its repair and recombination systems are well studied, and mutants are readily available. Using this approach, Levin *et al.* have demonstrated that bleomycin is indeed generating abasic sites *in vivo* (35). Several other studies have demonstrated sensitivity in *lexA* and *recA* deficient *E. coli* (36; 37), (demonstrating that the SOS response is important for resistance to bleomycin). In

addition, polymerase beta-based short patch pathways (base excision repair) and aphidicolin-sensitive long patch pathways have been shown to be involved in the repair of bleomycin-induced damage in human fibroblasts (38). Little, however, has been done to examine the formation and repair of double strand breaks.

PART D: RECOMBINATIONAL REPAIR

The mechanisms by which double strand breaks are repaired is, in and of itself, an important topic of investigation. In *E. coli,* a recombinational repair system is believed to be responsible for the repair of double strand breaks (39). Recombinational repair, which is also responsible for "tolerance" of DNA lesions encountered by the replication fork, involves three steps (See Diagram 8): initiation, the Holliday junction formation, and then resolution. Recognition of strand breaks and daughter strand gaps require separate systems of enzymes. Once repair is initiated, however, the two repair systems converge. Thus, the same enzymes are used for Holliday junction formation and resolution for both double strand breaks and daughter strand gaps.

D1. Initiation

Recombinational repair is initiated by the recognition and binding of the DNA damage, which is either a double strand break or a daughter strand gap (from the arrest of the replication fork). In the case of double strand break, an enzyme complex of RecB and

RecC (an ATPase, dsDNA exonuclease, DNA helicase, chi-specific endonuclease) binds double strand breaks and then chews back blunt ends to form overhangs. The actions of this complex are regulated by RecD, which when knocked out leaves cells recombination proficient. However, the *recBCD* triple mutant has the same phenotype as *recBC*, which demonstrates deficiency in double strand end repair (40).

RecF is also an initiator of recombinational repair; however, its role seems to be limited (although this is still unclear) to the repair of daughter strand. RecF binds ss DNA and brings RecA to the site of damage. It has been shown to bind RecO and RecR to promote RecA binding to ss DNA. *E. coli* mutants deficient in *recF* are deficient in daughter strand gap repair (41).

D2. Holliday Junction Formation

Once the initiator binds, RecA is recruited to the site to initiate recombinational repair. RecA, an ATPase, forms helical fliaments, and is a catalyst of homologous pairing and strand exchange. *E. coli* mutants deficient in RecA are deficient in recombination (42).

D3. Resolution of the Holliday Junction

Belonging to what are thought to be separate pathways, RuvABC and RecG are both involved in the resolution of the Holliday junction (43; 44). RuvA has been shown to bind Holliday junctions and, in doing so recruits RuvB. RuvB is an ATPase, and

promotes branch migration, which is then resolved by RuvC, which cleaves the Holliday junction. RecG shares similar functions as RuvABC, although it does not have the same cleaving abilities as RuvC and it is thought to promote branch migration and translocation of three strand junctions. Both *recG* and *ruvAB* mutants have slight deficiencies in recombination, but the *recGruvAB* double mutant (which is identical to the *recGruvC* mutant in phenotype) is severely deficient. This result suggests that the RecG and RuvAB proteins have overlapping functions but are not fully interchangeable (45).

Chapter II Materials and Methods

In order to determine whether the recombinational repair pathway is important for repair of DNA damaged by bleomycin, mutants deficient in recombinational repair were tested for sensitivity to bleomycin. Sensitivity of these mutants to bleomycin treatment was examined under two conditions: conditions that promote cell division, and conditions that arrest it. This Chapter describes the details on how these experiments were carried out.

PART A: *ESCHERICHIA COLI* STRAINS USED

The recombinational repair *E. coli* mutants were used to probe the mechanism of bleomycin genotoxicity. Six different mutants were examined; specifically the triple mutants *recBCD* and *ruvABC,* double mutant *recGruvC*, and single mutants *recF*, *recG*, *ruvC*. These strains were provided by Dr. Martin Marinus (University of Massachusetts Medical School). The wild type strain, used as a control, is the common AB1157 originally created by DeWitt *et al.* (45). Details about the genotypes of each of these strains can be found in Table 1.

PARTB: BLEOMYCIN SOURCE AND USAGE

Bleomycin sulfate (Blenoxane), a mixture of bleomycin sulfate salts isolated from *Streptomyces verticillus*, was purchased from Sigma. The copper content of this sample was less than 0.1%. Half of the experiments were done with a culture tissue-tested batch of bleomycin (denoted BLM*) (p. 1716, Sigma catalogue of 1998). The other half of the experiments were done with a batch of bleomycin that was not culture tissue-tested (p.190, Sigma catalogue of 1998). BLM was dissolved in 10 mM HEPES, KOH, pH 7.6, at a concentration of 0.49 mM, and 0.24 mM for the second BLM* aliquot. The concentration was determined by spectrophotometry, where E_{290}= 14,000 M^{-1} and the molecular weight of BLM = 1440 g/mol (46).

PART C: GROWTH OF CELLS AND DRUG TREATMENT

C1. Treatment of Non-Dividing Cells:

The basic procedure used to probe mutant *E. coli* cells for sensitivity to bleomycin was adapted from a procedure developed by Levin *et al.* (35). Cell cultures of various *E. coli* strains were grown overnight at 37°C (spinning) in standard LB media (Bactotryptone, Bactoyeast Extract, NaCl, pH 7.0) absent of any antibiotics. The following day 10 µl of cells were diluted into 10 ml of fresh LB medium. These cells

were then grown at 37°C (spinning) until cells were in exponential growth phase (OD_{600} = 0.3-0.6), which generally took about 3.5 hrs. At this point cells were transferred into sterile 50 ml tubes and centrifuged for 15 minutes at 9000 rpm. The supernatant was then poured off and the pellet re-suspended in 4 ml of M9 medium (Na_2HPO_4, KH_2PO_4, NaCl, Na_4Cl, $CaCl_2$). The concentration of cells was then calculated, where ((OD_{600} x 6.7)-0.3)= cells/ml. M9 was added until the concentration was 1.2 x 10^8 cells/ml. Aliquots of 450 µl of cells were treated with BLM at a final concentration of 8.3, 16.7, 33.4 and 50 µM (where 50 µM = 72 µg /ml). These cultures were then incubated at 37°C in a water bath for two hours (Note that the volume of BLM/HEPES mixture added to cells was approximately 50 µl for each experiment). Reactions were then stopped by diluting the cells in M9 medium, which was done by adding 10 µl of cells to 990 µl of M9. Cells, diluted further into various concentrations, were then plated onto LB plates in order to determine colony-forming units, a measurement of survival.

C2. Treatment of Dividing Cells

The same procedure as was used for treatment of non-dividing cells was used to treat dividing cells, with the following alterations:

1. The cells were grown to early exponential phase, where OD=0.2-0.3 (thus, the concentration was 1-1.7 x 10^8 cells);
2. Aliquots of 450µl of these cells were then treated directly (not spun down or placed in M9);

3. BLM* (a new batch, see above) at a final concentration of 0.8, 1.6, 3.2, 4.8 and 7 μM (depending on the experiment) was added to these exponential phase, dividing cells. Note that the second batch, with a stock concentration of 0.24 mM, was often diluted by one fourth or one fifth in order to keep a volume of BLM*/HEPES mixture added to cells at approximately 50 μl as above;
4. Cells were treated at 37°C for 1 hour, either in the water bath (as above) or in a shaker (see below). The reaction was stopped by removing 100 μl of cells and adding this portion to 900 μl of M9. Survival was measured as described above.

C3. Treatment of Shaking Dividing Cells

Some of the dividing cells (see treatment of dividing cells above) were placed in a rotating 37°C incubator (rather than placed in the water bath) during treatment with BLM*. For these cells, 900 μl were treated with 100 μl of the BLM*/HEPES mixture with a finial BLM* concentration of 0.8, 1.6, 3.2 and 4.8 μM. This 1000 μl volume was then placed in a 5 ml Falcon tube (with a culture tube cover so that air can pass through). The tube was placed in a large culture tube with an eppendorf tube in the bottom (to hold the falcon tube in place). This double tube setup was then placed in a rotator in an incubator at 37°C for one hour. The cells were then treated as described in the previous procedure.

C4. How Sensitivity was Measured

Sensitivity of the various mutants was measured by comparing the fraction of survival of each mutant to the fraction of survival of the wild type strain. The fraction of survival was measured by dividing the number of cells that survived for each dose of bleomycin by the number of the same type of cells that survived when no bleomycin was added. The fraction of survival was then plotted as a function of bleomycin concentration. The difference between fraction of survival of wild type and mutant at the third dose of bleomycin treatment estimates sensitivity.

Chapter III Results

This study of consists of four general experiments. In the first, the sensitivities of various recombination/repair mutants were investigated in conditions that arrest cell division (specifically, treatment in M9, a salt solution deficient in sugars and other nutrients) (Part A). The effects of treating wild type *E. coli* cells under conditions that promote cell division (specifically treatment in LB, a nutrient rich liquid broth, and treatment in LB, shaking) were then examined (Part B). The last two experiments examined how these two conditions, treatment in LB and shaking, affected the sensitivities of the panel of recombination/repair deficient mutants (Part C and Part D).

PART A: SENSITIVITY OF NON-DIVIDING MUTANT CELLS TO BLEOMYCIN:

The importance of RecB (which has the same phenotype as *recBCD*) in the repair of bleomycin-induced damage has been demonstrated in a previous study (47). This study shows that DNA reformation found in wild type *E. coli* cells is abolished in *recB* mutants. Results in Figure 1 clearly support these earlier findings, demonstrating over one log increased killing in the *recBCD* mutant as compared to wild type (at a dose of 30 μM BLM). The *recGruvC* mutant was also shown to be as sensitive to bleomycin as *recBCD*. Interestingly, neither *recG, ruvABC* nor *ruvC* deficiencies alone demonstrated

sensitivity. The *recF* mutant also demonstrated no sensitivity to bleomycin. These results have been summarized in Table 2.

PART B: DIVIDING CELLS DEMONSTRATE INCREASED SENSITIVITY TO BLEOMYCIN

In this first experiment, (Figure 1) cells were treated under conditions where they were not dividing. Bleomycin is known to be more effective against dividing cells (48). Therefore, this same experiment was conducted a second time, only under conditions where the cells were allowed to divide during treatment with bleomycin. The results of this experiment, presented in Figure 2, demonstrated that *E. coli* cells were much more sensitive to bleomycin when they were dividing (approximately two and a half logs of increased killing). However, shaking them while they were being treated (which should increase division rate by providing oxygen) did not seem to increase sensitivity further (Figure 2). Whether increasing oxygen increased cell division rate, however, was not measured.

PART C: SENSITIVITY OF DIVIDING MUTANT CELLS TO BLEOMYCIN

Although a general increase in sensitivity of dividing cells to bleomycin was predictable, a change in relative sensitivity of the various mutants was not (Figures 3 and 4). Figure 3 demonstrates that *ruvABC*, which was not sensitive to bleomycin in M9, was extremely sensitive in LB. In fact, the sensitivity in LB was equal to that of *recBCD* and *recGruvC*, the sensitivity of which seems to have increased to about 2.5 log of increased killing as compared with the wild type strain. The *ruvC* and *recG* mutants also demonstrated a slight increase in sensitivity having about a half a log of increased killing as compared with the wild type strain (Figure 4). Interestingly, the sensitivity of *ruvC* and *recG* were approximately equal in magnitude. The *recF* mutant, on the other hand, remained insensitive to bleomycin when treated in M9 (Figure 4). The results have been summarized in Table 2. It is important to note, however, that the data from Figure 4 were obtained from a single experiment, and these results thus need to be confirmed.

PART D: EFFECTS OF SHAKING ON SENSITIVITY OF DIVIDING CELLS

If cell division is a factor in the differential sensitivities seen above, then shaking the cells (which is known to increase *E. coli* dividing rate) during treatment with bleomycin could affect the sensitivity of various mutants. Results in Figure 2 demonstrate that shaking also did not increase sensitivity of wild type. In Figures 5 and 6, we see that shaking did not seem to affect the relative sensitivity of the various mutants. However, data from Figure 5 and 6 also need to be confirmed.

Chapter IV Discussion

The results of this study, presented in Chapter III, examined two systems, bleomycin function *in vivo* and recombinational repair, under two conditions, when cells were dividing and when cells were not. This final Chapter discusses these results. Part A examines the results from the first experiment, where the sensitivities of various recombination deficient mutants to bleomycin were observed under conditions where cells were not dividing (Chapter III, A1. and Figure 1). Conclusions about both bleomycin damage and the mechanism of recombinational repair are inferred (Chapter IV PartA). In the following section, how cell division during treatment with bleomycin changes the function of these two systems (Part B-D) is discussed. Part G summarizes general conclusions about the bleomycin-induced damage *in vivo* and the mechanism by this damage is repaired. The final section (G2) proposes future experiments.

PART A: *RECBCD* AND *RECGRUVC* ARE IMPORTANT FOR REPAIR

A1. Bleomycin Induces Double Strand Breaks *in Vivo*

RecBCD specifically binds to double strand breaks. From the results in Figure 1 the *recBCD* mutant demonstrated significant sensitivity to bleomycin. This suggested that bleomycin was indeed causing double strand breaks *in vivo*. This notion is supported

by the fact that *recGruvC* mutants also demonstrated high sensitivity (Figure 1). At the same time, sensitivity of *recBCD* and *recGruvC* indicates that recombinational repair was important for repair of the lesions caused by bleomycin (Figure 1). Insensitivity of *recF* suggested that it was the double strand break recombinational repair system (rather than the daughter strand gap pathway) that was required for repair of bleomycin-induced damage.

A2. Daughter Strand Gap Repair was Not Required

RecF is critical for the initiation of daughter strand gap repair, a mechanism that allows the cell to "tolerate" or by-pass lesions during replication. The lack of sensitivity of the *recF* mutants to bleomycin thus suggested that bleomycin was not creating lesions that interrupt replication, and that the cell cannot repair (which would require bypass in order to continue replicating). Recall, however, that in Figure 1, the cells were not replicating. Daughter strand gap repair is required specifically during replication. Thus in order to be sure that RecF (and thus daughter strand gap repair) was not involved in the repair of bleomycin-induced damage, the sensitivity of *recF* mutants had to be tested under cell division permissive conditions. Results in Figures 4 and 6 demonstrate that *recF* remained insensitive when the cells were in LB (and therefore replicating). This result further suggested that the daughter strand gap repair system is not important for repair of bleomycin-induced damage.

A3. RuvABC and RecG Have Redundant Functions

Both RecG and RuvABC are involved in resolution of the Holliday junction. Although RecG and RuvABC are thought to resolve Holliday junctions through independent pathways, they are also thought to have some overlapping functions (45). Both the *recG* and *ruvABC* mutants have slight deficiencies in recombination, but the *recGruvAB* double mutant (which is identical to the *recGruvC* mutant in phenotype) is severely deficient (45).

Results from Figure 1 suggested that under the conditions where cells were not dividing, the functions of *recG* and *ruvABC* were completely overlapping. This is shown by the fact that the *recGruvC* double mutant was shown to be highly sensitive to bleomycin, while none of the individual mutants, *ruvABC*, *ruvC* nor *recG,* were sensitive. Perhaps under these conditions, the type of repair required was of a type that either RecG or RuvABC can carry out. Under different conditions, however, where more damage or different types of damage are induced, new functions of RecG and RuvABC are required making them no longer interchangeable. This is what was seen in Figures 3 and 4 and will be discussed in a later section.

A4. Sensitivity of *recGruvC* is Equal to That of *recBCD*

Two inferences can be made from the observation that, in Figure 1, *recGruvC* exhibited the same level of sensitivity as *recBCD*. The first is that it supports the idea that they are part of the same pathway. Knocking one out entirely eliminates the ability

to repair double strand breaks (39). The second is that neither of these enzymes was being used to repair other lesions created by bleomycin (such as daughter strand gap repair). For if, for example, daughter strand gap repair was being engaged, then one would have expected higher sensitivity of the *recGruvC* mutant than *recBCD*.

PART B: CELL DIVISION INCREASES SENSITIVITY TO BLEOMYCIN

Bleomycin is known to be more effective on dividing cells than non-dividing cells (thus providing a possible reason explaining why it is effective in cancer treatment). Therefore it seemed important to see if the sensitivity of the wild type *E. coli* cells to bleomycin would increase when the cells were treated in LB, where they would continue to grow, versus M9, which, lacking nutrients necessary for division, slows or halts cell growth. The results in Figure 2 demonstrated that, in fact, cells treated in LB, instead of M9, were, at 5 μM BLM, 3 fold more sensitive than cells grown in M9.

There can be two general reasons for this increased sensitivity. The first is that there was an increase in the number of lesions. The second is that each lesion was more detrimental to the dividing cell than the non-diving cell.

B1. Bleomycin Enters Cells More Efficiently?

There are, once again, several reasons why more lesions could be formed. It is possible that under rapid growth conditions bleomycin was more effective at inducing

damage. For example, when cells are dividing more iron or oxygen could be available for bleomycin activation. A second possibility is that more bleomycin was getting into the cells. Bleomycin is a large molecule and the exact mechanism by which it enters cells is unknown, although some evidence suggests that it is actively transported into the cells (49). Evidence that supports the idea that it is actively transported is the fact that after several hours of treatment, intracellular concentrations of bleomycin remain lower than the extra-cellular concentrations (50). Perhaps, during cell division, bleomycin is able to enter cells more easily. This is another possible explanation for increased toxicity when cells are treated with BLM in LB.

B2. Each Lesion is More Detrimental In Dividing Cells

The second general cause for increase sensitivity could be that each lesion was more detrimental to the cell when the cell was dividing then when it was not. When cells are dividing there is less time to repair damage. In addition, the process of attempting to replicate this damaged or "intercalated" DNA could lead to damage or cell death, which would not occur were the cells not being replicated. In order to investigate this possibility further, I decided to alter another variable that should increase cell replication, namely the oxygen level.

B3. Increasing Oxygen Has No Effect on Sensitivity to BLM

Cell growth rate is not only dependent on available sugar and other nutrients but on oxygen as well. Oxygen acts as the terminal electron acceptor in respiration. If media, in which *E. coli* cells are growing, are not supplemented with oxygen during growth (i.e., by shaking), oxygen levels are depleted with in 15 minutes (51). Thus, in order to enhance cell growth further, cells, incubated in LB, were shaken during treatment (rather than being placed in a water bath). Although the addition of sugar enhanced sensitivity to bleomycin drastically, shaking, as seen in Figure 2, had no obvious effect.

There are two ways to explain the fact that shaking had no effect. One explanation could be that the method of shaking was not effective in increasing oxygen levels significantly to alter cell division. However, this method is the standard method of increasing oxygen in LB during *E. coli* growth. Another method by which lesions could have been increased is the following. As stated earlier bleomycin is believed to enter cells via some type of transport mechanism. It is known that the sugar moieties on bleomycin are crucial for this form of transport (52). One possibility is that bleomycin is taken up as a "modified" sugar so that when the cells are in LB, channels important for transporting sugar into the cell are opened, allowing bleomycin to enter the cell, along with the sugars, at a much higher rate. This theory could be tested easily by fluorescent or radio labeling of bleomycin and examining rate of uptake into the cells in different media.

PART C: TREATMENT IN DIVIDING CELLS INDUCES NEW REPAIR PATHWAYS

Increased sensitivity of dividing cells to bleomycin may be due to increased number of lesions. In this case, all of mutants sensitive to bleomycin in non-dividing cells should demonstrate increased sensitivity. Likewise, the mutants insensitive in non-dividing cells should remain insensitive. However, increased sensitivity may be due to alterations in the types of damage that bleomycin is inducing. In this case, the specific mutants that are sensitive to bleomycin may change. In order to determine if treatment of dividing cells (versus non-dividing) actually changes the types of mutations induced by bleomycin, the various recombination/repair mutants were treated with bleomycin in LB and examined for sensitivity.

C1. A New Function for RuvAB

In non-dividing conditions the phenotypes of *recG*, *ruvABC* and *ruvC* were equal (namely no sensitivity to bleomycin). The high sensitivity of *recGruvC* mutants suggested that either *recG* or the *ruvABC* complex was needed, but that functionally one could replace the other. The fact that RuvA, RuvB and RuvC work together to resolve Holliday junctions would suggest the *ruvC* and *ruvABC* mutants are equivalent. This hypothesis was supported by the fact that both the *ruvC* and *ruvABC* strains were insensitive to bleomycin. From this result one could conclude that, functionally, the *recG*

is equivalent to the *ruvAB* mutant, which is equivalent to *ruvC* (see Chart 2). However, the results in Figures 3 and 4 suggest that *ruvAB* has a function independent of *recG* and *ruvC*. This function is essential for repair of damage done by bleomycin only under conditions where cells are dividing.

C2. RuvAB is Essential and Independent of RuvC

When dividing cells were treated with bleomycin, the sensitivity of the *ruvABC* strain went from zero to a sensitivity exactly equal to that of *recGruvC* and *recBCD* (two and a half logs of killing as compared with wild type). This observation suggested that a function of *ruvABC*, which was not essential in non-dividing cells (Figure 1), became essential to repairing lesions induced by bleomycin when dividing cells were treated (Figure 3). Although the sensitivities of *recG* and *ruvC* (which were, interestingly enough, equal) also increased, the sensitivities of these two mutants was still less than that of *ruvABC*, *recGruvC* and *recBCD* by almost two logs. Thus in these conditions *ruvC* and *ruvABC* no longer shared the same phenotype. From these results, one can infer that it is RuvAB (rather than RuvABC) that acquired this new function important for repair of bleomycin-induced damage. Sensitivity of the *ruvAB* mutant to bleomycin should be examined under these conditions to confirm this result.

C3. RuvABC and RecG are Not Exchangeable

Overall, these data suggested that under these conditions both of the resolution pathways in recombinational repair (*recG* and *ruvABC*) were now needed to protect the cell against damage induced by bleomycin. Whether this was due to an increase in the number of lesions or an alteration in the types of damage caused by each molecule of bleomycin is unclear. The fact that sensitivity of *recGruvC* and *recBCD* mutants increases in dividing cells suggested that the general amount of damage repaired by double strand break recombinational repair increases. The fact that *recF* mutants remained insensitive to bleomycin suggested that at least damage requiring daughter strand gap repair was not invoked.

PART D: SHAKING CELLS HAS NO EFFECT ON SENSITIVITY TO BLEOMYCIN

The ability of bleomycin to cause double strand breaks was dependent on the availability of oxygen. Thus shaking the cells, which increases oxygen levels in the medium could theoretically increase the ability of bleomycin to cause double strand breaks. The lack of increased sensitivity or alteration in sensitivity therefore suggested two points. The first is that the oxygen availability is not altered (i.e., all of the increased oxygen was used by the *E. coli* cell for division). The second possibility was that

increasing oxygen in this manner did not alter the chemistry of bleomycin and its interaction with DNA. The fact that shaking had no effect on the pattern of sensitivity of the various recombination/repair mutants (Figures 5 and 6) suggested that in fact, shaking did not alter the types of damage induced by bleomycin. Data in Figures 5 and 6 have only been tested a few times, however and need to be re-tested in order to confirm this conclusion.

PART E: BLEOMYCIN INDUCES SENSITIVITY OF *E. COLI* TO HEPES BUFFER: LIPID PEROXIATION?

One of the side effects of bleomycin treatment is alveolar cell damage and subsequently pulmonary inflammation (53). This is not thought to be caused by damage of DNA in lung alveolar cells, but rather by damage of the lipid membranes of the cells. Bleomycin is known to be able to induce lipid peroxidation (54). During this study, this "other" function of bleomycin seems to have been revealed.

During one experiment instead of diluting cells in M9 (as usual), they were accidentally diluted in HEPES. Although all of the zero bleomycin added cells grew normally (counting 2-5x10^8 cells) (data not shown), all of the cells treated with bleomycin, from 0.8-4.8 μM, were 100% killed. This result suggested that bleomycin somehow made the cells vulnerable to this new environment. Considering bleomycin's known ability to induce lipid peroxidation (54), it was possible that in this experiment

bleomycin actually created holes in the cell walls. This "poration" then enabled HEPES, which is N-2-hydroxyl ethyl piperazine-N'-2-ethane sulfonic acid in salt and buffer, to flow into or suck water out of the cell. This lipid peroxidation activity of bleomycin should be investigated further as it could significantly affect its mechanism of cytotoxicity *in vivo*.

PART F: CONCLUDING THOUGHTS

F1. Conclusions About the Genotoxicity of Bleomycin *In Vivo:*

From this study, several inferences can be made about the genotoxicity of bleomycin. Results from this study indicated that bleomycin induced double strand breaks *in vivo*. At the same time, bleomycin did not seem to create lesions that block replication and required daughter strand gap repair.

In this study, bleomycin was shown to be more effective against dividing cells. Increased sensitivity of dividing cells to bleomycin could be due to increased uptake of bleomycin by dividing cells. However, alterations in the sensitivity of the various mutants suggested that new types of repair systems were required when dividing cells were treated. This result suggested that, in dividing cells, new types of damage were induced. Perhaps the same lesions created in non-dividing cells were more detrimental to the cells when they were replicating their DNA. However, a large increase in DNA

damage could also have been overwhelming the repair system used in non-dividing cells, requiring the involvement of a new set of enzymes in dividing cells.

The fact that shaking, which is known to increase cell division, had no effect on the sensitivity of cells to bleomycin suggested that it is not cell division that caused an increase in sensitivity. This suggests the theory that sugar may be important for uptake of bleomycin. However, this is all speculative. Why bleomycin is more effective in dividing cells needs to be investigated further.

F2. Conclusions On The Mechanism of Recombinational Repair

From this study new insights are also gained about the roles of the various recombinational repair enzymes in the repair of bleomycin-induced damage. RuvABC and RecG seemed to be interchangeable for the types of damage induced when treated in M9. In addition, under these conditions, *ruvABC* and *ruvC* seem to be operationally the same mutant. The equal sensitivity of the recBCD and recGruvC mutants confirmed the model proposed by Cox that these two enzymes function as part of the same pathway (RecBCB functioning upstream of RecG and RuvABC) (39). However, when dividing cells were treated, RuvABC took on a role that was essential and seemingly independent of both RuvC and RuvG. This result suggested that the function of these enzymes may be multi-modal.

F3. Clinical Significance of Results

The results of my work are consistent with experience of usage of bleomycin in the clinical world. Bleomycin is generally used clinically in combination with other chemotherapeutic drugs such as cisplatin. Damage induced by cisplatin is also addressed by recombinational pathways, to an even greater extent than bleomycin (55). If cells were treated by both agents, their recombination system would be severely taxed and possibly overwhelmed, especially in rapidly growing cells where bleomycin damage seemed to affect at least two recombination pathways at once (*recG* and *ruvABC*). A better understanding of how these drugs target dividing cells and affect the repair system will enable the design of more effective drugs for combating cancer.

PART G: FUTURE EXPERIMENTS

The results of this study lead to many questions that should be further investigated. Experiments investigating the genotoxicity of bleomycin will be described first. Then, in the following section, experiments examining the mechanism of recombinational repair are proposed.

G1. Testing The Mechanism of Bleomycin Genotoxicity

From this study it is clear that bleomycin caused double strand breaks *in vivo*. Studies by Levin and Demple have also demonstrated that bleomycin induces abasic sites *in vivo,* requiring repair by endonuclease IV (35). At this point it would be very interesting to determine, *in vivo,* the relative importance of these two types of damages. One way to do this would be to examine an endonuclease IV deficient, *recBC* deficient triple mutant, for example. The results of this experiment would also indicate whether abasic sites lead to double strand breaks.

The toxicity of bleomycin was much higher in dividing cells. One possibility is that in dividing cells bleomycin was actually causing a higher number of double strand breaks. One way to examine if levels of damage were actually increased DNA would be to examine the sensitivity of *recA*, *lexA, recN* deficient mutants to bleomycin under dividing and non-dividing conditions. These three genes are important for the SOS response and thus would indicate whether the general SOS response was increased when cells were treated in M9, LB or LB-shaking. The levels of DNA damage should also be examined directly to see if one actually gets increased damage in dividing cells.

It is possible that bleomycin enters dividing cells more efficiently than non-dividing cells. In order to test this hypothesis, fluorescence labeling could be used to visualize transport of bleomycin into dividing versus non-dividing cells. The effects of sugar on transport of bleomycin into cells could also be tested in this manner.

Our results suggested that shaking, and thus increasing oxygen, has no effect on the genotoxicity of bleomycin. However, it is known from *in vitro* studies of bleomycin

(see Chapter 1, B4) that availability of oxygen affects the types of DNA damage induced by bleomycin. In order to examine further the effects of oxygen on the genotoxicity of bleomycin, oxygen could be bubbled into M9 medium during treatment of cells with bleomycin. Under these conditions, sensitivity of the various recombinational repair mutants should then be examined. The results of this experiment would indicate the importance of oxygen to bleomycin's cytotoxicity. This would be helpful for understanding its function as a chemotherapeutic agent, as cancer cells tend to suffer from hypoxia.

G2. Testing the Mechanism of Recombinational Repair

Results from this study suggest that RuvAB may have a role that is independent of ruvC. This role was observed in bleomycin-treated dividing cells. When non-dividing cells were treated however, the *ruvABC* and *ruvC* mutants had the same phenotype. In order to confirm this suspicion, the sensitivity of the *ruvAB* mutants (versus the *ruvABC* mutants) should be examined under both cell division permissive and non-permissive conditions. For this same reason the *ruvABrecG* mutant, which has been claimed to have the same phenotype as *ruvCrecG* (45), should also be tested for sensitivity to bleomycin in both cell division permissive and non-permissive conditions.

These studies will improve our understanding of how *E. coli* repair double strand breaks. Although the exact mechanism of repair may be different in humans, understanding how these repair machines work in *E. coli* can give new insights as to how human cells repair double strand breaks.

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Diagrams

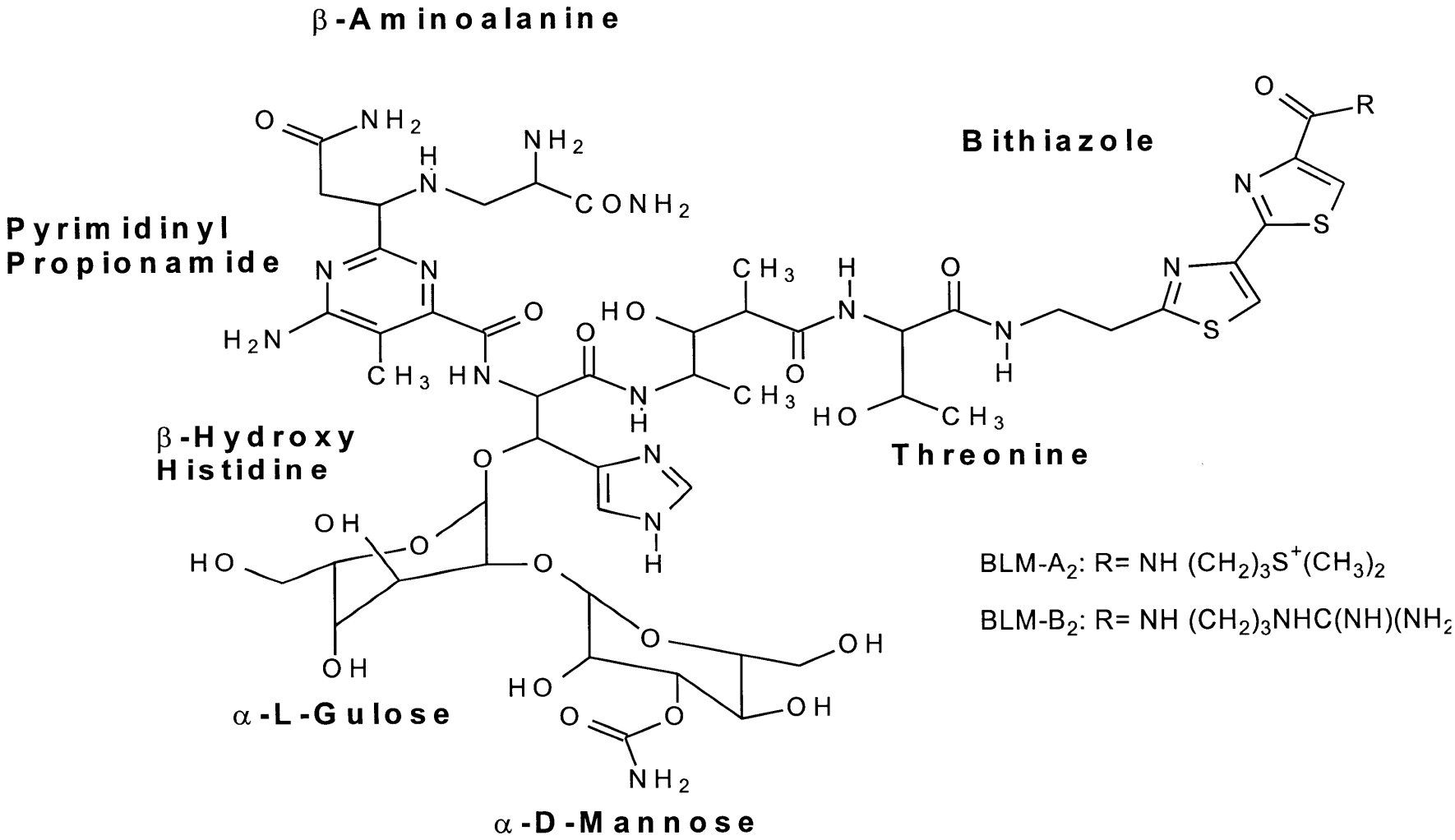

Diagram 1: Structure of Bleomycin and Its Six Domains: the metal-complexing beta-hydroxymidazole; DNA binding bithiazole; its terminal amine which distinguishes various forms of BLM; the linker region which determines efficiency of double strand breaks; disaccharides important for uptake and, the pyrimidinyl propinonamide, which effects sequence specificity. (Diagram was adapted from Wu *et al.* (1996) J. Am. Chem. Soc., 118: 1268-1280)

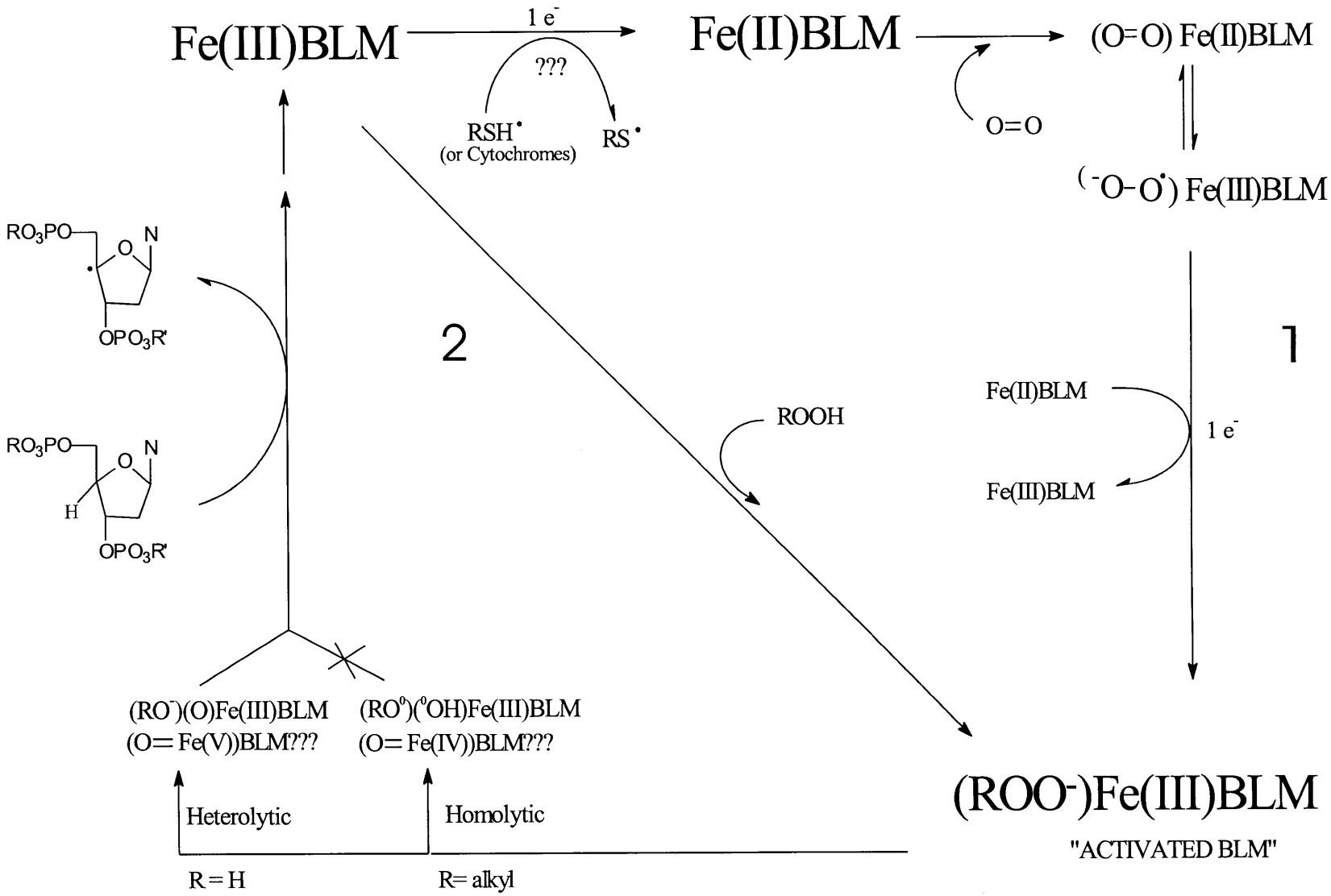

Diagram 2: Bleomycin Activation Pathways. Labels 1 and 2 indicate the two main pathways for activation of Bleomycin. (Diagram was adapted from Dedon, P., Goldberg, I., J. Am. Chem.Soc. (1992) 5: 311-331)

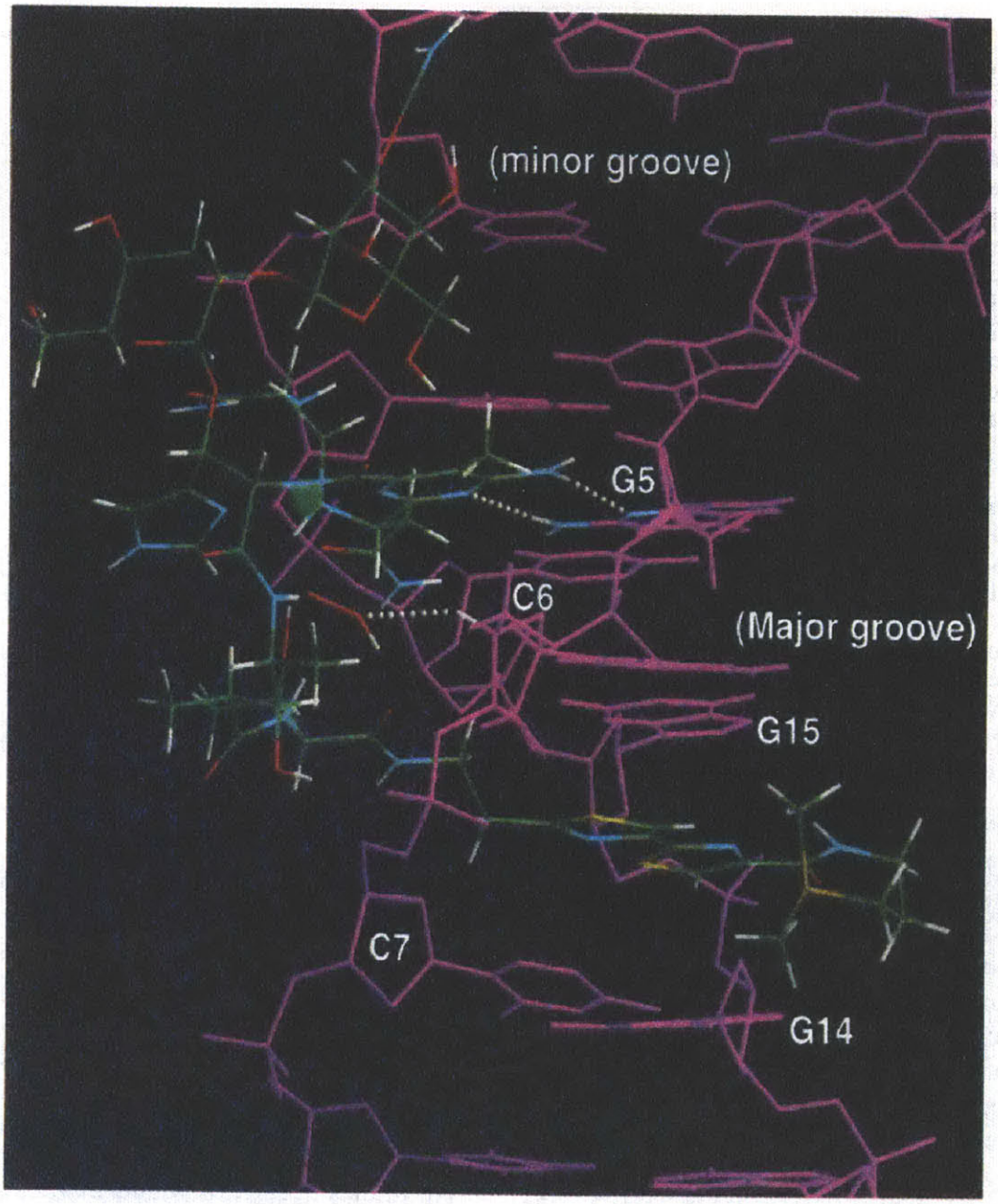

Diagram 3: Structure of Co-BLM A2, colored green (atoms colored by element C =green, O=red, N=blue, S=yellow) bound to DNA (purple, C6-H4' = white). Damaged strand is in the foreground, running 5' to 3' from the upper right to the lower left corner. The dotted lines indicate the H-bond interactions between the pyrimidinyl propionamide moiety of CoBLM and the G5 of the DNA. Also indicated is the proximity of the distal oxygen of the hydroperoxide ligand to the C6-H4' (2.5 angstroms). (Figure was taken from Wu, W., Vanderwall, D., Turner, C., Kozarich, J., Stubbe, J., J. Am. Chem. Soc. (1996), 118, 1281-1294)

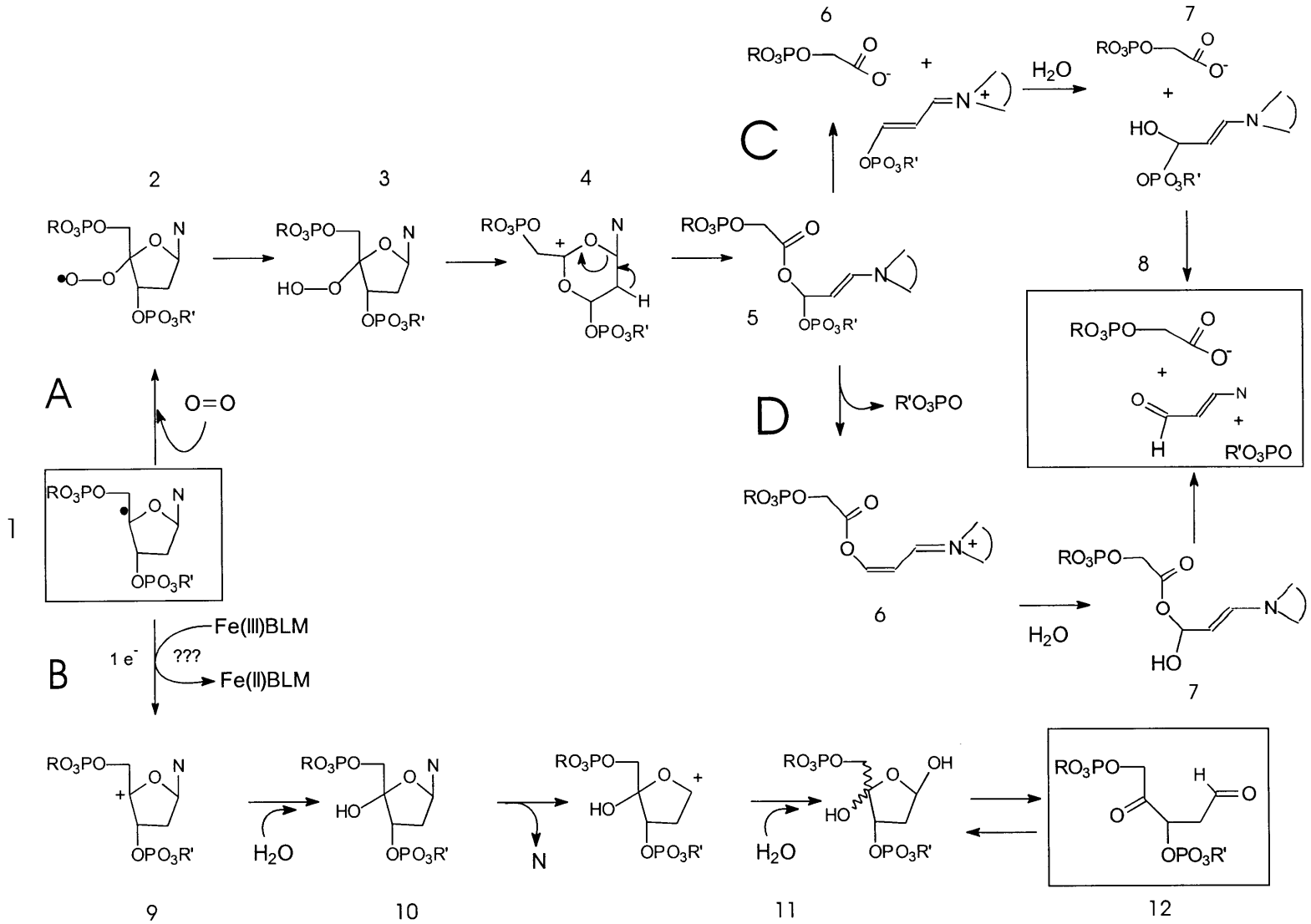

Diagram 4: Proposed Mechanism of Bleomycin-induced DNA Degradation. Bleomycin initiates degradation via hydrogen atom abstraction from the 4′ carbon of the deoxyribose. This initial, hydrogen abstracted, sugar and the final products of degradation are boxed. (Diagram was adapted from Dedon, P., Goldberg, I., J. Am. Soc. (1992) 5, 311-331)

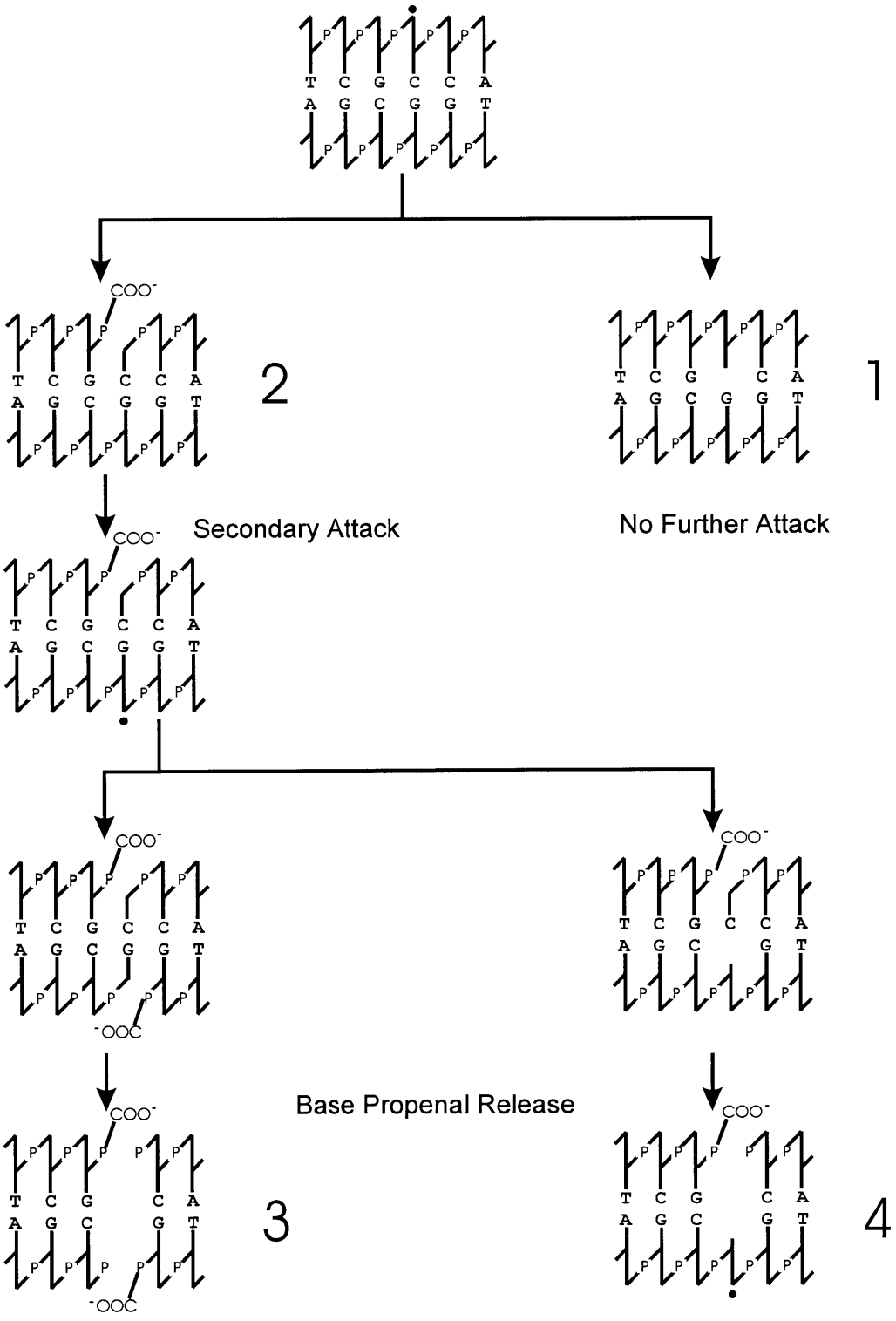

Diagram 5: Four Types of Damage Induced by Bleomycin. Bleomycin can create abasic sites (1), single strand breaks (2), double strand breaks (3) and single strand breaks opposing abasic sites (4). (Diagram was adapted from Povirk, L., Mutation Research (1996) 71-89)

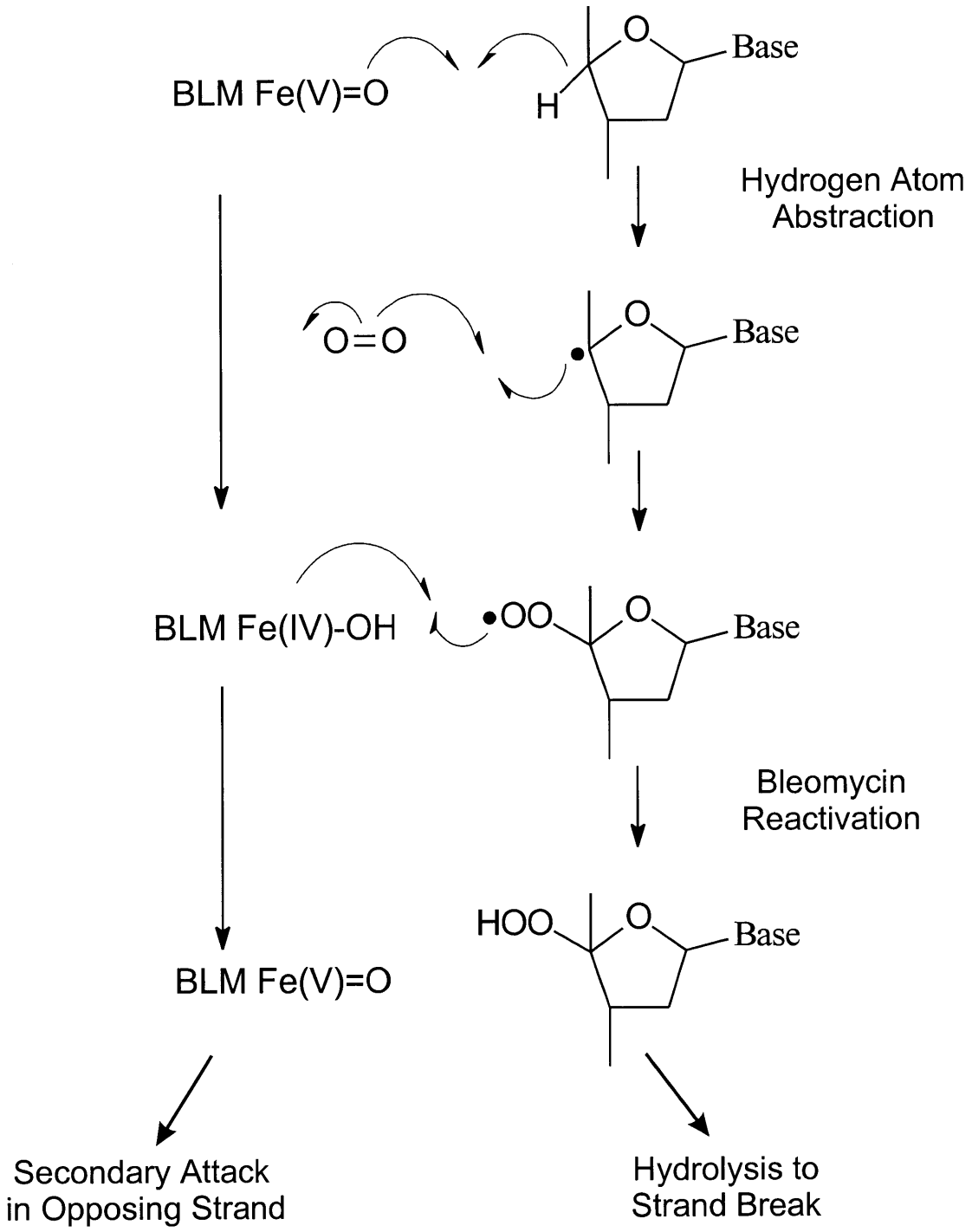

Diagram 6: Proposed Mechanims of Bleomycin Reactivation Enabling Double Stranded Lesions. (Diagram was adapted from Absalon, M.J. Kozarich,J. and Stubbe, J., Biochemistry (1995) 34, 2076-2086)

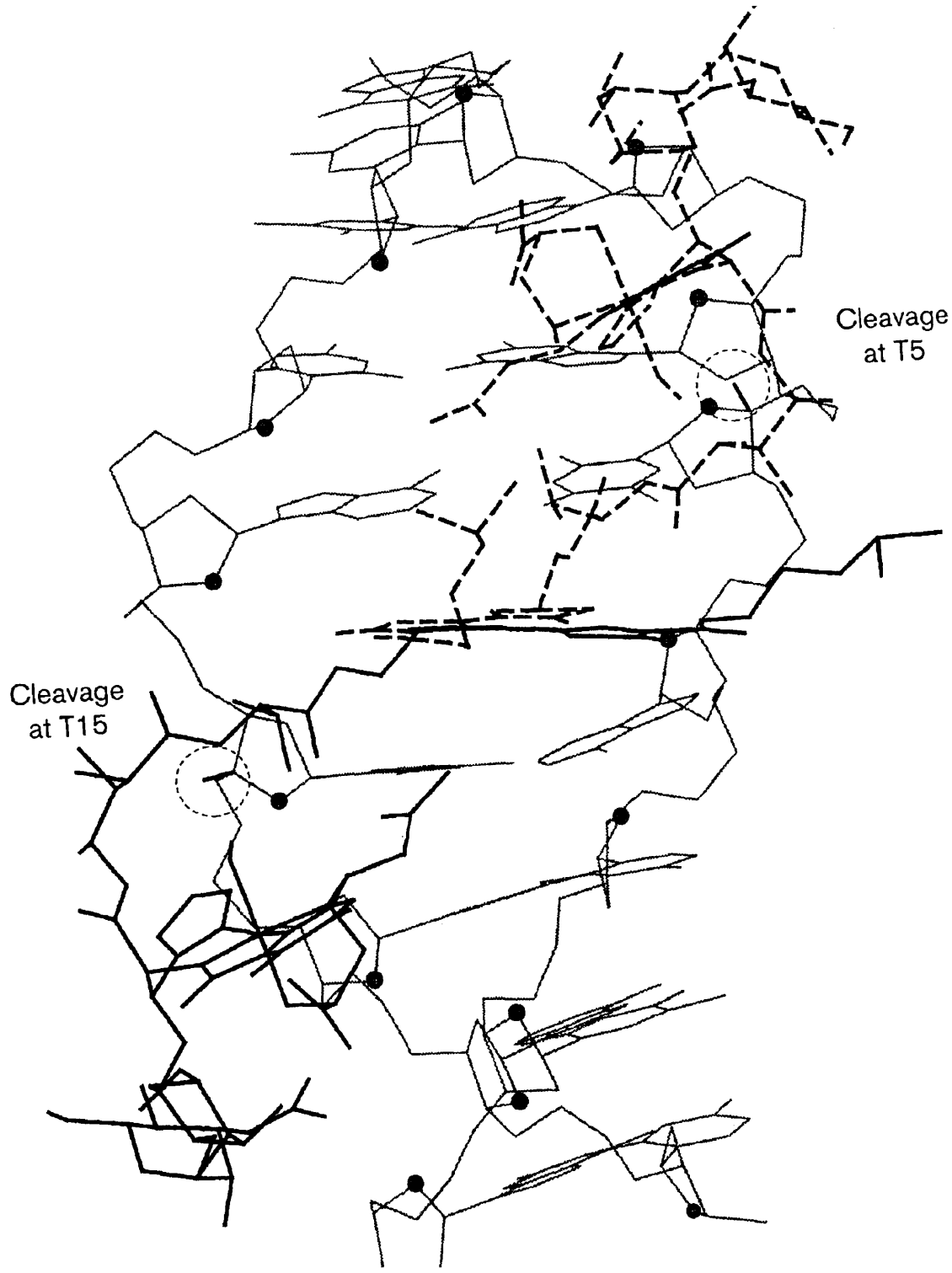

Diagram 7: Model of Bleomycin Flipping During Double Strand Cleavage. The translocation of the metal-binding domain of HOO-CoBLM from T5 (dashed lines) to T15 (solid lines) occurs by a rotation around the (B-C4')-(B-C2) bond and the repositioning of the bithiazole. The 4' hydrogens at T5 and T15 are circled and the O4' of DNA is shown as a gray sphere. (Diagram was adapted from Valderwall *et al.* Chem. Biol., (1997) 4: 373-387)

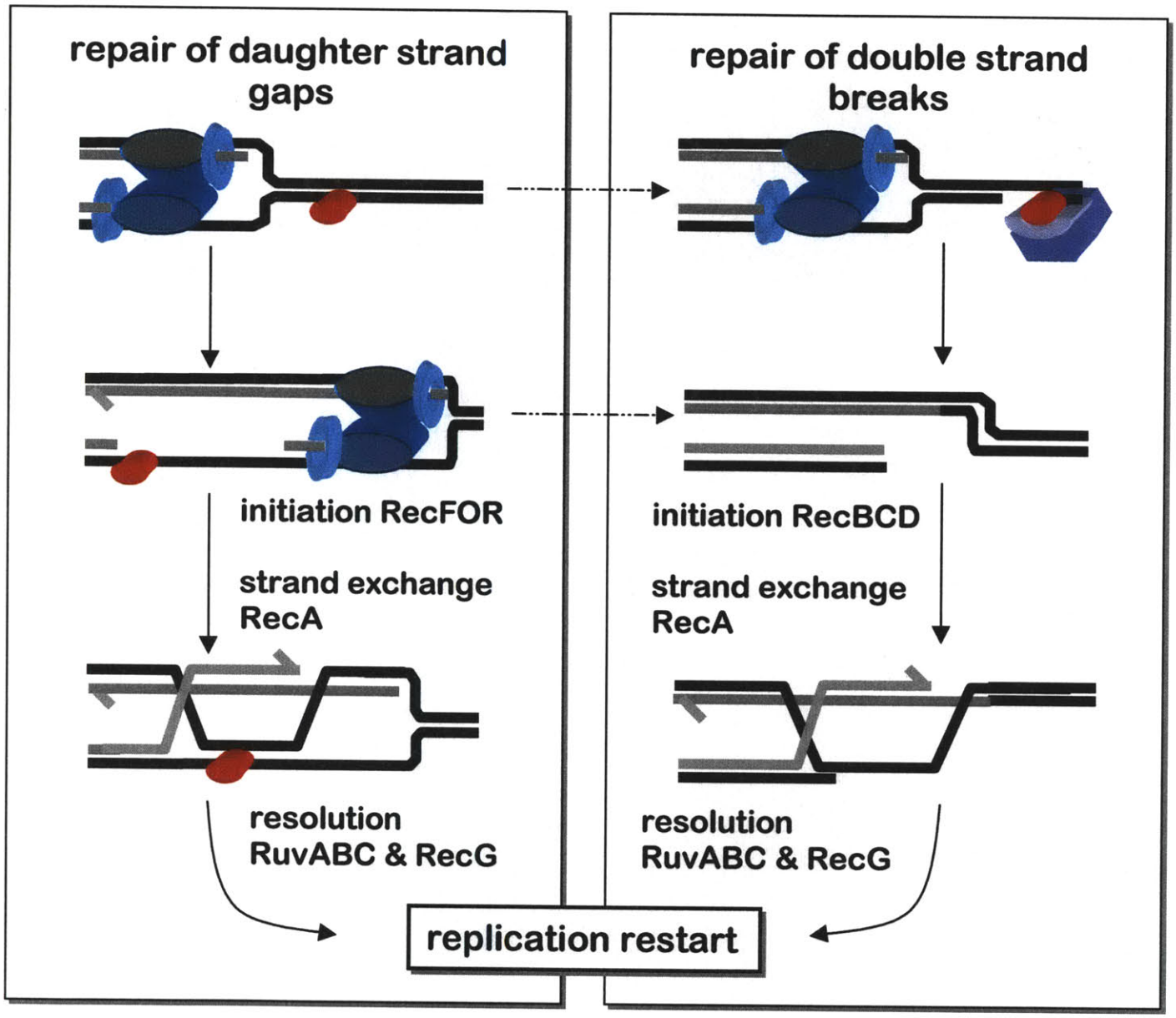

Diagram 8: The Two Pathways of Recombination/Repair. Although daughter strand gap (on the left) and double strand break (right) are initiated with different enzymes (RecFOR vs RecBCD), they share the same enzymes for Holliday junction formation (RecA) and resolution (RuvABC or RecG). The diagram was adapted from Michel Cox (Cox, M. Proc. Natl. Acad. Sci. USA (1997) 94 11764-1176).

Tables

Table 1: Recombination/Repair Mutants Used for Examining the Mechanism of Bleomycin Genotoxicity

Repair/Recombination Status	Strains	Genotype
WT	AB1157	F-thr-ara-14leuB6 DE(gpt-proA)62 lacY1 tsx-33 glnV44(AS) galK2(Oc) rfbD1mg1-51rpoS39g(Am)rspL31(Str) KdgK51xylA5mtl-1argE3(Oc)thi-1
recBCD	KM21	ΔrecC ph recBrecD::Kan^R
recF	JCN239	*recF*143
ruvABC	AM547	ruvA60::Tn10 ruvB52 *ruvC*53
ruvC	CS85	*ruvC*53
recG	N3793	*ruvC*53 *recG*258
recGruvC	N3398	*ruvC*53 *recG*258

Wild type strain was isolated by DeWitt and Adelbeg EA., (1962) Genetics 47, pg. 577. All of the mutants strains were kindly provided by M. Marinus, Univeristy of Massachusetts Medical School, Worchester MA.

Table 2: Relative Sensitivities of Wild Type and Recombination Mutants to Bleomycin: A Qualitative Summary of the Results in Figures 1, 3 and 4.

Relative Sensitivity	Non- Dividing Cells	Dividing Cells
0	WT, *recF*, *recG*, *ruvAB*, *ruvC*	WT, *recF*
+		*ruvC*, *recG*
++	*recBCD*, *recGruvC*	
++++		*ruvABC*, *recBCD*, *recGruvC*

The relative sensitivity of each strain as compared with the wild type strain was estimated at doses of bleomycin of: between 20 to 50 μM for non-dividing cells and 2 to 5 μM for dividing cells. A relative sensitivity of "O" means that the strains were as sensitivite to bleomycin as wild type. The + means that strains were more sensitive to bleomycin than wild type by about half a log. Each additional + represents an additional half a log of increased sensitivity as compared to sensitivity of the wild type strain.

Figures

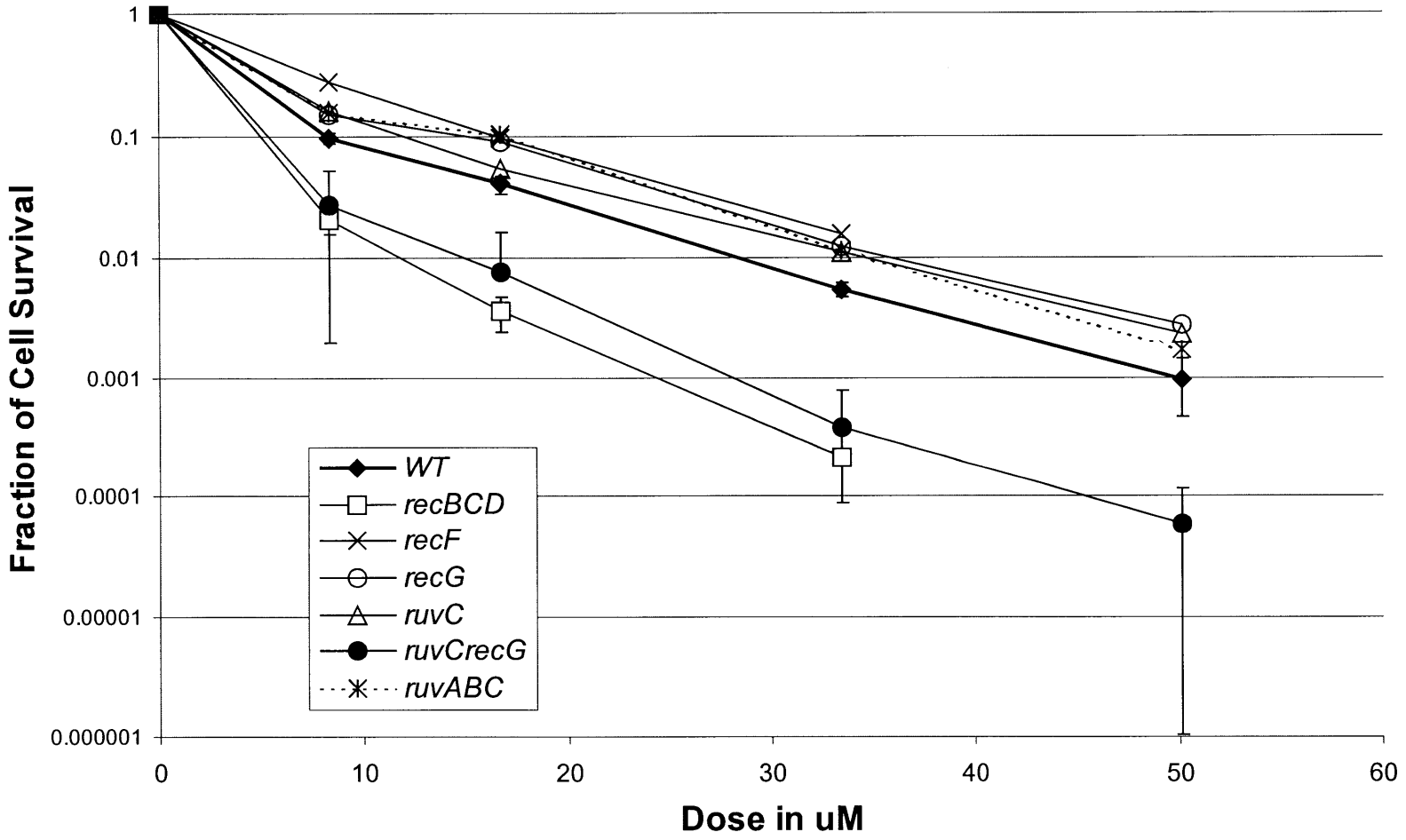

FIGURE 1: SENSITIVITY OF MUTANTS TO BLM IN NON-DIVIDING CONDITIONS. Cultures of *E. coli* strains were grown to the exponential phase as described in the text. These cells were then centrifuged and the pellets re-suspended in M9 to a final concentration of 1.2×10^8 cell/ml. These cells were then treated with bleomycin at concentrations of 8.3, 16.7, 33.4 and 50 uM (where 50 uM = 72 ug/ml) for 2 hrs. For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Each point represents the average of between three to six independent experiments. The error bars represent standard error of the mean.

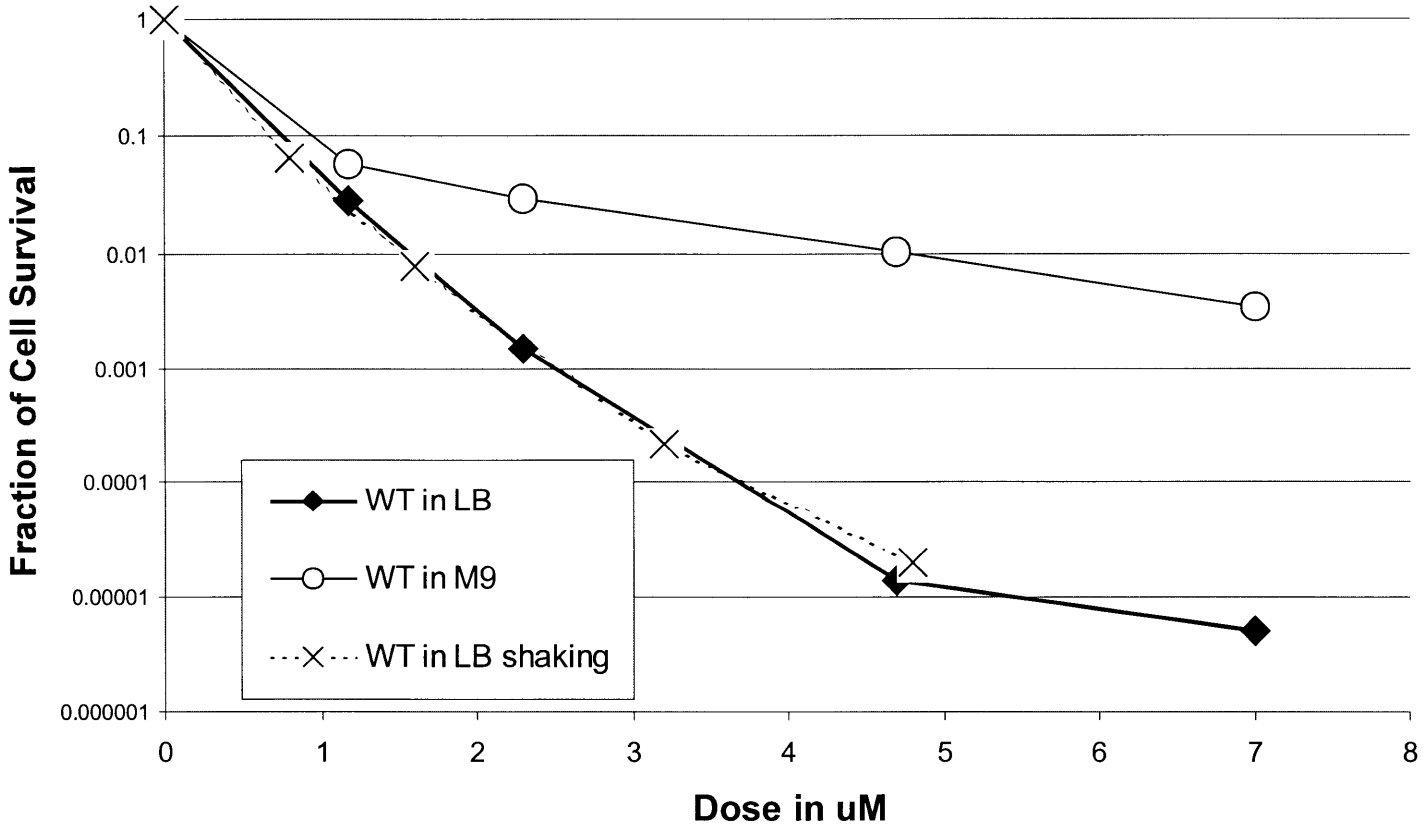

FIGURE 2: SENSITIVITY OF WILD TYPE *E. COLI* TO BLM IN NON-DIVIDING VS. DIVIDING CONDITIONS. Cultures of *E. coli* strains AB1157 were grown to the exponential phase as described in the text. These cells were then treated with bleomycin at concentrations of 1.2, 2.3, 4.7 and 7 uM (where 7uM = 11 ug/ml), for 2 hrs when treated in M9 or 1 hr when treated in LB. When the cells were shaken, concentrations of 0.8, 1.6, 3.2 or 4.8 uM of bleomycin were used (1 hr incubation). For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Data are from one experiment but the same results have been observed in other experiments where wild type was used as a control (see other figures).

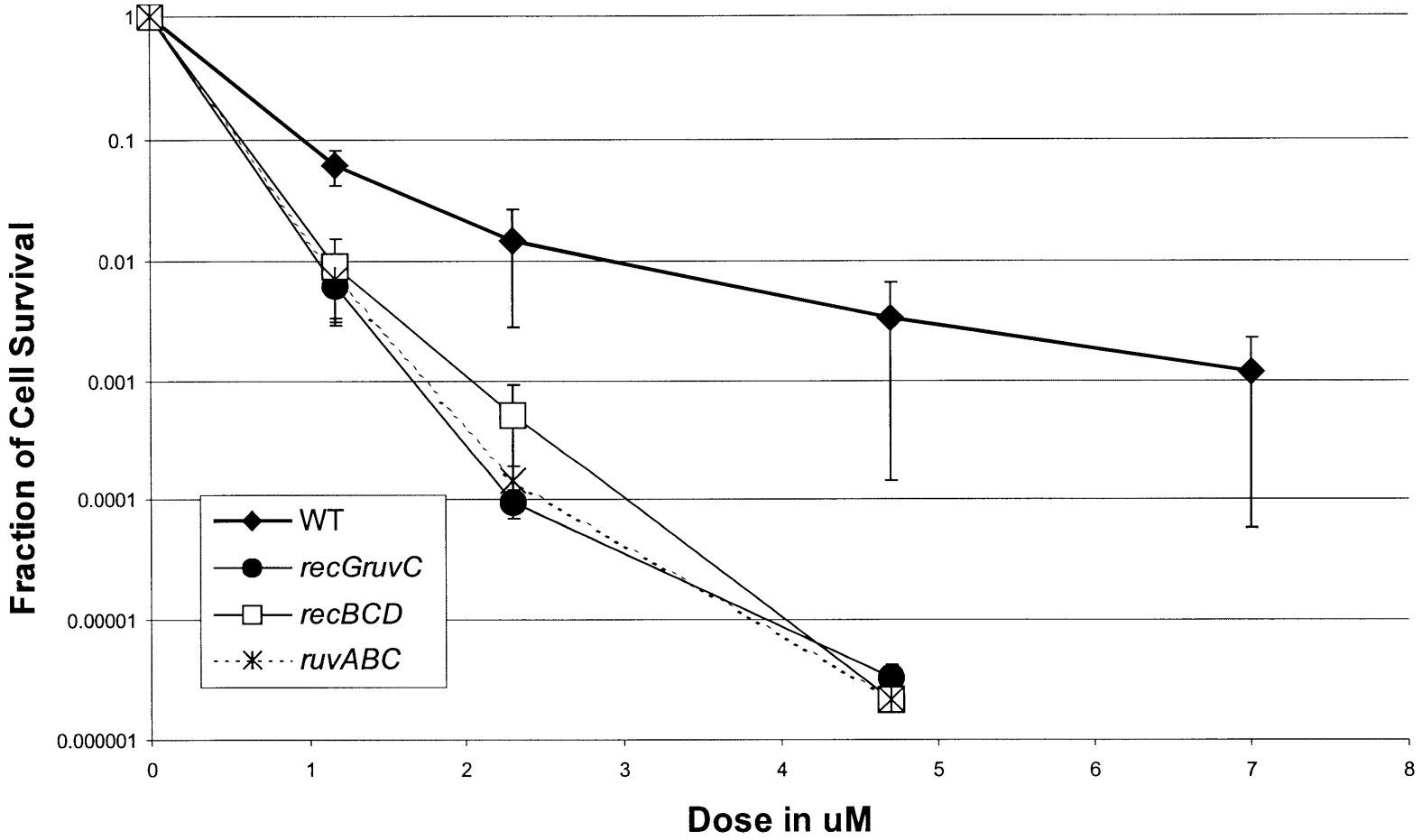

FIGURE 3: SENSITIVITY OF *RECGRUVC*, *RECBCD,* AND *RUVABC* MUTANTS TO BLM IN DIVIDING CONDITIONS. Cultures of *E. coli* strains were grown to the exponential phase as described in the text. These cells were then treated with BLM at concentrations of 1.2, 2.3, 4.7 and 7 uM (where 7 uM = 11 ug/ml) for 1hr. For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Each point represents the average of between three to six independent experiments. The error bars represent standard error of the mean.

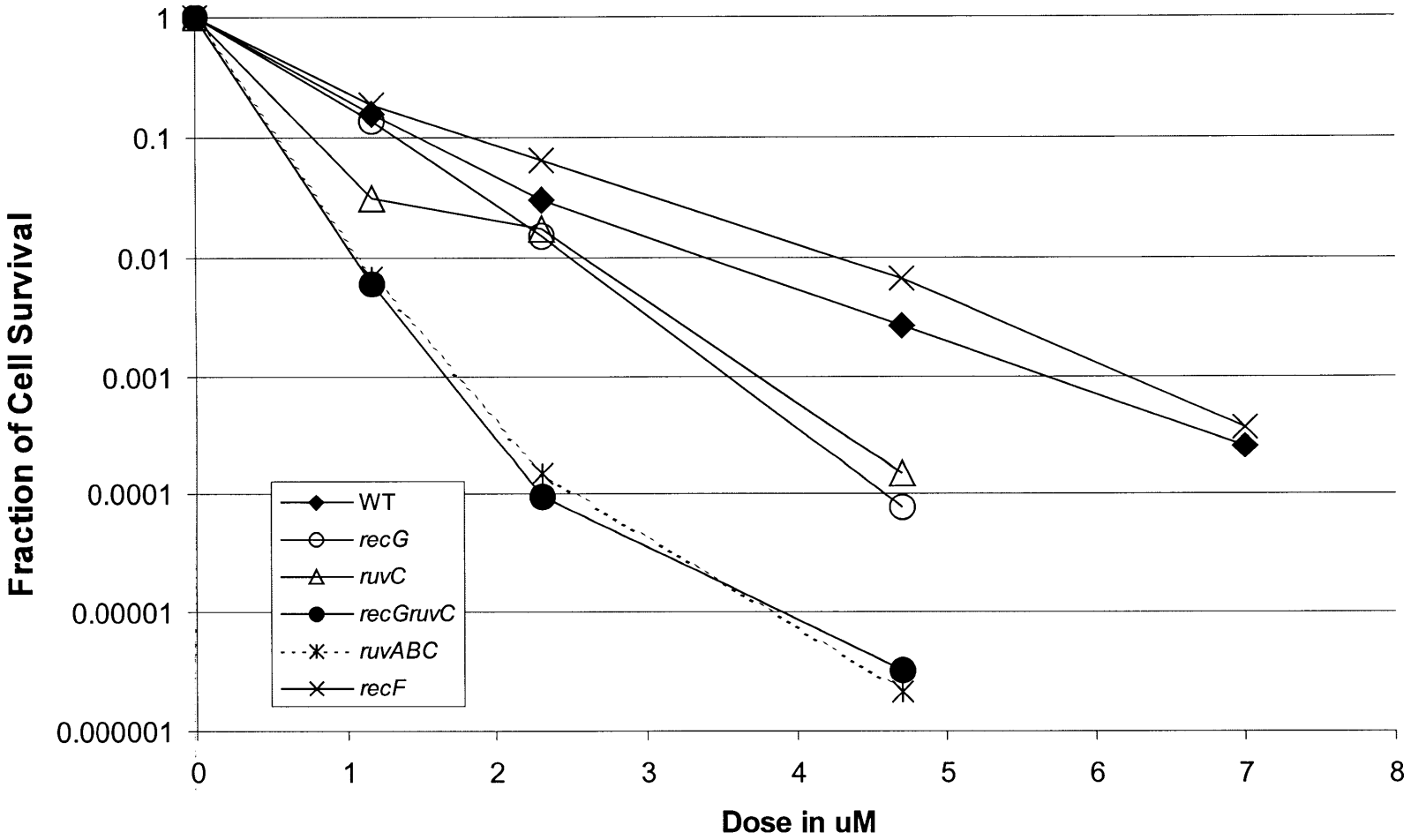

FIGURE 4: SENSITIVITY OF *RECG, RUVC, RECF* MUTANTS TO BLM IN DIVIDING CONDITIONS. Sensitivities of *recGruvC* and *ruvABC* mutants were plotted for comparison. Cultures of *E. coli* strains were grown to the exponential phase as described in the text. These cells were then treated with BLM at concentrations of 1.2, 2.3, 4.7 and 7 uM (where 7uM = 11 ug/ml) for 1 hr. For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Data are from one experiment and *need* to be duplicated.

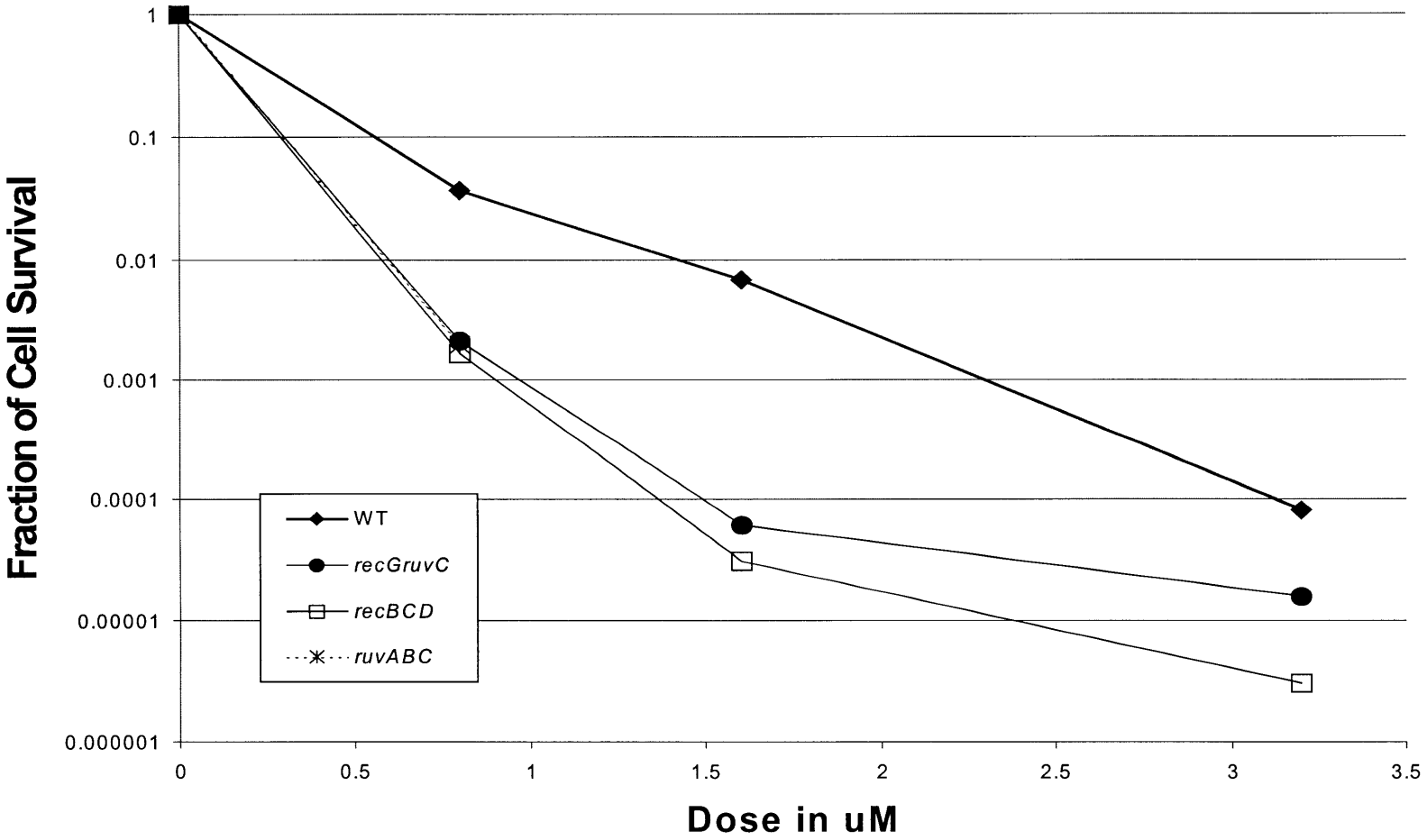

FIGURE 5: SENSITIVITY OF *RECGRUVC*, *RECBCD* AND *RUVABC* MUTANTS TO BLM IN DIVIDING AND SHAKING CONDITIONS. Cultures of *E. coli* strains were grown to the exponential phase as described in the text. These cells were then treated with bleomycin at concentrations of 0.8, 1.6, 3.2, and 4.8 uM (where 4.8 uM = 6.9 ug /ml) for 1 hr while being shaken. For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Data are from one experiment and thus *need* to be duplicated.

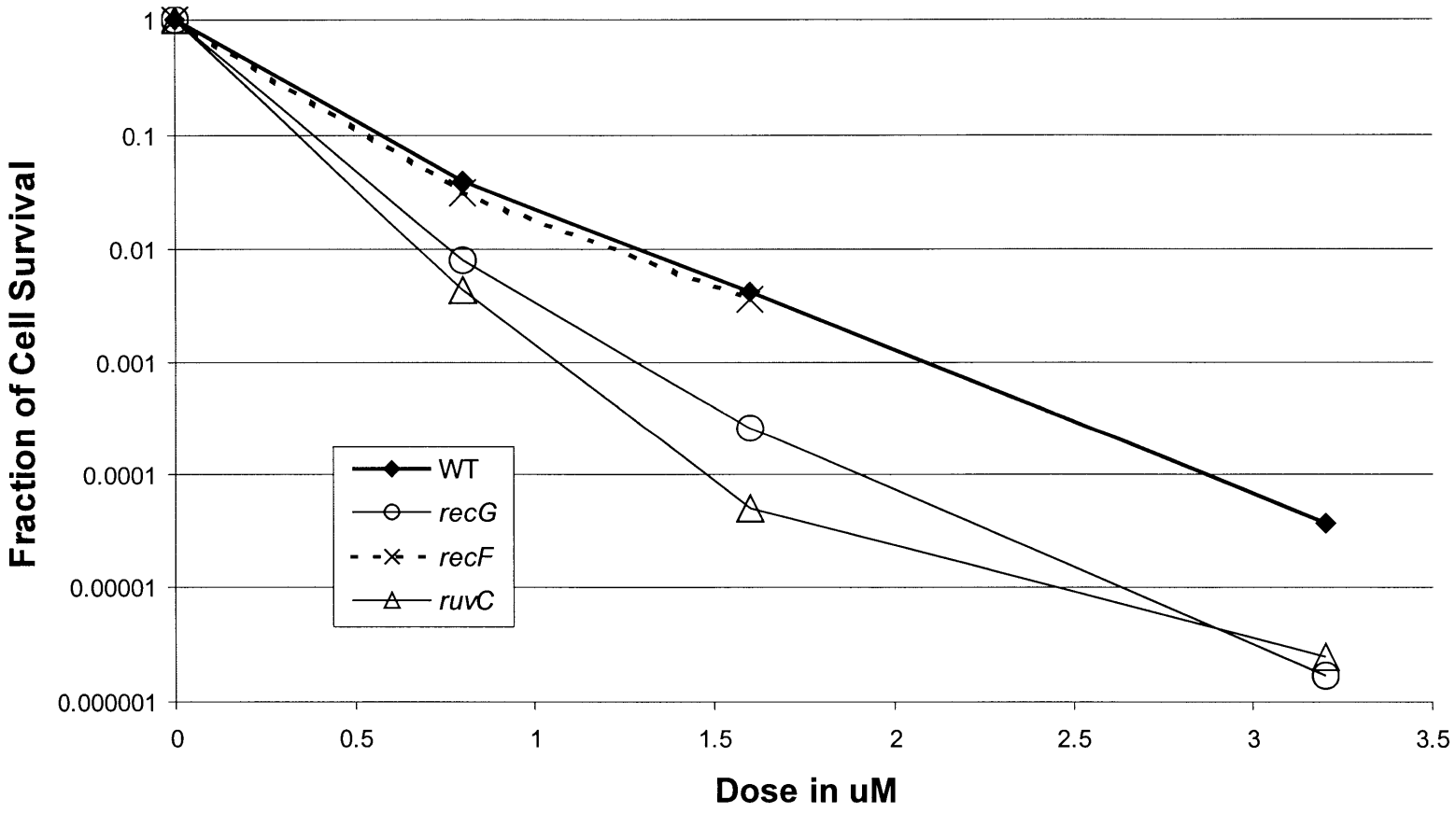

FIGURE 6: SENSITIVITY OF *RECG*, *RUVC* AND *RECF* TO BLM IN DIVIDING AND SHAKING CONDITIONS. Cultures of *E. coli* strains were grown to the exponential phase as described in the text. These cells were then treated with bleomycin at concentrations of 0.8, 1.6, 3.2 and 4.8 uM (where 4.8 uM = 6.9 ug/ml) for 1 hr while being shaken. For each point, an aliquot of cells was diluted into M9 and plated in order to determine colony-forming units (46). Data are from one experiment but have been duplicated in two experiments. Additional experiments should be done to confirm these results.