Effects of Bleomycin on Growth Kinetics and Survival of *Saccharomyces cerevisiae*: a Model of Repair Pathways

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In order to analyze the roles of some repair genes in the processing of bleomycin-induced DNA damage and, especially, the interrelationships among the involved repair pathways, we investigated the potentially lethal effect of bleomycin on radiosensitive mutants of *Saccharomyces cerevisiae* defective in recombination, excision, and *RAD6*-dependent DNA repair. Using single, double, and triple *rad* mutants, we analyzed growth kinetics and survival curves as a function of bleomycin concentration. Our results indicate that genes belonging to the three epistasis groups interact in the repair of bleomycin-induced DNA damage to different degrees depending on the concentration of bleomycin. The most important mechanisms involved are recombination and postreplication repair. The initial action of a potentially inducible excision repair gene could provide intermediate substrates for the *RAD6*- and *RAD52*-dependent repair processes. Interaction between *RAD6* and *RAD52* genes was epistatic for low bleomycin concentrations. *RAD3* and *RAD52* genes act independently in processing DNA damage induced by high concentrations of bleomycin. The synergistic interaction observed at high concentrations in the triple mutant *rad2-6 rad6-1 rad52-1* indicates partial independence of the involved repair pathways, with possible common substrates. On the basis of the present results, we propose a heuristic model of bleomycin-induced DNA damage repair.

Bleomycin (BLM) is a radiomimetic glycopeptide antibiotic used extensively in tumor therapy (32, 48). This drug binds to DNA and through a free-radical-based mechanism produces DNA base loss and single- and double-strand breaks (43, 44). In different lines of eukaryotic cells, BLM produces inhibition of DNA synthesis (10) as well as cell cycle G2 delay (19, 38). With phages, bacteria, and yeast cells, it was demonstrated that this drug is recombinogenic and mutagenic (11, 23, 39, 42). Analysis of BLM-induced mutagenesis in repackaged lambda phage suggests SOS repair dependence (33).

BLM-induced DNA damage in prokaryotic and eukaryotic cells is partially reparable (3, 22, 27, 40). For human fibroblasts, a long-patch mode of excision repair of BLM-induced damage was recently described (4, 5). Using haploid and diploid radiosensitive strains of *Saccharomyces cerevisiae*, Moore (22) showed that all *rad* single mutants sensitive to BLM tested, with the exception of *rad15*, are sensitive to X rays. Likewise, BLM-sensitive (*blm*) mutants exhibit cross-sensitivity to ionizing radiation and hydrogen peroxide (26). These results suggest that some aspects of the repair of BLM and ionizing radiation DNA damage in yeast cells may be similar. Furthermore, DNA ligase (the product of the *CDC9* gene) is required in the overall process of restoring full-size DNA molecules from DNA fragments produced by BLM (24, 25).

We have recently shown that BLM induces at least one error-prone type of repair in *S. cerevisiae* and that the *PSO4* (*XS9*) gene acts as a mutation-triggering factor (39). Furthermore, the combination of UV light and BLM produces different degrees of sensitization depending on dose ranges, suggesting overlapping lesion specificity in involved repair pathways (excision and recombination) (1).

The aim of the present work is to further analyze the roles of some repair genes in processing potentially lethal BLM-induced DNA damage and, especially, the interrelationships among the involved repair processes. Using single, double, and triple radiosensitive (*rad*) mutants defective in three known DNA repair functions (recombination, excision, and postreplication repair) (8, 9, 12, 13), we surveyed the effect of BLM on growth kinetics and survival. Our results indicate that genes belonging to the three epistasis groups interact in the repair of BLM-induced DNA damage to different degrees depending on the concentration of BLM. The most important mechanisms involved are recombination and postreplication repair. The initial action of a potentially inducible excision repair gene could provide intermediate substrates to the *RAD6*—and *RAD52*-dependent repair processes.

**MATERIAL AND METHODS**

**Yeast strains.** The haploid strains of *S. cerevisiae* used in this study were kindly provided by J. A. P. Henriques. The strains employed were wild-type XV-185-4c (*MATa ade2-1 arg4-17 his1-7 lys1-1 trp3-48 hom3-10 RAD*); the single mutants *rad2-6, rad3-e5, rad6-1*, and *rad52-1*; the double mutants *rad2-6 rad6-1, rad6-1 rad52-1*, and *rad3-e5 rad52-1*; and the triple mutant *rad2-6 rad6-1 rad52-1*. The *rad* mutants were all derived from crosses of the original mutants with the wild-type strain XV-185-4c. The original source of the *rad* alleles and phenotypic analysis of all the strains were previously described (14, 29).

Since suppression of the *rad6-1* mutant is frequently observed (20, 37), UV sensitivity was tested as described by Nunes et al. (30). We obtained the expected sensitivity for a nonsuppressed *rad6-1* strain (20). Moreover, previously BLM-treated logarithmic and stationary cells of the *rad6-1* strain were exposed again to various concentrations of BLM in fresh nutrient liquid medium. Growth curves similar to those shown in Fig. 2A were obtained. These tests indicated

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FIG. 1. Growth kinetic curves of haploid strain $RAD^+$ cultured in liquid YED medium containing different concentrations of BLM. $\bullet$, control; $\bigcirc$, B1 (1 $\mu$g/ml); $\square$, B2 (3.75 $\mu$g/ml); $\triangle$, B3 (7.5 $\mu$g/ml).

FIG. 2. Growth kinetic curves of haploid strains rad6-1 (A), rad52-1 (B), rad6-1 rad52-1 (C), and rad2-6 rad6-1 rad52-1 (D), cultured in liquid YED medium containing different concentrations of BLM. $\bullet$, control; $\bigcirc$, B1 (1 $\mu$g/ml); $\square$, B2 (3.75 $\mu$g/ml); $\triangle$, B3 (7.5 $\mu$g/ml). In panel A, the curve corresponding to B1 does not differ significantly from the curve corresponding to the control. t/h, time (in hours).

the lack of suppression of the rad6-1 mutant employed in this study.

**Media.** The liquid medium YED contained 0.5% yeast extract (Difco) and 1% dextrose (Difco). Cells were plated on YED medium (YEDA) solidified by 2% agar (Difco) (30).

**BLM treatments.** BLM for clinical use (Kayaku Co. Ltd., Tokyo, Japan) was employed. Stock solutions of BLM were prepared with sterile distilled water. For growth kinetic experiments, BLM was added to liquid YED medium at different concentrations ($B1 = 1 \mu$g/ml, $B2 = 3.75 \mu$g/ml, and $B3 = 7.5 \mu$g/ml) just before the inoculation of yeast cells. A preliminary test showed that higher concentrations of the drug resulted in complete growth inhibition of all strains under our experimental conditions. For survival experiments, solid YEDA medium containing BLM was prepared according to a previously described method (1, 22, 26). In short, BLM was added to cooled YEDA immediately after pouring and was gently mixed. Plates containing different
concentrations of the drug (3.75 to 30 μg/ml) were employed. Although plates were used within 24 h after preparation, similar surviving fractions were obtained with plates used 10 days after preparation. Hence, BLM was stable during the incubation time of the experiments. Independent survival determinations showed that this BLM treatment performed in solid YED medium is equivalent to exposing the same cell samples in liquid YED medium containing BLM to continuous agitation for 0.4 h at 30°C and then washing and plating the cells on nutrient YEDA.

Growth kinetic assay. Growth was initiated at a cell concentration of 0.5 to 1 × 10⁶ cells per ml by the inoculation of fresh cells (previously grown in YED to early stationary phase) in 10 ml of YED with or without BLM. Cells were grown at 30°C, pH 5.5 ± 0.3, with continuous aeration and agitation. Growth was monitored through counting (by means of a hemocytometer) the number of cells per ml (N) as a function of the incubation time. The standard error of the counts was estimated as ±4.5% of N. Growth was also monitored by observing the optical density by using the corresponding calibration curves. Data from at least three reproducible experiments are given in the figures.

Survival determinations. Cultures in YED medium were initiated as described above, and cell samples in the mid-log phase of growth (N = 10⁷ cells per ml) were collected, except for the rad52-I and rad6-I rad52-1 mutants. Cell samples of both strains in early log phase (N = 3 × 10⁶ cells per ml) were collected, since survival determinations performed during the partial synchronization observed during growth kinetic studies (Fig. 2B and C) were similar to those of cells in the stationary phase of growth (data not shown).

Growth kinetic curves of haploid strains rad3-e5 (A) and rad3-e5 rad52-1 (B), cultured in liquid YED medium containing different concentrations of BLM. •, control; □, B2 (3.75 μg/ml); Δ, B3 (7.5 μg/ml). The curves corresponding to B1 (1 μg/ml) do not differ significantly from the curves corresponding to the control. t/h, time (in hours).

RESULTS

Growth kinetics of cell populations. The growth curves of haploid yeast cell populations of wild-type R4D⁺ and single, double, and triple rad mutants in liquid YED medium without and with BLM are shown in Fig. 1 to 4. The number of cells per ml (N) versus time (t) was plotted on semi-log coordinates. For simplicity, experimental points were plotted up to 35 h, since no further modification in growth thereafter was observed. To analyze these growth curves quantitatively, the following kinetic parameters were calculated: the maximal number of cells per ml reached in the stationary phase of growth (Nmax), the doubling time of the population (DT) during the exponential phase of growth, and the lag phase time (tlag), which corresponds to the mean time elapsed before the onset of cell division. Table 1 summarizes the growth kinetic parameters of the strains studied cultured without BLM and in the presence of various concentrations of the drug (B2 = 3.75 μg/ml and B3 = 7.5 μg/ml).

Differences among growth kinetic parameters of strains cultured without BLM (control samples), most probably due to different genetic backgrounds, were observed (Table 1). Hence, the growth kinetics of each strain cultured with BLM were compared with the corresponding values of the control without BLM treatment. The observed DT values of all studied strains were in accordance with those reported previously (18). As described in Materials and Methods, results indicated the lack of suppression of the rad6-1 mutation. The rad52-I and the rad6-1 rad52-1 mutants exhibited partial synchronization in the log phase of growth, thereafter growing at the same rate until entering the stationary phase (Fig. 2B and C). The reason for this partial synchronization is unknown.

The growth kinetics of all strains used in this study were modified to different degrees by continuous exposure of the strains to various concentrations of BLM. The most sensitive strain was the triple mutant rad2-6 rad6-1 rad52-1, since it showed no growth at any BLM concentration tested (Fig. 2D). The rad52-I single mutant and rad6-1 rad52-1 double
mutant displayed intermediate sensitivities. In fact, the growth of both mutants was completely blocked at B2 (3.75 µg/ml) and B3 (7.5 µg/ml), and it was modified at B1 (1 µg/ml) (Fig. 2B and C). At the latter BLM concentration, the rad6-1 rad52-1 double mutant showed a twofold increase in the DT and both mutants exhibited a 50% decrease in the Nmax and no variation in the tlag. Interestingly, in the presence of the drug, partial synchronization was not observed. The growth kinetics of the other strains studied were significantly modified at B2 and B3 (Fig. 1, 2A, 3, and 4). In fact, analysis of the kinetic parameters showed an increase in the DT and a decrease in the Nmax (Table 1). An increase in the tlag as a function of BLM concentration was observed with wild-type RAD+ (Fig. 1) and with all mutant strains that exhibited growth (Fig. 2A, 3, and 4). This increase was more significant in the rad3-e5 rad52-1 double mutant (14-fold at B2) (Fig. 3B) and in the rad6-1 single mutant (6-fold at B3) (Fig. 2A) than in the corresponding controls.

Survival curves to BLM. To determine independence, epistasis and/or synergistic interactions among the repair processes studied (2, 12), we measured the survival of cells continuously exposed to various concentrations of BLM. All survival curves presented two components of different slopes. The exception was the rad6-1 single mutant, which showed a single exponential course.

Regarding the survival curves of the single mutants (Fig. 5), the rad52-1 mutant showed the maximal level of sensitivity to low concentrations of BLM (0 µg/ml ≤ BLM < 15 µg/ml). For higher concentrations (15 µg/ml ≤ BLM ≤ 30 µg/ml), the most important gene involved in BLM sensitivity was RAD6. RAD52 had an intermediate effect. Single mutants defective in excision repair were not significantly affected by the drug.

The functional relationships among the RAD2, RAD6, and RAD52 genes can be inferred from Fig. 6 and 7. For low BLM concentrations, the rad6-1 rad52-1 double mutant showed an epistatic interaction between the corresponding genes, since the double mutant was as sensitive as the most sensitive single mutant. For high BLM concentrations, this double mutant exhibited a survival rate higher than that of the rad6-1 single mutant (Fig. 6). The rad2-6 rad6-1 double mutant was highly resistant, since no difference in survival with respect to the RAD+ strain and the rad2-6 mutant was observed (Fig. 7). Among the examined strains, the triple mutant rad2-6 rad6-1 rad52-1 showed the highest level of BLM sensitivity, especially at high concentrations of the

![Figure 4](http://jb.asm.org/)

**FIG. 4.** Growth kinetic curves of haploid strains rad2-6 (A) and rad2-6 rad6-1 (B) cultured in liquid YED medium containing different concentrations of BLM. ●, control; □, B2 (3.75 µg/ml); △, B3 (7.5 µg/ml). The curves corresponding to B1 (1 µg/ml) do not significantly differ from the curves corresponding to the control. t/h, time (in hours).

**TABLE 1.** Growth kinetic parameters of yeast strains cultured in the presence of various concentrations of BLM in YED medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>BLM concn (µg/ml)*</th>
<th>tlag</th>
<th>DT</th>
<th>Nmax</th>
</tr>
</thead>
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<tr>
<td>RAD+</td>
<td>0</td>
<td>2.5</td>
<td>1.5</td>
<td>9.8</td>
</tr>
<tr>
<td>rad2-6</td>
<td>3.75</td>
<td>4.5</td>
<td>2.0</td>
<td>6.2</td>
</tr>
<tr>
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<td>0.75</td>
<td>2.75</td>
<td>6.6</td>
</tr>
<tr>
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<td></td>
<td>1.5</td>
<td>1.75</td>
<td>5.3</td>
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<tr>
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<td>3.5</td>
<td>6.2</td>
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</table>

* Parameters are explained in the text. The tlag and the DT were measured in hours (→ tlag → ∞; DT = 0, lack of growth). The Nmax was measured as the number of cells per ml and divided by 107. N0 is the initial number of cells in the culture per ml (see Fig. 1 to 4).
drug (Fig. 6). In this case, the observed interaction was synergistic.

For high BLM concentrations, the sensitivity of the double mutant involving the RAD3 and RADS2 genes exhibited additivity of the sensitivities of the corresponding single mutant genes (Fig. 8).

DISCUSSION

Present results show that BLM-induced lethal DNA damage is processed by the recombinational, RAD6-dependent, and excisional repair mechanisms. The function of the known epistasis groups (8, 9, 12, 13) is dynamic and depends on the BLM concentration range. According to survival and growth kinetic analysis, at low BLM concentrations (0 ≤ BLM < 15 μg/ml) the most important gene involved in the repair of potentially lethal damage is RADS2, whereas for high BLM concentrations (15 μg/ml ≤ BLM ≤ 30 μg/ml), the RAD6 gene plays the most important role. It must be emphasized that the survival curves were obtained by plating on increasing concentrations of BLM, and therefore the results may not be strictly comparable to those of experiments in which treatment is given briefly and then ended.

It is noteworthy that all strains studied except rad6-1 exhibited biphasic survival curves with a resistant second component (Fig. 5 through 8). Mammalian cells also show biphasic survival curves with BLM (6, 47). This biphasic response could be due to the induction of repair enzymes. Interestingly, in our laboratory it has been recently shown that the resistant component of these curves depends at least in part on BLM-inducible repair (39). Moreover, the RAD6 gene is induced by DNA damage during the log phase of growth (21). Other possible explanations for the observed resistant second component are the presence of cells with different uptakes of the drug (40) and differential target availabilities. More experimental work is necessary in order to discriminate between these possibilities.

The exponential survival curve of the rad6-1 mutant as well as the significant increase in the t_{50} (Fig. 2A and 5) could be related not only to RAD6 postreplication repair activities (20, 34, 41) but also to its regulatory functions (20, 21, 41).

The fact that the growth of the rad52-1 mutant under continuous exposure to BLM is either defective or completely blocked (Fig. 2B), as well as its sensitivity with low BLM concentrations (Fig. 5), indicates the important role of the RADS2 gene product in processing at least part of BLM-induced lethal damage. A functional RADS2 gene is required for almost all mutagen-induced double-strand break repair (15, 31, 35). Our results agree with those reported previously (22) and with the described sensitivities of rad52-1 mutants to agents which, like BLM, induce strand

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**FIG. 5.** Surviving fraction (S) as a function of BLM concentrations ([B]) of exponential-phase populations. ○, RAD+; ●, rad2-6; ▽, rad3-e5; +, rad6-1; ●, rad52-1.

**FIG. 6.** Surviving fraction (S) as a function of BLM concentrations ([B]) of exponential-phase populations. ○, RAD+; ●, rad2-6; +, rad6-1; ●, rad52-1; □, rad6-1 rad52-1; +, rad2-6 rad6-1 rad52-1.
breaks. These include X rays (15, 35), neocarzinostatin (28), and phleomycin (24).

Regarding the excision-defective mutants studied, growth kinetic parameters of rad2-6 and rad3-e5 showed minor modifications upon BLM treatment. The latter is partially inhibited at 7.5 μg of BLM per ml (Table 1; Fig. 3A and 4A). Moreover, high survival with BLM was observed with both mutants (Fig. 5). These facts indicate a low input flow through the corresponding excision pathway in the presence of other functioning repair processes. Since the RAD3 product has DNA helicase and ATPase activities (45, 46), this gene could have an accessory role in the repair of BLM damage, perhaps by unwinding the DNA duplex during repair.

Concerning interactions among repair processes (8, 9, 12, 13, 18), our results showed epistasis between the RAD6 and RADS2 genes at low BLM concentrations. In fact, according to results obtained by other authors, RAD6 functions represent an earlier step required for repair, mutagenesis, recombination, and meiosis (16, 21, 41). RAD6 and RADS2 gene products acting on the same repair pathway were also reported in studies with the antitumoral agent BD-40 (29).

For high BLM concentrations, rad6-1 rad52-1 as well as rad2-6 rad6-1 double mutants displayed significantly higher rates of survival than the rad6-1 single mutant (Fig. 6 and 7). This resistance could be explained by the function of alternative repair pathways. Indeed, in the double mutant rad2-6 rad6-1, repair of BLM-induced damage could take place through the recombinational pathway. Recent results from Schiestl et al. (37) indicated that the SRS2 suppressor of rad6-1 acts by channeling DNA lesions into the RADS2 repair pathway. For the rad6-1 rad52-1 double mutant, suppression of the rad6 mutation seems less probable, since it depends on the RADS2 recombinational pathway and the excision repair pathway is not required (37). The difference between the survival rate of the rad6-1 rad52-1 double mutant and that of the triple mutant rad2-6 rad6-1 rad52-1 is a measure of repair due to the RAD2 gene product. It has been shown that the RAD2 gene is inducible by BLM (36). In consequence, we could speculate that the relatively high resistance of the double mutant rad6-1 rad52-1 is due to the initial action of the potentially inducible RAD2 gene, which could provide intermediate substrates to be processed by later steps of the repair pathways. In this context, the RAD24 gene is involved in excision as well as in recombination repair of UV and X-ray damage (7). XS genes (XS6, XS8, and XS9 = PS04) are also involved in both types of repair (13, 30). Although differential repair is the most likely explanation for the results, it is not the only one possible. The high rates of survival for both double mutants could also be explained by leakiness of the corresponding mutations, genetic factors varying between the strains (e.g., auxotrophic alleles), as well as differences in their abilities to take up or metabolize BLM.

Additive interaction between the RAD3 and RADS2 genes at high BLM concentrations (Fig. 8) indicates the independence of these repair processes. A similar type of interaction was described for UV irradiation (2).

The combination of rad2, rad6, and rad52 mutations confers synergistic inactivation (Fig. 7), indicating independence and possible common substrates of the involved repair processes. Interestingly, recent evidence indicates that the expression of DNA repair genes belonging to these three different epistasis groups is independently regulated (17).

We propose a model according to the facts discussed above that describes DNA repair of BLM-induced damage (Fig. 9).
ACKNOWLEDGMENTS

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REFERENCES


FIG. 9. Heuristic model of DNA repair pathways according to the present results. BLM-induced DNA damage (strand breaks and base loss) can be processed by the excision, recombinational, and RAD6-dependent repair pathways. The magnitude of repair fluxes depends on BLM concentration. The RAD52 product gene plays a central role at low concentrations of this drug, whereas at high concentrations, the RAD6 product is more important in processing BLM-induced DNA damage. The arrowheads in the RAD6 group represent different categories of loci dependent on the RAD6 gene. Upon the mutation of RAD2 and RAD6 genes, repair could take place through the recombinational pathway. The initial action of a potentially inducible repair gene could provide intermediate substrates to the RAD6- and RAD52-dependent repair processes. RAD3 and RAD52 genes are independent in processing DNA damage induced by high BLM concentrations. These three main repair processes are partially independent, with possible common steps and substrates.


