ISOLATION AND PARTIAL CHARACTERIZATION OF MUTANTS OF *SACCHAROMYCES CEREVISIAE* ALTERED IN SENSITIVITIES TO LETHAL EFFECTS OF BLEOMYCINS

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(Received for publication July 21, 1980)

Two of eight mutants (*bmr*) isolated in *Saccharomyces cerevisiae* on the basis of their increased resistance to lethal effects of antitumor bleomycins (BM), and about two-thirds of 180 yeast mutants (*bms*) isolated on the basis of their increased sensitivities to cell-killing by phleomycins (PM) or BM were sensitive to one or more of the agents UV, X-rays or hydrogen peroxide. Thus these mutants are likely to be altered in processes acting directly or indirectly on DNA damage. The remaining six *bmr* mutants and approximately 60 *bms* mutants appear as resistant as the parent strain to cell-killing by UV or X-rays, and are likely therefore, to be altered in cell wall or membrane function. A genetic basis for the phenotypes of some of the *bmr* and *bms* mutants has been established.

Antitumor bleomycins (BM) inhibit DNA synthesis and replication of viruses, bacteria, fungi, mammalian cells, and several tumor cell types[1~4]. Discovery of the selective antitumor effect of BM on squamous cell carcinoma[5] led to its present widespread use clinically in the treatment of cancers.

Present information regarding the action of BM on cells *in vitro* and *in vivo* suggests the antibiotic's toxicity could be due to several parameters at the cellular level. These include membrane-related factors and the local physiological environment of the cells which affect penetration of BM into cells, relative activity of cells to inactivate BM, cellular capacities to repair DNA damage, and extra- or intracellular factors which can enhance or suppress cell-killing effects of BM. Studies of mutants altered in their resistance to lethal effects of BM could provide information regarding patterns of cross-sensitivity or cross-resistance which potentially might relate to modes of action of BM, early (*e.g.* DNA and membrane) and late (*e.g.* lethal and genetic) effects of BM, and designs of chemotherapy regimens. We have isolated several classes of mutants of the eucaryote, *Saccharomyces cerevisiae*, on the basis of their altered resistance to lethal effects of bleomycin, or the structurally-similar antibiotic phleomycin (PM), and this report is devoted to a summary of some of their characterizations. Yeast's well-characterized genetic system, stable haploid and diploid cycle, rapid growth, clonability, and flexibility for genetic and biochemical characterizations, make it suitable for isolating and characterizing mutant strains.

Materials and Methods

Yeast strain

Mutants were derived from haploid strain CM1069-40 (α, *ade*2, *trp*5, *ilvl*, *cycl*)[6].

This paper is based on work performed in part under Contract No. DE-AC02-76EV03490 with the U.S. Department of Energy at the University of Rochester Department of Radiation Biology and Biophysics and has been assigned Report No. UR-3490-1888.

Antibiotics

Phleomycin (lot no. 64L1276) and bleomycin (several lots) preparations were generous gifts from Bristol Laboratories (Syracuse, NY). For most experiments, PM was dissolved in 50 mM Tris-Cl (pH 7.5) or deionized water (pH 6.7) just prior to use. For storage at −20°C in small quantities, PM was dissolved in 50 mM Tris-Cl (pH 7.5) at 0.1 mg/ml; it loses activity in solution when kept at room or cold temperatures, but it can be repeatedly thawed and refrozen with only negligible loss in activity. BM was dissolved just prior to use in deionized water (pH 6.7) or 50 mM phosphate buffer (pH 7.5).

Media

Routine, nonsynthetic media contained 1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose and 0.08 mg/ml adenine sulfate (YPAD). Synthetic media contained 2% dextrose, 0.16% Bacto yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and amino acid supplements. PM or BM was added to cooled sterile media containing 1% or 1.5% Agar Noble (Difco); media were used within 24 hours after preparation.

Following exposures of stationary-phase cells to antibiotic in water or 50 mM phosphate buffer up to one hour, washed cells were appropriately diluted and plated on YPAD and synthetic media to assess viabilities.

Treatments

For quantitative survival assays, strains were grown to stationary phase in liquid YPAD, and washed; cells and buds were counted in hemacytometers, appropriately diluted, and plated on media with 0, 0.05, 0.1, 0.175, 0.25 and 0.5 μg/ml PM, or 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 μg/ml BM. Washed cells were treated in suspension at $1 \sim 2 \times 10^7$ cells per ml deionized water at 23°C. For irradiation, washed cells were plated and exposed in open petri plates to UV light (38, 75, 113, 188 and 263 Jm^{-2}) or X-rays (10, 20, 30, 40, 50, 75, 100 kr). Colonies were counted after incubations of 36~96 hours at temperatures appropriate for each strain. Each strain was tested at least twice, but more often 3~5 times in separate experiments of each type.

Sensitivities of all strains were compared at several UV, X-ray, and drug doses at each of a range of temperatures by careful uses of numerous fresh master plates, as described in detail previously[6]. Hydrogen peroxide (H_2O_2; 80, 100, 120, 140, 160 mg/ml) was added to YPAD adjusted to pH 7.5 with 50 mM phosphate buffer; media were stored in the dark and plated under gold 15W F15T8 Westinghouse fluorescent lamps.

Sources of UV and X-irradiation

These have been described previously[6].

Mutageneses

Treatments with 2% ethyl methanesulfonate (EMS) were carried out in 50 mM phosphate buffer (pH 7); reactions were terminated with sodium thiosulfate. Cells were exposed to X-rays in open Petri plates.

Results

By conventional techniques of crossing, sporulation and dissection, we constructed meiotic segregants containing desired genetic and fermentation markers, and other characteristics required for quantitative studies involving several types of growth media. Mutants were isolated from one of these strains, CM1069-40.

Bleomycin-resistant (*bmr*) Mutants

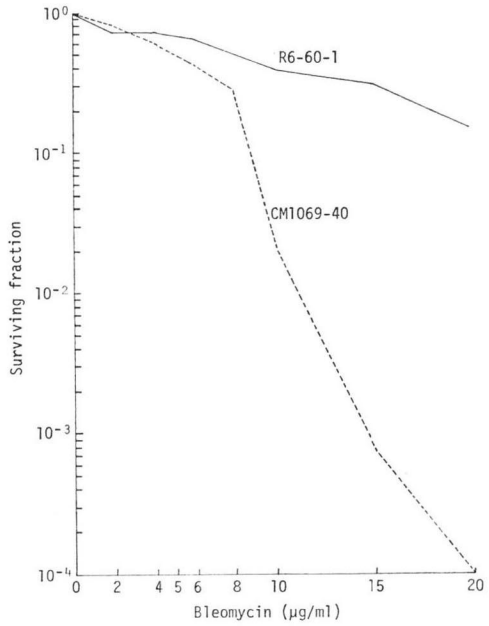
A two-step selection was designed for isolation of mutants more resistant than their parent to cell-killing by bleomycin. Fresh late stationary phase, washed CM1069-40 cells were UV-irradiated (0.1%~1% survival) on the surfaces of synthetic complete media. After post-treatment incubation in the dark at 30°C for 18 hours, sterile penicillin discs were applied to the center of each plate. BM

Table 1. Mutants more resistant than CM1069-40 to cell-killing by bleomycin.

Phenotype	Number of mutants
As resistant as CM1069-40 to killing by UV and X-rays	6
More sensitive than CM1069-40 to killing by UV and X-rays	1
More sensitive than CM1069-40 to killing by UV; as resistant as CM1069-40 to killing by X-rays	1

Fig. 1. Comparison of bleomycin-induced cell-killing of a bleomycin-resistant (*bmr*) mutant strain, R6-60-1, and its isogenic parent.

Cells were exposed to antibiotic in deionized water (pH 6.7). A typical experiment is illustrated.



(Bristol, Lot No. C4177) was dissolved in sterile 50 mM phosphate buffer (pH 7.5), then applied to discs in *ca.* amounts of 0, 375, 750, 1,250 and 1,875 μg. Incubation was continued in the dark at 30°C for 3 days. Colonies were selected and subcloned on synthetic complete, buffered media containing 0, 30 and 60 μg/ml. Only the several subclones of each of 8 original resistant mutants which subsequently grew on both 30 and 60 μg/ml were selected for further characterizations. Once it was determined that less killing resulted from exposures to drug in the presence of phosphate buffer[6], (C. MOORE, manuscript submitted for publication), buffer was eliminated from media and cells in suspension were treated in deionized water, pH 6.7. Lower drug concentrations could then be used.

An example of the large difference in resistance to cell killing between one of these mutants and its parent when growing in the presence of low concentrations of BM is shown in Fig. 1. Treatments of the two strains with about 5 μg/ml BM or less resulted in similar killing, but higher doses clearly distinguished the mutant strain and its parent.

Characterizations of segregants from a backcross involving R6-60-1 clearly indicated the basis for the altered resistance to killing by BM can be attributed to a single gene which appears not to confer an altered resistance to killing by either UV or X-rays (C. MOORE, unpublished results).

Like R6-60-1, five of the other bleomycin-resistant mutants were as resistant as the parent strain to cell-killing by UV and X-rays (Table 1). The remaining two were UV-sensitive, one also being X-ray sensitive.

Isolation of Bleomycin-sensitive (*bms*) Mutants

Bleomycin-sensitive mutants were isolated directly on the basis of their increased sensitivities to lethal effects of PM and BM after treatments with ethyl methanesulfonate (EMS) resulting in 30~50% survival, or X-rays resulting in 0.5%~10% survival. Over 2,500 independent

Table 2. Responses to lethal effects of antibiotic, UV and X-rays of bleomycin-sensitive mutants isolated from CM1069-40.

Phenotype	Number of mutants	Fraction of total
Cross-sensitive to BM, PM and UV	7	25 %
Cross-sensitive to BM, PM and X-rays	6	21 %
Cross-sensitive to BM, PM, UV and X-rays	7	25 %
Sensitive only to BM and PM	8	29 %
Total characterized	28	100 %

clones grown on YPAD at 23°C, transferred to 16- or 32-spot master plates, and replicated to obtain uniform cell concentrations in suspension wells, were tested at ranges of drug doses and at incubations at 23°, 30° and 37°C for dose- and temperature-dependent growth inhibition. Bleomycin-sensitive isolates were subcloned and 10 subclones of each mutant were tested for sensitivities to cell-killing by 7 drug, 6 UV, 6 X-ray, and 8 H_2O_2 doses. The range of increased sensitivities to each agent was two to eighty-fold over the parental strain, with increases of two- to four-fold being the most common over the dose ranges tested.

Characterizations of 28 independent isolates are summarized in Table 2. These mutants distributed themselves approximately equally into four classes, based on their cross-sensitivities to UV, X-rays, or both. Three or four mutants, or about one-half, in each class were also sensitive to hydrogen peroxide.

From one of the mutant strains (E16-11) more sensitive than its parent to cell-killing by PM and BM, but not to irradiation or H_2O_2, additional substrains even more sensitive to PM-induced cell-killing were isolated after X-ray treatment resulting in 1% survival. The rationale for this approach had been based on the initial supposition that the membranes or thick cell wall of yeast might be imprevious to low doses of phleomycin and bleomycin, especially at late stationary phase. Thus, at least one mutation of the type characterized in E16-11 would be required to remove such a barrier, similar to the *blm*-mutation in one or more of the bleomycin-sensitive mutants isolated in *Escherichia coli* K12 which maps in a membrane cluster[7]. To confirm the genetic basis for increased sensitivity to killing by BM and confirm that a single gene shown is responsible, the mutant E16-11 was crossed to a related strain of opposite mating type, the diploid was sporulated, and each meiotic segregant was tested for its sensitivity to killing by BM and radiation. The responses of meiotic segregants from six complete tetrads are summarized in Table 3, and demonstrate single-gene segregation for enhanced bleomycin-sensitivity, with no evidence of segregation for UV or X-ray sensitivity. Other genetic markers segregating in the pedigree were tested for 2: 2 segration as well. Further, two revertants (EII15-4 and EII43-10) isolated

Table 3. Properties of segregants from a backcross of *bms* mutant strain E16-11 to a related strain with resistance to killing by BM like normal (*BMS*) strains.

Strain number	Sensitivity to BM
CM1069-40	R
CM1068-9	R (resistance like CM1069-40 to killing by BM)
E16-11	S (more sensitive than CM1069-40 to killing by BM)
CM1069-40×E16-11	R (Diploid no.=CM-1172)
CM1172-1A	R
-1B	R
-1C	S
-1D	S
CM1172-2A	S
-2B	S
-2C	R
-2D	R
CM1172-3A	R
-3B	S
-3C	S
-3D	R
CM1172-4A	R
-4B	S
-4C	S
-4D	R
CM1172-5A	R
-5B	S
-5C	S
-5D	R
CM1172-6A	S
-6B	R
-6C	S
-6D	R

"S" denotes reductions in colony-forming ability of 0.5~3 orders of magnitude when grown at 30° or 37°C on nonsynthetic complete medium containing 7.5 to 15 μg/ml BM.

from bleomycin-sensitive E16-11 were as resistant as CM1069-40 to cell killing by bleomycin, UV and X-rays; EII15-4 is somewhat more resistant than CM1069-40 at high BM test doses and at 23°C.

Table 4 summarizes 152 independent isolates from *bms* strain E16-11. Forty-three percent exhibited enhanced sensitivity to X-rays, UV or both, 35% exhibited enhanced sensitivity to H_2O_2, and 10% exhibited cross-sensitivities to PM and BM, H_2O_2, UV and X-rays.

Table 4. Supersensitive mutants isolated from E16-11.

Sensitive to	Number of mutants	Fraction of total
PM, BM, and H_2O_2	28	18 %
PM, BM, and UV	20	13 %
PM, BM, UV, H_2O_2	8	5 %
PM, BM, X-rays	9	6 %
PM, BM, H_2O_2, X-rays	2	2 %
PM, BM, UV, and X-rays	11	7 %
H_2O_2, UV and X-rays	15	10 %
BM, PM only	59	39 %
Total	152	100 %

Discussion

One or both of the confirmed *bmr* mutants sensitive to killing by irradiation could be altered in processes acting on DNA damage, and thus are of interest to examine further in molecular studies. Since the other six *bmr* mutants appear as resistant as the parent strain to cell-killing by UV and X-rays, it is concluded that increased resistance to cell-killing by BM is not associated with either increased or decreased resistance to cell-killing by UV or X-rays. A cell wall or membrane component to the action of BM on yeast could be altered in the six *bmr* mutants with unchanged sensitivities to killing by radiation.

In contrast to our finding, all PM-resistant mutants isolated from *E. coli* B by GRIGG[8] were UV-sensitive. These PM-resistant *E. coli* were also unable to reactivate UV-irradiated phage T1. Together with the finding that *E. coli* B excision-defective mutants are more resistant than wild-type to lethal effects of PM[8], this indicates the association in *E. coli* B of dark-repair processes and resistance to lethality of PM. This association was not found among excision defective mutants of yeast[6] or *E. coli* K12[9]. The difference between *E. coli* B and K12 is likely related in part to membrane permeability, rather than to major differences between cellular capacities to cope with phleomycin-induced DNA damage. An effect of a *fil* mutation[10] possibly present in *E. coli* B strains could also be a factor.

One of two PM-resistant mutants isolated in the slime mold *Physarium polycephalum* was UV-sensitive[11]. A recent report by BRABBS and WARR[12] described the isolation and characterization of five clones of Chinese hamster ovary cells, about twice as resistant to killing by 2~10 μg/ml BM as parental cell cultures. Two clones lost their increased resistance after subculturing in drug-free medium. Resistance was stable in three of the clones, one of which, unlike the parental culture, was cross-resistant to the drugs puromycin and vinblastine sulphate, and showed potentiation of BM action by Tween 80. Thus, membrane permeability could be altered in this clone. No test for radiation sensitivity was described. A bleomycin-resistant strain isolated in *Bacillus subtilis*, also not tested for sensitivity to killing by radiation, was found to be cross-resistant to mitomycin C, but not to daunomycin[13].

Cross-sensitivities of many of the *bms* mutants isolated directly from CM1069-40 to cell-killing by one or more of H_2O_2, UV and X-rays, suggest the increased sensitivities to cell-killing could be related to DNA damage and its repair. Of particular interest is the identification of a new category of mutants which are PM- (*bms*) and UV-sensitive, but not X-ray sensitive (Table 2). None of the *rad* mutants of yeast, isolated in other laboratories on the basis of increased sensitivities to UV or X-rays in comparison to normal (*RAD*$^+$) strains, possessed this phenotype. Although genetic analyses are incomplete, it is known at the present time that single gene mutations in the *bms* mutants commonly confer the cross-sensitivities. Moreover, selection for increased sensitivity to PM among mutagenized clones from radiation-resistant, bleomycin-sensitive E16-11 resulted in a large proportion (61%) of mutants cross-sensitive to cell-killing by one or more of agents H_2O_2, UV and X-rays. As expected, two or more genes segregate in pedigrees involving these mutants. Properties of some of the 180 mutants, in

addition to those tabulated in Tables 2 and 4, include 60~75% reductions in viability, inability to grow at 37°C, temperature-dependent growth inhibition by one or several irradiative or chemical agents, and increased or decreased frequencies of spontaneous, BM, UV or X-ray induced reversion of one, two or three test markers[14] (C. MOORE, unpublished experiments).

The bleomycin-sensitive mutation spontaneously borne in *E. coli* B is not related to defective DNA repair[15,16]. All bleomycin-sensitive mutants isolated from *E. coli* K12 (strain AB1157) were as resistant as the parent to UV irradiation[7,16], indicating these mutations also are not likely to be related to DNA repair. That the *blm-* mutation in one or more of the K12 mutants maps in a membrane cluster[7] suggests the mutation affects membrane permeability. Moreover, *hcr*$^-$ (*uvrB*$^-$, AB1185) and *recA*$^-$ (AB2463) single mutants of K12 respond to BM similar to wild-type (AB1157), while *blm2A*$^-$ *recA*$^-$ and *blm2A*$^-$ *rec(B,C)*$^-$ are far more sensitive to BM than either *blm2A*$^-$ or *blm2A*$^-$ *hcr*$^-$, which respond similarly[15,7,16]. These characterizations are comparable to those by NAKAYAMA[9] of excision-defective and recombination-deficient K12 mutants after phleomycin treatments. It is likely, as well, that at least some of the bleomycin-sensitive yeast mutants not cross-sensitive to cell-killing by additional test agents, possess altered membrane properties which render them more vulnerable to cytotoxicity due to enhanced drug permeability. Alternatively, such mutants could affect the activity of phleomycin or bleomycin taken into the cell. It is certainly possible, however, that cell-survival after PM- or BM-treatment depends upon at least one component of DNA repair not required for survival after treatments with other test agents.

Acknowledgments

The assistance of JAMES CALLAHAN, ANN SCHMICK, DOUGLAS KNIPPLE, WILLIAM HANNON and RICH JOSEPHSON in the experiments is gratefully acknowledged. This work was supported in part by grants CA25609 from the National Institutes of Health, VC-190 from the American Cancer Society and BMS75-13172 from the National Science Foundation, and in part by the U. S. Energy Research and Development Administration at the University of Rochester Biomedical and Environment Research Project. This paper has been assigned Report No. UR-3490-1888.

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