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Potentially Lethal Damage Repair Is Due to the Difference of DNA Double-Strand Break Repair under Immediate and Delayed Plating Conditions

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Cells plated immediately after irradiation on nutrient agar (immediate plating) exhibit a lower survival than cells which are kept under nongrowth conditions before plating (delayed plating). The difference between the survival curves obtained after immediate plating and delayed plating is considered to exhibit the cell's capacity to repair potentially lethal damage. In yeast evidence has been presented previously for the DNA double-strand break (DSB) as the molecular lesion involved in the repair of potentially lethal damage observed at the cellular level. Radiation-induced DSB are repaired in cells plated on nutrient agar, i.e., under growth conditions, as well as in cells kept under nongrowth conditions. In this paper DSB repair under growth and nongrowth conditions is studied with the help of the yeast mutant rad54-3 which is temperature conditional for DSB repair. It is shown that the extent of repair of potentially lethal damage can be varied by shifting the relative fractions of repair of DSB under growth conditions versus nongrowth conditions. Repair of DSB in cells plated on nutrient agar is promoted when glucose is substituted by Na-succinate as an energy source. As a result the immediate plating survival curve approaches the delayed plating survival curve, thus reducing the operationally defined repair of potentially lethal damage. We show that this reduced potentially lethal damage repair is caused, however, by a higher amount of DSB repair in cells immediately plated on succinate agar as compared to glucose agar.

INTRODUCTION

Postirradiation incubation of cells under nongrowth conditions before plating on nutrient agar (delayed plating, DP) in general results in an increased survival compared to that observed when cells are plated immediately after irradiation (immediate plating, IP). The difference between the immediate and delayed plating survival curves is often referred to as the delayed plating effect or liquid holding recovery of colony-forming ability in the case of microorganisms and is the operational definition of potentially lethal damage repair (PLDR) in the case of mammalian cells (*1*).

It has been shown previously that the increase in survival of yeast cells held after irradiation under nongrowth conditions is accompanied by the disappearance of double-strand breaks (DSB) from the DNA (*2*). This concomitant rejoining of DSB with potentially lethal damage repair suggests DSB as possible candidates for these *potentially lethal lesions* whose repair leads to an increased survival of cells during delayed

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plating procedures. More recently, strong evidence for a causal relationship between rejoining of DSB and potentially lethal damage repair of irradiated cells has been reported (*3*). This concerns results obtained with the radiosensitive diploid yeast mutant rad54-3 which is temperature conditional with respect to DSB rejoining (*4*). At the temperature permissive for DSB rejoining, potentially lethal damage repair occurred as judged by an increase in survival of irradiated rad54-3 cells after prolonged holding under nongrowth conditions. In contrast, at the temperature restrictive for DSB rejoining the extent of potentially lethal damage repair was negligible (*3*).

In this paper evidence is presented showing that the amount of potentially lethal damage repaired depends on the fraction of DSB repaired in cells under growth versus nongrowth conditions.

At the outset we would like to make a clear distinction between potentially lethal damage (PLD) and its repair (PLDR), both operationally defined and observed at the cellular level, and potentially lethal lesions, which we define as DSB, and their repair which are measured at the molecular level or indirectly by measuring survival of the rad54-3 mutant cells (see Materials and Methods).

MATERIALS AND METHODS

Strains of Saccharomyces cerevisiae

The diploid wild-type strain 211 (*5*) and the diploid temperature-conditional radiosensitive mutant rad54-3 (g580) were used in these experiments. The mutant strain was generously donated by Dr. J. Game. The mutant rad54-3 is unable to repair DSB at the restrictive temperature of 36°C; however, at the permissive temperature of 23°C DSB are repaired (*4*). In contrast, at both temperatures rad54-3 cells repair base damage and single-strand breaks as judged by their uv response at both temperatures (*6*). When grown at 36°C after irradiation, survival of cells is low, and an exponential survival curve is observed (see Fig. 3). In this case about one DSB per cell corresponds to one lethal event (*7*). In contrast, when grown at 23°C survival is high and a shouldered survival curve is obtained (see Fig. 2).

This indicates that in the mutant rad54-3 DSB repair is the principal determinant of survival, and a high survival can be assumed to be indicative of DSB repair having taken place without measuring the repair of these lesions directly.

Immediate and Delayed Plating Conditions

Stationary cells of strain 211 were grown on and harvested from nutrient agar plates incubated at 30°C as described previously (*8*). Cells were washed twice in phosphate buffer (67 mmol/l), pH 6.0, and irradiated. After irradiation cells were plated either immediately on both YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 0.75% Oxoid agar) and YP-sodium-succinate agar (containing 2.7% sodium-succinate instead of glucose) or aerated cell suspensions were liquid-held for 72 h at 30°C in a double-concentrated phosphate buffer (130 mmol/liter), pH 6.0, before plating on both types of nutrient agar. Doublings of 211 cells during liquid holding treatment are avoided under these conditions (*9*). Plated cells were grown at 30°C for 4 days (YPD) and 6 days (YP-sodium-succinate) and macrocolonies were counted.

Stationary cells of the mutant rad54-3 were grown on and harvested from YPD agar plates incubated at 30°C. Cells were washed twice in phosphate buffer (67 mmol/liter), pH 7.0, and sonicated (Branson B15) to separate clumps. After irradiation, either cells were plated immediately on both YPD and YP-sodium-succinate agar or aerated cell suspensions were liquid-held for 72 h in phosphate buffer before plating on both types of nutrient agar. DSB repair on nutrient agar plates and during liquid holding treatment was permitted when irradiated cells were incubated at 23°C and inhibited at 36°C. Plated cells were incubated at 23°C for 4 days and 36°C for 8 days on YPD agar, and at 23°C for 6 days and 36°C for 10 days on YP-sodium-succinate agar and macrocolonies were counted.

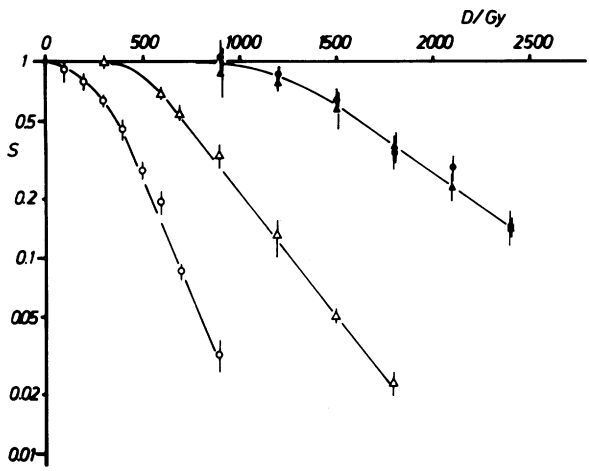

FIG. 1. Survival curves of wild-type yeast (211) obtained after immediate plating of irradiated cells on YPD (○) or YP-Na-succinate agar (△) and after delayed plating (cells incubated in phosphate buffer for 72 h before plating) on YPD (●) or YP-NAa-succinate agar (▲).

Irradiation

Cells were irradiated in phosphate buffer (67 mmol/liter) at temperatures between 0 and 4°C with 30 MeV electrons (mean restricted LET = 0.1 keV/μm) as described elsewhere (*10*).

RESULTS

Immediate and Delayed Plating Survival Curves of Wild-Type Yeast Obtained on Nutrient Agar Plates Containing Different Energy Sources

The shape of the immediate plating survival curve of wild-type yeast cells (211) can be modified by providing different energy sources in the nutrient agar. This is demonstrated in Fig. 1 for the commonly used YPD agar (energy source = glucose) and for the YP-succinate agar in which glucose has been substituted by Na-succinate. The immediate plating survival curve observed on succinate agar exhibited a shoulder width of D_q = 475 Gy and a D_0 for the exponential part of the survival curve of 345 Gy, whereas the corresponding values obtained for the immediate plating survival curve on YPD agar are D_q = 250 Gy and D_0 = 190 Gy. However, when irradiated wild-type cells were kept under nongrowth conditions for 72 h at 30°C before plating on either type of nutrient agar, the resulting delayed plating survival curves were identical (D_q = 1190 Gy; D_0 = 620 Gy) (see Table I). Thus plating wild-type cells immediately after irradiation on succinate agar results in a D_q 1.9 times higher and a D_0 1.8 times higher than observed after immediate plating on YPD agar. In contrast, no increase in these values was obtained after delayed plating of irradiated cells on succinate agar.

Immediate and Delayed Plating Survival Curves of the rad54-3 Mutant Obtained on Nutrient Agar Plates Containing Different Energy Sources

The results described above prompted the same type of experiments using the rad54-3 mutant to investigate the possible involvement of DSB and their repair in

TABLE I

Shoulder Widths D_q and D_0 Values of Survival Curves Obtained after Immediate (IP) and Delayed Plating (DP) on YPD and YP-Na-Succinate Agar

	YPD		*YP-Na-succinate*	
Strain	D_q/*Gy*	D_0/*Gy*	D_q/*Gy*	D_0/*Gy*
Wild type 211	IP: 250 DP: 1190	IP: 190 DP: 620	IP: 475 DP: 1190	IP: 345 DP: 620
rad54-3/rad54-3 at temperature permissive for DSB rejoining (23°C)	IP: 15 DP: 80	IP: 59 DP: 218	IP: 74 DP: 150	IP: 112 DP: 220
rad54-3/rad54-3 at temperature restrictive for DSB rejoining (36°C)	— —	IP: 16 DP: 21	— —	IP: 16 DP: 21

this effect. Immediate and delayed plating survival curves of rad54-3 were measured at both the permissive (23°C) and restrictive (36°C) temperatures for DSB rejoining. At the temperature permissive for DSB rejoining (Fig. 2) rad54-3 exhibited a pattern of immediate plating survival curves on both types of nutrient agar similar to that observed with wild-type cells. Survival of rad54-3 cells was higher when cells were plated immediately after irradiation on succinate agar compared to YPD agar (D_q-74 Gy (succinate agar) and D_q = 15 Gy (YPD agar); D_0 = 112 Gy (succinate agar) and D_0 = 59 Gy (YPD agar (*11*))). Irradiated rad54-3 cells kept for 72 h at the temperature permissive for DSB rejoining before plating at 23°C showed liquid holding recovery of colony-forming ability. However, unlike wild-type cells, a somewhat higher survival was obtained after plating on succinate agar (solid triangles in Fig. 2) compared

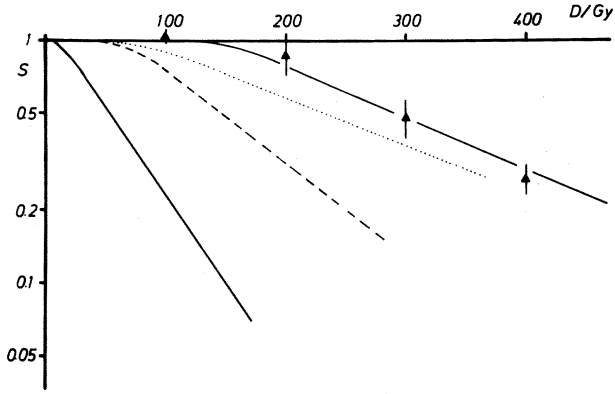

FIG. 2. Survival curves of the temperature-conditional rad54-3 mutant obtained after immediate plating of irradiated cells on YPD (solid line (*11*)) or YP-Na-succinate agar (dashed line (*11*)) and after delayed plating (cells held in phosphate buffer for 72 h before plating) on YPD (dotted line (*3, 14*)) or YP-Na-succinate agar (▲) at the permissive temperature (23°C) for DSB rejoining.

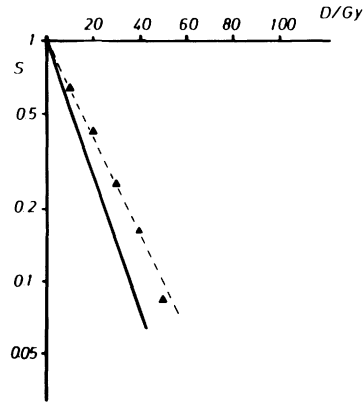

FIG. 3. Survival curves of the temperature-conditional rad54-3 mutant obtained after immediate plating of irradiated cells on YPD or YP-Na-succinate agar (solid line (*11*)) and after delayed plating (cells held in phosphate buffer for 72 h before plating) on YPD (dashed line (*3*)) or YP-Na-succinate agar (▲) at the temperature restrictive (36°C) for DSB rejoining.

to YPD agar (*3*) (see Table I). Thus, after immediate plating on succinate agar of irradiated rad54-3 cells and subsequent incubation of cells at the temperature permissive for DSB rejoining the observed D_q was 4.8 times and the D_0 1.9 times higher than observed after immediate plating on YPD agar. After delayed plating on succinate agar the D_q increased by a factor of 1.9 compared to plating on YPD agar, whereas the D_0 remained unchanged.

The immediate and delayed plating survival curves of rad54-3 at the temperature restrictive for DSB rejoining are shown in Fig. 3. No difference was detected between the survival curves obtained after plating irradiated cells immediately on either YPD or succinate agar (*11*). Although there was a slight increase in survival after 72 h liquid holding treatment at 36°C (see Discussion), the survival curves were identical when cells were delayed before being plated on YPD (3) or succinate agar (see Table I).

DISCUSSION

Immediate Plating Survival Curves

Survival of cells plated immediately after irradiation depends on the energy source supplied in the nutrient agar. Substitution of glucose (YPD) by Na-succinate (YP-Na-succinate) was found to enhance radioresistance of yeast wild-type cells (Fig. 1) and of the rad54-3 mutant at the temperature permissive for DSB repair (Fig. 2). Yeast cells plated on nutrient agar containing Na-succinate exhibit an increased post-irradiation lag before entering S phase compared to cells plated on glucose containing nutrient agar (*12*). For example, in our study the lag phase of strain 211 increased from 3.6 h (YPD) to 8.4 h on YP-Na-succinate. It has been reported that an increased lag before entry into the S phase provides more time for repair of potentially lethal lesions, thereby resulting in an enhanced survival of cells (*12, 13*). The studies with the rad54-3 mutant cells suggest that the radiation-induced potentially lethal lesions which are repaired during the lag on the nutrient agar after immediate plating are

DSB. This can be deduced from a comparison of experimental results presented in Fig. 2 and Fig. 3 showing that on YP-Na-succinate agar an enhanced radioresistance of rad54-3 cells is observed when DSB repair is permitted (Fig. 2), but not when it is restricted (Fig. 3). Apparently when DSB rejoining is restricted, the additional repair time provided by plating cells on succinate agar does not result in a higher survival, although under these conditions rad 54-3 cells are capable of repairing other types of lesions such as base damage and single-strand breaks, adduced from their response to uv light (*6*).

Summarizing the experimental findings discussed so far we can say that the radioresistance of yeast cells plated immediately after irradiation depends strongly on the repair of potentially lethal lesions occurring on the nutrient agar plate. From our results with the rad54-3 mutant we deduce that these potentially lethal lesions are DSB.

Delayed Plating Survival Curves

When irradiated wild-type yeast cells are held under nongrowth conditions at 30°C for 72 h before plating, no difference between the survival curves obtained on YPD or succinate agar can be detected (Fig. 1). We interpret this result to indicate that enough of the potentially lethal lesions are repaired during the 72 h treatment to nullify any difference in survival when cells are plated on either nutrient agar.

For the temperature-conditional mutant rad54-3 a postirradiation 72 h treatment under nongrowth conditions at the temperature permissive for DSB rejoining (23°C) followed by plating of cells results in a somewhat lower survival on YPD agar compared to succinate agar incubated at 23°C (Fig. 2). This may be explained by the slower repair of DSB in rad54-3 cells compared to wild-type cells.[1] Thus, for rad54-3 cells, the 72-h treatment under nongrowth conditions may be too short for sufficient repair of DSB. We therefore interpret the higher survival of cells on succinate agar (Fig. 2, closed triangles) as a continuation of repair of the remaining DSB in rad54-3 cells after plating.

Cells of the rad54-3 mutant, however, yielded the same delayed plating survival curves on both types of nutrient agar when the 72-h postirradiation treatment under nongrowth conditions and incubation of plated cells were performed at the temperature restrictive for DSB repair (Fig. 3). We observed for these cells a slightly higher survival after the delayed plating procedure compared to that after immediate plating. The reason for this seems to be the repair of a few DSB under nongrowth conditions even at 36°C due to the presence of repair enzymes in these cells which had been pregrown at 30°C. This is indicated by the finding that the immediate and delayed plating survival curves of rad54-3 are identical at 36°C for cells pregrown at 36°C (results not shown).

From the results discussed so far it can be stated that a change of the energy source in the nutrient agar had a larger effect on survival of cells subjected to the immediate plating procedure than with the delayed plating protocol.

[1] M. Budd, *The Genetics of X-Ray Induced Double-Strand Break Repair in Saccharomyces cerevisiae.* Ph.D. thesis, Lawrence Berkeley Laboratory, University of California, 1982 (LBL-14684).

DSB Repair under Growth versus Nongrowth Conditions and the Extent of Repair of Potentially Lethal Damage

Potentially lethal damage has been defined as damage, the lethal expression of which may be modified by alterations in postirradiation conditions (*1*). The repair of this damage can be measured by the differences in survival observed after immediate and delayed plating regimes of irradiated yeast or mammalian cells. A small difference between an immediate and delayed plating survival curve operationally indicates a small capacity of cells to repair potentially lethal damage during the postirradiation period under nongrowth conditions before plating. However, studies on the molecular lesions involved in the cellular phenomenon of potentially lethal damage repair have revealed a somewhat different picture.

From direct measurements in yeast of radiation-induced DSB and their repair and from survival studies with DSB repair proficient and deficient yeast cells, it had been concluded previously that the potentially lethal lesion is the DSB whose repair results in the "repair of potentially lethal damage" operationally observed at the cellular level (*2, 3, 14*). Moreover, it has been shown for various eukaryotic cell types that radiation-induced DSB are not only repaired under nongrowth (*15, 16*) but also under growth conditions (*4, 15–23*). However, experimental evidence suggests that the fidelity of DSB repair under nongrowth conditions seems to be higher than that observed under growth conditions (*2, 24*), yielding higher survival of irradiated cells after delayed compared to immediate plating.

In view of these findings at the molecular level we suggest that the extent of potentially lethal damage repair at the cellular level may be determined by the proportion of DSB repaired in irradiated cells under growth conditions versus nongrowth conditions. Thus shifting the fraction of DSB repair occurring under growth versus nongrowth conditions might lead to a change of the extent of potentially lethal damage repair operationally observable. Evidence supporting this view is presented in this paper by measuring the temperature-dependent survival of irradiated rad54-3 cells which is indicative of the temperature-dependent repair of DSB in these cells. It is shown that a higher survival of irradiated cells plated on succinate agar versus glucose agar is obtained (Figs. 1, 2) which—as deduced from the rad54-3 data—is interpreted to be due to more repair of DSB in cells on the succinate plate (Figs. 2, 3). This effect is observed under immediate plating conditions whereas it is absent (wild-type cells, Fig. 1) or small (rad54-3 cells, Fig. 2) under delayed plating conditions. As a consequence of this repair of DSB, occurring under the immediate plating protocol during the increased lag before cells enter the S phase, wild-type and rad54-3 cells plated on succinate agar show a reduced level of potentially lethal damage repair as it is operationally defined; i.e., there is less difference in the immediate and delayed plating survival curves under these conditions.

Similarly, mammalian cell lines *in vitro* exhibiting only a small amount of potentially lethal damage repair as deduced from survival studies might be considered to have a correspondingly small capacity to repair potentially lethal damage. However, in view of the findings presented here, a small amount of potentially lethal damage repair observed in an immediate plating/delayed plating type experiment nevertheless involves a relatively large fraction of potentially lethal lesions, i.e., DSB, being

repaired in immediately plated cells, resulting in a relatively high survival of cells after immediate plating in these systems. Therefore, in mammalian cell systems where little potentially lethal damage repair is operationally observed, this may be not due to a reduced capacity to repair potentially lethal damage but rather to an extensive repair of DSB under immediate plating conditions, which reduces the difference between immediate and delayed plating survival curves.

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