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# Exponential or Shouldered Survival Curves Result from Repair of DNA Double-Strand Breaks Depending on Postirradiation Conditions[1]

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The yeast mutant *rad54-3* is temperature conditional for the rejoining of DNA double-strand breaks, but cells do proliferate at both the restrictive and permissive temperatures. Thus, after irradiation with 30 MeV electrons, survival curves can be obtained which may or may not involve double-strand break rejoining under certain experimental conditions. Because of this special property of *rad54-3* cells, it was possible to demonstrate that rejoining of radiation-induced double-strand breaks under nongrowth conditions yields exponential survival curves the slopes of which decrease as a function of the rejoining time. These survival data suggest that, under nongrowth conditions, the rejoining of double-strand breaks is an unsaturated process and lacks binary misrepair. In contrast, whenever rejoining of double-strand breaks occurs under growth conditions, shouldered survival curves are observed. This is true for immediate plating as well as for delayed plating survival curves. It is proposed that it is the unsaturated rejoining of double-strand breaks under nongrowth conditions, lacking binary misrepair, which is responsible for potentially lethal damage repair. 

## INTRODUCTION

Postirradiation incubation of cells under nongrowth conditions before plating on nutrient agar (delayed plating, DP) results in a better survival as compared to plating of cells immediately after irradiation (immediate plating, IP). The differences between the two shouldered survival curves are attributed to the repair of potentially lethal damage (PLD repair) occurring during the postirradiation holding of cells under nongrowth conditions.

The DNA double-strand break (DSB) is considered to be the main type of molecular lesion induced by ionizing radiation which leads to cell killing. In yeast, induction of DSBs is stochastic and proportional to radiation dose (*1*), and an exponential survival curve is observed when DSB repair is absent (*2, 3*). Experimental evidence suggests that one unrepaired DSB is lethal for a cell (*2, 4–8*). In addition, there may be a second mechanism by which DSBs confer lethality, namely that of DSBs interacting with each other to yield misrepaired DNA molecules (binary misrepair) (*3, 9, 10*).

[1] Dedicated to Professor W. Pohlit on the occasion of his 60th anniversary.



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PLD repair in yeast has been shown to be attributable to the rejoining of DSBs during the postirradiation holding of cells under nongrowth conditions (*11–13*). To gain more insight into the processes of PLD repair and particularly the role of DSB repair therein, use was made of the yeast mutant *rad54-3* which is temperature conditional for DSB rejoining (*14*). In this mutant DSB rejoining functions can be separated from growth functions by switching cells to the restrictive temperature for DSB rejoining. With the help of this mutant DSB rejoining processes occurring during the holding of irradiated cells under nongrowth conditions can be separated from those occurring under growth conditions, i.e., on the nutrient agar plate. In wild-type cells, survival curves involving repair of DSBs solely under nongrowth conditions cannot be obtained because subsequent DSB repair cannot be prevented in cells when these are plated on nutrient agar for survival assay (*12*). However, with the mutant *rad54-3* survival curves comprising rejoining of DSBs under (1) nongrowth conditions only, (2) growth conditions only, and (3) nongrowth followed by growth conditions, which is the experimental schedule for a PLD-repair-type experiment, can be obtained.

## MATERIALS AND METHODS

### *Survival Assays*

Survival assays were performed with the diploid strain *rad54-3* which is temperature conditional for DSB rejoining and conditionally radiation sensitive (*14*). This strain was kindly given to us by Dr. J. Game. At 36°C this strain is unable to rejoin DSBs, whereas at 23°C DSBs can be rejoined (*14*). Cells are able to proliferate at both temperatures and to yield macrocolonies, although growth is slower at 36°C. Stationary-phase cells were grown on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) at 36°C and harvested after 7 days. Cells were washed twice in 67 m*M* phosphate buffer, pH 7.0, and sonicated to separate clumps (Branson B15). After irradiation cells were subjected to one of two possible treatment schedules.

1. To allow DSB rejoining solely under nongrowth conditions: Cells were kept under nongrowth conditions (67 m*M* phosphate buffer, pH 7.0) for various periods at the temperature permissive for DSB rejoining (23°C). After this treatment cells were plated on nutrient agar (YPD) and grown to macrocolonies at the temperature restrictive for DSB rejoining (36°C).

2. To allow DSB rejoining under growth conditions: Cells were plated immediately after irradiation on nutrient agar and kept for various periods at the temperature permissive for DSB rejoining. Then plates were shifted to the temperature restrictive for DSB rejoining and incubated until macrocolonies could be counted.

### *Irradiation Procedure*

Cells were irradiated in buffer at ice temperature. The conditions of irradiation with 30 MeV electrons (mean restricted LET = 0.1 keV/$\mu$m) have been described elsewhere (*1*).

## RESULTS

### *Exponential Survival Curves Involving DSB Repair under Nongrowth Conditions*

On the basis of exponential survival curves observed for irradiated yeast cells unable to rejoin DSBs (*2, 3*), unsaturated, dose-independent rejoining of DSBs under nongrowth conditions should yield exponential survival curves, the slopes of which decrease with increasing extent of repair of DSBs. In wild-type yeast cells, such exponential survival curves solely involving repair of DSBs under nongrowth conditions

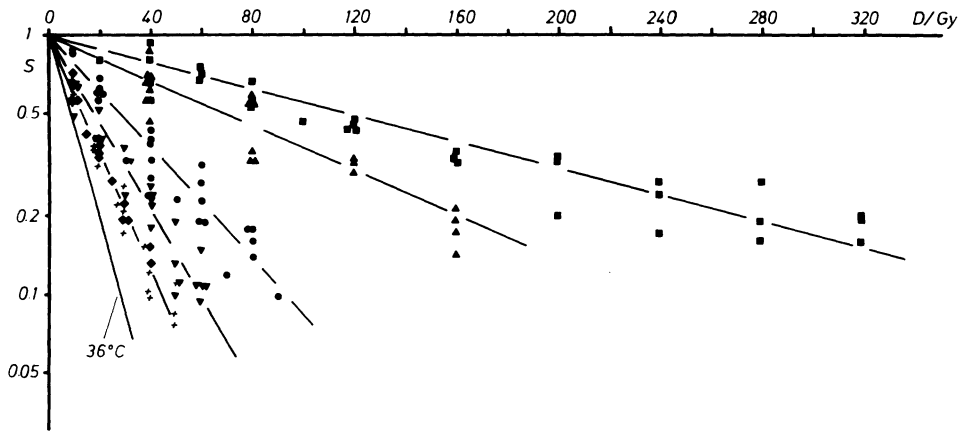


FIG. 1. Exponential survival curves involving DSB rejoining under nongrowth conditions. Cells of *rad54-3* were held after irradiation under nongrowth conditions at the temperature permissive for DSB rejoining (23°C) for 0 (experimental data points shown in (*17*)), 5.5 (+), 8 (♦), 16 (▼), 24 (●), 72 (▲), and 115 h (■). For survival assay cells were grown on nutrient agar plates at the restrictive temperature for DSB rejoining (36°C). The solid curves were fitted by regression analysis.

cannot be obtained, because DSB repair occurs both during postirradiation holding under nongrowth conditions (*11*) *and* on nutrient agar (i.e., under growth conditions) after plating cells for survival assay in a "delayed plating" (or liquid holding recovery) experiment (*6, 12*). However, survival data involving DSB repair under nongrowth but not under growth conditions on the nutrient agar plate can be obtained with the help of the yeast mutant *rad54-3*. This mutant being temperature conditional for DSB rejoining (*14*) allows DSB rejoining to be switched on and off by choosing the appropriate temperature. Cells of *rad54-3* were held after irradiation under nongrowth conditions at the temperature permissive for DSB rejoining (23°C) for various periods followed by plating them on nutrient agar where they grew to macrocolonies at the temperature restrictive for DSB rejoining (36°C). The resultant survival curves are shown in Fig. 1. Within a dose range of up to 320 Gy exponential survival curves of *rad54-3* cells are observed for treatments under nongrowth conditions up to 115 h. The slopes of these exponential survival curves decrease with increasing time for rejoining DSBs. These experiments were not extended to longer periods of treatment because the plating efficiency became lower than 50% beyond 115 h.

*Shouldered Survival Curves Involving DSB Rejoining under Growth Conditions*

A second set of survival experiments was performed to study the possible effect of incubation of irradiated cells in growth medium on the shapes of survival curves. Cells of *rad54-3* were plated immediately after irradiation on nutrient agar (i.e., under growth conditions). DSB rejoining was allowed at 23°C and stopped after various periods by transferring the plates to the restrictive temperature (36°C). Growth was continued at this temperature until macrocolonies could be counted. Figure 2 shows the results of these experiments. An exponential survival curve was obtained for irra-

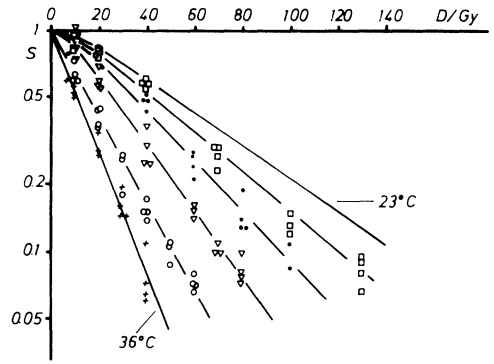


FIG. 2. Shouldered survival curves involving DSB rejoining under growth conditions. Cells of *rad54-3* were plated immediately after irradiation on nutrient agar. DSB rejoining was allowed at 23°C for various periods and stopped by shifting cells to the temperature restrictive for DSB rejoining (36°C), where they remained for macrocolony formation. For comparison, the exponential survival curve involving no rejoining of DSBs (i.e., plated cells were always kept at 36°C) and the shouldered survival curve involving maximum time of rejoining of DSBs (i.e., plated cells were always kept at 23°C) are given. Incubation was at 23°C to allow DSB rejoining for 60 min (+), 150 min (○), 5.5 h (▽), 10 h (●), and 20 h (□). The curves were fitted by regression analysis.

diated cells plated on nutrient agar without allowing DSB rejoining (i.e., plates were kept at 36°C until macrocolony formation) (*3*). After only 60 min at the temperature permissive for DSB rejoining no increase in survival of *rad54-3* cells could yet be observed. However, after 105 and 150 min at 23°C cell survival gradually increased, and after 5.5 h at 23°C a shoulder-type survival curve appeared. In general, with increasing time for DSB rejoining under growth conditions, survival of *rad54-3* cells increased, the resultant survival curves exhibiting both a gradual decrease of the slope *and* an increase of the shoulder width.

It should be mentioned that DSB rejoining under growth conditions resulting in the formation of shouldered survival curves can be observed in the relatively low dose range of up to about 130 Gy (Fig. 2), and exponential survival curves resulting from DSB rejoining under nongrowth conditions could be followed up to 320 Gy (Fig. 1).

### *Shouldered Survival Curves Involving DSB Rejoining under Nongrowth followed by Growth Conditions*

According to the classical potentially lethal damage-repair-type experiment, rejoining of DSBs in *rad54-3* cells was allowed during postirradiation holding under nongrowth conditions at 23°C followed by rejoining of DSBs under growth conditions on the nutrient agar plate at 23°C. The results of these experiments have been published already: shouldered survival curves were observed and radioresistance increased with holding of irradiated cells under nongrowth conditions at 24, 48, and 72 h (*13*). In Fig. 3 data are shown for the 72 h-delayed plating experiment (*13*) together with survival data involving DSB rejoining for 72 h under nongrowth conditions only (from Fig. 1) and the survival curve lacking DSB repair (*2*) to illustrate the new insight into the processes underlying PLD repair.

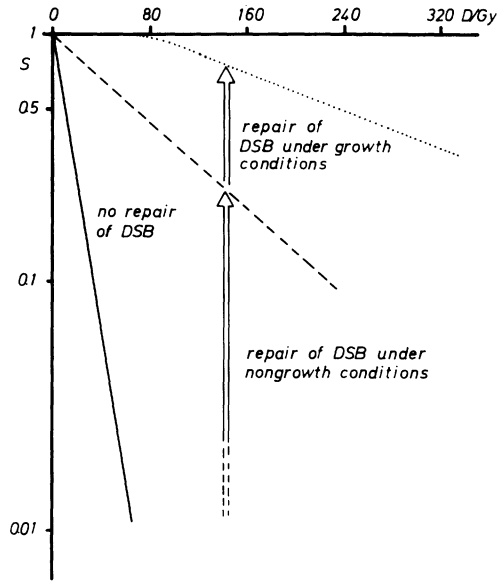


FIG. 3. Mechanism of potentially lethal damage repair. The survival curves of *rad54-3* cells involving no repair of DSBs, or exclusive rejoining of DSBs under nongrowth conditions, or rejoining of DSBs under both nongrowth followed by growth conditions are presented. The solid exponential survival curve involves no repair of DSBs; i.e., cells were plated on nutrient agar for macrocolony growth at the restrictive temperature for DSB rejoining (original data in (*9*), Fig. 1). The dashed exponential survival curve involves rejoining of DSBs exclusively under nongrowth conditions for 72 h at the permissive temperature for DSB rejoining, since cells had subsequently grown to macrocolonies at the restrictive temperature for DSB rejoining (Fig. 1, this paper). The dotted shouldered delayed plating survival curve involves both rejoining of DSBs under nongrowth conditions for 72 h and subsequent rejoining of DSBs under growth conditions, since cells were always held at the permissive temperature for DSB rejoining (original data in (*13*), Fig. 1). In wild-type cells only the delayed plating survival curve can be observed.

### *Kinetics of Increase in Survival of Irradiated rad54-3 Cells for Various Postirradiation Treatments*

Figure 4 shows the kinetics of increase in survival of *rad54-3* cells irradiated with 60 Gy and subjected to DSB rejoining solely under nongrowth conditions or solely under growth conditions. In addition, three data points which originate from previously published delayed plating survival curves of *rad54-3* cells (*13*) have been included.

An increase in survival as a function of the period of treatment at the temperature permissive for DSB rejoining was observed for all postirradiation treatment applied. Although the kinetics of increase in survival was faster under growth than under nongrowth conditions, the maximum increase in survival achieved under growth conditions was smaller than that under nongrowth conditions. However, the highest increase in survival was achieved when rejoining first occurred under nongrowth conditions, yielding a time-dependent increase in survival which rose further after subsequent incubation under growth conditions. Figure 4 also shows that virtually all the potentially lethal lesions (which we consider as DSBs) induced by a dose of 60 Gy

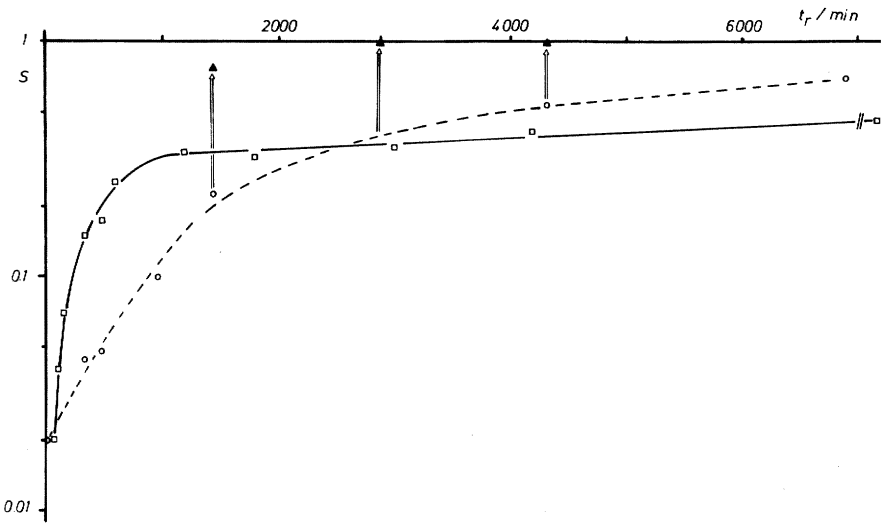


FIG. 4. Comparison of the kinetics of increase in survival of irradiated rad54-3 cells under various postirradiation conditions. Kinetics of increase in survival of *rad54-3* cells irradiated with 60 Gy followed by postirradiation treatment allowing DSB rejoining (a) solely under nongrowth conditions (○) (i.e., 23°C under nongrowth followed by 36°C under growth conditions on nutrient agar), or (b) growth conditions (□) (i.e., 23°C, nutrient agar). Also included are three survival points (▲) from Fig. 1 in (*13*) observed after allowing DSB rejoining for 24, 48, and 72 h at 23°C under nongrowth conditions, followed by further rejoining of DSBs under growth conditions in cells plated and incubated on the nutrient agar at 23°C. This treatment corresponds to a typical delayed plating experiment performed with wild-type cells where DSB rejoining under nongrowth conditions cannot be uncoupled from DSB rejoining under growth conditions. The arrows indicate the additional increase in survival due to DSB rejoining continuing under growth conditions on the nutrient agar.

(surviving fraction = 0.02 after "immediate plating" at the temperature restrictive for DSB rejoining (36°C)) are repaired provided that conditions for repair are optimum, since 100% survival was observed after a 48 h treatment of cells under nongrowth conditions at the temperature permissive for DSB rejoining followed by incubation under growth conditions at the same temperature.

## DISCUSSION

In yeast DSBs are induced stochastically and proportional to radiation dose (*1*) yielding, in the absence of DSB repair, an exponential survival curve the $D_0$ of which corresponds to a dose which induces an average of about one DSB per cell. This has been adduced as evidence for the unrepaired DSB as a lethal event (*2, 8*). Starting from this exponential survival curve involving no repair of DSBs, a series of exponential survival curves is obtained provided that *rad54-3* cells are allowed to rejoin DSBs only under nongrowth conditions. The slopes of these curves decrease with increasing time for DSB rejoining (Fig. 1). These exponential survival curves suggest that DSB rejoining under nongrowth conditions is an unsaturated, dose-independent process which lacks binary misrepair; i.e., there is no interaction between two DSBs yielding

a lethal lesion. If binary misrepair would occur under these conditions, the resulting survival curves were bent down. The exponential survival curves are observed in the dose range up to 320 Gy; this dose corresponds on the average to only about 20 DSBs per diploid *rad54-3* cell (*13*).

A very different picture is observed, however, when irradiated cells are allowed to rejoin DSBs under growth conditions (Fig. 2). Here, again starting from an exponential survival curve involving no DSB rejoining, with increasing time of incubation of irradiated cells under growth conditions at the temperature permissive for DSB rejoining, shouldered survival curves gradually develop with increasing shoulder width ($D_q$) and decreasing slope. Thus treatment of irradiated cells allowing DSB rejoining under nongrowth conditions yields exponential survival curves up to 320 Gy (Fig. 1), whereas DSB rejoining under growth conditions leads to shouldered survival curves up to 130 Gy (Fig. 2), a dose at which only about eight DSBs per cell are induced (*13*). These results contradict the view that the shoulder of a survival curve is determined by the shoulder of the *induction* curve of DSBs (as measured by the neutral elution technique) (*15*) rather than by repair processes. It has been shown previously (Fig. 1 in (*13*)) that a combination of these two postirradiation treatments typically performed in a PLD-repair-type experiment, i.e., incubation of irradiated *rad54-3* cells under nongrowth conditions followed by incubation under growth conditions (on the nutrient agar plate), also results in shouldered survival curves when both treatments are performed at the permissive temperature for DSB rejoining. Our findings lead to a better understanding of the processes involved in PLD repair. This is demonstrated in Fig. 3 for a delayed plating experiment with *rad54-3* cells for a 72-h period of rejoining of DSBs under nongrowth conditions followed by DSB rejoining under growth conditions. Starting from the exponential survival curve involving no DSB repair, most of the DSBs are rejoined during a 72-h period under nongrowth conditions at the permissive temperature in an unsaturated reaction, free of binary misrepair, resulting in an exponential survival curve which exhibits a decreased slope. This is an intermediate step in the PLD repair process which cannot be observed in wild-type cells. Upon subsequent plating of cells on the nutrient agar, however, the exponential survival curve is converted into a shouldered curve; the reason for this is not clear at the moment. We propose that it is this unsaturated rejoining of DSBs, lacking binary misrepair, which causes the better survival of irradiated cells in a PLD-type experiment.

The question then arises as to why rejoining of DSBs under growth conditions should yield shouldered survival curves. One explanation could be that DSB rejoining under growth conditions is a saturated reaction. There are no data yet available for the half-time constant of DSB rejoining under growth conditions in yeast. However, it has been reported for EAT cells that DSB rejoining under growth conditions as measured by either the neutral sucrose sedimentation technique or the unwinding method follows an exponential function of time with a $T_{1/2}$ of 1.6 or 1.8 h, respectively (*16, 17*). Thus these experimental data so far available on DSB rejoining under growth conditions indicate that first order kinetics apply and give no evidence for saturated kinetics. Contradictory to this, Wheeler and Wierowski (*18*) conclude from their results obtained by measuring the *in situ* rejoining of total breaks (i.e., single- and double-strand breaks) in undifferentiated and differentiated brain cells, that the

second, slow and dose-dependent component of rejoining—which presumably may reflect the rejoining of DSBs—is a saturated process. Recently, Wheeler and Nelson (*19*) presented evidence suggesting that the bending down of the shouldered survival curve of undifferentiated brain cells is due to the transition of the unsaturated reaction of rejoining of total DNA breaks to a saturated reaction for increasing doses of radiation. The results obtained with *rad54-3* cells cannot be simply interpreted by such a mechanism, since the shouldered survival curves observed for DSB rejoining under growth conditions (Fig. 2) are obtained in a dose range ($D \leq 130$ Gy) *lower* than that where exponential survival curves observed for DSB rejoining under nongrowth conditions are obtained ($D \leq 320$ Gy) (Fig. 1). On the basis of the ratio of the number of substrates (i.e., DSBs) to the number of repair enzymes, in the dose range up to 130 Gy, exponential survival curves reflecting unsaturated repair kinetics would be expected rather than shouldered survival curves and—at higher doses up to 320 Gy—saturation of the repair reaction would yield shouldered rather than exponential survival curves. If saturation of the repair process would account for the shouldered survival curves in the dose range up to 130 Gy, where not more than on the average eight DSBs per cell are induced by radiation, one would have to postulate that under growth conditions, to increase the ratio of the number of DSBs to the number of repair enzymes, either repair enzymes become inactive or that the number of DSBs increases by the addition of secondary DSBs, which are formed in the course of repair of other radiation-induced lesions (*20*).

A second possible explanation to account for shouldered survival curves is, under conditions where DSBs are rejoined according to an unsaturated kinetics, that the rejoining of DSBs may be more error-prone when it occurs under growth conditions than when it occurs under nongrowth conditions. There could be an enhanced probability of interaction between at least two DSBs yielding misrejoined DNA molecules which would result in cell death. This is analogous to "exchange" chromosomal aberration formation observed in mammalian cells and which is known to arise from DSBs (*21*). It is also known that the accelerated rejoining of DSBs under growth conditions is more error-prone in the sense that more exchange aberrations (*22,* see discussion p. 63) and more cell killing (*11,* see discussion) occur. Thus it seems possible that interaction between DSBs in irradiated cells held under growth conditions resulting in binary misrepair may be enhanced for various reasons, such as lack of time for correct rejoining due to the progression of cells through the cell cycle with its many synthesizing activities, especially DNA replication, and/or enhanced formation of secondary DSBs as a result of repair of other DNA lesions (*20*). Under such circumstances more DSBs per time unit may be present in a cell, resulting in an enhanced interaction between DSBs.

In Fig. 4 we have compared the kinetics of the increase in survival after different postirradiation treatments of *rad54-3* cells irradiated with a dose of 60 Gy, which corresponds on the average to only four DSBs per cell (*13*): firstly, nongrowth conditions, secondly, growth conditions, and thirdly, the combination of nongrowth followed by growth conditions. Although the increase in survival is slower under nongrowth conditions, a higher level of survival is observed under nongrowth conditions as compared to growth conditions after longer periods of DSB rejoining. Thus it seems that under nongrowth conditions cells have more time available, enabling

them to rejoin correctly DSBs, whereas under growth conditions DSB rejoining is more rapid but also more error-prone. Furthermore, Fig. 4 shows that virtually all DSBs induced by a dose of 60 Gy can be rejoined correctly, provided *rad54-3* cells meet the appropriate conditions. This is the case when cells are held after irradiation under nongrowth conditions and subsequently under growth conditions. This combination of treatments results in the maximum achievable rejoining of DSBs which cannot be reached by either treatment alone.

In conclusion, the data presented here show that rejoining of DSBs under nongrowth conditions is an unsaturated, dose-independent reaction, which lacks binary misrepair, yielding exponential survival curves. In contrast, shouldered survival curves arise as a consequence of DSB rejoining under growth conditions. In a PLD-repair-type experiment, where postirradiation rejoining of DSBs under nongrowth conditions is followed by DSB rejoining under growth conditions on the nutrient agar plate, the unsaturated rejoining of DSBs under nongrowth conditions lacking binary misrepair results in an intermediary, exponential survival curve and is responsible for the better survival of delayed plated cells. The shouldered form of the delayed plating survival curve is brought about by the subsequent DSBs rejoining under growth conditions.

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