

Radioresistance of *Deinococcus radiodurans*: Functions Necessary To Survive Ionizing Radiation Are Also Necessary To Survive Prolonged Desiccation

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Forty-one ionizing radiation-sensitive strains of *Deinococcus radiodurans* were evaluated for their ability to survive 6 weeks of desiccation. All exhibited a substantial loss of viability upon rehydration compared with wild-type *D. radiodurans*. Examination of chromosomal DNA from desiccated cultures revealed a time-dependent increase in DNA damage, as measured by an increase in DNA double-strand breaks. The evidence presented suggests that *D. radiodurans*' ionizing radiation resistance is incidental, a consequence of this organism's adaptation to a common physiological stress, dehydration.

The *Deinococcaceae* are a small family of non-spore-forming bacteria which exhibit a remarkable capacity to resist the lethal effects of ionizing radiation (10, 11, 18). Well-aerated, exponential-phase cultures of members of this family will survive 5,000 Gy of gamma radiation without loss of viability (14), and survivors are routinely recovered from cultures exposed to as much as 20 kGy (1, 7). Of the five species that make up the *Deinococcaceae*, *Deinococcus radiodurans* has been most extensively studied, and it has been determined that the radioresistance of this species is a direct result of its ability to efficiently repair the DNA damage generated during irradiation (10, 11, 18). In other words, the extreme radioresistance of *D. radiodurans*—and presumably the other deinococci—appears to be the result of an evolutionary process that selected for organisms that could tolerate massive DNA damage. The reasons for *D. radiodurans*' ionizing radiation resistance are obscure, however. The deinococci's radioresistance cannot be an adaptation (i.e., an evolutionary modification of a character under selection) to ionizing radiation, because there is no selective advantage to being ionizing radiation resistant in the natural world. There are no terrestrial environments that generate such a high flux of ionizing radiation (20). It must therefore be assumed that the deinococci's radioresistance is an incidental use of the cell's DNA repair capability. In this study, we ask why the DNA repair capabilities of *D. radiodurans* evolved, and we provide evidence suggesting that they were built by selection for desiccation resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All *D. radiodurans* strains were grown at 30°C in TGY broth (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) or on TGY agar (TGY broth with 1.5% agar).

Quantifying desiccation resistance. Cells from an exponential-phase culture of each strain examined were collected by centrifugation, washed in 4 volumes of 10 mM MgSO₄, and resuspended in an equal volume of 10 mM MgSO₄. A 100- μ l aliquot of this suspension was spotted on a sterile glass coverslip, placed inside a sterile petri dish, and dried at 25°C in a desiccator over anhydrous CaSO₄ containing a visual indicator. The desiccators were sealed, and the dried cultures were stored undisturbed at 25°C for 6 weeks. Relative humidity within the

desiccators was measured as less than 5% with a membrane hygrometer. Samples were revived by washing the cells free of the plate in 1 ml of 10 mM MgSO₄ and plating on TGY agar (7). Plates were placed in a 30°C incubator and scored for survivors 5 days later.

Monitoring the appearance of DNA damage by pulsed-field gel electrophoresis. The cultures used to prepare chromosomal DNA were concentrated by centrifugation and washed in butanol-saturated 0.5 M EDTA (pH 8.0) to remove the outer membrane and make the cell wall susceptible to lysozyme. Butanol-stripped cells were concentrated by centrifugation, suspended in 0.5 M EDTA, heated to 65°C for 30 min, and resuspended in 0.05 M EDTA before being embedded in an equal volume of 1.6% low-melting-point preparative-grade agarose in 0.05 M EDTA. Agarose plugs containing the embedded cells were soaked overnight in a 1-mg/ml lysozyme solution in 0.05 M EDTA (pH 8.0) at 45°C. The lysozyme solution was removed by aspiration and replaced with a 2-mg/ml pronase E solution in 10 mM Tris-HCl-0.5 M EDTA-1% laurylsarcosine (pH 8.0). The agarose plug was soaked in this solution overnight at 45°C. Chromosomal DNA liberated by this treatment was separated by clamped homogeneous electric field pulsed-field gel electrophoresis with 0.5 \times TBE (Tris-borate-EDTA) at 1.4 V/cm² and 13°C with a 10- to 60-s ramp for 22 h.

RESULTS

Ionizing radiation-sensitive mutants of *D. radiodurans* are also sensitive to desiccation. It is possible to isolate ionizing radiation-resistant bacteria from natural microflora by selecting for desiccation resistance (16), indicating that at least a subset of the cellular functions necessary to survive exposure to ionizing radiation are also necessary to survive desiccation. Since dehydration induces DNA damage in bacteria (2, 4, 5), it was considered likely that the ability to repair DNA damage was one of these functions. To test this possibility, 41 ionizing radiation-sensitive derivatives of *D. radiodurans* were evaluated for the ability to survive desiccation. Forty of these strains are designated IRS_n, and they were derived from *D. radiodurans* 302, a *uvrA1* derivative of the type strain *D. radiodurans* R1 (19). The remaining strain, designated rec30, was derived directly from R1 (13). Strain 302 is as resistant to ionizing radiation as R1, displaying approximately 90% survival following exposure to 5,200 Gy of gamma radiation when irradiated during exponential-phase growth (8). The ionizing radiation-sensitive strains are from 2- to 300-fold more sensitive to gamma radiation than their parent strains at this dose (8).

The effect of dehydration on the viability of the ionizing radiation-sensitive strains is illustrated in Fig. 1, which shows the mean percent survival of individual strains desiccated for 6 weeks. The radioresistant strains R1 and 302 exhibited approximately 63% survival. In contrast, every ionizing radiation-sensitive strain tested was substantially more sensitive to des-

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TABLE 1. Bacterial strains and plasmids

| <i>D. radiodurans</i> strain | Description | Reference |
|------------------------------|---|-----------|
| R1 | ATCC 13939 | 1 |
| 302 | As R1 but <i>uvrA1</i> (<i>mtcA</i>) ^a | 12 |
| rec30 | As R1 but <i>recA</i> | 13 |
| IRS1-IRS49 | As 302 but ionizing radiation sensitive | 19 |

^a The *mtcAB* region of the *D. radiodurans* chromosome has recently been sequenced by Minton and colleagues (9) and shown to contain a single gene whose product is homologous with the UvrA protein of *E. coli*. In light of this new information, these investigators have recommended that the *mtcA* mutation of *D. radiodurans* 302 be designated *uvrA1*.

iccation. Only three of these strains demonstrated greater than 10% survival, and the most desiccation-sensitive strains were from 100- to 250-fold less viable than their parents. From these data, it is apparent that *D. radiodurans*' ionizing radiation resistance and desiccation resistance are functionally interrelated phenomena and that by losing the ability to repair ionizing radiation-induced cellular damage, *D. radiodurans* is sensitized to the lethal effects of desiccation.

Dehydration induces DNA DSBs. It has been reported that there is a substantial reduction in the molecular weight of *D. radiodurans* R1 chromosomal DNA when it is isolated from cultures that have been exposed to a vacuum for 12 days (5). As illustrated in Fig. 2, *D. radiodurans* R1 cultures that have been desiccated over CaSO₄ also suffer extensive DNA damage. Intact chromosomal DNA could not be detected in R1 cultures desiccated for 6 weeks (Fig. 2, lane 3). Instead, a wide band appeared near the 50-kb size marker, indicating that a large number of DNA double-strand breaks (DSBs) were introduced either during desiccation or upon rehydration of these cultures. Untreated R1 cultures did not show evidence of DNA DSBs (Fig. 2, lane 2). If it is assumed that the R1 genome is 3 Mbp (6) and that all the fragments in lane 3 are 50

kb or smaller, a minimum of 60 DSBs would be required to generate the pattern observed in lane 3. The extent of DNA damage observed following desiccation is comparable to what occurs when R1 cultures are exposed to high doses of gamma radiation (Fig. 2, lane 4). Other lethal stresses, including heat (Fig. 2, lane 6) and starvation during prolonged storage in stationary phase (Fig. 2, lane 7), have no obvious effect on the size of chromosomal DNA. Even a massive dose of UV radiation (Fig. 2, lane 8) generates only enough damage to produce a faint smear on the gel. Although we did not formally investigate the formation of desiccation-induced DNA DSBs, the similarities between the gel patterns produced by the chromosomal DNA isolated from desiccated cultures and that isolated from gamma-irradiated cultures suggest that radical chemistry is involved in their formation.

Dehydration-induced DNA DSBs accumulate as a function of time. The damage inflicted during desiccation accumulates slowly. DNA DSBs are not obvious in *D. radiodurans* cultures until 8 days after desiccation (Fig. 3). Beyond 28 days, it is difficult to detect intact chromosomal DNA, and by 42 days, only low-molecular-weight DNA is apparent. Cultures of the *Escherichia coli* K-12 strain AB1157 also accumulate DNA DSBs with the same kinetics as *D. radiodurans* (Fig. 4), suggesting that the DNA DSBs observed when *D. radiodurans* is dried are solely a consequence of desiccation and not a function of physiological changes peculiar to *D. radiodurans*.

At least one other factor also influences the ability of *D. radiodurans* to survive dehydration. The survival curves of the *E. coli* and *D. radiodurans* cultures used to generate the data shown in Fig. 3 and 4 are plotted in Fig. 5. The viability of the *E. coli* cultures declined dramatically upon desiccation. There was a 1,000-fold reduction in survivors by the second day of desiccation, and by day 14, it was not possible to detect viable organisms in the dried *E. coli* cultures; that is, there was less than 1 viable organism per 100 μ l of original culture. The rapid loss of viability in the dried *E. coli* cultures was not accompa-

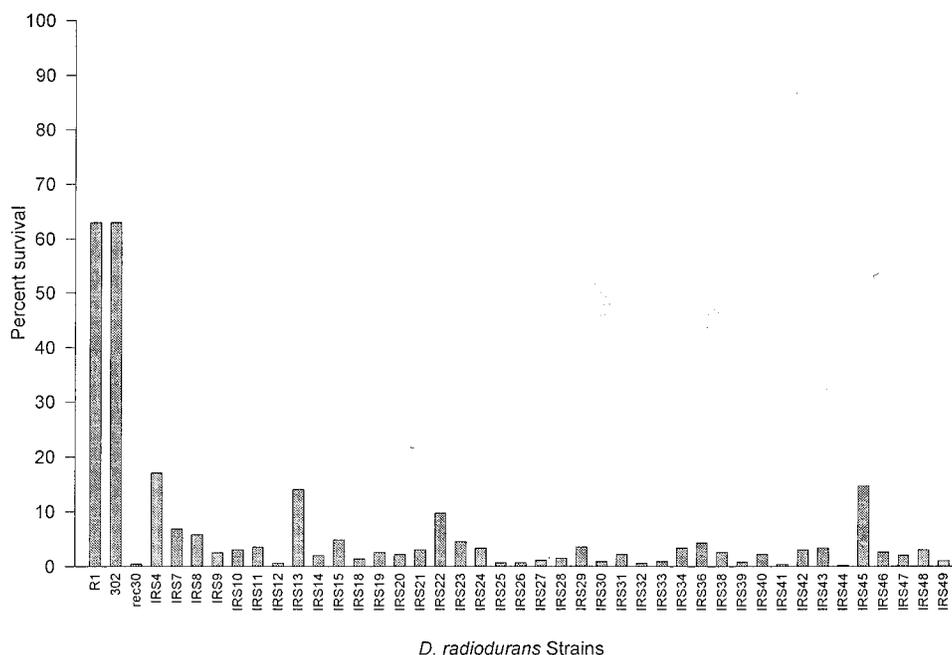


FIG. 1. Effect of 6 weeks of desiccation on the survival of ionizing radiation-sensitive strains of *D. radiodurans*. Values are the mean percent survival in two separate trials, with three replicates per trial. Each survival value reported is relative to that strain's titer immediately prior to desiccation.

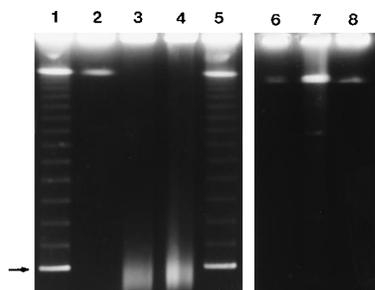


FIG. 2. Accumulation of chromosomal DNA DSBs in *D. radiodurans* R1 cultures subjected to desiccation. Lanes 1 and 5, lambda ladder size standards (Bio-Rad Laboratories, Richmond, Calif.). The arrow indicates the 48.5-kb marker. Lane 2, *D. radiodurans* R1 chromosomal DNA prepared from an untreated culture; lane 3, R1 chromosomal DNA prepared from a culture that had been desiccated for 6 weeks. Cultures were desiccated as described in the legend to Fig. 1. Lane 4, R1 chromosomal DNA prepared from a culture that had been exposed to 5,200 Gy of gamma radiation. Cells were irradiated at 22°C with a ^{60}Co source at a dose rate of 14.4 Gy/min. Lane 6, R1 chromosomal DNA isolated from a culture that was heat treated at 52°C for 68 min, conditions which define the 90% lethal dose for *D. radiodurans* R1, as described by Bridges et al. (3). Heat treatment was carried out by incubating a thin-walled polypropylene screw-cap tube containing 5 ml of exponential-phase cell culture in a water bath. Lane 7, R1 chromosomal DNA isolated from a UV-irradiated cell culture. Five milliliters of exponentially growing cells were concentrated to 500 μl , placed into a disposable petri dish, and swirled, uncovered, under a germicidal lamp at a dose rate of 25 $\text{J}/\text{m}^2/\text{s}$ until a total dose of 5,000 J/m^2 was obtained. Lane 8, R1 chromosomal DNA isolated from cells held in stationary phase in liquid medium at 30°C for 100 days.

nied by overt evidence of DNA DSBs (Fig. 4), indicating that multiple DNA DSBs were not responsible for the observed lethality.

There was no loss of viability in *D. radiodurans* cultures during the first 14 days of desiccation. Over the next 28 days, however, there was a 1.1 log reduction in viability, a value inconsistent with the data presented in Fig. 1, where 63% survival was reported in a nearly identical experimental protocol. The *D. radiodurans* R1 cultures used to generate the data in Fig. 5 exhibited only 17% survival after 6 weeks of desiccation. The two experiments differed only in the number of times that the desiccator was opened during each trial. The desiccator was sealed throughout the 6 weeks of desiccation during the studies represented in Fig. 1 and 2, but it was opened at each time point examined in Fig. 3 and 4. This suggested that periodically opening the desiccator was harmful to the dried cells.

To evaluate this possibility, the experiment shown in Fig. 1 was repeated, but the desiccator was opened for 30 min at the beginning of each week of the 6-week trial. Survival of the R1

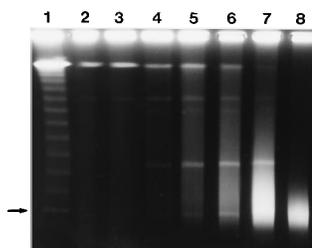


FIG. 3. Appearance of DNA DSBs in a desiccated *D. radiodurans* R1 culture as a function of time. Lane 1, lambda ladder size standards. The arrow indicates the 48.5-kb marker. Lane 2, *D. radiodurans* R1 chromosomal DNA prepared from an untreated culture. Lanes 3 to 8, R1 chromosomal DNA preparations obtained from cultures after 1, 4, 8, 14, 28, and 42 days of desiccation, respectively.

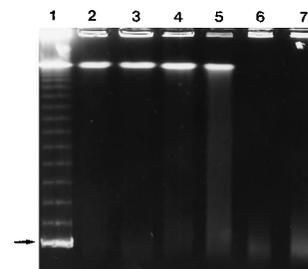


FIG. 4. Appearance of DNA DSBs in a desiccated *E. coli* AB1157 culture as a function of time. Lane 1, lambda ladder size standards. The arrow indicates the 48.5-kb marker. Lane 2, AB1157 chromosomal DNA prepared from an untreated culture. Lanes 3 to 7, AB1157 chromosomal DNA preparations obtained after 2, 5, 16, 28, and 42 days of desiccation, respectively.

and 302 strains (Fig. 6) was reduced to 13% when this modified protocol was followed, a value consistent with the 17% survival reported in Fig. 5. The ionizing radiation-sensitive strains exhibited a similar reduction in survival, but they remained between 10- and 100-fold less viable than their parent strains when rehydrated. The physical basis of this effect was not investigated, but it was noted that as the desiccator was opened, the relative humidity within the system increased to approximately 60%, and upon resealing the system, it took from 30 to 45 min for it to return to less than 5%. It is therefore possible to lower the survival of desiccated cultures of *D. radiodurans* by subjecting them to cycles of desiccation and partial rehydration.

DISCUSSION

The ionizing radiation resistance of *D. radiodurans* has been difficult to explain from an evolutionary point of view, because the selective advantage of this extreme character is not obvious. The average outdoor terrestrial absorbed dose rate in air from gamma radiation is $5 \times 10^{-8} \text{ Gy h}^{-1}$, or 0.4 mGy year $^{-1}$, and the highest reported absorbed dose rate, measured in thorium-rich monazite sands found near Guarapari, Brazil, is only 175 mGy year $^{-1}$ (20), far too low to be considered the selective force that could build the degree of radioresistance

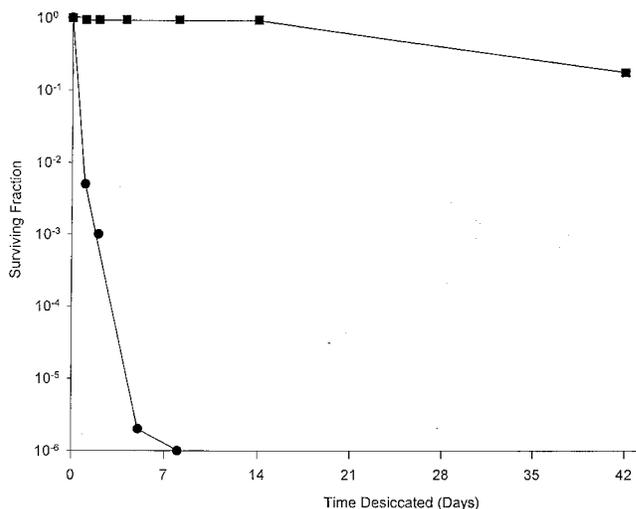


FIG. 5. Representative survival curves for *D. radiodurans* R1 (squares) and *E. coli* AB1157 (circles) as a function of time of desiccation over CaSO_4 . Values are the means of a single trial, with three replicates per trial.

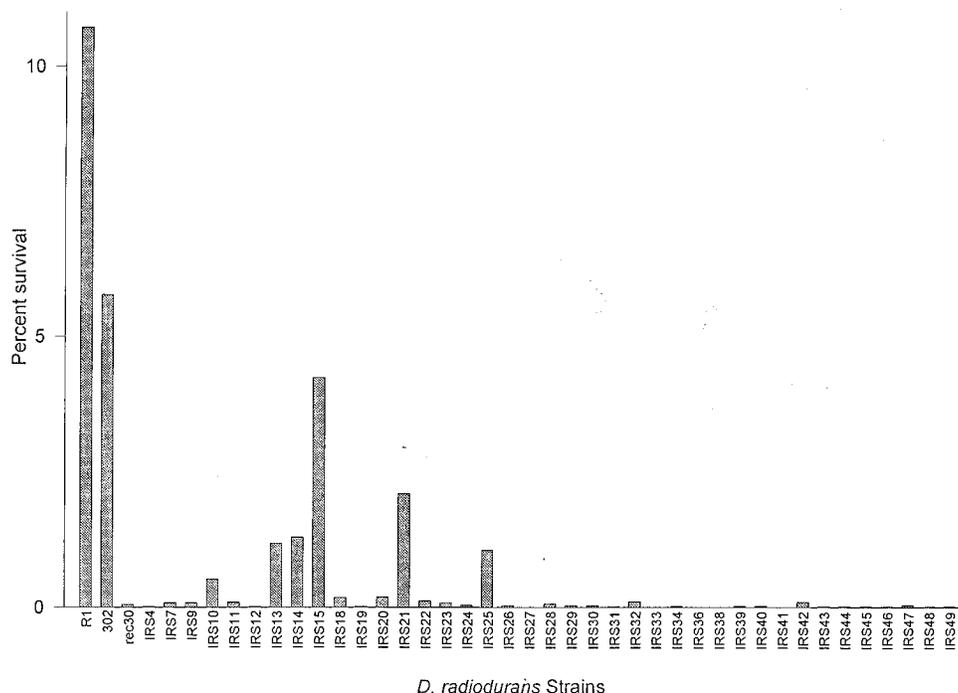


FIG. 6. Effect of opening the desiccator on the survival of ionizing radiation-sensitive strains of *D. radiodurans*. Values are the mean percent survival in four separate trials, with three replicates per trial. Cultures were desiccated as described in Materials and Methods except that at the beginning of each week in the 6-week trial, the desiccator was opened to ambient humidity for 30 min.

associated with wild-type *D. radiodurans*. It has therefore been assumed that the radiotolerance of *D. radiodurans* is a fortuitous consequence of an evolutionary process that permitted this bacterium to cope with an environmental stress other than ionizing radiation.

In this study, we have established that *D. radiodurans*' cellular responses to ionizing radiation and dehydration significantly overlap by showing that 41 ionizing radiation-sensitive strains of *D. radiodurans* were also sensitive to desiccation (Fig. 1). We infer that the loss of DNA repair ability in the ionizing radiation-sensitive strains was responsible for the desiccation sensitivity of these strains, and we offer three interrelated arguments to support this inference. First, prolonged dehydration causes extensive DNA damage (Fig. 2 and 3), with 6 weeks of desiccation resulting in a minimum of 60 DNA DSBs, indicating that to survive desiccation, the *D. radiodurans* cell requires extraordinary DNA repair capability. Second, there is evidence from studies of *E. coli* that desiccation resistance is influenced by the DNA repair ability of the strain being dehydrated. Asada et al. (2) have shown that AB2480, a *recA uvrA* isogenote of AB1157, is substantially more sensitive to drying under a vacuum than is AB1157. We assume that if DNA repair capability contributes to the survival of a desiccation-sensitive organism such as *E. coli*, it should contribute to the survival of a desiccation-resistant organism as well. Finally, nine of the strains represented in Fig. 1 express characterized DNA repair defects. IRS26, IRS27, IRS47, and *rec30* are recombination-defective mutants (8, 13). IRS7, IRS33, and IRS38 express inactive forms of the deinococcal homolog of *E. coli* DNA polymerase I (8), and genetic evidence indicates that IRS18 and IRS41 express proteins unable to appropriately regulate repair of DNA damage. For these strains, the loss of DNA repair capability resulted in sensitivity to desiccation. The remaining IRS strains have not been characterized, but it

is assumed that most are unable to adequately repair ionizing radiation-induced DNA damage.

The role of DNA repair in the desiccation resistance of *D. radiodurans* is not unlike that ascribed to DNA repair in spores of *Bacillus* spp. (17). During their dormancy, spores accumulate DNA damage that cannot be repaired until the spore germinates. The lack of water within the spore prevents enzymatic activity and therefore DNA repair. As demonstrated in Fig. 3 and 4, there is the potential for DNA damage to accumulate in vegetative cells during desiccation, and since we have not observed any evidence of DNA repair in *D. radiodurans* cultures while they are desiccated (unpublished observations), we assume that desiccation-induced DNA damage cannot be repaired until the organism is rehydrated. Desiccation-resistant vegetative organisms must be able to either inhibit the formation of DNA damage while dehydrated or repair it upon rehydration. In *D. radiodurans*, it is the capacity to repair DNA damage which appears to be most developed.

In *E. coli*, dehydration-induced lethality (Fig. 5) did not appear to correlate with the formation of DNA DSBs (Fig. 4), suggesting that the differences between *D. radiodurans* and *E. coli* extend beyond the ability of each to repair DNA damage. This was not unexpected, since dehydration adversely affects the membranous and proteinaceous components of the cell as well as its nucleic acids (15). One would predict that *D. radiodurans*, being desiccation resistant, has mechanisms that contend with or inhibit all dehydration-induced cellular damage and that *E. coli* does not have such mechanisms. Pending further investigation, we assume that the loss of viability observed when the desiccator is repeatedly opened is due either to an increase in DNA damage caused by the cycles of dehydration or to the disruption of a part of *D. radiodurans*' defense against dehydration that is not related to DNA damage repair.

We conclude that *D. radiodurans* is an organism that has

adapted to dehydration and that its DNA repair ability is a manifestation of that evolutionary process. We believe that *D. radiodurans* is ionizing radiation resistant because it is resistant to desiccation and because desiccation resistance appears to require extensive DNA repair.

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