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# Extremely Radiation-Resistant Mutants of a Halophilic Archaeon with Increased Single-Stranded DNA-Binding Protein (RPA) Gene Expression

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Extremely halophilic archaea are highly resistant to multiple stressors, including radiation, desiccation and salinity. To study the basis of stress resistance and determine the maximum tolerance to ionizing radiation, we exposed cultures of the model halophile *Halobacterium* sp. NRC-1 to four cycles of irradiation with high doses of 18–20 MeV electrons. Two independently obtained mutants displayed an LD<sub>50</sub> > 11 kGy, which is higher than the LD<sub>50</sub> of the extremely radiation-resistant bacterium *Deinococcus radiodurans*. Whole-genome transcriptome analysis comparing the mutants to the parental wild-type strain revealed up-regulation of an operon containing two single-stranded DNA-binding protein (RPA) genes, VNG2160 (*rfa3*) and VNG2162, and a third gene of unknown function, VNG2163. The putative transcription start site for the *rfa3* operon was mapped ~40 bp upstream of the ATG start codon, and a classical TATA-box motif was found centered about 25 bp further upstream. We propose that RPA facilitates DNA repair machinery and/or protects repair intermediates to maximize the ionizing radiation resistance of this archaeon. © 2007 by Radiation Research Society

## INTRODUCTION

Extremely halophilic archaea are among the most extremophilic microorganisms, and they display tolerance to multiple stressors (1). Halophilic archaea are found in brines containing saturating levels of sodium chloride such

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as in saltern crystallizer ponds that are exposed to intense solar radiation. To survive under these conditions, they have developed remarkable resistance to UV radiation and have a highly efficient photoreactivation repair system (2). In addition, this class of microorganisms has been shown to survive prolonged exposure to desiccation and may live in brine inclusions in ancient underground salt deposits (3). Their resistance to desiccation and radiation may also be responsible for their prolonged survival in outer space, where they have been recovered after longer exposure than any other vegetative cells tested (4).

Among the extremely halophilic archaea, *Halobacterium* sp. NRC-1 is a well-characterized model organism with a completely sequenced genome and post-genomic tools for the study of numerous cellular pathways (5). This microbe is easily cultured in the laboratory and is amenable to genetic studies through a facile gene knockout system and whole genome DNA microarrays (6). As a result, *Halobacterium* sp. NRC-1 has been used for a wide range of studies, including UV and ionizing radiation tolerance (7–13). *Halobacterium* sp. NRC-1 is able to efficiently repair extensive double-strand DNA breaks in its genomic DNA, produced both by desiccation and by ionizing radiation, as well as cyclopyrimidine dimers and 6-4 pyrimidone photoproducts produced by UV radiation.

The genome of *Halobacterium* sp. NRC-1 contains a 2-Mbp chromosome and two related extrachromosomal replicons of 365 and 191 kbp (14). The genome contains homologs of both bacterial and eukaryotic DNA repair genes (15, 16). Some of the predicted proteins involved in nucleotide excision repair, such as Rad2 (FEN1/XPG), Rad3 (XPD), Eif4A (RAD1/XPF) and Rad 25 (XPB), are more closely related to eukaryotic proteins than to bacterial proteins. However, other proteins, including the UvrABC excision repair system, are more similar to the bacterial counterparts (17). The presence of homologs to bacterial excision repair genes is unusual for the archaea, and recent genetic knockout studies have established their importance in dark repair in *Halobacterium* sp. NRC-1 (18). Genetic

studies have shown that the active photoreactivation system of *Halobacterium* sp. NRC-1 is mediated by a photolyase, coded by the *phr2* gene (2). In a transcriptome analysis of *Halobacterium* sp. NRC-1 exposed to UV radiation, genes likely involved in homologous recombination, including *radA1*, were shown to be significantly induced, suggesting the involvement of homologous recombination in repair of DNA damage (12, 19).

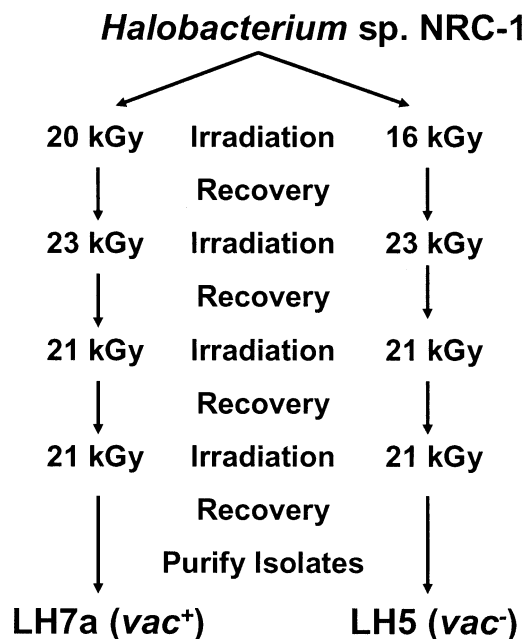
*Halobacterium* sp. NRC-1 has recently been shown to have high resistance to  $\gamma$  radiation (20). The resistance was attributed to the ability to repair double-strand breaks as well as to the protective properties of specific membrane pigments to oxidative damage. The high salinity in which this organism and other halophiles grow also provides protection against damage by decreasing the oxygen content and hence levels of damaging oxygen-produced radicals (21). Given the already considerable resistance to radiation exhibited by this organism, it was unclear whether genetic changes would allow survival after higher challenging doses of radiation. The radiation resistance of some bacterial species has been increased up to 20-fold by repeatedly exposing growing cultures to doses of radiation that allow very few survivors (22, 23). In these cases, however, the natural resistance of the starting organisms to both radiation and desiccation was very low in comparison to *Halobacterium*. In addition, the relatively low rate of dose delivery from these radiation sources necessitated longer times for delivery of the higher doses, during which DNA repair mechanisms may have been operating. It is likely that simultaneous mutation of many genes involved in DNA repair would be required in these cases for changes in resistance.

Here we report the isolation of extremely radiation-resistant mutants of *Halobacterium* sp. NRC-1, which are the most radiation-resistant organisms currently known, by selection for isolates displaying increased resistance to electron-beam radiation. Through transcriptome analysis, we have identified the gene expression difference in these mutants that is likely responsible for increased radiation resistance.

## MATERIALS AND METHODS

### Strains and Growth Conditions

The strains of *Halobacterium* used were derivatives of strain NRC-1 (Fig. 1). *Deinococcus radiodurans* R1 (ATCC 13939) was a gift from John Battista at Louisiana State University. *Halobacterium* cultures were grown in CM<sup>+</sup> medium (24) at 42°C with shaking, without supplemental illumination. *D. radiodurans* was grown in TGY (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) at 30°C with aeration (25). For solid medium, 2% agar was added. *Halobacterium* plates were incubated at 42°C for 7 days, then removed and allowed to continue to grow at room temperature until no new colonies appeared. *D. radiodurans* plates were incubated at 30°C for 3–5 days. Liquid cultures for irradiation were inoculated from purified isolates on solid medium. Stock cultures were maintained in glycerol at –80°C. For short-term use, purified cultures were maintained on stock plates at 4°C. Growth rates were monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) of cultures in liquid medium over



**FIG. 1.** Selection of *Halobacterium* strains LH5 and LH7a from parent strain NRC-1 through irradiation–growth cycles. Independent aliquots of a stationary-phase culture of *Halobacterium* sp. NRC-1 were irradiated at the doses indicated and allowed to recover by inoculation into fresh medium prior to subsequent irradiations.

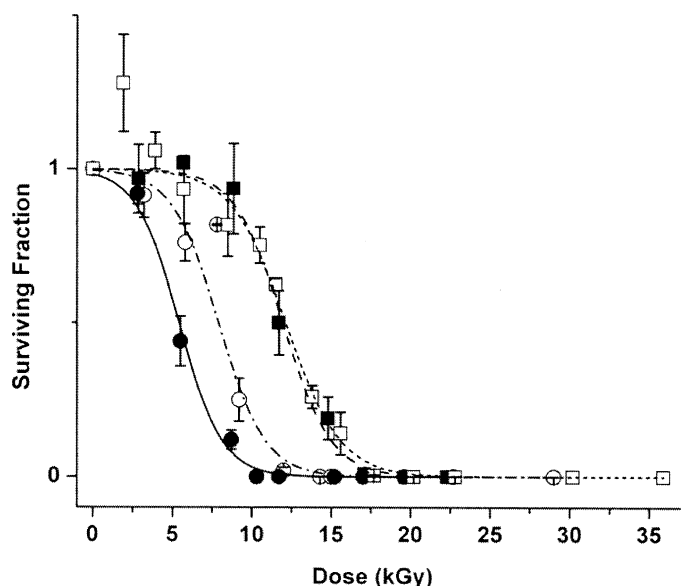
time. Samples of growing cultures were diluted and plated at various times to ensure that vastly different cell sizes, the presence of nonviable cells, or differences in gas vesicle production did not skew the OD<sub>600</sub> readings. For determination of survival, irradiated liquid cultures were immediately diluted and spread on plates. Survival after irradiation was determined by comparing surviving cells to an unirradiated control.

### Irradiation and Dosimetry

The 20 MeV pulsed electron LINAC delivered 18–20 MeV electrons at a peak current of 80 mA, with a peak dose rate of  $2.5 \times 10^5$  Gy/s and an average dose rate of 30 Gy/s. The pulse width was 2 ms, and the repetition rate was 60 Hz. Samples were irradiated at room temperature in 0.2-ml thin-walled PCR tubes held 2.5 m from the beam port. At this distance, the uniformity of the beam was within 10% of the peak dose over a circular area of 10 cm diameter, which encompassed the sample holder size. Samples were located on isocontours of dose with sample dose variations less than 1%. Beam location was determined before and after irradiations with PIN diodes, and the sample doses delivered were measured using a GEX Corporation thin-film dosimetry system and GEX B3 radiochromic film (26).

### Irradiation–Growth Cycles

A stationary-phase culture ( $4 \times 10^8$  cells/ml) of *Halobacterium* NRC-1 in 0.1-ml aliquots was exposed to electrons from a 20 MeV LINAC at doses of  $16.4 \pm 1.6$  and  $19.5 \pm 2$  kGy. These aliquots were diluted into 2 ml of CM<sup>+</sup> medium and incubated with shaking for 2–5 days at 42°C until growth was visible. Glycerol was added to 15%, and 1 ml of each suspension was frozen at –80°C. Prior to the next irradiation, the frozen stocks were used to inoculate 2 ml of CM<sup>+</sup> medium and grown for 2 days at 42°C. Aliquots of these cultures were irradiated at  $23 \pm 2.3$  kGy. Each was then diluted into 2 ml broth and incubated until growth was visible. Once again, 0.1-ml aliquots of these cultures were irradiated at  $20.6 \pm 2.1$  kGy. Fifty microliters of each irradiated sample was diluted into 2 ml CM<sup>+</sup> medium and grown for 7 days, until growth was visible.



**FIG. 2.** Survival of exponential-phase *Halobacterium* sp. NRC-1, LH5 and LH7a compared to *Deinococcus radiodurans* R1 after electron-beam irradiation. Values from multiple experiments were binned according to dose. Error bars represent the errors on the mean survival per bin. NRC-1 (●), *D. radiodurans* (○), LH5 (■), and LH7a (□). Individual data points were fitted to the Boltzmann curves shown. NRC-1 (—), *D. radiodurans* (- - -), LH5 (- - -), and LH7a (- - - -).

These cultures were irradiated in 0.1-ml aliquots at  $21 \pm 2.1$  kGy. Fifty microliters of each aliquot was diluted into 1 ml CM<sup>+</sup> medium until growth was visible, approximately 7 days. These two cultures were streaked on solid medium and individual isolates were purified four times. LH5 was derived from the cultures originally irradiated at 16.4 kGy and LH7a from the culture irradiated at 19.5 kGy. This procedure is summarized in Fig. 1.

#### Cell Irradiation and Survival Measurement

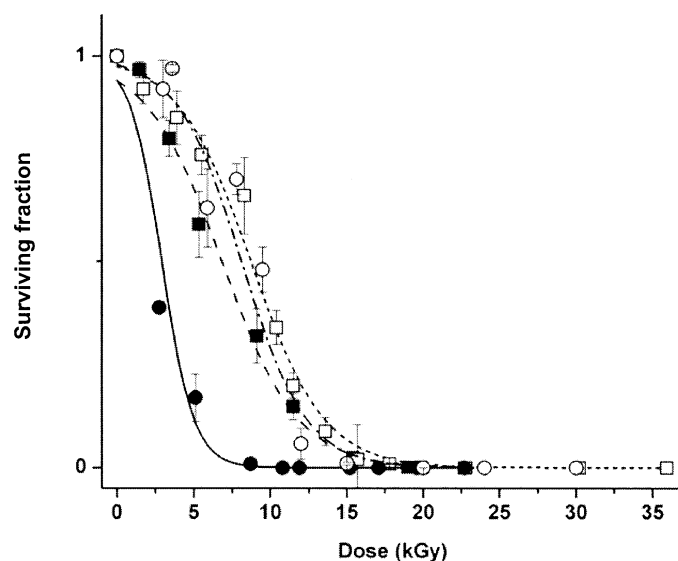
Each strain was grown in liquid culture to the appropriate density and culture state (exponential or stationary phase). In an individual experiment, a culture was divided into aliquots and subjected to a predetermined set of doses, including no radiation. The aliquots were diluted in growth medium immediately after irradiation and plated in duplicate or triplicate. In a given experiment, the multiple platings of the unirradiated (0 Gy) aliquot were counted and averaged to give the initial cell density in colony-forming units per milliliter (cfu/ml). This value represented 100% survival and was used as the basis of comparison for all irradiated aliquots of that culture. The duplicate or triplicate platings of each irradiated aliquot were averaged to determine cfu/ml at that dose. The surviving fraction for that dose was determined by dividing the cfu/ml by the cfu/ml representing 100% survival (0 Gy, or initial cell density) of that culture. For each strain, multiple experiments (independent cultures) were performed on at least two different days, and the surviving fractions were combined (Figs. 2 and 3).

#### Survival Curve Fitting

For each strain, surviving fractions from multiple irradiations were combined and plotted in OriginPro 7 (OriginLab Corporation, Northampton, MA). The resulting data were fitted to a sigmoidal Boltzmann function,

$$\left[ \frac{(A_1 + A_2)}{1 + e^{(X - X_0)/dx}} \right] + (A_2),$$

with two parameters fixed at  $A_2 = 0$  and  $A_1 = 1$ . Here  $X_0$  is the LD<sub>50</sub>



**FIG. 3.** Survival of stationary-phase *Halobacterium* sp. NRC-1, LH5 and LH7a compared to *Deinococcus radiodurans* R1 after electron-beam irradiation. Values from multiple experiments were binned according to dose. Error bars represent the errors on the mean survival per bin. NRC-1 (●), *D. radiodurans* (○), LH5 (■), and LH7a (□). Individual data points were fitted to the Boltzmann curves shown. NRC-1 (—), *D. radiodurans* (- - -), LH5 (- - -), and LH7a (- - - -).

and  $dx$  is the width of the transition from surviving fraction = 1 to surviving fraction = 0 (see Figs. 2 and 3). Individual values from multiple irradiations (ranging in number from 30 to 67; see Table 1 in Supplementary Information) for each strain were combined and used for curve fitting. Bins were selected for clarity of presentation, with each bin (with one exception; see Table 1 in Supplementary Information) containing a minimum of two data points and an average of five. The binned values were used for y-error determination in Figs. 2 and 3, which is calculated as the error on the mean surviving fraction of each bin. For clarity, the binned data points and standard error are presented in Figs. 2 and 3; however, the curve in these figures was generated from the fit of the unbinned data points.

#### Microarray Analysis

*Halobacterium* sp. strain NRC-1 and derivatives LH5 and LH7a were grown under aerobic conditions to early exponential growth phase (OD<sub>600</sub> of 0.15 to 0.3). Cells were harvested by chilling the incubation vessels in an ethanol-dry ice bath for 1 min followed by centrifugation of the culture (8,000g, 5 min, 2°C) in chilled beakers. Total RNA was isolated immediately after harvest at 25°C using an Agilent Total RNA isolation kit (Agilent Technologies, Palo Alto, CA), and DNA was hydrolyzed using amplification grade DNase (Sigma-Aldrich, St. Louis, MO). Lysis of cells was carried out directly in lysis buffer. Two treatments with DNase (Invitrogen, Carlsbad, CA) were carried out to remove contaminating DNA from the RNA preparations. To minimize biological noise, RNA preparations from three 50-ml cultures of each strain grown under identical conditions were pooled to equal parts for cDNA synthesis. cDNA was prepared from 7 µg total RNA with Super Script III reverse transcriptase (Invitrogen) and Cy3- or Cy5-dCTP (Amersham Biosciences, Piscataway, NJ), and purified after alkaline hydrolysis of RNA on Qiagen mini-elute columns (Qiagen, Valencia, CA). The labeled cDNA targets were mixed with hybridization buffer and control targets and hybridized to microarray slides assembled into a hybridization chamber (Agilent) for 17 h at 60°C in the dark. After hybridization, the slides were washed as described in the Agilent protocol and scanned for the Cy3 and Cy5 fluorescent signals with an Agilent DNA-microarray scanner (Model

no. G2565BA). Transcriptome profiling of cells was carried out in duplicate. Relative mRNA levels were determined by parallel two-color hybridization to oligonucleotide (60-mer) microarrays representing 2474 open reading frames (ORFs) representing 97% of unique *Halobacterium* sp. NRC-1 ORFs according to Müller and DasSarma (10). Previous results showed that differences in the relative intensity of the channels could be adjusted for by intensity-dependent LOWESS normalization (10, 27). Image processing and statistical analysis were carried out using Feature Extraction Software Version 7.1 (Agilent). Log ratios for each feature were calculated and the significance of the log ratio was assessed by calculating the most conservative log ratio error and significance value (*P* value) using a standard error propagation algorithm (Agilent) and a universal error model (Rosetta Biosoftware, Seattle, WA).

#### Reverse-Transcriptase PCR (RT-PCR)

Total RNA was isolated as described above. DNA was removed from the RNA by three treatments with RNase-free DNase I (Qiagen). cDNA was synthesized from 0.2 to 0.8 µg RNA and 2 pmol of specific primer with SuperScript II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen) as described by the supplier. The PCR amplification mixtures contained 6 µl cDNA, 200 nM of each primer, 200 µM dNTP, 1 U *Taq* polymerase in PCR buffer with 1.5 mM MgCl<sub>2</sub>. For amplification of parts of *rfa3-rfa8* and *rfa8-ral*, and to map the transcriptional start site of *rfa3*, the following primer pairs were used (positions in the NRC-1 chromosome of primers in parentheses): -29F (1594051-1594069)/-29R (1594539-1594556); +3F (1594082-1594103)/+3R (1594603-1594622); *rfa3-rfa8*F (1594829-1594848)/*rfa3-rfa8*R (1595225-1595244); *rfa8-ral*F (1595510-1595534)/*rfa8-ral*R (1595754-1595777). PCR parameters were as follows: 3 min at 92°C, 30 cycles of 0.45 min at 94°C, 1 min at 50°C for the primer pair -29F/-29R, 1 min at 58°C for +3F/+3R, and 1 min at 60°C for the other two pairs, 1 min at 72°C, followed by 7 min at 72°C.

## RESULTS

### Isolation of Extremely Radiation-Resistant Mutants of *Halobacterium* sp. NRC-1

Using a series of irradiation-growth enhancements based on a procedure used by Davies and Sinskey on *Salmonella typhimurium* (22), we enriched a culture of *Halobacterium* sp. NRC-1 for individuals with increased tolerance to high doses of electron-beam radiation. The two isolates chosen for further study, LH5 and LH7a, were derived from independently irradiated and derived cultures (Fig. 1). These strains were also phenotypically distinct. Strain LH5 had lost the gas vesicle production capability of the parent NRC-1, due to an IS element insertion into the *gvpJ* gene (data not shown) rendering colonies translucent, whereas strain LH7a retained the gas vesicle-positive phenotype (opaque colonies) of the parent strain, NRC-1.

### Characterization of Radiation Resistance in *Halobacterium* sp. NRC-1 and Radiation-Resistant Mutants LH5 and LH7a

To determine the extent of the radiation resistance acquired by the two mutants, exponential-phase cultures (OD<sub>600</sub> < 0.2) of these strains and the parent strain NRC-1 were irradiated with 18–20 MeV electrons from a standard medical LINAC over a range of doses. For comparison, we also measured the survival of *D. radiodurans*

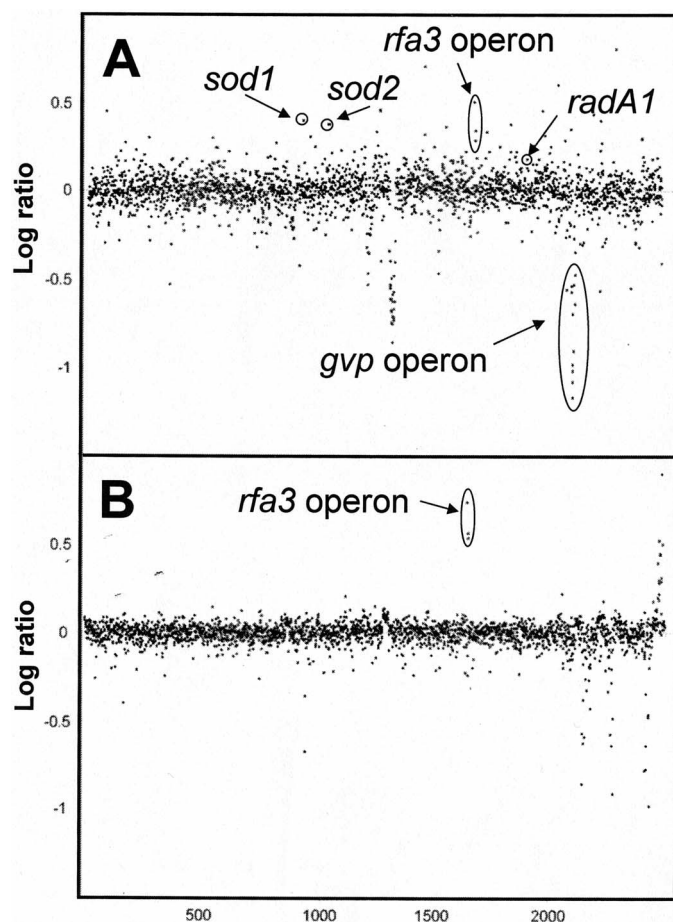
R1, one of the most radiation-resistant organisms known (28). Exponentially growing *Halobacterium* wild-type parent strain, NRC-1, had an LD<sub>50</sub> of 5.4 kGy (±0.2). The LD<sub>50</sub> of *D. radiodurans*, irradiated under the same conditions, was 7.9 kGy (±0.2). In contrast, the LD<sub>50</sub> for the *Halobacterium* isolate LH5 was 11.9 kGy (±0.4) and that of LH7a was 12.1 kGy (±0.3). Moreover, survivors of LH5 and LH7a were reproducibly detected at doses as high as 25 kGy, where there were few or no survivors of *D. radiodurans*. The graph showing surviving fractions and fitting of the data for the exponentially growing cultures is presented in Fig. 2.

Since microorganisms generally display greater resistance in stationary phase (29–31), each strain was also tested in stationary phase (Fig. 3). We confirmed the observation (20) that *Halobacterium* sp. NRC-1, unlike most other microbes, shows greater resistance when actively growing and tested the response of LH5 and LH7a at this growth density. As expected, the LD<sub>50</sub> of NRC-1 in the stationary phase was significantly less (2.9 kGy ± 0.2) than the value for the exponential-phase cultures (5.4 kGy). Both radiation-resistant isolates also showed decreased resistance when in the stationary phase. The LD<sub>50</sub> of LH5 decreased to 6.8 kGy (±0.3) and that of LH7a decreased to 8.7 kGy (±0.2).

One simple explanation for the increased radiation resistance of strains LH5 and LH7a would be reduced growth rate. Reduction in growth rate has been shown to increase survival of *D. radiodurans* after irradiation (29, 30). Given the radiation doses received by these *Halobacterium* cells, it is highly likely that each individual contains numerous mutations, some of which may affect the growth rate. To rule out the latter possibility, we verified that the doubling times of LH5 and LH7a were the same as that of the parent strain NRC-1 by measuring the OD<sub>600</sub> of cultures grown under standard conditions (data not shown).

### Transcriptional Analysis of Strains LH5 and LH7a

In principle, the molecular basis for increased radiation resistance of strains LH5 and LH7a may be attributed to one or more mutations in the genome. These mutations may result in increased activity of proteins involved in protection against and/or repair of radiation-induced damage. Alternatively, regulatory elements that govern expression of genes involved in protection or repair may be affected. To test for the latter scenario on a genome-wide scale, we used previously described *in situ* synthesized oligonucleotide arrays (10) to compare the transcriptomes of the mutant strains LH5 and LH7a to that of the wild-type strain NRC-1 under our standard conditions (see the Materials and Methods). In strain LH5, 47 and 72 genes out of 2474 ORFs (97% genome coverage) displayed more than a 1.5-fold (*P* < 0.01) increase or reduction, respectively, in transcript level compared to the wild type (Fig. 4; Table 2 in Supplementary Information). Most interestingly, genes in-



**FIG. 4.** Whole-genome microarray hybridization results comparing mutant strains LH5 (panel A) and LH7a (panel B) with the wild-type strain (NRC-1). For each ORF represented on the array, the logarithm<sub>10</sub> of the hybridization ratio of mutant cells (Cy5-labeled cDNA) to wild-type cells (Cy3-labeled cDNA) is displayed in black marks on the y axis. The location of ORFs within the entire 2.6-Mb genome maps is shown on the x axis. Expression ratios of selected genes are indicated.

involved in homologous recombination, e.g., *radA1* (archaeal homolog of *rad51/recA*) and *rfa3*, *rfa8*, *ral* (single-stranded DNA-binding protein complex), and protection against reactive oxygen species, e.g., *sod1* and *sod2* (Mn-dependent superoxide dismutases), were induced in LH5. As expected from the gas vesicle-deficient phenotype of LH5, the *gvp* (gas vesicle protein) genes (7) had much lower expression levels in that strain. In LH7a, 11 and 36 genes had transcript levels that were significantly increased or decreased, respectively (Fig. 4; Table 2 in Supplementary Information). Among those, *rfa3*, *rfa8* and *ral* were the only genes that had induced transcript levels in both LH5 and LH7a (2.1- to 3.7-fold and 3.4- to 5.5-fold, respectively; Table 2 in Supplementary Information).

#### Transcription and Promoter Analysis of the *rfa3* Operon

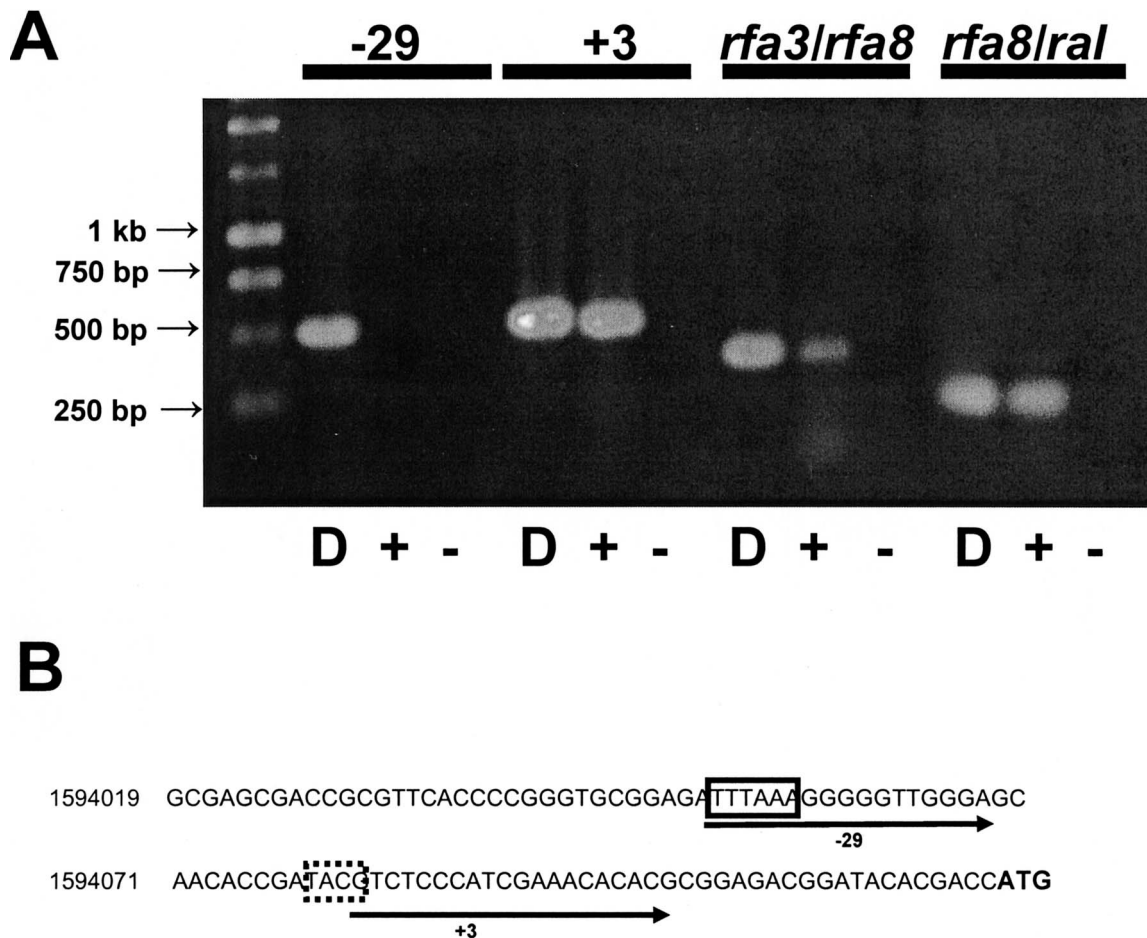
To gain further insight into the transcriptional organization and regulation of the *rfa3*, *rfa8* and *ral* gene region, we used RT-PCR analysis first to determine whether *rfa3*

and *rfa8* and *rfa8* and *ral* are transcriptionally linked and likely form an operon. Primer pairs flanking the two intergenic regions produced amplification products (Fig. 5A), confirming that transcription proceeds from *rfa3* through *rfa8* to *ral*, resulting in a polycistronic transcript. To determine the transcriptional start site, two primer pairs were designed, with one set (−29F and R) having one end 29 bp upstream of the predicted transcription start point and the other within the coding region of the gene, while the second set (+3F and R) had one end 3 bp within the predicted transcription start point and the other within the coding region of the gene (see Fig. 5B for the upstream primer positions). The results of RT-PCR showed that the second set (+3F and R) produced a PCR product, whereas the first set did not (−29F and R) (Fig. 5A). These results are consistent with the location of the transcription start point at ~40 bp upstream of the ATG start codon. Although this codon corresponds to the second ATG in the predicted *rfa3* ORF from genome annotation (13), subsequent alignment of *rfa3* orthologs (data not shown) in other archaea showed it to be consistent with the translation start. A classical TATA-box motif (TTTAAA) was found centered about 25 bp upstream of the putative transcriptional start site mapped (Fig. 5B).

## DISCUSSION

We have examined the ability of the model haloarchaeon *Halobacterium* sp. NRC-1 and its mutants to tolerate ionizing radiation and addressed the mechanism of the increased radiation resistance of two derivatives, LH5 and LH7a, using DNA microarrays. Our results indicate that these organisms are extremely resistant to ionizing radiation, most likely reflecting their natural environment, where frequent exposure to intense solar radiation and prolonged exposure to desiccation would have similar DNA-damaging effects. Although the degree of resistance of the wild-type *Halobacterium* sp. NRC-1 is slightly lower than that of some of the most radiation-resistant microorganisms known, such as *D. radiodurans*, its LH5 and LH7a mutant derivatives are even more highly resistant than any known natural isolates, including the thermophilic species *Rubrobacter radiotolerans* (32). The mechanism of acquisition of increased radiation resistance was apparent from transcriptomic analysis of the LH5 and LH7a mutants compared to the NRC-1 wild-type parent. Coincidence of induction of the *rfa3* operon in two independent radiation-resistant mutants strongly suggested that the single-stranded DNA-binding protein RPA is critical to the phenotype.

The naturally high radiation resistance of *Halobacterium* may reflect the high salinity of the growth medium. The standard growth medium for *D. radiodurans* lacks the protective salt concentrations necessary for growth of the halophile *Halobacterium* (33). Comparisons in identical medium were impossible, because the two microorganisms cannot coexist in a single medium. Interestingly, many of



**FIG. 5.** Analysis of *rfa3* operon in *Halobacterium* sp. NRC-1. Panel A: Agarose gel electrophoresis of reverse transcriptase-PCR assays with primers targeting upstream regions of the predicted transcriptional start site, -29 and +3, as well as intergenic regions of *rfa3* and *rfa8* and *rfa8* and *ral*, respectively. “D” stands for genomic DNA as template, “+” for assays with RNA as template and conducted with reverse transcriptase, and “-” for assays with RNA as template conducted without reverse transcriptase. Sizes of products were as predicted (for -29, 506 bp; for +3, 541 bp; for *rfa3/rfa8*, 416 bp; for *rfa8/ral*, 268 bp). Panel B: Upstream region of *rfa3* in *Halobacterium* sp. NRC-1. Box with solid line, TATA box; box with broken line, haloarchaeal transcriptional start site consensus [(T/C)(A/G)NG]; arrows, positions of primers -29F and +3F; start codon is in bold. Absolute nucleotide positions are given.

the high-dose survivors of the *Halobacterium* isolates displayed a significant delay in growth (not shown) and required extended incubation times to reach macroscopic colony formation size. Upon reisolation, however, these survivors formed colonies at the same rate as unirradiated controls, and there was no evidence of long-term postirradiation growth delay. This phenomenon was not observed for the irradiated *D. radiodurans* cultures.

Microorganisms generally display increased sensitivity to radiation when actively growing. The lower level of resistance during exponential phase has been attributed to saturation of the repair machinery during rapid growth (29). Although most microbial species tested, including *D. radiodurans*, have been reported to show higher resistance during stationary phase, there are examples of the reverse situation (30, 31). Recently, *Halobacterium* NRC-1 has been reported to show maximum resistance to  $\gamma$  radiation when in the exponential phase (20). We confirmed this ob-

servation for NRC-1 with electrons and demonstrated that the same is true for the enhanced isolates.

Although reduction in growth rate has been shown to increase survival of *D. radiodurans* after irradiation (29, 30), we ruled out this explanation for increased radiation resistance of strains LH5 and LH7a by verifying doubling times. Moreover, as shown, greater resistance is observed in exponentially growing *Halobacterium* cells, both wild-type and mutants, than in stationary-phase cells.

Our LD<sub>50</sub> for stationary-phase *Halobacterium* NRC-1 (2.9 kGy) was slightly lower than that estimated from the data of Kotteman *et al.* (20) who used a <sup>60</sup>Co  $\gamma$ -radiation source (approximately 3.3 kGy). This may reflect the differences in radiation types (electron beam and  $\gamma$  rays), dose rates (30 Gy/s and 121 Gy/s), radiation delivery (pulsed and continuous), uncertainty in dosimetry, and culture conditions (such as the oxygenation state of the saturated cultures).

Comparison of the transcriptome of wild-type strain NRC-1 to the LH5 and LH7a mutants using DNA microarrays and further characterization by RT-PCR showed that an operon containing three genes, *rfa3*, *rfa8* and *ral*, was up-regulated in both mutant strains. The predicted proteins encoded by the *rfa3* and *rfa8* genes (34 kDa and 21 kDa) are homologs to the family of single-strand DNA-binding proteins (COG1599 and 3390). The encoded proteins may form, together with the *ral* gene product (15 kDa), the large, medium and small subunits of a protein complex similar to the eukaryotic single-strand DNA-binding protein RPA (which contains 70-, 32- and 14-kDa subunits) (34). Given the wide-ranging roles played by RPA in DNA repair (nucleotide excision, base excision, mismatch and double-strand break repair), as well as in DNA replication and recombination, induction of these genes may be highly relevant to almost all aspects of DNA metabolism in our isolated mutants (35, 36).

The closely related archaeal and eukaryotic proteins are designated replication protein A (RPA) while the bacterial proteins are termed SSB (for single-strand DNA-binding protein) (35). The homologous SSB protein from *D. radiodurans* has recently been crystallized and its three-dimensional structure determined (37). This protein is present under nonstressed conditions at levels approximately tenfold higher than the homologous protein in *E. coli*. Exposure to ionizing radiation increases the level almost fourfold. This increase correlates with our observation of increased inherent levels of expression from the *rfa3* operon in LH5 and LH7a and suggests a common mechanism for resistance to high levels of DNA damage through increased stabilization of exposed single-strand DNA. The genome of *D. radiodurans* has recently been shown to reassemble after radiation damage through a mechanism involving DNA replication and homologous recombination, processes that generate extensive regions of single-strand DNA requiring protection by SSB (38). Regulation of levels of these ubiquitous proteins may be a general survival mechanism in cells from diverse organisms.

Structural analyses have shown that all RPAs and SSBs contain at least one conserved nonspecific single-strand DNA-binding domain, called an oligonucleotide/oligosaccharide-binding (OB) fold (34–37). The Rfa3 protein of *Halobacterium* contains one OB fold (positioned at <sup>84</sup>VDI-TSI<sup>157</sup>) and also one nucleic acid-binding Zn-finger motif (<sup>189</sup>RCP- HGE<sup>209</sup>). The Rfa8 protein contains one OB fold (<sup>48</sup>VFV-ITM<sup>130</sup>), and the Ral protein contains a coiled-coil region (<sup>74</sup>SKS-TDS<sup>111</sup>). Orthologous genes (66–70% amino acid sequence identity) were found in the genomes of the related haloarchaea *Haloarcula marismortui* and *Haloferax volcanii*. Similar operons are also present in members of the euryarchaeal families Thermococcales and Methanosarcinales (39, 40). However, there is no significant sequence similarity of the small subunit proteins from members of the three families.

In a previous study, the *rfa3* operon was also found to

be induced after irradiation of the *Halobacterium* sp. NRC-1 wild-type strain with UVC light (12). This observation, combined with the striking up-regulation of this key protein reported here in two independently isolated mutant strains in the absence of inducing doses of radiation, indicates that increased transcript levels of the *rfa3* operon are advantageous during repair of both non-ionizing and ionizing radiation-induced DNA damage in *Halobacterium*. The proteins encoded by this operon may therefore be involved in the recognition or resolution of a common damage repair intermediate.

The role of the RPA protein in archaea DNA repair is of significant interest. Our findings, and those of Cubeddu and White using the crenarcheota, *Sulfolobus solfataricus*, suggest that regulation of these proteins is important for DNA repair in archaea (41). However, previous work describing mutants with increased radiation resistance in *E. coli* and *Salmonella* did not show similar effects in these bacteria (22, 23). In *Saccharomyces cerevisiae*, of the numerous mutations involved in recombination, replication and repair, including many in the RPA genes, none has been shown to increase the inherent resistance in this organism. Therefore, diverse mechanisms may be at play and will require further analysis for fuller understanding. By further increasing the doses to these resistant strains, we may be able to determine the biological “limit” to the amount of radiation to which cells can adapt. Given the similarity between the archaeal and eukaryotic DNA repair systems, this information may be relevant to our understanding of human cell response to high doses of radiation, such as occurs during cancer therapy, and allow intervention strategies to be developed.

#### SUPPLEMENTARY INFORMATION

Supplementary Table 1: Electron-beam doses delivered and mean survival of *Halobacterium* strains and *Deinococcus radiodurans*: <http://dx.doi.org/10.1667/RR0935.1.s1>.

Supplementary Table 2: Log ratios and *P* values for gene expression comparisons between two mutant *Halobacterium* strains (LH5 and LH7a) and the parent strain NRC-1: <http://dx.doi.org/10.1667/RR0935.1.s2>.

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