

Radiation Desiccation Response Motif-Like Sequences Are Involved in Transcriptional Activation of the Deinococcal *ssb* Gene by Ionizing Radiation but Not by Desiccation[∇]

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Single-stranded-DNA binding protein (SSB) levels during poststress recovery of *Deinococcus radiodurans* were significantly enhanced by ⁶⁰Co gamma rays or mitomycin C treatment but not by exposure to UV rays, hydrogen peroxide (H₂O₂), or desiccation. Addition of rifampin prior to postirradiation recovery blocked such induction. *In silico* analysis of the *ssb* promoter region revealed a 17-bp palindromic radiation/desiccation response motif (RDRM1) at bp –114 to –98 and a somewhat similar sequence (RDRM2) at bp –213 to –197, upstream of the *ssb* open reading frame. Involvement of these *cis* elements in radiation-responsive *ssb* gene expression was assessed by constructing transcriptional fusions of edited versions of the *ssb* promoter region with a nonspecific acid phosphatase encoding reporter gene, *phoN*. Recombinant *D. radiodurans* strains carrying such constructs clearly revealed (i) transcriptional induction of the *ssb* promoter upon irradiation and mitomycin C treatment but not upon UV or H₂O₂ treatment and (ii) involvement of both RDRM-like sequences in such activation of SSB expression, in an additive manner.

Tolerance to high doses of ionizing radiation (X rays and gamma rays), which cause extensive and lethal DNA damage, is rare among life forms. However, species of the Gram-positive, pink-orange aerobic bacterium *Deinococcus* have been known to survive exposure to extremely high doses of ionizing radiation, UV rays, mitomycin C, desiccation, and other DNA-damaging agents (4, 30). Members of the family *Deinococcaceae*, comprising over 30 species, inhabit diverse environments and survive 5 kGy of ⁶⁰Co gamma rays without any loss of viability (31). Some of the strains are known to survive doses as high as even 15 kGy or more (13). The phenomena underlying such extremophilic behavior of deinococci, though not entirely clear, fall into three major categories. These are (i) a unique condensed organization of the genome, which minimizes DNA damage and facilitates rapid postirradiation repair (9, 11, 23, 43); (ii) highly proficient, regular, and novel DNA repair mechanisms (6, 8) aided by proteins unique to deinococci (6, 38); and (iii) very capable enzymatic/nonenzymatic cleaning systems to scavenge reactive oxygen species (ROS) and protect proteins from oxidative damage (9, 10) or to degrade and resynthesize damaged proteins (22) in order to quickly alleviate the radiation toxicity and restore cellular homeostasis. Consequently, the conventional enzymes responsible for postirradiation recovery (PIR) survive and function with far better efficiency in deinococci (26). New mechanisms continue to be elaborated (20, 22, 42).

Genomes of at least three highly radioresistant *Deinococcus* spp. have already been completely sequenced (14, 26, 39).

These species are (i) *D. radiodurans*, the species of the first prototype strain isolated from irradiated meat cans 50 years ago and the most studied so far (1); (ii) the moderately thermophilic *D. geothermalis*, isolated from a hot spring in Italy (17); and (iii) the most recent, *D. deserti*, obtained from surface sands of the Sahara desert (13). *In silico* analyses of the genomes of these species have defined a minimal set of genes required for extreme radiation/desiccation resistance (RDR) in the genus *Deinococcus* (14, 26). Comparative genomics supplemented by microarray and proteomic analyses have revealed enhanced expression of several genes in *Deinococcus* spp. immediately following irradiation (24, 25, 38). As expected, this set is dominated by genes related to DNA end protection, replication, recombination, and repair, but it also includes genes encoding chaperones, proteases, RNA binding proteins, RNA ligases, transcription factors, membrane transporters, Krebs cycle enzymes, transposases, superoxide dismutases/thioredoxin/peroxidases, and even tellurium resistance and plant LEA-like proteins (5, 10, 14, 19, 20, 25, 26, 29, 32, 38).

Mechanisms which facilitate radiation-induced gene expression have been investigated for a very few selected genes (such as *recA*) in deinococci and remain poorly understood. *D. radiodurans recA* expression is induced by the PprI protein, which, however, does not bind the *recA* promoter (15, 18, 21). LexA, the well-known repressor of *recA* expression in most bacteria, is not induced upon irradiation in *D. radiodurans* and does not regulate postirradiation *recA* induction (33). In most cases, the radiation-enhanced gene expression appears to be due to transcriptional activation (24, 25), but the corresponding regulatory *cis* elements and *trans*-acting proteins have remained largely unexplored so far. Critical examination of deinococcal genomes recently identified a 17-bp palindromic sequence (T, T, A/C, T/C, G, T/C, N, N, T/A, N, A, A/G, C,

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TABLE 1. Plasmids and primers used in the study

Plasmid or primer	Description ^a	Reference, source, or comment
Plasmids		
pRAD1	<i>E. coli</i> - <i>D. radiodurans</i> shuttle vector (copy no., 7–10 per cell in <i>D. radiodurans</i>)	28
pRN1	pRAD1 with <i>phoN</i> cloned at NdeI and BamHI sites; Cb ^r Cm ^r	Lab collection
pTZ57R/T	PCR product cloning vector (2,886 bp, Ap ^r) for blue-white selection of transformants	MBI Fermentas
pTAPssb102	102-bp sequence upstream of <i>D. radiodurans</i> <i>ssb</i> cloned in pTZ57R/T	This study
pSN2	pRN1 with 112-bp fragment from pTAPssb102 subcloned at NruI and NdeI sites	This study
pSN3	pRN1 with 132-bp sequence upstream of <i>D. radiodurans</i> <i>ssb</i> ORF cloned at XbaI and NdeI sites	This study
pSN4	pRN1 with 351-bp sequence upstream of <i>D. radiodurans</i> <i>ssb</i> ORF cloned at XbaI and NdeI sites	This study
Primers		
P1	5'-CCGAGAAGGATTACAATCTAGAACG-3'	XbaI
P2	5'-CGGAGTGGGAAGATCTAGAAAGGCCTG-3'	XbaI
P3	5'-GCGAGCTCATAATTGACTCTGCTTGTACTATCTAGTG-3'	SacI
P4	5'-CATGCCTCGGGCCATATGAAATTC-3'	NdeI
P5	5'-GGAGCGGATAACAATTTACACA-3'	P5 and P6 flank multiple-cloning site, 58 bp on either side in pRad1
P6	5'-AACGCGGCTGCAAGAATGGTA-3'	

^a The underlined sequence corresponds to the restriction site incorporated in the primer.

G/A, T/G, A, A) upstream of several radiation-induced genes in all three *Deinococcus* spp. (14, 26). The most conserved nucleotides are shown in bold, while the center of dyad symmetry lies around the ninth nucleotide, which is T/A. The sequence, first described by Makarova et al. as a radiation/desiccation response motif (RDRM), was found upstream of 29 genes in *D. radiodurans* and 25 genes in *D. geothermalis* (26). Subsequently, the motif has also been reported upstream of 25 genes in *D. deserti* by de Groot et al., (14), who have preferred to describe it as radiation response motif (RRM). The radiation and desiccation resistance (RDR) regulon common to all three *Deinococcus* spp. thus includes about 25 genes, all of which possess RDRM sequences upstream of their open reading frames (ORFs). Prominent genes/operons in this set include several DNA replication/recombination/repair-related genes, such as *ssb*, *gyrA* and *gyrB*, *cinA/ligT/recA*, *uvrA*, *uvrB* and *uvrD*, *mutS*, *recQ*, *ruvB*, *pprA*, *ddrA*, *ddrB*, *ddrD*, and others (6, 14, 26). In *D. radiodurans* and in *D. deserti*, for which transcriptome data are available, all the genes of the RDR are also upregulated during postirradiation recovery (14). Strangely, however, the involvement of RDRM sequences *per se* in desiccation/radiation induction of deinococcal genes has never been verified experimentally.

The present study assessed the role of RDRM-like sequences in radiation-induced gene expression, taking the single-stranded-DNA binding protein (SSB)-encoding gene, *ssb*, as a test case. The *ssb* gene in *D. radiodurans* harbors two RDRM-like sequences, RDRM1 and RDRM2, located between bp -114 and -98 and bp -213 and -197, respectively (16, 39; <http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gdr>). Using truncated versions of the *ssb* promoter carrying none, one, or both of the RDRM-like sequences fused to the reporter gene *phoN*, we have demonstrated that both RDRM sequences are involved in radiation-induced *ssb* expression, in an additive way. Our data also show that desiccation or exposure to UV or hydrogen peroxide treatment does not influence *ssb* expression in *D. radiodurans*.

MATERIALS AND METHODS

Bacterial strains and growth condition. *Deinococcus radiodurans* strain R1 was maintained in TGY (1% tryptone, 0.1% glucose, 0.5% yeast extract) medium at 32°C under agitation at 150 rpm. *Escherichia coli* strain DH5α [F⁻ *recA41 endA1 gyrA96 thi-1 hsdR17* (r_K⁻ m_K⁺) *supE44 relA lacU169*] was maintained in Luria-Bertani (LB) medium at 37°C under agitation at 150 rpm. Bacterial growth was measured spectrophotometrically as turbidity (optical density at 600 nm [OD₆₀₀]). The antibiotics used for selection of recombinants were chloramphenicol (3 μg ml⁻¹) for *D. radiodurans* and carbenicillin (100 μg ml⁻¹) for *E. coli* DH5α.

PCR amplification, cloning, and transformation. The pRN1 vector, generated previously in our laboratory, contains a promoterless *phoN* gene (Table 1) and was used for cloning of all the putative promoter fragments. Genomic DNA isolation from *D. radiodurans* and plasmid isolation from *E. coli* were carried out as described previously (28, 37). The primers used for PCR were designed based on the corrected published sequences of the *ssb* gene (16) and the *Deinococcus* genome (39; <http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gdr>). All PCR products were purified using a QiaexII gel extraction kit (Qiagen, Hilden, Germany). For construction of plasmids pSN3 and pSN4, the 132-bp and 351-bp DNA sequences upstream of the *ssb* open reading frame (ORF) (see Fig. 3b and c) were PCR amplified using primers shown in Table 1. The PCR products were gel purified, restriction digested with XbaI-NdeI, and ligated at same sites in pRN1. Plasmid pSN2 was generated in two steps. First, 102 bp of DNA upstream of the *ssb* ORF was PCR amplified using primers P3 and P4 and ligated in the pTZ57R/T PCR product cloning vector (Table 1), yielding pTAPssb102. Next, plasmid pTAPssb102 was restriction digested with SmaI-NdeI to release the 112-bp insert, which was gel purified and ligated to pRN1 at NruI-NdeI sites to obtain pSN2. All the plasmid constructs were transformed in *E. coli* DH5α cells and screened on plates containing carbenicillin (100 μg ml⁻¹). Plasmids isolated from *E. coli* DH5α were used to transform *D. radiodurans* cells as described earlier (28). All recombinant clones were also screened by colony PCR and confirmed by sequencing using primers P5 and P6 (Table 1). Deinococcal transformants expressing the *phoN* reporter gene were screened on histochemical plates (2, 36) containing phenolphthalein diphosphate (PDP) (1 mg ml⁻¹), methyl green (MG) (10 μg ml⁻¹), and chloramphenicol (Cm) (3 μg ml⁻¹). The PhoN-positive colonies exhibited a green color.

Treatment with various stresses. Wild-type and recombinant *D. radiodurans* cells (carrying plasmid pSN2, pSN3, pSN4, or pRN1) were grown overnight at 32°C to an OD₆₀₀ of 4 to 5/ml and subjected to one of the following stress treatments: (i) 2 to 8 kGy of gamma radiation from a ⁶⁰Co source (GC220; Atomic Energy of Canada Limited, Canada) (0.36 kGy h⁻¹), (ii) 20 μg ml⁻¹ of mitomycin C (Sigma, St. Louis, MO) for 15 min at 32°C, (iii) 50 or 100 mM hydrogen peroxide (Ranchem, New Delhi, India) for 1 h at 32°C, or (iv) 2.5 or 5 kJ m⁻² of UV (Philips) (5 J m⁻² s⁻¹). UV stress was applied to 4 ml of culture

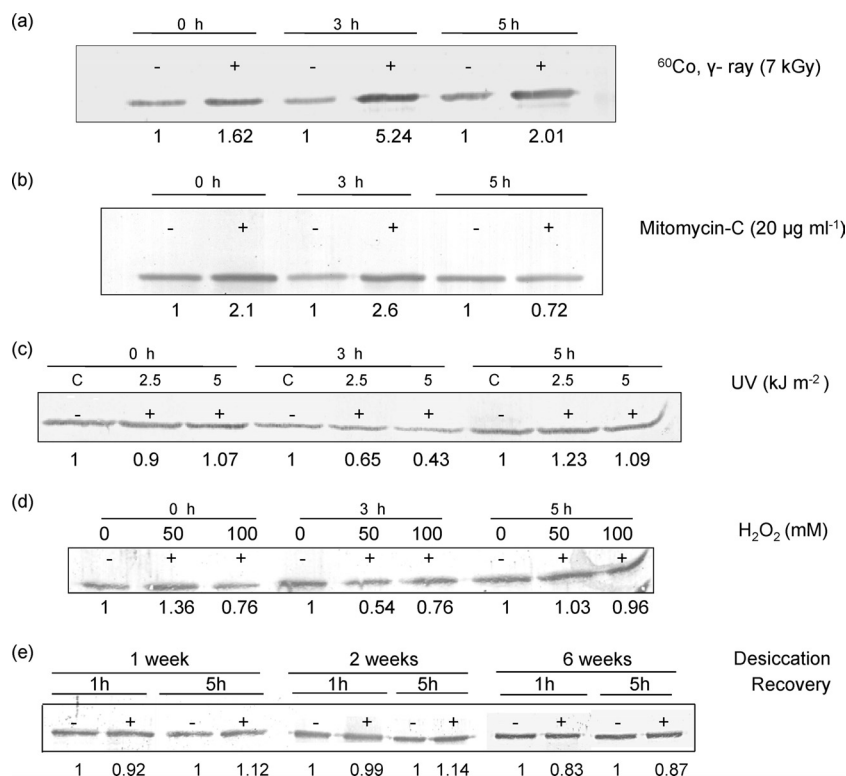


FIG. 1. Effect of DNA-damaging agents on deinococcal SSB levels. Stationary-phase deinococcal cells were exposed to the specified dose or concentration of either gamma rays (a), mitomycin C for 15 min (b), UV rays (c), or H_2O_2 for 1 h (d) or were dehydrated in a desiccator using fused calcium chloride as desiccant for the specified duration (e). After the stress, cells were allowed to recover in TGY medium, and proteins were extracted at the specified time points during poststress recovery. Equal amounts of proteins (30 μg) were electrophoretically resolved by 12% SDS-PAGE and electroblotted on nitrocellulose membrane. SSB levels in stressed (+) and unstressed (-) samples were immunodetected using anti-*D. radiodurans* SSB antibody and quantitated. The numbers below the lanes depict quantitation of SSB levels by densitometry.

spread in a 9-cm sterile petri plate to form a <1-mm layer and to minimize absorption by the TGY medium. For desiccation stress, *D. radiodurans* cells grown overnight were filtered and subjected to 1, 2, or 6 weeks of desiccation using fused calcium chloride in desiccators stored at 23°C. Following stress treatments, cells were washed three times with fresh TGY medium, inoculated in TGY at a final OD_{600} of 0.5, and allowed to recover. Periodically, samples were removed for various analyses and either used directly for biochemical assays or snap-frozen in liquid nitrogen and stored at -70°C for Western blots or zymograms.

Western blotting and immunodetection of SSB. For assessment of transcriptional activation of *ssb* expression, wild-type *D. radiodurans* cells grown overnight ($\text{OD}_{600} = 5 \text{ ml}^{-1}$) were divided into two sets. To one set, rifampin (50 $\mu\text{g ml}^{-1}$) was added prior to gamma radiation of 7 kGy and washed off before PIR; to the second set, rifampin was added after irradiation but before PIR of cells. An additional treatment had TGY medium with rifampin (50 $\mu\text{g ml}^{-1}$) which was irradiated separately and added during PIR of irradiated cells. All treatments had their corresponding unirradiated controls.

For quantitation of SSB levels, proteins were extracted from wild-type *D. radiodurans* after 3 h of poststress recovery. Protein extracts (30 to 100 μg) from control or stressed samples were resolved by 12% SDS-PAGE and electroblotted on a nitrocellulose membrane. The primary antibody used was anti-*D. radiodurans* SSB antibody at a 1:200 dilution and incubated overnight, while the secondary antibody used was anti-rabbit IgG (Sigma, St. Louis, MO) coupled to alkaline phosphatase at a 1:10,000 dilution and incubated for 1.5 to 2 h. The blot was developed using the substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Biochemicals, Germany).

Assays for reporter gene activity. *phoN* was used as a reporter gene for the first time in this study. PhoN is a periplasmic nonspecific acid phosphatase that cleaves organic phosphate to release phosphoric acid, optimally at pH 5 to 7 (7). PhoN activity expressed from the *Pssb* promoter was monitored in the following two ways: (i) by zymogram analyses of stressed cells after 3 h of PIR, as described earlier (2, 36), with activity staining of PhoN protein on zymograms carried out

using NBT-BCIP and (ii) by spectrophotometric analysis of cell-bound PhoN activity as *p*-nitrophenol (pNP) released from *p*-nitrophenylphosphate (pNPP) (Sisco Research Laboratories, Mumbai, India) at 405 nm. Acid phosphatase enzyme activity of cells is reported as nmol of pNP liberated $\text{min}^{-1} \text{OD}_{600} \text{ unit}^{-1}$ (7).

RESULTS

Effects of DNA-damaging agents on deinococcal *ssb* expression. *D. radiodurans* cells exposed to gamma rays (7 kGy) or mitomycin C (20 $\mu\text{g ml}^{-1}$ for 15 min) showed significant induction of immunodetectable SSB levels on Western blots during poststress recovery (Fig. 1a and 1b). The increase in SSB levels displayed a time dependence, with maximum enhancement observed at 3 h of poststress recovery following exposure to both stressors (Fig. 1a and b). Exposure to high doses of UV rays (2.5 to 5 kJ m^{-2}) or hydrogen peroxide (50 to 100 mM) did not influence cellular SSB levels in *D. radiodurans* (Fig. 1c and d). Prolonged desiccation of 1 to 6 weeks also did not affect SSB levels (Fig. 1e).

Effect of gamma irradiation on *ssb* expression in *D. radiodurans*. A distinct radiation dose-dependent increase in SSB levels, compared to those in the unirradiated controls, was observed during postirradiation recovery in *D. radiodurans*, resulting in 6.01-fold-higher SSB levels at a 7-kGy dose after 3 h of postirradiation recovery (PIR) (Fig. 2a). The observed radiation-induced SSB enhancement at 7 kGy was abolished if rifampin was added during PIR (Fig. 2b, lanes 4 and 5) but was

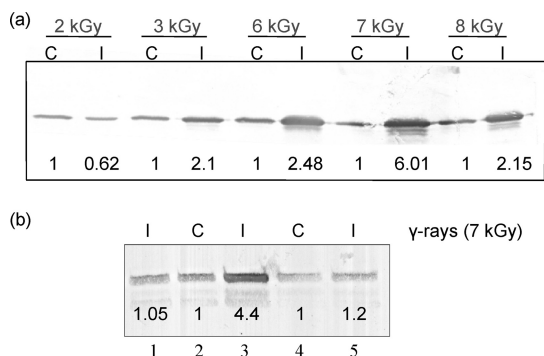


FIG. 2. Effect of ionizing radiation on deinococcal SSB levels. (a) Stationary-phase *D. radiodurans* cells were exposed to specified dose of ^{60}Co gamma rays. (b) Cells were irradiated (7 kGy) in the presence of $50\ \mu\text{g ml}^{-1}$ of rifampin, which was either added during irradiation and washed off before recovery (lanes 2 and 3), added during PIR (lanes 4 and 5), or irradiated (7 kGy) (I) and added during PIR (lane 1), and compared with unirradiated control (C) cells. Posttreatment cells were allowed to recover in TGY medium for 3 h. Protein extracts (100- μg blot [a] or 30- μg blot [b]) were electrophoretically resolved. Other details were as described for Fig. 1.

seen in cells irradiated in the presence of rifampin but recovered in the absence of transcriptional inhibitor (Fig. 2b, lane 3). Addition of irradiated rifampin during PIR also inhibited the radiation-induced SSB enhancement (Fig. 2b, lane 1), indicating that irradiation did not inactivate rifampin.

Bioinformatic analysis of DNA sequence upstream of the deinococcal *ssb* gene. The *ssb* gene in *D. radiodurans* is located 119 bp downstream of the annotated *rpsF* gene and is followed

by the *rpsR* gene, located 56 bp downstream of *ssb* (Fig. 3a). *In silico* analysis of the 351-bp region upstream of the *ssb* ORF using BROM software (Softberry) predicted the putative -10 and -35 promoter-like sequences, while a manual sequence search revealed the Shine Dalgarno (SD) sequence upstream of the *ssb* ORF (Fig. 3b). A 17-bp predicted conserved radiation/desiccation response motif ($5'$ -TTATGTCAT TGACATAA- $3'$) was observed between bp -114 and -98 (RDRM1), and a not-as-conserved but similar motif ($5'$ -AAC CGCCATCGCCAGCA- $3'$) was observed between bp -213 and -197 (RDRM2) upstream of the start of the *ssb* ORF (Fig. 3b). This places RDRM1 in the intergenic region between the *rpsF* and *ssb* genes and RDRM2 within the *rpsF* gene, 74 bp upstream of the stop codon (Fig. 3b and c).

Construction of *Pssb-phoN* transcriptional fusions. In order to assess the possible role played by the two RDRM-like sequences in regulation of *ssb* expression, transcriptional fusions of RDRM carrying DNA sequences were constructed with the *phoN* gene (Fig. 3c), which encodes a nonspecific acid phosphatase. For this, we used an existing plasmid, pRN1 (Table 1). Specified lengths of *ssb* promoter regions were cloned in pRN1 (as described in Materials and Methods) to generate recombinant plasmids pSN2, pSN3, and pSN4 (Fig. 3c; Table 1). The plasmid pSN4 carried both RDRM1 and RDRM2, pSN3 carried only RDRM1, and pSN2 carried only the last 5 bases of the RDRM1 sequence (Fig. 3b and c).

Assessment of reporter (PhoN) activity expressed from the deinococcal *ssb* promoter. Differences among various clones were visualized using activity staining on zymograms. In-gel enzyme activity of PhoN in clone pSN4 showed strong induc-

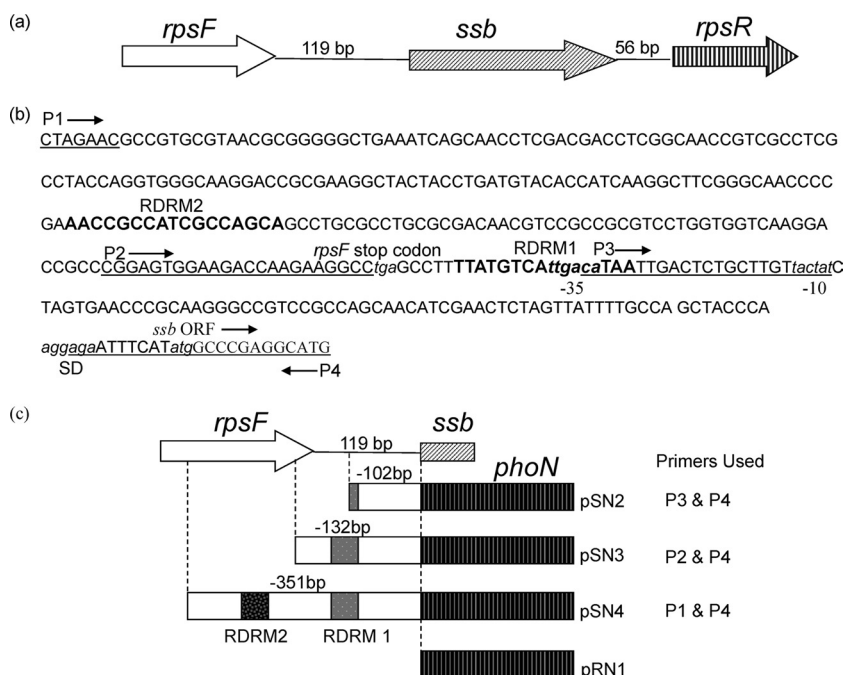


FIG. 3. Organization of the *ssb* gene in *D. radiodurans* and various constructs. (a) Genomic location of the *ssb* gene in *D. radiodurans*. (b) Nucleotide sequence of the putative promoter region of *ssb* from the *D. radiodurans* genome, showing various *cis* elements. RDRM1 and RDRM2 sequences are shown in bold. A putative SD sequence, -10 - and -35 -like sequences, the *rpsF* stop codon, and the *ssb* start codon are italicized and labeled. Primer sequences are underlined. The first codon of the *D. radiodurans* *ssb* ORF is shown. (c) Schematic representation of various *Pssb-phoN* transcriptional fusions and the primers used.

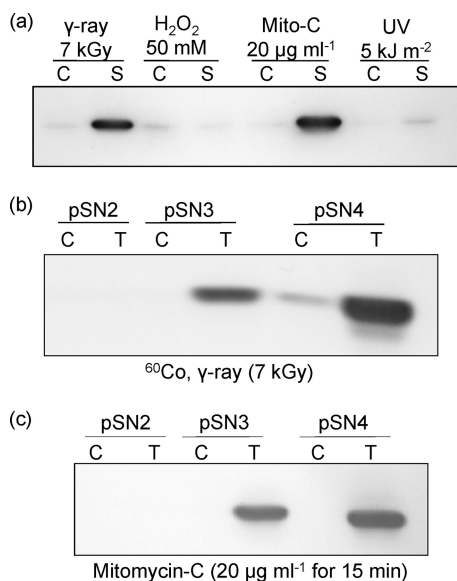


FIG. 4. Zymogram analysis of PhoN reporter activity expressed from the deinococcal *ssb* promoter during poststress recovery. (a) The recombinant *D. radiodurans* strain carrying the construct pSN4 was exposed to different stresses and allowed to recover in fresh TGY medium for 3 h under the usual growth conditions. Proteins were extracted in nonreducing buffer and electrophoretically resolved by 12% SDS-PAGE. Activity bands were developed by incubating the gel with NBT-BCIP in acetate buffer. (b and c) Recombinant *D. radiodurans* strains carrying either pSN2, pSN3, or pSN4 were exposed to 7 kGy of ⁶⁰Co gamma rays or 20 µg ml⁻¹ of mitomycin C for 15 min. Other details were as described for panel a.

tion of the reporter gene after exposure of cells to gamma rays or mitomycin C but not upon exposure to UV or H₂O₂ stress (Fig. 4a). The gamma ray/mitomycin C induction of various *Pssb-phoN* constructs was rigorously assessed further (Fig. 4b

and c). For both the stresses, the highest in-gel enzyme activity was observed in clone pSN4, followed by clone pSN3, while only basal-level activity was seen in clone pSN2 (Fig. 4b and c). The relative induction of *Pssb*-driven PhoN expression in various clones was further confirmed and quantitated by spectrophotometric analysis of PhoN enzyme activity, using pNPP as the substrate (Fig. 5). The reporter gene activity (PhoN) in both clones pSN3 and pSN4 increased steadily with time. For both gamma rays and mitomycin C, clone pSN4 cells showed much higher activity (~6- to 8-fold after 5 h) than pSN3 (~4- to 5-fold after 5 h) when equivalent cells were tested (Fig. 5). Clone pSN2 showed only basal PhoN activity and did not respond to irradiation or mitomycin C treatment.

DISCUSSION

The single-stranded-DNA binding protein (SSB), in conjunction with the RecA protein, is involved in all important DNA-related cellular activities, such as replication, transcription, recombination, and repair, in bacteria. Several earlier studies have shown that RecA is normally expressed at a low basal level in *D. radiodurans* but that its levels significantly increase during recovery from radiation stress (12, 25). The present data reveal that SSB levels in *D. radiodurans* follow a similar kinetics and are induced by irradiation in a time-dependent (Fig. 1a) and dose-dependent (Fig. 2a) manner. Such enhanced SSB levels during PIR are attained by transcriptional activation of the *ssb* gene. This is borne out by the facts that (i) addition of the bacterial transcription inhibitor rifampin before commencement of PIR blocks such enhancement, (ii) cells irradiated in the presence of rifampin but recovered after washing off of rifampin show enhanced SSB levels, and (iii) irradiation does not inactivate rifampin and addition of irradi-

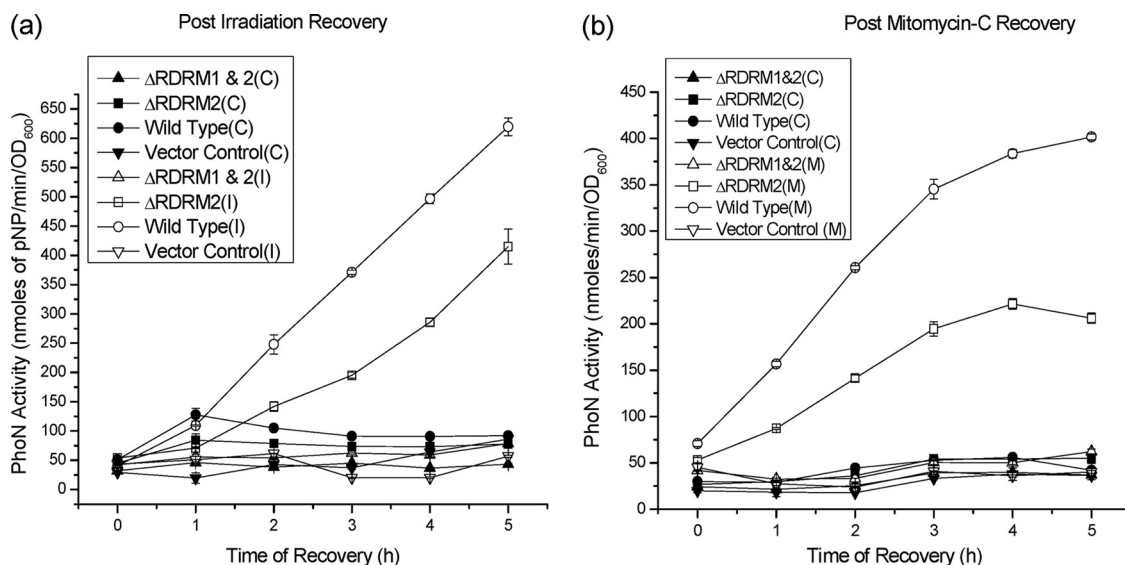


FIG. 5. Biochemical assays of PhoN reporter activity expressed from the deinococcal *ssb* promoter during poststress recovery. Recombinant *D. radiodurans* strains carrying plasmid pSN2 (Δ RDRM1 and Δ RDRM2), pSN3 (Δ RDRM2), pSN4 (wild type), or pRN1 (vector control) were exposed to either 7 kGy of ⁶⁰Co gamma rays (a) or 20 µg ml⁻¹ mitomycin C for 15 min (b). After the stress, cells were washed and allowed to recover in TGY for 5 h. Aliquots of 100 µl were removed periodically for cell-based acid phosphatase assays using pNPP as the substrate. PhoN activity in untreated control (C), irradiated (I), or mitomycin C-treated (M) cells during recovery is shown.

Comparative genomics had very accurately predicted the possible involvement of RDRM-like sequences in radiation/desiccation-responsive expression of several genes in the three deinococcal species studied so far (6, 14, 26), though such a role had never been experimentally verified. The present work has revealed the importance and involvement of RDRM-like sequences in radiation-induced *ssb* gene expression and its insensitivity to desiccation in *D. radiodurans*. In the future, if this feature is found to be common to other genes/operons which harbor upstream RDRM-like sequences, it may be more prudent to rename this regulatory element the radiation response motif (RRM), instead of using the name RDRM given earlier.

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