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Characterization of the role of the RadS/RadR two-component system in the radiation resistance of *Deinococcus radiodurans*

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Deinococcus radiodurans shows extraordinary tolerance to DNA damage, and exhibits differential gene expression and protein recycling. A putative response regulator, the DRB0091 (RadR) ORF, was identified from a pool of DNA-binding proteins induced in response to gamma radiation in this bacterium. *radR* is located upstream of *drB0090*, which encodes a putative sensor histidine kinase (RadS) on the megaplasmid. Deletion of these genes both individually and together resulted in hypersensitivity to DNA-damaging agents and a delayed or altered double-strand break repair. A Δ*radRradS* double mutant and a Δ*radR* single mutant showed nearly identical responses to gamma radiation and UVC. Wild-type RadR and RadS complemented the corresponding mutant strains, but also exhibited significant cross-complementation, albeit at lower doses of gamma radiation. The *radS* transcript was not detected in the Δ*radR* mutant, suggesting the existence of a *radRS* operon. Recombinant RadS was autophosphorylated and could catalyse the transfer of γ phosphate from ATP to RadR *in vitro*. These results indicated the functional interaction of RadS and RadR, and suggested a role for the RadS/RadR two-component system in the radiation resistance of this bacterium.

INTRODUCTION

Deinococcus radiodurans is characterized by its extreme tolerance to DNA-damaging agents, including radiation and desiccation (Makarova *et al.*, 2001; Blasius *et al.*, 2008). Efficient biphasic DNA double-strand break (DSB) repair (Daly *et al.*, 1994; Daly & Minton, 1996) and a strong oxidative stress tolerance (Markillie *et al.*, 1999; Slade & Radman, 2011) are among the mechanisms attributed to these phenotypes. DSB repair is primarily supported by extended synthesis-dependent strand annealing (ESDSA) (Zahradka *et al.*, 2006) and the RecF pathway of homologous recombination (Misra *et al.*, 2006; Slade *et al.*, 2009). *D. radiodurans* cells exposed to gamma radiation show a relatively longer lag phase. During this period cell division stops, and resumes presumably after DSB repair has been accomplished (Cox & Battista, 2005). This might indicate the existence of an uncharacterized checkpoint regulation between DNA repair and cell division. A bacterium exposed to gamma radiation adjusts its transcriptome and proteome by regulating gene expression (Liu *et al.*, 2003; Tanaka *et al.*, 2004) and protein recycling (Joshi *et al.*, 2004). The SOS response, a classical example of a DNA-damage response in bacteria (Shimoni *et al.*, 2009; Walker, 1996), is missing in *D. radiodurans* (Bonacossa de Almeida *et al.*, 2002; Narumi *et al.*, 2001). Recently, the involvement of a eukaryotic-type Ser/Thr protein kinase (eSTPK) in gamma radiation resistance and DSB repair of *D. radiodurans* has been demonstrated (Rajpurohit & Misra, 2010). The roles of Ser/Thr protein kinases in DNA damage-induced signalling processes are better understood in eukaryotes (Zhou & Elledge, 2000). Further, the genome of this bacterium encodes a large number of putative transcription factors, response regulators and different types of protein kinases, including 20 putative histidine kinases (Leonard *et al.*, 1998; White *et al.*, 1999; Makarova *et al.*, 2001; Kim & Forst, 2001). Thus, the contribution of these unique proteins to the extraordinary radiation resistance of this bacterium can be envisaged.

Here, we report the functional characterization of the DR_B0090 and DR_B0091 ORFs, encoding a putative sensor histidine kinase (hereafter referred to as RadS) and a cognate response regulator (hereafter referred to as RadR),

Abbreviations: DSB, double-strand break; PIR, post-irradiation; TCS, two-component system.

Three supplementary figures and a supplementary table are available with the online version of this paper.

respectively, and their involvement in the radiation resistance and DSB repair of *D. radiodurans*. Δ*radS* and Δ*radR* mutants showed a higher sensitivity to DNA damage and oxidative stress produced by UV (254 nm) and gamma radiation, hydrogen peroxide and desiccation as compared with the wild-type. The Δ*radRradS* double mutant and the Δ*radR* single mutant showed nearly identical responses to both gamma and UVC radiation. The expression of wild-type RadS and RadR on a plasmid showed almost complete functional complementation in the respective mutants. Purified RadS showed autokinase activity and could phosphorylate RadR *in vitro*. These results suggest that the *radR* and *radS* genes are organized in a bicistronic operon and that the RadS/RadR two-component system (TCS) plays an important role in the radiation resistance of *D. radiodurans*.

METHODS

Bacterial strains, plasmids and growth conditions. *D. radiodurans* R1, a generous gift from Professor J. R. Battista, Louisiana State University, Baton Rouge, LA, USA, was grown in TGY medium (0.5 % Bacto tryptone, 0.1 % glucose, 0.3 % Bacto yeast extract), as described earlier (Mattimore & Battista, 1996). Plasmids pET28a+ (Novagen) and pRADgro (Misra *et al.*, 2006) and their derivatives were maintained in *Escherichia coli* strain DH5α in Luria–Bertani (LB) broth or on LB agar plates supplemented with kanamycin (25 μg ml^{-1}) and ampicillin (100 μg ml^{-1}), respectively. All molecular biology grade chemicals were purchased from Sigma Chemical Co., Roche Biochemicals, New England Biolabs and Bangalore Genei. *Deinococcus* mutants were grown in the presence of kanamycin (8 μg ml^{-1}) and chloramphenicol (5 μg ml^{-1}), as required. Other recombinant techniques used were as described by Sambrook & Russell (2001).

Isolation of DNA-binding proteins using a deinococcal DNA–cellulose matrix. Genomic DNA was isolated from both unirradiated and gamma-irradiated *D. radiodurans* R1 as described earlier (Battista *et al.*, 2001). DNA was sonicated at 30 Hz, one stroke of 2 s with 0.2 μm duty cycles, and was UV cross-linked with cellulose (DNA–cellulose). Free DNA was washed with buffer containing 50 mM NaCl. The cell-free extracts were prepared from unirradiated cells and from cells after 0, 15, 30, 45 and 60 min irradiation, and were incubated with the DNA–cellulose matrix for 60 min. The proteins bound to DNA–cellulose were packed into a column and washed with buffer A (20 mM Tris/HCl, pH 7.5, 20 %, v/v, glycerol, 0.5 mM EDTA, pH 8, 0.5 mM PMSF, 0.5 mM DTT) containing 50 mM NaCl, to remove the unbound proteins until the A_{280} was nearly zero. The bound proteins were eluted with a buffer containing 0.7 M NaCl and dialysed in buffer A supplemented with 50 %, v/v, glycerol and stored at −20 °C until further use.

Generation of deletion mutants of *D. radiodurans*. The Δ*radS* and Δ*radR* mutants were generated using strategies described previously (Khairnar *et al.*, 2008). The primers used in this study are listed in Supplementary Table S1. In brief, the 1 kb upstream of the initiation codon and 1 kb downstream from the stop codon of DR_B0090 (*radS*) were PCR-amplified using primers HK90F1 and HK90R1 for upstream, and primers HK90F2 and HK90R2 for downstream fragments. Similarly, 1 kb upstream and 1 kb downstream from DR_B0091 (*radR*) were PCR-amplified using RR91F1 and RR91R1 primers for the upstream and RR91F2 and RR91R2 for the downstream fragments, separately. The upstream fragments of both genes were cloned at *Apa*I and *Eco*RI sites into pNOKOUT, and downstream fragments were cloned at *Bam*HI and *Xba*I sites to yield pNOKHK90 and pNOKRR91, respectively. Similarly, the upstream fragment of *radR* and the downstream fragment of *radS* were cloned into pNOKOUT to yield pNOKradRS. These recombinant plasmids were linearized with *Xmn*I and transformed into *D. radiodurans*, and transformants were grown for several generations in TGY broth containing kanamycin (8 μg ml^{-1}). The complete replacement of wild-type alleles(s) with the neomycin phosphotransferase gene (*nptII*) was confirmed by PCR amplification using gene-specific primers, and the single mutants were designated Δ*radS* and Δ*radR* (Supplementary Fig. S1) and the double mutant Δ*radRradS*.

Construction of RadS and RadR expression plasmids. The 1.3 kb *radS* and 0.66 kb *radR* genes were PCR-amplified from the genomic DNA of *D. radiodurans* using gene-specific primers and cloned into pRADgro at the *Apa*I and *Hin*dIII sites (Misra *et al.*, 2006) to obtain pGroHK90 and pGroRR91, respectively. The recombinant plasmids were transformed into *D. radiodurans* and the respective deletion mutants as described earlier (Meima *et al.*, 2001). The transformants were scored on TGY agar plates containing chloramphenicol (8 μg ml^{-1}). The *radS* and *radR* genes were PCR-amplified using sequence-specific primers and cloned at the *Nde*I and *Hin*dIII sites into pET28a+ to yield pETHK90 and pETRR91, respectively. These plasmids were transformed into *E. coli* BL21 (DE3) pLysS for expression and purification of recombinant proteins.

Cell survival studies. *D. radiodurans* and its derivatives were grown in TGY medium at 32 °C to the late-exponential phase and treated with different doses of gamma radiation at a dose rate of about 5.86 kGy h^{-1} (G5000, ^{60}Co, Board of Radiation and Isotopes Technology, Department of Atomic Energy, India), and UV (254 nm) radiation at 0.295 J s^{-1} m^{-2} (Gentec PSV-3303 laser power meter), as described previously (Kota & Misra, 2006). For desiccation, the cells were incubated at 5 % humidity for different time intervals as described earlier (Mattimore & Battista, 1996). For hydrogen peroxide, the cells were treated with different concentrations of H_2O_2 for 30 min with shaking. Appropriate dilutions were plated on TGY agar plates and the c.f.u. ml^{-1} was recorded after 48 h of incubation at 32 °C.

DNA strand break repair studies. The cells were irradiated with 6.5 kGy of gamma radiation at a dose rate of 5.86 kGy h^{-1} and aliquots were collected at different time intervals post-irradiation (PIR). The DNA fragments were separated by PFGE using the modified protocols described by Mattimore & Battista (1996). In brief, the cells were washed with PBS-saturated butanol for 30 min at 25 °C, followed by a wash in 50 % ethanol for 5 min. Cells were embedded in 0.7 % agarose and plugs were incubated in lysis buffer I (5 mg lysozyme ml^{-1}, 5 mM EDTA, pH 8) for 5 h at 37 °C, followed by overnight incubation at 50 °C in lysis buffer II (0.5 M EDTA, pH 8, 1 % sodium sarcosine, 2 mg proteinase K ml^{-1}). The plugs were washed four times with TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0) and then washed three times for 1 h each with *Not*I buffer at 50 °C. Subsequently, the plugs were transferred to fresh tubes containing 30 U *Not*I in its buffer and incubated overnight at 37 °C. The DNA fragments were analysed on 1 % multipurpose agarose (Roche Biochemicals) and visualized by staining with ethidium bromide.

Purification of recombinant protein and *in vitro* protein phosphorylation. *E. coli* BL21(DE3) pLysS cells harbouring pETHK90 and pETRR91 plasmids were induced with 200 μM IPTG and proteins were analysed by 10 % SDS-PAGE. Recombinant proteins were purified by immobilized metal-chelating affinity

chromatography (IMAC), using modified protocols described previously (Misra *et al.*, 1998). In brief, the IPTG-induced cells were suspended in sample buffer (100 mM NaH_2PO_4, 10 mM Tris/HCl, 8 M urea, pH 8.0) and incubated at 37 °C for 30 min with mild agitation. The clear supernatant was passed through an IMAC column pre-equilibrated with sample buffer and washed with sample buffer adjusted to pH 6.3. Proteins were eluted with sample buffer at pH 5.9 and analysed by 10 % SDS-PAGE. Pure fractions were pooled, adjusted to pH 8.0 and passed through a fresh IMAC column. Protein refolding was done with a slow gradient of 8–0 M urea containing 0–5 mM DTT in sample buffer, and the proteins were eluted with elution buffer (10 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 300 mM imidazole). Purity was checked by SDS-PAGE, and pure fractions were dialysed in buffer containing 10 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$ and 50 % (v/v) glycerol.

The kinase activity was checked by incubating the recombinant RadS and RadR separately and together in the presence of [γ-^{33}P]ATP, as described elsewhere (Shrivastava *et al.*, 2007). In brief, both the proteins were incubated in a reaction mixture containing 50 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM cold ATP and 200 μCi (7.4 MBq) [γ-^{33}P]ATP for different time intervals at 25 °C. RadS and RadR were taken separately as controls. A dose-dependent assay was carried out with a constant RadS concentration (20 μM) and an increasing concentration of RadR under similar conditions. Both RadS alone and RadS with RadR were incubated for different time periods in the presence of [γ-^{32}P]ATP. The reaction products were analysed by 8 % SDS-PAGE at 25 °C. The gel was stained with Coomassie brilliant blue and dried, and signals were recorded by autoradiography and by scanning on a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

A putative response regulator can be identified amongst the radiation-induced proteins

Cell-free extracts were prepared from unirradiated as well as gamma radiation-treated cells collected at 15 min intervals up to 1 h PIR, and DNA-binding proteins were isolated using DNA–cellulose columns, as described in Methods. Both SDS-PAGE and 2D PAGE analysis showed the differential expression of several proteins, which were identified by MALDI MS (data not shown). A protein of ~28 kDa that was induced in the first 15 min and maintained at higher levels up to 1 h PIR (Fig. 1) showed a peptide mass fingerprint (Supplementary Fig. S2) that matched with the protein encoded by the DR_B0091 ORF. A domain search analysis using www.expasy.ch/prosite indicated that this protein had a CheY-homologous receiver domain at the N terminus and a transcriptional regulatory protein domain at the C terminus (Supplementary Fig. S3). This suggested that the DRB0091 protein could interact with both protein and DNA simultaneously. The CheY receiver domain at the N terminus of such proteins undergoes phosphorylation at a specific aspartate residue and plays an important role in the signal transduction mechanism (Appleby *et al.*, 1996). DR_B0090 (*radS*) is located downstream from DR_B0091 (*radR*) on the megaplasmid, and encodes a putative histidine kinase (White *et al.*, 1999). It was predicted that

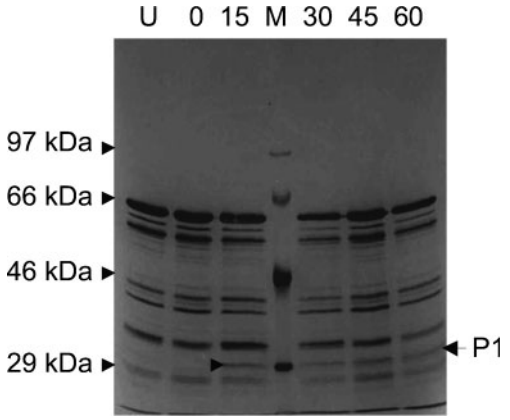

Fig. 1. SDS-PAGE analysis of dsDNA-binding proteins (DBPs) during the early phase of PIR recovery of *D. radiodurans* R1. Total DBPs were isolated from gamma-irradiated cells as described in Methods. DBPs from an unirradiated sample (U) and after different periods PIR (0, 15, 30, 45 and 60 min) were separated by SDS-PAGE. A protein (P1) showing inducible synthesis from 15 min PIR onwards was identified using MALDI-TOF MS data, as shown in Supplementary Fig. S2.

the aspartyl group present at position 51 of RadR, similar to the CheY receiver domain, was the probable site for phosphorylation by RadS or other histidine kinases in this bacterium. Functional domain analysis of RadS showed the presence of HATPase and HisKA domains, and a transmembrane domain (between amino acids 7 and 29) that overlapped with the signal peptide of the protein (http://smart.embl-heidelberg.de). RadS also contains a HAMP linker domain normally reported from bacterial sensor proteins such as histidine kinases, adenylyl cyclases, methyl-accepting proteins and phosphatases, and chemotaxis proteins (http://pfam.sanger.ac.uk/family/PF00672.18). Protein kinases containing HAMP linker domains are integral membrane proteins and are involved in two-component signal transduction processes (Aravind & Koonin, 1999) in bacteria. The HATPase domain contains the ATP-binding motif and a characteristic G-X-G motif, while the HisKA domain has a site for phosphorylation and a dimer interface motif. These characteristics suggest that RadS and RadR are a histidine sensor kinase and its cognate response regulator, respectively. The possibility that RadR is phosphorylated by RadS and that the functional interaction of these proteins contributes to the radiation resistance of this bacterium were further investigated.

The Δ*radS* and Δ*radR* mutants are hypersensitive to both oxidative stress and DNA damage

The *radS* and *radR* genes were deleted individually and together from the genome of *D. radiodurans*, and mutants were designated Δ*radS*, Δ*radR* and Δ*radS*Δ*radR*, respectively. The cell survival of single mutants was determined in response to various DNA-damaging agents such as gamma

radiation, UVC (254 nm), H_2O_2 and desiccation. The tolerance of these cells decreased by nearly two orders of magnitude in response to gamma radiation (Fig. 2a), three orders of magnitude to UVC (Fig. 2b), 10-fold to 25 mM hydrogen peroxide (Fig. 2c) and nearly three orders of magnitude to desiccation at 5 % humidity (Fig. 2d) as compared with wild-type cells. The cell survival of the *radS* mutant decreased more rapidly when cells were treated with a dose of up to 2 kGy of gamma radiation as compared with doses above 2 kGy. This indicates the possibility that RadS controls significant pathways associated with radiation resistance and DSB repair. The role of another response regulator, DR_2418, in the gamma radiation resistance of *D. radiodurans* has also been shown recently (Wang *et al.*, 2008). These results suggest the involvement of the RadS/RadR system in the DNA damage tolerance of *D. radiodurans*.

Wild-type proteins show functional complementation *in trans*

To confirm that the phenotypes of the Δ*radS* and Δ*radR* mutants were due to the absence of the respective proteins, the wild-type RadS and RadR were expressed on plasmids pGroHK90 and pGroRR91, respectively, in both the single and the double mutants of *D. radiodurans*. The effects of these proteins on the gamma radiation response of the mutants were evaluated. The Δ*radS* and Δ*radR* mutants expressing the respective proteins recovered nearly 80–90 % of wild-type tolerance to gamma radiation (Fig. 3a, b). Interestingly, the Δ*radS* mutant expressing RadR and the Δ*radR* mutant expressing RadS on plasmids also showed a dose-dependent recovery of DNA damage tolerance. At lower doses up to 4 kGy, the recovery of gamma radiation resistance was significant, and RadR was

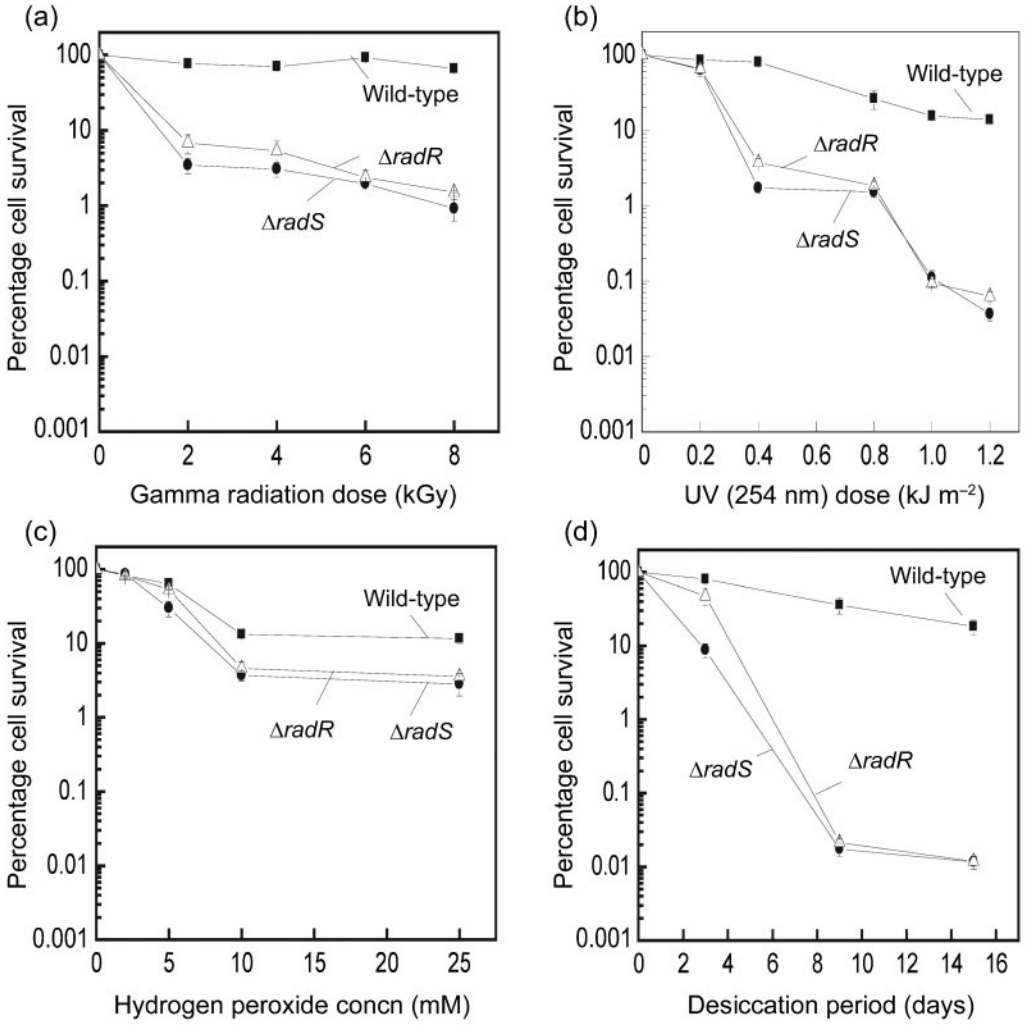

Fig. 2. DNA damage response of wild-type and mutant derivatives of *D. radiodurans*. The cell survival of wild-type, Δ*drB0090* (Δ*radS*) and Δ*drB0091* (Δ*radR*) mutants was measured with gamma radiation (a), UV (b), hydrogen peroxide (c) and desiccation (d) treatments. A cell survival of 100 % for the wild-type, Δ*radR* and Δ*radS* mutants corresponds to 2.8×10^7, 2.1×10^7 and 1.8×10^7 cells ml^{-1}, respectively. Experiments were repeated at least three times in triplicates; error bars, SD.

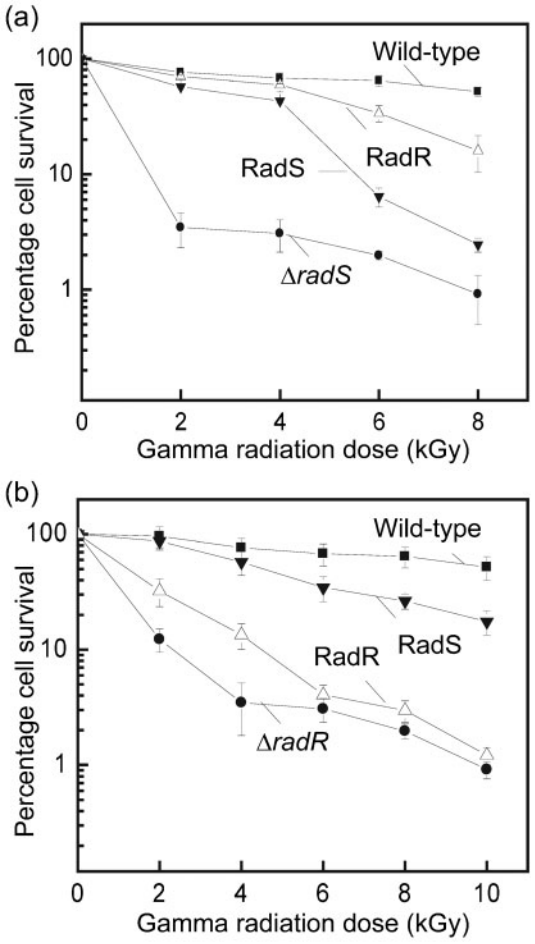

Fig. 3. Functional complementation in mutants. Δ*radS* (a) and Δ*radR* (b) mutants expressing wild-type *radS* (RadS) and *radR* (RadR) genes were treated with different doses of gamma radiation, and cell survival was compared with that of the wild-type. A cell survival of 100 % corresponds to 3.1×10^7, 1.5×10^7 and 2.7×10^7 cells ml^{-1} for the wild-type, mutants and complemented cells, respectively. Experiments were repeated at least three times in triplicates; error bars, SD.

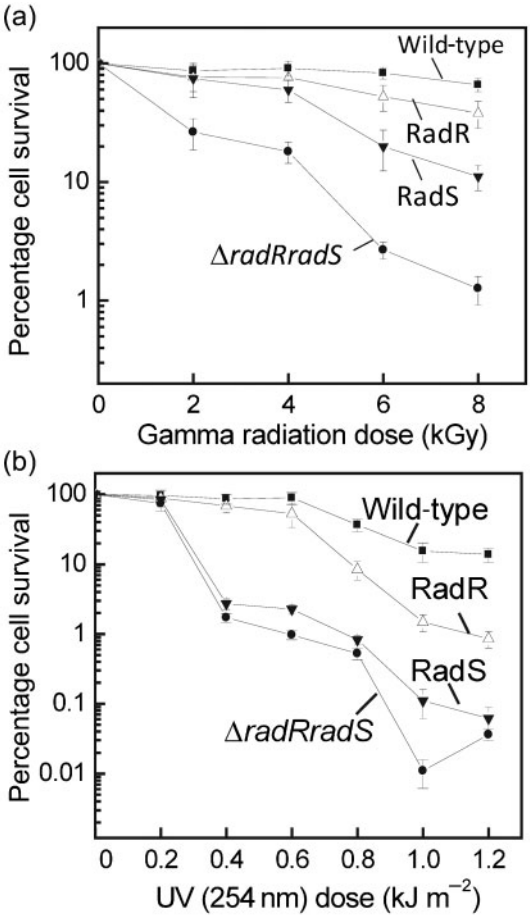

Fig. 4. Effect of DNA damage on cell survival of the Δ*radRradS* double mutant. The Δ*radRradS* mutant and its derivatives expressing wild-type *radS* (RadS) and *radR* (RadR) alleles were treated with different doses of gamma radiation (a) and UV (254 nm) (b), and cell survival was monitored as described in Methods and compared with that of the wild-type. A cell survival of 100 % for the wild-type, double mutant and its derivatives expressing RadR and RadS corresponds to 8.2×10^7, 1.2×10^7, 3.6×10^7 and 2.6×10^7 cells ml^{-1}, respectively. Experiments were repeated at least three times in triplicates; error bars, SD.

able fully to restore the wild-type resistance to gamma radiation in the *radS* mutant. However, above 4 kGy, the levels of complementation in the respective mutants were significantly lower (Fig. 3). Furthermore, the deletion of the *radR–radS* (drB0091–drB0090) operon showed a response to UVC and gamma radiation nearly identical to that of the *radR* single mutant (Fig. 4). Interestingly, the expression of the wild-type alleles of *radS* and *radR* separately in a Δ*radRradS* double mutant also showed functional complementation very similar to the effects of these proteins in the Δ*radR* mutant (Fig. 4a). However, the Δ*radRradS* mutant expressing the RadR protein showed a near-complete recovery of UVC resistance at lower doses, although the recovery was significantly lower at higher doses (Fig. 4b). Unlike RadS complementation with respect to gamma radiation resistance, the double mutant showed a low level of RadS complementation with respect to UVC resistance (Fig. 4b). This indicated that the effects of RadS on gamma radiation- and UVC-induced DNA damage repair are different.

Genetic modifications that lead to differential responses of *D. radiodurans* to gamma and UVC radiation have been reported previously (Misra *et al.*, 2006; Khairnar *et al.*, 2008). The expression of transgenes cloned in pRADgro under the *groESL* promoter has been shown in both *E. coli* (Kota & Misra, 2006) and *D. radiodurans* (Misra *et al.*, 2006; Khairnar *et al.*, 2008). The functional complementation of the *radS* mutation by overexpression of RadR might lead one to speculate either that RadR is activated by other

histidine kinase(s) and/or that there is a dose-dependent effect of RadR, which might have residual activity in its unphosphorylated form. However, the complementation by wild-type RadS, though partial, in the Δ*radR* and Δ*radRradS* mutants is intriguing, and the molecular mechanism underlying the support by RadS of the loss of RadR is not clear. However, the possibility exists that RadS phophorylates other regulatory protein(s), which might complement the functional loss of RadR/RadS in these mutants. Since the genome of *D. radiodurans* encodes a large number of stress-responsive regulatory proteins, the possibility that some of these proteins are phosphorylated by RadS and functionally complement the functions of RadR cannot be ruled out. Transcriptome studies of the *radS* mutant confirmed the expression of *radR*, while the reverse was not observed (data not shown). Further, we observed that the mere absence of RadS in the Δ*radS* mutant was able to affect the expression of various genes in *D. radiodurans* exposed to gamma radiation (GEO accession numbers GSE17720, GSM442437, GSM442435). This might indicate that although RadS functions through RadR as its cognate response regulator, it also functions through other proteins, which possibly contribute to the expression of genes responsible for radiation resistance. Although further studies are required to test these hypotheses, the dose-dependent effects of TCSs and the functional redundancy of such proteins have been reported in bacteria. These results therefore suggested that RadS and RadR are expressed in a bicistronic operon and have roles in the radiation resistance of *D. radiodurans*.

Both the Δ*radS* mutant and the Δ*radR* mutant show defective DSB reassembly

To understand the molecular mechanisms underlying the roles of these proteins in the radiation resistance of *D. radiodurans*, the DSB repair of Δ*radS* and Δ*radR* mutants was monitored by PFGE. Interestingly, although the two mutants showed a nearly identical response to gamma radiation, for unknown reasons the DSB repair kinetics were slightly different. The Δ*radS* mutant showed a delayed recovery of the *Not*I pattern of its genome as compared with the wild-type, while the Δ*radR* mutant showed the faster appearance of several *Not*I fragments, although the pattern (Mattimore & Battista, 1996) was different from that of the wild-type (Fig. 5): at least one large band and a few smaller-size *Not*I fragments were missing. This may indicate a genomic rearrangement that has made the *radR* deletion mutant sensitive to gamma radiation. PFGE carried out with undigested genomic DNA supported this

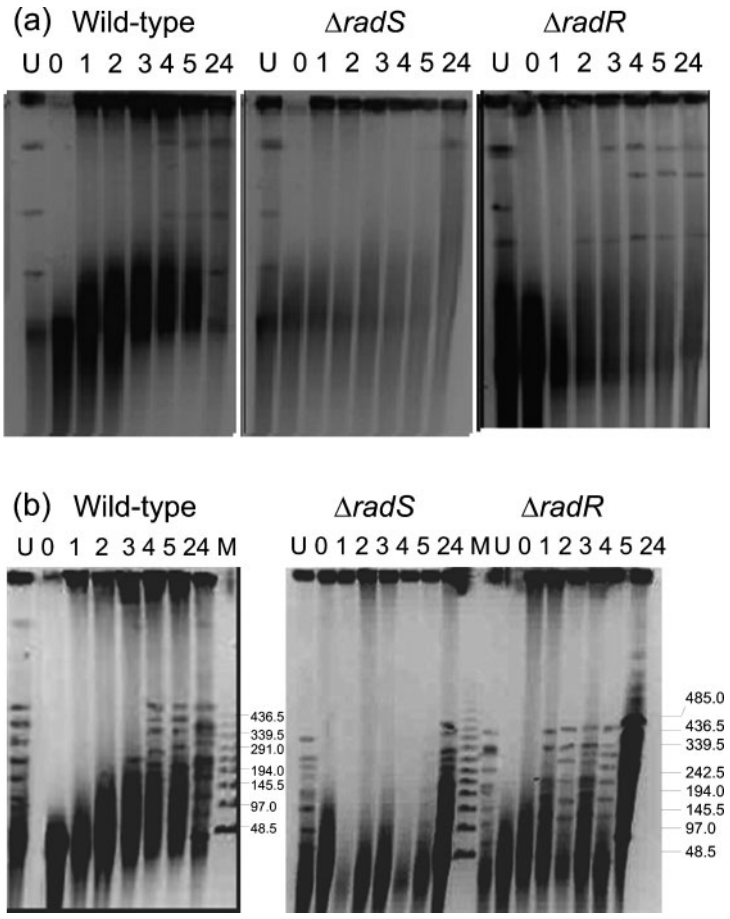

Fig. 5. DNA strand break repair kinetics during PIR recovery. The wild-type and deletion mutants of the *radS* (Δ*radS*) and *radR* (Δ*radR*) genes were treated with 6.5 kGy of gamma radiation, and aliquots were drawn at different times (0, 1, 2, 3, 4, 5 and 24 h) of PIR recovery. Both undigested (a) and *Not*I-digested (b) genomic DNA was independently analysed by PFGE and compared with unirradiated controls (U) and PFGE size markers (M).

speculation. The patterns of DNA bands, presumably representing the banding of intact genomes in unirradiated cells of the wild-type, Δ*radS* and Δ*radR* mutants, were similar (Fig. 5). Upon gamma irradiation followed by PIR recovery, these cells showed distinct patterns. The Δ*radS* mutant showed no sign of recovery, while the wild-type recovered its typical pattern. The Δ*radR* mutant showed a different pattern of recovered DNA signatures (Fig. 5a, compare lanes 4, 5 and 24 of Δ*radR* with those of the wild-type). These results might lead one to postulate a role for RadR in maintaining genome integrity during the PIR recovery of this bacterium. Recently, the possibility of genomic rearrangement in recombination repair mutants recovering from radiation stress has been indicated (Repar *et al.*, 2010; Hickman *et al.*, 2010). Delayed DSB repair leading to gamma radiation sensitivity as observed in the Δ*radS* mutant agrees with previous findings in which the loss of gamma radiation resistance was due to a defect in the DSB repair of *D. radiodurans* (Misra *et al.*, 2006; Slade *et al.*, 2009; Khairnar *et al.*, 2008). The differential effects of *radS* and *radR* deletions on DSB repair, despite the fact that both mutants show a near-identical response to gamma radiation, is interesting and intriguing. Both these mutants showed a growth pattern almost identical to that of the wild-type under standard culture conditions (data not shown), suggesting that these deletions have an insignificant effect on the normal growth of this bacterium but do have a role in response to DNA damage.

Recombinant RadR is phosphorylated by recombinant RadS in solution

The recombinant RadS and RadR proteins were purified to near homogeneity (Fig. 6). The kinase activity of RadS was checked in the presence and absence of purified RadR as a probable substrate. RadS showed autophosphorylation in the presence of [γ-^{33}P]ATP, but RadR phosphorylation was observed only in the presence of RadS (Fig. 6). When increasing amounts of RadR were incubated with [γ-^{33}P]ATP in the absence of RadS, the protein did not show phosphorylation (Fig. 6b), while RadR incubated with RadS showed phosphorylation. A time-course assay supported the conclusion that RadS phosphorylates RadR *in vitro* (Fig. 6c). These results suggested the functional interaction of RadS with RadR, and that RadR is a substrate for RadS *in vitro*. The level of RadS phosphorylation reduced significantly when it was incubated with a higher molar ratio of RadR (data not shown), and also on longer incubation with twice the molar concentration of RadR (Fig. 6c). An increased phosphorylation of RadR by RadS and a reduction in the signal of RadS both at the higher molar ratio of RadR and on longer incubation might argue in favour of substrate-driven forward kinetics for the phosphotransfer reaction catalysed by RadS. Surprisingly, the recombinant RadS purified from *E. coli* showed low activity *in vitro*. However, the low activity of a mycobacterial membrane histidine kinase purified from *E. coli* has been shown (Shrivastava *et al.*, 2007). These results suggest that RadS is a

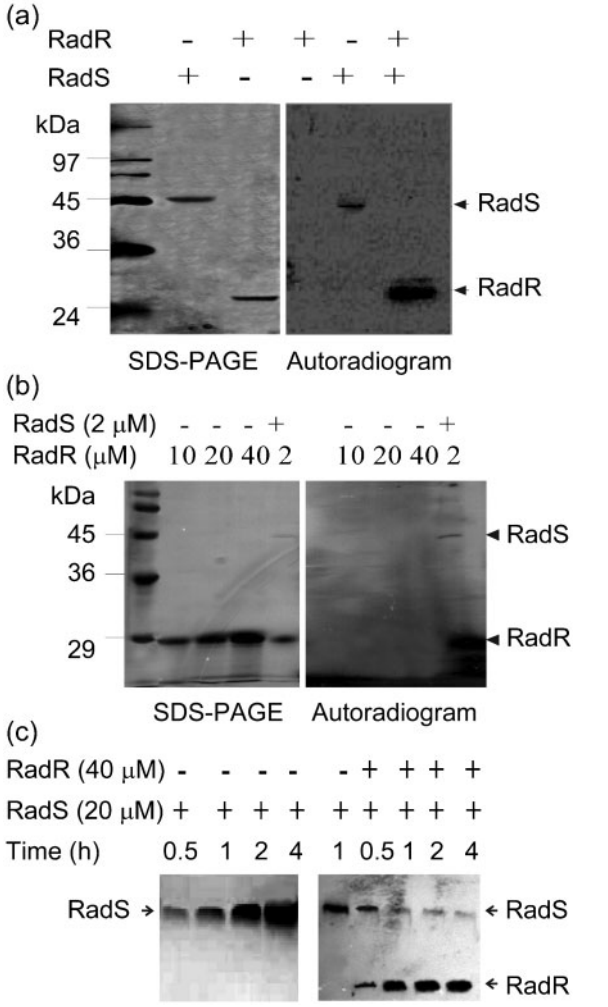

Fig. 6. Functional characterization of RadS and RadR proteins *in vitro*. Purified RadS was incubated with RadR in different combinations in the presence of [γ-^{33}P]ATP at 37 °C for 6 h (a), and with increasing concentrations of RadR in the presence of [γ-^{32}P]ATP (b). To determine the time-dependent autokinase and transkinase activities of RadS, purified RadS was incubated alone and in the presence of RadR for different time intervals in the presence of [γ-^{33}P]ATP (c). The products were analysed and proteins were visualized by Coomassie staining (SDS-PAGE), and phosphoproteins were visualized by autoradiography (Autoradiogram).

histidine kinase which can phosphorylate a cognate response regulator, RadR, expressed from the same operon.

Both membrane-bound and cytoplasmic signal transduction protein kinases and their cognate response regulators have been identified in bacteria (Parkinson, 1993; Mascher *et al.*, 2006). Roles for histidine kinases and their cognate response regulators in sensing heavy metal stress by inducing the cascade of protein phosphorylation have been reported. *D. radiodurans* has been shown to accumulate a higher amount of Mn as compared with Fe and Cu (Ghosal *et al.*, 2005), and the physiological significance of this in the

bacterial response to oxidative and radiation stress has been reported (Shashidhar *et al.*, 2010; Bagwell *et al.*, 2008). RadS shows 37 % identity to the histidine kinase CusS, involved in sensing copper toxicity in *E. coli* (Rensing & Grass, 2003). Classical TCSs, consisting of a sensor protein kinase and a response regulator regulating the differential expression of a gene in response to nutritional and other abiotic stresses, with the exception of DNA damage, have been reported in bacteria (Parkinson, 1993; Inouye & Dutta, 2003). On the other hand, the DNA damage response-mediated regulation of the cell cycle and DSB repair have been well documented in eukaryotes (Sancar *et al.*, 2004; Lavin & Kozlov, 2007).

Here, we report findings that suggest a role for a histidine kinase (RadS) and its cognate response regulator (RadR) in the high tolerance to DNA damage of *D. radiodurans*. Both autophosphorylation of RadS and its ability to phosphorylate RadR *in vitro* indicate the stress-responsive nature of the RadS/RadR system. Recently, the involvement of protein phosphorylation in DNA metabolism and radiation resistance has been shown in bacteria. The role of pyrroloquinoline-quinone (PQQ), an inducer of protein kinase activity, in radiation resistance and DSB repair (Rajpurohit *et al.*, 2008), the co-existence of DNA repair proteins with protein kinases and phosphoproteins in a multiprotein DNA-processing complex (Kota & Misra, 2008), and the role of a response regulator, DR_2418, in the regulation of *recA* and *pprA* expression (Wang *et al.*, 2008), have been reported in *D. radiodurans*. Also, a DNA damage-responsive membrane protein kinase with a role in radiation resistance and DSB repair has been characterized in *D. radiodurans* (Rajpurohit & Misra, 2010), and gamma radiation-induced changes in the levels of cyclic AMP and ATP, and the activity of stress-responsive enzymes such as adenylyl cyclase, protein kinases and phosphodiesterases, have been shown in this bacterium (Kamble *et al.*, 2010). The functional interaction of PQQ with a periplasmic protein kinase with a role in radiation tolerance (Khairnar *et al.*, 2007), and the role of a TCS in the expression of DNA recombination and repair genes, have also been reported in *E. coli* (Oshima *et al.*, 2002; Zhou *et al.*, 2003). The phosphorylation of the single strand DNA-binding protein (SSB) of *Bacillus subtilis* and its effect on the DNA-binding activity of SSB have been demonstrated (Mijakovic *et al.*, 2006). These findings support the involvement of protein phosphorylation and the roles of protein kinases in the bacterial response to DNA damage. The results presented here suggest that RadS/RadR form a TCS that contributes to the extraordinary tolerance of *D. radiodurans* to radiation resistance and DNA damage, and to our knowledge is the first report showing the role of a TCS in the radiation resistance of a prokaryote.

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