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Cooperation of PprI and DrRRA in response to extreme ionizing radiation in *Deinococcus radiodurans*

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Deinococcus radiodurans possesses extreme resistance to ionizing radiation, and has been engineered for the remediation of toxic components in radioactive environments. We have previously shown that PprI (also named IrrE) and DrRRA are essential for the DNA protection and repair pathways that respond to ionizing radiation stress in this species. Here, we investigated the combined roles of PprI and DrRRA in resistance to gamma radiation (800 Gy). The double mutant, Δ*drRRA* Δ*pprI*, was more sensitive to gamma rays than either Δ*drRRA* or Δ*pprI* single mutants, and exhibited an elevated level of intracellular protein carbonylation, an extended delay in genome reconstitution and reduced transcriptional levels of certain DNA protection and repair proteins, such as *kat*, *sod*, *recA* and *pprA*. Interestingly, the induction of DrRRA by ionizing radiation was partially inhibited by the deletion of *pprI*. Taken together, these results suggest that DrRRA and PprI might have collaborative roles in the response of *D. radiodurans* to extreme ionizing radiation.

***Deinococcus*, DNA repair, anti-oxidation, ionizing radiation**

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Deinococcus radiodurans possesses an exceptional tolerance to the killing effects of highly stressful agents, such as ionizing radiation, ultraviolet (UV) light, hydrogen peroxide and desiccation. Exponentially growing *D. radiodurans* is able to survive a 15 kGy dose of acute ionizing radiation, which is approximately 50–100 times higher than the tolerance of *Escherichia coli* [1]. Biotechnologies have been developed based on this radiation-resistant bacterium to degrade pollutants in radioactive mixed waste environments [2–5].

Like other extreme microorganisms, *D. radiodurans* employs various strategies to overcome stress [6–12], and several mechanisms for its extreme resistance to ionizing radiation have been proposed [13–16]. A pool of distinctive genes involved in the stress response has been investigated to explain the extreme resistance of *D. radiodurans* [17–23]. Among them, two regulators are noteworthy. One is *pprI* (inducer of pleiotropic proteins promoting DNA repair, also named *irrE*), a general switch for downstream DNA repair and protection pathways [24,25]. Extensive biochemical and genetic research on *pprI* has generated a compelling picture of its *in vivo* function. Our recent research suggests that PprI participates in the regulation of several pathways, including DNA repair, stress response, energy metabolism, transcriptional regulation, signal transduction, protein turnover and chaperoning [21]. Exogenous expression of *pprI* in *E. coli* promotes DNA repair and offers oxidative damage protection, thereby enhancing radioresistance [26]. Another stress response gene is *drRRA* (*Deinococcus radiodurans* response regulator A), a novel response regulator that modulates multiple pathways, including antioxidation and DNA repair [20]. Deletion of this gene renders the bacteria highly

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sensitive to radiation, oxidative stress, and desiccation. Both *pprI* and *drRRA* play crucial roles in regulating downstream genes involved in DNA damage protection and repair pathways upon ionizing radiation stress. However, whether these two genes act cooperatively *in vivo* is not known.

In this study, we constructed a double-knockout strain lacking the *pprI* and *drRRA* genes to investigate their combined roles in cellular resistance. The functions of PprI and DrRRA upon exposure to extreme ionizing radiation were collaborative.

1 Materials and methods

1.1 Bacterial strains and growth conditions

D. radiodurans (ATCC 13939) was grown at 30°C in TGY medium (0.5% Bacto tryptone, 0.1% glucose, 0.3% Bacto yeast extract) or on TGY plates supplemented with 1.5% Bacto-agar. When necessary, media were supplemented with 50 μg mL^{-1} kanamycin and 3.4 μg mL^{-1} chloramphenicol. *D. radiodurans* cells were transformed using the modified $CaCl_2$ technique [4]. All strains and plasmids used in this work are listed in Table 1.

1.2 Construction of the Δ*pprI* Δ*drRRA* double-knockout strain

Δ*pprI* and Δ*drRRA* single mutant strains were constructed using the deletion mutagenesis technique with a kanamycin cassette as described previously [20,27]. To obtain a double-knockout mutant with different antibiotic-resistance genes, we replaced the kanamycin cassette in Δ*drRRA* with a chloramphenicol cassette (Figure 1(a)). Briefly, primers P1 (5′-GGGTCATGCAGAAGAACTCGG-3′) and P4 (5′-TGGCTCGCACTCCAGACAAT-3′) were used to amplify the fragment containing the sequences flanking the kanamycin cassette from genomic Δ*drRRA* DNA. This fragment was ligated into the pMD18-T Simple vector lacking the multiple cloning sites (TaKaRa, Japan) to generate pTMRK. This plasmid was then digested with *Bam*H I and *Hin*d III to remove the kanamycin cassette, and the chloramphenicol cassette from pKATcat [28] was ligated in its place to generate pTMRC. Primers P1 and P4 were used to amplify a fragment containing the chloramphenicol cassette and the upstream and downstream *drRRA* fragments. This fragment was then transformed into competent, exponential-phase Δ*pprI* single mutant cells. Candidate double-mutants resistant to both kanamycin and chloramphenicol were selected on TGY plates with antibiotics, and verified by PCR, DNA sequencing and Western blotting (Figure 1(b) and (c)).

1.3 Western blotting analysis

D. radiodurans cells, either irradiated or non-irradiated, were harvested and disrupted. Supernatants were separated by SDS-PAGE, transferred to nitrocellulose, and detected with antisera against DrRRA or PprI (rabbit IgG, laboratory stock) [20,27]. As a control, *D. radiodurans* GroEL was detected with *E. coli* GroEL antiserum (Sigma, USA).

1.4 *D. radiodurans* survival assay

The gamma radiation survival assay was performed as described previously [20]. Briefly, aliquots of cells were irradiated at room temperature for 1 h with ^{60}Co gamma rays at doses from 100 to 6000 Gy. After treatment, cells were plated on TGY plates and incubated at 30°C for 3 d before enumeration of colonies.

1.5 Measurement of intracellular protein carbonylation levels

Intracellular protein carbonylation was measured as described previously with some modifications [29]. Briefly, cells grown to A_{600} = 1.0 were harvested and gamma-irradiated with 800 Gy. Freshly treated cells were lysed by

Table 1 Strains and plasmids used in this study

Strains	Description	Source or reference
D. radiodurans R1	ATCC 13939	Lab stock
DH5α host strain	For plasmid construction	Invitrogen, US
Δ*pprI*	*D. radiodurans* R1 *pprI* deletion mutant harboring kanamycin cassette	[27]
Δ*drRRA*	*D. radiodurans* R1 *drRRA* deletion mutant harboring kanamycin cassette	[20]
Δ*pprI* Δ*drRRA*	*D. radiodurans* R1 *pprI* and *drRRA* double deletion mutant harboring kanamycin and chloramphenicol cassettes	This study
Plasmids	Description	Source or reference
pMD18-T Simple	TA-cloning vector	TaKaRa, Japan
pKATcat	pUC19 vector with *Bam*HI-*Hin*dIII fragment which contains a *cat* gene	[28]
pTMRK	pMD18-T Simple derivative carrying kanamycin cassette with flanking upstream and downstream fragments of *drRRA*	This study
pTMRC	pMD18-T Simple derivative carrying chloramphenicol cassette with flanking upstream and downstream fragments of *drRRA*	This study

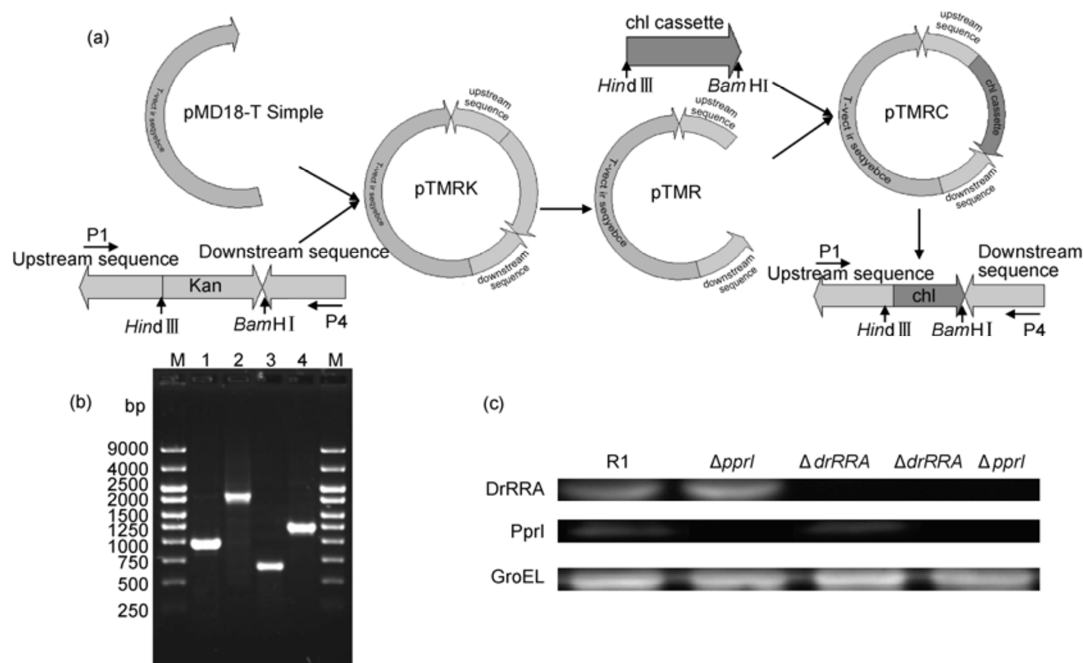

Figure 1 Construction and verification of the Δ*pprI* Δ*drRRA* double mutant. (a) A simple and convenient method for antibiotic-resistance cassette substitution. A fragment containing the kanamycin cassette and flanking *drRRA* sequences was amplified and ligated into the pMD18-T Simple vector to generate pTMRK. The kanamycin cassette was then digested and replaced by the chloramphenicol cassette, with the upstream and downstream fragments unchanged. Primers P1 and P4 were used for amplifying the tripartite ligation products. (b) Verification of the double mutant Δ*pprI* Δ*drRRA* by PCR. Lanes M, DNA molecular weight marker; Lanes 1 and 2, PCR products from *D. radiodurans* R1 and the double mutant with the upstream and downstream sequences of *pprI*; Lanes 3 and 4, PCR products from *D. radiodurans* R1 and the double mutant with the upstream and downstream sequences of *drRRA*. (c) Verification of the double mutant Δ*pprI* Δ*drRRA* by Western blotting. R1, wild type; Δ*pprI*, *ppr1*-deficient; Δ*drRRA*, *drRRA*-deficient; Δ*pprI* Δ*drRRA*, the double mutant. Total protein (100 μg) from cell extracts was loaded in each lane. Anti-GroEL antibody was used as the loading control.

sonication in phosphate-buffered saline (PBS) containing 1 mmol L^{-1} phenylmethanesulfonyl fluoride (PMSF) and 0.5 mmol L^{-1} ethylene diamine tetraacetic acid (EDTA). The protein concentration of the supernatant was determined using a Bradford Protein Assay Kit (Beyotime, China). Cell-free extracts containing 10 mg mL^{-1} of total protein were incubated with 400 μL of 10 mmol L^{-1} 2,4-dinitro- phenyl hydrazine (DNPH, dissolved in 2 mol L^{-1} HCl) for 1 h in the dark, and the mixture was vortexed once every 10 min. Protein was precipitated with ice-cold 20% (w/v) trichloroacetic acid (TCA), washed with ethyl acetate in ethanol (V:V=1:1) to remove the excess DNPH, and dissolved in 1.25 mL of guanidine hydrochloride (6 mol L^{-1}). The absorbance of the supernatant was determined at 370 nm. Protein carbonyl content was expressed as μmol mg^{-1} protein after subtracting the values obtained from unirradiated controls.

1.6 Pulsed-field gel electrophoresis (PFGE)

Sample preparation and PFGE analysis were performed essentially as described previously [20,30]. Briefly, bacteria (A_{600} = 0.4) were exposed to 800 Gy gamma radiation and incubated in fresh TGY broth. After 0, 90, 180 and 360 min of irradiation, cells were added to agarose plugs for sequential digestion with lysozyme in 0.05 mol L^{-1} EDTA, and proteinase K in 0.5 mol L^{-1} EDTA. The plugs were washed extensively with Tris-EDTA (TE) buffer, digested with *Not* I and subjected to PFGE.

1.7 RNA isolation and quantitative real-time PCR

Bacteria grown to A_{600} = 0.4 were irradiated with 800 Gy, incubated at 30°C for 30 min, and harvested. Total RNA was extracted using a Trizol kit (Invitrogen, USA), and treated with RNase-free DNase I (Promega, USA). RNA quality and quantity were evaluated using a NanoDrop Spectrophotometer (NanoDrop, USA). First-strand cDNA synthesis was carried out in 20 μL reactions with 1 μg purified RNA, and 3 μg random hexamers. The Quant SYBR Green PCR Kit (Tiangen, China) was used according to the manufacturer's instructions, and the constitutively expressed housekeeping gene *DR0089* was used as a normalization factor. mRNA levels of 8 antioxidant and DNA repair-related genes were measured.

2 Results

2.1 The double mutant showed increased sensitivity to gamma-radiation

Using the simple and convenient antibiotic-resistance sub-

stitution method described above, we engineered the Δ*pprI* Δ*drRRA* double mutant harboring kanamycin and chloramphenicol resistance gene cassettes. Simultaneous disruption of *pprI* and *drRRA* resulted in higher sensitivity to gamma radiation compared with either of the single deletion mutants (Figure 2(a)). The survival fraction of the Δ*pprI* Δ*drRRA* strain was approximately 10% after 250 Gy of acute irradiation, while both single mutants were only slightly affected at this dosage. The survival fraction decreased with increasing irradiation dose. When the dosage was increased to 800 Gy, the survival of Δ*pprI* Δ*drRRA* decreased to about 0.3%. By comparison, the Δ*drRRA* single mutant showed 20% survival, while the Δ*ppr1* single mutant showed 3% survival. At dosages higher than 2 kGy, the double mutant still displayed a higher sensitivity, though all the response curves began to flatten.

2.2 Level of protein carbonylation was elevated in the double mutant

Carbonylation levels of proteins in gamma-irradiated cells (800 Gy) were analyzed. As indicated in Figure 2(b), both single mutants exhibited higher levels of protein carbonylation compared with the wild-type R1 after gamma irradiation. The protein carbonyl content measured in wild-type R1, and in the Δ*pprI* and Δ*drRRA* mutants in response to gamma radiation was (6.43±0.26), (13.95±1.82) and (8.43±1.11) μmol mg^{-1}, respectively, while it rose markedly in the double mutant, reaching 20.59±1.34 μmol mg^{-1}. This result suggests that the mutant lacking both PprI and DrRRA is more sensitive to protein oxidative damage than either of the single mutants.

2.3 Increased delay of genome reconstitution following gamma irradiation in the double mutant

We compared the genome reconstitution process in wild-type R1 and in the three mutants under low dosage gamma irradiation (800 Gy). The reconstitution rate of the Δ*drRRA* single mutant was close to that of the wild type, while the Δ*pprI* single mutant recovered more slowly (Figure S1). As illustrated in Figure 2(c), an intact genome was formed 180 min after radiation exposure in Δ*pprI* cells. However, the recovery process was extended to 360 min in the double-knockout cells. Therefore, the simultaneous deletion of *pprI* and *drRRA* resulted in delayed genome reconstitution of *D. radiodurans*.

2.4 Induction of radiation-response genes was attenuated in the double mutant

Transcriptional levels of essential anti-oxidation and DNA

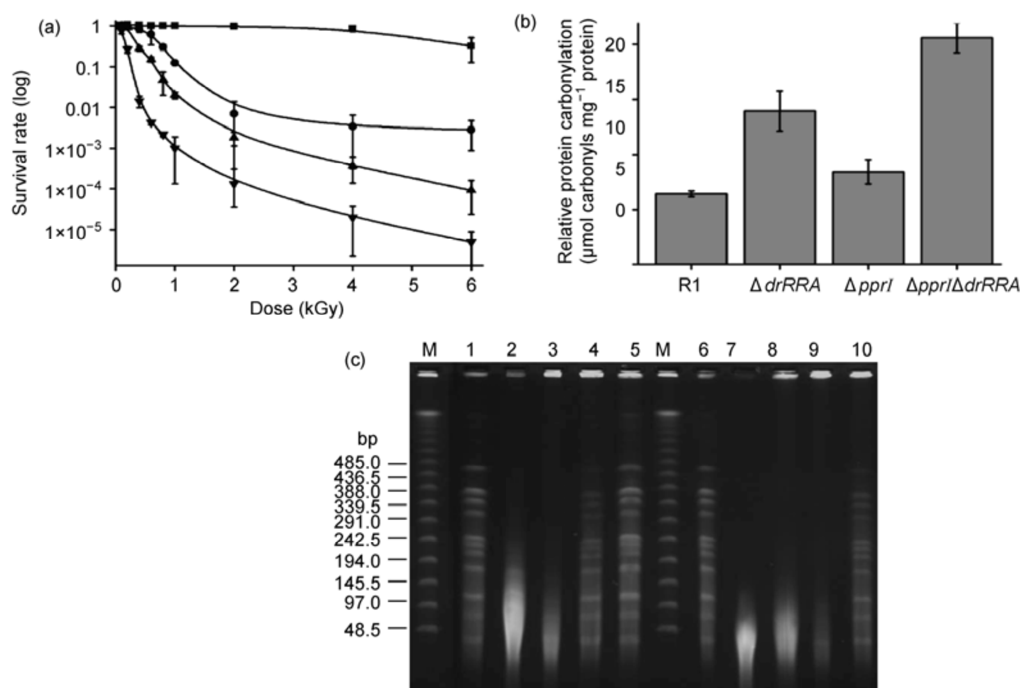

Figure 2 Combined effect of *pprI* and *drRRA* in the double mutant. (a) Survival curves for *D. radiodurans* following exposure to gamma radiation. Squares, wild-type R1; circles, Δ*drRRA* single mutant; upright triangles, Δ*pprI* single mutant; inverted triangles, Δ*pprI* Δ*drRRA*, double mutant. Values are the mean ± standard deviation of four independent experiments. (b) Comparison of intracellular protein carbonylation levels in wild-type and mutant strains after 800 Gy gamma radiation. After subtraction of the untreated background, values are the mean ± standard deviation of three independent experiments. (c) Genome recovery of Δ*pprI* and Δ*pprI* Δ*drRRA* following gamma radiation (800 Gy). The Δ*pprI* Δ*drRRA* double mutant showed a delay in intact genomic DNA restoration compared with the Δ*pprI* mutant after irradiation. M, Lambda ladder PFG marker (NEB); lanes 1 and 6, unirradiated Δ*pprI* and Δ*pprI* Δ*drRRA*, respectively; lanes 2–5, Δ*pprI* 0, 90, 180 and 360 min post-irradiation; lanes 7–10, Δ*pprI* Δ*drRRA* 0, 90, 180 and 360 min post-irradiation.

repair genes were evaluated in the double mutant. As shown in Table 2, the levels of certain antioxidation-related genes, such as *sod* (superoxide dismutase) (*DR1279* and *DR1546*) and *kat* (catalase) (*DR1998*) were clearly decreased in the double mutant relative to the single mutants and the wild type. Transcript levels of the DNA repair-related genes, *recA* (recombinase A) and *pprA* (pleiotropic protein promoting DNA repair), displayed a similar pattern: both genes were transcribed less in the double mutant compared with either of the single mutants, although transcript levels were not obviously reduced in the Δ*drRRA* single mutant after low dose gamma irradiation. To exclude the possibility of artifactual changes causing lethality, transcript levels of certain genes in the Δ*pprI* Δ*drRRA* double mutant were compared with those in a Δ*recA* mutant, which is extremely sensitive to gamma rays [31] (Table S1). *PprA* and *recA* are two essential elements located in diverse pathways in *D. radiodurans*. Both are greatly induced in response to gamma radiation [32]. *PprA* was significantly down-regulated in the double mutant, while it was dramatically up-regulated in the Δ*recA* mutant after gamma radiation. This suggested that the fold change in the double mutant was indeed related to gene regulation by PprI and DrRRA, and not due to decreased cellular survival.

2.5 Effect of PprI on DrRRA expression

The above results indicate that PprI and DrRRA collaborate in mediating cellular resistance to stress. In addition, subsequent investigation showed that PprI imposes an effect on DrRRA. As shown in Figure 3, DrRRA is moderately induced by gamma radiation in wild-type R1. However, the induction of DrRRA in the Δ*pprI* mutant was only half that of the wild type at 1 hour post-irradiation. In contrast, the levels of PprI in the Δ*drRRA* mutant remained unchanged, both after gamma radiation and under normal conditions. Thus, PprI might partially affect the expression of DrRRA in response to gamma radiation, indicating roles in a common pathway in addition to the non-overlapping functions described above.

3 Discussion

Previous studies have shown that anti-oxidative metabolism is a process critical to extreme radioresistance [14]. As an important type of oxidative modification, protein carbonylation could severely impair the integrity of both the genome and proteome, resulting in altered radioresistance [33].

Table 2 Real-time PCR of DNA repair and anti-oxidation-related genes in the mutants relative to wild-type R1 after gamma radiation (800 Gy)

Locus	Annotation[a)]	Fold change (±SD)		
		Δ*drRRA* (+γ)/R1(+γ)	Δ*pprI* (+γ)/R1(+γ)	Δ*pprI* Δ*drRRA* (+γ)/R1(+γ)
DR1279	Superoxide dismutase Mn (SodA)	−2.78 (±0.14)	−2.77 (±0.19)	−5.16 (±0.18)
DRA0202	Superoxide dismutase CU-ZN (SodC)	−5.02 (±0.79)	−2.74 (±0.21)	−3.82 (±0.82)
DR1546	Copper/zinc-superoxide dismutase (SodC)	−3.89 (±0.19)	−2.77 (±0.08)	−8.38 (±0.15)
DR0146	Catalase (KatA)	−1.29 (±0.01)	−1.67 (±0.15)	−3.01 (±0.20)
DRA0259	Catalase with C-terminal domain similar to FABB domain (KatE)	−3.40 (±0.26)	−3.69 (±0.22)	−4.45 (±0.11)
DR1998	Catalase (KatE)	−5.18 (±0.18)	−2.47 (±0.19)	−10.07 (±0.06)
DR2340	Recombinase (RecA)	−1.65 (±0.20)	−11.16 (±0.14)	−23.98 (±0.11)
DRA0346	predicted protein (PprA)	−1.58 (±0.21)	−9.85 (±0.30)	−13.23 (±0.21)

a) Functional annotation is based on KEGG (http://www.genome.jp/kegg/).

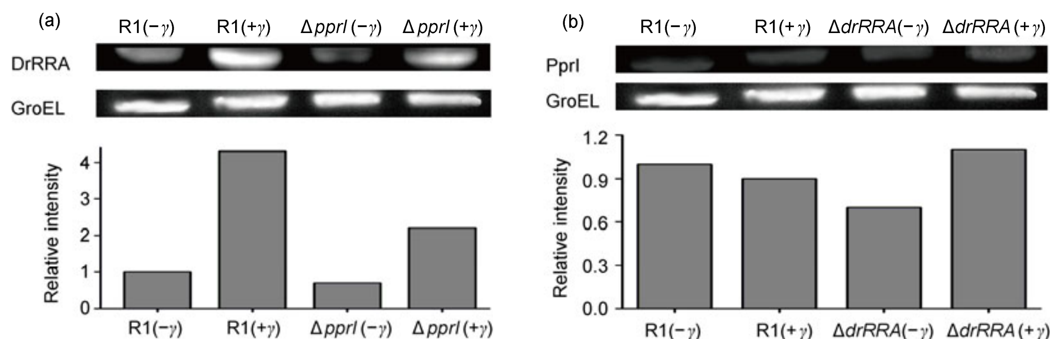

Figure 3 Western blot analysis of DrRRA (a) and PprI (b) levels in wild type and mutants. R1, wild type; Δ*pprI*, *ppr1*-deficient; Δ*drRRA*, *drRRA*-deficient. Minus γ (−γ), non-irradiated; plus γ (+γ), post-gamma radiation. Total protein (100 μg) from cell extracts was loaded in each lane. Anti-GroEL antibody was used as the loading control.

Recently, it has been shown that the extraordinary robustness of *D. radiodurans* depends on efficient proteome protection [34]. In our study, intracellular protein carbonylation levels in the double mutant were significantly higher compared with the single mutants after treatment with gamma radiation, suggesting a combined effect of PprI and DrRRA on inhibition of *in vivo* protein carbonylation. In particular, KAT and SOD are regarded as major ROS-scavenging proteins that prevent DNA damage caused by hydrogen peroxide and superoxide anion radicals, thus contributing to the extreme radioresistance of *D. radiodurans* [32]. Here, transcriptional levels of the two superoxide dismutases and of one catalase were further reduced in the double mutant compared with the single mutants, demonstrating that PprI and DrRRA might operate additively in the defense of *in vivo* oxidation through common downstream elements.

Extreme ionizing radiation causes numerous double- and single-strand breaks, as well as damage to the nucleotide bases. These lesions are lethal to most organisms [8,35]. Strikingly, *D. radiodurans* is able to efficiently reconstitute hundreds of short fragments shattered by gamma ray bombardment into a functional genome with high fidelity. However, if essential genes in this process are disrupted, the ability of DNA repair might be reduced, delaying the restoration of the completed genome [20,21,30,36,37]. In this work, an extended delay was apparent in the double-mutant cells relative to the Δ*pprI* and Δ*drRRA* single mutants following gamma irradiation. Specifically, induction of two essential DNA repair and protection genes, *recA* and *pprA*, were dramatically down-regulated in the double mutant after gamma irradiation, compared with transcript levels in the single mutants. This down-regulation might contribute to the delayed genome reconstitution and reduced radioresistance of the double knockout cells, because sufficient levels of *recA* and *pprA* are necessary for DNA double-strand break repair in *D. radiodurans* [36,38,39]. We propose that PprI and DrRRA coordinate common anti-oxidation protection/repair related elements to cope with extreme gamma radiation.

In addition, our results reveal that deletion of *pprI* in the wild type leads to a reduced accumulation of DrRRA protein following gamma radiation. Therefore, the production of *pprI* might influence the expression of *drRRA* when this bacterium suffers DNA damage and oxidation stress. The newly discovered *D. radiodurans* cold-shock protein, PprM (modulator of the PprI-dependent DNA damage response), is also involved in the response to radiation, mediated by PprI [22]. We hypothesize that PprI might be a general DNA damage response regulator, governing a group of sub-regulators to finely tune the complicated cellular processes induced upon stress so as to survive the extreme gamma radiation.

This study shows a close cooperation between DrRRA and PprI. We postulate that PprI and DrRRA belong to different pathways, but that they also produce additive effects on certain common elements, so as to collaborate in the response to extreme gamma-irradiation. When DNA damage occurs, PprI might be activated, resulting in induced expression of a series of DNA repair and protection genes, such as *recA* and *pprA*. DrRRA might also affect the above elements and other targets involved in DNA repair and anti-oxidation. Meanwhile, PprI may partially influence the expression of DrRRA *in vivo*. Thus, PprI and DrRRA act in concert through common and specific cellular components to ensure the extreme radioresistance of *D. radiodurans*.

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Supporting Information

Figure S1 Genome recovery of wild type R1, the mutants Δ*drRRA* and Δ*pprI* following gamma-radiation (800 Gy). The mutant Δ*drRRA* showed no delay in intact genomic DNA restoration compared with wild type R1 after gamma-radiation. However, the mutant Δ*pprI* showed a 45-min-delay relative to wild type R1. λ-ladder was the PFGE molecular marker (NEB). Lane C, unirradiated cells; lanes 1–6, post-irradiation time 0, 45, 90, 180, 270 and 360 min.

Table S1 Real-time PCR of some anti-oxidation and DNA repair related genes in the double mutant relative to the mutant Δ*recA* after gamma-radiation (800 Gy)

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