RNA-Binding Domain is Necessary for PprM Function in Response to the Extreme Environmental Stress in *Deinococcus radiodurans*

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Abstract *Deinococcus radiodurans* was considered as one of the most radiation-resistant organisms on Earth because of its strong resistance to the damaging factors of both DNA and protein, including ionizing radiation, ultraviolet radiation, oxidants, and desiccation. PprM, as a bacterial cold shock protein homolog, was involved in the radiation resistance and oxidative stress response of *D. radiodurans*, but its potential mechanisms are poorly expounded. In this study, we found that PprM was highly conserved with the RNA-binding domain in *Deinococcus* genus through performing phylogenetic analysis. Moreover, the paper presents the analysis on the tolerance of environmental stresses both in the wild-type and the *pprM/pprM* RBD mutant strains, demonstrating that *pprM* and RNA-binding domain disruptant strain were with higher sensitivity than the wild-type strain to cold stress, mitomycin C, UV radiation, and hydrogen peroxide. In the following step, the recombinant PprM was purified, with the finding that PprM was bound to the 5'-untranslated region of its own mRNA by gel mobility shift assay in vitro. With all these findings taken into consideration, it was suggested that PprM act as a cold shock protein and its RNA-binding domain may be

involved in reaction to the extreme environmental stress in *D. radiodurans*.

Keywords *Deinococcus radiodurans* · PprM · Environmental stress · RNA-binding domain

Introduction

Deinococcus radiodurans is unparalleled among all known species when it comes to its ability to stand up the DNA-damaging conditions, which contain ionizing radiation, UV light and desiccation [1–3]. This bacterium can survive in case of suffering from acute exposure to 15-kGy β -ray and chronic γ -radiation at a dose rate of 60 Gy/h without the significant death of any cell [4, 5], while only 5 Gy of γ -radiation will be able to kill a human and nearly 200–800 Gy of γ -radiation will kill *E. coli* [6]. Ionizing irradiation will lead to the DNA double-strand breaks (DSBs) which are lethal to nearly all the organisms. However, *D. radiodurans* can survive even after undergoing over 200 genomic DSBs caused by ionizing radiation; meanwhile, the genome is reassembled accurately before the beginning of the next cycle of the cell division [2]. It has been determined that *D. radiodurans* possesses almost a complete set of DNA repair pathways, such as recombination repair, mismatch repair, base excision repair, and non-homologous end-joining repair [1, 7]. Apart from the efficient DNA repair mentioned above, radiation tolerance for *D. radiodurans* might result from the protection of proteins against oxidation [1, 7]. In addition, a highly condensed nucleoid structure of *D. radiodurans* is beneficial to the DNA damage repair after suffering the exposure to ionizing radiation [8].

Wei Li and Yun Ma have contributed equally to this work.

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Both the transcriptomic and proteomic analyses have concluded that a large number of genes and proteins play an important part in the radiation tolerance of *D. radiodurans*. PprI (also referred to as IrrE) coding by DR_0167, as a global regulator, is known for its ability to regulate several pathways for its extreme resistance, such as DNA damage response, oxidative stress response, energy metabolism, and signal transduction [9]. PprI leads to the expression of a number of genes, including *recA*, *pprA* and other radiation-responsive genes after exposing to the gamma radiation [9, 10]. Moreover, PprI plays a key role in the regulation network of cellular resistance following radiation damage, while at the same time it is able to regulate the DNA damage response. PprM (DR_0907), as a modulator of the PprI-dependent DNA damage response to radiation stress, is involved in repressing the production of PprA and was proved to be essential for KatE1 production under normal cultural conditions [11, 12]. As shown from the nucleotide sequence analyses, PprM is endowed with high sequence identity to bacterial cold shock protein homolog [11]. However, the proteomic analysis suggested that PprM was a heat shock-induced protein rather than a cold shock-induced protein [13]. These results indicated that PprM protein possesses many potential and important functions, which further investigation is required. In this study, we constructed *pprM* deletion and *pprM* RNA-binding domain deletion mutants to investigate the physiological role of PprM in *D. radiodurans*.

Materials and Methods

Bacterial Strains, Plasmids, Medium and Growth Conditions

Deinococcus radiodurans R1 (ATCC 13939) was grown in TGY medium (0.5% Bactotryptone, 0.3% Bacto Yeast Extract, 0.1% Glucose) at 30 °C or on TGY plates supplemented with 1.5% (w/v) agar. The *E. coli* strain BL 21(DE3) and JM109 was cultured in LB medium at 37 °C. Ampicillin (100 µg/ml), and kanamycin (50 µg/ml) were added to the medium if required. *E. coli* and *D. radiodurans* were grown as batch cultures in LB broth or TGY broth, with the shaking at 220 rpm. All strains and plasmids used in this study were listed in Table 1.

Construction of *pprM/pprM*-RBD Gene Mutants

As described above, gene mutation targeting *pprM* and RNA-binding domain were performed with modifications [14, 15]. To construct *pprM* gene mutants, the DNA fragments upstream and downstream of *pprM* were amplified by PCR with the employment of *D. radiodurans* genomic

DNA, the oligonucleotide-primer sets DR_0907 UF/DR_0907UR and DR_0907 DF/DR_0907 DR respectively. The PCR fragments were amplified by overlapping PCR through the oligonucleotide primer sets DR_0907 UF and DR_0907 DR. Besides, the final PCR fragments and pk18mobsac B plasmid were handled by *EcoR* I and *BamH* I respectively, after which they were ligated by T₄ DNA ligase so as to construct pk18mobsac B- Δ *pprM* plasmid. Then, the pk18mobsac B- Δ *pprM* plasmid was transformed into the wild-type *D. radiodurans* cells in the phase of the exponential growth and grew at solidified TGY medium with 50 µg/ml kanamycin. Subsequently, the single recombination positive clones were obtained and confirmed by PCR using the oligonucleotide primer sets DR_0907 UF/DR_0907 DR. Furthermore, the second round of screening *pprM* mutants XR1 was carried out using the TGY plates supplemented with 10% sucrose. The positive clones were picked and grew at solidified TGY medium supplemented with kanamycin and kanamycin-free mediums, respectively. The mutant XR1 that grew in kanamycin-free medium and did not grow in kanamycin-containing plate has been obtained. Finally, the mutant XR1 was confirmed by PCR and sequencing. For the gene mutation of *pprM* RNA-binding domain, the similar methods were constructed pk18mobsac B- Δ *pprM* to yield pk18mobsac B- Δ RBD through the oligonucleotide primer sets DR_0907RBD UF/DR_0907RBD UR, DR_0907RBD DF/DR_0907RBD DR and DR_0907RBD UF/DR_0907RBD DR. Subsequently, the pk18mobsac B- Δ RBD plasmid was transformed into wild-type *D. radiodurans*. Then, *pprM* RNA-binding domain mutants XRBD were screened by using 10% of sucrose. Besides, mutant XRBD was determined by PCR using the oligonucleotide primer sets DR_0907RBD F/DR_0907RBD R and sequencing. All the nucleotide primers applied in this research showed in Supplementary Table 1.

Protein Expression and Purification

The coding sequence of *pprM* (DR_0907) has been amplified through PCR from *D. radiodurans* genomic DNA and inserted into pGEX-6p-1 vector at the *EcoR* I and *Xho* I sites so as to create plasmid pGEX-6p-1-*pprM*. The plasmid pGEX-6p-1-*pprM* was transferred into *E. coli* BL21 (DE3) and expressed recombinant PprM contained an N-terminal GST-Tag. The *E. coli* BL21 (DE3) clones were cultivated at 37 °C in LB medium with 100 µg/ml ampicillin to an optical density at 600 nm of 0.6–0.8. The protein expression was triggered at 30 °C for 6 h after added isopropyl- β -D-thioga-lactopyrano side (IPTG) at a final concentration of 0.3 mM. The recombinant protein was purified by One-Stop GST-Tagged Protein Miniprep

Table 1 Strains and plasmids used in this study

Strains and plasmids	Description	Source
Strains		
<i>D. radiodurans</i>		
R1	Wild-type (ATCC 13939)	Lab stock
XR1	R1 but <i>pprM</i> (DR_0907) deleted	This study
XRBD	R1 but <i>pprM</i> Δ RNA-binding domain	This study
YR1	XR1 with pRADK- <i>pprM</i>	This study
<i>E. coli</i>		
JM109	Host for cloning vectors	Lab stock
BL21(DE3)	Host for gene expression	Lab stock
Plasmids		
pk18mobsac B	A suicide plasmid for gene mutation	ATCC
pk18mobsac B- Δ <i>pprM</i>	pk18mobsac B containing the DNA fragment for Δ <i>pprM</i>	This study
pk18mobsac B- Δ RBD	pk18mobsac B containing the DNA fragment for Δ RBD	This study
pGEM-T Easy	Plasmids for gene subclones	Promega
pGEX-6p-1	Plasmids for gene expression	Lab stock
pGEX-6p-1- <i>pprM</i>	pGEX-6p-1 containing the wild-type <i>pprM</i> gene	This study
pRADK- <i>pprM</i>	pRADK containing the wild-type <i>pprM</i> gene	Lab stock

Pack (Tiandz Inc, China). Subsequently, the products were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue.

Gel-Shift Assay for *pprM* mRNA and PprM

As described above, gel-shift assay was performed with some modifications [16]. To obtain the 5'-UTR in the potential new start site of *pprM* mRNA (new start site was showed in Supplementary Fig. 1), a transcription in vitro was carried out through the T7 RNA production system (Promega, USA) in line with the instructions provided by the manufacturer. The purified recombinant PprM (5 μ g) was incubated with 5'-UTR of *pprM* (0.2 μ g) in PBS buffer (including 140 mM NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate and 10 mM KCl, pH 7.4) at 4 °C for 2 h after gentle mixing. Hereafter, the admixtures were mixed with the loading buffer, and analyzed on 1.5% agarose gels electrophoresis at 80 V. Gels were stained with ethidium bromide and Coomassie Brilliant Blue respectively.

Growth Curves and Survival Tests

The growth curves of the wild-type, *pprM* complement and *pprM/pprM* RBD mutation strains were determined to the optical density at 600 nm (OD₆₀₀), with all strains cultivated in 4 ml of liquid TGY medium until the OD₆₀₀ reached 0.8. Moreover, the cells were diluted with 100 ml of fresh TGY medium to 1:50. In addition, the cultivations were established at 30 and 15 °C with the shaking rate of 220 rpm, followed by the detection of the OD₆₀₀ every 2 h.

In the MMC sensitivity test, cells were diluted to 50 times and treated at a final concentration of 20 μ g/ml MMC, and then smeared evenly across the surface of TGY plates every 5 min. In the UV sensitivity test, cells at the mid-log phase were diluted with fresh TGY medium. Besides, samples were taken 200 μ l plated onto the TGY agar plates, and then suffered from the UV radiation (800 J/m²) with different exposure durations. In the H₂O₂ sensitivity assay, all cultivations with an OD₆₀₀ of $\sim$ 1.0 were treated in different concentrations (including 10, 20, 30, 40, and 50 mM) of H₂O₂ for 30 min at room temperature. Besides, an excess of catalase was added to deactivate H₂O₂. Following, these *D. radiodurans* cells were diluted quantitatively 50-fold with fresh TGY medium and taken 200 μ l plated onto the TGY agar plate. All the plates were cultivated at 30 °C for 3 days to complete the colonies count. The data represent both the means and standard deviations of three independent experiments.

Results

Amino Acid Sequence Analysis of PprM Proteins in *Deinococcus*

Figure 1a showed the phylogenetic relationship PprM homologs from the genus *Deinococcus* included *Deinococcus radiodurans*, *Deinococcus geothermalis*, *Deinococcus deserti*, *Deinococcus maricopensis*, *Deinococcus proteolyticus*, *Deinococcus peraridilitoris* and *Deinococcus gobiensis*. The phylogenetic tree contained three subfamilies and PprM (DR_0907) belongs to a

Fig. 1 Amino acid sequence analysis of PprM proteins in *Deinococcus*. **a** Molecular phylogenetic relationship of PprM homologs among *Deinococcus radiodurans*, *Deinococcus geothermalis*, *Deinococcus deserti*, *Deinococcus maricopensis*, *Deinococcus proteolyticus*, *Deinococcus peraridilitoris* and *Deinococcus gobiensis*. Multiple alignment and phylogenetic tree were built using the MEGA program

distinct clade of subfamily together with PprM homologs from *Deinococcus geothermalis*. Interestingly, *Deinococcus radiodurans* (DR_0907), *Deinococcus proteolyticus* (Deipr_0868) and *Deinococcus peraridilitoris* (Deipe_0736) only had one PprM homolog; *Deinococcus geothermalis* (Dgeo_1006, Dgeo_0638), *Deinococcus maricopensis* (Deima_1320, Deima_1260) and *Deinococcus gobiensis* (DGo_PA0041, DGo_CA1136) owned two PprM homologs, while *Deinococcus deserti* (Deide_3p00840, Deide_2p00490, Deide_09930) included three PprM homologs. As showed from the result of this multiple alignment, PprM homologs from *Deinococcus* with high sequence identity, and all of them contained two RNA-binding motifs and four DNA-binding sites (Fig. 1b). Besides, PprM homologs consisted of a rich content of aromatic residues, which was essential for various proteins binding to RNA. Based on the data, these proteins were highly conserved and could bind RNA with *Deinococcus*. The annotated N terminus of PprM protein from *D. radiodurans* (DR_0907) was different from that of the other *Deinococci*. It was suggested by the nucleotide sequence analyses that translation initiation may occur at an AUG codon downstream of the annotated start codon of DR_0907, which would create a protein being 47 residues

[17, 18]. **b** Multiple amino acid sequence alignment of PprM. Sequences was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>). RNA-binding domains, RNP1 and RNP2 were boxed. Asterisks indicate identical residues, dashes indicate gaps in the alignment, and triangles indicate DNA-binding sites

shorter than the previously annotated one (Supplementary Fig. 1).

Delete RNA-Binding Domain of PprM Cause *D. radiodurans* Cells Growth Slowed Down Sharply

PprM was considered as a cold shock protein homolog with RNA-binding domain in *D. radiodurans*. For further investigated whether PprM and its RNA-binding domain was involved in tolerance cold stress of *D. radiodurans*, comparison had been made among the cell viability of the wild type (R1), a pprM mutant strain (XR1) and a pprM RNA-binding domain mutant strain (XRNP) at 30 and 15 °C, respectively. As showed in Fig. 2a, the loss of PprM and its RNA-binding domain was retarded the cells growth sharply under normal cultural conditions. However, PprM and its RNA-binding domain disrupted strains were barely growing at 15 °C (Fig. 2b). These results suggested that PprM may play an important role in growing and the resistance to cold stress of *D. radiodurans*.

Fig. 2 Cell growth curve of *D. radiodurans* strains. **a** Culture temperature was 30 °C, **b** Culture temperature was 15 °C. R1, wildtype strain; XR1, *pprM*-knockout R1strain; XRBD, *pprM*

Δ RNA-binding domain R1 strain, Data represent the mean and standard deviation of three independent experiments

PprM Protein Bound to 5'-Untranslated Region of its Own mRNA in Vitro

As revealed in the bioinformatic analyses, PprM was a cold shock protein homolog and contained RNA-binding domain. To detect whether PprM with RNA-binding activity, RPI-Pred online software was employed to predict the interactions between PprM protein and the 5'-UTR of its own mRNA on the basis of the sequence and structural information, the result proved the possibility of the interaction (Data not show). To further investigate whether PprM possessed RNA-binding activity, the wild-type PprM protein with a GST-tag at N-terminal has thus been purified (Fig. 3a). Therefore, the 5'-UTR of the *pprM* mRNA was transcribed in vitro (Fig. 3b). In the gel mobility shift assay, the 5'-UTR of the *pprM* mRNA was significantly retarded by PprM protein (Fig. 3c). It was suggested from

the result that PprM protein may bind to 5'-untranslated region of its own mRNA in vitro.

Deleted RNA-Binding Domain of PprM Resulted in *D. radiodurans* Cells Sensitive to DNA-Damaging Agents

To determine whether the RNA-binding activity of PprM contributes to its extreme resistance capability, the survived XR1 strain and XRNP strain were researched under the stress of DNA-damaging agents. The *pprM*-deficient strain XR1 and RNA-binding domain mutant strain XRNP have displayed markedly higher sensitivity to mitomycin C (Fig. 4a), UV radiation (Fig. 4b) and hydrogen peroxide (Fig. 4c) than that of the wild-type strain. Compared with the wild-type *D. radiodurans* strain which almost fully restores the resistance to DNA-damaging agents, the *pprM*-deficient strain XR1 and RNA-binding domain mutant

Fig. 3 Gel mobility shift assay for the binding of PprM to RNA. **a** Purification of GST-PprM protein. Samples were subjected to 15% SD-PAGE and then stained with Coomassie Brilliant Blue. Lane M, Protein Ladder (Beyotime); lanes 1–2, GST- PprM protein. **b** Transcription 5'-UTR of *pprM* mRNA in vitro. Lane M, DNA Ladder (CWBIO); lane 1, DNA template of 5'-UTR of *pprM* mRNA; lanes

2–3, transcription products of 5'-UTR in vitro; **c** RNA-binding assay of PprM by gel mobility shift assay. Lane 1, transcription products of 5'-UTR (0.2 µg); lanes 2–3, mixture of transcription products of 5'-UTR (0.2 µg) and GST- PprM protein (0.5 µg). **d** Gel in **c** stained with Coomassie Brilliant Blue

Fig. 4 Survival fractions of *D. radiodurans* strains under **a** Mitomycin C, **b** UV radiation and **c** hydrogen peroxide. R1, wildtype strain; XR1, *pprM*-knockout R1 strain; YR1, XR1 with pRADK-

pprM; XRBD, *pprM* Δ RNA-binding domain R1 strain. Data represent the mean and standard deviation of three independent experiments

strain XRNP have restored only partial resistance. Besides, the resistance of *pprM*-complementary strain YR1 was between the wild-type *D. radiodurans* and the *pprM* mutant strain, which indicated the importance of RNA-binding domain of PprM for the normal function and plays an important role in the extreme resistance.

Discussion

Due to the rapid decrease of the temperature, bacterial cold shock proteins were induced to regulate the tolerance to cold stress as well as other biological processes under normal growth conditions [19]. PprM proteins, as homologs of bacterial cold shock protein [11, 20], have high sequence identity on the basis of the sequence analyses (Fig. 1). In this study, the *pprM* mutant strain and *pprM* RNA-binding domain mutant strain were barely grown under the cultural conditions of 15 °C (Fig. 2), suggesting that PprM plays a critical role in resisting cold stress which needs RNA-binding domain. In the previous study, the cold shock protein was reported that bound its own 5'-untranslated region and regulate gene expression in *Escherichia coli* [21]. Therefore, the paper presents the analysis on the RNA-binding capacity of PprM, finding it also bound to the 5'-UTR of its mRNA in vitro (Fig. 3). This result shows that PprM with RNA-binding activity is similar to other bacteria cold shock protein [21]. However, the previous data of proteomic analysis indicated that the expression of PprM was achieved significantly increase under the induction of the heat shock [13]. Thence, further investigations will be necessary to delineate the cold shock role of PprM in *D. radiodurans*.

PprM is a modulator of the PprI-dependent DNA damage response [11]. PprI has been reported that as a global regulator, can regulate the DNA damage response and the cellular survival networks after the radiation damage [10]. PprI induced the production of RecA and PprA in response

to the ionizing radiation [9]. RecA and PprA are key DNA repair proteins that play important roles in response to radiation stress in *D. radiodurans* [22]. Interestingly, PprM was found involved in repressing the emergence of PprA under irradiation [11], which indicated that PprI and PprM were participated in positively and negatively regulating the expression of PprA, respectively. In addition, PprM is necessary to produce KatE1 by the inhibition of *katE1* negative regulators in *D. radiodurans* under the normal cultural condition [12]. All these results demonstrate that PprM can play a regulatory role without suffering the cold shock. Besides, the previous studies have indicated that the *pprM* mutant strain displayed higher sensitivity to gamma rays than that of the wild-type [11]. Here, it was found that *pprM* and *pprM* RNA-binding domain mutant strain also demonstrated higher sensitivity to mitomycin C (Fig. 4a), UV radiation (Fig. 4b) and H₂O₂ (Fig. 4c) than the wild-type. These results indicated that PprM contribute to the extreme environmental stress resistance of *D. radiodurans*.

PprM is the only cold shock protein homolog in *D. radiodurans*. For decades, only a few studies have reported the biological function of PprM and we know little about it. Based on our findings, it is proposed that the RNA-binding domain may play an essential role in exerting PprM functions to adapt to the extreme environmental stresses. However, the role and molecular mechanism of PprM in resistance to extreme environmental stress remain still unresolved. Additional studies are required to elucidate the regulatory mechanisms of PprM and its RNA-binding domain during stress responses in *D. radiodurans*.

Conclusion

Our results suggest that PprM is a cold shock protein and its RNA-binding domain is necessary for PprM function in reaction to the extreme environmental stress in *D. radiodurans*.

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Compliance with Ethical Standards

Conflicts of interest All authors declare no conflicts of interest.

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