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ORIGINAL ARTICLE

Knockout of *pprM* Decreases Resistance to Desiccation and Oxidation in *Deinococcus radiodurans*

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Abstract *Deinococcus radiodurans* has attracted a great interest in the past decades due to its extraordinary resistance to ionizing radiation and highly efficient DNA repair system. Recent studies indicated that *pprM* is a putative pleiotropic gene in *D. radiodurans* and plays an important role in radioresistance and antioxidation, but its underlying mechanisms are poorly elucidated. In this study, *pprM* mutation was generated to investigate resistance to desiccation and oxidative stress. The result showed that the survival of *pprM* mutant under desiccation was markedly retarded compared to the wild strain from day 7–28. Furthermore, knockout of *pprM* increases the intercellular accumulation of ROS and the sensibility to H_2O_2 stress in the bacterial growth inhibition assay. The absorbance spectrum experiment for detecting the carotenoid showed that deinoxanthin, a carotenoid that peculiarly exists in Deinococcus, was reduced in the *pprM* mutant in the *pprM* mutant. Quantitative real time PCR showed decreased expression of three genes viz. *CrtI* (DR0861, 50%),*CrtB* (DR0862, 40%) and *CrtO* (DR0093, 50%), which are involved in deinoxanthin synthesis, and of Dps (DNA protection during starving) gene (DRB0092) relevant to ion combining and DNA protection in cells. Our results suggest that *pprM* may affect antioxidative ability of *D. radiodurans* by regulating the synthesis of deinoxanthin and the concentration of metal ions. This may provide new clues for the treatment of antioxidants.

Keywords *Deinococcus radiodurans* · *pprM* · Deinoxanthin · Antioxidantion

Introduction

D radiodurans is an orange-pink, non-pathogenic bacteria well-known for the robust resistance to a range of ambient stresses such as ionizing radiation, UV, oxidatives and desiccation [1]. PprM is a radiation stress response protein, which deletion increases the sensitivity of *D. radiodurans* to γ-rays and UV and also regulates the catalase KatE1 in *D. radiodurans* [2]. However, the detailed underlying molecular mechanism about *pprM* against envirionment stresses or attacks remains poorly understand. We previously used the overlap-PCR to knockout the *pprM* gene and constructed a *pprM* mutant strain (Δ*pprM*) (detailed procedures are not mentioned in this article and is in press). When exploring the functional role that *pprM* plays in *D. radiodurans,* we found that the Δ*pprM* present a lighter color than the wild type.

Reactive oxygen species (ROS) is a by-product of water after radiation. When facing extremely situations, more ROS would be produced in cells. The free oxygen radicals would damage DNA, RNA and proteins, thus the vitality of

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life would be threatened. Scavenging of ROS ability reflect the capacity of anti-oxidation in cells. *D. radiodurans* posseses a robust system on withstanding oxidizing material, which system includes the enzyme and non-enzyme components. Superoxide dismutase (SOD) and catalase (CAT) are two important antioxidant enzymes. The activity of SOD is 6 times and the CAT is 30 times higher than *E. coli* [3]. The main non-enzyme component includes carotenoid. Deinoxanthin is a carotenoid that peculiarly exists in *Deinococcus* and is related to the strain's color [4]. It could protect DNA from the attacks and make tremendous contributions to the oxidation resistance [5]. Previous research discovered that *CrtI* (DR0861), *CrtB* (DR0862) and *CrtO* (DR0093) are three main genes that influence the biosynthesis of deinoxanthin, the deletion of these genes resulted the bacterial to be colorloss and also brings hypersensitivity to oxidative stresses [6]. The highly ratio of Mn(II)/Fe(II) in cells is another non-enzyme mechanism that facilitates the defence against oxidative damage. The abundance of Mn ions assist in removing ROS, however, the Fenton action of Fe^{2+} would bring huge damages to cells. Dps is a functional protection protein which can combine with Fe^{2+} and oxidize Fe^{2+} to Fe^{3+}, and also has high affinity in binding with DNA, consequently prevent hydroxyl free radical from damaging DNA. These prominent features allow Dps detoxify Fe^{2+} and H_2O_2 simultaneously [7].

Based on our experimental results, we hypothesized that *pprM* may facilitate the antidesiccation ability and the decrease in antioxidation and the knockout of *pprM* may result in the reduction of deinoxanthin and Dps through inhibiting gene expression of no-enzyme antioxidant relevant to their synthesis.

Material and Method

Bacterial Strains and Growth Conditions

The wild type *D. radiodurans* R1 (CGMCC 1.633) was purchased from the China General Microbiological Culture Collection Center, and the *pprM* mutant were stored in laboratory and all cultivated at 30 °C in TGY broth (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) or on TGY agar plates (1.5% agar) [8].

Desiccation Assay

The desiccation procedure was modified [9], as the allochroic silicagel was placed into a sterile beaker and used as desiccant to create a desiccative condition. Both the wild type and the *pprM* mutant were incubated for 48 h. The bacterial cultures were centrifuged at 6,000 rpm for 15 min and resuspended with 1XPBS. Four small EP tubes (1.5 ml) were added with 100 µl suspension, then subjected to 7, 14, 21 and 28 days of desiccative condition. After the desiccative time, the dehydrated cells were resuspended with sterilized water and diluted to 10^{-1}–10^{-5} times, 6 µl of each diluted culture was spotted on the TGY medium and incubated at 30 °C for 3 days.

H_2O_2 Oxidation Assay

The detection of sensitivity to H_2O_2 was analyzed by using the disc inhibition assay [10]. The bacteria were cultivated in TGY broth for about 48 h 1 ml of each culture was spread-plated onto TGY agar plates by using a spreading rod, then 4 sterilized filter paper discs with 6 mm diameter were placed on the agar surface. 10 µl of H_2O_2 with different concentration (50, 100, 150 and 200 mM) was dropped on the paper disc respectively. The diameter of the growth inhibition zone was measured after cultivated at 30 °C for 3 days. (The wider of the sterility zone, the more sensitive to H_2O_2).

Intracellular ROS Accumulation Assay

ROS generation assay was performed according to previous description with some modification [11]. In brief,100 µL of exponential phase cultures were washed 3 times with 1XPBS, then resuspended with 1 ml 1XPBS containing 1 µL DCFH-DA(10 µM) and incubated at 37 °C for 20 min that avoided light with shaking. The cells were washed twice by 1XPBS and resuspended in 1 ml 1XPBS. Each cell suspension was equally dispensed into two parts, one of which was exposed to 2KGy of γ radiation and the other part was placed under room temperature as control. ROS would oxidize DCFH into DCF which is fluorescent. Thus, the fluorescence intensities that were measured using a fluorescence spectrophotometer (Shimadzu Japan) with an excitation wavelength of 485 nm and emission wavelength of 525 nm would reflect the ROS accumulation in cells.

Carotenoid Assay

The wild type and the Δ*pprM* were cultivated to exponential phase for about 48 h, then 1% of the cultures were dropped into 50 mL TGY fresh medium at 30 °C. Both of cultures were centrifuged 6,000 rpm,4 °C for 10 min.Cells were collected and washed by dd H_2O for twice. The supernatant were discarded and the substrates were dissolved by pre-cooled acetone and methanol (V/V = 7:2), after shaking under shadow for 30 min, the solution were centrifuged under 10,000 rpm for 10 min and the supernatants were collected. The procedures were repeated twice

or until the color fades. The supernatants were detected on the spectrophotometer (Shimadzu Japan), absorbance spectrum is from 350–600 nm.

RNA Isolation, Quantitative Real-Time PCR (QRT-PCR) Experiment

The wild and Δ*pprM* were cultivated for about 40 h in an appropriate condition until OD = 0.4, total RNA was extracted from the bacterial cultures with Trizol Reagent (Invitrogen Life Technologies) according to manufacture's manual. Complementary DNA was synthesized in 20 μL of reaction containing 2 μg of RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo). Gene expression in RNA level was evaluated using quantitative PCR with Quant SYBR Green PCR kit (Roche). Triplicate reactions were carried out for each sample. The primers used in QRT-PCR were listed in Table 1. The 16sRNA was used as a reference for normalization. Fold change was determined using the $2^{\Delta\Delta CT}$ method.

Results and Discussion

pprM Mutant Strain is More Sensitive to Desiccation

Desiccation would occur extensive damages on DNA directly, and also increase the formation of ROS [12]. Our data (Fig. 1) illustrate that even though prolonged desiccation would bring about a side effect, the wild type still stayed viability after 7 or 14 days. However, while the cultures were diluted to different concentration, the clone of *pprM* mutant is scarce, presenting a significant survival retardation from the early stage (7 days) to the prolonged stage (28 days) against the wild *D. radiodurans* strain under desiccation.

pprM Mutant Strain is More Sensitive to H_2O_2 stress

When exposed to H_2O_2 stress, oxidative damage would occur to cells. We treat the bacteria under variation of H_2O_2 concentration, this assay demonstrated that the wild *D. radiodurans* shows a well-behaved resistance, however the Δ*pprM* appears a remarkable inhibition (Supplementary Fig. 1 shows the inhibited zone around the colony under all concentration). and the diameter was wider as the H_2O_2 concentration increased (measured in Table 2).

Table 1 Primers used in this study

Primers	Sequences (5′-3′)
DR0862-F	ACGAGTACCGCGACATGGA
DR0862-R	GTGAGCTGCATCGCCTGA
DR0093-F	GAGCTGCTCGTTCCAGTCCTT
DR0093-R	ACCCTATGTTTCACGCTTCCG
DRB0092-F	GCATCTGCCACTGGTACTTCTC
DRB0092-R	CACTACGAGGGCATCAGCAA
16sRNA-F	GCGACGATACATAGCCGACCT
16sRNA-R	TCCATTGCCGAAGATTCCCTA

Differences in ROS Level Between the Wild Type and the Δ*pprM*

Excessive ROS would be generated after irradiation and attack cells inevitably. The detection of fluorescence intensity demonstrated that the ROS accumulation in the *pprM* mutant is nearly twice higher before exposed to radiation, and is almost 4 times higher after 2 k Gy irradiation against the wild type (Fig. 2). This suggested that the deletion of *pprM* would affect the ROS scavenging ability,and reconfirming that *pprM* is involved in oxidative stress response.

Deletion of *pprM* Leads to Decreased Carotenoid Level

Since the wild type and Δ*pprM* had difference in color, we attempted to detect the content of caroteniod in these two strains. Deinoxanthin is the main caroteniod in *D. radiodurans* and has a absorbance shoulder at around 480 nm [13]. According to the results, the wild type exhibited a higher peak than the *pprM* mutant (Fig. 3), which demonstrating that the deletion of *pprM* leading to the deficiency in deinoxanthin synthesis in *D. radiodurans.*

The Expression of Deinoxanthin Synthesis Related Genes and Dps Gene Decreased in the *pprM* Mutant

As the *pprM* mutant was deficient in the content of deinoxanthin, we found that *CrtI* (DR0861), *CrtB* (DR0862) and *CrtO* (DR0093), which are three main genes that involved in the biosynthesis of deinoxanthin, presented a lower transcriptional level in the Δ*pprM* (DR0861 is 0.5 fold, DR0862 is 0.4 fold, DR0093 is 0.4 fold and DRB0092 is 0.5 fold expressed) compared to the wild type through QRT-PCR assays (Fig. 4). In addition, *dps-2* (DRB0092), a gene that relevant to oxidant stress and linked to the regulation of ratio of Mn/Fe [14], was also decreased (0.5 fold) in the Δ*pprM*, which hints that *pprM* may regulate the expression of the components that involved in responding ambient stresses.

Discussion

Deinococcus radiodurans exhibits extraordinary resistance to external stresses owing to collaboration of multiple regulating systems. In it's genome, about 1/3 of the genes

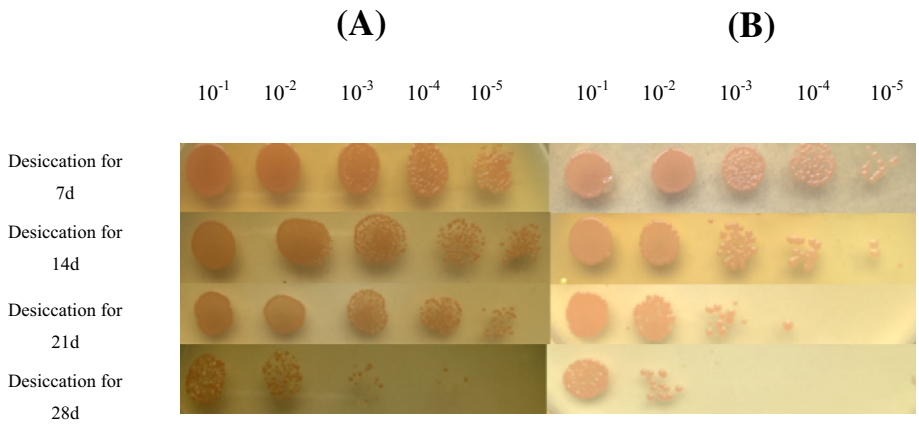

Fig. 1 The survival of *D. radiodurans* on TYG agar plate after exposed to desiccation. **a** wild type and **b** *pprM* mutant. The different colony spot on same *row* had different cell concentration with same desiccative days and the different colony spot on the same *column* had the same concentration with different desiccative days

Table 2 The diameters of inhibition zone

H_2O_2 (mM)	Diameter (mm)		
	WT ($\bar{x} \pm s$)	Δ*pprM* ($\bar{x} \pm s$)	*p
50	3 ± 0.3	6 ± 0.2	0.01
100	4 ± 0.4	9 ± 0.2	0.01
150	5 ± 0.3	13 ± 0.4	0.01
200	8 ± 0.5	17 ± 0.4	0.01

The assay was repeated for three times *$p < 0.05$

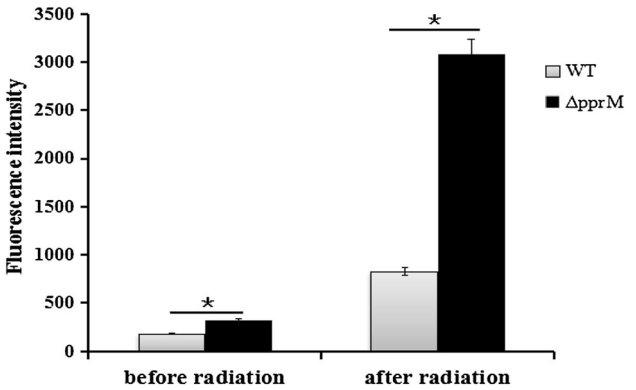

Fig. 2 Fluorescence intensity in wild type (*grey bar*) and the Δ*pprM* (*black bar*) before & after radiation The average and standard deviations was from three independent experiments. *represents $p < 0.05$

play significant roles in radioprotection and DNA repair. *pprI* is considered to be a general switch in the protection of *D. radiodurans*. The PprI protein was found to accelerate the expression of 210 genes after irradiation, and 21 of which are DNA damage repair genes [15]. PprM is a a homolog of a cold shock protein (CSP) and also a damage responsor that regulated by *pprI* [16]. Since early research states that desiccation is a significant factor that contribute *D. radiodurans* evolving to be such a robust organism and the resistance to oxidative stress is closely related to its

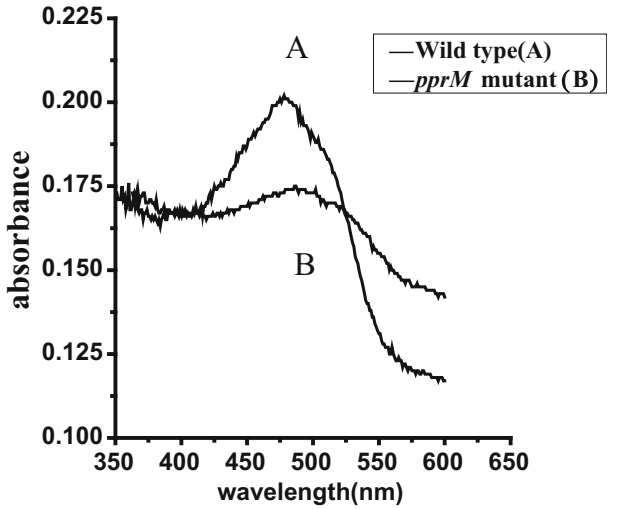

Fig. 3 Absorbance of carotenoid from the wild type (*a*) and the *pprM* mutant (*b*)

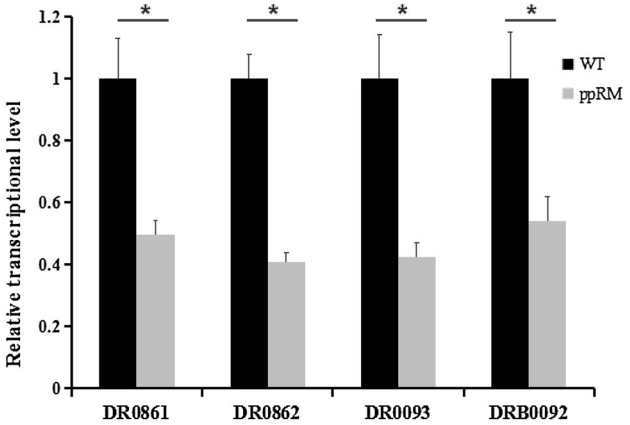

Fig. 4 The expression of genes in the wild-type (*black bar*) and Δ*pprM* (*grey bar*). Assays were measured and the standard deviation of three independent experiments conducted in duplicate. *$p < 0.05$

survival, we investigate the *pprM* mutant's resistance to stress conditions. Our results demonstrate that *pprM* gene knockout would affect the resistance ability to desiccation

and hydrogen peroxide, indicating that *pprM* is involved in stress tolerance in *D. radiodurans*.

ROS is a reverse component generated under extremely harsh conditions. Ghosal et al. reported that 80% of the damages occurred on DNA derived from ROS [17]. The detection of ROS accumulation experiment showed that more ROS accumulated in the Δ*pprM*, suggesting that *pprM* may participate in quenching harmful ROS to protecting cells from oxidation, thus complementing the mechanism how *pprM* plays its role in resistance ability.

Carotenoid is well acknowledged for it's prominent ability in antioxidation in cells. Deinoxanthin is a kind of carotenoid that only exsists in *Deinococcus*. It owns 13 unsaturated double bond, which made contribution to antioxidative ability and could also exhibit protection on proteins and DNA [18]. Since the Δ*pprM* has a lower content of deinoxanthin,and the QRT-PCR results showed that the genes *DR0862, DR0861, DR0093* which were related to biosynthetic pathway of deinoxanthin were down regulated in the Δ*pprM,* it can be assumed that *pprM* may influence deinoxanthin synthesis in an indirect way.

The abundance of Mn(II) attributed a lot to antioxidantive ability in *D. radiodurans*. High concentration of manganese ion improved the progress of removing superoxide and facilitated the compacted of DNA [19]. Recent study found that PprI could cleave the inhibitor protein DdrO, a transcription factor that suppresses DNA damage response genes' expression, when the Mn^{2+} is abundant, which also backed up the importance of Mn in *D. radiodurans* laterally [20]. However the excessive Fe^{2+} may produce cytotoxicity, thus, the highly ratio of Mn(II)/Fe(II) in cells is another non-enzyme mechanism that facilitates the defense against oxidative damage. Since the expression of *dps* gene is depressed in the *pprM* mutant, we inferred that *pprM* may also exert its function in regulating the intracellular ratio of Mn/Fe in *D. radiodurans*.

As the underlying resistance mechanism is more complex than we can anticipate, we propose that *pprM* itself may be a stress response protein which could exert function on oxidation resistance or prevent damages on DNA. Additionally, since the analysis of structure shows that *pprM* embracing RNA binding domains, and could regulate the expression of some stress response genes in a transcription level, we denounce that *pprM* may act as a pleiotropic regulator in *D. radiodurans* in an indirect way.

pprM could assist in resisting ambient stresses, which endows it to be an excellent model for research of mechanisms and applications of antioxidation and antidesiccation, and could be applied in agriculture and industry. Carotenoid had been widely applied in medical condition, it can enhance immunity and accelerate metabolism to prevent human diseases [21]. But the underlying benefits of carotenoid still need further research. Some scholars transformed deinoxanthin into cells, demonstrating that this protein can induce the apoptosis of tumor cells [22]. Our results showed that *pprM* knockout not only reduced the resistance to oxidative and desiccative, but also lead to the decrease of deinoxanthin and dsp through inhibiting their gene expression. This may provide a new clue for the prevention and treatment of irradiation and oxidative damages, and also for developing new antioxidant and antidesiccative agents in application.

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