The Journal of Microbiology (2010) Vol. 48, No. 4, pp. 473-479
DOI 10.1007/s12275-010-0043-8

DRA0336, Another OxyR Homolog, Involved in the Antioxidation Mechanisms in *Deinococcus radiodurans*[§]

Longfei Yin[1], Liangyan Wang[1], Huiming Lu[1], Guangzhi Xu[1], Huan Chen[1,2], Hongdan Zhan[1], Bing Tian[1]*, and Yuejin Hua[1]*

[1]*Key Laboratory for Nuclear-Agricultural Sciences of Chinese Ministry of Agriculture and Zhejiang Province, Institute of Nuclear-Agricultural Sciences, Zhejiang University, Hangzhou 310029, P. R. China*
[2]*Zhejiang Institute of Microbiology, Hangzhou 310027, P. R. China*

(Received February 1, 2010 / Accepted March 26, 2010)

A novel OxyR (DR0615) with one conserved cysteine that senses hydrogen peroxide in *Deinococcus radiodurans* had been identified in our previous work. Comparative genomics revealed that *D. radiodurans* possesses another OxyR homolog, $OxyR_2$ (DRA0336). In this study, we constructed the deletion mutant of $oxyR_2$ and the double mutant of both the OxyR homologs to investigate the role of OxyR in response to oxidative stress in *D. radiodurans*. Deletion of $oxyR_2$ resulted in an obviously increased sensitivity to hydrogen peroxide, and the double mutant for *oxyR* and $oxyR_2$ was significantly more sensitive than any of the two single mutants. The total catalase activity of the double mutant was lower than that of any of the single mutants, and reactive oxygen species (ROS) accumulated to a greater extent. DNA microarray analysis further suggested that $oxyR_2$ was involved in antioxidation mechanisms. Site-direct mutagenesis and complementation analysis revealed that C_{228} in $OxyR_2$ was essential. This is the first report of the presence of two OxyR in one organism. These results suggest that *D. radiodurans* OxyR and $OxyR_2$ function together to protect the cell against oxidative stress.

***Keywords*:** *Deinococcus*, OxyR, $OxyR_2$, antioxidation mechanism, oxidative stress

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, and superoxide anion radical produced in bacterial cells are byproducts of the aerobic respiratory chain process, and several environment stresses including radiation and desiccation. Oxidative stress is highly toxic for cells and cause impairment in cellular function by oxidation of important biomolecules (Imlay, 2003). Bacteria have evolved multiple systems to protect themselves from ROS. Some ROS sensors including OxyR, PerR, and OhrR were identified to respond to ROS by distinct peroxide-sensing mechanism (Mongkolsuk and Helmann, 2002). OxyR in *Escherichia coli* has been well defined and reviewed (Storz and Imlay, 1999; Pomposiello and Demple, 2001). OxyR is a redox-sensitive protein belonging to the LysR family of DNA binding transcriptional regulators (Christman *et al.*, 1989). OxyR regulates the expression of hydrogen peroxide-inducible genes, including *katG* (hydroperoxidase I), *ahpCF* (alkyl hydroperoxide reductase), *gorA* (glutathione reductase), *grxA* (glutaredoxin 1), *dps*, *oxyS* (regulatory RNA), *fur* repressor, *trxC* (thioredoxin 2), *dsbG* (disulfide bond chaperone-isomerase), *hemH* (heme biosynthetic gene), six-gene *suf* operon, *fhuF* (protein required for iron uptake), and *uxuA* (mannonate hydrolase) (Zheng *et al.*, 2001a, 2001b). The molecular basis for the regulatory function of OxyR has been elucidated. First, the conserved site Cys-199 in the OxyR of *E. coli* is activated for reaction with H_2O_2, leading to the formation of an unstable Cys-sulphenic acid (Cys-SOH) intermediate. Then Cys-199 reacts with another conserved site, Cys-208, to form an intramolecular disulfide bond (Zheng *et al.*, 1998). The disulfide bond is reduced by GrxA and glutathione (Aslund *et al.*, 1999). The crystal structures of the regulatory domain of OxyR, in both its reduced and oxidized forms, have been resolved. It was demonstrated that the regulation is realized through a reversible disulfide bond formation with a folded domain (Choi *et al.*, 2001).

The bacterium *Deinococcus radiodurans* is renowned for its extreme resistance to several abiotic stress including ionizing radiation, ultraviolet light, desiccation, and oxidative stress (Makarova *et al.*, 2001). Different mechanisms contributing to resistance to ionizing radiation have been proposed (Cox and Battista, 2005; Blasius *et al.*, 2008). However, only modest research has been done on the response mechanism to oxidative stress. Recently we have demonstrated that a novel OxyR (DR0615) in *D. radiodurans* with only one cysteine residue can sense hydrogen peroxide stress (Chen *et al.*, 2008). Using *E. coli* OxyR as the sequence query, we could identify another putative homolog of OxyR (DRA0336), hereafter referred as $OxyR_2$, in *D. radiodurans*. To the best of our knowledge, there has been no report of the presence of two *oxyR* genes in one organism. Therefore, it is worthwhile to investigate the role of this gene in oxidative stress.

In the present study, we constructed a null mutant of the gene $oxyR_2$ (dra0336) and a double deletion mutant of the *oxyR* and $oxyR_2$. Our results showed that $OxyR_2$ and OxyR are

* For correspondence. E-mail: (B. Tian) tianbing@zju.edu.cn, (Y. Hua) yjhua@zju.edu.cn; Tel/Fax: +86-571-8697-1703
§ Supplemental material for this article may be found at http://www.springer.com/content/120956

involved in the antioxidation process by regulating different genes under oxidative stress.

Materials and Methods

Bacterial strains, plasmids, media, and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary data (Table 1). The wild type and its derivatives were grown aerobically at 30°C in TGY (0.5% tryptone, 0.3% yeast extract, and 0.1% glucose) broth or on agar plates when required. *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth or on LB plates at 37°C. When required, ampicillin, kanamycin, and chloramphenicol were added to achieve final concentrations of 100, 20, and 3 μg/ml, respectively.

Construction of the *oxyR*$_2$ deletion mutant strain

Null mutants were constructed by double crossover recombination of a kanamycin resistance cassette into the genome, with some modifications of the method as described previously (Gao *et al.*, 2005). Briefly, all primers (listed in Supplementary data Table 2) were designed using the genome sequence information of *D. radiodurans* R1 available on NCBI (www.ncbi.nlm.nih.gov). The upstream fragment was amplified by PCR, with the primer set PF_1 and PR_1 containing *Bam*HI restriction site in primer PR_1. Similarly, the downstream fragment was PCR-amplified with the primer set PF_2 and PR_2, containing *Hin*dIII restriction site in primer PF_2. The upstream and downstream fragments were digested with *Bam*HI and *Hin*dIII, respectively, and ligated to the *Bam*HI-*Hin*dIII predigested fragment of the kanamycin resistance cassette obtained from pRADK (Gao *et al.*, 2005). The ligation product was used as a template for PCR amplification of the full length PCR product, with the primers set PF_1 and PR_2. The resulting PCR product was ligated into the pMD18T-Easy vector (TaKaRa, Japan) to yield pTKAN, which was then transformed into *D. radiodurans* R1, as described previously (Kitayama *et al.*, 1983). Mutant strains were selected on TGY agar plates supplemented with kanamycin (20 μg/ml). The *dra0336* gene mutant was designated as $MOxyR_2$.

Construction of the *oxyR-oxyR*$_2$ double mutant

The double mutant was constructed based on the *oxyR* mutant MOxyR (Chen *et al.*, 2008). In brief, the chloramphenicol resistance cassette was obtained from pKatCAT (Funayama *et al.*, 1999). Plasmid pTKAN, digested with *Bam*HI and *Hin*dIII to obtain the fragment containing the upstream and downstream of the *dra0336* gene, was ligated with the *Bam*HI-*Hin*dIII fragment of the chloramphenicol resistance cassette to generate pTCHL. This plasmid was transformed into MOxyR. Double mutant strains were selected on TGY agar plates supplemented with kanamycin (20 μg/ml) and chloramphenicol (3 μg/ml). The double mutant was named DM. The identity and truthfulness of all mutants were verified by DNA sequencing and size estimation of the PCR product and the restriction digestion product by agarose gel electrophoresis.

Complementation of the $MOxyR_2$ mutant of *D. radiodurans*

The 915 bp $OxyR_2$ coding DNA fragment was PCR-amplified from the genomic DNA of *D. radiodurans* using the primer pair, PFcom and PRcom. The PCR products were cloned into pMD18T, to yield the pTC plasmid. This plasmid was restriction-digested with *Nde*I and *Bam*HI to yield the DNA fragment containing the coding sequence of $OxyR_2$, and cloned into pRADK plasmid which had been digested with the same enzymes, to yield the pRADC plasmid. The truthfulness of the clones was confirmed by partial DNA sequencing and restriction digestion analysis. This plasmid was transformed into the $MOxyR_2$ strain, and the resulting variant was designated as $MOxyR_2$-C.

PCR site-direct mutagenesis of *oxyR*$_2$ at C_{228}, C_{272}, and C_{290}

PCR mutagenesis was performed as reported previously (Fuangthong and Helmann, 2002). Briefly, the PCR fragment was obtained with the primer PFcom and the corresponding mutagenic antisense primer MC_{228}-PR (the mutated bases are underlined in Supplementary data Table 1). The other PCR fragment was cloned with primer PRcom and the corresponding mutagenic sense primer MC_{228}-PF that is complementary to the mutagenic antisense primer MC_{228}-PR. Then, both PCR products were denatured, annealed and amplified, using primers PFcom and PRcom. This product of the PCR-amplified was

Table 1. Part of the down-regulated genes in $MOxyR_2$ are shown compared with R1 after H_2O_2 treatment

Locus	Annotation	Fold change	QRT-PCR	*P* value
DR0780	hypothetical protein	8.37		0.0000
DRB0016	hemin ABC transporter, ATP-binding protein	2.92	2.32	0.0003
DR0330	hypothetical protein	2.36		0.0005
DR1175	sensor histidine kinase-response regulator	2.34		0.0005
DR0537	hypothetical protein	2.23		0.0006
DRA0259	catalase	2.18	2.41	0.0007
DRB0031	chromosome partitioning ATPase, putative, ParA family	2.14		0.0018
DR1540	isocitrate dehydrogenase (*icd*)	1.90		0.0029
DR2554	hypothetical protein	1.84		0.0036
DRB0062	hypothetical protein	1.83		0.0059
DR1219	ferrous iron transport protein B (*feoB*)	1.82		0.0066
DR0971	electron transfer flavoprotein, beta subunit (*etfB*)	1.79		0.0080
DRB0107	ribonucleotide reductase, NrdI family	1.74		0.0086
DR1505	NADH dehydrogenase I, B subunit (*nuoB*)	1.72	1.78	0.0122
DR0970	electron transfer flavoprotein, alpha subunit (*etfA*)	1.64	1.83	0.0163
DRA0019	N-acetyltransferase	1.59		0.0217

cloned into the pMD18T vector, producing the plasmid $pTMC_1$. Similarly, $pTMC_2$ and $pTMC_3$ were constructed using mutagenic primers MC_{272}-PF and MC_{272}-PR, MC_{290}-PF and MC_{290}-PR, respectively. The above plasmids were digested with *Nde*I and *Bam*HI, and ligated to pRADK that had been predigested with the same restriction enzymes. The resulting plasmids were named $pRADMC_1$, $pRADMC_2$, and $pRADMC_3$, and were transformed into $MOxyR_2$ strain. The resulting strains were named $MOxyR_2$-MC_1, $MOxyR_2$-MC_2, and $MOxyR_2$-MC_3, respectively. The sequences of the $oxyR_2$ were verified by sequencing.

Measurement of cell survival rate

The survival rate of *D. radiodurans* exposed to hydrogen peroxide was determined by the method described previously (Carbonneau *et al.*, 1989). Briefly, cells in the early stationary phase (OD_{600}=1.0) were diluted to an appropriate concentration with phosphate buffer. A 30% hydrogen peroxide solution was added into the cell suspension to obtain final concentrations of 0, 10, 20, 30, and 40 mM, and the cells were incubated for 30 min with shaking at 30°C. Then, sufficient catalase (Sigma, USA) was added (100 mg/ml) to inactive the hydrogen peroxide. After the treatment, the cells were spread on TGY agar plates, and the colonies were enumerated after incubating at 30°C for 3 days. Finally, the survival rate was expressed as the number of colonies obtained from treated samples as a percentage of the number for untreated controls.

Measurement of catalase activity in protein preparations

The cells were collected by centrifugation and disrupted on ice with an ultrasonicator at an output of 450 W for 10 min. The debris was removed by centrifugation and the concentration of the soluble protein was determined using the Bradford method. Catalase activity was assayed by measuring the decomposition rate of hydrogen peroxide at 240 nm as described previously (Chen *et al.*, 2008).

Measurement of the accumulation of reactive oxygen species

The oxidant-sensitive probe DCHF-DA was used to determine the intracellular ROS levels in *D. radiodurans*, as described previously (Chen *et al.*, 2008). The fluorescence intensity of the samples was measured using a fluorescence spectrophotometer (Shimadzu, Japan), with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. ROS values are given in arbitrary units of fluorescence.

RNA isolation, DNA microarray assay, and quantitative real-time PCR (QRT-PCR)

The wild type and mutant strain were cultured in 100 ml TGY to the mid-exponential phase. Then, the cultures were divided into two parts, a final concentration of 20 mM hydrogen peroxide was added into one part for 30 min, and the other part was used as a control. The cells were collected by centrifugation. RNA isolation, DNA microarray assay, and QRT-PCR were performed as described previously (Chen *et al.*, 2007). Briefly, after liquid nitrogen grinding, total RNA was isolated using TRIZOL reagent (Invitrogen, USA). Then, DNA was removed by treatment with RNase-free DNaseI (Promega, USA). The RNA concentration was determined by measuring the A_{260}/A_{280} ratio with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA). Hybridization probes and microarray hybridization were prepared as described previously. The microarrays were scanned using the GenePix 4000B and hybridization signals were quantified by GenePix pro 5.1. Normalization and statistical analysis were carried out in the R computing environment (2.2.0, Raqua on Windows) using the linear models of microarray data package (Limma). Global LOESS normalization was used to normalize all data. Three replicated samples were used in microarray experiments. All the reactions in QRT-PCR were performed according to the manufacturer's instructions of the SYBR Green PCR kit (Bioer, China). The housekeeping gene *dr0089*, which was unaffected in our treatment, was used as a reference. The amplification and detection for specific products were performed using Mx3000P with the real-time PCR Detection System (Stratagene, USA).

Results and Discussion

Identification of *D. radiodurans* $OxyR_2$

Recently *D. radiodurans* OxyR (DR0615), has been identified and characterized (Chen *et al.*, 2008). Analysis of the *D. radiodurans* R1 genome (Lin *et al.*, 1999) using the BLAST program with the *E. coli* OxyR amino acid sequence as a query sequence revealed the presence of another putative homolog DRA0336 ($OxyR_2$). The deduced amino acid sequence of *D. radiodurans* $OxyR_2$ shares 37% identity with *D. radiodurans* OxyR and 28% identity with *E. coli* OxyR. The $OxyR_2$ amino acid sequence exhibits a probable helix-turn-helix motif in the DNA binding domain, which mediates DNA binding and promoter recognition. As shown in Fig. 1, R4, L32, S33, and R50 are four of the five most conserved residues in the DNA binding domain and E255 involved in tetramerization is also conserved (Kullik *et al.*, 1995). D142 and R273 are in the possible activating region (Wang *et al.*, 2006). L124 and A233 are necessary for forming a hydrophobic core (Choi *et al.*, 2001). These important sites are conserved in $OxyR_2$. Cysteine residue C_{228} of $OxyR_2$ is also conserved with the C_{208} of *E. coli* OxyR. C_{208} and C_{199} are essential for redox activity in *E. coli* OxyR (Zheng *et al.*, 1998).

The $oxyR_2$ gene is responsible for the sensitivity to hydrogen peroxide

The $oxyR_2$ mutant was constructed with the strategy described in 'Materials and Methods'. Agarose gel electrophoresis analysis indicated that the kanamycin resistance cassette completely replaced the coding region of $oxyR_2$ and the mutant is homozygous (Supplementary data Figs. 1A and B). To investigate whether *D. radiodurans* $oxyR_2$ is necessary for the resistance to oxidative stress, a cell survival assay with H_2O_2 treatment was performed using the wild type R1 and mutant $MOxyR_2$, the results showed that mutant $MOxyR_2$ is significantly more sensitive compared to the wild type (Fig. 2A). Trans-expression of the $OxyR_2$ gene in mutant $MOxyR_2$ could fully restore the H_2O_2 resistance (Fig. 2A). This suggested that $MOxyR_2$ mutant phenotypes were not due to the absence of any proteins downstream to $MOxyR_2$, but the absence of $oxyR_2$.

Double mutant is more sensitive to hydrogen peroxide than any single mutant

The mutant strain MOxyR of *D. radiodurans* shows signify-cantly reduced resistance to H_2O_2 compared with the wild type strain R1 (Chen *et al.*, 2008). To determine whether *oxyR* and $oxyR_2$ have functional redundancy in the resistance of H_2O_2, the *oxyR* and $oxyR_2$ double mutant was constructed (Supplementary data Fig. 1C). The survival of double mutant under

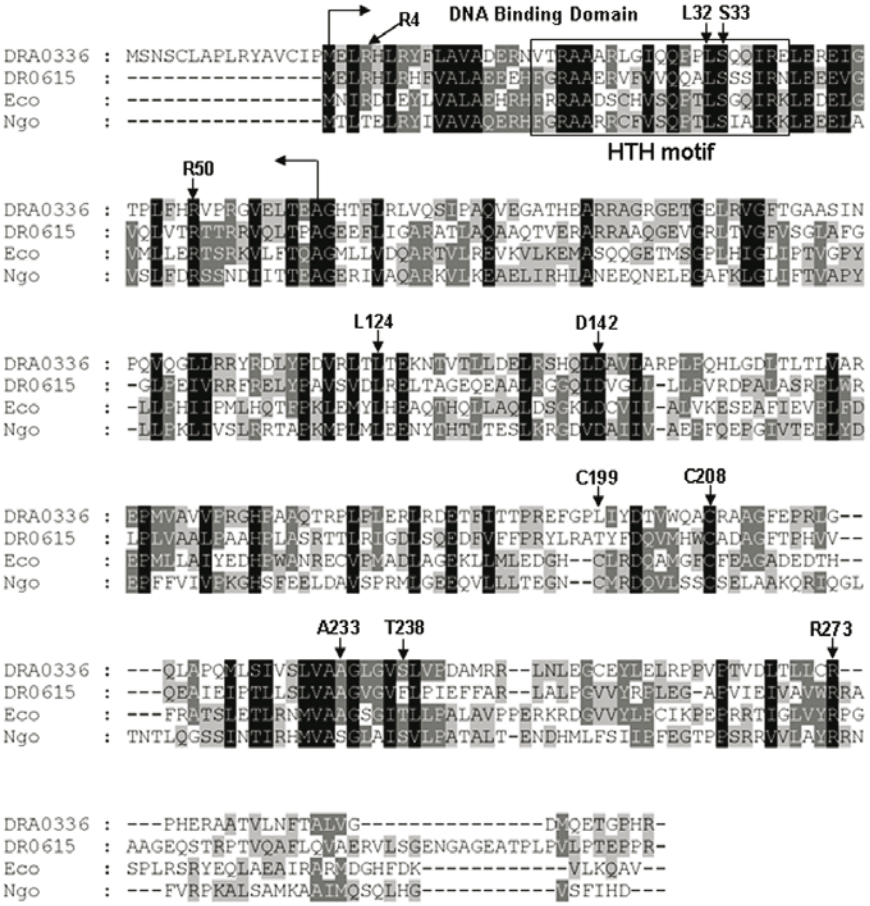

Fig. 1. Multiple amino acid sequence alignment of OxyR homologs. The multiple sequence alignment was generated by using the program CLUSTAL X. The results were prepared using Genedoc (copyright Karl Nicholas). The identical amino acids are highlighted in black and the similar ones are highlighted in grey. The DNA binding domain is shown between the two arrows and the helix-turn-helix (HTH) motif is boxed. DRA0336; DR0615, Eco, and Ngo denote the $OxyR_2$ of *D. radiodurans*, *D. radiodurans* OxyR, *E. coli* OxyR, and *Neisseria gonorrhoeae* OxyR, respectively. The numbering is based on the *E. coli* OxyR sequence.

the stress of H_2O_2 was examined. The double mutant was significantly more sensitive to H_2O_2 than any of the single mutants (Supplementary data Fig. 2B), indicating that both *oxyR* and *oxyR*$_2$ in *D. radiodurans* are necessary to survive H_2O_2 stress.

ROS levels increase in the mutants

Both single (OxyR, $OxyR_2$) and double mutant were sensitive to H_2O_2 treatment. Therefore it has been hypothesized that mutants might have reduced free radical scavenging activity compared to the wild type. To test this hypothesis, we measured the total intracellular ROS levels. Both of the single mutant strains accumulated much more ROS than the wild type R1, and the double mutant strain DM accumulated the highest level of ROS (Fig. 3). Furthermore, ROS accumulation increased in all the strains followed by the H_2O_2 treatment, compared with those without H_2O_2 treatment, and the DM still had the higher levels of ROS. These results were consistent with the phenotypic data that DM is the most sensitive strain to H_2O_2.

Deletion of *oxyR*$_2$ attenuates the enzymatic activity of catalase

Catalase plays a crucial role in decomposition of hydrogen peroxide or peroxides. To study whether deletion of *oxyR*$_2$ affect the catalase activity in *D. radiodurans*, we measured the total activity of catalase in cell-free extract of *D. radiodurans*. We found that catalase activity can be induced followed by H_2O_2 treatment (Fig. 4). The absence of any one OxyR homolog, or both, had no influence on catalase activity in all the mutant strains, in absence of the treatment with H_2O_2, but did prevent the induction of catalase activity when the cells were exposed to H_2O_2 (Fig. 4). In addition, the total catalase activity of DM had almost no increase after the treatment with H_2O_2. However, $MOxyR_2$ still showed a moderate induction of catalase activity. These results suggest that the deletion of *oxyR*$_2$ attenuates the induction of catalase activity in $MOxyR_2$.

Additionally, we measured catalase activity induction against ionizing radiation. The result of total catalase activity against ionizing radiation is almost similar to that against H_2O_2 (Supplementary data Fig. 2).

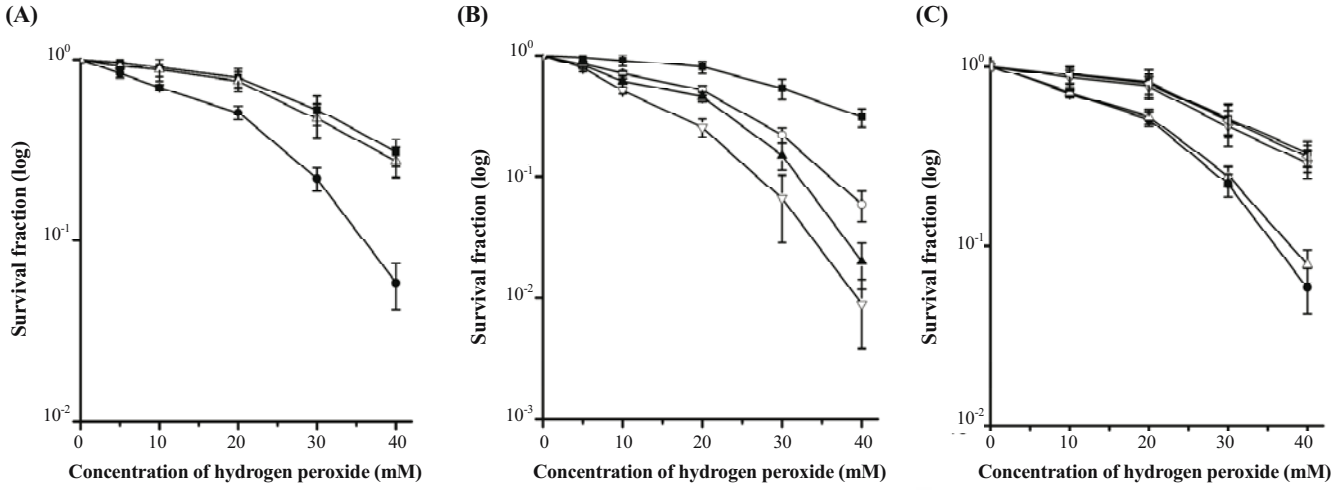

Fig. 2. Survival curves for *D. radiodurans* following exposure to H_2O_2. (A) Wild type, R1 (■); *oxyR*$_2$ knockout mutant, MOxyR$_2$ (●); MOxyR$_2$ complemented with the *oxyR*$_2$ gene, MOxyR$_2$-C (△). (B) R1 (■); MOxyR$_2$ (○); *oxyR* knockout mutant, MOxyR (▲); *oxyR* and *oxyR*$_2$ double mutant, DM (▽). (C) R1 (■); MOxyR$_2$ (●); MOxyR$_2$ complemented with the *oxyR*$_2$ gene with the site mutation C_{228}A, MOxyR$_2$-C1 (△); MOxyR$_2$ complemented with the *oxyR*$_2$ gene with the site mutation C_{272}A, MOxyR$_2$-C2 (▽); MOxyR$_2$ complemented with the *oxyR*$_2$ gene with the site mutation C_{290}A, MOxyR$_2$-C3 (○). Values are Means±SD of three independent experiments.

Transcriptional profile of MOxyR$_2$ versus R1 after treatment with 20 mM H_2O_2

The results of the intracellular ROS accumulation assay indicated that the DM accumulated the higher levels of ROS than any single mutant. Deletion of OxyR repressed catalase *katE* (*dr1998*) and genes associated with ROS production (e.g., *dr0343*, *dr1505*, and *dr1493*) (Chen *et al.*, 2008). Therefore, to test the role of OxyR$_2$ in *D. radiodurans* under H_2O_2 treatment, the whole genome expression profile of MOxyR$_2$ was performed in comparison with R1 after treatment with 20 mM H_2O_2 during the exponential growth phase. Gene expression changes were verified by QRT-PCR under the same conditions as applied in the microarray assay, and the expression pattern was the same as that in the microarray data (Table 1). Supplementary data Tables 3 and 4 showed the genes that changed at least 1.5-fold ($p<0.05$). Table 1 shows the genes associated with ROS generation. Interestingly, we found that two flavoproteins genes (*dr0970* and *dr0971*), one catalase gene *katG* (*dra0259*), *hutD* (*drb0016*), and *feoB* (*dr1219*) were repressed in MOxyR$_2$ (Table 1). *katG* (*dra0259*) as a ROS scavenger was repressed significantly. This was consistent with the results of the measurement of the total catalase activity. On the other hand, flavoprotein is regarded as one of the major sources generating ROS within the respiratory chain (Seaver and Imlay, 2004; Ghosal *et al.*, 2005). The repression of two flavoproteins genes might be a response to the higher ROS accumulation in MOxyR$_2$. Iron is an important nutrient in all organisms, but it poses the problem of toxicity to cell and poor solubility. *D. radiodurans*, like most bacteria, has evolved various mechanisms to solve this problem, such as a highly efficient iron transport system (Lin *et al.*, 1999). At high level of ROS accumulation, the ferrous iron transport gene

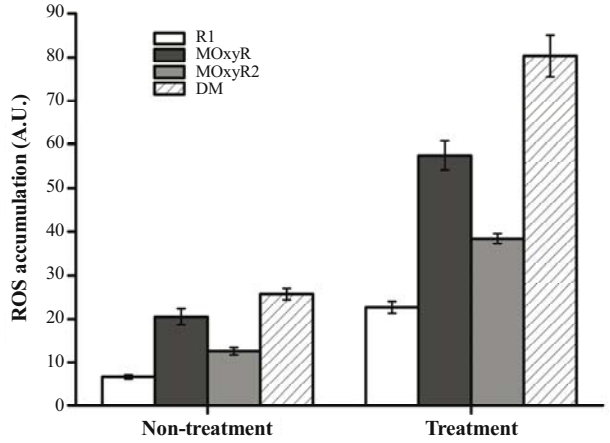

Fig. 3. Levels of intracellular ROS in R1, MOxyR, MOxyR$_2$, and DM after H_2O_2 treatment (Treatment) or not (Non-treatment). Values are Means±SD of three independent experiments.

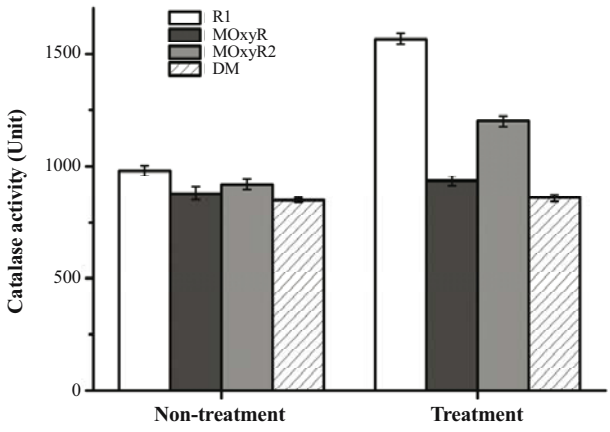

Fig. 4. Catalase activity of R1, MOxyR, MOxyR$_2$, and DM after H_2O_2 treatment (Treatment) or not (Non-treatment). Results are Means±SD of three independent experiments.

(*dr1219*) and the hemin transport gene (*drb0016*) were repressed in MOxyR$_2$, which might be a cell response to avoid the generation of harmful free radical (e.g., hydroxyl radical) through the Fenton reaction [unbound Fe(II) reacting with H_2O_2]. In addition, a number of hypothetical genes are changed significantly (Supplementary data Tables 3 and 4). In future work the function of these genes under oxidative stress needs to be studied. Previous investigations had confirmed that *D. radiodurans* has very complex antioxidation regulating process, and expresses several proteins, PprI, PprA, OxyR, RecX, RecD, and DrRRA that are involved in the antioxidation process (Hua *et al.*, 2003; Sheng *et al.*, 2005; Kota and Misra, 2006; Zhou *et al.*, 2007; Chen *et al.*, 2008; Wang *et al.*, 2008). This study suggests that OxyR$_2$ is involved in this process.

C_{228} is a crucial site for survival in H_2O_2 stress

To test the role of cysteine residues in the activity of *oxyR$_2$* and in the survival of H_2O_2 stress, we constructed the site-direct mutagenesis strains MOxyR$_2$-MC$_1$, MOxyR$_2$-MC$_2$, and MOxyR$_2$-MC$_3$, in which C_{228}, C_{272}, and C_{290} of OxyR$_2$ were mutated into alanine, respectively. Mutation of C_{228} remarkably diminished the resistance of the cell to H_2O_2, while the mutation of C_{272} or C_{290} had no significant influence on H_2O_2 resistance compared with the wild type strain (Fig. 2C). OxyR$_2$ may belong to the 1-Cys OxyR family, similar to DrOxyR, but it is distinct from the typical 2-Cys OxyR family, such as the OxyR in *E. coli*. It is perhaps because of this difference that the *oxyR$_2$* could not complement the defect of *E. coli oxyR* mutant strain GS09 (our unpublished data).

In this study we demonstrated that OxyR$_2$ is one of the essential components for antioxidant processes in *D. radiodurans*. Further research needs to identify and establish whether there are any interactions between OxyR and OxyR$_2$.

Acknowledgements

Authors would like to give thanks to Mr. Yogendra Singh Rajpurohit for critical reading of the manuscript. This work was supported by a grant from the National Hi-Tech Development Program (2007AA021305), a key project from the National Natural Science Foundation of China (30830006), a grant from the National Natural Science Foundation of China (30870035), a project from Science and Technology Department of Zhejiang Province of China (2006E10058), and the project "Application of Nuclear Techniques in Agriculture" from the Chinese Ministry of Agriculture (200803034).

References

Aslund, F., M. Zheng, J. Beckwith, and G. Storz. 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. USA* 96, 6161-6165.

Blasius, M., S. Sommer, and U. Hubscher. 2008. *Deinococcus radiodurans*: what belongs to the survival kit? *Crit. Rev. Biochem. Mol. Biol.* 43, 221-238.

Carbonneau, M.A., A.M. Melin, A. Perromat, and M. Clerc. 1989. The action of free radicals on *Deinococcus radiodurans* carotenoids. *Arch. Biochem. Biophys.* 275, 244-251.

Chen, H., Z. Xu, B. Tian, W. Chen, S. Hu, and Y. Hua. 2007. Transcriptional profile in response to ionizing radiation at low dose in *Deinococcus radiodurans*. *Prog. Nat. Sci.* 17, 529-536.

Chen, H., G. Xu, Y. Zhao, B. Tian, H. Lu, X. Yu, Z. Xu, N. Ying, S. Hu, and Y. Hua. 2008. A novel OxyR sensor and regulator of hydrogen peroxide stress with one cysteine residue in *Deinococcus radiodurans*. *PLoS ONE.* 3, e1602.

Choi, H., S. Kim, P. Mukhopadhyay, S. Cho, J. Woo, G. Storz, and S. Ryu. 2001. Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105, 103-113.

Christman, M.F., G. Storz, and B.N. Ames. 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. *Proc. Natl. Acad. Sci. USA* 86, 3484-3488.

Cox, M.M. and J.R. Battista. 2005. *Deinococcus radiodurans* - the consummate survivor. *Nat. Rev. Microbiol.* 3, 882-892.

Fuangthong, M. and J.D. Helmann. 2002. The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc. Natl. Acad. Sci. USA* 99, 6690-6695.

Funayama, T., I. Narumi, M. Kikuchi, S. Kitayama, H. Watanabe, and K. Yamamoto. 1999. Identification and disruption analysis of the *recN* gene in the extremely radioresistant bacterium *Deinococcus radiodurans*. *Mutat. Res.* 435, 151-161.

Gao, G., H. Lu, L. Huang, and Y. Hua. 2005. Construction of DNA damage response gene *pprI* function-deficient and function-complementary mutants in *Deinococcus radiodurans*. *Chin. Sci. Bull.* 50, 311-316.

Ghosal, D., M.V. Omelchenko, E.K. Gaidamakova, V.Y. Matrosova, A. Vasilenko, A. Venkateswaran, M. Zhai, and *et al*. 2005. How radiation kills cells: survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress. *FEMS Microbiol. Rev.* 29, 361-375.

Hua, Y., I. Narumi, G. Gao, B. Tian, K. Satoh, S. Kitayama, and B. Shen. 2003. PprI: a general switch responsible for extreme radioresistance of *Deinococcus radiodurans*. *Biochem. Biophys. Res. Commun.* 306, 354-360.

Imlay, J.A. 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol.* 57, 395-418.

Kitayama, S., S. Asaka, and K. Totsuka. 1983. DNA double-strand breakage and removal of cross-links in *Deinococcus radiodurans*. *J. Bacteriol.* 155, 1200-1207.

Kota, S. and H.S. Misra. 2006. PprA: A protein implicated in radioresistance of *Deinococcus radiodurans* stimulates catalase activity in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 72, 790-796.

Kullik, I., J. Stevens, M.B. Toledano, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. *J. Bacteriol.* 177, 1285-1291.

Lin, J., R. Qi, C. Aston, J. Jing, T.S. Anantharaman, B. Mishra, O. White, M.J. Daly, K.W. Minton, J.C. Venter, and D.C. Schwartz. 1999. Whole-genome shotgun optical mapping of *Deinococcus radiodurans*. *Science* 285, 1558-1562.

Makarova, K.S., L. Aravind, Y.I. Wolf, R.L. Tatusov, K.W. Minton, E.V. Koonin, and M.J. Daly. 2001. Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Microbiol. Mol. Biol. Rev.* 65, 44-79.

Mongkolsuk, S. and J.D. Helmann. 2002. Regulation of inducible peroxide stress responses. *Mol. Microbiol.* 45, 9-15.

Pomposiello, P.J. and B. Demple. 2001. Redox-operated genetic switches: the SoxR and OxyR transcription factors. *Trends Biotechnol.* 19, 109-114.

Seaver, L.C. and J.A. Imlay. 2004. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *J. Biol. Chem.* 279, 48742-48750.

Sheng, D., G. Gao, B. Tian, Z. Xu, Z. Zheng, and Y. Hua. 2005. RecX is involved in antioxidant mechanisms of the radioresistant bacterium *Deinococcus radiodurans*. *FEMS Microbiol. Lett.* 244,

251-257.

Storz, G. and J.A. Imlay. 1999. Oxidative stress. *Curr. Opin. Microbiol.* 2, 188-194.

Wang, X., P. Mukhopadhyay, M.J. Wood, F.W. Outten, J.A. Opdyke, and G. Storz. 2006. Mutational analysis to define an activating region on the redox-sensitive transcriptional regulator OxyR. *J. Bacteriol.* 188, 8335-8342.

Wang, L., G. Xu, H. Chen, Y. Zhao, N. Xu, B. Tian, and Y. Hua. 2008. DrRRA: a novel response regulator essential for the extreme radioresistance of *Deinococcus radiodurans*. *Mol. Microbiol.* 67, 1211-1222.

Zheng, M., F. Aslund, and G. Storz. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279, 1718-1721.

Zheng, M., X. Wang, B. Doan, K.A. Lewis, T.D. Schneider, and G. Storz. 2001a. Computation-directed identification of OxyR DNA binding sites in *Escherichia coli*. *J. Bacteriol.* 183, 4571-4579.

Zheng, M., X. Wang, L.J. Templeton, D.R. Smulski, R.A. LaRossa, and G. Storz. 2001b. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol.* 183, 4562-4570.

Zhou, Q., X. Zhang, H. Xu, B. Xu, and Y. Hua. 2007. A new role of *Deinococcus radiodurans* RecD in antioxidant pathway. *FEMS Microbiol. Lett.* 271, 118-125.