

## Characterization and Radio-resistant Function of Manganese Superoxide Dismutase of *Rubrobacter radiotolerans*

Hiroaki TERATO<sup>1\*</sup>, Katsuyuki SUZUKI<sup>2</sup>, Nobuhiro NISHIOKA<sup>2</sup>, Atsushi OKAMOTO<sup>2</sup>, Yuka SHIMAZAKI-TOKUYAMA<sup>1</sup>, Yuko INOUE<sup>1</sup> and Takeshi SAITO<sup>3</sup>

### ***Rubrobacter radiotolerans*/Superoxide dismutase/Radical scavenger/Radio-resistance.**

*Rubrobacter radiotolerans* is the most radio-resistant eubacterium without spore-formation in the life cycle, and its D<sub>37</sub> is 16,000 Gy against gamma-rays. To understand the molecular mechanism of the high radio-resistance, we purified and characterized superoxide dismutase (SOD) of this organism as enzymatic radical scavenger, and then analyzed its genetic information. The purified SOD protein formed homotetramerization of 24,000 Da-monomer, while maintaining its enzymatic activity against potassium cyanide and hydrogen peroxide. We obtained a partial amino acid sequence of the protein and cloned the gene from it. Sequence analysis of the cloned gene indicated that the protein showed a similarity to other bacterial manganese SODs (Mn-SODs). Sequencing for adjacent regions of the gene showed that the gene had promoter elements with an open reading frame for putative PAS/PAC sensor protein at the 5'-adjacent region. Introduction of the gene into *Escherichia coli* cells lacking intrinsic SOD genes restored the cellular enzymatic activity and resistance to methyl viologen, indicating the gene at work. A mutant cell harboring this gene also became resistant against gamma-rays. The present results suggest that the protein in question is the Mn-SOD of *R. radiotolerans*, a good candidate as a radio-protection factor for this bacterial radio-resistance.

### INTRODUCTION

We know that ionizing radiation wreaks immense damage on living organisms. Most multicellular eukaryotes such as human are killed with just a few Gy of gamma-rays. Common unicellular prokaryotes prove to be more resistant, but the tolerance level is at a mere few tens of Gy. On the other hand, highly radio-resistant organisms have been found on the earth, which are all bacteria. The first report of such radio-resistant bacteria was about *Micrococcus radiodurans*, now referred to as *Deinococcus radiodurans*, according to subsequent taxonomic studies.<sup>1,2)</sup> *D. radiodurans* without sporulation shows that its D<sub>37</sub> is 7,000 Gy of gamma-rays, a value that is about a hundred times higher than that of *Escherichia coli*. Several dozens of eubacteria, of various

taxa, with similar radio-resistant property have since been found and listed in the "radio-resistant bacteria" group. We define eubacteria as radio-resistant, regardless of their taxis, if they show that the D<sub>37</sub> is over several thousand Gy against gamma-rays and have no sporulation in the life cycle. In searching for such eubacteria, *Arthrobacter radiotolerans* was dredged from a radioactive hot spring in Japan.<sup>3)</sup> A subsequent taxonomic study redefined this species as *Rubrobacter radiotolerans*, the current name.<sup>4)</sup>

*R. radiotolerans* is the most highly radio-resistant bacterium with a D<sub>37</sub> of 16,000 Gy against gamma-rays. This value is approximately 200 times higher than that of *E. coli*, and 2.1 times higher still than that of *D. radiodurans*. As a radio-resistant mechanism of *R. radiotolerans*, we have regarded the reddish color of the cell to be of possible importance for some time. We noted that the major red pigment in the cell was bacterioruberin, one of carotenoids.<sup>5)</sup> Carotenoid is a candidate for a radio-protecting agent, because it works as a radical scavenger with its conjugate double bonds absorbing deleterious radicals and reactive oxygen species.<sup>6,7)</sup> We showed that amount of damage in DNA molecules irradiated by gamma-rays in a test tube was reduced when this pigment coexisted.<sup>8,9)</sup> Also the colorless mutant of *Halobacterium salinarum* became more sensitive to gamma-rays than the wild type, bacterioruberin having originally been reported

\*Corresponding author: Phone: +81-952-34-2413,

Fax: +81-952-34-2015,

E-mail: terato@cc.saga-u.ac.jp

<sup>1</sup>Analytical Research Center for Experimental Sciences, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan; <sup>2</sup>Faculty of Engineering, Kinki University, 1 Takaya Umenobe, Higashi-Hiroshima 739-2116, Japan; <sup>3</sup>Research Reactor Institute, Kyoto University, 2 Asashiro-Nishi, Kumatori 590-0494, Japan.

doi:10.1269/jrr.11105

from this halophilic archaeon.<sup>8,10)</sup> We thus demonstrated bacterioruberin's radio-protection ability *in vitro* and *in vivo*. Additionally, we pointed out that *R. radiotolerans* showed less yields of DNA-double strand breaks (DNA-DSBs) after irradiation of gamma-rays.<sup>11)</sup> Sucrose density gradient centrifugation showed that 14 DNA-DSBs per genome of *R. radiotolerans* were produced at 32 kGy of gamma-rays. This yield would be equivalent to 4.6–5.8% of DNA-DSBs per genome of *D. radiodurans*, if irradiated at the same dose.<sup>12,13)</sup> Radio-resistance is commonly derived from two major pathways, i.e., protection and repair. The results above indicate that the radio-resistance of *R. radiotolerans* is mainly based on the high protective ability for DNA unlike *D. radiodurans* mainly dependent on the DNA repair.<sup>14)</sup>

Considering the extreme degree of radio-resistance of *R. radiotolerans*, radio-protective capacity of bacterioruberin was rather modest.<sup>8,9)</sup> Some other protective agents were therefore assumed to be involved for the radio-resistance to become so high. In this study, we examined superoxide dismutase (SOD) of *R. radiotolerans*, as one of enzymatic radical scavengers. We extracted and purified a SOD protein from cells of *R. radiotolerans*. The SOD protein was determined to be a manganese SOD (Mn-SOD) of this organism after its biochemical properties and the genetic information were analyzed. We introduced the cloned gene into *E. coli* strain lacking two intrinsic SOD genes. The cloned gene functioned to restore the radio-resistance of the host cell. Our present results suggest that the SOD is likely to be of major driving force behind the radio-resistance of *R. radiotolerans*.

## MATERIALS AND METHODS

### *Bacteria, plasmid and chemicals*

*Rubrobacter radiotolerans* P-1 (JCM 2153T) was provided by RIKEN Bio-Resource Center through National Bio-Resource Project of Japan. *E. coli* GC4468 (K12 F<sup>-</sup>  $\Delta$ lac4169 *rpsL*) and QC1726 (GC4468 F(*sodA-lacZ*)49 F(*sodB-kan*)1-D<sub>2</sub> Cm<sup>r</sup> Km<sup>r</sup>) strains were generous gifts.<sup>15)</sup> Plasmid DNA pACYC184 for *E. coli* was obtained from National Institute of Genetics through National Bio-Resource Project of Japan.<sup>16)</sup> Anion exchanger diethylaminoethyl cellulose DE-52 was from Whatman for chromatography. Sephacryl S-300 was from GE healthcare for gel filtration. BCA protein assay reagent to determine protein concentration was from Pierce. Cytochrome C, xanthine and bovine erythrocyte copper/zinc SOD (Cu, Zn-SOD) were from Sigma. Xanthine oxidase was from Roche. Methyl viologen was from Nacalai Tesque. The chemicals used in this study were obtained from Wako Chemicals except for ones described in the text.

### *Protein purification*

*R. radiotolerans* was grown as in the previous reported

manner.<sup>11)</sup> Fifty gram-wet cells of this bacterium, which was suspended in 500 mL of 10 mM sodium phosphate buffer (pH7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.6 M sucrose, 1 mg mL<sup>-1</sup> achromopeptidase and 1 mg mL<sup>-1</sup> lysozyme, was sonicated at 125 W for total 10 min with appropriate intervals on ice. The cell suspension after removing cell debris by centrifugation was eluted through a DE-52 column with a linear gradient (0–500 mM) of sodium chloride in 10 mM sodium phosphate buffer (pH7.8) with 0.1 mM EDTA. Then the enzymatic active fraction was eluted through a hydroxyapatite column with a linear gradient (10–100 mM) of sodium phosphate buffer (pH7.8) containing 0.1 mM EDTA. Subsequently the active fraction was subjected to Sephacryl S-300 gel filtration with 50 mM sodium phosphate buffer (pH7.8) containing 150 mM NaCl, and then the second DE-52 column chromatography.

### *Protein assays*

For protein characterization, diverse gel electrophoreses were used in this study. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was to evaluate protein purification with silver staining.<sup>17)</sup> Native-PAGE was to examine the multimerization with coomassie brilliant blue (CBB)-R250 staining and with nitro blue tetrazolium staining for in-gel enzymatic activity.<sup>18,19)</sup> To analyze isoelectric point of the protein, 1  $\mu$ g of the purified protein with 6% (v/v) Ampholite pH3.5–10 (Amersham) was subjected on a Rectangel electrophoresis apparatus (Atto) at 5 mA for 30 min, 3 mA for 30 min and 1 mA for 5 h in cathodic 25 mM sodium hydroxide (NaOH) and anodic 10 mM phosphoric acid buffers. The electrophoresed gel was sliced for 1 cm-width along electrophoretic direction. Then, respective crushed slices were immersed in 2 mL of water for 2 h at 25°C, and then pH of respective suspensions were measured for isoelectric point. The position of protein was detected by CBB-R250 staining with another aliquot of the gel. For protein sequencing, 1  $\mu$ g of the purified protein was separated by SDS-PAGE. The gel stained with Serva Blue-G (Serva) was transblotted to polyvinylidene fluoride membrane (BioRad) with 1 mM *N*-cyclohexyl-3-aminopropanesulfonic acid-NaOH buffer (pH11) and 1% (v/v) methanol at 150 mA cm<sup>-2</sup> for 30 min on Semidry blotting apparatus (BioRad). The membrane piece containing the protein was subjected to the Protein sequencer Type 477A (Applied Biosystems).

SOD enzymatic assay was based on a previously reported procedure with cytochrome C.<sup>20)</sup> Absorbance of 3 mL reaction mixture containing appropriate amount of SOD protein was monitored at 550 nm for one min by a thermo-controlled spectrophotometer (Shimadzu UV1200/TCC-240A). One unit of SOD activity was defined on the basis of 50%-inhibition of absorbance change. For definition of protein species, samples were pretreated with 1 mM potas-

sium cyanide (KCN) for up to 60 min, and with up to 175  $\mu\text{M}$  hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 60 min before measurement of the activity.

### Gene cloning and sequencing

Chromosomal DNA of *R. radiotolerans* was extracted by a common procedure.<sup>21)</sup> For cell lysis, 0.4 mg  $\text{mL}^{-1}$  achromopeptidase was added. Polymerase chain reaction (PCR) of the chromosomal DNA was performed with AmpliTaq Gold (Applied Biosystems) and two primers, Prim5'-2 and Prim3'-2, which were based on N-terminal amino acid sequence of the purified protein and C-terminal conserved region of prokaryotic Mn-SOD sequence, VWEHAYYI, respectively.<sup>22)</sup> The partial 519 bp-PCR product was used for the following full-length gene cloning. 6-9 kbp-fragments of the chromosome DNA digested with both *Pst*I and *Bst*YI contained similar sequence by southern hybridization for the 519 bp-amplified product as the probe (data not shown). Therefore, the 6-9 kbp-fragments were recovered from electrophoresed gel and used for full length cloning of the gene. The fragments were ligated with the *Sau*3AI cassette of LA PCR *in vitro* Cloning Kit (Takara Bio) at the *Bst*YI end. Those products were introduced into pTAC1 vector with TA PCR Cloning Kit with Jet (BioDynamics Laboratory). Consecutive PCRs with CassettePrimer-C1/RRSOD-S3 and CassettePrimer-C2/RRSOD-S6 for the vector-introduced DNA fragment were performed for cloning of the full-length gene and downstream region. Cloning of upstream region of the gene was performed in a similar manner except for some alterations mentioned below. The chromosomal DNA was digested with only *Pst*I. Then 6-9 kbp-products were ligated with *Pst*I cassette at both ends, and amplified by two consecutive PCRs with appropriate primer sets. DNA sequencing for each product was performed by ABI PRISM 310 Genetic Analyzer with BigDYE Sequencing Kit (Applied Biosystems). Sequence data was analyzed by Blast and Clustal W.<sup>23,24)</sup> Oligodeoxyribonucleotides (ODNs) for cloning and sequencing primers were prepared by chemical synthesis with phosphoramidite units. ODNs used in this study are listed in Supplemental data 1.

### Functional complementation test for radio-resistance

The cloned DNA fragment containing the full-length gene was digested with both *Eco*RI and *Nco*I, and then introduced into pACYC184 vector digested with same restriction enzymes to construct pRrSOD. This plasmid was introduced into the competent cell of *E. coli* QC1726. The *E. coli* cells exponentially growing aerobically in LB media at 37°C were irradiated with gamma-rays up to 500 Gy on <sup>60</sup>Co-gamma source of Kyoto University Research Reactor Institute. The dose rate was 10 Gy  $\text{min}^{-1}$ . Irradiated cells were plated on LB agar plates with approximate dilution. After 1 day incubation at 37°C, the growing colonies were counted to calculate surviving fraction. To evaluate operation of the

introduced gene in *E. coli* cell, SOD activity of the crude cell extract was measured by SOD Test Kit (Wako Chemicals) based on a procedure with nitro blue tetrazolium.<sup>19)</sup> For the same purpose, sensitivity of *E. coli* cells for methyl viologen, a reagent producing superoxide ( $\cdot\text{O}_2^-$ ), was also assessed. The exponentially growing cells were exposed to methyl viologen up to 18 mg  $\text{mL}^{-1}$  for 1 h. Then, cells were diluted and plated on LB agar plates for colony counting.

## RESULTS AND DISCUSSION

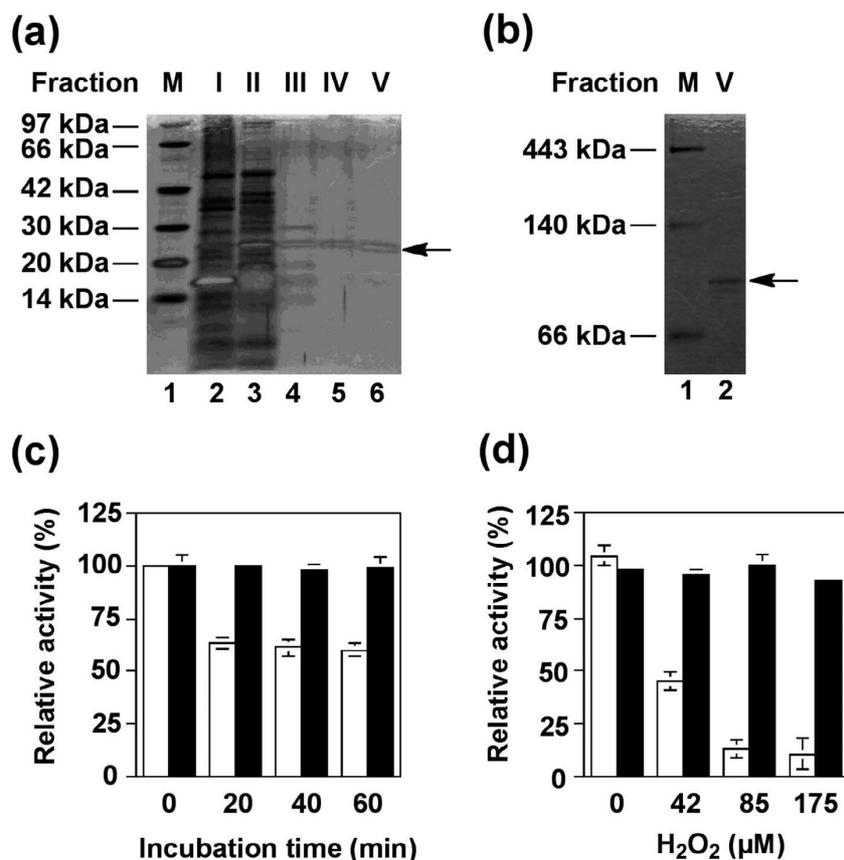
The SOD protein was sufficiently purified after four consecutive chromatographic purification steps (Table 1). The purification chromatograms are shown in Supplemental data 2. The obtained protein through whole purification (Fraction V) showed a single band at 24,000 Da on SDS-PAGE gel with silver staining, which was also supporting evidence for sufficient purification (Fig. 1a). Therefore Fraction V was used for all following experiments in this study. The protein showed different mobility on native-PAGE, which corresponded to 89,600 Da (Fig. 1b). The duplicated gel stained with in-gel activity showed that the corresponding protein band in native-PAGE gel was certainly the purified SOD (data not shown). Apparent mass on native-PAGE gel suggested that the active protein was composed of four homogeneous subunits under physiological conditions. Electrophoresing pattern of the protein indicated that the isoelectric point was 4.43. The final purified protein showed 21165.6 U  $\text{mg}^{-1}$  as its specific activity based on the assay method with cytochrome C.<sup>20)</sup> This specific activity indicated that purification was done adequately as in other previous reports about bacterial SODs.

Enzymatic activity of the purified SOD was resistant to KCN and  $\text{H}_2\text{O}_2$ . The protein was not exhausted for 60 min-incubation with 1 mM KCN before activity measurement (Fig. 1c). Meanwhile bovine erythrocyte Cu, Zn-SOD

**Table 1.** Purification table of the SOD of *R. radiotolerans*

Fraction	Protein amount (mg)	Total activity (U)	Specific activity (U $\text{mg}^{-1}$ )	Yield (%)	Purification (fold)
I	296	12335	41.7	100	1.0
II	55.9	8462.1	151.3	68.6	3.6
III	13.6	6834.1	502.1	55.4	12.1
IV	0.500	1768.5	3537.1	14.3	84.9
V	0.062	1316.2	21165.6	10.7	508.0

I, Crude cell extract; II, active fraction through the first DE-52 chromatography; III, active fraction through hydroxyapatite chromatography; IV, active fraction through gel filtration; V, active fraction through the second DE-52 chromatography. Activity unit is based on the assay procedure described in text.<sup>20)</sup>



**Fig. 1.** (Upper) Protein assays of SOD from *R. radiotolerans*: (a) SDS-PAGE of respective fractions through the purifying operation with silver staining, and (b) native-PAGE of the purified fraction with CBB-R250 staining. Numbers at the left represent molecular masses of the molecular markers (lane M). Arrows indicate the band corresponding to the purified protein. Roman numerals above the gel figures indicate respective fractions through the purifying operation. (Lower) Enzymatic activity of SOD from *R. radiotolerans* and of bovine erythrocyte Cu, Zn-SOD with inhibitors: (c) enzymatic activity after pre-incubation with 1 mM KCN for up to 60 min, and (d) enzymatic activity of SOD proteins after pre-incubation with various concentrations of H<sub>2</sub>O<sub>2</sub> for 60 min. Fraction V was used in both experiments as the final purified SOD protein from *R. radiotolerans*. White and black columns represent bovine erythrocyte Cu, Zn-SOD and SOD of *R. radiotolerans*, respectively. Data in experiments (c) and (d) were standardized with respective values at 0 min in (c). Data were averages of three independent experiments. Standard deviations are indicated with error bars.

decreased its enzymatic activity to 60.1% after 20 min-incubation with the same concentration of KCN. The protein was also indicative of being resistant for 60 min-incubation with up to 175  $\mu$ M H<sub>2</sub>O<sub>2</sub>, but Cu, Zn-SOD was greatly reduced to 10.6% in activity under the same condition with 175  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fig. 1d). Of the three SOD species with different metal cofactors, i.e., one with copper plus zinc (Cu, Zn-SOD), one with iron (Fe-SOD) and one with manganese (Mn-SOD), Cu, Zn-SOD showed sensitivity to KCN,<sup>25)</sup> and both Cu, Zn-SOD and iron SOD were sensitive to H<sub>2</sub>O<sub>2</sub>.<sup>26)</sup> All these biochemical characteristic results indicated that the SOD purified from *R. radiotolerans* has properties similar to known manganese SODs. The property of resistance to H<sub>2</sub>O<sub>2</sub> is thought to be consistent with the radio-protection mechanism.

We also obtained amino acid sequence of the N-terminal region of the purified protein as "AYELPPLPYDYNALE." The N-terminal methionine is thought to be detached from the protein after translation. This sequence is certainly similar to other bacterial Mn-SODs.<sup>22)</sup> We then cloned the gene from the obtained sequence and the sequence "VWE-HAYYI" as a highly conserved region close to the C-terminal active site of prokaryotic Mn-SOD.<sup>22)</sup> Finally, the full-length gene was obtained with its open reading frame (ORF) containing 618 nucleotides and the deduced protein sequence with 206 amino acids (Fig. 2a). Molecular mass of the protein is calculated to be 23463.6 Da with this deduced amino acid sequence. It is consistent with the mass of the subunit estimated from the SDS-PAGE result mentioned above. The deduced amino acid sequence conserves four residues as

(a)

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1 ATGGCTTACGAATTGCCCGCGCTCCCGTACGACTACAACGCGCTCGAACCGACAATAGAC 60
1 MetAlaTyrGluLeuProProLeuProTyrAspTyrAsnAlaLeuGluProThrIleAsp 20

61 GAGCAGACGATGCACCTCCACCACGAGAAGCACCACAACACCTACCTACCAACCTGAAC 120
21 GluGlnThrMetHisLeuHisHisGluLysHisHisAsnThrTyrLeuThrAsnLeuAsn 40

121 GCCGCGCTCGAGAAGCACCCGGACGCCGACCCGGGCGACGCCGAGGAGCTGATGCGCAAC 180
41 AlaAlaLeuGluLysHisProAspAlaAspProGlyAspAlaGluGluLeuMetArgAsn 60

181 ATAGACTCCGTCCCGGACGACATAAAGACCGCGGTCCGCAACAACGGCGGGGGTACGTA 240
61 IleAspSerValProAspAspIleLysThrAlaValArgAsnAsnGlyGlyGlyTyrVal 80

241 AACCACAACCTTCTTCTGGCAGATCATGTCCCCGACGGCGGCGGCGAGCCGACCGGCGAC 300
81 AsnHisAsnPhePheTrpGlnIleMetSerProAspGlyGlyGlyGluProThrGlyAsp 100

301 CTCGCGCAGGCGATAAACGACGCCCTACGGCTCCTTCGACGCCTTCAAGGAGAGTGGGGC 360
101 LeuAlaGlnAlaIleAsnAspAlaTyrGlySerPheAspAlaPheLysGluGluTrpGly 120

361 AAGGTCCGGGGTCCCGGACGCGTCTTCGGTTCGGGTGGGTCTGGCTTATAGCCAACCCC 420
121 LysValGlyAlaProGlySerValPheGlySerGlyTrpValTrpLeuIleAlaAsnPro 140

421 GACGGTCCGTACGATCGAGAAGACCGCAAACCAGGACTCCCCCTCATGGAGGGCAAG 480
141 AspGlySerValThrIleGluLysThrAlaAsnGlnAspSerProLeuMetGluGlyLys 160

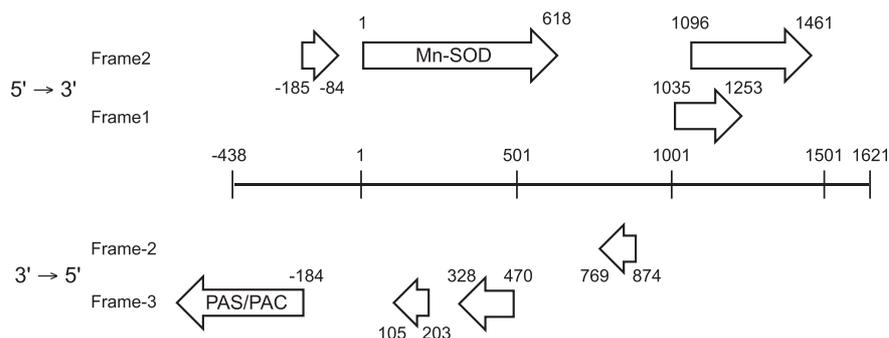
481 ACCCCGTCCTGGGGTTCGACGTCTGGGAGCAGCCTACTACCTCAAGTACCAGAACCGT 540
161 ThrProValLeuGlyLeuAspValTrpGluHisAlaTyrTyrLeuLysTyrGlnAsnArg 180

541 CGTCCCAGTACATCGAGAACTGGTGAACGTCGTTAACTGGGAAGAGGCCGAGCGCCGC 600
181 ArgProGluTyrIleGluAsnTrpTrpAsnValValAsnTrpGluGluAlaGluArgArg 200

601 TACCAGGAGATCACCCGCTAG
201 TyrGlnGluIleThrArg***

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(b)



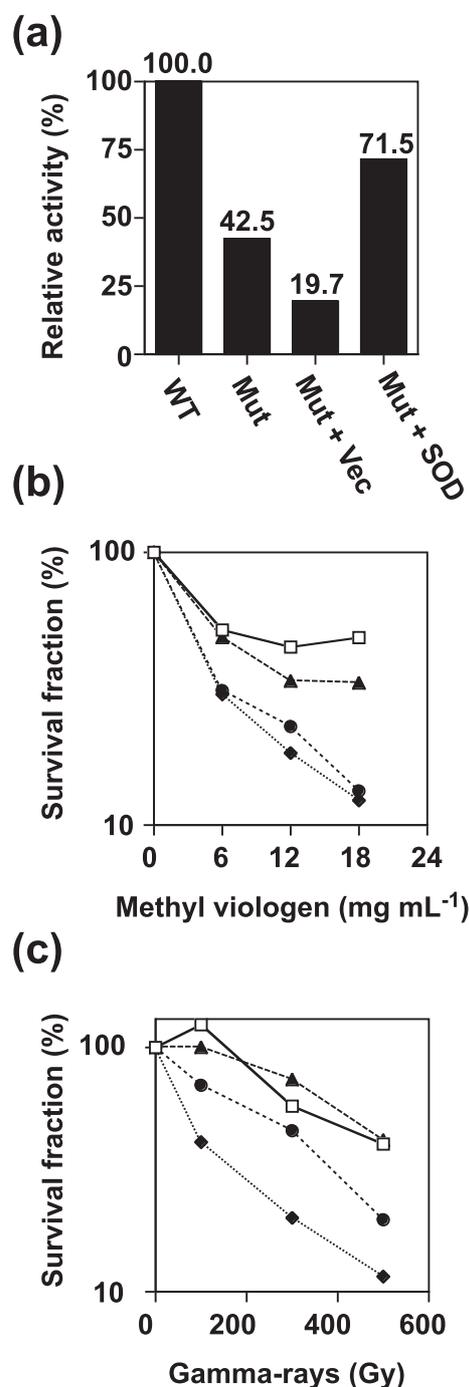
**Fig. 2.** Gene structure of Mn-SOD of *R. radiotolerans*: (a) nucleotide sequence (upper line) and deduced amino acid sequence (bottom line) of Mn-SOD gene of *R. radiotolerans*, and (b) genomic region of *R. radiotolerans* containing Mn-SOD gene and adjacent areas analyzed in this study. Four conserved amino acid residues as ligands to metal cofactor are underlined in (a). ORFs found in the analysis are indicated as arrow outlines pointing in transcriptional orientations. Each pair of numbers close to arrow indicates start and end positions of the corresponding ORF. Numbers are all from the start position of Mn-SOD gene. Except for Mn-SOD gene and putative PAS/PAC sensor protein, other ORFs shown in this figure have no information homologous to any genes. The nucleotide sequence data has been deposited in DDBJ database under the accession number AB086393.

ligands to the metal cofactor in Mn-SODs (Fig. 2a).<sup>27)</sup> It has certain homology to other prokaryotic Mn-SODs, too (Supplemental data 3). The ORF has promoter sequences in 5'-upstream from the first codon, such as -10 and -35 elements showing GAGAAG and TAGAAG, respectively. Another

ORF is found further in the 5'-upstream region, which is a gene for a putative PAS/PAC sensor protein like *Rubrobacter xylanophilus*. PAS domains are shown in many proteins responsible for sensing of oxygen, redox, light or metals.<sup>28)</sup> Later, PAC domain was detected as a similar motif to PAS

domain, and they have since been referred to as PAS/PAC domains.<sup>29</sup> It is interesting that the ORF of putative PAS/PAC sensor protein is close to the Mn-SOD gene on the genome of *R. radiotolerans* like *R. xylanophilus*, because these two products seem to be involved in redox regulation. We need further study to clarify function of the PAS/PAC proteins in the two *Rubrobacter* species. This ORF has both -10 and -35 elements and starts from 184-nucleotide upstream of the first codon of SOD gene. Both genes are oppositely oriented on the chromosomal DNA. On the other hand, 3'-downstream region of SOD gene containing three ORFs has no similarity to *R. xylanophilus*. Although the deduced SOD protein sequence shows certain homology to other prokaryotic Mn-SOD species, the best score for homology is not for the congeneric *R. xylanophilus*. The identity and similarity between *R. radiotolerans* and *R. xylanophilus* are 63% and 78%, respectively (Supplemental data 3). Taking into account the fact there is no similarity in their 3'-adjacent regions of Mn-SOD genes as opposed to their 5'- counterparts (Fig. 2b), we can consider the evolutionary and environmental acquisitions of radio-resistance property of radio-resistant species. *Rubrobacter* species reported here were found at globally separated hot waters: Japanese (*R. radiotolerans*),<sup>3</sup> Portuguese (*R. xylanophilus*)<sup>30,31</sup> and Taiwanese (*Rubrobacter taiwanensis*).<sup>32</sup> Those congeneric species are thought to have acquired their individual character over relatively long separated periods in their respective habitats. And those habitats are filled with a variety of environment factors, such as ionizing radiation and high temperature, which can enhance a given genetic alteration.

To evaluate involvement of SOD in radio-resistance, the gene was introduced into *E. coli* cells lacking whole intrinsic SOD genes. The exogenous SOD gene from *R. radiotolerans* seemed to function in *E. coli* cell, because the gene introduced into it improved its SOD activity (Fig. 3a). The activity assay for crude cell extracts indicated that *E. coli* QC1726 lacking two endogenous SOD genes (*sodA* and *sodB*) showed only 42.5% of the wild type GC4468. Introduction of the cloned SOD gene into QC1726 improved the activity level up to 71.5% of the wild type. The empty vector pACYC184 did not recover the enzymatic activity. Rather, it further decreased the activity to 19.7% of the wild type. Intracellular existence of empty vector sometimes affects the cell viability with its burden of additional replication. It seems to bear recovery of the enzymatic activity in the cell harboring full length SOD gene. We found that introduction of the SOD gene with another plasmid vector, pTrc99A, showed similar recovery of the activity (data not shown). As expected, the introduced gene led the host cell into increased resistivity for methyl viologen, a reagent producing  $\cdot\text{O}_2^-$  (Fig. 3b). The mutant QC1726 cell became more sensitive to methyl viologen than the wild type GC4468. As with the enzymatic activity of crude cell extract, QC1726 harboring



**Fig. 3.** Function of SOD gene introduced into *E. coli* cells: (a) SOD activity of *E. coli* crude cell extracts. Examined were *E. coli* GC4468 as wild type for intrinsic *sodA* and *sodB* (WT), QC1726 lacking entire SOD function due to those two genes (Mut), QC1726 carrying pRrSOD (Mut + SOD), and pACYC184 (Mut + Vec). Data are standardized with that of the wild type. Numbers at respective columns are relative activity (%). Survival fractions for methyl viologen (b) and gamma-rays (c) of *E. coli* cells. GC4468 (open square), QC1726 (closed diamond), QC1726 carrying pRrSOD (closed triangle), and pACYC184 (closed circle) were examined.

the exogenous SOD gene restored the resistance to 67.7% of resistivity of the wild type, but not the empty vector. These results indicate that SOD gene from *R. radiotolerans* manifests its native activity in the recipient *E. coli* cells.

The mutant QC1726 cell also exhibited more sensitivity to gamma-rays (Fig. 3c). Against gamma-rays, the introduced SOD gene achieved the recovery of radio-resistance of the mutant cell; the sensitivity of QC1726 harboring the exogenous gene became equivalent to that of the wild type. The empty vector had insignificant effect in this case, too. The slight improvement of survival with the empty vector might be derived from retardation of the cell division rate, which is due to addition of the redundant genetic materials. Thus, the SOD gene from *R. radiotolerans* simultaneously provided recovery of both SOD activity and radio-resistance for the mutant host cell lacking intrinsic SOD gene. There are significant studies that have demonstrated the radio-protecting ability of SOD *in vitro* and *in vivo*, supporting our conclusion.<sup>33–35</sup> The primary products from irradiated water molecule are hydrogen radical (H·), hydroxy radical (·OH) and hydrated electron ( $e^-_{aq}$ ), but not  $\cdot O_2^-$ . However  $\cdot O_2^-$  is generated with H· or  $e^-_{aq}$  under oxic condition.  $\cdot O_2^-$  shows relatively inactive character itself, but it can produce the most reactive ·OH in cooperation with H<sub>2</sub>O<sub>2</sub>, which is one of indirect products. This process is referred to as the Haber-Weiss reaction, which requires presence of catalytic metal ions such as Fe<sup>2+</sup>.<sup>36</sup> Thus  $\cdot O_2^-$  certainly contributes to radiation effects in a common organic system, which is usually under oxic condition, where such metal ions abound; the effect is termed as oxygen effect.<sup>37</sup> SOD can reduce radiation effect by inhibiting the oxygen effect via its ability to remove  $\cdot O_2^-$ .<sup>38,39</sup> The SOD is thought to be in part responsible for the high radio-resistant character of *R. radiotolerans*.

Conclusively, we purified and characterized Mn-SOD of *R. radiotolerans*, and analyzed the gene structure and radio-protecting function in this study. Our present results indicate that the Mn-SOD exerts capability for the protection of *R. radiotolerans* from ionizing radiation. We now know only two radio-protecting factors of this species, SOD and carotenoid, and definitely need further investigation to shed light on the total picture of this species' radio-resistance.

#### ACKNOWLEDGMENT

We appreciate generous gift of bacterial strains provided by Prof. Tatsuo Nunoshiba at Tohoku University (presently at International Christian University). We also acknowledge both RIKEN Bio-Resource Center and National Institute of Genetics through National Bio-Resource Project of Japan for other resources. This work was carried out in part under the Visiting Researchers Program of Kyoto University Research Reactor Institute. It was partly supported by Chugoku Electric Research Foundation (HT).

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Received on June 19, 2011

Revision received on July 22, 2011

Accepted on July 25, 2011

J-STAGE Advance Publication Date: October 5, 2011