The Journal of Microbiology, April 2009, p. 172-177 Vol. 47, No. 2
DOI 10.1007/s12275-008-0221-0


# Iso-Superoxide Dismutase in *Deinococcus grandis*, a UV Resistant Bacterium

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(Received September 10, 2008 / Accepted February 9, 2009)

***Deinococcus grandis*** **possesses two types of superoxide dismutase (SOD, E. C. 1.15.1.1.) that show distinct electrophoretic behavior, one that migrates slowly and the other that migrates rapidly (SOD-1 and SOD-2, respectively). In this study, SOD-1 was uniformly and abundantly detected, regardless of growth phase, whereas SOD-2 was not detected during early growth, but was detectable from the exponential growth phase. In addition, a substantial increase in SOD-2 was observed in cells that were treated with potassium superoxide or UV, which suggests that SOD-2 is an inducible protein produced in response to stressful environments. Insensitivity of SOD-1 to both $H_2O_2$ and cyanide treatment suggests that SOD-1 is MnSOD. However, SOD-2 would be FeSOD, since it lost activity in response to $H_2O_2$ treatment, but not to cyanide. Localization studies of *D. grandis* iso-SODs in sucrose-shocked cells suggest that SOD-1 is a membrane-associated enzyme, whereas SOD-2 is a cytosolic enzyme. In conclusion, SOD-1 seems to be an essential constitutive enzyme for viability and SOD-2 appears to be an inducible enzyme that is probably critical for survival upon UV irradiation and oxidative stress.**

***Keywords*****:** *Deinococcus grandis*, iso-SODs, membrane bound SOD, inducible cytosolic SOD

The genus *Deinococcus*, which belongs to Deinococcus/Thermus phylum, has many peculiar characteristics, such as a thick wall comprised of several distinct layers including an outer membrane-like structure, a membrane-bound carotenoid pigment, and the presence of ornithine in muramic acid cross-bridge of peptidoglycan (Brooks and Murray, 1981; Rainey *et al*., 1997; Battista and Rainy, 2001). The most unusual feature of *Deinococcus* species, however, is their extreme resistance to UV, ionizing radiation, and strong oxidative stress (Battista *et al.*, 1999; Yun and Lee, 2000; Battista and Rainy, 2001). Members of the genus *Deinococcus*, which includes not only Gram-positive coccus, but also Gram-negative rod bacteria, have been isolated from a variety of natural habitats (Oyaizu *et al*., 1987; Ferreira *et al*., 1997; Hirsch *et al*., 2004; Suresh *et al*., 2004; De Groot *et al*., 2005; Rainey *et al.*, 2005, 2007; Zang *et al*., 2007). For the insight of UV resistant nature of the genus *Deinococcus*, a numerous investigation was made not only on the repairing systems of damaged DNA (Evans and Moseley, 1983; Agostini *et al*., 1996; Carroll *et al.*, 1996; Minton, 1996; Bauche and Laval, 1999; Narumi *et al*., 2004), but also on scavenging enzymes of reactive oxygen species (ROS) generated by radiation or oxidative stress (Oh and Lee, 1998; Yun and Lee, 2000; Yun and Lee, 2004; Kobayashi *et al*., 2006; Seo and Lee, 2006; Sung and Lee, 2007). A report on the SOD profiles of some mesophilic *Deinococcus* showed that there were multiple forms of SOD in *D. radiodurans* (three iso-SODs), *D. proteolyticus* (two iso-SODs), and *D. grandis* (two iso- SODs) (Yun and Lee, 2001). To gain an insight on the roles and nature of iso-SODs in Deinococcal bacteria, the production pattern of iso-SODs in *D. grandis* under stressful conditions and their sub-cellular localization were currently investigated in this study.

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## Materials and Methods

### Bacterial culture and chemicals

*D. grandis* ATCC 43672 (American Type Culture Collection, USA) was cultured in TYGM medium containing 1% tryptone, 0.5% yeast extract, 0.2% glucose, and 0.2% L-methionine at 30°C with continuous aeration at 150 rpm (Yun and Lee, 2004). Bacterial growth was monitored by measuring the $OD_{600}$ (DU-65 Spectrophotometer, Beckman, USA). Chemicals including potassium superoxide, hydrogen peroxide solution, nitro blue tetrazolium, riboflavin, pyrogallol, cacodylic acid, KCN, and materials for gel electrophoresis were purchased from Sigma Chemical Co. Most of the medium constituents were bought from Difco Laboratory.

### Preparation of cell-free extracts

The cells harvested by centrifugation at 4,500×g for 20 min (Supra 22K, Hanil, Korea) were suspended in 50 mM potassium phosphate buffer (pH 7.0), after which they were washed three times with the same buffer. The cell-free extracts were obtained from sonicated-preparations of the cells at 4°C (5 sec pulse on, 15 sec pulse off for a total 5 min, Fisher sonic dismembrator, Fisher Scientific, USA) and then by centrifugation at 12,000×g for 20 min (Yun and Lee, 2004).

### SOD assay and protein quantification

SOD activity was assayed using the pyrogallol autooxidation

method (Marklund and Marklund, 1974). Briefly, 1 ml of 50 mM Tris-Cl buffer (pH 8.2) containing 50 mM of cacodylic acid and 1 mM diethylenediamine-pentaacetic acid (EDPA) was placed in a cuvette. Then, either 10 μl of 50 mM potassium phosphate buffer (pH 7.0) (E*std*) or 10 μl of cell-free extract (E*test*) was added. Subsequently, 20 μl of substrate solution containing 10 mM pyrogallol and 0.05 mM hydrochloric acid was added to the enzyme assay mixture. After briefly shaking the reaction mixture, the absorbance of the reaction mixture at 420 nm was recorded for 210 sec at ambient temperature. The difference ($\triangle E$) in absorbance between these two readings was then used to calculate the SOD activity as follows: ($\triangle Estd$-$\triangle Etest$)×2.06 ×100/$\triangle Estd$. One unit of SOD was defined as the amount of enzyme required to induce 50% inhibition of the pyrogallol autooxidation rate at 420 nm. The protein concentration was measured by the method described by Lowry *et al*. (1951), using BSA as a standard.

### Gel electrophoresis and SOD activity staining on polyacrylamide gel

Proteins in cell-free extract were resolved by polyacrylamide gel electrophoresis (PAGE) (Gersten, 1996). Visualization of the SOD bands resolved on the gel was conducted by the SOD activity staining method (Beauchamp and Fridovich, 1971; Kroll *et al*., 1991). Briefly, the gels were soaked in a solution of 490 μM nitro blue tetrazolium (NBT) for 20 min, then in a solution containing 14 mM tetramethylenediamine (TEMED), 14 μM riboflavin, and 36 μM phosphate potassium phosphate (pH 7.8) for 15 min. The gel was then illuminated using a fluorescent lamp for 5∼15 min to visualize the white achromatic zone of SOD activity on the blue background. The SOD bands on the gel were analyzed by densitometry using a Kodak eletrophoresis documentation and analysis system (Kodak ID Image Analysis, Japan). The sensitivity of the SODs on the gel to chemicals was evaluated by soaking the gel in 50 mM potassium phosphate buffer (pH 7.8) containing either 20 mM $H_2O_2$ or 10 mM KCN for 60 min at room temperature prior to activity staining of the SOD (Kroll *et al*., 1991; Yun and Lee, 2004).

### SOD production in cells under stressful conditions

After the cell culture reached an $OD_{600}$=0.3∼0.4, cells were treated with different concentrations of hydrogen peroxide or potassium superoxide (0∼20 mM) and then allowed to incubate for an additional 60 min. Then, the cells were harvested and disrupted by ultrasonication to prepare cell-free extracts. For UV irradiation, cells ($OD_{600}$=0.3∼0.4) were harvested and resuspended in 50 mM potassium phosphate buffer (pH 7.0) and then subjected to UV irradiation (UV lamp, Minuvis, Desaga, Germany). The UV dose was measured using a VLX 3 W radiometer (Radiometer, France).

### Cellular localization of iso-SODs

Cells harvested at the stationary growth phase were suspended in 30 mM Tris-HCl buffer (pH 8.0) containing 20% sucrose and 1 mM of EDTA at a ratio of 1 g of wet cells to 80 ml of buffer (Yun and Lee, 2000). After 10 min of incubation at 30°C, centrifugation was followed to fractionate the supernatant (sucrose-shocked fluid) and cell debris-pellet. The proteins in the supernatant (sucrose-shocked fluid) were dialyzed against 50 mM potassium phosphate buffer (pH 7.0) and then concentrated several fold by ultra-filtration (PM-10 membrane, Amicon, USA). The loosely bound proteins on the sucrose shocked cell debris-pellet were then released by rapidly suspending the pellet in cold distilled water followed by 10 min of incubation in an ice bath, after which the cell-debris pellet was collected by centrifugation. Proteins in this pellet were released by sonication and the SODs in each fraction were resolved by PAGE.

## Results and Discussion

The *Deinococcus*, the strict aerobic bacteria, are extraordinarily resistant to UV as well as ionizing radiation and oxidative stress. Superoxide dismutase along with catalase is an essential enzymes involved in protecting organisms from the toxicity of ROS that are generated as a result of normal aerobic respiration and by environmental stress such as UV- and ionizing radiations (Walkup and Kogoma, 1989; Fridovich, 1995; Wang and Schellhorn, 1995; Halliwell and Gutteridge, 1999). The SOD activity in *D. grandis* began to increase from the late exponential growth phase, and the

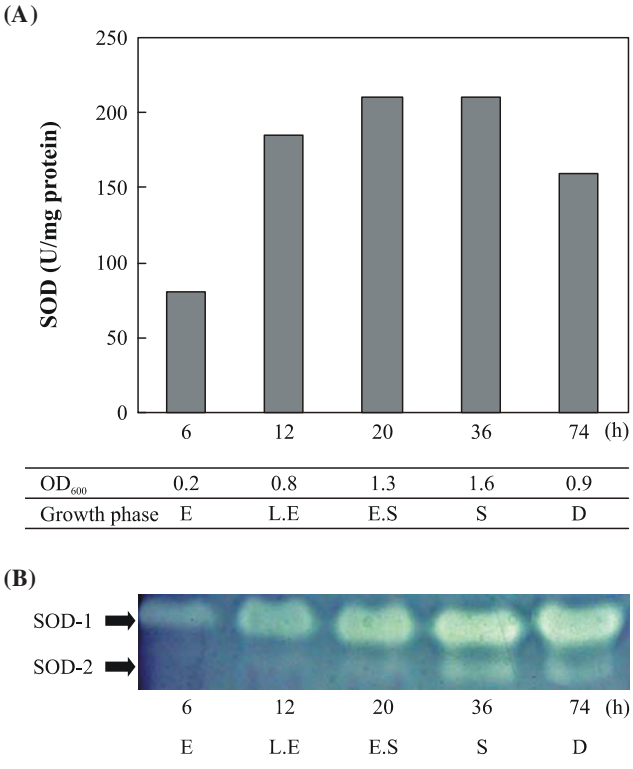


**Fig. 1.** Production of SOD by *D. grandis* during growth. (A) Growth and SOD activity of *D. grandis*. Exponentially grown *D. grandis* in TYGM medium was subcultured into a new TYGM to give $OD_{600}$=0.1. (B) Proteins in cell-free extracts were resolved on 10% gel. Activity staining of SOD band was made by Chou and Tan method (1990). E, exponential phase; L.E, late exponential phase; E.S, early stationary phase; S, stationary phase; D, death phase. SOD activity represents an average of triplicates. This data is a representative of several experiments.

level of SOD at stationary phase was remarkably increased, but it was slightly reduced during the death phase. A substantial increase of SOD in actively growing *D. grandis* is quite conceivable because SOD is the enzyme involved in the scavenging of superoxide radicals generated during normal aerobic respiration. Electrophoretic resolution of the SOD in cell-free extracts prepared with *D. grandis* cultures in each growth phase revealed that SOD-1 was constitutively present irrespective of the growth phases, whereas SOD-2 was observed at the late exponential phase as shown in Fig. 1. Densitometry of each of the SOD bands (data not shown) on the gels showed that the SOD-1 activity at the stationary phase was a few times higher than that of SOD-2. he growth dependent production of SOD-2 in cells after the early stationary phase would be attributable to the accumulation of endogenous oxidants due to an increase in respiratory activity. Similar observations of increased SOD activity in aging cells or cells under internal oxidative stress have been well documented in other organisms (Chou and Tan, 1990; Schnell and Steinman, 1995; St. John and Steinman, 1996; Park and Han, 1997). The presence of iso-SODs in living organisms, including eukaryotes and procaryotes has also been exclusively reported (Benov and Fridovich, 1994; Sadosky *et al*., 1994; Fridovich, 1995; Schnell and Steinman, 1995; St. John and Steinman, 1996; Li *et al*., 2002; Yun and Lee, 2004). When production studies regarding changes of *D. grandis* iso-SODs in response to exogenous oxidative stress, such as

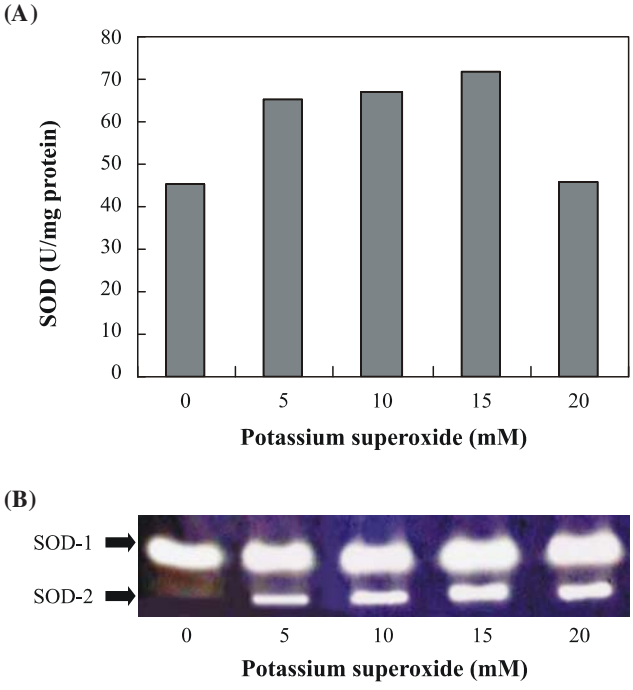


**Fig. 2.** Production of SOD by *D. grandis* treated with potassium superoxide. (A) Cells in the exponential phase ($OD_{600}$=0.3~0.4) were treated with potassium superoxide for 60 min and SOD activity was measured. (B) Proteins in cell-free extracts of potassium superoxide treated-cells were resolved on 10% gel and followed by activity staining. SOD activity represents an average of triplicates. This data is a representative of several experiments.

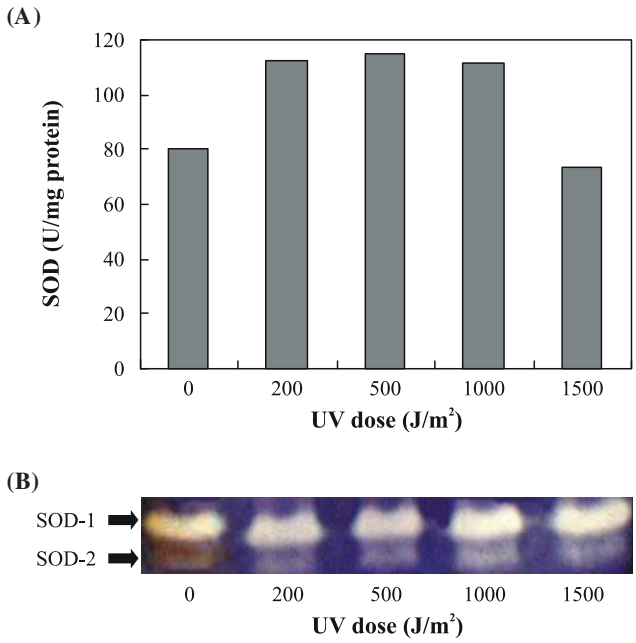


**Fig. 3.** Production of SOD by *D. grandis* irradiated with UV. (A) SOD activity in *D. grandis* treated with different UV dose. Cells at the exponential phase ($OD_{600}$=0.3~0.4) were irradiated with the given UV dose and incubated for 60 min. (B) Proteins in cell-free extracts were resolved on 10% polyacrylamide gel and SOD bands were visualized by activity staining. SOD activity represents an average of triplicates. This data is a representative of several experiments.

superoxide- and peroxide-treatment or UV irradiation were conducted, the production pattern of SODs was somewhat dissimilar. The treatment of the cells in the mid-exponential growth phase with potassium superoxide caused an increase in total SOD activity (ca. 45% increase at 5~15 mM potassium superoxide) as shown in Fig. 2A. As shown in Fig. 2B, band intensities of SOD-1 resolved on gel were rather similar except ca. 15% increase of SOD-1 intensity of the cells treated with 15 mM potassium superoxide when estimated by a densitometer. Interestingly, an apparent increase in SOD-2 was occurred in the cells treated with even 5 mM potassium superoxide. Similar results of SOD production were obtained in cells exposed to UV, and SOD-2 production was equally dependent on UV and oxidative stress (Fig. 3). However, in cells treated with various concentrations of hydrogen peroxide, we observed that the level of SOD-1 remained nearly constant and that of SOD-2 was not increased at any concentration (Fig. 4), unlikely the induction of SOD-2 in cells exposed to superoxide or UV. This is not surprising since the peroxide ion is the product of SOD activity dismutating superoxide radical in the ROS scavenging system (Fridovich, 1995).

All SODs are metalloenzymes, regardless their origins. The metallo-forms of SOD in bacteria are rather diverse. For instance, a single MnSOD in *Bacillus subtilis* (Inaoka *et al*., 1998) and NiSOD in *Streptomyces* spp. (Youn *et al*., 1996) were reported, whereas the iso-SODs MnSOD, FeSOD, and CuZnSOD were detected in *Escherichia coli* (Benov and

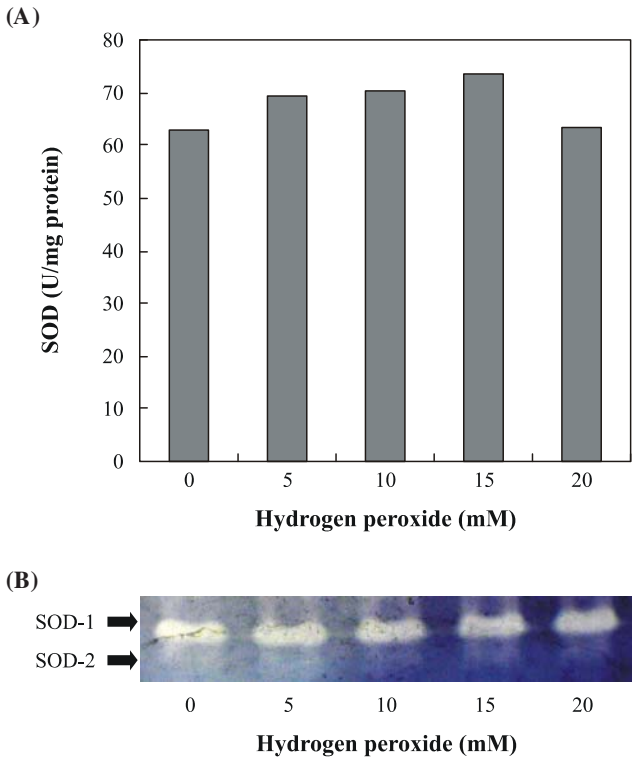


**Fig. 4.** Production of SOD by *D. grandis* treated with hydrogen peroxide. (A) Cells in the exponential phase ($OD_{600}$=0.3~0.4) were treated with hydrogen peroxide for 60 min and specific activity of SOD in the hydrogen peroxide treated cells was measured. (B) Proteins in cell-free extracts of the hydrogen peroxide treated-cells were resolved on 10% gel and followed by activity staining. SOD activity represents an average of triplicates. This data is a representative of several experiments.

Fridovich, 1994). Simple distinction of the metallo-form of SODs is possible by evaluating the sensitivity of enzymes to cyanide ($CN^-$) or $H_2O_2$ treatment. Cyanide is a reversible inhibitor of SODs that exerts a dose-dependent effect whereas $H_2O_2$ is an irreversible inhibitor (Fridovich, 1995). It is known that MnSOD is insensitive to not only cyanide ($CN^-$) but also $H_2O_2$, whereas CuZnSOD is sensitive to both chemicals, and FeSOD is sensitive to $H_2O_2$ but not to $CN^-$ (Kroll *et al*., 1991; Yun and Lee, 2004). When PAGE

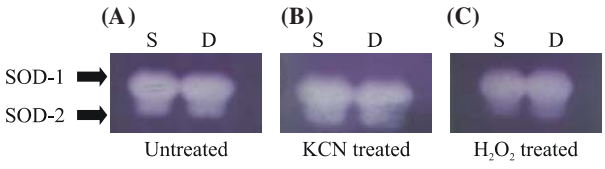


**Fig. 5.** Susceptibility of *D. grandis* SODs to $H_2O_2$ and KCN. Activity staining of SOD on 10% gel prior to either 50 mM KCN (B) or 20 mM $H_2O_2$ treatment (C). Each well was loaded with 6.8 μg of protein in *D. grandis* cell-free extracts obtained from either stationary (S) or death (D) phase. MnSOD is not susceptible to both $H_2O_2$ and KCN, whereas FeSOD is inactivated by $H_2O_2$, but not by KCN treatment.

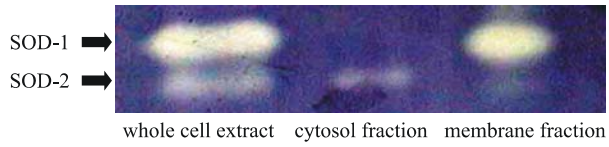


**Fig. 6.** Cellular localization of iso-SODs of *D. grandis.* Resolution of *D. grandis* iso-SODs in each fraction by PAGE followed by activity staining. Each well was loaded with 10 μg protein.

gels resolved proteins in cell-free extract prepared from the stationary culture of *D. grandis* were treated with either $H_2O_2$ or KCN and followed the SOD activity staining, activity of SOD-1 was not affected by such treatments, suggesting that SOD-1 is MnSOD. However, a strong inhibition of SOD-2 by $H_2O_2$ treatment was observed, but no inhibition of SOD activity in response to KCN treatment (Fig. 5). The strong inhibition of SOD-2 by $H_2O_2$ as well as the insignificant change of enzyme activity by KCN suggests that the SOD-2 is FeSOD. However, metal analysis of these SODs in purified enzyme preparation is required to confirm the nature of the metal cofactor. For this reason, *D. grandis* SODs are currently being purified. In general, SOD isoenzymes show differentiated physiological roles through expression under different situation as well as through different sub-cellular localization (Kroll *et al*., 1991; Sadosky *et al*., 1994; Schnell and Steinman, 1995; St. John and Steinman, 1996; Li *et al*., 2002; Dufernez *et al*., 2006). Electrophoretic resolution of *D. grandis* SODs in the fractions of sucrose-shocked cells revealed that SOD-2 was present in the supernatant of sucrose-shocked cells, whereas SOD-1 was present in the cell pellet fraction (Fig. 6). These results clearly indicate that SOD-1 was a membrane-associated enzyme, whereas SOD-2 was a cytosolic enzyme. Briefly, the differential expression and subcellular localization of iso-SODs in *D. grandis* suggests that the membrane associated SOD-1 is an essential constitutive enzyme required during the normal aerobic growth and that a cytosolic SOD-2 is an inducible enzyme which is probably critical in withstanding UV irradiation and oxidative stress.

## Acknowledgements

This work was supported by a Chungbuk National University grant in 2006. We thank to Ms. E.-J. Park for her assistance.

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