Production of Superoxide Dismutase by *Deinococcus radiophilus*

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The production of superoxide dismutase (SOD) varied in *Deinococcus radiophilus*, the UV resistant bacterium, depending upon different phases of growth, UV irradiation, and superoxide treatment. A gradual increase in total SOD activity occurred up to the stationary phases. The electrophoretic resolution of the SOD in cell extracts of *D. radiophilus* at each growth phase revealed the occurrence of MnSOD throughout the growth phases. The SOD profiles of *D. radiophilus* at the exponential phase received oxidative stress by the potassium superoxide treatment or UV irradiation also revealed the occurrence of a single SOD. However, these treatments caused an increase in SOD activity. The data strongly suggest that *D. radiophilus* has only one species of SOD as a constitutive enzyme, which seems to be a membrane-associated protein.

Keywords: Constitutive enzyme, *Deinococcus radiophilus*, Membrane-associated SOD, Oxidative stress, Superoxide dismutase, UV resistance

Introduction

Superoxide dismutases (SODs, EC1.15.1.1) protect cells from the toxicity of superoxide radicals (O\(^2^-\)) which are generated in cells by various causes, such as aerobic respiration, UV, ionizing radiations (X-ray, \(\gamma\)-ray, cosmic ray), cigarette smoking, and some redox-cycling drugs (Fridovich, 1995; Halliwell and Gutteridge, 1998; Cho and Song, 2000; Storz and Zheng, 2000). All SODs are metalloenzymes such as MnSOD, FeSOD, and Cu/Zn SOD. Also, an occurrence of NiSOD was reported in the *Streptomyces* species (Youn et al., 1996). Metal ions at the active site of SODs are different, depending upon cell types; Cu/ZnSOD is found exclusively in cytosols of eukaryotes and rarely in prokaryotes. In contrast, MnSOD is present in both the prokaryotes and mitochondria of eukaryotes; FeSOD is found in the cytosol of prokaryotes, as well as in the chloroplasts of some green plants (Halliwell and Gutteridge, 1998).

The most peculiar feature of the *Deinococcus* species is their extreme resistance against UV and ionizing radiations and oxidative stress (Evans and Moseley, 1983; Murray, 1986; Mueller et al., 1996; Battista, 1997). Although it could be speculated that the unusual radio-resistance is attributed to their morphological characteristics (including a thick cell wall of several distinct layers and membrane-bound carotenoid pigment), one could easily assume that the operation of the efficient scavenging systems against the reactive oxygen species (ROS) and the repairing potential of the damaged cellular components that are mediated by toxic oxidants are highly engaged in the extreme radio-resistance of *Deinococcus*. Extensive studies on UV resistance in regard to the repairing genes for damaged DNA (Evans and Moseley, 1983; Gutman et al., 1993; Daly et al., 1994; Agostini et al., 1996; Narumi et al., 1997; Bauche and Laval, 1999; Kim et al., 2002) and the entire genome analysis of *D. radiodurans* that was reported in recent years (White et al., 1999; Makarova et al., 2001) would be insufficient to understand the radio-resistant nature of *Deinococcus*. Therefore, attention has been paid to the scavenging enzymes against the reactive oxygens in relation to the UV resistance. Also, studies on hydroperoxidases (catalases) that eliminate peroxide radicals were conducted in *D. radiophilus* that possess three isotypes of catalases (Lee and Lee, 1995; Oh and Lee, 1998; Yun and Lee, 2000). Nevertheless, other ROS-scavenging enzymes in *Deinococcus* have not yet been investigated. Since SODs, along with catalases, play key roles in protecting cells against toxic oxidants, the production of SOD in *Deinococcus radiophilus* was investigated.

Materials and Methods

Bacterial culture and chemicals *Deinococcus radiophilus* ATCC 27603 was cultured in a TYGM medium (1% tryptone,
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0.5% yeast extract, 0.2% glucose, and 0.2% L-methionine) at 30°C with continuous aeration at 150 rpm (Soung and Lee, 2000; Yun and Lee, 2001). *Pseudomonas aeruginosa* was grown in a Luria-Bertani (LB) medium. Bacterial growth was recorded with an optical density at 600 nm (OD$_{600}$) (DU-65 Spectrophotometer, Beckman). Most of the medium constituents and chemicals including riboflavin, nitro blue tetrazolium (NBT), tetramethylethylene diamine (TEMED), KCN, pyrogallol, cacodylic acid, diethylenediamine-pentacetic acid (EDP A) potassium superoxide, and materials for polyacrylamide gel electrophoresis were purchased from the Difco Lab. (Detroit, USA) and Sigma Chemical Co. (St. Louis, USA), respectively.

**SOD assay and protein quantification** The SOD activity in the cell-free extracts that were prepared by ultrasonic disruption was assayed by the method of pyrogallol autooxidation. The pyrogallol autooxidation rate by superoxide radicals, which is generated chemically from cacodylic acid and EDP A, was measured in the presence of the SOD enzyme. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of the pyrogallol autooxidation rate at 420 nm (Marklund and Marklund, 1974). The protein concentration was determined by the method of Lowry et al. (1951).

**Gel electrophoresis** Proteins in the cell-free extract that was prepared by ultrasonic disruption (Soung and Lee, 2000; Yun and Lee, 2001) were resolved by nondenaturing polyacrylamide gel electrophoresis (PAGE) in a Tris-glycine buffer (Gersten, 1996).

**Activity staining of SOD** Visualization of the SOD bands that were resolved on a polyacrylamide gel was made by a method (Beauchamp and Fridovich, 1971) modified by Chou and Tan (1990). The gels were soaked in 490 uM NBT for 20 min, then in a solution that contained 28 mM TEMED, 28 uM riboflavin, and 36 mM potassium-phosphate buffer (pH 7.8) for 15 min. Then, the gels were illuminated with a fluorescent lamp for 5-15 min to visualize white bands of SOD activity in the blue background. Density of the SOD bands on the gel was measured by densitometry with either gel Doc 1000 (Molecular analyst software, version 1.4.1) from Bio-RAD (Hercules, USA), or Kodak electrophoresis documentation and analysis system (KODAK 1D Image Analysis, Tokyo, Japan).

Distinction between the FeSOD and MnSOD bands was made by soaking the gels for 60 min at room temperature in a solution of 20 mM H$_2$O$_2$ and 50 mM potassium phosphate (pH 7.8) before activity staining for SOD. This treatment caused the selective inactivation of FeSOD (Chou and Tan, 1990).

**SOD induction by either superoxide treatment or UV irradiation** The survival of *D. radiophilus* upon exposure to potassium superoxide and UV irradiation was studied prior to an induction study of SOD by superoxide treatment and UV irradiation. Exponentially-grown *D. radiophilus* was subcultured into a new TYGM medium in order to give an initial OD$_{600}$ of 0.1. When the culture reached an OD$_{600}$ of 0.2, potassium peroxide was added to the culture with final concentrations from 0-100 mM. The culture was then further incubated for 60 min at 30°C. Properly-diluted cell cultures with 50 mM potassium phosphate buffer (pH 7.0) were plated on TYGM agar in triplicate for the colony-forming units (CFU). The cells of the culture that reached an OD$_{600}$ = 0.2 were harvested by centrifugation. They were washed three times and resuspended in 50 mM potassium phosphate buffer, pH 7.0. The cell suspension in a Petri dish (without lid) was UV irradiated with a UV lamp (Minuvis, Desaga, Heidelberg, Germany). The UV dose (0-1500 J/m²) was measured using a VLX-3W radiometer (Vilber Lourmat, Marne La Vallee, France). Ten ml of the irradiated cell suspension was placed in the test tube, then vortexed and subjected in order to measure CFU. For comparison, a parallel experiment was run with *P. aeruginosa* that was grown in a LB medium. CFUs of *D. radiophilus* and *P. aeruginosa* were recorded at 48 and 24 h of incubation on the TYGM and LB agar plates, respectively. For an induction study of SOD, the cultures of *D. radiophilus* (OD$_{600}$= 0.2) that received different concentrations of potassium superoxide (0-20 mM) were further incubated for 60 min at 30°C. Then, the cell-free sonic extract was prepared with the cells that were harvested. The UV-irradiated cells, as previously described, were allowed a 60 min incubation at 30°C before preparation of the cell-free sonic extract.

**Cellular localization of SOD** The cells that were harvested at the stationary phase were suspended in 30 mM Tris-HCl buffer (pH 8.0) that contained 20% sucrose and 1 mM EDTA at a ratio of 1 g of wet cells in 80 ml of the buffer (Yun and Lee, 2000). After a 10 min incubation at 30°C, the supernatant and cell pellet were fractionated by centrifugation. The proteins on the supernatant (sucrose-shocked fluid) that was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) were concentrated to several folds by ultrafiltration (PM-10 membrane, Amicon, Beverly, USA). The cell pellet was followed by a cold-wash step; suspension of the pellet in cold-distilled water allowed a 10 min incubation in an ice bath in order to release the loosely-bound proteins on the pellet surface. The cold wash supernatant and cell pellet were fractionated by centrifugation. Proteins in the sucrose-shocked cell pellet were released by sonic disruption. SOD in each preparation was resolved by PAGE, followed by activity staining. Since catalase-2 and catalase-3 of *D. radiophilus* are known as members of membrane and cytosol proteins, respectively (Yun and Lee, 2000), then catalase activity staining of the gel is simultaneously done for the references of fractionation.

**Results and Discussion**

SOD is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species that are generated during aerobic respiration for energy production. The SOD activity of *D. radiophilus* showed a gradual increase along with growth phases, as shown in Fig. 1. The growth-dependent increase of SOD activity in aging cells could be attributed to the accumulation of endogenous oxidants during the prolonged respiratory activity, as reported in other organisms, including *E. coli*, *Caulobacter crescentus*, *Streptomyces coelicolor*, *Arcobacter nitrofigilis*, and *Schizosaccharomyces pombe* (Farr and Kogoma, 1991; Schnell and Steinman, 1995; Kim et al., 1996; Park and Han,...
The electrophoretic resolution of SOD in the cell-free extracts that were prepared with *D. radiophilus* cultures during each growth phase revealed the occurrence of single SOD (Fig. 2). The SOD band at the stationary phase was about 20% denser when compared with that at the early exponential phase. The *Deinococcus* species, the obligate aerobics, are extraordinarily resistant to UV- and ionizing-radiations. For example, *D. radiodurans* can survive exposure up to 30,000 Gy of ionizing radiation (Madigan *et al.*, 2000). In addition to the deleterious effect of UV radiation on DNA, the UV light attributes various cell damages to generating-reactive oxygen intermediates (Tyrrell, 1991). The high resistance of *D. radiophilus* to exogenous superoxide and UV radiation is depicted in Fig. 3, there is a nearly 50% survival, even with a 60 mM potassium superoxide treatment (Fig. 3A) and 1,000 J/m² of UV dose (Fig. 3B). Treatment of cells with varied concentrations of potassium superoxide caused a differential increase of SOD, that is, ca. 60% increase of the level of untreated cells at 15 mM KO₂ (Fig. 4A). However, an intensity of the SOD band that was obtained from the 15 mM KO₂ treated-cells was about 20% denser when compared with that of the non-treated cells. The discrepancy in the increase of SOD between the specific activity in cell-free extract and resolution on the gel could be due to the different systems of assay. A similar result of the SOD production was obtained when the cells were exposed to UV (Fig. 5). All of these data suggest that *D. radiophilus* SOD is a constitutive enzyme whose activity is inclined to increase with environmental stresses. *D. radiophilus* SOD was
Production of SOD in *D. radiophilus* reported as MnSOD in a previous paper (Yun and Lee, 2001), and it was reconfirmed by sensitivity studies (data not shown). MnSOD is insensitive to both hydrogen peroxide and cyanide. In contrast, CuZnSOD is sensitive to hydrogen peroxide as well as cyanide. FeSOD is sensitive to hydrogen peroxide but not to cyanide (Chou and Tan, 1990).

Reports on the iso-enzymes that show distinct physiological roles with different cellular localizations in prokaryotic cells were made in many instances (Gregory *et al.*, 1973; Goldberg and Hochman, 1989; Kim *et al.*, 1996; St. John and Steinman, 1996; Yun and Lee, 2000; Li *et al.*, 2002). In *D. radiophilus*, the membrane-associated catalase-2 is a constitutive enzyme, whereas the cytosolic catalase-3 is an inducible enzyme that responds to environmental stress (Yun and Lee, 2000). Another example of these instances is a recent report on the induction of FeSOD during heterocyst differentiation and localization of MnSOD in the thylakoid membrane in Cyanobacteria *Anabaena* sp. (Li *et al.*, 2002). Electrophoretic resolution of the protein in the preparations that were obtained from the sucrose-shocked cells of *D. radiophilus* revealed the omni-presence of SOD in both the cytosol and membrane-associated fractions. However, there was an obvious difference in SOD abundance between the two fractions, ca. three-fold denser in the intensity of the SOD band (145,000...
densitometer unit, DU) in the membrane fraction when compared with that of the cytosol fraction (56,000 DU) (Fig. 6). However, we consider that D. radiophilus SOD is a membrane-associated protein because of its abundance and clear presentation of iso-catalases that are employed as the reference proteins of each fraction. We assume that SOD in cytosol could be attributed to either the not-yet membrane-incorporated SOD that occurred in cytosol, or SOD that is released from the membrane into the cytosol fraction during osmotic shock by sucrose. All of our data suggests that D. radiophilus possesses a sole MnSOD, which is a constitutive enzyme and responsible for scavenging the superoxide radicals either internally or externally. Therefore, one might easily assume the immense role of this MnSOD to D. radiophilus in surviving environmental stresses, including UV irradiation and oxygen stress. Further studies of the regulation of ROS-scavenging enzymes (including SOD, hydroperoxidase, glutathione reductase, and others in Deinococcus species) are required in order to understand its radioresistant physiology.

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References

identification of mutation sites of two mitomycin-sensitive strains and the first discovery of insertion sequence element from deinobacteria. Gene 198, 115-126.


