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Occurrence of Thioredoxin Reductase in *Deinococcus* Species, the UV resistant Bacteria

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The occurrence of thioredoxin reductase (NAD(P)H: oxidized-thioredoxin reductase, EC 1.6.4.5, TrxR) in five mesophilic species of *Deinococcus* was investigated by PAGE. Each species possessed a unique TrxR pattern, for example, a single TrxR characterized *D. radiopugnans* while multiple forms of TrxR occurred in other Deinococcal spp. Most of TrxRs occurring in *Deinococcus* showed dual cofactor specificity, active with either NADH or NADPH, although the NADPH specific-TrxR was observed in *D. radiophilus* and *D. proteolyticus*.

Keywords: UV resistant *Deinococcus* spp., thioredoxin reductase (TrxR), multiplicity of TrxR, NADH/NADPH dependent TrxR, NADPH specific-TrxR

Among the many features of *Deinococcus*, the most unusual feature is extreme resistance to UV and ionizing radiation, and oxidative stress (Murray, 1986; Battista, 1997; Yun and Lee, 2003). Since the extreme resistance of *Deinococcus* toward UV and oxidative insults can be attributed to protective systems against reactive oxygen species (ROSs) and the ability to repair the damaged cellular molecules, any information gain on antioxidant components as well as the reparative systems would contribute to improved understanding of radiation and oxidative resistance (Halliwell and Gutteridge, 1999; Storz and Zheng, 2000). Although extensive information on the repairing genes of damaged DNA (Evans and Moseley, 1983; Gutman *et al.*, 1993; Agostini *et al.*, 1996; Narumi *et al.*, 1997; Bauche and Laval, 1999; Kim *et al*, 2002) and on genome analysis of *D. radiodurans* (Makarova *et al.*, 2001) is now available, it remains quite uncertain for UV and oxidative-resistance of *Deinococcus* species. Thus, we have investigated intensively on scavenging enzymes for the toxic oxygen derivatives in relation to the UV resistance. And our reports on the detoxifying enzymes of ROS such as hydroperoxidases and superoxide dismutases in *Deinococcus* would be found elsewhere (Oh and Lee, 1998; Soung and Lee, 2000; Yun and Lee, 2000; Yun and Lee, 2001, 2003, 2004). However, little attention has been paid to a number of antioxidant small molecules such as the carotenoids and thiol compounds and their potential participation in a resistance mechanism. Many thiol compounds including glutathione, mycothiol, and thioredoxin (Trx) are known to play a role in protecting bacterial cells from endobiotic and exobiotic electrophiles (Halliwell and Gutteridge, 1999; Arnér and Holmgren, 2000; Kim *et al.*, 2004). However, the production of these compounds among prokaryotes is quite variable. For example, glutathione appears to be largely restricted to Cyanobacteria, purple bacteria, whereas mycothiol was reported in *Actinomycetes* including *Streptomyces* (Park *et al.*, 2006) and thioredoxin in a number of Proteobacteria, *Chlamydia*, and in anaerobic amino acid degrading Gram-positive bacteria (Newton *et al.*, 1996; Harms *et al.*, 1998). Thioredoxin (Trx) is a relatively small single polypeptide (10-12 kDa) containing two adjacent -SH groups in its reduced form (Trx-SH), which becomes an oxidized form (Trx-S_2) by donating electron to oxidoredox proteins. The oxidized Trx is then converted to the reduced form of Trx *in vivo* by activity of Trx reductase (TrxR) using NAD(P)H as cofactor (Halliwell and Gutteridge, 1999; Mustacich and Powis, 2000).

Among the numerous roles of thioredoxin (Trx) in the redox regulatory systems (Buchnan *et al.*, 1994; Rigobells *et al.*, 1998; Arnér and Holmgren, 2000), the involvement of the dithiol form of thioredoxin

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(Trx-SH) in the antioxidant systems by repairing oxidative protein damage and by direct reaction with H_2O_2 in both prokaryotes and eukaryotes is widely recognized (Scandalios, 1997; Halliwell and Gutteridge, 1999; Arnér and Holmgren, 2000).

Despite vast roles of TrxR in cellular redox homeostasis and antioxidant defenses by recycling oxidized Trx to the reduced Trx (May *et al.*, 2002), information on antioxidant thiol compounds, including thioredoxin, is not available for the extremely UV resistant *Deinococcus* spp. Thus, we believed it is worthwhile to investigate the thioredoxin system consisting of thioredoxin (Trx), thioredoxin reductase (TrxR), and reduced pyridine nucleotides as it would occur in *Deinococcus*. We report here evidence for the occurrence of trxR in the mesophilic *Deinococcus*.

The strains of *Deinococcus radiodurans* ATCC 13939, *D. radiophilus* ATCC 27603, *D. grandis* ATCC 43672, *D. proteolyticus* ATCC 35074, and *D. radiopugnans* ATCC 19172 were cultured in TYGM medium containing 1% tryptone, 0.5% yeast extract, 0.2% glucose, and 0.2% methionine (Yun and Lee, 2003) at 30°C with continuous shaking (150 rpm). Bacterial growth was monitored at OD_{600} (DU-65 Spectrophotometer, Beckman, USA). Thioredoxin reductase (TrxR, E.C. 1.8.1.9) activity in sonic cell-free extracts was measured by monitoring for a 5 min interval, the change in absorbance at 412 nm resulting from the reduction of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) to TNB (2-nitro-5-thiobenzoic acid). The natural substrate of TrxR, i.e., thioredoxin, is difficult to obtain and very expensive, so that activity of TrxR is usually assayed using DTNB (as an alternative substrate) in the reduction assay, a substitution that has been proved to be sufficiently specific (Holmgren and Bjornstedt, 1995). The reaction mixture consists of an aliquot of enzyme preparation, 1 mM of EDTA, 5 mM DTNB, 0.2 mM of NAD(P)H maintained at 25°C (Gromers, *et al.*, 1998; Arnér *et al.*, 1999; Davioud- Charvet *et al.*, 1999; Stefankava *et al.*, 2006). One unit of TrxR is defined as the activity of enzyme yielding 1 μmol of TNB ($\varepsilon_{412} = 13.6 \times 10\ mM^{-1}\ cm^{-1}$) per min. Protein was quantified by the Lowry method (Lowry *et al.*, 1951). Proteins in the cell lysates prepared by ultrasonic disruption were resolved by PAGE on 8.0% (w/v) gel in a Tris- glycine buffer, pH 8.8 (Gersten, 1996). The TrxR bands resolved on gels were subjected to activity staining by incubation in 250 mM Tris-HCl buffer (pH 8.4) containing 1.5 mM NAD(P)H, and 2.0 mM DTNB at room temperature for 20 min. An additional 30 min incubation of gels followed in the same staining solution but containing 40 μM DCIP (2, 6- dichloroindophenol), and 1.0 mM MTT (3-[4, 5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolim bromide) (Ye *et al.*, 1997). A final confirmation of TrxR activity on gel was carried out with thioredoxin, a product of recombinant *E. coli* (Sigma, USA), in place of DTNB as the authentic substrate of TrxR.

TrxR, a flavoprotein disulfide oxidoreductase catalyzing reduction of Trx-S_2 to Trx-$(SH)_2$, is an enzyme central to cellular thiol metabolism and also plays a role in protection against oxidative stress (Becker *et al.*, 2000; Mustacich and Powis, 2000; Nordberg and Arnér, 2001). Two types of TrxR, H-TrxR (dimer of ~ 55 kDa of subunit) and L-TrxR (dimmer of ~35 kDa of subunit) have been characterized. H-TrxR occurs in Eukarya, whereas L-TrxR has been found in Archaea, Bacteria and some Eukarya including fungi, plants, and the protozoan parasite, *Entamoeba* (Hirt *et al.*, 2002). Extensive information is available on L-TrxR for a number of Proteobacteria, *Chlamydia*, *Mycobacterium,* and *Streptomyces*, but in few gram-positive bacteria (Hirt *et al.*, 2002). Resolution of the proteins in the cell lysates made from *Deinococcus* cultures at log and stationary phases revealed multiple forms of TrxR with different electrophoretic mobilities (Fig. 1). These TrxRs also showed differing specificities towards NADPH and NADH (Fig. 1A and B). *D. radiopugnans* was characterized by a single TrxR active with both NADH and NADPH, whereas the other species yielded multiple forms of TrxR. All TrxRs observed in the *Deinococcus* spp. seemed to be constitutive, regardless their requirements of NADH or NADPH, however, one species of three iso-TrxRs occurring in *D. grandis* was considerably increased in its NADH dependent activity at the stationary phase. Most of the TrxRs occurring in *Deinococcus* have dual activities involving both NADH and NADPH. Interestingly, the TrxR specifically active with only NADPH was observed in *D. radiophilus* and *D. proteolyticus* in addition to the NADH/NADPH dependent TrxR (Fig. 2). The multiplicity of TrxR observed in the mesophilic *Deinococcus* species is depicted in Table 1. These observations would serve to provide the fundamental information for investigation of TrxR in the *Deinococcus* species, the extraordinary resistant bacteria toward UV and oxidative stress.

Environmental factors, particularly oxidative stress, are known to influence the level of TrxR to exert a protective role against cellular oxygen toxicity (Rigobells *et al.*, 1998; Ejima *et al.*, 1999; Arnér and Holmgren, 2000; Mustacich and Powis, 2000). In comparing TrxR activities of each *Deinococcus* species at the exponential and stationary phases, a slightly higher TrxR activity was detected in cells at the stationary than at the exponential phase. The increase in TrxR level at the stationary phase was likely reflection of oxidative stress due to intrinsic by-products of aerobic metabolism. The TrxR level among the Deinococcal

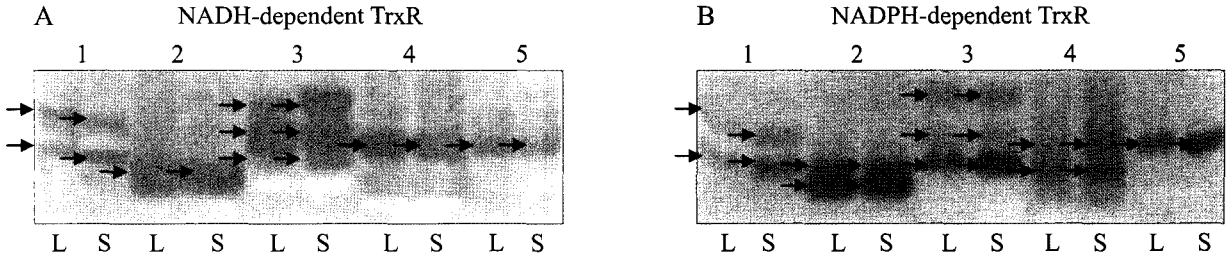

Fig. 1. Electrophoretic profiles of thioredoxin reductase of *Deinococcus* spp. Each well was loaded with 50 μg of protein in cell-free extracts of *Deinococcus* cultures. Activity staining of TrxR on 8.0% (w/v) polyacrylamide gel was performed as described in the text. Lanes, 1. *D. radiodurans*, 2. *D. radiophilus*, 3. *D. grandis*, 4. *D. proteolyticus*, 5. *D. radiopugnans*. L: log phase, S: stationary phase.

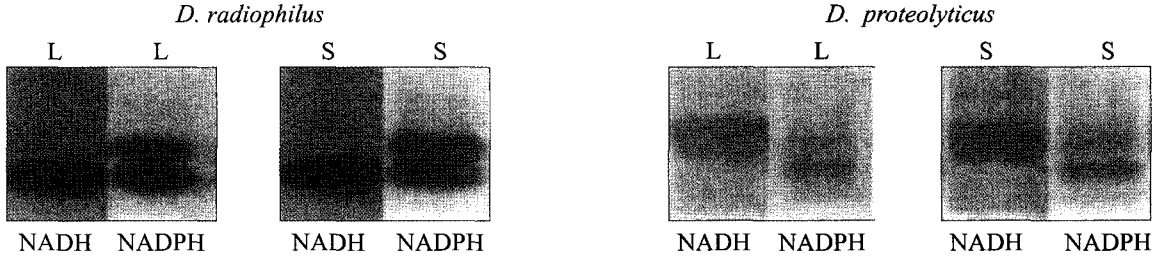

Fig. 2. Iso-TrxR showing different reduced pyridine nucleotide specificity. Each well was loaded with 50 μg of protein in cell-free extracts of *D. radiophilus* or *D. proteolyticus* cultures. Activity staining of TrxR resolved on 8.0% (w/v) polyacrylamide gel was performed in presence of either NADH or NADPH as described in Materials and Methods. L: log phase, S: stationary phase.

Table 1. Cofactor (NADH/NADPH) dependency of *Deinococcal* TrxRs

Species	Log phase	Stationary phase	Remark
D. radiodurans	u-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
	l-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
D. radiophilus	u-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
	l-NADPH specific	NADPH specific	constitutive
D. grandis	u-NADH/NADPH dependent	NADH*/NADPH* dependent	inducible*/ constitutive
	m-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
	l-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
D. proteolyticus	u-NADH/NADPH dependent	NADH/NADPH dependent	constitutive
	l-NADPH specific	NADPH specific	constitutive
D. radiopugnans	NADH/NADPH dependent	NADH/NADPH dependent	constitutive

* : increase in NADH and NADPH activities.
u, m, and l: upper, middle and lower bands on gels, respectively (refer Fig. 1)

species varied, i.e. the NADH-dependent TrxR of *D. radiophilus* and *D. grandis* at stationary phase seemed to be two or three-fold higher than that of *D. radiopugnans*, which showed the lowest activity (Fig. 3). One may assume that a little discrepancy occurring between levels of TrxR activity and the intensity of TrxR band on gel is reflection of two different assay systems. An observation of the higher level of TrxR in *D. radiophilus* is rather interesting, despite a report that *D. radiodurans* is thought to be the most UV resistant of the Deinococcal species (Battista, 1997). A direct correlation between the levels of TrxR and their resistance to UV radiation and to oxidative stress within the *Deinococcus* species, however, is yet to be

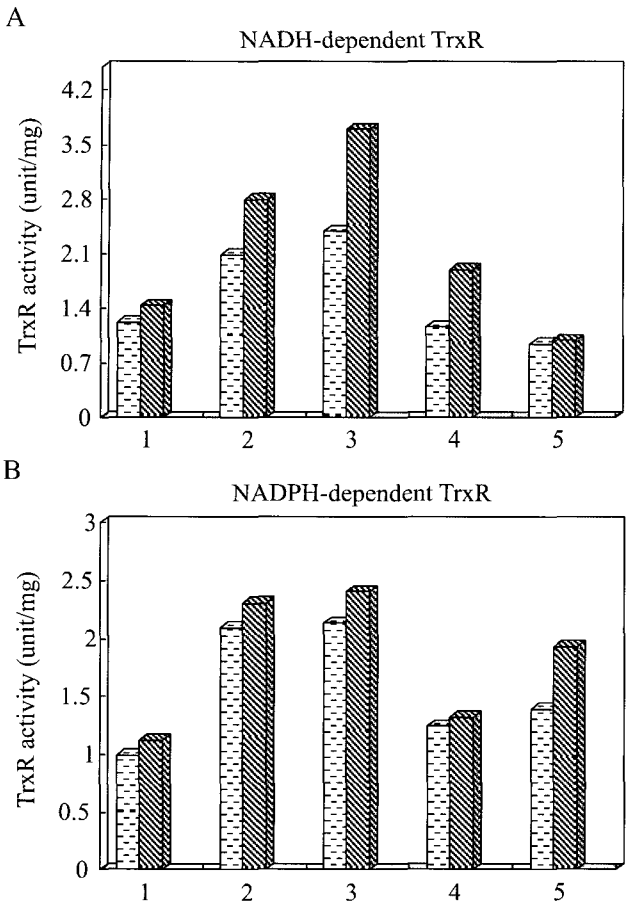

Fig. 3. Thioredoxin reductase activity of *Deinococcus* spp. during the growth phase. TrxR activities of cell cultures were assayed as described in the text. These figures are representative of results among four separate experiments. 1. *D. radiodurans*, 2. *D. radiophilus*, 3. *D. grandis*, 4. *D. proteolyticus*, 5. *D. radiopugnans*. □: log phase culture, ■: stationary phase culture.

confirmed.

The genus *Deinococcusis* is characterized by a peculiar set of organisms that show an extreme resistance to ionizing and UV radiation and some other properties (Murray, 1986; Battista, 1997). The phylogenetic diversity of the five species of mesophilic *Deinococcus* was determined by their 16S ribosomal DNA sequence comparison (Rainey *et al.*, 1997). Among *Deinococcus* genus, four species are gram-positive cocci and one species is a gram-negative rod. Because of the similarities in their morphology and biochemical-physiological properties of the *Deinococcus* gram-positive species to each other, it is laborious to distinguish them from one another, particularly when there is the cross-contamination. Thus, a relatively simple technique would be convenient in identifying the Deinococcal species from each other as like multiple isozyme analysis in the identification of *Leishmania* spp. (Kreutzer *et al.*, 1983) and allozyme analysis in the differentiation of *Saccharomyces paradoxus* (Naumov *et al.*, 1997). Without exception, every *Deinococcus* species possesses multiple TrxR regardless of the log or stationary growth phases. Therefore, we suggest that the unique electrophoretic profiles of the TrxRs for each mesophilic species of *Deinococcus* would be useful in their identification. Such information could be coupled with their profiles of catalases and SODs (Soung and Lee, 2000; Yun and Lee, 2001). Our ongoing investigation on the Deinococcal TrxRs is intended to additional and valuable information on the thioredoxin system for the prokaryotic TrxR.

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