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Note

Heavy metal toxicity mitigation by iron-containing superoxide dismutase 2 of *Streptomyces coelicolor* A3(2)

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Streptomyces coelicolor A3(2)의 철 함유 superoxide dismutase 2에 의한 중금속 독성 완화

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Bacterial growth inhibition by lead, zinc and cadmium was measured by using modified Tris minimal medium. The toxicity against *Escherichia coli* strain was in the order of zinc> cadmium> lead, and the *Escherichia coli* strain overexpressing iron-containing superoxide dismutase 2 of *Streptomyces coelicolor* A3(2) was found to have resistance to heavy metals.

Keywords: *Streptomyces coelicolor* A3(2), heavy metal, resistance, iron superoxide dismutase 2

Heavy metals are toxic to bacteria and the biochemical principles of their toxicities have been studied (Duxbury, 1981; Nies, 1999; Banjerdkij *et al*., 2005; Duruibe *et al*., 2007; Mamtani *et al*., 2011). Several mechanisms of heavy metal resistance have also been studied. Among them, metal-binding metallothioneins have been thought to be most suitable for bioremediation. Bacterial metallothioneins, similar to eukaryotic metallothioneins, are cysteine-rich proteins that sequestrate toxic heavy metals by forming complexes with them (Bruins *et al*., 2000). Other heavy metal-binding proteins with high anionic amino acid or histidine contents have been identified in prokaryotes, e.g., the cadmium-binding protein (Capasso *et al*., 1996) and the nickel-binding protein (Olson and Maier, 2000). We found that the extracellular iron containing SOD (FeSOD) of *Streptomyces subrutilus* P5 absorbed lead ions (So *et al*., 2001) and could induce the lead resistance of recombinant *E. coli* producing this FeSOD (Kim *et al*., 2014). In this paper, we extend our study to *S. coelicolor* A3(2), the model strain in streptomycetes molecular biology, which possesses two FeSODs (FeSOD1 and FeSOD2) together with nickel containing SOD (NiSOD) (Chung *et al*., 1996). The amino acid sequences of the FeSODs from *S. coelicolor* A3(2) and *S. subrutilus* P5 share a same signature amino acid sequence and the common N and C terminal domains. FeSOD2 of *S. coelicolor* A3(2) shows slightly higher amino acid sequence similarity (80%) to FeSOD of *S. subrutilus* P5 as compared to FeSOD1 (78%), and we could expect that the heterogenous expression of this gene in *E. coli* also mitigate heavy metal toxicity. Therefore, we cloned and expressed the FeSOD2 gene (*sodF2*) in a recombinant strain of *E. coli* M15 [pREP4], referred to as *E. coli-sodF2*/A3(2), following basically the same experimental protocols as we described previously (Kim *et al*., 2014). The wild type strain M145 of *S. coelicolor* A3(2) was used as the source of *sodF2* (GenBank accession number, AF099015). The forward primer with a *Bam*H1

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restriction site, 5′-GGATCCGCCATCTACACGCTTC-3′, and the reverse primer with a *Hin*dIII restriction site, 5′-AAGCT TGCCCTTGACGGGGAT-3′ were designed for amplification of a DNA fragment (654 bp) containing the entire sequence of *sodF2* (Bioneer Co.). The *sodF2* fragment was inserted into *Bam*H1/*Hin*dIII sites of the expression vector, pQE30. The induction and purification of 6xHis-tagged SOD were performed (Qiagen Korea), and the enzyme activity of the 6xHis-tagged SOD was confirmed by the negative band staining on non-denaturing polyacrylamide gel using nitroblue tetrazolium and riboflavin (Beauchamp and Fridovich, 1971).

The *E. coli-sodF2*/A3(2) was activated for one day in LB broth, at 200 rpm, 37°C to be used as inoculums (5% v/v) for fresh cultures until logarithmic growth phase (OD_{600} = ca. 0.6), then 1 mM IPTG was treated for one hour to induce the SOD production. The induced culture was inoculated into the Tris minimal broth (1% v/v) composed of glucose (11 mM), NH_4Cl (5 mM), K_2SO_4 (0.5 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (10 mM), Tris-HCl (100 mM), IPTG (1 mM), pH 6.5, and each toxic heavy metal ions [0.05 mM of $Pb(NO_3)_2$, 0.1 mM of $ZnCl_2$, and 0.1 mM of $CdCl_2$, respectively], and the OD_{600} value was determined after 20 h incubation (18 h for lead). These heavy metal concentrations did not interfere with the OD_{600} measurements because no agglutination reactions by medium components and heavy metals occurred. Following the same protocol, we estimated also the heavy metal tolerance of the recombinant *E. coli* M15[pREP4] producing FeSOD of *S. subrutilus* P5, referred to as *E. coli-sodF*/P5, which already showed the enhanced lead tolerance in our previous study (Kim *et al*., 2014).

Many reports have determined the survival rates based on the viable cell counting on agar plates to describe heavy metal resistance of bacteria. This approach, however, are time-consuming and needs somewhat laborious plate counting. On the other hand, the optical density at 600 nm (OD_{600}) has also been used to analyze growth in the presence of heavy metal ions (Ong *et al*., 2015). In using OD_{600} as the indicator of bacterial growth it should be confirmed that there is no precipitation in culture broth, because it interrupts accurate turbidity measurement. We first choose a relatively simple Tris minimal medium which was originally used for the study of Mg effects on the expression of a transcription factor in *Salmonella* strains (Norte *et al*., 2003) and tested which components could cause the turbidity due to precipitation reaction. Because we found that phosphate ion was the only one component to cause strong turbidity when mixed with cadmium-, lead-, or zinc ion, phosphate was omitted from the Tris minimal medium. Besides, pH of the minimal medium was adjusted to 6.5 to increase the solubility of the heavy metal ions and the concentrations of each heavy metal ions were reduced to such low levels to avoid precipitation even in the absence of phosphate; 0.1 mM for cadmium, 0.05 mM lead, and 0.1 mM zinc ion. Using this modified liquid Tris minimal medium we could measure OD_{600} of the overnight cultures to evaluate the heavy metal resistance.

The growths of non-induced cultures were compared with the induced cultures as described in the following text. As shown in Fig. 1, growth of the non-induced *E. coli-sodF2*/A3(2) was remained at 28% with zinc, 67% with cadmium, and 87% with lead, compared with the growth of the same strain without heavy metal. Similar results were for the *E. coli-sodF*/P5. The non-induced cultures showed clearly decreased growth due to heavy metal ions; the sequence of toxicity was zinc > cadmium > lead. It is interesting that the toxicity of zinc was stronger than

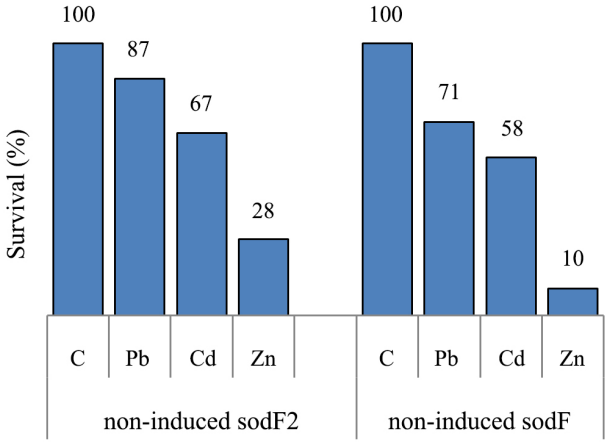

Fig. 1. The growth inhibition of the non-induced recombinant *E. coli* strains caused by heavy metal ions. The recombinant *E. coli* strains were grown in Tris minimal medium containing each heavy metal ion without IPTG treatment. The relative OD_{600} (%) measured at 20 h (18 h for lead) were given, compared with that of the culture grown without heavy metal ions. The values presented are the result of one experiment. Through repeated experiments, we confirmed that the same tendency appears.
sodF2; *S. coelicolor* A3(2)
sodF; *S. subrutilus* P5
C (control); the non-induced growth in the absence of added heavy metal ions.

that of cadmium in this study, while cadmium has usually been known to be more toxic to bacteria (Nies, 1999). There was an interesting report that zinc toxicity greatly increased if zinc existed as zinc-chloride (Zn-Cl) complex compared with $ZnSO_4$ (Babich and Stotzky, 1978). Because zinc was dissolved as $ZnCl_2$ in this study, zinc toxicity should be exhibited more strongly. In addition, by speciation of cadmium ions, the proportion of free cadmium ions, which are highly toxic among cadmium species, could be very low, while the less toxic chloro-complex was dominant in Tris minimal medium (Rahtnayake *et al*., 2013). Therefore, the weak toxicity of cadmium in this study was assumed to be a result of cooperation of modified metal ions. Unlike cadmium and lead, which are not essential for cells, zinc is required for variety of important biochemical processes such as enzyme function and structural stability, constituent of DNA binding proteins, DNA replication and gene expression (Choudhury and Srivastava, 2001). Recent studies have revealed that zinc inhibits manganese uptake (McDevitt *et al*., 2011) and key enzymes of glycolysis (Ong *et al*., 2015). Therefore, 0.1 mM of zinc ions used in this study might be enough to show its stronger toxic effect than cadmium.

The growth of the recombinant *E. coli-sodF2*/A3(2) indicated that the production of FeSOD2 of *S. coelicolor* A3(2) was closely linked to the increased survival in the presence of each heavy metal ions: OD_{600} was increased by 1.1 times in the presence of lead, by 1.8 times in the presence of cadmium, by 2.4 times in the presence of zinc, respectively (Fig. 2). In the case of *E. coli-sodF*/P5, resistance to heavy metals was also clearly increased by FeSOD production: 1.5 times for lead, 1.6 times for cadmium, and 7 times for zinc (Fig. 2). The Zn detoxification effect of FeSOD of *S. subrutilus* P5 was clearly high, but at present, we could not explain whether this high Zn detoxification effect is due to the specific structure of FeSOD based on the amino acid sequence information. The increase in resistance to lead was relatively weak. In this experiment, the concentration of lead was only half that of other heavy metals, so the change in toxicity and resistance might be less evident. The lead resistance of recombinant *E. coli-sodF2*/A3(2) was measured again in the presence of higher lead concnetration of 0.1 mM by plate counting method. The induced and non-induced *E. coli* strains were suspended in MES buffer (50 mM, pH 6.5), containing 0.1 mM lead, respectively, and allowed to stand for certain period of times as indicated in Fig. 3. Then, the number of surviving bacteria was measured by colony counting on agar plates after serial dilution. As shown in Fig. 3, the FeSOD2

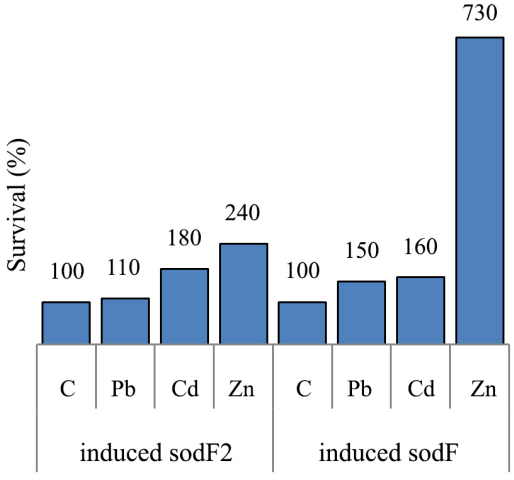

Fig. 2. The growth increase of the recombinant *E. coli* strains in the presence of heavy metal ions by the induction of each *sod* gene expression. The relative OD_{600} (%) measured at 20 h (18 h for lead) are given, compared with that of the non-induced culture grown with heavy metal ions. The values presented are the result of one experiment. Through repeated experiments, we confirmed that the same tendency appears.
sodF2; *S. coelicolor* A3(2)
sodF; *S. subrutilus* P5
C; the non-induced growth in the presence of heavy metal ions.

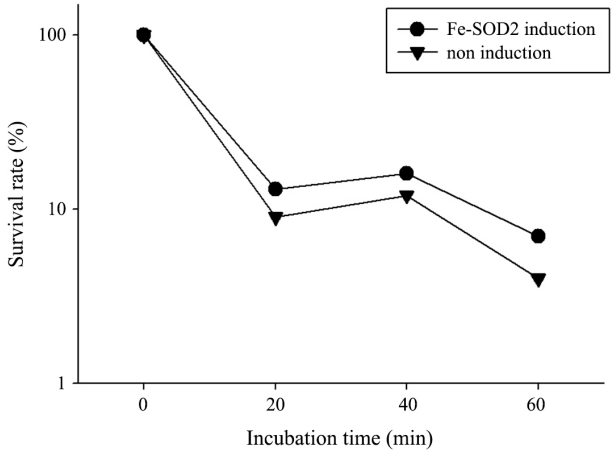

Fig. 3. Survival of the recombinant *E. coli-sodF2*/A3(2) induced and non-induced for FeSOD2 production in the presence of 0.1 mM lead ions. The recombinant *E. coli* cells carrying *sodF2* of *S. coelicolor* A3(2) were grown to log phase (OD_{600}= ca. 0.6) in LB and induced for FeSOD2 production by 1.0 mM IPTG. Non-induced cells were also prepared without IPTG treatment. The cells were harvested and resuspended into the sterile MES buffer (50 mM, pH 6.5) containing 0.1 mM Pb^{2+} and the survival rates were determined at the indicated times by serial dilution and plate counting. 100% corresponds to $(2.2 \pm 0.2) \times 10^6$ cells/ml.

production-induced strain showed 1.4~1.8 times higher survival rates at all time points than the non-induced strains. Therefore, it could be concluded that, although it was relatively weak, the lead resistance should be attributed to FeSOD2. As we have previously described that the sequestration of heavy metals by SOD should be the reason for the heavy metal tolerance, we also determined the lead binding capacity of the FeSOD2 of *S. coelicolor* A3(2). Lead ions (87 and 130 μM) and the purified 6xHis-tagged SOD protein (3.3×10^{-2} μM) were mixed into 50 mM MES buffer (pH 6.5), incubated at 30°C for 30 min, and filtrated by centrifugation using centricon YM-10 (Amicon Bioseparations, Millipore) at 4°C, 4,000 × *g* for 40 min. The eluates were assayed for residual lead using an inductively coupled plasma (ICP) mass spectrometer (PQ3, VG Elemental, Winsford Cheshire) at the Korea Basic Science Institute (KBSI). The numbers of lead ions per FeSOD2 protein were calculated from the difference between the initial and residual concentrations. The measurements were carried out twice and the representative data were presenting 104 g-atom Pb/molSOD, which was somewhat lower than that (123 g-atom Pb/molSOD) of FeSOD of *S. subrutilus* P5 (Kim *et al*., 2014). This low level of lead binding capacities might be reflected in the lower lead resistance of *E. coli* with FeSOD2 of *S. coelicolor* A3(2) than *E. coli* with FeSOD from *S. subrutilus* P5. Our study did not reveal which features of FeSOD2 are related to heavy metal binding. Since no known heavy metal binding motif has been found in the SOD protein, it is necessary to study the specific molecular structure as revealed in soy protein in the future (Liu *et al*., 2013).

As shown above, FeSOD2 is also closely related to heavy metal resistance in *S. coelicolor* A3(2). In addition, it was possible to quantitatively analyze the resistance of heavy metals by measuring the growth of bacteria in phosphate omitted Tris minimal medium containing each heavy metal.

적 요

납, 아연, 카드뮴에 의한 미생물 생장 저해를 변형된 Tris minimal medium을 사용하여 측정하였다. *Escherichia coli* 균주에 대한 독성의 세기는 아연 > 카드뮴 > 납 순으로 나타났고 *Streptomyces coelicolor* A3(2)의 철 함유 superoxide dismutase 2를 과발현하는 *E. coli* 균주는 중금속 저항성이 증가되었음을 알 수 있었다.

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