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Identification and Molecular Characterization of Superoxide Dismutase Genes in *Pseudomonas rhodesiae* KK1 Capable of Polycyclic Aromatic Hydrocarbon Degradation

Dong-Heon Lee[1], Kye-Heon Oh[2], Seung Il Kim[3] and Hyung-Yeel Kahng[1]*

[1]*Department of Environmental Education, Sunchon National University, 255 Jung-Ang Ro, Suncheon 540-742, Korea*
[2]*Department of Life Science, Soonchunhyang University, P.O. Box 97, Asan, Chung-Nam 336-600, Korea*
[3]*Proteomics Team, Korea Basic Science Institute, Daejeon 305-333, Korea*

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Pseudomonas rhodesiae KK1 has been reported to degrade polycyclic aromatic hydrocarbons (PAHs), such as anthracene, naphthalene, and phenanthrene, which are considered major environmental contaminants. Interestingly, antioxidant genes, including superoxide dismutase, are known to be expressed at different levels in response to environmental contaminants. This study was performed to identify the superoxide dismutase gene in strain KK1, which may be indirectly involved with degradation of PAHs, as well as to investigate the expression pattern of the superoxide dismutase gene in cells grown on different PAHs. Two types of superoxide dismutase genes responsible for the antioxidant defense mechanism, Mn-superoxide dismutase (*sodA*) and Fe-superoxide dismutase (*sodB*), were identified in *P. rhodesiae* KK1. The *sodA* gene in strain KK1 shared 95% similarity, based on 141 amino acids, with the Mn-sod of *P. fluorescens Pf*-5. The *sodB* strain, based on 135 amino acids, shared 99% similarity with the Fe-sod of *P. fluorescens Pf*-5. Southern hybridization using the sod gene fragment as a probe showed that at least two copies of superoxide dismutase genes exist in strain KK1. RT-PCR analysis revealed that the *sodA* and *sodB* genes were more strongly expressed in response to naphthalene and phenanthrene than to anthracene. Interestingly, *sodA* and *sodB* activities were revealed to be maintained in cells grown on all of the tested substrates, including glucose.

Key words : Antioxidant enzyme, PAHs, *Pseudomonas rhodesiae* KK1, RT-PCR, SOD

Introduction

Recently, studies on the roles of antioxidant enzymes such as catalase and superoxide dismutase for degradation of pollutants have been attracted by environmental microgiologists [5, 10, 17, 21]. Polycylcic aromatic hydrocarbons (PAHs) are representative ones which have been reported cytotoxic, mutagenic, and potentially carcinogenic [2]. These chemicals also might play a role of environmental stressors to microorganisms. Reactive oxygen species (ROS) such as H_2O_2, OH^- and O_2^- are strong oxidants and often produced in microorganisms during metabolism of PAHs. Mechanisms for production and the removal of ROS in microorganisms have been studied for ages by many microbiologists, resulting in elucidation of the gene structures and functions of catalases and superoxide dismutases which are involved with removal of ROS [3, 11, 12, 16, 20]. Strong oxidative stress caused by high concentration of ROS might be lethal to most organisms, because many antioxidant enzymes including catalases and superoxide dismutases so far identified are not able to function at such high concentration.

It has been reported that *sodA* activities are inducible under oxidative stress in *Pseudomonas* strains [15, 20]. The superoxide dismutase (SOD) is among the microbial defense systems against oxidative stress from ROS. ROS may not only be harmful or damaging to microbial cells, but it may decrease the survival rate of microorganisms in environment. Antioxidant enzymes including SOD have been known to play critical roles of scavenging ROS. Microorganisms frequently face oxidative stresses caused by the pollutants themselves or intermediates generated during biodegradation processes even though they can utilize a pollutant as a substrate. Methyl-tert butyl ether was found to induce the expression of two types of superoxide dismutase (SodM and SodF) in *Pseudomonas putida* KT2440 [13]. The overexpression

***Corresponding author**
Tel : +82-61-750-3385, Fax : +82-61-750-3308
E-mail : kahng@sunchon.ac.kr

of these antioxidant enzymes may be effective in scavenging the ROS generated during naphthalene degradation in *P. putida* KT2440 [10]. *Pseudomonas rhodesiae* KK1 has been reported to be able to utilize PAHs such as anthracene, naphthalene and phenanthrene [9]. This study focuses on the identification of SOD as well as on the analysis of relative transcriptional expression of antioxidant enzymes responding to PAHs in *P. rhodesiae* KK1.

Material and Methods

Cell growth and PAHs-induced stress

Pseudomonas rhodesiae KK1 cells were pre-grown in LB medium at 30°C for 18 hr, and 1 ml of the culture was transferred to a set of flasks containing 100 ml of the same medium, and further grown at 30°C until the optical density reached 0.5-0.6 at 600 nm. The grown cells were recovered by centrifugation at 4°C, 4,000x g for 10 min, washed two times with BM buffer (pH 6.8) containing 0.1 g $CaCl_2 \cdot H_2O$, 0.1 g $FeCl_3$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g NH_4NO_3, 0.2 g KH_2PO_4, 0.8 g K_2HPO_4 in 1 liter distilled water, and suspended with BM buffer. In order to obtain cells stressed by PAH the same amount of the suspended cells was transferred to the same buffer which contains either glucose, anthracene, naphthalene, or phenanthrene, and incubated at 30°C, 180 rpm for 6-hr. Besides, cells were exposed to one of the three oxidative agents of 0.5 mM H_2O_2, 0.5 mM tert-butyl hydroperoxide, 0.2 mM menadione and 0.2 mM paraquat in order to induce oxidative chemical stress in KK1 cells. After the addition of the oxidative stressors, cell growth continued at 30°C for 15 min or 30 min, and the cells were then collected by centrifugation for 20 min at 4,000 xg for the test of superoxide dismutase activity. The effect of the three PAHs on the activity of superoxide dismutase was investigated by the ferric cyanide stain method on 7.5% non-denaturing polyacrylamide slab gel [14].

Superoxide dismutase activity test

For the test of enzyme activities, cells of strain KK1 were incubated in 100 ml LB medium at 30°C until cell growth reached to early-stationary phase. Cells were harvested by centrifugation at 4°C, 10,000× g for 30 min, washed three times with 50 mM phosphate buffer (pH 7.0) and disrupted with Mini-Bead Beater™ Cell Disruptor (Biospec products Co., Bartlesville, OK, U.S.A.). Preparation of crude cell extract was performed according to the method previously published [16]. Superoxide dismutase activity was measured according to the method mentioned previously [1]. A reaction mixture containing 3 ml of 100 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 12 mM L-methionine, 75 μM nitroblue tetrazolium chloride (NBT), 2 μM riboflavin, and 0-50 μl of enzymatic extract was exposed to illumination from a 30-W fluorescent lamp for 15 min at 15, 20, 30, 40, and 50°C to start the photochemical reduction of NBT to blue formazan, which was measured as the increase in absorbance at 540 nm using an ELISA microplate reader. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photo reduction in comparison with tubes without the tissue extract that were kept in the dark. All the activity tests were performed in three times.

Cloning and identification of superoxide dismutase genes

DNA was extracted by using the Wizard genomic DNA purification kit (Promega Co., Madison, WI, U.S.A.) and used for the amplification of superoxide dismutase and 16S rRNA from strain KK1. The superoxide dismutase gene fragments were amplified through the PCR using a set of degenerate primers, sod-F (5′-AAR CAY CAY CAR ACN TAY GT-3′) - sod-R (5′-TAR TAN SHR TGY TCC CA-3′) designed in this study. The amplified gene sequences were compared and analyzed with relevant gene sequences available from GenBank database (http://www.ncbi.nlm.nih.gov/blast/) to draw the phylogenetic affiliation using CLUSTAL W software as mentioned previously [16].

RNA extraction and transcriptional expression analysis by RT-PCR

Total RNA was extracted from KK1 cells grown on PAH such as anthracene, naphthalene and/or phenanthrene using RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.) with RNase-free DNase, and quantified at 260 nm by spectrophotometer for synthesis of cDNA. KK1 cells grown on glucose were also used for RNA extraction as the positive control. cDNA was constructed using ™Reverse Transcription System (Promega Co., Madison, WI, U.S.A.) according to the method provided by the manufacturer. One microgram of total RNA and 0.5 μg/μl of random primer were mixed in a microfuge tube, and the mixed solution was adjusted to 5 μl by nuclease-free water. It was heated at 70℃ for 5 min, cooled on ice for 5 min, and the solution was added to 15 μl of the prepared reaction solution [Nuclease-Free Water, imProm-

II™ 5X reaction buffer, 10 mM dNTP (final concentration 0.5 mM), recombinant RNasin® ribonuclease inhibitor, im-Prom-II™ reverse transcriptase]. The final reaction solution was incubated at 25℃ for 5 min, followed by sequential incubation at 42℃ for 60 min for cDNA synthesis. The reaction was stopped by heating at 70℃ for 15 min. PCR was performed using 1 μl cDNA as template and the reaction solution [10X buffer 2.5 μl, 25 mM $MgCl_2$ 2 μl, 10 mM dNTP 0.5 μl, 10 pmol primer set 2.5 μl, *Taq* polymerase (5 U/μl) 0.25 μl], and the final volume was adjusted to 25 μl with nuclease-free water. RT-PCR was performed using a set of following primers: sodAF (5′-GGT GGG CAT GCC AAC CAT TCG-3′) - sodAR (5′-GTA GGT ARG TTC CCA CAC ATC-3′) for superoxide dismutase A, and sodBF (5′-GCT CAG GTC TGG AAC CAC ACC-3′) – sodBR (5′-GTA TGC GTG TTC CCA GAC GTC-3′) for superoxide dismutase B. And, KK1-16F (5′-CAG ACT CCT ACG GGA GGC A-3′) - KK1-16R (5′-CGT GGA CTA CCA GGG TAT C-3′) for 16S rRNA gene were also amplified as the positive control in the RT-PCR for the analysis of transcriptional expression according to the method published previously [14].

Results and Discussion

Enzyme activity of superoxide dismutase in *Pseudomonas rhodesiae* KK1

P. rhodesiae KK1 has the degradation ability for PAHs such as anthracene, naphthalene and phenanthrene [9]. Negative stain-based analysis of superoxide dismutase (SOD) in cell extracts of strain KK1 grown on BM medium containing glucose, anthracene, naphthalene, and/or phenanthrene revealed the existence and expression of Sod (Fig. 1). SOD activity was observed in the similar level in all the cells grown with glucose and PAHs, even though there was a little difference in SOD activity on PAHs-induced cells. This result suggested that a sod gene is constitutively expressed. In order to further analyze the expression pattern of sod gene under different conditions, sod genes in strain KK1 were investigated using molecular techniques

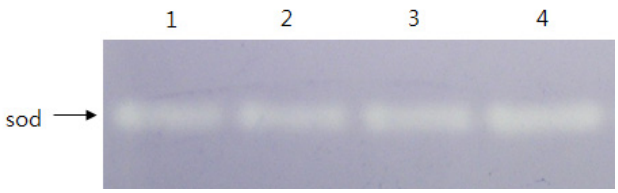

Fig. 1. Activity of superoxide dismutase in *P. rhodesiae* KK1. Cells were pre-grown on LB medium and collected. Cells grown to 0.5-0.6 at 600 nm were transferred to BM medium containing PAHs or oxidative stressors and incubated at 160 rpm at 30℃. Glucose was used as control. Total protein (10ug) isolated from cells following 6h incubation was used activity staining. A, PAH-induced. Lanes 1, glucose; 2, anthracene; 3, naphthalene; 4, phenanthrene.

Polycyclic aromatic ring-hydroxylating dioxygenase gene in *Pseudomonas rhodesiae* KK1

A 300-bp aromatic ring-hydroxylating gene for α-subunit of dioxygenase responsible for degradation of polycyclic aromatic hydrocarbons in strain KK1 was obtained by PCR amplification using a set of degenerate primers, DioF and DioR [9]. Sequence analysis of the gene revealed naphthalene 1,2-dioxygenase composed of 94 amino acids, which shared 98.9% similarity with nahAc of *Pseudomonas fluorescens* PC20 [7], 96.8% with *nahAc* of *Pseudomonas putida* G7 [8], 95.7% with *ndoC2* of *Pseudomonas fluorescens* ATTC 17483 [4], 93.6% with *pahAc* of *Pseudomonas putida* OUS82 [24], and 86.2% with *nahAc* of *Pseudomonas balearica* SP1402 [4] and *Pseudomonas stutzeri* AN11 [4] (Fig. 2). A part of the amplified PAH-ring hydroylating gene sequence was used for the analysis of the transcriptional gene expression using RT-PCR as well as for the comparative analysis of transcriptional gene expression of PAH-ring hydroxylating dioxygenase and sod genes.

Identification and analysis of SOD genes in *Pseudomonas rhodesiae* KK1

The approximately 420-bp putative superoxide dismutase gene fragment amplified by PCR was found to contain two types of superoxide dismutase in *P. rhodesiae* KK1. One of them was Mn-superoxide dismutase (*sodA*) composed of 423-bp and 141 amino acids, and the other is Fe-superoxide dismutase (*sodB*) composed of 405-bp and 135 amino acids (Fig. 3, Fig. 4).

Both Mn- and Fe-SOD have been found in many prokaryotic bacteria including *Pseudomonas* species [6, 13, 15, 19, 20]. Multialignment analysis based on 141 amino acids showed that *sodA* in strain KK1 shared 95% similarity with Mn-sod of *P. fluorescens Pf*-5 [19], 92% with Mn/Fe-Sod of *P. fluorescens* PfO-1 [22], 89% with of Mn-sod of *P. syringae pv. tomato str.* DC3000 [12] and 88% with Mn-sod of *P. aeruginosa* PAO1 [23]. The *sodB* shared 99% similarity, based on 135 amino acids, with *Fe-sod* of *P. fluorescens Pf*-5 [19] and super-

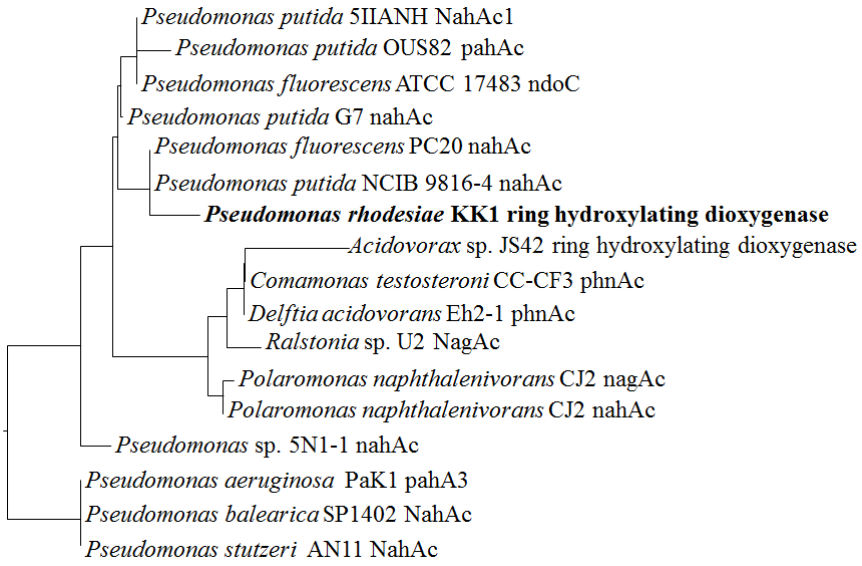

Fig. 2. Phylogenetic analysis of ring-hydroxylating dioxygenase genes from *P. rhodesiae* KK1 and other bacterial strains based on multiple alignment of the deduced amino acid sequence.

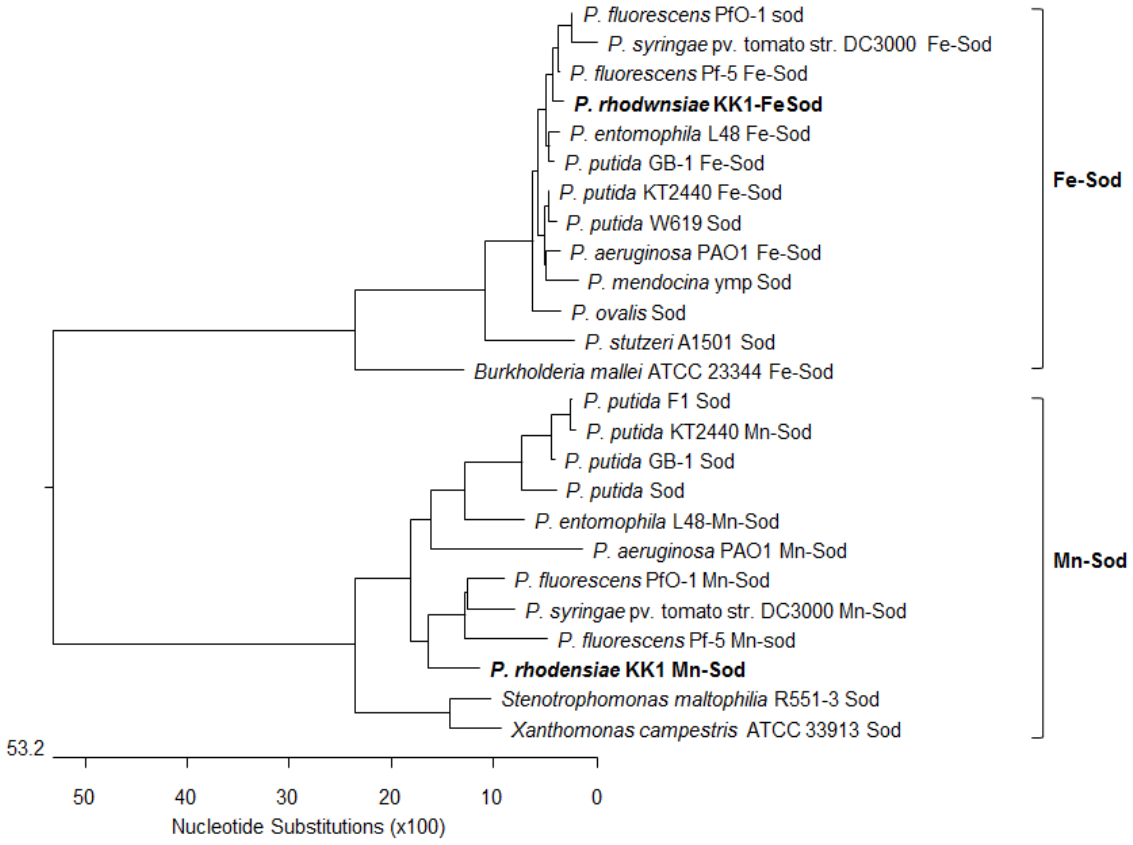

Fig. 3. Phylogenetic analysis of two types of superoxide dismutase (FeSOD and MnSOD) genes in *P. rhodesiae* KK1 and other bacterial strains based on deduced amino acid sequences.

oxide dismutase of *P. fluorescens* PfO-1 [22], 97% with *Fe-sod* of *P. putida* KT2440 [18], and 94% with *Fe-sod* of *P. stutzeri* A1501. When twelve Fe-SOD and/or Mn-SOD homologues in *Pseudomonas* species are aligned, highly conserved residues which have identical amino acid at the same position were found in several regions as highlighted in grey shading in Fig. 4. The N-terminal and C-terminal domain regions are more densely conserved than the central domain region. It is noticeable that the amino acid residues highly conserved in either Fe- or Mn-SOD, but different between the two

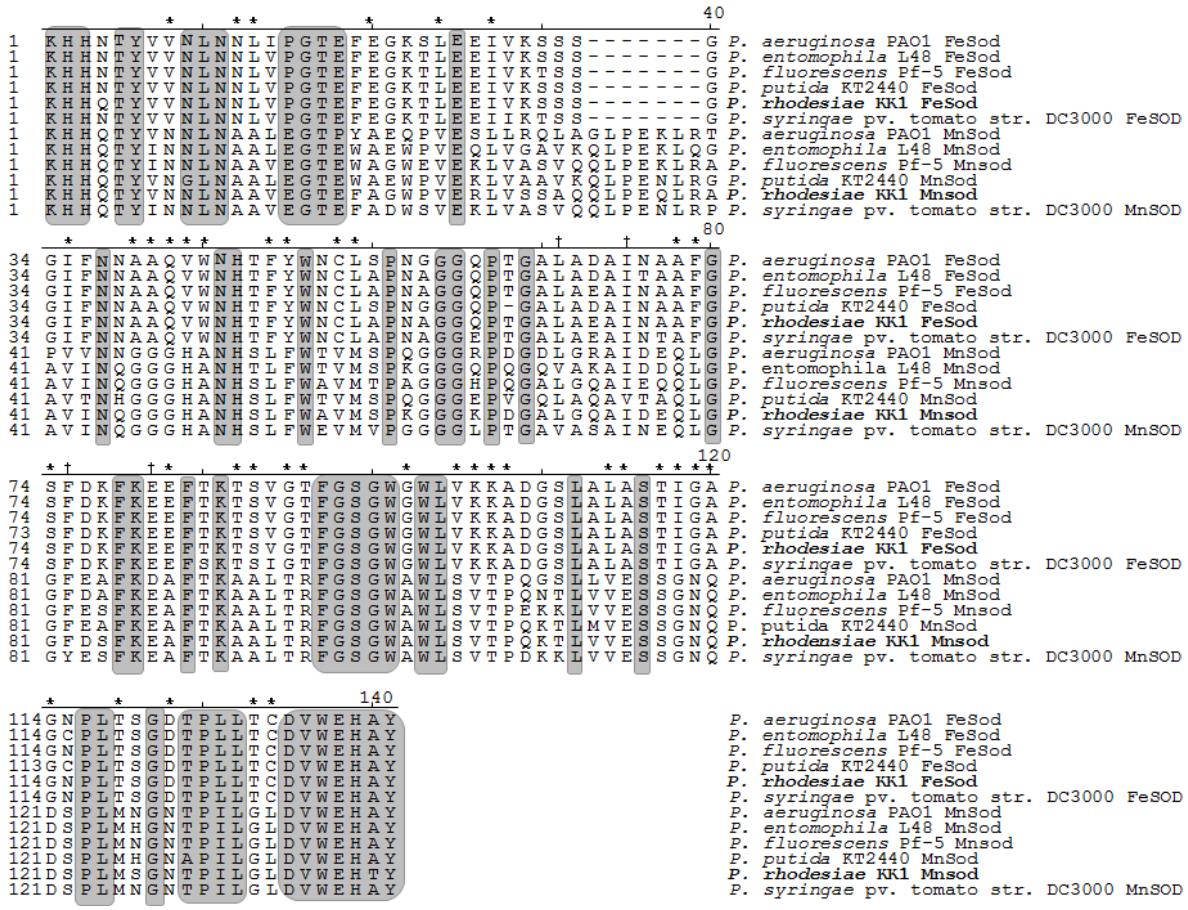

Fig. 4. Multialignment of two types of superoxide dismutase (FeSOD and MnSOD) genes based on deduced amino acid sequences in *P. rhodesiae* KK1 with those found in other bacteria. Identically conserved residues in twelve MnSOD and FeSOD homologues are highlighted in grey shading. The amino acid residues identically conserved in either MnSOD or FeSOD, but different between the two genes are indicated with asterisks. Gaps are represented by dashes and were introduced to maximize the alignment. The multiple sequence alignment analysis was carried out using the Clustal method within the MEGALIGN program of Lasergene.

genes were found in forty regions as indicated with an asterisk. This result suggested that there are several more conserved regions unique to Fe- or Mn-SOD.

Southern hybridization using the PCR antioxidant gene fragment as a probe showed that at least more than two copies of superoxide dismutase genes exist in strain KK1 (Fig. 5). Restriction patterns with superoxide dismutase gene signals were found at 20 kb- and 1.8 kb-*Apa*I fragments, 20 kb-*Bam*HI fragment, 8.0 kb-, 3.0 kb- and 0.5 kb-*Eco*RI fragments, 20 kb-*Hin*dIII fragment, 4.0 kb- and 2.5 kb-*Pst*I fragments, and 4.0 kb- and 2.7 kb-*Sal*I fragments. These facts suggested that at least more than two copies of superoxide dismutase exist in strain KK1. These results were consistent with the previous studies [6, 18, 19, 23], in that more than two copies of superoxide dismutase genes have been found in many *Pseudomonas* species such as *P. putida*, *P. fluorescens*, *P. syringae* and *P. aeruginosa*.

Effect of PAHs on transcriptional expression of SOD genes in *Pseudomonas rhodesiae* KK1

The transcriptional gene expression pattern of SOD genes in response to PAHs in *P. rhodesiae* KK1 cells was investigated based on RT-PCR analysis, along with that of the ring-hydroxylation gene responsible for the cleavage of aromatic ring. It was found that the ring-hydroxylating gene expression in the transcriptional level was more stimulated in KK1 cells grown with naphthalene and phenanthrene than glucose and anthracene, suggesting that expression of ring-hydroxylating gene for the degradation of PAHs in strain KK1 might be quickly stimulated by naphthalene and phenanthrene (Fig. 6). Interestingly, the PAH ring-hydroxylating gene product is found in the basic level in the glu-

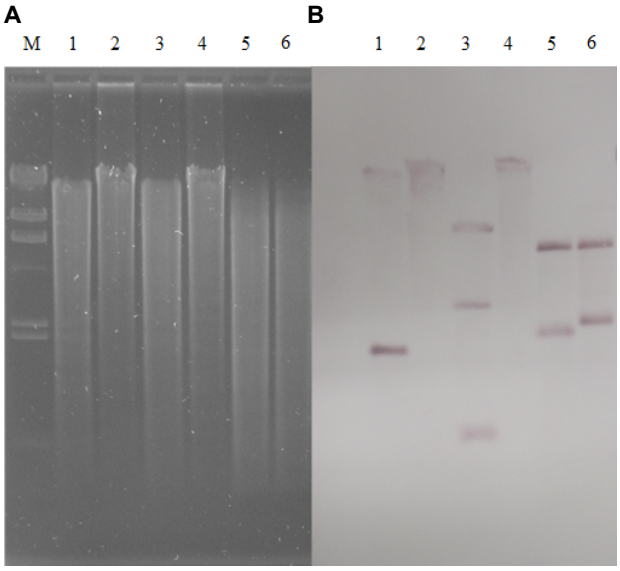

Fig. 5. Southern hybridization of superoxide dismutase genes using total genomic DNA from *Pseudomonas rhodesiae* KK1. The DNA fragments digested with several restriction enzymes such as *Apa*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I were hybridized with the superoxide dismutase gene DNA fragment obtained from the PCR amplification. Restriction patterns of total genomic DNA of strain KK1 are shown in left side of the panel (A), while right side shows the signals hybridized with superoxide dismutase gene probe labeled with Dig DNA labeling kit (B). Lanes M, DNA size marker (λ- *Hind*Ⅲ); 1, Genomic DNA digested with *Apa*I, 2, *Bam*HI; 3, *EcoR*I; 4, *Hin*dⅢ; 5. *Pst*I; 6, *Sal*I.

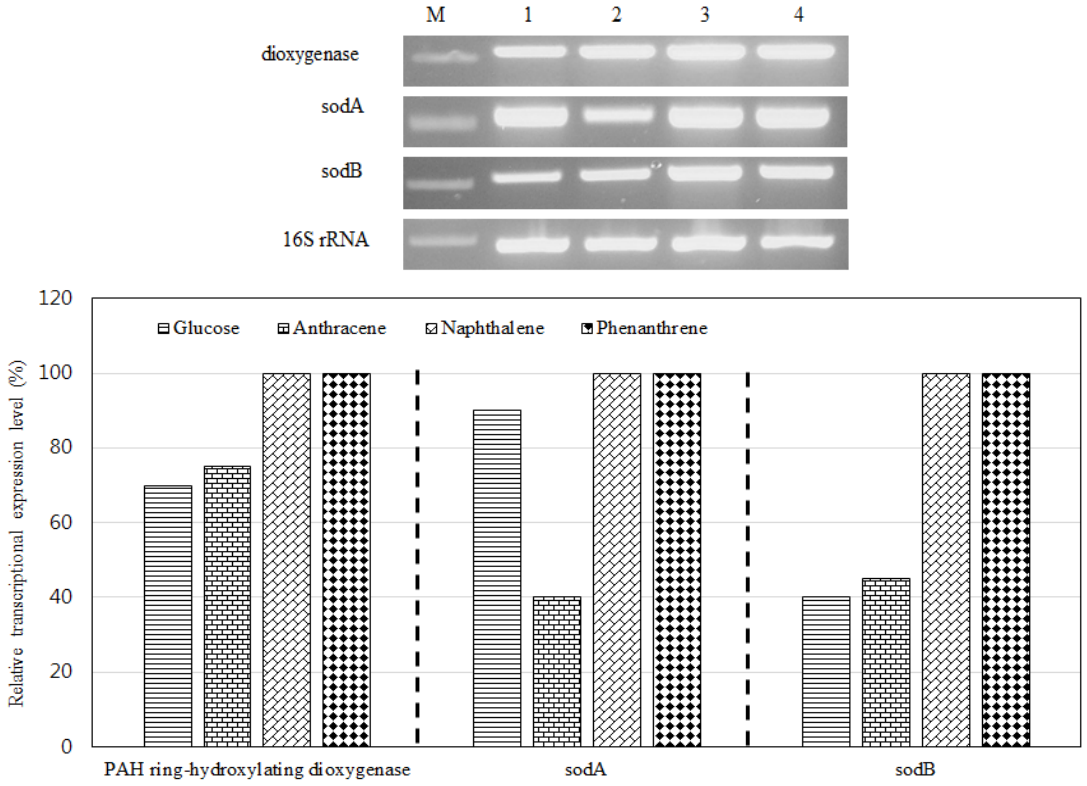

Fig. 6. Transcriptional expression analysis of ring-hydroxylating enzyme (dioxygenase)- and/or superoxide dismutase isomers (*sodA* and *sodB*)-encoding genes of *Pseudomonas rhodesiae* KK1 using RT-PCR with total RNA from cells incubated with glucose (lane 1), anthracene (lane 2), naphthalene (lane 3) or phenanthrene(lane 4) as a substrate. M, size marker. The expression level was relatively determined based on the amount of transcriptional products.

cose grown-cells without PAHs. Relative transcriptional level of the PAH ring-hydroxylating gene grown with glucose and anthracene was approximately 65% and 70%, respectively. Transcriptional gene expressions of Mn-SOD (*sodA*) and Fe-SOD (*sodB*) genes were commonly more strongly stimulated in response to naphthalene and phenanthrene than anthracene. It is notable that *sodA* gene is expressed in glucose-grown cells in the similar level with those grown with naphthalene and phenanthrene. Whereas the transcriptional expression level of *sodB* gene was lowest in

cells grown with glucose. Vattanaviboon *et al* [26] reported the constitutive expression of Fe-SOD in *Vibrio harveyi*, whereas Mn-SOD was expressed at the stationary phase and could be induced by a superoxide generator. However, SodA1 gene in *Bacillus anthracis,* which is cambialistic for magnesium and iron, was found to be constitutively expressed [25]. It is remarkable that our findings suggested the possibility Mn-SOD gene might be under constitutive expression, warranting further study with other substrates such as fructose and citrate.

In conclusion, the expression pattern of *sodA* and *sodB* genes is very similar in KK1 cells exposed to PAHs, but different in KK1 cells grown with glucose. Interestingly, *sodA* gene from KK1 cells grown with glucose was found to be transcriptionally expressed in the similar level with cells grown with naphthalene and phenanthrene, while *sodB* gene was not. These facts suggested that SODA might play a more important role in cells exposed to naphthalene and phenanthrene for the removal ROS generated in cells during oxidative metabolism even though both SODA and SODB are responsible for the degradation of ROS.

Acknowledgments

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초록 : PAH를 분해할 수 있는 *Pseudomonas rhodesiae* KK1의 SOD 유전자의 동정 및 분자학적 특성 분석

이동헌[1] · 오계헌[2] · 김승일[3] · 강형일[1]*
([1]순천대학교 환경교육과, [2]순천향대학교 생명과학과, [3]한국기초과학지원연구원 프로테옴팀)

Pseudomonas rhodesiae KK1은 이미 주요한 환경오염물질인 anthracene, naphthalene, phenanthrene과 같은 다환성 방향족 화합물(PAHs)을 분해할 수 있음을 보고한 바 있다. 흥미롭게도, superoxide dismutase를 비롯한 항산화 유전자는 환경오염물질에 반응하여 다른 수준으로 발현됨이 알려져 있다. 본 연구는 균주 KK1에서 PAHs 분해에 간접적으로 관계될 것으로 여겨지는 superoxide dismutase 유전자의 존재를 동정하고 세 가지 PAHs를 기질로 하여 생장한 세포에서 superoxide dismutase 유전자의 발현 양상을 조사하고자 수행하였다. *P. rhodesiae* KK1에서 항산화 기작에 관여하는 두 가지지 형의 superoxide dismutase인 Mn-superoxide dismutase (*sodA*)와 Fe-superoxide dismutase (*sodB*) 유전자를 동정하고 그 특성을 규명하였다. 균주 KK1에서 발견된 *sodA* 유전자는 141개의 아미노산 유전자를 기준으로 *P. fluorescens Pf*-5의 Mn-sod와 95%, *sodB* 유전자는 135개 아미노산을 기준으로 *P. fluorescens Pf*-5의 Fe-sod와 99%의 가장 높은 상동성을 나타내었다. *sod* 유전자 단편을 탐침자로 사용한 Southern 혼성화 반응 결과 적어도 두 개 이상의 superoxide dismutase 유전자가 균주 KK1에 존재함을 규명하였다. RT-PCR 분석을 통해 *sodA* 및 *sodB* 유전자들은 anthracene보다 naphthalene과 phenanthrene에 반응하여 더 강하게 발현함을 보여주었다. 포도당과 PAHs를 기질로 사용하여 생장한 세포에서 *sodA*와 *sodB* 유전자는 활성 상태로 존재함이 밝혀졌다.