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Effects of Gene Expression of *Photobacterium leiognathi* CuZn Superoxide Dismutase(PSOD) by *lacZ* Promoter Control under Oxidative Stress

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The effects of PSOD expression on lacZ-sodP fusion (pYK4) was explored in *Escherichia coli* sodA sodB mutants (QC774) under oxidative stress. In this system, although β-galactosidase activity was not fully induced by isopropyl-1-thio-β-galactosidase (IPTG) and was inhibited by glucose, functional PSOD was under lacZ promoter control and was induced by IPTG, lactose, PQ and copper ions. Finally, the results show that higher PSOD expression level was consistently important in defending against superoxide radicals.

KEY WORDS superoxide dismutase, oxygen radical, gene expression, *lac* promoter

Organisms that live in an oxygen environment require defenses against toxic reduction states of oxygen, including the superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical (OH). A very large effort has been expended to define the cellular processes that generate toxic oxygen species and to identify the types of damage that result (8).

SODs are metalloenzyme of which the sole known function is to eliminate, through a dismutation reaction ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) (20), the superoxide ($O_2^-$) produced in all living organisms exposed to oxidative stress. Although these enzymes are believed to have important role in preventing deleterious effects in the cell, a necessary next step is to understand the regulatory mechanism for expression of this system.

P. leiognathi possesses two isoenzymes, a copper and an iron (26, 28) containing SOD, respectively. It has been known that PSOD can be enhanced by redox-oxygen compounds such as paraquat (PQ) (1, 13, 24) and plumbagin (13, 14, 17), and by copper ion under aerobic condition (25). Previously, Moody and Hassan (23) proposed that MnSOD is negatively regulated by an iron containing repressor protein at the level of transcription, whereas Pugh and Fridovich (27) suggested regulation by autogeneous repression via apo-SOD.

Recently Kim (17) reported on the expression of construct the fusion gene, pYK4, which has a functional PSOD gene with a deleted periplasmic targeting signal sequence. This plasmid resulted in an in-frame fusion such that expression of PSOD is under the control of the lacZ gene regulatory region.

If so, how can PSOD be expressed and be controlled by lac repressor or inducer under oxidative stress? In addition, as shown in the previous study, copper ion induction of PSOD in the presence of IPTG is important in the understanding of the SOD biosynthetic regulatory mechanism. Finally, the cellular responses to oxidative stress need to be understood quantitatively.

To elucidate the effect of the β-galactosidase inducer IPTG on the lacZ-sodP gene fusion, the magnitude of the genetic response by PSOD expression to oxidative-mediated stress in *E. coli* SOD deficient mutants was explored.

MATERIALS AND METHODS

Bacterial strains and Plasmids

The *Escherichia coli* K-12 sodA sodB double mutant strain QC774[GC4468 ϕ(sodA-lacZ)49Cm^r ϕ(sodB-Kan)1 −Δ2Km^r] and wild type strain GC 4468(F Δlac4169 rspL) were described previously (5). Plasmid pYK4 (17) was employed as shown in Fig. 1.

Media and Growth conditions

Rich medium was LB and minimal medium was M9 salts (21) supplemented with 0.4% glucose (M9G) and thymine (1 μg/ml). Precultures in LB

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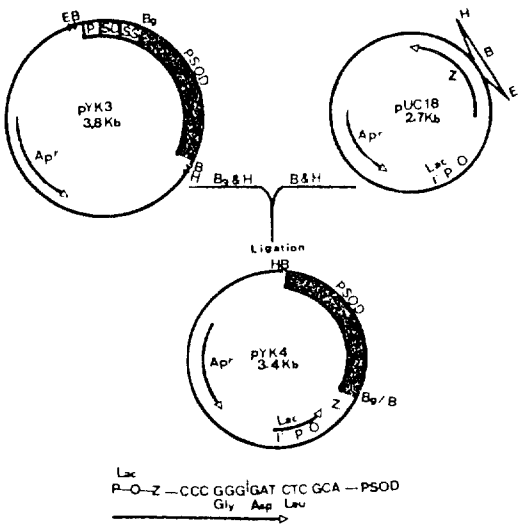

Fig. 1. *Construction of a functional PSOD gene with deleted periplasm-targeting signal sequence. The single BglII site of pYK3 separates the PSOD signal-sequence and enzyme coding regions. The BglII-HindIII fragment from pYK3 was ligated into the multiple cloning site of BamHI-HindIII digested pUC18. This resulted in an in-frame fusion such that expression of PSOD (now cytoplasmic) if under the control of the lacZ promoter.Explanation of the fusion region: The functional wildtype PSOD begins with Gln-Asp-Leu. In predicted fusion peptide Gln has been replaced with the first 12 amino acids (residue 12=Gly) of the pUC18 multiple cloning site.*

or M9G inoculated from single colonies were grown at 37°C in the presence of appropriate antibiotics when carrying resistance markers (20 μg/ml: Cm, 40 μg/ml: Ap, 40 μg/ml: Km). All cells were cultured with vigorous shaking(rpm, 250) at 37°C. To test cell survival on minimal medium, cells were grown overnight with vigorous aeration (250 rpm) at 37°C in LB broth supplemented with appropriate antibiotics. Overnight cultures were diluted 1:100 into 20 ml of fresh LB in 250 ml Erlenmyer flasks and were monitored for optical density OD_{600}nm=0.1. Then isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1.0×10^{-3}–3.0×10^{-3} M), glucose (10%), lactose (50 mM), paraquat (PQ, 0.1 mM) and $CuSO_4$ (0.1 mM) were added, respectively. To investigate the cellular response, cells were cultured for six hours. To investigate the sensitivity of growth rates to paraquat, after two hours growth, various prior exposures were washed with LB medium, and cultures treated with paraquat (0.1 mM) were examined at OD_{600} nm for 4 hours. Six hours-cultured cells (100 μl) were also plated on LB agar containing paraquat concentrations from 0.1 to 0.6 mM. Finally cell survival was determined by counting colonies after two days. For paraquat-sensitivity disk/diffusion plating assay, samples from previous 6 hours-cultures were spread onto M9G agar supplemented with 0.2% casamino acids. The other details were followed as described by Kim (17) including sensitivity to paraquat determined by measurement of inhibition zone.

Crude extracts and enzyme assays

Transformants (5 ml) obtained with pYK4 were harvested and crude extracts were prepared by cycles of freezing and thawing as described by Carlioz and Touati (5). These procedures were modified in that no lysozyme is included in the disruption medium and each freeze period was preceded by a 30 second interval of vortexing in the presence of equal volume of 0.5 mm glass beads. Cellular debris and glass beads were removed by centrifugation, and total protein concentration was estimated by the method of Lowry *et al.* (18) and was determined by the coomassie blue method (3). Enzyme activity on 12% polyacrylamide slab gels was visualized by using the method of Beauchamp and Fridovich (2). For quantitative determination of SOD activity, Pyrogallol method (19) was employed as described previously. β-galactosidase activity was measured, unless otherwise stated, on whole cells grown in LB medium as described by Miller (21). The other details were performed as described by Kim (17).

RESULTS

Assessment of sensitivity of QC774(pYK4) strains to oxidative stress

E. coli sodA sodB double mutant cells (QC774) were grown under aerobic conditions on minimal glucose medium. They exhibit increased sensitivity to agents of oxidative stress such as paraquat, and grow more slowly than wild type cells on rich medium (5, 25). This sensitivity was inhibited by increasing concentration of IPTG (Fig. 2, 3, 4, Table 1) and by growth in lactose (Fig. 3, Table 1). However, this was enhanced by adding glucose (Fig. 2, Table 1). Furthermore, the growth rates were directly proportional to IPTG induction on LB medium containing paraquat (Fig. 2, 4, Table 1). This determination was confirmed by assays of PSOD with different concentrations of IPTG and β-galactosidase activity (Table 2). This observation suggested that β-galactosidase could induce the PSOD and thereby this PSOD function is more efficient against oxidative stress. PSOD activity was induced approximately 3 fold as compared with the control. However, β-galactosidase was negligible at the level of induction. Most notably, cells carrying pYK4 at high-

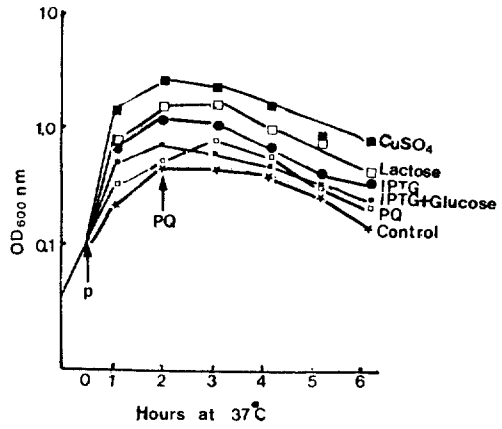

Fig. 2. *Sensitivity of QC774(pYK4) on various growth conditions under oxidative stress.*
P: pretreated point by various substances

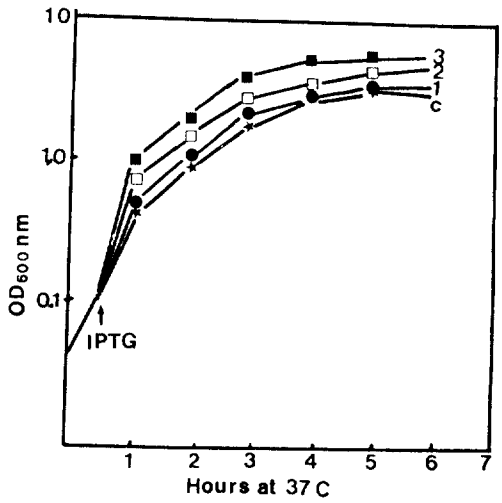

Fig. 3. *Growth rates in LB medium of QC774(pYK4) cells on IPTG induction.*
Symbols; ☆, controls(QC774); ●, 1.0×10^{-3} M IPTG; □, 2.0×10^{-3} M IPTG; ■, 3.0×10^{-3} M IPTG- treated cells. Numbers indicates the times of IPTG treated-concentrations.

er levels of IPTG exhibited significantly higher growth rates. This is important in understanding the effect of glucose on SOD biosynthesis. Similar results were obtained in survivorship experiments in which cells were plated directly onto LB agar containing 0.1 to 0.6 mM paraquat, and in experiments where cells were plated at high density in the presence of paraquat-soaked filter-paper disk (Table 1).

Effect of copper ion

The growth rate to oxidative stress of *E. coli*

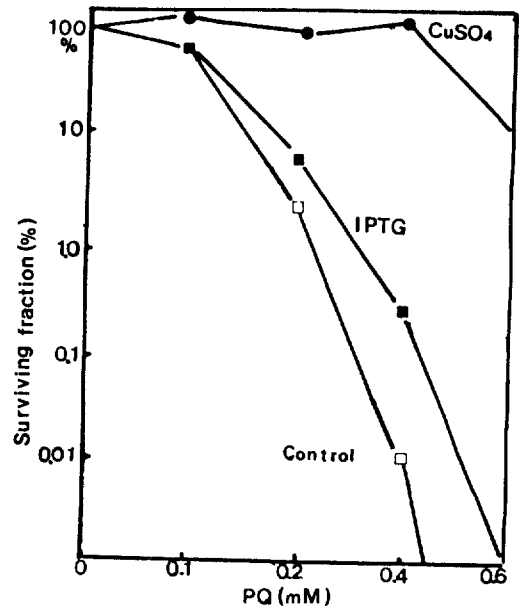

Fig. 4. *Effects of IPTG (3×) and copper ions on survival of QC774(pYK4) in the presence of high PQ concentrations. Details are described in methods.*

Table 1. *Response of QC774(pYK4) cells to plating minimal medium and to the paraquat disk/diffusion assay*

Under various growth conditions	Relative zone of inhibition
QC774(pYK4)	
Control	0.78
+Glucose	0.82
+IPTG+Glucose	0.79
+IPTG(3×)	0.65
+$CuSO_4$	0.54
+Lactose	0.53
QC774	1.00
GC4468	0.41

sodA sodB mutant cells with pYK4 was significantly increased by the addition of $CuSO_4$ (0.1 mM) in LB growth medium (Fig. 2, 3, Table 1). The sensitivity of cells to the effect of added copper with increasing concentrations of paraquat on LB agar medium was dramatically reduced (Fig. 4, Table 1). This result suggests that copper participates in the redox-sensitive control of the production of PSOD under oxidative stress. Thus the external copper ions can be transported efficiently into the cytoplasm and thereby they inserted into the metal prosthetic group for the metal-binding site of PSOD.

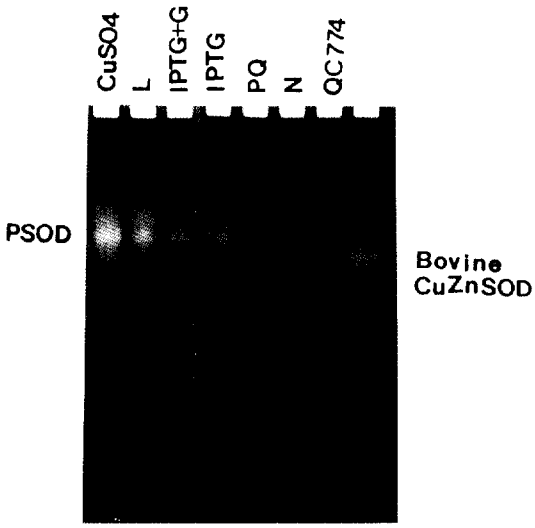

Fig. 5. *PSOD expression of QC774(pYK4) cells. Each lane received 40 μg of total cell protein L (lactose); G (glucose); N (untreated). Bovine CuZnSOD was used as a standard (Sigma). Details are described in methods.*

Table 2. *Effect of various compounds on β-galactosidase and PSOD expression in lacZ-sodP fusion*

Treatments	β-galactosidase (u/mg)	PSOD (u/mg)
	QC774 / QC774 (pYK4)	QC774 / QC774 (pYK4)
glucose	4.6 / 4.8	none / 20
lactose	5.4 / 5.7	/ 80
paraquat	4.3 / 3.8	/ 30
IPTG-1×	4.8 / 5.2	/ 78
2×	4.7 / 5.2	/ 80
3×	4.8 / 5.2	/ 84
$CuSO_4$	4.1 / 3.9	/ 90
none	4.0 / 3.5	/ 23

Effect of expression of PSOD by lacZ promoter control

pYK4 carried a copy of the PSOD gene from which the nucleotide sequence encoding the membrane-transport signal peptide had been deleted. The plasmid was contained a cytosolic fusion peptide in which the transport signal of PSOD is replaced by the first 12 amino acids of the pUC18-encoded lacZ alpha peptide (17). Therefore this lac promoter has a mutant operator that can not bind a repressor molecule because the lac operator occupies the first 26bp of the lacZ gene and has been partially deleted. Finally, the sodP structural gene contiguous with operator mutation was under the control of the lacZ promoter (Fig. 1) and thereby was expressed constitutively. The PSOD of *E. coli* sodA sodB mutant cells carrying pYK4 was induced by IPTG, lactose, PQ and copper ions (Fig. 2, 4, Table 1). Interestingly, the lacZ promoter was also insensitive to paraquat and the PSOD expression was increased three and five-fold by increasing concentration of IPTG and by addition of copper ions, respectively (Fig. 5, Table 2). However, the effective expression was repressed in the presence of glucose (Fig. 5, Table 2). Increasing concentrations of paraquat, which generates O_2^- in *E. coli* sodA sodB encoded pYK4 resulted in increasing differential rates of PSOD biosynthesis (data not shown). This suggests that cellular responses to deleterious effects were a consistently important variable at the level of PSOD expression. In such a fusion, expression of β-galactosidase was at a low, negligible level and was slightly induced by IPTG, paraquat and copper ions (Table 2). Nevertheless, PSOD expression was fully induced by these inducers. This suggests that PSOD expression was not under a simple control mechanism in this organism and can be regulated multifunctionally such as post-transcriptional (30) or by the soxR regulon (7).

DISCUSSION

The results presented above show that *E. coli* sodA sodB mutants encoded pYK4 are capable of inducing an active PSOD. Although lac operator of pYK4 was mutated, it was insensitive to oxidative stress. A low level of PSOD expression in the absence of any induction was observed either as the result of lac promoter or O^c mutation. Hallewell *et al.* (12) reported the expression of human CuZnSOD in *E. coli* using the tacI promoter. The authors obtained normal specific SOD activity. In contrast to my result, the different level of expression is due to the different promoter system. This principle can be further supported by the previous investigation of the low level of expression by using a vector containing a thermoinducible PL promoter and β-lactamase-derived ribosomal binding site (29).

Touati (5) reported previously sodA-lacZ gene fusions respond accurately to MnSOD inducer when the sodA gene was transcribed from tac promoter. In this system, MnSOD was induced only under tac promoter control and was induced by IPTG. Our results are in agreement in Touati's proposal (30). However, β-galactosidase did not affect SOD expression as compared with the former result. Furthermore, β-galactosidase was expressed at a low level and was not fully induced by IPTG, paraquat and copper ions (Table 2). It is thought this is due to deletion of the larger fragment of lacZ structural gene. Copper

induction of PSOD was significant in *E. coli* sodA sodB cells. This suggestes that copper binding regulatory protein ACE1 (23, 24) or upstream activation sequences($UAS_{cup}1$), such as in yeast, *Saccharomyces cerevisiae* (10), may exist in our system. Although the increase in the availability of glucose was followed by an increase in superoxide dismutase and in growth rates (15), the results (Fig. 3, 5 and Table 2) demonstrate that glucose paralleled the PSOD expression levels and growth survival rates. In the presence of glucose, both levels were strongly inhibited. This suggests that uptake and retention of paraquat by cells encoded pYK4 were dependent upon a carbon source such as in *E. coli* B cells (22) or can be controlled by the cyclic AMP receptor if pYK4 has a catabolite-sensitive operon. This should be further studied since the results can be interpreted in terms of the requirement of paraquat for energy-dependent active transport (electrons to support the redox cycling of paraquat) or perhaps can not be induced by a catabolite-sensitive operon. If the increasing uptake and retention of paraquat was due to glucose or elevation of external pH on internal pH of cells, membrane electrochemical gradient would be increased and thereby could affect the PSOD activity and its function (22). Effect of survivals on the lethality of paraquat was consistently in agreement in PSOD expression levels. In particular, copper ions were very effective in inducing PSOD. This remarkable induction of PSOD activity and function is thought to be due to neutralization of free radicals by metal copper cation (9), but it remains to be determined. Furthermore, the extensive overlap among several global responses such as heat shock (6) and oxyR (7, 11) in the pYK4 system remains to be investigated.

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초 록: 산소압하에서 *lacZ* Promoter 조절에 의한 *Photobacterium leiognathi* CuZn Superoxide Dismutase(PSOD)의 유전인자 발현 효과
김영곤 (조선대학교 자연과학대학 생물학과)

산화압 조건에서 *Escherichia coli* sodA sodB 돌연변이체(QC774) 내에서 lacZ-sodP의 유전인자 융합에 따른 PSOD의 발현 효과가 탐구되었다. 본 조직에 있어 비록 β-galactosidase 활성도는 IPTG에 의해 유도되지 않고 glucose에 억제되었을지라도 기능적인 PSOD는 lacZ promoter 조건하에 있었고 IPTG, lactose, PQ 그리고 copper 이온에 의해 유도되었다. 결과적으로 더 높은 PSOD의 발현 정도가 superoxide radicals을 방어하는데 변함없이 중요했다.