Expression and Regulation of the sodF Gene Encoding Iron- and Zinc-Containing Superoxide Dismutase in Streptomyces coelicolor Müller

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Streptomyces coelicolor Müller contains two superoxide dismutases (SODs), nickel-containing (NiSOD) and iron- and zinc-containing SOD (FeZnSOD). The sodF gene encoding FeZnSOD was isolated by using PCR primers corresponding to the N-terminal peptide sequence of the purified FeZnSOD and a C-terminal region conserved among known FeSODs and MnSODs. The deduced amino acid sequence exhibited highest similarity to Mn- and FeSODs from Propionibacterium shermanii and Mycobacterium spp. The transcription start site of the sodF gene was determined by primer extension. When the sodF gene was cloned in pLI702 and introduced into Streptomyces lividans TK24, it produced at least 30 times more FeZnSOD than the control cells. We disrupted the sodF gene in S. lividans TK24 and found that the disruptant did not produce any FeZnSOD enzyme activity but produced more NiSOD. The expression of the cloned sodF gene in TK24 cells was repressed significantly by Ni, consistent with the regulation pattern in nonoverproducing cells. This finding suggests that the cloned sodF gene contains the cis-acting elements necessary for Ni regulation. When the sodF mRNA in S. coelicolor Müller cells was analyzed by S1 mapping of both 5’ and 3’ ends, we found that Ni caused a reduction in the level of monocistronic sodF transcripts. Ni did not affect the stability of sodF mRNA, indicating that it regulates transcription. S. lividans TK24 cells overproducing FeZnSOD became more resistant to oxidants such as menadione and lawsone than the control cells, suggesting the protective role of FeZnSOD. However, the sodF disruptant survived as well as the wild-type strain in the presence of these oxidants, suggesting the complementing role of NiSOD increased in the disruptant.

In aerobic organisms, reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical are produced as by-products of normal metabolism. They are also generated when cells are exposed to environmental insults including redox-cycling agents and radiation. To protect cells against oxidative stresses caused by reactive oxygen species, living organisms have evolved complex oxidative defense and repair systems (10, 13). Superoxide dismutase (SOD) is considered one of the key enzymes in the oxidative defense system, catalyzing the conversion of superoxide anion to hydrogen peroxide and molecular oxygen. The reaction is catalyzed by cyclic oxidation and reduction of the transition metal in the active site of SODs (13). Depending on the type of metal cofactors, SODs are classified into four groups: MnSOD containing manganese, FeSOD containing iron, CuZnSOD containing copper and zinc, and NiSOD containing nickel (22, 46, 47).

Most bacteria possess two types of SODs in the cytosol, mostly FeSOD and MnSOD. They are either dimers or trimers of identical subunits and have nearly identical primary sequences and tertiary structures, suggestive of evolution from a common ancestor (18). Despite the structural similarity, most of them have strict metal specificity (45). However, several bacteria such as Propionibacterium shermanii, Streptococcus mutans, and Bacteroides gingivalis were found to use the same apoprotein to produce either active MnSOD or FeSOD (2, 28, 29). This type of SOD is called cambialistic SOD. FeSOD and MnSOD have no similarity in sequence and structure with CuZnSOD, suggestive of independent evolution. Periplasmic CuZnSOD has been found in several bacteria, including Photobacterium leiognathi, Caulobacter crescentus, Escherichia coli, and some pseudomonads (5, 11, 39–41). NiSOD, which has been purified from several Streptomyces spp., is distinct from the above three groups of SODs on the basis of amino acid composition, N-terminal amino acid sequence, and immunocross-reactivity (22, 46, 47).

The regulation of expression of SOD genes has been characterized in several bacteria (45). In E. coli, the expression of the MnSOD gene (sodA) is induced by oxygen and markedly increases in response to paraquat, a superoxide-generating agent (45). The regulation is exerted both transcriptionally and posttranscriptionally (44). The transcriptional regulation of MnSOD is mediated by several global regulators such as SoxRS, Fur, Fnr, ArcA, and integration host factor (8, 45). In contrast, the transcription of the FeSOD gene (sodB) is mostly constitutive and thus far has been suggested to be regulated only by the fur locus (31). On the other hand, the periplasmic CuZnSOD increases at least 100-fold during the stationary phase partly due to the increase in transcription (19). Regarding the role of each SOD in the bacterial cell, it is suggested that MnSOD and FeSOD protect cells against superoxide originating from intracellular sources, whereas periplasmic CuZnSOD protects cells against external superoxide, judged from its localization (35). It is not certain whether the two cytosolic SODs are functionally equivalent. A study using controlled expression of E. coli MnSOD and FeSOD genes from an inducible promoter suggested that MnSOD is more effective in preventing DNA damage whereas FeSOD is more effective in protecting superoxide-sensitive enzymes (16). It has been sug-
Streptomyces coelicolor contains NiSOD and FeZnSOD in the cytosol (22). Purified FeZnSOD is a homotrimer of 22.2-kDa subunits containing 0.36 mol of iron and 0.26 mol of zinc per mol of subunit. Its N-terminal amino acids and enzymatic characteristics suggest that it is similar to either FeSOD or MnSOD. We have found that expression of the two SODs in S. coelicolor is regulated by Ni in such a way that Ni induces NiSOD expression and represses FeSOD expression (22, 23). To understand the regulation of FeZnSOD at more defined levels of gene expression, we have isolated the gene (sodF) encoding FeZnSOD and analyzed its expression.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** S. coelicolor ATCC 10147 (Müller) and *Streptomyces lividans* TK24 cells were grown as described previously (17, 22). For liquid culture, YE (1% glucose), 0.5% Bacto Peptone, 0.3% malt extract (supplied by PoscoChem). The mixture was subjected to 30 cycles of denaturation at 94°C, annealing for 1 min at 50 to 55°C, and extension for 1 min (supplied by the manufacturer with 5 mM deoxynucleoside triphosphates and 1U of RNasin (Promega) extended by Moloney murine leukemia virus reverse transcriptase (Promega). To prepare the S1 probe buffer supplied by the manufacturer with 5 mM deoxynucleoside triphosphates and 1 μg of Ranisin μl⁻¹. The extended product was analyzed on a 6% polyacrylamide gel containing 7 M urea. The extended product was analyzed on a 6% polyacrylamide gel containing 7 M urea.

**sodF** gene. Genomic DNA from *S. coelicolor* Müller was digested with *Sal*I and *Bgl*II and electrophoresed on a 0.8% agarose gel. Several gel slices containing *Sal*I fragments in the range of 0.5 to 0.7 kb which hybridized with the *sodF* PCR fragment were cut out of the gel. The DNA was eluted and purified from each gel slice by using a GeneClean kit II (Bio101), electrophoresed on a 0.8% agarose gel, and confirmed for hybridization with the PCR product or oligonucleotide FC. The best-hybridizing DNA fraction was ligated with pUC18 and transformed into E. coli DH5α. Transformants were screened by colony hybridization as described by Hopwood et al. (17). The overlapping clones containing a 3.2-kb *Sal*I fragment and a 0.8-kb *Bgl*II/*Pvu*I fragment were named pEK11 and pEK12, respectively. These two clones were found to contain the *sodF* open reading frame (ORF) truncated at the C and N termini, respectively. pEK13 with a 1.3-kb DNA insert encompassing the entire *sodF* ORF was constructed by recombining the 0.8-kb *Pvu*I/*Sal*I fragment from pEK11 and the 0.5-kb *Sal*I/*Bgl*II fragment from pEK12 into pUC18 at the Smal site.

**Detection of SOD activity.** Preparation of cell extracts, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), activity staining of SOD in the gel, and the activity assay for SOD were done as described previously (4, 22, 39). The activity assay for SOD activity in the nondenaturing gel was detected by its ability to deplete superoxide, which can reduce nitroblue tetrazolium (39).
Expression of the sodF gene in E. coli. PCR was done on a pEK13 template, using a universal primer and the mutagenic primer FS3 (5'-CCGGATCCGG CTCTACAGC-3'), which corresponds to the N-terminal sequence of the sodF ORF with an NcoI site created (underlined). The PCR product was doubly digested with NcoI and HindIII and cloned into pET-21d (Novagen), generating pET-SODF3. E. coli BL21(DE3)pLysS cells were transformed with pET-SODF3 or pET-21d. Freshly grown transformant cells were induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h before harvest. Cell extracts of the transformant were plated on NA medium containing 50 μg/ml G418. Freshly grown transformant cells were induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h before harvest. Cell extracts of the transformant were plated on NA medium containing 50 μg/ml G418.

Disruption of the sodF gene in S. lividans TK24. To obtain the internal fragment of the sodF gene, PCR was done with FS1 (5'-GCG-9) and FS2 (5'-GACAAGCACCACGCC-9), which corresponds to the N-terminal sequence of the sodF gene. PCR was done with FS4 (5'-GCG-9) and FS3 (5'-GACAAGCACCACGCC-9), which corresponds to the C-terminal sequence of the sodF gene. The PCR fragment was subcloned into pUC18, resulting in pEK13. The cloned PCR product was used as a probe to isolate the sodF gene from S. coelicolor M145. The probe hybridized to a specific fragment of the sodF gene in S. coelicolor DNA digested with either SalI or BglII/PvuII (data not shown). The hybridizing DNA containing the entire sodF ORF was cloned into pUC18, resulting in pEK13.

The nucleotide and deduced amino acid sequences of the 1,312-bp DNA fragment in pEK13 are shown in Fig. 1. It contains an ORF beginning with codons for the same N-terminal peptide sequence of the purified FeZnSOD except for the initiating methionine. The predicted polypeptide with a serine at the N terminus consists of 212 residues with a predicted molecular mass of 23.4 kDa, in close agreement with the estimated size of the purified FeZnSOD protein (22.2 kDa). The deduced amino acid sequence of FeZnSOD was compared with those of other known SODs (Fig. 2). It exhibited the highest similarity (57% identity and 73% similarity) to the cambialistic SOD from P. shermanii, in agreement with the previous comparison with N-terminal peptide sequences (22). It showed about 70% similarity to either Fe- or MnSODs from Mycobacterium spp. Four residues which are known to be ligands for the metal cofactor (indicated with arrows in Fig. 2) were found at conserved positions (32, 38).

Transcription start site of the sodF gene. To determine the transcription start site of the sodF gene, primer extension analysis was carried out with RNAs isolated from S. coelicolor Müller cells (Fig. 3). A single species of extended cDNA of 68 nucleotides was detected with the FS4 oligonucleotide primer (Fig. 1), localizing the transcription start site to 38 nucleotides upstream from the translation start codon (Fig. 3, lane PE). A putative promoter element at the −10 region (TACGCGT) was found, similar to the consensus −10 hexamer sequence of promoters (TAGAPuT) recognized by the major sigma factor σs of S. coelicolor (Fig. 1) (20, 42). However, the sequence near the −35 region did not match any known consensus promoter sequences. A putative ribosome binding site was localized 9 nucleotides upstream from the start codon (Fig. 3).

Expression of the sodF gene in S. lividans and E. coli. To confirm the functional expression of the sodF gene in Strepto-
myces, the 1.3-kb PvuII fragment containing the sodF gene from pEK13 was cloned into pIJ702 at the SacI/SphI site to generate pIJ3SODF and introduced into S. lividans TK24. TK24 cells contained similar SOD enzymes as S. coelicolor Muller, as demonstrated by activity staining (Fig. 4, lanes 1 and 2) and immunoblotting with antibodies against either FeZnSOD or NiSOD (data not shown). Cells containing pIJ3SODF produced at least 30 times more FeZnSOD activity than cells containing the parental vector (pIJ702) (Fig. 4, lanes 2 and 3). In FeZnSOD-overproducing cells, however, NiSOD expression was repressed (lane 3). Assuming high homology between the sodF genes from S. coelicolor and S. lividans, we tried disrupting the sodF gene in S. lividans TK24 as described in Materials and Methods. The disruptant cells thus obtained exhibited no FeZnSOD activity, as expected (Fig. 4, lane 4). On the other hand, the activity of NiSOD increased in the sodF disruptant cells. The expression and disruption of the sodF gene were confirmed by immunoblotting analysis (Fig. 4B).

We next used the pET overexpression system and constructed pET-SODF3 as described in Materials and Methods. In this process, the second amino acid serine is bound to the transcription start site. Total RNAs isolated from S. coelicolor Muller cells grown in YEME were analyzed by primer extension with primer FS4 (lane PE). The DNA sequence ladder (lanes A, G, C, and T) was obtained with the same primer and plasmid pEK13, containing the entire sodF gene, as a template. Part of the DNA sequence is shown on the left, and the sodF transcription start site is indicated by a bent arrow.
result suggests that the cloned sodF gene contains the cis-acting sequence element which responds to the repressive action of Ni.

**Regulation of sodF transcription in S. coelicolor Müller by nickel.** To elucidate the step(s) where Ni exerts the regulatory role in sodF gene expression, we examined the changes in sodF mRNA in *S. coelicolor* Müller by S1 mapping analysis. Using the S1 probe labeled at the 5’ position of the BgII-cut end, we detected an S1-protected fragment of the predicted size (480 nucleotides) in the absence of Ni (Fig. 6A, lane 1). When nickel was added to the medium (YEME), the level of sodF transcripts decreased about 30-fold within 1 h (Fig. 6A, lane 2).

A similar reduction in sodF mRNA induced by Ni was observed when RNAs from cells grown in Ni-rich YEMES and Ni-deficient YEME media were analyzed (Fig. 6A, lanes 3 and 4). This finding confirms the previous observation by Kim et al. (22) of the reduced synthesis of FeZnSOD in YEMES media and demonstrates that the regulation is at the level of transcripts. When the S1 probe labeled at the 3’ end of SalI site was used, a protected fragment of 190 nucleotides was detected (Fig. 6B, lane 1). This allows the localization of the transcription termination site of the sodF gene to 90 nucleotides downstream from the translation stop codon, suggesting that sodF mRNA is monocistronic. The same extent and kinetics of reduction in transcripts (data not shown).

To test the specificity of Ni in this regulation, we examined the effects of other metals, including Cd, Cu, and Fe. Figure 6C represents an S1 mapping analysis of sodF mRNA from cells treated with 0.1 to 100 µM NiCl2 or 100 µM (each) CdCl2, CuCl2, or FeCl3. The results demonstrate that Ni is effective even at 1 µM, reducing the level of sodF mRNA more than 10-fold (Fig. 6C; compare lanes 1, 2, and 3). On the other hand, Cd, Cu, and Fe had no effect in regulating the level of sodF mRNA (lanes 5 to 7).

We tested whether Ni changes the stability of mRNA by S1 mapping the 3’ end of the sodF mRNA obtained at various time intervals after treatment of *S. coelicolor* Müller cells with rifampin (300 µg ml⁻¹). The result demonstrated that the half-life of sodF mRNA was about 18 min and was not affected by Ni (data not shown). This result clearly indicates that Ni specifically represses the expression of the sodF gene at the transcriptional level.

**Role of FeZnSOD in protecting *S. lividans* cells against superoxide-generating oxidants.** We examined the effect of overproducing or eliminating FeZnSOD on the survival of *S. lividans* cells upon treatment with various oxidants known to generate superoxide radicals. When spores from cells containing either pIJ702 or pIJSODF were plated on NA medium containing various concentrations of menadione or lawsone, we observed that FeZnSOD-overproducing cells survived better than the control cells (Fig. 7), suggesting that FeZnSOD plays a protective role against these oxidants. The effect of FeZnSOD overproduction was less pronounced when cell survival was tested against plumbagin (data not shown). On the contrary, the sodF disruptant survived as well as the wild type in the presence of these oxidants (data not shown). This result was not unexpected, since sodF mutant cells produced more NiSOD than the wild type. It is most likely that the increased production of NiSOD compensated for the loss of FeZnSOD in protecting cells against superoxide-generating oxidants.

**DISCUSSION**

Most bacterial cytosolic SODs are Fe- and MnSODs which usually contain 0.5 to 1.0 g-atom of either Fe or Mn per monomorphic subunit (18). However, several SODs are known to contain both Fe and Zn. They all exist as tetramers and include SODs from *Methanobacterium bryantii* (26), *Nocardia asteroides*, which also contains Mn (3), *Thermoplasma acidophilum* (36), and *Streptomyces* spp. (22, 47). FeZnSOD from *S. coelicolor* has been reported to exhibit typical FeSOD-like characteristics, judged from both absorption spectra and sensitivity to inhibitors (21, 22). The primary sequence of the SodF polypeptide reveals the highest similarity to the cambialistic SOD from *P. shermanii* and both Fe- and MnSODs from *Mycobacterium* spp. It has been suggested that one of the ligands for the metal cofactor can influence the metal specificity of either Mn- or FeSOD. X-ray crystallographic structure of FeSOD from My-
cobacterium tuberculosis showed that the fifth ligand of iron, a hydroxide ion, interacts with His145, which is replaced with glutamine in Mycobacterium leprae MnSOD (9). In FeZnSOD of S. coelicolor Müller, histidine was found at the corresponding position, consistent with the presence of iron as the cofactor.

Expression of the two SODs in S. coelicolor has been demonstrated to be affected dramatically by the presence of Ni and metal chelators (21, 22). Ni increases and decreases the synthesis of NiSOD and FeZnSOD, respectively. The regulatory role of Ni is on the transcriptional level for both NiSOD (23) and FeZnSOD as demonstrated in this study. So far, Ni-dependent transcriptional regulation has been reported only for the hydrogenase gene expression in Bradyrhizobium japonicum (24, 25). The transcriptional regulator which responds to Ni has not yet been identified. Ni repression of the cloned sodF gene on a multicopy plasmid suggests the presence of a cis-acting regulatory site within the 1.3-kb cloned fragment. The localization of the cis-acting element is expected to allow identification of the Ni-responsive factor. Other than Ni, the depletion of Fe by chelator desferrioxamine decreases the expression of FeZnSOD (21). This finding is consistent with the observation that the sodB gene for FeSOD in E. coli is positively regulated by Fur. Since a Fur-like regulator has been recently identified in S. coelicolor (12), regulation of the sodF gene by Fe needs to be investigated further in this respect.

Expression of SODs in S. coelicolor increases only slightly (less than twofold) upon treatment with superoxide-generating oxidants such as paraquat, plumbagin, and menadione (21) and also increases about twofold in the stationary phase. This contrasts with the expression of catalase, another oxidative defense enzyme, a subset of whose isozymes increases significantly as cells enter the stationary phase, differentiate, or are treated with oxidants such as H$_2$O$_2$ (7, 27). Our observation suggests that the amount of SODs is rather strictly controlled in S. coelicolor, in contrast with catalases. The compensating Ni-regulated expression of NiSOD and FeZnSOD as well as the increased expression of NiSOD in a sodF disruptant support this idea. The compensating expression of the two SODs also suggests that the two enzymes have some roles in common, at least for protecting cells against oxidants as demonstrated in the sodF disruptant. The elucidation of the specific role of each SOD requires further investigation of SOD mutants under various environments.

**ACKNOWLEDGMENTS**

This work was supported by a grant from the Korea Science and Engineering Foundation to the Research Center for Microbiology, Seoul National University. E.-J. Kim was the recipient of the postdoctoral fellowship from the Research Institute of Basic Sciences, Seoul National University.

We thank J.-S. Choi for expert assistance in preparing antibodies.

**REFERENCES**


