Original

Identification of a gene coding for manganese superoxide dismutase from the cellular slime mold *Dictyostelium discoideum*

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SUMMARY

The soil-dwelling social amoeba *Dictyostelium discoideum* is known to be unusually resistant to oxidative stress. However, little is known about how *D. discoideum* cells protect themselves from the stress. We found a gene coding for manganese superoxide dismutase (MnSOD) in the database of *D. discoideum*. The gene, designated *sodE*, encodes a polypeptide 37% identical to *Escherichia coli* MnSOD. A plasmid expressing the truncated form of SodE, pSOD5, was constructed to determine whether SodE suppressed *E. coli* JI132 (*sodA sodB*). Experimental results showed that JI132 transformed by pSOD5 was more tolerant of menadione than was JI132 carrying a control vector and that the mutation rate of JI132 carrying pSOD5 was lower than that of JI132 harboring the control vector.

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INTRODUCTION

Reactive oxygen species (ROS) are significant source of cellular and DNA damage. Organisms express various enzymes, such as superoxide dismutases (SODs), that detoxify ROS in order to protect themselves. SODs convert superoxide anions to H_2O_2, which is then disproportionated to water by catalases or peroxidases. SODs are divided into four subfamilies according to cofactors: copper-/zinc-containing SOD (Cu/ZnSOD), manganese-containing SOD (MnSOD), iron-containing SOD (FeSOD) and nickel-containing SOD (NiSOD) (Fridovich, 1995; Miller, 2004). Because of their important role, SODs are conserved widely in prokaryotes and eukaryotes.

The social amoeba *Dictyostelium discoideum*, which feeds on bacteria in soil, is highly resistant to oxidative stress (Garcia et al., 2000). Thus, *D. discoideum* is thought to be a system suitable for studying mechanisms that function in protection of organisms from ROS. Four Cu/ZnSODs have been identified from *D. discoideum*. They were designated SodA (Garcia et al., 2000), SodB (Tsuji et al., 2002), SodC (Tsuji et al., 2003) and SodD (Akaza et al., 2002). In addition, four proteins that are predicted to be SODs can be found in *D. dis-*

coideum databases.

We describe here the results of analysis of one of latter four SODs. The amino acid sequence of the protein, SodE, is similar to that of MnSODs. A truncated form of SodE partially suppressed *Escherichia coli* (*sodA sodB*).

MATERIALS AND METHODS

Sequence analysis

The genomic sequence of the gene for SodE was found in DictyBase, an online database for *D. discoideum* (http://dictybase.org). The ID number for the gene is DDB0202901. The nucleotide sequence of the cDNA was found in a *Dictyostelium* cDNA database (http://dictycdb.biol.tsukuba.ac.jp). The clone number of the cDNA is SSA566. The program used in the analysis of amino acid sequences was DDBJ ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html).

Strains

The *E. coli* strains used in this study were JI132 (*sodA sodB*) and its parental strain AB1157 (Imlay and Linn, 1987). *D. discoideum* used was axenic strain Ax2. Ax2 cells were maintained in an HL5 liquid medium (Ashworth and Watts, 1970; Watts and Ashworth, 1970).

Menadione treatment of *D. discoideum*

Ax2 cells were grown in an HL5 medium until cell density reached 1 - 2 x 10^6 cells /ml. The cells were collected by centrifugation, washed with a buffer (1.66 g NaH_2PO_4 in 1 L H_2O, pH adjusted to 6.1 with KOH) and suspended in the buffer at a density of 1 x 10^6 cells /ml. The cells were then treated with 1 - 15 mM menadione for 1 h, washed with the buffer, and incubated with a fresh HL5 medium for 20 h. Then the dye phloxine B was added to the cell culture at a final concentration of 0.01%. Stained cells (dead cells) and unstained cells were counted under a microscope to determine cell viability. For gene expression analysis, cells were exposed to 5 mM menadione for 1 h and harvested for RNA preparation.

H_2O_2 treatment of *D. discoideum*

Ax2 cells were grown in an HL5 medium until cell density reached 1 - 2 x 10^6 cells /ml. The cells were collected by centrifugation, washed, and suspended in the buffer at a density of 1 x 10^6 cells / ml. The cells were then treated with 0.5 mM H_2O_2 for 1 h and subjected to RNA purification.

RT-PCR

Changes in gene expression following the treatment were analyzed by RT-PCR. A One Step RT-PCR Kit (Takara Shuzo, Kyoto, Japan) was used. Total RNA samples extracted from Ax2 cells were used as templates. Amplification of *rnlA* (Ogawa et al., 2000) and *actin15* (Knecht et al., 1986) was conducted as an experimental control. Primers used were 5'-GGGTCACAATCAAATTCATATACTCTTCCAGATTTACC (forward primer for *sodE*), 5'- CCTAACCAACCCCAACCAGAACCTTGAATTGCAGTAG (reverse primer for *sodE*), 5'-TTACATTTATTAGACCCGAAACCAAGCG (forward primer for *rnlA*), 5'-TTCCCTTTAGACCTATGGACCTTAGCG (reverse primer for *rnlA*), 5'-ATGGATGGTGAAGATGTTCAAGC (forward primer for *actin15*) and 5'-TCTGTGGACAATTGATGGACCAG (reverse primer for *actin15*).

Plasmids used in the assays

A plasmid carrying SodE cDNA was provided by *Dictyostelium* cDNA Project Japan. The plasmid pSOD5 was constructed from an *Nde*I-*Not*I fragment of the cDNA clone, an *Nde*I-*Not*I fragment of pYSN1 (Hasegawa et al., 2004) and a linker prepared from oligonucleotides (5'-TAATGTCATATACTCTTCCGGATTTACCG and 5'-TACGGTAAATCCGGAAGAGTATATG-

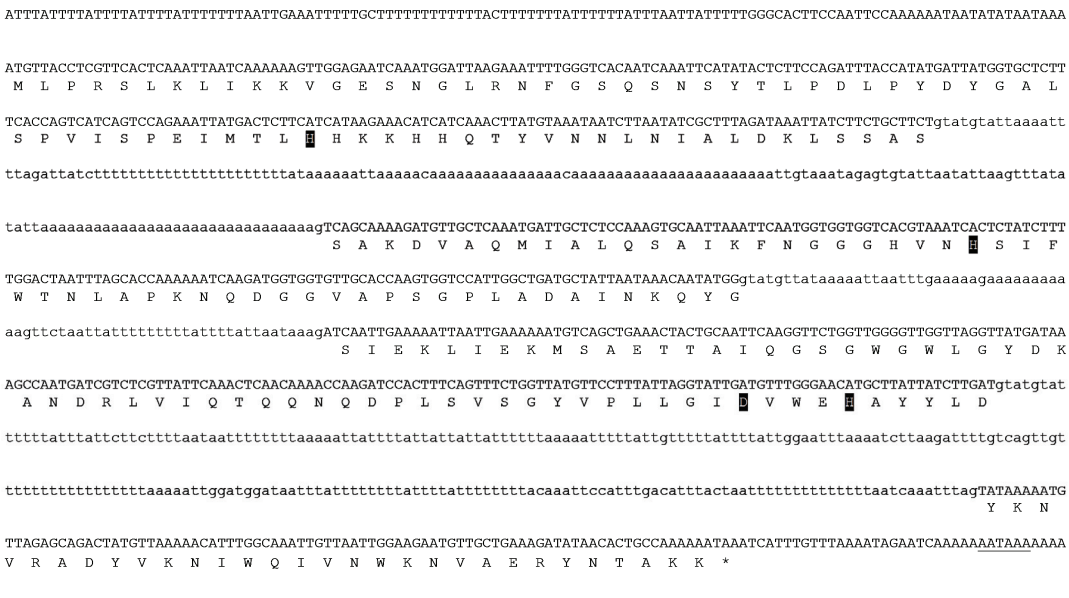

Fig. 1. Sequence analysis of SodE. Nucleotide sequences of the genomic DNA and cDNA were found in the DictyBase and cDNA database, respectively. Residues that function as ligands for the metal ion are shown in white letters. Consensus poly(A) signals are underlined.

ACAT). This plasmid was designed to express a truncated form of SodE in which Leu-2 to Asn-26 were deleted under the control of the *lac* promoter. A plasmid pUC18 was used as control.

Susceptibility tests

Disc assay: Test cells were spread on an LB agar plate and then a paper disc loaded with 10 µl of 1 M menadione was placed on the center of the plate. After 24-h incubation for JI132(pUC18) and 16-h incubation for other transformants, diameters of the inhibition zones formed on the bacterial lawns were measured to determine the sensitivity of the test cells to the drug. Minimum inhibitory concentration (MIC) assay: Menadione was incorporated into LB in serial two-fold concentrations from 1 to 4096 mg/l. The inoculated tubes were incubated at 37 degrees C. The MIC of the agent was defined as the lowest concentration that inhibited visible growth of the bacteria.

Mutation rate of *E. coli*

Cultures of *E. coli* cells were spread on an LB plate containing 100 mg/l rifampicin and incubated at 37 degrees C. The titer of rifampicin-resistant colonies was determined after 40-h incubation for JI132(pUC18) and 16-h incubation for other transformants. The same cultures were diluted and plated in the absence of rifampicin and tittered after the incubation.

RESULTS

Sequence analysis of SodE

The gene *sodE* coding for SodE is mapped on chromosome 2. A comparison of the nucleotide sequences of the genomic DNA and cDNA revealed that the open reading frame is interrupted by three introns (Fig. 1). SodE consists of 226 amino acid residues.

Alignment showed that the amino acid sequence

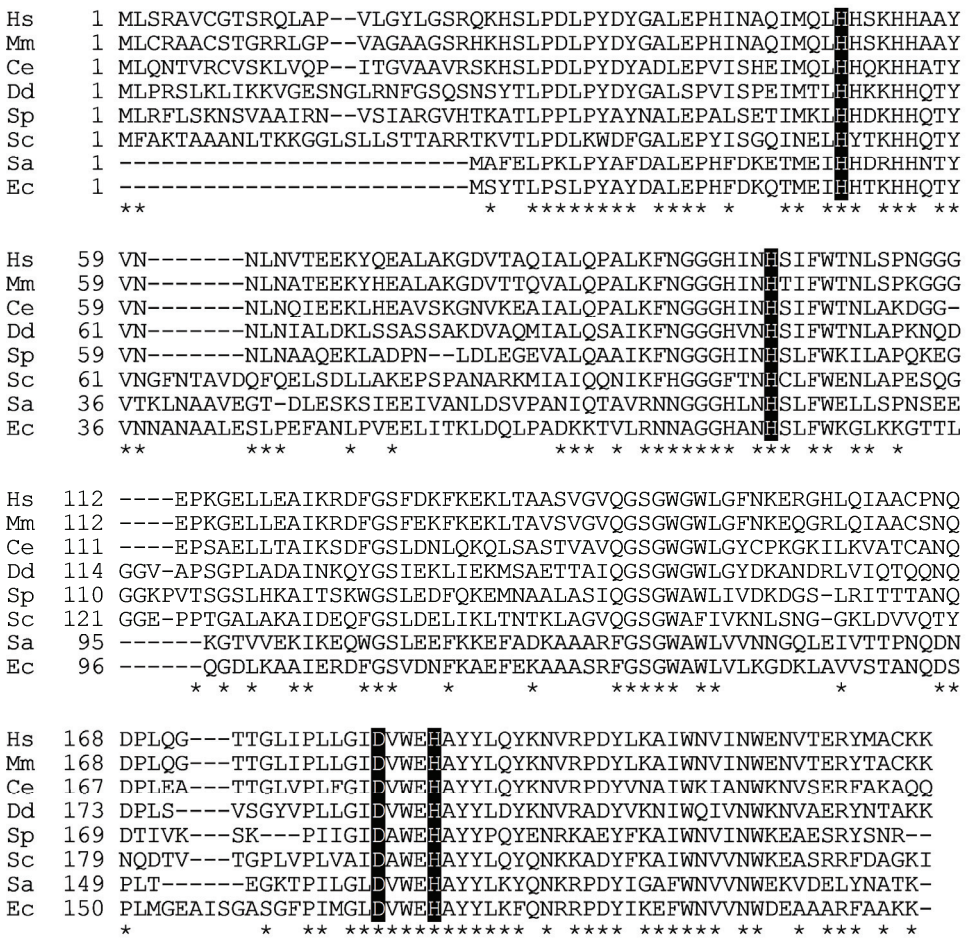

Fig. 2. Alignment of the MnSod proteins. *D. discoideum* SodE is presented as Dd. Residues conserved in 5 or more species are indicated by asterisks. Residues coordinating the metal center are shown in white letters. Accession numbers are AAH12423 (*H. sapiens* presented as Hs), P09671 (*M. musculus* presented as Mm), BAA12821 (*C. elegans* presented as Ce), Q9UQX0 (*S. pombe* presented as Sp), CAA26092 (*S. cerevisiae* presented as Sc), AAD17309 (*S. aureus* presented as Sa) and AAB03041 (*E. coli* presented as Ec).

is highly conserved in MnSODs (Fig. 2). The identity of the amino acid sequence of SodE to that of the homologs are 52% (*Homo sapiens*), 53% (*Mus musculus*), 54% (*Caenorhabditis elegans*), 47% (*Schizosaccharomyces pombe*), 41% (*Saccharomyces cerevisiae*), 41% (*Staphylococcus aureus*) and 37% (*E. coli*). Three histidines and one aspartate that function as ligands for the metal ion are conserved (Edwards et al., 1998).

Response of the gene to oxidative stress

We determined viability of *D. discoideum* cells treated with menadione sodium bisulfite (water-soluble menadione), a superoxide-generating reagent, as described in Materials and Methods. We found that the treatment of 5 mM menadione was sublethal for *D. discoideum*, resulting in 95% viability after 1 h (Fig. 3A).

D. discoideum cells were exposed to 5 mM menadione for 1 h and subjected to RT-PCR analysis.

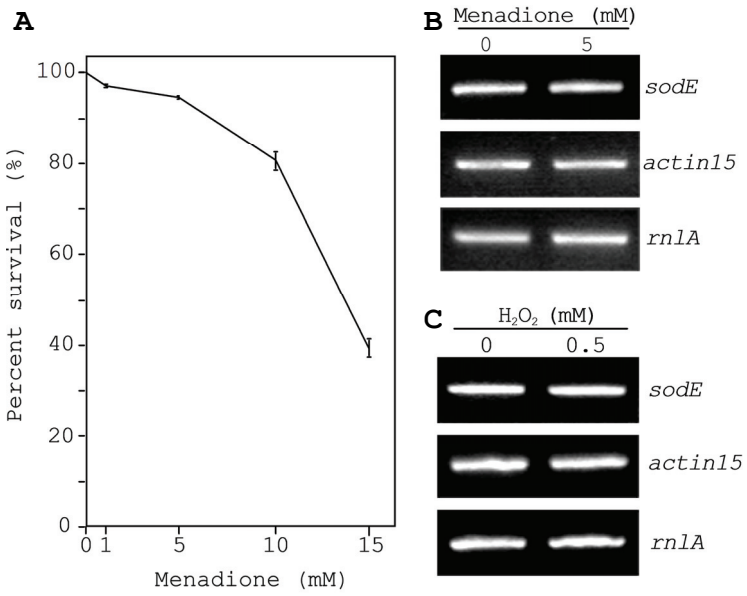

Fig. 3. Responses of the gene to menadione and H_2O_2. (A) Viability of *D. discoideum* Ax2 following treatment with menadione. (B) Response of *sodE* to menadione. Ax2 cells were exposed to 5 mM menadione for 1 h and subjected to RNA preparation for RT-PCR analysis. Reaction products amplified 14 cycles (*sodE*), 16 cycles (*actin15*) and 14 cycles (*rnlA*) are shown. (C) Response of *sodE* to H_2O_2. Cells were exposed to 0.5 mM H_2O_2 for 1 h and subjected to RNA preparation. Expression levels were analyzed by RT-PCR. Reaction products amplified 20 cycles (*sodE*), 16 cycles (*actin15*) and 14 cycles (*rnlA*) are shown.

RT-PCR products amplified 14 - 24 cycles after reverse transcription were resolved by gel electrophoresis, and their signal intensities were analyzed. RT-PCR products of *rnlA* and *actin15* are shown as controls. No significant difference was found between the expression levels of *sodE* in exposed cells and control cells (Fig. 3B). An experiment was also carried out to determine whether the expression level is changed by treatment with 0.5 mM H_2O_2 for 1 h, a sublethal condition for *D. discoideum* (Garcia et al., 2000). The expression level following the treatment was almost the same as that of the control cells (Fig. 3C).

These results demonstrate that *sodE* is not induced by oxidative stress.

Suppression of *sod* mutant of *E. coli*

We tested whether SodE suppresses an *sod* mutant of *E. coli*. For the assay, pSOD5 was constructed as described in Materials and Methods. This plasmid expressed SodE in which Leu-2 to Asn-26 were deleted under the control of the *lac* promoter (Fig. 4).

Sensitivity of *E. coli* cells to menadione was determined by comparing the sizes of inhibition zones as described in Materials and Methods. Diameters of the inhibition zones formed on lawns of JI132(pSOD5) were smaller than those formed on lawns of JI132(pUC18) (Fig. 5).

We also determined MIC of menadione for the test cells by the two-fold dilution method. MIC values observed in AB1157(pUC18) and JI132 (pUC18) were 2048 mg/l and 64 mg/l, respectively, while MIC of the agent for JI132(pSOD5) was 512 mg/l. These results indicate that JI132 expressing SodE was more tolerant of menadione than was JI132 carrying a control vector.

We next determined the rate of mutation to rifampicin resistance of *E. coli* cells as described in Materials and Methods. We found that the mutation rate of JI132(pUC18) was over 30-times higher than that of AB1157(pUC18), while the mutation rate of JI132(pSOD5) was over 3-times higher than that of AB1157(pUC18) (Table 1). These results suggest that SodE contributes to the protection of DNA from ROS.

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              -35                      -10       +1
---CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCA

    SD
CACAGGAGAAACATAATGTCATATACTCTTCCGGATTTACCGTATGATTAT-----GCCAAAAAATAA---
                M  S  Y  T  L  P  D  L  P  Y  D  Y       A  K  K  *
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Fig. 4. Sequence of the truncated *sodE* and its upstream region on pSOD5. DNA coding for the truncated form of SodE was placed downstream of the *lac* promoter. The promoter (–35 and –10), transcription initiation site (+1) and SD are indicated. The linker is boxed.

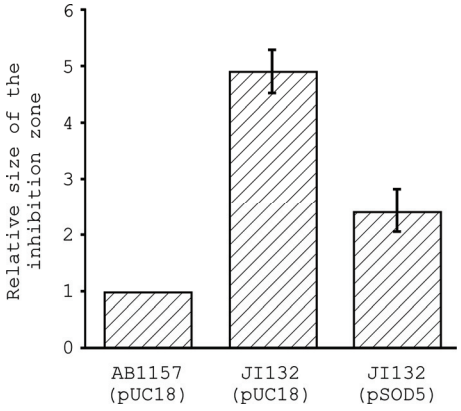

Fig. 5. Sensitivity of *E. coli* to menadione. Relative sizes of the inhibition zones are shown. Four sets of experiments were carried out.

Table 1. Rate of mutation to rifampicin resistance. Three sets of experiments were carried out.

Cells tested	Rate of mutation to rifampicin resistance (mutants/10^8 cells)
AB1157 (pUC18)	0.57 ± 0.22
JI132 (pUC18)	20.19 ± 6.46
JI132 (pSOD5)	1.94 ± 0.95

DISCUSSION

Alignment showed high degree of similarity between SodE and MnSODs. The experimental results presented in this report indicate that SodE functioned as an SOD in *E. coli*. We concluded from these results that SodE is MnSOD of *D. discoideum*.

MnSOD is conserved both in prokaryotes and eukaryotes. SodE and other eukaryotic proteins carry a long N-terminal extension that is not observed in bacterial SODs. The N-terminal region in eukaryotic MnSODs is thought to contain the mitochondria targeting signal. Thus, SodE would localize in mitochondria.

ROS are produced endogenously as by-products of normal aerobic metabolism. The mitochondrial electron transport chain is the most prolific source of cellular ROS. Therefore, MnSOD that localizes in mitochondria would play an essential role in the protection of mitochondrial and cellular components against ROS, although it is not induced by oxidation stress. Further experiments to determine the importance of SodE in *D. discoideum* are in progress.

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REFERENCES

Akaza, Y., Tsuji, A. and Yasukawa, H. (2002) Analysis of the gene encoding copper/zinc superoxide dismutase homolog in *Dictyostelium discoideum*. Biol. Pharm. Bull., 25, 1528-1532.

Ashworth, J. M. and Watts, D. J. (1970) Metabolism of the cellular slime mould *Dictyostelium discoideum* grown in axenic culture. Biochem. J., 119, 175-182.

Edwards, R. A., Baker, H. M., Jameson, G. B., Whittaker, M. M., Whittaker, J. W. and Baker, E. N. (1998) Crystal structure of *Escherichia coli* manganese superoxide dismutase at 2.1-Å resolution. J. Biol. Inorg. Chem., 3, 161-171.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., 64, 97-112.

Garcia, M. X., Foote, C., van Es, S., Devreotes, P. N., Alexander, S. and Alexander, H. (2000) Differential developmental expression and cell type specificity of *Dictyostelium* catalases and their response to oxidative stress and UV-light. Biochim. Biophys. Acta, 1492, 295-310.

Hasegawa, Y., Wakabayashi, M., Nakamura, S., Kodaira, K., Shinohara, H. and Yasukawa, H. (2004) A homolog of *Escheriachia coli* RecA in mitochondria of the cellular slime mold *Dictyostelium discoideum*. DNA Repair, 3, 515-525.

Imlay, J. A. and Linn, S. (1987) Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol., 169, 2967-2976.

Knecht, D. A., Cohen, S. M., Loomis, W. F. and Lodish, H. F. (1986) Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high-copy transformation vectors. Mol. Cell. Biol., 6, 3973-3983.

Miller, A. F. (2004) Superoxide dismutases: active sites that save, but a protein that kills. Curr. Opin. Chem. Biol., 8, 162-168.

Ogawa, S., Yoshino, R., Angata, K., Iwamoto, M., Pi, M., Kuroe, K., Matsuno, K., Morio, T., Urushihara, H., Yanagisawa, K. and Tanaka, Y. (2000) The mitochondrial DNA of *Dictyostelium discoideum*: complete sequence, gene content and genome organization. Mol. Gen. Genet., 263, 514-519.

Tsuji, A., Akaza, Y., Kodaira, K. and Yasukawa, H. (2002) Copper/zinc superoxide dismutases in *Dictyostelium discoideum*: amino acid sequences and expression kinetics. J. Biochem. Mol. Biol. Biophys., 6, 215-220.

Tsuji, A., Akaza, Y., Nakamura, S., Kodaira, K. and Yasukawa, H. (2003) Multinucleation of the *sodC*-deficient *Dictyostelium discoideum*. Biol. Pharm. Bull., 26, 1174-1177.

Watts, D. J. and Ashworth, J. M. (1970) Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J., 119, 171-174.