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Growth and development of *Dictyostelium discoideum* overexpressing manganese superoxide dismutase

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SUMMARY

Dictyostelium discoideum, a soil-dwelling eukaryotic microorganism, encodes a manganese superoxide dismutase named SodM (formerly called SodE). Fluorescence of SodM-GFP fusion protein was detected in mitochondria of *D. discoideum*, indicating that SodM is a mitochondria-localizing superoxide dismutase. Expression of *sodM* was observed throughout the life cycle. Maximum level of gene expression was observed at the aggregation stage. An *sodM*-overexpressing *D. discoideum*, *sodM*^{oe}, grew faster and reached higher cell density in a liquid medium than did the wild type. Multicellular structures formed by *sodM*^{oe} on a non-nutrient agar were larger in size than those formed by the wild type. These results indicate that SodM plays important roles both in growth and development of *D. discoideum*.

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INTRODUCTION

Oxidative stress resulting from the generation of reactive oxygen species (ROS) is a significant source of cellular damage. ROS are produced endogenously as by-products of normal aerobic metabolism. Mitochondrial electron transport chain is the most prolific source of cellular ROS (Halliwell and Gutteridge, 1989). To detoxify ROS, organisms express various enzymes such as superoxide dismutase (SOD), catalase and peroxidase. SOD converts superoxide anions to H_2O_2, which is then disproportionated to water by catalase or peroxidase.

Eukaryotes generally contain manganese-containing SOD (MnSOD) in mitochondria. This enzyme is thought to play an important role in survival by protecting cellular components from ROS. For instance, the growth of a *Saccharomyces cerevisiae* mutant in which the gene for mitochondrial MnSOD was disrupted was inhibited in an aerobic condition although this mutant grew as rapidly as the parental strain in the absence of oxygen (Van Loon et al., 1986).

On the other hand, a MnSOD-overexpressing

S. cerevisiae showed an increased chronological life span, as assayed by the ability of stationary (G0-arrested) cultures to maintain viability over time (Harris et al., 2003). However, the MnSOD-overexpressing yeast showed a shorter replicative life span, as assayed by the number of daughters produced by each actively dividing mother yeast. The reduction in replicative life span is thought to result from a defect in segregation of mitochondria from mother to daughter. These results indicated that overexpression of MnSOD affected growth of *S. cerevisiae*.

The effect of overexpression of MnSOD on cell growth has been analyzed not only in the lower eukaryote but also in mammalian cells. Inhibition of cell growth resulting from overexpression of MnSOD was observed in rat glioma cells (Zhong et al., 1996) and NIH/3T3 cells (Li et al., 1998). Several studies have demonstrated that overexpression of MnSOD by transfection of MnSOD cDNA suppresses the malignant phenotype and metastasis of cancer cells (Church et al., 1993; Safford et al., 1994; Li et al., 1995). In addition, promotion of cell differentiation as a result of overexpression of MnSOD has been reported (St Clair et al., 1994). These observations demonstrate that mitochondrial MnSOD functions both in growth and differentiation of eukaryotic cells.

The cellular slime mold *Dictyostelium discoideum* is a suitable model for studying molecular mechanisms controlling growth and differentiation. *D. discoideum* is a unicellular organism in the presence of nutrients but initiates a developmental process to form a multicellular structure under a starved condition. *D. discoideum* cells aggregate into groups of 10^5 cells. Each cell group organizes into a structure called a slug that consists of prestalk cells (cells that will make up the stalk) and prespore cells (cells that will differentiate into spores). Then the slug changes its form to a fruiting body consisting of stalk cells and spores (Loomis, 1975). *D. discoideum* completes its developmental process within 24 h under a starved condition.

D. discoideum encodes an MnSOD named SodM (formerly called SodE; Akaza et al., 2006). A previous study has shown that *sod*-deficient *Escherichia coli* expressing SodM exhibited increased survival after treatment with a superoxide-generating agent and that the transformed bacteria had decreased frequency of mutation.

The cellular localization of SodM, the developmental expression pattern of the gene for the protein, *sodM*, and the characterization of an *sodM*-overexpressing strain of *D. discoideum*, *sodM* ^{oe}, are described in this report.

MATERIALS AND METHODS

Strains and culture condition

D. discoideum Ax2 was used as a parental strain in this study. Ax2 and a transformant derived from Ax2 were cultured in an HL5 liquid medium (Watts and Ashworth, 1970) and HL5 supplemented with 5 mg/l blasticidin S (BS), respectively. For plaque formation, cells were spread on a nutrient agar plate with *E. coli* WA802 (*metB*1 *lac3 lacY1 galK*2 *galT*22 *supE hsdR*2). For development, cells were harvested, washed with an Na/K buffer (1.66 g NaH_2PO_4 in 1 L H_2O, pH adjusted to 6.1 with KOH), plated on non-nutrient agar at a density of 1.0×10^6 cells/cm^2, and incubated. Cell culture and incubation were carried out at 22 °C.

RT-PCR and primers

RT-PCR was carried out by the use of a One-Step RT-PCR Kit (Takara Bio, Shiga, Japan). The reaction conditions used were in accordance with the instruction manual. The primers used to amplify *sodM* mRNA and *rnlA* (Ogawa et al., 2000), an internal marker, were described in a previous report (Akaza et al., 2006). DNA fragments were amplified by 14 to 24 cycles (*sodM*) or 6 to 10 cycles (*rnlA*) of PCR following reverse transcrip-

tion, and signal intensities were analyzed before they were saturated.

Plasmids

A plasmid expressing a GFP fusion protein was constructed by the following procedure. A *Sal*I - *Pvu*II fragment of pSSA566, a cDNA clone of SodM, was inserted into *Sal*I - *Sma*I of pEGFP-N1 (Clontech, CA, USA) to fuse genes encoding a part of SodM (Met^{1} – Ser^{141}) and GFP. Then the fusion gene was placed downstream of the *actin15* promoter on pTIKL-Bsr-Exp, an *E. coli – D. discoideum* shuttle vector carrying a gene cassette for BS resistance (Larochelle et al., 1997). A plasmid for *sodE* overexpression was constructed by placing the full length of the SodE cDNA downstream of the *actin15* promoter on pTIKL-Bsr-Exp.

Others

Restriction enzymes, calf intestine alkaline phosphatase and T4 DNA ligase were purchased from Toyobo (Osaka, Japan). MitoRed, a dye for mitochondria staining, was purchased from Dojindo (Kumamoto, Japan).

RESULTS

Mitochondria localization of SodM

SodM carries a long N-terminal extension that is not observed in bacterial MnSODs (Akaza et al., 2006). This region is thought to contain the cellular localization signal. To determine cellular localization of SodM, *D. discoideum* Ax2 was transformed by a plasmid carrying the gene for SodM (Met^{1} – Ser^{141})-GFP fusion protein. Ax2 cells harboring the plasmid were maintained in an HL5 medium supplemented with BS and subjected to microscopic analysis. Prior to the analysis, the cells were treated with MitoRed to stain mitochondria. *D. discoideum* carries 200-250 copies of mitochondrial DNA (Fukuhara, 1982), suggesting that each amoeba contains 250 or less mitochondria. The microscopic analysis showed fluorescence of GFP in mitochondria, indicating that SodM is a mitochondria localizing protein (Fig. 1).

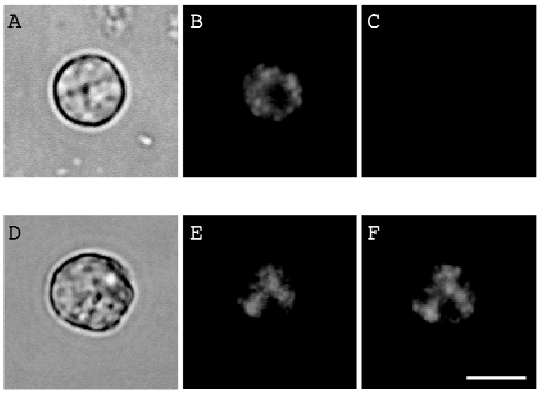

Fig. 1. Distribution of SodM-GFP in *D. discoideum* cells. Ax2 (A - C) and Ax2 expressing SodM-GFP fusion protein (D - F) were photographed under a microscope. A and D: Visible light. B and E: Fluorescence of MitoRed. C and F: Fluorescence of GFP. The bar indicates 10 µm.

Developmental expression pattern

D. discoideum is a unicellular organism in the presence of nutrients but initiates a developmental process to form a multicellular structure under a starved condition. Expression levels of *sodM* during the life cycle of *D. discoideum* were analyzed by RT-PCR. Amplification of *rnlA* was conducted as a control. Expression of the gene was observed both in growing cells and developing cells. The maximum level of expression was observed at the aggregation stage (Fig. 2).

Establishment of *sodM*-overexpressing strain

A plasmid carrying the full length of *sodM* under the *actin15* promoter was constructed to overexpress SodM in *D. discoideum.* Ax2 was transformed by the plasmid and incubated in an HL5 medium supplemented with BS for selection. A BS-resistant clone was isolated from the culture and subjected to assays. Expression levels of *sodM* in the Ax2 and the BS-resistant strain were analyzed by RT-PCR. The reaction products were

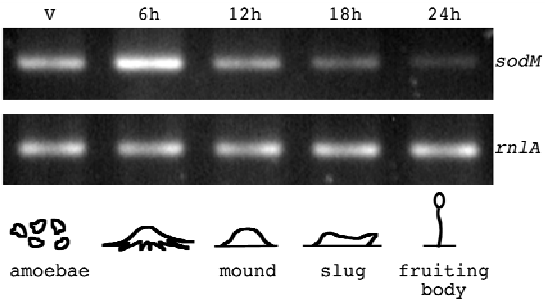

Fig. 2. Developmental expression pattern of *sodM*. Change in gene expression was analyzed by RT-PCR. Total RNA samples extracted from the cells in the growth phase (v) and developmental phases (6 – 24 h) were used as templates. Amplification of *rnlA* was performed as a control. Reaction products amplified by 20 cycles (*sodM*) or 7 cycles (*rnlA*) after reverse transcription are shown. Illustration showing developmental process of *D. discoideum* is presented at the bottom.

electrophoresed and their signal intensities were analyzed. The level of gene expression in the BS-resistant strain was approximately 3-fold higher than that in the wild type (Fig. 3). Almost the same results were obtained from experiments conducted two times. These results indicate that the strain established in this work is an *sodM*-overexpressing strain (*sodM*^{oe}).

Cell growth of *sodM*^{oe}

Experiments were carried out to determine whether overexpression of SodM affects cell growth. Ax2 and *sodM*^{oe} were inoculated into an HL5 liquid medium and an HL5 supplemented with BS, respectively, and growth of the cells was monitored. The results showed that *sodM*^{oe} grew faster than did the wild type in the liquid medium. In addition, *sodM*^{oe} reached higher cell density in the medium than did the wild type (Fig. 4A).

Growth of Ax2 and that of *sodM*^{oe} on a bacterial lawn were also analyzed. *D. discoideum* amoebae were spread on an agar plate with bacteria and incubated. The predatory amoebae grew and formed plaques on the bacterial lawn (Fig. 4B). Plaques formed by *sodM*^{oe} were larger than those formed by Ax2, indicating that *sodM*^{oe} grew faster than the wild type on the bacterial lawn. These results indicate that overexpression of SodM promotes cell growth.

Multicellularity and development of *sodM*^{oe}

Development of *sodM*^{oe} was compared with that of Ax2. Amoebae grown in an HL5 liquid medium were collected, washed, plated on a non-nutrient agar, and incubated. In this experiment, amoebae were plated at almost the same number and same cell density. Mounds formed on the plate 12 h after plating were photographed (Fig. 5A). As shown in the figure, mounds formed by *sodM*^{oe} were larger than those formed by Ax2, whereas the number of mounds formed by *sodM*^{oe} was smaller than the number of mounds formed by Ax2.

At 24 h, fruiting bodies were observed on the same plate (Fig. 5B). The height of the fruiting bodies (*h* in Fig. 5B) formed by Ax2 and that formed by *sodM*^{oe} were 576.9 +/- 112.7 and 708.0 +/- 107.1 μm, respectively. The values are means +/- S.D. from 37 fruiting bodies formed by Ax2 and 35 fruiting bodies formed by *sodM*^{oe}. Fruiting bodies formed by *sodM*^{oe} were taller than those formed by Ax2 ($p < 0.01$). The sizes of sporangia (*l* in Fig. 5B) of the same fruiting bodies were also measured. The sizes of sporangia formed by Ax2 and *sodM*^{oe} were 72.2 +/- 15.4 and 89.8 +/- 20.6 μm, respectively. There was a significant difference between sizes of sporangia formed by the two strains ($p < 0.01$).

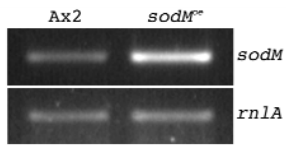

Fig. 3. Establishment of *D. discoideum sodM*^{oe} strain. Expression levels of *sodM* in *D. discoideum* cells were analyzed by RT-PCR. Total RNA samples purified from the amoebae were used as templates for the reaction. Reaction products amplified by 20 cycles (*sodM*) or 7 cycles (*rnlA*) after reverse transcription are shown.

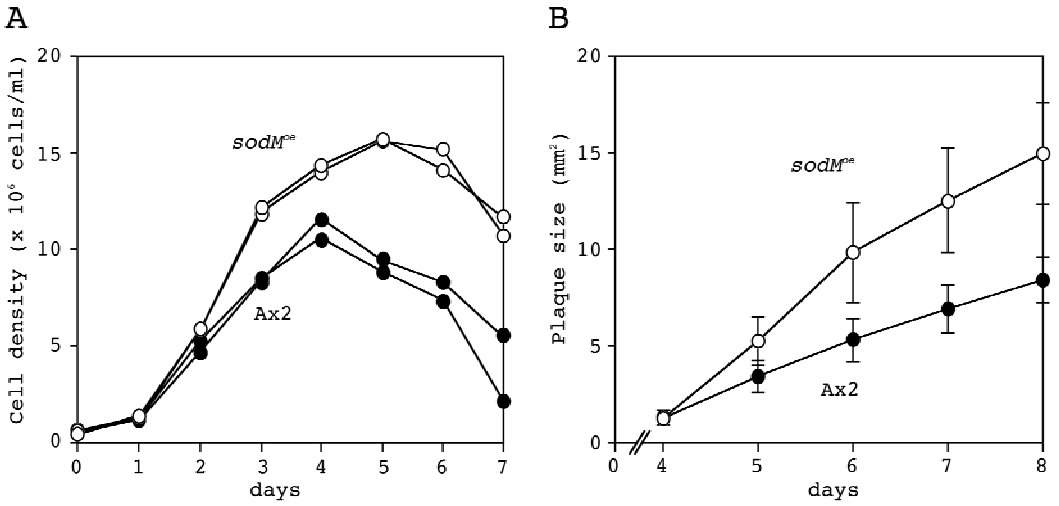

Fig. 4. Growth of *sodM*^{oe}. (A) Growth of *D. discoideum* amoebae in an HL5 medium. Amoebae were grown in the liquid medium at 22 °C, sampled at 24-h intervals, and counted under a microscope. Two sets of experiments were carried out. (B) Growth of amoebae on a bacterial lawn. *D. discoideum* amoebae were spread on agar plates with *E. coli* and incubated at 22 °C. Plaques were photographed and their sizes were measured. The values are means +/- S.D. from 10 plaques.

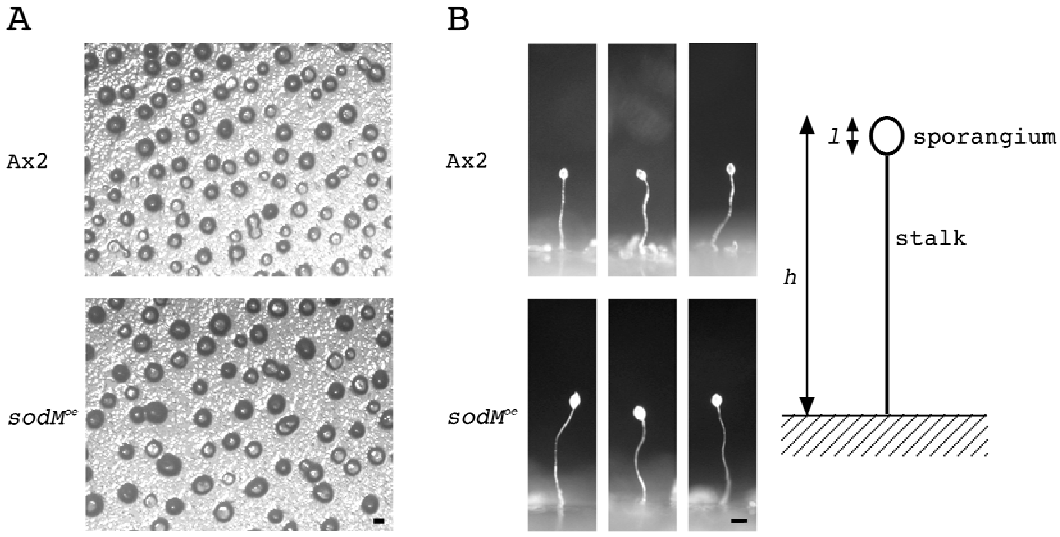

Fig. 5. Multicellular structures formed by *sodM*^{oe}. (A) Mounds formed by Ax2 and *sodM*^{oe}. Amoebae were plated on non-nutrient agar and incubated for 12 h. Mounds formed on the agar plates were photographed under a microscope. The bar indicates 100 µm. (B) Fruiting bodies formed by Ax2 and *sodM*^{oe}. Amoebae were plated on non-nutrient agar and incubated for 24 h. Fruiting bodies formed on the agar plates were photographed under a microscope. Illustration of a fruiting body is presented. Height of the fruiting body (*h*) and size of the sporangium (*l*) were measured. The bar indicates 100 µm.

DISCUSSION

The lower eukaryotic microorganism *D. discoideum* encodes mitochondrial MnSOD. Expression of the gene for the protein was observed both in the vegetative stage and developmental stage, suggesting that SodM plays important roles throughout the life cycle of this organism. The maximum level of expression was observed at the aggregation stage, suggesting that aggregating amoebae generate more ROS than do cells of other stages.

A stable *sodE*-overexpressing strain was established and subjected to analysis. The *sodM*^{oe} grew faster than its parental strain, indicating that overexpression of SodM accelerated the cell cycle. In addition, the cell density of *sodM*^{oe} in the medium was higher than that of the wild type. ROS generated in the cells would be efficiently detoxified by the increased amount of SodM. Efficient detoxification of cellular ROS would contribute to promotion of cell growth.

Differences between *sodM*^{oe} and the wild type were observed not only in the growth phase but also in the developmental phase. Mounds formed by *sodM*^{oe} were larger than those formed by Ax2, while the number of mounds formed by *sodM*^{oe} was smaller than that formed by Ax2. These results indicate that the number of cells comprising a mound formed by *sodM*^{oe} is greater than the number of cells in a mound formed by Ax2.

The sizes of fruiting bodies formed by the two strains were different. The experimental results demonstrated that fruiting bodies formed by *sodM*^{oe} were approximately 1.2-fold taller than those formed by Ax2. *D. discoideum* forms a fruiting body to hold the spore mass as high off the ground as possible in order to disperse spores. If the fruiting body is too short, spores will not be dispersed over a large area. On the other hand, if the fruiting body is too high, the stalk will bend and spores will not be dispersed. Thus, regulation of the height of the fruiting body is important for optimal spore dispersal. The experimental results presented here suggest that MnSOD plays an important role in height regulation of *D. discoideum*. Further analysis to determine the function of SodM in height regulation is in progress.

The height of fruiting bodies formed by *sodM*^{oe} was approximately 1.2-fold greater than that of fruiting bodies formed by Ax2. The vertical axis of sporangia formed by *sodM*^{oe} was also approximately 1.2-fold longer than that of sporangia formed by Ax2. These results suggest that there is no significant difference between the proportions of fruiting bodies formed by the wild type and *sodM*^{oe}. For further analysis, experiments are in progress to determine the ratio of prespore cells to prestalk cells in a multicellular structure formed by *sodM*^{oe}.

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