Journal of Bioscience and Bioengineering
VOL. 111 No. 6, 613–617, 2011

www.elsevier.com/locate/jbiosc

Molecular structure and gene analysis of Ce^{3+}-induced methanol dehydrogenase of *Bradyrhizobium* sp. MAFF211645

Nanung Agus Fitriyanto,[1] Mako Fushimi,[2] Mika Matsunaga,[2] Ambar Pertiwiningrum,[3] Tomonori Iwama,[2] and Keiichi Kawai[2,*]

The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501–1193, Japan,[1] Faculty of Applied Biological Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan,[2] and Faculty of Animal Science, Gadjah Mada University, Jl. Agro No. 1, Karangmalang, Yogyakarta, Indonesia[3]

Received 24 September 2010; accepted 26 January 2011
Available online 19 February 2011

The molecular structure and nucleotide sequence of Ce^{3+}-induced methanol dehydrogenase (MDH) of *Bradyrhizobium* sp. MAFF211645 were investigated. The addition of 30 μM Ce^{3+} to 1/10 nutrient broth containing 0.5% methanol remarkably increased MDH activity. Furthermore, La^{3+} increased MDH activity, but other heavier rare earth and metal elements did not have the same effect. MDH increased by Ce^{3+} was purified by sequential column chromatography, and the purified MDH migrated as a single band with an apparent molecular weight of 68 kDa on SDS-PAGE. The apparent molecular weight of native MDH was estimated to be 108,000 by gel chromatography. The MDH was comprised of two identical subunits. N-terminal 23-amino acid sequence, 1-NDELHKMAQNPKDWVMPAGDYAN-23, of the purified MDH exhibited 91.3% identity to that of the MDH large subunit-like protein encoded by *mxaF'* of *Bradyrhizobium japonicum* USDA110. Nucleotide sequencing of the MDH gene of strain MAFF211645 yielded a deduced amino acid sequence comprising 601 amino acid residues, an N-terminal signal peptide, and a mature MDH comprising 578 amino acid residues with a predicted molecular mass of 62,918 Da. Further analysis of the deduced amino acid sequence of mature MDH revealed that the functional amino acids in its active site, such as two adjacent Cys residues, and bacterial quinoprotein signatures 1 and 2 were conserved. These results indicate that Ce^{3+}-induced MDH encoded by *mxaF'* may be involved in methanol metabolism in *Bradyrhizobium* sp. MAFF211645.

[**Key words:** *Bradyrhizobium*; Methanol dehydrogenase; Rare earth element; Ce^{3+}; *mxaF'*]

Methanol dehydrogenase [EC 1.1.2.7] (MDH) is a key periplasmic enzyme that catalyzes the oxidation of methanol to formaldehyde. MDH of methylotrophs is a quinoprotein containing pyrroloquinoline quinone (PQQ) and Ca^{2+} at its active site (1,2). Complete gene analysis of *Methylobacterium extorquens* AM1 revealed that two genes encoded the structural proteins of MDH. These gene *mxaF* and *mxaI*, encode the large (α) and small (β) subunits of MDH, respectively (3,4). MDHs of *M. extorquens* (5), *Methylobacterium* sp. HG-1 (6), and *Methylobacillus glycogens* (7) were demonstrated to possess a heterotetrameric structure ($\alpha_2\beta_2$). However, the role of β-subunit remains unclear (4). In addition to *mxaF*, *xoxF* (synonymous for *mxaF'*) of *M. extorquens* AM1 (8), *Methylibium petroleiphilum* PM1 (9), and *Beggiatoa alba* (10), and *mxaF'* of *Bradyrhizobium japonicum* USDA110 (11) were predicted to encode the MDH large subunit-like protein common to putative PQQ-dependent dehydrogenase.

Rare earth elements (REEs) have been widely used as raw materials in high-technology products (12). In China, REE mixtures containing mainly La^{3+} and Ce^{3+} have been used as microfertilizers to improve crop yield (13) and animal growth promoters (14). Although many studies from China have reported the beneficial effects of REEs on higher organisms, the physiological mechanism of these effects has not yet been clarified. Most biochemical investigations of the effects of REE utilize a substitute or competitive antagonist for Ca^{2+} because the ionic radius of REE is similar to that of Ca^{2+}. Moreover, Ca^{2+}-dependent enzymes and other proteins may be inhibited by REEs (15).

A few studies report the effects of REEs on microbial activity. La^{3+} changed the outer membrane structure of *Escherichia coli*, resulting in a change in membrane permeability (16). Some REEs such as La^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Tb^{3+}, and Dy^{3+} were demonstrated to inhibit the active transport of Ca^{2+} in *Mycobacterium phlei* (17). However, REE effects on microbial activity have not yet been sufficiently clarified.

To elucidate the effect of Ce^{3+}, a representative REE, on microbial activity, we screened Ce^{3+}-responsive microorganisms. We isolated a soil bacterium, and identified it as *Bradyrhizobium* sp. MAFF211645. This bacterium produced exopolysaccharide (EPS) around its colonies when Ce^{3+} was present (18). It is of interest that Ce^{3+} affects microbial activity. During the study on the effects of Ce^{3+} on EPS production from this strain by proteome analysis using 2-dimentional differential gel electrophoresis, we found that the levels of several proteins were increased when Ce^{3+} was added to the culture

* Corresponding author. Tel./fax: +81 58 293 2905.
E-mail address: kawaik@gifu-u.ac.jp (K. Kawai).

1389-1723/$ - see front matter © 2011, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2011.01.015

medium. Among them, the N-terminal 15-amino acid sequence of one protein exhibited 86.7% (13 aa/15 aa) identity to that of the deduced amino acid sequence of the MDH large subunit-like protein encoded by *mxaF'* (accession no. Q98GY2) of *B. japonicum* USDA110 (11). In addition, *mxaF'* was also found in the genomes of *Bradyhrizobium* sp. BTAi1 (accession no. A5EP13), *Bradyrhizobium* sp. ORS278 (accession no. A4YZ34) and other *Bradyrhizobium* spp. Although the condensed resting cells of the wild-type strain of *B. japonicum* USDA110 could oxidize methanol, those of an *mxaF* deletion mutant (in this published report (19), *mxaF* should be read as *mxaF'* (accession no. Q89GY2) that corresponds to blr6213 located on the genome of *B. japonicum* USDA110) could not oxidize methanol. These results suggest that the MDH large subunit-like protein encoded by *mxaF'* may be involved in methanol metabolism in *Bradyrhizobium* spp. However, the MDH large subunit-like protein functions as an active MDH in methanol oxidation remain unclear. In addition, no report on MDH from *Bradyrhizobium* spp. is present. To elucidate the role of Ce^{3+} in methanol metabolism in *Bradyrhizobium* sp. MAFF211645, we examined the effects of Ce^{3+} on MDH, a key enzyme of methylotrophic metabolism in this bacterium.

In this study, we describe the induction, molecular structure, and nucleotide sequence of Ce^{3+}-induced MDH of *Bradyrhizobium* sp. MAFF211645.

MATERIALS AND METHODS

Materials REEs (Ultra-pure grade, trivalent chloride form) were purchased from Wako Chemical Industries Ltd. (Osaka, Japan). Ultrafree-MC microcentrifuge filter unit 5000 Da MWCO, phenazine ethosulfate, and 2, 6-dichlorophenolindophenol (DCPIP) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Nitro blue tetrazolium was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2-Morpholino-ethanesulfonic acid monohydrate (MES) was purchased from Dojindo Laboratory (Kumamoto, Japan). PD-10, HiTrap SP Sepharose HP (1 ml), and Mono S 5/50 GL columns were purchased from GE Healthcare (Uppsala, Sweden). The BenchMark protein ladder was purchased from Invitrogen (Carlsbad, CA, USA). The BCA protein assay kit was purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were of analytical grade and commercially available.

Strains and culture conditions *Bradyrhizobium* sp. MAFF211645 and 1/10 nutrient broth (0.1% meat extract, 0.1% peptone, 0.05% NaCl, pH 7.2) were used in this study. Bacterium was cultured with shaking at 30°C. The growth was monitored by colony-forming unit (CFU) ml^{-1} or by absorbance t 600 nm. CFU was determined as follows. Culture broth was withdrawn, diluted with sterile water, and spread on a nutrient agar plate. The plates were cultured for 7 days at 30°C. Colonies that appeared on the agar plates were counted.

Preparation of cell-free extract Cells were harvested at late log phase by centrifugation at 8000 ×*g* for 20 min, washed with 20 mM Tris–HCl buffer, pH 8.0, and suspended in the same buffer. Cells were lysed with sonication, cell debris was removed by centrifugation at 16,000 ×*g* for 20 min. The supernatant was used as the cell-free extract.

Purification of Ce^{3+}-induced MDH Cell-free extract (2.5 ml) was applied to a PD-10 column that had been previously equilibrated with 25 mM MES buffer, pH 5.0. The column was eluted with the same buffer. The eluate was applied to a HiTrap Sepharose HP column that had been previously equilibrated with 25 mM MES buffer, pH 5.5. The column was eluted with the same buffer containing 1 M NaCl at a flow rate of 1.0 ml min^{-1}. The active fractions were pooled and concentrated using the Ultrafree-MC microcentrifuge filter unit. The concentrate was applied to a Mono S 5/50 GL column using the SCL-6B SPD-6AV C-R6A high-performance liquid chromatography system (Shimadzu Co., Kyoto, Japan). The proteins were eluted by a gradient time program using 0–30% of 25 mM MES buffer, pH 5.5, containing 1 M NaCl at a flow rate of 1.0 ml min^{-1}. The active fractions were pooled and concentrated as describe above.

Enzyme and protein assay MDH activity was determined according to the method of Day and Anthony (20). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of DCPIP min^{-1} using $\varepsilon_{600} = 19.1\ mM^{-1}\ cm^{-1}$ (21). The protein concentration was determined using BCA protein assay kit according to the manufacturer's instructions (22).

SDS-polyacrylamide gel electrophoresis (PAGE) SDS-PAGE was performed according to the method of Laemmli with 12% polyacrylamide gels under denaturing conditions (23). Proteins were stained with 0.25% Coomassie Brilliant Blue (CBB) R-250.

Molecular weight estimation of native Ce^{3+}-induced MDH The molecular weight of native Ce^{3+}-induced MDH was estimated by gel chromatography using the Superdex 200 10/300 GL column. Column was equilibrated and eluted with 50 mM Tris–HCl, pH 8.0, containing 0.1 M NaCl at flow rate 0.3 ml min^{-1}. The molecular weight standards were ferritin (440,000), aldolase (158,000), conalbumin (75,000), and ovalbumin (43,000).

Determination of N-terminal amino acid sequence After SDS-PAGE, proteins were electrophoretically transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using the Transblot SD cell (Bio-Rad, Hercules, CA, USA) at 25 V for 4 h. The membrane filter was stained with CBB R-250 and destained with 50% methanol. The protein band on the filter was excised and subjected to N-terminal amino acid sequence analysis using a gas-phase protein sequencer (Procise™ Protein Sequencing System) connected to an on-line model 610A (Applied Biosystems, Foster City, CA, USA).

DNA manipulations Genomic DNA was isolated by a standard method (24) and the quality and concentration of the isolated DNA were evaluated by 0.7% agarose gel electrophoresis (25). The gene encoding Ce^{3+}-induced MDH was amplified by polymerase chain reaction (PCR) using Blend *Taq* (Toyobo, Osaka, Japan) and degenerate primers. The primers were prepared based on the N-terminal amino acid sequence of purified Ce^{3+}-induced MDH and a conserved sequence of genes of other bacteria, highly homologous to *mxaF'* from *B. japonicum* USDA110. The following primers were used. Forward primers: mdh-F1, 5′-ATGTCGGGTGATCCTCCGACTTAGT-3′; mdh-F2, 5′-CAACGACGAACTCCACAAGA-3′; mdh-F3, 5′-AGCGCCTACGACATCAAGACCGGCA-3′. Reverse primers: mdh-R1, 5′-GCGAGTCCTTCAACGCCGGCGCGTA-3′; mdh-R2, (5′-GGCAGCGAGAACACGGTGAGCGTTC-3′); mdh-R3, 5′-GAACGGCTCGTAGTCCATGC-3′. PCR mixture (20 μl) was consisted of template DNA, Blend *taq* buffer, dNTPs, *Taq* DNA polymerase, and primers. PCR was performed as follows. After incubation of the PCR mixture for 90 s at 94°C the DNA was amplified using 35 cycles. Each cycle consisted of denaturation for 10 s at 98°C, annealing for 30 s at 55°C, extension for 2 min at 72°C and then incubation for 5 min at 72°C. The amplified DNA fragment was evaluated by agarose gel electrophoresis. The PCR product was precipitated by ethanol, dissolved in HiDi™ Formaldehyde (Applied Biosystems), and then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Comparative analyses of nucleotide and deduced amino sequences were performed through database searches using the BLAST program (http://www.ncbi.nml.nih.gov).

Nucleotide sequence accession number The nucleotide sequence data of Ce^{3+}-induced MDH obtained in this study has been submitted to DDBJ under the accession number AB548312.

RESULTS

Cell growth in the presence of Ce^{3+} and/or methanol Concentration of Ce^{3+} in agricultural soils in Australia and China is estimated to be a range of about 10 to 50 μM (26). Therefore, we have used Ce^{3+} at 30 μM. The addition of 30 μM Ce^{3+} and/or 0.5% methanol to the broth did not provide any stimulatory or inhibitory effects on cell growth of strain MAFF211645 when the cell growth was monitored by CFU ml^{-1}. However, cell growth was inhibited by more than 50 μM Ce^{3+}. High concentration of Ce^{3+} seems to inhibit the cell growth. Using absorbance at 600 nm to monitor the culture, the absorbance of culture broth remarkably increased only when both Ce^{3+} and methanol were added to the broth. This increase in absorbance was found to be due to EPS production by this strain, some amount of it was produced in broth.

MDH activity of cells grown in the presence of REEs and metal ions We examined MDH activity in a cell-free extract of *Bradyrhizobium* sp. MAFF211645 that was grown in the presence of 30 μM Ce^{3+} and/or 0.5% methanol. As shown in Fig. 1, we detected weak MDH activity in the cell-free extract of cells grown in the presence of 30 μM Ce^{3+} (0.03 U/mg of protein). In addition, the cells grown in the presence of 0.5% methanol showed very low activity (0.014 U/mg of protein). Surprisingly, the cells grown in the presence of both 30 μM Ce^{3+} and 0.5% methanol exhibited very high MDH activity (0.64 U/mg of protein). The addition of La^{3+} also produced a similar result (0.64 U/mg of protein), whereas Pr^{3+} slightly increased MDH activity (0.12 U/mg of protein). However, other heavier REEs including Gd^{3+}, Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, Yb^{3+}, and Lu^{3+} as well as metal elements such as Mg^{2+}, K^{+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Sr^{2+}, and Ba^{2+} did not increase MDH activity. The addition of Ce^{3+} or La^{3+} to cell-free extract did not increase the MDH activity. These results indicate that Ce^{3+} or La^{3+} induced specifically MDH activity of *Bradyrhizobium* sp. MAFF211645.

Purification and some properties of Ce^{3+}-induced MDH Cell-free extract was prepared from cells that were grown in the presence of Ce^{3+}, as a representative REE. We purified the Ce^{3+}-induced MDH. Table 1 summarizes the results of purification. The Ce^{3+}-induced MDH