February 2006
Master's Thesis

Cloning and Expression of *mxaJ* and *mxaG* genes and Their interaction with Methanol dehydrogenase

Graduate School of Chosun University

Department of Bio Materials Engineering

Phan Trong Nhat

Cloning and Expression of *mxaJ* and *mxaG* genes and Their interaction with Methanol dehydrogenase

February 2006

Graduate School of Chosun University

Department of Bio Materials Engineering

Phan Trong Nhat

Cloning and Expression of *mxaJ* and *mxaG* genes and Their interaction with Methanol dehydrogenase

Advisor: Prof. Si Wouk Kim

Thesis submitted for the degree of Master of Science

November 2005

Graduate School of Chosun University

Department of Bio Materials Engineering

Phan Trong Nhat

Thesis submitted in partial fulfillment of the requirement for the Award of the degree of Master of Science

Approved by the Guidance Committee:

Professor Jin Cheon Yu
Chosun University _____

Professor Eun Hee Cho
Chosun University _____

Professor Si Wouk Kim
Chosun University _____

November 2005

Graduate School of Chosun University

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
CBR	coomassie brilliant blue R-250
DCPIP	2.6-dichlorophenol indophenol
DMA	dimethylamine
DMF	dimethylformamide
DMS	dimethylsulfate
DMSO	dimethylsufoxide
IPTG	isopropylthiogalactoside
MDH	methanol dehydrogenase
NBT	nitro blue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PES	phenazine ethosulfonnate
PMS	phenazine methosulfonate
PMSF	phenylmethylsulfonyl fluoride
PQQ	pyrroloquinoline quinon
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
SMM	standard mineral base medium
TMA	trimethylamine
TMBZ	3, 3', 5, 5'-tetramethylbenzidine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

ABSTRACT

Cloning and Expression of *mxaJ* and *mxaG* genes and Their interaction with Methanol dehydrogenase

Phan Trong Nhat

Advisor: Prof. Si Wouk Kim, Ph. D.

Department of Bio Materials Engineering,

Graduate School of Chosun University

Methylophaga aminosulfidovorans SK1 is a marine methylotrophic bacterium that is capable of growing in the presence of methanol as a sole carbon and energy source. Oxidation of methanol to formaldehyde in *M. aminosulfidovorans* SK1 is catalyzed by the periplasmic quinoprotein methanol dehydrogenase (MDH) and cytochrome c_L as its primary electron acceptor. Genomic DNA fragments, which relating the methanol oxidation (*mxaFJGIR* genes), were cloned and sequenced fully. I cloned a gene designated as *mxaG* that encoded cytochrome c_L and expressed the recombinant gene in *Escherichia coli* under anaerobic conditions. During purification of methanol dehydrogenase (MDH) from *M. aminosulfidovorans* SK1, I obtained two different fractions (MDH I and MDH II) which had MDH activities. MDH I and MDH II fractions

showed different biochemical features such as molecular weight and pI. Each α-subunit of MDH I and MDH II was identical except that MDH II fraction had 30kDa unknown protein. Sequence analysis of *N*-terminal amino acid of the protein represented that 30 kDa unknown peptide may be the product of *mxaJ* gene. In this study, we compared the characters of the recombinant and wild type cytochrome c_L, purified 30 kDa unknown peptide, and studied interaction between *mxaJ* and methanol dehydrogenase in *M. aminosulfidovorans* SK1 via yeast two – hybrid system.

I. Introduction

Methylotrophic bacteria are a group of bacteria which are able to grow at the expense of compounds containing one or more carbon atoms but no carbon to carbon bonds as energy and carbon sources (1, 2). Methanol and methane are two important substrates of methylotrophic bacteria in aquatic and terrestrial environment. Methanol is produced during the decomposition of plant polymers and is probably excreted from living leaves and by methane oxidizing bacteria, whereas methane is mainly produced by methanogenic Archaea in anoxic environments. Methylotrophs can oxidize methanol to carbon dioxide through sequential reactions catalyzed by a series of enzymes including methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FAD), that have been reviewed in detail by Murrell et al (3).

$$CH_3OH \xrightarrow{MDH} HCHO \xrightarrow{FADH} HCOOH \xrightarrow{FAD} CO_2$$

All of these methylotrophs use the enzyme MDH to oxidize methanol to formaldehyde, releasing electrons are accepted by cytochrome *c*. The *c* - type cytochromes are electron transport proteins involved in respiratory processes of almost all organisms. The distinguishing feature of this protein family is the presence of a haem group covalently linked to the polypeptide via one or two thioether bonds (2). In gram - negative methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic MDH, which has pyrroloquinoline

quinone (PQQ) as a prosthetic group and cytochrome c_L as its primary electron acceptor (4). MDH and cytochrome c_L are present at high concentrations in periplasm and constitute the first part of a methanol oxidation electron transport chain. A type cytochrome c_H is also involved in methanol oxidation and cytochrome c_H is oxidized by a terminal oxidase (5):

Methanol → MDH → cytochrome c_L → cytochrome c_H → terminal oxidase

Most methylotrophic bacteria contain at least two soluble *c*-type cytochromes whose molecular weights, isoelectric points and absorption spectra are different from each other. The *c* - type cytochromes can be classified based on the isoelectric point and usually the isoelectric point of cytochrome c_L is lower than that of cytochrome c_H (5).

The gram - negative marine methylotrophs that are able to utilize methanol but unable to utilize methane have been first described by Yamaoto *et al* (6). Methylophaga has been proposed as a new genus of the gram - negative marine methylotrophs. Methylophaga is a moderate halophilic genus possessing the ribulose monophosphate (RuMP) pathway for carbon assimilation. The phenotypic properties of marine methylotrophic bacteria of methylophaga are similar to those of terrestrial strains of gram - negative, obligate or restricted facultative methylotrophs except for sodium chloride tolerance. This means that it is difficult to divide taxonomically the soil and marine methylotrophic bacteria possessing RuMP pathway. Methylophaga species are distinguished from the

other RuMP pathway methylobacteria by their requirements for Na^+, Mg^+ and vitamine B_{12}. The only point that can use to classify them is the low level of DNA - DNA homology between them. Therefore, only a limited number of marine strains have been described so far as follows *Methylophaga marina*, *M. thalassica*, *M. limanica*, *M. sulfidovorans* and *M. alcalica*. It indicates that some more strains of marine methylotrophic bacteria should be isolated and characterized.

M. aminosulfidovorans SK1 is a marine methylotrophic bacterium that is capable of growing in the presence of methanol as a sole carbon and energy source. The oxidation of methanol to formaldehyde in the gram - negative, pink - pigmented, marine methylotroph is catalyzed by the periplasmic quinoprotein methanol dehydrogenase (7, 8, 9). MDH has been isolated and purified from several different strains of microorganisms including *Methylobacterium extorquens* AM1 (10), *Methylobacterium extorquens* (11), *Methylosinus trichosporium* OB3b (12), *Hyphomicrobium X* (13), *Hyphomicrobium methylovorum* GM2 (14) and *Methylosinus* sp. WI 14 (15). X - ray crystallographic studies on MDH from *M. extorquens* (16, 17), *Methylophilius* W3A1 (18) and *Paracoccus dentrificans* (19) demonstrated that MDH exists in an $\alpha_2\beta_2$ tetramer. MDH is an $\alpha_2\beta_2$ tetramer of approximate subunit molecular weight 60 and 8 kDa (20), and contains two molecules of pyrroloquinoline quinine (PQQ) per tetramer as a prosthetic group. The primary specific electron

acceptor for essential MDH activity is cytochrome c_L and calcium ion (Ca_2^{++}), which then transfers reducing equivalents by way of another cytochrome (C_H) to the membrane – bound cytochrome oxidase (21, 22, 23, 24).

The methanol oxidation (Mxa) system of *M. extorquens* AM1 has proved to be complex, and to date 24 genes (*mxa* genes) have been shown to play a role in MDH synthesis, assembly, or regulation or PQQ synthesis in this microorganism (25). The Mxa system has also been studied in a number of other gram - negative methylotrophs, including *Methylobacterium organophilum* XX (26), *Methylobacterium organophilum* DSM 760 (25) and *Paracoccus denitrificans* (27). Overlap in gene designations has caused confusion, and so a new unified nomenclature for the methanol oxidation genes has been introduced. The *mxa* genes in *M. extorquens* AM1 are clustered in different loci. The largest of these, the *mxa* locus, contains several *mox* genes arranged in three clusters, *mxaFJGIR*, *mxaAKL* and *mxaB. mxaF* and *mxaI* encode the large (α) MDH subunit of 60 kDa and small (β) MDH subunit of 8 kDa, respectively, which form the $\alpha_2\beta_2$ heterodimer (20). *mxaG* encodes the cytochrome c_L electron acceptor, but the functions of *mxaJ* and *mxaR* remain unknown. However, in *Acetobacter methanolicus*, a 32kDa polypeptide with similarity to MxaJ peptide has been isolated in association with MDH at a single molecule per tetramer ($\alpha_2\beta_2\gamma$). Therefore, it was proposed that MxaJ might play a role in vivo in electron transfer of methanol oxidation system, in enabling correct structural

conformation of MDH, or in correct assembly of the PQQ, Ca^{2+}, and MDH (28, 29). Phenotypic characterization of a *mxaJ* deletion mutant constructed in *P. denitrificans* also suggested a chaperonin – like role for MxaJ. Van Spanning et al constructed a *P. denitrificans mxaR* insertion mutant and proposed that the cytoplasmic MxaR has a role in the regulation of formation of active MDH (30). In *M. extorquens* AM1, *mxaB* has been shown to be required for transcription of *mxaF* (31). Finally, *mxaA*, *mxaK*, and *mxaL* have been shown to encode polypeptides essential for correct incorporation of calcium ion in to MDH (32, 33). MDH isolated from strains defective in these genes is inactive, lacks Ca^{2+} ion, and has PQQ in a fully oxidized form rather than the normal semiquinone form. The absorption spectra of these inactive MDHs suggest that PQQ is bound differently in the absence of Ca^{2+}. However, incubation with high levels of calcium salts (0.5 to 10 mM) in vitro restores MDH activity. It was proposed that MxaA, MxaK, and MxaL either maintain a high level of calcium in the periplasm, binding Ca^{2+} and by inserting it into the MDH, or stabilize a configuration of MDH that permits incorporation of Ca^{2+} at low concentrations (34).

The yeast two – hybrid assay is a system for identifying and analyzing protein – protein interactions. Since the original description in 1989, the technique has provided insight into many biological pathways. A variety of adaptations to the technique have been developed that allow analysis of protein – DNA, protein – RNA, or small molecule – protein interactions. Recent developments now allow

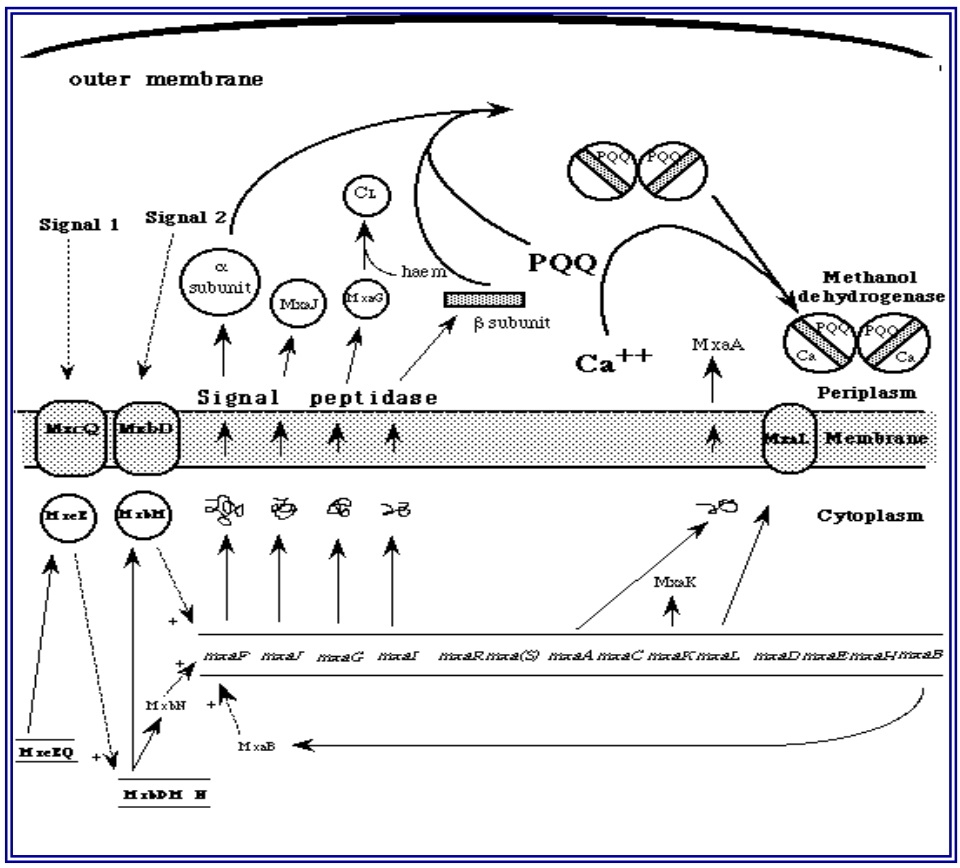


Fig. 1. A model for the expression of methanol dehydrogenase in *Methylobacterium extorquens* AM1

the use of these technologies to perform global analyses of all such interactions that occur in cells. The principle of the yeast two – hybrid system: Two plasmids are constructed, the bait – encoding protein X fused to the C – terminus of a transcription factor DNA – binding domain (BD) and the prey – encoding protein Y fused to an activation domain (AD). Alternatively, the prey can consist of proteins encoded by an expression library. Each plasmid is introduced into an appropriate yeast strain either by co-transformation, sequential transformation, or by yeast mating. Only if proteins X and Y physically interact with one another are the BD and AD brought together to reconstitute a functionally active transcription factor that binds to upstream specific activation sequences (UAS) in the promoters of the reporter genes, and to activate their expression (35, 36).

In this study, I described the cloning and expression of the gene, *mxaG*, which encoded cytochrome c_L from *M. aminosulfidovorans* SK1. Under strict anaerobic conditions, the expression of *mxaG* gene can be achieved in *E. coli* DH5α enough to isolate the protein. I have purified the expressed protein and compared it with the wild type cytochrome c_L isolated from *M. aminosulfidovorans* SK1.

On the other hand, the genes encoding the α and β subunits of MDH (*mxaF* and *mxaI*) and cytochrome c_L (*mxaG*) are located in a same operon together with *mxaJ*. However, the role of *mxaJ* gene has been poorly characterized. To solve this problem, we carried out yeast-two hybrid system. The *mxaF*, *mxaI* and *mxaJ* genes were ligated with binding domain (pGBKT7) vector and activation domain

(pGADT7) vector and expressed in yeast cells AH-109. *In vivo* binding assay of yeast-two hybrid system were performed to confirm the interaction between α and β subunits of MDH and *mxaJ* product.

II

Cloning and Expression of *mxaG* gene from *Methylophaga aminosulfidovorans* SK1 in *Escherichia coli*

A. Materials and Methods

1. Bacteria strains and plasmids

The plasmids used in this study are pGEM – T Easy (Promega Corporation) and pASK-IBA6 vector (IBA BioTAGnology); an *E. coli* strain DH5α was used as a host for amplification and transformation of recombinant plasmid.

2. Growth conditions

M. aminosulfidovorances SK1 was incubated in the standard mineral base medium (SMM – table 1) containing 1% (v/v) methanol.

E. coli DH5α cells were grown on LB (Broth acc. To Miller: casein peptone 10 g, yeast extract 5 g, sodium chloride 10 g, distilled water 1 *l* and 50 μg/ml ampicillin) and TYS medium (1% trypton, 1% yeast extract, 0.5% sodium chloride and 50 μg/ml ampicillin).

3. Isolation of *M. aminosulfidovorances* SK1 chromosomal DNA

Total DNA from *M. aminosulfidovorances* SK1 was obtained by a modification of the procedure of Goldberg and Ohman. *M. aminosulfidovorances* SK1 was incubated in SMM medium containing 1% methanol at 30°C to the mid-logarithmic phase. The cells from a 50 ml culture were harvested by centrifugation at 10,000 x g for 15 min at 4°C and washed twice in PBS buffer. Suspended in 10 ml of solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-

HCl, pH 8.0). 1 ml of lysozyme (50 mg/ml), 0.5 ml RNase (2 mg/ml) and 0.1 ml proteinase K (50 mg/ml) was added, and the mixture was incubated at 37 °C for 1 hour. Then, 0.6 ml of 10% SDS solution was added and the mixture was incubated at 37 °C for 1 h. And 1 ml of mercaptoethanol was added and 0.5 time volume with 3 M sodium acetate solution (pH 5.2) to the aqueous phase and centrifugation at 10,000 x g for 15 min. A 0.6 time volume of isopropanol was layered above the mixture at room temperature. The two phases were mixed with a glass rod, thus spooling the chromosomal DNA. The rod with DNA was blotted of excess liquid and placed in 3 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 4 °C overnight to dissolve the DNA.

4. Construction of cloning and expression plasmids

The *mxaG* gene from *M. aminosulfidovorans* SK1 was cloned from the chromosomal DNA using the following primer set (5'-AAG CTCGAG TTATAAAAGTGGGATGCC-3' and 5'-TCTAGAAGGAAATTTTTTAAAT CATGACTATCAAAAC-3'). The underlined sequences indicate *XhoI* and *XbaI* sites, respectively. The amplified gene was ligated with pGEM-T Easy vector (Promega Corporation). The *E. coli* cells containing *mxaG* gene were recognized by white/blue selection. The vector containing *mxaG* gene was digested with the *XhoI* and *XbaI*, and the DNA fragment with *mxaG* gene was

Table 1. Composition of standard mineral base medium (SMM)

Components	Amounts
NaH_2PO_4	2.34 g
K_2HPO_4	6.1 g
$(NH_4)_2SO_4$	1 g
$NaNO_3$	1 g
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.01 g
$ZnSO_4.7H_2O$	0.5 mg
$MnSO_4.H_2O$	0.5 mg
$CuSO_4.5H_2O$	0.1 mg
$Co(NO_3)_2$	0.1 mg
$Na_2B_4O_{7.}10H_2O$	0.1 mg
$NaMoO_4.2H_2O$	2 mg
Ferric EDTA solution*	0.1 ml
NaCl	30 g
Distill water	1 l

** The ferric EDTA solution was made by combining a solution containing 17.9 g of sodium EDTA and 3.23 g of KOH dissolved in 186 ml distill water and a solution containing 13.7 g of $FeSO_4.7H_2O$ in 364 ml of distill water. The mixture was bubbled overnight with air, and stored in a brown glass bottle.*

cloned into the expression plasmid pASK-IBA6 (IBA BioTAGnology). This recombinant plasmid (pASK-*mxaG*) was transformed into *E. coli* strain DH5α cells by heat shock at 42 °C for exactly 90 sec. An *E. coli* strain DH5α was transformed with this vector and incubated at 37 °C overnight on LB agar plate containing ampicillin.

5. Expression and purification of recombinant cytochrome c_L from *E. coli*

A fresh colony of *E. coli* strain DH5α, containing pASK-*mxaG* plasmid, was inoculated into TYS medium and grown overnight under aerobic conditions. The pre-culture was inoculated into the 5L TYS medium under strict anaerobic condition. The condition was maintained by continuous flushing of the growth chamber with N_2 (figure 2). The growth medium was supplemented with 50μg/ml ampicillin, 200ng/l tetracycline, 10mM KNO_3 and induced with 0.1mM IPTG when OD600 reached 0.5 - 0.6. The cells were harvested by centrifugation at 14,000 x g for 10 min at 4 °C. The pellet was resuspended in 10 ml of precooled buffer P (500 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) with 1 mg/ml lysozyme and put in ice for 30 min followed by centrifugation at 14,000 x g for 5 min at 4 °C. The clear supernatant was applied to Ni-NTA affinity column for purification of the recombinant protein with 6xHis-tag using the QIA express system. Haem staining was carried out by using the method of Thomas et al (37). Recombinant cytochrome c_L containing 6xHis-tagged proteins

was bound to Ni-NTA resin. The bound protein was washed with 250 mM Tris-HCl buffer containing 10 mM imidazole and 300mM NaCl and eluted with a linear gradient of 20 - 250 mM imidazole in the same buffer. Fractions containing respective proteins were pooled and concentrated. Next, the 6X his tagged protein was removed to get the recombinant cytochrome c_L (figure 3B). Finally, the recombinant cytochrome c_L was compared with native cytochrome c_L about molecular weight, absorption spectra and the rates of electron transfer.

6. Purification of native cytochrome c_L

M. aminosulfidovorans SK1 cells from the late exponential growth phase were harvested at 10,000 x g for 10 min at 4 ℃ and later suspended in 25 mM Tris/HCl buffer, pH 8.0 at 4 ℃ and were disrupted by sonicator. Unbroken cells and debris were removed by centrifugation at 14,000 x g for 20 min at 4 ℃. The resulting supernatant was centrifuged at 100,000 x g for 90 min, and the supernatant was used as a soluble fraction. Purification of cytochrome c_L was carried out at 4 ℃ from the soluble fraction. Solid $(NH_4)_2SO_4$ was slowly added to this protein solution to obtain 50% saturation. After removal of the precipitate by centrifugation at 15,000 x g for 30 min, $(NH_4)_2SO_4$ was added to obtain 80% final saturation. The precipitated proteins were centrifuged and redissolved in the same buffer. After dialysis for 16 h, proteins were concentrated with Centricon (YM-10, Amicon, Bedford, USA) and Aquacide II (Calbiochem, Darmstadt,

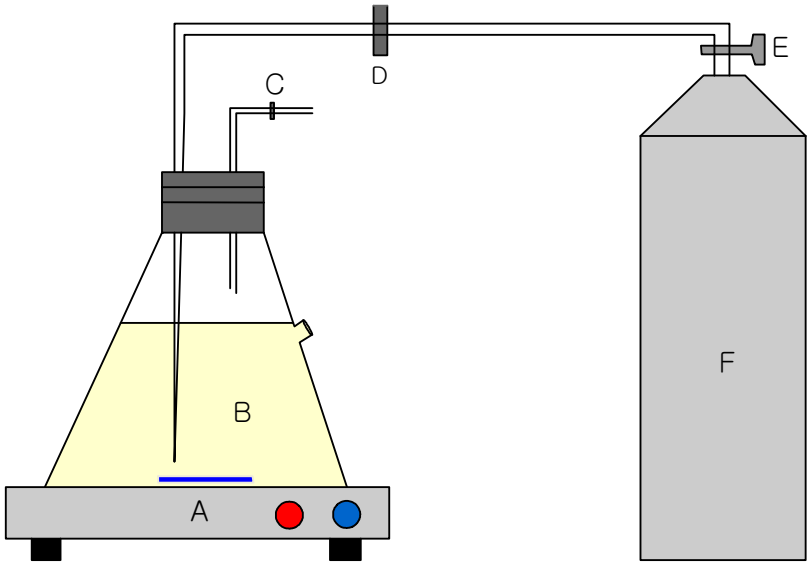


Fig. 2. Schematic diagram of strict anaerobic culture system for expression of recombinant cytochrome c_L in *E. coli* strain DH5α. A: stirrer, B: 5 *l* flask, C & D: 0.2 μm air filter, E: gas valve and F: nitrogen gas tank.

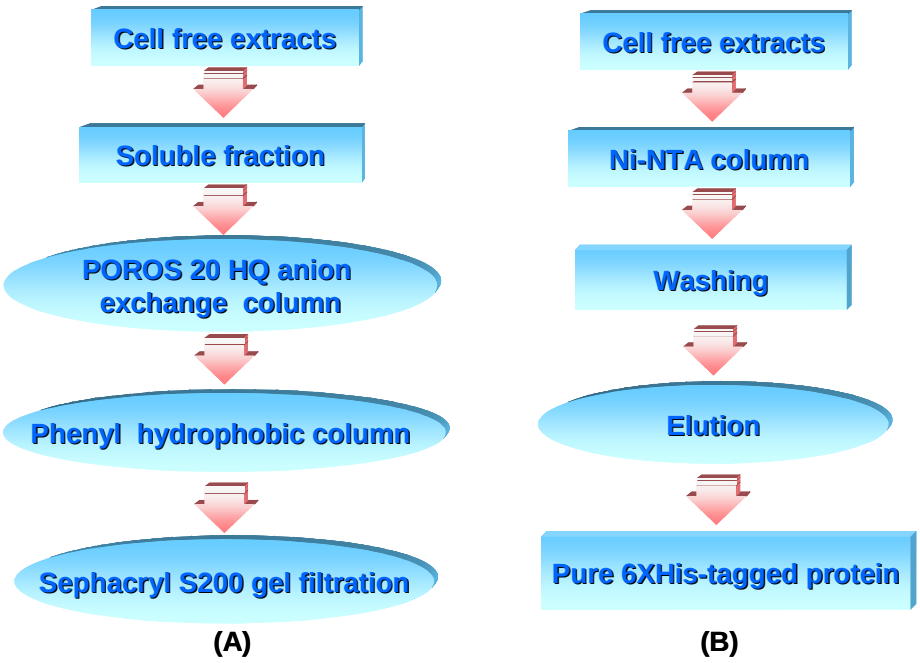


Fig. 3. Purification steps of native (A) and recombinant cytochrome c_L (B).

Germany). The concentrate was applied to a POROS 20 HQ column, which was previously equilibrated with standard buffer. Cytochrome c_L was eluted with a linear gradient of 0-1M NaCl in standard buffer. Fractions containing respective proteins were pooled, concentrated, and applied to an Hydrophobic interaction chromatography, which was equilibrated with standard buffer containing 1.5M $(NH_4)_2SO_4$. After that fractions containing respective proteins were pooled, concentrated, and applied to Gel Filtration, which was equilibrated with standard buffer containing 0.15M NaCl. Finally the native cytochrome c_L was purified and used to the next experiment steps. All steps of purifying the native cytochrome c_L were showed in the figure 3B.

7. SDS and native-polyacrylamide gel electrophoresis

Tricine - SDS-PAGE was performed as described by Schagger and Jagow (38). Proteins were separated on a 12% gel and stained with Coomassie brilliant blue R-250. Native - PAGE was performed using the method of Laemmli (39) but without SDS in the system and no treatment of samples.

8. TMBZ staining

TMBZ staining of native polyacrylamide gels were stained for peroxidase activity. Prepare 20 ml TMBZ activity staining solution (included 6 ml of 6.3 mM TMBZ freshly solution and 14 ml of 0.25 M CH_3COONa, pH 5.0). When

the native - polyacrylamide gel electrophoresis were finished, the gels were stained with 20 ml TMBZ activity staining solution in dark for 1 to 2 h. After that put 170 μl H_2O_2 and mixed for 20 sec and washed by 0.25 M CH_3COONa, pH 5.0 containing 30% isopropanol to remove any precipitated TMBZ and to clear the gel background and enhance the staining intensity.

9. Materials

Restriction enzymes, DNA ligase, and Tag polymerase used in this study were purchased from Takara (Takara Shuco Co., Kyoto, Japan). Reagents for SDS-PAGE, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal), Isopropylthiogalactoside (IPTG), 3, 3', 5, 5'-tetramethylbenzidine (TMBZ) were purchased from Sigma.

B. Results and discussion

Figures 4 to 8 showed the steps to construct of cloning and expression vector, in which *mxaG* gene was ligated with an expression vector pASK-IBA6. Yields of obtained recombinant cytochrome c_L were about 0.3 mg/l of culture. Figure 9 showed the SDS - PAGE analysis of wild type and recombinant cytochrome c_L. The molecular weight of recombinant cytochrome c_L was the same as that of wild-type cytochrome c_L (21 kDa). Figure 10 (A) and (B) show the native PAGE and the haem staining of two cytochromes c_L, respectively.

Table 2 and figure 9 compared the absorption spectra of reduced wild type and recombinant cytochrome c_L. Both of the proteins had the same three absorption peaks at 551.0, 521.0 and 415.5 nm. This result showed that both of two proteins contained the same hame concentration. Figure 12 showed the electron flow rates from MDH to wild type or recombinant cytochrome c_L which was oxidized by potassium ferricyanide prior to the reaction. The oxidized cytochrome c_L was reduced by MDH and absorbance at 551.0 nm increased in a time - dependent manner. The electron transfer was finished completely within 20 sec.

The electron flow between electron carriers in methanol oxidation was determined by using the artificial electron transport system in which potassium ferricyanide was used as a final electron acceptor. The decrease of absorbance at 420 nm by the reduction of potassium ferricyanide was monitored. Figure 13 showed the rates of electron transfer from MDH to potassium ferricyanide via native and recombinant cytochromes c_L. Regardless of the type of cytochrome c_L, the reduction of potassium ferricyanide was accomplished within 20 min. This means that recombinant cytochrome c_L could act as an electron carrier in the methanol oxidation.

In summary, I have constructed, expressed and characterized the recombinant cytochrome c_L of *M. aminosulfidovorans* SK1 in *E. coli* strain DH5α under strict anaerobic conditions. The biochemical and spectroscopic properties of recombinant cytochrome c_L were the same as those of wild type cytochrome c_L.

This result will be useful to understand the roles of *mxaG* gene in *mxaFJGIR* genes cluster of *M. aminosulfidovorans* SK1

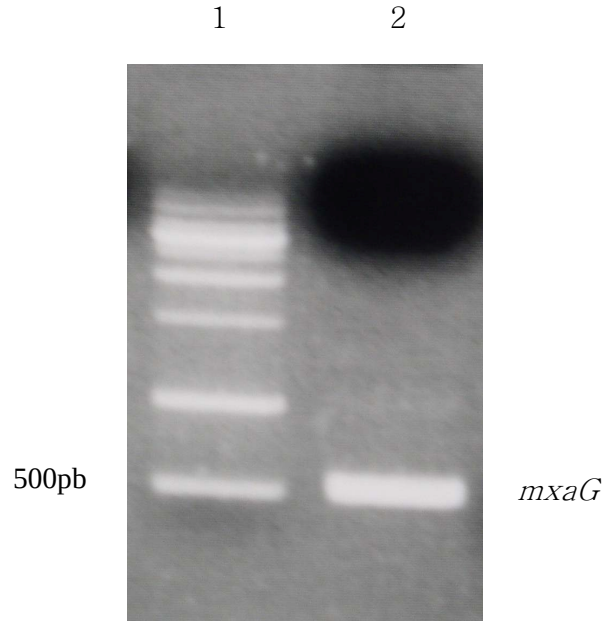


Fig. 4. PCR of *mxaG* gene on 1% agarose gel. Template: 50 ng chromosome DNA of *M. aminosunfidovorans* SK1. Primers (see page 11) and PCR conditions: 94°C for 30 sec to denaturation, 55°C for 30 sec to annealing and 72°C for 1 min to extention for 30 cycle. Lane 1: 1kb DNA maker; lane 2: *mxaG* gene.

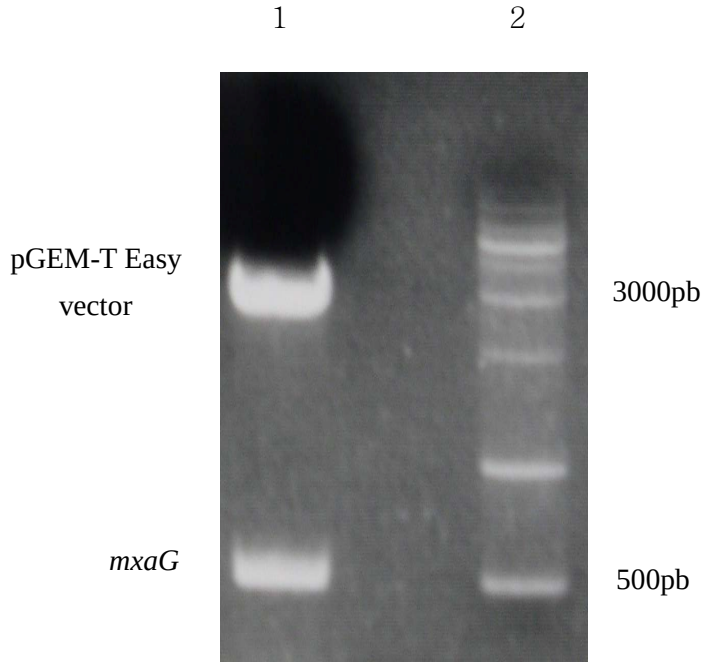


Fig. 5. pGEM- *mxaG* plasmids were digested by *XhoI* and *XbaI* and separated on 1% agarose gel. Lane 1: *mxaG* gene and pGEM-T Easy vector; lane 2: 1kb DNA maker.

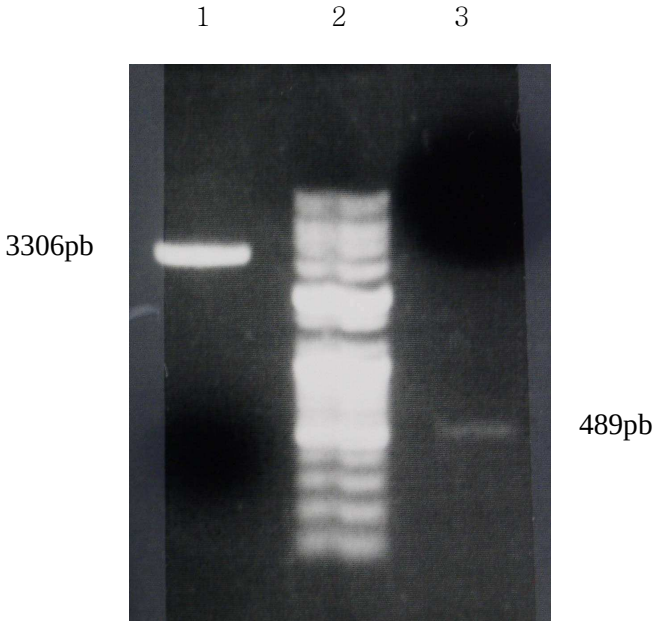


Fig. 6. pASK-IBA6-*mxaG* plasmids were cut by *XhoI* and *XbaI* and separated on 1% agarose gel. Lane 1: pASK-IBA6; lane 2: 100 bp plus DNA ladder and lane 3: *mxaG* gene.

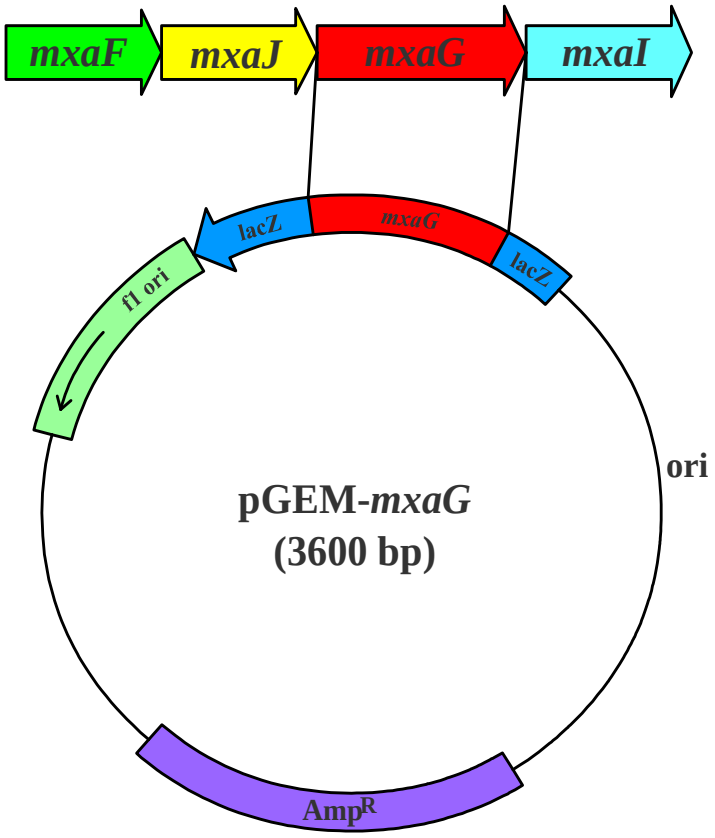


Fig. 7. The construction of the pGEM-*mxaG* for cloning.

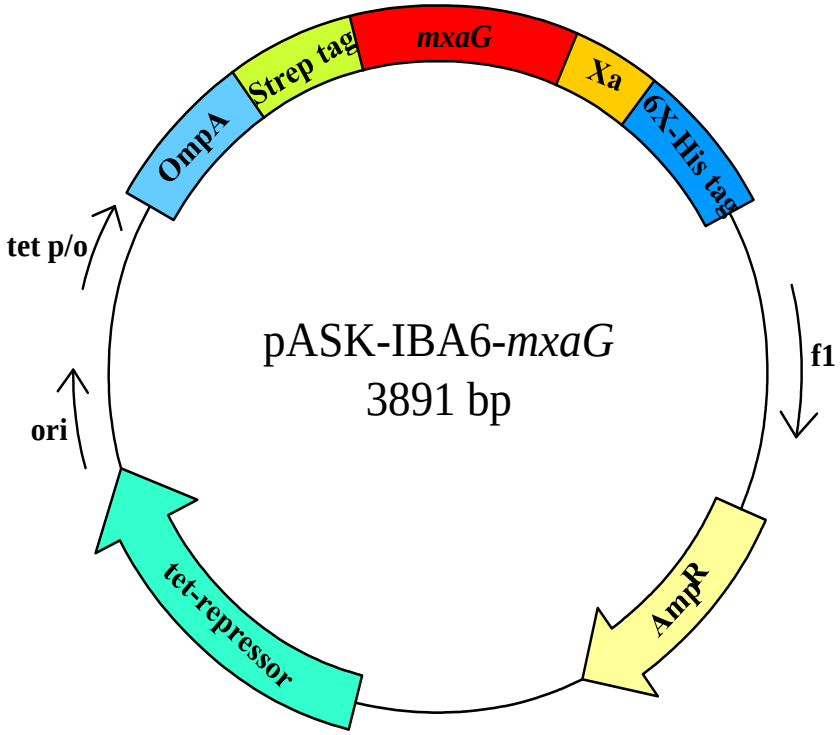


Fig. 8. Map of the expression plasmid for *mxa*G with 6X-His tag.

Table 2. Comparison of the wild type and recombinant cytochrome c_L

	Wild type cytochrome c_L	**Recombinant cytochrome c_L**
Molecular weight (kDa)	21	21
Absorption maxima (nm)		
Ferrocytochrome (α)	551.0	551.5
Ferrocytochrome (β)	521.5	521.0
Ferrocytochrome (γ)	415.5	415.5

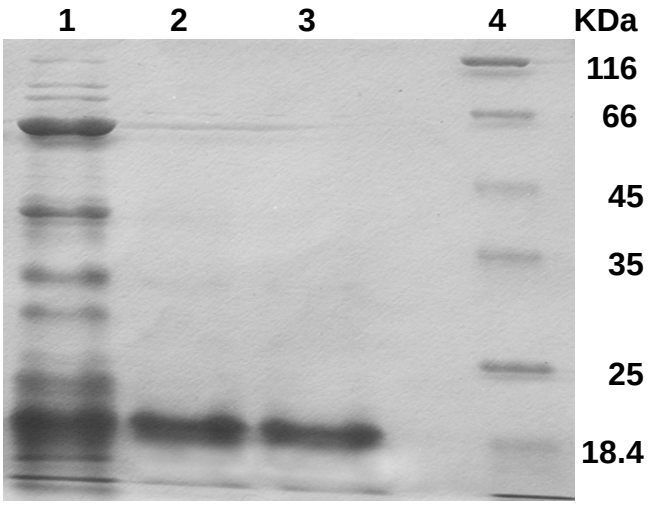


Fig. 9. SDS-PAGE analysis of wild type and recombinant cytochrome c_L. Proteins were separated on a 12.5% gel and stained with 0.1% CBR. Lane 1: cell - free extract; lane 2: purified wild type cytochrome c_L; lane 3: purified recombinant cytochrome c_L; lane 4: protein size marker.

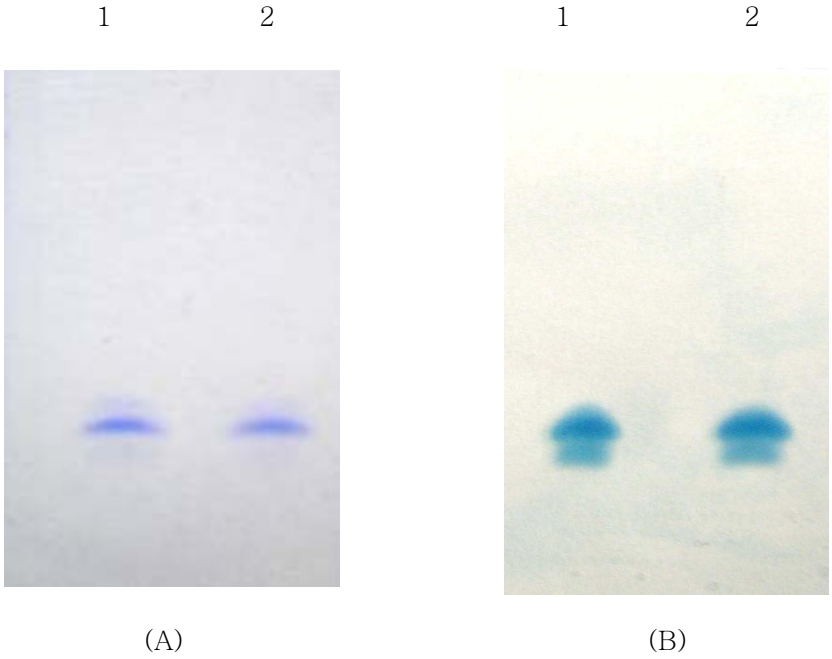


Fig. 10. Native PAGE analysis of wild - type and recombinant cytochrome c_L. Proteins were separated on a 12.5 % gel without SDS and stained with CBR (A) or TMBZ (B). Lane 1: wild type cytochrome c_L; lane 2: recombinant cytochrome c_L.

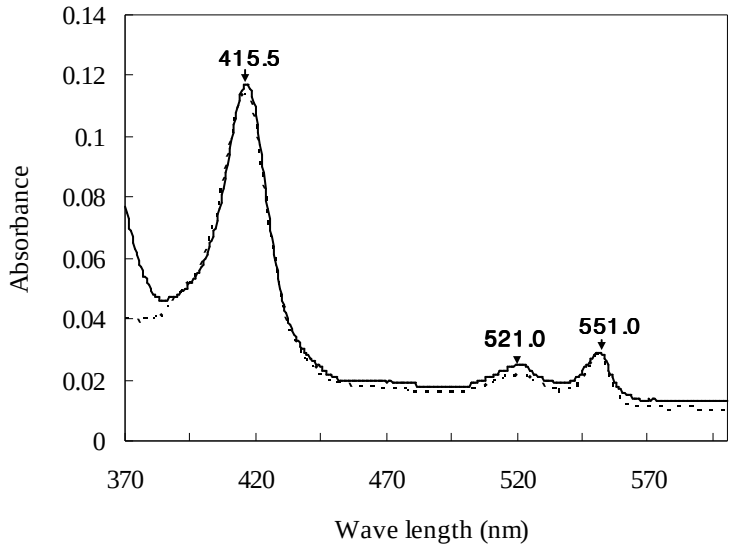


Fig. 11. Comparison of reduced absorption spectra of wild type cytochrome c_L (——) and recombinant cytochrome c_L (-----).

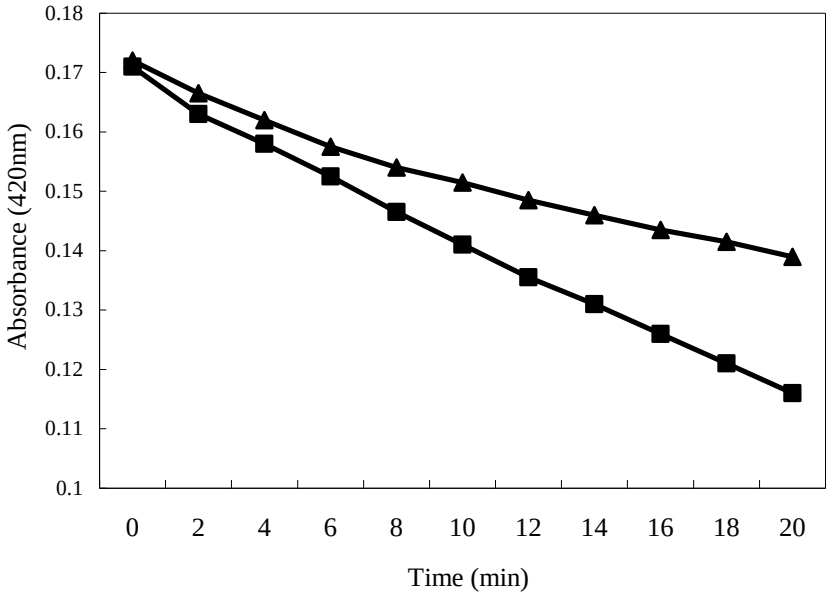


Fig. 12. Reduction of potassium ferricyanide with MDH and cytochrome c_L (wild type or recombinant type). Reduction rates were measured by the decrease of absorbance at 420 nm at room temperature. The assay systems differed from each other as follows: (▲), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, 25 μM wild type cytochrome c_L; (■), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, 25 μM recombinant cytochrome c_L. Reactions were started by the addition of MDH (20 μM).

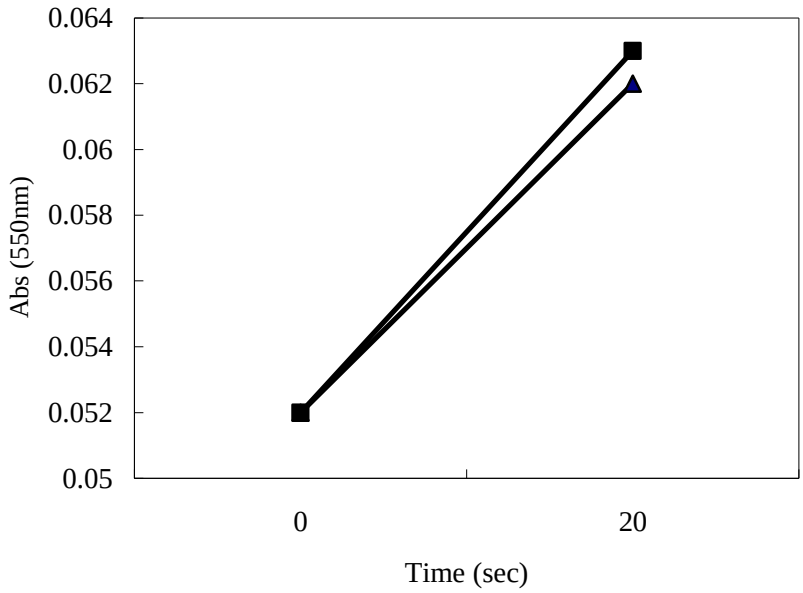


Fig. 13. Comparison of reduction rates of wild type and recombinant cytochrome c_L. Wild type and recombinant cytochrome c_L (25 μM) were oxidized with 3 μM potassium ferricianide and the oxidant was removed by passage through a PD - 10 column. Reaction rates were measured by the increase of absorbance at 551.0 nm. Each reaction mixture contained oxidized wild-type cytochrome c_L (▲) or recombinant cytochrome c_L (■), and reactions were started by the addition of MDH (20 μM).

III

The interaction of MxaJ peptide with Methanol dehydrogenase

A. Materials and Methods

1. Bacteria strains and plasmids

The AH-109 competent yeast cells and pGBKT7-DNA BD vector & pGADT7 AD vector were purchased from Clontech. An *E. coli* strain DH5α was used as host cells for amplification and transformation of recombinant plasmid. The plasmids used in this study are pGEM – T Easy (Promega Corporation).

2. Purification of Methanol dehydrogenase

2.1. Cell free extract of M. aminosulfidovorans SK1

M. aminosulfidovorans SK1 was cultivated in SMM medium. Cells from the late exponential growth phase were harvested at 10,000 x g for 10 min at 4°C and washed by PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM K_2HPO_4, pH 7.0). Later cells were suspended in Tris-HCl buffer (25 mM, pH 7.0, standard buffer) at 4 °C and were disrupted by sonication. When using sonication cytoplasm and periplasmim soluble fractions were obtained. Unbroken cells and debris were removed by centrifugation at 14,000 x g for 20 min at 4 °C. The resulting supernatant was ultra centrifuged at 100,000 x g for 90 min, and the supernatant was used as a soluble fraction.

On the other hand, cells were suspended in pre-cooled buffer P (10 mM Tris, 500 mM sucrose, 1 mM EDTA and 1 mg/ml lyzozyme, pH 8.0) at 4 °C on ice for 30 min. MDH were located in periplasmic membrane so using buffer P to get

periplasmic soluble fractions. After centrifugation at 14,000 x g, 4 °C for 5 min, the clear supernatants were used to purify MDH.

2.2. Purification of MDH

* *Sonication:* The soluble fraction that getting from ultra - centrifugation was continued. The solid $(NH_4)_2SO_4$ was slowly added to this soluble fraction to obtain 50% saturation. After removal of the precipitate by centrifugation at 15,000 x g for 30 min, further $(NH_4)_2SO_4$ was added to obtain 80% final saturation. The precipitants were centrifuged and dissolved in the standard buffer. After dialysis for 16h, they were concentrated with Centricon (YM-10, Amicon, Bedford, USA) and Aquacide II (Calbiochem, Darmstadt, Germany). The concentrate was applied to a POROS 20 HQ anion exchange column which was equilibrated with standard buffer. MDH were eluted with a linear gradient of 0 to 1 M NaCl in standard buffer. MDH containing fractions were pooled, concentrated, and applied to a hydrophobic interaction column which was equilibrated with standard buffer containing 1.5M $(NH_4)_2SO_4$, pH 8.0. After that fractions containing respective proteins were pooled, concentrated, and applied to a gel filtration which was equilibrated with standard buffer containing 0.15M NaCl.

* *P - lysis:* The clear supernatant was applied to POROS 20 HQ anion exchange column which was equilibrated with standard buffer. MDH protein was

eluted with a linear gradient of 0 to 1 M NaCl in standard buffer. Fractions containing respective proteins were pooled, concentrated, and applied to an hydrophobic interaction column which was equilibrated with standard buffer containing 1.5 M $(NH_4)_2SO_4$, pH 8.0. Protein concentration was determined using Bradford reagent with bovine serum albumin as standard.

3. MDH Assay

MDH activity was determined by a spectrophotometric assay with PES and DCPIP as electron acceptors at 30 °C. The component of assay buffer contained 100 mM Tris-HCl, 1 mM KCN, 15 mM NH_4Cl, 6.7 mM CH_3OH, 0.04 mM DCPIP and 1.1 mM PES, pH 9.0. The reaction was initiated by the addition of MDH. MDH activity was determined by measuring the decrease in absorbance of reduced DCPIP (ε = 1.91x 10^7 cm M^{-1}) at 600 nm. One unit of activity was defined as 1 μmol DCPIP reduced per minute under the assay conditions.

4. SDS and Native polyacrylamide gel electrophoresis

Tricine-SDS-PAGE was performed as described by Schagger and Jagow (38). Proteins were separated on a 15% gel and stained with CBR. Native-PAGE was performed using the method of Laemmli (39) but without SDS in the system and no treatment of samples.

5. MDH Activity Staining

Prepare 30 ml MDH activity staining solution contained 100 mM Tris – HCl, pH 9.0 (26 ml), 1.0 mM KCN (1.0 ml), 15 mM NH_4Cl (1.0 ml), 6.7 mM CH_3OH (1.0 ml), 0.04 mM NBT (60 μl), 1.1 mM PES (0.011035 g). After native - polyacrylamide gel electrophoresis were finished, the gels were stained with 30 ml MDH activity staining solution in dark for 15 min.

6. Western Blot Analysis

Transfer buffer (20 mM glycine, 25 mM Tris-HCl, 20% CH_3OH). TBS-T solution (20 mM Tris-HCl, 0.136 mM NaCl, 0.1% Tween 20, pH 7.4). 5% blocking solution (1.5 g Skim milk, 30 ml TBS-T solution). Detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5). 3M paper, PVDF membrane and SDS-PAGE gel were run by Semi-dry transfer system (Southam Warwickshire, England) at 110 mA for 2 h. Transfer membrane was put into 20 ml 5% blocking solution at room temperature for 1 hour and washed by TBS-T solution 3 times for 30 min. Next put in to primary antibody: 5% blocking solution (1:5000) at room temperature for 1 h and washed by TBS-T solution 3 times for 45 min, washing. Next put into anti-rabbit alkaline phosphatase: 5% blocking solution (1: 5000) at room temperature for 1 h and washed by TBS-T solution 3 times for 45 min. Finally, transfer membrane was detected by 5 ml detection buffer containing 10.5 μl NBT (50 mg/ml) and 165 μl BCIP (10 mg/ml) for 15 sec.

7. MxaJ peptide purification from SDS-PAGE gels by sonication extraction

After the bands of MxaJ peptide were localized in 15% gel in SDS-PAGE condition, they were cut out with a razor blade and washed (three times for 5 min) with 2 ml of 250 mM Tris buffer (250 mM EDTA, pH 7.4), followed by three rinses of 5 min with distillated water. The water was removed with a Pasteur pipet and the gel slices were chopped finely with a spatula. Then 1.0 ml of 20 mM Tris buffer, pH 7.4, containing 0.1% (v/v) SDS (the ratio of buffer volume to gel piece volume was approximately 2:1) was added. The samples were sonicated for 10 min, in an ice bath (five or six passes of 30 sec). To separate the sonicated gel from the extraction buffer, 1.5 ml of sample was applied to a Sephadex G-25 resin, equilibrated with 20 mM Tris buffer, pH 7.4, containing 0.1% (v/v) SDS. Prior to the application of the sample, the column was dried by means of centrifugation. The purified proteins were rerun by SDS-PAGE and stained with 0.1% CBR or silver nitrate for densitometric analysis and determination of the percentage recovery (40).

8. Analysis of *N* – terminal amino acid sequence of MxaJ peptide

SDS-PAGE and electrophoretic transfer of peptides onto a PVDF membrane were done. The peptide on the membrane was stained with 0.1% CBR for 1 min. After the membrane containing peptide was visualized, cut out, and the *N*-terminal sequence was directly analyzed by a gas-phase protein sequenator.

9. Polymerase chain reaction (PCR)

PCR were carried by GeneAmp 2400 PCR system (Applied Biosystem, Roche) with the primer sets for *mxaF, mxaI* and *mxaJ* genes. The sequence of primer sets were showed at table 3 and PCR was carried out under the following conditions: 94 °C for 45 sec to denaturalize, 55 °C for 30 sec to annealing and 72 °C for 2 min to extent for 30 cycle with *mxaF*; and 94 °C for 30 sec to denaturalize, 55 °C for 30 sec to annealing and 72 °C for 1 min to extent for 30 cycle with *mxaI* and *mxaJ*. The PCR products were analyzed on 1% agarose gel that contained 0.5 μg/ml of ethidium bromide. PCR products were eluted from the gel using the Gel Extraction Kit from NucleoGen and ligated to the pGEM-T Easy vectors. The ligation mixture was transformed into *E. coli* strain DH5α cells. The transformed cells were plated on LB medium plate that contained 40 μg/ml of X-gal and 50 μg/ml of ampicillin at 37 °C. The pGEM-T Easy vectors that contained *mxaF, mxaI* or *mxaJ* were double digested with *EcoRI* and *XhoI* or with *EcoRI* and *PstI*. The digested products were analyzed on 1% agarose gel and eluted from the gel using the Gel Extraction Kit from NucleoGen. Those DNA fragments were ligated with pGADT7 and pGBKT7 vectors.

Table 3. PCR primer sets for *mxaF, mxaI* and *mxaJ* genes

Order	Primer name	Sequences
1	*mxaF*–start - *EcoRI*	5’-GAATTCATGAGAGAAATGCATCATAGC-3’
2	*mxaF* – stop – *XhoI*	5’-CTCGAGCATGCCGTATTCACCTAAGC-3’
3	*mxaF* – stop – *PstI*	5’-CTGCAGCATGCCGTATTCACCTAAGC-3’
4	*mxaI* –start - *EcoRI*	5’-GAATTCATGATGTTGTCAGGTGCAAC-3’
5	*mxaI* – stop – *XhoI*	5’-CTCGAGTTAATAAACCCATTTACCTG-3’
6	*mxaI* – stop – *PstI*	5’-CTGCAGTTAATAAACCCATTTACCTG-3’
7	*mxaJ* –start - *EcoRI*	5’-TTAGAATCCAATACCAGCACGCTCAAA-3’
8	*mxaJ* – stop – *XhoI*	5’-AAGCTCGAGTTATAAAAGTGGGATGCC-3’
9	*mxaJ* – stop – *PstI*	5’-CTGCAGTTATAAAAGTGGGATGCC-3’

10. Construction of pGADT7 and pGBKT7 vectors with *mxaF, mxaI* or *mxaJ* genes

The principle of the yeast two - hybird system was showed in the figure 14. The pGBKT7 vector expresses proteins fused to amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD). In yeast, fusion proteins are expressed at high levels from the constitutive ADH1 promoter (P_{ADH1}). pGBKT7 also contains multiple cloning sites (MCS) with unique restriction sites in frame with the 3' end of the GAL4 DNA-BD for constructing fusion proteins with a bait protein. pGBKT7 replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2 μ ori, respectively. The vector carries the Kan^r for selection in *E. coli* and the TRP1 nutritional marker for selection in yeast. The p GBKT7 vectors were double digested with *EcoRI* and *PstI* . The digested products were analyzed on 1% agarose gel that contained 0.5 μg/ml of ethidium bromide and were eluted from the gel using the gel extraction kit from NucleoGen. Those pGBKT7 vectors were ligated with *mxaF, mxaI* and *mxaJ* genes. The ligation mixture was transformed into DH5α cells. The transformed cells were plated on LB plate that contained 50 μg/ml of kanamycin at 37 °C.

The pGADT7 vector expresses proteins fused to amino acids 768-881 of the GAL4 activation domain (AD). In yeast, fusion proteins are expressed at high levels from the constitutive ADH1 promoter (P_{ADH1}). The MCS of pGADT7 has unique restriction sites in frame with the 3'-end of the GAL4 AD for constructing

a fusion protein with either a protein of interest or a fusion protein library. The fusion protein is targeted to the yeast nucleus by the SV40 nuclear localization sequences that have been added to the activation domain sequence. pGADT7 replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2 μ ori, respectively. The vector carries Amp^r for selection in *E. coli* and the LEU2 nutritional marker for selection in yeast. The $pGADT_7$ vectors were double digested with *EcoRI* and *XhoI*. The digested products were analyzed on 1% agarose gel that contained 0.5 μg/ml of ethidium bromide and were eluted from the gel using the gel extraction kit from NucleoGen. Those pGADT7 vectors were ligated with *mxaF, mxaI* and *mxaJ* genes. The ligation mixture was transformed into DH5α cells. The transformed cells were plated on LB medium plate that contained 50 μg/ml of ampicillin at 37 °C.

The pGBKT7 and pGADT7 vectors, that contained *mxaF, mxaI* and *mxaJ* genes, were transformed into AH109 competent cells.

11. Yeast transformation procedures

Add 0.1 μg of plasmid DNA with 150 μl of yeast competent cells to a fresh 1.5 ml tube and mix well by vortexing. Add 0.6 ml of sterile PEG/LiAc solution to each tube and vortex at high speed for 10 sec to mix. Incubate at 30℃ for 30 min with sharking at 200 rpm. Add 70 μl of DMSO and mix well by gentle inversion. Do not vortex. Heat shock for 15 min in a 42°C water bath. Chill cells

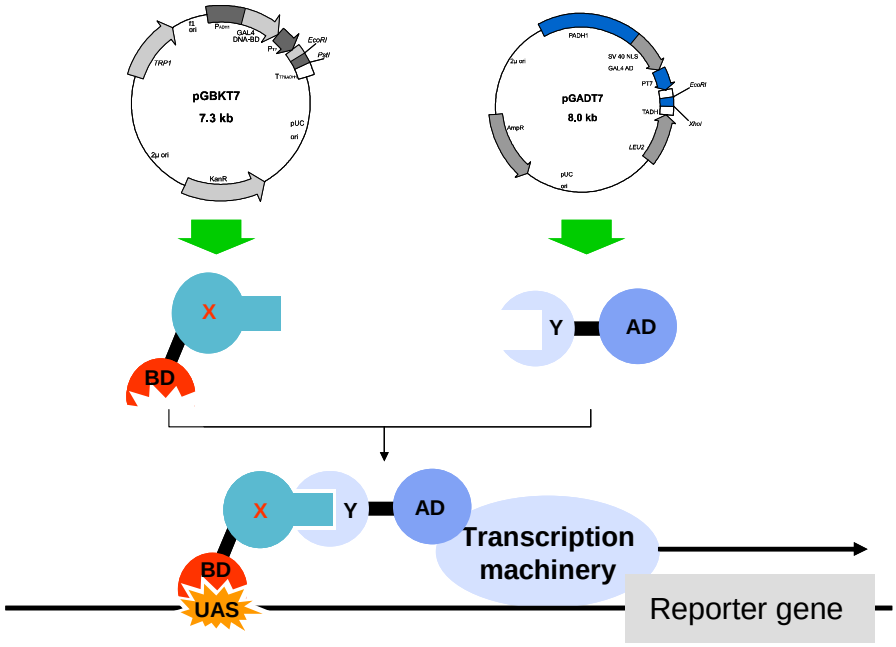


Fig. 14. The principle of the yeast two-hybird system. Two plasmids are constructed, the bait-encoding protein X fused to the *C*-terminus of a transcription factor DNA-binding domain (BD) and the prey-encoding protein Y fused to an activation domain (AD). Alternatively, the prey can consist of proteins encoded by an expression library. Each plasmid is introduced into an appropriate yeast strain either by co-transformation, sequential transformation, or by yeast mating. Only if proteins X and Y physically interact with one another are the BD and AD brought together to reconstitute a functionally active transcription factor that binds to upstream specific activation sequences (UAS) in the promoters of the reporter genes, and to activate their expression.

on ice for 1-2 min and centrifuge cells for 5 sec at 14.000 x g at room temperature. Remove supernatant and suspend cells in 0.5 ml of sterile 1X TE buffer. Plate 100 μl on SD agar plate that will select for the desired transformation. Incubate plates, up-side-down, at 30°C until colonies appear.

12. Materials

Restriction enzymes, DNA ligase, Tag polymerase and alkaline phosphatase were purchased from Takara (Takara Shuco Co., Kyoto, Japan). Phenylmethylsulfonyl fluoride (PMSF), phenazine ethosulfonnate (PES), 2.6-dichlorophenol indophenol (DCPIP) were purchased from Sigma. Polyvinylidene difluoride (PVDF) membrane was purchased from BIORAD.

B. Results and discussion

In the previous study, I found that the difference between MDH I and MDH II fractions was the presence of MxaJ peptide. MDH II contained MxaJ peptide. I wanted to test a possibility that MDH and MxaJ peptides interact each other. During cultivation of *M. aminosulfidovorans* SK1, I have investigated the expression of MDH and MxaJ peptide and measured the specific MDH activity. Figure 15 showed the elution profile of MDH through POROS 20 HQ anion exchange chromatography. The column was equilibrated with 25 mM Tris-HCl, pH 7.0 buffer as standard buffer. MDH protein was eluted with a linear gradient

of 0 M to 1 M NaCl in standard buffer. All fractions containing MDH were analyzed with MDH activity using DCPIP as a terminal electron acceptor. The fractions from 45 to 52 were run on native PAGE 8% gel to make the DMH activity staining using PES as an artificial primary electron acceptor. Figure 16 presented the MDH activity staining of fractions from POROS 20 HQ anion exchange column chromatography. This result showed that all fractions from 45 to 52 had MDH activity. Figure 17 showed the Western blot analysis of MDH with ant - MDH antibody and MxaJ peptide with anti - MxaJ. The fractions from 45 to 48 have not contained MxaJ protein while the fractions from 49 to 52 have remained MxaJ protein, but all of them have MDH activity. Figure 18 showed the growth curve of *M. aminosulfidovorances* SK1 and MDH specific activity was expressed through all growth stage. MDH specific activity was determined by a spectrophotometric assay with PES and DCPIP as electron acceptors. This result showed the highest MDH specific activity at the late exponential phase and early stationary phase. MDH was fully expressed through all growth stage as the same amount, while MxaJ peptide expression decreased substantially at early stationary phase. Otherwise, the specific activity of MDH at early stationary phase also decreased significantly. The inhibitory effect of anti - MxaJ antibody on the activity of MDH was also represented. Therefore, the expression of MxaJ peptide maybe related with the activity of MDH catalyzing methanol to formaldehyde. To understand the presence of MDH and MxaJ peptide, *M.*

aminosulfidovorans SK1 were cultivated on fructose, trimethylamine and methanol. SDS-PAGE analysis of crude extracts and Western blot analysis MxaJ peptide with anti-MxaJ (figure 20) shown that MDH and MxaJ peptide were presented at the same time. It suggests that there existed an interaction between MDH and MxaJ peptides. MxaJ peptide was run on 15% SDS-PAGE and transfer onto a PVDF membrane. The peptide on the membrane was stained with 0.1% CBR for 1 min and then washed. After the membrane containing peptide was visualized (figure 21), it was cut out, and the *N*-terminal sequence was directly analyzed by a gas-phase protein sequenator. Twelve amino acids in the N-terminal of the 30kDa peptide were determined and compared with the amino acid sequences of the *N*-terminal portion in the expected mature protein of the *mxaJ* products of *Hyphomicrobium methylovorum* and *Methylovorus sp.* SS1. As shown in figure 22, 5 amino acid residues in the sequence were identical with those of *H. methylovorum* and *M. sp.* SS1.

Figure 23 showed the PCR of *mxaF, mxaI* and *mxaJ* genes with the primers were presented the table 3. After gel elution, the PCR products were ligated with pGEM-T Easy vectors and transformed into *E. coli* strain DH5α. The plasmids were double digested with *EcoRI* and *XhoI* or *EcoRI* and *PstI* (figure 24) to get *mxaF, mxaI* and *mxaJ* genes. Finally, these genes were ligated with $pGBKT_7$ (digested by *EcoRI* and *PstI*) or $pGADT_7$ (digested by *EcoRI* and *XhoI*) and transformed into *E. coli* DH5α. And they were checked again after plasmid

isolation from *E. coli*. (figure 27). Figure 25 and 26 showed the plasmid construction of *mxaF, mxaJ* and *mxaI* with the pGBKT7 plasmids adjacent to the GAL4 BD DNA and the pGADT7 plasmid adjacent to the GAL4 DNA-binding domain.

In summary, two forms of MDH (I and II) were purified from M. *aminosulfidovorans* SK1. MDH II was MDHI containing an extra 30 kDa protein. The α subunits of both MDH I and MDH II were the same. Thirty kDa peptide was likely to be a product of mxaJ by *N* - teriminal analysis. The purified MDH and over expressed MxaJ peptide could interact in methanol oxidation. MxaJ peptide and α subunit of MDH were detected on the Western blot anti-MxaJ antibody and with anti-MDH antibody, respectively. Western blot analysis of MxaJ with anti-MxaJ antibody was done by growing time - course. The amount of MxaJ gradually decreased into cells to enter the stationary phase.

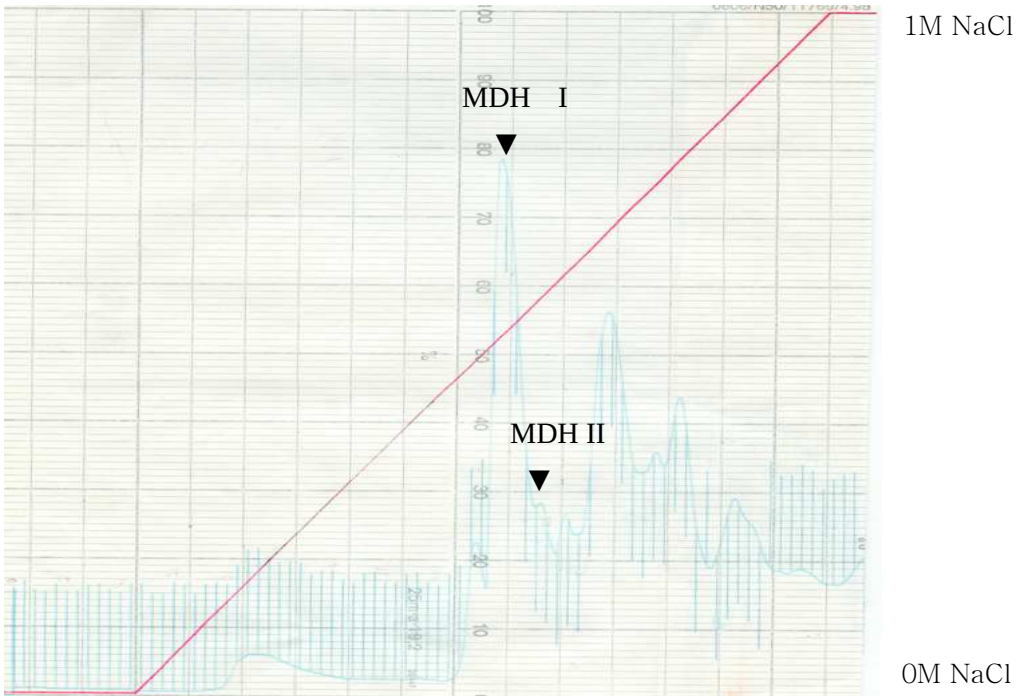


Fig. 15. Elution profile of MDH through POROS 20 HQ anion exchange chromatography. The column was equilibrated with standard buffer. MDH protein was eluted with a linear gradient of 0 M to 1 M NaCl in standard buffer.

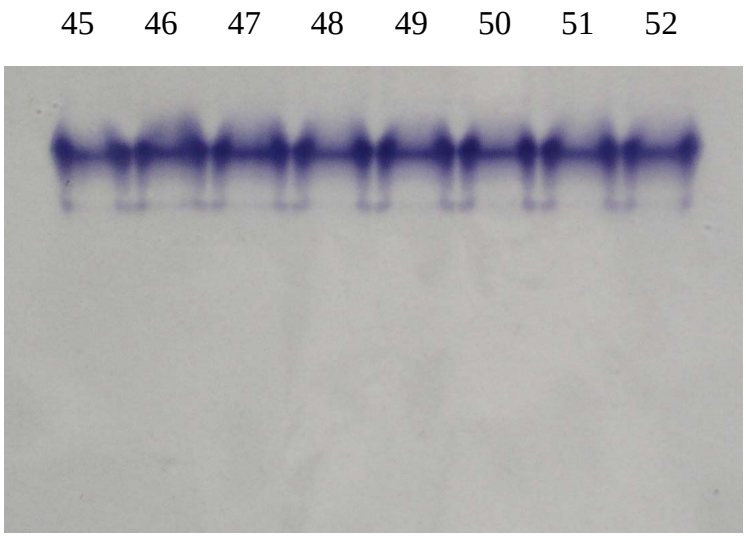


Fig. 16. MDH activity staining. Fractions from POROS 20 HQ anion exchange chromatography. Prepare 30 ml MDH activity staining solution contained 100 mM Tris – HCl, pH 9.0 (26 ml), 1.0 mM KCN (1.0 ml), 15 mM NH4Cl (1.0 ml), 6.7 mM CH3OH (1.0 ml), 0.04 mM NBT (60 μl), 1.1 mM PES (0.011035 g). Native - PAGE was stained with 30 ml MDH activity staining solution in dark for 15 min.

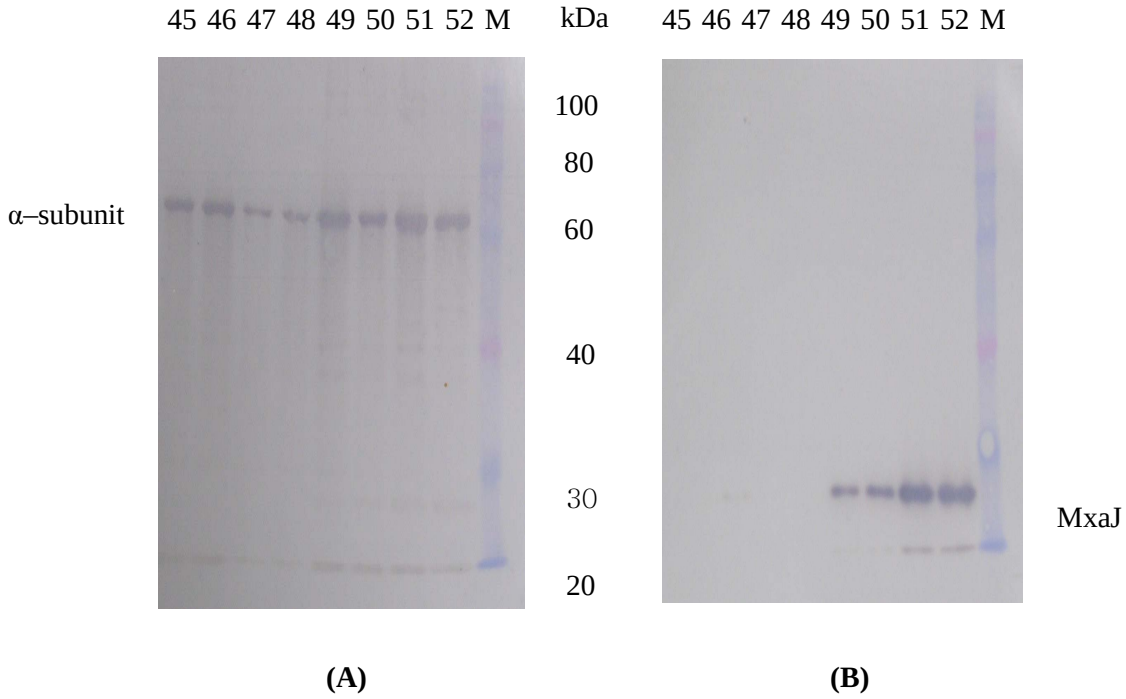


Fig. 17. Western blot assay. Western blot analysis of α-subunit of MDH (A) with anti-MDH antibody and MxaJ peptide (B) with anti-MxaJ. Transfer membrane was detected by 5 ml detection buffer containing 10.5μl NBT (50 mg/ml) and 165μl BCIP (10 mg/ml) for 15 sec.

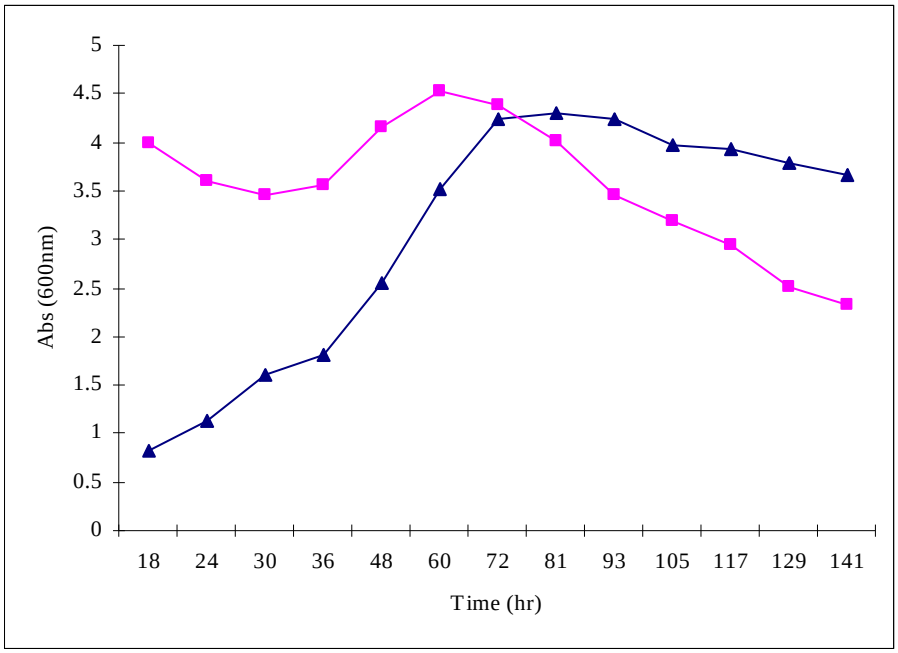


Fig. 18. Relationship between growth curve (▲) and MDH specific activity (■). MDH activity was determined by a spectrophotometric assay with PES and DCPIP as electron acceptors.

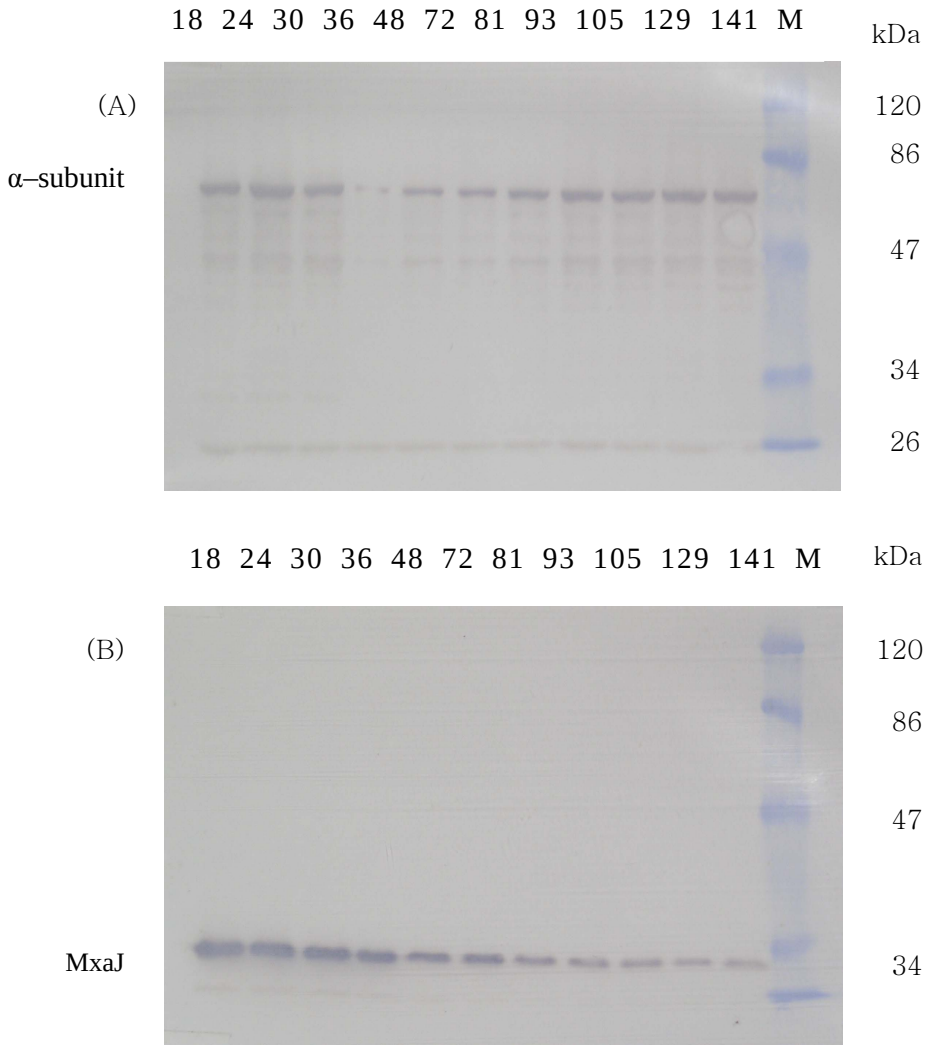
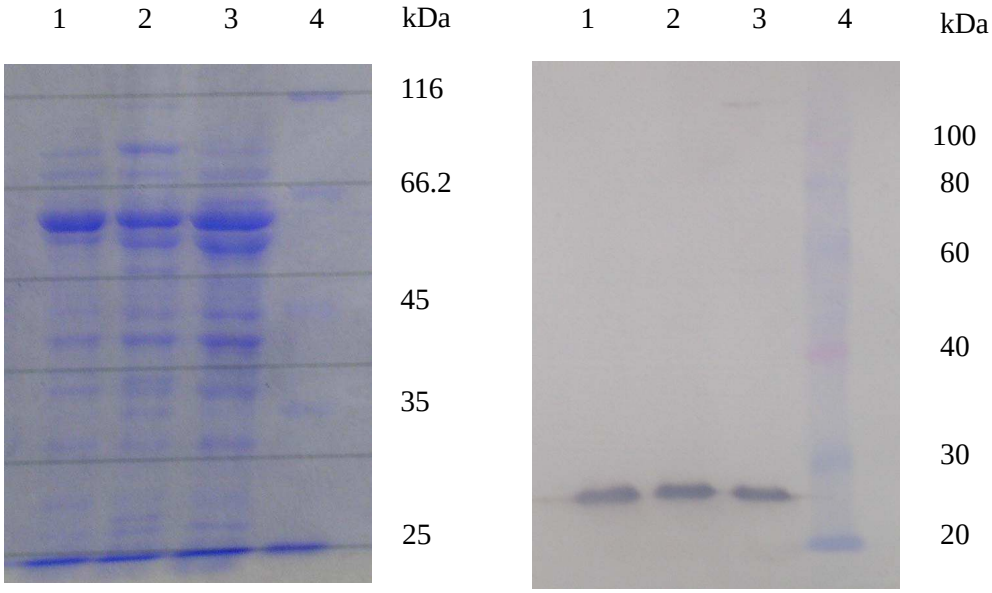


Fig. 19. Western blot analysis of pattern of MDH and MxaJ proteins with anti-MDH polyclonal antibody (A), and anti-MxaJ ploYclonal antibody (B). Second antibody: anti-rabbit AP. Transfer membrane was detected by 5 ml detection buffer containing 10.5 μl NBT (50 mg/ml) and 165 μl BCIP (10 mg/ml) for 15 sec.



(A) **(B)**

Fig. 20. SDS-PAGE analysis and Western blot of cells free extracts of *M. aminosulfidovorans* SK1 growing on different carbon sources. Lane 1: on methanol; lane 2: TMA; lane 3: fructose; lane 4: protein size marker. (A) Proteins were separated on a 15% gel and stained with 0.1% CBR. (B) Western blot analysis MxaJ peptide with anti-MxaJ. Transfer membrane was detected by 5 ml detection buffer containing 10.5 μl NBT (50 mg/ml) and 165 μl BCIP (10 mg/ml) for 15 sec.

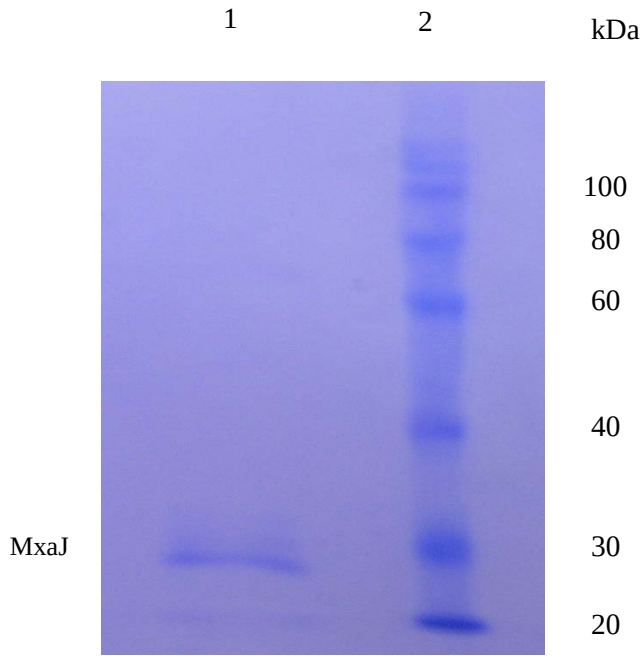


Fig. 21. MxaJ peptide was run on 15% SDS-PAGE and transfer onto a PVDF membrane. The peptide on the membrane was stained with 0.1% CBR for 1 min and then washed. After the membrane containing peptide was visualized, it was cut out, and the *N*-terminal sequence was directly analyzed by a gas-phase protein sequenator. Lane 1: MxaJ peptide, lane 2: protein marker.

M. aminosulfidovorans SK1	NTSNVKLCAAXD
Hyphomicrobium methylovorum	DTSALRVCAAAN
Methylovorus sp SS1	TSADNPLRICAG

Fig. 22. Comparison of *N*-terminal amino acids sequence of 30kDa protein type II MDH with those of the expected mature *mxaJ* products from *Hyphomicrobium methylovorum* and *Methylovorus sp. SS1*.

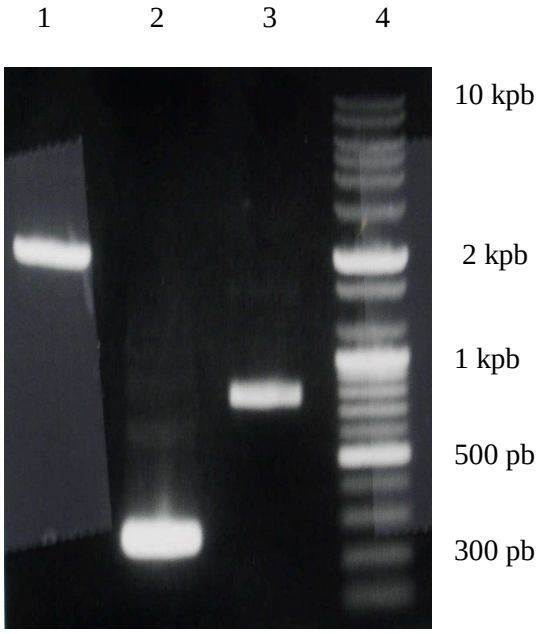


Fig. 23. PCR of *mxaF, mxaI* and *mxaJ* were separated on 1% agarose gel.. Template: 50 ng chromosome DNA of *M. aminosunfidovorans* SK1. Primers (see table 3) and PCR conditions: 94 °C for 45 sec to denaturation, 55 °C for 30 sec to annealing and 72 °C for 2 min to extention for 30 cycle with *mxaF*; and 94 °C for 30 sec to denaturation, 55 °C for 30 sec to annealing and 72 °C for 1 min to extention for 30 cycle with *mxaI* and *mxaJ*. Lane 1: *mxaF* gene; lane 2: *mxaI* gene; lane 3: *mxaJ* gene and lane 4: 100pb DNA plus ladder.

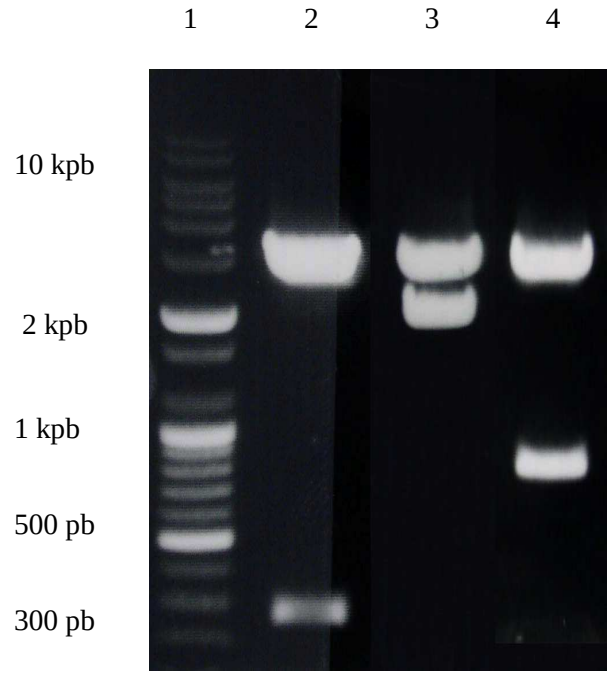


Fig. 24. Double digested by *EcoRI* and *XhoI* and separated on 1% agarose gel. Lane 1: 100pb DNA plus ladder; lane 2: *mxaI* gene and pGEM-T Easy vector; lane 3: *mxaF* gene and pGEM-T Easy vector; lane 4: *mxaJ* gene and pGEM-T Easy vector.

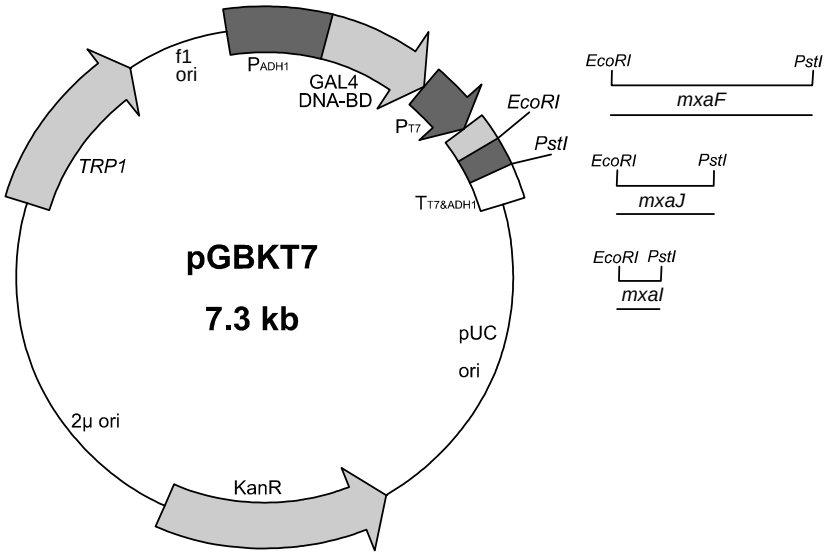


Fig. 25. Plasmid construction: *mxaF, mxaJ* and *mxaI* were inserted into the pGBKT7 plasmids adjacent to the GAL4 BD DNA.

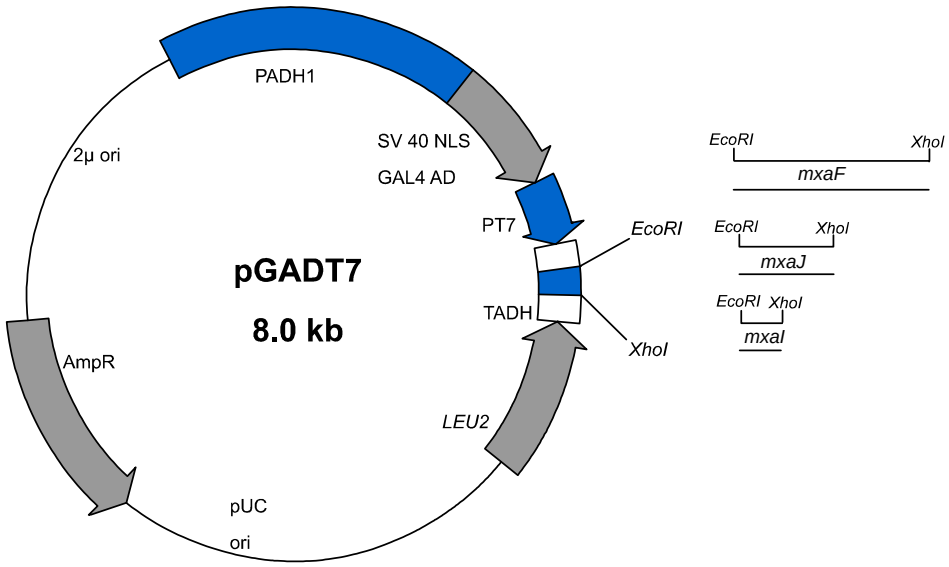


Fig. 26. Plasmid construction: *mxaF, mxaJ* and *mxaI* were inserted into the pGADT7 plasmids adjacent to the GAL4 DNA-binding domain.

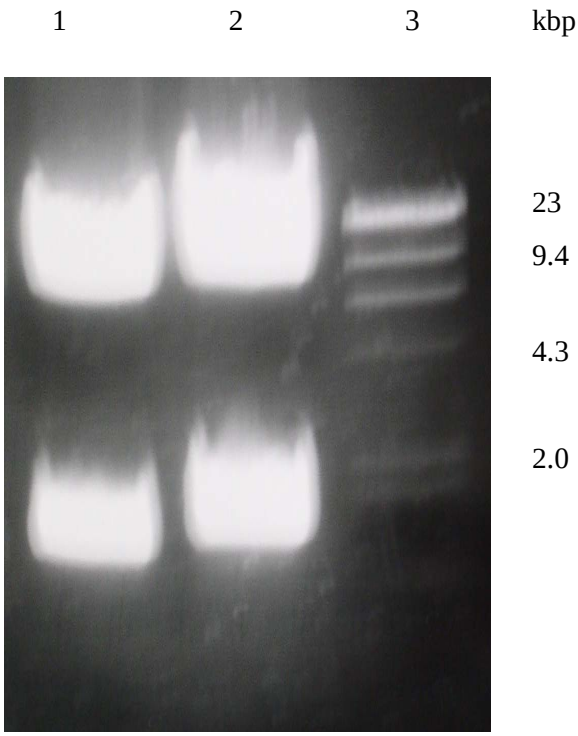


Fig. 27. pGBKT7, pGADT7 and *mxaF* gene were double digested. Lane 1: pGBKT7 vector and *mxaF* gene were cut by *EcoRI* and *Pst1*, lane 2: pGADT7 vector and *mxaF* gene were cut by *EcoRI* and *XhoI*, lane 3: λ Hind III marker.

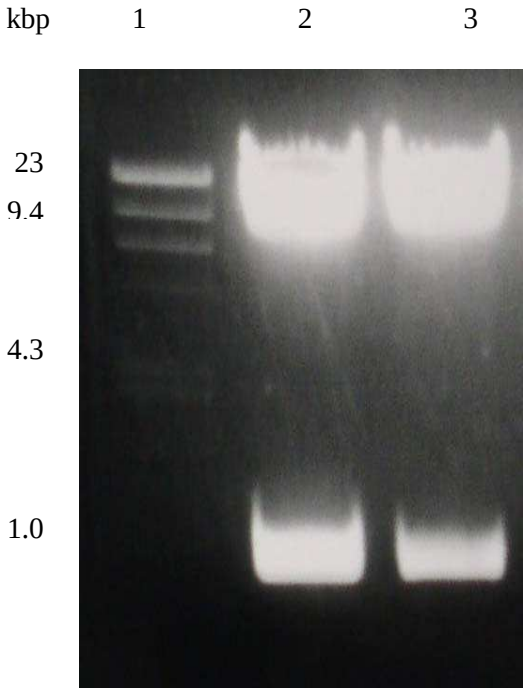


Fig. 28. pGBKT7, pGADT7 and *mxaJ* gene were double digested. Lane 1: λ Hind III marker. lane 2: pGBKT7 vector and *mxaJ* gene were cut by *EcoRI* and *Pst1*, lane 3: pGADT7 vector and *mxaJ* gene were cut by *EcoRI* and *XhoI*,

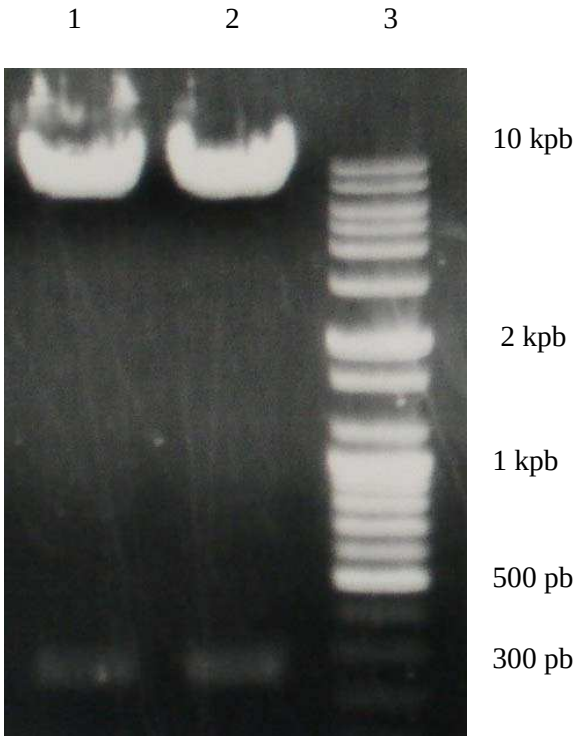


Fig. 29. pGBKT7, pGADT7 and *mxaI* gene were double digested. Lane 1: pGBKT7 vector and *mxaI* gene were cut by *EcoRI* and *Pst1*, lane 2: pGADT7 vector and *mxaI* gene were cut by *EcoRI* and *XhoI*, lane 3: 100pb DNA ladder.

IV. References

[1]. Anthony, C.. The biochemistry of Methylotrophs. *Academic Press* Ltd., London. 1982.

[2]. Anthony, C.. “Bacterial oxidation of methane and methanol.” *Adv. Microb Physiol*. ***27***: 113-210, 1986.

[3]. Murrell, J. C., McDonald, I. R. and Gilbert, B.. “Regulation of expression of methane monooxygenase by copper ions.” *Trends Microbiol* ***8***: 221-225, 2000.

[4]. Moore, G. R., and G. W. Pettigrew.. Cytochrome *c*: Evolutionary, structural and physicochemical aspects. NY: Springer-Verlag. 1990.

[5]. Koh, M. J., C. S. Kim, Y. A. Kim, H. S. Choi, E. H. Cho, E. Kim, Y. M. Kim, S. W. Kim.. “Properties of electron carriers in the process of methanol oxidation in a new restricted facultative marine methylotrophic bacterium, *Methylophaga* sp. MP.” *J. Microbiol. Biotechnol.* ***12***: 476-482, 2002.

[6]. Yamamoto, M., Yoshihiro, S., Kageaki, K., Rokuro, O. and Taiji, I.. “Isolation and characterization of marine methanol - utilizing bacteria.” *J. Ferment. Technol.* **56**: 451- 458, 1978.

[7]. 김시욱, “토양에서 배양한 절대메탄올자화세균의 메탄올 산화에 관한연구.” 박사학위 논문. 연세대학교 생물학과.

[8]. Morris, C. J., Kim, Y. M., Perkins, K. E, and Lidstrom, M. E.. “Identification

and nucleotide sequences of *mxaA, mxaC, mxaK, mxaL* and *mxaD* genes from *Methylobacterium extorquens* AM1." *J Bacteriology*, ***177***: 6825-6831, 1995.

[9]. Laukel, M., Rossignol, M., Borderies, G., Volker, U. and Vorholt, J. A.. "Comparison of the proteome of *Methylobacterium extorquens* AM1 grown under methylotrophic and nonmethylotrophic conditions." *Proteomics*, ***4***: 1247-1264, 2004.

[10]. Day, D. J., Anthony, C.. "Methanol dehydrogenase from *Methylobacterium extorquens* AM1." *Methods Enzymol*, ***188***: 210-216, 1990.

[11]. Liu. Q., Kirchhoff, J. R., Faehnle, C. R., Viola, R. E. and Hudson, R. A.. "A rapid method for the purification of methanol dehydrogenase from *Methylobacterium extorquens*," *Protein Expr. Purif.*. xxx, xxx-xxx, 2005.

[12]. Parker, M. W., Cornish, V. and Best, D. J.. "Purification, crystallization and preliminary X-ray diffraction characterization of methanol dehydrogenase from *Methylosinus trichosporium* OB3b." *Eur. J. Biochem.* ***164***: 223-227, 1987.

[13]. Duine, J. A., Frank, J. and Westerling. J.. "Purification and properties of methanol dehydrogenase from *Hyphomicrobium x*." *Biochim. Biophys. Acta*. ***524***: 277-287, 1978.

[14]. Tanaka, Y., Yoshida, T., Watanabe, K., Izumi, Y. and Mitsunaga, T.. "Cloning and analysis of methanol oxidation genes in the methylotroph

Hyphomiceobium methylovorum GM2." *FEMS Micro. Letters*, ***154***: 397-401, 1997.

[15]. Grosse, S., Voigt. C., Wendlandt, K. D. and Kleber, H. P.. "Purification and properties of methanol dehydrogenase from *Methylosinus* sp. WI 14." *J. Basic Microbiol*. ***38***: 189-196, 1998.

[16]. Anthony, C. and Williams, P.. "The structure and mechanism of methanol dehydrogenase." *Biochim. Biophys. Acta* ***1647***: 18-23, 2003.

[17]. Ghosh, M., Anthony, C., Harlos, K., Goodwin, M. G. and Blake, C.. "The refined structure of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens* at 1.94 angstrom." *Structure* ***3***: 177-187, 1995.

[18]. Xia, Z. X., Dai, W. W., Zhang, Y. F., White, S. A., Buyd, G. D. and Mathews, F. S.. "Determination of the gene sequence and the three dimentional structure at 2.4 angstrom resolution of methanol dehydrogenase from *Methylophinus* W3A1." *J. Mol. Biol.* ***259***: 480-501, 1996.

[19]. Xia, Z. X., Dai, W. W., He, Y. N., White, S. A., Mathews, F. S. and Davidson, V. L.. "X - ray structure of methanol dehydrogenase from *Paracoccus denitrificans* and molecular modeling of its interactions with cytochrome *c*-551i." *J. Biol. Inorg. Chem*, ***8***: 843-854, 2003.

[20]. Nunn, D. N., Day, D. and Anthony, C.. "The second subunit of methanol dehydrogenase of *Methylobacterium extorquens* AM1." *Biochem. J.*, ***260***: 857-862, 1989.

[21]. Munn, D. N. and Anthony, C.. “The nucleotide sequence and deduced amino acid sequence of the cytochrome c_L gene of *Methylobacterium extorquens* AM1, a novel class of c-type cytochrome.” *Biochem. J.*, ***256***: 673-676, 1988.

[22]. Anthony, C.. “The c - type cytochromes of methylotrophic bacteria.” *Biochim. Miophys. Acta*, ***1099***: 1-15, 1992.

[23]. Anthony, C., Ghosh, M. and Blake, C. C.. “The structure and function of methanol dehydrogenase and related quinoproteins containing pyrrolo-quinoline quinone.” *Biochem. J.*, ***304***: 665-674, 1994.

[24]. White, S., Boyd, G., Mathews, F. S., Xia, Z. X., Dai, W. W., Zhang, Y. F. and Davidson, V. L.. “The active site structure of the calcium - containing quinoprotein methanol dehydrogenase.” *Biochemistry*, ***32***: 12955-12958, 1993.

[25]. Lidstrom, M. E., Anthony, C., Biville, F., Gasser, F., Goodwin, P., Hanson, R. S. and Harms, N. “New unified nomenclature for genes involved in the oxidation of methanol in gram - negative bacteria.” *FEMS Microbiol Lett.* ***117***: 103-106. 1994.

[26]. Bastien, C., Machlin, S., Zhang, Y., Donaldson, K. and Hanson, R. S.. “Organization of genes required for the oxidation of methanol to formaldehyde in three type II methylotrophs.” *Appl. Environ. Microbiol*, ***55***: 3124-3130, 1989.

[27]. Harms, N., Reijnders, W. N., Anazawa, H., Van der Palen, C. J., Van Spanning, R. J., Oltmann, L. F. and Stouthamer, A. H.. "Identification of a two - component regulatory system controlling methanol dehydrogenase synthesis in *Paracoccus denitrificans." Mol Microbiol.* ***8***: 457-470, 1993.

[28]. Matsushita, K., Takahashi, K. and Adachi, O.. "A novel quinoprotein methanol dehydrogenase containing an additional 32-kilodalton peptide purified from *Acetobacter mathanolicus*: identification of the peptide as a MoxJ product." *Biochemistry* ***32***: 5576-5582, 1993.

[29]. Machlin, S. M., Tam, P. E., Bastien, C. A., and Hanson, R. S.. "Genetic and physical analyses of *Methylobacterium organophilum* XX genes encoding methanol oxidation." *J. Bacteriol*. ***170***: 141-148, 1988.

[30]. Van Spanning, R. J. M., Wansell, C. W., Boer, T. D., Hazelaar, M. J., Anazawa, H., Harms, N., Oltmann, L. F. and Stouthamer, A. H.. "Isolation and characterization of the *moxJ, moxG, moxI,* and *moxR* genes of *Paracoccus denitrificans*: Inactivation of *moxJ, moxG,* and *moxR* and the resultant effect on methylotrophic growth." *J. Bacteriol*, ***173***: 6948-6961, 1991.

[31]. Morris, C. J. and Lidstrom, M. E.. "Cloning of a methanol – inducible *moxF* promoter and its analysis in *moxB* mutant of *Methylobacterium extorquens* AM1rif." *J. Bacteriol*. ***174***: 4444-4449, 1992.

[32]. Nunn, D. N. and Lidstrom, M. E.. "Isolation and complementation analysis

of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1." *J. Bacteriol.* ***166***: 581-590, 1986.

[33]. Nunn, D. N. and Lidstrom, M. E.. "Phenotypic characterization of 10 methanol oxidation mutant classes *Methylobacterium* sp. strain AM1." *J. Bacteriol.* ***166***: 591-597, 1986.

[34]. Richardson, I. W. and Anthony, C.. "Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion." *Biochem. J.* ***287***: 709-715, 1992.

[35]. Causier, B.. "Studying the interactome with the yeast two - hybird system and mass spectrometry." *Mass Spec Rev*. ***23***: 350-367, 2004.

[36]. Coates, P. J. and Hall, P. A.. "The yeast two-hybird system for identifying protein – protein interactions." *J. Pathology*. ***199***: 4-7, 2003.

[37]. Thomas, P. E., D. Ryan, and W. Levin.. "An improved staining procedure for the detection of the peroxidase activity of cytochrome P450 on sodium dodecyl sulphate polyacrylamide gels." Anal. Biochem. ***75***: 168-176, 1976.

[38]. Schagger, H. and Jagow, G. V.. "Tricine - sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa." *Anal Biochem*, ***166***: 368-379, 1987.

[39]. Laemmli, U. K.. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature*, ***227***: 680-685, 1970.

[40]. Retamal, C. A., Thiebaut, P. and Alves, E. W.. “Protein purification from polyacrylamide gels by sonication extraction.” *Analytical Biochemistry*, ***268***: 15-20, 1999.