

Isolation and Molecular Analysis of Methanol Oxidation Genes in an Obligate Methylophilic Bacterium, *Methylobacillus* sp. Strain SK-5

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Abstract Methanol dehydrogenase (MDH) is a key enzyme in the process of methanol oxidation in methylophilic bacteria. However, information on MDH genes from genus *Methylobacillus* is limited. In this study, a 6.5-kb *Hind*III DNA fragment of *Methylobacillus* sp. SK-5 chromosomal DNA was isolated from the genomic library of the strain by using a degenerate oligonucleotide probe that was designed based on *N*-terminal amino acid sequence of the MDH α subunit purified from the strain. Molecular analysis of the fragment revealed four tightly clustered genes (*mxafJGI*) involved in the methanol oxidation. The first and fourth genes were very similar to *mxaf* (77% identity for nucleotides and 78% identity for amino acids) and *mxal* (67% identity for nucleotides and 68% identity for amino acids) genes, respectively, from *Methylovorus* sp. SS1. Genes *mxaf* and *mxal* encode α and β subunits of MDH, respectively. The two subunits were identified from purified MDH from *Methylobacillus* sp. SK-5. A dendrogram constructed by comparison of amino acid sequences of MDH α subunits suggests that Mxaf from *Methylobacillus* sp. SK-5 belongs to a subfamily cluster of MDH α subunits from β -subgroup *Proteobacteria*. The subfamily cluster is separated from the other subfamily that consists of α - and γ -subgroup *Proteobacteria*. This study provided information on MDH genes from a methylophilic bacterium in β -subgroup *Proteobacteria*, which would aid to better develop a gene probe to detect one-carbon metabolizing bacteria.

Key words: Methanol dehydrogenase (MDH), *mxaf*, PQQ, *Methylobacillus*

Methylophilic bacteria are capable of growth on reduced one-carbon (C1) compounds (e.g., methanol, methane,

methylamine, etc.) as the sole carbon and energy source. In Gram-negative methylophilic bacteria, methanol is oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MDH, EC 1.1.99.8), which has pyrroloquinoline quinone (PQQ) as a prosthetic group and cytochrome c_1 as its primary electron acceptor. MDH consists of two identical dimers of large and small subunits in an $\alpha_2\beta_2$ conformation [4, 7, 9, 14, 17].

Methanol oxidation genes have so far been analyzed mainly in α -subgroup *Proteobacteria* such as *Methylobacterium extorquens* AM1 and *Paracoccus denitrificans*. Among 32 genes known to be required for methanol oxidation [15], two chromosomal genes encode MDH; *mxaf* and *mxal* encode large (α) and small (β) subunits of MDH, respectively.

Use of *mxaf* as a potential gene probe to detect methylophilic and methanotrophic bacteria has been studied, based on conserved amino acid sequence identified by comparative analysis of known sequences of the MDH α subunit [18]. So far, complete *mxaf* genes have been isolated from only five methylophilic bacteria, and their nucleotides were sequenced; *M. extorquens* AM1 [2, 3], *M. organophilum* XX [16], *P. denitrificans* PD1207 [12], *Hyphomicrobium methylovorum* GM2 [23], and *Methylophilus methylophilus* W3A1 [27]. The former four strains belong to the α -subgroup of *Proteobacteria* while the latter belong to the β -subgroup. Despite the ecological significance of genus *Methylobacillus* as a dominant population in an aerated lagoon used for wastewater treatment [28], information on the MDH gene in the genus is limited. A complete nucleotide sequence of *mxaf* from a methylophilic strain belonging to the genus has not been reported. Therefore, it is necessary to accumulate more information on methanol oxidation genes from phylogenetically diverse bacteria to better develop gene probes to detect methylophilic and methanotrophic bacteria in the environment.

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In this study, we isolated methanol oxidation genes (corresponding to *mxoFJGI*) from a β -subgroup methylotrophic bacterium, *Methylobacillus* sp. SK-5, and analyzed them at the molecular level, particularly focusing on *mxoF* [18]. The information obtained in this study would aid to develop a functional gene probe to detect methylotrophs and methanotrophs.

MATERIALS AND METHODS

Strains and Plasmid

Methylobacillus sp. SK-5 was previously isolated from soil by virtue of its ability to utilize methanol as a carbon and energy source [13]. The strain was grown in NMS mineral salt solution [26] with 1% (v/v) methanol at 30°C. *E. coli* DH5 α cells were cultured at 37°C on LB medium and used as a host strain for the gene cloning experiment [20, 21, 22]. Vector plasmid pUC19 was used for the cloning experiment and recombinant DNA was introduced into *E. coli* DH5 α by electroporation [8]. Transformed *E. coli* DH5 α cells were grown on LB medium supplemented with ampicillin (50 μ g/ml).

Chemicals and Enzymes

All chemicals were purchased from Sigma Chemical Company (St. Louis, U.S.A.) unless otherwise specified. DEAE-Toyopearl was obtained from Supelco (Bellefonte, U.S.A.) while Mono-S and Superose 12 were from Amersham Biosciences (Uppsala, Sweden). Standard proteins for SDS-PAGE were purchased from Sigma.

[α -³²P]dCTP purchased from Amersham Biosciences was used for radiolabeled gene probe preparations. Restriction endonucleases, T₄ DNA ligase, and bacterial alkaline phosphatase were obtained from Takara Shuzo Co., Ltd. (Shiga, Japan).

Enzyme Purification

Methylobacillus sp. SK-5 cells were harvested when the optical density of the culture reached 1.0 at 600 nm. Harvested cells were washed with 20 mM Tris-HCl buffer (pH 8.0) and disrupted by two passages through French Pressure Cell (SLM Instruments, Urbana, U.S.A.). Cell debris was removed by centrifugation at 15,000 \times g for 15 min, while the membrane fraction was removed by further centrifugation at 100,000 \times g for 60 min at 4°C. The supernatant was used for enzyme (MDH) purification at 4°C. Ammonium sulfate was slowly added to the supernatant to give 60% saturation. After removal of the precipitate by centrifugation (15,000 \times g, 60 min), (NH₄)₂SO₄ was further added to give 85% saturation. The precipitated proteins after centrifugation were dissolved in 20 mM Tris-HCl (pH 8.0) buffer and subjected to dialysis. The dialysate was concentrated with Centricon (Millipore,

Bedford, MA, U.S.A.) and applied to a DEAE-Toyopearl column equilibrated with 20 mM Tris-HCl buffer. Pre-eluted MDH with 20 mM Tris-HCl buffer was applied to a Mono-S column that was equilibrated with phosphate buffer (pH 6.0) and eluted with a 0–100 mM linear gradient of NaCl in phosphate buffer. Fractions containing the enzymatic activities were pooled, concentrated, and applied to a FPLC Superose 12 HR 10/30 column which was equilibrated with 20 mM Tris-HCl buffer containing 0.15 M NaCl. The purified MDH was determined by 15% SDS-PAGE.

Enzyme Assay

MDH activity was determined by a modified spectrophotometric method [4] by measuring decrease of absorbance of the substance, 2,6-dichlorophenol indophenol (DCPIP), at 600 nm. Protein concentration was determined by the Bradford method [6].

Determination of N-Terminal Amino Acid Sequence

Purified MDH was separated into its subunits by 15% SDS gel electrophoresis and blotted to a polyvinylidene fluoride membrane. N-Terminal amino acid sequence was determined by a sequencer (Model 473A, Applied Biosystems) method of Edman and Begg [9].

DNA Extraction and Cloning

Genomic DNA of *Methylobacillus* sp. SK-5 was extracted by the method of Goldberg and Ohman [10] and plasmid DNA was isolated by the alkaline lysis method [5]. Extracted genomic DNA was partially digested with *Hind*III and subjected to agarose gel electrophoresis. DNA fragments in the desired size range (approximately 5–10 kb) were cut out from the agarose gel and extracted using a QIAGEN Gel Extract Kit (QIAGEN, Valencia, CA, U.S.A.). *Hind*III-digested plasmid pUC19 was treated with bacterial alkaline phosphatase and ligated to fractionated genomic DNA with T₄ ligase. Ligated DNA was transformed into competent *E. coli* DH5 α cells.

Transformants (ampicillin-resistant white colonies) containing gene *mxoF* were screened using a degenerate oligonucleotide probe, 1F [AACCA(A/G)GA(C/T)CTGCA(A/G)AACCTG]. The probe was designed based on N-terminus amino acid sequence of the MDH α subunit purified from *Methylobacillus* sp. SK-5. Probe F1 (100 pmol) was 5'-end labeled with [α -³²P] ATP and T₄ kinase according to the manufacturer's instruction. Colony hybridization was performed using the labeled F1 probe. Hybridization was performed at 42°C for 16 h with hybridization buffer containing 6 \times SSC (0.15 M sodium chloride and 0.015 M sodium citrate), 1 \times Denhardt solution, 100 μ g/ml yeast tRNA, and 0.05% sodium pyrophosphate. Final washes were performed at increasing temperatures (55 to 65°C) in a 1 \times SSC. Autoradiographs were obtained by exposing hybridized filters to Fuji RX films for 16 to 24 h.

Nucleotide Sequence Determination and Analysis

The isolated 6.5-kb *Hind*III DNA fragment was sequenced by a DNA sequencer (Applied Biosystems Model 373A, Foster City, U.S.A.) using a *Taq* DyeDeoxy Terminator Cycle sequencing kit. Custom-made oligonucleotides were used for primer walking. The resulting DNA sequence was screened against GenBank database. BLAST (version 2.0) [1] was used to search the most similar sequences in the database. DNA sequences were aligned and analyzed by DNASIS program (Hitachi, Tokyo, Japan). Evolutionary distances were calculated using the program CLUSTAL X and a dendrogram was constructed by using the program CLUSTAL X [24].

RESULTS AND DISCUSSION

Properties of MDH

The native MDH had a molecular mass of 130 kDa and consisted of two identical subunits in an $\alpha_2\beta_2$ conformation. Each of the α and β subunits had molecular masses of 63 and 8 kDa, respectively. Purified MDH from *Methylobacillus* sp. SK-5 showed broad substrate specificity with alcohols. Compared to its activity with methanol, its activity on alcohols containing more than three carbons was relatively high, while its activity on formaldehyde was relatively low (only 7% of MDH activity with methanol) (Table 1). MDH from other strains showed 78% or more activity with formaldehyde, compared to its activity with methanol [11]. Activity of MDH from *Methylobacillus* sp. SK-5 increased with carbon number in the alcohols tested in this study, showing the highest activity with 1-octanol. MDH from *Methylocystis* sp. GB25 showed decreasing activity with higher carbon number in the alcohols [11].

Isolation of Methanol Oxidation Genes of *Methylobacillus* sp. SK-5

The genomic library of *Methylobacillus* sp. SK-5 was constructed. A total of 1,200 recombinant clones containing

Table 1. Substrate specificity of methanol dehydrogenase from *Methylobacillus* sp. SK-5.

Substrate ^a	Relative activity (%) ^b	Substrate ^a	Relative activity (%) ^b
Methanol	100	1-Hexanol	144
Ethanol	100	1-Heptanol	152
1-Propanol	97	1-Octanol	159
1-Butanol	118	Formaldehyde	7
1-Pentanol	119		

^aFinal concentration of each substrate was 6.7 mM.

^b100% corresponds to 1.5 unit/mg of protein. One unit is defined as the amount of enzyme that catalyzes the reduction of 1 μ mol DCPIP per minute. Enzyme activities were obtained as average values from three independent experiments.

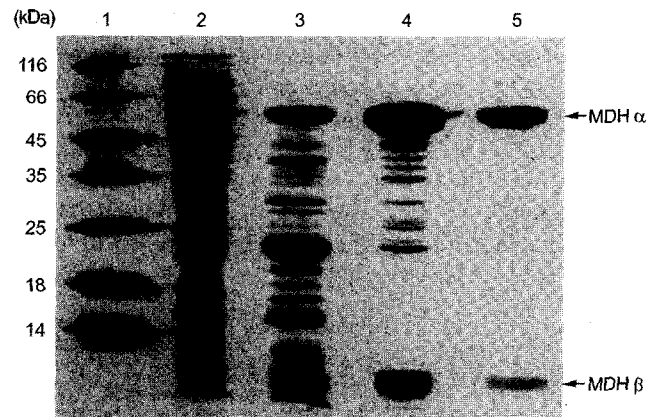


Fig. 1. SDS-polyacrylamide gel electrophoresis of MDH from *Methylobacillus* sp. SK-5 during purification steps.

Lane 1, size markers; lane 2, cell free extracts obtained from ultracentrifugation of *Methylobacillus* sp. SK-5 lysate; lane 3, DEAE-Toyoperal pool; lane 4, Mono-S pool; lane 5, purified MDH after gel filtration.

*Hind*III DNA fragments from the strain were screened by colony hybridization using the probe 1F, a degenerate oligonucleotide probe designed based on the *N*-terminal amino acid sequence of MDH α subunit purified from *Methylobacillus* sp. SK-5 (Fig. 1). One clone showing a positive signal from the hybridization was obtained as a result of the screening. The clone was designated pMDH and used for further study.

Clone pMDH contained a *Hind*III DNA insert in the size of approximately 6.5 kb (Fig. 2). A restriction map of pMDH was obtained. Nucleotide sequence of the entire 6.5-kb *Hind*III DNA fragment was determined as described in Materials and Methods.

Nucleotide Sequence Analysis of MDH Gene and Intervening Genes

Nucleotide sequence analysis revealed that the *Hind*III DNA fragment of pMDH contained four complete open reading frames (ORF) involved in methanol oxidation (Figs. 2, 3). The four ORF were designated genes *mx*aF, *mx*aJ, *mx*aG, and *mx*aI, since they showed high similarity to genes *mx*aFJGI from *Methylovorus* sp. SS1 (unpublished, GenBank accession No. AAD56237) in the GenBank

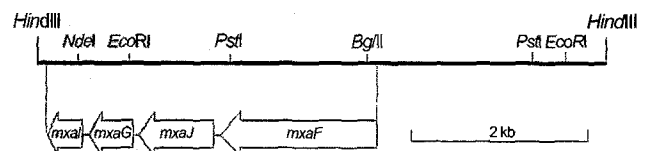


Fig. 2. Location of genes *mx*aFJGI in 6.5-kb *Hind*III DNA fragment isolated from *Methylobacillus* sp. SK-5 chromosome. Arrows indicate transcriptional directions of the genes. Restriction sites in the fragment are indicated. The *lac* promoter on the cloning vector pUC19 is located on the left of the map.

database. The putative *mxoF* (encoding MDH α -subunit) and *mxoI* (encoding MDH β -subunit) showed the highest similarities with *mxoF* and *mxoI* from the strain SS1: *mxoF*, 77% identity for nucleotides and 78% identity for amino acids; *mxoI*, 67% identity for nucleotides and 68% identity for amino acids. Genes *mxoF* and *mxoI* from *Methylobacillus* sp. SK-5 were separated by two intervening genes, *mxoJ* (encoding a protein with unknown function)

and *mxoG* (encoding cytochrome c_1). Their identity with the strain SS1 was as follows: *mxoJ*, 55% for nucleotides and 47% for amino acids; *mxoG*, 67% for nucleotides and 68% for amino acids. Cytochrome c_1 is the electron acceptor for MDH [7].

The newly identified *mxoF*, *mxoJ*, *mxoG*, and *mxoI* gene ORF were 1.8, 0.8, 0.5, and 0.3-kb long, respectively (Fig. 3). They were transcribed in the same direction (Fig. 2).

ATACAGCGG	GTGCAACGCC	CCCGGGTACA	AGCCAATAAG	AAATTGAGCA	ACGCCGGATG	60	<i>MxoJ</i>		
CTGGCGAGCG	GTTATCAGTT	TATCCACCOC	GATTATGTAT	CGGGATATGG	TGCCITTGCTT	120	ATGCATATTG	TGATAAGAA	ATCCTGTATC
GCTGCAATGA	GCTGACCOCG	ATTGCTGTGA	CCAATGAAGA	AAACGCCACA	AGGGCAGCCT	180	<i>M H I V D K K S C I S A V K A A L V G V</i>		
TGTGGCGTT	TTTATTTAGC	GGCAGTTCAA	CATAATCCTT	AGGCGTTTCG	GGTGATTTTC	240	GCCGGAATGC	TGGCCTTGCA	GCGCCAGGCG
CCGAAGGGGA	TATTTTAAAT	ATAGCAATAT	GC0CCAGCCA	TTTAGAGGGA	GAGTCACTAG	300	<i>A G M L A L O A O A A D E L R V C A G K</i>		
CAGTTTAGCT	ATAAGCCCTG	TAAATATCAT	GCTCCCGCGC	AAGGCTGGGT	CACGACATTG	360	GACGAGTTGC	CTTACTCAA	TGACAAGCAA
GTGTGAATAC	TTTCTATCT	AATCGGCAAT	CTTTCATCTG	TTGCACTTGT	TACAAATTGT	420	D E L P Y S N D K Q Q G F E N E I A K V		
TAAAATTCT	TTTGCOCGAG	TAACAACCGA	TCGGCAGGGA	GTTTGTAAATC	AAACCAGAAAT	480	GTGGCAAGG	CCATGAACCG	CAAGGCTCA
GAGGAGAAGT	TCT					492	V G K A M N R K V S F V W W S D A R Y S		
							GTCCAGGAT	TTCTTGACAA	GAAGCAGTGT
<i>mxoF</i>							V R D F L D K K Q C D V L L G L D K G D		
ATGAAAGGCA	GAGTAACCCA	CGTCGGTATC	AGCGCTGCTG	TCAGCAGTCT	GCTGGTGCCT	552	GCACGGTCC	TGAATACCAA	GACCTACTAT
<i>M K G R V T H V G I S A A V S S L L V L</i>						612	P R V L N T K T Y Y K S G Y V F V T R K		
GCGACGATGC	AAGGTGCTCA	GGCAAAACCA	GATCTGCAAA	ATCTGACAAA	GAACGCAGAC	672	GACAGGGAAA	TAGATATTAC	CTCTGGGAT
<i>A T M O G A O A N Q D L Q N L T K N A D</i>						732	D R E I D I T S W D H P Y L K E R N F R		
AACTGGGCTT	TGCAAAACCG	TAACACTACT	GGTCAGCACA	ATAGCAGACT	GAGCCAAATC	792	CTGGGTTCT	TACCTGACAG	TCCTGCCAAG
N W A L Q T G N Y T G Q H N S T L S Q I						852	GACATGTTGC	ACTATGTGAC	CGAGCTGACC
AATAAGGCA	ACGTCAAGAA	CCTGAAGGCA	GCTTGGTCTT	TC0CCACCGG	CGTGTTCGAC	912	D M F D Y L T E L T D F K S T R N R Y I		
N K G N V K N L K A A W S F S T G V L H						972	AAATGTAGC	AGCCGAGCT	GGTCAATGAC
GGTCAEBAAG	GCGGCCATT	GGTGAATGGC	GACATGATGT	ACATCCACAG	TGGTTC0CCT	1032	K I D E R K L V N D V I T M K L H A A A		
G H E G A P L V I G D M M Y I H S A F P						1092	ATTTGGGGCC	CGACAGTTCG	AAAGTATGTG
AACAACACAT	TCGCTGTTAA	CTTGAATGAC	CCTGGCCTGA	TTG0CCTGCCA	GCACAAGCCA	1152	I W G P T V A K Y V A E S P T P L N M V		
N N T F A V N L N D P G V I A W G H K P						1212	CTGATCGAAG	ACAACGCCAC	GCGTGCCAAC
AAGCAAAATCG	CTTC0GTTAA	GGCTGTGCA	TGTTCCGACA	TCGTTAA0CG	CGGTTTGGCT	1272	L I E D N A T R A N G S K I P M Q Y E V		
K Q I A S V K A V A C C D I V N R G L A						1332	G T A A T G G G C	T A C G G C T T G G	CGACGAA0CC
TACGGCGA0G	ACAAGTCTG	CAAGACTCAG	CTGGACGGCA	AGCTGGTTGC	CCTCGATGCC	1392	V M G V R L G D E A L K A E L D N A I T		
Y G D G K I V K T Q L D G K L V A L D A						1452	TCTAGCCAGC	AAGAGATTGA	TCCGATTCTC
AACTCCGGTA	ABGTGCTGTG	GGAAATCGAA	GATATGATC	CTAAGGTTGG	TGCAACACTG	1512	S S Q Q E I D A I L K R E H I P L L P I		
K S G K V V W E I E V C D P K V G A T L						1572	TA0TTTTTTT	GTAACCTCCGG	GGTAGTAA
ACCCAAGCTC	CATTGATTGT	AAAGAACTACT	GTTCTGGTTG	GCTGTC0CGG	TGCTGAG0CTG	1632			
T Q A P F I V K N T V L V G C S G A E L						1692			
GGTGTTCGTC	GTGCTGTTAA	CTCCTTCAAC	CTGAAGACCG	GCGAACTGCA	ATGGC0TGCC	1752			
G V R G A V N S F N L K T G E L Q W R A						1812			
TTTGCAACTG	GTCCGACGCA	ABAGTGGCC	TTGGCCAA0A	ACTTCAACAG	CGACAATCCA	1872			
F A T G P D E E V R L A K K N F N S D N P						1932			
CACACGGTCT	AATTGGCCTG	GGGCTGAAA	ACCTGGGAAG	GTGATGCATG	GAAGATCGGT	1992			
H Y G Q F G L G L K T W E G D A W K I G						2052			
GGCGGCACCA	ACTGGGCTG	GTAC0GCTTAC	GATC0TAAGC	TGAACCTGTT	CTACTACGGT	2112			
G G T N W G W Y A Y D P K L N L F Y Y G						2172			
TC0CGTAACC	CAGCGCCATG	GAAGCAAA0C	ATGGCTCCTG	GGCAACA0A	GTGGACCATG	2232			
S G N P A P W N E T M R R P G D N K W T M						2292			
ACTATCTGGG	CGCGTACCT	GGACACAGGG	GAAGCCAA0T	GGGGTACCA	AAAGACTCCT	2352			
T I W A R D L D T G E A K W G Y Q K T P						2379			
CACGACGAGT	GGGACTTCGC	TGG0CGTGAAC	CAAATGATCC	TTTCCGATCA	CAAGGTTGAT				
H D E W D F A G V N Q M I L S D H K V D									
GGCAAGGTAA	CTCCTCTGCT	GACCACATCG	GACC0TAA0C	GCATCATGTA	CAC0CGTAA0G				
G K V T P L L T H I D R N G I M Y T L N									
CGTACAAGCG	GTAACCTGAT	CCAGGCTGCC	AAGGTTGATC	CAGCCGTA0A	CGTGTTC0AAG				
R D N G N L V Q A A K V D P A V N V F K									
AAAGTTGACC	TCAAGACAGG	TACGCCAGTA	CGTGACCCTG	AGTTCAGCAC	ACGCATGGAC				
K V D L K T G T P V R D P E F S T R M D									
CACAAGAGCA	CCAACGATG	TC0TTC0GCG	ATGGGTTTCC	ACAACCAAGG	TCTGGATGCA				
H K S T N V C P S A M G F H N Q G L D A									
TTGGATCTTG	ACGAGCCTAT	CGTTTACGCA	GGCCTGAACC	ACATTTGTAT	GGATTTGGGAG				
L D L D E P I V Y A G L N H I C M D W E									
COCTTCATGC	TGCCCTACCG	TGCCGGTACG	TTCTTCGTAG	GTGCAACCCT	GCGCATGTAC				
P F M L P Y R A G Q F F V G A T L A M Y									
CCTGGACCTA	GCGGCCAAC	CAAGAAGGAA	ATGGGTCAAG	TACGCGCCAT	GGACATCGTG				
P G P S G P T K K E M G Q V R A M D I V									
ACAGGCAAGT	ACAAGTGGAC	CAAGTGGGAG	AAGTTCGCTG	TTTGGGCGGG	TACTCTCGCT				
T G K Y K W T K W E K F A V W G G T L A									
ACCAAGGGCG	GCGTGGTTGC	CTACAACCG	CTTGATGGCT	ACATCAAGGC	CCTGACAAGG				
T K G G V A Y N T L D G Y I K A L D K									
GACAACGGCA	AGGAACGTGT	GAAGTTCAAG	ATGCCATCCG	GTGGTATCCG	TGCTCCTATG				
D N G K E L W K F K M P S G G I G A P M									
ACCTTACCAGT	TCAAGGGTAA	GCAATACATC	GGCTCCATGT	ACGGTGTAGG	TGGTTGGCCT				
T Y Q F K G K Q Y I G S M Y G V G G W P									
GGCGTTGGTC	TGTTATTCGA	CCTGACC0AT	CCTAG0GCTG	GCTG0GGTGC	TGTAGGTTGCC				
G V G L V F D L T D P S A G L G A V G A									
TTCAAGGAGC	TGACAGACCA	CACCTCAAATG	GGCGGTGGCC	TGATGGTGT	CAGCCTGTAA				
F K E L Q N H T Q M G G G L M V F S L									
TTTCAAGTAA	GCAAGCTGAT	GTGATATTCC	GGGGGAGCAG	GTTCGCCCGG	GTATCTAAGT				
GAAATTCGGT	TTTGAATTG	AGTACAC							

Fig. 3. The complete nucleotide sequence of genes *mxoF*, *mxoJ*, *mxoG*, and *mxoI*. The genes *mxoF*, *mxoJ*, *mxoG*, and *mxoI* encode methanol dehydrogenase (MDH) α subunit, a protein with unknown function, cytochrome c_1 , and MDH β subunit, respectively. The nucleotide sequences of the genes were deposited in GenBank under the accession no. AF494424. Underlined nucleotide sequences are putative ribosomal binding sites. Putative signal sequence of each gene is italicized and arrows indicate inverted repeats. The asterisks indicate stop codons.

The intergenic regions separating *mxajfjgi* were 87-, 25-, and 98-bp long, respectively. An inverted repeat was found in each of the intervening regions between *mxaf* and *mxaj* and between *mxag* and *mxal* (Fig. 3). They might form a step-loop or hairpin structure in the mRNA.

The gene order, *mxajfjgi* found in *Methylobacillus* sp. SK-5, were identical with those in the other four strains belonging to α -subgroup of *Proteobacteria* (*M. extorquens* AM1 [2, 3], *M. organophilum* XX [16], *P. denitrificans* PD1207 [12], *H. methylovorum* GM2 [23]). Gene *mxaf* in *P. denitrificans* forms a gene cluster with the *mxaj*, *mxag*, *mxal*, *mxar*, and *mxas* genes in that order [25]. Genes

mxajfjgirs in *M. extorquens* AM1 were found upstream of gene cluster *mxackld* [19]. Gene *mxaf* forms a gene cluster with the *mxajgirs* in *H. methylovorum* GM2 [23].

Deduced amino acids of gene *mxaf* from *Methylobacillus* sp. SK-5 were 599 amino acids (Fig. 3). The *N*-terminal amino acid sequence (NQDLQNLTKNADNVALQTG) of purified MDH α subunit was identified in the region of N29-G47 of the gene *mxaf*. The *N*-terminal region, M1-A28, had a feature of the signal sequence previously found in *mxaf* from *H. methylovorum* GM2 [23], which serves to translocate the enzyme into the periplasm during biosynthesis.

<i>M. sp.</i> SK-5	MKGRVTHVG	SAAVSSLLVL	ATMGGAGANQ	D-LQNLTKNA	DNWALQTGNY	TGQHNSTLSQ	INKGNVKNLK	69
<i>M. methylophilus</i> W3A1			MADA	D-LDKQVNTA	GAWPIATGGY	YSQHNSPLAQ	INKSNVKNVK	43
<i>M. sp.</i> SS1	MKARITATGF	--AVAGLALS	AMLPVSAAAA	DSLEALGANP	NNWTMTQGDY	TGQHYSLRSQ	ITNGVKNLE	68
<i>M. extorquens</i> AM1	MSRFVTS	VSAAMLALAP	AALSSGAYAN	DKLVELSKSD	DNWVMPGKNY	DSNFSDLKQ	INKGNVQLR	68
			* *	*	*	* * *	* * * * *	
<i>M. sp.</i> SK-5	AAWS-FSTGV	LHGHEGAPLV	IGDMMYIHSA	FPNNTFAVNL	NDPGVIWQH	KPKQIASVKA	VACCDIVNRG	138
<i>M. methylophilus</i> W3A1	AAWS-FSTGV	LNGHEGAPLV	IGDMMYVHSA	FPNNTYALNL	NDPGKIVWQH	KPKQDASTKA	VMCCDVDRG	112
<i>M. sp.</i> SS1	AGLGHFSTGL	LTGHEGAPLV	IGDMMYINTP	FPNNTFALNL	AEPEKIVWQH	KPKQDASVKA	VACCDIVNRG	138
<i>M. extorquens</i> AM1	PAWT-FSTGL	LNGHEGAPLV	VDGKMYIHST	FPNNTFALGL	DDPGTILWQD	KPKGNPAARA	VACCDIVNRG	137
	****	* *****	**	***** *	* * * *	****	* * * * * *	
<i>M. sp.</i> SK-5	LA--YGDGK-	---IVKTQLD	GKLVALDAKS	GKVVWEIEVC	DPKVGATLQ	APFIVKNTVL	VGCSGAELGV	202
<i>M. methylophilus</i> W3A1	LA--YGAGG-	---IVKKQAN	GHLALDAKT	GKINWEVEVC	DPKVGSTLQ	APFVAKDTVL	MGCSGAELGV	176
<i>M. sp.</i> SS1	LA--YDDGH-	---IFKTQLD	GHLVASDAKT	GKELWKMENC	DPAVGSTITQ	APFVAKGKVL	VGCSGAELGV	202
<i>M. extorquens</i> AM1	LAYWPGDGKT	PALILKTQLD	GNAALNAET	GETVWVENS	DIKVGSTLTI	APYVVKDKVI	IGSSGAELGV	207
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<i>M. sp.</i> SK-5	RGAVNSFNLK	TGELQWRAFA	TGPDEEVLRA	KNFNSDNPHY	GQFGLGLKTW	EGDAWKIGGG	TNWGWYAYDP	272
<i>M. methylophilus</i> W3A1	RGAVNAFDLK	TGELKWRAFA	TGSDDSVRLA	KDFNSANPHY	GQFGLGKTKW	EGDAWKIGGG	TNWGWYAYDP	246
<i>M. sp.</i> SS1	RGYVTAYDQK	TGELVWRSFA	TGPDNELNLA	KDFNKDNPHY	GQMGGLGKTKW	EGEGWKIGGG	TNWGWYAYDP	272
<i>M. extorquens</i> AM1	RGYLTAYDVK	TGEOVWRAFA	TGPDKDLLLA	SDFNINKPHY	GQKGLGTGTW	EGDAWKIGGG	TNWGWYAYDP	277
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<i>M. sp.</i> SK-5	KLNLFFYYGSG	NPAPWNETMR	PGDNKWTMTI	WARDLDTGEA	KWGYQKTPHD	EWDFAGVNGM	ILSDHK-VDG	341
<i>M. methylophilus</i> W3A1	KLNLFFYYGSG	NPAPWNETMR	PGDNKWTMTI	WGRDLDTGMA	KWGYQKTPHD	EWDFAGVNGM	VLTDQP-VNG	315
<i>M. sp.</i> SS1	KLNLFFYYGSG	NPAPWNETMR	PGDNKWTMTI	WARDVDTGAA	KWGYQKTPHD	EWDFAGVNGM	ILTDQA-VNG	341
<i>M. extorquens</i> AM1	GTNLIFYGTG	NPAPWNETMR	PGDNKWTMTI	FGRDADTGEA	KFGYQKTPHD	EWDFAGVNGM	MLSEKDKDG	347
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<i>M. sp.</i> SK-5	KVTPLLTHID	RNGIMYTLNR	DNGNLVQAAK	VDPAVNVFKK	VDLKTGTPVR	DPEFSTRMDH	KSTNVCPDSAM	411
<i>M. methylophilus</i> W3A1	KMTPLLSHID	RNGILYTLNR	ENGNLVAEK	VDPAVNVFKK	VDLKTGTPVR	DPEFSTRMDH	KGTNICPSAM	385
<i>M. sp.</i> SS1	KTQPLLTHVD	RNGIMYTLNR	TGSIYVQAAK	VDPAVNVFKK	VDLKTGLPVR	DPEFSTRMDH	KGTNICPSAM	411
<i>M. extorquens</i> AM1	KARKLLTHPD	RNGIVYTLDR	TDGLVLSANK	LDDTVNVFKS	VDLKTGTPVR	DPEYSTRMDH	LAKDICPSAM	417
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<i>M. sp.</i> SK-5	GFHNQGLDAL	DLDEPIVYAG	LNHICMDNEP	FMLPYRAGGF	FVGATLAMYP	GPNGPTK--K	EMGQVRAMD	479
<i>M. methylophilus</i> W3A1	GFHNQGLDSD	DPSRRTFYFG	LNHICMDNEP	FMLPYRAGGF	FVGATLAMYP	GPNGPTK--K	EMGQIRAFDL	453
<i>M. sp.</i> SS1	GFHNQGLDAY	DPSRRTFYFG	LNHICMDNEP	FMLPYRAGGF	FVGATLAMYP	GPNGPTK--K	EMGQVLRAMD	479
<i>M. extorquens</i> AM1	GYHNQGHDSY	DPKRELFMIG	LNHICMDNEP	FMLPYRAGGF	FVGATLAMYP	GPNGPTK--K	EMGQVLRAMD	487
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<i>M. sp.</i> SK-5	VTGKYKWTW	EKFAVWGGTL	ATKGLLVAYN	TLDGYIKALD	KDNGKELWKF	KMPSSGIGAP	MTYQFKGKY	549
<i>M. methylophilus</i> W3A1	TTGKAKWTW	EKFAVWGGTL	YTKGLLVHVA	TLDGYIKALD	KDNGKELWKF	KMPSSGIGAP	MTYSFKGKY	523
<i>M. sp.</i> SS1	VTGEVKTW	EKFSVWGGTL	ATKGLLVFYN	TLDGNIKALD	KTNKKEIKWF	KMPSSAIGAP	MSYAKGKY	549
<i>M. extorquens</i> AM1	ITGDYKWEK	ERFAVWGGTM	ATAGDLVYFG	TLDGYLKARD	SDTGDLWKF	KIPSSAIGAP	MTYTHKGTQ	557
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<i>M. sp.</i> SK-5	IGSMYGVGGW	PGVGLVFDLT	DPSAGLVAVG	AFKELQNHQ	MGGGLMVFSL			599
<i>M. methylophilus</i> W3A1	IGSMYGVGGW	PGVGLVFDLT	DPSAGLVAVG	AFRELQNHQ	MGGGLMVFSL			573
<i>M. sp.</i> SS1	IATNYGVGGW	PGVGLVFDLT	DPSAGLVAVG	AFKELQNYTQ	MGGGVVVFGL			599
<i>M. extorquens</i> AM1	VAIYGVGGW	PGVGLVFDLA	DPTAGLVAVG	AFKELQNYTQ	MGGGVVVFSL			607
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Fig. 4. Amino acid sequence alignment of four methanol dehydrogenase α subunits.

Source strain of each sequence is as follows: *M. sp.* SK-5, *Methylobacillus* sp. SK-5; *M. methylophilus* W3A1, *Methylophilus methylophilus* W3A1; *M. sp.* SS1, *Methylobacterium extorquens* AM1. Symbol * indicates conserved amino acids in the active sites of all methanol dehydrogenases. Overlined residues indicate potential amino acids involved in tryptophan-docking motifs. The asterisks indicate conserved amino acid residues in the alignment.

The cleavage site occurred after the sequence Ala-Xxx-Ala, a feature conserved in all of the signal peptides for periplasmic Mxa polypeptides [3]. The putative *mxoF* promoter sequence (-35 -AAAGAGC-, -10 -TAGAA-) observed in *M. extorquens* AM1, *M. organophilum*, and *P. denitrificans* was not found in the 5' upstream region of *mxoF* isolated from *Methylobacillus* sp. SK-5 [23].

The deduced amino acid sequence of gene *mxoG* encoding cytochrome *c_L* showed the typical heme-binding site (-CSGCH-) at position 79 through 83 (Fig. 3), which was formed by two closely linked cysteine residues and a consecutive histidine residue [20].

Comparative Analysis of Deduced Amino Acid Sequences of *mxoF* Genes

A methylotrophic bacterium *Methylobacillus* sp. SK-5 is an obligate methanol-oxidizer [13]. MDH is involved in a key step in methanol oxidation, catalyzing conversion of methanol to formaldehyde that serves as an intermediate of both assimilative and dissimilative metabolism in methylotrophs. Considering that MDH is a tetramer composed of $\alpha_2\beta_2$ and its active site is in α subunit, the gene encoding the α subunit is a potential source to develop a functional gene probe that can be used to detect C1-utilizing bacteria.

The deduced amino acid sequences of genes corresponding to *mxoF* were aligned for comparison (Fig. 4). The amino

acid sequences showed key amino acids (Asn 283, Glu 323, Arg 352, and Asn 415) at the active site of the MDH α subunit. The key amino acids in the active site are completely conserved among all MxaF sequences reported. Potential amino acids involved in the tryptophan docking motifs were also found. They are suggested to form a planar stabilizing girdle of interactions around the periphery of the subunit [18].

The deduced amino acid sequence of gene *mxoF* from *Methylobacillus* sp. SK-5 was compared with those of other MDH α subunits to construct a dendrogram to show their phylogenetic relationship (Fig. 5). The dendrogram contained two subfamilies of the proteins. One subfamily (lower part of the dendrogram) contained MDH α subunits of α - and γ -subgroups *Proteobacteria*, while the other (upper part of the dendrogram) contained those of β -subgroups *Proteobacteria*. The dendrogram clearly showed a separate cluster of MDH α subunits of β -subgroup *Proteobacteria*.

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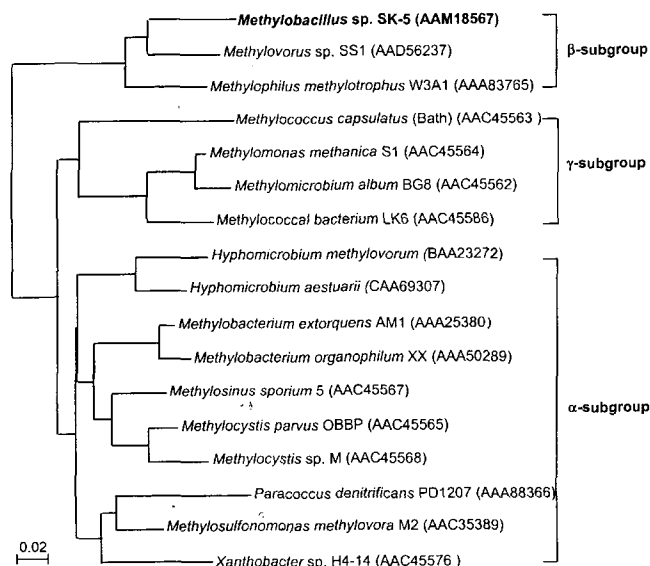


Fig. 5. Phylogenetic relationship of *mxoF* gene products (partial, 172 amino acids; accession number in brackets).

The dendrogram was generated by comparison of derived amino acid sequences of methanol dehydrogenase (MDH) α subunits, using the program CLUSTAL X. Since only 5 complete sequences of MDH α subunits have been reported, the partial sequence (172 amino acids) of each source was compared. The sequences were retrieved from the GenBank database. Scale bar represents 2% sequence divergence. Phylogenetic positions of each strain based on 16S rDNA genes are indicated by α , β , and γ for the subgroups of *Proteobacteria*.

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