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Purification, Characterization, and Cloning of Trimethylamine Dehydrogenase from *Methylophaga* sp. Strain SK1

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Abstract Trimethylamine dehydrogenase (TMADH, EC 1.5.99.7), an iron-sulfur flavoprotein that catalyzes the oxidative demethylation of trimethylamine to form dimethylamine and formaldehyde, was purified from *Methylophaga* sp. strain SK1. The active TMADH was purified 12.3-fold through three purification steps. The optimal pH and temperature for enzyme activity was determined to be 8.5 and 55°C, respectively. The V_{max} and K_m values were 7.9 nmol/min/mg protein and 1.5 mM. A genomic DNA of 2,983 bp from *Methylophaga* sp. strain SK1 was cloned, and DNA sequencing revealed the open reading frame (ORF) of the gene coding for TMADH. The ORF contained 728 amino acids with extensive identity (82%) to that of *Methylophilus methylotrophus* W_3A_1.

Keywords: trimethylamine, trimethylamine dehydrogenase, *tmd* gene, *Methylophaga* sp. strain SK1

INTRODUCTION

Trimethylamine is toxic to animals including humans because of its tissue-corrosive and tissue-penetrative properties. Trimethylamine has also been shown to inhibit the synthesis of macromolecules such as DNA, RNA, and proteins, and to have teratogenic effects on animal embryos [1]. Elimination of trimethylamine from contaminated environments by using microorganisms, therefore, would be beneficial to mankind. Methylotrophic bacteria are a group of microbes that can grow aerobically on compounds that contain one or more carbon atoms, but not compounds having carbon to carbon bonds. Obligate methylotrophs will grow only on C_1 compounds, whereas facultative methylotrophs are able to grow on a variety of multicarbon substrates.

Three different enzymes capable of oxidizing trimethylamine have been identified in methylotrophs. The majority of methylotrophs that use trimethylamine as a sole carbon source utilize trimethylamine monooxygenase to generate trimethylamine-*N*-oxide, which is subsequently converted to dimethylamine and formaldehyde by trimethlyamine-*N*-oxide demethylase catalysis [2,3]. In obligate methylotrophs and in some restricted facultative methylotrophs, however, trimethylamine is oxidized by a nicotinamide-independent trimethylamine dehydrogenase (TMADH, EC 1.5.99.7) [2]. TMADH is a complex iron-sulfur flavoprotein enzyme that transfers electrons to a soluble flavoprotein known as electron transferring flavoprotein [4]. In one case, a nicotinamide-dependent TMADH has been reported [5]. Since Colby and Zatman first purified the nicotinamide-independent TMADH [6], this enzyme, obtained from *Methylophilus methylotrophus* W_3A_1, has been extensively studied. The TMADH is composed of two identical 83,000-dalton subunits, each of which folds into three different structural domains [7]. The largest domain, at the NH_2 terminus of the molecule, is folded as an eight-stranded parallel α/β barrel and contains the [4Fe-4S] and covalently bound FMN cofactors [8]. The medium and smaller domains bear a striking resemblance to the FAD and NADPH-binding domains of glutathione reductase.

We have isolated a novel marine methylotrophic bacterium, *Methylophaga* sp. strain SK1, from seawater obtained near Mokpo, Korea [9]. This bacterial strain could be cultivated aerobically in a media containing trimethylamine as a source of carbon and energy and interestingly could also grow on methanol, monomethylamine, dimethylamine, dimethyl sulfide, glucose, and fructose (a restricted facultative methylotroph) substrates. In this study, we report on the purification and characterization of TMADH from *Methylophaga* sp. strain SK1 in our efforts to develop a biosensor that detects trimethylamine generated from decaying fishes. We also report on our cloning of the gene for the enzyme and the function of the gene.

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MATERIALS AND METHODS

Bacteria, Medium, and Culture Conditions

Methylophaga sp. strain SK1 was cultivated on an artificial seawater medium [9] containing 0.45% (w/v) trimethylamine HCl as carbon and energy sources. The pre-cultured cells were inoculated into a 100 L fermentor (KF-100L, KoBioTech, Korea) containing 70 L of medium. Cells were incubated at 30°C and aerated by continuous air flow (35 L/min). Cells from the late exponential growth phase were harvested by centrifugation at 10,000 × g, washed with 25 mM Tris-HCl buffer (pH 8.0, standard buffer), and kept frozen at −80°C. *E. coli* DH5α cells were cultured at 37°C on Luria-Bertani medium and used as a host strain for the gene cloning experiments [10-14]. Vector plasmid pUC19 was used for cloning and recombinant DNA was introduced into *E. coli* DH5α by electroporation [15]. Transformed *E. coli* DH5α cells were grown on LB medium supplemented with ampicillin (50 μg/mL).

Enzyme Purification

All purification procedures were carried out at 4°C. Harvested cells were disrupted by two passages through a French Pressure Cell (SLM Instruments, USA). Cell debris was removed by centrifugation at 15,000 × g for 15 min, and when necessary the membrane fraction was removed by further centrifugation at 100,000 × g for 60 min. Ammonium sulfate was slowly added to the supernatant to achieve 60% saturation. After removal of the precipitate by centrifugation at 5,000 × g for 60 min, additional ammonium sulfate was added to give 80% saturation. After centrifugation, the precipitated proteins were dissolved in standard buffer and dialyzed. The dialysate was concentrated using a Centricon (Millipore, USA) and applied to a POROS 20 HQ column (PerSeptive Biosystems, USA) equilibrated with standard buffer. After elution with standard buffer, the bound TMADH was released using a linear gradient of 0 to 1.0 M NaCl dissolved in standard buffer; TMADH eluted in the range of 0.4 to 0.5 M NaCl. Fractions containing TMADH were collected and dialyzed against standard buffer and the supernatant applied to a hydrophobic column (High prep 16/10 Phenyl, Amersham Bioscience) equilibrated with standard buffer containing 1.5 M ammonium sulfate. After the unabsorbed materials were washed from the column using equilibrating buffer, the adsorbed proteins were eluted with a linear gradient of ammonium sulfate (from 1.5 to 0 M) in standard buffer. TMADH was eluted by ammonium sulfate free buffer solution. Each active fraction was pooled, concentrated and applied to a Sephacryl S200 (HiPrep™ 16/60, Amersham Bioscience) which was equilibrated with 20 mM Tris-HCl buffer containing 0.15 M NaCl. The purity of TMADH was assessed by 15% SDS-PAGE.

Enzyme Assay

Protein concentration was determined by the Bradford method [16] using bovine serum albumin as standard. TMADH activity was assayed photometrically at 30°C by a two-dye linked assay system; phenazine ethosulfate (PES) was used as an artificial electron acceptor and 2,6-dichlorophenol indophenol (DCPIP) as terminal acceptor [2]. The reaction mixture (3 mL) contained 100 mM Tris-HCl buffer (pH 8.0), 4.6 mM trimethylamine, 1.1 mM PES, 0.04 mM DCPIP, 1 mM KCN, 5 μM sodium hydrosulfate, and 10 μM sodium bicarbonate. Reactions were started by adding the TMADH and reduction of the DCPIP was measured by the decrease in absorbance at 600 nm. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1 mol DCPIP per min.

Molecular Weight Determination

The molecular weights of native TMADH was determined by gel filtration on a Sephacryl S200 column using the following proteins as standards: β-amylase (200,000 Da); alcohol dehydrogenase (150,000 Da); bovine serum albumin (66,000 Da); carbonic anhydrase (29,000 Da); and cytochrome *c* (12,400 Da). The molecular weight of the subunits was determined by SDS-PAGE as described by Laemmli [17] using 15% polyacrylamide gels; electrophoresis was carried out at a constant current of 50 mA for 90 min at room temperature.

Activity Staining

Native 8% polyacrylamide gels were used to identify activity by staining the purified TMADH. After electrophoresis, the gels were treated with staining solution until the blue-black band was observed. The staining solution (30 mL) contained 100 mM Tris-HCl buffer (pH 8.0), 4.6 mM trimethylamine, 1.1 mM PES, 0.04 mM nitro blue tetrazolium, 1 mM KCN, 5 μM sodium hydrosulfate, and 10 μM sodium bicarbonate [18].

DNA Extraction and the Cloning of a *tmd* Gene

Genomic DNA of *Methylophaga* sp. SK1 was extracted by the method of Goldberg and Ohman [19], and plasmid DNA was isolated by the alkaline lysis method [20]. Extracted genomic DNA was fully digested with *Hind*III and subjected to agarose gel electrophoresis. DNA fragments in the desired size range were cut out from the agarose gel and extracted using a Qiagen Gel Extract Kit (Qiagen, USA). *Hind*III digested plasmid pUC19 was treated with bacterial alkaline phosphatase and ligated to fractionated genomic DNA using T4 ligase. Ligated DNA was transformed into competent *E. coli* DH5α cells. Transformant (ampicillin-resistant white colony) containing the trimethylamine gene (*tmd* gene) were screened using degenerate PCR (*tmdF* primer 5′-AARGCYGAAGGCGGNTGG, and *tmdR* primer 5′-GCCNCCGTAYTYGTC KGTRC, probe F1). This primer was designed based on the known amino acid sequence of TMADH from *M. methylotrophus* W_3A_1. Probe F1 (100 pmol) was 5′-end labeled with [α-^{32}P] ATP and T_4 kinase according

Table 1. Purification of TMADH from *Methylophaga* sp. strain SK1

	Total protein (mg)	Total Activity* (U)	Specific activity (U/mg protein)	Purification fold (%)	Yield (%)
Cell free extract	67.02	12.53	0.19	1.00	100
Soluble fraction	42.16	10.04	0.24	1.27	80
POROS 20 HQ	4.62	5.05	1.19	6.36	44
Phenyl hydrophobic	0.61	1.32	2.18	11.66	11
Sephacryl S200	0.11	0.25	2.29	12.27	2

*One activity unit is defined as the amount of enzyme that catalyzes the reduction of 1 mol DCPIP per minute. Enzyme activities were obtained as average values from three independent experiments.

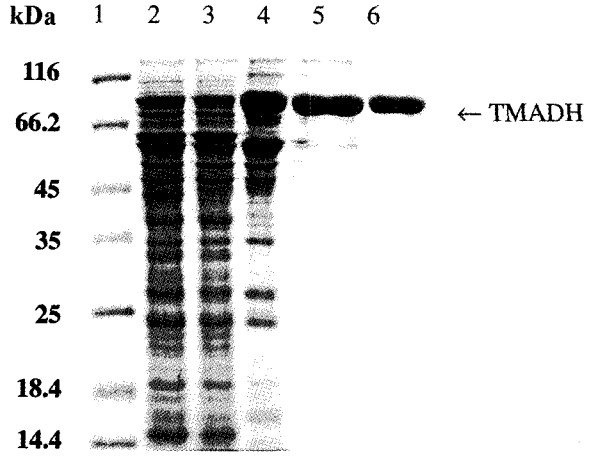

Fig. 1. SDS-PAGE of TMADH from *Methylophaga* sp. strain SK1. Lane 1, molecular size marker; lane 2, cell free extract; lane 3, soluble fraction; lane 4, POROS 20 HQ column; lane 5, phenyl hydrophobic column; lane 6, purified TMADH with Sephacryl S200 column.

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 × SSC (0.15 M sodium chloride and 0.015 M sodium citrate), 1 × Denhardt solution, 100 μg/mL yeast tRNA, and 0.05% sodium pyrophosphate. Final washes were performed at increasing temperatures (55 to 65°C) in a 1 × SSC. Autoradiographs were subsequently obtained by exposing the hybridized filters to Fuji RX films for 16 to 24 h.

Nucleotide Sequence Determination and Analyses

The isolated 3 kb *Hind*III fragment was sequenced by a DNA sequencer (Applied Biosystems Model 373A, USA) using a Taq DyeDeoxy Terminater Cycle sequencing kit. Custom-made oligonucleotides (Bioneer, Korea) were used for DNA sequencing and cloning. The Basic Local Alignment Search Tool (BLAST, version 2.0) network service [21] was used to search similar sequences in the international database (NCBI). DNA sequences were aligned and analyzed by the DNASIS program (Hitachi, Japan).

RESULTS AND DISCUSSION

Purification of TMADH

TMADH has been suggested to be a peripheral membrane protein of the methylotrophs and localized in the membranes periplasmic face [22]. However, after disruption of the cells using a French press, the TMADH activity of *Methylophaga* sp. strain SK1 was found exclusively in the soluble fraction. The purification steps of TMADH are summarized in Table 1. TMADH was purified more than 12-fold through a three step process, with a final yield of 2% and a specific activity of 2.29 units per mg protein. Gel filtration on Sephacryl S200 was necessary to obtain a single band in SDS-PAGE (Fig. 1). Solutions of the purified TMADH stored in 50 mM potassium phosphate buffer (pH 7.0) showed an approximate 20% loss of activity during 24 h at 4°C.

Relative Molecular Mass and Isoelectric Point

The molecular mass of TMADH, by gel filtration, was estimated as 143 kDa. The molecular mass of the subunit in SDS-PAGE appeared to be 80 kDa (Fig. 1). The *tmd* gene of 2,187 bp encodes a protein of 728 amino acids with an apoenzyme subunit molecular mass of 81.6 kDa (Fig. 2). The calculated subunit molecular mass, therefore, is in good agreement with the SDS-PAGE value approximating 80 kDa. Together these results suggest that the enzyme is a dimer consisting of two identical subunits. The isoelectric point determination for the enzyme was determined to be 7.2.

Substrate Specificity

Purified TMADH of *Methylophaga* sp. strain SK1 showed narrow substrate specificity with methylated amines (Table 2). Enzyme activities toward substrates such as dimethylamine (DMA), diethylamine (DEA), and benzylamine were 85.1, 59.9, and 49.1% of that toward trimethylamine, respectively. Moreover, the TMADH demonstrated no activity with primary amines such as monomethylamine and *n*-butylamine, and tertiary triethyla-mine (TEA). Similarly, the TMADH of *M. methylotrophus* W_3A_1 was only able to oxidize substrates such as DMA, DEA, and TEA [3]. A detailed study on the sub-

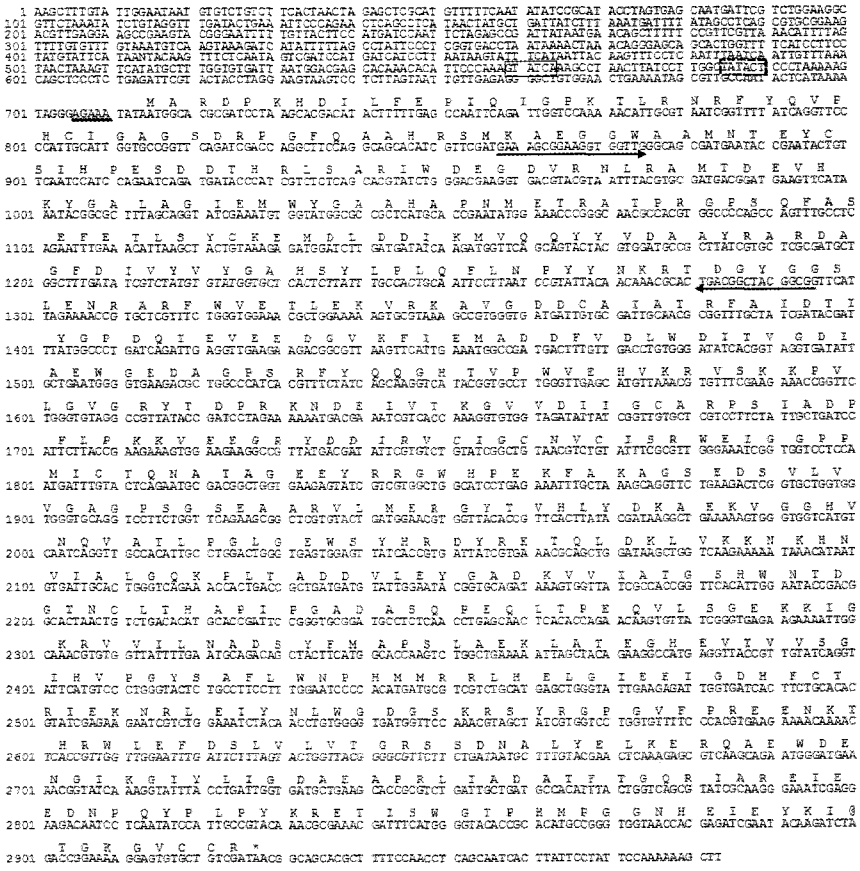

Fig. 2. The entire nucleotide sequence and deduced amino acid sequence of *tmd* gene. 2,983 bp genomic fragment which contain entire TMADH ORF was isolated by colony hybridization with 437 bp fragment as a probe. Potential Shine-Dalgarno sequences (underlined) was found at −10 bp of the upstream of *tmd* gene. A putative promoter region of TMADH has the following conserved sequences; "GTATCA" sequence at −35 (boxed), "TATACT" sequence at −10 regions (dot boxed) from a putative transcription start site A (bold). Termination codons are indicated by an asterisk, and the potential Shine-Dalgarno sequences are underlined. Arrows indicated primer sequences.

Table 2. Substrate specificities of TMADH

Substrates		Relative activity[a] (%)	K_m (mM)
Tertiary amine	Trimethylamine	100.0	1.5
	Triethylamine	0	
Secondary amine	Dimethylamine	85.1	3.9
	Diethylamine	59.9	21.5
Primary amine	Monomethylamine	0	
	n-Butylamine	0	152.3
	Benzylamine	49.1	

[a]Relative activity in the presence of trimethylamine was taken as 100%.

strate specificity of the enzyme has been carried out over a wider range of substrates for the TMADH of bacterium 4B6 [6]. The TMADH could oxidize both tertiary amines (such as TMA, TEA (very low activity), diethylmethylamine) and secondary amines (such as DMA, DEA); by contrast, primary, quaternary, and poly-amines were not substrates. The V_{max} and K_m values for TMADH from *Methylophaga* sp. strain SK1 were 7.9 nmol/min/mg protein and 1.5 mM, respectively.

Effects of pH and Temperature

The effect of pH on TMADH activity was studied in the pH range of pH 7 to 10. Similar to the TMADH from bacterium 4B6 [2], the TMADH of *Methylophaga* sp. strain SK1 showed a high activity at pH 8.5 (Fig. 3A). The TMADH activity was assessed at various temperatures in 100 mM Tris-HCl buffer (pH 8.5) (Fig. 3B), and was determined to peak at 55°C. After 60 min of incubation at 60°C, the enzymes activity remained at 50% (Fig. 4).

Effects of Divalent Cations

The effects of several divalent cations on TMADH activities were tested under the assay conditions noted in Table 3. TMADH was strongly inhibited by Fe^{2+} and to-

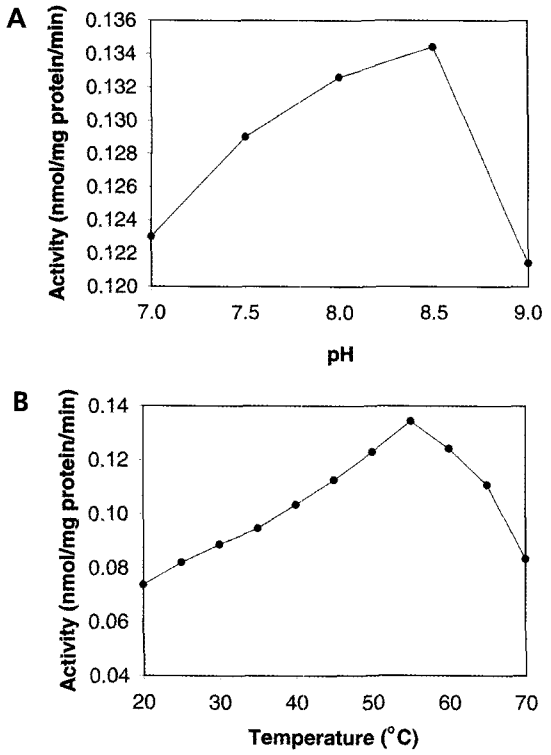

Fig. 3. (A) Effects of pH and (B) temperature on the activity of TMADH.

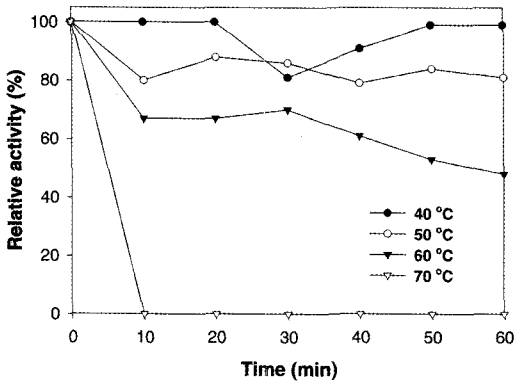

Fig. 4. Effect of temperature on the stability of TMADH.

tally inhibited by Cu^{2+}, Co^{2+}, and Mn^{2+}. However, Cd^{2+}, Mg^{2+}, Ca^{2+}, and Ba^{2+} did not suppress enzyme activity.

Isolation of the *tmd* Gene from *Methylophaga* sp. Strain SK1

The genomic library of *Methylophaga* sp. strain SK1 was constructed, and we have isolated a 437 bp DNA fragment that encodes a portion of the trimethylamine dehydrogenase gene (specifically amino acids from 49 to 194) from the *Methylophaga* sp. strain SK1 using PCR. This fragment was subsequently used as a probe to isolate the *tmd* gene from the genomic library. A total of 1,200 recombinant clones from the library were screened by colony hybridization using this probe to find a single clone with a positive signal. The clone contained an insert measuring 3 kb. The nucleotide sequence of the entire 3 kb-*Hind*III DNA fragment was determined as described in Materials and Methods.

Table 3. Effects of divalent cations on TMADH activity

	Concentration (mM)	Relative activity (%)
Control	–	100
Cd^{2+}	1	173
Mg^{2+}	1	123
Ca^{2+}	1	120
Ba^{2+}	1	115
Zn^{2+}	1	90
Fe^{2+}	1	40
Cu^{2+}	1	3
Co^{2+}	1	0
Mn^{2+}	1	0

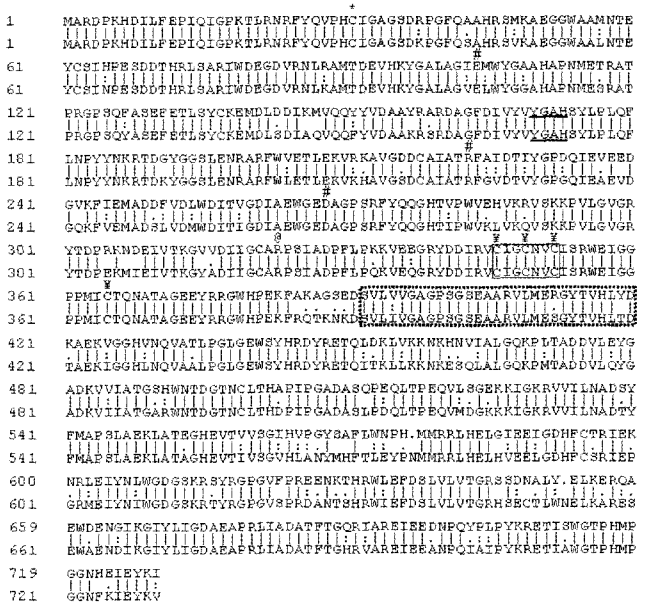

Fig. 5. Alignment of TMADH from *Methylophaga* sp. strain SK1 (upper line) with that from *M. methylotrophus* W_3A_1 (lower line, Swiss-Plot accession number P16099), Identity = 82.83% (603/728), Similar residues = 13.74% (100/728). The box indicates the 4Fe-4S binding motif (*C-X-X-C-X-X-C*). The dotted box indicates ADP-binding site. Underlined residue indicate substrate-binding region. Symbols on the amino acid, *: S-6-FMN cysteine; #: FMN-binding site; @: FMN phosphate group binding site; ¥: iron-sulfur (4Fe-4S) binding group.

Nucleotide Sequence Analysis of the TMADH Gene

DNA sequencing of the 3 kb insert revealed the open reading frame (ORF) of the *tmd* gene, and 5′ untranslated

Table 4. Sequence alignment of the *C-X-X-C-X-X-C* motif of trimethylamine dehydrogenase with the TMADH family in SWISS-PROT/TREMBL and closely similar ferredoxins

Protein	Species	Residue No.[a]	Sequence	Accession No.[b]
TMADH	*Methylophaga* sp. strain SK1	345	**C**IG**C**NV**C**	In this study
TMADH	*M. methylotrophus* W_3A_1	345	**C**IG**C**NV**C**	P16099
DMADH	*Hypermicrobium* X	352	**C**IG**C**NV**C**	Q48303
BaiC[c]	*Eubacterium* sp.	332	**C**IG**C**DQG**C**	P19410
Ferredoxin	*C. vinosum*	8	**C**IN**C**NV**C**	P00208
Ferredoxin II	*R. capsulata*	39	**C**ID**C**GV**C**	P18082

[a]Residue No. indicates the starting position of the 4Fe-4S binding motif in the primary sequence.
[b]Swiss-Plot accession number (http://www.expasy.ch).
[c]BaiC: Bile acid-inducible operon protein C.

region (Fig. 2). The ORF was comprised of 2,187 base pairs coding for 728 amino acids (GenBank accession number DQ675455). The ORF had the highest match to the *tmd* gene from *M. methylotrophus* W_3A_1 (82% identity) [23,24]. A putative ribosomal binding site (AGAAA) was also found (underlined in Fig. 2). A supposed promoter region of the gene has been assigned; "GTATCA" sequence at −35, "TATACT" sequence at −10 regions from an accepted transcription start site "A".

Comparative Analysis of Deduced Amino Acid Sequences of the *tmd* Gene

TMADH is a homodimeric protein with identical subunits. Each subunit contains the following cofactors: a covalently linked 6-*S*-cysteinyl FMN (linking the flavin via its 6-position to Cys30 of the polypeptide), a bacterial ferredoxin type [4fe-4S] center, and an equivalent of tightly bound ADP having no known function [25-27]. The deduced amino acids of the *tmd* gene from *Methylophaga* sp. strain SK1 (728 amino acids) were compared with those of *M. methylotrophus* W_3A_1 (Fig. 5) wherein the amino acid sequences matched putative FMN-binding sites (Cys-30, Glu-103, Arg-222, Asp-267, and Arg-322). The incorporation of FMN and covalent attachment were important in TMADH. Flavinylation proceeds via a nucleophilic attack by the thiolate of Cys-30 at C-6 of the isoalloxazine ring of enzyme-bound FMN [28]. Potential amino acids involved in the 4Fe-4S binding motif (Cys-345, Cys-348, Cys-351, and Cys-364) were also found by our analyses. The first three of these cysteines are separated by two groups of two residues, Ile-Gly and Asx-Val, respectively. This *C-X-X-C-X-X-C* motif has been found in several [4Fe-4S] ferredoxins [24] (Table 5). The amino acids in the motif are completely conserved among the TMADH and dimethylamine dehydrogenases [29]. In addition, this deduced amino acid showed an ADP-binding site at positions 391 through 420. Several other interacting residues had conservative changes.

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