The Journal of Microbiology, February 2008, p. 45-50 Vol. 46, No. 1
DOI 10.1007/s12275-008-0017-2

Isolation and Taxonomic Characterization of a Novel Type I Methanotrophic Bacterium

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(Received January 15, 2008 / Accepted January 31, 2008)

A methane-oxidizing bacterium was isolated from the effluent of manure and its molecular and biochemical properties were characterized. The isolate was aerobic, Gram-negative, and non-motile. The organism had a type I intracytoplasmic membrane structure and granular inclusion bodies. The outer cell wall surface (S-layers) was tightly packed with cup-shaped structures. Colonies were light yellow on nitrate mineral salt agar medium. In addition, the organism was catalase and oxidase positive. The isolate used the ribulose monophosphate (RuMP) pathway for carbon assimilation, and was able to utilize methane and methanol as a sole carbon and energy source, however, it could not utilize any other organic compounds that were tested. The cells grew well in a mixture of methane and air (methane:air=1:1, v/v) in a compulsory circulation diffusion system, and when grown under those conditions, the optimum pH was approximately 7.0 and the optimal temperature was 30°C. In addition, the specific growth rate and generation time were 0.13 per h and 5.43 h, respectively, when grown under the optimum conditions. The major ubiquinone was Q-8, and the G+C mol% of the DNA was 55.3. Phylogenetic analyses based on the 16S rRNA gene sequence comparisons showed that this bacterium belongs to a group of type I methanotrophs, and that it is most closely related to *Methylomicrobium*, with a sequence similarity of 99%. Therefore, the isolate was named *Methylomicrobium* sp. HG-1.

***Keywords*:** Methanotrophic bacteria, intracytoplasmic membrane structure, ribulose monophosphate (RuMP) pathway, *Methylomicrobium*

Methane-oxidizing bacteria (methanotrophs) are widespread in nature and play an indispensable role in the global carbon cycling of methane. The atmospheric concentration of methane, which is an important global warming gas, has been increasing for many decades (Whittenbury *et al*., 1970; Hanson and Hanson, 1996). Methanotrphs are a ubiquitous group of microorganisms that possess the unique ability to utilize methane as the sole source of carbon and energy, and are therefore considered to be important regulators of atmospheric methane fluxes in nature (Reeburgh *et al*., 1993; Mancinelli, 1995; Fjellbirkeland *et al*., 2001). Methanotrophs are aerobic bacteria that convert methane to methanol in the first step of their metabolic pathway using methane monooxygenase (MMO). In addition, methanol dehydrogenase (MDH), which is an enzyme responsible for the oxidation of methanol to formaldehyde, is assimilated into cellular biomass or oxidized to CO_2, thereby providing reducing power for biosynthesis. *In vitro*, MDH is coupled to the electron transport chain at the level of cytochrome *c* (Ro *et al*., 2000; Brantner *et al*., 2002; Koh *et al*., 2002; Kim *et al*., 2005, 2006).

Methanotrophs have been difficult to identify because information regarding their phenotypic and chemotaxonomic properties is limited. This has led to nomenclatural problems, especially concerning the assignment of species to genera. However, several studies have evaluated the species and genus organization of the methanotroph groups using a more thorough polyphasic taxonomic approach, which has led to redefinition of several species and genera (Bowman *et al*., 1995).

The current classification separates all known methanotrophs into three groups (Types I, II, and X) based on multiple criteria, including cell morphology, the arrangement of intracytoplasmic membranes (ICM), the pathway for formaldehyde assimilation, the DNA G+C content and the major cellular fatty acid profiles (Whittenbury *et al*., 1970; Higgins *et al*., 1981; Bowman *et al*., 1993, 1995; Kaluzhnaya *et al*., 2001). Type I methanotrophs include three broadly homologous clusters of species, referred to as *Methylococcacea*, and it has been proposed that this group should also contain the genera *Methylococcus*, *Methylomicrobium*, *Methylobacter*, and *Methylomonas* (Bowman *et al*., 1995). The type II methanotrophs contain closely related groups that belong to the genera *Methylocystis* and *Methylosinus*. In addition, a new group, type X, was added to accommodate methanotrophs similar to *Methylococcus capsulatus* that, like type I methanotrophs, utilize ribulose monophosphate (RuMP) as the primary pathway for formaldehyde assimilation. Type X

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methanotrophs are also distinguished from type I methanotrophs because they possess low levels of ribulose-bisphosphate carboxylase, which is involved in the serine pathway by which carbon assimilation occurs (Hanson *et al*., 1996). Due to the ability of methanotrophs to catalyze a large number of biotransformations, they have attracted the interest of scientists studying the development of biological methods for degradation of toxic chemicals and the use of bacteria containing MMO for the production of chemicals with commercial values (Han *et al*., 1999; Han and Semrau, 2000).

In this study, we isolated, characterized, and phylogenetically positioned a novel strain of type I methanotrophic bacterium that grows rapidly in the presence of methane.

Materials and Methods

Isolation and culture conditions

A novel strain of bacteria was isolated from the effluent of manure in Goksung, Republic of Korea using the modified nitrate-mineral-salt (NMS) medium described by Higgins *et al.* (1981). The bacterium was cultured using medium in which the pH had been adjusted to 7.0 at an agitation speed of 150 rpm at 30°C.

An appropriate dilution of the liquid culture samples was spread onto a NMS plate that contained 1.5% (w/v) noble agar (Difco), and then placed in an airtight box. A mixture of methane and air (1:1, v/v) was then passed through a 0.2 μm pore air filter and injected into the box. Control plates were also incubated in the absence of methane to determine if the samples were contaminated with non-methane oxidizing colonies. The plates were incubated at 30°C in the dark and then observed at 3 day or 1 week intervals over 3∼4 weeks. In addition, the gas mixture was exchanged every 3 days. Single colonies that formed on the NMS agar plates were transferred onto fresh NMS agar plates and then re-incubated for another week. Isolates were considered to be pure if they were morphologically similar in appearance. Methane-oxidizing colonies generally appeared after 5∼7 days, and small, non-methane-oxidizing colonies also appeared on plates that were incubated with and without methane. The purity of the methanotrophic bacteria was ascertained by phase-contrast microscopic observation. For long-term storage, 500 μl of 50% (v/v) glycerol was added to 1 ml of late exponential phase liquid culture, and the mixed suspension was then frozen at -70°C.

The pH range at which growth could occur was determined to range from 4.0 to 10.0 and the growth temperature range was determined to range from 10 to 45°C. In addition, the tolerance to concentrations of NaCl ranging from 1 to 5% (w/v) was also evaluated.

Morphology and electron microscopy

Cell morphology was determined using 5-day-old liquid culture medium by Gram-staining, with detailed morphological examinations being made using electron microscopy. Briefly, the cells in the NMS medium were initially fixed with glutaraldehyde, which was added to the medium at a final concentration 2% (v/v), during the late-exponential phase. The cells were then subjected to an additional fixation using 1% (w/v) OsO_4 in 0.1 M cacodylate buffer, followed by two water washes. After dehydration in a series of alcohols, the cells were embedded with Epon resin and then polymerized at 60°C for 18 h. Sections of 70∼80 nm were then cut using an ultramicrotome (LKB 2128 Ultratome; LKB) and viewed on a transmission electron microscope (JEOL JEM-2000F X2) operating at 80 kV.

Utilization of carbon and nitrogen sources

The ability of the isolate to utilize the following carbon sources at a concentration of 0.1% (w/v or v/v) was tested in the presence of potassium nitrate, which was used as a nitrogen source: ethanol, formate, formamide, urea, sodium succinate, malate, yeast extract, $_{D}$-glucose, $_{D}$-fructose, methylamine, casitone, peptone, tryptone, and methanol. In addition, the ability of the isolate to grow on methanol was tested in a liquid medium that contained concentrations of methanol ranging from 0.1∼5.0% (v/v). Volatile liquid carbon sources were filter sterilized and then added to the basal medium after autoclaving. This solution was then dispensed in 3 ml volumes into 20 ml glass vials. Nitrogen sources were tested similarly in the presence of methane using NMS medium in which the potassium nitrate was replaced with one of the following compounds at a concentration of 0.1% (w/v): ammonium molybdate, ammonium amidosulfate, ammonium vanadate, ammonium dihydrogen phosphate, potassium nitrate, formamide, glycine, urea, yeast extract, ammonium oxalate, L-alanine, L-glutamine, L-glutamic acid, L-tryptophan, L-asparagine, and casitone. All test preparations were incubated for 1 week in duplicate. For carbon and nitrogen source tests, growth was confirmed by comparison with negative controls. Sensitivity to antibiotics was examined by plating the cells onto agar-solidified medium and then placing BBL sensi-discs containing the following antibiotics (μg/ml) on the medium: Penicillin (10), Amoxicillin (30), Piperacillin (100), Aztreonam (30), Imipenem (10), Cefamandole (30), Cefazolin (30), Cefotaxime (30), Gentamicin (10), Amikacin (30), Netilmicin (30), Tobramycin (10), Tetracycline (30), Erythromycin (15), Chloramphenicol (30), Vancomycin (30), Ciprofloxacin (5), Clindamycin (2), and Sulfamethoxazole (23.75). Growth under methane was assessed after 5 days.

Chemotaxonomic characterization

Fatty acid methyl esters were prepared from a biomass harvested from the NMS medium after 7 days of incubation at 30°C, and then analyzed using a gas chromatograph (GC-14A, Shimadzu) according to the instructions provided by the Microbial Identification System (MIDI). Ubiquinones were extracted and purified according to the method described by Collins (1985). Analysis of the ubiquinones was conducted using a HPLC apparatus (Agilent 1200, USA) equipped with a C-18 reverse-phase column. DNA was extracted using the method described by Marmur (1961), and the DNA G+C content was determined following the method described Tamaoka and Komagata (1984) using a HPLC apparatus (Agilent 1200, USA) equipped with a C-18 reverse-phase column.

Enzyme assay

The activity of hexulose-6-phosphate synthase (HSP), the

key enzyme involved in the RuMP pathway, was measured following a modification of the method described by Nash (1953). Briefly, the HPS activity was assayed at 37°C by measuring the decrease in the amount of formaldehyde that occurred after the reaction of the added formaldehyde with the ribulose 5-phosphate formed in the reaction mixture. One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of formaldehyde per min.

Analysis of 16S rRNA and *pmoA* gene sequences

Chromosomal DNA from the isolate was obtained using the following method: Two liters of batch culture (Kim and Kim, 2006) were pelleted and resuspended in 5 ml of solution I (50 mM Tris; pH 8.0, 25% sucrose). Next, 0.5 ml of lysozyme (20 mg/ml in 0.25 mM Tris; pH 8.0) was added and the solution was then incubated for 1 h at 37°C. After incubation for 1 h at 37°C, 1ml of 0.25 M EDTA (pH 8.0) was added, followed by incubation for 1 h at 37°C. Finally, Sarkosyl was added to a final concentration of 1% (v/v), and the mixture was incubated at 37°C for 30 min and then at 60°C for 5 to 30 min until lysis was complete. The lysate was then subjected to CsCl gradient centrifugation for 16 h (Sambrook *et al*., 1989; Gilbert *et al*., 2000). The 16S rRNA gene was then amplified by PCR using the following primers: 16SF; 5'-GAGTTTGATCCTGGCTCAG-3', *E. coli* 16S rRNA position 9-27 and 16SR; 5'-AGAAAGGAGGTGATC CAGCC-3', *E. coli* 16S rRNA position 1542~1525. The samples were subjected to the following conditions in a Roche Diagnostic System (Applied Biosystems, *GeneAmp* PCR system 2400): initial denaturation at 94°C for 5 min; 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°C), and extension (1.5 min at 72°C); and a final extension at 72°C for 5 min. The *pmoA* gene was amplified by PCR using primers *pmoA*F; 5'-TTCTGGGGNTGGACN TAYTTYCC-3' and *pmoA*R; 5'-CCNGARTAYSTHMGNAT GGTNGA-3' following the method described by Steinkamp *et al.* (2001). PCR for *pmoA* gene amplification was performed in 50 μl reaction mixtures in 0.3 ml microcentrifuge tubes using a Roche Diagnostic System.

16S rRNA and the deduced *pmoA* sequences were com-

Table 1. Morphological and biochemical properties of the strain HG-1

Characteristics	Strain HG-1
Morphology	Short-rod
Gram reaction	Negative
Size	1×1.5 μm
Colony color	Light yellow
Motility	Negative
Exospore	Absent
Cyst	Absent
pH	
Growth range	6.5~9.0
Optimal	7.0
Temperature	
Growth range	25~35
Optimal	30
Oxidase	Positive
Catalase	Positive
Carbon assimilation pathway	RuMP
Specific growth rate (μ)	0.13 h^{-}
Generation time (G)	5.43 h
Quinone system	Q-8
DNA G+C mol %	55.3

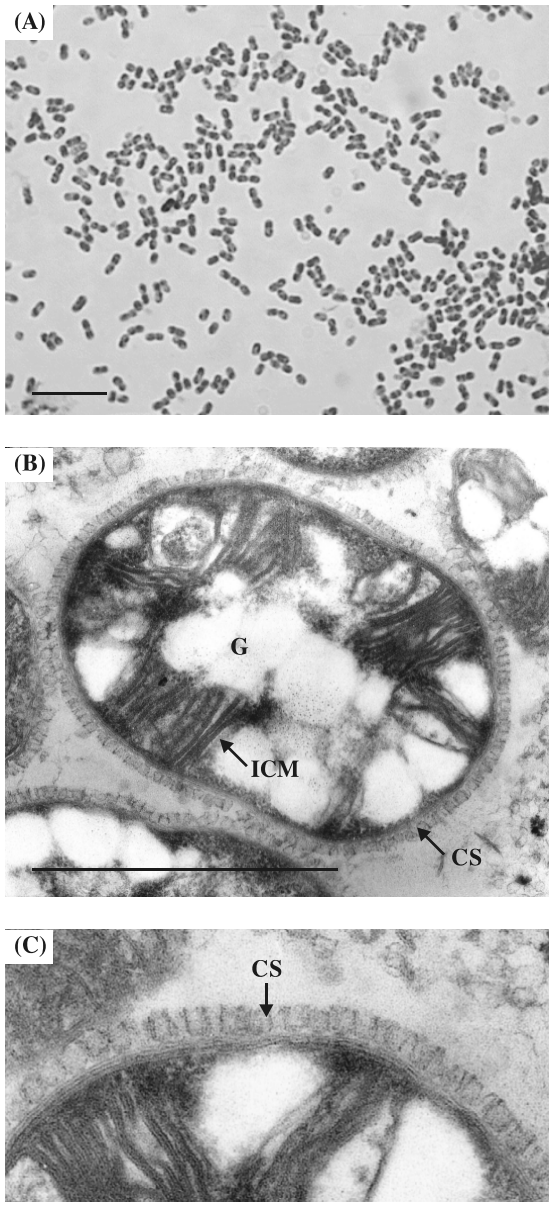

Fig. 1. Phase-contrast micrograph (A) and transmission electron micrograph (B) of the isolate: (A) phase-contrast micrograph; (B) thin section of the cell with cup-shaped structures (CS) on the outer membrane surface and large granular inclusion bodies (G); (C) enlarged cup-shaped structures. Bar: 10 μm (A) and 1 μm (B).

pared with available sequences in the GenBank database using the NCBI tool, BLAST. Next, the DNA sequences were aligned to representative sequences of closely related organisms using the CLUSTAL X 1.81 program. Phylogenetic trees were then constructed using the Tree-View 1.6.6 program.

Nucleotide sequence accession numbers

The 16S rRNA gene and partial *pmoA* gene nucleotide sequences were deposited in the NCBI GenBank nucleotide sequence database under the accession numbers AF495887 and AF495888, respectively.

Results and Discussion

Morphology and ultrastructure

The isolate was a non-motile, Gram-negative, non-spore forming short rod that formed light-yellow, opaque, round colonies when grown on NMS agar medium. However, the isolate did not form cyst-like structures that are often found in *Methylobacter* species, which is a key differential trait that divides *Methylomicrobium* from *Methylobacter* (Wise *et al.,* 2001). The isolate was catalase and oxidase positive and reproduced by binary fission (Table 1). In addition, the intracytoplasmic membrane structure of the isolate appeared to be comprised of stacks of vesicular disks, which are the typical appearance of type I membranes (Fig. 1). The isolate also had tightly packed cup-shaped structures on the outer cell wall surface, similar to those found on haloalkaliphilic and haloalkalitolerant methanotrophs such as *Methylomicrobium alcaliphilum*, *M. modestohalophilum*, and *M. buryatense*, which are known to live in environments with high osmolarity and pH (Sorokin *et al*., 2000; Kaluzhnaya *et al*., 2001). Furthermore, similar structures that are not identical to S-layers have also been found in *M. album* BG8, which is a neutrophilic and nonhalophilic methanotroph (Jeffries and Wilkinson, 1978). However, the taxonomic importance of bacterial S-layers is still obscure, although it has been proposed that they are related to the osmoadaption mechanisms in haloalkaliphilic methanotrophs (Khmelenina *et al*., 1999).

Physiological and biochemical characteristics

The physiological and biochemical characteristics of the isolate are shown in Table 1. The optimum pH and temperature were 7 and 30°C, respectively, and NaCl was not required

Table 2. Comparison of major fatty acids of type I methanotroph genera with the strain HG-1

Fatty acid	*Methylomonas*	*Methylobacter*	*Methylomicrobium*	*Methylococcus*	Strain HG-1
14:0	22±3	9±2	1±1	1±1	1.7
16:1ω8c	30±11	0	16±3	0	14.6
16:1ω7c	11±4	57±1	17±3	28±10	14.0
16:1ω6c	9±4	5±1	10±4	3±2	8.1
16:1ω5c	4±2	7±1	6±1	3±2	21.2
16:1ω5t	12±4	11±1	20±10	<1	15.9
16:0	7±2	8±1	15±3	44±8	2.4

Values are percentages of the total phospholipid fatty acids (for genera : Mean±SD) ; data are from Bowman *et al.* (1995).

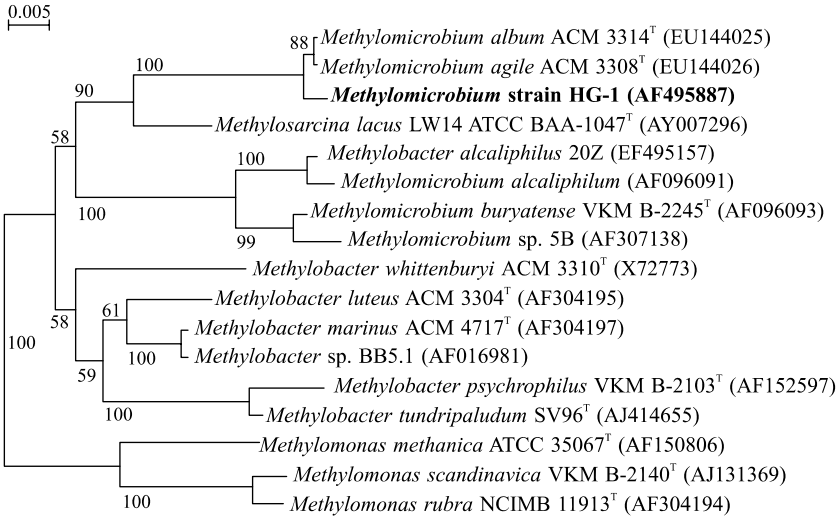

Fig. 2. Phylogenetic analysis of the 16S rRNA gene sequences of type I methanotrophs and the isolate. The dendrogram shows the results of analysis using CLUSTAL X and TREE-VIEW. Bar, 0.005 substitutions per base position. Bootstrap analysis from 100 replicates is shown (values less than 50% are not shown).

for growth. In addition, the isolate could not utilize any carbon sources evaluated except for methane and methanol, and no growth was observed on nutrient-rich media. However, the isolate was able to grow well using a novel culture system (compulsory circulation diffusion system) that contained a mixture of methane and air (methane:air=1:1, v/v) (Kim and Kim, 2006), and under these conditions it was found to have a specific growth rate and generation time of 0.13 per h and 5.43 h, respectively. The strain was also capable of growth in NMS medium that contained a high concentration of methanol (3%). This result shows that methanol is not inhibitory to the growth of the isolate, which is uncommon for the type I methanotrophs. The isolate could also utilize urea, yeast extract, potassium nitrate and ammonium-containing chemicals as nitrogen sources, however, the isolate was not able to fix nitrogen. In addition, the isolate was resistant to erythromycin, chloramphenicol and clindamycin, but sensitive to other antibiotics tested. Additionally, the isolate was positive for hexulose phosphate synthase, the key enzyme involved in the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation. The isolate also possessed a Q-8 with ubiquinone system. The G+C content of the isolate's DNA, as determined by HPLC, was 55.3 mol%, and, as seen in Table 2, the predominant cellular fatty acids of the isolate were unsaturated $C_{16:1\omega8c}$ (14.6%), $C_{16:1\omega7c}$ (14.0%), $C_{16:1\omega5c}$ (21.2%) and $C_{16:1\omega5t}$ (15.9%). The profiles for strain HG-1 suggest that, based on the significant amounts of $C_{16:1\omega8c}$, $C_{16:1\omega7c}$, and $C_{16:1\omega5t}$ that were detected, the strain is most similar to the genus *Methylomicrobium*. However the strain contained four times more $C_{16:1\omega5c}$ than other *Methylomicrobium* species (Table 2), and when compared with other type I methanotrophic genera, the major cellular fatty acids profile of the isolate was unique.

Phylogenetic analysis

The 16S rRNA gene and partial *pmoA* gene sequences of the isolate were determined, and the phylogenetic tree derived from these data revealed that the isolate branched together with a cluster of *Methylomicrobium* species within the *Gammaproteobacteria* (Fig. 2). The relatively high level of 16S rRNA gene sequence identity between the isolate and members of the genus *Methylomicrobium* indicated that they were closely related, with the closest relatives being *M. album* ACM 3314^T [formerly *Methylobacter albus* (Bowman *et al*., 1993)] (99%), *M. agile* ACM 3308^T (99%), and *Methylosarcina lacus* LW14 ATCC BAA-1047^T (96%). Lower sequence similarities were found when the sequence of the isolate was compared with those of all other species with validly published names in the genus *Methylobacter*, including *M. whittenburyi* ATCC 51738^T (94%), *M. luteus* ATCC 49878^T (94%), and *M. alcaliphilus* (94%). Phylogenetic analysis of the deduced amino acid sequence of *pmoA* using CLUSTAL X showed that the *pmoA* sequence of the isolate branched within the *Methylosarcina*/*Methylobacter* group of *pmoA* sequences, however, there is a shortage of *pmoA* sequences from type strains of these groups of methanotrophs, therefore, the precise position of the *pmoA* sequence from strain HG-1 could not be determined (data not shown).

Based on the phenotypic and genotypic characteristics of the isolate, it was preliminarily classified as a species of the genus *Methylomicrobium.* The isolate is closely related to *Methylomicrobium album*, however, some features are different. For example, strain HG-1 was capable of growth on NMS medium that contained a high methanol concentration (3%), whereas *M. album* cannot grow on media that contains greater than 0.1% methanol. In addition, strain HG-1 contained a higher concentration of $C_{16:1\omega5c}$ than *M. album*, and the phylogenetic similarity of the deduced *pmoA* sequence obtained from strain HG-1 and that of *M. album* was not very high. Therefore, we propose the name *Methylomicrobium* sp. HG-1 for this isolate.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRI) (No. KRF-2004-202-C00472).

References

Bowman, J.P., L.I. Sly, P.D. Nichols, and A.C. Hayward. 1993. Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus*, and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Syst. Bacteriol.* 43, 735-753.

Bowman, J.P., L.I. Sly, and E. Stackebrandt. 1995. The phylogenetic position of the family *Methylococcaceae*. *Int. J. Syst. Bacteriol.* 45, 182-185.

Brantner, C.A, C.C. Remsen, H.A. Owen, L.A. Buchholz, and M. Collins. 2002. Intracellular localization of the particulate methane monooxygenase and methanol dehydrogenase in *Methylomicrobium album* BG8. *Arch. Microbiol.* 178, 59-64.

Collins, M.D. 1985. Analysis of isoprenoid quinones. *Methods Microbiol*. 18, 329-366.

Fjellbirkeland, A., V. Torsvik, and L. Ovreås. 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF*, and 16S rRNA sequences. *Antonie Van Leeuwenhoek* 79, 209-217.

Gilbert, B., I.R. McDonald, R. Finch, G.P. Stafford, A.K. Nielsen, and J.C. Murrell. 2000. Molecular analysis of the *pmo* (particulate methane monooxygenase) operons from two type II methanotrophs. *Appl. Environ. Microbiol.* 66, 966-975.

Han, J.I., S. Lontoh, and J.D. Semrau. 1999. Degradation of chlorinated and brominated hydrocarbons by *Methylomicrobium album* BG8. *Arch. Microbiol.* 172, 393-400.

Han, J.I. and J.D. Semrau. 2000. Chloromethane stimulates growth of *Methylomicrobium album* BG8 on methanol. *FEMS Microbiol. Lett.* 187, 77-81.

Hanson, R.S. and T.E. Hanson. 1996. Methanotrophic bacteria. *Microbiol. Rev.* 60, 439-471.

Higgins, I.J., D.J. Best, R.C. Hammond, and D. Scott. 1981. Methane-oxidizing microorganisms. *Microbiol. Rev.* 45, 556-590.

Jeffries, P. and J.F. Wilkinson. 1978. Electron microscopy of cell wall complex of *Methylomonas albus*. *Arch. Microbiol*. 119, 227-229.

Kaluzhnaya, M., V. Khmelenina, B. Eshinimaev, N. Suzina, D. Nikitin, A. Solonin, J. Lin, I. McDonald, C. Murrell, and Y. Trotsenko. 2001. Taxonomic characterization of new alkaliphilic and alkalitolerant methanotrophs from soda lakes of the southeastern transbaikal region and description of *Methylomicrobium buryatense* sp. nov. *Syst. Appl. Microbiol.* 24, 166-176.

Khmelenina, V.N., M.G. Kalyuzhnaya, V.G. Sakharovsky, N.E.

Suzina, Y.A. Trotsenko, and G. Gottschalk. 1999. Osmoadaptation in halophilic and alkaliphilic methanotrophs. *Arch. Microbiol*. 172, 321-329.

Kim, H.G. and S.W. Kim. 2006 Purification and characterization of a methanol dehydrogenase derived from *Methylomicrobium* sp. HG-1 cultivated using a compulsory circulation diffusion system. *Biotechnol. Bioprocess. Eng*. 11, 134-139.

Kim, H.G., T.N. Phan, T.S. Jang, M.J. Koh, and S.W. Kim. 2005. Characterization of *Methylophaga* sp. strain SK1 cytochrome c_L expressed in *Escherichia coli. J. Microbiol.* 43, 499-502.

Koh, M.J., C.S. Kim, Y.A. Kim, H.S. Choi, E.H. Cho, E.B. Kim, Y.M Kim, and S.W. Kim. 2002. 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.

Mancinelli, R.L. 1995. The regulation of methane oxidation in soil. *Annu. Rev. Microbiol.* 49, 581-605.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* 3, 208-218.

Nash, T. 1953. The colorimetric estimation of formaldehyde by means of the hantzsch reaction. *Biochem. J.* 55, 416-421.

Reeburgh, W. S., S.C. Whalen, and M.J. Alperin. 1993. The role of methylotrophy in the global methane budget, p. 1-14. *In* J.C. Murrell and D.P. Kelly (eds.). Microbial growth on C_1 compounds. Intercept. Andover, UK.

Ro, Y.T., E.B. Kim, and Y.M. Kim. 2000. Enzyme activities related to the methanol oxidation of *Mycobacterium* sp. strain JC1 DSM 3803. *J. Microbiol.* 38, 209-217.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning; a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, USA.

Sorokin, D.Y, B.E. Jones, and J.G. Kuenen. 2000. An obligate methylotrophic, methane-oxidizing *Methylomicrobium* species from a highly alkaline environment. *Extremophiles* 4, 145-155.

Steinkamp, R., W. Zimmer, and H. Papen. 2001. Improved method for detection of methanotrophic bacteria in forest soils by PCR. *Curr. Microbiol.* 42, 316-322.

Tamaoka, J. and K. Komagata. 1984. Determination of DNA base composition by reversed phase high-performance liquid chromatography. *FEMS Microbiol. Lett*. 25, 125-128.

Whittenbury, R., K.C. Phillips, and J.F. Wilkinson. 1970. Enrichment, isolation, and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* 61, 205-218.

Wise, M.G., J.V. McArthur, and L.J. Shimkets. 2001. *Methylosarcina fibrata* gen. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type I methanotrophs. *Int. J. System. Evol. Microbiol.* 611-621.