System. Appl. Microbiol. 24, 166–176 (2001)

http://www.urbanfischer.de/journals/sam

SYSTEMATIC AND APPLIED MICROBIOLOGY

Taxonomic Characterization of New Alkaliphilic and Alkalitolerant Methanotrophs from Soda Lakes of the Southeastern Transbaikal Region and description of *Methylomicrobium buryatense* sp.nov.

MARINA KALUZHNAYA[1], VALENTINA KHMELENINA[1], BULAT ESHINIMAEV[1], NATALIA SUZINA[1], DIMITRY NIKITIN[1], ALEXANDER SOLONIN[1], JU-LING LIN[2], IAN MCDONALD[2], COLIN MURRELL[2] AND YURI TROTSENKO[1]

[1]G.K.Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino, Moscow region, Russia
[2]Department of Biological Sciences, University of Warwick, Coventry, UK

Received April 9, 2001

Summary

Five strains of obligate methanotrophic bacteria (4G, 5G, 6G, 7G and 5B) isolated from bottom sediments of Southeastern Transbaikal soda lakes (pH 9.5–10.5) are taxonomically described. These bacteria are aerobic, Gram-negative monotrichous rods having tightly packed cup-shaped structures on the outer cell wall surface (S-layers) and Type I intracytoplasmic membranes. All the isolates possess particulate methane monooxygenase (pMMO) and one strain (5G) also contains soluble methane monooxygenase (sMMO). They assimilate methane and methanol via the ribulose monophosphate pathway (RuMP). The isolates are alkalitolerant or facultatively alkaliphilic, able to grow at pH 10.5–11.0 and optimally at pH 8.5–9.5. These organisms are obligately dependent on the presence of sodium ions in the growth medium and tolerate up to 0.9–1.4 M NaCl or 1 M $NaHCO_3$. Although being mesophilic, all the isolates are resistant to heating (80 °C, 20 min), freezing and drying. Their cellular fatty acids profiles primarily consist of $C_{16:1}$. The major phospholipids are phosphatidylethanolamine and phosphatidylglycerol. The main quinone is Q-8. The DNA G+C content ranges from 49.2–51.5 mol %. Comparative 16S rDNA sequencing showed that the newly isolated methanotrophs are related to membres of the *Methylomicrobium* genus. However, they differ from the known members of this genus by DNA-DNA relatedness. Based on pheno- and genotypic characteristics, we propose a new species of the genus *Methylomicrobium* - *Methylomicrobium buryatense* sp. nov.

Key words: Siberian soda lakes – alkaliphilic and alkalitolerant methanotrophs – *Methylomicrobium buryatense* sp. nov.

Introduction

Obligate methane-utilising bacteria or methanotrophs are specialized group of microorganisms able to utilise methane as the sole carbon and energy source. Methanotrophs are very important microorganisms with regard to their potential for the production of bulk and fine chemicals, as biodegradation and bioremediation agents, and in the greenhouse effect by lowering emission of methane into the atmosphere. Methanotrophs are widespread in nature and inhabit soils, wetlands, fresh and marine waters, lakes and sediments, and are mostly neutrophilic and mesophilic (WHITTENBURY et al., 1970, TROTSENKO, 1976; HANSON and HANSON, 1996). Nevertheless, in the last decade investigations of extreme environments with high or low pH, temperatures, salinities etc have led to the discovery of a variety of extremophilic and extremotolerant methanotrophs (reviewed by TROTSENKO and KHMELENINA, 2001).

Continental soda lakes are extreme habitats characterized by unfavorable physicochemical conditions (high values of pH, mineralization and low concentrations of oxygen). Although alkaline lakes are confined to specific geographic regions, they comprise more than 80% of all inland lake waters by volume, and soda lakes have existed throughout the geological record. Alkaliphilic microbial communities of soda lakes have shown to be composed of major trophic groups and contain representatives of the main branches of the phylogenetic tree of the prokaryotes

0723-2020/01/24/02-166 $ 15.00/0

(ZAVARZIN et al., 1999; GRANT and JONES, 2000). Methane is a major product of the terminal stage of the destruction of organic matter in saline soda lakes and is formed at up to 10–80 nM CH_4 d^{-1} (OREMLAND et al., 1982; NAMSARAEV et al., 1999). The methane thus originated could be further oxidized to CO_2 by methanotrophic communities (JOYE et al., 1999). Potential methane oxidation rates in soda lakes of the southern Transbaikal region were measured and constituted up to 50 nM CH_4 d^{-1} (KHMELENINA et al., 2000a).

Recent molecular ecology studies on several soda lakes have shown the occurrence of obligate methanotrophs (KALYUZHNAYA et al., 1999). The first alkaliphilic methanotroph was isolated from Tuva soda lakes and was tentatively identified as *Methylobacter alcaliphilus* (KHMELENINA et al., 1997). Subsequently several publications described the isolation and initial characterization of alkaliphilic methanotrophs from a highly alkaline lake Magadi in Kenyan (SOROKIN et al., 2000) and soda lakes of the southern Transbaikal region (KALYUZHNAYA et al., 1999; KHMELENINA et al., 2000a). Although the new isolates were preliminarily assigned to the genus *Methylomicrobium*, their precise taxonomic status remained unknown. Here, on the basis of pheno- and genotypic characteristics, we proposed the Transbaikal methanotrophic isolates to be classified as a new species *Methylomicrobium buryatense*.

Materials and Methods

Bacterial strains and culture conditions

Methods for the enrichment and pure cultures isolation have been described earlier (KHMELENINA et al.,1997; KALYUZHNAYA et al. 1999). All isolated cultures were grown in mineral medium of the following composition (g/l): KNO_3, 1; KH_2PO_4, 0.35; $Na_2HPO_4 \cdot 12H_2O$, 0.65; $NaHCO_3$, 8.4; Na_2CO_3, 3.0; NaCl, 7.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02 and 1 ml trace elements (pH 9.5) (KHMELENINA et al., 1997). Liquid cultures were grown in 750 ml flasks containing 100 ml of the above medium with shaking (200 rpm/min) for 2–3 days under methane-air atmosphere (2:1) at 26 °C. Cultures were maintained on a solidified mineral medium containing 1.5% ($^w/_v$) Difco agar. Plates were incubated under the same methane-air mixture for 5–10 days.

The methanotrophs *Methylosinus trichosporium* OB3b, NCIMB 11131, *Methylomicrobium album* BG8 NCIMB 11123 obtained from the Russian Culture Collection of Microorganisms (VKM) and *Methylomicrobium* sp. AMO 1 (SOROKIN et al., 2000) were cultivated under methane in NMS medium (WHITTENBURY et al., 1970).

Electron microscopy

For preparation of thin sections, exponentially grown cells were prefixed in the presence of ruthenium red (LUFT, 1964). Ultrathin sections of cells and negatively stained preparations were prepared and examined in a Jeol JEM 100B (Japan) electron microscope as described earlier (KHMELENINA et al., 1999).

Physiological and biochemical characterization

• Effect of pH, temperature and salinity: The effect of pH on growth rates was investigated by cultivating bacteria in liquid basal medium supplemented with 0.75% $^w/_v$ NaCl. The following buffers were used: NaH_2PO_4/Na_2HPO_4 (pH 4.2-8.5), $NaHCO_3/Na_2CO_3$ (pH 9–10.5) and Na_2HPO_4/KOH (pH 11) at a final concentration 0.05 M. 10 ml of cultures grown at the appropriate pH were used for inoculations. The temperature range for growth and the effect of NaCl concentrations (0–1.5 M) on the growth rates were examined in liquid medium at pH 9.5. Aliquots were taken and optical density at 600 nm (OD_{600}) was measured at 6 h intervals. $NaHCO_3/Na_2CO_3$ was replaced by 2-[N-Cyclohexylamino]ethanesulfonic acid (CHES)-KOH buffer (pH 9.5, 0.05 M) for determination of carbonate-dependence of bacterial growth. Also, $KHCO_3/K_2CO_3$ (pH 9.5, 0.05 M) instead of $NaHCO_3/Na_2CO_3$ and KCl instead of NaCl were used for studying the sodium dependence of bacterial growth. Their growth rates were calculated according to Pirt (1975).

• Substrate utilization: The ability of the isolates to utilize various carbon substrates was tested by the use of liquid basal medium supplemented with autoclaved or filter-sterilized substrates (mono-, di- and trimethylamine, formate, formaldehyde, tetramethylammonium chloride, formamide, acetate, pyruvate, citrate, malate, succinate, D-glucose, D-xylose, D-arabinose, maltose, sucrose, mannitol, ethanol, glycerol and yeast extract at a final concentration of 0.5 g l^{-1}. The ability of the strains to grow on methanol was tested in liquid medium at concentrations of 0.1–9% ($^v/_v$).

Nitrogen sources were tested using agar medium in which KNO_3 was replaced by one of the following compounds at a concentration of 0.05% ($^w/_v$): $NaNO_2$, $(NH_4)_2SO_4$, methylamine, urea, glycine, L-alanine, L-proline, L-glutamate, L-aspartate, L-tryptophan and yeast extract. For N_2 fixation experiments, a nitrate-free medium was used.

• Phenotypic characterization: Heat resistance was tested by heating cell suspensions at 80 °C for 20 min, followed by growth at optimal pH and salinity in liquid cultures for 6 days. To test freeze-drying resistance, the cell suspensions in mineral growth medium were frozen at –20 °C and then lyophilized. Desiccation resistance was assessed according to WHITTENBURY et al. (1970). Sensitivity to antibiotics was examined by spreading cells into agar solidified medium and placing Difco discs containing the following antibiotics (μg ml^{-1}): neomycin (30), kanamycin (30), ampicillin (10), erythromycin (15), linkomycin (2), gentamycin (10), novobiocin (30), nalidixic acid (30), streptomycin (10). Growth under methane was assessed after 5 days.

The concentrations of formaldehyde and formate in the culture liquid was measured by the methods of Nash (1953), and Lang and Lang (1960), respectively. The rate of methane oxidation by cells was determined using an LP-7 polarograph (Czech Republic) as described elsewhere (KALYUZHNAYA et al., 1999). Screening for methanotrophs containing sMMO was performed by the naphthalene oxidation test (BODROSSY et al., 1995). sMMO activity in whole cell suspensions was measured by a colorimetric method (PHELPS et al., 1992).

• Cell-free extracts preparation and enzyme assays: Cultures were grown to mid-log phase under methane at optimal salinity and pH and then harvested by centrifugation at 5 000 g for 20 min. The cells (1 g) were washed once in 50 mM Tris HCl buffer, pH 7.5 containing 0.1 M NaCl, and suspended in the same buffer (4 ml) followed by MSE (150 W, 20 kHz) sonication in ice-cooling cuvettes (3×30 sec) and sedimented at 30 000 g for 30 min. The supernatants were used for enzyme assays by previously published methods (TROTSENKO et al., 1986; TROTSENKO and SHISHKINA, 1991; KALYUZHNAYA et al., 1999).

• Cellular fatty acids, phospholipids and quinone system analyses: Cellular fatty acids profiles for strain B5 were analysed by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zelkullturen). Cultures for phospholipid analysis were grown at optimal salinity (0.13 M NaCl) at different pH in the presence of ^{14}C-methane (Amersham, 0.2 μCi/μmol). ^{14}C-phospholipids were extracted and analyzed as previously described (KHMELENINA et al., 1999). Quinones were extracted and purified according to Collins (1985) and their analysis was done by using a Finnigan MX-1310 (Germany) mass spectrometer.

DNA analysis

• DNA G+C content determination and DNA-DNA hybridization: DNA was isolated and the G+C content was determined by the thermal denaturation method (SAMBROOK et al., 1989).

For DNA-DNA hybridization the DNAs (1 μg/ml) obtained from all the new isolates and *Methylomicrobium album* BG8, *Methylomicrobium* AMO1 and *Methylobacter alcaliphilus* 20Z were labelled with the ^{3}H-dNTP by using the "Nick translation Kit" No. 5500 (Amersham Pharmacia Biotech). DNA (1 mg) was immobilized on 0.22 μm nylon membranes („Hiiu Kalur", Estonia) and hybridized to various radioactive labeled probes (specific activity 5×10^6 dpm/μg). The hybridization mixture (SAMBROOK et al., 1989) was incubated for 24 h at optimal conditions:

T_{inc}= 0.51 GC% + 47 – 0.61·(% formamide).

PCR was used to amplify the *mmo*X and *pmo*A genes from the chromosomal DNA of all the new strains. Designed primers for *mmo*X1/*mmo*X2 (MIGUEZ et al., 1997) and *mmo*X882/1403 (McDONALD et al.,1995) were used to amplify 369 bp and 564 bp fragments of *mmo*X genes. The primers A189/A682 were used to amplify a 525 bp portion of *pmo*A genes (HOLMES et al., 1995).

• Sequencing of 16S rDNA: 16S rDNAs of strains 4G, 5G, 6G, 7G and 5B were amplified by thermostable DNA-polymerase (USB) via PCR in a mixture containing 1x*Taq*Pol buffer (USB), 0.2 μg chromosomal DNA, 20 pM oligonucleotide primers pA and pH' (EDWARDS et al., 1989), 2.5 mM dNTP, 2.5 mM $MgCl_2$ and 2 U *Taq*^{r}. After denaturation at 94 °C for 5 min, the reaction mixture was subjected to 30 thermal cycles (52 °C, 1 min; 72 °C, 1.5 min; 94 °C, 1 min). PCR products were sequenced using the FemtoMol Kit (Promega). 16S rDNA was sequenced in both strands by Sanger's method using Taq polymerase (Promega) and pA, pC, pE, pD′ , pF′, and pH′ primers (EDWARDS et al., 1989). All the procedures were done according to the manufacturer's protocol. The determined 16S rDNA sequences were aligned against those of closely related strains with the use of the CLUSTAL program (version 1.60). Pairwise evolutionary distances were computed by the correction of Jukes and Cantor (FELSENSTEIN, 1989) using the DNADIST program and phylogenetic tree constructed via FITCH program of the PHYLIP (version 3.5) software package.

The 16S rDNA sequences determined in this study have been deposited in the EMBL Nucleotide Sequence Database under the accession numbers AF096093 (strain 4G), AF096092 (strain 5G and 7G) and in the GenBank as AF307139 (strain 5B). The accession numbers of the sequences used as references and registered in the GenBank are the following: *Methylomicrobium pelagicum* (X72772), *Methylomicrobium album* (X72777), *Methylomicrobium agile* (X72767), *Methylobacter whittenburyi* (X72773), *Methylobacter luteus* (X72772), *Methylobacter vinelandii* (L20841), *Methylobacter bovis* (L20839), *Methylomonas rubra* (M95662), *Methylomonas sp.* 761 (L20846), *Methylomonas methanica* (AF15086), *Methylomonas aurantiaca* (X72776), *Methylomonas fodinarum* (X72778), *Methylococcus thermophilus* (X73819), *Methylococcus sp.* JB140 (X72769), *Methylococcus capsulatus* (Bath) (X72771), *Methylococcus capsulatus* (Texas) (X72771), *Methylocaldum gracile* (U89298), *Methylocaldum szegediense* (U89300), *Methylocaldum tepidum* (U89297).

• PCR amplification and sequence analysis of pmoA gene: Amplification of the *pmo*A gene from strain 5B was performed using the primer sets A189 and A682. The sequence of these primers and PCR amplification conditions were described by HOLMES et al (1995). Reaction products were checked for size and purity on 1% (w/v) agarose gels and then ligated into the pCR II vector supplied with the TA cloning kit (InVitrogen, San Diego, USA), according to the manufacturer's instructions.

Small-scale preparations of plasmids were done as described previously (SAUNDERS and BURKE, 1990). DNA sequencing reactions were carried out by cycle sequencing using the ABI PRISM Dye-Terminator Kit (PE Applied Biosystems, Warrington, Cheshire, UK). Primers used for sequencing the *pmo*A gene clone were the M13–40 forward and M13 reverse primers (Gibco-BRL, Paisley, Scotland). Inferred polypeptide sequences, PmoA, were aligned manually with sequences obtained from the GenBank database and dendrograms were constructed using the programs PROTDIST, PROTPARS, and FITCH from the PHYLIP v3.5c package (FELSENSTEIN, 1993). New gene sequences have been deposited in GenBank under the accession numbers: *pmoA* sequence of strain 5B, AF307138.

Results

Morphology and ultrastructure

Cells of all five strains are large motile rods 0.7–1.3 μm in width and 1.2–3.0 μm in length with a single flagellum (Fig. 1a). They reproduce by binary fission and do not obviously form cysts or other resting bodies. Cell walls of typical Gram-negative structure and Type I internal membrane arrangements were seen in ultrathin sectioned cells (Fig. 1b). Cells of all strains had tightly packed cup-shaped structures on the outer cell wall surface. These structures were clearly seen on sectioned preparations of the cells prefixed by ruthenium red, indicating that they contain polysaccharides.

Cultural and physiological properties

Colonies of the new isolates grown at 30 °C for 5 days on mineral medium agar plates under a methane-air atmosphere were white to slightly cream, uniform, glistening convex with entire edges, 1–2 mm in diameter. The new isolates grew over a wide pH range and revealed different pH optima. Strain 5G grew at pH 6.0 to 10.0, with a relatively high rate at pH 8.0-8.5 (t_d = 6.9 h); strains 4G and 6G grew at pH 6.0–11.0 (optimally at pH 9.5 with t_d values of 7.7 h and 6.3 h, respectively), strain 5B grew at pH range of 6.8 to 10.5 (optimally at pH 9.5 with t_d value 10.2 h). Strain 7G grew at pH 6.0–11.0 and optimally at pH 7.5 (t_d=5.8 h).

All the isolates required 0.13 M sodium ion concentration in order to support the highest growth rate but no growth occurred in sodium-free medium (when NaCl was omitted and $KHCO_3/K_2CO_3$ or CHES buffers were used instead of sodium carbonate buffer). Strains 7G and 5B were capable of growth at salinities up to 5% ($^w/_v$) NaCl, however, strains 4G, 5G and 6G could grow at a salt concentration as high as 8% ($^w/_v$) NaCl. All the strains grew optimally at 0.1–0.3 M $NaHCO_3/Na_2CO_3$. Strains 5B and 6G were capable of growth in 1 M sodium carbonate buffer (Fig. 2). All the strains grew well in microaerobic conditions.

Being mesophilic, the new isolates grew at the temperatures ranging from 7 to 37 °C, with the optimum at 28–30 °C. Strain 5G was capable of growth over the widest range of temperature (4–45 °C). Remarkably, most of the isolates were shown to be heat resistant (capable of survival after heating at 80 °C for 20 min) and dessication, as well as freeze-drying resistant.

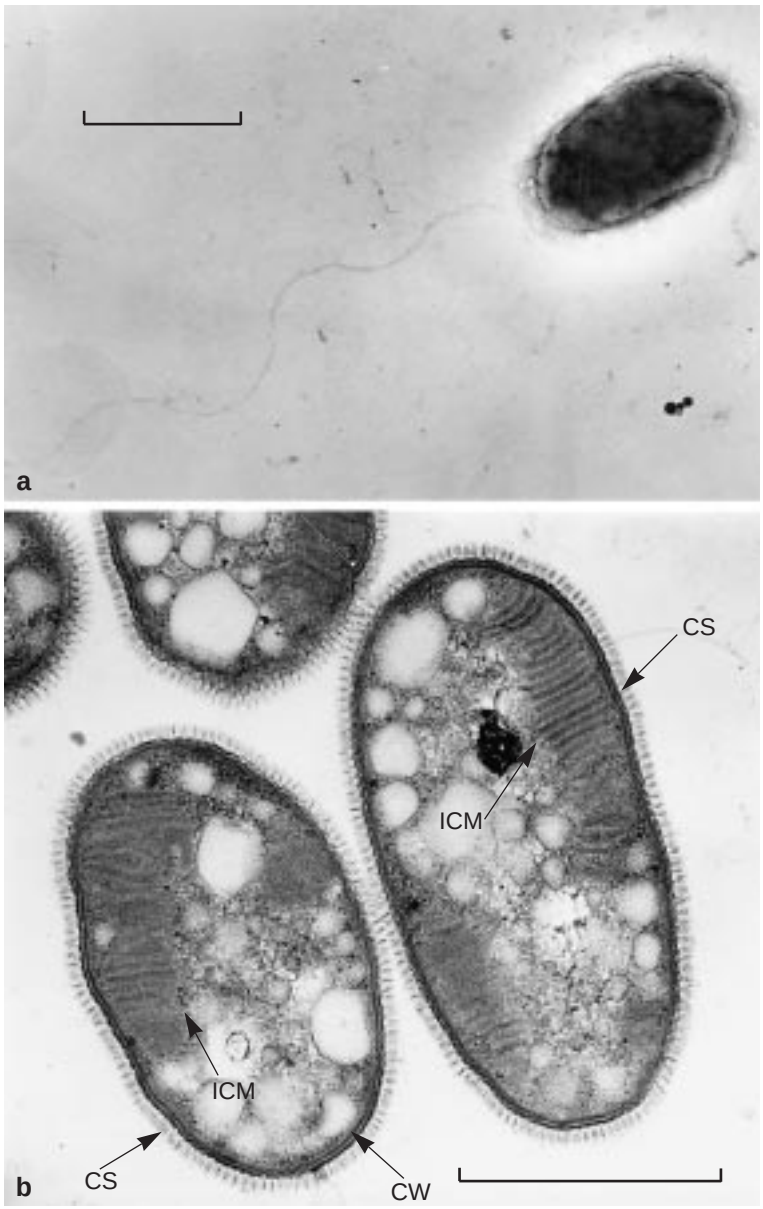

Fig. 1. Cell morphology of strain 5B: a – negatively stained cell with single flagellum; b – thin sectioned cell showing cell wall structure (CW), internal membrane arrangements (ICM) and cup-shaped structures on the outer cell wall surface (CS). Bar 1 μm (all panels).

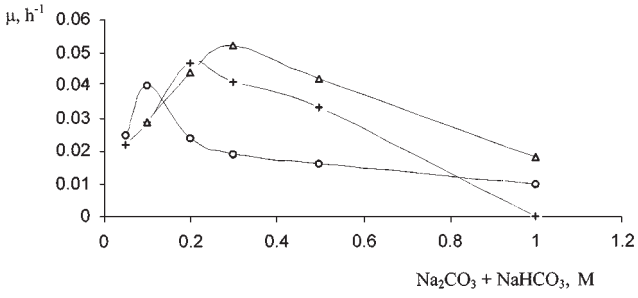

Fig. 2. Effect of different concentrations of sodium carbonate buffer on growth rate of strains 4G (+), 6G (□) and 5B (○). The cultures were grown in the presence of 0.75% NaCl.

Methane or methanol but no other one-carbon or multi-carbon compounds tested (see "Methods") supported growth of the isolates, thus indicating that these bacteria are obligate methanotrophs. All the strains were capable of growth in medium containing extremely high methanol concentrations of up to 7% ($^{v}/_{v}$). The maximum growth rate was obtained at 1% ($^{v}/_{v}$) methanol. Growth of the strains on methanol, was accompanied by accumulation up to 1.2 mM formaldehyde and 8 mM formate in the growth medium (Fig.3), however, no formaldehyde was detected in the medium when grown on methane. The haloalkalitolerant isolates produced high amounts of slime, especially during cultivation on methanol.

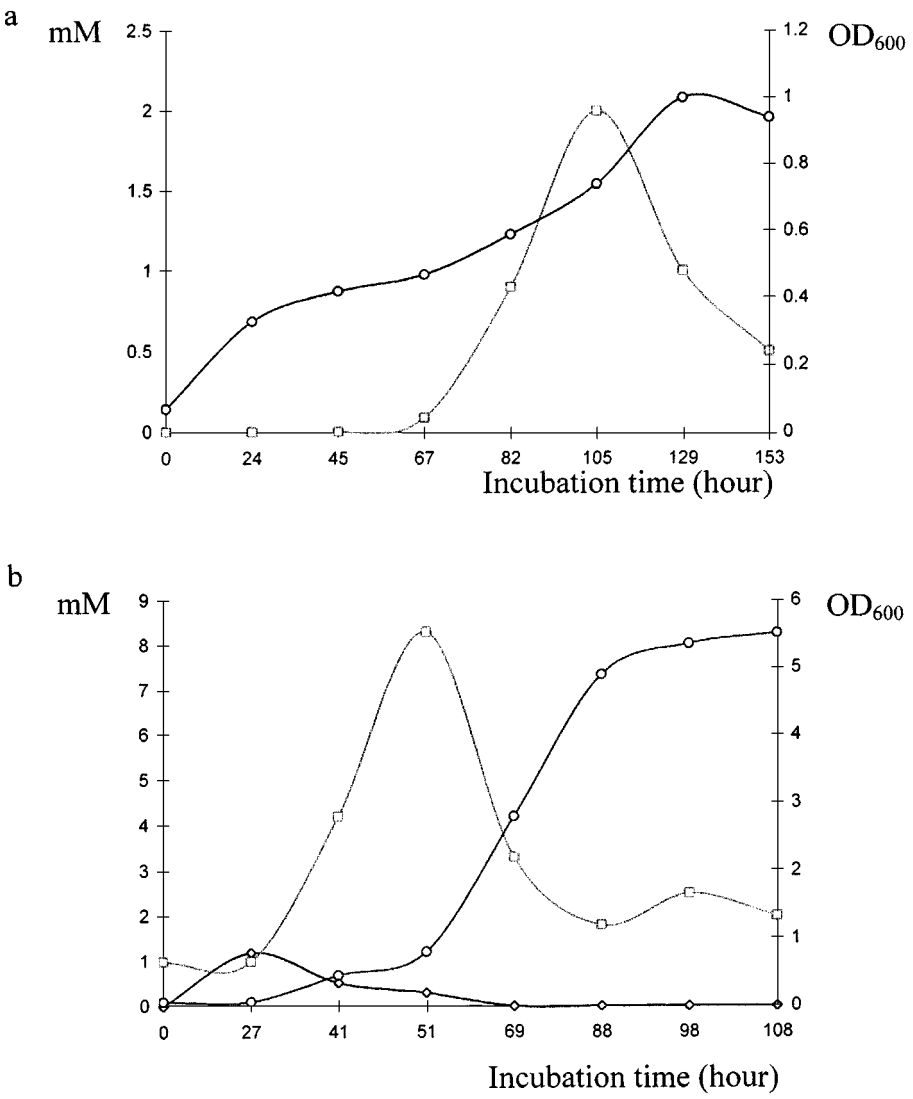

Fig. 3. Accumulation of formaldehyde (◇) and formate (□) during growth (○) of strain 5B on methane (a) and methanol (b).

All the strains used nitrates, nitrites, tryptophan, urea and yeast extract as nitrogen sources and none of them were able to fix dinitrogen. Also, all the strains were capable of growth in neutral medium containing ammonium salts as a sole nitrogen source but only two of them (strains 5B and 6G) grew under alkaline conditions (pH 9.5) in the presence 0.3 mM NH_4^+. All the strains were resistant to gentamycin, streptomycin and kanamycin, but sensitive to neomycin, novobiocin and nalidixic acid. In addition, strains 5B and 6G were tolerant to erythromycin and ampicillin, while strains 4G and 7G were resistant to linkomycin.

Metabolic characteristics

Cell suspensions of the haloalkaliphilic isolates oxidised methane with relatively high rates (180–200 nmol min^{-1} mg^{-1} of protein) and exhibited a high affinity for methane (K_s= 0.9–2.9 μm). Only strain 5G was capable of oxidising naphthalene (150 nmol min^{-1} mg^{-1} of protein) after growth in copper-free medium, thus implying the presence of sMMO.

As seen from Table 1, cell-free extracts of all strains contained activity of the hexulosephosphate synthase and the other enzymes of the RuMP pathway such as pyrophosphate (PPi)-dependent 6-phosphofructokinase and fructose-1,6-bisphosphate aldolase. Also, activities of the pentose phosphate cycle enzymes (glucose-6-phosphate and 6-phosphogluconate dehydrogenases) and Entner-Doudoroff pathway (2-keto-3-deoxy-6-phosphogluconate aldolase) were present. Alternatively, the serine pathway key enzymes (hydroxypyruvate reductase and serine-glyoxylate aminotransferase) as well as the ribulosebisphosphate cycle enzymes (ribulose bisphosphate carboxylase/oxygenase and phosphoribulokinase) were not found. Activities of α-ketoglutarate dehydrogenase was also not

Table 1. Enzyme activities in cell extracts of the new isolates (nmol · min^{-1} · mg^{-1} protein).

Enzyme	Co Cofactor	Strains				
		4G	5G	6G	7G	5B
Particulate methane monooxygenase	NADH	24	30	20	35	32
Soluble methane monooxygenase *		0	150	0	0	0
Methanol dehydrogenase	PMS	18	207	10	40	93
Formaldehyde dehydrogenase	PMS	13	5	3	4	17
Formate dehydrogenase	PMS	288	275	4	114	243
	NAD	203	137	12	47	81
Hexulosephosphate synthase		364	430	463	230	60
Ribulose bisphosphate carboxylase		0	0	0	0	0
Phosphoribulokinase		0	0	0	0	0
Hydroxypyruvate reductase	NAD(P)H	0	0	0	0	0
Serine-glyoxylate aminotransferase	NAD(P)H	0	0	0	0	0
6-Phosphofructokinase	PPi	372	482	140	321	30
	ATP	0	0	0	0	0
Fructose-1,6-bisphosphate aldolase		175	221	70	150	40
2-Keto-3-deoxy-6-phosphogluconate aldolase		34	32	18	28	30
Glucose-6-phosphate dehydrogenase	NAD	11	0	0	0	0
	NADP	7	10	15	7	11
6-Phosphogluconate dehydrogenase	NAD	10	20	3	13	15
	NADP	14	0	3	0	0
Pyruvate dehydrogenase	NAD	27	4	4	4	15
α-Ketoglutarate dehydrogenase	NAD	0	0	0	0	0
Isocitrate lyase		0	0	0	0	0
Malate syntase		0	0	0	0	0

P* – measurements were done with cell suspensions. PMS = phenazine methosulfate.

Table 2. Cellular fatty acid composition of the *Methylomicrobium* species.

Fatty acid	% of total	
	Strain 5B	*Methylomicrobium* spp*
14:0	4.71	0.7–1.8
i15:0	0	0–7.3
15:0	0.87	0
a15:0	0.95	0
16:1w8c	0	12.1–19.0
16:1w7c	50.98	14.1–19.9
16:1w6c	0	5.9–14.4
16:1w5c	15.07	5.6–7.4
16:1w5t	0	5.6–28.2
16:0	20.95	11.3–18.1
16:0 3OH	0.87	0
18:2w6,9c	1.03	0
17:0	0	0–0.1
18:1w9c	1.22	0
18:1w7c	0.61	0–26.5
18:0	1.36	0–2.8
18:0 3OH	1.38	0

* Data from Bowman et al. (1993).

detectable thus indicating the operation of the incomplete tricarboxylic acid cycle. The glyoxylate cycle enzymes were also absent. In general, the metabolic pattern of the novel isolates is rather similar to that of type I methanotrophic bacteria.

Table 3. Cell phospholipids (% of total) of strains 5G and 7G grown at different pH.

Phospholipids	Strain 5G		Strain 7G	
	pH 7.0	pH 9.0	pH 6.8	pH 9.0
Phosphatidic acid	0.01	4	0.01	1.2
Phosphatidylserine	2.2	7.6	5.6	4.7
Phosphatidylethanolamine	95.0	63.0	72	65
Phosphatidylglycerol	2.4	18	13	28
Cardiolipin	traces	traces	2.7	2.4

Membrane lipids

The cellular fatty acid profile of strain B5 is presented in Table 2. Comparison with data published for the other representatives of type I methanotrophs (BOWMAN et al., 1995) showed the highest similarity of the cell fatty acid composition between the strain B5 and *Methylomicrobium* species. $C_{16:1}$ acids were also prevailing in isolates 5G, 4G, 6G and 7G.

The major polar lipids in the all haloalkalitolerant methanotrophs studied were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Also, phosphatidylserine (PS) and phosphatidic acid (PA) were found in all isolates. The pH dependence of phospholipid composition was studied for strains 5G and 7G (Table 3). An increase in PG level and decrease in PA were observed

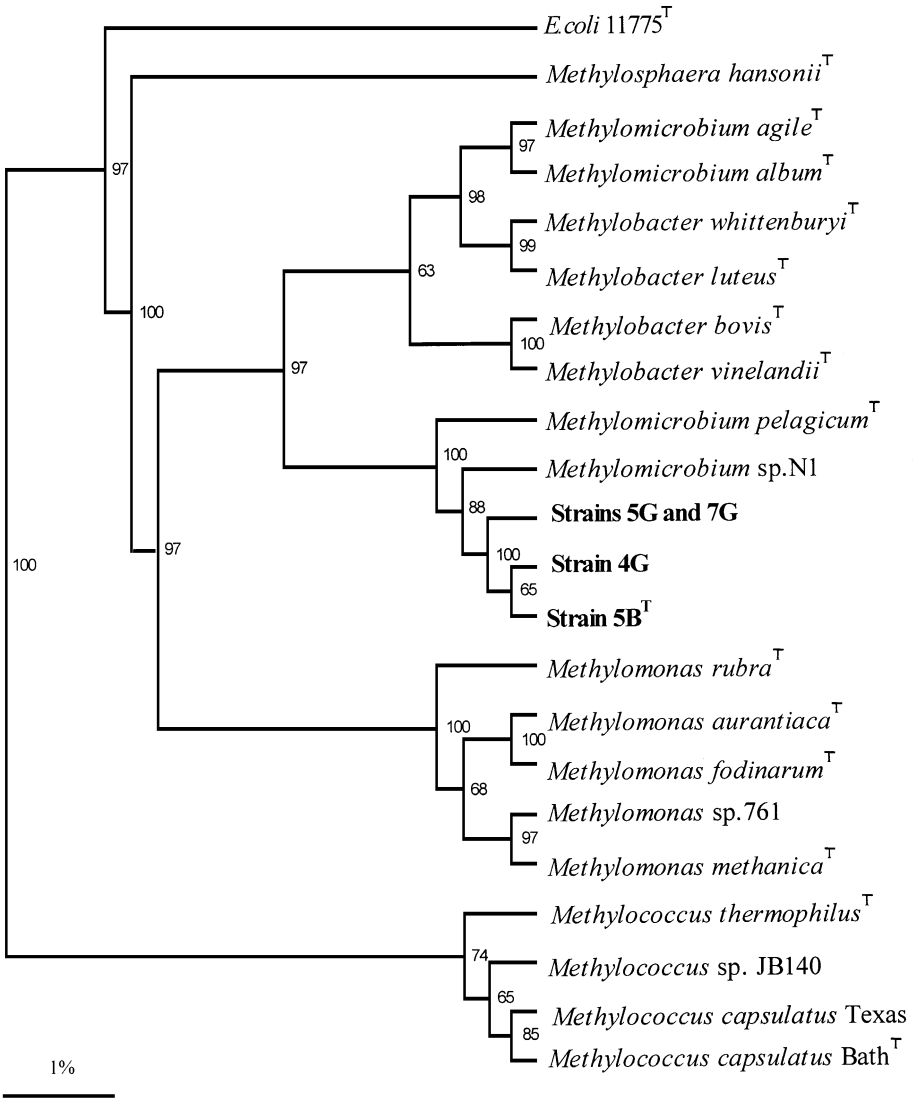

Fig. 4. Phylogenetic position of new alkaliphilic strains among methanotrophs of the gamma- *Proteobacteria*. Pairwise evolutionary distances were computed by the correction of Jukes and Cantor using the DNADIST program and phylogenetic tree constructed via FITCH program of the PHYLIP (version 3.5) software package. Bar = 1% of evolutionary difference.

in response to elevated pH in the growth medium. A major ubiquinone was found to be Q-8.

Genomic characteristics

Virtually identical G+C contents in DNA (49.2–51.9 mol%) were found for the strains studied. These values were close to those obtained for the many other representatives of type l methanotrophs. The DNA-DNA hybridization analysis showed that all the isolates were closely related to each other because of the high homology of their DNAs (74–84%). However, the level of DNA-DNA hybridization of the isolates with *Methylomicrobium album* BG8 did not exceed 6% (Table 3).

Phylogenetic analysis

16S rDNA sequencing analysis showed 95% similarity with *Methylomicrobium pelagicum* by comparison of 1460–1520 available bases and only 94% similarity with *M. album* (Fig.4). The sequences were compared with those of *Methylomicrobium* species available in the GenBank data base.

The *pmo*A-specific PCR primers amplified the correct DNA fragment from all the strains (Fig.5a). No PCR-product was obtained with template DNA extracted from these strains using *mmo*X882/*mmo*X1403-primers. However, by using *mmo*X1/*mmo*X2 primers, the expected PCR products were obtained for strain 5G DNA (Fig.5b).

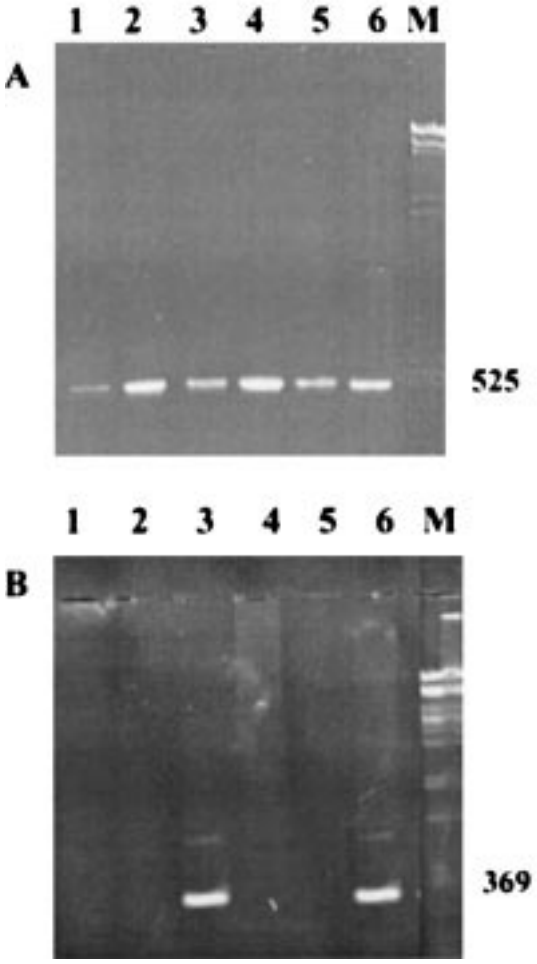

Fig. 5. PCR amplification of *pmo*A (a) and *mmo*X (b) genes of particulate and soluble MMO from alkaliphilic methanotrophic isolates: 1 – strain 5B; 2 – strain 4G; 3 – strain 5G; 4 – strain 6G; 5 – strain 7G; 6(a) – *Methylomicrobium album* BG8^T; 6(b) – *Methylosinus trichosporium* OB3B^T

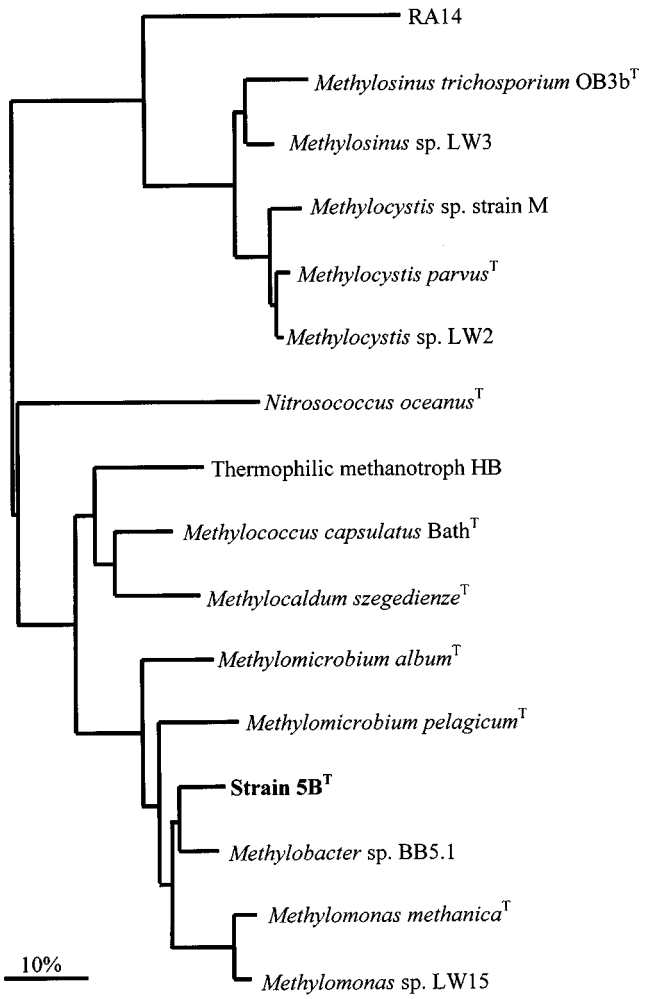

Fig. 6. Phylogenetic analysis of the derived PmoA sequence from the alkaliphilic methanotroph strain 5B. The dendrogram shows the results from analysis using PROTDIST and FITCH. The bar represents 10% sequence divergence, as determined by measuring the lengths of horizontal lines connecting any two species.

Phylogenetic analysis of the derived amino acid sequence of *pmo*A from strain 5B (Fig.6), using the PROTDIST, PROTPARS and FITCH programs of the PHYLIP package (FELSENSTEIN, 1993), showed that the *pmoA* sequence from 5B branched within the *Methylomicrobium/Methylobacter* group of *pmo*A sequences, however the shortage of *pmo*A sequences from type strains of these groups of methanotrophs means that the precise position of the *pmo*A sequence from strain 5B can not be determined.

Table 4. DNA-DNA hybridisation between the new strains and *Methylomicrobium* species.

Strain	Level (%) of hybridisation with labelled DNA from the strains:						
	5B	4G	5G	6G	7G	AMO1	*M.album* BG8^T
M.album BG8	1	1	1	1	1	1	100
AMO1	29	24	25	47	33	100	
7G	83	72	77	82	100		
6G	72	82	78	100			
5G	84	80	100				
4G	83	100					
5B	100						

Table 5. Major characteristics of the new alkaliphilic isolates and *Methylomicrobium* species.

Characteristics	Strain B5	Strain 4G	Strain 5G	Strain 6G	Strain 7G	*M. pelagicum*[a]	*M. album* BG 8[a]
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Size (μm)	1–1.3 × 2.0–3.0	1–1.3 × 2.0–3.0	1–1.3 × 2.0–3.0	1–1.3 × 2.0–3.0	0.7–1 × 1.2–2.5	0.5 × 0.8–1.2	0.6–1.0 × 0.5–3.0
Motility (flagellum)	+	+	+	+	+	+	+
Pigmentation	White	White	White	White	White	White	White
Exopolysacharide production	+	–	+	+	–	nd	nd
Intracytoplasmic membranes	Type I	Type I	Type I	Type I	Type I	Type I	Type I
S-layers	Cup-shaped	Cup-shaped	Cup-shaped	Cup-shaped	Cup-shaped	–	Cup-shaped
sMMO	–	–	+	–	–	–	–
pMMO	+	+	+	+	+	+	+
C_1-assimilation pathway	RuMP	RuMP	RuMP	RuMP	RuMP	RuMP	RuMP
pH for growth	6.8 – 10.5	6.0–10.0	6.0–10.0	6.0–11.0	5.5	5.5–9.0	
Optimum 9.3	6.0–10.0	8.0–8.5	8.0–8.5	8.0–8.5	7.5		
NaCi for growth, % $^{w}/_{v}$	0 – 5	0.2–8	0.2–8	0.2–8	0.2–5	0.2–5	1–2.5
Optimum	0.75	0.75	0.75	0.75	0.75		
Temperature for growth, °C	8–37	7–45	7–45	7–45	15–37	20–30	20–37
Optimum, °C	30	30	30	30	28	25	30
Heat resistance	+	+	+	+	+	–	–
Desiccation resistance	+	+	+	+	+	–	–
Carbon source utilization							
methanol 0.1%	+	+	+	+	+	+	+
7.0%	+	+	+	+	+	nd	nd
methylamine 0.05%	–	–	–	–	–	+/–	+/–
Cardiolipin	+	+	–	+	+	nd	nd
Phosphatydilcholine	–	–	–	–	–	nd	nd
Main quinone	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8
16S rDNA gene homology, %	100	98	98	98	98	95	94
G + C, mol %	51.5	50.6	50.6	50.6	50.6	49	54-56
Source of isolation	soda lake	soda lake	soda lake	soda lake	soda lake	sea water	garden

Data from references: [a]Bowman et al., 1993; 1995; nd–not determined.

Discussion

Five strains of Type I obligate methanotrophs were isolated from different southern Transbaikal soda lakes having high pH values (pH 9.5–10.5). Due to the high homology of their DNAs (72–84%) as well as high similarity of the 16S rRNA genes (98%) all the isolates were referred to as one species. 16S rDNA sequence analysis also showed a relatively high similarity between our isolates and the marine methanotroph *M. pelagicum* (96%). Moreover, many other features of taxonomic value, i.e. cell morphology, ICM arrangement, C_1-assimilation pathway, the cellular fatty acids and phospholipid composition as well as the absence of resting stage formation were similar (Table 5). Unfortunately, *M. pelagicum* was not available for this study and hence no DNA-DNA hybridization was performed between these organisms. Along with *M. pelagicum*, the genus *Methylomicrobium* includes at least two species, the neutrophilic nonhalophiles *M. album* BG8 and *M. agile* (BOWMAN et al., 1995). However, very low DNA homology was found between our isolates and *M. album* BG8 (1%).

Since no cysts or cyst-like structures were detected, an intriguing feature of the new isolates was their resistance to heat and desiccation treatments. Such environmentally significant resistance may be related to an intracellular accumulation of the compatible solutes: sucrose, ectoine and 5-oxoproline (KHMELENINA et al. 1999, 2000b). Compatible solutes are known to serve as protectants for cells not only against osmotic stress but also against heating, freezing and drying (LIPPERT and GALINSKI, 1992; LOUIS et al., 1994). Therefore, the synthesis of compatible solutes by these new isolates may aid their survival in the severe climate of southern Siberia (up to +40 °C in summer and –40 °C in winter) where some soda lakes experience complete freezing and drying.

Additionally, the new strains showed some differences in their physiological properties especially with regard to the pH and salinity ranges as well as pH optima for growth, antibiotic resistance and the occurrence of sMMO genes. All the isolates needed sodium ions for growth and showed the highest growth rates in the almost equal sodium salts concentration (0.13 M NaCl or $NaHCO_3$) although they were distinct in their halotolerance. Surprisingly, when grown in alkaline media, all the isolates were more halotolerant than those grown in neutral media (KALYUZHNAYA et al., 1999).

Also, extremely high methanol concentrations were found to support the growth of haloalkalitolerant methanotrophs studied. It is worthy that rather high tolerance to methanol has been observed for *M. pelagicum* (SIEBURTH et al., 1987). Presumably, formaldehyde accumulation may inhibit growth of most methanotrophs on methanol. The biochemical rationale for such a striking tolerance to methanol by these novel methanotrophs remains to be explored.

All the isolates form the additional glycoprotein structures as cup-shaped subunits on their outer cell wall surfaces. The taxonomic significance of bacterial S-layers is still obscure. The analogous, but not identical, S-layers were also revealed in *M. album* BG8 (JEFFRIES and WILKINSON, 1978). The role of S-layers in neutrophilic methanotrophs is unknown. In haloalkaliphilic methanotrophs it was proposed to be a part of their osmoadaptation mechanisms (KHMELENINA et al., 1999).

From both the 16S rDNA and the PmoA analysis it can be seen that the alkaliphilic strain 5B groups closely with, but distinctly separate from, the *Methylomicrobium* and *Methylobacter* 16S rRNA and PmoA sequences respectively, suggesting that these novel alkaliphilic methanotrophs may belong to a new taxon. This would be supported by the DNA/DNA hydridization results showing that these strains are quite different from *Methylomicrobium album* BG8.

The major properties of the new isolates are summarized in Table 5. Based on their phenotypic and genotypic characteristics, these methanotrophs may be classified as a new species *Methylomicrobium buryatense*.

Description of Methylomicromium *buryatense* sp. nov.

Methylomicromium buryatense sp. nov. **(**bur.ya.ten'se N.L. neut.adj. *buryatense* pertaining to the Buryat region). Gram-negative rods, 0.7–1.3 μm in diameter and 1.2–3.0 μm in length, and reproduce by binary fission. Cells are motile by single polar flagellum. These bacteria form S-layers as cup-shaped units on outer cell wall surface and have Type I intracytoplasmic membranes as stacks of vesicular discs. Colonies on mineral salts medium under methane-air atmosphere are white to slightly cream, uniform, glistening convex with entire edges, 1–2 mm in diameter. Utilize only methane and methanol via the RuMP cycle. Nitrate, nitrite, urea, yeast extract, tryptophan and ammonia are used as the nitrogen sources. Able to grow within the temperature range 15–37 °C (4–45 °C for strain 5G) at pH 6.0–11.0 and salinity from 0.2 to 5% ($^w/_v$) NaCl. Optimal growth conditions: 28-30 °C, 0.5–0.75% ($^w/_v$) NaCl, pH 8.5–9.5. Resistant to heating (80 °C, 20 min), freezing and drying. The major phospholipids are phosphatidylethanolamine and phosphatidylglycerol. The major fatty acids are $C_{16:0}$ and $C_{16:1}$. Ubiquinone Q-8 is the major quinone. The mol% G+C of the DNA is 49.6–51.5 (T_m). The strains were isolated from soda lakes of southern Transbaikal region. The type strain *Methylomicrobium buryatense* $5B^T$ is deposited in the Russian Culture Collection of Microorganisms as VKM B-2245. This species also includes the strains 4G, 5G, 6G and 7G.

Acknowledgments

We are grateful to N.G. Starostina (Institute of Biochemistry and Physiology of Microorganisms, Pushchino) and D.Y. SOROKIN (Institute of Microbiology, Moscow) for fruitful collaboration.

The work was in part supported by the RFBR grant 01-04-48511 and NERC grant GT5/99/FS11.

References

BODROSSY, L., MURRELL, J. C., DALTON, H., KALMAN, M., PUSKAS, L. G., KOVACS, K. L.: Heat-tolerant methanotrophic bacteria from the hot-water effluent of a natural-gas field. Appl. Environ. Microbiol. 61, 3549–3555 (1995).

BOWMAN, J. P., SLY, L. I., NICHOLS, P. D., HAYWARD, A. C.: 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. (1993).

BOWMAN, J. P., SLY, L. I., STACKEBRANDT, E.: The phylogenetic position of the family *Methylococcaceae*. Int. J. Syst. Bacteriol. 45, 182–185 (1995).

COLLINS, M. O.: Analysis of isoprenoid quinones, p. 329–366. In: Methods in Microbiology (G. Gottschalk, ed.) New York, Acad Press, 18 (1985).

EDWARDS, U., ROGALL, T., BLOCKER, H., EMDE, M. and BOTTGER, E. C.: Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic. Acids. Res. 17, 7843–7853 (1989).

FELSENSTEIN, J.: PHYLIP (phylogenetic interference package) version 3.5c. Department of Genetics, University of Washington, Seattle, USA (1993).

GRANT, W. D., JONES, B. E.: Alkaline environments, p. 126–133. In: Encyclopedia of Microbiology. (J. Lederberg, ed). 2nd edition. San Diego, Acad. Press, 3 (2000).

HANSON, R. S., HANSON, T. E.: Methanotrophic bacteria. Microbiol. Rev. 60, 437–471 (1996).

HOLMES, A. J., COSTELLO, A., LIDSTROM, M. E., MURRELL, J. C.: Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. 132, 203–208 (1995).

JEFFRIES, P., WILKINSON J. F.: Electron microscopy of cell wall complex of *Methylomonas albus*. Arch. Microbiol. 119, 227–229 (1978).

JOYE, S. B., CONNELL, T. L., MILLER, L. G., OREMLAND, R. S., JELLISON, R. S.: Oxidation of ammonia and methane in an alkaline, saline lake. Limnol. Oceanograph. 44, 178–188 (1999).

KALYUZHNAYA, M. G., KHMELENINA, V. N., SUZINA, N. E., LYSENKO, A. M., TROTSENKO, Y.A.: New methanotrophic isolates from soda lakes of the southeastern Transbaikal region. Mikrobiologiya. (English translation). 68, 677–685 (1999).

KHMELENINA, V.N., KALYUZHNAYA, M. G., STAROSTINA, N. G. SUUZINA, N.E., TROTSENKO, Y.A.: Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva soda lakes. Curr Microbiol 35, 1–5 (1997).

KHMELENINA, V. N., KALYUZHNAYA, M. G., SAKHAROVSKY, V .G. SUZINA, N. E., TROTSENKO, Y. A., GOTTSCHALK, G.: Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch. Microbiol. 172, 321–329 (1999).

KHMELENINA, V. N., ESHINIMAEV B. TS., KALYUZHNAYA, M. G., TROTSENKO Y. A.: Potential Activity of methane and ammonium oxidation by methanotrophic communities from the soda lakes of Southern Transbaikal. Mikrobiologiya. (English translation). 69, 460–465 (2000a).

KHMELENINA, V. N., SAKHAROVSKY, V. G., RESHETNIKOV, A. S., TROTSENKO, Y. A.: Synthesis of osmoprotectants by halophilic and alkaliphilic methanotrophs. Mikrobiologiya. (English translation). 69, 381–386 (2000b).

LANG, E., LANG, H.: Spezifische Farbreaktion zum direkten Nachweise der Ameisensaure. Z. Anal. Chem. 260, 8–10 (1960).

LIPPERT, K., GALINSKI, E. A.: Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotech. 37, 61–65 (1992).

LOUIS, P., TRUPER, H. G., GALINSKI, E. A.: Survival of *Escherichia coli* during drying and storage in the presence of compatible solutes. Appl. Microbiol. Biotechnol. 41, 684–688 (1994).

LUFT, J. H.: Electron microscopy of cell extrageneous coats as revealed by ruthenium red staining. J. Cell Biol. 23, 54A–55A (1964).

MCDONALD, I. R., KENNA, E. M., MURRELL, J. C.: Detection of methylotrophic bacteria in enviromental samples with the PCR. Appl. Environ. Microbiol. 61, 116–121 (1995).

MIGUEZ, C. B., BOURGUE, D., GREEN, C. W., GROLEAU, D.: Detection and isolation of methanotrophic bacteria possessimg soluble methane monooxygenase (sMMO) genes using the polymerase chain reaction (PCR). Microbiol. Ecol. 33, 21–31 (1997).

NAMSARAEV B. B. ZHILINA, T. N., KULYROVA, A. B., GORLENKO, V. M.: Methanogenesis in soda lakes of south eastern Siberia. Mikrobiologiya. (English translation). 68, 671–676 (1999).

NASH T.: The colorimetric estimation of formaldehyde by means of the Hantsch reaction. Biochem. J. 55, 416–421 (1953).

OREMLAND, R. S., MARSH, L., DESWARAIS, D. L.: Methanogenesis in Big Soda Lake. Appl. Environ. Microbiol. 43, 462–468 (1982).

PHELPS, P. A., AGARWAL, S. K., SPEITEL, G. E., GEORGIOU, G.: *Methylosinus trichosporium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl. Environ. Microbiol. 58, 3701–3708 (1992).

PIRT, J. S.: Principles of Microbe and Cell Cultivation. Oxford, Blackwell Scientific Publications. (1995).

SAMBROOK, J., FRITSCH, E. F., MANIATIS, T.: Molecular Cloning. A Laboratory Manual. (N. Irwin et al., eds). 2nd edition. New York, Cold Spring Harbor Laboratory Press. (1989).

SAUNDERS, S. E., BURKE. J. F.: Rapid isolation of miniprep DNA for double strand sequencing. Nucleic Acids Res, 18, 4948–4948. (1990).

SOROKIN, D. Y., JONES, B. E., KUENEN, J. G.: A novel obligately methylotrophic, methane-oxidizing *Methylomicrobium* species from a highly alkaline environment. Extermophiles. 4, 145–155 (2000).

SIEBURTH, J. M., JOHNSON, P. W., EBERHARDT, M. A., SIERACKI, M. E., LIDSTROM, M. E., LAUX, D.: The first methane-oxidizing bacterium from the upper mixing layer of the deep ocean: *Methylomonas pelagica* sp. nov. Curr. Microbiol. 14, 285–293 (1987).

TROTSENKO, Y. A.: Isolation and characterization of obligate methanotrophic bacteria, p. 329–336. In: Microbial production and utilization of gases (H_2, CO_2, CH_4). (H. G. SCHLEGEL et al., eds). Goettingen, Goltze. (1976).

TROTSENKO, Y. A., DORONINA, N. V., GOVORUKHINA, N. I.: Metabolism of non-motile obligately methylotrophic bacteria. FEMS Microbiol. Letters 33, 293–297 (1986).

TROTSENKO, Y. A., KHMELENINA, V. N.: Extremophilic and extremotolerant methanotrophic bacteria. Arch. Microbiol. (submitted).

TROTSENKO, Y. A., SHISHKINA, V. N.: Studies on phosphate metabolism in obligate methanotrophs. FEMS Microbiol. Rev. 87, 267–271 (1991).

WHITTENBURY, R., DAVIES, S. L., DAWEY, J. F.: Exospores and cysts formed by methane-utilizing bacteria. J. Gen. Microbiol. 61, 219 (1970).

ZAVARZIN, G. A., ZHILINA, T. N., KEVBRIN, V. V.: The alkalipilic community and its functional diversity. Mikrobiologiya. (English translation). 68, 503–521 (1999).

Corresponding author:

YURI A. TROTSENKO, G. K. SKRYABIN Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino, Moscow region, 142290, Russia

Tel: ++7-095-925 7478; Fax: ++7-095-956 3370;

e-mail: trotsenko@ibpm.serpukhov.su