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Methylophaga alcalica sp. nov., a novel alkaliphilic and moderately halophilic, obligately methylotrophic bacterium from an East Mongolian saline soda lake

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A moderately haloalkaliphilic and obligately methylotrophic bacterium (strain M39^T) with the ribulose monophosphate pathway of carbon assimilation is described. Cells of this methanol and methylamine utilizer are aerobic, Gram-negative, asporogenous, motile short rods, multiplying by binary fission. It is auxotrophic for vitamin B_{12} and requires $NaHCO_3$ or NaCl for growth in alkaline medium. Its cellular fatty acid profile consists primarily of straight-chain saturated $C_{16:0}$ and unsaturated $C_{16:1}$ and $C_{18:1}$ acids. The major ubiquinone is Q-8. The dominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol. Diphosphatidylglycerol is also present. Optimal growth conditions are 25–29 °C, pH 9·0–9·5 and 3–4 % (w/v) NaCl. Cells accumulate the cyclic amino acid ectoine as the main compatible solute. The DNA G+C content is 48·3 mol%. Based on 16S rDNA sequence analysis and DNA–DNA relatedness (25–30 %) with the type strains of marine methylotrophs belonging to the genus *Methylophaga*, the novel isolate M39^T (=VKM B-2251^T =ATCC BAA-297^T) was classified as the type strain of *Methylophaga alcalica* sp. nov.

INTRODUCTION

Soda lakes represent the major type of naturally occurring highly alkaline (pH ⩾12) environment characterized by substantial buffering capacity (Grant & Jones, 2000). Their multicomponent microbial communities possess steady trophic relationships and an almost closed cycle of organic matter (Zavarzin *et al.*, 1999). Recently, Kalyuzhnaya *et al.* (1999, 2001) isolated several haloalkaliphilic/-tolerant methanotrophs from the South Siberian soda lakes and demonstrated that intermediates of methane oxidation (methanol, formaldehyde and formate) are excreted into the culture medium. Logically, the trophic relationships between haloalkaliphilic heterotrophs, oligotrophs and methanotrophs must also include aerobic methylobacteria, which provide C_1 units for the recycling of carbon present in the products of incomplete methane oxidation into the common pool of organic matter in these alkaline ecosystems (Trotsenko & Khmelenina, 2002a, b). Very recently, we isolated a novel alkalitolerant, facultatively methylotrophic bacterium with the ribulose bisphosphate (RuBP) pathway and classified it as *Ancylobacter natronum* (Doronina *et al.*, 2001). However, no alkaliphilic methylobacteria with the ribulose monophosphate (RuMP) pathway have yet been isolated.

To date, four genera have been described for neutrophilic, obligate and restricted facultatively methylotrophic bacteria with the RuMP pathway of C_1 assimilation: *Methylobacillus* (Yordy & Weaver, 1977; Urakami & Komagata, 1986), *Methylophilus* (Jenkins *et al.*, 1987), *Methylovorus* (Govorukhina & Trotsenko, 1991) and *Methylophaga* (Janvier *et al.*, 1985; Urakami & Komagata, 1987a; de Zwart *et al.*, 1996). *Methylophaga* species are distinguished from the other RuMP-pathway methylobacteria by their requirements for Na^+, Mg^{2+} and vitamin B_{12}, tolerance of NaCl and low DNA G+C content (38·0–49·0 mol%). Here, we report the first isolation and taxonomic characterization of an alkaliphilic and moderately halophilic, RuMP-pathway obligate methylotroph belonging to the genus *Methylophaga*. The name *Methylophaga alcalica* sp. nov. is proposed for this isolate.

Abbreviations: NMS, nitrate/mineral salts; RuBP, ribulose bisphosphate; RuMP, ribulose monophosphate.

The GenBank accession number for the 16S rDNA sequence of strain M39^T is AF384373.

METHODS

Bacterial strains. *Methylophaga marina* ATCC 35842^T, *Methylophaga thalassica* ATCC 33146^T (Janvier *et al.*, 1985) and *Methylophaga sulfidovorans* RB-1^T (=LMD 95.210^T =VKM B-2175^T) (de Zwart *et al.*, 1996) were used as reference strains. These methylobacteria were grown in ASW medium (Janvier *et al.*, 1985). Strain M39^T was isolated and grown as described below.

Enrichment and isolation. Samples of bottom sediment were taken from Lake Khotontyn, an East Mongolian saline soda lake, having pH 9·8–10·2 and total salt content of 360 g l^{-1} (45 g Na_2CO_3, 34 g $NaHCO_3$ and 65 g NaCl l^{-1}). Salinity and alkalinity measurements were conducted according to the US Environmental Protection Agency (1983).

Samples (0·5 g) were placed into 750 ml Erlenmeyer flasks with 50 ml nitrate/mineral salts (NMS) medium containing (l^{-1}): KNO_3, 1·0 g; KH_2PO_4, 1·0 g; $MgSO_4.7H_2O$, 0·22 g; NaCl, 30·0 g; ferric citrate, 30 mg; $CaCl_2.2H_2O$, 30 mg; $MgCl_2.4H_2O$, 5·0 mg; $ZnSO_4.7H_2O$, 5·0 mg; and $CuSO_45H_2O$, 0·5 mg. The pH was adjusted to 9·5 by addition of a mixture of $NaHCO_3/Na_2CO_3$ (final concentration 0·1 M). Samples were incubated with 1 % (v/v) methanol at 29 °C, with shaking at 120 r.p.m. for 1 week. After several transfers in NMS medium over 3–5 days, the enrichments were serially diluted and grown on NMS agar [2 % (w/v) agar; Difco]. After several successive re-isolations of single colonies, the presence of heterotrophic colonies was checked by spreading samples of methanol-grown liquid culture into plates of Difco nutrient agar with pH 7·0 and 9·5 and plates of NMS agar with 2 % (v/v) methanol. The above procedures resulted in isolation of a pure culture of the methylotrophic strain M39^T. The strain was stored in liquid medium NMS for 10 days, on agar slants at 4 °C for 2 weeks or freeze-dried with a protectant (skimmed milk) for over a year.

Identification methods. Cell morphology, Gram staining, motility and flagellation were studied on cultures grown on NMS agar. Nitrate reduction was tested in liquid NMS medium after 1, 2 and 3 days incubation. Indole production from 1 mM L-tryptophan was determined with the Salkowski reagent (Gordon & Weber, 1951). Starch hydrolysis was tested by using an iodine solution after 3 days incubation over NMS agar or K media (Doronina *et al.*, 2000) containing 0·2 % (w/v) soluble starch. The oxidase activity test was performed with a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was detected by pouring a 3 % (v/v) H_2O_2 solution into colonies on NMS agar. Halotolerance was tested by inoculating the cells in liquid NMS or K medium with various concentrations of NaCl (0·05–14 %, w/v). Growth at different temperatures and pH was tested in liquid NMS and K media. Utilization of a wide range of growth substrates was also determined in these media after cultivation for 2 weeks with methanol replaced by the other carbon compounds (more than 50 were tested). Organic acids, amino acids and methylated amines were added at concentrations of 0·05–0·3 % (w/v), while carbohydrates and alcohols were added at concentrations of 0·2–0·5 % (w/v or v/v). To test alternative nitrogen sources, KNO_3 was replaced by other nitrogen compounds. Methane utilization was tested in an atmosphere containing methane and air (1 : 1, v/v) in 700 ml conical flasks containing 100 ml NMS medium and fitted with rubber stoppers. Hydrogen utilization was tested by the same procedure but under atmosphere of $H_2/O_2/CO_2$ (7 : 2 : 1, by vol.).

Chemotaxonomic properties. Fatty acids were extracted from lyophilized cells. To 30 mg of dry biomass, 200 µl of a 5·4 M solution of anhydrous HCl in methanol was added and the mixture was heated at 70 °C for 2 h. The fatty acid methyl esters and aldehyde derivatives obtained were extracted twice with 100 µl hexane. The extract was dried and silylated in 20 µl *N,O*-bis(trimethylsilyl) trifluoroacetamide for 15 min at 65 °C. A 1 µl portion of the reaction mixture was analysed with a model HP-5985B GC-MS system (Hewlett Packard) equipped with a capillary column (25 × 0·25 mm) consisting of fused quartz an Ultra-1 non-polar methylsilicone phase. An initial temperature of 170 °C was ramped to 270 °C at a rate 5 °C min^{-1}. The column was baked after each run for 2 min at 300 °C. The injector and detector temperatures were respectively held at 250 and 300 °C. Data processing was carried out with an HP-1000 computer by using the standard programs of the GC-MS system (Hewlett Packard). Cellular phospholipids were determined as described previously (Govorukhina & Trotsenko, 1989). Ubiquinones were extracted and purified according to Collins (1985). Their analysis was done by using a Finnigan MX-1310 GS-MS system.

For identification and evaluation of intracellular organic solutes, exponentially growing cells (100 mg) at 0·25–1·5 M NaCl were suspended in 2 ml methanol and allowed to stand for at least 1 h at room temperature and then centrifuged (10 000 ***g***, 15 min). The pellet was re-extracted as described above. The supernatants were combined and the solvent was removed under vacuum. The dried methanol extracts were dissolved in 1 ml 0·01 M maleic acid in D_2O and were used for ^{1}H-NMR analysis on a WP-80SY NMR spectrometer (Khmelenina *et al.*, 1999; Doronina *et al.*, 2000). The dried methanol extracts were also dissolved in 1 ml 0·3 M sodium citrate buffer (pH 3·3) and used for amino acid analysis on a Biotronic LC 3000 amino acid analyser. The cytoplasmic volume of strain M39^T at 0·8 M NaCl was estimated using ^3H_2O and ^{14}C-methoxyinulin to monitor the permeable and impermeable volumes as described by Khmelenina *et al.* (1999).

Enzyme assays were done as described previously (Trotsenko *et al.*, 1986; Doronina *et al.*, 1995).

Electron microscopy. An aliquot of cell suspension was mounted on a Formvar-coated copper grid and stained with 0·2 % (w/v) phosphotungstic acid (pH 7·2). Samples were prefixed with 1·5 % (v/v) glutaraldehyde in 0·05 M cacodylate buffer (pH 7·2) and washed three times with 1 % (w/v) OsO_4 in 0·05 M cacodylate buffer (pH 7·2) for 3 h at 20 °C for thin sectioning. After dehydration in a series of alcohols, the cells were embedded in Spurr epoxy resin and sectioned with an LKB 2128 Ultratome. Ultrathin sections were mounted on copper grids and double-stained with uranyl acetate and lead citrate. Negatively stained preparations and thin sections were imaged in a JEOL JEM-100B transmission electron microscope at an operating voltage of 60 kV.

DNA isolation and characterization. DNA was isolated and purified according to Marmur (1961). DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with a Beckman DU-8B spectrophotometer at a heating rate of 0·5 °C min^{-1} and calculated according to Owen & Lapage (1976). DNA from *Escherichia coli* K-12 was used as the standard. DNA–DNA hybridization was done on nitrocellulose membrane filters (0·22 µm) according to Denhardt (1966) in an incubation mixture containing 0·15 M NaCl and 0·015 M trisodium citrate (pH 7·0) with 20 % (v/v) formamide at a temperature at 59 °C for 24 h. [1′,2′,5′-^{3}H]dCTP and an N 5500 nick-translation kit (Amersham) were used for labelling of the DNA probe.

16S rDNA sequencing and phylogenetic analysis. 16S rDNA of strain M39^T was amplified by PCR using a thermostable DNA polymerase (USB) in a mixture containing 1× *Taq*Pol buffer (USB), 0·2 µg chromosomal DNA, 20 pM oligonucleotide primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH′ (5′-AAGGAGGTGATCCAGCTCGT-3′) (Edwards *et al.*, 1989), 2·5 mM dNTPs, 2·5 mM $MgCl_2$ and 2 U *Taq* polymerase. 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 by using the FemtoMol kit (Promega). 16S rDNA was sequenced on both strands by the dideoxy chain-termination method using *Taq* polymerase (Promega) and primers pA, pC (5′-CTACGGGAGGCAGCAGTGGG-3′), pE (5′-AAACTCAAAGGAATTGACGG-3′), pD′ (5′-GTATTACCGCGGCTGCTG-3′), pF′ (5′-ACGAGCTGACGACAGCCATG-3′) and pH′ (Edwards *et al.*, 1989). All procedures were done according

to the manufacturers' protocols. Primers were labelled by T4 polynucleotide kinase (Fermentas) with [γ-^{32}P]ATP (Nuklid-Trans) according to Sambrook *et al.* (1989). Sequencing reactions were performed in a mixture containing 1 × DNA Seq buffer (Promega), 0·2 μg amplified 16S rDNA, 2 pM labelled oligonucleotide primers, ddNTP/dNTP mix (Promega) and 2 U sequencing grade *Taq* polymerase; 30 rounds of sequencing PCR were performed (94 °C, 2 min; 42 °C, 15 s; 72 °C, 30 s; 94 °C, 15 s). The 16S rDNA sequences determined were aligned against those of closely related strains with the use of the program CLUSTAL (version 1.60). Pairwise evolutionary distances were computed by the Jukes & Cantor correction (Felsenstein, 1993) using the program DNADIST and a phylogenetic tree was constructed via the program FITCH from the PHYLIP software package (version 3.5).

RESULTS

Morphology

Cells of strain M39^T are Gram-negative, asporogenous rods, 0·6–0·8 × 1·4–2·8 μm, motile by a single polar flagellum, occurring singly or rarely in pairs (Fig. 1a, b). Reproduction occurred by binary fission. No capsule or intracellular complex membranes were formed. The main morphological feature of the cells is the occurrence of a thick (about 20 nm) granular layer between the cytoplasmic and outer membranes (Fig. 1b), which is usual for marine methylobacteria of the genus *Methylophaga* (Janvier *et al.*, 1985).

Phenotypic characteristics

When grown on agar medium, colonies were 0·5–2 mm in diameter after 4 days at 29 °C, translucent, white to slightly cream and round with glossy surfaces and entire edges. Cells did not grow on nutrient or peptone broth supplemented with 3 % (w/v) NaCl at pH 7·0 or 9·5. Nitrate was reduced to nitrite. Hydrolysis of starch was not observed. Indole was produced from L-tryptophan. Strain M39^T required vitamin B_{12} for growth. The isolate was a strict aerobe that produced catalase, oxidase and urease. Nitrate, glutamate, urea (medium NMS), methylamine and ammonium (medium K with NaCl) were utilized as nitrogen sources. Growth occurred at temperatures between 4 and 35 °C; the optimum growth temperature was 25–29 °C. The optimum pH for growth was 9·0–9·5; no growth was detected below pH 7·0 (medium K with NaCl) or pH 8·0 (medium NMS) or above pH 11·5. Strain M39^T grew well at 3–4 % NaCl, but did not grow at NaCl concentrations above 10 % (w/v). The generation time was about 0·4 h^{-1} on medium NMS [pH 9·5 with 0·5 % (v/v) methanol, 3 % (w/v) NaCl and 20 mg vitamin B_{12} l^{-1}]. Strain M39^T could utilize methanol and methylamine as carbon and energy sources, but not di- and trimethylamines, organic acids, sugars, amino acids, C_2–C_6 alcohols or gas mixtures of $CO_2/H_2/O_2$ or CH_4/O_2.

As seen from Table 1, the dominant cellular fatty acids of strain M39^T were straight-chain saturated $C_{16:0}$ and unsaturated $C_{16:1}$ and $C_{18:1}$ acids. The presence of 3-hydroxy fatty acids was observed, but not 2-hydroxy fatty acids. Strain M39^T had cellular fatty acid composition type A and is similar to group 11 of the marine methylobacteria (Urakami & Komagata, 1987b). Analysis of the cellular phospholipids revealed the presence of phosphatidylethanolamine and mono- and diphosphatidylglycerol in strain M39^T. The major ubiquinone was Q-8.

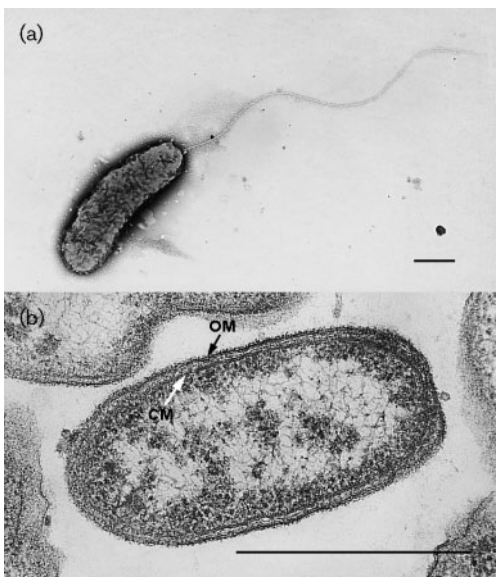

Fig. 1. Electron micrographs of cells of strain M39^T. (a) Negatively stained cell. (b) Ultrathin section showing cell wall structure. The periplasmic space appears as a thick layer with a granular structure located between the outer membrane (OM) and the cytoplasmic membrane (CM). Bars, 0·5 μm.

Table 1. Cellular fatty acid composition of strain M39^T grown on methanol

Fatty acid	Proportion (% of total fatty acids)
Straight-chain acids	
$C_{12:0}$	2·48
$C_{14:0}$	0·24
$C_{15:0}$	0·20
$C_{16:0}$	30·14
$C_{16:1}$	30·41
$C_{17:0}$	1·02
$C_{17:1}$	0·03
$C_{17:2}$	0·06
$C_{18:0}$	1·69
$C_{18:1}$	32·10
Cyclopropane acids	
$C_{17:0}$ cyclo	1·17
$C_{19:0}$ cyclo	0
Hydroxy acids	
3-OH $C_{10:0}$	0·23
3-OH $C_{14:0}$	0·21
3-OH $C_{16:0}$	0·03

Table 2. Intracellular organic solute pools in strain M39^T grown at various osmolarities

Experiments were carried out at pH 9.

NaCl concentration (M)	Solute content [μg (mg cell dry wt)$^{-1}$]		
	Ectoine	Glutamate	Sucrose
0·25	0	30	0
0·83	160	45	0
1·5	235	70	35

Intracellular organic solutes

Cells of strain M39^T grown at various NaCl concentrations were examined for the accumulation of organic solutes. The ^{1}H-NMR spectrum showed that ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine 4-carboxylic acid) and glutamate were the major cytosolic constituents in cells grown at 0·8–1·5 M NaCl, whereas sucrose was detected in cells grown at 1·5 M NaCl (Table 2). Chromatographic analysis of non-hydrolysed samples of the cell extract revealed glutamate as the major amino acid. The cytoplasmic pool of these three organic solutes in cells was considerably influenced by the salt concentration, thus implying their osmoprotective role. The cytoplasmic volume of strain M39^T grown at 0·8 M NaCl was estimated as 1·2 μl H_2O (mg cell dry wt)$^{-1}$. Consequently, the total intracellular solute pools of cells grown at 0·2 or 1·5 M NaCl were calculated as 0·2 and 1·9 M, respectively, sufficient to balance the osmotic equilibrium.

Metabolic characteristics

The enzyme profile of methanol-grown cells (Table 3) indicates that strain M39^T can oxidize methanol to formaldehyde by pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase. Direct oxidation of formaldehyde via formate to CO_2 appears to be a minor route, since the activities of formaldehyde and formate dehydrogenases are low. Formaldehyde is assimilated through the RuMP cycle (Entner–Doudoroff variant), as confirmed by the occurrence of 3-hexulose-phosphate synthase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. The RuMP cycle rearrangement reactions are catalysed by transketolase and transaldolase. Glucose-6-phosphate dehydrogenase is active with both NAD^+ and $NADP^+$. Rather high levels of these enzymes indicate the preferential oxidation of formaldehyde to CO_2 via the dissimilatory hexulose-phosphate cycle. Neither the serine nor ribulose-bisphosphate pathway of C_1 assimilation operates, since the appropriate specific enzymes, hydroxypyruvate reductase and serine-glyoxylate transaminase and ribulose-bisphosphate carboxylase, are absent. The tricarboxylic acid cycle is deficient in 2-oxoglutarate dehydrogenase. The absence of isocitrate lyase and malate synthase indicates that the glyoxylate shunt is inoperative in strain M39^T. Primary assimilation of NH_4^+ occurs via the glutamate cycle (GS/GOGAT system) and by reductive amination of 2-oxoglutarate.

Table 3. Activities of enzymes of primary and intermediate metabolism in cell extracts of strain M39^T grown on methanol

Activities are given as nmol min^{-1} (mg protein)$^{-1}$. Abbreviations: GSH, reduced glutathione; PMS, phenazine methosulfate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

Enzyme	Co-factor(s)	Activity
Methanol dehydrogenase	PMS	781
Formaldehyde dehydrogenase	PMS	29
Formaldehyde dehydrogenase	NAD^+, GSH	0
Formate dehydrogenase	PMS	4
Formate dehydrogenase	NAD^+	0
3-Hexulose-phosphate synthase		263
Hydroxypyruvate reductase	NAD(P)H	0
Ribulose-1,5-bisphosphate carboxylase		0
Glucose-6-phosphate dehydrogenase	NAD^+	300
Glucose-6-phosphate dehydrogenase	$NADP^+$	362
6-Phosphogluconate dehydrogenase	NAD^+	0
6-Phosphogluconate dehydrogenase	$NADP^+$	238
Transaldolase		74
Transketolase		223
KDPG aldolase		75
Citrate synthase		20
2-Oxoglutarate dehydrogenase	NAD^+	0
Isocitrate dehydrogenase	NAD^+	193
Isocitrate dehydrogenase	$NADP^+$	0
Isocitrate lyase		0
Malate synthase		0
Glutamate dehydrogenase	NADH	59
Glutamate dehydrogenase	NADPH	52
Glutamate synthase	NADH	59
Glutamate synthase	NADPH	44
Glutamine synthetase	ATP, Mn^{2+}	462

Genotypic characteristics

The DNA G+C content of strain M39^T was estimated via T_m as 48·3 mol%. The level of DNA relatedness between the novel isolate and reference strains of the genus *Methylophaga* (*M. marina* ATCC 35842^T, *M. thalassica* ATCC 33146^T and *M. sulfidovorans* LMD 95.210^T) did not exceed 25–30 %, consistent with their assignment to separate species of the genus *Methylophaga*. A total of 1359 nucleotides of 16S rDNA sequence were determined for strain M39^T, corresponding to positions 7–1449 of the *E. coli* sequence. The sequences were compared with those of representatives of the α-, β- and γ-subclasses of the *Proteobacteria*, including the validly published methylotrophic genera. In preliminary trials, a total of 98 sequences were used and several phylogenetic trees were generated. Phylogenetic analysis employing different algorithms showed similar results (Fig. 2). Hence, strain M39^T

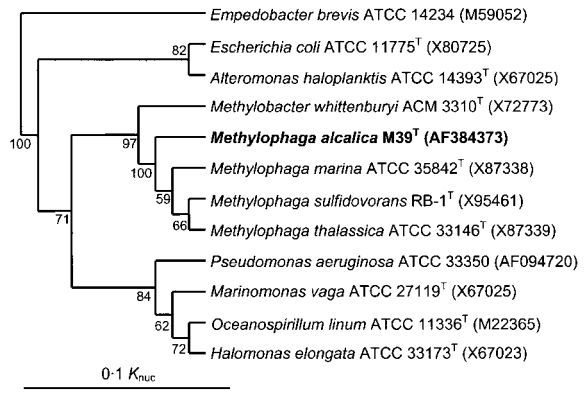

Fig. 2. Phylogenetic position of *Methylophaga alcalica* M39^T among methylotrophs of the γ-*Proteobacteria*. Pairwise evolutionary distances were computed by the correction of Jukes & Cantor using programs of the PHYLIP package (version 3.5). 16S rDNA of *Empedobacter brevis* was used as an outgroup. Numbers at branch points are bootstrap values from 100 replicates. Bar, 10 % evolutionary difference.

appeared to be related to members of the γ-*Proteobacteria*, supported by signature analysis (Woese, 1987). According to 16S rDNA sequence analysis, strain M39^T had similarity of 94–95 % to *M. marina*, *M. thalassica* and *M. sulfidovorans*. The similarity between strain M39^T and other members of the γ-*Proteobacteria*, including methylotrophic species, fell in the range 87·1–92·6 %.

DISCUSSION

The specific feature of the shallow salt lakes of the Mongolian steppe is their dynamic water regimen, often resulting in fluctuations of the salt concentration within a short period (Zavarzin *et al.*, 1999). It is well established that bacteria growing at high salinity generally accumulate one or more specific compatible solutes intracellularly in order to counteract the outflow of water molecules. These compatible solutes comprise a heterogeneous group of organic compounds including polyols, sugars, amino acids, betaine and ectoine (Galinski, 1995). Strain M39^T not only grew over a wide range of NaCl concentrations, but also required Na^+ for growth and tolerated relatively high external salinity by synthesis of low-molecular-mass organic solutes, namely ectoine, glutamate and sucrose. Recently, ectoine was found as a major osmolyte in the serine-pathway aerobic methylobacteria belonging to the genus *Methylarcula* (Doronina *et al.*, 2000). Our calculations based on NMR data and the intracellular water content showed that the levels of ectoine, glutamate and sucrose in strain M39^T grown at 1·5 M NaCl may respectively reach 1·4, 0·45 and 0·08 M, being totally sufficient to maintain osmotic equilibrium between the cytoplasm and the saline environment.

Strain M39^T is an obligate methylotroph with several common characteristics that allow its classification as a *Methylophaga* species. So far, the genus *Methylophaga* has three validly described species, *M. marina*, *M. thalassica* and *M. sulfidovorans*, and includes moderately halophilic neutrophilic bacteria that utilize C_1 compounds through the RuMP pathway. The genus *Methylophaga* was found to be clearly separated from other methylotrophic bacteria and formed a distinct branch within the γ-subclass of *Proteobacteria* (Janvier & Grimont, 1995). In comparison with known members of the genus *Methylophaga*, the cells of strain M39^T are larger, have a higher G+C content and grow optimally in alkaline medium, corresponding to the characteristics of their natural habitat. Strain M39^T is capable of growth on methanol and methylamine, but not on any other tested C_1 or C_n substrate, while *M. marina* and *M. thalassica* grow on fructose and *M. sulfidovorans* grows on dimethylsulfide (Table 4).

Strain M39^T exhibited only 25–30 % DNA–DNA relatedness to known *Methylophaga* species. In the phylogenetic tree derived from 16S rDNA sequences (Fig. 2), strain M39^T consistently branched together with the cluster of the *Methylophaga* species within the γ-*Proteobacteria*. The relatively high level of 16S rDNA sequence identity (94–95 %) between strain M39^T and members of the genus *Methylophaga* indicated their close relationship. Thus, the phenotypic and genotypic data allow the separation of strain M39^T as a distinct genospecies of the genus *Methylophaga*. Consequently, strain M39^T is classified as the type strain of a novel *Methylophaga* species, *Methylophaga alcalica* sp. nov.

Description of *Methylophaga alcalica* sp. nov.

Methylophaga alcalica (al.ca′li.ca. N.L. n. *alcali* alkali, from Arabic n. *al qaliy* soda ash; N.L. adj. *alcalica* from alkaline media).

Gram-negative rods, 1·4–2·8 μm in length and 0·6–0·8 μm in diameter. Multiply by binary fission. Cells are motile by single polar flagella and have a thick (20–30 nm) periplasmic space. Colonies on mineral salts/methanol agar are white to slightly cream, 1–2 mm in diameter. Strictly aerobic and auxotrophic for vitamin B_{12}. Moderately haloalkaliphilic. Able to grow at 4–35 °C, at pH 8·0–11·0 on NMS medium and at pH 7·0–10·5 on K medium with 0·05–10 % (w/v) NaCl and grows optimally at 25–29 °C, pH 9·0–9·5, 3–4 % (w/v) NaCl. Accumulates ectoine and glutamate intracellularly as the main osmoprotectants. Does not grow on peptone/yeast extract medium with or without NaCl at pH 7·0 or 9·5. Catalase- and oxidase-positive. Reduces nitrate to nitrite. Produces indole from tryptophan. Obligate methylotroph that utilizes only methanol and methylamine via the RuMP pathway. Tricarboxylic acid cycle is incomplete at the level of 2-oxoglutarate dehydrogenase; the glyoxylate shunt enzymes are absent. Nitrate, glutamate, urea and ammonia are used as nitrogen sources. The prevailing cellular fatty acids are $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$. The major ubiquinone is Q-8. Cardiolipin is present. DNA G+C content is 48·3 mol% (T_m). The type strain, strain

Table 4. Major differentiating characteristics of *Methylophaga* species

Species are listed as: 1, *M. alcalica* sp. nov. VKM B-2251^T; 2, *M. marina* ATCC 35842^T; 3, *M. thalassica* ATCC 33146^T; 4, *M. sulfidovorans* LMD 95.210^T. All taxa are positive for growth on methanol and methylamine.

Characteristic	1	2	3	4
Cell size (μm)	0·4 × 1·2–2·8	0·2 × 1	0·2 × 1	0·2 × 0·9
Growth temperature (°C):				
Range	4–35	10–40	8–42	17–35
Optimum	25–29	30–37	30–37	22
pH for growth:				
Range	7·0–11·0	5·0–9·0	5·0–9·0	6·0–9·0
Optimum	9·0–9·5	7·0–7·5	7·0–7·5	7·4–7·8
NaCl concentration for growth (%):				
Maximum	10	12	12	12
Optimum	3–4	1–4	1–4	1·5–2·5
Growth on:				
Dimethylsulfide	−	−	−	+
Fructose	−	+	+	−
DNA G+C content (mol%)	48·3	43·0	44·0	42·4

M39^T (=VKM B-2251^T =ATCC BAA-297^T), was isolated from the East Mongolian saline soda lake Khotontyn.

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