Classification of halo(alkali)philic and halo(alkali)tolerant methanotrophs provisionally assigned to the genera *Methylobacter* and *Methylobacter* and emended description of the genus *Methylobacter*

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The taxonomic positions of four aerobic, obligately halo(alkali)philic/-tolerant, methanotrophic bacteria previously affiliated with the genera *Methylobacter* ('*Methylobacter alcaliphilus*' strains 20Z and 5Z) and *Methylobacter* (*Methylobacter* strains AMO1 and NI) were investigated. Phylogenetic analysis of 16S rRNA gene sequences indicated that the strains form a separate branch within the type I methanotrophic bacteria and are closely related to *Methylobacter pelagicum*. DNA–DNA hybridization data revealed relatively low levels of relatedness of *Methylobacter* sp. AMO1 and *Methylobacter* sp. N1 with each other and with previously described species of the genera *Methylobacter* and *Methylobacter* (<55%), indicating that they may represent novel species. Based on the results presented here and on previously reported morphological and physiological characteristics, we classify these halotolerant and halophilic methanotrophic strains as representing novel species within the genus *Methylobacter*: *Methylobacter alcaliphilus* sp. nov. (type strain 20Z^T = VKM B-2133^T = NCIMB 14124^T; reference strain 5Z = VKM B-2180), *Methylobacter japonense* sp. nov. (type strain NI^T = VKM B-2462^T = FERM BP-5633^T = NBRC 103677^T) and *Methylobacter kenyense* sp. nov. (type strain AMO1^T = NCCB 97157^T = NCIMB 13566^T = VKM B-2464^T). The genus *Methylobacter* has been emended in its description.

Methanotrophic bacteria (methanotrophs) are a physiological subgroup of aerobic bacteria capable of utilization of methane as a single source of carbon and energy (Whittenbury *et al.*, 1970). Methanotrophs are distributed

ubiquitously in nature and play an important role in global carbon cycling. Specifically, they control methane emissions from a variety of environments (Higgins *et al.*, 1980; Whalen & Reeburgh, 1990; Hanson & Hanson, 1996). The growing interest in environmental cycling of methane has resulted in characterization and description of novel methanotrophs (Bowman *et al.*, 1997; Trotsenko & Khmelenina, 2002a; Wise *et al.*, 2001; Warttinen *et al.*, 2006; Dedysh *et al.*, 2007). Over the past few decades, our knowledge of the diversity and distribution of methanotrophic bacteria in

Abbreviation: ICM, intracytoplasmic membrane.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain 20Z^T, strain 5Z, *Methylobacter albus* BG8^T and *Methylobacter agile* ATCC 35068^T are EF495157, AF096091, EU144025 and EU144026, respectively.

natural saline environments has been significantly expanded (Trotsenko & Khmelenina, 2002b; Lin *et al.*, 2004). It has been shown that methane-oxidizing microbial communities of saline and alkaline environments, such as saline ponds and soda lakes, are dominated by gammaproteobacterial (type I) methanotrophs (Trotsenko & Khmelenina, 2002b; Lin *et al.*, 2004; Nercessian *et al.*, 2005). Several species of halophilic/-tolerant type I methanotrophic bacteria have been isolated in pure culture and characterized (Khmelenina *et al.*, 1997; Kalyuzhnaya *et al.*, 1998, 1999; Sorokin *et al.*, 2000; Kaluzhnaya *et al.*, 2001; Heyer *et al.*, 2005). Some of the new isolates were described as novel taxa of methanotrophic bacteria, such as *Methylohalobius crimeensis* (Heyer *et al.*, 2005) and *Methylococcoides buryatense* (Kaluzhnaya *et al.*, 2001), while others were tentatively referred to as members of *Methylobacter* or *Methylococcoides* (Khmelenina *et al.*, 1997; Fuse *et al.*, 1998; Kalyuzhnaya *et al.*, 1998; Sorokin *et al.*, 2000).

The alkaliphilic, halophilic strains 20Z and 5Z (Khmelenina *et al.*, 1997) and AMO1 (Sorokin *et al.*, 2000) were isolated from highly alkaline soda lakes of Tuva (Russian Federation) and Kenya (Africa), respectively. Strain NI was isolated from marine mud (Hiroshima, Japan) (Fuse *et al.*, 1998). A great deal of information regarding the structural, biochemical and molecular mechanisms of adaptation of these methanotrophic isolates to high pH and salt has been accumulated (Khmelenina *et al.*, 1999; Trotsenko & Khmelenina, 2002a, b; Reshetnikov *et al.*, 2006). These extremophilic methanotrophs became attractive models for commercial production of fine chemicals from methane, such as ectoine, a multifunctional natural stabilizer (Trotsenko *et al.*, 2005). They also have potential for biotechnological applications, such as bioremediation of trichloroethylene, dimethylsulphide and dimethyldisulphide (Fuse *et al.*, 1998; Sorokin *et al.*, 2000). Overall, detailed characterization of the

physiological, morphological and phenotypic properties of these halophilic methanotrophs has indicated that they are related to each other (Trotsenko & Khmelenina, 2002b). Here we compare the halo(alkalo)philic/-tolerant strains in terms of 16S rRNA gene sequence phylogeny and DNA-DNA relatedness.

Strains used in this study are listed in Table 1. *Methylococcoides album* ATCC 33003^T and *Methylococcoides agilis* ATCC 35068^T were obtained from the ATCC and were grown on basal nitrate-mineral salts (NMS) medium (Whittenbury *et al.*, 1970). Strain AMO1 was obtained from the NCIMB as strain NCIMB 13566. Other strains were from the laboratory collections of Y. T. (strains 20Z and 5Z), H.F. (strain NI) and M.L. (*Methylobacter marinus* A45^T). Strains 20Z, 5Z, AMO1 and *Methylococcoides buryatense* 5G were grown on NMS medium to which 0.1 M NaHCO₃, 0.01 M Na₂CO₃ and 0.3 M NaCl were added. *Methylobacter marinus* A45^T was grown on basal NMS medium to which 0.3 M NaCl was added. *Methylococcoides* sp. NI was grown on artificial seawater medium (Cho & Giovannoni, 2003). All strains were grown under a methane/air (50:50) atmosphere. The G + C content of *Methylococcoides* sp. NI was determined by HPLC separation as described by Katayama-Fujimura *et al.* (1984) and was found to be 49.9 mol% (*n*=3).

The 16S rRNA gene sequences of strains NI^T and AMO1^T have been determined in previous studies (Fuse *et al.*, 1998; Sorokin *et al.*, 2000). The 16S rRNA genes of strains 20Z^T and 5Z were amplified by PCR using the pA' and pH' primer set (Edwards *et al.*, 1989). PCR products were sequenced on both strands by using [γ -³²P]ATP-labelled primers pA, pC, pE, pD, pF and pH (Edwards *et al.*, 1989) and the *fmol* DNA Cycle Sequencing System (Promega), according to the manufacturer's protocol. The sequencing reactions were then subjected to PAGE, followed by

Table 1. Strains of moderately (halo)alkaliphilic methanotrophs

pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase. All strains use the ribulose monophosphate cycle.

Strain	Growth optimum	C ₁ oxidation	DNA G+C content (mol%)	Reference(s)
Halophiles/neutrophiles				
<i>Methylococcoides pelagicum</i> AA-23 ^{T*}	pH 6.8†, 0.5–2 % NaCl	pMMO	49.1	Sieburth <i>et al.</i> (1987); Bowman <i>et al.</i> (1995)
<i>Methylococcoides</i> sp. NI	pH 8.1†, 3–5.6 % NaCl	pMMO/ sMMO	49.9‡	Fuse <i>et al.</i> (1998)
Halo(alkali)philes				
' <i>Methylobacter alcaliphilum</i> ' 20Z and 5Z	pH 9.0, 1–3 % NaCl	pMMO	47.8	Khmelenina <i>et al.</i> (1997)
<i>Methylococcoides buryatense</i> strains 4G, 5G, 6G, 7G and 5B ^T	pH 7.5–9.5, 0.75 % NaCl	pMMO/ sMMO	49–51	Kaluzhnaya <i>et al.</i> (2001)
<i>Methylococcoides</i> sp. AMO1	pH 10, 3 % Na ⁺	pMMO	51	Sorokin <i>et al.</i> (2000)

*No strain of this species is available.

†Reported growth conditions; the pH optimum was not determined.

‡Determined in this study.

autoradiography. Since sequences of 16S rRNA genes available from GenBank for *Methylobacterium album* ACM 3314^T (GenBank accession no. X72777) and *Methylobacterium agile* ACM 3308^T (X72767) have several missing bases, we amplified and resequenced 16S rRNA gene fragments for these type strains as described previously (Miller *et al.*, 2005). The sequences were aligned using the CLUSTAL W program (Higgins *et al.*, 1996) and phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 2004). Distance and parsimony methods were employed, with 1000 bootstrap analyses.

Sequences of strains AMO1^T, 20Z^T and NI^T displayed relatively high similarity levels (96–97 %) to each other and were closely related to the sequences of *Methylobacterium pelagicum* ACM 3505^T (96–98 %) and *Methylobacterium buryatense* 5B^T (97 %). On the phylogenetic tree all the sequences clustered together, forming a monophyletic group, with high bootstrap values (Fig. 1). The sequences showed lower similarity with the sequences of other halophilic methanotrophic bacteria, such as *Methylobacter marinus* A45^T (<96 %), *Methylosphaera hansonii* AM11^T (<89 %) and *Methylhalobium crimeensis* 10Ki^T (<91 %). It is notable that sequences of two strains presently classified as *Methylobacterium agile* (ATCC 35068^T) and *Methylobacterium album* (BG8^T) (Bowman *et al.*, 1995) were separated from the sequences of halo(alkali)philic representatives of this genus, *Methylobacterium pelagicum* ACM 3505^T (Sieburth *et al.*, 1987; Bowman *et al.*, 1995) and *Methylobacterium buryatense* 5B^T (Kaluzhnaya *et al.*, 2001), and fell instead together with sequences representing the *Methylosarcina* clade (Bowman *et al.*, 1993) with high bootstrap values (Fig. 1). Close relationships between *Methylosarcina* species and non-halophilic members of the genus *Methylobacterium* were already demonstrated by

phylogenetic analysis of particulate methane monooxygenase (*pmoA*) gene sequences (Wise *et al.*, 2001). *Methylobacter* species were also divided between three major branches: the first was formed by psychrophilic/-tolerant species (*Methylobacter psychrophilus* and *Methylobacter tundripaludum*), the second cluster includes *Methylobacter luteus*, *Methylobacter marinus* and '*Methylobacter vinelandii*' 87, while *Methylobacter whittenburyi* falls within the *Methylobacterium*/*Methylosarcina* clade. These data indicate that a reclassification of non-halophilic species of the genus *Methylobacterium*, as well as significant revision of the genera *Methylobacter* and *Methylosarcina*, is needed.

To clarify further the taxonomic status of the strains in question, we performed DNA–DNA hybridization analyses. DNA was extracted as described previously (Kalyuzhnaya *et al.*, 1999) and DNA–DNA hybridization experiments were carried out as described by Johnson (1994). *Methylobacterium pelagicum* was not included in these experiments, as it is no longer available in culture. DNA–DNA relatedness of the halo(alkali)philic/-tolerant methanotrophs is shown in Table 2. These data are in agreement with the 16S rRNA gene phylogeny and indicate that strains AMO1^T, 20Z^T and NI^T are more closely related to each other (28–54 %) and to *Methylobacterium buryatense* 5G (22–54 %) than to *Methylobacterium agile* ATCC 35068^T and *Methylobacterium album* BG8^T (less than 20 %). However, the modest level of hybridization between the strains in question indicated that they represent three different species. These data are consistent with the previously described phenotypic differences between the strains (Khmelenina *et al.*, 1997; Kaluzhnaya *et al.*, 2001). Overall genomic characterization confirmed that strains 20Z^T, AMO1^T and NI^T should be assigned to the genus *Methylobacterium* within novel species, for which we

Fig. 1. 16S rRNA gene sequence-based distance dendrogram showing the phylogenetic positions of strains AMO1^T, 20Z^T and NI^T in relation to other type I and type X methanotrophs. The 16S rRNA gene sequence of *Escherichia coli* K-12 was used as an outgroup. Bar, 0.1 changes per nucleotide sequence position. GenBank accession numbers are given in parentheses.

Table 2. DNA–DNA relatedness (%) between strains 20Z^T, AMO1^T and NI^T and reference strains

Strain	1	2	3	4	5	6
1. <i>Methylobacter</i> sp. 20Z ^T	100					
2. <i>Methylomicrobium</i> sp. AMO1 ^T	21	100				
3. <i>Methylomicrobium</i> sp. NI ^T	45	40	100			
4. <i>Methylomicrobium buryatense</i> 5G	55	29	54	100		
5. <i>Methylomicrobium album</i> ATCC 33003 ^T	18	17	15	17	100	
6. <i>Methylomicrobium agile</i> ATCC 35068 ^T	16	17	17	19	47	100

propose the names *Methylomicrobium alcaliphilum* sp. nov., *Methylomicrobium kenyense* sp. nov. and *Methylomicrobium japonense* sp. nov., respectively.

Description of *Methylomicrobium alcaliphilum* sp. nov.

Methylomicrobium alcaliphilum (al.ca.li.phi'lum. N.L. n. *alcali* from French n. *alcali* alkali; Gr. adj. *philos* loving; N.L. neut. adj. *alcaliphilum* loving alkaline conditions).

Previously known as '*Methylobacter alcaliphilum*'. Gram-negative cells, varying from rods as a major form to cocci and ellipsoids, 1.2–1.3 × 2.0–3.0 µm. Reproduces by binary fission. Motile by a single polar flagellum. Cells have type I intracytoplasmic membranes (ICM) and could form surface (S)-layers consisting of cup-shaped subunits, 38 nm in height and 33 nm in diameter, arranged in *p6* symmetry. Colonies are white to slightly cream, uniform, regularly convex, soft with entire edges. Grows well in the pH range 7.2–9.5. Does not grow below pH 7.0. Sodium ions (at 0.05 M) are required for growth. Tolerates up to 1.5 M NaCl. Accumulates ectoine as a compatible solute during growth in saline media. Methane-consuming activity is fastest at pH near 9. The G+C content of DNA of the type strain is 47.9 mol% (*T_m*). Major phospholipid fatty acids are iso-C_{16:0}, C_{16:0} and C_{14:0}.

The type strain is strain 20Z^T (=VKM B-2133^T =NCIMB 14124^T), isolated from surface sediment of the soda lake Shara-Nur, Tuva, Russian Federation. Reference strain 5Z (=VKM B-2180) was isolated from the soda lake Khadyn, Tuva.

Description of *Methylomicrobium kenyense* sp. nov.

Methylomicrobium kenyense (ken.yen'se. N.L. neut. adj. *kenyense* pertaining to Kenya).

Gram-negative, large, ovoid rods, sometimes coccoid, 1–1.5 × 2–3 µm. Motile by means of flagella. A small fraction of cells may possess up to three peritrichous flagella, while the majority possess a single flagellum. Cells have type I ICM and form an additional subunit S-layer, loosely

associated with the cell wall. S-layers consist of small subunits, 1.8 nm in diameter and 1.5 nm high, arranged on the cell surface in a hexagonal symmetry. Grows well in the pH range 9–10.5. Sodium ions are required for growth. Tolerates up to 1.1 M total sodium ion concentration. Ammonia, nitrate and nitrite can be used as nitrogen sources. Oxidizes ammonium to nitrite above pH 10.0. Methane-oxidizing and ammonium-oxidizing activities are maximal at pH near 10. The G+C content of DNA of the type strain is 50.9 mol% (*T_m*).

The type strain, strain AMO1^T (=NCCB 97157^T =VKM B-2464^T =NCIMB 13566^T), was isolated from surface sediments of a Kenyan soda lake.

Description of *Methylomicrobium japonense* sp. nov.

Methylomicrobium japonense (ja.pa.nen'se. N.L. neut. adj. *japonense* pertaining to Japan).

Gram-negative, rod-shaped cells, 0.9–1.2 × 1.8–2.8 µm. Reproduces by binary fission. Motile by a single polar flagellum. Cells have type I ICM. Colonies are white to slightly cream, uniform, regularly convex, soft with entire edges. Slightly halophilic; grows between 0.03 and 1.5 M NaCl, optimally at 0.4–0.8 M NaCl. Grows well between 15 and 37 °C; does not grow at or below 4 °C or at or above 45 °C. The G+C content of DNA of the type strain is 49.9 mol% (HPLC method). The major fatty acid is C_{16:1} (72–76 %), other significant phospholipid fatty acids are C_{14:0} (3 %), C_{16:0} (11–17 %) and C_{16:0} 3-OH (0–6 %). Some strains contain soluble methane monooxygenase (sMMO). Liquid culture is often flocculating.

The type strain, NI^T (=VKM-B-2462^T =FERM BP-5633^T =NBRC 103677^T), was isolated from marine mud (Hiroshima, Japan).

Emended description of the genus *Methylomicrobium*

Cells are short rods, 0.5–1.5 × 1.5–3.0 µm. Motile by a single polar flagellum or three peritrichous flagella. Reproduce by binary fission and unable to form cysts or spores. Cells contain type I ICM as stacks of vesicular discs. Cells may possess a thin slime capsule or regular glycoprotein S-layers arranged in *p2* or *p6* symmetries. Obligate methanotrophs utilizing methane or methanol as carbon and energy sources but not other C₁ or C_n compounds. Assimilate formaldehyde via the ribulose monophosphate pathway. Utilize nitrate and ammonium salts as nitrogen sources. Mesophilic, with optimal growth at 25–30 °C. Some strains are alkalitolerant or alkaliphilic, growing well in the pH range 9–10.5, and require sodium ions for growth. Possess a particulate MMO, and some strains may also contain a soluble MMO. The most abundant fatty acids are C_{16:1}ω7c, C_{16:1}, C_{16:0}, C_{14:0} and C_{16:1}ω8c (only for *Methylomicrobium album* and

Methylococcobium agile). The primary quinone is ubiquinone-8 (Q-8). The G+C content of the DNA is 46–51 mol%. Member of the family *Methylococcaceae* in the *Gammaproteobacteria*. The type species is *Methylococcobium agile*.

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