Methylohalobius crimeensis gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea

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A novel genus and species are proposed for two strains of methanotrophic bacteria isolated from hypersaline lakes in the Crimean Peninsula of Ukraine. Strains 10Ki T and 4Kr are moderate halophiles that grow optimally at 1–1·5 M (5·8–8·7 %, w/v) NaCl and tolerate NaCl concentrations from 0·2 M up to 2·5 M (1·2–15 %). This optimum and upper limit are the highest for any methanotrophic bacterium known to date. The strains are Gram-negative, aerobic, non-pigmented, motile, coccoid to spindle-shaped bacteria that grow on methane or methanol only and utilize the ribulose monophosphate pathway for carbon assimilation. They are neutrophilic (growth occurs only in the range pH 6·5–7·5) and mesophilic (optimum growth occurs at 30 °C). On the basis of 16S rRNA gene sequence phylogeny, strains 10Ki T and 4Kr represent a type I methanotroph within the ‘Gammaproteobacteria’. However, the 16S rRNA gene sequence displays <91·5 % identity to any public-domain sequence. The most closely related methanotrophic bacterium is the thermophilic strain HB. The DNA G + C content is 58–7 mol%. The major phospholipid fatty acids are 18 : 1ω7 (52–61 %), 16 : 0 (22–23 %) and 16 : 1ω7 (14–20 %). The dominance of 18 : 1 over 16 : 0 and 16 : 1 fatty acids is unique among known type I methanotrophs. The data suggest that strains 10Ki T and 4Kr should be considered as belonging to a novel genus and species of type I methanotrophic bacteria, for which the name Methylohalobius crimeensis gen. nov., sp. nov. is proposed. Strain 10Ki T (= DSM 16011T = ATCC BAA-967T) is the type strain.

INTRODUCTION

The 11 presently recognized genera of aerobic methanotrophic bacteria are divided into two groups: type I and type II. These groups differ in many ways, including phylogenetic affiliation (the ‘Gammaproteobacteria’ versus the ‘Alphaproteobacteria’), carbon-assimilation pathway (ribulose monophosphate pathway versus serine pathway), the dominant phospholipid fatty acids (PLFAs) (14 : 0, 16 : 0 and 16 : 1 versus 18 : 1) and the geometric arrangement of intracytoplasmic membranes (Hanson & Hanson, 1996; Bowman, 2000). Aerobic methanotrophic bacteria inhabit environments where both methane (CH4) and oxygen (O2) are present, such as surface sediments of marine and freshwater aquifers. In hypersaline wetlands, high rates of CH4 production can occur in anaerobic sediment, primarily through the methanogenic degradation of methylamines and methanol (Giani et al., 1984; Ollivier et al., 1994). Aerobic surface sediments and waters from these environments are therefore potential habitats for methanotrophic bacteria.

Hypersaline environments present unique challenges for microbial growth. Most halophilic members of the Bacteria maintain osmotic pressure by accumulating organic solutes in the cytoplasm, a strategy requiring a large energy input into biosynthesis. This metabolic cost may explain the absence of some low-energy-yielding biogeochemical processes in environments with very high salt contents, but aerobic methane oxidation should remain an energetically viable mode of existence (Oren, 1999). Nevertheless, aerobic methane oxidation is not always detectable in hypersaline lakes. The process could not be detected in surface microbial mats or surface sediments of Solar Lake, Egypt (8·5 % w/v salt content) (Conrad et al., 1995), of solar salterns in Egypt.
(13.2% salt) (Conrad et al., 1995) or of Lake Sivash in Ukraine (15–33% salt) (Slobodkin & Zavarzin, 1992). However, a sensitive 14CH4 labelling technique permitted the observation of methane oxidation in the surface water, the thin microbial mat (1–2 mm) and the aerobic surface sediment of diverse hypersaline lakes in Ukraine (8–33% salt) (Sokolov & Trotsenko, 1995; Khmelenina et al., 1996) and Tuva (9–20% salt) (Khmelenina et al., 1996). Aerobic methane oxidation was also detectable in the aerobic surface waters of two stratified hypersaline lakes in the USA: Mono Lake (7–10% salt) (Joye et al., 1999) and Big Soda Lake (12.5% salt) (Iversen et al., 1987). However, the rates were very low and did not effectively reduce CH4 efflux through the epilimnion. Halophilic or halotolerant aerobic methanotrophic bacteria are therefore present and active in some, but perhaps not all, hypersaline environments.

The type I methanotrophic bacterium Methylobacterium pelagicum (Sieburth et al., 1987; Bowman et al., 1993), which was isolated from sea water, possesses a slightly to moderately halophilic phenotype [in the system of Kushner (1978), a halophile has an obligate requirement for NaCl and exhibits maximum growth at <0.5 M for a slight halophile, 0.5–2.5 M for a moderate halophile or >2.5 M for an extreme halophile]. Slightly to moderately halophilic methanotrophic bacteria have also been isolated from alkaline soda lakes in Kenya (Sorokin et al., 2000) and central Asia (Khmelenina et al., 1996, 1997; Kalyuzhnaya et al., 1999), from pH-neutral Lake Sasyk in Ukraine (Kalyuzhnaya et al., 1998) and from various marine waters (Table 1). Methylohalobius hansonii, isolated from Antarctic lakes, is reported to require sea water for growth, but its potentially halophilic phenotype has not been closely investigated. All other known halophilic methanotrophic bacteria, according to 16S rRNA phylogeny, form a single phylogenetic radiation within the ‘Gammaproteobacteria’ and probably all represent species of Methylobacterium (Fig. 1). The maximum salt tolerances of these halophilic Methylobacterium spp. are typically <8% NaCl (1–4 M) (Table 1). It is therefore questionable whether they can account for methane oxidation activity in many hypersaline lakes, which can contain as much as 33% salt (Sokolov & Trotsenko, 1995). The present paper reports on the detection and isolation of novel halophilic methanotrophic bacteria from hypersaline lakes in Ukraine.

### METHODS

#### Sites and sampling
Samples were taken from two thalassohaline hypersaline lakes (Kirleutskoe and Krugloe) located near the town of Krasnoperekopsk in the Crimean Peninsula, Ukraine. The lakes are shallow (generally <2 m deep) and unstratified. The sediment is covered, in places, with a crust of crystallized salt (0–1 cm in depth). Below this is a thin (5–20 mm), aerobic, light-brown-coloured surface sediment layer; anaerobic, black, FeS-containing muck occurs further down. Surface aerobic sediments (the upper 20 mm pooled from several areas) were sampled in September 1990 and again in August 2001.

Analysis of a water sample taken from Lake Kirleutskoe in May 1990 (analysis was by Brom, JSC, Krasnoperekopsk, Ukraine) determined the following ion ratios (weights as percentages of total salts): 0.79% Ca2+, 5.33% Mg2+, 29.5% Na+, 57.0% CI–, 0.21% Br–; 7.06% SO42–, 0.045% HCO3– and 0.057% CO32–. Other characteristics of the lakes are outlined in Table 2. Salt content was determined using a density aerometer by comparison with sea water. Dissolved CH4 was measured as described by Heyer & Suckow (1985). Gas bubbles released from disturbed sediment were collected in a water-filled inverted funnel and measured by GC with flame-ionization detection, using a Chromatron model GCHF 18.3-4 system (1 m Porapak Q column, 25°C oven, N2 carrier gas). Potential methane consumption rates were estimated in sealed 140 ml serum vials containing 2 ml surface sediment in lake water, without a gas headspace. Water (5 ml)

### Table 1. Some characteristics of known halotolerant and halophilic methanotrophic bacteria

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>NaCl range (M)</th>
<th>pH range (optimum)</th>
<th>Source</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylobacterium pelagicum AA-23T</td>
<td>0.3–0.8</td>
<td>NA</td>
<td>Sea water, Sargasso Sea</td>
<td>Bowman et al. (1993); Sieburth et al. (1987)</td>
</tr>
<tr>
<td>Methylobacterium sp. IR1</td>
<td>0.2–0.7 [0.25–0.34]</td>
<td>[7–6]</td>
<td>Sea water, Plymouth Sound</td>
<td>Bowman et al. (1993); Lees et al. (1991)</td>
</tr>
<tr>
<td>Methylohalobius hansonii AM6T, AM11</td>
<td>Require sea water</td>
<td>NA</td>
<td>Ace Lake, Antarctica</td>
<td>Bowman et al. (1997)</td>
</tr>
<tr>
<td>Methylobacterium sp. AMO-1 'Methylobacterium alcaliphilus' SZ, 20Z</td>
<td>Up to 1.1</td>
<td>6.0–11 [9–5]</td>
<td>Soda lakes, Kenya</td>
<td>Sorokin et al. (2000)</td>
</tr>
<tr>
<td>'Methylobacterium modestoalcalophilus' 1OS</td>
<td>0.03–1.5 [0.4]</td>
<td>5.5–8.5 [6–5]</td>
<td>Lake Sasyk, Ukraine</td>
<td>Khmelenina et al. (1996, 1997)</td>
</tr>
<tr>
<td>Methylobacterium burlatense 5B, 4G, 5G, 6G, 7G</td>
<td>0.1–4 [0.14]</td>
<td>6.0–11 [8–0.8.5]</td>
<td>Lake Krugloe, Ukraine</td>
<td>Kalyuzhnaya et al. (1999, 2001)</td>
</tr>
<tr>
<td>Methylohalobius crimeensis</td>
<td>0.2–2.5 [1–1.5]</td>
<td>6.5–7.5 [7]</td>
<td>Lake Kirleutskoe, Ukraine</td>
<td>This study</td>
</tr>
<tr>
<td>Methylohalobius crimeensis 10KiT</td>
<td>0.2–2.5 [1–1.5]</td>
<td>6.5–7.5 [7]</td>
<td>Lake Kirleutskoe, Ukraine</td>
<td>This study</td>
</tr>
</tbody>
</table>

NA, Data not available.
was replaced with CH₄-saturated water, and individual sample vials (n = 2 or 3) were used at 0, 48 and 96 h for determination of dissolved CH₄, as described by Heyer & Suckow (1985). Methane-production rates for anaerobic sediment (below 20 mm depth) were estimated from the accumulation of CH₄ over 14 days in the gas headspace of duplicate 140 ml serum vials containing 100 ml of a 1:1 slurry of anaerobic sediment and lake water, which were capped and the headspace flushed well with N₂. Methane production and consumption rates were estimated by linear regressions and calculated assuming a 2-cm-deep methanotrophic layer and a 10-cm-deep methanogenic layer.

**Table 2.** Some physical and biological characteristics of Lake Krugloe and Lake Kirleutskoe

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Kirleutskoe</th>
<th>Krugloe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₄ concentration in water (µM)</td>
<td>0·29−2·46</td>
<td>0·08</td>
</tr>
<tr>
<td>CH₄ mixing ratio in sediment gas bubbles (p.p.m.v.)</td>
<td>121 000</td>
<td>224−376</td>
</tr>
<tr>
<td>Water pH</td>
<td>7·8</td>
<td>7·5</td>
</tr>
<tr>
<td>Sediment pH (2001)</td>
<td>6·8</td>
<td>7·5</td>
</tr>
<tr>
<td>Salt content:</td>
<td>% (w/v)</td>
<td></td>
</tr>
<tr>
<td>M*</td>
<td>26·5</td>
<td>23·4</td>
</tr>
<tr>
<td>Potential CH₄ oxidation rate ± SEM (µmol m⁻² h⁻¹) (at 45−50 µM CH₄)</td>
<td>410 ± 60·6</td>
<td>137 ± 26·6</td>
</tr>
<tr>
<td>Potential CH₄ production rate ± SEM (µmol m⁻² h⁻¹)</td>
<td>15 ± 4·7</td>
<td>ND</td>
</tr>
<tr>
<td>Most probable number, medium A with 0−20% added NaCl [cells (g dry weight sediment)⁻¹]</td>
<td>3−40</td>
<td>0−23</td>
</tr>
</tbody>
</table>

*Calculated on the basis of the salt composition of Lake Kirleutskoe (see Methods).
Most probable number counts were done, as previously described, in basal salts medium A (Horz et al., 2002) supplemented with NaCl at 0, 5, 10 or 20 % (w/v).

Enrichment and isolation of strains 10KiT and 4Kr. Ten different media were made for the enrichment and isolation of methanotrophic bacteria: medium A (Horz et al., 2002) supplemented with 5, 10, 15, 20 or 25 % (w/v) NaCl and medium S supplemented with 5, 10, 15, 20 or 25 % total dissolved salts. Medium S was designed to mimic the salt composition of the lakes. It contained (1\(^{-1}\)) 0.5 g NH\(_4\)NO\(_3\), 0.5 g NaHPO\(_4\), 2H\(_2\)O, 0.1 g K\(_2\)HPO\(_4\), 0.005 g FeSO\(_4\), 7H\(_2\)O and 1 ml trace elements solution, as in medium A (Horz et al., 2002), to which different salts were added to recreate the ratios of the different ions determined from the above analysis of Lake Kirleutskoe. A 10 % salt-strength medium S contained (1\(^{-1}\)) 66.0 g NaCl, 10.45 g Na\(_2\)SO\(_4\), 0.10 g Na\(_2\)CO\(_3\), 0.06 g NaHCO\(_3\), 0.28 g NaBr, 20.89 g MgCl\(_2\), and 2.21 g CaCl\(_2\). The pH of all media was adjusted to about 7-5 with NaOH. The pH was tested after autoclaving and was always in the range 7-3-7-9.

Enrichments were performed at 30 °C either in 120 ml serum vials (25 ml medium) capped with butyl-rubber stoppers and shaken at 140 r.p.m. on a rotary shaker, or in 20 ml test tubes (5 ml medium) incubated unshaken in closed glass desiccators. The incubation atmosphere in each case was a CH\(_4\)/CO\(_2\)/air gas mixture (20 : 1 : 79) at 1 bar. Vials were capped with butyl-rubber stoppers to prevent loss of vaporized substrates and were shaken at 120 r.p.m. at 30 °C. Further potential growth substrates were tested in unshaken cultures of medium A (6-5 % NaCl) supplemented with one of the following carbon sources (0-05 %, w/v): formate, formamide, glucose, fructose, sucrose, lactose, galactose, xylose, sorbose, maltose, raffinose, arabinose, ribose, lactate, oxalate, citrate, mannitol or sorbitol. Growth was examined after 21 days incubation by comparison with a negative control (incubated without a carbon source). Nitrogen sources were tested by replacing NH\(_4\)Cl in medium A (incubation under CH\(_2\)) with one of the following (0-05 %, w/v): NaNO\(_2\), NH\(_4\)OH, (NH\(_4\))\(_2\)SO\(_4\), KNO\(_3\), glycine, l-alanine, l-serine, l-isoleucine, l-proline, l-cystine, l-methionine, l-glutamine, l-histidine, l-arginine, l-lysine or yeast extract. Growth was examined after 21 days incubation by comparison with a negative control (N-free medium).

Enzyme assays. To test for soluble methane monooxygenase activity, the naphthalene-oxidation assay (Graham et al., 1992) was performed on 1–2-week-old cultures on plates of medium A (6-5 % NaCl) made with and without copper in the trace elements. We also attempted to amplify partial fragments of the mmaX gene (encoding a subunit of soluble methane monooxygenase) and of the mfh gene (encoding dinitrogenase reductase H), using primers and PCR thermal cycling profiles as described by Auman et al. (2000) and Auman et al. (2001), respectively. PCR mixtures contained 0-5 μM each primer, 1× Premix F (Epicenter Technologies) and 1 U Taq polymerase (QBioGene). PCRs were run on a GeneAmp PCR System 9700 temperature gradient cycler (Perkin-Elmer Applied Biosystems).

For preparation of cell-free extracts, cells in the exponential phase were harvested (10 700 g, 20 min) and resuspended in 0-05 M Tris/HCl (pH 7-2). Cells were sonicated in a Branson Ultrasonic SA sonicator (Carouge) four times at 30 s each at 150 W and 20 kHz, with cooling on ice. The breaking of the cell envelopes was verified microscopically. The cell debris were centrifuged at 14 300 g for 10 min at 4 °C and the supernatant was used for the spectrophotometric detection of hydroxy pyruvate reductase activity, as described by Large & Quayle (1963), and of hexulose phosphate synthase activity, as described by Dahl et al. (1972).

Comparative sequence analysis. DNA was extracted from strains 10KiT and 4Kr using a mechanical disruption procedure (Hencel et al., 2000). PCR-mediated amplifications of the 16S rRNA gene from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for Escherichia coli 16S rRNA) and of a partial fragment of the pmaA gene (encoding a subunit of particulate methane monooxygenase), and DNA sequencing on an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems), were performed as described previously (Heyer et al., 2002). Sequences were aligned to the ARB program database (Strunk & Ludwig, 1996) and phylogenetic trees were constructed using TREE-FUZZLE, a quartet maximum-likelihood method (Schmidt et al., 2002).
**Cellular fatty acid profiles and DNA G+C content.** Fatty acid methyl esters were extracted from cells grown in liquid medium A (5 % NaCl) in the exponential growth phase (4–7 days growth with daily re-gassing of the headspace) and analysed by gas chromatography as described by Kämpfer & Kroppenstedt (1996). Analysis of the DNA G+C content was performed as described by Groth et al. (1996).

**RESULTS AND DISCUSSION**

**Characterization of sampling sites**

Salt contents of lake water in 1990 were 23–26 % (Table 2). Above-ambient concentrations of CH$_4$ were measured in lake water and in sediment gas bubbles, demonstrating the occurrence of methanogenesis in situ. Both methane production and methane oxidation could be measured in sediment incubations, indicating that both halophilic (or halotolerant) methanogenic and methanotrophic florae were present. However, estimated most probable number counts of methanotrophic bacteria were very low, i.e. between 1 and 40 cells (ml aerobic sediment)$^{-1}$. Counts were similar regardless of the lake sampled or the salt content of the medium used (0–20 % NaCl).

**Enrichment and isolation of strains 10KiT and 4Kr**

Isolates were obtained from sediments of Lake Krugloe and Lake Kirleutskoe from both sampling dates, in both unshaken and shaken enrichments of media A and S at 5 and 10 % salt. Sequences of the pmoA genes for each isolate were obtained and were all found to be identical. It was therefore assumed that all isolates were very similar, so only two were selected for closer characterization: 10KiT from Lake Kirleutskoe (medium A, 5 % NaCl) and 4Kr from Lake Krugloe (medium A, 5 % NaCl). These strains were isolated in 1991 and maintained for over 10 years by serial transfer. The purity of the strains was verified by examining cultures using phase-contrast microscopy. No growth occurred on nutrient agar (+5 % NaCl) or on plates of medium A (+5 % NaCl) incubated without CH$_4$ in the gas phase. In addition, growth did not occur on any of the multicarbon substrates tested (see Methods), indicating that the cultures were not contaminated by heterotrophic satellites.

**Cultural and morphological characteristics**

Colonies of 4Kr grown on plates (2 weeks) were 0.5–1 mm in diameter, round, convex, cream-coloured, smooth and shiny, with entire edges and a soft surface. Colonies of 10KiT were somewhat smaller (0.2–0.5 mm) and bright white instead of cream-coloured. When grown on methane, strain 4Kr was motile and formed coccolid cells or short (sometimes spindle-shaped) rods that were 1 μm (0.5–1.6 μm) in width and 2.1 μm (1.6–3.2 μm) in length. Strain 10KiT was motile, formed coccolid cells or short rods that were 1.1 μm (0.8–1.3 μm) in width and 2.2 μm (1.6–3.2 μm) in length. Cells grown on methanol were often larger and more coccolid-shaped. Cells were Gram-negative. Cells of older cultures of strain 10KiT, especially after several consecutive transfers on solid medium, contained highly refractive inclusions (Fig. 2). Most cells were non-motile, but motility was occasionally observed. Cells usually occurred in pairs but occasionally formed short chains of three to eight cells. Staining for Azotobacter-type cysts was negative. No exosporules or cysts were evident in cell preparations, even after 3 weeks incubation. However, in old cultures, rare enlarged, highly refractive cells were observed.

A typical type I intracytoplasmic membrane arrangement, consisting of bundles of two to ten parallel membranes located along the cell periphery, was evident in both strains (Fig. 3). The membrane bundles in strain 10KiT were generally smaller than those in strain 4Kr. Large inclusions of low electron density, probably comprising poly-β-hydroxybutyrate granules, were also observed in some cells (Fig. 3b).

The specific growth rate (μ) in medium A (6.5 % NaCl) under 10–20 % (v/v) CH$_4$, calculated from increases in OD$_{600}$ in the exponential phase (<6 days) of growth (mean of duplicate vials), was 0.019 h$^{-1}$ for strain 4Kr (equivalent to a doubling time of 36 h) and 0.028 h$^{-1}$ for strain 10KiT (equivalent to a doubling time of 25 h).

**Physiological and biochemical characteristics**

Both strains grew best in medium containing 1–1.5 M NaCl, and continued to grow at NaCl concentrations up to 2.5 M (Fig. 4). Neither strain grew in medium A without added NaCl: the slightly positive OD$_{600}$ values at 0 % NaCl were caused by the inoculum and did not increase during the growth period. The pH range for growth was very narrow, being pH 6.5–7.5. Optimal growth occurred at 30 °C. Growth occurred at temperatures from 15 °C (the lowest temperature tested) to 42 °C, but not at 50 °C.

Isolates 10KiT and 4Kr have a higher tolerance of dissolved salts than that of any known halophilic Methylohalobius species, and, unlike most halophilic Methylohalobius species, they prefer neutral rather than alkaline pH values (Table 1). Theoretically, their salt tolerance is sufficient for...
them to be active and growing in hypersaline systems in which very little or no aerobic methane oxidation can be measured, e.g. in Solar Lake (Conrad et al., 1995) and in Mono Lake (Joye et al., 1999). This suggests that rates of methane oxidation in these hypersaline habitats are constrained by other environmental factors, such as alkaline pH or inhibitory compounds. On the other hand, methane-oxidation activity was measured in Lake Krugloe and Lake Kirleutskoe when salt contents were 3·4–3·8 M (23·4–26·5 %), considerably above the growth threshold of the isolates. We tested whether strains 10KiT and 4Kr, when grown under 1 M NaCl and then transferred to higher salt concentrations, continued to oxidize CH4 (data not shown), but we found that the upper limit of activity was the same as the growth threshold (about 2·5 M). Therefore, in theory, the strains studied should be active in the lakes only during periods of lower salinity, such as after rainfall. The discrepancy between their upper limit of salt tolerance and the salt content of the lakes during sampling suggests either that uncultivated methanotrophic bacteria were present that had an even more halophilic phenotype than strains 4Kr and 10KiT, or that ecological interactions in the sediment allowed strains like 10KiT and 4Kr to display a greater salt tolerance than was observed in pure culture. The former option is supported by the estimation of only low population densities of culturable methanotrophic bacteria from most probable number counts, although it is possible that media were not optimal or that the handling of samples involved an osmotic shock that destroyed many cells.

Both strains grew at all methanol concentrations tested (0·1–0·5 %), as well as on methane. No growth occurred on any of the other substrates tested. No growth was observed in N-free medium after 3 weeks incubation. In support of

Fig. 3. Transmission electron micrographs of thin sections of strains 10KiT (a, b) and strain 4Kr (c, d). IM, Intracytoplasmic membrane; PHB, poly-β-hydroxybutyrate. Bars, 1 μm.

Fig. 4. Growth of isolates 10KiT (solid bars) and 4Kr (open bars) in different concentrations of NaCl, measured as OD600 values after 1 week of growth. Data are means of triplicates±SEM. The small positive OD600 values at 0 % NaCl were equal to the initial OD600 (i.e. of the inoculant).
the conclusion that the strains could not fix atmospheric nitrogen, a *nifH* gene was not detectable in a PCR assay. The inorganic nitrogen sources NH$_4$Cl, NH$_2$OH and (NH$_4$)$_2$SO$_4$ supported growth of both strains. l-Alanine and KNO$_3$ also supported weak growth of strain 4Kr, but the other nitrogen sources tested did not support growth.

Soluble methane monooxygenase activity was not detectable with a colorimetric assay, nor was an *mmoX* gene amplifiable using a PCR system that detects most known *mmoX* genes of methanotrophic bacteria (Auman et al., 2000; Heyer et al., 2002). Both 4Kr and 10Ki$^T$ displayed activity of hexulose phosphate synthase, the key enzyme of the ribulose monophosphate pathway, but there was no detectable activity of the serine-pathway enzyme hydroxy-xyruvate reductase. The DNA G+C content of strain 10Ki$^T$ was 58.6 mol% and that of strain 4Kr was 58.8 mol%.

Isolates 10Ki$^T$ and 4Kr possess a unique PLFA profile compared with all other known methanotrophic bacteria (Table 3). The profile is dominated by an 18:1 fatty acid (18:1ω7c) as in type II methanotrophic bacteria, but also contains large amounts of 16-carbon fatty acids, as in other type I methanotrophic bacteria. The 18:1 fatty acids are generally minor constituents in other type I methanotrophic bacteria, although they can contribute as much as 14-7 % of the total in some *Methylococcus* strains and as much as 26.5 % in some *Methyloomonas* strains (Bowman et al., 1993). Type I methanotrophic bacteria instead contain primarily 16:0 or 16:1 fatty acids, plus 14:0 acids in *Methyloomonas* species (Bowman et al., 1993). The predominant PLFA in the novel strains (18:1ω7c) is also the predominant PLFA in the acidophilic type II methanotrophic bacteria *Methylolopsis* and *Methylolocella*, making up >75 % of the total PLFA content in these species (Dedysh et al., 2004). PLFAs are often used as biomarkers to distinguish between type I and type II methanotrophic bacteria in natural systems (Bowman, 2000). However, it must be recognized that this technique is based on generalized differences that are not always valid. The novel strains possess a PLFA profile that is intermediate with respect to typical patterns of type I and type II methanotrophic bacteria.

### Comparative sequence analysis

A new taxonomic position for the isolates is supported by phylogenetic analysis as well as by their unique phenotypic traits. A BLAST search of GenBank found no 16S rRNA gene sequence with >91.5 % identity to isolate 10Ki$^T$ or isolate 4Kr. The most closely related sequences (based on uncorrected distances) from pure cultures were of *Methylothermus* sp. HB (GenBank accession no. U89299; 90-2 % identity), *Rhabdochromatium marinum* (accession no. X84316; 90-7 % identity) and *Thiorhodococcus minor* CE2203$^T$ (accession no. Y11316; 90-3 % identity). Given the guideline of 3 % 16S rRNA gene sequence difference to define a novel genus and species (Stackebrandt & Goebel, 1994), the low identity of the novel isolates with respect to any known bacterial species demonstrates that they represent a novel genus.

The 16S rRNA-based phylogeny shows that the isolates belong to a novel phylogenetic branch of the *Gamma proteobacteria*, and are only distantly related to previously known halophilic *Methylomicrobium* strains (Fig. 1). Strains 4Kr and 10Ki$^T$ have identical 16S rRNA gene sequences. The closest evolutionary relative is the thermophilic methanotrophic bacterium *Methylothermus* strain HB. Unlike strains 4Kr and 10Ki$^T$, which are mesophilic, *Methylothermus* strain HB is a thermophile with a growth optimum of 62–65 °C (Bodrossy et al., 1999). The evolutionary relationships of these organisms are intriguing and suggest that this phylogenetic cluster may be composed of diverse extremophilic species. Unfortunately, there is as yet no thorough description of *Methylothermus* available.

The phylogenetic position of the isolates is supported by comparative sequence analysis of partial derived PmoA amino acid sequences (Fig. 5). PmoA has been demonstrated to have phylogenetic value in distinguishing methanotrophic bacterial species (Holmes et al., 1995; Heyer et al., 2002). The PmoA-based phylogeny agrees with the 16S rRNA-based phylogeny as to the relationship between strains 4Kr and 10Ki$^T$ and other methanotrophic bacteria, and again calculates that the thermophilic

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**Table 3.** Cellular PLFA compositions of isolates 10Ki$^T$ and 4Kr as percentages of total PLFAs


<table>
<thead>
<tr>
<th>PLFA</th>
<th>10Ki$^T$</th>
<th>4Kr</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.22</td>
<td>–</td>
<td>0–3.8</td>
<td>–</td>
</tr>
<tr>
<td>14:0</td>
<td>2.45</td>
<td>1.35</td>
<td>0–7–24.6</td>
<td>0–4.1</td>
</tr>
<tr>
<td>15:0</td>
<td>0.48</td>
<td>0.31</td>
<td>0–12.7</td>
<td>0–0.7</td>
</tr>
<tr>
<td>16:1ω8c</td>
<td>–</td>
<td>–</td>
<td>0–41.3</td>
<td>–</td>
</tr>
<tr>
<td>16:1ω7c</td>
<td>19–60</td>
<td>14–20</td>
<td>7–7–57.4</td>
<td>0–3–14.2</td>
</tr>
<tr>
<td>16:1ω6c</td>
<td>–</td>
<td>–</td>
<td>0–18</td>
<td>0–0.3</td>
</tr>
<tr>
<td>16:1ω5c</td>
<td>–</td>
<td>–</td>
<td>0–9.0</td>
<td>0–0.2</td>
</tr>
<tr>
<td>16:1ω5t</td>
<td>–</td>
<td>–</td>
<td>0–28.2</td>
<td>–</td>
</tr>
<tr>
<td>16:0</td>
<td>23–02</td>
<td>22–80</td>
<td>4–3–56</td>
<td>0–7–7.7</td>
</tr>
<tr>
<td>17:0 cyclo</td>
<td>0.67</td>
<td>–</td>
<td>0–15.1</td>
<td>0–6.5</td>
</tr>
<tr>
<td>17:0</td>
<td>0.29</td>
<td>0.26</td>
<td>0–1.4</td>
<td>0–0.4</td>
</tr>
<tr>
<td>18:1ω8c</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0–73.6</td>
</tr>
<tr>
<td>18:1ω7c</td>
<td>51–87</td>
<td>60–53</td>
<td>0–26.5</td>
<td>14–8–82.2</td>
</tr>
<tr>
<td>18:0</td>
<td>0.47</td>
<td>0.55</td>
<td>0–2.8</td>
<td>0–0.5</td>
</tr>
</tbody>
</table>

*A peak identified as 16:0 2-OH was also detected (at 0-93 %), but the identity was not verified.*
methanotrophic bacterium ‘Methylothermus’ strain HB is the closest neighbour. In comparison with the large 16S rRNA gene sequence difference (9–8 %) between ‘Methylothermus’ and Methylohalobius, which demonstrates a genus-level difference, the two organisms have very closely related PmoA amino acid sequences. This difference (7–6 %) is comparable to species-level or even strain-level differences in other methanotrophic bacteria. However, when the nucleotide-based pmoA phylogeny is calculated, the difference increases dramatically to 20 %, similar to genus-level differences among other methanotrophic bacteria. The pmoA gene in these extremophiles may be under extremely strong purifying selection, such that genetic mutations causing amino acid changes in PmoA are more rapidly removed from the gene pool than in other methanotrophic bacteria.

The phylogenetic separation of the two known halophilic groups of methanotrophic bacteria (Methylococcus species versus strains 10KiT and 4Kr) suggests that they evolved a halophilic phenotype independently. Strains 10KiT and 4Kr are more closely related to the type X subset of type I methanotrophic bacteria (Methylococcus and Methylocaldum species) than to other genera, according to both 16S rRNA and PmoA phylogenies. On the basis of its placement within the ‘Gammaproteobacteria’, the new genus Methylohalobius should be considered as belonging to the type I methanotrophic bacteria, or the family Methylocaldaceae. However, it is not yet clear whether this family is monophyletic. This is especially the case with Methylohalobius and ‘Methylothermus’, which may be phylogenetically more closely related to some non-methanotrophic bacteria than to other methanotrophic bacteria. These relationships are not yet clear because of the scarcity of pure-culture sequences.

Description of Methylohalobius gen. nov.

Methylohalobius (Me.thy.lo.ha.lo’i.us. N.L. neut. n. methyl, methyis the methyl group; Gr. masc. n. halos, halos salt; Gr. masc. n. bios life; N.L. masc. n. Methylohalobius salt-requiring, methyl-using bacterium).

Gram-negative, polymorphic, coccoid and spindle-shaped cells that are 1 μm (0.5–1.6 μm) in width and 2.1 μm (1.6–3.2 μm) in length. Reproduce by normal cell division. Cells occur singly, in pairs or in short chains, but do not form rosettes. Motile. Resting stages (exospores, Azotobacter-type cysts, or lipid cysts) not present. Possess a typical type I intracytoplasmic membrane system. Do not possess soluble methane monooxygenase. Moderate halophiles growing from 0–2° up to 2.5 M NaCl (optimum 1–0–1.5 M). Temperature optimum for growth is 30 °C (range <15 to >42 °C; no growth occurs at 50 °C). Grow between pH 6–5 and 7.5. Grow on methane and methanol. Utilize C₁ compounds via the ribulose monophosphate pathway. Do not fix atmospheric nitrogen. The major PLFA is 18 : 1v7.

The DNA G + C content of the type strain of the type species is 58–7 mol%. Phylogenetically belongs to the ‘Gammaproteobacteria’ (type I methanotrophic bacteria or Methylocaldaceae); the closest methanotrophic bacterial neighbours are the thermophilic methanotroph strain HB and Methylocaldum capsulatus Bath. Contains one species, the type species Methylohalobius crimeensis. Habitats are pH-neutral hypersaline wetlands.

Description of Methylohalobius crimeensis sp. nov.

Methylohalobius crimeensis (cri.me.en’sis. N.L. masc. adj. crimeensis pertaining to Crimea).
Description as for the genus. The type strain is strain 10K\textsuperscript{T} (=DSM 16011\textsuperscript{T} = ATCC BAA-967\textsuperscript{T}), which was isolated from hypersaline Lake Kirleutskoe in the Crimean Peninsula, Ukraine. The species also includes strain 4Kr.

**ACKNOWLEDGEMENTS**

J. H. was supported, in part, by the Bundesministerium für Bildung und Forschung through the BIOLOG project. We would like to thank Dr Reiner Hedderich for assisting with enzyme tests, Dr Angela Smirnova for acting as interpreter, Claudia Knief for assistance with molecular work and Nina Ringleff for technical assistance. PLFA and G+C analyses were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

**REFERENCES**


