Methylosarcina fibrata gen. nov., sp. nov. and Methylosarcina quisquiliarum sp. nov., novel type I methanotrophs

Mark G. Wise,1† J Vaun McArthur2 and Lawrence J. Shimkets1

Author for correspondence: Lawrence J. Shimkets. Tel: +1 706 542 2681. Fax: +1 706 542 2674. e-mail: shimkets@arches.uga.edu

Two novel species of obligate methane-oxidizing bacteria, isolated from landfill soil, were characterized. Both strains were unusual in that some members of the population grew in irregularly shaped, refractile cell packets that resembled sarcina-like clusters. Electron microscopy revealed that the cell packets were covered with a slime layer and the cells contained many large granular inclusion bodies. The individual cells of each strain were sometimes motile and had differing morphologies. Isolate AML-C10T was always coccoidal in shape, and the cells were covered with extracellular fibrils. Isolate AML-D4T was pleomorphic, changing from rod to coccidal form, sometimes exhibiting an unusual fusiform morphology. AML-D4T lacked the extensive fibrillar matrix observed with AML-C10T. Both strains utilized only methane and methanol as carbon sources. In stationary phase, the cells of each strain swelled in size and formed cysts. Aside from morphological differences, strains could also be distinguished from each other by cellular protein patterns, as well as by temperature and pH tolerances. 16S rDNA phylogenetic analysis showed that these are type I methanotrophs (family: Methylococcaceae) most closely related to the Methylobacter/Methylomicrobium clade, although they form a monophyletic grouping supported by moderately high bootstrap values. By 16S rDNA database searches, the most similar species to both isolates were Methylobacter spp. However, partial particulate methane monooxygenase sequence analysis suggested that these bacteria might be more closely related to Methylomicrobium than Methylobacter. Furthermore, cellular fatty acid profiles of the strains more closely resemble those of Methylomicrobium, although the absence of significant levels of 16:1ω5c argues for the uniqueness of these two strains. On the basis of the results described here, it is proposed that a new genus should be created, Methylosarcina gen. nov., harbouring two species, Methylosarcina fibrata sp. nov. (type species) and Methylosarcina quisquiliarum sp. nov. The type strains are AML-C10T (= ATCC 700909T = DSM 13736T) and AML-D4T (= ATCC 700908T = DSM 13737T), respectively.

Keywords: Methylosarcina gen. nov., methanotrophs, landfills

INTRODUCTION

The obligate methane-oxidizing bacteria, known as methanotrophs, play a key role in the global consumption of methane (Hanson, 1998; King, 1992). Methane is efficient at absorbing and re-emitting infrared radiation, and its accumulation contributes to global warming (Topp & Hanson, 1991). Methanotrophs are also of interest due to their ability to degrade some environmental contaminants, like the common solvent trichloroethylene (DiSpirito et al., 1992; Tsien et al., 1989).
Methane-oxidizing bacteria have historically been divided into two groups, the type I and the type II methanotrophs, based primarily on their biochemistry, morphological features and phylogenetic placement (Hanson & Hanson, 1996). 16S rRNA sequence analysis of the type I (or group I) methanotrophs shows them to form a coherent cluster within the \( \gamma \)-subclass of the Proteobacteria (Bowman et al., 1995). These bacteria utilize the ribulose monophosphate pathway for formaldehyde assimilation and possess distinctive disc-shaped bundles of intracytoplasmic membranes (Hanson et al., 1992). The type I methanotrophs have been taxonomically united to form the family Methylomocaceae, of which four genera were originally distinguished on the basis of phospholipid fatty acid patterns, 16S rDNA analysis and phenotypic traits: *Methylococcus*, *Methylomonas*, *Methylobacter*, *Methylocmicribium* (Bowman et al., 1993, 1995). More recently, the ecological and phylogenetic diversity of the *Methylomocaceae* has expanded considerably as members of a psychrophilic genus, *Methylospheera* (Bowman et al., 1997), and thermophilic genera, *Methyllocaldum* and 'Methylothermus' (Bodrossy et al., 1997, 1999), have been described.

Previously, we reported on the use of an extinction-dilution enhancement method to isolate novel methanotrophs from landfill cover soil in the southeastern USA (Wise et al., 1999). 16S rDNA analysis of two type I isolates, designated AML-C10\(^\text{T}\) and AML-D4\(^\text{T}\), showed that they were members of a novel methanotrophic phylotype as defined by clone sequences retrieved from directly extracted soil DNA. The purpose of this work was to formally characterize these two strains. On the basis of this characterization, particularly the morphological, fatty acid and phylogenetic data, we propose a new genus, *Methylosarcina* gen. nov., with the description of *Methylosarcina fbrata* sp. nov. (type species) and *Methylosarcina quisquiliarum* sp. nov.

**METHODS**

**Bacterial strains.** AML-C10\(^\text{T}\) and AML-D4\(^\text{T}\) were isolated from cover soil collected at the Athens-Clarke County Municipal Landfill in August, 1998. The extinction-dilution enrichment procedure used to isolate these strains has been previously described (Wise et al., 1999). Both strains were maintained in an approximately 25% (v/v) methane (balance: air) atmosphere on NMS medium (Hanson et al., 1992) at 30 °C. For long-term storage, 800 µl 50% (v/v) glycerol was added to 1 ml of late-exponential-phase liquid culture, and the mixed suspension was frozen at −70 °C.

**Morphological characterization.** Original morphological observations were made with wet mounts viewed by phase-contrast microscopy. Cyst formation was observed by the method of Vela & Wyss (1964) and staining with acridine orange (Tchan & New, 1984). Capsules and slime layers surrounding cells and cell packets were detected by negative staining with nigrosin. The heat resistance of cysts was assessed by heating 3-week-old cyst-containing cultures at 80 °C for 20 min, and then checking for growth. Desiccation resistance was assessed by modifying the method of Whittenbury et al. (1970b). Briefly, 100 µl cells from approximately 3-week-old liquid cultures was examined microscopically for the presence of cysts, then transferred to 0.5 ml centrifuge tubes and placed uncapped in a desiccation chamber and allowed to dry. At 1 week intervals, dried cell material was reinoculated into NMS to check for growth. For all tests, growth was monitored spectrophotometrically (OD\(_{660}\)) with a Beckman DU-50 series spectrophotometer. Since the strains often formed flocculent particles, it was necessary to confirm growth by phase-contrast microscopy.

**Electron microscopy.** Detailed morphological examinations were made with electron microscopy as described previously (Arnold & Shimkets, 1998). Briefly, the cells in NMS medium were initially fixed with glutaraldehyde (added to the medium at a final concentration 2%, v/v) at late-exponential phase. The secondary fix was 1% osmium tetroxide in 0.1 M cacodylate buffer, followed by two water washes, and then a 10% ethanol series dehydration. After dehydration, samples for scanning electron microscopy were placed on 0.1% carbon-coated, 300 mesh Formvar and carbon-coated. Exponential-phase cells were allowed to dry on the grids and stained with 2% phosphotungstic acid.

**Physiological and biochemical tests.** The following carbon sources were tested for their ability to support growth [each at 0.1%, w/v (or v/v for liquids), in NMS]: acetate, nutrient broth, sucrose, formamide, casitone, yeast extract, formate, glucose, methanol, dimethylamine, triethylamine, pyruvate, ethanol, formaldehyde, citrate and methanol. Nitrogen source utilization was tested by omitting nitrate from the NMS medium and substituting with the following nitrogen sources (at 0.1%): NH\(_4\)Cl, peptone, l-glutamic acid, t-glutamate, t-aspartate, fumaric acid, glutathione, methanamine, dimethylamine and yeast extract. Cells were also inoculated into N-free medium to test for the ability to fix \( \text{N}_2 \).

The ability of the organisms to grow at pH values ranging from 4.0 to 9.0 was tested in modified NMS. For the lower pH values, the medium was buffered with citrate-phosphate buffer (after establishing that citrate could not be used as a carbon source). Growth at the higher pH values was tested in NMS adjusted with Tris buffer. Growth was tested at 22, 25, 32, 37 and 45 °C. Tolerance to salt was assessed by adding NaCl (a range of 0–2.5%, w/v, at 0.5% increments) to the medium.

The oxidase, catalase, Gram stain and nitrate reduction tests were performed according to standard methods (Benson, 1985).

**Enzyme assays.** AML-C10\(^\text{T}\) and AML-D4\(^\text{T}\) were grown to late-exponential phase and lysed by sonication. The presence of the following enzymes was assayed spectrophotometrically at room temperature: hydroxypyruvate reductase (Large & Quayle, 1963), hexulose phosphate synthase (Dahl et al., 1972) and ribulose-1,5-diphosphate carboxylase (Anderson & Fuller, 1969). The presence of the soluble and particulate methane monooxygenase genes was determined using PCR as described previously (Wise et al., 1999).
Fatty acid analysis. Lipids were extracted from late-exponential-phase cells according to the method of Bligh & Dyer (1959). Fatty acid methyl esters were prepared by methanolation of the samples in methanolic 1 M HCl at 80 °C for several hours. The solvent was then evaporated and the fatty acids were dissolved in hexane and analysed by GC-MS. The double-bond positions of the various unsaturated fatty acids were determined by preparing dimethyl disulfide derivatives of the fatty acid methyl esters (Yruela et al., 1990). The methyl esterified dimethyl disulfide adducts of the fatty acids were analysed by GC-MS. This analysis was performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany.

DNA–DNA hybridization. DNA–DNA hybridization between AML-C10T and AML-D4T was carried out as described by De Ley et al. (1970) with the modifications described by Huss et al. (1983) and Escara & Hutton (1980). The analysis was performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany.

Phylogenetic analysis. Extraction of genomic DNA, amplification and sequencing of the 16S rDNA were described previously (Wise et al., 1999). Sequences were aligned using the PILEUP program that is part of the University of Wisconsin's Genetic Computer Groups (GCG) Sequence Analysis Software Package and corrected manually according to primary and secondary structure similarity. Phylogenetic trees were constructed using the fastDNAml program (Olsen et al., 1994; Felsenstein, 1981), which employs a maximum-likelihood algorithm, and with the DNAPARS program, which uses a parsimony method (Hillis et al., 1993). The fastDNAml program was run remotely via the Worldwide Web at the Pasteur Institute (URL: http://bioweb.pasteur.fr). The DNAPARS program is part of the PHYLIp suite of phylogenetic analysis programs (version 3.572, from J. Felsenstein, University of Washington). Bootstrap analysis for 100 resamplings was performed with both algorithms to provide confidence estimates for tree topologies (Felsenstein, 1985). The GenBank/EMBL/DDBJ accession numbers for sequences on the tree are as follows: Escherichia coli, Z83204; Methylococcus thermophilus ACM 35855, X73819; Methylococcus capsulatus (Bath) ACM 3302, X72771; Methylococcus capsulatus (Bath) ACM 1292T, X72770; Methylophaera hansonii AM6T, U67929; Methylomonas fodinaria ACM 3268T, X72778; Methylomonas aurantiaca JB103T, X72776; Methylomonas parvus ACM 3308T, X72767; Methylomicroidium agile ACM 3308T, X72767; Methylomicroidium album VKM-BG8T, X72777; Methylomicroidium pelagicum ACM 3505T, X72775; Methyllobacter whittenburyi ACM 3310T, X72773; Methyllobacter marinus A45T, M95658; Methyllobacter luteus ACM 3304T, M95657; Methyllobacter sp. BB5.1, AF016981. For Methylomonas methylamica St1, the 16S rRNA sequence was obtained from C. R. Woese and is available at the Ribosomal Database Project (URL: http://www.cme.msu.edu/RDP) as ‘Mlm.metha1’.

A portion of the pmoA gene was amplified with the primers pmof1 (5'-GGGGGAACCTTCTGGGGTTGGGAC-3') and pmor (5'-GGGGGRCICAGTCTTACCGCAA-3') (Cheng et al., 1999) and sequenced as previously described (Wise et al., 1999). Alignment of translated gene sequence was performed using GCG’s PILEUP program. The phylogenetic tree was constructed using the NEIGHBOR-JOINING application (Saitou & Nei, 1987) from a matrix of pairwise genetic distances as calculated by the PROTDIST program (Felsenstein, 1988). Both programs are part of PHYLIp. Bootstrap analysis was performed for 100 resamplings. The GenBank/EMBL/DDBJ accession numbers for the sequences used are as follows: Methylocystis parvus OBBP5, U31651; Methylococcus capsulatus (Bath) ACM 3302, L40804; Methylocaldum szegediense OR2T, U89303; Nitrosospira.
Fig. 2. Scanning (a–c) and transmission (d, e) electron micrographs of AML-C10¹; (a) aggregation of cells; (b) high-magnification view of cell cluster showing fibrillar matrix; (c) individual cells, diplococci, and tetrads with extracellular fibrils; (d) thin section through cell packet showing a closely associated compact layer surrounding cells (arrow) and secondary diffuse, loosely associated material (arrow with asterisk), bar 0.5 µm; (e) high-magnification thin section through a tetrad showing fine structure, including large inclusion bodies, bar 0.25 µm.
**RESULTS**

**Morphological and cultural characteristics**

Colonies of AML-C10<sup>T</sup> and AML-D4<sup>T</sup> on solidified NMS medium were circular, light brownish to buff in colour, and had regular margins. After extended incubation on plates, both strains formed raised, wrinkled colonies. In liquid culture, both strains often grew in a flocculent manner, and the flocculent particles settled rapidly when shaking was ceased. When examined by phase-contrast microscopy, the particles were refractile and resembled irregularly shaped sarcina-like clusters (Fig. 1). Negative staining with nigrosin revealed that these aggregations were covered in a diffuse slime layer. Individual cocccid-shaped cells, diplococci and tetrads, of which many were highly motile, were also observed in actively growing AML-C10<sup>T</sup> cultures. Individual AML-D4<sup>T</sup> cells were pleomorphic; they were originally observed as ovoid-, rod- or fusiform-shaped cells, but eventually converted to a coccal form (see below). Some AML-D4<sup>T</sup> cells in the population were also motile. In both strains, motile cells possessed one, or rarely two, polar flagella as revealed by negative staining and electron microscopy.

Scanning electron micrographs of methane-grown exponential-phase AML-C10<sup>T</sup> cultures revealed clusters of cells that divided in more than one plane (Fig. 2a). High magnification showed that the cells in the packets were surrounded with what appears to be an extracellular matrix of fibrils (Fig. 2b). Individual AML-C10<sup>T</sup> cells had a diameter of approximately 0.8–1.5 µm (Fig. 2c) and were also covered with fibrils. Thin sections taken through the cell packets revealed a compact electron-opaque layer surrounding the cell membranes, and what appears to be a second more compact electron-opaque layer surrounding the cell (Fig. 2d). Individual cells contained many large inclusion bodies (Fig. 2e). Occasionally stacked membranes, typical of type 1 methanotrophs, were observed.

The pleomorphic nature of strain AML-D4<sup>T</sup> is evident in the scanning electron micrographs of methane-grown exponential-phase cells presented in Fig. 3. As originally observed, AML-D4<sup>T</sup> sometimes exhibited an unusual fusiform morphology, distinguished by a bulbous protrusion from the centre of the cell (Fig. 3a). In such cultures, cells were also present as rods and sometimes chains of ovoid-shaped cells. Some rods were as long as 6 µm (Fig. 3b). After repeated subculture in liquid NMS, however, this strain converted to a form in which growth was exclusively in the sarcinal packets, with a few loose individual cocci and diplococci present (Fig. 3c). In this sarcinal form, very few motile cells were observed and the cells lacked the extensive fibrilar matrix noted in AML-C10<sup>T</sup> (Fig. 3d). Fusiform-shaped cells (along with rods and cocci) were also sometimes observed as members of isolated colonies on NMS plates. Interestingly, when these colonies were inoculated in liquid NMS the cells grew exclusively in the sarcinal form.

Thin sections through AML-D4<sup>T</sup> sarcinal packets showed the cells to be similar in ultrastructure to AML-C10<sup>T</sup>, as both contain large granular inclusion bodies (Fig. 3e). However, the stacks of internal membranes were more obvious in AML-D4<sup>T</sup> cells than in AML-C10<sup>T</sup> (Fig. 3f). Also, AML-D4<sup>T</sup> lacked the compact layer present in AML-C10<sup>T</sup>; instead, packets are surrounded with what appears to be capsular material (Fig. 3e).

After extended incubation, both strains formed large cells that eventually differentiated into cysts. In strain AML-C10<sup>T</sup>, individual cells or cells in the aggregates swelled up in size to 2–3 µm (although some cells as large as 4 µm in diameter were seen) and formed cysts. AML-D4<sup>T</sup> cells similarly rounded up and swelled to 2–4 µm before encystment. Cysts were most often present singly or in pairs, but were sometimes part of large clusters. The cysts of both strains were heat-sensitive (80 °C for 20 min) and did not survive 1 week of desiccation.

Under optimal conditions in NMS medium with methane serving as carbon source, AML-C10<sup>T</sup> had an doubling time of approximately 3.5 h. AML-D4<sup>T</sup> grew slower, with an approximately 6.5 h doubling time. AML-C10<sup>T</sup> consistently grew to a higher turbidity (approximate maximum OD<sub>660</sub> = 0.80) with many single cells and diplococci present at late-exponential phase. AML-D4<sup>T</sup> showed a maximum OD<sub>660</sub> of approximately 0.45 when grown on methane, with fewer individual cells being produced. It was noted that with extended subculture growth in the cell packets became increasingly rare for strain AML-C10<sup>T</sup>. Instead, liquid culture became more evenly dispersed, and the culture was dominated by highly motile individual cells, diplococci and tetrads. Strain AML-D4<sup>T</sup> retained the tendency to grow in the clusters throughout 1 year of subculturing.

**Physiological and biochemical characteristics**

Neither AML-C10<sup>T</sup> nor AML-D4<sup>T</sup> was able to utilize any of the carbon sources tested for growth except methane and methanol. No growth was observed on nutrient-rich media. Strain AML-C10<sup>T</sup> grew as well on methanol as on methane. Strain AML-D4<sup>T</sup>, however, preferred methane to methanol as sole source of carbon and energy. Nitrate, ammonia, peptone, urea, yeast extract, l-glutamine, l-aspargin, l-aspartate and l-glutamate were accepted as nitrogen sources by both strains. Neither strain utilized l-glycine, methyamine or dimethylaniline. No growth was observed in nitrogen-free medium (i.e. only N<sub>2</sub> present), even under low oxygen tensions. It was noted that complex nitrogen sources, like yeast extract or peptone, sig-
Fig. 3. For legend see facing page.
Table 1 Major fatty acids of type I methanotroph genera and novel isolates

Values are percentages of the total phospholipid fatty acids (for genera: mean±standard deviation); data are from Bowman et al. (1995), except AML-C10<sup>T</sup> and AML-D4<sup>T</sup>, this study. NR, Not reported; ND, not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Methylomonas</th>
<th>Methyllobacter</th>
<th>Methylophilus</th>
<th>Methylococcus</th>
<th>AML-C10&lt;sup&gt;T&lt;/sup&gt;</th>
<th>AML-D4&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2.7</td>
<td>3.8</td>
</tr>
<tr>
<td>14:0</td>
<td>22±3</td>
<td>9±2</td>
<td>1±1</td>
<td>1±1</td>
<td>2±1</td>
<td>1±2</td>
</tr>
<tr>
<td>16:1ω&lt;sub&gt;8c&lt;/sub&gt;</td>
<td>30±11</td>
<td>0</td>
<td>16±3</td>
<td>0</td>
<td>14±1</td>
<td>15±9</td>
</tr>
<tr>
<td>16:1ω&lt;sub&gt;7c&lt;/sub&gt;</td>
<td>11±4</td>
<td>57±1</td>
<td>17±4</td>
<td>28±10</td>
<td>24±8</td>
<td>32±2</td>
</tr>
<tr>
<td>16:1ω&lt;sub&gt;6d&lt;/sub&gt;</td>
<td>9±4</td>
<td>5±1</td>
<td>10±4</td>
<td>3±2</td>
<td>6±7</td>
<td>9±1</td>
</tr>
<tr>
<td>16:1ω&lt;sub&gt;5e&lt;/sub&gt;</td>
<td>4±2</td>
<td>7±1</td>
<td>6±1</td>
<td>3±2</td>
<td>ND</td>
<td>0±7</td>
</tr>
<tr>
<td>16:1ω&lt;sub&gt;5f&lt;/sub&gt;</td>
<td>12±4</td>
<td>11±1</td>
<td>20±10</td>
<td>&lt;1</td>
<td>300</td>
<td>18±9</td>
</tr>
<tr>
<td>16:0</td>
<td>7±2</td>
<td>8±1</td>
<td>15±3</td>
<td>44±8</td>
<td>19±7</td>
<td>18±3</td>
</tr>
</tbody>
</table>

Fig. 4. Cellular protein patterns after SDS-PAGE of whole-cell extracts of AML-C10<sup>T</sup> (lane 2) and AML-D4<sup>T</sup> (lane 3). Molecular masses of the standards (lane 1) are indicated in kDa on the left.

Fig. 3. Scanning (a–d) and transmission (e, f) electron micrographs of AML-D4<sup>T</sup>: (a) ovoid-, rod- and fusiform-shaped cells; (b) high-magnification image of cells as long as 6 μm; (c) growth in sarcinal form; (d) high-magnification view of growth in sarcinal form – note lack of fibrils; (e) thin section through a sarcinal packet showing capsular material (arrows) surrounding cell clusters, bar 1 μm; (f) high-magnification view of a tetrad showing inclusion bodies and stacks of intracytoplasmic membranes (arrows), bar 0.25 μm.

Figuratively stimulated growth and increased the frequency of motile cells. AML-C10<sup>T</sup> and AML-D4<sup>T</sup> were Gram-negative, oxidase-negative and catalase-positive. Both strains reduced nitrate to nitrite, although no growth could be detected under anaerobic conditions with nitrate as electron acceptor and methane or methanol as electron donor and carbon source. Overall, AML-C10<sup>T</sup> seemed to have a wider physiological tolerance to temperature and pH. Both strains grew at 22 °C and 32 °C, but only AML-C10<sup>T</sup> grew at 37 °C. Neither strain could grow at 45 °C. AML-C10<sup>T</sup> and AML-D4<sup>T</sup> could grow at pH 5.5 and 9.0; however, only AML-C10<sup>T</sup> grew at pH 5.0. This is in agreement with the enrichment procedure used to isolate these two strains. As described previously, AML-C10<sup>T</sup> and other isolates with identical 16S rDNA gene sequence dominated enrichments at pH 4.8, whereas enrichments at pH 6.8 selected for AML-D4<sup>T</sup> and other isolates with identical 16S rDNA sequence (Wise et al., 1999). Both strains could grow when 1.0% NaCl was added to the medium, but not 1.5%.

Both AML-C10<sup>T</sup> and AML-D4<sup>T</sup> were positive for hexulose phosphate synthase, the key enzyme of the ribulose monophosphate pathway for formaldehyde assimilation. Hydroxyxypyruvate reductase, the key enzyme of the serine cycle for formaldehyde assimilation, was not detected. No ribulose-1,5-bisphosphate carboxylase could be detected. As reported previously, neither strain yielded any PCR product with primers specific for the alpha subunit of the soluble methane monooxygenase hydroxylase component (mnoX gene) (Wise et al., 1999).

Cellular fatty acid and protein analysis

The whole-cell fatty acid composition was determined by the GC-MS technique for both isolates. Both isolates contained significant amounts of 16:1ω<sub>8c</sub>, 16:1ω<sub>7c</sub>, 16:1ω<sub>6d</sub>, 16:1ω<sub>5f</sub> and 16:0. Interestingly, unlike members of other methanotroph genera studied...
to date, all of the 16:1ω5 fatty acid was in the trans-configuration in strain AML-C10\textsuperscript{T}, and only trace amounts of the cis-isomer were found in strain AML-D4\textsuperscript{T}. Table 1 compares the fatty acid profile of the novel isolates to the profiles of related genera belonging to the Methylococcaceae.

Protein patterns of strains AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T} after separation by SDS-PAGE and staining with Coomassie blue are shown in Fig. 4. As expected, the strains had some proteins in common; however, they exhibited a considerable heterogeneity, especially in the 26–39 kDa range.

DNA base composition

The G + C content of the DNA of strains AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T}, as determined by HPLC, was 54.1 ± 0.2 mol% (mean ± standard deviation; \( n = 3 \)) and 54.3 ± 0.3 mol% (\( n = 3 \)), respectively.

DNA–DNA hybridization

DNA–DNA hybridization studies revealed that the level of relatedness between AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T} was 52.0%. Thus the two strains should be considered different genospecies.

Phylogenetic analysis

The 16S rRNA and partial \textit{pmoA} gene sequences of strains AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T} were determined in a previous study (Wise et al., 1999). AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T} are 99.9% identical to each other over the length of the 16S rRNA gene. 16S rRNA gene database searches revealed that the strains are most identical to \textit{Methylobacter} sp. strain BB5.1, a NaCl-requiring species isolated from estuary sediment (Smith et al., 1997). \textit{Methylobacter} sp. strain BB5.1 is 95.9% and 95.4% identical to AML-C10\textsuperscript{T} and AML-D4\textsuperscript{T}, respectively. The phylogenetic tree shown in Fig. 5 reflects the relationship of the novel strains to some
other members of the Methylococcaceae. The consensus tree drawn by both the maximum-likelihood and parsimony algorithms had the same topology; both isolates cluster together in a group distinct from the Methylobacter/Methylomicrobium clade with moderately high bootstrap support.

Primer pair pmoA/pmo (Cheng et al., 1999) gave the expected 330 bp product with both strains and the derived amino acid sequence was used in a phylogenetic analysis. The partial pmoA protein sequences of AML-C10T and AML-D4T were both most similar to that of Methylibacterium album at 97.3% and 94.5% amino acid similarity, respectively. This relationship is evident in the tree shown in Fig. 6.

DISCUSSION

We are aware of only one report in the literature that describes methanotrophic bacteria that form sarcinal-type packets. A methane-oxidizing strain, named Methylococcus mobilis, was isolated from a chemostat inoculated with sewage sludge and shown to form cell packets similar in appearance to AML-C10 (Hazeu et al., 1980). Indeed, transmission electron micrographs of AML-C10T show a striking similarity to those of Methylococcus mobilis; the cells of both strains are surrounded by a compact layer that seems to cement the cells together. Also both possess a secondary, more diffuse, slime layer, and large inclusion bodies. However, on the basis of the description of Methylococcus mobilis, isolate AML-C10T differs in at least three respects: (i) AML-C10T, unlike Methylococcus mobilis, will utilize urea as a nitrogen source, (ii) the minimum doubling time of AML-C10T (3.5 h) is much shorter than that reported for Methylococcus mobilis (14 h), and the mol% G + C was reported to be 56.3 ± 0.4 for Methylococcus mobilis whereas we measured a value of 54.1 ± 0.2 for AML-C10T. Furthermore, the occurrence of large, spherical cysts was not reported by Hazeu. Unfortunately, Methylococcus mobilis is no longer extant, so definitive macromolecule sequence comparison is impossible.

Methanotrophic bacteria that exhibit pleomorphology in a manner similar to isolate AML-D4T have also been previously observed. Whittenbury et al. (1970a) described an isolate named ‘Methylobacter chroococcum’ (no longer extant) that changed from rod to coccal form and back again. ‘Methylobacter chroococcum’ also formed large cells, like AML-D4T, and the photomicrographs provided by Whittenbury show some resemblance to AML-D4T. ‘Methylobacter chroococcum’ in ‘filamentous’ form looks quite similar to the fusiform-shaped AML-D4T, as both possess the bulbous protrusion from the centre of the cell (Whittenbury et al., 1970a). In coccal form, ‘Methylobacter chroococcum’ made tetrads, as does AML-D4T. However, there are some notable differences between AML-D4T and the description of ‘Methylobacter chroococcum’. ‘Methylobacter chroococcum’ was reported to have a pale-pink colony colour and was non-motile. Also Whittenbury reported that ‘Methylobacter chroococcum’ did not grow when 0.1% (w/v) methanol was added to the medium as a carbon source, although it could grow when methanol was added slowly in the vapour phase. Our strain grows on 0.1% methanol, albeit poorly.

Phylogenetic analysis of the 16S rRNA gene shows that the lineage defined by AML-C10T and AML-D4T is related to, but diverges from, the Methylobacter/Methylomicrobium clade. This divergence is supported by moderately high bootstrap values in trees constructed using both maximum-likelihood and maximum-parsimony algorithms. By 16S rDNA sequence similarity, both isolates are most identical to members of the Methylobacter: the three most identical species in the databases are Methylobacter sp. strain BB5.1, followed by Methylobacter luteus and Methylobacter whittenburyi. However, analysis of the deduced amino acid sequence of part of the pmoA gene suggests that the particulate methane monooxygenase genes present in AML-C10T and AML-D4T are more like that in Methylibacterium album than any member of the Methylobacter. Bowman et al. (1995) has proposed that the best criterion for classifying methanotrophs at the genus level is fatty acid profiles. The profiles for AML-C10T and AML-D4T suggest, again, these strains are most like the genus Methylomicrobium, as significant amounts of 16:1o8c, 16:1o7c and 16:1o5t were detected (although the virtual absence of 16:1o5c argues for the unique status of these two strains). Much of the phenotypic data, on the other hand, implies that the two strains in question are more similar to the Methylobacter. Cyst formation is a key differential trait that divides Methylobacter from Methylomicrobium; AML-C10T and AML-D4T both make cyst-like structures, although they appear to be somewhat different than Methylobacter cysts in that they do not confer desiccation resistance for 1 week. Also, these strains have the typical light-brownish colour of most Methylobacter colonies, whereas Methylomicrobium colonies are non-pigmented or opaque white. Taken as a whole, these data do not allow for definitive classification of AML-C10T and AML-D4T into either Methylobacter or Methylomicrobium, therefore we suggest that the creation of a new genus is warranted.

AML-C10T and AML-D4T were recently shown to be the only cultured members of the predominant type I methanotrophic phylotype present in the cover soil of the Athens-Clarke County Municipal Landfill in Athens, Georgia, USA (Wise et al., 1999). A large number of similar 16S rDNA clone sequences were retrieved from DNA extracted directly from the landfill soil, suggesting that many genetically related, but not-yet-cultured, species are present in this habitat. These bacteria may be particularly well adapted to the high methane concentrations typical in landfill soils. Although we realize that the taxonomic description could change as further bacteria from this phylotype
are isolated, we propose the formation of a new genus on the basis of the characterization of the cultured strains described here. The following is a description of *Methylosarcina* gen. nov., containing two species, *Methylosarcina fibrata* sp. nov. and *Methylosarcina quisquiliarum* sp. nov.

**Description of Methylosarcina gen. nov.**

*Methylosarcina* (Me.thyl.o.sar.ci'na. N.L. neut. n. methylum the methyl group; L. fem. n. sarcina pack, bundle; N.L. fem. n. Methylosarcina methane-utilizing bundle of cells).

Members are Gram-negative, oxidase-negative and catalase-positive. Light brown to buff coloured colonies are present on NMS agar. Cell morphology can vary from coccoid to rod, or fusiform shape. In liquid culture, growth can be flocculating or evenly dispersed. Cells often grow in sarcinal-like aggregations covered by a capsule or diffuse slime layer. Individual cells, diplococci and tetrads are occasionally motile by means of one, or rarely two, polar flagella. In stationary phase, cells tend to increase in size and eventually form desiccation- and heat-sensitive cysts. Cells utilize only methane and methanol as carbon sources. Nitrate, ammonia, peptone, urea, yeast extract, l-glutamine, l-asparagine, l-aspartate and l-glutamate are accepted as nitrogen sources. No nitrogen fixation occurs. Strictly aerobic. Ribulose bisphosphate carboxylase and soluble methane monooxygenase are absent. Phylogenetic analysis of 16S rDNA places members in the family *Methylococcaceae*, most closely related to the genera *Methylobacter* and *Methylococcus*. Major fatty acids are 16:1ω7c, 16:1ω5t and 16:0. 16:1ω5c is absent or present in trace amounts. G+C content is approximately 54 mol%. Type species is *Methylosarcina fibrata*.

**Description of Methylosarcina fibrata* sp. nov.**

*Methylosarcina fibrata* (fi.br'a ta. L. fem. n. fibra a fibre or filament; M.L. fem. adj. *fibrata* covered with fibres or fibrils).

Characteristics are the same as given in the genus description with the following amendments. Cells are pleomorphic; originally observed as ovoid- or rod-shaped, sometimes fusiform with a bulbous protrusion from the middle. In this form, cell length ranges from 1 to 6 µm and width approximately 1 µm. In sarcinal form, cells grow in packets covered with a capsular material. Individual cells in this form are coccoid in shape (approx. 1 µm in diameter). Cells do not make an extensive fibrillar matrix. Liquid culture is flocculating. Cells grow at pH 5–5.90 and 22–32°C. G+C content is 54.3±0.3 mol%. Type strain is AML-D4T (ATCC 700908T = DSM 13737T).

**Description of Methylosarcina quisquiliarum* sp. nov.**

*Methylosarcina quisquiliarum* (quis.qui.li.arum. L. plur. fem. n. quisquiliae rubbish, trash, etc., denoting that strains were isolated from a landfill site).

Characteristics are the same as given in the genus description with the following amendments. Cells are pleomorphic; originally observed as ovoid- or rod-shaped, sometimes fusiform with a bulbous protrusion from the middle. In this form, cell length ranges from 1 to 6 µm and width approximately 1 µm. In sarcinal form, cells grow in packets covered with a capsular material. Individual cells in this form are coccoid in shape (approx. 1 µm in diameter). Cells do not make an extensive fibrillar matrix. Liquid culture is flocculating. Cells grow at pH 5–5.90 and 22–32°C. G+C content is 54.3±0.3 mol%. Type strain is AML-D4T (ATCC 700908T = DSM 13737T).

**Acknowledgements**

The authors would like to thank: Drs John P. Shields and Russ Carlson for technical assistance, Professor Dr Hans G. Trüper for help with bacterial nomenclature, and Dr William B. Whitman for providing helpful comments on the manuscript.

This research was supported by Financial Assistance award DE-FC09-96SR18546 from the US Department of Energy to the University of Georgia Research Foundation and by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

**References**


