Methyloversatilis universalis gen. nov., sp. nov., a novel taxon within the Betaproteobacteria represented by three methylotrophic isolates

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Methylotrophs are micro-organisms that are able to grow on single-carbon compounds. Methylotrophy is widespread within the bacterial world and is found within members of the Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria and members of the Firmicutes (Lidstrom, 2001). Culture-independent approaches to evaluate the potential for methylotrophy in freshwater Lake Washington (Washington State, USA), via detection and analysis of the genes for tetrahydrofolate-linked formaldehyde oxidation (fae and fhcD), have suggested the presence of species not closely related to known methylotrophs, based on gene sequence divergence (Kalyuzhnaya et al., 2004, 2005; Nercessian et al., 2005). This paper describes isolation of pure cultures of two bacterial strains from Lake Washington (designated FAM5T and 500) possessing divergent methylotrophy genes, and their comparison with a previously described strain (EHg5).

Strain EHg5 was isolated from soil contaminated with chemical industrial wastes (Estarreja, Portugal), after enrichment on methanol, as described by De Marco et al. (2004). Strains FAM5T and 500 were isolated from the top layer of Lake Washington sediment as part of the present study. Sediment samples were collected from a 63-m-deep site as described previously (Kalyuzhnaya et al., 2004). Samples (1 ml) of the upper 1.0 cm of the sediment were inoculated into 250-ml flasks containing 50 ml filtered (0.22-μm filter; Millipore) Lake Washington water (LWW) supplemented with 5 mM formaldehyde solution (37% formaldehyde stock solution stabilized with 10–15% methanol; Fisher Scientific). The flasks were incubated for 2–3 weeks at room temperature with shaking (125 r.p.m.). In subsequent enrichments, 10 ml of the previous enrichment culture was diluted 1:10 (to a total of 50 ml) in the same medium. Flasks were incubated at room temperature with shaking for 1 week. Strain FAM5T was obtained as a
colony after the third enrichment culture was plated onto a solid medium (LWW solidified with 1.8% Bacto agar and supplemented with 10 mM formaldehyde solution) and incubated at room temperature for 1 week. The isolate was further purified by triplicate streaking on LWW agar supplemented with 0.1% methanol. Strain 500 was obtained from the same sediment sample as follows. A sediment sample (5 ml) was homogenized for 5 min by using a PRO 200 homogenizer (at setting 3; Pro Scientific Inc.) and diluted in LWW (to obtain 1–10 cells per 200 μl) supplemented with 5 mM formaldehyde solution. The diluted samples were transferred into 96-well microplates (200 μl per well) and the microplates were covered with lids and incubated for up to 3 months at room temperature. Microplate wells were routinely checked visually, and the contents of those showing microbial growth were transferred to 5 ml LWW supplemented with formaldehyde and plated on LWW agar medium supplemented with 0.1% methanol. Pure cultures were obtained by streaking the resulting colonies onto fivefold-diluted basal mineral medium (Harder et al., 1973) supplemented with vitamin B12 (0.1 μg ml⁻¹). Vitamin B12 dependence is widespread within the Betaproteobacteria (Willems et al., 1992), and a vitamin-dependent pattern of the cultures in question was initially observed based on their association on plates with potentially B12-producing alphaproteobacteria (data not shown). The purity of the cultures was monitored by microscopy and by growth tests using media with and without vitamin B12.

All strains were routinely grown in basal salts medium supplemented with 0.1% methanol and vitamin B12. Negatively stained preparations and thin sections of cells of strain FAM5T were viewed using a 1200 Ex II transmission electron microscope (JEOL) at an operating voltage of 80 kV. For long-term storage at −80 °C, cells were suspended in the basal salts medium supplemented with 10% DMSO. Phenotypic characterization of the isolates was performed as described by Smibert & Krieg (1994). Growth rates were determined by monitoring cell density at 600 nm using a Bioscreen C plate reader (MBR) or in 5-ml tube or 250-ml flask bulk cultures. Specific growth rates were calculated as means of triplicate determinations. The optimal concentration of methanol for growth was tested using the plate reader at the following concentrations (v/v): 0-01, 0-05, 0-1, 0-2, 0-3, 0-5, 1, 2, 3, 4 and 5%. All other substrates were tested at a concentration of 0.1% (w/v). The following substrates were tested as carbon sources: methylamine, dimethylamine, trimethylamine, methylsuccinate, formaldehyde, formate, vanillin, choline, betaine, sarcosine, oxalate, acetate, pyruvate, glycolate, citrate, malate, succinate, glucose, maltose, fructose, mannitol, ethanol, acetone, toluene, glycerol, serine, alanine, asparagine, arginine and aspartate. The following substrates were tested as nitrogen sources: KNO3, NH4Cl, methylamine, dimethylamine, trimethylamine, guanidine, glycine, serine, valine, alanine, cysteine, asparagine, aspartate, arginine, methionine, histidine, proline, glutamate, phenylalanine, tryptophan, peptone, yeast extract, threonine, urea and N2. Sensitivity to antibiotics was examined by spreading cells onto basal mineral medium agar plates and placing onto them Difco discs that contained a variety of antibiotics (see below). The effect of antibiotics on cell growth was assessed after 1 week of incubation. Cellular phospholipid fatty acid analyses were performed by the CCUG, University of Göteborg, Sweden. Analyses of the respiratory quinones were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Chromosomal DNA was extracted and purified as described previously (Kalyuzhnaya et al., 2005a). The G+C content was determined by HPLC separation as described by Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were performed using the competition procedure described by Johnson (1994). 16S rRNA gene fragments were amplified as described by Lane (1991) and sequenced using the BigDye 3.1 termination sequencing kit (Applied Biosystems). Reaction analyses were performed by the Department of Biochemistry Sequencing Facility at the University of Washington. DNA sequences were aligned using the CLUSTAL W program (Higgins et al., 1996). Phylogenetic analysis was carried out using the PHYLIP package (Felsenstein, 2003). The distance method was employed, and 100 bootstrap analyses were performed.

Cells of strains EHg5, FAM5T and 500 were Gram-negative, straight or slightly curved rods (1.2 ± 0.4 × 0.4 ± 0.1 μm) (Fig. 1). Cells were non-motile, reproduced by binary fission and did not form resting bodies. Colonies of isolates FAM5T and 500 were white (turning brown with age), circular, convex, brittle and up to 2 mm in diameter. In liquid cultures, cells of these strains tended to clump

![Fig. 1. Electron micrograph of thin-sectioned cells of strain FAM5T showing the cell wall. Bar, 0.25 μm.](image-url)
together, forming white flakes. Isolate EHg5 formed circular colonies, 1–2 mm in diameter, that were caramel in colour, circular and smooth. Cells of strain EHg5 did not form aggregates in liquid culture. All isolates required vitamin B₁₂ for growth. The isolates were able to use all the substrates tested (see above) with the following exceptions. Strain FAM5ᵀ did not use acetate, pyruvate or aspartate as carbon sources or alanine, cysteine or N₂ as nitrogen sources. Strain 500 did not use mannitol or aspartate as carbon sources or KNO₃, phenylalanine, tryptophan or N₂ as nitrogen sources. Strain EHg5 did not utilize vanillin, choline, sarcosine, oxalate, acetate or mannitol as carbon sources or cysteine, asparagine, aspartate, histidine, proline, glutamate, peptone, yeast extract, threonine or N₂ as nitrogen sources. All three isolates grew in the presence of up to 5% methanol, the maximal growth rate occurring at 0–0.5–1% methanol (isolate EHg5) or 0–0.01–0.1% (isolates FAM5ᵀ and 500). pH and temperature optima were tested in liquid basal medium supplemented with 0.1% methanol. Growth occurred at pH 6.5–9, with optimum growth at pH 7.5 (isolate EHg5) or 8–0 (isolates FAM5ᵀ and 500). The isolates grew within a temperature range of 10–42°C, with optimum growth at 37°C (isolates FAM5ᵀ and EHg5) or 30°C (isolate 500). The strains were oxidase- and catalase-positive. Tests for nitrate reduction, urease activity, sugar fermentation and indole production were negative. All isolates were resistant to (μg ml⁻¹): ampicillin (10), erythromycin (15), chloramphenicol (30), SXT (sulphamethoxazole/trimethoprim, 27·75+1·25), vancomycin (5) and penicillin (10), but were sensitive to amoxicillin (25), tetracycline (30), kanamycin (30), nalidixic acid (30), gentamycin (10) and tetracycline (30), streptomycin (10), neomycin (30) and penicillin (10).

Cell extract preparation and enzyme activity measurements were conducted as described by Miller et al. (2005). In addition, tests for methanol dehydrogenase were conducted in the presence of NAD (1 mM) as an electron acceptor, at pH 7 and 9–5. Methanoloxidase was assayed in the following reaction mixture (1 ml): 50 mM MOPS/KOH buffer (pH 7·5), 0·17 mM o-dianisidine, 5 mM methanol, 2 U horseradish peroxidase ml⁻¹ and cell extract (all reagents were from Sigma). Activity was monitored spectrophotometrically by observing absorbance at 436 nm (εo-dianisidine = 8·31 × 10⁵ mol⁻¹ cm⁻¹; Bergmeyer et al., 1974). Tests for methanol dehydrogenase and methanol oxidase were negative. Tests for methylamine dehydrogenase in methylamine-grown cells were also negative. PCR amplification of the mxaF gene with specific primers (McDonald & Murrell, 1997) produced negative results. Genes for the tetrahydromethanopterin-linked formaldehyde oxidation pathway (fae and fhcD) were successfully amplified using the primers described by Kalyuzhnaya & Chistoserdova (2005). No activities were detected for key enzymes of the ribulose monophosphate (RuMP) cycle for formaldehyde assimilation, but activities of key enzymes of the cycle (serine glyoxylate aminotransferase and hydroxyproline reductase) were found.

For fatty acid analysis, the strains were grown on trypticase soy agar (TSA; Difco). In general, the fatty acid profiles were similar for the three isolates (Table 1). The major fatty acids were C₁₆:1ω7c (51–56%) and C₁₆:0 (29–31%). Cyclopropaeanoanoic acid was detected only in strain FAM5ᵀ. Fatty acid C₁₉:1ω6c was found in strains FAM5ᵀ and 500, while C₁₅:0 was unique to strain EHg5 and C₁₈:0 was unique to strain 500. The major quinone of all three isolates was ubiquinone Q8. DNA G+C contents were 63·5±0·6, 65·5±0·5 and 63·9±0·2 mol% for isolates EHg5, FAM5ᵀ and 500, respectively. The genomic relatedness of the new isolates was investigated using DNA–DNA hybridization. Isolates FAM5ᵀ and 500 were shown to be closely related (93% DNA–DNA relatedness), whereas isolate EHg5 shared only 44 and 59% relatedness with isolates FAM5ᵀ and 500, respectively.

Based on phylogenetic analysis of 16S rRNA gene fragment sequences, the three isolates were closely related to each other. The sequences for isolates FAM5ᵀ and 500 were identical, and showed 99.85% similarity with the sequence for isolate EHg5. The new isolates were only distantly related to the well-characterized methylotrophs of the family Methylophilaceae (<89% sequence similarity; Jenkins & Doroty, 1987; Doronina et al., 2005) and to a recently characterized methylotroph within the Burkholderiales, Methylibium petroleiphilum PM1ᵀ (<90%; Nakatsu et al., 2006). Closest relationships to recognized bacteria were with the cholesterol-oxidizing denitrifier Sterolibacterium denitrificans Chol-1Sᵀ (93%; Tarlera & Denner, 2003) and the nitrogen-fixing Derxia gummosa IAM 13946ᵀ (92·8%; Xie et al., 2001). Isolates FAM5ᵀ and 500 were similar to the well-characterized methylotrophs of the family Methylophilaceae (<89% sequence similarity; Jenkins & Doroty, 1987; Doronina et al., 2005) and to a recently characterized methylotroph within the Burkholderiales, Methylibium petroleiphilum PM1ᵀ (<90%; Nakatsu et al., 2006). Closest relationships to recognized bacteria were with the cholesterol-oxidizing denitrifier Sterolibacterium denitrificans Chol-1Sᵀ (93%; Tarlera & Denner, 2003) and the nitrogen-fixing Derxia gummosa IAM 13946ᵀ (92·8%; Xie et al., 2001). Isolates FAM5ᵀ and 500 were similar

Table 1. Fatty acid profiles and major quinones of strains EgH5, FAM5ᵀ and 500 compared with those of Sterolibacterium denitrificans Chol-1Sᵀ

<table>
<thead>
<tr>
<th>Component</th>
<th>EHg5</th>
<th>FAM5ᵀ</th>
<th>500</th>
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<tr>
<td>C₁₀:0 3-OH</td>
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<td>2·3</td>
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<tr>
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<td>–</td>
<td>NR</td>
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<td>–</td>
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<td>NR</td>
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<td>C₁₈:1ω7c/12c/9t</td>
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& Yokota, 2004) (Fig. 2). However, the 16S rRNA gene sequences of the isolates characterized here were closely related (97–99 % sequence similarity) to a large number of sequences obtained from a variety of environmental samples, such as from drinking water (Williams et al., 2004), activated sludge (Loy et al., 2005), biofilms (Cole et al., 2004), gold and diamond mines (GenBank accession no. AY187895), subsurface waters (accession no. DQ336969), contaminated groundwater (Connon et al., 2005) and soils (accession no. AM159490). With regard to cultivable unclassified members of the Rhodocyclaceae, the three isolates were closely related to isolates HTCC379 (99 % sequence similarity), W8 (99 %) (Connon et al., 2005) and Thauera sp. MG70 (98 %) (Gomila et al., 2005).

Many members of the family Rhodocyclaceae possess biotechnological or agricultural potential, such as the ability to degrade halogenated compounds (Song et al., 2000), enhanced biological phosphate removal (Bond et al., 1995), denitrification and reduction of (per)chlorates (Achenbach et al., 2001; Coates et al., 1999) and nitrogen fixation (Xie & Yokota, 2004). It was shown that species of the Rhodocyclales represent the majority of bacterial populations in waste-treatment plants and bioreactors (Bond et al., 1995; Juretschko et al., 2002); however, none has yet been characterized as showing methylotrophy, and many members of the Rhodocyclales have been assumed to be recalcitrant to cultivation (Loy et al., 2005). Culture-independent studies of bacteria from Lake Washington employing PCR surveys (Nercessian et al., 2005; Kalyuzhnaya & Chistoserdova, 2005) or metagenomic analysis (Kalyuzhnaya et al., 2005b) have indicated that a broad group of members of the Betaproteobacteria not closely related to any known methylotrophic groups represented a significant portion of the total microbial community. Routine strategies for isolating methylotrophic bacteria, employing classic C1 substrates such as methanol, methylamine or formate, have not resulted in enrichment of the betaproteobacteria in question, as they were readily out-competed by the well-characterized methylotrophic 'specialists', members of the genera Methylophilus, Methylophilaceae, Methylobacterium and Ralstonia (M. G. Kalyuzhnaya, unpublished results). However, betaproteobacterial species related to members of the Burkholderiales and Rhodocyclales were enriched after supplementation of LWW with formaldehyde solution. Experiments with the resulting pure cultures demonstrated that all these isolates could utilize a broad range of C1 compounds, as exemplified by the strains described here. Therefore, our data indicate that supplementation with formaldehyde and/or vitamin B12 could be used for selective enrichment of methylotrophic representatives of the Burkholderiales and Rhodocyclales from complex microbial communities.

Although the new isolates grew on C1 compounds at rates similar to those reported for representatives of the Methylophilaceae, we were not able to detect activities of typical primary oxidation enzymes such as methanol dehydrogenase or methylamine dehydrogenase, by using standard assay conditions. These results suggest that these novel methylotrophs may possess alternative primary oxidation systems. The isolates described here also differ from representatives of the Methylophilaceae by possessing activities of the serine cycle enzymes but lacking activities of key enzymes of the RuMP cycle. The methylotrophic representative of the Burkholderiales, Methylibium petroleiphilum, also possesses the genes for the serine cycle and lacks key genes of the RuMP cycle (K. Hristova, personal communication). Analysis of the genome of Methylibium petroleiphilum PM1T

Fig. 2. Phylogenetic tree constructed using neighbour-joining analysis showing the positions of isolates FAMS, 500 and EHG5 within the Betaproteobacteria, based on 16S rRNA gene sequences. Strain numbers and GenBank accession numbers (in parentheses) are shown. Bootstrap support values of >90 % are indicated by filled circles, 70–90 % by shaded circles and 55–70 % by open circles. Bar, 10 substitutions per 100 nucleotide positions.
(http://genome.jgi-psf.org/finished_microbes/methe/methe. home.html) showed that it lacks the typical mxaFJGI gene cluster encoding the subunits of methanol dehydrogenase and the associated cytochrome c (M. G. Kalyuzhnaya and L. Chistoserdova, unpublished). These results indicate that the metabolic patterns of C1 utilization of the new isolates must be similar to those of representatives of the Rhodocyclales and Burkholderiales, but they differ significantly from the typical metabolic scheme employed by representatives of the Methylphilaceae. In contrast to other members of the family Rhodocyclaceae, the isolates described here were not able to fix nitrogen or to reduce nitrate.

The low 16S rRNA gene sequence similarity with recognized members of the Betaproteobacteria (<94%) warrants the placement of the strains described here within a new genus within the family Rhodocyclaceae. Although isolate EHG5 shared relatively low levels of DNA-DNA relatedness with the two Lake Washington isolates, the phenotypic and metabolic characteristics of the three isolates were very similar. Based on these results, we propose that all three strains represent a single taxon within the Rhodocyclaceae, for which we propose the name Methyloversatilis universalis gen. nov., sp. nov.

Description of Methyloversatilis gen. nov.

Methyloversatilis [Me.thy’lo.vers.at-il’is. N. Gr. n. methyl (from Gr. n. methu wine and Gr. n. hulè wood) methyl radical; L. adj. versatilis versatile; N.L. fem. n. versatilis versatile methyl (utilizer), reflecting the versatile trophic abilities of the first isolates].

Cells are Gram-negative, non-motile rods that multiply by binary fission. Able to utilize a variety of C1 and multicarbon compounds. Assimilate C1 units via the serine pathway. The major fatty acids are C16:1ω9c and C16:0. The major quinone is Q8. The G+C content of the DNA is 64–65 mol%. The type and only species is Methyloversatilis universalis.

Description of Methyloversatilis universalis sp. nov.

Methyloversatilis universalis (u.ni.ver.sa’lis. L. fem. adj. universalis universal, reflecting the ubiquitous distribution of the first isolates in the environment).

Cells are 1.2 ± 0.4 × 0.4 ± 0.1 μm in size and occur singly or in pairs. Growth occurs at pH 6–9, with optimal growth at pH 7.5–8. Temperature optimum is 30–37°C. Utilizes methanol, methylated amines, formaldehyde and formate, as well as a variety of multicarbon compounds. Oxidase- and catalase-positive. Negative for nitrate reduction, urease activity, sugar fermentation and indole production. Resistant to ampicillin, erythromycin, chloramphenicol, SXT, vancomycin and penicillin, but sensitive to amoxicillin, cephalothin, kanamycin, nalidixic acid, tetracycline, streptomycin, neomycin and gentamicin.

The type strain, FAMS\(^T\) (=CCUG 52030\(^T\) = JCM 13912\(^T\)), was isolated from freshwater Lake Washington (USA).

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References


