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

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The taxonomic positions and phylogenetic relationships of two new methylotrophic isolates from Lake Washington (USA) sediment, FAM5^T and 500, and the previously described methylotrophic strain EHg5 isolated from contaminated soil in Estarreja (Portugal) were investigated. All three strains were facultative methylotrophs capable of growth on a variety of C_1 and multicarbon compounds. Optimal growth occurred at pH 7·5–8 and 30–37 °C. The major fatty acids were $C_{16:1}\omega7c$ and $C_{16:0}$. The major quinone was ubiquinone Q8. Neither methanol dehydrogenase nor methanol oxidase activities were detectable in cells grown on methanol, suggesting an alternative, as-yet unknown, mechanism for methanol oxidation. The isolates assimilated C_1 units at the level of formaldehyde, via the serine cycle. The DNA G+C content of the strains ranged between 64 and 65 mol%. 16S rRNA gene sequence similarity between the three new isolates was 99·85–100 %, but was below 94 % with other members of the *Betaproteobacteria*, indicating that the isolates represent a novel taxon. Based on physiological, phenotypic and genomic characteristics of the three isolates, a new genus, *Methyloversatilis* gen. nov., is proposed within the family *Rhodocyclaceae*. The type strain of *Methyloversatilis universalis* gen. nov., sp. nov. is FAM5^T (=CCUG 52030^T=JCM 13912^T).

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 tetrahydromethanopterin-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 FAM5^T 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 FAM5^T 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 FAM5^T was obtained as a

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Abbreviations: LWW, Lake Washington water; RuMP, ribulose monophosphate; SXT, sulphamethoxazole plus trimethoprim.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *fae* and *fhcD* gene sequences of strain FAM5^T are DQ442273, DQ457109 and DQ457110, and that for the 16S rRNA gene sequence of strain 500 is DQ923115.

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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 B_{12} (0·1 μg ml^{-1}). Vitamin B_{12} 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 B_{12}-producing alphaproteobacteria (data not shown). The purity of the cultures was monitored by microscopy and by growth tests using media with and without vitamin B_{12}.

All strains were routinely grown in basal salts medium supplemented with 0·1 % methanol and vitamin B_{12}. Negatively stained preparations and thin sections of cells of strain FAM5^T 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: KNO_3, NH_4Cl, methylamine, dimethylamine, trimethylamine, guanidine, glycine, serine, valine, alanine, cysteine, asparagine, aspartate, arginine, methionine, histidine, proline, glutamate, phenylalanine, tryptophan, peptone, yeast extract, threonine, urea and N_2. 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, FAM5^T 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 FAM5^T 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

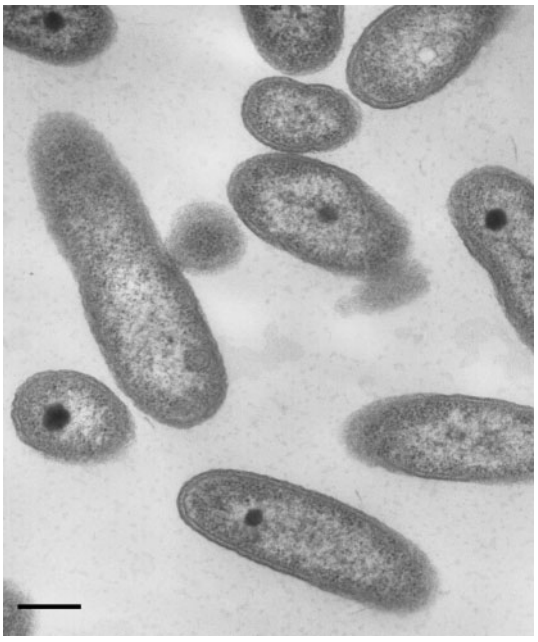

Fig. 1. Electron micrograph of thin-sectioned cells of strain FAM5^T showing the cell wall. Bar, 0·25 μm.

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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 B_{12} for growth. The isolates were able to use all the substrates tested (see above) with the following exceptions. Strain FAM5^T did not use acetate, pyruvate or aspartate as carbon sources or alanine, cysteine or N_2 as nitrogen sources. Strain 500 did not use mannitol or aspartate as carbon sources or KNO_3, phenylalanine, tryptophan or N_2 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 N_2 as nitrogen sources. All three isolates grew in the presence of up to 5 % methanol, the maximal growth rate occurring at 0·05–1 % methanol (isolate EHg5) or 0·01–0·1 % (isolates FAM5^T 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 FAM5^T and 500). The isolates grew within a temperature range of 10–42 °C, with optimum growth at 37 °C (isolates FAM5^T 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), cephalothin (30), kanamycin (30), nalidixic acid (30), tetracycline (30), streptomycin (10), neomycin (30) and gentamicin (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. Methanol oxidase 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 ($\varepsilon_{o\text{-dianisidine}} = 8{\cdot}31 \times 10^3$ mol l^{-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 serine cycle (serine glyoxylate aminotransferase and hydroxypyruvate 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 $C_{16:1}\omega7c$ (51–56 %) and $C_{16:0}$ (29–31 %). Cyclopropaneoctanoic acid was detected only in strain FAM5^T. Fatty acid $C_{19:1}\omega6c$ was found in strains FAM5^T and 500, while $C_{15:0}$ was unique to strain EHg5 and $C_{18:0}$ 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, FAM5^T and 500, respectively. The genomic relatedness of the new isolates was investigated using DNA–DNA hybridization. Isolates FAM5^T and 500 were shown to be closely related (93 % DNA–DNA relatedness), whereas isolate EHg5 shared only 44 and 59 % relatedness with isolates FAM5^T 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 FAM5^T 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* PM1^T (<90 %; Nakatsu *et al.*, 2006). Closest relationships to recognized bacteria were with the cholesterol-oxidizing denitrifier *Sterolibacterium denitrificans* Chol-1S^T (93 %; Tarlera & Denner, 2003) and the nitrogen-fixing *Derxia gummosa* IAM 13946^T (92·8 %; Xie

Table 1. Fatty acid profiles and major quinones of strains EgH5, FAM5^T and 500 compared with those of *Sterolibacterium denitrificans* Chol-1S^T

Values for fatty acids are percentages of total fatty acids. Data for *S. denitrificans* Chol-1S^T were taken from Tarlera & Denner (2003). RQ, Rhodoquinone; −, not detected; NR, not reported.

Component	EHg5	FAM5^T	500	*S. denitrificans* Chol-1S^T
Fatty acids				
$C_{10:0}$ 3-OH	2·7	3·3	3·0	14·0
$C_{12:0}$	3·8	3·8	3·3	5·3
$C_{12:0}$ 3-OH	3·1	2·6	2·3	NR
$C_{14:0}$	1·7	2·1	2·3	0·9
$C_{15:0}$	0·8	−	−	0·8
$C_{16:1}\omega7c$	56·1	51·2	52·6	33·8
$C_{16:0}$	29·0	30·2	31·1	21·5
$C_{17:0}$ cyclo	−	2·2	−	NR
$C_{18:0}$	−	−	0·5	NR
$C_{18:1}\omega7c/12t/9t$	2·8	3·8	3·6	14·5
$C_{18:1}\omega9c$	−	−	0·5	NR
$C_{19:1}\omega6c$	−	0·8	0·9	NR
Major quinone(s)	Q8	Q8	Q8	Q-8/RQ-8

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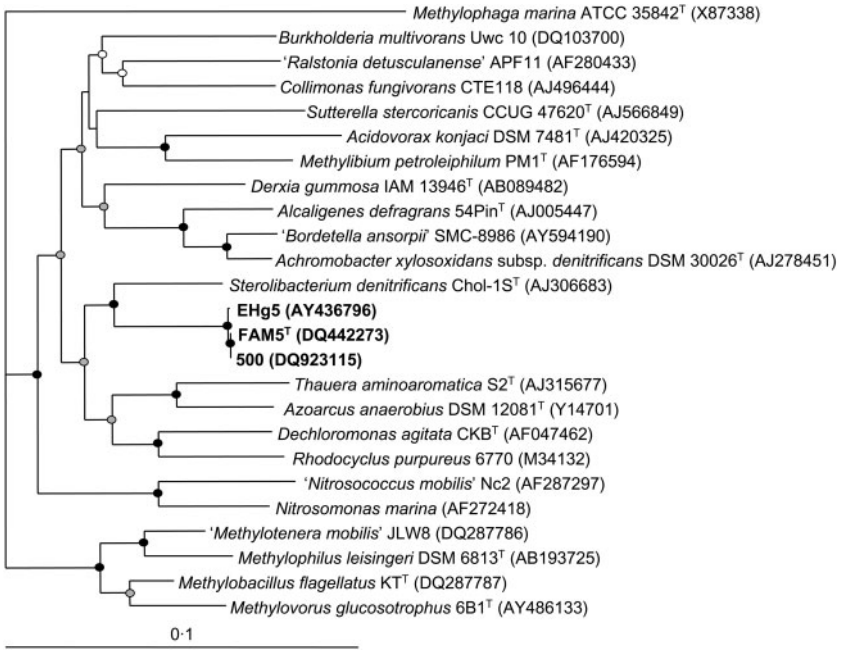

Fig. 2. Phylogenetic tree constructed using neighbour-joining analysis showing the positions of isolates FAM5^T, 500 and EHg5 within the *Betaproteobacteria*, based on 16S rRNA gene sequences. Strain numbers and GenBank accession numbers (in parentheses) are shown. Bootstrap support values of >90 % are indicated by filled circles, 70–90 % by shaded circles and 55–70 % by open circles. Bar, 10 substitutions per 100 nucleotide positions.

& 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 C_1 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*, *Methylopila*, *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 C_1 compounds, as exemplified by the strains described here. Therefore, our data indicate that supplementation with formaldehyde and/or vitamin B_{12} could be used for selective enrichment of methylotrophic representatives of the *Burkholderiales* and *Rhodocyclales* from complex microbial communities.

Although the new isolates grew on C_1 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* PM1^T

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(http://genome.jgi-psf.org/finished_microbes/metpe/metpe.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 C_1 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 *Methylophilaceae*. 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.ver.sa.ti′lis. N. Gr. n. *methyl* (from Gr. n. *methu* wine and Gr. n. *hulê* wood) methyl radical; L. adj. *versatilis* versatile; N.L. fem. n. *Methyloversatilis* 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 C_1 and multicarbon compounds. Assimilate C_1 units via the serine pathway. The major fatty acids are $C_{16:1}\omega7c$ and $C_{16:0}$. 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 $1{\cdot}2\pm0{\cdot}4\times0{\cdot}4\pm0{\cdot}1$ 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, FAM5^T (=CCUG 52030^T=JCM 13912^T), was isolated from freshwater Lake Washington (USA).

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