Novel methylotrophic isolates from lake sediment, description of *Methylotenera versatilis* sp. nov. and emended description of the genus *Methylotenera*

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Phylogenetic positions, and genotypic and phenotypic characteristics of three novel methylotrophic isolates, strains 301^T, 30S and SIP3-4, from sediment of Lake Washington, Seattle, USA, are described. The strains were restricted facultative methylotrophs capable of growth on single carbon compounds (methylamine and methanol) in addition to a limited range of multicarbon compounds. All strains used the *N*-methylglutamate pathway for methylamine oxidation. Strain SIP3-4 possessed the canonical (MxaFI) methanol dehydrogenase, but strains 301^T and 30S did not. All three strains used the ribulose monophosphate pathway for C1 assimilation. The major fatty acids in the three strains were C_{16:0} and C_{16:1ω7c}. The DNA G + C contents of strains 301^T and SIP3-4 were 42.6 and 54.6 mol%, respectively. Based on 16S rRNA gene sequence phylogeny and the relevant phenotypic characteristics, strain SIP3-4 was assigned to the previously defined species *Methylovorus glucosotrophus*. Strains 301^T and 30S were closely related to each other (100% 16S rRNA gene sequence similarity) and shared 96.6% 16S rRNA gene sequence similarity with a previously described isolate, *Methylotenera mobilis* JLW8^T. Based on significant genomic and phenotypic divergence with the latter, strains 301^T and 30S represent a novel species within the genus *Methylotenera*, for which the name *Methylotenera versatilis* sp. nov. is proposed; the type strain is 301^T (=VKM B-2679^T=JCM 17579^T). An emended description of the genus *Methylotenera* is provided.

Methylotrophic bacteria belonging to the family of *Methylophilaceae* are widespread across a variety of terrestrial, freshwater and estuary ecosystems (Jenkins & Jones, 1987; Govorukhina & Trotsenko, 1991; Doronina *et al.*, 2005; Kalyuzhnaya *et al.*, 2006; Giovannoni *et al.*, 2008). We have recently demonstrated that *Methylophilaceae*, and especially species of the genus *Methylotenera*, are important functional types in environmental cycling of C1 compounds in Lake Washington sediment (Kalyuzhnaya *et al.*, 2008b, 2009). Although a number of closely related *Methylotenera* strains (95–97% 16S rRNA similarities) appear to be present in this ecosystem, only one *Methylotenera* strain has been

formally described so far (Kalyuzhnaya *et al.*, 2006). In this study, a dilution-plating approach was used that resulted in isolation of three novel strains (301^T, 30S and SIP3-4) of the family *Methylophilaceae*. Two of these strains were characterized in detail, in terms of their phenotypic, physiological, biochemical and genomic properties.

Lake Washington sediment samples were collected as described previously (Kalyuzhnaya *et al.*, 2004). To isolate strains 301^T and 30S, 1 ml aliquots of sediment samples were mixed with 9 ml filtered lake water, and serial dilutions were plated onto plates containing lake water-based medium solidified with agar (2%; Difco) supplemented with 5 mM methylamine. After 2 weeks incubation at room temperature, individual colonies were restreaked onto fresh agar plates. Two types of colonies were observed: large opaque milky-white (named strain 301^T) and small translucent (named strain 30S). Each type of colony was plated

Abbreviation: RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession number for the complete genome sequence of *Methylotenera versatilis* 301^T is CP002056.

Two supplementary figures are available with the online version of this paper.

separately and pure cultures of strains 301^T and 30S were obtained after six passages on solid media.

Strain SIP3-4 was isolated from a methanol enrichment culture. A sample of lake sediment (3 ml) was mixed with 50 ml lake water supplemented with methanol (0.1 %) and nitrate (10 mM). The enrichment culture was incubated for 2 days at 30 °C and reinoculated (1/100) into 50 ml of the same culture medium. A pure culture of isolate SIP3-4 was obtained by serial dilutions of a third passage of the enrichment culture.

Purity of the cultures was monitored by microscopy, 16S rRNA gene amplification and sequencing, and was ultimately confirmed by complete genome sequencing for strains 301^T and SIP3-4 (the genomes will be described in a separate publication). A pure culture of strain SIP3-4 was routinely maintained on a basal salts medium supplemented with methanol or methylamine (Harder *et al.*, 1973). Pure cultures of strains 301^T and 30S were routinely maintained on a diluted (0.2 ×) basal salts medium supplemented with 0.1 % methylamine and a vitamin stock solution (Kalyuzhnaya *et al.*, 2008a). Cells of strain SIP3-4 were prepared for scanning electron microscopy as described previously (Kalyuzhnaya *et al.*, 2006) and these were viewed using an FEI Sirion scanning electron microscope (http://depts.washington.edu/ntuf/facility/sirion_sem.php). Cells of strain 301^T were prepared as described in Supplementary material (available in IJSEM Online) and viewed using a Hitachi s800 field emission scanning electron microscope at the University of Hawaii (<http://www5.pbrc.hawaii.edu/bemf/site/>). For long-term storage at -80 °C, cells were suspended in the basal salts media given above and supplemented with 10 % DMSO.

Phenotypic characterization of the isolates was carried out essentially as described previously (Smibert & Krieg, 1994). Sensitivity to antibiotics was examined by spreading cells onto mineral medium agar plates and placing BBL SensiDiscs (BD Diagnostic Systems) on them; the effect of antibiotics was assessed after 2 weeks incubation. Cellular fatty acid (phospholipid fatty acid) analysis was carried out by Microbial Insights, Inc. (Rockford, TN, USA; <http://www.microbe.com/>). Chromosomal DNA was extracted and purified as described previously (Kalyuzhnaya *et al.*, 2006). Whole genome sequencing, genome assembly and gene annotation were carried out by the Department of Energy Joint Genome Institute (Walnut Creek, CA, USA; <http://www.jgi.doe.gov/>) essentially as described previously (Lykidis *et al.*, 2010). 16S rRNA gene sequences were aligned using the program CLUSTAL W (Higgins *et al.*, 1996) and phylogenetic analysis was carried out using the PHYLIP package (Felsenstein, 2004).

Cells of strain 301^T were oval or rod-shaped ($1.0 \pm 0.2 \times 0.5 \pm 0.1$ µm in size), sometimes forming long filaments (up to 10–15 µm) (Fig. 1a). Cells grown on methylamine-supplemented solid medium for 3 days were non-motile; however, genome analysis revealed that the strain possessed genes for flagellum biosynthesis similar to the ones present

Fig. 1. Scanning electron micrographs of cells of strains 301^T (a) and SIP3-4 (b). Bars, 1 µm.

in the genomes of motile members of the family *Methylophilaceae* (Chistoserdova *et al.*, 2007; Kalyuzhnaya *et al.*, 2008b). Colonies of strain 301^T were white (slightly yellowish in old cultures), mucoid, undulate, circular, convex, viscous and up to 5 mm in diameter. The isolate grew well on solid media but not in liquid culture. Only cultures incubated without shaking showed some growth. Strain 301^T grew well on methylamine, fructose and betaine. Weak growth was observed on solid agar medium supplemented with methanol, ethanol or pyruvate. In addition, the isolate could grow on a complex medium containing 0.5 % bacto-peptone, 0.05 % yeast extract and vitamins (ATCC medium 36; <http://www.lgcstandards-atcc.org/>). Arabinose, formate, acetate, succinate, malate, mannitol, methionine, valine, threonine, vanillin, phenol, DMSO and trimethylamine did not support growth. Ammonium, nitrate and urea were used as sources of nitrogen. pH and temperature optima of the isolate were tested on solid basal media supplemented with 0.1 % methylamine. Growth occurred at pH 4.2–8.0 and 4–30 °C, with optimum growth at pH 5.8–6.5 and 18–21 °C.

Cells of strain 30S were motile and oval-shaped ($0.8 \pm 0.2 \times 0.6 \pm 0.1$ µm in size). On agar plates supplemented with methylamine, the strain formed small, smooth, circular, slightly greenish, translucent colonies with butyrous texture. Strain 30S was able to utilize methylamine and fructose but not betaine. With respect to other sources of

carbon and nitrogen as well as temperature and pH optima, strain 30S behaved like strain 301^T. The 16S rRNA gene and fragments of the *mdgA* (*N*-methylglutamate dehydrogenase, small subunit), *fae* (formaldehyde-activating enzyme) and *soxA* (sarcosine oxidase) genes of this strain were identical to those of strain 301^T, indicating a very close relatedness between the two isolates. Thus, only strain 301^T was further characterized.

Cells of strain SIP3-4 were motile, slightly curved rods, $1.4 \pm 0.4 \times 0.3 \pm 0.1$ μm in size (Fig. 1b). During growth on solid media supplemented with methanol or methylamine, the isolate formed circular, smooth colonies with entire margins, 1–2 mm in diameter and caramel in colour. The isolate grew well in liquid culture, with doubling times of 3.5 h on methanol, 17 h on methylamine and 15 h on glucose. Fructose, arabinose, formate, acetate, ethanol, pyruvate, succinate, malate, mannitol, methionine, valine, threonine, betaine, vanillin, phenol, DMSO and trimethylamine did not support growth. pH and temperature optima of strain SIP3-4 were tested in liquid mineral media

supplemented with 0.1 % methanol. Growth occurred at pH 4.2–8.0 and 9–37 °C, with optimum growth at pH 6.5 and 30 °C.

All isolates were resistant to penicillin (10 U) and sensitive to kanamycin (30 μg), ampicillin (10 μg), streptomycin (100 μg), neomycin (30 μg) and erythromycin (15 μg).

Cell extracts were prepared and enzyme activities were determined as described previously (Miller *et al.*, 2005). Enzyme activities were monitored spectrophotometrically by observing absorbance at either 340 nm (extinction coefficient of NADH = 6.22×10^3 mol l⁻¹ cm⁻¹) or 600 nm (extinction coefficient of DCPIP = 8.31×10^3 mol l⁻¹ cm⁻¹; Bergmeyer *et al.*, 1974). The novel isolates were similar to other representatives of the family *Methylophilaceae* in that they possessed high activity of a key enzyme of the ribulose monophosphate (RuMP) cycle, hexulose phosphate synthase [200 ± 20 nmol min⁻¹ (mg protein)⁻¹] and lacked activities of key enzymes of the serine cycle (serine glyoxylate aminotransferase and hydroxypyruvate reductase). Whole genome sequence analyses confirmed that strains SIP3-4

Table 1. Characteristics of the novel strains and related bacteria of the family *Methylophilaceae*

Taxa: 1, strain 301^T; 2, *Methylotenera mobilis* JLW8^T; 3, *Methylobacillus*; 4, SIP3-4; 5, *Methylovorus*; 6, *Methylophilus*; 7, *Methylophilales* bacterium HTCC2181. NR, Not reported; ND, not determined; TR, trace; +/–, variable depending on strain.

Character	1	2	3*†	4	5†‡	6*†	7§
Genome size (Mb)	3.06	2.55	2.97	3.08	NR	NR	1.30
DNA G + C content (mol%)	42.6	45.5	53–62 (55.7)	54.6	56–58	50–53	37.9
Plasmids	–	–	–	2	NR	NR	–
Copies of rRNA gene cluster	3	2	2	2	NR	NR	1
Methylamine dehydrogenase (<i>mauFBEDACJG</i>)	–	+	+	–	+/–	+/–	–
NMG pathway (<i>mgdABCDgmasAmgsABC</i>)	+	–	+	+	+/–	+/–	–
Methanol dehydrogenase (<i>mxoA</i>)	–	–	+	+	+	+	–
RuMP pathway	+	+	+	+	+	+	+
Complete TCA cycle	–	–	–	–	–	–	–
Fatty acids:							
C _{16:0}	38	32	41–43	49	47–49	32–48	NR
C _{16:1} ω7c	56	66	32–36	45	32–35	46–53	NR
C _{16:1} ω7t	2.2	0	3–8	0.2	2–3	4–12	NR
cyclo-C _{17:0}	TR	TR	5–8	2.7	10–13	1–2	NR
C _{18:0}	2	0.3	0.7–1.2	0.5	0.6–0.7	0.6–4.0	NR
C _{18:1} ω7c	0.4	0.7	5	1.7	1–2	0.7–2.0	NR
DNA–DNA identity (% of genes with identity ≥ 70 %):							
301 ^T	100	41.3	11.3	16.2	ND	ND	3.2
SIP3-4		20.6	33.9	100	ND	ND	2.6
JLW8 ^T		100	15.4		ND	ND	5.0
<i>Methylobacillus flagellatus</i> KT ^T			100		ND	ND	2.0

*Data from Jenkins & Jones (1987).

†Data from Govorukhina & Trotsenko (1991).

‡Data from Doronina *et al.* (2005).

§Data from Giovannoni *et al.* (2008).

||Data from Chistoserdova *et al.* (2007).

and 301^T possessed all the genes for the RuMP cycle and lacked key genes of the serine cycle (Table 1). Neither α -ketoglutarate dehydrogenase nor the glyoxylate shunt genes/enzymes were present.

Strain SIP3-4 displayed high methanol dehydrogenase activity [300 ± 30 nmol min⁻¹ (mg protein)⁻¹] and genome analysis (<http://genome.jgi-psf.org/>) showed that it possessed the typical *mxhFJGI* gene cluster encoding the subunits of methanol dehydrogenase and the associated cytochrome *c*. Strain 301^T lacked *mxhFJGI* in its genome (data not shown). Tests for methylamine dehydrogenase in cells grown in methylamine (0.1 %, w/v) were negative for both strains. Accordingly, the genomes lacked recognizable homologues of the *mau* genes (*mauFBEDAC*). However, both strains were positive for *N*-methylglutamate dehydrogenase activity, a key enzyme of the *N*-methylglutamate pathway [75 ± 10 and 50 ± 10 nmol min⁻¹ (mg protein)⁻¹ for strains 301^T and SIP3-4, respectively]. Gene clusters highly similar to the ones described recently, encoding *N*-methylglutamate dehydrogenase, *N*-methylglutamate synthase and γ -glutamyl methylamide synthase (Latypova *et al.*, 2010), were identified in the genomes of isolates 301^T and SIP3-4 (data not shown).

For fatty acid analysis, the isolates were grown on agar plates supplemented with methylamine. In general, the

fatty acid profiles of the cultures were typical of other representatives of the family *Methylophilaceae* (Table 1), the major fatty acids being C_{16:1 ω 7c} and C_{16:0}. As with other methylotrophs of the *Methylobacillus*/*Methylovorus* subgroup, strain SIP3-4 possessed cyclo-C_{17:0} (approx. 3 %). Only trace amounts of the C₁₇ fatty acids were detected in strain 301^T, which is typical for representatives of the *Methylophilus*/*Methylotenera* subgroup.

Based on genome sequence data, DNA G + C contents of the novel isolates were 42.6 mol% (strain 301^T) and 54.6 mol% (strain SIP3-4). 16S rRNA gene phylogeny is depicted in Fig. 2. Of the formally characterized bacteria, the novel isolates were related, as expected, to methylotrophs of the family *Methylophilaceae* (Jenkins & Jones, 1987; Doronina *et al.*, 2005) with strain SIP3-4 being most closely related to the type strains of *Methylovorus glucosotrophus* and *Methylovorus mays* (99.0 % sequence similarity) and strain 301^T being most closely related to the type strain of *Methylotenera mobilis* (96.6 % sequence similarity).

Genome relatedness of the novel isolates to each other and to the most related organisms with sequenced genomes was investigated via *in silico* DNA–DNA hybridization. The details of this method are described in the Supplementary material (available in IJSEM Online). Only 41.3 % of the genes of strain 301^T were found to have ≥ 70 % identity

Fig. 2. Phylogenetic tree showing the relationship of isolates 301^T and SIP3-4 to representatives of the families *Methylophilaceae* and *Rhodocyclaceae* within the betaproteobacteria, based upon 16S rRNA gene sequences. The 16S rRNA gene sequence of *Methylophaga marina* ATCC 35842^T, a gammaproteobacterium, was used as outgroup. The tree was constructed for nearly full-length 16S rRNA gene sequences (1368–1371 bp) using the maximum-likelihood method (DNAML, PHYLIP version 3.69). Bootstrap analysis was performed with 1000 replicates. Bootstrap values over 50 % are shown. Strain collection accession numbers and GenBank accession numbers (in parentheses) are shown.

with the genes of the type strain *Methylothermobacter mobilis* JLW8^T. These data, along with physiological tests, suggest that strain 301^T represents a novel species within the genus *Methylothermobacter*, for which the name *Methylothermobacter versatilis* is proposed, in light of the versatile trophic abilities of the isolate.

Strain SIP3-4 was distantly related to both *Methylobacillus flagellatus* KT^T and *Methylothermobacter mobilis* JLW8^T, with 33.9% and 20.6% of genes sharing $\geq 70\%$ identity, respectively. As no genome sequence for a *Methylovorus* representative is available, the phylogenetic affiliation of strain SIP3-4 was determined based on phenotypic characteristics and 16S rRNA gene phylogeny, and the strain was assigned to the species *Methylovorus glucosotrophus*. The characteristics of the strain fit with the current description of the genus and the species (Doronina *et al.*, 2005).

Emended description of the genus *Methylothermobacter*

Methylothermobacter [Me.thy.lo.te'ner.a. N.L. *methylum* (from French *méthyle*, back-formation from French *méthylène*, coined from Gr. n. *methu* wine and Gr. n. *hulê* wood) the methyl group radical; N.L. pref. *methylo-* pertaining to the methyl radical; L. fem. adj. *tenera* delicate; N.L. fem. n. *Methylothermobacter* a methyl group-oxidizing delicate bacterium].

Gram-negative rods. Some strains are motile. Do not form resting bodies and multiply by binary fission. Utilize methylamine. In addition, may utilize methanol, betaine, fructose, ethanol and pyruvate as sole sources of carbon and energy. Some strains are positive for urease and nitrate reduction. Oxidize methylamine via methylamine dehydrogenase or via *N*-methylglutamate pathway and assimilate C1 units via the RuMP pathway. The major fatty acids are C_{16:1}ω7c and C_{16:0}. The DNA G+C content is in the range 42.6–45.5 mol%. Type species is *Methylothermobacter mobilis*.

Description of *Methylothermobacter versatilis* sp. nov.

Methylothermobacter versatilis (ver.sa'ti.li.s. L. fem. adj. *versatilis* versatile).

General characteristics are as for the genus. Facultative methylotroph, grows at pH 4.2–8.0, with optimum growth at pH 5.8–6.5. Temperature optimum is 18–21 °C. Cells are oval or rod-shaped, 1.0 ± 0.2 × 0.5 ± 0.2 µm in size, and may form small cell clusters. In addition to methylamine, may utilize fructose, betaine, methanol, pyruvate and ethanol. Grows on a complex medium containing 0.5% bacto-peptone, 0.05% yeast extract and vitamins. Ammonium, nitrate and urea may serve as sources of nitrogen. The major fatty acids are C_{16:1}ω7c and C_{16:0}.

The type strain is 301^T (=VKM B-2679^T=JCM 17579^T), isolated from sediment from freshwater Lake Washington (USA). The DNA G+C content of the type strain is 42.6 mol%.

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