Methyloversatilis thermotolerans sp. nov., a novel thermotolerant facultative methylothroph isolated from a hot spring

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A newly isolated facultatively methylothrophic bacterium (strain 3tT) was investigated. Cells of the isolate were Gram-stain-negative, non-motile rods that multiplied by binary fission. The strain utilized methanol, methylamine and a variety of multicarbon compounds as a carbon and energy source. Growth occurred at pH 6.5–8.5 (optimally at 7.0–7.5) and at 10–45 °C (optimally at 30–37 °C). The major fatty acids of methanol-grown cells were C16 : 1ω7c and C16 : 0. The predominant phospholipids were phosphatidylethanolamine and phosphatidylglycerol. The major ubiquinone was Q-8. Strain 3tT possessed pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase and assimilated C1 units at the level of formaldehyde and CO2 via the serine cycle. The DNA G+C content of the strain was 63.6 mol% (Tm). On the basis of 16S rRNA gene sequence similarity (98.1 %) and rather low DNA–DNA relatedness (30 %) with the type strain of the type species of the genus Methyloversatilis (Methyloversatilis universalis FAM5T), and physiological and biochemical characteristics, the isolate was classified as a representative of a new species of the genus and named Methyloversatilis thermotolerans 3tT (=VKM B-2692T=CCUG 61694T=DSM 25156T).

Strains of the genus Methyloversatilis are Gram-negative, non-motile rod-shaped and facultatively methylothetic bacteria able to grow on one-carbon compounds such as methanol or methylamine as the sole carbon and energy source, as well as on a wide range of multicarbon substrates (Kalyuzhnaya et al., 2006). Members of the genus Methyloversatilis have been identified as the dominant methanol- and ethanol-assimilating bacteria in lab scale denitrifying reactors using DNA stable isotope probing (Baytshtok et al., 2009). Methyloversatilis universalis FAM5T was used as a model organism to characterize the effects of growth on methanol and ethanol in anoxic chemostats (Lu et al., 2012). At the time of writing, the genus Methyloversatilis comprised the only recognized species, Methyloversatilis universalis FAM5T, isolated from Lake Washington (USA) sediment (Kalyuzhnaya et al., 2006). Here we describe isolation of pure culture of bacterial strain 3tT from a hot spring of the Uzon Caldera, Kamchatka (50 °C, pH 6.8, depth 10 cm) and its comparison with Methyloversatilis universalis FAM5T.

Abbreviations: PMS, Phenazine methosulfate; PQQ, pyrroloquinoline quinone.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA and mxaF gene sequences of strain 3tT are KC782839 and KC782840, respectively.

One supplementary figure is available with the online version of this paper.

A water sample (2 ml) was inoculated into a 400 ml flask containing 50 ml sterile medium K supplemented with methanol (0.5 %, v/v) and 0.2 ml vitamin mixture in methanol (1 μg each of B12, biotin and thiamine). Medium K contained (g l−1): KH2PO4, 2.0; (NH4)2SO4, 2.0; MgSO4.7H2O, 0.025; NaCl, 0.5; FeSO4.7H2O, 0.002; pH 7.2. The flask was incubated for 1 week at 40 °C with shaking (180 r.p.m.). In subsequent enrichments, 10 ml of the initial enrichment culture was diluted 1 : 10 (to a total of 50 ml) with the same medium. Flasks were incubated at 40 °C with shaking for 3 days. The suspension of the enrichment culture was plated to obtain single colonies onto K agar medium (2 % agar; Difco) with 0.5 % methanol. The isolated bacterial colonies were reincultured on methanol agar slants, transferred into liquid medium, and then again grown on a solid medium for purification. The reisolated bacterial colonies were reincultured on a slant agar. The purity of the isolated strain 3tT was controlled by light (ICM-405; OPTON) and electron (JEM-100B; JEOL) microscopy, as well as by the uniformity of colonies grown on K agar medium with methanol.

The type strain of the type species of the genus Methyloversatilis, Methyloversatilis universalis FAM5T (=VKM B-2588T=CCUG 52030T=JCM 13912T) was used as a reference strain. Cell morphology, Gram staining, motility and flagellation were studied for the isolate grown on K agar medium with methanol (0.5 %, v/v). Motility...
was observed by light microscopy (ICM-405; OPTON) and by negatively stained preparations. Negatively stained preparations and thin sections were imaged in a JEOL JEM-100B transmission electron microscope at operating voltages of 60 and 80 kV, respectively (Doronina et al., 1998). The bacterial strain was stored in liquid mineral medium for 1 month, on agar slants at 4 °C for 2 weeks or in a freeze-dried state with a protectant (skimmed milk) for over a year. Catalase activity was determined by bubble production in 3% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-p-phenylenediamine using a Bactident Oxidase strip (Merck). Other biochemical tests were performed by inoculating API 20NE and API 20E strips (bioMérieux) following the manufacturer’s instructions and incubating for 48 h at 28 °C. Indole production from 1 mM L-tryptophan was determined with Salkowski reagent (0.05M FeCl3 in 35% HClO4) on medium K, in which (NH4)2SO4 was replaced with 0.5% (w/v) KNO3. The temperature range for growth was estimated by growing the isolate in liquid K medium at different temperatures (4–50 °C). The effect of pH was investigated at an optimum temperature of 30 °C in the range pH 5.0–10.0. Various concentrations of methanol (0.1–7.0%, v/v) were tested in a liquid medium K. Halotolerance was tested by inoculating the cells into liquid medium K with various concentrations of NaCl (1–3%). Aliquots were taken at 12 h intervals and the OD600 was measured on a UV-1700 spectrophotometer (Shimadzu) in triplicate. The specific growth rate was calculated from increases in OD600 during the exponential growth phase. Utilization of a wide range of growth substrates was determined after cultivation on K medium for 2 weeks with methanol replaced by other carbon compounds and by using API 20E/20NE strips. Carbohydrates, organic acids, amino acids and methylated amines were added at concentrations of 0.05–0.3% (w/v), while alcohols were added at concentrations of 0.2–0.5% (v/v). To test alternative nitrogen sources, (NH4)2SO4 was replaced by the other nitrogen compounds (1%, w/v) such as peptone, nitrate, urea, glutamate and serine. This strain was not able to grow on mineral medium without nitrogen sources and could not reduce atmospheric nitrogen. A PCR amplification test using primers specific for the nifH gene (Fedorov et al., 2008) was negative in our strain, thus suggesting its inability to fix nitrogen. Methane utilization was tested under an atmosphere of methane and air (1:1, v/v) in flasks (700 ml) fitted with rubber stoppers and containing 100 ml mineral K medium. Autotrophic growth was tested by the same procedure, but under an atmosphere of H2/O2/CO2 (7:2:1, by vol.). Utilization of dichloromethane was tested as described previously (Doronina et al., 1995).

Enzyme assays of primary and intermediary pathways of C1 metabolism were done as described by Trotsenko et al., 1986. Enzyme activities were expressed as nmol of substrate transformed or the product formed in 1 min per mg of protein. Protein was determined by the method of Lowry et al. (1951).

For analysis of cellular fatty acids the strain was grown at 30 °C in liquid medium K. After 48 h of growth in exponential phase the cells were harvested by centrifugation (5000 g, 20 min), washed with distilled water and freeze-dried. The fatty acids in freeze-dried cells were extracted, saponified and esterified followed by GC-MS analysis of fatty acid methyl esters according to the instructions of the MIDI Sherlock MIS system (library TSBA6; version 6.0B) (Sasser, 1990).

Polar lipids were extracted from 500 mg cell biomass (exponentially growing cells) with chloroform/methanol (1:2, v/v) by stirring for 1 h in an ice bath, followed by centrifugation at 5000 g for 20 min; two phases were separated during this period. The extraction procedure was performed twice and the upper phases were collected and combined. Then, 2 ml chloroform and 2 ml distilled water were added to the supernatant and the resulting mixture was subjected to careful shaking for 15 min in an ice bath. The mixture was then centrifuged and three phases were separated. The lowest phase with polar lipids was withdrawn, evaporated at 30 °C and dissolved in 200 ml chloroform. Polar lipids were separated by two-dimensional TLC (Kieselgel 60, 10×10 cm; Merck) by using chloroform/methanol/water (65:25:4, by vol.) in the first direction, followed by chloroform/methanol/acetic acid/water (85:12:15:4, by vol.) in the second direction. Plates were sprayed with various specific reagents for detection of different polar lipids (Kates, 1972). Phospholipid standards from Sigma were used during comparative analysis.

DNA was isolated and purified according to Marmur (1961). The DNA G+C content was determined by using the thermal denaturing (Tm) method with a Beckman DU-8B spectrophotometer at a heating rate of 0.5 °C min−1 and calculation was made according to the protocol of Owen & Lapage (1976) using the equation: mol G+C=(Tm×2.08)−106.4. The DNA of Escherichia coli K-12 was used as the standard. DNA–DNA hybridization experiments were performed as described by De Ley et al. (1970). PCR-mediated amplification of the 16S rRNA gene was performed using primers described by Lane (1991). DNA sequencing was performed using ABI PRISM BigDye Terminator v. 3.1 reagents, followed by analysis of the reaction products in an automatic sequencer (3730 DNA Analyzer; Applied Biosystems). The preliminary screening for sequences similarities was done with BLAST (http://www.ncbi.nlm.nih.gov/blast). The 16S rRNA gene sequence determined was then aligned against those of closely related strains obtained from the recent GenBank releases using the CLUSTAL W software (Thompson et al., 1994). Positions of sequence uncertainty were omitted and a total of 1415 nt were used in the analysis. Phylogenetic relationships were determined by the neighbour-joining method and the programs of the TREECON package (version 1.3b) (Van de Peer & De Wachter, 1994), with bootstrap analysis of 1000 trees. Phylogenetic trees were also reconstructed using the maximum-likelihood algorithm implemented in the PHYLIP software package (Felsenstein,
The stability of the topology of the phylogenetic tree was assessed by using the bootstrap method with 1000 repetitions. A sequence similarity matrix was determined by using BioEdit 7.0.9.0 (Hall, 1999).

The \textit{mxaF} gene encodes the large subunit of the classical pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase, which catalyses the oxidation of methanol to formaldehyde in a majority of extant Gram-negative methylotrophic bacteria (Anthony & Williams, 2003). Using primers f1003 and r1561 and the protocol developed by McDonald & Murrell (1997) we amplified an approximately 550 bp \textit{mxaF} gene fragment from DNA of strain 3t\textsuperscript{T}.

The \textit{mxaF} gene amplicon was purified using a Promega column and it was sequenced on an automatic sequencer (3730 DNA Analyser; Applied Biosystems) by using ABI PRISM BigDye Terminator v. 3.1 reagents. Processing and translation of the nucleotide sequence to the amino acid sequence was performed by using Gene Runner (version 3.05; Hastings Software). The MxaF sequence determined (184 aa) was then aligned using the \textsc{Clustal W} software. Phylogenetic analyses were carried out by using the TREECON programs of the package with bootstrap analysis of 100 trees (Van de Peer & De Wachter, 1994). A phylogenetic tree based on \textit{mxaF} amino acid sequences showing the phylogenetic position of the new strain among methylotrophic bacteria was reconstructed by using the neighbour-joining method implemented by the TREECON software package (version 1.3b). A phylogenetic tree was also reconstructed by the maximum-likelihood method using the software package \textsc{MEGA} version 5 (Tamura et al., 2011).

Cells of the strain 3t\textsuperscript{T} were Gram-stain-negative, non-motile, non-sporulating, straight or slightly curved rods (0.3–0.4 × 1.2–2.6 \textmu m) (Fig. 1). Reproduction occurred by binary fission. After 60 h of incubation on solidified medium K with 0.5\% (v/v) methanol and vitamin B\textsubscript{12} (20 \textmu g l\textsuperscript{-1}) at 29 °C, colonies were white (turning brown with age), convex and circular with entire edges, 0.5–1.0 mm in diameter. The isolate required vitamin B\textsubscript{12} for growth. Strain 3t\textsuperscript{T} did not grow on complex media such as Luria–Bertani (LB) (Sambrook & Russell, 2001) or trypti-case soy broth or agar (Sigma). Tests for oxidase and catalase were positive. Negative for cellulase, pectinase and sugar fermentation. Strain 3t\textsuperscript{T} was able to grow on the following substrates as sole carbon and energy sources: methanol, methylamine, D-glucose, fructose, sucrose, maltose, lactose, raffinose, D-rhamnose, D-glucosamine, acetate, succinate, fumarate, acetone, ethanol, glycerol and L-serine. According to API 20NE tests, our strain was positive for nitrate reduction, indole production and assimilation of D-glucose, and negative for arginine dehydrolase, urease, \beta-glucosidase (ascellin hydrolysis), gelatinase, \beta-galactosidase (p-nitrophenyl \beta-D-galactopyr- anosidase) and assimilation of L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate, capric acid, adipic acid, malic acid and trisodium citrate. According to API 20E tests, our strain was positive for indole production and assimilation of D-glucose, L-rhamnose and sucrose, and all other reactions in this test were negative.

Strain 3t\textsuperscript{T} grew slowly on formate and formaldehyde at 0.05\% (w/v) and was unable to use sarcosine, methane, dichloromethane, citrate, glutamate or H\textsubscript{2}/CO\textsubscript{2}. The isolate grew in the presence of up to 5\% methanol, with maximal growth rate occurring at 0.05–0.5\% methanol, and used \((\text{NH}_4)\text{SO}_4\) and KNO\textsubscript{3} as nitrogen sources. Peptone, urea, glutamate and serine were not used as nitrogen sources. Growth occurred at pH 6.5–8.5 with optimum growth at pH 7.0–7.5. Strain 3t\textsuperscript{T} grew within a temperature range of 10–45 °C, with optimum growth at 30–37 °C.

Sensitivity to antibiotics was examined by placing Difco discs on cells spread on agar plates containing medium K. The effect of antibiotics on cell growth was assessed after 70 h. No inhibition zones were detected around the discs.
containing (μg ml⁻¹) erythromycin (15), penicillin (10), neomycin (30), chloramphenicol (30), oxacillin (5) or lincomycin (10). Strain 3tT was sensitive to novobiocin (30), gentamicin (10), kanamycin (30), nalidixic acid (30), streptomycin (10) and tetracycline (30).

The major cellular fatty acids of methanol-grown cultures of strains 3tT and FAM5T were C₁₆:₁ω7c (49–60 %) and C₁₆:₀ (29–32 %). 11-Methyl-C₁₈:₁ was detected only in strain 3tT (Table 1). Major phospholipids of strains 3tT and FAM5T were phosphatidylethanolamine and phosphatidylglycerol. Diphosphatidylglycerol and phosphatidylserine were also detected (Fig. 2). The major ubiquinone was Q-8. The DNA G+C content of strain 3tT was 63.3 mol% (Tₘ).

In order to establish the routes for primary C₁ metabolism in the isolated strain, enzymic activities were determined in extracts of cells grown in the presence of methanol. The strain possessed PQ-dependent methanol dehydrogenase, which required alkaline pH and ions for in vitro activity with phenazine methosulfate (PMS) as an artificial electron acceptor (Table 2). Two formaldehyde- and formate-oxidizing enzymes were present, i.e. NAD-dependent and PMS-linked. Formaldehyde and CO₂ were assimilated via the serine pathway as confirmed by the presence of hydroxypropane reductase and serine-glyoxylate aminotransferase. The ribulose monophosphate and ribulose bisphosphate pathways were not involved in primary C₁ assimilation due to the absence of the appropriate key enzymes (hexulose-phosphate synthase and ribulose-bisphosphate carboxylase). Primary assimilation of ammonia occurred by both reductive amination of α-ketoglutarate and the glutamate cycle (GS/GOGAT system).

A nearly complete 16S rRNA gene sequence (1415 nt) of strain 3tT was obtained. Sequence similarities to the related type strains were calculated using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The neighbour-joining phylogenetic tree (Fig. 3) reconstructed from 16S rRNA gene sequences showed that strain 3tT belongs to the genus *Methyloversatilis*; the strain showed 98.3 % 16S rRNA gene sequence similarity to *Methyloversatilis universalis* FAM5T.

**Table 1.** Cellular fatty acid compositions (% total fatty acids) of strain 3tT and *Methyloversatilis universalis* FAM5T grown on methanol for 48 h at 30 °C

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>3tT</th>
<th>FAM5T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₀:₀ 3-OH</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>C₁₂:₀</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>C₁₂:₀ 3-OH</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>C₁₄:₀</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>C₁₆:₁ω7c</td>
<td>49.8</td>
<td>59.7</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>29.5</td>
<td>31.8</td>
</tr>
<tr>
<td>C₁₇:₀ cyclo</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>C₁₈:₁ω7c</td>
<td>8.9</td>
<td>2.3</td>
</tr>
<tr>
<td>11-Methyl C₁₈:₁ω7c</td>
<td>7.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Comparison of the amino acid sequence of MxaF showed high levels of similarity between strain 3tT and *Methyloversatilis universalis* FAM5T (GenBank accession no. WP_020164049), *Methyloversatilis* spp. RZ18-153 (ACB73291) and FAM1 (Metunv1DRAFT_1436 PRJNA159997) (97.3 % sequence similarity), species of the genus *Methyllobacillus* (68.9–69.7 %), *Methylbipila capsulata* ATCC 700176T (CAL47581) (71.3 %), and species of the genus *Methylphilus* (67.7–67.9 %), as well as the species of the genus *Methyllobacterium*; *M. nodulans ORS 2060* (AF220764), *M. radiotolerans* JCM 2831T (YP_001756860), *M. extorquens* DSM 6343 (AI878068), *M. populi* BJ001T (YP_001927264) (Fig. S1). Other strains representing *Methyloversatilis universalis* FAM500 (Metunv3DRAFT_2315 ARWH01000001) and EH5g (Metunv2DRAFT_Scaffold1.1 NZ_KB900539.1) showed 51.4 % similarity to strain 3tT and probably have another type of PQQ-dependent methanol/ethanol dehydrogenase. The recently sequenced genome of *Methyloversatilis universalis* FAM5T helped to identify features of this organism related to the consumption of single- and multi-carbon compounds (Kittichotirat et al., 2011). As is known, *Methyloversatilis universalis* FAM5 possesses, together with three homologues of methanol dehydrogenase (XoxF1, XoxF2 and XoxF3), the PQQ-dependent methanol/ethanol dehydrogenase Medh2 system that is involved in the oxidation of both methanol and ethanol (Lu et al., 2012). Thus the phylogenetic tree based on amino acid sequences of MxaF supports the phylotypic position of strain 3tT among species of the genus *Methyloversatilis* (Fig. S1).

Strain 3tT shared several important characteristics with *Methyloversatilis universalis* FAM5T. These are chemo-organo-trophic and facultatively methylotrophic bacteria with the serine pathway of C₁ assimilation and their morphology, major phospholipids, cellular fatty acid profiles and quinone components are similar. But, we found at least seven different phenotypic characteristics between strains 3tT and FAM5T (Table 3). Based on phenotypic, genotypic and phylogenetic properties, strain 3tT is considered to represent a novel species of the genus *Methyloversatilis*, for which the name *Methyloversatilis thermotolerans* sp. nov. is proposed.

**Description of *Methyloversatilis thermotolerans* sp. nov.**

*Methyloversatilis thermotolerans* (ther.mo.to’lerans. Gr. n. thermē heat; L. part. adj. tolerans tolerating; N.L. part. adj. thermotolerans tolerant of heat).

Cells are Gram-stain-negative, non-motile rods (0.3–0.4 x 1.2–2.6 μm), that multiply by binary fission. Colonies on solidified medium K with 0.5 % (v/v) methanol and vitamin B₁₂ are white (turning brown with age), convex and circular with entire edges, 0.5–1.0 mm.

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Utilizes methanol and methylamine, as well as a variety of multi-carbon compounds (sugars and organic acids). Requires vitamin B12 for growth. Growth is not observed on complex media such as LB medium or trypticase soy medium, or in medium K with methane, dichloromethane, citrate or sacrosine. Growth is observed at 10–45 °C (optimal at 30–37 °C) and pH 6.5–8.5 (optimal at 7.0–7.5). No growth occurs in the presence of 3 % (w/v) NaCl. Tests for activities of oxidase and catalase and nitrate reduction are positive. Negative for pectinase, cellulase, protease, arginine dehydrolase, β-galactosidase and sugar fermentation. Produces indole from L-tryptophan on medium K with methanol as the sole carbon and energy source and under ammonium limitation. The major cellular fatty acids are C_{16:1}v_{7}c, C_{16:0}. The dominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol. The major ubiquinone is Q-8. C_{1} compounds are assimilated via the serine pathway. Methanol is oxidized to formaldehyde by methanol dehydrogenase. Resistant to erythromycin, penicillin, neomycin, chloramphenicol, oxacillin and lincomycin, but sensitive to novobiocin, gentamicin, kanamycin, nalidixic acid, streptomycin and tetracycline.

Table 2. Enzyme activities in a cell-free extract of the strain 3tT grown on methanol

No activity of the following enzymes was observed: ribulose-bisphosphate carboxylase/oxygenase, 3-hexulose-phosphate synthase, isocitrate dehydrogenase (NAD\(^+\)). Standard deviations of the data were ± 10 %.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor(s)</th>
<th>Enzyme activity [nmol min(^{-1}) (mg protein(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol dehydrogenase</td>
<td>PMS</td>
<td>110</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase</td>
<td>PMS</td>
<td>160</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>NAD(^+)</td>
<td>52</td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>NADH</td>
<td>420</td>
</tr>
<tr>
<td>Serine-glyoxylate aminotransferase</td>
<td>NADH</td>
<td>120</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>NADP(^+)</td>
<td>53</td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase</td>
<td>NAD(^+)</td>
<td>12</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>NADH</td>
<td>62</td>
</tr>
<tr>
<td>Glutamate synthase</td>
<td>NADH</td>
<td>13</td>
</tr>
<tr>
<td>Glutamine synthase</td>
<td>NADH</td>
<td>52</td>
</tr>
<tr>
<td>Glutamate synthase</td>
<td>ADP, Mn(^{2+})</td>
<td>44</td>
</tr>
</tbody>
</table>
The type strain, 3tT (=VKM B-2692T=CCUG 61694T=DSM 25156T), was isolated from the hot spring of the Uzon Caldera, Kamchatka (Russia). The DNA G+C content of the type strain is 63.6 mol% (Tm).

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**References**


