Methylophilus glucosoxydans sp. nov., a restricted facultative methylotroph from rice rhizosphere

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Two restricted facultatively methylotrophic strains, designed B^T and P, were isolated from rice roots. The isolates were strictly aerobic, Gram-negative, asporogenous, mesophilic, neutrophilic, motile rods that multiplied by binary fission and were able to synthesize indole-3-acetate. The cellular fatty acid profiles of the two strains were dominated by C_{16:0}, C_{16:1 ω 7c} and C_{16:0} 2-OH. The major ubiquinone was Q-8. The predominant phospholipids were phosphatidylethanolamine and phosphatidylglycerol. Cardiolipin (diphosphatidylglycerol) was absent. The two strains assimilated methanol carbon at the level of formaldehyde via the ribulose monophosphate cycle (2-keto-3-deoxy-6-phosphogluconate variant). They lacked α -ketoglutarate dehydrogenase and glutamate dehydrogenase. They assimilated ammonium via the glutamate cycle enzymes glutamine synthetase and glutamate synthase. The DNA G + C contents of strains B^T and P were 52.5 and 51.5 mol% (*T_m*), respectively. The level of DNA–DNA reassociation between these strains was 78 %, indicating that they belong to one species. Phylogenetic analysis of strain B^T based on 16S rRNA and methanol dehydrogenase (*mxhF*) gene sequences showed a high level of similarity to members of the genus *Methylophilus*. As the two isolates were clearly distinct from all recognized members of the genus *Methylophilus* based on phenotypic data and levels of DNA–DNA relatedness (30–46 %), they are considered to represent a novel species, for which the name *Methylophilus glucosoxydans* sp. nov. is proposed; the type strain is B^T (=VKM B-1607^T=CCUG 59685^T=DSM 5898^T).

The genus *Methylophilus* comprises non-halophilic, obligately and restricted facultatively methylotrophic bacteria having the ribulose monophosphate (RMP) pathway of formaldehyde assimilation. At the time of writing, the genus comprised five recognized species: *Methylophilus methylotrophus* (Jenkins *et al.*, 1987), *M. leisingeri* (Doronina & Trotsenko, 1994), *M. rhizosphaerae* (Madhaiyan *et al.*, 2009), and *M. flavus* and *M. luteus* (Gogleva *et al.*, 2010). The ability to grow on methanol is common to the type strains of these species. No NADP⁺-specific isocitrate dehydrogenase or glutamate dehydrogenase activities were observed for any *Methylophilus* species and diphosphatidylglycerol was absent from their phospholipid profile. However, these metabolic characteristics were not determined for *M. rhizosphaerae* CBMB127^T (Madhaiyan *et al.*, 2009).

Abbreviations: KDPG, 2-keto-3-deoxy-6-phosphogluconate; MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; RMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mxhF* gene sequences of strain B^T are HM001269 and HM001270, respectively.

One supplementary figure and one supplementary table are available with the online version of this paper.

We previously isolated two colourless restricted facultatively methylotrophic strains, designated B^T and P, from the rhizosphere of rice (*Oryza sativa* L.) sampled from Vietnam (Doronina *et al.*, 1988). Here, we describe the characterization of these strains, and show that they represent a single novel species of the genus *Methylophilus*.

Strains B^T and P were grown on 'medium K', containing (per litre): 2.0 g KH₂PO₄, 2.0 g (NH₄)₂SO₄, 0.025 g MgSO₄·7H₂O, 0.5 g NaCl and 0.002 g FeSO₄·7H₂O (pH 7.2). Solid medium K was prepared by adding 2.0 % (w/v) agar (Difco).

M. methylotrophus NCIMB 10515^T, *M. leisingeri* DSM 6813^T, '*M. quaylei*' VKM B-2338, *M. flavus* Ship^T (=VKM B-2547^T) and *M. luteus* Mim^T (=VKM B-2548^T) were used as reference strains. DNA from *M. rhizosphaerae* CBMB127^T was used for DNA–DNA hybridization experiments. Cell morphology, Gram staining and flagellation were studied in strains B^T and P grown on solid medium K with methanol (0.5 %, v/v). Motility was observed by light microscopy (OPTON.ICM-405) of exponentially growing cultures in liquid medium. Negatively stained preparations and thin sections were imaged by using a JEOL JEM-100B transmission electron microscope at operating voltages of 60 and 80 kV, respectively (Ivanova *et al.*, 2007). Biochemical tests

were carried out by using API 20NE, API 20 E and API 50CH strips (bioMérieux) following the manufacturer's instructions. Indole production from 1 mM L-tryptophan was determined with Salkowski reagent (0.05 M FeCl₃ in 35 % HClO₄) on mineral liquid medium K in which (NH₄)₂SO₄ was replaced with 0.5 % (w/v) KNO₃. The indole compounds from the culture medium of the novel isolates were identified by TLC and HPLC primarily as indole-3-acetic acid. Additionally, the auxin structure was confirmed based on MS spectra (Finnigan MAT 8430). Halotolerance was tested by inoculating the cells into liquid medium K with various concentrations of NaCl (0–3 %). Growth at 4–43 °C, various pH and with 0.5–5.0 % (v/v) methanol was tested in liquid medium K. Utilization of dichloromethane was tested as described previously (Doronina *et al.*, 2001). Enzyme assays were performed as described by Doronina & Trotsenko (1994). Fatty acid extraction from 40–60 mg cell mass was carried out by whole-biomass acid methanolysis with 0.4 ml of 1.2 M HCl in methanol by heating to 80 °C for 1 h. Resulting fatty acid methyl esters were extracted twice with 0.2 ml hexane and processed on an Agilent Technologies AT-5850/5973 GC-MS system with a cross-linked methyl silicone capillary column (HP-5ms). The oven temperature was programmed from 140 to 320 °C at 7 °C min⁻¹. Then, 1–2 µl derivatized sample was injected into the GC at 280 °C. Fatty acids and other lipid components were ionized by electron impact at 70 eV after separation in the GC column and were analysed in scan mode. The quadrupole mass spectrometer used has a resolution of 0.5 mass units over the whole mass range of 2–950 Da. The sensitivity of the GC-MS system was 0.01 ng methyl stearate. Each substance was confirmed by its mass spectrum and by NIST mass spectral database library searches. 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 with 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 µl 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. Ubiquinones were determined as described by Doronina *et al.* (2003).

DNA was isolated and purified according to Marmur (1961). The DNA G + C content was determined by thermal

denaturation (*T*_m) as described by Ivanova *et al.* (2007). DNA–DNA hybridization experiments were performed as described by De Ley *et al.* (1970). PCR-mediated amplification of the 16S rRNA gene was performed by using the primers described by Lane (1991). PCR was carried out as described previously (Doronina *et al.*, 2001). 16S rRNA gene sequencing was carried out with the same primers as described by Doronina *et al.* (2001). DNA sequencing was performed by using ABI PRISM BigDye Terminator v. 3.1 reagents, followed by analysis of reaction products on an automated ABI PRISM 3730 DNA sequencer (Applied Biosystems). Preliminary screening for similarity was done with the program BLAST (<http://www.ncbi.nlm.nih.gov/blast>). The 16S rRNA gene sequences determined were aligned against those of closely related strains obtained from GenBank by using the software CLUSTAL W (Thompson *et al.*, 1994). A total of 1387 nt was 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 100 trees. Phylogenetic trees were constructed by using various algorithms implemented in the TREECON and PHYLIP software packages (Felsenstein, 1989). A sequence similarity matrix was determined by using BioEdit 7.0.9.0 (Hall, 1999).

The *mxoF* gene encodes the large subunit of the classical pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase (MDH), which catalyses the oxidation of methanol to formaldehyde in the majority of extant Gram-negative methylotrophic bacteria (Anthony & Williams, 2003). Using primers f1003 and r1561 and the protocol developed by McDonald & Murrell (1997), we were able to amplify an approximately 550 bp *mxoF* gene fragment from DNA of strain B^T. The *mxoF* gene amplicons were purified by using Promega columns and were sequenced on a CEQ2000 XL automatic sequencer (Beckman Coulter) by using the enzyme CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter). Processing and translation of nucleotide sequences to amino acid sequences were performed by using Gene Runner (version 3.05) (Hastings Software). The *mxoF* gene sequence was aligned by using the software CLUSTAL W. Phylogenetic analyses were carried out by using the TREECON package (Van de Peer & De Wachter, 1994) with bootstrap analysis of 100 trees. Phylogenetic trees were constructed by using various algorithms implemented in the TREECON software package.

Cells of strains B^T and P were Gram-negative, asporogenous, motile rods (0.3–0.5 × 1.0–2.5 µm) with a single polar flagellum. Reproduction occurred by binary fission. The predominant cellular fatty acids of strains B^T and P were C_{16:1}ω7c (43–49 %) and C_{16:0} (34–35 %). C_{16:0} 2-OH and minor amounts of C_{10:0} 3-OH, C_{18:0} and C_{17:0} cyclo fatty acids were also detected (Supplementary Table S1, available in IJSEM Online). The fatty acid profiles of strains B^T and P were similar to those of members of the genus *Methylophilus* (Gogleva *et al.*, 2010). Data for the reference type strains were determined in the present study, except for *M. rhizosphaerae*

CBMB127^T, which are taken from Madhaiyan *et al.* (2009). As members of the genus *Methylophilus* have rather high generation times (0.3–0.5 h⁻¹) when grown in the presence of methanol (0.5–1.0 %, v/v), for analysis we used exponentially growing cells. In contrast, Madhaiyan *et al.* (2009) extracted cellular fatty acids from cultures grown for 4–5 days, i.e. in stationary growth phase, which may explain the differences in the proportions of some components. The major ubiquinone of strains B^T and P was Q-8. Analysis of cellular phospholipids revealed the presence of phosphatidylethanolamine, phosphatidylglycerol and a small amount of phosphatidylcholine (Supplementary Fig. S1, available in IJSEM Online). Cardiolipin (diphosphatidylglycerol) was not found. Strains B^T and P had a similar phospholipid composition.

The enzyme profiles of methanol-grown cells indicated that strains B^T and P oxidize methanol to formaldehyde by PQQ-linked MDH (Table 1). Direct oxidation of CH₂O via formate to CO₂ appears to be a minor route, as activities of formaldehyde dehydrogenase and formate dehydrogenase were low. Formaldehyde oxidation and assimilation through the RMP cycle were confirmed by the presence of 3-hexulose-phosphate synthase. Strains B^T and P showed the 2-keto-3-deoxy-6-phosphogluconate (KDPG) variant of the RMP

cycle as activity of KDPG aldolase was detected but activity of fructose-1,6-bisphosphate aldolase was absent. Glucose-6-phosphate dehydrogenase was active with both NAD⁺ and NADP⁺. Neither the serine nor the ribulose bisphosphate pathways of C₁ assimilation operate, as the appropriate specific enzymes, namely hydroxypyruvate reductase, serine-glyoxylate transaminase and ribulose bisphosphate carboxylase, were absent. The tricarboxylic acid cycle is incomplete given that these strains were deficient in α -ketoglutarate dehydrogenase. The absence of isocitrate lyase and malate synthase indicated that the glyoxylate shunt does not operate in strains B^T and P. They assimilate NH₄⁺ via the glutamate cycle, which was confirmed by the presence of glutamine synthetase and glutamate synthase. Activity of glutamate dehydrogenase was absent in the two strains.

The DNA G + C contents of strains B^T and P were 52.5 and 51.5 mol% (T_m), respectively.

In the phylogenetic tree derived from 16S rRNA gene sequences (Fig. 1), strain B^T consistently branched together with the cluster of *Methylophilus* species within the *Betaproteobacteria*. Relatively high levels of 16S rRNA gene sequence similarity (97.2–98.5 %) between strain B^T and the type strains of members of the genus *Methylophilus* showed

Table 1. Enzyme activities [nmol min⁻¹ (mg protein)⁻¹] in cell extracts of the novel strains grown on methanol

Standard deviations of the data were ± 10 %.

Enzyme	Cofactor*	Strain B ^T	Strain P
Methanol dehydrogenase	PMS	380	289
Formaldehyde dehydrogenase	PMS	0	0
	NAD ⁺	35	12
Formate dehydrogenase	PMS	28	16
	NAD ⁺	0	0
3-Hexulosephosphate synthase		180	190
Hydroxypyruvate reductase	NAD(P)H	0	0
Serine-glyoxylate transaminase	NAP(P)H	0	0
Ribulose-1,5-bisphosphate carboxylase		0	0
Glucose-6-phosphate dehydrogenase	NAD ⁺	530	440
	NADP ⁺	1780	1400
6-Phosphogluconate dehydrogenase	NAD ⁺	140	130
	NADP ⁺	180	130
Fructose-1,6-bisphosphate aldolase		0	0
6-Phosphofructokinase	ATP	0	0
KDPG aldolase		85	110
α -Ketoglutarate dehydrogenase	NAD ⁺	0	0
Isocitrate dehydrogenase	NADP ⁺	0	0
	NAD ⁺	178	148
Isocitrate lyase		0	0
Malate synthase		0	0
Glutamate dehydrogenase	NAD(P)H	0	0
Glutamate synthase	NADH	17	14
Glutamine synthetase	ATP, Mn ²⁺	27	25

*PMS, Phenazinmethosulfate.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain B^T among obligate and restricted facultative methylotrophs of the *Betaproteobacteria*. Numbers at branch points are bootstrap values based on 100 replicates. Bar, 5 nt substitutions per 100 nt.

a close relationship. Comparative analysis of amino acid sequences of MxaF confirmed these data, revealing high levels of similarity between strain B^T and *M. methylotrophus* ATCC 35528^T (98.2 %) as well as *M. flavus* VKM B-2547^T, *M. luteus* VKM B-2548^T and '*M. quaylei*' VKM B-2338 (97.0–97.1 %) (Fig. 2). The level of DNA–DNA relatedness between strains B^T and P was 78 %. Levels of DNA–DNA relatedness between the novel strains and *M. methylotrophus* NCIMB 10515^T, *M. leisingeri* DSM 6813^T, '*M. quaylei*' VKM B-2338, *M. rhizosphaerae* CBMB127^T, *M. flavus* VKM B-2547^T and *M. luteus* VKM B-2548^T were 30–46 % (results of three replicates; SD $\pm$ 3 %). Differential characteristics of strain B^T and the type strains of recognized *Methylophilus* species are summarized in Table 2.

On the basis of the data presented here, strains B^T and P are considered to represent a single novel species of the

genus *Methylophilus*, for which the name *Methylophilus glucosoxydans* sp. nov. is proposed. The formal taxonomic description is given below.

Description of *Methylophilus glucosoxydans* sp. nov.

Methylophilus glucosoxydans (glu.co.so.xy'dans. N.L. n. *glucosum* glucose; N.L. part. adj. *oxydans* oxidizing; N.L. part. adj. *glucosoxydans* oxidizing glucose).

Gram-negative rods that are 0.3–0.5 $\times$ 1.0–2.5 μ m in size. Cells multiply by binary fission and are motile by means of a single polar flagellum. Colonies on mineral salts/methanol agar are white and 2 mm in diameter. Strictly aerobic, restricted facultative methylotroph that utilizes only methanol and glucose as growth substrates. Able to grow at 10–34 $^{\circ}$ C, at pH 6.5–8.0 and with 0.05–0.50 % (w/v) NaCl

Fig. 2. Neighbour-joining tree based on MxaF amino acid sequences showing the phylogenetic position of strain B^T among methylotrophic bacteria. Numbers at branch points are bootstrap values based on 100 replicates. Bar, 5 amino acid substitutions per 100 amino acids.

Table 2. Differential characteristics of strain B^T and members of the genus *Methylophilus*

Strains, 1, B^T; 2, *M. flavus* VKM B-2547^T (data from Gogleva *et al.*, 2010); 3, *M. luteus* VKM B-2548^T (Gogleva *et al.*, 2010); 4, *M. methylotrophus* NCIMB 10515^T (Jenkins *et al.*, 1987); 5, *M. leisingeri* DSM 6813^T (Doronina & Trotsenko, 1994); 6, '*M. quaylei*' VKM B-2338 (Doronina *et al.*, 2005); 7, *M. rhizosphaerae* CBMB127^T (Madhaiyan *et al.*, 2009). No glutamate or NADP⁺-specific isocitrate dehydrogenases activities were observed for all strains and diphosphatidylglycerol was absent in the phospholipid profile; these characteristics were not determined for *M. rhizosphaerae* CBMB127^T. All strains were able to grow on methanol. Data were obtained under the same cultural conditions and by using standardized methodology except for *M. rhizosphaerae* CBMB127^T. ND, Not determined.

Characteristic	1	2	3	4	5	6	7
Flagellation	+	–	–	+	–	–	–
Colony pigmentation	White	Yellow	Yellow	Greyish white	Pale pink	White	White
Type of methylotrophy	Restricted facultative	Obligate	Restricted facultative	Restricted facultative	Restricted facultative	Obligate	Restricted facultative
Growth substrates							
Methylamine	–	–	–	+	–	–	+
Dimethylamine	–	–	–	+	–	–	–
Trimethylamine	–	–	–	+	–	–	+
Dichloromethane	–	–	–	–	+	–	+
Glucose	+	–	+	+	+	–	–
Fructose	–	–	–	–	–	–	+
Urease	–	–	–	+	+	+	ND
Acetoin production	–	+	+	–/+	–	ND	ND
Nitrate reduction	+	–	–	ND	ND	+	+
Fructose-1,6-bisphosphate aldolase	–	+	+	–	–	–	ND
Optimum temperature (°C)	28–30	19–24	24–26	30–37	30–35	25–29	28
Optimum pH	7.0–7.6	7.2–7.8	7.2–7.8	6.5–7.2	6.8–7.2	6.5–7.5	6.8
DNA G + C content (<i>T_m</i>) (mol%)	52.5	50.7	54.5	50.3	50.2	54.0	47.9
Isolation source	Rhizosphere of rice (<i>Oryza sativa</i> L.)	Phyllosphere of <i>Rosa</i> <i>cinnamomea</i> L.	Phyllosphere of <i>Tussilago farfara</i> L.	Activated sludge	Wastewater	Contaminant of <i>Methylocystis</i> <i>methanolicus</i>	Rhizosphere of rice (<i>Oryza sativa</i> L.)

(optimally at 28–30 °C, at pH 7.0–7.6 and with 0.05 % NaCl). Growth factors are not required. Urease- and oxidase-positive. Nitrate is reduced to nitrite. Produces indole (indole-3-acetic acid) from tryptophan on medium with nitrate as nitrogen source. Formaldehyde is assimilated via the RMP pathway (Entner–Doudoroff variant). Assimilates NH_4^+ via the glutamate cycle. The tricarboxylic cycle is incomplete due to the absence of α -ketoglutarate dehydrogenase. Glyoxylate shunt enzymes are absent. Nitrate and ammonia are used as nitrogen sources. The predominant cellular fatty acids are $\text{C}_{16:1\omega7c}$ and $\text{C}_{16:0}$. $\text{C}_{16:0}$ 2-OH and minor amounts of $\text{C}_{10:0}$ 3-OH, $\text{C}_{18:0}$ and $\text{C}_{17:0}$ cyclo are also found. The major ubiquinone is Q-8. The predominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol. Cardiolipin (diphosphatidylglycerol) is not found. Does not grow on peptone-yeast extract medium.

The type strain is B^T (=VKM B-1607^T=CCUG 59685^T=DSM 5898^T), isolated from rhizosphere of rice (*Oryza sativa* L.) sampled from Vietnam. The DNA G+C content of the type strain is 52.5 mol% (T_m). Strain P (=VKM B-1608), isolated from a similar source, is a second strain of the species.

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