Methylophilus flavus sp. nov. and *Methylophilus luteus* sp. nov., aerobic, methylotrophic bacteria associated with plants

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Novel yellow, obligately methylotrophic and restricted facultatively methylotrophic bacteria, respectively designated strains Ship^T and Mim^T, with the ribulose monophosphate pathway of C₁ assimilation are described. Cells were strictly aerobic, Gram-negative, asporogenous, non-motile rods that multiply by binary fission, were mesophilic and neutrophilic and synthesized indole-3-acetic acid and exopolysaccharide. The predominant cellular fatty acids were C_{16:0} and C_{16:1}. The major ubiquinone was Q-8. The predominant phospholipids were phosphatidylethanolamine and phosphatidylglycerol; diphosphatidylglycerol was absent. The two strains 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 Ship^T and Mim^T were 50.7 and 54.5 mol% (*T_m*), respectively. The level of 16S rRNA gene sequence similarity between these strains was very high (99.8 %) but they shared a low level of DNA–DNA relatedness (44 %). Based on 16S rRNA gene sequence analysis and low levels of DNA–DNA relatedness with the type strains of recognized species of the genus *Methylophilus* (31–36 %), strains Ship^T and Mim^T are considered to represent novel species of the genus *Methylophilus*, for which the names *Methylophilus flavus* sp. nov. (type strain Ship^T = DSM 23073^T = VKM B-2547^T = CCUG 58411^T) and *Methylophilus luteus* sp. nov. (type strain Mim^T = DSM 22949^T = VKM B-2548^T = CCUG 58412^T) are proposed.

Knowledge of the biodiversity of aerobic methanol-oxidizing bacteria has been extended significantly in recent years (Kolb, 2009). At the time of writing, the group of obligately and restricted facultatively methylotrophic methanol-utilizing bacteria with the ribulose monophosphate (RuMP) pathway of formaldehyde assimilation comprises four genera. Terrestrial obligately and restricted facultatively methylotrophic bacteria assigned to the genera *Methylobacillus* (Yordy & Weaver, 1977; Urakami & Komagata 1986), *Methylophilus* (Jenkins *et al.*, 1987) and *Methylovorus* (Doronina *et al.*, 2005a) are clearly

distinguished from marine methylotrophic bacteria belonging to the genus *Methylophaga* (Urakami & Komagata, 1987) based on physiological characteristics and DNA G + C content. Because of their similar morphology and metabolic organization, the main taxonomic criteria used to classify obligately and restricted facultatively methylotrophic bacteria are their genomic and phylogenetic characteristics.

Here, we describe two newly isolated methylotrophic strains, designated Ship^T and Mim^T, having the RuMP pathway and show that they represent two novel species of the genus *Methylophilus*.

Strains Ship^T and Mim^T were isolated from the phyllosphere of dog rose (*Rosa cinnamomea* L.) and coltsfoot (*Tussilago farfara* L.), respectively, sampled from the city park in Pushchino (Moscow region, Russia). The strains were grown on medium K [(l⁻¹): 2.0 g KH₂PO₄, 2.0 g (NH₄)₂SO₄, 0.025 g MgSO₄ · 7H₂O, 0.5 g NaCl and 0.02 g FeSO₄ · 7H₂O; pH 7.2]. Solidified medium K was prepared by adding 2.0 % (w/v) agar (Difco).

Representative members of the genus *Methylophilus*, namely *Methylophilus methylotrophus* NCIMB 10515^T,

Abbreviations: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA/*mxhF* gene sequences of strains Ship^T and Mim^T are FJ872108/FJ872110 and FJ872109/FJ872111, respectively. The accession numbers for the *mxhF* gene sequences of *M. methylotrophus* NCIMB 10515^T and '*M. quayle*' MT are FJ904267 and FJ904268.

Tables giving the cellular fatty acid compositions of strains Ship^T and Mim^T and related strains and levels of DNA–DNA relatedness between strains Ship^T and Mim^T and related strains, electron micrographs and a dendrogram based on deduced *MxhF* amino acid sequences are available as supplementary material with the online version of this paper.

M. leisingeri DSM 6813^T, '*M. quaylei*' MT and *M. rhizosphaerae* CBMB127^T, were used as reference strains. Cell morphology, Gram staining, motility and flagellation were studied for strains Ship^T and Mim^T grown on medium K agar with methanol (0.5%, v/v). Negatively stained preparations and thin sections were imaged on 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 by using Salkowski reagent on medium K in which (NH₄)₂SO₄ was replaced with 0.5% (w/v) KNO₃. The indole compounds from culture medium of the new isolates were identified by TLC and HPLC; the main component was indole-3-acetic acid. Additionally, auxin structure was confirmed by MS (Finnigan MAT 8430). Halotolerance was tested by inoculating cells into liquid medium K with various concentrations of NaCl (0–3%). Growth at different temperatures (4–43 °C), pH and methanol concentrations (0.5–5%, v/v) was tested in liquid medium K. Utilization of dichloromethane was tested as described previously (Doronina *et al.*, 1995). Sensitivity to antibiotics was examined by placing Difco discs on cells spread on agar plates. The discs contained the following antibiotics (µg): gentamicin (10), neomycin (30), streptomycin (10), ampicillin (10), nalidixic acid (30), lincomycin (2), oxacillin (10), neomycin (30) or novobiocin (5). The effect of antibiotics on cell growth was assessed after 2 days. Cellular fatty acids, polar lipids and ubiquinones were determined as described by Ivanova *et al.* (2007). The sugar composition of the extracellular polysaccharide was determined as described previously (Doronina *et al.*, 2003). DNA was isolated and purified according to Marmur (1961). The DNA G+C content was determined by using thermal denaturation (*T*_m) as described by Ivanova *et al.* (2007). DNA reassociation analysis was performed according to Johnson (1985). PCR amplification and sequencing of the 16S rRNA gene was carried out as described by Lane (1991). Preliminary screening for similarity was via BLAST searches (<http://www.ncbi.nlm.nih.gov/blast>). The 16S rRNA gene sequences of strains Ship^T and Mim^T were aligned against those of closely related strains obtained from GenBank by using CLUSTAL W software (Thompson *et al.*, 1994). Positions of sequence uncertainty were omitted and a total of 1430 and 1418 nt (strains Ship^T and Mim^T, respectively) were used in the analysis. Phylogenetic relationships were determined with the neighbour-joining method and programs within the TREECON package (version 1.3b) (Van de Peer & De Wachter, 1994), with bootstrap analysis based on 100 replicates. 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 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 *mxoF* gene fragment of approximately 550 bp from DNA of strains Ship^T and Mim^T, *M. methylotrophus* NCIMB 10515^T and '*M. quaylei*' MT. The *mxoF* gene amplicons were purified by using Promega columns and they 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, Inc.). The *mxoF* gene sequence determined was aligned by using CLUSTAL W software. Phylogenetic analyses were carried out by using programs in the TREECON package (Van de Peer & De Wachter, 1994) with bootstrap analysis based on 100 trees. Phylogenetic trees were constructed by using various algorithms implemented in the TREECON software package.

Cells of strains Ship^T and Mim^T were Gram-negative, asporogenous, non-motile rods (0.2–0.3 × 1.9–2 µm) that occurred singly, in pairs or in small groups (see Supplementary Fig. S1, available in IJSEM Online). Reproduction occurred by binary fission.

The predominant cellular fatty acids of strains Ship^T and Mim^T were C_{16:1ω7} (58.1 and 52.3%, respectively) and C_{16:0} (30.8 and 32.4%); 2-OH and 3-OH fatty acids were also found (Supplementary Table S1). The data indicated that strains Ship^T and Mim^T have cellular fatty acid profiles similar to those of recognized *Methylophilus* species. The major ubiquinone was Q-8. Analysis of cellular phospholipids revealed the presence of phosphatidylethanolamine and phosphatidylglycerol; diphosphatidylglycerol was not found. Strains Ship^T and Mim^T had similar phospholipid compositions.

The enzyme reaction profiles of methanol-grown cells indicated that strains Ship^T and Mim^T oxidize methanol to formaldehyde by PQQ-linked MDH (Table 1). Direct oxidation of formaldehyde via formate to CO₂ appears to be a minor route, as activities of formaldehyde dehydrogenase and formate dehydrogenase were low. Formaldehyde assimilation through the RuMP cycle was confirmed by the presence of 3-hexulose phosphate synthase. Remarkably, strains Ship^T and Mim^T have two variants of the RuMP cycle; activity of 2-keto-3-deoxy-6-phosphogluconate aldolase was detected along with activities of fructose-1,6-bisphosphate aldolase and ATP-dependent 6-phosphofructokinase. 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 (hydroxypyruvate reductase, serine-glyoxylate transaminase and ribulosebisphosphate carboxylase) were absent. The tricarboxylic acid cycle is incomplete, because these strains were deficient in α-ketoglutarate dehydrogenase. The absence of isocitrate lyase

Table 1. Enzyme activities in cell extracts of strains Ship^T and Mim^T grown on methanol

Enzyme	Cofactor(s)*	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Ship ^T	Mim ^T
Methanol dehydrogenase	PMS	159	140
Formaldehyde dehydrogenase	PMS	27	11
	NAD ⁺ , GSH	19	10
Formate dehydrogenase	PMS	29	17
	NAD ⁺	1	10
3-Hexulosephosphate synthase	–	187	110
Hydroxypyruvate reductase	NAD(P)H	0	0
Serine-glyoxylate transaminase	NAD(P)H	0	0
Ribulose-1,5-bisphosphate carboxylase	–	0	0
Glucose-6-phosphate dehydrogenase	NAD ⁺	362	650
	NADP	383	450
6-Phosphogluconate dehydrogenase	NAD ⁺	26	61
	NADP	16	42
Fructose-1,6-bisphosphate aldolase	–	82	74
6-Phosphofructokinase	ATP	3	2
	PP _i	0	0
Keto-3-deoxy-6-phosphogluconate aldolase	–	52	39
α-Ketoglutarate dehydrogenase	NAD ⁺	0	0
Isocitrate dehydrogenase	NADP	0	0
	NAD ⁺	12	10
Isocitrate lyase	–	0	0
Malate synthase	–	0	0
Glutamate dehydrogenase	NAD(P)H	0	0
Glutamate synthase	NADH	32	23
Glutamine synthetase	ATP, Mn ²⁺	19	60

*GSH, Glutathione; PMS, phenazine methosulfate; PP_i, pyrophosphate.

and malate synthase indicates that the glyoxylate shunt does not operate in strains Ship^T and Mim^T. They assimilate ammonium via the glutamate cycle, which was confirmed based on the presence of glutamine synthetase and glutamate synthase. Activity of glutamate dehydrogenase was absent in the two strains.

The DNA G+C content of strains Ship^T and Mim^T was 50.7 and 54.5 mol% (*T_m*), respectively (Table 2). In the phylogenetic tree derived from 16S rRNA gene sequences, strains Ship^T and Mim^T consistently branched with the *Betaproteobacteria* (Fig. 1). Relatively high levels of 16S rRNA gene sequence similarity (98.0–99.0%) were found between the novel strains and the type strains of recognized *Methylophilus* species, indicating their close relationship. Comparative analysis of deduced amino acid sequences of the MxaF protein confirmed these relationships; high levels of similarity were found between strains Ship^T and Mim^T and *M. methylophilus* NCIMB 10515^T (97.0–97.6%) as well as '*M. quaylei*' MT (97.6–97.8%) (Supplementary Fig. S2).

The level of DNA–DNA relatedness between strains Ship^T and Mim^T was only 44%. Levels of DNA–DNA relatedness between the novel strains and *M. methylophilus* NCIMB

10515^T, '*M. quaylei*' MT and *M. leisingeri* DSM 6813^T were 31–36% (Supplementary Table S2), suggesting that they are not affiliated at the species level. Phenotypically, strains Ship^T and Mim^T were similar to recognized members of the genus *Methylophilus*, being obligately and restricted facultatively methylotrophic rods, having the RuMP cycle of C₁ assimilation and activity of NADP-specific isocitrate dehydrogenase and lacking glutamate dehydrogenase and diphosphatidylglycerol in the phospholipid profile.

Strains Ship^T and Mim^T can be distinguished clearly from recognized members of the genus *Methylophilus* based on yellow colony pigmentation, the presence of fructose-1,6-bisphosphate aldolase, optimum temperature for growth and source of isolation. Strains Ship^T and Mim^T can be differentiated from *M. rhizosphaerae* CBMB127^T based on 16S rRNA gene sequence similarity (98.7 and 98.8%, respectively) as well as by their inability to utilize methane, ethanol, dichloromethane, methylamine, trimethylamine, fructose and succinate, and to hydrolyse gelatin (Madhaiyan *et al.*, 2009). Utilization of methane by *M. rhizosphaerae* CBMB127^T needs to be confirmed by the presence of well-developed intracellular membranes as well as the *pmoA* gene. In the formal taxonomic description of

Table 2. Characteristics of strains Ship^T and Mim^T and the type strains of recognized *Methylophilus* species

Strains: 1, Ship^T; 2, Mim^T; 3, *M. methylotrophus* NCIMB 10515^T (data from Jenkins *et al.*, 1987); 4, *M. leisingeri* DSM 6813^T (Doronina & Trotsenko, 1994); 5, '*M. quaylei*' MT (Doronina *et al.*, 2005b); 6, *M. rhizosphaerae* CBMB127^T (Madhaiyan *et al.*, 2009). All strains were negative for activity of glutamate dehydrogenase and NADP-specific isocitrate dehydrogenase and lacked diphosphatidylglycerol in the phospholipid profile (these characteristics were not reported for *M. rhizosphaerae* CBMB127^T). All strains were able to grow on methanol. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Flagellation	–	–	+	–	–	–
Colony pigmentation	Yellow	Yellow	Greyish white	Pale pink	White	White
Methylotrophy type*	O	RF	RF	RF	O	RF
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)	19–24	24–26	30–37	30–35	25–29	28
Optimum pH	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</i> _m) (mol%)	50.7	54.5	50.3	50.2	54.0	47.9
Isolation source	Phyllosphere (<i>R. cinnamomea</i>)	Phyllosphere (<i>T. farfara</i>)	Activated sludge	Wastewater	Contaminant of <i>Methylocystis methanolicus</i>	Rice rhizosphere

*O, Obligate; RF, restricted facultative.

M. rhizosphaerae, data regarding phospholipid composition and pathways of C₁ and nitrogen assimilation are absent, as are data for motility.

The characteristics of strains Ship^T and Mim^T and the type strains of recognized *Methylophilus* species are summarized in Table 2. Based on the data presented, strains Ship^T and Mim^T are considered to represent novel species of the genus *Methylophilus*, for which the names *Methylophilus flavus* sp. nov. and *Methylophilus luteus* sp. nov., respectively, are proposed.

Description of *Methylophilus flavus* sp. nov.

Methylophilus flavus (fla'vus. L. masc. adj. *flavus* yellow).

Obligately methanol-utilizing, Gram-negative, asporogenous, non-motile rods (0.2–0.3 × 1.9–2.0 µm) that occur singly, in pairs or in small groups. Reproduces by binary fission. Colonies on mineral salts/methanol agar are yellow, circular, 2–3 mm in diameter with a wavy edge, flat, semitransparent, shining and rough. Strictly aerobic, producing catalase and oxidase; urease-negative. Optimal growth occurs at 19–24 °C, at pH 7.2–7.8 and in the presence of 0.05 % (w/v) NaCl. Produces indole-3-acetic

acid from tryptophan on medium with nitrate as nitrogen source. Acetoin is produced, gelatin is not liquefied and H₂S is not produced. No vitamins or other growth factors are required. Oxidizes methanol by PQQ–MDH to formaldehyde and assimilates methanol via the RuMP pathway. The tricarboxylic cycle is incomplete; α-ketoglutarate dehydrogenase activity is absent. Glyoxylate shunt enzymes and ribulose-bisphosphate carboxylase are absent. Assimilates ammonium via the glutamate cycle. Nitrate and ammonia are used as nitrogen sources. The predominant cellular fatty acids are C_{16:0} and C_{16:1}. The major ubiquinone is Q-8. The predominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol; diphosphatidylglycerol is absent. Cells form extracellular polysaccharide consisting of rhamnose, glucose and mannose (2.59:47.9:51.21 %). Resistant to streptomycin and oxacillin, but sensitive to ampicillin, novobiocin, nalidixic acid, gentamicin, neomycin and lincomycin. Unable to grow on peptone-yeast extract medium. The DNA G + C content of the type strain is 50.7 mol% (*T*_m).

The type strain, Ship^T (=DSM 23073^T =VKM B-2547^T =CCUG 58411^T), was isolated from the phyllosphere of dog rose sampled from the city park in Pushchino, Moscow.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains Ship^T and Mim^T among obligate and restricted facultative methylotrophs of the *Betaproteobacteria*. Numbers at branch points are bootstrap values based on 100 replicates. Bar, 10 substitutions per 100 nucleotide positions.

Description of *Methylophilus luteus* sp. nov.

Methylophilus luteus (lu.te'us. L. masc. adj. *luteus* yellow).

Restricted facultative methylotroph that utilizes methanol and glucose. Gram-negative, asporogenous, non-motile rods (0.2–0.3 × 1.9–2 µm) that occur singly, in pairs or in small groups. Reproduces by binary fission. Colonies on mineral salts/methanol agar are yellow, circular, 3–4 mm in diameter with plane edges, convex, non-transparent and smooth. Strictly aerobic, producing catalase and oxidase; urease-negative. Optimal growth occurs at 24–26 °C, at pH 7.2–7.8 and in the presence of 0.05 % (w/v) NaCl. Produces indole 3-acetic acid from tryptophan on medium with nitrate as nitrogen source. Acetoin is produced, gelatin is not liquefied and H₂S is not produced. No vitamins or other growth factors are required. Oxidizes methanol by PQQ-MDH to formaldehyde and assimilates methanol via the RuMP pathway. The tricarboxylic cycle is incomplete; α-ketoglutarate dehydrogenase is absent. Glyoxylate shunt enzymes and ribulose-bisphosphate carboxylase are absent. Assimilates ammonium via the glutamate cycle. Nitrate and ammonia are used as nitrogen sources. The predominant cellular fatty acids are C_{16:0} and C_{16:1}. The major ubiquinone is Q-8. The predominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol; diphosphatidylglycerol is absent. Cells form large amounts of extracellular polysaccharide. Unable to grow on peptone-yeast extract medium. The DNA G+C content of the type strain is 54.5 mol% (T_m).

The type strain, Mim^T (=DSM 22949^T =VKM B-2548^T =CCUG 58412^T), was isolated from the phyllosphere of coltsfoot sampled from the city park in Puschino, Moscow.

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