***Methylopila capsulata* gen. nov., sp. nov., a novel non-pigmented aerobic facultatively methylo-trophic bacterium**

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A new genus, *Methylopila*, and one new species are described for a group of seven strains of facultatively methylo-trophic bacteria with the serine pathway of C₁ assimilation. These bacteria are aerobic, Gram-negative, non-spore-forming, motile, colourless rods that multiply by binary fission. Their DNA base content ranges from 66 to 70 mol% G+C. Their cellular fatty acid profile consists primarily of C_{18:1n7} *cis*-vaccenic and C_{19:0} cyclopropane acids. The major hydroxy acid is 3-OH C_{14:0}. The main ubiquinone is Q-10. The dominant cellular phospholipids are phosphatidylethanolamine and phosphatidylcholine. The new isolates have a low level of DNA–DNA homology (5–10%) with the type strains of the serine pathway methylobacteria belonging to the genera *Methylobacterium*, *Aminobacter*, *Hyphomicrobium* and *Methylo-rhabdus*. Another approach, involving 16S rRNA gene sequence analysis of strain IM1^T, has shown that the new isolates represent a separate branch within the α -2 subclass of the *Proteobacteria*. The type species of the new genus is *Methylopila capsulata* sp. nov., with the type strain IM1^T (= VKM B-1606^T).

Keywords: serine pathway methylo-trophs, *Methylopila capsulata* gen. nov., sp. nov.

INTRODUCTION

The group of aerobic Gram-negative asporogenous facultatively methylo-trophic bacteria (methylobacteria) having the serine pathway and capable of growth on a variety of reduced C₁ substrates, except methane, is very heterogeneous. However, so far only four of the previously described genera have been validly published: *Hyphomicrobium*, *Methylobacterium*, *Aminobacter* and *Methylo-rhabdus*. As known, the representatives of the genus *Hyphomicrobium* form hyphae and multiply by budding (Hirsch, 1984), the genus *Methylobacterium* was emended for the pink-pigmented facultative methylobacteria (PPFM) (Patt *et al.*, 1976; Bousfield & Green, 1985). The genus *Aminobacter* was proposed for non-pigmented, budding, methylamine-utilizing bacteria unable to grow on methanol (Urakami *et al.*, 1992) and the new genus *Methylo-rhabdus* was suggested for

colourless non-motile dichloromethane-utilizing bacteria (Doronina *et al.*, 1995). Nevertheless, it seemed evident that a taxonomic structure of the serine pathway facultative methylobacteria should include some new taxa to cover their phenotypic and genotypic diversity. Previously, we described three novel strains of colourless facultative methylobacteria having an intermediate position between the genera *Methylobacterium* and *Aminobacter* and proposed the generic name *Methylo-microbium* for these strains (Govorukhina *et al.*, 1989; Doronina & Trotsenko, 1992). Since the genus '*Methylo-microbium*' (Govorukhina *et al.*, 1989) has not been validated, this generic name was subsequently used for a group of Type I methanotrophs (Bowman *et al.*, 1995). In view of the fact that our extended study of '*Methylo-microbium capsulatus*' (Govorukhina *et al.*, 1989) showed that this organism did not belong to the genus *Methylo-microbium* (Bowman *et al.*, 1995) it would be appropriate to place this organism in a new genus, for which we propose the name *Methylopila*. Our studies of seven non-pigmented strains of facultatively methylo-trophic bacteria which utilize the serine pathway indicate that they should be placed in a single

Abbreviations: PMS, phenazine methosulfate; PPFM, pink-pigmented facultative methylobacteria.

The GenBank accession number for 16S rDNA sequence for *Methylopila capsulata* IM1^T is AF004844.

species, for which we propose the name *Methylopila capsulata*.

METHODS

Bacterial strains. Pure cultures of seven bacterial strains (IM1^T, UZ, 14, 9AA, 10AA, 26AA and 37AA) utilizing methanol and methylamine as well as some multicarbon compounds as growth substrates were isolated from soil samples taken from Uzbekistan (IM1^T, 14, UZ) and Kazakhstan (AA) as described earlier (Govorukhina *et al.*, 1989; Doronina & Trotsenko, 1992). *Methylobacterium organophilum* XX ATCC 27886^T was kindly supplied by R. S. Hanson (University of Minnesota, USA). K. Komagata (Tokyo University, Japan) provided *Protomonas* strains (Urakami & Komagata, 1984) that have been reclassified later into the genus *Methylobacterium* (Bousfield & Green, 1985) as *Methylobacterium extorquens* NCIB 9399^T (= TK 0001^T) and *Methylobacterium rhodesianum* NCIB 10611 (= TK 0016). *Hyphomicrobium zavarzinii* IFAM ZV-622^T was obtained from G. A. Zavarzin (Institute of Microbiology, Moscow, Russia). *Aminobacter aminovorans* ATCC 23314^T (= JCM 7852^T = NCIB 9039^T), *Aminobacter aminovorans* ATCC 23819 (strain MA = NCIB 11590) (Urakami *et al.*, 1992), *Methylobacterium multivorans* ATCC 51890^T (strain DM13^T = VKM B-2030^T) and *Pseudomonas aeruginosa* ATCC 10145^T were used as the reference strains.

The methylotrophic cultures were grown on a liquid mineral medium K, containing 2.0 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.5 g NaCl, 0.125 g MgSO₄ · 7H₂O, 0.002 g FeSO₄ · 7H₂O, and 1 l distilled water; the pH of this medium was adjusted to 7.2. Substrates were sterilized and added separately. Methanol or methylamine were added to a concentration of 0.5% (v/v) or 0.3% (w/v), respectively. Flasks were shaken at 29 °C. *Pseudomonas aeruginosa* was grown with 0.3% (w/v) glucose. The bacterial strains were maintained on medium K solidified with Difco Bacto agar or PYG agar (pH 7.0) (Urakami *et al.*, 1992).

Identification methods. Cell morphology, Gram staining, motility and flagellation were determined by using the cultures grown on solid medium K. Production of a fluorescent pigment was tested on King A and B agarized media. Nitrate reduction was tested in liquid medium K in which (NH₄)₂SO₄ was replaced with 0.5% (w/v) KNO₃ after 1, 3 and 5 d incubation. The methyl red and Voges-Proskauer (acetoin) reactions were tested in glucose/phosphate/peptone broth. Indole production was determined with Kovacs' reagent in 1% Tryptone broth. Hydrogen sulfide production was tested on triple-sugar iron agar for 2 weeks. Gelatin hydrolysis was observed in stab cultures in yeast extract-peptone medium containing 12% gelatin after 4 weeks incubation at 22 °C. Starch hydrolysis was determined by using an iodine solution on medium K agar containing 0.2% soluble starch after 1, 3 and 10 d incubation.

Ammonia production was tested in peptone water by Nessler's reagent. Oxidase activity test was performed with a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride. Urease activity was observed on Christensen's medium. Catalase activity was detected by pouring a 3% H₂O₂ solution into colonies on solid medium K or PYG agar. Halotolerance was tested by inoculating the cells into liquid medium K with various concentrations of NaCl (1–3%). Growth at different temperatures and pH values were tested in liquid medium K. Utilization of a wide range of growth substrates (> 50) was also determined in liquid

medium K after 2 weeks cultivation with methanol being replaced by the other carbon compounds. Organic acids and amino acids were added at concentrations of 0.05–0.3%, while carbohydrates and alcohols were added at concentrations of 0.2–0.5%. To test alternative nitrogen sources (NH₄)₂SO₄ was replaced by the other nitrogen compounds.

Methane utilization was tested in an atmosphere containing CH₄ and air (1:1, v/v) in 700 ml conical flasks containing 100 ml medium K and fitted with rubber stoppers. Hydrogen utilization was tested by the same procedure but in an atmosphere of H₂ + O₂ + CO₂ (7:2:1, by vol.). Utilization of mono- and dichloromethane was tested as described earlier (Doronina *et al.*, 1995). Liquid cultures were grown at 30 °C on a rotary shaker at 180 r.p.m.

Phospholipid and cellular fatty acid composition. Phospholipid composition of the cells was determined according to the methods described previously (Govorukhina & Trotsenko, 1989). To determine the cellular fatty acid composition the cultures were grown on methanol or methylamine with 2% agar medium K on Petri plates for 48 h at 29 °C. Several milligrams of cells were transferred with a loop to a screw-top microreaction jar containing 150 µl of a 20% aqueous tetramethylammonium hydroxide solution, the mixture was heated at 100 °C for 20 min and cooled to room temperature, and 100 µl of acetonitrile was added. The jar was shaken, 20 µl of the top (acetonitrile) layer was withdrawn and evaporated in a stream of nitrogen at room temperature, 20 µl 20% tetramethylammonium hydroxide solution was added, the mixture was heated at 100 °C for 1 min, and a 0.2–0.5 µl portion was introduced into the chromatograph injector (heated to 385 °C). The analysis was carried out in a Hewlett-Packard 5830A chromatograph, using a 15 m × 0.2 mm glass capillary column filled with silicone OV-101 sorbent.

Poly-β-hydroxybutyrate was analysed according to Braunneg *et al.* (1978) by using a Pye Unicam 104 gas chromatograph. Ubiquinones were extracted and purified according to Collins (1985). Their analysis was done by using a Finnigan MX-1310 mass spectrometer. Enzyme assays were done as previously described (Meiberg & Harder, 1978; Trotsenko *et al.*, 1986; Doronina *et al.*, 1995).

Electron microscopic analysis. To obtain thin sections, bacterial cells were fixed for 1 h at 4 °C in 0.05 M sodium cacodylate buffer (pH 7.2) containing 1.2% (v/v) glutaraldehyde, washed three times in the same buffer and refixed in 1% (w/v) OsO₄ in 0.05 M cacodylate buffer for 4 h at 4 °C. After dehydration in a series of alcohols, the cells were embedded in Spurr epoxy resin and sectioned with an LKB 2128 Ultratome. The thin sections were stained with 1% uranyl acetate and then with 2% lead citrate for 10 min and mounted on copper grids. To obtain negatively contrasted preparations, the cells were stained in 0.3% (w/v) phosphotungstic acid (pH 7.2). Micrographs were taken with a JEM 100B transmission electron microscope at an operating voltage of 60 kV.

DNA isolation and characterization. DNA was isolated and purified according to Marmur (1961). DNA G+C contents were determined by the thermal denaturation method with a Beckman DU-8B spectrophotometer at a heating rate of 0.5 °C min⁻¹ and were calculated according to Owen & Lapage (1976). The DNA from *Escherichia coli* K-12 was used as the standard. DNA-DNA hybridization was done on nitrocellulose membrane filters (0.22 µm) at optimal conditions (incubation in 50% formamide at 50 °C for 24 h with the use of deoxy [1',2',5'-³H]CTP and the nick trans-

lation kit N 5500 (Amersham) as described earlier (Doronina *et al.*, 1987).

16S rDNA sequence determination and analysis. Genomic DNA was extracted and the 16S rRNA gene was selectively amplified by PCR, using the following primers: 5'-AGAG-TTGATCCTGGCTCAG-3' as forward and 5'-TACGG-TTACCTTGTTACGACTT-3' as the reverse (Lane, 1991). The PCR reaction was carried out in a final volume of 100 µl, with 1 µg DNA template, 200 µM each primer, 200 µM each dNTP, 3 U Tet-z polymerase (BioMaster, Russia) and reaction buffer (100 mM Tris/HCl pH 8.3, 500 mM KCl, 2 mM MgCl₂). The temperature cycling was done by using 30 cycles of the following thermal profile: 1 min at 94 °C, 1 min at 42 °C, 1 min at 72 °C. The final extension was carried out at 72 °C for 6 min. The PCR products were purified using the PCR-prep kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene was sequenced in both directions with the use of forward and reverse primers listed by Lane (1991). DNA sequencing was carried out by using Sequenase version 2 of the VSB kit (USB) with minor modifications.

Phylogenetic analysis. The sequence of strain IM1^T was aligned manually with sequences obtained from the database of small subunit rRNAs (Larsen *et al.*, 1993). The sequence was compared with members of the *Proteobacteria*: *Methylobacterium agile* ACM 3308^T, X72767; *Methylococcus capsulatus* Texas^T, X72770; *Methylobacter luteus* VKM-53B^T, M95657; *Methylomonas methanica* ACM 3307^T, RDP 653; *Methylophaga marina* ATCC 35842^T, X87338; *Pseudomonas aeruginosa* ATCC 25330, M34133; *Methylophilus methylotrophus* NCIB 10515^T, L15475; *Methylobacillus glycogenes* ATCC 29475^T, M95652; *Rhodobacter sphaeroides* IFO 12203, D16425; *Paracoccus denitrificans* LMG 4218^T, X69159; *Hyphomicrobium vulgare* ATCC 27500, X53182; *Methylosinus trichosporium* OB3b^T, M29024; *Methylocystis parvus* OBBP^T, M29026; *Rhodospseudomonas viridis* ATCC 19567, D25314; *Blastobacter denitrificans* LMG 8443, X66025; *Methylobacterium organophilum* NCIMB 11278^T, D32226; *Xanthobacter agilis* SA35, D16425; *Blastobacter capsulatus* ATCC 43294, X73042; *Brucella abortus* 11/19, X13695; *Acidomonas methanolica* IMET 10945^T, D30770; *Bosea thiooxidans* BI-42^T, X81044; *Ancylobacter aquaticus* DSM 101^T, M27803; *Thiobacillus novellus* IAM 12100, D32247; and *Bacillus subtilis* 168, X00007, as outgroup. Regions that were either not sequenced in one or more reference organisms or had alignment uncertainty, i.e. positions 1–45, 70–100, 183–218, 451–480, 998–11048 and 1376–3' terminus (*E. coli* nomenclature) were omitted from the analyses, so in total 1078 nucleotides were used in the analysis. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by use of the correction of Jukes & Cantor (1969). A tentative unrooted phylogenetic tree was constructed by using a 'maximum topological similarity' algorithm (Chumakov & Yushmanov, 1988) and the program TREE of the GENEBEE package (Brodsky *et al.*, 1991). The rooted-by-outgroup phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with bootstrap analysis of 100 trees and using the TREECON package programs (Van de Peer & De Wachter, 1994).

RESULTS

Morphology

The bacterial isolates were represented by Gram-negative, asporogenous, colourless rods (0.5–0.7) ×

Fig. 1. Electron micrographs of cells of strain IM1^T. (a) Negatively stained preparation. Note the subpolar location of the single flagellum; a large amount of capsular material is evident. (b, c) Ultrathin sections; different stages of cell division. The arrow in (b) indicates a part of the capsule covered with spikes. (c) Constrictions in the final stage of cell division. Bars, 0.5 µm.

(0.8–1.5) µm in size, with a cell shape sometimes approaching coccoid. Reproduction occurred by binary fission. The separating daughter cells were equal in size to the parent cells and had one lateral flagellum (Fig. 1a). After division, the cells remained

Table 1. Cellular fatty acid composition of colourless and pink-pigmented serine pathway methylobacteria (% of total fatty acids)

Bacteria were grown on agarized synthetic medium with 0.5% (v/v) methanol or 0.3% (w/v) methylamine for 24 h at 29 °C.

Strain	n16:0	16:1*	n18:0	c18:1 ω 9	c18:1 ω 7	t18:1 ω 7	cy19:0	cMe18:1	Hydroxy acids 3-OH†		
									C _{12:0}	C _{14:0}	C _{16:0}
IM1 ^T	5.5	0.6	4.5	1.0	68.6	7.8	8.4	1.4			1.2
UZ	2.8	1.1	3.4	0.7	67.6	4.8	15.1	1.3			1.3
14	3.8	1.1	3.6	0.7	65.3	6.8	14.1	1.2			1.2
<i>Aminobacter aminovorans</i> ATCC 23314 ^T	9.8	1.2	7.6	0.6	55.7	6.5	3.1	0	0.4		
<i>Methylobacterium multivorans</i> ATCC 51890 ^T	15.4	1.8	4.0	0.8	52.8	8.4	16.7	3.1			1.1
<i>Methylobacterium organophilum</i> ATCC 27886 ^T	2.8	0.4	7.7	0.7	76.8	9.4	0.3	0			0.8
<i>Methylobacterium extorquens</i> NCIB 9399 ^T	5.4	7.0	9.4	1.3	66.2	8.3	0.8	0			0.5
<i>Methylobacterium rhodesianum</i> NCIB 10611 ^T	4.5	0.6	8.3	1.0	73.7	8.6	0.3	0			1.0
<i>Hyphomicrobium zavarzinii</i> IFAM ZV-622 ^T	4.7	0.9	8.1	0.6	80.4	5.4	5.0	0		2.3	1.7

* Total hexadecenoic acids shown, including palmitoleic, palmitelaidinic and 16:1 with position of double bond not established.

† Analysed as methoxy derivative form.

connected by a constriction apparently formed by the outer membrane (Fig. 1b, c). The bacteria studied had a distinct capsule covered with spikes (Fig. 1a).

Physiological characteristics

Colonies on glucose-potato agar (2 d) were punctate, round, convex, lustrous, white, translucent, uniform in consistency and mucoid. Streak cultures on meat-peptone agar were distinct and white, with even edges, a smooth surface and a mucoid consistency. Growth was moderate and odourless. Growth on agarized medium with methanol was profuse, diffuse and mucoid, and the colonies had even edges and a smooth surface. The strains hydrolysed starch and gelatin (weakly), but not cellulose and casein milk, although they caused the latter to become alkaline (pH 6.5–8.8). Acidification was observed on glucose-containing medium without gas formation. All strains were strict aerobes, although they reduced nitrates to nitrites slowly (2 weeks). They did not form indole or ammonia, but evolved hydrogen sulfide on meat-infusion broth. Methyl red and Voges–Proskauer tests were negative. No water-soluble fluorescent pigment was produced on King A and B media. Urease and oxidase were present, catalase activity was very low.

The strains grew well on mineral salt liquid medium with methanol (optimal concentration 0.5%) or methylamine (0.3%) as the carbon and energy sources. Additional growth factors (yeast autolysate, biotin, thiamin, nicotinic acid and riboflavin) were not required. Bacterial growth was also supported by some alcohols (ethanol, butanol, glycerol and sorbitol), organic acids (pyruvic, malic, succinic and fumaric acids), carbohydrates (maltose, glucose, fructose, sucrose and arabinose), as well as some amino acids (glutamate and sarcosine). Strains IM1^T, UZ, 9AA, 10AA, 26AA and 37AA did not grow on methane, mono- and dichloromethane, citrate, methionine, asparagine, lysine, arginine, histidine, glycine, valine,

betaine or under the gas mixture of CO₂ + H₂ + O₂. Strain 14 also failed to grow on butanol, sorbitol and fructose, but could utilize *N,N*-dimethylglycine. All strains utilized methylamine, some amino acids, urea, peptone, ammonium salts and nitrates as nitrogen sources. The optimum growth temperature was 28–30 °C, while pH optimum was 7.0–7.2. Growth did not occur in the presence of 3% NaCl. The generation time was 7–12 h on medium K with 0.5% CH₃OH.

Chemotaxonomic characteristics

Table 1 shows cellular fatty acid profiles for the new isolates and members of the genera *Methylobacterium*, *Aminobacter*, *Methylobacterium* and *Hyphomicrobium*. The methylation of *cis*-vaccenic acid (c_{18:1 ω 7}) to lactobacillic acid (cy_{19:0}) must be considered as a remarkable difference between colourless methylobacteria and the PPFMs. The virtual absence of lactobacillic acid in the fatty acid spectra of the PPFMs has also been reported by other authors (Urakami & Komagata, 1984). Another feature of the fatty acid spectra of all the strains is the occurrence of *cis*-methyloctadec-11-enoic acid, an isomer of lactobacillic acid which is also formed by methylation of *cis*-vaccenic acid. This fatty acid is synthesized by some Gram-negative stalked bacteria (*Prosthecomicrobium*, *Caulobacter*, *Asticacaulis*), but it is completely absent in members of the genera *Methylobacterium*, *Aminobacter* and *Hyphomicrobium* (Andreev *et al.*, 1986). The PPFMs, *Methylobacterium multivorans* and the new isolates contained primarily 3-OH C_{14:0} acid, whereas *Hyphomicrobium* spp. contained 3-OH C_{16:0} and *Aminobacter* strains contained large amounts of 3-OH C_{12:0} acid (Urakami *et al.*, 1992). Remarkably, *Hyphomicrobium zavarzinii* had ubiquinone Q-9, while all the other serine pathway methylobacteria possessed ubiquinone Q-10.

As seen from Table 2, the major phospholipids in the serine pathway facultative methylobacteria are phosphatidylethanolamine (12–50%) and phospho-

Table 2. Phospholipid composition of colourless and pink-pigmented serine pathway methylobacteria

Abbreviations: PEA, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; LL, lysolycetene; 8–12, unidentified phospholipids.

Strain	Phospholipid (%)											
	PEA	PG	DPG	PA	PS	PC	LL	8	9	10	11	12
IM1 ^T	40	12	8	3	1	18	7	5	0	0	5	0
UZ	42	11	8	3	0	17	6	4	0	0	6	0
14	23	20	5	3	0	36	4	2	2	2	1	2
<i>Aminobacter aminovorans</i> ATCC 23314 ^T	37	16	10	2	0	25	1	1	2	0	5	3
<i>Methylobacterium multivorans</i> ATCC 51890 ^T	24	13	4	1	0	43	2	2	2	1	3	3
<i>Methylobacterium organophilum</i> ATCC 27886 ^T	12	21	5	2	1	47	6	2	0	1	1	1
<i>Methylobacterium extorquens</i> NCIB 9399 ^T	26	14	4	2	3	35	2	2	0	4	4	4

Table 3. Enzyme activities in cell extracts of the new isolates of methylobacteria grown on different substrates

PMS, phenazine methosulfate; PEP, phosphoenolpyruvate. There was no activity with any of the following enzymes and cofactors: formaldehyde dehydrogenase (NAD⁺), formate dehydrogenase (NAD⁺), isocitrate dehydrogenase (NAD⁺), isocitrate lyase and pyruvate carboxylase.

Enzyme (cofactor)	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]					
	Strain IM1 ^T		Strain UZ		Strain 14	
	Methanol	Succinate	Methanol	Succinate	Methanol	Succinate
Methanol dehydrogenase (PMS)	362	118	250	71	310	102
N-Methylglutamate dehydrogenase (PMS)	0*	0	0*	0	0*	0
Methylamine dehydrogenase (PMS)	226*	21	180*	14	210*	22
Formaldehyde dehydrogenase (PMS)	77	9	68	7	82	10
Formate dehydrogenase (PMS)	129	22	98	11	135	15
Hydroxypyruvate reductase (NADH)	1930	497	1910	329	2100	310
Hydroxypyruvate reductase (NADPH)	759	387	720	250	815	200
Serine-glyoxylate aminotransferase (NADH)	237	90	240	52	330	100
Malate lyase (ATP, CoA)	52	19	49	15	67	11
Glycerate kinase (ATP)	28	12	34	10	35	12
Pyruvate dehydrogenase (NAD ⁺)	47	63	52	74	49	76
Citrate synthase	10	35	15	55	11	65
Isocitrate dehydrogenase (NADP ⁺)	41	72	49	84	56	90
Malate dehydrogenase (NADH)	51	215	68	290	70	325
Malate dehydrogenase (NADPH)	55	94	72	110	69	120
α-Ketoglutarate dehydrogenase (NAD ⁺)	8	14	8	17	10	21
Succinate dehydrogenase (PMS)	27	88	30	85	25	76
PEP carboxylase						
+ Acetyl-CoA	88	31	85	28	90	27
– Acetyl-CoA	39	19	35	15	42	15

* Methylamine-grown cells.

tidylcholine (17–47%). Strains IM1^T, UZ, 14, 9AA, 26AA and 34AA have been shown (Trotsenko *et al.*, 1993) to accumulate a large amount of poly-β-

hydroxybutyrate (20–40%, dry cell weight) under nitrogen limitation and excess of carbon source (methanol).

Table 4. DNA–DNA relatedness between colourless and pink-pigmented serine pathway methylbacteria

Strain	Hybridization (%) with labelled DNA from strains:					
	IM1 ^T	UZ	ATCC 23314	ATCC 51890	ATCC 27886	NCIB 9399
IM1 ^T	100	78	5	5	4	4
UZ	83	100	5	5	3	3
14	80	88	6	4	3	4
9AA	78	76	6	5	3	3
10AA	86	81	5	5	3	3
26AA	80	78	4	5	3	3
37AA	80	82	5	5	3	3
<i>Aminobacter aminovorans</i> ATCC 23314 ^T	4	4	100	4	3	3
<i>Methylobacterium multivorans</i> ATCC 51890 ^T	5	5	5	100	4	3
<i>Methylobacterium organophilum</i> ATCC 27886 ^T	5	4	5	4	100	16
<i>Methylobacterium extorquens</i> NCIB 9399 ^T	5	5	4	3	15	100
<i>Hyphomicrobium zavarzinii</i> IFAM ZV-622 ^T	4	4	4	4	3	3

Metabolic characteristics

To establish the routes for primary and intermediary metabolism in our isolates, the enzyme activities were determined in extracts of cells grown on methanol, methylamine or succinate. Since similar results were obtained for all the isolates tested the enzyme activities are presented for only three strains. As shown in Table 3 they oxidize methanol and methylamine to CO₂ through formaldehyde and formate by the appropriate dehydrogenases active with phenazine methosulfate (PMS). The enzymes of the *N*-methylglutamate pathway were not detected. The serine pathway specific enzymes (hydroxypyruvate reductase and serine-glyoxylate aminotransferase) were found. Due to the absence of the isocitrate lyase (ICL) activity the strains consequently employed the ICL-negative variant of the serine pathway. Alternatively, the key enzymes of the ribulose monophosphate and ribulose biphosphate pathways, i.e. 3-hexulose phosphate synthase, ribulose biphosphate carboxylase and phosphoribulokinase were not present. The new isolates had low or zero activities for the pentose phosphate pathway enzymes (glucose-6-phosphate and 6-phosphogluconate dehydrogenases as well as 2-keto-3-deoxy-6-phosphogluconate aldolase). However, they contained a complete set of enzymes of the tricarboxylic acid cycle playing primarily a biosynthetic role during methylotrophic growth. CO₂ was fixed by phosphoenolpyruvate carboxylase stimulated by acetyl-CoA. Pyruvate carboxylase was absent.

Genomic characteristics

The G + C contents of the DNA of strains IM1^T, UZ, 14, 9AA, 10AA, 26AA and 37AA were estimated via melting point (*T_m*) as 66.5–69.2 mol %. Because DNA hybridization remains the optimal method for measuring the degree of relatedness between highly related organisms (Stackebrandt & Goebel, 1994), the

degree of genomic similarity between the strains and members of the other taxa was assessed by DNA–DNA hybridization (Table 4). The DNA hybridization values among the new strains were very high (76–90 %), making it possible to assign all of them to the same taxonomic cluster (genus and species). On the other hand, they had a low degree of DNA hybridization with the representatives of the genera *Methylobacterium*, *Aminobacter*, *Hyphomicrobium*, *Methylobacterium* and *Pseudomonas*.

Comparative sequence analysis

The 1331 nucleotides of the strain IM1^T 16S rRNA gene were sequenced, corresponding to positions 8–1406 of the *E. coli* sequence. The phylogenetic analysis employing different algorithms showed similar results. The branching pattern of the methylotrophic strains correlated perfectly with previous results of Bratina *et al.* (1992) and Janvier & Grimont (1995). According to our data strain IM1^T appears to be related to the members of the α -subclass of *Proteobacteria*. The affiliation of this strain to the α -subclass is supported by signature analysis. Some features characteristic for the 16S rDNA molecule of the α -subclass, such as a shortened helix in nucleotide positions 184–193 (*E. coli* numbering) and all specific signature positions (Woese, 1987) were found in the sequences analysed.

The matrix of full sequence similarity values for strain IM1^T and representative members of the *Proteobacteria* indicate that for strain IM1^T the highest degree of relationship is found with certain members of the α -2 subclass, e.g. *Xanthobacter agilis* (92.5 % similarity) and *Brucella abortus* (92.7 % similarity). These values, however, are not necessarily an indication of specific relationship with these organisms, because they belong to different clusters of the α -2 subclass of *Proteobacteria*. In the unrooted tree constructed (data not shown), strain IM1^T forms a

Fig. 2. Rooted-by-outgroup phylogenetic tree showing relationships among *Methylopila capsulata* strain IM1^T and other members of the Proteobacteria including methylotrophic genera. *Bacillus subtilis* is the outgroup. Numbers refer to bootstrap values (more than 50). Bar indicates a distance of 0.05.

separate branch within the α -2 subclass. In the rooted-by-outgroup tree (Fig. 2), the bootstrap value is too low (Woese, 1987) for the branching point of strain IM1^T. Hence, strain IM1^T is a new branch of the α -2 subclass of Proteobacteria.

DISCUSSION

The new isolates were distinguished clearly from members of the genus *Pseudomonas sensu stricto*, on the basis of the following characteristics: utilization of C₁-compounds (methanol and methylated amines), quinone system, cellular fatty acid composition (Urakami *et al.*, 1992), and low level of DNA–DNA hybridization. As mentioned above, only four generic names have been validly published for Gram-negative serine pathway methylobacteria: *Methylobacterium* (Patt *et al.*, 1976), *Aminobacter* (Urakami *et al.*, 1992), *Hyphomicrobium* (Hirsch, 1984), and *Methylorhabdus* (Doronina *et al.*, 1995). However, our isolates differed from the PPFMs belonging to the genus *Methylobacterium* by some morphological features, colour of colonies, hydrolysis of gelatin and starch, cellular fatty acid composition and low DNA similarity. The new strains were distinguished from non-pigmented members of the genus *Aminobacter* by methanol utilization, primary C₁-metabolism, cellular fatty acid composition, DNA G+C content and low DNA similarity levels. They also differed from members of the genus *Hyphomicrobium* by their inability to form hyphae, quinone system, primary methylamine metabolism, cellular fatty acid composition and low DNA similarity and from *Methylorhabdus multivorans* by morphological features, the presence of oxidase and low DNA similarity. Based on these results, our isolates are close neighbours and might be placed in a separate taxon, for which we propose the generic name *Methylopila*, with the type species *Methylopila capsulata*. The major characteristics differentiating the genus *Methylopila* from the other related genera of

serine pathway methylobacteria are summarized in Table 5.

The results of our 16S rDNA sequence analysis showed that the genus *Methylopila* forms a distinct branch within the α -subclass of the Proteobacteria, separated from known genera of aerobic methylotrophic bacteria. Phylogenetic relationships that were derived from the 16S rDNA sequence analysis correlate well with the results of 5S rDNA sequencing (Boulygina *et al.*, 1993). Genotypic data also demonstrated that a new genus was clearly separated from all other described taxa of the serine pathway methylobacteria. On the basis of the data presented here and that published previously by Govorukhina *et al.* (1989) we propose that the seven isolates studied be placed in a new genus, for which we propose the name *Methylopila* gen. nov. The genus contains a single species, which is automatically the type species of the genus. The strains, currently placed within the genus *Methylopila* were originally described under the name '*Methylomicrobium capsulatum*' (Govorukhina *et al.*, 1989), and we therefore propose that the new species be named *Methylopila capsulata* sp. nov.

Description of the genus *Methylopila* gen. nov.

Methylopila (Me.thyl.o.pi.la. M.L.n. methyl the methyl group; Gr. adj. pila ball or sphere; M.L. *Methylopila* methyl-using sphere).

When grown on methanol mineral salt medium, the cells are motile rods, usually 0.5–0.7 μ m in diameter and 1.0–1.3 μ m in length, which occur singly or in pairs. Gram-negative. Non-pigmented. Endospores are absent. Multiply by fission. No prosthecae detected. Colonies are white. Pyocyanin and fluorescein are not produced. Growth is supported by nutrient agar and PYG agar. The methyl red and Voges–Proskauer tests are negative. Oxidase- and urease-positive. Acids are produced from sugars

Table 5. Major differentiating characteristics of the serine pathway facultative methylobacteria belonging to various genera

Character	<i>Methylobacterium</i>	<i>Aminobacter</i>	<i>Hyphomicrobium</i>	<i>Methylorhabdus</i>	<i>Methylopila</i>
Morphology (flagella)	+	+	+	—	+
Reproduction by:					
Budding	—	+	+	—	—
Division	+	—	—	+	+
Hyphae formation	—	—	+	—	—
Oxidase	+	+	—	—	+
Catalase	+	+	+	+	—
Carotenoids	+	—	—	—	—
Methylamine metabolism:					
Amine dehydrogenase	+	—	—	+	+
N-Methylglutamate	+	+	+	—	—
Formaldehyde dehydrogenase (NAD ⁺ /GSH)	—	—	—	+	—
Isocitrate lyase	—	+	+	—	—
Cyclopropane acid cy _{19:0}	Trace	+	+	+	+
Major ubiquinone	Q-10	Q-10	Q-9	Q-10	Q-10
Utilization of methanol	+	—	+	+	+
DNA G + C content (mol %)	60–70	62–64	61–65	66–67	66–70

oxidatively, but not fermentatively. Nitrates are reduced to nitrites slowly. Chemo-organotrophic and facultatively methylotrophic assimilating C₁ compounds by the isocitrate lyase[−] serine pathway. Vitamins are not required for growth. Ammonium salts, nitrate, urea, peptone, some amino acids and methylated amines are utilized as nitrogen sources. Strictly aerobic, with respiratory metabolism. Good growth occurs between pH 6.5 and 7.5, but not above pH 9.0 and below pH 5.0. Grows well at 28 and 35 °C but not at 42 °C. No growth in the presence of 3% NaCl. The cell fatty acid profile is characterized by the presence of 60–70% *cis*-vaccenic acid (C_{18:1ω7}). The major hydroxy acid is 3-OH C_{14:0}. The quinone system is ubiquinone Q-10. Dominant phospholipids are phosphatidylethanolamine and phosphatidylcholine. The DNA G + C content ranges from 66 to 70 mol %. The levels of DNA–DNA hybridization with members of the genera *Methylorhabdus*, *Methylobacterium*, *Aminobacter*, *Hyphomicrobium* and *Pseudomonas* are less than 10%. The genus *Methylopila* belongs to the α-2 subclass of the *Proteobacteria*, as revealed by comparative sequence analysis of the 16S rDNA, and is phylogenetically distinct from the other methylotrophic genera. The type species is *Methylopila capsulata*.

Description of *Methylopila capsulata* sp. nov.

Methylopila capsulata (cap.su.la'ta. L.n. capsule a small chest, capsule; M.L. adj. *capsulata* having a capsule).

In addition to the characteristics that define the genus, it has the characteristics described below. Cells are motile pleomorphic rods or cocci occurring singly or in

pairs and having a capsule covered with spikes. Colonies grown on nutrient agar or glucose-potato agar are round; viscous, semitransparent, convex, even edged, 2 mm in diameter. Cells accumulate poly-β-hydroxybutyrate. Does not form indole or NH₃. Catalase activity is very low. Produce H₂S during growth in nutrient broth. Utilizable carbon sources are methanol, methylated amines, butanol, ethanol, glycerol, maltose, sucrose, L-arabinose, D-fructose, D-glucose, succinate, fumarate, pyruvate. Gelatin and starch are hydrolysed weakly. Methanol and methylamine are oxidized by the appropriate PMS-linked dehydrogenases to formaldehyde which is assimilated by the isocitrate lyase[−] serine pathway. The mol % G + C of the DNA is 67.2 (T_m). The type strain IM1^T was isolated from soil of Tashkent city (Uzbekistan) and is deposited in the Russian Collection of Microorganisms as *Methylopila capsulata* VKM B-1606^T. The species also includes strains UZ, 14, 9AA, 10AA, 26AA and 37AA.

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