Phylogenetic position and emended description of the genus *Methylovorus*

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The genus *Methylovorus*, currently represented by the restricted facultative methylotroph *Methylovorus glucosotrophus* Govorukhina and Trotsenko 1991 and the obligate methylotroph *Methylovorus mays* Doronina *et al.* 2001, is here established by direct sequencing of amplified 16S rRNA genes and DNA–DNA hybridization to be clearly separated from the extant ribulose monophosphate (RuMP) pathway methyllobacteria and to form a distinct branch within the β -Proteobacteria.

To date, four genera have been described for the group of obligately and restricted facultatively methylotrophic bacteria (methyllobacteria) with the ribulose monophosphate (RuMP) pathway of C_1 assimilation: *Methylophaga* (Janvier *et al.*, 1985; de Zwart *et al.*, 1996; Doronina *et al.*, 2003a, b), *Methylobacillus* (Yordy & Weaver, 1977; Urakami & Komagata, 1986), *Methylophilus* (Jenkins *et al.*, 1987) and *Methylovorus* (Govorukhina & Trotsenko, 1991).

The genus *Methylovorus* was proposed for restricted facultative methyllobacteria isolated from activated sludge, mud, soil and pond water. On the basis of some phenetic characteristics and DNA–DNA relatedness, five isolates were described as one species, *Methylovorus glucosotrophus*. Another restricted facultative methylotroph, *Methylovorus* sp. strain SS1, was isolated from Malaysian soil samples (Seo & Kim, 1993) but not identified to the species level. More recently, we isolated from maize phyllosphere an obligate methylotroph which was classified as the novel species *Methylovorus mays* (Doronina *et al.*, 2000, 2001). It synthesized phytohormones, cytokinins and auxins (Ivanova *et al.*, 2000, 2001), and exerted a beneficial effect on the *in vitro* growth and morphogenesis of plants (Kalyaeva *et al.*, 2001). More recently, we also demonstrated that *Methylovorus glucosotrophus* 6B1^T is able to produce indole-3-acetic acid (Doronina *et al.*, 2002). In the light of these novel results, it seems reasonable to revise the formal description of the genus *Methylovorus*. Besides, the phylogenetic position of the *Methylovorus* species was not previously investigated. Here, we report some novel

characteristics and phylogenetic analysis of the genus *Methylovorus*.

DNA of *Methylovorus glucosotrophus* 6B1^T (=ATCC 49758^T) and *Methylovorus mays* C^T (=NCIMB 13992^T) was isolated and purified according to Marmur (1961) and the 16S rRNA genes were amplified and sequenced (Lane, 1991). The determined 16S rRNA gene sequences were aligned against those of representative taxa of methyllobacteria by using the CLUSTAL program. Positions of sequence uncertainty were omitted; in total, 1415 nucleotides were used in the analysis. Phylogenetic relationships were determined by the neighbour-joining method and the programs of the TREECON package (Van de Peer & De Wachter, 1994), by maximum-likelihood by using the PUZZLE program (Strimmer & von Haeseler, 1996) and by maximum-parsimony using the program DNAPARS from the PHYLIP package (Felsenstein, 1989) with bootstrap analysis of 100 trees.

The sequences were compared to representatives of the α -, β - and γ -subclasses of the Proteobacteria, including the methylotrophic genera with validly published names. In preliminary trials, a total of 98 sequences were used and several phylogenetic trees were generated. The phylogenetic analyses employed different algorithms, with similar results. According to 16S rRNA gene sequence analysis, strains 6B1^T and C^T were closely related, with a similarity level of 98.9%. The highest degree of similarity was found to species of *Methylophilus* (92.6–94.3%) and *Methylobacillus* (92.1–94.3%) (Fig. 1). Thus, strains 6B1^T and C^T are phylogenetically separated from other representatives of the β -Proteobacteria at the generic level. Moreover, the level of DNA–DNA relatedness between *Methylovorus glucosotrophus* 6B1^T and *Methylovorus mays* C^T was 56%. However, they had a very low degree of DNA–DNA hybridization (5–8%) with *Methylophilus methylotrophus* NCIMB 10515^T and *Methylobacillus glycogenes* ATCC

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Abbreviation: RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 6B1^T and C^T are respectively AY486133 and AY486132.

Fig. 1. Phylogenetic position of *Methylovorus glucosotrophus* 6B1^T and *Methylovorus mays* C^T among related micro-organisms based on 16S rRNA gene sequences. Pairwise evolutionary distances were computed by the Jukes–Cantor correction using programs of PHYLIP (version 3.5) package. *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. Bar, 2% evolutionary difference. Numbers at branch points are bootstrap values from 100 replicates.

29475^T. DNA–DNA hybridization was carried out according to the method of Denhardt (1966).

Major differential characteristics of the RuMP-pathway obligate and restricted facultative methylobacteria are summarized in Table 1.

Emended description of the genus
***Methylovorus* Govorukhina and Trotsenko 1991**

Methylovorus [Me.thy.lo.vo'rus. N.L. n. *methyl* the methyl radical; N.L. masc. adj. *vorus* (sic) consuming; N.L. masc. n. *Methylovorus* (sic) the methyl-consumer].

Gram-negative rods, 0.4–0.6 × 1.0–1.4 µm. Motile by a single polar flagellum. Do not form endospores or complex intracellular membranes, either sheath or prosthecae. Some strains produce slime. Multiply by binary fission. No aggregation or pigmentation in liquid medium. Colonies on methanol/mineral salt agar incubated for 2 days at 30 °C are circular, 1–2 mm in diameter, with entire edges, convex and translucent to opaque, creamy or milky in colour. No growth or very poor growth on nutrient agar and in nutrient broth at 30–40 °C. No growth under an atmosphere of CH₄ + O₂ or H₂ + CO₂ + O₂. No growth in the

presence of 3% (w/v) NaCl. Optimal pH for growth, 7.0–7.5, and temperature, 35–40 °C. Strictly aerobic with respiratory metabolism. Obligate or restricted facultative methylotrophs. Utilize methanol as the carbon and energy source. Some strains can grow poorly on methylated amines and glucose. Nitrates, ammonium salts, methylated amines and glutamate serve as nitrogen sources. Acetoin, H₂S and NH₃ are not produced in test medium. Urease-, catalase- and oxidase-positive. Peroxidase is variable. Do not degrade cellulose, gelatin or Tween 80. Indole is formed from L-tryptophan in mineral medium with methanol as the sole carbon and energy source and with KNO₃ as a nitrogen source. Ammonium ions inhibit tryptophan deamination. Assimilate C₁ compounds through the RuMP pathway (Entner–Doudoroff variant) and ammonia via the glutamate cycle (glutamate synthase and glutamine synthetase). Neither α-ketoglutarate dehydrogenase nor the glyoxylate shunt enzymes are present. 6-Phosphogluconate dehydrogenase is specific for NAD⁺ (not NADP). The prevailing cellular fatty acids are C_{16:0} and C_{16:1ω7}. The major ubiquinone is Q₈. The dominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin). Belong to the β-subclass of the *Proteobacteria*. Habitat: activated sludge, mud, soil,

Table 1. Major differential characteristics of obligate and restricted facultative methylobacteria with the RuMP pathway of C₁ assimilation

Members of all four genera exhibit NAD⁺-specific isocitrate dehydrogenase activity. GS/GOGAT, Glutamine synthetase/glutamate synthase.

Characteristic	<i>Methylovorus</i>	<i>Methylophilus</i>	<i>Methylobacillus</i>	<i>Methylophaga</i>
DNA G + C content (mol%)	56–58	50–53	53–62	38–49
Growth at 3% (w/v) NaCl	–	–	–	+
Vitamin requirement	–	–	–	B ₁₂
Ammonia assimilation	GS/GOGAT	GS/GOGAT	Glutamate dehydrogenase	Glutamate dehydrogenase, GS/GOGAT
NADP-specific isocitrate dehydrogenase	–	–	+	+
NADP-specific 6-phosphogluconate dehydrogenase	–	+	+	+
Diphosphatidylglycerol	+	–	+	+
Optimal growth temperature (°C)	35–40	30–37	29–42	25–32
Subclass of <i>Proteobacteria</i>	β	β	β	γ

pond water and plants. The G+C content of the DNA is 56–58 mol% (T_m). The type species is *Methylovorus glucosotrophus* Govorukhina and Trotsenko 1991.

Emended description of *Methylovorus glucosotrophus* Govorukhina and Trotsenko 1991

Methylovorus glucosotrophus [glu.cos.o.tro'phus. N.L. n. *glucosum* glucose; Gr. adj. *trophikos* nursing, tending or feeding; N.L. masc. adj. *glucosotrophus* (sic) feeding on glucose].

Morphology and general characteristics are as given for the genus. Growth is possible at 20–45 °C and pH 6.5–8.5, with optima at 35–37 °C and pH 7.2. Restricted facultative methylotroph. Methanol and glucose are used as sole carbon and energy sources. Generation times when grown on methanol and glucose are respectively 2 and 17 h. No vitamins or any additional growth factors are required. Hydrolyses starch. The G+C content of the DNA is 55.8 mol% (T_m).

The type strain, strain 6B1^T (=ATCC 49758^T=VKM B-1745^T=NCIMB 13222^T), was isolated from wastewaters in Alma-Ata (Kazakhstan). The GenBank accession number for the 16S rRNA gene sequence of the type strain is AY486133.

Emended description of *Methylovorus mays* Doronina et al. 2001

Methylovorus mays (mays. N.L. n. *mays* maize, the name of the host plant *Zea mays* L.).

Morphology and general characteristics are as given for the genus. Growth is possible at 20–45 °C and pH 6.5–8.5, with optima at 35–40 °C and pH 7.0–7.5. Obligate methylotroph utilizing methanol as the sole carbon and energy source. Generation time is 2 h. No vitamins or additional growth factors are required. Does not hydrolyse starch. Phytosymbiont producing phytohormones: cytokinins (zeatin and zeatine riboside) and auxins (indole-3-acetic acid and indole-3-lactic acid). Promotes germination of seeds and stimulates plant growth and morphogenesis. The G+C content of the DNA is 57.2 mol% (T_m).

The type strain, strain C^T (=VKM B-2221^T=NCIMB 13922^T), was isolated from the phyllosphere of *Zea mays* L. in Moscow region (Russia). The GenBank accession number for the 16S rRNA gene sequence of the type strain is AY486132.

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