32 The Family *Methylophilaceae*

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Abstract
Methylophilaceae, a family within the order *Methylophilales*, embraces the genera *Methylophilus* (type genus), *Methylobacillus*, *Methylovorus*, and *Methylotenera*. Betaproteobacterial obligate and restricted facultative methylotrophs capable of utilizing methanol or methylamine as a sole source of carbon and energy. Do not use methane (methylobacteria). Gram-negative rods, multiply by binary fission. Assimilate C_1 compounds via the ribulose monophosphate (Quayle) cycle. Major fatty acids are $C_{16:1\omega7c}$ and $C_{16:0}$. However, obligate methylobacteria possess similar morphology and metabolic organization. Thus, the main criteria used to clarify obligate methylobacteria into separate genera and species are their genomic and phylogenetic characteristics. On the other hand, members of the family are defined by some chemotaxonomic and biochemical properties, such as specific phospholipids and enzymes which are used for the delineation of genera. Members of the family are mainly found in activated sludge, mud, river, lake and pond waters, and plants.

Taxonomy: Historical and Current

Short Description of the Family

Me. thy. lo. phi. la' ce. ae. M.L. masc. n. *Methylophilus* type genus of the family, *-aceae* ending to denote family, M.L. fem. pl. n. *Methylophilaceae* the *Methylophilus* family.

Phylogenetically a member of the order *Methylophilales*, class *Betaproteobacteia* (Garrity et al. 2005). The family contains the type genus *Methylophilus* (Jenkins et al. 1987), *Methylobacillus* (Yordy and Weawer 1977: emended by Urakami and Komagata 1986), *Methylovorus* (Govorukhina and Trotsenko 1991: emended by Doronina et al. 2005a), *Methylotenera* (Kalyuzhnaya et al. 2006).

Members of the family are not halophilic obligate or restricted facultative methylotrophs, assimilate one-carbon compounds via the 2-keto-3-deoxy-6-phospogluconate (KDPG) variant of the ribulose monophosphate (RuMP) pathway. Gram-negative rods, multiply by binary fission. Motile by means of one or several polar or subpolar flagella or nonmotile. Do not form resting bodies. Do not grow in TGY, LB, and Nutrient media. Methane is not used. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Major cellular fatty acids are $C_{16:1\omega7c}$ and $C_{16:0}$. The major phospholipid is phosphatidylethanolamine. Ubiquinone Q-8 is the predominant isoprenoid quinone. The phylogenetic distance between the four genera is about 93–96 % 16S rRNA gene sequence similarity.

Phylogenetic Structure of the Family and Its Genera

Analysis of the nearly complete sequences of the 16S rRNA genes indicate that methylobacteria of the family *Methylophilaceae* are separated from sequences of species representing the genera *Methylophilus*, *Methylobacillus*, *Methylovorus*, and *Methylotenera* (Fig. 32.1).

E. Rosenberg et al. (eds.), *The Prokaryotes – Alphaproteobacteria and Betaproteobacteria*, DOI 10.1007/978-3-642-30197-1_243,

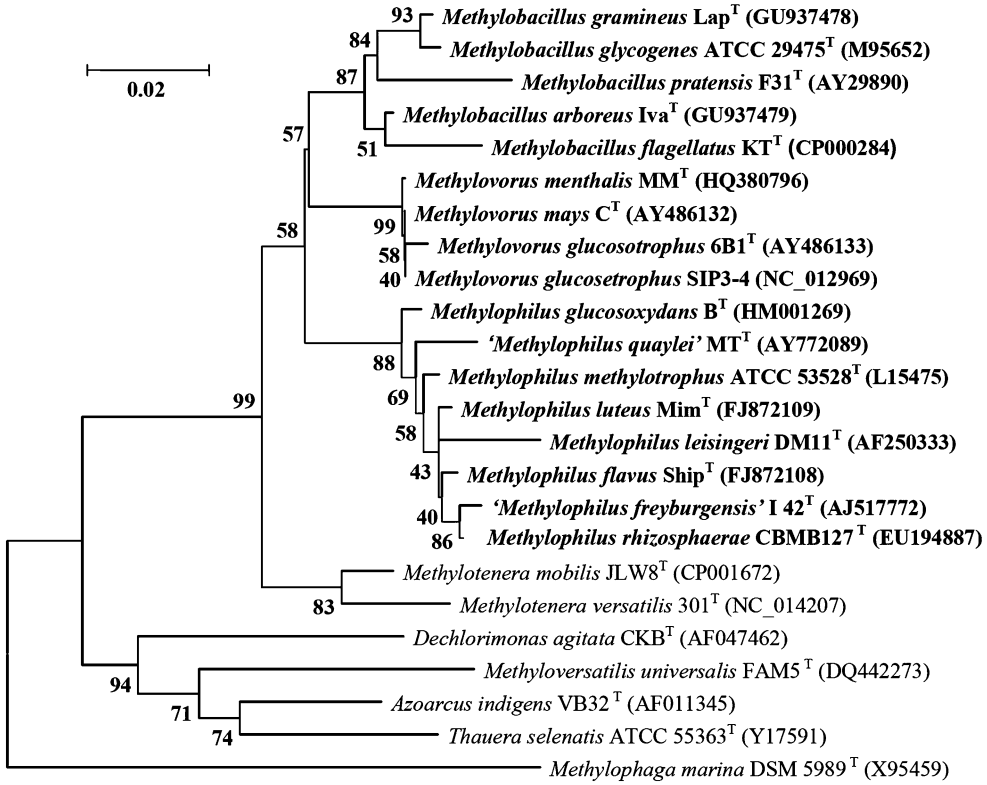

Fig. 32.1

***Maximum likelihood* phylogenetic tree showing the relationship of representatives of the *Methylophilaceae* based on 16S rRNA gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)**

Functional gene—the *mxaF* gene encodes the large subunit of the classical pyrroloquinoline quinone–linked methanol dehydrogenase which is found in majority of extant Gram-negative methylobacteria (Anthony and Williams 2003). Phylogenetic tree based on mxaF amino acid sequences show high levels of similarity between the members of the family *Methylophilaceae* (Fig. 32.2).

Molecular Analyses

DNA-DNA Hybridization Studies

Almost all descriptions of *Methylophilus*, *Methylobacillus*, and *Methylovorus* species include results of DNA-DNA hybridization (DDH) studies. Levels of DNA-DNA relatedness between *Methylophilus* strains—*M. methylotrophus* NCIMB 10515^T, *M. leisingeri* DSM 6813^T, *M. quaylei* VKM B-2338^T, *M. rhizosphaerae* CBMB127^T, *M. flavus* VKM B-2547^T, *M. luteus* VKM B-2548^T, and *M. glucosoxydans* VKM B-1607^T were 28–46 % (Madhaiyan et al. 2009; Doronina et al. 2012).

DNA-DNA relatedness between the reference strains of the genus *Methylobacillus* (*M. glycogens* ATCC 29475^T, *M. flagellatus* DSM 6875^T, *M. pratensis* NCIMB 13994^T, *M. gramineus* VKM B-2591^T, *M. arboreus* VKM B-2590^T) were in the range 38–45 % (Gogleva et al. 2011).

DDH relatedness between *Methylovorus glucosetrophus* VKM B-1745^T and *Methylovorus mays* VKM B-2221 was 56–58 % (Doronina et al. 2000). The levels of DNA-DNA homology between *Methylovorus menthalis* VKM B-2663^T and *M. glucosetrophus* VKM B-1745^T and *M. mays* VKM B-2221^T were 40 and 58 %, respectively (Doronina et al. 2011).

Moderate DDH relatedness of 12–18 % between type strains of the genera *Methylophilus*, *Methylobacillus*, and *Methylovorus* is a proof for intrageneric membership (Govorukhina and Trotsenko 1991; Doronina and Trotsenko 1994).

Genome Comparison

The complete genome sequence of five species branching within the 16S rRNA gene tree of *Methylophilaceae* have been released: the type strain of *Methylobacillus flagellatus* KT^T (Chistoserdova et al. 2007), *Methylotenera mobilis* JLW8^T, *Methylotenera versatilis* 301^T, and *Methylovorus glucosetrophus* SIP3-4 (Lapidus et al. 2011) and unclassified *Methylophilales* strain HTCC 2181^T

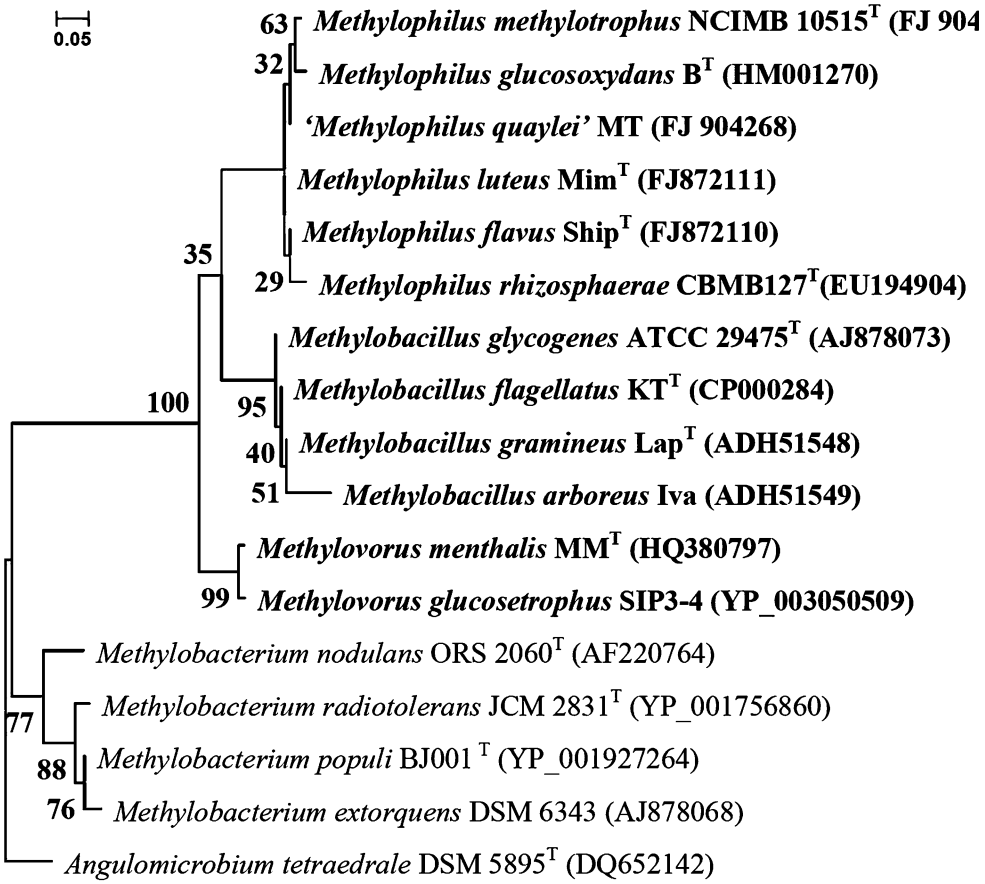

Fig. 32.2

Maximum likelihood tree based on *mxa*F amino acid sequences showing the phylogenetic position members of *Methylophilaceae* among methylotrophic bacteria. The numbers at the branch points are bootstrap values from 1,000 replicates. Bar, 5 % of evolutionary distance (5 amino acid substitutions per 100 amino acids). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (see previous *Fig. 32.1*)

(Giovannoni et al. 2008) (*Tables 32.1–32.3*). The genome of *Methylobacillus flagellatus* KT^T represented by a single circular chromosome of approximately 3 Mbp, potentially enoding a total of 2,766 proteins.

Based on genome analysis as well as the results of mutational analyses, their methylotrophy is enabled by methanol and methylamine dehydrogenases and their specific electron transport chain components, the tetrahydromethanopterin (H_4MPT)-linked formaldehyde oxidation pathway and the assimilatory and dissimilatory RuMP cycles, and by a formate dehydrogenase. Some of the methylotrophy genes are present in more than one (identical or nonidentical copy). The obligate dependence on single-carbon compounds appears to be due to the incomplete tricarboxylic acid cycle (TCA), as no genes potentially encoding alpha-ketoglutarate, malate or succinate dehydrogenases are identifiable. The genome of *M. flagellatus* was compared in terms of methylotrophy functions to the previously sequenced genomes of three methylotrophs, *Methylobacterium extorquens* (an alphaproteobacterium, 7 Mbp) (Chistoserdova et al. 2003), *Methylibium petroleiphilum* (a betaproteobacterium, 4 Mbp) (Kane et al. 2007), and *Methylococcus capsulatus* Bath (a gammaproteobacterium, 3.3 Mbp) (Ward et al. 2004). Strikingly, metabolically and/or phylogenetically, the methylotrophy functions in *M. flagellatus* were more similar to those in *M. capsulatus* and *M. extorquens* than to the ones in the more closely related *Methylibium petroleiphilum* species, providing the first genomic evidence for the polyphyletic origin of methylotrophy in *Betaproteobacteria*.

Comprehensive proteomics to assess the expressed portion of the genome of *Methylobacillus flagellatus* was implemented (Chistoserdova et al. 2009; Chistoserdova 2011; Hendrickson et al. 2010). A total of 1,671 proteins (64 % of the inferred proteome) were detected, including all the predicted essential proteins. Nonrandom patterns observed with the nondetectable proteins appeared to the corresponding silent genomic islands, as inferred through the functional profiling and genome localization. The protein contents in methylamine- and methanol-grown cells showed a significant overlap, confirming the commonality of methylotrophic metabolism downstream of the primary oxidation reactions. The new insights into methylotrophy include detection of proteins for the *N*-methylglutamate pathway of methylamine

Table 32.1
Genome statistics and general features of *Methylophilaceae* species

Strain	Genome size (bp)	%GC DNA	No. of:				Mean coding sequence length (bp)	% Coding regions	Reference
			Proteins encoded	rRNA operons	$tRNA_s$	Replicons			
Methylotenera mobilis JLW8	2,547,570	45.51	2,348	2	46	1	975.63	89.96	Lapidus et al. (2011)
Methylotenera versatilis 301	3,059,871	42.64	2,800	3	47	1	993.59	90.26	Lapidus et al. (2011)
Methylovorus glucosetrophus SIP3-4	3,082,007	54.61	2,922	2	48	3	966.10	91.51	Lapidus et al. (2011)
Methylobacillus flagellatus KT	2,971,517	55.72	2,759	2	46	1	973.73	90.61	Chistoserdova et al. (2007)
Methylophilales strain HTCC2181	1,304,428	37.93	1,338	1	36	1	923.45	95.00	Giovannoni et al. (2008)

Table 32.2
Relationships between the five *Methylophilaceae* strains compared on the basis of 16S rRNA gene identity and on percentage of common proteins (Lapidus et al. 2011)

Strain	Gene or protein identity[a] with:				
	Mt. mobilis JLW8	*Mt. versatilis* 301	*Mv. glucosetrophus* SIP3-4	*Mb. flagellatus* KT	*Methylophilales* strain HTCC2181
Mt. mobilis JLW8		96.6	94.3	93.8	94.6
Mt. versatilis 301	51.6		93.5	93.6	94.3
Mv. glucosetrophus SIP3-4	33.6	29.6		96.5	93.9
Mb. flagellatus KT	29.6	25.9	38.7		92.9
Methylophilales strain HTCC2181	17.0	16.9	15.7	15.2	

[a]16S rRNA identity is shown in the upper right and percentage of common proteins (at 70 % identity) in the lower left

oxidation that appears to be auxiliary. Two alternative enzymes for the 6-phosphogluconate dehydrogenase reaction (GndA and GndB) and the formate dehydrogenase reaction (FDH1 and FDH4) were detected. Mutant analysis revealed that GndA and FDH4 are crucial for the organism's fitness, while GndB and FDH1 are auxiliary.

Comparative analysis revealed that the core genome of *Methylophilaceae* may be as small as approximately 600 genes, while the pangenome may be as large as approximately 6,000 genes. Significant divergence between the genomes in terms both gene content and gene and protein conservation was uncovered, including the varied presence of certain genes involved in methylotrophy. Overall, data demonstrate that metabolic potentials can vary significantly between different species of *Methylophilaceae*, including organisms inhabiting the very same environment. These data suggest that genetic divergence among the members of the family may be responsible for their specialized and nonredundant functions in C1 cycling, which in turn suggests means for their successful coexistence in the specific ecological niches.

Phenotypic Analyses

The main features of members of *Methylophilaceae* are listed in *Tables 32.4–32.7*.

Methylophilus Jenkins et al. 1987, 447[VP]; Doronina et al. 2010, 2012

Me.thy.lo.phi'lus. Fr.n. *methyle* the methyl radical; Gr.adj. *philos* loving; M.L. masc.n. *Methylophilus* methyl radical loving.

Gram-negative, asporogenous, motile by polar flagella or nonmotile rods that are 0.2–0.6 × 1.0–2.5 μm in size. Some strains produce exopolysaccharide. Colonies on methanol—minimal salt

Table 32.3

Major pathways and enzymes for carbon and nitrogen metabolism predicted from genomes (Lapidus et al. 2011)

Enzyme or pathway[a]	*Mt. mobilis* JLW8	*Mt. versatilis* 301	*Mv. glucosetrophus* SIP3-4	*Mb. flagellatus* KT	*Methylophilales* strain HTCC2181
RuMP cycle	+	+	+	+	+
Gnd enzymes	GndB	GndB	GndA	GndA, GndB	GndA
MADH	+	–	–	+	–
NMG pathway	–	+	+	+	–
H_4MPT pathway	+	+	+	+	–
H_4F pathway	+	+	+	+	+
Fae homologs	Fae2	Fae2, Fae3	Fae2, Fae3	Fae2, Fae3	–
FDH2	+	+	+	+	+
FDH4	+	–	+	+	–
MDH (MxaFJGI)	–	–	+	+	–
PQQ synthesis	+	+	+	+	+
pqqA (gene copies)	5	4	5	3	3
MxaRSACKL copies	2	2	3	3	2
XoxF (copies)	2	3	4	4	1
NapA/NirBD	+	+	+	+[b]	–
AniA/Nor	+	–	–	–	–
Urea metabolism	–	+	+	+	–
Choline degradation	–	+	–	–	–
MCA cycle	+	+	–	–	+

[a]*RuMP* ribulose monophosphate, *Gnd* 6-phosphogluconate dehydrogenase, *H_4MPT* tetrahydromethanopterin, *FDH* formate dehydrogenase, *MDH* methanol dehydrogenase, *MADH* methylamine dehydrogenase, *NMG N*-methylglutamate, *XoxF* homolog of the large subunit of MDH, *FDH* formate dehydrogenase, *NapA/NirBD* assimilatory nitrate reduction pathway, *AniA/Nor* denitrification pathway, *MCA* methylcitric acid

[b]Nonorthologous

agar plates incubated for 2 days at 29 °C—are pigmented or nonpigmented, 1–4 mm in diameter, with entire edge, convex, translucent to opaque, smooth. No or extremely poor growth on nutrient or blood agar; no hemolysis. Optimal temperature 24–37 °C; no growth occurs at 4 °C and 45 °C. Optimal pH 6.5–7.8. Aerobic. Metabolism respiratory; very little or no acid is produced from glucose. Methanol is oxidized as the sole carbon and energy source by all the strains. In addition, a very limited range of other carbon compounds such as methylated amines, formate, glucose, and fructose may be utilized as sole carbon and energy sources. Methane is not used. No vitamins or other growth factors are required. Catalase- and oxidase positive. Nitrate and ammonium salts are used as nitrogen sources. Produce indole from tryptophan on medium with nitrate as nitrogen source. The predominant cellular fatty acids are $C_{16:0}$ and $C_{16:1}$. The major isoprenoid quinone component is ubiquinone with eight isoprene units (Q-8). The predominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol; diphosphatidylglycerol (cardiolipin) is absent.

Formaldehyde is assimilated via the RuMP pathway. Assimilate NH_4^+ via the glutamate cycle. The tricarboxylic acid cycle is incomplete due to the absence of α-ketoglutarate dehydrogenase. Glyoxylate shunt enzymes are absent. The mol% G + C of DNA is 48–54.

The type species is *Methylophilus methylotrophus* Jenkins, Byron, and Jones 1987. The type strain is AS1 (ATCC 53528^T = DSM 46235^T = LMG 6787^T = NCIMB 10515^T = VKM B-1623^T).

Comparison of selected characteristics of members of the genus *Methylophilus* are listed in ❯ *Table 32.4*.

Methylobacillus Yordy and Weawer, 1977, 254^{VP}, Emend. Urakami and Komagata 1986, 509

Meth.yl.o.ba.cil'lus. Fr. *methyle* the methyl radical; L.dim.n. *bacillus* a small rod; M.L. masc.n *Methylobacillus* methyl rodlet.

Cells are Gram-negative, asporogenous rods 0.3–0.6 × 0.8–2.0 μm motile or nonmotile, multiplying by binary fission, mesophilic and neutrophilic. Most strains are obligate methylotrophs; however, some strains can also use fructose. Colonies are shiny, smooth, raised, entire, white to light yellow, 1–4 mm in diameter on methanol-containing agar. Methanol is oxidized as the sole carbon and energy source by all strains. Aerobic, having a strictly respiratory type of metabolism with

Table 32.4
Comparison of selected characteristics of members of the genus *Methylophilus*

Characteristic	1	2	3	4	5	6	7
Flagellation	+	–	–	+	–	–	–
Colony pigmentation	White	Yellow	Yellow	Grayish 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	+	–	+	+	+/–[a]	–	–
Fructose	–	–	–	–	–	–	+
Urease	–	–	–	+	+	+	+
Acetoine 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
G + C content (*T*m) (mol%)	52.5	50.7	54.5	50.3	50.2	54.0	47.9
Isolation source	Rhizosphere of rice (*Oryza sativa* L.)	Phyllosphere of *Rosa cinnamomea* L.	Phyllosphere of *Tussilago farfara* L.	Activated sludge	Wastewater	Contaminant of *Methylocystis methanolicus*	Rhizosphere of rice (*Oryza sativa* L.)

Strains 1 *M. glucosoxydans* VKM B-1607^T (Doronina et al. 2012), 2 *M. flavus* VKM B-2547^T (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 and Trotsenko 1994), 6 *M. quaylei* VKM B-2338 (Doronina et al. 2005b), 7 *M. rhizosphaerae* CBMB127^T (Madhaiyan et al. 2009). No glutamate and 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
[a]Dichloromethane dehalogenase locates on a plasmid

oxygen as the terminal electron acceptor. No vitamins or other growth factors are required. Nitrate and ammonia are used as the nitrogen sources. The prevailing cellular fatty acids are straight–chain saturated $C_{16:0}$ and unsaturated $C_{16:1}$ acids. The major ubiquinone is Q-8. The predominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (diphosphatidylglycerol). Ammonia is assimilated by glutamate dehydrogenase. The tricarboxylic acid cycle is incomplete due to the absence of α-ketoglutarate dehydrogenase. Glyoxylate shunt enzymes are absent. The mol. G + C of DNA is 50–61. The type species is *Methylobacillus glycogenes* Yordy and Weaver 1977. The type strain TK 0113 = Yordy and Weaver T-11 (= ATCC 29475^T = DSM 5685^T = JCM 2850^T = LMG 6082^T = NCIMB 11375^T = VKM B-2060^T).

Table 32.5 lists the differentiating properties of *Methylobacillus* species.

Methylovorus Govorukhina and Trotsenko 1991, 161^{VP}, Emend Doronina, Ivanova, and Trotsenko, 2005, 903

Me.thy.lo.vo'rus. N.L. *methyl* the methyl radical; N.L. masc.n adj. *vorus* consuming; N.L. masc.n. *Methylovorus* 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

Table 32.5

Major characteristics that allow differentiation among members of the genus *Methylobacillus* (Gogleva et al. 2011)

Characteristic	*M. arboreus* Iva^T VKM B-2590	*M. gramineus* Lap^T VKM B-2591	*M. flagellatus* DSM 6875^T	*M. glycogenes* ATCC 29475^T	*M. pratensis* F31^T VKM B-2224
Flagellation	1	1–4	1–4	–	1
Utilization of methylamine	–	–	+	+	+
Isocitrate dehydrogenase $NADP^+$	+	+	–	+	+
Urease	–	–	+	+	+
Acetoine production	+	+	–	–	–
Starch hydrolysis	+	+	+	–	+
Nitrate reduction	–	–	+	+	+
Growth at:					
37 °C	+	+	+	+	+
42 °C	–	–	+	–	–
3 % (w/v) NaCl	+	–	–	–	–
pH optimum	7.9–8.5	7.2–7.8	7.2–7.3	6.0–8.0	6.5–7.5
DNA G + C content (mol%)	54.0	50.5	55.5	53.2	61.5

Table 32.6

Characteristics of the type strains of the genus *Methylovorus* (Doronina et al. 2011)

Character	*M. menthalis* VKM B-2663	*M. mays* VKM B-2221^T	*M. glucosotrophus* VKM B-1745^T
Flagella	1	1	1
Methylotrophy type	Obligate	Obligate	Restricted facultative
Growth substrates:			
Methanol	+	+	+
Methylamine	–	–	–
Dimethylamine	–	–	–
Trimethylamine	–	–	–
Glucose	–	–	+
Fructose	–	–	–
Ammonium assimilation	Glutamate cycle (glutamate synthase/glutamine synthetase)		
6-phosphogluconate dehydrogenase ($NADP^+$)	–	–	–
Optimal growth temperature (°C)	24–26	35–40	35–37
pH optimum	8.5–9.0	7.0–7.5	7.2
G + C content (*Tm*), mol%	54.5	57.2	55.8
Source of isolation	*Mentha arvensis* L.	*Zea mays* L.	Wastewater

produce slime. Multiply by binary fission. No aggeregation or pigmentation in liquid medium. Colonies on methanol mineral salt agar incubated for 2 days at 30 °C are circular, 0.5–2.0 mm in diameter, with entire edges, convex and translucent to opaque, creamy or milky in color. No growth under an atmosphere of $CH_4 + O_2$ or $H_2 + CO_2 + O_2$. No growth in the presence of 3 % NaCl. Strictly aerobic with respiratory metabolism. Obligate or restricted facultative methylotrophs. Utilize methanol as the carbon and energy source. Some strains can grow poorly on glucose. Nitrates, ammonium salts, and glutamate serve as the nitrogen sources. Acetoine, H_2S, and NH_3 are not produced in test medium. Urease-, catalase-, and oxidase-positive. Peroxidase is variable. Do not degrade cellulose, gelatin, Tween 80. Indole is formed from L-tryptophan in mineral medium with methanol as the sole carbon and energy source with KNO_3 as a nitrogen source. Ammonium ions inhibit tryptophan

Table 32.7
Characteristics of the type strains of the genus *Methylotenera* (Kalyuzhnaya et al. 2012)

Character	*M. mobilis* JLW8^T	*M. versatilis* 301^T
Genome size (Mb)	2.55	3.06
DNA G + C content (mol%)	45.5	42.6
Plasmids	–	–
Copies of rRNA gene cluster	2	3
Methylamine dehydrogenase (*mau*FBEDACJG)	+	–
NMG pathway (*mgd*ABCD*gmas*A*mgs*ABC)	–	+
Methanol dehydrogenase (*mxa*FI)	–	–
Complete TCA cycle	–	–
Growth using:		
Methanol	–	+
Methylamine	+	+
Glucose	–	–
Fructose	–	+
Pyruvate	–	+
Ethanol	–	+
Nitrate as source of nitrogen	–	+

deamination. Assimilate C_1 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 $C_{16:0}$ $C_{16:1\omega7}$. The major ubiquinone is Q-8. The predominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. Cardiolipin is present.

The G + C content of DNA is 54–58 mol%. The type species is *Methylovorus glucosotrophus* Govorukhina and Trotsenko 1991. The type strain is 6B1 (VKM B-1745^T = ATCC 49758^T = DSM 6874^T = NCIMB 13222^T = UCM B-1745^T). *Table 32.5* lists characteristics of the type strains of the genus *Methylovorus*.

Methylotenera Kalyuzhnaya, Bowerman, Lara, Lidstrom, and Chistoserdova 2006, 289^{VP}, Emend. Kalyuzhnaya, Beck, Vorob'ev, Smalley, Kunkell, Lidstrom, Chistoserdova, 2012

Me.thy.lo.te'ner.a. N.L. *methylum* (from French *me´thyle*, back-formation from French *méthylène*, coined from Gr. n. *methu* wine and Gr. n. *hulê* wood) the methyl group radical; N.L. pref. *methylo-* pertaining to the methyl radical; L. fem. adj. *tenera* delicate; N.L. fem. n. *Methylotenera* a methyl group-oxidizing delicate bacterium.

Gram-negative rods. Some strains are motile. Do not form resting bodies and multiply by binary fission. Utilize methylamine. In addition, may utilize methanol, betaine, fructose, ethanol, and pyruvate as the sole sources of carbon and energy. Some strains are positive for urease and nitrate reduction. Oxidize methylamine by methylamine dehydrogenase or via *N*-methylglutamate pathway and assimilate C_1 compounds via the RuMP pathway. The major cellular fatty acids are $C_{16:1\omega7c}$ and $C_{16:0}$. The DNA G + C content is in the range 42.6–45.5 mol%.

The type species is *Methylotenera mobilis* Kalyuzhnaya et al. 2006. The type strain is JLW8 (= ATCC BAA-1282^T = DSM 17540^T).

Comparison of selected characteristics of the type strains of the genus *Methylotenera* are listed in *Tables 32.7* and *32.8*.

Colonies of *Methylotenera mobilis* JLW8^T (= ATCC BAA-1282^T = DSM 17540 ^{T}) were cream to light brown and 1–2 mm in diameter when grown at 30 °C for 4–7 days. No pigmentation was observed when cells were grown in liquid culture (Kalyuzhnaya et al. 2006).

Colonies of *Methylotenera versatilis* 301^T were white (slightly yellowish in old cultures), mucoid, undulate, circular, convex, viscous, and up to 5 mm in diameter. The isolate grew well on solid media but not in a liquid culture. Only cultures incubated without shaking showed some growth (Kalyuzhnaya et al. 2012).

Isolation, Enrichment, and Maintenance Procedures

Isolation, Enrichment

Strains of *Methylophilus methylotrophus* have been isolated from activated sludge, mud, river, and pond water (MacLenman et al., British patent 1370892). The type strain AS1^T was isolated from activated sludge.

Methylophilus leisingeri DM11^T was isolated in Switzerland from groundwater contaminated with dichloromethane (Gaelli and Leisinger 1985; Doronina and Trotsenko 1994). 20 ml groundwater was passed through a 0.2 µm pore diameter membrane filter, and the filter was incubated in 50 ml mineral medium containing 10 mM dichloromethane. The medium contained (g/l): $K_2HPO_4 \cdot 3H_2O$, 4.1; KH_2PO_4, 1.4; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2. Prior autoclaving, the pH of the medium was adjusted to 7.2. After sterilization dichloromethane was added, and 1 l of the medium was supplemented with 1 ml trace element solution (g/l): $FeSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 1.0; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.25; H_3BO_3, 1.0; $CuCl_2 \cdot 2H_2O$, 0.25; $ZnCl_2$, 0.25; NH_4VO_3, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.25; and $Ca(NO_3)_2$, 0.25. The bacteria were grown at 29 °C in 750-ml Erlenmeyer flasks containing 100 ml of the medium on a rotary shaker at 180 rpm. The flasks were tightly closed with rubber stoppers. Since the pH of the medium decreased when dichloromethane was used, dichloromethane was added in three portions to a final concentration of 10 mM (0.85 g/l) each after the pH of the medium had been adjusted to 7.2 by NaOH. Pure culture was isolated by the method of

Table 32.8
Differential characteristics of methylobacteria of the family *Methylophilaceae*

Characteristic	1	2	3	4
Flagellation	+/−	+/−	+	+/−
Colony pigmentation	White, yellow, grayish white, pale pink	White, light yellow	White	Cream, light brown, white, slightly yellowish
Growth substrates:				
Methanol	+	+	+	+/−
Methylamine	+/−	+/−	+/−	+
Glucose	+/−	+/−	+/−	−
Fructose	+/−	+/−	−	+/−
Methylamine dehydrogenase	+/−	+/−	−	+/−
N-methylglutamate pathway	+/−	+/−	−	+/−
Methanol dehydrogenase	+	+	+	−
Ammonia assimilation:				
Glutamate cycle	+	−	+	ND
Glutamate dehydrogenase	−	+	−	ND
Presence of 6-phosphogluconate dehydrogease ($NADP^+$ linked)	+	+	−	ND
Presence of diphosphatidylglycerol	−	+	+	ND
DNA G + C content (mol%)	48–54	50–61	54–57	42–46

Taxa: 1 *Methylophilus* (data from Doronina et al. 2012), 2 *Methylobacillus* (Gogleva et al. 2011), 3 *Methylovorus* (Doronina et al. 2011), 4 *Methylotenera* (Kalyuzhnaya et al. 2012)
"+" Positive "+/−" variable "−" negative *ND* not determined

exhausting plating of enrichment onto the same medium, containing 2.0 % purified agar Difco and 0.1 g/l of Bromothymol Blue. Petri plates were incubated in a 2.5 l desiccator, 0.2 ml portions of dichloromethane being added after 24 h. Colonies of bacteria that decompose dichloromethane are surrounded by yellow zones due to formation of HCl.

Methylophilus quaylei MT^T was isolated as an airborne contaminant during cultivation of methanotroph *Methylocystis methanolicus* on methanol (Doronina et al. 2005b). The sample of mixed liquid culture grown on medium "K" containing gl^{-1}: KH_2PO_4—2.0; $(NH_4)_2SO_4$—2.0; $MgSO_4 \cdot 7H_2O$—0.025; NaCl—0.5; $FeSO_4 \cdot 7H_2O$—0.002, pH 7.2 in Petri dish ($h = 5$ mm) was UV incubated for 10 min. After UV treatment, the culture was serially diluted and grown on "K" agar (Difco 2.0 % w/v) with 2 % (v/v) CH_3OH. A successive isolation of a single colonies resulted in the isolation of a pure culture of obligately methylotrophic strain MT^T, which could not grow on Difco nutrient agar or in atmosphere of methane: air (1:1).

Methylophilus rhizosphaerae $CBMB127^T$ was isolated from rhizosphere soils of rice cultivars (*Oryza sativa* L. cv O-dae and Nam-pyeoung, respectively) on selective ammonium mineral salt (AMS) medium (Whittenbury et al. 1970) with 0.5 % methanol. Cells were maintained on nutrient agar (NA, Difco) with 1 % (v/v) methanol or on AMS medium (Madhaiyan et al. 2009).

Strains *Methylophilus flavus* $Ship^T$ and *Methylophilus luteus* 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) (Gogleva et al. 2010). The strains were grown on medium "K." Solidified medium "K" was prepared by adding 2.0 % (w/v) Difco agar.

Methylophilus glucosoxydans B^T was isolated from rhizosphere rice (*Oryza sativa* L.) sampled from Vietnam on "K" agar with 2.0 % methanol (Doronina et al. 2012).

An enrichment culture of *Methylobacillus glycogenes* $T\text{-}11^T$ was prepared by adding a small amount of partially decayed tomato to a liquid mineral salt medium containing 2 % methanol (v/v) (Yordy and Weaver 1977). The bacterium was isolated from the enrichment culture by streaking for isolated colonies on a mineral salt agar medium containing 2 % methanol. A colony was picked and cultured at 30 °C with shaking (200 rpm) in a liquid mineral salt medium (pH 7.0), containing 2 % methanol. The procedure was repeated until a pure culture was obtained.

The strain *Methylobacillus flagellatus* KT^T was isolated by the same procedure from sewage (Govorukhina et al. 1987). *Methylobacillus pratensis* $F31^T$ was isolated on mineral salt medium, containing 0.5 % (v/v) methanol from meadow grass (*Poa trivialis* L.) sampled from the city park in Helsinki (Finland) (Doronina et al. 2004).

Stains *Methylobacillus arboreus* Iva^T and *Methylobacillus gramineus* Lap^T were isolated from willow buds (*Salix fragilis* L.) and phyllosphere of silverweed (*Potentilla anserina* L.), respectively, sampled from the city park in Pushchino (Moscow Region, Russia) (Gogleva et al. 2011).

Strain of the genus *Methylovorus* were isolated from activated sludge, mud, soil, pond water, and plants (Govorukhina and Trotsenko 1991; Seo and Kim 1993; Doronina et al. 2000, 2005a, 2011).

Methylovorus glucosotrophus $6B1^T$ was isolated from pond water (Govorukhina and Trotsenko 1991). *Methylovorus mays* C^T was isolated from maize phyllosphere (*Zea mays* L.) (Doronina et al. 2000). *Methylovorus menthalis* MM^T was isolated from the root of corn mint (*Mentha arvensis* L.) (Doronina et al. 2011).

The root was washed three times with sterile distilled water and placed into an Erlenmeyer flask (750 ml) with 200 ml of K medium and 0.5 % (v/v) methanol. After three transfers for 2 days on a rotary shaker (180 rpm) at 29 °C, the suspension of the methylobacterial enrichment culture was plated to obtaining

single colonies (exhaustive inoculation) onto agarized "K" medium with methanol. Isolated methylobacterial colonies were reinoculated on agar slants, transferred into liquid medium, and then again on solid medium for exhaustive inoculation. Reisolated methylobacterial colonies were reinoculated on slant agar. The purity of the isolated culture was controlled by light and electron microscopy, as well as by the uniformity of colonies on the agarized medium with methanol.

An obligate methylamine utilizer, *Methylotenera mobilis* JLW8^T, was isolated from Lake Washington sediment (Washington State, USA) after enrichment in a basal salt medium (Harder et al. 1973) diluted fivefold and supplemented with 0.1 % methylamine (Kalyuzhnaya et al. 2006). Also the restricted facultative methylotroph *Methylotenera versatilis* 301^T was isolated from this ecosystem by a dilution-plating approach (Kalyuzhnaya et al. 2012). To isolate strain 301^T, 1 ml aliquot of sediment samples was mixed with 9 ml filtered lake water, and serial dilutions were plated onto plates containing lake water-based medium solidified with agar (2 %; Difco) supplemented with 5 mM methylamine. After 2 weeks incubation at room temperature, individual colonies were restreaked onto fresh agar plates.

Maintenance Procedures

Generally, strains of the family *Methylophilaceae* are maintained on basal salts media as agar slants at 4 °C for 1 month. Some members of the genus *Methylophilus* must be subculturing every 10 days. For long-term storage at −20 °C or −80 °C, cells are suspended in the basal salts media and supplemented with 10 % DMSO or 20 % glycerol. Long-term preservation methods include freeze-drying in skim milk and maintenance in liquid nitrogen at −196 °C.

Ecology

Strains of the family *Methylophilaceae* are obligate or restricted facultative methylotrophs (methylobacteria) capable of growth on single-carbon compounds (methanol, methylamines, dichloromethane) and play an important role for the aerobic conversion of C1 compounds in different ecological niches. They have been isolated from activated sludge, mud, river, lake and pond waters, plant rhizosphere and phyllosphere. Association of aerobic methylobacteria with plants are permanent and results from the fact that methylobacteria consume methanol released by plant into the environment through leaf stomata (Nemecek-Marshall et al. 1995; Fedorov et al. 2011). Methanol is formed during demethylation of cell wall pectin under active growth of plant cells. Plants are therefore the main source of methanol in the biosphere (Galbally and Kirstine 2002). Association between plants and methylotrophs is mutually advantageous, because methylobacteria stimulate plant growth and development due to production of bioactive substances: phytohormones (auxins, cytokinins) and vitamins (Fedorov et al. 2011).

Methylophilus rhizosphaerae (Madhaiyan et al. 2009), *Methylophilus flavus* and *Methylophilus luteus* (Gogleva et al. 2010), *Methylophilus glucosoxydans* (Doronina et al. 2012), *Methylobacillus pratensis* (Doronina et al. 2004), *Methylobacillus arboreus* and *Methylobacillus gramineus* (Gogleva et al. 2011), *Methylovorus mays* (Doronina et al. 2000), and *Methylovorus menthalis* (Doronina et al. 2011) were isolated from plants.

Pathogenicity

No reports of the family *Methylophilaceae* causing overt or opportunistic infections in humans, animals, or insects have been published. The members of this family are obligate or restricted facultative methylotrophs and unable to grow on blood agar or other complicated media.

The type strain *Methylophilus methylotrophus* AS1 is resistant to penicillin, oleandomycin, and susceptible to nalidixic acid and streptomycin (Jenkins et al. 1987). *Methylophilus leisingeri* DM11^T (VKM B-2013^T = DSM 6813^T) is resistant to erythromycin and sensitive to nalidixic acid, novobiocin, and kanamycin (Doronina and Trotsenko 1994). *Methylophilus flavus* Ship^T (VKM B-2547^T = DSM 23073^T = CCUG 58411^T) is resistant to streptomycin and oxacillin and sensitive to ampicillin, novobiocin, nalidixic acid, gentamicin, neomycin, and lincomycin (Gogleva et al. 2010). *Methylobacillus flagellatus* KT^T (ATCC 51484 = DSM 6875 = VKM B-1610) is resistant to streptomycin, erythromycin, ampicillin, neomycin, and lincomycin and sensitive to novobiocin, nalidixic acid, kanamycin, and gentamicin (Govorukhina et al. 1987). *Methylobacillus arboreus* Iva^T (VKM B-2590^T = CCUG 59684^T = DSM 23628^T) is resistant to ampicillin, oxacillin, novobiocin, streptomycin, and neomycin and sensitive to nalidixic acid, gentamicin, kanamycin, and lincomycin (Gogleva et al. 2011). *Methylobacillus gramineus* Lap^T (=VKM B-2590^T= =CCUG 59683^T = DSM 23629^T) is resistant to novobiocin, nalidixic acid, and neomycin and sensitive to ampicillin, oxacillin, gentamicin, streptomycin, kanamycin, and lincomycin (Gogleva et al. 2011).

Mehylovorus glucosetrophus 6B1^T (=ATCC 49758^T = VKM B-1745^T = NCIMB 13222^T) is resistant to neomycin, lincomycin, erythromycin, and ampicillin, but sensitive to novobiocin, nalidixic acid, kanamycin, and streptomycin (Govorukhina and Trotsenko 1991).

Methylovorus mays C^T (=VKM B-2221^T = NCIMB 13922^T) is resistant to ampicillin and lincomycin and sensitive to gentamicin, kanamycin, nalidixic acid, neomycin, novobiocin, streptomycin, and erythromycin (Doronina et al. 2000).

Methylotenera mobilis JLW8^T (=ATCC BAA-1285^T = DSM17540^T) is sensitive to gramicidin, kanamycin, and tetracycline (Kalyuzhnaya et al. 2006). *Methylotenera versatilis* 301^T (=JCM 17579^T = VKM B-2679^T) is resistant to kanamycin, ampicillin, streptomycin, neomycin, and erythromycin (Kalyuzhnaya et al. 2012).

Application

Forage protein methylobacteria of the genera *Methylophilus* and *Methylobacillus* are characterized by higher values of specific growth rate, economic coefficient, protein and lysine content, and favorable composition of elements and fatty acids (MacLennan et al. 1974; Doronina and Trotsenko 1986; Large and Bamforth 1988; Trotsenko et al. 2005). For all the reasons above, they are preferable for large-scale cultivation and forage protein production.

Exopolysaccharides (EPSs)

Methylobacteria of the genera *Methylobacillus*, *Methylophilus*, and *Methylovorus* synthesize EPSs from methanol; of note, EPSs yield may be regulated by varying the composition of the medium and growth conditions (Doronina et al. 2005b; Gogleva et al. 2010).

Phytosymbiosis

Aerobic methylobacteria are ubiquitous in the phyllosphere and rhizosphere of plants and often colonize their seeds (Trotsenko et al. 2001; Fedorov et al. 2011). Biological testing (using *Amarantus caudatus* L. seedlings), TLC, HPLC, and solid-phase enzyme immunoassays established the presence of zeatin and zeatin riboside in the culture liquid of *Methylovorus mays* (Ivanova et al. 2000). It was also demonstrated that representatives of the genera *Methylophilus*, *Methylobacillus*, and *Methylovorus* synthesize indole compounds, particularly indoleacetic acid (IAA) when grown in media containing methanol or methylamine in the presence of 5 mM L-tryptophan (Doronina et al. 2002; Gogleva et al. 2010, 2011).

The synthesis of auxins in methylobacteria depended on the composition of the medium: when $(NH_4)_2SO_4$ was replaced by KNO_3, the amount of IAA synthesized increased three- to fivefold.

Methylovorus mays exerted a beneficial effect on the growth and morphogenesis of tobacco, potato, and fiber flax grown in vitro (Kalyaeva et al. 2001). This obligate methylobaterium stimulated morphogenesis and antifungal resistance of Chinese cabbage *Brassica chinensis* L. (Doronina et al. 2009). The ability of methylobacteria to stimulate the growth and morphogenesis of plants indicates their promise in experimental plant physiology and agrobiotechnology.

Enzymes

Enzymological studies demonstrated that a number of enzymes of methylobacteria exhibit very high activity. These results provided a basis for isolating pure preparations of various dehydrogenases and other enzymes, which have a potential as research reagent and analytical tools: glucose-6-phosphate dehydrogenase (EC 1.1.1.49; 250U/mg protein) (Sokolov et al. 1980); $NADP^+$ glutamate:dehydrogenase (EC 1.4.1.4; 180 U/mg protein) (Sokolov and Trotsenko 1987).

Biodegradation of Toxic Compounds

Methylobacteria of the family *Methylophilaceae* degrade a broad spectrum of highly toxic compounds: methanol, formaldehyde, methylated amines, and dichloromethane. The culture of *Methylobacillus* sp. is appropriate for elimination of methanol from industrial sewage (cellulose sulfate manufacturing facilities) (Trotsenko et al. 2005).

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