Use of 16S rRNA Analysis To Investigate Phylogeny of Methylophilic Bacteria

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Small-subunit rRNAs from 24 gram-negative methylophilic bacteria have been sequenced. A phylogenetic tree was constructed on the basis of sequence similarities by using a weighted least-mean-square difference method. The methylophilic bacteria were separated into coherent clusters in which bacteria in each group shared physiological characteristics.

Methylophilic bacteria are a diverse group of microbes that utilize one-carbon compounds more reduced than carbon dioxide as sole energy sources. In addition, these organisms assimilate carbon from the primary substrate at the oxidation level of formaldehyde for the synthesis of cell material (2, 25). Bacteria able to grow on methane are a subset of methylophilic bacteria called methanotrophs. One of the criteria for the traditional classification of methanotrophs is the pathway used for formaldehyde assimilation (2, 8, 22). The two dissimilar pathways used by methylophilic bacteria are the ribulose monophosphate (RuMP) and serine pathways. Methanotrophs are further differentiated on the basis of arrangement of intracytoplasmic membranes, predominance of specific membrane phospholipid fatty acids, and other properties into groups I, II, and X (5, 7, 21). Methanotrophs also possess a utilitarian mixed-function oxidase, methane monooxygenase (MMO), that effects the conversion of methane to methanol. This enzyme has a broad substrate specificity and catalyzes the oxidation of a wide variety of aliphatic, alicyclic, and aromatic hydrocarbons (21). There are two forms of MMO—soluble and

membrane bound (also known as particulate MMO) (14). The soluble MMO is able to oxidize a wider range of compounds than the particulate enzyme (3, 4) and has been observed to be expressed only in group II methanotrophs of the genus "Methylosinus" and in one group X methanotroph (3, 19).

Methylophilic bacteria may play an important role in two current environmental concerns—global warming and groundwater contamination. Methane is an abundant organic gas in the atmosphere, and its concentration has been increasing at the alarming rate of 1% per year for the last 150 to 200 years (16). This gas absorbs infrared radiation more efficiently than carbon dioxide and may contribute more than carbon dioxide to global warming in the next century. Understanding the role of methanotrophs in the global carbon cycle is imperative. Recent research has demonstrated that soluble MMO oxidizes several groundwater pollutants, including trichloroethylene, vinyl chloride, and other halogenated hydrocarbons (10, 18). Other methylophilic bacteria have been demonstrated to metabolize the common industrial solvent dichloromethane (12). With such diverse organisms and potentially im-

TABLE 1. Descriptions and sources of methylophilic bacteria sequenced

Organism	Carbon source for growth	Pathway for formaldehyde assimilation	Source ^a	Reference
<i>Methylobacter</i> sp. strain A4	Methane	RuMP	M. Lidstrom	15
" <i>Methylobacter alba</i> " BG8	Methane	RuMP	R. Whittenbury	22
<i>Methylobacter lutea</i> ^b	Methane	RuMP	NCIMB 11914	11
" <i>Methylobacter rubra</i> "	Methane	RuMP	NCIMB 11913	5
" <i>Methylobacter flagellatus</i> KT1	Methanol	RuMP	Y. Totsenko	6
<i>Methylobacter glycogenes</i>	Methanol	RuMP	ATCC 29475	24
<i>Methylobacter methanolic</i>	Methanol	RuMP	ATCC 21852	22
<i>Methylobacter methylovora</i>	Methanol	RuMP	ATCC 21852	22
" <i>Methylosinus</i> " sp. strain B	Methane	Serine	This laboratory	19
" <i>Methylosinus</i> " sp. strain LAC	Methane	Serine	This laboratory	1
" <i>Methylosinus sporium</i> "	Methane	Serine	Y. Totsenko	22
<i>Methylobacterium</i> sp. strain M27	Methanol	Serine	NCIMB 9686	2
<i>Methylobacterium extorquens</i>	Methanol	Serine	NCIMB 9399	2
Strain PK-1	Methanol	Serine	S. Hirano	9
Strain PR-6	Methanol	Serine	S. Hirano	9

^a Strains were obtained directly from the indicated culture collection or individuals. ATCC, American Type Culture Collection, Rockville, Md.; NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland; S. Hirano, University of Wisconsin, Madison; M. Lidstrom, California Institute of Technology, Pasadena; Y. Totsenko, Russian Academy of Sciences, Puschino, Moscow Region; R. Whittenbury, University of Warwick, Coventry, England.

^b This strain was originally designated *Methylococcus luteus* NCIMB 11914. The name was changed here to reflect the findings of previous work (11) and data included in this paper.

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FIG. 1. Unrooted phylogenetic relationship among methylotrophic bacteria and other representatives within the class *Proteobacteria*. The abbreviations on the tree represent the following organisms: α -subclass reference organisms *Agrobacterium tumefaciens* (A. tume.) and *Rhodospirillum rubrum* (R. rubrum); α -subclass methylotrophs *Methylobacterium* sp. strain DM4 (M. sp. DM4), *Methylobacterium* sp. strain M27 (M. sp. M27), *Methylobacterium extorquens* (M. ext.), *Methylobacterium extorquens* AM1 (M. ext. AM1), *Methylobacterium organophilum* XX (M. org. XX), strain PK-1 (PK-1), and strain PR-6 (PR-6); α -subclass methanotrophs *Methylocystis parvus* OBBP (M. par. OBBP), "*Methylosinus*" sp. strain B (M. sp. B), "*Methylosinus*" sp. strain LAC (M. sp. LAC), "*Methylosinus methanica*" 81Z (M. meth. 81Z), "*Methylosinus sporium*" (M. spor.), and "*Methylosinus trichosporium*" OB3b (M. t. OB3b); β -subclass reference organism *Pseudomonas testosteroni* (P. test.); β -subclass methylotrophs "*Methylobacillus flagellatus*" KT1 (M. flag.), *Methylobacillus glycogenes* (M. gly.), "*Methylobacterium methanotrophus*" (M. meth. AS1), "*Methylobacterium methanotrophus*" (M. meth. AS1), and *Methylobacterium methanotrophus* AS1 (M. meth. AS1); γ -subclass reference organism *Escherichia coli*; γ -subclass methanotrophs *Methylococcus capsulatus* (M. cap.), *Methylomonas* sp. strain A4 (M. sp. A4), *Methylomonas alba* BG8 (M. alba BG8), *Methylomonas lutea* (formerly *Methylococcus luteus*) (M. luteus), *Methylomonas methanica* (M. meth.), and *Methylomonas rubra* (M. rubra).

portant applications, development of a useful taxonomic system is essential.

Tsuji et al. have demonstrated that it is possible to distinguish among and classify methylotrophic bacteria by using 16S rRNA sequence analysis (20). Furthermore, oligonucleotide signature probes based on the sequences were defined and applied to identify physiological groups of methylotrophic bacteria (17). The present work extends the sequence data base by considering methylotrophs and methanotrophs which utilize different pathways for formaldehyde assimilation.

Table 1 describes the salient properties of 15 new methylotrophs from which 16S rRNAs were sequenced. Isolation and reverse transcriptase sequencing of 16S rRNA from the organisms, data analysis, and phylogenetic tree construction were performed as previously described (20).

The sequences were determined with minimal ambiguities to over 90% of completion for each organism. Table 2 shows the percent homology determined for each pairing. Nine previously reported methylotroph 16S rRNA sequences (20) were included in the calculations. All the values were used to develop a phylogenetic tree (Fig. 1). The tree also includes representative organisms from subdivisions within the class *Proteobacteria* (13, 23): *Agrobacterium tumefaciens*, α -2; *Escherichia coli*, γ ; *Pseudomonas testosteroni*, β ; and *Rhodospirillum rubrum*, α -1.

The serine pathway methanotrophs and methylotrophs form tight, separate clusters within the α -2 subdivision. The genus "*Methylosinus*" forms a coherent group. The relationship between these organisms correlates with the observation that the members of this group produce soluble MMO, the enzyme responsible for trichloroethylene degradation (3,

19). Furthermore, the placement of strains PK-1 and PR-6, two pink-pigmented, methanol-utilizing methylotrophs isolated from garden bean plant leaves (9), with the pink-pigmented serine pathway methylotrophs of the genus *Methylobacterium* shows the utility of this analysis in rapidly classifying methylotrophs.

The RuMP pathway methylotrophs also separated into clusters. Four methanotrophs from the *Methylomonas* species group together but with less cohesiveness than the serine pathway methanotrophs. The RuMP methylotrophs unable to grow on methane were found in a different cluster with *Methylophilus methylotrophus* AS1. The four new RuMP pathway methylotrophs sequenced for this study belong to the genera *Methylomonas* and *Methylobacillus*. The homologies, however, do not match the genus designations. *Methylobacillus glycogenes* is more closely related to "*Methylomonas methylovora*" and "*Methylomonas methanolica*" than to "*Methylobacillus flagellatus*."

The information presented in this paper expands the data base of 16S rRNA sequences for methylotrophs. Moreover, the results support classical taxonomic groupings based on phenotype (22) and provide a useful and rapid tool for evaluating phylogenetic distribution of new isolates.

Nucleotide sequence accession numbers. The new nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the following accession numbers: "*Methylobacillus flagellatus*" KT1, M95651; *Methylobacillus glycogenes*, M95652; *Methylobacterium* sp. strain M27, M95653; *Methylobacterium* sp. strain PK-1, M95654; *Methylobacterium* sp. strain PR-6, M95655; *Methylobacterium extorquens*, M95656; *Methylococcus luteus*, M95657; *Methylomonas* sp. strain A4, M95658; "*Methylomonas alba*" BG8, M95659; "*Methylomonas methanolica*," M95660; "*Methylomonas methylovora*," M95661; "*Methylomonas rubra*," M95662; "*Methylosinus*" sp. strain B, M95663; "*Methylosinus*" sp. strain LAC, M95664; and "*Methylosinus sporium*," M95665.

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