Taxonomic studies on methylotrophic bacteria by 5S ribosomal RNA sequencing

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Nucleotide sequences of 5S ribosomal RNA (rRNA) isolated from 19 strains of Gram-negative methylotrophic bacteria were determined. Comparison of these sequences allowed construction of a tentative phylogenetic tree and showed that the bacteria analysed belong to the Proteobacteria and fell into several clusters, including obligate methanotrophs, obligate methylotrophs and several groups of facultative methylotrophs. Taxonomic relations between methylotrophic and non-methylotrophic bacteria are discussed, and the polyphyletic nature of methylotrophy as a taxonomic feature is highlighted.

Introduction

Methylotrophic bacteria are those which can use methane and its derivatives as a source of energy and carbon. Some methylotrophic bacteria can use C₁ compounds only (obligate methylotrophs), while others can also use multi-carbon components as growth substrates (facultative methylotrophs). Like autotrophs, these bacteria are able to synthesize all cellular components from C₁-substrates, but they also use these substrates as a source of energy. In this respect they are intermediate between true autotrophs and true heterotrophs. Despite progress in the study of the biochemistry and physiology of these bacteria, the systematics of methylotrophs remains obscure. A major problem is whether the feature of methylotrophy is monophyletic or polyphyletic in origin, and consequently whether methylotrophs should be placed in a single taxon or be split into different taxonomic units. Comparative analysis of sequences of proteins and nucleic acids, primarily ribosomal RNAs (rRNAs), has recently become a powerful tool for bacterial classification (Woese, 1987). Determination of 5S rRNA sequences allows extensive phylogenetic screening to be performed in a relatively short time (Chumakov, 1987). This paper describes the results of this approach in the study of phylogeny and systematics of methylotrophic bacteria.

Methods

Strains and growth conditions. Nineteen strains of methylotrophic bacteria were used (Table 1). Obligate methanotrophic bacteria were cultivated as described by Galchenko & Nesterov (1981). *Methylophilus methylotrophus* was grown in mineral medium (Loginova *et al.*, 1981) containing 0.5% (v/v) methanol, pH 7.0, with aeration at 30 °C. *Pseudomonas stutzeri* was grown as described earlier (Trojan *et al.*, 1978). All other strains of facultative methylotrophic bacteria were grown in Hirsch's medium (Hirsch & Conti, 1964) with 0.5% (v/v) methanol.

Isolation and sequencing of 5S rRNA. Approximately 1 g of wet cells at the end of the exponential phase were suspended in 0.01 M-sodium acetate buffer, pH 5.1, and extracted with hot (60 °C) phenol and 0.5% SDS (Chumakov, 1987). RNA was precipitated and washed with ethanol. Approximately 15 µg RNA was 3'-end-labelled *in vitro* with cytidine 3',5'-[5'-³²P]diphosphate (pCp, 'Izotop', USSR; 1000 Ci mmol⁻¹, 50 µCi per sample; 1 Ci = 37 GBq) using T4 phage RNA ligase (England & Uhlenbeck, 1978) ('Ferment', USSR; 80 units per sample). Labelled RNAs were fractionated by high-voltage electrophoresis through 8% (w/v) polyacrylamide gels (Maniatis *et al.*, 1982). 5S RNA was eluted and sequenced by the chemical method of Peattie (1979) with minor modifications (Chumakov, 1987). Sequencing gels (Maniatis *et al.*, 1982) were run in 60 × 30 × 0.19 mm slabs at 4500 V.

Phylogenetic analysis. 5S ribosomal RNA sequences were aligned as described by Wolters & Erdman (1988) and used to calculate the mutation distance matrix. Tentative phylogenetic trees were constructed using the novel 'maximum topological similarity' (MTS) method (Chumakov & Yushmanov, 1988; Yushmanov & Chumakov, 1988). The algorithm described in these papers was implemented in a 'C' language computer program in the UNIX operating system and run on a Wicat S-150 personal computer.

1. *M. capsulatus* ATCC 19069 (220182) -GCCUGGCGGCCAGAGCGAGCGGGAACCCGAUCCCAUCCCGAC
 2. '*M. agile*' A20 (220180) -GCUUGGUGACCAUAGCGAGCGUGAACCACCCGAUCCCAUCCCGAA
 3. '*M. rubra*' 15S (220179) -GCUUGGUGACCAUAGCGAGCGUGAACCACCCGAUCCCAUCCCGAA
 4. *M. methanica* 12 (220178) -NCUUGGUGACCAUAGCGAGCGUGRACCACCCGAUCCCAUCCCGAA
 5. '*M. capsulatus*' Y (220184) -NYUUGGUGACCAUAGCGAGCGUGACCCACCCGAUCCCAUCCCGAA
 6. '*M. trichosporium*' 4E (220200) NNCUUGGUGACUUAUAGUGAGCGUGAACCACCCGAUCCCAUCCCGAA
 7. '*M. parvus*' 492 (220199) NNCUUGGUGACCAUAGCGAGCGUGAACCACCCGAUCCCAUCCCGAA
 8. *M. methylotrophus* NCIB 10515 (220195) --CUUGGGGACAAUAGCGGUUUGGAACCACCCCUUCCCAUCUCGAA
 9. *Acetobacter* sp. MB 58 (220202) GAUCUGGUGGCCAUGGCGGGAGCAAAUCAGCCGAUCCCAUCCCGAA
 10. *Acetobacter* sp. 914 (220204) NNUUGAUGGCCAUGGCGGGAGCAAAUCACCCGAUCCCAUCCCGAA
 11. '*H. vulgare*' NP-160 (220151) GACCUGGUGAUUAUGGCGGGUGGUCUGCACCCGAUCUCAUCCCGAA
 12. *Hyphomicrobium* sp. G10 (220149) GACCUGGUGAUUAUGGCGGGUGGUCUGCACCCGAUCCCAUCCCGAA
 13. *M. organophilum* NP-220 (220135) --CC-GGCGGCCUGAGCGGUGUGCCAGAACCAGAUCCCAUCUCGAA
 14. *M. extorquens* AM1 (220142) -GCCUGGCGGCCUGAGCGGUGUGGAAGAACCAGAUCCCAUCUCGAA
 15. '*T. mutans*' U2 (220132) ---CUGGUGGCCAUUGCGAGGAGCCUGAACCCGACUCCAUCCCGAA
 16. '*R. vacuolatum*' Rv (220131) ---CUGGUGGCCAUUGCGAGGAGCCCCAAUUCGAUCUCACUCCCGAA
 17. '*B. viscosus*' D7 (220130) -GCCUGGUGGCUAUGGCGAGGAGUCCGAACCAGAUCCCAUCCCGAA
 18. *A. aquaticus* Ma (220133) --CCUGGUGGCCAUUGCGAGGAGCCCCAACCCGAUCCCAUCCCGAA
 19. *P. stutzeri* 8 (220144) -GCCUGGUGGCUAUGGCGAGGAGGCCUAACCAGAUCCCAUCCCGAA
1. CUCGGAAGUGAAACCGCUUAGCGCCGAUGAUAGUGCAAGAAUA-CCUGUGCGAAAGUAGGGAACUGCCAGGCUC
 2. CUCGGAAGUGAAACCGCUUAGCGCCGAUGAUAGUGCGACAGGUUGUCGUGCGAAAGUAGGGAUUGUMAAGCANN
 3. CUCGGAAGUGAAACCGCUUAGCGCCGAUGAUAGUGUGGCGAGGUUGCCAUGUGAAAGUAGGUGCAUCGCMAGCUN
 4. CUCGGAAGUGAAACCGCUUUGCGCCGAUGAUAGUGUGGCGAGUUGCCAUGUGAAAGUAGGUGCAUCGCMAGCUN
 5. CUCGGAAGUGAAACCGCUUAGUGCCGAUGAUAGUGUGGCAAUUUGCCAUGCGAAAGUAGGGAUUGCMAAAN--
 6. CUCGGAAGUGAAACCGCUUAGCGCCGAUGAUAGUGUGGCGUUGCCAUGCGAAAGUAGGGAUUGCMAAGYN-
 7. CUCGGAAGUGAAACCGCUUAGCGCCGAUGAUAGUGUGGCGUUGCCAUGCGAAAGUAGGGAUUGCMAAGCN-
 8. CAGGCGCGUGAAACGAACUUGCGCCAAUGAUAGUGUACU-CUU-CGUAUGCGAAAGUAGGUGCAUCCCAAGCC-
 9. CUCGGCCGUGAAAUUGCCCCAGCGCCCAUGAUACUCUGCCUCA-AGGACCGGAAAAGUCGGUGCGCCGAGAYY-
 10. CUCGGCCGUGAAAUUGCCCCAGCGCCCAUGAUACUCUGCCUCA-AGGACCGGAAAAGUCGGUGCGCCGAGAUCC-
 11. CUCGGCCGUGAAACCGCCAGCGCCCAUGGUACUUCGUCUUA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 12. CUCGGCCGUGAAACCGCCAGCGCCCAUGGUACUUCGUCUUA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 13. CUCGGCCGUGAAACACACACAGCGCCCAUGGUACUGUGUCUUA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 14. CUCGGCCGUGAAACACACAGCGCCCAUGGUACUGUGUCUUA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 15. CUCGGCCGUGAAACUCCUCAGCGCCCAUGGUACUAGUCUCA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 16. CUCGGCCGUGAAACUCCUCAGCGCCCAUGGUACUAGUCUCA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 17. CUCGGCCGUGAAACUCCUCAGCGCCCAUGGUACUUCGUCUCA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 18. CUCGGCCGUGAAACUCCUCAGCGCCCAUGGUACUAGUCUCA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU
 19. CUCGGCCGUGAAACUCCUCAGUGCCCAUGGUACUAGUCUUA-AGCAGCGGAGAGUAGGUGCGUGCCAGGUCU

Fig. 1. Nucleotide sequences of 5S rRNA of 19 methylotrophic bacteria, determined in this study. The EMBL accession numbers of the sequences are shown in parentheses.

Results and Discussion

Several obligate methylotrophic bacteria which use only methane or methanol as carbon and energy sources were chosen for 5S rRNA sequencing. Other bacteria in this study were facultative strains, which use C_1 compounds as well as multi-carbon compounds as growth substrates. All the bacteria used in this study were Gram-negative.

5S rRNA sequences of the methylotrophic bacteria determined in this investigation are shown in Fig. 1. These sequences were compared with published data (Wolters & Erdman, 1988) by calculating the mutation distance matrix. The distance matrix can be used to construct tentative phylogenetic trees using different approaches, such as cluster analysis, maximum parsimony methods, compatibility methods, etc. We used a new procedure, based on the so-called maximum topological similarity principle (Chumakov & Yushmanov, 1988). This approach has an important advantage over many

others in that it invokes no a priori assumptions about the mode of the evolutionary process (such as maximum parsimony in the respective method, or equal rates of mutation in different evolutionary lineages in cluster methods). The tentative unrooted tree showing the relations among the methylotrophic bacteria studied is shown in Fig. 2. The methylotrophic bacteria fell into several clusters: obligate methanotrophs, obligate methylotrophs, and several groups of facultative methylotrophs.

The phylogenetic relations within groups of obligate methano- and methylotrophic bacteria revealed by comparative 5S rRNA sequence analysis do not support the current rudimentary classification of these organisms. According to this classification, obligate methane-oxidizing bacteria are divided into five genera, comprising two groups. The first includes *Methylomonas*, *Methylococcus* and *Methylobacter* and is characterized by organisms which assimilate C_1 compounds via the ribulose monophosphate pathway and which possess

Table 1. *Strains used*

Name	Strain no.*	Reference
<i>Methylococcus capsulatus</i>	ATCC 19069	Galchenko <i>et al.</i> (1986)
' <i>Methylomonas agile</i> '	A20 ^a	Whittenbury <i>et al.</i> (1970)
' <i>Methylomonas rubra</i> '	15S ^a	Romanovskaya <i>et al.</i> (1978)
<i>Methylomonas methanica</i>	12 ^b	Galchenko <i>et al.</i> (1986)
' <i>Methylobacter capsulatus</i> '	Y ^a	Whittenbury <i>et al.</i> (1970)
' <i>Methylosinus trichosporium</i> '	4E ^a	Romanovskaya <i>et al.</i> (1978)
' <i>Methylocystis parvus</i> '	492 ^a	Romanovskaya <i>et al.</i> (1978)
<i>Methylophilus methylotrophus</i>	NCIB 10515	Byrom & Ousby (1975)
<i>Acetobacter</i> sp.	MB 58 ^c	Babel & Muller (1977)
<i>Acetobacter</i> sp.	914 ^c	Andrianova <i>et al.</i> (1986)
<i>Hyphomicrobium vulgare</i>	NP-160 ^b	Nikitin <i>et al.</i> (1986)
<i>Hyphomicrobium</i> sp.	G10 ^b	Nikitin <i>et al.</i> (1986)
<i>Methylobacterium organophilum</i>	NP-220 ^b	Patt <i>et al.</i> (1976)
<i>Methylobacterium extorquens</i>	AM1 ^c	Peel & Quayle (1961)
' <i>Tuberoidobacter mutans</i> '	U2 ^b	Slabova & Nikitin (1986)
' <i>Renobacter vacuolatum</i> '	Rv ^b	Nikitin <i>et al.</i> (1987)
' <i>Blastobacter viscosus</i> '	D7 ^b	Nikitin <i>et al.</i> (1986)
<i>Ancylobacter aquaticus</i>	Ma ^d	Nikitin <i>et al.</i> (1987)
<i>Pseudomonas stutzeri</i>	8 ^c	Troyan <i>et al.</i> (1978)

indicate that these groupings are doubtful, since all these genera were recovered in a single cluster within subdivision γ of the Proteobacteria (Stackebrandt *et al.*, 1988). The composition of the genus *Methylomonas* is not well defined (Galchenko *et al.*, 1986), and 5S rRNA sequence comparison revealed heterogeneity within this

Fig. 3. Tentative phylogenetic tree, constructed by the maximum topological similarity method, showing the position of methylotrophic (filled circles) and non-methylotrophic (open circles) bacteria.

genus. The sequences of *M. methanica* and '*M. rubra*' 5S rRNA differ significantly from those of '*M. agile*' and '*M. albus*'. Obviously, the taxonomy of *Methylobacter* requires further study.

As a whole, the obligate methane-oxidizing bacteria comprise an identifiable taxonomic entity distinct from all other methylotrophic bacteria. Another feature that enables us to cluster these bacteria together is the presence of the 4-5S RNA (data not shown). This small RNA is essential for protein synthesis in *Escherichia coli* (Brown & Fournier, 1984) and was found to be abundant in subdivision γ of the Proteobacteria, but was apparently lacking from bacteria outside this group (Chumakov, 1987).

Obligate and restricted facultative methylobacteria ('*Methanomonas methylotropha*', '*Methanomonas glucosidans*', *Methylobacter methylotrophus* and '*Methylobacter clara*') formed another well-defined group within the β subdivision of the Proteobacteria (Fig. 2). This group consists of two subgroups, the composition of which was also confirmed by DNA/DNA hybridization and fatty acid composition data (Andreev *et al.*, 1986; Govorukhina *et al.*, 1986). The results of a more extensive phylogenetic study of this group will be published elsewhere.

Several isolated branches were formed by the facultative methylobacteria. All these groups are related to subdivision α of the Proteobacteria. The separate position of the genus *Acetobacter* seems to be in good agreement with the properties of this genus: unlike most other methylotrophs it grows in media of low pH. Two other groups were represented by *Hyphomicrobium* (hypha-forming budding heterotrophic bacteria) and *Methylobacterium* (facultative methylotrophic bacteria), both of which possess similar fatty acid compositions, and utilize C_1 compounds via the serine pathway. The separate position of this latter group compared to other groups of facultative methylotrophs was also proposed by others on the basis of their phenotypic properties (Hood *et al.*, 1987).

The last branch of facultative methylotrophs was formed by representatives of different genera: *Blastobacter*, *Ancylobacter*, *Pseudomonas*, '*Renobacter*', '*Tuberoiodobacter*' (Fig. 2). The latter two genera do not yet have formal taxonomic standing; the data presented in this paper help to resolve the uncertainty of their taxonomic position. This group of methylotrophs is quite heterogeneous from the formal taxonomic viewpoint, but its constituent genera (except *Pseudomonas*) are capable of budding cell division and growth in autotrophic condi-

tions by oxidizing molecular hydrogen. These properties argue for their inclusion in a single group. Moreover, the similarity of their 5S rRNA sequences suggests that representatives of this group may be included in a single (probably new) genus. Obviously, this reclassification will require more detailed comparison of the phenotypic properties of these bacteria.

Fig. 3 shows a tentative phylogenetic tree including both methylotrophic and non-methylotrophic bacteria. It should be noted that bacteria utilizing C_1 compounds do not all form a single line of descent on the tree, but rather are intermingled with bacteria lacking this property. The ability to utilize C_1 compounds allows methylotrophs to fill various ecological and trophic niches, and hence they might be strongly selected for under certain conditions. The data in this paper indicate that obligate methane-oxidizing bacteria, and bacteria that obligately utilize other C_1 compounds, form two independent and probably monophyletic groups. On the other hand, facultative methylotrophy is probably of polyphyletic origin and as such should not be used as a heavily weighted taxonomic feature. Nevertheless, we feel that a definite conclusion on this point cannot be made only on the basis of 5S rRNA sequence comparison. It is highly desirable to study the molecular phylogeny of the key enzymes of C_1 metabolism in order to compare it with the phylogeny of the rRNA. Moreover, it is evident that 5S rRNA analysis can be regarded only as a source of preliminary phylogenetic information, due to the short length of this molecule. The use of 16S and 23S rRNA will permit more definite conclusions. Nevertheless, the ease of 5S rRNA sequence determination, and the lack of significant differences in results of 5S and 16S rRNA comparison in examples studied so far, make 5S rRNA a suitable molecule for rapid phylogenetic screening.

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