16S ribosomal RNA sequence analysis for determination of phylogenetic relationship among methylotrophs

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16S ribosomal RNAs (rRNA) of 12 methylotrophic bacteria have been almost completely sequenced to establish their phylogenetic relationships. Methylotrophs that are physiologically related are phylogenetically diverse and are scattered among the purple eubacteria (class Proteobacteria). Group I methylotrophs can be classified in the β- and the γ-subdivisions and group II methylotrophs in the α-subdivision of the purple eubacteria, respectively. Pink-pigmented facultative and non-pigmented obligate group II methylotrophs form two distinctly separate branches within the α-subdivision. The secondary structures of the 16S rRNA sequences of ‘Methylocystis parus’ strain OBBP, ‘Methylosinus trichosporium’ strain OB3b, ‘Methylsporovibrio methanica’ strain 81Z and Hyphomicrobiun sp. strain DM2 are similar, and these non-pigmented obligate group II methylotrophs form one tight cluster in the α-subdivision. The pink-pigmented facultative methylotrophs, Methylobacterium extorquens strain AM1, Methylobacterium sp. strain DM4 and Methylobacterium organophilum strain XX form another cluster within the α-subdivision. Although similar in phenotypic characteristics, Methylobacterium methylothrophicus strain AS1 and methylotrophic species DM11, which do not utilize methane, are similar in 16S rRNA sequence to bacteria in the β-subdivision. The methane-utilizing, obligate group I methanotrophs, Methylococcus capsulatus strain BATH and Methylomonas methanica, are placed in the γ-subdivision. The results demonstrate that it is possible to distinguish and classify the methylotrophic bacteria using 16S rRNA sequence analysis.

Introduction

Approximately 10³ megatons of methane are produced globally each year by anaerobic micro-organisms. The majority of this methane (80–90%) is consumed within one metre of the earth’s surface by a diverse group of methane-oxidizing bacteria known as methylotrophs (Anthony, 1982). Methylotrophs utilize reduced one-carbon compounds as carbon and energy sources and assimilate formaldehyde as a major source of cellular carbon (Anthony, 1982). Recently, methylotrophs have received considerable interest because several of them possess the enzymes dichloromethane dehalogenase or methane monooxygenase, which degrade various environmentally important pollutants, e.g. halogenated alkanes, alkenes, and mono- and poly-substituted aromatic compounds (Higgins et al., 1984; Scholtz et al., 1988). Unfortunately, it is difficult to distinguish and identify many methylotrophs by means of classical taxonomy. This paper presents an alternative method for identifying these methylotrophs using a molecular biological approach.

Whittenbury & Dalton (1983) proposed a classification scheme in which obligate methane-oxidizing bacteria are separated into groups I, II, and type X based on the pathways utilized for formaldehyde assimilation, intracytoplasmic membrane structures formed, types of resting stages formed, DNA base ratios, growth temperatures, and several other characteristics. Group I methanotrophs utilize the efficient ribulose monophosphate (RuMP) pathway for formaldehyde assimilation, lack a complete tricarboxylic acid cycle, possess bundles

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Abbreviations: PPFM, pink-pigmented facultative methylotroph; RuMP, ribulose monophosphate.

The 16S rRNA sequences of methylotrophs reported in this study have been submitted to GenBank and have been assigned the accession numbers M29021 to M29029.
of vesicular disc-shaped intracytoplasmic membranes, and contain DNA with a G + C content of 50–54 mol %. Type X methanotrophs, e.g. *Methyloccocus capsulatus*, contain DNA with a higher G + C content (62.5 mol %) and grow at 45°C. They are the only methanotrophs known to assimilate carbon dioxide via the Benson-Calvin cycle utilized by autotrophic bacteria. The majority of formaldehyde is assimilated via the RuMP pathway and small amounts via the serine pathway (Dalton & Whittenbury, 1976). Group II methyloptrophs utilize the serine pathway for formaldehyde assimilation, possess a complete tricarboxylic acid cycle, contain paired intracytoplasmic membranes around the cell periphery and contain DNA with a G + C content of approximately 62.5 mol %. The predominant fatty acid portion of phospholipids of group I and type X methanotrophs contains 16 carbon atoms, while those of group II methyloptrophs contain 18 carbon atoms (Whittenbury & Dalton, 1980).

Methyloptrophic bacteria that do not oxidize methane include the methanol- and methylamine-utilizing methylotrophs and those that use methylated sulphur compounds and chlorinated derivatives of methane. These bacteria are more diverse in morphology and physiological characteristics than the methanotrophs. Gram-positive and Gram-negative strains, and many facultative methanol- and methylamine-utilizers, as well as obligate methylotrophs that utilize methanol, methylamine or both, are known (Anthonv, 1982).

The Gram-negative bacteria that utilize methanol and methylamine contain catabolic and assimilatory sequences, except for methane monooxygenase, similar to methane-utilizing bacteria. They can be divided into two physiological groups based on the pathway for formaldehyde assimilation.

Jenkins & Jones (1987) made a taxonomic study of 115 obligate, facultative and restricted facultative methyloptrophs. Morphology, physiology, mol % G + C values of DNA, isoprenoid quinone content and polar lipid contents of the strains were examined. Their results indicated that the obligate methanol-utilizing bacteria exhibited a high degree of phenotypic similarity and were taxonomically distinct from other methanol-utilizing bacteria.

Facultative methanol- and methylamine-utilizing methyloptrophs have over the years been given various generic names, such as *Vibrio, Flavobacterium*, *Protaminobacter, Pseudomonas*, and finally *Methylobacterium* (Green & Bousfield, 1981; Whittenbury et al., 1970). According to Green & Bousfield (1981), pink-pigmented facultative methyloptrophs (PPMFs) are morphologically similar, utilize the serine pathway for formaldehyde assimilation, and are clustered within a single group of phenotypically similar bacteria with five sub-clusters. Thus, Green & Bousfield (1982) suggested that all PPFMs should be included in the genus *Methylobacterium*. Green & Gibson (1984) later stated that the PPFMs are probably heterogeneous and suggested that molecular biological approaches are needed to resolve the taxonomic dilemma.

Realizing the limitation of conventional taxonomic approaches, Whittenbury & Krieg (1984) pleaded with researchers to refrain from publishing proposals of new genera and new species of methane oxidizers until a firm basis for a taxonomy was established. They challenged researchers to apply molecular biological techniques, e.g. 16S rDNA sequencing, in order to design a useful taxonomic system for methyloptrophs.

Ribosomal RNA (rRNA) appears to be the choice for molecular biological approaches to phylogeny. As opposed to DNA, rRNAs are abundant, comprising approximately 20% of the dry weight of an *Escherichia coli* cell (Ingraham et al., 1983) making it easier to isolate these molecules. The rRNAs are universally distributed, evolutionarily homologous, functionally important, conserved in nucleotide sequences and overall secondary structure, and lack artifacts of lateral transfer between contemporary organisms (Pace et al., 1986). rRNA sequences are independent of growth conditions (Gibson et al., 1979) and are useful for comparing even physiologically dissimilar organisms (Woese, 1987). Of SS, 16S and 23S rRNAs, the SS and 16S molecules have received the most attention, largely for historical and technical reasons (Pace et al., 1986). Although SS rRNA has been utilized to study phylogenetic relatedness of methyloptrophs (Chumakov, 1987; Horis & Osiwa, 1979; Wolfrum & Stolp, 1987) Pace et al. (1986) and Woese (1987) found it to be too small (about 120 nucleotides) to be useful for phylogenetic inferences. Structural analysis of 16S rRNA (about 1500 nucleotides) enabled Woese and his co-workers to infer phylogenetic relationships among a wide variety of organisms and established evolutionary distinctions between archaeobacteria and eubacteria (Woese, 1987; Fox et al., 1980).

**Methods**

*Micro-organisms.* *Methylococcus capsulatus* strain BATH was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, UK; *Methylocystis parvus* strain OBBP and *Methylomus trichosporium* strain OB3b were kindly provided by R. Whittenbury, University of Warwick, Coventry, UK; *Methylophilus extorquens* strain AM1 was obtained from P. Goodwin, North East Surrey College of Technology, Surrey, UK; *Methylphilus methylotrophus* strain AS1 was obtained from Imperial Chemical Industries, Billingham, UK; *Methylobacterium* sp. strain DM4, methylotrophic species DM11, and *Hyphomicrobium* sp. strain DM2 were obtained from T. Leisinger, Mikrobiologisches Institut ETH-Zentrum/LFY, Zürich, Switzerland. The other strains are isolates of R. S. Hanson and are available from the Gray Freshwater Biological Institute.
Methylomonas methanica, Methylobacterium extorquens strain AM1, Methylo bacterium organophili um strain XX and M ethylobacterium sp. strain DM4 are all pink-pigmented and, with the exception of Methylomonas methanica, are all group II facultative methylotrophs (Patt et al., 1976; Kohler-Staub et al., 1987). 'Methylcystis parus' strain OBBP, 'Methylosinus trichosporium' strain OB3b and 'Methylosporovibrio methanica' strain 81Z are non-pigmented and are obligate group II methylotrophs. Methyloccus capsulatus strain BATH, Methylomonas methanica and Methylosinus methylotrphus strain AS1 are group I obligate methylotrophs. Methyloccus capsulatus is a unique micro-organism which possesses ribulose bisphosphate carboxylase and has been described as a type X methanotroph (Whittenbury & Dalton, 1980). While an obligate methylotroph, Methylomonas methylotrphus strain AS1 does not utilize methane as sole carbon and energy source and does not normally possess the intracellular membrane associated with methane assimilation in the group I organisms. Methylotrophic species DM11 is an uncharacterized bacterium that appears to belong to group I and is capable of growth on dichloromethane, methanol and methylamine (Scholtz et al., 1988). Strain DM2 is a Hyphomicrobiurn species (Kohler-Staub et al., 1987).

Culture conditions. Methylotrophic bacteria were grown in a minimal salts medium (Dalton & Whittenbury, 1976) with either methane or methanol as the sole carbon source. Methyloccus capsulatus, 'Methylcystis parus' strain OBBP, M ethylosinus methanica, Methylo philus methylotrphus strain AS1, 'Methylosinus trichosporium' strain OB3b and 'Methylosporovibrio methanica' strain 81Z were grown in the presence of methane and air (25:75, v/v), and that other bacteria with 0-5% (v/v) methanol. With the exception of Methyloccus capsulatus, which was incubated at 37°C, all bacteria were grown with continual agitation at 30°C. Stock cultures, kept at ~70°C in 35% glycerol, were used to inoculate cultures. The cultures were activated by transferring them into 10 ml liquid medium, which was incubated until visible growth was observed (approximately 1-2 weeks). Active cultures were used to inoculate cultures. The cultures were activated by transferring them into 10 ml liquid medium, which was incubated until visible growth was observed (approximately 1-2 weeks). Active cultures were then transferred into 100 ml minimal salts medium and incubated for 3-7 d. Cells were harvested by centrifugation and were washed twice with distilled water. Cell pellets (approximately 100 mg wet wt) were transferred into 2.5 ml micro screw-cap tubes (no. 72.693, Sarstedt, FRG) and stored frozen until used. In order to confirm the results, most of the bacteria were grown twice and total RNA was isolated from both cultures for 16S rRNA sequencing.

Sequencing 16S rRNAs. Cell lysis and total RNA isolation from methylotrophs was accomplished by the hot phenol/sodium lauryl sulphate extraction method described by Stahl et al. (1984). The 16S rRNA in bulk cellular RNA preparations was used for sequencing analysis without further purification.

Eight synthetic 16S rRNA-specific oligonucleotide primers, which mimic conserved sequence regions of the 16S rRNA molecules of E. coli (Pace et al., 1986), were used. These primers are complementary to the E. coli 16S rRNA sequence positions 109–125, 342–357, 513–517, 690–704, 906–920, 1100–1115, 1392–1406 and 1515–1539, respectively. The primers anneal to the specific 'priming sites' on the template. The primer sequences were elongated by use of a reverse transcriptase (Lane et al., 1985) and the resulting DNA sequences determined by the dideoxynucleotide sequencing method (Sanger et al., 1977).

Phylogenetic tree. All manipulations of the sequence data, including alignment, were performed on a VAX 8600 computer (VMS operating system) using programs written by Olsen (1983). The homology calculation and phylogenetic tree construction were conducted on a SUN 3/160 workstation (UNIX operating system) using programs of De Soete (1983). This method partially corrects the observed number of sequence differences for multiple and back mutations (Jukes & Canter, 1969). For phylogenetic tree construction, the program uses a steepest-descent method, utilizing the weighted least-mean-square difference criterion. This method statistically weighs the difference between the estimated evolutionary distance separating each pair of sequences and the corresponding tree distance for optimum tree construction (Olsen et al., 1986).

Results and Discussion

Over 90% of 16S rRNA molecules were sequenced for the majority of the methylotrophic bacteria examined. Typical secondary structures of 16S rRNA sequences of the groups I and II methylotrophs, represented by Methylphilus methylotrphus strain AS1 and 'Methylosinus trichosporium' strain OB3b, are presented in Figs 1 and 2, respectively. Complete sequences are not required for phylogenetic characterization of most organisms, however. The phylogenetic trees inferred from partial sequences of the 16S rRNA have been demonstrated to have topologies identical to those obtained from complete sequences (Lane et al., 1985).

Phylogenetic relationship of methylotrophs

A phylogenetic tree summarizing the relationship among methylotrophic bacteria and other representatives of the purple eubacteria is presented in Fig. 3. Segment lengths are drawn proportionally to evolutionary distance as estimated from sequence divergence. The phylogenetic relationships among the methylotrophs computed using the steepest-descent method (Olsen, 1983), the least-squares algorithm (De Soete, 1983), and the maximum likelihood method (Felsenstein, 1981) are essentially similar, although there are some subtle differences in evolutionary distance.

As might be expected, the morphologically and physiologically diverse group of methane-utilizing bacte ria included in the family Methylococcaceae (Whittenbury & Krieg, 1984) is not an evolutionarily coherent group but rather scattered among the purple eubacteria (Fox et al., 1980). This purple eubacterial group (class Proteobacteria; Stackebrandt et al., 1988), which may be divided into the α-, β- and δ-subdivisions (Woese, 1987), includes rhizobacteria, agrobacteria and rickettsi as in addition to enteric bacteria, vibrios, pseudomonads, and other groups.

The group I methylotrophs could be sub-divided into two groups, belonging to the β- and γ-subdivisions, respectively; the group II methylotrophs all fell into the α-subdivision. Comparison with the 11 signature nucleotide sequences used by Woese et al. (1984a, b) for classification of micro-organisms also confirmed this subdivision. The α- and β/γ-subdivisions may be differentiated by the differences in 16S rRNA sequences, specifically between the nucleotide positions
Fig. 1. Secondary structure of the 16S rRNA sequence of *Methylophilus methylotrophus* strain AS1, an obligate group I methylotrophic bacterium.
Fig. 2. Secondary structure of 16S rRNA sequence of 'Methylosinus trichosporium' strain OB3b, an obligate group II methanotrophic bacterium.
The phylogenetic relationship among methylotrophic bacteria and other representatives of the purple eubacteria. The methylotrophs shown are: *Methylobacterium extorquens* strain AM1, *Methylobacterium* sp. strain DM4, *Methylobacterium organophilum* strain XX, *Methylocystis parvus* strain OBBP, *Methylosinus trichosporium* strain OB3b, *Methylosporovibrio methanica* strain 812, *Methylomonas methanica* strain BATH, *Methylomonas* methanica and *Methylphilus methylotrophus* strain AS1. Methylotrophic species DM11 and *Hyphomicrobium* sp. strain DM2 are not shown since confidence in their segment lengths is significantly lower than those of others, due to the limited number of nucleotides sequenced. Reference micro-organisms shown are as follows. α-Subclass: *Rhodospirillum rubrum, Rhodospirillum fulvum, Rhodopseudomonas globiformis, Agrobacterium tumefaciens, Rhodomicrobium vannielii, Rhodopseudomonas palustris, Rhodopseudomonas acidophila, Pseudomonas diminuta, Rhodobacter capsulatus, Erythrobacter sp. OC101, Erythrobacter longus*. β-Subclass: *Rhodocyclus gelatinosa, Pseudomonas (Comamonas) testosteroni, Alcaligenes faecalis, Pseudomonas cepacia, Spirillum volutans, Nitrosonomas europaea, Neisseria gonorrhoeae*. γ-Subclass: *Escherichia coli*. The scale bar represents an observed percentage sequence dissimilarity, which was defined as $U/(M + U + G/2) \times 100$, where $M$ is the number of identical residues, $U$ is the number of dissimilar residues, and $G$ is the number of the sequences containing an alignment gap (Olsen et al., 1986).

180 and 220 (*E. coli* designate) (Woese, 1987). Figs 4(a) and 4(b) show the partial secondary structures of the 16S rRNA sequences of *Methylomonas methanica*, a group I obligate methylotroph, and *Methylocystis parvus* strain OBBP, a group II obligate methylotroph, illustrating the differences between the α- and β/γ-subdivisions.

It has been reported that members of the β-subdivision differ from those of the α-subdivision in cytochrome c: cytochromes of the β-subdivision are of the small-subunit group, while cytochromes of the α-subdivision are of the medium or large group (Woese, 1987). Thus, it may be of interest to confirm if such differences in cytochrome hold for methylotrophs of the two subdivisions.

**The α-subdivision**

Within the α-subdivision, the PPFMs and non-pigmented obligate group II methylotrophs form two separate branches. The non-pigmented group II methylotrophs, *Methylocystis parvus* strain OBBP, *Methylosinus trichosporium* strain OB3b, *Methylosporovibrio methanica* strain 812 and *Hyphomicrobium* sp. strain DM2 form one cluster (Fig. 3). Sequence differences among methylotrophs of this cluster are under 7%. All but *Hyphomicrobium* sp. strain DM2 are obligate group II methanotrophs and are obligate aerobes. *Hyphomicrobium* sp. strain DM2 utilizes methanol, methylamine and dichloromethane as carbon and energy sources and is
Fig. 4. Partial secondary structures of the 16S rRNA sequences, highlighting differences among methylotrophs. (a, b) Sequence differences between nucleotide positions 180 and 220 (E. coli designate) of a group I methylotroph, Methylomonas methanica (a) and a group II methylotroph, 'Methylocystis parus' strain OBBP (b), illustrating the differences between the α- and the βγ-subdivisions of the purple eubacteria. (c, d) Sequence differences between nucleotide positions 440 and 490 (E. coli designate) of the group II PPFMs Methylobacterium extorquens strain AM1 (c) and Methylobacterium organophilum strain XX (d), both of which belong to the α-subdivision. (e, f) Sequence differences between nucleotide positions 60 and 110 (E. coli designate) of Methylococcus capsulatus strain BATH (α-subdivision; e) and Methylophilus methylotrophus strain AS1 (β-subdivision; f), illustrating the differences within the group I methylotrophs.
capable of anaerobic growth with nitrate as a terminal electron acceptor.

The secondary structures of the 16S rRNA of 'Methylcocystis parus' strain OBBP, 'Methylosinus trichosporium' strain OB3b and 'Methylosporovibrio methanica' strain 81Z are essentially similar. A typical secondary structure of 16S rRNA sequence of the α-subdivision, that of 'Methylosinus trichosporium' strain OB3b, is shown in Fig. 2.

The PPFMs, Methylobacterium extorquens strain AM1, Methylobacterium sp. strain DM4 and Methylobacterium organophilum strain XX, form another cluster within the α-subdivision (Fig. 3). Although these organisms were found in the same cluster, there are quite distinct differences in the secondary structures of 16S rRNA between Methylobacterium extorquens strain AM1 and Methylobacterium organophilum strain XX, specifically in the region between nucleotides 450 and 480 (E. coli designate). The secondary structure of M. extorquens strain AM1 includes an additional stem and loop structure (Fig. 4c, d).

Comparison with the signature nucleotide sequences used by Woese et al. (1984a) to further subdivide microorganisms into four separate α-subgroups indicated that the methylo trophs that belong in these two clusters are phylogenetically related to those classified in the α-2 subgroup. The α-1, α-2, α-3 and α-4 subgroups include such bacteria as Agaspasilirum itersonii, Azospirillum brasilense and Rhodospirillum rubrum; Agrobacterium tumefaciens and Pseudomonas diminuta; Rhodopseudomonas palustris and Paracoccus denitrificans; and Erythrobacter longus, respectively (Woese, 1984a).

The γ-subdivision

The group I obligate methanotrophs Methylococcus capsulatus and Methylococcus methanica fall into the γ-subdivision. The former is a type X methanotroph and the latter is a group I methanotroph; they share the RuMP pathway but differ in DNA base content, optimum growth temperature and morphology (Whittenbury & Krieg, 1984). Sequence differences between these methanotrophs are over 20%. None of the reference microorganisms belonging to the γ-subdivision fell close to the branch of these methanotrophs, however. The nucleotide sequence AACUCAAAUG, unique to the γ-subdivision (Woese et al., 1985) is also absent in these two methanotrophs. Comparison with nucleotide sequences specific to the γ-subdivision (Woese et al., 1985) indicated that the 16S rRNA sequences of Methylococcus capsulatus and Methylococcus methanica are clearly distinct from those in the γ-1 and γ-2 subgroups; comparisons with those in the γ-3 subgroup were inconclusive. Further detailed studies are needed to classify these methanotrophs precisely into subgroups.

The γ-1, γ-2, and γ-3 subgroups include the following bacteria: Nitrosococcus oceanus; Legionella; and E. coli, Pseudomonas aeruginosa and Proteus vulgaris, respectively (Woese et al., 1985).

The β-subdivision

The group I obligate methylotrophs Methylophilus methylotrophus strain AS1 and methylotrophic species DM11, on the other hand, fall into the β-subdivision. Distant separation of this group of obligate methylotrophs from the obligate methanotrophs that belong to the γ-subdivision may be supported by the fact that Methylophilus methylotrophus strain AS1 does not utilize methane or normally possess intracellular membranes typical of group I methanotrophs. The major difference in the secondary structures of 16S rRNA sequences of these methylotrophs, typified by Methylococcus capsulatus strain BATH (γ-subdivision) and Methylophilus methylotrophus strain AS1 (β-subdivision) is highlighted in Fig. 4(e, f).

According to the signature nucleotide sequences specific to the β-subdivision (Woese et al., 1984b), Methylophilus methylotrophus strain AS1 and methylotrophic species DM11 are similar in 16S rRNA sequence to bacteria in the β-3 subgroup. A secondary structure of 16S rRNA of Methylophilus methylotrophus strain AS1 is presented in Fig. 1. The β-subdivision includes Aquaspirillum aquaticum and Pseudomonas (Comamonas) testosteroni; Alcaligenes faecalis and Pseudomonas cepacia; Nitrosomonas europaea and Spirillum volutans in the β-1, β-2 and β-3 subgroups, respectively (Woese et al., 1984b).

Concluding remarks

Until recently, the biochemical markers used to define microbial community structure have been confined to cell envelope and membrane components. These markers offer some opportunities for categorizing populations but are inadequate for varieties of microorganisms with similar metabolic traits. Using conventional taxonomic approaches, Green and co-workers (Green & Bousfield, 1982; Green & Gibson, 1984) concluded that Methylobacterium organophilum strain XX was virtually indistinguishable from other PPFMs. The original description of Methylobacterium organophilum strain XX included its ability to grow on methane, a characteristic not found in other PPFMs (Patt et al., 1974). However, this characteristic was readily lost and difficult to reproduce. The two strains utilized most for genetic studies are Methylobacterium organophilum strain XX and Methylobacterium extorquens strain AM1.
Although similar in phenotypic characteristics, these two strains are clearly distinguishable by their 16S rRNA sequences (Fig. 4c, d).

The present study demonstrates that it is possible to distinguish and classify the methylo trophic bacteria using 16S rRNA sequence analysis. Our results also indicate that phylogenetic relationships based on 16S rRNA sequences reflect the classical taxonomic classification systems based on phenotypic characteristics for methylo trophs. Thus, 16S rRNA sequence analysis should be a useful tool for detailed classification of methylo trophs. Unique sequences within the 16S rRNA molecules of methylo trophs identified in our study may be utilized to construct species- or group-specific probes for taxonomic, commercial and environmental applications.

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