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 SS 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 (Troyan 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 SS 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 (3'CP, 'Izotop', USSR; 1 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). SS RNA was eluted and sequenced by the chemical method of Peattie et al., 1977) using T4 phage RNA ligase (England & Uhlenbeck, 1978) ('Ferment', USSR; 80 units per sample). Sequencing gels (Maniatis et al., 1982) were run in 60 × 30 × 0-19 mm slabs at 4500 V.

Phylogenetic analysis. SS 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.
Results and Discussion

Several obligate methylotrophic bacteria which use only methane or methanol as carbon and energy sources were chosen for 16S rRNA sequencing. Other bacteria in this study were facultative strains, which use C1 compounds as well as multi-carbon compounds as growth substrates. All the bacteria used in this study were Gram-negative.

16S 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 methanoo- methylotrophic bacteria revealed by comparative 16S rRNA sequence analysis do not support the current rudimentary classification of these organisms. According to this classification, obligate methanooxidizing bacteria are divided into five genera, comprising two groups. The first includes Methylocystis, Methylococcus and Methylobacter and is characterized by organisms which assimilate C1 compounds via the methanooxidizing enzyme. The second includes Methylococcus, Methylocystis and Methylobacter and is characterized by organisms which assimilate C1 compounds via the ribulose monophosphate pathway and which possess
bundles of disk-shaped vesicles distributed throughout the cell. The second includes *Methylosinus* and *Methylocystis*, which assimilate C₁ compounds via the serine pathway and possess paired membranous vesicles located at the periphery of the cells. SS rRNA comparison and DNA/DNA hybridization data (Doronina et al., 1988) 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 SS rRNA sequence comparison revealed heterogeneity within this

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**Table 1. Strains used**

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylococcus capsulatus</em></td>
<td>ATCC 19069</td>
<td>Galchenko et al. (1986)</td>
</tr>
<tr>
<td>'Methylomonas agile'</td>
<td>A20</td>
<td>Whittenbury et al. (1970)</td>
</tr>
<tr>
<td>'Methylomonas rubra'</td>
<td>155</td>
<td>Romanovskaya et al. (1978)</td>
</tr>
<tr>
<td><em>Methylomonas methanica</em></td>
<td>12°</td>
<td>Galchenko et al. (1986)</td>
</tr>
<tr>
<td>'Methylomonas capsulatus'</td>
<td>Y°</td>
<td>Whittenbury et al. (1970)</td>
</tr>
<tr>
<td>'Methylosinus trichosporium'</td>
<td>4E°</td>
<td>Romanovskaya et al. (1978)</td>
</tr>
<tr>
<td>'Methylocystis parvis'</td>
<td>492°</td>
<td>Romanovskaya et al. (1978)</td>
</tr>
<tr>
<td><em>Methylphilus methylotrophus</em></td>
<td>NCIB 10515</td>
<td>Byrom &amp; Ousby (1975)</td>
</tr>
<tr>
<td>Acetobacter sp.</td>
<td>MB 58°</td>
<td>Babel &amp; Müller (1977)</td>
</tr>
<tr>
<td>Acetobacter sp.</td>
<td>914°</td>
<td>Andrianova et al. (1986)</td>
</tr>
<tr>
<td>Hyphomicrobium vulgar</td>
<td>NP-160°</td>
<td>Nikitin et al. (1986)</td>
</tr>
<tr>
<td>Hyphomicrobium sp.</td>
<td>G10°</td>
<td>Nikitin et al. (1986)</td>
</tr>
<tr>
<td><em>Methylobacterium organophilum</em></td>
<td>NP-220°</td>
<td>Patt et al. (1976)</td>
</tr>
<tr>
<td><em>Methylobacterium extorquens</em></td>
<td>AM1°</td>
<td>Peel &amp; Quayle (1961)</td>
</tr>
<tr>
<td>'Tuberoidobacter mutans'</td>
<td>U2°</td>
<td>Slabova &amp; Nikitin (1986)</td>
</tr>
<tr>
<td>'Bacillus vacuolatmum'</td>
<td>RV°</td>
<td>Nikitin et al. (1987)</td>
</tr>
<tr>
<td>'Blastobacter viscosus'</td>
<td>D7°</td>
<td>Nikitin et al. (1986)</td>
</tr>
<tr>
<td>Ancylobacter aquaticus</td>
<td>Ma°</td>
<td>Nikitin et al. (1987)</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>8°</td>
<td>Troyan et al. (1978)</td>
</tr>
</tbody>
</table>

* Strain collections: a, Collection of Institute of Microbiology and Virology, Kiev; b, Collection of Institute of Microbiology, Moscow; c, Collection of Microbiology Department, Moscow-State University; d, Collection of University of Göttingen, FRG.
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.

Several isolated branches were formed by the facultative methylobacteria. All these groups are related to subdivision $\alpha$ 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*, *Tuberoïdobacter* (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-

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Obligate and restricted facultative methylobacteria ('*Methanomonas methyllovora*', '*Methanolomonas glucose-oxidans*', *Methylophilus methylotrophus* and '*Methylo-

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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 C1 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 C1 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 C1 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 C1 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.

References


