Contribution of Genome Characteristics to Assessment of Taxonomy of Obligate Methanotrophs

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The DNAs of a variety of obligate methanotrophic bacteria were analyzed for base composition and nucleotide distribution. Genome molecular weights were determined for representative strains. Similarity maps attained by plotting DNA base composition versus nucleotide distribution and genome molecular weight showed that related species formed distinct clusters. Group I methanotrophs were found to form three clusters in both nucleotide distribution and genome size analyses. The first cluster consisted of five Methylococcus species, Methyloccus methanicus, Methylococcus fohnarum, Methylococys aurantiaca, "Methylococcus rubra," and "Methylococcus agile." The other clusters included both Methylococys species and Methyloccus species, indicating the heterogeneity within these genera. One cluster contained low-G+C-content Methyloccus strains and included Methyloccus whitneyi, Methyloccus bovis, Methyloccus vinelandii, Methyloccus luteus, and "Methyloccus uranilicus." The type strains of Methylococys pelagica and "Methyloccus alba" and a marine methanotrophic strain also clustered with the low-G+C-content Methyloccus group rather than with the genus Methyloccus. "Methyloccus gracilis" also appeared to be genetically distinct from the true Methylococcus species and clustered with the high-G+C-content Methyloccus strains. This cluster included Methyloccus capsulatus, Methyloccus thermophilus, and other moderately thermophilic group I methanotrophic strains. The group II methanotrophs belonging to the genera "Methyllosinus" and "Methyllobacter" formed separate generic clusters according to genome molecular weight data but not according to nucleotide distribution data.

Methanotrophic bacteria are a diverse group of gram-negative bacteria which obligately utilize C1 compounds, including methane, methanol, and methylamine, as sole sources of carbon and energy (3, 5, 11, 27). They are unable to utilize as sole energy sources any compounds with carbon-carbon bonds. The taxonomy of methanotrophs is based mainly on morphological characters, and the systematics of this group remains confused and incomplete (26). Studies on biochemical pathways, ultrastructure of intracytoplasmic membranes (3, 11), 16S rRNA sequences (25), and fatty acid contents (1, 2, 20) have shown that methanotrophs form two major groups (groups I and II). The group I methanotrophs include the genera Methyloccus and Methyloccus and are characterized by the presence of stacked vesicular disks of intracytoplasmic membrane, the ribulose monophosphate pathway for C1 compound incorporation, and mainly 16:1 and 16:0 fatty acids; these organisms appear to belong to the γ subdivision of the proteobacteria. The group II methanotrophs (the genera "Methyllosinus" and "Methyllobacter") are characterized by intracytoplasmic membranes that are arranged peripherally in the cell parallel to the cytoplasmic membrane, the serine pathway for C1 compounds, and mainly 18:1 fatty acid and belong to the α subdivision of the proteobacteria. Currently only the group I genera Methyloccus and Methyloccus have valid taxonomic status (17, 22). The other genera are not on the Approved Lists of Bacterial Names (17, 22) and have no standing in bacterial nomenclature. Chemotaxonomic and immunological analyses (4, 6, 8, 15, 16) have clearly shown that the group I methanotrophs can be separated into three groups.

The physicochemical properties of DNA have proved to be useful as a means of characterizing and identifying budding and hyphal bacteria (9, 13). DNA compositional nucleotide distribution studies (7) basically involve measuring the shape of DNA melting profiles. Two measures can be obtained. The first measure is the DNA melting transition width, which is indicative of the nucleotide distribution in a bacterial genome. The nucleotide distribution (σg + σr) is determined from the time interval required for DNA to denature one standard deviation to the left (σg) and right (σr) of the melting temperature and is expressed as G+C content. The second measure is the ratio (σg/σr), which is a measure of the skewness or asymmetry of the melting curve. The results of De Ley (7) showed that nucleotide distributions within bacterial genera appear to be consistent and thus can be used effectively in taxonomic studies. Likewise, the use of DNA renaturation techniques has proved to be a useful means to accurately determine genome molecular weight (10). These properties of DNA were used in this study in an attempt to observe the inter- and intrageneric relationships of various representatives of methane-utilizing bacteria and to assess the taxonomy of these organisms as a guide to further study.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Cultivation. Methane-utilizing strains were grown on solidified NMS medium (5) under a methane-air-CO2 (5:4:1) atmosphere. Marine methanotrophic strain A4 was cultivated on NMS medium supplemented with 0.5% NaCl and 5 ml of Staley vitamin solution (24) per liter. Methylococys pelagica was grown on NMS medium prepared with artificial seawater (derived from Difco marine agar 2216 [catalog no. 0979]), supplemented with 5 ml of vitamin solution per liter, and solidified with 1% agarose (type V) (21). Most strains were incubated in the dark at 28°C; the exceptions were Methyloccus capsulatus strains, "Methyloccus gracilis" strains, and Methyloccus sp. strains JB87, JB137, JB146, and JB173, which were incubated at 45°C.
DNA extraction. Cultures were grown on agar plates for 3 to 5 days. Cell growth was harvested from plates with distilled water and washed twice with saline-EDTA (0.1 M disodium EDTA, 0.15 M NaCl; pH 8.0) by using centrifugation at 10,000 × g for 10 min. Cells of “Methylosinus” and “Methylocystis” strains were pretreated with a solution containing 1 mg of lysozyme per ml for 1 h at 37°C before lysis. All strains were lysed by adding 2% (final concentration) sodium dodecyl sulfate at 60°C. DNAs were extracted and purified from cell lysates by using a modified Marmur technique (23).

DNA analyses. DNA samples were analyzed by using a
FIG. 1. Similarity map of obligately methanotrophic bacteria based on DNA base compositions and nucleotide distributions. The numbers are the laboratory numbers for strains given in Table 1.

RESULTS AND DISCUSSION

The DNA base composition, nucleotide distribution, and genome molecular weight for each of the methanotrophs which we studied are shown in Table 1. Similarity maps constructed by plotting nucleotide distribution versus DNA base composition (G+C content) (Fig. 1) and genome molecular weight versus DNA base composition (G+C content) (Fig. 2) were created from the DNA analysis data.

Nucleotide distributions. The group I methanotrophs formed three clusters on the nucleotide distribution-DNA base composition map. Strains of *Methylococcus* spp. produced two separate clusters. The first cluster contained *Methylococcus capsulatus*, *Methylococcus thermophilus*, and other moderately thermophilic strains with DNAs having high G+C contents (59 to 66 mol%) and transition widths of 10.2 to 11.5 mol% G+C. Strains of "*Methylomonas gracilis*" also clustered with these strains. The low-G+C-content *Methylococcus* cluster also included "*Methylomonas alba*," *Methylomonas pelagica* (21), and marine methan-
otrophic strain A4 of Lidstrom (14). The species *Methylomonas methanica, Methylomonas fodinarum, Methylomonas aurantiaca*, "*Methylomonas rubra,*" and "*Methylomonas agile*" exhibited DNA melting transition widths of 10.5 to 12.1 mol% G+C and formed a single cluster. The DNA base compositions of the members of this cluster ranged from 51 to 60 mol% G+C.

The group II methanotrophs (members of the genera "*Methylosinus*" and "*Methylocystis*") exhibited similar nucleotide distributions and DNA base compositions and formed two overlapping clusters (Fig. 1). For these strains the G+C contents ranged from 62 to 67 mol% and the transition widths ranged from 7.5 to 9.7 mol% G+C.

**Genome molecular weights.** The genome molecular weights of the methanotrophs which we studied were useful for demonstrating relationships between taxa; these relationships were consistent with the relationships determined by using nucleotide distribution data.

The *Methylomonas* species again fell into separate clusters. The genome sizes of *Methylomonas methanica* strains were similar to those of *Methylomonas fodinarum, Methylomonas aurantiaca, "Methylomonas rubra," and "Methylomonas agile*" strains, varying from $1.8 \times 10^9$ to $2.6 \times 10^9$ daltons. On the other hand, "*Methylomonas gracilis*" appeared to be genotypically similar to *Methylococcus thermophilus* and *Methylococcus capsulatus* (Table 1), with which it shares phenotypic characteristics that are common to other moderately thermophilic group I methanotrophs (19, 20) and are not present in most mesophilic group I methanotrophs. These characteristics include the ability to fix nitrogen and the ability to fix CO$_2$ via Calvin-Benson cycle enzymes in the presence of methane (18, 19, 26). *Methylomonas pelagica* also appeared to be unrelated to *Methylomonas methanica* and clustered with the low-G+C-content *Methylococcus* strains because of its significantly smaller genome size and the broad DNA melting transition widths of these organisms. The genome size of "*Methylomonas alba*" overlapped the genome sizes determined for members of the *Methylomonas* cluster, but this organism had a significantly higher transition width, which was similar to the transition widths determined for the low-G+C-content *Methylococcus* strains. These observations indicate that the description given previously for the genus *Methylomonas* (19) may be imprecise; this description has led to the assignment of morphologically similar but genotypically unrelated species to the genus. *Methylococcus capsulatus, Methylococcus thermophilus,* and moderately thermophilic strains JB137, JB140, and JB146 have genome sizes ($2.4 \times 10^9$ to $3.0 \times 10^9$ daltons) that are considerably larger than the genome sizes of the other.
species of the genus *Methylococcus* (19), which also have lower G+C contents (55 mol% or less). The latter species have genome sizes that range from $1.6 \times 10^9$ to $2.0 \times 10^9$ daltons. The genus *Methylococcus* was defined on the basis of purely phenotypic characteristics (19) and is clearly genotypically heterogeneous. The results of several limited studies of both phenotypic and chemotaxonomic properties (1, 2, 6, 8, 20) support this view.

The group II methanotrophs (the genera "*Methylosinus*" and "*Methylocystis*") can be distinguished by their different genome molecular weights. "*Methylocystis*" genome sizes range from $2.0 \times 10^9$ to $2.5 \times 10^9$ daltons and are on the average 50 to 100% larger than the genome sizes of "*Methylosinus*" strains (range, $1.3 \times 10^9$ to $1.5 \times 10^9$ daltons).

This study of the physicochemical properties of genomic DNAs provided information that is helpful for assessing the taxonomic relationships among the genera and species of the obligately methanotrophic bacteria; in particular, it demonstrated the genetic heterogeneity in the genera *Methylomonas* and *Methylococcus*. This approach was undertaken as the first stage of an extensive taxonomic study of these organisms, and the results highlight the areas that need nomenclatural revision.

REFERENCES