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EXPERIMENTAL ARTICLES

Phylogenetic Analysis of Dichloromethane-Utilizing Aerobic Methylotrophic Bacteria

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Abstract—The phylogenetic relationships of 12 aerobic dichloromethane-degrading bacteria that implement different C_1-assimilation pathways was determined based on 16S ribosomal RNA sequences and DNA–DNA hybridization data. The restricted facultative methylotroph *"Methylophilus leisingerii"* DM11 with the ribulose monophosphate pathway was found to belong to the genus *Methylophilus* cluster of the beta subclass of Proteobacteria. The facultative methylotroph *Methylorhabdus multivorans* DM13 was assigned to a separate branch of the alpha-2 group of Proteobacteria. *Paracoccus methylutens* DM12, which utilizes C_1-compounds via the Calvin cycle, was found to belong to the alpha-3 group of Proteobacteria (more precisely, to the genus *Paracoccus* cluster). Thus, phylogenetic analysis confirmed the taxonomic status of these recently characterized bacteria. According to the degree of DNA homology, several novel strains of methylotrophic bacteria were divided into three genotypic groups within the alpha-2 group of the Proteobacteria. Genotypic group 1, comprising strains DM1, DM3, and DM5 through DM9, and genotypic group 3, comprising strain DM10, were phylogenetically close to the methylotrophic bacteria of the genus *Methylopila*, whereas genotypic group 2 (strain DM4) was close to bacteria of the genus *Methylobacterium*. The genotypic groups obviously represent distinct taxa of methylotrophic bacteria, whose status should be confirmed by phenotypic analysis.

Key words: methylotrophs, aerobic dichloromethane degraders, phylogeny, DNA–DNA hybridization, 16S rRNA genes.

Dichloromethane, or methylene chloride (CH_2Cl_2), is a highly toxic and volatile compound that is widely used in industry as a solvent and refrigerant and is one of the most abundant pollutants of natural waters and the atmosphere. In 1995, the annual production of dichloromethane in western European countries was as large as 135000 tons [1]. This compound is a component of municipal and industrial sewage [2]. Long-term contact with dichloromethane leads to various occupational diseases. At concentrations greater than 10 mg/l, CH_2Cl_2 impairs the ecological status of water bodies. All this explains interest in the research of microorganisms capable of degrading this pollutant. It should be noted that we were unable to reveal dichloromethane degraders among more than 200 methylotrophic bacteria and yeasts available in culture collections, although such degraders (strains DM1 through DM14) were isolated from habitats with a permanent selective pressure of CH_2Cl_2 [3–5]. The relevant works published until now have mainly concentrated on the investigation of the dehalogenation of dichloromethane, while the metabolic pathways involved and the taxonomic position of the known dichloromethane-utilizing bacteria were not conclusively determined. Some of these strains were found to be gram-negative facultative methylotrophs with the serine pathway of C_1-metabolism, presumably belonging to the genera *Hyphomicrobium* (strain DM2) [6] and *Methylobacterium* (strain DM4) [7]. The only strain with the serine pathway that was characterized in depth is DM13, which is the type strain of the new species *Methylorhabdus multivorans* [8]. Two other well characterized strains, DM11 and DM12, implement the ribulose monophosphate and ribulose bisphosphate metabolic pathways, respectively. Strain DM11 was classified as a representative of the new species "*Methylophilus leisingerii*" [9], and strain DM12 as a representative of *Paracoccus methylutens* [10]. The phylogeny of dichloromethane-utilizing bacteria, including the aforementioned strains, has not yet been studied.

The aim of the present work was to appreciate the phylogenetic relationships of dichloromethane-utilizing methylotrophic bacteria.

MATERIALS AND METHODS

Strains and cultivation conditions. Strains DM1 and DM3 through DM10 were obtained from Th. Leis-

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inger (Mikrobiologisches Institut ETH, Zurich). Strains DM11, DM12, and DM13 were isolated from enrichment cultures [8–10]. Bacteria were cultivated as described earlier [8].

DNA analysis. DNA was isolated and purified by routine procedure [11]. The nucleotide composition of DNA was determined by the thermal denaturation method (heating rate 0.5 deg/min) using a Beckman DU-8B spectrophotometer (United States). DNA–DNA hybridization was carried out on Synpore nitrocellulose filters (Czech Republic) [12] using [1',2',5'-^{3}H]-cytidine-5'-triphosphate, an enzyme kit for nick-translation (Amersham, United Kingdom), and the following reference strains: *Methylopila capsulata* VKM B-1606^T, *Methyloarcula marina* VKM B-2159^T, *Aminobacter aminovorans* NCIB 9059^T, *Methylobacterium organophilum* ATCC 27886^T, *M. extorquens* NCIB 9399^T, *M. rhodesianum* NCIMB 10611, *M. mesophilicum* ATCC 29983^T, *M. radiotolerans* ATCC 27329^T, *M. rhodinum* ATCC 14821^T, *M. aminovorans* JCM 8240^T, *M. zatmanii* NCIMB 12243, and *M. fujisawaence* NCIMB 12417^T.

16S rDNA amplification and sequencing. The 16S rRNA gene was selectively amplified in vitro using the forward bacterial primer 5'-GTTTGATCCTGGCTCAG-3' (11–27 according to *E. coli* numbering) and the reverse universal primer 5'-TACGGTTACCTTGTTACGACTT-3' (1492–1513 according to *E. coli* numbering) [13]. The reaction mixtures (100 μl) contained standard concentrations of dNTPs and equimolar amounts of primers A and H in 10 mM Tris–HCl buffer (pH 8.3) with 1.5 mM $MgCl_2$, 50 mM KCl, and 0.001% gelatin. Each of the 30 PCR cycles performed was a standard three-step reaction, with DNA denaturation at 94°C for 30 s, primer annealing at 40°C for 60 s, and DNA synthesis at 72°C for 150 s. PCR amplification products were purified on low-melting-point agarose and Promega columns, and were sequenced in both directions using forward and reverse universal primers and the Sequenase kit (Biochemicals, Cleveland, Ohio, USA).

The 16S rRNA gene sequences of strains DM4, DM6, DM9, DM11, DM12, and DM13 have been submitted to the GenBank database under the accession numbers AF227127, AF227126, AF250333, AF250334, and AF004845, respectively.

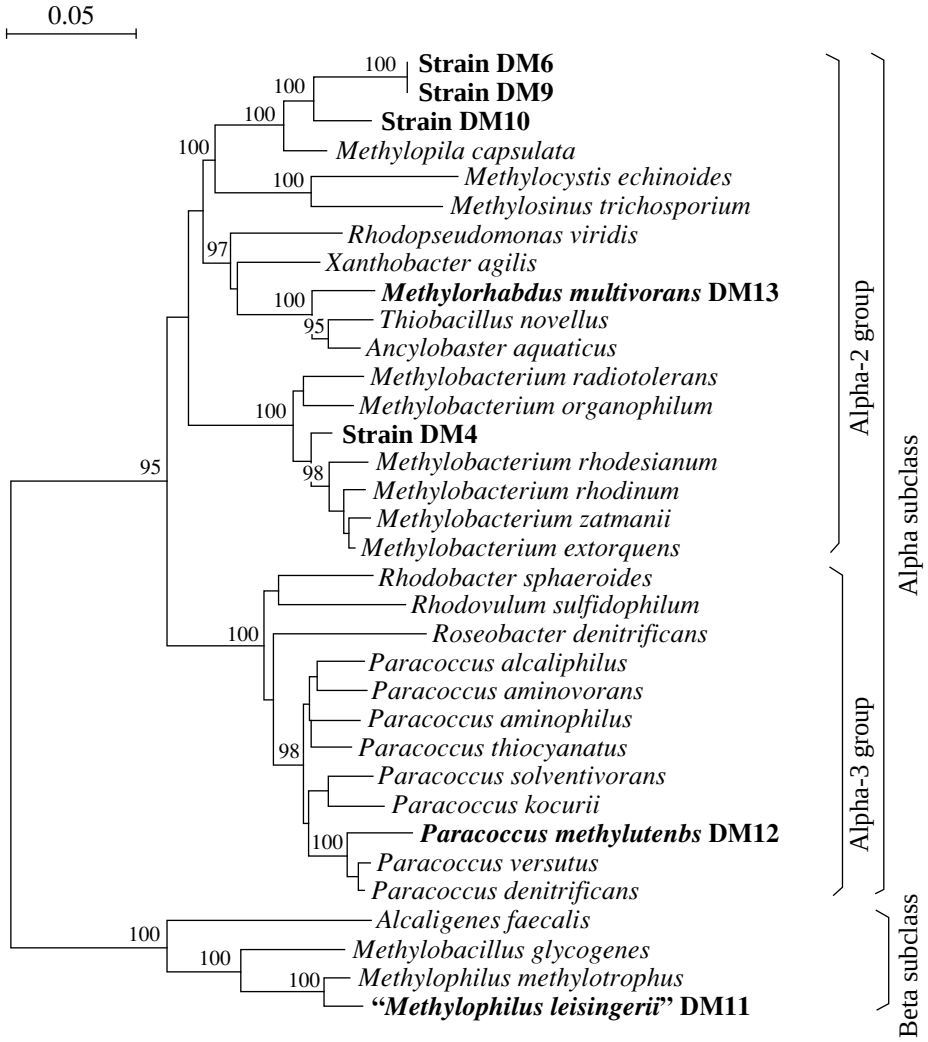
Analysis of 16S rDNA sequences. The 16S rRNA gene sequences were preliminary analyzed using the database and software resources of the Ribosomal Database Project [14]. For a more precise evaluation of the phylogenetic relationship between the bacterial strains studied, the 16S rDNA sequences were manually aligned with the respective sequences of bacteria (including methylobacteria) of various genera belonging to the alpha and beta subclasses of Proteobacteria, which are available in the latest version of the GenBank database (parenthesized are accession numbers): *Methylopila capsulata* IM1^T (AF004844), *Methylophilus methylotrophus* NCIB 10515^T (L15475), *Methylobacillus glycogenes* ATCC 29475^T (M95652), *Methylobacterium extorquens* JCM 2802 (D32224), *M. rhodinum* JCM 2811 (D32229), *M. organophilum* NCIMB 11278^T (D32226), *M. zatmanii* NCIMB 12243 (L20804), *M. radiotolerans* JCM 2831 (D32227), *M. rhodesianum* JCM 2810 (D32228), *Methylosinus trichosporium* OB3b^T (M29024), *Methylocystis parvus* OBBP^T (M29026), *Xanthobacter agilis* SA35 (D16425), *Ancylobacter aquaticus* DSM 101^T (M27803), *Thiobacillus novellus* IAM 12100 (D32247), *Rhodopseudomonas viridis* ATCC 19567 (D25314), *Rhodobacter sphaeroides* IFO 12203 (D16425), *Paracoccus denitrificans* LMG 4218^T (X69159), *P. kocurii* JCM 7684 (D32241), *P. aminovorans* JCM 7685 (D32240), *P. versutus* IAM 1281 (D32243), *P. solventivorans* DSM 6637 (Y07705), *P. thiocyanatus* THI011 (D32242), *P. alkaliphilus* JCM 7364 (D32238), *P. aminophilus* JCM 7686 (D32239), *Roseobacter denitrificans* OCH 114 (M96746 and AF004844), *Rhodovulum sulfidophilum* W-4^T (D13475), and *Alcaligenes faecalis* ATCC 8750^T (M22508).

After the sequences for which not all nucleotide positions were determined had been excluded from consideration, a total of 1325 nucleotide positions were compared. Evolutionary distances, which were expressed as the number of nucleotide substitutions per 100 nucleotides, were calculated according to Jukes and Cantor [15]. An unrooted phylogenetic tree was constructed with the aid of the algorithm implemented in the TREECON software package [16]. To assess the reliability of branching points, 100 alternative trees were subjected to bootstrap analysis.

RESULTS

Genotypic analysis. In the nucleotide composition of their DNAs, all of the investigated strains (DM1, DM3–DM10) comprised a homogeneous group (the G+C contents of the DNAs were within a narrow range of 66.3 to 68.8 mol %) and were close to *Methylorhabdus multivorans* DM13, "*Methylophilus leisingerii*" DM11, and *Paracoccus methylutens* DM12 strains, which were investigated earlier [8–10].

In the similarity of the total DNA sequences, these strains were divided into three genotypic groups. Genotypic group 1 included strains DM1, DM3, and DM5 through DM9 with a high level of DNA homology (from 81 to 98%). The degree of DNA homology between the bacteria of this group and other dichloromethane-utilizing strains, including *Methylorhabdus multivorans* DM13, "*Methylophilus leisingerii*" DM11, and *P. methylutens* DM12, as well as the methylotrophic bacteria of the genera *Aminobacter, Methylopila*, and *Methyloarcula*, did not exceed 15%. Genotypic group 2 included one strain, DM4, which exhibited lower than 10% DNA similarity to the reference strains but was relatively close in this parameter to bacteria of the genus *Methylobacterium* (14–57% DNA



Phylogenetic tree showing the position of dichloromethane-degrading methylotrophic bacteria within the Proteobacteria. Figures indicate bootstrap values (values of less than 95 are not shown). The scale bar represents 5 nucleotide substitutions per 100 nucleotides (evolutionary distances).

homology). Genotypic group 3 also included one strain, DM10, whose degree of DNA homology with the reference strains did not exceed 10%.

Phylogenetic analysis. For phylogenetic analysis, we chose three dichloromethane-degrading strains, DM11, DM12, and DM13, which represent new taxa of methylotrophic bacteria [8–10], and four strains, DM6, DM9, DM4, and DM10, which represent three aforementioned DNA homology groups. The 16S rDNA sequences of all the strains studied were determined almost completely, except strain DM6, for which only a fragment of about 450 nucleotide was sequenced (approximately between the positions 50–500, according to *E. coli* numbering).

Preliminary phylogenetic analysis showed that all the strains studied belonged to Proteobacteria; however, they did not form a monophyletic cluster. To gain better insight into the phylogenetic relationships of these strains, we constructed a phylogenetic tree of these strains and related reference strains based on 16S rDNA sequences (see figure).

The phylogenetic analysis of the 16S rDNA sequences showed that strain DM11 of the species "*Methylophilus leisingerii*" belongs to the beta subclass of Proteobacteria, since the specific features of the primary and secondary structure of its 16S rDNA are typical of this phylogenetic group [17]. On the phylogenetic tree, strain DM11 formed a monophyletic cluster with the related species *Methylophilus methylotrophus* (97.5% similarity of 16S rDNA sequences), while the degree of its similarity to another methylotrophic representative of the beta subclass, *Methylobacillus glycogenes*, was considerably lower (90.8%).

DM13, the type strain of the species *Methylorhabdus multivorans* [8], was found to belong to the alpha subclass of Proteobacteria [17]; it was closer to the

autotrophic members of the alpha-2 group, *Ancylobacter aquaticus* (96.2% similarity of 16S rDNA sequences) and *Thiobacillus novellus* (95.7% similarity), than to the methylotrophic *Xanthobacter agili* (92.6% similarity).

Strain DM12, a representative of the new species *Paracoccus methylutens* [10], was classified into the alpha-3 group of Proteobacteria, where it formed a monophyletic cluster with species of the genus *Paracoccus* (93.7–97.4% similarity of 16S rDNA sequences). This degree of similarity corresponds to the interspecific similarity level typical of the genus *Paracoccus* (93.7–99.2%).

Strains DM6 and DM9 of genotypic group 1 had identical 16S rDNA sequences (100% similarity) and were affiliated to the alpha-2 group of Proteobacteria, although the degree of similarity to other members of this group, including methylotrophs, was relatively low (no more than 89.5%). The recently described facultative methylotroph *Methylopila capsulata* IM1 [18] turned out to be the closest relative of strains DM6 and DM9 (95.5% similarity of 16S rDNA sequences). These three methylotrophs formed a monophyletic cluster with strain DM10 of genotypic group 3 (95.4% similarity).

Strain DM4 of genotypic group 2 and species of the genus *Methylobacterium* formed a phylogenetic cluster within the alpha-2 group of Proteobacteria. The members of this cluster had a 95.0–98.4% similarity of their 16S rDNA sequences, which corresponded to the typical value of interspecific similarity in this genus (94.5–99.7%).

DISCUSSION

The results of phylogenetic analysis agree well with the results of phenotypic and genotypic studies, according to which the dichloromethane-degrading strains DM4 and DM12 were ascribed to the genera *Methylophilus* and *Paracoccus*, respectively [9, 10]. At the same time, the degree of phylogenetic similarity of these strains to the known species of these genera (less than 97.5% similarity of 16S rDNA sequences) was insufficient to identify these strains with any of these species [19]. Therefore, phylogenetic analysis proved the validity of the classification of strains DM4 and DM12 as new species.

Strain DM13 was sufficiently close to some autotrophic proteobacteria of the alpha-2 group phylogenetically, but not phenotypically [8]: *Ancylobacter aquaticus* is not a methylotroph and *Thiobacillus novellus* can utilize C_1-compounds, but its important feature is the oxidation of sulfur compounds. Generally, members of the alpha-2 group are phenotypically dissimilar, but are characterized by a low divergence of their 16S rDNA sequences. For instance, the phylogenetic divergence of *Ancylobacter aquaticus* and *Thiobacillus novellus* species is as low as 2.8%. The relatively low degree of phylogenetic relations between strain DM13 and the methylotroph *Xanthobacter agili* corresponds to their phenotypic differences, including differences in the C_1-metabolism pathways. Thus, phylogenetic analysis confirmed the classification of strain DM13 as a member of the new species *Methylorhabdus multivorans* [8], which probably comprises a new branch of the alpha-2 group of Proteobacteria.

The degree of similarity of 16S rDNA sequences of strains DM1, DM3, and DM5 through DM9 (genotypic group 1) corresponds to typical intraspecific values [20]; this suggests that they may comprise a new species. Strain DM10 (genotypic group 3) probably comprises another new species. Phylogenetically, these two presumptive species are close to the known methylotrophs of the genus *Methylopila* [18]. However, the conclusive affiliation of the two species to this genus requires detailed phenotypic analysis.

According to the results of genotypic, phylogenetic, and preliminary phenotypic analyses [7], strain DM12 refers to the genus *Methylobacterium*. The degree of similarity of the total DNA and 16S rDNA sequences is insufficient to identify this strain with any known species of this genus [19, 20]. Therefore, strain DM12 may also represent a new species.

The isolation of dichloromethane-utilizing methylobacteria from the soils that have long been contaminated with this compound allowed Stucki *et al.* to suggest that the dichloromethane-degrading capacity is not an inherent but an acquired property of methylotrophs [6]. The phylogenetic diversity of dichloromethane-degrading strains established in this study does not contradict this assumption. It is known that the ability to utilize dichloromethane is due to the presence of dichloromethane dehalogenase, an enzyme that turned out to be identical in the four methylotrophic strains studied, including DM1, DM2, and DM4 [21]. Based on this finding, some researchers suggested that this enzyme has evolved recently in parallel with the accumulation of dichloromethane in the medium, and was then disseminated among other methylotrophs through horizontal gene transfer. If this were so, at least some of the methylobacteria maintained in collections would possess the ability to degrade dichloromethane. However, we failed to detect dichloromethane-utilizing methylotrophs among the collection strains. Moreover, all twelve dichloromethane-degrading strains subjected to phylogenetic analysis in the scope of this study turned out to be representatives of new taxa of aerobic methylobacteria.

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