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Full Paper

Reclassification of *Methylobacterium chloromethanicum* and *Methylobacterium dichloromethanicum* as later subjective synonyms of *Methylobacterium extorquens* and of *Methylobacterium lusitanum* as a later subjective synonym of *Methylobacterium rhodesianum*

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Phylogenetic analysis based on 16S rDNA sequences was performed on all type strains of the 14 validly described *Methylobacterium* species to ascertain the genealogic relationships among these species. The results showed that type strains of *Methylobacterium* were divided into two monophyletic groups whose members were distinct species with sequence similarity values greater than 97.0% between any two of the members in the same group. Only *M. organophilum* JCM 2833^T and ATCC 27886^T were not divided into those two groups. In particular, strains of *M. dichloromethanicum* and *M. chloromethanicum* exhibited extremely high similarity values (99.9 and 100%, respectively) with the type strain of *M. extorquens*. To clarify the relationships among *Methylobacterium* species in more detail, phylogenetic analysis based on the 5′ end hyper-variable region of 16S rDNA (HV region), ribotyping analysis, fatty acid analysis, G+C content analysis and DNA-DNA hybridization experiments was performed on 58 strains of *Methylobacterium* species. Results of the ribotyping analysis and the phylogenetic analysis based on HV region sequences indicated that many *Methylobacterium* strains, including *M. 'organophilum'* DSM 760^T, have been erroneously identified. The DNA G+C content of *Methylobacterium* strains were between 68.1 and 71.3%. Results of whole-cell fatty-acid profiles showed that all strains contained 18 : 1ω7c as the primary fatty acid component (82.8–90.1%), with 16 : 0 and 18 : 0 as minor components. *M. dichloromethanicum* DSM 6343^T, *M. chloromethanicum* NCIMB 13688^T, and *M. extorquens* IAM 12631^T exhibited high DNA-DNA relatedness values between each other (69–80%). *M. lusitanum* NCIMB 13779^T also showed a close relationship with *M. rhodesianum* DSM 5687^T at DNA-DNA relatedness levels of 89–92%. According to these results, many *Methylobacterium* strains should be reclassified, with *M. dichloromethanicum* and *M. chloromethanicum* regarded as a synonym of *M. extorquens*, and *M. lusitanum* a synonym for *M. rhodesianum*.

Key Words——hypervariable region; *Methylobacterium*; *Methylobacterium chloromethanicum*; *Methylobacterium dichloromethanicum*; *Methylobacterium extorquens*; *Methylobacterium lusitanum*; *Methylobacterium rhodesianum*

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Introduction

In 1976, the genus *Methylobacterium* was proposed and defined by the type species *M. organophilum*; a

gram-negative, methane-utilizing bacteria (Patt et al., 1976). Following this, Green and Bousfield (1983) showed that the phenotype of *Methylobacterium organophilum* was highly similar to methane-non-utilizing, pink-pigmented, facultatively methylotrophic (PPFM) bacteria. They proposed the new species *Methylobacterium rhodinum*, *Methylobacterium radiotolerans* and *Methylobacterium mesophilicum*, which were methane-non-utilizing organisms, and amended the description of the genus *Methylobacterium* (Green and Bousfield, 1983). *Methylobacterium* strains produce carotenoid pigment and bacteriochlorophyll in their cells, and they belong to a group of aerobic bacteriochlorophyll-containing (ABC) bacteria (Hiraishi and Shimada, 2001). Currently, the genus *Methylobacterium* consists of 14 valid species, *Methylobacterium extorquens* (Bousfield and Green, 1985), *Methylobacterium rhodesianum*, *Methylobacterium zatmanii* and *Methylobacterium fujisawaense* (Green et al., 1988), *Methylobacterium aminovorans* (Urakami et al., 1993), *Methylobacterium thiocyanatum* (Wood et al., 1998), *Methylobacterium dichloromethanicum* (Doronina et al., 2000), *Methylobacterium chloromethanicum* (McDonald et al., 2001), *Methylobacterium suomiense*, and *Methylobacterium lusitanum* (Doronina et al., 2002). The taxonomic study based on phenotypic properties (Green and Bousfield, 1982) showed that *Methylobacterium* species were divided into two physiological groups. One of them contained the metabolically less reactive organisms, such as *M. extorquens*, *M. rhodesianum*, *M. rhodinum* and *M. zatmanii*, and the other group was composed of metabolically more active organisms such as *M. fujisawaense* and *M. radiotolerans*. However, except for the properties of carbon- and energy-source utilization, other phenotypic and chemotaxonomic characteristics are quite similar among all *Methylobacterium* species (Hiraishi et al., 1995). Thus the analysis of 16S rDNA sequence and DNA-DNA relatedness were found to be important for the identification of *Methylobacterium* strains (Bratina et al., 1992; Hood et al., 1987; Tuji et al., 1990).

Members of the genus *Methylobacterium* are distributed in a wide variety of natural environments (Green and Bousfield, 1982, 1983), including soil, dust, air, and fresh water. These bacteria also inhabit human-made environments, such as potable water supplies, bathrooms, and washstands, where they sometimes produce pink ropy masses of growth. Because many *Methylobacterium* strains are resistant to chlorination and disinfection with ultraviolet beam (Furuhata et al., 1989, 1993; Hiraishi et al., 1995), they survive after sterilization and contaminate the food manufacturing environment. Furthermore, *Methylobacterium* strains are slow growers and can survive and replicate on poor carbon sources (Hiraishi et al., 1995).

Against this background, we have examined the use of the 5′ end hyper-variable region (HV region) sequence of 16S rDNA (Goto et al., 2000, 2002a, b, 2004) as a tool for the rapid identification of *Methylobacterium* species. During these studies, inconsistencies were discovered regarding the phylogenetic classification of several *Methylobacterium* species. Firstly, the 16S rDNA sequences used as reference data in previous studies (Doronina et al., 2000, 2002; McDonald et al., 2001) were unreliable because they contained many gaps and unidentified base positions due to either sequencing errors or ambiguities. Secondly, there were no data on the type strains of *M. aminovorans*. Thirdly, DNA-DNA hybridization experiments (Doronina et al., 2000, 2002) were performed using the strain JCM 2810 as the reference strain of *M. rhodesianum* when it is known that strain JCM 2810 is not the type strain for this species. For these reasons, and to enable a fast and accurate method for the rapid identification and grouping of *Methylobacterium* species, it is necessary to define the taxonomic identities of all valid *Methylobacterium* species.

In this paper, we re-determined 16S rDNA sequences of 14 validly described *Methylobacterium* species and investigated their DNA-DNA relatedness, and verified taxonomic identities of each *Methylobacterium* species. In addition to these results, on the basis of physiological and biochemical characteristics, fatty acid analysis, and ribotypes patterns, it was strongly suggested that some species should be reclassified.

Materials and Methods

Strains and culture conditions. The strains used in this study were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), IAM (IAM culture collection, Institute of Molecular and Cellular Bioscience, The University of Tokyo, Tokyo, Japan), JCM (Japan Collection of Microorganisms, RIKEN Bio

Resource Center, Saitama, Japan), and NCIMB (The National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK). In order to confirm whether type strains with an ambiguous taxonomic status were genuine, multiple type strains were obtained from different culture collections and examined in a number of phylogenetic studies. *Methylobacterium* strains were grown in Nutrient agar at 30°C. *Rhodopseudomonas palustris* DSM 123^T was cultivated as recommended in the DSM strain catalogue.

Sequencing of 16S rDNA and hypervariable region. Nearly complete 16S rDNA sequences of the test strains were determined using a 16S rRNA Gene Kit following the protocol of the manufacturer (Applied Biosystems, Foster City, CA, USA). For 39 strains of *Methylobacterium* species, 5′ end hyper-variable region of 16S rDNA were amplified and sequenced using the universal primers, which were previously reported as an index for rapid identification of species in the genus *Bacillus* (Goto et al., 2000). DNA corresponding to the HV region was amplified with the primer set according to the method of Sadaie et al. (1997). PCR products were purified using PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, CA, USA). Sequencing reactions were performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the amplification primers. Sequence reaction products were purified with AutoSeq^{TM} G-50 (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). A model ABI 310 automatic DNA sequencer (Applied Biosystems) was used for sample electrophoresis and data collection.

Phylogenetic analysis. Sequence analysis was performed using Gene Works (version 2.0, IntelliGenetics, Inc., Mountain View, CA, USA) and the GenBank/EMBL/DDBJ databases. Multiple sequence alignment was performed using CLUSTAL W version 1.81 (Thompson et al., 1994). Evolutionary distances were calculated by the Kimura's two-parameter model (Kimura, 1980), without alignment gaps and unidentified base positions taken into account during distance calculation, and phylogenetic trees were constructed from the distance data by using the neighbor-joining method of Saitou and Nei (1987). The robustness for individual branches was estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985). The GenBank/EMBL/DDBJ accession numbers used for phylogenetic analysis are presented in Fig. 1. The sequence of *M. thiocyanatum* DSM 11490^T was not included in the phylogenetic analysis based on HV region sequences due to it containing 4 polymorphic sites. When these unidentified base positions were removed from subsequent phylogenetic calculations, the informative positions were lost from the final data set.

Ribotyping analysis. The 16S rDNA sequences for the two type strains of each of the species *M. rhodesianum* (DSM 5687^T and NCIMB 12249^T), *M. extorquens* (IAM 12631^T and NCIMB 9399^T), *M. organophilum* (ATCC 27886^T and JCM 2833^T), and *M. radiotolerans* (DSM 1819^T and IAM 12098^T) were identical, hence only one type strain of each species was used for ribotype analysis. Strains were grown for 2 days on Nutrient agar. Automated ribotyping was performed under the conditions described for the RiboPrinter microbial characterization system (DuPont-Qualicon, Wilmington, DE, USA). The banding patterns for *Eco*RI ribotyping were read using GelConvert software (DuPont-Qualicon). Cluster analysis of the ribotype patterns was based on the UPGMA method (Sokal and Michener, 1958) using the software BioNumerics 3.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Genomic DNA extraction and DNA-DNA hybridization. Genomic DNA was extracted and purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the protocols of the manufacturer. For DNA-DNA hybridization, genomic DNA was extracted using the Qiagen Blood & Cell Culture DNA Maxi Kit (Qiagen GmbH, Hilden, Germany), according to the Qiagen Genomic DNA Handbook 09/97, and purified by equilibrium centrifugation on a CsCl-ethidium bromide gradient (Treisman, 1989) using the Optimum^{TM} MAX Ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Desalting was performed using Ultrafree-4 Centrifugal Filter Unit (Millipore, Bedford, MA, USA). DNA-DNA relatedness values were determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and microplates. Each hybridization experiment was repeated at least three times. Hybridization was performed at the optimal and stringent temperatures for renaturation, which were 50°C and 65°C, respectively, for 3 h.

Chemotaxonomic characterization. Cells for fatty acid analysis were grown on Trypticase soy agar (Difco) at 30°C for 2 days. Cells were saponified, methylated to create fatty acid methyl esters and extracted following the Sherlock Microbial Identification

System version 4.02 (MIDI). The G+C content of DNA was determined using HPLC as described previously (Tamaoka and Komagata, 1984).

Phenotypic characterization. Fifteen type strains were examined. Cells grown aerobically on Nutrient medium at 30°C for 1 day were subjected to a set of 70 phenotypic tests. This set of tests consisted of carbon source acidification for 49 organic compounds, biochemical analysis for 8 enzymes, and carbon source utilization using 12 organic compounds. Cells were incubated at 30°C during all tests. These tests were performed with the API50CH and API20NE test strips (bioMérieux, Marcy-l'Etoile, France) respectively; the final reading was made after 3 weeks of incubation for the API50CH and 3 days for the API20NE. Utilization of sodium citrate was tested on Simmons citrate medium (Eiken Chemical Co., Ltd., Tokyo, Japan) for 3 days.

Microbial degradation characteristics of chloromethane and dichloromethane. Eight strains composed of *M. extorquens* HV cluster were examined. Cells were pre-cultivated on Nutrient agar at 30°C for 3 days and thrice washed in sterile saline. The suspensions of cells were added to 5.0 ml of the basal medium (Green and Bousfield, 1982) containing 200 ppm dichloromethane solution or replacing 1.0 ml air to chloromethane gas in a 20 ml serum vial of gas phase as a sole carbon source. The vials were sealed with a rubber stopper and an aluminum cap, and incubated at 30°C for 2 days. The residual amounts of dichloromethane or chloromethane gas in the vials were measured with GC-MS, and the concentrations of chloride ion in the liquid phase of vials were measured with CE. A control mixture without microorganisms was also incubated under the same conditions to monitor the spontaneous degradation of dichloromethane and chloromethane.

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences obtained in this study are AB175629–175648 and AB175650 as detailed in Fig. 1.

Results

Comparative sequence analysis of 16S rDNA and phylogenetic analysis

Nineteen almost complete 16S rDNA sequences (1,433–1,435 bp) for the type strains of 14 validly published *Methylobacterium* species were determined. The level of sequence similarity ranged from 94.9 to 100% among these strains. The 16S rDNA-based phylogenetic tree (Fig. 1) showed that there were two groups (Groups A and B) whose members were

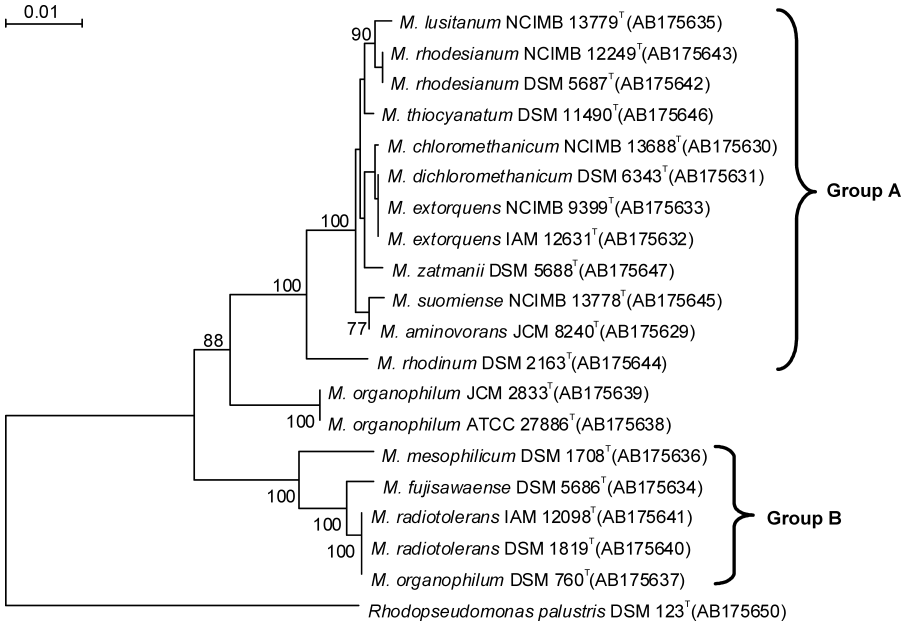

Fig. 1. Neighbor-joining tree of *Methylobacterium* species based on 16S rDNA sequences.

Rhodopseudomonas palustris DSM 123^T served as an outgroup. The final dataset included 1,426 unambiguously aligned sites. Bootstrap values (expressed as percentage of 1,000 replications) greater than 70% are shown at the branch points.

distinct species with sequence similarities greater than 97.0% between any two of members in the same group. Group A was composed of ten species, *M. lusitanum*, *M. rhodesianum*, *M. thiocyanatum*, *M. zatmanii*, *M. chloromethanicum*, *M. dichloromethanicum*, *M. extorquens*, *M. aminovorans*, *M. suomiense* and *M. rhodinum*. Nine species except for *M. rhodinum* in Group A were closely related to each other with 16S rDNA similarity values of higher than 99.0%. In particular, *M. dichloromethanicum* and *M. extorquens* revealed identical 16S rDNA sequences, while *M. chloromethanicum* had only one nucleotide different to *M. extorquens*. On the other hand, Group B was composed of five strains of *M. mesophilicum*, *M. fujisawaense*, *M. radiotolerans* and *M. 'organophilum'* DSM 760^T. Moreover, *M. 'organophilum'* DSM 760^T had identical 16S rDNA sequence to the type strain of *M. radiotolerans*, whereas it showed low similarity values (96.4%) to ATCC 27886^T and JCM 2833^T of *M. organophilum* which do not belong to either group A or B.

Moreover, some species showed many discrepancies between 16S rDNA sequences which were determined in this study and those previously reported. For example, in *M. dichloromethanicum* DSM 6343^T there were 23 sequence differences, 1 gap and 39 ambiguous nucleotides between the sequences for AF227128 and AB175631. Similarly, in *M. chloromethanicum* NCIMB 13668^T there were 20 sequence differences, 3 gaps and 2 ambiguous nucleotides between the sequences for AF198624 and AB175630. In *M. zatmanii* DSM 5688^T there were 4 sequence differences, 8 gaps and 14 ambiguous nucleotides between the sequences for L20804 and AB175647. In *M. lusitanum* NCIMB 13779^T there were 14 differences, 8 gaps and 11 ambiguous nucleotides between the sequences for AY009403 and AB175635. In *M. suomiense* NCIMB 13778^T there were 12 sequence differences, 2 gaps and 11 ambiguous nucleotides between the sequences AY009404 and AB175645. We obtained these strains twice and analyzed in duplicate, and yet the results were found to be consistently the same.

Phylogenetic analysis based on HV region

To confirm whether the previously reported 5′end hyper-variable region (HV region) sequence of 16S rDNA used for the differentiation of *Bacillus* species (Goto et al., 2000) is effective for *Methylobacterium* species, we determined the HV region sequence (237–239 bp) of the 58 strains of *Methylobacterium* species and constructed phylogenetic tree based on 236 common nucleotide positions (Fig. 2). As a result, many strains showed high similar HV region sequences with different named type strains. For example, in the case of *M. extorquens* strains, all except 4 strains scattered to three HV clusters of *M. fujisawaense*, *M. rhodesianum* and *M. zatmanii*. *M. lusitanum* NCIMB 13779^T had the same sequence as the type strain of *M. rhodesianum* (DSM 5698^T and NCIMB 12249^T). The sequence from *M. thiocyanatum* showed high similarity (>99.9%) with *M. rhodesianum* NCIMB 9144, and *Methylobacterium* sp. NCIMB 9141 and NCIMB 9145.

Ribotyping analysis

In order to verify the phylogenetic relationships among *Methylobacterium* species, 55 strains were subjected to ribotyping analysis using *Eco*RI (Fig. 3). The result showed that the strains in the same HV cluster had similar ribotypes, and they were separated from the strains of other HV clusters at the deep positions of the roots in the phylogenetic dendrogram. However, the electrophoresis patterns of each strain showed some diversity.

DNA base composition and DNA-DNA relatedness

DNA G+C contents and DNA-DNA hybridizations were examined using 55 tested strains. As shown in Table 1, the DNA G+C content of the strains tested were 68.1–71.3% (average=69.4%), and there was little difference among these strains. DNA-DNA hybridizations were examined to resolve relationships among the type strains in more detail, and to verify the genetic relationship of the strains within the HV clusters. As shown in Table 2, the DNA-DNA relatedness values within each HV cluster exhibited high similarities of greater than 69%. *M. chloromethanicum* NCIMB 13688^T, *M. dichloromethanicum* DSM 6343^T and *M. extorquens* IAM 12631^T displayed high levels of DNA-DNA relatedness (69–80%), and low levels of DNA-DNA relatedness to the other type strains. *M. lusitanum* NCIMB 13779^T and *M. rhodesianum* DSM 5687^T exhibited high DNA-DNA relatedness (>89%). For *M. organophilum*, DNA-DNA relatedness between DSM 760^T and JCM 2833^T was distantly related at the 3% value, while strain DSM 760^T showed the highest level of DNA-DNA relatedness (95%) with *M. radiotolerans* IAM 12098^T.

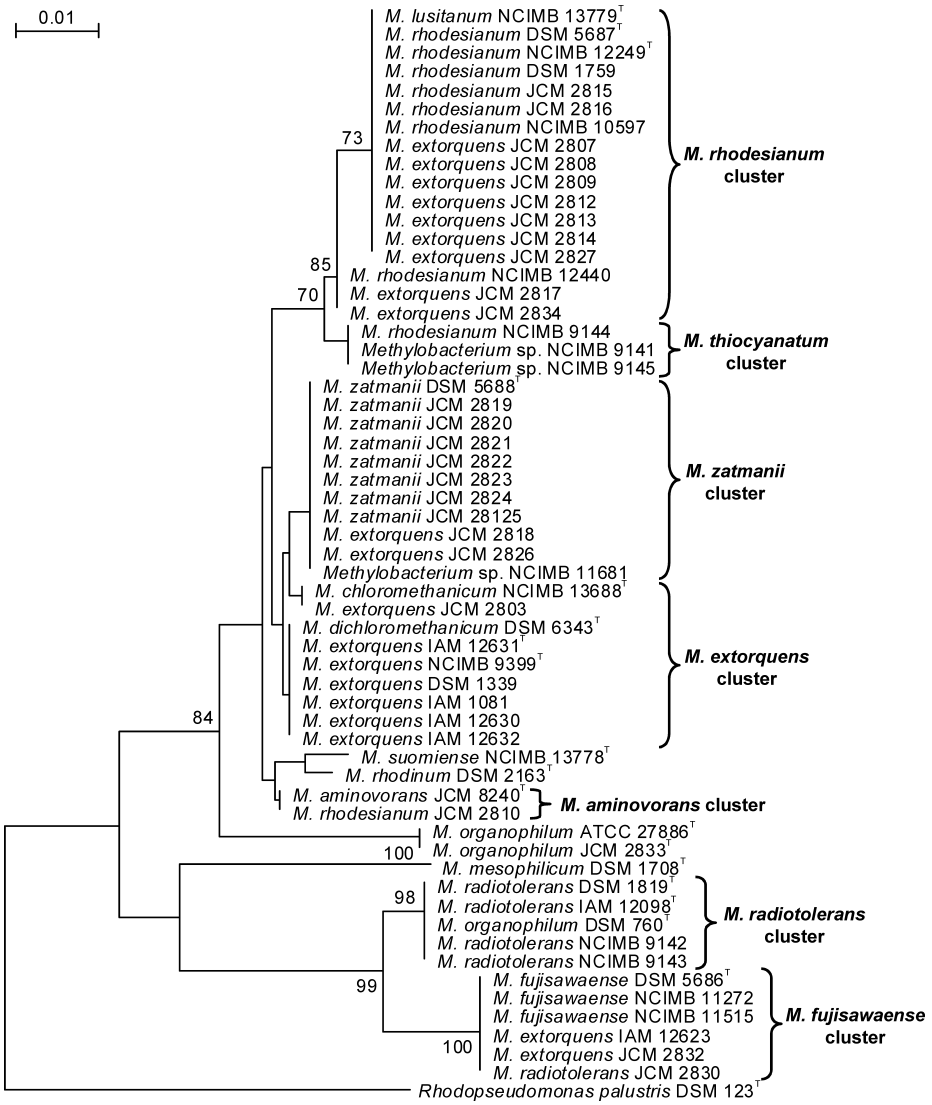

Fig. 2. Phylogenetic relationship among 58 strains of *Methylobacterium* species, inferred from the sequences of 5′ end hyper-variable region of 16S rDNA (HV region: 236 bp).

The phylogenetic tree constructed using the neighbor-joining method. *Rhodopseudomonas palustris* DSM 123^T was used as the outgroup. Bootstrap values (expressed as percentage of 1,000 replications) greater than 70% are shown at the branch points.

Fatty acid analysis

As shown in Table 1, all strains contained 18 : 1ω7c as the primary fatty acid component (average=87.0%), with the minor components 14 : 0 3OH, 16 : 1ω7c, 16 : 0, 18 : 0 and 18 : 0 3OH. Notably, eight strains which were located in the *M. extorquens* cluster (Fig. 2) were characterized by a lower ratio of 18 : 1ω7c (78.0–81.0%) and a higher ratio of 16 : 1ω7c (6.3–13.6%) compared to other tested strains.

Physiological and biochemical characteristics

Differential characteristics among *Methylobacterium* species using the API20NE and API50CH system are summarized in Table 3. All strains were positive or weakly positive for urease, acidification of glycerol, D- and L-arabinose, ribose and D- and L-xylose. All strains were negative for indole, glucose oxidation, arginine dihydrolase, β-gulcosidase, protease, β-galactosidase, utilized D-mannitol, *N*-acetyl-D-glucosamine, maltose, *n*-capric acid, phenyl acetate, acidification of erythritol, adonitol, β-methyl-xyloside, L-sorbose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose,

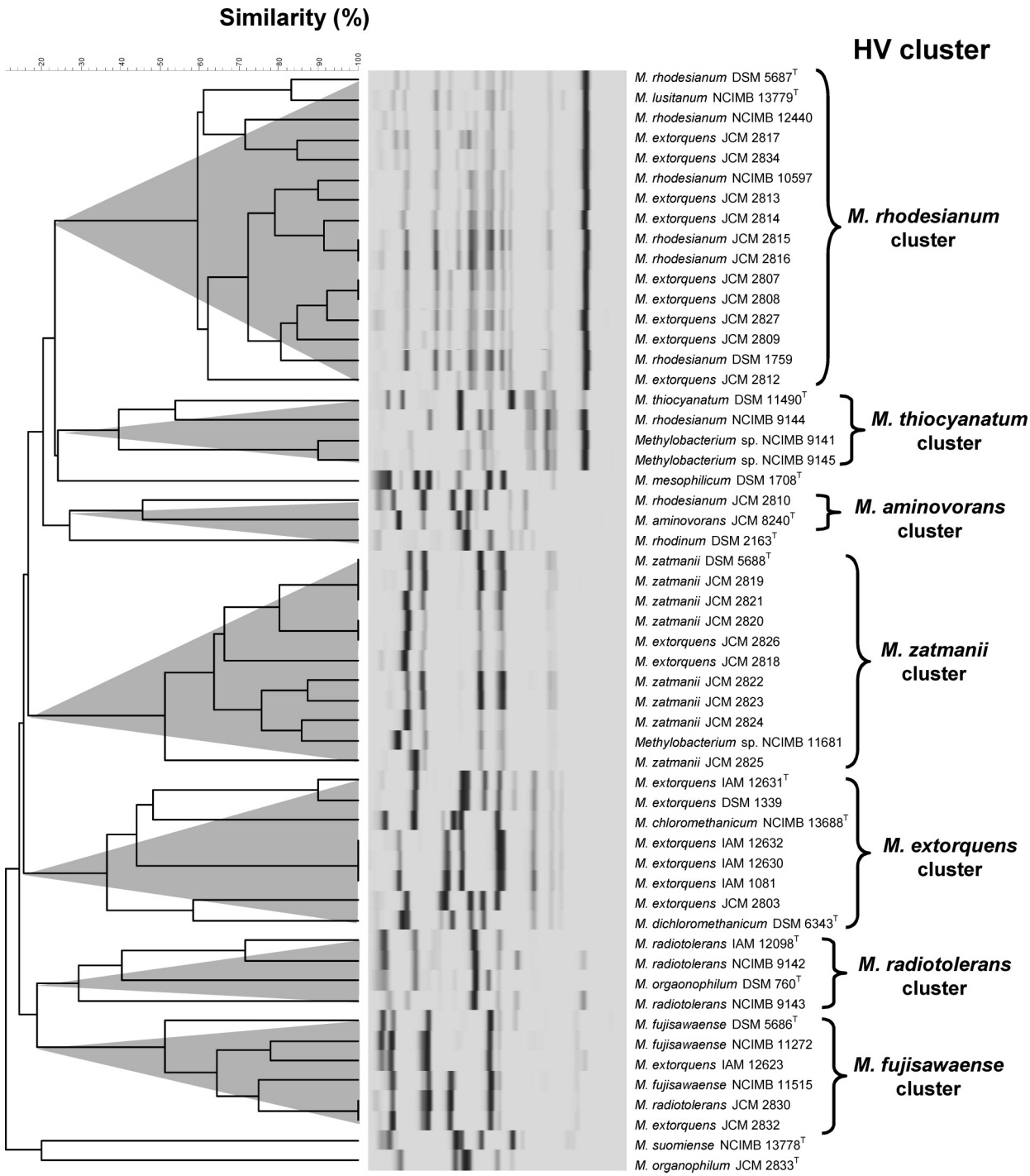

Fig. 3. UPGMA phylogenetic dendrogram obtained from cluster analysis of *Eco*RI ribotypes among 55 strains of *Methylobacterium* species.

melibiose, saccharose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-genetiobiose, D-turanose, D- and L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate. Regarding *M. organophilum*, strain JCM 2833^T clearly differed from strain DSM 760^T in physiological and biochemical characteristics, although their origins were the same and they were expected to show the same character.

Degradation of dichloromethane and chloromethane

As shown in Table 4, compared with the control and other strains, only *M. dichloromethanicum* DSM 6343^T

Table 1. Fatty acid composition and DNA G+C content of *Methylobacterium* species.

HV cluster	G+C mol%	Fatty acid						
		14 : 0 3OH	16 : 1ω7c	16 : 0	18 : 1ω7c	18 : 0	18 : 0 3OH	Sum[a]
M. rhodesianum cluster (n=15)	69.3±0.5	2.0±0.3	ND	2.9±0.6	88.1±1.7	4.8±1.5	1.5±0.3	ND
M. lusitanum NCIMB 13779^T	69.1	2.0	ND	3.2	86.9	5.8	2.1	ND
M. thiocyanatum cluster (n=4)	69.2±0.2	1.4±0.2	tr	3.2±0.5	90.1±0.6	2.4±0.5	1.1±0.1	tr
M. zatmanii cluster (n=11)	69.2±0.2	1.8±0.2	1.1±0.2	2.0±0.3	88.8±0.6	4.2±0.6	1.4±0.1	1.1±0.2
M. extorquens cluster (n=6)	68.5±0.2	1.8±0.4	9.2±2.5	2.8±0.7	80.0±2.1	3.7±0.8	1.3±0.1	9.5±2.2
M. dichloromethanicum DSM 6343^T	68.1	1.6	6.3	4.2	78.0	4.7	1.4	9.0
M. chloromethanicum NCIMB 13688^T	68.4	1.5	7.4	3.6	81.0	4.3	1.3	7.4
M. aminovorans cluster (n=2)	68.4±0.3	1.8±0.5	3.7±0.4	2.7±0.3	84.7±1.0	5.7±0.1	1.4±0.4	3.7±0.4
M. rhodinum JCM 2163^T	70.1	1.4	5.9	2.3	82.8	5.8	1.1	5.9
M. suomiense NCIMB 13778^T	68.1	1.5	4.6	3.3	84.1	4.6	tr	4.6
M. organophilum JCM 2833^T	69.6	1.9	0.6	4.1	83.7	5.8	2.5	0.6
M. mesophilicum DSM 1708^T	69.2	tr	2.1	5.3	85.6	4.3	tr	2.1
M. radiotolerans cluster (n=3)	71.2±0.2	tr	1.1±0.1	4.5±0.5	89.2±0.9	3.0±0.3	tr	1.1±0.1
M. organophilum DSM 760^T	71.3	tr	1.0	4.0	89.9	2.8	tr	1.0
M. fujisawaense cluster (n=6)	70.2±0.4	1.0±0.2	2.2±0.5	3.7±0.1	89.0±0.7	2.4±0.5	tr	2.2±0.5

Means±SD are given. tr, trace (less than 1.0%); ND, not detected.

[a] Fatty acid that could not be separated by GC using the Microbial Identification System (Microbial ID) software were considered summed features. It contains 16 : 1ω7c and/or 15 : 0 iso 2OH.

decreased the amount of the dichloromethane gas to less than 10% and produced chloride ion. The result shows that only *M. dichloromethanicum* DSM 6343^T had dichloromethane degrading ability. On the other hand, results of fixed quantity analysis of chloromethane degrading ability showed that compared to the control, no strains decrease a chloromethane gas or increased chloride ion in the medium.

Discussion

When the 16S rDNA sequences determined here were compared with previously reported sequences in public databases, they showed incongruity, though both sequences originated from the same strain. Furthermore, the phylogenetic trees in a previously published paper (Doronina et al., 2002) showed a great deal of topological conflicts with that of present study (data not shown). In this study, the sequence similarity values among *M. extorquens*, *M. chloromethanicum* and *M. dichloromethanicum* showed 99.9 or 100% (Fig. 1), suggesting cospecific among them. In fact, this supposition is principally supported by the DNA-DNA relatedness data (Table 2). Thereby, these discrepancies between past studies and the present study may be due to low quality sequences having been used in the past taxonomic study of *Methylobacterium*.

Stackebrandt and Goebel (1994) showed that any two strains in the same group having less than 97% levels of 16S rDNA sequences similarity would not exhibit more than 60% levels of DNA-DNA relatedness. In this study, *Methylobacterium* species showed extremely high similarity in 16S rDNA sequences, and they were divided into two groups (Group A and B) on the basis of more than 97% sequences similarity levels between any two distinct species in the same group. In order to clarify the relationships among *Methylobacterium* species in more detail, phylogenetic analysis based on HV region sequences and ribotypes patterns were also performed on 58 strains of *Methylobacterium* species. Although the strains comprising each cluster were almost the same in both results (Figs. 2 and 3), many strains of *Methylobacterium* species did not cluster with their respective type strains as the nearest neighbors. From the above result, it was regarded that many strains may have been erroneously identified and further polyphasic taxonomic studies were necessary to clarify the taxonomic relationships among *Methylobacterium* species.

Table 2. DNA-DNA relatedness among 55 strains of *Methylobacterium* species.

	Strain	DNA-DNA hybridization (%) with:														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*M. lusitanum* NCIMB 13779^T	100	89	31	25	42	16	34	44	15	9	6	4	2	0	0
2	*M. rhodesianum* DSM 5687^T	92	100	34	17	35	24	28	43	9	9	5	3	3	5	5
	M. rhodesianum DSM 1759	92	83	36	11	24	25	23	39	9	4	3				
	M. rhodesianum JCM 2815	92	83	33	17	22	23	22	38	7	6	8				
	M. rhodesianum JCM 2816	85	85	33	16	23	23	20	34	8	5	6				
	M. rhodesianum NCIMB 10597	94	87	39	19	32	27	30	43	13	7	6				
	M. extorquens JCM 2807	88	93	41	11	26	24	25	34	8	8	2				
	M. extorquens JCM 2808	87	90	31	13	25	23	23	31	6	8	1				
	M. extorquens JCM 2809	87	89	18	12	28	25	26	32	6	8	2				
	M. extorquens JCM 2812	87	90	36	18	24	24	25	37	12	9	7				
	M. extorquens JCM 2813	87	95	36	18	26	22	23	42	16	5	5				
	M. extorquens JCM 2814	84	90	16	19	22	21	25	35	5	11	6				
	M. extorquens JCM 2827	81	87	24	12	29	24	26	37	5	9	1				
	M. extorquens JCM 2817	90	90	29	15	20	28	23	32	9	10	3				
	M. extorquens JCM 2834	87	78	35	19	20	24	21	33	13	2	5				
	M. rhodesianum NCIMB 12440	93	80	31	25	18	23	27	27	12	9	6				
3	*M. thiocyanatum* DSM 11490^T	33	25	100	19	23	30	34	11	14	11	4	5	1	0	4
	M. rhodesianum NCIMB 9144	39	20	80	20	26	29	29	14	9	10	3				
	Methylobacterium sp. NCIMB 9141	44	21	74	20	32	39	43	24	8	8	2				
	Methylobacterium sp. NCIMB 9145	46	19	73	17	28	32	35	21	7	12	3				
4	*M. zatmanii* DSM 5688^T	20	18	12	100	16	16	18	16	10	8	11	4	2	4	1
	M. zatmanii JCM 2819	22	17	25	97	14	13	15	10	5	8	11				
	M. zatmanii JCM 2820	19	20	24	97	12	13	14	10	4	8	9				
	M. zatmanii JCM 2821	22	15	24	100	13	14	14	11	7	7	11				
	M. zatmanii JCM 2822	29	19	26	98	15	15	16	10	10	12	11				
	M. zatmanii JCM 2823	31	20	18	100	11	15	22	17	8	9	11				
	M. zatmanii JCM 2824	28	19	18	100	16	18	20	15	8	10	11				
	M. zatmanii JCM 2825	27	19	20	100	14	15	17	17	7	9	12				
	M. extorquens JCM 2818	23	19	27	96	13	16	16	10	3	7	11				
	M. extorquens JCM 2826	34	28	31	100	15	17	17	16	4	8	9				
	Methylobacterium sp. NCIMB 11681	31	7	10	80	6	16	19	8	6	7	13				
5	*M. chloromethanicum* NCIMB 13688^T	22	17	19	13	100	70	72	22	10	6	2	2	2	2	3
	M. extorquens JCM 2803	36	18	29	24	74	80	78	23	9	4	2				
6	*M. dichloromethanicum* DSM 6343^T	38	22	26	17	69	100	76	26	9	4	4	3	3	4	2
7	*M. extorquens* IAM 12631^T	37	33	37	11	75	73	100	33	9	4	4	3	5	4	2
	M. extorquens DSM 1339	36	26	28	14	80	82	93	28	4	7	4				
	M. extorquens IAM 1081	31	25	28	13	80	80	77	27	6	4	6				
	M. extorquens IAM 12630	33	25	29	12	80	78	74	27	5	4	4				
	M. extorquens IAM 12632	37	23	27	17	73	80	74	26	7	6	3				
8	*M. aminovorans* JCM 8240^T	37	36	22	12	17	27	31	100	7	10	1	3	1	0	3
	M. rhodesianum JCM 2810	37	46	29	21	37	27	29	88	7	10	4				
9	*M. rhodinum* JCM 2163^T	11	11	14	8	9	8	8	13	100	14	1	2	3	6	3
10	*M. suomiense* NCIMB 13778^T	4	10	11	13	4	6	6	11	20	100	1	2	1	6	1
11	*M. organophilum* JCM 2833^T	9	8	5	8	0	7	6	3	3	3	100	2	3	3	1
12	*M. radiotolerans* IAM 12098^T	0	0	2	3	0	2	1	0	4	3	1	100	86	37	9
13	*M. organophilum* DSM 760^T	1	3	1	0	2	0	1	1	3	2	2	95	100	46	14
	M. radiotolerans NCIMB 9142												90	70	24	6
	M. radiotolerans NCIMB 9143												82	76	31	5
14	*M. fujisawaense* DSM 5686^T	2	2	6	7	3	3	3	1	4	1	1	45	31	100	15
	M. fujisawaense NCIMB 11272												37	30	87	5
	M. fujisawaense NCIMB 11515												37	30	92	9
	M. extorquens IAM 12623												38	28	88	12
	M. extorquens JCM 2832												38	28	92	10
	M. radiotolerans JCM 2830												36	31	89	15
15	*M. mesophilicum* DSM 1708^T	0	0	1	3	0	1	1	0	2	1	1	15	15	8	100

Strains: 1, *M. lusitanum* NCIMB 13779^T; 2, *M. rhodesianum* DSM 5687^T; 3, *M. thiocyanatum* DSM 11490^T; 4, *M. zatmanii* DSM 5688^T; 5, *M. chloromethanicum* NCIMB 13688^T; 6, *M. dichloromethanicum* DSM 6343^T; 7, *M. extorquens* IAM 12631^T; 8, *M. aminovorans* JCM 8240^T; 9, *M. rhodinum* JCM 2163^T; 10, *M. suomiense* NCIMB 13778^T; 11, *M. organophilum* JCM 2833^T; 12, *M. mesophilicum* DSM 1708^T; 13, *M. organophilum* DSM 760^T; 14, *M. radiotolerans* IAM 12098^T; 15, *M. fujisawaense* DSM 5686^T.

Table 3. Physiological and biochemical characteristics of the type strains of *Methylobacterium* species.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Acid from :															
D-Xylose	+	+	+	W	+	+	+	W	+	+	+	+	+	+	+
D-Lyxose	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
L-Fucose	W	W	+	+	+	+	+	W	W	+	−	W	+	+	+
D-Tagatose	W	W	W	W	W	W	W	W	W	W	W	−	−	−	−
D-Fructose	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−
Galactose	−	−	W	−	−	−	−	−	+	+	+	+	+	+	+
D-Glucose	−	−	W	−	−	−	−	−	+	+	+	+	+	+	+
D-Fucose	−	−	W	−	−	−	−	−	−	W	−	+	+	+	+
D-Mannose	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Urease	+	+	+	+	+	+	+	+	+	+	+	+	W	+	+
Nitrate reduction to nitrite	W	−	−	−	−	−	−	+	−	W	−	−	−	+	−
Utilization of :															
DL-Malic acid	+	+	+	+	+	−	+	+	+	−	+	−	+	+	+
Glucose	−	−	−	W	+	W	W	−	+	−	W	+	W	+	+
L-Arabinose	−	−	−	W	−	−	−	−	−	−	−	+	+	+	+
D-Mannose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W
Gluconate	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
Adipic acid	−	−	−	−	−	−	−	−	−	−	−	−	W	+	+
Citric acid	−	−	−	−	−	−	−	−	−	+	−	W	+	+	+

Strains: 1, *M. lusitanum* NCIMB 13779^T; 2, *M. rhodesianum* DSM 5687^T; 3, *M. thiocyanatum* DSM 11490^T; 4, *M. zatmanii* DSM 5688^T; 5, *M. chloromethanicum* NCIMB 13688^T; 6, *M. dichloromethanicum* DSM 6343^T; 7, *M. extorquens* IAM 12631^T; 8, *M. aminovorans* JCM 8240^T; 9, *M. rhodinum* JCM 2163^T; 10, *M. suomiense* NCIMB 13778^T; 11, *M. organophilum* JCM 2833^T; 12, *M. mesophilicum* DSM 1708^T; 13, *M. organophilum* DSM 760^T; 14, *M. radiotolerans* IAM 12098^T; 15, *M. fujisawaense* DSM 5686^T. +, positive; −, negative; W, weakly positive.

Table 4. Fixed quantity of the amount of dichloromethane utilized by eight strains that compose *M. extorquens* HV cluster.

	CH_2Cl_2 conc. (%)	Cl^- ion conc. (ppm)
Control	100	nd
M. chloromethanicum NCIMB 13688^T	103	nd
M. extorquens JCM 2803	102	nd
M. dichloromethanicum DSM 6343^T	6	239
M. extorquens IAM 12631^T	98	nd
M. extorquens DSM 1339	101	nd
M. extorquens IAM 1081	102	nd
M. extorquens IAM 12630	100	nd
M. extorquens IAM 12632	103	nd

The amount of residual dichloromethane gas in the gas phase was shown in the ratio to the control. The amount of produced chloride ion in the liquid phase was shown in the concentration.

nd: below detection limit of 12.5 ppm.

The DNA G+C content of all strains (Table 1) showed similar values between 68.1–71.3%, indicating the genus *Methylobacterium* to be a homogenous group. The DNA G+C content values determined here for *M. chloromethanicum* NCIMB 13688^T, *M. dichloromethanicum* DSM 6343^T, *M. lusitanum* NCIMB 13779^T and *M. suomiense* NCIMB 13778^T revealed higher values (1–4%) than the results published previously. This discrepancy could probably be due to the different methods employed in this study (HPLC) compared to those used in the previous study (thermal denaturation method). Furthermore, the whole-cell fatty-acid analysis (Table 1) showed that the eight strains of *M. extorquens* cluster could be distinguished from the strains of other HV clusters on the point of a lower ratio of 18 : 1ω7c and a higher ratio of 16 : 1ω7c, but the other strains had similar fatty acid compositions and contents. The results indicated that it was difficult to identify all *Methylobacterium* species based on the

fatty acid profiles. On the other hand, the type strains were roughly divided into two groups on the basis of the differential characteristics of utilization as sole carbon source (Table 3). This tendency corresponded to the previous study (Green and Bousfield, 1982), but there were no remarkable differences among tested strains to distinguish each species based on the phenotypic characteristics in this study.

With the minimum level of DNA-DNA relatedness between strains required to define a species recommended as being 70% (Wayne et al., 1987), the data shown in Table 2 support the separation of *Methylobacterium* strains into 11 distinct species in accordance with this value. The clustering pattern on the basis of DNA-DNA relatedness values was identical to the clusters formed by HV region sequences and ribotypes patterns (Figs. 2 and 3), respectively. Thus these results show that many strains had been erroneously identified based on these genetic data. The details of species having more than 70% DNA-DNA relatedness are shown as follows.

M. chloromethanicum NCIMB 13688^T, *M. dichloromethanicum* DSM 6343^T and *M. extorquens* IAM 12631^T exhibited extremely high DNA-DNA relatedness values and were distinct from other type strains of *Methylobacterium* (Table 2). This result differed from the results obtained by Doronina et al. (1996, 2000) and McDonald et al. (2001), who found that strain CM4^T (=NCIMB 13688^T) and strain DM4^T (=DSM 6343^T) had an intermediate level of DNA-DNA relatedness (57–68.5%) to the type strain of *M. extorquens*. Furthermore, *M. lusitanum* NCIMB 13779^T and *M. rhodesianum* DSM 5687^T, also had more than 70% levels of DNA-DNA relatedness (Table 2). This result clearly differs from the results obtained by Doronina et al. (2002), who found only 27% DNA-DNA relatedness between strain F20^T (=NCIMB 13779^T) and *M. rhodesianum* JCM 2810. The discrepancy between these data could be due to the fact that Doronina et al. (2002) used *M. rhodesianum* JCM 2810 as the reference strain of *M. rhodesianum*, although it is not the type strain. In this study, it was confirmed by the results of phylogenetic analysis of the HV region sequences (Fig. 1) and the DNA-DNA hybridization experiment (Table 2) that strain JCM 2810 should be reidentified as *M. aminovorans* rather than *M. rhodesianum*. Concerning the type strains of *M. organophilum*, JCM 2833^T and DSM 760^T exhibited low level DNA-DNA relatedness (Table 2). Though strain JCM 2833^T exhibited low level relatedness to all other type strains, strain DSM 760^T exhibited extremely high level relatedness to the type strain of *M. radiotolerans*. This result was supported by 16S rDNA phylogenetic tree and physiological characteristics (Fig. 1 and Table 3). *M. organophilum* ATCC 27886^T and DSM 760^T have been deposited by the author who proposed this species (Patt et al., 1976), while strain JCM 2833^T was obtained from the ATCC. Since strain ATCC 27886^T and JCM 2833^T have an identical 16S rDNA sequence (Fig. 1), they are likely to be the same strain. Compared to reference data of phylogenetic analysis (Doronina et al., 2002; Urakami et al., 1993; Wood et al., 1998), JCM 2833^T is thought to be the authentic type strain. Based on these facts, it is regarded that DSM 760^T had been mislabeled.

In the previous reports (Doronina et al., 2000; McDonald et al., 2001), *M. chloromethanicum* CM4^T and *M. dichloromethanicum* DM4^T were described as new species, based on the intermediate levels of DNA-DNA relatedness and their growth characteristics in chloromethane or dichloromethane, which differed from *M. extorquens*. With quantitative analysis, we were able to confirm that only *M. dichloromethanicum* had the ability to degrade dichloromethane, and the tested strain was the authentic strain of *M. dichloromethanicum*. However, it is known that the dichloromethane utilization character of *M. dichloromethanicum* DM4 correlates with possession of an intact 120 kb plasmid (Galli and Leisinger, 1988). At the same time, it suggests that dichloromethane utilization could be lost if the growth condition of *M. dichloromethanicum* had been changed and the plasmid was lost from the cell. On the other hand, contrary to expectations, *M. chloromethanicum* NCIMB 13688^T rarely reduced chloromethane gas with release of chloride ion as well as other strains. We have not been able to show their ability to utilize chloromethane. In our opinion, these changeable or non-reproducible characteristics should not be used for species definition.

Grouping of *Methylobacterium* strains on the basis of DNA-DNA relatedness values was mostly consistent with the clustering seen in the phylogenetic tree based on the HV region sequences. Therefore the HV region can be regarded as a useful marker for the rapid identification and grouping of *Methylobacterium* species. On the other hand, for a definitive determination of *Methylobacterium* species, DNA-DNA hybridization

and phylogenetic analysis based on 16S rDNA sequences are also very important. At present, the taxonomic status of *Methylobacterium* species is confused due to the existence of erroneous 16S rDNA sequence data on public databases. In order to avoid the proposal of erroneous new species, the taxonomic status of *Methylobacterium* species should be corrected on the basis of the polyphasic evidence presented here.

In conclusion, on the basis of all data considered in this study (DNA-DNA relatedness, phylogenetic analysis of 16S rDNA, phenotypic, and chemotaxonomic characterizations), *M. extorquens*, *M. chloromethanicum* and *M. dichloromethanicum* should be unified as one species, and *M. rhodesianum* and *M. lusitanum* as one species. According to the international rule (Rule 24b of the Bacteriological Code, Lapage et al., 1992) in which the oldest legitimate epithet has the priority, we propose that *M. chloromethanicum* and *M. dichloromethanicum* should be considered as a synonym of *M. extorquens*, and *M. lusitanum* be considered as a synonym of *M. rhodesianum*. Furthermore, strains of *M. extorquens* JCM 2807, JCM 2808, JCM 2809, JCM 2812, JCM 2813, JCM 2814, JCM 2817, JCM 2827 and JCM 2834 should be reidentified as *M. rhodesianum*. The strains *M. rhodesianum* NCIMB 9144, *Methylobacterium* sp. NCIMB 9141 and NCIMB 9145 should also be reidentified under species *M. thiocyanatum*. In addition, this study has found that strains of *M. extorquens* JCM 2818, JCM 2826 and *Methylobacterium* sp. NCIMB 11681 were reidentified as *M. zatmanii*, the strain *M. rhodesianum* JCM 2810 reidentified as *M. aminovorans*, strains of *M. extorquens* IAM 12623 and JCM 2832, and the strain *M. radiofolerans* JCM 2830 were renamed as species *M. fujisawaense*, and strain *M. organophilum* DSM 760 reidentified as *M. radiotolerans*.

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