Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov.

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Using a combination of 16S rRNA analysis and antigenic fingerprinting consisting of new and published data, the phylogenetic position of 18 thermophilic isolates currently classified as *Methanobacterium* species was reinvestigated. The results were verified by independent methods, including, where applicable, plasmid and phage typing. Comparative analysis of 16S rRNA data for 30 strains belonging to the order *Methanobacteriales* strongly suggested that mesophilic and thermophilic *Methanobacterium* isolates are distantly related and should be assigned to separate genera. For the thermophilic strains the genus *Methanothermobacter* was initially proposed by Boone, Whitman and Rouvière. Furthermore, the results support a reclassification of 15 isolates in three species within the proposed genus: (i) *Methanothermobacter thermautotrophicus* comb. nov., containing eight isolates, six of which are able to utilize formate (type strain ΔH^T); (ii) *Methanothermobacter wolfeii* comb. nov., containing four formate-utilizing isolates (type strain DSM 2970^T); (iii) *Methanothermobacter marburgensis* sp. nov., containing three obligately autotrophic isolates (type strain Marburg^T). Of the nine isolates formerly referred to as *Methanobacterium thermoformicium*, six were reclassified as *Methanothermobacter thermautotrophicus* and three as *Methanothermobacter wolfeii*.

INTRODUCTION

Methanogenic archaea share the unique ability to produce methane from a limited number of one- and two-carbon substrates. However, they show a remark-

able diversity based on the sequence of their 16S rRNA, a phylogenetic tool that helped define the group (Fox *et al.*, 1977). Additional characteristics typically used to classify methanogens include morphology, nutritional versatility, growth temperature, cell wall structure and G+C content of chromosomal DNA (Boone & Maestrojuan, 1993). Using those criteria, the genus *Methanobacterium* was defined to include rod-shaped, mesophilic and thermophilic ob-

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ligate autotrophs (growth on H_2/CO_2), some of which were also capable of utilizing formate for growth. Thermophilic *Methanobacterium* species are ubiquitous in a number of environments such as anaerobic digesters, digested sludge and hot springs. Up to eight distinct species have been described: *Methanobacterium thermoautotrophicum* (Zeikus & Wolfe, 1972; Zeikus, 1972), *Methanobacterium wolfei* (Winter *et al.*, 1984), *Methanobacterium thermoformicicum* (Zhilina & Ilarionov, 1984), *Methanobacterium defluvii* (Kotelnikova *et al.*, 1993b), *Methanobacterium thermophilum* (Laurinavichyus *et al.*, 1988), *Methanobacterium thermoflexum* (Kotelnikova *et al.*, 1993b), *Methanobacterium thermoalcaliphilum* (Blotevogel *et al.*, 1985) and *Methanobacterium thermoaggregans* (Blotevogel & Fischer, 1985).

The need for a taxonomic revision has been recognized for many years by several authors. Based on the results of the Gram stain, Prévot (1980) proposed to separate mesophilic and thermophilic species of *Methanobacterium* and to rename the thermophiles *Zeikusella*. DNA–DNA hybridization studies revealed a surprising lack of similarity between two isolates of *Methanobacterium thermoautotrophicum*, strains ΔH^T and Marburg^T (Brandis *et al.*, 1981), a finding later confirmed and extended with antigenic fingerprinting data (Touzel *et al.*, 1992), and eventually corroborated by phylogenetic analysis of 16S rRNA sequences (Nölling *et al.*, 1993b). The latter two studies revealed the heterogeneity of two species: *Methanobacterium thermoautotrophicum*, consisting of obligately autotrophic strains, and *Methanobacterium thermoformicicum*, consisting of formate utilizers. Three groups were recognized: (i) one containing *Methanobacterium thermoautotrophicum* ΔH^T and *Methanobacterium thermoformicicum* strains Z-245, FTF, THF, CSM3, FF1 and FF3; (ii) *Methanobacterium thermoformicicum* strains CB12, SF-4 and HN4; (iii) *Methanobacterium thermoautotrophicum* Marburg^T. Recently, the inability of strain *Methanobacterium thermoautotrophicum* ΔH^T to grow on formate could be traced to the lack of formate-dehydrogenase-encoding genes in its chromosome at the location where those genes are located in *Methanobacterium thermoformicicum* strain Z-245, while the 'autotroph' *Methanobacterium wolfei* was found to use formate as a growth substrate (Nölling & Reeve, 1997). On the other hand, taxonomic reanalysis of *Methanobacterium thermoalcaliphilum* suggested that it is in fact a synonym of *Methanobacterium thermoautotrophicum* (Kotelnikova *et al.*, 1993a).

In 1993, Boone, Whitman and Rouvière provided molecular taxonomic evidence that the thermophilic species of *Methanobacterium* should be moved into a separate genus, suggesting *Methanothermobacter* as the name (Boone *et al.*, 1993). In the meantime, this new genus name has not been formally proposed or used. A recent survey of the database of 16S rRNA sequences identified several new entries of thermophilic *Methanobacterium* species, *Methanobacterium defluvii*,

Methanobacterium thermoflexum (Kotelnikova *et al.*, 1993b), *Methanobacterium thermophilum* (Laurinavichyus *et al.*, 1988) and *Methanobacterium thermoautotrophicum* strain ZH3 (Stettler *et al.*, 1994), and prompted us to update the growing body of data available. To complete the survey of available isolates, we also included *Methanobacterium wolfei*, *Methanobacterium thermoautotrophicum* strain Hveragerdi and strain *Methanobacterium thermoautotrophicum* JW510, which are deposited at the German Collection of Microorganisms and Cell Cultures (DSM).

Our analysis relied on three independent data sets: analysis of 16S rRNA sequences, antigenic fingerprinting experiments, and data pertaining to extrachromosomal elements (plasmids and phages). Taken together, our results support the two proposals mentioned above, i.e. (i) the need to create a new genus for thermophilic isolates of *Methanobacterium*, and (ii) a classification of 15 thermophilic isolates into three species.

METHODS

Archaeal strains and growth conditions. *Methanobacterium thermoautotrophicum* strains Marburg^T (DSM 2133^T) and ZH3 (DSM 9946), *Methanobacterium wolfei* (DSM 2970^T) and *Methanobacterium thermoformicicum* FF3 (Nölling *et al.*, 1991) were from our respective strain collections. The following strains were obtained from DSMZ: *Methanobacterium thermoautotrophicum* Hveragerdi (DSM 3590), *Methanobacterium thermoautotrophicum* JW510 (DSM 1910), *Methanobacterium thermoformicicum* Z-245 (DSM 3720), *Methanobacterium thermoformicicum* CB12 (DSM 3664), *Methanobacterium defluvii* (DSM 7466), *Methanobacterium thermophilum* (DSM 6529) and *Methanobacterium thermoflexum* (DSM 7268). Phage $\Psi M2$ (Jordan *et al.*, 1989; Pfister *et al.*, 1998) was from our strain collection. Strains were grown at 55–60 °C on a rotary shaker operated at 90 r.p.m. in standard media described for *Methanobacterium thermoautotrophicum* strain ΔH^T (Nölling *et al.*, 1991) and *Methanobacterium thermoautotrophicum* strain Marburg^T (Schönheit *et al.*, 1979); the latter medium was supplemented with 8 µM sodium tungstate and 5·8 µM sodium selenite.

DNA preparation, amplification and determination of 16S rRNA sequences. Chromosomal DNA of *Methanobacterium* isolates was obtained from a 250 ml culture by the method of Jarrell *et al.* (1992). After ethanol precipitation, DNA was suspended in a suitable buffer and RNA was removed by digestion for 10 min at 37 °C with RNase A. For PCR amplification, 20–50 ng DNA was subjected to a 30-cycle program consisting of 1 min steps for denaturation (94 °C), annealing (42–45 °C) and amplification (72 °C); the final cycle included a 10 min amplification step. *Taq* polymerase (Fermentas) and the following oligonucleotides were used (restriction sites underlined): Bam16S, 5′-CAC<u>GGATCC</u>GAACGGCTCAGTAACACG-3′ and Pst16S, 5′-GTG-<u>CTGCAG</u>GGGCTACCTTGTTACGACT-3′. Amplified DNA fragments were separated by agarose gel electrophoresis, and the expected 1·4 kb fragments were excised, isolated, digested with *Bam*HI and *Pst*I, and cloned in the vectors pUC18 (Yanisch-Perron *et al.*, 1985) or pBluescript. rDNA sequences were determined by a chain-termination method with fluorescent detection at Microsynth (Balgach,

Switzerland). Duplicates were obtained from separate PCR reactions and from different batches of chromosomal DNA to exclude cross-contaminations.

Antigenic fingerprinting. The partial antigenic fingerprint was determined by indirect immunofluorescence and a quantitative slide immunoenzymic assay with a panel of S probes for rod-shaped methanogens, *Methanobrevibacter smithii* PS and ALI; *Methanobacterium thermoautotrophicum* GC1 and ΔH^T; *Methanobacterium bryantii* MoH; *Methanobacterium formicicum* MF; and *Methanosphaera stadtmanae* MCB3 (Conway de Macario *et al.*, 1982; Macario & Conway de Macario, 1983, 1985). The cells were cultivated and harvested from 30 ml culture by centrifugation (10000 *g*, 5 min) at 4 °C, and the pellet was resuspended in 1 ml saline containing 1·5 % (w/w) formaldehyde and processed for immunological analysis.

Phage typing: infection by phage ΨM2. Strains *Methanobacterium thermoformicicum* FF3 and *Methanobacterium thermoautotrophicum* ZH3 pregrown in Marburg medium were diluted 1:10 in the same medium, and different volumes of a fresh ΨM2 lysate of strain *Methanobacterium thermoautotrophicum* Marburg^T were added (0·05–1 ml). Before inoculation, the ΨM2 lysate had been centrifuged at 10000 *g* and filtered through a 0·45 μm filter to remove intact host cells. A control culture containing no lysate was prepared for both strains. In addition, strain *Methanobacterium thermoautotrophicum* Marburg^T as a positive lysis control and strain *Methanobacterium thermoautotrophicum* ΔH^T as a negative control were subjected to the same manipulations. All culture vials were pressurized with 2 bar of a H_2/CO_2 mixture (80/20%, v/v) and incubated for up to a week at 55 °C on a rotary shaker operated at 90 r.p.m.

Presence of chromosomally integrated ΨM2 sequences. Chromosomal DNA of strains *Methanobacterium thermoautotrophicum* Marburg^T, *Methanobacterium thermoautotrophicum* ZH3, *Methanobacterium thermoautotrophicum* ΔH^T, *Methanobacterium thermoformicicum* Z-245, *Methanobacterium thermoformicicum* FF3, *Methanobacterium thermoformicicum* THF, *Methanobacterium wolfei* DSM 2970^T and *Methanobacterium thermoformicicum* CB12 were prepared as described above (Jarrell *et al.*, 1992), digested with *Bam*HI, separated by agarose gel electrophoresis in a 0·7% gel, blotted onto Hybond-N membrane, hybridized with random-primed, DIG-labelled total ΨM2 DNA at 68 °C for 12 h and subsequently developed according to the manufacturer (Roche Diagnostics).

RESULTS

Phylogenetic analysis of the 16S rRNA of thermophilic *Methanobacterium* species

A total of 16 16S rRNA sequences of thermophilic *Methanobacterium* species was found in the GenBank and EMBL databases as of October 12, 1998. In addition to the 11 sequences reported earlier (Nölling *et al.*, 1993b), data are now available for *Methanobacterium thermoautotrophicum* strain ZH3 (Stettler *et al.*, 1994), *Methanobacterium wolfei* (Stettler *et al.*, 1995), *Methanobacterium defluvii* and *Methanobacterium thermoflexum* (Kotelnikova *et al.*, 1993b) and *Methanobacterium thermophilum* (Laurinavichyus *et al.*, 1988). However, the available sequence of *Methanobacterium wolfei* lacks variable region VRI

(Nölling *et al.*, 1993b), and those of *Methanobacterium defluvii*, *Methanobacterium thermoflexum* and *Methanobacterium thermophilum* differ from those of all other isolates at several positions not located in known variable regions. Therefore, we decided to resequence the variable regions of the 16S rRNA gene of those four strains; in addition, our survey included two phylogenetically uncharacterized isolates, strains *Methanobacterium thermoautotrophicum* Hveragerdi (Butsch & Bachofen, 1981) and *Methanobacterium thermoautotrophicum* JW510 (DSM 1910), to reach a total of 18 thermophilic isolates. The sequenced portions compared in the alignment of Fig. 1 included bases 101–660 and 1070–1435 according to Ostergaard *et al.* (1987).

Analysis of partial 16S rRNA sequences suggests that all 18 sequences can be roughly divided into three groups, as proposed earlier (Nölling *et al.*, 1993b): (i) a group of sequences from *Methanobacterium thermoautotrophicum* strains Marburg^T, ZH3 and now with a third member, strain Hveragerdi; (ii) a group of sequences from *Methanobacterium wolfei* and *Methanobacterium thermoformicicum* strains CB12, SF-4 and HN4; (iii) a group of 11 sequences from the ΔH group recognized previously (Nölling *et al.*, 1993b), plus sequences from *Methanobacterium defluvii*, *Methanobacterium thermoflexum*, *Methanobacterium thermophilum* and strain *Methanobacterium thermoautotrophicum* JW510.

The sequence group around strain *Methanobacterium thermoautotrophicum* Marburg^T expanded with strain *Methanobacterium thermoautotrophicum* Hveragerdi, which had, like *Methanobacterium thermoautotrophicum* strain ZH3, an additional C between positions 193 and 194. This prompted us to resequence that portion of the strain *Methanobacterium thermoautotrophicum* Marburg^T 16S rRNA molecule, and we found no difference at that position (herein referred to as 193b) with strains *Methanobacterium thermoautotrophicum* Hveragerdi and ZH3. Because this change was the only difference between the 16S rRNA of strains *Methanobacterium thermoautotrophicum* Marburg^T and ZH3, both molecules may now be identical. Eight signature bases were found for the Marburg group of sequences: U122, G167, C193b, G199, G556, U1084, G1085 and U1394 (Fig. 1).

In the *Methanobacterium wolfei* group, the type strain has a U at position 901 instead of a C in strain *Methanobacterium thermoautotrophicum* Marburg^T and strain *Methanobacterium thermoformicicum* HN4 has an A at position 1066 instead of a G (Nölling *et al.*, 1993b). Five signature bases were found for this group: A164, A166, C188, C1396 and C1397.

The ΔH group now includes four new strains, with strain *Methanobacterium thermoautotrophicum* JW510 as the closest relative of strain *Methanobacterium thermoformicicum* THF. Our results indicate that at the 16S rRNA level there is no support for assigning *Methanobacterium defluvii*, *Methanobacterium thermo-*

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                                              VRI
              121                                                    #           209
Marburg       cUgggauaaccccgggaaacuggggauaaaccUggauaggugaUgCGgccuggaaugguGcuucaccGaaacaCccuUcGgggugcccaa
ZH3           ..........................................................................................
Hveragerdi    .C........................................................................................
CB12          .C.................................C.........A.AU....................C.....-...C.-..........
SF-4          .C.................................C.........A.AU....................C.....-...C.-..........
HN4           .C.................................C.........A.AU....................C.....-...C.-..........
wolfei        .C.................................C.........A.AU....................C.....-...C -..........
JW510         .C.................................C.............UU..........U............-......-..........
THF           .C.................................C..............U..........U............-......-..........
CSM3          .C...............................................U..........U............-......-..........
FF1           .C...............................................U..........U............-......-..........
Z-245         .C...............................................U..........U............-......-..........
defluvii      .C.................................C.............U..........U............-......-..........
ΔH            .C.................................C.............U..........U............-......-..........
FF3           .C.................................C.............U..........U............-......-..........
FTF           .C.................................C.............U..........U............-......-..........
thermoflexum  .C.................................C.............U..........U............-...-..C.-..........
thermophilum  .C.................................C.............U..........U............-......-..........


              231          261          551          1071                  1211          1271
Marburg       gguAguuggu   cCaucaucgg   aucccGcagc   cagcggGacccuUUGggguu   cgacacUgaa    acUcgcccuc
ZH3           ..........   ..........   ..........   ....................   ..........    ..........
Hveragerdi    ..........   ..........   ..........   ......A.............   ..........    ..........
CB12          ..........   ..........   .....A....   ......A......AU.....    ..........    ..........
SF-4          ..........   ..........   .....A....   ......A......AU.....    ..........    ..........
HN4           ..........   ..........   .....A....   ......A......AU.....    ..........    ..........
wolfei        ..........   ..........   .....A....   ......A......AU.....    ..........    ..........
JW510         ..........   .G........   .....A....   ......A......AU.....    ......C...    ..........
THF           ..........   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
CSM3          ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
FF1           ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
Z-245         ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
defluvii      ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
ΔH            ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
FF3           ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
FTF           ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
thermoflexum  ...U......   .G........   .....A....   ......A......AU.....    ......C...    ..C.......
thermophilum  ...U......   .G........   .....A....   ......A.....CAU.....    ......C...    ..C.......


                                    ___VRII__
              1311         1381
Marburg       caUuaucgcg   gaugaggccacaGUaUUUUgCugug
ZH3           ..........   ..........................
Hveragerdi    ..........   ..........................
CB12          ..........   ..............C.CC...U....
SF-4          ..........   ..............C.CC...U....
HN4           ..........   ..............C.CC...U....
wolfei        ..........   ..............C.CC...U....
JW510         ..........   ..............C....G......
THF           ..........   ..............C....G......
CSM3          ..C.......   ..............AC...C..U....
FF1           ..C.......   ..............AC...C..U....
Z-245         ..C.......   ..............AC...C..U....
defluvii      ..C.......   ..............AC...C..U....
ΔH            ..C.......   ..............AC...C..U....
FF3           ..C.......   ..............AC...C..U....
FTF           ..C.......   ..............AC...C..U....
thermoflexum  ..C.......   ..............AC...C..U....
thermophilum  ..C.......   ..............AC...C..U....
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Fig. 1. Alignment of variable regions of 16S rRNA sequences from thermophilic *Methanobacterium* strains. Regions VRI and VRII designate highly variable regions. Nucleotides identical to those in the sequence of strain *Methanobacterium thermoautotrophicum* Marburg[T] are represented by dots, sequence differences by the corresponding nucleotide, and gaps by dashes. An additional nucleotide found upon resequencing strain *Methanobacterium thermoautotrophicum* Marburg[T] 16S rRNA gene is marked by #. This analysis was performed on two non-contiguous segments of the 16S rRNA sequences, corresponding to bases 101–660 and 1070–1435 according to the numbering of Ostergaard *et al.* (1987); those segments cover variable regions VRI and VRII as well as most of the other positions at which sequence variations have been detected. Sequences were aligned using the program PILEUP (GCG package; gap creation penalty 1·0, gap extension penalty 2·0). Strain names and accession numbers: *Methanobacterium thermoautotrophicum* Marburg[T], X15364; ZH3, Z37156; ΔH[T], X68720; *Methanobacterium thermoformicicum* Z-245, X68712; FTF, X68713; THF, X68711; CSM3, X68716; FF1, X68714; FF3, X68715; CB12, X68717; SF-4, X68718; HN4, X68719.

philum and *Methanobacterium thermoflexum* to separate species. In our analysis, *Methanobacterium thermophilum* differed from strain *Methanobacterium thermoautotrophicum* ΔH[T] only at bases 308 (A instead of G) and 1083 (C instead of U), and *Methanobacterium thermoflexum* only at base 197 (C instead of U), while *Methanobacterium defluvii* was entirely identical to strain *Methanobacterium thermoautotrophicum* ΔH[T] except at three bases unresolved (N) in the *Methanobacterium thermoautotrophicum* ΔH[T] sequence. Therefore, we propose to revise their status and incorporate them into the ΔH group. Strains

Methanobacterium thermoformicicum THF and *Methanobacterium thermoautotrophicum* JW510 stand apart from all other isolates assigned to this group, and their exact phylogenetic position may have to be revised later, should these outlying branches receive new members. Three signature bases were found for the ΔH group: U180, G262 and C1217. Strains *Methanobacterium thermoformicicum* THF and *Methanobacterium thermoautotrophicum* JW510 considered as a subgroup have only one signature base, G1399, while the ΔH group minus THF and JW510 has five: U234, C1273, C1313, A1393 and C1398.

Similarity values between pairs of 16S rRNA sequences were calculated for all isolates of this study, plus a number of strains belonging to the order *Methanobacteriales*. In Table 1, they were grouped according to the partitioning suggested by the analysis of 16S rRNAs. Two conclusions can be derived from those data: (i) similarity scores within each of the three groups identified by sequence alignment ($> 99\%$ except for strains *Methanobacterium thermoautotrophicum* JW510 and *Methanobacterium thermoformicicum* THF in the ΔH group) are significantly higher than scores between any group pair ($97.9 \pm 0.7\%$); (ii) similarity scores of all three groups with respect to mesophilic *Methanobacterium* species are markedly lower ($89.9 \pm 0.8\%$) than any of the above scores, indicating that the three groups around strains *Methanobacterium thermoautotrophicum* ΔH[T], *Methanobacterium wolfei* DSM 2970[T] and *Methanobacterium thermoautotrophicum* Marburg[T] are more closely related to one another than to mesophilic *Methanobacterium* species.

Phylogenetic trees constructed with the data in Table 1 are shown in Fig. 2. Both trees are based on partial rRNA sequences (927 bases) that were available for all isolates in this study (Fig. 2a) and for other strains of the order *Methanobacteriales* (Fig. 2b). The latter were used both as outlying sequences and to illustrate the position of thermophiles with respect to mesophiles. As expected since the same data were used, tree A graphically supports the view that thermophilic isolates should be distributed into three species, and tree B illustrates the genus-wide distance separating thermophilic and mesophilic strains of *Methanobacterium*. Since essentially all the variable positions were included in the sequences analysed here, it is unlikely that the same analysis performed with complete sequences would result in a significantly different picture.

Antigenic fingerprinting

The antigenic fingerprinting results published previously (Touzel *et al.*, 1992) consistently supported the grouping of thermophilic *Methanobacterium* strains into three distinct clusters. To complete the survey of antigenic groupings, fingerprinting of representative strains of all three groups was performed, namely strains *Methanobacterium wolfei* DSM 2970[T] (a

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Table 1. Similarity scores returned by the program FASTA for alignments of 16S rRNA sequences of *Methanobacteriales* according to the proposed three-species model

For this analysis, two non-contiguous segments of the 16S rRNA sequences, corresponding to bases 101–660 and 1070–1435 according to the numbering of Ostergaard *et al.* (1987), were assembled. In addition to the 18 sequences included in Fig. 1, the following sequences were retrieved (accession no. in parentheses): *Methanobacterium bryantii* DSM 863 (M59124), *Methanobacterium bryantii* RiH2 (AF028688), *Methanobacterium formicicum* DSM 1312 (M36508), *Methanobacterium formicicum* FCam (AF028689), *Methanobacterium subterraneum* A8p (X99044), *Methanobacterium subterraneum* C2BIS (X99045), *Methanothermus fervidus* (M32222), *Methanosphaera stadtmanae* (M59139), *Methanobrevibacter ruminantium*, *Methanobrevibacter arboriphilicus* (both from the Ribosomal Database Project site), *Methanobrevibacter filiformis* (U82322) and *Methanobrevibacter curvatus* (U62533).

	1	2	3	4	5	6	7
1 *Methanobacterium thermoautotrophicum* ΔH[T]	99.4 ± 0.6	98.2 ± 0.4	97.9 ± 0.6	89.9 ± 0.8	87 ± 2	85.4 ± 0.5	87.1 ± 0.5
2 *Methanobacterium wolfei* DSM 2970[T]		99.8 ± 0.2	97.6 ± 0.4	89.7 ± 0.6	87 ± 2	84.7 ± 0.3	86.9 ± 0.3
3 *Methanobacterium thermoautotrophicum* Marburg[T]			99.5 ± 0.5	89.8 ± 0.6	87 ± 2	84.9 ± 0.2	86.9 ± 0.3
4 Mesophiles*				97 ± 3	89 ± 2	87.1 ± 0.3	83.5 ± 0.5
5 *Methanobrevibacter*†					95 ± 5	86.0 ± 1.2	81.6 ± 0.6
6 *Methanosphaera stadtmanae*						100	78.9
7 *Methanothermus fervidus*							100

*Mesophiles refers to *Methanobacterium formicicum* DSM 1312, *Methanobacterium formicicum* FCam, *Methanobacterium subterraneum*, *Methanobacterium bryantii* DSM 863 and *Methanobacterium bryantii* RiH2.

† *Methanobrevibacter arboriphilicus*, *Methanobrevibacter ruminantium*, *Methanobrevibacter filiformis* and *Methanobrevibacter curvatus*.
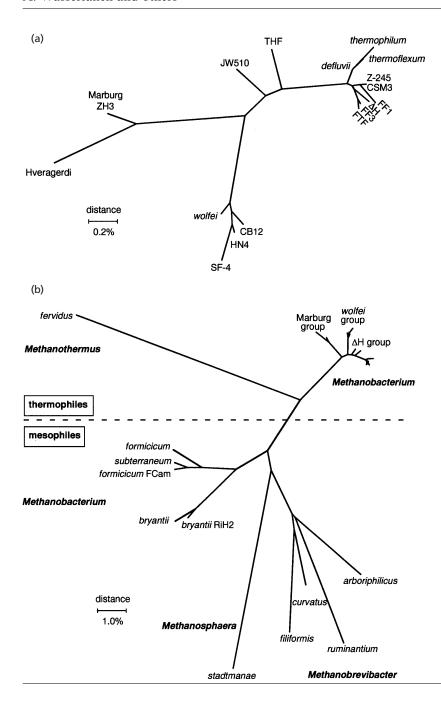

Fig. 2. Phylogenetic trees showing (a) the thermophilic members of the genus *Methanobacterium*, and (b) their position within the order *Methanobacteriales*. For this analysis, two non-contiguous segments of the 16S rRNA sequences, corresponding to bases 101–660 and 1070–1435 according to Ostergaard *et al.* (1987), were assembled and then aligned using the program PILEUP of the GCG package (gap creation penalty 1·0, gap extension penalty 2·0). The alignment was manually corrected to remove extra bases or fill gaps with Ns when those discrepancies were observed only in a minority of the sequences (we observed that unedited sequences confused the programs used to calculate distances). All edited sequences were 927 bases long. Distances were computed with the CLUSTAL W package at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/) using the neighbour-joining model, and fed to the program DRAWTREE of the PHYLIP package at the Pasteur Institute (http://bioweb.pasteur.fr/seqanal/interfaces/drawtree-simple.html).

proposed type strain), *Methanobacterium thermoauto-trophicum* JW510, *Methanobacterium thermoformi-cicum* FF3, *Methanobacterium defluvii*, *Methanobacterium thermophilum*, *Methanobacterium thermoflexum* and *Methanobacterium thermoautotrophicum* ZH3. The strains were antigenically fingerprinted at seven positions using seven S-probes as previously described (Macario & Conway de Macario, 1983): 1 and 10, *Methanobrevibacter smithii* PS and ALI, respectively; 2, *Methanobacterium formicicum* MF; 4, *Methanobacterium bryantii* MoH; 11 and 12, *Methanobacterium thermoautotrophicum* GC1 and ΔHᵀ, respectively; and 27, *Methanosphaera*

stadtmanae MCB3. Each strain has a unique antigenic fingerprint that differs from the others including the reference strains (Table 2). None of the strains was antigenically related to the mesophile *Methanobacterium formicicum* MF in spite of the fact that some, like *Methanobacterium thermoformicicum* Z-245, grow on formate. Similarly, none was related to the mesophile *Methanobacterium bryantii* MoH. *Methanobacterium defluvii* and *Methanobacterium thermophilum* are antigenically more related to each other than to the other isolates. Except for *Methanobacterium thermoautotrophicum* Marburgᵀ and *Methanobacterium thermoflexum*, all the strains were

Table 2. Partial antigenic fingerprinting of thermophilic *Methanobacterium* species analysed and related reference methanogens

The immunizing strains for the antisera were as follows: S-probe 1, *Methanobrevibacter smithii* PS; S-probe 2, *Methanobacterium formicicum* MF; S-probe 4, *Methanobacterium bryantii* MoH; S-probe 10, *Methanobrevibacter smithii* ALI; S-probe 11, *Methanobacterium thermoautotrophicum* GC1; S-probe 12, *Methanobacterium thermoautotrophicum* ΔH^T; S-probe 27, *Methanosphaera stadtmanae* MCB3. The intensities of the reactions ranged from 0 [no reaction (values omitted for clarity)] to 4 (maximum intensity).

Strain	Reaction with S-probe						
	1	**2**	**4**	**10**	**11**	**12**	**27**
Reference							
Methanobrevibacter smithii PS	4			4			
Methanobrevibacter smithii ALI	3			4			
Methanobacterium thermoautotrophicum GC1					4	2	
Methanobacterium thermoautotrophicum ΔH^T					2	4	
Methanobacterium formicicum MF		4					
Test							
Methanobacterium thermoautotrophicum Marburg^{T*}	1			3			
Methanobacterium thermoautotrophicum ZH3					1	1	
Methanobacterium thermoformicicum CB12*					2	1	
Methanobacterium wolfei DSM 2970^T					3		
Methanobacterium thermoformicicum THF*					4	3	
Methanobacterium thermoautotrophicum JW510					4		
Methanobacterium thermoformicicum Z-245*					3	2	
Methanobacterium thermoformicicum FTF*					3	3	
Methanobacterium thermoformicicum FF3					4	1	
Methanobacterium defluvii	3				4	2	
Methanobacterium thermophilum	4				4	4	
Methanobacterium thermoflexum	3						

* Data from Touzel *et al.* (1992).

antigenically related to either *Methanobacterium thermoautotrophicum* GC1 or ΔH^T, or to both. In conclusion, fingerprinting results clearly indicate that mesophilic and thermophilic *Methanobacterium* isolates are antigenically different. However, among thermophilic isolates they are less informative at the group level, where significant differences are observed (cf. strains *Methanobacterium thermoautotrophicum* Marburg^T and ZH3 on the one hand and *Methanobacterium thermoautotrophicum* ΔH^T, *Methanobacterium defluvii* and *Methanobacterium thermoflexum* on the other hand). The reasons for this antigenic diversity within groups recognized at the 16S rRNA level are not clear but may have taxonomic relevance.

Plasmid and phage typing of thermophilic *Methanobacterium* strains

Plasmid DNA was reported in three isolates of the $\Delta H/Z$-245 group and in two isolates of the Marburg group. In contrast, there is no report about plasmids in the *Methanobacterium wolfei* group. Interestingly, all three plasmids from the $\Delta H/Z$-245 group, pFV1, pFZ1 and pFZ2, are structurally related, suggesting that they share a common replicon, pFZ1 (Nölling *et al.*, 1991, 1992). Similarly, the DNA sequences of plasmids pME2001 and pME2200 from strains *Methanobacterium thermoautotrophicum* Marburg^T and ZH3, respectively, reveal a close evolutionary relationship with each other (Stettler *et al.*, 1994), but not with the pFZ1 plasmid family (data not shown). Consistent with the existence of two types of replicon in *Methanobacterium*, portions of plasmids pFV1 and pFZ1 were detected by Southern hybridization in total DNA of plasmid-free strains *Methanobacterium thermoautotrophicum* ΔH^T, *Methanobacterium thermoformicicum* THF, *Methanobacterium thermoformicicum* FF1, *Methanobacterium thermoformicicum* FF3 and CSM3 belonging to the $\Delta H/Z$-245 group, but not in the DNA of strains *Methanobacterium thermoformicicum* CB12 and SF-4 belonging to the *Methanobacterium wolfei* group or in the DNA of strain *Methanobacterium thermoautotrophicum* Marburg^T. Strain *Methanobacterium thermoformicicum* HN4 of the *Methanobacterium wolfei* group was the only isolate giving a hybridization signal with one of the probes (Nölling *et*

Table 3. Main phenotypic traits of type strains of thermophilic *Methanobacterium* isolates

Sources: *Methanobacterium thermoautotrophicum* ΔH[T], Zeikus & Wolfe (1972); *Methanobacterium thermoautotrophicum* Marburg[T], Schönheit *et al.* (1980); *Methanobacterium wolfei* DSM 2970[T], Winter *et al.* (1984).

	ΔH[T]	Marburg[T]	DSM 2970[T]
Source	Sewage sludge	Sewage sludge	Sewage sludge and river sediment
Morphology	0.4×3–120 µm	0.35×3–20 µm	0.4×2.5 µm
Growth substrates	H_2/CO_2*	H_2/CO_2	H_2/CO_2, formate*
Temperature			
Range	40–75 °C	45–70 °C	37–74 °C
Optimum	65–70 °C	65 °C	55–65 °C
pH			
Range	6.0–8.8	5.0–8.0	6.0–8.2
Optimum	7.2–7.6	6.8–7.4	7.0–7.5
NaCl concn (g l^{-1})			
Range	0.1–35†	0.1–35†	NA†
Optimum	0.6†	0.5†	Up to 10
G+C mol%	50±2‡	48±0.5‡	61§

* Formate can be used as growth substrate by most of the isolates phenotypically closely related to the type strain ΔH[T]; unlike earlier reports, *Methanobacterium wolfei* grows on formate (J. Nölling, unpublished data).

† Data from Ciulla *et al.* (1994) and Perski *et al.* (1981); NA, data not available.

‡ Additional data from Brandis *et al.* (1981); Kotelnikova *et al.* (1993a); Touzel *et al.* (1992).

§ 48.5 mol% in Kotelnikova *et al.* (1993a).

al., 1993c). However, in the absence of a transformation system for those thermophiles, it is not possible to infer whether plasmids carrying the pFV1 replicon cannot replicate in hosts harbouring plasmids of the pME2001 family and vice versa.

Phage ΦF1 specifically infects the following strains of the ΔH/Z-245 group: *Methanobacterium thermoautotrophicum* ΔH[T], *Methanobacterium thermoformicicum* strains Z-245, FTF, CSM3, FF1 and FF3 (but not THF). In contrast, *Methanobacterium thermoformicicum* strains CB12, SF-4 and HN4 of the *Methanobacterium wolfei* group and strain *Methanobacterium thermoautotrophicum* Marburg[T] were not infected (Nölling *et al.*, 1993a). Conversely, phage ΨM2, which is specific for strain *Methanobacterium thermoautotrophicum* Marburg[T], did not infect strains *Methanobacterium thermoautotrophicum* ΔH[T], *Methanobacterium thermoformicicum* Z-245, *Methanobacterium thermoformicicum* THF and *Methanobacterium thermoformicicum* FF3 (ΔH/Z-245 group), *Methanobacterium thermoformicicum* CB12 and *Methanobacterium wolfei* DSM 2970[T] (*Methanobacterium wolfei* group) and *Methanobacterium thermoautotrophicum* ZH3 (Marburg group). It has been shown that strain *Methanobacterium thermoformicicum* THF harbours a restriction/modification system with target sites on the genome of ΦF1, thus possibly explaining the phage-resistant phenotype of strain *Methanobacterium thermoformicicum* THF (Nölling *et al.*,

1993a). A similar reasoning can be made to explain the failure of phage ΨM2 to infect cells of strain *Methanobacterium thermoformicicum* THF since the complete phage sequence is now available (Pfister *et al.*, 1998). The resistance of *Methanobacterium thermoautotrophicum* strain ZH3 to infection by phage ΨM2 may be due to inefficient phage binding related to differences in the cell surface properties of strains *Methanobacterium thermoautotrophicum* ZH3 and *Methanobacterium thermoautotrophicum* Marburg[T], as indicated by the antigenic fingerprinting results. *Methanobacterium wolfei* spontaneously lyses when starved for hydrogen (König *et al.*, 1985) and harbours an integrated, defective prophage (Stettler *et al.*, 1995). Thus, the host range of phages of the ΨM family may not be limited to isolates of the Marburg group, although no productive infection has yet been documented in any isolate other than strain *Methanobacterium thermoautotrophicum* Marburg[T] itself.

Plasmid content and phage susceptibility are somewhat relative phenotypes due to frequent exceptions. However, our data suggest the existence of distinct plasmid and phage families in accordance with the proposed speciation model.

DISCUSSION

The genus *Methanothermobacter* was recently proposed to include several, if not all, thermophilic

strains of *Methanobacterium* (Boone *et al.*, 1993). This proposal was based on analysis of partial 16S rRNA sequences, which returned less than 93–95 % similarity with their mesophilic counterparts. Our own results, summarized in Table 1 and Fig. 2, confirm that view, and our similarity scores of approximately 90 % undoubtedly support the creation of a new genus, *Methanothermobacter*. In addition, they indicate a certain level of discrepancy among thermophilic isolates, which led us to propose the creation of three species with type strains ΔH[T], DSM 2970[T] and Marburg[T], respectively (Table 3; see also Touzel *et al.*, 1992). To some extent, this model is supported by antigenic fingerprints, and plasmid as well as phage typing. However, those markers are not necessarily distinctive phenotypes in methanogens (see, for example, Keswani *et al.*, 1996) and those three species are difficult to define using growth characteristics and habitats, for example. Isolates grouped together with strain Marburg[T] on the basis of their 16S rRNA signatures grow as straight to smoothly curved rods in chains significantly shorter than those formed by cells of isolates of the ΔH group.

DNA–DNA reassociation experiments performed by several groups also clearly indicate phenotypic differences between strain *Methanobacterium thermoautotrophicum* ΔH[T], strain *Methanobacterium wolfei* DSM 2970[T] and strain *Methanobacterium thermoautotrophicum* Marburg[T] (Brandis *et al.*, 1981; Kotelnikova *et al.*, 1993a; Touzel *et al.*, 1992). This is strong evidence in support of our proposal for a new species with strain Marburg[T] as a type strain. However, using in part the same phenotype, Kotelnikova *et al.* (1993a, b) recently described three new thermophilic species, *Methanobacterium defluvii*, *Methanobacterium thermoflexum* and *Methanobacterium thermophilum*. In contrast, our analysis of 16S rRNA indicates a very close relationship of those three strains with isolates of the ΔH group. In keeping with the conclusions of Stackebrandt & Goebel (1994), we therefore propose to transfer those three thermophilic strains to the genus *Methanothermobacter* while keeping their status as separate species, viz. as *Methanothermobacter defluvii*, *Methanothermobacter thermoflexus* and *Methanothermobacter thermophilus*.

Description of *Methanothermobacter* gen. nov. (David R. Boone, personal communication)

Methanothermobacter (Me.tha.no.ther.mo.bac′ter. N.L. neut. n. *methanum* methane; Gr. adj. *thermos* hot; M.L. masc. n. *bacter* equivalent of Gr. dim. neut. n. *bakterion* rod, staff; M.L. masc. n. *Methanothermobacter* thermophilic methane rod).

Curved or crooked slender rods, moderately long to filamentous, 0·3–0·5 μm wide. Endospores not formed. Cells stain Gram-positive, and ultrastructure appears typically Gram-positive, but cell walls are composed of pseudomurein. Non-motile. Cells produce fimbriae. Very strictly anaerobic. Fastest growth between 55 and 65 °C. Energy metabolism by reduction of CO_2 to CH_4, with H_2 as electron donor; some cells can also use formate as electron donor. Sulfur is reduced to sulfide, but this reaction does not yield energy for growth. Ammonia is the sole nitrogen source, and sulfide may serve as sulfur source. The DNA G+C content is 32–61 mol %. Type species: *Methanothermobacter thermautotrophicus* comb. nov.

Emended description of *Methanothermobacter thermautotrophicus* comb. nov. [formerly *Methanobacterium thermoautotrophicum* (corrig.) Zeikus and Wolfe 1972, 712[AL]]

Methanothermobacter thermautotrophicus (therm.au. to.tro.phi′cus. Gr. adj. *thermos* hot; Gr. pref. *auto* self; Gr. adj. *trophikos* one who feeds; N.L. masc. adj. *thermautotrophicus* thermophilic and autotrophic).

Cells are slender, cylindrical, irregularly crooked rods that are 0·35–0·5 μm wide and 3–7 μm long and frequently occur in filaments that are 10–120 μm long. Gram-positive. Non-motile. Endospores not formed. Deep colonies in roll tubes are tannish white, roughly spherical, diffuse and filamentous. Growth is rapid in mineral medium with CO_2 as the sole carbon source, NH_3 as the sole nitrogen source, sulfide as the sole sulfur source and H_2/CO_2 as the sole energy source. Growth on formate as the sole carbon and energy source is possible for some strains. Not stimulated by organic additions, although acetate may be assimilated. The DNA G+C content is 48–50 mol % as determined by thermal denaturation. Some strains harbour a plasmid, and some are infected by lytic phages. Habitat: thermophilic, anaerobic digesters, hot springs. The type strain is strain ΔH[T] (=DSM 1053[T] = ATCC 29096[T]), which was isolated from an anaerobic sewage sludge digester; this strain does not grow on formate. The reference strain includes strain Z-245 (= DSM 3720) Zhilina and Ilarionov 1984, which grows on formate.

Emended description of *Methanothermobacter wolfeii* comb. nov. (formerly *Methanobacterium wolfei* Winter and Lerp 1984, 465[AL])

Methanothermobacter wolfeii (wolf′e.i.i. M.L. gen. n. *wolfeii* of Wolfe who pioneered the research on methanogenesis).

Cells are slender, cylindrical, sometimes crooked rods that are 0·35–0·5 μm wide and 2·5 μm long and occur singly or in pairs, or in longer chains. Gram-positive. Non-motile. Endospores not formed. Colonies on agar plates are 1–2 mm in diameter, yellowish and convex. Growth is rapid in mineral medium with NH_3 as the sole nitrogen source, sulfide as the sole sulfur source and H_2/CO_2 or formate as the sole carbon and energy sources. The type strain is stimulated by addition of tungsten and spontaneously lyses when deprived of energy. The DNA G+C content of the type strain is 61 mol % as determined by thermal denaturation.

Strains are devoid of extrachromosomal elements. The type strain harbours a chromosomally integrated, defective prophage. Habitat: mesophilic and thermophilic sludge or digesters. The type strain is strain DSM 2970^T (= ATCC 43096^T), which was isolated from a mixture of sewage sludge and river sediment.

Description of *Methanothermobacter marburgensis* sp. nov. (formerly *Methanobacterium thermoautotrophicum*)

Methanothermobacter marburgensis (mar.bur.gen′sis. German n. *Marburg* name of a city in Germany; M.L. masc. suffix *-ensis* pertaining to/originating from a locality; M.L. n. *marburgensis* from Marburg).

Cells are slender, cylindrical rods that are 0·30–0·4 μm wide and 3·0–3·5 μm long and frequently occur in pairs or chains up to 20 μm long. Gram-positive. Non-motile. Endospores not formed. Colonies on agar plates are 1–4 mm in diameter, white or slightly yellowish, and convex. Growth is rapid in mineral medium with CO_2 as the sole carbon source, NH_3 as the sole nitrogen source, sulfide as the sole sulfur source and H_2/CO_2 as the sole energy source. Growth on formate is not possible. Not stimulated by organic additions, although acetate may be assimilated. The DNA G+C content of the type strain is 48 mol% as determined by thermal denaturation. Strains may harbour one plasmid, and the type strain is infected by lytic phages. Habitat: mesophilic sewage sludge and hot springs. The type strain is strain Marburg^T (= DSM 2133^T), which was isolated from mesophilic sewage sludge.

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