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 ∆H³); (ii) *Methanothermobacter wolfeii* comb. nov., containing four formate-utilizing isolates (type strain DSM 2970³); (iii) *Methanothermobacter marburgensis* sp. nov., containing three obligately autotrophic isolates (type strain Marburg³). Of the nine isolates formerly referred to as *Methanobacterium thermoformicicum*, six were reclassified as *Methanothermobacter thermotrophicus* and three as *Methanothermobacter wolfeii*.

**Keywords:** Archaea, *Methanobacterium*, *Methanothermobacter*, phylogeny, 16S rRNA

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**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-

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ligate autotrophs (growth on H₂/CO₂), 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 thermosudetense (Zeikus & Wolfe, 1972; Zeikus, 1972), Methanobacterium wolfei (Winter et al., 1984), Methanobacterium thermophilicum (Zhilina & Ilarionov, 1984), Methanobacterium defluviit (Kotelnikova et al., 1993b), Methanobacterium thermophilum (Laurinavichyus et al., 1988), Methanobacterium thermoflexum (Kotelnikova et al., 1993b), Methanobacterium thermoacidiphilum (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, Prévôt (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 ΔH⁷ and Marburg³ (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 thermosudetense, consisting of obligately autotrophic strains, and Methanobacterium thermophilicum, consisting of formate utilizers. Three groups were recognized: (i) one containing Methanobacterium thermosudetense ΔH⁷ and Methanobacterium thermophilicum strains Z-245, FTF, THF, CSM3, FF1 and FF3; (ii) Methanobacterium thermophilicum strains CB12, SF-4 and HN4; (iii) Methanobacterium thermosudetense Marburg³. Recently, the inability of strain Methanobacterium thermosudetense ΔH⁷ 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 thermophilicum 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 thermoacidiphilum 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 defluviit, 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 extra-chromosomal 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**

**Archaean strains and growth conditions.** Methanobacterium thermoautotrophicum strains Marburg³ (DSM 2133⁷) and ZH3 (DSM 9946), Methanobacterium wolfei (DSM 2970⁷) and Methanobacterium thermophilum 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 thermophilicum Z-245 (DSM 3720), Methanobacterium thermophilicum CB12 (DSM 3664), Methanobacterium defluviit (DSM 7466), Methanobacterium thermophilum (DSM 6529) and Methanobacterium thermoflexum (DSM 7268). Phage PM2 (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 ΔH⁷ (Nölling et al., 1991) and Methanobacterium thermoautotrophicum strain Marburg³ (Schönheit et al., 1979); the latter medium was supplemented with 8 µM sodium tungstate and 58 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); the final cycle included a 10 min amplification step. Taq polymerase (Fermentas) and the following oligonucleotides were used (restriction sites underlined): Bam16S, 5‘-CACGATCCGAACGGGCTCTAGTAAACAGC-3‘ and Pst16S, 5‘-GTGGTCTGACGGGCTACCTTGTTACGACT-3‘. Amplified DNA fragments were separated by agarose gel electrophoresis, and the expected 1.4 kb fragments were excised, isolated, digested with BamHI and PstI, 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,
Taxonomy of thermophilic Methanobacterium species

Antigenic fingerprinting. The partial antigenic fingerprint was determined by indirect immunofluorescence and a quantitative slide immunoenzymic assay with a panel of 8 probes for rod-shaped methanogens, Methanobrevibacter smithii PS and AL1; Methanobacterium thermoautotrophicum GC1 and ΔH2; Methanobacterium bryantii MoH; Methanobacterium formicicum MF; and Methanospirillum 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/v) 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 Marburg7 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 culture control containing no lysate was prepared for both strains. In addition, strain Methanobacterium thermoautotrophicum Marburg7 as a positive lysis control and strain Methanobacterium thermoautotrophicum ΔH2 as a negative control were subjected to the same manipulations. All culture vials were presurized with 2 bar of a H2/CO2 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 Marburg7, Methanobacterium thermoautotrophicum ZH3, Methanobacterium thermoautotrophicum ΔH2, Methanobacterium thermoformicicum Z-245, Methanobacterium thermoformicicum FF3, Methanobacterium thermofomricicum THF, Methanobacterium wolfei Z-245, and Methanobacterium thermofomricicum CB12 were prepared as described above (Jarrell et al., 1992), digested with BamHI, 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 thermoformicium (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 thermoformicium 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 Marburg7, 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 thermophilum, Methanobacterium thermophilum and strain Methanobacterium thermoautotrophicum JW510.

The sequence group around strain Methanobacterium thermoautotrophicum Marburg7 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 Marburg7 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 Marburg7 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 Marburg7 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-
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\(^1\) are represented by dots, sequence differences by the corresponding nucleotide, and gaps by dashes. An additional nucleotide found upon resequencing strain Methanobacterium thermoautotrophicum Marburg\(^1\) 16S rRNA gene is marked by \(\ast\). 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, gap extension penalty 2). Strain names and accession numbers: Methanobacterium thermoautotrophicum Marburg\(^1\), X15364; ZH3, Z37156; ∆HT, X68720; Methanobacterium thermoformicum 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 ∆H\(^T\) 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 ∆H\(^T\) except at three bases unresolved (N) in the Methanobacterium thermoautotrophicum ∆H\(^T\) sequence. Therefore, we propose to revise their status and incorporate them into the ∆H group. Strains

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Methanobacterium thermoautotrophicum THF and Methanobacterium thermoformicicum 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 thermoautotrophicum THF and Methanobacterium thermoformicicum 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 THF and Methanobacterium thermoformicicum THF in the ΔH group) are significantly higher than scores between any group pair (97.9 ± 0.7%); (ii) similarity scores of all three groups with respect to mesophilic Methanobacterium species are markedly lower (89.9 ± 0.8%) than any of the above scores, indicating that the three groups around strains Methanobacterium thermoautotrophicum ΔH⁴, Methanobacterium wolfei DSM 2970⁳ and Methanobacterium thermoautotrophicum Marburg⁴ 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⁳ (a

#### 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 RH2 (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).

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*Mesophiles refers to Methanobacterium formicicum DSM 1312, Methanobacterium formicicum FCam, Methanobacterium subterraneum, Methanobacterium bryantii DSM 863 and Methanobacterium bryantii RH2.

†Methanobrevibacter arboriphilicus, Methanobrevibacter ruminantium, Methanobrevibacter filiformis and Methanobrevibacter curvatus.
proposed type strain), Methanobacterium thermoautotrophicum JW510, Methanobacterium thermoformicum FF3, Methanobacterium defluvii, Methanobacterium thermophilum, Methanobacterium thermoflexum and Methanobacterium thermoaototrophicum 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 thermoaototrophicum GCl and ΔH, 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 thermoformicum 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 thermoaototrophicum Marburg and Methanobacterium thermoflexum, all the strains were

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, gap extension penalty 2). 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).
Taxonomy of thermophilic *Methanobacterium* species

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

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<td><em>Methanobacterium thermoflexum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* 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 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 ΔH/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 ΔH/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* Marburg T and ZH3, respectively, 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* 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* 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* 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* 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).
Table 3. Main phenotypic traits of type strains of thermophilic Methanobacterium isolates

Sources: Methanobacterium thermoautotrophicum ΔH\textsuperscript{T}, Zeikus & Wolfe (1972); Methanobacterium thermoautotrophicum Marburg\textsuperscript{T}, Schönheit et al. (1980); Methanobacterium wofei DSM 2970\textsuperscript{T}, Winter et al. (1984).

<table>
<thead>
<tr>
<th>Source</th>
<th>AH\textsuperscript{T}</th>
<th>Marburg\textsuperscript{T}</th>
<th>DSM 2970\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Sewage sludge</td>
<td>Sewage sludge</td>
<td>Sewage sludge and river sediment</td>
</tr>
<tr>
<td>Growth substrates</td>
<td>0.4 × 3–120 µm H\textsubscript{2}/CO\textsubscript{2} \textsuperscript{*}</td>
<td>0.35 × 3–20 µm H\textsubscript{2}/CO\textsubscript{2}</td>
<td>0.4 × 2.5 µm H\textsubscript{2}/CO\textsubscript{2}, formate \textsuperscript{*}</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>40–75 °C</td>
<td>45–70 °C</td>
<td>37–74 °C</td>
</tr>
<tr>
<td>Optimum</td>
<td>65–70 °C</td>
<td>65 °C</td>
<td>55–65 °C</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6.0–8.8</td>
<td>5.0–8.0</td>
<td>6.0–8.2</td>
</tr>
<tr>
<td>Optimum</td>
<td>7.2–7.6</td>
<td>6.8–7.4</td>
<td>7.0–7.5</td>
</tr>
<tr>
<td>NaCl concn (g l\textsuperscript{-1})</td>
<td>0–1–35\textsuperscript{†}</td>
<td>0–1–35\textsuperscript{†}</td>
<td>NA\textsuperscript{†}</td>
</tr>
<tr>
<td>G + C mol%</td>
<td>50 ± 2\textsuperscript{‡}</td>
<td>48 ± 0.5\textsuperscript{‡}</td>
<td>61\textsuperscript{§}</td>
</tr>
</tbody>
</table>

\* Formate can be used as growth substrate by most of the isolates phenotypically closely related to the type strain ΔH\textsuperscript{T}; unlike earlier reports, Methanobacterium wofei 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 ΨF1 specifically infects the following strains of the ΔH/Z-245 group: Methanobacterium thermoautotrophicum AH\textsuperscript{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\textsuperscript{T} were not infected (Nölling et al., 1993a). Conversely, phage ΨM2, which is specific for strain Methanobacterium thermoautotrophicum Marburg\textsuperscript{T}, did not infect strains Methanobacterium thermoautotrophicum ΔH\textsuperscript{T}, Methanobacterium thermoformicicum Z-245, Methanobacterium thermoformicicum THF and Methanobacterium thermoformicicum FF3 (AH/Z-245 group), Methanobacterium thermoformicicum CB12 and Methanobacterium wofei DSM 2970\textsuperscript{T} (Methanobacterium wofei 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 ΨF1, 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\textsuperscript{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\textsuperscript{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 AH, DSM 2970 and Marburg, 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 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* AH, strain *Methanobacterium wolfei* DSM 2970 and strain *Methanobacterium thermoautotrophicum* Marburg (Brandis *et al*., 1981; Kotelnikova *et al*., 1993a; Touzel *et al*., 1992). This strong evidence in support of our proposal for a new species with strain Marburg 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 thermophilum*.

**Description of Methanothermobacter gen. nov.**

(David R. Boone, personal communication)


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 fimbiae. Very strictly anaerobic. Fastest growth between 55 and 65 °C. Energy metabolism by reduction of CO₂ to CH₄, with H₂ 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 thermotrophicus* comb. nov.

**Emended description of Methanothermobacter thermotrophicus comb. nov. (formerly Methanothermobacter thermoaautotrophicum (corrig.) Zeikus and Wolfe 1972, 712⁴])**


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 CO₂ as the sole carbon source, NH₃ as the sole nitrogen source, sulfate as the sole sulfur source and H₂/CO₂ 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 AH (=DSM 1053³ = ATCC 29096³), 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 Methanothermobacter wolfei Winter and Lerp 1984, 465⁴)]**

*Methanothermobacter wolfei* (wolf’e.i.i. M.L. gen. n. *wolfei* 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 NH₃ as the sole nitrogen source, sulfide as the sole sulfur source and H₂/CO₂ 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 2970T (= ATCC 43096T), 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/or 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 media. The type strain is isolated from a mixture of sewage sludge and river sediment.

Growth is rapid in mineral medium with CO₂ as the sole carbon source, NH₃ as the sole nitrogen source, sulphide as the sole sulphur source and H₂/CO₂ 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 digester sludge. The type strain is strain MarburgT (= DSM 2133T), which was isolated from mesophilic sewage sludge.

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