DNA Relatedness among Some Thermophilic Members of the Genus *Methanobacterium*: Emendation of the Species *Methanobacterium thermoautotrophicum* and Rejection of *Methanobacterium thermoformicum* as a Synonym of *Methanobacterium thermoautotrophicum*

JEAN PIERRE TOUZEL,^{1*} EVERLY CONWAY DE MACARIO,² JÖRK NÖLLING,³
WILLEM M. DE VOS,³ TATJANA ZHILINA,⁴ AND A. M. LYSENKO⁴

*Institut National de la Recherche Agronomique, Station de Technologie Alimentaire, B.P. 39, F-59651 Villeneuve d'Ascq Cedex, France*¹; *Wadsworth Center for Laboratories and Research, New York State Department of Health, and Department of Biomedical Sciences, School of Public Health, State University of New York, Albany, New York 12201-0509*²; *Bacterial Genetics Group, Department of Microbiology, Agricultural University of Wageningen, NL-6703 CT Wageningen, The Netherlands*³; and *Institute of Microbiology, Russian Academy of Sciences, Prospect 60-Let Oktyabrya, 7/2, Moscow 117811, Russia*⁴

DNA reassociation was used to determine levels of relatedness among four thermophilic *Methanobacterium* strains that are able to use formate and between these organisms and two representative strains of *Methanobacterium thermoautotrophicum*, strain ΔH^T (= DSM 1053^T = ATCC 29096^T) (T = type strain) and strain Marburg (= DSM 2133). Three homology groups were delineated, and these groups coincided with the clusters identified by antigenic fingerprinting. The first group, which had levels of cross hybridization that ranged from 73 to 99%, included *M. thermoautotrophicum* ΔH^T , *Methanobacterium thermoformicum* Z-245, *Methanobacterium* sp. strain THF, and *Methanobacterium* sp. strain FTF. The second and third groups were each represented by only one strain, *Methanobacterium* sp. strain CB-12 and *M. thermoautotrophicum* Marburg, respectively (cross-hybridization levels, 13 to 30 and 29 to 33%, respectively). Our results indicate that the name *M. thermoformicum* should be rejected as it is a synonym of *M. thermoautotrophicum*. The taxonomic positions of strains Marburg and CB-12 need further investigation.

Rod-shaped, thermophilic, formate-utilizing, methane bacteria have been isolated from globally dispersed geographical locations. On the basis of differences of 10 to 15°C in temperature optima and temperature ranges compared with *Methanobacterium thermoautotrophicum*, a characteristic that has been used previously to separate species in the genera *Methanogenium* and *Methanococcus*, and on the basis of formate utilization, Zhilina and Ilarionov (24, 25) proposed the name *Methanobacterium thermoformicum* for their isolate Z-245. This name has been validated (8a). However, the taxonomic position of this organism has never been clearly assessed. The purpose of this work was to use DNA-DNA hybridization for taxonomic assignment of four thermophilic formate-utilizing strains of the genus *Methanobacterium*, including isolate Z-245, which had been grouped previously by antigenic fingerprinting.

MATERIALS AND METHODS

Strains, media, and culture conditions. The strains used in this study are listed in Table 1. Most of these organisms were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; strains CB-12 and THF were kindly provided by David Boone and Steve Zinder, respectively. The methanogens were cultivated on BCYT medium as previously described (19). The medium was buffered with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-

sulfonic acid) or PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] unless otherwise indicated.

The anaerobic gas was an H₂-CO₂ mixture (80:20). Sterile media were inoculated with previous cultures (2%, vol/vol). Organisms were mass cultured in 2-liter fermentors containing the same media with continuous sparging of H₂-CO₂ and H₂S.

G+C contents of the DNAs. Frozen cells were thawed and disrupted with a French press at 76,000 kPa, and the DNA was then purified by phenol extraction and hydroxylapatite adsorption as described by Johnson (9). The thermal denaturation procedure was used to determine the DNA guanine-plus-cytosine (G+C) content. The DNA was dissolved in a saline citrate solution (0.15 M NaCl plus 0.015 M trisodium citrate 2-hydrate; pH brought to 7 with 0.1 N HCl). The *A*₂₆₀ and the cell temperature were digitally recorded at 0.5-min intervals with a model SP 800 spectrophotometer (Kontron, Zürich, Switzerland) equipped with a model PD 415 temperature programmer (Huber, Offenbourg-Elgersweier, Germany). The melting temperature was computed by using the method of the median as described by Ferragut and Leclerc (7).

DNA homologies. All of the DNA samples were sonicated at 0°C for 1 min with a Labsonic 1510 instrument (Braun, Melsungen, Germany) by using a 10-W energy setting and a 4-mm probe. Sonication was performed a total of four times (1 min each), with 1-min rests between the sonic treatments.

Nick translation was performed with a Bethesda Research Laboratories kit by using the manufacturer's protocol and deoxy [1',2',5'-³H]cytidine 5'-triphosphate as the radioac-

* Corresponding author.

TABLE 1. Sources of strains

Strain	Other designation	Geographical area	Reference
<i>M. thermoautotrophicum</i> ΔH^T	DSM 1053 ^T	Urbana, Ill.	22
<i>M. thermoautotrophicum</i> Marburg	DSM 2133	Marburg, Germany	8
<i>M. thermoformicum</i> Z-245	DSM 3720	USSR	24
<i>Methanobacterium</i> sp. strain CB-12	DSM 3664	Chengdu, People's Republic of China	23
<i>Methanobacterium</i> sp. strain THF	DSM 3848	Ithaca, N.Y.	26
<i>Methanobacterium</i> sp. strain FTF	DSM 3012	Lille, France	19

tive label. After nick translation, the labeled DNA was separated from excess nucleotides by chromatography on Sephadex G-50 on a spun column as described by Maniatis et al. (13). The S1 nuclease-resistant cores in the radioactive probes were eliminated by chromatography on a hydroxyapatite column in a Pasteur pipette (bed dimensions, 0.6 by 5 cm). The DNA sample was denatured by heating it for 10 min in a boiling bath. The phosphate concentration was brought to about 0.1 M, and the sample was deposited on the top of the column. Fractions (5 drops) that were eluted by phosphate buffer (0.12 M, pH 6.8) were monitored for radioactivity and pooled. The pooled fractions were freeze-dried and dissolved in 0.1 ml of water. The phosphate buffer was replaced by TES (Tris-EDTA-saline) buffer by spun-column chromatography on Sephadex G-50.

The reassociation reaction mixtures contained 5 μ l (0.010 to 0.025 μ g, 6,000 to 12,000 cpm) of labeled DNA, 100 μ l (40 μ g) of unlabeled DNA, 35 μ l of water, and 280 μ l of a buffer containing 600 mM NaCl, 15 mM EDTA (pH 8), 15 mM Tris buffer (pH 8), 0.0225% sodium dodecyl sulfate, and 75% formamide. The DNA mixtures were denatured by heating them in boiling water for 10 min. Hybridizations were conducted for 18 h at 35°C. The single-stranded DNAs were eliminated by incubating the preparations for 1 h at 30°C in the presence of a suitable dilution of S1 nuclease (9), and the hybrids were precipitated with an equal volume of 10% trichloroacetic acid, collected on Whatman type GF/F fiber-glass discs, washed five times with ice-cold trichloroacetic acid and then with ethanol, dried, and counted with a Betamatic liquid scintillation counter (Kontron) in the presence of 10 ml of Insta-gel scintillation mixture.

DNA hybrid thermal stability. The thermal stabilities of the DNA-DNA hybrids were estimated by using the S1 nuclease procedure described by Johnson (9). The temperatures used to obtain the dissociation profiles were 65 through 100°C.

Antigenic fingerprinting. Antibody S-probes were prepared as previously described (11) from antisera against the reference methanogens listed in Table 4. Antigenic fingerprinting was done as described previously (10) by using indirect immunofluorescence and the quantitative slide immunoenzymatic assay (12). Levels of antigenic relatedness

among the strains and between the strains and reference methanogens were determined by performing a comparative analysis of antigenic fingerprints with a reference table (12).

Nucleotide sequence accession numbers. The nucleotide sequences of *M. thermoautotrophicum* ΔH^T and *M. thermoformicum* Z-245 5S rRNAs have been deposited in the EMBL Nucleotide Sequence Database under accession numbers X62857 and X62858, respectively.

RESULTS AND DISCUSSION

The G+C contents of the six test strains (Table 3) ranged from 47 to 50 mol%. The values obtained for strains ΔH^T (T = type strain) and Marburg (48.6 and 47.7 mol%, respectively) were in close agreement with the values obtained by Brandis et al. (4), who used the same method (48.6 and 47.6 mol%, respectively). The previously reported values for the G+C content of strain ΔH^T were 52 and 49.7 mol% as determined by buoyant density in CsCl (1, 22) and 49.4 mol% as determined by thermal denaturation (14).

Levels of DNA homology were determined by using the DNA of each of the six strains as a reference. [³H]DNA probes hybridized at levels of 71 to 82% with their homologous DNAs and at levels of 3 to 7% with unrelated DNA from salmon sperm. Table 3 shows that strains ΔH^T , Z-245, FTF, and THF formed a homogeneous group with more than 73% homology. However, strain Marburg was noticeably distant from this group (level of homology, less than 35%). Strain CB-12 exhibited less than 30% homology with strain Marburg and with each of the other strains.

The groups described above coincided with the clusters identified by antigenic fingerprinting. Table 4 shows that strains Z-245, FTF, and THF formed a cluster of related organisms that were close to reference strains ΔH^T and GC1. On the other hand, strain CB-12 was antigenically less closely related to strains ΔH^T and GC1, and strain Marburg was not related to either of these strains.

Strains ΔH^T , Z-245, FTF, and THF clearly belong to the same species since they share more than 82% DNA homology. This conclusion is in agreement with the proposal of Priest et al. (18), who stated that, "Although there is no universally accepted level of DNA homology which delineates a bacterial species, most workers agree that strains within a species should share at least 50 to 60% homology." This grouping was further supported by the results of a ΔT_m study which showed that the most heterogeneous hybrid was the hybrid between strain ΔH^T and strain THF, which had a ΔT_m of less than 5°C (Table 2) (ΔT_m is the difference between the melting temperature of a homologous hybrid and the melting temperature of a heterologous hybrid). Similar conclusions about the relationship between *M. thermoautotrophicum* ΔH^T and *M. thermoformicum* Z-245 were reached by Chumakov et al. (6) on the basis of data on 5S RNA sequences, which differed in only six nucleotides (Fig.

TABLE 2. Reassociation of DNA from *M. thermoautotrophicum* ΔH^T

Source of unlabeled DNA	% Relative binding at:		ΔT_m at 65°C
	65°C	75°C	
<i>M. thermoautotrophicum</i> ΔH^T	100	90	0
<i>M. thermoformicum</i> Z-245	74	68	0.5
<i>Methanobacterium</i> sp. strain THF	66	50	4.4
<i>Methanobacterium</i> sp. strain FTF	74	72	0.7

TABLE 3. Levels of DNA homology, melting temperatures, and G+C contents

Strain ^a	Formate use	Melting temp (°C)	G+C content (mol%)	% DNA homology with reference DNA from strain:					
				ΔH^T	Z-245	THF	FTF	Marburg	CB-12
ΔH^T	—	89.32	48.6 $\pm$ 0.5	100 ^b	97	82	90	31	27
Z-245	+	89.39	48.8 $\pm$ 0.3	83	100	73	84	29	25
THF	+	89.74	49.6 $\pm$ 0.4	88	77	100	74	30	23
FTF	+	89.58	49.2 $\pm$ 0.3	99	100	82	100	31	24
Marburg	—	89.94	47.7 $\pm$ 0.5	35	29	33	31	100	13
CB-12	+	88.82	47.4 $\pm$ 0.4	30	26	27	25	27	100

^a *M. thermoautotrophicum* ΔH^T and Marburg, *M. thermoformicum* Z-245, and *Methanobacterium* spp. strains CB-12, THF, and FTF.

^b Homologous values are reported as 100%.

1), and by Bezrukova et al. (3), who found that strain Z-245 is a serotype of *M. thermoautotrophicum* ΔH^T .

The same grouping of the formate-utilizing strains was obtained by Nölling et al. (16), who studied plasmid contents and hybridization of total DNAs with the *fdhA* gene from *Methanobacterium formicum* JF. Furthermore, preliminary results (17) from a sequence analysis of the 16S rRNA genes from *M. thermoformicum* strains and *M. thermoautotrophicum* ΔH^T and Marburg are in agreement with the results presented here but are in contrast with the findings of Yamamoto et al. (20), who proposed that the formate-utilizing thermophilic methanobacteria should be separated into two groups on the basis of immunological cross-reactions with their antisera.

In our proposal a well-known and well-studied strain, *M. thermoautotrophicum* ΔH^T , which has never been shown to grow on formate, is now included in a group that contains three formate-utilizing strains.

The inability to grow on formate may be explained by the fact that the genes for formate utilization are either not present or not functional. For *M. thermoautotrophicum* ΔH^T and Marburg the latter possibility seems to be the case since it has been shown recently by probing with the *M. formicum* *fdh* operon (kindly provided by J. Ferry) that there are homologous sequences in the genomes of *M. thermoautotrophicum* ΔH^T and Marburg (15).

TABLE 4. Antigenic fingerprints of the strains analyzed and related reference methanogens

Reference strain	Test strain	Reaction with S-probe ^a :											
		1	2	4	5	8	9	10	11	12	21	22	27
ΔH^T		0	0	1	0	0	0	0	2	4	0	0	1
GC1		0	0	1	0	0	0	0	4	2	0	0	1
	Z-245	0	0	0	0	0	0	0	3	2	0	0	0
	FTF	0	0	0	0	0	0	0	3	3	0	0	0
	THF	0	0	0	0	0	0	0	4	3	0	0	0
	CB-12	0	0	0	0	0	0	0	2	1	0	0	0
PS		4	0	0	0	0	0	4	0	0	0	0	0
ALI		3	0	0	0	0	0	4	0	0	0	0	0
	Marburg	1	0	0	0	0	0	3	0	0	0	0	0

^a The immunizing strains for the antisera were as follows: S-probe 1, *Methanobrevibacter smithii* PS; S-probe 2, *Methanobacterium formicum* MF; S-probe 4, *Methanobacterium bryantii* MoH; S-probe 5, *Methanobacterium bryantii* MoHG; S-probe 8, *Methanobrevibacter ruminantium* M1; S-probe 9, *Methanobrevibacter arboriphilicus* DH1; S-probe 10, *Methanobrevibacter smithii* ALI; S-probe 11, *Methanobacterium thermoautotrophicum* GC1; S-probe 12, *Methanobacterium thermoautotrophicum* ΔH^T ; S-probe 21, *Methanobrevibacter arboriphilicus* AZ; S-probe 22, *Methanobrevibacter arboriphilicus* DC; and S-probe 27, *Methanospaera stadtmaniae* MCB3. The intensities of the reactions ranged from 0 (no reaction) to 4 (maximum intensity).

It is obvious that a negative characteristic has limited taxonomic significance. Benstead et al. (2) have recently shown that a mesophilic *Methanobacterium* strain which was originally described as unable to utilize formate could be grown on formate under defined conditions. These authors indicated that "the ability to utilize formate is of limited taxonomical significance" in that case.

Therefore, we propose that the description of *M. thermoautotrophicum* should be modified in order to accommodate the formate-utilizing strains and that the name *M. thermoformicum* should be rejected as a synonym of *M. thermoautotrophicum*. Strain ΔH is maintained as the type strain, and strain Z-245 is included in the list of reference strains, whereas strain Marburg is removed from this list. An emended description of the species is given below. Further work will be needed to assess the correct positions of *Methanobacterium* sp. strains Marburg and CB-12; the present hybridization data indicate that these strains should not be classified in the species *M. thermoautotrophicum*. The idea that strains ΔH^T and Marburg should be placed in different species has been discussed by Brandis-Heep (5) and has been supported by Zeikus (21).

Emended description of *Methanobacterium thermoautotrophicum* (corrig.) Zeikus and Wolfe 1972, 712^{AL}. *Methanobacterium thermoautotrophicum* (ther.mo.au.to.tro.phi'cum. Gr. adj. *thermos*, hot; Gr. pref. *auto*, self; Gr. adj. *trophikos*, one who feeds; N.L. neut. adj. *thermoautotrophicum*, thermophilic and autotrophic) cells are slender, cylindrical, irregularly crooked rods that are 0.35 to 0.6 μ m wide and 3 to 7 μ m long and frequently occur in filaments that are 10 to 120 μ m long. Gram positive. Nonmotile. Endospores are not formed. Fimbriae are present.

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, sulfide 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 and is just as rapid as growth on H₂-CO₂. Not stimulated by organic additions, although acetate may be assimilated.

The G+C content of the DNA is 48 to 50 mol% (as determined by thermal denaturation). Some strains harbor plasmids.

Habitat: thermophilic, anaerobic digesters.

The type strain is strain ΔH (= DSM 1053 = ATCC 29096), which was isolated from an anaerobic sewage sludge digester; this strain does not grow on formate.

The reference strains include strains YTB (= DSM 1850) Zeikus et al. 1980 and Z-245 (= DSM 3720) Zhilina and Ilarionov 1984; the latter strain grows on formate.

	1	11	21	31	41	51	
ΔH	GUUGCGGUC	AUGGCGUGGG	GUUUAUACCU	GAUCUCGUUU	CGAUCUCAGU	AGUUAAGUCC	
Z-245			A				
	61	71	81	91	101	111	121
ΔH	UGCUGCGUUG	UGGGUGUGUA	CUGCGGUUUU	UUGCUGUGGG	AAGCCCACUU	CACUGCCAGA	CAA
Z-245							C ACCC

FIG. 1. Alignment of 5S ribosomal RNA sequences from *M. thermoautotrophicum* ΔH^T and *M. thermoformicum* Z-245. The sequences from the 5' end to the 3' end are shown. For strain Z-245 only the nucleotides that differ from the strain ΔH^T nucleotides are shown. Data from reference 6.

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