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Methanogens: Reevaluation of a Unique Biological Group

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INTRODUCTION

The discovery by Italian physicist Alessandro Volta (49) in 1776 that "combustible air" was being formed in the sediments of streams, bogs, and lakes rich in decaying vegetation led to subsequent discoveries by Béchamp, Popoff, Tappeneiner, Hoppe-Seyler, Söhngen, and Omelianski that defined a microbial basis for the origin of methane gas (11). Within the past decade studies have established the widespread and fundamental role of the methane-producing bacteria in anaerobic degradation processes in nature (63, 118, 119, 124, 126). More elusive has been an understanding of the systematic relationships among the methane bacteria and their relationship to other procaryotes. On one hand, the methanogens are a morphologically diverse group of organisms; on the other, they are a physiologically coherent group of strict anaerobes, sharing the common metabolic capacity to produce methane. The uncertainty concerning their systematic relationships was reflected in early taxonomic schemes which initially dispersed the methanogenic bacteria among the better characterized bacterial groups according to their morphologies. Later, Barker (11) emphasized the highly unique physiology of the group and thereby clustered the methanogens into a single family, the *Methanobacteriaceae*. Although Barker's regrouping is presently recognized (20), the scheme provides limited insight into the relationships among the various methanogenic species. Methanogens metabolize only a restricted range of substrates and are poorly characterized with respect to other metabolic, biochemical, and molecular properties. Of the three genera proposed in the most recent edition of *Bergey's Manual of Determinative Bacteriology* (20), only one genus is moderately well characterized and contains more than one species that is available in pure culture. Both gram-negative and gram-positive cells are represented in that genus; morphologies vary from short, lancet-shaped cocci to long, filamentous rods; and deoxyribonucleic acid (DNA) base composition ranges widely from 28 to 51 moles percent guanine plus cytosine (mol% G + C).

The dilemma posed by seemingly contradictory characters (in this case an apparent unifying physiology versus diverse morphologies) and a dearth of phenotypic characters to define relationships are characteristic features of bacterial classification for the last century. Our inability to formulate a constant basis for bacterial systematics stems from the conceptual view of microbial relationships taken by early taxonomists. In contrast to a phylogenetic definition of relationships found between the higher eucaryotes, bacterial systematics historically became fixed into an empirical definition of relationships. This approach reflected both the lack of genealogical information and the need for a practical scheme—one in which a number of simple phenotypic characters were sufficient to identify an organism. The problems of an empirical approach were concisely summarized by R. Y. Stanier and C. B. van Niel in 1941 (93):

> ... an empirical system is largely unmodifiable because the differential characters employed are arbitrarily chosen and usually cannot be altered to any great extent without disrupting the whole system. Its sole ostensible advantage is its greater immediate practical utility; but if the differential characters used are not mutually exclusive (and such mutual exclusiveness may be difficult to attain when the criteria employed are purely arbitrary) even this advantage disappears. [P. 438–439]

The subsequent history of bacterial systematics has confirmed the insight of these statements; a constant reshuffling of taxa and the invention of new taxa have been the inevitable outcome of the introduction of new methods for classifying bacteria.

van Niel (106) proposed that the issues could be simply resolved by shifting our emphasis from "indication of relationships" among the bacteria to "means of identification." In this light, any taxonomic scheme is an effective tool for ascertaining the identity of an organism. For this purpose, van Niel encouraged the development of multiple-determinant schemes based on a wide range of differential characters (106). This attitude is to some extent reflected in the most recent edition of *Bergey's Manual*, where the major clusters of organisms are grouped into sections, or "parts." Whereas relationships are specified on the basis of certain characteristics within the group, there is no indication of relationships between the various parts. Such an approach, although important as a means of identification, provides a limited basis for bacterial systematics. The concept of genealogical relationships is a starting point for any true understanding of microbial diversity. The advantages of a phylogenetic or natural definition of relatedness were summarized by Stanier and van Niel (93):

Even granting that the true course of evolution can never be known and that any phylogenetic system has to be based to some extent on hypothesis, there is good reason to prefer an admittedly imperfect natural system to a purely empirical one. A phylogenetic system has at least a rational basis, and can be altered and improved as new facts come to light; its very weaknesses will suggest the type of experimental work necessary for improvement. [P. 438]

Indeed, certain groups, such as the methanogens, can only be approached by developing a measure of their relationships.

The capacity to sequence macromolecules provides the key to a natural basis for bacterial classification. Macromolecular sequences are in effect historical records. These "semantides" (informational macromolecules) (136) can be read through comparative analysis to reveal genealogical relationships (111). A number of approaches have compared amino acid sequences of proteins, such as cytochrome *c* and ferredoxin, to elucidate phylogenetic groupings—e.g., the phylogeny constructed for the purple nonsulfur bacteria on the basis of cytochrome *c* (3). These approaches were limited, however, by the distribution among organisms of the protein in question and by the fact that in many cases one had to compare functionally related but not equivalent molecules, making interpretation of the extent of sequence difference uncertain. Schwartz and Dayhoff (84) have combined published sequence data from 5S ribosomal ribonucleic acid (rRNA), cytochrome *c*, and ferredoxin to present a composite phylogeny for a limited number of eucaryotes and procaryotes. Although a number of assumptions are required to integrate such a diverse range of sequence information, the approach represents a significant contribution to the beginnings of a bacterial phylogeny.

A more direct phylogenetic approach is based on the comparative analysis of 16S rRNA. The ribosome is of ancient origin, is universally distributed, and appears to be functionally equivalent over the broad range of bacteria (12, 75, 120). The 16S rRNA primary structure is sufficiently constrained that it has not changed extensively with time (114). It has regions both of extreme conservation and of variability, making it useful in establishing distant as well as relatively close relationships. Moreover, the molecule is large enough (1,540 nucleotides) to be a "statistical ensemble," which makes it a more certain indicator of phylogenetic relationships than are smaller molecules (e.g., 5S rRNA).

"Comparative cataloging" of 16S rRNA has been used to detect the phylogenetic relationships among the chloroplasts of *Lemna* (C. R. Woese, unpublished data), *Euglena* (122), *Porphyridium* (15, 16), and the cyanobacteria (work done in the laboratory of W. F. Doolittle; 16, 17, 29) clearly delineating the procaryotic nature of the eucaryotic chloroplast. The technique has been used (36) to confirm and extend the classically defined genus *Bacillus*, based originally on spore morphology and related structures (41). To date, well over 100 species of procaryotes have been characterized in terms of their 16S rRNA oligonucleotide catalogs, including representatives from the genera *Acetobacterium*, *Acholeplasma*, *Acinetobacter*, *Actinomyces*, *Aeromonas*, *Alcaligenes*, *Aphanocapsa*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Bdellovibrio*, *Bifidobacterium*, *Brevibacterium*, *Cellulomonas*, *Chlorobium*, *Chloroflexus*, *Chromatium*, *Clostridium*, *Corynebacterium*, *Dactylosporangium*, *Escherichia*, *Eubacterium*, *Geodermatophilus*, *Halobacterium*, *Halococcus*, *Lactobacillus*, *Leptospira*, *Leuconostoc*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Mycoplasma*, *Myxococcus*, *Nocardia*, *Paracoccus*, *Pasteurella*, *Pediococcus*, *Peptococcus*, *Photobacterium*, *Planococcus*, *Propionibacterium*, *Pseudomonas*, *Rhodomicrobium*, *Rhodopseudomonas*, *Rhodospirillum*, *Ruminococcus*, *Sphaerotilus*, *Spirillum*, *Spirochaeta*, *Spiroplasma*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Sulfolobus*, *Synechococcus*, *Thermoactinomyces*, *Thermoplasma*, *Treponema*, and *Vibrio* (36, 61, 80, 81, 115, 117, 121, 123; Woese, unpublished data).

Recently, Fox et al. (35) applied comparative cataloging of 16S rRNA to a variety of methanogenic bacteria. The methanogens were shown to be a unique, coherent biological grouping phylogenetically distant from the typical bacteria. Subsequent studies on DNA structure (69), intermediary metabolism (32, 38, 39, 97, 109, 108), lipid composition (64, 101, 101a, 102), and cell wall composition (52, 54–56) have confirmed and amplified these results. Herein, we formally present a new taxonomic scheme for the methanogens based on their phylogenetic relatedness as revealed by 16S rRNA comparisons. In the scheme the species are arranged into a simple pattern consistent with their now known phenotypic properties. The present phylogenetic analysis makes it apparent that the generally accepted taxonomic classification of bacteria at the higher levels (40) is not structured to include the methanogens.

GROWTH OF METHANOGENS

Techniques for Growth of Methanogens

Recent advances in the culture of methanogens are based on the pioneering efforts of Hun-

gate (50, 51). The versatility of these anaerobic procedures is well documented (19, 21, 31, 47, 48, 51, 60, 67). The technique used in this study for routine growth of methanogens in a pressurized atmosphere of 80% H_2 and 20% CO_2 has been described by Balch and Wolfe (7). The system consists of three components: a serum bottle or glass tube modified to accommodate a thick rubber septum (Fig. 1), a Freter-type anaerobic chamber (5), and a gassing manifold (Fig. 2). Medium without reducing agent (Table 1) is brought to a boil under an atmosphere of 80% N_2 and 20% CO_2 by use of the Hungate technique (51). Reducing agent is added, and the flask is stoppered and transferred into the anaerobic chamber, where the medium is dispensed into tubes. Tubes are sealed in the chamber. When transferred outside the chamber, the gas phase in each tube is exchanged for an 80% H_2–20% CO_2 gas mixture by the use of a gassing manifold (Fig. 2).

This procedure offers several advantages over the classical techniques. Medium which is dispensed into tubes in an anaerobic chamber is exposed to a uniform, low-oxygen (2 μl/liter) environment. The thick rubber stopper (Fig. 1), when crimped into place, provides a seal at both positive and negative pressures and withstands numerous needle penetrations. The use of syringes for all transfers offers a maximal degree of protection against oxygen and bacterial contaminants, since the tube is never opened. The use of elevated pressure decreases the necessity for frequent addition of substrate as the methane bacteria consume the gas mixture. When necessary, tubes can be repressurized aseptically in a few seconds (Fig. 3) (7). In general, the technique requires a minimal investment of time to yield consistent, quantitative results.

The system, when applied to cultivation of methanogens in 100- to 200-ml amounts of medium (Fig. 4), renders the use of shake flasks (21) obsolete. The bottle offers all the advantages of the tube technique; it eliminates the requirement for continuous flushing with a gas mixture and subsequent loss of sulfide from the medium, which may produce erratic growth responses for certain methanogens.

Methanogens are easily cultivated on agar plates by use of the anaerobic chamber. Reduced, sterile, 2% agar medium is poured into plastic petri plates maintained in the chamber for at least 12 h to remove dissolved oxygen. After inoculation, plates are transferred into a cylinder (Fig. 5). Outside the chamber the cylinder is pressurized to 2 atmospheres with an 80% H_2–20% CO_2 gas mixture in a manner similar to that described above for tubes (7). Growth

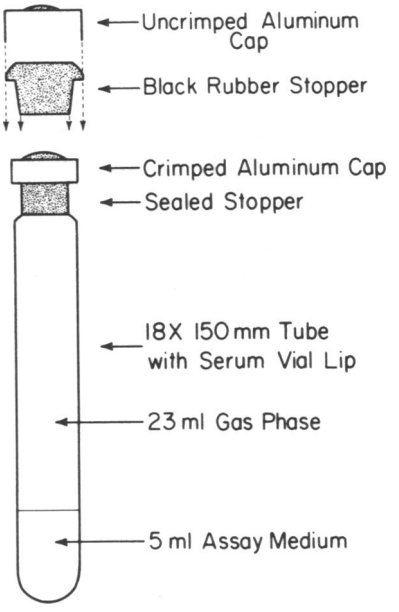

FIG. 1. *Serum tube (67) and specially manufactured black rubber stopper (Bellco Glass, Inc., Vineland, N.J.; no. 2048-00150 serum tube and no. 2048-11800 stopper) used in the pressurized tube technique (7). The stopper is crimped into place with a one-piece aluminum seal (no. 224183; Wheaton Scientific Div., Wheaton Industries, Millville, N.J.).*

can be followed by methane production or by the decrease in cylinder pressure as observed on the pressure gauge (Fig. 5). Edwards and McBride (31) describe an alternative ultralow-oxygen chamber built inside the anaerobic chamber for cultivation.

Principal Media

Three basis media (Table 1) were used to culture the methanogens. *Methanospirillum hungatii, Methanosarcina barkeri* (strains MS, 227, and W), *Methanobacterium formicicum, Methanobacterium arbophilicum, Methanobacterium ruminantium* strain PS, and *Methanobacterium* strains M.o.H., M.o.H.G., AZ, and DC were grown in medium 1. *M. ruminantium* strain M1 and *Methanobacterium mobile* were cultured in medium 1 containing only 2.5 g of $NaHCO_3$, the medium being additionally supplemented with (in grams per liter): valeric, isovaleric, α-methylbutyric, and isobutyric acids, 0.5 each; 2-mercaptoethanesulfonic acid, 0.0001. For culture of *M. mobile*, an unidentified partially purified factor found in rumen fluid was added (W. E. Balch, unpublished data). *Methanobacterium thermoautotrophicum* was cultured in medium 2. Black Sea isolate JR1, Cariaco isolate

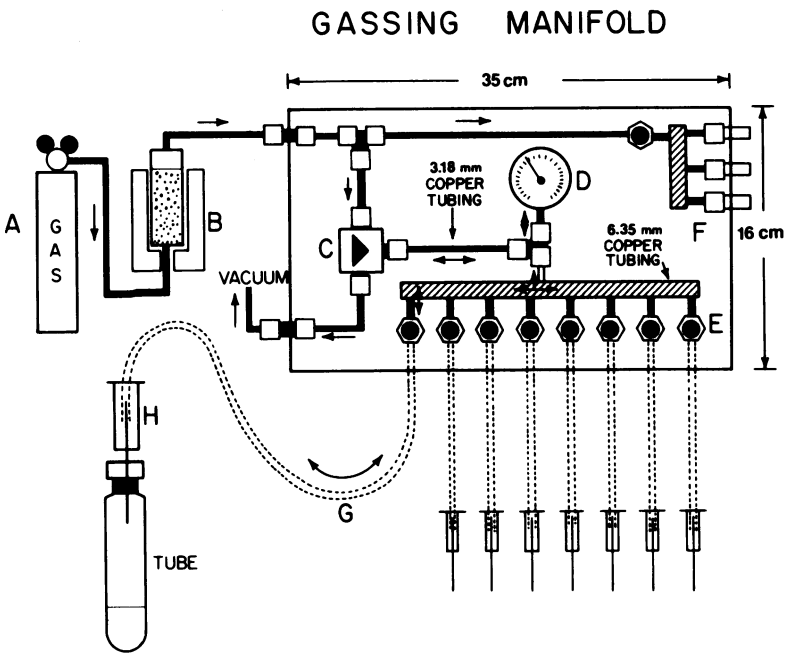

FIG. 2. *Gassing manifold with apparatus for supply of oxygen-free gas (7). (A) Gas mixture tank; (B) reduced copper column (oxygen scrubber) with heater; (C) three-way valve with Swagelok fittings (Crawford Fitting Co., Solon, Ohio); (D) vacuum-pressure gauge; (E) Nupro-Fine metering valves (Whitney Co., Oakland, Calif.); (F) alternate tubing connectors for gassing probes which may be used in the normal Hungate procedure (51); (G) thin-bore polyethylene tubing (Bel-Art Products, Pequannock, N.J.); (H) Vacutainer-Holder Needle (Becton, Dickenson & Co., Rutherford, N.J.). Squares indicate Swagelok brass fittings. Sections of copper tubing 6.35 and 3.18 mm thick are indicated by the shaded and solid regions, respectively.*

JR1, *Methanococcus* strain PS, and *Methanococcus vannielii* were cultured in medium 3. The substrate for growth as well as the gas phase for all media was a mixture of 80% H_2 and 20% CO_2 (Linde Specialty Gas Div., Union Carbide Corp., New York, N.Y.) at 2 atmospheres of pressure. Media could be stored for up to 2 weeks before use.

Strain Histories

The sources and histories of methanogens used in this study are summarized in Table 2. Where available, the culture collection number of the German collection of microorganisms (Deustche Sammlung von Mikroorganismen [DSM], D-3400, Göttingen, Federal Republic of Germany) is given to facilitate requests for cultures that are maintained by H. Hippe.

Deoxyribonucleic Acid Base Composition Determination (Moles Percent Guanine plus Cytosine)

Methanobacterium strains AZ and DC, *M. ruminantium* strains M1 and PS, *Methanobacterium* strains M.o.H. and M.o.H.G., *M. formicicum*, *M. thermoautotrophicum*, and *M. barkeri* strains MS and 227 were incubated for 30 min at 60°C in 2% sodium dodecyl sulfate, followed by disruption in a French pressure cell at 3,000 lb/in^2. *M. mobile*, *M. vannielii*, *Methanococcus* strain PS, Cariaco isolate JR1, and Black Sea isolate JR1 were disrupted by freezing and thawing. DNA was purified from the lysed cells by the method of Marmur (65); the buoyant density of the DNA was determined by cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge. The base ratio was calculated by the method of Schildkraut et al. (83). DNA from *Micrococcus lysodeikticus* (a gift from C. L. Hershberger) was used as a standard.

GENERATION OF 16S RIBOSOMAL RIBONUCLEIC ACID OLIGONUCLEOTIDE CATALOGS

Labeling and Ribosomal Ribonucleic Acid Isolation

The procedures for determination of the oli-

TABLE 1. *Compositions of standard media*[a]

Medium	Component														
	Mineral 1[b] (ml)	Mineral 2[c] (ml)	Mineral 3[d] (ml)	NaCl (g)	NH_4Cl (g)	Trace minerals[e] (ml)	Trace vitamins[f] (ml)	$FeSO_4 \cdot 7H_2O$ (g)	$Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$ (g)	$NaHCO_3$ (g)	Sodium acetate (g)	Sodium formate (g)	Yeast extract (Difco) (g)	Trypticase (BBL)	L-Cysteine hydrochloride·H_2O (g)	$Na_2S \cdot 9H_2O$ (g)
1	50	50				10	10	0.002		5.0	2.5	2.5	2.0	2.0	0.5	0.5
2		25			1.25	10	10		0.02	7.5					0.6	0.6
3			500	18		10	10		0.002	5.0	1.0		2.0	2.0	0.5	0.5

[a] Ingredients are added to distilled water to give a final volume of 1 liter. Cysteine and Na_2S are added after boiling the medium under an 80% N_2–20% CO_2 gas mixture, the final gas phase of tubed medium being an 80% H_2–20% CO_2 gas mixture at two atmospheres of pressure.

[b] Contains 6 g of K_2HPO_4 per liter of distilled water.

[c] Contains, in grams per liter of distilled water: KH_2PO_4, 6; $(NH_4)_2SO_4$, 6; NaCl, 12; $MgSO_4 \cdot 7H_2O$, 2.6; $CaCl_2 \cdot 2H_2O$, 0.16.

[d] Contains, in grams per liter of distilled water: KCl, 0.67; $MgCl_2 \cdot 2H_2O$, 5.5; $MgSO_4 \cdot 7H_2O$, 6.9; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.28; K_2HPO_4, 0.28.

[e] Contains, in grams per liter of distilled water (pH to 7.0 with KOH): nitrilotriacetic acid, 1.5; $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot 2H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4$ or $CoCl_2$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01. Dissolve nitrilotriacetic acid with KOH to pH 6.5; then proceed to add minerals.

[f] Contains, in milligrams per liter of distilled water: biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pantothenate, 5; vitamin B_{12}, 0.1; *p*-aminobenzoic acid, 5; lipoic acid, 5.

gonucleotide catalogs of rRNA have been described extensively (36, 82, 105, 116). In essence, the technique involves labeling cells with [^{32}P]orthophosphate at a final concentration of 0.5 to 1.0 mCi/ml. For this purpose, media (Table 1) depleted in phosphate were prepared. Yeast extract and Trypticase were dephosphorylated by addition of magnesium acetate (0.35 g) to 100 ml containing 2 g each of yeast extract and Trypticase. The solution was titrated to pH 8.5 with concentrated ammonium hydroxide, held for 1 h on ice, and centrifuged for 10 min at 48,000 × *g* to remove the magnesium phosphate precipitate. Cells grown for three to four generations in the presence of [^{32}P]were collected by centrifugation, washed, and opened by passage through a French pressure cell at 10,000 lb/in^2. Total rRNA was obtained by phenol extraction, and the 16S rRNA was separated by polyacrylamide gel electrophoresis followed by elution and purification with Whatman CF-11 cellulose column chromatography as indicated previously (6).

Determination of Oligonucleotide Catalog

Purified 16S rRNA was digested with ribonuclease (guanyloribonuclease) T_1, the resulting oligonucleotides being resolved by two-dimensional paper electrophoresis (82) with modifications (105, 116). Briefly, the analysis involves an initial electrophoretic separation on cellulose acetate, followed by a replica transfer by blotting to diethylaminoethyl cellulose paper, with subsequent orthogonal electrophoresis. The resulting oligonucleotide "fingerprint" (or primary pattern) comprises a pattern of "isopleths." Each isopleth is a coherent group defined by the uridine content per oligonucleotide. Within each isopleth, oligonucleotides are displayed by order of size, and within each size "isocline" they are separated to some extent on the basis of composition. Figure 6 is a representative fingerprint of this type. The sequences of the various oligonucleotides are determined by secondary and tertiary enzymatic procedures (105, 116).

Analysis of Data

The resulting oligonucleotide catalogs are examined by a standard taxonomic analysis (4). An association coefficient (S_{AB}) for each binary couple is defined as follows: $S_{AB} = 2N_{AB}/(N_A + N_B)$, where N_A and N_B are the total numbers of nucleotides (found in hexamers and larger) in catalogs *A* and *B*. N_{AB} represents the number of nucleotides in oligomers common to the two catalogs. S_{AB} values range from 1.0 (complete sequence homology) to 0.02 (the random coincidence level). The values are an underestimate

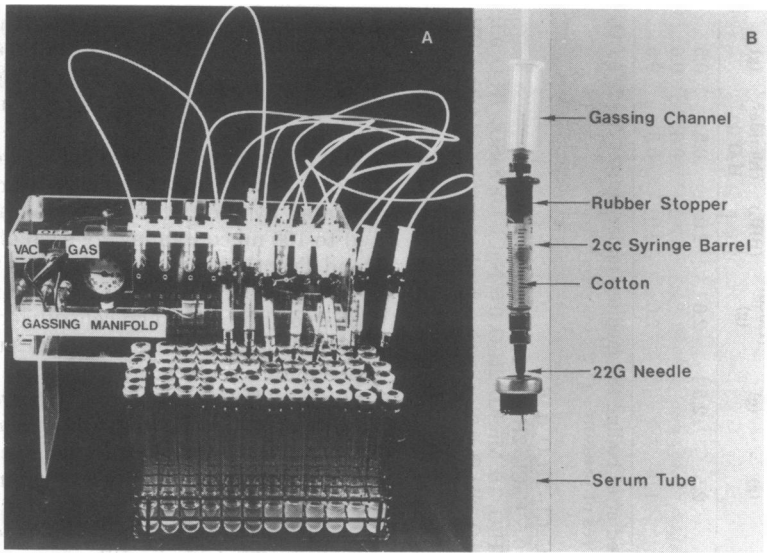

FIG. 3. *(A) Use of the gassing manifold to aseptically repressurize cultures during growth (7). (B) Detailed view of a sterile gassing syringe connected to a tube and gassing channel.*

of the actual percent homology between two catalogs, because related but nonidentical oligomers are not considered. The matrix of S_{AB} values for each binary comparison over a given set of organisms is used to generate a dendrogram by average linkage (between the merged groups) clustering (4).

S_{AB} is a measure of the sequence homology between two rRNA's. Branching patterns are generally independent of whether single, average, or complete linkage clustering techniques are used (4). Moreover, analysis of the data in terms of families of related oligonucleotides (36; G. E. Fox, unpublished data) yields similar branching patterns.

COMPARATIVE CATALOGING OF 17 METHANOGENS

The 17 organisms whose 16S rRNA oligonucleotide catalogs are listed in Table 3 include the major types of methanogens now in pure culture. These lists include previously unpublished 16S rRNA oligonucleotide catalogs for 7 methanogens: *Methanobacterium* strains AZ and DC, *Methanobacterium* strain M.o.H.G., *M. mobile*, *M. barkeri* strain 227, *M. vannielii*, and *Methanococcus* strain PS. The extent of their relatedness to all methanogens thus far examined (6, 35) can be seen in Fig. 7; this dendrogram is constructed from the S_{AB} values calculated for individual binary comparisons given in Table 4. Values for three other groups (the enteric-*Vibrio*, *Bacillus*, and cyanobacteria) are averages of several representative species and are included in this dendrogram for perspective.

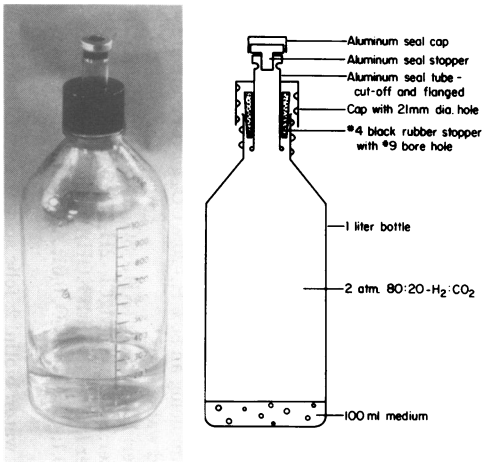

FIG. 4. *Modified 1-liter storage bottle (no. 219760; Wheaton Scientific Co.) used for growing cells in 100- to 200-ml liquid volumes at 2 atmospheres of pressure (9). The cap has a 21-mm-diameter hole to allow an aluminum seal tube (Bellco Glass), which has been cut and flanged, to protrude from the bottle. The tube is sealed in the bottle with a no. 4 black rubber stopper with a no. 9-size hole. The tube and stopper are held in place by screwing the bottle cap down. The bottle is pressurized after autoclaving and before use. For safety, the bottle is maintained in a stainless steel canister after pressurization (9).*

A dendrogram published when 10 methanogens had been characterized showed the group to possess two major divisions (35); we now see at least three (Fig. 7). The first division contains the *Methanobacterium* species with the excep-

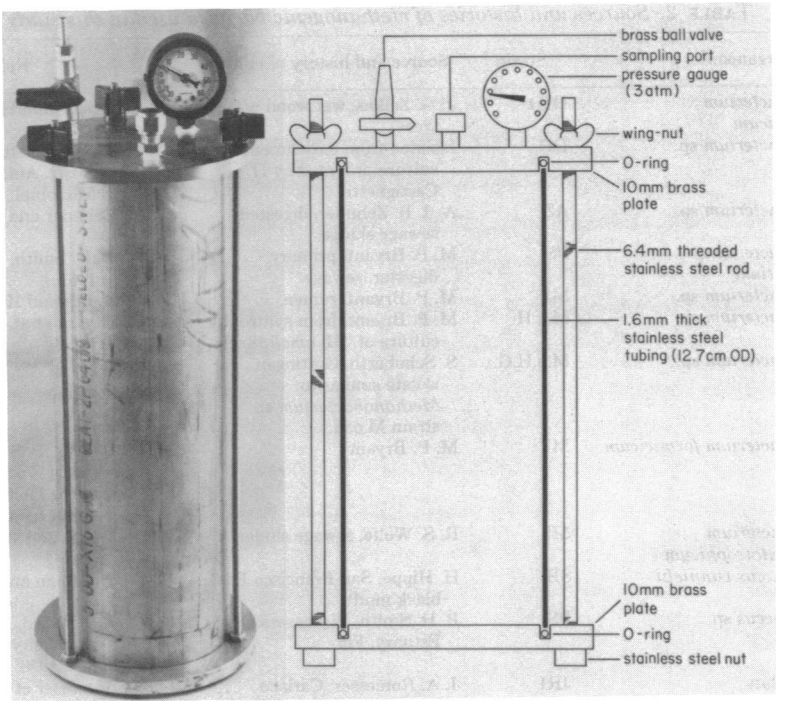

FIG. 5. *Pressure cylinder used to incubate agar plates at an elevated pressure of 2 atmospheres of an 80% H_2–20% CO_2 gas mixture. The cylinder length is designed to fit into the air lock of a Freter (5)-type anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.).*

tion of *M. mobile*; the second contains *Methanococcus* species; and the third contains *Methanosarcina*, *Methanospirillum*, *M. mobile*, and two new marine isolates (Black Sea isolate JR1 and Cariaco isolate JR1). Divisions I and III have two subgroups each (see Fig. 7): IA comprises coccobacillus-like, gram-positive rods; IB comprises long, gram-positive rods; IIIA comprises various gram-negative forms, and IIIB contains the gram-positive sarcina-like methanogens.

Although Fig. 7 was generated by average linkage clustering analysis between the merged groups (4), application of complete linkage or single linkage or both gives a dendrogram of near identical topology to that shown (G. E. Fox, unpublished analysis). It is apparent that the choice of clustering procedure does not have a significant effect on the branching orders reported here. The standard deviations about the means for all binary comparisons involved in a cluster are presented as brackets about the S_{AB} values (Fig. 7).

DEVELOPMENT AND OUTLINE OF A NEW TAXONOMIC TREATMENT BASED ON 16S RIBOSOMAL RIBONUCLEIC ACID

The methanogenic bacteria are recognized as a separate family in Part 13 of the 8th edition of *Bergey's Manual of Determinative Bacteriology* (20). Table 5 presents the current key to the genera of the family *Methanobacteriaceae*. The major determinative feature is morphology. The result of such a qualitative approach is a treatment which provides little insight into the relationships between the various species. A solution to the present inadequate taxonomic status is provided by the results obtained from comparative cataloging of 16S rRNA. The degree of sequence homology found among the 16S rRNA's isolated from different organisms provides a quantitative basis for establishing relationships.

The methanogens represent a coherent phylogenetic grouping quite distinct from other typical bacteria. Just how distinct they may be is indicated in Fig. 7; even the enteric-*Vibrio* species and the cyanobacteria appear closely related by comparison. By use of these same criteria, it is apparent that the methanogens as a group are very diverse. The deepest branches are as distinct from one another as the gram-negative enteric-*Vibrio* group is from the gram-positive bacilli (Fig. 7). We propose that the *Methanobacteriaceae* Barker 1956 be redefined to contain only a limited range of methanogenic species. S_{AB} values derived for all species provide

TABLE 2. *Sources and histories of methanogenic bacteria used in this study*

Organism[a]	Strain	Source and history of culture	DSM no.	Reference
1. *Methanobacterium arbophilicum*	DH1	J. G. Zeikus, wet wood of trees	1125	Zeikus and Henning (130)
1a. *Methanobacterium* sp.	DC	Isolated from enrichment culture provided by D. Castignetti	1536	D. Castignetti and D. A. Klein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, O14, p. 247
2. *Methanobacterium* sp.	AZ	A. J. B. Zehnder, digested sewage sludge	744	Zehnder and Wuhrmann (125)
3. *Methanobacterium ruminantium*	PS	M. P. Bryant, primary digester, sewage	861	P. H. Smith, Abstr. Bacteriol., 1961
4. *Methanobacterium* sp.	M1	M. P. Bryant, rumen	1093	Smith and Hungate (88)
5. *Methanobacterium* sp.	M.o.H.	M. P. Bryant, from syntrophic culture of *"M. omelianskii"*	863	Bryant et al. (23)
6. *Methanobacterium* sp.	M.o.H.G.	S. Schoberth, Göttingen isolate similar to *Methanobacterium* sp. strain M.o.H.	862	
7. *Methanobacterium formicicum*	MF	M. P. Bryant	1535	C. G. T. P. Schnellen, Ph.D., thesis, Technical, University of the Delft, Delft, the Netherlands, 1947
8. *Methanobacterium thermoautotrophicum*	ΔH	R. S. Wolfe, sewage sludge	1053	Zeikus and Wolfe (131)
9. *Methanococcus vannielii*	SB	H. Hippe, San Francisco Bay black mud	1224	Stadtman and Barker (90)
10. *Methanococcus* sp.	PS	P. H. Smith, Waccasassa Estuary, Fla.	1537	J. M. Ward, Ph.D. thesis, University of Miami, Miami, Fla., 1970
11. Cariaco isolate	JR1	J. A. Romesser, Cariaco Trench sediment, Venezuela	1497	Romesser et al. (81a)
12. Black Sea isolate	JR1	J. A. Romesser, Black Sea sediment	1498	Romesser et al. (81a)
13. *Methanospirillum hungatii*	JF1	J. G. Ferry, sewage sludge	864	Ferry et al. (33)
14. *Methanobacterium mobile*	BP	P. H. Smith, rumen	1539	Paynter and Hungate (79)
15. *Methanosarcina barkeri*	MS	M. P. Bryant, Urbana, Ill., sewage sludge digester	800	C. G. T. P. Schnellen, Ph.D. thesis
15a. *Methanosarcina barkeri*	227	R. A. Mah, Los Angeles, Calif. sewage sludge digester	1538	Mah et al. (62)
15b. *Methanosarcina barkeri*	W	R. A. Mah, gas-vacuolated strain		R. A. Mah, Abstr. Annu. Meet. Am. Soc. Microbiol., 1977, I32, p. 160

[a] Organism numbers correspond to those in Table 4.

the basis for description of three orders, four families, and seven genera. These categories reflect taxa characteristic of the typical bacteria.

Orders

Figure 8 presents a new taxonomic treatment of the methanogens based on comparative cataloging of 16S rRNA's. At the highest level the methanogens are divided into three new orders. Order I, *Methanobacteriales* (group I in Fig. 7); order II, *Methanococcales* (group II in Fig. 7); and order III, *Methanomicrobiales* (group III in Fig. 7). Separate groupings at least at the rank of order are justified by the low S_{AB} values observed (0.22 to 0.28) between the proposed orders (Fig. 7 and 8). These S_{AB} values are comparable to those separating the typical gram-negative and gram-positive bacteria.

Families

Order I, *Methanobacteriales*, and order II, *Methanococcales*, contain one family each, an emended description of the *Methanobacteriaceae* Barker 1956 and the *Methanococcaceae*, respectively. Order III contains two new families, *Methanomicrobiaceae* and *Methanosarcinaceae* (Fig. 8). The two families in order III are justified on the basis of the S_{AB} value relating groups IIIA and IIIB ($S_{AB} = 0.35$) in Fig. 7. The S_{AB} value is similar to that obtained in the binary comparison of the phototrophic bacterium *Chromatium* sp. (family *Chromatiaceae*) with *Rhodopseudomonas sphaeroides* (family *Rhodospirillaceae*), 0.34, or that of *Bifidobacterium breve* (family *Actinomycetaceae*) and *Streptomyces griseus* (family *Streptomycetaceae*), 0.35 (Woese, unpublished data).

Genera

The family *Methanobacteriaceae* in order I is proposed to contain two genera; an emended description of the genus *Methanobacterium*

Kluyver and van Niel 1936 and a new genus, *Methanobrevibacter* (Fig. 8). Comparative cataloging of 16S rRNA of the genus *Bacillus* supports this separation (36). For example, in the binary comparisons of *Bacillus subtilis* with *Bacillus cereus*, *Bacillus pasteurii*, and *Sporolactobacillus inulinus*, one obtains S_{AB} values of 0.73, 0.65, and 0.56, respectively. The S_{AB} value of 0.5 obtained in the binary comparison of groups IA and IB (Fig. 7) is of sufficient depth to justify two genera.

The *Methanococcales* pose a dilemma. An S_{AB} of 0.60 is observed in the binary comparison of the two species cataloged (Table 4). This value is not substantially different than that observed for groups IA and IB (Fig. 7). Since only two species are presently described, we conservatively propose that the family *Methanococcales* contain an emended description of the single genus *Methanococcus* (Kluyver and van Niel) Barker 1936. This assessment of their relationship may have to be modified as more isolates become available.

The family *Methanomicrobiaceae* in order III is proposed to contain three genera: *Methanogenium* Romesser and Wolfe 1979, *Methanospirillum* Ferry, Smith, and Wolfe 1974, and a new genus, *Methanomicrobium*. The relative positions of the three genera with respect to each other are presently not clear. For example, in Table 4 average S_{AB} values of 0.51 and 0.52 are observed in the binary comparisons of the two species in *Methanogenium* (Cariaco isolate JR1 and Black Sea isolate JR1) with the *Methanospirillum* sp. (*M. hungatii*) and the *Methanomicrobium* sp. (*M. mobile*), respectively. An S_{AB} value of 0.36 is observed in the binary comparison of the *Methanomicrobium* sp. (*M. mobile*) and *Methanospirillum* sp. (Table 4). These data suggest that the genera *Methanomicrobium* and *Methanospirillum* are more distant from each other but equidistant from *Methanogenium*. The clustering technique used in Fig. 7 defines a slightly different topology. Additional isolates are needed to clarify the relationships among the three genera.

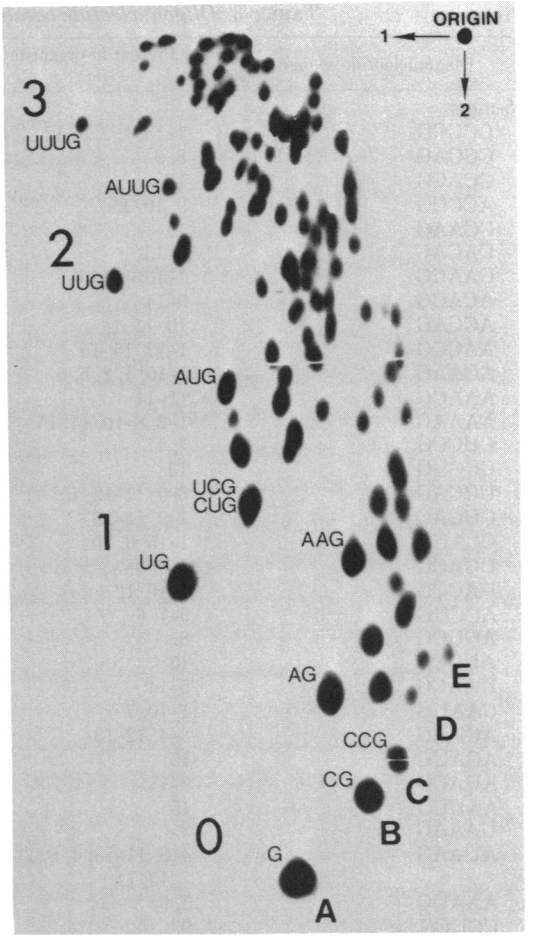

FIG. 6. *Representative 16S rRNA oligonucleotide fingerprint (primary), using ribonuclease T_1. Isopleths, with their characteristic uridine (U) content are indicated by numbers. Size isoclines within each isopleth are indicated by large letters. The oligonucleotide compositions of representative oligomers is so indicated, where G is guanosine, A is adenosine, and C is cytosine.*

Species

The compositions of the proposed genera are as follows (Fig. 8, Table 6). In the family *Methanobacteriaceae* the genus *Methanobacterium* Kluyver and van Niel 1936 is emended to contain only three species. These include *M. formicicum* Schnellen 1947 (C. T. G. P. Schnellen, Ph.D. thesis, Technical University of the Delft, Delft, the Netherlands, 1947) as the neotype species, *M. thermoautotrophicum* Zeikus and Wolfe 1972, and a new species, *M. bryantii*, formerly *Methanobacterium* strain M.o.H. Bryant 1971. The relationship of *M. bryantii* to *M. formicicum* and *M. thermoautotrophicum* is defined by the average S_{AB} value of 0.6 (Fig. 7), a value similar to that found in defining separate *Bacillus* species (36). *M. bryantii* is named for Marvin P. Bryant, who pioneered studies in the separation and characterization of this species from the *Methanobacillus omelianskii* syntrophic association (23). An S_{AB} value of 0.93 was obtained in the binary comparison of *M. bryantii* and *Methanobacterium* strain M.o.H.G. Such high S_{AB} values are found for closely related members of a genus or species. For example, S_{AB} values of

TABLE 3. *Oligonucleotide catalogs for 16S rRNA's of 17 methanogens*[a]

Oligonucleotide sequence	Present in organism no.:	Oligonucleotide sequence	Present in organism no.:
5-mers		UUUCG	7, 9, 11, 13, 14
CCCCG	1-15; 1, 2, 6, 12	UUCUG	4
CCCAG	8, 9	UCUUG	12, 13
CCACG	15	CUUUG	1-6, 15
ACCCG	9, 10, 15	UUUAG	3, 10-11, 14; 14
CCAAG	13	UUAUG	7, 13; 13
CACAG	9, 13	UUUUG	3, 10, 13
CAACG	1-15; 12, 13	6-mers	
ACACG	9-14	CCCCAG	7-10
ACCAG	10, 11, 14	CCCAAG	8, 10, 15
AACCG	1-12, 15; 15	CAACCG	12, 13
ACAAG	1-10; 1, 2, 5, 6	ACCACG	11-13
AAACG	11-14	ACACCG	8, 11-13, 15
AAAAG	1-2, 8-10, 14, 15	AAACCG	12, 13, 15
CUCCG	7, 11	CCACAG	14
CCCUG	13	AAAACG	14
UCCAG	8-9, 11-12, 15	CCCACG	9, 10
CUCAG	1-9, 11-15	CCCUCG	5-6, 12, 15
CCAUG	1-15; 9, 10	CCUCAG	5-6
UCACG	1-3, 5-7	CUCCAG	5-6
UACCG	1-10, 12	UCCCAG	3, 11-15
ACCUG	5-7; 6	CCACUG	5-7, 13
ACUCG	8	ACCUCG	13
AUCCG	13	CCUAAG	1-6, 15
UAACG	5-14	CUCAAG	5-8
CAAUG	1-10; 7	CCAUAG	7, 14
ACUAG	3-4, 12-13	CAUACG	1-2
ACAUG	15	ACACUG	5-7, 11-14
AUACG	9-11	AACCUG	1-10, 15; 1, 2
AAUCG	15	AAUCCG	9-11, 13-15; 14
UAAAG	3	CUAAAG	11-14
AUAAG	4-8, 11-15; 4, 8, 11-13; 11	UAAACG	1-8, 12-13, 15
AAAUG	7, 9	ACUAAG	9-10, 13
CCUCG	9	ACAAUG	1-15
CUAAG	2, 9-10; 10	AUAACG	15
UCCCG	14	AAUACG	1-8, 15
CACUG	14	AACAUG	15; 15
UUCCG	1-8, 12; 7	AAACUG	1-15; 12, 13, 14
CUUCG	5-6, 8, 10, 12, 14	AAAUCG	1-4, 11
UCCUG	1-10; 7, 9	AAUAAG	1-3, 5-10, 14
CCUUG	1-2, 9-10	CCCUAG	2
CUCUG	8, 10, 12	UACACG	14
UCUAG	11	CCUACG	9-10
UUCAG	5-6, 9-14; 13	CCAUCG	9-10
CUAUG	6	AUCCCG	9-10
UACUG	11-15; 12-15; 14	AACUAG	9-10
UAUCG	11-12	CCCUUG	8, 15
ACUUG	1-10, 15	CCUCUG	11-14
AUCUG	4-7, 11-12	UCCCUG	1-15; 9
AUUCG	3-4, 15	CCUUAG	7, 11, 12
UUAAG	1-15; 1-3, 5, 7-10, 12, 15	CUCUAG	1-4
		CUUCAG	13
UAAUG	1-3, 5-6, 9-10, 15; 3	UCCUAG	1-3
AUAUG	4, 7, 13	UUCCAG	1-8
AAUUG	1-15; 1-3, 6, 8, 15	CCUAUG	4, 10
AUUAG	1-15; 1-8, 11, 13-14; 11	CUACUG	1-4, 8-9
		UCACUG	4, 10-14
UAUAG	14	CUAUCG	11-15

TABLE 3—*Continued*

Oligonucleotide sequence	Present in organism no.:	Oligonucleotide sequence	Present in organism no.:
UCAUCG	11, 13	AACAUCG	15
CAUCUG	11	AAAUCCG	11-13
ACUCUG	11-12	UAAAAAG	1-2, 4-8
ACCUUG	5-10, 14	UAAACAG	9
AUCCUG	1-8, 11-15	CUCACCG	14
UCUAAG	11-12, 14	ACCCUCG	14
UUACAG	12	UCCCAAG	14
UAUCAG	13	CAACUCG	14
UAUACG	11	CCCUUAG	1-2, 4-8
UAAUCG	1-15	CAUCCUG	11-15
AUACUG	4, 9-12, 14-15	UACUCCG	11
ACAUUG	1-2	AUCUCCG	12
AACUUG	4	ACCUUCG	13
AAUCUG	5-6, 8, 11-14	UCCUAAG	11
UAAAUG	7	UUACCAG	1-3, 5-8, 15
AUUAAG	1-8, 11-12, 14	CUAACUG	4, 7
AAUAUG	13	UAACUCG	1-4, 7, 9-12, 15
UUACCG	14	AUUCCAG	11, 14
CUCAUG	9	AUCAUCG	8
CACUUG	9-10; 10	AAUCUCG	4
CAUAUG	9-10	AACCUUG	8
CCUUCG	10	UCUAAAG	10, 15
CCUUUG	1-3, 5-6, 9	CUUAAAG	10-13
CUUUCG	15	CAAUAUG	15
UCUCUG	1-3, 5-8	AUACUAG	1
UUCCUG	5-6	AAUCUAG	1-3, 7, 11-12, 14
UCUUAG	5-6	AAAUCUG	15
CUAUUG	1-4, 7-10	UAAAAUG	15
UUACUG	15	UAACCUG	5
UAUUCG	4	CCCUAUG	9-10, 14
AUUCUG	3, 12-13, 15	UAAAUAG	10
ACUUUG	3	UCACCUG	9
UAUAUG	12	CUCCUUG	1-6, 8-15
UCCUUG	10	UCCCUUG	13
UCUAUG	10	UUCUCCG	11, 14
CUUUUG	1-7; 1, 2	CUCUUAG	3
UCUUUG	2, 7	UACUUCG	12
UUUUUG	11	UACUCUG	15
7-mers		UCAUAUG	15
ACCCACG	1-8, 11-14	UAAUCUG	7
ACCACCG	11, 14	AAUUUAG	4
AACCCCG	11, 14	UUAACUG	14
CCAACAG	11, 12, 14	AUCCUUG	9-10
CAACACG	1, 3, 6, 8	CAUUAUG	10
CAAACCG	12, 13	UUCUUCG	15
AAAAAAG	10	UCUCUUG	11, 12, 14
CCCUACG	1-15	CUUUAUG	15
CCCACUG	15	UUUAUCG	1-2
UCCACCG	7, 8	UAUUUCG	1
CCACCUG	10, 15	AUUAUUG	15
CCCUAAG	11, 12	CUUUUAG	10
UCACACG	4	UAUUCUG	2
CUACACG	2, 5, 7, 9-15	UUCUUUG	5-8
UAACCCG	6, 8	UAUUUUG	4
AUACCCG	1-13, 15	UUUUUUG	1-4
AACCUCG	12	8-mers	
CCUAAAG	1-9	CCACAACG	1-3
UAACACG	1-8, 11-15	ACCCCAAG	1-2, 5-6, 9-10
AUAACCG	11	AAACCCCG	13
AAUCCAG	12-13, 15	UCCACCAG	13

TABLE 3—*Continued*

Oligonucleotide sequence	Present in organism no.:
CCCACAUG	11-12, 14
CUCAACCG	12
ACCCUCAG	11
ACCACCUG	1-2, 4-8, 12, 15
UAACACCG	1-8, 10, 15
AUCCCAAG	3-4
AAAUCCCG	1-2
AUACCCCG	14
AAACCUAG	9
CCCUCAUG	1-2, 4, 7
UACUCCCG	7
AU(CCUC)CG	5-6
CCUAUCAG	15
CCUAACUG	5-6
CUUAACCG	7, 11, 13
UAAUCCCG	13
CUACAAUG	1-15
UACUACAG	15
UAAUACCG	9, 11-14
AUUACCAG	4
AUAACCUG	5, 8, 11-12, 15
ACAAUCUG	13
AAAUCCUG	1-3, 8, 11-13
AUAAACUG	4-8
AUAAAUAG	3
UAACUACG	14
CUACCCUG	9-10
UACUCCAG	9
AAUCUCCG	9
(CU,CCUU)CG	7
AUCCUUCG	7
UCUAACUG	1-2
CUUAACUG	3-6, 8, 14
UAAUCCUG	1-4, 8
UCUAAAUG	1-2
UUAAAUCG	15
CAUAUAUG	15
AAAUCUUG	15
AAAUUCUG	3-4, 14
AUAAAUUG	1-2
UACUUCAG	14
AUAAUCUG	14
CUUUUCAG	8
UUCUCAUG	3
UUUAAUCG	13
UAUCAUUG	13
UUUAAAUG	3-4
AUAUUUAG	5
UUUAAUUG	1-12, 14-15
UUUUUUCG	2, 3-4
UUUUAUUG	1
9-mers	
CCCACCAAG	5-7, 9-10
CACACACCG	1-15
CACACAACG	14, 15
CCCAACAAG	11-14
AACCCCAAG	8
AAACCCAAG	7
CCUCACCAG	12
CCUACCAAG	8
CCUACAACG	15
AUAACCCCG	8, 12, 14-15
AAACCUCCG	1-8
CACACUAAG	1-8
AUAAACCCG	8
UACUCCCAG	1-8
UAAUCCCCG	11
AAUCCCCUG	1-2, 4-8
CUUACCAAG	1-4
(UC)ACACAUG	4
(UC)ACAAUCG	3-4
UCAUAACCG	7
CUAAUACCG	4
ACCCUUAAG	11
AUAACCCUG	13
AUAACCCUG	1-7
AUAAUACCG	5-7
AUAUACAAG	13
CUUAACCAG	9-10
CACUCAUAG	9-10
UACUCCAAG	10
UCUUACCAG	15
UCACUAUCG	8
UAAUCCCUG	15
UAAUCCUCG	12
AAUUUCCCG	15
AAUCCUCUG	3
UCAUAAUCG	1, 2, 5, 6
CUAAUACUG	1, 2
CAUCAUAUG	15
AUAAUUCCG	15
AAUUAUCCG	11-14
UUUAAAACG	11
UAAACUAUG	11, 14
AUAAUACUG	3
AAUCUCCUG	9, 10
UAAACUCUG	9
CCUAUCUAG	10
AUAACCUUG	10
CUAUUACUG	13
UUAAAUUCG	1, 2
UUUAAUAAG	2, 3
UAAUCCUUG	14
UAAACUUUG	10
UUAUAUUCG	3
UAUUUCUAG	13
UUUAUUAAG	1
AUUUUUAAG	14
CUUUUAUUG	8
UUUUUCCUG	2
UUUUUAUUG	3, 7
UUUUUUUCG	1
10-mers	
CACAACCACG	4-6, 8, 13
AAUAACCCCG	11
ACCACCUAUG	9, 10, 13
AAUCUCACCG	12
AAAUCUCACG	7, 14

TABLE 3—*Continued*

Oligonucleotide sequence	Present in organism no.:
UAACUCAAAG	12
AAACUUAAAG	1–15
ACCUUACCUG	15
UUACCAUCAG	4
UACCUACUAG	15
AAUCACUUCG	6
AACCCUUAUG	8
UAAAUAACUG	13
UAACUCAUAG	10
CAUAACCUUG	9
UUCUUCACCG	8
ACUCUACUUG	13
CUUAACUAUG	1, 2
AUACUAUUAG	2, 3, 5–7
AAUCAUUUCG	5
UUCCCUAUUG	7
UCUUCUUAAG	7
AUUUUUUUCG	1
AUUUCCUUUG	2
UUUUCUUUUG	5, 6
11-mers	
CACCACAACAG	12
ACAACUCACCG	15
AAAUCCCACAG	8
CAUCUCACCAG	9–11, 13
UAACUCACCCG	13
AAAUCUCACCG	11, 13
AAACACCUUCG	8
AAAUCCCAUAG	5, 6
UCCCUCCCCUG	15
CAUAUCCUCCG	15
AAAUCCUAUAG	4
CAUCUUACCAG	14
UUUCAACAUAG	11, 13
A(UA,UCA,CUA)UG	8
CUUUUAUCAAG	9
UCUCCUAACUG	14
UUUCAAAAUAG	14
UUUCAAUAUAG	15
CUUUUCUUAAG	1, 2, 4
CUUUUCAUUAG	3
UUCUUUAAUCG	11
UUUCUUCUCCG	10
AUUCUUUUCUG	9
UUUUUUUCCUG	1
UUUUUUUUAAG	3
12-mers	
CCACCCAAAAAG	1–3, 7–9
(AC,U,CCC)AAAAAG	4
UCAAACCACCCG	12–13, 15
UCACACCACCCG	9
UCAAACCAUCCG	11
UCACACCAUCCG	10
ACAUCUCACCAG	1–8
CCACUCUUAACG	5, 6–8
CUCAUUAACACG	9
CCAUUCUUAACG	1–4
CUCAACUAUUAG	15
CUCAUUAUCACG	10
CCACUAUUAUUG	11
CAAUUAUUCCUG	3
UUCAUAAUACUG	9, 10
CCACUUUUAUUG	12
(AA,UUUA,UA)UUCG	2
CCAUUUUUAUUG	2, 5–6
(CUA, CUUUUA)UUG	4
13-mers	
UAAACUACACCUG	15
(CAA, CCA)CAUUCUG	8
UAAUACUCCAUAG	13
UUUCAAAAUAACG	12
ACCACUCUUAUUG	9, 10
ACCACUUUUAUUG	14
AUAAUUUUUCCUG	4
(UUU,CUU,CU)AAAUG	6
UUUAAAUCCUCUG	9, 10
14-mers	
AAAACUUUACCAUG	13
UCAAACCAUCUUAG	14
UUUAAAACACAUCG	14
AAAACUUUACAAUG	11–12, 14, 15
AACUACUUUCUCCG	9
AAUAAUCUUUUCUG	9
AUUUUU(CCU,CU)UUG	3
15-mers	
UCUAAAACACACCUG	12
AUAACCUACCCUUAG	1–4
AUAACCUAACCUUAG	7
AUAAUACUCCUAUAG	10
AAUAAUACCCUAUAG	12
AAUAAUACUCCAUAG	11
AUAAUCUACCCUUAG	6
UCUUCUAUUUAAAUG	5
UUAACUUAACCUCAG	9
AUAAUUCUCCAUAAG	9
16-mers	
UAAUCCCCUAAACCAG	8
AAAUCCUAUAAUCCCG	7
AAUCUCCUAAACAUAG	5, 6
CAAUCUCUUAAACCUG	11
UAAUCUCCUAAACCUG	7
UUAACUUACCCUCAAG	10
AAAUCCUAUAAUCCUG	5, 6
17-mers	
UCAAUCUCCUAAAC-CUG	14
CAAUCUUUUAAA-CCUAG	4
UAAU(CCU,CU)AA-ACUUAG	1–3
AUAAU(CCU,CU)-AAACCUG	13
18-mers	
AACAAUCUCCUAAAC-CUG	12
CAAAUCUCCUAAACC-CUG	10

TABLE 3—*Continued*

Oligonucleotide sequence	Present in organism no.:	Oligonucleotide sequence	Present in organism no.:
24-mers		Posttranscriptionally modified sequences[b]	
(AAACA,UAAUCUCA)-CCCAUCCUUAG	15	1. a. ĊCCG	1–8
		b. ĊCĊG	9, 10
Termini, 5′ end		c. C(Ċ,C)G	11–15
pAG	7, 8	d. AΨUAG	9, 10
pAAUCCG	5, 6	2. CCĠCG	15
pAAUCUG	1, 2, 4	3. a. ÀÀCCUG	1–10
pAUUCUG	3, 11–15	b. ÀÀUCUG	11–15
pAUUCCG	9, 10	4. a. UÀÀCAAG	1–10
Termini, 3′ end		b. UAÀCAAG	11–15
$AUCACCUCCU_{OH}$	1–8, 11–15	5. a. AUN̂CAACG	1–10
$AUCACCUCC_{OH}$	9, 10	b. ACN̂CAACG	11–15

[a] The first column is the oligonucleotide sequence (C, cytosine; G, guanosine; A, adenosine; U, uridine); the second column shows organisms in which that sequence is found. Organisms are designated by number (see Table 4) as follows: 1, *M. arbophilicum* and *Methanobacterium* sp. DC; 2, *Methanobacterium* sp. AZ; 3, *M. ruminantium* PS; 4, *M. ruminantium* M1; 5, *Methanobacterium* sp. M.o.H.G.; 6, *Methanobacterium* sp. M.o.H.; 7, *M. formicicum*; 8, *M. thermoautotrophicum*; 9, *M. vannielii*; 10, *Methanococcus* sp. PS; 11, Cariaco isolate JR1; 12, Black Sea isolate JR1; 13, *M. hungatii*; 14, *M. mobile*; 15, *M. barkeri* strains MS and 227. Multiple occurrences of a sequence in a given organism are denoted by repeating the organism's number in the second column, e.g., "1–4, 6–8; 3, 7; 3" signifies a double occurrence in organism 7 and a triple occurrence in organism 3.

[b] The posttranscriptional modifications indicated by the superscript dots are identified as described in footnote *a* of Table 13. Ψ, Pseudouridine.

0.88, 0.87, and 0.87 are generated in the binary comparisons of *Bacillus brevis* strains 1028 (NRS) and 8185 (ATCC), 1028 and 953 (NRS), and 8185 and 953, respectively (Woese, unpublished data). *Methanobacterium* strain M.o.H.G. is designated *M. bryantii* strain M.o.H.G.

The genus *Methanobrevibacter* contains three species: *M. ruminantium* as type species, *M. arboriphilus*, and a new species, *M. smithii*, formerly *Methanobacterium ruminantium* strain PS Smith 1961 (P. H. Smith, Abstr. Bacteriol., A40, p. 60, 1961). *M. arboriphilus*, formerly *Methanobacterium arbophilicum (sic)* Zeikus and Henning 1975, and *M. ruminantium*, formerly *Methanobacterium ruminantium* strain M1 Smith and Hungate 1958, are justified as separate species based on the S_{AB} values presented in Fig. 7. *M. smithii* is named for Paul H. Smith, who isolated the organism in his pioneering work on the ecology and physiology of the methanogenic bacteria. *Methanobacterium* sp. strain AZ Zehnder and Wuhrmann 1977 yields an S_{AB} value of 0.84 in the binary comparison with *M. arboriphilus* (Fig. 7, Table 4). The 16S rRNA catalog of *Methanobacterium* sp. strain DC was identical to the *M. arboriphilus* catalog. We have tentatively designated both organisms as strains of *M. arboriphilus* (Fig. 8, Table 6) until information on more features is available.

In the family *Methanococcaceae* the genus

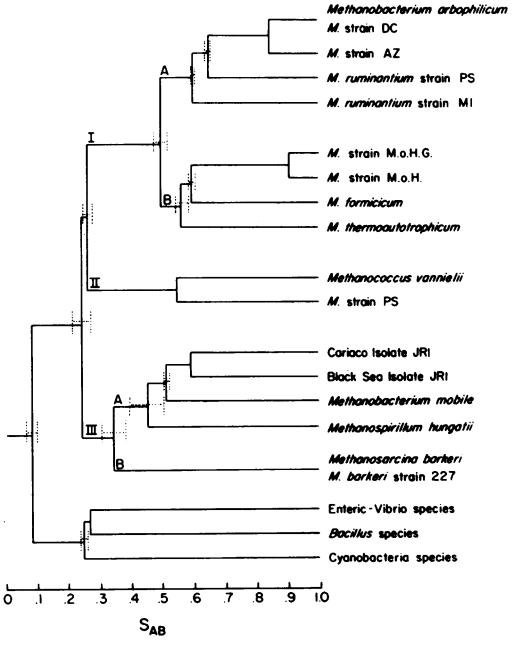

FIG. 7. *Dendrogram of relationships of methanogens and typical bacteria. The figure was constructed by average linkage clustering (between the merged groups) from the S_{AB} values given in Table 4. The standard deviation about the mean for all binary comparisons involved in a given cluster is indicated by the dotted lines.*

TABLE 4. *S_{AB} values (lower sector) for each indicated binary comparison and total number of nucleotides in sequence hexamers and larger that are common to both catalogs (upper sector)*[a]

Organism	Organism																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *M. arbophilicum, Methanobacterium* sp. DC		540	389	372	307	314	308	316	174	155	151	159	123	129	156	58	58	52
2. *Methanobacterium* sp. AZ	0.84		426	386	342	335	325	309	181	162	158	166	130	136	163	60	59	49
3. *M. ruminantium* PS	0.62	0.67		371	283	290	288	293	166	147	163	169	141	157	166	58	59	49
4. *M. ruminantium* M1	0.60	0.62	0.62		297	297	285	296	148	159	148	156	131	144	155	71	68	50
5. *Methanobacterium* sp. M.o.H.G.	0.51	0.56	0.48	0.51		533	354	340	165	164	150	170	156	155	176	61	60	52
6. *Methanobacterium* sp. M.o.H.	0.52	0.55	0.49	0.51	0.93		347	346	158	157	135	155	149	148	161	61	60	52
7. *M. formicicum*	0.51	0.53	0.49	0.49	0.62	0.61		323	175	168	157	163	135	157	150	62	59	53
8. *M. thermoautotrophicum*	0.51	0.50	0.49	0.50	0.58	0.60	0.56		165	158	151	174	151	151	184	63	58	50
9. *M. vannielii*	0.29	0.30	0.28	0.25	0.29	0.28	0.31	0.28		352	132	115	121	126	119	46	31	26
10. *Methanococcus* sp. PS	0.25	0.26	0.24	0.27	0.28	0.27	0.29	0.26	0.60		137	120	126	124	139	57	37	29
11. Cariaco isolate JR 1	0.24	0.25	0.27	0.25	0.26	0.23	0.27	0.25	0.23	0.23		352	296	320	207	33	42	48
12. Black Sea isolate JR 1	0.26	0.27	0.28	0.26	0.29	0.27	0.28	0.29	0.20	0.20	0.59		305	292	261	39	57	54
13. *M. hungatii*	0.20	0.21	0.24	0.22	0.27	0.26	0.23	0.26	0.21	0.21	0.50	0.52		214	201	45	52	51
14. *M. mobile*	0.21	0.22	0.26	0.24	0.26	0.25	0.27	0.25	0.22	0.21	0.54	0.49	0.36		197	40	48	55
15. *M. barkeri* MS, 227	0.25	0.25	0.27	0.25	0.29	0.27	0.25	0.30	0.20	0.22	0.34	0.43	0.33	0.32		60	64	46
16. Enteric-*Vibrio* species	0.10	0.08	0.08	0.10	0.10	0.09	0.09	0.10	0.07	0.08	0.05	0.06	0.07	0.10	0.10		157	145
17. *Bacillus* species	0.10	0.10	0.10	0.14	0.10	0.11	0.11	0.12	0.06	0.06	0.08	0.10	0.10	0.10	0.08	0.27		151
18. Cyanobacteria species	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.06	0.06	0.08	0.09	0.08	0.10	0.11	0.24	0.26	

[a] Values given for the enteric-*Vibrio* species, *Bacillus* species, and cyanobacteria species represent averages obtained from 11 (L. Zablen, Ph.D. thesis, University of Illinois, Urbana, 1975), 7 (36), and 4 (16) individual species, respectively.

Methanococcus (Kluyver and van Niel) Barker 1936 is emended to contain two species: *M. vannielii* Stadtman and Barker 1951 as the neotype species, and a new species, *M. voltae*, formerly *Methanococcus* strain PS (J. M. Ward, M.S. thesis, University of Miami, Miami, Fla. 1970). *M. voltae* is named for the Italian physicist Alessandro Volta, who discovered the combustible nature of gas from anaerobic sediments.

In the family *Methanomicrobiaceae*, the genus *Methanomicrobium* is proposed to contain the single member *M. mobile*, formerly *Methanobacterium mobile* Paynter and Hungate 1968. Results of comparative cataloging suggest that this organism is unrelated to the species of group I *Methanobacteriaceae* (S_{AB} = 0.24) in Fig. 7. It is sufficiently distinct from other members of the *Methanomicrobiaceae* (Fig. 7) to be placed in a new genus (Fig. 8).

The genus *Methanogenium* contains the species *M. cariaci* (formerly Cariaco isolate JR1) as the type species and *M. marisnigri* (formerly Black Sea isolate JR1) (Fig. 7). These are two new marine isolates recently described by Romesser et al. (81a).

The genus *Methanospirillum* contains the single species *M. hungatei* as the type species, formerly *M. hungatii* (*sic*) Ferry, Smith, and Wolfe 1974. The organism was named in honor of R. E. Hungate (33). (Incorrect spelling necessitates the correction of the species name from *hungatii* to *hungatei* at this time.)

In the family *Methanosarcinaceae*, the genus *Methanosarcina* (Kluyver and van Niel) Barker 1956 is emended to contain the single member *Methanosarcina barkeri* Schnellen 1947 (Schnellen, Ph.D. thesis) as the neotype species. Three other isolates of *Methanosarcina* were obtained for 16S rRNA analysis (Table 2). *M. barkeri* strain 227 (62, 86) was indistinguishable from *M. barkeri* strain MS (Table 4, Fig. 7). Partial characterization of *M. barkeri* strain W (R. A. Mah, M. R. Smith, and L. Baresi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I32, p. 160), a gas-vacuolated strain, indicated an S_{AB} value between 0.8 and 0.9 in the binary comparisons with strains 227 and MS. We conservatively propose a strain designation for both isolates.

The proposed taxonomic treatment for the methanogens is summarized in Table 6 and Fig. 8. All references for the remainder of this paper are to the new designations, unless specified otherwise.

EVIDENCE IN SUPPORT OF THE NEW TAXONOMIC TREATMENT

Recent evidence provided by studies on cell wall structure and composition, lipid distribu-

tion, intermediary metabolism, and nucleic acid composition substantiate the hierarchy of relationships presented in Fig. 7 and 8.

Cell Wall Structure and Composition

An examination of the chemical composition of the cell wall, Gram reaction, cell morphology, and cell ultrastructure support the proposed taxonomic treatment (Table 7). Electron micrographs of cell ultrastructure have been presented in detail elsewhere (33, 52, 54, 56, 59, 81a, 126, 128, 132, 133).

Order I, *Methanobacteriales*. In the family *Methanobacteriaceae*, species of the genus *Methanobacterium* possess fimbriae (27) and are straight to irregularly crooked gram-positive rods often forming filaments. The species of the genus *Methanobrevibacter* are gram-positive, lancet-shaped cocci or short rods which form pairs, chains, or irregular clumps depending on the growth conditions. *M. arboriphilus* and *M. smithii* each possess a single flagellum (27).

A unifying characteristic of the family is a gram-positive envelope (59, 125, 126, 128, 130, 132). Members of the genus *Methanobacterium* have a sharply defined, smooth, gram-positive cell wall 15 to 20 nm in width when viewed in ultrathin sections of whole cells (59, 128). In the genus *Methanobrevibacter*, ultrathin sections of whole cells of *M. ruminantium* reveal a distinctive triple layer in which a cell wall 30 to 40 nm in width consists of an inner electron-dense layer adjacent to a thick, more electron-transparent middle layer and a rough, irregular outer layer (59, 128). Ultrathin sections of isolated sacculi from representative members of the family show only one electron-dense layer 10 to 15 nm in width (56). This corresponds to the inner layer of *M. ruminantium* and the smooth layer of *Methanobacterium*. The isolated sacculi are indistinguishable morphologically from the cell wall sacculi of peptidoglycan-containing gram-positive bacteria (56).

Kandler and König (56) have shown the chemical composition of cell wall sacculi isolated from representative species of the *Methanobacteriaceae* to consist of a polymer containing three L-amino acids and *N*-acetylated sugars. In general, the sacculi contained L-lysine–L-alanine–L-glutamate–*N*-acetylglucosamine (or *N*-acetylgalactosamine and/or *N*-acetyltalosaminuronic acid) in a molar ratio of 1:1.2:2:1 (56; H. König and O. Kandler, Arch. Microbiol., in press). No muramic acid or D-amino acids were found. Further analysis of the cell wall structure of *M. thermoautotrophicum* (König and Kandler, in press) defines a peptidoglycan polymer which differs strikingly from that of typical bacteria in the absence of muramic acid and the presence of *N*-acetyltalosaminuronic acid and only L-amino acids (Fig. 9). Historically, the cell wall polymer of typical bacteria was referred to as murein, a muramic acid-containing peptidoglycan. This term has been replaced in the literature by the more general term peptidoglycan.

TABLE 5. *Key to the genera of family Methanobacteriaceae Barker 1956 (from the 8th edition of Bergey's Manual of Determinative Bacteriology [20])*

I. Rods or chain-forming, lancet-shaped coccoids. Genus I. *Methanobacterium*
II. Cocci other than chain-forming lancets.
A. Large cocci in packets. Genus II. *Methanosarcina*
B. Cocci occurring singly, in pairs, or in clumps. Genus III. *Methanococcus*

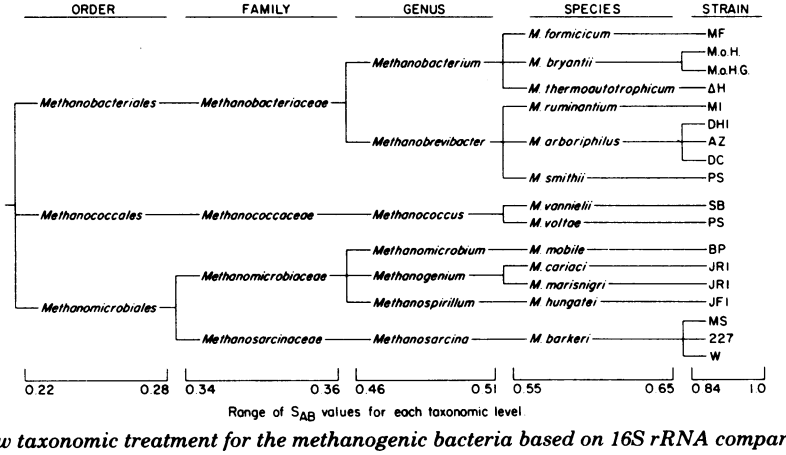

FIG. 8. *New taxonomic treatment for the methanogenic bacteria based on 16S rRNA comparative cataloging.*

TABLE 6. *Proposed taxonomic scheme of the methanogenic bacteria based on comparative cataloging of 16S rRNA*

Methanogen	Type strain	Reference	DSM no.	Former designation	Taxonomic descriptions
Order I. *Methanobacteriales* (type order)					
Family I. *Methanobacteriaceae*					
Genus I. *Methanobacterium* (type genus)					
1. *Methanobacterium formicicum* (neotype species)	MF[a]	see Table 2	1535	*Methanobacterium formicicum*	20, 22, 59, 74, 87, 128
2. *Methanobacterium bryantii*	M.o.H.[b]	23	863	*Methanobacterium* sp. strain M.o.H.	20, 22, 23, 59
Methanobacterium bryantii strain M.o.H.G.		see Table 2	862	*Methanobacterium* sp. strain M.o.H.G.	
3. *Methanobacterium thermoautotrophicum*	ΔH[b]	131	1053	*Methanobacterium thermoautotrophicum*	20, 99, 128, 131, 132
Genus II. *Methanobrevibacter*					
1. *Methanobrevibacter ruminantium* (type species)	M1[a]	88	1093	*Methanobacterium ruminantium* strain M1	7, 18, 20, 22, 59, 76, 88, 96
2. *Methanobrevibacter arboriphilus*	DH1[b]	130	1125	*Methanobacterium arbophilicum*	130
Methanobrevibacter arboriphilus strain AZ		125	744	*Methanobacterium* sp. strain AZ	110, 125
Methanobrevibacter arboriphilus strain DC		see Table 2	1536	*Methanobacterium* strain DC	
3. *Methanobrevibacter smithii*	PS[b]	see Table 2; 87	861	*Methanobacterium ruminantium* strain PS	20, 22, 87
Order II. *Methanococcales*					
Family I. *Methanoccaceae*					
Genus I. *Methanococcus*					
1. *Methanococcus vannielii* (neotype species)	SB[b]	90	1224	*Methanococcus vannielii*	20, 52, 53, 90
2. *Methanococcus voltae*	PS[b]	see Table 2	1537	*Methanococcus* sp. strain PS	
Order III. *Methanomicrobiales*					
Family I. *Methanomicrobiaceae* (type family)					
Genus I. *Methanomicrobium* (type genus)					
1. *Methanomicrobium mobile* (type species)	BP[b]	79	1539	*Methanobacterium mobile*	20, 79
Genus II. *Methanogenium*					
1. *Methanogenium cariaci* (type species)	JR1[b]	81a	1497	Cariaco isolate JR1	81a
2. *Methanogenium marisnigri*	JR1[b]	81a	1498	Black Sea isolate JR1	81a
Genus III. *Methanospirillum*					
1. *Methanospirillum hungatei*	JF1[b]	33	864	*Methanospirillum hungatii*	20, 33, 34, 78, 127, 128
Family II. *Methanosarcinaceae*					
Genus II. *Methanosarcina* (type genus)					
1. *Methanosarcina barkeri* (type species)	MS[a]	see Table 2	800	*Methanosarcina barkeri*	20, 107–109
Methanosarcina barkeri strain 227		see Table 2	1538	*Methanosarcina barkeri* strain 227	62, 86
Methanosarcina barkeri strain W		see Table 2		*Methanosarcina barkeri* strain W	

[a] Proposed neotype strain.
[b] Actual type strain.

TABLE 7. *Summary of major features associated with cell wall structure and composition*[a]

Order	Family	Genus	Gram reaction	Morphology	Cell wall composition
I	I	*Methanobacterium*	+	Long rods	Pseudomurein
		Methanobrevibacter	+	Short rods	
II	I	*Methanococcus*	−	Regular to irregular coccus	Protein subunits with trace glucosamine
III	I	*Methanomicrobium*	−	Short, curved rod	Protein subunits
		Methanogenium	−	Highly irregular coccus	
		Methanospirillum	−	Long, curved rod	Protein subunits with external sheath
	II	*Methanosarcina*	+	Irregular coccus in packets	Heteropolysaccharide

[a] Cell wall composition description from Kandler and Konig (56), Kandler and Hippe (55) and Jones et al. (52).

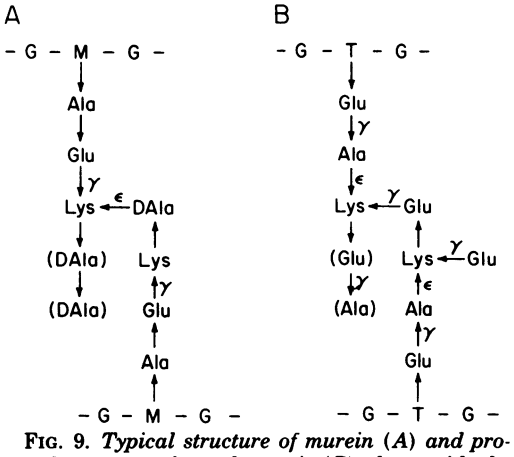

FIG. 9. *Typical structure of murein (A) and proposed structure of pseudomurein (B), the peptidoglycan polymer found in cell walls of M. thermoautotrophicum (König and Kandler, Arch. Microbiol., in press). G, N-Acetylglucosamine; M, N-acetylmuramic acid; T,N-acetyltalosaminuronic acid.*

To distinguish between the two polymer types, the structure in methanogens is referred to as pseudomurein (54).

Individual differences in the cell wall compositions of the *Methanobacteriaceae* are mostly restricted to neutral sugar content (56). However, *M. ruminantium* was shown to differ substantially with respect to the polypeptide component (56). L-Threonine completely replaced L-alanine, and the position of *N*-acetylglucosamine was taken by *N*-acetylgalactosamine. In addition, both *M. ruminantium* and *M. arboriphilus* cell walls were unique in their high phosphate contents (56).

Order II, *Methanococcales*. The two gram-negative species in the genus *Methanococcus* are highly motile, irregular to regular coccoid cells (0.3 to 5 μm in diameter), depending on growth conditions. Electron micrographs of the outer surfaces by Jones et al. (52) showed a regular array of protein subunits. Ultrathin sections of whole cells revealed a single layer of wall material 18 nm thick (52). Only one freeze-fracture plane was observed in the plasma membrane-cell wall complex, presumably passing through the hydrophobic region of the plasma membrane, as no fractures were observed to occur in the wall itself (52). Treatment of freeze-dried cells with 2% sodium dodecyl sulfate at 100°C for 30 min or disintegration of cells with glass beads followed by incubation with trypsin resulted in complete solubilization (O. Kandler, personal communication). Amino acid analysis of the total acid hydrolysate of whole cells did not yield muramic acid or diaminopimelic acid (52). Trace amounts of glucosamine were found in both *M. vannielii* and *M. voltae* (O. Kandler, unpublished data).

Order III, *Methanomicrobiales*. Genera in the *Methanomicrobiales* exhibit a more diverse range of morphological properties. In the gram-negative family *Methanomicrobiaceae*, the member of the genus *Methanomicrobium* is a highly motile, short, slightly curved rod (0.7 by 1.5 to 2.0 μm) distinct from other methane bacteria. No evidence for a rigid sacculus was found by Kandler and König (56). Freeze-dried cells treated with sodium dodecyl sulfate or disintegrated with glass beads followed by incubation with trypsin were completely solubilized (56). Muramic acid or amino sugars were not detected in the total acid hydrolysate of whole cells (Kandler, personal communication).

The genus *Methanogenium* includes poorly motile, highly irregular coccoid cells (1 to 2 μm in diameter). Ultrathin sections of whole cells (81a) showed a cell wall 10 nm in width closely

opposed to the cytoplasmic membrane with a periodic surface pattern similar to that of *M. vannielii* (52). In contrast to *M. vannielii*, salt is required for cell stability (81a). Treatment of freeze-dried cells with sodium dodecyl sulfate or disintegration with glass beads followed by incubation with trypsin resulted in complete solubilization of the cells (56). Muramic acid or amino sugars were not detected in total acid hydrolysates of whole cells (56).

The genus *Methanospirillum* consists of motile, regularly curved, long rods that form continuous spiral filaments. Ultrathin sections of whole cells show a well-defined, double-layered cell envelope (33, 127, 128). It consists of an electron-dense outer sheath (composed of subunits arranged in stacks perpendicular to filament length) adjacent to a more electron-dense inner layer which envelopes individual cells that are separated by a "spacer" element (127). Cell wall preparations of *M. hungatei* yielded no rounded sacculi, only cylindrical sheaths of variable length and of brittle structure (56). Total acid hydrolysates of the sheath revealed a complex spectrum of 18 amino acids, but not amino sugars, or sugars. The sheath was resistant to sodium dodecyl sulfate and trypsin treatments. It was concluded that the "cell wall" preparation represented only the outer layer (56), which is not involved in septum formation (127, 128). The inner layer was destroyed by the isolation procedure used, a result similar to those obtained for other genera in the *Methanomicrobiaceae*. No muramic acid, diaminopimelic acid, or amino sugars were detected in the total acid hydrolysate of whole cells (56).

Members of the family *Methanosarcinaceae* are irregular, gram-positive cocci that form packets of varying size. These clumps are large enough to present a grainy appearance to the naked eye. Division planes of the cells in the packet are not necessarily perpendicular. Ultrathin sections showed a very thick (500 nm), amorphous outer layer which often appeared laminated (128). Zhilina (133) reported a triple-layered appearance of the cell wall in a gas-vacuolated strain. Results of chemical analyses of isolated cell walls indicated that they consist of an acid heteropolysaccharide that contains galactosamine, neutral sugars, and uronic acids (55). No muramic acid, glucosamine, glutamic acid, or other amino acids typical of peptidoglycan were found in the two strains examined (55).

A summary of the major features associated with cell wall structure and composition and their relationship to the proposed new taxonomic treatment is presented in Table 7. The chemical differences from the muramic acid-containing peptidoglycan of typical bacteria are reflected in the lack of sensitivity of methanogens to antibiotics, such as penicillin, cycloserine, and vancomycin (52, 55, 56; Balch, unpublished data), as well as to the inability of lysozyme to effect cell wall lysis (52, 56).

Methanogens are represented by a diverse range of both gram-positive and gram-negative cell wall types. A comparison of cell wall composition and structure to reaction of the Gram stain reveals a strict correlation between the presence of a thick, rigid sacculus and a gram-positive reaction, a negative Gram reaction being correlated with the absence of a rigid sacculus (52, 54–56). These results correlate well with cell wall structure and Gram reaction in peptidoglycan-containing bacteria, supporting the assumption that the Gram reaction does not depend on the chemical nature of the cell wall but rather on its physical properties.

Lipid Composition

A survey of the lipid composition of the methanogenic bacteria shows that ether-linked polyisoprenoid (branched) chain lipids are the predominant components (Table 8) (64, 101, 101a, 102). Of the total lipids of whole cells, 20 to 30% are found to be neutral lipids, and 70 to 80% are polar lipids. Tornabene and Langworthy (101) and Makula and Singer (64) have shown that the polar lipids are principally isopranyl ethers of glycerol and are found to consist primarily of C_{20} phytanyl and C_{40} biphytanyl glycerol ethers (structures I and II, Fig. 10). The distribution between the major lipid ethers in nine representative methanogens is shown in Table 8. Both the C_{20} phytanyl and the C_{40} biphytanyl glycerol ethers were found in the *Methanobactericeae* and in the one genus examined of the *Methanomicrobeaceae* (*Methanospirillum*). Trace or no detectable levels of the C_{40} biphytanyl glycerol ethers were found in the *Methanococcaceae* or the *Methanosarcinaceae* (Table 8) (101).

The neutral lipids comprised a wide range (C_{14} to C_{30}) of isoprenoid hydrocarbons (101a). The principle compounds were C_{30}, C_{25}, and C_{20} acyclic isoprenoid hydrocarbons with a continuous range of hydroisoprenoid derivatives. No C_{35} or C_{40} compounds were detected. All the methanogens with the exception of the *Methanosarcinaceae* contained the C_{30} bifarnesyl or squalene components (Table 8; structure III, Fig. 10). The C_{25} and C_{20} isoprenoid hydrocarbon components were found in five and three of the nine methanogens examined, respectively (101a).

Thus, methanogens lack saponifiable lipids

TABLE 8. *Distribution of the major neutral and polar lipids in the methanogenic bacteria*[a]

Order	Family	Genus	Species	Major lipids: Neutral (isoprenoid hydrocarbons)	Major lipids: Polar (isopranyl glycerol ethers)
I	I	*Methanobacterium*	*M. formicicum*	ND[b]	C_{20} + C_{40} ethers
			M. bryantii	$C_{30}H_{50}$, $C_{30}H_{52}$	C_{20} + C_{40} ethers
		Methanobrevibacter	*M. thermoautotrophicum*	$C_{30}H_{50}$, $C_{30}H_{52}$, $C_{30}H_{54}$, $C_{25}H_{50}$	C_{20} + C_{40} ethers
			M. ruminantium	$C_{30}H_{50}$, $C_{30}H_{52}$, $C_{30}H_{54}$, $C_{30}H_{56}$	C_{20} + C_{40} ethers
			M. arboriphilus (AZ)	$C_{30}H_{50}$	C_{20} + C_{40} ethers
			M. smithii	$C_{30}H_{50}$, $C_{30}H_{52}$	C_{20} + C_{40} ethers
II	I	*Methanococcus*	*M. vannielii*	$C_{30}H_{50}$, $C_{25}H_{42}$	C_{20} ethers
			M. voltae	$C_{30}H_{52}$, $C_{30}H_{54}$, $C_{30}H_{56}$	C_{20} ethers
III	I	*Methanomicrobium*		ND	ND
		Methanogenium		ND	ND
		Methanospirillum	*M. hungatei*	$C_{30}H_{50}$, $C_{30}H_{52}$	C_{20} + C_{40} ethers
	II	*Methanosarcina*	*M. barkeri*	$C_{25}H_{46}$, $C_{25}H_{48}$, $C_{25}H_{50}$, $C_{25}H_{52}$	C_{20} ethers

[a] Data presented are from Makula and Singer (64), Tornabene and Langworthy (101), and Tornabene et al. (101a, 102).

[b] ND, Not determined.

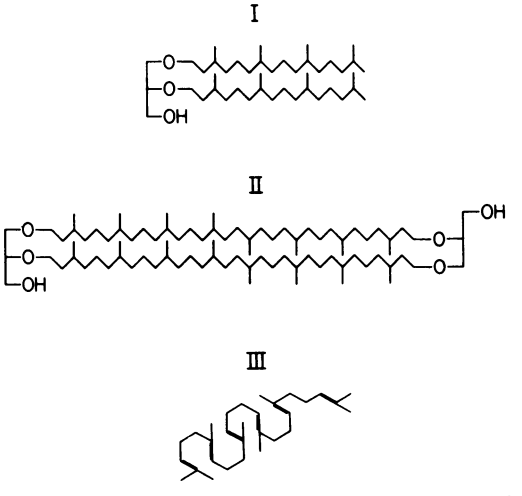

FIG. 10. *Structures of C_{20} diphytanyl glycerol diether (I) and C_{40} dibiphytanyl diglycerol tetraether (II), principal components of the polar lipid fraction of methanogenic bacteria. Structure III is $C_{30}H_{50}$ (squalene), a common isoprenoid hydrocarbon found in the neutral lipid fraction of methanogens.*

that are characteristic of typical bacteria. Instead, the major lipid components are ether-linked polyisoprenoid (branched) chain lipids. The C_{30} (squalene) isoprenoid hydrocarbon components dominate the neutral lipid fraction, whereas C_{20} phytanyl and C_{40} biphytanyl glycerol ethers dominate the polar fraction. The latter are ether-linked analogs of the ester-linked glycolipids and phospholipids found in typical bacteria. These findings provide the first evidence for the microbial synthesis of multi-branched isoprenoid hydrocarbons in the carbon range comparable to that found in sediments and petroleum and may have major implications in the interpretation of biogeochemical evolution (101).

Deoxyribonucleic Acid Base Composition (Moles Percent Guanine plus Cytosine)

Table 9 provides comparative data on the DNA base composition of 21 methanogen strains. The DNA mol% G + C content varies from 27.5 to 61%. However, each of the proposed individual groups shows a restricted range. The species of the genus *Methanobrevibacter* define the range of 27.5 to 32 mol% G + C, whereas the species of the genus *Methanobacterium* range from 32.7 to 40.7 mol% G + C for the mesophiles and 49.7 mol% G + C for the thermophile. The latter value may reflect the unique habitat of *M. thermoautotrophicum*. The two species in the genus *Methanococcus* have mol% G + C contents of 30.7 and 31.1. This is in contrast to the two marine cocci in the genus *Methanogenium*, where the mol% G + C contents are 51.6 and 61.2. The remaining genera in the *Methanomicrobiales* exhibit a range of mol% G + C contents (Table 9). The patterns observed reinforce the unique status of each genus.

Substrates for Growth and Methane Production

Table 9 summarizes the substrates for growth and methane production by methanogenic bacteria. All methanogens thus far examined oxidize hydrogen and reduce carbon dioxide to methane. Some species metabolize formate. Poor growth on carbon monoxide has been reported for some strains (25, 27). The members of the *Methanosarcinaceae* will additionally metabolize meth-

TABLE 9. *Summary of DNA base composition (mol% G + C) and substrates that serve as sole electron donors for methanogenesis and growth*

Order	Family	Species	mol% G + C[a]	Reference	Substrates for growth and CH_4 production[b]
I	I	*Methanobacterium formicicum*	40.7[c] (42.0)	126	H_2, formate
		Methanobacterium bryantii	32.7[c] (38.0)	131	H_2
		Methanobacterium bryantii strain M.o.H.G.	33.2[c]		H_2
		Methanobacterium thermoautotrophicum	49.7[c] (52.0)	131	H_2
		Methanobrevibacter ruminantium	30.6[c]		H_2, formate
		Methanobrevibacter arboriphilus	27.5	130	H_2
		Methanobrevibacter arboriphilus strain AZ	31.6[c]		H_2
		Methanobrevibacter arboriphilus strain DC	27.7[c]		H_2
		Methanobrevibacter smithii	31.0[c] (32.0)	126	H_2, formate
II	I	*Methanococcus vannielii*	31.1[c]		H_2, formate
		Methanococcus voltae	30.7[c]		H_2, formate
III	I	*Methanomicrobium mobile*	48.8[c]		H_2, formate
		Methanogenium cariaci	51.6	81a	H_2, formate
		Methanogenium marisnigri	61.2	81a	H_2, formate
		Methanospirillum hungatei	45.0	33	H_2, formate
		Methanospirillum hungatei strain GP1	46.5	78	H_2, formate
	II	*Methanosarcina barkeri*	38.8[c] (43.5)	108	H_2, CH_3OH, CH_3NH_2, acetate
		Methanosarcina barkeri strain 227	38.8[c]		H_2, CH_3OH, CH_3NH_2, acetate
		Methanosarcina barkeri strain W	40.5[d]		H_2, CH_3OH, CH_3NH_2, acetate
		Methanosarcina barkeri strain UBS	43.5	109	H_2, CH_3OH, CH_3NH_2, acetate
		Methanosarcina barkeri strain Z	51.0	135	H_2, CH_3OH, CH_3NH_2, acetate

[a] Values not done in this laboratory are presented in parentheses.
[b] CO_2 is reduced to CH_4 when H_2 is the substrate.
[c] Unpublished data, methods used to determine mol% G + C were as described in Deoxyribonucleic Acid Base Composition Determination (Moles Percent Guanine plus Cytosine).
[d] Unpublished data provided by R. A. Mah, using bouyant density method of Schildkraut et al. (83).

anol, methylamine (dimethylamine, trimethylamine, and ethyldimethylamine), and acetate (46, 62, 86, 89, 107–109) as the sole electron donor for growth and methane production. These results support the separation of the *Methanosarcina* sp. from other members of the *Methanomicrobiales* into at least a separate family.

Metabolic Pathways

Coenzyme M (2-mercaptoethanesulfonic acid). The oxidation of hydrogen and the reduction of CO_2 to produce methane by methanogens involves a highly unique biochemistry. Taylor and Wolfe (97) have identified a new coenzyme, coenzyme M (2-mercaptoethanesulfonic acid) involved in methyl transfer reactions in methane bacteria. It is the smallest of all known coenzymes and exceptional in its high sulfur content and acidity. The cofactor is required by methylcoenzyme M reductase, an enzyme present in all methanogens (R. P. Gunsalus, Ph.D. thesis, University of Illinois, Urbana, Ill., 1977) and active in the terminal steps of CO_2 reduction to CH_4 (42–44, 66, 97).

In a survey to determine the biological distribution of coenzyme M among nonmethanogens (8), a broad range of procaryotic organisms and eucaryotic tissues were examined; coenzyme M was not detected. In contrast, coenzyme M was found in high levels in all methanogens available in pure culture. An average intracellular concentration of 0.2 to 2 mM coenzyme M was observed (8). It was concluded that the coenzyme does not play a general role in other methyl transfer reactions; its importance lies in its central role

in methanogens, organisms which are pivotal for normal biodegradation in the cecum, rumen, sludge digesters, and aquatic sediments.

Coenzyme F_{420}. The structure of F_{420}, a low-potential electron carrier, was recently elucidated by Eirich et al. (32). Evidence suggests that F_{420} is the flavin mononucleotide analog 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5'-phosphate, which has an *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid side chain attached in a phosphodiester linkage (Fig. 11). F_{420} has been detected in all methanogens but has not been detected elsewhere (L. D. Eirich, Ph.D. thesis, University of Illinois, Urbana, Ill., 1978). F_{420} participates as an electron carrier in the nicotinamide adenine dinucleotide phosphate-linked hydrogenase and formate dehydrogenase systems in methanogens (34, 103, 104, 108). Recently, Zeikus et al. (129) have demonstrated that F_{420} is reduced by coenzyme A-dependent pyruvate and α-ketoglutarate dehydrogenases in extracts of *M. thermoautotrophicum*, a reaction normally mediated by ferredoxin in most bacteria (70). In addition to F_{420}, chromophoric factors F_{342} and F_{430} have been described in *M. thermoautotrophicum* (45). Their role is presently unknown. However, both F_{420} and F_{342} are useful for tentative identification of methanogens by fluorescent microscopy (28, 68). Cytochromes, menaquinones, or ubiquinones have not been detected in methanogenic bacteria to date (35, 100, 129).

Intermediary metabolism. The pathway of CO_2 fixation into cell carbon in methanogens remains to be elucidated. Taylor et al. (98), Weimer and Zeikus (108), and Zeikus et al. (129) have been unable to demonstrate key enzymes for the serine pathway (hydroxypyruvate reductase), the hexulose pathway (hexulosephosphate synthetase), or the reductive pentose phosphate pathway (ribulose-1,5-bisphosphate carboxylase) in cell-free extracts of *M. thermoautotrophicum* or *M. barkeri*. Analysis of short-term $^{14}CO_2$ fixation products yielded similar results (26). Taylor et al. (98) found $^{14}CO_2$ labeling patterns inconsistent with either the reductive tricarboxylic acid pathway or the total synthesis of acetate from CO_2, a pathway typical of certain clostridia. Zeikus et al. (129) demonstrated the presence of key oxidoreductases of the tricarboxylic acid cycle in *M. thermoautotrophicum* with the exception of isocitrate dehydrogenase. *M. barkeri* was found to lack fumarate reductase and α-ketoglutarate dehydrogenase (109). The authors concluded that either additional key enzymes remain to be detected in each organism or that a new pathway of autotrophic CO_2 fixation is operative.

Acetate has been shown to be assimilated into cell carbon in a wide range of methanogens (22, 39, 62, 86, 89, 98, 107–109), providing up to 60% of total cell carbon in cells grown in the presence of acetate. Fuchs et al. (39) recently examined acetate assimilation into alanine, aspartate, and glutamate in *M. thermoautotrophicum*. Cell carbon synthesis from labeled acetate was consistent with a pathway of pyruvate (alanine) synthesis from 1 C_2 compound and 1 CO_2, oxaloacetate (aspartate) synthesis from 1 C_2 compound and 2 CO_2, and α-ketoglutarate (glutamate) synthesis from 1 C_2 compound and 3 CO_2 via oxaloacetate, malate, fumarate, and succinate (Fig. 12). Weimer and Zeikus (109) found acetate assimilation into cell carbon in *M. barkeri* to differ from that in *M. thermoautotrophicum*; synthesis of α-ketoglutarate occurred via oxaloacetate, citrate, and isocitrate from 2 C_2 compounds and 1 CO_2 (Fig. 12). The authors suggested that the differences observed in intermediary metabolism may reflect the phylogenetic divergence between the two organisms (109).

Although acetate is an important precursor to cell carbon, the net synthesis of acetate from CO_2 in autotrophically grown cells remains an enigma. Fuchs and Stupperich (38) recently demonstrated the absence of a complete reductive carboxylic acid cycle in *M. thermoautotrophicum* grown on H_2 and CO_2 in the presence of [^{14}C]succinate. Only glutamate was found to be significantly labeled, excluding the possibility that oxaloacetate and pyruvate can be synthesized from succinate via isocitrate and citrate or other intermediates of the tricarboxylic acid cycle. The results suggested that a cyclic mechanism for regeneration of CO_2 acceptor molecules (e.g., acetyl coenzyme A) from citric acid cycle intermediates is not operative.

Ribosomal and Transfer Ribonucleic Acids of Typical Bacteria and Methanogens

rRNA sequences of methanogens are not closely related to those of typical bacteria. The methanogen 16S (23S) rRNA sequences bear no more relationship to those of typical bacteria than they do to the eucaryotic 18S (23S) rRNA's (30; D. Stahl, Ph.D. thesis, University of Illinois, Urbana, Ill., 1978). In the secondary structure of their 5S rRNA's (37) methanogens again seem no closer to the typical bacteria than to the eucaryotic examples (K. Luehrsen and C. R. Woese, unpublished data). The posttranscriptional modification patterns in 16S rRNA (see Tables 3 and 13) and 23S rRNA (Stahl, Ph.D. thesis) of methanogens differ strikingly from those of typical bacteria in spite of the fact that

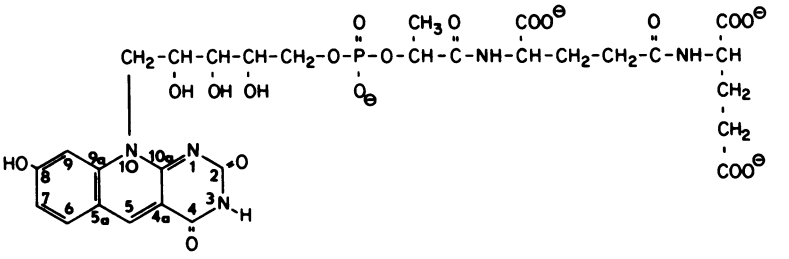

FIG. 11. *Structure of F_{420}.*

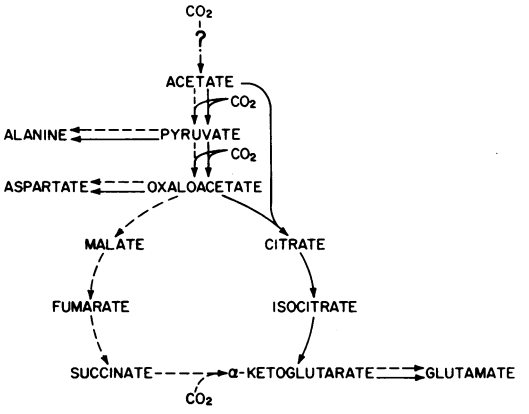

FIG. 12. *Proposed pathway of CO_2 fixation and acetate assimilation into cell carbon in M. thermoautotrophicum (dotted lines) and M. barkeri (solid lines). Data presented are from Fuchs et al. (39), Fuchs and Stupperich (38), and Weimer and Zeikus (109).*

the modifications tend to occur in the same locales in the two cases.

Methanogen transfer RNAs are unique in that they do not contain the universal common arm sequence GTΨCG; rather, they contain an analog, either GΨΨĊG or GÜΨCG (the superscript dot denotes a modification of the base, but Ü ≠ T). Best (14) reports no evidence in *M. vannielii* transfer RNAs for either of the modified bases ribothymidine and 7-methylguanosine, bases typical of *Escherichia coli*. R. Gupta and C. R. Woese (unpublished data) have confirmed these results and extended them to all the groups of methanogens. However, in disagreement with Best (14), they find no evidence for the presence of dihydrouridine except in the case of *M. barkeri*.

Genome Sizes of Typical Bacteria and *Methanobacterium thermoautotrophicum*

Mitchell et al. (69) have examined the DNA organization of *M. thermoautotrophicum*. The DNA of the methanogen was found to be as homogeneous as that of *E. coli* based on thermal melting profiles. In addition, results of buoyant density centrifugation in CsCl indicated that satellites were absent, and results of renaturation studies indicated the DNA to be of one class. On the basis of three different DNA renaturation kinetic experiments, the complexity (genome size) of the methanogen DNA was found to be two to three times smaller than that of *E. coli*. Assuming the *E. coli* genome to be 2.7×10^9 daltons, this yields an average value of 1.1×10^9 daltons for the genome size of *M. thermoautotrophicum*. These results are impressive, considering that *M. thermoautotrophicum* can be grown autotrophically with hydrogen as the energy source and CO_2 as the sole carbon source.

FORMAL DETAILED PROPOSAL: REVISION OF CLASSIFICATION AND NOMENCLATURE

In the most recent taxonomic treatment of the group (11, 20), the methanogens were placed in a single family, the *Methanobacteriaceae*. The treatment is no longer tenable in light of recent information. Below, we present revised definitions of the family *Methanobacteriaceae*, the genus *Methanobacterium*, the genus *Methanococcus*, and the genus *Methanosarcina* as well as definitions of the proposed new orders, families, genera, and species outlined above. Wherever possible, current nomenclatural usage is retained to maintain the historical development of the group.

Order I, *Methanobacteriales*

Methanobacteriales Balch and Wolfe (ord. nov.) Me.tha.no.bac.ter.i.al'es. M.L. fem. pl. n. *Methanobacteriaceae* type family of order; *-ales* ending to denote order; M. L. fem. pl. n. *Methanobacteriales* the *Methanobacteriaceae* order.

Cells are short, lancet-shaped cocci to long, filamentous rods. Cells are typically gram positive, although some strains are reported to be gram variable. Cell wall structure appears typically gram positive, when viewed in ultrathin section, but does not contain muramic acid. Pseudomurein is the predominant peptidogly-

can polymer. Ether-linked polyisoprenoid (branched) chain lipids are the predominant lipid components. The *Methanobacteriales* are very strict anaerobes which obtain energy for growth by the oxidation of H_2 and sometimes formate or CO or both with the reduction of CO_2 to methane. Cells contain coenzyme M and coenzyme F_{420}. They form a highly specialized physiological group which does not utilize carbohydrates, proteinaceous material, or organic compounds other than formate or CO as energy sources. They are widely distributed in nature, being found in anaerobic habitats, such as sediments of natural waters, soil, anaerobic sewage digesters, the gastrointestinal tracts of animals, and ecosystems where geothermally produced H_2 accumulates.

Family I, *Methanobacteriaceae.* *Methanobacteriaceae* Barker 1956 (emend.). Me.tha.no.bac.ter.i.a'ce.ae. M. L. neut. n. *Methanobacterium* type genus of family; *-aceae* ending to denote family; M. L. fem. pl. n. *Methanobacteriaceae* the *Methanobacterium* family.

Only one family, *Methanobacteriaceae*, is accepted in the order *Methanobacteriales*; the description of the family is the same as that of the order. The family *Methanobacteriaceae* contains two genera.

Genus I, *Methanobacterium.* *Methanobacterium* Kluyver and van Niel 1936 (emend.). Me.tha.no.bac.ter'i.um. M. L. n. *methanum* methane; Gr. n. *bakterion* a small rod; M. L. neut. n. *Methanobacterium* the methane (-producing) rodlet.

Cells are straight to irregularly crooked long rods (0.5 to 1 μm in width) often forming filaments. Cells are nonmotile, mesophiles to thermophiles. Cells possess fimbriae. DNA base composition range can be expected to include 33 to 41 mol% G + C (determined for three strains) for the mesophiles. The one thermophilic strain is 50 mol% G + C. The type species, *M. soehngenii* Barker 1936, was never obtained in pure culture (10) and is not included on the list of approved names (1). The neotype species of the genus *Methanobacterium* is therefore designated to be *M. formicicum* Schnellen 1947 (Schnellen, Ph.D. thesis). The species *M. formicicum* Schnellen 1947 and *M. thermoautotrophicum* Zeikus and Wolfe 1972 are described elsewhere (20, 22, 59, 74, 87, 99, 103, 131, 132). *M. bryantii* sp. nov., formerly *Methanobacterium* strain M.o.H. (23), is described below.

Methanobacterium bryantii. *Methanobacterium bryantii* Balch and Wolfe (sp. nov.). bry.an.ti.i'. M. L. gen. n. *bryantii* of Bryant; named for M. P. Bryant for his pioneering work in the separation and characterization of this organism from the *M. omelianskii* syntrophic culture.

The original description by Bryant et al. (23) is supplemented with material of Bryant et al. (22) and Langenberg et al. (59). Cells are slender and cylindrical with blunt, rounded ends, often forming chains and filaments with unevenly crooked cells. Cells are gram positive to gram variable. Cell width is from 0.5 to 1.0 μm with chains up to 10 to 15 μm in length. Cells are nonmotile and possess fimbriae. Surface colonies, which can reach 1 to 5 mm in diameter, are flat with diffuse to filamentous edges and have a characteristic gray to light gray-green appearance. Deep colonies are rounded and filamentous. Strains tend to clump in liquid culture. Cells grow optimally at 37 to 39°C at pH 6.9 to 7.2. Cells utilize H_2 as the sole energy source. Formate is not a substrate for growth and methanogenesis as found in *M. formicicum*. NH_4^+ is essential as the main source of cell nitrogen. Acetate, cysteine, and B-vitamins are highly stimulatory for growth. The DNA base composition of the type strain (M.o.H.) is 32.7 mol% G + C.

The type strains for the species in the genus *Methanobacterium* are designated *M. thermoautotrophicum* strain ΔH (DSM 1053) (131) and *M. bryantii* strain M.o.H. (DSM 863) (23). As the original strain of *M. formicicum* is not extant, *M. formicicum* strain MF (isolated from sewage sludge by M. P. Bryant) is designated the neotype strain (DSM 1535).

Genus II, *Methanobrevibacter.* *Methanobrevibacter* Balch and Wolfe (gen. nov.). Me.tha.no.bre.vi.bac'ter. M. L. n. *methanum* methane; L. adj. *brevis* short; M. L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod, staff; M. L. masc. n. *Methanobrevibacter* the short methane (-producing) rod.

Cells are lancet-shaped cocci to short rods which often form pairs or chains 0.5 to 1.0 μm in width. Cells are nonmotile to poorly motile with an optimal growth range of 37 to 39°C. DNA base composition range can be expected to include 27.5 to 32 mol% G + C (determined for five strains). *M. ruminantium* (Smith and Hungate 1958) Balch and Wolfe (comb. nov.) is designated the type species for the genus *Methanobrevibacter*, as it was the first species characterized. The rumen strain M1 has been nutritionally characterized by Bryant et al. (18, 22). *M. arboriphilus* (Zeikus and Henning 1975) Balch and Wolfe (comb. nov.) is described elsewhere (112, 127, 132). *M. smithii* sp. nov., formerly *M. ruminantium* strain PS Smith 1961 (P. H. Smith, Abstr. Bacteriol., A40, p. 60, 1961), is described below.

Methanobrevibacter smithii. *Methanobrevibacter smithii* (Smith 1961) Balch and Wolfe (sp. nov.). smith.i.i'. M. L. gen n. *smithii* of Smith; named for P. H. Smith for his pioneering work on the ecology and physiology of the methanogenic bacteria.

The original description by Smith (P. H. Smith, Abstr. Bacteriol., A40, p. 60, 1961) is supplemented with material of Bryant et al. (22) on a sludge strain (PS) previously considered to be *M. ruminantium*. Cells are short, lancet-shaped to oval cocci, 0.5 to 1.0 μm in width and 1.0 to 1.5 μm in length. Cells frequently occur in pairs and long chains resembling streptococci. Cells are nonmotile and strongly gram positive. Surface colonies are translucent, convex, and circular with entire margins and usually yellow white in appearance. Cells may use either H_2 or formate as a substrate for energy and growth. Cells grow optimally at 37 to 39°C. DNA base composition of the type strain is 30.6 mol% G + C. *M. smithii* requires ammonia as a nitrogen source and acetate as a major carbon source for growth. The species is morphologically identical to *M. ruminantium* with the exception that *M. smithii* has a single polar flagellum but is nonmotile when viewed in wet mount. The major differences are physiological and nutritional. *M. smithii* can be cultured on a simple chemically defined medium in the absence of α-methylbutyrate, amino acids, and coenzyme M, which are essential for the growth of *M. ruminantium* (7, 22, 96). *M. smithii* grows optimally from pH 6.9 to 7.4, whereas *M. ruminantium* grows optimally from pH 6.3 to 6.8.

The type strains for species in the genus *Methanobrevibacter* are designated *M. arboriphilus* strain DH1 (DSM 1125) (130) and *M. smithii* strain PS (DSM 861) (20, 22, 87; Smith, Abstr. Bacteriol., A40, p. 60, 1961). *M. ruminantium* strain M1 (DSM 1093) (18, 20, 22, 59, 88, 128) is designated the neotype strain, because the original strain of Smith and Hungate (88) was not maintained.

Order II, *Methanococcales*

Methanococcales Balch and Wolfe (ord. nov.). Me.tha.no.coc.cal'es. M. L. fem. pl. n. *Methanococcaceae* type family of order; *-ales* to denote order; M. L. fem. pl. n. *Methanococcales* the *Methanococcaceae* order.

Cells are regular to irregular cocci, 1.0 to 5.0 μm in diameter, and gram negative with a single layer of protein subunits external to the plasma membrane. Muramic acid is not detected. Ether-linked polyisoprenoid (branched) chain lipids are the predominant lipid components. The *Methanococcales* are very strict anaerobes which obtain energy for growth by the oxidation of H_2 or formate with reduction of CO_2 to CH_4. Cells contain coenzyme M and coenzyme F_{420}. They form a highly specialized physiological group which does not utilize carbohydrates, proteinaceous material, or organic compounds other than formate as energy sources. Widely distributed in nature, being found in anaerobic habitats, such as sediments of natural waters.

Family I, *Methanococcaceae.* *Methanococcaceae* Balch and Wolfe (fam. nov.). Me.tha.no.coc.ca'ce.ae. M. L. neut. n. *Methanococcus* (Kluyver and van Niel) Barker 1936 type genus of the family; *-aceae* ending to denote a family; M. L. fem. pl. n. *Methanococcaceae* the *Methanococcus* family.

Only the one family, *Methanococcaceae*, is accepted in the order *Methanococcales*; the description of the family is the same as that of the order. The family *Methanococcaceae* contains one genus.

Genus I, *Methanococcus.* *Methanococcus* (Kluyver and van Niel) Barker 1936 (emend.). Me.tha.no.coc'cus. M. L. n. *methanum* methane; M. L. n. *coccus* a spherical cell; M. L. masc. n. *Methanococcus* the methane coccus.

Cells are fragile, regular to irregular cocci (0.5 to 5 μm in diameter) that occur singly or in pairs. Cells are highly motile, with an optimal growth range from 32 to 40°C. DNA base composition range can be expected to include 30.7 to 31.1 mol% G + C (determined for two strains). The type species, *Methanococcus mazei* Barker 1936, was never obtained in pure culture (10) and is not included in the list of approved names (1). The neotype species of the genus *Methanococcus* is therefore designated to be *M. vannielii* Stadtman and Barker 1951 and is described in detail elsewhere (20, 52, 53, 90). *M. voltae* sp. nov., formerly *Methanococcus* strain PS (Ward, M. S. thesis) is described below.

Methanococcus voltae. *Methanococcus voltae* Balch and Wolfe (sp. nov.). vol'tae. M. L. gen. n. *voltae* of Volta; named for the Italian physicist Alessandro Volta for discovery of the combustible nature of gas from anaerobic sediments.

The original description of J. M. Ward (M.S. thesis) is supplemented with unpublished data of W. E. Balch. Cells are highly motile regular to irregular cocci (0.5 to 3.0 μm in diameter). Morphology is dependent on the physiological state of the cell and on the ionic strength of the medium. Surface colonies can reach 1 to 3 mm in diameter and are clear and convex with smooth edges. Subsurface colonies are cream colored, with rough-textured interiors and irregular edges. Cells grow optimally from 32 to 40°C,

pH 6.7 to 7.4. H_2 and formate serve as the sole energy sources for growth and methane production. DNA base composition of the type strain is 30.7 mol% G + C. Cells are sensitive to low osmotic environments; cells require 1.2 to 4.8% NaCl for optimal growth. A summary of the major phenotypic properties which distinguish *M. voltae* and *M. vannielii* is presented in Table 10.

The type strains for species in the genus *Methanococcus* are designated *M. vannielii* strain SB (DSM 1224) (52, 53, 90) and *M. voltae* strain PS (DSM 1537) (Ward, M.S. thesis).

Order III, *Methanomicrobiales*

Methanomicrobiales Balch and Wolfe (ord. nov.). Me.th.no.mi.cro.bi.al'es. M. L. fem. pl. n. *Methanomicrobiaceae* type family of order; *-ales* ending to denote order; M. L. fem. pl. n. *Methanomicrobiales* the *Methanomicrobiaceae* order.

Cells are cocci to rods, gram negative or gram positive, nonmotile to motile. In ultrastructure, a wide range of cell wall enclosures are observed, none of which contain muramic acid. Ether-linked polyisoprenoid (branched) chain lipids are the predominent lipid components. These very strict anaerobes obtain energy for growth by oxidation of H_2 or formate with the reduction of CO_2 to CH_4 or via fermentation of such compounds as methanol, methylamine (dimethylamine, trimethylamine, and ethyldimethylamine), and acetate with the formation of methane and CO_2. Cells contain coenzyme M and F_{420}. The *Methanomicrobiales* form a highly specialized physiological group which does not utilize carbohydrate, proteinaceous materials, or organic compounds other than those listed above as energy sources. They are widely distributed in nature, being found in anaerobic habitats, such as sediments of natural waters, soil, anaerobic sewage digesters, and the gastrointestinal tracts of animals.

Family I, *Methanomicrobiaceae.* *Methanomicrobiaceae* Balch and Wolfe (fam. nov.). Me.tha.no.mi.cro.bi.a'ce.ae. M. L. neut. n. *Methanomicrobium* type genus of the family; *-aceae* ending to denote a family; M. L. fem. pl. n. *Methanomicrobiaceae* the *Methanomicrobium* family.

Cells are gram negative, cocci to straight, slightly curved rods. Cells oxidize H_2 or formate as the sole energy source for growth and methane production. Three genera are included in the family *Methanomicrobiaceae.*

Genus I, *Methanomicrobium.* *Methanomicrobium* Balch and Wolfe (gen. nov.). Me.tha.no.mi.cro.bi'um. M. L. n. *methanum* methane; Gr. adj. *micros* small; Gr. adj. *bios* life; M. L. neut. n. *Methanomicrobium* the methane (-producing) small life.

Cells are short, straight to slightly curved rods (0.7 by 1.5 to 2.0 μm) with rounded ends. Cells are highly motile, with a growth optimum of 38 to 40°C. Only H_2 serves as the substrate for growth and methane production. DNA base composition of the lone species is 48.8 mol% G + C. The type species is *M. mobile* (Paynter and Hungate 1968) Balch and Wolfe (comb. nov.). Detailed descriptions of this genus are presented elsewhere (20, 79). The type strain is strain BP (DSM 1539) (79).

Genus II, *Methanogenium.* *Methanogenium* Romesser and Wolfe 1979. Me.tha.no.gen.i'um. M. L. n. *methanum* methane; Gr. suff. v. *-genes* producing; M. L. neut. n. *Methanogenium* the methane producer.

Cells are highly irregular cocci (0.5 to 2.5 μm in diameter) that occur singly. Cells are poorly motile. The optimal growth temperature is 25 to 30°C. Both H_2 and formate serve as substrates for growth and methane production. Marine forms are sensitive to low osmotic environments; cells require 1.5 to 3.0% NaCl for growth. DNA base composition range can be expected to include 51.2 to 61 mol% G + C (determined for two strains). Only two species, *M. cariaci* Romesser and Wolfe 1979 and *M. marisnigri* Romesser and Wolfe 1979, are available, and they are described in detail elsewhere (81a). The type strains for the species are designated *M. cariaci* strain JR1 (DSM 1497) and *M. marisnigri* strain JR1 (DSM 1498) (81a).

Genus III, *Methanospirillum.* *Methanospirillum* Ferry, Smith, and Wolfe 1974. Me.tha.no.spi.ril'lum. M. L. n. *methanum* methane; Gr. n. *speira* a spiral; M. L. neut. n. *Methanospirillum* the methane(-producing) spiral.

Cells are slowly motile, regularly curved rods (0.5 by 7 μm) often forming long spiral filaments. Cells have a distinctive double-wall appearance when viewed in thin section. Cells lack a rigid cell wall sacculus, the outer layer being a proteinaceous sheath. Both H_2 and formate are substrates for growth and methane production. DNA base composition range can be expected to include 45 to 46.5 mol% G + C (determined for two strains). One species is included in the genus *Methanospirillum, M. hungatei* Ferry, Smith, and Wolfe 1974 (*M. hungatii* [*sic*] Ferry, Smith, and Wolfe 1974). A complete description of this species is presented elsewhere (33, 34, 78, 127). *M. hungatei* Ferry, Smith, and Wolfe 1974 (33) is designated the type species of the genus *Methanospirillum.* The type strain is strain JF1 (DSM 864) (33).

TABLE 10. *Comparison of known phenotypic properties of the new species M. voltae to M. vannielii (52, 53, 90)*[a]

Species	Phenotypic property																				
	Morphology	Size (μm)	Temp optimum (°C)	Generation time (38°C; Table 1, medium 3) (h)	pH optimum	NaCl (%)		Growth on:				Growth factors	Flagella	HS-CoM	F_{420}	Muramic acid	Isoprenylglycerol ethers		C_{30} isoprenoid hydrocarbons	Gram reaction	mol% G + C
						Optimum	Range	H_2/CO_2	Sodium formate	Sodium acetate	Methanol						C_{20}	C_{40}			
M. voltae	Regular to irregular coccus	0.5–3.0	32–40	3	6.7–7.4	4.0	0.5–5.0	+	+ (pH 7)	−	−	Yeast extract (Difco) is stimulatory	+	+	+	−	+	−	+	−	30.7
M. vannielii	Regular to irregular coccus	1–4	36–40	8	7.0–9.0	2.4	0–3.2	+	+ (pH 8)	−	−	Yeast extract (Difco) and Se or W are highly stimulatory	+	+	+	−	+	−	+	−	31.1

[a] Data presented for *M. voltae* are from the original description by J. M. Ward (M.S. thesis) supplemented with the unpublished data of W. E. Balch, obtained by using modifications of medium 3 in Table 1.

Family II, *Methanosarcinaceae*. *Methanosarcinaceae* Balch and Wolfe (fam. nov.). Me.tha.no.sar.cin.a'ce.ae. M. L. neut. n. *Methanosarcina* type genus of the family; *-aceae* ending to denote family; M. L. fem pl. n. *Methanosarcinaceae* the *Methanosarcina* family.

The *Methanosarcinaceae* are large, spherical to pleomorphic gram-positive cells (1.5 to 2.5 μm in diameter) often forming packets of varying size. Cell division planes are not necessarily perpendicular. Cells are nonmotile mesophiles to thermophiles. Energy for growth is obtained by the oxidation of H_2 with the reduction of CO_2 to methane or by the metabolism of methanol, methylamine (dimethylamine, trimethylamine, and ethyldimethylamine), and acetate with the formation of methane and CO_2 as end products; ammonia is an additional product from degradation of the amines. The cell wall is characteristically thick (500 nm), being composed of an acid heteropolysaccharide (55). Gas-vacuolated forms have been reported (133; Mah et al. Abstr. Annu. Meet. Am. Soc. Microbiol., 1977, I32, p. 160).

Genus I, *Methanosarcina*. *Methanosarcina* (Kluyver and van Niel) Barker 1956 (emend.). Me.tha.no.sar.ci'na. M. L. n. *methanum* methane; L. n. *Sarcina* a generic name; M. L. fem. n. *Methanosarcina* the methane sarcina.

Only one genus, *Methanosarcina*, is included in the family *Methanosarcinaceae*; the description of the genus is the same as that of the family. DNA base composition range can be expected to include 39 to 51 mol% G + C (determined for five strains). The type species, *M. methanica* (Smit) Kluyver and van Niel 1936, was never obtained in pure culture and is not included in the list of approved names (1). We therefore designate *M. barkeri* Schnellen 1947 (Schnellen, Ph.D. thesis) the neotype species of the genus *Methanosarcina*. A complete description of this genus is presented elsewhere (11, 20, 46, 62, 86, 107–109, 128, 133–135). The type strain is strain MS (DSM 800) (a sewage sludge isolate of M. P. Bryant).

A key showing the main differential characteristics of the taxa under consideration is presented in Table 11.

OVERVIEW

Resolution of the Methanogen Group

rRNA is a semantide of sufficiently broad distribution, constant function, and conserved sequence to serve as a reliable indicator of phylogenetic relationships over a wide spectrum of organisms. The results of comparative cataloging of the 16S rRNA's from representative species of methanogens defines a comprehensive and detailed taxonomy which reflects the hierarchy observed in Fig. 7, an arrangement consistent with other classical determinative characters, including morphology, Gram reaction, mol% G + C, cell wall composition, and lipid distribution.

Cluster analysis demonstrates the methanogens to be a major group. The most distant relationships—having S_{AB} values in the range 0.2 to 0.3—are as distant as those observed among the gram-positive bacteria, enteric bacteria, and cyanobacteria, for example. The methanogens are also as phenotypically varied as are the typical bacteria. It is reasonable, then, that taxa at least at the level of class (or division) be assigned to the methanogen group. We consider the present taxonomic assignments (based on a limited range of known species) to be conservative estimates of the levels of genealogical and phenotypic relatedness within the entire group.

Taxonomic categories within the proposed methanogen group were chosen to reflect those characteristic of typical bacteria. Present taxa may have to be revised upwards as more information becomes available. For example, the relationships of the *Methanosarcinaceae* to other methanogens are defined by a single species. A less conservative interpretation of the S_{AB} value in Fig. 7 would suggest that the *Methanosarcinaceae* alone deserve the rank of order. This is supported by a range of distinctive phenotypic differences circumscribing the group. A similar situation applies to some of the lower taxa. *M. arboriphilus* strain AZ or *M. barkeri* strain W may deserve the rank of separate species on the basis of several unique characters. Interspecies and interstrain differences are poorly defined at this time. Additional methanogenic isolates, when compared to known species on the basis of phenotypic properties, immunological properties, and relevant molecular markers (e.g., 16S rRNA sequence, protein sequence[s], and nucleic acid hybridization), should provide the necessary insight to refine the existing taxa and define new ones.

Systematics within the procaryotes is, in one sense, necessarily arbitrary; no consistent definition has been available for the assignment of the rank of species or any of the higher taxa. The degree of subdivision of a group often reflects the intensity with which it has been studied, rather than its true depth. The subjectivity of bacterial taxonomic levels was recognized by Benecke in 1912 (13):

> What, then are species, i.e., the lowest of the taxonomic units? The answer is: that which the investigator who proposes the species wishes to include in

TABLE 11. *Determinative key to species of the methanogenic bacteria based on simple phenotypic characters*

I. Gram-positive to gram-variable rods or lancet-shaped cocci often forming chains and filaments.
Order I. *Methanobacteriales*
Family I. *Methanobacteriaceae*
A. Slender, straight to irregularly crooked long rods often occurring in filaments.
Genus I. *Methanobacterium*
1. Mesophilic.
a. Methane produced from formate.
Methanobacterium formicicum
b. Methane not produced from formate.
Methanobacterium bryantii
2. Thermophilic
Methanobacterium thermoautotrophicum
B. Short rods or lancet-shaped cocci which often occur in pairs or chains.
Genus II. *Methanobrevibacter*
1. Cells form short, nonmotile rods which do not utilize formate.
Methanobrevibacter arboriphilus
2. Chain-forming, lancet-shaped cocci that produce methane from formate and require acetate as a carbon source.
a. Growth requirement for 2-mercaptoethanesulfonic acid and D-α-methyl butyrate.
Methanobrevibacter ruminantium
b. Do not have an obligate growth requirement for 2-mercaptoethanesulfonic acid or D-α-methyl-butyrate.
Methanobrevibacter smithii
II. Gram-negative cells or gram-positive cocci occurring in packets.
A. Gram-negative, regular to slightly irregular cocci often forming pairs.
Order II. *Methanococcales*
Family I. *Methanococcaceae*
Genus I. *Methanococcus*
1. Cells inhibited by addition of 5% NaCl to medium.
Methanococcus vannielii
2. Cells not inhibited by addition of 5% NaCl to medium.
Methanococcus voltae
B. Gram-negative rods or highly irregular cocci occurring singly.
Order III. *Methanomicrobiales*
Family I. *Methanomicrobiaceae*
1. Straight to slightly curved, motile, short rods.
Genus I. *Methanomicrobium*
Methanomicrobium mobile
2. Irregular coccoid cells.
Genus II. *Methanogenium*
a. Cells require acetate.
Methanogenium cariaci
b. Cells do not require acetate.
Methanogenium marisnigri
3. Regularly curved, slender, motile rods, often forming continuous spiral filaments.
Genus III. *Methanospirillum*
Methanospirillum hungatei
C. Gram-positive coccoid cells which usually occur in packets and ferment methanol, methylamine, and acetate.
Family II. *Methanosarcinaceae*
Genus I. *Methanosarcina*
Methanosarcina barkeri

accordance with his scientific tact. Obviously it is impossible to answer the question in any other way because Nature herself does not create species, but only individuals with their descendants, so-called "clones." And the systematist collects such clones into groups or bundles which he calls "species." How big or how little he wants to make his packets—that depends upon his scientific attitude, which may vary for different individuals. [P. 212-213]

Although the present approach does not provide an absolute definition of these "packets," it

promises to develop a consistent, relative measure of taxonomic categories. Such an approach should provide a basis for interpretation of microbial diversity.

Phylogenetic Relationship of Methanogens to Other Organisms

It is customary to view an organism as being either procaryotic or eucaryotic. This distinction was originally formulated by Chatton in 1937 (24) and refined by Stanier, van Niel, Murray, Allsopp, and others (2, 71-73, 91, 92, 94). Initially, cytological features, such as organelles, formed the basis for the distinction, and the procaryote was identified in an essentially negative way—by its not possessing the characteristically eucaryotic features. At this point the procaryote-eucaryote distinction was organizational, or taxonomic. Detailed molecular characterizations have subsequently provided a far more extensive basis for making the distinction. However, in the process there has occurred a subtle and unrecognized shift in the concept. What was initially a purely organizational distinction has now become a mutually exclusive genealogical dichotomy: *Procaryotae* and *Eucaryotae* are accepted today as the two primary phylogenetic categories.

If one considers methanogens only in a general organizational sense, then they are clearly procaryotes. They represent a variety of sizes and shapes typical of bacteria. They lack the eucaryotic nuclear membrane and organelles. At the gross molecular level their ribosomes resemble bacterial ribosomes in overall size and size of their main component parts. Yet the methanogens appear to challenge procaryote-eucaryote as a phylogenetic dichotomy. In terms of rRNA sequence homologies, the methanogens are no closer to ordinary bacteria than they are to the "cytoplasmic aspect" (18S rRNA) of the eucaryotic cell (112). This conclusion, based initially on the semantide rRNA, has been confirmed and extended in terms of phenotypic properties (8, 14, 32, 35, 38, 39, 45, 52, 54-56, 64, 69, 95, 96, 101, 101a, 102, 108, 109, 113, 119). Such a finding demands that we reexamine some basic assumptions of bacterial systematics. Can we maintain the present empirical definition of the subdivisions of the *Procaryotae* (40) and rationally incorporate the methanogens? If not, how do we provide a systematic hierarchy of the typical bacteria, methanogens, and eucaryotes which is both deterministically and genealogically consistent?

The definition of the kingdom *Procaryotae* (72) has recently been supplemented by Gibbons and Murray (40); it constructs a hierarchy of relationships for the major bacterial groups on the basis of cell wall type (Gram reaction). The procaryotes are formally subdivided into three new divisions: division I, *Gracilicutes*, comprises all the gram-negative murein cell wall types (inclusive of the cyanobacteria); division II, *Firmacutes*, comprises the gram-positive murein cell wall types; and division III, *Mollicutes* (30), comprises the mycoplasmas, organisms without cell walls. In addition, Gibbons and Murray suggest that a fourth division, "mendocutes," may be needed for organisms which "do not have a clear-cut type of cell wall containing peptidoglycan" (40); this last would include the methanogens.

This proposal is commendable in that it is the

TABLE 12. *Degree of similarity between 16S rRNA catalogs of methanogens and other archaebacteria (represented by Halobacterium), the typical bacteria, and the eucaryotic 18S rRNA*[a]

Group	Group								
	1	2	3	4	5	6	7	8	9
1. *Halobacterium* species	291								
2. Order I methanogens	170	343							
3. Order II methanogens	167	153	265						
4. Order III methanogens	119	163	126	352					
5. Cyanobacteria	51	50	49	26	277				
6. *Rhodospirillaceae* species	58	79	51	62	147	263			
7. *Bacillus* species	54	58	51	32	151	177	333		
8. Enteric-*Vibrio* species	43	61	43	52	145	194	157	372	
9. Eucaryotic 18S rRNA	56	60	48	51	38	37	35	33	339

[a] For every pair of catalogs the number of nucleotides in sequence hexamer and larger that are common to both catalogs is determined. The numbers shown are averages for all such catalog pairs between any two groups of organisms. (Posttranscriptionally modified nucleotides are for this purpose treated as unmodified sequences.) The values given for *Halobacterium*, the cyanobacteria, *Rhodospirillaceae*, *Bacillus*, and the enteric-*Vibrio* groups represent averages obtained from 2, 5, 5, 10, and 10 individual species, respectively. The value given for the eucaryotic 18S rRNA represents an average obtained from three cell types (*Lemna*, yeast, and L-cells). Nucleotide numbers on the diagonal represent total numbers of nucleotides common to each group.

first attempt to establish formally an encompassing taxonomy of the bacteria on the basis of a unifying, molecularly defined characteristic. However, in the short time since its publication our perception of the evolutionary significance of the cell wall has changed dramatically. Due largely to the work of Kandler and his associates (54–56), we now know that among organisms recognized as bacteria, the murein wall (gram positive or gram negative) is but one of several basically different wall types. The pseudomurein wall, the polysaccharide wall, and the protein subunit wall also exist (54–56). All the latter wall types are represented among the methanogens. Is it correct then, to consider the murein wall type to be somehow primary and thus the basis for structuring a classification of all bacteria? Or should all wall types be considered phylogenetically equivalent and a classification scheme be devised that gives each type the same weight? These questions cannot be answered on the basis of cell wall type per se; cell wall type, though taxonomically (deterministically) useful, appears insufficient to define a bacterial phylogeny.

Use of semantide analysis (136) has proven to be a reliable indicator of phylogenetic relatedness (3, 17, 36, 84, 111, 122). On the basis of comparative rRNA studies, Woese and Fox (112) have suggested that the categories *Euccaryotae* and *Procaryotae* be viewed as different "levels of organization"—as "domains" that are not phylogenetically comparable. The procaryote genealogically can be directly compared to the eucaryote only when the latter is conceptually dissected into the sum of its phylogenetically separate parts (e.g., mitochrondrion, chloroplast, cytoplasm [18S rRNA]). The term "urkingdom" or "primary kingdom" was coined to designate the highest phylogenetic category in the "procaryotic domain."

Three urkingdoms have been proposed (112); one, the "eubacteria," comprises all bacteria that possess murein cell walls (and includes the cyanobacteria and most of the wall-less mycoplasmas); a second is defined by that aspect of the eucaryotic cell represented in the cytoplasmic 18S rRNA; whereas a third, the "archaebacteria," includes the methanogens. This latter category is also known to contain the extreme halophiles (halobacteria), and two thermoacidophiles, *Sulfolobus* and *Thermoplasma* (61, 115; Table 12). Woese et al. (115) recently summarized their unique and unifying molecular properties. None have murein cell walls, all have membranes whose lipid components are the highly unusual ether-linked polyisoprenoid (branched) chain lipids, and they possess characteristic transfer RNAs and rRNA's in their translation machinery (Table 13). The members of the archaebacteria are as ecologically and biochemically diverse as the eubacteria (115). Indeed, existence of archaebacteria phenotypically distinct from the methanogens argues against an interpretation of the 16S rRNA data suggesting that the rate of rRNA sequence change in the methanogens is for some reason different, e.g., more rapid, than that found in the eubacteria.

Given the novelty of the procaryotic domain concept, we do not feel justified in proposing here a formal taxonomic rank at the highest level. There can no longer be any doubt that the methanogens and their relatives are a distinct group of organisms. In any taxonomic scheme, whether designed as a "means of identification" (72) or as an "indication of relationships" (72),

TABLE 13. *Posttranscriptionally modified sequences in methanogens and their likely counterparts in other bacteria*

	Sequence[a]	Occurrence in: Methanogen order: I	II	III	Extreme halophiles	Typical bacteria (%)
1. a.	Ċ-CCG	+	−	−	−	None
b.	Ċ-CĊG	−	+	−	−	None
c.	C(Ċ,C)G	−	−	+	+	None
d.	Ċ-CCG	−	−	−	−	>95
2.	CCĠCG	−	−	±[b]	−	>95
3. a.	ÅÅCCUG	+	+	−	−	~30
b.	ÅÅUCUG	−	−	+	+	None
c.	ÅÅG	−	−	−	−	~60
4. a.	UÅÅCAAG	+	+	−	−	None
b.	UAÅCAAG	−	−	+	+	None
c.	ÙAACAAG	−	−	−	−	>95
5. a.	AUÑCAACG	+	+	−	−	None
b.	ACÑCAACG	−	−	+	+	None
c.	AAĠĊAACG	−	−	−	−	>95
d.	AUĠĊAACG	−	−	−	−	>95

[a] The modifications, to the extent they are known, are the following. C modifications: Ċ- in 1a and 1b is tentatively identified as 2′*O*MeC; Ċ- in 1d is 4Me2′*O*MeC; Ċs in 1b and c are unidentified, are sensitive to pancreatic nuclease, and are probably the same, but they are distinguishable from Ċ in 5c, which is 5MeC. A modifications: all Às in 3 are N^6Me_2A; as are the second Às in 4a and b; the initial Å in 4a is unique but unidentified. G modifications: Ġ in 2 is 7MeG, identified by its being quantitatively destroyed by concentrated ammonium hydroxide at room temperature (N. Leonard, personal communication). Ġ in 5c and d is either 2MeG (most cases) or another, unidentified modification (cleaved by T_1 nuclease); Ñ in 5a and b may be a G derivative in that it is slowly cleaved by T_1 nuclease. U modifications: Ù in 4c is unidentified; it is cleaved by pancreatic nuclease at a reduced rate.

[b] Found only in *Methanosarcina* sp.

it is clear the methanogens and their relatives must ultimately be assigned a rank equivalent to that covering all typical bacteria.

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