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Evolution

Classification of methanogenic bacteria by 16S ribosomal RNA characterization

(comparative oligonucleotide cataloging/phylogeny/molecular evolution)

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ABSTRACT **The 16S ribosomal RNAs from 10 species of methanogenic bacteria have been characterized in terms of the oligonucleotides produced by T_1 RNase digestion. Comparative analysis of these data reveals the methanogens to constitute a distinct phylogenetic group containing two major divisions. These organisms appear to be only distantly related to typical bacteria.**

The methane-producing bacteria are a poorly studied collection of morphologically diverse organisms that share the common metabolic capacity to grow anaerobically by oxidizing hydrogen and reducing carbon dioxide to methane (1–3). Their relationships to one another and to other microbes remain virtually unknown. Protein and nucleic acid primary structures are perhaps the most reliable indicators of phylogenetic relationships (4–6). By using a molecule, such as the 16S ribosomal RNA, that is readily isolated, ubiquitous, and highly constrained in sequence (7), it is possible to relate even the most distant of microbial species. To date, approximately 60 bacterial species have been characterized in terms of their 16S ribosomal RNA primary structures (refs. 6–9, unpublished data). We present here results of a comparative study of the methanogens by this method, which shows their relationships to one another and to typical bacteria.

METHODS

Methanobacterium ruminantium strain *PS*, *Methanobacterium* strain *M.o.H.*, *Methanobacterium formicicum*, and *Methanosarcina barkeri* were provided by M. P. Bryant. *Methanobacterium arbophilicum* (10) was obtained from J. G. Zeikus. Two new marine isolates, Cariaco isolate JR-1 and Black Sea isolate JR-1, were provided by J. A. Romesser. *Methanospirillum hungatii* (11) and the above methanogens were cultivated in the following low-phosphate medium (values in g/liter): $(NH_4)_2SO_4$, 0.22; NaCl, 0.45; $MgSO_4 \cdot 7H_2O$, 0.09; $CaCl_2 \cdot H_2O$, 0.06; $FeSO_4 \cdot 7H_2O$, 0.002; resazurin, 0.001; sodium formate, 3.0; sodium acetate, 2.5; $NaHCO_3$, 6.0; trace mineral solution and vitamin solution (12), 10 ml each; and dephosphorylated yeast extract (Difco) and Trypticase (BBL), 2.0 each. For growth of marine isolates, NaCl was added to a final concentration of 15 g/liter. Procedures for preparation of media, growth of organisms, ^{32}P labeling, extraction of labeled 16S ribosomal RNA, and analysis of T_1 RNase digests of this RNA have been published (13–17).

The resulting oligonucleotide catalogs were examined with standard clustering techniques (18). An association coefficient for each binary couple is defined as follows: $S_{AB} = 2N_{AB}/(N_A + N_B)$, in which N_A, N_B, and N_{AB} are the total number of residues represented by hexamers and larger in catalog *A* and in catalog *B* and their overlap of common sequences, respectively. The association coefficient, S_{AB}, so defined provides what is generally an underestimate of the true degree of homology between two catalogs because related but nonidentical oligomers are not considered. The matrix of S_{AB} values for each binary comparison among the members of a given set of organisms is used to generate a dendrogram by average linkage (between the merged groups) clustering. The resulting dendrogram is, strictly speaking, phyletic because no "ancestral catalog" has been postulated. However, it is clear from the molecular nature of the data that the topology of this dendrogram would closely resemble, if not be identical to, that of a phylogenetic tree based upon such ancestral catalogs.

RESULTS

The 10 organisms whose 16S ribosomal RNA oligonucleotide catalogs are listed in Tables 1 and 2 cover all of the major types of methanogens now in pure culture except for 2; we have been unable to obtain a culture of *Methanococcus vannielii* (19), and *Methanobacterium mobile* (20) has proven difficult to grow and label. The sequences in Table 1 bear little resemblance to those for typical bacteria (refs. 6–9; unpublished data). Fig. 1 is a dendrogram derived from the S_{AB} values in Table 3. It can be seen that the methanogens comprise two major divisions. The first contains the *Methanobacterium* species; the second contains *Methanosarcina*, *Methanospirillum*, and the two marine isolates. Each division has two subgroups: group IA comprises coccobacillus-like Gram-positive rods, IB comprises long Gram-positive rods, and IIA comprises various Gram-negative forms; group IIB contains one member, a Gram-positive sarcina. Table 2 lists the post-transcriptionally modified sequences found in these RNAs. Most of the modifications are unique to the methanogens, and variations in their pattern correlate strongly with the grouping shown in Fig. 1, providing independent evidence for this grouping.

DISCUSSION

Because of their diverse morphologies and different Gram reactions, some microbiologists have considered the methanogens to be a heterogeneous group of organisms. Their scattered classification in the seventh edition of *Bergey's Manual* reflected this attitude. On this view, the commonality of their biochemistry, if it required explanation, could be rationalized in terms of a reticulate evolution, involving an appropriate plasmid. However, the above evidence indicates that this type of relationship among the methanogens is certainly not the case. The basis for classification used herein—i.e., ribosomal RNA—is

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Table 1. Oligonucleotide catalogs for 16S rRNA of 10 methanogens

Oligonucleotide sequence	Present in organism number
5-mers	
CCCCG	1–10;1,5,8
CCCAG	6
CCACG	10
ACCCG	10
CCAAG	9
CACAG	9
CAACG	1–10;8–9
ACACG	7–9
ACCAG	7
AACCG	1–10;10
ACAAG	1–6;1,5
AAACG	7–9
AAAAG	1,6,9–10
CUCCG	4,7
CCCUG	9
UCCAG	6–8,10
CUCAG	1–10
CCAUG	1–10
UCACG	1–2,4–5
UACCG	1–6,8
ACCUG	4–5;5
ACUCG	6
AUCCG	9
UAACG	4–9
CAAUG	1–6;4
ACUAG	2–3,8–9
ACAUG	10
AUACG	7
AAUCG	10
UAAAG	2
AUAAG	3–10;3,6–9;7
AAAUG	4
UUCCG	1–6,8;4
CUUCG	5–6,8
UCCUG	1–6;4
CCUUG	1
CUCUG	6,8
UCUAG	7
UUCAG	5,7–9;9
CUAUG	5
UACUG	7–10;8–10
UAUCG	7–8
ACUUG	1–6,10
AUCUG	3–5,7–8
AUUCG	2–3,10
UUAAG	1–10;1–2,4,6,8,10
UAAUG	1–2,5,10;2
AUAUG	3–4,9
AAUUG	1–10;1–2,4–6,9
AUUAG	1–10;1–7,9;7
UUUCG	4,7,9
UUCUG	3
UCUUG	8–9
CUUUG	1–3,5,10
UUUAG	2,7
UUAUG	4,9;9
UUUUG	2,9
6-mers	
CCCCAG	4,6
CCCAAG	6,10
CAACCG	8–9
ACCACG	7–9
ACACCG	6–10
AAACCG	8–10
CCCUCG	5,8,10
CCUCAG	5
CUCCAG	5
UCCCAG	2,7–10
CCACUG	4–5,9
ACCUCG	9
CCUAAG	1–3,5,10
CUCAAG	4–6
CCAUAG	4
CAUACG	1
ACACUG	4–5,7–9
AACCUG	1–6,10;1
AAUCCG	7,9–10
CUAAAG	7–9
UAAACG	1–6,8–10
ACUAAG	9
ACAAUG	1–10
AUAACG	10
AAUACG	1–6,10
AACAUG	10;10
AAACUG	1–10;8–9
AAAUCG	1–3,7
AAUAAG	1–2,4–6
CCCUUG	6,10
CCUCUG	7–9
UCCCUG	1–10
CCUUAG	4,7–8
CUCUAG	1–3
CUUCAG	9
UCCUAG	1–2
UUCCAG	1–6
CCUAUG	3
CUACUG	1–3,6
UCACUG	3,7–9
CUAUCG	7–10
UCAUCG	7,9
CAUCUG	7
ACUCUG	7–8
ACCUUG	4–6
AUCCUG	1–10
UCUAAG	7–8
UUACAG	8
UAUCAG	9
UAUACG	7
UAAUCG	1–10
AUACUG	3,7–8,10
ACAUUG	1
AACUUG	3
AAUCUG	5–9
UAAAUG	4
AUUAAG	1–8
AAUAUG	9
CCUUUG	1–2,5
CUUUCG	10
UCUCUG	1–2,4–6
UUCCUG	5
UCUUAG	5
CUAUUG	1–4,6
UUACUG	10
UAUUCG	3
AUUCUG	2,8–10
ACUUUG	2
UAUAUG	8
CUUUUG	1–5;1
UCUUUG	1,4
UUUUUG	7
7-mers	
ACCCACG	1–9
ACCACCG	7
AACCCCG	7
CCAACAG	7–8
CAACACG	1–2,5–6
CAAACCG	8–9
CCCUACG	1–10
CCCACUG	10
UCCACCG	4–6
CCACCUG	10
CCCUAAG	7–8
UCACACG	3
CUACACG	4,7–10
UAACCCG	5–6
AUACCCG	1–10
AACCUCG	8
CCUAAAG	1–6
UAACACG	1–10
AUAACCG	7
AAUCCAG	8–10
AACAUCG	10
AAAUCCG	7–9
UAAAAAG	1,3–6
CCCUUAG	1,3–6
CAUCCUG	7–10
UACUCCG	7
AUCUCCG	8
ACCUUCG	9
UCCUAAG	7
UUACCAG	1–2,4–6,10
CUAACUG	3–4
UAACUCG	1–4,7–8,10
AUUCCAG	7
AUCAUCG	6
AAUCUCG	3
AACCUUG	6
UCUAAAG	10
CUUAAAG	7–9
CAAUAUG	10
AUACUAG	1
AAUCUAG	1–2,4,7–8
AAAUCUG	10
UAAAAUG	10
CUCCUUG	1–3,5–10
UCCCUUG	9
UUCUCCG	7
CUCUUAG	2
UACUUCG	8
UACUCUG	10
UCAUAUG	10
UAAUCUG	4
AAUUUAG	3
UUCUUCG	10
UCUCUUG	7–8
CUUUAUG	10
UUUAUCG	1
UAUUUCG	1
AUUAUUG	10
UUCUUUG	4–6
UAUUUUG	3
UUUUUUG	1–3
8-mers	
CCACAACG	1–3,5–6,9–10
ACCCCAAG	1,5
AAACCCCG	9
UCCACCAG	9
CCCACAUG	7–8
CUCAACCG	8
ACCCUCAG	7
ACCACCUG	1,3–6,8,10
UAACACCG	1–6,10
AUCCCAAG	2–3
AAAUCCCG	1
CCCUCAUG	1,3–4
UACUCCCG	4
AU(CCUC)CG	5
CCUAUCAG	10
CCUAACUG	5
CUUAACCG	4,7,9
UAAUCCCG	9
CUACAAUG	1–10
UACUACAG	10
UAAUACCG	7–9
AUUACCAG	3
AUAACCUG	6–8,10

Table 1 continues on following page.

Table 1. (continued)

Oligonucleotide sequence	Present in organism number
ACAAUCUG	9
AAAUCCUG	1–2,6–9
AUAAACUG	3–6
AUAAAUAG	2
(CU,CCUU)CG	4
AUCCUUCG	4
UCUAACUG	1
CUUAACUG	2–3,5–6
UAAUCCUG	1–3,6
UCUAAAUG	1
UUAAAUCG	10
CAUAUAUG	10
AAAUCUUG	10
AAAUUCUG	2–3
AUAAAUUG	1
CUUUUCAG	6
UUCUCAUG	2
UUUAAUCG	9
UAUCAUUG	9
UUUAAAUG	2–3
UUUAAUUG	1–8,10
UUUUUUCG	2–3
UUUUAUUG	1
9-mers	
CCCACCAAG	4–5
CACACACCG	1–10
(CCA,CAA)CAG	8
CCCAACAAG	7–9
AACCCCAAG	6
AAACCCAAG	4
CCUCACCAG	8
CCUACCAAG	6
CCUACAACG	10
AUAACCCCG	6,8,10
AAACCUCCG	1–6
CACACUAAG	1–6
AUAAACCCG	6
UACUCCCAG	1–3,5–6
UAAUCCCCG	7
AAUCCCCUG	1,3–6
CUUACCAAG	1–3
(UC)ACACAUG	3
(UC)ACAAUCG	2–3
UCAUAACCG	4
CUAAUACCG	3
ACCCUUAAG	7
AUAAUCCCG	9
AUAACCCUG	1–5
AUAAUACCG	4–5
AUAUACAAG	9
UCUUACCAG	10
UCACUAUCG	6
UAAUCCCUG	10
UAAUCCUCG	8
AAUUUCCCG	10
AAUCCUCUG	2
UCAUAAUCG	1,5
CUAAUACUG	1
CAUCAUAUG	10
AUAAUUCCG	10
AAUUAUCCG	7–9
UUUAAAACG	7
UAAACUAUG	7
AUAAUACUG	2
CUAUUACUG	9
UUAAAUUCG	1
UUUAAUAAG	2
UUAUAUUCG	2
UAUUUCUAG	9
UUUAUUAAG	1
CUUUUAUUG	6
UUUUUAUUG	2,4
UUUUUUUCG	1
10-mers	
AAUAACCCCG	7
ACCACCUAUG	9
AAUCUCACCG	8
AAAUCUCACG	4
UAACUCAAAG	8
AAACUUAAAG	1–10
ACCUUACCUG	10
UUACCAUCAG	3
UACCUACUAG	10
AAUCACUUCG	5
AACCCUUAUG	6
UAAAUAACUG	9
UUCUUCACCG	6
ACUCUACUUG	9
CUUAACUAUG	1
AUACUAUUAG	2,4–5
UUCCCUAUUG	4
UCUUCUUAAG	4
AUUUUUUUCG	1
UUUUCUUUUG	5
11-mers	
ACAACUCACCG	10
AAAUCCCACAG	6
CAUCUCACCAG	7,9
UAACUCACCCG	9
AAAUCUCACCG	7,9
AAACACCUUCG	6
AAAUCCCAUAG	5
UCCCUCCCCUG	10
CAUAUCCUCCG	10
AAAUCCUAUAG	3
UUUCAACAUAG	7,9
A(UA,UCA,CUA)UG	6
UUUCAAUAUAG	10
CUUUUCUUAAG	1,3
CUUUUCAUUAG	2
UUCUUUAAUCG	7
UUUUUUUCCUG	1
UUUUUUUUAAG	2
12-mers	
CCACCCAAAAAG	1–2,4,6
UCAAACCACCCG	8–10
UCAAACCAUCCG	7
ACAUCUCACCAG	1–6
CCACUCUUAACG	4–6
CCAUUCUUAACG	1–3
CUCAACUAUUAG	10
CCACUAUUAUUG	7
CAAUUAUUCCUG	2
CCACUUUUAUUG	8
CCAUUUUUAUUG	5
(CUA,CUUUUA)UUG	3
13-mers	
UAAACUACACCUG	10
(CAA,CCA)CAUUCUG	6
UAAUACUCCAUAG	9
UUUCAAAAUAACG	8
AUAAUUUUUCCUG	3
(UUU,CUU,CU)AAAUG	5
14-mers	
AAAACUUUACCAUG	9
AAAACUUUACAAUG	7–8,10
AUUUUU(CCU,CU)UUG	2
15-mers	
UCUAAAAACACACCUG	8
AUAACCUACCCUUAG	1–3
AUAACCUAACCUUAG	4
AAUAAUACCCUAUAG	8
AAUAAUACUCCAUAG	7
AUAAUCUACCCUUAG	5
16-mers	
UAAUCCCCUAAACCAG	6
AAAUCCUAUAAUCCCG	4
AAUCUCCUAAACAUAG	5
CAAUCUCUUAAACCUG	7
UAAUCUCCUAAACCUG	4
AAAUCCUAUAAUCCUG	5
17-mers	
CAAUCUUUUAAACCUAG	3
UAAU(CCU,CU)AAACUUAG	1–2
AUAAU(CCU,CU)AAACCUG	9
18-mer	
AACAAUCUCCUAAACCUG	8
24-mer	
(AAACA,UAAUCUCA)— CCCAUCCUUAG	10
termini	
5′ end	
pAG	4,6
pAAUCCG	5
pAAUCUG	1,3
pAUUCUG	2,7–10
3′ end	
$AUCACCUCCU_{OH}$	1–10

First column is oligonucleotide sequence; second column shows organisms in which that sequence is found. Organisms are designated by number (see Fig. 1) as follows: 1, *M. arbophilicum;* 2, *M. ruminantium* strain *PS;* 3, *M. ruminantium* strain *M-1;* 4, *M. formicicum;* 5, *M.* sp. strain *M.o.H.;* 6, *M. thermoautotrophicum;* 7, Cariaco isolate JR-1; 8, Black Sea isolate JR-1; 9, *Methanospirillum hungatii;* 10, *Methanosarcina barkeri.* Multiple occurrences of a sequence in a given organism are denoted by repeating the organism's number in column 2: e.g., *1–4,6–8;3,7,,3* signifies a double occurrence in organism 7 and a triple occurrence in organism 3.

independent of particular biochemistries and, as representative of the cellular information processing systems, should be considered idionomonic of the organism. By means of this approach we have shown not only that methanogens are a coherent phylogenetic grouping but also that they are quite distinct from other bacteria as well. Just how distinct they may be is indicated in Fig. 1; even enterics and blue-green algae appear closely related by comparison.

Table 2. Post-transcriptionally modified sequences and likely counterparts

Sequence	Occurrence in methanogens IA	IB	IIA	IIB	Occurrence in typical bacteria
1. ÅÅCCUG	+	+	−	−	30%
AÅUCUG	−	−	+	+	None
AÅG	−	−	−	−	55%
2. UÅÅCAAG	+	+	−	−	None
UAÅCAAG	−	−	+	+	None
UAACAAG	−	−	−	−	>95%
3. AUṄCAACG	+	+	−	−	None
ACṄCAACG	−	−	+	+	None
AXĠĊAACG	−	−	−	−	>90%
4. ṄCCG	+	+	−	−	None
C(Ċ,C)G	−	−	+	+	None
N′CCG	−	−	−	−	>95%
5. CCĊCG	−	−	−	+	>95%

Post-transciptionally modified sequences in methanogens and their likely counterparts in the bacteria that have been examined. In group 1, Å is *N*-6-diMe (21), identified by electrophoretic mobilities of Å and ÅÅ and by total resistance to U_2 nuclease. In group 2, U̇ is partially resistant to pancreatic nuclease, the first Å when modified is still U_2 nuclease sensitive; the second Å is *N*-6-diMe. Ṅ in group 3 is resistant to pancreatic nuclease but is electrophoretically U-like. X stands for U or A. In group 4, Ṅ and Ṅ′ are not cleaved by endonucleases; ṄC and Ṅ′C are electrophoretically distinguishable; Ċ is cleaved by pancreatic nuclease and has C-like electrophoretic properties. In group 5, Ċ (21, 22) is not cleaved by pancreatic nuclease and is readily deaminated by NH_4OH.

A phylogenetic distinction of this apparent magnitude is suspect unless substantiated by other evidence. In fact, a distinction of this magnitude reasonably demands that there be many and striking differences in corresponding phenotypes. Consider the following points.

(*i*) Methane production involves a highly unique biochemistry. In probing its details, the biochemist is beginning to uncover an unusual spectrum of coenzymes. For example, coenzyme M, involved in methyl transfer in methane formation, is the smallest of all known coenzymes; it is unique in its sulfur content and acidity (24). One of us (W.E.B.) has examined a wide variety of tissues and organisms for the presence of this cofactor and found it to be confined to the methanogens. Similarly, coenzyme F_{420}, which handles low-potential electrons, is present in all methanogens but so far is not found elsewhere (25).

(*ii*) We have been unable to detect cytochromes in these organisms, and R. Thauer obtained no evidence for the presence of quinones in *M. thermoautotrophicum* (personal communication). The extent to which their overall biochemistry is unique remains to be determined.

(*iii*) All other bacterial cell walls so far examined, with the single exception of the extreme halophiles, contain peptidoglycan (26, 27). However, cell walls of the methanogens (eight examples) do not contain this compound (ref. 28; O. Kandler, personal communication).

(*iv*) Table 2 shows that the pattern of base modification in 16S ribosomal RNA in methanogens is, for the most part, different from that in typical bacteria. This holds for the 23S rRNA as well (D. Stahl, personal communication). Moreover, methanogens are the first major group of organisms characterized (prokaryote or eukaryote) whose tRNAs lack the so-called "common sequence," TΨCG. Division I methanogens contain a ΨΨĊG sequence, whereas in division II it becomes U̇ΨĊG (the dot above a base signifies an unidentified modification; U̇ ≠ T) (L. Magrum and D. Stahl, unpublished data).

It should be noted that three of these four points appear to be completely unrelated to the production of methane or to the requirement of a strictly anaerobic niche. These differences become the more impressive when it is realized that methanogens have been characterized but little in terms of their general biochemistry and molecular biology, and not at all genetically. It would appear that methanogens ultimately may have to be classified as a systematic group distinct from other bacteria (inclusive of the blue-green algae).

Although it cannot be unequivocally concluded that methanogens represent the most ancient divergence yet encountered in the bacterial line of descent, the possibility is certainly likely. How ancient, then, could the methanogenic phenotype be? It may well be older than the blue-green algal one, which fossil evidence suggests to be close to 3 billion years (29). On the assumption that equivalent S_{AB} values measure the same physical time, the most ancient divergence within the methanogens proper ($S_{AB} \sim 0.25$) is comparable to that which separates blue-green algae from most of the other bacteria (Fig. 1). Methanogens might then have existed at a time when an anaerobic atmosphere, rich in carbon dioxide and hydrogen, enveloped the planet and, if so, could have played a pivotal role in this planet's physical evolution.

Note Added in Proof: Preliminary characterization of *Methanobacterium mobile*, a motile, Gram-negative, short rod, places this organism in group IIA. *Methanobacterium* sp. strain AZ (30) has been shown to be a strain of *M. arbophilicum*; $S_{AB} = 0.87$ for the pair.

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Table 3. S_{AB} values for each indicated binary comparison

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *M. arbophilicum*	—												
2. *M. ruminantium PS*	.66	—											
3. *M. ruminantium M-1*	.60	.60	—										
4. *M. formicicum*	.50	.48	.49	—									
5. *M.* sp. *M.o.H.*	.53	.49	.51	.60	—								
6. *M. thermoautotrophicum*	.52	.49	.51	.54	.60	—							
7. Cariaco isolate JR-1	.25	.27	.25	.26	.23	.25	—						
8. Black Sea isolate JR-1	.26	.28	.26	.28	.27	.29	.59	—					
9. *Methanospirillum hungatii*	.20	.24	.21	.23	.23	.22	.51	.52	—				
10. *Methanosarcina barkeri*	.29	.26	.24	.24	.26	.25	.33	.41	.34	—			
11. Enteric-vibrio sp.	.08	.08	.11	.09	.09	.10	.05	.06	.07	.10	—		
12. *Bacillus* sp.	.10	.10	.14	.11	.11	.12	.08	.10	.10	.08	.27	—	
13. Blue-green sp.	.10	.10	.10	.10	.10	.11	.08	.09	.08	.11	.24	.26	—

The values given for enteric-vibrio sp., *Bacillus* sp., and blue-green sp. represent averages obtained from 11 (9), 7 (6), and 4 (23) individual species, respectively.

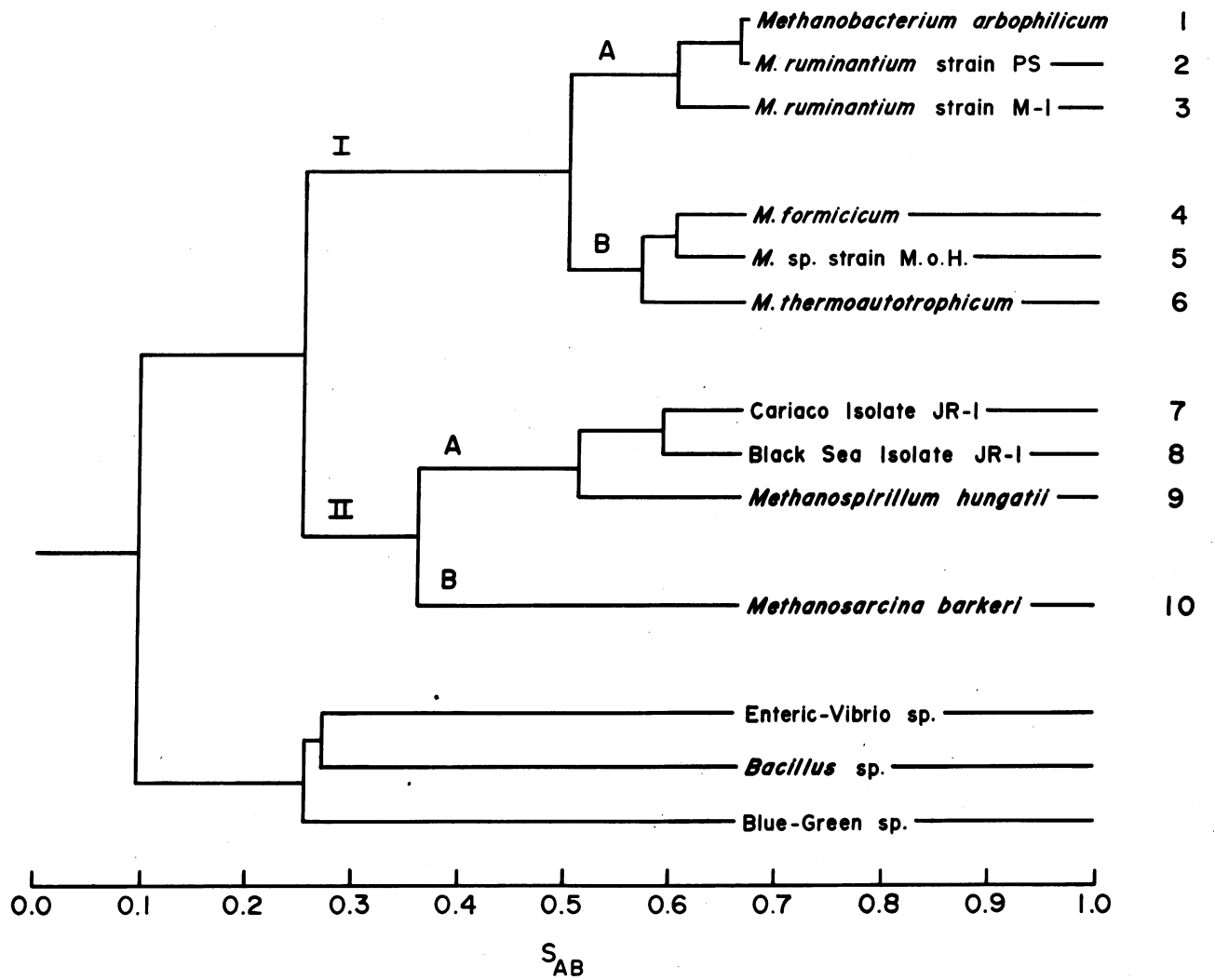

FIG. 1. Dendrogram of relationships of methanogens and typical bacteria. The figure was constructed by average linkage clustering (between the merged groups) from the S_{AB} values given in Table 3.

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