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An Ancient Divergence among the Bacteria

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Summary. The 16S ribosomal RNAs from two species of methanogenic bacteria, the mesophile *Methanobacterium ruminantium* and the thermophile *Methanobacterium thermoautotrophicum*, have been characterized in terms of the oligonucleotides produced by digestion with T_1 ribonuclease. These two organisms are found to be sufficiently related that they can be considered members of the same genus or family. However, they bear only slight resemblance to "typical" Procaryotic genera; such as *Escherichia, Bacillus* and *Anacystis.* The divergence of the methanogenic bacteria from other bacteria may be the most ancient phylogenetic event yet detected – antedating considerably the divergence of the blue green algal line for example, from the main bacterial line.

Key words: Comparative cataloging – Methanogenic bacteria – Phylogeny – 16S ribosomal RNA – Evolution

Introduction

Bacteria clearly played a central role in shaping and reflecting the environmental parameters of the primitive Earth. An understanding of their phylogeny is essential to any attempt to reconstruct the primitive scenario. Without such a background our concept of Precambrian evolution must remain a purely speculative one.

The structure of the bacterial phylogenetic tree is not readily revealed by comparative biochemistry or morphology. Although some progress has been made the general outline of the tree remains largely unknown. In addition, what information is available is primarily qualitative and is thus of limited value to the molecular paleontologist. However, the approach pioneered by Zuckerkandl and Pauling (1965), the reading of

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the historical trace retained in macromolecular sequence, holds great promise for the bacteria, in spite of their antiquity and in spite of the fact that they possess a relatively rapid biological clock, i.e., they fix mutations at a relatively rapid rate (Drake, 1974). All bacteria contain macromolecules, the constraints on whose primary structures are sufficiently complex that evidence of extremely ancient events are still retained therein.

It was suggested several years ago that the ribosomal RNAs might be the proper "semantides" through which to approach bacterial phylogeny (Sogin et al., 1972). Not only is the translation function ubiquitous, but ribosomal RNAs appear functionally equivalent (interchangeable) over a wide range of bacteria (Nomura et al., 1968; Lee and Evans, 1971; Bellemare et al., 1973; Wrede and Erdman, 1973), implying considerable sequence homology. The conjecture has proven correct.

To date, approximately 40 bacterial species including at least one representative of each of the following genera; *Escherichia, Aeromonas, Vibrio, Acinetobacter, Alcaligenes, Pseudomonas, Rhodopseudomonas, Rhodospirillum, Chromatium, Bacillus, Corynebacterium, Micrococcus, Lactobacillus, Clostridium, Mycoplasma, Acholeplasma, Anacystis, Aphanocapsa* have been characterized by comparative cataloging of their 16S rRNAs. This sample is broadly representative of the bacteria, although not exhaustively so. All of these ribosomal RNAs exhibit considerable sequence homology despite the antiquity of their divergences (Woese et al., 1975).

In the present communication 16S rRNA catalogs are reported for two species of the methanogenic bacteria, the mesophile *Methanobacterium ruminantium* and thermophile *Methanobacterium thermoautotrophicum.* These anaerobes are of particular interest to the evolutionist because of their unique energy metabolism – the generation of methane from carbon dioxide and hydrogen. In that the latter two gases are considered to have been prevalent in the early Precambrian atmosphere these organisms could represent an ancient divergence in the line of bacterial descent.

Materials and Methods

Methanobacterium ruminantium strain *M1* was obtained from M.P. Bryant, Department of Dairy Science, University of Illinois, and cultivated in the following low-phosphate medium (values in g/l): $(NH_4)_2SO_4$, 0.45; NaCl, 0.9; $MgSO_4 \cdot 7H_2O$, 0.18; $CaCl_2 \cdot 2H_2O$, 0.12; $FeSO_4 \cdot 7H_2O$, 0.002; resazurin, 0.001; sodium formate, 2.0; sodium acetate, 2.5; $NaHCO_3$, 2.5; L-cysteine $\cdot H_2O$, 0.5; $Na_2S \cdot 9H_2O$, 0.5; 2-mercaptoethane sulfonic acid, 0.0001; and dephosphorylated yeast extract (Difco) and Trypticase (BBL), 2.0 each. Isobutyric, α-methyl butyric, isovaleric, and valeric acids were each added at a final concentration of 0.05 % (v/v); Tween 80 was added at 0.002 %. *Methanobacterium thermoautotrophicum* (Zeikus and Wolfe, 1972) was cultivated in the following phosphate-free medium (values in g/l): $(NH_4)_2SO_4$, 0.15; NaCl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.06; $CaCl_2 \cdot 2H_2O$, 0.04; $FeSO_4$ $(NH_4)_2SO_4 \cdot 6H_2O$, 0.02; NH_4Cl, 1.25; resazurin, 0.001; $NaHCO_3$, 7.5; L-cysteine $\cdot H_2O$, 0.6; $Na_2S \cdot 9H_2O$, 0.6; trace mineral solution, 10.0 ml and vitamin solution (Wolin et al., 1963), 10.0 ml. Tween 80 was added at a final concentration of 0.002 %.

Media were prepared in a 500 ml round bottom flask under an 80 % nitrogen and 20 % carbon dioxide gas mixture by the Hungate technique as modified by Bryant and

Robinson (1961). Organisms were grown under a 80 % hydrogen and 20 % carbon dioxide gas mixture at 3 atmospheres pressure in 125 ml serum bottles as described by Balch and Wolfe (1976). To a 15 ml culture in early log phase, ^{32}P ortho-phosphate in a 0.3 % L-cysteine · H_2O – 0.3 % Na_2S · $9H_2O$ solution under a nitrogen gas phase was introduced anaerobically at a final concentration of 0.5 – 1.0 mc/ml.

After 3-5 generations in log phase growth the cells were harvested by centrifugation. Cell lysis was effected by passage through a French press at 10,000 psi. The 16S rRNA was obtained by direct phenol extraction of the lysate (Gierer and Schramm, 1956) followed by electrophoresis on a 3.6 % polyacrylamide gel (Bishop et al., 1967), the 16S rRNA band being located by radioautography and removed from the appropriate gel slice by electrophoresis (Luehrsen, unpublished). A final desalting on Whatman Chromedia CF-11 (Doolittle and Pace, 1971) was performed.

Two dimensional electrophoretic fingerprints of ribonuclease T_1 digests of each 16S rRNA were prepared according to the method of Sanger and coworkers with modifications introduced by this laboratory (Sanger et al., 1965; Uchida et al., 1974). The oligonucleotides were not dephosphorylated. Secondary and tertiary characterizations leading to sequence of individual spots on the primary fingerprint were done by the methods of Uchida et al. (1974) and Woese et al. (1976).

Results and Discussion

Table 1 lists the catalogs of oligonucleotide digestion products for the two 16S rRNAs, indicating in addition the presence or absence of each sequence in any of several large groups of "typical" bacteria.

Table 1. List of oligonucleotides found in *M. ruminantium.* (column 1) and *M thermoautotrophicum* (column 2). Their occurrence in *Bacillus* species, seven examples, (column 3); blue green algae, four species, (column 4); and *Enteric-Vibrio* species, eleven examples, (column 5) is indicated by a + (present in $\gtrsim$ 80%), a ± (present in 30–50%) or a – (present in < 30%). Uncertainty as to the number of occurrences of a particular oligonucleotide are indicated by 0-1, 1-0, 1-2, etc., where the first number of the couple should be taken as the more likely. Sequence uncertainties are enclosed in brackets

pentamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species
CCCCG	1	1	+	+	+
CCCAG	0	1	+	+	+
CACCG	1	0-1	+	+	+
CAACG	1	1	+	+	+
AACCG	1	1	+	+	+
ACAAG	1	1	+	+	+
AAAAG	0-1	1-2	+	+	+
CUCCG	0-1	0	[+]	+	+
UACCG	1	1	+	+	+
UCCAG	0	1	+	+	+
CUCAG	1	1	+	+	+
CCAUG	1	1	+	+	+
ACUCG	0	1	+	+	+
UAACG	1	1	+	+	+
CAAUG	1	0	+	+	+
ACUAG	1	2	+	+	+
AUAAG	2	0	+	+	+
UUCCG	1	1	+	+	+
UCCUG	1	1	+	+	+
CUCUG	0	1	+	+	+
CUUCG	1	1	+	+	+
ACUUG	1	1	+	+	+
AUUCG	1	0	+	+	+
AUCUG	1	0	+	+	+
UUAAG	2	2	+	±	±
AUUAG	2	2	+	±	±
AAUUG	1	0	+	+	+
AUAUG	1	0	+	+	+
UUCUG	1	0	+	+	+
CUUUG	1	0	+	+	+

Table 1 (continuation)

nonamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species
CACACACCG	1	?	-	-	-
AACCCCAAG	0	1	-	-	-
Unsequenced	0	1			
CCUACCAAG	0	1	-	-	-
AUAACCCCG	0	1	-	-	-
AAACCUCCG	1	1	-	-	-
CACACUAAG	1	1	-	-	-
AUAAACCCG	0	1	-	-	-
UACUCCCAG	1	1	-	-	-
AAUCCCCUG	1	1	-	-	-
CUUACCAAG	1	0	-	-	-
CUACACAUG(?)	1	0	-	-	-
CUAAUACCG	1	0	+	±	+
(UC)ACAAUCG	1	0	-	-	-
AUAACCCUG	1	0	-	-	-
UCACUAUCG	0	1	-	-	-
CUUUUAUUG	0	1	-	-	-

decamer					
[CCA,CAA,CA]CG	1	?	-	-	-
AAACUUAAAG	1	1	-	-	-
UUACCAUCAG	1	0	-	-	-
AACCCUUAUG	0	1	-	-	-
UUCUUCACCG	0	1	-	-	-

undecamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species
AAAUCCCACAG	0	1	-	-	-
AAACACCUUCG	0	1	-	-	-
AAAUCCUAUAG	1	0	-	-	-
A[UA,UCA,CUA]UG	0	1	-	-	-
CUUUUCUUAAG	1	0	-	-	-

duodecamer and larger					
ACAUCUCACCAG	1	1	-	-	-
CCACUCUUAACG	0	1	-	-	-
CCAUUCUUAACG	1	0	-	-	-
[CUA,CUUUUA]UUG	1	0	-	-	-
UAAUUUUUCCUG(?)	1	0	-	-	-
[CAA,CCA]CAUUCUG	0	1	-	-	-
AUAACCUACCCUUAG	1	0	-	-	-
UAAUCCCCUAAACCAG	0	1	-	-	-
CAAUCUUUUAAACCUAG	1	0	-	-	-

post transcriptionally modified	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species
*NCCG	1	1	-	-	-
**AACCUG	1	1	-	-	+
**UAACAAG	1	1	-	-	-
*AUNCAACG	1	1	-	-	-
5' terminus					
pAAUCUG	1	0	-	-	-
pAG	0	1	-	-	-
3' terminus					
AUCACCUCCU OH	1	1	-	-	-

Table 1 (continued)

hexamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species	heptamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species	octamer	M. ruminantium	M. thermoautotrophicum	Bacillus species	Blue-green species	Enteric-Vibrio species
CCCCAG	0	1	+	+	+	ACCCACG	1	1	+	+	+	UAACACCG	1	1	+	+	+
CCCAAG	0	1	+	+	+	[CA,CAA]CG	0	1	+	+	+	ACCACCUG	1	1	+	+	+
AACCCG	0	0-1	+	+	+							AUCCCAAG	1	0	+	+	+
ACACCG	0	1	+	+	+	UCCACCG	0	1	+	+	+						
						CCCUACG	1	1	+	+	+	CCCUCAUG	1	1	+	+	+
CUCAAG	1	1	+	+	+	UCACACG	1	0	+	+	+	CUACAAUG	1	1	+	+	+
AACCUG	1	1	+	+	+	UAACCCG	0	1	+	+	+	AUUACCAG	1	0	+	+	+
UAAACG	1	1	+	+	+	AUAGCCG	1	1	+	+	+	AUAACCUG	0	1	+	+	+
AAUACG	1	1	+	+	+	UAACACG	1	1	+	+	+	AAAUCCUG	0	1	+	+	+
ACAAUG	1	1	+	+	+	CCUAAAG	1	1	+	+	+	AUAAACUG	1	1	+	+	+
AAACUG	1	1	+	+	+	UAAAAAG(?)											
AAAUCG	1	0	+	+	+							UAAUCCUG	1	1	+	+	+
AAUAAG	0	1	+	+	+	CCCUUAG	1	1	+	+	+	CUUAACUG	1	1	+	+	+
						UUACCAG	0	0	+	+	+	AAAUUCUG	1	0	+	+	+
UCCCUG	1	1	+	+	+	UAACUCG	1	0	+	+	+						
CCCUUG	0	1	+	+	+	CUAACUG	1	0	+	+	+	CUUUUUCAG	0	1	+	+	+
UUCCAG	1	1	+	+	+	AUCAUCG	0	1	+	+	+	UUUAAAUG	1	0	+	+	+
CUCUAG	1	0	+	+	+	AACCUUG	0	1	+	+	+						
UCACUG	1	0	+	+	+	AAUCUCG	1	0	+	+	+	UUUAAAUUG	1	1	+	+	+
CUACUG	1	1	+	+	+												
CCUAUG	1	0	+	+	+	CUCCUUG	1	1	+	+	+	UUUUUUUCG	1	0	+	+	+
AUCCUG	1	1	+	+	+	AAUUUAG	1	0	+	+	+						
ACCUUG	0	1	+	+	+												
UAAUCG	1	1	+	+	+	UUCUUUG	0	1	+	+	+						
AUACUG	1	0	+	+	+	UAUUUUG	1	0	+	+	+						
AACUUG	1	0	+	+	+												
AAUCUG	0	1	+	+	+	UUUUUUG	1	0	+	+	+						
AUUAAG	1	1	+	+	+												
UCUCUG	0	1	+	+	+												
UAUUCG	1	0	+	+	+												
CUAUUG	1	1	+	+	+												
CUUUUG	1	0	+	+	+												

Although there are not many sequences in Table 1 common to the 16S rRNAs of the methanogenic bacteria and other bacterial 16S rRNAs, their number is sufficient to demonstrate a primary structural relationship (Pechman and Woese, 1972). In addition, the following points should be noted: 1) the 3′ terminal segment, $G/AUCACCUCCU_{OH}$ is of the general form $G/AUCACCUCCUN_X$ common to most bacterial 16S rRNAs (where N_X stands for $0-5$ additional pyrimidine residues, sometimes terminating in A_{OH}) (Shine and Dalgarno, 1975; Woese et al., 1975; Woese et al., unpublished). 2) Those sequences (>5 nucleotides) common to one methanogenic species and one or more of the other bacterial groups tend to be found in the second methanogenic species as well. And 3) although not indicated in the table, all of the post-transcriptionally modified oligonucleotides found in methanogenic bacteria appear to have counterparts in the 16S rRNA catalogs of the other bacteria. $\overset{*}{N}CCG$ in the methanogenic bacteria appears to correspond to the sequence $\overset{*}{G}CCG$ reported in all other bacteria (Fellner and Sanger, 1968; Woese et al., 1975). $UAA\overset{*}{C}AAG$ has a $\overset{*}{U}AACAAG$ counterpart in other bacteria (Woese et al., 1975), while $AU\overset{*}{N}CAACG$ may have the counterpart $AU\overset{*}{G}CAACG$. (There exist uncertainties in the *E. coli* 16S rRNA sequence regarding the placing of $AU\overset{*}{G}$ in relation to CAACG.)

The catalogs of the two methane producers do bear a strong resemblance to one another. Their binary relatedness coefficient (Fox et al., 1977; Bonen and Doolittle, 1976) is 0.49, a value one observes for distantly related members within an ancient genus or family.

The extent of relatedness to other bacterial groups can be seen in Figure 1, a phylogenetic tree constructed from the binary association coefficients of the organisms included in Table 2. It is clear that the methane producers stand quite apart from these otherwise diverse bacterial groups. This can also be seen in Table 1, where among oligonucleotides hexamer and larger, there occur no more than 6 sequence coincidences between a methane producer and any of the bacterial groups. This number of coincidences exceeds twelve for any binary comparison not involving a methanogenic species.

In view of these results the evolutionary divergence between the methanogenic bacteria and the procaryotes appears to be extremely ancient—antedating any others so far detected. Unfortunately, for the present this divergence cannot be readily

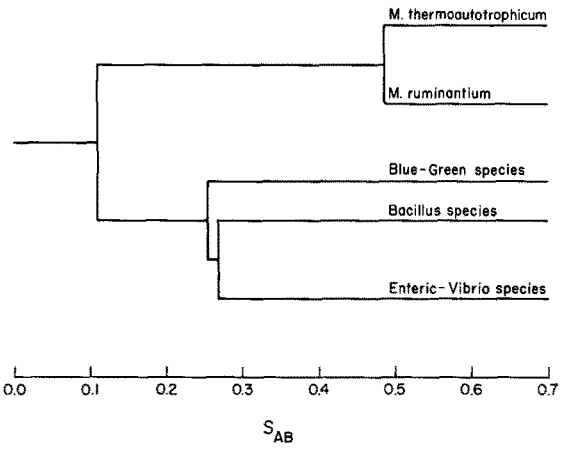

Fig. 1. A dendrogram obtained by average linkage clustering is shown for the two methanogenic bacteria and the three groups of organisms included in Table 1. The binary association coefficient, S_{AB}, has been introduced previously (Fox et al., 1977; Bonen and Doolittle, 1976). Briefly, it is the ratio of twice the number of nucleotide residues contained in identical oligomers between two catalogs (of size 6 or larger) to the total number of nucleotide residues represented by oligomers 6 and larger in two combined catalogs

related to real time. However, it is generally accepted that rocks over 2.5 billion years old show evidence of organisms that seem to be blue-green algae (Schopf, 1972) i.e. the bluegreen divergence from the bacterial line of descent should be at least this old.

The phylogeny of methanogenic bacteria is not yet sufficiently explored to speculate beyond this point. If their phenotype were indeed an ancient one (i.e., it does not represent a "recently" evolved biochemical complexion) then the group itself would be expected to contain some ancient divergence – divergences represented by relatedness coefficients considerably lower than those seen between *M. ruminatium* and *M. thermoautotrophicum.*

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