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Taxonomic Relations between Archaebacteria Including 6 Novel Genera Examined by Cross Hybridization of DNAs and 16S rRNAs

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Summary. DNAs from 16 species of archaebacteria including 6 novel isolates were hybridized with 16S rRNAs from 7 species representing different orders or groups of the urkingdom of archaebacteria.

The yields, normalized for the number of genes per μg of DNA, and the temperature stabilities of all hybrids were determined and related to each other.

A taxonomic tree constructed from such fractional stability data reveals the same major divisions as that derived from comparative cataloging of 16S rRNA sequences. The extreme halophiles appear however as a distinct order besides the three known divisions of methanogens.

The methanogens, the halophiles and *Thermoplasma* form one of two clearly recognizable branches of the archaebacterial urkingdom. The order represented by *Sulfolobus* and the related novel order *Thermoproteales* form the other branch.

Three novel genera, *Thermoproteus, Desulfurococcus* and the "stiff filaments" represent three families of this order.

The extremely thermophilic methanogen *Methanothermus fervidus* belongs to the *Methanobacteriales*. SN1, a methanogen from Italy, appears as another species of the genus *Methanococcus*. Another novel methanogen, M3, represents a genus or family of the order *Methanomicrobiales*.

Key words: Archaebacteria – Taxonomy – Evolution – DNA – 16S rRNA – Hybridization – Phylogeny – *Thermoproteales*

Introduction

Three novel extremely themophilic, anaerobic, sulfur respiring types of organisms isolated from Icelandic Solfataras, represented by 1. the genus *Thermoproteus*, 2. the related "stiff filaments" (both Zillig et al. 1981a) and 3. the genus *Desulfurococcus* (Zillig et al. 1981b), have been recognized as archaebacteria (Woese et al. 1978; Fox et al. 1980). The component patterns of the DNA dependent RNA polymerases of *Thermoproteus* and *Desulfurococcus* (Zillig et al. 1981c) and the comparison of the T1-RNAase oligonucleotide catalogs of the 16S rRNAs (Fox et al. 1977) from *Thermoproteus* and *Sulfolobus* (E. Stackebrandt, C.R. Woese and W. Zillig, unpublished) yield evidence for a relation between the novel thermoacidophiles and *Sulfolobus* (Brock et al. 1972) though their exact taxonomic position within the urkingdom of the archaebacteria and their relation with each other remain to be examined.

As indicated by the nature of its envelope and by its T1-RNAase oligonucleotide catalog, the extremely thermophilic methanogen *Methanothermus fervidus*, also isolated from an Icelandic hot spring (Stetter et al. 1981) is related to *Methanobacterium*. Its DNA dependent RNA polymerase shows however no immunochemical cross reaction with an antibody against the enzyme from *Methanobacterium*. Therefore, an independent determination of the phylogenetic distance between *Methanothermus* and *Methanobacterium* appeared instructive.

The taxonomic positions of two novel methanogens, SN1 and M3, isolated from Italian sources are yet undetermined.

The method applied in establishing the urkingdom of the archaebacteria, comparative cataloging of 16S rRNA sequences (Fox et al. 1977), would require the determination of 6 new oligonucleotide catalogues.

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Table 1. List of the species of archaebacteria from which DNAs and RNAs ($^{+}$) were obtained, their sources and culture conditions, and the buffers and lysis procedures used in the preparation of nucleic acids

Species	Strain, designation, culture collection, source	Culture medium and conditions	Wash buffer	Lysis procedure		
Methanococcus voltae^{+}	PS, DSM 1537	Balch et al. 1979 medium 3, pH 6.9	salt basis of medium	1% SDS,	50 mM Tris 20 mM NaCl	pH 7.8
SN1	SN1, Stufe die Nerone, Italy	Balch et al. 1979 medium 3, pH 6.9	salt basis of medium	1% SDS,	50 mM Tris 10 mM EDTA	pH 8.0
Methanobacterium thermoautotrophicum^{+}	Δ H, R.S. Wolfe, Urbana	Balch et al. 1979 medium 2, pH 7.0	salt basis of medium	glass beads	50 mM Tris 20 mM NaCl	pH 7.8
Methanothermus fervidus	V24S, Hveradalir, Iceland DSM 2088	Stetter et al. 1981	salt basis of medium	french press	50 mM Tris 10 mM EDTA	pH 8.0
Methanogenium marinigris^{+}	JRI, DSM 1498	Balch et al. 1979 medium 3, pH 6.9	salt basis of medium	1% SDS	50 mM Tris 20 mM NaCl	pH 7.8
Methanosarcina barkeri	MS, R.S. Wolfe, Urbana	Balch et al. 1979 medium 3, pH 6.9		1% SDS,	50 mM Tris 10 mM EDTA	pH 8.0
M3	M3					
Halobacterium halobium^{+}	RI, D. Oesterhelt, Martinsried	Oesterhelt and Stoeckenius 1974	salt basis of medium	0.5% SDS	TA buffer	pH 7.5
Halococcus morrhuae	DSM 1309	Reistad 1970		glass beads	TA buffer,	pH 7.5
Thermoplasma acidophilum^{+}	122-1B2, DSM 2978 E.A. Freundt, Aarhus	Darland et al. 1970	0.05 M sodium acetate pH 5	0.5% SDS,	0.05M sodium acetate	pH 5
Sulfolobus acidocaldarius^{+}	DSM 639	Brock et al. 1972	salt basis of Brock's medium pH 5.5	0.05% triton X 100	TA buffer	pH 7.5
Sulfolobus spec.$^{+}$	B6, Beppu, Japan	Brock et al. 1972 + 1% S_8	salt basis of Brock's medium pH 5.5	0.05% triton X 100	TA buffer	pH 7.5
Thermoproteus tenax^{+}	Kra 1, Krafla, Iceland	Zillig et al. 1981		0.5% SDS,	TA buffer	pH 7.5
"Stiff filaments"	Hvv 3, Hveravellir, Iceland	Zillig et al. 1981		0.5% SDS,	TA buffer	pH 7.5
Desulfurococcus mucosus	O7, Askja, Iceland	Zillig et al. 1981b		0.05% triton X 100	TA buffer	pH 7.5

A fast and relatively simple procedure for the determination of phylogenetic distances, based on cross hybridization of labelled 16S rRNAs with filter bound DNAs has been described by De Ley and De Smedt (1975).

In this paper a phylogenetic tree of the archaebacteria containing all above mentioned novel genera is derived from cross hybridization data. On this basis the taxonomic positions of the three novel sulfur respiring types of thermophiles and the three new methanogens have been elucidated. The significance of the differences of this tree from that based on comparative cataloging data shall be discussed.

Materials and Methods

Organisms

The strain designations, culture collection designations and/or sources of all organisms, from which DNAs and 16S rRNAs have been prepared, and the media used for mass cultures are listed in Table 1.

Preparation of DNAs

5 to 10 g of bacterial mass (wet weight) were suspended in 25 to 50 ml of the buffers listed in column 5 of Table 1 and lysed as indicated there. TA buffer is 0.05M tris HCl, 0.01M EDTA; 0.01M β mercaptoethanol, 0.022M NH_4Cl pH 7.5.

The DNA was prepared from the crude extracts by a phenol procedure as described previously (Zillig et al. 1980). All DNAs were banded by CsCl equilibrium gradient centrifugation.

Preparation of 16S rRNAs

For the preparation of ^{32}P labelled 16S rRNAs, 20 to 160 ml cultures of the 8 organisms marked with a star in column 1 of Table 1 were grown in the media listed in column 3 of Table 1 except that normal yeast extract was replaced by low phosphate yeast extract containing 20 nmoles phosphate per g. The latter was prepared by precipitation of phosphate with a slight excess of magnesium sulfate at pH 10.5. If required the final phosphate concentration was adjusted to $2x10^{-5}M$ by addition of free phosphate. 25 μC per ml of carrier free orthophosphate (New England Nuclear or Amersham Buchler, Braunschweig) were added after inoculation of the medium. Cells were harvested in the late exponential or the stationary phase of growth about 2 to 3 days after inoculation by 15 minutes centrifugation at

Table 2. Temperatures of half melting (= TMs), cpm/µg DNA and correction factors for the calculation of fractional yields from these of all hybrids of 14 DNAs with 7 16S rRNAs of different archaebacteria. The values in brackets in the *Sulfolobus* column are for the annealing of the DNA from *Sulfolobus* acidocaldarius DSM 639 with the homologuous RNA

RNA from	*Methanococcus*		*Methanobacterium*		*Methanogenium*		*Halobacterium*		*Thermoplasma*		*Sulfolobus* B6		*Thermoproteus*		Divisions for calculation of fractional yields
DNA from	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	TM°C	cpm/µg DNA	
Methanococcus	78.2	1015	73.1	50	71.7	60	67.1	354	67.6	254	71.7	36	72.5	26	1015
SN1	77.1	276	75.8	57	69.7	37	68.4	239	67.6	156	71.9	38	72.5	20	677
Methanobacterium	71.8	200	78.5	770	71.7	57	70.5	489	67.9	312	68.7	46	72.1	44	770
Methanothermus	72.3	250	75.4	212	70.4	69	70.8	462	69.8	450	72.6	101	72.1	80	1136
Methanogenium	68.0	28	71.5	13	81.4	292	69.5	120	71.1	59	64.6	27	68.4	15	292
Methanosarcina	68.3	67	70.8	31	73.3	61	70.6	278	68.7	177	64.5	41	69.6	19	573
M3	67.8	120	71.4	45	72.6	60	69.3	593	–	–	–	–	–	–	1217
Halobacterium	67.1	41	70.0	14	71.5	25	85.1	1015	66.0	109	68.7	12	67.0	17	1015
Halococcus	67.5	31	70.6	12	68.5	17	75.8	516	67.0	61	67.6	9	67.5	14	1015
Thermoplasma	68.5	58	69.8	15	67.6	18	66.1	163	81.1	2038	67.9	41	65.6	18	2038
Sulfolobus (DSM 639)	68.4	40	67.4	11	64.8	16	67.1	80	67.4	104	81.7 (86.0)	119	73.7 (334)	39	334
Thermoproteus	67.1	39	69.7	9	67.0	18	68.4	83	67.4	108	72.0	75	87.5	220	220
"Stiff filaments"	– –	–	–	–	72.4	40	–	–	68.4	178	75.2	166	78.2	261	440
Desulfurococcus	66.0	74	68.9	12	66.6	23	68.1	109	67.2	128	74.6	65	74.2	108	301

5000 cpm in a Heraeus-Christ minifuge and washed once with the buffers listed in column 4 of Table 1. They were suspended in 1–2 ml of the buffers listed in column 5 of Table 1 and lysed as listed there. The lysates were shaken with 1 volume of phenol for 3 minutes. Then, 1 volume of chloroform was added, the mixtures were shaken for another 3 minutes and centrifuged for 5 minutes as above. The aqueous phases were reextracted twice with 2 volumes of chloroform. After addition of 10 µg per ml of yeast sRNA (Boehringer) and 0.3M sodium acetate (final concentration) the nucleic acids were precipitated with 2 volumes of ethanol. After 16 hours at -20°C, they were pelleted in 30 minutes as above, washed once with cold absolute ethanol, dried and dissolved in water. They were separated by a modification of the acidic urea agarose gel electrophoresis procedure described by Rosen et al. (1975) and visualized by autoradiography.

The 16S rRNA bands were excised and eluted electrophoretically as described by Goldfarb and Daniel (1981). The 16S rRNAs were precipitated from the eluates and then washed and dried as described above for the total nucleic acids. They were dissolved in 5xSSC containing 25% formamide.

Hybridisation

Hybridisations to 1 to 6 nitro-cellulose filters of 0.6 cm diameter carrying 2 to 9 µg each of the different DNAs were performed in 200 µl aliquots of 5xSSC containing 25% formamide and 166000 cpm corresponding to 60 µg of RNA per filter in 1 ml Eppendorf tubes for 16 hours at 60°C. In the case of hybridisation of *Thermoplasma* DNA with *Thermoplasma* rRNA this was found to be a saturating amount of RNA. The supernatant was then removed by centrifugation through holes pinched into the bottoms of the tubes. The filters were washed twice with 0.5 ml each of 5xSSC and then subjected to digestion in 0.5 ml each of a solution of 10 µg RNAase A (Worthington) and 50 units RNAase T1 (Worthington) per ml 5xSSC for 45 minutes at 37°C, and finally washed 2 times with 0.5 ml each of 5xSSC. The digestion was repeated. The filters were suspended in 0.3 ml 5xSSC containing 25% formamide. The filter bound radioactivity was determined as Cerenkov counts. It ranged between more than 70000 and a few hundred cpm.

The filters in 0.3 ml 5xSSC and 25% formamide were then heated successively to 65, 70, 75, 80, 85, 90 and 95°C for 15 minutes each. After each heating period, the buffer was replaced and the residual filter bound radioactivity was determined as Cerenkov counts.

Finally, the filter bound DNA was determined as described by Burton (1968).

Results

^{32}P labelled 16S rRNAs from members of 7 known or presumed orders of the archaebacterial urkingdom were hybridized with 14 different DNAs from archaebacteria, of which 6 were from the species of undetermined taxonomic position mentioned in the introduction. Two sets of data were determined, namely A) the cpm per µg of DNA hybridized in RNAase stable form at 60°C in 5xSSC containing 25% formamide and B) the temperature stability curves for all hybrids (Table 2). The data of set A which have been obtained in RNA excess, depend both on genome size and on the number of rRNA genes per genome. Without further information, it is not possible to determine both parameters separately. However, expression of the data as fractions of the yields of the hybridizations with the homologuous DNAs, which are taken to be 1, related all values to each other and standardized them for the number of 16S rRNA genes (Table 3).

In the case of hybridizations with DNAs for which homologuous RNAs were not available, correction factors were determined empirically. The basis is the observation that the yield ratios of hybrids containing DNAs of different members of one order appear rather constant independent of the RNA probe as long as this is from a different order.

Table 3. 1. Spans between the temperature of hybridization (60°C) and the TMs of the hybrids expressed as fractions of the spans between 60°C and the TMs of the hybrids with the homologuous DNAs (≡ 1) (= fractional stabilities, fr. st. = TM -60°C/TMhom. -60°C), and 2. yields of hybridization expressed as fractions of yields of the hybridization with the homologuous DNA. For calculation of data see Materials and Methods

RNA from	*Methanococcus*		*Methanobacterium*		*Methanogenium*		*Halobacterium*		*Thermoplasma*		*Sulfolobus* B6		*Thermoproteus*	
DNA from	fr. st.	yield	fr. st.	yield	fr. st.	yield	fr. st.	yield	fr. st.	yield	fr. st.	yield	fr. st.	yield
Methanococcus	1	1	0.71	0.05	0.55	0.06	0.28	0.35	0.36	0.25	0.45	0.04	0.24	0.03
SN1	0.94	0.4	0.85	0.08	0.45	0.05	0.33	0.35	0.36	0.23	0.46	0.05	0.24	0.03
Methanobacterium	0.65	0.26	1	1	0.55	0.07	0.42	0.64	0.37	0.40	0.33	0.06	0.25	0.06
Methanothermus	0.68	0.22	0.83	0.19	0.49	0.06	0.44	0.40	0.46	0.39	0.48	0.09	0.26	0.07
Methanogenium	0.44	0.10	0.62	0.05	1	1	0.38	0.42	0.53	0.20	0.17	0.10	0.18	0.05
Methanosarcina	0.46	0.12	0.58	0.05	0.62	0.11	0.42	0.50	0.41	0.31	0.17	0.07	0.19	0.03
M3	0.43	0.10	0.62	0.04	0.59	0.16	0.37	0.50	—	—	—	—	0.18	0.02
Halobacterium	0.39	0.04	0.54	0.01	0.54	0.02	1	1	0.28	0.11	0.33	0.01	0.14	0.02
Halococcus	0.41	0.03	0.57	0.01	0.40	0.02	0.63	0.51	0.38	0.06	0.29	0.01	0.14	0.01
Thermoplasma	0.47	0.03	0.53	0.01	0.36	0.01	0.24	0.08	1	1	0.30	0.02	0.11	0.01
Sulfolobus (DSM 639)	0.46	0.12	0.40	0.03	0.22	0.05	0.28	0.24	0.35	0.31	0.83 (1.)[a]	0.36 (1.)[a]	0.49	0.12
Thermoproteus	0.39	0.18	0.52	0.04	0.33	0.08	0.33	0.38	0.35	0.49	0.46	0.34	1	1
"Stiff filaments"	–	–	–	–	0.31	0.09	–	–	0.40	0.40	0.58	0.38	0.67	0.60
Desulfurococcus	0.33	0.25	0.48	0.04	–	0.08	0.32	0.36	0.34	0.42	0.56	0.21	0.57	0.30

[a] For RNA of Sulfolobus DSM 639 with DNA of Sulfolobus DSM 639

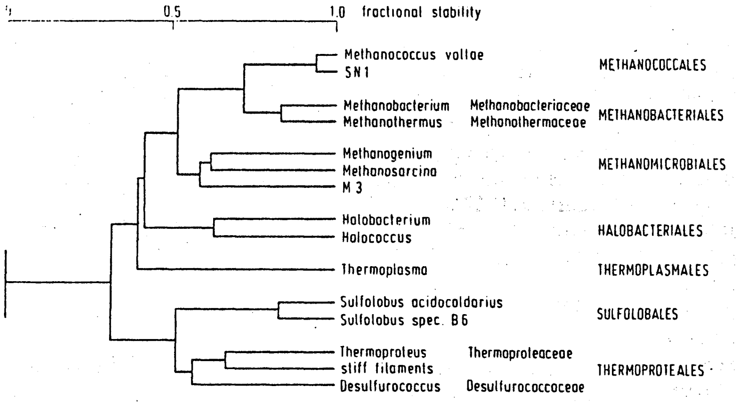

Fig. 1. Dendrogram of fractional stabilities of hybrids between 16S rRNAs and DNAs of archaebacteria. Construction of dendrogram from the values listed in Table 3 as described in Materials and Methods.

From the melting curves obtained from set B of the data temperatures of 50% stability of all hybrids, termed TMs, were determined. To relate them to each other, the spans between the temperature of hybridization, 60°C, and the TMs of the homologuous pairings were taken to be 1 and the TMs of other hybrids containing the same RNA were expressed as fractions thereof. The figures obtained in this way, termed fractional stabilities (see Table 3) could be used in the same manner as the fractional yields or for example S_{AB} values (Fox et al. 1977) for measuring distances between the species from which RNA and DNA had been obtained, thus allowing the construction of a dendrogram. It should, however, be pointed out that a linear relationship between the fractional stability and the phylogenetic distance or perturbation of homology cannot be assumed so that such a dendrogram expresses the order, but not the magnitudes of taxonomic relations.

None of the RNA probes representing different divisions of the archaebacterial kingdom yielded a stable hybrid with DNAs from *E.coli, Saccharomyces cerevisiae* and calf thymus.

The fractional yields listed in Table 3 are only in two RNA series, from Halobacterium and from Thermoplasma, in the same range as the fractional stabilities. The few high values in the other series are for pairings of the RNA with DNAs either of close relatives, e.g. *Methanogenium-Methanosarcina, Sulfolobus acidocaldarius* DSM 639-*Sulfolobus* B6, or of presumed close relatives, e.g. *Methanobacterium-Methanothermus*, or they may indicate closeness (see below), e.g. *Methanococcus*-SN1, *Methanogenium*-M3, *Thermoproteus*-stiff filaments-*Desulfurococcus-Sulfolobus*. The other values in these series are low but very similar for DNAs from related or presumably related organisms. The fractional yields of reciprocal pairings are often quite different. An explanation is offered in the discussion. The orders of the fractional yields in the different RNA series correspond to those of the fractional stabilities only for the highest values.

The yield data could thus only be used as additional evidence for close relation and the quantitative evaluation was confined to the fractional stabilities. As the fractional yields, within a given RNA series, these agree closely for related or probably related organisms. As those, in a given RNA series, they are highest for the DNAs of organisms closely or probably closely related to the RNA donor. But, in addition, they are similar in reciprocal pairings.

Because intra order distances were normally measured in but one direction (with one RNA), the fractional stabilities of pairings of close relatives were immediately used as intra order branching levels.

Since the distances between different members of one order and different members of another order are very similar to each other (Table 3), inter order distances were determined by averaging all measured distances between members of the different orders including values for reciprocal pairings. The sequence of branching levels in constructing a dendrogram including all species studied, was determined by proceeding in the direction of decreasing relationship and averaging corresponding distances.

The significance of all intraorder branchings and of some interorder ramifications like those between the *Methanococcales*, the *Methanobacteriales* and the *Methanomicrobiales*, and that between the *Thermoproteales* and the order represented by *Sulfolobus* is strengthened by the fractional yield data. In addition, close correlations within groups are evident in all but the homologuous RNA series from the small divergence of both the fractional yields and the fractional stabilities of the hybrids containing DNAs of different members of a group.

The affiliation of a species to an order can just the reverse be immediately recognized in the corresponding RNA series from the deviation of the respective fractional yields and fractional stabilities from those measured in that series for members of all other orders. Furthermore, both values are considerably higher than those appertaining other organisms, and may approach 1.

SN1 appears so close to *Methanococcus voltae* that it can be assumed to belong to the same genus. The distance of M3 to *Methanogenium* and *Methanosarcina* indicates that it represents at least another genus, if not another family of the order *Methanomicrobiales*. The distances between the different members of the order *Thermoproteales* is in the same range. We therefore consider them different families within this order. *Desulfurococcus* is less closely related to *Thermoproteus* than the stiff filaments.

Discussion

The method applied here for the determination of the taxonomic positions of several novel species of archaebacteria has been developed by De Ley and De Smedt (1975). They have already pointed out that only the stabilities and not the yields of hybrids furnish reliable information for the determination of taxonomic relations. This is also clear from our data (Table 2), especially from the strong drop of the fractional yields with increasing phylogenetic distance and from the striking differences of reciprocal values. These observations could be explained by assuming that during hybridization, an equilibrium between hybridized RNA and free RNA with native secondary and tertiary structure is established which, among other parameters, depends on the stability of the secondary and tertiary structure of the particular rRNA in relation to that of the hybrid. The following formulation, in which ts means tertiary structure intact, ss means no tertiary structure, explains this model: $\mathrm{RNAts} + \mathrm{DNA} \rightleftarrows \mathrm{RNAss} + \mathrm{DNA} \leftrightarrows \mathrm{RNAss} - \mathrm{DNA}$. Many of the rRNAs used in these hybridization experiments, as for example those from the highly thermophilic organisms *Thermoproteus* and *Sulfolobus*, should have quite stable tertiary structures under hybridization conditions. These show the described behaviour markedly. On the other hand, the fractional yields of hybrids containing the rRNA of *Halobacterium*, an organism growing around 40°C and with a DNA of high GC content, are in the same range as the corresponding fractional stabilities.

It must be emphasized that because of the lack of relevant theoretical background, the treatment of the data is entirely empirical. The approach used especially the normalisation to the span between the hybridization temperature and the TMs of the homologuous hybridization accounts for the large differences between the TMs of different homologuous pairings and allows to treat the data like S_{AB} values.

Dendrograms have been constructed as well with weighted information as from all data available. The differences are small. The essential conclusions remain the same.

The appointment of species of unknown taxonomic position to orders on the basis of hybridization experiments is particularly significant because their intraorder hybrids have always a high fractional stability, are always obtained in high fractional yields and are related to different hybridization probes by yield and stability data very close to those of other members of that order.

The division of the urkingdom of the archaebacteria into two different branches, that of the methanogens and that of the thermoacidophiles, follows clearly from our data. The order of association, and even the association itself, of the extreme halophiles and particularly of *Thermoplasma* with the branch of the methanogens is less significant. *Thermoplasma* has a rather low relation with members of both branches and might thus even represent a third branch between the others.

In contrast to their position in the tree obtained by comparative cataloging of 16S RNA (Fox et al. 1980), in the dendrogram derived from the hybridiza-