J Mol Evol (1986) 24:167–173

Journal of Molecular Evolution

Hybridization Homology: A New Parameter for the Analysis of Phylogenetic Relations, Demonstrated with the Urkingdom of the Archaebacteria*

H.-P. Klenk, B. Haas, V. Schwass, and W. Zillig

Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

Summary. Ribosomal RNAs of 17 species of archaebacteria were hybridized to corresponding and non-corresponding nitrocellulose bound DNAs. The temperature stability of these hybrids and the amount of bound rRNA were determined.

A formula was derived to correct the hybridization yields for the different genome lengths and numbers of rRNA operons per genome. This correction made it possible to determine hybridization homologies, as functions of velocity constants, which could then be used in a similar way as sequence homologies.

The results were consistent with those from 16S rRNA total sequence data. No correlation was found between the hybridization homologies and the temperature stabilities of the hybrids.

This new method is faster and simpler than the method based on total 16S rRNA sequence determination although it provides less total information. Its application to archaebacterial phylogeny has shown the *Thermococcales* to represent a third branch of the kingdom beside the branch of the methanogens + halophiles, and that of the *Thermoproteales* + *Sulfolobales*. The method has also provided a detailed description of the phylogeny of the *Sulfolobales* showing their origin within the *Thermoproteales*.

Key words: Ribosomal RNAs — Cross-hybridization — Hybridization yield — Fractional hybridization — Hybridization homology — Phylogenetic tree — Phylogeny — Evolution — Archaebacteria

Offprint requests to: H.-P. Klenk

*Presented at the FEBS Symposium on Genome Organization and Evolution, held in Crete, Greece, September 1–5 1986

Introduction

Ribosomal RNAs are excellent marker molecules for the investigation of phylogenetic relations (Noller and Woese 1981). They are ubiquitous, evolutionarily highly conservative, easy to purify, and now readily analyzed by two molecular biological methods: sequencing and hybridization. Sequence comparisons have been widely used for the determination of phylogenetic relations (Woese and Olsen 1986). These methods, however, require special expertise and are time-consuming and complex compared to hybridization methods. In this paper a relatively quick and technically easy procedure is presented which provides results on the basis of hybridizations which correlate well with those of sequence comparisons.

Hybridization techniques have been improved and simplified since Hall and Spiegelman (1961) described the first procedures. In 1975 De Ley and De Smedt described a procedure for DNA-rRNA hybridizations which was subsequently used in many phylogenetic studies (e.g., Mordarski et al. 1980; Zillig et al. 1980; Garvie and Farrow 1981; Tu et al. 1982; Schlotterbeck 1984). This method uses the melting point of the DNA-rRNA hybrids as a measure for the phylogenetic relations between organisms. The hybridization yield was used only in similarity maps for the discrimination of hybrids with

identical melting points. A quantitative evaluation of cross-hybridization experiments solely on the basis of yields has been regarded as impossible because the hybridization yields appeared inconsistent with each other. We found that this problem could be overcome by correcting yield data for the different genome lengths and operon numbers.

A second major problem in doing hybridization with rRNA is its extensive renaturation during incubation due to the stability of its secondary structure (Woese et al. 1980). Baharaeen and coworkers (1983) solved this problem by synthesis of complementary DNA from fragmented 25S rRNA using reverse transcriptase. A simpler solution used by Schlotterbeck (1984), and in this paper, involves direct hybridization of rRNA fragments obtained by alkaline cleavage.

A third problem which was solved by the alkaline cleavage method was the failure to obtain radioactive labelling of the rRNAs of some thermoacidophilic archaebacteria in vivo. Fragments of these rRNAs can be easily labelled with the method described here.

Materials and Methods

Organisms

DNA and rRNA were isolated as described below from the following archaebacteria: *Halobacterium halobium, Methanolobus tindarius*; *Methanothermus fervidus* DSM 2088; *Methanobacterium thermoautotrophicum* DSM 1053; *Methanococcus vannielii* DSM 1224; *Thermoplasma acidophilum* DSM 1728; *Thermococcus celer* DSM 2161; *Calduplex woesei* DSM 3773, isolate AN1; *Desulfurococcus mucosus* DSM 2163; *Thermoproteus tenax* DSM 2978; *Sulfolobus* sp. B12; *Sulfolobus* sp. B6/2; *Sulfolobus acidocaldarius* DSM 639; *Sulfolobus brierleyi* DSM 1651; *Desulfurolobus ambivalens.*

Preparation of DNA

Five to ten g of frozen cells were suspended in 5 volumes of 100 mM NaCl, 1 mM Na_2-EDTA, 20 mM Tris-Cl, pH 8.0. The pH was adjusted to 8.0–8.5 and the cells were lysed with SDS (Sodium laurylsulfate, final concentration 1%) and Triton-X-100 (final cencentration 0.1%). The DNA was isolated from the crude extract by the phenol procedure as previously described (Zillig et al. 1980). All DNAs were banded twice in CsCl density gradients and were transferred to nitrocellulose filters (Tu et al. 1982).

Preparation of Total rRNAs

Five to seven g of frozen cells were suspended in 5 volumes of the same buffer as in the DNA preparation described above with the addition of 25 mM $MgCl_2$. Cells were opened as before and after a 30 min incubation at 37°C with 0.2 μg DNaseI/ml the remaining DNA was fragmented by sonication. Cell debris was pelleted and the ribosomes were isolated from the supernatant by a number of centrifugation steps. In the first step the supernatant was layered over a cushion of 20% sucrose in 0.5 M Tris-Ac, pH 7.5, 22 mM NH_4Cl, 10 mM Na_2-EDTA, 20 mM $MgCl_2$ and 0.07% β-mercaptoethanol and centrifuged for 5 h at 50,000 rpm at 20°C in the Beckman 50 Ti rotor. The pellet was resuspended in the same buffer as before except that the $MgCl_2$ concentration was reduced from 20–1 mM. This suspension was layered over a sucrose gradient of 7.5–30% sucrose, 5–10% glycerol, 50 mM Tris-Ac, pH 7.5, 50 mM $MgCl_2$, 0.4 M KCl, 0.07% β-mercaptoethanol. It was centrifuged for 9 h at 20°C at 27,000 rpm in the Beckman SW27 rotor. The gradient was fractionated with a peristaltic pump while monitoring the OD_{260}. The two peaks with the 50S- and 30-ribosomal subunits were pooled and phenolized.

Fragments were produced by 30 min cleavage in a borate buffer (2.5 M H_3BO_3, 1.2 M NaOH) at 70°C. They were labelled with γ-^{32}P-ATP and polynucleotide kinase (Silberklang et al. 1979). Unreacted mono-nucleotides and smaller fragments were removed over a Sephadex-G-100 column.

Hybridization

The nitrocellulose filters with the DNAs were prehybridized overnight at 50°C in 2× SSC containing 20% formamide, 1 g/l salmon sperm DNA, 0.5 g/l *Saccharomyces cerevisiae* tRNA, 2 mM ATP and 0.1% SDS. For each rRNA sample a dilution series ranging from 0.5–50 μg labelled rRNA in 1 ml 2× SSC with 20% formamide was prepared. Each cap contained nitrocellulose filters with various DNAs and a control filter without DNA. Hybridization was done at 50°C for 48 h. It was followed by three RNA digestion periods of 30 min each with 15 μg/ml RNase A and 75 U/ml RNase T_1 in 2× SSC at 37°C. After the third RNase digestion a plateau of rRNA remaining on the nitrocellulose filters was reached. After the RNase digestions, the nitrocellulose filters were separated, the RNA was determined by scintillation counting and the DNA by a modified Burton reaction (Giles and Myers 1965; Meijs and Schilperoort 1971).

Melting Curves of the Hybrids

Hybridization for the melting curves was done with 7 μg rRNA/ml for 48 h. The melting of the DNA-rRNA hybrids was done as described by Tu et al. (1982).

Hybridization Yield

Nucleic acid hybridizations depend on the collision of two molecules with complementary sequences. Anderson and Young (1985) described general mathematical techniques for handling the kinetics of hybridizations. We have transformed and applied one of their kinetic equations for DNA-RNA hybridizations:

$$d(\mathrm{DR})/dt = k_1 \times (\mathrm{D}) \times (\mathrm{R}) - k_2(\mathrm{DR}) \qquad (1)$$

where (D) = concentration of filter bound DNA; (R) = concentration of RNA in the hybridization solution; (DR) = concentration of the hybrid; k_1 = velocity constant of hybrid formation and k_2 = velocity constant of hybrid decay.

This equation can be simplified under the following conditions: (1) The hybridization temperature 50°C is far below the melting point of most hybrids. Therefore k_2 is small compared with k_1; (2) The concentration of free RNA in the hybridization solution decreased only 1–2% during the reaction. Therefore (R) can be considered as constant; (3) At low RNA concentrations and/or short reaction times, conditions far from allowing saturation, the concentration of free DNA remains practically constant; (4) Under these conditions, the concentration of hybrids

(DR) is very low compared with (R) or (D). The second term of Eq. (1) can then be neglected.

$$d(\mathrm{DR})/dt = k_1 \times (\mathrm{R}) \times (\mathrm{D}) \tag{2}$$

After integration over time and taking (DR) = 0, when t_0 = 0 Eq. (2) becomes:

$$(\mathrm{DR})/(\mathrm{D}) = k_1 \times (\mathrm{R}) \times t \tag{3}$$

This value expressed as percent is the "hybridization yield" (Y):

$$\mathrm{Y} = 100 \times (\mathrm{DR})/(\mathrm{D}) \tag{4}$$

The hybridization mixture contains many different rRNA fragments, which arose during cleavage in borate buffer. (DR) is therefore the sum of the different hybrids of all those RNA fragments. The term R_i represents one of those fragments. From this follows:

$$(\mathrm{R}) = \sum_{i=1-n} (\mathrm{R}_i) \tag{5}$$

$$(\mathrm{DR}) = \sum_{i=1-n} (\mathrm{DR}_i) \tag{6}$$

Equations (3) and (5) substituted into Eq. (4) give:

$$\mathrm{Y} = 100 \times t \times \sum_{i=1-n} k_{1i} \times (\mathrm{R}_i) \tag{7}$$

To increase the accuracy of the hybridization yield determination values were taken from regression curves at 1 µg RNA/ml for each dilution series. This concentration was chosen because it is low enough to avoid saturation and high enough for reliable signals in scintillation counting.

Fractional Hybridization

Each hybridization solution contains nitrocellulose filters with DNAs from different species. The hybridization yields of those DNAs with the RNA in the solution can be compared with the yield of the corresponding self-hybridization of this RNA with the DNA from the same organism. The term D_a represents the DNA which is homologous to the RNA in the hybridization solution (RNA R_a); D_b represents the DNA of any other organism; $\mathrm{D}_a\mathrm{R}_a$ = self-hybridization; $\mathrm{D}_b\mathrm{R}_a$ = cross-hybridization:

$$\mathrm{D}_a\mathrm{R}_a/\mathrm{D}_a = t \times \sum_{i=1-n} k_{1iaa} \times (\mathrm{R}_{ai}) \tag{8}$$

$$\mathrm{D}_b\mathrm{R}_a/\mathrm{D}_b = t \times \sum_{i=1-n} k_{1iba} \times (\mathrm{R}_{ai}) \tag{9}$$

k_{1iaa} = velocity constant of self-hybrid formation between DNA_a and RNA_a-fragment$_i$, k_{1iba} = velocity constant of cross-hybrid formation between DNA_b and RNA_a-fragment$_i$. The relation between any cross-hybridization and self-hybridization can be expressed as:

$$(\mathrm{D}_b\mathrm{R}_a/\mathrm{D}_b)/(\mathrm{D}_a\mathrm{R}_a/\mathrm{D}_a) = \sum_{i=1-n} k_{1iba} \times (\mathrm{R}_{ai})/\sum_{i=1-n} k_{1iaa} \times (\mathrm{R}_{ai}) \tag{10}$$

The fraction of $(\mathrm{R}_{ai})/(\mathrm{R}_a)$ is a parameter for the share of k_{1i} within k_1. Since all hybridizations with one RNA are done within the same hybridization mixture, $(\mathrm{R}_{ai})/(\mathrm{R}_a)$ is only a normalization for the length distribution of the RNA fragments. The distribution of RNA fragment lengths should be the same for all RNAs as long as the cleavage in the borate buffer is reproducible.

k_{1ba} depends on the sequence homology of the rRNA operon between the two organisms which should be compared. The fraction k_{1iba}/k_{1iaa} is near 1 for closely related organisms and decreases with increasing sequence difference between the rRNA operons of the two organisms.

The relation between the hybridization yields of any cross-hybridization and the corresponding self-hybridization is defined as "fractional hybridization" (BA%) expressed in percent:

$$\mathrm{BA\%} = 100 \times \sum_{i=1-n} k_{1iba} \times (\mathrm{R}_{ai})/\sum_{i=1-n} k_{1iaa} \times (\mathrm{R}_{ai}) \tag{11}$$

Hybridization Homology

The fractional hybridization depends not only on the sequence homology of the rRNA operons, but also on the genome lengths and the numbers of rRNA operons per genome of the two organisms.

$1(a)$ is the genome length of DNAa; $n(a)$ is the number of rRNA operons per genome of DNAa. $1(b)$ and $n(b)$ are the corresponding parameters of the DNAb. The fractional hybridization of DNAa in a solution of RNAb AB% is therefore:

$$\mathrm{AB\%} = \mathrm{H} \times \frac{n(a) \times 1(b)}{1(a) \times n(b)} \tag{12}$$

And the corresponding BA% can be expressed as:

$$\mathrm{BA\%} = \mathrm{H} \times \frac{n(b) \times 1(a)}{1(b) \times n(a)} \tag{13}$$

H is the proportionality factor which relates the hybridization yield with the genome length and the number of rRNA operons per genome.

Multiplying Eqs. (12) and (13) leads to the hybridization homology:

$$\mathrm{AB\%} \times \mathrm{BA\%} = \mathrm{H}^2 \tag{14}$$

or:

$$\mathrm{H} = \sqrt{\mathrm{AB\%} \times \mathrm{BA\%}} \tag{15}$$

H is therefore the *hybridization homology* between the rRNA operons of two DNAs, calculated solely from the hybridization yields of the two possible cross-hybridizations, but considering the different genome lengths and numbers of rRNA operons per genome.

Genome Length per rRNA Operon

The hybridization homology is not the only parameter which can be calculated from the hybridization yields. The relation of the genome lengths per rRNA operon $(1(a)/n(a)$ and $1(b)/n(b))$ between two organisms can also be calculated with the hybridization yields:

$$\mathrm{AB\%/BA\%} = (n(a)/1(a) \times 1(b)/\mathrm{n}(b))^2 \tag{16}$$

or:

$$n(a)/1(a) \times 1(b)/n(b) = \sqrt{\mathrm{AB\%/BA\%}} \tag{17}$$

There are two applications for Eq. (17): (1) If the number of rRNA operons per genome for an organism is known, it is possible to calculate the genome length provided this organism can be related to a second organism for which both parameters are known; (2) With this parameter is is possible to calculate the hybridization homology with only one known fractional hybridization instead of two in Eq. (15) when the relation of the genome lengths per rRNA operon between those two organisms is known:

$$\mathrm{H} = \mathrm{BA\%} \times n(a)/1(a) \times 1(b)/n(b) \tag{18}$$

Calculating Phylogenetic Distances

For the construction of a phylogenetic tree the hybridization homologies are to be transformed into distance values. The basis of this transformation was the observation of Bonner et al. (1973) that for each 10% base mismatch the kinetic velocity constant of hybrid formation will decrease to one half of its original value. From this observation a formula for the distance (S) can be deduced:

$$S = 10/1n2 \times \ln(100/H) \qquad (19)$$

The value 2 in this formula has not been exactly determined. Therefore, the distance S only approximately corresponds to percent-sequence difference. (Fig. 1).

Construction of the Phylogenetic Tree

From the distance values a tree can be constructed as described by Fitch and Margoliash (1967).

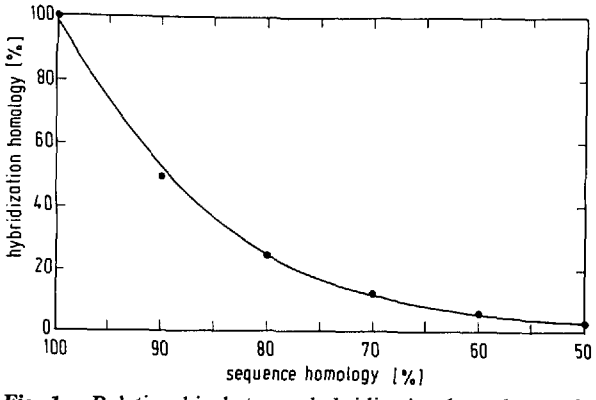

Fig. 1. Relationship between hybridization homology values and sequence homology values

Results

^{32}P labelled total rRNAs from 17 species of the archaebacterial urkingdom were hybridized with the DNAs of those organisms. Two sets of data were determined: the hybridization homologies and the melting points of the DNA-rRNA hybrids. The homologies were calculated from the yields as described in Eqs. (15) and (18). The determination of 96 out of the possible 136 distances between the 17 organisms was more than sufficient for the construction of a phylogenetic tree. Table 1 shows the hybridization homologies and the distances calculated from the hybridization homologies with Eq. (19).

In the cases of *Thermoproteus tenax* and *Methanococcus vannielii* not only the total rRNAs were isolated and hybridized against the DNAs of each other but also the pure 16S and 23S rRNAs. The hybridization homology for the 16S rRNAs was higher than that for the 23S rRNAs, but the hybridization homology for the whole operon, calculated from the values for the single rRNAs, corrected for their different length, is in good agreement with the value obtained for the total rRNA.

For 6 out of the 17 organisms used the total sequence of the 16S rRNA is known (Woese and Olsen 1986). The correlation between the distances calculated from the hybridization homologies of the total rRNAs and the distances from the percent-sequence homologies of the 16S rRNAs is shown in Fig. 2. The deviations of some of the points in the diagram may be explained with the different RNA

Table 1. Hybridization homologies and corresponding phylogenetic distances*

	H. ha	M. ti	T. ac	M. th	M. fe	M. va	T. ce	I. AN	C. wo	D. mu	T. te	D. am	S. br	S. ac	S. so	S. B12	S. B6/2
H. halobium	x	24.3	14.7	29.4	16.6	17.5	26.6	17.4	6.0	13.0	9.7	3.7	13.9	14.5	12.6	11.9	5.9
M. tindarius	20.4	x	10.2	17.4	14.4	15.7	16.7			12.5	8.2		5.4	5.4		5.5	
T. acidophil.	27.7	32.9	x	12.9	7.4	12.9	16.6	12.7	6.0	7.8	8.8	4.6	4.2	7.2	7.9	5.4	4.0
M. thermoaut.	17.7	25.3	29.5	x	55.1	21.5	31.5			22.6	14.7	14.7	9.5	16.0		15.5	
M. fervidus	25.9	28.0	37.6	8.6	x	18.4				18.5	11.6			11.0		11.5	
M. vannielii	25.1	26.7	29.6	22.2	24.5	x	22.9		7.5	15.9	7.2			8.2	8.6	8.8	5.4
T. celer	19.1	25.8	26.0	16.7		21.3	x	69.1	23.2	28.9		12.4	15.5	23.4	13.4	26.7	21.0
Isolat AN1	25.3		29.8				5.3	x	24.2	39.8	16.8				13.8		
C. woesei	40.6		40.6			37.4	21.1	20.5	x	14.7	8.4				3.8		
D. mucosus	29.5	30.0	36.8	21.5	24.3	26.5	17.9	13.3	27.6	x	32.5			34.0	27.8	25.5	22.5
T. tenax	33.7	36.1	35.2	27.7	31.1	38.1		25.8	35.8	16.2	x	19.6	15.3		17.8	26.8	32.3
D. ambival.	47.6		44.4	27.7			30.1				23.5	x		33.9		37.0	
S. brierleyi	28.4	42.2	45.8	33.9			26.8				27.1		x	17.1	39.9		29.4
S. acidocald.	27.8	42.0	38.0	26.4	31.9	36.1	20.9			15.6		15.6	25.5	x	28.5		
S. solfatar.	29.9		36.7			35.3	29.1	28.6	47.3	18.5	24.9		13.3	18.1	x	50.6	17.7
S. sp. B12	30.7	42.0	42.1	26.9	31.2	35.1	19.1			19.7	19.0	14.3			9.8	x	31.5
S. sp. B6/2	40.8		46.5			42.0	22.5			21.4	16.3		17.7		25.0	16.7	x

* The upper right part of the table contains the hybridization homologies in percent, the lower left part shows the corresponding phylogenetic distances (S). H.ha = *Halobacterium halobium*, M.ti = *Methanolobus tindarius*, T.ac = *Thermoplasma acidophilum*, M.th = *Methanobacterium thermoautotrophicum*, M.fe = *Methanothermus fervidus*, M.va = *Methanococcus vannielii*, T.ce = *Thermococcus celer*, I.AN = isolate AN1, C.wo = *Calduplex woesei*, D.mu = *Desulfurococcus mucosus*, T.te = *Thermoproteus tenax*, D.am = *Desulfurolobus ambivalens*, S.br = *Sulfolobus brierleyi*, S.ac = *Sulfolobus acidocaldarius*, S.so = *Sulfolobus solfataricus*, S.B12 = *Sulfolobus* sp. B12, S.B6/2 = *Sulfolobus* sp. B6/2

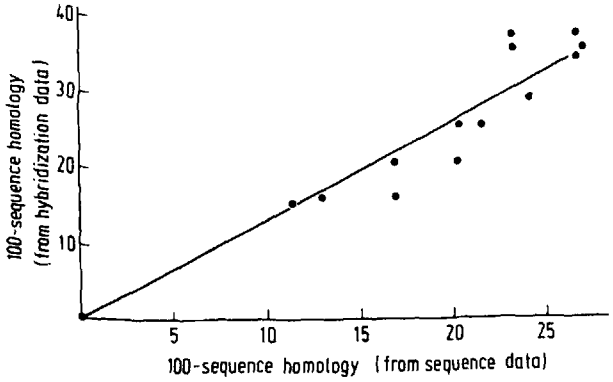

Fig. 2. The correlation between distance values calculated from sequence and hybridization data. The phylogenetic distance (S) is defined as 100%-sequence homology

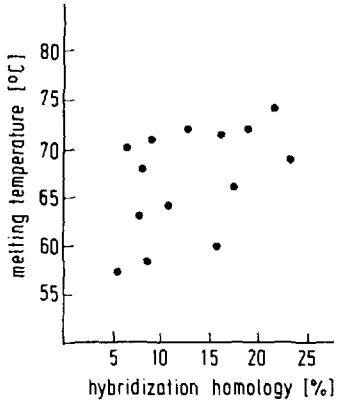

Fig. 3. The correlation between the hybridization homologies and the melting temperatures of the corresponding cross-hybrids. The rRNA used in this example was from *Methanococcus vannielii*, the DNAs were from *Methanococcus vannielii* and 13 other archaebacteria

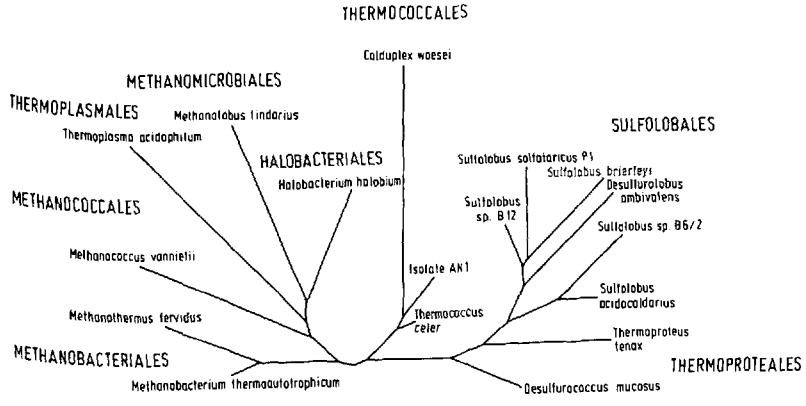

Fig. 4. Phylogenetic tree of the archaebacteria. The organisms on both sides of the tree have the same mean distance to the vortex at the center of the tree

material (total rRNA against 16S rRNA). The diagram shows that the two methods of distance determination are generally in reasonable agreement.

As demonstrated for a few examples in Fig. 3, the T_m-values for the DNA-rRNA hybrids do not correlate with the hybridization homologies. Therefore T_m-values have not been used for the construction of a phylogenetic tree.

The distance values of Table 1 were used for the construction of a phylogenetic tree. The optimized tree shown in Fig. 4 had a standard deviation of 10.7%. A not yet optimized version of this tree, with a standard deviation of 12.0%, has previously been shown in Zillig et al. (in press).

The tree shows *Calduplex woesei* (Zillig et al. in press), the isolate AN1 (Morgan and Daniel 1982) and *Thermococcus celer* (Zillig et al. 1983) in a third, intermediary branch of the archaebacterial urkingdom between the established branches of the methanogens + halophiles and the *Thermoproteales* + *Sulfolobales*. It gives a detailed picture of the *Sulfolobales*, including *Desulfurolobus* (Zillig et al. 1986a), and their relation to the *Thermoproteales*.

The hybrization yields could also be used for the determination of genome length. In the case of *Halobacterium halobium* genome length and number of rRNA operons per genome are known (Moore et al. 1969). For some other organisms the fractional hybridization with *Halobacterium halobium* was measured and the number of rRNA operons per genome was known (Neumann et al. 1983). Table 2 shows the calculated genome lengths for these organisms.

Discussion

The described hybridization procedure produces results comparable to those of the more laborious sequencing methods and is readily applicable to phylogenetic and taxonomic problems. It requires little specialized equipment, only small amounts of cells for isolating rRNAs and DNAs, which can be stored for usage in successive cross-hybridizations, and allows easy handling of low doses of radioactivity for fast and reproducible in vitro labelling of RNA frag-

Table 2. Calculated genome lengths of archaebacteria in million base pairs

Methanobacterium thermoautotrophicum	6.1
Methanococcus vannielii	6.0
Sulfolobus sp. B12	4.7
Halobacterium halobium	4.1
Thermococcus celer	4.0
Thermoplasma acidophilum	2.9
Thermoproteus tenax	2.7
Desulfurococcus mucosus	2.0

ments. The distance values obtained are of similar magnitude and are proportional to those from sequence data. The main disadvantages compared to sequence comparison are less information, no "signatures" (Woese and Olsen 1986) are obtained, the requirement of reference DNAs and rRNAs for each assignment and the relatively large errors in distance values caused by small errors in the case of low hybridization homologies. The ease of the method, however, compensates these drawbacks. DNA-DNA hybridization has a much shorter range and other methods like immunochemical cross-reactions and determining lineages of feature designs are not quantitative.

The alkaline cleavage of the rRNAs solves the problem caused by the stability of the secondary structure of rRNA and eliminates the difficulties of in vivo labelling of some organisms. The melting points of hybrids between DNA and fragmented RNA are in the same range as those with unfragmented RNAs, but prehybridization was necessary for the observation of stable hybrids.

The concentration at which the hybridization yields were compared, 1 μg rRNA/ml, was empirically determined. This concentration has proved to be high enough for significant label in bound rRNA and low enough to avoid the influence of unspecific adsorption of labelled material to the nitrocellulose filters.

The hybridization time can be reduced to about 16 h, so that hybridization can be done overnight. RNA dilution series are not necessary. To save time and material, it is sufficient to perform multiple hybridizations with a standard amount (1 μg RNA/ml) to decrease the error of the single measurement.

The new method has proved its validity by the assignment of several novel isolates to their phylogenetic position. The phylogenetic tree of the archaebacteria obtained in this way resembles that obtained by the comparison of total sequences of 16S rRNAs except in a few details, e.g., the relative branching order of *Methanococcales* and *Methanobacteriales* and of *Desulfurococcales* and *Thermoproteus*. The new third branch of the archaebacteria contains the shortest offshoot, *Thermococcus celer* (Woese and Olsen 1986) and the long offshoot of *Calduplex woesei*, which gives this branch a depth comparable to those of the other two branches. The facultative sulfur reducing and sulfur oxidizing *Desulfurolobus* appears in the middle of the *Sulfolobales* which arise from within the *Thermoproteales*.

References

Anderson LM, Young D (1985) Quantitative filter hybridisation in nucleic acid hybridisation. In: Hames B, Higgins SJ (eds) Nucleic acids hybridisation. IRL Press, Oxford, pp 74–111

Baharaeen S, Melcher U, Vishniac HS (1983) Complementary DNA: 25S ribosomal RNA hybridization: an improved method for phylogenetic studies. Can J Microbiol 29:546–551

Bonner TI, Brenner DJ, Neufield BR, Britten RJ (1973) Reduction in the rate of DNA reassociation by sequence divergence. J Mol Biol 81:123–135

De Ley J, De Smedt J (1975) Improvements of the membrane filter method for DNA: rRNA hybridization. Antonie van Leeuwenhoek 41:287–307

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Garvie EI, Farrow JAE (1981) Sub-division within the genus *Streptococcus* using deoxyribonucleic acid: ribosomal ribonucleic acid hybridization. Zbl Bact Hyg I Orig C 2:299–310

Giles KW, Myers A (1965) An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 206: 4979

Hall BD, Spiegelman S (1961) Sequence complementarity of T_2-DNA and T_2-specific RNA. Proc Natl Acad Sci USA 47: 137–146

Meijs WH, Schilperoort RA (1971) Determination of the amount of DNA on nitrocellulose membrane filters. FEBS Letters 12: 166–168

Moore RL, McCarthy BJ (1969) Base sequence homology and renaturation studies of the deoxyribonucleic acid of extremely halophilic bacteria. J Bacteriol 99:255–262

Mordarski M, Goodfellow M, Tkacz A, Pulverer G, Schaal KP (1980) Ribosomal ribonucleic acid similarities in the classification of *Rhodococcus* and related taxa. J Gen Microbiol 118:313–319

Morgan HW, Daniel RM (1982) Proceedings of the XIII international congress of microbiology, Boston, Mass, USA, August 1982

Neumann H, Gierl A, Tu J, Leibrock J, Staiger D, Zillig W (1983) Organization of the genes for ribosomal RNA in archaebacteria. Mol Gen Genet 192:66–72

Noller HF, Woese CR (1981) Secondary structure of 16S ribosomal RNA. Science 212:403–411

Schlotterbeck E (1984) DNS: rRNS Hybridisierung mit hilfe *in vitro* markierter rRNS. Diploma Thesis, Technische Universität München

Silberklang H, Gillum AM, Rajbhandary UL (1979) Use of in vitro ^{32}P labeling in the sequence analysis of nonradioactive tRNAs. Methods Enzymol 59G:58–109

Tu J, Prangishvilli D, Huber H, Wildgruber G, Zillig W, Stetter KO (1982) Taxonomic relations between archaebacteria including 6 novel genera examined by cross hybridization of DNAs and 16S rRNAs. J Mol Evol 18:109–114

Woese CR, Magrum LJ, Gupta RB, Siegel RB, Stahl DA, Kop J, Crawford N, Brosius J, Gutell R, Hogan J, Noller HF (1980) Secondary structure model for bacterial 16S ribosomal RNA: phylogenetic, enzymatic and chemical evidence. Nucleic Acids Res 8:2275–2293

Woese CR, Olsen J (1986) Archaebacterial phylogeny: perspectives on the urkingdom. System Appl Microbiol 7:161–177

Zillig W, Stetter KO, Wunderl S, Schulz W, Priess H, Scholz I (1980) The *Sulfolobus*—"*Caldariella*" group: taxonomy on the basis of the structure of DNA—dependent RNA polymerases. Arch Microbiol 125:259–269

Zillig W, Tu J, Holz I (1981) *Thermoproteales*—a third order of thermoacidophilic archaebacteria. Nature 293:85–86

Zillig W, Holz I, Janekovic D, Schäfer W, Reiter W-D (1983) The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. System Appl Microbiol 4:88–94

Zillig W, Yeates S, Holz I, Böck A, Rettenberger M, Gropp F, Simon G (1986) *Desulfurolobus ambivalens*, sp. nov. gen. nov., an autotrophic archaebacterium facultatively oxidizing or reducing sulfur. System Appl Microbiol 8:197–203

Zillig W, Holz I, Klenk H-P, Trent J, Wunderl S, Janekovic D, Imsel E, Haas B (1987) *Pyrococcus woesei*, gen. nov. sp. nov, an extremely thermophilic marine archaebacterium, representing a novel order, *Thermococcales*. System Appl Microbiol (in press)

Note added in proof: The organism here termed *Calduplex woesei* has been found to belong to the genus *Pyrococcus* and has been renamed *Pyrococcus woesei*.