
Organization of rRNA structural genes in the archaeobacterium *Thermoplasma acidophilum*

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ABSTRACT

In the archaeobacterium *Thermoplasma acidophilum*, each of the structural genes for 5S, 16S and 23S rRNA occur once per genome. In contrast to those of eubacteria and eukaryotes, they appear unlinked. The distance between the 16S and the 23S rDNA is at least 7.5 Kb, that between 23S and 5S rDNA at least 6 Kb and that between 16S and 5S rDNA at least 1.5 Kb. No linkage between those genes has been found by the analysis of recombinant plasmids carrying Bam HI and Hind III rDNA fragments as by hybridizing those plasmids to fragments of *Thermoplasma* DNA generated by 6 individual restriction endonucleases, recognizing hexanucleotide sequences.

INTRODUCTION

On the basis of comparative sequence analysis of 16S (or 18S) rRNA (1,2) and some other features at the molecular level, the prokaryotic domain has been divided into two distinct kingdoms, the eubacteria and archaeobacteria (3), as different from each other as from the eukaryotes or, rather, eukaryotic cytoplasm. This paper reports on the organization of the rRNA genes (rDNA) of the archaeobacterium *Thermoplasma acidophilum*, first isolated by Darland et al. (4). The genome of *Thermoplasma* contains about 1.7×10^3 Kb (5). On the basis of partial sequencing of the 16S rRNAs (1), 16S rRNA-DNA cross hybridization (6) and RNA polymerase component patterns (7), the organism has been classified as representative of an isolated genus in the thermoacidophilic branch of the archaeobacterial kingdom.

All known eubacterial rDNAs are organized as operons with the typical gene sequence 5'-16S-23S-5S-3' (8). The *E. coli* chromosome contains at least seven copies of such rrn operons. Chloroplast rDNAs have the same gene sequence (9,10,11). In

contrast, eukaryotic rDNA transcription units have the gene sequence 5'-18S-5.8S-28S-3' (12,13). They are transcribed by RNA polymerase A. The 5S rRNA is transcribed from a separate transcription unit by RNA polymerase C (14). In mitochondria, the larger and the smaller rDNA (corresponding both to the 23S and the 16S rRNA genes of eubacteria respectively) are either closely linked as in animal cells (15) or separated by a large spacer (16,17,18,19). 5S rDNA exists only in the mitochondrial genomes of flowering plants. In maize, it is linked to the 18S rDNA, which is separated by 16 Kb from the 28S rDNA (20).

MATERIALS AND METHODS

1. Organisms and Plasmids

Thermoplasma acidophilum, 122-1B2 was obtained from E. A. Freundt, Aarhus, Denmark. E. coli C600. Plasmid pBR322. pRB 224, a pBR322 derivative carrying a fragment of Thermoplasma 23S rDNA, was a generous gift of U. Rexer and R. Schnabel from our laboratory.

2. Growth of Thermoplasma

For growth and labeling of Thermoplasma, the medium described by Darland, et al. (4) was modified by omission of inorganic phosphate and substitution of normal by low phosphate yeast extract prepared in the same manner as the low phosphate peptone by Pinkerton, et al. (21).

3. Gels for RNA

5S rRNA and tRNAs were prepared by electrophoresis through a 10% polyacrylamide gel containing 6 M urea, 0.1% SDS in Tris-borate buffer (22).

For the analysis and preparation of RNA, especially of the large rRNAs, the acid-urea agarose gel described by Rosen et al. (23) was modified. Two buffers were used: buffer A, 0.25 M citric acid-0.5 M β -alanine, pH 3.6, buffer B, 0.25 M citric acid. For every 10 ml of 2% gel, 0.2 g of agarose was dissolved in 2.23 ml of water by heating. 6.67 ml of 9 M urea solution (pre-warmed 50°C) was added. After thorough mixing and rapid cooling to room temperature, 0.75 ml of buffer B, 0.25 ml of buffer A and 0.1 ml of 10% SDS were added. After pouring, the gel was kept at 4°C for at least 4 hr. Before application, 10 μ l

each of the samples were mixed with 2 μ l of 10x sample buffer (containing 1 mM EDTA, 50% sucrose and 2 mg/ml each of xylene cyanol blue, bromophenol blue and orange G) and 7.5 mg of urea heated to 70°C for 2 min. After chilling on ice, 2 μ l of a mixture of 1 volume buffer A and 4 volumes buffer B were added. The electrophoresis was carried out for 16 hr at 5V/cm and 4°C. The running buffer was 10-fold diluted buffer A containing 0.1% SDS.

4. Gels for DNA

DNA fragments larger than 200 bp were fractionated in horizontal agarose gel slabs (24). Hind III digested T5 phage DNA fragments were used as size standards.

Small DNA fragments (20-2000 bp) were fractionated in a modified 5% polyacrylamide-0.5% agarose composite gel as described by Peacock and Dingman (22). To avoid the running front, Tris-phosphate buffer (24) was used instead of Tris-borate buffer. Hae III and/or Alu I digested pBR322 DNA fragments were used as size standards (25).

5. Preparation of Thermoplasma Ribosomes

TEMN buffer, containing 20 mM Tris, 25 mM MgCl₂, 40 mM NaCl and 0.1 mM EDTA, pH 7.8, was routinely employed for Thermoplasma ribosome preparation. Thermoplasma cells were washed twice with 50 mM Na-acetate buffer pH 5.0, then lysed by suspension in TEMN buffer containing 0.01% (final concentration) triton X-100. After low speed centrifugation, the clear cell lysate was centrifuged in a Beckman 60-Ti rotor at 50,000 rpm and 15°C for 6 hr. The ribosome pellet was dissolved in TEMN buffer by shaking at 4°C.

The ribosomes were purified by centrifuging in a 10-30% sucrose gradient in the Beckman SW-41 rotor, for 150-180 min at 35,000 rpm and 15°C.

6. Preparation of rRNAs

The chloroform-phenol-SDS method (26,27) was used for preparation of RNAs from ribosomes. 16S and 23S rRNA were precipitated with 3 M Na-acetate (end concentration) from the aqueous phase (28). The 5S rRNA was recovered from the 3 M Na-acetate supernatant by ethanol precipitation. The RNAs were further purified by gel electrophoresis.

7. Preparation of Labeled rRNA Probes

The ^{32}P -labeled rRNA probes were prepared either by 5'-labeling in vitro (29) or by growing the cells in the medium described above but containing 25 $\mu\text{Ci/ml}$ carrier free ^{32}P phosphate (Amersham). The rRNAs were purified from the ribosomes by phenol extraction followed by gel electrophoresis. For the elution of labeled RNA probes from gels the glycerol gradient electrophoresis method (30) was modified. Instead of sodium chloride, sodium acetate was used. After electrophoresis the gradient was fractionated in 0.3 ml samples.

8. Preparation of DNAs

The Thermoplasma DNA was prepared from the clear cell lysate by phenolization, and then banded twice by equilibrium CsCl centrifugation.

Plasmids were isolated as described by William et al. (31). The ethidium bromide bound to the supercoiled plasmid DNA was extracted with isopropanol.

9. Nick-translation

Nick-translation was carried out according to Rigby et al. (32). 0.1 ml of reaction mixture contained 1 μg of DNA, 50 μCi of $\alpha\text{-}^{32}\text{P}\text{-dCTP}$ (Amersham, sp. act. 400 Ci/mmol) and 10 U of DNA polymerase.

10. Blotting and Hybridization

For hybridization experiments, DNA fragments were usually transferred from gels and covalently linked to DBM (diazobenzyl-oxymethyl) paper (33,34). Fragments larger than 200 bp were transferred onto nitrocellulose (Schleicher & Schuell, BA 85, 0.45 μm) using the method of Southern (35).

For quenching the contaminating rRNA(s) in hybridization experiments, an excess of cold 23S rRNA was added to labeled 16S rRNA probes and an excess of both cold 23S and 16S rRNA was added to labeled 5S rRNA probes. Hybridizations were performed in 50% formamide-5xSET (20xSET containing 1 M Tris, 3 M NaCl and 20 mM EDTA, pH 7.8) at 42°C overnight.

11. Determination of Gene Copy Number

The rRNA gene copy number was determined by quantitative RNA-DNA hybridization as described by De Ley and De Smedt (36). Small filters containing 7-10 μg of immobilized Thermoplasma DNA

were incubated with increasing amounts each of ^{32}P -labeled 23S, 16S and 5S rRNAs (sp. act. about $4 \mu\text{Ci}/\mu\text{g}$) in 2xSET, 25% formamide and the quenching rRNA(s) at 60°C for 16 hr. The filters were then washed with 2xSET and submitted to digestion with $10 \mu\text{g}$ of RNase A and 50 U of RNase T1. The weight of RNA corresponding to the radioactivity remaining on the filters was calculated. The amount of DNA on the filters was determined by the diphenylamine reaction according to Burton (37).

12. Shotgun Cloning

BamH I- or Hind III-digested Thermoplasma DNA and pBR322 were ligated under the conditions described by Dugaiczky et al. (38). Calcium-treated E. coli C600 was transformed with the ligated DNA (39). The transferred cells were incubated at 37°C for 45 min after addition of LB medium.

13. Colony Hybridization

Clones containing Thermoplasma rDNA were identified by colony hybridization (40) using ^{32}P -labeled Thermoplasma rRNAs as hot probes. After hybridizing, the nitrocellulose sheets were treated with RNase A until the radioactivity in the supernatant reached a minimum plateau.

RESULTS

1. The Number of rRNA Gene Copies in the Thermoplasma Genome

The copy number of rRNA genes in the Thermoplasma genome was determined by quantitative rRNA-DNA hybridization.

The saturation of $10 \mu\text{g}$ of Thermoplasma DNA was reached with about 11 ng 23S, 6 ng 16S and 0.5 ng 5S rRNA respectively (Fig. 1). The molecular weight of the 23S rRNA is 1.2×10^6 , that of the 16S rRNA 0.48×10^6 , and that of the 5S rRNA 4×10^4 respectively, that of the Thermoplasma chromosome 1×10^9 . Thus there is only one copy for each of the three rRNA genes within the Thermoplasma genome.

2. Analysis of rDNA Organization in the Thermoplasma Chromosome

To determine the linkage relationship between the 23S, 16S and 5S rRNA genes, Thermoplasma DNA was digested separately with the restriction enzymes Bam HI, Bgl I, Cla I, Eco RI, Hind III and Pst I. The digests were fractionated in 1% agarose gels and the fragment patterns transferred onto DBM paper which was then

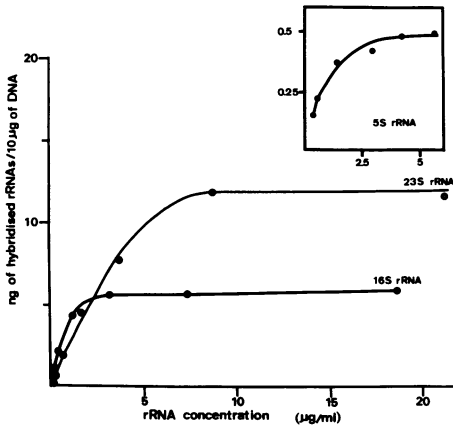


Fig. 1 RNA saturation curves for the hybridization of Thermo-plasma DNA with 5S, 16S and 23S rRNAs.

subjected to hybridization with 5'-³²P-labeled rRNA probes.

The hybridization patterns are shown in Fig. 2. The sizes of the hybridizing fragments are summarized in Table 1(a). Fig. 2 shows 1) The 23S rRNA gene is digested into two fragments each by Bam HI, Bgl I, Hind III and Pst I, whereas it remains intact in the Cla I and Eco RI digests. 2) 16S and 5S rRNA each hybridizes to one (different) fragment only in each of the digests. 3) No DNA fragment hybridizes with more than one of the three rRNAs, i.e. no linkage relationship between 23S, 16S and 5S rRNA genes can be found in any of the six digests.

Examination of the sizes of the hybridizing DNA fragments listed in Table 1(a) suggests that each of the three rRNA genes is far apart from the others. Since no linkage has been found, the sizes of the spacers could be larger than the average fragment size generated by these six enzymes, i.e. 4.1 Kb (4⁶ bp). Restriction enzymes with hexanucleotide recognition sequences should have on the average at least one cleavage site in the spacers of this size. Assuming that all three genes are linked in one operon, it follows from the sizes of the fragments listed in Table 1, that the minimal size of such an operon is 15 Kb, independent of the gene order. For example, the 5S rRNA hybridizes to an 11.2 Kb Bgl I fragment. Even if the 23S and 16S rRNA genes lie just beyond the ends of this fragment, the total size of the operon must be greater than 15 Kb assuming that the 5S rRNA gene lies between the 23S and 16S ones. If one assumes

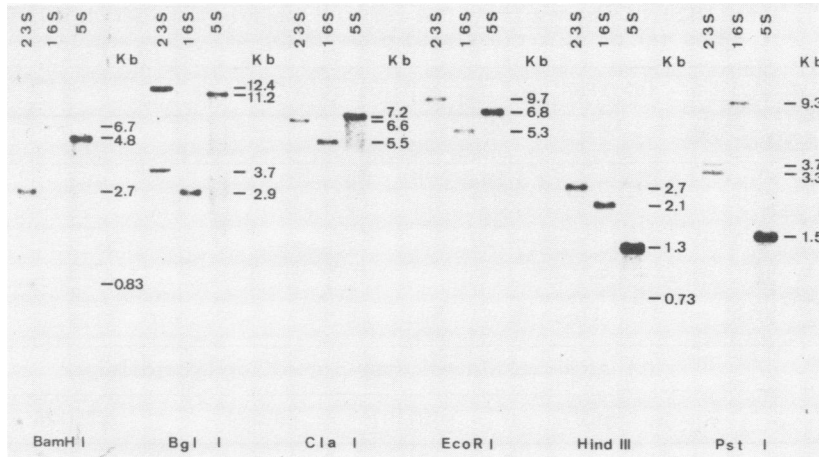


Fig. 2 Hybridization of 5'-³²P-labeled *Thermoplasma* 23S, 16S and 5S rRNAs to restriction endonuclease digested DNA.

that the 16S or 23S rRNA gene lies in the middle, a similar argument can be made from the Pst I and Eco RI digests respectively.

3. Cloning of *Thermoplasma* rDNA and Restriction Mapping

In order to analyse *Thermoplasma* rDNA structure more clearly, Bam HI and Hind III rDNA fragments were cloned into pBR322. Four different plasmids carrying *Thermoplasma* rDNA were isolated. The two from the Bam HI clones are 23S rDNA clones, containing pTB82 and pTB52. One of the two Hind III clones, containing pTH1101, carries 23S rDNA, the other, containing pTH 126, 16S rDNA. No plasmid carrying 5S rDNA has been isolated.

The cloned rDNA fragments have been physically mapped by Bam HI, Bgl I, Cla I, Eco RI, Hind III, Pst I and Taq I (Fig. 3) restrictions. pTB52 is the only plasmid which contains two inserted fragments. The overlaps of the inserts of the plasmids pTB82, pTB52, pTH1101 and pRB224 as shown in Fig. 3 are consistent with the restriction pattern and the hybridization data. Fig. 3(a) shows a common Pst I site in the overlapping inserts of pTB82, pTB52 and pTH1101; and another Pst I site on the far side of the insert of pRB224. The two Pst I sites are about 3.2 Kb apart. Two Bgl I sites in the region covered by the cloned rDNA fragments are separated by about 3.5 Kb. There is an

Table 1. Sizes (Kb) of *Thermoplasma* DNA restriction fragments which hybridize to rRNAs (a) or nick-translated plasmids (b)

enzyme used for digesting <i>Thermoplasma</i> DNA	(a) rRNA used for hybridization			
	23S	16S	5S	
Bam HI	2.7, 0.83	6.7	4.8	
Hind III	2.7, 0.73	2.1	1.3	
Bgl I	12.4, 3.7	2.9	11.2	
Cla I	6.6	5.5	7.2	
Eco RI	9.7	5.3	6.8	
Pst I	3.7, 3.3	9.3	1.5	

	(b) nick-translated plasmid used for hybridization			
	pTB52	pTH1101	pRB224	pTH126
Bgl I	3.7, 1.1★	12.4, 3.7	12.4	2.9
Cla I	6.6, 2.42★	6.6	6.6	5.5, 0.67★
Eco RI	9.7	9.7	9.7, 2.12★	5.3
Pst I	3.7, 3.3	3.7, 3.3	3.3, 3.0★	9.25

★ denotes those fragments which are only found in the hybridization of nick-translated plasmids.

Eco RI site on one side of the region, and a Cla I site on the other. Of these enzymes only Cla I cuts the insert of pTH126, which contains the 16S rRNA gene (Fig. 3(b)).

Taq I sites have been recognized by comparing the restriction patterns of the pBR322 vector and the recombinant plasmids digested by 1) Taq I, 2) Taq I + Bam HI 3) Taq I + Hind III, and 4) Taq I + Bam HI + Hind III. The Taq I sites neighboring either both ends of the inserts, or a Bam HI site or a Hind III site can be directly identified. Taq I sites not in the neighborhood of ends or the Bam HI and the Hind III site can be detected by hybridization, if the stretches between them and other known sites contain rRNA-hybridizing fragments, assuming the absence of intervening sequences in rDNA. This appears to be the case because all Taq I sites identified in this way are located in the terminal sequences of the rRNA genes. The positions of some Taq I sites situated between rDNAs and insert ends have not been identified; in these cases the sizes (bp) of the corresponding restriction fragments are given below the stretch between the two neighboring known Taq I sites. One of the Taq I sites in the cloned 16S rDNA has been recognized as a

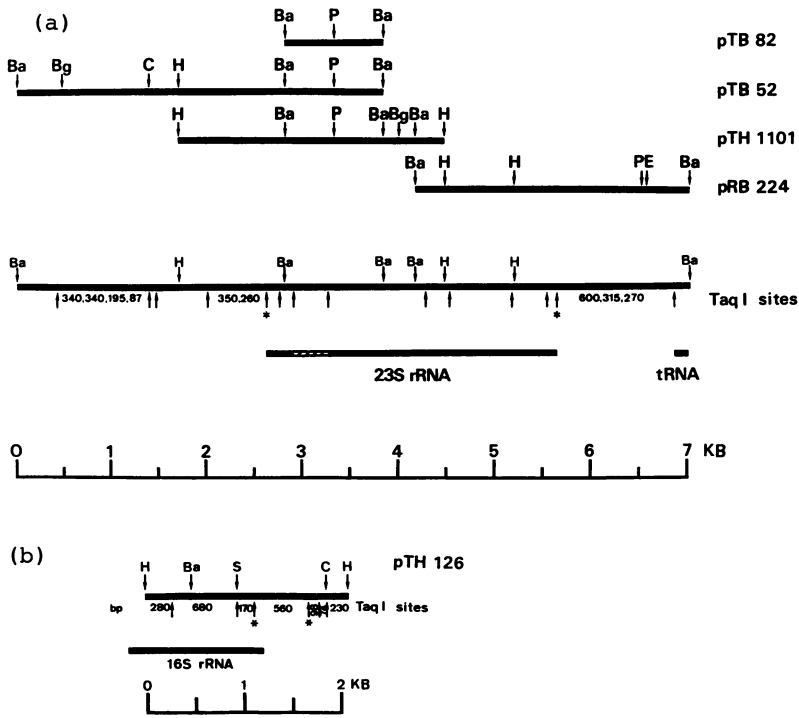


Fig. 3 Physical maps of the Bam HI (Ba), Bgl I (Bg), Cla I (C), Eco RI (E), Hind III (H), Pst I (P), and Taq I restriction sites and the positions of the rRNA genes and tRNA gene(s) in the cloned *Thermoplasma* rDNA fragments. * denotes the Taq I sites which have been identified after rRNA-DNA hybridization (see Fig. 4). The sizes of fragments flanked by two unidentified Taq I cleavages are shown between the neighboring known sites.

Sal I site (Fig. 3(b)). There is no Sal I site in the cloned 23S rDNA fragments.

4. Location of the 16S and 23S rDNA in the Cloned DNA Fragments

To determine the precise positions of rRNA genes in the cloned DNA fragments, 5'-³²P-labeled rRNAs were hybridized to the electrophoretically separated fragments of the plasmids trebly digested by Taq I, Bam HI and Hind III.

Fig. 4 shows the comparison between the restriction fragments and hybridization patterns. The restriction maps of the regions covered by the cloned fragments and the positions of the 23S and

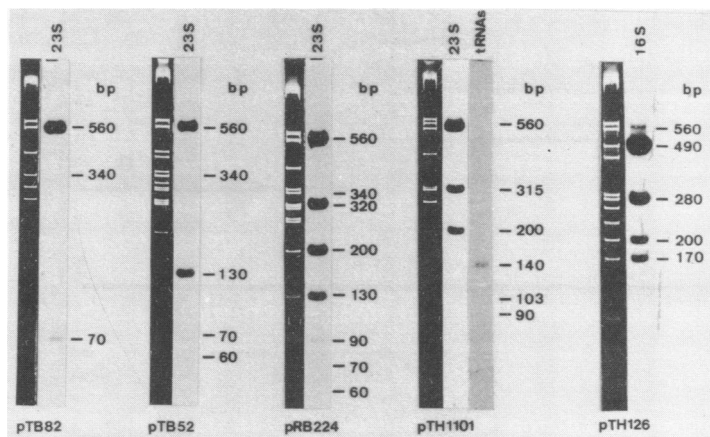


Fig. 4 Hybridization of *Taq* I digested *Thermoplasma* rDNA-carrying plasmids with 5'-³²P-labeled rRNAs and tRNAs. Only the cases yielding positive hybridization are shown.

the 16S rDNA therein are shown in Fig. 3(a) and 3(b) respectively.

The inserts of pTB82, pTB52, pTH1101 and pRB224 cover a DNA fragment with a size of more than 7 Kb. This DNA fragment contains an entire 23S rRNA gene of about 3 Kb, and a tRNA gene or a part of it at one terminus of the region. The distance between the 23S rRNA gene and the tRNA gene is about 1.2 Kb. In this spacer and in the DNA sequence on the other side of the 23S rRNA gene, no 16S or 5S rRNA gene has been detected. A DNA sequence of 340 bp in the inserts of pTB82, pTB52 and pTH1101 (generated by *Taq* I, corresponding to the hatched region on 23S rRNA in Fig. 3(a)) was strongly hybridized by 23S rRNA in some experiments but not in others. This is assumed to be an artifact caused by the secondary structure of a short sequence in 23S rRNA (for example a hairpin) in this region.

The pTH126 has an insert of about 2.1 Kb which contains most of the 16S rRNA gene, i.e. 1.2 Kb. No 23S or 5S rDNA or tRNA gene has been found in the rest of this cloned DNA fragment.

5. Linkage Relationship Analysis of rRNA Genes in the *Thermoplasma* Genome with the Recombinant Plasmids

The whole 5S rRNA gene and a small part of the 16S rRNA gene have so far not been cloned. Linkages between the three rRNA

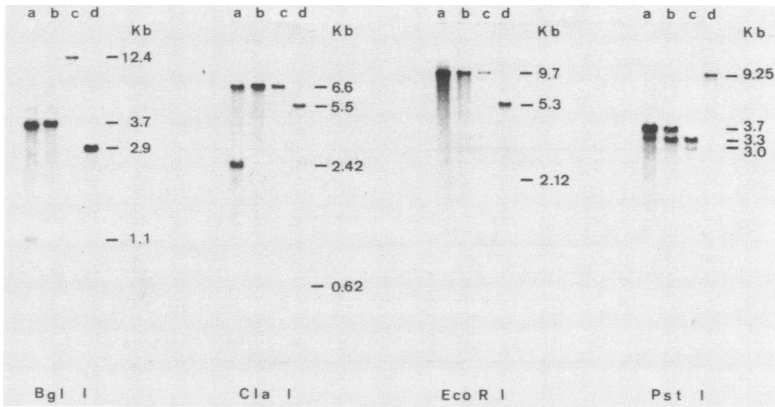


Fig. 5 Hybridization of restricted *Thermoplasma* DNA with the nick-translated *Thermoplasma* rDNA-carrying plasmids pTB52 (track a), pTH1101 (track b), pRB224 (track c) and pTH126 (track d).

genes have not been found in the cloned fragments. The hybridization of nick-translated plasmids to electrophoretically separated Bgl I, Cla I, Eco RI and Pst I fragments of *Thermoplasma* DNA can expand the analysis into the regions flanking the cloned DNA sequences up to the next sites for these restriction enzymes (remember that the rDNA has been cloned with Bam HI and Hind III).

The result is shown in Fig. 5; the sizes of the hybridizing bands are summarized in Table 1(b). In addition to the known rDNA containing fragments listed in Table 1(a), some new bands appear (see Table 1(b), marked with stars (*)). These represent fragments flanking the cloned sequences which do not hybridize with 23S and 16S rRNA, but with the plasmids carrying rDNA. By relating the sizes of these bands to the known Bgl I, Cla I, Eco RI and Pst I restriction sites in the inserts (Fig. 3), the next sites for these enzymes to either side of the cloned region have been determined (Fig. 6).

Fig. 6 shows the thus expanded 16S rRNA-gene containing region of about 6.3 Kb, with the next Cla I sites at the ends. It contains two DNA fragments of about 3.4 (left) and 1.5 Kb (right) respectively, to either side of the 16S rRNA gene. The detected 23S rRNA-gene containing DNA sequence of about 20 Kb

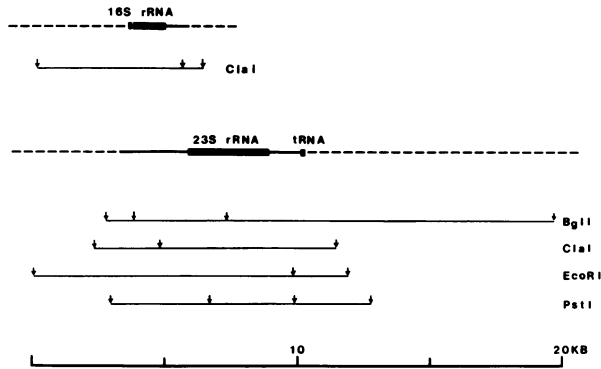


Fig. 6 Schematic representation of the fragments flanking the cloned *Thermoplasma* rDNA-containing regions. The cloned *Thermoplasma* rDNA-containing regions are represented by continuous lines. The fragments flanking the regions detected by hybridizing restricted *Thermoplasma* DNA with nick-translated rDNA carrying plasmids are represented by broken lines. The positions of the 23S rRNA gene, the 16S rRNA gene, and tRNA gene(s) are represented by bars.

between an Eco RI site (left) and a Bgl I site (right) contains DNA fragments of about 6 (left) and 11 Kb (right) respectively flanking the 23S rRNA gene. No fragment hybridizing both with the 16S rDNA-carrying plasmid and the group of 23S rDNA-carrying plasmids has been discovered. Thus the distance between the 16S rRNA gene and the 23S rRNA gene in the *Thermoplasma* genome should be at least as large as the sum of the sizes of the shorter DNA fragments flanking both rRNA genes in Fig. 6, i.e. 7.5 Kb (1.5 + 6 Kb).

By comparing the hybridization patterns in Fig. 2 with those in Fig. 5 and the sizes of the hybridized fragments in Table 1(a) and (b), no linkage relationships (common hybridized bands) between 5S and 23S or 5S and 16S rRNA genes have been found. This means that if all six enzymes would cut *Thermoplasma* DNA just beside the 5S rRNA gene, the distances between the 5S and the 23S genes would be at least 6 Kb and that between the 5S and the 16S rRNA genes at least 1.5 Kb.

DISCUSSION

It is questionable if there is only one promoter for all

three apparently unlinked rRNA genes in Thermoplasma. Even in Neurospora mitochondria, where a spacer of about 5 Kb separates the 19S rRNA gene and the 25S rRNA gene (41,42), separate precursors for both rRNA have been clearly demonstrated by Green et al. (43,44), i.e. the two rRNA genes are transcribed from two independent transcription units. The spacers between Thermoplasma rRNA genes are larger (with the possible exception of the spacer between 16S and 5S rRNA genes, which must still be at least 1.5 Kb). As expected for the existence of separate transcription units, no common transcript containing more than one rRNA has been observed even after short ^{32}P -pulse labeling of Thermoplasma cells. Two precursors of 16S rRNA, with molecular weights of 0.59×10^6 and 0.51×10^6 , do not hybridize to 5S rRNA-gene containing DNA fragments (data not shown). The rDNA organization of another archaebacterium Halobacterium halobium (45) is completely different from that of Thermoplasma. Hofman et al. reported the occurrence of one set of rDNA per Halobacterium chromosome with the gene sequence 5'-16S-23S-5S-3' as always found in eubacteria. No common precursor for 16S and 23S rRNA was detected. So far these are the only cases where archaebacterial rDNA organization has been described. The question remains whether in archaebacteria the genes coding for the three rRNAs are generally located in more than one transcription unit, or only Thermoplasma which has a rather isolated position in the thermoacidophilic branch of the archaebacterial urkingdom, possesses a unique rDNA organization. This question will be answered by studying other archaebacteria.

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