Divergent Evolution of 5S rRNA Genes in *Methanococcus*

Günter Wich[a], Lionel Sibold[b], and August Böck[a]

[a] Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Straße 1a, D-8000 München 19, Bundesrepublik Deutschland
[b] Unité de Physiologie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, Paris, Rue du Dr. Roux 28, F-75724 Paris Cedex 15, France

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

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The organization of genes for 5S rRNA in the methanogenic archaebacterium *Methanococcus (M.) voltae* and their nucleotide sequences have been determined. *M. voltae* possesses three 5S rRNA genes, one of them is organized in an rRNA transcriptional unit coding for 16S-23S-5S rRNA. The other two are associated with seven tRNA genes in a putative transcriptional unit composed of 5′-tRNA^{Thr}-tRNA^{Pro}-tRNA^{Tyr}-tRNA^{Lys} – 5S rRNA-tRNA^{Asp}-tRNA^{Lys} – 5S rRNA-tRNA^{Asp}-3′. Coding regions plus spacers of the tRNA^{Lys}-5S rRNA-tRNA^{Asp} block of this gene cluster occur twice with identical sequence. The 5S rRNA from this cluster displays considerable sequence divergence to the rRNA operon-linked 5S rRNA gene. Comparison of the *M. voltae* 5S rRNA sequences with those from *M. vannielii* revealed that the operon-linked genes on one hand and the tRNA-linked 5S genes on the other share a greater sequence homology than the two types of genes within each of the two organisms. This indicates an independent evolution of the two sets of 5S rRNA genes without selective pressure from other ribosomal components or, alternatively, lateral gene transfer.

Introduction

Genes for stable RNA species are excellent model systems for the analysis of genome organization and gene expression. Since they are universal cell components and since they have been strongly conserved during evolution their sequence comparison has provided a wealth of information on the evolution of macromolecules and of organisms [1].

One of the peculiar features of the organization of stable RNA genes in archaebacteria concerns the high diversity in which these genes are organized on the chromosome. They may be linked in typical eubacterial-type transcriptional units as in extreme halophiles [2, 3] or as in methanogens like *Methanobacterium* [4, 5] or they may be completely unlinked as in *Thermoplasma* [4]. Other organisms like *Methanococcus (M.)* possess a "mixed" type of rRNA gene organization. In *M. vannielii,* for example, there are four 16S-23S-5S rRNA operons [6] and, in addition, a single "extra" 5S rRNA gene clustered in a transcriptional unit with seven genes for tRNA [7]. The four operon-linked 5S rRNA genes have an identical sequence which differs considerably from that of the "extra" 5S rRNA gene. The products of both gene types are functional since analysis of 5S rRNA extracted from the ribosomes indicated the presence of the products of both genes.

The high sequence polymorphism within a single genome could be the result of either a lateral gene transfer event, *e.g.* of the "extra" 5S rRNA gene, or of the independent evolution of the two types of genes after a gene duplication event. To gain information on this interesting question we have analyzed the organization and the primary structure of the 5S rRNA genes from *M. voltae,* an organism closely related to *M. vannielii.*

Materials and Methods

Strains and Plasmids

Methanococcus (M.) voltae strain PS (DSM 1537) was obtained from the German Collection of Microorganisms, Göttingen, F.R.G. Chromosomal DNA fragments from *M. voltae* were cloned into plasmid pUC9. For subcloning, plasmid vector pUC19 was used [8]. Recipient *E. coli* strains in transformation experiments were *E. coli* 7902, *E. coli* JM105 and *E. coli* JM101, respectively.

Recombinant DNA techniques

A method for isolation of high molecular weight chromosomal DNA from *Methanococcus* cells has

Reprint requests to Dr. A. Böck.

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been described [9]. Standard recombinant DNA techniques like preparation of plasmid DNA, restriction-enzyme analysis, recovery of DNA fragments from agarose gels and radioactive labelling of DNA fragments were carried out as given by Maniatis *et al.* [10].

Hybridization experiments with DNA and RNA were performed as described by Southern [11]. The modification of Johnson *et al.* [12] was used to lower unspecific background. DNA-DNA-hybridizations were carried out at 65 °C; for RNA-DNA-hybridizations the conditions were 37 °C and presence of 50% formamide. Detection of recombinant plasmids containing 5S rRNA genes was by colony hybridization [13].

DNA sequence analysis

Sequence analysis was carried out by the chemical cleavage method [14, 15]. Alternatively, the chain termination method was used [16], following the modification of Chen and Seeburg [17] for double stranded DNA. Both DNA strands were sequenced throughout.

Purification of RNA

16S rRNA and 23S rRNA were prepared from 30S and 50S ribosomal subunits of *Methanococcus* as outlined by Jarsch *et al.* [6]. 5S rRNA and tRNA were purified from bulk RNA of *Methanococcus* by electrophoresis in urea/polyacrylamid gels [18]. The separated RNA species were recovered by electroelution. The 5′ ends of RNA were labelled with polynucleotide kinase and [γ^{32}P]ATP according to Maniatis *et al.* [10].

Results and Discussion

Genomic organization of 5S rRNA genes in Methanococcus voltae

Chromosomal DNA from *M. voltae* was digested with the restriction endonucleases *Eco*RI and *Hind*III, respectively. The fragments generated were size-separated in agarose gels, transferred to nitrocellulose filters [11] and hybridized to electrophoretically purified 5S rRNA of *M. vannielii*, a closely related organism. For each restriction enzyme two DNA fragments were found to hybridize with 5S rRNA, a 4.2 kb and 2.65 kb *Hind*III and a 11 kb and 8.5 kb *Eco*RI fragment (Fig. 1). The hybridization intensities of the fragments differed: the 4.2 kb *Hind*III fragment and the 8.5 kb *Eco*RI fragment showed much stronger hybridization signals. Longer exposure (72 h instead of 3 h as for Fig. 1) of the autoradiograph yielded an additional, very weak *Hind*III band of 5.2 kb (not shown).

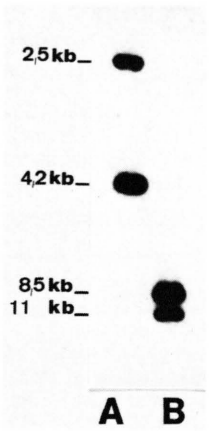


Fig. 1. Autoradiograph of a Southern blot of *M. voltae* genomic DNA cut with *Hind*III (lane A) and *Eco*RI (lane B), respectively, and hybridized to 5′[^{32}P]-labelled 5S rRNA. The length of the hybridizing DNA fragments is indicated.

The three *Hind*III restriction fragments were cloned into the vector pUC9 and screened by colony hybridization [13]. Positive clones carrying the 4.2 kb *Hind*III fragment (pS1), the 2.65 kb *Hind*III fragment (pS2) and the weakly hybridizing 5.2 kb fragment (pS3) were obtained.

To determine the genetic organization of the genes cloned, the recombinant plasmids were hybridized to purified 16S, 23S, 5S rRNA and to tRNA from *M. vannielii* (not shown). The 2.65 kb and the 5.2 kb *Hind*III fragments of pS2 and pS3 hybridized with 23S rRNA, the 5.2 kb fragment showed additional hybridization with 16S rRNA and bulk tRNA. No significant 5S hybridization activity could be obtained with pS3; the weak signal mentioned above is, therefore, most probably caused by a minor contamination of tRNA in the purified 5S rRNA. The 4.2 kb *Hind*III fragment of pS2 hybridized only with 5S rRNA and bulk tRNA.

The organization of ribosomal RNA genes of *M. voltae* consistent with the plasmid hybridization patterns described is given in Fig. 2a and 2b. *M. voltae* possesses only one rRNA operon, combining genes for 16S, 23S and 5S rRNA. This operon is cut

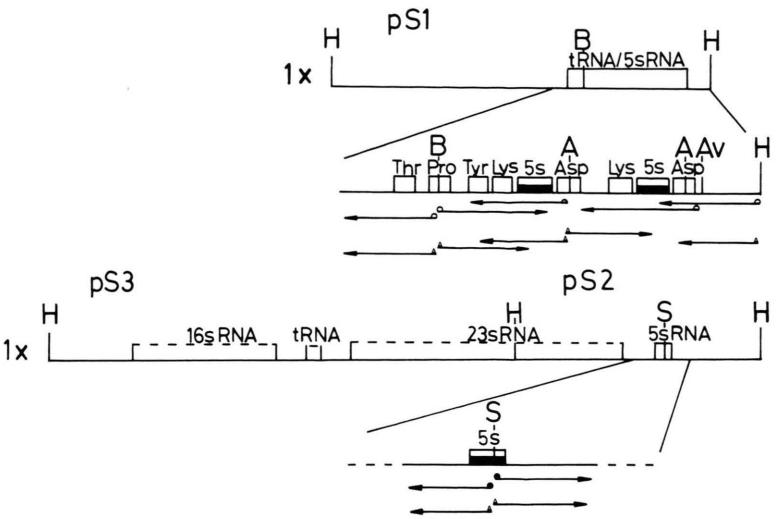


Fig. 2. Cloning and sequencing strategy of the rRNA genes of *M. voltae* and their putative organization. The sequenced regions are drawn out enlarged. Coding regions for 5S rRNA and tRNA genes are boxed. The broken lines in part (b) indicate the approximate location of the 16S and 23S rRNA genes from *M. voltae* as suggested by hybridizations (see text). The amino acid designations denote the presumed specificity of the respective tRNA genes. Arrows give the sequencing strategy: triangles indicate 3′ labelled, unfilled semicircles 5′ labelled restriction sites for sequencing by the Maxam-Gilbert procedure [17]. For sequences marked by filled circles the chain termination sequencing method [16] was used. Abbreviation for restriction sites are: (A) *Ava*I; (Av) *Ava*II; (B) *Bam*HI; (H) *Hind*III; (S) *Sca*I.

in two pieces by a *Hind*III site within the 23S rRNA gene. This *Hind*III site is conserved in the 23S rRNA gene of the closely related methanogen *M. vannielii* [19]. The two fragments are present in pS2 (5S rRNA gene and 3′ half of the 23S rRNA gene) and pS3 (5′ half of the 23S rRNA gene and the 16S rRNA gene). A tRNA gene is presumably located in the 23S-16S rRNA intercistronic spacer.

In addition to the transcriptional unit for 16S-23S-5S rRNA, *M. voltae* contains an unlinked 5S rRNA cistron which is carried by pS1. The respective fragment of pS1 also hybridized with bulk tRNA.

The rRNA gene organization of *M. voltae* delineated from these results resembled that of *M. vannielii* [6] in that both organisms possess eubacterial type transcriptional units for 16S-23S-5S rRNA and additional "extra" 5S rRNA genes; however, in contrast to *M. voltae, M. vannielii* contains four rRNA operons.

Sequence analysis of the "extra" 5S rRNA gene

A physical map and the sequencing strategy for the 5S rRNA hybridizing part of the *Hind*III insert of plasmid pS1 are presented in Fig. 2a. The detailed analysis of the DNA sequence revealed the presence of seven tRNA gene-like structures and two complete 5S rRNA sequences (see Fig. 2a and 3). The amino acid specificities of the seven tRNAs deduced from the anticodon sequences are: $tRNA^{Thr}$ (UGU); $tRNA^{Pro}$ (UGG); $tRNA^{Tyr}$ (GUA); $tRNA^{Lys}$ (UUU) and $tRNA^{Asp}$ (GUC).

As shown in Fig. 3, the sequence block $tRNA^{Lys}$-5S rRNA-$tRNA^{Asp}$ and its intercistronic spacers are exactly repeated within the tRNA-5S rRNA cluster. To prove that this structure indeed exists in the chromosomal DNA of *M. voltae* and is not due to a cloning artifact, rehybridization experiments were carried out. *M. voltae* chromosomal DNA was digested with *Hind*III and double digested with *Hind*III and *Ava*I, respectively, and the fragments were electrophoresed side by side with the respective fragments from plasmid pS1. Hybridization was performed with the gel-purified *Ava*I fragment, which contains one of the $tRNA^{Lys}$-5S rRNA-$tRNA^{Asp}$ repeats (results not shown). The *Hind*III digest of chromosomal DNA delivered a fragment, which had the same length as the corresponding cloned fragment. The *Hind*III/*Ava*I double digest delivered two genome fragments, one identical to the 3.5 kb *Hind*III-*Ava*I fragment and one identical to the 0.38 kb *Ava*I-*Ava*I fragment of the cloned restriction fragment of pS1 (see Fig. 2a). The arrangement of 5S and tRNA genes on plasmid pS1 (see Fig. 3), therefore, exactly reflects their chromosomal organization.

In *M. vannielii,* an "extra" 5S rRNA gene is also combined with tRNA genes in an operon-like structure [7]. Both the order of the genes and the sequence of the coding regions are highly conserved.

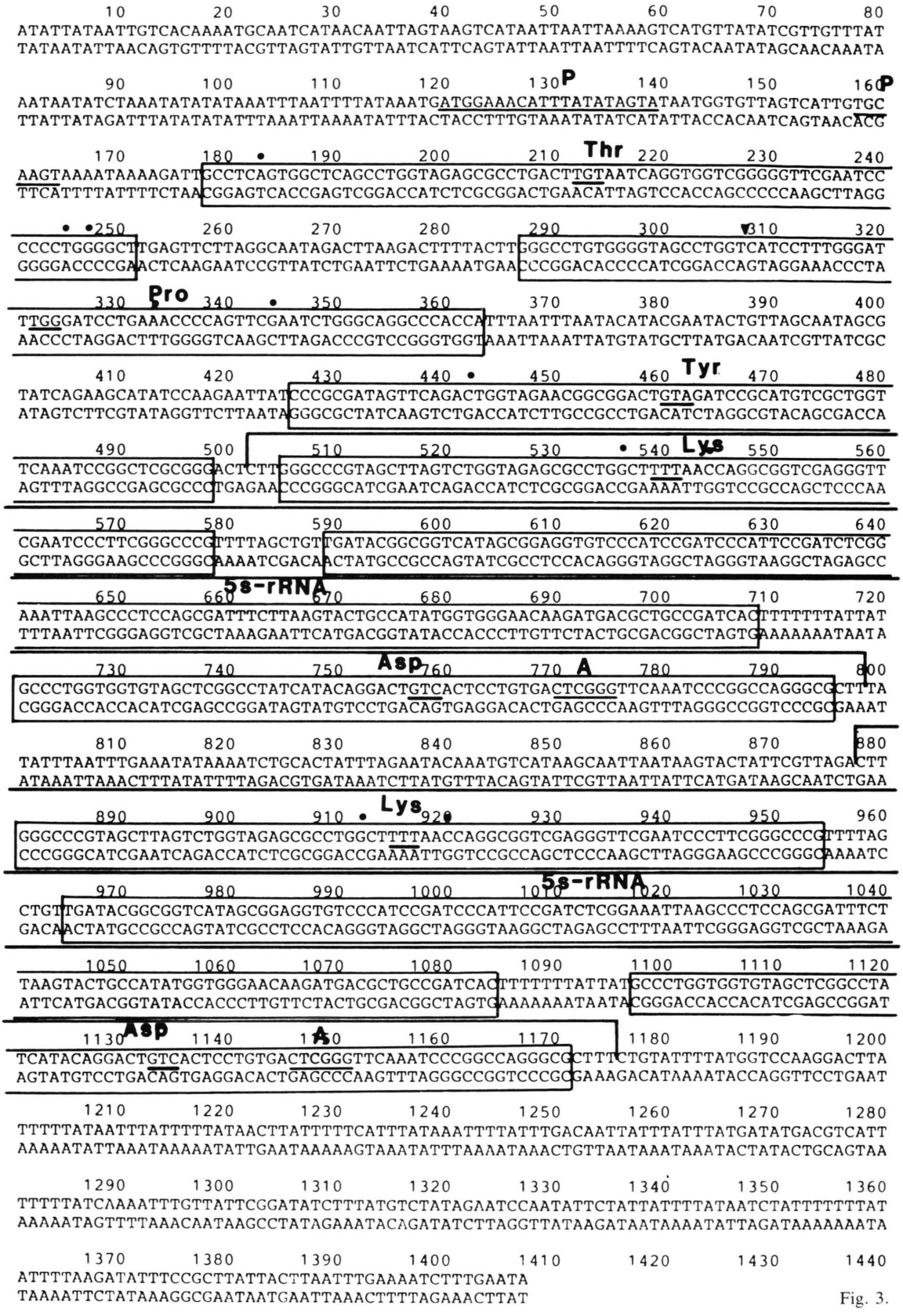


Fig. 3.

The only difference between the gene arrangement in the tRNA/5S rRNA clusters of *M. voltae* and *M. vannielii* is that just the $tRNA^{Lys}$ and the $tRNA^{Asp}$ gene sequence is tandemly repeated in the operon of *M. vannielii* [7]. A possible reason for the existence of the duplicated "extra" 5S rRNA gene in *M. voltae* could reside in the fact that this organism possesses only one rRNA operon-linked (*versus* 4 of *M. vannielii*) 5S rRNA gene. There is, however, no information yet on any selective advantage of a surplus of 5S rRNA genes over those for 16S and 23S rRNA.

As mentioned above the coding regions of the 5S rRNA/tRNA operon in *M. voltae* and *M. vannielii* are highly conserved. The coding regions for $tRNA^{Thr}$ and $tRNA^{Pro}$ differ from those of *M. vannielii* in three positions, those for $tRNA^{Lys}$ and $tRNA^{Tyr}$ in two and one position, respectively; the sequences for $tRNA^{Asp}$ are identical. The sequence differences presumably do not alter the secondary structure of the respective tRNA molecules since they either occur in loop regions or – when in stems – are accompanied by a compensatory change.

It is interesting to note that the $tRNA^{Pro}$ gene, as in *M. vannielii* [7], seems to encode the 3′ terminal CCA end in the DNA structure. Since the homology is very low outside of the coding regions this conservation clearly indicates that this CCA is indeed transcribed into the mature tRNA structure. This is in contrast to tRNA genes sequenced so far from other archaebacteria (for review see [20]).

5S rRNA genes

There is high sequence heterogeneity between the rRNA operon-linked and the "extra" 5S rRNA genes of *M. vannielii* [7, 21]. They differ in no less than 13 positions. Such a degree of heterogeneity in rRNA genes of a single organism is unique for procaryotes but has been reported for some eukaryotic systems (for review see [22]).

To extend this analysis to the two types of 5S rRNA genes in *M. voltae,* the operon-linked 5S rRNA gene of pS2 was subcloned into plasmid vector pUC18 using a *Sca*I restriction site; the sequence of the cloned operon-linked 5S rRNA gene was determined.

As shown in Fig. 4a, the 5S rRNA genes of *M. voltae* also display considerable sequence polymorphism. The "extra" 5S rRNA gene diverges from that of the rRNA operon-linked one in eleven positions (Fig. 4a, 4b). None of these differences results in an altered secondary structure (Fig. 4a).

As shown in Fig. 4b and 4c, the sequences of the operon-linked 5S rRNA genes on one hand and of the "extra" 5S rRNA genes on the other are highly conserved in the two species. Fig. 4c demonstrates the unusual fact that the two types of 5S rRNA genes in one organism (the operon-linked 5S rRNA gene and the "extra" 5S rRNA gene) are less related to each other than the respective 5S rRNA gene types in the two organisms.

There are several conclusions which can be drawn from this observation:

(i) The different relative ratio of operon-linked and "extra" 5S rRNA gene copies, one operon-linked and two "extra" 5S rRNA genes in *M. voltae versus* four operon-linked and one "extra" 5S rRNA gene in *M. vannielii,* suggests that the products of both 5S rRNA gene types, despite of the considerable differences in primary structure, are functionally equivalent.

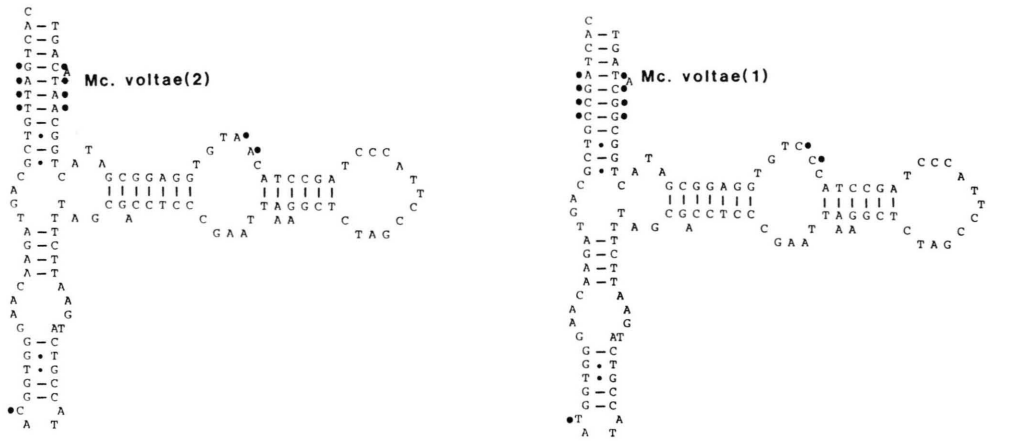
(ii) All operon-linked 5S rRNA genes of *M. vannielii* have an identical sequence [19]. Since the products of both types of 5S rRNA genes are functionally equivalent the conservation of the operon-linked 5S genes is not due to biological pressure on the primary structure but to an rRNA operon specific mechanism, *e.g.* randomization of mutations by recombination events.

(iii) The relationship between the different gene types suggests that they must have evolved in an ancestral methanogen before division into the two species by duplication of an ancestral 5S rRNA gene and divergent development, or by horizontal gene transfer.

(iv) The surprisingly high sequence heterogeneity of 5S rRNA genes in a single organism restricts the value of phylogenetic trees based on 5S rRNA, espe-

Fig. 3. Sequence of the tRNA/5S rRNA gene cluster of *M. voltae.* Regions coding for tRNA and for 5S rRNA genes are boxed. The amino acid specificities of the tRNAs are given above the sequence. Dots indicate positions in the tRNA nucleotide sequences which differ from the sequences of the equivalent genes in *M. vannielii* [7]. The anticodon triplet of each tRNA is underlined. Two sequence boxes which are homologous to the putative promoter consensus sequence of stable RNA genes from *M. vannielii* [23] are marked. The *Ava*I restriction sites (A) used for rehybridization experiments (see text) are indicated.

4a



4b

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TGATACGGCGGTCATAGCGGAGGTGTCCCATCCGATCCCATTCCGATCTCGGAAATTAAGCCCTCCAGCGATTTCTTAAGTACTGCCATATGGTGGGAACAAGATGACGCTGCCGATCAC   M.voltae(1)
TGATACGGCGGTCATAGCGGGGGTGTAACATCCGATCCCATCCCGATCTCGGAAATTAAGCCCTCCAGCGATTCCTTAAGTACTGCTATCTAGTGGGAACAAGGTGACGCTGCCGATCAC   M.vannielii(1)
TGACATAACGGTCATAGCGGAGGTGTAACATCCGATCCCATTCCGATCTCGGAAATTAAGCCCTCCAGCGATTTCTTAAGTACTGCCATACGGTGGGAACAAGATGACGCTGTTAGTCAC   M.voltae(2)
TGACATAACGGTCAAAGCGGAGGTGTAACATCCGATCCCATCCCGATCTCGGAAATTAAGCCCTCCAGCGATTCTTTAAGTACTGCTATCTAGTGGGAACAAAGTGACGCCGTTAGTCAC   M.vannielii(2)
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4c

	a)	b)	c)	d)
a) Mc.voltae(1)	-	9	11	20
b) Mc.vannielii(1)	93	-	16	13
c) Mc.voltae(2)	91	86.5	-	11
d) Mc.vannielii(2)	83	89	91	-

Fig. 4. Primary and secondary structure of the 5S rRNA gene products from *M. voltae:* (a) Secondary structure of the "extra" 5S rRNA (1) and the operon-linked 5S rRNA gene transcripts (2). Differences in primary structures are marked by dots. (b) Comparison of the 5S rRNA-types of *M. voltae* and *M. vannielii.* Numbers in brackets denote the gene type: (1), "extra" 5S rRNA genes; (2), operon-linked 5S rRNA genes. Bases different from the *M. voltae* "extra" 5S rRNA gene transcript are indicated. Two regions which are highly conserved in the "extra" 5S rRNAs and the operon-linked 5S rRNAs, respectively, are boxed. (c) 5S rRNA sequence homologies between *M. voltae* and *M. vannielii.* Numbers in the upper right-hand triangle denote the differences in primary structure. Numbers in the lower left-hand triangle are percent sequence homologies.

cially when sequences are compared between organisms possessing different copy numbers of these genes.

Intercistronic spacers

The tRNA genes and the 5S rRNA genes encoded by pS1 are organized in an identical transcriptional orientation and they are separated by only short spacers (Fig. 5). Whereas the coding regions are highly homologous the spacer regions differ both in length and nucleotide sequence. Where conserved features are present within the spacers they are considered to possess a biological functions:

(i) Two homology boxes are present in the 5′ flanking regions. These conserved sequence motifs meet the consensus sequence of the promotor postulated for transcription of stable rRNA genes in *Methanococcus* [20, 23].

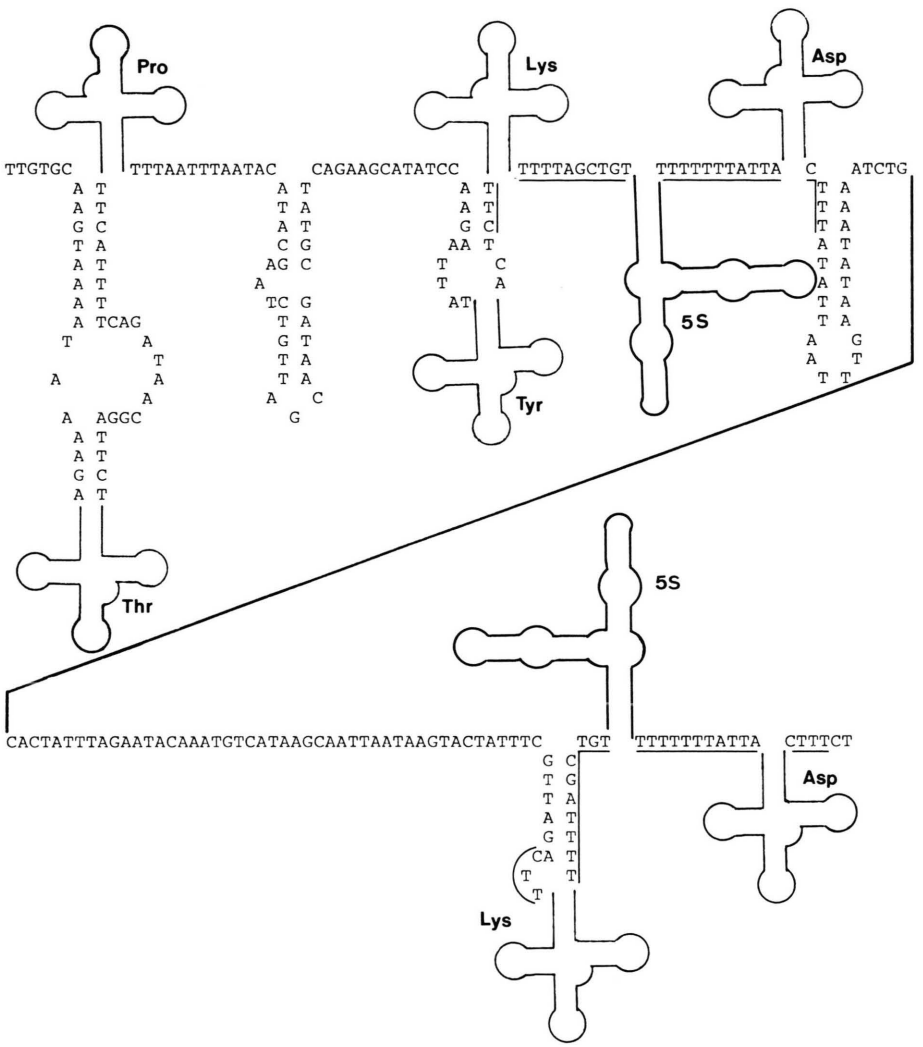

Fig. 5. Possible secondary structure of the tRNA/5S rRNA operon transcript. The sequence of the $tRNA^{Lys}$-5S rRNA-$tRNA^{Asp}$ gene block which is tandemly repeated is underlined.

(ii) The secondary structure of the putative 5S rRNA/tRNA transcript tends to separate the mature tRNA sequences by stem/loop structures (Fig. 5). Similar potential recognition sites for tRNA processing have been found for many procaryotic systems (for review see [20]).

(iii) The tRNA and 5S rRNA gene flanking regions in *M. voltae* and in *M. vannielii* share a high A−T content but otherwise very low homology. Nevertheless, a short conserved sequence motif seems noteworthy: the coding sequences for $tRNA^{Asp}$, which are part of the repeated sequence blocks in the *M. voltae* tRNA/5S rRNA cluster and in the *M. vannielii* cluster are followed in each of the four examples by a conserved sequence of four bases, namely $^{5'}CTTT^{3'}$. Homology drops down immediately after this four-base-motif. An identical sequence was found surrounding the $tRNA^{Ala}$ gene, which is located within the 16S−23S rRNA gene spacer in *M. vannielii* and was thought to participate in processing of this tRNA gene [21].

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