Nucleic Acids Research, 2001, Vol. 29, No. 22 **4699–4706**

Post-transcriptional modification in archaeal tRNAs: identities and phylogenetic relations of nucleotides from mesophilic and hyperthermophilic *Methanococcales*

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Received July 10, 2001; Revised and Accepted September 19, 2001

ABSTRACT

Post-transcriptional modifications in archaeal RNA are known to be phylogenetically distinct but relatively little is known of tRNA from the *Methanococci*, a lineage of methanogenic marine euryarchaea that grow over an unusually broad temperature range. Transfer RNAs from *Methanococcus vannielii*, *Methanococcus maripaludis*, the thermophile *Methanococcus thermolithotrophicus*, and hyperthermophiles *Methanococcus jannaschii* and *Methanococcus igneus* were studied to determine whether modification patterns reflect the close phylogenetic relationships inferred from small ribosomal subunit RNA sequences, and to examine modification differences associated with temperature of growth. Twenty-four modified nucleosides were characterized, including the complex tricyclic nucleoside wyosine characteristic of position 37 in tRNA^{Phe} and known previously only in eukarya, plus two new wye family members of presently unknown structure. The hypermodified nucleoside 5-methylaminomethyl-2-thiouridine, reported previously only in bacterial tRNA at the first position of the anticodon, was identified by liquid chromatography-electrospray ionization mass spectrometry in four of the five organisms. The ribose-methylated nucleosides, 2′-*O*-methyladenosine, N^2,2′-*O*-dimethylguanosine and N^2,N^2,2′-*O*-trimethylguanosine, were found only in hyperthermophile tRNA, consistent with their proposed roles in thermal stabilization of tRNA.

INTRODUCTION

Despite evolutionary constraints placed on tRNA structure to produce overall correct folding of the molecule for accurate translation of the universal genetic code, tRNA sequences and post-transcriptional modifications vary considerably and in a mutually interactive—and poorly understood—fashion across the three phylogenetic domains of life. More than 80 modified ribonucleosides have been identified in tRNAs (1); these include a small group of modifications observed in tRNAs of almost all organisms (2). In addition to this conserved core, Archaea, Bacteria and Eucarya each make phylogenetically characteristic modifications to their tRNAs following transcription.

In their biologically active forms, proteins and RNAs must adopt specific secondary and tertiary structures that may be entropically unfavorable. Consequently, at high temperatures the macromolecules are subject to denaturation, forming entropically favored, but biologically inactive and potentially deleterious products. Although proteins are frequently processed post-translationally by covalent modification, these modifications are usually not important determinants of protein stability. In contrast, modifications introduced into RNA after transcription have been shown to exert clear effects on stability (3,4). Whereas modifications in the anticodon region of tRNAs can dramatically alter codon specificity (5) and aminoacyl-tRNA synthetase recognition (6), others appear to provide significant stabilization to the folded tRNA (7–9 and references therein). This effect is substantial in hyperthermophilic Archaea growing at temperatures that would otherwise denature unmodified tRNAs (4).

In the mesophilic euryarchaeon *Haloferax volcanii*, for which extensive tRNA sequence data are available (10,11), ~11% of the nucleotides in each tRNA are modified. Most of these modifications consist of base or ribose methylations of

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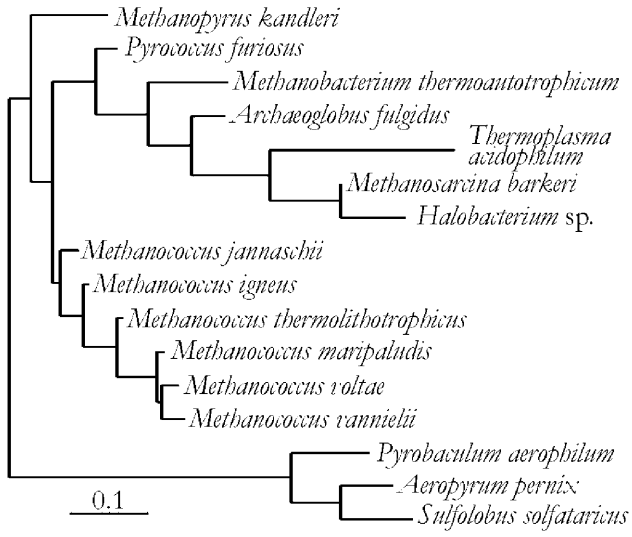

Figure 1. Phylogeny of small subunit ribosomal RNA genes from *Methanococcales* and Archaea for which genomic sequence information is available. This tree was extracted from the full prokaryotic tree (release 7.1) of the Ribosomal Database Project (63). The scale bar represents 0.1 nucleotide replacements per position.

nucleosides whose sequence locations are generally conserved among tRNA species. Analyses of tRNA hydrolysates from other archaea including the thermophiles *Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*, *Thermoplasma acidophilum*, *Sulfolobus solfataricus*, *Pyrodictium occultum*, *Thermoproteus neutrophilus*, *Pyrococcus furiosus* and *Pyrolobus fumarii* have identified numerous modified nucleotides, including many unique to the Archaea (4,12–16).

The *Methanococcales* comprise an order of marine Archaea that use H_2, CO_2 and formate (some species) to perform methanogenesis. All species are motile, irregular cocci and most grow chemolithotrophically. Despite their morphological and physiological similarities, the species are ecologically, genomically and phylogenetically diverse (Fig. 1) (17). The mesophilic *Methanococci* (such as *Methanococcus vannielii*, *Methanococcus voltae* and *Methanococcus maripaludis*) grow at ambient temperatures and were isolated from marine marshes and estuaries. Thermophilic *Methanococcus thermolithotrophicus*, initially isolated from geothermally heated marine sediment (18), grows optimally at 65°C. In contrast, the hyperthermophilic species *Methanococcus jannaschii* and *Methanococcus igneus*, isolated from submarine hydrothermal vents, grow optimally near 91°C (19). This broad range of optimal growth temperatures among a phylogenetically related group of organisms has made the *Methanococcales* an ideal model system for comparative analyses of thermal adaptation (17,20,21).

An early survey of nucleosides from *M.vannielii* tRNAs identified significant amounts of 1-methyladenosine (m^1A), 1-methylguanosine (m^1G), N^2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine (m^2_2G), pseudouridine (ψ) and 2′-*O*-methylcytidine (Cm), with trace quantities of 2-thiocytidine (s^2C) and 4-thiouridine (s^4U) (12). In contrast to analyses of bacterial and eukaryotic tRNA nucleosides, no significant 7-methylguanosine (m^7G) or 5-methyluridine (m^5U) was detected in *M.vannielii* tRNAs, which supported the distinctiveness of the Archaea as a fundamental phylogenetic domain. Results described here greatly extend this inventory of modified nucleosides in the *Methanococci* by identifying rare and hypermodified nucleosides in five species of the genus *Methanococcus*. This study was designed in part to provide a close comparison of the effect of clear differences in wild-type temperature of growth among closely related archaea, to gain insights into which modifications play thermostabilizing roles.

MATERIALS AND METHODS

Cell sources and growth of cultures

Methanococcus jannaschii JAL-1^T (DSM 2661) was a gift from the laboratory of R. Wolfe, University of Illinois. *Methanococcus maripaludis* JJ^T (DSM 2067) was a gift from W. Whitman, University of Georgia, Athens. *Methanococcus thermolithotrophicus* SN-1^T (DSM 2095) was provided by B. Mukhopadhyay, University of Illinois, originally from K. O. Stetter. *Methanococcus vannielii* SB^T (DSM 1224) culture was purchased from the Oregon Collection of Methanogens (OCM 148). *Methanococcus jannaschii* and *M.igneus* were grown anaerobically in defined minimal medium at 82–85°C under a pressurized headspace of 200 kPa H_2:CO_2 (80:20, v:v) (22). The medium contained 476 mM NaCl, 14 mM $MgCl_2$, 14 mM $MgSO_4$, 22 mM NH_4Cl, 4.6 mM KCl, 0.8 mM K_2HPO_4, 10 mM $NaHCO_3$, 5 mM $CaCl_2$, 10 µM $Fe(NH_4)_2(SO_4)_2$, 2 µM Na_2MoO_4, 2 µM Na_2WO_4, 2 µM Na_2SeO_4, 4 µM resazurin, 1 mM Na_2S and trace minerals (23,24). The concentrations of trace minerals were 73 µM trisodium nitriloacetic acid, 5.1 µM $MnCl_2$, 8.4 µM $CoCl_2$, 7.3 µM $ZnCl_2$, 3.1 µM $CuSO_4$ and 4.2 µM $NiCl_2$ (25). *Methanococcus maripaludis* and *M.vannielii* were grown in the same medium at 37°C and adjusted with NaOH to pH 7.5. *Methanococcus thermolithotrophicus* was grown in the same medium at 65°C.

Extraction and purification of tRNAs

Total RNA was isolated by the guanidinium-acidic phenol–chloroform extraction procedure (26). Extractions were performed aerobically under normal laboratory lighting conditions. Cells (0.5 g) were suspended in 5 ml extraction buffer containing 4 M guanidine isothiocyanate, 0.5% (w:v) sodium *N*-laurylsarcosine, 25 mM sodium citrate (pH 7.0) and 110 mM 2-mercaptoethanol. Cells were dispersed by passage through a syringe with a 22 gauge needle. Five hundred microliters of 2 M sodium acetate (pH 4.1) was added with 5 ml unbuffered phenol. This solution was mixed on a rotating shaker at 4°C for at least 8 h. One ml of chloroform:isoamyl alcohol (49:1, v:v) solution was added with mixing followed by incubation on ice for 15 min. After centrifugation at 3000 *g* for 15 min, the aqueous phase was re-extracted with 3 ml of chloroform:isoamyl alcohol (49:1). After centrifugation at 3000 *g* for 15 min, the aqueous phase was decanted, mixed with 2 vol of cold ethanol and stored at –20°C for at least 3 h. Precipitated total RNA was recovered by centrifugation at 17 000 *g* and the pellet was dried at room temperature.

DEAE–Sephadex (Pharmacia) chromatography at 4°C was used to purify tRNAs (27). A dried pellet of total RNA was resuspended in 1 ml binding buffer containing 100 mM NaCl

and 10 mM Tris–HCl (pH 7.0). Passage through a syringe with a 22 gauge needle was used to disrupt the polysaccharide matrix that co-purified with RNA from some organisms. Insoluble material was removed by centrifugation at 16 000 *g* for 5 min and the soluble fraction was applied to a syringe column containing 1.5 ml bed volume DEAE–Sephadex equilibrated in binding buffer. The column was washed with 5 vol of binding buffer followed by 5 vol of wash buffer (250 mM NaCl and 10 mM Tris–HCl pH 7.0). Fractions were collected from an isocratic elution with 1 M NaCl and 10 mM Tris–HCl (pH 7.0) and were then precipitated with 2 vol of cold ethanol for at least 3 h at –20°C. Precipitated tRNA was pelleted by centrifugation for 15 min at 6000 *g*, washed with 80% ethanol and then dried under vacuum using a SpeedVac apparatus (Savant). tRNA pellets were resuspended in distilled water and assayed spectrophotometrically by absorbance at 260 nm. Pooled fractions were analyzed by urea denaturing polyacrylamide gel electrophoresis (PAGE) followed by ethidium bromide staining. tRNAs were dried under vacuum using a SpeedVac apparatus (Savant).

Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) of total enzymatic digests of tRNA

Unfractionated tRNAs were hydrolyzed to nucleosides using nuclease P1, venom phosphodiesterase and bacterial alkaline phosphatase as described previously (28). Analysis of nucleosides was carried out with an LC/MS system consisting of a Quattro II triple-quadrupole mass spectrometer equipped with a Z-spray ion source (Micromass) and an HP 1090 liquid chromatograph with a photodiode array detector (Hewlett-Packard). Tuning of the mass spectrometer was performed with 10 mM adenosine solution. High-performance liquid chromatography (HPLC) separations of nucleosides were made by using a Luna C-18 reversed-phase column, 2.0 × 250 mm (Phenomenex), thermostatted at 40°C, with 5 mM ammonium acetate pH 5.3, and acetonitrile/water (40:60, v:v) at a flow rate of 0.3 ml/min without splitting (29). Source and desolvation temperatures were 130 and 350°C, respectively. All instrument control and data processing were performed with the MassLynx 3.4 software (Micromass). Procedures and interpretation of data for qualitative analysis of nucleosides in RNA hydrolysates were essentially as described in detail for a similar LC/MS system (29).

RESULTS

Nucleosides resulting from total tRNA digestion were separated and identified by LC/MS as shown by representative data sets from three organisms in Figure 2. Great selectivity in nucleoside assignments results from the combined use of HPLC relative retention times and ESI mass spectra, and does not require chromatographic separation of individual nucleosides (29). The dynamic range of detection of modified nucleosides using this method is ~1 in 1000 to 1 in 10 000 total nucleosides. In addition to the four canonical nucleosides in methanococcal tRNAs, these analyses identified 26 modified nucleosides as listed in Table 1, including two new nucleosides of unknown structure, designated N_{422} (unknown nucleoside of molecular mass 422 Da) and demethylwyosine (imG*). Systematic

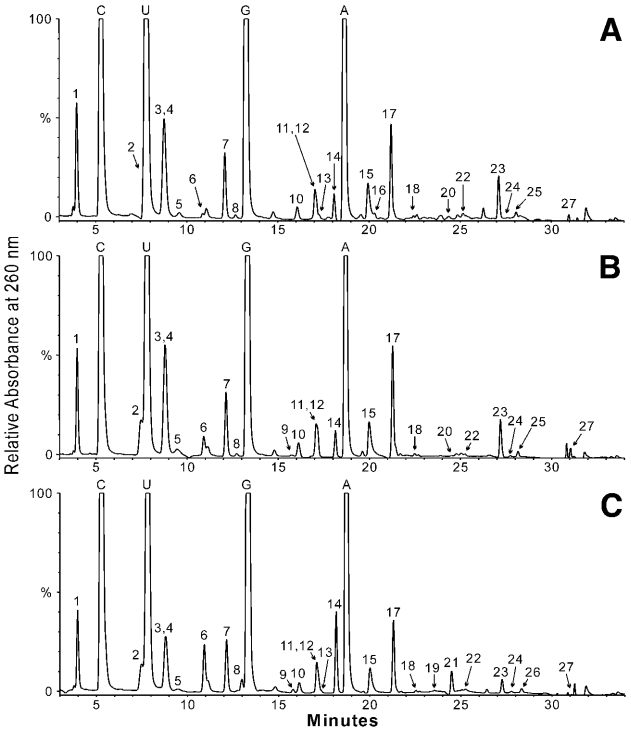

Figure 2. LC/MS analysis of nucleosides in unfractionated tRNA isolates from *Methanococci.* (**A**) *Methanococcus maripaludis*, (**B**) *M.thermolithotrophicus* and (**C**) *M.igneus*. Nucleoside identities were established from mass spectra and relative retention times: 1, pseudouridine; 2, 1-methyladenosine; 3, 2-thiocytidine; 4, 1-methylpseudouridine; 5, 5-methylaminomethyl-2-thiouridine; 6, 5-methylcytidine; 7, 2′-*O*-methylcytidine; 8, inosine; 9, 2′-*O*-methyluridine; 10, 4-thiouridine; 11, 1-methylinosine; 12, 1-methylguanosine; 13, 2′-*O*-methylguanosine; 14, N^2-methylguanosine; 15, archaeosine; 16, unknown nucleoside N_{422}; 17, N^2,N^2-dimethylguanosine; 18, N^6-threonylcarbamoyladenosine; 19, 2′-*O*-methyladenosine; 20, N^6-methyladenosine; 21, N^2,2′-*O*-dimethylguanosine; 22, demethylwyosine (isomer not known); 23, N^6-hydroxynorvalylcarbamoyladenosine; 24, wyosine; 25, 2-methylthio-N^6-threonylcarbamoyladenosine; 26, N^2,N^2,2′-*O*-trimethylguanosine; 27, N^6,N^6-dimethyladenosine (contaminant; see text).

names and structures for each nucleoside can be found on the world-wide web at: http://medlib.med.utah.edu/RNAmods/).

Unexpectedly, the highly modified uridine anticodon derivative 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) (structure in Fig. 3), known previously to occur only in bacteria (2), was found in four of the five methanococcal species examined (Table 1). Rigorous identification was made as shown in Figure 4, based on relative retention times and mass spectra of the 9.4 min eluate compared with synthetic mnm^5s^2U (data not shown). In particular, loss of methylamine from the base ion to form *m/z* 141 ion is characteristic of the methylaminomethyl side chain on uridine (30). Three ribose-methylated nucleosides were observed only in tRNAs from the hyperthermophiles *M.igneus* and *M.jannaschii*: 2′-*O*-methyladenosine (Am), N^2,2′-*O*-dimethylguanosine (m^2Gm) and N^2,N^2,2′-*O*-trimethylguanosine (m^2_2Gm). Unknown nucleosides N_{422} (M_r 422) and imG* (M_r 321) both exhibit UV absorption spectra characteristic of members of the tricyclic wyosine nucleoside family (31): UV λ_{max} (HPLC) 233, 287 nm and UV λ_{max} (HPLC) 228, 282 nm, respectively. The molecular mass of the nucleoside

Table 1. Modified nucleosides identified in tRNA from selected *Methanococci*

	Organism				
	M.maripaludis	*M.vannielii*	*M.thermolithotrophicus*	*M.igneus*	*M.jannaschii*
Growth temperature	37°C	37°C	65°C	85°C	85°C
Nucleoside[a]					
ψ	+	+	+	+	+
m^1A	+	+	+	+	+
s^2C	+	+	+	+	
$m^1\psi$	+	+	+	tr	tr
mnm^5s^2U	+	+	+	+	+
m^5C	+	+	+	+	+
Cm	+	+	+	+	+
I[b]	+	+	+	+	+
Um	tr		tr	+	+
s^4U	+	+	+	+	+
m^1I	+	+	+	+	+
m^1G	+	+	+	+	+
Gm	+	tr	+	+	+
m^2G	+	+	+	+	+
G^+	+	+	+	+	+
N_{422}[c]	+	+			+
m^2_2G	+	+	+	+	+
t^6A	+	+	+	+	+
Am				+	+
m^6A[d]	+	+	+	tr	+
m^2Gm				+	+
imG*[c]	+	+	+	+	+
hn^6A	+	+	+	+	+
imG	+	+	+	+	+
ms^2t^6A	+	+	+	tr	+
m^2_2Gm				+	+

[a]Nucleosides are listed in the HPLC elution order shown in Figure 2.
[b]Presence of I resulting from artifactual deamination of A is possible.
[c]Structure unknown; see text for discussion.
[d]Occurrence (in part) via rearrangement of m^1A is possible.
Um, 2-*O*-methyluridine; m^6A, N^6-methyladenosine; tr, trace amount; assignment tentative.

designated imG* is 14 Da lower than that of wyosine (imG) (M_r 335) originally discovered in yeast tRNA, and is consistent with an identity corresponding to a demethylwyosine in which the base contains only one methyl group. Three isomeric structures are, therefore, possible for imG* as a consequence of three potential methylation sites. The structure determinations of N_{422} and imG* are presently being pursued.

Among the most abundant modified nucleosides (Fig. 2) are those found in the TψCG loop [1-methylpseudouridine, ($m^1\psi$) ψ, Cm, 1-methylinosine (m^1I) and m^1A], in the loop corresponding to the bacterial dihydrouridine loop [archaeosine (G^+)] and in the hinge regions that join the four stems of the molecule [m^2G, m^2_2G and 5-methylcytidine (m^5C)]. These modifications were identified previously in the sequences of fractionated tRNAs from *H.volcanii* (10). Other observed modifications include purine nucleosides at position 37 (adjacent to the anticodon triplet), and consist of m^1G, N^6-threonylcarbamoyladenosine (t^6A), 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A) and imG. This group may also include N^6-hydroxynorvalylcarbamoyladenosine (hn^6A), an adenosine N^6-carbamoyl-linked norvaline derivative, which has been earlier identified in thermophilic bacteria and archaea, as well as in *M.vannielii* (32).

N^6,N^6-Dimethyladenosine (m^6_2A) was observed in small amounts in all methanococcal RNA preparations (see peak 27 in Fig. 2), but is not a known tRNA nucleoside. It is often found in trace amounts in tRNA isolates (P.F.Crain, S.C.Pomerantz and J.A.McCloskey, unpublished observations), but is judged to arise from a fragment of 16S rRNA in which its presence is ubiquitous. This conclusion was supported by its detection by MS in an RNase T1 digest of the *M.igneus* tRNA preparation as the unique and highly conserved (33) 16S rRNA fragment $m^6_2Am\ ^6_2A$ CCUGp (M_r 1994.3; data not shown).

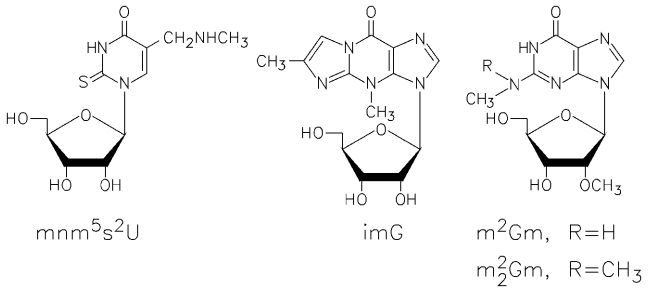

Figure 3. Structures of *Methanococcus* tRNA nucleosides mnm^{5}s^{2}U, imG, m^{2}Gm and m$^2_2$Gm.

DISCUSSION

Distribution and phylogenetic relationships of modified nucleosides in methanococcal tRNAs

This overall diversity of the 26 different modifications observed is relatively high, and compares with 15 in *H.volcanii* (10), the most extensively studied archaeon with respect to both identity and sequence locations of tRNA modifications, and ~26 in *Escherichia coli* tRNA (34) and 29 in *Salmonella typhimurium* (35). Nucleoside mnm^{5}s^{2}U, which was identified in four of the five organisms studied, was unknown previously in the Archaea. In bacterial tRNA it occurs in the first position of the anticodon, in tRNAs that code for Glu and Lys (36), and is a multistep reaction product of the *asuE* and *trmE* genes (30,37). It is an important identity element at position 34 in *E.coli* tRNA^{Glu}, required for efficient aminoacylation and for specific codon recognition (38,39). The striking clustering of mnm^{5}s^{2}U in the *Methanococci* suggests the possibility of horizontal gene transfer with the bacteria. However, relatively little comprehensive information exists concerning the phylogenetic distribution of mnm^{5}s^{2}U in bacteria, which would be useful in making inter-domain comparisons. Recent studies of mnm^{5}s^{2}U in the anticodon stem–loop of tRNA^{Lys} using nuclear magnetic resonance (NMR) showed that this residue exerts considerable influence in stabilizing the stacking of the anticodon nucleotides and on the characteristic backbone U-turn at residues 33–34 (40). Much of the influence of mnm^{5}s^{2}U derives from the steric effect of sulfur at C-2, a powerful promoter of the C3′-*endo* sugar conformation (9). This thiolated nucleotide is the substrate for a selenium insertion enzyme that synthesizes the structurally related 5-methylaminomethyl-2-selenouridine in tRNAs of several bacteria and in the archaeon *M.vannielii* (41). Although mnm^{5}se^{2}U was not observed in the present study, it is readily susceptible to photochemical degradation under the conditions used for tRNA isolation.

Four of the modified nucleosides shown in Table 1 are unique to archaea: m$^1\psi$, a T-loop surrogate for similarly shaped m^{5}U-54 (42); archaeosine (G$^+$), a 7-deazaguanosine derivative (43) that is conserved at position 15 and occurs widely in many tRNA isoacceptors (10,36) and the ribose-methylated species m^{2}Gm and m$^2_2$Gm, characteristic of archaeal hyperthermophiles (see discussion in following section). The tricyclic nucleoside wyosine (imG, Fig. 3) was known previously only in eukaryal tRNA, where it is restricted presumably to position 37, adjacent to the 3′ end of the anticodon in tRNA^{Phe}, as are other members of the wye ('Y') family

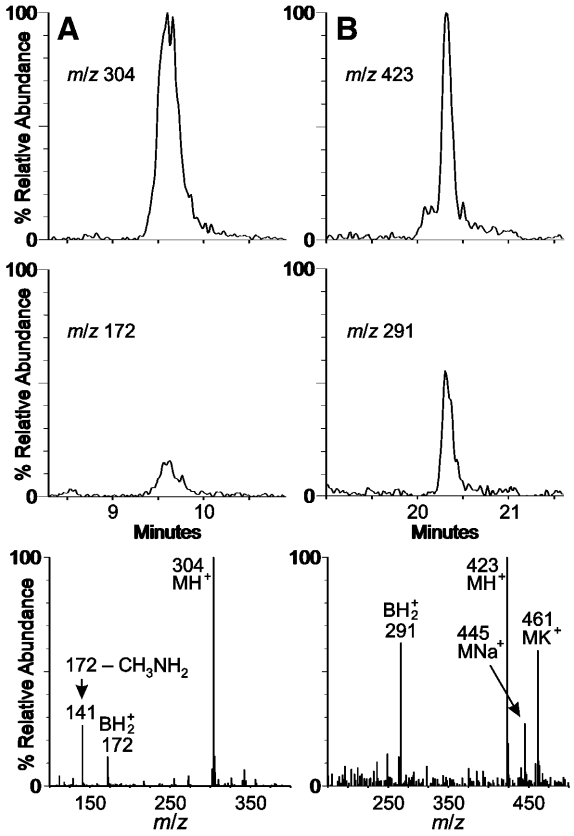

Figure 4. Detection of nucleosides in *M.maripaludis* tRNA. (**A**) 5-Methylaminomethyl-2-thiouridine and (**B**) unknown nucleoside of molecular mass 422. Ion chromatograms corresponding to MH$^+$ (protonated molecule) and BH_2^+ ions (nucleoside fragment ion derived from the base moiety, B, and corresponding to the protonated free base) are shown in the top and middle panels, respectively; ESI mass spectra recorded at 9.7 and 20.3 min are shown in the bottom panels.

(36). The only member of the wyosine family previously known outside of eukaryal tRNA was designated mimG (42), a methylated derivative of imG, which occurs widely in the phylum Crenarchaeota (15). Its occurrence suggests that enzymes for biosynthesis of the unique tricyclic base structure (which is derived from guanine; 44) might also occur in the Euryarchaea. This possibility is borne out by the occurrence of the three 'Y' derivatives imG, imG* and N_{422} in the *Methanococci*, and appears to extend an earlier conclusion that post-transcriptional modifications of tRNA nucleosides in the Archaea are, in terms of structural motifs, generally more eukaryotic than bacterial (14).

Other nucleosides listed in Table 1 that contain base modifications that are shared with eukarya but not bacteria (2) are m^{5}C, m^{1}I, and m^{2}G and m$^2_2$G, and their ribose methylated derivatives that are unique to the archaeal thermophiles. Three nucleosides in the *Methanococci* that exhibit base modifications shared only with bacteria, in addition to mnm^{5}s^{2}U, are s^{2}C (which is somewhat uncommon in archaea), s^{4}U and the hypermodified norvaline derivative of A, hn^{6}A. Thus, a total of nine tRNA nucleosides found in the *Methanococci* contain typically eukaryal base modifications whereas four are otherwise found only in bacteria. This tendency supports the broad

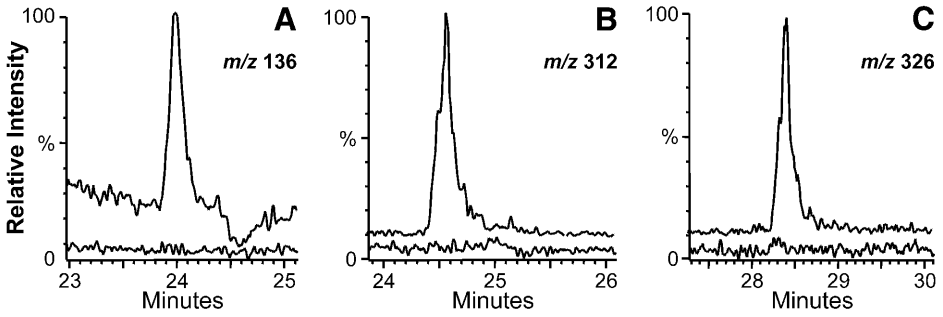

Figure 5. Comparisons of three nucleosides that distinguish hyperthermophilic and mesophilic *Methanococci*. Ion chromatograms in each panel: top trace *M.igneus*; bottom trace *M.maripaludis*. (**A**) Base ion (BH_2^+) for detection of 2′-*O*-methyladenosine. (**B**) Molecular ion (MH^+) for detection of N^2,2′-*O*-dimethylguanosine. (**C**) Molecular ion (MH^+) for detection of N^2,N^2,2′-*O*-trimethylguanosine.

conclusion from examination of full genomic sequences, that genes encoding information processing functions in Archaea (those dealing with replication, transcription and translation) most closely resemble those found in Eukarya (45,46). Along analogous lines, it will be interesting to establish whether amino acid sequences of the archaeal enzymes that produce common modification motifs (e.g. the ribosylmethyltransferases) are more similar to eukaryal homologs than to corresponding bacterial homologs, as has been studied for the complex system of genes responsible for ψ biosynthesis (47).

The diversity of structural modifications in methanococcal nucleosides characteristic of tRNA position 37 in the anticodon loop is notable: seven of 26 species listed in Table 1 (m^1G, N_{422}, t^6A, ms^2t^6A, hn^6A, imG and imG*). It has been proposed that the enzyme responsible for formation of m^1G-37, tRNA(m^1G37)methyltransferase, is part of a minimal set of genes required for life (48). The highly conserved extent of purine modification at position 37 has long been known (49). However, there has been heightened recent interest due to the implication of modification at this site in maintenance of correct translational reading frame (50), as well as to a greater understanding of the influence of N-37 modification on anticodon loop structure (9).

Comparison of hyperthermophilic and mesophilic tRNA modifications

In mesophiles, RNA structure is stabilized by elevated G + C content of stems, which in turn loosely correlates with optimal growth temperature (7,51), and with overall levels of post-transcriptional modification (52,53). However, in the case of thermophiles, stem G + C content essentially maximizes, and modification becomes relatively more important. The effect of modification has been determined directly by correlation between culture temperature for a given organism and increase in RNA T_m (4,54,55), or has been implied from increased levels of specific modifications, such as ribose methylation at *O*-2′ in tRNA (54) and rRNA (56), as a consequence of increased culture temperature.

The organisms selected for the present study were chosen in part to determine whether there are differences in tRNA modification patterns among relatively closely related species that grow optimally at very different temperatures, thus minimizing differences due to phylogeny alone. This requirement is met through comparisons of tRNA from *M.jannaschii* and *M.igneus* (86–91°C) and *M.maripaludis* (37°C), whose phylogenetic relationships are shown in Figure 1. As seen in Figure 2 and Table 1, the modification profiles among the methanococcal tRNA nucleosides are relatively similar with regard to both identity and abundance. To some extent this observation reflects the fact that tRNA-encoding genes from the mesophilic species *M.maripaludis* are 86–93% identical to their homologs in the hyperthermophile *M.jannaschii*. The similar abundance of modified nucleosides suggests similarity of modification sites as well, and demonstrates a correlation between tRNA modification patterns and phylogenetic closeness as defined by SSU rRNA sequences. However, a clear difference is observed between the two hyperthermophiles *M.igneus* and *M.jannaschii* and the remaining organisms (including *M.thermolithotrophicus*) with respect to the occurrence of three nucleosides: m^2Gm, m_2^2Gm and Am. Selected ion chromatograms from the LC/MS analyses of *M.igneus* and *M.maripaludis* tRNAs are representative of the data as a whole and demonstrate complete absence of the three nucleosides in tRNA from the mesophile (Fig. 5). Another mesophilic *Methanococcus*, *M.voltae*, also lacks these three modifications (J.Konisky, P.F.Crain and J.A.McCloskey, unpublished experiments). Thermodynamically, 2′-*O*-methylated nucleosides favor the C3′-*endo* ribose pucker, which minimizes steric interferences of the base and C3′-phosphate with the ribose 2′-*O*-methyl group (57). In a pentofuranose ring, this conformation necessarily affects the backbone torsion angle and correlates with a low (anti-) glycosidic bond angle. Because this conformation matches that of nucleotides in an A-form double helix, the 2′-*O*-methyl group decreases the entropic costs of forming the duplex structure, and thereby stabilizes the base-paired form (58). Therefore, ribose methylation of specific nucleotides is a common and frequently convergent means of stabilizing the RNAs of archaeal thermophiles.

Among the 2′-*O*-methylated nucleosides, m^2Gm and m_2^2Gm have thus far been identified only in hyperthermophilic archaea (15), and are likely located primarily at position 26, at the junction of the dihydrouridine and anticodon stems (see discussion in 4). In yeast $tRNA^{Phe}$, m_2^2G -26 pairs with A-44, mediating the coaxial stacking of these two stems (59,60). NMR studies of nucleosides show that, as with other 2′-*O*-methylations, m_2^2Gm favors the C3′-*endo-anti* conformation, and that the effect is enhanced by the steric interference

between the guanine N^2-methyl groups and the 2′-*O*-methyl (61). Thus, the ribose methylations in m^2Gm and m^2_2Gm are expected to increase the thermal stability of the helical structure involved in coaxial stacking of the stems (4), although the magnitude of this effect cannot be distinguished from other stabilizing features. In the comparison of *Pyrococcus furiosus* cells grown at 100 versus 70°C, m^2_2Gm and its precursor m^2_2G were three times and one-third (respectively) as abundant in the high temperature cells as in the low temperature cells, consistent with ribose methylation being an active thermal adaptation (4). Similar results were observed with the thermotolerant bacterium *Bacillus stearothermophilus* where cells grown at 70°C had three times the 2′-*O*-methylated nucleosides as cells grown at 50°C (54). As pointed out by a reviewer, the relatively lower amount of m^2_2Gm and other ribose-methylated species in *M.igneus* tRNA compared with that from *P.furiosus* (4) suggests that the ribose methylation mechanism of stabilization may be relatively less important in the present case. However, it is notable that the levels of m^2Gm and m^2_2G modification in *M.jannaschii* (chromatogram not shown) are nearly double those in *M.igneus*, while still appearing to be lower than those earlier found in *P.furiosus*.

It is intriguing that Am is common in the tRNAs of archaeal hyperthermophiles (15), but the locations of the modification are unknown. At low frequency, the amino acid acceptor stems of some eukaryotic tRNAs contain Am (36), and an S100 extract from *P.furiosus* was demonstrated to methylate an adenosine at position 6 in an *in vitro* transcript of a *H.volcanii* tRNA gene (62). It is possible that no recurring location will be found; with the exception of Cm at position 56, no widely conserved sites of ribose methylation are known in the Archaea. Until modification sites are identified experimentally, we will not speculate on the roles that Am might play in the stability of the tRNAs of hyperthermophiles.

ACKNOWLEDGEMENTS

Authentic 5-methylaminoethyl-2-thiouridine was obtained from K. Murao, Jichi Medical School. The authors are grateful to R. S. Wolfe, W. B. Whitman, B. Mukhopadhyay and K. O. Stetter for strains used in this study. This work was supported by NIH grant GM29812 (J.A.M.), by Department of Energy grant DE-FG02-84ER13241 (J.K.), NIH grant GM22854 (D.S.), and by grant NAG5-8479 from the National Aeronautics and Space Administration (G.J.O. and C.R.Woese).

REFERENCES

1. Rozenski,J., Crain,P.F. and McCloskey,J.A. (1999) The RNA modification database-1999 update. *Nucleic Acids Res.*, **27**, 196–197.
2. Motorin,Y. and Grosjean,H. (1998) Appendix 1: Chemical structures and classification of posttranslationally modified nucleosides in RNA. In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC, pp. 543–549.
3. Hall,K.B., Sampson,J.R., Uhlenbeck,O.C. and Redfield,A.G. (1989) Structure of an unmodified tRNA molecule. *Biochemistry*, **28**, 5794–5801.
4. Kowalak,J.A., Dalluge,J.J., McCloskey,J.A. and Stetter,K.O. (1994) Role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. *Biochemistry*, **33**, 7869–7876.
5. Curran,J.F. (1998) Modified nucleosides in translation. In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC, pp. 493–516.
6. Giegé,R., Sissler,M. and Florentz,C. (1998) Universal rules and idiosyncratic features in tRNA identity. *Nucleic Acids Res.*, **26**, 5017–5035.
7. Watanabe,K., Oshima,T., Iijima,K., Yamaizumi,Z. and Nishimura,S. (1980) Purification and thermal stability of several amino acid-specific tRNAs from an extreme thermophile, *Thermus thermophilus* HB8. *J. Biochem. (Tokyo)*, **87**, 1–13.
8. Agris,P.F. (1996) The importance of being modified: roles of modified nucleosides and Mg^{2+} in RNA structure and function. *Prog. Nucleic Acid Res. Mol. Biol.*, **53**, 79–129.
9. Davis,D.R. (1998) Biophysical and conformational properties of modified nucleosides in RNA (nuclear magnetic resonance studies). In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC, pp. 85–102.
10. Gupta,R. (1984) *Halobacterium volcanii* tRNAs. Identification of 41 tRNAs covering all amino acids, and the sequences of 33 class I tRNAs. *J. Biol. Chem.*, **259**, 9461–9471.
11. Gupta,R. (1986) Transfer RNAs of *Halobacterium volcanii:* sequences of five leucine and three serine tRNAs. *System. Appl. Microbiol.*, **7**, 102–105.
12. Best,A.N. (1978) Composition and characterization of tRNA from *Methanococcus vannielii*. *J. Bacteriol.*, **133**, 240–250.
13. Gupta,R. and Woese,C.R. (1980) Unusual modification patterns in the transfer ribonucleic acids of archaebacteria. *Curr. Microbiol.*, **4**, 245–249.
14. McCloskey,J.A. (1986) Nucleoside modification in archaebacterial transfer RNA. *System. Appl. Microbiol.*, **7**, 246–252.
15. Edmonds,C.G., Crain,P.F., Gupta,R., Hashizume,T., Hocart,C.H., Kowalak,J.A., Pomerantz,S.C., Stetter,K.O. and McCloskey,J.A. (1991) Posttranscriptional modification of transfer RNA in thermophilic archaea. *J. Bacteriol.*, **173**, 3138–3148.
16. McCloskey,J.A., Liu,X.-H., Crain,P.F., Bruenger,E., Guymon,R., Hashizume,T. and Stetter,K.O. (2000) Posttranscriptional modification of transfer RNA in the submarine hyperthermophile *Pyrolobus fumarii*. *Nucleic Acids Symp. Ser. No.* **44**, 267–268.
17. Boone,D.R., Whitman,W.B. and Rouvière,P. (1993) Diversity and taxonomy of methanogens. In Ferry,J.G. (ed.), *Methanogenesis*. Chapman & Hall, New York, NY, pp. 35–80.
18. Huber,H., Thomm,M., König,H., Thies,G. and Stetter,K.O. (1982) *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. *Arch. Microbiol.*, **132**, 47–50.
19. Stetter,K.O. (1996) Hyperthermophilic procaryotes. *FEMS Microbiol. Rev.*, **18**, 149–158.
20. Haney,P.J., Badger,J.H., Buldak,G.L., Reich,C.I., Woese,C.R. and Olsen,G.J. (1999) Thermal adaptation analyzed by comparison of protein sequences from mesophilic and extremely thermophilic *Methanococcus* species. *Proc. Natl Acad. Sci. USA*, **96**, 3578–3583.
21. Haney,P.J., Stees,M. and Konisky,J. (1999) Analysis of thermal stabilizing interactions in mesophilic and thermophilic adenylate kinases from the genus *Methanococcus*. *J. Biol. Chem.*, **274**, 28453–28458.
22. Balch,W.E., Fox,G.E., Magrum,L.J., Woese,C.R. and Wolfe,R.S. (1979) Methanogens: re-evaluation of a unique biological group. *Microbiol. Rev.*, **43**, 260–296.
23. Rajagopal,B.S. and Daniels,L. (1986) Investigations of mercaptans, organic sulfides, and inorganic sulfur compounds as sulfur sources for the growth of methanogenic bacteria. *Curr. Microbiol.*, **14**, 137–144.
24. Mukhopadhyay,B., Johnson,E.F. and Wolfe,R.S. (1999) Reactor-scale cultivation of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* to high cell densities. *Appl. Environ. Microbiol.*, **65**, 5059–5065.
25. Daniels,L., Belay,N. and Rajagopal,B.S. (1986) Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. *Appl. Environ. Microbiol.*, **51**, 703–709.
26. Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
27. Gupta,R. (1995) Preparation of transfer *RNA*, aminoacyl-tRNA synthetases, and tRNAs specific for an amino acid from extreme halophiles. In DasSarma,S. and Fleischmann,E.M. (eds), *Archaea: A Laboratory Manual* (*Halophiles*). Cold Spring Harbor Press, Plainview, NY, pp. 119–131.
28. Crain,P.F. (1990) Preparation and enzymatic hydrolysis of RNA and DNA for mass spectrometry. *Methods Enzymol.*, **193**, 782–790.
29. Pomerantz,S.C. and McCloskey,J.A. (1990) Analysis of RNA hydrolyzates by LC/MS. *Methods Enzymol.*, **193**, 796–824.

30. Hagervall,G., Edmonds,C.G., McCloskey,J.A. and Björk,G.R. (1987) Transfer RNA (5-methylaminomethyl-2-thiouridine) methyltransferase from *Escherichia coli* K-12 has two enzymatic activities. *J. Biol. Chem.*, **262**, 8488–8495.
31. Kasai,H., Goto,M., Ikeda,K., Zama,M. and Mizuno,Y. (1976) Structure of wye (Yt base) and wyosine (Yt) from *Torulopsis utilis* phenylalanine transfer ribonucleic acid. *Biochemistry*, **15**, 898–904.
32. Reddy,D.M., Crain,P.F., Edmonds,C.G., Gupta,R., Hashizume,T., Stetter,K.O., Widdel,F. and McCloskey,J.A. (1992) Structure determination of two new amino acid-containing derivatives of adenosine from tRNA of thermophilic bacteria and archaea. *Nucleic Acids Res.*, **20**, 5607–5614.
33. van Knippenberg,P.H., Van Kimmenade,J.M. and Heus,H.A. (1984) Phylogeny of the conserved 3′ terminal structure of the RNA of small ribosomal subunits. *Nucleic Acids Res.*, **12**, 2595–2604.
34. Gehrke,C.W. and Kuo,K.C. (1990) Ribonucleoside analysis by reversed-phase high performance liquid chromatography. In Gehrke,C.W. and Kuo,K.C. (eds), *Chromatography and Identification of Nucleosides*, *Part A*, Vol. 45A. Journal of Chromatography Library, Elsevier, New York, NY, pp. A3–A64.
35. Buck,M., Connick,M. and Ames,B.N. (1983) Complete analysis of tRNA-modified nucleosides by high performance liquid chromatography: the 29 modified nucleosides of *Salmonella typhimurium* and *Escherichia coli* tRNA. *Anal. Biochem.*, **129**, 1–13.
36. Sprinzl,M., Horn,C., Brown,M., Ioudovitch,A. and Steinberg,S. (1998) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, **26**, 148–153.
37. Sullivan,M.A., Cannon,J.F., Webb,F.H. and Bock,R.M. (1985) Antisuppressor mutation in *Escherichia coli* defective in biosynthesis of 5-methylaminomethyl-2-thiouridine. *J. Bacteriol.*, **161**, 368–376.
38. Sakamoto,K., Kawai,G., Watanabe,S., Niimi,T., Hayashi,N., Muto,Y., Watanabe,K., Satoh,T., Sekine,M. and Yokoyama,S. (1996) NMR studies of the effects of the 5′-phosphate group on conformational properties of 5-methylaminomethyluridine found in the first position of the anticodon of *Escherichia coli* tRNA(Arg)4. *Biochemistry*, **35**, 6533–6538.
39. Madore,E., Florentz,C., Giegé,R., Sekine,S., Yokoyama,S. and Lapointe,J. (1999) Effect of modified nucleotides on *Escherichia coli* $tRNA^{Glu}$ structure and on its aminoacylation by glutamyl-tRNA synthetase. Predominant and distinct roles of the mnm^5 and s^2 modifications of U34. *Eur. J. Biochem.*, **266**, 1128–1135.
40. Sundaram,M., Crain,P.F. and Davis,D.R. (2000) Synthesis and characterization of the native anticodon domain of *E.coli* $tRNA^{Lys}$: simultaneous incorporation of modified nucleosides mnm^5s^2U, t^6A, and pseudouridine using phosphoramidite chemistry. *J. Org. Chem.*, **65**, 5609–5614.
41. Wittwer,A.J., Tsai,L., Ching,W.M. and Stadtman,T.C. (1984) Identification and synthesis of a naturally occurring selenonucleoside in bacterial tRNAs: 5-[(methylamino)methyl]-2-selenouridine. *Biochemistry*, **23**, 4650–4655.
42. McCloskey,J.A., Crain,P.F., Edmonds,C.G., Gupta,R., Hashizume,T., Phillipson,D.W. and Stetter,K.O. (1987) Structure determination of a new fluorescent tricyclic nucleoside from archaebacterial tRNA. *Nucleic Acids Res.*, **15**, 683–693.
43. Gregson,J.M., Crain,P.F., Edmonds,C.G., Gupta,R., Hashizume,T., Phillipson,D.W. and McCloskey,J.A. (1993) Structure of the archaeal transfer RNA nucleoside G*-15 (2-amino-4,7-dihydro-4-oxo-7-β-D-ribofuranosyl-1*H*-pyrrolo[2,3-*d*]pyrimidine-5-carboximidamide (archaeosine)). *J. Biol. Chem.*, **268**, 10076–10086.
44. Garcia,G.A. and Goodenough-Lashua,D.M. (1998) Mechanisms of RNA-modifying and -editing enzymes. In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC, pp. 135–168.
45. Olsen,G.J. and Woese,C.R. (1997) Archaeal genomics: an overview. *Cell*, **89**, 991–994.
46. Doolittle,W.F. and Logsdon,J.M.,Jr (1998) Archaeal genomics: do archaea have a mixed heritage? *Curr. Biol.*, **8**, R209–211.
47. Watanabe,Y. and Gray,M.W. (2000) Evolutionary appearance of genes encoding proteins associated with box H/ACA snoRNAs: Cbf5p in *Euglena gracilis*, an early diverging eukaryote, and candidate Gar1p and Nop10p homologs in archaebacteria. *Nucleic Acids Res.*, **28**, 2342–2352.
48. Björk,G.R., Jacobsson,K., Nilsson,K., Johansson,M.J., Bystrom,A.S. and Persson,O.P. (2001) A primordial tRNA modification required for the evolution of life? *EMBO J.*, **20**, 231–239.
49. Auffinger,P. and Westhof,E. (1998) Location and distribution of modified nucleotides in tRNA. In Grosjean,H. and Benne,R. (eds), *Modification and Editing of RNA*. ASM Press, Washington, DC, pp. 569–576.
50. Björk,G.R., Durand,J.M., Hagervall,T.G., Leipuviene,R., Lundgren,H.K., Nilsson,K., Chen,P., Qian,Q. and Urbonavicius,J. (1999) Transfer RNA modification: influence on translational frameshifting and metabolism. *FEBS Lett.*, **452**, 47–51.
51. Galtier,N. and Lobry,J.R. (1997) Relationships between genomic G + C content, RNA secondary structures, and optimal growth temperature in prokaryotes. *J. Mol. Evol.*, **44**, 632–636.
52. Sampson,J.R. and Uhlenbeck,O.C. (1988) Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*. *Proc. Natl Acad. Sci. USA*, **85**, 1033–1037.
53. Derrick,W.B. and Horowitz,J. (1993) Probing structural differences between native and *in vitro* transcribed *Escherichia coli* valine transfer RNA: evidence for stable base modification-dependent conformers. *Nucleic Acids Res.*, **21**, 4948–4953.
54. Agris,P.F., Koh,P. and Söll,D. (1973) The effect of growth temperature on the *in vivo* ribose methylation of *Bacillus stearothermophilus*. *Arch. Biochem. Biophys.*, **154**, 277–282.
55. Watanabe,K., Shinma,M. and Oshima,T. (1976) Heat-induced stability of tRNA from an extreme thermophile, *Thermus thermophilus*. *Biochem. Biophys. Res. Commun.*, **72**, 1137–1144.
56. Noon,K.R., Bruenger,E. and McCloskey,J.A. (1998) Posttranscriptional modifications in 16S and 23S rRNAs of the archaeal hyperthermophile *Sulfolobus solfataricus*. *J. Bacteriol.*, **180**, 2883–2888.
57. Kawai,G., Yamamoto,Y., Kamimura,T., Masegi,T., Sekine,M., Hata,T., Iimori,T., Watanabe,T., Miyazawa,T. and Yokoyama,S. (1992) Conformational rigidity of specific pyrimidine residues in tRNA arises from posttranscriptional modifications that enhance steric interaction between the base and the 2′-hydroxyl group. *Biochemistry*, **31**, 1040–1046.
58. Yokoyama,S., Inagaki,F. and Miyazawa,T. (1981) Advanced nuclear magnetic resonance lanthanide probe analyses of short-range conformational interrelations controlling ribonucleic acid structures. *Biochemistry*, **20**, 2981–2988.
59. Kim,S.H., Suddath,F.L., Quigley,G.J., McPherson,A., Sussman,J.L., Wang,A.H., Seeman,N.C. and Rich,A. (1974) Three-dimensional tertiary structure of yeast phenylalanine transfer RNA. *Science*, **185**, 435–440.
60. Robertus,J.D., Ladner,J.E., Finch,J.T., Rhodes,D., Brown,R.S., Clark,B.F.C. and Klug,A. (1974) Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature*, **250**, 546–551.
61. Kawai,G., Ue,H., Yasuda,M., Sakamoto,K., Hashizume,T., McCloskey,J.A., Miyazawa,T. and Yokoyama,S. (1991) Relation between functions and conformational characteristics of modified nucleosides found in tRNAs. *Nucleic Acids Symp. Ser.*, 49–50.
62. Constantinesco,F., Motorin,Y. and Grosjean,H. (1999) Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities *in vitro*. *Nucleic Acids Res.*, **27**, 1308–1315.
63. Maidak,B.L., Cole,J.R., Lilburn,T.G., Parker,C.T.,Jr, Saxman,P.R., Stredwick,J.M., Garrity,G.M., Li,B., Olsen,G.J., Pramanik,S., Schmidt,T.M. and Tiedje,J.M. (2000) The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.*, **28**, 173–174.