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

James A. McCloskey*, David E. Graham1, Shaolian Zhou, Pamela F. Crain, Michael Ibba2, Jordan Konisky1, Dieter Söll2 and Gary J. Olsen1

Departments of Biochemistry and Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112-5820, USA, 1Department of Microbiology, University of Illinois, Urbana, IL 61801, USA and 2Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA

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 tRNAPhe 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, N2,2′-O-dimethylguanosine and N2,N2,2′-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

*To whom correspondence should be addressed at: University of Utah, 30 South 2000 East, Room 311A, Salt Lake City, UT 84112-5820, USA. Tel: +1 801 581 5581; Fax: +1 801 581 7457; Email: james.mccloskey@m.cc.utah.edu

Present addresses:
David E. Graham, Department of Biochemistry, Virginia Polytechnic and State University, Blacksburg, VA 24061-0308, USA
Michael Ibba, Center for Biomolecular Recognition, IMBG, The Panum Institute, Blegdamsvej 3c, Copenhagen N, DK-2200, Denmark
Jordan Konisky, Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892, USA
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, Pyrococcus 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 H₂, CO₂ and formate (some species) to perform methanogenesis. All species are motile, irregular cocci and the group of organisms has made the Archaea an ideal model system for comparative analyses of thermal adaptation among closely related archaea, to gain insights into which modifications play thermostabilizing roles.

**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.

**MATERIALS AND METHODS**

**Cell sources and growth of cultures**

*Methanococcus jannaschii* JAL-1T (DSM 2661) was a gift from the laboratory of R. Wolfe, University of Illinois. *Methanococcus maripaludis* JJJT (DSM 2067) was a gift from W. Whitman, University of Georgia, Athens. *Methanococcus thermolithotrophicus* SN-1T (DSM 2095) was provided by B. Mukhopadhyay, University of Illinois, originally from K. O. Stetter. *Methanococcus vannielii* SBT (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 H₂/CO₂ (30:20, v:v) (22). The medium contained 476 mM NaCl, 14 mM MgCl₂, 14 mM MgSO₄, 22 mM NH₄Cl, 4.6 mM KCl, 0.8 mM K₃HPO₄, 10 mM NaHCO₃, 5 mM CaCl₂, 10 µM Fe(NH₄)₂(SO₄)₂, 2 µM Na₂MoO₄, 2 µM Na₂WO₄, 2 µM Na₂SeO₃, 4 µM resazurin, 1 µM Na₂S and trace minerals (23,24). The concentrations of trace minerals were 73 µM trisodium nitroloacetic acid, 5.1 µM MnCl₂, 8.4 µM CoCl₂, 7.3 µM ZnCl₂, 3.1 µM CuSO₄ and 4.2 µM NiCl₂ (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 cell suspension was added with mixing followed by incubation on ice 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 mlbinding buffer containing 100 mM NaCl
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 nucleo-

sides 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.
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 (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_{222} (unknown nucleoside of molecular mass 422 Da) and dimethylwyosine (imG*). Systematic 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 (mmm\textsuperscript{5}U) (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\textsuperscript{5}s\textsuperscript{2}U (data not shown). In particular, loss of methylamine from the base ion to 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 thermophilic methanococci M.\textit{igneus} and \textit{M.jannaschii}: \textit{2′-O-methyladenosine} (Am), N\textsuperscript{2,2′-O-dimethylguanosine} (m\textsuperscript{2}Gm) and N\textsuperscript{2,2′-O-trimethylguanosine} (m\textsuperscript{2}Gm). Unknown nucleosides N\textsubscript{422} (M\textsubscript{422}) and imG* (M\textsubscript{321}) both exhibit UV absorption spectra characteristic of members of the tricyclic wyosine nucleoside family (31): UV λ\textsubscript{max} (HPLC) 233, 287 nm and UV λ\textsubscript{max} (HPLC) 228, 282 nm, respectively. The molecular mass of the nucleoside
designated imG* is 14 Da lower than that of wyosine (imG) (Mᵣ 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 N422 and imG* are presently being pursued.

Among the most abundant modified nucleosides (Fig. 2) are those found in the TψCG loop [1-methylpseudouridine, (m₁ψ) ψ, Cm, 1-methylinosine (m₁I) and m₁A], in the loop corresponding to the bacterial dihydrouridine loop [archaeosine (G⁺)] and in the hinge regions that join the four stems of the molecule [m₂G, m₂₂G and 5-methylcytidine (m₅C)]. These modifications were identified previously in the sequences of fractionated tRNAs from M.thermolithotrophicus (10). Other observed modifications include purine nucleosides at position 37 (adjacent to the anticodon triplet), and consist of m₁G, N⁶-threonylcarbamoyladenosine (t₆A), 2-methylthio-N⁶-threonylcarbamoyladenosine (ms₂t₆A) and imG. This group may also include N⁶-hydroxyarginine (hn6A), an adenosine N⁶-carbamoyl-linked norvaline derivative, which has been earlier identified in thermophilic bacteria and archaea, as well as in M.vannielii (32).

N⁶,N⁶-Dimethyladenosine (m₆₂A) 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 m₆₂Am₆₂A CCUGp (Mᵣ 1994.3; data not shown).

---

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

<table>
<thead>
<tr>
<th>Nucleosideᵃ</th>
<th>M.maripaludis</th>
<th>M.vannielii</th>
<th>M.thermolithotrophicus</th>
<th>M.igneus</th>
<th>M.jannaschii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temperature</td>
<td>37°C</td>
<td>37°C</td>
<td>65°C</td>
<td>85°C</td>
<td>85°C</td>
</tr>
<tr>
<td>Nucleosideᵃ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ψ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₁A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>s₂ψ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₁ψ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mm₅'s₄U</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₁C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cm</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Um</td>
<td>tr</td>
<td>tr</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>s₄U</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₁I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₁G</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N₄₂²</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₂G</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>t₆⁴A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Am</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₅²A⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₂Gm</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>imG⁺⁺⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>h₄₆A⁺⁺⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>imG⁺⁺⁺⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ms₂t₆⁴A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>m₂₂Gm</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ᵃNucleosides are listed in the HPLC elution order shown in Figure 2.
ᵇPresence of I resulting from artificial deamination of A is possible.
ᶜStructure unknown; see text for discussion.
ᵈOccurrence (in part) via rearrangement of m₁A is possible.

Um, 2-O-methyluridine; m₅²A, N⁶-methyladenosine; tr, trace amount; assignment tentative.
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 mmn^5s2U, 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 mmn^5s2U in the Methanococci suggests the possibility of horizontal gene transfer with the bacteria. However, relatively little comprehensive information exists concerning the phylogenetic distribution of mmn^5s2U in bacteria, which would be useful in making inter-domain comparisons. Recent studies of mmn^5s2U 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 mmn^5s2U 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 mmn^5s2U 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^1ψ, a T-loop surrogate for similarly shaped m^1U-54 (42); archaeosine (G^p), 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^2Gm and m^2^2Gm, 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 wy family (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 N422 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^5C, m^1I, and m^2G and m^2^2G, 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 mmn^5s2U, are s^2C (which is somewhat uncommon in archaea), s^4U and the hypermodified norvaline derivative of A, hn^6A. 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
between culture temperature for a given organism and increase modification has been determined directly by correlation modification becomes relatively more important. The effect of thermophiles, stem G + C content essentially maximizes, and transcriptional modification (52,53). However, in the case of content of stems, which in turn loosely correlates with optimal In mesophiles, RNA structure is stabilized by elevated G + C modifications codon loop structure (9).

The diversity of structural modifications in methanococcal nucleosides characteristic of tRNA position 37 in the anti-codon loop is notable: seven of 26 species listed in Table 1 (m1G, N122, tAU, ms2t6A, hm6A, imG and imG*). It has been proposed that the enzyme responsible for formation of mG-37, tRNA(mG37)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 anti-codon 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 Tm (4,54,55), or has been implied from increased levels of specific modifications, such as ribose methylation at O-2' in tRNA (54) and tRNA (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: m2Gm, m3Gm 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 interferes 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 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, m2Gm and m3Gm 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 tRNA35, m2G -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, m3Gm favors the C3'-endo-anti conformation, and that the effect is enhanced by the steric interference
between the guanine N2-methyl groups and the 2'-O-methyl (61). Thus, the ribose methylation in m3Gm and m2Gm 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, m3Gm and its precursor m3G 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 m2Gm 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 m3Gm and m2G 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. Whitan, 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.Woece).

REFERENCES
