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Influence of Temperature on tRNA Modification in Archaea: *Methanococcoides burtonii* (Optimum Growth Temperature [T_{opt}], 23°C) and *Stetteria hydrogenophila* (T_{opt}, 95°C)

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We report the first study of tRNA modification in psychrotolerant archaea, specifically in the archaeon *Methanococcoides burtonii* grown at 4 and 23°C. For comparison, unfractionated tRNA from the archaeal hyperthermophile *Stetteria hydrogenophila* cultured at 93°C was examined. Analysis of modified nucleosides using liquid chromatography-electrospray ionization mass spectrometry revealed striking differences in levels and identities of tRNA modifications between the two organisms. Although the modification levels in *M. burtonii* tRNA are the lowest in any organism of which we are aware, it contains more than one residue per tRNA molecule of dihydrouridine, a molecule associated with maintenance of polynucleotide flexibility at low temperatures. No differences in either identities or levels of modifications, including dihydrouridine, as a function of culture temperature were observed, in contrast to selected tRNA modifications previously reported for archaeal hyperthermophiles. By contrast, *S. hydrogenophila* tRNA was found to contain a remarkable structural diversity of 31 modified nucleosides, including nine methylated guanosines, with eight different nucleoside species methylated at O-2′ of ribose, known to be an effective stabilizing motif in RNA. These results show that some aspects of tRNA modification in archaea are strongly associated with environmental temperature and support the thesis that posttranscriptional modification is a universal natural mechanism for control of RNA molecular structure that operates across a wide temperature range in archaea as well as bacteria.

The posttranscriptional processing of tRNA produces a diverse wealth of modified nucleotides (29, 30, 41), most of which occur at conserved RNA sequence locations in all three phylogenetic domains (4, 45). Many of the functional roles of these modifications, in addition to other factors, such as G-C and metal ion content, are associated with their influence on secondary and tertiary structures in RNA (1, 14, 43). Thus, RNA modifications offer an important means of mediation of RNA structure across the entire temperature range of natural habitats for microorganisms: in low-temperature organisms, a degree of conformational flexibility in tRNA must be maintained during translation, while in the case of thermophiles, protection against environmental temperatures which may exceed the melting point of unmodified base-paired stems is required (27, 50). For example, it has been shown with increases in growth temperature for a single species (2, 27, 51) or through comparison of closely related organisms growing optimally at different temperatures (32) that selected stabilizing tRNA modifications are associated with increased culture temperature. By contrast, in bacterial psychrophiles, low levels of modification have been reported, with the exception of dihydrouridine (10), a modified tRNA nucleoside which is associated with enhancement and maintenance of molecular flexibility at low temperatures (12).

From a phylogenetic perspective, it is interesting that the nucleoside structural motifs used for RNA stabilization at higher temperatures are known to be different in bacterial thermophiles (13, 24) and archaeal thermophiles (17), but tRNA modifications in low-temperature archaea have not previously been examined. We report here a detailed study of the identities and levels of nucleoside modifications in unfractionated tRNA from the psychrotolerant archaeon *Methanococcoides burtonii* (20). Cultures were grown at 23°C (the optimum growth temperature) and at 4°C (closer to the natural habitat temperature of 1 to 2°C [20]) in order to examine the overall level of tRNA modifications in comparison with that in bacteria, as well as recently studied mesophilic and thermophilic methanococci (32), and to assess the influence of a significant reduction in culture temperature upon modification. For contrast within the archaeal domain, we examined tRNA from the hyperthermophile *Stetteria hydrogenophila* (25) cultured at 93°C, near the growth optimum of 95°C. In both cases nucleoside modifications were measured by analysis of total enzymatic digests of tRNA using combined LC/MS, a definitive method for structural identification of RNA nucleoside modifications (7, 39).

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MATERIALS AND METHODS

Abbreviations and symbols used. Systematic names and structures for each nucleoside can be found on the World Wide Web at http://medlib.med.utah.edu/RNAmods. Abbreviations and symbols used are as follows: D, dihydrouridine; Ψ, pseudouridine; Um, 2′-*O*-methyluridine; $m^1\Psi$, 1-methylpseudouridine; s^2U, 2-thiouridine; s^4U, 4-thiouridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; m^5C, 5-methylcytidine; m^5Cm, 5,2′-*O*-dimethylcytidine; Cm, 2′-*O*-methylcytidine; m_2^4Cm, N^4,N^4,2′-*O*-trimethylcytidine; ac^4C, N^4-acetylcytidine; ac^4Cm, N^4-acetyl-2′-*O*-methylcytidine; m^1A, 1-methyladenosine; m^2A, 2-methyladenosine; m^6A, N^6-methyladenosine; m_2^6A, N^6,N^6-dimethyladenosine; t^6A, N^6-threonylcarbamoyladenosine; m^6t^6A, N^6-methyl-N^6-threonylcarbamoyladenosine; hn^6A, N^6-hydroxynorvalylcarbamoyladenosine; $hn^6A + CH_3$, monomethyl derivative of hn^6A; ms^2t^6A, 2-methylthio-N^6-threonylcarbamoyladenosine; ms^2hn^6A, 2-methylthio-N^6-hydroxynorvalylcarbamoyladenosine; Am, 2′-*O*-methyladenosine; I, inosine; m^1I, 1-methylinosine; m^1G, 1-methylguanosine; m^2G, N^2-methylguanosine; m^7G, 7-methylguanosine; Gm, 2′-*O*-methylguanosine; m_2^2G, N^2,N^2-dimethylguanosine; m^2Gm, N^2,2′-*O*-dimethylguanosine; m_2^2Gm, N^2,N^2,2′-O-trimethylguanosine; m_3G, trimethylguanosine; $m^{2,7}Gm$, N^2,7,2′-*O*-trimethylguanosine; o^8G, 8-oxoguanosine; imG, wyosine; imG2, isomer of imG; imG*, demethyl isomer of wyosine; mimG, methylwyosine; G^+, archaeosine; MH^+, molecular ion, corresponding to the protonated molecule; BH_2^+, base fragment ion, corresponding to the protonated free base of a nucleoside; SME medium, synthetic marine medium; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography-electrospray ionization mass spectrometry; T_m, melting temperature.

Cell sources and culture conditions. *M. burtonii* (DSM 6242) was isolated from Ace Lake in Antarctica, where the in situ temperature is annually 1 to 2°C (20). It has an optimal growth temperature of 23°C and an upper growth temperature limit of approximately 28°C (20). To prepare biomass for tRNA extraction, cells were grown anaerobically in a modified methanogen growth medium (47) at 4 and and 23°C, the cells were harvested by centrifugation (10,000 × *g*), and the pellet was lyophilized.

S. hydrogenophila (DSM 11227) was cultivated in half-strength SME medium as described previously (25). Mass cultures were grown in a 100-liter enamel-coated fermentor at 93°C at pH 6.0. The agitation rate was 100 rpm, and the gassing rate was 2 liters/min (H_2/CO_2 = 80:20).

Isolation and enzymatic digestion of tRNA. tRNAs were isolated as described previously (5) and totally digested to nucleosides using nuclease P1, phosphodiesterase I, and bacterial alkaline phosphatase (8), usually on a scale of several tens of micrograms of tRNA.

Analysis of tRNA digests by LC/MS. Nucleoside mixtures produced by enzymatic hydrolysis of tRNA were analyzed using an LC/MS system consisting of a Quattro II (Micromass) triple-quadrupole mass spectrometer with a Z-spray ion source interfaced to an HP 1090 (Hewlett-Packard) liquid chromatograph with a photodiode array detector, with both instruments under the control of MassLynx 3.4 (Micromass) software. Under typical operating conditions, mass spectra of quality suitable for nucleoside identification using this system can be obtained at limits of detection of approximately one modified nucleoside per 5×10^3 total nucleosides in a digest of unfractionated tRNA, a ratio constrained largely by HPLC column loading limits.

Chromatographic separation of nucleosides (see Fig. 1 and 2) was carried out with a Luna C_{18} (Phenomenex) reversed-phase column (2.0 by 250 mm), thermostatted at 40°C, with a buffer gradient system composed of 5 mM ammonium acetate (pH 5.3) (buffer A) and acetonitrile-water (40:60, vol/vol) (buffer B) at a flow rate of 0.3 ml/min, essentially as described previously (19). Analysis of dihydrouridine in *M. burtonii* tRNAs (see Fig. 3) was made with a Develosil RP-Aqueous (C_{30}) (Phenomenex) reversed-phase column (250 by 2 mm), with the same buffer system as above but using a multilinear gradient from 100% A to 100% B over 24 min. The procedures for the LC/MS experiments and the interpretation of resulting data for identification of nucleosides were similar to those detailed earlier for a thermospray, rather than electrospray, ionization-based protocol (39).

Some LC/MS experiments were carried out by using deuterated chromatographic mobile phases, such as D_2O and ND_4COCH_3, to allow complete exchange of labile hydrogen (protium) atoms in nucleosides by deuterium during chromatography (18). Labeled HPLC solvents were prepared by closely following an earlier protocol (39), or for small volumes and limited use by simply diluting concentrated unlabeled reagents with D_2O. The mass shifts resulting from deuterium exchange, when unambiguous values were obtained, were used to verify nucleoside structure assignments and to distinguish isomers differing in the number of exchangeable hydrogen atoms.

Calculation of relative tRNA modification levels. Crude estimates of overall modification levels for each tRNA were calculated from HPLC peak areas from UV detection at 260 nm, using MassLynx chromatography software routines. Based on total peak areas of 100% (including unmodified A, U, G, and C), the sum of modified nucleoside peak areas for each organism is presented as a portion of the total. Although individual nucleosides exhibit different molar absorptivities, for example in purines versus pyrimidines, the aggregate absorptivities and molar ratios of purines and pyrimidines are considered sufficiently similar to allow crude estimates of modification levels to be derived in this fashion. Responses from deoxynucleosides resulting from residual DNA impurities, readily recognized from their mass spectra, were excluded from the calculations.

Estimates of the average numbers of residues of modified nucleosides m^2G, m^2Gm, m_2^2G, and m_2^2Gm per tRNA molecule were calculated as follows. Percents A, U, G, and C were calculated from respective HPLC peak areas using the following relative 260 nm UV response values, which were established from an equimolar nucleoside digest that had been analyzed by HPLC using the same buffered gradient as for LC/MS measurements: A, 1.0; U, 0.685; G, 0.80; and C, 0.49. Individual nucleoside abundances were then referenced via HPLC peak areas to percent G, assuming a molar absorptivity (ε) ratio of 0.86 for $\varepsilon_G:\varepsilon_{m_2^2G}$ (22) (and therefore for $\varepsilon_G:\varepsilon_{m_2^2Gm}$ as well), and a molar absorptivity ratio of 1.22 for $\varepsilon_G:\varepsilon_{m^2G}$ (and therefore for $\varepsilon_G:\varepsilon_{m_2^2Gm}$), all at 260 nm (22). The residue-per-tRNA abundance values were calculated assuming 76 nucleotides per tRNA molecule.

RESULTS

Modified nucleosides identified by LC/MS analysis are summarized in Table 1, representing assignments shown in Fig. 1 and 2. In selected cases (see below), UV spectra of HPLC effluents on line, and mass spectra of deuterium-exchanged nucleosides, were used to distinguish isomers. All nucleoside assignments shown were made primarily on the basis of their mass spectra and relative HPLC retention times (39) and are considered definitive. Eighteen ribonucleosides were detected in digested tRNA isolates from *M. burtonii* and 34 were detected in tRNA from *S. hydrogenophila*, of which one in *M. burtonii* (m_2^6A) and three in *S. hydrogenophila* (o^8G, m_2^4Cm, and m_2^6A) were judged not to be native nucleosides from tRNA (see below). Therefore, a total of 17 nucleosides from *M. burtonii* tRNA and 31 nucleosides from *S. hydrogenophila* tRNA were characterized. All isolates were found to contain 2′-deoxyribonucleosides resulting from DNA impurities, which were readily identified from their characteristic mass spectra and relative retention times (39).

***M. burtonii* nucleosides.** No qualitative difference in identities of nucleosides between the cultures grown at 4 and 23°C was observed. Relative modification levels from 4 and 23°C cultures were measured as 1.9 and 2.6%, respectively. In consideration of the low levels of modification involved, the difference between these values is not considered significant. Likewise, no differences in the modification patterns exhibited in Fig. 1A and 1B are apparent. Ion current responses from dihydrouridine and uridine at the two culture temperatures are shown in Fig. 3. The relative D levels of 1.5% (4°C) and 1.7% (23°C) are experimentally indistinguishable.

The detection of D, a modified nucleoside having essentially no UV-absorbing chromophore and thus not detectable with a conventional UV detector, was based on mass analysis using the MH^+ ion, *m/z* 247 (Fig. 3A and C). For this purpose a C_{30} reversed-phase HPLC column was used in which D (M_r, 246) separates completely from Ψ (M_r, 244). Use of this column avoids overlapping (in time) signals from D and the second isotope peak of Ψ (both *m/z* 247), as found with the more

TABLE 1. Summary of ribonucleosides identified in unfractionated tRNA

Nucleoside	Result for organism at growth temp			
	Methanococcoides burtonii (4°C)	*Methanococcoides burtonii* (23°C)	*Stetteria hydrogenophila* (90°C)	*Methanococcus igneus* (85°C)[a]
Uridines				
D	+	+		
Ψ	+	+	+	+
Um			+	+
$m^1\Psi$	+	+		tr
s^4U	+	+		+
Cytidines				
s^2C				+
m^5C			+	+
m^5Cm			+	
Cm	+	+	+	+
ac^4C			+	
ac^4Cm			+	
Adenosines				
m^1A	+	+	+	+
m^2A			+	
m^6A	+	+	+	+
t^6A	+	+	+	+
hn^6A	+	+	+	+
$hn^6A + CH_3$[b]			+	
ms^2t^6A			+	+
ms^2hn^6A	+	+	+	
Am			+	+
I			+	+
m^1I			+	+
Guanosines				
m^1G	+	+	+	+
m^2G	+	+	+	+
m^7G	+	+	+	
Gm	+	+	+	+
m_2^2G	+	+	+	+
m^2Gm			+	+
m_2^2Gm			+	+
m_3G[b]			+	
$m^{2,7}Gm$			+	
imG			+	+
imG2[b]			+	
imG*[b]	+	+		+
mimG			+	
Other nucleosides				
G^+	+	+		+
Unknown N415[b]			+	

[a] From reference 32.
[b] Complete structure unknown.

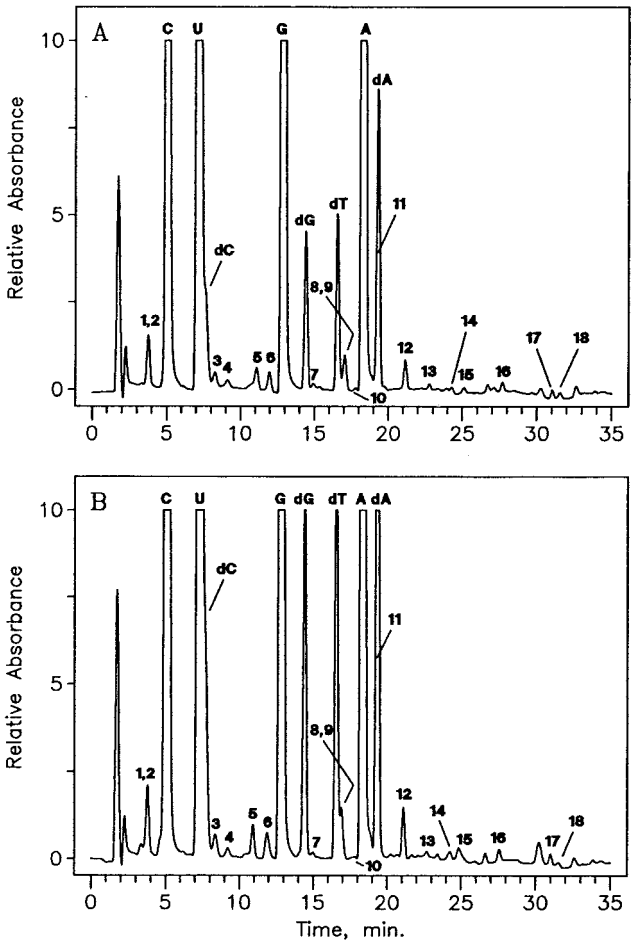

FIG. 1. LC/MS analysis of nucleosides from unfractionated tRNA of *M. burtonii* cultured at 4°C (A) or 23°C (B). Components: 1, D; 2, Ψ; 3, $m^1\Psi$; 4, m^1A; 5, m^7G; 6, Cm; 7, s^4U; 8, m^1G; 9, Gm; 10, m^2G; 11, G^+; 12, m_2^2G; 13, t^6A; 14, m^6A; 15, imG*; 16, hn^6A; 17, m_2^6A (non-tRNA nucleoside; see the text); 18, ms^2hn^6A.

commonly used C_{18} reversed-phase system (39). The D/uridine ion current ratios shown in Fig. 3 reflect an average D level of between one and two residues per tRNA molecule. This estimate is based on comparison with D and uridine ion current signals from unfractionated *Escherichia coli* tRNA in which the amounts of the two nucleosides had been accurately determined by a stable-isotope dilution method (11) and the assumption that uridine contents of *E. coli* and *M. burtonii* tRNAs are similar.

s^4U (component 7) was distinguished from the 2-thio isomer by its UV spectrum: λ_{max}, 331 nm for component 7; the values in the literature (21) are 331 nm for s^4U and 275 nm for s^2U. The 17-min HPLC peak in Fig. 1A and B is composed of two nucleoside isomers (components 8 and 9) with the same molecular mass (M_r, 297) corresponding to monomethyl guanosines and having essentially the same retention times (39). They are recognized and assigned as m^1G and Gm, as indicated by presence of both protonated base ions *m/z* 166 and 152, respectively, occurring within approximately the same elution profile (7). Similarly, the hypermodified nucleoside G^+ (component 11 in Fig. 1) elutes in the leading edge of a much larger amount of 2-deoxyadenosine from DNA impurity but is easily mass detected by using its unusual molecular ion (MH^+, *m/z* 325) and base ion (BH_2^+, *m/z* 193) mass values. The complex amino acid-containing nucleoside hn^6A (component 16 in Fig. 1) was distinguished from an isomer known in bacterial tRNA (m^6t^6A), which differs from hn^6A by the placement of a methyl group in the amino acid side chain (40). Differentiation between the two isomers was based on further fragmentation of the base ion (BH_2^+, *m/z* 295), whose mass is common to both possibilities. Formation of *m/z* 136 from adenine and *m/z* 134 from protonated hydroxynorvaline, rather than *m/z* 150 (methy-

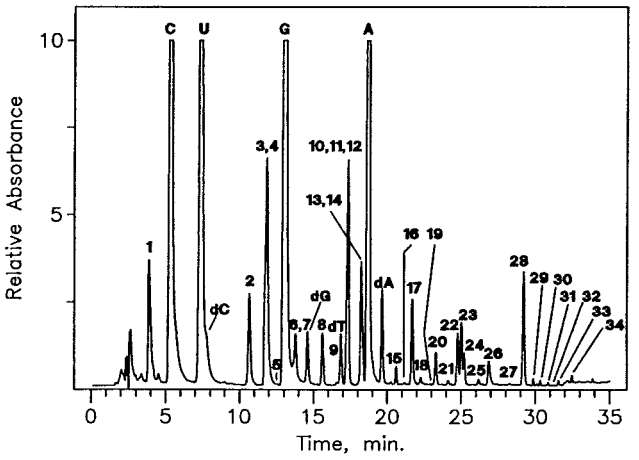

FIG. 2. LC/MS analysis of nucleosides from unfractionated tRNA of *S. hydrogenophila* cultured at 90°C. Components: 1, Ψ; 2, m^5C; 3, m^1A; 4, Cm; 5, I; 6, m^7G; 7, o^8G (non-tRNA nucleoside; see the text); 8, Um; 9, m^5Cm; 10, m^1I; 11, m^1G; 12, Gm; 13, ac^4C; 14, m^2G; 15, t^6A; 16, m_3G isomer of unknown structure; 17, m_2^2G; 18, Am; 19, m_2^4Cm (non-tRNA nucleoside, see the text); 20, ac^4Cm; 21, m^2A; 22, m^6A; 23, m^2Gm; 24, hn^6A; 25, ms^2t^6A; 26, $m^{2,7}Gm$; 27, imG; 28, m_2^2Gm; 29, $hn^6A + CH_3$; ms^2hn^6A; 30, 31, imG isomer of unknown structure; 32, unknown nucleoside N415; 33, m_2^6A (non-tRNA nucleoside, see text); 34, mimG.

ladenine) and *m/z* 120 (threonine), as would be required for the isomer m^6t^6A, indicated the identity of component 16 as hn^6A. One nucleoside with a partially known structure was encountered in *M. burtonii* tRNA, designated imG* in Fig. 1 and Table 1. This nucleoside (M_r, 321), previously found in tRNAs from five methanococci (32), is a member of the imG tricyclic nucleoside family, as shown by its characteristic UV spectrum (26) (UV λ_{max}, 228 and 282 nm). The molecular mass corresponds to a nucleoside having two fewer methyl groups than the archaeal nucleoside mimG (M_r, 349), which has three methyls (31). The structure of nucleoside imG* is under investigation. Component 17 in Fig. 1, m_2^6A, is a common minor product in tRNA preparations, arising as an easily detected impurity derived from 16S rRNA, in which its presence is unique and ubiquitous in highly conserved sequences (48, 49). Unannotated peaks in Fig. 1 were shown from their mass spectra not to be nucleosides.

***S. hydrogenophila* nucleosides.** Nucleosides released by hydrolysis of *S. hydrogenophila* tRNA are represented in a chromatogram of unusual complexity (Fig. 2). o^8G (component 7 in Fig. 2) coelutes with m^7G (MH^+, *m/z* 298; BH_2^+, *m/z* 168) and was identified following deuterium labeling (*m/z* 300 shift to 308; *m/z* 168 shift to 174), which showed seven exchangeable hydrogen atoms in the neutral molecule, one more than in guanosine. Component 7 therefore exhibits 16 Da of modification in the base compared with guanosine and no modification in ribose. Assignment of the likely structure as o^8G was

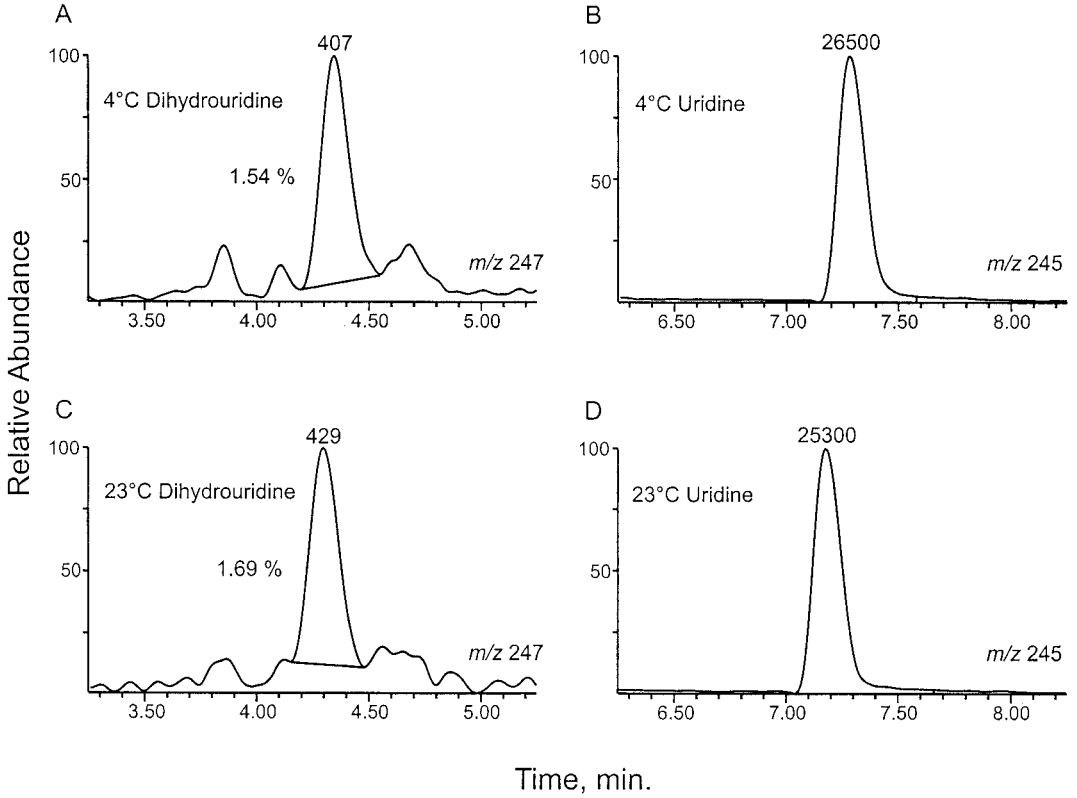

FIG. 3. LC/MS detection of D in tRNA from *M. burtonii* cultured at 4°C (A) and 23°C (C), based on response using the MH^+ ion, *m/z* 247. Relative peak areas (407 and 429 units) compared with corresponding responses from uridine (26,500 and 25,300 units) (B and D) show relative levels of D of 1.54% at 4°C and 1.69% at 23°C. The baselines used for area integrations are indicated in panels A and C.

confirmed by rigorous comparison against authentic material (gift of T. Hashizume, University of Utah): HPLC retention time, 13.7 min; MS (MH^+, *m/z* 300; BH_2^+, *m/z* 168). This unexpected component is viewed as a likely product of adventitious oxidation of guanosine, as might result from xenobiotic or metabolic oxidation processes (for discussion and leading references to the sources of this nucleoside in RNA, see references 23 and 52). Support for what we view as the unlikely authenticity of o^8G as being native to tRNA would require at a minimum its localization to specific tRNA sequence sites, which was not undertaken. Similar observations and conclusions regarding trace amounts of o^8G in some archaeal hyperthermophile tRNAs have been previously made (J. A. McCloskey and K. O. Stetter, unpublished results).

The assignments of site of cytosine methylation in components 2 and 9 as position C-5 (i.e., m^5C and m^5Cm) rather than as the likewise biologically plausible N^4- or N-3 methyl isomers, were made by deuterium labeling. Component 2 showed five exchangeable hydrogens in the neutral molecule and three in the neutral base, while component 9 showed four and three, respectively. Those values mandate methylation at C-5. Substitution at heteroatom positions (N^4-or N-3) would result in one fewer exchangeable hydrogen in each case.

The unusual diversity of modifications exhibited by *S. hydrogenophila* tRNA includes m^2A, not previously reported for any type of archaeal RNA (34). Deuterium exchange experiments were used to confirm the assignments shown for the methyladenosine isomers m^2A and m^6A (Fig. 2) made initially by relative retention times and mass spectra. Their isotopic exchange patterns likewise differ due to C versus N alkylation sites, respectively: m^2A contains five exchangeable hydrogens in the neutral molecule (component 21), while m^6A contains four (component 22).

The presence of both methylguanosine isomers m^1G and Gm (components 11 and 12 in Fig. 2) was established by using the base ions as in the case of *M. burtonii* (Fig. 1) described previously. All other assignments of multiple components eluting in unresolved HPLC peaks were made on the basis of molecular ion and base ion mass values and relative retention times, as appropriate (39).

Three additional nucleosides with partially known structures and one with an unknown structure were found in the *S. hydrogenophila* digest. In all four cases, the characteristic 132-mass-unit spacing between MH^+ and BH_2^+ ions (due to loss of ribose) was used to identify each component as a ribonucleoside, without a substitution in ribose. Component 16 (Fig. 1) (UV λ_{max}, 261.5 nm) is judged from its molecular mass (M_r, 325) and base mass as likely to be a base-trimethylated derivative of guanosine. The potential mass isomer acetylguanosine (not a known RNA component) cannot be excluded, although its elution position would likely be much later. Component 29 is assigned as a methyl homolog of the complex amino acid-containing nucleoside hn^6A (40), based on a molecular mass of 440, which is 14 higher than that of hn^6A (M_r, 426). Upon deuterium exchange the MH^+ ion *m/z* 441 shifted to *m/z* 449, requiring seven exchangeable hydrogens in the neutral molecule. This shift value is the same as for nucleoside hn^6A, consistent with substitution by the additional methyl group to be on carbon rather than a heteroatom (N or O).

Two nucleosides observed are considered likely to arise as minor impurities from small-subunit rRNA: m_2^6A and m_2^4Cm (components 33 and 19 in Fig. 2). The arguments concerning the origin of m_2^6A are presented above. The novel trimethylcytidine m_2^4Cm was recently discovered and characterized in trace amounts in a tRNA isolate from the hyperthermophilic archaeon *Aeropyrum pernix*; it was concluded to arise as an impurity from 16S rRNA, where it was found in greater abundance (R. Van Wagoner, E. Bruenger, G. M. Caballero, N. Nomura, Y. Sako, and J. A. McCloskey, unpublished data), and is therefore similarly assigned in the present case to rRNA.

TABLE 2. Structural diversity and modification levels of nucleosides in selected unfractionated tRNAs

Organism	Culture temp (°C)	No. of modified tRNA nucleosides detected	% Modification[a]	Reference
Methanococcoides burtonii	4	17[b]	1.9	This study
Methanococcoides burtonii	23	17[b]	2.6	This study
Stetteria hydrogenophila	90	31[c]	13	This study
Methanococcus maripaludis	37	23	9.3	32
Methanococcus igneus	85	24	9.6	32
Escherichia coli	37	26	5.1	21; this study
ANT-300[d]	5	13	~6.6[e]	10

[a] Crude estimate based on HPLC peak areas; see the text.
[b] One additional nucleoside was detected (m_2^6A) and judged to be not native to tRNA; see the text.
[c] Three additional nucleosides were detected and judged to be not native to tRNA (o^8G, m_2^4Cm, and m_2^6A); see the text.
[d] A psychrophilic marine bacterium (35) which is a species of *Moritella* (36).
[e] Estimate calculated by using unpublished HPLC chromatograms associated with an earlier report (10).

DISCUSSION

The overall differences found between tRNA modifications in the two archaea studied are dramatic, particularly in three respects. First, the modification level in the psychrotolerant archaeon *M. burtonii* of approximately 2% is the lowest of which we are aware in any organism, including the bacterial psychrophile ANT-300 (Table 2). This comparison also considers *Mycoplasma capricolum* tRNAs, which are often considered undermodified, based on the complete set of published tRNA sequences (3). Within the domain *Archaea*, and based on the same measurement criteria, modification of *M. burtonii* tRNA is significantly lower than in the mesophilic archaeal methanogen *Methanococcus maripaludis* (32). We conclude that tRNA adaptation to cold in archaea parallels, and could be more pronounced than, that observed for cold-adapted bacteria (10), but additional organisms should be studied. (Comments on the differences between two culture temperatures in *M. burtonii* are presented below.)

By contrast, the 13% tRNA modification level found in the hyperthermophile *S. hydrogenophila* is exceptional, compared for example to that in the archaeal thermophile *Methanococcus igneus* (Table 2), which grows at 85°C. These observations support the overall thesis that posttranscriptional modification in tRNA serves in part to provide crucial elements of control in

fine-tuning of tertiary structure for optimal function (16, 43), which is of paramount importance in thermophiles (27, 50) but less so at significantly lower temperatures (10), as in the notable case of *M. burtonii*.

It is important to note that the overall extent of modification is also subject to the influence of phylogeny, the effects of which cannot always be clearly distinguished from those associated with temperature. An unusual but notable example is the similarity of modification levels between *M. maripaludis* and *M. igneus* (Table 2), which grow optimally at very different temperatures. However, if the levels of specific modifications closely tied to thermostability are considered, a clearer picture of temperature effects emerges: e.g., the trimethylated guanosine m_2^2Gm, believed to be a stabilizing entity at tRNA stem junctions, is present in *M. igneus* (85°C) but absent in *M. maripaludis* (37°C) (32). (Further discussion of m_2^2Gm is given below in the section on *S. hydrogenophila*.) For comparison of organisms not closely related the phylogeny, temperature distinction is also confounded by the present lack of sufficient data to gain a refined picture, particularly with regard to bacterial thermophiles, for comparison with a large body of data on archaeal thermophiles (17, 27, 33).

The differences between *M. burtonii* and *S. hydrogenophila* in overall modification, when considered in terms of the numbers of structural motifs employed at the nucleoside level (Table 2), are similarly great. The finding of 31 different modified nucleosides in *S. hydrogenophila* tRNAs is the greatest number of which we are aware, based on earlier reports of tRNA modification in various archaeal hyperthermophiles (17, 32, 33) and eukarya, including unfractionated calf liver tRNA (21). The occurrence of nine different methylated guanosines is particularly striking. The structural diversity of nucleosides in *M. burtonii*, 17 modified nucleosides, is greater than that in the bacterium ANT-300 but still significantly below that of other representative organisms (Table 2). The strong apparent correlation between temperature of growth and number of modification motifs recruited by the RNA-processing enzymes seems to be more pronounced at higher temperatures, at least within the archaea. This probably reflects the essential need for mechanisms of tRNA stabilization at growth temperatures generally above the melting points of unmodified tRNA base-paired stems (a summary of T_m values in relation to G+C content and the role of modification can be found in reference 27). Additional comparisons of modification patterns, between closely related archaeal mesophiles and thermophiles from selected methanococci, have been reported (32).

M. burtonii is shown (Fig. 3) to contain significant amounts of D, a nucleoside associated with maintenance of polynucleotide flexibility in RNA (12). This modification is absent in *S. hydrogenophila* and in most other archaeal hyperthermophiles and mesophiles examined (17), unlike its wide occurrence in eukarya and bacteria, including the bacterial thermophiles (45). Within archaea, the finding of D in abundance only in *M. burtonii* supports and extends the interpretation of its functional importance in psychrophilic bacteria (10). The occurrence of more than one D residue per tRNA molecule in *M. burtonii* is about the same as that in *E. coli* (1.79 mol%) (11) but is greater than that in other archaea studied to date. Thus, posttranscriptional enzymatic processing to form D appears to be a common mechanism for adaptation to the cold in both archaea and bacteria.

Quantitative data for D incorporation is not available for other cold-adapted archaea. Recently, however, putative D synthase genes were identified in the partial genome sequences of *M. burtonii* and the psychrophilic methanogen *Methanogenium frigidum* (44). This indicates that D incorporation may be a general characteristic of cold-adapted archaea. No convincing evidence was obtained that levels of D are responsive to a decrease in growth temperature (Fig. 3), although it will be interesting to examine regulation of expression of the D synthase gene.

We know of no previous effort to document the presence versus the absence of D or the level of D as a function of temperature in tRNA from any given organism. Thus, there is no evidence for a response opposite to that found for bacterial (2, 51) and archaeal (27) thermophiles (increase in abundance of selected stabilizing modifications with culture temperature). However, it was shown that in small D-containing oligonucleotides the fractional population of the inherently more flexible C-2′-*endo* ribose conformer of D increases at lower temperatures (12). Therefore, in contrast to a presence-versus-absence mechanism for preservation of conformational flexibility at lower temperatures, the influence of D in those organisms may reside to some extent in a thermodynamic preference for favored ribose conformers in existing D residues present in the D and T loops.

Both *M. burtonii* and *S. hydrogenophila* contain tRNA modifications that have been formed potentially under the direction of small RNAs known under the generic name of guide RNAs (15, 38). These modifications are pseudouridines (in both organisms) and ribose-methylated nucleosides (in *S. hydrogenophila*). Modification targeting by sRNAs has been studied mostly with regard to rRNA modification in eukarya but has been reported to operate also in some archaeal tRNAs (15, 38, 46). The extent to which these mechanisms of modification operate in the present case is unknown, and progress is confounded by the absence of knowledge regarding isoacceptor tRNA sequences, which remains a worthwhile area for future investigation.

Modifications in *M. burtonii*. The strikingly low levels of tRNA modification that occur in *M. burtonii* include nine nucleosides that are common to RNA in all three phylogenetic domains (34) (*Archaea*, *Bacteria*, and *Eukarya*) and thus constitute a minimalist core of modifications that probably evolved prior to the separation of these domains. These modified species are D, Ψ, and the simple monomethylated nucleosides Cm, Gm, m^1G, m^7G, m^1A, and m^6A. Two of the modifications, $m^1\Psi$ and G^+, are unique hallmarks of archaeal modification that occur widely but are each highly conserved at single sequence locations, tRNA positions 54 and 15, respectively (45). The remaining nucleosides are evenly divided from the perspective of phylogenetic commonality: s^4U, hn^6A, and ms^2hn^6A are shared with bacteria, while the guanosine derivatives m^2G, m_2^2G, and imG* are otherwise characteristically eukaryotic (34). Notably absent in tRNA from *M. burtonii* is the complex anticodon nucleoside mnm^5s^2U, recently detected in four mesophilic and thermophilic methanococci (32). However, mnm^5s^2U is usually considered a bacterial modification (in tRNA-Glu and -Lys [45]) which is otherwise unknown in

archaeal tRNA and may be restricted only to the methanococci. Overall, these findings substantially add to our limited understanding of the mechanisms of adaptation in psychrotolerant archaea (6).

Modifications in *S. hydrogenophila*. A significant fraction of the unusual number and diversity of modifications in *S. hydrogenophila*, 9 of 31 nucleosides, are modified by methylation at O-2′ of ribose and are therefore assumed to exert influences through points of regional stabilization of the folded tRNA molecule. This effect is attributed to thermodynamic stabilization of the C-3′-*endo* sugar conformation, thus avoiding base–O-2′ steric interactions in the alternate C-2′-*endo* conformation (14), resulting in higher T_ms (9) and reduced dynamic motion at elevated temperatures. Previous studies have demonstrated increased levels of ribose methylation as a function of culture temperature, in both tRNA (2, 27, 28) and rRNA (37). Two nucleosides found in *S. hydrogenophila*, ac^4Cm and m_2^2Gm (and the related modification m^2Gm), were reported to be temperature responsive in tRNA from the archaeal hyperthermophile *Pyrococcus furiosus* (27). The family of structurally related nucleosides, m^2G, m_2^2G, m^2Gm, and m_2^2Gm, are from known archaeal tRNA sequences, conserved at only two locations, positions 10 and 26 (45). Nucleotides at these locations are at the junctions of the acceptor and D-loop stems and of the D-loop and anticodon stems, respectively, where they play crucial roles in the control and stabilization of the tertiary L fold of the tRNA (42). The unusual abundance of m_2^2Gm in Fig. 2, taking UV molar absorptivities into account, corresponds to an average of approximately 0.6 m_2^2Gm residues per tRNA molecule, while the estimated sum of the four modifications is approximately two residues per molecule. This implies that essentially all tRNAs from *S. hydrogenophila* are modified at both positions, in contrast to *M. burtonii* (Fig. 1, components 10 and 12), in which an average of only ~0.2 residues per molecule are modified in this fashion. This result further extends the implication of these residues in tRNA stabilization in the archaeal thermophiles (27, 32) and their greatly reduced role in tRNA from *M. burtonii*.

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