Posttranscriptional Modification of tRNA in Psychrophilic Bacteria

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Posttranscriptional modification in tRNA is known to play a multiplicity of functional roles, including maintenance of tertiary structure and cellular adaptation to environmental factors such as temperature. Nucleoside modification has been studied in unfractionated tRNA from three psychrophilic bacteria (ANT-300 and Vibrio sp. strains 5710 and 29-6) and one psychrotrophic bacterium (Lactobacillus bavaricus), Based on analysis of total enzymatic hydrolysates by liquid chromatography-mass spectrometry, unprecedented low amounts of modification were found in the psychrophiles, particularly from the standpoint of structural diversity of modifications observed. Thirteen to 15 different forms of posttranscriptional modification were found in the psychrophiles, and 10 were found in L. bavaricus, compared with approximately 29 known to occur in bacterial mesophiles and 24 to 31 known to occur in the archaela hyperthermophiles. The four most abundant modified nucleosides in tRNA from each organism were dihydrouridine, pseudouridine, 7-methylguanosine, and 5-methyluridine. The molar abundances of the latter three nucleosides were comparable to those found in tRNA from Escherichia coli. By contrast, the high levels of dihydrouridine observed in all three psychrophiles are unprecedented for any organism in any of the three phylogenetic domains. tRNA from these organisms contains 40 to 70% more dihydrouridine, on average, than that of the mesophile E. coli or the psychrotroph L. bavaricus. This finding supports the concept that a functional role for dihydrouridine is in maintenance of conformational flexibility of RNA, especially important to organisms growing under conditions where the dynamics of thermal motion are severely compromised. This is in contrast to the role of modifications contained in RNA from thermophiles, which is to reduce regional RNA flexibility and provide structural stability to RNA for adaptation to high temperature.

The structural diversity (31) and multiplicity of roles played by posttranscriptional modification in RNA, particularly tRNA (3, 4, 59), has been clearly established. Despite the fact that 80 different modified nucleosides are presently known (8) to occur in tRNA from organisms representing all three phylogenetic domains (31), nearly all knowledge of posttranscriptional modification in RNA is derived from studies of mesophiles and thermophiles. Of 521 reported tRNA sequences (46), the only apparent exception is the initiator tRNA from the arthropod Euphausia superba, which inhabits the Antarctic Sea (52). We are unaware of previous investigations reporting modification in tRNA from any psychrophilic microorganism, despite the fact that greater than 80% of the biosphere is characterized by temperatures below 5°C. Nevertheless, interest in these organisms and their biotechnological potential has increased substantially in recent years (25, 32), and they represent a fundamentally important but understudied segment of the biosphere. Psychrophilic microorganisms have an optimum temperature for growth at 15°C or lower, a maximum temperature for growth at or below 20°C, and a minimal temperature for growth at 0°C or lower (35). Examples include the presently studied organisms ANT-300 (growth range, −5 to 12°C; optimum, 7°C) (36), strain 29-6 (growth range, −5 to 9°C; optimum, 7°C) (21), and strain 5710 (growth range, −5 to 11°C; optimum, 4 to 8°C) (21). Psychrophilic microorganisms are restricted to permanently cold habitats such as oceans (the temperature of most ocean water is around 3°C) and polar areas. The ecological distribution of psychrophiles and psychrotrophs has been reviewed by Baross and Morita (2). Psychrophiles are defined as those organisms that do not meet the definition stated above and can be thought of as mesophiles (maximum temperature of growth above 20°C) capable of growth at or around 0°C. An example is Lactobacillus bavaricus (growth range, 2 to 37°C) (47).

Compelling evidence for biochemical adaptation of psychrophiles to low temperatures comes primarily from studies of psychrophilic proteins and membrane lipids. It is known, for example, that decreasing the culture temperature of a psychrophile increases levels of unsaturated phospholipids and neutral lipids to maintain membrane fluidity at low temperatures (22, 23, 38). Cold adaptation of psychrophilic proteins has been demonstrated by subtilisin from Bacillus sp. strain TA41 (12) and α-amylase from Alteromonas haloplanktes A23 (16) having higher catalytic efficiencies (Kcat/Km) than their mesophilic counterparts. This characteristic is associated with an increase in molecular flexibility that allows catalytic conformational changes to occur with less energy input, compensating for the reduction of reaction rates at low temperatures (12, 16). Although some general effects of variations in culture temperature on nucleic acid levels, ribosome structure and function,
and tRNA structure in psychrophilic bacteria (32) and fungi (45) have been reported, very little is known regarding cold adaptation of nucleic acids through posttranscriptional processes in psychrophilic microorganisms.

The role of posttranscriptionally modified nucleosides in RNA of thermophilic bacteria (54, 55) and archaea (26, 28) in enforcing conformational stability of RNA has been documented. By contrast, there is no extant literature on the extent of posttranscriptional modification of any RNA in psychrophilic microorganisms or whether specific modified nucleosides are produced in the RNA of these organisms to preserve biological activity and structural integrity at low temperature.

This paper reports results of a detailed study of nucleoside modification patterns in the unfractionated tRNA from three psychrophilic bacteria, ANT-300 and Vibrio sp. strains 5710 and 29-6, and one psychrotrophic bacterium, L. bavaricus.

MATERIALS AND METHODS

Organisms, media, and culture conditions. All materials used for culturing, including pipette tips, centrifuge bottles, moclucation loops and media, were held at 5°C or lower before use.

Cultures of ANT-300 (36), a heterotrophic, psychrophilic marine bacterium which is an unnamed species of Martiella (37), were grown in Difco Marine Broth 2216, modified by the addition of 1 g of select yeast extract (Gibco BRL, Gaithersburg, Md.) per liter. The cells were grown under aerobic conditions in 700-ml batch cultures cultivated in 1,000-ml wide-mouth Erlenmeyer flasks. Cells were shaken at 135 rpm on a rotary shaker at 5°C until an optical density at 540 nm of 0.8 to 1.0 was attained. The cells were then harvested by centrifugation at 7,000 rpm for 10 min, washed with cold, sterile water, and immediately suspended in a cold solution of 0.15 M sodium chloride, 0.05 M sodium acetate, and 0.01 M magnesium acetate (pH 4.5) prior to extraction of tRNA.

Samples of strains 29-6 and 5710, psychrophilic marine bacteria tentatively classified as Vibrio strains (21), were collected during a dive in Sagami Bay, Kanagawa, Japan. The collection was carried out by the Shinkai 2000 (strain 29-6) and Shinkai 6500 (strain 5710) submersible vessels belonging to the Japan Marine Science and Technology Center. Sample collection was done with a remote-controlled arm attached to the vessel, and the mud suspensions were poured into a sterilized collection cylinder. Mud samples were divided into fractions on the ship immediately after the submersible vessel ascended to the surface. The samples, kept on ice, were then spread onto modified LB medium plates (1% [wt/vol] Difco Tryptone, 0.5% [wt/vol] Difco Yeast Extract, 3% [wt/vol] sodium chloride, [wt/vol] 1.5% agar) and incubated at 4°C. Bacterial colonies on the plates were picked and examined further. Strain 29-6 was isolated from a sample collected at a depth of 447 m in the Sea of Japan (21). Strain 5710 was isolated from a sample collected at a depth of 2,250 m in Suruga Bay, Japan (21).

These bacteria were grown in 100 ml of modified LB medium in 500-ml Erlenmeyer flasks shaking constantly at 10 rpm in water baths filled with 20 to 50% (wt/vol) antifreeze-water at 5°C. Cell growth was monitored by the increase in optical density at 660 nm. Cells were harvested by centrifugation at 5,000 × g for 10 min, and the supernatant was removed. The collected cells were freeze-dried and stored at −80°C.

Cultures of L. bavaricus DSM 20269, a psychrotrophic bacterium and species of the subgenus Streptobacterium (47), were grown in Lactobacillus MR5 medium (catalog no. 0881; Difco) at 4°C. The cells were harvested by centrifugation during the late logarithmic growth phase. The cell yield was 4.5 g/liter. The collected cells were frozen and stored at −70°C.

tRNA. tRNA was extracted from ANT-300, strains 29-6 and 5710, and L. bavaricus as described by Kuchino et al. (29). Final purification of tRNA was accomplished by silica-based anion-exchange chromatography using a Nucleobond AX500 cartridge.

Enzymatic digestion of tRNA. tRNA was hydrolyzed to nucleosides by using nuclease P1, venom phosphodiesterase, and bacterial alkaline phosphatase as described by Crain (7).

Directly combined liquid chromatography-mass spectrometry (LC-MS). Analysis of nucleosides in tRNA digests was carried out with a mass spectrometer consisting of a noncommercial quadrupole mass analyzer, with a thermal spray high-performance liquid chromatography (HPLC) interface (Vestec Corp., Houston, Tex.), controlled by a Vector/One data system (Teknivent, St. Louis, Mo.). HPLC separations were made by using a Supelcosil LC-18S reversed-phase column, 4.6 by 250 mm, and a 3 cm Brownlee Spheri-5 C18 precolumn. The HPLC gradient elution system of Buck et al. (5) with 0.25 M ammonium acetate (pH 6.0) and acetonitrile was used with minor modifications in the gradient profile (40). Thermostop ionization mass spectra were acquired every 1.7 s during the 40-min chromatographic separation. The instrument, procedures, and interpretation of data for characterization of nucleosides in RNA hydrolysates have been described in detail elsewhere (40).

Measurement of nucleoside molar ratios. Using weighed amounts of authentic nucleosides as standards, the molar proportions of pseudouridine, 5-methyluridine, and 7-methylguanosine relative to A, U, G, and C were determined from HPLC chromatographic peak heights, using UV detection in conjunction with standard absorbance curves for each nucleoside. G-C content and U mole percent calculations in tRNA from each organism were measured in the same way. Molar percentages of dihydrouridine in tRNA from the four bacteria were measured by using isotope dilution LC-MS as described (9).

RESULTS

Determination of posttranscriptional modification in tRNA from ANT-300, 29-6, 5710, and L. bavaricus by LC-MS. The identities of posttranscriptionally modified nucleosides in enzymatic hydrolysates of tRNA from the four organisms studied were established by LC-MS. HPLC chromatograms corresponding to the LC-MS analysis of each are presented in Fig. 1. The nucleoside identities indicated were established from relative HPLC retention times compared with catalog values (40) and from continuously recorded mass spectra. The ratio of UV absorbances at two wavelengths (A247/A280) was used as an additional criterion for nucleoside identification. All assignments made in Fig. 1, with the exception of dihydrouridine, were corroborated by the alignment of characteristic mass spectral ion profiles with UV detection peak profiles. Dihydrouridine elutes from the chromatograph on the leading edge of pseudouridine but is not detected by UV absorbance. It is, however, readily detected by mass spectrometry (15). A selected ion chromatogram for m/z 247, corresponding to the protonated molecular ion (MH+), of dihydrouridine, along with a simultaneously recorded mass spectrum taken from the analysis of nucleosides in a tRNA digest from strain 29-6 is shown in Fig. 2. Similar mass spectral data (not shown) demonstrating the presence of dihydrouridine were obtained for the other three tRNA digests shown in Fig. 1.

All four organisms showed exceptional undermethylation of their tRNAs relative to mesophiles and thermophiles. ANT-300, 29-6, 5710, and L. bavaricus have 13, 15, 14, and 10 modified nucleosides, respectively, in their unfractionated tRNAs. G-C contents in tRNA from these organisms ranged from 54 to 59% as measured from HPLC chromatographic peak heights. Molar percentages of the four most abundant of the observed modified nucleosides are shown in Table 1.

DISCUSSION

The involvement of tRNA in protein synthesis is a fundamentally important process in which tRNA interaction is required with a number of molecules in the cell, including elongation factors, initiation factors, aminoacyl-tRNA synthetases, RNA processing enzymes, and the ribosome. tRNA is also involved in additional diverse activities such as chlorophyll and heme biosynthesis, cell wall biosynthesis, and priming of retroviral RNA-directed DNA synthesis (44).

Posttranscriptional processing of tRNA produces numerous modified nucleosides (31) which appear to play a variety of roles in fine-tuning tRNA structure and function and the intermolecular interactions in which it participates (3, 4). The effects of certain posttranscriptional modifications on specific tRNA functions, such as enforcement of correct codon recognition (59), and on the structural stability of RNA (26, 28, 54, 55) have been well documented. Further, it is known that tRNA molecules completely lacking modified nucleosides do not adopt the native conformation without compensation from high magnesium concentrations (20).

The most extensive structural range of posttranscriptional modifications is found in tRNA from the hyperthermophilic archaea (15). A number of these modifications serve to stabi-
lize the C-3'-endo form of ribose in the polynucleotide chain, effectively stabilizing the A-type helical conformation that is common to RNA (26, 27, 57). They also promote stability through hydrogen bonding and enhanced base stacking (13), and they provide nuclease protection by ribose methylation (1). These modifications thereby provide significantly increased structural integrity to tRNA in these organisms, many of which grow optimally near the boiling point of water (48).

**Nucleosides in tRNA of psychrophilic bacteria.** Although much is known regarding posttranscriptionally modified nucleosides in mesophiles and thermophiles, posttranscriptional processing of tRNA from psychrophilic microorganisms has not been previously examined. G-C contents in tRNA from ANT-300, 29-6, 5710 (C), and *L. bavaricus* (D). Detection was done with UV at $A_{254}$. The mass spectrometrically detected elution position of dihydrouridine is indicated in each panel, although there was no UV response (see the text and Fig. 2). A listing of nucleoside symbols and chemical names is given in reference 31. Peak identities are indicated. Asterisks designate nonnucleoside impurities.

![Chromatographic separation of nucleosides from LC-MS analysis of enzymatic digests of 40 μg of unfractionated tRNA from ANT-300 (A), 29-6 (B), 5710 (C), and *L. bavaricus* (D). Detection was done with UV at $A_{254}$. The mass spectrometrically detected elution position of dihydrouridine is indicated in each panel, although there was no UV response (see the text and Fig. 2). A listing of nucleoside symbols and chemical names is given in reference 31. Peak identities are indicated. Asterisks designate nonnucleoside impurities.](https://jb.asm.org/)

**FIG. 1.** Chromatographic separation of nucleosides from LC-MS analysis of enzymatic digests of 40 μg of unfractionated tRNA from ANT-300 (A), 29-6 (B), 5710 (C), and *L. bavaricus* (D). Detection was done with UV at $A_{254}$. The mass spectrometrically detected elution position of dihydrouridine is indicated in each panel, although there was no UV response (see the text and Fig. 2). A listing of nucleoside symbols and chemical names is given in reference 31. Peak identities are indicated. Asterisks designate nonnucleoside impurities.

![Detection of dihydrouridine in strain 29-6 tRNA. (A) Reconstructed ion chromatogram for m/z 247 corresponding to the MH⁺ ion for dihydrouridine; (B) mass spectrum recorded at 3.2 min in panel A, showing the MH⁺ ion corresponding to dihydrouridine.](https://jb.asm.org/)

**FIG. 2.** Detection of dihydrouridine in strain 29-6 tRNA. (A) Reconstructed ion chromatogram for m/z 247 corresponding to the MH⁺ ion for dihydrouridine; (B) mass spectrum recorded at 3.2 min in panel A, showing the MH⁺ ion corresponding to dihydrouridine.
TABLE 1. Mole percentages of selected modified nucleosides in tRNA from five organisms

<table>
<thead>
<tr>
<th>Modification</th>
<th>Mol % (residues/100 nucleotides)</th>
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<tbody>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>Dihydrouridine</td>
<td>1.79</td>
</tr>
<tr>
<td>Pseudouridine</td>
<td>2.96</td>
</tr>
<tr>
<td>5-Methyluridine</td>
<td>1.10</td>
</tr>
<tr>
<td>7-Methylguanosine</td>
<td>0.645</td>
</tr>
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* Value from reference 10.

(∼77°C [data not shown]) to be similar to that of the mesophile *Escherichia coli* (75°C) (53).

With regard to modification patterns, however, we find that tRNA from psychrophilic and psychrotrophic bacteria is in general significantly hypomodified compared to that of organisms which grow optimally at higher temperatures. The psychrophiles and psychrotroph studied here contain, on average, 10 to 15 modified nucleosides (Fig. 1), compared with approximately 29 for mesophilic bacteria such as *Salmonella typhimurium* (5) and *E. coli* (18) or as many as 31 for the archaeal hyperthermophile *Pyrocococcus species* (53), which grows optimally at 100°C (15, 34). All of the posttranscriptionally modified nucleosides identified in the presently studied bacteria are known to be involved in the maintenance of basic structural characteristics of tRNA molecules. The X-ray crystallographic structure of yeast tRNA^Met^ shows 2'-O-methylguanosine, pseudouridine, 5-methyluridine, and 7-methylguanosine to be involved in forming tertiary hydrogen bonds essential for maintaining the universally conserved L-shaped three-dimensional structure of tRNA (42). Pseudouridine is also known to be important in specific interactions with aminoacyl-tRNA synthetases (3) and in maintenance of helix stability through base stacking (13). The modified nucleosides 2-thiocytidine, 2'-O-methylcytidine, and 2'-O-methylguanosine, and each of the adenosine derivatives identified in this study (Fig. 1), are known to be involved in fine-tuning anticodon loop structure and the fidelity of codon recognition (59).

No modified nucleosides that are unique to the bacterial psychrophiles were found in this study. Of the modified nucleosides identified, 2-thiocytidine, 2'-methyladenosine, and 5'-methyl-N^2^-threonylcarbamoyladenosine are unique to the eu-bacteria (31). 5-Methylcytidine occurs in a number of archaea and eukaryotes (19) but until now has been identified only in bacteria, in thermophiles such as *Pyrococcus furiosus* (34) which are deeply branched in the universal phylogenetic tree.

5-Methylcytidine is a modified nucleoside known to play a role in Mg^{2+} binding to tRNA (11). Its presence in tRNA from psychrophilic bacteria suggests a role in maintenance of the integrity of Mg^{2+} binding domains in a relatively flexible molecule (see below). Interestingly, N^6^-isopentenyladenosine, which is considered generally as a eukaryotic modification (31), is also present in strains 29-6 and 5710 and in *L. bavaricus* (but A is absent in ANT-300 [data not shown in Fig. 1A]). 2-Thio-5-methyluridine (54, 55) and the various hypermodified nucleosides known to significantly increase stability of tRNA from thermophiles at elevated temperatures (28) are predictably absent in tRNA from psychrophiles. This absence is understandable in organisms growing at temperatures close to the freezing point of water, where potential thermal denaturation of tRNA is not at issue.

All of these findings together suggest that the modified nucleosides which are present in tRNA from ANT-300, 29-6, 5710, and *L. bavaricus* can be considered as representing a basal level of modification necessary for enforcing the universally conserved three-dimensional L-shaped conformation of tRNA, and the fidelity of tRNA function in protein synthesis (43), without sacrificing conformational flexibility.

**Cold adaptation of tRNA from psychrophilic bacteria.** Temperature is an environmental parameter that plays an important role in the biochemical adaptation and evolution of living organisms (24). It seems likely that microorganisms which grow at or below the freezing point of water have adapted over evolutionary time to their environment by development of molecular mechanisms to ensure conformational flexibility and dynamic motion in biological macromolecules, to compensate for the reduction of reaction rates and structural freedom at low temperatures. Indeed, it is known that psychrophiles maintain membrane fluidity by incorporating increased levels of unsaturated fatty acids into their lipid bilayers (22, 23, 38). In addition, psychrophiles enforce conformational flexibility in proteins via the elimination of salt bridges, hydrophobic clusters, interactions among aromatic side chains, and of proline residues in loop regions (12, 16).

One purpose of this study was to determine how tRNA from psychrophiles may be similarly adapted for function at low temperatures by posttranscriptional formation of specific modified nucleosides which promote conformational flexibility and dynamic motion in RNA. An additional purpose, if such nucleosides are present, was to establish whether they occur at significantly higher levels in the tRNA of psychrophiles versus that of mesophiles or thermophiles. The four most abundant modified nucleosides in tRNA from the four organisms studied here are dihydrouridine, pseudouridine, 7-methylguanosine, and 5-methyluridine. Molar abundance values for the latter three nucleosides are very similar to those found in *E. coli* (Table 1). A comparison of molar percentages of dihydrouridine in the five organisms, however, indicates that the molar abundance of this nucleoside, measured quantitatively by LC-MS, are unprecedented: levels in the three psychrophilic bacteria are 40 to 70% greater than that found in the mesophile *E. coli* (9) or the psychrotroph *L. bavaricus*. Sequence locations of dihydrouridine in isoaccepting tRNAs of the four organisms studied are not known but are presumably mainly within positions 14 to 21 of the D loop, a generally conserved modification pattern in bacterial tRNAs (19, 46). The uniquely nonplanar base of dihydrouridine which results from the absence of C-5-C-6 double bond resists stacking (49, 51), normally a common mechanism of stabilization in RNA. In addition, recent nuclear magnetic resonance studies of dihydrouridine 3'-monophosphate and the oligonucleotide ApDpA indicate that dihydrouridine is unusual in that the C-2'-endo sugar conformation (the principal conformer of DNA) is favored and that this effect is propagated to the 5'-neighboring residue in the oligonucleotide (10). The structure of dihydrouridine, showing the nonplanar base, and C-2'-endo sugar conformation are illustrated in Fig. 3. The C-2'-endo conformer is inherently more flexible (58) and accommodates a wider range of glycosyl torsion angles (50) than the dominant RNA conformer, C-3'-endo.

Thermodynamic data from the nuclear magnetic resonance conformational studies of Dp and ApDpA indicate that the structural effect of replacement of U by D is enhanced at low temperatures (10), thereby magnifying the advantage which psychrophiles receive from relatively high levels of U-D modification in their tRNA for maintenance of local conformational flexibility at low temperatures. For instance, at 25°C, the fractional populations of C-2'-endo and C-3'-endo conformers of dihydrouridine in ApDpA are 91 and 9%, respectively; at 5°C, the values shift to 96 and 4%, corresponding to an in-
crease in the [C-2’-endo]/[C-3’-endo] equilibrium constant from 10.8 at 25°C to 22.3 at 5°C (10). Even though tRNA from psychrophilic bacteria contains unprecedented high levels of dihydrouridine, the fact that the melting temperatures of tRNA from psychrophiles are similar to that of E. coli is not surprising based on what is known regarding the locations of dihydrouridine residues in loop regions of tRNA molecules (46, 56). Optical melting experiments measure the stability of helical structure in nucleic acids. Additional flexibility in loops conferred by a high molar abundance of dihydrouridine, therefore, would not be reflected in such an experiment, if the localized flexibility conferred by dihydrouridine is not significantly propagated to the base-paired helical domains of tRNA.

The role of dihydrouridine, therefore, is to confer local and not global structural flexibility to RNA molecules.

In conclusion, the present finding of significantly elevated levels of dihydrouridine in tRNA from psychrophiles, together with studies of the conformational characteristics of dihydrouridine (10), supports a conclusion that the role of this modified nucleoside is in promotion of greater local conformational flexibility and dynamic motion in RNA, providing an advantage in organisms growing under conditions where thermal motion, enzymatic reaction rates, and intermolecular interactions of biomolecules are compromised.

Dihydrouridine is highly conserved in tRNA of eukaryotes and bacteria (19, 46) but not in archaea, where it is generally absent or present in low amounts (15, 33). It has been observed by using LC-MS techniques in the deeply branched bacterial thermophiles T. maritima (15) and Aquifex pyrophilus (6), but the levels have not been measured quantitatively. Insufficient evidence is in hand to speculate about whether the presence of dihydrouridine bears on the contentious issue (17, 30, 39) of whether early organisms at the base of the phylogenetic tree were psychrophiles or thermophiles. In this respect, it will be of interest to determine whether tRNA from psychrophilic archaea (14) contains dihydrouridine, but to date the only successful cultivation has been in symbiosis with a marine sponge (41).

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