Post-transcriptional modifications in the small subunit ribosomal RNA from *Thermotoga maritima*, including presence of a novel modified cytidine

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ABSTRACT

Post-transcriptional modifications of RNA are nearly ubiquitous in the principal RNAs involved in translation. However, in the case of rRNA the functional roles of modification are far less established than for tRNA, and are subject to less knowledge in terms of specific nucleoside identities and their sequence locations. Post-transcriptional modifications have been studied in the SSU rRNA from *Thermotoga maritima* (optimal growth 80°C), one of the most deeply branched organisms in the Eubacterial phylogenetic tree. A total of 10 different modified nucleosides were found, the greatest number reported for bacterial SSU rRNA, occupying a net of ~14 sequence sites, compared with a similar number of sites recently reported for *Thermus thermophilus* and 11 for *Escherichia coli*. The relatively large number of modifications in *Thermotoga* offers modest support for the notion that thermophile rRNAs are more extensively modified than those from mesophiles. Seven of the *Thermotoga* modified sites are identical (location and identity) to those in *E. coli*. An unusual derivative of cytidine was found, designated N-330 (M_r 330.117), and was sequenced to position 1404 in the decoding region of the rRNA. It was unexpectedly found to be identical to an earlier reported nucleoside of unknown structure at the same location in the SSU RNA of the archaeal mesophile *Haloferax volcanii*.

Keywords: post-transcriptional modification; *Thermotoga maritima*; *Haloferax volcanii*; small ribosomal subunit RNA; liquid chromatography–mass spectrometry; electrospray ionization

INTRODUCTION

Relatively little is known concerning the specific roles of post-transcriptional modifications in rRNA (summarized

by Decatur and Fournier 2002), in contrast to tRNA, in which a number of modification functions are slowly being unraveled (Agris 2004). In the case of rRNA, the influence of nucleotide modification on ribosome assembly and maturation (Bachelierie and Cavaillé 1998; Maden 1998; Mason 1998) is perhaps the best documented (Vaughan et al. 1967; Cunningham et al. 1990, 1991; Rydén-Aulin et al. 1993; Sirum-Connolly and Mason 1993; Sirum-Connolly et al. 1995; Green and Noller 1996), but mechanistic details at the molecular level have been scant. There is strong circumstantial evidence in the archaeal thermophiles that ribose methylation at O2' provides a significant mechanism for thermal stabilization of rRNA (Noon et al. 1998) and of archaeal tRNA (Kowalak et al. 1994; Noon et al. 2003), primarily through reduced flexibility resulting from strong thermodynamic enforcement of the C3'-endo sugar conformation (Kawai et al. 1992). Also, from the standpoint of function, selective methylation of rRNA nucleotides provides, in some instances, an effective mechanism for

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Abbreviations: LC/ESI-MS, combined liquid chromatography–electrospray ionization mass spectrometry; Ψ, pseudouridine; m¹U, 3-methyluridine; Cm, 2'-O-methylcytidine; m⁴Cm, N⁴,O-2'-dimethylcytidine; m⁵C, 5-methylcytidine; m²G, N²-methylguanosine; m⁷G, 7-methylguanosine; Am, 2'-O-methyladenosine; m²A, N⁶,N⁶-dimethyladenosine; MS/MS, tandem mass spectrometry; M³⁻, molecular ion having three negative charges, corresponding to loss of three protons from the neutral molecule M, i.e., (M – 3H)³⁻ (analogous definitions for other charge states); MH⁺, protonated molecule ion; BH₂⁺, protonated base ion in which B is the base fragment attached to ribose and BH corresponds to the neutral base; CID, collision-induced dissociation.

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conferring resistance to antibiotics that target the bacterial ribosome (Douthwaite et al. 2005).

Many of the structures and sequence locations of rRNA modifications are relatively well conserved (Maden 1990; Rozenski and McCloskey 2005) and exhibit distinct differences among the three principal evolutionary domains, Archaea, Bacteria, and Eukarya. The complete modification map for SSU rRNA of the bacterial thermophile *Thermus thermophilus* was recently completed (Guymon et al. 2006) and found to exhibit a notably lower level of modification than that of the archaeal thermophile *Sulfolobus solfataricus* (Noon et al. 1998) growing in the same temperature range (70°C–75°C), and a pattern of modification (nucleoside structures and sequence locations) unexpectedly similar to that in the mesophile *Escherichia coli*. For a tabulation of *E. coli* rRNA modifications and leading references see <http://medlib.med.utah.edu/RNAmods/>.

We have examined the SSU rRNA modifications in a second bacterial thermophile, *Thermotoga maritima* (optimal growth at 80°C) (Huber et al. 1986), an organism placed by small subunit ribosomal RNA (SSU rRNA) phylogeny as one of the most deeply rooted organisms in the Eubacterial tree. Analysis of the complete *Thermotoga* genome sequence (Nelson et al. 1999) as well as earlier data (Aravind et al. 1998) led to conclusions that extensive lateral gene transfer between *T. maritima* and Archaea have occurred, reflected in the unusually high percentage (24%) of genes in *T. maritima* that are similar to those in Archaea. Examination of *T. maritima* SSU rRNA in the present study was intended in part to establish whether its modifications are to any extent archaeal in nature, and thus reflecting possible horizontal transfer of RNA modification enzymes. Further, this study serves to extend the knowledge base of bacterial SSU rRNA modification patterns (Rozenski and McCloskey 2005; Guymon et al. 2006) beyond *E. coli* and *T. thermophilus*, the only bacteria for which complete SSU modification maps are available, and beyond organisms for which RNase T1 catalog data were reported (Rozenski and McCloskey 2005) in which modifications were sometimes reported to occur, often without chemical identity of the modified nucleoside or firm knowledge of its sequence location in the 16S RNA molecule.

RESULTS

Modified nucleoside content of *T. maritima* 16S rRNA

Identities and approximate numbers of modified nucleosides, determined by LC/ESI-MS analysis of total nucleoside digests of 16S rRNA (Supplemental Fig. S1, at <http://library.med.utah.edu/mccloskey>) are: Ψ (3.8 residues), m^3U (1.0), m^4Cm (1.2), m^5C (0.73), Cm (0.82), unknown nucleoside N-330 (UV molar absorptivity not known, so stoichiometry not estimated), m^2G (2.3), m^7G (1.1), Am (trace level to 1.3; see text), and m_6^2A (2.1).

Identification and sequence location of modified residues in *T. maritima* 16S rRNA

RNA modifications represented by changes in mass from the parent nucleotide were mapped. The locations of pseudouridines, which are mass silent, were not measured beyond the estimation of 3.8 net residues in the molecule. Chromatographic separation of RNase T1 digestion products of *T. maritima* SSU rRNA is shown in Figure 1, with the corresponding mass data for modified oligonucleotides given in Table 1. A section of the RNase U2 chromatogram in which modified oligonucleotides were eluted is shown in Supplemental Figure S2.

To a certain extent the procedures used for identification and sequence placement of modified residues are analogous to those recently used and described in a study of *T. thermophilus* SSU rRNA (Guymon et al. 2006). Therefore, descriptions of results for seven modified residues in the present work can be found in the Supplemental Data section. These are: m^7G -527 (Supplemental Fig. S3), m^2G -966 and m^5C -967 (Supplemental Fig. S4), m^2G -1051 (Supplemental Table S2), m^4Cm -1402 (Supplemental Fig. S5), Cm -1409 (Supplemental Figs. S6,S7), and 1518- m_6^2A -1519 placement. Results for N-330–1404, m^3U -1498, and Am -1499 follow below.

N-330–1404

The presence of a novel structurally unknown nucleoside of M_r 330 at position 1404 is indicated from the following four lines of evidence. (1) The sequencing spectrum of T1 oligonucleotide M_r 1393 (Supplemental Fig. S5) can be assigned only if a unique nucleotide residue mass of 392 (N-330p minus H_2O) is used in the third nucleotide position. (2) The base[−] (m/z 197) and N > p[−] (m/z 391) monomer fragment ion signals (Supplemental Table S1) were assigned from the data in Supplemental Figure S5 and time aligned as shown in Supplemental Figure S8. Ions

FIGURE 1. HPLC separation of RNase T1 digestion products of *T. maritima* 16S rRNA, annotated to show modified oligonucleotides determined from mass shifts in conjunction with data in Table 1. UV absorbance detection at 260 nm.

TABLE 1. Modified nucleosides in RNases T1 and U2 hydrolysis products from *Thermotoga maritima* 16S rRNA

Modification	RNase T1			RNase U2		
	Sequence ^a	M _r meas. (error) ^b	Monomer ions, m/z ^c	Sequence ^a	M _r meas. (error) ^b	Monomer ions, m/z ^c
m ⁷ G-527	525-CCm ⁷ GCGp ^d -529	1637.29 (0.05)	164	525-CCm ⁷ GCGG>p-530	1964.31 (0.03)	
m ² G-966	964-AUm ² Gp-966	1012.16 (0.01)	164, 358, 376	965-Um ² Gm ³ CUA>p-969	1619.26 (0.04)	164, 358, 376, 124, 336
m ⁵ C-967	967-m ⁵ CUAAGp-971	1646.25 (0.01)	124, 336			
m ² G-1051	1051-m ² G>p-1051	359.03 (0.03)	164	1049-UGm ² GUG>p ^d -1053	1661.25 (0.04)	164, 358
m ⁴ Cm-1402, N-330–1404	1402-m ⁴ CmCN-330Gp ^d -1405	1393.28 (0.01)	124, 350, 197 (N-330 base), 391 (N-330>p)	1399-CCCGm ⁴ CmCN-330GUCA>p-1408	3270.53 (0.06)	
Cm-1409	1406-UCAmCmGp ^d -1410	1622.24 (0.01)	336, 398 pCm>p	Mass not found through tracking		
m ³ U-1498,	1498-m ³ UAmACAAGp ^d -1504	2318.43 (0.07)	125, 319, 337,	1495-UCCGm ³ UA>p-1499	1605.26 (0.06)	125, 319, 337, 342
Am-1499			225, 342	1495-UCCGm ³ UAm>p-1499	1620.16 (0.17) avg.	
m ² A-1518, 1519	1518-m ² Am ² AGp-1520	1077.23 (0.01)	162, 356, 374	Mass not found through tracking		

^a*E. coli* sequence numbering used throughout.

^bMass was experimentally measured; error is found minus calculated mass for the modified oligonucleotide shown.

^cSee also Supplemental Table S1.

^dSequenced by mass spectrometry.

corresponding to the modified residue $m^4\text{Cm}$, whose presence is indicated from the total nucleoside analysis data in Supplemental Figure S1, were also time aligned. The exact coelution pattern of ions from N-330 and $m^4\text{Cm}$ is shown in Supplemental Figure S8, and supports the presence of both of those nucleosides in the T1 oligonucleotide M_r 1393. The characteristic bacterial nucleoside $m^4\text{Cm}$ serves as an internal marker for position 1402 (Rozenski and McCloskey 2005) and correlates with the full oligonucleotide sequence derived by mass spectrometry in Supplemental Figure S5. (3) The presence of a base-modified unknown nucleoside of M_r 330 (MH^+ , m/z 331; BH_2^+ , m/z 199; λ_{max} 270) eluting at 3.85 min in the total nucleoside digest of *Thermotoga* 16S rRNA, Supplemental Figure S1. (4) Modification of the universal C-1404 was also reported in several other bacterial SSU rRNAs (although not in *E. coli*) (Rozenski and McCloskey 2005), but not the identity of the modification.

$m^3\text{U}$ -1498 and Am-1499

Assignment of the unusual tandem methylation sites 1498 and 1499 was made primarily through the T1 product M_r 2318 (Table 1). The experimental mass 2318.43, using the corresponding gene sequence to calculate allowable RNA sequences, solely designates the unique sequence segment as shown in Table 1 corresponding to nucleotides 1498–1504 with two methyl groups. The sequence mass spectrum of this T1 fragment (Fig. 2) requires, through the presence of a clear y_1 – y_5 ion series (McLuckey et al. 1992), that the methyl groups be confined to the first two nucleotides. The abundant methyluracil base ion m/z 125, in conjunction with the total nucleoside census (Supplemental Fig. S1) then implies $m^3\text{U}$ in position 1498 and Am, the only methylated A in the rRNA, at position 1499. (No methyladenine base

ion, m/z 148, was observed). This methylation pattern was supported by the finding in the U2 digest of the 3'-truncated version of this sequence but having an extension of three nucleotides on the 5' end, M_r 1620 (see Table 1). However, the assignment of Am-1499 in these RNase products appeared to be at odds with the absence of the methylribose cyclic phosphate ion (m/z 225) in the spectrum, usually a strong indicator of presence of a ribose-methylated nucleotide (Phillips and McCloskey 1993). To address this point the product ion mass spectrum of chemically synthesized $m^3\text{UAmACAAGp}$ was acquired. It appeared essentially identical (data not shown) to the mass spectrum displayed in Figure 2, including the absence of ion m/z 225. The sequence of the Am-containing region is therefore assigned as shown in Table 1. The occurrence of Am is further supported by its presence in the total nucleoside digest (Fig. S1), with MH^+ and BH_2^+ ions appearing at the expected retention time (Pomerantz and McCloskey 1990). Interestingly, a second culture of *T. maritima* showed an otherwise very similar overall modification data set, but with the 1495–1499 fragment occurring primarily as M_r 1605.2 (see Table 1), i.e., with one fewer methyl group. We interpret this finding, taken in conjunction with variable amounts of Am found in nucleoside total digests from two different *T. maritima* cultures, as an indication that the level of Am-1499 is both substoichiometric and variable in the rRNA. To our knowledge, this is the first report of Am in RNA from bacteria; however, using LC/ESI-MS we have observed Am in unfractionated tRNA from *E. coli* MRE 600 (F. Qiu and J. McCloskey, unpubl., 1998).

3'-Terminal RNase T1 fragment from *T. maritima* 16S rRNA

The terminal fragment is a 15-mer, expected to contain the anti-Shine–Dalgarno mRNA-binding sequence. Because of the recent unusual finding of adjacent pseudouridines at 1540 and 1541 in the 3'-tail of *T. thermophilus* (Guymon et al. 2006), the *Thermotoga* 3'-terminal fragment was subjected to a Ψ detection protocol (Pomerantz and McCloskey 2005) using LC/ESI-MS/MS. No Ψ was detected. The terminal fragment was mass measured as 4591.7 (4591.60 calc.), and MS-sequenced as AUCACCUCCUUCUAOH (Supplemental Fig. S9), thus revealing the molecular terminus as AOH-1545 (*E. coli* numbering) in the processed 16S molecule.

Presence of nucleoside N-330 in *Haloferax volcanii* 16S rRNA

The discovery of a new modified nucleoside in the *Thermotoga* SSU at the same position previously described as containing a modified cytidine in *H. volcanii* 16S rRNA (Gupta et al. 1983) prompted a revisit of our previously

FIGURE 2. Product ion mass spectrum from RNase T1 fragment M_r 2318, showing $m^3\text{U}$ -1498 adjacent to Am-1499. For clarity, only peaks used for sequence assignment are annotated. Sequence coverage from the four sequence ion series (a–B, d– H_2O , w, and y) (McLuckey et al. 1992) is shown by horizontal bars.

published study of the *Haloferax* SSU RNA modifications (Kowalak et al. 2000). When the ion chromatograms for the diagnostic MH^+ and BH_2^+ ions for N-330 nucleoside (m/z 331 and 199, respectively) were extracted from our *Haloferax* total nucleoside digest data, their presence was confirmed by the appearance of these two ions in a peak exactly coeluting at 3.6 min (Fig. 3). If N-330 is, in fact, the “C*” nucleotide previously reported, the calculated mass of the predicted *Haloferax* N-330-containing T1 oligonucleotide (CCC*Gp) would be M_r 1365.7. Reconstructed ion chromatograms were therefore generated using the M^- and M^{2-} ions (m/z 1364.7 and m/z 681.8, respectively), and for the B^- ion for the base of N-330 (m/z 197). As shown in Supplemental Figure S10, each of the three ions forms a peak eluting at 16.0 min, indicating N-330 to be the modified C* nucleotide found by Woese and colleagues (Gupta et al. 1983).

DISCUSSION

Modification identities and levels in *T. maritima* SSU RNA

The finding of 10 different modified nucleosides at a net occupancy level of ~ 14 sequence sites marks *T. maritima*, and similarly *T. thermophilus* (Guymon et al. 2006), as the most extensively modified bacterial SSU RNAs presently known. Examination of a number of catalogued RNase T1 modification maps, although by their nature less complete than the full modification maps from *E. coli* (leading citations in ref. Bakin et al. 1994; see also <http://medlib.med.utah.edu/RNAmods>), and *T. thermophilus* (Guymon et al. 2006) SSU RNAs, suggests that the levels and identities of modifications are perhaps narrower and more

conserved in Bacteria than in the Archaea and Eukarya. For example, modification levels in Archaeal 16S RNA range from five residues in *H. volcanii* (Gupta et al. 1983) to ~ 38 in *S. solfataricus* (Noon et al. 1998). Modification at ~ 8 –11 SSU RNA sequence sites appears to be most common in bacteria (*E. coli* has 11) while the elevation by about 30% of modification levels in the bacterial thermophiles (versus mesophiles) supports the conclusion that post-transcriptional modification in general serves to support structural stabilization of RNA (Sampson and Uhlenbeck 1988; Derrick and Horowitz 1993; Kowalak et al. 1994). The presence of 11 modifications was recently reported for *T. thermophilus* 23S rRNA (Mengel-Jorgensen et al. 2006), compared with ~ 23 in *E. coli* LSU RNA. This relative concentration is significantly lower than expected, based on our finding of 14 sites in the *Thermus* 16S RNA. However, the methodology used (Mengel-Jorgensen et al. 2006) was not designed to provide a complete census of modifications that required placement. As in the case of *T. thermophilus* (Guymon et al. 2006), rRNA modification levels in *Thermotoga* are characteristically much lower than in the Archaeal thermophiles growing at about the same temperature (Noon et al. 1998), with considerably less reliance on ribose O-2' methylation as a major means of structural stabilization. The finding of a net 3.8 residues of Ψ in *Thermotoga* SSU RNA is notable in that Ψ has been shown to play a strong, although often overlooked role in RNA stabilization (Davis 1995, 1998), and the level in *Thermotoga* appears to be the highest concentration of Ψ reported in bacterial SSU RNAs (Ofengand and Rudd 2000).

The availability of three bacterial SSU RNA modification maps, *E. coli* (Brosius et al. 1978), *Thermus* (Guymon et al. 2006), and now *Thermotoga*, when coupled with information from T1 catalogs (Rozenki and McCloskey 2005) permits an estimation of the most highly conserved modification sites that are unique to bacteria, even though most of the T1 catalog data identify sites but not chemical structures of modifications. In bacteria these sites are C*-967 in h31 and U*-1498 in the center of the decoding region in h44. An exception to the bacterial uniqueness of a modified C-967 appears to occur in the archaeon *Thermoproteus tenax* (Woese et al. 1984). The position 967 modification is reported to be m^5C in all four cases in which the nucleoside structure has been established (*E. coli*, *Thermus*, *Thermotoga*, and *Proteus*). The adjacent modification m^2G -966, implicated in P-site tRNA binding (von Ahsen and Noller 1995; Korostelev et al. 2006), represents an essentially universal SSU modification site in all phylogenetic domains (Rozenki and McCloskey 2005) and is represented by an interesting diversity of modified nucleoside structures in Archaea and Eukarya (Kowalak et al. 2000). The identity of U*-1498 has been established specifically as m^3U in the same four organisms. The unusual dimethylcytidine m^4Cm -1402 is unique to bacterial rRNA (Limbach et al. 1994), but this site appears to be

FIGURE 3. Demonstration of presence of modified nucleoside N-330 in a total nucleoside digest of *H. volcanii* 16S rRNA (from previously acquired data) (Kowalak et al. 2000), detected from reconstructed ion chromatograms (RIC) for characteristic positively charged ions. (A) UV absorbance at 260 nm. (B) RIC for the MH^+ ion of N-330 (m/z 331). (C) RIC for the protonated base fragment ion of N-330 (m/z 199).

modified in fewer than half of the reported cases (Rozenski and McCloskey 2005). In *T. thermophilus* the m⁴Cm residue serves to stabilize the third tRNA nucleotide by H binding of its phosphate to the 4-methylamino moiety (Korostelev et al. 2006).

Modifications at the functional center of the ribosome

As deduced in earlier literature, modification sites in bacterial SSU RNAs tend to occur, in three-dimensional space, near the decoding center of the RNA (Brimacombe et al. 1993; Decatur and Fournier 2002). Four of the 16S modifications (m²G-966, m⁵C-1400, m⁴Cm-1402, and m³U-1498) were determined by X-ray crystallography to support interaction between 16S RNA and the P-site codon and anticodon stem-loop (Korostelev et al. 2006). These observations reflect the net importance of modification to efficient ribosomal function, as has been stated (Decatur and Fournier 2002). The high level of modification in the upper portion of helix 44 in SSU RNA occurs at the interface with the LSU RNA, forming a cavity in which translation occurs. These modifications in h44 include six methyl groups each in *Thermotoga* and *Thermus*, opposite an analogous concentration of modifications on the 23S side of the *Thermus* LSU (Mengel-Jorgensen et al. 2006). Mengel-Jorgensen et al. (2006) have concluded that the occurrence of modifications in the 23S RNA of *Thermus* principally at the RNA–RNA interface suggests that they play a role in modulating the RNA–RNA interface contact. Their conclusions are supported by the locations of modifications in 16S RNA from *Thermotoga* (present work), *Thermus* (Guymon et al. 2006), and a series of studies on *E. coli* (Brimacombe et al. 1993; Decatur and Fournier 2002), showing that the ribosomal subunit interface is intimately associated with post-transcriptional modifications.

Modified nucleoside N-330–1404 in the decoding region of the RNA

Unknown N-330 is remarkable in two ways: first, in terms of its structural properties as inferred thus far; second, its unexpected occurrence in another phylogenetic domain, at the same location in the SSU RNA of the archaeal mesophile *H. volcanii*. Interestingly, C-1404 has been reported as modified but with unknown structure in the RNase T1 SSU maps of five bacteria (Rozenski and McCloskey 2005), and two archaea, *H. volcanii* (Gupta et al. 1983) and *S. solfataricus* (Woese et al. 1984). C-1404 pairs with essentially universal G-1497 (Cannone et al. 2002) near the top of h44. N-330 is implied to be a derivative of cytidine by the corresponding gene sequence (Gupta et al. 1983). The molecular mass of 330 Da is unique among all known modified nucleosides in RNA (see <http://medlib.med.utah.edu/RNAmods>) and suggests its

structure to be more complex than any of the nine known modified cytidines in rRNA (Rozenski and McCloskey 2005). The accurate molecular mass of N-330 was measured as 330.117 ± 0.002 using a Micromass Q-ToF mass spectrometer. This value is consistent with an elemental composition of C₁₂H₁₈N₄O₇ (calc. 330.1175), thus requiring a side chain having one N atom, with three Ns accounted for by the cytosine heterocycle. Unfortunately, the exact chemical structure of N-330–1404 cannot be further pursued due to laboratory closure and retirement of the corresponding author.

It is interesting to consider the possibility that the structures and distributions of post-transcriptionally modified nucleosides—the specific products of a sizable array of RNA modification enzymes—could, in some instances, be indicators of past horizontal transfer of DNA coding for modification enzymes. This notion is supported by the sharp lines of phylogenetic demarcation of some modified nucleoside families (Limbach et al. 1994; Motorin and Grosjean 1998). The possibility of lateral transfer was earlier raised (McCloskey et al. 2001) in light of the strong clustering of the (otherwise bacterial) anticodon nucleoside mnm⁵s²U in tRNAs from the *Methanococci*, a lineage of methanogenic marine archaea. Interestingly, nucleoside N-330 is the only rRNA nucleoside occurring in *Thermotoga* that is shared only by bacteria and archaea, all others (with the exception of m⁴Cm-1402, which is uniquely bacterial) being found in all three phylogenetic domains. Nucleoside N-330 might therefore be, in part, the product of RNA modification enzyme genes transferred horizontally between *Haloferax* and *Thermotoga*. This speculation is based on the report of extensive lateral transfer between *Thermotoga* and archaea (Aravind et al. 1998; Logsdon and Faguy 1999), such that almost one-quarter of the *Thermotoga* genome (24%) was found to be archaeal in nature (Nelson et al. 1999). Work in the area of RNA modification enzymes (Leung et al. 1998), however, while extensive in some notable cases, such as the yeast tRNA modification enzymes (Johansson and Byström 2005) and the pseudouridine synthases (Ofengand and Fournier 1998), by its nature generally lags behind knowledge of modified nucleoside structures (now numbering about 106; see <http://medlib.med.utah.edu/RNAmods>) and their phylogenetic distributions. Pursuit of this possibility that N-330 is a product of lateral gene transfer will require knowledge of the chemical structure of N-330, and also of the sequences of specific enzymes responsible for its formation.

MATERIALS AND METHODS

Thermotoga maritima MSB8 cells were grown at 80°C at The University of Georgia Bioexpression and Fermentation Facility, under the supervision of T.E. Davies, and RNA from this source was acquired in two ways. First, purified 16S rRNA was obtained as a gift from V. Ramakrishnan (Department of Biochemistry, University of Utah; present address MRC Laboratory of Molecular

Biology, Cambridge, U.K.). Second, *T. maritima* 30S ribosomal subunits were obtained from the same source and 16S RNA was extracted from the 30S ribosomes with TRI Reagent (Chomczynski and Sacchi 1987) following the manufacturer's protocol. 16S rRNAs (typically, 100 µg; 200 pmol) were digested totally to nucleosides with nuclease P1, phosphodiesterase I, and BAP (Crain 1990). Aliquots of RNA (5–100 pmol) were digested with 1000 units of RNase T1 (Ambion) in 10 mM Tris+ 1 mM EDTA, pH 7 for 30 min at 37°C or 45 min at 55°C. Alternatively, the RNA was digested with RNase U2 (Industrial Research Ltd.) in 20 mM diammonium citrate (DAC), pH 5, 1 mM EDTA, or in 20 mM DAC, pH 5, 1 mM EDTA, 8 M urea, with 5–10 units RNase U2 for 15 min at 60°C, then an additional 5–10 units of enzyme was added and digestion continued for 15 min. Details of the mass spectrometry methods used in this study can be found in the Supplemental Data section.

SUPPLEMENTAL DATA

The following supplemental material can be found at <http://library.med.utah.edu/mccloskey>: LC/ESI-MS analysis of nucleosides in *Thermotoga* 16S rRNA; partial chromatogram from LC/ESI-MS analysis of RNase U2 digest of *Thermotoga* 16S rRNA; assignments for monomer ions used in Table 1; data and discussion for placements of m⁷G, m²G (two each), m⁵C, m²A (two each), and Cm; additional comments on nucleoside N-330; sequence mass spectrum of T1 oligonucleotide M_r 4591.7; mass spectrometry Materials and Methods.

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REFERENCES

- Agris, P.F. 2004. Decoding the genome: A modified view. *Nucleic Acids Res.* **32**: 223–238.
- Aravind, L., Tatusov, R.L., Wolf, Y.I., Walker, D.R., and Koonin, E.V. 1998. Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. *Trends Genet.* **14**: 442–444.
- Bachellerie, J.-P. and Cavaillé, J. 1998. Small nucleolar RNAs guide the ribose methylations of eukaryotic rRNAs. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 255–272. ASM Press, Washington, DC.
- Bakin, A., Kowalak, J.A., McCloskey, J.A., and Ofengand, J. 1994. A single pseudouridine residue in *E. coli* 16S RNA is located at position 516. *Nucleic Acids Res.* **22**: 3681–3684.
- Brimacombe, R., Mitchell, P., Osswald, M., Stade, K., and Bochkariov, D. 1993. Clustering of modified nucleotides at the functional center of the ribosome. *FASEB J.* **7**: 161–167.
- Brosius, J., Palmer, M.L., Kennedy, P.J., and Noller, H.F. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci.* **75**: 4801–4805.
- Cannone, J.J., Subramanian, S., Schnare, M.N., Collett, J.R., D'Souza, L.M., Du, Y., Feng, B., Lin, N., Madabusi, L.V., Muller, K.M., et al. 2002. The comparative RNA web (CRW) site: An online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* **3**: 2.
- Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
- Crain, P.F. 1990. Preparation and enzymatic hydrolysis of RNA and DNA for mass spectrometry. *Methods Enzymol.* **193**: 782–790.
- Cunningham, P.R., Weitzmann, C.J., Nègre, D., Sinning, J.G., Frick, V., Nurse, K., and Ofengand, J. 1990. In vitro analysis of the role of rRNA in protein synthesis: Site-specific mutation and methylation. In *The ribosome: Structure, function, and evolution* (eds. W.E. Hill et al.), pp. 243–252. ASM Press, Washington, DC.
- Cunningham, P.R., Richard, R.B., Weitzmann, C.J., Nurse, K., and Ofengand, J. 1991. The absence of modified nucleotides affects both in vitro assembly and in vitro function of the 30S-ribosomal subunit of *Escherichia coli*. *Biochimie* **73**: 789–796.
- Davis, D.R. 1995. Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Res.* **23**: 5020–5026.
- Davis, D.R. 1998. Biophysical and conformational properties of modified nucleosides in RNA (nuclear magnetic resonance studies). In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 85–102. ASM Press, Washington, DC.
- Decatur, W.A. and Fournier, M.J. 2002. rRNA modifications and ribosome function. *Trends Biochem. Sci.* **27**: 344–351.
- Derrick, W.B. and Horowitz, J. 1993. Probing structural differences between native and *in vitro* transcribed *Escherichia coli* valine transfer RNA: Evidence for stable base modification-dependent conformers. *Nucleic Acids Res.* **21**: 4948–4953.
- Douthwaite, S., Fourmy, D., and Yoshizawa, S. 2005. Nucleotide methylations in rRNA that confer resistance to ribosome-targeting antibiotics. In *Fine-tuning of RNA functions by modification and editing* (ed. H. Grosjean), pp. 285–307. Springer-Verlag, Berlin.
- Green, R. and Noller, H.F. 1996. In vitro complementation analysis localizes 23S rRNA posttranscriptional modifications that are required for *Escherichia coli* 50S ribosomal subunit assembly and function. *RNA* **2**: 1011–1021.
- Gupta, R., Lanter, J.M., and Woese, C.R. 1983. Sequence of the 16S ribosomal RNA from *Halobacterium volcanii*, an archaebacterium. *Science* **221**: 656–659.
- Guymon, R., Pomerantz, S.C., Crain, P.F., and McCloskey, J.A. 2006. The influence of phylogeny on posttranscriptional modification of rRNA in thermophilic prokaryotes: The complete modification map of 16S rRNA of *Thermus thermophilus*. *Biochemistry* **45**: 4888–4899.
- Huber, R., Langworthy, T.A., König, H., Thomm, M., Woese, C.R., Sleytr, U.B., and Stetter, K.O. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch. Microbiol.* **144**: 324–333.
- Johansson, M.J.O. and Byström, A.S. 2005. Transfer RNA modifications and modifying enzymes in *Saccharomyces cerevisiae*. In *Fine-tuning of RNA functions by modification and editing* (ed. H. Grosjean), pp. 87–120. Springer-Verlag, Berlin.
- Kawai, G., Yamamoto, Y., Kamimura, T., Masegi, T., Sekine, M., Hata, T., Iimori, T., Watanabe, T., Miyazawa, T., and Yokoyama, S. 1992. Conformational rigidity of specific pyrimidine residues in tRNA arises from posttranscriptional modifications that enhance steric interaction between the base and the 2'-hydroxyl group. *Biochemistry* **31**: 1040–1046.
- Korostelev, A., Trakhanov, S., Laurberg, M., and Noller, H.F. 2006. Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell* **126**: 1065–1077.
- Kowalak, J.A., Dalluge, J.J., McCloskey, J.A., and Stetter, K.O. 1994. Role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. *Biochemistry* **33**: 7869–7876.
- Kowalak, J., Bruenger, E., Crain, P.F., and McCloskey, J.A. 2000. Identities and phylogenetic comparisons of post-transcriptional

- modifications in 16S ribosomal RNA from *Haloferax volcanii*. *J. Biol. Chem.* **275**: 24484–24489.
- Leung, H.-C.E., Hagervall, T.G., Björk, G.R., and Winkler, M.E. 1998. Genetic locations and database accession numbers of RNA-modifying and -editing enzymes. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 561–565. ASM Press, Washington, DC.
- Limbach, P.A., Crain, P.F., and McCloskey, J.A. 1994. Summary: The modified nucleosides of RNA. *Nucleic Acids Res.* **22**: 2183–2196.
- Logsdon, J.M. and Faguy, D.M. 1999. Evolutionary genomics: *Thermotoga* heats up lateral gene transfer. *Curr. Biol.* **9**: R747–R751.
- Maden, B.E.H. 1990. The numerous modified nucleosides in eukaryotic ribosomal RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **39**: 241–303.
- Maden, B.E.H. 1998. Intracellular locations of RNA-modifying enzymes. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 421–440. ASM Press, Washington, DC.
- Mason, T.L. 1998. Functional aspects of the three modified nucleotides in yeast mitochondrial large-subunit rRNA. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 273–280. ASM Press, Washington, DC.
- McCloskey, J.A., Graham, D.E., Zhou, S., Crain, P.F., Ibba, M., Konisky, J., Söll, D., and Olsen, G.J. 2001. Post-transcriptional modification in archaeal tRNAs: Identities and phylogenetic relations of nucleotides from mesophilic and hyperthermophilic *Methanococcales*. *Nucleic Acids Res.* **29**: 4699–4706.
- McLucky, S.A., Van Berkel, G.J., and Glish, G.L. 1992. Tandem mass spectrometry of small, multiply charged oligonucleotides. *J. Am. Soc. Mass Spectrom.* **3**: 60–70.
- Mengel-Jorgensen, J., Jensen, S.S., Rasmussen, A., Poehlsgaard, J., and Kirpekar, F. 2006. Modifications in *Thermus thermophilus* 23S ribosomal RNA are centered in regions of RNA–RNA contact. *J. Biol. Chem.* **281**: 22108–22117.
- Motorin, Y. and Grosjean, H. 1998. Appendix 1: Chemical structures and classification of posttranscriptionally modified nucleosides in RNA. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 543–549. ASM Press, Washington, DC.
- Nelson, K.E., Clayton, R.A., Gill, S.R., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Nelson, W.C., Ketchum, K.A., et al. 1999. Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of *Thermotoga maritima*. *Nature* **399**: 323–329.
- Noon, K.R., Bruenger, E., and McCloskey, J.A. 1998. Posttranscriptional modifications in 16S and 23S rRNAs of the archaeal hyperthermophile *Sulfolobus solfataricus*. *J. Bacteriol.* **180**: 2883–2888.
- Noon, K.R., Guymon, R., Crain, P.F., McCloskey, J.A., Thomm, M., Lim, M., and Cavicchioli, R. 2003. Influence of temperature on tRNA modification in Archaea: *Methanococcoides burtonii* (T_{opt} 23°C) and *Stetteria hydrogenophila* (T_{opt} 95°C). *J. Bacteriol.* **185**: 5483–5490.
- Ofengand, J. and Fournier, M.J. 1998. The pseudouridine residues of rRNA: Number, location, biosynthesis, and function. In *Modification and editing of RNA* (eds. H. Grosjean and R. Benne), pp. 229–253. ASM Press, Washington, DC.
- Ofengand, J. and Rudd, K.E. 2000. Bacterial, archaeal, and organellar rRNA pseudouridines and methylated nucleosides and their enzymes. In *The ribosome: Structure, function, antibiotics, and cellular interactions* (eds. R.A. Garrett et al.), pp. 175–189. ASM Press, Washington, DC.
- Phillips, D.R. and McCloskey, J.A. 1993. A comprehensive study of the low energy collision-induced dissociation of dinucleoside monophosphates. *Int. J. Mass Spectrom. Ion Process.* **128**: 61–82.
- Pomerantz, S.C. and McCloskey, J.A. 1990. Analysis of RNA hydrolyzates by LC/MS. *Methods Enzymol.* **193**: 796–824.
- Pomerantz, S.C. and McCloskey, J.A. 2005. Detection of the common RNA nucleoside pseudouridine in mixtures of oligonucleotides by mass spectrometry. *Anal. Chem.* **77**: 4687–4697.
- Rozenski, J. and McCloskey, J.A. 2005. The small subunit rRNA modification database. *Nucleic Acids Res.* **33**: D135–D138.
- Rydén-Aulin, M., Shaoping, Z., Kylsten, P., and Isaksson, L.A. 1993. Ribosome activity and modification of 16S RNA are influenced by deletion of ribosomal protein S20. *Mol. Microbiol.* **7**: 983–992.
- Sampson, J.R. and Uhlenbeck, O.C. 1988. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro. *Proc. Natl. Acad. Sci.* **85**: 1033–1037.
- Sirum-Connolly, K. and Mason, T.L. 1993. Functional requirement of a site-specific ribose methylation in ribosomal RNA. *Science* **262**: 1886–1889.
- Sirum-Connolly, K., Peltier, J.M., Crain, P.F., McCloskey, J.A., and Mason, T.L. 1995. Implications of a functional large ribosomal RNA with only three modified nucleotides. *Biochimie* **77**: 30–39.
- Vaughan, M.H., Soeiro, R., Warner, J.R., and Darnell, J.E. 1967. Role of RNA methylation in rRNA maturation. *Proc. Natl. Acad. Sci.* **58**: 1527–1534.
- von Ahlsen, U. and Noller, H.F. 1995. Identification of bases in 16S rRNA essential for tRNA binding at the 30S ribosomal P site. *Science* **267**: 234–237.
- Woese, C.R., Gupta, R., Hahn, C.M., Zillig, W., and Tu, J. 1984. The phylogenetic relationships of three sulfur dependent archaeobacteria. *Syst. Appl. Microbiol.* **5**: 97–105.

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