RNA (2002), *8*:202–213. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S135583820101336X

Posttranscriptional modifications in the A-loop of 23S rRNAs from selected archaea and eubacteria

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ABSTRACT

Posttranscriptional modifications were mapped in helices 90–92 of 23S rRNA from the following phylogenetically diverse organisms: *Haloarcula marismortui, Sulfolobus acidocaldarius, Bacillus subtilis,* and *Bacillus stearothermophilus.* Helix 92 is a component of the ribosomal A-site, which contacts the aminoacyl-tRNA during protein synthesis, implying that posttranscriptional modifications in helices 90–92 may be important for ribosome function. RNA fragments were isolated from 23S rRNA by site-directed RNase H digestion. A novel method of mapping modifications by analysis of short, nucleotide-specific, RNase digestion fragments with Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) was utilized. The MALDI-MS data were complemented by two primer extension techniques using reverse transcriptase. One technique utilizes decreasing concentrations of deoxynucleotide triphosphates to map 2′-*O*-ribose methylations. In the other, the rRNA is chemically modified, followed by mild alkaline hydrolysis to map pseudouridines (Ψs). A total of 10 posttranscriptionally methylated nucleotides and 6 Ψs were detected in the five organisms. Eight of the methylated nucleotides and one Ψ have not been reported previously. The distribution of modified nucleotides and their locations on the surface of the ribosomal peptidyl transferase cleft suggests functional importance.

Keywords: 2′-*O*-ribose methylation; A-site; MALDI mass spectrometry; modified ribonucleotides; pseudouridine; ribosomes

INTRODUCTION

In the ribosome, the A-loop of 23S rRNA (helix 92 loop) contacts aminoacyl-tRNA positioned in the A-site through base pairing between C75 of the A-site-bound tRNA and G2553 of 23S rRNA (*Escherichia coli* numbering is used here and in the following). This is evidenced by both mutagenesis studies (Kim & Green, 1999) and X-ray crystallography (Nissen et al., 2000). The A-loop (U2552-C2556) constitutes a part of the peptidyl transferase loop in domain V of 23S rRNA, and is a component of the rRNA cleft where the peptidyl transferase reaction takes place. The functional importance of this loop is emphasized by several studies. First, tRNA bound in the A-site elicits acyl-dependent U2555 and terminal A-dependent G2553 footprints in the A-loop (Moazed & Noller, 1989). Second, puromycin, an antibiotic that binds to the A-site, footprints at G2553 (Rodriguez-Fonseca et al., 2000) and reacts with a peptidyl-tRNA analog even when crosslinked to G2553 (Green et al., 1998). Third, a selection experiment shows that modification of U2555 interferes with transfer of the peptidyl moiety to puromycin (Bocchetta et al., 1998). Finally, mutations at G2550, U2552, G2553, and G2557 reduce the efficiency of peptidyl transferase in vitro (Porse & Garrett, 1995; Kim & Green, 1999) and mutations at U2555 increase frame shifting errors and miscoding by the ribosome (O'Connor & Dahlberg, 1993). The five residues in the A-loop are phylogenetically highly conserved although transversions occur in archaea at positions U2554 and U2555.

Of the 96 different types of posttranscriptionally modified nucleotides that have been reported in RNA, 30 are found in rRNA (Rozenski et al., 1999). Modified nucleotides, especially 2′-*O*-ribose methylations (Maden, 1990) and pseudouridines (Ψs; Ofengand et al., 1995) are frequently found in eukaryotic rRNA. Their locations are specified by small nucleolar RNAs (snoRNAs), which anneal to the rRNA and thereby mediate modifications at specific positions (reviewed by Maxwell & Fournier, 1995; Smith & Steitz, 1997). There are considerably fewer modified nucleotides in prokary-

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otes, where modifications are mediated by RNA site-specific enzymes. The number of Ψs varies from 55 in the large ribosomal subunit in humans, over five in *Bacillus subtilis*, four in *Halobacterium halobium* (an archaea), to one in *Saccharomyces cerevisiae* mitochondria (reviewed by Ofengand & Fournier, 1998). Particularly the A-loop of eukaryotic 23S rRNAs is heavily posttranscriptionally modified with three modified nucleotides in a row.

The function of the posttranscriptional modifications in rRNA is enigmatic. The observation that they cluster in conserved regions of functionally important rRNA domains implies that the modifications may be important for ribosome function (Maden, 1990; Brimacombe et al., 1993). They have been suggested to be involved in processing of RNA, folding of RNA, stabilizing RNA structures, providing alternative hydrogen bonding capabilities and so forth. A distinct effect of specific rRNA modifications is observed where they confer antibiotic resistance by interfering with binding of the antibiotic. Few modifications seem to be essential because functionally active *Bacillus stearothermophilus* and *Thermus aquaticus* ribosomes can be assembled from in vitro-transcribed RNA devoid of modifications (Green & Noller, 1999; Khaitovich et al., 1999) although modifications in a 80-nt-long fragment of 23S rRNA were necessary for assembly of active *E. coli* ribosomes (Green & Noller, 1996). In addition, a clear effect of 2′-*O*-ribose methylation of U2552 in the A-loop has been demonstrated by *E. coli* deficient in this methylation, which showed decreased growth rate, reduced protein synthesis activity, and an increased proportion of ribosomal subunits (Caldas et al., 2000).

An important goal in elucidating the role of modified ribonucleotides is to determine the extent to which the modifications are phylogenetically conserved in certain positions. We studied five organisms representing gram-negative/gram-positive eubacteria, crenarchaeota/euryarchaeota archaea and high/low temperature growing organisms to determine the degree of conservation in their A-loop region modification pattern and thereby gained an understanding of their importance and function. Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) in combination with digestion by RNases (Kirpekar et al., 2000) was used to locate posttranscriptional modifications in the A-loop subfragment of 23S rRNA. As this approach left some ambiguity regarding the position of modifications in the primary structure, we combined it with a primer extension method identifying 2′-*O*-ribose methylations. Finally, we screened the helices 90–92 region for Ψs by a 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMCT) modification procedure (Bakin & Ofengand, 1993), as this derivatization is mass silent and cannot be detected directly by MALDI-MS. The identified modifications are related to the X-ray crystal structure of *Haloarcula marismortui* 50S ribosomal subunit (Ban et al., 2000), and their possible functions are discussed.

RESULTS

MALDI-MS

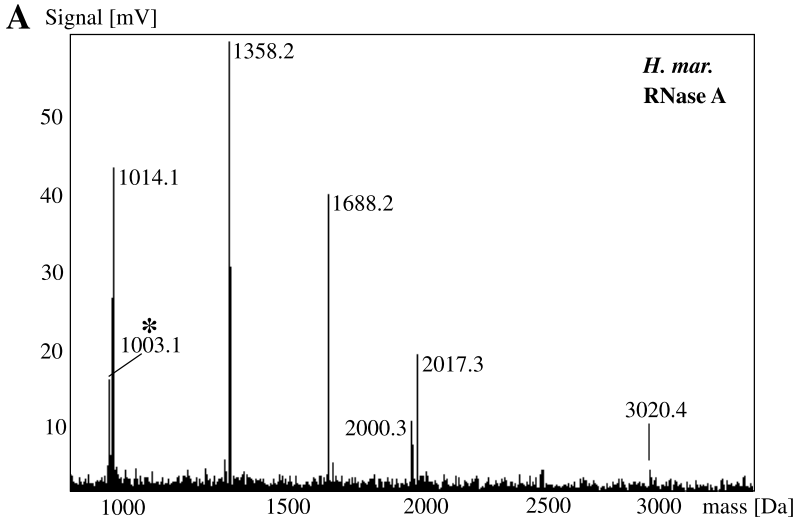
The mapping of posttranscriptional modifications in large RNA molecules such as 23S rRNA is not trivial, but MALDI-MS analysis of RNA subfragments requires only a few picomoles with an optimal size of approximately 100 nt in length. Therefore, we isolated defined RNA fragments from 23S rRNA by site-directed RNase H digestion. RNA fragments containing helices 90–92 from 23S rRNA and from in vitro RNA transcripts were purified by cleavages with RNase H, followed either by extraction from polyacrylamide gels or with a streptavidin/biotin system as described in Materials and Methods.

The isolated fragments were digested to completion with the pyrimidine-specific RNase A or the guanosine phosphate specific RNase T1, yielding two different pools of subfragments 1–15 nt in length. These were analyzed with MALDI-MS, and modified nucleotides were identified by comparing measured masses of digested fragments with theoretical values derived from the gene sequence of the rRNA. Mono- and dinucleotides are not considered in the MALDI-MS spectra, because their mass region is dominated by signals from the matrix, buffers, and so forth.

MALDI-MS analysis was performed on both native rRNA and in vitro RNA transcripts with the same sequence to assist the interpretation of the spectra. No data for the in vitro transcripts are shown. Nucleotides and fragments are numbered according to the relevant organism followed by the corresponding *E. coli* number(s) indicated in parentheses. Nm indicates a 2′-*O*-ribose methylation whereas mN indicates a methylation at an unknown position in the nucleotide.

Analysis of the A-loop region from *E. coli* 23S rRNA was performed as a test of the method because all modifications in the 23S rRNA from this organism have been mapped previously. One fragment from each RNase digest showed a mass increment of 14.0 Da compared with the calculated mass, corresponding to the presence of one methylated nucleotide. Ordering of all observed fragments into the sequence of helices 90–92 unambiguously determined the modified fragments to derive from $C_{2551}mUGU_{2554}$, thereby verifying a methylation at U2552 in the A-loop of *E. coli* 23S rRNA (data not shown).

As an example of MALDI-MS spectra, the A-loop region from *H. marismortui* is shown in Figure 1A,B. All masses from relevant peaks are summarized in Figure 1C together with the positions assigned to the corresponding fragments. The RNase A and T1 digests each reveal a fragment with an increased mass of



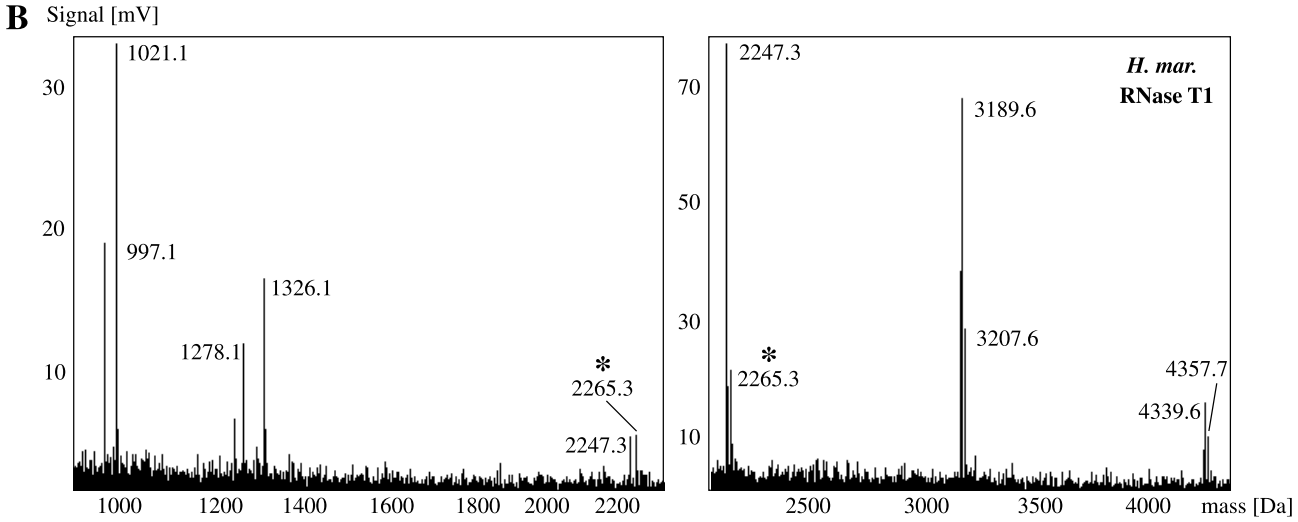


FIGURE 1. The MALDI-MS spectra obtained from the analysis of *H. marismortui* 23S rRNA helices 90–92 digested with RNase A (**A**) or RNase T1 (**B**). All fragments contain a phosphate at the 3′ end. Each spectrum contains one peak deviating from the theoretical mass denoted by *. The RNase T1 spectrum is divided into two segments because the entire mass range could not be accommodated into a single spectrum. **C**: Summary of the assignment of the observed peaks to cognate *H. marismortui* sequences. The doublet peaks with a 18-Da mass difference in the RNase T1 digestion originates from species with identical sequences harboring either 2′-3′-cyclic phosphate (c.p.) or normal phosphate. *E. coli* numbering is indicated for the modified fragments.

C

RNase A			**RNase T1**		
Mass	Sequence	Position *H.mar.*	Mass	Sequence	Position *H.mar.*
1014.1	GGU	2543-2545	4339.6	UUCCCUCCAUCCUG c.p.	2545-2558
2000.3	AGAAGC	2566-2571	4357.6	UUCCCUCCAUCCUG	2545-2558
1358.2	GGGC	2572-2575	1278.1	CCCG	2559-2562
2017.3	AAGGGU	2576-2581	997.1	CAG	2565-2567
1688.2	GAGGU	2582-2586	1021.1	AAG	2568-2570
1003.1*	UGU or	2587-2589	1326.1	CAAG	2575-2578
		(E.c.2552-2554)	2247.3*	UUGUUCG c.p.	2586-2592
1003.1*	GUU	2588-2590	2265.3*	UUGUUCG	2586-2592
		(E.c.2553-2555)			(E.c.2551-2557)
3020.4	AAAGGAGGU	2599-2607	3189.6	CCUAUUAAAG c.p.	2593-2602
			3207.6	CCUAUUAAAG	2593-2602

28.0 Da at 1003.1 Da and 2265.3 Da, respectively, compared to the theoretical mass of the digest. Each modified fragment contains an expected, but uncleaved, nucleotide substrate, which denotes a modification on G2588 (E.c. 2553), and on either U2587 (E.c. 2552) or U2589 (E.c. 2554). These modifications render the named nucleotides resistant to RNase digestion.

The corresponding region of 23S rRNA from *B. subtilis, B. stearothermophilus*, and *Sulfolobus acidocaldarius* was investigated in a similar way (spectra not shown) and a summary of fragments with excess mass compared to the theoretical mass from all the organisms is presented in Table 1.

B. subtilis and *B. stearothermophilus* have identical sequences in helices 90–92 in 23S rRNA and showed very similar MALDI-MS spectra. The analysis of the RNase A digest did not reveal any fragments deviating from the theoretical masses. Analysis of the RNase T1 digest revealed a 2250.3 Da fragment from both organisms, which corresponds to a fragment methylated at G2581 (E.c. 2553) as it is protected against RNase T1 digestion. In addition, a 1967.3 Da fragment was observed in the RNase T1 digest of *B. subtilis*, indicating a fragment carrying a methylation at the internal G2602 residue (E.c. 2574). A minor fragment with a mass of 1303.2 Da corresponds to an unmethylated $U_{2599}ACG_{2602}$ (E.c. 2571–2574) fragment, and therefore G2602 is only partially methylated. Though MALDI-MS is generally not suited for quantifications, the peak intensities indicated that the degree of methylation is higher than 75%.

Finally, we analyzed the *S. acidocaldarius* 23S rRNA helices 90–92 fragment, where positioning of the modified nucleotides was complicated because of a redundant fragment pattern. The RNase A digestion revealed three fragments, each indicating the presence of a methylation (Table 1). Only the 3378.5 Da fragment can be assigned to a unique methylated sequence. The RNase T1 digest also revealed three methylated fragments (Table 1). The 4729.5 Da fragment carries two methylations, and a monomethylated 4715.5-Da fragment corresponding to the same sequence was also observed. This indicates the presence of a partial methylation, but it cannot be mapped unambiguously, due to the many RNase A targets (pyrimidines) in the fragment. The inhibition of RNase T1 digestion at G_{2649} (E.c. 2509; see Table 1) shows that this residue is fully methylated. Combining data from the RNase A and T1 digestions localized the methylation to the U_{2651}-U_{2662} (E.c. 2511–2522) sequence. The methylation site in the A_{2704}-C_{2713} (E.c. 2564–2573) sequence can also be narrowed down because the nonmethylated U_{2702}-G_{2707} (E.c. 2562–2567) fragment from the RNase T1 spectrum overlaps this fragment, and therefore the methylation site is positioned in the fragment G_{2708}-C_{2713} (E.c. 2568–2573). The localization of the methylated 988.1-Da fragment(s) in helices 90–92 is ambiguous because of multiple sequence repeats, but the methylation(s) can be restricted to three possible locations: a methylation at $U_{2646}C_{2647}$ (E.c. 2506–2507) and/or $U_{2673}G_{2674}$ (E.c. 2534–2535) and/or $U_{2692}G_{2693}$ (E.c. 2552–2553).

TABLE 1. The MALDI-MS fragments from helices 90–92 of 23S rRNA from all investigated organisms, which show masses corresponding to modified fragments.[a]

Organism	Size	RNase A	Position	E.c. position
H. marismortui	1003.1 Da	2m(GUU or UGU)	2587–2589 or 2588–2590	2552–2554 or 2553–2555
S. acidocaldarius	988.1 Da	m(UGC, GUC, GCU or CGU)		
	1027.2 Da	m(GGC)		
	3378.5 Da	m(AAAGGGGAGC)	2704–2713	2564–2573
E. coli	989.1 Da	m(GUU or UGU)		
Organism	Size	RNase T1	Position	E.c. position
H. marismortui	2265.3 Da	2m(UUGUUCG)	2586–2578	2551–2557
S. acidocaldarius	988.1 Da	m(CUG or UCG)		
	4715.5 Da	m(GCUCUUCCCACCCUG)[b]	2649–2663	2509–2523
	4729.5 Da	2m(GCUCUUCCCACCCUG)	2649–2663	2509–2523
B. stearothermophilus	2250.3 Da	m(CUGUUCG)	2579–2585	2551–2557
B. subtilis	1967.3 Da	m(UACGCG)[b]	2599–2604	2571–2576
	2250.3 Da	m(CUGUUCG)	2579–2585	2551–2557
E. coli	988.2 Da	m(UCG or CUG)		

[a]The *H. marismortui* fragments and one *S. acidocaldarius* fragment had a mass increase of 28.0 Da corresponding to dimethylated fragments (denoted 2m) whereas all others have a 14.0-Da mass increase corresponding to a single methyl group (denoted m). The position of the fragments are shown with both organism and *E. coli* numbering whenever it could be explicitly determined from the MALDI-MS data. Underlined Gs are not cut by RNAse T1, and are thus methylated.

[b]Fragments from a partially methylated sequence region.

Detection of 2′-*O*-ribose methylations

The results from the MALDI-MS analysis were complemented by a method utilizing primer extension on rRNA templates (Maden et al., 1995). Some posttranscriptional modifications act as a steric hindrance, causing reverse transcriptase to pause. This results in an increased frequency of termination preceding the modified position and sometimes additionally at the position (Maden et al., 1995). This effect is enhanced for progressively diminished dNTP substrate concentrations at positions carrying a 2′-*O*-ribose methylation, resulting in concentration-dependent stops (cds) upon primer extension. However, RNA secondary structure can also give rise to an increased frequency of termination and thus cause cds as well as concentration-independent stops (non-cds). Comparing primer extension reactions on native rRNA with extension on unmodified in vitro RNA allows distinction between cds arising from 2′-*O*-ribose methylations and RNA secondary structure. Cds caused by RNA secondary structure are observed with both native rRNA and in vitro RNA templates, while cds caused by 2′-*O*-ribose methylations only occur in the native rRNA. 2′-*O*-ribose-methylated nucleotides in secondary structure give rise to stronger cds than those from unmodified structured RNA. Gel autoradiograms from representative primer extension analyses from *H. marismortui*, *B. subtilis*, *B. stearothermophilus*, *S. acidocaldarius*, and *E. coli* rRNA templates are shown in Figure 2. The primer extensions assisted in identifying some of the methylations as 2′-*O*-ribose methylation and also helped to pinpoint the exact position in the sequence in cases where MALDI-MS could not distinguish between different possibilities. A summary of all data is presented in Figure 3.

In *E. coli*, a clear cds at position U2552 is present in native 23S rRNA whereas only a very faint band is observed in the corresponding T7 transcript, indicating a 2′-*O*-ribose methylation at U2552 (Fig. 2) in agreement with the MALDI-MS analysis. This is in accordance with previous mapping of a ribose methylation at this position by Branlant et al. (1981).

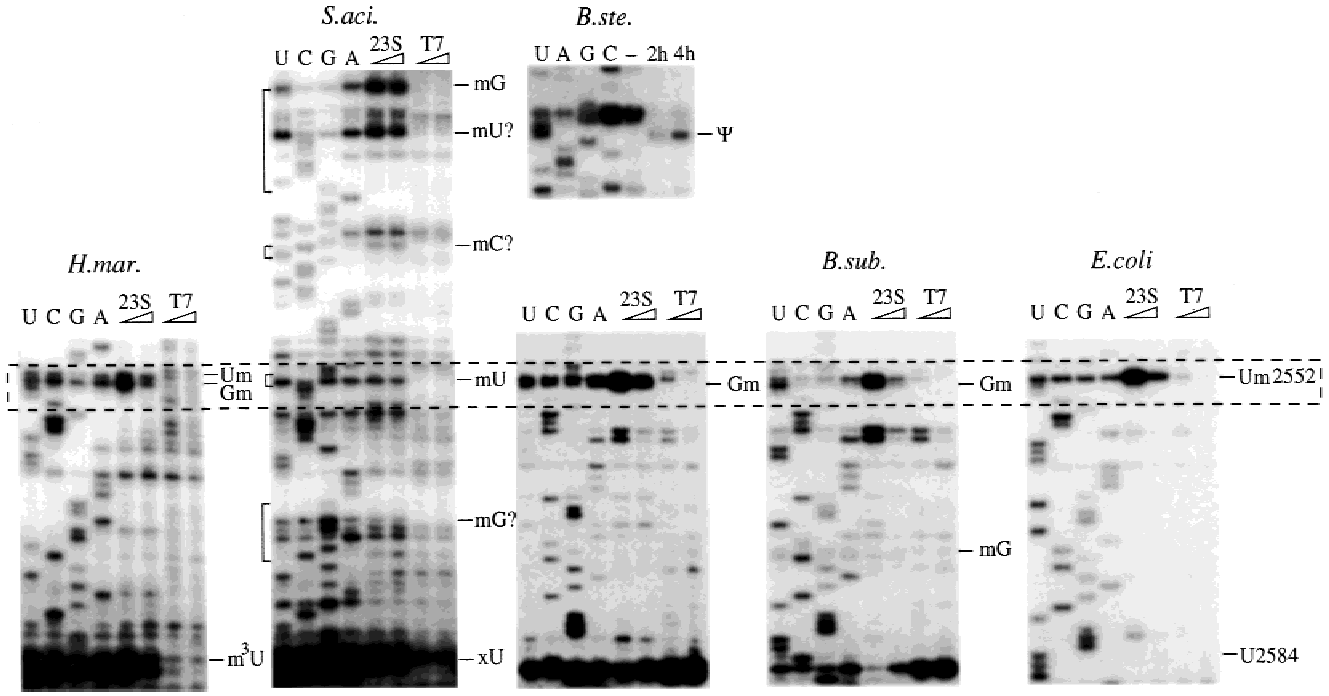


FIGURE 2. Autoradiograms showing primer extension analyses to detect modifications. UCGA indicate sequencing lanes. Triangles denote increasing concentrations of dNTP. 23S are primer extensions on 23S rRNA and those labeled T7 are from in vitro transcripts of domain V. The identified modifications are indicated: m denotes methylation, Nm denotes 2′-*O*-ribose methylation. The dashed lines shows A-loop nucleotides. In *H. marismortui* (*H.mar.*), Um2587 (E.c. 2552), Gm2588 (E.c. 2553), and m^3U2619 (E.c. 2584) are indicated. In *S. acidocaldarius* (*S.aci.*), several ambiguous methylated positions were detected by MALDI-MS, and these are indicated with brackets. Probable 2′-*O*-ribose methylations are observed at G2649 (E.c. 2509) and U2692 (E.c. 2552), and possible modifications, marked with question marks, are observed at U2653, C2673, and G2708 (E.c. 2513, 2533, and 2568, respectively). A presumed modification at U2724 (E.c. 2584) is also indicated (xU). In the Ψ assay of *B. stearothermophilus* (*B.ste.*), - indicates no CMCT and 2h and 4h indicate CMCT followed by alkaline treatment in 2 and 4 h, respectively. A Ψ at 2594 (E.c. 2457) is shown. Cds at G2581 (E.c. 2553) and U2582 suggest 2′-*O*-ribose methylations. As the MALDI-MS only suggested a methylation at G2581 (E.c. 2553), both the cds bands are probably caused by 2′-*O*-ribose methylation at G2581 (E.c. 2553). The same situation is observed in *B. subtilis* (*B.sub.*), which also has a faint non-cds, which corresponds to the partial methylation detected with MALDI-MS at G2602 (E.c. 2574).

A

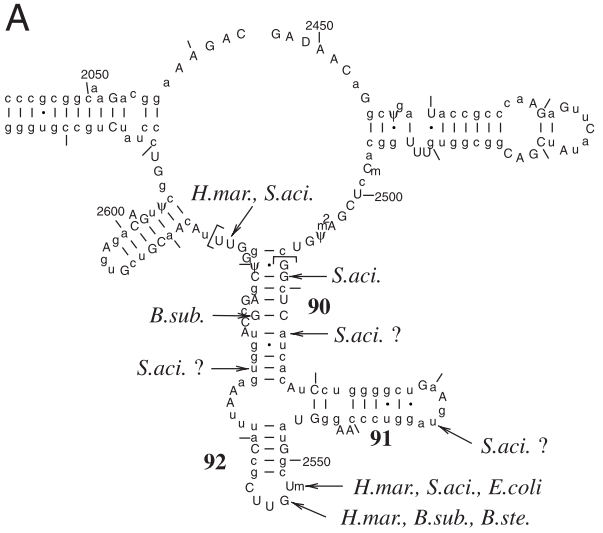


FIGURE 3. **A**: *E. coli* secondary structure of part of 23S rRNA domain V with nucleotides modified in *E. coli* shown in the sequence and universally conserved nucleotides (>90%) shown in upper case letters (Gutell Lab; Comparative RNA Web Site URL: http://www.rna.icmb.utexas.edu/). Brackets indicates the RNA region investigated for modifications. Arrows indicate the posttranscriptional modifications mapped in *H. marismortui*, *B. subtilis*, *B. stearothermophilus*, and *S. acidocaldarius*. A question mark indicates possible methylated position, which could not be explicitly determined. **B**: Summary of the modified nucleotides found in helices 90–92 displayed alongside modifications in *Homo sapiens* (*H. sap.*; Maden, 1990), hamster mitochondria (mit.; Baer & Dubin, 1981) and yeast mitochondria (Sirum-Connolly et al., 1995). The A-loop positions are marked with dashed lines.

B

Position (*E. coli*)	*H. mar.*	*S. aci.*	*B.ste.*	*B. sub.*	*E. coli*	*H. sap.*	Hamster mit.	Yeast mit.
2509		mG						
2510						Cm		
2511						Ψ		
2513		mU?						
2525						Ψ		
2533		mC?						
2548						Gm		
2552	Um	mU			Um	Um	Um	Um
2553	Gm		Gm	Gm		Gm	Gm	
2554						Ψ		
2568		mG?						
2574				mG				
2575						Ψ		
2576						Am		
2580					Ψ			Ψ
2584	m^3U	xU				m^3U		

The investigation of helices 90–92 from *H. marismortui* with primer extension analysis revealed two cds, denoting G2588 (E.c. 2553) and perhaps U2587 (E.c. 2552) to be 2′-*O*-ribose methylated. The MALDI-MS analysis identified a methylation at both U2587, and G2588 or U2589. Altogether this determines the presence of Um2587 and Gm2588. This is confirmed by X-ray data from the crystal structure of the 50S subunit from *H. marismortui* (D. Klein, P. Nissen, T.A. Steitz, P.B. Moore, pers. comm.).

Likewise, in the analyses of the *B. subtilis* and *B. stearothermophilus*, two cds at G2581 (E.c. 2553) and U2582 suggest 2′-*O*-ribose methylation. The MALDI-MS reveals only one methylation at G2581 (E.c. 2553), so a single 2′-*O*-ribose methylation here probably caused both cds. A faint non-cds is present from *B. subtilis*, corresponding to the partial methylation detected with MALDI-MS at G2602 (E.c. 2574). The non-cds nature of this band suggest that the modification is not a 2′-*O*-ribose methylation, but a m^1G, a m^2G, or a m^7G methylation.

Primer extension analysis on 23S rRNA from *S. acidocaldarius* shows several non-cds and some possible cds: Although various dNTP concentrations in addition to those shown in Figure 2 were used, no clear cds were observed. The MALDI-MS data was ambiguous with regard to the methylated positions; these ambiguities are indicated with brackets next to the sequence in

Figure 2. The primer extension analysis indicates some modifications inside these regions. In summary, our results suggest the A-loop region of *S. acidocaldarius* 23S rRNA to be methylated at G2649 (E.c. 2509) and U2692 (E.c. 2552) and at one or more of the following positions: U2653 (E.c. 2513), C2673 (E.c. 2533), and G2708 (E.c. 2568; Omer et al., 2000 numbering). In a study of snoRNA homologs in *S. acidocaldarius*., G2649 (E.c. 2509), G2666 (E.c. 2526), and U2692 (E.c. 2552) in the helices 90–92 region were pointed out as targets for 2′-*O*-ribose methylations (Omer et al., 2000). That work tested G2649 and U2692 with primer extension, and reported a cds at G2649 but no stops at U2692. We observed stops at both of these positions, but not as cds. The existence of complementary snoRNA homologs together with the primer extension stops and peaks in the MALDI-MS spectrum corresponding to a methylation all point to G2649 (E.c. 2509) and U2692 (E.c. 2552) as being 2′-*O*-ribose methylated. Contrary to this, neither our MALDI-MS nor primer extension analysis showed any indication of a modification at G2666 (E.c. 2526).

The very strong stop at the bottom of the autoradiograms for *H. marismortui* and *S. acidocaldarius* are likely to be caused by some kind of modification, as no band or a much weaker band is observed at the analogous sequence in the T7 RNA transcripts. Further investigation of this position in *H. marismortui* with MALDI-MS (described in Materials and Methods) showed that position U2619 (E.c. 2584) is methylated.

Mapping of Ψs

The A-loop region and its vicinity were also investigated for the presence of Ψs, which have the same mass as their uridine precursors, and are therefore not detectable with the MALDI-MS method used here. rRNA was treated with CMCT, which reacts with the N_3 position in uracil, the N_1 position in guanine, and both the N_1 and N_3 positions in Ψ. Subsequent mild alkaline treatment of the RNA leaves only the Ψ modified, which gives rise to a distinct stop at the 3′ adjacent nucleotide by primer extension analysis (Bakin & Ofengand, 1993). The positions of Ψ2605, Ψ2580, and Ψ2504 in *E. coli* (Bakin & Ofengand, 1993), Ψ2520 (E.c. 2492) in *B. subtilis* (Ofengand & Bakin, 1997), and Ψ2594 (E.c. 2457) in *S. acidocaldarius* (Massenet et al., 1999) were verified (data not shown). In addition, no Ψs were detected in *H. marismortui* (data not shown), but we detected Ψ2520 (E.c. 2492) in *B. stearothermophilus*, as shown in Figure 2.

DISCUSSION

The A-loop is known from eukaryotic 28S-like RNA to be a hot spot for modifications. Human, mouse, rat, chicken, *Xenopus laevis*, and *Saccharomyces carlsbergensis* (Eladari et al., 1977; Veldman et al., 1981; Maden, 1988) all have the modified triad UmGmΨ (E.c. 2552–2554) in this loop. Of the five organisms investigated here, only the *E. coli* 23S rRNA has been systematically mapped for posttranscriptional modifications.

Identification of modified nucleotides by MALDI-MS

We employed MALDI-MS as the initial method to identify modifications in discrete fragments of 23S rRNAs isolated by site-directed RNase H digestion. In contrast to the primer extension methods also used in this study, mass spectrometry will detect all types of modifications (except Ψs) and partial modifications might also be revealed. Combining the data from the RNase A and T1 digests made it possible to locate the modifications in the primary RNA sequence in many cases. Some modifications, for example, 2′-*O*-ribose methylations, inhibit RNase A and T1 digestions (Maden, 1990), whereas other modifications do not. Only a few systematic studies are available on how different modifications inhibit RNase cleavage (e.g., Lankat-Buttgereit et al., 1987), so the precise nature of a modification cannot be inferred from the cleavage pattern. Due to the heterogeneity of the RNase H cleavage, some unexpected peaks might appear in the MALDI-MS spectra. Additionally, as seen in Figure 1, peaks may arise from intermediates of RNase T1 digestion containing a cyclic 2′-3′phosphate. The use of unmodified T7-RNA fragments of the same sequence that were treated the same way helped in overcoming these problems.

Primer extension analysis with varying dNTP concentrations

The primer extension in the A-loop region of 23S rRNAs with varying dNTP concentrations served two purposes. It was primarily used to distinguish between 2′-*O*-ribose methylations and nucleotide base methylations, although not all 2′-*O*-ribose methylations will confer cds (Maden et al., 1995). Second, in cases where MALDI-MS could not exactly pinpoint the modified position in the RNA sequence, the primer extension assay aided the interpretation. The unmodified RNA served as an important control, because several bands will appear from template structure and hydrolysis. The method has been used extensively to detect 2′-*O*-ribose methylations mediated by snoRNAs in eukaryotes and by snoRNA homologs in archaea, but its reliability is limited, especially without unmodified control RNA. A study of *Pyrococcus* had problems with a high G/C content and very stable RNA secondary structures (Gaspin et al., 2000). This might also play a role in our study of *S. acidocaldarius*, where no clear cds were detected, although several stops, specific for the 23S rRNA template, were observed. Alternatively, no

2′-*O*-ribose methylation should be present in this region in *S. acidocaldarius*, which is unlikely because 17 ribose methylations have been predicted in *S. acidocaldarius* 23S rRNA by the alignment of snoRNA homologs (Omer et al., 2000). Seven of these positions were tested with the dNTP assay and four positions were confirmed as being 2′-*O*-ribose methylated, and three others were not. As described above, we have taken the existence of complementary snoRNA homologs, together with primer extension stops and MALDI-MS peaks corresponding to methylations, as strong indications for 2′-*O*-ribose-methylation.

Modifications at positions equivalent to *E. coli* U2584

In *H. marismortui*, we identified a methylation at U2619 (E.c. 2584) with MALDI-MS giving rise to a very strong primer extension stop (Fig. 2). Although the methylation could not be further identified with the methods used in this study, the electron density maps of the *H. marismortui* large ribosomal subunit reveal that U2619 is N_3 methylated (D. Klein, P. Nissen, T.A. Steitz, P.B. Moore, pers. comm.). Lack of this modification has been shown to confer sparsomycin-resistance (Lázaro et al., 1996). Stops at the corresponding position have previously been observed from both eukaryotes and archaea (Ofengand & Bakin, 1997). In *Homo sapiens*, the modification is also a m^3U (Maden, 1990). Therefore, the strong stop in the primer extension of *S. acidocaldarius* 23S rRNA at U2724 (E.c. 2584), relative to T7 RNA (xU in Fig. 2) is probably also due to a m^3U. Primer extension stops were not observed at this position in *E. coli*, whereas *B. stearothermophilus* and *B. subtilis* show strong stops in T7 RNA that are not caused by modification, but likely by structure.

Are modifications common and/or specific features?

The exact positions of the modifications in helices 90–92 vary considerably between the different organisms, but all have modifications somewhere in the first 3 nt of the A-loop as indicated in Figure 3. The positions equivalent to U2552 is modified in *E. coli*, *H. marismortui*, and *S. acidocaldarius*. This nucleotide position is considered to be universally 2′-*O*-ribose methylated but our results from *B. subtilis* and *B. stearothermophilus* show that they do not carry a 2′-*O*-ribose methylation at this position. The adjacent nucleotide, G2553, is 2′-*O*-ribose methylated in *H. marismortui*, *B. subtilis*, and *B. stearothermophilus*. Mitochondria have a modification pattern similar to that of archaea and eubacteria, whereas three A-loop nucleotides are modified in all examined eukaryotic sequences. The consensus from bacteria and archaea appears to be that modifications are always present in the A-loop at positions equivalent to U2552 and/or G2553. All organisms investigated so far have one to three modifications in the first 3 nt in this loop. Also, the modifications seem to be 2′-O-ribose methylations and Ψs. Otherwise there is no consensus as to the location of modifications in other parts of the helices 90–92.

Sites of 23S RNA modifications in the 50S subunit

E. coli contains 23 posttranscriptionally modified nucleotides including 13 methylations, 8 Ψs, 1 methyl-Ψ, and 1 dihydrouridine (Branlant et al., 1981; Smith et al., 1992; Kowalak et al., 1995, 1996; Ofengand & Bakin, 1997). When displaying these modifications in the 2.4 Å X-ray crystal structure of the *H. marismortui* 50S subunit, as shown in Figure 4A, it is evident that all the modifications group near the center of the ribosome. The posttranscriptional modifications in red space fill representation are clearly clustered around the peptidyl transferase center, which is illustrated by the green transition state analog CCdApPuro. Three Ψs (*E. coli* 1911, 1915, and 1917) are not displayed in Figure 4A because they are not included in the crystal structure. The recent 5.5 Å X-ray structure of the complete *Thermus thermophilus* ribosome shows that these three positions are also close to the peptidyl transferase center in a loop, which forms part of an intersubunit bridge (Yusupov et al., 2001). The localization of the modifications suggests involvement in the peptide elongation process, more than a role in ribosomal maturation, where a more even distribution of modifications throughout the rRNA would be expected.

The modified nucleotides in the A-loop

The positions of the three *H. marismortui* modifications identified in this study are all close to the peptidyl transferase center. In the stereo view (Fig. 4B), the *H. marismortui* 23S rRNA helices 90–92 are shown with positions Um2587, Gm2588, and m^3U2619 (E.c. 2552, 2553, and 2584) in red, illustrating their close proximity to the transition state analog, CCdApPuro (in green). The CCdApPuro spans both the A- and P-sites with the CCdA-moiety resembling the CCA 3′ end of P-site-bound tRNA, and the p (phosphate) connecting the A-site and the P-site, imitating the tetrahedral carbon of the native peptidyl transferase intermediate (Welch et al., 1995; Nissen et al., 2000).

All A-loops seem to contain 2′-*O*-ribose methylations and/or Ψs (Fig. 3B). NMR studies on 2′-*O*-ribose methylations on nucleotide and oligonucleotide models have demonstrated that 2′-*O*-ribose methylations results in thermodynamic stabilization of the C3′-endo ribose conformation in both pyrimidines and purines (Kawai et al., 1992). Ψ enhances local RNA stacking in single- and double-stranded regions, by favoring the C3′-endo con-

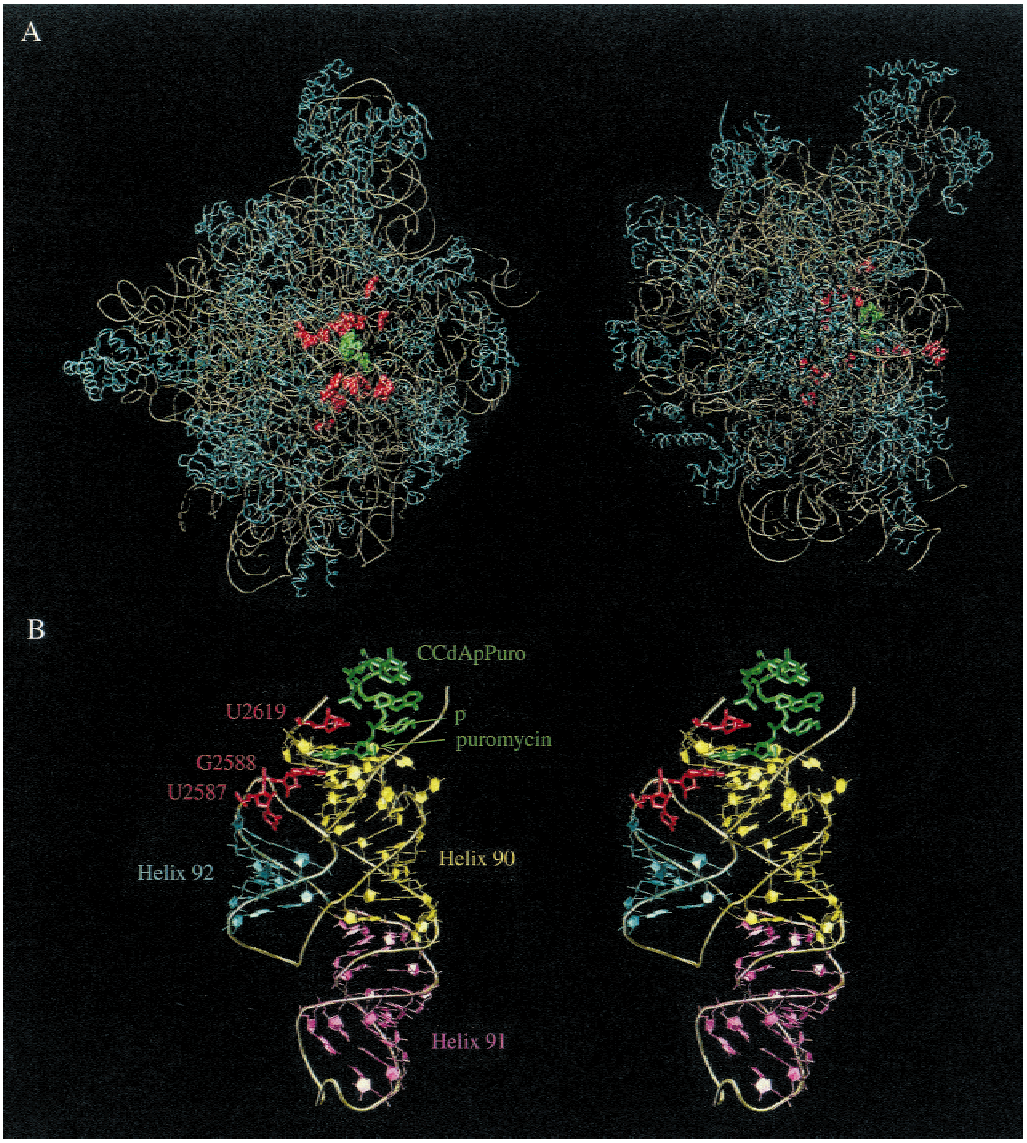


FIGURE 4. **A**: The *H. marismortui* 50S subunit high-resolution structure (Ban et al., 2000) with the rRNA backbone shown in gold and ribosomal proteins in blue (left: crown view; right: side view with the subunit interface to the right). Twenty of the 23 known modified positions in 23S rRNA from *E. coli* are indicated with red (space filled representation) at the corresponding positions in *H. marismortui*. The CCdApPuro substrate analog is shown in green to indicate the peptidyl transferase centre. **B**: Stereo view of the *H. marismortui* 23S rRNA helices 90–92 encompassing Um2587, Gm2588, and m_3U2619 (E.c. 2552, 2553, and 2584) shown in red and CCdApPuro in green. Bases involved in base pairing within helix 90, 91, and 92 are shown in yellow, purple, and cyan, respectively. The molecular visualization was done using VMD (Humphrey et al., 1996).

formation of the ribose ring, which restricts Ψ to the anticonformation (Davis, 1998). Furthermore, Ψ is an isomer of uridine with the N_1 atom available for hydrogen bonding. Both 2′-*O*-ribose methylations and Ψs have a stabilizing effect on RNA by favoring the C3′-endo ribose conformation, which diminishes the distance between the bases and enhances stacking, making the RNA helix more rigid. However, many modified positions are not located within helical regions. Recent NMR studies of the *E. coli* A-loop structure in a 19-nt fragment have shown that 2′-*O*-ribose methylation of U2552 has little overall effect on the structure of the A-loop but just affects the local configuration of C2556 and U2555 within the loop (Blanchard & Puglisi, 2001). It should be noted that this structure differs from the *H. marismortui* 50S and the *T. thermophilus* backbone conformations for the helices 90–92 region.

The *H. marismortui* 23S rRNA A-loop folds back on helix 90 contacting the nucleotides at the base of the helix, forming a triple base pair between the Gm2588 (E.c. 2553) and the C2542:G2617 (E.c. 2507:2582) base pair (Ban et al., 2000), but Gm2588 (E.c. 2553) is also claimed to base pair with C75 of tRNA in A-site (Nissen et al., 2000). The other 2′-*O*-ribose-methylated nucle-

otide in the A-loop, Um2587 (E.c. 2552), is suggested to form a triple base pair with U1996 (E.c. 1955) and C2591 (E.c. 2556; Ban et al., 2000). All five bases in the A-loop are pointing into the peptidyl transferase groove and form an almost continuous stack on helix 92 with only one break in the stacking between Um2587 (E.c. 2552) and Gm2588 (E.c. 2553). We suggest that the 2′-*O*-ribose methylations are anchors, which help the A-loop nucleotides stack in a manner that is optimal for function.

MATERIALS AND METHODS

Growth of cells and isolation of rRNA

E. coli MRE 600 and *B. subtilis* 168 cells were grown at 37 °C in LB growth medium (Sambrook et al., 1989) to an optical density of $A_{550} = 0.6$. *H. marismortui* cells were grown at 37 °C in SW-25 growth medium (Lázaro et al., 1996) to an optical density of $A_{600} = 1.2$. *S. acidocaldarius* cells were grown at 70 °C in NOB8 medium (Brock et al., 1972), with the addition of 0.1% yeast extract, to an optical density of $A_{600} = 1.2$. *B. stearothermophilus* cells were provide by Professor R.A. Garrett. All cells were harvested by cooling on ice followed by centrifugation. *E. coli*, *B. subtilis*, *B. stearothermophilus*, and *S. acidocaldarius* cells were resuspended in TMN buffer (20 mM Tris-HCl, pH 7.75, 10 mM $MgCl_2$, 100 mM NH_4Cl), and *H. marismortui* cells were resuspended in KCl lysis buffer (70 mM HEPES-KOH, pH 7.8, 3 M KCl, 60 mM $MgAc_2$). Resuspended cells were treated with RNase-free DNase I (Roche), and the cells were opened by vortexing 6 × 30 s with an equal volume of glass beads. The lysate was recovered by centrifugation, 9 mM EDTA was added, and it was layered on a 40 mL 10–40% sucrose gradient made in TMN with 1 mM $MgCl_2$ and centrifuged 19 h at 42,000 × *g* in an SW28 rotor (Beckman, Palo Alto). The gradient was analyzed on a Pharmacia GradiFrac system, and the fractions containing 50S ribosomal subunits were pooled and recovered by precipitation with 0.7 vol of ethanol and resuspended in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The 50S subunits were extracted with phenol and chloroform, and the mixture of 5S and 23S rRNA was recovered by precipitation with 2.5 vol of ethanol and resuspended.

The numbering of nucleotides in the various 23S rRNAs is as used by Ban et al. (2000; *H. marismortui*), Omer et al. (2000) and Massenet et al. (1999—whose numbering differs by 3 nt; *S. acidocaldarius*), Ofengand and Bakin (1997; *B. subtilis*), and Genbank accession number X01387 (*B. stearothermophilus*).

Plasmid cloning and in vitro transcription of 23S rRNA domain V

The domain V region of 23S rRNA from *B. subtilis*, *B. stearothermophilus*, *S. acidocaldarius*, and *H. marismortui* were amplified by PCR using the DNA oligonucleotides: 5′-AACT GCAGCTCGGTGAAATTATAGTAC-3′ and 5′-CGGGATCCC CCGGTCCTCTCGTAC-3′, 5′-CGGGATCCCCGGTGAAATT ATAC-3′ and 5′-GGAATTCCCCGGTCCTCTCGTAC-3′, 5′-CGGGATCCCCCTGTTCCTCTCGAT-3′ and 5′-AACTGCAG CCTCGTGAACGCCCT-3′, and 5′-CGGAATTCCCCGGTGA ACTGTACGTTCC-3′ and 5′-CGGGATCCCGTAGTTCCTCT CGTACTATACG-3′, respectively. The PCR fragments were cloned downstream of the T7 promoter of pUT719, (Østergaard et al., 1998) after treatment with *Bam*HI and *Pst*I for cloning from *B. subtilis* and *S. acidocaldarius*, and *Bam*HI and *Eco*RI were used for cloning from *B. stearothermophilus*. The *H. marismortui* PCR fragment was cloned downstream of the T7 promoter of pGEM3 (Promega) using *Eco*RI and *Bam*HI. Restriction enzymes were purchased at Amersham Pharmacia and used as recommended by the supplier. The ligated constructs were transfected into competent *E. coli* DH1 or TG1 cells (Sambrook et al., 1989). All constructs were verified by sequence analysis of the inserted domain V.

In vitro transcripts of 23S rRNA domain V were obtained by transcription with T7 RNA polymerase as previously described (Vester & Douthwaite, 1994). Plasmid p625dV containing the cloned 23S rRNA domain V from *E. coli* (Vester et al., 1998), *B. subtilis*, *S. acidocaldarius*, and *H. marismortui* were linearized with *Bam*HI, and the plasmids from *B. stearothermophilus* were linearized with *Eco*RI. Radioactive transcripts were obtained by adding [α-^{32}P]UTP (Amersham Pharmacia) to the transcription mixture.

Isolation of specific RNA fragments

Isolation of RNA fragments for MALDI-MS analysis was obtained by RNase H (USB) cleavage of RNA regions flanking helices 90–92 of the cloned and native 23S rRNA domain V. The cleavage was directed by hybridizing the DNA oligonucleotides 5′-CGACGGTCTAAACCCAGCTCACGACC-3′ and 5′-CCGACATCGAGGTAGCAGCC-3′ to position E.c. 2569–2595 and 2489–2509 in RNase H buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 2 mM DTT) for 2 min at 80 °C and slow cooling to 50 °C, followed by incubating with 0.5 U of RNase H for 10 min at 50 °C.

The RNA fragment from *H. marismortui* was recovered by hybridizing a biotinylated DNA oligonucleotide 5′-Biotin-C6-AAAAAAAAAAGGCGAACAACCTCACC-3′ covering the E.c. positions 2544–2559, which was isolated using streptavidin-coated Dynabeads (M-280 streptavidin. Dynal). The Dynabeads were washed twice in 1 M NH_4OAc and twice in water, after which the fragments were separated from the biotinylated DNA oligonucleotide by heat denaturation. The isolation procedure was monitored by running identical procedures on radioactive in vitro transcripts using PAGE that allowed the identification of the RNA fragment on autoradiograms.

The RNA fragments from the other organisms were recovered by a different method. RNase H cleaved radioactive in vitro transcripts, nonradioactive in vitro transcripts, and native rRNA from *E. coli*, *B. subtilis*, *B. stearothermophilus*, and *S. acidocaldarius* were run on denaturing 8% polyacrylamide gels. An autoradiogram identified the fragments of interest and was used as a stencil to excise the bands containing the RNA fragments derived from the nonradioactive in vitro transcripts and native rRNA. The fragments were extracted in 2 M NH_4OAc and the fragments were recovered by precipitation with an equivalent volume of ethanol and isopropanol and dissolved in water.

Isolation of an rRNA fragment containing U2619 from *H. marismortui* was achieved by hybridizing a complementary DNA oligonucleotide 5′-CGACGGTCTAAACCCAGCTC

ACGACC-3′ covering E.c. positions 2569–2595, and digesting the single-stranded RNA with mung bean nuclease (Stratagene) at 37 °C for 2 h. After extraction with phenol and chloroform, the DNA/RNA hybrid fragment containing U2619 (and remaining double-stranded RNA fragments) was precipitated with ethanol, resuspended, and treated with RNase-free DNase I (Roche). The DNase I was removed by phenol extraction and ethanol precipitation, and the RNA was dissolved in water. The U2619-containing RNA fragment was isolated by hybridization of a biotinylated DNA oligonucleotide 5′-Biotin-C6-GACGGTCTAAACCCAGCTCACGAC-3′, followed by isolation with Dynabeads as described above.

MALDI-MS analysis of RNase digested fragments

Detection of posttranscriptional modifications using MALDI-MS was done as described by Kirpekar et al. (2000). The fragments must be free of contaminating RNA, such as full-length 23S rRNA, RNase H semidigested 23S rRNA, 16S rRNA, 5S rRNA, and tRNA, which would give extra signals in the MALDI-MS spectra. One to two picomoles of in vitro and rRNA fragments encompassing helices 90–92 from *E. coli*, *B. subtilis*, *B. stearothermophilus*, *S. acidocaldarius*, and *H. marismortui* were completely digested either with RNase A (Sigma) for 1–4 h or RNase T1 (USB) for 16 h in 50 mM 3-hydroxypicolinic acid (3-HPA) in 5% acetonitrile. The RNA must be free of alkali metals and Tris, which can appear as adducts, complicating the MALDI-MS analysis.

The 1–2-μL sample was mixed on the target with 0.7 μL matrix (0.5 M 3-HPA in 50% acetonitrile) and a small amount of NH_4-loaded ion exchange beads (Nordhoff et al., 1993). The sample was allowed to crystallize by air drying and the ion exchange beads were carefully removed. The samples were analyzed with a Bruker Reflex II MALDI-MS instrument.

Detection of 2′-*O*-ribose methylations and Ψs by primer extension analysis

Detection of 2′-*O*-ribose methylations and Ψs was done essentially as previously described by Maden et al. (1995) and Bakin and Ofengand (1993). The modifications were monitored by primer extension analysis using reverse transcriptase (AMV, Finnzymes) and 5′-[^{32}P]-labeled oligodeoxynucleotide primers (Stern et al., 1988). The following primers were used for the extension reactions: 5′-TCCGGTCCTCT CGTACT-3′ for *E. coli*.; 5′-TAGAGTCCGACCTGTC-3′ for *S. acidocaldarius*; 5′-TAGCAGCCGACCTGT-3′ for *H. marismortui*; and 5′-CGCGACGGATAGGGAC-3′ for *B. subtilis* and *B. stearothermophilus.* The cDNA products of the primer extension reactions were separated on 6% polyacrylamide sequencing gels. The positions of the stops in cDNA synthesis were visualized on a gel autoradiogram and identified by reference to dideoxynucleotide sequencing reactions on 23S rRNA run in parallel. dNTP concentrations used in the primer extension reactions to identify 2′-*O*-ribose methylation were as follows: *B. subtilis* and *B. stearothermophilus*: 0.010 mM, 0.025 mM, and 0.500 mM. *S. acidocaldarius* and *H. marismortui*: 0.010 mM, 0.025 mM, and 0.500 mM.

The detection of Ψ was done by treating the rRNA with CMCT (Sigma). Subsequent mild alkaline treatment of the RNA leaves predominantly the Ψ modified, which gives rise to a distinct stop 1 nt 3′ from the Ψ position on autoradiograms of primer extension reactions.

ACKNOWLEDGMENTS

We thank K.S. Long and S.M. Poulsen for critical reading of the manuscript and L.B. Johansson for excellent technical assistance. Professor R.A. Garrett and Dr. Q. She are thanked for *B. subtilis* and *S. acidocaldarius* cells. Dr. E. Nordhoff is thanked for initial MALDI-MS analysis with the detection of the methylation at U2619 in *H. marismortui* 23S rRNA. FK was supported by the Carlsberg Foundation and through the Danish Biotechnology Instrument Centre.

Received August 16, 2001; returned for revision October 4, 2001; revised manuscript received October 31, 2001

REFERENCES

Baer RJ, Dubin DT. 1981. Methylated regions of hamster mitochondrial ribosomal RNA: Structural and functional correlates. *Nucleic Acids Res 9*:323–337.

Bakin A, Ofengand J. 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: Analysis by the application of a new sequencing technique. *Biochemistry 32*:9754–9762.

Ban N, Nissen P, Hansen J, Moore PB, Steitz TA. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science 289*:905–920.

Blanchard SC, Puglisi JD. 2001. Solution structure of the A loop of 23S ribosomal RNA. *Proc Natl Acad Sci USA 98*:3720–3725.

Bocchetta M, Xiong L, Mankin AS. 1998. 23S rRNA positions essential for tRNA binding in ribosomal functional sites. *Proc Natl Acad Sci USA 95*:3525–3530.

Branlant C, Krol A, Machatt MA, Pouyet J, Ebel JP, Edwards K, Kossel H. 1981. Primary and secondary structures of *Escherichia coli* MRE 600 23S ribosomal RNA. Comparison with models of secondary structure for maize chloroplast 23S rRNA and for large portions of mouse and human 16S mitochondrial rRNAs. *Nucleic Acids Res 9*:4303–4324.

Brimacombe R, Mitchell P, Osswald M, Stade K, Bochkariov D. 1993. Clustering of modified nucleotides at the functional center of bacterial ribosomal RNA. *FASEB J 7*:161–167.

Brock TD, Brock KM, Belly RT, Weiss RL. 1972. *Sulfolobus*: A new genus of sulphur-oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol 84*:54–68.

Caldas T, Binet E, Bouloc P, Richarme G. 2000. Translational defects of *Escherichia coli* mutants deficient in the Um(2552) 23S ribosomal RNA methyltransferase RrmJ/FTSJ. *Biochem Biophys Res Commun 271*:714–718.

Davis DR. 1998. Biophysical and conformational properties of modified nucleosides in RNA (Nuclear Magnetic Resonance studies). In: Grosjean H, Benne R, eds. *Modification and editing of RNA*. Washington, DC: American Society for Microbiology Press. pp 85–102.

Eladari ME, Hampe A, Galibert F. 1977. Nucleotide sequence neighbouring a late modified guanylic residue within the 28S ribosomal RNA of several eukaryotic cells. *Nucleic Acids Res 4*:1759–1767.

Gaspin C, Cavaille J, Erauso G, Bachellerie JP. 2000. Archaeal homologs of eukaryotic methylation guide small nucleolar RNAs: Lessons from the *Pyrococcus* genomes. *J Mol Biol 297*:895–906.

Green R, Noller HF. 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.

Green R, Noller HF. 1999. Reconstitution of functional 50S ribo-

somes from in vitro transcripts of *Bacillus stearothermophilus* 23S rRNA. *Biochemistry 38*:1772–1779.
Green R, Switzer C, Noller HF. 1998. Ribosome-catalyzed peptide-bond formation with an A-site substrate covalently linked to 23S ribosomal RNA. *Science 280*:286–289.
Humphrey W, Dalke A, Schulten K. 1996. VMD: Visual molecular dynamics. *J Mol Graph 14*:27–38.
Kawai G, Yamamoto Y, Kamimura T, Masegi T, Sekine M, Hata T, Iimori T, Watanabe T, Miyazawa T, 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.
Khaitovich P, Tenson T, Kloss P, Mankin AS. 1999. Reconstitution of functionally active *Thermus aquaticus* large ribosomal subunits with in vitro-transcribed rRNA. *Biochemistry 38*:1780–1788.
Kim DF, Green R. 1999. Base-pairing between 23S rRNA and tRNA in the ribosomal A site. *Mol Cell 4*:859–864.
Kirpekar F, Douthwaite S, Roepstorff P. 2000. Mapping posttranscriptional modifications in 5S ribosomal RNA by MALDI mass spectrometry. *RNA 6*:296–306.
Kowalak JA, Bruenger E, Hashizume T, Peltier JM, Ofengand J, McCloskey JA. 1996. Structural characterization of U*-1915 in domain IV from *Escherichia coli* 23 S ribosomal RNA as 3-methylpseudouridine. *Nucleic Acids Res 24*:688–693.
Kowalak JA, Bruenger E, McCloskey JA. 1995. Posttranscriptional modification of the central loop of domain V in *Escherichia coli* 23 S ribosomal RNA. *J Biol Chem 270*:17758–17764.
Lankat-Buttgereit B, Gross HJ, Krupp G. 1987. Detection of modified nucleosides by rapid RNA sequencing methods. *Nucleic Acids Res 15*:7649.
Lázaro E, Rodriguez-Fonseca C, Porse B, Urena D, Garrett RA, Ballesta JP. 1996. A sparsomycin-resistant mutant of *Halobacterium salinarium* lacks a modification at nucleotide U2603 in the peptidyl transferase centre of 23 S rRNA. *J Mol Biol 261*:231–238.
Maden BE. 1988. Locations of methyl groups in 28 S rRNA of *Xenopus laevis* and man. Clustering in the conserved core of molecule. *J Mol Biol 201*:289–314.
Maden BE. 1990. The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol 39*:241–303.
Maden BE, Corbett ME, Heeney PA, Pugh K, Ajuh PM. 1995. Classical and novel approaches to the detection and localization of the numerous modified nucleotides in eukaryotic ribosomal RNA. *Biochimie 77*:22–29.
Massenet S, Ansmant I, Motorin Y, Branlant C. 1999. The first determination of pseudouridine residues in 23S ribosomal RNA from hyperthermophilic Archaea *Sulfolobus acidocaldarius*. *FEBS Lett 462*:94–100.
Maxwell ES, Fournier MJ. 1995. The small nucleolar RNAs. *Annu Rev Biochem 64*:897–934.
Moazed D, Noller HF. 1989. Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell 57*:585–597.
Nissen P, Hansen J, Ban N, Moore PB, Steitz TA. 2000. The structural basis of ribosome activity in peptide bond synthesis. *Science 289*:920–930.
Nordhoff E, Cramer R, Karas M, Hillenkamp F, Kirpekar F, Kristiansen K, Roepstorff P. 1993. Ion stability of nucleic acids in infrared matrix-assisted laser desorption/ionization mass spectrometry. *Nucleic Acids Res 21*:3347–3357.
O'Connor M, Dahlberg AE. 1993. Mutations at U2555, a tRNA-protected base in 23S rRNA, affect translational fidelity. *Proc Natl Acad Sci USA 90*:9214–9218.
Ofengand J, Bakin A. 1997. Mapping to nucleotide resolution of pseudouridine residues in large subunit ribosomal RNAs from representative eukaryotes, prokaryotes, archaebacteria, mitochondria and chloroplasts. *J Mol Biol 266*:246–268.
Ofengand J, Bakin A, Wrzesinski J, Nurse K, Lane BG. 1995. The pseudouridine residues of ribosomal RNA. *Biochem Cell Biol 73*:915–924.
Ofengand J, Fournier MJ. 1998. The pseudouridine residues of rRNA: Number, location, biosynthesis and function. In: Grosjean H, Benne R, eds. *Modification and editing of RNA*. Washington, DC: American Society for Microbiology Press. pp 229–253.
Omer AD, Lowe TM, Russell AG, Ebhardt H, Eddy SR, Dennis PP. 2000. Homologs of small nucleolar RNAs in Archaea. *Science 288*:517–522.
Østergaard P, Phan H, Johansen LB, Egebjerg J, Østergaard L, Porse BT, Garrett RA. 1998. Assembly of proteins and 5 S rRNA to transcripts of the major structural domains of 23 S rRNA. *J Mol Biol 284*:227–240.
Porse BT, Garrett RA. 1995. Mapping important nucleotides in the peptidyl transferase center of 23 S rRNA using a random mutagenesis approach. *J Mol Biol 249*:1–10.
Rodriguez-Fonseca C, Phan H, Long KS, Porse BT, Kirillov SV, Amils R, Garrett RA. 2000. Puromycin-rRNA interaction sites at the peptidyl transferase center. *RNA 6*:744–754.
Rozenski J, Crain PF, McCloskey JA. 1999. The RNA Modification Database: 1999 update. *Nucleic Acids Res 27*:196–197.
Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Sirum-Connolly K, Peltier JM, Crain PF, McCloskey JA, Mason TL. 1995. Implications of a functional large ribosomal RNA with only three modified nucleotides. *Biochimie 77*:30–39.
Smith CM, Steitz JA. 1997. Sno storm in the nucleolus: New roles for myriad small RNPs. *Cell 89*:669–672.
Smith JE, Cooperman BS, Mitchell P. 1992. Methylation sites in *Escherichia coli* ribosomal RNA: Localization and identification of four new sites of methylation in 23S rRNA. *Biochemistry 31*: 10825–10834.
Stern S, Moazed D, Noller HF. 1988. Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension. *Methods Enzymol 164*:481–489.
Veldman GM, Klootwijk J, de Regt VC, Planta RJ, Branlant C, Krol A, Ebel JP. 1981. The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Res 9*:6935–6952.
Vester B, Douthwaite S. 1994. Domain V of 23S rRNA contains all the structural elements necessary for recognition by the ErmE methyltransferase. *J Bacteriol 176*:6999–7004.
Vester B, Nielsen AK, Hansen LH, Douthwaite S. 1998. ErmE methyltransferase recognition elements in RNA substrates. *J Mol Biol 282*:255–264.
Welch M, Chastang J, Yarus M. 1995. An inhibitor of ribosomal peptidyl transferase using transition-state analogy. *Biochemistry 34*:385–390.
Yusupov MM, Yusupova GZ, Baucom A, Lieberman K, Earnest TN, Cate JH, Noller HF. 2001. Crystal structure of the ribosome at 5.5 Å resolution. *Science 292*:883–896.