FEBS Letters 462 (1999) 94–100 FEBS 22957

The first determination of pseudouridine residues in 23S ribosomal RNA from hyperthermophilic *Archaea Sulfolobus acidocaldarius*

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Received 16 October 1999

Abstract We describe the first identification of pseudouridine (Ψ) residues in ribosomal RNA (23S rRNA) of an hyperthermophilic *Archaea Sulfolobus acidocaldarius*. In contrast to *Eucarya* rRNA, only six Ψ residues were detected, which is rather close to the situation in *Bacteria*. However, three modified positions (Ψ_{2479}, Ψ_{2535} and Ψ_{2550}) are unique for *S. acidocaldarius*. Two Ψ residues at positions 2060 and 2594 are universally conserved, while one other Ψ (position 2066) is also common to *Eucarya*. Taken together the results argue against the conservation of Ψ-synthases between *Archaea* and *Bacteria* and provide a basis for the search of snoRNA-like guides for Ψ formation in *Archaea*.

Key words: Pseudouridine; rRNA; SnoRNA; Pseudouridine-synthase; *Archaea*; *Sulfolobus acidocaldarius*

1. Introduction

The rRNAs and tRNAs of all organisms and the *Eucarya* UsnRNAs examined so far undergo extensive post-transcriptional modification (for review see [1–4]). The most frequent chemical modifications in rRNAs are methylations of the 2′-hydroxyl of the ribose moieties (2′-*O*-methylation) and isomerisations of uracil residues into pseudouridines (Ψ). The Ψ residues in rRNA of the large ribosomal subunit (LSU) have been mapped for several of species from the *Eucarya* and *Bacteria* domains (*Homo sapiens*, *Mus musculus*, *Saccharomyces cerevisiae*, *Escherichia coli*, etc.), for one *Archaea* (*Halobacterium halobium*), and also for mitochondria and chloroplasts of several *Eucarya* cells [5–10]. The number of Ψ residues per rRNA molecule varies widely from *Bacteria* and *Archaea* to *Eucarya* (from nine Ψ residues in *E. coli* LSU rRNA to 55 Ψ residues in its human counterpart). However, all detected Ψ residues are concentrated in three domains of the LSU rRNA (domains II, IV and V). These domains are located at or are functionally linked to the peptidyl transferase centre (PTC) ([5,6,11]; for review see [2]). Despite the clustering of Ψ residues in the functional site of the ribosome, the exact importance of these Ψ residues in ribosomal assembly and peptidyl transferase reaction remains unknown. Several Ψ sites present in LSU rRNAs are evolutionarily conserved. The situation is quite different for the Ψ residues present in the rRNA of the small ribosomal subunit (SSU). In this rRNA, the Ψ residues are mostly present at sites specific for each organism and no apparent clustering of these modified nucleotides in defined areas of SSU rRNA was observed (for review see [2])

Synthesis of Ψ residues in RNA molecules is catalysed by a large family of enzymes, the RNA:Ψ-synthases. Recent progress in studies of Ψ residue formation demonstrated that *Bacteria* and *Eucarya* cells do not share the same process to modify U residues in rRNAs. Indeed, in *Bacteria*, Ψ formation is ensured by site- or multisite-specific rRNA:Ψ-synthases. Several such enzymes have recently been characterised in *E. coli* and *Bacillus subtilis* [12–18]. Two multisite-specific *E. coli* enzymes, RluC and RluD, catalyse Ψ formation at six distinct sites in 23S rRNA (positions 955, 2504 and 2580, and 1911, 1915 and 1917, respectively) [14–16]. Three other Ψ-synthases, RluB in *B. subtilis* and RsuA and RluE in *E. coli*, each modify a unique U residue into a Ψ residue [13,18,19]. The first discovered rRNA:Ψ-synthase in *E. coli*, RluA, displays a dual substrate specificity, since it is implicated in the formation of Ψ_{746} in 23S rRNA and of Ψ_{32} in tRNAs [12,17].

In *Eucarya*, pre-rRNA maturation involves many different small nucleolar RNAs (snoRNAs) of two different classes: the H/ACA and the C/D snoRNAs. Most of these H/ACA and C/D snoRNAs are implicated in selection of sites of Ψ formation and 2′-*O*-methylations, respectively, by site-specific base pairing with the pre-rRNA (for review see [2,4]). Specific H/ACA snoRNA guides have been found for at least 23 pseudouridylation sites out of 30 in yeast 26S rRNAs [20] and, in most cases, the direct implication of the corresponding snoRNA in modification at a given site was proved experimentally [21,22]. In yeast, this snoRNA-guided modification system comprises several protein components and a unique Ψ-synthase, Cbf5p [23,24]. *S. cerevisiae* Cbf5p and the tRNA:Ψ_{55}-synthase Pus4p are both homologous to the *E. coli* tRNA:Ψ_{55}-synthase TruB [23,25]. Cbf5p counterparts have been previously reported for a variety of *Eucarya*, such as *Kluyveromyces lactis* [26], *Drosophila melanogaster* (Nop60p) [27], rat (NAP 57) [28] and human (DKC1) [29].

Although the *Archaea* and *Bacteria* appear rather similar in terms of general genome organisation, some of the *Archaea* genes show greater sequence similarity to *Eucarya* than to *Bacteria* counterparts (for review see [30]). Up to now, it is not known whether the *Archaea* domain also possesses RNA-guided modification systems. Several observations argue for the existence of only C/D-type RNPs, but not for H/ACA-type RNPs in *Archaea* [31]. Indeed, the *S. cerevisiae* C/D snoRNAs are associated with proteins Nop1p (fibrillarin in

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Abbreviations: Ψ, pseudouridine; nt, nucleotides; RT, reverse transcriptase; CMCT, *N*-cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimide metho-*p*-toluolsulfonate; PTC, peptidyl transfer centre; LSU, large ribosomal subunit; SSU, small ribosomal subunit

0014-5793/99/$20.00
PII: S0014-5793(99)01524-0

human), Nop56p and Nop58p [32–35]. A gene homologous to the yeast *NOP1* gene was detected in three methanogenic *Archaea* [36,37], and a homologue of the yeast *NOP58* gene was found in the *Archaea Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum* and *Methanococcus jannaschii* [31,37–39]. The estimated number of 2′-*O*-methylations in *Sulfolobus solfataricus* LSU rRNA (about 44 sites) [40] is much higher than that found in *Bacteria* (four sites in *E. coli* LSU rRNA), and quite similar to that observed in *Eucarya* (55 sites in yeast LSU rRNA, for review see [4]). This huge number of 2′-*O*-methylation sites in *Archaea* rRNAs suggests that this type of post-transcriptional modification in *Archaea* may be RNA-guided like in *Eucarya*. As far as rRNA pseudouridylation is concerned, the situation with *Archaea* seems to be different. H/ACA snoRNA guides directing pseudouridylation of rRNA in *S. cerevisiae* are found to be associated with the Gar1p protein [24,41–43] and no homologous protein has been reported up to now in *Archaea* [31]. In fact, the only observation in favour of an RNA-guided pseudouridylation system in *Archaea* is the finding of an open reading frame, conserved in all studied *Archaea* genomes, that shows 62% sequence similarity with Cbf5p and only 29% with TruB [24]. The authors concluded that the *Archaea* protein is most likely a Cbf5p and not a TruB homologue.

Thus, the question remains open about the existence of a RNA-guided system for Ψ synthesis in *Archaea* rRNAs. To search for the presence of genes encoding H/ACA-like guide RNAs in the *Archaea* genomes, one has first to identify the positions of Ψ residues in rRNAs from these organisms. Up to now, the location of Ψ residues in LSU rRNAs was determined for only one halophilic *Archaea* (*H. halobium*) [10]. In this paper we report the mapping of Ψ residues in the LSU rRNA of a hyperthermophile *Archaea*, *Sulfolobus acidocaldarius*. Six Ψ residues were detected in the studied rRNA regions, which is slightly higher than the 3.7 ± 0.1 mol/mol of Ψ residues detected by chromatography/mass spectrometry in the *S. sulfolobus* LSU rRNA [40] and the four Ψ residues localised in the *H. halobium* LSU rRNA. Only a few modification positions are conserved between *Bacteria* and *Archaea*, while the others seem to be specific for *Archaea* or common to *Archaea* and *Eucarya*. These observations will be discussed in terms of the evolution of Ψ-synthase activities.

2. Materials and methods

2.1. RNA preparation

S. acidocaldarius cells were a generous gift of P. Forterre (Orsay, France). The method of RNA preparation was adapted from Aiba et al. [44], 0.3–0.6 g of cells were washed with 10 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.2 buffer and centrifuged for 10 min at 4000 rpm. Cells were resuspended in 300 μl of 1 mM EDTA, 0.5% SDS, 20 mM sodium acetate pH 5.5 buffer, treated with 300 μl of phenol, incubated for 10 min at 65°C and centrifuged. The extraction was done twice, recovered RNAs were treated with equal volumes of chloroform/isoamyl alcohol (24:1) and ethanol precipitated in the presence of 0.3 M NaCl.

2.2. Mapping of Ψ residues in the S. acidocaldarius LSU rRNA

The *N*-cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimide metho-*p*-toluolsulfonate (CMCT) modification protocol was adapted from Bakin and Ofengand [8], with the modification described previously [45]. Modification by CMCT was performed with 10 μg of *S. acidocaldarius* total RNA.

Positions of CMCT modifications were identified by primer extension analysis, using the AMV RT (Life Science, USA), in the conditions described by Mougin et al. [46]. The oligonucleotides complementary to the following regions of the *S. acidocaldarius* LSU rRNAs were used as primers: for domain II, nucleotides (nt) 1121–1139 (primer 1), for domain IV, nt 2158–2178 (primer 2) and nt 2072–2091 (primer 3) and for domain V, nt 2575–2592 (primer 4), nt 2638–2656 (primer 5) and nt 2736–2751 (primer 6). The hybridisation conditions were optimised to improve the yield of reverse transcrip-

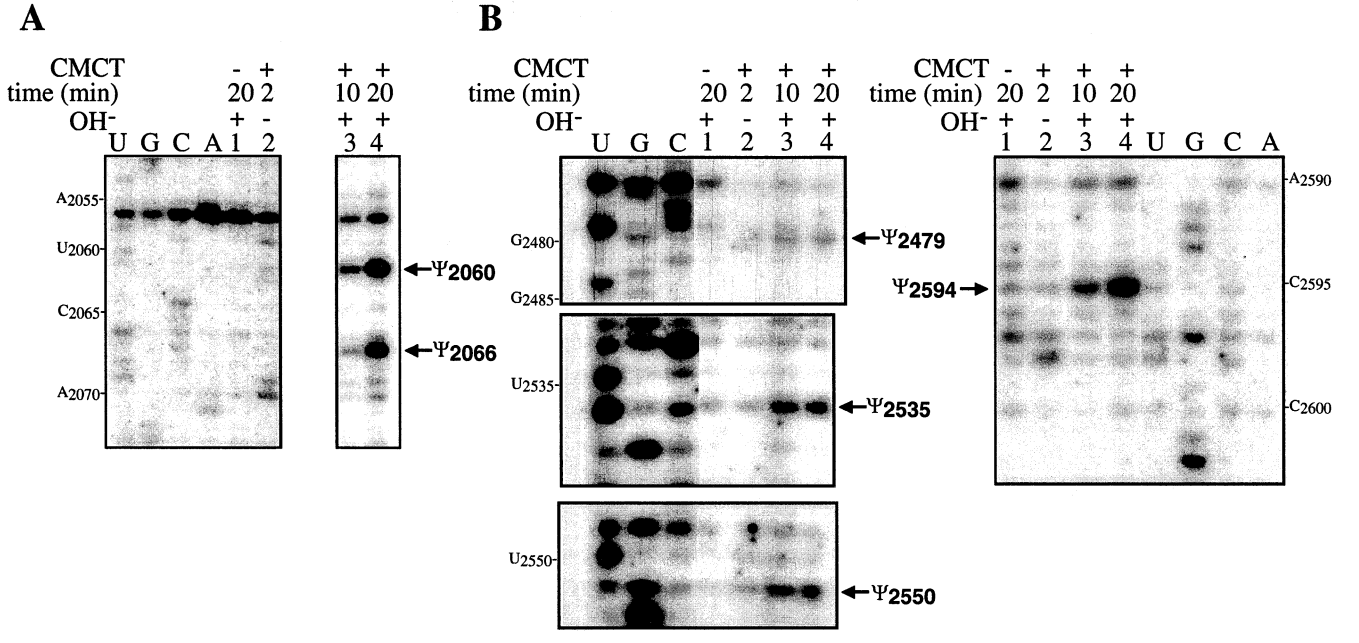

Fig. 1. Primer extension analysis of Ψ residues in domains IV (A) and V (B) of the *S. acidocaldarius* LSU rRNA. Modification of total *S. acidocaldarius* RNA by CMCT was done for 2, 10 and 20 min (lanes 2, 3 and 4, respectively). Experimental conditions were as described in Section 2. In lanes 3 and 4, the CMCT-modified RNA was subjected to alkaline treatment at pH 10.4. A control extension experiment was made without CMCT treatment (lane 1). Lanes U, G, C and A correspond to the RNA sequencing ladder. The reverse transcription stops, in lanes 3 and 4, indicate the presence of Ψ residues at these positions and are shown by arrows.

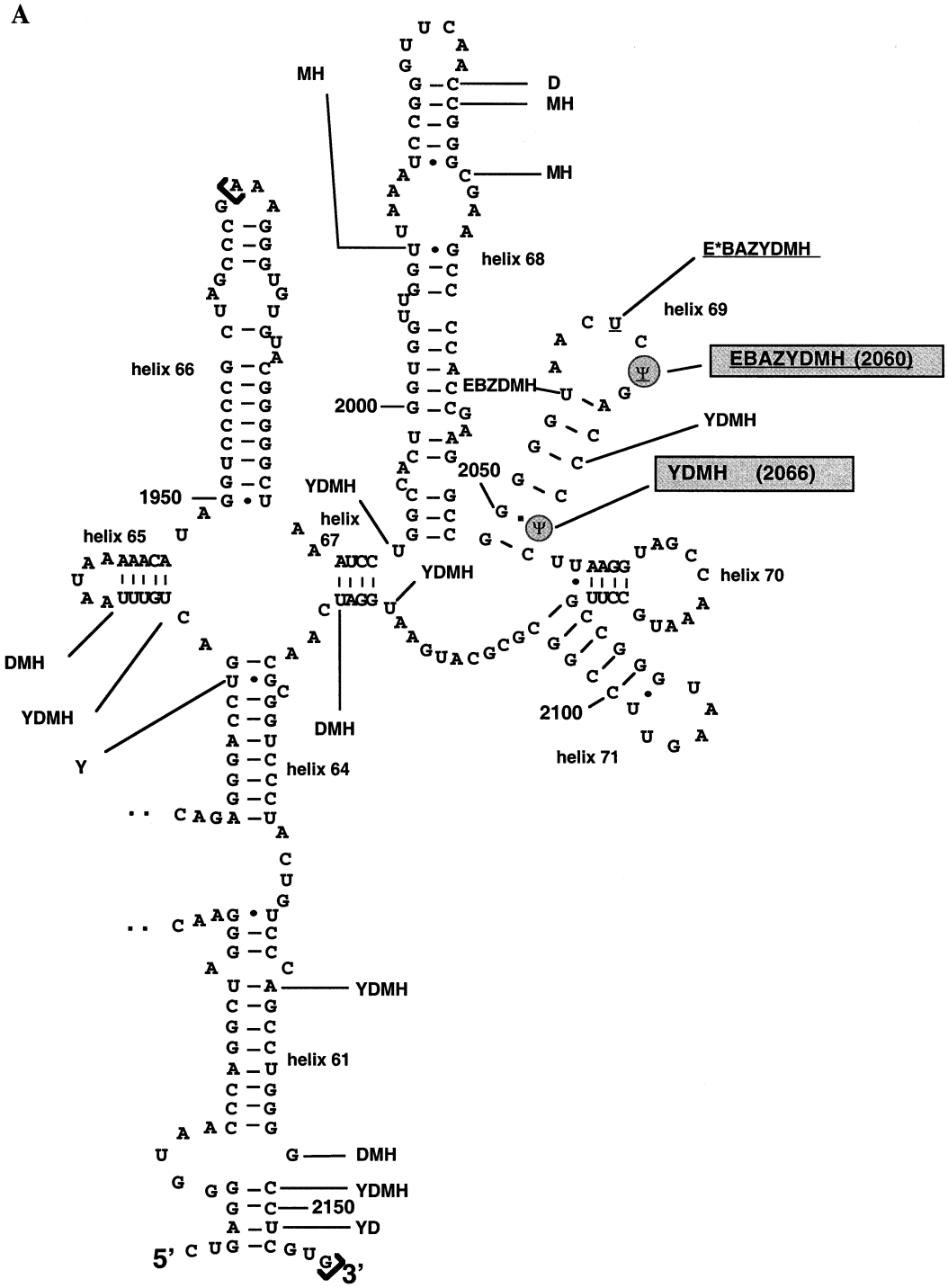

Fig. 2. Location of Ψ residues in domains IV (A) and V (B) of the *S. acidocaldarius* LSU rRNA and comparison with the locations of Ψ residues in LSU rRNAs of other species. The sequence shown is that of *S. acidocaldarius* [59]. The six Ψ residues detected in this study are shown in grey. The positions of the Ψ residues found in other organisms are indicated by: E, *E. coli*; B, *B. subtilis*; A, *H. halobium*; Y, *S. cerevisiae*; D, *D. melanogaster*; M, *M. musculus*; H, *H. sapiens*; Z, *Zea mays* chloroplast; Ym, Mm, Tm and Hm, *S. cerevisiae*, *M. musculus*, *Trypanosoma brucei* and *H. sapiens* mitochondria, respectively (for review see [2]). The asterisk (*) indicates an N^3-methyl Ψ residue [48]. Nucleotide positions, starting from the 5′-terminal nucleotide of *S. acidocaldarius* LSU rRNA, are indicated. The regions analysed by primer extension analysis are between brackets.

tion. Primers 1, 2 and 4 were annealed for 10 min at 65°C followed by a 10 min incubation in ice, while oligonucleotides 3, 5 and 6 were hybridised for 30 s at 94°C followed by incubations for 5 min at 65°C and 5 min at 55°C. Primers were 5′-end labeled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase. RNA sequencing was done with 40 μg of total *S. acidocaldarius* RNA.

3. Results and discussion

3.1. General strategy

Mapping Ψ residues in the *S. acidocaldarius* LSU rRNA was done on a total RNA fraction using the chemical method

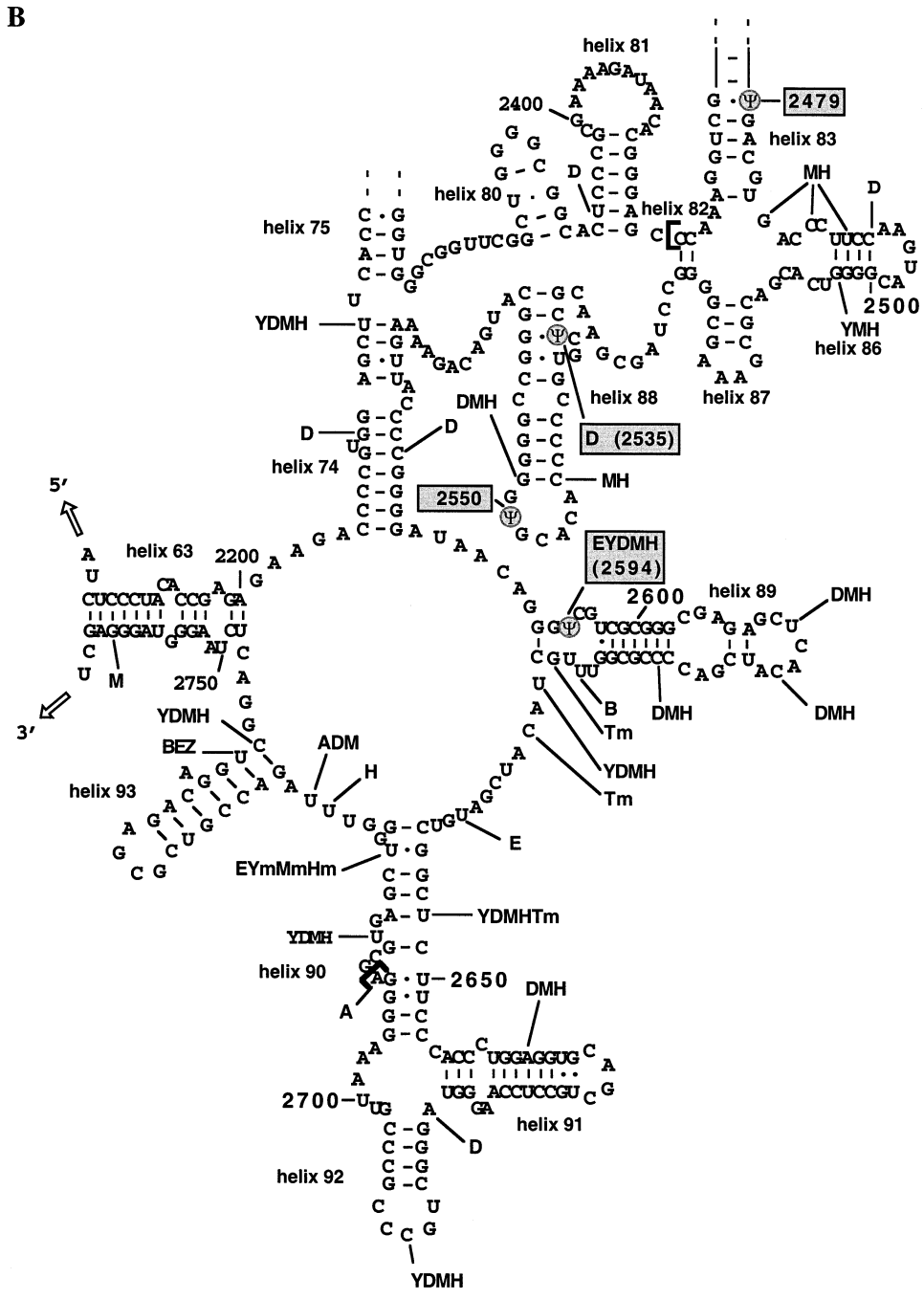

Fig. 2 (*continued*).

based on the alkaline-resistant modification of Ψ residues by CMCT, followed by their detection by primer extension analysis [8]. Due to the high GC content of *S. acidocaldarius* LSU rRNA, the specific conditions of hybridisation had to be determined for each oligonucleotide primer (see Section 2). The Ψ mapping in *S. acidocaldarius* LSU rRNA was done for the LSU rRNA regions that were found to contain most of the Ψ residues in the other species studied ([5,6,8–10]; for review see [2]). Thus, three regions were analysed by CMCT treatment followed by primer extension analysis: nt 1000–1120 in domain II, 1966–2155 in domain IV and 2419–2708 in domain V.

The results of primer extension analysis show that the region 1000–1120 in domain II of *S. acidocaldarius* 23S rRNA does not contain Ψ residues. It is noteworthy that *E. coli* is the only prokaryote found to have two Ψ residues in this domain [5,10]. Domains II of *B. subtilis* and *H. halobium* LSU rRNA were found to be devoid of Ψ residues [10].

3.2. Two Ψ residues are found in S. acidocaldarius domain IV 23S rRNA

We detected two Ψ residues in domain IV of the *S. acidocaldarius* 23S rRNA, at positions 2060 and 2066, respectively (Fig. 1A). Fig. 2A shows the location of these two Ψ residues in *S. acidocaldarius* domain IV as compared to locations of Ψ residues found in other species. All the pseudouridylation sites

detected in domain IV of the LSU rRNAs from *S. acidocaldarius*, *H. halobium* and *Bacteria* are clustered in a single stem-loop structure (numbered 69 according to the nomenclature of [47]. In contrast, in the cytoplasmic LSU rRNA from *Eucarya*, Ψ residues are more widely distributed in domain IV (Fig. 2A). The two Ψ residues underlined in Fig. 2A were found in all studied LSU rRNAs, except the mitochondrial ones, whose domain IV does not contain Ψ residues [10] and, as shown by the present data, a U residue is present at position 2058 in *S. acidocaldarius* LSU rRNA. The activity required to convert U_{2058} into a Ψ residue is probably absent in *S. acidocaldarius*. In *E. coli*, the Ψ residue corresponding to the *S. acidocaldarius* U_{2058} is a hypermodified N^3-methyl Ψ residue [48]. The presence of this residue generates a strong RT stop upon primer extension analysis, even in the absence of CMCT treatment [8]. This is not the case for position 2058 in the *S. acidocaldarius* LSU rRNA. CMCT clearly reacts with uridine residue at this position (Fig. 1A, lane 2) and the CMCT modification is alkaline-labile (Fig. 1A, lanes 3 and 4). Interestingly, the second Ψ residue detected in stem-loop structure 69 of the *S. acidocaldarius* LSU rRNA was found only in *Eucarya* species (*S. cerevisiae*, *D. melanogaster*, *M. musculus* and *H. sapiens*, Fig. 2A). The presence of a Ψ residue at this position was never detected in LSU rRNAs from *Bacteria* or *Archaea*. Finally, the third Ψ residue present in stem-loop structure 69 of *E. coli* and *B. subtilis*, which is rather well conserved in *Eucarya* LSU rRNAs (position 1911 in *E. coli*), is not detected in *S. acidocaldarius* (position 2054). Since unmodified U residue is present at this position in *S. acidocaldarius* rRNA this means that the corresponding enzymatic activity is absent. Clearly, stem-loop structure 69 is the target for Ψ-synthases in all living organisms. However, positions of modifications vary between *Bacteria*, *Archaea* and *Eucarya*. They vary even from one *Archaea* to the other. It should be pointed out that in *E. coli*, stem-loop structure 69 of domain IV was shown to be located at the active site of the 50S subunit. It is protected against nuclease degradation upon association of the 30S and 50S subunits [49] and, as shown by cross-linking of the *E. coli* 23S rRNA segment from position 1912 to 1920 (corresponding to nt 2055–2063 in *S. acidocaldarius*) to the decoding centre of 16S rRNA [50], it is juxtaposed to the decoding centre of the 30S subunit. These regions of 23S and 16S rRNAs are not only close in space, but also functionally related, since fidelity of codon recognition is disturbed upon mutation at the positions in *E. coli* 23S rRNA corresponding to positions 2057 and 2059 in the *S. acidocaldarius* 23S rRNA (Fig. 2A) [51]. In addition, chemical footprinting of 23S rRNA revealed a protection of the bases from stem-loop structure 69 by the tRNA located at the P site [52]. The strong phylogenetic conservation of one of the Ψ residues in the terminal loop of stem-loop structure 69 suggests a functional importance of this modification for ribosome function.

3.3. Four Ψ residues are found in S. acidocaldarius domain V 23S rRNA

The analysis of a large region of the *S. acidocaldarius* domain V reveals the presence of four pseudouridylation sites at positions 2479, 2535, 2550 and 2594 (Fig. 1B). The strong RT stops observed at the positions 2535, 2550 and 2594 suggest a complete conversion of U into Ψ. In contrast, based on the low intensity of the RT stop at position 2479 (Fig. 1B), the pseudouridylation at this position may be partial. As found for domain IV, pseudouridylation sites in domain V are tightly clustered in *Bacteria*l LSU rRNAs, whereas the numerous Ψ residues found in domain V of the *Eucarya* LSU rRNAs are rather dispersed in the entire domain (Fig. 2B) ([10], for review see [2]). Four Ψ residues were detected in domain V of the *E. coli* LSU rRNA. They are clustered in the area expected to play an important role for the peptidyl transferase activity [8]. Only one of these four Ψ residues is present in domain V of the *S. acidocaldarius* LSU rRNA (position 2594). Modification at this position is highly conserved in *Bacteria*, *Archaea* and *Eucarya*. This conserved Ψ residue is located at or very close to the peptidyl transferase centre. Indeed, it is found in stem-loop structure 89, proposed to be implicated in ribosomal subunit interaction, decoding function and peptidyl transferase activity ([51,53]; for review see [54,55]). It is possible that this conserved Ψ residue is implicated in one of these ribosomal functions.

The three other Ψ residues that we detected in the *S. acidocaldarius* LSU rRNA are located in another area of domain V (positions 2479–2550) (Figs. 1B and 2B). Based on studies in *E. coli*, this part of domain V is likely to be functionally important. It should be in close contact with the 3′ end of the tRNA at the A-site, since the terminal loop of stem-loop structure 80 (Fig. 2B), containing a Gm residue in *E. coli*, was proposed to base pair with the CCA at the 3′ extremity of tRNAs [56]. Two out of the three Ψ residues detected in this area of domain V are specific for the *S. acidocaldarius* LSU rRNA (positions 2479 and 2550) and one was found only in *D. melanogaster* LSU rRNA (position 2535) (see Fig. 2B).

3.4. Which kind of Ψ-synthases can form the detected Ψ residues?

The total number of Ψ residues detected in *S. acidocaldarius* LSU rRNA is quite similar to that found for *Bacteria* like *E. coli* or *B. subtilis*. However, their location shows differences as compared to that in *Bacteria*. Among the Ψ residues that we detected in the *S. acidocaldarius* LSU rRNA, two are highly conserved in *Bacteria* and *Eucarya* (positions 2594 and 2060), one of them was also detected in *H. halobium* ($Ψ_{2060}$) (for review see [2]). The third Ψ residue detected in the *S. acidocaldarius* LSU rRNA ($Ψ_{2066}$) is highly conserved in *Eucarya* but absent in *Bacteria*. Finally, among the three remaining ones, two have never been detected in any other species ($Ψ_{2479}$ and $Ψ_{2550}$) and one ($Ψ_{2535}$) was detected only in *D. melanogaster*. In spite of this variability of modified positions, all the Ψ residues detected in *S. acidocaldarius* LSU rRNA are concentrated in the parts of the LSU rRNA domains IV and V, where Ψ residues were detected in the *Bacteria* and *Eucarya* LSU rRNAs. Hence, in all *Bacteria* and *Archaea* species studied, the same regions are the target of Ψ-synthase activities. However, the locations of modified positions are variable. How can this situation be explained in relation to Ψ-synthase specificities?

In *E. coli*, the three Ψ residues in loop 69 and its border (positions equivalent to positions 2054, 2058 and 2060 in *S. acidocaldarius*) are synthesised by the same Ψ-synthase RluD [14,16]. Since only one or two of these Ψ residues are found in *S. acidocaldarius* and *H. halobium*, respectively, the corresponding *Archaea* Ψ-synthases should differ in their substrate specificity as compared to the *E. coli* RluD enzyme. In *E. coli*, deletion of the *RLUD* gene was found to result in a severe

growth inhibition [16]. Based on our observation of the strong conservation of the modification at position 2060 (*S. acidocaldarius* numbering), the growth defect observed in the *E. coli rluD*-deleted strain may be due to the absence of Ψ formation at the position corresponding to Ψ_{2060}. However, one cannot exclude the possibility that RluD protein catalyses the formation of other essential Ψ residues or performs some other critically important functions unrelated to its Ψ-synthase activity.

In human, formation of Ψ residues in loop 69 is guided by the H/ACA snoRNA U19. This single RNA is predicted to direct Ψ formation at two sites equivalent to *S. acidocaldarius* U_{2058} and Ψ_{2060} [57]. Thus, if the formation of this residue in *S. acidocaldarius* is RNA-guided like in *Eucarya*, this RNA should lack the ability to direct Ψ synthesis at position 2058.

The formation of Ψ residue at position 2594 in *E. coli* LSU rRNA is predicted to be catalysed by the Ψ-synthase RluE [19] and is guided by the H/ACA snoRNAs snR34 in yeast or U65 in human. These two guide RNAs are also involved in the formation of a second Ψ residue at the position equivalent to U_{2648} in *S. acidocaldarius* [22]. Thus, again, if *S. acidocaldarius* Ψ formation in LSU rRNA was RNA-guided, the guide RNA would differ from that in *Eucarya*, directing Ψ formation only at one position. In spite of the strong conservation of the modification at position 2594, it should be noted that deletion of the snR34 gene has no effect on growth [58]. Taking individually post-transcriptional modifications of rRNAs were not found to be essential. However, they may bring selective advantages explaining their conservation.

The comparison of the Ψ sites common for *E. coli* and *S. acidocaldarius* shows that only one Ψ-synthase (RluE) may be conserved between *Bacteria* and *Archaea*. However, the homology search, based on the motif II sequence conserved in all the known Ψ-synthases [19], detected only two putative Ψ-synthase genes in *Archaea* genomes, in contrast to 10 enzymes in *E. coli*. In addition, the two *Archaea* proteins found in this way are members of the TruA or TruB families, acting on tRNAs at positions 38–40 and 55 respectively. No genes related to the RluA family, to which RluE belongs, were detected. In other words, based on the present data and current knowledge, the Ψ-synthase activities responsible for the Ψ formation in *Archaea* LSU rRNA seem to be different from those characterised in *Bacteria*. Either these activities are carried by purely protein enzymes that differ in specificity from those found in *Bacteria* or the specificity of the *Archaea* enzyme depends upon RNA guides. If such RNAs are present they must be quite less numerous than those found in *Eucarya*. Furthermore, they should be different among *Archaea*, since *H. halobium* and *S. acidocaldarius* share only one common modified position. Using the characteristic features of *Eucarya* snoRNA guides, one may now search for putative RNA guides for Ψ synthesis in the genomes of *S. acidocaldarius* and *H. halobium*.

Acknowledgements: This work was supported by laboratory funds from the Ministère de la Recherche et de l'Enseignement supérieur and the French Centre National de la Recherche Scientifique. S. Massenet and I. Ansmant are pre-doctoral fellows supported by a fellowship from the Ministère de la Recherche et de l'Enseignement supérieur. Y. Motorin is supported by an Associated Researcher position at the CNRS ('Poste Rouge'). P. Forterre is acknowledged for providing us with *S. acidocaldarius* cells.

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