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Deficiency of the tRNA^{Tyr}:Ψ35-synthase aPus7 in Archaea of the Sulfolobales order might be rescued by the H/ACA sRNA-guided machinery

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ABSTRACT

Up to now, Ψ formation in tRNAs was found to be catalysed by stand-alone enzymes. By computational analysis of archaeal genomes we detected putative H/ACA sRNAs, in four Sulfolobales species and in *Aeropyrum pernix*, that might guide Ψ35 formation in pre-tRNA^{Tyr}(GUA). This modification is achieved by Pus7p in eukarya. The validity of the computational predictions was verified by *in vitro* reconstitution of H/ACA sRNPs using the identified *Sulfolobus solfataricus* H/ACA sRNA. Comparison of Pus7-like enzymes encoded by archaeal genomes revealed amino acid substitutions in motifs IIIa and II in Sulfolobales and *A. pernix* Pus7-like enzymes. These conserved RNA:Ψ-synthase-motifs are essential for catalysis. As expected, the recombinant *Pyrococcus abyssi* aPus7 was fully active and acted at positions 35 and 13 and other positions in tRNAs, while the recombinant *S. solfataricus* aPus7 was only found to have a poor activity at position 13. We showed that the presence of an A residue 3′ to the target U residue is required for *P. abyssi* aPus7 activity, and that this is not the case for the reconstituted *S. solfataricus* H/ACA sRNP. In agreement with the possible formation of Ψ35 in tRNA^{Tyr}(GUA) by aPus7 in *P. abyssi* and by an H/ACA sRNP in *S. solfataricus*, the A36G mutation in the *P. abyssi* tRNA^{Tyr}(GUA) abolished Ψ35 formation when using *P. abyssi* extract, whereas the A36G substitution in the *S. solfataricus* pre-tRNA^{Tyr} did not affect Ψ35 formation in this RNA when using an *S. solfataricus* extract.

INTRODUCTION

In all domains of life, pseudouridine residues (Ψ) are the most frequent post-transcriptionally modified residues in RNAs. They are universally found in ribosomal RNA (rRNAs) and in tRNAs (1,2). U to Ψ conversions are catalysed either by stand-alone enzymes [specific RNA:Ψ-synthases, (3)] or by small ribonucleoprotein particles [H/ACA snoRNPs or H/ACA scaRNPs in eukarya, and H/ACA sRNPs in Archaea, (4)]. H/ACA RNPs contain a small RNA that defines the targeted U residue by base-pair interaction with the RNA substrate (5–8). Archaeal and eukaryal H/ACA RNPs contain 4 proteins: Nop10, Gar1, L7ae/Nhp2p and Cbf5/Dyskerin (9). CBF5 belongs to the TruB family of RNA:Ψ-synthases. Whereas aCBF5 alone has no activity on rRNAs (5–8), recent data showed an *in vitro* activity of aCBF5 at position 55 in tRNAs. This activity does not require the presence of a guide RNA (10–12).

The additional free N_1-H of the Ψ nucleobase allows the formation of an additional hydrogen bond, either in *cis*, within the RNA molecule, or in *trans*, with another RNA molecule or a protein. For instance, residue Ψ35 in the eukaryal cytoplasmic tRNA^{Tyr}(GUA) allows the formation of an hydrogen bond with the O2′ of residue U33, which stabilizes the anticodon loop structure (13,14). Furthermore, substitution of the C-N bond between the ribose and the nucleobase by a C-C bond limits the flexibility of the ribose-phosphate backbone and favours RNA-RNA base-pair interactions (13–15).

In all organisms, pseudouridylations were found to occur at numerous positions in tRNAs (seven in *Escherichia coli*, at least 15 in *Saccharomyces cerevisiae* and even more in higher eukaryotes) (1). Formation of residue Ψ55 in the TΨC loop is the most frequent

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pseudouridylation in tRNAs. It is likely involved in the recognition of elongator tRNA at the A site of ribosomes. Pseudouridylations are also frequently found at tRNA positions 13, 27, 28 and 39 (1). As it was found in all eukaryal $tRNA^{Tyr}$(GUA) and not in bacterial $tRNA^{Tyr}$(GUA), residue Ψ35 in the anticodon loop of $tRNA^{Tyr}$(GUA) is considered as an eukaryal conserved specific modification. It plays an important role in mRNA decoding in eukarya (16,17). Identification of modified residues in archaeal RNAs, especially tRNAs, is far less advanced than in bacteria and eukarya. Studies mainly focused on archaeal tRNA-specific post-transcriptional modifications, such as methylations (m^1I58, Gm18) and archaeosine (18–20). For some hyperthermophilic Archaea, global profiles of post-transcriptional modifications in tRNAs were studied by the HPLC/MS approach (2). However, the locations of the modified residues were not defined. The full set of tRNA molecules was only sequenced for the mesophilic euryarchaeote *Haloferax volcanii* (1,21) and this halophilic organism may not be representative of the whole archaeal domain, since this domain includes organisms growing in a large variety of conditions (22). However, the data obtained for the complete set of *Halobacterium volcanii* tRNAs and for a few tRNAs of other archaeal species, show that U to Ψ conversions occur at several positions in archaeal tRNAs.

Up to now, in bacteria and eukarya, all pseudouridylations in tRNAs were shown to be catalysed by stand-alone enzymes and most of the tRNA:Ψ-synthases in *E. coli* and in *S. cerevisiae* were identified (for review, 3,23). Some of them have quite narrow substrate specificities, as is the case for the bacterial TruB, TruD and TruC enzymes (modification at a single position for each of them, 55, 13 and 65, respectively) (24–26). The yeast Pus4p, Pus8p (cytoplasmic) and Pus9p (mitochondrial) enzymes also have narrow specificities (positions 55 for Pus4p and 32 for Pus8p and Pus9p) (27–29). In contrast, the TruA bacterial tRNA:Ψ-synthases acts at several sites in tRNAs (30), and RluA modifies both tRNAs and rRNAs (31). The eukaryal Pus1p and Pus7p enzymes also have very broad specificities. Pus1p acts at eight distinct positions in tRNAs (32,33) and at position 44 in U2 snRNA (34). Pus7p converts U into Ψ in U2 snRNA (position 35) (35), several tRNAs (position 13), the pre-$tRNA^{Tyr}$(GUA) (position 35) (36) and 5S rRNA (position 50) (37).

The number of rRNA:Ψ-synthases is highly reduced in eukarya compared to bacteria, since several stand-alone specific rRNA:Ψ-synthases are replaced by the H/ACA snoRNP system. Altogether, 11 and 10 distinct RNA:Ψ-synthases were characterized in *E. coli* and in *S. cerevisiae*, respectively. Five of them act on tRNAs in *E. coli* (24–26,30,31), while eight of them act on tRNAs in *S. cerevisiae* (27–29,33,36,38,39).

Studies on RNA:Ψ-synthases in archaea are far less advanced. One puzzling observation is the detection of only a very limited number of putative genes for RNA:Ψ-synthases in archaeal genomes, when taking into account the known signatures of RNA:Ψ-synthases. The only putative genes, which are found in all species, encode a TruD/Pus7 homologue, aCBF5 (TruB homologue) and PusX/Pus10 which can both act at position 55 in tRNAs (9–12). In euryarchaeal species an additional gene encoding a member of the TruA family was also detected (9). The activities of the TruD/Pus7 and the TruA family members have not been studied in details.

According to the present knowledge on pseudouridylations in archaeal tRNAs, the most frequently modified positions are 13, 39, 54 and 55. *In vitro* tests made with a *Pyrococcus furiosus* cellular extract and *H. volcanii* on *S. cerevisiae* tRNA transcripts revealed the presence of tRNA:Ψ-synthase activities acting at positions 39 and 55 (40). As mentioned above, aCBF5 and/or Pus10 might be the catalyst(s) at position 55. Pus10 was also recently found to be the catalyst for Ψ formation at position 54 (41). The enzymes acting at the other positions still remain to be identified. Based on the specificities identified for the *E. coli* TruD enzyme and its yeast and plant Pus7 counterparts, archaeal Pus7-like enzymes may catalyze modification at position 13 in some tRNAs and perhaps at position 35 in $tRNAs^{Tyr}$(GUA) which are expressed with an intron. Indeed, modification at this position in $tRNA^{Tyr}$(GUA) was found to be intron dependent in eukarya (42,43). Therefore, the absence of Ψ35 in mature $tRNA^{Tyr}$(GUA) in the *H. volcanii* pre-$tRNA^{Tyr}$(GUA) was up to now expected to be explained by the absence of intron in this tRNA (21). Finally, based on the limited number of putative RNA:Ψ-synthases found in archaea and the detection of tRNA specific C/D sRNPs in archaea (44), one can reasonably ask the question whether there may also be some tRNA-specific H/ACA sRNPs in archaea explaining why, in spite of a limited number of RNA:Ψ-synthases, archaeal tRNAs contain several Ψ residues. An alternative explanation can be a very broad specificity of tRNA:Ψ-synthases in these organisms.

Here, by a computational search in archaeal genomes, using a recently developed approach (45), we found H/ACA sRNAs which may guide Ψ35 formation in the pre-$tRNA^{Tyr}$(GUA). However, these H/ACA sRNAs were only found in species of the Sulfolobales and Desulfurococcales orders. We demonstrated their activity by *in vitro* reconstitution of the H/ACA sRNPs. On the other hand, comparison of the amino acid sequences of Sulfolobales and Desulfurococcales aPus7 enzymes with those of other archaeal species revealed amino acid substitutions in the active site. We verified the defect of activity of the Sulfolobales enzymes by production of recombinant aPus7 enzymes from *Pyrococcus abyssi* and *Sulfolobus solfataricus*. Interestingly, we found that the recombinant *P. abyssi* aPus7 enzyme has a broad specificity *in vitro*. It modifies tRNAs at position 13 and at other positions as well as intron-less and intron-containing $tRNAs^{Tyr}$(GUA) at position 35. To get more information on the catalyst acting at position 35 in tRNAs in *P. abyssi* and *S. solfataricus*, we compared the activity of *P. abyssi* and *S. solfataricus* extracts on WT and A36G variants of the *P. abyssi* $tRNA^{Tyr}$(GUA) and *S. solfataricus* pre-$tRNA^{Tyr}$(GUA), respectively.

MATERIALS AND METHODS

DNA constructs for the production of aPus7 proteins and their variants

The DNA fragments coding the WT aPus7 proteins from *P. abyssi* and *S. solfataricus* were obtained by PCR amplification using genomic DNAs from these two species. The forward and reverse primers used for amplification generated a NheI and BamHI restriction site, respectively. After digestion with NheI and BamHI, the amplified DNA was inserted into plasmid pET28b digested by the same nucleases. The variants aPus7 proteins were produced by site-directed mutagenesis (QuickChange kit, Stratagene, USA). The sequences of the oligonucleotides used for the PCR amplifications and site-directed mutagenesis are available on request.

Purification of recombinant proteins

Wild-type and variant aPus7 proteins were produced in *E. coli* as His_6-tagged protein fusions and purified by affinity chromatography on Ni–NTA agarose (Qiagen, France). Induction of protein production was done by addition of 1 mM IPTG in the LB media supplemented by 50 μg/ml of kanamycin. Cell pellets were suspended in Lysis buffer (20 mM Tris–HCl, pH 7.7; 300 mM NaCl; 1 mM DTT; 10 mM Imidazole) and sonicated. After removal of cell debris, the resulting cell-free extracts were directly loaded onto Ni–NTA agarose. After a washing step with 20 mM imidazole in Lysis buffer, His_6-tagged proteins were eluted in Lysis buffer containing 250 mM of imidazole. Purified proteins were stored at room temperature in the elution buffer supplemented with 10% glycerol, or at –80°C by adjusting the glycerol concentration to 50%.

The *P. abyssi* aCBF5, aNOP10, aGAR1 and L7Ae were produced as GST fusion proteins as previously described (7). They were stored at –80°C in the following buffer, 50 mM Tris–HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol.

In vitro transcription of tRNAs, H/ACA sRNA and the sRNP target RNA

Wild-type and mutated $tRNA^{Asp}$(GUC) and $tRNA^{Tyr}$(GUA) from *P. abyssi* and WT and mutated $tRNA^{Asp}$(GUC) and pre-$tRNA^{Tyr}$(GUA) from *S. solfataricus* as well as the Sso1 H/ACA sRNA from *S. solfataricus* were produced by *in vitro* transcription of linearized plasmids. The DNA templates coding for those tRNAs and sRNA were amplified by PCR using genomic DNAs of the *P. abyssi* strain GE5 or the *S. solfataricus* strain P2. Forward primers used for amplification bear the sequence for a T7 RNA polymerase promoter. Amplified products were cloned in plasmid pUC18 using its SmaI restriction site. Linearization was performed with the BstNI restriction enzyme. $tRNA^{Asp}$(GUC) and $tRNA^{Tyr}$(GUA) variants were produced by site-directed mutagenesis (QuickChange kit, Stratagene, USA). All *in vitro* produced RNA transcripts were purified by electrophoresis on an 8M urea 8% polyacrylamide gel. For the pseudouridylation assays, the corresponding RNA transcripts were uniformly labelled with [α–^{32}P]ATP or other [α–^{32}P]NTPs. Conditions for transcription and labelling were described previously (46).

Preparation of cellular extracts

About 10^{10} cells from *P. abyssi* or *S. solfataricus* grown to exponential phase and centrifuged, were re-suspended in the extraction buffer (25 mM Tris–HCl, pH 7,5; 25 mM KCl; 5 mM $MgCl_2$; 10% Glycerol; 2 mM DTT). The cells were sonicated for 5 min followed by centrifugation at 10 000 *g*. The concentration of glycerol was adjusted to 20% for storage at –20°C.

In vitro pseudouridylation assay

The tRNA:Ψ35-synthase activity of purified recombinant aPus7 enzymes or H/ACA sRNP complexes was measured by the nearest-neighbour approach in the conditions described previously (7,46). The sRNA guided activity of reconstituted H/ACA sRNPs was tested by mixing of ∼4 pmol of Sso1 sRNA with ∼150 fmol of the [α-^{32}P]ATP-labelled tRNA in buffer D (20 mM HEPES, pH 7.9, 150 mM KCl; 1.5 mM MgCl2; 0.2 mM EDTA) along with aCBF5, aNOP10, aGAR1 and L7Ae proteins at a 200 nM concentration (7). The non-RNA-guided reaction of aPus7 enzymes on tRNA was tested on ∼50 fmol of [α-^{32}P]ATP-labelled tRNAs in buffer Psi (100 mM Tris–HCl, pH 8.0; 100 mM $AcNH_4$; 5 mM $MgCl_2$; 2 mM DTT; 100 μM EDTA). The activity assays with cell-free extracts were performed with the same amount of labelled tRNA in buffer D in the presence of a similar amount of *P. abyssi* and *S. solfataricus* cellular extracts. For both the sRNA-guided and non RNA-guided reactions, samples were incubated at 55°C, 65°C or 80°C and the reaction was started by addition of the proteins (200 nM each). Activity assays with cellular extracts were carried out at 65°C. Then, the RNAs were extracted and digested by RNAse T2. The resulting 3′-phosphate-mononucleotides were fractionated by 2D thin-layer cellulose chromatography (47). The radioactivity in the spots was quantified using the ImageQuant software after exposure of a Phosphorimager screen. The amount of Ψ residue formed was determined taking into account the total number of U residues in the tRNA.

Mapping of pseudouridylation sites in tRNAs

Pseudouridine mapping in RNAs was done using modification by *N*-cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimide metho-*p*-toluolsulfonate (CMCT) followed by an alkaline treatment for specific modification of Ψ residues (48). Modifications were performed with 0.5 μg of *in vitro* transcribed *S. solfataricus* pre-$tRNA^{Tyr}$. The modified residues were identified by primer extension with reverse transcriptase using the AMV RT (QBiogene, USA) in the conditions previously described (49). The 5′-labelled primer used was complementary to the 14 nts at the 3′-end of the tRNA. RNA sequencing was done in parallel using 4 μg of *in vitro* transcribed RNA.

RESULTS

Some archaeal H/ACA sRNAs are predicted to guide Ψ formation in tRNAs

By applying the computational method that we recently developed for the search of H/ACA sRNA genes in archaeal genomes (45), we found five putative H/ACA sRNA motifs, which might direct U to Ψ conversion at position 35 in pre-tRNAs^{Tyr}(GUA) that contain an intron (Figure 1A). One of these unusual H/ACA motifs was detected in a species of the Desulfurococcales order, *Aeropyrum pernix* (sRNA Ape7), the four other ones were found in four species of the Sulfolobales order, namely *S. solfataricus* (sRNA Sso1), *Sulfolobus tokodaii* (sRNA Sto1), *Sulfolobus acidocaldarius* (sRNA Sac1) and *Metallosphaera sedula* (sRNA Mse2). These five putative H/ACA motifs fitted to the rules established based on our recent exhaustive analysis of the *P. abyssi* H/ACA sRNAs (45): they all contain a K-turn structure which is linked to the pseudouridylation pocket by a 5- or 6-bp long helix 2. They also contain the conserved ACA trinucleotide at the 3′-end of the stem–loop structure (Figure 1A). Interestingly, each DNA sequences coding the Sso1, Sto1 and Sac1 H/ACA motifs is directly flanked by a DNA sequence corresponding to a C/D box motif. In *S. solfataricus* this C/D motif corresponds to the already characterized Sso-159 C/D box sRNA (50). The yet uncharacterized C/D motifs detected in *S. tokodaii* and *S. acidocaldarius* were denoted Sto-sR4 and Sac-sR39, respectively. By using a DNA probe complementary to the Sso1 H/ACA motif, we verified the presence of the Sso1 H/ACA sRNA in total RNA extracted from *S. solfataricus* cells. The northern blot analysis (Figure 1B) revealed the presence of a long form of the RNA that likely contains both the H/ACA and the C/D motifs, and a short form likely corresponding to the H/ACA motif alone. Therefore, the Sso1 H/ACA sRNA is expressed in *S. solfataricus*.

The predicted RNA base-pair interactions between the five putative H/ACA motifs detected and their predicted pre-tRNA^{Tyr}(GUA) targets involved both the anticodon stem–loops and the intron sequences (Figure 1C). These putative interactions fitted to the criteria that we recently established for efficient H/ACA sRNA–target RNA interaction (45). Indeed, as found in *Pyrococcus* species or in *Thermococcus kodakarensis*, the distance between the targeted U residue and the ACA motif is of 14 nt and a stable base-pair interaction is formed between the 3′-guide sequence and the target RNA (Figure 1C). Noticeably, the predicted interactions of the pre-tRNAs^{Tyr}(GUA) with the 3′ guide sequences of the Sac1 and the Mse2 H/ACA motifs are exceptionally long as compared to the previously characterized interactions (7,8,11,50–52).

Based on computational analysis using the RNAMOT program (45), no other possible target sites in rRNAs or tRNAs were found for the Mse2, Sso1, Sto1 and Sac1 H/ACA motifs. However, the Ape7 motif might guide pseudouridylation at position 2444 in 23S rRNA, as previously found for the *P. abyssi* Pab91 H/ACA sRNA (7). Therefore, we concluded that the Mse2, Sso1, Sto1 and Sac1 H/ACA motifs likely guide pseudouridylation in pre-tRNA^{Tyr}(GUA) and this may also be the case for the Ape7 H/ACA motif.

The reconstituted H/ACA Sso1 sRNP catalyses Ψ35 formation in pre-tRNA^{Tyr} transcripts

For an experimental confirmation of the pseudouridylation guiding capability of the identified H/ACA motifs, we used the *in vitro* reconstitution assay established for archaeal H/ACA sRNPs (46). Taking into account the strong sequence conservation of both the H/ACA sRNP proteins and the H/ACA sRNA characteristic features in archaea, we hypothesized that an H/ACA sRNP reconstituted by using the Sso1 H/ACA RNA and recombinant *P. abyssi* H/ACA sRNP proteins produced in *E. coli* may be active. EMSA experiments, indeed confirmed the association of an Sso1 H/ACA RNA transcript with the recombinant *P. abyssi* L7Ae (L), aCBF5 (C), aNOP10 (N) and aGAR1 (G) proteins. The association patterns were similar to those previously obtained for the *P. abyssi* Pab91 sRNA (7) (data not shown). Therefore, as the target U residue in the *S. solfataricus* (Sso) pre-tRNA^{Tyr}(GUA) was followed by an A residue (Figure 2A), we tested the activity of the reconstituted particles by the nearest neighbour approach, using an [α-^{32}P]ATP-labelled Sso pre-tRNA^{Tyr}(GUA) transcript (46). Digestion by RNase T2 allowed the detection of Ψp residues released from ΨA dinucleotide sequences. As a control, an assay was also performed in the absence of Sso1 RNA (Figure 2B). Fractionation by 2D thin-layer chromatography of the resulting 3′-phosphate mononucleotides showed that, in spite of the presence of several UA dinucleotides in the Sso pre-tRNA^{Tyr}(GUA), the ratio of U to Ψ conversion in this RNA in the presence of the reconstituted sRNP was close to 1 mole/mole of RNA (Figure 2B). In addition, no modification was obtained in the absence of Sso1 RNA. These data were in agreement with the expected U to Ψ conversion at position 35 in the transcript. To complete the demonstration, we repeated the experiment on an Sso pre-tRNA^{Tyr}(GUA) with a U to C mutation at position 35. As expected, no U to Ψ conversion was detected in this RNA (Figure 2B). We also verified that the U to Ψ conversion in the WT Sso pre-tRNA^{Tyr}(GUA) occurred at position 35 by CMCT–RT analysis. This is illustrated by detection of an RT-stop at position 36 in the primer extension analysis (Figure 2C). Therefore, we concluded that in association with *P. abyssi* H/ACA sRNP proteins, the Sso1 H/ACA RNA is capable to guide U to Ψ conversion at position 35 in an *in vitro* transcribed pre-tRNA^{Tyr}(GUA).

Archaeal species with a putative tRNA-specific guide sRNA show sequence variations in the Pus7-like enzyme

As mentioned in the introduction, U to Ψ conversion at position 35 in pre-tRNA^{Tyr}(GUA) is catalysed by the Pus7 stand-alone enzyme in yeast (36). By genomic sequence analysis we found putative genes for aPus7-like enzymes in the archaeal species whose genomes were completely sequenced. When the amino acid sequences of the highly conserved motifs I, II and III of RNA:Ψ-synthases were aligned for all the putative aPus7-like proteins

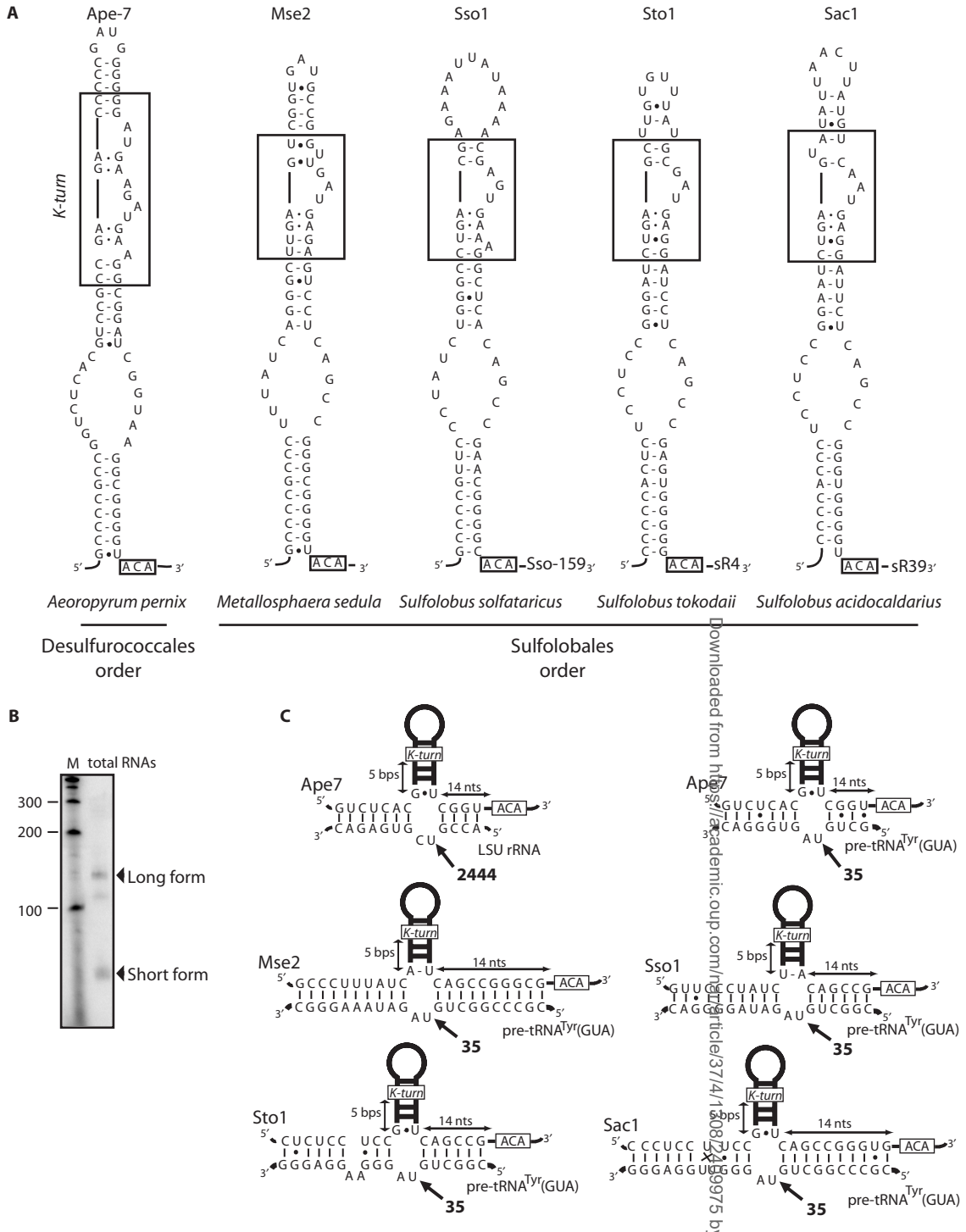

Figure 1. Putative H/ACA sRNA guides for Ψ35 modification in pre-tRNA^{Tyr}(GUA) found in Sulfolobales species and in *A. pernix*. (**A**) Structures of the putative H/ACA sRNAs involved in Ψ35 formation in the pre-tRNA^{Tyr}(GUA) of *A. pernix*, *M. sedula*, *S. solfataricus*, *S. tokodaii* and *S. acidocaldarius*. Characteristic K-turns and H/ACA sequences are boxed. The identity of the C/D box sRNAs present at the 3′ extremities of the Sso1 (Sso-159), Sto1 (sR4) and Sac1 (sR39) H/ACA sRNAs are indicated. (**B**) Northern blot analysis of Sso1 sRNA expression in *S. solfataricus*. Total RNA was fractionated by electrophoresis in an 8% polyacrylamide gel containing 8 M urea, transferred to Nylon membrane (Hybond N+, GE Healthcare, USA) and hybridized with an Sso1 sRNA-specific ^{32}P-labelled oligonucleotide. Sizes of molecular weight markers are given on the left of the autoradiogram. Arrows indicate the positions of the short-form RNA which may correspond to the free Sso1 H/ACA sRNA (H/ACA) and of the full-length transcript probably containing the 3′ terminal C/D box sRNA (H/ACA + C/D). (**C**) Proposed base-pair interactions of the Ape7, Mse2, Sso1, Sto1 and Sac1 H/ACA sRNAs with their proposed target pre-tRNAs^{Tyr}(GUA), and potential interaction between the Ape7 sRNA and its 23S rRNA target. An arrow indicates the site of U to Ψ conversion. The distance between the pseudouridylation pocket and the K-turn and that between the pseudouridylation pocket and the ACA box are shown.

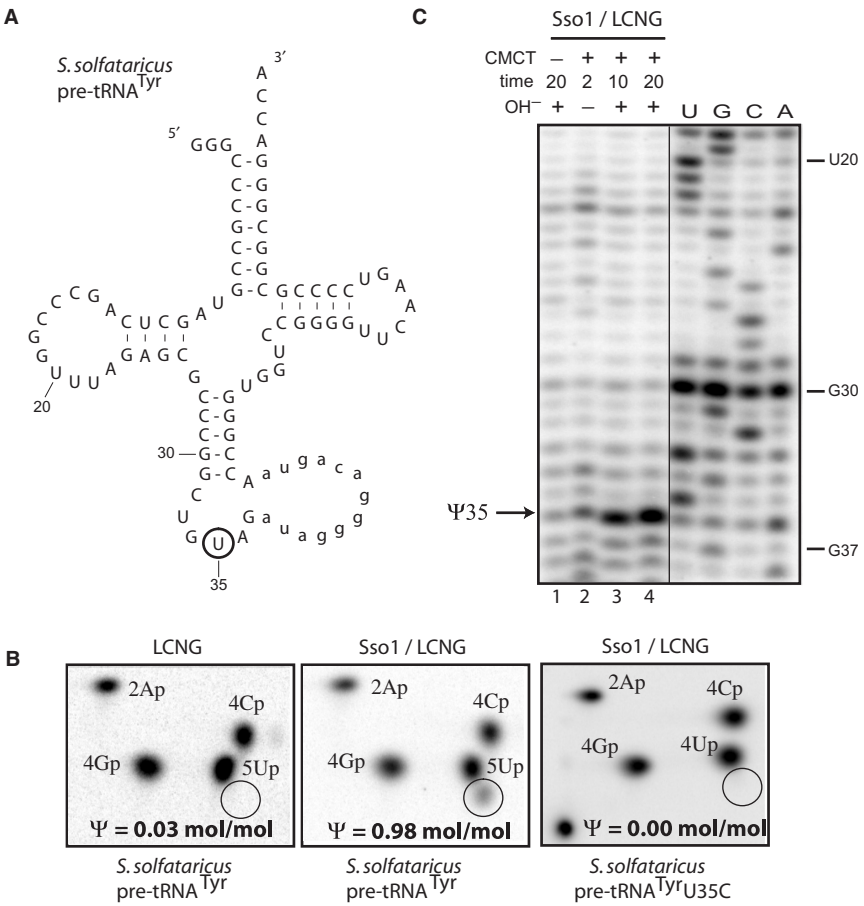

Figure 2. *In vitro* Sso1 sRNA-guided Ψ35 formation in the Sso pre-tRNA^{Tyr}(GUA). (**A**) 2D structure of the *S. solfataricus* pre-tRNA^{Tyr}(GUA). The targeted U35 residue is circled. The sequence of the intron present in the anticodon loop is represented by small letters. (**B**) *In vitro* reconstitution of the Sso1 H/ACA sRNP and test of its activity at position Ψ35 in Sso pre-tRNA^{Tyr}(GUA). [α-^{32}P]ATP-labelled pre-tRNA^{Tyr} (GUA) or its U35C variant was incubated for 90 min at 65°C in the presence of the L7Ae, CBF5, NOP10 and GAR1 recombinant proteins each at a 200 nM concentration (LCNG), in the presence (Sso/LCNG) or the absence (LCNG) of the Sso1 H/ACA sRNA. Modified transcripts were then digested by RNase T2 and the resulting 3′-NMPs were analysed by 2D TLC (47). Positions corresponding to the pseudouridine spot are circled on the autoradiograms. The 3′-NMP composition of the RNase T2 digest, as well as the molar amount of Ψ residue formed by mole of RNA are also indicated on the 2D TLC autoradiograms. (**C**) CMCT–RT mapping of the Ψ residue formed upon treatment of the *S. solfataricus* pre-tRNA^{Tyr}(GUA), in conditions allowing the reconstitution of an active Sso1 H/ACA sRNPs (Sso1/LCNG). The modified RNA was treated by CMCT, for 2, 10 and 20 min with (+) or without (–) subsequent alkaline treatment (OH$^-$) and analysed by primer extension. A control experiment was performed in the absence of CMCT treatment. Lanes U, G, C and A correspond to the sequencing ladders obtained with the same oligonucleotide. An arrow shows the position of the RT stop corresponding to Ψ35.

(Figure 3A), we found a strong conservation of these motifs in the archaeal Pus7-like enzymes, except for the enzymes of the four Sulfolobales and the *A. pernix* species that contain putative tRNA-specific H/ACA sRNAs. These sequence divergences concern motifs II and III. Motif II contains the catalytic Asp residue (53) (Figure 3A and B). It also contains a conserved pair of Arg-His residues (54). The catalytic Asp residue is found in motifs II of the four Sulfolobales species and *A. pernix*, but the Arg-His pair (R78-H79 in *P. abyssi*) is replaced by an Ala(or Thr)-Asn (or Cys) pair (A90/N91 in *S. solfataricus*) (Figure 3A and B). Substitution of the Arg-His pair in motif II by other residues is also observed in a few other archaeal species that do not contain a tRNA specific H/ACA sRNA. However, in these species the highly conserved amino acids of motif IIIa are present, especially the conserved basic residue (*N. maritimus* and *C. symbosium*) which is replaced by an isoleucine in the four Sulfolobales species studied (Ile in *S. solfataricus*) and in *A. pernix* (Figure 3A). Furthermore, other amino acid substitutions are also observed in motifs II and IIIa of these five species. Altogether, these observations suggested an altered activity or specificity of the aPus7-like enzymes in *S. solfataricus*, *S. tokodaii*, *S. acidocaldarius*, *M. sedula* and *A. pernix*.

A pre-tRNA^{Tyr}(GUA):Ψ35-synthase activity is present in *S. solfataricus* cellular extracts

As no Ψ35 had yet been detected in archaeal pre-tRNA^{Tyr}(GUA) or tRNA^{Tyr}(GUA), the first question was to know whether such modification can occur in archaea. We failed to analyse directly the cellular Sso tRNA^{Tyr}(GUA) by CMCT treatment in total RNA,

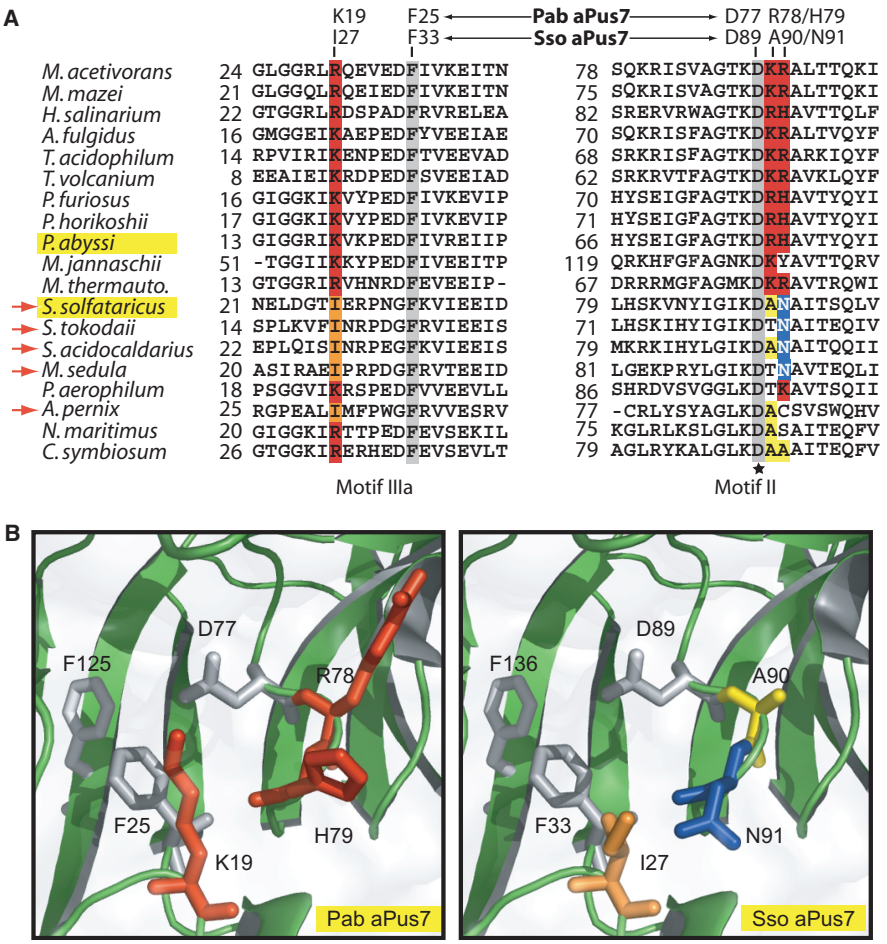

Figure 3. Presence of unusual Pus7-like enzymes in Sulfolobales species and *A. pernix*. (**A**) Multiple sequence alignment of Pus7 proteins from various archaeal species. Only the regions of motif IIIa, and motif II which contains the catalytic D residue, are shown. Different colours are used to highlight each of the conserved residues in motifs IIIa and II. The names of the organisms are indicated on the left, red arrows show species that contain a pre-tRNA^{Tyr}-specific H/ACA sRNA. (**B**) 3D structure modelization of the Pab aPus7 (left) and Sso aPus7 (right) active sites. Modelization was done by using the crystal structure of *M. mazei* TruD (PDB 1Z2Z). Only highly conserved amino acids in the catalytic sites of RNA:Ψ-synthases are represented in the Pab and Sso aPus7 models, the K27I, R90A and H91N substitutions in Sso aPus7 can be seen.

because of the difficulty to find an efficient and specific primer for extension analysis. Therefore, we tested whether a pre-tRNA^{Tyr}(GUA):Ψ35-synthase activity exists in *S. solfataricus* extracts. To this end, the *in vitro* transcribed Sso pre-tRNA^{Tyr}(GUA) and its U35C variant, both labelled by [α-^{32}P] ATP incorporation, were incubated for 90 min at 65°C in a *S. solfataricus* cellular extract. Then, by using the same approaches as above, namely RNase T2 digestion followed by 2D thin-layer chromatography, we detected the formation of 0.60 mol of Ψ residue per mole of RNA in the WT pre-tRNA, whereas no modification was detected in the U35C pre-tRNA variant (Figure 4, top panels). Formation of a Ψ residue at position 35 in the WT Sso pre-tRNA^{Tyr} substrate in the Sso extract was also verified by CMCT-RT analysis (data not shown). Importantly, when using this extract, no Ψ formation was detected in the heterologous *P. abyssi* (Pab) tRNA^{Tyr}(GUA), which is naturally synthesized without intron (Figure 4). In contrast, when the same kind of experiment was performed using a *P. abyssi* cellular extract and the *in vitro* transcribed WT or U35C variant Pab tRNA^{Tyr}(GUA), 0.77 mol of Ψ residues was formed per mole of WT Pab tRNA^{Tyr}(GUA) and this amount was reduced to 0.42 mol of Ψ/mol of RNA after U35C substitution (Figure 4, bottom panels). Presence of spots corresponding to several modified residues others than Ψ residues, in particular m^{1}G37, Cm56, m^{1}I57 and m^{1}A58 were detected in these experiments performed with total cellular extracts. This was an indication for the high quality of these extracts in terms of RNA modification activities. Hence, taken together, the data demonstrated that a specific pre-tRNA^{Tyr}(GUA):Ψ35-synthase activity exists in *S. solfataricus*, while in *P. abyssi* cells, at least one or more RNA:Ψ-synthases capable to modify tRNA^{Tyr} at position 35 and at one or more positions are present (one of the possible additional modified position in this tRNA was position 13). Although we did not prove directly the presence of Ψ35 residues in tRNAs^{Tyr} extracted from *P. abyssi* and *S. solfataricus*, we demonstrated the presence of catalysts able to achieve this modification in cellular extracts from these two species. This was a strong indication for the presence of a tRNA:Ψ35-synthase activity in these two species.

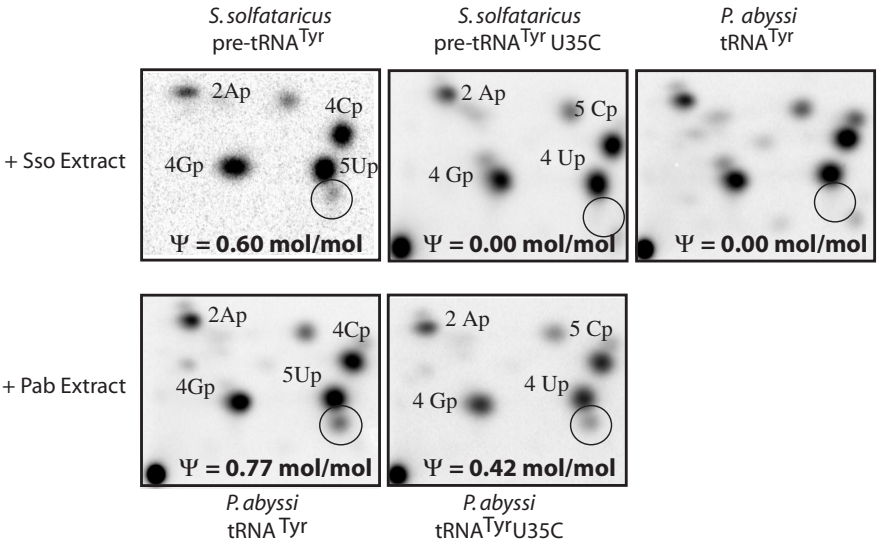

Figure 4. Test of the activity of Sso and Pab cellular extracts on the Sso pre-tRNA^{Tyr}(GUA) and Pab tRNA^{Tyr}(GUA). The [α-^{32}P]ATP-labelled Sso pre-tRNA^{Tyr} (GUA) and Pab tRNA^{Tyr}(GUA) together with their U35C variants were incubated for 90 min at 65°C in the presence of a Sso or Pab cellular extracts. Formation of residue Ψ35 was tested as described in the legend to Figure 2B, by 2D-TLC analysis after digestion of the RNA by the RNase T2. The molar amounts of Ψ residue formed per mole of RNA are indicated on the autoradiograms of the five 2D-TLCs. Additional spots visible on the 2D-TLC autoradiograms correspond to formation of methylated nucleotides naturally present in many archaeal tRNAs (m^{1}G37, Cm56, m^{1}I57 and m^{1}A58). Reproducible results were obtained in three independent experiments.

In contrast to Pab Pus7-like, the Sso Pus7-like fails to form Ψ35 in the Sso pre-tRNA^{Tyr} (GUA) *in vitro*

As mentioned above, the Sso Pus7-like enzyme contains the catalytic Asp residue in motif II. Therefore, despite the amino acid substitutions found in this motif and motif IIIa, we could not exclude the possibility that it carries the pre-tRNA^{Tyr}(GUA):Ψ35-synthase activity which was detected in the extract. To test for this possibility, the recombinant *S. solfataricus* (Sso) Pus7-like enzyme and, as a control the *P. abyssi* (Pab) Pus7-like enzyme, were produced in *E. coli* as His$_6$-tagged fusions and purified. The activity of the two recombinant proteins were tested on *in vitro* transcribed Sso pre-tRNA^{Tyr}(GUA) and Pab tRNA^{Tyr}(GUA) (Figure 5A) that were both labelled by incorporation of [α-^{32}P]ATP. In order to preserve the tRNA and pre-tRNA 2D and 3D structures, enzymatic activities were first tested by incubation for 1 h at 55°C of the RNA substrates with the recombinant enzymes (conditions are given in 'Materials and methods' secion). When the Sso Pus7-like recombinant enzyme was used, no Ψ formation was detected, as well in the Sso pre-tRNA^{Tyr}(GUA) as in the Pab tRNA^{Tyr}(GUA) (Figure 5B). In contrast, the Pab Pus7-like recombinant enzyme formed average amounts of 1.47 and 1.91 mol of Ψ residue per mole of Sso pre-tRNA^{Tyr} and Pab tRNA^{Tyr}, respectively (mean values of three distinct experiments). When a U to C substitution was generated at position 35 in these RNAs, the above average values were reduced to 0.71 and 0.97, respectively, indicating that modification did occur at position 35 and also at one or more positions (Figure 5B).

As both *P. abyssi* and *S. solfataricus* grow at high temperatures (98°C and 80°C, respectively), we tested whether the activity of the *P. abyssi* enzyme was stable at 80°C. However, after 1 h of incubation at this temperature the tRNA and pre-tRNA structures were likely unfolded, we were aware that this unfolding will probably modify the Pab Pus7 RNA:Ψ-synthase specificity. Indeed, 3.47 mol of Ψ/mol of Sso pre-tRNA^{Tyr}(GUA) were formed in these conditions (Figure 5B). The five UA dinucleotides in this RNA were probably all modified to some extent in the absence of RNA 3D and 2D structures. In contrast, even at this high temperature no modification was formed in this tRNA when using the Sso Pus7-like recombinant enzyme (Figure 5B).

Altogether, the data revealed the absence of activity of the Sso Pus7-like recombinant enzyme at position 35 in the Sso pre-tRNA^{Tyr}(GUA). More generally, this enzyme was not active on any of the UA dinucleotides in this pre-tRNA at any of the conditions tested. In contrast, our data demonstrated the capability of the Pab Pus7-like recombinant enzyme to modify position 35 in both intron-containing and intron-less tRNAs^{Tyr}. In addition, upon unfolding of the RNA structure this enzyme could modify a large number of sites in RNAs.

Like eukaryal enzymes the Pab Pus7-like recombinant enzyme modifies tRNAs at both positions 13 and 35

As the yeast Pus7 enzyme catalyzes Ψ13 formation in some cytoplasmic tRNAs, in particular the tRNA^{Asp}(GUC), we tested whether the Sso and Pab Pus7-like enzymes can catalyse Ψ13 formation in the *in vitro* transcribed Sso tRNA^{Asp}(GUC), Pab tRNA^{Asp}(GUC) and the Pab tRNA^{Tyr}(GUA) that all contain a U residue at position 13 (Figure 5A). Here again, in order to preserve the RNA structure, incubation of transcripts labelled by [α-^{32}P]ATP incorporation with the recombinant proteins were performed at 55°C. We found that the recombinant Pab Pus7-like enzyme catalyses the formation of average amounts of 1.08 and 1.15 mol of Ψ/mol of WT Pab

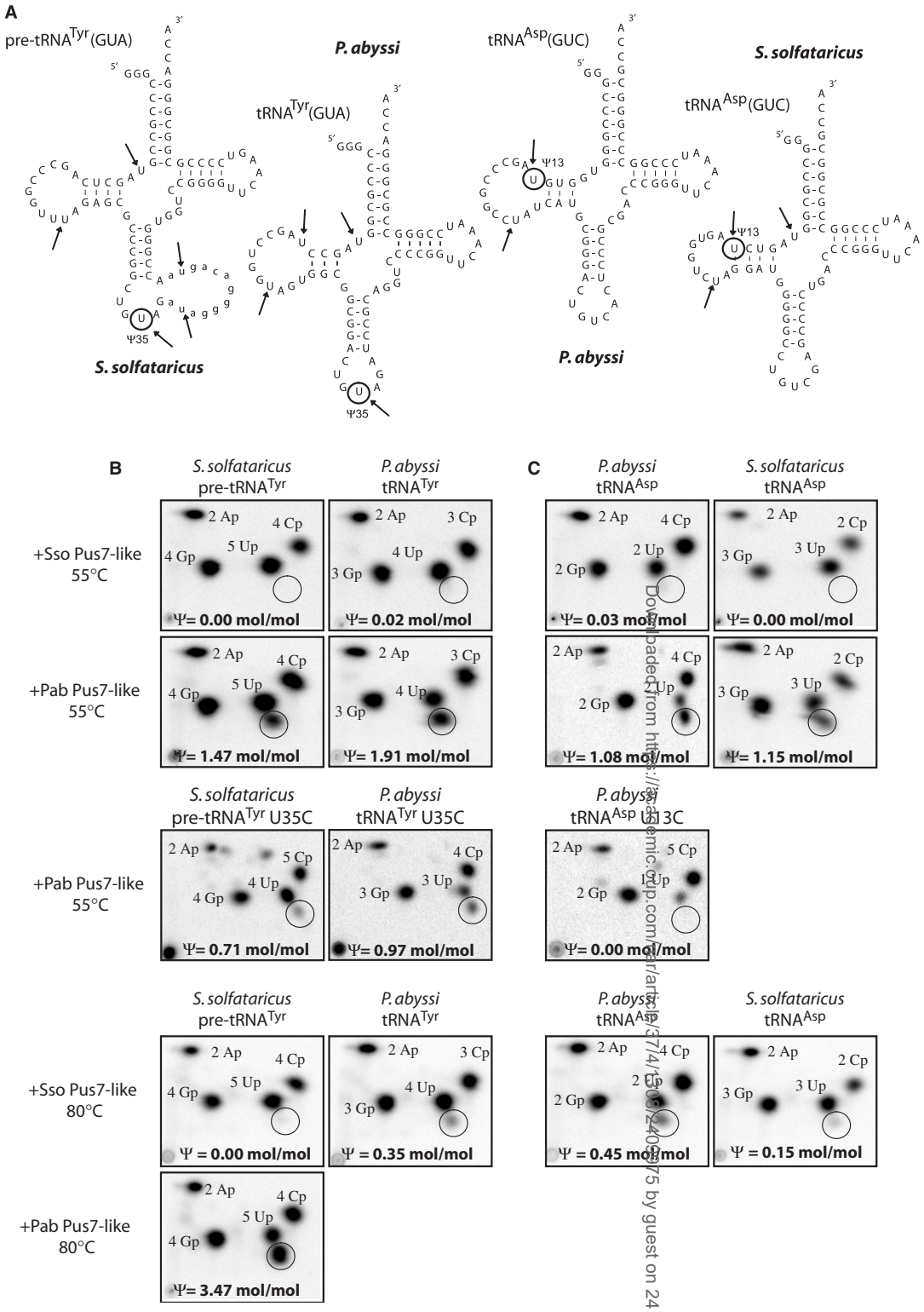

Figure 5. Test of the activity of the recombinant Pab and Sso Pus7-like enzymes on different Sso and Pab tRNAs and pre-tRNA substrates. (**A**) 2D structures of the *P. abyssi* $tRNA^{Tyr}$(GUA) and $tRNA^{Asp}$(GUC) and *S. solfataricus* pre-$tRNA^{Tyr}$(GUA) and $tRNA^{Asp}$(GUC). The UA dinucleotides present in these RNAs are indicated by arrows. The potential U35 target sites in mature and pre-$tRNA^{Tyr}$(GUA) and the U13 target sites in the Pab $tRNA^{Tyr}$(GUA) and the Sso and Pab $tRNAs^{Asp}$(GUC) are circled. (**B**) Tests of the *in vitro* activity of the recombinant Sso Pus7-like and Pab Pus7-like enzymes on the Sso pre-$tRNA^{Tyr}$(GUA) and Pab $tRNA^{Tyr}$(GUA) and their respective U35C variants. All the transcripts were [α-^{32}P]ATP-labelled and incubated for 90 min at 55°C or 80°C with the recombinant proteins (Pab Pus7-like or Sso Pus7-like) as indicated on the left of the panel. Modified RNAs were analysed as described in Figure 2. The molar amounts of Ψ residue formed per mole of RNA in the experiments are given for each 2D-TLC autoradiogram. Reproducible results were obtained in three independent experiments. (**C**) *In vitro* activity tests with the Sso and Pab recombinant Pus7-like enzymes on the Sso and Pab $tRNA^{Asp}$(GUC) and the U13C Pab $tRNA^{Asp}$ variant, same legend as for (B). (**D**) The Pab Pus7-like enzyme modifies only UA dinucleotides. The *P. abyssi* $tRNA^{Tyr}$(GUA) was labelled by incorporation of [α-^{32}P] ATP, CTP, UTP or GTP. Formation of Ψ residue by Pab aPus7 was tested after 90 min of incubation at 55°C.

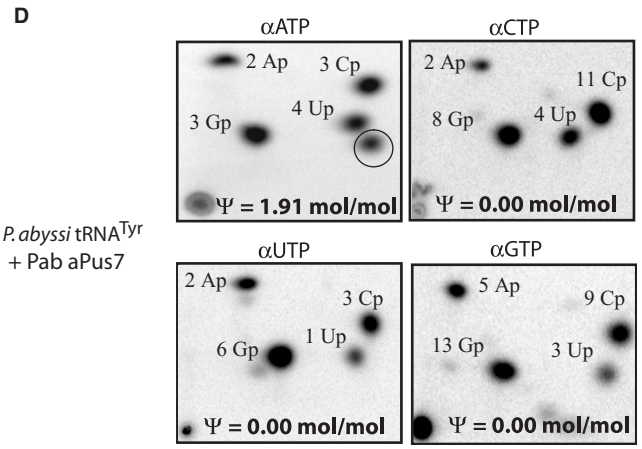

Figure 5. Continued.

$tRNA^{Asp}$(GUC), Sso $tRNA^{Asp}$(GUC), respectively (Figure 5C). As shown above, 1.91 mol of Ψ were formed per mole of Pab $tRNA^{Tyr}$(GUA) (Figure 5B). U13C substitution in the Pab $tRNA^{Asp}$(GUC) completely abolished Ψ formation in this tRNA (Figure 5C), while U13C substitution in the Pab $tRNA^{Tyr}$(GUA) decreased Ψ formation in this tRNA to 1.31 mol/RNA mole (Figure S2), showing that that the Pab Pus7-like recombinant enzyme modifies these two tRNAs at position 13. Here again, no Ψ formation was detected in the three tested tRNAs when using the recombinant Sso Pus7-like recombinant enzyme at 55°C (Figure 5C). When incubations were performed at 80°C, the Pab enzyme formed more than 2 mol of Ψ/mol of Sso $tRNA^{Asp}$(GUC). Therefore, here again at this high temperature each of the UA dinucleotides was probably modified. Interestingly, at 80°C, the Sso recombinant enzyme modified at low yields each of the three tRNAs that contain a U13 residue. However, we cannot conclude that these *in vitro* data obtained at very high temperature reflect the activity of this enzyme at position 13 *in cellulo*, since in the above assays performed in Sso cellular extract at 65°C, no pseudouridylation in UA dinucleotides of the Pab $tRNA^{Tyr}$(GUA) was detected (Figure 4).

Taken together, these data demonstrated an activity of the recombinant Pab Pus7-like enzyme at position 13 in tRNAs and a low activity at high temperature of the recombinant Sso Pus7-like enzyme on tRNAs containing a U residue at position 13.

Modifications by the Pab Pus7-like enzyme mainly occurs in UA dinucleotides

In yeast modification by Pus7p was found to occur in a conserved sequence ($Pu_{-4}(G/c)_{-3}U_{-2}N_{-1}ΨA_{+1}Pu_{+2}$) (36) including an A residue at position +1 as referred to the modified residue. Above, we tested the activity of the recombinant Pab Pus7-like enzyme at UA dinucleotides. It was interesting to check whether this enzyme can modify UU, UC or UG dinucleotides or has the same requirement for a 3′ A residue as the eukaryal enzyme. To answer this question, the Pab $tRNA^{Tyr}$(GUA) was labelled by [α-^{32}P]GTP, [α-^{32}P]CTP or [α-^{32}P]UTP incorporation. No modification was detected when these NTPs were used for RNA labelling (Figure 5D). Similarly, no Ψ residue was formed in an [α-^{32}P]GTP-labelled Sso $tRNA^{Tyr}$ (data not shown). Therefore, we concluded that the Pab Pus7-like enzyme, like yeast Pus7p, has a marked preference for UA dinucleotides as compared to UU, UC and UG dinucleotides. Based on the numerous common properties found between the Pab Pus7-like enzyme and yeast Pus7p, we will now denote this enzyme Pab aPus7.

Different properties of the $tRNA^{Tyr}$:Ψ35-synthase in Sso and Pab cellular extracts

Based on the pre-$tRNA^{Tyr}$(GUA) and $tRNA^{Tyr}$(GUA):Ψ35-synthase activities that we found in the *P. abyssi* and *S. solfataricus* extracts, respectively, and on the *in vitro* properties of the reconstituted *S. solfataricus* H/ACA sRNP and the recombinant Pab Pus7, we assumed that the $tRNA^{Tyr}$(GUA) from both *S. solfataricus* and *P. abyssi* contains a Ψ35 residue. To get additional support to the idea that *P. abyssi* likely require aPus7 for Ψ35 formation in tRNA, whereas *S. solfataricus* may use an H/ACA sRNA-guided system, we took advantage of the requirement of a 3′ A residue for efficient U to Ψ conversion by Pab aPus7 and the absence of this requirement for U to Ψ conversion catalysed by H/ACA sRNPs, which was also experimentally demonstrated (data not shown). Indeed, based on the above characterized specificity of the Pab aPus7 enzyme, we expected that an A36G substitution in the Pab $tRNA^{Tyr}$(GUA) transcript would abolish the activity of this enzyme at position 35 in this tRNA. In contrast, the same A36G base substitution was not expected to affect the activity of the Sso1 H/ACA sRNP at position 35 in the Sso pre-$tRNA^{Tyr}$(GUA) (residue A36 as well as the target U35 residue are not base-paired with the guide RNA, see Figure 1). In order to release labelled Ψp from position 35 after RNase T2 digestion, the variant A36G Pab $tRNA^{Tyr}$(GUA) and A36G Sso pre-$tRNA^{Tyr}$(GUA) transcripts were labelled by [α-^{32}P]GTP incorporation. As expected (Figure 6A), despite the presence of four UG dinucleotides in the A36G Pab $tRNA^{Tyr}$ transcript, no Ψ formation was detected after incubation of this RNA, either with Pab aPus7 (55°C) or with a Pab cellular extract (65°C). In contrast, formation of nearly 1 mol of Ψ/mol of RNA was observed when using the reconstituted Sso1 H/ACA sRNP in the presence of the Sso A36G pre-$tRNA^{Tyr}$ (GUA) variant (65°C). In addition, similar levels of U to Ψ conversions were detected upon incubation of the [α-^{32}P]ATP labelled Sso pre-$tRNA^{Tyr}$(GUA) or the [α-^{32}P]GTP labelled Sso A36G pre-$tRNA^{Tyr}$(GUA) transcript in an Sso cellular extract (65°C) (Figure 6B).

Taken together these data were in agreement with the idea that in a *P. abyssi* cellular extract, Pab aPus7 may be responsible for Ψ35 formation in the Pab $tRNA^{Tyr}$(GUA), whereas in an *S. solfataricus* cellular extract, the Sso H/ACA sRNP may catalyse Ψ35 formation in the Sso pre-$tRNA^{Tyr}$(GUA). To try to get a more formal proof of the H/ACA sRNP activity on the pre-$tRNA^{Tyr}$ in *S. solfataricus*, we prepared Sso cellular extracts devoid of RNAs by treatment with micrococcal nuclease.

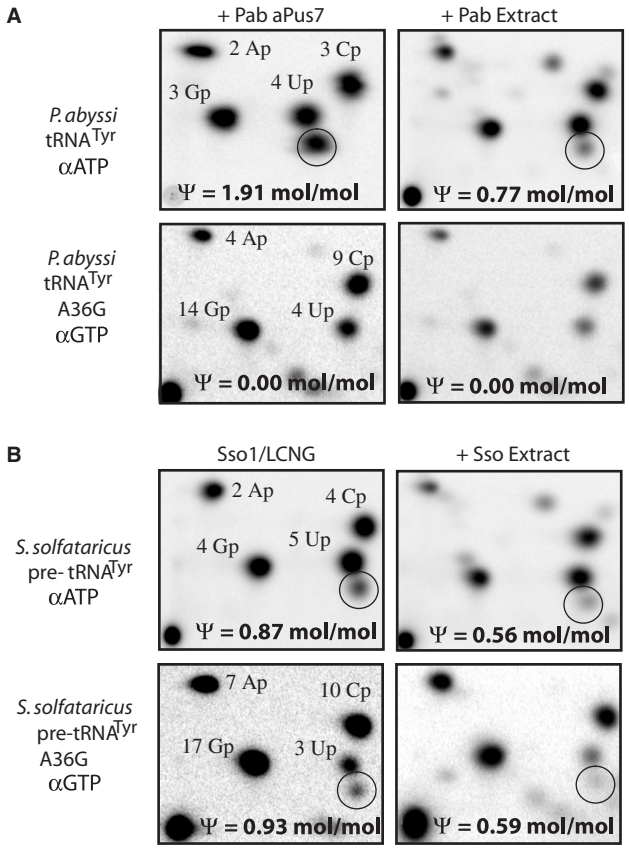

Figure 6. Formation of Ψ35 in $tRNA^{Tyr}$(GUA) is likely catalysed by Pab aPus7 in *P. abyssi,* while the Sso1 H/ACA sRNP might be the catalysts in *S. solfataricus*. (**A**) Modification of the [α-^{32}P]ATP-labelled WT $tRNA^{Tyr}$(GUA) and its [α-^{32}P]GTP-labelled A36G variant by recombinant Pab aPus7 and in a Pab cellular extract was tested by the nearest neighbour approach. Incubations with the recombinant proteins were performed for 90 min at 55°C and with the extract for 90 min at 65°C. Additional spots on the TLC chromatograms corresponding to the formation of m^1G37, Cm56, m^1I57 and m^1A58 are indicated. The molar amounts of Ψ residue formed per mole of RNA are given. (**B**) The activity at 65°C of the reconstituted Sso1 sRNP (Sso1/LCNG) and an Sso cellular extract was tested on the WT Sso pre-$tRNA^{Tyr}$(GUA) and its U36G variant. The Sso1 H/ACA sRNP was reconstituted and its activity was tested as described in the legend to Figure 2B.

The activity of these extracts was tested on the Sso pre-$tRNA^{Tyr}$ after nuclease inactivation using various chelators of divalent cations. As expected, no residual $tRNA^{Tyr}$:Ψ35-synthase activity was detected. However, when the same experiment was performed as a control using the *P. abyssi* cellular extract, we also detected a strong decrease of $tRNA^{Tyr}$:Ψ35-synthase activity after treatment. Most probably, the chelators used to inhibit micrococcal nuclease affected enzyme activity and/or RNA substrate structure. Therefore, in the absence of a clear control we had no direct proof that the Sso $tRNA^{Tyr}$:Ψ35-synthase activity detected in the extract was strictly dependent on the H/ACA sRNA. However, altogether, most of our *in vitro* observations are in agreement with the fact that the deficiency that we observed for the aPus7 tRNA:Ψ synthase activity in *S. solfataricus* might be compensated by the presence of a specific H/ACA sRNA guide in this species.

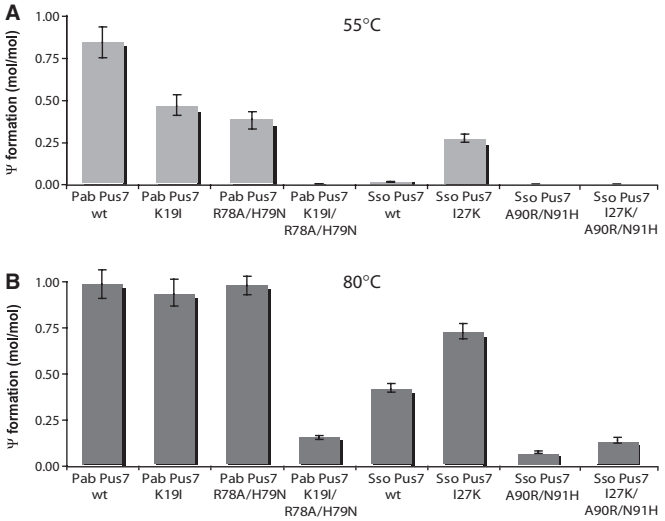

Figure 7. Test of the activity of Pab aPus7 and Sso aPus7 recombinant WT or variant proteins on the [α-^{32}P]ATP-labelled *P. abyssi* $tRNA^{Asp}$(GUC). Enzymatic tests were performed as described in the legend to Figure 5, except that we used point-mutated variants of the enzymes, Pab aPus7 K19I, R78A/H79N, and with the three R78A, H79N and K19I mutations and Sso aPus7 I127K, A90R/N91H and with the three I127K, A90R and N91H mutations. Incubations with the enzymes were performed both at 55°C (**A**) and at 80°C (**B**) and the molar amounts of Ψ residue formed per mole of $tRNA^{Asp}$ are given in a histogram. Error bars correspond to the standard deviations in three independent experiments.

Important roles of the conserved basic residue in motif IIIa and the KR/RH pair in motif II of archaeal aPus7

We tested which of the observed amino acid substitutions in motifs II and IIIa of Sso aPus7 might explain its lower activity compared to Pab aPus7. In order to dissociate the effect of amino acid substitutions in motif IIIa from amino acid substitutions in motif II, the K27I substitution found in motif IIIa of the Sso aPus7 was transferred into the Pab aPus7 enzyme by site-directed mutagenesis (variant Pab K19I). We also transferred the R90A/H91N sequence found in motif II of Sso aPus7 in Pab aPus7 (variant R78A/H79N). Finally, we produced a Pab aPus7 variant mutated in both motifs (K19I + R78A/H79N). Conversely, we tested whether we could restore some activity in Sso aPus7 by individual I27K substitution in motif IIIa, double A90R and N91H substitutions in motif II or by combination of these three amino acid substitutions. Variant Pab aPus7 and Sso aPus7 were produced as recombinant proteins in *E. coli* and purified using their His_6-tag sequence. As WT Pab aPus7 modifies only position 13 in Pab $tRNA^{Asp}$, this substrate was used to test the activity of the variant proteins. As above, each test of activity was first done at 55°C in order to preserve the RNA structure. The mean values obtained in three independent experiments are shown in Figure 7. Both the K19I and R78A/H79N substitutions in Pab aPus7 decreased the activity at position 13 in the Pab $tRNA^{Asp}$ at 55°C (Figure 7A), and combination of the three amino acid substitutions completely abolished this activity. Restoration of an RH pair in motif II of Sso aPus7 had almost no effect on its activity

at 55°C, whereas restoration of the conserved basic residue in motif IIIa (I27K variant) allowed a significant increase of U to Ψ conversion (Figure 7A). However, the combination of all three substitutions had no positive effect. Interestingly, at 80°C (Figure 7B), the individual K19I or R78A/H79N substitutions in the Pab aPus7 had almost no negative effect on its activity towards the Pab tRNA^{Tyr}(GUA). However, their combination nearly abolished this activity. As described above (Figure 5C), the WT Sso aPus7 showed some activity on the Pab tRNA^{Asp}(GUA) at 80°C. The I27K substitution in this enzyme noticeably increased this activity (0.71 mol of Ψ/mol of tRNA, against 0.45 for the WT enzyme). However, here again the A78R and N79H substitutions had no positive effect on this activity.

Therefore, both *S. solfataricus* and the R78/H79 pair in motif II are important for activity of Pab aPus7 at position 13 in tRNAs and activity of Sso aPus7 can be increased by introduction of the basic residue in motif IIIa. Taken together, the data point out the strong functional importance of the basic residue in motif IIIa of aPus7 enzymes.

DISCUSSION

In this work, we described for the first time a family of H/ACA sRNAs that are likely dedicated to tRNA pseudouridylation and, in order to explain their presence, we made the first detailed analysis of the activity of archaeal Pus7-like enzymes.

Like the eukaryal enzymes, the *P. abyssi* aPus7 enzyme is active at positions 13 and 35 in tRNAs

The archaeal aPus7 enzymes belong to the TruD family of tRNA:Ψ-synthases. Whereas *E. coli* TruD only modifies position 13 in tRNA^{Glu} (25), our *in vitro* data reveal that, like its eukaryal counterpart (35,36), Pab aPus7 likely modifies tRNAs at positions 13 and 35 *in cellulo*. Our data also suggest that this enzyme can modify some other positions in archaeal tRNAs.

Consistent with our observations, 13 out of the 42 sequenced *H. volcanii* tRNAs contain a Ψ13 residue (21). Surprisingly, whereas both in plants and in yeast, the U35 to Ψ35 conversion in tRNA^{Tyr}(GUA) is strictly intron dependent (42,43,55), here we observed the efficient Ψ35 formation by both Pab aPus7 and a Pab cellular extract in an intron-less Pab tRNA^{Tyr}(GUA) transcript. This strongly suggests that this modification can also occur on an intronless tRNA *in cellulo*. Up to now, Ψ35 formation in intronless tRNA was only observed in the mitochondrial tRNA^{Asn}(GUU) from the starfish *Asteria amurensis* (56). Recent data revealed that only half of the archaeal tRNA^{Tyr}(GUA) encoding genes contain an intron (57,58). Based on the present data, this absence of intron may have a limited influence on the presence of a Ψ35 residue in tRNAs^{Tyr}.

Modification by *P. abyssi* aPus7 preferentially occurs at UA dinucleotides

Previous comparisons of the *S. cerevisiae* Pus7p RNA substrates revealed that this enzyme modifies U residues that belong to a highly conserved sequence (Pu_{-4}(G/c)$_{-3}U_{-2}N_{-1}\Psi A_{+1}Pu_{+2}$) (36). A similar consensus sequence was also proposed to be required for Ψ35 formation in plant pre-tRNAs^{Tyr}(GUA) (43). In addition, most of the tRNA sequences surrounding Ψ35 residue in plant and animal tRNAs fit to the above consensus. In agreement with this observation, our data strongly suggest that the presence of an A residue located 3′ to the target uridine is required for Pab aPus7 activity. In addition, at the two positions where we observed an efficient *in vitro* U to Ψ conversion by Pab aPus7 at 55°C, positions 13 in the Pab tRNA^{Asp}(GUC) and positions 35 in the Pab tRNA^{Tyr}(GUA) and Sso pre-tRNA^{Tyr}(GUA), respectively, the modified sequence fits to the above consensus (Figure 5A). However, by site-directed mutagenesis of UA dinucleotides in the Pab tRNA^{Tyr} (GUA), we demonstrated that in addition to position 35 and 13 which are modified at high yields, position 22 that does not fit to the consensus sequence is partially modified at 55°C by the recombinant enzyme (about 0.22 mol Ψ/mol RNA) (Figure S2). Therefore, the presence of the target U residue in a (Pu_{-4}(G/c)$_{-3}U_{-2}N_{-1}\Psi A_{+1}Pu_{+2}$) sequence is not as strictly required for archaeal aPus7 as it is for eukaryal Pus7. Only the presence of an A_{+1} residue may be strictly required. Accordingly, the sequence alignment of archaeal tRNAs known to contain a Ψ13 residue (56) shows that, whereas an A_{+1} residue is always conserved, the U_{-2} residue, found in all substrates of eukaryal Pus7-like enzymes, is found only in about one-third of the modified archeal tRNAs. This may also explain why when *in vitro* assays were performed in conditions where tRNA structure was melted (80°C), Pab aPus7 modified almost all UA dinucleotides in the substrate. Recent results obtained for the *P. abyssi* tRNA:m^{5}C-methyltransferase Trm4 demonstrated that its interaction with a protein called Archease modulates both its solubility and its substrate specificity *in cellulo* (59). Therefore, we cannot exclude the possibility that in its cellular context the aPus7 specificity is maintained at the very high growth temperature of *P. abyssi* by interaction with cellular proteins. Altogether, these observations suggest lower sequence constraints for aPus7 activity as compared to eukaryal enzymes.

Peculiar aPus7 proteins are found in some crenarchaeote species

By sequence alignment of archaeal Pus7-like proteins with yeast Pus7p and *E. coli* TruD, the Sulfolobales Pus7-like enzymes were all found to display several amino acid substitutions in the highly conserved motifs IIIa and II. According to the four available Sulfolobales genomic sequences, this peculiar sequence of aPus7 seems to be a general feature in this archaeal order. In contrast, among the four species of the Desulfurococcales order, whose complete genomic sequences are available, only *A. pernix* was found to carry amino acid substitutions in the aPus7 motif IIIa.

As expected by the location of motifs IIIa and II at the active sites of RNA:Ψ-synthases and their strong conservation in these enzymes (60,61) alterations of these motifs

in the Sulfolobales aPus7 proteins strongly decrease their activity. Our data show that among the numerous amino acid substitutions found in the Sulfolobales motifs IIIA and II, replacement of the conserved basic residue in motif IIIa by an Ile residue (Ile27 in Sso aPus7, Lys19 at the corresponding position in Pab aPus7) has the strongest negative effect on the activity. This strong effect can be explained by the necessity for a salt bridge formation between this conserved lysine residue and the catalytic aspartic acid residue (Asp77 in Pab aPus7) (Figure 3AB). This interaction was predicted to facilitate the nucleophilic attack by the COO^- group of the Asp residue at position C6 of the uridine base (53). The Ile27 residue in Sso aPus7 cannot form this salt bridge (Figure 3). This probably explains the almost complete inactivity of Sso aPus7 at 55°C. Our observation that the Sso aPus7 activity is markedly increased when the conserved basic residue in motif IIIa is restored reinforces this hypothesis.

Whereas, no activity of Sso aPus7 was detected at position 35 in the Sso pre-tRNA^{Tyr}(GUA), even at 80°C, the Sso aPus7 enzyme is not completely inactive since we detected some activity at high temperature on some tRNAs that contain a U residue at position 13. At high temperature, due to the higher flexibility of the protein backbone, other amino acids than the mutated ones may contribute to Asp89 activation in the active site. We can imagine that a low activity of Sso aPus7 at position 13 in tRNAs may be sufficient to ensure a minimum level of modification at this position *in vivo*. In contrast, Sso aPus7 may be unable to ensure sufficient modification at position 35 in tRNA^{Tyr}, explaining why a specific H/ACA sRNA dedicated to Ψ35 formation in tRNA^{Tyr} appeared in Sulfolobales and *A. pernix*. The lower negative effect of amino acid substitutions in motif II (RH/KR) on aPus7 activity compared to substitution of the basic conserved amino acid in motif IIIa, is consistent with the absence of Ψ35-specific H/ACA sRNA in Desulfurococcales species that display mutations in the aPus7 RH/KR conserved pair in motif II.

The high functional importance of Ψ35 in archaeal tRNA^{Tyr} may explain the requirement for an H/ACA sRNA in Sulfolobales

The need for a guide RNA for Ψ35 formation in Sulfolobales tRNA^{Tyr} may also be related to a high functional importance of Ψ35 in tRNA^{Tyr}(GUA). As mentioned in the 'Introduction' section, residue Ψ35 reinforces the stability of the anticodon stem–loop by interaction with residue U33 (13). This structural stabilization may increase the aminoacylation efficiency of tRNA^{Tyr}(GUA) by its cognate aminoacyl-tRNA synthetase (16) and also increases the stability of the codon-anticodon interaction during mRNA translation on the ribosome (16,41). This was shown by extensive studies on suppressor tRNA^{Tyr}. For instance, the absence of Ψ35 in SUP6 tRNA^{Tyr}(UUA) was found to decrease its *in vivo* ability to read-through the ochre stop codon UAA (42). Similarly, the presence of residue Ψ35 increased the tRNA^{Tyr} suppression activity on ochre (UAA) and amber (UAG) stop codons in the Tobacco Mosaic Virus RNA (17). One can imagine that the stabilization of codon–anticodon interactions is particularly important for organisms growing at very high temperatures like the hyperthermophilic Archaea. Therefore, it is reasonable to expect an important role of residue Ψ35 in the codon–anticodon interaction during mRNA decoding in these organisms.

Sulfolobales and *A. pernix* are the first organisms found to contain H/ACA RNA guides that may guide tRNA modification

Up to now, in Bacteria as well as in Eukarya, all tRNA post-transcriptional modifications were shown to be catalysed by enzymes that do not require a guide RNA. In contrast, an important implication of C/D box sRNAs in tRNA 2′-*O*-methylations was already demonstrated in Archaea (44). The present study describes for the first time H/ACA sRNAs that may direct pseudouridylation in tRNAs. By using the computational approach that we developed recently (45), we tested whether other H/ACA sRNAs dedicated to tRNA modification may exist in 48 available complete archaeal genomic sequences. No other tRNA-specific H/ACA sRNAs were found. Therefore, the H/ACA sRNAs identified in Sulfolobales and *A. pernix* may constitute a unique example of adaptive evolution: an H/ACA sRNA that was probably initially dedicated to rRNA modification may have been deviated from its original target to complement the deficiency of a tRNA:Ψ35-synthase. The shift from a non-guided to a guided post-transcriptional modification machinery was already observed for 2′-*O*-methylation at position 56 in archaeal tRNAs. Formation of Cm56 in the tRNA TΨ-loop is catalysed by the specific tRNA:2′-*O*-methyltransferase (aTrm56) in Archaea (62). The aTrm56 protein is missing in the crenarchaeote *Pyrobaculum aerophilum*, in which the modification is achieved by an additional C/D sRNP.

Links between tRNA and rRNA modifications in Sulfolobales and *A. pernix*

Interestingly, the Ape7 sRNA, in addition to guide tRNA modification, has also conserved the possibility to guide Ψ formation at position 2444 in 23S rRNA (Figure 1). The U residue at the equivalent position in *P. abyssi* 23S rRNA is modified by the Pab91 sRNA (7). The Ape7 sRNA may have gained an additional activity at position 35 of the pre-tRNA^{Tyr}(GUA) after deleterious mutations in aPus7. In contrast, based on computational analysis, the four Sulfolobales H/ACA sRNAs that guide tRNA modification are not expected to guide any modification in rRNAs. Interestingly, both their 5′- and 3′- guide sequences form unusually long interactions with the pre-tRNA^{Tyr} (GUA) (Figure 1) and some base compensatory mutations are found between the guide and target RNAs when base-pair interactions are compared in the four species. A strong link still exists between these H/ACA sRNAs and rRNA maturation, since the genes encoding Sso1, Sto1 and Sac1 H/ACA sRNAs also encode C/D box sRNAs (Figure S1, Supplementary Data). Our observation of a common transcript containing the

H/ACA and C/D sRNAs is in agreement with the recent finding that the Sso-159 C/D sRNA is expressed as a 120-nt-long RNA (50). The Sso-159 sRNA was proposed to guide 2′-*O*-methylation at position U2621 in 23S rRNA. The C/D motifs of the Sto1 sR4 and Sac1 sR39 C/D box sRNAs can also guide 2′-*O*-methylation at the same positions in 23S rRNA (data not shown). In addition, by computational approach, we could predict that these 3 C/D sRNAs may also guide 2′-O-methylation of 23S rRNA at positions 2645 and 2648 (*S. solfataricus* numbering). Altogether, this study suggests that sRNA sequences which have a capacity of rapid evolution may represent a flexible adaptive reservoir for RNA modification in archaea.

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REFERENCES

1. Rozenski,J., Crain,P.F. and McCloskey,J.A. (1999) The RNA Modification Database: 1999 update. *Nucleic Acids Res.*, **27**, 196–197.
2. McCloskey,J.A., Graham,D.E., Zhou,S., Crain,P.F., Ibba,M., Konisky,J., Soll,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.
3. Hamma,T. and Ferre-D'Amare,A.R. (2006) Pseudouridine synthases. *Chem. Biol.*, **13**, 1125–1135.
4. Ye,K. (2007) H/ACA guide RNAs, proteins and complexes. *Curr. Opin. Struct. Biol.*, **17**, 287–292.
5. Ni,J., Tien,A.L. and Fournier,M.J. (1997) Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell*, **89**, 565–573.
6. Ganot,P., Bortolin,M.L. and Kiss,T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell*, **89**, 799–809.
7. Charpentier,B., Muller,S. and Branlant,C. (2005) Reconstitution of archaeal H/ACA small ribonucleoprotein complexes active in pseudouridylation. *Nucleic Acids Res.*, **33**, 3133–3144.
8. Baker,D.L., Youssef,O.A., Chastkofsky,M.I., Dy,D.A., Terns,R.M. and Terns,M.P. (2005) RNA-guided RNA modification: functional organization of the archaeal H/ACA RNP. *Genes Dev.*, **19**, 1238–1248.
9. Watanabe,Y. and Gray,M.W. (2000) Evolutionary appearance of genes encoding proteins associated with box H/ACA snoRNAs: cbf5p in *Euglena gracilis*, an early diverging eukaryote, and candidate Gar1p and Nop10p homologs in archaebacteria. *Nucleic Acids Res.*, **28**, 2342–2352.
10. Roovers,M., Hale,C., Tricot,C., Terns,M.P., Terns,R.M., Grosjean,H. and Droogmans,L. (2006) Formation of the conserved pseudouridine at position 55 in archaeal tRNA. *Nucleic Acids Res.*, **34**, 4293–4301.
11. Muller,S., Fourmann,J.B., Loegler,C., Charpentier,B. and Branlant,C. (2007) Identification of determinants in the protein partners aCBF5 and aNOP10 necessary for the tRNA:Ψ55-synthase and RNA-guided RNA:Ψ-synthase activities. *Nucleic Acids Res.*, **35**, 5610–5624.
12. Gurha,P., Joardar,A., Chaurasia,P. and Gupta,R. (2007) Differential roles of archaeal box H/ACA proteins in guide RNA-dependent and independent pseudouridine formation. *RNA Biol.*, **4**, 101–109.
13. Auffinger,P. and Westhof,E. (1997) RNA hydration: three nanoseconds of multiple molecular dynamics simulations of the solvated tRNA(Asp) anticodon hairpin. *J. Mol. Biol.*, **269**, 326–341.
14. Massenet,S., Mougin,A. and Branlant,C. (1998) Posttranscriptional modifications in the U small nuclear RNAs. In Grosjean,H. and Benne,R. (eds), *The Modification and Editing of RNA.*, ASM Press, New York, pp. 201–227.
15. Arnez,J.G. and Steitz,T.A. (1994) Crystal structure of unmodified $tRNA^{Gln}$ complexed with glutaminyl- tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry*, **33**, 7560–7567.
16. Bare,L.A. and Uhlenbeck,O.C. (1986) Specific substitution into the anticodon loop of yeast tyrosine transfer RNA. *Biochemistry*, **25**, 5825–5830.
17. Zerfass,K. and Beier,H. (1992) Pseudouridine in the anticodon G ΨA of plant cytoplasmic $tRNA^{Tyr}$ is required for UAG and UAA suppression in the TMV-specific context. *Nucleic Acids Res.*, **20**, 5911–5918.
18. Gregson,J.M., Crain,P.F., Edmonds,C.G., Gupta,R., Hashizume,T., Phillipson,D.W. and McCloskey,J.A. (1993) Structure of the archaeal transfer RNA nucleoside G*-15 (2-amino-4,7-dihydro-4-oxo-7-beta-D-ribofuranosyl-1H-pyrrolo[2,3-d]pyrimidine-5-carboximi dam ide (archaeosine)). *J. Biol. Chem.*, **268**, 10076–10086.
19. Grosjean,H., Constantinesco,F., Foiret,D. and Benachenhou,N. (1995) A novel enzymatic pathway leading to 1-methylinosine modification in *Haloferax volcanii* tRNA. *Nucleic Acids Res.*, **23**, 4312–4319.
20. Ishitani,R., Nureki,O., Fukai,S., Kijimoto,T., Nameki,N., Watanabe,M., Kondo,H., Sekine,M., Okada,N., Nishimura,S. *et al.* (2002) Crystal structure of archaeosine tRNA-guanine transglycosylase. *J. Mol. Biol.*, **318**, 665–677.
21. Gupta,R. (1984) *Halobacterium volcanii* tRNAs. Identification of 41 tRNAs covering all amino acids, and the sequences of 33 class I tRNAs. *J. Biol. Chem.*, **259**, 9461–9471.
22. Chaban,B., Ng,S.Y. and Jarrell,K.F. (2006) Archaeal habitats–from the extreme to the ordinary. *Can. J. Microbiol.*, **52**, 73–116.
23. Ansmant,I. and Motorin,I. (2001) Identification of RNA modification enzymes using sequence homology. *Mol. Biol.*, **35**, 248–267.
24. Del Campo,M., Kaya,Y. and Ofengand,J. (2001) Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*. *RNA*, **7**, 1603–1615.
25. Kaya,Y. and Ofengand,J. (2003) A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA*, **9**, 711–721.
26. Nurse,K., Wrzesinski,J., Bakin,A., Lane,B.G. and Ofengand,J. (1995) Purification, cloning, and properties of the tRNA Ψ55 synthase from *Escherichia coli*. *RNA*, **1**, 102–112.
27. Becker,H.F., Motorin,Y., Planta,R.J. and Grosjean,H. (1997) The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of psi55 in both mitochondrial and cytoplasmic tRNAs. *Nucleic Acids Res.*, **25**, 4493–4499.
28. Ansmant,I., Motorin,Y., Massenet,S., Grosjean,H. and Branlant,C. (2001) Identification and characterization of the tRNA:Ψ31-synthase (Pus6p) of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **276**, 34934–34940.
29. Behm-Ansmant,I., Grosjean,H., Massenet,S., Motorin,Y. and Branlant,C. (2004) Pseudouridylation at position 32 of mitochondrial and cytoplasmic tRNAs requires two distinct enzymes in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **279**, 52998–53006.
30. Kammen,H.O., Marvel,C.C., Hardy,L. and Penhoet,E.E. (1988) Purification, structure, and properties of Escherichia coli tRNA pseudouridine synthase I. *J. Biol. Chem.*, **263**, 2255–2263.
31. Wrzesinski,J., Nurse,K., Bakin,A., Lane,B.G. and Ofengand,J. (1995) A dual-specificity pseudouridine synthase: An *Escherichia*

coli synthase purified and cloned on the basis of its specificity forΨ746 in 23S RNA is also specific for Ψ32 in $tRNA^{phe}$. *RNA*, **1**, 437–448.
32. Simos,G., Tekotte,H., Grosjean,H., Segref,A., Sharma,K., Tollervey,D. and Hurt,E.C. (1996) Nuclear pore proteins are involved in the biogenesis of functional tRNA. *EMBO J.*, **15**, 2270–2284.
33. Motorin,Y., Keith,G., Simon,C., Foiret,D., Simos,G., Hurt,E. and Grosjean,H. (1998) The yeast tRNA:pseudouridine synthase Pus1p displays a multisite substrate specificity. *RNA*, **4**, 856–869.
34. Massenet,S., Motorin,Y., Lafontaine,D.L., Hurt,E.C., Grosjean,H. and Branlant,C. (1999) Pseudouridine mapping in the *Saccharomyces cerevisiae* spliceosomal U small nuclear RNAs (snRNAs) reveals that pseudouridine synthase pus1p exhibits a dual substrate specificity for U2 snRNA and tRNA. *Mol. Cell. Biol.*, **19**, 2142–2154.
35. Ma,X., Zhao,X. and Yu,Y.T. (2003) Pseudouridylation (Ψ) of U2 snRNA in *S.cerevisiae* is catalyzed by an RNA-independent mechanism. *EMBO J.*, **22**, 1889–1897.
36. Behm-Ansmant,I., Urban,A., Ma,X., Yu,Y.-T., Motorin,Y. and Branlant,C. (2003) The *Saccharomyces cerevisiae* U2 snRNA:pseudouridine-synthase Pus7p is a novel multisite-multisubstrate RNA:Ψ-synthase also acting on tRNAs. *RNA*, **9**, 1371–1382.
37. Decatur,W.A. and Schnare,M.N. (2008) Different mechanisms for pseudouridine formation in yeast 5S and 5.8S rRNAs. *Mol. Cell. Biol.*, **28**, 3089–3100.
38. Lecointe,F., Simos,G., Sauer,A., Hurt,E.C., Motorin,Y. and Grosjean,H. (1998) Characterization of yeast protein Deg 1 as Pseudouridine synthase (Pus 3) catalysing the formation of Ψ_{38} and Ψ_{39} in tRNA anticodon loop. *J. Biol. Chem.*, **273**, 1316–1323.
39. Behm-Ansmant,I., Branlant,C. and Motorin,Y. (2007) The *Saccharomyces cerevisiae* Pus2 protein encoded by YGL063w ORF is a mitochondrial tRNA:Ψ27/28-synthase. *RNA*, **13**, 1641–1647.
40. Constantinesco,F., Motorin,Y. and Grosjean,H. (1999) Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities in vitro. *Nucleic Acids Res.*, **27**, 1308–1315.
41. Gurha,P. and Gupta,R. (2008) Archaeal Pus10 proteins can produce both pseudouridine 54 and 55 in tRNA. *RNA*, **14**, 2521–2527.
42. Johnson,P.F. and Abelson,J. (1983) The yeast $tRNA^{Tyr}$ gene intron is essential for correct modification of its tRNA product. *Nature*, **302**, 681–687.
43. Szweykowska-Kulinska,Z. and Beier,H. (1992) Sequence and structure requirements for the biosynthesis of pseudouridine (Ψ35) in plant pre-$tRNA^{Tyr}$. *EMBO J.*, **11**, 1907–1912.
44. Omer,A.D., Ziesche,S., Decatur,W.A., Fournier,M.J. and Dennis,P.P. (2003) RNA-modifying machines in archaea. *Mol. Microbiol.*, **48**, 617–629.
45. Muller,S., Charpentier,B., Branlant,C. and Leclerc,F. (2007) A Dedicated Computational Approach for the Identification of Archaeal H/ACA sRNAs. *Methods Enzymol.*, **425**, 355–387.
46. Charpentier,B., Fourmann,J.B. and Branlant,C. (2007) Reconstitution of Archaeal H/ACA sRNPs and test of their activity. *Methods Enzymol.*, **425**, 389–405.
47. Keith,G. (1995) Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie*, **77**, 142–144.
48. Bakin,A. and 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.
49. Motorin,Y., Muller,S., Behm-Ansmant,I. and Branlant,C. (2007) Identification of modified residues in RNAs by reverse transcription-based methods. *Methods Enzymol.*, **425**, 21–53.
50. Tang,T.H., Polacek,N., Zywicki,M., Huber,H., Brugger,K., Garrett,R., Bachellerie,J.P. and Huttenhofer,A. (2005) Identification of novel non-coding RNAs as potential antisense regulators in the archaeon *Sulfolobus solfataricus*. *Mol. Microbiol.*, **55**, 469–481.
51. Thebault,P., de Givry,S., Schiex,T. and Gaspin,C. (2006) Searching RNA motifs and their intermolecular contacts with constraint networks. *Bioinformatics*, **22**, 2074–2080.
52. Muller,S., Leclerc,F., Behm-Ansmant,I., Fourmann,J.B., Charpentier,B. and Branlant,C. (2008) Combined in silico and experimental identification of the Pyrococcus abyssi H/ACA sRNAs and their target sites in ribosomal RNAs. *Nucleic Acids Res.*, **36**, 2459–2475.
53. Kaya,Y., Del Campo,M., Ofengand,J. and Malhotra,A. (2004) Crystal structure of TruD, a novel pseudouridine synthase with a new protein fold. *J. Biol. Chem.*, **279**, 18107–18110.
54. Huang,L., Pookanjanatavip,M., Gu,X. and Santi,D.V. (1998) A conserved aspartate of tRNA pseudouridine synthase is essential for activity and a probable nucleophilic catalyst. *Biochemistry*, **37**, 344–351.
55. Nishikura,K. and De Robertis,E.M. (1981) RNA processing in microinjected Xenopus oocytes. Sequential addition of base modifications in the spliced transfer RNA. *J. Mol. Biol.*, **145**, 405–420.
56. Tomita,K., Ueda,T. and Watanabe,K. (1999) The presence of pseudouridine in the anticodon alters the genetic code: a possible mechanism for assignment of the AAA lysine codon as asparagine in echinoderm mitochondria. *Nucleic Acids Res.*, **27**, 1683–1689.
57. Mallick,B., Chakrabarti,J., Sahoo,S., Ghosh,Z. and Das,S. (2005) Identity elements of archaeal tRNA. *DNA Res.*, **12**, 235–246.
58. Sprinzl,M. and Vassilenko,K.S. (2005) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, **33**, D139–D140.
59. Auxilien,S., El Khadali,F., Rasmussen,A., Douthwaite,S. and Grosjean,H. (2007) Archease from *Pyrococcus abyssi* improves substrate specificity and solubility of a tRNA m^5C methyltransferase. *J. Biol. Chem.*, **282**, 18711–18721.
60. Hoang,C. and Ferre-D'Amare,A.R. (2004) Crystal structure of the highly divergent pseudouridine synthase TruD reveals a circular permutation of a conserved fold. *RNA*, **10**, 1026–1033.
61. Del Campo,M., Ofengand,J. and Malhotra,A. (2004) Crystal structure of the catalytic domain of RluD, the only rRNA pseudouridine synthase required for normal growth of *Escherichia coli*. *RNA*, **10**, 231–239.
62. Renalier,M.H., Joseph,N., Gaspin,C., Thebault,P. and Mougin,A. (2005) The Cm56 tRNA modification in archaea is catalyzed either by a specific 2'-O-methylase, or a C/D sRNP. *RNA*, **11**, 1051–1063.