



HAL
open science

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

Fabrice Leclerc, Christiane Branlant, Alan Urban, Sébastien Muller, Yuri Motorin, Arnaud Hecker

► To cite this version:

Fabrice Leclerc, Christiane Branlant, Alan Urban, Sébastien Muller, Yuri Motorin, et al.. Deficiency of the tRNA^{Tyr:Ψ35}-synthase aPus7 in Archaea of the Sulfolobales order might be rescued by the H/ACA sRNA-guided machinery. *Nucleic Acids Research*, 2009, 37 (4), pp.1308-1322. 10.1093/nar/gkn1037 . hal-03992159

HAL Id: hal-03992159

<https://hal.science/hal-03992159>

Submitted on 16 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Sébastien Muller¹, Alan Urban¹, Arnaud Hecker², Fabrice Leclerc¹, Christiane Branlant^{*1} and Yuri Motorin¹

¹Laboratoire de Maturation des ARN et Enzymologie Moléculaire, UMR 7567 CNRS-UHP Nancy Université, BP 239, 54506 Vandoeuvre-les-Nancy Cedex, FRANCE

²Institut de Génétique et Microbiologie, Université Paris-Sud, IFR115, UMR8621-CNRS, 91405 Orsay, FRANCE

Short running title : Pseudouridine Ψ35 formation in archaeal tRNAs

*Corresponding author :

Christiane BRANLANT

Laboratoire Maturation des ARN et Enzymologie Moléculaire

UMR 7567 CNRS-UHP Nancy I

Faculté des Sciences, BP 239

54506 Vandoeuvre-les-Nancy Cedex, FRANCE

Tel. + 33 (0).3.83.68.43.03

Fax + 33 (0).3.83.68.43.07

e-mail christiane.branlant@maem.uhp-nancy.fr

Keywords : Archaea / pseudouridine / RNA:pseudouridine-synthase / tRNA / sRNA-guide

ABSTRACT

The presence of a pseudouridine residue at position 35 (Ψ 35) is a characteristic feature of all known eukaryotic tRNA^{Tyr}(GUA). Ψ 35 formation in *S. cerevisiae* pre-tRNA^{Tyr}(GUA) is catalyzed by the RNA: Ψ -synthase Pus7, which also acts at position 13 in several tRNAs and at position 35 in U2 snRNA. In Bacteria, tRNA: Ψ -synthase TruD catalyzes the formation of pseudouridine residue at position 13 in tRNAs, but not at position 35. Here we found that the archaeal homologue (aPus7) of eukaryal Pus7 and bacterial TruD is able to modify tRNAs at position 13 and also position at 35 in both intron-containing and intron less tRNA^{Tyr}(GUA). Surprisingly, in several species of the Sulfolobales and Desulfurococcales orders, substitutions of amino acids, which are important for Pus7 activity, abolish Pus7 activity at position 35 in tRNA^{Tyr}(GUA). Despite the absence of tRNA: Ψ 35-synthase activity of Pus7 in these species, cell-free extract conserves the activity towards Ψ 35, complementing the deficiency of endogenous aPus7. In these species, we found that Ψ 35 formation in tRNA^{Tyr}(GUA) is insured by aCBF5-based machinery associated with a specific H/ACA sRNA that guides U35 modification. We demonstrated this activity by using both *in vitro* reconstitution assays and cellular extracts. This is the first demonstration that an H/ACA sRNA can guide pseudouridine formation in tRNAs.

INTRODUCTION

In all domains of life, pseudouridine (Ψ) formation is the most frequent post-transcriptional modification of RNAs. These U to Ψ conversions are catalysed by two major pathways: either by a stand-alone enzyme (specific RNA: Ψ -synthase), that can have narrow or large specificity (for review, Hamma & Ferre-D'Amare, 2006), or by a highly specific small ribonucleoprotein particle (H/ACA snoRNP or H/ACA scaRNP in eucarya, and H/ACA sRNP in Archaea) (Lafontaine *et al.*, 1998; Watkins *et al.*, 1998). H/ACA RNPs contain a small RNA that defines the targeted U residue by base-pair interaction with the RNA substrate (Charpentier *et al.*, 2007). As well in Eukarya as in Archaea, H/ACA sRNPs also contain 4 proteins: Nop10, Gar1 L7ae/Nhp2p and Cbf5/Dyskerin, which carries the activity of RNA: Ψ -synthase. Recent data showed that the aCBF5/aNOP10/aGAR1 complex in Archaea may also catalyze tRNA pseudouridylation without guide RNA (Charpentier *et al.*, 2007; Muller *et al.*, 2007a).

Pseudouridine residues are universally found in ribosomal RNA (rRNAs) and in tRNAs (Sprinzl & Vassilenko, 2005). In Eukarya, their presence was also detected in the spliceosomal UsnRNAs and some snoRNAs (for review, Massenet *et al.*, 1998)). Conversion of U into Ψ residue in RNAs modifies the local conformation of RNA molecules. Indeed, the additional free N₁-H position on the nucleobase allows the formation of an additional base-pair interactions, either in *cis* within the RNA molecule, or in *trans* with another RNA molecule or a hydrogen bond interaction with a protein. Secondly, substitution of the C-N bond between the ribose and the nucleobase by a C-C bond limits the flexibility of the ribose conformation that also favours RNA-RNA base-pair interactions (Auffinger & Westhof, 1997; Massenet *et al.*, 1998).

In all organisms, tRNA molecules can be pseudouridylated at multiple sites (7 positions in *E. coli*, at least 15 positions in *S. cerevisiae*, and even more in higher eukaryotes) (Sprinzl &

Vassilenko, 2005). Among the Ψ residues in tRNAs, the eukaryal specific Ψ 35 residue in the anticodon loop of tRNA^{Tyr}(GUA) was shown to play an important role in mRNA decoding (Tomita *et al.*, 1999). Up to now, for both Bacteria and Eukarya, pseudouridylation in tRNAs were shown to be catalysed by numerous stand-alone enzymes and most of the tRNA: Ψ -synthases in *E. coli* and in *S. cerevisiae* have been identified (for review, Ansmant & Motorin, 2001; Hamma & Ferre-D'Amare, 2006). Some of them were found to have quite narrow specificity, as is the case for the bacterial TruB, TruD and TruC enzymes. They are responsible for U to Ψ conversion at one single site (positions 55, 13 and 65 in tRNAs, respectively). Other bacterial tRNA: Ψ -synthases have larger specificities, TruA acts at several sites in tRNAs (Kammen *et al.*, 1988), while RluA modifies both tRNAs and rRNAs (Raychaudhuri *et al.*, 1999). In contrast, in Eukarya, the numerous pseudouridylations in cytoplasmic rRNAs are catalysed by H/ACA snoRNPs. Therefore, in these species, tRNA: Ψ -synthases are either dedicated only to tRNA modification or act both on tRNAs and UsnRNAs (Pus1p and Pus7p) (Massenet *et al.*, 1999; Behm-Ansmant *et al.*, 2003). Here again, some of the tRNA: Ψ -synthases dedicated to tRNAs have a very narrow specificity, as is the case for Pus4p or Pus6p, Pus8p and Pus9p (acting at position 55, 31 et 32, respectively) (Becker *et al.*, 1997; Ansmant *et al.*, 2001; Behm-Ansmant *et al.*, 2004). Other enzymes may have very large spectrum of substrates, like Pus1p which acts at 8 distinct positions in tRNAs and at position 44 in U2 snRNA (Massenet *et al.*, 1999), or Pus7p that converts U into Ψ in 3 distinct types of RNAs, in U2 snRNA at position 35, in some tRNAs at position 13 and in the pre-tRNA^{Tyr}(GUA) at position 35 (Behm-Ansmant *et al.*, 2003). Altogether, 10 distinct RNA: Ψ -synthases were characterized in *E. coli* and in *S. cerevisiae*, however, in *E. coli* five of them act on tRNAs (Kammen *et al.*, 1988; Gutsell *et al.*, 2000; Wrzesinski *et al.*, 2000; Del Campo *et al.*, 2001; Kaya & Ofengand, 2003), while 8 tRNA-specific enzymes are present in *S. cerevisiae* (Simos *et al.*, 1996; Becker *et al.*, 1997; Motorin *et al.*, 1998;

Ansmant *et al.*, 2001; Behm-Ansmant *et al.*, 2003; Behm-Ansmant *et al.*, 2004; Hepfer *et al.*, 2005; Behm-Ansmant *et al.*, 2007).

Studies on archaeal tRNA:Ψ-synthases are far less advanced. Indeed, whereas rRNA pseudouridylation and 2'-*O*-methylation in Archaea were shown to be catalysed via sRNA-guides (Omer *et al.*, 2000; Dennis *et al.*, 2001), little is known on the tRNA modification systems. In addition, post-transcriptional modifications in tRNAs have only been identified in a limited number of archaeal species: all tRNA molecules were sequenced only for *Haloferax volcanii* (Gupta, 1984). However, this halophilic organism may not be good representative of the whole archaeal domain. Indeed, the diversity of the archaeal organisms is extremely wide, since Archaea includes organisms growing not only at normal salt concentration, pH, temperature and pressure, but also many species able to grow in various extreme conditions, in particular at high pressure and elevated temperatures (Chaban *et al.*, 2006). For some of these hyperthermophilic Archaea, global profile of post-transcriptional modifications in rRNA and/or tRNA was studied by the HPLC/MS approach (McCloskey *et al.*, 2001). However, the identified modified residues were not localized in individual RNA molecules. Only 2'-*O*-methylations in many archaeal tRNAs were localized, because they are catalyzed by sRNPs containing C/D box sRNAs (Renalier *et al.*, 2005). Therefore, studies on the tRNA modification enzymes in Archaea were essentially focused on a limited number of post-transcriptional modifications, which are specific of the archaeal domain, such as methylations (m¹I58, Gm18) and archaeosine (Gregson *et al.*, 1993; Grosjean *et al.*, 1995; Ishitani *et al.*, 2002; Roovers *et al.*, 2006; Muller *et al.*, 2007b). Recently peculiar catalysts for the universal pseudouridylation at position 55 in tRNAs were also identified (the aCBF5/aNOP10/aGAR1 complex or the newly identified PsuX/Pus10 enzyme) (Roovers *et al.*, 2006; Muller *et al.*, 2007b). One puzzling observation in Archaea, is a limited number of putative genes encoding RNA:Ψ-synthases that can be detected in the entirely sequenced

genomes using sequence homology with bacterial or eukaryotic counterparts. Only genes encoding homologs of the bacterial TruB, TruD/Pus7 and PsuX/Pus10 enzymes are found in all species. In euryarchaeota species an additional gene encoding a member of the TruA family was detected (Watanabe & Gray, 2000). The archaeal homologue of TruB is the aCBF5 enzyme involved in H/ACA sRNP formation. PsuX/Pus10 was recently found to modify position 55 in tRNAs. Therefore up to now, the activities of the TruD/Pus7 homologue and the TruA family member have never been studied.

Based on the data obtained for the *H. volcanii* tRNAs and some other archaeal species, several positions in archaeal tRNAs can be pseudouridylated. The most frequent ones are positions 13, 39, 54 and 55. Furthermore, *in vitro* tests made with a *Pyrococcus furiosus* cellular extract and *H. volcanii* tRNA transcripts confirmed the formation of Ψ residues at position 39 and 55 (Constantinesco *et al.*, 1999). As mentioned above, aCBF5 and Pus10 are both implicated in U to Ψ conversion at position 55. The enzymes acting at the other pseudouridylation sites still have to be identified. One reasonable candidate for modification at position 13 is the archaeal aPus7. In addition, as yeast Pus7p also modifies U35 in the pre-tRNA^{Tyr}(GUA) (Behm-Ansmant *et al.*, 2003), even though Ψ 35 was not found in the *H. volcanii* tRNA^{Tyr}(GUA) (Gupta, 1984), an activity of the aPus7 enzyme at position 35 in tRNA cannot be excluded. The absence of Ψ 35 in the *H. volcanii* tRNA^{Tyr}(GUA) could be due to the absence of intron in its gene. Indeed, Ψ 35 formation in both yeast and plant tRNAs^{Tyr} was found to be intron-dependent (Johnson & Abelson, 1983; Szweykowska-Kulinska & Beier, 1992). Taking into account a low number of tRNA: Ψ -synthases in most Archaea, Ψ residues in tRNAs from these organisms could be formed not only by stand-alone enzymes, but also by H/ACA sRNPs, like is the case of certain tRNA 2'-O-methylations catalysed by C/D box sRNPs.

Here, we tried to address several open questions concerning pseudouridylation of archaeal tRNAs. By using the computational method that we recently developed for the search of H/ACA sRNA motifs (Muller *et al.*, 2007a), we identified an H/ACA sRNA able to guide Ψ 35 formation in the pre-tRNA^{Tyr}(GUA) in some archaeal species of the Sulfolobales and Desulfurococcales orders. We demonstrated its activity by *in vitro* reconstitution of the corresponding H/ACA sRNP and by a series of tests using archaeal cellular extracts. In parallel, we observed that the species that express this particular H/ACA sRNA, possess Pus7 proteins bearing amino acid substitutions expected to affect their activity. Therefore, by using recombinant proteins, we compared the activities of aPus7 enzymes from *P. abyssi* and *S. solfataricus* on tRNA^{Asp}, tRNA^{Tyr}(GUA) and pre-tRNA^{Tyr}(GUA). *P. abyssi* does not express the tRNA^{Tyr}(GUA)-specific H/ACA sRNA, while it is present in *S. solfataricus*. We showed that Pab Pus7 can act at position 13 in tRNAs and at position 35 in both intron-less and intron-containing tRNA^{Tyr}(GUA). However, tRNA^{Tyr}(GUA) is not modified by enzyme originating from the species that express a H/ACA sRNA guiding U to Ψ conversion at position 35.

RESULTS

Some H/ACA sRNAs in Archaea 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 (Muller *et al.*, 2007a), we found putative H/ACA sequence sRNAs which may direct U to Ψ conversion in tRNAs. Interestingly, all of them were predicted to guide the conversion of the same U target: position 35 in the intron-containing pre-tRNAs^{Tyr}(GUA) (Fig. 1A and 1B). One of these unusual H/ACA sRNAs was detected in the archaea of the Desulfurococcales order, *Aeropyrum pernix* (sRNA Ape7) and four other ones were found in the four species of the Sulfolobales order, namely *Sulfolobus solfataricus*

(sRNA Sso1), *Sulfolobus tokodaii* (sRNA Sto1), *Sulfolobus acidocaldarius* (sRNA Sac1) and *Metallosphaera sedula* (sRNA Mse2). These 5 putative H/ACA RNA motifs all contain a K-turn located at the expected distance (5 bps) from the pseudouridylation pocket and the conserved ACA trinucleotide is present at the 3' -end of the stem-loop structure (Fig. 1). Interestingly, the DNA genes for the *S. solfataricus*, *S. tokodaii*, *S. acidocaldarius* H/ACA motifs are directly flanked by DNA sequences corresponding to C/D box sRNAs: the already characterized Sso-159 (from *S. solfataricus*) (Tang *et al.*, 2002) and C/D box sRNAs that we denoted Sto1 sR4 and Sac1 sR39 for *S. tokodaii* and *S. acidocaldarius*, respectively). By using the complementary DNA probe, we verified the presence of Sso1 H/ACA RNA in total RNA fraction extracted from *S. solfataricus* cells. The northern blot analysis shown in Figure 1C reveals the presence of a long form of this RNA, that likely contains both the H/ACA and the C/D motifs, and a short form corresponding to the H/ACA motif alone. Therefore, the putative Sso1 H/ACA sRNA is indeed expressed in *S. solfataricus*.

The predicted RNA-basepairing interactions between each of the putative H/ACA sRNAs and the corresponding pre-tRNA^{Tyr}(GUA) species involve both the anticodon stem-loop of tRNA and the intron sequences (Fig. 1B). These interactions all fit to the criteria that we recently established for efficient H/ACA sRNA-target RNA interaction (Muller *et al.*, 2007a). Indeed, as for the H/ACA sRNA-rRNA interaction found in *P. abyssi*, the distance between the K-turn and the internal loop is of 5 bps, the distance between the targeted U residue and the ACA motif is of 14 nts and a stable base-pair interaction is formed between the 3'-guide sequence and the target RNA (Fig. 1B). In case of the Sac1 and the Mse2 sRNAs, the predicted interaction with the 3' sequence is exceptionally long as compared to previously characterized H/ACA sRNAs that direct rRNA pseudouridylation (Muller *et al.*, in preparation). However, the interaction predicted for the 3'-guide sequence of the *A. pernix* sRNA has a lower stability (Fig. 1B). In favour of the biological significance of the predicted

H/ACA sRNA-tRNA interactions, we found that sequence variations in the Sulfolobales H/ACA 5'-guide elements correlated to sequence variations in the pre-tRNA^{Tyr}(GUA) intron, in order to preserve the possible interaction (Fig. 1B). No other possible target sites in other RNAs were found for the Mse2, Sso1, Sto1 and Sac1 H/ACA sRNAs. Only the Ape-7 motif can guide pseudourylation at position 2444 in 23S rRNA, as found previously for the *P. abyssi* Pab91 H/ACA sRNA (Fig. 1A) (Charpentier *et al.*, 2005).

Therefore we concluded that the Mse2, Sso1, Sto1 and Sac1 H/ACA sRNAs likely guide pseudourylation in the pre-tRNA^{Tyr}(GUA) and this may also be the case for the Ape-7 H/ACA sRNA.

An sRNA-guided Ψ35 formation is detected in pre-tRNA^{Tyr} *in vitro*

For an experimental confirmation of the pseudouridylation guiding capability of the identified H/ACA sRNAs at position 35 in the pre-tRNA^{Tyr}(GUA), we used the *in vitro* reconstitution assay established for archaeal H/ACA sRNPs (Charpentier *et al.*, 2007). We expected that the *P. abyssi* proteins should be active in a H/ACA sRNP reconstitution assay even if an heterologous H/ACA sRNA is used. To check this hypothesis, we used EMSA to test the association between an *in vitro* transcribed Sso1 H/ACA sRNA and various combinations of the four *P. abyssi* proteins (L7Ae (L), aCBF5 (C), aNOP10 (N) and aGAR1 (G)) produced in *E. coli* (data not shown). Association pattern obtained for Sso1 H/ACA sRNA was very similar to the pattern observed previously for the *P. abyssi* Pab91 sRNA (Charpentier *et al.*, 2005). Thus we tested the activity of the reconstituted particles on an [α -³²P]ATP-labeled *S. solfataricus* pre-tRNA^{Tyr}(GUA) transcript (2 hours incubation at 65°C in the presence of the guide RNA and the 4 LCNG proteins). Labelling by [α -³²P]ATP incorporation followed by RNase T2 digestion allowed the detection of the released Ψp by 2D thin layer chromatography (2D TLC). As a control, the assay was also performed in the absence of the

Sso1 RNA guide (Fig. 2B). In addition, in order to verify that Ψ 35 formation can occur in the Sso pre-tRNA^{Tyr} *in cellulo*, the pre-tRNA^{Tyr}(GUA) transcript was also incubated for 2 hours at 65°C in a *S. solfataricus* cellular extract. As shown in Figure 2B, Ψ formation was detected in the extract and after incubation in conditions of sRNP reconstitution, and it is strictly dependent on the presence of the Sso1 RNA. Although, several UA dinucleotides are present in the pre-tRNA^{Tyr}(GUA), only U35 was expected to be a target. To confirm this hypothesis, we used two complementary approaches. First, the unlabeled pre-tRNA^{Tyr}(GUA) was incubated in conditions for *in vitro* reconstitution of the Sso1 RNP activity and, in parallel, in the Sso in cellular extract. The two RNA samples were subjected to CMCT-RT analysis. The appearance of a reverse transcriptase stop at position 36 in the RT elongation profile confirmed Ψ formation at position 35 in both assays. Finally, a complete demonstration that modification occurred only at position 35 was obtained by repeating the reconstitution assay and the incubation in the Sso cellular extract with an [α -³²P]ATP-labeled pre-tRNA^{Tyr} containing a U35C base substitution. No formation of Ψ residue was detected in the 2D-TLC profile neither with the reconstituted H/ACA sRNPs nor with the cellular extract (Fig. 2B). Altogether, these data clearly demonstrated that the Sso1 H/ACA sRNA can guide Ψ 35 formation in the Sso pre-tRNA^{Tyr}(GUA).

The presence of an H/ACA sRNA guiding Ψ 35 formation in archaeal pre-tRNA^{Tyr} is accompanied by amino acid sequence variations in the Pus7 protein

The identification of an H/ACA sRNA directing modification at position 35 in the archaeal pre-tRNA^{Tyr}(GUA) was surprising, since genes encoding Pus7-like enzymes were detected in all archaeal genomes and, as mentioned above, Pus7 is responsible for this modification both in plants and in yeasts. Local sequence alignment of the Pus7-like proteins from various archaeal species representing most of the archaeal genera, together with the 5 Sulfolobales

and *A. pernix* species is shown on Fig. 3B. We observed a high degree of divergence in motifs IIIa and II for the four Sulfolobales and *A. pernix* Pus7 compared to the other archaeal species (Fig. 3B). This sequence divergence correlates with the presence of H/ACA sRNA guiding tRNA modification. Motif IIIa and II are highly conserved amino acid sequences in all RNA:Ψ-synthases and are located at the catalytic pocket (Kaya *et al.*, 2004). Motif II contains the catalytic Asp residue involved in U isomerisation (Huang *et al.*, 1998). As expected, most of the highly conserved residues in motifs IIIa and II (K19, F25, D77, R78, H79, according to the numbering in the *P. abyssi* Pus7 sequence) are found in the Pus7 amino acid sequences from species that do not express an H/ACA guide for position Ψ35 in pre-tRNA^{Tyr} (Fig. 3A). In contrast, in the Pus7 amino acid sequences from the 4 Sulfolobales species and *A. pernix*, the conserved basic residue in motif IIIa (K19 in *P. abyssi*) is replaced by an isoleucine residue (I27 in *S. solfataricus*) and the arginine-histidine pair in motif II (R78/H79 in *P. abyssi*) is changed into an alanine (or threonine) - asparagine (or cysteine) pair (A90/N91 in *S. solfataricus*) (Fig. 3B). Other amino acid substitutions are also observed around these conserved positions. However, the catalytic aspartic residue remains conserved (D89 in *S. solfataricus*). Altogether, these observations suggested an altered activity of the Pus7-like enzymes in *S. solfataricus*, *S. tokodaii*, *S. acidocaldarius*, *M. sedula* and *A. pernix*.

Pab Pus7 modifies tRNAs at position 13 and 35 while Sso Pus7 fails to catalyze Ψ35 formation

To check the effects of amino acid substitutions at the catalytic pocket of the *S. solfataricus* Pus7-like enzyme and more generally to test for the activity of the archaeal Pus7-like enzymes, we produced the *P. abyssi* (Pab) and the *S. solfataricus* (Sso) Pus7-like proteins as His₆-tagged fusions in *E. coli*. We used various RNA transcripts to test activities of purified recombinant proteins (Fig. 4A): two of them corresponded to *S. solfataricus* RNAs (Sso pre-

tRNA^{Tyr}(GUA) and Sso tRNA^{Asp}(GUC)) and two other ones to *P. abyssi* RNAs (Pab tRNA^{Tyr}(GUA) and Pab tRNA^{Asp}(GUC)). The two tRNA^{Asp} transcripts were used to detect the potential activity of the archaeal Pus7-like enzyme at position 13. The use of intronless tRNA^{Tyr} and a pre-tRNA^{Tyr} transcript with an intron was expected to bring information on the functional importance of an intron for modification at position 35. The *in vitro* activities of the recombinant proteins were tested by the nearest neighbour approach. As all the U residues expected to be modified were followed by an A residue, all the transcripts were labeled by incorporation of [α -³²P]ATP during transcription. Control experiments were performed with the *in vitro* transcribed tRNA^{Tyr} labeled by [α -³²P]GTP, [α -³²P]CTP or [α -³²P]UTP incorporation, as well as with an [α -³²P]GTP-labeled Sso tRNA^{Tyr}. Incubation conditions are given in Materials and Methods. The assays were first performed at 55°C, a temperature expected to preserve the intact tRNA 2D and 3D structure. As above, formation of Ψ residues was detected by 2D TLC after digestion of the RNA sample by RNase T2 (Fig. 4 B and C). Whereas no Ψ formation was detected in the control experiments using [α -³²P]GTP, CTP or UTP labeled RNAs (see panel C in Fig. 4), Ψ formation was detected for all the [α -³²P]ATP labeled substrates that we incubated with Pab Pus7p. This was a strong indication for the requirement of an A residue 3' to the targeted U residue for activity of Pab Pus7. In contrast, no Ψ formation was detected for any of the tested substrate when Sso Pus7 was used for incubation. This demonstrated the strong negative influence of the amino acid substitution found in the catalytic pocket of Sso Pus7 on the tRNA: Ψ -synthase activity of this enzyme.

We noticed that Pab Pus7 modified all four RNA transcripts but at variable levels (more than one mole of Ψ /mole tRNA for the Pab tRNA^{Tyr}, the Sso pre-tRNA^{Tyr} and the Sso tRNA^{Asp} transcripts and less than one mole of Ψ /mole tRNA for the Pab tRNA^{Asp} transcript (Fig. 4B)). To interpret these data, we had to take into account the fact that all Ψ residues formed 5' to an A residue were detected when [α -³²P]ATP labeled transcripts are used in assays. Therefore, to

test whether Pab Pus7 acts at position 13 and 35 in tRNAs like its eukaryal homologue, we generated a U13C substitution in the Pab tRNA^{Asp}(GUC) transcript and U35C substitutions in both the Pab tRNA^{Tyr}(GUA) and Sso pre-tRNA^{Tyr}(GUA) transcripts. As illustrated in Figure 4B, in spite of the presence of a two UA dinucleotides in the Pab tRNA^{Asp} molecule, U13C substitution in this tRNA completely abolished Ψ formation by Pab Pus7p. Therefore, we concluded that the Pab Pus7 enzyme, like its eukaryal counterpart, converts U into Ψ at position 13 in tRNAs. Similarly, in spite of the presence of numerous UA dinucleotides in the Pab tRNA^{Tyr} transcript (four altogether), only two moles Ψ /mole RNA were formed in the WT tRNA and only one in the tRNA mutant. Finally, whereas 5 UA dinucleotides are present in the Sso pre-tRNA^{Tyr} transcript, only 1.47 moles of Ψ were formed per RNA molecule and only half of this amount (0.71 mole Ψ /mole tRNA) was detected upon U35C substitution. Therefore, we concluded that the archaeal Pab Pus7 enzyme can modify position Ψ 35 in tRNAs, independently of the presence of an intron. In the molecule, other modifications in the U35C Pab tRNA^{Tyr} transcript may occur at position 8, 13 or 21. The 1.15 mole Ψ formed in Sso tRNA^{Asp} may correspond to U13 Ψ conversion plus a modification at one of the other UA dinucleotides. We detected only 0.77 mole of Ψ /RNA in Pab tRNA^{Tyr} transcript after incubation with the Pab cellular extract (Fig. 4). However, here again, the amount of Ψ formation was divided by a factor of 2 (0.42 mole of Ψ /mole tRNA) after U35C substitution. Taking into account the fact that efficiency of modification in the cell-free extract is lower compared to that obtained with purified recombinant enzymes, we considered that at least two uridines are likely modified upon incubation of the Pab tRNA^{Tyr} transcript in a Pab cellular extract and one of them is the residue U35.

Important roles of the conserved basic residue in motif IIIa and KR/RH pair in motif II of archaeal Pus7

The above data showed a very low or even undetectable *in vitro* activity at 55°C of the Sso Pus7 on the 4 tRNA substrates that we tested. Based on the amino acid sequence alignment of motifs IIIa et II of the archaeal Pus7 enzymes (Fig. 3), at least 3 amino acid substitutions could explain this inactivity of Sso Pus7. In order to dissociate the effect of the conserved basic residue in motif IIIa, from the effect of the substitutions in the conserved KR/RH pair of motif II, the K27I substitution found in the Sso motif IIIa was transferred into Pab Pus7 sequence by a site-directed mutagenesis (variant Pab K19I). We also transferred the R90A/H91N double substitution in the Sso motif II into Pab Pus7 sequence (variant R78A/H79N). In addition, we produced a Pab Pus7 variant enzyme mutated in both motifs (K19I + R78A/H79N). Conversely, we tested whether we could restore activity in Sso Pus7 by the individual I27K substitution in motif IIIa, the double A90R and N91H substitutions in motif II or by combination of these 3 amino acid substitutions. The variant Pab Pus7 and Sso Pus7 enzymes were produced as recombinant proteins in *E. coli* and purified using their His₆-tag sequence. As the WT Pab Pus7 modifies only position 13 in the Pab tRNA^{Asp}, this substrate was used to test the activity of the variant proteins. Each test of activity at 55°C was done in triplicate and the mean values obtained are shown in Figure 5. In accordance with the data mentioned above, about 0.8 mole of Ψ residue were formed in Pab tRNA^{Asp} by WT Pab Pus7, whereas a very low activity was detected with the WT Sso Pus7. Both the K19I and R78A/H79N substitutions in Pab Pus7 decreased the activity towards position 13 in the Pab tRNA^{Asp} transcript at 55°C (Fig. 5A). Furthermore, combination of the 3 amino acid substitutions completely abolished the activity of Pab Pus7 at this temperature. Restoration of an RH pair in motif II of Sso Pus7 had almost no effect on the Sso Pus7 activity, whereas restitution of the conserved basic residue in motif IIIa (I27K variant) allowed significant

increase of U to Ψ conversion. However, combination of all 3 substitutions had no positive effect. Therefore, we concluded that both K19 in motif IIIA and R78/H79 in motif II are important for Pab Pus7 enzyme activity at position 13 in tRNA. Some activity can be restored in the Sso Pus7 by introduction of the basic residue in motif IIIa..

As both *S. solfataricus* and *P. abyssi* grow at elevated temperatures (80°C and 100°C, respectively) we tested the activities of the WT and mutated enzymes at 80°C. Obviously, in these *in vitro* conditions, the tRNA 2D structure is expected to be considerably compromised. However, at this elevated temperature, we were able to detect some activity of the WT Sso Pus7 at position 13 in the Pab tRNA^{Asp} (Fig. 5B). The individual K19I or R78A/H79N substitutions had almost no effect on the Pab Pus7 activity at this temperature. However, their combination nearly abolished the enzymatic activity. In contrast, the I27K substitution in Sso Pus7 increased its activity at position 13 in the Pab tRNA^{Tyr} at 80°C (0.6 against 0.4 Ψ mole of Ψ / mole RNA for the variant and WT enzymes, respectively). However, here again the A78R and N79H substitutions had no positive effect on the Sso Pus7 activity. Therefore, we concluded that in any of the active site sequence context tested, and at any temperature, a basic residue in motif IIIa is important for Pus7 activity. Furthermore, at elevated temperatures the Sso Pus7 displays low, but detectable, activity at position 13. In contrast, according to the results obtained with the pre-tRNA^{Tyr} at 80°C (Fig. 6), even at this high temperature, it has no activity at position 35 in pre-tRNA, which probably explains the need for an H/ACA guided tRNA modification in *Sulfolobus*.

***P. abyssi* and *S. solfataricus* cellular extracts contain different enzymatic activities for Ψ 35 formation in tRNA^{Tyr}**

For a complete demonstration that *P. abyssi* uses Pab Pus7 for Ψ 35 modification in tRNA, whereas *S. solfataricus* uses an H/ACA sRNA-guided system to accomplish this modification, we designed the following strategy. It was based on our observation that the archaeal Pus7

enzymes require UA sequence context for U to Ψ conversion. We expected that introduction of an A36G substitution in the Pab tRNA^{Tyr} transcript should abolish U to Ψ conversion by Pab Pus7 at position 35 in this tRNA. In contrast, the same A36G base substitution should not affect modification machinery at position 35 in Sso pre-tRNA^{Tyr}, since H/ACA sRNA guide is not sensitive to the identity of the residue located 3' to the modified U (this residue as well as the target U residue are not base-paired, see Fig. 1). In order to release labeled Ψ p from position 35 after RNase T2 digestion, the variant A36G Pab tRNA^{Tyr} and A36G Sso pre-tRNA^{Tyr} transcripts were labeled by [α -³²P]GTP incorporation. As expected (Fig. 6A), in spite of the presence of 4 UG dinucleotides in the Pab tRNA^{Tyr} transcript, no Ψ formation was detected after incubation of this tRNA either with Pab Pus7 or with a Pab cellular extract. In contrast, formation of one Ψ residue was still observed upon reconstitution of the Sso1 H/ACA sRNP in the presence of the Sso A36G pre-tRNA^{Tyr} variant. In addition, the same level of U to Ψ conversion was detected upon incubation of the [α -³²P]ATP labeled WT Sso pre-tRNA^{Tyr} or the [α -³²P]GTP labeled Sso A36G pre-tRNA^{Tyr} transcripts in an Sso cellular extract (Fig. 6B).

Taken together these data brought a clear demonstration that in a Pab cellular extract, Pab Pus7 is solely responsible for Ψ 35 formation in the Pab tRNA^{Tyr}(GUA), whereas, in an Sso cellular extract the Sso H/ACA sRNP catalyses modification of the Sso pre-tRNA^{Tyr}(GUA) at the same position. Therefore, the deficiency of Pus7 activity that we found in *S. solfataricus*, is compensated by the presence of a specific H/ACA sRNA guide in this species. This is the first observation of an H/ACA sRNA guide dedicated to tRNA modification.

DISCUSSION

In this work, we performed a detailed analysis of the activity of archaeal Pus7 enzymes on tRNAs. We showed that the absence of fully active Pus7 in Sulfolobales can be compensated

by the presence of an H/ACA guide RNA that directs modification at position 35 in the pre-tRNA^{Tyr}(GUA).

The archaeal Pus7 enzymes is active at position 13 and 35 in tRNAs

The archaeal Pus7 enzymes belong to the TruD family of tRNA:Ψ-synthases. Whereas TruD in *E. coli* modifies only position 13 in tRNA^{Glu} (Kaya & Ofengand, 2003), here we show that, like its eukaryal counterpart, the archaeal Pab Pus7 enzyme can modify both positions 13 and 35 in tRNAs and maybe at some other positions (Ma *et al.*, 2003). Accordingly, 13 out of the 42 sequenced *H. volcanii* tRNAs, contain a Ψ residue at position 13 (Gupta, 1984). Except the universal Ψ55 residue, U13 is the most frequently pseudouridylated position in archaeal tRNAs. Interestingly, whereas both in plants and in yeast, the U to Ψ conversion at position 35 in tRNA^{Tyr} is strictly intron dependent (Nishikura & De Robertis, 1981; Johnson & Abelson, 1983; Szweykowska-Kulinska & Beier, 1992), here we observed the efficient formation of a Ψ35 residue in the intronless Pab tRNA^{Tyr}(GUA). Modification of U35 occurred both upon incubation with the recombinant Pab Pus7 protein and in a cellular extract. Therefore, according to these data, the point of view on Ψ35 formation in archaeal tRNA^{Tyr} should be revised. Indeed, archaeal tRNA^{Tyr} was not expected to be modified at U35 since bacterial TruD does not modify bacterial tRNAs at this position and archaeal tRNA genes were supposed to be intronless (Gupta, 1984).

However, recent data showed that about half of the tRNA^{Tyr}(GUA) genes in archaea contains an intron (Sprinzl & Vassilenko, 2005). In addition, here we demonstrate that Pab Pus7 can act on both tRNA^{Tyr}(GUA) precursor and on intronless tRNA. Therefore, tRNAs^{Tyr}(GUA) in archaea are likely pseudouridylated at position 35. Interestingly, before the present data, the only example of the formation of a Ψ35 residue in intronless tRNAs concerns the mitochondrial tRNA^{Asn}(GUU) from the starfish *Asteria amurensis* (Tomita *et al.*, 1999).

Archaeal Pus7 modifies RNA only at UA dinucleotides

A comparison of the *S. cerevisiae* Pus7p RNA substrates revealed that this enzyme modifies U residues which are comprised in a highly conserved sequence (Pu₋₄(G/c)₋₃U₋₂N₋₁ΨA₊₁Pu₊₂) (Behm-Ansmant *et al.*, 2003). A similar consensus sequence was proposed to be required for modification at position 35 of the plant tRNAs^{Tyr}(GUA) (Szweykowska-Kulinska & Beier, 1992). In addition, most of the tRNA sequences surrounding Ψ13 residues present in Bacteria, plant and animal tRNAs fit to the above consensus. Here, in agreement with this observation, we demonstrated that an A residue located 3' to the targeted U residue is strictly required for Pab Pus7 activity. In addition, at each of the 3 positions, where we could demonstrate an efficient U to Ψ conversion by Pab Pus7 (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 also fit to the above consensus. Remarkably, a second UA dinucleotide which is present in the Pab tRNA^{Asp} is not modified by Pab Pus7. The absence of modification may be explained by the divergence of the surrounding sequences. A pyrimidine (U residue), instead of a Pu residue is found at position +2 and a C residue, instead of a U residue is located at position -2. In the three other RNAs tested in the present work, more than one sequence was fitting the consensus sequence and this may explain the formation of Ψ residues at multiple sites during incubation with Pab Pus7. However, it should be noticed that when Pab Pus7 was incubated at high temperature (80°C) with the various tRNA substrates that we tested, a larger yield of pseudouridylation was observed, suggesting a strong decrease of the Pab Pus7 specificity in these conditions, where the tRNA and pre-tRNA 3D and 2D structures are likely disrupted. Recent results obtained for archaeal tRNA:m5C methyltransferase *P. abyssi* Trm4 demonstrate that interaction with Archease modulate both the solubility of the protein and its substrate specificity (Auxilien *et al.*, 2007). Similar

interactions between Pab Pus7 and its cellular environment may restrict the interaction of the modification enzyme with its RNA targets.

Peculiar Pus7 proteins are found in some crenarchaeota species

Sequence alignment showed that known Pus7 sequences from species of the Sulfolobales and the Desulfurococcales orders, that both belong to the Crenarchaeota kingdom, bear several amino acid substitutions in the highly conserved motifs IIIa and II. This may be a general feature of Sulfolobales Pus7, since all the species whose genomes were entirely sequenced displays this particularity. In contrast, this is not a general property of the Desulfurococcales order, since many other species, like *S. marinus*, *I. hospital* and *H. butylicus* have conventional Pus7 proteins. Both these motifs are located at the enzyme active site and may directly participate in catalysis. Mutations in these conserved motifs strongly decrease the Pus7 activity, and the observed negative effect can be explained on the basis of the 3D structures established for the closely related TruD protein (Hoang & Ferre-D'Amare, 2004) and other RNA:Ψ-synthases (Del Campo *et al.*, 2004). Indeed, the conserved lysine residue in motif IIIa (K19 in Pab Pus7) is expected to make a salt bridge with the catalytic aspartic acid residue (D77 in Pab Pus7, Fig. 3). This interaction facilitates the nucleophilic attack of the COO⁻ group on the C6 of the uridine base. In Sso Pus7, as the lysine residue in motif IIIa is replaced by an isoleucine (I27), a salt bridge cannot be formed with the catalytic D89 residue (Fig. 3). In consequence, only a poor activation of the COO⁻ group of D89 is expected. This probably explains almost complete inactivity of Sso Pus7 at 55°C which we observed for Sso Pus7. However, some activity of Sso Pus7 at position 13 in tRNAs was detected at 80°C. Thus, at high temperatures other amino acids of the catalytic site may contribute to D89 activation due to the higher flexibility of the protein backbone. Our observation that the Sso Pus7 activity is markedly increased when the conserved basic residue in motif IIIa is restored, also points out the functional importance of this residue for catalysis. In contrast, amino acid

residues in motif II (RH/KR) seem to be less important for U isomerization. Accordingly, some of the Desulfurococcales species, that do not contain Ψ 35-specific H/ACA sRNA, show mutations in the RH/KR conserved pair in motif II of Pus7. However, all these proteins conserved the basic residue of motif II, therefore these Pus7 enzymes are probably able to modify U at position 35 in tRNA^{Tyr}(GUA).

In contrast, no activity of Sso Pus7 at position 35 was detected, both at 55°C and at 80°C. This restricted specificity of Sso Pus7 towards tRNAs compared to Pab Pus7 may be explained by a difference in orientation of the tRNA substrate in substrate binding pocket. Indeed, modification of tRNA at position 13 implies the interaction with the tRNA D-stem, while U35 is located in the anticodon. Thus, some additional stabilizing RNA-protein interactions outside of the enzyme active site may compensate for the absence of proper catalytic functional groups. .

Even if Sso Pus7 activity at position 13 in tRNAs is very low, it may be sufficient to ensure a minimum level of modification at this position *in vivo*. In contrast, Sso Pus7 is probably unable to ensure sufficient modification at position 35 in tRNAs^{Tyr}, explaining why a specific H/ACA sRNA dedicated to Ψ 35 formation in tRNA^{Tyr} appeared in Sulfolobales and *A. pernix*

The high functional importance of Ψ 35 in archaeal tRNA^{Tyr} may explain the requirement for an H/ACA sRNA in Sulfolobales

The need for a guide RNA for Ψ 35 formation in Sulfolobales tRNA^{Tyr} may be related to a high functional importance of Ψ 35 in tRNA^{Tyr}. A great number of experiments were performed in order to define the function of this modified residue in tRNA^{Tyr}. First of all, residue Ψ 35 was shown to reinforce the stability of the anticodon stem-loop structure by establishment of a hydrogen bond between the N₁-H of its nucleobase and the ribose O₂' of the residue U33. This N₁-H...O₂' hydrogen bond is more stable than the weak C₅-H...O₂' bond

formed in the absence of pseudouridine (Auffinger & Westhof, 1997). This structural stabilization was proposed to influence the efficiency of tRNA^{Tyr} aminoacylation by its cognate aminoacyl-tRNA synthetase (Bare & Uhlenbeck, 1986). However, primary action seems to consist in the stabilisation of the codon-anticodon interaction. This was shown by intensive studies of suppressor tRNAs^{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 (Johnson & Abelson, 1983). 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 (Zerfass & Beier, 1992). 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 guides for tRNA modification

Up to now, as well in Bacteria as in Eukarya, all tRNA posttranscriptional modifications were shown to be catalyzed by enzymes that do not require a guide RNA. In contrast, an important implication of C/D box sRNAs for tRNA 2'-*O*-methylation was already demonstrated in Archaea (Renalier *et al.*, 2005). However, up to now, H/ACA sRNA dedicated to tRNAs had not been discovered. The H/ACA sRNAs that we found in four Sulfolobales species and *A. pernix* are the first example of a tRNA-specific H/ACA guide RNAs. By using the computational approach that we developed for the search of H/ACA sRNA genes in archaeal genomes (Muller, 2007 #49), we tested whether other genes for tRNA specific H/ACA sRNAs exist in the 48 available complete archaeal genome sequences. However, no other

examples of H/ACA sRNAs dedicated to tRNA modification were found. Therefore, we concluded that the H/ACA sRNA present in Sulfolobales and *A. pernix* constitute a unique example of adaptative evolution: an H/ACA sRNA that was probably initially dedicated to rRNA modification was deviated from its original target to complement the deficiency of a tRNA:Ψ³⁵-synthase. The shift from a non-guided to guided post-transcriptional machinery was already observed for 2'-*O*-methylation at position 56 in archaeal tRNAs (Renalier *et al.*, 2005). Formation of Cm56 in the tRNA TΨ-loop is catalysed by the specific tRNA: 2'-*O*-methyltransferase (aTrm56) in Archaea (Renalier *et al.*, 2005). Surprisingly, the homologous protein is missing in the crenoaarchaeon *Pyrobaculum aerophilum*, while the Cm56 modification is still present. The absence of aTrm56 in this organism is in fact compensated by the appearance of a C/D box sRNA dedicated to modification at this position (Renalier *et al.*, 2005). Hence, in this case, an additional C/D sRNA was used to achieve an important 2'-*O*-methylation which is normally catalyzed by a stand-alone enzyme. Thus, in order to complement the absence of an important RNA:Ψ-synthase, an H/ACA sRNA guided system was selected to insure modification of tRNAs, which are normally pseudouridylated by stand-alone enzymes. Altogether, these findings demonstrate the extreme plasticity of the RNA modification machinery in Archaea, and more generally, the adaptation potential constituted by the sRNA guides.

tRNA and rRNA modification may be tightly related in Archaea

The presence of tRNA-specific H/ACA sRNA guides in certain archaeal organisms demonstrate the tight relations existing between modification of various classes of RNA the cell. Indeed, in Archaea and eukaryotes sRNA-guided machinery is mostly dedicated to rRNA, but also to sn/snoRNA. In our study we characterized several tRNA-specific H/ACA sRNA in Sulfolobales. Interestingly, the Ape-7 sRNA, in addition to tRNA modification, has also conserved the possibility to guide Ψ formation at position 2444 in 23S rRNA (Fig. 1), U

residue in analogous position is modified by the Pab91 sRNA in *P. abyssi* (Charpentier *et al.*, 2005). The Ape-7 sRNA probably gained an additional activity at position 35 of the pre-tRNA^{Tyr}(GUA) after deleterious mutations in Pus7p. The Ape-7 sRNA with the two possible activities may correspond to a transitory situation. Note that base-pair interaction between the pre-tRNA^{Tyr} and the 5' guide sequence is strong but the interaction with the 3' guide has a lower stability. In contrast, the four other H/ACA sRNAs found in Sulfolobales do not guide anymore modification in rRNAs. They are strictly dedicated to tRNAs and interactions with both the 5' and the 3' guide sequences are extremely stable.

Remarkably, the genes encoding Sso1, Sto1 and Sac1 H/ACA sRNAs contain a segment encoding a C/D box sRNA at their 3'-extremities (Fig. 1). In this work we showed that a transcript containing both the H/ACA and the C/D sRNA is indeed expressed in *S. solfataricus*. This observation is in agreement with recent data showing that this C/D sRNA denoted Sso-159 is expressed as a 120-nt long RNA (Tang *et al.*, 2002). 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 (Fig. 7). In addition, we propose that the these 3 C/D sRNAs might also guide 2'-*O*-methylation of 23S rRNA at positions 2645 and 2648 in (*S. solfataricus* numbering, Fig. 7). Altogether, this study show that sRNA sequences which have a capacity of rapid evolution represent a flexible adaptative reservoir for RNA modification in archaea and probably also in Eukarya.

MATERIALS AND METHODS

DNA constructs for the production of Pus7 proteins and their variants

The DNA fragments encoding WT Pus7 proteins from *P. abyssi* and *S. solfataricus* were obtained by a PCR amplification using genomic DNA from these two species. Oligonucleotide primers, used for amplification contained NheI and BamHI restriction sites for forward and reverse primers, respectively. Amplified DNA was digested by NheI and BamHI and inserted into pET28b plasmid digested by the same restrictases. The variants of Pus7 proteins were produced by site-directed mutagenesis (QuickChange kit, Stratagene, USA). The sequence of the oligonucleotides used for the PCR amplifications and site-directed mutagenesis are available on request.

Purification of recombinant proteins

Wild-type and variant Pus7 proteins were produced in *E. coli* as His₆-tagged proteins and purified using affinity chromatography on Ni-NTA agarose (Qiagen, France). Induction of protein production was done by 1 mM IPTG in LB media supplemented by 50 µg/ml of kanamycin. Cell pellet was resuspended in Lysis buffer (20 mM Tris-HCl, pH7.7; 300 mM NaCl; 1 mM DTT ;10 mM Imidazole) and sonicated. Cell-free extract was directly loaded onto Ni-NTA agarose. After washing step at 20 mM of imidazole in the same buffer, His₆-tagged proteins were eluted in the Lysis buffer containing 250 mM of imidazole. Purified proteins were stored at ambient 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 (Charpentier *et al.*, 2005). 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, glycerol 10%.

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

Wild-type and mutated tRNA^{Asp} and tRNA^{Tyr} from *P. abyssi* and *S. solfataricus* and 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 *P. abyssi* strain GE5 or *S. solfataricus* strain P2. Forward primer used for amplification bears the sequence of T7 RNA polymerase promoter. Amplified products were cloned in pUC18 vector through SmaI restriction site. Linearization was performed using BstNI restriction endonuclease.

Templates encoding the minisubstrates derived from *S. solfataricus* tRNA^{Tyr} (see Figure 6) were obtained by annealing of two complementary oligonucleotides. Additional tRNA^{Asp} and tRNA^{Tyr} variants were produced by site-directed mutagenesis (QuickChange kit, Stratagene, USA). All synthetic RNA transcripts were purified by denaturing gel electrophoresis in 8% urea-PAAG. For the pseudouridylation assays, the corresponding RNA transcripts were uniformly labeled with [α -³²P]ATP or other [α -³²P]NTPs. Conditions for transcription and labeling were described previously (Charpentier *et al.*, 2007).

Cellular extracts

About 10¹⁰ cells from *P. abyssi* or *S. solfataricus* pellets grown to exponential phase were resuspended in the extraction buffer (25 mM Tris-HCl, pH 7,5 ; 25 mM KCl; 5 mM MgCl₂ ; 10 % Glycérol ; 2 mM DTT). The cells were sonicated for 5 min followed by centrifugation at 10 000 g. The concentration of glycerol was adjusted at 20% for storage at -20°C.

In vitro pseudouridylation assay

The tRNA:Ψ35-synthase activity of purified Pus7 or H/ACA sRNP complexes was measured by the nearest-neighbor approach in the conditions described previously (Charpentier *et al.*, 2005; Charpentier *et al.*, 2007). The sRNA guided activity of reconstituted H/ACA sRNPs was measured as previously described by mixing of ~ 4 pmol of Sso1 sRNA with ~150 fmol

of the [α - 32 P]ATP-labeled 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 (Charpentier *et al.*, 2005). The non RNA-guided reaction of Pus7 on tRNA was tested on ~50 fmol of [α - 32 P]ATP-labeled tRNAs in buffer Psi (100 mM Tris-HCl, pH8.0; 100 mM AcNH₄; 5 mM MgCl₂; 2 mM DTT; 100 μ M EDTA). The activity assays with cell-free extracts were performed with the same amount of labeled tRNA in buffer D in the presence of a similar amount of UDO_{260nm} of *P. abyssi* and *S. solfataricus* cellular extracts. For both 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 (Keith, 1995). 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 the site of pseudouridylation in tRNAs

Pseudouridine mapping in RNAs was done using the *N*-cyclohexyl-*N'*-(2-morpholinoethyl)-carbodiimide metho-*p*-toluolsulfonate (CMCT) modification protocol and primer extension with the reverse transcriptase (Bakin & Ofengand, 1993) for review see (Motorin *et al.*, 2007). CMCT modification was performed with 0.5 μ g of *in vitro* transcribed *S. solfataricus* tRNA^{Tyr}. Positions of alkali-resistant CMCT modifications were identified by primer extension analysis, using the AMV RT (QBiogene, USA) in the conditions previously described (Motorin *et al.*, 2007) using an oligonucleotides complementary to the 14 nt at the 3' end of the tRNA. Primers were 5'-end labeled using T4 polynucleotide kinase and [γ - 32 P]ATP (3000 Ci/mmol). RNA sequencing was done with 4 μ g of *in vitro* transcribed RNA (for details on the CMCT/RT technique, see (Motorin *et al.*, 2007)).

ACKNOWLEDGMENTS

S. Muller and A. Urban were a pre-doctoral fellow supported by the French Ministère de la Recherche et des Nouvelles Technologies (MNRT). The work was supported by the Centre National de la Recherche Scientifique (CNRS), the MNRT and the PRST « Bioingénierie » of Région Lorraine. We thank P. Forterre (IGM, Orsay, France) for helpful discussions.

BIBLIOGRAPHY

- Ansmant I, Motorin I. 2001. [Identification of RNA modification enzymes using sequence homology]. *Molekuliarnaia biologiya* 35:248-267.
- Ansmant I, Motorin Y, Massenet S, Grosjean H, Branlant C. 2001. Identification and characterization of the tRNA:Psi 31-synthase (Pus6p) of *Saccharomyces cerevisiae*. *J Biol Chem* 276:34934-34940.
- Auffinger P, 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.
- Auxilien S, El Khadali F, Rasmussen A, Douthwaite S, Grosjean H. 2007. Archease from *Pyrococcus abyssi* improves substrate specificity and solubility of a tRNA m5C methyltransferase. *J Biol Chem* 282:18711-18721.
- Bakin A, Ofengand J. 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center - analysis by the application of a new sequencing technique. *Biochemistry* 32:9754-9762.
- Bare LA, Uhlenbeck OC. 1986. Specific substitution into the anticodon loop of yeast tyrosine transfer RNA. *Biochemistry* 25:5825-5830.
- Becker HF, Motorin Y, Planta RJ, 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.
- Behm-Ansmant I, Branlant C, Motorin Y. 2007. The *Saccharomyces cerevisiae* Pus2 protein encoded by YGL063w ORF is a mitochondrial tRNA:Psi27/28-synthase. *RNA* 13:1641-1647.
- Behm-Ansmant I, Grosjean H, Massenet S, Motorin Y, 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.
- Behm-Ansmant I, Urban A, Ma X, Yu Y-T, Motorin Y, Branlant C. 2003. The *Saccharomyces cerevisiae* U2 snRNA:pseudouridine-synthase Pus7p is a novel multisite-multisubstrate RNA:Psi-synthase also acting on tRNAs. *RNA* 9:1371-1382.
- Chaban B, Ng SY, Jarrell KF. 2006. Archaeal habitats--from the extreme to the ordinary. *Can J Microbiol* 52:73-116.
- Charpentier B, Fourmann JB, Branlant C. 2007. Reconstitution of Archaeal H/ACA sRNPs and Test of their Activity. *Methods Enzymol* 425:389-405.
- Charpentier B, Muller S, Branlant C. 2005. Reconstitution of archaeal H/ACA small ribonucleoprotein complexes active in pseudouridylation. *Nucleic Acids Res* 33:3133-3144.
- Constantinesco F, Motorin Y, Grosjean H. 1999. Transfer RNA modification enzymes from *Pyrococcus furiosus*: detection of the enzymatic activities in vitro. *Nucleic Acids Res* 27:1308-1315.
- Del Campo M, Kaya Y, Ofengand J. 2001. Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*. *RNA* 7:1603-1615.
- Del Campo M, Ofengand J, 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.
- Dennis PP, Omer A, Lowe T. 2001. A guided tour: small RNA function in Archaea. *Mol Microbiol* 40:509-519.
- Gregson JM, Crain PF, Edmonds CG, Gupta R, Hashizume T, Phillipson DW, McCloskey JA. 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-carboximide (archaeosine)). *J Biol Chem* 268:10076-10086.
- Grosjean H, Constantinesco F, Foiret D, Benachenhou N. 1995. A novel enzymatic pathway leading to 1-methylinosine modification in *Haloflex volcanii* tRNA. *Nucleic Acids Res* 23:4312-4319.
- 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.
- Gutgsell N, Englund N, Niu L, Kaya Y, Lane BG, Ofengand J. 2000. Deletion of the *Escherichia coli* pseudouridine synthase gene *truB* blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. *RNA* 6:1870-1881.
- Hamma T, Ferre-D'Amare AR. 2006. Pseudouridine synthases. *Chem Biol* 13:1125-1135.
- Hepfer CE, Arnold-Croop S, Fogell H, Steudel KG, Moon M, Roff A, Zaikoski S, Rickman A, Komsisky K, Harbaugh DL, Lang GI, Keil RL. 2005. DEG1, encoding the tRNA:pseudouridine synthase Pus3p, impacts HOT1-stimulated recombination in *Saccharomyces cerevisiae*. *Mol Genet Genomics* 274:528-538.
- Hoang C, Ferre-D'Amare AR. 2004. Crystal structure of the highly divergent pseudouridine synthase TruD reveals a circular permutation of a conserved fold. *RNA* 10:1026-1033.

- Huang L, Pookanjanatavip M, Gu X, Santi DV. 1998. A conserved aspartate of tRNA pseudouridine synthase is essential for activity and a probable nucleophilic catalyst. *Biochemistry* 37:344-351.
- Ishitani R, Nureki O, Fukai S, Kijimoto T, Nameki N, Watanabe M, Kondo H, Sekine M, Okada N, Nishimura S, Yokoyama S. 2002. Crystal structure of archaeosine tRNA-guanine transglycosylase. *J Mol Biol* 318:665-677.
- Johnson PF, Abelson J. 1983. The yeast tRNA^{Tyr} gene intron is essential for correct modification of its tRNA product. *Nature* 302:681-687.
- Kammen HO, Marvel CC, Hardy L, Penhoet EE. 1988. Purification, structure, and properties of Escherichia coli tRNA pseudouridine synthase I. *J Biol Chem* 263:2255-2263.
- Kaya Y, Del Campo M, Ofengand J, Malhotra A. 2004. Crystal structure of TruD, a novel pseudouridine synthase with a new protein fold. *J Biol Chem* 279:18107-18110.
- Kaya Y, Ofengand J. 2003. A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA* 9:711-721.
- Keith G. 1995. Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie* 77:142-144.
- Lafontaine DL, Bousquet-Antonelli C, Henry Y, Caizergues-Ferrer M, Tollervey D. 1998. The box H + ACA snoRNAs carry Cbf5p, the putative rRNA pseudouridine synthase. *Genes Dev* 12:527-537.
- Ma X, Zhao X, Yu YT. 2003. Pseudouridylation (Psi) of U2 snRNA in S.cerevisiae is catalyzed by an RNA-independent mechanism. *EMBO J* 22:1889-1897.
- Massenet S, Motorin Y, Lafontaine DL, Hurt EC, Grosjean H, 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.
- Massenet S, Mougins A, Branlant C. 1998. Posttranscriptional modifications in the U small nuclear RNAs. In: Grosjean H, Benne R, eds. *The Modification and Editing of RNA*. New York: ASM Press. pp 201-227.
- McCloskey JA, Graham DE, Zhou S, Crain PF, Ibba M, Konisky J, Soll D, Olsen GJ. 2001. Post-transcriptional modification in archaeal tRNAs: identities and phylogenetic relations of nucleotides from mesophilic and hyperthermophilic Methanococcales. *Nucleic Acids Res* 29:4699-4706.
- Motorin Y, Keith G, Simon C, Foiret D, Simos G, Hurt E, Grosjean H. 1998. The yeast tRNA:pseudouridine synthase Pus1p displays a multisite substrate specificity. *RNA* 4:856-869.
- Motorin Y, Muller S, Behm-Ansmant I, Branlant C. 2007. Identification of Modified Residues in RNAs by Reverse Transcription-Based Methods. *Methods Enzymol* 425:21-53.
- Muller S, Charpentier B, Branlant C, Leclerc F. 2007a. A Dedicated Computational Approach for the Identification of Archaeal H/ACA sRNAs. *Methods Enzymol* 425:355-387.
- Muller S, Fourmann JB, Loegler C, Charpentier B, Branlant C. 2007b. Identification of determinants in the protein partners aCBF5 and aNOP10 necessary for the tRNA:Psi55-synthase and RNA-guided RNA:Psi-synthase activities. *Nucleic Acids Res* 35:5610-5624.
- Nishikura K, De Robertis EM. 1981. RNA processing in microinjected Xenopus oocytes. Sequential addition of base modifications in the spliced transfer RNA. *J Mol Biol* 145:405-420.
- Omer AD, Lowe TM, Russell AG, Ebhardt H, Eddy SR, Dennis PP. 2000. Homologs of small nucleolar RNAs in Archaea. *Science*, 288:517-522.
- Raychaudhuri S, Niu L, Conrad J, Lane BG, Ofengand J. 1999. Functional effect of deletion and mutation of the Escherichia coli ribosomal RNA and tRNA pseudouridine synthase RluA. *J Biol Chem* 274:18880-18886.
- Renalier MH, Joseph N, Gaspin C, Thebault P, Mougins 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.
- Roovers M, Hale C, Tricot C, Terns MP, Terns RM, Grosjean H, Droogmans L. 2006. Formation of the conserved pseudouridine at position 55 in archaeal tRNA. *Nucleic Acids Res* 34:4293-4301.
- Simos G, Tekotte H, Grosjean H, Segref A, Sharma K, Tollervey D, Hurt EC. 1996. Nuclear pore proteins are involved in the biogenesis of functional tRNA. *EMBO J* 15:2270-2284.
- Sprinzi M, Vassilenko KS. 2005. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 33:D139-140.
- Szweykowska-Kulinska Z, Beier H. 1992. Sequence and structure requirements for the biosynthesis of pseudouridine (psi 35) in plant pre-tRNA(Tyr). *EMBO J* 11:1907-1912.
- Tang TH, Bachelier JP, Rozhdetsvensky T, Bortolin ML, Huber H, Drungowski M, Elge T, Brosius J, Huttenhofer A. 2002. Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. *Proc Natl Acad Sci USA* 99:7536-7541.
- Tomita K, Ueda T, 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.

- Watanabe Y, Gray MW. 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 archaeobacteria. *Nucleic Acids Res* 28:2342-2352.
- Watkins NJ, Gottschalk A, Neubauer G, Kastner B, Fabrizio P, Mann M, Luhrmann R. 1998. Cbf5p, a potential pseudouridine synthase, and Nhp2p, a putative RNA-binding protein, are present together with Gar1p in all H BOX/ACA-motif snoRNPs and constitute a common bipartite structure. *RNA*, 4:1549-1568.
- Wrzesinski J, Bakin A, Ofengand J, Lane BG. 2000. Isolation and properties of *Escherichia coli* 23S-RNA pseudouridine 1911, 1915, 1917 synthase (RluD). *IUBMB Life* 50:33-37.
- Zerfass K, Beier H. 1992. Pseudouridine in the anticodon G psi A of plant cytoplasmic tRNA(Tyr) is required for UAG and UAA suppression in the TMV-specific context. *Nucleic Acids Res* 20:5911-5918.

FIGURE LEGENDS

Figure 1 Panel A: Structures of tRNA^{Tyr}(GUA) Ψ35-specific sRNA guides from *A. pernix*, *M. sedula*, *S. solfataricus*, *S. tokodaii* and *S. acidocaldarius*. Characteristic K-turns and H/ACA sequences are boxed. C/D box sRNAs present at the 3' extremities of Sso1 (sR159), Sac1 (sR4) and Sto1 (sR39) are indicated. Panel B: Proposed base-pairing of Ape7, Mse2, Sso1, Sto1 and Sac1 with their tRNA^{Tyr}(GUA) or LSU RNA targets. An arrow indicates the site of U to Ψ conversion. The distance between the pseudouridylation pocket and K-turn and ACA-box is shown. Panel C: Northern blot analysis of Sso1 expression in *S. solfataricus*. Total RNA was separated on urea – PAAG, RNAs were transferred to Nylon membrane (Hybond N+, GE Healthcare, USA) and hybridized with Sso1-specific [³²P]-labeled oligonucleotide. Molecular weight marker is given on the left. Arrows indicate the positions of free Sso1 sRNA guide (H/ACA) and its C/D-box 3'-extended form (H/ACA+C/D).

Figure 2 Sso1 sRNA-guided modification of Ψ35 in pre-tRNA^{Tyr}(GUA). Panel A: Cloverleaf structure of unmodified tRNA transcript corresponding to *S. solfataricus* tRNA^{Tyr}(GUA) precursor, position of U35 is circled. The sequence of the intron present in the anticodon loop of this tRNA is given in small letters. Panel B: *In vitro* reconstitution tests to reveal Sso1-guided Ψ35 formation. [³²P]ATP-labeled tRNA^{Tyr} precursor was incubated for 90 min at 65°C in the presence of L7Ae/CBF5/NOP10/GAR1 (LCNG), the H/ACA sRNA Sso1 and the four proteins (Sso1/LCNG) and *S. solfataricus* cell-free extract (Sso extract). Additional incubation in the Sso extract was performed with [³²P]ATP-labeled Sso pre-tRNA^{Tyr}(GUA) with U35->C substitution. Modified transcripts were digested by RNase T2 and the resulting 3'-NMPs were analysed by 2D TLC (Keith, 1995). Position of the

pseudouridine spot is circled. The 3'-NMP composition of the RNase T2 digest as well as molar proportion of the formed Ψ residue are indicated on each panel. Panel C: CMCT-RT mapping of the pseudouridine residue formed upon *S. solfataricus* tRNA^{Tyr}(GUA) modification in the reconstituted sRNPs (Sso1/LCNG) and in *S. solfataricus* cell-free extract (Sso extract). Modified RNA was treated by CMCT, for 2, 10 and 20 min with (+) or without (-) subsequent alkaline treatment (OH-) and analyzed 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 Ψ 35.

Figure 3 Panel A: Multiple sequence alignment of Pus7 proteins from various Archaea. Only the regions of Motif IIIa and Motif II containing the catalytic D residue are shown. Positions of the conserved motifs are given for each sequence. Arrows indicate the organisms possessing the tRNA^{Tyr}-specific H/ACA sRNA. Panel B: Differences found in the active sites of Pab Pus7 and Sso Pus7. 3D modelling of the active sites of Pab Pus7 (left) and Sso Pus7 (right) was done using the X-ray structure of *M. masei* TruD (PDB 1Z2Z). Only a highly conserved amino acids are shown.

Figure 4 Enzymatic activity of WT Pab Pus7, Sso Pus7 and Pab extract on different tRNA substrates. Panel A : Cloverleaf structures of tRNA^{Tyr}(GUA) and tRNA^{Asp}(GUC) from *P. abyssi* and tRNA^{Tyr}(GUA) precursor and tRNA^{Asp}(GUC) from *S. solfataricus*. All 5'-UA-3' contexts present in these tRNAs are indicated by arrows. Potential natural sites of Ψ formation (U13 in tRNA^{Asp} and U35 in tRNA^{Tyr}) are circled. Panels B: Results of *in vitro* activity tests performed with two *P. abyssi* tRNA substrates and their U35C and U13C variants. Panel C: Results of *in vitro* activity tests performed with two *S. solfataricus* tRNA substrates and their U35C variant of tRNA^{Tyr}. All

transcripts were [α - 32 P]ATP-labeled and incubated for 90 min with the recombinant proteins (Pab Pus7 or Sso Pus7) at 55°C or with Pab extract at 65°C. Modified RNAs were analysed as described in the legend to Figure 2. Panel D: Control incubations performed at 55°C with Pab Pus7 and *P. abyssi* tRNA^{Tyr} labeled by [α - 32 P]CTP, [α - 32 P]UTP and [α - 32 P]GTP. Incubation conditions as as for Panel B and C.

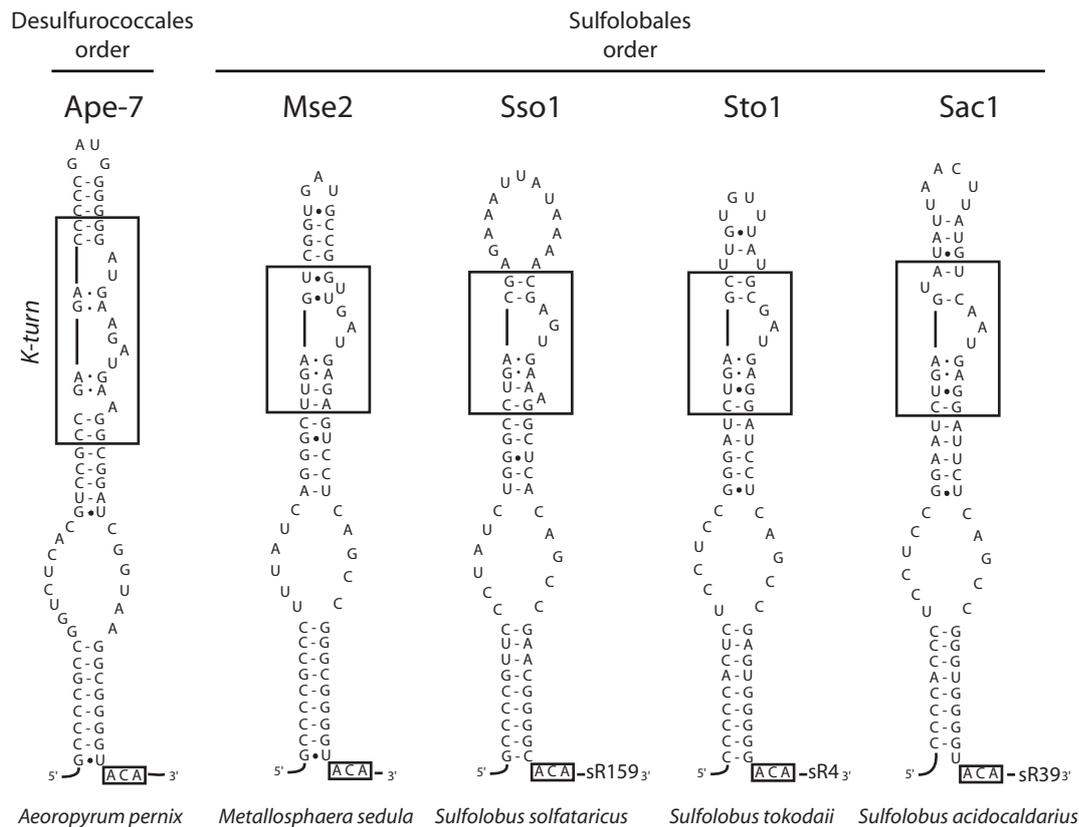
Figure 5 Enzymatic activity of Pab Pus7 and Sso Pus7 protein variants with [α - 32 P]ATP-labeled *P. abyssi* tRNA^{Asp}(GUC). Enzymatic tests were performed as described in the legend of Figure 4, except that point-mutated variants of Pab Pus7 and Sso Pus7 were used. Tests were done at 55°C (Panel A) and at 80°C (Panel B) and a molar ration of formed Ψ residue/tRNA is given in a histogram. Error bars were calculated using experiments done in triplicate and independent quantification of Ψ MP spot relative to four other NMP spots.

Figure 6 Pseudouridine Ψ 35-formation in WT and A36G variants of tRNA^{Tyr} from *P. abyssi* and pre-tRNA^{Tyr} from *S. solfataricus*. Incubations for 90 min with the recombinant Pab Pus7 were performed at 55°C, while the tests with *P. abyssi* (Pab), *S. solfataricus* (Sso) extracts and LCNG/Sso1 sRNP were performed at 65°C (the optimal temperature for cell-free extracts). [α - 32 P]ATP or [α - 32 P]GTP-labeled tRNA transcripts were used. Analysis of Ψ formation was done as described in the legend to Fig. 2. The 3'-NMP composition of the RNase T2 digest as well as molar proportion of the formed Ψ residue are indicated on each panel. Additional spots visible in [α - 32 P]ATP-labeled tRNA incubated in cellular extracts correspond to formation of methylated nucleotides naturally present in many archaeal tRNAs (m¹G37, Cm56, m¹I57 and m¹A58).

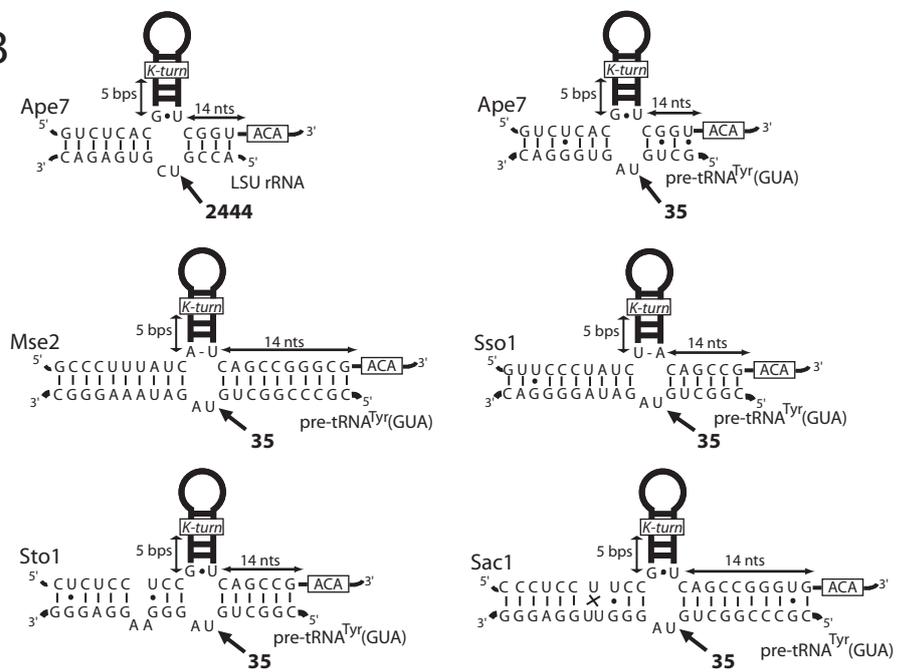
Figure 7 Panel A: Predicted secondary structure of the extended form of Sso1 sRNA containing both H/ACA and C/D motifs. K-turn, ACA-box, as well as C/D and C'/D'

boxes are indicated. Panel B: Predicted base-pairing between Sso1-sR159, Sto1-sR4 and Sac1-sR39 with the corresponding 23S rRNA targets. Two additional potential targets in 23S rRNA for sR159 are shown on the bottom.

A



B



C

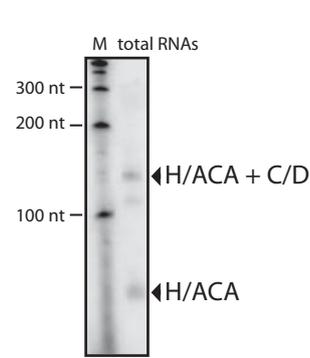


Figure 1

A

	K19	F25		Pab Pus7		D77 R78/H79
	I27	F33		Sso Pus7		D89 A90/N91
<i>M. acetivorans</i>	24	GLGGRLRQ	VEDEFIVKEITN	78	SQKRISVAGTKD	KRALTTQKI
<i>M. mazei</i>	21	GLGGQLRQ	EIEDEFIVKEITN	75	SQKRISVAGTKD	KRALTTQKI
<i>H. salinarium</i>	22	GTGGRLR	DSPADFRVRELEA	82	SRERVRWAGTKD	RHAVTTQLF
<i>A. fulgidus</i>	16	GMGGEIK	AEPEDFYVEEIAE	70	SQKRISFAGTKD	KRALTVQYF
<i>T. acidophilum</i>	14	RPVIRIK	ENPEDFTVVEEVAD	68	SRKRISFAGTKD	KRARKIQYF
<i>T. volcanium</i>	8	EEAIEIK	RDPEDFSVEEIIAD	62	SRKRVTFAGTKD	KRAVKLQYF
<i>P. furiosus</i>	16	GIGGKIK	VYPEDFIVKEVIP	70	HYSEIGFAGTKD	RHAVTYQYI
<i>P. horikoshii</i>	17	GIGGKIK	VYPEDFIVKEVIP	71	HYSEIGFAGTKD	RHAVTYQYI
<i>P. abyssi</i>	13	GIGGRIK	VKPEDFIVREIIP	66	HYSEIGFAGTKD	RHAVTYQYI
<i>M. jannaschii</i>	51	-TGGLIK	KYPEDFIVVEIITP	119	QRKHFGFAGNKD	KYAVTTQRV
<i>M. thermauto.</i>	13	GTGGRIK	VHNRDFEVEEIP-	67	DRRRMGFAGMKD	KRAVTRQWI
→ <i>S. solfataricus</i>	21	NELDGTI	ERPNGFKVIEEID	79	LHSKVNYIGIKD	ANAITSQLV
→ <i>S. tokodaii</i>	14	SPLKVF	INRPDGFVIEEIS	71	LHSKIHYIGIKD	TNAITEQIV
→ <i>S. acidocaldarius</i>	22	EPLQIS	INRPEGFKVIEEIS	79	MKRKIHYLGIKD	ANAITQQII
→ <i>M. sedula</i>	20	ASIRAEI	PRPDGFRVTEEID	81	LGEKPRYLGIKD	TNAVTEQLI
<i>P. aerophilum</i>	18	PSGGVIK	RSPEDFVVEEVLL	86	SHRDVSVGGLKD	TKAVTSQII
→ <i>A. pernix</i>	25	RGPEAL	IMFPWGFVRESRV	77	-CRLYSYAGLKD	ACSVSWQHV

Motif IIIa

Motif II

B

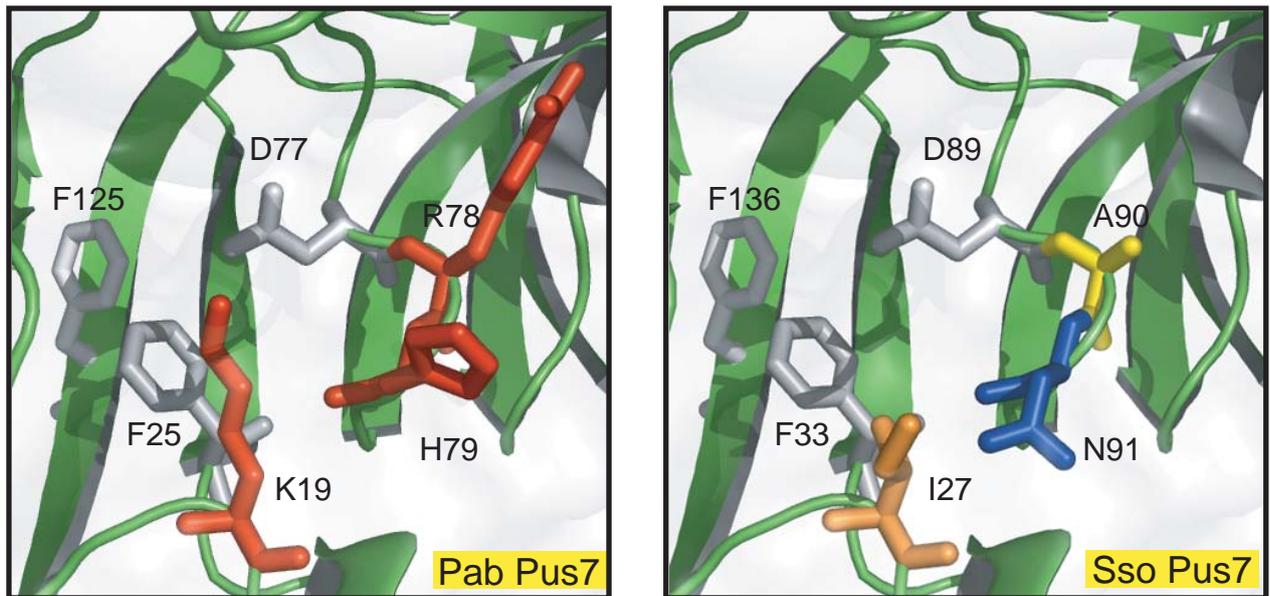


Figure 3

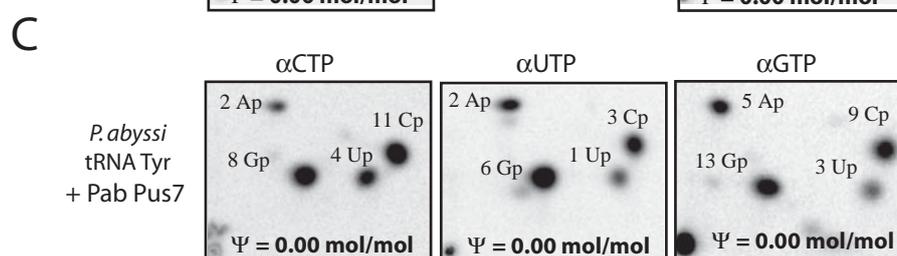
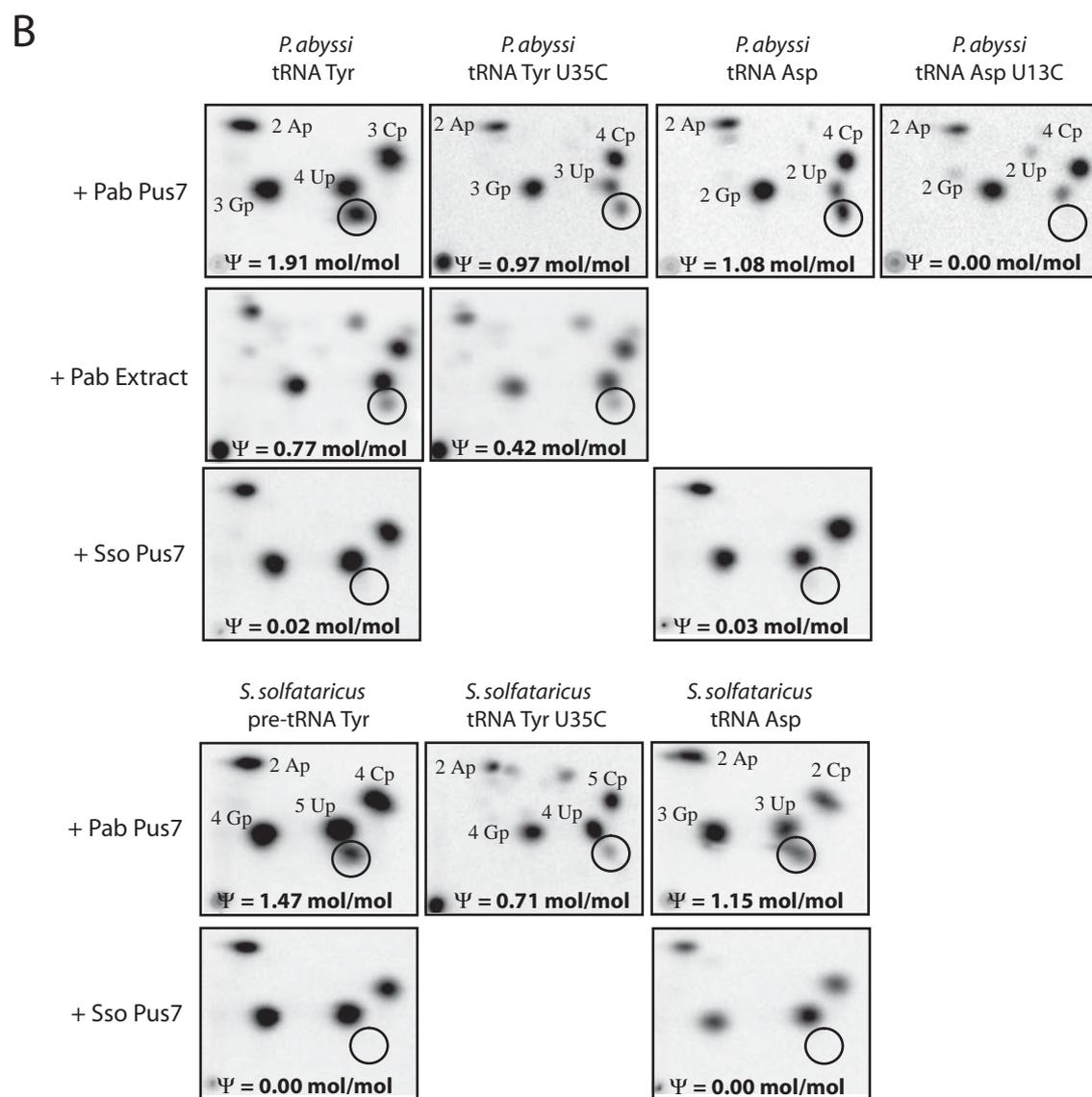
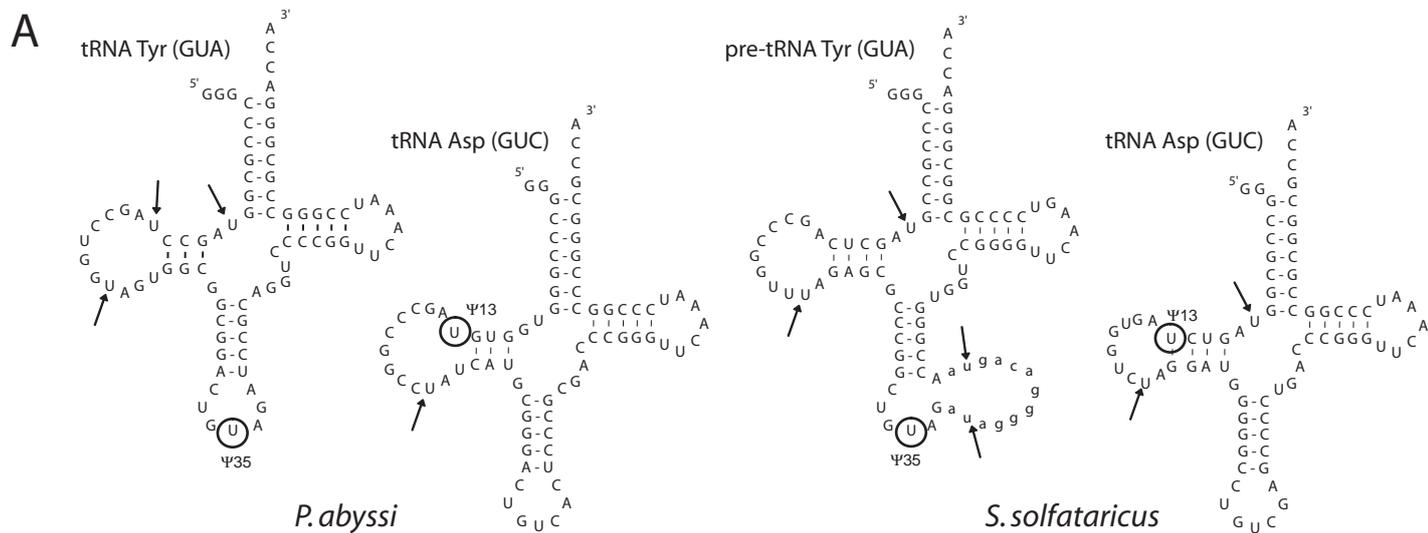
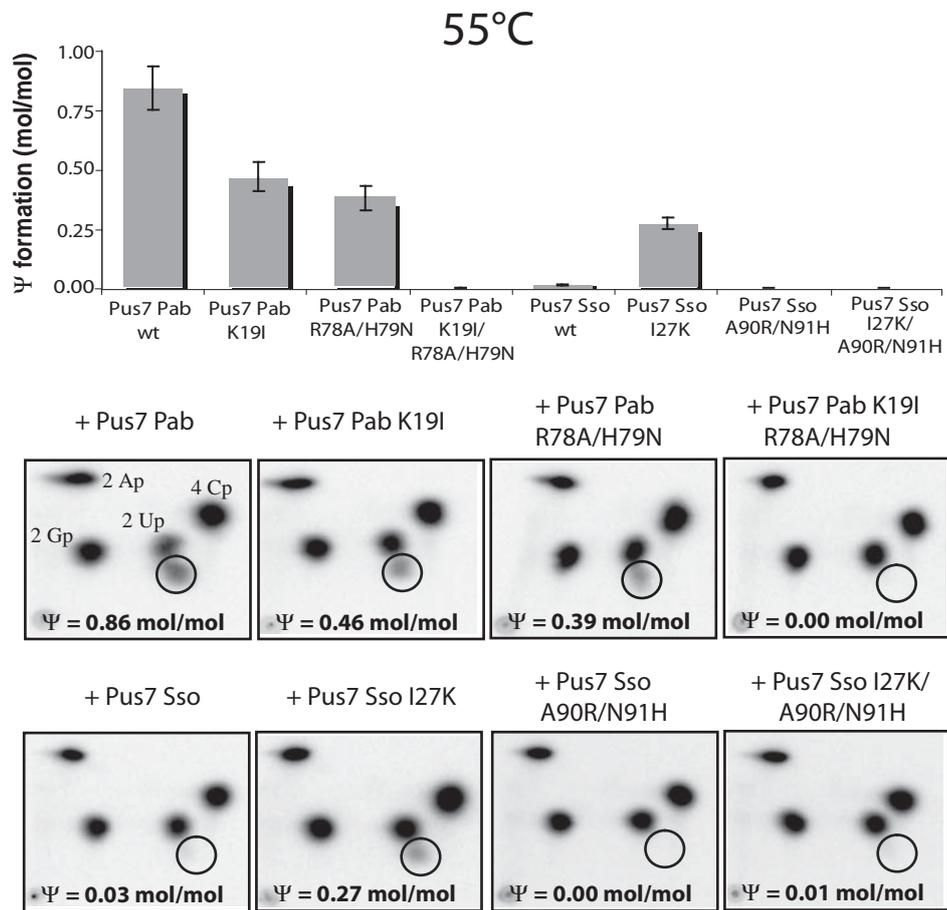


Figure 4

A



B

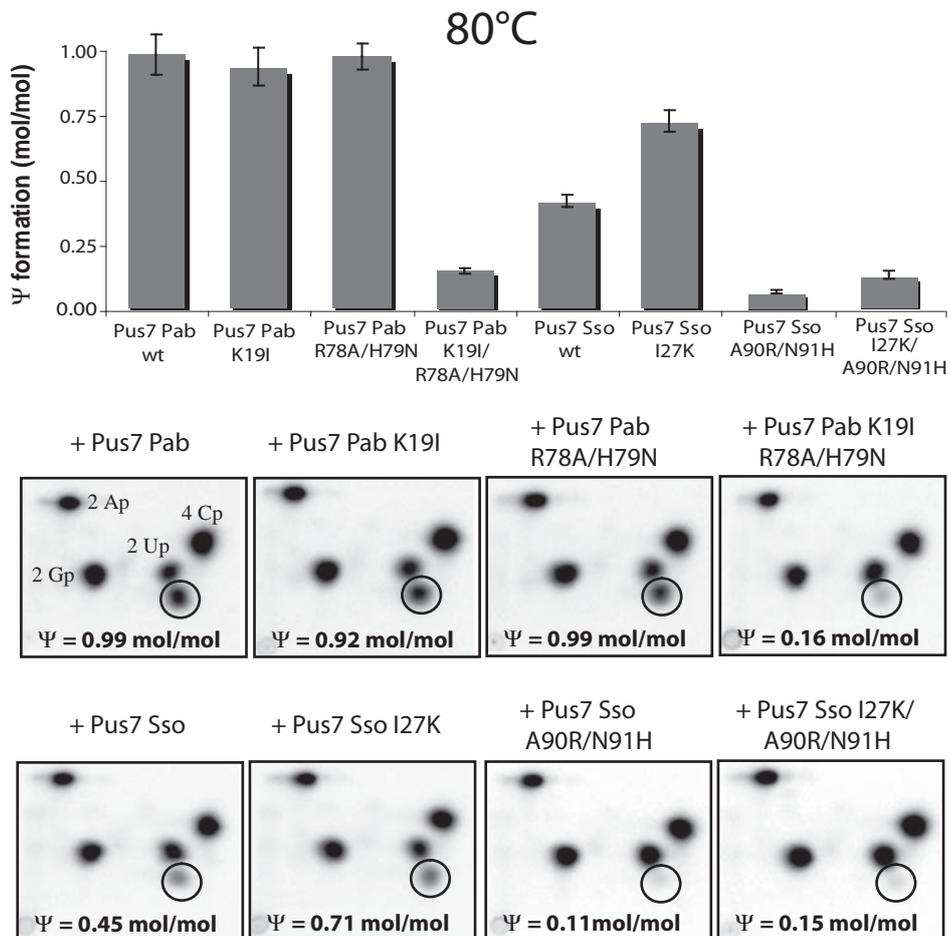


Figure 5

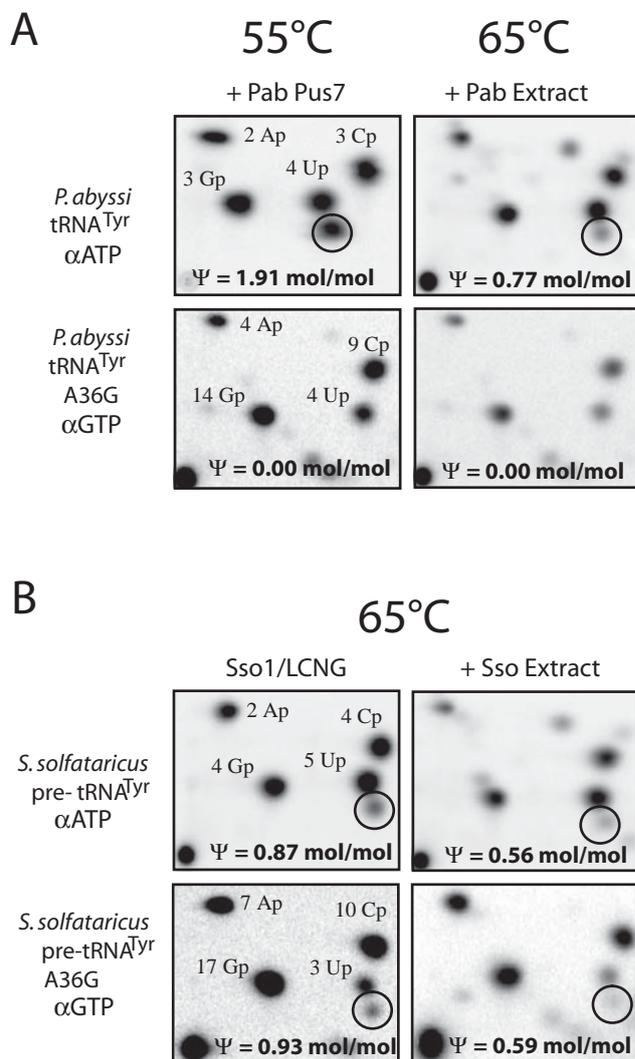


Figure 6

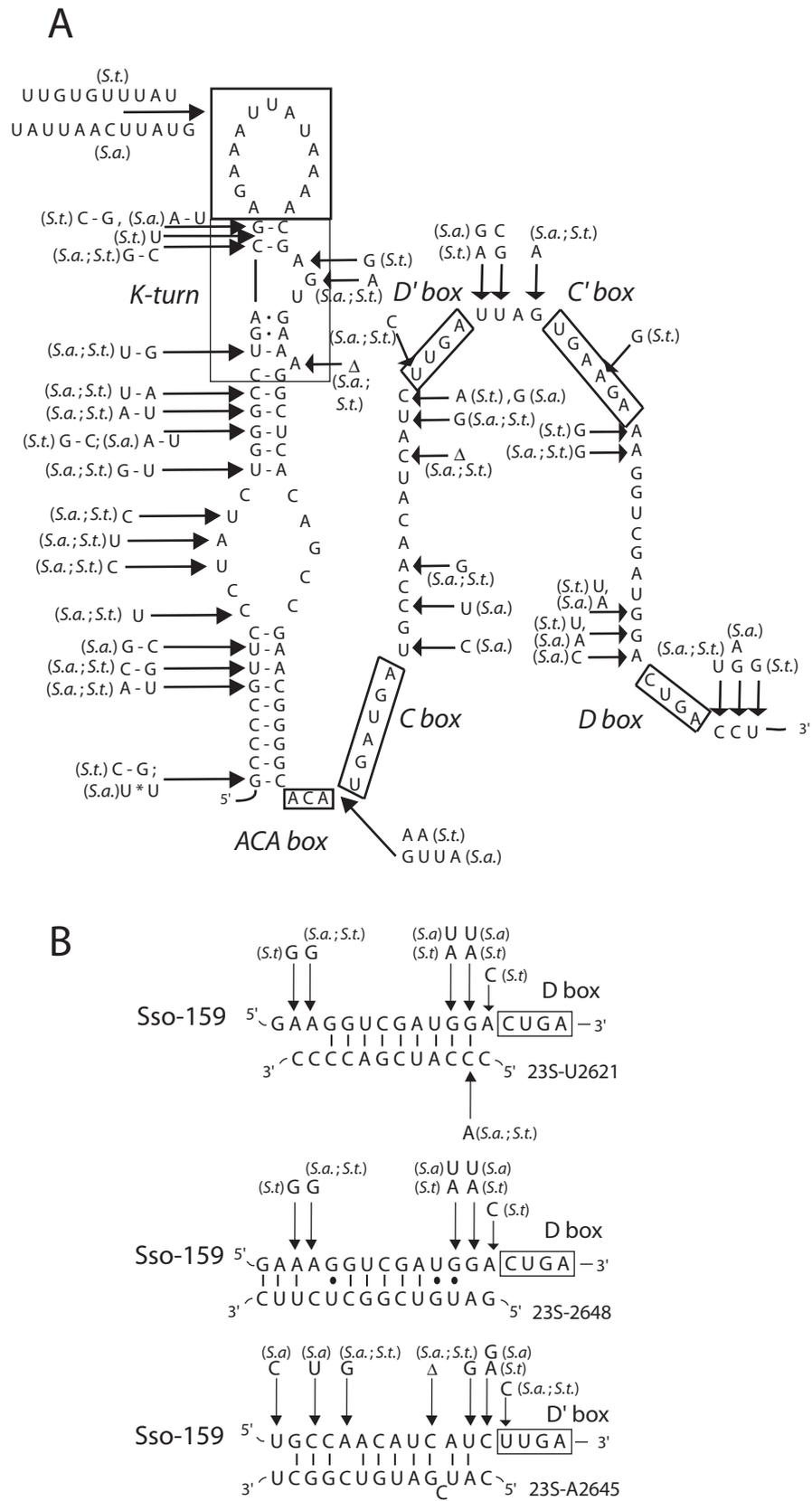


Figure 7