Thermostable RNase P RNAs lacking P18 identified in the *Aquificales*

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ABSTRACT

The RNase P RNA (*rnpB*) and protein (*rnpA*) genes were identified in the two *Aquificales Sulfurihydrogenibium azorense* and *Persephonella marina*. In contrast, neither of the two genes has been found in the sequenced genome of their close relative, *Aquifex aeolicus*. As in most bacteria, the *rnpA* genes of *S. azorense* and *P. marina* are preceded by the *rpmH* gene coding for ribosomal protein L34. This genetic region, including several genes up- and downstream of *rpmH*, is uniquely conserved among all three *Aquificales* strains, except that *rnpA* is missing in *A. aeolicus*. The RNase P RNAs (P RNAs) of *S. azorense* and *P. marina* are active catalysts that can be activated by heterologous bacterial P proteins at low salt. Although the two P RNAs lack helix P18 and thus one of the three major interdomain tertiary contacts, they are more thermostable than *Escherichia coli* P RNA and require higher temperatures for proper folding. Related to their thermostability, both RNAs include a subset of structural idiosyncrasies in their S domains, which were recently demonstrated to determine the folding properties of the thermostable S domain of *Thermus thermophilus* P RNA. Unlike 16S rRNA phylogeny that has placed the *Aquificales* as the deepest lineage of the bacterial phylogenetic tree, RNase P RNA-based phylogeny groups *S. azorense* and *P. marina* with the green sulfur, cyanobacterial, and δ/ε proteobacterial branches.

Keywords: *Aquificales*; *Sulfurihydrogenibium azorense*; *Persephonella marina*; RNase P; tRNA processing

INTRODUCTION

The *Aquificales* are a group of thermophilic bacteria, with *Aquifex aeolicus* being the best-known representative. 16S rRNA, as well as elongation factors Tu and G, phylogenies have suggested that *Aquifex* represents the deepest branch of the bacterial phylogenetic tree (Burggraf et al. 1992; Huber et al. 1992; Bocchetta et al. 2000). However, other phylogenetic approaches analyzing conserved small insertions and deletions in a variety of proteins (Gupta 2000; Griffiths and Gupta 2004) and those based on RNA polymerase β, β′, and σ^{70} subunits (Gruber and Bryant 1998; Klenk et al. 1999) have favored a close affiliation of *Aquifex* with the δ/ε division of the proteobacteria and the *Chlamydia-Cytophaga* group.

Although RNase P is the ubiquitous tRNA 5′-end maturation enzyme found in all kingdoms of life, neither a candidate gene for its RNA (*rnpB*) nor its protein subunit (*rnpA*) has been identified in the sequenced genome of *A. aeolicus* (Deckert et al. 1998; Swanson 2001). Likewise, no RNase P-like activity could be detected in cell lysates of the bacterium (Willkomm et al. 2002), and a recent RNomics approach also failed to reveal an RNase P RNA candidate (Willkomm et al. 2005).

To shed light on tRNA 5′-end maturation in the *Aquificales*, we analyzed close relatives of *A. aeolicus*, namely *Sulfurihydrogenibium azorense* and *Persephonella marina*, for which genome sequences had been made accessible by The Institute for Genomic Research (TIGR). Our results demonstrate that the two bacteria harbor a bacterial type A RNase P RNA, although with deviations from the bacterial consensus, the major one is the lack of P18. Despite the absence of the L18–P8 interdomain contact, both RNase P RNAs are more active and stable than *Escherichia coli* RNase P at higher temperatures (55°C–65°C). The RNase P protein genes (*rnpA*) of *S. azorense* and *P. marina* were identified as well and co-localize with the *rpmH* gene encoding ribosomal protein L34 as in the majority of bacteria (Hartmann and Hartmann 2003).

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Article published online ahead of print. Article and publication date are athttp://www.rnajournal.org/cgi/doi/10.1261/rna.242806.

RESULTS AND DISCUSSION

Identification of *rnpB* genes in *S. azorense* and *P. marina*

RNase P RNA (P RNA) genes (*rnpB*) were identified in the completed but yet unpublished genome sequences of *S. azorense* and *P. marina* (available at TIGR, www.tigr.org) using the program PatScan as described under Materials and Methods.

P RNA structures and kinetics

The secondary structures of the P RNAs from *P. marina* and *S. azorense* are shown in Figure 1. The two P RNAs are highly similar (average pairwise sequence identity ~65% in a structure-based alignment). Besides a few 1- or 2-nucleotide (nt) insertions/deletions, *P. marina* differs from *S. azorense* by two major expansions, one in P12 and the other in P16/17 to create the P16.1 element in the *P. marina* structure. The hallmark of the two *Aquificales* P RNAs is the absence of helix P18, a structural feature originally identified in the genus *Chlorobium* (Haas et al. 1994). Deletion of P18 eliminates one of the three tetraloop-helix interactions (L8/P4, L9/P1 L18/P8) that bridge S- and C-domains (Fig. 1). In both RNAs, helices P1 (16 or 18 bp) and P9 (6 bp) are extended, helix P14 has 10 instead of the usual 9 bp, and *S. azorense* P RNA also lacks the bulged nucleotide close to the base of P14. The apical P12 structures are idiosyncratic, and *P. marina* P RNA has a small stem–loop (P16.1) inserted between helices P16 and P17.

We analyzed the kinetics of the two P RNAs next to *E. coli* P RNA under single turnover conditions (E>>S) at 0.1 M Mg^{2+}, 0.1 M NH_4^+, and pH 6.0. P RNAs were preincubated in assay buffer for 5 min at 55°C and 55 min at 37°C to resolve potential folding traps as observed for the thermostable P RNA of *Thermus thermophilus* (R.K. Hartmann, unpubl.). Indeed, this preincubation protocol eliminated compacted conformers of *S. azorense* and *P. marina* P RNAs, which appeared as fast-migrating bands on native PAA gels (data not shown). In contrast,

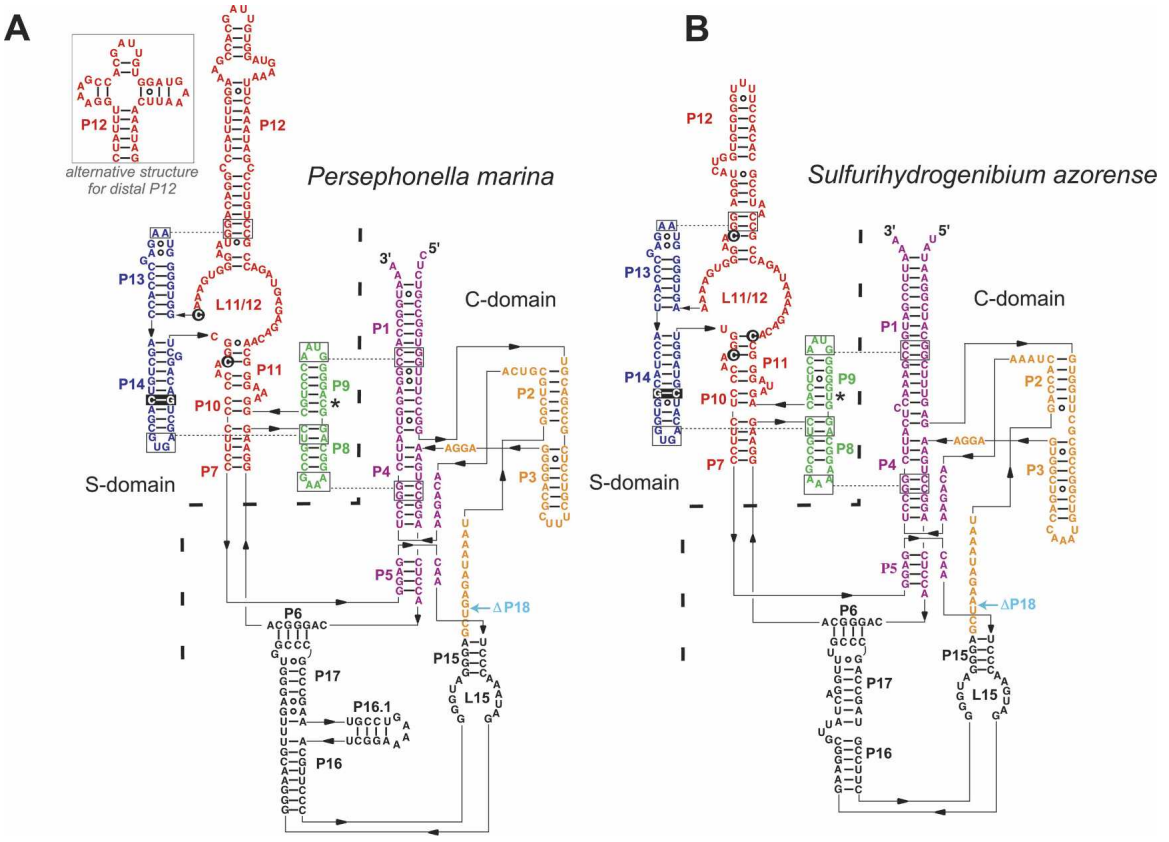

FIGURE 1. Secondary structure presentation of P RNAs from (*A*) *P. marina* (accession no. DQ529278) and (*B*) *S. azorense* (accession no. DQ529245), according to Tsai et al. (2003) and taking into account the data of Torres-Larios et al. (2005). The C-domain (catalytic domain) and the S-domain (specificity domain) are separated by the broken line. (P) Paired regions; (L) loop regions; (open boxes) tertiary contacts, connected by stippled lines. Highlighted nucleotides and asterisk next to helix P9 mark nucleotide identities and structural features also found in the thermostable S domain of *T. thermophilus* P RNA (Baird et al. 2006). For the distal part of P12 in the *P. marina* structure, an alternative cruciform structure is depicted. However, the long P12 stem–loop shown in the main structure contains an internal loop with a stabilizing sarcin-ricin motif in its bulge (5′-AGUA- and 5′–GAA-; Leontis and Westhof 1998; Zhong et al. 2006) and is energetically favored according to thermodynamic calculations (Hofacker 2003).

preincubation only for 1 h at 37°C incompletely (*S. azorense*) or completely (*P. marina*) failed to resolve these conformers. The kinetic results are summarized in Table 1. At 37°C and in comparison with *E. coli* P RNA, the *S. azorense* ribozyme cleaved the precursor $tRNA^{Gly}$ ($ptRNA^{Gly}$) substrate with about the same single turnover $K_{m(sto)}$, but at a three- to fourfold reduced maximum cleavage rate (k_{react}). Catalytic performance of *P. marina* P RNA was lower, with approximately a sevenfold increase in $K_{m(sto)}$ and a 31-fold decrease in k_{react}. Since the two *Aquificales* are thermophilic bacteria (growth optima ~70°C; Götz et al. 2002; Aguiar et al. 2004), we tested processing activity at two additional temperatures (55°C and 65°C), using P RNA concentrations (0.27 μM for *E. coli*, 0.27 μM for *S. azorense*, and 2.0 μM for *P. marina*) around the respective $K_{m(sto)}$ value determined at 37°C (see Table 1). For this purpose, P RNAs were preincubated in assay buffer for 1 h at 55°C or 65°C before addition of substrate and incubation at the same temperature. Whereas the rate of cleavage (k_{obs}) by *E. coli* P RNA decreased from 4.7 min^{-1} at 37°C to 2.8 min^{-1} at 55°C to 1.3 min^{-1} at 65°C, the two *Aquificales* P RNAs showed a higher substrate turnover at 55°C versus 37°C (Table 1). In the case of *S. azorense* P RNA, the cleavage rate at 65°C was still equal to that at 37°C, although substrate affinity is expected to be lower at 65°C than 37°C. Inspection of experimental endpoints (EP, maximum fraction of substrate that could be converted to mature tRNA during extended incubation times) obtained in the individual processing reactions revealed that ≤40% of substrate could be cleaved by *E. coli* P RNA at 65°C. In contrast, endpoints remained normal (>80%) for reactions catalyzed by the two *Aquificales* P RNAs (Table 1). These findings suggest that *E. coli* P RNA, but not *P. marina* and *S. azorense* P RNAs, traps a substantial fraction of the substrate in an inactive conformation at 65°C, which we interpret as an indication of partial un- or misfolding of *E. coli* P RNA at this temperature.

Holoenzyme activity assays

The two *Aquificales* P RNAs, and *E. coli* P RNA as a control, were reconstituted with the *E. coli* or *Bacillus subtilis* P protein and tested for processing activity in the presence of 2 mM Mg^{2+} under multiple turnover conditions at 37°C (Table 1). In general, bacterial P RNAs do not show RNA-alone activity under these conditions. Both *Aquificales* P RNAs could be activated by the two heterologous P proteins, *S. azorense* P RNA more efficiently than *P. marina* P RNA, and both much less efficiently than *E. coli* P RNA. Since the conformation of the *P. marina* P RNA was not uniform at 2 mM Mg^{2+}, as inferred from native PAGE (data not shown), we measured the activity of the reconstituted holoenzymes at 10 mM Mg^{2+}, with the pH lowered to 6.5 to suppress RNA-alone cleavage. Under these conditions, all P RNAs were homogeneously folded according to native PAGE analyses (data not shown), and RNA-alone

TABLE 1. Kinetic data for reactions catalyzed by P RNA alone and hybrid holoenzymes

	37°C				55°C		65°C	
P RNA	$K_{m(sto)}$ (μM)	k_{react} (min^{-1})	k_{obs} (min^{-1})	EP	k_{obs} (min^{-1})	EP	k_{obs} (min^{-1})	EP
E. coli	0.24 (±0.03)	9.7 (±0.6)	4.7 (±0.3)	0.92	2.8 (±0.25)	0.8	1.3 (±0.4)	0.4
S. azorense	0.28 (±0.05)	2.7 (±0.2)	1.4 (±0.1)	0.9	3.1 (±0.4)	0.93	1.25 (±0.2)	0.85
P. marina	1.97 (±0.39)	0.32 (±0.02)	0.15 (±0.01)	0.76	0.6 (±0.01)	0.92	0.06 (±0.01)	0.9

Holoenzymes		k_{obs} (min^{-1})	
P RNA	+ Protein	2 mM Mg^{2+}	10 mM Mg^{2+}
E. coli	*E. coli*	3.5 (±0.3)	4.8 (±0.58)
	B. subtilis	4.2 (±0.2)	4.8 (±1.1)
S. azorense	*E. coli*	0.05 (±0.003)	0.43 (±0.05)
	B. subtilis	0.24 (±0.06)	0.47 (±0.04)
P. marina	*E. coli*	0.028 (±0.003)	0.56 (±0.16)
	B. subtilis	0.014 (±0.001)	0.70 (±0.12)

RNA-alone reactions were performed under single-turnover conditions (E≫S) using trace amounts (<1 nM) of 5′-end labeled *T. thermophilus* $ptRNA^{Gly}$ as substrate in a reaction buffer containing 100 mM $Mg(OAc)_2$, 100 mM NH_4OAc, 0.1 mM EDTA, 50 mM MES pH 6.0 at the indicated temperatures; processing reactions were started by combining enzyme and substrate solutions; $K_{m(sto)}$ and k_{react} are the single turnover K_m and v_{max} values, k_{obs} values designate the cleavage rate at a single P RNA concentration (0.27 mM for *E. coli*, 0.27 mM for *S. azorense*, and 2.0 mM for *P. marina* RNase P RNA), and EP is the experimental endpoint (maximum fraction of substrate that could be converted to mature tRNA during extended incubation times). Assay conditions for holoenzyme reactions, performed under multiple turnover conditions: 150 mM NH_4OAc, 2 mM spermidine, 0.05 mM spermine, 4 mM β-mercaptoethanol, 10 nM P RNA, 40 nM P protein, and 100 nM $ptRNA^{Gly}$, 2 or 10 mM $Mg(OAc)_2$, 20 mM Hepes pH 7.4 (37°C) with 2 mM Mg^{2+}, and pH 6.5 with 10 mM Mg^{2+}; under holoenzyme conditions, no RNA-alone activity was observed in the time window of the assay, except for *E. coli* RNA alone at 10 mM Mg^{2+} (k_{obs} = 0.14 min^{-1}). In the holoenzyme reactions, k_{obs} is given as nanomoles of substrate converted per nanomole of RNase P RNA per minute. All values are based on at least three independent experiments. For more details, see Materials and Methods.

activity was low for the *E. coli* and not detectable for the *P. marina* and *S. azorense* P RNAs. At 10 mM Mg^{2+}, the activity of holoenzymes containing the *Aquificales* P RNAs increased relative to those with *E. coli* P RNA, and activity profiles for the two *Aquificales* P RNAs were now reversed, the chimeric *P. marina* holoenzymes being more active than the *S. azorense* counterparts. Our results illustrate that the Mg^{2+} requirements for folding and catalysis of the three P RNAs differ markedly, being highest for *P. marina* and higher for the *Aquificales* than for *E. coli* P RNA. We additionally tested if the *P. marina* and *S. azorense rnpB* genes might be able to replace the native *rnpB* gene in the *B. subtilis* mutant strain SSB318 (Wegscheid et al. 2006), but the results were negative (data not shown). Altogether, these results support the notion that the two *Aquificales* strains encode a canonical bacterial type A RNase P holoenzyme, however, with idiosyncratic features.

Identification of *rnpA* genes

The *rnpA* genes encoding the protein subunit of RNase P in *P. marina* and *S. azorense* were identified (Fig. 2A) by using a local version of tblastn (see Materials and Methods). Both *rnpA* genes are sandwiched between the *rpmH* gene encoding ribosomal protein L34 and the downstream *yidD-YidC* genes (Fig. 2B), which represent the genetic context found in most bacteria (Hartmann and Hartmann 2003). In the case of *S. azorense rnpA*, the only candidate start codon is a CUG immediately upstream of the *rpmH* stop codon (data not shown); CUG has been described as a low-efficiency start codon (O'Donnell and Janssen 2001). We also considered the possibility of a sequencing error in an A_9 stretch at the beginning of *S. azorense rnpA* (nucleotides 19–27 of *rnpA*, accession no. DQ529246). We thus sequenced a PCR fragment derived from *S. azorense* genomic DNA. However, the sequence as provided by TIGR turned out to be correct. An alignment with other bacterial P proteins (Fig. 2A) supported our annotation of *rnpA* genes.

Thermostability of *Aquificales* P RNAs

Thermostability of *P. marina* and *S. azorense* P RNAs despite the absence of P18 is a surprising finding of this study. The apical loop of P18 docks to P8 as one of the three major tertiary interactions (L8/P4, L18/P8, L9/P1) that bridge S- and C-domains (Fig. 1) in bacterial type A RNAs. The only other known bacterial P RNAs lacking P18 are from mesophilic *Chlorobia* (Haas et al. 1994), where RNA stability is expected to be less crucial than in thermophiles. Deletion of P18 from *E. coli* P RNA caused a 60-fold increase in K_m under multiple turnover conditions at 25 mM Mg^{2+} and 1 M NH_4^+ (Haas et al. 1994). The absence of the L18/P8 contact in the two *Aquificales* P RNAs may be compensated by strengthening the L9/P1 contact. Evidence for this possibility stems from the observation that helices P1 can be extended to 16 and 18 bp, respectively (Fig. 1), and P9 to 6 bp in both RNAs. Mesophilic P RNAs usually have 11 bp in P1 and 4–5 bp in P9, including a bulged nucleotide near the base of the stem in P1 as well as P9, which interrupts helix continuity (Massire et al. 1998; Fig. 3). Helix P9 is also stabilized in other thermostable P RNAs (5 bp and deletion of the bulge in *Thermotoga maritima*, and 5 G-C bp in *T. thermophilus*), as is P1 (12–14 bp in *T. thermophilus* and *T. aquaticus*, absence of the nucleotide bulge in P1 of *T. maritima*) (Massire et al. 1998; Fig. 3). Furthermore, the two *Aquificales* and

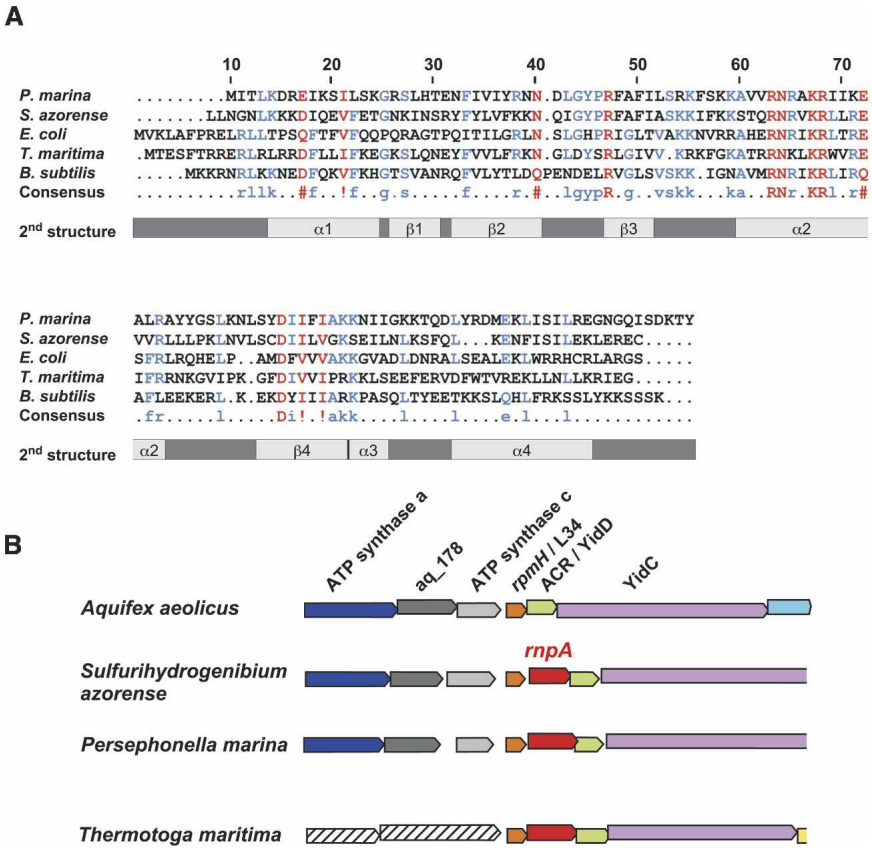

FIGURE 2. (*A*) Alignment of *S. azorense* and *P. marina* P protein sequences (accession nos. DQ529246 and DQ529279) with those of selected bacteria. (Red) Amino acid or type of amino acid conserved in all five sequences; (blue) conserved in at least three of the five sequences; (!)I or V; (#)D, E, N, or Q. Secondary structure elements are shown for the *T. maritima* P protein (Kazantsev et al. 2003). (*B*) Schematic illustration of the *rpmH/rnpA* gene context in the three *Aquificales* strains relative to *T. maritima* representing the mainstream of bacteria; accession nos. for the genomic regions of *S. azorense* and *P. marina*: DQ529246 and DQ529279.

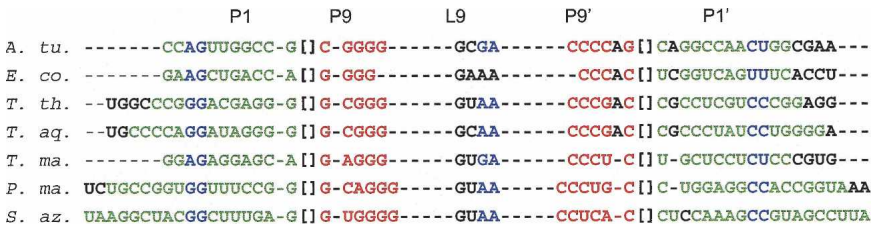

FIGURE 3. Partial alignment of P1, P9, and L9 sequences (P1 and P9 designate the 5′ halves and P1′ and P9′ the 3′ halves of the respective helix), according to Massire et al. (1998). (Blue) Nucleotides that take part in the tetraloop-helix interaction; (green) canonical pairings, including G-U pairs, within P1; (red) canonical pairings, including G-U pairs, within P9; (black) unpaired nucleotides, and all L9 nucleotides in *E. coli* RNase P RNA, as the geometry of this tertiary contact is unclear (Massire et al. 1998). RNase P RNAs from the two *Thermus* and the two *Aquificales* species are the only known examples of apical L9 5′-GYAA tetraloops interacting with two consecutive G-C bp in P1. (*A. tu.*) *Agrobacterium tumefaciens*, (*E. co.*) *E. coli*, (*T. th.*) *T. thermophilus*, (*T. aq.*) *T. aquaticus*, (*T. ma.*) *T. maritima*, (*P. ma.*) *P. marina*, (*S. az.*) *S. azorense*.

Thermus P RNAs share the feature of a 5′-GYAA L9 tetraloop and a P1 receptor site consisting of a G-C bp tandem, a combination not present in other bacteria (Massire et al. 1998; Fig. 3). Increased stiffness of P9 and its P1 receptor region in *P. marina*, *S. azorense*, and *Thermus* may stabilize the L9/P1 interaction, which in turn may partially compensate for the absence of the L18/P8 contact in the two *Aquificales* RNAs.

Tao Pan and coworkers have introduced 12 key base identities of the *T. thermophilus* S domain into that of *E. coli* (Baird et al. 2006). The *E. coli* S-domain mutant gained the same stability and Mg^{2+}-dependent folding cooperativity as the *T. thermophilus* S domain. Subsets of these *T. thermophilus* features are present in the two *Aquificales* P RNAs (Fig. 1, highlighted nucleotides, asterisk next to P9). This includes, for example, the stabilization of P9, P11, and P14 (for details, see Baird et al. 2006). These structural similarities to *T. thermophilus* P RNA may contribute as well to thermostability of the two *Aquificales* P RNAs.

Phylogenetic aspects

So far, no candidate genes for the RNA (*rnpB*) or the protein subunit (*rnpA*) have been identified in the sequenced genome of *A. aeolicus* (Deckert et al. 1998; Swanson 2001). However, in *S. azorense* and *P. marina*, *rnpA* is encoded with *rpmH*, as in the majority of bacteria. Surprisingly, the genetic context of *rpmH* genes in *A. aeolicus, S. azorense*, and *P. marina* is very similar except for the absence of *rnpA* in *A. aeolicus.* This not only holds for *yidD* and *yidC* downstream of *rpmH/rnpA*, a conserved trait in most bacteria, but also for the *rpmH* upstream region (Fig. 2B). Here, all three *Aquificales* encode a conserved hypothetical protein (aq_178 in *A. aeolicus*) and an ATP synthase subunit, a constellation not observed outside the *Aquificales* (Hartmann and Hartmann 2003). Thus, the apparent loss of genes for bacterial-like RNase P components is confined to the genus *Aquifex* or the *Aquificaceae*. We further inspected the genetic context of *rnpB* genes in *S. azorense* and *P. marina*, hoping that a conserved context may guide us to a so far unidentified *rnpB* gene in *A. aeolicus*. However, the context of *rnpB* already deviates entirely between *S. azorense* and *P. marina* (data not shown).

A number of phylogenies, in particular the one based on 16S rRNA, have positioned *Aquifex* and the *Aquificales* as the deepest branch of the bacterial phylogenetic tree (Burggraf et al. 1992; Huber et al. 1992; Bocchetta et al. 2000). Phylogenetic trees on the basis of RNase P RNA, which we constructed from a structure-based sequence alignment (Massire et al. 1998) using the program fastDNAml, unexpectedly did not corroborate an early origin of the *Aquificales* P RNAs (data not shown). With substantial variation in detail caused by small changes in the alignment, the P RNA trees all grouped the *Aquificales* with the green sulfur, cyanobacterial, and δ or ε proteobacterial branches, in general agreement with several protein-based phylogenies (Gruber and Bryant 1998; Klenk et al. 1999; Gupta 2000; Griffiths and Gupta 2004). The lack of P18 in the *P. marina* and *S. azorense* P RNAs, a feature they share with Archaea and Eukaryotes, thus seems to represent not an ancient, but a derived, trait.

MATERIALS AND METHODS

Bioinformatic analyses

Genes encoding RNase P RNA (*rnpB*) were identified in the completed but yet unpublished genome sequences of *S. azorense* and *P. marina* (available at TIGR, www.tigr.org) using PatScan (Dsouza et al. 1997). The pattern to describe the RNase P RNA-encoding genes consisted of several conserved sequence and structure-based pattern units. There are two main conserved sequence elements in the core structure: 5′-GAGGAANNUCNNNNC (designated as CR I) (Chen and Pace 1997) and 5′-AGNNNNAU…{2–67 nt}…ACANAA (designated as CR IV) (Chen and Pace 1997). These conserved sequence elements were surrounded by additional conserved structural elements to reduce the amount of false-positive hits. The pattern description was ("/" stands for line break):

%RNaseP Pattern description File

r1={au,ua,gc,cg,gu,ug}/p2=6…7/(G|3…4)/p3=1…6/3…72/r1∼p3[1,0,0]/GAGGA/p4=ANN/U/p6=CNNNN/C/p5=3…4/1…27/p7=3…7/50…300/r1∼p7[1,0,0]/r1∼p5[1,0,0]/NAA/p15=2…4/4…95/r1∼p15/G/2…67/AGNNNNAU/r1∼p2[1,0,0]/2…60/ACANAA/r1∼p6[1,0,0]/r1∼p4[1,0,0]/A

For calculation of phylogenetic trees, we structurally aligned the RNase P RNA sequences of *P. marina* and *S. azorense* and—as outgroups—*Homo sapiens* and *Thermococcus litoralis* or *Sulfolobus acidocaldarius* to the structural alignment of bacterial RNase P RNA sequences from Massire et al. (1998), using ConStruct (Luck et al. 1999) and making manual adjustments. After removal of all partial sequences (e.g., those lacking P1), phylogenetic trees were predicted using fastDNAml (Felsenstein 1981; Olsen et al. 1994).

The *rnpA* genes encoding the protein subunit of bacterial RNase P were identified by using a local version of tblastn, which is part of the BLAST package (Altschul et al. 1990). The RNase P protein sequence from *E. coli* (Acc. AAC76727) was used as a query to search for potential *rnpA* coding regions in the provided genomes. Finally, the resulting sequence(s) were used to query the NCBI BLAST servers for validation.

Secondary structure predictions were performed using Mfold (Zuker 2003). ClustalW (www.ebi.ac.uk/clustalw) was used for nucleic acid sequence alignments, and protein sequences were aligned with MultAlin (Corpet 1988).

Cloning of RNase P RNA candidates

For the construction of plasmids pUC19T7*Pma_rnpB* and pUC19T7*Saz_rnpB*, to be used as templates for in vitro transcription of the two P RNAs, respective DNA sequences were amplified by genomic PCR using primers 5′-GCGGGATCC*TAATACGACTCACTATAGG*ATATCTCTGCCGGTGGTTTCC-3′ and 5′-CGCCTTAAGACCGGTGGCCTCCAGTAA-3′ for *P. marina*, and 5′GCGGGATCC*TAATACGACTCACTATAGG*TATAAGGCTACGGCTTTGAG-3′ and 5′-CGCCTTAAGTTTAAGGCTACGGCTTTGGAG-3′ for *S. azorense*. PCR fragments were cloned into pUC19 via EcoRI and BamHI sites included in the primers (underlined); the sense primers additionally introduced a T7 promoter (in italics).

In vitro transcription and 5′-end labeling

RNAs were produced by run-off transcription with T7 RNA polymerase and subsequent gel purification as described in Busch et al. (2000). The substrate, *T. thermophilus* ptRNA^{Gly}, was transcribed from plasmid pSBpt3′HH (Busch et al. 2000), and *E. coli* P RNA from plasmid pDW98 (Smith et al. 1992) linearized with BsaAI. *S. azorense* and *P. marina* P RNAs were transcribed from plasmids pUC19T7*Saz_rnpB* and pUC19T7*Pma_rnpB*, both linearized with EcoRI, resulting in 20 and 17 extra nucleotides, respectively, beyond the 3′ ends shown in Figure 1. Substrate 5′-end labeling with [γ-^{32}P]-ATP and T4 polynucleotide kinase was performed as detailed in Heide et al. (1999).

Sequencing of PCR products and recombinant plasmids

DNA sequencing was performed by MWG-BIOTECH AG (Ebersberg, Germany).

Preparation of recombinant RNase P proteins

E. coli and *B. subtilis* RNase P proteins carrying an N-terminal His-tag (His-tagged peptide leader: MRGSHHHHHHGS, encoded in plasmid pQE-30 in *E. coli* JM109) were expressed essentially as described (Rivera-León et al. 1995). Cell cultures were grown in LB broth containing 100 μg/mL ampicillin to an OD_{600} of 0.6, and IPTG was added to a final concentration of 1 mM. Cells were harvested at 4°C after another 3 h of growth (OD_{600} ~2.5). The following steps were performed at 4°C or on ice, and all buffers contained 40 μg/mL of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Cells were suspended in 10–15 mL sonication buffer SB (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 0.1% triton X-100, 1 M NH_4Cl). After sonication (Branson Sonifier 250, output 20, duty cycle 50%, 15 min on ice), the sample was centrifuged for 30 min (4°C, 14,500*g*), and the supernatant was mixed with Ni-NTA agarose (400 μL for 2 L of cell culture), which had been prewashed twice with 10 mL SB buffer. The sample was then incubated for 1–2 h at 4°C under gentle mixing or rotating. The Ni-NTA agarose slurry was washed three times (centrifugation–resuspension cycles; centrifugation at 4°C and 8500 rpm in a desktop centrifuge) with ice-cold washing buffer (30 mM imidazol, 50 mM Tris-HCl, pH 8.0, 8 M urea, 0.1% triton X-100). Proteins were then eluted with 500 μL elution buffer (50 mM Tris-HCl, pH 7.0, 10% glycerol, 7 M urea, 20 mM EDTA, 0.3 M imidazol) for 45 min at 4°C under gentle shaking. Eluates were dialyzed twice for 1 h and subsequently overnight against volumes of 500 mL dialysis buffer (50 mM Tris-HCl, pH 7.0, 0.1 M NaCl, 10% glycerol, dialysis bags: Roth, molecular weight cut-off 12–14 kDa); during dialysis, a white precipitate formed. The contents of the dialysis tubes were transferred to a 2 mL Eppendorf tube and centrifuged for 20 min at 4°C and 12,000 rpm in a desktop centrifuge. The supernatant contained RNase P protein devoid of any P RNA contamination, whereas the pellet included traces of P RNA and was therefore discarded. All purification steps were monitored by 17% SDS-PAGE to assess the purity and concentration of RNase P proteins.

Kinetics

For single-turnover RNA-alone reactions, trace amounts (<1 nM) of 5′-end labeled *T. thermophilus* ptRNA^{Gly} substrate were preincubated in reaction buffer (100 mM $Mg[OAc]_2$, 100 mM NH_4OAc, 0.1 mM EDTA, 50 mM MES pH 6.0 [37°]) for 5 min at 55°C and 25 min at 37°C. Likewise, P RNAs were preincubated in the same buffer for 5 min at 55°C and 55 min at 37°C. Processing reactions were started by combining enzyme and substrate solutions and assayed at 37°C. For processing assays at 55°C and 65°C, P RNA solutions were preincubated for 1 h at 55°C or 65°C.

For holoenzyme cleavage assays, buffer KN (20 mM Hepes-KOH, pH 7.4, 2 mM $Mg[OAc]_2$, 150 mM NH_4OAc, 2 mM spermidine, 0.05 mM spermine, and 4 mM β-mercaptoethanol) (Dinos et al. 2004) was used to closely mimic physiological conditions. In addition, these assays were performed in the identical buffer, but at pH 6.5 and 10 mM $Mg[OAc]_2$. In vitro reconstitution of RNase P holoenzymes was performed as follows: RNase P RNAs were incubated in buffer KN for 5 min at 55°C and 50 min at 37°C, after which RNase P protein was added, followed by another 5 min at 37°C before addition of substrate. Aliquots of the cleavage reactions were withdrawn at various time points and analyzed by electrophoresis on 20% polyacrylamide/8 M urea gels. Data analysis and calculations were performed essentially as previously described (Busch et al. 2000).

ACKNOWLEDGMENTS

We thank the Deutsche Forschungsgemeinschaft for financial support (DFG HA1672/13-1) and the Wilhelm-Hahn-und-Erben-Stiftung for defraying publication charges. We also thank Anna-Louise Reysenbach (Portland, Oregon, USA) for the gift of *S. azorense* and *P. marina* genomic DNA, TIGR for making genomic sequences of the two organisms available before publication, and the reviewers for insightful comments.

Received July 28, 2006; accepted August 26, 2006.

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Thermostable RNase P RNAs lacking P18 identified in the *Aquificales*

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RNA 2006 12: 1915-1921 originally published online September 27, 2006
Access the most recent version at doi:10.1261/rna.242806