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The RNase P RNA from cyanobacteria: short tandemly repeated repetitive (STRR) sequences are present within the RNase P RNA gene in heterocyst-forming cyanobacteria

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ABSTRACT

The RNase P RNA gene (*rnpB*) from 10 cyanobacteria has been characterized. These new RNAs, together with the previously available ones, provide a comprehensive data set of RNase P RNA from diverse cyanobacterial lineages. All heterocystous cyanobacteria, but none of the non-heterocystous strains analyzed, contain short tandemly repeated repetitive (STRR) sequences that increase the length of helix P12. Site-directed mutagenesis experiments indicate that the STRR sequences are not required for catalytic activity *in vitro*. STRR sequences seem to have recently and independently invaded the RNase P RNA genes in heterocyst-forming cyanobacteria because closely related strains contain unrelated STRR sequences. Most cyanobacteria RNase P RNAs lack the sequence GGU in the loop connecting helices P15 and P16 that has been established to interact with the 3′-end CCA in precursor tRNA substrates in other bacteria. This character is shared with plastid RNase P RNA. Helix P6 is longer than usual in most cyanobacteria as well as in plastid RNase P RNA.

INTRODUCTION

RNase P is a ubiquitous ribonucleoproteic enzyme responsible for generation of the mature 5′-end of tRNA from precursor molecules by a single endonucleolytic cleavage (1–4). In bacteria the enzyme is composed of a single RNA molecule of 350–450 nt and a small basic protein. The RNA subunit is the catalytic subunit and it can cleave its substrates *in vitro* in the absence of protein under appropriate buffer conditions (5). This catalytic RNA has been extensively studied and a large set of sequences has been obtained (6). On the basis of this information as well as on biochemical data, secondary and tertiary structure models have been proposed (7–10). The RNase P RNA subunit has been characterized in several cyanobacteria (11–13). From this limited data set it could be concluded that cyanobacterial RNase P RNA conforms to the model proposed for bacterial RNase P but has several peculiarities, the most surprising being the presence of short tandemly repeated repetitive (STRR) sequences (14) within the coding sequence of *Anabaena* sp. PCC 7120 and *Calothrix* sp. PCC 7601 RNase P RNA (12). The STRR sequences increase the length of helix P12 (Fig. 1). In this work we describe 10 additional RNase P RNA sequences from diverse cyanobacteria that represent the main evolutionary lineages of this group of bacteria. The new sequences described here, together with those previously available from cyanobacteria and plastids, represent a comprehensive set of this broad group of organisms. All heterocystous strains contain STRR sequences that increase the length of helix P12. Deletion of the STRR sequences does not significantly affect *in vitro* enzymatic activity of the RNAs.

Most of the cyanobacterial RNase P RNAs lack a GGU sequence in the loop connecting P15 with P16 postulated to interact with the 3′-end RCCA sequence of substrates (15,16). This character is shared with the plastid RNase P RNAs and suggest a different mechanism of substrate interaction.

Furthermore, the comparative analysis described here supports an extension of helix P6 from 4 to 7 bp in most cyanobacteria.

MATERIALS AND METHODS

PCR amplification and sequencing of cyanobacteria *rnpB* genes

DNA was obtained from *Synechocystis* sp. PCC 6308, *Anabaena* sp. ATCC 29413, *Fischerella* sp. UTEX 1829, *Nostoc* sp. PCC 7107 and *Nostoc* sp. PCC 7413 as described (17) and 1 ng used for PCR reactions. PCR was done on whole cells in strains *Dermocarpa* sp. PCC 7437, *Oscillatoria* sp. PCC 7515, *Synechococcus* sp. PCC 6717, *Synechococcus* sp. PCC 7001 and *Synechococcus* sp. PCC 7003. In this case a small number of cells were dispersed in TE buffer and heated at 95°C for 10 min, centrifuged briefly and an aliquot of the supernatant used for PCR. PCR was done with *Taq* DNA polymerase and using degenerate oligonucleotide primers complementary to highly conserved sequences located near the 5′- and 3′-ends of bacterial RNase P RNA genes. The primers used were 59F (5′-GIIGAGGAAAGTC-CIIGCT-3′) and 347R (5′-RTAAGCCGGRTTCTGT-3′) (7; Fig. 1). The PCR products were purified on a 3% agarose gel and directly cloned in pGEM-T (Promega) and both strands sequenced with Sequenase 2.0 (US Biochemicals). To make sure that the sequences obtained did not contain mutations introduced by the

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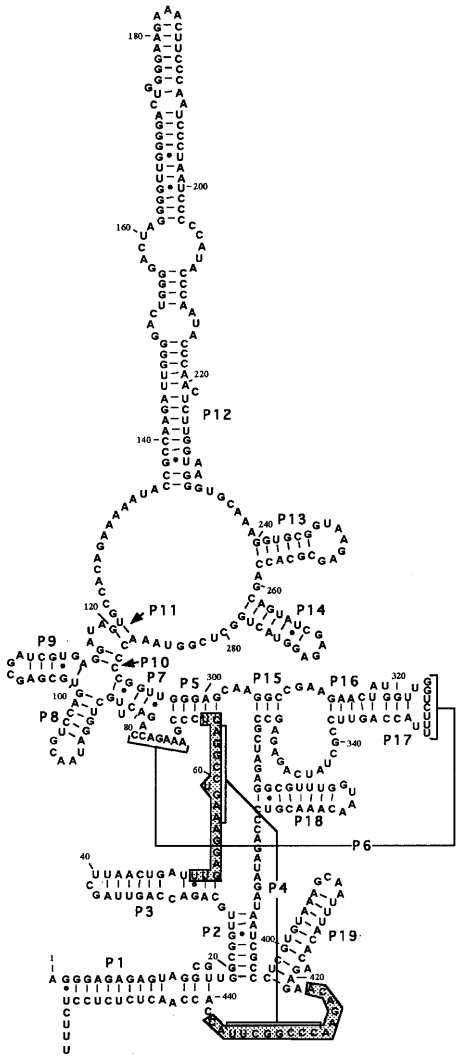

Figure 1. *Anabaena* sp. PCC 7120 RNase P RNA secondary structure model. Helices are designated according to Haas *et al*. (7). The two highly conserved regions used for PCR amplification are shaded.

PCR procedure, two independent amplifications were done for each strain and two clones of each were sequenced separately.

Sequence and structure analysis

All the available RNase P RNA sequences from cyanobacteria and plastids (Table 1) were aligned manually based on the conserved secondary structure elements. Sequences derived from PCR primers as well as the variable sequences in helix P12 (see Results) were excluded from the alignment. A phylogenetic tree based on the aligned RNase P RNA sequences was generated using the maximum likehood procedure of the PHYLIP package (18). All the RNase P RNA sequences and secondary structures are available from the Ribonuclease P (http://www.mbio.ncsu.edu/RNaseP/) (6), GenBank and EMBL databases. The accession numbers for the new sequences described in this work are X97388–X97397.

Table 1. Source of cyanobacteria and plastid RNase P RNA sequences

Organism	Accession no.	Reference
Non-heterocystous cyanobacteria		
Dermocarpa sp. PCC 7437 (partial)	X97396	This work
Oscillatoria sp. PCC 7515 (partial)	X97397	This work
Pseudanabaena sp. PCC 6903	X73135	13
Synechococcus sp. PCC 6301	X63566	11
Synechococcus sp. PCC 6717 (partial)	X97392	This work
Synechococcus sp. PCC 7001 (partial)	X97391	This work
Synechococcus sp. PCC 7003 (partial)	X97393	This work
Synechocystis sp. PCC 6308 (partial)	X97390	This work
Synechocystis sp. PCC 6803	X65707	12
Heterocystous cyanobacteria		
Anabaena sp. ATCC 29413 (partial)	X97389	This work
Anabaena sp. PCC 7120	X65648	12
Calothrix sp. PCC 7601	X65649	12
Fischerella sp. UTEX 1829 (partial)	X97388	This work
Nostoc sp. PCC 7107 (partial)	X97394	This work
Nostoc sp. PCC 7413 (partial)	X97395	This work
Unidentified cyanobacteria		
PS#4 (partial)	U28099	10
ESH183A (partial)	U28093	10
Plastids		
Cyanophora paradoxa cyanelle	U30821	35
Porphyra purpurea chloroplast	U38804	36

Mutagenesis

Deletion of the STRR sequences in helix P12 of *Anabaena* sp. PCC 7120 and of the equivalent region in helix P12 of *Synechocystis* sp. PCC 6803 was achieved by oligonucleotide-directed mutagenesis with the Altered Sites System (Promega). For this purpose a genomic fragment containing the *Anabaena* or *Synechocystis rnpB* genes (12) was cloned in the pALTER vector and mutagenesis done according to the manufacturer. The mutagenic oligonucleotides were 5′-GATAGTGCCACAGAAAAATACCGCCAAGAAACTTGGTAAGGGTGCAAAGGTGCGGTAAG-3′ for *Anabaena* and 5′-GACAGTGCCACAGAAAAATACCGCCCTTTTTAAGGGTAAGGGTGCAAAGGTGCGGTAAG-3′ for *Synechocystis*.

After mutagenesis the *rnpB* genes were amplified by PCR with a forward primer that contains an *Eco*RI site and the T7 promoter and overlaps the 5′-end of the coding sequence and a reverse primer that contains a *Dra*I site overlapping the 3′-end of the coding sequence and a *Hin*dIII site. A PCR fragment of the expected size was obtained, purified, digested with *Eco*RI and *Hin*dIII and ligated into pUC19 that had been treated with the same enzymes.

The primers used for PCR were as follows. *Anabaena*: forward, 5′-GGAATTCTAATACGACTCACTATAGGGAGAGAGTAGG-3′; reverse, 5′-CCCAAGCTTTAAAAGAGGAGAGAGTTG-3′. *Synechocystis*: forward, 5′-GGAATTCTAATACGACTCACTATAGAGAGTTAGGGAGG-3′; reverse, 5′-CCCAAGCTTTAAAAAAAGAGAGTTAGTC-3′.

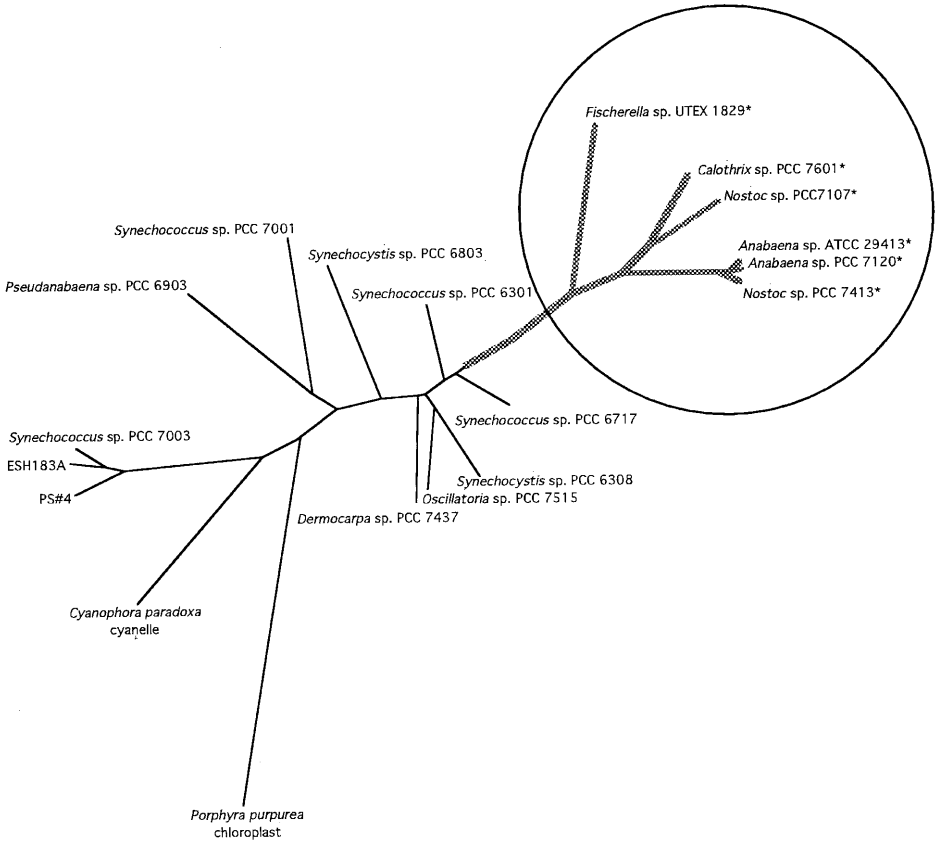

Figure 2. Phylogenetic tree based on RNase P RNA sequences from cyanobacteria and plastids. Stars indicate the sequences that contain STRR sequences in helix P12. Branch length is proportional to sequence divergence. The circled area contains the heterocyst-forming strains and has been enlarged 4-fold to help in visualization of the relationships among their RNase P RNAs.

Table 2. Structure of heterocyst-forming cyanobacterial RNase P RNAs

Strain	STRR sequence	STRR type
Anabaena sp. PCC 7120	ATTGGGG ACTGGGG ACTAGGG GTTGGGG ACTGGGG aagaa ACTTCCC AATCCCT AATCCCC CATACCC AATACCC	STRR1
Nostoc sp. PCC 7413	ATTGGGG CTAGGGA CTAGGGA CTGGGGA CTGGGGA agaa ACTTCCC AATCCCT AATCTCA GATGCTC GATCCCC	STRR1
Anabaena sp ATCC 29413	AACAATT CAAAATT CAAAATT CAAAATT CAAAATG aataat TTTGGAT TTTGAGT CTTAGTT ATGAATT CAATTTT	STRR6
Nostoc sp. PCC 7107	AACAAGT AAAAAGT AAAAAGCA AAAAAGC AAAAGAA acaaaaattt CTTTTTA CTTTTGC CCTTTAA CTTTTGC CTTTTTT	STRR6
Calothrix sp. PCC 7601	ATTAGTC ATTAGTC ATTTGTC ATTAGTC ATTTGGA aaa ACACAAA GGACAAA GGACAAA GAACAAA TGACCAA	STRR2
Fischerella sp. UTEX 1829	CAGTGAA CAGTGAA CAGTTAT CAGTGAA CAGTAAA taa CTGATAA CTGATAA CTGATAA CTGATAA CTGAAAA	STRR3

The portion of helix P12 containing the STRR sequences is shown. The STRR sequences in the first half of each helix are shown on the top line and the STRR sequences in the second half on the bottom line for each strain. Lower case represents sequences connecting both sets of repeats. The STRR types are defined according to Mazel *et al*. (14) and Jackman and Mulligan (24).

The wild-type *rnpB* genes were amplified with the same primers and cloned in the same way. All the constructions were confirmed by sequencing. The plasmids obtained after digestion with *Dra*I were used for *in vitro* run-off transcription with T7 RNA polymerase (19). The RNAs obtained would contain the same ends and sequence as the authentic *Anabaena* or *Synechocystis* RNase P RNAs or their mutant derivatives (ΔP12).

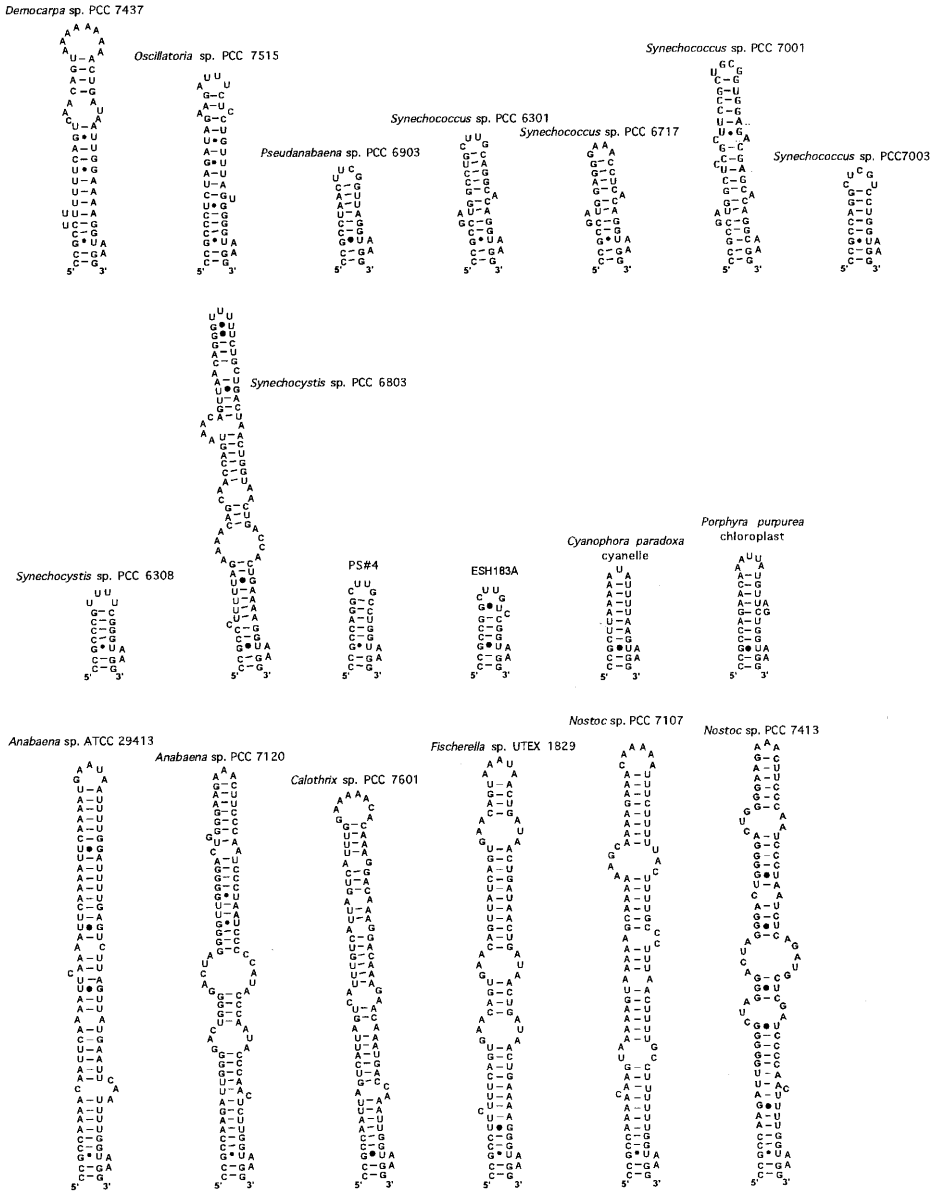

Figure 3. Sequence and possible secondary structure of helix P12 in cyanobacteria and plastid RNase P RNA.

Enzymatic assays and determination of K_m

The RNase P RNAs were resuspended in assay buffer and renatured by incubation at 65°C for 5 min and slowly cooled to room temperature before enzymatic assays. RNase P activity was assayed using *Escherichia coli* precursor $tRNA^{Tyr}$ (20) and kinetic data obtained by determination of initial reaction rates at different substrate concentrations as described (20), using a phosphorimager (Fuji) to quantify the radioactive bands on assay gels. For the holoenzyme reaction the assay buffer was 10 mM HEPES, pH 7.5, 10 mM magnesium acetate and 400 mM ammonium acetate. Holoenzyme was reconstituted with *in vitro* transcribed RNase P RNA and a 50-fold molar excess of purified RNase P protein from *E.coli* or *Synechocystis*. For the RNA alone reaction the buffer was similar but the magnesium acetate concentration was increased to 100 mM and 5% polyethyleneglycol was added.

RESULTS AND DISCUSSION

The new cyanobacteria RNase P RNAs characterized in this work were chosen to represent the main evolutionary lineages, as well as diverse morphological and physiological types. Four heterocyst-forming strains were used to give a larger representation of this group and evaluate the generality of the presence of STRR sequences in *rnpB* genes.

Cyanobacteria are a highly diverse group of bacteria with multiple morphological and physiological types (21). The phylogenetic relationships among them have been studied by sequence analysis of rRNA (22,23). One main conclusion of those studies is that heterocyst-forming strains constitute a monophyletic

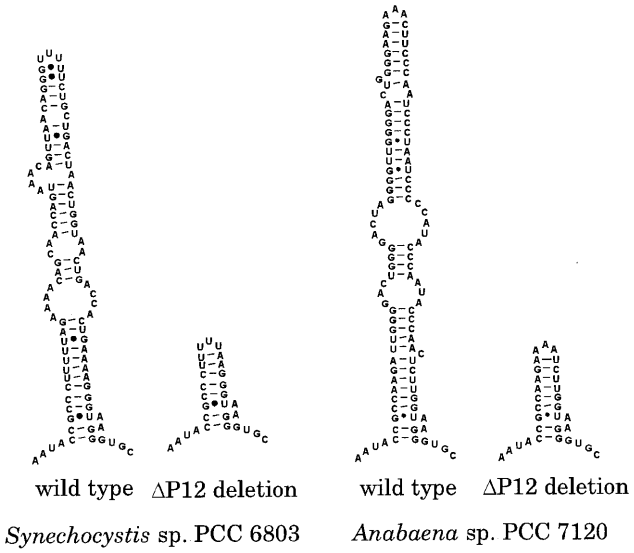

Figure 4. Mutagenesis of helix P12. The structure of helix P12 from *Synechocystis* 6803 and *Anabaena* 7120 as well as of the mutants derived from them is shown.

group of organisms. A phylogenetic analysis using the RNase P RNA sequences (Fig. 2) supports this fact. Also, the plastid RNase P RNAs branch within the cyanobacteria but are clearly more divergent. This could be due in part to the high AT content of the plastid genome.

P12 and STRR sequences

The heterocyst-forming strains share a unique character, i.e. the presence of 10 STRR sequences in helix P12, increasing the length of this helix (Fig. 3). STRR sequences are imperfect repeats of a heptanucleotide that are present in many copies throughout the genome of heterocyst-forming strains, usually (14,24) in intergenic regions. Their function is unknown. There is only one described example of STRR sequences interrupting a coding sequence (25). Recently sequences similar to STRR have been identified in some unicellular strains (26), but there is no information on their RNase P genes. The STRR sequences in helix P12 belong to different families (Table 2): STRR1 in *Anabaena* sp. PCC 7120 and *Nostoc* sp. PCC 7413; STRR6 in *Anabaena* sp. ATCC 29413 and *Nostoc* sp. PCC 7107; STRR2 in *Calothrix* sp. PCC 7601; STRR3 in *Fischerella* sp. UTEX 1829. It is striking that closely related strains contain STRR sequences of different families. For instance, *Anabaena* sp. PCC 7120 and *Anabaena* sp. ATCC 29413 RNase P RNAs are identical except for one substitution and one deletion, however, their helices P12 are unrelated due to the presence of different types of STRR sequences. This raises the question of the evolutionary origin of insertion of STRR sequences in RNase P RNA of heterocyst-forming cyanobacteria. The fact that STRR sequences are found in the genomes of many heterocyst-forming cyanobacteria would support the idea that they spread before diversification of this group of cyanobacteria. However, the presence of unrelated STRR sequences in sister strains *Anabaena* 7120 and *Anabaena* 29413 or *Calothrix* 7601 and *Nostoc* 7107 would support a more recent and independent origin of the insertions. This is difficult to

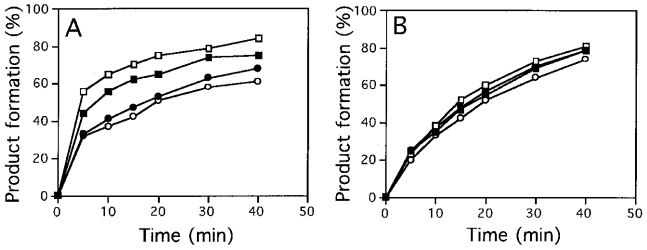

Figure 5. Enzymatic activity of RNase P holoenzymes reconstituted with different RNase P RNAs and the RNase P protein subunit from *E.coli* (**A**) or *Synechocystis* 6803 (**B**). Assays were done as described in Materials and Methods using 5 (A) or 25 nM (B) each RNA and and a 5-fold excess of protein with 16 pM precursor $tRNA^{Tyr}$ as substrate. The RNAs used were *Synechocystis* 6803 wild-type (○), *Synechocystis* 6803 ΔP12 deletion (●), *Anabaena* 7120 wild-type (□) and *Anabaena* 7120 ΔP12 deletion (■).

reconcile with parsimony criteria. One possible scenario is that the several families of STRR sequences invaded the genome of a common ancestor of heterocystous cyanobacteria but have spread and multiplied their copy number recently. Different STRR sequences have being amplified in different strains in a random fashion. That would explain why strains that have a very recent common ancestor contain different kinds of STRR sequences, not only within the *rnpB* gene, but also at other positions in the genome (24,25).

If STRR sequences have spread randomly through the genome it would be expected that their conserved position within the *rnpB* gene, inserted almost exactly at the same position in helix P12, is due to a specific role of the STRR sequences at that position, or at least their presence should not detrimentally affect enzymatic activity of the RNA. This question has been addressed by site-directed mutagenesis of *Anabaena* 7120 helix P12. A deletion was made of the STRR sequences in P12, shortening the length of the helix. An equivalent deletion was made in the *Synechocystis* RNA (Fig. 4). Previous studies in *E.coli* (27) have shown that deletion of helix P12 significantly affects enzymatic activity of RNase P RNA. No activity is detected in the presence of the protein and a much higher ionic strength is required for activity of the RNA alone. However, in those studies P12 was completely deleted, including the highly conserved structure at the base of the helix and the surrounding single-stranded nucleotides. In our case only the STRR sequences (or an equivalent region in *Synechocystis*) were deleted, therefore maintaining the conserved elements of the helix. On the other hand, the RNase P RNA from *Mycoplasma fermentans* naturally completely lacks helix P12 and is active (28). This suggests that helix P12 is not required for catalytic activity *in vivo*.

As shown in Table 3, the deletions introduced in P12 did not significantly affect the catalytic efficiency of the RNAs when assayed under the same buffer conditions. In *Synechocystis* the mutant has a higher K_m but also a slightly higher k_{cat}. In *Anabaena* the mutant has slightly lower K_m and k_{cat}. The protein subunit from *Anabaena* 7120 RNase P has not been purified, therefore it was not possible to study the effect of deletion of the STRR on the homologous holoenzyme. However, the proteins from *E.coli* and *Synechocystis* are available. Cyanobacterial RNase P RNA can reconstitute a functional holoenzyme with both protein subunits (29). Figure 5 shows that deletion of helix P12 does not affect activity of the reconstituted holoenzyme with

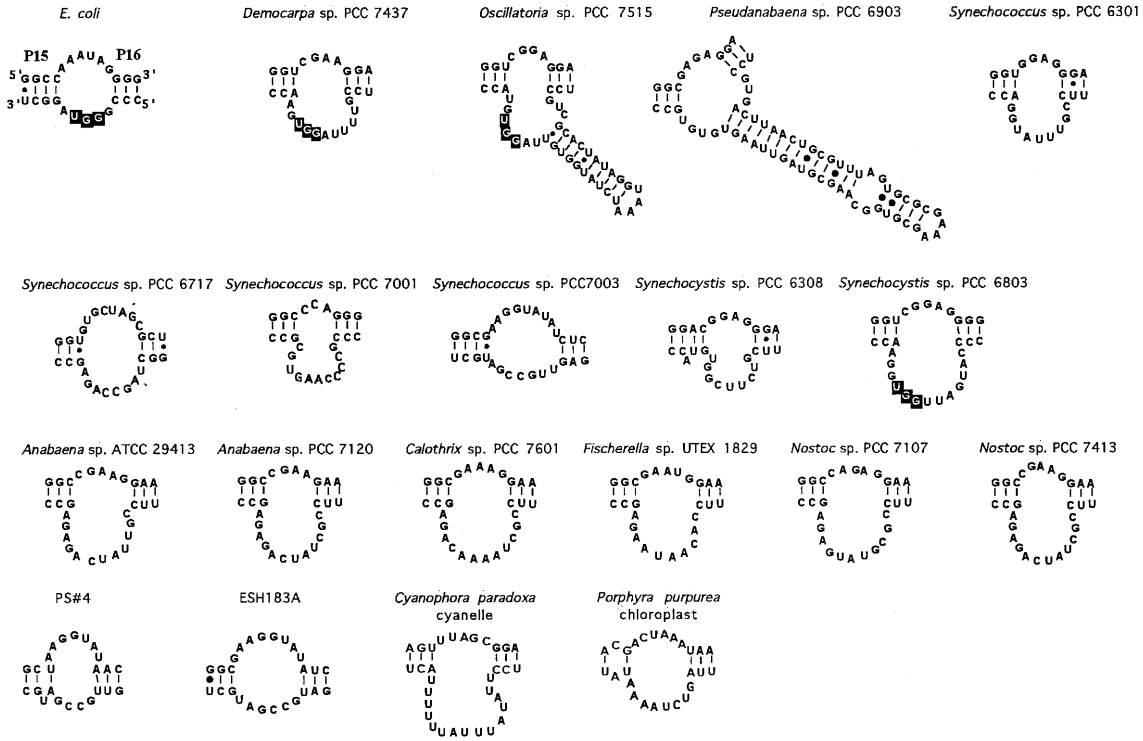

Figure 6. Structure of the loop connecting helix P15 and P16 in RNase P RNA. The GGU sequence is highlighted in the structures that contain it.

either the *E.coli* protein (Fig. 5A) or the *Synechocystis* protein (Fig. 5B). All RNAs show similar reaction rates under the conditions used. As previously described (29), holoenzymes reconstituted with the *E.coli* protein are more efficient in processing *E.coli* precursor $tRNA^{Tyr}$ than holoenzymes reconstituted with the *Synechocystis* protein (compare Fig. 5A and B; five times more holoenzyme was used in B than in A). These data suggest that the presence of the STRR sequences in P12 is not an essential requisite for RNase P activity. The assays described here were done with an *E.coli* precursor tRNA substrate. Of course, it cannot be excluded that the STRR sequences are required for processing of some specific substrate in *Anabaena*. Heterocyst differentiation might require a specific RNA processing step catalysed by RNase P that is not required in non-heterocystous strains. Several unconventional substrates for RNase P, including mRNAs, have been described recently (30,31) in bacteria.

Table 3. Kinetic parameters of RNase P RNA from *Synechocystis* 6803 and *Anabaena* 7120 and derived mutants

Enzyme	$K_m \pm$ SD (μM)	$k_{cat} \pm$ SD (per min)	k_{cat}/K_m
Synechocystis wild-type	0.14 ± 0.04	0.14 ± 0.04	1.0
Synechocystis DP12	0.45 ± 0.16	0.25 ± 0.07	0.55
Anabaena wild-type	9.82 ± 0.92	7.24 ± 0.36	0.74
Anabaena DP12	6.63 ± 0.77	5.46 ± 0.51	0.82

Kinetic parameters were calculated with *E.coli* pre-$tRNA^{Tyr}$ as substrate as described in Materials and Methods.

The loop connecting P15 and P16

A number of results have established that this region of the *E.coli* RNase P RNA is important for interaction with the substrate. The 'GGU motif' in the bottom half of this loop has been implicated in direct interaction with the conserved 3′-terminal 'RCCA' sequence of a tRNA precursor (15,16). The GGU motif as well as the structure of the loop is conserved among bacterial RNase P. However, the equivalent region in cyanobacteria is clearly different (Fig. 6) and most of the strains do not contain a GGU motif. Some of the strains, such as *Oscillatoria* 7515 and *Pseudanabaena* 6903, contain an extra helix in this region and the loop is in general larger than in *E.coli*. Some strains contain a GGU sequence in the loop but, due to the larger size of the loop, the GGU motif does not seem to be structurally equivalent to the *E.coli* GGU motif. It has been speculated (32) that cyanobacterial RNase P RNAs interact with their substrate differently than does *E.coli* RNase P RNA and that this might be related to the fact that cyanobacterial tRNA genes usually do not encode the 3′-terminal CCA sequence. In fact, and contrary to the effect of the 3′-terminal CCA sequence on RNase P activity in *E.coli* (33), the presence of the 3′-terminal CCA sequence seems to negatively affect its processing by the *Synechocystis* 6803 enzyme (A.Pascual and A.Vioque, unpublished observations).

Helix P6

The pseudoknot created by helix P6 is generally 4 bp long. However, in cyanobacteria it can be potentially extended to 7 bp in most strains, except in *Synechococcus* 7001, where it can be only 6 bp long, or 4 bp long in PS#4, ESH183A and *Synechococcus* 7003. Due to high conservation of the nucleotides

that could potentially increase the length of P6, there is little but significant support from compensatory substitutions for the extended helix. The extended helix contains three AU base pairs in all but three strains. Two of the three new base pairs are supported by just one compensatory substitution, while the third is an invariant AU base pair. In both published tertiary structure models of *E.coli* RNase P RNA (8,9) P6 is at the core of the structure connecting the domain formed by P7–P11 to the domain formed by P15–P17. An extended P6 can therefore increase the stability of the core structure. This extra stability may be required to compensate for the larger size of the loop connecting P15 and P16 that, everything else being similar, would negatively affect the connection of P16 with P7. In fact, the three strains whose P6 is only 4 bp long (PS#4, ESH183A and *Synechocystis* 7003) have a rather smaller loop between P15 and P16 than the others (Fig. 5).

Concluding remarks

The RNase P RNAs from cyanobacteria conform to the general structure established for bacterial RNase P RNAs but contain a number of features specific to this group of bacteria, as has been discussed above. The high variability in sequence and size of the loop connecting P15 with P16 and the absence in most strains of a 'GGU motif' suggest a mode of substrate interaction different from the model proposed for *E.coli*. The presence of highly variable STRR sequences in P12 in heterocyst-forming strains illustrates the plasticity of the cyanobacterial genome and suggests that they can be used as taxonomic tools (34): PCR of the *rnpB* gene can be used to identify closely related strains of cyanobacteria that cannot be distinguished by other means.

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