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SURVEY AND SUMMARY

Evolutionary variation in bacterial RNase P RNAs

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ABSTRACT

Sequences encoding RNase P RNAs from representatives of the last remaining classical phyla of Bacteria have been determined, completing a general phylogenetic survey of RNase P RNA sequence and structure. This broad sampling of RNase P RNAs allows some refinement of the secondary structure, and reveals patterns in the evolutionary variation of sequences and secondary structures. Although the sequences range from 100 to <25% identical to one another, and although only 40 of the nucleotides are invariant, there is considerable conservation of the underlying core of the RNA sequence. RNase P RNAs, like group I intron RNAs but unlike ribosomal RNAs, transfer RNAs or other highly conserved RNAs, are quite variable in secondary structure outside of this conserved structural core. Conservative regions of the RNA evolve by substitution of apparently interchangeable alternative structures, rather than the insertion and deletion of helical elements that occurs in the more variable regions of the RNA. In a remarkable case of convergent molecular evolution, most of the unusual structural elements of type B RNase P RNAs of the low G+C Gram-positive Bacteria have evolved independently in *Thermomicrobium roseum*, a member of the green non-sulfur Bacteria.

BACKGROUND

Ribonuclease P is the endoribonuclease responsible for the 5′ maturation of tRNA precursors (1–3, and references therein). RNase P is a ribonucleoprotein in all organisms, but is best understood in Bacteria, in which the RNA component of the enzyme is by itself catalytically proficient *in vitro*, i.e. it is a ribozyme (4). RNase P is present and essential in all cells and subcellular compartments that synthesize tRNA, but catalytic proficiency by the RNA alone has been demonstrated only for the bacterial RNAs (and in all such RNAs tested). The structure of bacterial RNase P RNA has been studied in detail, primarily using comparative methods (3,5–7).

Although recognizable RNase P RNAs are present in all organisms, they are more variable in both sequence and secondary structure than are the ribosomal RNAs and transfer RNAs. Bacterial and archaeal RNase P RNAs are more similar in sequence and structure than either is to the eukaryal RNAs (8–10). Bacterial RNase P RNAs examined so far fall into two main structural classes. These RNAs share a common ‘core’, and synthetic minimal RNase P RNAs consisting only of these core sequences and structures are catalytically proficient (11,12). Type A is the usual form of RNase P RNA in Bacteria,whereas type B RNAs are found only in the low G+C Gram-positive Bacteria, such as *Bacillus subtilis* (13). Structural variation between type A and B RNase P RNAs, and between the instances of each structure type, is predominated by variation in the presence or absence of helical elements and in variation of the size of the distal regions of these helices. However, there is additional variation in the form of small differences in the lengths of helices, loops and joining regions.

We have completed a broad phylogenetic survey by obtaining sequences encoding the RNA from the remaining classical Kingdoms of Bacteria (14): the spirochaetes, planctomycetes, Chlamydiae and the green non-sulfur Bacteria (Table 1). The purpose of this Survey and Summary is to describe new insights into the extent and patterns of evolutionary variation in RNase P RNA sequence and structure in Bacteria.

Table 1. Newly-obtained RNase P RNA-encoding sequences[a]

Species	Phylum	Accession number
Planctomyces maris	Planctomycetes	AF056384
Pirellula staleyi	Planctomycetes	AF056385
Leptospirillum weilii	Spirochaetes	AF056382
Leptospirillum bergpertersonii	Spirochaetes	AF056381
Borellia hermsii	Spirochaetes	AF056376
Thermoleophilum album	Green non-sulfur	AF056388
Chloroflexus aurantiacus	Green non-sulfur	AF056377
Herpetosiphon aurantiacus	Green non-sulfur	AF056380
Thermomicrobium roseum	Green non-sulfur	AF056389
Chlamydia psittaci	Chlamydiae	AF056378
Chlamydia trachomatis	Chlamydiae	AF056379
Pseudomonas testosteroni	Proteobacteria	AF056386
Thiobacillus thioparus	Proteobacteria	AF056390
Myxococcus xanthus	Proteobacteria	AF056383
Synechococus spp. PCC7942	Cyanobacteria	AF056387
Heliobacterium chlorum	Gram-positive	AF056391

[a]Cloned, sequenced and tested for catalytic function using previously described methods (9,13,37). All sequences and secondary structures are available on-line at the RNase P Database at http://www.mbio.ncsu.edu/RNaseP/ (43).

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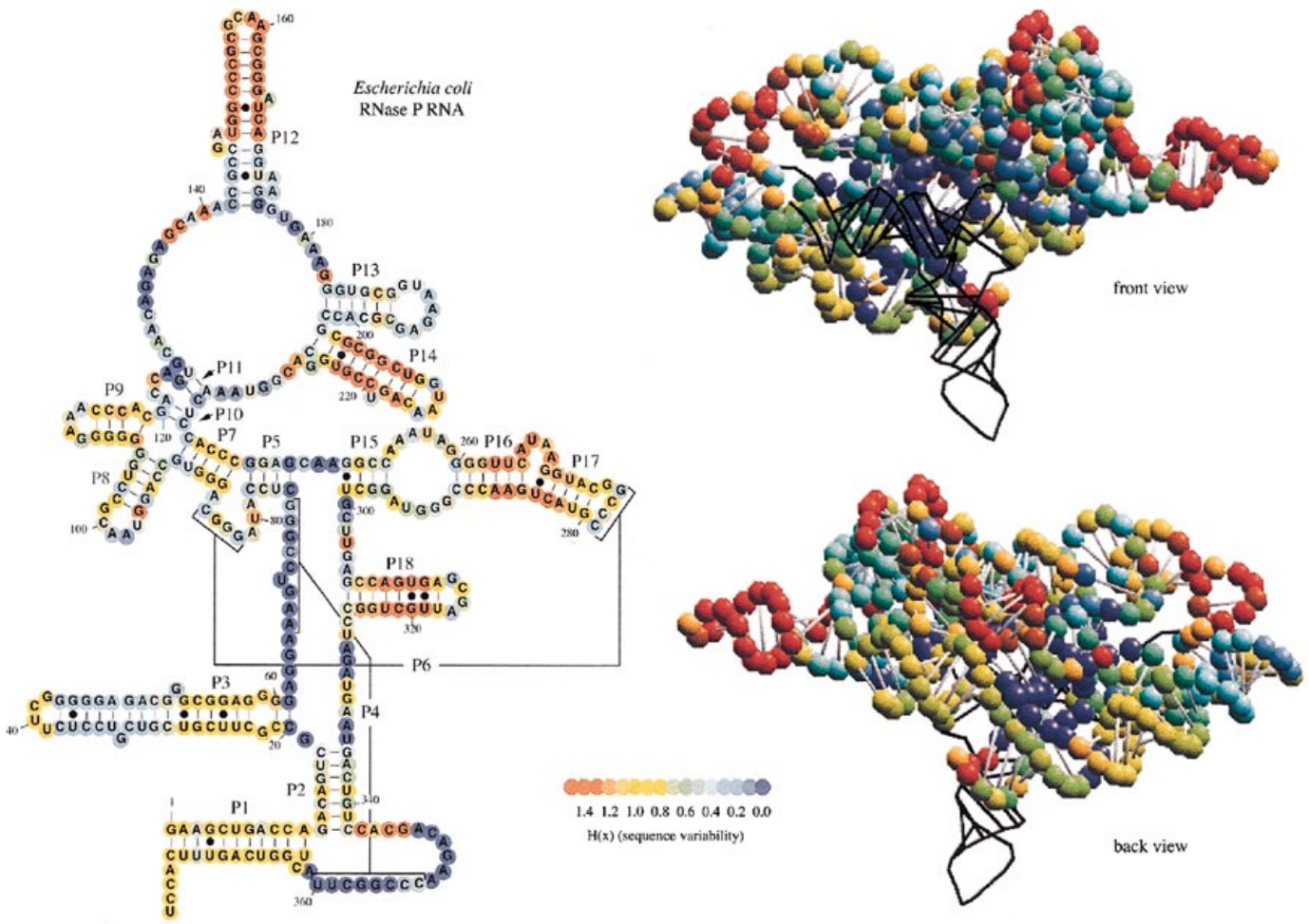

Figure 1. Sequence variation in bacterial RNase P RNAs. The secondary structure of the RNase P RNA of *E.coli*. Helices are labeled P1–P18 from 5′ to 3′ (37). Nucleotides are colored based on their conservation from blue (highly conserved) to red (highly variable) as assessed quantitatively by H(x) (15). A 'pseudoatom' representation of a model of the tertiary structure of the *E.coli* RNase P RNA (20), with a sphere at the location of the phosphate of each residue and the pre-tRNA precursor shown as a black 'stick figure', is likewise colorized. Highly-conserved bases are scattered throughout the sequence and secondary structure, but are concentrated in the vicinity of the pre-tRNA binding surface of the tertiary structure.

DISCUSSION

Conserved sequences

Only 40 nt in bacterial RNase P RNAs are absolutely conserved in the 145 sequences now available. However, this largely underestimates the extent of sequence conservation in these RNAs (Fig. 1); in reality, the majority of sequence positions are conservative. Sequence variation of any sequence position 'x' can be assessed quantitatively using the entropy coefficient H(x) (15). Sequence positions in the phylogenetically-conserved core of secondary structure are, on average, more conserved than those in the variably-present periphery of the RNA. However, there is considerable overlap in the range of sequence variation in these two portions of the RNA (Fig. 2). The extremely conservative positions [H(x) < 0.25] are found in the core of the RNA, and the most highly variable positions [H(x) > 1.25] are in the peripheral portions of the RNA, but outside of these extremes, there is no significant difference in the extent of variation in core and peripheral sequence positions.

There are several helices, both in the core and periphery of the RNA, that are conserved in sequence at the base and terminal loop but extremely variable in sequence along the length of the helix: P9, P14, P16/17 and P18 (Fig. 2). The proximal ends of these helices are located within important conserved sequence and structure, and interact at their terminal loops in secondary or tertiary contacts elsewhere in the molecule. The loop of P16/P17 interacts with nucleotides between P5 and P7 to form P6, closing a pseudoknot in the RNA secondary structure (16). P9, P14 and P18 all terminate in GNRA tetraloops that form tertiary contacts with base pairs in important helices in the RNA structure. Covariation data for all of these loops and their helical contact points is consistent with the analogous interaction present in the group I intron crystal structure (17). The loop of P9 interacts with base pairs near the distal end of P1 (18,19), constraining the orientation of this helix and by extension that of P4, that probably stacks onto the proximal face of P1 (20,21). In some organisms,

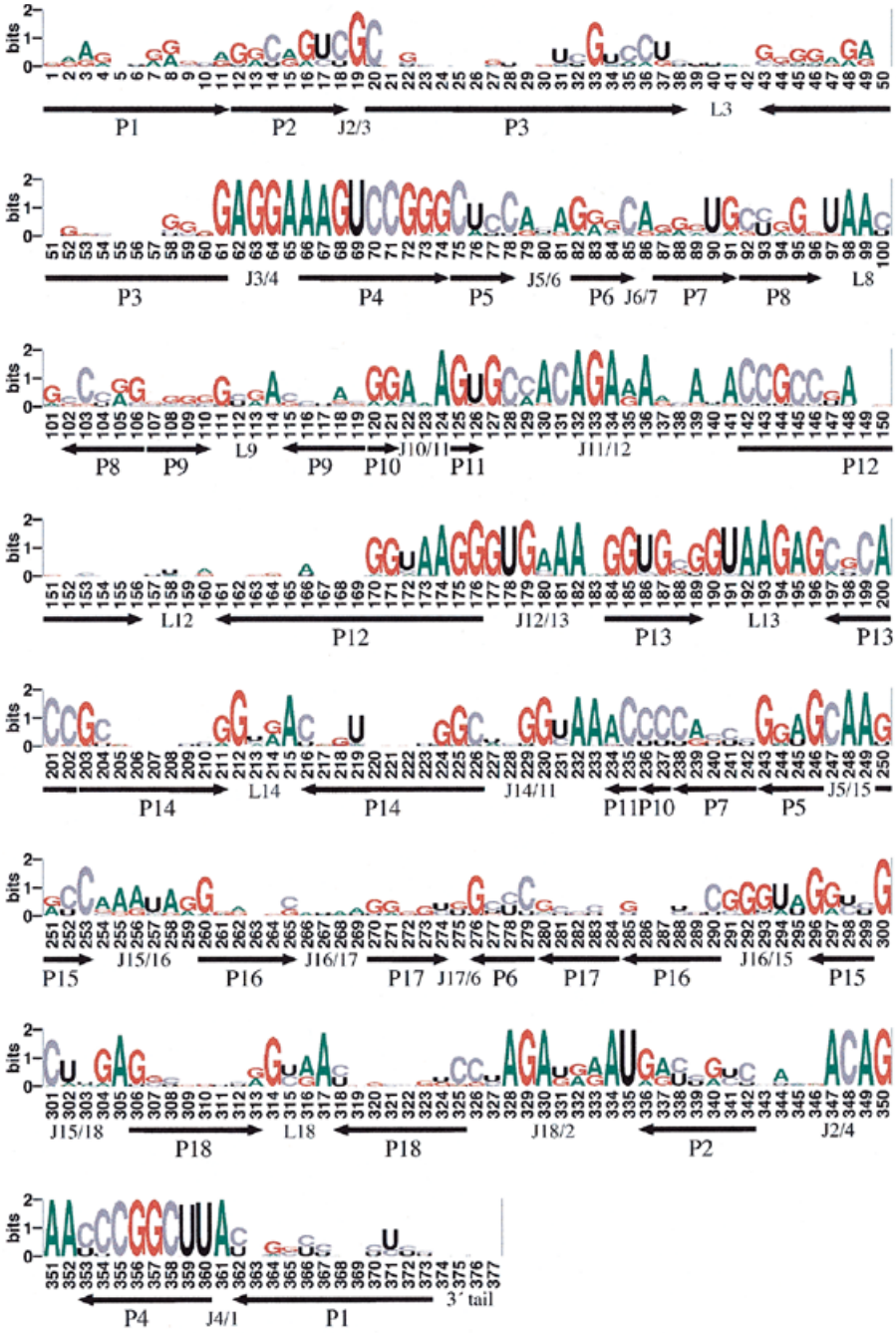

Figure 2. Sequence logo of 'type A' RNase P RNAs. Sequence logo, as described by (44) using Steve Brenner's 'Weblogo' at http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi . Nucleotide conservation is measured in bits of information, ranging from invariant bases (2 bits of information) to positions with equal occurrences of G, A, C and U (0 bits of information). Numbering is based on the *E.coli* RNA. The secondary structure is labeled P1–18 as in Figure 1; associated loops and joining regions are also labeled as previously described.

this tertiary interaction is replaced by a helix formed from nucleotides in the loop of P9 and nucleotides immediately 3′ to a shortened P1, creating an additional pseudoknot in the RNA (18). The GNRA terminal loops of both P14 and P18 interact with highly conserved base pairs in P8 (22), apparently stabilizing this region of the molecule, which has been implicated in recognition of the T-loop of pre-tRNA (23). The deletion of either helix P14 or P18 results in RNAs that are defective *in vitro*, but can be rescued biochemically by increased ionic strength, consistent with their presumed role in the structural stabilization of the RNA (24). These helices seem then to serve as 'connecting rods', constraining the relative distance and orientation of critical portions of the RNA.

Other extremely variable regions of the RNA include the distal region of P12, J15/16 and the 5′ portion on J2/4 (including P19 in RNAs that contain it). The lengths of these sequences vary, in some cases dramatically with the frequent insertion and deletion of large sequence elements, and so part of this apparent sequence

variation may be the result of difficulty in identifying homologous residues. It seems reasonable to consider these regions linkers, but it is also likely that helical elements in these regions serve to stabilize adjacent, conserved helices via stacking interactions.

Some helical elements are highly conserved in sequence, including the nucleotides that make up and flank P4, which are the most highly conserved in the molecule. Nucleotides in P8 are conserved, presumably because of their need to interact with L14 and L18 (the loops of P14 and P18) in addition to the structural requirements for substrate recognition (22,23). The base of P12 is very highly conserved, and the conservation of these sequences is tightly correlated with the presence of P13/P14; when P13/14 is absent, the sequences at the base of P12 are no longer conserved and the 2 nt bulge is absent (22). P13 is also highly conserved in sequence, implying that it interacts with the base of P12, but the nature of this interaction is not known. The conservation of the sequences of P10 and P11 may result from their structural importance and short length; temporary disruption of a single base pair midway through an evolutionary covariation would likely disrupt these 2 bp helices. The apparent conservation of the distal portion of P3 may be in part an artifact of the sequence collection—nearly all of the sequences that contain long P3s are from one phylogenetic group (the 'Proteobacteria'), that are disproportionately represented in the sequence collection. Truncation of this helix results in no detectable biochemical phenotype (24). However, the extent of conservation is intriguing, and genetic data suggest that this region may have some functional role (25).

It has been shown that the terminal base pairs of helices are critical for the stability of a helix (26), and so it might be expected that the nucleotides that make up the terminal base pairs of helices would be more conservative than those that make up the remainder of the helix. This is the case in RNase P RNA (Fig. 1); the terminal bases have H values that average 0.25 less than other base pairs in the same helix. The variability of terminal base pairs is comparable, overall, to sequences in unpaired regions of the RNA rather than the more variable nucleotides in the internal portions of helices.

Alternative structural motifs

Structural variation in RNase P RNA consists primarily of the presence or absence (i.e. insertion or deletion) of discrete helical elements peripheral to the phylogenetically-conserved functional 'core' of the RNA. The sporadic presence or absence of helices, and extreme variation in the size of the distal regions of certain helices, often occurs in regions of extreme sequence variability (the exception is P13). These elements are also quite variable in sequence and length, and are thought therefore to be located on the periphery of the RNA (27). For example, deletion of the distal portion of P3 in the *Escherichia coli* RNA had no measurable effect on the biochemical properties of the RNA *in vitro* (24). It is thought that these elements have little or no role in the structure or function of the RNA, perhaps performing minor stabilization of core helices (via helical stacking), or are simply tolerated by the RNA (in an evolutionary sense) as long as they do not interfere with its structure or function.

The use of alternative non-homologous structures occurs in more conservative regions of the RNA. There is evidence that these variably present helices are structural alternatives; i.e. that the presence of either of two possible helices is acceptable (e.g. P5.1 and P6/17, P10.1 and P13/14) (16,28). Alternative helices are thought to fulfill the same structural role, probably actually filling the same three-dimensional space. In instances where the functional role of these elements has been examined, they seem to be involved in important but not essential stabilization of the core structure of the RNA. For instance, *E.coli* RNase P RNAs lacking either P6/16/17 or P18 are severely defective when assayed in conditions optimal for the native RNA, but function nearly normally in terms of both K_m and K_{cat} at extremely high ionic strength (11,24).

Examination of the available collection of RNAs reveals that structural variation also occurs in the form of alternative (and presumably functionally interchangeable) forms of homologous structures; i.e. some regions come in distinct interchangeable versions. Three of these alternative motifs are described below. The use of distinct structural alternatives is likely to be a general characteristic of RNA structure, and the repeated exchange of these motifs suggests that there are a limited number of acceptable structural solutions that can be obtained readily by evolutionary processes.

P6 alternatives. Helix P6 completes a pseudoknot in the peripheral (non-core) structure of type A RNase P RNAs. In type B RNAs, helix P5.1 is thought to replace P6 and P17, in structure and function (16). P6, and the joining regions connecting it to the remainder of the RNA, vary in length and sequence, but occur predominantly in two forms (Fig. 3). In one form, such as that found in *E.coli*, P6 is invariably 4 bp in length and is preceded by the J5/6 sequence 'AYA' (Y = C or U; 90% of sequences) or more generally 'RNA' (R = A or G, N = any base; 98% of sequences). In this P6 motif, J6/7 is usually a single A (82% of sequences) or more generally R (94%). J17/6 is a single nucleotide, G or U; the other junction between P6 and P17 (J6/17) is usually direct (96%), but in a few cases is a single nucleotide. P17 varies from 4 to 7 nt in length, usually 5 or 6 (82%). In the other common structural motif for this region, P6 is connected to P5 (J5/6) and P7 (J6/7) by single purine nucleotides. In these RNAs, P6 varies in length from 4 to 8 bp in length, but is usually 6 or 7 (90%). The joining regions with P17 are more variable, with 1 or 2 nt in J17/6 and 0–2 nt in J6/17. P17 varies from 3 to 6 bp in length, but is usually 3 or 4 (93%). It has previously been proposed, on the basis of molecular models and analogy to H-type pseudoknots, that P6 and P17 are stacked (16). Variation in the lengths of P6 and P17 support this suggestion; the lengths of these helices are inversely correlated, i.e. the sum of the lengths of the helices is more conserved that the lengths of each helix individually.

These two structural motifs have interchanged several times in the evolution of modern bacterial RNase P RNAs, without any discernable alterations in the remainder of the molecule. Therefore, it seems that these two motifs are truly structural alternatives, and that the conversion from one to the other is a relatively simple evolutionary process. Some RNase P RNAs may represent evolutionary intermediates in the process, e.g. those of *Thermotoga* (P6 is 4 bp, but otherwise conforms to the other motif) and a scattering of α-purple Bacteria, cyanobacteria and spirochaetes that contain two purines in J5/6 and 7 or 8 bp P6s.

J15/16 alternatives. In most bacterial RNase P RNAs, P15 is a 4 bp helix and J15/16 is a symmetrical or nearly-symmetrical loop containing conserved sequences (Fig. 3), some of which are involved in recognition of the 3′-RCCA tail of tRNAs (29). These conserved sequences are also present in the type B RNAs,

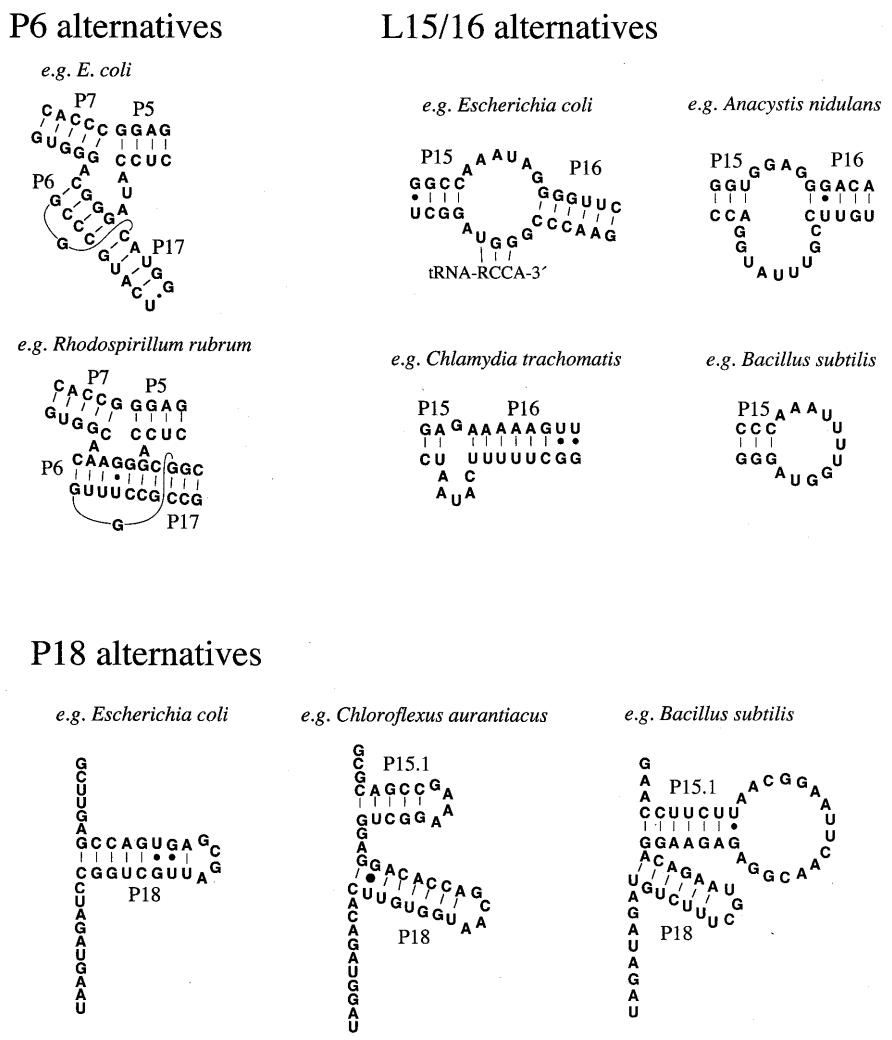

Figure 3. Alternative structural motifs. Alternative structures of homologous regions (as opposed to alternatives using non-homologous structural elements), or 'motifs', for P6, J15/16 and P18. See text for a description of the general features of each motif.

although in these RNAs P15 is only 3 bp and P16 and P17 are absent. In *Chlamydia*, however, P15 is short (2 bp) and J15/16 is a small asymmetric loop (30, and this study). In the cyanobacteria, P15 is 3 bp and J15/16 is a large asymmetric loop lacking the usually conserved nucleotides (31–35). Many of the cyanobacterial RNAs contain a large helical insertion in the J15/16 loop. Presumably these alternative structural motifs are also responsible for 3′-RCCA recognition by these RNase P RNAs, but if so the interactions must be quite different than in the common form of J15/16. In *E.coli*, a conserved GGU sequence forms base pairs with the two Cs and the preceding purine in the substrate (Fig. 3) (29,36); these conserved sequences are absent from the structural alternatives for PJ15/16.

P18 alternatives. P18 is a highly conserved structural element in most bacterial RNase P RNAs, consisting of an 8 bp stem–loop capped with a GNRA tetraloop (Fig. 2). P18 is connected to P15 and P2 by 6 and 10 nt joining regions, respectively, including highly conserved nucleotides. The GNRA tetraloop of P18 interacts with base pairs in P8 (22). P18 apparently functions as a scaffold, stabilizing the conserved core sequences at its base and P8 at its loop. Type B RNase P RNAs contain a pair of helices in place of P18 and the flanking 4 nt; which of these helices, if either, are homologous to P18 was unclear, since neither conforms to conserved P18 structure nor could form analogous tertiary contacts with P8. Some bacterial RNase P RNAs (i.e. those of *Chlorobium*) lack P18 and the flanking nucleotides entirely (37). Bacterial RNAs that lack standard P18 structure have longer than usual P8s with non-standard loop sequences, leading to the suggestion that these RNAs compensate for the absence of the L18/P8 tertiary stabilization with alternative tertiary contacts between the loop of P8 and some other unspecified part of the RNA (37).

All of the RNase P RNAs from the green non-sulfur Bacteria contain a pair of helices in place of P18. In *Chloroflexus aurantiacus* and *Herpetosiphon aurantiacus*, the 3′-most of these helices conforms to standard P18 structure; the 5′-most helix (P15.1) represents an insertion in otherwise standard type A structure. In *Thermoleophilum album* and *Thermomicrobium*

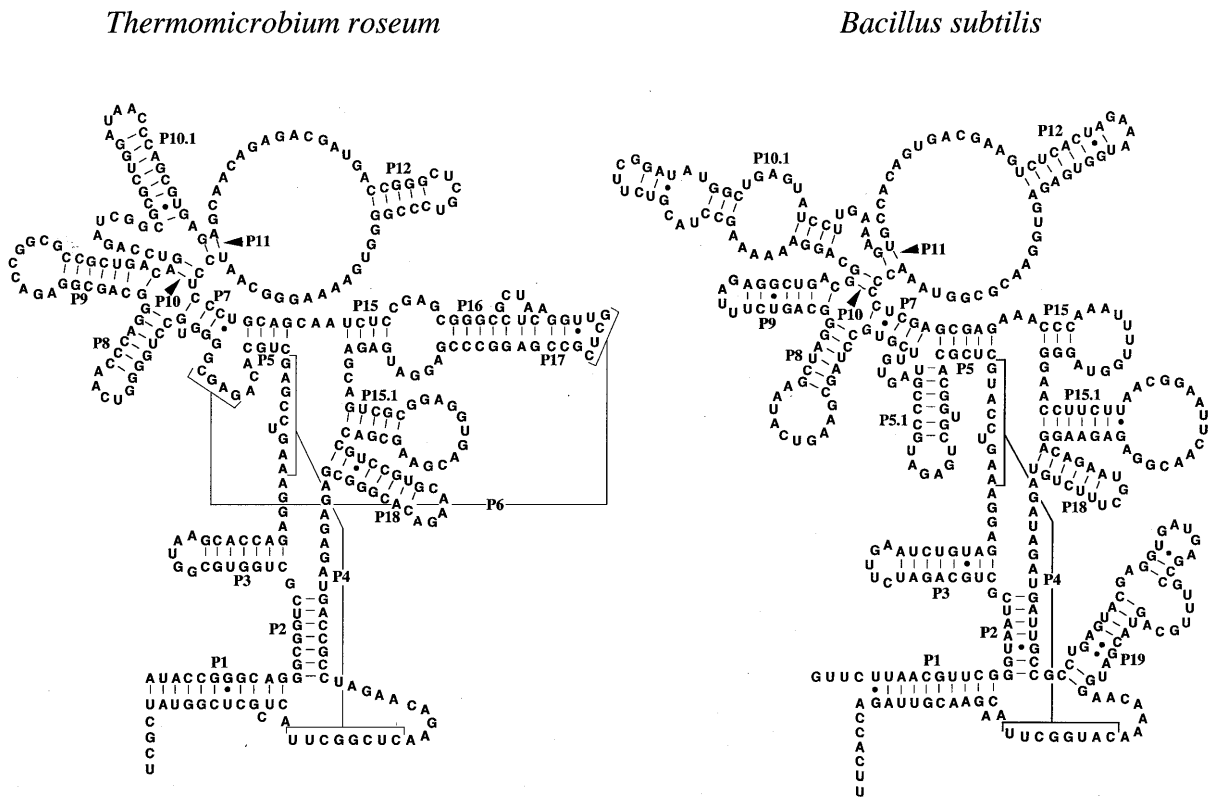

Figure 4. Evolutionary convergence of structure in *T.roseum* and type B RNase P RNAs. The secondary structures of the *T.roseum* (a member of the green non-sulfur Bacteria and relatives) RNase P RNA and that of a typical type B RNase P RNA from *B.subtilis* (a member of the low G+C Gram-positive Bacteria). These two RNAs are unrelated phylogenetically and in sequence, but share a number of unusual structural features as a result of convergent evolution (see text).

roseum (Fig. 4), however, P18 has diverged from the usual form, including the loss of 5 flanking nt and the potential tertiary contact with P8. These RNAs, and especially that of *T.roseum*, resemble type B RNAs in this region.

Frequent occurrence of a bulged nucleotide adjacent to the terminal base pair of a helix

Re-examination of the structure of P14 shows that an additional base pair is supported by the comparative data (this base pair has been discovered independently by Massire *et al.*; 20). The refined structure of P14 contains, in most RNAs, a single bulged nucleotide (most often G) on the 'exiting' strand adjacent to the terminal base pair at the proximal end of the helix. This structure seems thermodynamically unlikely, but is actually common in the RNase P RNA secondary structure. P1 generally contains a bulged nucleotide (variable in identity) after the basal base pair on the 'entering' strand (3′ in this case) of the RNA (13,19). P9 generally contains a bulged purine one basepair proximal to the base (on the 3′ strand, which is 'exiting' the helix in this case). The bulged nucleotides at the bases of P9 and P14 are structurally equivalent, and quite different than the bulged nucleotide at the base of P1. These bulged nucleotides are highly conserved in identity as well as structure, suggesting an important role in the structure of the RNA, although all are dispensable, at least from an evolutionary perspective. These helices (P1, P9 and P14) are all thought to be stacked onto important helices (P4, P8 and P13, respectively) on the face of the helix containing the bulged nucleotide (19–21,38). Perhaps the bulged nucleotide interacts with the adjacent unpaired sequences (there is no specific comparative data to support this hypothesis for any of these nucleotides) or adjusts the geometry of the terminal base pair favorably for the stacking interaction (38).

Convergent evolution of *T.roseum* and type B RNase P RNAs

The type B RNase P RNAs of the low G+C Gram-positive Bacteria differ in many ways from those of the common type A RNAs. In the low G+C Gram-positive Bacteria, this evolutionary change occurred abruptly, and no intermediates or partially restructured RNAs have been identified in this group, despite a diverse phylogenetic sampling (13). Surprisingly, the RNase P RNA of *T.roseum* contains most of the structural alterations present in type B RNAs (Fig. 4). This evolutionary convergence is limited to secondary structure; there is no discernable convergence in the sequences of these RNAs. Unlike the abrupt change in the Gram-positive Bacteria, the analogous changes in the RNase P RNA of *T.roseum* occurred step-wise, with intermediate forms preserved in the RNAs of modern organisms. All of the green non-sulfur Bacteria contain P15.1, and in *T.roseum* and *T.album*, the P18 motif resembles those of type B RNAs, including alteration of P8 (see above). The loss of the P13/14 element (absent in the β-proteobacteria as well as type B RNAs) and the aquisition of P10.1 in *T.roseum* results in an RNA that is only one step (replacement of P16/17/6 with P5.1) from standard type B structure. Therefore, the RNAs of the green

non-sulfur Bacteria create an evolutionary series connecting type A and type B RNase P RNA structure. The *T.roseum* RNA lacks the conserved length and GAAA tetraloop of P12 in most type B RNAs, and there is no known interaction between L12 and P10.1 (13,39). The *T.roseum* sequence also has complementarity between sequences in an enlarged L9 and the distal 3′ strand of P1, seen previously in some of the type B RNAs from *Mycoplasma*, that could form an additional helix, P21, and create a third pseudoknot in the RNA (40). Unlike the *Mycoplasma* sequences, the *T.roseum* RNA has the potential to form either a standard 11 bp P1 or P21. The convergent evolution of type B structure in more than one phylogenetic branch of the Bacteria implies that this restructuring process is a relatively easy evolutionary process, bolstering the suggestion that the tertiary structures of these RNAs are similar despite their differences in secondary structure (16).

Other bacterial groups

A few of the minor evolutionary groups of Bacteria remain to be examined with respect to RNase P RNA structure, including the thermophilic oxygen reducers (e.g. *Aquifex* and *Hydrogenobacter*), the *Leptospirillum* group, the Fibrobacteria and the Fusobacteria. The thermophilic oxygen reducers are of particular interest because they may represent both the earliest known divergence in the bacterial lineage and the most primitive bacterial group (at least in terms of rRNA divergence) (41). However, the complete sequence of the genome of *Aquifex aeolicus* (42) does not contain sequences with obvious similarity to known RNase P RNAs or proteins, suggesting that the enzyme is quite different in structure than in other organisms, perhaps lacking an RNA entirely. Transfer RNAs in *A.aeolicus* are nevertheless encoded in clusters and as part of ribosomal RNA operons, implying the need for RNase P activity.

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