

Structure and evolution of ribonuclease P RNA in Gram-positive bacteria

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ABSTRACT

The sequences and structures of RNase P RNAs of some Gram-positive bacteria, e.g. *Bacillus subtilis*, are very different than those of other bacteria. In order to expand our understanding of the structure and evolution of RNase P RNA in Gram-positive bacteria, gene sequences encoding RNase P RNAs from 10 additional species from this evolutionary group have been determined, doubling the number of sequences available for comparative analysis. The enlarged data set allows refinement of the secondary structure model of these unusual RNase P RNAs and the identification of potential tertiary interactions between P10.1 and L12, and between L5.1 and L15.1. The newly-obtained sequences suggest that RNase P RNA underwent an abrupt, dramatic restructuring in the ancestry of the low-G+C Gram-positive bacteria after the divergence of the branches leading to the 'Clostridia and relatives' and the remaining low-G+C Gram-positive species. The unusual structures of the RNase P RNAs of *Mycoplasma hyopneumoniae* and *M. floccularre* are apparently derived from RNAs with *Bacillus*-like structure rather than from intermediate, partially restructured ancestral RNAs. The structure of the RNase P RNA from the photosynthetic *Heliobacillus mobilis* supports the relationship of this specie with *Bacillus* and *Staphylococcus* rather than the 'Clostridia and relatives' as suggested by the sequences of their small-subunit ribosomal RNAs.

INTRODUCTION

Ribonuclease P (RNase P) is the endoribonuclease responsible for the removal of leader sequences from precursor tRNAs (see refs 1 and 2 for review). In bacteria, Archaea, Eucarya and mitochondria [but apparently not chloroplasts of vascular plants (3)], RNase P is a ribonucleoprotein. In all bacteria investigated, the RNA component of RNase P is catalytically active *in vitro*; i.e. it is a ribozyme.

The first two bacterial RNase P RNA sequences determined were those of *Escherichia coli* (4) and *Bacillus subtilis* (5), and the earliest reliable secondary structures were determined from

the comparative analysis of these sequences and those of a handful of each of their relatives (6). The expectation at the time was that additional sequences obtained from organisms from other phylogenetic groups of bacteria would be about as different from one another in sequence and secondary structure as are those of *E. coli* and *B. subtilis*. This was not the case; RNase P RNA sequences are now available from all of the 11 major bacterial phylogenetic lineages, and except for those of *Bacillus* and its relatives, all of these RNAs are similar to those of *E. coli* and relatives (1,7). It was fortuitous that the first two available bacterial RNase P RNA sequences represented each of the major structure classes.

The common bacterial RNase P RNA secondary-structure class (which for the purpose of discussion we term 'type A', for ancestral), such as that present in *E. coli*, has been examined in detail by comparative analysis of over 100 sequences (8), and preliminary models of its three-dimensional structure have been proposed (9,10). Phylogenetic analysis of bacterial RNase P RNAs suggests that this common structure class is the ancestral form of the bacterial RNA (11). Nearly every base pair in this structure can be confirmed by the occurrence of complementary covariation in the base pairs, and several tertiary interactions have been identified. The unusual RNase P RNAs of *Bacillus* and its relatives (termed 'type B', for *Bacillus*-like) are less well-understood. The type A and B RNase P RNAs share a common core of essential secondary structure, but differ in their suite of additional elements. It has been proposed that the tertiary structures of these two classes of RNase P RNAs closely resemble one another (12). Several lines of evidence are consistent with this: (i) the similarity of the kinetic properties of the *E. coli* and *B. subtilis* RNase P RNAs, (ii) the observation that the RNAs bind to and respond to both their homologous and heterologous RNase P protein subunits, and (iii) the steric potential of the alternative secondary structure elements to adopt similar three-dimensional structures. Determination of the structural and functional roles of the type-specific structural elements will require better understanding of the structure and evolutionary background of the type B RNase P RNAs. Therefore, we have determined sequences encoding RNase P RNAs from a broad range of Gram-positive bacteria for comparative analysis. In addition to structural information, these sequences shed light on the evolutionary transition from one structural type of RNA to another, and on phylogenetic relationships within the Gram-positive bacteria.

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MATERIALS AND METHODS

Nucleic acid purification

Clostridium sporogenes, *Lactobacillus acidophilus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Streptococcus faciem* were grown in trypticase soy broth (Difco) at 30°C (all cultures from the Indiana University stock culture collection). *Clostridium innocuum* (ATCC 14501) was grown anaerobically in beef liver infusion broth (ATCC medium 38) at 37°C. Cell paste from *Eubacterium thermomarinus* strain ES2 was a gift from Robert Kelly (North Carolina State University). DNAs were purified as previously described (13). *Heliobacillus mobilis* and *Heliobacterium chlorum* DNAs were gifts from Howard Gest and Carl Bauer (Indiana University) and *Acholeplasma laidlawii* DNA was a gift from Carl Woese (University of Illinois).

Southern hybridization

Southern analysis of *Bam*HI and *Pst*I restriction endonuclease-digested genomic DNAs was performed as previously described (13). The probes used were partially-hydrolyzed, uniformly ³²P-labeled run-off transcripts of the *B.subtilis* or *Streptomyces bikiniensis* RNase P RNA genes prepared as previously described (13).

PCR amplification

Polymerase chain reactions (14) were performed using buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM each dGTP, dCTP, dATP and dTTP, 0.05% Nonidet P40 and 200 ng each primer oligonucleotide. In some cases, reactions contained 5% acetamide. The primers used were 59FBam (CGGGATCCG-IIGAGGAAAGTCCIIIGC) and either 347REco (CGGAATTC-RTAAGCCGGRTTCTGT) or 347RXba (GCTCTAGATAAGC-CRYGTTYTGT). The amplifications included an initial 2 min 94°C incubation, 30 or 40 amplification cycles (92°C for 1.5 min, 50°C for 1.5 min, 72°C for 0.5 min each cycle), and a final 7 min 72°C incubation.

Cloning of RNase P RNA-encoding genes

The genes encoding RNase P RNA from *C.sporogenes*, *E.thermomarinus*, *H.mobilis*, *H.chlorum* and *M.luteus* were obtained by PCR amplification using primers 59FBam and 347REco. The genes encoding RNase P RNA from *A.laidlawii*, *C.innocuum*, *L.acidophilus*, *S.aureus*, *S.faecalis* and *S.faciem* were obtained in the same way using primers 59FBam and 347RXba. Restriction endonuclease-digested PCR products were separated by electrophoresis in 3% low-melting agarose gels (NuSieve GTG agarose, FMC, Rockland, ME). Agarose plugs containing the DNA bands were excised, melted (65°C), and used directly in ligation reactions containing restriction endonuclease-digested pBluescript KS⁺ DNA (Stratagene). In the case of *C.innocuum*, the amplified DNA was digested only with *Xba*I and cloned into *Xba*I/*Eco*RV-digested vector DNA. In the case of *H.chlorum*, only a small fragment of the gene was cloned due to the presence of an *Eco*RI site in the amplified DNA.

Sequence determination

The nucleotide sequences encoding RNase P RNAs were determined from double-stranded plasmid DNAs by the dideoxy

chain termination method (15) with Sequenase version 2.0 (Amersham, Arlington Heights, IL) using M13 universal, M13 reverse, 59FBam, 347REco, 347RXba, 174F (AGGGTCAAAN-GGTGSGGTAAGAG) and 174R (CTCTTACCSCACCNTTTC-ACCCT) primers. 7-Deaza-dGTP was used to alleviate band compressions in sequencing gels.

Sequence/structure analysis

RNase P RNA sequences were aligned manually using SeqApp (Don Gilbert, Indiana University). Comparative analysis of secondary structure was performed as previously described was identified using Covariation (16). Sequences derived from PCR primers were excluded from the analysis. Phylogenetic trees based on the RNase P RNA sequences or those of small-subunit ribosomal RNAs (using sequence alignments from the Ribosomal Database project) were generated using the DeSoete algorithm in GDE (17). Trees of the type A and type B RNase P RNAs were calculated separately and merged with a minimal representative tree. All RNase P RNA sequences, alignments and secondary structures are available electronically from the Ribonuclease P Database (<http://www.mbio.ncsu.edu/RNaseP/>) (18). The accession numbers for these sequences are U64877-U64887.

RESULTS

RNase P RNA-encoding genes

The Gram-positive bacteria are comprised of two major phylogenetic groups; the 'low G+C' group, of which *Bacillus* is a member, and the 'high G+C' group, which includes *Streptomyces*. Southern analyses of genomic DNAs were used to qualitatively assess sequence similarity between the type A *S.bikiniensis* and type B *B.subtilis* RNase P RNAs and those of various other members of the Gram-positive bacteria (Fig. 1). DNA from *C.sporogenes*, *E.thermomarinus* and *M.luteus* hybridized to the type A *S.bikiniensis* probe, whereas DNA from the remaining species (*S.aureus*, *S.faecalis*, *L.acidophilus*, *M.fermentans*, *A.laidlawii* and *H.chlorum*) hybridized to the type B *B.subtilis* probe (Fig. 1). Positive hybridization by these probes was mutually exclusive. The probe hybridized in each case to single bands of genomic DNA except where digests appeared to be incomplete, suggesting that RNase P RNA is encoded by single-copy genes in these organisms.

To determine if the sequence similarities identified by hybridization corresponded to differences in secondary structure types, and to obtain sequences for detailed analysis, RNase P RNA-encoding (*mpB*) genes from these organisms were amplified by polymerase chain-reaction using degenerate primers complementary to consensus sequences in the highly conserved helix P4, cloned, and their nucleotide sequences were determined. In those instances of genomic DNAs that hybridized to the *B.subtilis* probe, with the exceptions of the *H.chlorum*, amplification was only achieved with primers 59FBam and 347RXba, the latter primer being specifically designed to accommodate unusual nucleotides in the 3' strand of P4 of the RNase P RNAs of the available type B RNase P RNAs. In the instances of genomic DNAs that hybridized to the *S.bikiniensis* probe, and in the case of *H.chlorum*, amplification was obtained only using primers 59FBam and 347REco, the latter primer being designed to accommodate all type A RNase P RNA gene sequences but not those of the available type B RNAs.

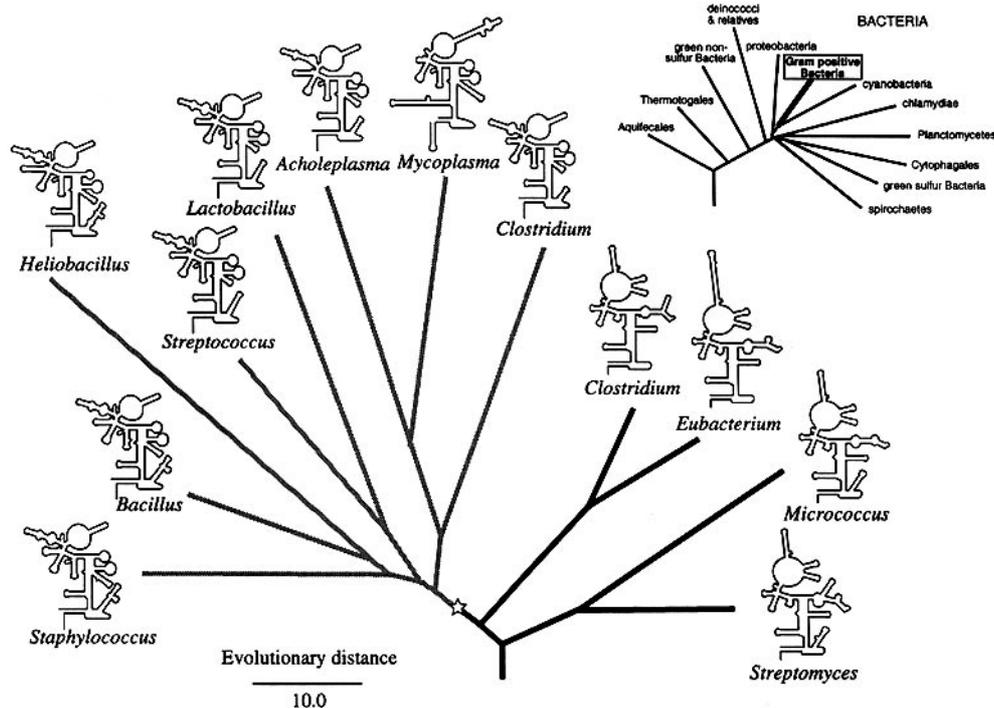


Figure 1. Evolution of RNase P RNA sequence and structure in Gram-positive bacteria. A representative phylogenetic tree based on RNase P RNA sequences is shown with line drawings of the secondary structures of the RNase P RNAs. The genera are represented by (clockwise) *Staphylococcus aureus*, *Bacillus subtilis*, *Heliobacillus mobilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Acholeplasma laidlawii*, *Mycoplasma hyopneumoniae*, *Clostridium innocuum*, *Clostridium sporogenes*, *Eubacterium thermomarinus*, *Micrococcus luteus* and *Streptomyces bikiniensis*. Line segments in grey connect those species whose DNA hybridized with *B.subtilis* RNase P RNA and not *S.bikiniensis* RNase P RNA; black line segments indicate those species whose DNAs hybridized with *S.bikiniensis* RNase P RNA and not *B.subtilis* RNase P RNA. The star indicates the position in the tree separating the type A and type B RNase P RNA structures; subtrees of each structure type were merged at this branch and so evolutionary distances through this branch are arbitrary. A phylogenetic tree based on small-subunit ribosomal RNA of the main evolutionary groups of the bacteria (24) is shown in the upper right-hand corner for reference.

Sequence and structure analysis

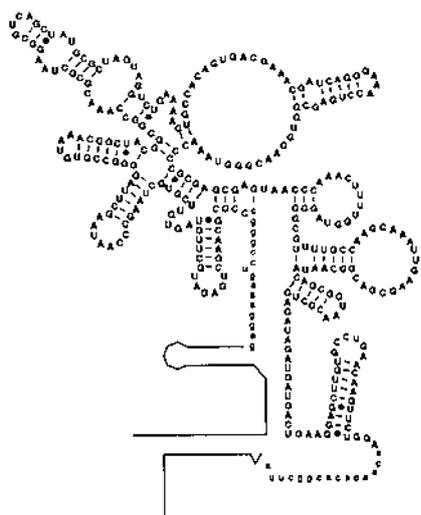
The newly obtained Gram-positive sequences were aligned with the database of bacterial RNase P RNAs, and secondary structures were inferred by comparative analysis (Fig. 2) (19). All of the RNase P genes isolated from DNA that hybridized to the *S.bikiniensis* probe were found to be type A RNase P RNAs; those that hybridized to the *B.subtilis* probe were found to be type B RNase P RNAs. The type B RNAs contained all of the secondary structural elements unique to the *Bacillus* RNAs (P5.1, P10.1, P15.1, P15.2), and lacked all of those elements absent in the *Bacillus* RNAs (P6, P13, P14, P16, P17, P18). The model for the secondary structure of the type B RNase P RNA is supported in detail by base–base covariation in 72% of the pairings proposed in the consensus model (Fig. 3). All helices except the P10 and P11 ‘minihelices’ are supported by the occurrence of covariation of at least two base-pairings. Helices P9, P10.1, P12 and P19, which are minimally present or absent in the consensus, are well-supported within those groups in which they occur; of the 113 base-pairings proposed in the *B.subtilis* RNase P RNA, 93 (82%) are specifically supported by phylogenetic covariation. Sequence variation within the context of this secondary structure is similar [assessed by the entropy coefficient H_x (20)] to that found in other phylogenetic groups of similar evolutionary depth; having abruptly and dramatically changed in sequence and structure, these RNAs apparently resumed evolutionary divergence at rates similar to those of RNase P RNAs in other evolutionary groups.

One element of the type B secondary structure model, P10.1, has undergone revision in this model. Sequence variation in this helix in the newly-obtained sequences is inconsistent with the previous model, in which the structure was based only on thermodynamic predictions consistent with the few sequences available at the time (21). The refined structure of P10.1 (Fig. 2) is well-supported by base covariations. This structure resembles similar helices in group I and group II intron RNAs that are thought to be involved in a tertiary interaction with a GAAA tetraloop (22,23). In addition, the proximal base pair of P1 (A11:U362 in *E.coli*; G14:C384 in *B.subtilis*) has been changed in the model; the same 5' base is now Watson–Crick base paired to the nucleotide one base 5' than in the previous model. This base pair in the previous model was not Watson–Crick (13) and is not supported by the additional sequences obtained since the pair was originally proposed. The spacing between P4 and P1 is now conserved (one base, always ‘A’) in the refined model, and most RNAs contain a bulged nucleotide 3' to the proximal base pair of P1.

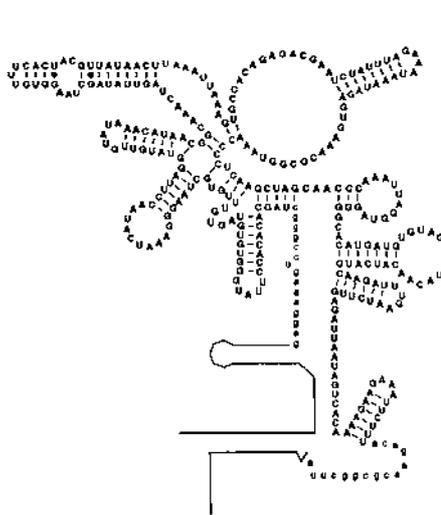
Phylogenetic analysis

The evolutionary relationships of the bacterial species were estimated by analysis of the RNase P RNA sequence alignment. The resulting phylogenetic tree (Fig. 1) is consistent with phylogenetic relationships inferred on the basis of small-subunit ribosomal RNAs (17) with the single exception of the placement of the photosynthetic Gram-positive bacteria. The photosynthetic Gram-positive bacteria have been considered a separate lineage,

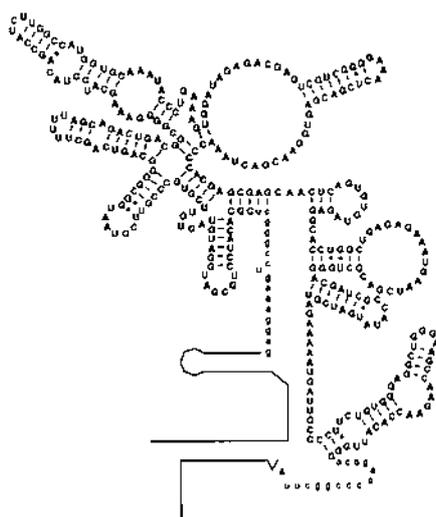
Lactobacillus acidophilus



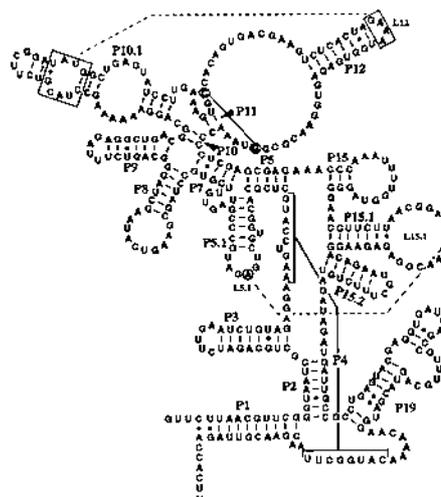
Acholeplasma laidlawii



Heliobacillus mobilis



Bacillus subtilis



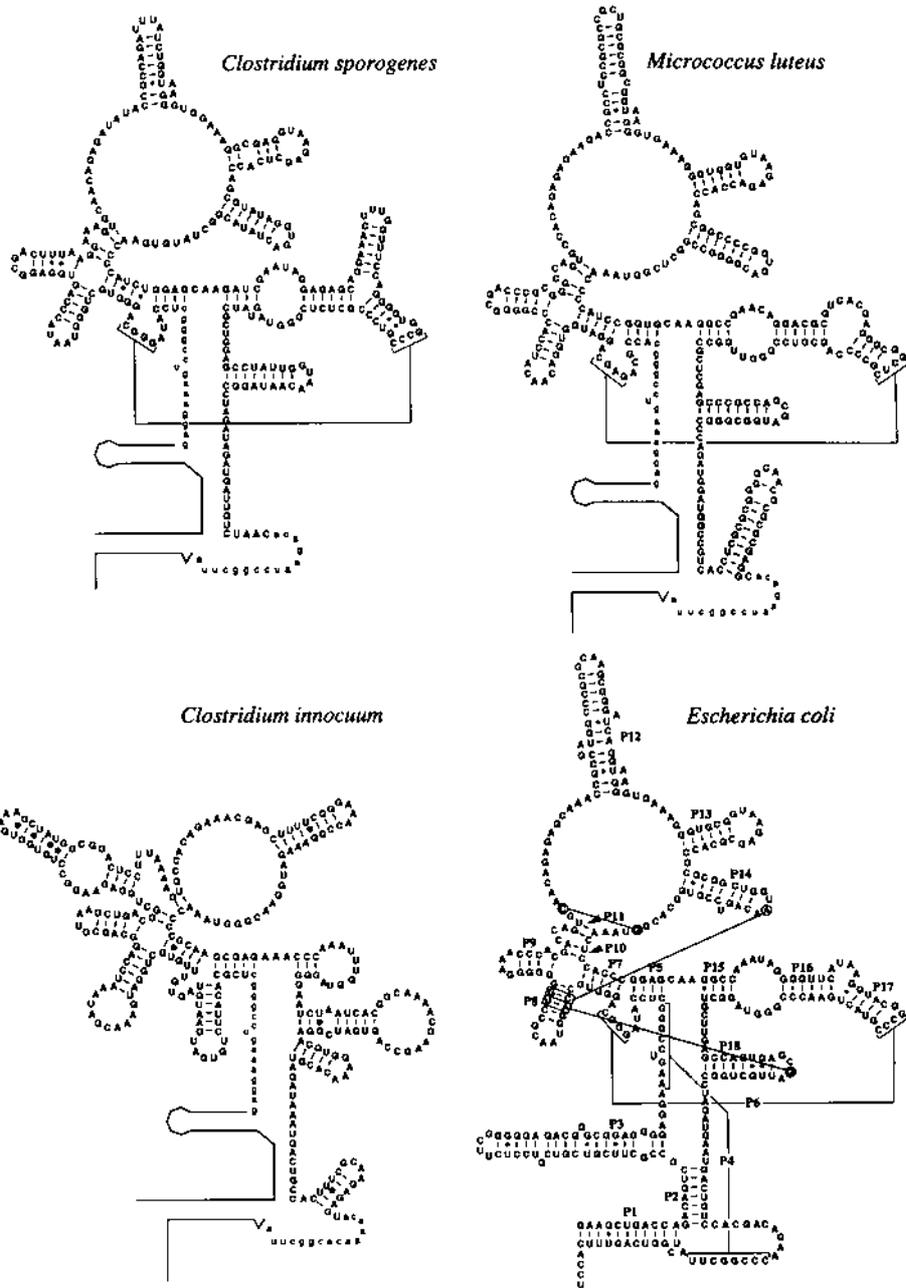
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distinct from both the low- and high-G+C subdivisions of Gram-positive bacteria, based primarily on preliminary small-subunit nuclease T1 catalog data and phenotypic characteristics (24). According to more recent small-subunit rRNA sequence comparisons (17), the photosynthetic species would be members of the ‘Clostridia and relatives’ group within the low G+C Gram-positive bacteria (of which *C.sporogenes* and *E.thermophilus* are members), inconsistent with its type B RNase P RNA. In the tree based on RNase P RNA sequences, *H.mobilis* appears as a distinct, rapidly-evolving branch within the ‘*Bacillus–Lactobacillus–Streptococcus* group’ of the low G+C subdivision. The sequence similarity, estimated phylogenetic relationships, and secondary structure details of the *H.mobilis* RNase P RNA all show that this organism is a member of the group of organisms with type B RNase P RNAs rather than type A RNase P RNAs,

as was suggested (but not resolved) by ribosomal RNA sequence analysis.

DISCUSSION

The sequence and structure of RNase P RNA underwent a dramatic restructuring in the common ancestor of the ‘*Bacillus–Lactobacillus–Streptococcus*’ and ‘*Mycoplasma* and relatives’ groups of the low G+C Gram-positive bacteria (Fig. 1). This alteration was apparently abrupt, at least from the broad perspective of bacterial evolution; there is no evidence of partially restructured RNAs amongst the diverse sequences examined. The RNase P RNAs from *Mycoplasma* spp. [except that of *M.genitalium*, taken from the complete genome sequence (25)] are distinct from those of the other *Bacillus*-like RNAs (26), but



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seem to have diverged from a more *Bacillus*-like ancestry rather than from a structural intermediate between the ancestral and *Bacillus*-like RNA classes. The *C. innocuum*, *A. laidlawii* and *M. genitalium* RNAs resemble those of *Bacillus* rather than those of the previously-determined *Mycoplasma* spp. despite the phylogenetic affiliation of these organisms. The *Mycoplasma* spp. RNase P RNAs (except that of *M. genitalium*) are distinct in sequence and lack P10.1, one of the unique elements of the type B RNAs; the sequence and presence of P10.1 in the *C. innocuum*, *A. laidlawii* and *M. genitalium* RNAs imply that the unusual features of the other *Mycoplasma* RNAs are derived secondarily from a *Bacillus*-like RNA.

It has previously been suggested that the absence in type B RNase P RNAs of P6, a helix that creates a pseudoknot in type A

RNAs, is compensated by the presence of P5.1, which could replace both P6 and P17 (also absent in type B RNAs) in the architecture of the RNAs (12). However, the replacement of P6 and the associated P16 and P17 in the *E. coli* RNA with the *B. subtilis* P5.1 does not result in restoration of the biochemical phenotype associated *in vitro* with the loss of P6/P16/P17 (27). It seems likely that P5.1 docks to an additional element, the analog of P16 in type A RNAs, to complete the structural replacement; this analog has yet to be identified. The comparative data suggest that the most likely candidate for this structural analog of P16 is P15.1; P5.1 and P15.1 are the only structural elements that are present in all RNase P RNAs that lack the P6/P16/P17 element. Although the number of type B RNase P RNA sequences is small for an analysis of tertiary contacts, there is significant covariation

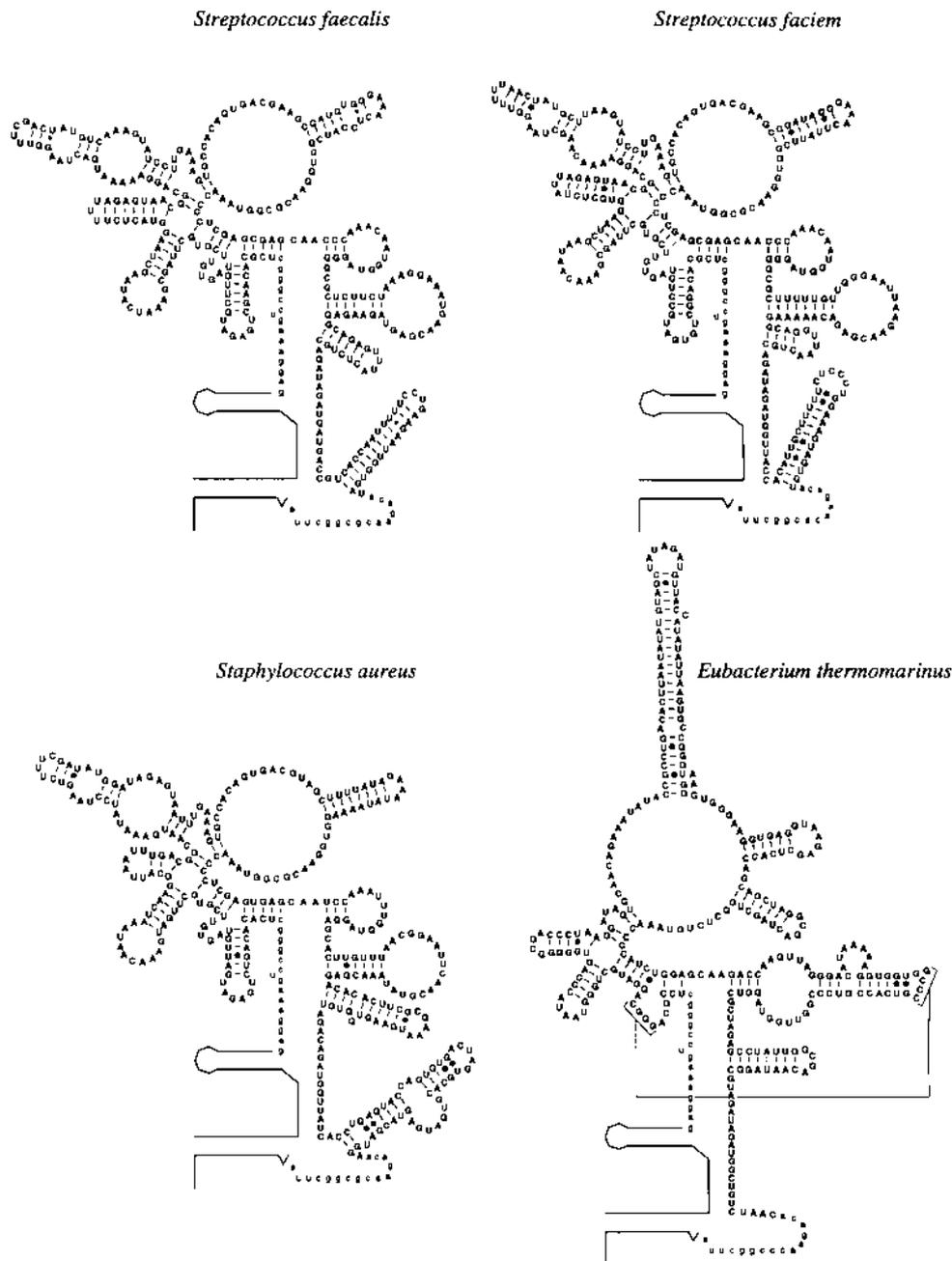


Figure 2. RNase P RNA secondary structures. Sequences shown in lower case indicate the position of the oligonucleotide primers used to amplify the gene sequences. Lines indicate regions of the encoded RNA distal to the PCR primers and therefore not available for analysis. The *B.subtilis* and *E.coli* RNase P RNAs are included for reference, and helices in these RNAs are labeled P1–P19. Tertiary interactions (see text) are also shown; the L12:P10.1 (28) and L5.1:L15.1 (this study) interactions are shown in grey because of their relatively weak comparative support and because they have not been confirmed by other methods.

between nucleotide A71 (*B.subtilis* numbering) in L5.1 and nucleotide U283 in L15.1 (Fig. 2), consistent with interaction between these loop's sequences.

The redefined secondary structure of P10.1 is interesting because it contains a structure and sequence motif that has the potential to form a tertiary interaction with GAAA tetraloops. This tertiary interaction was originally identified in group I intron RNAs (22), and has been confirmed biochemically in both group I and group II intron RNAs (23), although the physical nature of the interaction has yet to be determined. The presence of the sequence motifs and the possibility of the tertiary interaction in

the type B RNase P RNAs has already been pointed out (28). The most likely GAAA tetraloops in these RNAs to form the interaction is L12 because: (i) the length of P12 is invariant in all of the type B RNAs in which the P10.1 motif is present (suggesting a length-dependent loop contact); (ii) these loops are invariably the requisite GAAA, never an alternative GNRA or other loop sequence in those RNAs with the appropriate P10.1 sequence; and (iii) in those type B RNAs that lack P10.1 (*M.flocculara*, *M.hyopneumoniae* and *M.fermentans*), both the length and sequences of L12 are no longer conserved (i.e. the presence of the two required motifs covary phylogenetically).

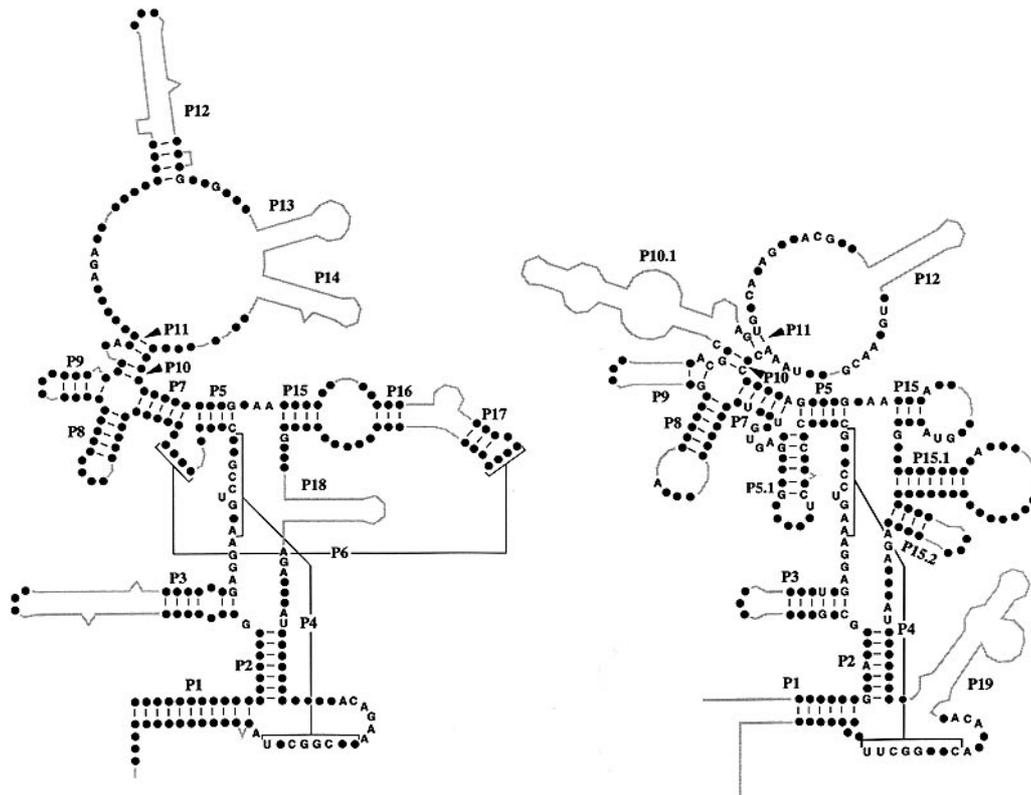


Figure 3. Consensus type A and type B RNase P RNAs. The type A consensus RNA (right) and the type B consensus RNA (left). Only nucleotide positions that are present in all instances of each type of RNA are shown, as letters (A, G, C or U) if invariant or dots (●) if variable in identity. Grey lines indicate elements of the *E. coli* (type A) or *B. subtilis* (type B) RNAs that are absent in some of the RNAs of each type and are therefore absent in the consensus. The type A consensus is based on 116 sequences (18). The type B consensus is based on 14 sequences, including all of those type B RNAs shown in Figure 2, and those of *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus brevis* (6), *Mycoplasma fermentans* (38), *Mycoplasma floccularre*, *Mycoplasma hyopneumoniae* (26) and *Mycoplasma genitalium* (25).

The *Bacillus brevis* RNA (6) lacks both sequence motifs, but has the appropriate motifs to form an alternative tertiary interaction involving the same loop sequence (in this case GCGA) with adjacent A=U and G=C base pairs, in the appropriate location in P10.1. The *C. innocuum* RNA has the P12 loop GAAA sequence but lacks the P10.1 motif. The region of P10.1 where the interaction would presumably take place are unusual in *C. innocuum*, however, in that the region is predominated by G-U pairs. It seems likely that the tertiary contact is maintained in the *C. innocuum* RNA by unusual helix geometry in this helix that may mimic the structure of the homologous region in other RNAs. In both *B. brevis* and *C. innocuum*, the length of P12 is the same (8 base pairs) as in those RNAs that can form the 'standard' group I intron-like tertiary interaction.

The nature of the evolutionary pressure that resulted in the dramatic alteration of RNase P RNA structure in the low G+C Gram-positive bacteria is not clear. Although exhaustive comparisons are not available, no clear difference in catalytic activity, kinetic properties, or substrate differentiation between the type A *E. coli* and type B *B. subtilis* RNase P RNAs have been demonstrated. Moreover, since their tertiary architectures seem to be equivalent and the proteins are interchangeable, the alteration in RNase P RNA from the A to the B type of structure may then be an evolutionary 'neutral' mutation. One interesting apparent difference in tRNA biogenesis between the low G+C Gram-positive bacteria and most other bacteria is the encoding of the 3'-terminal CCA sequence. The 3'-CCA sequence is generally included in the

RNA-encoding genes of most Bacteria, but in *B. subtilis* and *M. genitalium* the tRNA-encoding genes generally do not encode the 3'-CCA (how widespread this feature is among relatives of these organisms is not known). For instance, in *Haemophilus influenzae* (29) and *M. genitalium* (25), for which the genome sequences are available for complete comparison, 96% (54 of 56) and 6% (2 of 33), respectively, of tRNA-encoding genes include encoded 3'-CCA. However, it seems unlikely that the difference in RNase P RNA structure results directly from the presence or absence of encoded 3'-CCA; 3' processing of pre-tRNAs, including the post-transcriptional addition of CCA if required, usually precedes cleavage by RNase P (30). In any case, both the *B. subtilis* and *E. coli* RNase P RNAs respond similarly *in vitro* to alterations in the sequence or length of the 3'-terminus of substrate pre-tRNAs (31,32). It is also possible that the alteration in RNase P RNA structure is in response to a change in a substrate other than pre-tRNA. In all bacteria examined, except *Bacillus* spp., the signal recognition particle (SRP) RNA is a 4.5S RNA that is homologous only to the core domain of the much larger 7S SRP RNAs of Eucarya (a.k.a. eucaryotes) and Archaea (a.k.a. archaeobacteria) (33,34). Surprisingly, SRP RNA in *Bacillus* spp. is a large, 7S RNA similar to the eucaryal and archaeal RNAs (35). Both the 4.5S and 7S forms of bacterial SRP RNA are processed at their 5'-ends by RNase P (36,37). However, since *Mycoplasma* spp., which have type B RNase P RNAs, have typical 4.5S SRP RNAs (33), it is unlikely that the change in this substrate is responsible for the alteration in RNase P RNA structure.

Despite the differences in structure in the Gram-positive bacteria, phylogenetic trees based on these RNase P RNA sequences (Fig. 1) are generally consistent with those based on small-subunit ribosomal RNA sequences, which are commonly used for estimating phylogenetic relationships between organisms. Small-subunit rRNA analyses have not resolved the placement of the photosynthetic Gram-positive bacteria; it was originally thought that these organisms formed a group separate from either the high- or low-G+C subdivisions (termed the 'photosynthetic subdivision') (24). More recently, these organisms have been placed with the 'Clostridia and relatives', a group within the low G+C subdivision (17). Neither of these possibilities is consistent with the structure of the RNase P RNA from *H. mobilis* (Fig. 2) or the small sequence segment obtained from *H. chlorum*. Because the RNase P RNAs of *C. sporogenes* and *E. thermomarinus* (members of the clostridia and relatives subdivision) are of the ancestral type A, and the *H. mobilis* and *H. chlorum* RNAs are of the more recently derived type B, the heliobacteria must be related by ancestry to the *Bacillus-Lactobacillus-Streptococcus* and *Mycoplasma* and relatives evolutionary branches.

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