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Mycoplasma fermentans simplifies our view of the catalytic core of ribonuclease P RNA

ROBERT W. SIEGEL, AMY B. BANTA,[1] ELIZABETH S. HAAS,[2] JAMES W. BROWN,[2] and NORMAN R. PACE[3]

Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, Indiana 47405, USA

ABSTRACT

The catalytic RNA moiety of (eu)bacterial RNase P is responsible for cleavage of the 5′ leader sequence from precursor tRNAs. We report the sequence, the catalytic properties, and a phylogenetic-comparative structural analysis of the RNase P RNA from *Mycoplasma fermentans*, at 276 nt the smallest known RNase P RNA. This RNA is noteworthy in that it lacks a stem-loop structure (helix P12) that was thought previously to be universally present in bacterial RNase P RNAs. This finding suggests that helix P12 is not required for catalytic activity in vivo. In order to test this possibility in vitro, the kinetic properties of *M. fermentans* RNase P RNA and a mutant *Escherichia coli* RNase P RNA that was engineered to lack helix P12 were determined. These RNase P RNAs are catalytically active with efficiencies (k_{cat}/K_m) comparable to that of native *E. coli* RNase P RNA. These results show that helix P12 is dispensable in vivo in some organisms, and therefore is unlikely to be essential for the mechanism of RNase P action. The notion that all phylogenetically volatile structures in RNase P RNA are dispensable for the catalytic mechanism was tested. A synthetic RNA representing the phylogenetic-minimum RNase P RNA was constructed by deleting all evolutionarily variable structures from the *M. fermentans* RNA. This simplified RNA (Micro P RNA) was catalytically active in vitro with approximately 600-fold decrease in catalytic efficiency relative to the native RNA.

Keywords: phylogenetic analysis; RNA catalysis; RNase P; RNA structure

INTRODUCTION

Ribonuclease P (RNase P) is the ubiquitous RNA-processing endonuclease that forms the mature 5′ end of transfer RNA (tRNA) (for reviews see: Altman et al., 1993; Kirsebom, 1995; Pace & Brown, 1995). In Bacteria, RNase P is a ribonucleoprotein consisting of a large RNA (usually 350–400 nt) and a single, small protein (approximately 120 amino acids). The RNA component of bacterial RNase P RNA is a ribozyme, the catalyst in the processing reaction (Guerrier-Takada et al., 1983); in vitro, all bacterial RNase P RNAs investigated efficiently cleave pre-tRNA in the absence of the protein. In order to understand RNase P RNA and manipulate its mechanism, it is first necessary to solve its structure.

Phylogenetic-comparative structure analysis has resulted in the formulation of a highly refined secondary structure model for the bacterial RNase P RNA (Haas et al., 1994). Low-resolution, global tertiary structure models have been devised based on crosslinking and comparative studies (Harris et al., 1994; Westhof & Altman, 1994). Results of mutational (Darr et al., 1992; Waugh & Pace, 1993; Schlegl et al., 1994) and structure-mapping (Guerrier-Takada & Altman, 1993; Talbot & Altman, 1994) studies are generally consistent with the evolving RNase P RNA structural model.

The RNase P RNAs from different organisms differ remarkably in both sequence and length. Some blocks of sequence and structure are present in all types of RNase P RNA. Other structural elements, usually irregular hairpins, are not always present in the different types of the RNase P RNAs. The conserved elements are distributed throughout the RNA structure and are brought together in the tertiary structure to form the core of the ribozyme (Pace & Brown, 1995).

Reprint requests to: Norman R. Pace, Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, Indiana 47405; e-mail: nrpace@sunflower.bio.indiana.edu.

[1] Present address: Molecular Biotechnology, University of Washington, Seattle, Washington 98195, USA.

[2] Present address: Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695, USA.

[3] Address effective July 1, 1996: Department of Plant and Microbial Biology, Koshland Hall 111, University of California, Berkeley, California 94720, USA.

The blocks of sequence and structure that are present in every instance of bacterial RNase P RNA constitute a "phylogenetic-minimum" core structure. Because of their conservation, these sequences and structures are potentially involved in the mechanisms of substrate binding and catalysis. Conversely, structural elements not present in all instances of the ribozyme are not expected to be crucial for action. This notion has been tested by the fabrication and analysis of a simplified RNase P RNA containing structural elements previously considered to be absolutely conserved (Waugh et al., 1989). This RNA, a chimera of *Escherichia coli* and *Bacillus megaterium* RNase P RNA sequences and only 263 nt in length, was catalytically active in vitro with cleavage specificity indistinguishable from the parent molecules.

The genus *Mycoplasma* includes some of the smallest and simplest self-replicating prokaryotes (Herrmann, 1992). Mycoplasmas are representatives of the low G+C Gram-positive bacteria. It has been suggested that these organisms have undergone degenerative evolution, resulting in loss of a peptidoglycan-based cell wall and other properties, along with diminution of the genome (Woese et al., 1980). This process may also have resulted in a structural abbreviation of various molecules. As do other members of this genus, *Mycoplasma fermentans* has a restricted genome size of approximately 1 Mb (Neimark & Lange, 1990). Thus, it seemed possible that *M. fermentans* RNase P RNA might lack structural elements present in all other bacterial lineages and thereby provide information useful in refining our view of the catalytic core of this ribozyme.

In this paper, we report the structure and catalytic properties of RNase P RNA from *M. fermentans*. Phylogenetic–comparative structural analysis shows that the *M. fermentans* RNA is the smallest known naturally occurring RNase P RNA and also lacks a stem-loop structure (helix P12) previously considered to be universally present in bacterial RNase P RNAs. This finding suggests that helix P12 is not required for catalytic activity in all bacterial versions of this ribozyme. In order to test this hypothesis, the kinetic properties of the *M. fermentans* RNA and a mutant *E. coli* RNase P RNA engineered to lack helix P12 were determined. The results refine the phylogenetic-minimum RNase P RNA structure. We show that an RNA molecule containing only those evolutionarily conserved structural features is catalytically active in vitro with the same cleavage specificity as naturally occurring RNase P RNAs.

RESULTS

M. fermentans RNase P RNA-encoding gene

The RNase P RNA-encoding gene from *M. fermentans* was cloned and its nucleotide sequence determined. As detailed in the Materials and methods, a hybridization probe generated by PCR amplification of *M. fermentans* DNA with bacterial RNase P RNA-specific primers (Brown et al., 1996) was used to identify and clone the *M. fermentans* RNase P RNA gene from a size-selected genomic library. In Southern analyses with multiple restriction enzymes, the probe hybridized to a single DNA fragment (data not shown). This indicates that the *M. fermentans* RNase P RNA is encoded by a single-copy gene, as in previously examined bacteria (James et al., 1988; Haas et al., 1994). Phylogenetic–comparative analysis of the RNA-encoding sequence was used to determine the secondary structure of this novel RNA (Fig. 1). The *M. fermentans* RNA is generally typical of other low G+C Gram-positive bacteria, as expected from rRNA phylogeny (Weisburg et al., 1989). The *M. fermentans* RNA, however, lacks helix P12, replacing it with the simple spacer sequence AUU. The RNase P RNA encoding gene in *M. fermentans* also contains the corresponding sequence of a 3′ terminal helix, P20. This helix evidently is present in the RNase P RNA of both *M. hyopneumoniae* and *M. flocculare* (Svard et al., 1994). The latter RNAs, in contrast to the *M. fermentans* RNA, retain helix P12.

Kinetic characterization of RNase P RNAs lacking helix P12

Helix P12 is present in all known natural RNase P RNAs except that of *M. fermentans*. The universal presence of helix P12 previously indicated that it might be an essential structural element for the function of RNase P RNA. The absence of helix P12 from the *M. fermentans* RNA implies, however, that this structural element is not required for catalytic activity. This supposition was tested in the context of an RNase P RNA that normally contains P12 by engineering a mutant *E. coli* RNase P RNA (EcΔP12) in which this structural element is replaced with AUU, the corresponding spacer sequence in the *M. fermentans* RNA (Fig. 1). Because these RNase P RNAs are homologues, replacement of P12 in the *E. coli* RNA with nucleotides derived from *M. fermentans* should disrupt the global structure of the molecule minimally. In addition, two distinct forms of *M. fermentans* RNase P RNA were constructed to test the significance of helix P20 in the cleavage reaction (Fig. 1); Mfe302 RNA contains the helix, whereas Mfe276 RNA lacks this structure. The ionic requirements and kinetic properties of these three RNAs were compared to those of native *E. coli* RNase P RNA.

The monovalent salt (ammonium acetate) requirements of the individual RNAs are shown in Figure 2. All the RNase P RNAs lacking P12 have an increased salt optimum compared to the native *E. coli* RNA. Maximum activity for EcΔP12 RNA occurs at 1.5 M, whereas both *Mycoplasma* RNAs require 2 M monovalent salt. The cleavage specificities of these RNase P RNAs were investigated by comparison of the reaction products to

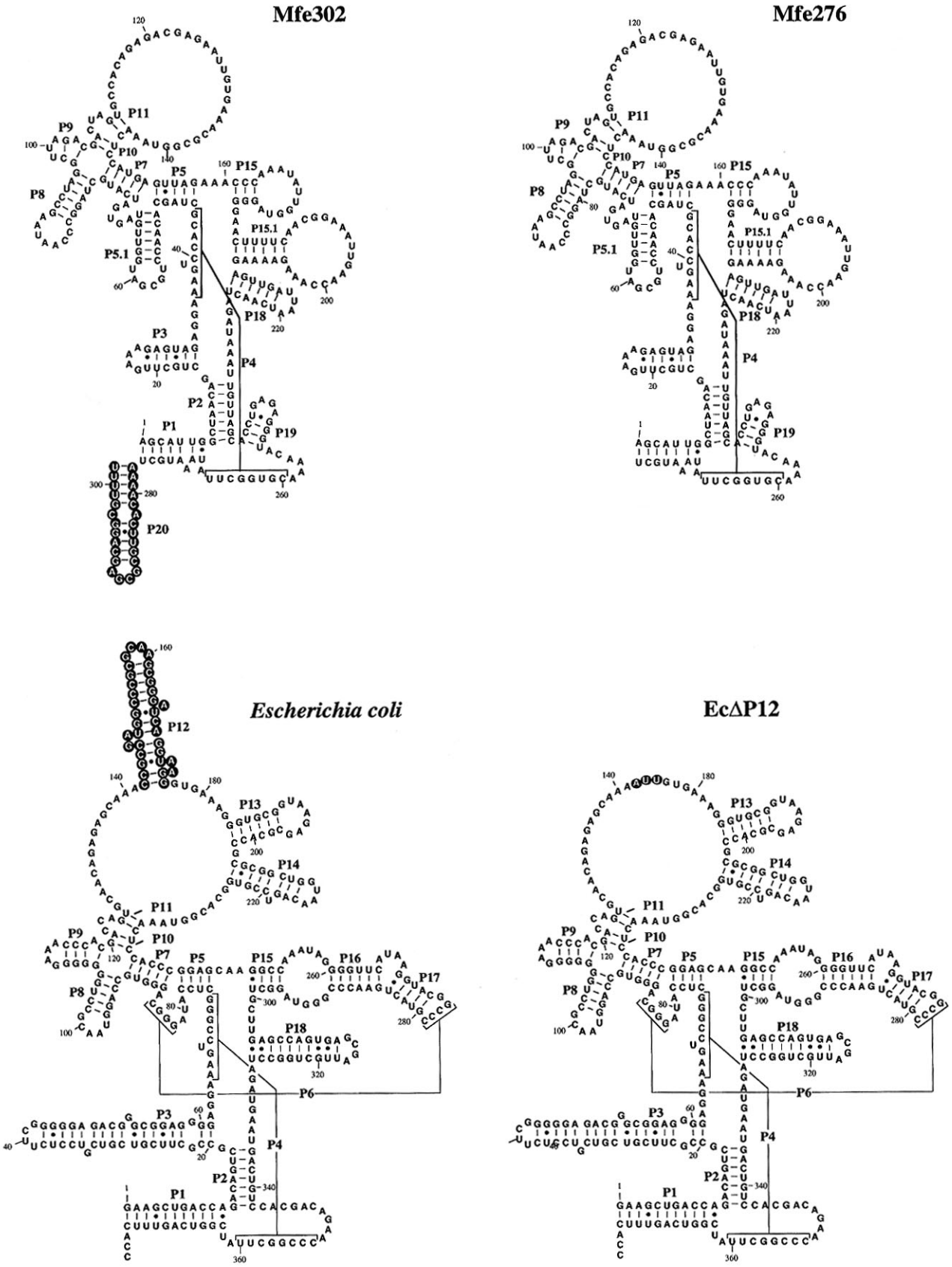

FIGURE 1. Secondary structure of *M. fermentans* RNase P RNA. Paired regions indicated by covariation analysis are depicted as P1–P20 (Haas et al., 1994). Two forms of the RNA are shown: Mfe302 RNA contains nt 277–302, comprising helix P20 (highlighted), whereas Mfe276 RNA was engineered to lack this structure. Helix P12 in native *E. coli* RNase P RNA and the nucleotides replacing this helix in EcΔP12 RNA are also highlighted.

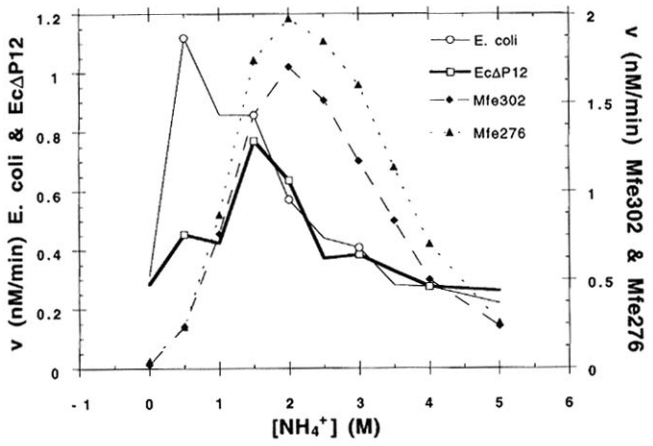

FIGURE 2. Influence of ammonium acetate on cleavage activity of RNAs lacking P12. Open circles indicate velocity (nM/min) for *E. coli* RNase P RNA; open squares, EcΔP12 RNA; filled diamonds, Mfe302 RNA; and filled triangles, Mfe276 RNA. Reactions contained 2 nM RNase P RNA, 25 mM MgOAc, 50 mM Tris-HCl, pH 8, 0.05% NP-40, 0.1% SDS, and either 233 nM (*E. coli*, EcΔP12) or 50 nM (Mfe302, Mfe276) of [^{32}P] pre-tRNA Asp at the indicated concentration of NH_4OAc.

those of native *E. coli* RNase P RNA, in which pre-tRNA is cleaved to form a 77-nt mature tRNA and a 33-nt 5′ leader fragment. EcΔP12, Mfe302, and Mfe276 all accurately cleave pre-tRNA in all ionic conditions tested (Fig. 3), as judged by the production of a single 33-nt 5′ fragment. The doublet of bands for the mature tRNA reflects 3′ heterogeneity due to addition of a nucleotide at the end of the transcript during run-off transcription by T7 polymerase. EcΔP12 RNA cleavage was mapped more precisely using 3′-terminal nucleotide analysis of the 5′ leader fragment. More than 98% of the 3′ termini corresponded to the nucleotide U, which is expected for cleavage at the authentic RNase P RNA cleavage site (data not shown). EcΔP12 therefore exhibits its precise cleavage at the appropriate phosphodiester bond.

The steady-state kinetic parameters of the RNAs lacking P12 were determined (Table 1). At 3 M NH_4^+/25 mM Mg^{2+}, these RNAs closely resemble native *E. coli* RNase P RNA in affinity for substrate (K_m), multiple-turnover catalytic rate (k_{cat}), and overall catalytic efficiency (k_{cat}/K_m). The removal of P12 does increase the optimal concentration of monovalent salt (from 0.5 M to ca. 2 M), indicating structural instability of the RNAs lacking P12: EcΔP12, Mfe302, and Mfe276 RNAs. This destabilization is reflected by a decreased affinity for substrate (increased K_m) at the lower ion concentration for each RNA lacking P12. Because the turnover rate of RNase P RNA is governed by product release (Reich et al., 1988), the reduced affinities result in more rapid product release and, therefore, higher k_{cat} values for those RNAs. The K_m defect is alleviated by increasing the monovalent salt concentration; at high salt, the mutant RNAs have K_m values nearly identical to native *E. coli* RNase P RNA. The kinetic data support the phylogenetic evidence that helix P12 is not critically important to the function of RNase P RNA. P12 does, however, contribute to the architectural stability of the RNA.

The *Mycoplasma* RNAs containing (Mfe302) or lacking (Mfe276) the 3′-terminal helix P20 have indistinguishable in vitro phenotypes. The RNAs have the same salt optimum and, at each ionic strength tested, the same kinetic profile (Fig. 2; Table 1). Although their affinities for substrate are similar to that of native *E. coli* RNase P RNA at 3 M monovalent salt, both Mfe302 and Mfe276 RNAs retain an elevated catalytic rate. Both versions of the *Mycoplasma* RNase P RNA are active at low ionic strength (100 mM NH_4^+ and 10 mM Mg^{2+}) in the presence of the *B. subtilis* protein subunit of the holoenzyme (data not shown). The fact that the Mfe302 and Mfe276 RNAs have the same kinetic properties indicates that helix P20 is not required in vitro for binding or catalysis by *M. fermentans* RNase P RNA.

Construction of a simplified catalytic RNA

Almost half of the length of any particular native RNase P RNA consists of blocks of sequence, usually helical domains, that are absent in other organisms. Because all bacterial RNase P RNAs are able to perform catalysis without the protein moiety, sequences and structures that are not present in at least one instance of the RNA are unlikely to be involved in the mechanism of action of the ribozyme. Conversely, the collection of sequences and structures present in all bacterial RNase P RNAs (Fig. 4A), a phylogenetic-minimum RNase P RNA, is expected to be catalytically competent. As described above, the structure of the *M. fermentans* RNase P RNA specifies that helix P12 is not essential for the minimum RNA. A synthetic version of a phylogenetic-minimum structure was constructed by eliminating all evolutionarily dispensable stem-loop structures from the *M. fermentans* RNA, the smallest naturally occurring RNase P RNA (Fig. 4B). These helices were substituted with homologously positioned sequence elements from native RNase P RNAs that do not contain the helical domains. The resulting RNA, Micro P RNA, which is only 211 nt in length, was tested for in vitro processing of pre-tRNA.

Cleavage activity of Micro P RNA

Micro P RNA is catalytically active in vitro; however, its optimum reaction conditions differ substantially from the native *M. fermentans* RNA on which it is based. Micro P RNA requires 300 mM divalent cation, far higher than the concentration required by the native RNA for maximum activity (Fig. 5A). The optimum monovalent cation concentration is 2.5 M, only slightly higher than that preferred by the *M. fermentans* RNA (Fig. 5B). Di-

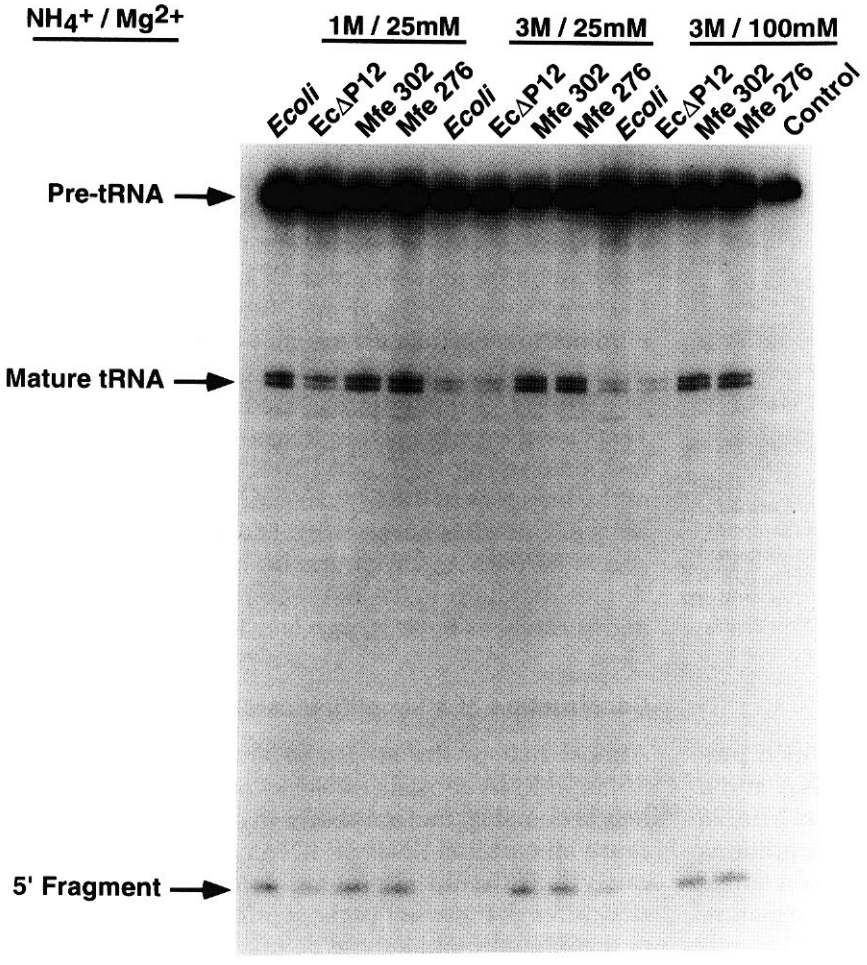

FIGURE 3. Specificity of pre-tRNA processing by RNAs lacking P12. Two nanomolar of each RNase P RNA was allowed to react 15 min at 37 °C in the presence of 50 mM Tris-HCl, pH 8, 0.05% NP-40, 0.1% SDS at the indicated monovalent and divalent ion concentration with 50 nM [^{32}P] pre-tRNA Asp. Products were resolved by denaturing PAGE. Control lane contained no enzyme.

valent cations likely provide structural support as well as serving as a cofactor required for catalysis (see Discussion). The accuracy of cleavage by Micro P RNA is comparable to that of the native *M. fermentans* RNase P RNA. Micro P RNA generates products of the expected sizes (Fig. 6), and nearest-neighbor analysis of the 5′ leader fragment found that >98% of the 3′ termini correspond to the nucleotide expected for accurate cleavage (data not shown). As with the earlier abbreviated RNase P RNA Min 1 RNA, Micro P RNA is not catalytically active at low ionic strength in the presence of the protein subunit (data not shown).

The steady-state kinetic properties of Micro P RNA at its optimal ionic strength (2.5 M monovalent/300 mM divalent) are compared to *M. fermentans* RNase P RNA in Table 2. Both affinity for substrate and catalytic rate of Micro P RNA are approximately 25-fold lower than the native RNA. These reductions result in a catalytic efficiency (k_{cat}/K_m) more than 600-fold lower than that of native RNase P RNA. The decline in cleavage proficiency of the synthetic RNA probably is due to the loss of global stability that results from removal of all phylogenetically variable helices (discussed below). Nevertheless, Micro P RNA cleaves substrate accurately

TABLE 1. Kinetic analysis of RNase P RNAs lacking helix P12.

	1 M NH_4^+/25 mM Mg^{2+}			3 M NH_4^+/25 mM Mg^{2+}			1 M NH_4^+/100 mM Mg^{2+}		
RNase P RNA	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
E. coli	6.8 ± 1.8	0.11 ± 0.003	17 ± 5.1	7.5 ± 1.5	0.11 ± 0.007	14 ± 2.1	9.5 ± 0.71	0.059 ± 0.0007	6.3 ± 0.56
EcΔP12	247 ± 23	0.43 ± 0.25	1.7 ± 0.8	18 ± 10	0.11 ± 0.04	6.5 ± 1.5	5.9 ± 5.7	0.065 ± 0.02	17 ± 13
Mfe 302	1,925 ± 35	51 ± 8	26 ± 4.8	14 ± 7.8	1.5 ± 0.14	120 ± 49	5.9 ± 4.4	0.42 ± 0.28	73 ± 7.1
Mfe 276	2,450 ± 350	62 ± 17	25 ± 3.3	20 ± 3.5	2.1 ± 0	105 ± 21	2.8 ± 0.92	0.26 ± 0.099	110 ± 81

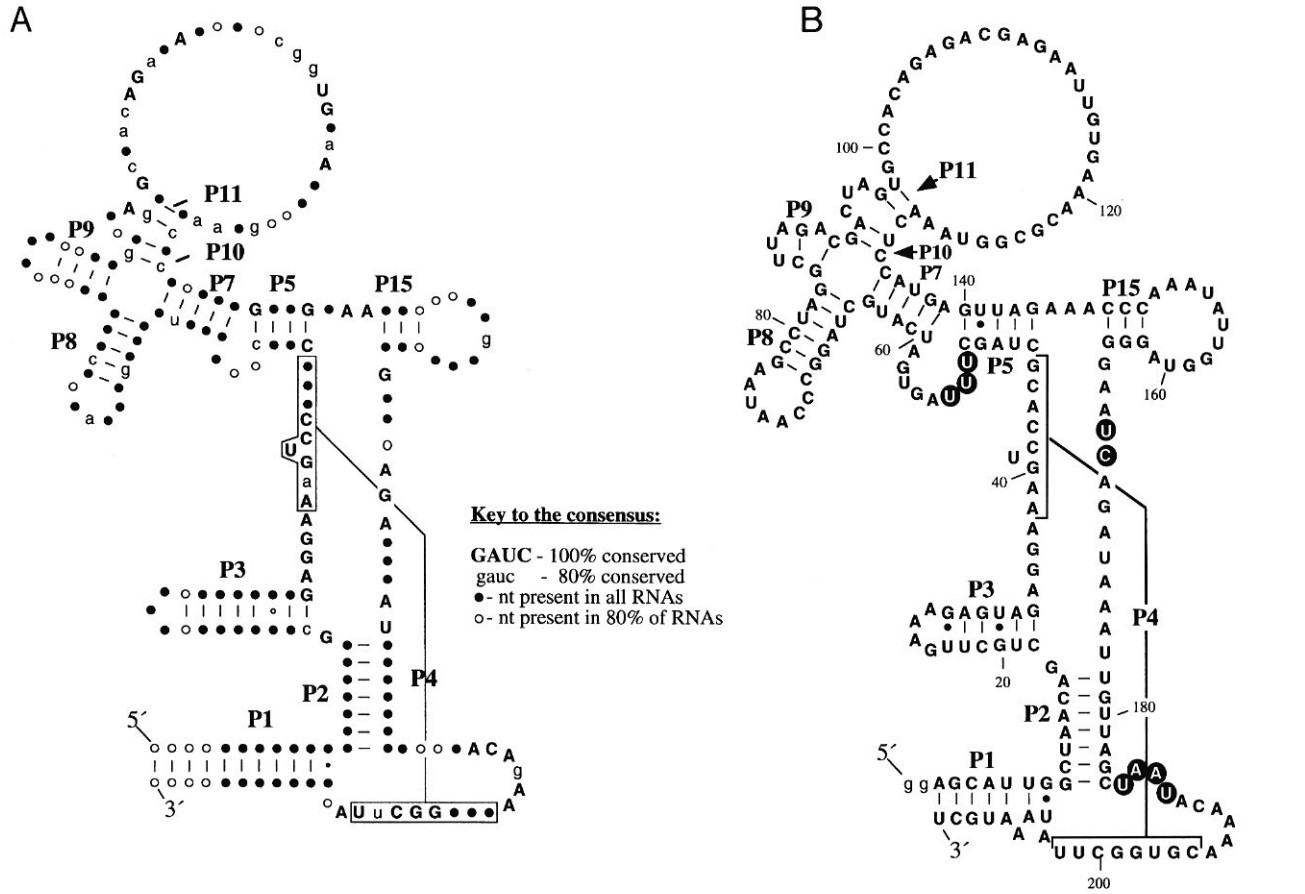

FIGURE 4. Evolutionarily conserved sequences and structures in bacterial RNase P RNA. **A:** Phylogenetic-minimum bacterial consensus RNase P RNA. Only the structural elements conserved in all known bacterial RNase P RNAs are included in the structure. Universally conserved nucleotides are in upper case letters; those that are at least 80% conserved, but not invariant, are in lower case letters. Nucleotides that are not conserved in identity but are universally present are denoted by filled circles; those present in at least 80% of the RNAs, but absent in at least one case, are denoted by open circles. The base pairings indicated by closed and open dots are, respectively, a conserved noncanonical (G·G or A·C) interaction and a pairing that is frequently G·G (e.g., in *E. coli*) rather than canonical. **B:** Proposed secondary structure of Micro P RNA based on *M. fermentans* RNase P RNA. Highlighted nucleotides represent residues used to replace a helix present in *M. fermentans* RNase P RNA. Helix P5.1 does not have an obvious phylogenetic replacement; however, this helix was replaced with uridylate residues, to match the number of nucleotides in that region of the *E. coli* RNA. The choice of U residues was to maximize flexibility in the local structure of the RNA. Helices P15.1 and P18 were replaced with nucleotides derived from *Chlorobium limicola*; and helix P19 with nucleotides from *M. hyopneumoniae*. Base pairs are indicated as described above.

and efficiently. This, together with the phylogenetic results, proves that it contains all the elements needed for processing pre-tRNA.

DISCUSSION

A detailed model for the secondary structure of bacterial RNase P RNA has been derived from phylogenetic-comparative analysis of numerous (>100) different sequences of the RNA (Brown et al., 1996). These RNAs each contain a core of conserved sequence and secondary structure that is evolutionarily modified in disparate organisms by the occurrence of discrete helical elements at distinct sites in the RNAs. Because these variable structures are not always present, they are unlikely to be involved in the basic functions of substrate-binding and catalytic action by this ribozyme. Conversely, sequences and structures that occur in all instances of the RNA are potentially involved in its mechanism of action.

We have studied the structure of *M. fermentans* RNase P RNA and found it to be unique in several respects. This RNA is richer in A+U (56%) than previously examined bacteria (e.g., 38.2% for *E. coli* RNase P RNA). More importantly, it lacks helix P12, previously considered a universal structural element in the bacterial type of RNase P RNA. The fact that helix P12 can be replaced with only three nucleotides indicates that this helix is a structural unit not intrinsically required for function in vivo. The absence of helix P12 conceivably could be compensated in some manner elsewhere in the molecule; one candidate might be helix P20.

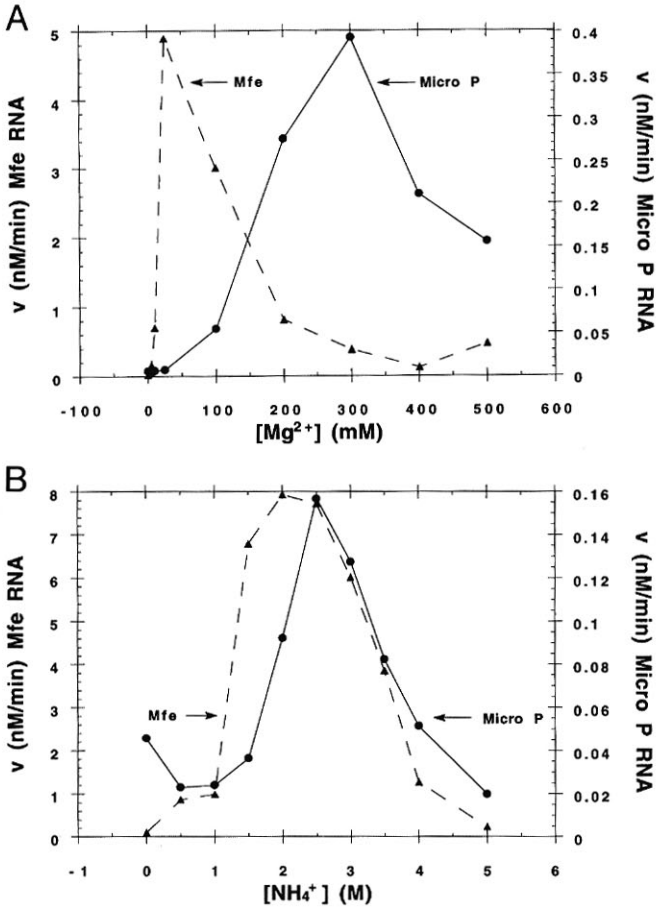

FIGURE 5. Optimal reaction conditions for Micro P RNA. **A:** Influence of magnesium acetate on activity. Filled circles and filled triangles indicate velocity (nm/min) for Micro P RNA and *M. fermentans* RNase P RNA, respectively. Reactions contained 3 M NH_4OAc, 50 mM Tris-HCl, pH 8, 0.05% NP-40, 0.1% SDS, 200 nM [^{32}P] pre-tRNA Asp, and either 10 nM (Micro P) or 2 nM (*M. fermentans*) RNase P RNA at the indicated MgOAc concentration. **B:** Influence of ammonium acetate on activity. Filled circles and filled triangles indicate velocity (nM/min) for Micro P RNA and *M. fermentans* RNase P RNA, respectively. Reactions contained 50 mM Tris-HCl, pH 8, 0.05% NP-40, 0.1% SDS, 200 nM [^{32}P] pre-tRNA Asp, and either 10 nM enzyme with 300 mM MgOAc (Micro P) or 2 nM enzyme with 25 mM MgOAc (*M. fermentans*) at the indicated NH_4OAc concentration.

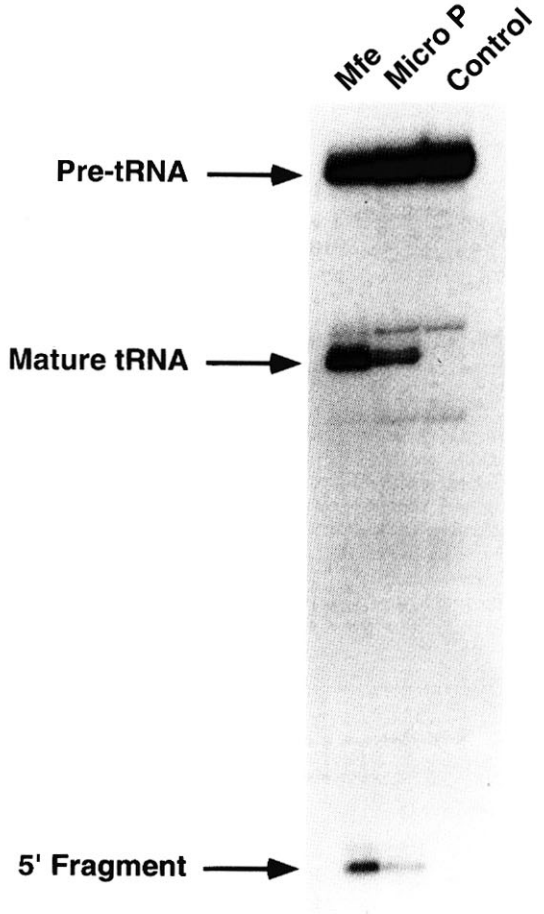

FIGURE 6. Micro P RNA accurately cleaves pre-tRNA. All reactions contained 2.5 M NH_4OAc, 100 nM [^{32}P] pre-tRNA Asp, 50 mM Tris-HCl, pH 8, 0.05% NP-40, and 0.1% SDS. The Micro P RNA reaction had 10 nM enzyme and 300 mM MgOAc and was incubated at 37 °C for 2 h. The *M. fermentans* reaction had 1 nM of enzyme and 25 mM MgOAc and was incubated at 37 °C for 20 min. Products were resolved by denaturing PAGE. Control lane contained no enzyme.

However, this helix has been observed in other RNase P RNAs that also contain helix P12 (Svard et al., 1994) and removal of P20 from the *M. fermentans* RNA renders an in vitro phenotype indistinguishable from the same RNA containing the helix. Because it is unlikely that native *E. coli* RNase P RNA contains both P12 as well as a functional replacement, the fact that EcΔP12 has native activity at its optimal ionic strength is consistent with the notion that the *M. fermentans* RNA does not contain a replacement for some catalytically important aspect of helix P12.

It is plausible that helix P20 is a component of a rho-independent terminator of the *M. fermentans* RNase P RNA gene and is retained in the mature RNase P RNA in vivo. Similar structures are reportedly contained in the RNA from other *Mycoplasma* species (Svard et al., 1994) and from *Thermus thermophilus* (Hartmann & Erdmann, 1991), whereas *E. coli* and *B. subtilis* RNase P RNAs are processed at their 3′ ends, removing the corresponding sequences (Gurevitz et al., 1983; Reich et al., 1986).

The absence of helix P12 in conjunction with the shortening of several other helices (P3, P9, and P19)

TABLE 2. Steady-state kinetic parameters for Micro P RNA.

RNase P RNA	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
Micro P[a]	1,900 ± 920	0.22 ± 0.073	0.125 ± 0.037
M. fermentans[b]	75 ± 28	5.7 ± 1.7	77 ± 5.6

[a] Assayed in 2.5 M monovalent/300 mM divalent ionic strength for 2 h at 37 °C.
[b] Assayed in 2.5 M monovalent/25 mM divalent ionic strength for 5 min at 37 °C.

makes the *M. fermentans* RNA the smallest native RNase P RNA so far identified. This size reduction, compared to the RNase P RNAs of other low G+C Gram-positive bacteria, correlates with the reduced size of the *M. fermentans* genome. However, this comparison between structural complexity and genome size is not always in good agreement. RNase P RNAs from other *Mycoplasma* species have been characterized (Svard et al., 1994) and more recently the entire genome of *M. genitalium* has been published, providing access to its RNase P RNA sequence (Fraser et al., 1995). All these *Mycoplasma* species have reduced genome sizes; however, none except *M. fermentans* has an abbreviated RNase P RNA. Moreover, the *M. fermentans* 16s rRNA also is somewhat smaller than those of other *Mycoplasma* species (Maidak et al., 1994).

The native and mutant RNAs lacking helix P12 require higher ionic strength for maximum activity than does the native *E. coli* RNase P RNA. This suggests that P12 contributes to the structure, the global stability of the RNAs. Mutations affecting the chemistry of the reaction are expected to be relatively independent of ionic strength. The high ionic strength could compensate for the absence of P12 by screening electrostatic (phosphate) repulsion, allowing RNAs lacking P12 to attain a near-native conformation due to the residual packing forces. Based on both the phylogenetic and kinetic results presented, we conclude that helix P12 is not essential in substrate binding or catalysis, but has a modest structural role in bacterial RNase P RNA (i.e., its deletion results in only a 10-fold decrease in catalytic efficiency at 1 M NH_4^+). The conserved nature of this helix indicates, however, that it has an important structural role in vivo in most bacterial RNase P RNAs.

The *M. fermentans* RNA refines our view of the phylogenetic-minimum RNase P RNA. In order to test the expectation that the minimum-conserved structure indeed is catalytically active, we designed and fabricated a synthetic version of the refined minimum structure. This RNA, Micro P RNA, was constructed by removing all phylogenetically dispensable helices from the native *M. fermentans* RNA, and shown to be catalytically active. Micro P RNA, roughly 60% of the size of the typical native RNase P RNA, must contain all the necessary nucleotides and structural elements needed for accurate processing of pre-tRNA. Because a majority of Micro P RNA consists of native *M. fermentans* sequence, this molecule is likely to maintain as-yet undiscovered tertiary interactions that help to quell the destabilizing influences of removing large blocks of sequence and their associated structure. The high level of activity of this minimal RNA attests to the utility of phylogenetic comparisons in the design of deletion endpoints throughout an RNA molecule.

Micro P RNA requires a substantially higher concentration of divalent cation for activity than does the parent molecule. It has been shown that Mg^{2+} is required for the catalytic mechanism of RNase P RNA, and is not required for proper folding of the ribozyme or substrate binding (Smith et al., 1992). However, other large catalytic RNAs may require divalent cations to achieve their proper tertiary structure (Pan et al., 1993; Smith, 1995). The increased divalent cation optimum for Micro P RNA suggests that Mg^{2+} may structurally stabilize its catalytic core.

The properties of Micro P RNA, including the requirement for very high ionic strength for activity, follow the trend seen in the properties of an earlier abbreviated RNase P RNA, Min 1 RNA (Waugh et al., 1989). This RNA, a chimera of the *E. coli* and *B. megaterium* sequences, was engineered to lack the several hairpin-like structures not present in both those types of the RNA. Although highly active, Min 1 RNA required higher monovalent salt for activity and was more thermally labile than the parent native RNAs. Micro P, lacking helices P12 and P18 contained in Min 1 RNA, has an even higher requirement for salt. The common theme is that removal of the helical appendages from the conserved core of the ribozyme results in structural destabilization. Three of the variably present elements, P14, P16/P17, and P18, are now thought to dock into the RNA superstructure, thereby stabilizing the global folding. The loop of P17 is involved in the long-range pairing P6 (Fig. 1); helices P14 and P18 are proposed to dock coaxially into P8 by forming minor groove, base-triple interactions involving GNRA tetraloops (Brown et al., 1996).

The variably present helical elements represent an RNA architectural theme that probably occurs in all large RNAs. Similar long-range interactions have been detected in both Group I and Group II introns (Jaeger et al., 1994; Costa & Michel, 1995). The helical elements form spanning struts, structural trusses, in which a helix rooted at one location in the molecule docks by its loop elsewhere, commonly through interactions involving a GNRA tetraloop. These long-range interactions would stabilize the tertiary superstructure of the catalytic core. Removal of these struts could result in distortion of the catalytic conformation due to electrostatic repulsion of RNA phosphates. High ionic strength is thought to counteract the distortion by screening the electrostatic repulsion, thereby allowing the tenuous tertiary structure of the core to assume the active conformation. Micro P RNA lacks helices present in Min 1 RNA and has even higher ionic requirements than does that particular RNA.

Simplified, synthetic versions of native RNase P RNAs have held some allure as experimental tools. Because they are smaller in size than native RNAs, they might seem relevant models for studies using analytical methods for which the native molecules are too large, for instance, spectroscopy and crystallography. Our current perspective on RNase P RNA structure suggests, however, that simplified versions of this mol-

ecule inevitably will prove to be intrinsically less stable than the native RNAs. Consequently, the abbreviated RNAs are not ideal subjects for most physical studies of global structure. On the other hand, small, well-defined subdomains of the RNA should be useful for analysis of local structure by physical methods. Comparative structure analysis identifies many of the subdomains that constitute the ribozyme. The comparative perspective illuminates the conserved, catalytic core of RNase P RNA in the context of the phylogenetic-minimum structure, and provides valuable experimental directions toward a better understanding of this unusual enzyme.

MATERIALS AND METHODS

Bacterial strains

M. fermentans strain PG18 (ATCC 19989) was a gift from K. Wise and K. Cleavinger. *E. coli* strain CJ236 (Kunkel, 1985) was used in site-directed mutagenesis experiments.

Oligonucleotides

59FBam: CGG GAT CCG IIG AGG AAA GTC CII GC; 347REco: CGG AAT TCR TAA GCC GGR TTC TGT; Mfe5′: GGG AAT TCT AAT ACG ACT CAC TAT AGG AGC ATT GGC TAA CAG CT; Mfe3′-302: AAC TGC AGG GAT GGC ACG CGT CAA AAC GCC TGC TCG CGC; Mfe3′-276: AAC TGC AGG GAT GGC ACG CGT CAG CAT TTA TAA GCC ACG; EcΔP12R: ACC GCA CCC TTT CAC AAT TTT GCT CTC TGT TGC; MicroPΔ5.1R: GGC CTA GCA TGA TCA CTA AAG CTA GCG TGG ACT TTC C; MicroPΔ15.1-19R: ATA AGC CAC GTT TTG TAT TAG CTA ACA ATT TAT CTG ATT CCC CTA CCA ATA TTT.

Plasmids

The following plasmids were used in this work: pUC19 was purchased from New England Biolabs, pBluescriptKS+ was purchased from Stratagene, and pTZ19R was purchased from United States Biochemical. pDW98 and pDW152 were provided by D. Waugh (Waugh et al., 1989). pEcP2 was provided by D. Smith (Darr et al., 1992).

Cloning of *M. fermentans* RNase P RNA gene

The cloning and screening methods used were as described (Brown et al., 1991). A fragment of the *M. fermentans* RNase P RNA-encoding gene, used as a probe in Southern blot analysis, was obtained by amplification of genomic DNA using oligonucleotide primers 59FBam and 347REco, specified above, under conditions as described (Angert et al., 1993). The RNase P RNA gene was cloned as a 4-kB *Hind* III/*Pst* I genomic DNA fragment in pBluescriptKS+. Transcription vectors that generate products with precise 5′ and 3′ termini in vitro were constructed by cloning DNA amplified from the above pBluescript clone using Mfe5′ and either Mfe3′-302 or Mfe3′-276 primers. Plasmids pMfe302 and pMfe276 were created by inserting the appropriate *Eco*R I/*Pst* I PCR fragment into the *Eco*R I/*Pst* I sites of pUC19. These DNA templates produce upon transcription two distinct *M. fermentans* RNase P RNAs, differing in the presence or absence of the 3′-terminal helix, P20.

Nucleic acid sequencing

Double-stranded plasmid DNAs were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 (United States Biochemical). The GenBank accession number of the sequence encoding the *M. fermentans* RNase P RNA is U41756.

Construction of mutant RNase P RNAs

Plasmid pEcΔP12 was created by using site-directed mutagenesis (Kunkel, 1985) as described (Sambrook et al., 1989). The mutagenic primer EcΔP12R was used to replace helix P12 (nt 142–176) with the sequence AUU in the *E. coli* RNase P RNA gene of pEcP2.

Plasmid pMicroP was constructed by first inserting an *Eco*R I/*Bsa*H I fragment from pMfe276 into pTZ19R to create pMfeTZ. Mutagenic primers MicroPΔP5.1R and MicroPΔ15.1-19R were used with this plasmid for site-directed mutagenesis as discussed above. The phylogenetic replacements are described in the legend to Figure 4B.

Preparation of RNA transcripts

RNAs were synthesized in vitro by run-off transcription using T7 RNA polymerase (gift of B. Pace). Transcription reactions followed the protocol of Milligan and Uhlenbeck (1989), except that NTP concentrations were raised to 5 mM each. RNAs were radiolabeled internally by the addition of 50 μCi of [α-^{32}P] GTP to the transcription mix; the concentration of unlabeled GTP was lowered to 0.2 mM in these reactions. Transcription products were resolved by gel electrophoresis and RNAs were visualized by UV shadow (Darr et al., 1992). The appropriate bands were excised, and RNA was eluted from the gel slices by diffusion (overnight at 25 °C) into a buffer consisting of 0.3 M sodium acetate, 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1% SDS. The RNA eluate was resuspended in H_2O after ethanol precipitation and quantitated by UV absorbance or specific radioactivity.

RNAs were synthesized from the following linearized plasmids (the restriction enzyme used to digest each plasmid is listed parenthetically): (1) pre-tRNA^{Asp}, pDW152 (*Bst*N I); (2) native *E. coli* RNase P RNA, pDW98 (*Sna*B I); (3) Mfe302 RNA, pMfe302 (*Fok* I); (4) Mfe276 RNA, pMfe276 (*Fok* I); (5) Micro P RNA, pMicroP (*Fok* I). To accommodate the T7 polymerase promoter sequence, two G residues were added preceding nt 1 of the *M. fermentans* and Micro P RNAs.

Kinetic analysis of the cleavage reactions

Enzyme activity was measured at 37 °C in the presence of 50 mM Tris-HCl, pH 8, 0.05% Nonident-40 (NP-40), 0.1% SDS, and the indicated monovalent and divalent salt concentrations. The concentrations of native, EcΔP12, and both *M. fermentans* RNAs in reactions were 0.5–2.0 nM and Micro P RNA was at 10 nM. Assays were conducted at various sub-

strate concentrations above and below K_m unless otherwise noted. Enzyme and substrate RNAs were mixed in reaction buffer lacking Mg^{2+} on ice, heated for 2 min at 65 °C to allow conformation sortment, and then shifted to reaction temperature (37 °C). Reactions were initiated by addition of $Mg(OAc)_2$ and quenched after the indicated times by addition of 3 volumes of ice-cold ethanol. After precipitation, precursor and product RNAs were resolved by electrophoresis through denaturing 8% polyacrylamide/7 M urea gels. Reaction products were quantified in dried gels using a PhosphorImager (Molecular Dynamics).

Reaction rate constants were obtained by quantifying the extent of cleavage as a function of time. Velocities represent initial rates under first-order reaction conditions with substrate in excess of enzyme and the fraction of substrate cleaved <20%. The kinetic parameters k_{cat} and K_m were extracted by plotting velocity versus velocity/[substrate] (Eadie/Hofstee). Values are expressed as a mean ± standard deviation derived from four to five independent experiments.

Terminal nucleotide analysis

3′-Nucleotide analysis of 5′ product in the cleavage reaction was performed following the protocol of Nishimura (1979) as described (Frank et al., 1994).

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