GANTT, JOHN ARTHUR. Comparative Analysis of RNase P. (Under the direction of Dr. James W. Brown.)

This Master's Thesis contains a description of the results of two research projects that were worked on over the course of two years. The first was an attempt to heterologously reconstitute the *Bacillus subtilis* RNase P RNA with the RNase P protein subunits of *Methanothermobacter thermoautotrophicus*, to identify a functional homology between one or more of the archaeal proteins with the *Bacillus subtilis* RNase P protein. Unfortunately, the reconstitution experiment could not be completed due to the poor quality of the pre-tRNA^{asp} substrate synthesized. The second project dealt with comparative analysis of bacterial RNase P RNA. Eleven new RNase P RNA genes were amplified, sequenced, and their secondary structures derived by comparative analysis.

COMPARATIVE ANALYSIS OF RNASE P

by

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BIOGRAPHY

I, John Gantt, was born in Houston, Texas on December 16, 1978. After moving a number of times my family settled in North Carolina in the small town of Morganton, where my parents still reside. In 1997 I was accepted into High Point University where I majored in Biology and minored in Chemistry and Mathematics. There I learned that diversity and understanding is a treasure and that GOD has a place in his heart for all of us. After graduating from HPU, I entered into the Graduate department of Microbiology at North Carolina State University. Upon completion of the Master of Science requirements I accepted a management position at Molecular Toxicology Inc. in Boone, North Carolina.

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Through the support of my family and friends I have completed this arduous task. I would like to thank Dr. James Brown and the Brown lab for their continual support in my scientific growth. Drs. Steve Libby and Amy Grunden, my committee members, have also been invaluable in my progression. I would like to dedicate this thesis and my work to my late grandmother, “Big Margaret”. She will be missed.

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RNASE P LITERATURE REVIEW

In 1970 Sidney Altman and his colleagues, through the use of mutant tRNA precursors, discovered an enigmatic enzyme ribonuclease P (RNase P), involved in the biosynthesis of tRNA (1). All transfer-RNAs are initially transcribed as precursors containing extra nucleotides 3′ and 5′ to the mature ends. These extra sequences are removed by a complex set of enzymes and the resulting mature transfer RNA is transported to the ribosome where it aids in protein synthesis (2, 23, 24). RNase P is the ubiquitous endoribonuclease responsible for removal of the 5′ leader sequence of precursor tRNA (pre-tRNA) (3, 4, 25). RNase P has now been characterized from representatives of all three Domains of life, Bacteria, Archaea, and Eucarya, and those from mitochondria and chloroplasts. RNase P is a ribozyme; the RNA is the catalytic subunit of the enzyme and the protein is a structural enhancer (4). In 1989 Sidney Altman and Thomas Cech shared the Nobel Prize in Chemistry for their discovery of the catalytic properties of RNA; for their discoveries that group I introns are self-splicing (Cech) and that the RNA subunit of RNase P is a true catalytic ribozyme (Altman) (1).

The RNase P Reaction

The RNase P reaction has been studied in Bacteria, Archaea, and Eucarya, but is best characterized and described in the bacterial ribozyme (RNA only) reaction. This enzymatic reaction consists of three basic steps; enzyme-substrate binding, scissile bond cleavage, and product release (6).

Although perhaps most critical in cleaving many types of pre-tRNAs, RNase P is also required to recognize and cleave precursors from a wide variety of natural RNA substrates, including 4.5S RNA, tmRNA, and the *his* mRNA (6, 26, 12). This wide range

of substrates poses a problem in that even pre-tRNAs show considerable sequence variation. Because it cannot rely solely on sequences for substrate recognition, RNase P instead recognizes the overall tertiary structure of pre-tRNA with a minimal determinant of the T-stem, T-loop and acceptor stems. (Figure 1a) (12). Pre-tRNA contains several conserved features proximal to the cleavage site that are crucial to the ribozyme reaction (3, 6, 11, 4, 27). The 3′ RCCA "tail" is known to be responsible for positioning and anchoring of the substrate phosphodiester bond into the active site of the RNase P by base-pairing to the GGU in J16/15; this enzyme: substrate interaction is known as the 73/294 interaction (10, 5, 3). In addition to the 73/294 interaction, it has recently been determined that the 2′ hydroxyl of U(-1) in pre-tRNA and A(248) in RNase P RNA interact. Zahler et al. shows that mutagenesis of A(248), in J5/15, resulted in considerable miscleavage of the pre-tRNA. Lastly the G(1)-C(72) base-pair is known to contribute to affinity and catalysis, although the details of the contribution remain undefined (3) (Figure 3).

Even though RNA-RNA associations play an important role in catalysis, the protein subunit of RNase P also plays a role in substrate binding. The protein subunit assists by directly contacting the 5′ leader in a sequence non-specific manner thus ameliorating dependence on the CCA tail of the pre-tRNA (11, 8, 29, 30). The RNase P holoenzyme (RNA plus protein) has the ability to discriminate between substrate and product because of the protein interaction to the 5′ leader and, as a result product inhibition by mature tRNA is minimized (28).

The RNase P enzymatic mechanism remains unclear, because a complete tertiary structure of the enzyme has not been determined (31). Chemical groups in the RNA subunit are known not to be directly involved in the attack on the scissile bond. RNase P

requires monovalent cations, preferably K^+ and NH_4^+, whose role is apparently in counteracting electrostatic repulsion between RNA phosphates (12, 32). Divalent cations, preferably Mg^{2+}, contribute directly to catalysis. Mg^{2+} is known to be coordinated by a phosphate at the cleavage site and oxygens on the RNA subunit, thus allowing a hydroxyl attack by water on the same phosphate that coordinated Mg^{2+} (Figure 1b) (12, 33, 34). This is an Sn2-type nucleophilic reaction.

After the 3′O of the precursor chain is displaced the products dissociate from the reaction, the 5′ leader is degraded, and the resulting mature-length tRNA is further modified and finally utilized by the translation apparatus (2).

Bacterial RNase P

A majority of the information about RNase P has been derived from Bacteria, primarily *E. coli* and *B. subtilis*, because of the opportunity to correlate catalytic activity of the RNA with structure (12). Bacterial RNase P is a heterodimer consisting of a catalytic RNA, 350-400nts, and a single protein cofactor, 13-14kDa, which are known under normal conditions to be both essential *in vivo*. However, at high ionic strength *in vitro,* the protein subunit is unnecessary for catalysis (13, 4).

The bacterial RNase P protein is encoded by the single-copy *rnpA* gene, that is part of a well-conserved operon with *rmpH*, encoding ribosomal protein L34. The RNase P protein functions on at least two levels. It was first thought that the proteins only role was to shield unfavorable electrostatic repulsion between the ribozyme and the substrate, because it was catalytically replaceable by high monovalent salt concentrations. Recently it has been found that the protein alleviates product inhibition, enhances the relative rate of product release, and extends the substrate range (4, 5, 11). The overall structure is similar

to those of two ribosomal proteins, S5 and elongation factor G. This suggests that the protein subunit either arose early in protein evolution and thus mediated the conversion from all-RNA to an RNP world, or that one of these proteins was later recruited to function in RNase P (14).

The RNase P protein structure is three α-helices surrounding a central β-sheet (Figure 4) (4). Two conserved sets of residues in the tertiary structure, including the RNR motif and a large central cleft, are thought to assist in the interaction of the pre-tRNA substrate and the RNase P RNA subunit. The RNR (Arg-Asn-Arg) motif, located in α2, is thought to specifically bind the RNase P RNA, although the details of the interaction are not known (13). Most Arg-rich sequences of RNA-binding proteins specifically recognize RNA, thus setting a precedence for the binding motif of RNR (35, 36). Other evidence includes mutagenesis of conserved residues in the RNR motif of the RNase P protein hindering enzymatic activity in *in vitro* experiments (37). The large central cleft, formed by packing of α1 against the β-sheet, is thought to form weak interactions with the single stranded 5' leaders of pre-tRNA. Photocrosslinking experiments showed that nucleotides –4 to –7 in the pre-tRNA are proximal to Phe16(α1), Phe20(α1), Val32(β2), Tyr34(β2), Ser49(β3), and Ile86(β4) in the cleft (38).

Neither the RNA nor the holoenzyme of RNase P has been crystallized for structural analysis successfully. A different approach has been taken to examine the RNA structure, in which similar RNA sequences from a variety of organisms are compared and structure is determined/inferred based on the covariations in the sequence, a process termed phylogenetic comparative analysis (12). Bacterial RNase P RNA secondary

structure has been thoroughly studied by comparative analysis, which requires a substantial number of sequences for high resolution (11). RNAs from Bacteria have fallen into two main structural categories; type A (ancestral type), represented by various groups of Gram-negative and high G+C Gram-positive Bacteria and bear considerable sequence and structural similarity to most archaeal RNase P RNAs; and type B, which are restricted to low G+C Gram-positives including *Bacillus* and relatives. Figure 4 illustrates the RNA secondary structures of *E. coli*, representing type A, and *B. subtilis*, representing type B, and shows that although these RNAs are the most disparate of the bacterial RNase P RNAs, a minimal consensus sequence and structure can be identified that contains all of the essential determinants of the RNA. These two types of RNAs are able to interchange proteins, *in vivo* and *in vitro*, indicating an overall similarity in tertiary structure (11).

A standard nomenclature based primarily on the type-A RNA, represented by that of *E. coli*, has been applied to RNase P RNAs. Base-paired helices are labeled "P" one through twenty, unpaired joining regions "J" between helices, and terminal loops "L" (11). Comparative analysis indicates that although type A RNAs lack P5.1, P10.1, and P15.1, type B lack P13, P14, P16, and P17, indicating that these helices may play interchangeable functional and structural roles (11, 39). It is known that the evolutionary change leading from type A to type B RNA occurred abruptly and no intermediates have been identified (15).

Eukaryotic RNase P

Nuclear

RNase P from the nuclear region has been purified from a variety of eukaryotes, but is best understood from humans and the yeast *Saccharomyces cerevisiae*. The single eukaryotic nuclear RNase P RNA contains a conserved catalytic core similar to the bacterial RNA (Figure 6), strongly suggesting that the nuclear RNA function is homologous to that of the bacterial RNA, and further that the nuclear RNase P RNA is also a ribozyme (6, 40, 41). However, with a protein complement of 50%, compared to 10% in Bacteria, the nuclear RNA has become absolutely dependent on protein for function. The purpose of this protein addition includes structural stabilization, spatial organization of pre-tRNA biosynthesis, RNase P holoenzyme maturation, and perhaps participation in undiscovered processing pathways (6, 16, 17).

The human and *S. cerevisiae* RNase P holoenzymes exhibit many similarities in sequence (Table 1), but also share a significant sequence similarity with the ribosomal RNA processing enzyme RNase MRP. Of the nine protein subunits of the *S. cerevisiae* RNase P, eight are shared with RNase MRP (5, 6).

Organellar

The endosymbiotic theory states that organelles are the direct descendents of Bacteria (42). Eukaryotic cells containing such organelles, including mitochondria and chloroplasts, contain distinct RNase P complexes in these organelles, although the data is still very controversial.

Of all the mitochondrial RNase P complexes, that of *S. cerevisiae* is the most well defined. The holoenzyme contains an RNA subunit (Rpm1r) of about 490 nts encoded in

the mitochondrial genome and a single protein subunit (Rpm2p) of 105kDa encoded by a nuclear gene (5, 6, 43). During the purification of the enzyme, any fragmentation of the RNA that occurs does not interfere with its processing activity, suggesting that the protein is primarily responsible for structural integrity of the enzyme. Rpm2p was also found to be involved in cell growth, interdependent of mitochondrial translation (44). Human mitochondrial RNase P has been suggested to be purely protein based and any RNA associations still are considered a contaminant. Alternatively, the mitochondrial enzyme may be identical to that of the nuclear RNase P RNA (17).

The chloroplast RNase P from spinach has been isolated and may be purely protein. The buoyant density in CsCl of 1.28g/mL, the micrococcal nuclease insensitivity, and the inability to detect an RNA component using sensitive assays confirms this finding (5). A lack of inhibition by phosphothioate at the scissile bond may indicate that the bacterial cleavage mechanism is not being used. Another unknown aspect of this complex is that it has not yet been proven essential for pre-tRNA maturation *in vivo* (6).

Unlike higher plants, primitive algae, such as *Cyanophora paradoxa*, contain cyanelles with traditional RNase P activity. These photosynthetic genomes encode a type A bacterial RNase P RNA, although this RNA is not catalytic in itself (18). This resembles some archaeal RNase P RNAs in that the RNA conforms to the bacterial consensus but are not true ribozymes. The proteins have not been characterized, but they constitute 80% of the holoenzyme mass. This is very similar to eukaryotes in that it is thought that the protein took over essential structural functions (6).

Archaeal RNase P

Archaeal RNase P has been an enigma compared to its eukaryal and bacterial homologs. The first characterized archaeal holoenzymes, those of *Haloferax volcanii* and *Sulfolobus acidocaldarius*, were found to be very different biochemically (22). *Haloferax volcanii*, a halophilic euryarchaeon, was found to be sensitive to micrococcal nuclease treatment and to have a buoyant density in Cs_2SO_4 of 1.61, and so seemed to be composed primarily of RNA and thus resembling the bacterial enzyme. However, it was later shown that Cs_2SO_4 inactivated the enzyme. The RNase P of *Sulfolobus acidocaldarius*, a thermophilic sulfur-oxidizing acid crenarchaeon, was found to be resistant to the micrococcal nuclease treatment and had a lower buoyant density in Cs_2SO_4 of 1.27, thus indicating that it was predominantly protein. Protein content indicated that this might resemble the eukaryal RNase P. Recently, two RNase P enzymes from distantly related methanogenic Archaea, *Methanothermobacter thermoautotrophicus* and *Methanocaldococcus jannaschii*, have been characterized and it was determined that the buoyant densities, and other properties, were so alike that they argued against the idea that archaeal RNase P enzymes are so dissimilar (1.39g/mL for *M. jannaschii* and 1.42g/mL for *M. thermoautotrophicus*) (20, 21, 22).

A compilation of a variety of archaeal RNase P RNAs have been sequenced, and it has been determined through comparative analysis that two structural types exist. The first is the common structural class, type A (ancestral type), represented by *M. thermoautotrophicus*, which is a subset of the larger type A class of bacterial-like RNase P RNAs. The primary differences between archaeal and bacterial type A RNAs are the absence of P18, P13/14 in Archaea, and the presence in Archaea of a conserved extended

P12. Various bacterial RNase P RNAs lack structural elements P18 or P13/14, but these are rare (20). The second type of archaeal RNase P RNA, type M, found only in *Archaeoglobus* and *Methanococcus*, lack P8, L15/P16/P17/P6 and contain alterations in P7 and P10/11 (Figure 7). Evolutionarily, the loss of these essential structures, *ie*. P8 which is involved in substrate T-loop recognition and L15 which is involved in 3′-RCCA recognition, may have required the protein subunits to take an additional role in substrate binding. Type M RNAs are not capable of catalysis in the absence of protein, unlike type-A RNAs under high ionic strength (20).

Four proteins have been recently identified in *M. thermoautotrophicus* with homologues in *M. jannaschii* that share sequence conservation to four of the nine RNase P proteins in *S. cerevisiae* (Table 1). Mth11p, Mth1618p, Mth687p, and Mth688p were identified as *bona fide* RNase P proteins by copurification with the RNase P holoenzyme and the ability of antibodies against each of the recombinant proteins to immunoprecipitate RNase P activity (19). The conservation of archaeal protein subunits in nuclear RNase P is consistent with the view that eukaryotic RNase P has an archaeal-like origin (17).

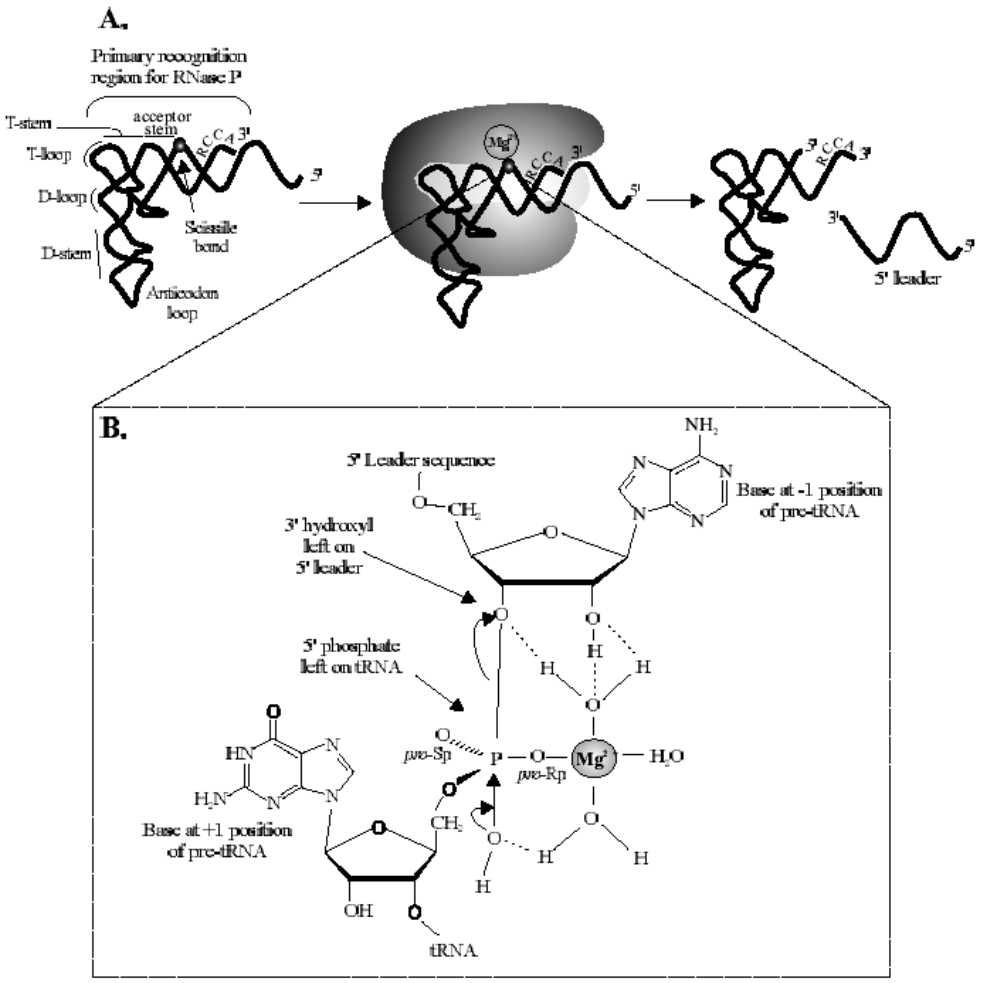


Figure 1: The RNase P reaction. (A) Three steps in the reaction: (1) holoenzyme and substrate recognition and binding via the acceptor stem, T-loop, and T-stem along with the conserved RCCA pre-tRNA sequence binding to the GGU sequence of the RNase P RNA, (2) cleavage of the scissile bond with Mg^{2+} and the protein cofactor, and (3) dissociation of the products. (B) Diagram of the putative trigonal pyramid intermediate. Taken from: Hall TA, Brown JW. 2001. The ribonuclease P family. Meth. Enz.. 341: 56-77.

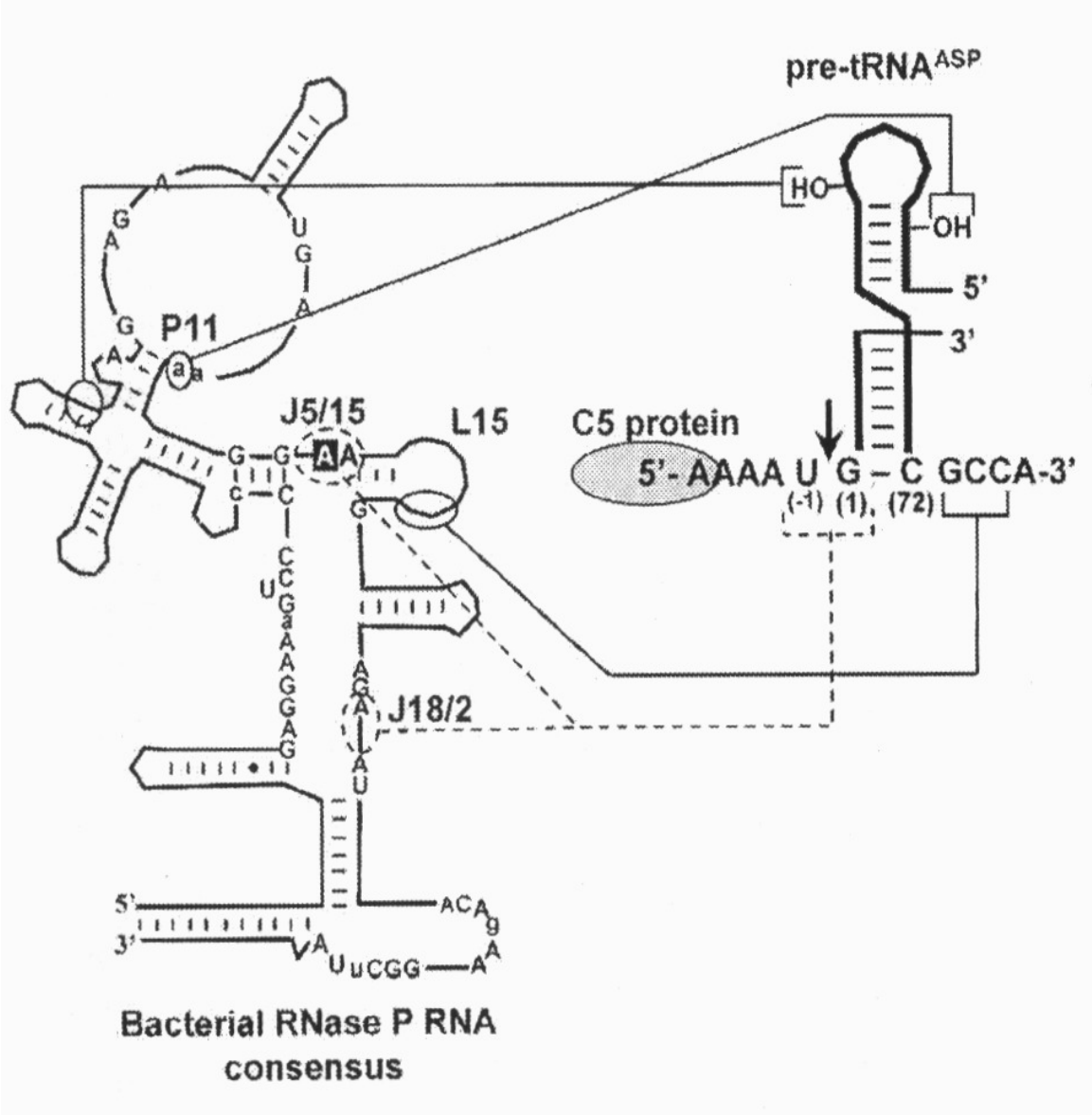


Figure 2: RNase P RNA:pre-tRNA interactions. The RCCA/GGU (73/294 interaction), N(-1)/A(248), and G(1)-C(72) interactions indicate all known information of enzyme: substrate binding. Taken from: Zahler NH, Christian EL, Harris ME. 2003. Recognition of the 5′ leader of pre-tRNA substrates by the active site of ribonuclease P. RNA 9: 734-745.

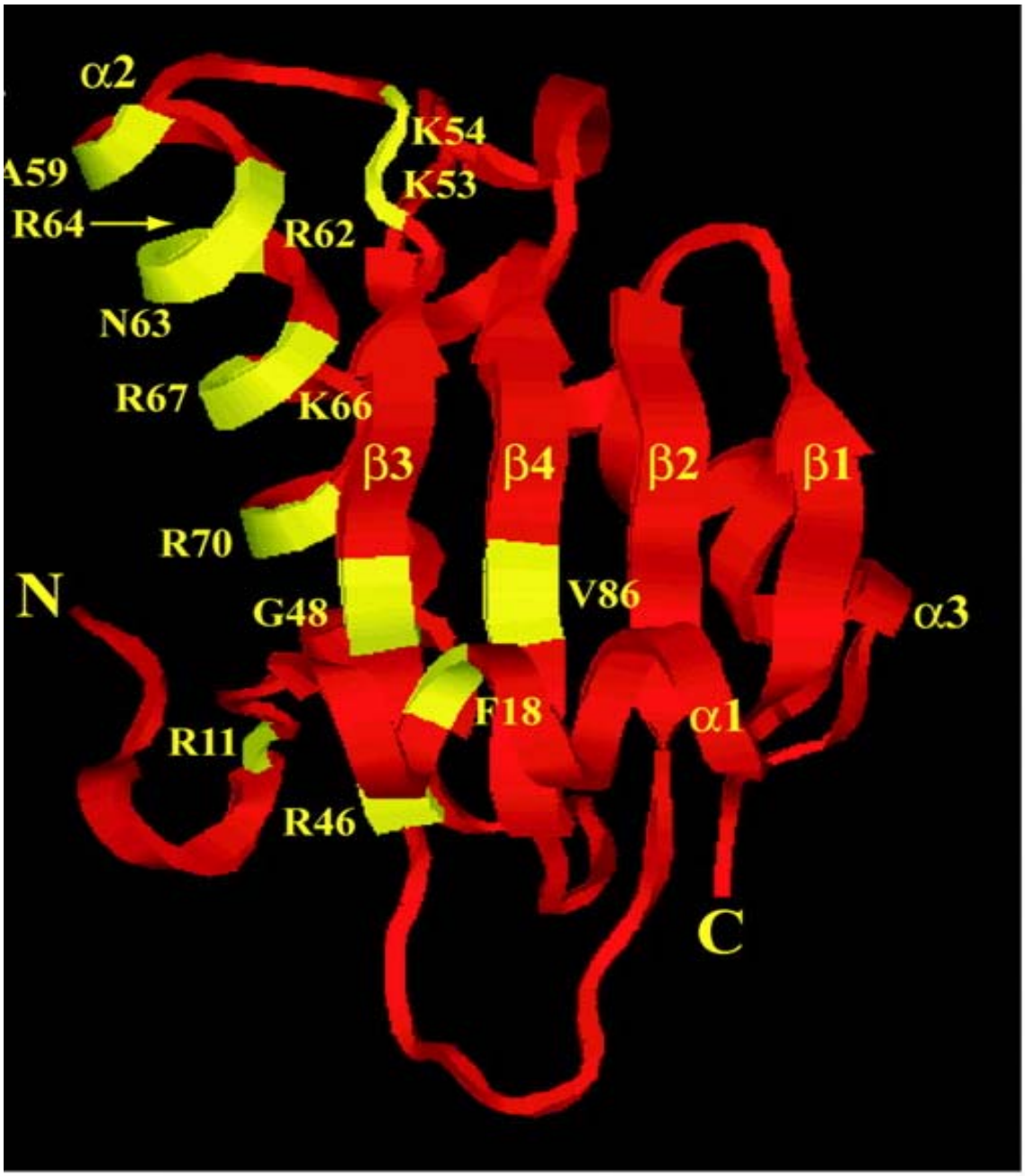


Figure 3: Bacterial RNase P protein. This illustrates the αβαβββα tertiary structure. Taken from: Jovanovic M, Sanchez R, Altman S, Gopalan V. 2002. Elucidation of structure-function relationships in the protein subunit of bacterial RNase P using a genetic complementation approach. Nucl. Acids Res. 30: 5065-5073.

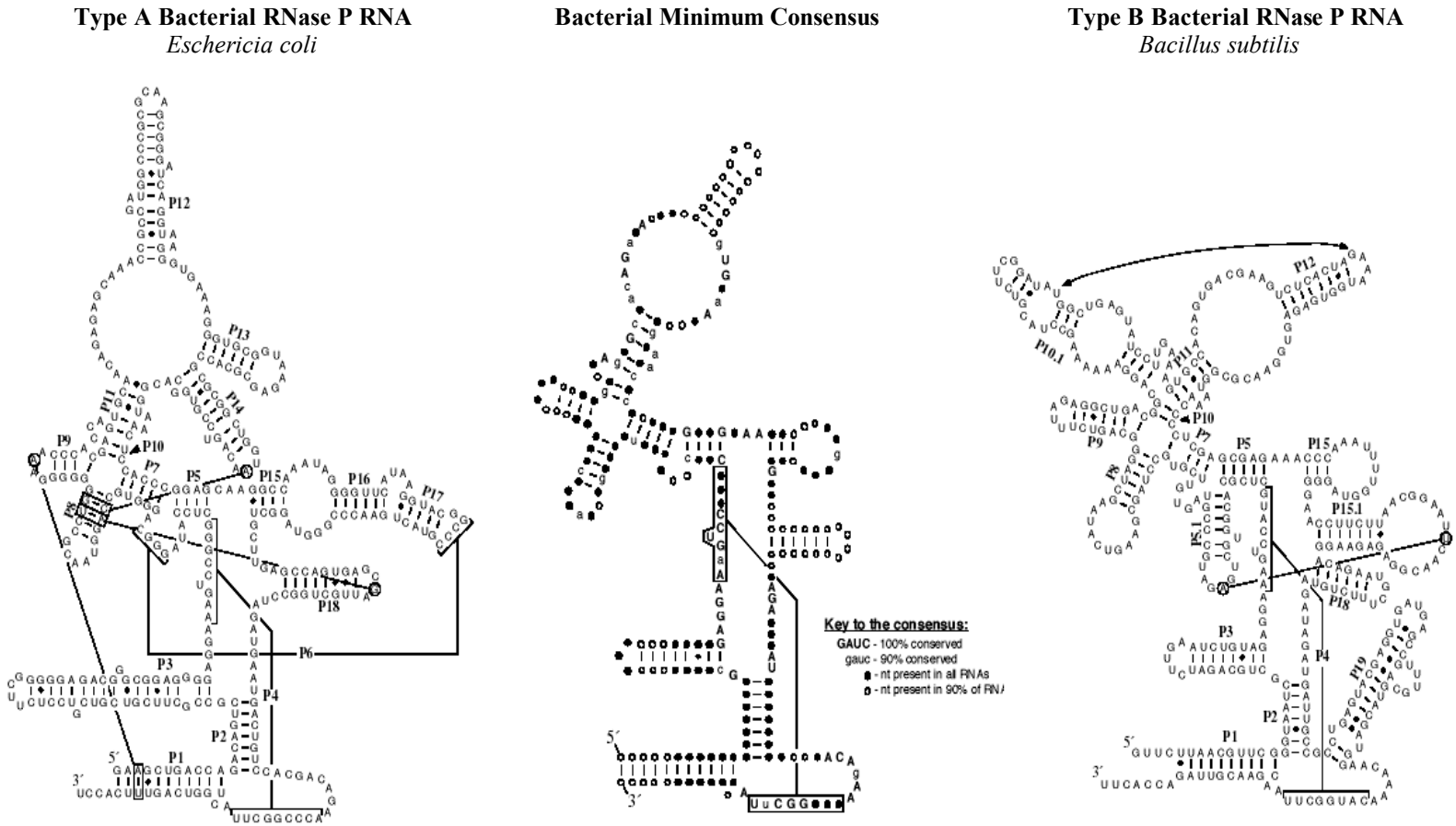


Figure 4: Bacterial type A RNase P RNA secondary structure, represented by *E.coli*, the minimum consensus bacterial RNase P RNA, and bacterial type B RNase P RNA secondary structure, represented by *Bacillus subtilis*. Adapted from: Pace NR, Brown JW. 1995. Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. J. Bacteriol. 177: 1919-1928.

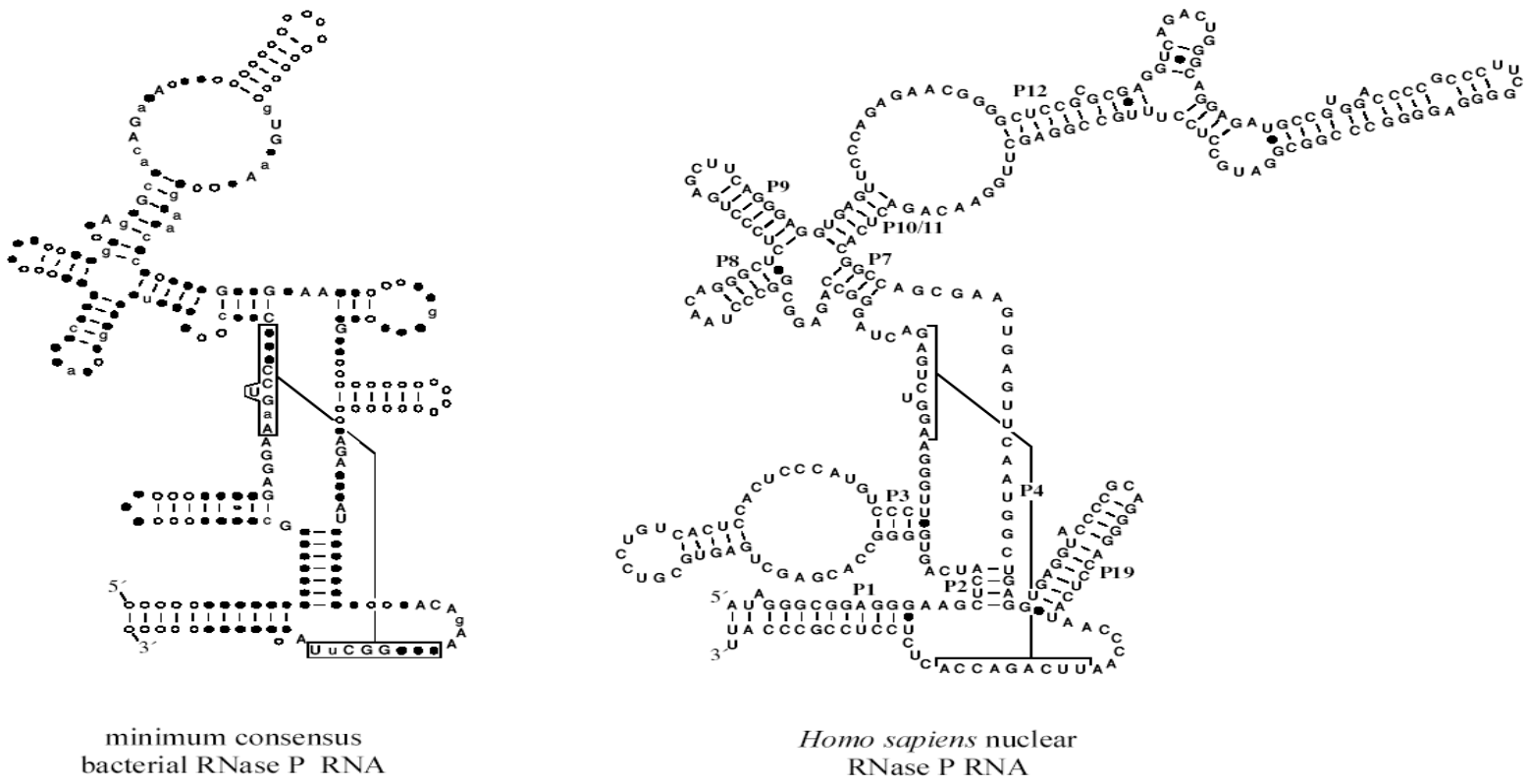


Figure 5: The minimum consensus bacterial RNase P RNA and the nuclear RNase P RNA secondary structure from *Homo sapiens*. Adapted from: Pace NR, Brown JW. 1995. Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. J. Bacteriol. 177: 1919-1928.
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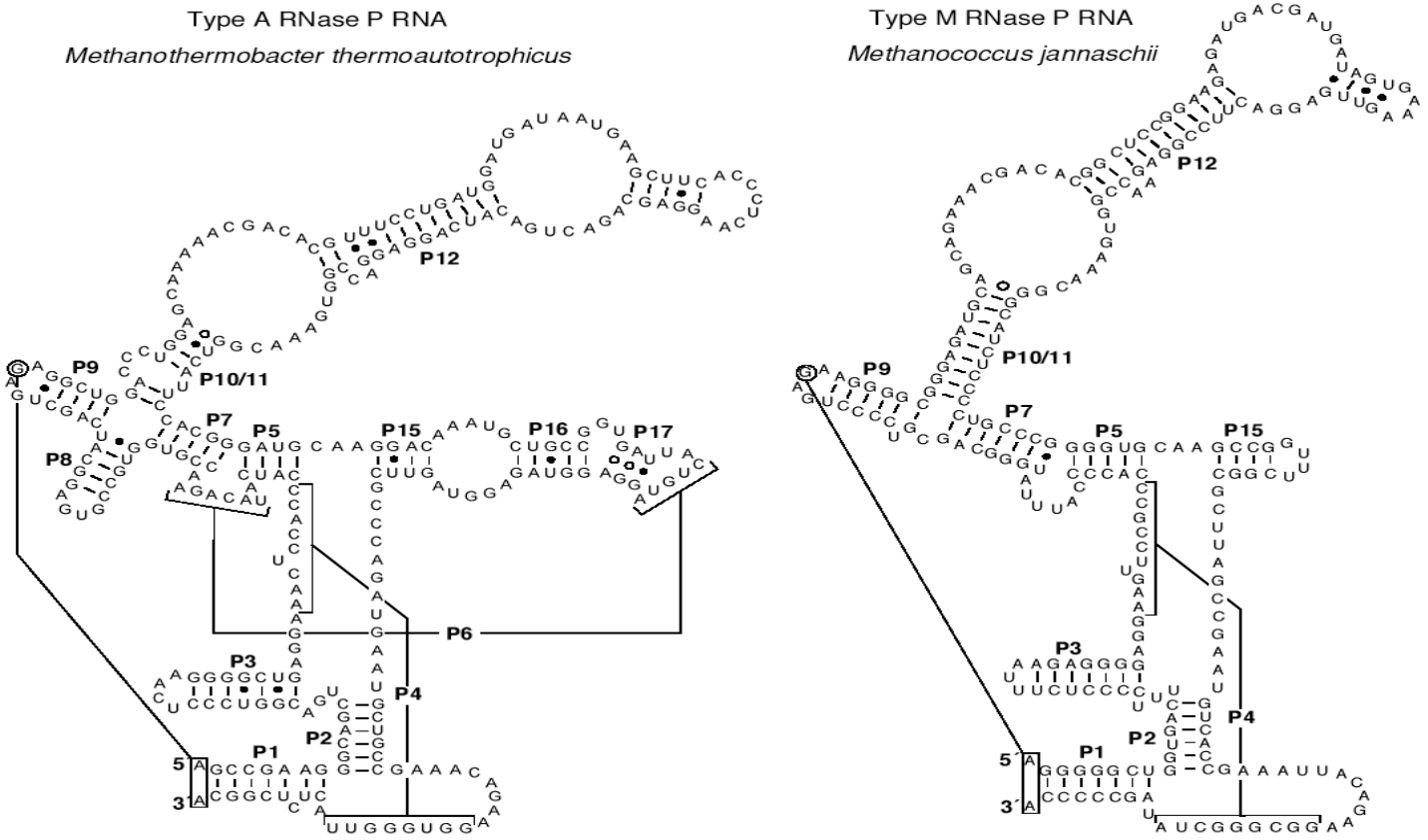


Figure 6: RNase P RNA secondary structures from *Methanothermobacter thermoautotrophicus* and *Methanocaldococcus jannaschii*. Taken from: Harris JK, Haas ES, Williams D, Frank DN, Brown JW. 2001. New insight into RNase P structure from comparative analysis of the archaeal RNA. RNA 7: 220-232.

Table 1: Comparison of subunits in RNase P and RNase MRP from yeast, human, and Archaea. Adapted from: Jarrous N. 2002. Human ribonuclease P: Subunits, function, and intranuclear localization. RNA 8: 1-7.
Xiao S, Scott F, Fierke CA, Engelke DR. 2002. Eukaryotic ribonuclease P: A plurality of ribonucleoprotein enzymes. Annu. Rev. Biochem. 71: 165-189.

	Yeast Gene		*Human Gene*			
Subunit Type	*RNase P*	*RNase MRP*	*RNase P*	*RNase MRP*	*Archaeal RNase P*	*Function/ Interaction*
RNA	RPR1		H1			Catalysis
RNA		NME1		(7-2)		Catalysis
Protein	POP1	POP1	hPOP1	hPOP1		Localization
Protein	POP3	POP3				
Protein	POP4	POP4	RPP29	RPP29	MTH11	Localization/ Substrate binding
Protein	POP5	POP5	hPOP5	hPOP5	MTH687	
Protein	POP6	POP6				
Protein	POP7	POP7	RPP20			ATPase/ helicase;
Protein	POP8	POP8				
Protein	RPP1	RPP1	RPP30	RPP30	MTH688	H1 RNA binding
Protein	RPR2		RPP21		MTH1618	Substrate binding/(ATPase/ helicase)
Protein		SNM1				
Protein			RPP38	RPP38		Localization/H1 RNA binding
Protein			RPP40			
Protein			RPP25			H1 RNA binding
Protein			RPP14			Substrate binding

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Chapter 1. RNase P proteins: The homology between that of archaeal and bacterial subunits.

ABSTRACT

No sequence similarity has been found between archaeal and bacterial protein subunits, and yet heterologous reconstitution between the *Bacillus subtilis* RNase P protein and some archaeal RNase P RNAs has been demonstrated. One of those archaeal RNAs is that of *Methanothermobacter thermoautotrophicus*, that has recently been shown to be associated with at least four protein subunits: Mth11p, Mth687p, Mth688p, and Mth1618p. By determining a functional interchangeability between one or more of these proteins with the *Bacillus subtilis* RNase P protein, an evolutionary relationship (homology) may be identified between the protein subunits of Archaea and Bacteria. Unfortunately, in this study, a relationship could not be identified due to poor quality pre-tRNA^{asp} substrate synthesized.

INTRODUCTION

RNase P is essential throughout the Domains of life, including subcellular organelles that are involved in tRNA biogenesis. RNase P is responsible for the removal of the 5′ leader sequence from all known precursor tRNAs (pre-tRNAs) (2, 4). The RNA subunit of RNase P by itself in Bacteria and some Archaea is capable of catalyzing the hydrolysis of pre-tRNA in the absence of its protein cofactor (at artificially high ionic strength), although this has not been demonstrated for any eukaryal RNase P RNA. The

RNA is the catalytic subunit of the enzyme (3). Most bacterial and archaeal RNase P RNAs have retained their ancestral type (type A) secondary structure. It has also been shown that the single bacterial RNase P protein can reconstitute enzymatic activity in the archaeal RNase P RNA of *M. thermoautotrophicus* (6). Four of the nine proteins subunits from the nuclear RNase P of *Saccharomyces cerevisiae* (Pop4p, Pop5p, Rpp1p, and Rpr2p) are homologous to four methanogenic archaeal RNase P proteins; in *M. thermoautotrophicus* these are Mth11p, Mth687p, Mth688p, and Mth1618p. (2, 6). These relationships between RNase P subunits in Bacteria, Archaea, and Eukarya suggest that heterologous reconstitution of enzymatic activity might be possible using the RNA and protein subunits from different Domains. Our goal in this study was to determine the functional homology between RNase P protein subunits found in Bacteria and Archaea. Unfortunately, due to complications in the RNase P assay the homology could not be assessed.

MATERIALS AND METHODS

Cloning and expression of recombinant Mth11p, Mth687p, Mth688p, and Mth1618p archaeal proteins

The Mth11, Mth687, Mth688, and Mth1618 ORFs were previously amplified from *Methanothermobacter thermoautotrophicus* genomic DNA and cloned as in-frame fusions into the pET16b (Novagen) plasmid creating amino-terminal 6X His-tagged constructs with T7 promoters and downstream ampicillin (AMP)-resistant markers (6). These plasmids were transformed into *E. coli* BL21(DE3) CodonPlus RIL (Stratagene), containing genomic chloramphenicol (CAM) resistance, and were plated onto AMP/CAM

(100μg/ml/34μg/ml) LB-plates to select for plasmid carriers. Mth11 was amplified using primers Mth11 5′*Bg1II* (GGAAGATCTGATAACCCCCAGGAATATTTTCAGGC) and Mth11 3′*BamHI* (CGCGGATCCTATGGTTTTCTAAATTTCTTC), Mth687 was amplified using primers Mth687 5′*XhoI* (CCGCTCGAGATGAAGATACTGCCACCAACACTG) and cMth687 3′*BamHI* (CGGGATCCTCAGTATTTATCTTTCTTGGAAGG), Mth688 was amplified using primers cMth688 5′*XhoI* (CCGCTCGAGTTGATTCCCCAAAGGATTCTG) and Mth688 3′*BamHI* (CGGGATCCTTAACTCTCAGGGAGGAGCC), and Mth1618 was amplified using primers Mth1618 5′*Bg1II* (GGAAGATCTGAGGAGAGGAAAGAGACCACG) and Mth1618 3′*BamHI* (CGCGGATCCTATCTGTCACTCTGGCTTTCCCC) at 100ng each, along with 10X reaction buffer (500mM KCl, 300mM Tris-Cl pH 8.3, and 15mM $MgCl_2$), 2.5μM each dNTPs, template, 3 units of Pfu Polymerase, and nuclease-free water up to volume in 50μl reactions (Perkin Elmer DNA Thermal Cycler).

The recombinant proteins were expressed via induction of the T7 promoter with 1mM IPTG at a culture O.D. of 0.5-0.7 at 600nm approximately 4-6 hours into growth. Four hours after induction with IPTG, cells from the 1L culture were harvested by centrifuging at 8,000 rpm for 30 min. in the Sorvall SS-34 rotor. Four milliliters of lysis buffer pH 8.0 (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole) per gram of cell material was then added to the cell pellet. One mg/ml of lysozyme was added and then incubated on ice for 30 min. They were then sonicated on ice and again centrifuged at 8,000 rpm for 30 min and the supernatant was collected. One ml of this cleared lysate was then mixed with four ml of Ni-NTA agarose (Qiagen) and mixed gently at 4°C for 60 min.

Each lysate-Ni-NTA mixture was loaded into a separate column and washed twice with 25mL of wash buffer pH 8.0 (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole) then eluted with elution buffer pH 8.0 (50mM NaH2PO4, 300mM NaCl, 250mM imidazole). Protein in fractions from each wash were electrophoresed in 12% SDS-PAGE gels. The gels were then stained, while shaking, with Coomassie-R250 and then destained, while shaking, to visualize the recombinant protein.

***In vitro* transcription**

α^{32}P labeled RNA transcripts of *Bacillus subtilis* (pre-$tRNA^{asp}$) were generated by run-off transcription from the T7 promoter on plasmid pDW128. Cleavage of pDW128 for run-off termination was achieved via restriction digest of pDW128 (David Waugh) using *Bst*NI. Reaction contents include transcription optimized 5X buffer(200mM Tris-Cl pH 7.9 at 25°C, 50mM NaCl, 30mM $MgCl_2$, and 10mM spermidine), 100mM DTT, 1.25µl RNasin, 10mM (ATP, CTP, UTP) and 2mM GTP, approximately 0.5µg of template, 50 units of recombinant T7 polymerase, and 100 µg $^{\alpha 32}$P labeled GTP. Reactions were incubated for 2 hours at 37°C. Products were separated on an 8% urea-polyacrylamide gel, visualized by autoradiography, excised and passively eluted into 10mM Tris pH 8, 1mM EDTA, and 0.1% SDS by shaking overnight at room temperature. The elutates were then phenol extracted, RNA was ethanol precipitated, and quantitated in the Beckman LS 6500 scintillation counter.

Plasmids containing the genes encoding RNase P RNAs (*E. coli* and *Methanothermobacter thermoautotrophicus*) were restriction digested and transcribed in a similar manner except that reactions contained 10mM GTP and no labeled GTP. *E. coli*

RNase P RNA containing plasmid pDW98 was digested with *Sna*BI while *M. thermoautotrophicus* RNase P RNA containing plasmid ΔH trimmed #18 was digested with *Bsa*I.

RNase P Ribozyme Assay

RNase P assay reactions of *E. coli* and *M. thermoautotrophicus* RNase P RNAs, were performed using a 90 µl premix of dried substrate (1 kcpm/reaction), 10X P Buffer (25mM $MgCl_2$, 0.05% nonidet P-40, and 50mM Tris) (9µl), 5M NH_4OAc (18µl for *E.coli* RNase P RNA and 54µl for *M. thermoautotrophicus* RNase P RNAs), and nuclease-free water (54µl for *E.coli* and 18µl for *M. thermoautotrophicus* RNase P RNAs) added to one µl of RNase P RNA prepared in serial 1:5 dilutions. Each 10µl reaction mixture was incubated for 10 min. at 37°C. The products were separated on an 8% urea-polyacryamide gel and visualized by phosphoimagery.

RESULTS AND DISCUSSION

Expression of recombinant Mth11, Mth687, Mth688, and Mth1618 Archaeal Proteins

After transformation of the recombinant protein plasmids into *E.coli* there was some difficulty in confirming the presence of the open reading frames (ORFs) by amplification. The initial method used after transformation to prepare the cells for expression of the recombinant proteins was: (1) plating onto LB (AMP-CAM), (2) checking for archaeal inserts in the plasmid via PCR amplification, (3) transferring to LB (AMP-CAM) broth, then again (4) checking for archaeal inserts in the plasmid via PCR. Possible toxicity resulting from the production of these archaeal proteins, as seen for

M. jannashii ORF 1625, may cause the cells to either die or lose the plasmid (personal communication C. Ellis). This protocol was optimized by excluding the testing for the presence of the insert via amplification, eliminating the need for repeated subcultivation. Induction of protein over-expression was achieved by addition of IPTG (Figure 1). Mth11p, Mth1618p, Mth687p, and Mth688p have been previously shown to run in 12% SDS-PAGE gels at 15.5, 23.5, 18 and 31.5 kDa, respectively. These proteins were to be used in gel-shift binding assay and the enzymatic cleavage assays, although time constraints made this impossible.

RNase P Ribozyme Assay

In a typical ribozyme reaction the RNase P RNA would remove the 5′ leader sequence from the pre-tRNA^{Asp} substrate (under high ionic strength), although *E. coli* and *M. thermoautotrophicus* RNase P RNAs ability to catalyze the cleavage of α^{32}P labeled *Bacillus subtilis* pre-tRNA^{Asp} substrate was impaired. More degradation bands were found than were to be expected in the 8% urea-polyacrylamide gel, attributed to poor substrate quality (Figure 2).

Titration, Gel-shift Binding, and Enzymatic Cleavage Assays

Three more assays were planned to be performed to determine the functional homology between the bacterial and archaeal protein subunits: (1) a gel-shift binding assay would be used to determine the organization of the archaeal protein subunits that bind to the bacterial RNA (Figure 4), (2) A titration assay would be used to determine the Kd of the bound and unbound bacterial RNase P RNA with archaeal proteins (Figure 3), (3) and an enzymatic cleavage assay would indicate whether one or more of the protein subunits could reconstitute enzymatic activity in the bacterial RNase P RNA. The latter experiment

would be the inverse of the *B. subtilis* protein: *M. thermoautotrophicus* RNA experiment already published (Figure 5). It remains to be shown which, if any, of the archaeal/nuclear RNase P proteins are functionally homologous to the bacterial protein.

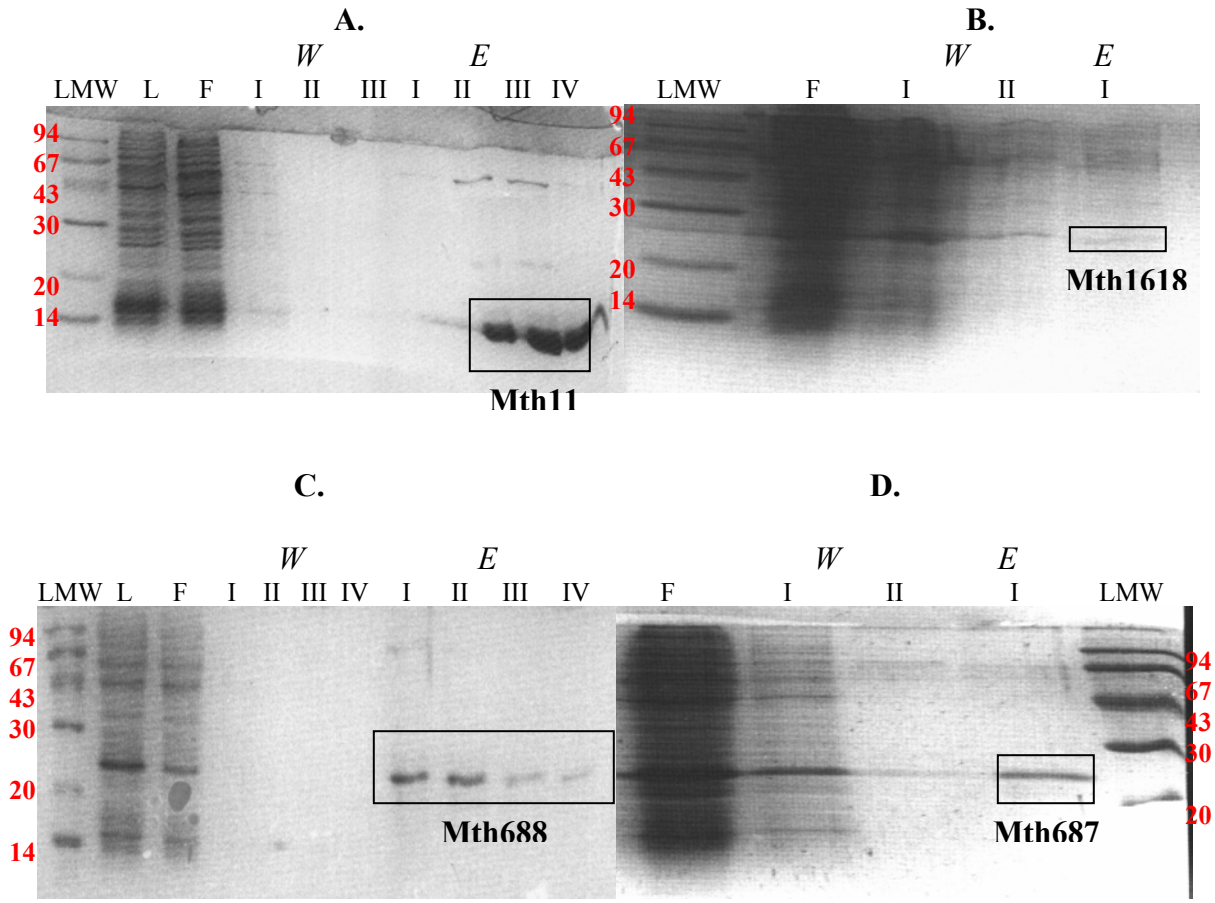


Figure 1: Expression of *M. thermoautotrophicus* RNase P recombinant proteins (A) 11p, (B)1618p, (C)688p, and (C)687p. (A) 12% SDS-PAGE gel of Mth11p, contents from left to right: low molecular weight marker (LMW), lysate (L), flow-through (F), wash (W) I-III, and elution (E) I-IV. (B) 12% SDS-PAGE gel of Mth1618p, contents from left to right: low molecular weight (LMW), flow-through (F), wash (W) I and II, and elution (E) I. (C) 12% SDS-PAGE gel of Mth688p, contents from left to right: low molecular weight marker (LMW), lysate (L), flow-through (F), wash (W) I-IV, and elution (E) I-IV. (D) 12% SDS-PAGE gel of Mth687p, contents from left to right: flow-through (F), wash (W) I and II, elution (E) I, and low molecular weight marker (LMW). The Low molecular weight marker is Phosphorylase at 94kDa, BSA at 67kDa, Ovalbumin at 43kDa, Carbonic Andydmi at 30kDa, Soybean Trypsin at 20.1kDa, and α-Lactolbumin at 14.4kDa.

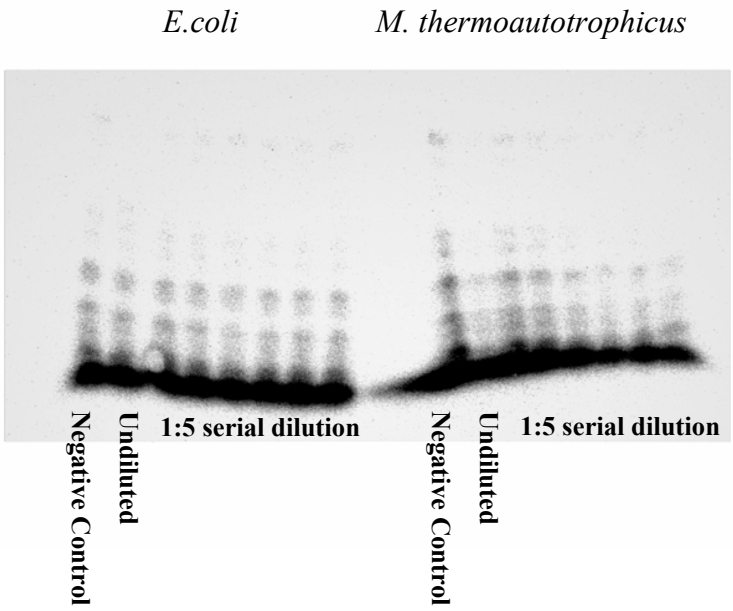


Figure 2: RNase P ribozyme assay. *E. coli* RNase P RNA ribozyme reaction on left and *M. thermoautotrophicus* RNase P RNA ribozyme reaction on right. Lane contents from left: (1) Negative control with no RNase P RNA, (2) Undiluted RNase P RNA, and (3-8) 1:5 serial dilutions of RNase P RNA.

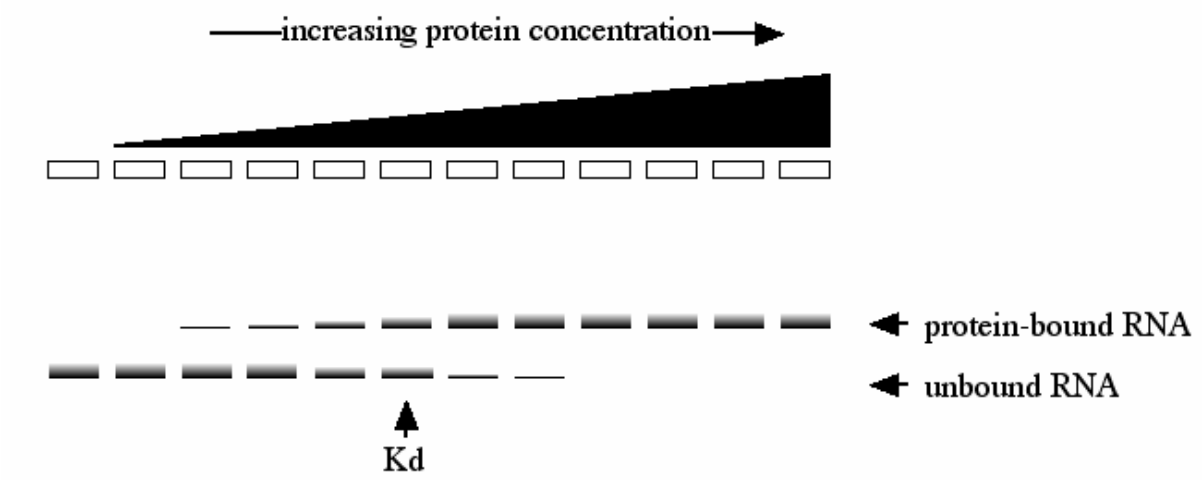


Figure 3: A titration gel-shift assay (shown diagrammatically). This is an example of a titration assay that would be used to determine the Kd of the bound and unbound bacterial RNase P RNA with archaeal proteins.

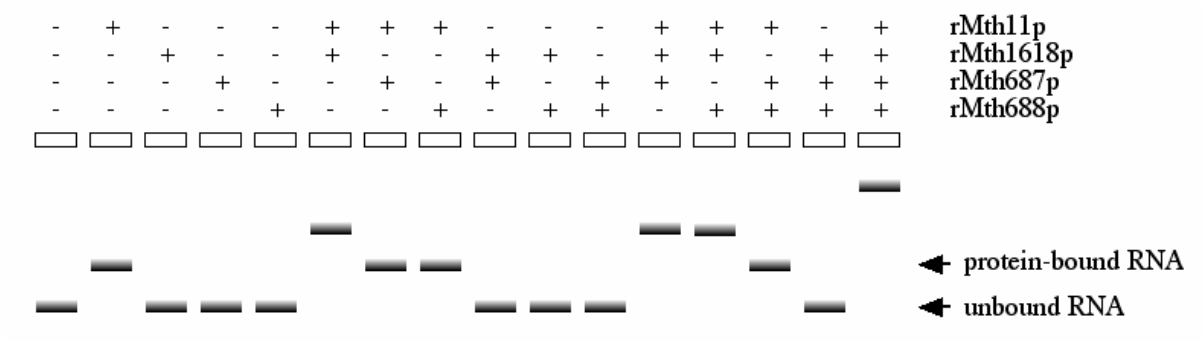


Figure 4: Gel-shift binding assay (shown diagrammatically). This is an example of how the archaeal proteins might bind to the bacterial RNase P RNA.

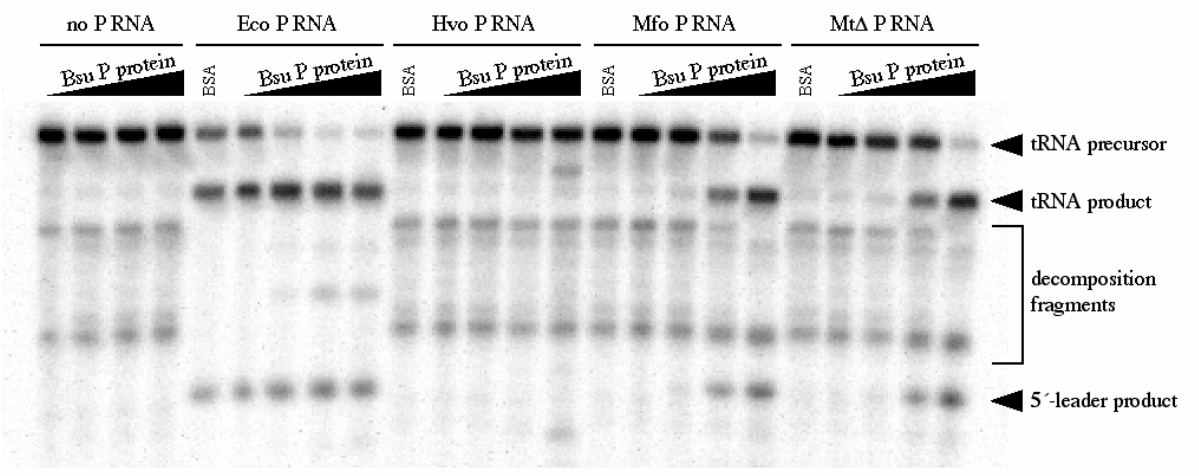


Figure 5: *B. subtilis* RNase P protein reconstituting activity in archaeal RNase P RNAs. Taken from: Pannucci JA, Haas ES, Hall TA, Harris JK, Brown JW. 1999. RNase P RNAs from some Archaea are catalytically active. Proc. Natl. Acad. Sci. USA. 96: 7803-7808.

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Chapter 2. Comparative analysis of the bacterial RNase P RNA structure

ABSTRACT

Eleven new RNase P RNA sequences from a range of Gram-negative and Gram-positive Bacteria have been amplified, sequenced, and their secondary structures derived through comparative analysis. These sequences and structures will contribute to the ongoing refinement of the model of RNase P RNA structure and evolution.

INTRODUCTION

Ribonuclease P (RNase P) is the endoribonuclease that catalyzes removal of the leader sequence in all known precursor-tRNAs (pre-tRNAs), generating the mature 5′ end of the tRNA. (1-3). In all organisms, RNase P is composed of conserved RNA and variable protein subunit(s). The RNA subunit (not the protein) is the catalyst; RNase P is a ribozyme. In Bacteria and most Archaea, the catalytic RNA is functional *in vitro* in the absence of protein. In Eukarya and other Archaea, the RNA depends absolutely on protein for function.

Construction of the RNase P RNA secondary structure is needed to understand the mechanisms of substrate recognition, catalytic activity, and evolution of the ribozyme (6). The first RNase P RNA sequences determined were those of *Escherichia coli* and *Bacillus subtilis*. Comparative analysis of the RNase P RNA sequences of these organisms and those of a small number of relatives led to the construction of the earliest meaningful secondary structure models (5). 427 secondary structures have now been determined, and ironically they fall into two types represented by the two dissimilar bacterial RNase P

RNAs that were first characterized, those of *Escherichia coli* and *Bacillus subtilis* (8). Type A, the ancestral type, is the most common type of RNase P RNA secondary structure and is represented by that of *E. coli.* This type can be found in among various groups of Gram-negative and Gram-positive Bacteria, whereas type B RNAs, represented by that of *B. subtilis*, are restricted to a subset of the low G+C Gram-positive Bacteria. The secondary structure of type B RNase P RNAs are quite different than their type A counterparts, but they contain a core of conserved sequence and structure (Figure 1). In addition, the ability to interchange protein subunits between *Escherichia coli* and *Bacillus subtilis* RNase P enzymes indicates an overall similarity in the tertiary structure of these RNAs (7).

In this study eleven new RNase P RNAs have been amplified and sequenced as part of the ongoing refinement of the RNase P RNA structure through comparative analysis. The organisms from which the RNase P RNAs originated were chosen from Carolina Biological Supply, because the genera were not represented in the RNase P Database.

MATERIALS AND METHODS

Growth of Organisms

Fifteen bacterial organisms were ordered from Carolina Biological Supply with the intent of characterizing their RNase P RNAs. The catalog number, organism, phylogenetic group, medium, and growth temperature can be found in Table 1. All of these organisms were grown with shaking and then stored at 4°C.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out in a Perkin Elmer GeneAmp PCR System 2400. The ReadyMix Taq PCR Reaction Mix (SIGMA), used to amplify RNase P, contained Taq DNA Polymerase in a 2X concentrate containing the nucleotides and reagents necessary for standard PCR: 25µl of 2X ReadyMix (1.5 units of Taq DNA Polymerase, 10mM Tris-HCl (pH 8,0), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 0.2mM dNTP), one µl each of appropriate forward and reverse primers (2ng/mL final concentration) (59*FBam* (CGGGATCCGIIGAGGAAAGTCCIIGC), 347*REco* (CGGAATTCRTAAGCCGGRTTCTGT), or 347*RXba* (GCTCTAGATAAGCCRYGTTYTGT) (I=Inosine, Y=C+T, R=A+G)), circa $1/10^{th}$ µl of cell material, and water up to a volume of 50µl. The amplifications were performed with an initial holding temperature of 94°C at 3 min. followed by 30 cycle sets of 94°C for 1.5 min., 50°C for 1.5 min., and 75°C for 2 min, and two final holding temperature of 75°C for 15 min. and 10°C until the tubes were collected for storage at 4ºC. PCR products were purified from reactions using the QIAquick PCR purification kit.

Sequencing

Sequences of the RNase P RNA genes were determined from double-stranded PCR products using an ABI Prism 377 sequencer (Iowa State University DNA Sequencing and Synthesis Facility). All purified PCR products were diluted to 5ng/100 bases/µl and primers (59*FBam*, 347*REco*, and 347*RXba*) were diluted to 5pmol/µl.

Sequence/Structure Construction and Analysis

The forward and reverse sequences of the RNase P RNA PCR products were assembled and edited using ABI Prism EditView. A BLAST Search of Genbank was performed using the forward sequence to identify the nearest related available sequence, and this guide secondary structure was obtained from the RNase P Database (8). Secondary structures were then constructed manually. Conserved elements were included by superimposing homologous regions within their related structure, while the structure of variable elements were predicted using MFOLD (Michael Zukar). The sequences were then added to the existing RNase P RNA alignment on the basis of their structures, using SeqApp and SeqPup (Don Gilbert, Indiana University). Phylogenetic trees were garnered using Phylip by the Neighbor-Joining Method (10).

RESULTS AND DISCUSSION

Of the fifteen organisms from Carolina Biological Supply, eleven RNase P RNAs were successfully amplified and sequenced. These organisms were chosen, because the genera were not represented in the RNase P Database. The RNase P RNA genes from these organisms were amplified with primers targeted to the conserved P4 region. Of these, ten were of the bacterial secondary structure type A: *Citrobacter freundii, Enterobacter aerogenes, Shigella flexneri, Acinetobacter calcoaceticus, Branhamella catarrhalis, Aquaspirillum serpens, Chromobacterium violaiceum, Spirillum volutans, Acetobacter aceti* and *Gluconobacter oxydans.* One was type B, that of *Sporosarcina ureae*. The type A RNAs were all from the Kingdom Proteobacteria, whereas *Sporosarcina ureae* is a member of the low G+C Gram-positive Bacteria.

The *Citrobacter freundii, Enterobacter aerogenes, Shigella flexneri, Acinetobacter calcoaceticus, Branhamella catarrhalis* sequence are consistent with their placement in the and categorized as χ-purple Bacteria (proteobacteria). The *Acinetobacter calcoaceticus* RNase P RNA structure was derived largely by comparison to Volunteer ESH7-9 RNase P sequence, although it contained a shorter P12 and a few nucleotide changes (Figure 2A). P12 in bacterial type A RNAs is an extremely variable region in sequence, length, and structure after the conserved base of the first five base-pairs that are thought to interact with P13 (9).

The *Branhamella catarrhalis* RNase P RNA was derived by comparison to the *Haemophilus influenza* RNase P RNA (Figure 2B). The helices are similar in length, but vary in sequence. This is typical for most RNase P RNAs in that there is significant sequence variability in the helices, indicating that the end-loops are more significant in substrate, protein, self, or cationic binding or that it is the base pairs rather than nucleotide identities that contribute to RNA structure and function. The last three χ-purple Bacteria are from a specific branch of the Kingdom known as Enterobacteriaceae, or enterics. These Bacteria are typically found in the intestinal tract of animals.

The *Enterobacter aerogenes* RNase P RNA was derived by comparison to that of *Klebsiella pneumoniae*, also an enteric (Figure 2C). Two nucleotide substitutions at the end-loop of P9 (L9) are the only differences between the RNAs of *E. aerogenes* and *K pneumoniae*. In bacterial RNase P RNAs, the GNRA L9 forms a tertiary interaction with the base-pairs in P1. The sequence obtained does not contain P1, so it is impossible to tell if this substitution would disrupt this contact (9).

The *Citrobacter freundii* RNase P RNA was most similar in sequence to that of another enteric, *Salmonella typhimurium* (Figure 2D). The only difference found was a nucleotide substitution in the four-nucleotide bulge between P16 and P17. This bulge is extremely variable in size and sequence, and probably serves only as a point of flexibility between P16 and P17.

The final enteric RNase P RNA was from *Shigella flexneri*, which was compared to that of *Escherichia coli* K-12 (Figure 2E). There is only sequence variation in P12, which is typical for very closely related RNase P RNAs. The conserved nature of the enteric RNase P RNAs as well as those of *Acinetobacter calcoaceticus* and *Branhamella catarrhalis* can be seen in the multiple-alignment of type A bacterial RNase P RNAs (Figure 3) and the phylogenetic tree based on this alignment (Figure 4).

The *Aquaspirillum serpens, Chromobacterium violaceum,* and *Spirillum volutans* RNase P RNA sequences are consistent with their placement in the β-purple bacteria (proteobacteria). The *Aquaspirillum serpens* RNase P RNA was modeled after the RNase P RNA of *Neisseria meningitidis* (Figure 2F). Although this RNA is its closest available relative, it contains multiple differences, including a number of nucleotide substitutions in the helices, a conserved GNRA tetra-loop in P12, and the presence of P19. Nucleotide substitutions in helices are common among different RNase P RNAs, and the GNRA tetraloop of P12, according to the sequence logo of type A RNase P RNAs (Figure 7), is not highly conserved. The most significant difference is the addition of P19, which where present is thought to serve to stabilize adjacent helices via stacking (probably P2) (1).

The *Chromobacterium violaceum* RNase P RNA was compared to the Volunteer ESH7-4 RNase P RNA sequence (Figure 2G). Both contain an unusual cruciform in P13,

while *C. violaceum* has an additional cruciform located in P12. The Volunteer ESH7-4 RNase P RNA sequence was previously considered to be of uncertain affiliation. The similarity of this RNA to that of *C. violaceum* indicates that this unknown RNase P RNA may belong to a β-purple bacterium.

Finally, the RNase P RNA of *Spirillum volutans,* a β-proteobacteria; was derived by comparison to the χ-purple bacteria *E. coli* (Figure 2H). Comparative analysis of a β-purple to that of a χ-purple bacteria may seem inappropriate, but this sequence is only distantly-related to any other known RNase P RNA. The RNase P RNAs from the β-purple Bacteria vary more in sequence and structure than the χ-purple bacteria. In the phylogenetic tree (Figure 4), the *S. volutans* RNA does not group with the other β-purple Bacteria, but seems to reside somewhere near Chlamydia or Spirochaetes, as does the α-purple bacterium *B. aphidicola*.

The *Acetobacter aceti* and *Gluconobacter oxydans* RNase P RNAs sequences are consistent with those of other α-purple Bacteria. The partial RNase P RNA from *Acetobacter aceti* is similar to that of the unknown RNase P RNA CP-A62 (Figure 2I). CP-A62 RNase P RNA has been considered to be of uncertain affiliation, although its similarity to *A. aceti* implies that it may be an α-purple Bacteria. Overall the structures are the same, with typical length variations in P17 and P19 (9). P10/11 helices in *Acetobacter aceti* and CP-A62 appear different, although the single-stranded RNA fork after P10/11 in CP-A62 contains base-pairs that would extend these helices, thus allowing further structural similarity. This mistake in CP-A62 could be fixed through MFOLD, a thermodynamically based RNA constructor program (Michael Zukar).

The RNase P RNA from *Gluconobacter oxydans* was derived by comparison to that of *Rhodospirillum rubrum* A348 RNase P RNA (Figure 2J). These RNAs share significant structural similarities, although there is sequence and length variation in P12 and P17/18/19. These two α-purple bacteria were found to coincide with typical α-purple RNase P RNAs (Figures 3 and 4).

The RNase P RNA of *Sporosarcina ureae* was the only type B RNase P RNA obtained. It is phylogenetically characterized as a low G+C Gram positive bacterium. The structure of the RNA was derived by comparison to that of *Bacillus subtilis* 168 (Figure 2K). Type B RNase P RNAs contain helices that differ from type A RNAs, *ie.* P5.1, P10.1, and P15.1. There are significant structural similarities between *S. ureae* and *B. subtilis*, with typical sequence variations (Figure 2K, 5). Known tertiary interactions are conserved.

The addition of eleven new partial RNase P RNAs to the already available 427 bacterial RNase P RNAs in the RNase P Database is crucial to the refinement of those and future RNase P RNAs through comparative analysis.

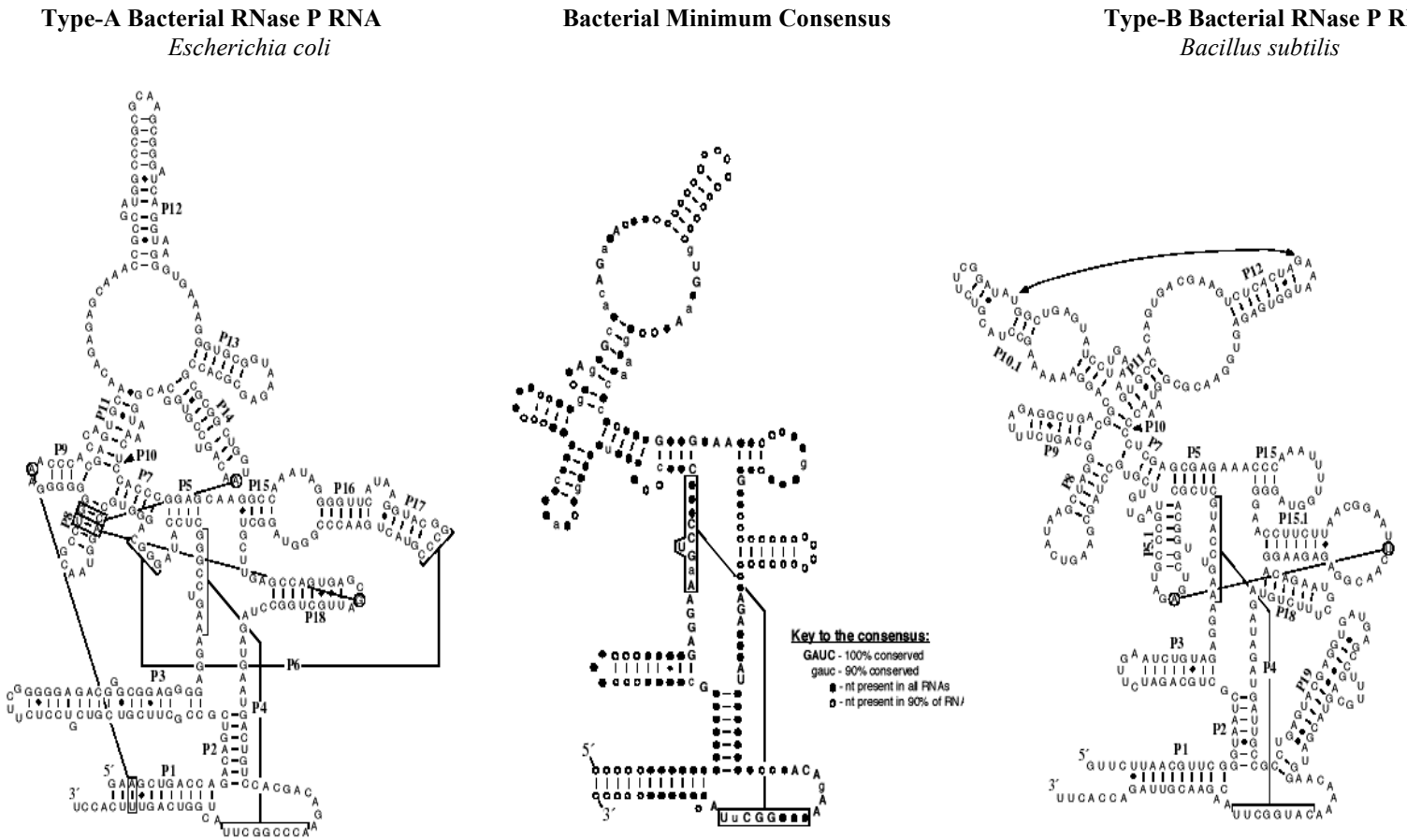


Figure 1: Bacterial type A RNase P RNA secondary structure, represented by *E.coli*, the minimum consensus bacterial RNase P RNA, and bacterial type B RNase P RNA secondary structure, represented by *Bacillus subtilis*.
Adapted from: Pace NR, Brown JW. 1995. Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. J. Bactertiol. 177: 1919-1928.

A.

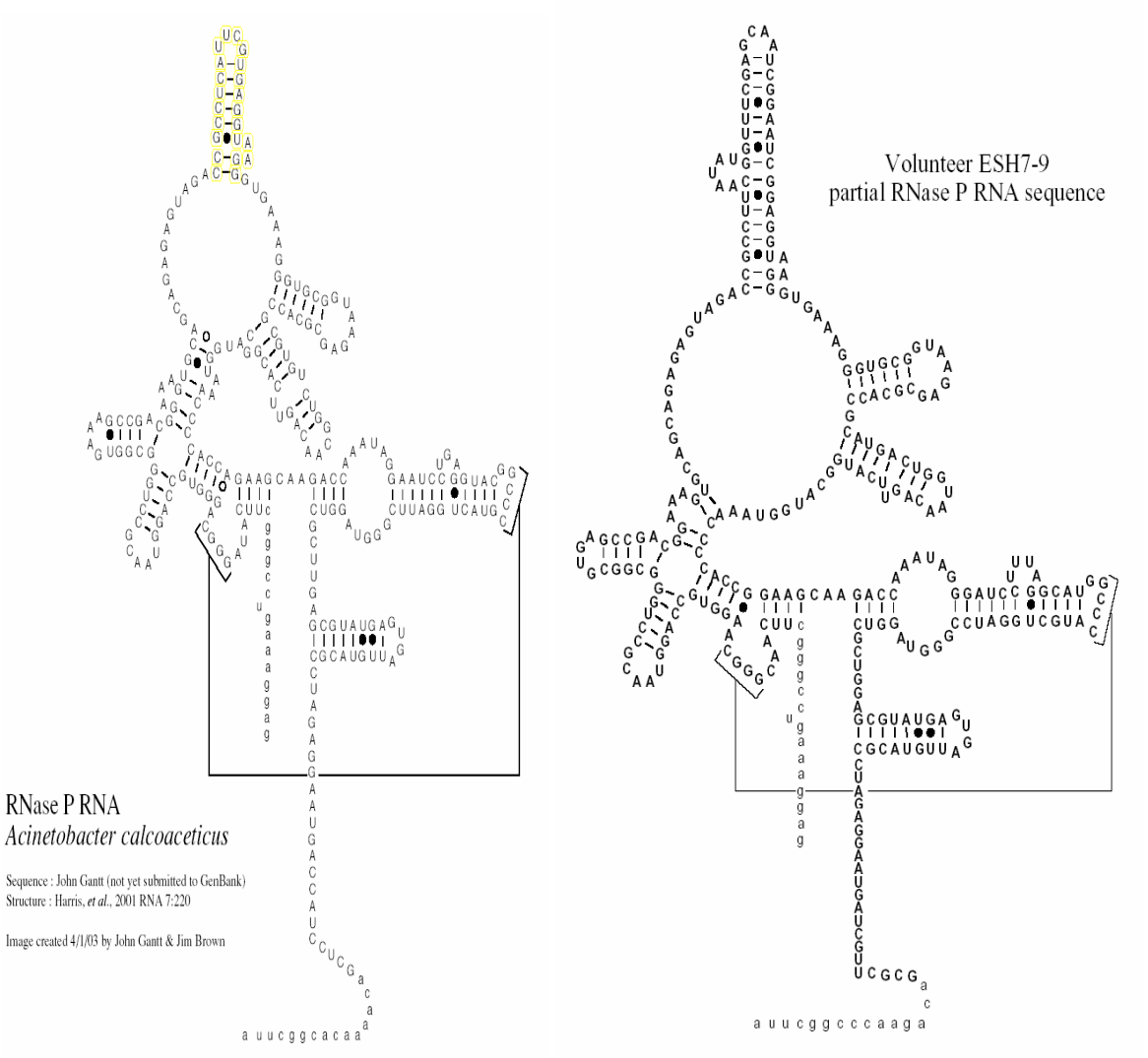


Figure 2: Comparative analysis of eleven new RNase P RNAs and their derivatives. Highlighted nucleotides show sequence differences. (A) *Acinetobacter calcoaceticus* and partial Volunteer ESH7-9, (B) *Branhamella catarrhalis* and *Haemophilus influenza*, (C) *Enterobacter aerogenes* and *Klebsiella pneumoniae*, (D) *Citrobacter freundii* and *Salmonella typhimurium*, (E) *Shigella flexneri* and *Escherichia coli*, (F) *Aquaspirillum serpens* and *Neisseria meningitidis,* (G) *Chromobacterium violaceum* and partial Volunteer ESH7-4, (H) *Spirillum volutans* and *Escherichia coli,* (I) *Acetobacter aceti* and CP-A62, (J) *Gluconobacter oxydans* and *Rhodospirillum rubrum*, and (K) *Sporocarsina ureae* and *Bacillus subtilis* RNase P RNA secondary structures.

B.

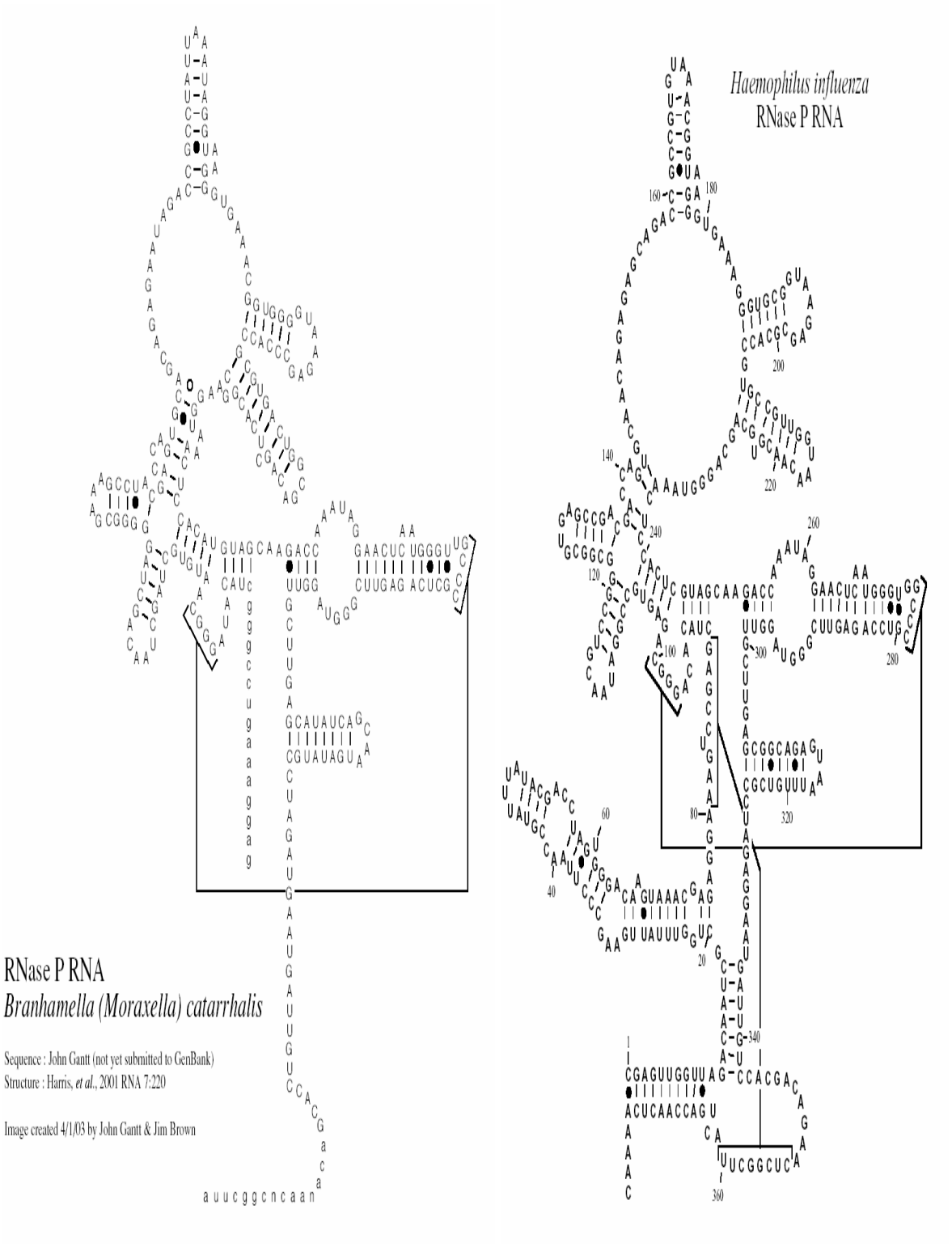


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C.

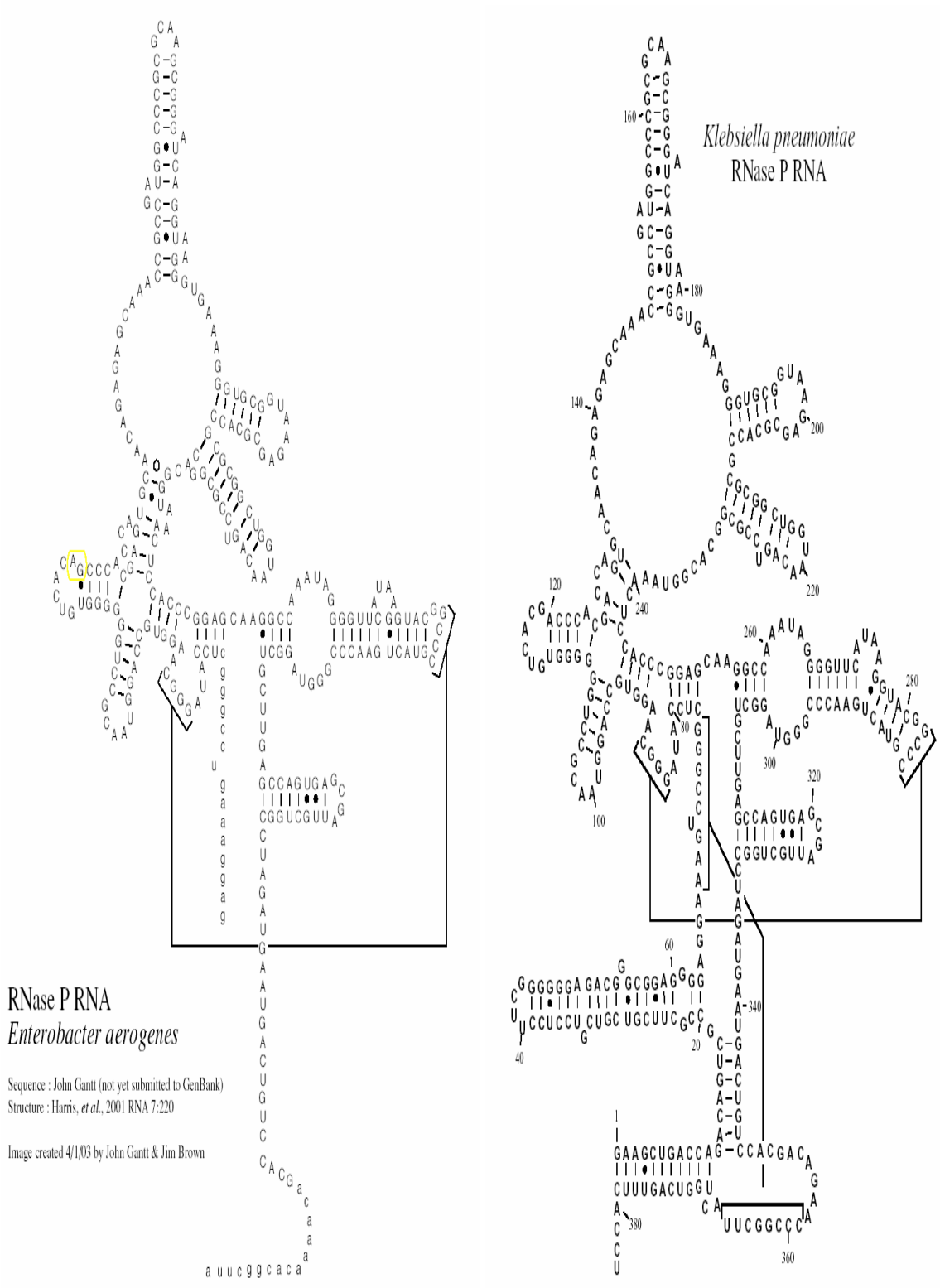


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D.

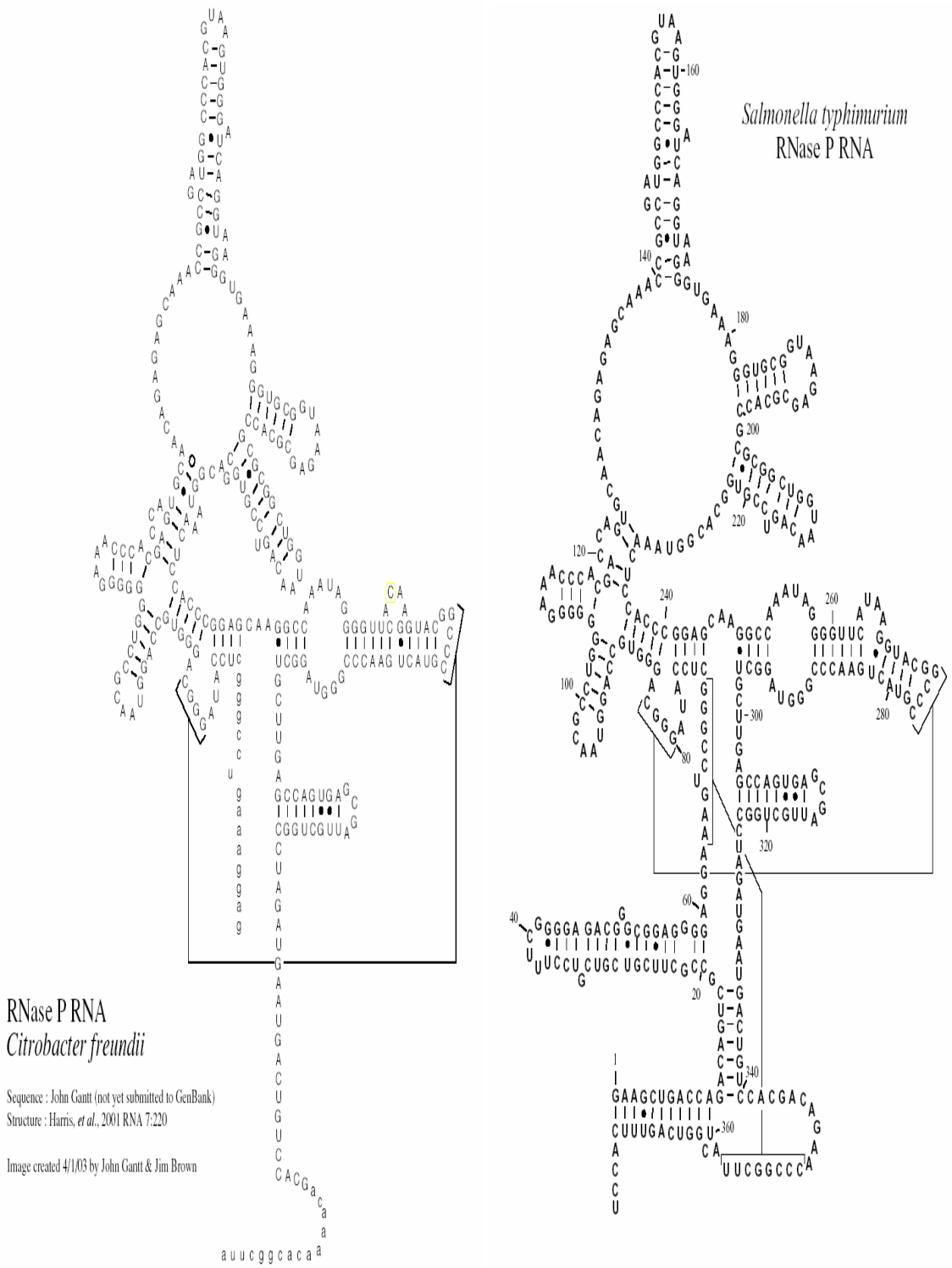


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E.

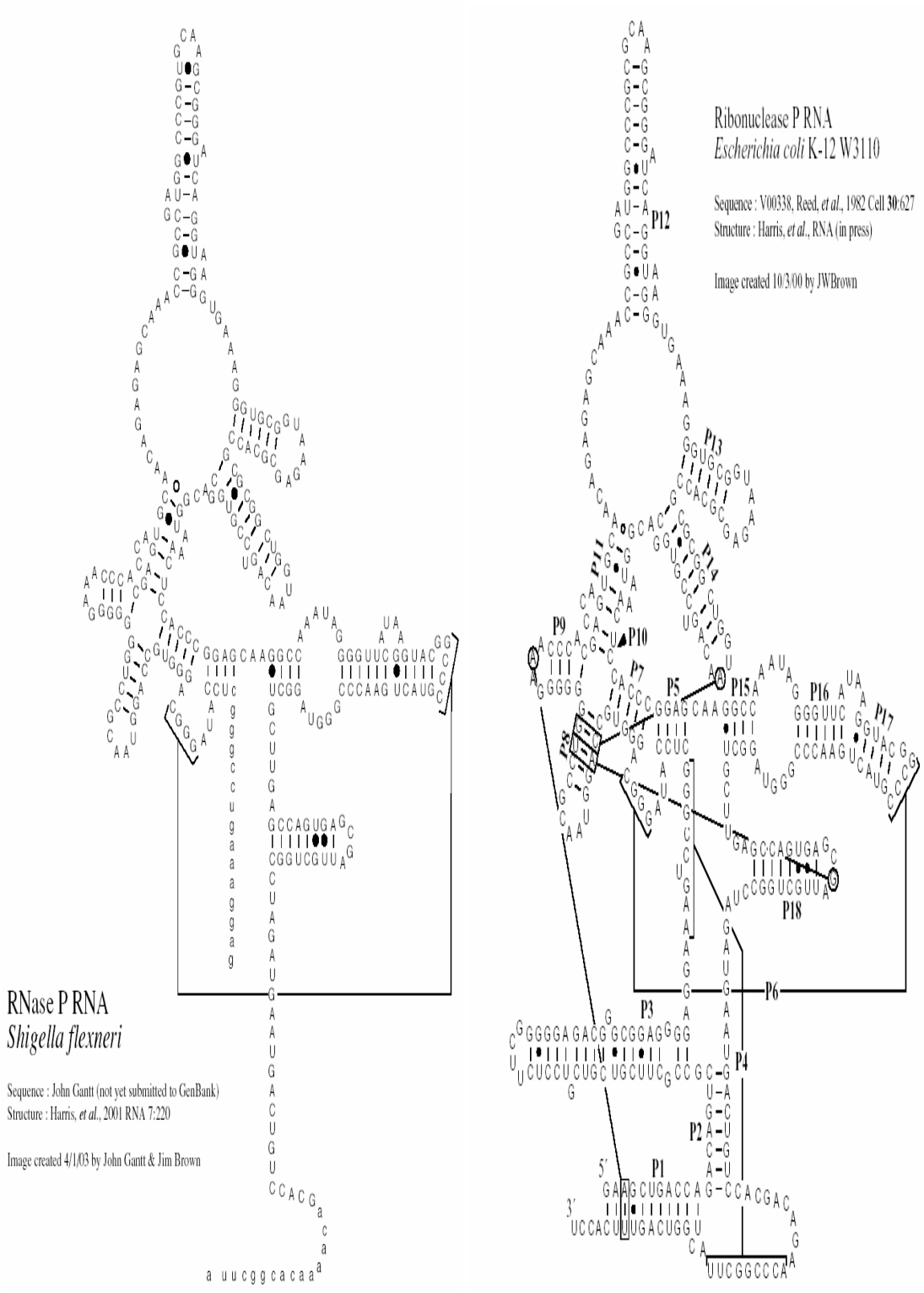


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F.

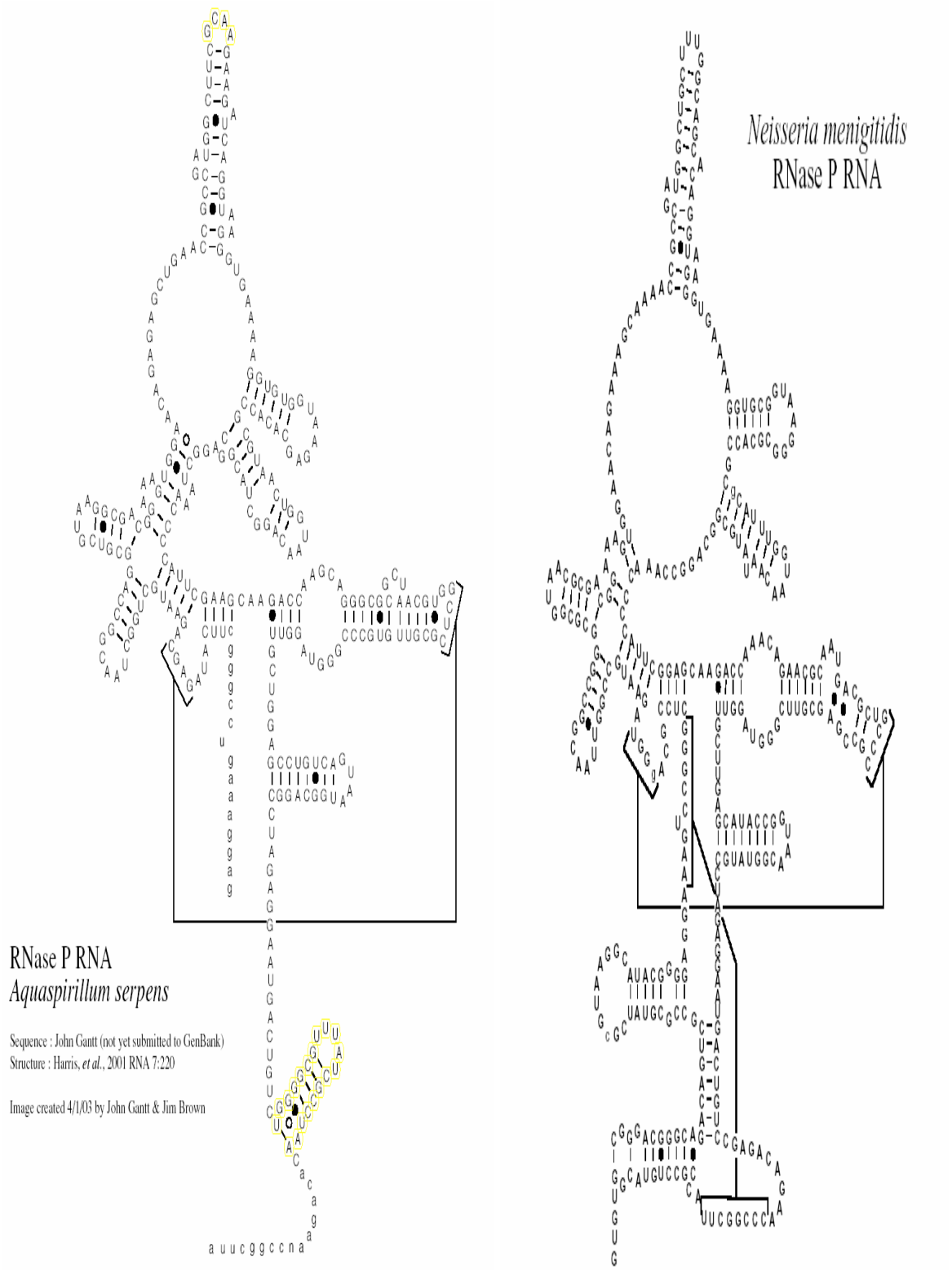


Figure 2 continued.

G.

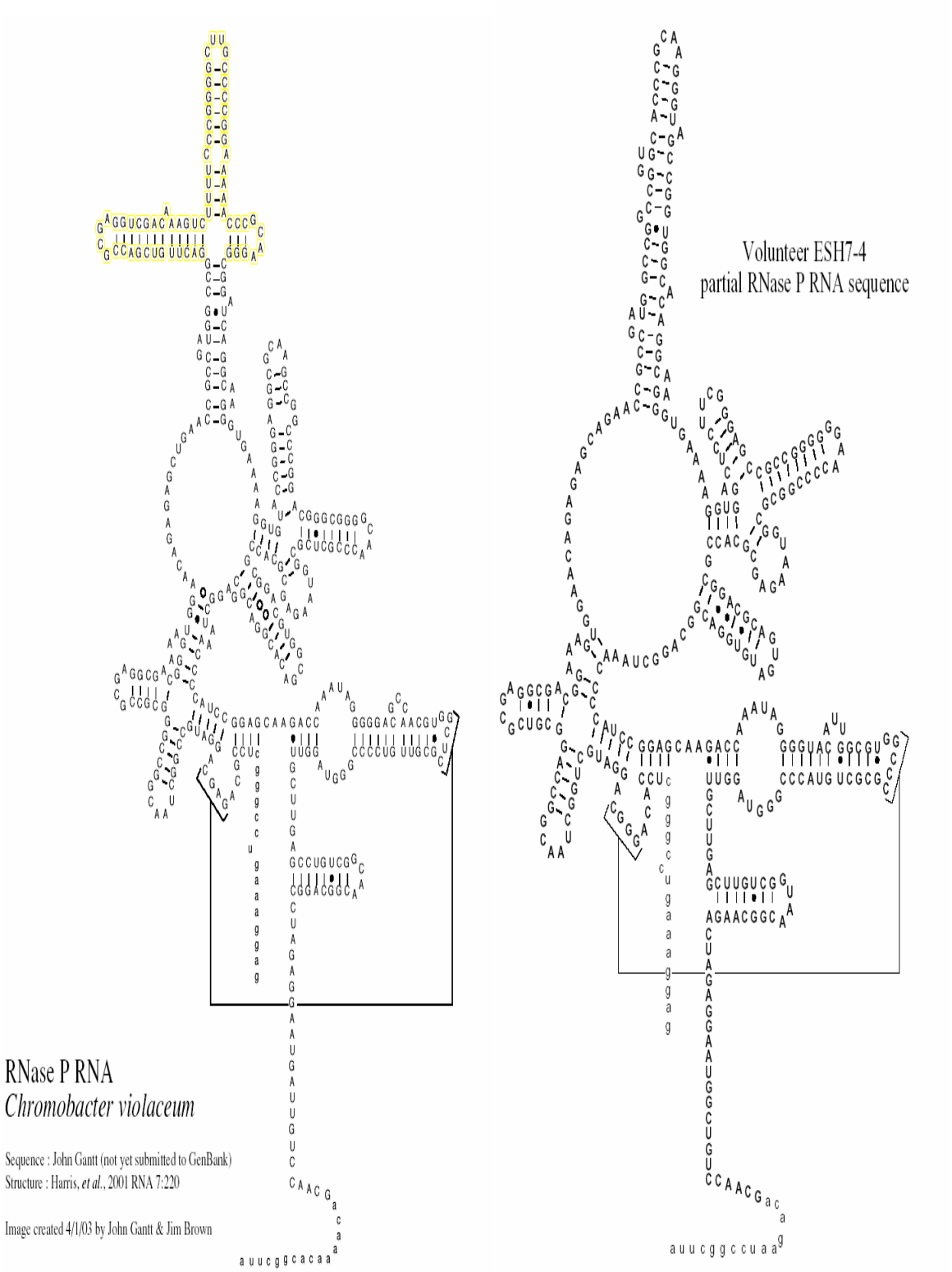


Figure 2 continued.

H.

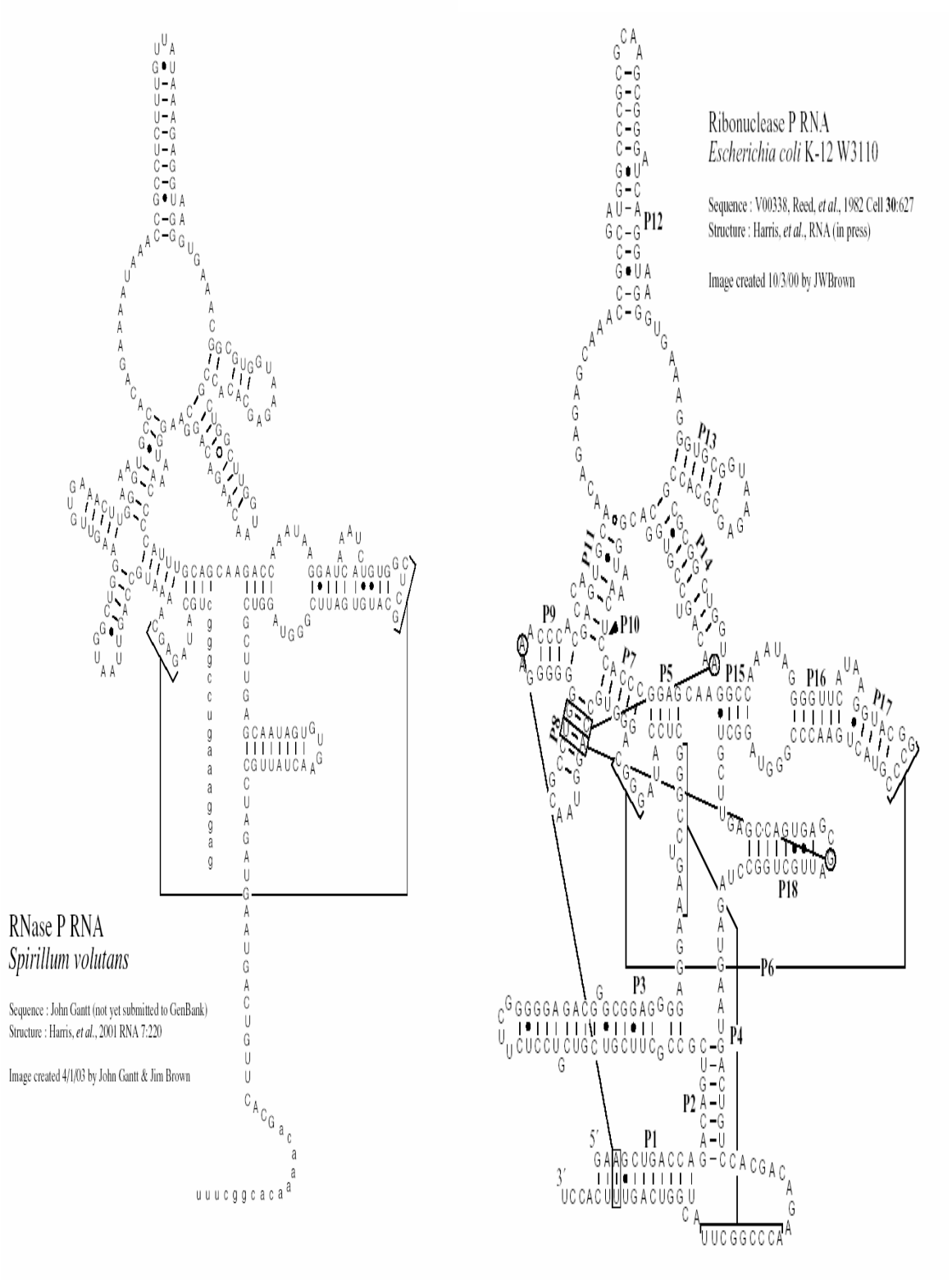


Figure 2 continued.

I.

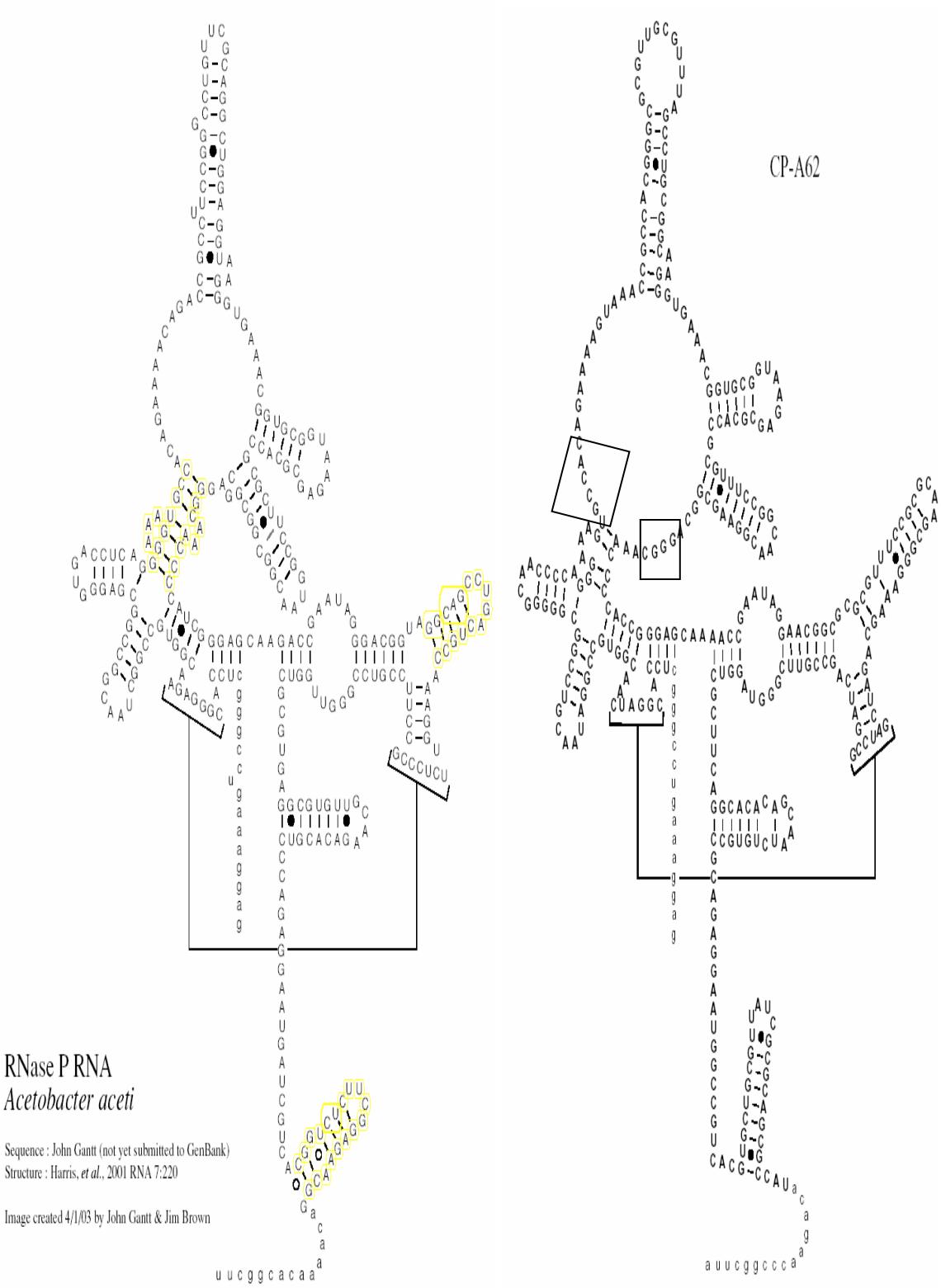


Figure 2 continued.

J.

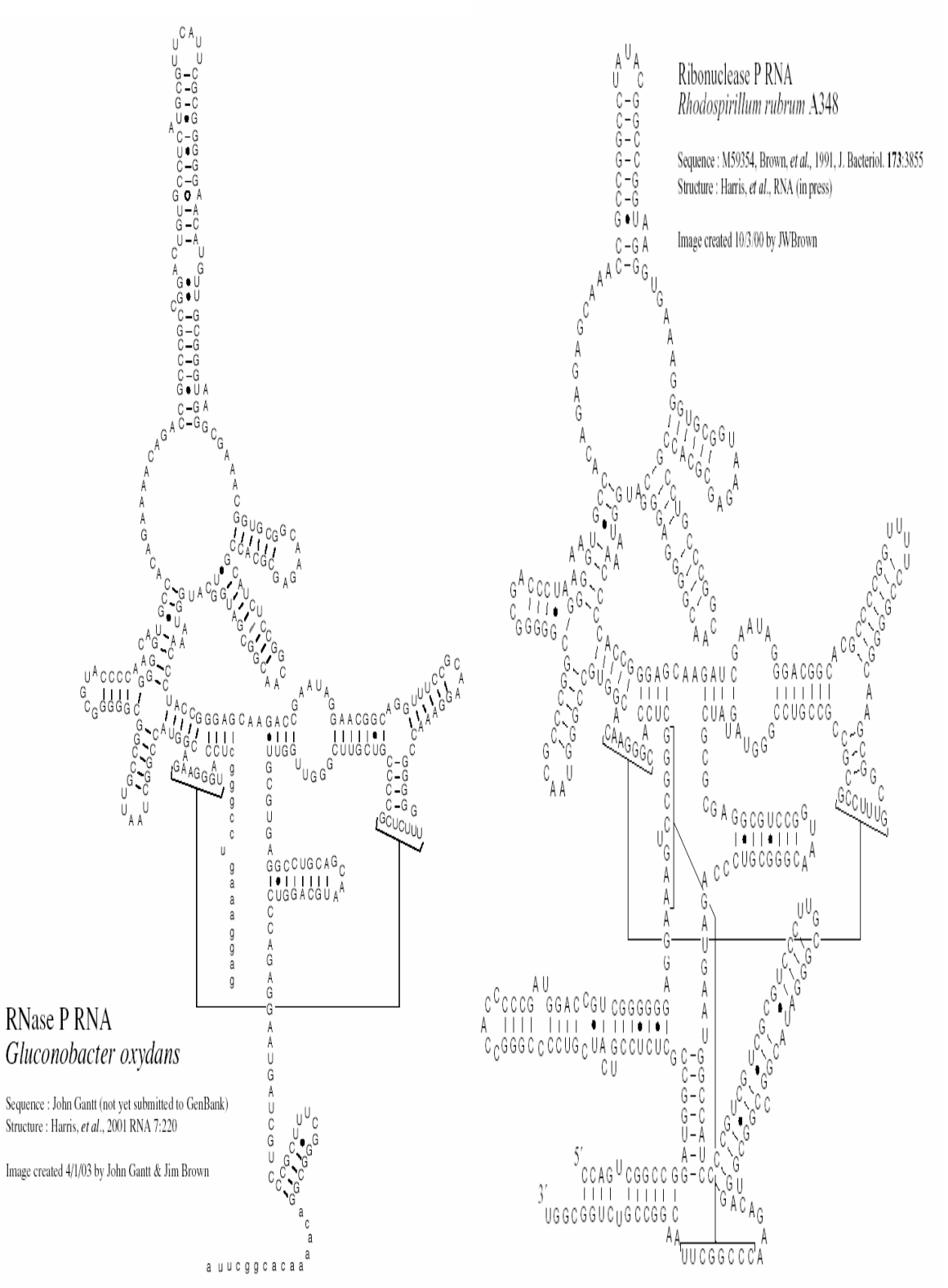
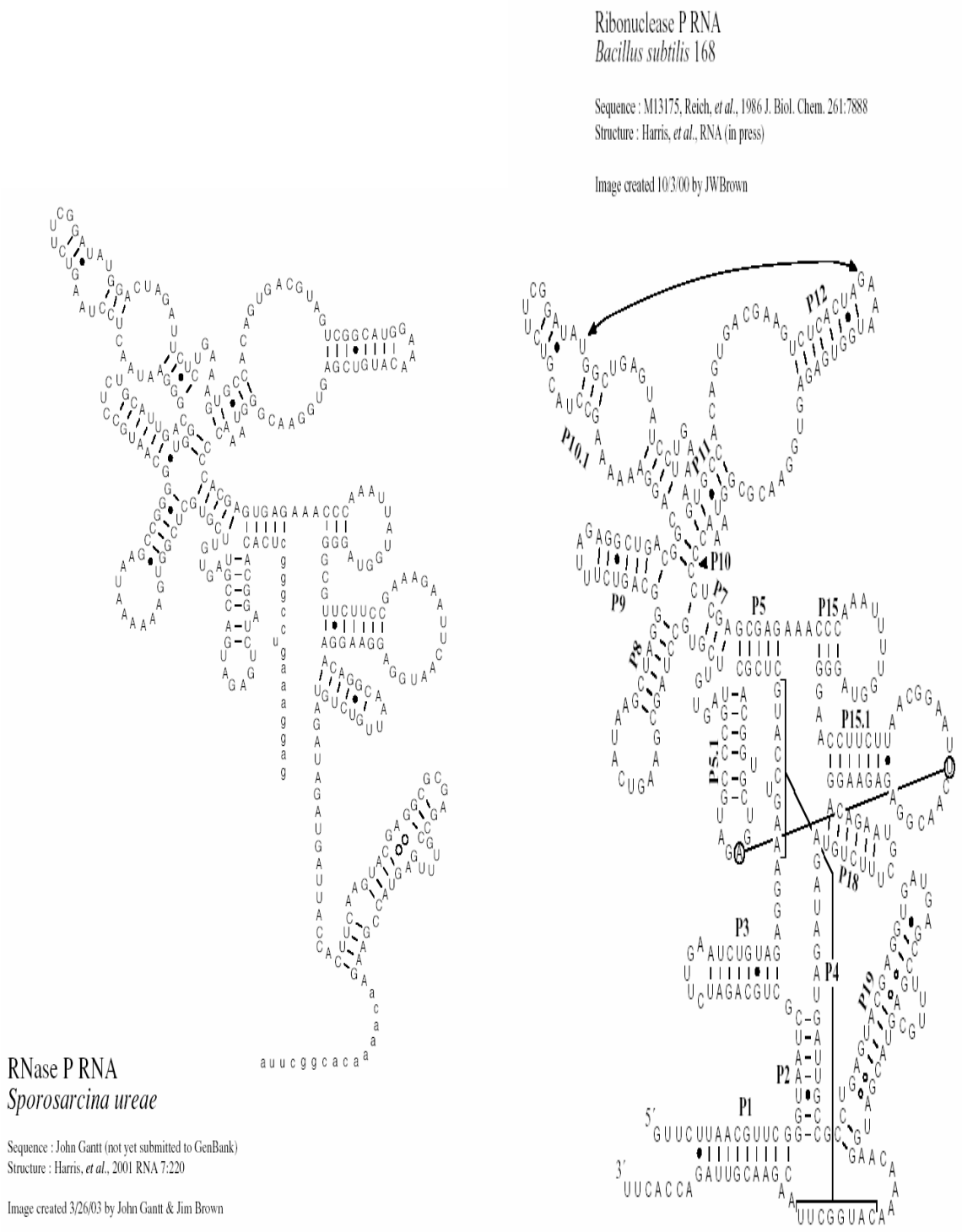


Figure 2 continued.

K.



Taken From:
Brown JW. 1999. The ribonuclease P database. Nucl. Acids Res. 27: 314.

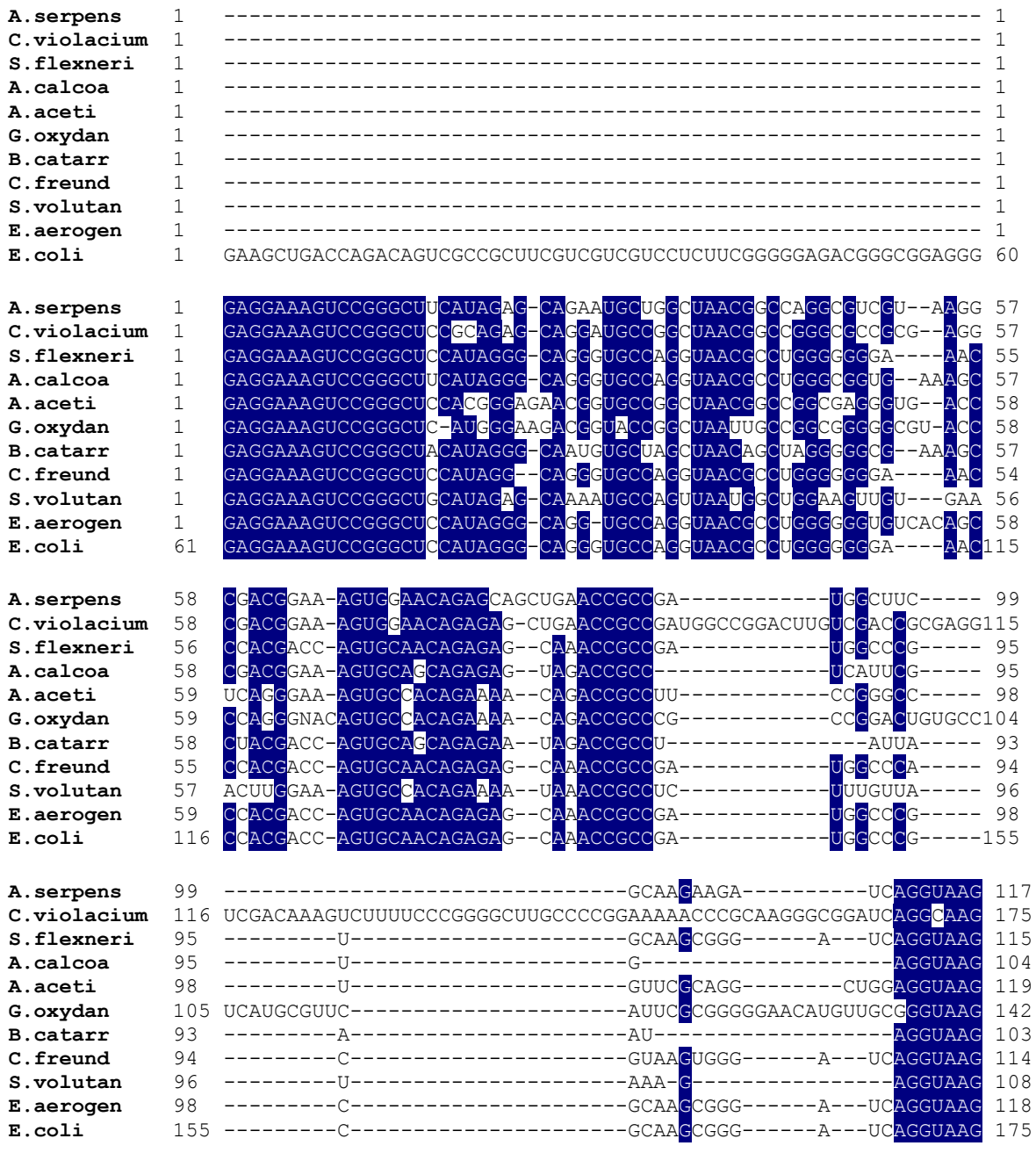


Figure 3: Type A bacterial RNase P RNA multiple sequence alignment. These sequences were aligned manually on the basis of secondary structure using Bioedit. Shaded regions indicate the most conserved nucleotide. *A. serpens* is *Aquaspirillum serpens* , *C. violaceum* is *Chromobacterium violaceum*, *S. flexneri* is *Shigella flexneri*, *A. calcoa* is *Acinetobacter calcoaceticus*, *A. aceti* is *Acetobacter aceti*, *G. oxydan* is *Gluconobacter oxydans*, *B. catarr* is *Branhamella catarrhalis*, *C. freund* is *Citrobacter freundii*, *S. volutan* is *Spirillum volutans*, *E. aerogen* is *Enterobacter aerogenes* and *E.coli* is *Escherichia coli.*

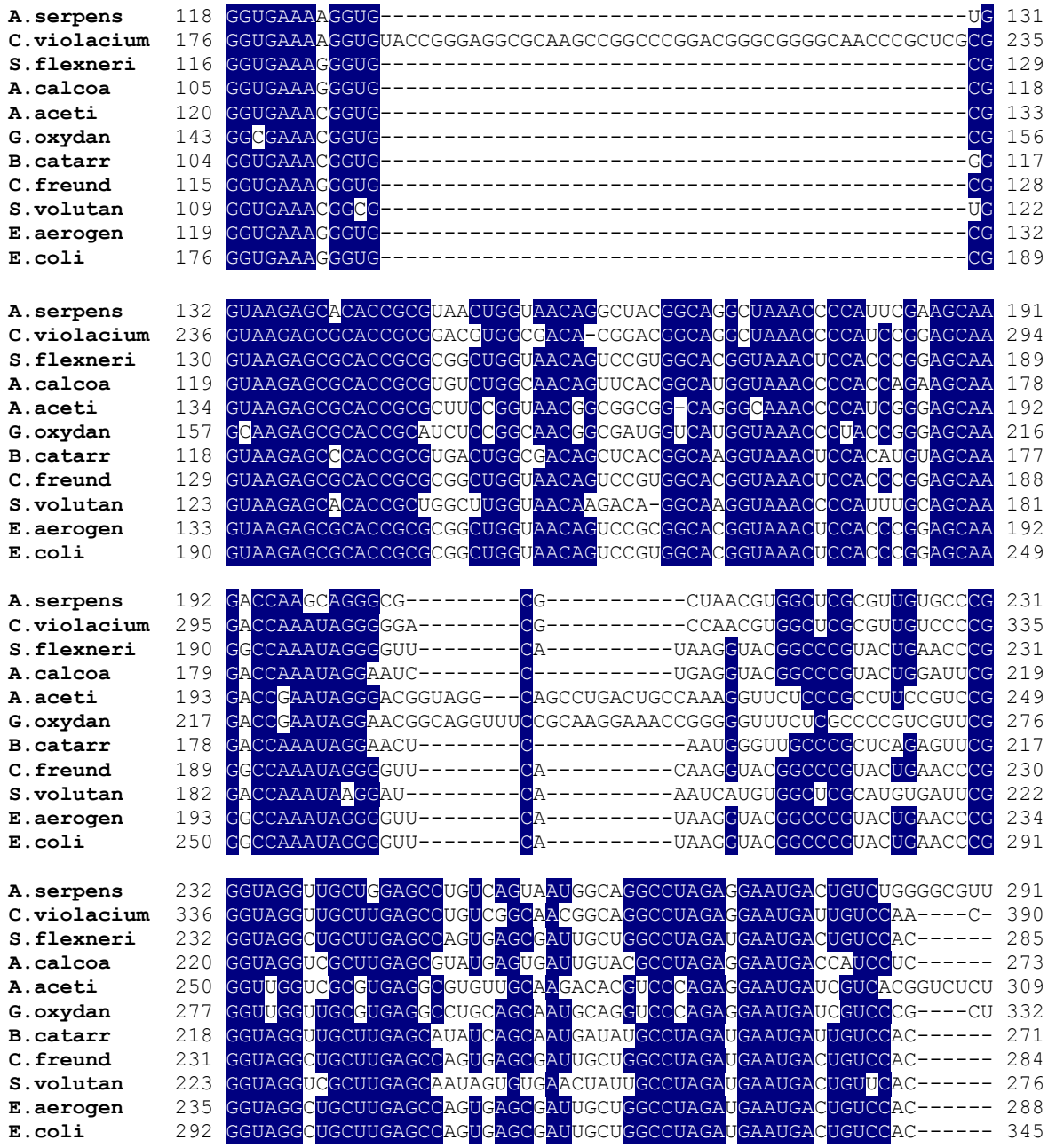


Figure 3 continued.

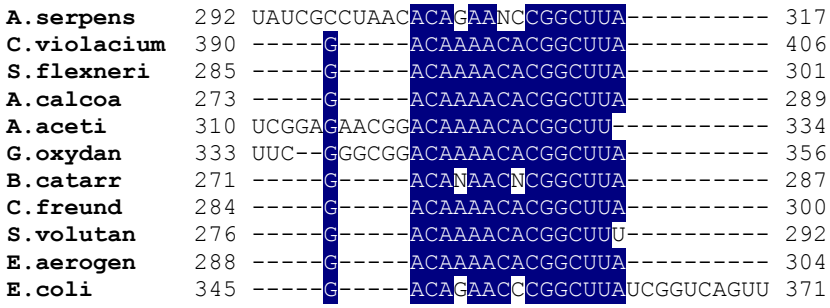


Figure 3 continued.

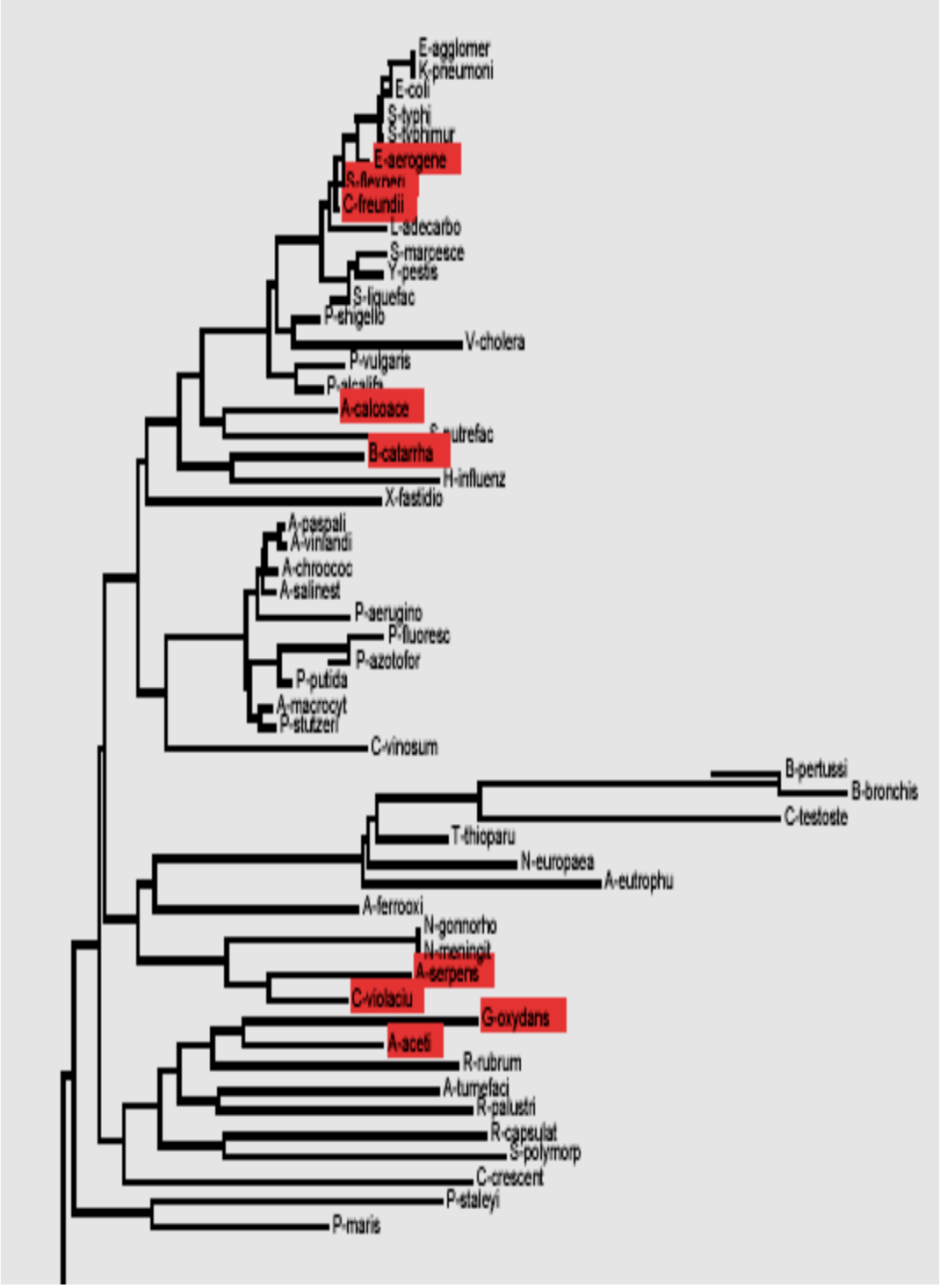


Figure 4: Type A bacterial RNase P RNA neighbor-joining tree. New RNase P RNAs are indicated by red highlight.

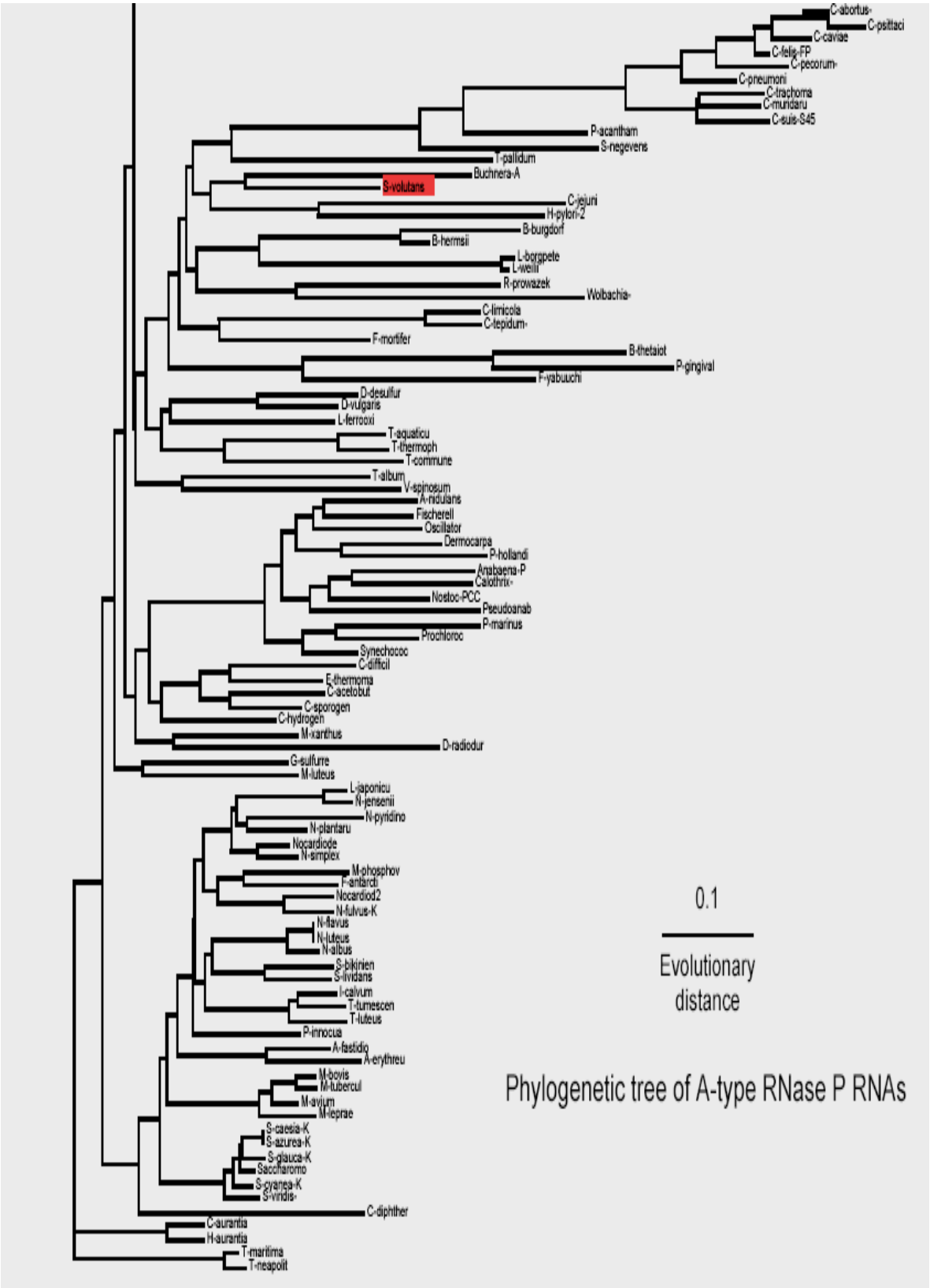


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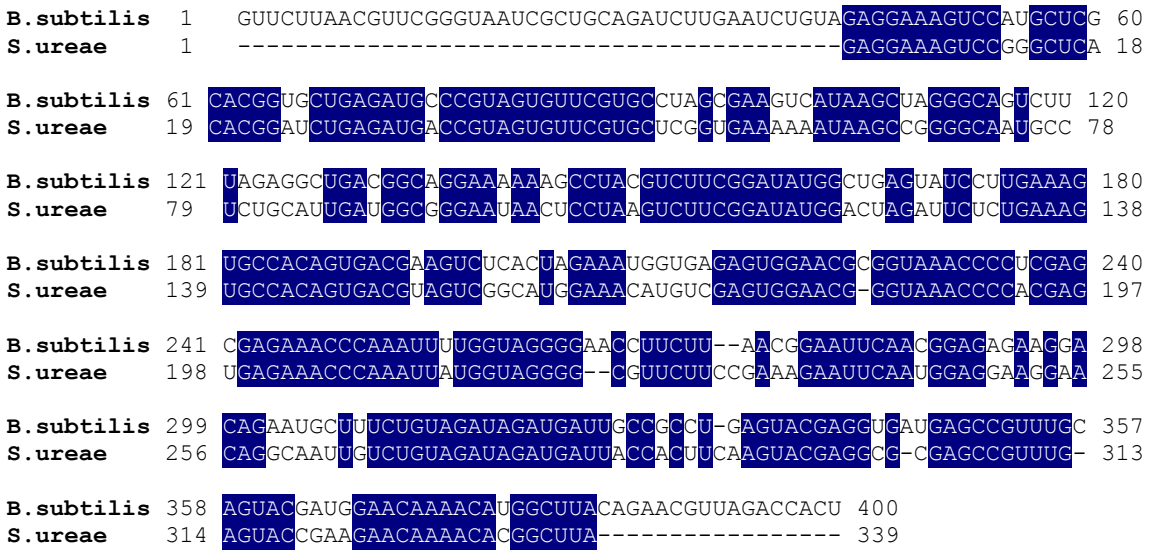


Figure 5: Type B bacterial RNase P RNA multiple sequence alignment. These sequences were aligned manually on the basis of secondary structure using Bioedit. *S. ureae* is *Sporosarcina ureae* and *B. subtilis* is *Bacillus subtilis*.

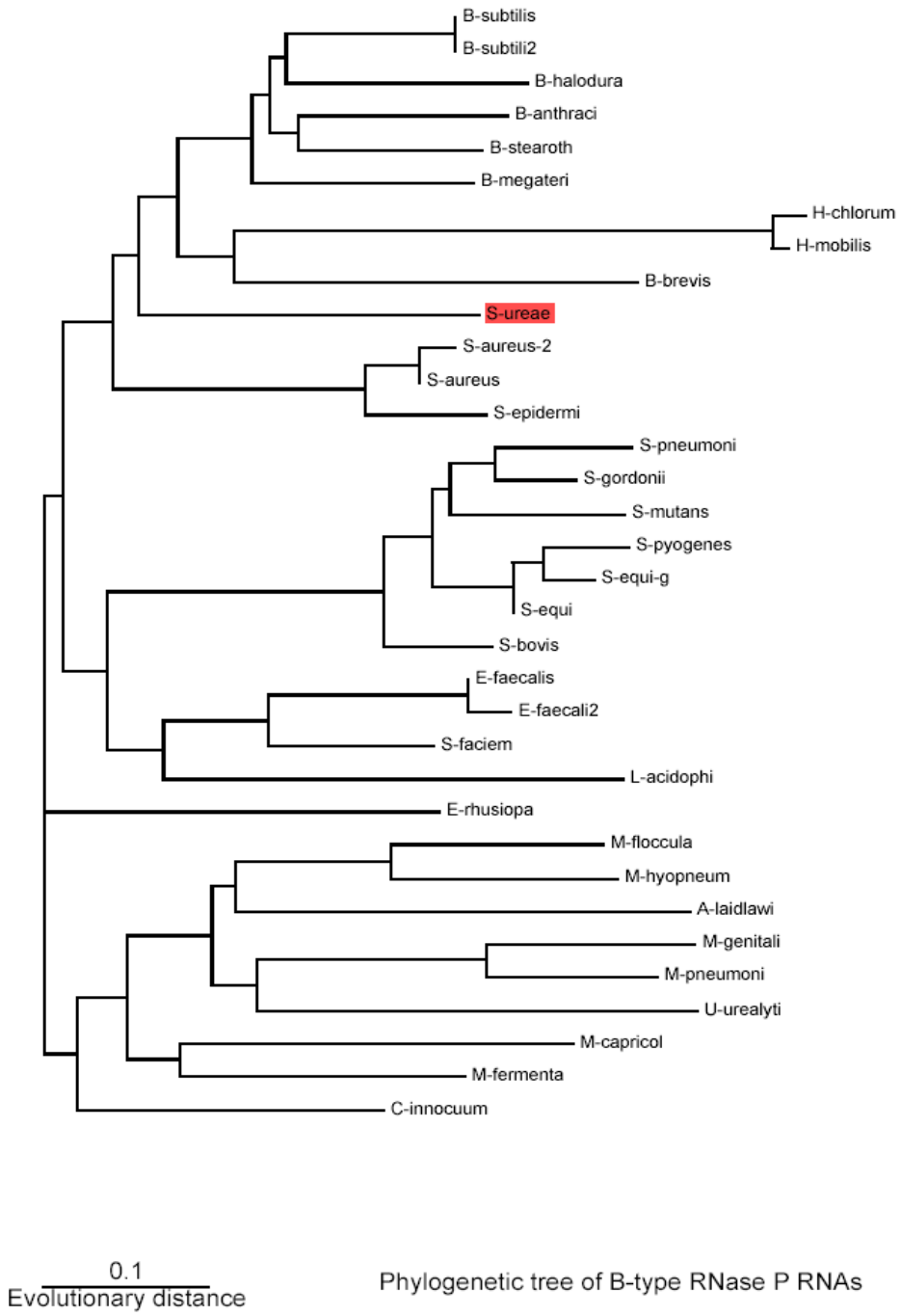


Figure 6: Type B bacterial RNase P RNA neighbor-joining tree.
The new RNase P RNA (*S.ureae*) is indicated by red highlight.

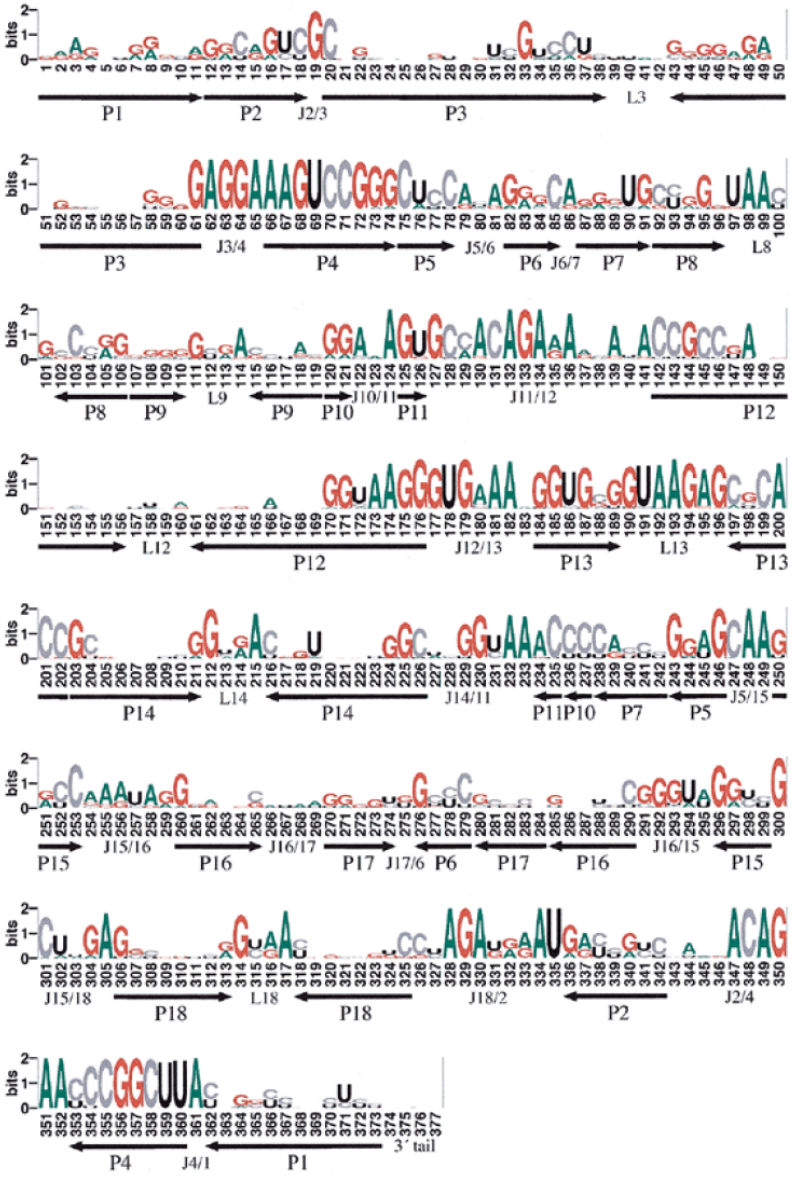


Figure 7: Sequence logo of type A RNase P RNAs. Numbering is based on the *E. coli* RNase P RNA. Taken from: Haas ES, Brown JW. 1998. Evolutionary variation in bacterial RNase P RNAs. Nucl.Acids Res. 26: 4093-4099.

Table 1: List of Organisms providing new RNase P RNAs.

Carolina Supply Catalog #	Organism	Phylogenetic Group	Medium	Growth Temp.
WW-15-4810	*Acetobacter aceti*	α-proteobacteria	Mannitol Special Agar	30°C
WW-15-4822A	*Acinetobacter calcoaceticus*	χ-proteobacteria	Nutrient Agar	37°C
WW-15-4828	*Aeromonas sobria*	χ-proteobacteria	Nutrient Agar	25°C
WW-15-4835A	*Alcaligenes faecalis*	β-proteobacteria	Nutrient Agar	37°C
WW-15-4838	*Aquaspirillum serpens*	β-proteobacteria	Nutrient Agar	30°C
WW-15-4840	*Arthrobacter globiformis*	High G+C Gram Positive	Nutrient Agar	30°C
WW-15-4929	*Branhamella catarrhalis*	χ-proteobacteria	Brain Heart Infusion Agar	37°C
WW-15-4931A	*Chromobacterium violaceum*	β-proteobacteria	Nutrient Agar	30°C
WW-15-4941	*Citrobacter freundii*	χ-proteobacteria	Nutrient Agar	37°C
WW-15-5030	*Enterobacter aerogenes*	χ-proteobacteria	Nutrient Agar	30°C
WW-15-5087	*Gluconobacter oxydans*	α-proteobacteria	Gluconobacter Agar	25°C
WW-15-5270	*Rhizobium leguminosarum*	α-proteobacteria	Rhizobium X Agar	25°C
WW-15-5470A	*Shigella flexneri*	χ-proteobacteria	Nutrient Agar	37°C
WW-15-5512	*Spirillum volutans*	β-proteobacteria	Peptone-Succinate Medium	30°C
WW-15-5518	*Sporosarcina ureae*	Low G+C Gram Positive	Nutrient Agar	25°C

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