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Convergent evolution of two different random RNAs for specific interaction with methionyl-tRNA synthetase

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ABSTRACT

Aminoacyl-tRNA synthetases (ARSs) recognize a specific sequence or structural characteristics of their cognate tRNAs. To contribute to the understanding how these recognition sites were selected, we generated two different RNA libraries containing either 42mer or 70mer random sequence and used them to select RNA aptamers that specifically bound to methionyl-tRNA synthetase (MRS) of *Mycobacterium tuberculosis*. The aptamer pools selected from the two RNA libraries showed strong binding affinity and selectivity to *M. tuberculosis* MRS compared to that of the homologous *Escherichia coli* MRS. The RNA aptamers selected from the two completely unrelated RNA pools shared the octamer sequence including CAU and the anticodon sequence of $tRNA^{Met}$. The secondary structure prediction suggested that the octamer motif in the selected aptamers would form a loop similar to the anticodon loop of $tRNA^{Met}$. The results suggest that the RNA loop containing CAU triplet could selected as a major recognition site for MRS during evolution more or less regarding, and also showed that species-specific ARS inhibitors can be obtained by *in vitro* evolution.

1. Introduction

As aminoacyl-tRNA synthetases ligate specific amino acids to their cognate tRNAs, the correct recognition of tRNAs by these enzymes determines the fidelity of protein synthesis. To maintain the specificity of the interaction between two molecules, ARSs developed an idiosyncratic pattern of tRNA recognition, and the specificity is thought to result from adaptive co-evolution of the enzyme and its cognate tRNA [1–4]. However, it is not understood how the tRNA recognition sites have been selected during evolution.

Interestingly, only limited tRNA sites have been used for specific recognition by ARSs and they are usually localized in the acceptor stem and anticodon loop. For instance, alanyl-tRNA synthetase recognizes the G3-U70 base pair and A73 discriminator nucleotide of its cognate tRNA [5–7]. In contrast, methionyl-tRNA synthetase interacts with the $tRNA^{Met}$ anticodon [8,9]. Although ARSs are grouped into two classes based on their structural features [10], the tRNA recognition pattern does not appear to be related to this classification [11,12], as both class I methionyl- and glutaminyl-tRNA [13], and class II aspartyl-tRNA synthetases [14] specifically interact with the anticodon of their cognate tRNAs. In addition, these enzymes use different structural domains for interacting with specific anticodons. While the two β-barrels in the C-terminal region of glutaminyl-tRNA synthetase are involved in the interaction with the $tRNA^{Gln}$ anticodon of [13], the α-helix and loop in the C-terminal domain of methionyl-tRNA synthetase is used for the interaction with the cognate anticodon [8]. In contrast, the N-terminal five stranded β-barrels of aspartyl-tRNA synthetase are used for anticodon recognition [14]. These data suggest that there should be many different ways for ARSs to specifically interact with the anticodon loop of tRNA.

In this study, we used two random RNA libraries of different size and sequence context to explore the structural motifs that are important for the specific interaction with methionyl-tRNA synthetase (MRS) isolated from a pathogenic microorganism, *Mycobaterium tuberculosis*. MRS consists of two distinct domains; the N-terminal domain is involved in the catalysis and acceptor stem interaction [15–17], whereas the C-terminal domain is responsible for the interaction with the CAU anticodon, which is the major recognition site [18]. This domain contains a helix and loop in which a highly conserved tryptophan is located. This tryptophan is critical for the specific interaction with the $tRNA^{Met}$ anticodon [8,19]. We have previously isolated the MRS structural gene from *M. tuberculosis* [20]. Its overall sequence and structure are homologous to the *Escherichia coli* MRS with 42% similarity and the enzyme cross-reacts with *E. coli* $tRNA^{Met}$ [20]. Nevertheless, we selected RNA molecules that could distinguish the two

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homologous MRSs by *in vitro* evolution and their sequence characteristics were analyzed.

2. Materials and methods

2.1. Preparation of methionyl-tRNA synthetase

M. tuberculosis and *E. coli* MRSs were expressed as a glutathione *S*-transferase (GST) fusion protein in *E. coli*. The structural genes for *M. tuberculosis* and *E. coli* MRSs were cloned into the *Bam*HI-*Eco*RI sites of pGEX4T-2 and the *Eco*RI-*Xho*I sites of pGEX4T-1, respectively. The fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) following the manufacturer's instructions. The purity of the GST-fused MRS was determined by SDS gel electrophoresis (data not shown).

2.2. Preparation of random sequence RNA libraries

The DNA templates were designed to generate RNA libraries containing 42mer and 70mer random sequences. The random 70mer sequence was flanked by the fixed sequences containing the T7 promoter and the *Sac*I site at the 5′ end and the *Bam*HI site at the 3′ end. The random 42mer template was also designed to contain the *Hind*III site and T7 promoter at the 5′ end and the *Bam*HI site at the 3′ end. Each of the oligonucleotides was made with double-stranded DNA and Klenow fragments.

Each of the RNA libraries was generated from the DNA templates by *in vitro* transcription. The reaction included 1–2 μg of the template DNAs in 8 mM Tris–HCl, pH 7.9, 1.2 mM $MgCl_2$, 0.4 mM spermidine and 2 mM NaCl containing 8.3 mM of each NTP in 20 μl, and was incubated in the presence of T7 polymerase (40 units, Ribomix, Promega) at 37 °C for 3 h. The template DNA was removed by the treatment of RNase-free DNase I (Promega) at 37 °C for 15 min. The resulting RNAs were separated from the reaction mixture by phenol extraction and a G-50 quick spin column (Boehringer Mannheim) followed by precipitation with ethanol. The quality of the RNA preparation was determined by running the sample on a denaturing polyacrylamide gel.

2.3. Selection of RNA aptamers

Different amounts of the purified *M. tuberculosis* MRS fused to GST (GST-MRS) or GST were added to 0.1 ml glutathione conjugated to Sepharose 4B and the beads were packed into a Spin-X column (Corning). The columns were then pre-equilibrated with a binding buffer containing 4 mM HEPES, pH 8.0, 80 mM ammonium acetate, 2 mM magnesium acetate, 0.002% NP-40 and 1% glycerol. The original RNA libraries (10 μg) were heated at 75 °C for 5 min, cooled down and then passed through the GST column to remove the RNA molecules that bound to GST. The eluted RNAs were loaded to the GST-MRS column. The RNAs that were not bound to GST-MRS were washed off with the binding buffer. The RNAs bound to GST-MRS were eluted with 15 mM reduced glutathione, and the RNAs were precipitated with ethanol. The precipitated RNAs were used as a template to generate cDNAs. Reverse transcription was initiated by adding avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, 25 units) to the reaction buffer containing 50 mM Tris–HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl and 1 mM DTT and incubated at 42 °C for 1 h. The cDNA was mixed with the specific primers (forward, 5′-CACTATAGGGGAGCTCGG-3′; reverse, 5′-AAGGATCCTCTGCAAAGC-3′) and amplified by polymerase chain reaction (PCR) with Taq polymerase in 10 mM Tris–HCl, pH 8.3, 50 mM KCl and 0.1 mg/ml gelatin. The amplified DNAs were first separated from the reaction mixture by phenol extraction and then purified using PCR product purification kit (Boehringer Mannheim). The amount of DNA obtained was determined by spectrophotometry at 260 nm and used for *in vitro* transcription. For the analysis of the aptamer sequences, the amplified 70mers were directly ligated into pGEM-T vector (Promega), and the 42mers were cut with *Hind*III and *Bam*HI and cloned to pBluescript II KS(+) (Stratagene). The plasmids were transformed into *E. coli* DH5α and re-isolated to determine the sequences of the inserted aptamers. The secondary structures of the selected RNA aptamers were predicted using the Mfold program [21].

2.4. Filter binding assay and determination of binding affinity

The radioactively labeled RNA aptamers were prepared by *in vitro* transcription at 37 °C for 90 min with T7 RNA polymerase in a reaction buffer containing 0.5 mM (A, G, U)TP, 12 μM CTP, [α-^{32}P] CTP 10 μCi (Amersham Pharmacia Biotech) in the presence of RNase inhibitor (RNasin 20 units, Promega). After removing DNA with RNase-free DNase I, the remaining RNAs were separated by phenol extraction and ethanol precipitation. The radioactive RNAs (0.75 nM) were mixed with different amounts of GST or MRS-GST of *M. tuberculosis* and *E. coli* in binding buffer as described above and incubated at 37 °C for 15 min. Then, the mixtures were loaded on a Hybond-C nitrocellulose membrane using a 96-well vacuum manifold filtration apparatus. The unbound RNAs were removed by vacuum suction and the filters were washed with binding buffer. The RNAs retained in the filter were quantified using a phosphor image analyzer (FLA-3000, Fuji). The data were used to make a Scatchard plot with $r = [\text{RNA-MRS}]/[\text{RNA}]_{\text{total}}$ on the *x*-axis and $r/[\text{MRS}]_{\text{total}}$ on the *y*-axis. The dissociation constant was determined from the slope of the plot.

2.5. Aminoacylation assay

The aminoacylation reaction of the *M. tuberculosis* or *E. coli* MRS was carried out in 20 mM HEPES buffer, pH 7.5, 0.1 mM EDTA, 150 mM NH_4Cl, 100 μg/ml BSA, 2 mM ATP, 4 mM $MgCl_2$, 20 μM [^{35}S] methionine, 4 μM *E. coli* total tRNA (Sigma). To determine the effect of the RNA aptamers on aminoacylation activity, the indicated amounts of RNAs were added to the aminoacylation reaction mixture containing 30 nM *M. tuberculosis* MRS or 3 nM *E. coli* MRS. The reaction was sampled at specific time intervals and spotted onto 3MM filter paper pre-soaked with 10% trichloroacetic acid containing 1 mM methionine. The filters were washed with cold 5% trichloroacetic acid containing 1 mM methionine and then with 95% ethanol. The amount of charged tRNA was determined by counting radioactivity on the dried filters using a liquid scintillation counter (Wallac 1409 DSA).

3. Results

3.1. Selection of RNA aptamers

Two different random 42mer and 70mer RNA libraries were used for the *in vitro* selection experiments. The DNA template used to prepare the random 70mer RNAs contained a T7 promoter sequence and *Sac*I site at the 5′ end and *Bam*HI site at the 3′ end (Supplementary Fig. 1). *In vitro* transcription of this template generated a 111mer (random 70mer flanked by the fixed sequences of 21mer and 20mer at the 5′ and 3′ ends, respectively). Another template for the random 42mer RNA contained *Hind*III site and the T7 promoter at the 5′ end and the *Bam*HI site at the 3′ end. The *in vitro* transcript from this template produced the 96mer containing the random

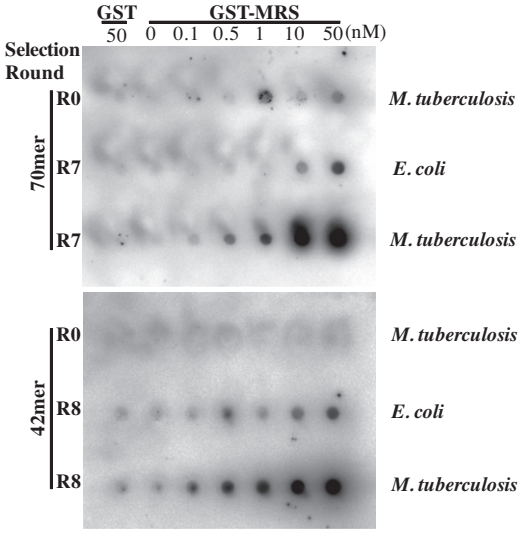

Fig. 1. Specific binding of the selected RNA aptamers to *M. tuberculosis* MRS. The original RNA library (R0) and the RNA aptamers obtained after 8 (R8) and 7 rounds (R7) of selection were radioactively labeled and mixed with the indicated concentrations of GST and GST-MRS of *M. tuberculosis* or *E. coli*. The mixture was filtered through nitrocellulose membrane, and the RNAs bound to each of the tested proteins were observed by autoradiography.

42mer sequence flanked by the fixed sequences of the 21mer and 33mer at the 5′- and 3′-ends, respectively.

The target protein, *M. tuberculosis* MRS, was expressed as GST-MRS and purified to homogeneity using glutathione affinity chromatography. Each of the RNA libraries was passed through a GSTS epharose column to remove RNAs bound to GST. The RNAs in the flowthrough were then loaded to GST-MRS Sepharose. The GST-MRS/RNA complex was eluted with reduced glutathione. The RNAs were then separated from GST-MRS by phenol extraction and amplified by PCR with specific primers. The amplified DNAs were subjected to *in vitro* transcription using T7 RNA polymerase and the resulting RNAs were used again for the next round of selection. The selection cycles were repeated 7 and 8 times for the 70mer and 42mer libraries, respectively, and the selected RNAs were used for further experiments.

3.2. Specific interaction of the selected aptamers with methionyl-tRNA synthetase of M. tuberculosis

The selected aptamers were compared with their original library for binding affinity and specificity to the target protein. The *M. tuberculosis* and *E. coli* MRSs were purified as GST fusion proteins. The selected aptamers were prepared after eight and seven rounds of selection for the 42mer and 70mer, respectively, and synthesized in the presence of [α-^{32}P] CTP. The original library and the selected aptamers were bound to GST or GST-MRSs of *M. tuberculosis* and *E. coli*, and binding was monitored by a filter binding assay. The selected 70 and 42mer aptamer pools bound to *M. tuberculosis* MRS in a dose-dependent manner (Fig. 1). In contrast, the selected aptamers did not bind to GST alone or showed poor affinity to *E. coli* MRS. The dissociation constants of the selected 70 and 42mer pools to *M. tuberculosis* MRS were 38.8 and 51.3 nM, respectively. We then tested whether the selected aptamers could specifically inhibit aminoacylation activity of the *M. tuberculosis* MRS. The selected aptamers both inhibited the catalytic activity of *M. tuberculosis* MRS, whereas *E. coli* MRS activity was little affected by addition of the selected aptamers (Fig. 2A and B). These data suggest that the selected aptamers could distinguish the two homologous prokaryotic MRSs.

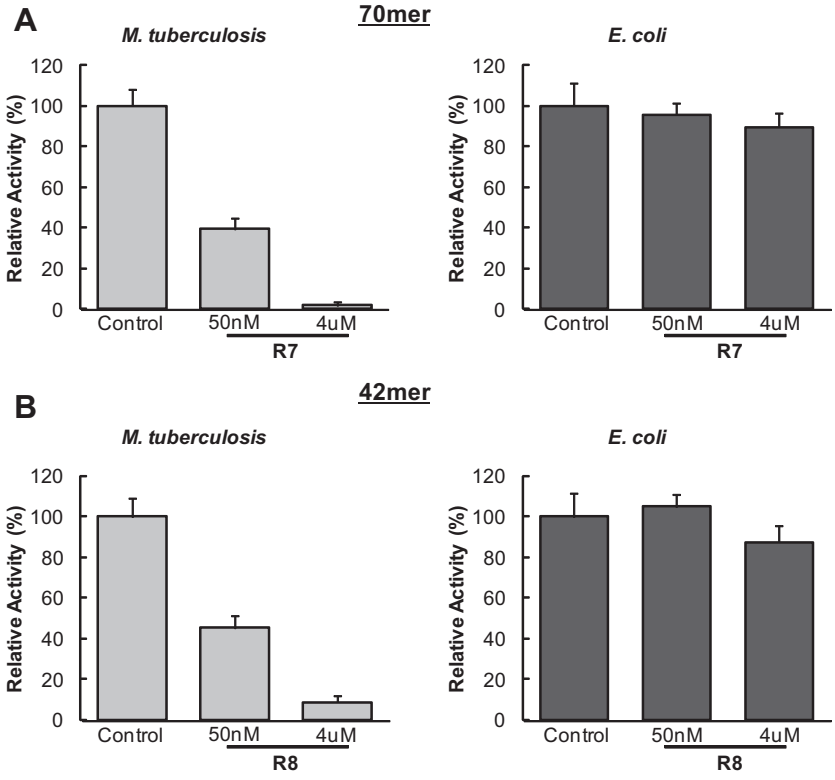

Fig. 2. The effect of the selected RNA aptamers on the aminoacylation activities of *M. tuberculosis* and *E. coli* MRSs. The indicated amounts of the selected RNAs from 70mer (A) and 42mer (B) were mixed with MRS of *M. tuberculosis* or *E. coli* and their effect on the aminoacylation activity was compared.

3.3. Sequence and binding characteristics of the selected aptamers

We then determined the sequences of the selected aptamers to examine whether the structural and sequence features important for specific recognition of *M. tuberculosis* MRS could be elucidated. A total of 29 and 18 RNAs were selected from the 70mer and 42mer libraries for sequencing, respectively. Although none of the selected 70mer RNAs were identical, they were classified into five

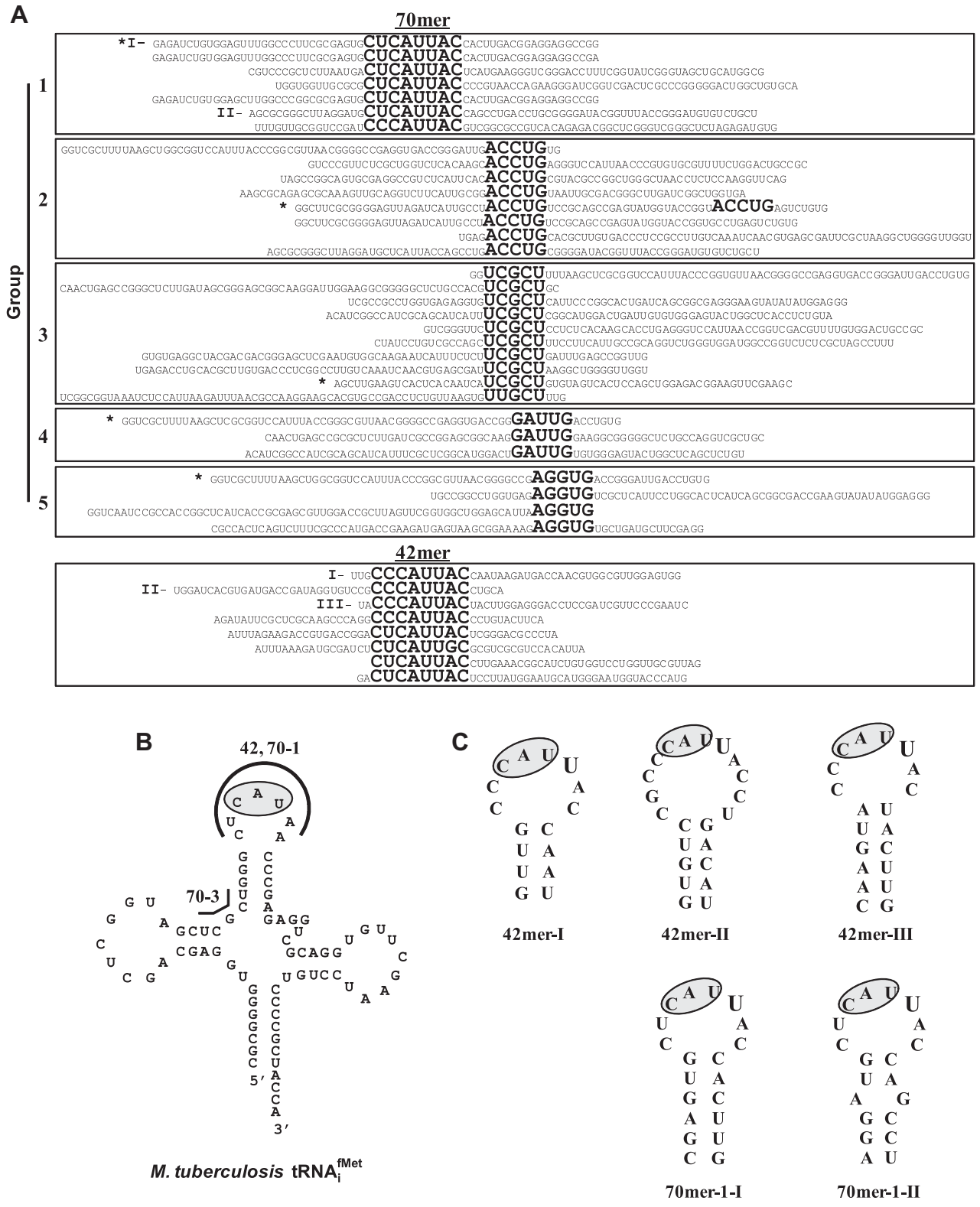

Fig. 3. Sequences and stem loop structure of the selected RNAs. (A) The selected RNA aptamers were grouped based on the consensus sequence motifs. While only one conserved motif was found in the selected 42mer RNAs, five different sequence motifs were identified from the 70mer pool. The conserved sequences are highlighted with large bold letters. (B) The locations of the conserved motifs found in the selected 70mer group 1 and group 3 RNAs are marked with lines in tRNA^{Met}. (C) The Met anticodon triplet CAU is highlighted with a gray oval in tRNA and the predicted RNA loop.

Table 1
K_d (nM) value of the selected aptamers.

Aptamers	*M. tuberculosis*	*E. coli*
70mer-1	11.2 ± 1.1	NB*
70mer-2	NB	NB
70mer-3	44.6 ± 3.8	NB
70mer-4	NB	NB
70mer-5	384.6 ± 5.9	344.8 ± 7.8
42mer-1	54.9 ± 4.3	NB

* NB: no binding at up to 500 nM of MRS.

different groups based on the conserved sequence motifs. In contrast, all of the selected 42mers contained only one type of the conserved sequence motif although their locations within the sequence varied (Fig. 3A).

To investigate whether the selected aptamers showed similar binding characteristics, one representative RNA was selected from each group (Fig. 3A, marked ∗) to determine its dissociation constant for *M. tuberculosis* and *E. coli* MRSs. Among the five tested RNAs, group 1 and 3 RNAs showed dissociation constants of 11.2 and 44.6 nM for *M. tuberculosis* MRS, respectively, whereas they did not bind to *E. coli* MRS (Table 1). In contrast, group 2 and 4 RNAs bound to neither of the two MRSs, whereas group 5 RNA bound to both with similar binding constants. These results suggest that the selected 70mer pool consisted of RNA species with different binding characteristics although the majority of them would prefer to bind *M. tuberculosis* MRS. The RNA sampled from the 42mer pool bound to the *M. tuberculosis* MRS with a dissociation constant of 54.9 nM but did not bind to the *E. coli* MRS. In addition, the dissociation constant using the pool of selected 42mer RNAs showed a similar result (data not shown). Thus, these results suggest that the RNAs of the selected 42mer pool are more homogeneous compared to the RNAs of the selected 70mer pool in the binding to the *M. tuberculosis* MRS.

Interestingly, group 1 RNAs of the 70mer pool showed the highest binding affinity to *M. tuberculosis* MRS and contain the C(U/C)CAUUAC octamer motif that was also found in the 42mer pool. This sequence motif was homologous to that located in the $tRNA^{Met}$ loop containing the CAU anticodon (Fig. 3B). Thus, we estimated the secondary structures based on the sequences of the selected aptamers containing this motif. The conserved motifs containing the CAU methionine anticodon, were localized in the loop region, which is similar to the $tRNA^{Met}$ anticodon loop (Fig. 3C). These results suggest that this loop in the selected aptamers could bind to the *M. tuberculosis* MRS in a similar manner to the $tRNA^{Met}$ anticodon loop.

4. Discussion

In vitro genetic selection of RNA has been used to isolate RNA aptamers with desired biological activities or to determine the sequence or structural motifs that are important for an interaction with target molecules [22–24]. This technique has been also applied to identify the RNA motifs or structures involved in specific interactions with tRNA synthetases [25–28]. Here we designed two random RNA libraries that did not have any sequence or structural similarity to tRNA and investigated whether a common RNA motif could be identified and whether the selected aptamers could distinguish the two homologous enzymes.

The two RNA libraries differed not only in the length of the random sequence region but also in the sequence and length of the fixed flanking region (Supplementary Fig. 1). Nevertheless, 7–8 rounds of iterative selection and amplification of these libraries against *M. tuberculosis* MRS enriched the target-binding RNA aptamers (Figs. 1 and 2). Both showed higher affinity to *M. tuberculosis* MRS than to *E. coli* MRS despite that the two enzymes are homologous. The selected aptamers might have explored the subtle structural difference between MRSs of the two species. The selected aptamers showed higher affinity than the cognate tRNA to the enzyme with a sub-micromolar range dissociation constant. Thus, these results suggest the possibility that RNA molecules with higher specificity and affinity to the target enzyme than tRNA could be generated by *in vitro* evolution.

Among the aptamers with several different sequence motifs in the selected 70mer RNAs (Fig. 3), the group 1 RNAs containing the conserved octamer showed the highest affinity to *M. tuberculosis* MRS (Table 1). The conserved sequence in group 1 was also found in the selected 42mer pool (Fig. 3A). Interestingly, the six bases in the conserved octamer sequence were identical to those in the $tRNA^{Met}$ anticodon loop (Fig. 3B and C). As MRS interacts with the $tRNA^{Met}$ anticodon, conservation of a similar sequence in the selected RNAs suggests that the aptamers could bind to MRS in a manner similar to the $tRNA^{Met}$ in this region. This possibility was supported by the observation that the group 3 RNAs also contained the conserved sequence present in $tRNA^{Met}$ (Fig. 3B). Thus, they are also expected to interact with MRS in a tRNA mode using this conserved region.

Interestingly, the conserved octamer sequence contained U next to CAU anticodon, whereas all of the initiator and elongator $tRNAs^{Met}$ of 18 different species contained A and G at this position (Fig. 3). To examine whether the conserved octamer was sufficient for the specific interaction with *M. tuberculosis* MRS and to determine whether U next to CAU actually plays a critical role in the specific interaction with *M. tuberculosis* MRS, we synthesized the sis-base RNA stem containing the octamer loop of CUCAUUAC or CUCAUAAC. The binding affinity and specificity of these synthetic RNAs were greatly decreased compared to that of the full-size selected aptamers (data not shown). These results suggest that the octamer sequence alone is not sufficient to maintain affinity to MRS. In addition, U next to CAU did not appear to be important for the species-specific interaction with *M. tuberculosis* MRS. Perhaps, the strong selection pressure for U next to CAU may be due to the difference in the position of CAU in the RNA aptamers and $tRNA^{Met}$ loops. While CAU in the aptamers is in one side of the loop, it is located at the center of the loop in the $tRNA^{Met}$. Perhaps, U is necessary to adjust CAU for the interaction with MRS. Several representative RNAs (group 1 of the 70mer and 42mers) were expected to form a long linear stem that did not look like the native tRNA (data not shown). Based on these results, it is likely that the conserved tRNA-like octamer makes a major contribution to the specific interaction with MRS, whereas the extra affinity and specificity may be added from the remainder of the conserved motif.

It is interesting that the same sequence motif homologous to the native $tRNA^{Met}$ anticodon loop was selected from the two different RNA pools, which are totally unrelated to the native tRNA. This result suggests a strong intrinsic selection pressure for the RNA loop containing CAU to make a specific interaction with MRS regardless of the sequence or structural context. It is thought that the specificity of tRNA recognition is determined by co-adaptation of tRNA synthetases and their cognate tRNAs [1]. We have previously shown that the anticodon-binding peptide in MRS accommodates multiple amino acid substitution without losing the ability to interact with $tRNA^{Met}$ as long as a few conserved amino acid residues are retained [19,29]. Thus, the results of this study show that the specific interaction between tRNAs and their cognate enzymes is highly localized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.116.

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