Three G·C Base Pairs Required for the Efficient Aminoacylation of tRNA^{Trp} by Tryptophanyl-tRNA Synthetase from *Bacillus subtilis*[†]

Feng Xu,[‡] Ge Jiang,[‡] Wei Li,[‡] Xinxia He,[‡,§] Youxin Jin,[*,‡] and Debao Wang[‡]

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China, and Zhejiang Normal University, Jinhua, Zhejiang 321004, China

ABSTRACT: Acceptor stem is an essential region in the recognition of tRNAs by their cognate aminoacyl-tRNA synthetase. In this study, a library containing 20 nt random region and tryptophanyl-tRNA synthetase (TrpRS) from *Bacillus subtilis* were used for in vitro selection to find a new structural feature in the tRNA^{Trp} acceptor stem sequence that is required for *B. subtilis* TrpRS recognition. After three rounds of selection, the TrpRS binding RNAs dominate the RNA pool. The aptamers share a common structure of three G·C base pairs, which was also found in the acceptor stem of wild-type *B. subtilis* tRNA^{Trp}. A series of tRNA^{Trp} variants was prepared by in vitro transcription, and their efficiencies of tryptophanylation (k_{cat}/K_M) were measured with the aid of TrpRS from *B. subtilis*. The mutants that possess the three G·C base pairs and G73 discriminator base exhibit almost the same aminoacylation efficiencies as *B. subtilis* tRNA^{Trp}, while the G73 discriminator base itself cannot confer efficient aminoacylation to the tRNA^{Trp} molecule. Thus, these three base pairs (G2·C71, G3·C70, and G4·C69) in the *B. subtilis* tRNA^{Trp} acceptor stem were established to be new identity elements, and their importance was between the previously characterized major element G73 and minor elements A1/U72 and G5/C68. The minimum set of identity elements that is required to confer efficient aminoacylation by *B. subtilis* TrpRS included G73, G2·C71, G3·C70, and G4·C69.

The translation of genetic information into proteins is essential for a proper biochemical functioning of the cell. The aminoacyl-tRNA synthetases (aaRS),[1] responsible for the specific selection of amino acids in the aminoacylation of tRNA, play a crucial role in this process (*1*). The aminoacyl-tRNA synthetase family can be divided into two groups primarily on the basis of the shared structural motifs in their catalytic domains (*2*). The tryptophanyl-tRNA synthetase (TrpRS) from *Bacillus subtilis* belongs to the class I aaRS family. It has an α_2 subunit structure and a molecular weight of 77000 (*3*).

A synthetase can recognize cognate tRNA(s) by a limited number of nucleotide residues (identity determinants) distributed within the tRNA structure. The crystal structures of tRNA−synthetase complexes have provided us with a precise view of the interactions between the identity determinants and their protein counterparts (*4−6*). As there are 60−70 similar L-shaped tRNA molecules in a cell, each tRNA structure must possess enough identity elements to permit

the cognate synthetase to aminoacylate the tRNA efficiently and to reject the 19 noncognate synthetases.

The identity elements of *B. subtilis* tRNA^{Trp} were characterized previously with in vitro aminoacylation assays (*7*). The identity elements recognized by *B. subtilis* tryptophanyl-tRNA synthetase were established to be the following: major elements, discriminator base G73 and anticodon; minor elements, A1/U72, G5/C68, and A9, in decreasing importance. The nonanticodon identity elements are mainly located in the acceptor stem of the *B. subtilis* tRNA^{Trp} sequence.

Recently, from another work in our laboratory, we observed that the aminoacylation efficiency of a human tRNA^{Trp} mutant was only 14% of wild-type *B. subtilis* tRNA^{Trp} when assayed by *B. subtilis* TrpRS (*8*), despite the fact that all of its previously characterized identity elements in acceptor stem were switched to those of *B. subtilis* tRNA^{Trp}. On the basis of this observation, we proposed that there are more elements that are required for *B. subtilis* TrpRS recognition distributed within tRNA^{Trp}. To confirm this notion, the SELEX (systematic evolution of ligands by exponential enrichment) method (*9, 10*) was employed in this work using immobilized *B. subtilis* TrpRS as the target. Because at least 11 tRNA synthetases can charge minihelices on the basis of the sequences of the acceptor stems of their cognate tRNAs (*11−14*) and the previously characterized identity elements which are mainly located in the acceptor stem of the *B. subtilis* tRNA^{Trp} sequence, we consider that the acceptor stem of tRNA^{Trp} plays an essential role in the recognition by *B. subtilis* TrpRS. To give an extensive
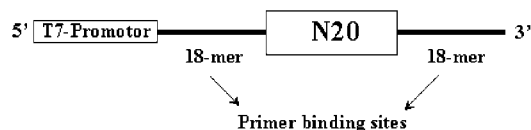
FIGURE 1: Design of the random 56-mer oligonucleotide used in the in vitro selection. N20 indicates a complete randomization over 20 nt positions. For primer sequences and restriction sites see Materials and Methods.

examination of the acceptor stem sequence, we designed a random library for in vitro selection. The 20 nt random region is long enough to mimic the acceptor stem sequence of tRNA[Trp].

MATERIALS AND METHODS

Plasmids and Reagents. The plasmid pKSW1 (*15*) that directs the overexpression of *B. subtilis* TrpRS was a kind gift from Dr. Xue Hong. Restriction endonucleases, RQ1 RNase-free DNase, the pGEM-T vector system, T4 DNA ligase, the *fmol* DNA sequencing system, and the RiboMAX large-scale RNA production system T7 were from Promega (Madison, WI). L-[5-³H]Tryptophan, [γ-³²P]ATP, [α-³²P]-UTP, Sepharose 4B, CNBr-activated Sepharose 4B, and DEAE-Sepharose CL-6B were from Amersham Pharmacia (Piscataway, NJ). L-Tryptophan was from Gibco BRL (Gaithersburg, MD). The mRNA selective PCR kit (version 1.1), EX Taq DNA polymerase, and RNasin were from Takara (Tokyo, Japan).

Enzyme Purification and Immobilization. B. subtilis TrpRS was purified to homogeneity as described previously (*15*) and immobilized on CNBr-activated Sepharose 4B as described in the instructions of the manufacturer.

Pool Construction. DNA pool and all primers were synthesized on a Beckman Oligo-1000 DNA synthesizer and purified by polyacrylamide gel electrophoresis under denaturing conditions. The synthesized DNA pool (Figure 1) included a cassette of 20 completely randomized nucleotides flanked by a set of primer sequences (5′-TAA TAC GAC TCA CTA TAG GGA ATT CTA TCC GGG TA-(N20)-CGA TCC AAA GCT TCG GCA- 3′; 3′ primer, 5′-TGC CGA AGC TTT GGA TCG-3′; 5′ primer, 5′-TTA TAC GAC TCA CTA TAG GGA ATT CTA TCC GGG TA-3′; the T7 promoter sequence is underlined).

In Vitro Selection. The synthetic ssDNA library was first PCR amplified to yield the dsDNA library, which was used as the template of in vitro transcription. The resulting RNA pool was subjected to three rounds of in vitro selection. All cycles of selection and amplification were performed as follows: gel-purified, α-³²P-labeled pool RNA was renatured by preincubation for 2 min at 70 °C and slowly cooled to room temperature. The annealed RNA pool was then incubated with immobilized TrpRS in a molar ratio of 10:1 (RNA:TrpRS) for 30 min with rotation at 22 °C in binding buffer (4 mM ATP, 1 mM DTT, 40 mM magnesium acetate, 140 mM Tris-HCl, pH 7.8, 400 units/mL RNasin). Following incubation, unbound RNA was removed by washing with binding buffer, and RNA bound to the protein was recovered by phenol extraction. Glycogen (20 μg) was added to the recovered RNA as a precipitation support. The RNA was ethanol precipitated, resuspended in TE buffer, and subjected to reverse transcription. The cDNA was PCR amplified, and the PCR−DNA was subjected to T7 in vitro transcription

to yield the RNA pool for the next selection cycle. To expedite the selection process, the selection stringency was increased in cycles 2 and 3 by changing the molar ratio of RNA:TrpRS to 30:1 and 100:1, respectively. The selection progress was monitored by pool binding activity assay. 5′-³²P-labeled pool RNA (0.1 μM) from one, two, or three cycles of selection and 0.1 μM longer 5′-³²P-labeled wild-type *B. subtilis* tRNA[Trp] (total length of 74 nt) were allowed to compete for binding to 10 nM immobilized *B. subtilis* TrpRS. After incubation for 30 min with rotation at 22 °C, unbound RNA was removed by washing with binding buffer, and bound RNA was phenol extracted and ethanol precipitated. Then the bound RNA was redissolved and subjected to electrophoresis on a 10% denaturing polyacrylamide gel. 5′-³²P-labeled pool RNA (0.1 μM) from one, two, or three cycles of selection was also incubated with Sepharose 4B as described above, and bound and unbound pool RNAs were recovered and counted by Wallac 1490 (Pharmacia) to calculate the binding ratio of pool RNA to Sepharose 4B. After three rounds of selection, the enriched RNA pool and initial RNA pool were reverse transcribed and sequenced following the protocol of the *fmol* DNA sequencing system (Promega, WI).

RNA Preparation. All of the tRNA variants were prepared by in vitro transcription (*16*). Mutagenesis of tRNAs was accomplished as described previously (*18*). All of the desired mutations were verified by sequencing. The transcripts were purified by 10% polyacrylamide gel electrophoresis under denaturing conditions. Before aminoacylation assay, transcripts were heated at 70 °C for 2 min and slowly cooled to room temperature.

Enzyme Activity Assay. B. subtilis enzyme was assayed at 22 °C in an aminoacylation mixture containing 4 mM ATP, 1 mM DTT, 1 μCi of L-[5-³H]tryptophan, 40 mM magnesium acetate, 140 mM Tris-HCl, pH 7.8, and 0.02 μM in vitro transcribed tRNA[Trp], in a total volume of 50 μL (*17*, *18*). One unit of aminoacylation activity was defined as the amount of enzyme needed to charge 1 pmol of tRNA[Trp] per minute under the assay condition. The kinetic constants were derived from Lineweaver−Burk plots.

RESULTS

Purification and Immobilization of B. subtilis TrpRS. B. subtilis TrpRS was overexpressed in *Escherichia coli* and purified to homogeneity. The purity of the enzyme was above 95% as determined by gel scan (Figure 2).

After the purification procedure, the enzyme was immobilized on CNBr-activated Sepharose 4B following the instructions of the manufacturer. About 0.5 mg of TrpRS was immobilized on 1 mL of CNBr-activated Sepharose 4B slurry. The specific activity of the immobilized TrpRS was 1670 units/mL.

In Vitro Selection. RNA molecules that bind to *B. subtilis* TrpRS were selected from an RNA pool of approximately 10¹² different sequences consisting of a 20 nt long random region and two flanking constant regions. A batch procedure was used in this work because it is proved to be advantageous in achieving specific binding to immobilized TrpRS. As compared to affinity chromatography with RNA in the mobile phase and TrpRS on the Sepharose matrix, the batch procedure allows a better control of volume for equilibration
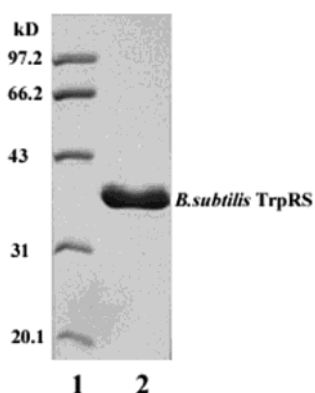
FIGURE 2: SDS−PAGE of purified TrpRS from *B. subtilis*. Electrophoresis was on a 0.1% SDS−12.5% gel stained by Coomassie blue. Lanes: 1, protein standard markers with molecular masses of 97.2, 66.2, 43, 31, and 20.1 kDa, respectively; 2, purified TrpRS from *B. subtilis*.
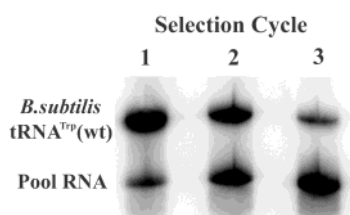

FIGURE 3: Assay of selection progress monitored by pool binding activity. 5′-^{32}P-labeled pool RNA (0.1 μM) from one, two, or three cycles of selection and 0.1 μM longer 5′-^{32}P-labeled wild-type *B. subtilis* tRNA^Trp (total length of 74 nt) were allowed to compete for binding to 10 nM immobilized *B. subtilis* TrpRS. The bound RNA was recovered and subjected to electrophoresis on a 10% denaturing polyacrylamide gel.

and washing and elution steps (*19*). TrpRS-bound aptamers were extracted from the immobilized enzyme, reverse transcribed to cDNA, amplified by PCR, and transcribed in vitro back to RNA to yield an enriched pool that was used as the input for the next selection cycle (*20*).

To monitor the selection progress, an assay of pool binding activity (modified from ref *20*) was performed. The wild-type *B. subtilis* tRNA^Trp was included in the assay as a specific competitor for *B. subtilis* TrpRS binding. Figure 3 shows the enrichment of the radiolabeled RNA pool (56-mer) for specific TrpRS binding in competition with the longer radiolabeled 74-mer wild-type *B. subtilis* tRNA^Trp in each selection cycle. After one round of selection, only a small amount of aptamers from the library that compete with the wild-type *B. subtilis* tRNA^Trp for *B. subtilis* TrpRS binding was detected. By round 2, binders from the library showed stronger binding activity than the wild-type *B. subtilis* tRNA^Trp. These molecules dominated the population after round 3. Two further rounds failed to produce any noticeable change of the binding activity as assayed by this method (data not shown). Meanwhile, the binding ratios of pool RNA from cycles 1, 2, and 3 to Sepharose 4B were all below 0.1%, which indicate that the pool RNA could not bind to Sepharose 4B specifically.

Sequence and Secondary Structure of the Selected RNAs. After three rounds of selection, the initial RNA pool and the third-round enriched RNA pool were reverse transcribed to cDNA and sequenced using the *fmol* DNA sequencing system (Promega, WI). Figure 4 shows the sequences of these
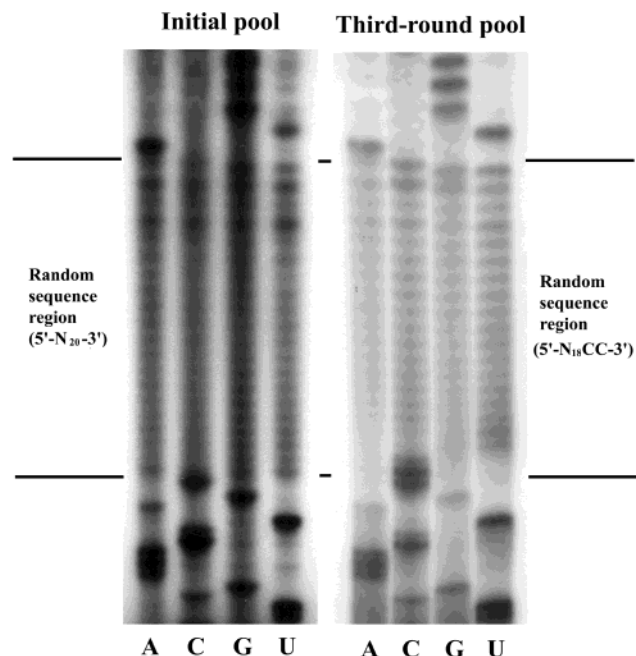

FIGURE 4: Sequence comparison between the initial pool and the third-round enriched RNA pool. Note that the last two nucleotides of the random sequence region evolved to be cytosines after three rounds of selection.
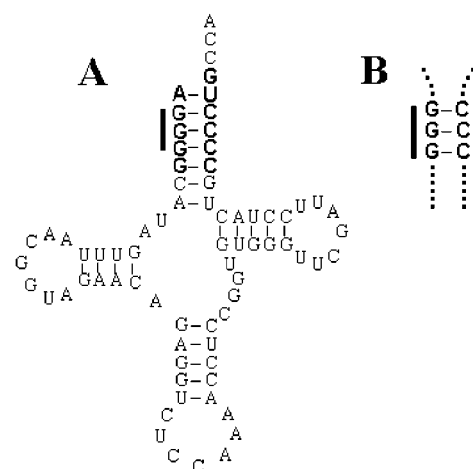

FIGURE 5: Secondary structure of *B. subtilis* tRNA^Trp and the conserved three G·C base pairs in the third-round enriched RNA pool. (A) Secondary structure of *B. subtilis* tRNA^Trp; the previously characterized identity elements are in a bold font. (B) The three conserved G·C base pairs in the third-round enriched RNA pool. Two cytosines, which were selected from the random sequences, are in a bold and italic font. Three G·C base pairs, which were found to be important in the selection, are indicated by a solid line.

two pools. The sequence of the initial pool shows no bias among the four nucleotides, A, C, G, and U, in the 20 nt randomized region. After three rounds of selection, an apparent bias to cytosine at the last two nucleotides of the random sequence region could be seen. Thus, a consensus sequence 5′-N$_{18}$CC-3′ was detected after three cycles of in vitro evolution. These two cytosines, together with another C presented in the 3′ constant region, could form three G·C base pairs to three G in the 5′ constant region (Figure 5B). Meanwhile, the wild-type *B. subtilis* tRNA^Trp has the same structure of these three base pairs (G2·C71, G3·C70, and G4·C69) in the acceptor stem (Figure 5A). On the basis of this observation, we propose that the three G·C base pairs
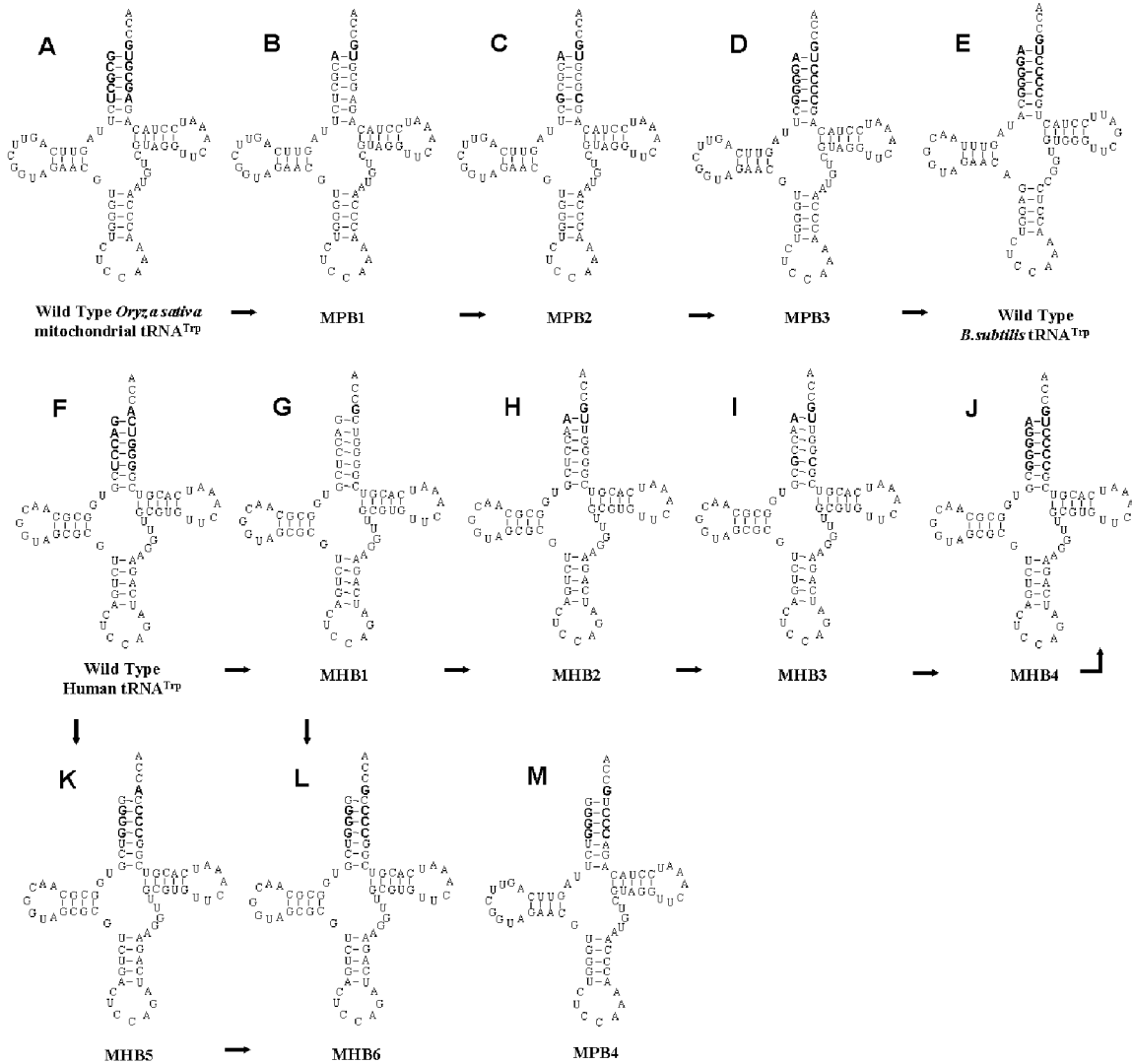
FIGURE 6: Secondary structure of the mutants and wild-type tRNA^{Trp}s. Mutated elements and their counterparts in wild-type tRNA^{Trp} are in a larger and bold font.

are an important structural feature that is required for the recognition by *B. subtilis* TrpRS. And then, we want to ask whether these base pairs are the new identity elements required for efficient aminoacylation by *B. subtilis* TrpRS.

Three G·C Base Pairs Required for Aminoacylation by B. subtilis TrpRS. Transfer RNA molecules prepared by in vitro transcription do not contain the modified nucleotides found in natural tRNAs. Despite this fact, most tRNA transcripts are efficient substrates for their cognate synthetase (*21−23*). In the *B. subtilis* TrpRS system (*7*), modified bases do not appear to contribute significantly to aminoacylation by *B. subtilis* TrpRS. Therefore, using in vitro transcribed tRNA as substrate, the aminoacylation activities of *B. subtilis* TrpRS could be evaluated.

Because the random pool RNA has no single-stranded CCA$_{OH}$ sequence at the 3′ end, it is not suitable for the aminoacylation assay. To test the importance of the three G·C base pairs in the aminoacylation of tRNA^{Trp}, two sets of single and multiple mutations were generated and assayed using *B. subtilis* TrpRS. One set of mutations is based on a mitochondrial tRNA^{Trp} from *Oryza sativa,* and another is based on human tRNA^{Trp} (Figure 6). Aminoacylation results (decrease in k_{cat}/K_M relative to that of the wild-type *B. subtilis* tRNA^{Trp}) are shown in Table 1. The individual kinetic

Table 1: Effect of Single and Multiple Nucleotide Mutations on Aminoacylation of tRNA^{Trp} Transcripts by *B. subtilis* TrpRS[a]

variant	k_{cat}/K_M (relative)	change in efficiency (x-fold)	variant	k_{cat}/K_M (relative)	change in efficiency (x-fold)
WTB	1.0	1.0	MHB1[b]	0.075	−13
WTP	0.0025	−400	MHB2[b]	0.11	−9.1
MPB1	0.0045	−222	MHB3[b]	0.14	−7.1
MPB2	0.013	−77	MHB4	0.95	−1.1
MPB3	0.87	−1.2	MHB5	0.05	−20
MPB4	0.42	−2.4	MHB6	0.48	−2.1
WTH[b]	0.0076	−132			

[a] The specificity constant (k_{cat}/K_M) for aminoacylation of tRNA^{Trp} variant transcripts was derived from a Lineweaver−Burk plot. k_{cat}/K_M is given relative to that of the wild-type *B. subtilis* tRNA^{Trp} transcript aminoacylated by *B. subtilis* TrpRS, which was set at 1.0. Concentrations of tRNA^{Trp} ranging from 0.1 to 20 μM were used, and the TrpRS concentration was 25, 50, or 100 nM. [b] These data were from ref *8*.

parameters measured for the wild-type tRNA were as follows: *B. subtilis* tRNA^{Trp} aminoacylated by *B. subtilis* TrpRS, $k_{cat} = 0.69$ s^{-1} and $K_M = 0.56$ μM. *B. subtilis* TrpRS weakly catalyzes the aminoacylation reaction of human tRNA^{Trp}, with a 132-fold decrease in k_{cat}/K_M relative to that of the wild-type *B. subtilis* transcript. A single mutation, A73G, greatly increased the k_{cat}/K_M value of human tRNA^{Trp}

by 10 times (from −132-fold to −13-fold; Figure 6F,G). Additional mutations G1A/C72U and U5G/G68C also had minor positive effects on *B. subtilis* TrpRS recognition (from −13-fold to −9.1-fold and −7.1-fold; Figure 6H,I) (*8*). At last, the multiple mutations of A2G/U71C, C3G/G70C, and C4G/G69C based on MHB3 significantly improved the aminoacylation efficiency by *B. subtilis* TrpRS to almost the same as wild-type *B. subtilis* tRNA^Trp (95% of the wild-type *B. subtilis* tRNA^Trp; Table 1). *O. sativa* mitochondrial tRNA^Trp is also a poor substrate for *B. subtilis* TrpRS, with a 400-fold decrease in aminoacylation efficiency as compared to wild-type *B. subtilis* transcript, despite the fact that it possesses the same discriminator base, G73, as *B. subtilis* tRNA^Trp. Mutations of G1A and U5G/A68C had little positive effects on *B. subtilis* TrpRS aminoacylation (from −400-fold to −222-fold and −77-fold; Figure 6B,C). The substitutions of C2G/G71C and C4G/G69C based on MPB2 greatly improved the aminoacylation efficiency from −77-fold to −1.2-fold as compared to wild-type *B. subtilis* tRNA^Trp (Figure 6C,D).

Although the three G·C base pairs have been proven to be important in the recognition and aminoacylation of tRNA^Trp by *B. subtilis* TrpRS, a subsequent test was carried out to identify whether these base pairs themselves could confer aminoacylation by *B. subtilis* TrpRS. We prepared three mutants for this assay (Figure 6K−M). Mutant MHB5 is not a good substrate for *B. subtilis* TrpRS; its aminoacylation efficiency is only 5% of the wild-type *B. subtilis* tRNA^Trp (Table 1). The substitution of A73G based on MHB5 greatly increased the k_{cat}/K_M value by 10 times (from −20-fold to −2.1-fold; Figure 6K,L). The multiple mutations of C2G/G71C and C4G/G69C based on wild-type *O. sativa* mitochondrial tRNA^Trp also dramatically increased the aminoacylation efficiency from −400-fold to −2.4-fold (Figure 6A,M).

DISCUSSION

Schimmel and Pouplana have argued that the acceptor stem and the anticodon loop of a tRNA molecule are in two domains with distinct function (*24*). The sequences and structures of tRNA acceptor stems can be thought of as an operational "RNA code" that is devoid of the anticodon trinucleotides of the genetic code (*25, 26*). Thus, aminoacylation specificity and efficiency generally depend on the sequences and structures of the acceptor stem (*27−35*).

The acceptor stem of *B. subtilis* tRNA^Trp was proven to be essential in the recognition by its cognate enzyme (*7, 8*). The nonanticodon identity elements are mainly located in the acceptor stem of *B. subtilis* tRNA^Trp (*7*). Substitutions of these identity elements lead to a sharp decrease in the aminoacylation efficiency of *B. subtilis* tRNA^Trp (*7, 8*). These facts establish that the *B. subtilis* TrpRS cannot recognize a tRNA molecule without these identity elements; on the other hand, these identity elements are not sufficient to make a tRNA molecule an excellent substrate for *B. subtilis* TrpRS. From a recent work in our laboratory, we found that a heterozygotic human tRNA^Trp with all of the previously characterized *B. subtilis* tRNA^Trp acceptor stem identity elements could only be aminoacylated to a level of 14% as compared to the wild-type *B. subtilis* tRNA^Trp. This fact indicates the existence of more identity elements distributed

within *B. subtilis* tRNA^Trp. Subsequently, two mutants of MPB1 and MPB2 on the basis of *O. sativa* mitochondrial tRNA^Trp were generated and assayed by *B. subtilis* TrpRS. The results of the aminoacylation assay provided further supports for our proposal. Although wild-type *O. sativa* mitochondrial tRNA^Trp has the same major identity element of G73 as *B. subtilis* tRNA^Trp, it is still a poor substrate for *B. subtilis* TrpRS (0.25% of the efficiency of the wild-type *B. subtilis* tRNA^Trp; Table 1). Mutant MPB2 has all of the previously characterized *B. subtilis* tRNA^Trp identity elements in the acceptor stem, but its aminoacylation efficiency is only 1.3% of the wild-type *B. subtilis* tRNA^Trp (Table 1). On the basis of these experimental data, we designed and constructed a random library for the SELEX experiment, hoping to find new structural features in the acceptor stem that contribute to the recognition of tRNA^Trp by *B. subtilis* TrpRS.

The in vitro selection against immobilized TrpRS continued for five rounds. After three rounds of selection, aptamers that specifically bound to TrpRS dominated the RNA pool (Figure 3). The fourth and fifth rounds failed to produce any noticeable increase of the binding activity. The selection process is rather fast (only three rounds to the highest binding activity). After sequencing the third-round RNA pool, we can provide two explanations to this result. First, we increased the selection stringency by changing the molar ratio of RNA:TrpRS from 10:1 to 30:1 and 100:1, which could expedite the selection process. Second, three G and one C in the selected three G·C base pairs already exist in the 5′ and 3′ constant region; it greatly facilitated the formation of these G·C base pairs and, therefore, accelerated the selection process. The three G and one C in the 5′ and 3′ constant regions are not designed by intention, but the RNA aptamers find them out and utilize them to expedite the evolution procedure.

Because the RNA molecule in the random library has no single-stranded CCA_OH sequence at the 3′ end, it is impossible to be a substrate in the aminoacylation assay. To confirm the importance of the three G·C base pairs in the aminoacylation of tRNA^Trp by *B. subtilis* TrpRS, additional mutants of MPB3 and MHB4 were generated and assayed by *B. subtilis* TrpRS. The aminoacylation efficiencies of MPB3 and MHB4 are almost the same as *B. subtilis* tRNA^Trp (87% and 95% of the *B. subtilis* tRNA^Trp). From the experimental data, we can draw the conclusion that the three G·C base pairs in the acceptor stem are essential to the efficient aminoacylation of tRNA^Trp by *B. subtilis* TrpRS. Other evidence for the three G·C base pairs as an important structural feature involved in the aminoacylation by *B. subtilis* TrpRS arose from *E. coli* tRNA^Trp (*7*). The aminoacylation efficiency of *E. coli* tRNA^Trp by *B. subtilis* TrpRS is 75% of the *B. subtilis* tRNA^Trp for it possesses the three G·C base pairs (G2·C71, G3·C70, and G4·C69) besides the previously characterized identity elements (G73, A1/U72, and G5/C68) of *B. subtilis* tRNA^Trp.

Subsequent aminoacylation assay of MHB5, MHB6, and MPB4 further confirms the importance of these successive G·C base pairs in aminoacylation of tRNA^Trp by *B. subtilis* TrpRS, but their importance could only be manifested on the basis of the G73 discriminator base (Table 1 and Figure 6K−M). Without G73, a tRNA^Trp molecule could not act as an excellent substrate for *B. subtilis* TrpRS. And, at the same time, if a tRNA^Trp molecule does not possess the three G·C
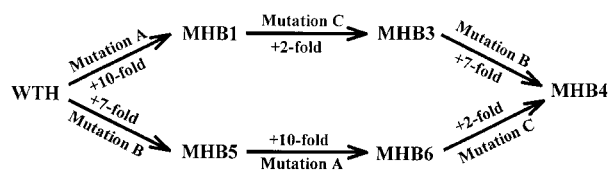
FIGURE 7: Effect of single and multiple nucleotide mutations on aminoacylation of human tRNA^{Trp} variants by *B. subtilis* TrpRS. Aminoacylation values were from this work and ref 8. Mutations A, B, and C were as follows: mutation A, A73G; mutation B, A2G/U71C, C3G/G70C, and C4G/G69C; mutation C, G1A/C72U and U5G/G68C.

base pairs, it could not be efficiently aminoacylated by *B. subtilis* TrpRS too (Table 1 and Figure 6C,I). Thus, we identified the minimum identity element set in the acceptor stem of tRNA^{Trp} that is required to confer efficient aminoacylation by *B. subtilis* TrpRS. This minimum set of identity elements includes G73, G2·C71, G3·C70, and G4·C69.

We searched a tRNA sequence database at http://medlib.med.utah.edu/RNAmods/trnabase/ and obtained 18 different tRNA^{Trp} sequences from bacteria. These 18 sequences were summarized into three groups on the basis of the nucleotides between positions 1−5 and 68−73 in tRNA^{Trp}s. The first group shares consensus sequences of AGGGG and CCCCUG. This group makes up 67% of the total sequences and includes the tRNA^{Trp} from *E. coli* and *B. subtilis*. The second group shares consensus sequences of AGGAG and CUCCUG. The third one shares GGGGG and CCCCCG sequences. Each of the last two groups makes up 16.7% of the total sequences. From the comparison of these sequences, we can find that G2·C71, G3·C70, G5/C68, and G73 are strictly conserved among bacterial tRNA^{Trp} sequences. A1·U72 and G4·C69 are also highly conserved (83.3% of the total sequences). These facts indicate the importance of these base pairs and nucleotides in the recognition of bacterial tRNA^{Trp} by their cognate enzymes.

From our present and previous work (8), we summarized the effects of three kinds of mutations (mutation A, A73G; mutation B, A2G/U71C, C3G/G70C, and C4G/G69C; mutation C, G1A/C72U and U5G/G68C) on aminoacylation of human tRNA^{Trp} variants by *B. subtilis* TrpRS (Figure 7). The effect of mutation B (+7-fold) was between mutation A (+10-fold) and mutation C (+2-fold). From these data, the three G·C base pairs (G2·C71, G3·C70, and G4·C69) were established to be new identity elements, and their importance is between the previously characterized major element G73 and minor elements A1/U72 and G5/C68 in the aminoacylation of tRNA^{Trp} by *B. subtilis* TrpRS.

ACKNOWLEDGMENT

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