Three G•C Base Pairs Required for the Efficient Aminoacylation of tRNA\textsuperscript{Trp} by Tryptophanyl-tRNA Synthetase from \textit{Bacillus subtilis}\textsuperscript{†}

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Received October 23, 2001; Revised Manuscript Received March 5, 2002

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 \textit{Bacillus subtilis} were used for in vitro selection to find a new structural feature in the tRNA\textsuperscript{Trp} acceptor stem sequence that is required for \textit{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 \textit{B. subtilis} tRNA\textsuperscript{Trp}. A series of tRNA\textsuperscript{Trp} variants was prepared by in vitro transcription, and their efficiencies of tryptophanylation (\textit{k}_{\text{cat}}/\textit{K}_M) were measured with the aid of TrpRS from \textit{B. subtilis}. The mutants that possess the three G•C base pairs and G73 discriminator base exhibit almost the same aminoacylation efficiencies as \textit{B. subtilis} tRNA\textsuperscript{Trp}, while the G73 discriminator base itself cannot confer efficient aminoacylation to the tRNA\textsuperscript{Trp} molecule. Thus, these three base pairs (G2\textsuperscript{-}C71, G3\textsuperscript{-}C70, and G4\textsuperscript{-}C69) in the \textit{B. subtilis} tRNA\textsuperscript{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 \textit{B. subtilis} TrpRS included G73, G2\textsuperscript{-}C71, G3\textsuperscript{-}C70, and G4\textsuperscript{-}C69.

The translation of genetic information into proteins is essential for a proper biochemical functioning of the cell. The aminoacyl-tRNA synthetases (aaRS),\textsuperscript{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 \textit{Bacillus subtilis} belongs to the class I aaRS family. It has an \textit{a}_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 \textit{B. subtilis} tRNA\textsuperscript{Trp} were characterized previously with in vitro aminoacylation assays (7). The identity elements recognized by \textit{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 \textit{B. subtilis} tRNA\textsuperscript{Trp} sequence.

Recently, from another work in our laboratory, we observed that the aminoacylation efficiency of a human tRNA\textsuperscript{Trp} mutant was only 14% of wild-type \textit{B. subtilis} tRNA\textsuperscript{Trp} when assayed by \textit{B. subtilis} TrpRS (8), despite the fact that all of its previously characterized identity elements in acceptor stem were switched to those of \textit{B. subtilis} tRNA\textsuperscript{Trp}. On the basis of this observation, we proposed that there are more elements that are required for \textit{B. subtilis} TrpRS recognition distributed within tRNA\textsuperscript{Trp}. To confirm this notion, the SELEX (systematic evolution of ligands by exponential enrichment) method (9, 10) was employed in this work using immobilized \textit{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 \textit{B. subtilis} tRNA\textsuperscript{Trp} sequence, we consider that the acceptor stem of tRNA\textsuperscript{Trp} plays an essential role in the recognition by \textit{B. subtilis} TrpRS. To give an extensive

\textsuperscript{†} This work was supported by the Natural Science Foundation of China (Grant 39730120), the Chinese Academy of Sciences (Grant KSCX 2-2-04), and a grant from Shanghai Institutes for Biological Sciences.

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§ Abbreviations: aaRS, aminoacyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; SELEX, systematic evolution of ligands by exponential enrichment.
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 tRNATrp.

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, RQI 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 Tag 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 GCC GTA-3′; 3′ primer, 5′-TTA TAC GAG TTA TAC GCA CCC GGG GGG-3′; 5′-TTA TAC GAC TCA TAG GGA ATT CTA TCC GCC GTA-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 tRNATrp (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 redisolved 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 tRNATrp 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 tRNATrp 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.
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 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, amplified by PCR, and sequenced using the fmol DNA sequencing system (Promega, WI). Figure 4 shows the sequences of these 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′-N18 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). Two cytosinosines, which were selected from the random sequences, were 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.

**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′-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. The bound RNA was recovered and subjected to electrophoresis on a 10% denaturing polyacrylamide gel.

**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 cytosinosines, 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.
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 CCAOH 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<sup>Trp</sup>, 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<sup>Trp</sup> from *Oryza sativa*, and another is based on human tRNA<sup>Trp</sup> (Figure 6). Aminoacylation results (decrease in $k_{cat}/K_M$ relative to that of the wild-type *B. subtilis* tRNA<sup>Trp</sup>) are shown in Table 1. The individual kinetic parameters measured for the wild-type tRNA were as follows: *B. subtilis* tRNA<sup>Trp</sup> aminoacylated by *B. subtilis* TrpRS, $k_{cat} = 0.69$ s<sup>-1</sup> and $K_M = 0.56$ μM. *B. subtilis* TrpRS weakly catalyzes the aminoacylation reaction of human tRNA<sup>Trp</sup>, 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<sup>Trp</sup>

### Table 1: Effect of Single and Multiple Nucleotide Mutations on Aminoacylation of tRNA<sup>Trp</sup> Transcripts by *B. subtilis* TrpRS<sup>a</sup>

<table>
<thead>
<tr>
<th>variant</th>
<th>$k_{cat}/K_M$ (relative)</th>
<th>change in efficiency (x-fold)</th>
<th>variant</th>
<th>$k_{cat}/K_M$ (relative)</th>
<th>change in efficiency (x-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTB</td>
<td>1.0</td>
<td>1.0</td>
<td>MHB1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.075</td>
<td>−13</td>
</tr>
<tr>
<td>WTP</td>
<td>0.0025</td>
<td>−400</td>
<td>MHB2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11</td>
<td>−9.1</td>
</tr>
<tr>
<td>MPB1</td>
<td>0.0045</td>
<td>−222</td>
<td>MHB3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14</td>
<td>−7.1</td>
</tr>
<tr>
<td>MPB2</td>
<td>0.013</td>
<td>−77</td>
<td>MHB4</td>
<td>0.95</td>
<td>−1.1</td>
</tr>
<tr>
<td>MPB3</td>
<td>0.87</td>
<td>−1.2</td>
<td>MHB5</td>
<td>0.05</td>
<td>−20</td>
</tr>
<tr>
<td>MPB4</td>
<td>0.42</td>
<td>−2.4</td>
<td>MHB6</td>
<td>0.48</td>
<td>−2.1</td>
</tr>
<tr>
<td>WTH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0076</td>
<td>−132</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> The specificity constant ($k_{cat}/K_M$) for aminoacylation of tRNA<sup>Trp</sup> 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<sup>Trp</sup> transcript aminoacylated by *B. subtilis* TrpRS, which was set at 1.0. Concentrations of tRNA<sup>Trp</sup> ranging from 0.1 to 20 μM were used, and the TrpRS concentration was 25, 50, or 100 nM. <sup>b</sup> These data were from ref 8.
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 MBH3 significantly improved the aminoaacylation efficiency by B. subtilis TrpRS to almost the same as wild-type B. subtilis tRNA\textsuperscript{Trp} (95% of the wild-type B. subtilis tRNA\textsuperscript{Trp}; Table 1). O. sativa mitochondrial tRNA\textsuperscript{Trp} is also a poor substrate for B. subtilis TrpRS, with a 400-fold decrease in aminoaacylation efficiency as compared to wild-type B. subtilis transcript, despite the fact that it possesses the same discriminator base, G73, as B. subtilis tRNA\textsuperscript{Trp}. Mutations of G1A and U5G/A68C had little positive effects on B. subtilis TrpRS aminoaacylation (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 aminoaacylation efficiency from −77-fold to −1.2-fold as compared to wild-type B. subtilis tRNA\textsuperscript{Trp} (Figure 6C,D).

Although the three G-C base pairs have been proven to be important in the recognition and aminoaacylation of tRNA\textsuperscript{Trp} by B. subtilis TrpRS, a subsequent test was carried out to identify whether these base pairs themselves could confer aminoaacylation by B. subtilis TrpRS. We prepared three mutants for this assay (Figure 6K−M). Mutant MBH5 is not a good substrate for B. subtilis TrpRS; its aminoaacylation efficiency is only 5% of the wild-type B. subtilis tRNA\textsuperscript{Trp} (Table 1). The substitution of A73G based on MBH5 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\textsuperscript{Trp} also dramatically increased the aminoaacylation efficiency from −400-fold to −2.4-fold (Figure 6A,M).

DISCUSSION

Schimmel and Poulahan 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, aminoaacylation specificity and efficiency generally depend on the sequences and structures of the acceptor stem (27−35).

The acceptor stem of B. subtilis tRNA\textsuperscript{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\textsuperscript{Trp} (7). Substitutions of these identity elements lead to a sharp decrease in the aminoaacylation efficiency of B. subtilis tRNA\textsuperscript{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\textsuperscript{Trp} with all of the previously characterized B. subtilis tRNA\textsuperscript{Trp} acceptor stem identity elements could only be aminoaacylated to a level of 14% as compared to the wild-type B. subtilis tRNA\textsuperscript{Trp}. This fact indicates the existence of more identity elements distributed within B. subtilis tRNA\textsuperscript{Trp}. Subsequently, two mutants of MPB1 and MPB2 on the basis of O. sativa mitochondrial tRNA\textsuperscript{Trp} were generated and assayed by B. subtilis TrpRS. The results of the aminoaacylation assay provided further supports for our proposal. Although wild-type O. sativa mitochondrial tRNA\textsuperscript{Trp} has the same major identity element of G73 as B. subtilis tRNA\textsuperscript{Trp}, it is still a poor substrate for B. subtilis TrpRS (0.25% of the efficiency of the wild-type B. subtilis tRNA\textsuperscript{Trp}; Table 1). Mutant MPB2 has all of the previously characterized B. subtilis tRNA\textsuperscript{Trp} identity elements in the acceptor stem, but its aminoaacylation efficiency is only 1.3% of the wild-type B. subtilis tRNA\textsuperscript{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\textsuperscript{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\textsubscript{OH} sequence at the 3′ end, it is impossible to be a substrate in the aminoaacylation assay. To confirm the importance of the three G-C base pairs in the aminoaacylation of tRNA\textsuperscript{Trp} by B. subtilis TrpRS, additional mutants of MPB3 and MBH4 were generated and assayed by B. subtilis TrpRS. The aminoaacylation efficiencies of MPB3 and MBH4 are almost the same as B. subtilis tRNA\textsuperscript{Trp} (87% and 95% of the B. subtilis tRNA\textsuperscript{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 aminoaacylation of tRNA\textsuperscript{Trp} by B. subtilis TrpRS. Other evidence for the three G-C base pairs as an important structural feature involved in the aminoaacylation by B. subtilis TrpRS arose from E. coli tRNA\textsuperscript{Trp} (7). The aminoaacylation efficiency of E. coli tRNA\textsuperscript{Trp} by B. subtilis TrpRS is 75% of the B. subtilis tRNA\textsuperscript{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\textsuperscript{Trp}.

Subsequent aminoaacylation assay of MBH5, MBH6, and MPB4 further confirms the importance of these successive G-C base pairs in aminoaacylation of tRNA\textsuperscript{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\textsuperscript{Trp} molecule could not act as an excellent substrate for B. subtilis TrpRS. And, at the same time, if a tRNA\textsuperscript{Trp} molecule does not possess the three G-C
base pairs, it could not be efficiently aminoacylated by B. subtilis TrpRS too (Table 1 and Figure 6C,1). Thus, we identified the minimum identity element set in the acceptor stem of tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{Trp}s. The first group shares consensus sequences of AGGGG and CCCCCUG. This group makes up 67% of the total sequences and includes the tRNA\textsuperscript{Trp} from E. coli and B. subtilis. The second group shares consensus sequences of AGGAG and CUCCUG. The third one shares GGGGG and CCCCUG 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Trp} by B. subtilis TrpRS.

ACKNOWLEDGMENT

We are grateful to Dr. Xue Hong (Hongkong University of Science and Technology) for helpful advice and technical support.

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