Modified nucleotides in *Bacillus subtilis* tRNA<sub>Trp</sub> hyperexpressed in *Escherichia coli*

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**ABSTRACT**

In the present study, modified nucleotides in the *B. subtilis* tRNA<sub>Trp</sub> cloned and hyperexpressed in *E. coli* have been identified by TLC and HPLC analyses. The modification patterns of the two isoacceptors of cloned *B. subtilis* tRNA<sub>Trp</sub> have been compared with those of native tRNA<sub>Trp</sub> from *B. subtilis* and from *E. coli*. The modifications of the A73 mutant of *B. subtilis* tRNA<sub>Trp</sub>, which is inactive toward its cognate TrpRS, were also investigated. The results indicate the formation of the modified nucleotides s<sup>4</sup>U8, Gm18, D20, Cm32, m<sup>8</sup>A/ms<sup>8</sup>mA37, T54 and 55 on cloned *B. subtilis* tRNA<sub>Trp</sub>. This modification pattern resembles the pattern of *E. coli* tRNA<sub>Trp</sub>, except that m<sup>7</sup>G is missing from the cloned tRNA<sub>Trp</sub>, probably on account of its short extra loop. In contrast, the pattern departs substantially from that of native *B. subtilis* tRNA<sub>Trp</sub>. Therefore, the cloned *B. subtilis* tRNA<sub>Trp</sub> has taken on largely the modification pattern of *E. coli* tRNA<sub>Trp</sub> despite the 26% sequence difference between the two species of tRNA, gaining in particular the Cm32 and Gm18 modifications from the *E. coli* host. A notable difference between the isoacceptors of the cloned tRNA<sub>Trp</sub> was seen in the extent of modification of A37, which occurred as either the hypomodified t<sup>5</sup>A or the hypermodified ms<sup>2</sup>mA form. Surprisingly, base substitution of guanosine by adenosine at position 73 of the cloned tRNA<sub>Trp</sub> has led to the abolition of the 2'-O-methylation modification of the remote G18 residue.

**INTRODUCTION**

Transfer RNAs play a pivotal role in protein synthesis, providing the adaptor molecules that accomplish the translation of nucleotidyl triplet codons into amino acids in the growing polypeptide chain. During this translation process, transfer RNAs interact with many different molecules in the cell including mRNA, ribosomes, initiation and elongation factors, peptidyl-tRNA hydrolase, cognate aminoacyl-tRNA synthetases, and a series of tRNA modifying enzymes. All tRNAs must conform to the same general constraints for size and shape in order to fit on the ribosome, and be recognized by the translation factors. At the same time, there must be critical differences between individual tRNAs that are essential for accurate recognition by their cognate aminoacyl-tRNA synthetases in order to maintain fidelity of translation. Such common as well as individual features of the various tRNAs may reside not only in their nucleotide sequences, but also in their tertiary structures. So far, there is still no systematic view of how a tRNA is recognized by the many molecules with which it interacts (1-3).

Recently, heterologous tRNA hyperexpression has been developed as an approach to gain insight into the structure-function relationships of tRNA. In this system, the wild type *Bacillus subtilis* tRNA<sub>Trp</sub> gene has been cloned and hyperexpressed in *Escherichia coli* (4). Upon reversed-phase HPLC fractionation of the corresponding gene products, up to four Trp-accepting peaks were revealed. Since tRNA<sub>Trp</sub> isoacceptors in all four peaks are trancribed from the same synthetic gene sequence, it is expected that they may differ from one another with respect to post-transcriptional modifications. It is indeed well known that multiple tRNA isoacceptors separable by chromatography often result from the presence or absence of base modifications (5-8). However, other factors such as the tryptophan charging (16) and the distinct conformations of the tRNA molecule may also contribute to the differential chromatographic mobility of different isoacceptors (9, 10, 16).

Since the cloned *B. subtilis* tRNA<sub>Trp</sub> expressed inside *E. coli* cells would be modified by *E. coli* modification enzymes, it is of great interest to determine to what extent its nucleotide modification pattern may resemble that of endogenous *E. coli* tRNA<sub>Trp</sub>. At present, no less than 37 *E. coli* and 16 *B. subtilis* tRNA sequences are known (11). Comparison of the modified nucleotides present at various positions in these two sets of tRNA furnishes an initial basis for predicting the modified nucleotides to be found on the cloned *B. subtilis* tRNA<sub>Trp</sub> (Figure 1). Comparison with the modified nucleotides actually occurring on the hyperexpressed *B. subtilis* tRNA<sub>Trp</sub> will therefore lead to a refinement of our understanding of the structural basis for the specificity of *E. coli* modification enzymes. A guanosine to adenosine base substitution at position 73 next to the 3'-CCA...
terminus of the cloned \textit{B. subtilis} tRNA$^{Trp}$ is known to prevent Trp-tRNA formation catalysed by \textit{B. subtilis} Trp-tRNA synthetase (15). The influence of this change at the discriminator base position on post-transcriptional modifications is included in the present study in order to probe the significance of this key functional residue on base-modification processes.

**MATERIAL AND METHODS**

**tRNA$^{Trp}$ fractionation**

\textit{B. subtilis} tRNA$^{Trp}$ was hyperexpressed in \textit{E. coli} and purified as described (4). This depended on the chemical synthesis of the \textit{Bacillus subtilis} tRNA$^{Trp}$ gene sequence derived from the sequence of the \textit{trmD} gene cluster sequence (12), plus a T7 promoter at the 5' end and a BsrNI restriction site at the 3' end, followed by ligation of the entire fragment into the phagemid pGEM9zf(−) (Promega Corp., USA) between the SfiI and HindIII sites. The resultant plasmid, pWWT, was cloned into \textit{Escherichia coli} JM109 under the control of a lac promoter. Hyperexpression of the gene to yield \textit{B. subtilis} tRNA$^{Trp}$ up to 28% of total tRNA was achieved upon induction of cells in exponential growth at A$_{600}$ of 0.2 by 1 mM isopropyl β-D-thiogalactopyranoside in minimal medium (M9-glycerol) for 4 hours. Total tRNA was extracted and fractionated by chromatography on a single Vydec C4 HPLC matrix (14). A plasmid, pWA73, carrying a \textit{B. subtilis} tRNA$^{Trp}$ mutant with guanosine replaced by adenosine at position 73 was cloned in the same way (15) and the \textit{in vivo} gene product, \textit{B. subtilis} tRNA$^{Trp}$ A73 mutant, was large-scale purified as for the wild type tRNA$^{Trp}$. In either instance, the HPLC tRNA fractions were collected by ethanol precipitation, or desalted and further concentrated by centrifugation in a Centricon-10 (from Amicon). The resulting tRNA samples were lyophilized. Further purification of the major peak of cloned \textit{B. subtilis} tRNA$^{Trp}$, 74-bases long, from contaminating host \textit{E. coli} tRNA$^{Trp}$, which is 76-bases in length and comigrated with the major peak of cloned \textit{B. subtilis} tRNA$^{Trp}$ in HPLC, was achieved by electrophoresis for 16 hours at 600 volts in a denaturing 12% polyacrylamide gel, pH 7.5. The major band of the cloned \textit{B. subtilis} tRNA$^{Trp}$ was excised, eluted and used as starting material for subsequent analysis.

Native \textit{E. coli} B tRNA$^{Trp}$ with a specific activity of 1700 pmol/equiv A$_{260}$ unit was purchased from Plenum Scientific Research Inc. (Hackensack, NY, USA).

**Identification of modified nucleotides**

Qualitative analysis of modified nucleotides was performed by thin layer chromatography (TLC) basically in accordance to Silberling et al. (17), with an additional elimination of excess γ$^{32}$P-ATP at the end of the kinase reaction by treatment with apyrase (Sigma Grade VIII, from potato) at 10 mU/0.5 μg tRNA for 30 minutes at 37°C (18). The various tRNAs$^{Trp}$ peaks (0.5 μg) were first subjected to complete T2 RNase (0.05 units, from Sigma, USA) digestion to yield 3'-monophosphonucleotides, then 5'-32P labelled using polynucleotide kinase (from Boehringer Mannheim, Germany) in the presence of γ$^{32}$P-ATP (3,000 Ci/mumole, from Amersham, England). After apyrase treatment, the 5'-32P-3'-P-nucleoside diphosphates were treated with 1 μg of P1 RNase (from Boehringer Mannheim, Germany) to obtain 5'-32P labelled monophosphonucleotides. Afterwards, the
samples were applied to two dimensional thin layer chromatography on CEL 400 cellulose plates (Schleicher & Schuell, Germany) developed in a solvent system composed of isobutyric acid/25% NH₄OH/H₂O (66/1/33, by volume) in the first dimension and 0.1 M sodium phosphate pH 6.8/ammonium sulfate/n-propanol (100/60/2, V/V/V) in the second dimension prior to autoradiography. The identification of radiolabelled spots was performed by comparison with reference maps (17–19).

Confirmation of the TLC identification, as well as quantitation, of the modified nucleosides were performed by HPLC after hydrolysis of the purified rRNA TTP. Each sample (20 μg) of tRNA dissolved in 50 μl of distilled water was digested overnight to completion at 37°C by 10 μl (0.3 U/μl) of P1 RNase (Boehringer Mannheim, Germany) in 30 mM acetate buffer pH 5.30, and 10 μl of 10 mM ZnCl₂. The solution of free 5'-phosphate nucleotides obtained was digested by 10 μl (0.1 U/μl) of bacterial alkaline phosphatase (BAP) (Sigma, St Louis, MO, USA) in 100 μl of 0.5 M Tris buffer pH 8.30 for 2 hours. The resultant hydrolysate was analyzed by HPLC using a Spectra-Physics liquid chromatograph equipped with a Spectra Focus detector (Spectra-Physics France, Les Ulis) and a Supelcosil LC 18S column, 250 mm x 4.6 mm (Supelco France, St Germain en Laye). Chromatography was carried out under the conditions for separation of ribonucleosides developed by Gehrke and Kuo (20). The instrumentation system consisted of a variable volume injector, a ternary solvent delivery system, a temperature-controlled column oven (maintaining a temperature of 26°C ± 0.2°C) and a forward scanning detector. A computer integrated the functions of each component and performed data processing, including post-run integrations as an important requirement for quantitative analysis. Each nucleoside component detected in the eluate was rigorously identified by comparison of its UV spectrum and retention time with ribonucleoside reference standards. All nucleosides present were quantified in terms of moles of residue per mole of tRNA molecule using the relative molar response (RMR) calculated for each nucleoside with BrG as internal standard.

In order to characterize a methylated dinucleotide (Nm-p-N') resistant to P1 nuclease, digestion with snake venom phosphodiesterase (SV-PDE) was employed. The total nuclease P1-BAP digest prepared as described above as well as the dinucleotide collected from HPLC eluates were hydrolyzed by a mixture of 20 μl of SV-PDE (0.001 U/μl), 10 μl of 10 mM magnesium acetate, and 50 μl of 0.5 M Tris buffer pH 8.30 for 4 hours at 37°C. The hydrolysate obtained was again analyzed by the HPLC protocol described above.

RESULTS

Heterologous tRNA TTP isoaceptors

When the wild-type B. subtilis tRNA TTP gene was hyperexpressed in E. coli in minimal medium, fractionation of total extracted tRNA by reversed-phase HPLC on a C4 column revealed up to four tryptophan accepting peaks, the major Peak I and the three minor Peaks II, III and IV (Figure 2A). These isoaceptors all migrated at same rate in denaturing 12% polyacrylamide gel (Figure 2B), indicating that they were all of equal length. The A73 mutant B. subtilis tRNA TTP obtained by hypexpression of the A73 mutant gene was also known to be of the same length as wild-type Peak I (15).

To examine the effects of growth-medium enrichment on the distribution of the isoaceptor peaks, Bacto-yeast extract and Bacto-tryptone were added to minimal medium culture of E. coli cells carrying pWWT 210 minutes after IPTG induction. The cells were grown for another 30 minutes prior to harvesting. Under these conditions, the isoaceptor Peak I was decreased significantly, while Peak II was increased, so that Peak II instead of Peak I became the major peak (Figure 3a). Peaks III and IV also remained evident, but these peaks were extensively diminished when treatment of the tRNA at pH 8 was carried out to strip away any tryptophan attached to the tRNA(Figure 3b). The letters I, II, III and IV denote the isoaceptor tRNA TTP peaks. (a) Unstripped tRNA; and (b) Stripped tRNA.

![Figure 3](image-url)

Figure 3. Effect of growth medium enrichment on expression of B. subtilis tRNA TTP isoaceptors. E. coli cells carrying pWWT were grown in minimal M9-glycerol medium to A₆₀₀ = 0.16 before addition of inducer IPTG to 1 mM. After 210 minutes, the culture (A₆₀₀ = 0.35) was enriched with Bacto-yeast extract (4 g/L) and Bacto-tryptone (6.4 g/L). Cells were harvested 30 minutes after enrichment at A₆₀₀ = 0.47. Total tRNA (0.73 mg) extracted was fractionated on HPLC C4 column (14) either without stripping, or after stripping off the amino acid on the tRNA by means of incubation of total extracted tRNA in 1.8 M TrisCl, pH 8, 37°C for 2 hours (13). Absorbance at 254 nm is shown by solid line, and Trp-accepting activity by dashed line. The letters I, II, III and IV denote the isoaceptor tRNA TTP peaks. (a) Unstripped tRNA; and (b) Stripped tRNA.

TLC identification of modified nucleotides

The TLC autoradiographs of the nucleotides of each of the four wild-type isoaceptors of B. subtilis tRNA TTP expressed in E. coli, the A73 mutant, and native E. coli tRNA TTP are represented in Figures 4a–4f. The presence of 2'-O-methylcytidine (Cm), 2'-O-methylguanosine (Gm), 5-methyluridine (Tu), dihydrouridine (D) and pseudouridine (Ψ) in all of the cloned B. subtilis tRNA TTP species examined was clearly demonstrated. The 4-thiouridine...
Figure 4. The TLC autoradiographs of $^{32}$P labelled nucleoside 5'-monophosphates in (a) wild-type *B. subtilis* tRNA$^{Trp}$ Peak I, (b) Peak II, (c) Peak III, (d) Peak IV, (e) A73 mutant and (f) native *E. coli* tRNA$^{Trp}$. A = 5'-$^{32}$P adenosine; C = 5'-$^{32}$P cytidine; U = 5'-$^{32}$P uridine; G = 5'-$^{32}$P guanosine; T = 5'-$^{32}$P 5-methyluridine; F = 5'-$^{32}$P pseudouridine; D = 5'-$^{32}$P dihydrouridine; Cm = 5'-$^{32}$P 2'-O-methylcytidine; Gm = 5'-$^{32}$P 2'-O-methylguanosine.

(sU) spot, between T and U on the TLC, was barely detectable, which is consistent with the known instability of this nucleotide (21). No gross difference was evident among the four wild-type isoacceptors. Only the intensity of the N6-isopentenyladenosine/2-methylthio-N6-isopentenyl-adenosine ($^{32}$A/$^{32}$A) spot varied between these isoacceptors. Interestingly, while the Gm spot was present in all four wild-type isoacceptors (Figures 4a–d), it was totally absent from the A73 mutant (Figure 4e). Otherwise the autoradiograph of the A73 mutant was similar to that of the wild-type with respect to the other modified nucleotides. No 7-methylguanosine (m7G) spot between Cm and C was detected in any of these species of cloned *B. subtilis* tRNA$^{Trp}$, although its presence in *E. coli* tRNA$^{Trp}$ was clear from a control analysis (Figure 4f) in agreement with previous observations (22). Because not all the modified nucleotides are equally susceptible to the enzymes used in the post-labeling of the nucleotides prior to TLC (17), the TLC analysis employed sufficed as a basis for the qualitative but not the quantitative assessment of the modified nucleotides in any given tRNA.

**Quantitation of modified nucleosides**

For the purpose of quantitation, HPLC separation was carried out for the nucleosides from the different molecular species of cloned *B. subtilis* tRNA$^{Trp}$ as well as native *E. coli* tRNA$^{Trp}$, of
Figure 5. HPLC profiles of ribonucleosides in tRNA<sup>Trp</sup> (a) cloned wild-type *B. subtilis* Peak I, (b) Peak II, (c) Peak III, (d) Peak IV, (e) A73 mutant and (f) native *E. coli* tRNA<sup>Trp</sup>. The quantitative estimates obtained were:

<table>
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<tr>
<th>tRNA&lt;sup&gt;Trp&lt;/sup&gt;</th>
<th>sU</th>
<th>Gm</th>
<th>D</th>
<th>Cm</th>
<th>N36</th>
<th>5A</th>
<th>m2A</th>
<th>m7G</th>
<th>T</th>
<th>Ψ</th>
</tr>
</thead>
<tbody>
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<td>0.2</td>
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<td>1.0</td>
<td>0.1</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Peak II</td>
<td>0.2</td>
<td>0.3</td>
<td>1.4</td>
<td>0.9</td>
<td>0.1</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Peak III</td>
<td>0.3</td>
<td>0.2</td>
<td>1.0</td>
<td>0.2</td>
<td>0.8</td>
<td>0.7</td>
<td>0.2</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Peak IV</td>
<td>0.2</td>
<td>0.2</td>
<td>1.3</td>
<td>0.6</td>
<td>0.4</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>A73 trace</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
<td>0.8</td>
<td>0.3</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td>trace</td>
<td>3.3</td>
<td>1.1</td>
<td>-</td>
<td>1.1</td>
<td>0.7</td>
<td>1.1</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

which the sequence is known (22) (Figure 5a–f). The amount of each modified nucleoside was expressed in terms of the molar ratio, or moles per mole of tRNA (Figure 6). The modified nucleosides T, Ψ and D gave molar ratios that were nearly stoichiometric, while s<sup>U</sup> and Cm gave molar ratios that were less than one. The Gm nucleoside was detected in each of the four wild-type *B. subtilis* tRNA<sup>Trp</sup> isoacceptors at a molar ratio of 0.2–0.3, and in native *E. coli* tRNA<sup>Trp</sup> in trace amounts. However, it was undetectable in the A73 mutant *B. subtilis* tRNA<sup>Trp</sup>, which is consistent with the absence of the Gm spot from the TLC plate for this mutant (Figure 4e). While the occurrence of the m<sup>7</sup>G peak was unambiguous in *E. coli*
Figure 6. Composition of modified ribonucleotides in the heterologously expressed wild-type isoacceptors I (the major in minimal growth medium), Peak II (the major in rich growth medium), the A73 mutant, and native B. subtilis tRNA^{Trp} (32). The bracketed representations of (s^U) and (Gm) indicate the partial, sub-stoichiometric amounts of these modifications observed.

tRNA^{Trp} (Figure 5f), this nucleoside was not detectable in any of the cloned wild-type or A73 mutant B. subtilis tRNA^{Trp} species.

The main difference between the various heterologously expressed B. subtilis tRNA^{Trp} species was found to reside in the content of i^6A/m^2i^6A (Figure 6). The molar ratio of i^6A/m^2i^6A was 0.9/0 for Peak I, 0/0.9 for Peak II, 0.2/0.7 for Peak III, 0/0.7 for Peak IV, and 1/0 for the A73 mutant form of Peak I.

A nucleoside peak eluting with a retention time of 36 minutes, and therefore referred to as N36, was observed in the P1 RNase-BAP digests of all the cloned species of B. subtilis tRNA^{Trp} including the A73 mutant (Figures 5a—e). After treatment with snake venom phosphodiesterase (SV-PDE), the N36 peak disappeared from the resulting hydrolysates, while the peak of Cm was significantly increased. This suggests that N36 could be a di- or oligo-nucleotide containing Cm, since such nucleotides are known to be partially resistant to P1 RNase. In confirmation, the N36 compound collected from the HPLC of wild-type Peak I yielded two nucleosides in equal molar amounts upon enzymatic hydrolysis with SV-PDE followed by BAP treatment (data not shown). These two nucleosides were readily identified as 2'-O-methylcytidine (Cm) and unmodified uridine (U) based on a comparison of their HPLC retention times and UV spectra with those of authentic Cm and U nucleosides. N36 was thus characterized as Cm-p-U. The sum of the molar ratios of Cm and N36 was found to be close to 1 for each of the cloned species of B. subtilis tRNA^{Trp} (Figure 6). Thus the variable, less than unity molar ratios of Cm could be explained by the partial hydrolysis of Cm-p-U.

DISCUSSION

Transfer RNAs undergo during their maturation extensive, diverse post-transcriptional modifications that vary with both the tRNA and the organism (23—25). The structural elements determining the specificity of different modifying enzymes are only beginning to be elucidated. A powerful approach for defining such structural specificity is to produce tRNAs of varying sequences inside the cell, and monitor the modifications imposed on them by the modifying enzymes. To facilitate analysis, the tRNAs should be hyperexpressed, so that relatively large quantities may be purified for biochemical and biophysical investigation. The heterologous hyperexpression of B. subtilis tRNA^{Trp} in E. coli satisfies these requirements.

In the present study, we have characterized the modification patterns of wild-type and A73 mutant B. subtilis tRNA^{Trp} produced in this system, and compared them to the patterns of native E. coli tRNA^{Trp} and of native B. subtilis tRNA^{Trp}. Both qualitative TLC and quantitative HPLC analyses demonstrated the stoichiometric formation of the T, Ψ and D modified...
nucleosides in all of the heterologously expressed *B. subtilis* tRNA^Trp^ species, indicating that the modification enzymes responsible for their formation are not rate limiting factors even under conditions of hypopexpression. This is not surprising in view of the fact that T, ¥ and D exist respectively in the TYC and D loops of almost all known tRNAs. The fact that only one ¥ per cloned *B. subtilis* tRNA^Trp^ was found, most probably originating from position 55, suggests that there is no significant occurrence of ¥ at position 13 (Figure 1).

The 7-methylguanosine (m^7^G) base is present at position 46 in 17 out of 20 *E. coli* tRNAs including tRNA^Trp^, but it is clearly absent from all of the cloned *B. subtilis* tRNA^Trp^. Therefore the *B. subtilis* tRNA^Trp^ gene transcript is not a substrate for the guanosine-7-methylase of *E. coli*. In this regard, it has been reported that guanosine-7-methylase actively recognizes G46 only when it is part of a 5-member extra loop (26–27). On this basis the inactivity of *B. subtilis* tRNA^Trp^ as a 7-methyl acceptor at G46 is probably due to G46 being a member of a 4-base extra loop, in contrast to the 5-base extra loop in the case of *E. coli* tRNA^Trp^.

Two forms of modified A, namely the hypomodified N6-isopentenylationenosine (i^6^A) and the hypermodified 2-methylthio-N6-isopentenylationenosine (ms^2^i^6^A) were isolated from cloned *B. subtilis* tRNA^Trp^ from the only likely location for these residues is position 37, the nucleotide 3' to the anticodon. The mechanism for the generation of these two nucleosides consists of a sequential conversion of A first to i^6^A, and the latter in turn to ms^2^i^6^A. Since all species of cloned *B. subtilis* tRNA^Trp^ contained a stoichiometric amount of N6-isopentenyl group at position-37, the i^6^A modification enzyme was not a limiting factor in *E. coli*. This was evidently not the case for the subsequent 2-methylthiolation of i^6^A37, which involves two modification enzymes in the formation of the hypermodified ms^2^i^6^A. Indeed, the extent of methylthiolation of A37 constituted the most prominent difference among the 4 wild-type isoacceptors. Although the exact basis for the incomplete formation of the 2-methylthiolate derivative remains to be established, at least two possibilities should be considered. First, the supply of the modifying enzyme(s) or their cofactor(s), such as iron (28) and/or cysteine (29), could be limiting. Secondly, the modification reaction could be regulated by growth condition (5–8, 30). Thus a shift-up of the growth medium from minimal to rich rapidly induced changes in the proportion of the different isoacceptors (Figure 3). The major tRNA^Trp^ peak changed from Peak I, containing only i^6^A, to Peak II, which contained a stoichiometric amount of ms^2^i^6^A. The presence of i^6^A37/ms^2^i^6^A37 as well as Cm32 in the anticodon loop is expected for the cloned *B. subtilis* tRNA^Trp^ no less than that of endogenous *E. coli* tRNA^Trp^, since both of these tRNA^Trp^ have exactly the same base sequence in the anticodon loop.

Besides s^U^ at position 8, which was difficult to quantitate owing to its instability (21), Gm is the only other modified nucleotide observed to be present at low levels (with a molar ratio of 0.2–0.3) in the cloned *B. subtilis* tRNA^Trp^ isoacceptors with the wild-type gene sequence (Figure 5). This nucleotide, found in 30% of *E. coli* tRNAs at position 18 in the D-loop, was detected only at trace levels in *E. coli* tRNA^Trp^ (ref. 22 and Figure 5f). It was completely undetectable in native *B. subtilis* tRNA^Trp^ (32). Thus the partial methylation of G18 in the cloned *B. subtilis* tRNA^Trp^ resembled neither native *E. coli* tRNA^Trp^ nor native *B. subtilis* tRNA^Trp^.

Observations with the thermostable tRNA-(guanosine-2'-)-methyltransferase from *Thermus thermophilus* suggested that the minimal requirement for that system to methylate the 2'-OH of G18 is the D 'loop-stem' structure, and possibly the presence of a s^U^ at position 8 (31). The difference in D-loop between *E. coli* tRNA^Trp^ and *B. subtilis* tRNA^Trp^ is that the former has an 8-member D-loop, whereas the latter bearing a deletion at base 17 has only 7 bases in its D-loop. The possibility exists that this structural difference may contribute to the higher level of Gm18 in *B. subtilis* tRNA^Trp^ expressed in *E. coli* compared to native *E. coli* tRNA^Trp^.

It is striking that the A73 mutant *B. subtilis* tRNA^Trp^ was completely lacking in Gm (Figure 4e and 5e), in contrast to the presence of this modification in all four wild-type isoacceptors (Figures 4a–d and 5a–d). This raises the question of whether it is the A73 base itself, or the global tRNA three-dimensional structure which A73 may influence, that represents the important recognition element for enzymatic 2'-methylatation at G18 by *E. coli* methylase. It is known that the G to A mutation at the discriminator position-73 of *B. subtilis* tRNA^Trp^ causes the tRNA^Trp^ to be inactive toward its cognate Trp-tRNA synthetase (15). Thus the replacement of G73 by A73 brought about a loss of recognition of the tRNA^Trp^ by Trp-tRNA synthetase, and also a loss of recognition by the Gm18 methylase.

Since the sum of Gm and Cm-p-U (N36) was one mole per mole of tRNA^Trp^, the formation of Cm was stoichiometric in all of the cloned species of *B. subtilis* tRNA^Trp^, as in the case of native *E. coli* tRNA^Trp^.

The compilation of tRNA sequences indicates position 32 to be the only expected location for Cm (Figure 1). This is supported by the characterization of N36 as 5'Cm-p-U3', which is the dinucleotide at positions 32 and 33 of *B. subtilis* tRNA^Trp^ (32). Interestingly, although Cm32 is fully formed in *B. subtilis* tRNA^Trp^ cloned and expressed in *E. coli*, it is absent from native *B. subtilis* tRNA^Trp^ (32), or indeed any other *B. subtilis* tRNAs of known sequence. In contrast, it exists in 4 out of 37 *E. coli* tRNAs, including tRNA^Trp^ (22). Therefore the structural elements recognized by the Cm32 modifying enzyme must be shared by the *B. subtilis* tRNA^Trp^ and *E. coli* tRNA^Trp^ sequences, despite the 26% base difference between these two sequences.

The modified nucleotides observed by Matsugi et al. (32) in native *B. subtilis* tRNA^Trp^ are D20, ¥31, i^6^A37, T54 and ¥55. Unlike *B. subtilis* tRNA^Trp^ expressed in *E. coli*, it lacks the Cm and Gm modifications. Since the same *B. subtilis* tRNA^Trp^ sequence is 2'-O-methylated at C32 and G18 inside *E. coli* but not inside *B. subtilis* cells, most likely *B. subtilis* does not contain the counterparts of the *E. coli* enzymes that methylate the 2'-OH of C32 and G18.

Overall, *B. subtilis* tRNA^Trp^ expressed in *E. coli* largely took on the characteristic post-transcriptional modifications of the host (Figure 6). The presence of Gm and Cm in the cloned *B. subtilis* tRNA^Trp^, although not in native *B. subtilis* tRNA^Trp^, furnishes a clear example in this regard. However, the post-transcriptional modifications of cloned *B. subtilis* tRNA^Trp^ were by no means identical to those of its *E. coli* counterpart. Thus m^7^G is present in *E. coli* tRNA^Trp^ at position 46, but not in the cloned *B. subtilis* tRNA^Trp^, possibly because the latter possesses a 4-member extra loop, instead of 5-member loop as in *E. coli* tRNA^Trp^.

It is also noteworthy that the ms^2^ group at A37 occurred to varying extents in the different tRNA^Trp^ isoacceptors. This modification was fully formed in Peak II, but completely lacking in Peak I. Since this represented the main difference between Peaks I and II, it is proposed that Peak II is derived from Peak I by methylthiolation at A37. The fact that the major isoacceptor was
Peak I in minimal growth medium, but Peak II in rich growth medium suggests that the incomplete methylation at A37 could be the result of overproduction of cloned tRNA<sub>Trp</sub> in minimal medium. Hence the conversion of i<sup>6</sup>A to ms<i>2</i><sup>i</sup>A<sub>6</sub> might be a rate-limiting process inside the cells. Since the hydrophobicity is a major fractionating parameter for reversed-phase HPLC as employed in this study, whereas the ms<sup>i</sup>A group is known to be highly hydrophobic, the differential chromatographic behavior of tRNA<sub>Trp</sub> Peaks I and II is probably due to the hydrophobicity difference between i<sup>6</sup>A37 and ms<i>2</i><sup>i</sup>A37.

Regarding the two minor peaks, Peak III was also enriched in i<sup>6</sup>A compared to Peak IV, whereas the latter was relatively enriched in ms<i>2</i><sup>i</sup>A. However, these peaks, being lower in quantities than Peak I and Peak II, might be more contaminated by host cell tRNAs. The relationship between Peaks III and IV, as well as their relationships to Peaks I and II, require further clarification, although comparison of HPLC profiles of total tRNA before and after stripping of the tryptophan charge on the tRNA (Figure 3) suggests that Peaks III and IV are the Trp-charged forms of Peaks I and II, respectively. It is well known that tRNA<sub>Trp</sub> could be right-shifted on the chromatogram by Trp-charging (16). As well, a number of investigators have found that some species of tRNA can exist in a physically denatured form that is chromatographically separable from the native form (9, 33–35). Therefore the possibility that Peaks III and IV included some denatured forms of Peaks I and II with an altered conformation also could not be excluded.

In conclusion, when B. subtilis tRNA<sub>Trp</sub> was expressed in the heterologous E. coli host, its modification pattern was determined by an interplay between its own base sequence and the specificity of the host modifying enzymes. Analysis of the observed modification has served to focus on the significance of a 5-base extra-loop to the 7-methylation of G46, uncover the unexpected importance of the G73 residue to the 2'-methylation of G18, and contrast the ability of E. coli to the inability of B. subtilis enzyme systems to catalyse the formation of Cm32 and Gm18 when presented with exactly the same substrate tRNA<sub>Trp</sub> sequence. These observations illustrate the utility of the heterologous expression approach, especially when quantitation of nucleoside modifications is facilitated by a hyperexpression of the cloned tRNA, in helping to unravel the complex structure-function relationships in post-transcriptional modification reactions. Such a heterologous tRNA expression system permits investigations into species specificity of post-transcriptional modification under physiological conditions in living cells.

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REFERENCES