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Life without tRNA\textsuperscript{Ile}\textsubscript{-}lysidine synthetase: translation of the isoleucine codon AUA in \textit{Bacillus subtilis} lacking the canonical tRNA\textsubscript{2}Ile

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ABSTRACT

Translation of the isoleucine codon AUA in most prokaryotes requires a modified C (lysidine or agmatidine) at the wobble position of tRNA\textsubscript{2}Ile to base pair specifically with the A of the AUA codon but not with the G of AUG. Recently, a \textit{Bacillus subtilis} strain was isolated in which the essential gene encoding tRNA Ile-lysidine synthetase was deleted for the first time. In such a strain, C\textsubscript{34} at the wobble position of tRNA\textsubscript{2}Ile is expected to remain unmodified and cells depend on a mutant suppressor tRNA derived from tRNA\textsubscript{1}Ile, in which G\textsubscript{34} has been changed to U\textsubscript{34}. An important question, therefore, is how U\textsubscript{34} base pairs with A without also base pairing with G. Here, we show (i) that unlike U\textsubscript{34} at the wobble position of all \textit{B. subtilis} tRNAs of known sequence, U\textsubscript{34} in the mutant tRNA is not modified, and (ii) that the mutant tRNA binds strongly to the AUA codon on \textit{B. subtilis} ribosomes but only weakly to AUG. These \textit{in vitro} data explain why the suppressor strain displays only a low level of misreading AUG codons \textit{in vivo} and, as shown here, grows at a rate comparable to that of the wild-type strain.

INTRODUCTION

The genetic code consists of 16 four-codon boxes in which the four codons in a box differ from one another in the 3’ terminal nucleotide. In 14 of the 16 boxes, all four codons either specify the same amino acid or are split into two sets of two codons; those ending in pyrimidines specifying one amino acid and those ending in purines specifying a different amino acid (1,2). The Wobble hypothesis of Crick proposes how a single tRNA with G in the first position of the anticodon (also called the wobble base) can read codons ending in U or C and how a tRNA with U (or a modified U) can read codons ending in A or G (3–5). The AUN codon box specifying isoleucine and methionine is unique in that three of the four codons, AUU, AUC and AUA, specify isoleucine, whereas the fourth codon, AUG, specifies methionine. This organization raises the question of how the AUA codon is read by an isoleucine tRNA without also reading the AUG codon for methionine.

The strategy used by various organisms to read isoleucine codons is kingdom-specific. Most eukaryotic cells contain two isoleucine tRNAs, the one with the anticodon IAU (tRNA\textsubscript{Ile}\textsubscript{IAU}; I = inosine) reads all three isoleucine codons following the Wobble hypothesis (3), whereas the other with the anticodon cAc (tRNA\textsubscript{Ile}\textsubscript{C9A9C9}; c = pseudo-uridine) is thought to read only AUA (6). A possible explanation for the presence of two tRNAs which can read AUA in eukaryotes is inefficient decoding of AUA by tRNA\textsubscript{Ile}\textsubscript{IAU} (7,8). Prokaryotes, which also contain two isoleucine tRNAs, have, however, evolved a different strategy for reading the three isoleucine codons. In most bacteria and archaebacteria, a tRNA with the anticodon GAU (tRNA\textsubscript{Ile}\textsubscript{GAU}), reads two of the isoleucine codons (AUU and AUC) following the Wobble hypothesis, whereas another tRNA with the anticodon C*AU reads the third isoleucine codon AUA. C* is derived from C and has been identified as lysidine in bacterial isoleucine tRNA (tRNA\textsubscript{2}Ile; L = lysidine) (9,10) and agmatidine in archaeal isoleucine tRNA (tRNA\textsubscript{2}Ile; C* = agmatidine) (11–13). In both cases, an amino acid, lysine (in bacteria) and a decarboxylated arginine (in archaebacteria), replaces the C2-oxo group of C\textsubscript{34}, the wobble base. The modification of C\textsubscript{34} to lysidine or agmatidine in tRNA\textsubscript{2}Ile results in a dual specificity switch of the tRNA in aminoacylation.
and in codon binding: while the unmodified tRNA with C34 is aminomethylated in vitro with methionine by methionyl-tRNA synthetase (MetRS) and reads the AUG codon, the modified tRNA is aminomethylated with isoleucine by isoleucyl-tRNA synthetase (IleRS) and reads the AUA codon (14–16).

Why have bacteria and archaea evolved a mechanism to use an isoleucine tRNA with a modified C34 in the anticodon to exclusively base pair with A instead of using an isoleucine tRNA with U34? Is it because a tRNA which contains U or a modified U in the wobble position cannot read the AUA codon without also misreading the AUG codon (4)? A possible answer to these questions could come from the analysis of codon recognition properties of isoleucine tRNAs from the very few bacterial and archaean species, whose genomes encode an isoleucine tRNA with the anticodon UAU (tRNAIleUAU) but not C*AU, such as Nanoarchaeum equitans, Korarchaeum sp., Mycoplasma mobile, Bifidobacterium adolescentis, Neorickettsia sennetsu and others [summarized in (17)]. These selected organisms are also distinguished by the absence of genes encoding tRNAIle-lysidine synthetase (TilS) in bacteria or tRNAIle-agmatidine synthetase (TiaS) in archaea, responsible for the biosynthesis of lysidine or agmatidine, respectively.

The recent isolation of a mutant tRNAIle gene in Bacillus subtilis in which the anticodon sequence GAT has been mutated to TAT (17) has provided us with the opportunity to study the properties, including the codon binding properties, of an isoleucine tRNA carrying U in the wobble position. This mutant tRNA, henceforth called mutant tRNAIleUAU, was isolated as a suppressor in a B. subtilis strain in which the gene encoding TilS had been deleted. In the absence of TilS, the wobble base of the isoleucine tRNA containing the CAU anticodon is expected to remain unmodified and cells depend on the mutant tRNAIleUAU for translation of the AUA codon. The availability of B. subtilis strains carrying the suppressor mutation in the isoleucine tRNA gene has allowed us to investigate (i) whether U34 in the mutant tRNA is modified, and (ii) whether the mutant tRNA is specific for AUA or also misreads AUG.

Here, we describe the characterization of two B. subtilis mutant strains lacking tilS and carrying the suppressor tRNA genes. We show that one of the mutant strains grows at a rate comparable to that of the wild-type strain. We also describe the purification and analysis of tRNAIle from this B. subtilis mutant strain. We have used biochemical and mass spectroscopic analyses to show that U34 in the wobble position of the mutant tRNAIleUAU is not modified. We also show that this mutant tRNA binds well to the AUA codon on B. subtilis ribosomes but binds only weakly to AUG, suggesting that it has an inherently low potential of misreading the AUG codon and of incorporating isoleucine in place of methionine. The B. subtilis strains deleted of tilS (17) used in this work represent the only examples of deletion of this essential gene. Therefore, in parallel, we have also purified and analyzed tRNAIle from one of the B. subtilis strains lacking tilS to confirm that it lacks the C34 to L34 base modification in the anticodon and has the expected aminomethylacylation and codon binding properties.

MATERIALS AND METHODS

Isolation of total tRNA from B. subtilis

Bacillus subtilis strains were grown in LB medium at 37°C with aeration; cells were harvested by centrifugation at an OD600 of 1.5–1.8 and immediately used for RNA isolation. All steps were carried out at 4°C unless otherwise noted. Bacillus subtilis cells from a 3 L-culture were pelleted and resuspended in 90 ml of extraction buffer [1 mM Tris–HCl or 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.5, 10 mM Mg(OAc)2]. A total of 100 ml of acid phenol (Ambion) was added and the suspension was placed on a nutator for 30 min. The mixture was centrifuged for 30 min at 10000g, the aqueous layer was adjusted to 1 M lithium chloride and left on ice for 2 h. After centrifugation (30 min, 10000g), tRNAs were recovered from the supernatant by precipitation with ethanol. The tRNA was washed extensively with 70% ethanol and resuspended in 10 mM HEPES, pH 7.5. Typically 250 to 350 A260 units of total tRNA, mostly free of rRNA, were obtained from a 3 L culture. The quality of the total tRNA preparation was confirmed by agarose gel electrophoresis and in vitro aminomethylation with isoleucine or methionine. For isolation of total tRNA under acidic conditions, cells were resuspended in ice-cold 0.1 M sodium acetate, pH 5.0. After extraction of total RNA using Trizol (Invitrogen) following the manufacturer’s instructions, large rRNAs were removed by precipitation with lithium chloride as described above. After several washes with ethanol, the tRNA was resuspended in 10 mM sodium acetate, pH 5.0, and stored at −80°C. A portion of the material was subjected to deacylation by the addition of Tris–HCl, pH 9.5, to a final concentration of 0.1 M. The deacylation reaction was performed at 37°C for 90–120 min; deacylated tRNAs were re-precipitated with ethanol and stored in 10 mM HEPES, pH 7.5.

Purification of isoleucine tRNAs from B. subtilis

Purification of individual isoleucine tRNAs was carried out essentially as described (18). Briefly, the tRNA of interest is purified in a two-step procedure involving (i) affinity chromatography using a 5’-biotinylated DNA oligonucleotide immobilized to streptavidin sepharose resin followed by (ii) purification of the highly enriched tRNA by electrophoresis on a native polyacrylamide gel. For ~400 A260 units of total tRNA, ~0.8 ml of streptavidin sepharose resin (Pharmacia) and 20 A260 units of the DNA oligonucleotide (IDT) complementary to nucleotides 54–76 of the tRNA of interest were used. tRNA and sepharose resin were mixed in a buffer containing 1.2 M NaCl, 30 mM HEPES, pH 7.5, and 15 mM ethylenediaminetetraacetic acid (EDTA) (6 × NHE). Following a denaturation step at 70°C for 30 min, the tRNA was allowed to bind to the oligonucleotide on the resin by lowering the temperature from 70°C to 30°C (~3°C/min). After 30 min at 30°C, the resin was washed...
several times with 3 × NHE buffer at 37°C. For elution of the bound tRNA, the resin was suspended in 0.5–1 ml of 0.1 × NHE buffer and incubated at 65°C for 5 min. The resin was centrifuged and the supernatant was collected as eluted tRNA. The elution step was repeated 10–15 times. Supernatant fractions containing tRNA were pooled, concentrated and used for electrophoresis on a native 10% polyacrylamide gel. The tRNA was detected by UV shadowing, eluted from the gel and dialyzed extensively against 5 mM ammonium acetate, pH 5.5. The dialyzed tRNA was concentrated by evaporation, precipitated with ethanol and the precipitate was washed several times with ethanol.

Analysis of *B. subtilis* tRNA by polyacrylamide gel electrophoresis and northern blotting

RNAs were analyzed by acid–urea polyacrylamide gel electrophoresis (PAGE) (19) and native PAGE (10–15% polyacrylamide in Tris/Borate/EDTA (TBE) buffer) followed by staining with ethidium bromide or northern blotting. The transfer of tRNA onto Hybond-XL (GE Healthcare) or Nytran SPC (Whatman) has been described (19). Northern blots were analyzed by autoradiography and phosphorimaging using Imagequant software.

Cloning and expression of *B. subtilis* IleRS and MetRS in *Escherichia coli*

The genes for *B. subtilis* MetRS and IleRS were amplified by polymerase chain reaction using *Pfu* Turbo DNA polymerase (Stratagene) from *B. subtilis* 168 genomic DNA and inserted into pET15b (Novagen) under control of the T7 promoter. *B. subtilis* MetRS and IleRS containing NH₂-terminal His₆-tags were expressed in *E. coli* BL21(DE3) and purified by affinity chromatography using Talon resin (Clontech) following the manufacturer’s protocol for batch–gravity flow purification of proteins.

*In vitro* aminoacylation and biotinylation of tRNA

0.002–0.5 A₂₆₀ of tRNA were aminoacylated *in vitro* with L-isoleucine or L-methionine as described below using purified *B. subtilis* IleRS or MetRS at a final concentration of 0.05 μM. Reaction mixtures contained: (i) for IleRS, 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 5 mM ATP, 0.1 μg/μl bovine serum albumin (BSA) and 100 μM L-isoleucine; and (ii) for MetRS, 50 mM imidazole, pH 7.6, 150 mM NH₄Cl, 15 mM MgCl₂, 10 mM ATP, 0.1 μg/μl BSA and 100 μM L-methionine. Incubation was at 37°C for 30–60 min. Reaction products were examined by acid–urea PAGE/northern blot analysis as described. Alternatively, *in vitro* aminoacylations were carried out in the presence of radiolabeled amino acids using 25 μM L¹⁴C-isoleucine (ARC), 5 μM L¹³H-isoleucine (ARC) or 20 μM L-methionine/L¹⁴C-methionine (Perkin Elmer). At various time points, aliquots were removed and analyzed by precipitation with trichloroacetic acid (TCA) followed by liquid scintillation counting of TCA-precipitable counts. *In vitro* biotinylation of tRNAs was as described before (12).

Template-dependent binding of aminoacylated tRNAs to ribosomes

The preparation of ribosomes from wild-type *B. subtilis* 168 and the binding of aminoacylated tRNAs to ribosomes were carried out essentially as described (20,21) with modifications as follows. For the preparation of ribosomes, *B. subtilis* cells were harvested in mid-log phase (OD₆₀₀ 0.5–0.6), resuspended in buffer A (20 mM HEPES, pH 7.4, 10 mM MgCl₂, 100 mM NH₄Cl and 6 mM 2-mercaptoethanol) and lysed by two passes through a French Press at 12000 psi. The resulting supernatant was centrifuged for 30 min at 30000g followed by ultracentrifugation at 100000g for 3 h. The ribosome pellet was washed with buffer B (20 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.5 M NH₄Cl and 6 mM 2-mercaptoethanol) by gentle shaking on ice. Ribosomes were re-pelleted and the final ribosome pellet was resuspended in buffer C (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 70 mM NH₄Cl, 30 mM KCl and 1 mM dithiothreitol), dialyzed extensively against the same buffer, divided into aliquots, flash frozen and stored at −80°C. All steps above were carried out at 4°C unless otherwise mentioned.

Prior to use, aliquots of frozen ribosome stock were thawed, “activated” by incubation at 42°C for 10 min and then cooled to room temperature (25°C). 2.5 μM ribosomes were pre-incubated with 0–300 μM mRNA (IDT) in buffer C for 5 min at room temperature in a 10 or 20 μl reaction. Radiolabeled aminoacylated tRNAs were added (~1500 cpm of ³H-Ile-tRNAs; 4000 cpm ³⁵S-Met-tRNAs), and incubation was continued for 30 min at room temperature. Reactions were terminated with 0.5 ml of ice-cold buffer C and filtered through nitrocellulose membranes (Millipore HA 0.45 μm). Preparation of ³H-Ile-tRNAs and ³⁵S-Met-tRNAs was as described above. The averages from three to five independent experiments are shown.

Analysis of ⁵'-³²P-labeled tRNA by partial RNase T1 and A digestion and alkali hydrolysis

Purified tRNAs were dephosphorylated with calf intestinal alkaline phosphatase (NEB) and subsequently labeled at the 5’ terminus with ³²P using T4-PNK (NEB) following standard procedures (22). After denaturing gel purification, ⁵'-³²P-labeled tRNA was mixed with non-radiolabeled tRNA and subjected to heat-denaturation at 65°C for 5 min in the respective reaction buffer suitable for partial RNase T1 or A digestion. Partial digestion of tRNA with RNase T1: 1 μg of tRNA in 50 mM Tris–HCl, pH 7.5, and 7 μM urea was pre-incubated at 50°C for 5 min before RNase T1 (10–15 U; Ambion) was added and samples were incubated at 50°C for 10 min. Partial digestion of tRNA with RNase A: 1 μg of tRNA in 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA was pre-incubated at 37°C for 5 min before RNase A (0.004 U; Ambion) was added and samples were...
incubated at 37°C for 5 min. Similarly, 1 μg tRNA was subjected to partial alkaline hydrolysis at 95°C for 2–4 min in a buffer containing 33 mM sodium carbonate/bicarbonate buffer, pH 9.2. Reactions were stopped by quick-freezing on dry ice, and reaction products were analyzed by denaturing 8–10% PAGE followed by autoradiography.

Analysis of 5′-32P-nucleotides by thin layer chromatography

For the analysis of 5′-32P-nucleotides isolated from 5′-32P-labeled fragments derived from nucleotides 34 to 37 of the anticodon loop, purified tRNAs were first subjected to partial alkali hydrolysis as described above, fragments produced were 5′-end labeled with 32P using T4-PNK (NEB) and run on a denaturing 8% polyacrylamide gel (23–26). Fragments corresponding to nucleotides 34–37 of the anticodon loop were eluted from the gel, digested with nuclease P1 and the 5′-terminal 32P-labeled nucleotide of each fragment was determined by thin layer chromatography (TLC) (22,27). The nuclease P1 (Sigma) digestion was carried out in 50 mM ammonium acetate, pH 5.0, for 6–8 h at 37°C. Samples were quick frozen on dry ice and lyophilized under vacuum. Cellulose F plates (Merck) were used for TLC analysis. Two different solvent systems were used for separation: (A) isobutyric acid:concentrated ammonia:water (66:1:33) (v:v:v) and (B) isopropanol:concentrated HCl:water (70:15:15) (v:v:v). For two dimensional analysis, samples were first run in solvent A for 16 h, plates were dried overnight and then run in solvent B for 28 h. TLC plates were analyzed by autoradiography. Non-radiolabeled nucleotide standards were added to samples and visualized by UV shadowing.

Mass spectral analysis of purified isoleucine tRNAs

For oligonucleotide sequence analysis, 1 μg of tRNA was digested with 50 U of RNase T1 (Worthington Biochemical Corp.) in 20 mM ammonium acetate, pH 5.3, for 2 h at 37°C. The digestion products were separated using a Thermo Surveyor HPLC system with a Waters XBridge C18 1.0 × 150 mm column at 40 μl/min with a gradient of 400 mM 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), 8.15 mM triethylamine (TEA), pH 7.0, and 400 mM HFIP, 8.15 mM TEA:methanol (50:50) (v:v), pH 7.0. The eluent was directed into a Thermo LTQ-XL using a capillary temperature of 275°C, spray voltage of 4.5 kV, sheath gas, auxiliary gas and sweep gas at 25, 14 and 10 arbitrary units, respectively. Collision-induced dissociation (CID) tandem mass spectrometry with a normalized collision energy of 35% was used in data-dependent mode to obtain sequence information from the RNase T1 digestion products as previously described (28). The data-dependent scan was performed on the most abundant ions and each ion was selected for CID for up to 15 scans for 30 s before it was put on a dynamic exclusion list for 30 s.

RESULTS

Characterization and growth phenotypes of B. subtilis strains lacking tilS

Wild-type B. subtilis 168 contains a total of four genes for two isoleucine tRNAs (29), three of which encode tRNA1Ile (anticodon: GAU; tRNA1IleGUA; Figure 1A) for translation of AUC and AUU codons, and one of which encodes tRNA2Ile (anticodon: CU, which is post-translationally modified to LAU, L = lysidine; tRNA2IleLAU; Supplementary Figure S1A) for translation of the AUA codon. Recently, two mutant strains of B. subtilis, JJS80 and EDJ958, were isolated in which the gene encoding TilS had been deleted (17). The resulting absence of TilS activity leads to a lack of modification of C34 to lysidine in tRNA1IleLAU, i.e. synthesis of tRNA1IleCAU, thereby rendering it inactive for translation of AUA codons. The survival of these B. subtilis strains was ensured through the presence of a mutant tRNA derived from one of the three tRNA1IleLAU genes with a change in the anticodon sequence from GAT to TAT (Supplementary Table S1), allowing for the synthesis of a new cellular tRNA1Ile species with a UAU anticodon (tRNA1IleUAU), most likely responsible for translation of AUA codons.

Bacillus subtilis mutant strains JJS80 and EDJ958 were grown in LB medium at 37°C and their growth was monitored over a 12 h period. The mutant strain JJS80, in which the trnQ gene carries the GAT to TAT mutation (Supplementary Table S1), grows almost wild-type like, while the mutant strain EDJ958, in which the trnB gene carries the same GAT to TAT mutation (Supplementary Table S1) grows slower and saturates at a lower cell density (Figure 1B). Total tRNA was isolated from both mutant strains and analyzed by PAGE followed by northern blot hybridization using a probe directed against the 5′ terminal portion of tRNA1Ile which detects both wild-type and mutant tRNA1Ile species. Separation on a 15% native polyacrylamide gel allowed the visualization of a slower migrating tRNA species consistent with the presence of the tRNA1IleUAU (Figure 1C). The abundance of tRNA1IleUAU varied substantially between JJS80 and EDJ958 and reflects most likely different transcription efficiencies for the different chromosomal loci from which the mutant tRNA1IleUAU is derived from in the respective strain. Since the mutant strain JJS80 consistently showed better growth than EDJ958 and produced more of tRNA1IleUAU, all experiments described below were carried out with JJS80.

Aminoacylation properties of tRNA1Ile and tRNA2Ile from wild-type B. subtilis 168 and mutant strain JJS80 lacking tilS

Total tRNA was isolated from B. subtilis JJS80, under acidic conditions, deacylated and re-aminocylated using purified recombinant aminoacyl-tRNA synthetases (Figure 2). tRNAs were analyzed by acid–urea PAGE/northern blotting. tRNA1Ile from both the wild-type and mutant strain are aminoacylated with isoleucine by IleRS (Figure 2A). Although tRNA1Ile and tRNA2Ile could
not be clearly separated under acid–urea PAGE conditions, it is noteworthy that total tRNA1Ile iso-
lar from JJS80 contains two additional faster migrating species, which most likely correlate with different modifications at position 37 (see below) and/or conformational variants of the mutant tRNA1IleUAU.

tRNA2Ile from the mutant strain showed a clear shift in mobility by acid–urea PAGE compared to wild-type tRNA2Ile, consistent with the lack of lysidine at position 34 (Figure 2B, compare lanes 1 and 6). Also, tRNA2Ile from the mutant strain showed a significant amount of deacylation during isolation and work-up compared to wild-type tRNA2Ile, possibly indicative of a change in amino acid specificity from isoleucine to methionine since the ester link between methionine and tRNA is much weaker than the ester link between isoleucine and tRNA (30). However, it is also possible that tRNA2Ile from the mutant strain is not as good a substrate for aminoacylation in vivo and, therefore, appears only partially aminoacylated. As expected, tRNA2Ile from the wild-type B. subtilis strain could be aminoacylated in vitro with isoleucine but not with methionine (Figure 2B, lanes 3 and 4), whereas tRNA2Ile from JJS80 could not be aminoacylated with isoleucine but could be aminoacylated instead with methionine (Figure 2B, lanes 8 and 9). Furthermore, the presence of
lysidine in wild-type tRNA$_{\text{Ile}}$$^{\text{GAU}}$ could be confirmed by reaction of the free NH$_2$ group in lysidine with an N-hydroxysuccinimide ester derivative of biotin giving rise to a clear mobility shift (12); in contrast, tRNA$_{\text{Ile}}$$^{\text{LAU}}$ from the mutant strain could not be biotinylated due to the lack of lysidine (Figure 2B, compare lanes 5 and 10).

**Purification of tRNA$_{\text{Ile}}$$^{\text{GAU}}$** and tRNA$_{\text{Ile}}$$^{\text{LAU}}$ from wild-type B. subtilis 168 and mutant strain JJS80 lacking tilS

Wild-type and mutant isoleucine tRNA$_{\text{Ile}}$$^{\text{GAU}}$ and tRNA$_{\text{Ile}}$$^{\text{LAU}}$ were purified from the _B. subtilis_ wild-type strain 168 and mutant strain JJS80, respectively. The extent of purification of various tRNAs was assessed by in vitro aminoacylation using _B. subtilis_ IleRS and MetRS, with $^{14}$C-isoleucine or $^{35}$S-methionine acceptance higher than 1200 pmole/A$_{260}$ unit for all tRNAs (Supplementary Table S2). The homogeneity of purified tRNAs was further verified by partial RNase T1 and RNase A digestion of $^5$P-labeled tRNAs (31–33) (Figure 3A and Supplementary Figure S1B), confirming that none of the samples contained detectable levels of contaminating tRNAs except for the desired mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$ (see below).

In general, 2.5–3 A$_{260}$ units of purified tRNA$_{\text{Ile}}$$^{\text{GAU}}$ and 0.5 A$_{260}$ of purified tRNA$_{\text{Ile}}$$^{\text{LAU}}$ or tRNA$_{\text{Ile}}$$^{\text{CAU}}$ were obtained from ~400 A$_{260}$ of total RNA. Although, wild-type tRNA$_{\text{Ile}}$$^{\text{GAU}}$ and mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$ could be separated by native PAGE (Figures 1C and 3B) under analytical conditions, we were unable to do so under preparative conditions. Therefore, most of the in vitro experiments described in this study, except in the last section, were carried out with a mixture of both wild-type and mutant tRNA, which herein is referred to as tRNA$_{\text{Ile}}$$^{\text{GAU/UAU}}$. Typically, 2.5 A$_{260}$ of purified tRNA$_{\text{Ile}}$$^{\text{GAU/UAU}}$ were obtained from ~400 A$_{260}$ of total RNA; based on the intensity of ethidium bromide-stained bands or northern blot (Figure 3B, lanes 2 and 4), this sample contained ~50% of the mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$ species as estimated by the analytical PAGE analysis.

**Characterization of tRNA$_{\text{Ile}}$$^{\text{LAU}}$ from B. subtilis lacking tilS**

The absence of lysidine at position 34 of the mutant tRNA$_{\text{Ile}}$$^{\text{CAU}}$ was further established by TLC analysis of the nucleotide at the wobble position as described in ‘Materials and Methods’ section. Figure 4A shows a clear difference in the mobility of lysidine originating from the wild-type tRNA$_{\text{Ile}}$$^{\text{LAU}}$ and the mobility of the unmodified C from the mutant tRNA$_{\text{Ile}}$$^{\text{CAU}}$. The mobility of the unmodified C was confirmed by non-radiolabeled standards that were run in parallel and visualized by UV shadowing.

Template-dependent binding of purified and aminoacylated $^3$H-Ile-tRNA$_{\text{Ile}}$$^{\text{LAU}}$ and $^{35}$S-Met-tRNA$_{\text{Ile}}$$^{\text{CAU}}$ to _B. subtilis_ ribosomes was performed with five different oligonucleotides; AUG AUU, AUG AUC, AUG AUG, AUG AUU and AUG UUU. Wild-type tRNA$_{\text{Ile}}$$^{\text{LAU}}$ showed binding to AUG and weaker binding to AUC and AUU; in contrast, tRNA$_{\text{Ile}}$$^{\text{CAU}}$ isolated from the tilS mutant strain showed binding only to AUG (Figure 4B and C).

**Characterization of U34 in the mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$**

As described above, mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$ was purified from JJS80 as a mixture of wild-type tRNA$_{\text{Ile}}$$^{\text{GAU}}$ and mutant tRNA$_{\text{Ile}}$$^{\text{UAU}}$ (tRNA$_{\text{Ile}}$$^{\text{GAU/UAU}}$; Figure 3B) and most in vitro experiments were carried out by direct side-by-side...
A comparison of the tRNA\textsubscript{1Ile} GAU and tRNA\textsubscript{1Ile} GAU=UAU. To verify the presence of tRNA\textsubscript{1Ile} GAU=UAU in the purified sample, purified 5\textsuperscript{32}P-labeled tRNAs were subjected to partial digestion with RNases T1 and A (31–33) (Figure 3A). While RNase T1 digests confirmed the presence of G at position 34 of both the tRNA\textsubscript{1Ile} GAU and the tRNA\textsubscript{1Ile} GAU=UAU preparations (Figure 3A, lanes 3 and 6), digests with RNase A confirmed the presence of U\textsubscript{34} in the tRNA\textsubscript{1Ile} GAU=UAU but not in the tRNA\textsubscript{1Ile} GAU preparation (Figure 3A, compare lanes 2 and 7).

The nature of U\textsubscript{34} in the mutant tRNA\textsubscript{1Ile} UAU was examined by mass spectral (MS) and TLC studies. First, purified tRNA\textsubscript{1Ile} GAU and tRNA\textsubscript{1Ile} GAU=UAU were completely digested with RNase T1 and the fragments produced were subjected to LC-MS/MS analysis. Due to the mutation in the anticodon from G\textsubscript{34} to U\textsubscript{34}, digestion with RNase T1 yields a different cleavage pattern for the wild-type and the mutant tRNA preparations (Table 1 and Supplementary Figure S2). In case of the wild-type tRNA, the anticodon loop is cleaved into two fragments, CCUGp and AUAAGp, while the mutant tRNA produces a mixture of three related 9-mers containing CCUUAUAAGp (Table 1). LC-MS/MS analysis showed clearly, that U\textsubscript{34} in the mutant tRNA is not modified (Table 1 and Supplementary Figure S3), unless it is modified to \textsuperscript{c}34, a mass-silent modification. Also, the modification at position 37 showed some interesting heterogeneity; in addition to \textsuperscript{t6A}, both \textsuperscript{m6A} and unmodified A are present in RNase T1 digests of the mutant but not of the wild-type tRNA (Table 1 and Supplementary Figure S3).

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Based on the results obtained from the RNase T1/MS analysis for the entire tRNA molecule (Supplementary Table S3), cloverleaf structures for wild-type and mutant tRNA\textsubscript{1Ile} were assembled (Figure 1A). These results were verified independently by TLC analyses of 5\textsuperscript{32}P-nucleotides isolated from 5\textsuperscript{32}P-labeled fragments derived from nucleotides 34 to 37 of the anticodon loop of both wild-type and mutant tRNA\textsubscript{1Ile} (Figure 3A and Supplementary Figure S3). Both, 1D and 2D-TLC corroborated and extended the results obtained from the RNase T1/MS analysis: (i) the mutant tRNA\textsubscript{1Ile} UAU contains an unmodified U\textsubscript{34} and not \textsuperscript{c}34, which separates clearly from U\textsubscript{34}(Figure 5A and Supplementary Figure S4); and (ii) the presence of unmodified A and two different modifications, \textsuperscript{m6A} and \textsuperscript{t6A}, at position 37 were confirmed in the mutant.

Table 1. RNA sequencing of the anticodon loop of wild-type tRNA\textsubscript{2Ile} GAU and mutant tRNA\textsubscript{2Ile} GAU=UAU by MS analysis of RNase T1 fragments

<table>
<thead>
<tr>
<th>tRNA fragment</th>
<th>Sequence</th>
<th>Observed m/z</th>
<th>Observed-expected m/z</th>
<th>Wild-type tRNA\textsubscript{2Ile} GAU</th>
<th>Mutant tRNA\textsubscript{2Ile} GAU=UAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>C31-G34</td>
<td>CCUGp</td>
<td>638.75</td>
<td>0.17</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A35-G39</td>
<td>AU[t6A]AGp</td>
<td>899.83</td>
<td>0.07</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C31-G39</td>
<td>CCUUUAU[m6A]Agp</td>
<td>1438.25</td>
<td>−0.03</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C31-G39</td>
<td>CCUUUAU[t6A]Agp</td>
<td>1511.75</td>
<td>−0.08</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Modified nucleosides: \textsuperscript{t6A} (\textsuperscript{N6-threonylcarbamoyladenosine}); \textsuperscript{m6A} (\textsuperscript{N6-methyladenosine}).

Note that the mutant tRNA\textsubscript{2Ile} UAU sample is a mixture of wild-type tRNA\textsubscript{2Ile} GAU and mutant tRNA\textsubscript{2Ile} GAU=UAU. Fragments that are unique within the mutant sample are highlighted in gray. The analysis of RNase T1-fragments derived from the entire tRNA molecule is shown in Supplementary Table S3.
Visualized by UV shadowing. Note that the mutant tRNA^Ile was confirmed with non-radiolabeled standards used as internal markers and a mixture of wild-type tRNA^Ile in 66:1:33 (v:v:v) solvent for the first dimension and an isopropanol:concentrated HCl:water (70:15:15) for the second dimension. The mobility of each nucleotide (pA, pC, pG, pU, pm6A, pt6A) was confirmed with non-radiolabeled standards used as internal markers and visualized by UV shadowing. Note that the mutant tRNA^Ile sample is a mixture of wild-type tRNA^Ile and mutant tRNA^Ile.

tRNA^Ile, whereas wild-type tRNA^Ile contained mostly t^A and some A (Figure 5B and Supplementary Figure S4). Since MS analysis of RNase T1 digests of wild-type tRNA^Ile indicated no unmodified A at position 37, the presence of a small amount of A at this position is most likely due to some contamination by the neighboring fragment on the polyacrylamide gel, carrying an A at position 38.

Codon binding properties of the mutant tRNA^Ile in the presence of B. subtilis ribosomes

Template-dependent binding of purified and aminoacylated wild-type and mutant 3H-Ile-tRNA^Ile to B. subtilis ribosomes was performed. As expected, wild-type tRNA^Ile showed binding to AUC and AUU codons and to a very minor extent to AUA (Figure 6A). In contrast, the mutant tRNA^Ile, which is a mixture of tRNA^Ile and tRNA^Ile, bound strongly to AUA, AUC and AUU (Figure 6B). Compared to the negative control (UUU-containing mRNA), the mutant tRNA also showed slightly elevated but weak binding to the methionine codon AUG (Figure 6B).

To remove the wild-type tRNA^Ile from the mixture of wild-type and mutant tRNA^Ile, tRNA^Ile was treated with RNase T1 in the presence of Mg^{++} under mild conditions to inactivate specifically the wild-type tRNA^Ile by cleavage at position 34 (35). RNase T1 treatment of tRNA^Ile produced a ‘nicked’ tRNA, which was inactive in both in vitro aminoacylation (Supplementary Figure S5A) and binding to ribosomes (Supplementary Figure S5C). The efficiency of RNase T1 cleavage at G34 is clearly demonstrated by the fact that aminoacylated wild-type Ile-tRNA^Ile lost essentially all ribosome binding activity subsequent to RNase T1-treatment (Supplementary Figure S5C; black bars). In contrast, binding of the mutant tRNA^Ile containing U34 to AUA is resistant to this treatment (Figure 6C). RNase T1 treatment of tRNA^Ile to a loss of isoleucine acceptance of ~40% (Supplementary Figure S5B), consistent with the level of contaminating wild-type tRNA^Ile species (Figure 3B). The resulting highly enriched mutant tRNA^Ile showed strong binding to AUA and weak binding to AUG and AUU, while binding to AUC was completely eliminated (Figure 6C; black bars).

Ribosome binding of the highly enriched tRNA^Ile sample was also carried out in the presence of an equimolar amount of competitor tRNA, in this case Met-tRNA^Met which reduced binding of Ile-tRNA^Ile to AUG, but not AUA or AUU, to background levels (Figure 6D; hatched bars). Weak binding to the AUA-containing mRNA leaves open the possibility that the mutant tRNA^Ile can base-pair, albeit weakly, with an AUU codon.

We also performed ribosome binding assays using the mutant tRNA^Ile and ribosomes isolated from E. coli. The mutant Ile-tRNA^Ile showed similar binding to the AUU codon with ribosomes from B. subtilis and E. coli. In contrast, binding of the mutant Ile-tRNA^Ile to the AUG codon was up about 3-fold with E. coli ribosomes (Supplementary Figure S6).

**DISCUSSION**

Characterization of the B. subtilis mutant tRNA^Ile

Strains of B. subtilis carrying a deletion in the gene encoding the essential tRNA^Ile modifying enzyme TilS can survive only in the presence of a mutant tRNA^Ile, whose anticodon wobble position has been changed from G34 to U34 (Supplementary Figure S4). The resulting highly enriched mutant tRNA^Ile showed strong binding to AUA and weak binding to AUG and AUU, while binding to AUC was completely eliminated (Figure 6C; black bars).

Ribosome binding of the highly enriched tRNA^Ile sample was also carried out in the presence of an equimolar amount of competitor tRNA, in this case Met-tRNA^Met which reduced binding of Ile-tRNA^Ile to AUG, but not AUA or AUU, to background levels (Figure 6D; hatched bars). Weak binding to the AUA-containing mRNA leaves open the possibility that the mutant tRNA^Ile can base-pair, albeit weakly, with an AUU codon.

We also performed ribosome binding assays using the mutant tRNA^Ile and ribosomes isolated from E. coli. The mutant Ile-tRNA^Ile showed similar binding to the AUU codon with ribosomes from B. subtilis and E. coli. In contrast, binding of the mutant Ile-tRNA^Ile to the AUG codon was up about 3-fold with E. coli ribosomes (Supplementary Figure S6).
lacking one or more of the determinants necessary for its modification by any of the U34 modifying enzymes in *B. subtilis*.

Interestingly, while the wild-type tRNA\textsubscript{1Ile} \textsubscript{GAU} contains the modified nucleoside \textit{t}6\textit{A} at position 37 next to the anticodon, the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} contains a mixture of A, \textit{t}6\textit{A} and \textit{m}6\textit{A} at this position. It is worth noting that several of the *B. subtilis* tRNAs, including tRNA\textsubscript{2Ile} \textsubscript{LAU} (36), contain \textit{m}6\textit{A} at position 37 (37). The presence of A, \textit{t}6\textit{A} and \textit{m}6\textit{A} at position 37 suggests that mutation of G34 to U34 has made the tRNA a poorer substrate for the \textit{t}6\textit{A} enzyme complex TsaB/C/D (38,39), so that A37 is either unmodified, modified to \textit{t}6\textit{A} or methylated to \textit{m}6\textit{A} by \textit{t}RNA\textsubscript{adenine N\textsubscript{6}-methyltransferase}, presumably a homolog of *E. coli* YfiC (40). In bacteria, archaea and eukaryotes, tRNAs that are substrates for the \textit{t}6\textit{A} enzyme usually contain G, C or a modified U in the anticodon wobble position (41). Therefore, why mutation of G34 to U34 has made the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} a poorer substrate for the \textit{t}6\textit{A} enzyme is not obvious, unless it is the lack of modification of U34.

**Figure 6.** Template-dependent binding of purified wild-type \textsuperscript{3H}Ile-tRNA\textsubscript{1Ile} \textsubscript{GAU} and mutant \textsuperscript{3H}Ile-tRNA\textsubscript{1Ile} \textsubscript{UAU} to ribosomes isolated from *Bacillus subtilis*. Oligonucleotides used were AUG AUA, AUG AUC, AUG AUG, AUG AUU and AUG UUU. (A) Wild-type tRNA\textsubscript{1Ile} \textsubscript{GAU} and (B) mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} sample; note that the mutant sample is a mixture of wild-type tRNA\textsubscript{1Ile} \textsubscript{GAU} and mutant tRNA\textsubscript{1Ile} \textsubscript{UAU}. (C) The mutant \textsuperscript{3H}Ile-tRNA\textsubscript{1Ile} \textsubscript{UAU} sample was treated with RNase T1 under native conditions to specifically inactivate the wild-type \textsuperscript{3H}Ile-tRNA\textsubscript{1Ile} \textsubscript{GAU} by cleavage at G34; the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} containing U34 is resistant to this treatment; (D) an equimolar amount of non-radioactive competitor Met-tRNA\textsubscript{2Ile} \textsubscript{CAU} was added to RNase T1-treated mutant tRNA. The oligonucleotide concentration in (C) and (D) was 200 \textmu M. Note that in (D) the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} sample was treated with RNase T1 first, followed by aminoacylation with \textsuperscript{3H}Ile, resulting in a doubling of \textsuperscript{3H}Ile-tRNA\textsubscript{1Ile} \textsubscript{UAU}-specific counts present in the ribosome binding experiments.

Aminoacylation of mutant *B. subtilis* tRNA\textsubscript{1Ile} \textsubscript{UAU}

Mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} is a good substrate for aminoacylation in vivo and in vitro by *B. subtilis* IleRS. Suzuki et al. showed recently that a particular arginine residue in *M. mobile* IleRS is important for aminoacylation of *M. mobile* tRNA\textsubscript{2Ile} \textsubscript{UAU} (42). It is, therefore, interesting to note that *B. subtilis* IleRS also has an arginine at the corresponding position, whereas *E. coli* IleRS has tryptophan.

Codon recognition properties of the *B. subtilis* mutant tRNA\textsubscript{1Ile} \textsubscript{UAU}

Binding experiments using *B. subtilis* ribosomes show that tRNA\textsubscript{1Ile} \textsubscript{UAU} binds mostly to the AUA codon and only weakly to the AUU and AUG codons (Figure 6C, AUA >>> AUU > AUG). Thus, the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} has an inherently low potential for misreading the methionine codon AUG and for inserting isoleucine in the place of methionine into proteins in vivo. This result explains the finding that the *B. subtilis* strain carrying the mutant tRNA\textsubscript{1Ile} \textsubscript{UAU} is viable and shows only a minimal level of
mis-incorporation of isoleucine in the place of methionine in a reporter protein (17). The level of mis-incorporation of isoleucine in the place of methionine of 10\(^{-3}\) to 1 appears to be well tolerated in B. subtilis, since the suppressor strain JSS80 grows at a rate comparable to that of the wild-type strain (Figure 1B). The ribosome binding results in vitro also indicate that the low level read-through of AUG by the mutant tRNA\(_{\text{UAU}}^{\text{Ile}}\) is more due to the inherently weak affinity of tRNA\(_{\text{UAU}}^{\text{Ile}}\) for the AUG codon than competition in vivo by the endogenous methionine tRNA.

The weak affinity of tRNA\(_{\text{UAU}}^{\text{Ile}}\) for the AUG codon on the ribosome is reminiscent of several tRNAs containing an unmodified U34 which read codons ending in A much better than to those ending in G (43–45). For example, transcripts of E. coli tRNA\(_{\text{Ser}}^{\text{Ser}}\), which contain U34 instead of the modified U normally present in the tRNA\(_{\text{Ser}}^{\text{Ser}}\); read the serine codon UCA just as well in an E. coli protein synthesis system but not UCG (46,47). Similarly, an anticodon stem loop fragment of human lysine tRNA carrying U34 binds to the lysine codon AAA but not to AAG on E. coli ribosomes (48). Yet another example comes from the analysis of human mitochondrial leucine tRNA, where replacement of 5-taurinomethyl modification of U normally present in the anticodon wobble position by U34 results in a severe deficiency in reading the leucine codon UUG without any effect on reading the UUA codon (49).

The highly preferential reading of codons ending in A by a tRNA with an unmodified U34 is, however, not universal. Although the U34 in the anticodon wobble position is almost always modified in tRNAs that have been sequenced (37,41,50), there are several exceptions. For various reasons including genome compactions, parasitic lifestyles etc., fungal, insect and vertebrate mitochondria and most Mycoplasma species contain a few number of tRNAs to read all the codons of the genetic code (51–57). In these cases, a single tRNA containing an unmodified U34 is used to read all four codons of a four-codon box specifying the same amino acid, instead of using at least two tRNAs for the same purpose as most bacterial, eukaryotic and archaeal systems do. Thus, mitochondrial and Mycoplasma ribosomes and tRNAs have coevolved to allow an unmodified U34 to base pair with U, C, A or G on the ribosome.

A number of studies including work presented here suggest that the codon reading properties of a tRNA containing an unmodified U34 depend upon several factors including the nature of the ribosome, the tRNA sequence and the tRNA anticodon loop structure. A recent example of both the ribosome and tRNA playing important roles in the decoding properties of a tRNA comes from an AUA-reading tRNA\(_{\text{UAU}}^{\text{Ile}}\) isolated from M. mobile (42). As mentioned above, in contrast to the AUA codon-specific tRNA\(_{\text{UAU}}^{\text{Ile}}\) isolated from most bacterial or archaea which contain lysidine or agmatidine, the M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\) contains an unmodified U (tRNA\(_{\text{UAU}}^{\text{Ile}}\)). Suzuki et al. have shown that the M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\) binds to the isoleucine codon AUG on M. mobile ribosomes. However, the same tRNA binds to both AUU and AUG on E. coli ribosomes. Thus, the M. mobile ribosome and tRNA\(_{\text{UAU}}^{\text{Ile}}\) have coevolved in such a way that the M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\), containing an unmodified U in the anticodon wobble position is restricted from binding to AUG, even though other M. mobile tRNAs containing an unmodified U34 read all four codons of a four-codon box. It would be interesting to see whether the M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\) binds only to AUU or to both AUU and AUG codons on ribosomes isolated from other Mycoplasma species. The specific binding of M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\) to AUU and not to AUG on M. mobile ribosomes highlights the importance of tRNA sequence and/or structure for the specificity of codon recognition. This may also apply to the mutant tRNA\(_{\text{UAU}}^{\text{Ile}}\) isolated from B. subtilis described in the present study, which binds preferentially to AUU, but only weakly to AUG on B. subtilis ribosomes. Similar to the M. mobile tRNA\(_{\text{UAU}}^{\text{Ile}}\), the B. subtilis mutant tRNA\(_{\text{UAU}}^{\text{Ile}}\) shows elevated binding to the AUG codon on E. coli ribosomes (Supplementary Figure S6), although the effect is not as pronounced as for the M. mobile tRNA (42).

Perhaps, the strongest evidence that tRNA sequence and/or structure can affect the codon reading properties of U34 comes from studies of a mutant E. coli glycine tRNA, which contains an unmodified U34 in the anticodon wobble position. Lagerkvist et al. have shown that this tRNA reads the glycine codons GGA and GGG on E. coli ribosomes but not the other glycine codons GGU or GGC (58). Remarkably, however, a single change of U32 in the anticodon loop to C32, found in Mycoplasma mycoides glycine tRNA, now allowed the tRNA to read all four glycine codons on E. coli ribosomes (59). In a reciprocal experiment, it was shown that while the M. mycoides glycine tRNA containing U34 can read all four glycine codons on E. coli ribosomes, a mutant tRNA in which C32 is changed to U32 reads only GGA and GGG but not GGU or GGC. These results show that like the mitochondrial and the Mycoplasma systems mentioned above, E. coli ribosomes also have the potential for allowing an unmodified U34 in the anticodon wobble position to base pair with U, C, A or G. However, whether this happens or not depends on the tRNA sequence and/or the tRNA anticodon loop structure (60), with the presence of C32 in the anticodon loop being a critical determinant in the case of E. coli and M. mycoides glycine tRNAs. The importance of the anticodon context in tRNA on efficiency and accuracy of codon reading was pointed out several years ago (61) and has since then been well established (62,63).

Finally, in experiments parallel to the work described in here, we have mutated C34, a precursor of agmatidine, in the anticodon wobble position of Haloarcula marismortui tRNA\(_{\text{UAU}}^{\text{Ile}}\) to U34 and have purified the mutant tRNA and studied its codon recognition properties using H. marismortui ribosomes. We have found that U34 in the mutant tRNA is modified and that the modified tRNA binds not only to AUU but also to AUG and AUU (AUU > AUG > AUU) (Mandal, D. and Köhrer, C. et al., unpublished data).
SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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