Life without tRNA\textsuperscript{Ile}-lysidine synthetase: translation of the isoleucine codon AUA in \textit{Bacillus subtilis} lacking the canonical tRNA\textsubscript{2}\textsuperscript{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}\textsuperscript{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\textsubscript{Ile}-lysidine synthetase was deleted for the first time. In such a strain, C34 at the wobble position of tRNA\textsubscript{2}\textsuperscript{Ile} is expected to remain unmodified and cells depend on a mutant suppressor tRNA derived from tRNA\textsubscript{1}\textsuperscript{Ile}, in which G34 has been changed to U34. An important question, therefore, is how U34 base pairs with A without also base pairing with G. Here, we show (i) that unlike U34 at the wobble position of all \textit{B. subtilis} tRNAs of known sequence, U34 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\textsuperscript{Ile}\.\textsubscript{IAU}; I = inosine) reads all three isoleucine codons following the Wobble hypothesis (3), whereas the other with the anticodon ψAψ (tRNA\textsuperscript{Ile}\.\textsubscript{ψAU}; ψ = pseudouridine) 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\textsuperscript{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 archaea, a tRNA with the anticodon GAU (tRNA\textsuperscript{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\textsuperscript{Ile}\.\textsubscript{LAU}; L = lysidine) (9,10) and agmatidine in archaeal isoleucine tRNA (tRNA\textsuperscript{Ile}\.\textsubscript{CAU}; C = agmatidine) (11–13). In both cases, an amino acid, lysine (in bacteria) and a decarboxylated arginine (in archaea), replaces the C2-oxo group of C34, the wobble base. The modification of C34 to lysidine or agmatidine in tRNA\textsuperscript{2}\textsubscript{Ile} results in a dual specificity switch of the tRNA in aminoacylation.
and in codon binding: while the unmodified tRNA with C34 is aminoaacylated in vitro with methionine by methionyl-tRNA synthetase (MetRS) and reads the AUG codon, the modified tRNA is aminoaacylated 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 (tRNA_{Ile}^{C34})

Biochemical and mass spectroscopic analyses to show that the AUA codon (14–16).

The availability of strains deleted of the isoleucine tRNA containing the CAU anticodon is expected to remain unmodified and cells depend on the isoleucine tRNA carrying the UAU anticodon. This mutant tRNA, henceforth called mutant tRNA_{Ile}^{C34}, was isolated as a suppressor in a B. subtilis strain. We have used biochemical and mass spectroscopic analyses to show that the AUA codon (14–16).

In parallel, we have also purified and analyzed tRNA_{Ile}^{C34} as the major isoleucine tRNA in the B. subtilis genome. We show that one of the mutant strains lacking the gene encoding TilS had the isoleucine tRNA with the anticodon sequence GAT (B. subtilis, Korarchaeum sp., 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 tRNA_{Ile}^{C34}-lysidine synthetase (TilS) in bacteria or tRNA_{Ile}^{C34}-agmatidine synthetase (TiaS) in archaea, responsible for the biosynthesis of lysidine or agmatidine, respectively.

The recent isolation of a mutant tRNA_{Ile}^{C34} 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 tRNA_{Ile}^{C34}, 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 tRNA_{Ile}^{C34} 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 tRNA_{Ile}^{C34} 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 tRNA_{Ile}^{C34} 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 tRNA_{Ile}^{C34} 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 aminoaacylation 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 OD_{600} 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 10000 g, the aqueous layer was adjusted to 1 M lithium chloride and left on ice for 2 h. After centrifugation (30 min, 10000 g), 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 A$_{260}$ 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 aminoaacylation 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 A$_{260}$ units of total tRNA, ~0.8 ml of streptavidin sepharose resin (Pharmacia) and 20 A$_{260}$ 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 × HNE). 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 SPV (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 L-14C-isoleucine (ARC), 5 μM L-3H-isoleucine (ARC) or 20 μM L-methionine/L-35S-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 (OD590 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 12,000 psi. The resulting supernatant was centrifuged for 30 min at 30,000g followed by ultracentrifugation at 100,000g 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 for collection of mass spectra 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; tRNA1Ile) for translation of AUC and AUU codons, and one of which encodes tRNA2Ile (anticodon: CAU, which is post-translationally modified to LAU; tRNA2Ile; 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 tRNA2Ile, (i.e. synthesis of tRNA2Ile) 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 tRNA1Ile 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 aUAU anticodon (tRNA1IUUAU), 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 trnO 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 32P-labeled nucleotide of each tRNA. The digestion products were separated by acid–urea PAGE followed by autoradiography. Non-radiolabeled nucleotide standards were added to samples and visualized by UV shadowing.

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-aminoacylated using purified recombinant aminoaecyl-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 tRNA isolated 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 tRNA\textsubscript{\text{1Ile}}\textsubscript{UAU}.

The tRNA\textsubscript{2Ile} from the mutant strain showed a clear shift in mobility by acid–urea PAGE compared to wild-type tRNA\textsubscript{2Ile}, consistent with the lack of lysidine at position 34 (Figure 2B, compare lanes 1 and 6). Also, tRNA\textsubscript{2Ile} from the mutant strain showed a significant amount of deacylation during isolation and work-up compared to wild-type tRNA\textsubscript{2Ile}, 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 tRNA\textsubscript{2Ile} from the mutant strain is not as good a substrate for aminoacylation in vivo and, therefore, appears only partially aminoacylated. As expected, tRNA\textsubscript{2Ile} 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 tRNA\textsubscript{2Ile} 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

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**Figure 1.** tRNA\textsubscript{1Ile} in Bacillus subtilis wild-type strain 168 and mutant strains JJS80 and EDJ958 lacking tilS. (A) Cloverleaf structure of wild-type tRNA\textsubscript{1Ile}\textsubscript{GAU}. The changes in mutant tRNA\textsubscript{1Ile}\textsubscript{UAU} are indicated. Location of modified nucleosides is based on TLC and LC-MS analysis presented in this study. D (dihydouridine); t'\text{A} (N\textsuperscript{2}-threonylcarbamoyladenosine); m\textsuperscript{6}A (N\textsuperscript{6}-methyladenosine); m\textsuperscript{7}G (7-methylguanosine); T (5-methyluridine); \text{ψ} (pseudouridine). (B) Growth analysis of B. subtilis wild-type and mutant strains. Cells were grown in LB medium at 37°C with aeration and cell growth was monitored over the course of 12 h. (C) Total tRNA was isolated from B. subtilis wild-type and mutant strains and analyzed by native PAGE (15%). 0.5 A\textsubscript{260} of total tRNA were applied per lane. tRNAs were detected by northern hybridization using a 5\textsuperscript{0}-32P-labeled DNA oligonucleotide directed against nucleotides 1–20 of tRNA\textsubscript{1Ile} which is identical in all tRNA\textsubscript{1Ile} species. A tRNA\textsubscript{1Ile} precursor species is indicated by asterisk.

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**Figure 2.** Acid–urea PAGE/northern blot analysis of isoleucine tRNAs from Bacillus subtilis wild-type and mutant strain JJS80 lacking tilS. (A) Analysis of tRNA\textsubscript{1Ile} from B. subtilis wild-type (WT; lanes 1–3) and mutant strain JJS80 (lanes 4–6). tRNA\textsubscript{1Ile} was visualized by northern hybridization using a 32P-labeled DNA oligonucleotide complementary to the 5\textsuperscript{0} portion of tRNA\textsubscript{1Ile}. (B) Analysis of tRNA\textsubscript{2Ile} from B. subtilis wild-type (WT; lanes 1–5) and mutant strain JJS80 (lanes 6–10). tRNA\textsubscript{2Ile} was visualized by northern hybridization using a 32P-labeled DNA oligonucleotide complementary to the 3\textsuperscript{0} portion of tRNA\textsubscript{2Ile}. 0.1 A\textsubscript{260} of total tRNA were applied per lane. ac, tRNA isolated under acidic condition; OH\textsuperscript{-}, deacylation by base treatment; Ile, in vitro aminoacylation using IleRS; Met, in vitro aminoacylation using MetRS; bio, in vitro biotinylation of lysidine.
lysidine in wild-type tRNA\textsuperscript{Ile}\textsubscript{GAU} could be confirmed by reaction of the free NH\textsubscript{2} group in lysidine with an N-hydroxysuccinimide ester derivative of biotin giving rise to a clear mobility shift (12); in contrast, tRNA\textsubscript{Ile}\textsubscript{CAU} from the mutant strain could not be biotinylated due to the lack of lysidine (Figure 2B, compare lanes 5 and 10).

**Purification of tRNA\textsubscript{Ile}\textsuperscript{1} and tRNA\textsubscript{Ile}\textsuperscript{2} from wild-type B. subtilis 168 and mutant strain JJS80 lacking tilS**

Wild-type and mutant isoleucine tRNA\textsubscript{Ile}\textsuperscript{1} and tRNA\textsubscript{Ile}\textsuperscript{2} 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 \(^{14}\text{C}\)-isoleucine or \(^{35}\text{S}\)-methionine acceptance higher than 1200 pmole/A\textsubscript{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 \(^5\text{S},^{32}\text{P}\)-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\textsubscript{Ile}\textsuperscript{1UAU} (see below).

In general, 2.5–3 A\textsubscript{260} units of purified tRNA\textsubscript{Ile}\textsuperscript{1GAU} and 0.5 A\textsubscript{260} of purified tRNA\textsubscript{Ile}\textsuperscript{2GAU} or tRNA\textsubscript{Ile}\textsuperscript{2CAU} were obtained from \(\sim\)400 A\textsubscript{260} of total RNA. Although, wild-type tRNA\textsubscript{Ile}\textsuperscript{1GAU} and mutant tRNA\textsubscript{Ile}\textsuperscript{1UAU} 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\textsubscript{Ile}\textsuperscript{1GAU-UAU}. Typically, 2.5 A\textsubscript{260} of purified tRNA\textsubscript{Ile}\textsuperscript{1GAU-UAU} were obtained from \(\sim\)400 A\textsubscript{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 \(\sim\)50% of the mutant tRNA\textsubscript{Ile}\textsuperscript{1UAU} species as estimated by the analytical PAGE analysis.

**Characterization of tRNA\textsubscript{Ile}\textsuperscript{2} from B. subtilis lacking tilS**

The absence of lysidine at position 34 of the mutant tRNA\textsubscript{Ile}\textsuperscript{2CAU} 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\textsubscript{Ile}\textsuperscript{2LAU} and the mobility of the unmodified C from the mutant tRNA\textsubscript{Ile}\textsuperscript{2CAU}. 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 \(^3\text{H}\)-Ile-tRNA\textsubscript{Ile}\textsuperscript{2AUA} and \(^{35}\text{S}\)-Met-tRNA\textsubscript{Ile}\textsuperscript{2CAU} to *B. subtilis* ribosomes was performed with five different oligonucleotides; AUG AAA, AUG AAC, AUG AUG, AUG AUU and AUG UUA. Wild-type tRNA\textsubscript{Ile}\textsuperscript{2LAU} showed binding to AAA, and weaker binding to AUC and AUU; in contrast, tRNA\textsubscript{Ile}\textsuperscript{2CAU} isolated from the *tilS* mutant strain showed binding only to AUG (Figure 4B and C).

**Characterization of U34 in the mutant tRNA\textsubscript{Ile}\textsuperscript{1}**

As described above, mutant tRNA\textsubscript{Ile}\textsuperscript{1UAU} was purified from JJS80 as a mixture of wild-type tRNA\textsubscript{Ile}\textsuperscript{1GAU} and mutant tRNA\textsubscript{Ile}\textsuperscript{1UAU} (tRNA\textsubscript{Ile}\textsuperscript{1GAU-UAU}; Figure 3B) and most *in vitro* experiments were carried out by direct side-by-side

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**Figure 3. Analysis of tRNA\textsubscript{Ile}\textsuperscript{1} purified from *Bacillus subtilis* wild-type and mutant strain JJS80.** (A) Characterization of purified tRNA\textsubscript{Ile}\textsuperscript{1GAU} from *B. subtilis* wild-type (WT; lanes 1–4) and tRNA\textsubscript{Ile}\textsuperscript{1GAU-UAU} from JJS80 (lanes 5–8) by RNase A and T1 analysis. The \(^5\text{S},^{32}\text{P}\)-labeled tRNA was partially digested with 0.004 U of RNase A (lanes 2 and 7) and 10 U of RNase T1 (lanes 3 and 6). Lanes 4 and 5 show a partial alkali digest; lanes 1 and 8 show undigested control reactions. \(^32\text{P}\)-labeled fragments were separated by denaturing PAGE and visualized by autoradiography. (B) Analysis of purified tRNA\textsubscript{Ile}\textsuperscript{1GAU} and tRNA\textsubscript{Ile}\textsuperscript{1GAU-UAU} by 15% native PAGE. tRNAs were visualized by staining with ethidium bromide (EtBr) and northern blot using a universal tRNA\textsubscript{Ile} probe. 0.01 A\textsubscript{260} of total tRNA were applied per lane.
comparison of the tRNA\textsubscript{1}Ile\textsuperscript{GAU} and tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{UAU}. To verify the presence of tRNA\textsubscript{1}Ile\textsuperscript{UAU} in the purified tRNA\textsubscript{1}Ile\textsuperscript{GAU} sample, purified 5\textsuperscript{32P}-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{1}Ile\textsuperscript{GAU} and the tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{UAU} preparations (Figure 3A, lanes 3 and 6), digests with RNase A confirmed the presence of U\textsubscript{34} in the tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{UAU} but not in the tRNA\textsubscript{1}Ile\textsuperscript{GAU} preparation (Figure 3A, compare lanes 2 and 7).

The nature of U\textsubscript{34} in the mutant tRNA\textsubscript{1}Ile\textsuperscript{UAU} was examined by mass spectral (MS) and TLC studies. First, purified tRNA\textsubscript{1}Ile\textsuperscript{GAU} and tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{UAU} were digested completely 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 AUAAAGp, while the mutant tRNA produces a mixture of three related 9-mers containing C

| tRNA fragment | Sequence       | Observed m/z | Observed-expected m/z | Wild-type tRNA\textsubscript{1}Ile\textsuperscript{GAU} | Mutant tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{UAU} |
|---------------|----------------|--------------|-----------------------|------------------------------------------------------|----------------------------------------------------------------------|
| C31-G34       | CCUGp          | 638.75       | 0.17                  | +                                                    | +                                                                      |
| A25-G39       | AU[t\textsuperscript{6A}]AGp | 899.83       | 0.07                  | +                                                    | +                                                                      |
| C31-G39       | CCUUAUAGp      | 1438.25      | 0.06                  | +                                                    |                                                                      |
| C31-G39       | CCUUAU[m\textsuperscript{6A}]AGp | 1445.25      | −0.03                 | +                                                    |                                                                      |
| C31-G39       | CCUUAU[t\textsuperscript{6A}]AGp | 1511.75      | −0.08                 | +                                                    |                                                                      |

Modified nucleosides: t\textsuperscript{6A} (N\textsuperscript{6}-threonylcarbamoyladenosine); m\textsuperscript{6A} (N\textsuperscript{6}-methyladenosine).

Note that the mutant tRNA\textsubscript{1}Ile\textsuperscript{UAU} sample is a mixture of wild-type tRNA\textsubscript{1}Ile\textsuperscript{GAU} and mutant tRNA\textsubscript{1}Ile\textsuperscript{GAU}=\textsuperscript{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.

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

These results were verified independently by TLC analyses of 5\textsuperscript{32P}-nucleotides isolated from 5\textsuperscript{32P}-labeled fragments derived from nucleotides 34 to 37 of the anticodon loop of both wild-type and mutant tRNA\textsubscript{1}Ile\textsuperscript{GAU} (Figure 5 and Supplementary Figure S4). Both, 1D and 2D-TLC corroborated and extended 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{1}Ile\textsuperscript{GAU} were assembled (Figure 1A).

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{1}Ile were assembled (Figure 1A). These results were verified independently by TLC analyses of 5\textsuperscript{32P}-nucleotides isolated from 5\textsuperscript{32P}-labeled fragments derived from nucleotides 34 to 37 of the anticodon loop of both wild-type and mutant tRNA\textsubscript{1}Ile\textsuperscript{GAU} (Figure 5 and Supplementary Figure S4). Both, 1D and 2D-TLC corroborated and extended the results obtained from the RNase T1/MS analysis: (i) the mutant tRNA\textsubscript{1}Ile\textsuperscript{UAU} contains an unmodified U\textsubscript{34} and not c\textsubscript{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, m\textsuperscript{6A} and t\textsuperscript{6A}, at position 37 were confirmed in the mutant tRNA\textsubscript{1}Ile\textsuperscript{GAU} sample.
tRNA1_{Ile}^\text{UAU}, whereas wild-type tRNA1_{Ile}^\text{GAU} contained mostly \( ^3\text{P} \)A and some A (Figure 5B and Supplementary Figure S4). Since MS analysis of RNase T1 digests of wild-type tRNA1_{Ile}^\text{GAU} 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 tRNA1_{Ile}^\text{UAU} in the presence of B. subtilis ribosomes

Template-dependent binding of purified and aminoacylated wild-type and mutant \(^3\text{H}\)-Ile-tRNA\text{Ile} to B. subtilis ribosomes was performed. As expected, wild-type tRNA1_{Ile}^\text{GAU} showed binding to AUC and AUU codons and to a very minor extent to AU (Figure 6A). In contrast, the mutant tRNA1_{Ile}^\text{GAU}, which is a mixture of tRNA1_{GAU}^\text{Ile} and tRNA1_{UAU}^\text{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 tRNA1_{Ile}^\text{GAU} from the mixture of wild-type and mutant tRNA1_{Ile}^\text{Ile}, tRNA1_{Ile}^\text{GAU/UAU was treated with RNase T1 in the presence of Mg\textsuperscript{++} under mild conditions to inactivate specifically the wild-type tRNA1_{Ile}^\text{GAU by cleavage at position 34 (35). RNase T1 treatment of tRNA1_{Ile}^\text{GAU 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-tRNA1_{Ile} lost essentially all ribosome binding activity subsequent to RNase T1 treatment (Supplementary Figure S5C; black bars). In contrast, binding of the mutant tRNA1_{Ile}^\text{UAU containing U34 to AUA is resistant to this treatment (Figure 6C). RNase T1 treatment of tRNA1_{Ile}^\text{UAU/UAU led to a loss of isoleucine acceptance of ~40% (Supplementary Figure S5B), consistent with the level of contaminating wild-type tRNA1_{Ile}^\text{GAU species (Figure 3B). The resulting highly enriched mutant tRNA1_{Ile}^\text{UAU 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 tRNA1_{Ile}^\text{UAU sample was also carried out in the presence of an equimolar amount of competitor tRNA, in this case Met-tRNA1_{Ile}^\text{GAU}, which reduced binding of Ile-tRNA1_{Ile}^\text{GAU 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 tRNA1_{Ile}^\text{UAU can base-pair, albeit weakly, with an AUA codon.}

We also performed ribosome binding assays using the mutant tRNA1_{Ile}^\text{UAU/UAU and ribosomes isolated from E. coli. The mutant Ile-tRNA1_{Ile}^\text{UAU showed similar binding to the AUA codon with ribosomes from B. subtilis and E. coli. In contrast, binding of the mutant Ile-tRNA1_{Ile}^\text{UAU to the AUG codon was up about 3-fold with E. coli ribosomes (Supplementary Figure S6).}

DISCUSSION

Characterization of the B. subtilis mutant tRNA1_{Ile}^\text{UAU}

Strains of B. subtilis carrying a deletion in the gene encoding the essential tRNA2 \( ^\text{He} \) modifying enzyme TilS can survive only in the presence of a mutant tRNA1_{Ile}^\text{Ile}, whose anticodon wobble position has been changed from G34 to U34 (tRNA1_{Ile}^\text{UAU}). This anticodon mutation allows the tRNA1_{Ile}^\text{UAU to act as a suppressor by reading the isoleucine codon AUA normally read by tRNA1_{Ile}^\text{UAU}. Two major questions of interest were: (i) is U34 at the anticodon wobble position of the mutant tRNA modified, and (ii) besides binding to the AUA codon for isoleucine, does the mutant tRNA also bind to the AUG codon for methionine? Through the analysis of tRNA1_{Ile}^\text{UAU using RNA sequencing and mass spectrometry, we have shown here that unlike the U34 in the anticodon wobble position of bacterial, eukaryotic and archaeal tRNAs, which are almost always modified, the U34 in tRNA1_{Ile}^\text{UAU is not. Thus, the mutant tRNA1_{Ile}^\text{UAU is...}
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{Ile}\textsuperscript{GAU} contains the modified nucleoside t\textsuperscript{6}A at position 37 next to the anticodon, the mutant tRNA\textsubscript{Ile}\textsuperscript{UAU} contains a mixture of A, t\textsuperscript{6}A and m\textsuperscript{6}A at this position. It is worth noting that several of the *B. subtilis* tRNAs, including tRNA\textsubscript{Ile}\textsuperscript{LAU} (36), contain m\textsuperscript{6}A at position 37 (37). The presence of A, t\textsuperscript{6}A and m\textsuperscript{6}A at position 37 of tRNA\textsubscript{Ile}\textsuperscript{UAU} suggests that mutation of G34 to U34 has made the tRNA a poorer substrate for the t\textsuperscript{6}A enzyme complex TsaB/C/D (38,39), so that A37 is either unmodified, modified to t\textsuperscript{6}A or methylated to m\textsuperscript{6}A by a tRNA adenine N\textsuperscript{6}-methyltransferase, presumably a homolog of *E. coli* YfiC (40). In bacteria, archaea and eukaryotes, tRNAs that are substrates for the t\textsuperscript{6}A enzyme usually contain G, C or a modified U in the anticodon wobble position (41). Therefore, why mutation of G34 to U34 should make the mutant tRNA\textsubscript{Ile}\textsuperscript{UAU} a poorer substrate for the t\textsuperscript{6}A enzyme is not obvious, unless it is the lack of modification of U34.

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

Mutant tRNA\textsubscript{Ile}\textsuperscript{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{Ile}\textsuperscript{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{Ile}**

Binding experiments using *B. subtilis* ribosomes show that tRNA\textsubscript{Ile}\textsuperscript{UAU} binds mostly to the AUU codon and only weakly to the AUA and AUG codons (Figure 6C, AUA \textsuperscript{*} AUU > AUG). Thus, the mutant tRNA\textsubscript{Ile}\textsuperscript{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{Ile}\textsuperscript{UAU} is viable and shows only a minimal level of

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Template-dependent binding of purified wild-type \textsuperscript{3}H-Ile-tRNA\textsubscript{Ile}\textsuperscript{GAU} and mutant \textsuperscript{3}H-Ile-tRNA\textsubscript{Ile}\textsuperscript{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{Ile}\textsuperscript{GAU} and (B) mutant tRNA\textsubscript{Ile}\textsuperscript{UAU} sample; note that the mutant sample is a mixture of wild-type tRNA\textsubscript{Ile}\textsuperscript{GAU} and mutant tRNA\textsubscript{Ile}\textsuperscript{UAU}. (C) The mutant \textsuperscript{3}H-Ile-tRNA\textsubscript{Ile}\textsuperscript{UAU} sample was treated with RNase T1 under native conditions to specifically inactivate the wild-type \textsuperscript{3}H-Ile-tRNA\textsubscript{Ile} by cleavage at G34; the mutant tRNA\textsuperscript{UAU} containing U34 is resistant to this treatment; (D) an equimolar amount of non-radioactive competitor Met-tRNA\textsubscript{Ile} was added to RNase T1-treated mutant tRNA. The oligonucleotide concentration in (C) and (D) was 200 mM. Note that in (D) the mutant tRNA\textsubscript{Ile}\textsuperscript{UAU} sample was treated with RNase T1 first, followed by aminoacylation with \textsuperscript{3}H-Ile, resulting in a doubling of \textsuperscript{3}H-Ile-tRNA\textsubscript{Ile}\textsuperscript{UAU}-specific counts present in the ribosome binding experiments.
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\(^{-3}\)/1 appears to be well tolerated in \textit{B. subtilis}, since the suppressor strain JJS80 grows at a rate comparable to that of the wild-type strain (Figure 1B). The ribosome binding results \textit{in vitro} also indicate that the low level read-through of AUG by the mutant tRNA\(^{34}\)\(_{1}\) is more due to the inherently weak affinity of tRNA\(^{34}\)\(_{1}\) for the AUG codon than competition \textit{in vivo} by the endogenous methionine tRNA.

The weak affinity of tRNA\(^{34}\)\(_{1}\) 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 \textit{E. coli} tRNA\(^{34}\)\(_{1}\), which contain U34 instead of the modified U normally present in the tRNA\(^{34}\)\(_{1}\) read the serine codon UCA just as well in an \textit{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 \textit{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 an unmodified 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 \textit{Mycoplasma} species contain a fewer 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 archaean systems do. Thus, mitochondrial and \textit{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 AUG-reading tRNA\(^{34}\)\(_{1}\) isolated from \textit{M. mobile} (42). As mentioned above, in contrast to the AUG codon-specific tRNA\(^{34}\)\(_{1}\) isolated from most bacteria or archaea which contain lysidine or agmatidine, the \textit{M. mobile} tRNA\(^{34}\)\(_{1}\) contains an unmodified U (tRNA\(^{34}\)\(_{1}\)). Suzuki et al. have shown that the \textit{M. mobile} tRNA\(^{34}\)\(_{1}\) binds to the isoleucine codon AUA but not to the methionine codon AUG on \textit{M. mobile} ribosomes. However, the same tRNA binds to both AUA and AUG on \textit{E. coli} ribosomes. Thus, the \textit{M. mobile} ribosome and tRNA\(^{34}\)\(_{1}\) have coevolved in such a way that the \textit{M. mobile} tRNA\(^{34}\)\(_{1}\) containing an unmodified U in the anticodon wobble position is restricted from binding to AUG, even though other \textit{M. mobile} tRNAs containing an unmodified U34 read all four codons of a four-codon box. It would be interesting to see whether the \textit{M. mobile} tRNA\(^{34}\)\(_{1}\) binds only to AUA or to both AUA and AUG codons on ribosomes isolated from other \textit{Mycoplasma} species. The specific binding of \textit{M. mobile} tRNA\(^{34}\)\(_{1}\) to AUA and not to AUG on \textit{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\(^{34}\)\(_{1}\) isolated from \textit{B. subtilis} described in the present study, which binds preferentially to AUA, but only weakly to AUG on \textit{B. subtilis} ribosomes. Similar to the \textit{M. mobile} tRNA\(^{34}\)\(_{1}\), the \textit{B. subtilis} mutant tRNA\(^{34}\)\(_{1}\) shows elevated binding to the AUG codon on \textit{E. coli} ribosomes (Supplementary Figure S6), although the effect is not as pronounced as for the \textit{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 \textit{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 \textit{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 \textit{Mycoplasma mycoides} glycine tRNA, now allowed the tRNA to read all four glycine codons on \textit{E. coli} ribosomes (59). In a reciprocal experiment, it was shown that while the \textit{M. mycoides} glycine tRNA containing U34 can read all four glycine codons on \textit{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 \textit{Mycoplasma} systems mentioned above, \textit{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 \textit{E. coli} and \textit{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 \textit{Haloarcula marismortui} tRNA\(^{34}\)\(_{1}\) to U34 and have purified the mutant tRNA and studied its codon recognition properties using \textit{H. marismortui} ribosomes. We have found that U34 in the mutant tRNA is modified and that the modified tRNA binds not only to AUA but also to AUGC and to AUU (AUA > AUG > AUU) (Mandal,D. and Köhler,C. et al., unpublished data).
SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES
1. Khorana,H.G. (1968) Nucleic acid synthesis in the study of the genetic code. Nobel Lectures. Elsevier Publishing Company.
2. Nirenberg,M. (1968) The genetic code. Nobel Lectures. Elsevier Publishing Company.
3. Crick,F.H. (1956) Codon-anticodon pairing: the wobble hypothesis. J. Mol. Biol., 19, 548–555.
4. Agris,P.F., Vendeix,F.A.P. and Graham,W.D. (2007) tRNA's of archaebacteria. Annu. Rev. Microbiol., 61, 1–13.
5. YokoYoshi,S., Kimura,Y., Grossman,H. and Bessho,Y. (2013) Life without tRNA^Arg-adenosine deaminase TadA: evolutionary consequences of decoding the four CGN codons as arginine in Mycoplasmas and other Mollicutes. Nucleic Acids Res., 41, 6531–6543.
6. Senger,B., Auxilien,S., Englisch,U., Cramer,F. and Fasiolo,F. (2001) Synthesis and characterization of AUA specific isoleucine transfer ribonucleic acid from Escherichia coli. Biochemistry, 30, 300–307.
7. Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Kato,M., Suzuki,T. and Nishimura,S. (1988) tRNA amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. Nature, 336, 179–181.
8. Curran,J.F. (1995) Decoding with the A:U wobble pair is inefficient. Nucleic Acids Res., 23, 683–688.
9. Harada,F. and Nishimura,S. (1974) Purification and characterization of AUA specific isoleucine transfer ribonucleic acid from Escherichia coli B. Biochemistry, 13, 300–307.
10. Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokoyama,S. (1988) Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. Nature, 336, 179–181.
11. Ikeda,Y., Kimura,S., Numata,T., Nakamuro,Y., Yokogawa,T., Ogata,T., Wada,T. and Suzuki,T. (2010) Agmatine-conjugated cytidine in a tRNA anticodon is essential for AUA decoding in archaea. Nat. Chem. Biol., 6, 277–282.
12. Köhrer,C., Strmivasan,G., Mandel,D., Mallick,B., Ghosh,Z., Chakrabarti,J. and RajBhandary,U.L. (2008) Identification and characterization of a tRNA decoding the rare AUA codon in Haloarcula marismortui. RNA, 14, 117–126.
13. Mandel,D., Köhrer,C., Su,D., Russell,S.P., Krivos,K., Castlederry,C.M., Blum,P., Limbach,P.A., Soll,D. and RajBhandary,U.L. (2010) Agmatine, an alternative way of RNA editing. Trends Biochem. Sci., 25, 168–174.
14. Grossjean,H. and Björk,G.R. (2004) Enzymatic conversion of cytidine to lysidine in anticodon of bacterial tRNA^Lys - an alternative way of RNA editing. Trends Biochem. Sci., 29, 165–168.
15. Suzuki,T. and Miyachi,K. (2010) Discovery and characterization of tRNA^Lys lysidine synthetase (TilS). FEBS Lett., 584, 272–277.
16. Phillips,G. and de Crey-Lagard,V. (2011) Biosynthesis and function of tRNA modifications in Archaea. Curr. Opin. Microbiol., 14, 335–341.
17. Fabret,C., Dervyn,E., Dalmais,B., Guillot,A., March,C., Grossjean,H. and Noirot,P. (2011) Life without the essential bacterial tRNA^Asp-lysidine synthetase TilS: a case of tRNA gene recruitment in Bacillus subtilis. Mol. Microbiol., 80, 1062–1074.
18. Suzuki,T. and Suzuki,T. (2007) Chaplet column chromatography: isolation of a large set of individual RNAs in a single step. Methods Enzymol., 425, 231–239.
19. Köhrer,C. and RajBhandary,U.L. (2008) The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. Methods, 44, 129–138.
20. Moazed,D. and Noller,H.F. (1989) Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. Cell, 57, 555–597.
21. Triana-Alonso,F.J., Spahn,C.M., Burkhardt,N., Rohrdanz,B. and Niehaus,K.H. (2000) Experimental prerequisites for determination of tRNA binding to ribosomes from Escherichia coli. Methods Enzymol., 317, 261–276.
22. Silberklang,M., Gillum,A.M. and RajBhandary,U.L. (1979) Use of in vitro ^32P-labeling in the sequence analysis of nonradioactive tRNAs. Methods Enzymol., 59, 58–109.
23. Stanley,J. and Vassilenko,S. (1978) A different approach to RNA sequencing. Nature, 274, 87–89.
24. Gupta,R.C. and Randerath,K. (1979) Rapid print-readout technique for sequencing of RNA's containing modified nucleotides. Nucleic Acids Res., 6, 3443–3458.
25. Kuchino,Y., Kato,M., Sugisaki,H. and Nishimura,S. (1979) Nucleotide sequence of starfish initiator tRNA. Nucleic Acids Res., 6, 3459–3469.
26. RajBhandary,U.L. (1980) Recent developments in methods for RNA sequencing using in vitro ^32P-labeling. Fed. Proc., 39, 2813–2821.
27. Silberklang,M., Gillum,A.M. and RajBhandary,U.L. (1977) The use of nuclease P1 in sequence analysis of end group labeled RNA. Nucleic Acids Res., 4, 4091–4108.
28. Menezes,S., Gaston,K.W., Krivos,K.L., Apolinario,E.E., Reich,N.O., Sowers,K.R., Limbach,P.A. and Perona,J.J. (2011) Formation of m^6G in Methanocaldococcus jannaschii tRNA catalyzed by the novel methyltransferase Trm14. Nucleic Acids Res., 39, 7641–7655.
29. Kunst,F., Ogawara,N., Moszer,I., Albertini,A.M., Aloni,G., Azavedo,V., Bertero,M.G., Bessieres,P., Bolotin,A., Borchert,S. et al. (1997) The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature, 390, 249–256.
30. Matthaei,J.H., Voigt,H.P., Keller,G., Neth,R., Schöch,G., Kübler,H., Amelunxen,F., Sander,G. and Parmeggiani,A. (1966) Specific interactions of ribosomes in decoding. Cold Spring Harb. Symp. Quant. Biol., 31, 25–38.
31. Simoncits,A., Brownlee,G.G., Brown,R.S., Rubin,J.R. and Guille,Y.H. (1977) New rapid gel sequencing method for RNA. Nature, 269, 833–836.
32. Donis-Keller,H., Maxam,A.M. and Gilbert,W. (1977) Mapping adenes, guanines, and pyrimidines in RNA. Nucleic Acids Res., 4, 2527–2538.
33. Lockard,R.E., Alzner-Deweerdt,B., Beckham,J.E., MacGee,J., Tabor,M.W. and RajBhandary,U.L. (1978) Sequence analysis of ^32P labeled tRNA and tRNA using polyacrylamide gel electrophoresis. Nucleic Acids Res., 5, 37–36.
34. Nishimura,S. (1979) Chromatographic mobilities of modified nucleotides. In: Schimmel,P.R., Söll,D. and Abelson,J.N. (eds), tRNA: Structure, Properties, and Recognition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 551–552.
35. Penswick,J.R. and Holley,R.W. (1965) Specific cleavage of the yeast alanine RNA into two large fragments. Proc. Natl Acad. Sci. USA, 53, 543–548.
36. Matsuura,J., Mitake,K. and Ishikawa,H. (1996) Characterization of a B. subtilis minor isoleucine tRNA derived from tDNA having a methionine anticodon CAT. J. Biochem., 119, 811–816.
37. Jühling,F., Mörl,M., Hartmann,R.K., Sprinzl,M., Stadler,P.F. and Pütz,J. (2009) tRNAdb 2009: Compilation of tRNA sequences and tRNA genes. Nucleic Acids Res., 37, D159–D162.
38. Deutsch,C., El Yacoubi,B., de Crecy-Lagard,V. and Iwata-Reuyl,D. (2012) Biosynthesis of threonylcarbamoyl...
adenoxyuridine (t^6A), a universal tRNA nucleoside. J. Biol. Chem., 287, 13666–13673.

39. Lauhon,C.T. (2012) Mechanism of N6-threonylcarbamoyladenosine (t^6A) biosynthesis: isolation and characterization of the intermediate threonylcarbamoyl-AMP. Biochemistry, 51, 8950–8963.

40. Golovina,A.Y., Sergiev,P.V., Golovin,A.V., Serebryakova,M.V., Demina,J., Govorun,V.M. and Donitsova,O.A. (2009) The sfoC gene of E. coli encodes an adenosine-N6 methyltransferase that specifically modifies A37 of tRNAVal(emo5UAC). RNA, 15, 1134–1141.

41. Björk,G.R. (1998) Modified nucleosides at positions 34 and 37 of tRNAs and their predicted coding capacities. In: Grosjean,H. and Benne,R. (eds), Modification and Editing of RNA. American Society for Microbiology, Washington, DC, pp. 577–581.

42. Taniguchi,T., Miyauchi,K., Nakane,D., Miyata,M., Muto,A., Nishimura,S. and Suzuki,T. (2013) Decoding system for the UAU codon by tRNA^{UAC} with the UAU anticodon in Mycoplasma mobile. Nucleic Acids Res., 41, 2621–2631.

43. Gustilo,E.M., Vendel,F.A. and Agris,P.F. (2009) Trna’s modifications bring order to gene expression. Curr. Opin. Microbiol., 11, 134–140.

44. Nasvall,S.J., Chen,P. and Björk,G.R. (2009) The wobble hypothesis revisited: uridine-5-oxyacetic acid is critical for reading of G-ending codons. RNA, 13, 2151–2164.

45. Johansson,M.J., Esberg,A., Huang,B., Björk,G.R. and Bystrom,A.S. (2008) Eukaryotic wobble uridine modifications specifically modify A37 of tRNA{Val}(emo5UAC).

46. Takai,K., Okumura,S., Hosono,K., Yokoyama,S. and Takaku,H. (1999) A single uridine modification at the wobble position of an E. coli gene of Mycoplasma capricolum boxes by adenosine or adenosine at the first anticodon position. J. Mol. Biol., 289, 6873–6877.

47. de Crecy-Lagard,V., Marck,C., Brochier-Armanet,C. and Grosjean,H. (2007) Comparative RNomics and modomics in Mollicutes: prediction of gene function and evolutionary implications. IUBMB Life, 59, 634–658.

48. Lustig,F., Boren,T., Claesson,C., Simonsson,C., Barciszewska,M., Samuelsson,T., Olofsson,B., Zinreich,S.J., de Crecy-Lagard,V. and Marck,C. (2006) Novel features in the genetic code and codon reading patterns in Neurospora crassa mitochondria based on sequences of six mitochondrial tRNAs. Proc. Natl Acad. Sci. USA, 103, 3167–3170.

49. Barrell,B.G., Anderson,S., Bankier,A.T., de Bruijn,M.H., Chen,E., Coulson,A.R., Drouin,J., Eperon,I.C., Nierlich,D.P., Roe,B.A., et al. (1980) Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc. Natl Acad. Sci. USA, 77, 3164–3166.

50. Bonitz,S.G., Berlani,R., Coruzzi,G., Li,M., Macino,G., Nobrega,F.G., Nobrega,M.P., Thalenfeld,B.E. and Tzagoloff,A. (1980) Codon recognition rules in yeast mitochondria. Proc. Natl Acad. Sci. USA, 77, 3167–3170.

51. Beckman,J.E., Sarnoff,J., Almey-DeWeerd,B., Yin,S. and RajBhandary,U.L. (1980) Novel features in the genetic code and codon reading patterns in Neurospora crassa mitochondria based on sequences of six mitochondrial tRNAs. Proc. Natl Acad. Sci. USA, 77, 3159–3163.

52. Andachi,Y., Yamao,F., Muto,A. and Osaka,S. (1989) Codon recognition patterns as deduced from sequences of the complete set of transfer RNA species in Mycoplasma capricolum. Resemblance to mitochondria. J. Mol. Biol., 209, 37–54.

53. Yasunaga,T., Nakane,D., Miyata,M., Muto,A., Nakane,K., Nakamura,S. and Suzuki,T. (2013) Decoding system for the UAU codon by tRNA^{UAC} with the UAU anticodon in Mycoplasma mobile. Nucleic Acids Res., 41, 2621–2631.

54. Barrell,B.G., Anderson,S., Bankier,A.T., de Bruijn,M.H., Chen,E., Coulson,A.R., Drouin,J., Eperon,I.C., Nierlich,D.P., Roe,B.A., et al. (1980) Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc. Natl Acad. Sci. USA, 77, 3164–3166.

55. Osawa,S., Jukes,T.H., Watanabe,K. and Muto,A. (1995) Translation of synonymous codons in families of Mycoplasma capricolum tRNAs with unmodified uridine or adenosine at the first anticodon position. J. Mol. Biol., 251, 486–492.

56. Inagaki,Y., Kojima,A., Bessho,Y., Horii,H., Ohama,T. and Osaka,S. (1995) Translation of synonymous codons in family boxes by Mycoplasma capricolum tRNAs with unmodified uridine or adenosine at the first anticodon position. J. Mol. Biol., 251, 486–492.

57. de Crecy-Lagard,V., Marck,C., Brochier-Armanet,C. and Grosjean,H. (2007) Comparative RNomics and modomics in Mollicutes: prediction of gene function and evolutionary implications. IUBMB Life, 59, 634–658.

58. Lustig,F., Boren,T., Guindy,Y.S., Elias,P., Samuelsson,T., Gehlke,C.W., Kuo,K.C. and Lagerkvist,U. (1989) Codon discrimination and anticodon structural context. Proc. Natl Acad. Sci. USA, 86, 6873–6877.

59. Lustig,F., Boren,T., Claesson,C., Simonsson,C., Barciszewska,M. and Lagerkvist,U. (1993) The nucleotide in position 32 of the tRNA anticodon loop determines ability of anticodon UCC to discriminate among glycine codons. Proc. Natl Acad. Sci. USA, 90, 3343–3347.

60. Olejnichazk,M. and Uhlenbeck,O.C. (2006) tRNA residues that have coevolved with their anticodon to ensure uniform and accurate codon recognition. Biochemistry, 45, 943–950.

61. Yarus,M. (1982) Translational efficiency of transfer RNA's: uses and limitations. J. Mol. Biol., 158, 849–859.

62. RajBhandary,U.L. (1980) Novel features in the genetic code and codon reading patterns in Neurospora crassa mitochondria based on sequences of six mitochondrial tRNAs. Proc. Natl Acad. Sci. USA, 77, 3159–3163.

63. Schmeing,T.M., Voorhees,R.M., Kelley,A.C. and Ramakrishnan,V. (2011) How mutations in tRNA distant from the anticodon affect the fidelity of decoding. Nat. Struct. Mol. Biol., 18, 432–436.