Life without tRNA$^{\text{Ile}}$-lysidine synthetase: translation of the isoleucine codon AUA in *Bacillus subtilis* lacking the canonical tRNA$_2^{\text{Ile}}$

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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$_2^{\text{Ile}}$ to base pair specifically with the A of the AU<u>A</u> codon but not with the G of AU<u>G</u>. Recently, a *Bacillus subtilis* strain was isolated in which the essential gene encoding tRNA$^{\text{Ile}}$-lysidine synthetase was deleted for the first time. In such a strain, C34 at the wobble position of tRNA$_2^{\text{Ile}}$ is expected to remain unmodified and cells depend on a mutant suppressor tRNA derived from tRNA$_1^{\text{Ile}}$, 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 *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 *B. subtilis* ribosomes but only weakly to AUG. These *in vitro* data explain why the suppressor strain displays only a low level of misreading AUG codons *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$_{\text{IAU}}^{\text{Ile}}$; I = inosine) reads all three isoleucine codons following the Wobble hypothesis (3), whereas the other with the anticodon ψAψ (tRNA$_{\psi A \psi}^{\text{Ile}}$; 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$_{\text{IAU}}^{\text{Ile}}$ (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$_{1\text{GAU}}^{\text{Ile}}$), 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$_{2\text{LAU}}^{\text{Ile}}$; L = lysidine) (9,10) and agmatidine in archaeal isoleucine tRNA (tRNA$_{2\text{C}^+\text{AU}}^{\text{Ile}}$; 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$_2^{\text{Ile}}$ results in a dual specificity switch of the tRNA in aminoacylation

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and in codon binding: while the unmodified tRNA with C34 is aminoacylated *in vitro* with methionine by methionyl-tRNA synthetase (MetRS) and reads the AUG codon, the modified tRNA is aminoacylated 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 archaeal species, whose genomes encode an isoleucine tRNA with the anticodon UAU (tRNA$_{UAU}^{Ile}$) 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 tRNA^{Ile}-lysidine synthetase (TilS) in bacteria or tRNA^{Ile}-agmatidine synthetase (TiaS) in archaea, responsible for the biosynthesis of lysidine or agmatidine, respectively.

The recent isolation of a mutant tRNA$_1^{Ile}$ 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 tRNA$_{1UAU}^{Ile}$, 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$_{1UAU}^{Ile}$ 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$_1^{Ile}$ 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$_{1UAU}^{Ile}$ 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$_2^{Ile}$ 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 aminoacylation 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 10 000g, the aqueous layer was adjusted to 1 M lithium chloride and left on ice for 2 h. After centrifugation (30 min, 10 000g), 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* aminoacylation 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 × 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 $3 \times$ NHE buffer at $37°C$. For elution of the bound tRNA, the resin was suspended in 0.5–1 ml of $0.1 \times$ 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 NH_2-terminal His_6-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 A_{260} 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 $MgCl_2$, 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 NH_4Cl, 15 mM $MgCl_2$, 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 (OD_{600} 0.5–0.6), resuspended in buffer A (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NH_4Cl and 6 mM 2-mercaptoethanol) and lysed by two passes through a French Press at 12 000psi. The cell lysate was cleared by two consecutive centrifugations for 15 min at 10 000g. 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 $MgCl_2$, 0.5 M NH_4Cl 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 $MgCl_2$, 70 mM NH_4Cl, 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 M 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′-^{32}P-nucleotides by thin layer chromatography

For the analysis of 5′-^{32}P-nucleotides isolated from 5′-^{32}P-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 ^{32}P 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 ^{32}P-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 tRNA$_1^{Ile}$ (anticodon: GAU; tRNA$_{1GAU}^{Ile}$; Figure 1A) for translation of AUC and AUU codons, and one of which encodes tRNA$_2^{Ile}$ (anticodon: CAU, which is post-translationally modified to LAU, L = lysidine; tRNA$_{2LAU}^{Ile}$; 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 tRNA$_{2LAU}^{Ile}$, (i.e. synthesis of tRNA$_{2CAU}^{Ile}$), 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 tRNA$_{1GAU}^{Ile}$ genes with a change in the anticodon sequence from GAT to TAT (Supplementary Table S1), allowing for the synthesis of a new cellular tRNA$_1^{Ile}$ species with a UAU anticodon (tRNA$_{1UAU}^{Ile}$), most likely responsible for translation of AUA codons.
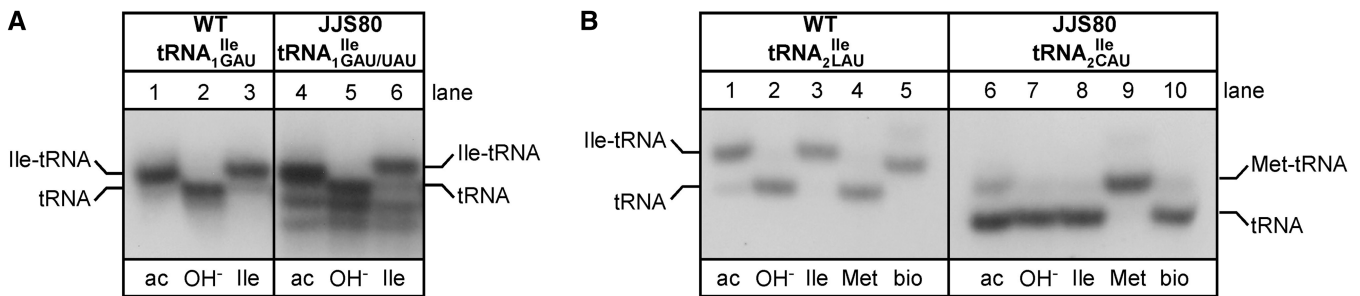
Bacillus subtilis mutant strains JJS80 and EDJ958 were grown in LB medium at 37°C and their growth was monitored over a 12 h period. The mutant strain JJS80, in which the *trnO* gene carries the GAT to TAT mutation (Supplementary Table S1), grows almost wild-type like, while the mutant strain EDJ958, 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 portion of tRNA$_1^{Ile}$, which detects both wild-type and mutant tRNA$_1^{Ile}$ species. Separation on a 15% native polyacrylamide gel allowed the visualization of a slower migrating tRNA species consistent with the presence of the tRNA$_{1UAU}^{Ile}$ (Figure 1C). The abundance of tRNA$_{1UAU}^{Ile}$ varied substantially between JJS80 and EDJ958 and reflects most likely different transcription efficiencies for the different chromosomal loci from which the mutant tRNA$_{1UAU}^{Ile}$ is derived from in the respective strain. Since the mutant strain JJS80 consistently showed better growth than EDJ958 and produced more of tRNA$_{1UAU}^{Ile}$, all experiments described below were carried out with JJS80.

Aminoacylation properties of tRNA$_1^{Ile}$ and tRNA$_2^{Ile}$ 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 aminoacyl-tRNA synthetases (Figure 2). tRNAs were analyzed by acid–urea PAGE/northern blotting. tRNA$_1^{Ile}$ from both the wild-type and mutant strain are aminoacylated with isoleucine by IleRS (Figure 2A). Although tRNA$_{1GAU}^{Ile}$ and tRNA$_{1UAU}^{Ile}$ could
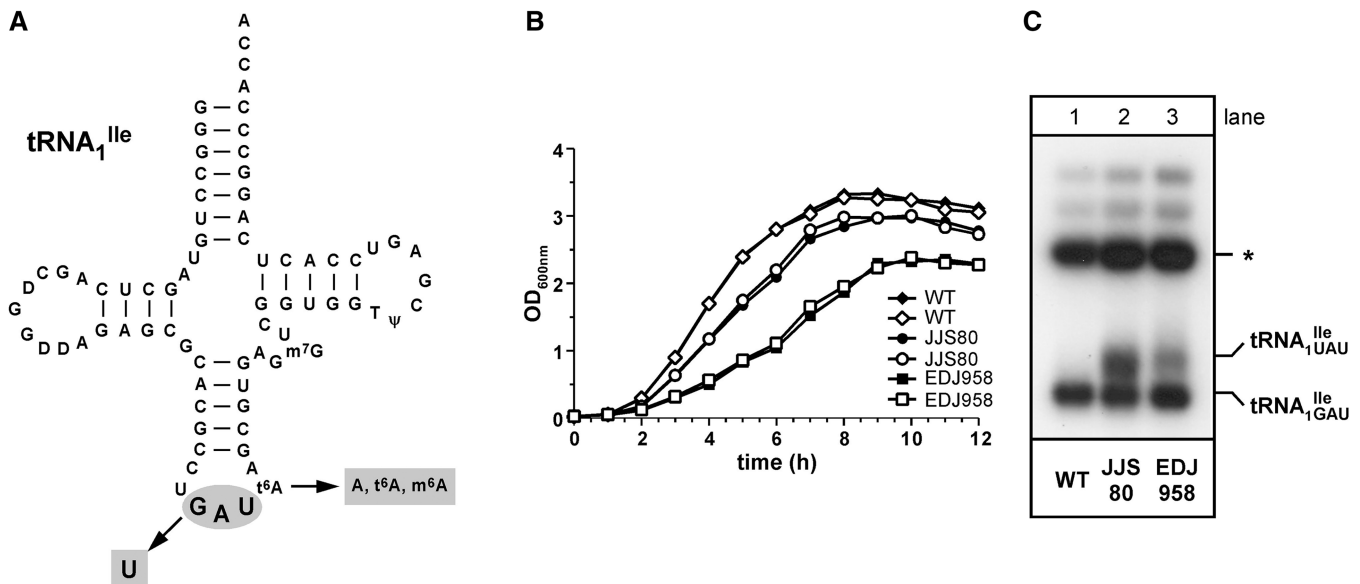
Figure 1. tRNA$_1^{Ile}$ in *Bacillus subtilis* wild-type strain 168 and mutant strains JJS80 and EDJ958 lacking *tilS*. (**A**) Cloverleaf structure of wild-type tRNA$_{1GAU}^{Ile}$. The changes in mutant tRNA$_{1UAU}^{Ile}$ are indicated. Location of modified nucleosides is based on TLC and LC-MS analysis presented in this study. D (dihydrouridine); t^{6}A (N^6-threonylcarbamoyladenosine); m^{6}A (N^6-methyladenosine); m^{7}G (7-methylguanosine); T (5-methyluridine); 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$_{260}$ of total tRNA were applied per lane. tRNAs were detected by northern hybridization using a 5′-^{32}P-labeled DNA oligonucleotide directed against nucleotides 1–20 of tRNA$_1^{Ile}$ which is identical in all tRNA$_1^{Ile}$ species. A tRNA$_1^{Ile}$ precursor species is indicated by asterisk.

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$_1^{Ile}$ from *B. subtilis* wild-type (WT; lanes 1–3) and mutant strain JJS80 (lanes 4–6). tRNA$_1^{Ile}$ was visualized by northern hybridization using a ^{32}P-labeled DNA oligonucleotide complementary to the 5′ portion of tRNA$_1^{Ile}$. (**B**) Analysis of tRNA$_2^{Ile}$ from *B. subtilis* wild-type (WT; lanes 1–5) and mutant strain JJS80 (lanes 6–10). tRNA$_2^{Ile}$ was visualized by northern hybridization using a ^{32}P-labeled DNA oligonucleotide complementary to the 3′ portion of tRNA$_2^{Ile}$. 0.1 A$_{260}$ of total tRNA were applied per lane. ac, tRNA isolated under acidic condition; OH$^-$, deacylation by base treatment; Ile, *in vitro* aminoacylation using IleRS; Met, *in vitro* aminoacylation using MetRS; bio, *in vitro* biotinylation of lysidine.

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$_{1UAU}^{Ile}$.

tRNA$_2^{Ile}$ from the mutant strain showed a clear shift in mobility by acid–urea PAGE compared to wild-type tRNA$_2^{Ile}$, consistent with the lack of lysidine at position 34 (Figure 2B, compare lanes 1 and 6). Also, tRNA$_2^{Ile}$ from the mutant strain showed a significant amount of deacylation during isolation and work-up compared to wild-type tRNA$_2^{Ile}$, 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$_2^{Ile}$ from the mutant strain is not as good a substrate for aminoacylation *in vivo* and, therefore, appears only partially aminoacylated. As expected, tRNA$_{2LAU}^{Ile}$ 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$_{2CAU}^{Ile}$ 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 $tRNA_{2LAU}^{Ile}$ 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, $tRNA_{2CAU}^{Ile}$ from the mutant strain could not be biotinylated due to the lack of lysidine (Figure 2B, compare lanes 5 and 10).

## Purification of $tRNA_1^{Ile}$ and $tRNA_2^{Ile}$ from wild-type *B. subtilis* 168 and mutant strain JJS80 lacking *tilS*

Wild-type and mutant isoleucine $tRNA_1^{Ile}$ and $tRNA_2^{Ile}$ 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 $5'$-^{32}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 $tRNA_{1UAU}^{Ile}$ (see below).

In general, 2.5–3 A_{260} units of purified $tRNA_{1GAU}^{Ile}$ and 0.5 A_{260} of purified $tRNA_{2LAU}^{Ile}$ or $tRNA_{2CAU}^{Ile}$ were obtained from ~400 A_{260} of total RNA. Although, wild-type $tRNA_{1GAU}^{Ile}$ and mutant $tRNA_{1UAU}^{Ile}$ 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 $tRNA_{1GAU/UAU}^{Ile}$. Typically, 2.5 A_{260} of purified $tRNA_{1GAU/UAU}^{Ile}$ 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 $tRNA_{1UAU}^{Ile}$ species as estimated by the analytical PAGE analysis.

Characterization of $tRNA_2^{Ile}$ from *B. subtilis* lacking *tilS*

The absence of lysidine at position 34 of the mutant $tRNA_{2CAU}^{Ile}$ 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 $tRNA_{2LAU}^{Ile}$ and the mobility of the unmodified C from the mutant $tRNA_{2CAU}^{Ile}$. 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-$tRNA_{2LAU}^{Ile}$ and ^{35}S-Met-$tRNA_{2CAU}^{Ile}$ to *B. subtilis* ribosomes was performed with five different oligonucleotides; AUG AUA, AUG AUC, AUG AUG, AUG AUU and AUG UUU. Wild-type $tRNA_{2LAU}^{Ile}$ showed binding to AUA, and weaker binding to AUC and AUU; in contrast, $tRNA_{2CAU}^{Ile}$ isolated from the *tilS*
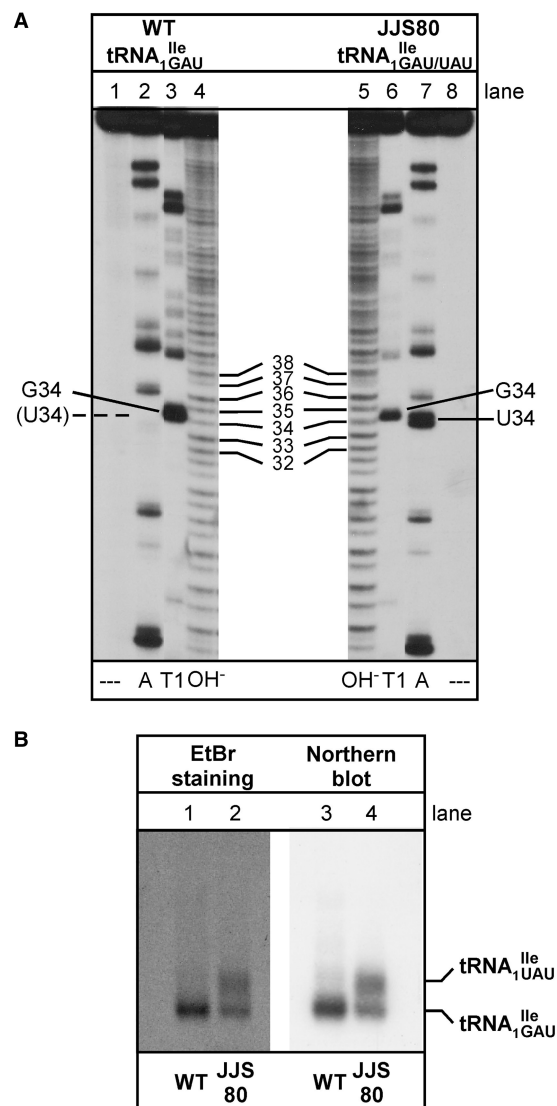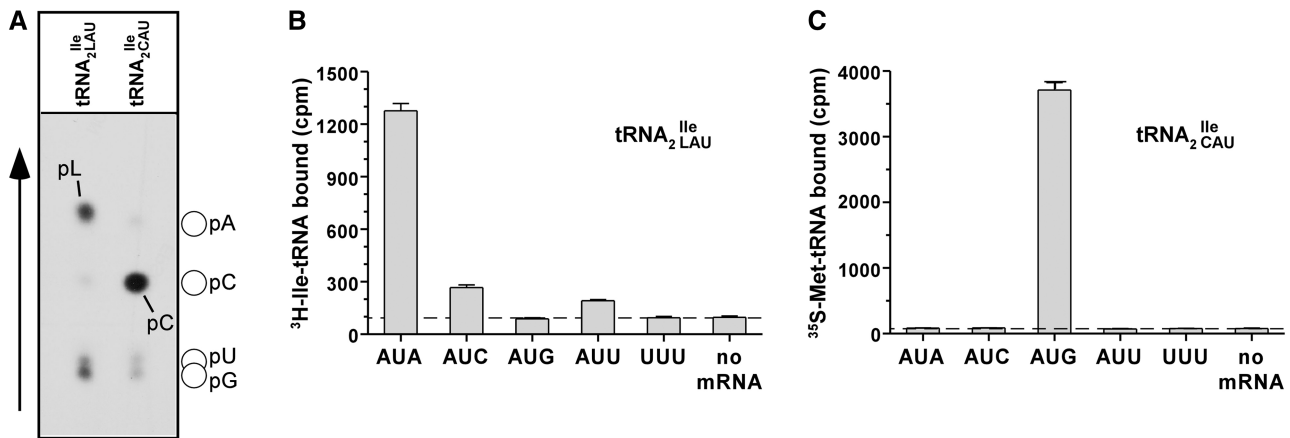

Figure 3. Analysis of $tRNA_1^{Ile}$ purified from *Bacillus subtilis* wild-type and mutant strain JJS80. (**A**) Characterization of purified $tRNA_{1GAU}^{Ile}$ from *B. subtilis* wild-type (WT; lanes 1–4) and $tRNA_{1GAU/UAU}^{Ile}$ from JJS80 (lanes 5–8) by RNase A and T1 analysis. The $5'$-^{32}P-labeled 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}P-labeled fragments were separated by denaturing PAGE and visualized by autoradiography. (**B**) Analysis of purified $tRNA_{1GAU}^{Ile}$ and $tRNA_{1GAU/UAU}^{Ile}$ by 15% native PAGE. tRNAs were visualized by staining with ethidium bromide (EtBr) and northern hybridization using a universal $tRNA_1^{Ile}$ probe. 0.01 A_{260} of total tRNA were applied per lane.

mutant strain showed binding only to AUG (Figure 4B and C).

Characterization of U34 in the mutant $tRNA_{1UAU}^{Ile}$

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

Figure 4. Lysidine is absent in tRNA$_2^{Ile}$ from *Bacillus subtilis* JJS80 lacking *tilS*. (**A**) 1D TLC analysis of the wobble position 34 in tRNA$_2^{Ile}$ purified from *B. subtilis* wild-type and JJS80. Purified wild-type tRNA$_{2LAU}^{Ile}$ and mutant tRNA$_{2CAU}^{Ile}$ were partially hydrolyzed by alkali, the 5′ termini of the fragments were ^{32}P-labeled using T4-PNK. ^{32}P-labeled fragments were subsequently digested with nuclease P1 and the nature of the 5′ terminal nucleotide was determined by TLC. The solvent used was isobutyric acid:concentrated ammonia:water (66:1:33) (*v:v:v*). The mobility of each nucleotide (pA, pC, pG, pU) was confirmed with non-radiolabeled standards used as internal markers and visualized by UV shadowing. (**B** and **C**) Template-dependent binding of purified wild-type ^{3}H-Ile-tRNA$_{2LAU}^{Ile}$ (B) and mutant ^{35}S-Met-tRNA$_{2CAU}^{Ile}$ (C) to ribosomes isolated from *B. subtilis*. Oligonucleotides used were AUG AUA, AUG AUC, AUG AUG, AUG AUU and AUG UUU; the oligonucleotide concentration was 200 µM.

Table 1. RNA sequencing of the anticodon loop of wild-type tRNA$_{1GAU}^{Ile}$ and mutant tRNA$_{1GAU/UAU}^{Ile}$ by MS analysis of RNase T1 fragments

tRNA fragment	Sequence	Observed *m/z*	Observed-expected *m/z*	Wild-type tRNA$_{1GAU}^{Ile}$	Mutant tRNA$_{1GAU/UAU}^{Ile}$
C31-G34	CCUGp	638.75	0.17	+	+
A35-G39	AU[t^{6}A]AGp	899.83	0.07	+	+
C31-G39	CCUUAUAAGp	1438.25	0.06		+
C31-G39	CCUUAU[m^{6}A]AGp	1445.25	−0.03		+
C31-G39	CCUUAU[t^{6}A]AGp	1511.75	−0.08		+

Modified nucleosides: t^{6}A (N^6-threonylcarbamoyladenosine); m^{6}A (N^6-methyladenosine).
Note that the mutant tRNA$_1^{Ile}$ sample is a mixture of wild-type tRNA$_{1GAU}^{Ile}$ and mutant tRNA$_{1UAU}^{Ile}$. 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.

comparison of the tRNA$_{1GAU}^{Ile}$ and tRNA$_{1GAU/UAU}^{Ile}$. To verify the presence of tRNA$_{1UAU}^{Ile}$ in the purified tRNA$_{1GAU/UAU}^{Ile}$ sample, purified 5′-^{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$_{1GAU}^{Ile}$ and the tRNA$_{1GAU/UAU}^{Ile}$ preparations (Figure 3A, lanes 3 and 6), digests with RNase A confirmed the presence of U34 in the tRNA$_{1GAU/UAU}^{Ile}$ but not in the tRNA$_{1GAU}^{Ile}$ preparation (Figure 3A, compare lanes 2 and 7).

The nature of U34 in the mutant tRNA$_{1UAU}^{Ile}$ was examined by mass spectral (MS) and TLC studies. First, purified tRNA$_{1GAU}^{Ile}$ and tRNA$_{1GAU/UAU}^{Ile}$ 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 G34 to U34, 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 C

CUUAUAAGp (Table 1). LC-MS/MS analysis showed clearly, that U34 in the mutant tRNA is not modified (Table 1 and Supplementary Figure S3), unless it is modified to ψ, a mass-silent modification. Also, the modification at position 37 showed some interesting heterogeneity; in addition to t^{6}A, both m^{6}A 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). 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$_1^{Ile}$ were assembled (Figure 1A).

These results were verified independently by TLC analyses of 5′-^{32}P-nucleotides isolated from 5′-^{32}P-labeled fragments derived from nucleotides 34 to 37 of the anticodon loop of both wild-type and mutant tRNA$_1^{Ile}$ (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$_{1UAU}^{Ile}$ contains an unmodified U34 and not ψ34, which separates clearly from U (34) (Figure 5A and Supplementary Figure S4); and (ii) the presence of unmodified A and two different modifications, m^{6}A and t^{6}A, at position 37 were confirmed in the mutant
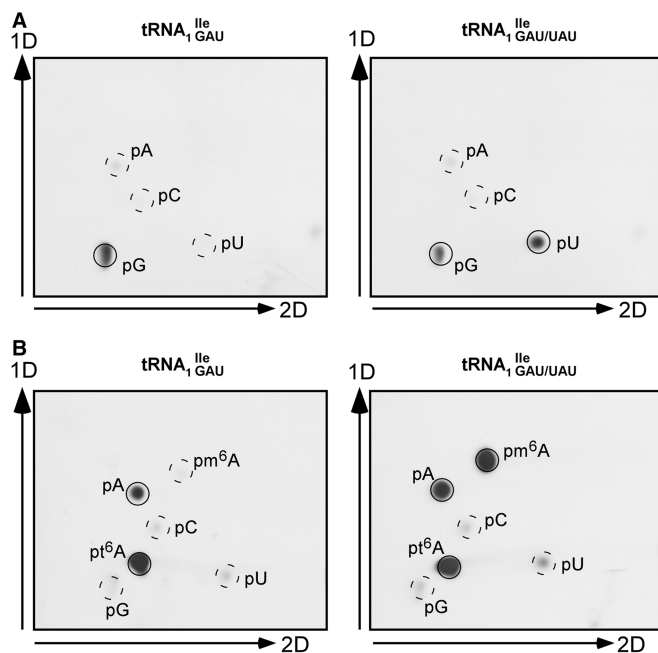
Figure 5. Characterization of nucleotides present at position 34 (**A**) and 37 (**B**) of purified wild-type and mutant *Bacillus subtilis* tRNA$_1^{Ile}$ by 2D TLC. Purified tRNAs were hydrolyzed and labeled with ^{32}P as described for Figure 4. The 5'-^{32}P-labeled nucleotides obtained after nuclease P1 digest were separated by 2D TLC, using an isobutyric acid:concentrated ammonia:water (66:1:33) (*v:v:v*) solvent for the first dimension and an isopropanol:concentrated HCl:water (70:15:15) (*v:v:v*) solvent for the second dimension. The mobility of each nucleotide (pA, pC, pG, pU, pm^{6}A, pt^{6}A) was confirmed with non-radiolabeled standards used as internal markers and visualized by UV shadowing. Note that the mutant tRNA$_1^{Ile}$ sample is a mixture of wild-type tRNA$_{1GAU}^{Ile}$ and mutant tRNA$_{1UAU}^{Ile}$.

tRNA$_{1UAU}^{Ile}$, whereas wild-type tRNA$_{1GAU}^{Ile}$ contained mostly t^{6}A and some A (Figure 5B and Supplementary Figure S4). Since MS analysis of RNase T1 digests of wild-type tRNA$_{1GAU}^{Ile}$ 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$_{1UAU}^{Ile}$ in the presence of *B. subtilis* ribosomes

Template-dependent binding of purified and amino-acylated wild-type and mutant ^{3}H-Ile-tRNA$_1^{Ile}$ to *B. subtilis* ribosomes was performed. As expected, wild-type tRNA$_{1GAU}^{Ile}$ showed binding to AUC and AUU codons and to a very minor extent to AUA (Figure 6A). In contrast, the mutant tRNA$_1^{Ile}$, which is a mixture of tRNA$_{1GAU}^{Ile}$ and tRNA$_{1UAU}^{Ile}$, 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$_{1GAU}^{Ile}$ from the mixture of wild-type and mutant tRNA$_1^{Ile}$, tRNA$_{1GAU/UAU}^{Ile}$ was treated with RNase T1 in the presence of Mg^{++} under

mild conditions to inactivate specifically the wild-type tRNA$_{1GAU}^{Ile}$ by cleavage at position 34 (35). RNase T1 treatment of tRNA$_{1GAU}^{Ile}$ 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$_{1GAU}^{Ile}$ lost essentially all ribosome binding activity subsequent to RNase T1-treatment (Supplementary Figure S5C; black bars). In contrast, binding of the mutant tRNA$_{1UAU}^{Ile}$ containing U34 to AUA is resistant to this treatment (Figure 6C). RNase T1 treatment of tRNA$_{1GAU/UAU}^{Ile}$ led to a loss of isoleucine acceptance of ~40% (Supplementary Figure S5B), consistent with the level of contaminating wild-type tRNA$_{1GAU}^{Ile}$ species (Figure 3B). The resulting highly enriched mutant tRNA$_{1UAU}^{Ile}$ 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$_{1UAU}^{Ile}$ sample was also carried out in the presence of an equimolar amount of competitor tRNA, in this case Met-tRNA$_{2CAU}^{Met}$, which reduced binding of Ile-tRNA$_{1UAU}^{Ile}$ to AUG, but not AUA or AUU, to background levels (Figure 6D; hatched bars). Weak binding to the AUU-containing mRNA leaves open the possibility that the mutant tRNA$_{1UAU}^{Ile}$ can base-pair, albeit weakly, with an AUU codon.

We also performed ribosome binding assays using the mutant tRNA$_{1GAU/UAU}^{Ile}$ and ribosomes isolated from *E. coli*. The mutant Ile-tRNA$_{1UAU}^{Ile}$ showed similar binding to the AUA codon with ribosomes from *B. subtilis* and *E. coli*. In contrast, binding of the mutant Ile-tRNA$_{1UAU}^{Ile}$ to the AUG codon was up about 3-fold with *E. coli* ribosomes (Supplementary Figure S6).

DISCUSSION

Characterization of the *B. subtilis* mutant tRNA$_{1UAU}^{Ile}$

Strains of *B. subtilis* carrying a deletion in the gene encoding the essential tRNA$_2^{Ile}$ modifying enzyme TilS can survive only in the presence of a mutant tRNA$_1^{Ile}$, whose anticodon wobble position has been changed from G34 to U34 (tRNA$_{1UAU}^{Ile}$). This anticodon mutation allows the tRNA$_{1UAU}^{Ile}$ to act as a suppressor by reading the isoleucine codon AUA normally read by tRNA$_{2LAU}^{Ile}$. 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 tRNA$_{1UAU}^{Ile}$ 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 tRNA$_{1UAU}^{Ile}$ is not. Thus, the mutant tRNA$_{1UAU}^{Ile}$ is
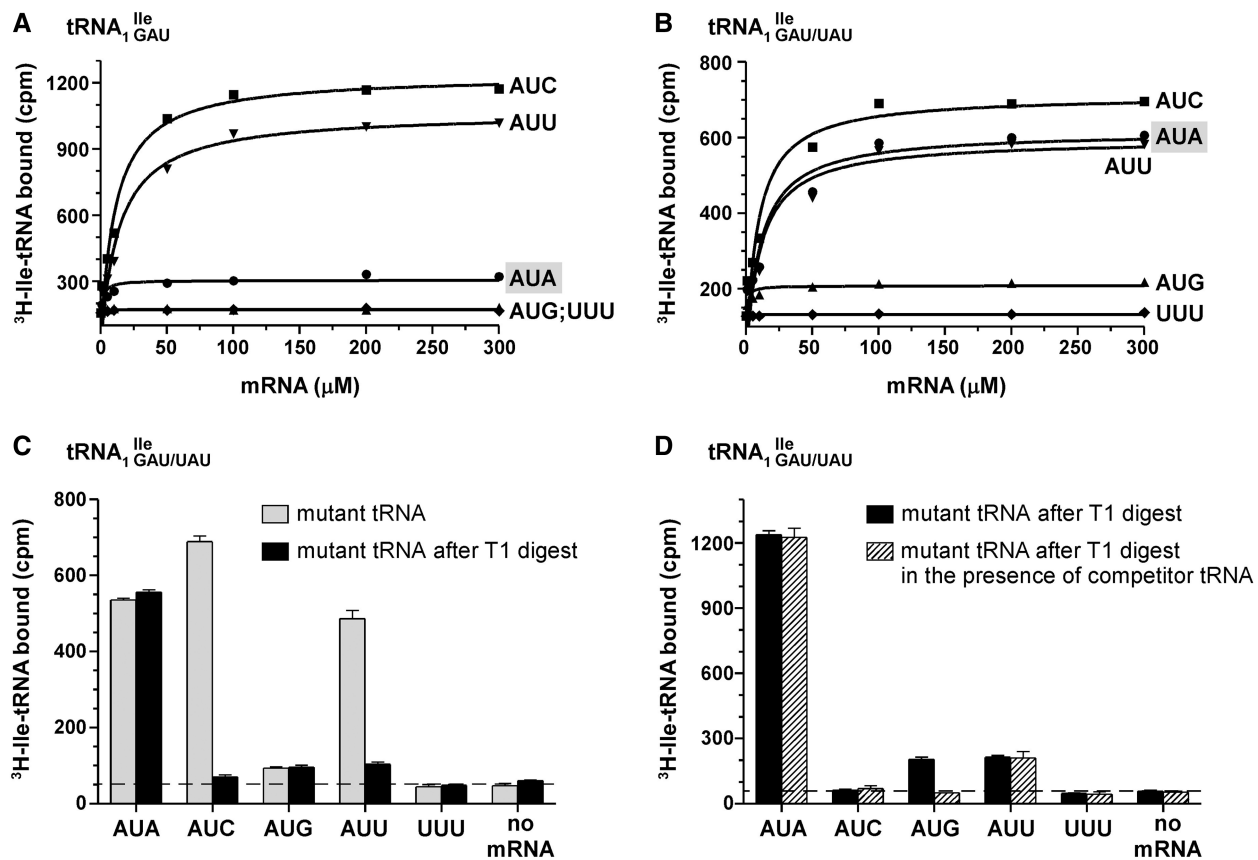
Figure 6. Template-dependent binding of purified wild-type ^{3}H-Ile-tRNA$_{1GAU}^{Ile}$ and mutant ^{3}H-Ile-tRNA$_{1GAU/UAU}^{Ile}$ to ribosomes isolated from *Bacillus subtilis*. Oligonucleotides used were AUG AUA, AUG AUC, AUG AUG, AUG AUU and AUG UUU. (**A**) Wild-type tRNA$_{1GAU}^{Ile}$ and (**B**) mutant tRNA$_{1GAU/UAU}^{Ile}$ sample; note that the mutant sample is a mixture of wild-type tRNA$_{1GAU}^{Ile}$ and mutant tRNA$_{1UAU}^{Ile}$. (**C**) The mutant ^{3}H-Ile-tRNA$_{1GAU/UAU}^{Ile}$ sample was treated with RNase T1 under native conditions to specifically inactivate the wild-type ^{3}H-Ile-tRNA$_1^{Ile}$ by cleavage at G34; the mutant tRNA$_1^{Ile}$ containing U34 is resistant to this treatment; (**D**) an equimolar amount of non-radioactive competitor Met-tRNA$_{2CAU}^{Ile}$ was added to RNase T1-treated mutant tRNA. The oligonucleotide concentration in (C) and (D) was 200 μM. Note that in (D) the mutant tRNA$_{1GAU/UAU}^{Ile}$ sample was treated with RNase T1 first, followed by aminoacylation with ^{3}H-Ile, resulting in a doubling of ^{3}H-Ile-tRNA$_{1UAU}^{Ile}$-specific counts present in the ribosome binding experiments.

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

Aminoacylation of mutant *B. subtilis* tRNA$_{1UAU}^{Ile}$

Mutant tRNA$_{1UAU}^{Ile}$ 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$_{2UAU}^{Ile}$ (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$_{1UAU}^{Ile}$

Binding experiments using *B. subtilis* ribosomes show that tRNA$_{1UAU}^{Ile}$ binds mostly to the AUA codon and only weakly to the AUU and AUG codons (Figure 6C, AUA >>> AUU > AUG). Thus, the mutant tRNA$_{1UAU}^{Ile}$ 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$_{1UAU}^{Ile}$ 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 $10^{-3}/1$ appears to be well tolerated in *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 *in vitro* also indicate that the low level read-through of AUG by the mutant $tRNA_{1 UAU}^{Ile}$ is more due to the inherently weak affinity of $tRNA_{1 UAU}^{Ile}$ for the AUG codon than competition *in vivo* by the endogenous methionine tRNA.

The weak affinity of $tRNA_{1 UAU}^{Ile}$ 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* $tRNA_1^{Ser}$, which contain U34 instead of the modified U normally present in the $tRNA_1^{Ser}$, read the serine codon UCA just as well in an *E. coli* protein synthesis system but not UCG (46,47). Similarly, an anti-codon 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 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 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 $tRNA_2^{Ile}$ isolated from *M. mobile* (42). As mentioned above, in contrast to the AUA codon-specific $tRNA_2^{Ile}$ isolated from most bacteria or archaea which contain lysidine or agmatidine, the *M. mobile* $tRNA_2^{Ile}$ contains an unmodified U ($tRNA_{2 UAU}^{Ile}$). Suzuki *et al.* have shown that the *M. mobile* $tRNA_{2 UAU}^{Ile}$ binds to the isoleucine codon AUA but not to the methionine codon AUG on

M. mobile ribosomes. However, the same tRNA binds to both AUA and AUG on *E. coli* ribosomes. Thus, the *M. mobile* ribosome and $tRNA_{2 UAU}^{Ile}$ have coevolved in such a way that the *M. mobile* $tRNA_{2 UAU}^{Ile}$ 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* $tRNA_{2 UAU}^{Ile}$ binds only to AUA or to both AUA and AUG codons on ribosomes isolated from other *Mycoplasma* species. The specific binding of *M. mobile* $tRNA_{2 UAU}^{Ile}$ to AUA 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 $tRNA_{1 UAU}^{Ile}$ isolated from *B. subtilis* described in the present study, which binds preferentially to AUA, but only weakly to AUG on *B. subtilis* ribosomes. Similar to the *M. mobile* $tRNA_{2 UAU}^{Ile}$, the *B. subtilis* mutant $tRNA_{1 UAU}^{Ile}$ 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* $tRNA_2^{Ile}$ 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 AUA but also to AUG and to AUU (AUA > AUG > AUU) (Mandal,D. and Köhrer,C. *et al.*, unpublished data).

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