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Title:**Recognition of tRNA^{Ile} with a UAU anticodon by isoleucyl-tRNA synthetase in lactic acid bacteria**

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Abbreviations

ARS, aminoacyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; L, lysidine; Tils, tRNA^{Ile}-lysidine synthetase; t⁶A, N⁶-threonylcarbamoyladenine; m⁶A, N⁶-methyladenine; TLC, thin-layer chromatography; SEM, standard error of the mean.

Abstract

In almost all eubacteria, the AUA codon is translated by tRNA^{Ile2} bearing lysidine (2-lysylcytidine; L) at the wobble position. L is introduced by tRNA^{Ile} lysidine synthetase (TilS) via post-transcriptional modification of the cytidine of tRNA^{Ile2}(CAU). *Lactobacillus casei* and *Lactobacillus plantarum* have *tilS* homologues and the tRNA^{Ile2}(CAU) genes. In addition, *L. casei* also has another tRNA^{Ile2} gene with a UAU anticodon. *L. plantarum* has a tRNA^{Ile}(UAU)-like RNA. Here we demonstrate that *L. casei* tRNA^{Ile2}(UAU) is charged with isoleucine by *L. casei* isoleucyl-tRNA synthetase (IleRS) but not by *L. plantarum* IleRS, even though the amino acid identity of these two enzymes is over 60%. It has been reported that, in *Mycoplasma mobile*, which has its tRNA^{Ile2}(UAU) but no *tilS* homologue, an Arg residue at position 865 of the IleRS is required for recognition of the UAU anticodon. This position is occupied by an Arg also in the IleRSs from both of the *Lactobacillus* species. Thus, other residues in *L. casei* IleRS should also contribute to the recognition of tRNA^{Ile2}(UAU). We found that a chimeric IleRS in which the N-terminal domain was replaced by the corresponding region of *L. plantatarum* IleRS has very low aminoacylation activity towards both tRNA^{Ile2}(UAU) and tRNA^{Ile1}(GAU). The A18G mutant had barely detectable aminoacylation activity towards either of the tRNAs^{Ile}. However, a double point mutant of A18G and G19N aminoacylated tRNA^{Ile1}(GAU), but not tRNA^{Ile2}(UAU). Our results suggest that, for *L. casei* IleRS, Ala18 and Gly19 also play a critical role in recognition of tRNA^{Ile2}(UAU).

Introduction

Accurate protein synthesis requires that aminoacyl-tRNA synthetases catalyze aminoacylation of their cognate amino acid to the 3' end of their cognate tRNA [1], and that the aminoacyl-tRNA correctly deciphers the genetic code. Two-codon sets ending in a purine (NNR) could in general be decoded by one tRNA species by virtue of the wobble interaction [2,3], while the AUR codons are translated differently, AUA into isoleucine (Ile) and AUG into methionine (Met). Hence, two different tRNAs are employed to distinguish and translate the two codons.

The wobble position of AUR-reading tRNAs not only determines the specificity for the third base of the codon but is also involved in tRNA discrimination during aminoacylation. In almost all eubacteria, the AUA codon is one of the most rarely used codon and is translated by tRNA^{Ile} bearing lysidine (2-lysylcytidine; L) at the wobble position [4–6]. Lysidine is introduced by tRNA^{Ile} lysidine synthetase (TilS) through post-transcriptional modification of cytidine at the wobble position of the tRNA^{Ile2}(CAU) precursor [7]. tRNA^{Ile2}(LAU) can be charged with isoleucine by IleRS. However the unmodified precursor tRNA^{Ile2}(CAU) is charged with Met by methionyl-tRNA synthetase (MetRS) and deciphers the Met AUG codon [6].

The tRNA^{Ile}(UAU) genes are only rarely found in eubacterial genomes [8–12]. *Mycobacterium mobile* has a tDNA with the TAT anticodon and lacks the genes for tRNA^{Ile2}(CAU) and *tilS* homologue. The tRNA^{Ile2}(UAU) molecule has an unmodified uridine at the wobble position and is charged with isoleucine by IleRS [10]. It is considered that the *M. mobile* ribosome rejects tRNA^{Ile2}(UAU) wobble base pairing with the AUG methionine codon. *M. mobile* IleRS aminoacylates *Escherichia coli* tRNA^{Ile2}(LAU) prepared *in vitro* [10]. On the other hand, IleRS of *E. coli* carrying the *tilS* gene does not efficiently recognize the UAU anticodon of tRNA^{Ile} [13].

In *Bacillus subtilis*, a suppressor mutant strain with a temperature-sensitive *tilS* gene has been obtained [14]. The *tilS*-suppressor strain has a mutation in one of the tRNA^{Ile1}(GAU) genes in which the wobble G has been changed into a T. The gene product of the mutant tRNA, tRNA^{Ile1}(GAU/UAU), is aminoacylated by IleRS and translates AUA codons, in the absence of the TilS activity.

Among the organisms that have tRNA^{Ile2}(UAU), the ones that also have the *tilS* gene are of interest [11,12]. If they have both tRNA^{Ile2}(UAU) and tRNA^{Ile2}(LAU), questions are raised as to whether both these tRNAs are charged with isoleucine. To clarify this issue, we have been using as model organisms *Lactobacillus plantarum* and *Lactobacillus casei*, which have *tilS* homologues and the tRNA^{Ile2}(CAU) genes in addition to the tRNA^{Ile2}(UAU) genes. They are lactic acid bacteria (LAB). LAB are widely used in fermentation and in various foods consumed by humans and other animals. Specific species or strains of LAB including *L. plantarum* and *L. casei* are known to have probiotic potential with health benefits [15–17]. In our previous report, we showed that the tRNA^{Ile}(UAU)-like gene of *L. plantarum* differs significantly from that of tRNA^{Ile} in terms of IleRS recognition determinants and is not aminoacylated by the IleRS [18]. For this reason, it was described as a “tRNA^{Ile}(UAU)-like gene”. *L. plantarum* IleRS also does not attach isoleucine to a mutant tRNA in which the anticodon of tRNA^{Ile2}(CAU) has been substituted with UAU [18]. In contrast, tRNA^{Ile2}(UAU) of *L. casei* has the recognition determinants, C4-G69 (G4-C69 in *L. casei* in tRNA^{Ile2}(UAU)), U12-A23, C29-G41, L34 (or G34), A35, U36, A37, A38 and A37, of bacterial IleRS [13], but it was not known if the tRNA can be aminoacylated by its cognate IleRS. In this report, the recognition mechanism of IleRS derived from these two types of LAB for tRNA^{Ile2}(UAU) has been compared and analyzed with reference

to the characterization and structural analysis data for related IleRSs that have been determined to date.

Results

Isoleucylation activity of IleRS from *L. plantarum* and *L. casei* for tRNA^{Ile} and mutant tRNA^{Ile}

L. plantarum appears to have the three tRNA^{Ile} species, although the RNA with the UAU anticodon has different characteristics from general tRNA^{Ile}. For instance, the sequence of the tRNA^{Ile}(UAU)-like RNA of *L. plantarum* differs significantly from that of general tRNA^{Ile} species in terms of IleRS recognition determinants and the presence of a long variable region (Fig. 1A). Our previous study also demonstrated that the tRNA^{Ile}(UAU)-like RNA was not charged with isoleucine by a *L. plantarum* S-100 fraction [18]. In the current experiments, recombinant versions of IleRS from *L. plantarum* were prepared, and the purified *Lp*-IleRS did not show aminoacylation activity for *Lp*-tRNA^{Ile}(UAU)-like RNA (Fig. 1B).

To test UAU anticodon recognition of the purified *L. plantarum* IleRS, mutant tRNA in which the anticodons of *Lp*-tRNA^{Ile1}(GAU) and *Lp*-tRNA^{Ile2}(CAU) were substituted with UAU were prepared (*Lp* indicates *L. plantarum*.) (Fig. 1C). The mutant tRNA transcripts of *Lp*-tRNA^{Ile1}(GAU/UAU) and *Lp*-tRNA^{Ile2}(CAU/UAU) were not aminoacylated by *Lp*-IleRS (Fig. 1D) consistent with the previous study using *L. plantarum* S-100 fraction [18]. In this experiment, tRNA^{Ile1}(GAU) was used as control tRNA and not tRNA^{Ile2}(LAU), because introduction of lysidine into the tRNA^{Ile2}(CAU) transcript by TilS was inefficient in our experiment.

L. casei also has three annotated tRNA^{Ile} species for which the anticodons are GAU, UAU and CAU. *L. casei* tRNA^{Ile2}(UAU) has IleRS recognition determinants and a more normal (canonical) secondary structure (Fig. 2A). For *L. casei* IleRS, the Ile charging activity was observed for *Lc*-tRNA^{Ile2}(UAU) (*Lc* indicates *L. casei*.) (Fig. 2B).

Taniguchi *et al.* suggested that for *Mycoplasma mobile* IleRS, the Arg residue at position 865 in the Zn-binding domain is important for efficient recognition of the UAU anticodon in the homogeneous tRNA^{Ile2}(UAU) [10]. An Arg is also found at the corresponding position for both *L. plantarum* and *L. casei* IleRSs. The amino acid identity of *Lp*- and *Lc*-IleRSs is over 60% (Fig. 3). However, *Lp*-IleRS charging activity for *Lc*-tRNA^{Ile2}(UAU) was found to be very low which corresponds about one-tenth to that of *Lc*-IleRS at the 10-min time point (Fig. 2C).

Analysis of chimeric IleRS proteins

To understand why *Lc*-IleRS could aminoacylate *Lc*-tRNA^{Ile2}(UAU) but *Lp*-IleRS could not, chimeric proteins of *Lc*- and *Lp*-IleRS were prepared as shown in Fig. 4A. IleRS can be divided into an N-terminal domain, a Rossman fold domain, a connective polypeptide 1 (CP1) editing domain, a CP2 domain, a helical domain, a C-terminal junction domain and a Zn-binding domain (Fig. 4A and 4B) [19]. Initially, we prepared two chimeric proteins named as N24-chimera and Zn-chimera. N24-chimera or Zn-chimera are *Lc*-IleRS proteins having the N-terminal domain or the Zn-binding domain of *Lp*-IleRS, respectively. In both the N24- and Zn-chimeric proteins, the Ile charging activities for *Lc*-tRNA^{Ile2}(UAU) were decreased compared to wild type *Lc*-IleRS. In particular, by replacing just the 24 amino acid residues of the N-terminal domain with the sequence of *Lp*-IleRS, the activity for *Lc*-tRNA^{Ile2}(UAU) of the N24-chimera and also for *Lc*-

tRNA^{Ile1}(GAU) was significantly decreased (Fig. 4C). A study of the structure of the complex of IleRS from *Staphylococcus aureus* and *Escherichia coli* tRNA^{Ile1}(Fig. 4B, PDB ID: 1FFY) demonstrated that the N-terminal domain and the Zn-binding domain interact with each other via the tRNA anticodon loop. We suspected that the decreased activity might be due to the combination of the heterologous N-terminal and Zn-binding domains derived from *Lc*- and *Lp*-IleRS. Therefore, N24/Zn-chimera in which both the N-terminal and Zn-binding domains are replaced by those of *Lp*-IleRS (Fig. 4A) was prepared. However, the N24/Zn-chimera lost almost all activity for both *Lc*-tRNA^{Ile2}(UAU) and *Lc*-tRNA^{Ile1}(GAU) (Fig. 4C). Because N24-chimera showed a significantly reduced activity and N24/Zn-chimera did not recover the activity, we focused the N-terminal region as the target of further analysis. N10-chimera in which the first 10 amino acid residues of the N-terminus were replaced with the corresponding region of *Lp*-IleRS was prepared to narrow down the region of *Lp*-IleRS important for activity. The N10-chimera showed isoleucine charging activity for both *Lc*-tRNA^{Ile2}(UAU) and *Lc*-tRNA^{Ile1}(GAU), although activity was lower than that of wild type *Lc*-IleRS (Fig. 4C). Furthermore, N24-*Lp*-IleRS chimera, which is a *Lp*-IleRS protein having the N-terminal domain of *Lc*-IleRS was also prepared and verified whether tRNA^{Ile2}(UAU) is aminoacylated. This protein has a low activity against tRNA^{Ile2}(UAU) that is not significantly different from that of *Lp*-IleRS WT (Fig. 2C and Fig. 4C). Moreover, we also prepared N351, N592 and N830 chimeric proteins in which N-terminal 351, 592, 830 amino acids were replaced by that of *Lp*-IleRS, respectively (Fig. 5A), however, these chimeric proteins have no or significantly lower Ile charging activities for either *Lc*-tRNA^{Ile2}(UAU) or *Lc*-tRNA^{Ile1}(GAU) (Fig. 5B).

Analysis of point mutant *Lc*-IleRSs

The results obtained with the chimeric IleRS proteins suggested that the amino acid residues required for tRNA^{Ile2}(UAU) isoleucylation were in the region between Lys11 and Glu24 of *Lc*-IleRS. In this region, six amino acid residues differ between *Lc*-IleRS and *Lp*-IleRS. We therefore prepared six single point mutants, namely S13K, P15K, A18G, G19N, K22V and N23K (Fig. 6A). All mutant proteins were prepared (Fig. 6B) and the initial velocities of isoleucylation of these mutants was measured using [¹⁴C]-isoleucine.

All the point mutant proteins and wild type IleRS showed lower isoleucine charging activities for *Lc*-tRNA^{Ile2}(UAU) than for *Lc*-tRNA^{Ile1}(GAU) (Fig. 6C). For the A18G mutant, we were unable to confirm efficient charging activity for both *Lc*-tRNA^{Ile2}(UAU) and *Lc*-tRNA^{Ile1}(GAU) (Fig. 6C and 6D). Further kinetic analysis showed that the k_{cat}/K_m value for tRNA^{Ile2}(UAU) was 7% of that for tRNA^{Ile1}(GAU) in wild type IleRS (Table 1). For either of the tRNAs, there was no significant difference in the relative k_{cat}/K_m value compared to wild type for all mutant proteins except for the A18G mutant. In the A18G mutant, the kinetic parameters for tRNA^{Ile2}(UAU) could not be determined. Also the k_{cat}/K_m value for tRNA^{Ile1}(GAU) was also only 1% of that of the wild type. From these results, we concluded that Ala18 is a crucial residue for the activity of *Lc*-IleRS. The A18G mutant protein also showed only a low activity against *Lc*-tRNA^{Ile1}(GAU), raising the question of why *Lp*-IleRS aminoacylates *Lc*-tRNA^{Ile1}(GAU) even though *Lp*-IleRS carries Gly18. Since the G19N mutant protein was almost as active as wild type *Lc*-IleRS (Fig. 6C, Table 1) and *Lp*-IleRS has Gly18 and Asn19 residues (Fig. 6A), an A18G/G19N double mutant was predicted to recover the aminoacylation activity at least against tRNA^{Ile1}(GAU). Mutation of A18G/G19N resulted in recovery of the isoleucine charging activity for *Lc*-tRNA^{Ile1}(GAU) with

a reduced K_m value (Fig. 6E and Table 1). As a result, the relative k_{cat}/K_m value for tRNA^{Ile1}(GAU) increased 120-fold compared to that of the A18G mutant (Table 1). However, in the A18G/G19N double mutant, the Ile charging activity for *Lc*-tRNA^{Ile2}(UAU) remained low (Fig. 6E), and the kinetic parameters for tRNA^{Ile2}(UAU) could not be determined. Thus, it was found that the Ala18 and Gly19 residues play an important role in recognition of the anticodon UAU of tRNA^{Ile2}.

Isolation and analysis of *L. casei* tRNA^{Ile}

Experiments described above revealed that *in vitro* transcribed *Lc*-tRNA^{Ile2}(UAU) could be aminoacylated by *Lc*-IleRS. It is known that the *N*⁶-threonylcarbamoyladenosine (t⁶A) modification at position 37 in tRNA^{Ile} plays an important role in efficient isoleucylation by *E. coli* IleRS [20]. Therefore, we tested aminoacylation using *Lc*-tRNA^{Ile2}(UAU) isolated from *L. casei* cells. Using a solid phase DNA probe method, the tRNA^{Ile} species were isolated (Fig. 7A) from *L. casei* small RNA fraction [21], and the Ile accepting activities of the isolated tRNA measured using *Lc*-IleRS. We had predicted that the Ile accepting activity of the isolated tRNA^{Ile} would increase compared to the transcribed tRNA^{Ile}. As expected, the Ile accepting activities of the isolated *Lc*-tRNA^{Ile1}(GAU) and *Lc*-tRNA^{Ile2}(LAU) was higher than that of the *in vitro* transcribed *Lc*-tRNA^{Ile1}(GAU). However, for the isolated *Lc*-tRNA^{Ile2}(UAU), only about one quarter of the activity of the transcribed tRNA^{Ile2}(UAU) could be detected (Fig. 7B).

Analysis of modified nucleosides of native *Lc*-tRNA^{Ile2}(UAU)

Since aminoacylation efficiency for the isolated *Lc*-tRNA^{Ile2}(UAU) was extremely poor, we confirmed using Kuchino's post labeling method [22] whether the wobble position was modified and whether t⁶A modification was present at position 37. No modification was detected in the wobble position in our experiment while t⁶A was detected at position 37 in *Lc*-tRNA^{Ile2}(UAU) (Fig. 8A and 8B).

Discussion

In *E. coli* which lacks a tRNA^{Ile2}(UAU) gene and has a *tilS* homologue, Trp905 is found at the position corresponding to Arg865 of *M. mobile* IleRS (Fig. 9A). Since W905R mutant of *E. coli* IleRS efficiently recognizes both tRNA^{Ile2}(UAU) and tRNA^{Ile2}(CAU), it has been proposed that the basic amino acid residue allows recognition of a pyrimidine at the wobble position of tRNA^{Ile2} [10]. A comparison of the amino acid sequences of IleRS from *M. mobile*, *E. coli*, *L. casei* and *L. plantarum* demonstrates that the amino acid residues that corresponds to Arg865 of *M. mobile* IleRS are also Arg in both IleRSs of *L. casei* and *L. plantarum* (Fig. 9A). *Lp*-IleRS did not aminoacylate the tRNA^{Ile2}(CAU) without lysidine at the wobble position [18]. *Lp*-IleRS rejects both tRNAs^{Ile2} despite that the key Arg residue in the Zn-binding domain is present. Unlike *Lp*-tRNA^{Ile2}(UAU), *Lc*-tRNA^{Ile2}(UAU) has a typical tRNA^{Ile} primary structure and has all the bases required for recognition by bacterial IleRS. In fact, *Lc*-IleRS can aminoacylate *Lc*-tRNA^{Ile2}(UAU) efficiently. Nonetheless, *Lp*-IleRS has significantly weaker aminoacylation efficiency for *Lc*-tRNA^{Ile2}(UAU). What is the origin of such a difference in substrate recognition between *Lc*-IleRS and *Lp*-IleRS? The results using the chimeric IleRS proteins (Fig. 4C) showed that the N24 chimera is able to aminoacylate *Lc*-tRNA^{Ile1}(GAU) with reduced efficiency, however, the activity

for *Lc*-tRNA^{Ile2}(UAU) was significantly reduced. On the contrary, the N10-chimera showed aminoacylation activities for both tRNA^{Ile1}(GAU) and tRNA^{Ile2}(UAU), indicating that the segment of amino acids 11 to 24 contains residues crucial for the recognition of *Lc*-tRNA^{Ile2}(UAU). Within this region, six amino acids differ between *L. plantarum* and *L. casei*. These amino acids were mutated one by one into *L. plantarum* counterpart residues. Among these mutant proteins, A18G had severe defect on the aminoacylation activity not only for *Lc*-tRNA^{Ile2}(UAU) but also for *Lc*-tRNA^{Ile1}(GAU). The activities of the other five mutant proteins for *Lc*-tRNA^{Ile2}(UAU) were not significantly different from that of wild type *Lc*-IleRS (Fig. 6C, Table 1). The structure of the complex of *S. aureus* IleRS and *E. coli* tRNA^{Ile1}[19] suggests that Ala18 is not likely to contact the tRNA directly. Instead, the neighboring amino acid (Arg17) establishes direct contact with A37 of tRNA (Fig. 9B). This region of the N-terminal sequence of IleRS sits tightly on the conserved Tyr645, Arg646 and Arg649 of the helical domain. The methyl group of Ala18 may help to position Arg17 to be positioned to interact with tRNA anticodon. Lack of the methyl group of Ala18 in A18G mutant might therefore change the positioning and/or dynamics of residue Arg17 during interaction with A37 of tRNA. In agreement with this idea, our results show that Ala18 is required for *Lc*-IleRS interaction with *Lc*-tRNA^{Ile}.

As A18G mutation abolished the aminoacylation activity even for tRNA^{Ile1}(GAU), we further asked how *Lp*-IleRS that has a glycine at position 18 is able to aminoacylate tRNA^{Ile1}(GAU). A comparison of IleRSs of some Lactobacillus species show that Asn19 co-appears with Gly18 (Fig. 9A). Therefore, we investigated whether the activity against tRNA^{Ile1}(GAU) could be restored by introducing A18G-G19N double mutant. As expected, the double mutant showed the Ile charging

activity only for tRNA^{Ile1}(GAU), and only slight activity was measured for tRNA^{Ile2}(UAU). Thus, Gly18 and Asn19 would be avoid interaction with inactive tRNA such as tRNA^{Ile2}(UAU).

Modified nucleosides of tRNA often alter tRNA recognition by cognate aminoacyl-tRNA synthetases [23]. In general, tRNAs which are modified post-transcriptionally by tRNA modification enzymes are better substrates for aminoacyl-tRNA synthetases than *in vitro* transcribed tRNA. For example, in *E. coli*, the tRNAs^{Ile} containing L34 and t⁶A37 modifications are better substrates than the tRNAs^{Ile} without the modified nucleoside(s) for IleRS [13,20,24]. Furthermore, N⁴-acetylcytidine (ac⁴C) modification at the wobble position of tRNA^{Met} is a positive determinant for MetRS and prevents it from becoming a substrate for IleRS [24]. Moreover, modified nucleosides in the anticodon of tRNA can fine tune the codon anticodon interaction during translation [25]. However, *Lc*-IleRS showed little aminoacylation activity towards tRNA^{Ile2}(UAU) purified from *L. casei* cells (Fig. 7B). In our nucleoside analysis of *Lc*-tRNA^{Ile2}(UAU), modified nucleosides were not detected in the anticodon of *Lc*-tRNA^{Ile2}(UAU), but t⁶A was detected at position 37 (Fig. 8). In *B. subtilis*, a mixture of t⁶A and m⁶A was found at position 37 of mutant tRNA^{Ile1} in which the anticodon sequence GAU was mutated to UAU [26]. It has been suggested that the mutation of G to U in the wobble position makes the tRNA a poor substrate for the t⁶A enzyme complex Tsab/C/D. It is notable that tRNA^{Ile2}(LAU) also contains m⁶A37 in *B. subtilis* [27]. From our analysis using Kuchino's post labeling method [28], there is no m⁶A37 modification in *Lc*-tRNA^{Ile2}(UAU). As far as *Lc*-IleRS is concerned, unmodified tRNA^{Ile2}(UAU) transcript is a better substrate than the isolated *Lc*-tRNA^{Ile2}(UAU). The experimental results suggest some of the modified nucleosides in *Lc*-tRNA^{Ile2}(UAU) play a role as a negative determinant for *Lc*-IleRS.

Both tRNA^{Ile2}(UAU) and tRNA^{Ile2}(LAU) are present in *L. casei*, both of them were aminoacylated by *Lc*-IleRS and thus could be utilized for the AUA codon decoding. However, in *L. casei*, the AUA codon is rare, and tRNAs^{Ile2}, especially tRNA^{Ile2}(UAU), are minor tRNAs. In fact, we obtained less tRNA^{Ile2}(UAU) than tRNA^{Ile2}(LAU) after purification. Considering this fact and the poor aminoacylation efficiency of the isolated *Lc*-tRNA^{Ile2}(UAU), it is expected that few *Lc*-tRNA^{Ile2}(UAU) molecules are aminoacylated in the cell, and that *Lc*-tRNA^{Ile2}(LAU) is preferentially used for AUA codon decoding. In the case of *L. casei*, we have preliminary evidence that some of the modified nucleosides in *Lc*-tRNA^{Ile2}(UAU) interfere with *Lc*-IleRS recognition. It is possible that tRNA^{Ile2}(UAU) avoids being aminoacylated so as not to be used in the AUG codon translation.

In conclusion, we demonstrated that tRNA^{Ile2}(LAU) and tRNA^{Ile2}(UAU) are expressed in *L. casei* and that the tRNA^{Ile2}(UAU) is aminoacylated by *Lc*-IleRS. The Ala18 and Gly19 of *Lc*-IleRS play a critical role in recognition of tRNA^{Ile2}(UAU). However, we found that native tRNA^{Ile2}(UAU) from *L. casei* cells is hardly aminoacylated by *Lc*-IleRS. The result suggests that some of the modified nucleosides in *Lc*-tRNA^{Ile2}(UAU) might be a negative determinant for *Lc*-IleRS.

Materials and methods

Bacterial culture

The culture source of *Lactobacillus plantarum* WCFS1 and *Lactobacillus casei* BL23 were kind gifts from Dr. Ro Osawa (Kobe University) and Luis Bermudez-Humaran (French National Institute for Agricultural Research), respectively. *L. plantarum* and *L. casei* were grown on Man-Rogas-Sharpe (MRS; Difco™ Lactobacilli MRS broth) agar (containing 1.5% agar, w/v) or cultured statically in MRS broth at 37 °C.

Recombinant IleRSs

For expression of recombinant IleRS in *E. coli* cells, *ileS* genes were amplified by PCR from genomic DNAs of *L. plantarum* or *L. casei* using KOD Fx neo DNA polymerase (Toyobo) and primers (Table S1). A hexahistidine (6xHis)-tag was added to the N-terminus of the obtained PCR fragments. Each fragment and a pET22b PCR fragment was assembled using NEBuilder HiFi assembly (New England BioLabs). The original 6xHis sequence of pET22b (Novagen) was removed in the amplified PCR fragment. The resulting expression plasmid was named pET22b-plant-IleRS or pET22b-casei IleRS.

Chimeric IleRS proteins, N10-, N24-, Zn-, N24/Zn-, 351-, 592-, 830- and N24-Lp-IleRS chimera were prepared as follows. For example, to construct the expression vector of the N10-chimera, the gene DNA sequence corresponding to ten amino acid residues from the N-terminus of *L. plantarum* IleRS and the subsequent downstream sequence of *L. casei* IleRS were amplified by PCR (Fig. 4A and Table S1). These DNA fragments were fused into one DNA fragment by overlap PCR and then the obtained DNA fragment was assembled with a pET22b PCR product using NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs.)

The mutations S13K, P15K, A18G, G19N, K22V, N23K and A18G/G19N were introduced into the pET22b-casei IleRS by site-directed mutagenesis using primer sets shown in Table S1.

The His-tagged IleRS proteins of *L. plantarum* and *L. casei* were expressed in *E. coli* Rosetta2 (DE3) (Novagen) at 37°C, in the presence of 1 mM IPTG (isopropyl- β -D-1-thiogalactopyranoside) for expression of wild type IleRS and chimeric proteins or 0.5 mM IPTG for point mutant proteins. The cells were then grown at 37°C for 4 h in the case of wild type IleRS and chimera proteins or for 2 h in the case of point mutant proteins. Collected cells were stored at -80°C. The cells were resuspended in buffer A (20 mM Tris-HCl (pH7.5), 500 mM NaCl, 6 mM β -mercaptoethanol, and 25 mM imidazole) and lysed by sonication. The resulting cell lysate was then centrifuged at 8,000g for 20 min and the supernatant was centrifuged again. The second supernatant was applied to a Ni-Sepharose High Performance column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A and then eluted with buffer A containing 25, 50, 100 and 250 mM imidazole in a stepwise elution. The fractions with 100 mM and 250 mM imidazole elution that contained the His-tagged IleRS were collected and dialyzed against buffer B (50 mM Tris-HCl (pH7.5), 5 mM NaCl, 5 mM MgCl₂ and 6 mM β -mercaptoethanol). The dialyzed IleRS fraction was applied to a Q-Sepharose Fast Flow column (GE Healthcare) equilibrated with buffer B and then a stepwise elution performed with buffer B containing 100, 200, 400 and 500 mM NaCl. The IleRS was eluted in 400 mM NaCl buffer B and was concentrated using an Amicon Ultra-15 centrifugal filter device (MWCO=10,000) (Millipore). The purified IleRS was stored at -30°C in stock buffer (50 mM Tris-HCl (pH7.5), 50 mM KCl, 5 mM MgCl₂ and β -mercaptoethanol) containing 50% glycerol. The concentration of

purified IleRS was determined using a Bradford assay. The purity of the protein was monitored by SDS-PAGE.

Isoleucylation analysis

Transfer RNA transcripts were prepared by run-off transcription using T7 RNA polymerase [29] and synthetic DNA oligo nucleotides shown in Supplementary Table 1. Isoleucylation assay of wild type IleRS, chimeric IleRS proteins and point mutant IleRS proteins was performed using tRNA transcripts and [¹⁴C(U)]-Ile (11.03 GBq/mmol) from Moravek Biochemicals, Inc.. The assay procedure was previously described [21]. Amino acid charging activity was measured by a filter assay at 37°C using 0.1 A₂₆₀ units of tRNA transcripts or 0.01 A₂₆₀ units of isolated tRNAs^{lle}. The radioactivity was quantitated on a liquid scintillation counter (AccuFlex LSC-8000, HITACHI).

Kinetic analysis of isoleucylation

An isoleucylation assay to measure the apparent kinetic parameters for tRNA was carried out at 37°C in 40 µl reaction mixture containing 100 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 2 mM ATP (pH7.0), 20 mM KCl, 11 µM [¹⁴C(U)]-Ile (9.62-14.76 GBq/mmol) (Moravek Biochemicals, Inc.), 0.0125-1 µM tRNA transcripts and 230 nM recombinant wild type IleRS or mutant IleRS proteins. The incubation time was varied depending on the activity of proteins. The reaction mixture was spotted onto a Whatman 3MM cellulose filter, and the reaction was stopped by immersing the filter in cold 5% trichloroacetic acid. Radioactivity derived from [¹⁴C(U)]-Ile was measured on a

liquid scintillation counter. The apparent kinetic parameters were determined by a Lineweaver-Burk plot.

RNA isolation

L. casei total RNA was extracted using ISOGEN II (Nippon Gene) according to the manufacturer's manual with slight modifications. Frozen *L. casei* cells (1 g) collected in early log phase were ground in a chilled mortar for 10-20 min. The ISOGEN II (4 mL) was added to the mortar, and cells were further ground for 10-20 min. The cell suspension was transferred to a tube, and then treated according to the ISOGEN II manual. *Lc*-tRNA^{Ile2}(UAU) was isolated from the extracted small RNA using a solid-phase DNA probe method described in our previous report [21]. The sequences of 3'-biotinylated DNA probes are described in Table S1. The isolated tRNA species were analyzed by 10% PAGE (7 M urea).

Nucleoside analysis of native *L. casei* tRNA^{Ile2}(UAU)

Anticodon nucleotides of isolated *Lc*-tRNA^{Ile2}(UAU) were analyzed using Kuchino's post labeling method [22] with slight modifications as follows. Limited cleavage of the isolated *Lc*-tRNA^{Ile2}(UAU) (0.01 A₂₆₀ units) was performed with formamide at 90°C for 2 min. The 5' end of the samples recovered by ethanol precipitation were phosphorylated using T4 polynucleotide kinase (New England BioLabs.) and [γ -³²P]-ATP (PerkinElmer). The 5' labelled samples were analyzed by 15% PAGE (7 M urea, 40 cm gel) and then the anticodon nucleotides were identified by 2D-TLC [30,31]. The 1st solvent system of the 2D-TLC was isobutylic acid : ammonia water:

water, 66:1:33, v/v/v. and the 2nd solvent system was isopropanol : HCl : water, 70:15:15, v/v/v.

5'-³²P-labeled nucleotides were monitored using a Typhoon FLA7000 (GE Healthcare).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

CT designed research. GU, YF, TY, NI, HF and CT performed experiments. CT, GU, SY and KT analyzed data. CT, SY and KT wrote the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Oligonucleotide sequences of primers and probes

Figure legends

Fig. 1. Aminoacylation activities of a recombinant *Lp*-IleRS. (A) Cloverleaf structure of *Lp*-tRNA^{Ile}(UAU)-like RNA. (B) Aminoacylation activities of *Lp*-IleRS were measured for *Lp*-tRNA^{Ile1}(GAU) and *Lp*-tRNA^{Ile}(UAU)-like RNA. (C) Cloverleaf structures of *Lp*-tRNA^{Ile1}(GAU) and *Lp*-tRNA^{Ile2}(CAU). Anticodons GAU or CAU indicated in gray were substituted with UAU in mutant tRNA^{Ile} transcripts. (D) The aminoacylation activities of *Lp*-IleRS recombinant protein

were determined against *Lp*-tRNA^{Ile1}(GAU) (blue circles), the *Lp*-tRNA^{Ile1}(GAU/UAU) mutant (orange triangles) and the *Lp*-tRNA^{Ile2}(CAU/UAU) mutant (green squares).

Fig. 2. Aminoacylation activities of *Lp*-IleRS and *Lc*-IleRS. (A) The cloverleaf structures of *Lc*-tRNA^{Ile2}(UAU) (left) and *Lc*-tRNA^{Ile1}(GAU) (right). Red characters are the nucleotides identified as positive determinants in bacterial IleRS. Surrounded characters by a red square are discriminators of bacterial IleRS. (B) The Ile charging activity of *Lc*-IleRS recombinant protein was measured against *Lc*-tRNA^{Ile1}(GAU) and *Lc*-tRNA^{Ile2}(UAU). (C) The Ile charging activity of *Lp*- and *Lc*-IleRS recombinant proteins were measured against *Lc*-tRNA^{Ile2}(UAU).

Fig. 3. Sequence alignment of *Lp*-IleRS and *Lc*-IleRS. The numbering of residues is based on *L. plantarum* IleRS. A part of Zn-binding domain of IleRS is expanded.

Fig.4. Aminoacylation activities of chimeric IleRS proteins. (A) Domain structure of *Lc*-IleRS. Domain names are as described in the reference [19]. Orange and Blue areas are the amino acid sequences of *Lc*-IleRS and *Lp*-IleRS, respectively. (B) Crystal structure of *Staphylococcus aureus* IleRS complexed with *Escherichia coli* tRNA^{Ile1}(GAU) (PDB ID: 1FFY). L-shaped tRNA is shown in gray. (C) Aminoacylation activities of four chimeric IleRS proteins were measured for *Lc*-tRNA^{Ile1}(GAU) (blue circles) and *Lc*-tRNA^{Ile2}(UAU) (red circles).

Fig. 5. Aminoacylation activities of chimera IleRS proteins. (A) Blue and orange areas are the amino acid sequences of *Lp*-IleRS and *Lc*-IleRS, respectively. (B) Aminoacylation activities of

Lp-IleRS wild type (red circles), chimera-351 (green triangles), 592 (orange squares) and 830 (blue rhombus) were measured for *Lc*-tRNA^{Ile1}(GAU) (left panel) and *Lc*-tRNA^{Ile2}(UAU) (right panel).

Fig. 6. Aminoacylation activities of *Lc*-IleRS mutant proteins. (A) Comparison of N-terminal region of *Lp*- and *Lc*-IleRS. The amino acid residues indicated by stars in *Lc*-IleRS were substituted with corresponding amino acids of *Lp*-IleRS. (B) 12% SDS-PAGE analysis of *Lc*-IleRS mutant proteins. Purified proteins (1 μ g each) were analyzed. The gel was stained with Coomassie Brilliant Blue. (C) Aminoacylation activities of wild type and *Lc*-IleRS mutants were measured for *Lc*-tRNA^{Ile1}(GAU) (blue bars) and *Lc*-tRNA^{Ile2}(UAU) (red bars). Ile incorporation into tRNA transcripts at a 5-min period are shown by bars. (D)(E) Ile charging activities of the A18G single mutant (D) or A18G/G19N double mutant (E) were measured for *Lc*-tRNA^{Ile1}(GAU) (blue circles) and *Lc*-tRNA^{Ile2}(UAU) (red circles), respectively.

Fig. 7. Aminoacylation activities of *Lc*-IleRS for isolated tRNAs^{Ile}. (A) Purification of *L. casei* small RNAs and isolation of tRNA^{Ile2}(UAU), tRNA^{Ile1}(GAU) and tRNA^{Ile2}(LAU) from *L. casei* BL23. Purified RNAs were separated on a 10% polyacrylamide (7 M urea) gel and detected by Toluidine blue staining. (B) Ile acceptance activities of native (red bars) and *in vitro* transcribed tRNAs (blue bars). The activities were determined using *Lc*-IleRS. Error bars are SEM for three independent experiments.

Fig. 8. Sequence analysis of anticodon region in *Lc*-tRNA^{Ile2}(UAU). (A) The isolated *Lc*-tRNA^{Ile2}(UAU) from *L. casei* BL23 was partially cleaved by formamide. The 5'-end of each fragment was labeled with radio isotope using gamma-³²P-ATP. The RNA fragments were separated by 15% PAGE (7 M urea). The RNA fragments on the 3' side cleaved at U33, U34, A35, U36 and A37 are shown in the gel. (B) RNA fragments cleaved by formamide were purified from gel and digested with nuclease P1, and then their 5'-nt were analyzed by 2D-TLC. The 1st solvent system of the 2D-TLC was isobutylic acid : aqueous ammonia : water, 66:1:33, v/v/v. and the 2nd solvent system was isopropanol : HCl : water, 70:15:15, v/v/v. Nucleotides at positions from 34 to 37 are shown. The arrow in the lower right panel indicates spots of p⁶A at position 37. (C) Clover-leaf structure of *Lc*-tRNA^{Ile2}(UAU).

Fig. 9. Sequence alignment and structure of bacterial IleRS. (A) Sequence alignment of the N-terminal domain and the Zn-binding domain of some bacterial IleRS. The numbering of residues is based on *L. casei* IleRS. Possession of a tRNA^{Ile2}(UAU) gene and *tilS* homologue in the organisms is shown by plus or minus. (B) Close-up view of the anticodon region of tRNA^{Ile1}(GAU) and *S. aureus* IleRS (PDB ID: 1FFY) [19]. The numbering of residues is based on *S. aureus* IleRS.

Table legends

Table 1. *K_m* and *k_{cat}* values of wild type and mutant protein of *Lc*-IleRS for *Lc*-tRNA^{Ile1}(GAU) and *Lc*-tRNA^{Ile2}(UAU). *K_m* values (\pm standard error) are given in μ M and *k_{cat}* values (\pm standard error) in turnovers per second.

Table 1. Kinetic parameters of isoleucylation by wild type *Lc-IleRS* and mutated *Lc-IleRS*s

<i>Lc-IleRS</i>	<i>Lc-tRNA^{Ile1}</i> (GAU)			<i>Lc-tRNA^{Ile2}</i> (UAU)		
	K_m (μM)	k_{cat} ($\times 10^{-3}\text{sec}^{-1}$)	k_{cat} / K_m (<i>relative</i>)	K_m (μM)	k_{cat} ($\times 10^{-3}\text{sec}^{-1}$)	k_{cat} / K_m (<i>relative</i>)
WT	0.12 \pm 0.03	1.1 \pm 0.15	1.0	0.37 \pm 0.17	0.24 \pm 0.06	1.0
S13K	0.15 \pm 0.07	1.3 \pm 1.8	0.9	0.26 \pm 0.15	0.31 \pm 0.12	1.8
P15K	0.25 \pm 0.01	1.5 \pm 0.13	0.7	0.12 \pm 0.06	0.17 \pm 0.05	2.3
A18G	1.6 \pm 1.0	0.15 \pm 0.060	0.01	ND	ND	ND
G19N	0.085 \pm 0.09	1.4 \pm 0.77	1.8	0.062 \pm 0.05	0.23 \pm 0.09	5.7
K22V	0.13 \pm 0.02	0.95 \pm 1.7	0.8	0.27 \pm 0.10	0.22 \pm 0.08	1.3
N23K	1.0 \pm 0.45	4.3 \pm 1.7	0.5	0.28 \pm 0.06	0.33 \pm 0.05	1.8
A18G/G19N	0.050 \pm 0.11	0.54 \pm 0.65	1.2	ND	ND	ND

ND, Kinetic assay could not be performed in any conditions.

Fig. 1

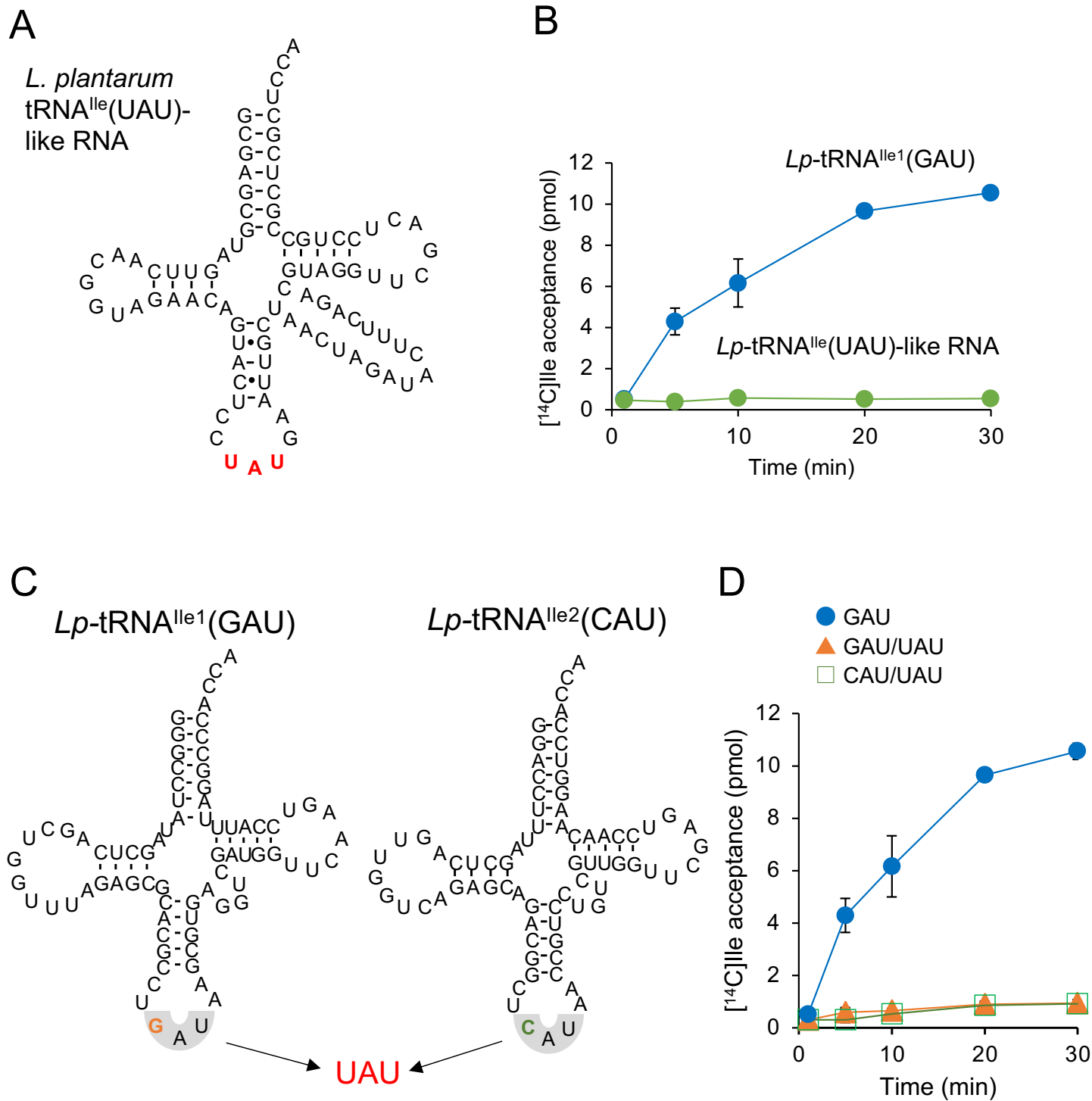
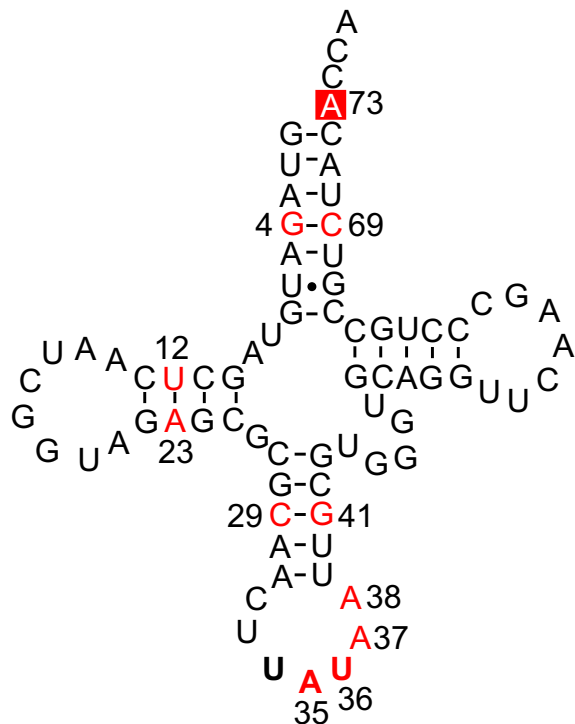


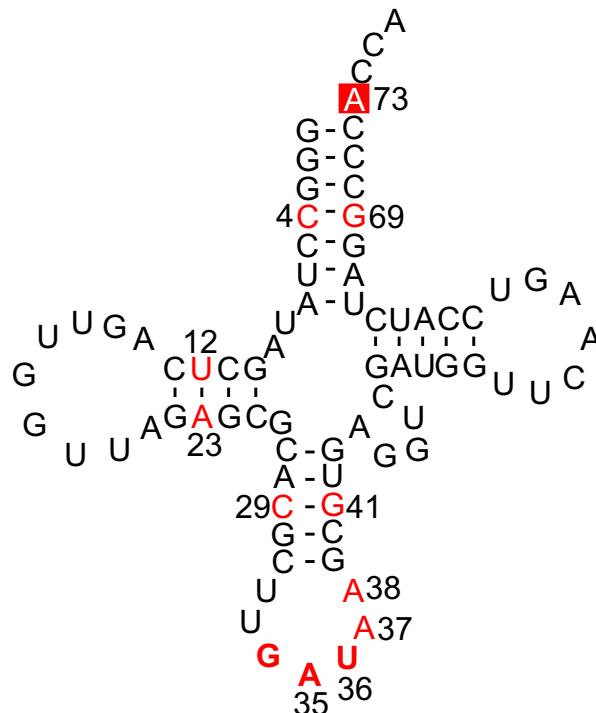
Fig. 2

A

Lc-tRNA^{Ile2}(UAU)

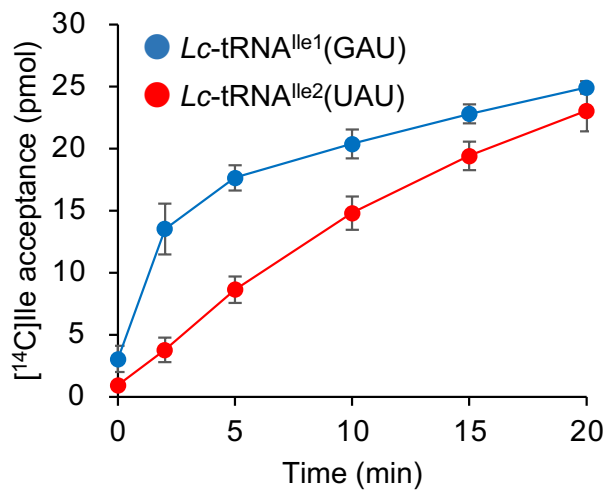


Lc-tRNA^{Ile1}(GAU)



B

Lc-IleRS



C

Lc-tRNA^{Ile2}(UAU)

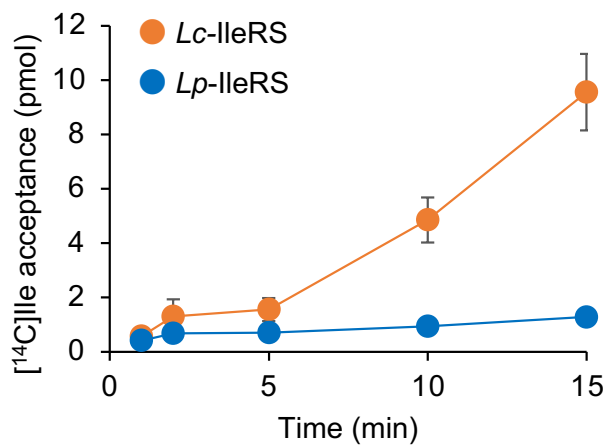
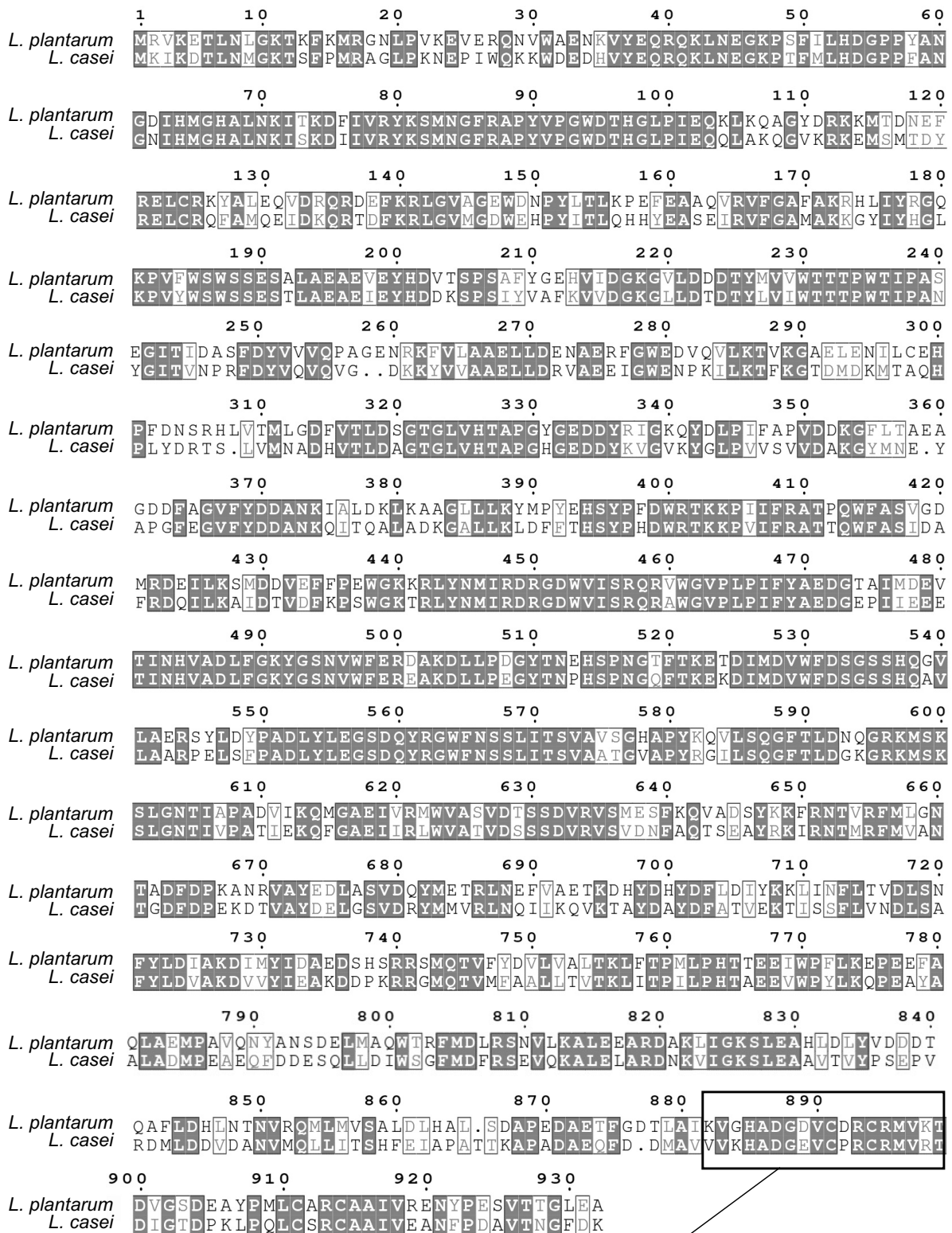
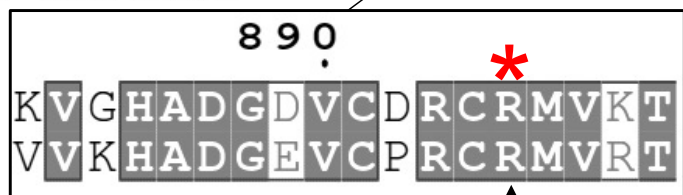


Fig. 3



L. plantarum
L. casei



Arg895

Fig. 4

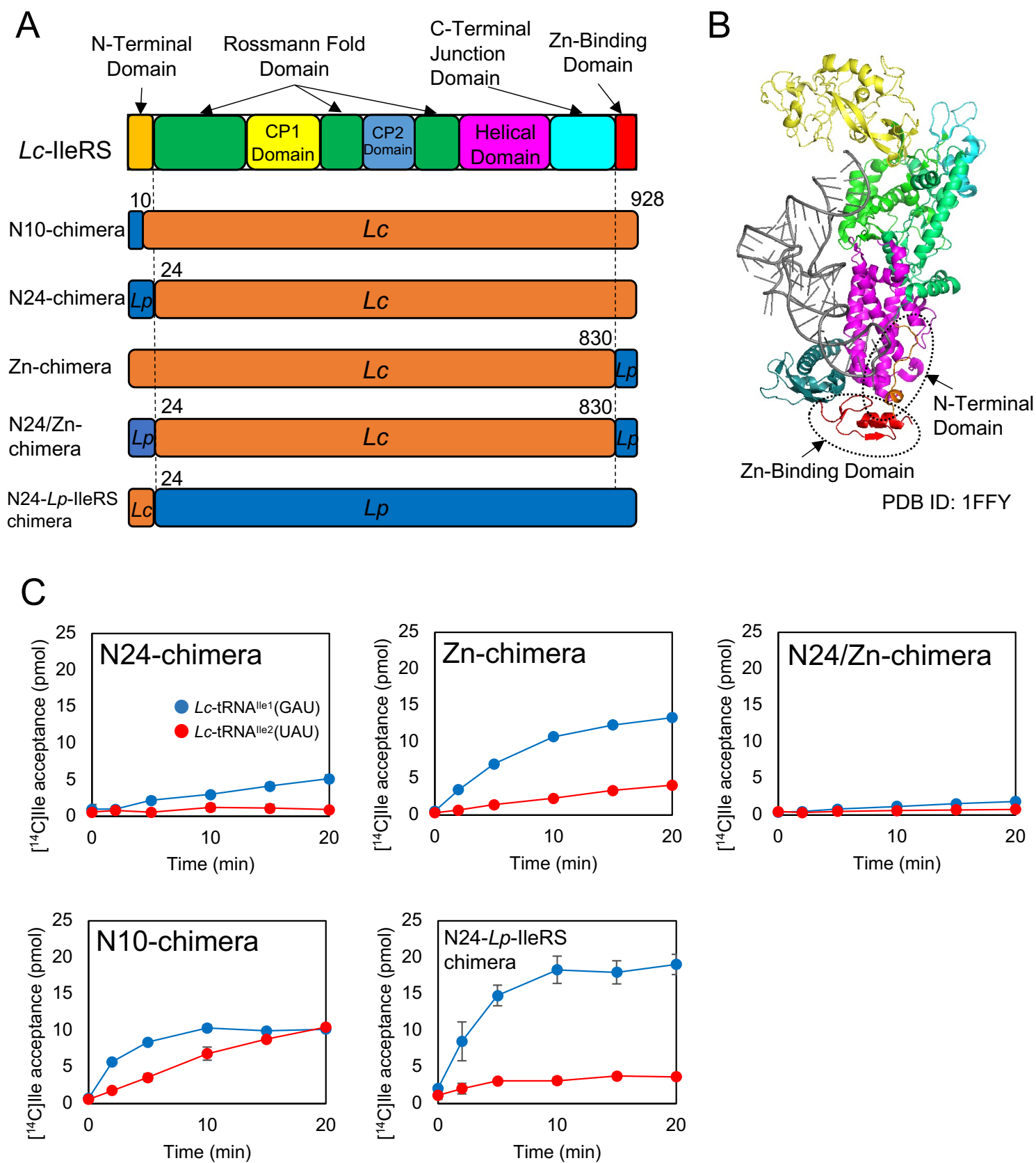
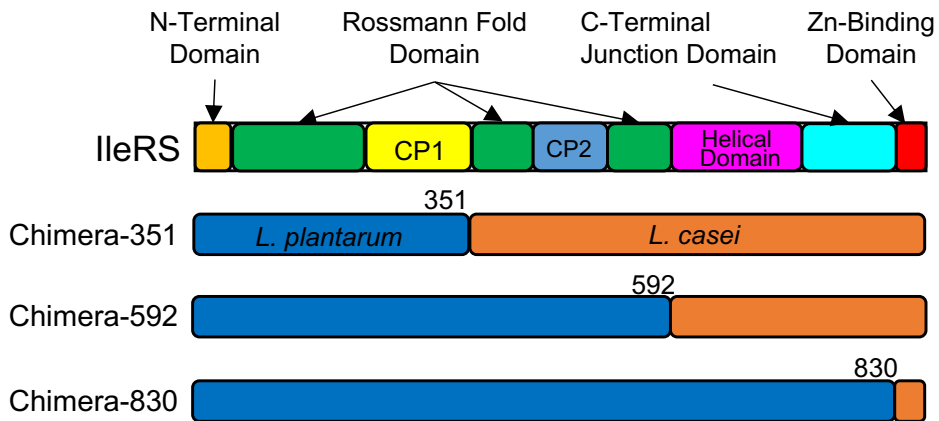


Fig. 5

A



B

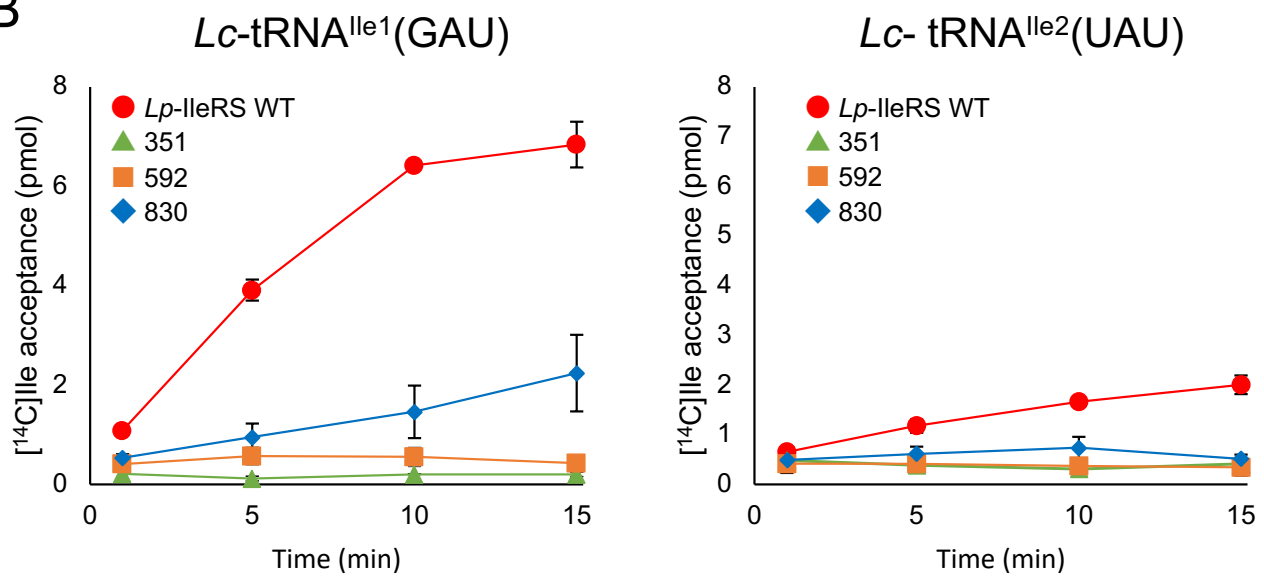


Fig. 6

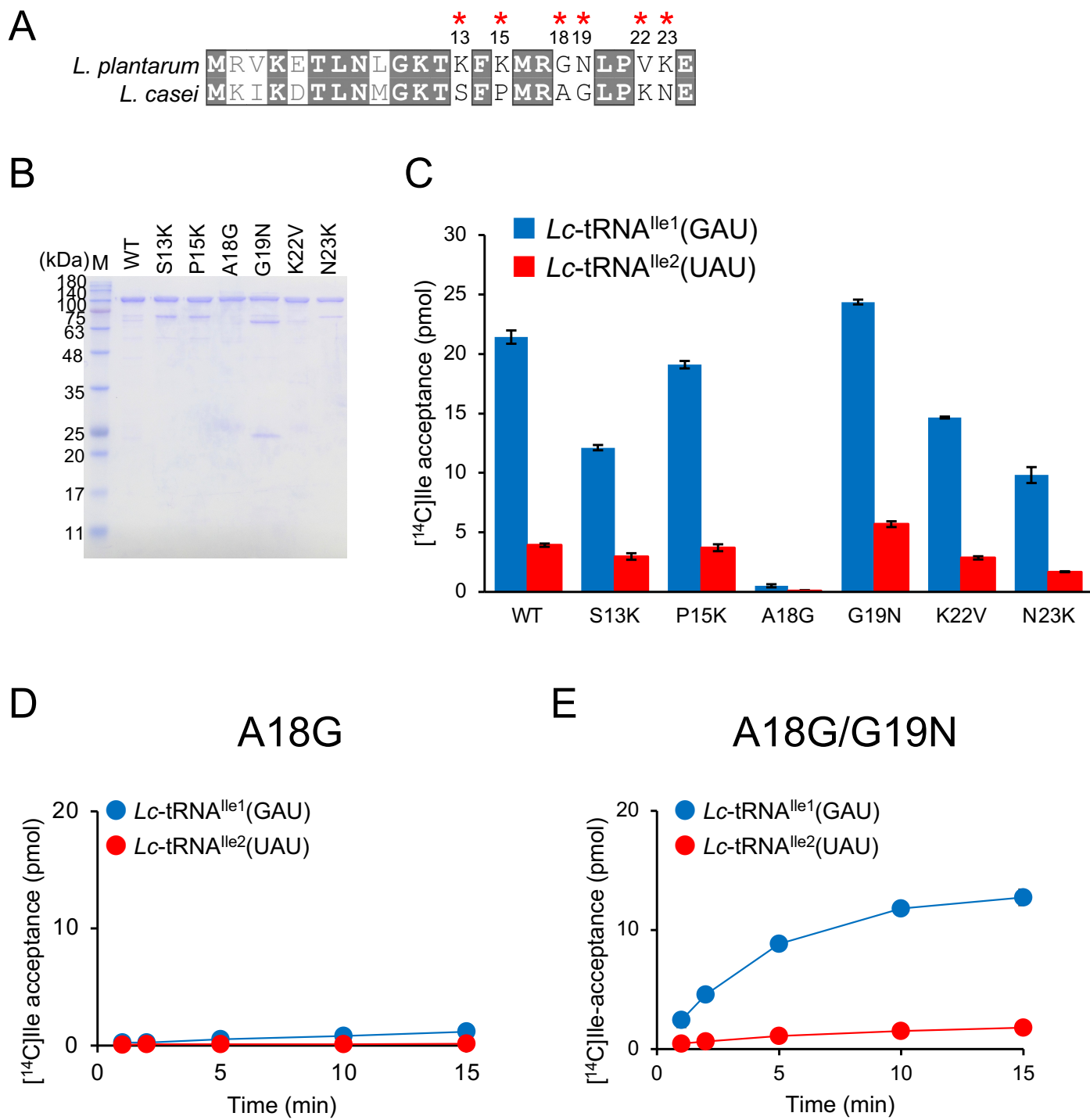
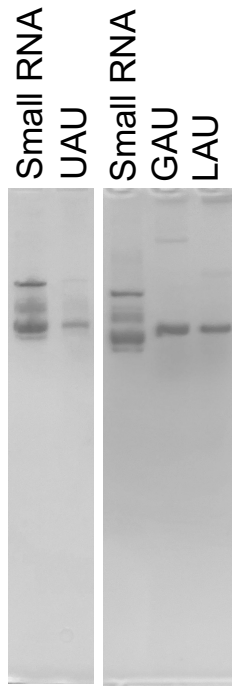


Fig. 7

A



B

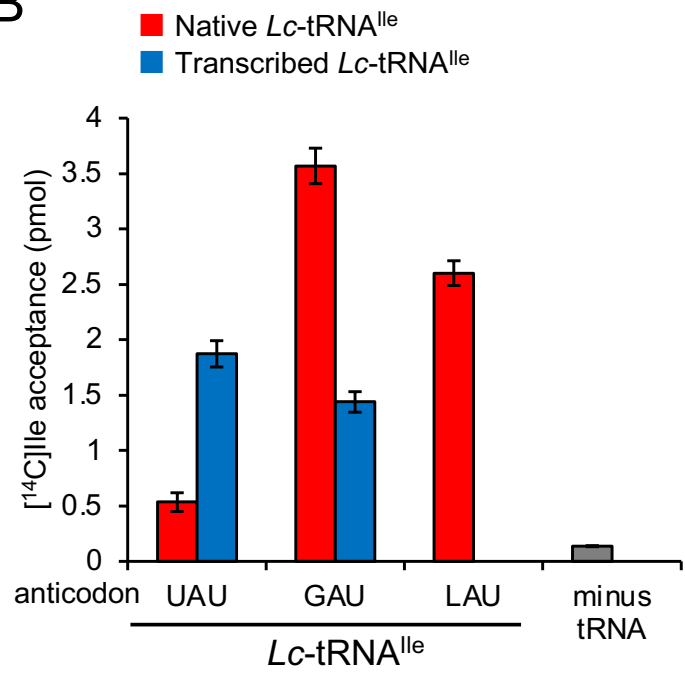


Fig. 8

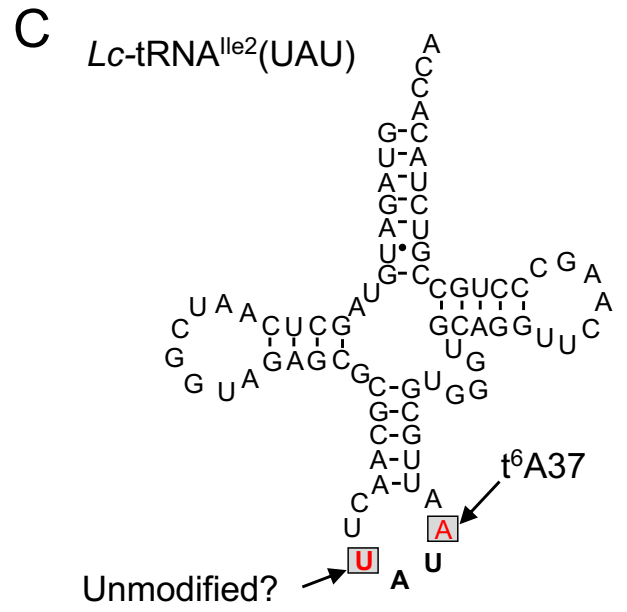
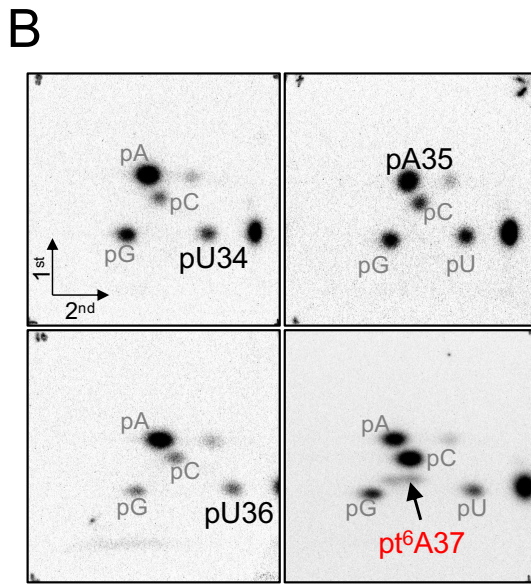
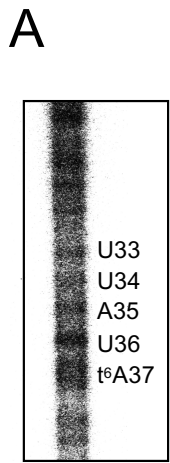


Fig. 9

A

		N-terminal domain of IleRS												Zn-binding domain of IleRS											
TAT	<i>tilS</i>	1	10	17	18	19	24	880	891																
+	+	<i>L. casei</i>	. . MKIKD	TLNMGKTSFPM	RAGL	LPKNEPIW		HADGEV	CP	RCR	MV	RTDI	I												
-	+	<i>L. plantrum</i>	. . MRVKE	TLNLGKTKFK	MRGN	LPVKEVER		HADGDV	CD	R	MV	KTDV	V												
-	+	<i>L. acidophilus</i>	. . MRIKD	TLNLGKTKFK	MRGN	LPVREAEW		HAEGEV	CP	RCR	MIR	TDI	I												
-	+	<i>L. helveticus</i>	. . MKIKD	TLNLGKTKFK	MRGN	LPVREAEW		HAEGEV	CP	RCR	MIR	TDI	I												
-	+	<i>L. delbruechii</i>	. . MRVKD	TLNLGKTKFK	MRGN	LPKREAEW		RAEGEV	CP	RCR	MY	RTDL	L												
-	+	<i>L. johnsonii</i>	. . MRVKD	TLNLGKTKFK	MRGN	LPVREAEW		HAAGEV	CP	RCR	RT	TTDV	V												
+	-	<i>M. mobile</i>	MKNKYKE	TLNIPQDVLP	MRAN	LIEKELKF		NYEGHL	CE	R	RI	YDFI	I												
+	-	<i>M. haemofelis</i>	. . MSFKN	TLNIPSTKFP	MRAN	LVENSTKY		KSALPK	CA	R	KR	HE . . .													
+	-	<i>M. suis</i>	. MSSLKD	TLFLPKTAFS	MKAN	LVQKQKSF		KSLNTP	CS	R	LL	YCHKE	'												
+	+	<i>F. balearica</i>	. MVDYKT	TLNLPETDFP	MRGN	LAQREPEM		KTEAAK	CE	R	WH	HRAEV	V												
-	+	<i>E. coli</i>	. MSDYKS	TLNLPETGFP	MRGD	LAKREPGM		KAEGEK	CP	R	WH	YTQDV	V												
-	+	<i>S. aureus</i>	. . MDYKE	TL LMPKTD	MRGG	LPNKEPQI		HADGEK	CE	R	W	NYS	EDL												

B

