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LeuRS can leucylate type I and type II tRNA^{Leu}s in *Streptomyces coelicolor*

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ABSTRACT

Transfer RNAs (tRNAs) are divided into two types, type I with a short variable loop and type II with a long variable loop. Aminoacylation of type I or type II tRNA^{Leu} is catalyzed by their cognate leucyl-tRNA synthetases (LeuRSs). However, in *Streptomyces coelicolor*, there are two types of tRNA^{Leu} and only one LeuRS (*Sco*LeuRS). We found that the enzyme could leucylate both types of *Sco*tRNA^{Leu}, and had a higher catalytic efficiency for type II *Sco*tRNA^{Leu}(UAA) than for type I *Sco*tRNA^{Leu}(CAA). The results from tRNA and enzyme mutagenesis showed that *Sco*LeuRS did not interact with the canonical discriminator A73. The number of nucleotides, rather than the type of base of the variable loop in the two types of *Sco*tRNA^{Leu}s, was determined as important for aminoacylation. *In vitro* and *in vivo* assays showed that the tertiary structure formed by the D-loop and TψC-loop is more important for *Sco*tRNA^{Leu}(UAA). We showed that the leucine-specific domain (LSD) of *Sco*LeuRS could help LeuRS, which originally only leucylates type II tRNA^{Leu}, to aminoacylate type I *Sco*tRNA^{Leu}(CAA) and identified the crucial amino acid residues at the C-terminus of the LSD to recognize type I *Sco*tRNA^{Leu}(CAA). Overall, our findings identified a rare recognition mechanism of LeuRS to tRNA^{Leu}.

INTRODUCTION

Transfer RNAs (tRNAs), short non-coding RNAs of ∼70–100 bases, act as adaptors by linking nucleotide sequences and amino acids through codon-anticodon pairing (1,2). To function as a substrate for proteins synthesis, tRNA is first charged with the corresponding amino acid by its cognate aminoacyl-tRNA synthetase (AARS), and then the aminoacyl-tRNA is delivered into the ribosome to biosynthesize proteins (1). The fidelity of the specific attachment of the amino acid with its cognate tRNA is governed by AARS, leading to the need to decipher the recognition set of tRNA by its cognate AARS.

tRNAs have a characteristic secondary structure consisting of the accepting stem, D stem-loop, anticodon stem-loop, variable loop and TψC stem-loop (2). Mature tRNAs are categorized into type I and II, based on the length of variable loop located between the anticodon stem and the TψC-stem (3). Type I tRNAs have a short variable loop, and type II tRNAs are characterized by a long variable loop (more than 10 nt) in most prokaryotes and in the cytoplasm of eukaryotes. tRNA^{Leu}, which can be leucylated by the cognate leucyl-tRNA synthetase (LeuRS), along with tRNA^{Ser} and tRNA^{Tyr}, belongs to the type II tRNA. However, in eukaryotic mitochondria, tRNA^{Leu} has a short variable loop, belonging to the type I tRNA, and can be leucylated by mitochondrial LeuRS. Cross-recognition between tRNA^{Leu} and LeuRS from the cytoplasm and mitochondria is blocked (1). This specific structure of the tRNA plays an important role in the recognition of its cognate AARS.

The identity elements of type II tRNA^{Leu} have been widely studied. The most important elements within *Escherichia coli* tRNA^{Leu} isoacceptors, which contribute to recognition and aminoacylation by the cognate LeuRSs, are A73 (4–6), A14 (4) and several structural features from the core of the tRNA^{Leu} molecules that are involved in tertiary interactions (A15-U48 (5,7), G18/G19-U55/C56 (4,7–8), U54-A58 (8) and the position of residue 47n in the variable region (6)). In *Aquifex aeolicus*, the recognition set of tRNA^{Leu} is similar to that of *Ec*tRNA^{Leu}s (9). In the human cytoplasmic leucylation system, the discriminator base A73; three base pairs C3-G70, A4-U69 and G5-C68 in the acceptor stem; nucleotide C20a in the D-loop; and the variable region are major contributing elements to aminoacylation (10–12). In addition to the common A73 discriminator, in the yeast cytoplasmic leucylation system, the tRNA^{Leu} anticodon loop is the major determinant (13). A previous study showed that human mitochondrial LeuRS (hmLeuRS) rec-

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ognizes human mitochondrial tRNA^{Leu} (hmtRNA^{Leu}) by the size of its anticodon loop and the length of its anticodon stem, rather than the specific nucleotides at positions 34–36 (14). In addition, footprinting experiments showed that the acceptor stem binds with its cognate AARS (14). The type I hmtRNA^{Leu} shares the discriminator A73 with type II tRNA^{Leu} (15).

Based on sequence homology and the structures of the catalytic active sites, AARSs are divided into two classes. LeuRS is a class I AARS and catalyzes the aminoacylation of tRNA^{Leu} (16). The reactions catalyzed by LeuRSs comprise a two-step process: The amino acid leucine is first activated with adenosine triphosphate (ATP) to synthesize the Leu-AMP intermediate; the Leu moiety of the intermediate is subsequently transferred to the tRNA^{Leu} bearing the cognate nucleotide triplet at its synthetic domain (17). LeuRS consists of a typical Rossmann dinucleotide-binding fold in the catalytic domain, an editing domain (CP1), ZN1 domain, leucine specific domain (LSD), anticodon binding domain (ABD) and a C-terminal domain (CTD) (16,18–20). The LSD, which is connected to the signature sequence KMSKS motif of class I AARS via a β-ribbon, modulates their aminoacylation activities (21–23). Crystal structure studies have also revealed that the LSD, together with the adjacent catalytically crucial KMSKS loop, plays a critical role in stabilizing A76 of tRNA^{Leu} and Leu-AMP during aminoacylation reactions (22,24). Our previous study showed that the LSD of *E. coli* LeuRS (*Ec*LeuRS) participates in tRNA^{Leu} recognition, favors the binding of tRNAs harboring a large loop in the variable region, and modulates the aminoacylation and proofreading functional cycle (23).

Genome mining of *Streptomyces coelicolor* revealed that in this bacterium, there are five tRNA^{Leu} isoacceptors, among them four, (*Sco*tRNA^{Leu}(UAA), *Sco*tRNA^{Leu}(GAG), *Sco*tRNA^{Leu} (CAG) and *Sco*tRNA^{Leu}(UAG)), have a long variable loop like type II tRNA^{Leu}s from bacteria, and one, *Sco*tRNA^{Leu}(CAA), has a short variable loop likes type I tRNA. It is interesting that in one actinomyces species, two types of tRNA^{Leu} coexist in the cytoplasm without membrane separation (25,26). However, in *S. coelicolor*, there is only one *leuS* gene encoding LeuRS. Can this one *Sco*LeuRS recognize and leucylate both type I and type II *Sco*tRNA^{Leu}? What is the difference in their recognition and catalysis by *Sco*LeuRS? Determining the recognition mechanism of *Sco*LeuRS of two types *Sco*tRNA^{Leu} is interesting and important.

In the present study, we choose *Sco*tRNA^{Leu}(UAA) among the four type II *Sco*tRNA^{Leu} isoacceptors as the representative to study its aminoacylation. *Sco*tRNA^{Leu}(CAA), *Sco*tRNA^{Leu}(UAA) and their mutants were successfully transcribed and purified *in vitro*. In additional, the genes encoding *Sco*tRNA^{Leu}(CAA) and *Sco*tRNA^{Leu}(UAA) were cloned into *E. coli*, and the two *Sco*tRNA^{Leu}s were isolated and purified from the transformants. Similarly, *Sco*LeuRS was obtained from the *E. coli* transformants containing *leuS*. The affinity and kinetics of *Sco*LeuRS for the two types of *Sco*tRNA^{Leu}s were measured and compared. An unprecedented role of ACCA at the 3′ end of the accepting stem for leucylation of *Sco*tRNA^{Leu}s was identified. Moreover, we showed that the LSD of *Sco*LeuRS helps another LeuRS that only leucylates type II tRNA^{Leu}, leucylate type I *Sco*tRNA^{Leu}(CAA). Our results highlighted the distinct determinants resulting from the scaffold structure of type I and II tRNAs for aminoacylation, and the structural flexibility of LeuRS to adapt to this difference between the two types of tRNAs, further indicating the co-evolution of this tRNA and its cognate synthetase.

MATERIALS AND METHODS

Materials

L-leucine, dithiothreitol (DTT), ATP, cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), 5′-GMP, Tris–HCl, $MgCl_2$, NaCl, β-mercaptoethanol (β-Me) and 3 M sodium acetate (NaAc) solution (pH 5.2) were purchased from Sigma-Aldrich Co. LLC (St Louis, MO, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from AMRESCO (OH, USA). [^{3}H] Leucine was obtained from PerkinElmer Inc. (Waltham, MA, USA). Q5 high-fidelity DNA polymerase was purchased from New England Biolabs (Ipswich, MA, USA). A DNA fragment rapid purification kit and a plasmid extraction kit were obtained from Yuanpinghao Biotech (Tianjin, China). Oligonucleotide primers were synthesized by Biosune (Shanghai, China). The KOD-neo-plus DNA polymerase and KOD-plus mutagenesis were obtained from TOYOBO (Osaka, Japan); and all genes and their variants were confirmed by DNA sequencing performed by Biosune (Shanghai, China). Protein standard markers, T4 ligase, pyrophosphatase, and restriction endonucleases were obtained from Thermo Scientific (Waltham, MA, USA). The nickel-nitrilotriacetic acid Superflow was purchased from Qiagen (Hilden, Germany). Amicon ultra-15 filters were obtained from Merck (Darmstadt, Germany). Competent *E. coli* Top10 and BL21 (DE3) cells were prepared in our laboratory. *Escherichia coli* ET12567, used to perform intergeneric conjugation from *E. coli* to *S. coelicolor,* was a kind gift from Prof. Wei-Hong Jiang's laboratory, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. The T7 RNA polymerase was purified from an overproducing strain maintained in our laboratory (27).

Gene cloning, mutagenesis and expression; and protein purification

All primers used to amplify genes are shown in Supplementary Table S1. The *Sco*LeuRS gene was amplified from *S. coelicolor* A3(2) M145 genomic DNA, which was a kind gift from Prof. Wei-Hong Jiang, and inserted into pET28a vector via the restriction enzymes *Nde*I and *Hind*III. We purchased the *Streptomyces azureus* strain from the China Center of Industrial Culture Collection and cultured it in our laboratory. The *S. azureus* genomic DNA was extracted using an Ezup Column Bacteria Genomic DNA purification kit from Sangon Biotech (Shanghai, China). The *S. azureus* LeuRS gene (*Saz*LeuRS) was amplified from genomic DNA and inserted into pET28a via the restriction enzymes *Nde*I and *Hind*III. The gene encoding

*Spur*LeuRS, which was synthesized according to the sequence from EBI-EMBL ensemble genome database (28), gene annotation ADL19_14010, and inserted between *Nde*I and *Xho*I sites in pET30a, was optimized for expression in *E. coli*. All constructs were confirmed by DNA sequencing at Biosune. The recombinant plasmids containing genes encoding *Ec*LeuRS and hmLeuRS, pET30a-EcleuS (29) and pET22b(+)-hmleuS-40 (30), respectively, were constructed in our laboratory.

The definition of the LSD domain of *Sco*LeuRS and *Spur*LeuRS was based on the crystal structure of *Ec*LeuRS (Val^{569} – Ser^{618}, PDB ID: 4AQ7) and sequence alignment. The LSD domain (Val^{594} – Glu^{638}) of *Spur*LeuRS was substituted by the LSD domain (Ile^{696} – Gly^{733}) from *Sco*LeuRS to obtain *Spur*LeuRS-*Sco*LSD. We further constructed multiple alanine replacement mutants, listed in Supplementary Figure S8. Five single site mutants at the terminus of the LSD residues, *Spur*LeuRS-*Sco*LSD-R730A, -L731A, -L732A, -G733A and -L732I, and one double-site mutant, *Spur*LeuRS-*Sco*LSD–L732A/G733A, were constructed by gene mutagenesis using pET30a-*Spur*LeuRS-*Sco*LSD as a template. The numbers in the names of the mutants indicated the position of residues in *Sco*LeuRS. The genes encoding all the mutants were confirmed by DNA sequencing.

All constructs were transformed into *E. coli* BL21 (DE3) cells to produce the proteins from the transformants. A single colony of each transformant was chosen and cultured in 500 ml of 2 × YT medium at 37°C. When the cells reached mid-log phase (A600 = 0.6), expression of the recombinant protein was induced by the addition of 0.2 mM IPTG for 12 h at 16°C. Protein purification was performed according to a previously described method (31), except that the buffers were: buffer A (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 10 mM imidazole, 10% glycerol and 5 mM β-Me), buffer B (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 20 mM imidazole, 10% glycerol and 5 mM β-Me), buffer C (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 250 mM imidazole, 10% glycerol and 5 mM β-Me), and buffer D (20 mM Tris–HCl, pH 8.0, 150 mM NaCl and 2 mM β-Me). The protein concentrations were determined using UV absorbance at 280 nm, and the molar absorption coefficient was calculated according to the sequence of each protein (32).

Preparation of $tRNA^{Leu}$

The DNA sequence of the T7 promoter and the *Sco*$tRNA^{Leu}$(CAA) and *Sco*$tRNA^{Leu}$(UAA) genes were obtained by ligating three chemically synthetized DNA fragments for each strand, which were then ligated into plasmid pTrc99b (pre-cleaved with *Eco*RI/*BamH*I) to construct pTrc99b-T7-*Sco*$tRNA^{Leu}$(CAA), pTrc99b-T7-*Sco*$tRNA^{Leu}$(UAA) and other *Sco*$tRNA^{Leu}$ isoacceptors using previously published methods (33). Amplification of the template and transcription of tRNA were performed according to a previously described method (31). The corresponding mutants were prepared using the same method as that for the wild-type. The accepting capacity of *Sco*$tRNA^{Leu}$(CAA) and -(UAA) are 1507 and 1574 pmol/A_{260}, respectively. The tRNA construct of *Ec*$tRNA^{Leu}$(UAA) and the tRNA construct of hmtRNA^{leu}(UUR) were constructed, and *in vitro* transcription was performed as reported previously (34,35); their amino acid accepting activity was ∼1500 pmol/A_{260}.

Aminoacylation assay

Leucylation of *Sco*$tRNA^{Leu}$ was performed at 37°C in a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 6 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 20 μM [^{3}H] leucine, 1 nM *Sco*LeuRS and 5 μM *Sco*$tRNA^{Leu}$ or its variants. The procedure was performed as previously described (29). Leucylation of *Sco*$tRNA^{Leu}$ by *Saz*LeuRS and *Spur*LeuRS was performed under the same conditions; by *Spur*LeuRS-*Sco*LSD and its variant was under the same conditions except for 10 nM enzyme in the mixture; and that by *Ec*LeuRS and hmLeuRS was performed as described previously (19,36). The kinetic parameters of *Sco*LeuRS for *Sco*$tRNA^{Leu}$ and its variants during aminoacylation were determined in the presence of varying concentrations of $tRNA^{Leu}$ (0.5–32 μM).

Actinomyces strain, growth conditions and complementation plasmid construction

The *S. coelicolor* J1501 strain was a kind gift from Dr Mei-Feng Tao, Shanghai Jiaotong University. J1501 was grown on an MS plate for spore collection. Other strains were grown on R2YE plates with the required growth supplements: Histidine at 50 μg ml^{-1} and uracil at 7.5 μg ml^{-1}. In all cases, *S. coelicolor* was grown at 30°C. For knockout of *bldA* (encoding *Sco*$tRNA^{Leu}$(UAA)), the cosmid SCE25AT::apra was also a gift of Dr Tao, and the intergeneric mating was conducted as described before (37), yielding J1501△*bldA*::apra. The plasmid pMS82, which was a present from Dr Maggie Smith, York University, UK, was used to construct a complementation plasmid. The *bldA* gene, together with the putative promoter region (38), was amplified from the *S. coelicolor* genome yielding a 387 bp product. The polymerase chain reaction product was digested with *Hind*III and *Kpn*I, and cloned into the respective sites of vector pMS82 to obtain pMS82bldA. Other mutants were constructed by gene mutagenesis using pMS82bldA as a template. Conjugation of the plasmids from the donor strain to J1501△*bldA* was performed according to the Practical Streptomyces Genetics handbook. For the time-course experiment, development was assessed through visual inspection.

RESULTS

*Sco*LeuRS has the same affinity for the two types of *Sco*$tRNA^{Leu}$s, but has a different aminoacylation activity

To study the interaction between *Sco*LeuRS and *Sco*$tRNA^{Leu}$s *in vitro*, we first obtained *Sco*LeuRS from *E. coli* transformants expressing its gene and then purified the protein to ∼95% homogeneity using affinity chromatography on Ni-NTA superflow (Supplementary Figure S1A). The transcripts of *Sco*$tRNA^{Leu}$(CAA) and -(UAA) were obtained *in vitro* (Supplementary Figure S1B). To compare the thermal stabilities of the two types

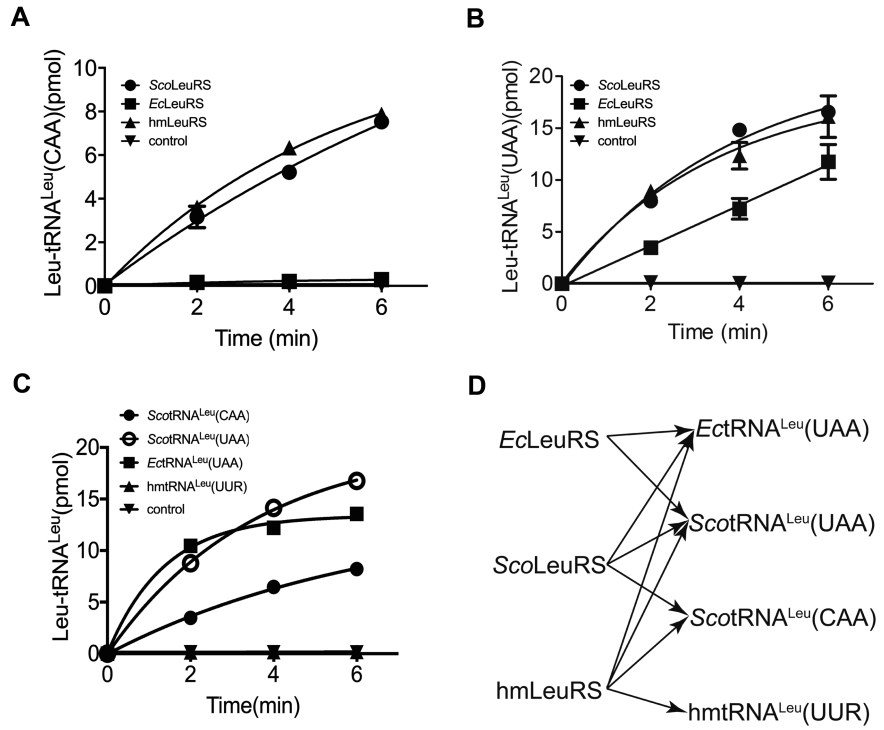


Figure 1. Cross-recognition between LeuRSs and tRNA^{Leu}s from various species. (**A** and **B**) Aminoacylation of 1 nM *Sco*LeuRS, 1 nM *Ec*LeuRS, 500 nM hmLeuRS or no LeuRS for 5 μM *Sco*tRNA^{Leu}(CAA) (A) or 5 μM *Sco*tRNA^{Leu}(UAA) (B), respectively. (**C**) Aminoacylation of 1 nM *Sco*LeuRS for 5 μM *Sco*tRNA^{Leu}(UAA), 5 μM *Sco*tRNA^{Leu}(CAA), 5 μM hmtRNA^{Leu}(UUR), 5 μM *Ec*tRNA or no tRNA, respectively. The data shown represent averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols. (**D**) Schematic demonstration of cross-recognition. The arrows indicate the ability to leucylate the indicated tRNA^{Leu}.

of *Sco*tRNA^{Leu}s, UV melting curves were obtained. Notably, the melting temperature of *Sco*tRNA^{Leu}(CAA) was 55.6 ± 0.8°C, which was slightly higher than that of *Sco*tRNA^{Leu}(UAA) (52.5 ± 0.1°C) (Supplementary Figure S2).

The reaction conditions for the leucylation of the two *Sco*tRNA^{Leu}s by *Sco*LeuRS were optimized. The optimal pH value was 8.5, and the optimal concentration of Mg^{2+} and the magnesium/ATP ratio were 6 mM and 1.5, respectively (Supplementary Figure S3). The kinetic parameters of *Sco*LeuRS for the transcripts of the two types *Sco*tRNA^{Leu}s were assayed and are shown in Table 1. The K_m and k_{cat} values of *Sco*LeuRS for *Sco*tRNA^{Leu}(CAA) were lower than those for *Sco*tRNA^{Leu}(UAA), indicating that *Sco*LeuRS has a slightly lower affinity for type II *Sco*tRNA^{Leu} and a higher catalytic rate of its leucylation. Therefore, the catalytic efficiency (k_{cat}/K_m) of *Sco*LeuRS for *Sco*tRNA^{Leu}(CAA) is less than half that for *Sco*tRNA^{Leu}(UAA). The kinetic parameters of *Sco*LeuRS for the *Sco*tRNA^{Leu}(CAA) and *Sco*tRNA^{Leu}(UAA) overexpressed and isolated from *E. coli* showed comparable results (data not shown).

*Sco*LeuRS aminoacylates prokaryotic tRNA^{Leu}, but not eukaryotic mitochondrial tRNA^{Leu}

The above data showed that *Sco*LeuRS can leucylate both type I *Sco*tRNA^{Leu}(CAA) and type II *Sco*tRNA^{Leu}(UAA). Considering that hmLeuRS is the only reported LeuRS that can leucylate type I tRNA^{Leu}s and *Sco*LeuRS has higher homology with well-studied *Ec*LeuRS, we asked whether *Sco*LeuRS, hmLeuRS and *Ec*LeuRS could cross-recognize their tRNA^{Leu}s?

The aminoacylation activity of hmLeuRS and *Ec*LeuRS for the two *Sco*tRNA^{Leu}s showed that hmLeuRS had similar leucylation activity for both types of *Sco*tRNA^{Leu}s compared with *Sco*LeuRS; *Ec*LeuRS could catalyze aminoacylation of *Sco*tRNA^{Leu}(UAA) with 50% activity; however, it could not leucylate *Sco*tRNA^{Leu}(CAA) completely (Figure 1A and B). The sequence alignment of these three LeuRSs showed that *Sco*LeuRS and hmLeuRS have the same domains as *Ec*LeuRS; however, *Sco*LeuRS has the longest N-terminus among them (Supplementary Figure S4), suggesting that domains in the N-terminus may play important roles in the aminoacylation of type I tRNA^{Leu}s. Moreover, *Ec*LeuRS has a lower activity for type II *Sco*tRNA^{Leu}(UAA) despite the high homology, which suggested that *Sco*tRNA^{Leu}s may have different recognition sets from *Ec*tRNA^{Leu}s.

We also examined the aminoacylation of *Sco*LeuRS for type II *Ec*tRNA^{Leu}(UAA) and type I hmtRNA^{Leu}(UUR). *Sco*LeuRS could aminoacylate type II *Ec*tRNA^{Leu}(UAA) with a similar reaction rate to that of *Sco*tRNA^{Leu}(UAA), but not type I hmtRNA^{Leu}(UUR) (Figure 1C), implying that *Sco*tRNA^{Leu}s and hmtRNA^{Leu}s have distinct identity elements.

Table 1. Aminoacylation kinetics constants of transcripts of *Sco*LeuRS for *Sco*tRNA^{Leu}(CAA), (UAA) and their mutants

*Sco*tRNA^{Leu}	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	k_{cat}/K_m (relative %)
(CAA) type I	0.16 ± 0.03	1.00 ± 0.01	6.25	100
-A73C	0.45 ± 0.01	0.98 ± 0.07	2.17	35
-A73G	0.24 ± 0.08	1.24 ± 0.05	5.17	83
-A73U	0.22 ± 0.08	1.93 ± 0.09	8.81	141
-C74A	nm	nm	nm	nm
-C74G	nm	nm	nm	nm
-C74U	nm	nm	nm	nm
-C75A	0.20 ± 0.02	0.25 ± 0.01	1.25	20
-C75G	0.13 ± 0.03	0.06 ± 0.01	0.46	7
-C75U	0.19 ± 0.01	0.10 ± 0.01	0.53	8
(UAA) type II	0.24 ± 0.03	3.40 ± 0.22	14.2	100
-A73C	0.21 ± 0.01	2.02 ± 0.04	9.62	68
-A73G	0.31 ± 0.01	2.90 ± 0.07	9.35	66
-A73U	0.22 ± 0.01	2.75 ± 0.60	12.5	88
-C74A	nm	nm	nm	nm
-C74G	nm	nm	nm	nm
-C74U	nm	nm	nm	nm
-C75A	0.45 ± 0.05	0.63 ± 0.05	1.39	10
-C75G	0.20 ± 0.08	0.01 ± 0.002	0.07	0.5
-C75U	0.68 ± 0.07	0.47 ± 0.06	0.69	5

The results are the averages of three independent repeats, with the standard deviations indicated.

C74 of *Sco*tRNA^{Leu}(CAA) and *Sco*tRNA^{Leu}(UAA) instead of A73 is the identity element

The aminoacylation assay implied that *Sco*tRNA^{Leu}s may choose different identity elements to those of hmtRNA^{Leu}(UUR) and *Ec*tRNA^{Leu}(UAA). A73 of hmtRNA^{Leu}(UUR) and *Ec*tRNA^{Leu}(UAA) is an canonical identity element (4–6,15). To determine whether it plays the same role in *Sco*tRNA^{Leu}(CAA) and -(UAA), a series of tRNA mutants was constructed (Figure 2B and D).

First, we mutated A73 to G, U, and C in the two *Sco*tRNA^{Leu}s, respectively, and the leucine accepting activities of these mutants were assayed (Table 1). For *Sco*tRNA^{Leu} (CAA), the K_m values of *Sco*LeuRS for all the mutants at A73 increased, indicating a weaker interaction between the tRNA and the enzyme; the k_{cat} values for A73G and A73U increased, although that for A73C only declined slightly. For *Sco*tRNA^{Leu}(UAA), the K_m values of *Sco*LeuRS for A73G increased, showing a weaker interaction between the tRNA and the enzyme, although that for A73C and A73U decreased slightly, indicating a stronger interaction; the k_{cat} values declined for the three mutants to varying degrees. However, *Sco*LeuRS still could recognize all six mutants at A73 (three for each *Sco*tRNA^{Leu}) and had relatively higher catalytic efficiency (Table 1). The data showed that A73 was not an identity element in *Sco*tRNA^{Leu}(CAA) and -(UAA) for *Sco*LeuRS.

Next, we determined whether A73 is an identity element in all isoacceptors of *Sco*tRNA^{Leu}. The replacement mutants at A73 of all other *Sco*tRNA^{Leu}-(GAG), -(CAG) and -(UAG) were constructed and their accepting activities were assayed (Supplementary Figure S5A–C). For all mutants at A73, *Sco*LeuRS maintained obvious charging capacities. The above results showed that in all *Sco*tRNA^{Leu} isoacceptors, A73 is not a discriminator, unlike other most tRNA^{Leu}s.

The C74C75A76 at the 3′ end of all tRNAs is common, and A is a common nucleotide for accepting amino acid. The A73 next to C74C75A76 is no longer identity element of *Sco*tRNA^{Leu}s; therefore, we determined whether other bases at the 3′ end play important roles. Mutagenesis of C74 and C75 of *Sco*tRNA^{Leu}s was performed and the accepting activities of the tRNA mutants were assayed (Table 1). The accepting capacities of both *Sco*tRNA^{Leu}(CAA) and -(UAA) were lost completely when C74 changed to the other three nucleotides, suggesting that this position is essential for both type I and type II *Sco*tRNA^{Leu}s (Table 1). However, our previous study showed that *Ec*LeuRS could still leucylate C74 mutants of *Ec*tRNA^{Leu}, although with lower activities, which suggested that C74 is not as significant in *Ec*tRNA^{Leu}s as it is in *Sco*tRNA^{Leu}s (29). The mutants at C75 of both *Sco*tRNA^{Leu}(CAA) and -(UAA) were also constructed. The kinetic parameters of *Sco*LeuRS for the tRNA C75 mutants showed that although both the binding and catalysis of *Sco*LeuRS for the two mutants was weakened, C75 in the two types *Sco*tRNA^{Leu}s was not crucial to their accepting capacities compared with those of C74 (Table 1).

Based on the tertiary structure of *Ec*LeuRS, Arg416 and Arg418 in the highly conserved motif ^{416}R/KLRDWGVSRQRYWG^{429} interact with A73 of *Ec*tRNA^{Leu}(UAA) (Figure 3A) (22). Our constructed mutants *Ec*LeuRS-R416A and -R418A showed obviously decreased aminoacylation activity (Figure 3B), indicating that disrupting the interaction between the residues of *Ec*LeuRS and A73 of tRNA^{Leu} abrogated the activity of *Ec*LeuRS; thus confirming that A73 is crucial to recognition by *Ec*LeuRS, which has been reported previously (4–6). The high sequence homology between *Sco*LeuRS and *Ec*LeuRS identified the homologous residues of Arg416 and Arg418 in *Ec*LeuRS as Arg517 and Arg519 in *Sco*LeuRS, which were changed to alanine. The leucylation activities of *Sco*LeuRS-R517A and -R519A for *Sco*tRNA^{Leu}(CAA) and -(UAA) were even higher than that of the wild-type *Sco*LeuRS (Figure 3C and D), suggesting that the interaction of Arg517 and Arg519 with

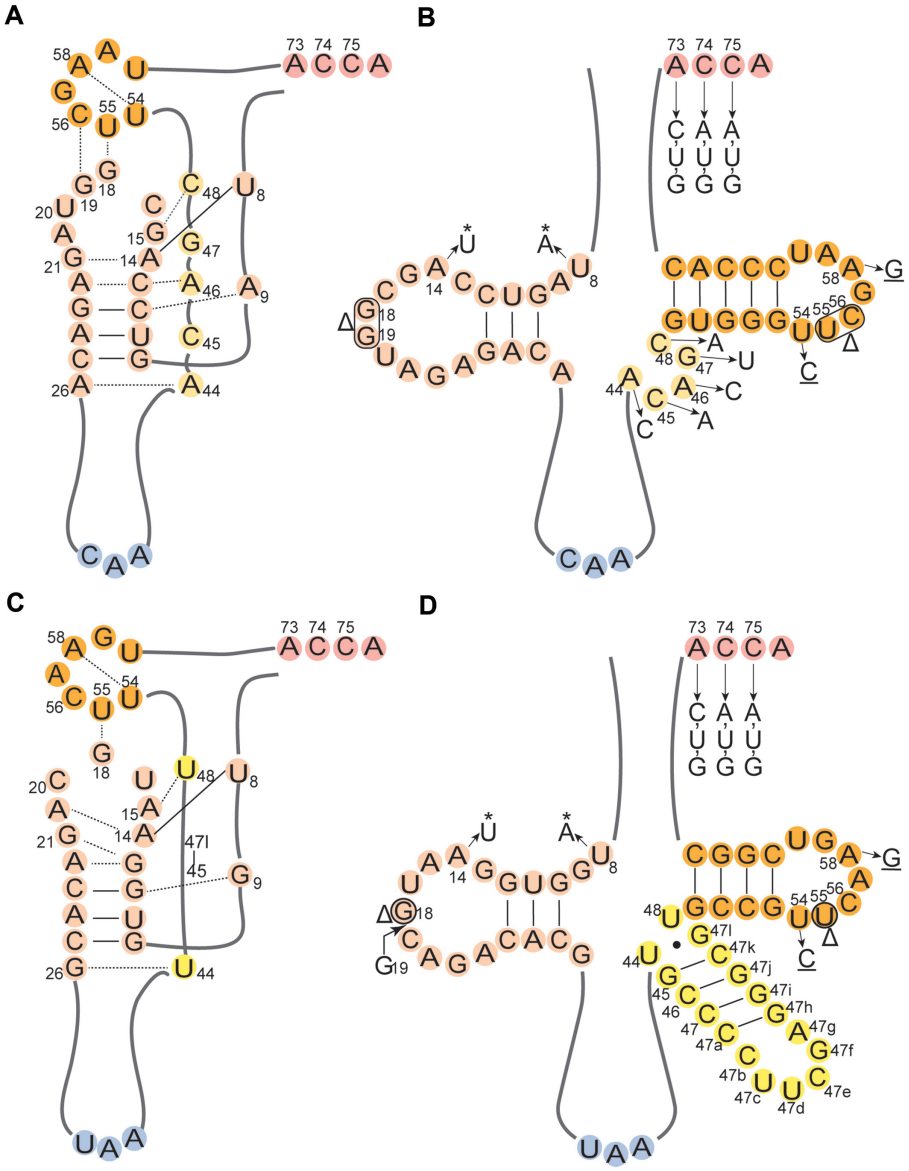


Figure 2. Summary of *Sco*tRNA^{Leu} constructs used in this study. (**A**) The L-shaped tertiary structure of *Sco*tRNA^{Leu}(CAA) based on the structure of *Staphylococcus aureus* tRNA^{Ile} (PDB ID: 1FFY), (**C**) that of *Sco*tRNA^{Leu}(UAA) based on the structure of *Ec*tRNA^{Leu}(UAA) (with *Ec*LeuRS) (PDB ID: 4AQ7) and *Tt*tRNA^{Leu}(CAG) (PDB ID: 2BYT); dotted lines indicate the tertiary interactions. (**B**) The variants derived from *Sco*tRNA^{Leu}(CAA), (**D**) those derived from *Sco*tRNA^{Leu}(UAA); the arrows indicate the mutation locations, asterisks indicate the double substitution mutant(A14U/U8A); triangles indicate the deletion mutation (in *Sco*tRNA^{Leu}(CAA) ΔG18/ΔG19 and ΔU55/ΔC56, or in *Sco*tRNA^{Leu}(UAA) ΔG18 and ΔU55); and the underlines indicate the double substitution mutant (U54C:A58G). SVL, variable loop of *Sco*tRNA^{Leu}(CAA), colored as dark yellow. LVL, variable loop of *Sco*tRNA^{Leu}(UAA), colored as light yellow.

A73 of *Sco*tRNA^{Leu}(CAA) and -(UAA) is not important for the activity of *Sco*LeuRS, and confirming that A73 in the two types of *Sco*tRNA^{Leu}s is not the identity element, unlike the *Ec*LeuRS-tRNA^{Leu} system.

In the *Ec*LeuRS and *Ec*tRNA^{Leu}(UAA) complex, C74 of the tRNA contacts with Arg424 of *Ec*LeuRS, which is the homolog of Arg525 of *Sco*LeuRS (Figure 4A) (22). *Ec*LeuRS-R424A resulted in the loss of half the aminoacylation activity of *Ec*LeuRS (Figure 4B), indicating that this residue is not crucial to aminoacylation. However, the aminoacylation activity of *Sco*LeuRS-R525A for both types *Sco*tRNA^{Leu}s was completely lost (Figure 4C and D). These results suggested that this Arg residue of *Sco*LeuRS is crucial to recognize C74 in both types of *Sco*tRNA^{Leu}s and is important for the activity of the enzyme.

To check whether the shift in the identity element from A73 to C74 is universal for all *Sco*tRNA^{Leu}s, we mutated C74 and C75 of type II *Sco*tRNA^{Leu}(GAG) into other nucleotides, and obtained the same results as those for *Sco*tRNA^{Leu}(UAA) (Supplementary Figure S5D).

The above results indicated that the interaction of *Sco*LeuRS with both types of *Sco*tRNA^{Leu}s was similar, and that C74 of *Sco*tRNA^{Leu}s is a crucial base at 3′ ACCA end instead of A73 in *Sco*tRNA^{Leu}s.

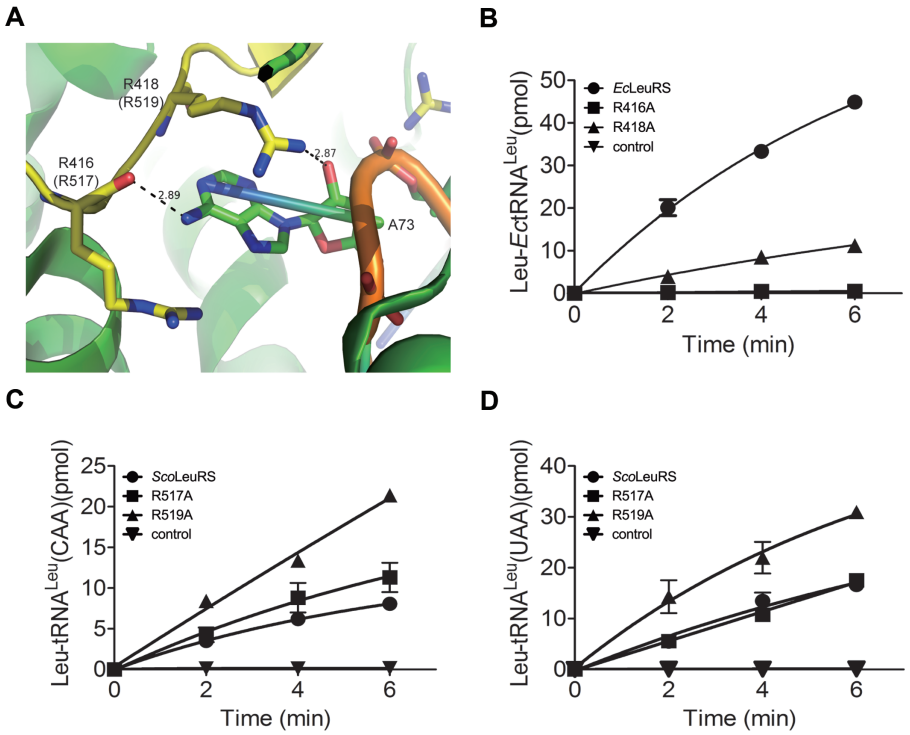


Figure 3. The interaction between residues in *Sco*LeuRS and A73 in *Sco*tRNA^{Leu}s. (**A**) Crystal structure of *Ect*RNA^{Leu} (orange in the cartoon mode) in complex with *Ec*LeuRS (green in the cartoon mode) during the aminoacylation conformation (PDB ID: 4AQ7, Ref. 22). Residues R416 and R418 (R517 and R519) of a conserved motif (yellow) are numbered and shown in the stick model with their distances to A73 of *Ect*RNA^{Leu}. (**B**) Aminoacylation of 1 nM *Ec*LeuRS-WT, -R416A, -R418A or no enzyme, respectively, for 5 μM *Ect*RNA^{Leu}(UAA). (**C** and **D**) Aminoacylation of 1 nM *Sco*LeuRS-WT, -R517A, -R519A or no enzyme, respectively, for 5 μM *Sco*tRNA^{Leu}(CAA) (C) or 5 μM *Sco*tRNA^{Leu}(UAA) (D). The data shown represent the averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.

The number of nucleotides of the variable loop in *Sco*tRNA^{Leu}s is important for aminoacylation

The variable loop of *Sco*tRNA^{Leu}(CAA) contains only five nucleotides, A^{44}C^{45}A^{46}G^{47}C^{48} (SVL); however, *Sco*tRNA^{Leu}(UAA), like other tRNA^{Leu}s, has a long variable loop with seventeen nucleotides (LVL), which could form five base pairs, U44·G47l, G45-C47k, C46-G47j, C47-G47i and C47a-G47h (Figure 2). We replaced the SVL of *Sco*tRNA^{Leu}(CAA) with the LVL of *Sco*tRNA^{Leu}(UAA) to produce a chimeric mutant *Sco*tRNA^{Leu}(CAA)-LVL. The catalytic efficiency of *Sco*LeuRS for this chimeric tRNA mutant decreased, with a larger K_m value (Table 2). Then, the nucleotides in the SVL of *Sco*tRNA^{Leu}(CAA) were gradually deleted to form four deletion mutants, named *Sco*tRNA^{Leu}(CAA)-SVLΔ1nt, -SVLΔ2nt, -SVLΔ3nt and -SVLΔ4nt (their names are shown in Figure 5E). Except for mutant *Sco*tRNA^{Leu}(CAA)-SVLΔ1nt (deletion of A44), which retained a slight accepting activity, the other three completely lost their accepting activities (Figure 5A). Then, five substitution mutants in the SVL of *Sco*tRNA^{Leu}(CAA) (*Sco*tRNA^{Leu}-A44C, -C45A, -A46C, -G47U and -C48A) were constructed. The K_m values of *Sco*LeuRS for A44C and C48A changed only slightly, and the k_{cat} values decreased. The K_m values for C45A decreased slightly; however, the k_{cat} value increased. The K_m values for A46C and G47U increased markedly; however, the k_{cat} values also increased. These data indicated that although the affinity of LeuRS for these tRNA mutants varied, the substitution tRNA mutants affected the catalytic efficiency only slightly (Table 2). The variable loop forms a compact core of seven base layers with the D-stem/loop (39) (Figure 2A); therefore, these results implied that the number of nucleotides on the variable loop, rather than the type of base, is more significant for this structure.

To understand the function of the LVL of *Sco*tRNA^{Leu}(UAA), the LVL was replaced with the SVL of *Sco*tRNA^{Leu}(CAA) to obtain another chimeric mutant *Sco*tRNA^{Leu}(UAA)-SVL. The K_m values of *Sco*LeuRS for the chimera increased 16-fold compared with that for *Sco*tRNA^{Leu}(UAA), indicating a weaker affinity of the enzyme for the tRNA mutant; however the k_{cat} value doubled, the catalytic efficiency decreased to 14% of that for *Sco*tRNA^{Leu}(UAA), and was one third of that recorded for *Sco*tRNA^{Leu}(CAA) with the same variable loop (Table 2). The data showed that other structural elements of *Sco*tRNA^{Leu} play important roles in the catalysis by *Sco*LeuRS. A series of deletion mutants in the LVL were constructed to investigate the role of the LVL in recognition by *Sco*LeuRS (Figure 5E). Successive deletions of five base pairs in the LVL produced five mutants, which all maintained their accepting capacity (Figure 5B). The catalytic rate of *Sco*LeuRS for the tRNA mutants, which were deleted for the first and second base pair in the LVL, was even higher than that for *Sco*tRNA^{Leu}(UAA) (Figure 5B). The mutant of *Sco*tRNA^{Leu}(UAA) deleted for C47b and A47g in the LVL (LVLΔ2nt) or deleted for two

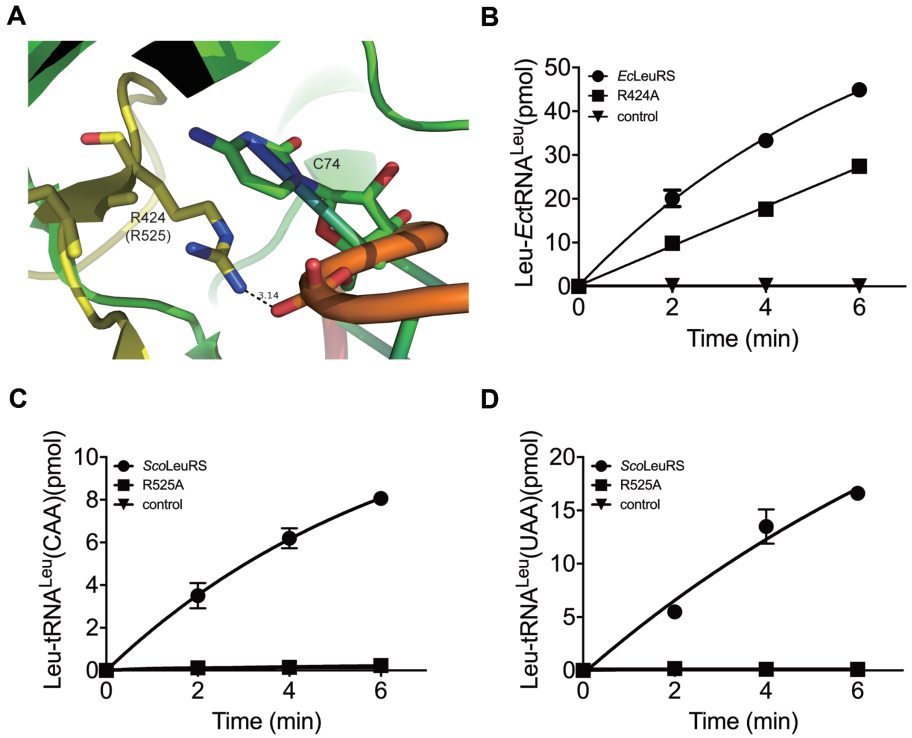
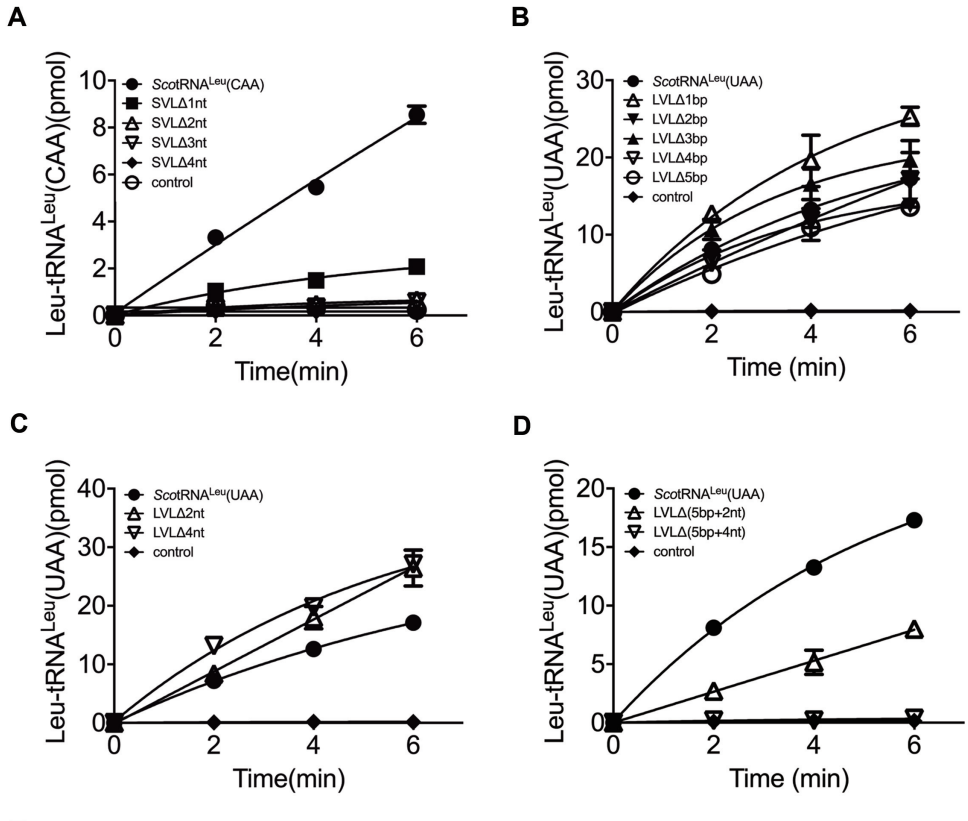


Figure 4. The interaction between residues in *Sco*LeuRS and C74 in *Sco*tRNA^{Leu}s. (**A**) Crystal structure of *Ec*tRNA^{Leu} (orange in the cartoon mode) in complex with *Ec*LeuRS (yellow in the cartoon mode) during the aminoacylation conformation (PDB ID: 4AQ7, Ref. 22). Residues R424 (R525) of a conserved motif (yellow) are numbered and shown in the stick model with their distances to C74 of *Ec*tRNA^{Leu}. (**B**) Aminoacylation of 1 nM *Ec*LeuRS-WT, -R424A or no enzyme, respectively, for 5 μM *Ec*tRNA^{Leu}(UAA). (**C** and **D**) Aminoacylation of 1 nM *Sco*LeuRS-WT, -R525A or no enzyme, respectively, for 5 μM *Sco*tRNA^{Leu}(CAA) (C) or 5 μM *Sco*tRNA^{Leu}(UAA) (D). The data shown represent the averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.

Table 2. Aminoacylation kinetics constants of transcripts of *Sco*LeuRS for *Sco*tRNA^{Leu}(CAA), (UAA) and their mutants

*Sco*tRNA^{Leu}	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	k_{cat}/K_m (relative %)
(CAA) type I	0.16 ± 0.03	1.00 ± 0.01	6.25	100
-LVL	0.75 ± 0.30	0.83 ± 0.03	1.10	18
-A44C	0.16 ± 0.03	0.88 ± 0.12	5.34	85
-C45A	0.14 ± 0.03	1.14 ± 0.17	8.41	130
-A46C	0.40 ± 0.04	1.43 ± 0.03	3.58	58
-G47U	0.54 ± 0.02	2.70 ± 0.43	5.00	80
-C48A	0.10 ± 0.04	0.65 ± 0.05	6.50	104
-A14U	2.33 ± 0.37	2.02 ± 0.35	0.86	14
-A14U/U8A	4.41 ± 0.93	2.22 ± 0.09	0.50	7.9
-ΔG18/ΔG19	0.18 ± 0.01	1.27 ± 0.11	7.03	113
-ΔU55/ΔC56	0.50 ± 0.07	3.00 ± 0.32	6.00	96
-U54C:A58G	0.47 ± 0.15	2.56 ± 0.28	5.49	87
(UAA) type II	0.24 ± 0.03	3.40 ± 0.22	14.2	100
-SVL	3.80 ± 0.14	7.70 ± 0.01	2.03	14
-A14U	1.27 ± 0.09	0.38 ± 0.01	0.30	2
-A14U/U8A	3.83 ± 0.19	0.54 ± 0.06	0.14	1
-▽G19	0.09 ± 0.02	1.85 ± 0.14	21.5	151
-ΔG18	4.28 ± 0.30	12.95 ± 0.25	3.03	21
-ΔU55	1.24 ± 0.13	10.31 ± 0.53	8.31	65
-U54C:A58G	0.20 ± 0.01	0.72 ± 0.11	3.60	25

The results are the averages of three independent repeats, with the standard deviations indicated.



E

Structure	name	accepting activity	Structure	name	accepting activity
^{48}C G $_{44}$A C A	*Scot*RNA^{Leu}(CAA) wild type	++++	^{48}UGCGGGAG C $_{44}$UGCCCCU U	*Scot*RNA^{Leu}(UAA) wild type	++++
^{48}C G $_{44}$A C A	SVLΔ1nt	+	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ1bp	++++++
^{48}C G $_{44}$A C A	SVLΔ2nt	-	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ2bp	++++
^{48}C G $_{44}$A C A	SVLΔ3nt	-	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ3bp	++++
^{48}C G $_{44}$A C A	SVLΔ4nt	-	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ4bp	+++
^{48}UGCGGGAG C $_{44}$UGCCCCU U	*Scot*RNA^{Leu}(UAA) wild type	++++	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ5bp	+++
^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ(5bp+2nt)	++	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ2nt	++++++
^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ(5bp+4nt)	-	^{48}UGCGGGAG C $_{44}$UGCCCCU U	LVLΔ4nt	++++++

Figure 5. Aminoacylation activity of *Sco*LeuRS and mutants of *Scot*RNA^{Leu} in variable arms. (**A**) Aminoacylation of 1 nM *Sco*LeuRS for 5 μM *Scot*RNA^{Leu}(CAA) -WT, -SVLΔ1nt, - SVLΔ2nt, - SVLΔ3nt, - SVLΔ4nt or no tRNA, respectively. (**B**) Aminoacylation of 1 nM *Sco*LeuRS for 5 μM *Scot*RNA^{Leu}(UAA) -WT, -LVLΔ1bp, - LVLΔ2bp, - LVLΔ3bp, - LVLΔ4bp, - LVLΔ5bp or no tRNA, respectively. (**C**) Aminoacylation of 1 nM *Sco*LeuRS for 5 μM *Scot*RNA^{Leu}(UAA) -WT, -LVLΔ2nt, -LVLΔ4nt or no tRNA, respectively. (**D**) Aminoacylation of 1 nM *Sco*LeuRS for 5 μM *Scot*RNA^{Leu}(UAA) -WT, -LVLΔ(5bp+2nt), -LVLΔ(5 bp + 4 nt) or no tRNA, respectively. The data shown represent the averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols. (**E**) A summary of the accepting activities of the mutants. The sequence of the variable loop is shown, and the red letters indicate the nucleotides that were deleted in the mutants.

more, U47c and G47f (LVLΔ4nt), still retained a higher accepting activity (Figure 5C). When the five base pairs of LVLΔ2nt were deleted, the accepting activity of the mutant LVLΔ(5bp+2nt) with only four nucleotides, 47d, -e, -f, -g in the LVL, decreased markedly; LVLΔ(5 bp + 4 nt) deleted for two more nucleotides, with only two nucleotides, 47d and 47e, in the LVL, lost its accepting activity completely (Figure 5D).

Notably, the accepting capacity of *Scot*RNA^{Leu}(CAA) and -(UAA) were lost completely when nucleotide 48 (C48 in *Scot*RNA^{Leu}(CAA) and U48 in *Scot*RNA^{Leu}(UAA)) were deleted (data not shown). Consequently, all deletion mutants retained nucleotide 48. Taken together, these results suggested that a length of five nucleotides (including nucleotide 48) in the variable loop of either

*Sco*tRNA^{Leu}(CAA) or -(UAA) is the shortest length required for relatively high aminoacylation.

The elbow structure plays different roles in two types of *Sco*tRNA^{Leu}

Most tRNAs have a similar L-shaped tertiary structure, which complicates the ability of AARSs to discriminate their cognate tRNAs from other tRNA species. Previous studies have shown that the U8:A14 tertiary structure base pair of *Ec*tRNA^{Leu}(CAG) and hmtRNA^{Leu}(UUR) is the identity element (4,15). The elbow structure base pairs between the D- and TψC-loops of *Ec*tRNA^{Leu} (G18:U55, G19:C56 and U54:A58) play important roles in aminoacylation (8). The possible tertiary base pairs of *Sco*tRNA^{Leu}s are shown in Figure 2 A and C. To determine whether the tertiary structure base pairs mentioned above play an important role in recognition by *Sco*LeuRS, several mutations at these sites were constructed.

In either *Sco*tRNA^{Leu}(CAA) or -(UAA), substitution of A14 with U to break U8:A14 decreased their leucine accepting capacities drastically (Table 2). Although an additional U8A mutation on *Sco*tRNA^{Leu}(CAA)-A14U or *Sco*tRNA^{Leu}(UAA)-A14U maintained the A8:U14 base pair, the accepting activities of the mutants also decreased (Table 2), indicating that leucylation of *Sco*tRNA^{Leu}(CAA) or -(UAA), like hmtRNA^{Leu}(UAA) and *Ec*tRNA^{Leu}, requires a precise U8:A14 pair. The K_m values of *Sco*LeuRS for the above four *Sco*tRNA^{Leu} mutants increased, indicating that the binding of *Sco*LeuRS with the four mutants was weakened compared with their cognate wild-type *Sco*tRNA^{Leu}s and that the tertiary structure base pair between U8 and A14 is important to maintain the interaction between the tRNA and the enzyme. The k_{cat} values of the enzyme for *Sco*tRNA^{Leu}(CAA)-A14U and -A14U/U8A increased, whereas those of *Sco*tRNA^{Leu}(UAA)-A14U and -A14U/U8A decreased obviously, implying that the U8:A14 tertiary structure base pair has different roles in the catalysis of *Sco*LeuRS. The catalytic efficiency (K_m/k_{cat}) of *Sco*LeuRS for the two *Sco*tRNA^{Leu}(UAA) mutants and two -(CAA) mutants decreased by varying degrees (12 and 7.9% for (CAA), 2 and 1% (UAA), respectively) compared with their cognate wild-type tRNA (Table 2). The results showed that the U8:A14 base pair in both *Sco*tRNA^{Leu}s is significant for their aminoacylation, and especially, it is more crucial in *Sco*tRNA^{Leu}(UAA).

Our previous work indicated that G18:U55 in *Ec*tRNA^{Leu} is not as important as that of G19:C56, and the base pair U54:A58 in the TψC-loop plays a more important role in aminoacylation (8). To compare the different contributions of these elbow structure base pairs of *Sco*tRNA^{Leu}(CAA) to aminoacylation, several deletion and substitution mutants were constructed and their kinetic parameters were assayed (Table 2). The K_m and k_{cat} values of *Sco*LeuRS for the deletion mutant *Sco*tRNA^{Leu}(CAA)-ΔG18/ΔG19 were slightly higher than those of the wild-type, leading to higher catalytic efficiency, although this mutant was predicted to have an impaired tertiary interaction. The K_m and k_{cat} values for another double deletion mutant, *Sco*tRNA^{Leu}(CAA)-ΔU55/ΔC56, and a double substitution mutant -U54C:A58G both increased, leading to only a slight change in catalytic efficiency. These results indicated that the elbow structure formed by these three base pairs does not play a crucial role in aminoacylation of *Sco*LeuRS for *Sco*tRNA^{Leu}(CAA).

Compared with the D-loop sequence of *Sco*tRNA^{Leu}(CAA), G19 is missing in that of *Sco*tRNA^{Leu}(UAA). The tertiary structure base pairs between the TψC-loop and the D-loop in *Sco*tRNA^{Leu}(UAA) only contain G18:U55 and U54:A58; however, it still retains a stable tertiary structure because of its higher accepting activity. We inserted G19 to form *Sco*tRNA^{Leu}(UAA)-▽G19, which forms a new G19:C56 base pair and should stabilize the elbow structure of the L-shaped *Sco*tRNA^{Leu}(UAA). The k_{cat} value of *Sco*LeuRS for this mutant decreased by about 50%, the K_m value decreased by about one third, and the catalytic efficiency of the enzyme for the mutant increased by 50%, indicating that the more stable the elbow structure, the stronger the affinity of *Sco*LeuRS for this mutant, leading to higher catalytic efficiency. As expected, deletion of G18 or U55 to break the hydrogen bond between them decreased the accepting capacity of these mutants. The K_m values of the two deletion mutants increased, indicating weakening of the binding of *Sco*LeuRS to these mutants. However, the k_{cat} values increased, suggesting a higher catalytic rate. The aminoacylation of the substitution mutant U54C:A58G declined, with a loss in its k_{cat} value, indicating that this tertiary structure base pair at the elbow of the L-shape in *Sco*tRNA^{Leu}(UAA) is more important than that in *Sco*tRNA^{Leu}(CAA).

In vivo complementation assay of *Sco*tRNA^{Leu}(UAA) matched the results *in vitro*

Earlier studies showed that in *S. coelicolor*, mutation in the gene *bldA* encoding *Sco*tRNA^{Leu}(UAA) led to a 'bald' phenotype, in which the bacteria lack aerial hyphae and spores, as well as antibiotic production (37,40–43). Complementation of *S. coelicolor* with a functional *bldA* rescued this deficiency, which could be observed by production of the dark-blue pigment actinorhodin (38). To investigate whether the mutations that did not affect aminoacylation could restore the phenotype, a bald strain, J1501Δ*bldA*::apra, was constructed as previously described (37) and introduced with different mutant genes of *Sco*tRNA^{Leu}(UAA) which were recombined into the integrative vector pMS82, as described in the 'Materials and Methods' section (44). Wild-type *bldA* was used as a positive control and an empty vector as the negative control. The *bldA* gene contains 5′-flank and 3′-flank regions of pre-mature tRNA and a full sequence of *Sco*tRNA^{Leu}(UAA). We mutated the given site of *Sco*tRNA^{Leu}(UAA) in *bldA* to generate pMS82bldA mutants. If the tRNA transcribed by the pMS82bldA mutants could be successfully leucylated, then the mRNA containing UUA could be translated to Leu from Leu-tRNA^{Leu} during protein synthesis, otherwise the Leu-tRNA^{Leu} and correct translation could not be performed (Figure 6A). Successful restoration of the bald phenotype of J1501Δ*bldA*::apra was inferred not only from the production of aerial mycelia and spores (data not shown), but also by the appearance of antibiotics and production of dark-blue pigment actinorhodin, as shown in Figure 6B.

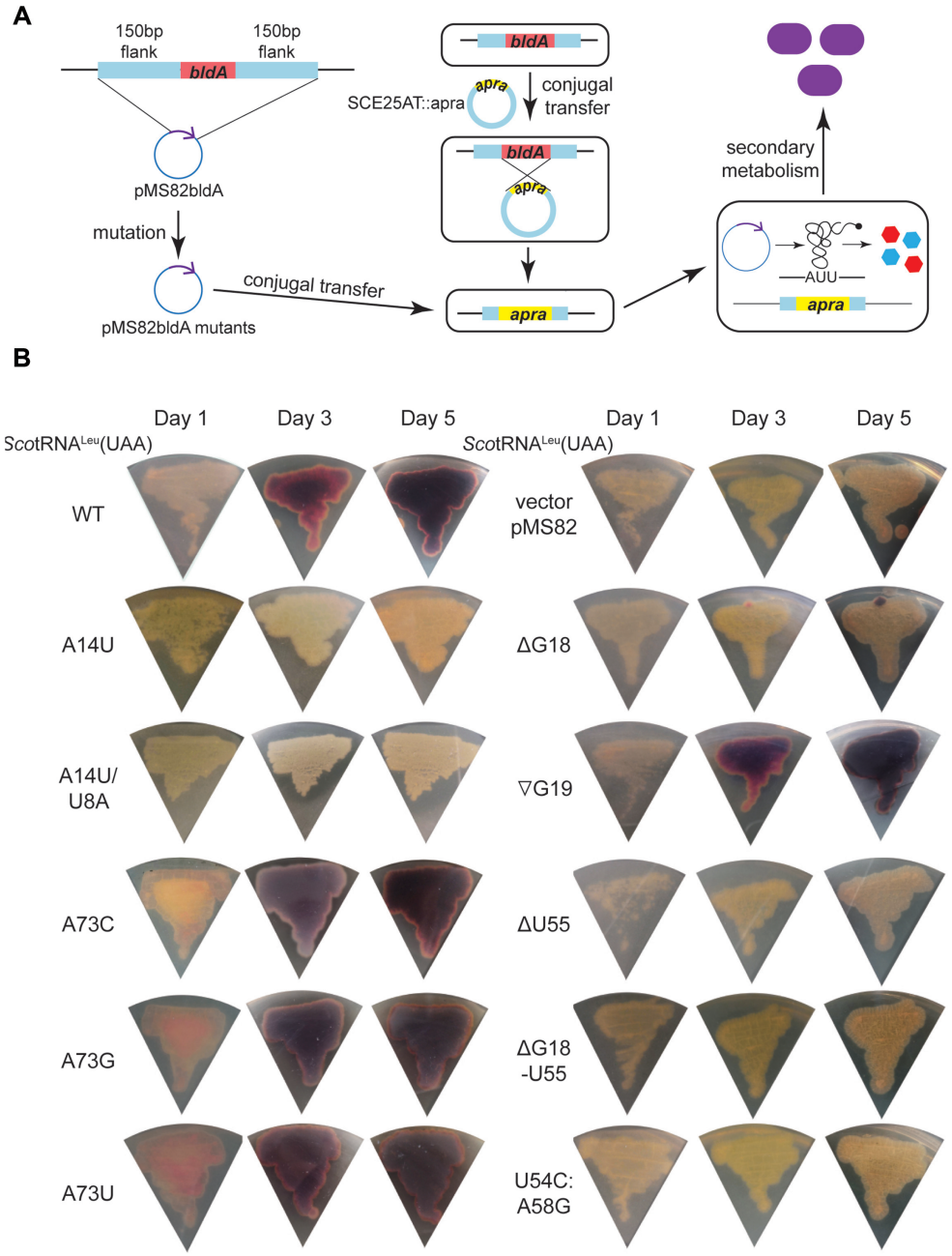


Figure 6. (**A**) Schematic representation of the complementation assay. (**B**) Complementation assay of the *Streptomyces coelicolor* knockout strain J1501Δ*bldA*::apra by different *bldA* genes and mutant constructs. The various constructs harbored on the integrative pMS82 plasmid were introduced into *S. coelicolor*, which were grown on R5 plates at 30°C for 5 days. The plates were photographed at the indicated times. The pMS82 empty vector was introduced as a negative control.

As expected, the wild-type *bldA* construct could restore the phenotype and the empty vector could not. Consistent with the results of the aminoacylation assay, no detectable difference was observed when comparing the negative control with the A14U and A14U/U8A mutants. Full restoration was observed for the three A73 mutants. Deletion of G18 and the crucial pair G18-U55, as well as substitution of U54-A58 to C54-G58, also failed to recover the *bald* phenotype. Inserting G19 induced a higher level of acitnorhodin production, which was consistent with the higher catalytic efficiency of *Sco*LeuRS for *Sco*tRNA^{Leu}(UAA)-▽G19. These results matched the *in vitro* results for *Sco*tRNA^{Leu} (UAA). However, we observed no restoration using △U55, although the *in vitro* assay of this mutant detected 65% of the accepting activity of the wild-type, which was probably caused by the lack of a ψ modification on this nucleotide (45).

Taken together, our results showed that the A14-U8 pair of the two types of *Sco*tRNA^{Leu}s is a mutual identity element, similar to other tRNA^{Leu}s. The structural stability of

the elbow formed by the D-loop and the TψC-loop is more important for type II *Sco*tRNA^{Leu}(UAA) than for type I *Sco*tRNA^{Leu}(CAA).

The LSD of *Sco*LeuRS is responsible for aminoacylation of type I tRNA^{Leu}

*Sco*LeuRS could leucylate the two types of *Sco*tRNA^{Leu} with different lengths of variable loops; therefore, to reveal which domain of *Sco*LeuRS contributes to the charging ability to the two types of *Sco*tRNA^{Leu}, we replaced various domains from *Sco*LeuRS in *Ec*LeuRS, which only charges type II tRNA^{Leu}. However, none of the chimeras of LeuRSs from *Ec*LeuRS and *Sco*LeuRS, including *Ec*LeuRS-*Sco*CTD (C-terminal domain), -*Sco*LSD (leucine specific domain), and -*Sco*CP1 (CP1 domain) could charge both type I and II *Sco*tRNA^{Leu} (data not shown), indicating the effect of the large conformational change in these chimeric enzymes from *S. coelicolor* (phylum Actinobacteria) and *E. coli* (phylum Proteobacteria) in the active site. We then turned to LeuRSs from other *Streptomyces* species to make the chimeric enzymes.

We screened the genomes of several *Streptomyces* strains and found that in the genomes of *S. azureus* and *Streptomyces purpurogeneiscleroticus,* there is no gene for type I tRNA^{Leu}, meaning that the LeuRS from these two *Streptomyces* strains probably cannot aminoacylate type I tRNA^{Leu}. *Streptomyces azureus* LeuRS (*Saz*LeuRS) and *S. purpurogeneiscleroticus* LeuRS (*Spur*LeuRS) were obtained from *E. coli* transformants harboring their genes (Supplementary Figure S6A). We found that *Saz*LeuRS could leucylate *Sco*tRNA^{Leu}(CAA); however *Spur*LeuRS could not, like *Ec*LeuRS (Supplementary Figures S6C and D). Phylogenetic analysis showed that *Spur*LeuRS is located on the same branch with *Ec*LeuRS (Supplementary Figure S6B). Therefore, chimeric enzymes substituted with various domains of *Spur*LeuRS with that of *Sco*LeuRS, separately, might help to identify the domain responsible for *Sco*LeuRS's ability to charge two types of *Sco*tRNA^{Leu}.

We replaced the CTD (Leu805–Val870) of *Spur*LeuRS with its *Sco*LeuRS counterpart (Arg901–Ala967), and CP1 of *Spur*LeuRS (Ser234–Arg439) with its *Sco*LeuRS counterpart (Ser306–Arg517) (Supplementary Figure S7A). Neither of the chimeric enzymes could leucylate *Sco*tRNA^{Leu}(CAA) (Supplementary Figure S7B and C). However, *Spur*LeuRS-*Sco*LSD, in which the LSD of *Spur*LeuRS (45 amino acid residues from Val594 to Glu638) was replaced with its *Sco*LeuRS counterpart (38 amino acid residues from Ile696 to Gly733) (Figure 7C), gained aminoacylation activity for type I tRNA^{Leu} (Figure 7B).

We then identified the residues on *Sco*LSD that affected the aminoacylation activity of *Spur*LeuRS-*Sco*LSD for type I *Sco*tRNA^{Leu}. The homology of LSDs is relatively low among various species (Supplementary Figure S9A); therefore, to narrow down our search, eight multi-alanine peptides were substituted for eight sequences from the N- to the C-terminus of *Sco*LSD, as shown in Figure 8. The leucylation activity of these substitution mutants for the two types of *Sco*tRNA^{Leu}s was assayed. Four substitution mutants, *Spur*LeuRS-*Sco*LSD-(696–699)A, -(700–703)A, -(709–714)A and-(715–720)A, could not charge either *Sco*tRNA^{Leu}(UAA) or *Sco*tRNA^{Leu}(CAA), indicating that the structure of these mutant enzymes was disrupted. The other four mutants retained their ability to charge both type I and II *Sco*tRNA^{Leu}. Among the eight mutants, three, *Spur*LeuRS-*Sco*LSD-(704–708)A, -(721–725)A and -(726–729)A, showed high aminoacylation activity toward *Sco*tRNA^{Leu}(UAA); however, their aminoacylation of *Sco*tRNA^{Leu}(CAA) was reduced by about 50% (Figure 8; Supplementary Figure S8B and C). At last, the last mutant, *Spur*LeuRS-*Sco*LSD-(730–733)A at the C-terminus of *Sco*LSD, retained its activity for *Sco*tRNA^{Leu}(UAA), but drastically lost its activity for *Sco*tRNA^{Leu}(CAA) (Figure 7D and E). Thus, these sequences of *Sco*LSD are probably important for the recognition of *Sco*tRNA^{Leu}(CAA).

A number of single-point mutants at these sites of *Sco*LSD were constructed. The aminoacylation assays revealed that residues from *Spur*LeuRS-*Sco*LSD-(704–708)A, -(721–725)A and -(726–729)A and three residues from *Spur*LeuRS-*Sco*LSD-(730–733)A showed no loss of aminoacylation activity of *Sco*tRNA^{Leu}(CAA) (Supplementary Figure S9). However, *Spur*LeuRS-*Sco*LSD-L732A retained 75% aminoacylation of *Sco*tRNA^{Leu}(UAA), but showed less than half the activity toward *Sco*tRNA^{Leu}(CAA) (Figure 7D and E). The aminoacylation activity of the L732I mutants was not influenced, suggesting the size and hydrophobicity of side chain of L732 is important for charging type I *Sco*tRNA^{Leu}. Notably, mutation of L732 was not sufficient to cause a reduction in activity to the same extent as *Spur*LeuRS-*Sco*LSD-(730–733)A. To test whether another amino acid residue among the four residues would strengthen the negative effect on the recognition of the enzyme to *Sco*tRNA^{Leu}(CAA), we mutated both L732 and G733 to A. This double mutation indeed triggered a loss of leucylation of *Sco*tRNA^{Leu}(CAA), however the mutant enzyme still leucylated *Sco*tRNA^{Leu}(UAA).

In summary, the LSD is the key fragment that helps *Streptomyces* LeuRS to gain the ability to charge type I *Sco*tRNA^{Leu}. The C-terminal residues of the LSD play an important role in distinguishing the two types of *Sco*tRNA^{Leu}.

DISCUSSION

Coexistence of two types of tRNA^{Leu} in a single compartment

Type I tRNA^{Leu} does not exist in the same compartment with type II tRNA^{Leu} in most cases, especially in eukaryotes. The most conventional example is human cells: Type II tRNA^{Leu} exists in the cytoplasm, while type I tRNA^{Leu} exists in the mitochondria, and there is a cognate LeuRS that leucylates each tRNA^{leu}. In prokaryotic bacteria, almost all tRNA^{Leu}s belong to type II tRNA, having a long variable loop and are leucylated by their cognate LeuRSs. The present study is the first to show that one LeuRS can leucylate two types of tRNA^{Leu} in one actinomyces cell.

Notably, in eukaryotes, the two types of tRNA^{Leu} are separated by a membrane and have their own cognate LeuRS, and have optimal catalysis conditions, such as human cell cytoplasm for the hctRNA^{leu}–hcLeuRS system and

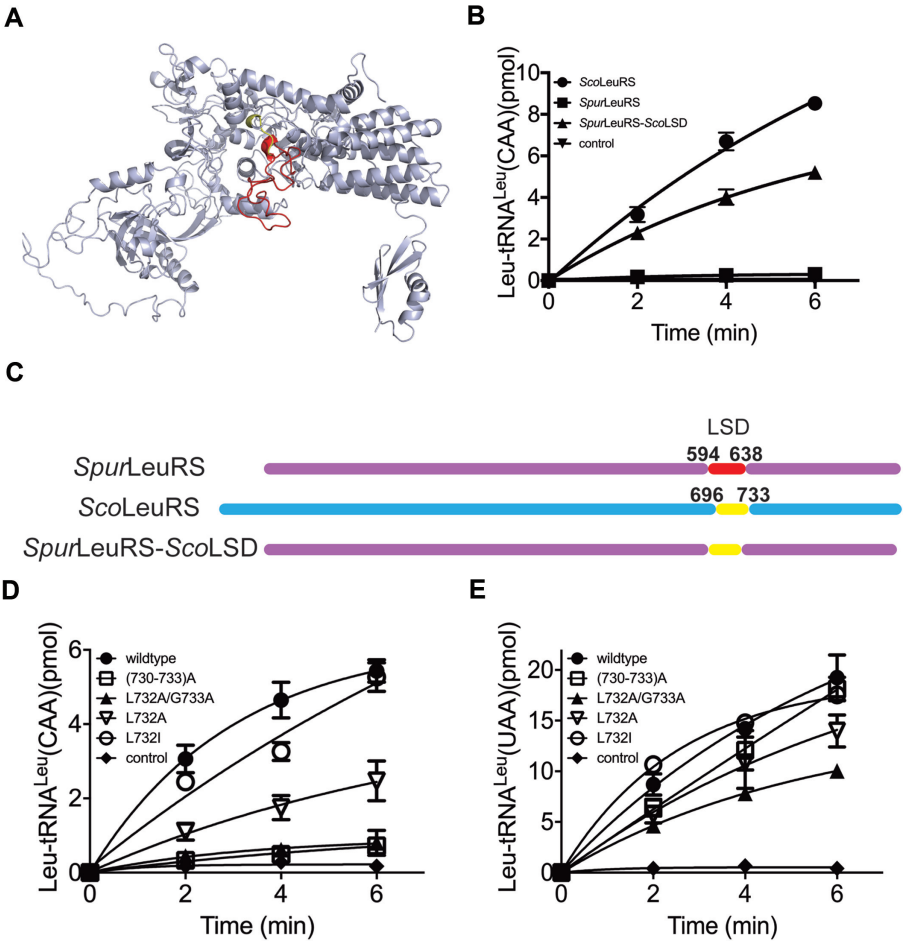


Figure 7. The effects of *Sco*LeuRS-LSD in recognizing type I *Sco*tRNA^{Leu}s. (**A**) Three-dimensional view of the *Sco*LeuRS model showing the LSD (red) and KMSKS motifs (yellow) in the aminoacylation state. (**B**) Aminoacylation of 1 nM *Sco*LeuRS, 1 nM *Spur*LeuRS, 10 nM *Spur*LeuRS-*Sco*LSD or no enzyme, respectively, for 5 μM *Sco*tRNA^{Leu}(CAA). (**C**) Schematic demonstration of the detailed fusion sites of *Spur*LeuRS-*Sco*LSD. The definition of LSD domain was based on the crystal structure of *Ec*LeuRS and sequence alignment. *Numbers* represent the beginning and end of each LSD domain in the context of the full-length enzyme. (**D** and **E**) Aminoacylation of 10 nM *Spur*LRS-*Sco*LSD-WT, -(730–733)A, -L732A/G733A, -L732A, -L732I or no enzyme, respectively, for 5 μM *Sco*tRNA^{Leu}(CAA) (D) or 5 μM *Sco*tRNA^{Leu}(UAA) (E). The data shown represent averages of three independent experiments and the corresponding standard errors. Some error bars are hidden by the symbols.

the human mitochondrial environment for hmtRNA^{Leu}–hmLeuRS system. However, *S. coelicolor* appears to overcome this micro-environment differences. The results showed that *Sco*tRNA^{Leu}(CAA) and -(UAA) display similar basic features. *Sco*tRNA^{Leu}(UAA) has a slightly lower T_m but twice the catalytic velocity of *Sco*tRNA^{Leu}(CAA), yet their binding affinities for *Sco*LeuRS are equal. The higher T_{tm} of *Sco*tRNA^{Leu}(CAA) might result from the additional G19-C56 pair. We also performed mis-charging and editing assays with non-cognate norvaline; the data showed that *Sco*LeuRS's mischarging and editing of two types *Sco*tRNA^{Leu} were comparable (data not shown). How *Sco*LeuRS binds two types of *Sco*tRNA^{Leu} and why *Sco*LeuRS shows a distinct catalytic velocity prompted our interest.

However, the result of a cross-species recognition experiment indicated the specificity of LeuRS from *Streptomyces*, as it is the only prokaryotic LeuRS that could leucylate both type I tRNA^{Leu} and type II tRNA^{Leu}. The alignment analysis showed that *Sco*LeuRS has a longer sequence than other previously reported prokaryotic LeuRSs, suggesting that its more flexible structure may help to leucylate the two types of tRNA^{Leu}. Notably, *Sco*LeuRS cannot charge hmtRNA^{Leu}(UUR) even though the tRNA^{Leu} is a type I tRNA. We suspected that the micro-environment might affect this leucylation process. However, we cannot rule out the possibility that *Sco*LeuRS did not adapt to the special structure of hmtRNA^{Leu}(UUR).

The identity elements of two types of *Sco*tRNA^{Leu}

Previous studies indicated that each type of tRNA^{Leu} has its own identity elements. *Sco*tRNA^{Leu}(UAA) and *Sco*tRNA^{Leu}(CAA) have some identity elements that are shared by earlier reported tRNA^{Leu}s, such as the A14-U8 pair for both *Sco*tRNA^{Leu}s. However, this is the first report that the A73 residue of *Sco*tRNA^{Leu}s is not the identity element recognized by its cognate *Sco*LeuRS. Mutagenesis of *Sco*LeuRS also showed that the activity of *Sco*LeuRS was not affected by interaction with A73,

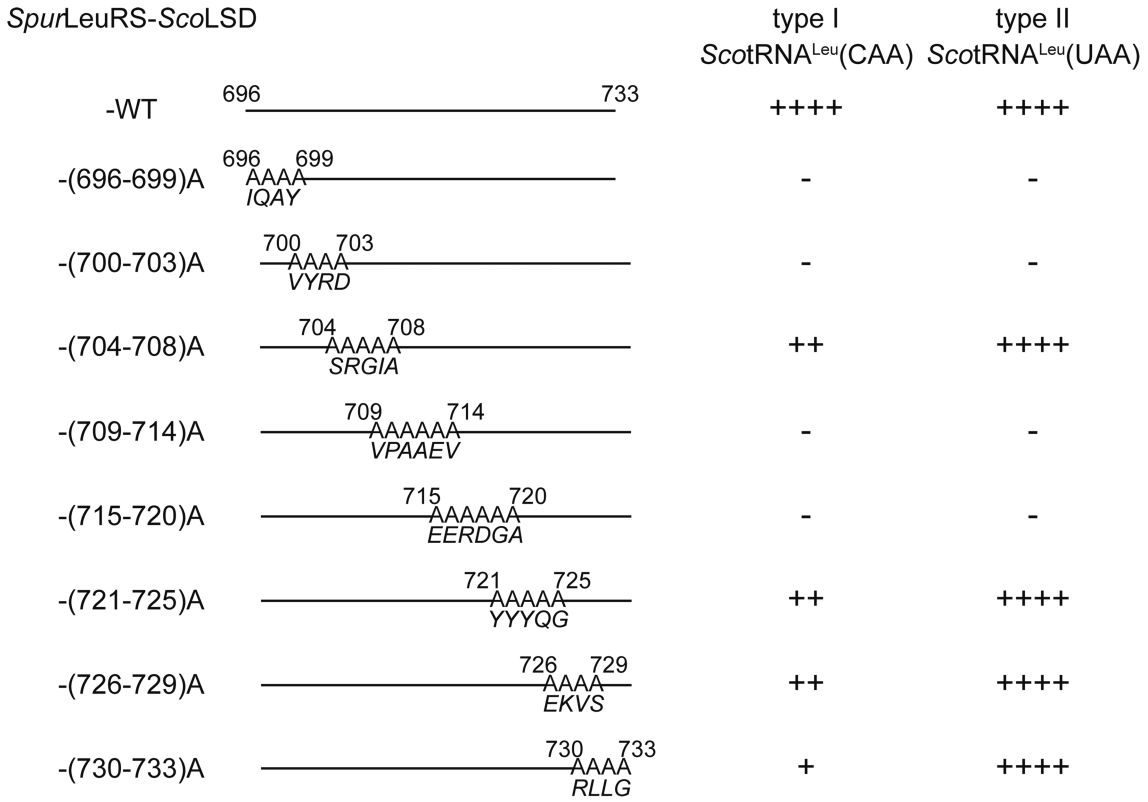


Figure 8. The aminoacylation of *Sco*tRNA^{Leu}(CAA) by multiple alanine mutants of *Spur*LeuRS-*Sco*LSD. Schematic demonstration of detailed replacement sites of *Spur*LeuRS-*Sco*LSD multiple alanine mutants. The *italic* fonts indicate the original sequence of *Sco*LSD. Aminoacylation of each mutant for *Sco*tRNA^{Leu} (CAA) and (UAA) is listed on the right compared with that of the wild-type.

as it is in *Ec*LeuRS. The structure of the aminoacylation state of *Ec*LeuRS-*Ec*tRNA^{Leu}(UAA) showed the A73 on *Ec*tRNA^{Leu}(UAA) was flipped and interacted with a conserved peptide, ^{416}R/KLRDWGVSRQRYWG^{429} (22). In the *Sco*LeuRS-*Sco*tRNA^{Leu} system, A73 may not be flipped to interact with this peptide. The crystal structure of *Ec*LeuRS- *Ec*tRNA^{Leu}(UAA) also displayed the interaction between A73 or the G1-U72 pair of tRNA and peptide 290–298 of CP1 domain (22). However, our data suggested that mutants *Sco*LeuRS-V385A, -E386A and -R387A (in which the residues are homologs of A291, E292 and A293 of *Ec*LeuRS, respectively. See Supplementary Figure S10A.) could leucylate *Sco*tRNA^{Leu} similarly to the wild-type (data not shown). In addition, the residues of peptide 290–298 in *Ec*LeuRS display low homology to those of *Sco*LeuRS (Supplementary Figure S10A). Hence, the structure formed by these residues of *Ec*LeuRS may differ from those of *Sco*LeuRS. Our data suggested that C74 in the CCA tail remains tightly bound to LeuRS and is important for leucylation of *Sco*tRNA^{Leu}s. Thus, C74 seems to be the new identity element for recognition of *Sco*LeuRS.

The tertiary structure of tRNA associates the D/TψC-loop interaction with a compact core of seven base layers (39). Our results suggested that D/TψC-loop interaction plays different roles regarding different types of *Sco*tRNA^{Leu}, even though the base pairs are similar. The core of seven base layers, which require the residues to form a variable loop, appeared distinct from *Sco*tRNA^{Leu}(CAA) and -(UAA). Previous crystal structure studies indicated that the variable loop in type II tRNA would comprise a hairpin, while type I tRNA would not (22,24,46). The complex of *Ec*LeuRS-*Ec*tRNA^{Leu}(UAA) implied an interaction between the CTD of *Ec*LeuRS and the 47f-47i nucleotide of *Ec*tRNA (22); the complex of *Thermus thermophilus* LeuRS (*Tt*LeuRS) and *Tt*tRNA^{Leu}(CAG) implied an interaction between R826 of *Tt*LeuRS and C47e of *Tt*tRNA, although this tRNA was deleted for two base pairs of the variable loop (24). Our data further confirmed that deletion of all base pairs on the variable loop did not harm the aminoacylation of *Sco*tRNA ^{Leu}(UAA). The residues that contacted with tRNA in *Ec*LeuRS-*Ec*tRNA^{Leu} system had a relatively low homology with those of *Sco*LeuRS (Supplementary Figure S10B); therefore, we suspected that there is no contact between CTD of *Sco*LeuRS and the variable loop of *Sco*tRNA^{Leu}. The variable loop participates in the core of seven base layers in type I *Sco*tRNA^{Leu}(CAA) more than it does in *Sco*tRNA^{Leu}(UAA). However, altering the bases of the variable loop of *Sco*tRNA^{Leu}(CAA) showed no drastic loss of amino acid accepting activity. We concluded that the 5 nt length of the variable loop is important for aminoacylation.

The unique linkers in *Sco*LeuRS to help to aminoacylate two types of tRNA^{Leu}

LeuRS is a multi-domain class at AARS that comprises a main enzyme body (Rossmann-fold catalytic domain

and class Ia anticodon-binding domain) and four flexibly linked additional domains, termed ZN1, CP1, LSD and CTD (17). We swapped the other three linked domains - CP1, CTD and LSD of *Sco*LeuRS, to another *Spur*LeuRS that could not leucylate type I *Sco*tRNA^{Leu} to form three chimeric LeuRSs. Notably, aminoacylation assays of chimeric *Spur*LeuRS-*Sco*CP1 showed drastic loss of activity for both *Sco*tRNA^{Leu}(CAA) and -(UAA), indicating that the CP1 domain plays a crucial role in aminoacylation. Previous studies of *Ec*LeuRS and *Tt*LeuRS hinted us that CP1 is a flexible structure (22,24,47), probably acting as an important regulating domain during aminoacylation and engaging in cross-talk with other domains (48). We suspected that a CP1 domain from other species would fail for this function, thus abrogating the aminoacylation activity of LeuRS. The CTD domain of *Ec*LeuRS was also suggested to contact with C56 of TψC loop, A20a of D loop and 47f to 47i of the variable loop of tRNA by crystal structure analysis (22). However, the results implied that the chimeric enzyme *Spur*LeuRS-*Sco*CTD did not charge this type I *Sco*tRNA^{Leu}. Moreover, *Sco*LeuRS showed high activity for mutant *Sco*tRNA^{Leu}(CAA)-ΔU55/ΔC56 (Table 2). We concluded that the CTD does not specifically contribute to aminoacylation of *Sco*tRNA^{Leu}. We then asked which domain of *Sco*LeuRS contributes to aminoacylation of type I *Sco*tRNA^{Leu}? Our results showed that only the LSD help *Spur*LeuRS to gain the ability to charging type I *Sco*tRNA^{Leu}. The crystal structure of *Ec*LeuRS showed that the LSD is crucial for positioning the conserved catalytic signature sequence, the KMSKS loop, during aminoacylation reactions (20,24). However, the structure of the LSD varies from species to species. The three-dimensional structure of the *Tt*LeuRS showed its LSD exhibits five β-strands and two short α-helices (24). In comparison, the LSD of *Ec*LeuRS contains an additional extended β-hairpin (22). This diversity implies that the LSD might be related to LeuRS's species specificity. Our results indicated that the LSD from *Sco*LeuRS contributes the capacity for charging type I tRNA^{Leu}. However, how the LSD works when an armless tRNA^{Leu} binds with LeuRS is not clear. The biochemical experiments showed that a gross deletion of LSD abolished the aminoacylation activity of *Ec*LeuRS (21). A previous crystal structure study showed that coupling of the LSD with the KMSKS loop located the tRNA in the correct place (22), suggesting that the KMSKS loop goes through a conformational change from an open state to a semi-open state; therefore, one could hypothesize that the LSD acts as a regulatory peptide to adjust the position of KMSKS to bind with tRNA^{Leu}. For type I tRNA^{Leu}, the LSD in *Sco*LeuRS helps KMSKS to bind and then performs aminoacylation.

Our results further showed that mutation of both L732 and G733 at the C-terminus of the LSD in *Sco*LeuRS caused *Spur*LeuRS-*Sco*LSD to lose its charging capability of tRNA^{Leu}(CAA). Interestingly, L732 and G733 link the LSD and KMSKS motifs. Additionally, leucine and glycine are both flexible residues that can better adjust the location of adjacent peptides. The loss of the aminoacylation activity of LeuRS after substituting both L732 and G733 might have resulted from an inability to correctly locate the KMSKS motif. The hypothesis that LSD properly locates KMSKS to leucylate type I tRNA^{Leu} further supported our original assumption that *Sco*LeuRS contains a regulatory peptide to adjust each domain to the right position to bind type I tRNA^{Leu}.

The coevolution of LeuRS and tRNA^{Leu}

While analyzing the genomes of *Streptomyces* strains, we noticed that some strains have LeuRSs highly similar to *Sco*LeuRS, but lack a type I tRNA^{Leu}. A model, *Saz*LeuRS, showed its capability of charging *Sco*tRNA^{Leu}(CAA), suggesting one of them evolved before the other. However, no reasonable explanation has been proposed for this observation so far. We may have a provided clue as to why type I tRNA^{Leu}, such as *Sco*tRNA^{Leu}(CAA) and hmtRNA^{Leu}(UUR), exist. The ancient tRNA^{Leu} probably belonged to type I tRNA and then evolved to type II tRNA, for whom LeuRS has a higher catalytic efficiency. The type I tRNA^{Leu} disappeared in response to some unknown pressure; however, some *Streptomyces* and human mitochondria did not encounter such pressure and thus retained the type I tRNA^{Leu}. Despite this unsolved question, we provided evidence showing that mitochondria originated from prokaryotes. The LeuRS from human mitochondria also recognized and charged two types of *Sco*tRNA^{Leu}. The structural stability at the elbow region of the type I *Sco*tRNA^{Leu}(CAA) is not so important to aminoacylation as it is for type II *Sco*tRNA^{Leu}(UAA), which also illustrated the structural flexibility of type I tRNA^{Leu}, a feature of hmtRNA^{Leu}(UUR) showed in earlier studies (14,15).

Another subtle clue that these two types of *Sco*tRNA^{Leu} probably function similarly came from previous *in vivo* studies related to secondary metabolism. *Sco*tRNA^{Leu}(UAA) is encoded by a secondary metabolism regulatory gene in *S. coelicolor*, *bldA* (40). The *bldA* knockout strain of *S. coelicolor* displayed a phenotype that lacked spores and antibiotics production (49). *bldA* is widespread among *Streptomyces* species. In this study, we proved that a functional *bldA* gene is essential for secondary metabolism. A *bldA* gene with a G19 insertion, whose accepting activity was elevated *in vitro*, showed higher production of actinorhodin, suggesting a modified tRNA^{Leu} could help to refine the metabolic process.

A previous study showed that a single TTA codon is mistranslated efficiently in *Streptomyces clavuligerus* and the authors hypothesized that this codon might be translated by tRNA^{Leu}(CAA) (50), given that there is a former report showing that this isoacceptor also reads UUA codons in the absence of a UUA reader in *Escherichia coli* (51). Furthermore, previous data demonstrated that the modes of accumulation of *Sco*tRNA^{Leu}(CAA) and *Sco*tRNA^{Leu}(UAA) are similar (38). Most interestingly, *Sco*tRNA^{Leu}(CAA) is the only isoacceptor that can replace *Sco*tRNA^{Leu}(UAA) after altering its anticodon to UAA (38). Taken together, the results suggested that these two isoacceptors are related. However, *Sco*tRNA^{Leu}(CAA), unlike *Sco*tRNA^{Leu}(UAA), cannot be deleted from the *S. coelicolor* genome. Our effort to knockout the gene encoding *Sco*tRNA^{Leu}(CAA) failed. This is probably related to the high codon usage of UUG (the codon deciphered by *Sco*tRNA^{Leu}(CAA)). There are 6706 UUG codons in the

S. coelicolor genome, but only 145 UUA codons (the codon deciphered by *Sco*tRNA^{Leu}(UAA)). In addition, the higher G+C content in the *S. coelicolor* genome makes UUA a rare codon, unlike UUG. In present study, the biochemical evidence suggests how these two types of *Sco*tRNA^{Leu} resemble each other. Thus, we hypothesized that evolutionarily, *Sco*tRNA^{Leu}(CAA) and (UAA) were likely to have the same function until *Sco*tRNA^{Leu}(UAA) evolved a more stable structure to completely replace *Sco*tRNA^{Leu}(CAA).

In summary, our results provided several comparisons between these two isoacceptors. *Sco*tRNA^{Leu}(CAA) has lower amino accepting capacity than *Sco*tRNA^{Leu}(UAA). The major difference between *Sco*tRNA^{Leu} and other reported tRNA^{Leu} is that they abrogate the canonical discriminator A73. However, C74 shows more contact with *Sco*LeuRS. They share the U8-A14 tertiary base pair as the identity elements. In addition, the more stable the tertiary structure at the elbow of L-shape, the higher the accepting activity of *Sco*tRNA^{Leu}(UAA). The stem part in the long variable loop of *Sco*tRNA^{Leu}(UAA) is not necessary for its leucylation; however, the number of nucleotides in the variable loop of either type I or type II *Sco*tRNA^{Leu} is crucial to their charging activities. By swapping domains, we showed that the LSD is specific and crucial for *Sco*LeuRS charging of *Sco*tRNA^{Leu}(CAA) and identified the key amino acid residues at C-terminus of the LSD for that function. Our study provided a deeper understanding of how two types of tRNAs work together in a prokaryotic single cell.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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