LeuRS can leucylate type I and type II tRNA\textsuperscript{Leu} 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\textsuperscript{Leu} is catalyzed by their cognate leucyl-tRNA synthetases (LeuRSs). However, in Streptomyces coelicolor, there are two types of tRNA\textsuperscript{Leu}, and only one LeuRS (ScoLeuRS). We found that the enzyme could leucylate both types of ScotRNA\textsuperscript{Leu}, and had a higher catalytic efficiency for type II ScotRNA\textsuperscript{Leu(UAA)} than for type I ScotRNA\textsuperscript{Leu(CAA)}. The results from tRNA and enzyme mutagenesis showed that ScoLeuRS 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 ScotRNA\textsuperscript{Leu}, was determined as important for aminoacylation. \textit{In vitro} and \textit{in vivo} assays showed that the tertiary structure formed by the D-loop and T\textsubscript{Ψ}C-loop is more important for ScotRNA\textsuperscript{Leu(UAA)}. We showed that the leucine-specific domain (LSD) of ScoLeuRS, which originally only leucylates type II tRNA\textsuperscript{Leu}, helped LeuRS to aminoacylate type I ScotRNA\textsuperscript{Leu(CAA)} and identified the crucial amino acid residues at the C-terminus of the LSD to recognize type I ScotRNA\textsuperscript{Leu(CAA)}. Overall, our findings identified a rare recognition mechanism of LeuRS to tRNA\textsuperscript{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 T\textsubscript{Ψ}C 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 T\textsubscript{Ψ}C-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\textsuperscript{Leu}, which can be leucylated by the cognate leucyl-tRNA synthetase (LeuRS), along with tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Try}, belongs to the type II tRNA. However, in eukaryotic mitochondria, tRNA\textsuperscript{Leu} has a short variable loop, belonging to the type I tRNA, and can be leucylated by mitochondrial LeuRS. Cross-recognition between tRNA\textsuperscript{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\textsuperscript{Leu} have been widely studied. The most important elements within \textit{Escherichia coli} tRNA\textsuperscript{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\textsuperscript{Leu} molecules that are involved in tertiary interactions (A15-U48 (5,7), G18-U55, C56 (4,7–8), U54-A58 (8) and the position of residue 47n in the variable region (6)). In \textit{Aquifex aeolicus}, the recognition set of tRNA\textsuperscript{Leu} is similar to that of \textit{EctRNA}\textsuperscript{Leus} (9). In the human mitochondrial leucylation system, the discriminator base A73; three base pairs C3-G70, A4-U69 and G5-C68 in the acceptor stem; nucleotide C20 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\textsuperscript{Leu} anticodon loop is the major determinant (13). A previous study showed that human mitochondrial LeuRS (hmLeuRS) rec-
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 Rossman 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 (EcLeuRS) 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, (ScotRNA^{Leu}(UAA), ScotRNA^{Leu}(GAG), ScotRNA^{Leu}(CAG) and ScotRNA^{Leu}(UAG)), have a long variable loop like type II tRNA^{Leu} from bacteria, and one, ScotRNA^{Leu}(CAA), has a short variable loop like 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 ScotLeuRS recognize and leucylate both type I and type II ScotRNA^{Leu}? What is the difference in their recognition and catalysis by ScotLeuRS? Determining the recognition mechanism of ScotLeuRS of two types ScotRNA^{Leu} is interesting and important.

In the present study, we choose ScotRNA^{Leu}(UAA) among the four type II ScotRNA^{Leu} isoacceptors as the representative to study its aminoacylation. ScotRNA^{Leu}(CAA), ScotRNA^{Leu}(UAA) and their mutants were successfully transcribed and purified in vitro. In addition, the genes encoding ScotRNA^{Leu}(CAA) and ScotRNA^{Leu}(UAA) were cloned into E. coli, and the two ScotRNA^{Leu} were isolated and purified from the transformants. Similarly, ScotLeuRS was obtained from the E. coli transformants containing leuS. The affinity and kinetics of ScotLeuRS for the two types of ScotRNA^{Leu} were measured and compared. An unprecedented role of ACCA at the 3′ end of the accepting stem for leucylation of ScotRNA^{Leu} was identified. Moreover, we showed that the LSD of ScotLeuRS helps another LeuRS that only leucylates type II tRNA^{Leu}, leucyl type I ScotRNA^{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, MgCl2, 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). [3H] 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). 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, 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 ScotLeuRS 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 NdeI and HindIII. 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 (SacLeuRS) was amplified from genomic DNA and inserted into pET28a via the restriction enzymes NdeI and HindIII. The gene encoding...
SpurLeuRS, which was synthesized according to the sequence from EBI-EMBL ensemble genome database (28), gene annotation ADL19_14010, and inserted between NdeI and XhoI 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 EcLeuRS and hmLeuRS, pET30a-EcLeuS (29) and pET22b(+)-hmleuS-40 (30), respectively, were constructed in our laboratory.

The definition of the LSD domain of ScoLeuRS and SpurLeuRS was based on the crystal structure of EcLeuRS (Val169 – Ser178, PDB ID: 4AQ7) and sequence alignment. The LSD domain (Val939 – Gln1038) of SpurLeuRS was substituted by the LSD domain (Ile666 – Gly753) from ScoLeuRS to obtain SpurLeuRS-ScoLSD. We further constructed multiple alanine replacement mutants, listed in Supplementary Figure S8. Five single site mutants at the terminus of the LSD residues, *Sco* LeuRS-ScoLSD-R730A, -L731A, -L732A, -G733A and -L732I, and one double-site mutant, SpurLeuRS-ScoLSD-L732A/G733A, were constructed by gene mutagenesis using pET30a-SpurLeuRS-ScoLSD as a template. The numbers in the names of the mutants indicated the position of residues in ScoLeuRS. 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 chanced and cultured in 500 ml of 2x 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**

The DNA sequence of the T7 promoter and the *ScotRNA* Leu(CAA) and *ScotRNA* Leu(UAA) genes were obtained by ligating three chemically synthesized DNA fragments for each strand, which were then ligated into plasmid pTrc99b (pre-cleaved with EcoRI/BamHI) to construct pTrc99b-T7-ScotRNA*Leu*(CAA), pTrc99b-T7-ScotRNA*Leu*(UAA) and other *ScotRNA* 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 acceptor capacity of *ScotRNA* Leu(CAA) and -UAA are 1507 and 1574 pmol/A260, respectively. The tRNA construct of Ec*RNA* 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/A260.

**Aminoacylation assay**

Leucylation of *ScotRNA Leu* was performed at 37°C in a reaction mixture containing 50 mM Tris–HCl (pH 8.5), 6 mM MgCl₂, 2 mM DTT, 4 mM ATP, 20 μM [³H] leucine, 1 nM ScoLeuRS and 5 μM ScotRNA Leu or its variants. The procedure was performed as previously described (29). Leucylation of ScotRNA Leu by pScLeuRS and SpurLeuRS was performed under the same conditions; by *Spur* LeuRS-ScoLSD and its variant was under the same conditions except for 10 mM enzyme in the mixture; and that by EcLeuRS and hmLeuRS was performed as described previously (19,36). The kinetic parameters of *ScoLeuRS* for *ScotRNA* Leu and its variants during aminoacylation were determined in the presence of varying concentrations of tRNA*Leu* (0.5–32 μM).

**Actinomycetes 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 a 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 *ScotRNA 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**

*ScoLeuRS* has the same affinity for the two types of *ScotRNA Leu* variants, but has a different aminoacylation activity

To study the interaction between ScoLeuRS and *ScotRNA Leu* variants in vitro, we first obtained ScoLeuRS from *E. coli* transformants expressing its gene and then purified the protein to ~95% homogeneity using affinity chromatography on Ni-NTA superflo (Supplementary Figure S1A). The transcripts of *ScotRNA 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\textsuperscript{Leu} from various species. (A and B) Aminoacylation of 1 nM ScoLeuRS, 1 nM EcLeuRS, 500 nM hmLeuRS or no LeuRS for 5 μM ScoRNA\textsuperscript{Leu}(CAA) (A) or 5 μM ScoRNA\textsuperscript{Leu}(UAA) (B), respectively. (C) Aminoacylation of 1 nM ScoLeuRS for 5 μM ScoRNA\textsuperscript{Leu}(UAA), 5 μM ScoRNA\textsuperscript{Leu}(CAA), 5 μM hmRNA\textsuperscript{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\textsuperscript{Leu}.

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

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

ScoLeuRS aminoacylates prokaryotic tRNA\textsuperscript{Leu}, but not eukaryotic mitochondrial tRNA\textsuperscript{Leu}

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

The aminoacylation activity of hmLeuRS and EcLeuRS for the two ScoRNA\textsuperscript{Leu}s showed that hmLeuRS had similar leucylation activity for both types of ScoRNA\textsuperscript{Leu}s compared with ScoLeuRS; EcLeuRS could catalyze aminoacylation of ScoRNA\textsuperscript{Leu}(UAA) with 50% activity; however, it could not leucylate ScoRNA\textsuperscript{Leu}(CAA) completely (Figure 1A and B). The sequence alignment of these three LeuRSs showed that ScoLeuRS and hmLeuRS have the same domains as EcLeuRS, indicating that ScoLeuRS and hmLeuRS have the same domains as EcLeuRS; however, ScoLeuRS 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\textsuperscript{Leu}s. Moreover, EcLeuRS has a lower activity for type II ScoRNA\textsuperscript{Leu}(UAA) despite the high homology, which suggested that ScoRNA\textsuperscript{Leu}s may have different recognition sets from EcRNA\textsuperscript{Leu}s.

We also examined the aminoacylation of ScoLeuRS for type II EcRNA\textsuperscript{Leu}(UAA) and type I hmtRNA\textsuperscript{Leu}(UUR), ScoLeuRS could aminoacylate type II EcRNA\textsuperscript{Leu}(UAA) with a similar reaction rate to that of ScoRNA\textsuperscript{Leu}(UAA), but not type I hmtRNA\textsuperscript{Leu}(UUR) (Figure 1C), implying that ScoRNA\textsuperscript{Leu}s and hmtRNA\textsuperscript{Leu}s have distinct identity elements.
The results are the averages of three independent repeats, with the standard deviations indicated.

Table 1. Aminoacylation kinetics constants of transcripts of ScoLeuRS for ScotrRNA^{Leu(CAA), (UAA)} and their mutants

| ScotrRNA^{Leu} | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹μM⁻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 A73 next to C74C75A76 is no longer identity element of ScotrRNA^{Leu}; therefore, we determined whether other bases at the 3’ end play important roles. Mutagenesis of C74 and C75 of ScotrRNA^{Leu} was performed and the accepting activities of the tRNA mutants were assayed (Table 1). The accepting capacities of both ScotrRNA^{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 ScotrRNA^{Leu} (Table 1). However, our previous study showed that EcLeuRS could still leucylate C74 mutants of EcRNA^{Leu}, although with lower activities, which suggested that C74 is not as significant in EcRNA^{Leu} as it is in ScotrRNA^{Leu} (29). The mutants at C75 of both ScotrRNA^{Leu}(CAA) and -(UAA) were also constructed. The kinetic parameters of ScoLeuRS for the tRNA C75 mutants showed that although both the binding and catalysis of ScoLeuRS for the two mutants was weakened, C75 in the two types ScotrRNA^{Leu} was not crucial to their accepting capacities compared with those of C74 (Table 1).

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

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

The aminoacylation assay implied that ScotrRNA^{Leu} may choose different identity elements to those of hmtRNA^{Leu(UUR)} and EctRNA^{Leu(UAA)}. A73 of hmtRNA^{Leu(UUR)} and EctRNA^{Leu(UAA)} is an canonical identity element (4–6,15). To determine whether it plays the same role in ScotrRNA^{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 ScotrRNA^{Leu}, respectively, and the leucine accepting activities of these mutants were assayed (Table 1). For ScotrRNA^{Leu(CAA)}, the $K_m$ values of ScoLeuRS 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 ScotrRNA^{Leu(UAA)}, the $K_m$ values of ScoLeuRS 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, ScoLeuRS still could recognize all six mutants at A73 (three for each ScotrRNA^{Leu}) and had relatively higher catalytic efficiency (Table 1). The data showed that A73 was not an identity element in ScotrRNA^{Leu(CAA)} and -(UAA) for ScoLeuRS.

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

The C74C75A76 at the 3’ end of all tRNAs is common, and A is a common nucleotide for accepting amino acid.
A73 of ScoRNA\textsubscript{Leu}(CAA) and -(UAA) is not important for the activity of ScoLeuRS, and confirming that A73 in the two types of ScoRNA\textsubscript{Leu}s is not the identity element, unlike the EcLeuRS-tRNA\textsubscript{Leu} system.

In the EcLeuRS and EcRNA\textsubscript{Leu}(UAA) complex, C74 of the tRNA contacts with Arg424 of EcLeuRS, which is the homolog of Arg525 of ScoLeuRS (Figure 4A) (22). EcLeuRS-R424A resulted in the loss of half the aminoaacylation activity of EcLeuRS (Figure 4B), indicating that this residue is not crucial to aminoaacylation. However, the aminoaacylation activity of ScoLeuRS-R525A for both types ScoRNA\textsubscript{Leu}s was completely lost (Figure 4C and D).

These results suggested that this Arg residue of ScoLeuRS is crucial to recognize C74 in both types of ScoRNA\textsubscript{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 ScoRNA\textsubscript{Leu}s, we mutated C74 and C75 of type II ScoRNA\textsubscript{Leu}(GAG) into other nucleotides, and obtained the same results as those for ScoRNA\textsubscript{Leu}(UAA) (Supplementary Figure S5D).

The above results indicated that the interaction of ScoLeuRS with both types of ScoRNA\textsubscript{Leu}s was similar, and that C74 of ScoRNA\textsubscript{Leu}s is a crucial base at 3' ACCA end instead of A73 in ScoRNA\textsubscript{Leu}s.
Figure 3. The interaction between residues in ScoLeuRS and A73 in ScotRNA\textsuperscript{Leu}\textsubscript{s}. (A) Crystal structure of Ec\textit{tRNA}\textsuperscript{Leu} (orange in the cartoon mode) in complex with Ec\textit{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 Ec\textit{tRNA}\textsuperscript{Leu}.

The number of nucleotides of the variable loop in ScotRNA\textsuperscript{Leu}\textsubscript{s} is important for aminoacylation

The variable loop of ScotRNA\textsuperscript{Leu}(CAA) contains only five nucleotides, A\textsuperscript{44}C\textsuperscript{45}A\textsuperscript{46}G\textsuperscript{47}C\textsuperscript{48} (SVL); however, ScotRNA\textsuperscript{Leu}(UAA), like other tRNA\textsuperscript{Leu}s, has a long variable loop with seventeen nucleotides (LVL), which could form five base pairs, U\textsuperscript{44}-G\textsuperscript{47}, G\textsuperscript{45}-C\textsuperscript{47}, C\textsuperscript{46}-G\textsuperscript{47}, G\textsuperscript{47}-i and G\textsuperscript{47}-h (Figure 2). We replaced the SVL of ScotRNA\textsuperscript{Leu}(CAA) with the LVL of ScotRNA\textsuperscript{Leu}(UAA) to produce a chimeric mutant ScotRNA\textsuperscript{Leu}(CAA)-LVL. The catalytic efficiency of ScoLeuRS for this chimeric tRNA mutant decreased, with a larger $K_m$ value (Table 2). Then, the nucleotides in the SVL of ScotRNA\textsuperscript{Leu}(CAA) were gradually deleted to form four deletion mutants, named ScotRNA\textsuperscript{Leu}(CAA)-SVL\textsubscript{1nt}, -SVL\textsubscript{2nt}, -SVL\textsubscript{3nt} and -SVL\textsubscript{4nt} (their names are shown in Figure 5E). Except for mutant ScotRNA\textsuperscript{Leu}(CAA)-SVL\textsubscript{1nt} (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 ScotRNA\textsuperscript{Leu}(CAA) (ScotRNA\textsuperscript{Leu}, A44C, -C45A, -A46C, -G47U and -C48A) were constructed. The $K_m$ values of ScoLeuRS for A44C and C48A changed only slightly, and the $k_{cat}$ value 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 ScotRNA\textsuperscript{Leu}(UAA), the LVL was replaced with the SVL of ScotRNA\textsuperscript{Leu}(CAA) to obtain another chimeric mutant ScotRNA\textsuperscript{Leu}(UAA)-SVL. The $K_m$ values of ScoLeuRS for the chimera increased 16-fold compared with that for ScotRNA\textsuperscript{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 ScotRNA\textsuperscript{Leu}(UAA), and was one third of that recorded for ScotRNA\textsuperscript{Leu}(CAA) with the same variable loop (Table 2). The data showed that other structural elements of ScotRNA\textsuperscript{Leu} play important roles in the catalysis by ScoLeuRS. A series of deletion mutants in the LVL were constructed to investigate the role of the LVL in recognition by ScoLeuRS (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 ScoLeuRS for the tRNA mutants, which were deleted for the first and second base pair in the LVL, was even higher than that for ScotRNA\textsuperscript{Leu}(UAA) (Figure 5B). The mutant of ScotRNA\textsuperscript{Leu}(UAA) deleted for C47b and A47g in the LVL (LVL\textsubscript{2nt}) or deleted for two
Figure 4. The interaction between residues in ScoLeuRS and C74 in ScotRNA{Leu}_{4}^{59}. (A) Crystal structure of Ec{tRNA}_{4}^{59} (orange in the cartoon mode) in complex with EcLeuRS (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}_{4}^{59}. (B) Aminoacylation of 1 nM EcLeuRS-WT, -R424A or no enzyme, respectively, for 5 μM Ec{tRNA}_{4}^{59}(UAA). (C and D) Aminoacylation of 1 nM ScoLeuRS-WT, -R525A or no enzyme, respectively, for 5 μM Sco{tRNA}_{4}^{59}(CAA) (C) or 5 μM Sco{tRNA}_{4}^{59}(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 ScoLeuRS for Sco{tRNA}_{4}^{59}(CAA), (UAA) and their mutants

| Sco{tRNA}_{4}^{59} | K_m (μM) | k_cat (s^{-1}) | k_cat/K_m (s^{-1}μM^{-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 |
| -γC19            | 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.
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+4nt) with only four nucleotides, 47d, -c, -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 ScoLeuRS(CAA) and -(UAA) were lost completely when nucleotide 48 (C48 in ScoRNALeu(CAA) and U48 in ScoRNALeu(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
ScotRNA\textsuperscript{Leu}\textsubscript{(CAA)} or -(UAA) is the shortest length required for relatively high aminoacylation.

**The elbow structure plays different roles in two types of ScotRNA\textsuperscript{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 EctRNA\textsuperscript{Leu}(CAA) and hmtRNA\textsuperscript{Leu}(UUR) is the identity element (4,15). The elbow structure base pairs between the D- and T\textsubscript{2}C-loops of EctRNA\textsuperscript{Leu} (G18:U55, G19:C56 and U54:A58) play important roles in aminoacylation (8). The possible tertiary base pairs of ScotRNA\textsuperscript{Leus} 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 ScotLeuRS, several mutations at these sites were constructed.

In either ScotRNA\textsuperscript{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 ScotRNA\textsuperscript{Leu}(CAA)-A14U or ScotRNA\textsuperscript{Leu}(UAA)-A14U maintained the A8:U14 base pair, the accepting activities of the mutants also decreased (Table 2), indicating that leucylation of ScotRNA\textsuperscript{Leu}(CAA) or -(UAA), like hmtRNA\textsuperscript{Leu}(UAA) and EctRNA\textsuperscript{Leu}, requires a precise U8:A14 pair. The K\textsubscript{m} values of ScotLeuRS for the above four ScotRNA\textsuperscript{Leu} mutants increased, indicating that the binding of ScotLeuRS with the four mutants was weakened compared with their cognate wild-type ScotRNA\textsuperscript{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\textsubscript{cat} values of the enzyme for ScotRNA\textsuperscript{Leu}(CAA)-A14U and -A14U/U8A increased, whereas those of ScotRNA\textsuperscript{Leu}(UAA)-A14U and -A14U/U8A decreased obviously, implying that the U8:A14 tertiary structure base pair has different roles in the catalysis of ScotLeuRS. The catalytic efficiency (K\textsubscript{m}/k\textsubscript{cat}) of ScotLeuRS for the two ScotRNA\textsuperscript{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 ScotRNA\textsuperscript{Leu}s is significant for their aminoacylation, and especially, it is more crucial in ScotRNA\textsuperscript{Leu}(UAA).

Our previous work indicated that G18:U55 in EctRNA\textsuperscript{Leu} is not as important as that of G19:C56, and the base pair U54:A58 in the T\textsubscript{2}C-loop plays a more important role in aminoacylation (8). To compare the different contributions of these elbow structure base pairs of ScotRNA\textsuperscript{Leu}(CAA) to aminoacylation, several deletion and substitution mutants were constructed and their kinetic parameters were assayed (Table 2). The K\textsubscript{m} and k\textsubscript{cat} values of ScotLeuRS for the deletion mutant ScotRNA\textsuperscript{Leu}(CAA)-ΔG18/AG19 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\textsubscript{m} and k\textsubscript{cat} values for another double deletion mutant, ScotRNA\textsuperscript{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 ScotLeuRS for ScotRNA\textsuperscript{Leu}(CAA).

Compared with the D-loop sequence of ScotRNA\textsuperscript{Leu}(CAA), G19 is missing in that of ScotRNA\textsuperscript{Leu}(UAA). The tertiary structure base pairs between the T\textsubscript{2}C-loop and the D-loop in ScotRNA\textsuperscript{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 ScotRNA\textsuperscript{Leu}(UAA)-\textsuperscript{G} G19, which forms a new G19:C56 base pair and should stabilize the elbow structure of the L-shaped ScotRNA\textsuperscript{Leu}(UAA). The k\textsubscript{cat} value of ScotLeuRS for this mutant decreased by about 50%, the K\textsubscript{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 for ScotLeuRS 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\textsubscript{m} values of the two deletion mutants increased, indicating weakening of the binding of ScotLeuRS to these mutants. However, the k\textsubscript{cat} values increased, suggesting a higher catalytic rate. The aminoacylation of the substitution mutant U54C:A58G declined, with a loss in its k\textsubscript{cat} value, indicating that this tertiary structure base pair at the elbow of the L-shape in ScotRNA\textsuperscript{Leu}(UAA) is more important than that in ScotRNA\textsuperscript{Leu}(CAA).

**In vivo complementation assay of ScotRNA\textsuperscript{Leu}(UAA) matched the results in vitro**

Earlier studies showed that in S. coelicolor, mutation in the gene bldA encoding ScotRNA\textsuperscript{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, J1501ΔbldA::apra, was constructed as previously described (37) and introduced with different mutant genes of ScotRNA\textsuperscript{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 ScotRNA\textsuperscript{Leu}(UAA). We mutated the given site of ScotRNA\textsuperscript{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-tRNALeu\textsuperscript{Leu} during protein synthesis, otherwise the Leu-tRNALeu\textsuperscript{Leu} and correct translation could not be performed (Figure 6A). Successful restoration of the bald phenotype of J1501ΔbldA::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 J1501ΔbldA::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 ScoLeuRS for ScoRNA^{Leu(UAA)}-▽G19. These results matched the in vitro results for ScoRNA^{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 ScoRNA^{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 ScotRNA^{Leu}(UAA) than for type I ScotRNA^{Leu}(CAA).

The LSD of ScotLeuRS is responsible for aminocoylation of type I tRNA^{Leu}

ScotLeuRS could leucylate the two types of ScotRNA^{Leu} with different lengths of variable loops; therefore, to reveal which domain of ScotLeuRS contributes to the charging ability to the two types of ScotRNA^{Leu}, we replaced various domains from ScotLeuRS in EcLeuRS, which only charges type II tRNA^{Leu}. However, none of the chimeras of LeuRSs from EcLeuRS and ScotLeuRS, including EcLeuRS-ScotCTD (C-terminal domain), -ScotLSD (leucine specific domain), and -ScotCP1 (CP1 domain) could charge both type I and II ScotRNA^{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 purpurigenescleroticus, 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 (SazLeuRS) and S. purpurigenescleroticus LeuRS (SpurLeuRS) were obtained from E. coli transformants harboring their genes (Supplementary Figure S6A). We found that SazLeuRS could leucylate ScotRNA^{Leu}(CAA); however SpurLeuRS could not, like EcLeuRS (Supplementary Figures S6C and D). Phylogenetic analysis showed that SpurLeuRS is located on the same branch with EcLeuRS (Supplementary Figure S6B). Therefore, chimeric enzymes substituted with various domains of SpurLeuRS with that of ScotLeuRS, separately, might help to identify the domain responsible for ScotLeuRS’s ability to charge two types of ScotRNA^{Leu}.

We replaced the CTD (Leu805–Val870) of SpurLeuRS with its ScotLeuRS counterpart (Arg901–Ala967), and CP1 of SpurLeuRS (Ser234–Arg439) with its ScotLeuRS counterpart (Ser306–Arg517) (Supplementary Figure S7A). Neither of the chimeric enzymes could leucylate ScotRNA^{Leu}(CAA) (Supplementary Figure S7B and C). However, SpurLeuRS-ScotLSD, in which the LSD of SpurLeuRS (45 amino acid residues from Val594 to Glu638) was replaced with its ScotLeuRS 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 ScotLSD that affected the aminoacylation activity of SpurLeuRS-ScotLSD for type I ScotRNA^{Leu}. The homology of LSDs is relatively low among various species (Supplementary Figure S9A); therefore, to narrow down our search, eight multialanine peptides were substituted for eight sequences from the N- to the C-terminus of ScotLSD, as shown in Figure 8. The leucylation activity of these substitution mutants for the two types of ScotRNA^{Leu} was assayed. Four substitution mutants, SpurLeuRS-ScotLSD-(696–699)A, -(700–703)A, -(709–714)A and -(715–720)A, could not charge either ScotRNA^{Leu}(UAA) or ScotRNA^{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 ScotRNA^{Leu}. Among the eight mutants, three, SpurLeuRS-ScotLSD-(704–708)A, -(721–725)A and -(726–729)A, showed high aminoacylation activity toward ScotRNA^{Leu}(UAA); however, their aminoacylation of ScotRNA^{Leu}(CAA) was reduced by about 50% (Figure 8; Supplementary Figure S8B and C). At last, the last mutant, SpurLeuRS-ScotLSD-(730–733)A at the C-terminus of ScotLSD, retained its activity for ScotRNA^{Leu}(UAA), but drastically lost its activity for ScotRNA^{Leu}(CAA) (Figure 7D and E). Thus, these sequences of ScotLSD are probably important for the recognition of ScotRNA^{Leu}(CAA).

A number of single-point mutants at these sites of ScotLSD were constructed. The aminoacylation assays revealed that residues from SpurLeuRS-ScotLSD-(704–708)A, -(721–725)A and -(726–729)A and three residues from SpurLeuRS-ScotLSD-(730–733)A showed no loss of aminoacylation activity of ScotRNA^{Leu}(CAA) (Supplementary Figure S9). However, SpurLeuRS-ScotLSD-L732A retained 75% aminoacylation of ScotRNA^{Leu}(UAA), but showed less than half the activity toward ScotRNA^{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 ScotRNA^{Leu}. Notably, mutation of L732 was not sufficient to cause a reduction in activity to the same extent as SpurLeuRS-ScotLSD-(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 ScotRNA^{Leu}(CAA), we mutated both L732 and G733 to A. This double mutation indeed triggered a loss of leucylation of ScotRNA^{Leu}(CAA), however the mutant enzyme still leucylated ScotRNA^{Leu}(UAA).

In summary, the LSD is the key fragment that helps Streptomyces LeuRS to gain the ability to charge type I ScotRNA^{Leu}. The C-terminal residues of the LSD play an important role in distinguishing the two types of ScotRNA^{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...
the human mitochondrial environment for hmtRNA_{Leu}–hmLeuRS system. However, *S. coelicolor* appears to overcome this micro-environment differences. The results showed that ScoRNA_{Leu}(CAA) and -UAA display similar basic features. ScoRNA_{Leu}(UAA) has a slightly lower T_m but twice the catalytic velocity of ScoRNA_{Leu}(CAA), yet their binding affinities for ScoLeuRS are equal. The higher T_m of ScoRNA_{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 ScoLeuRS’s mischarging and editing of two types ScoRNA_{Leu} were comparable (data not shown). 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 the two types of tRNA_{Leu}. Notably, ScoLeuRS 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 ScoLeuRS did not adapt to the special structure of hmtRNA_{Leu}(UUR).

The identity elements of two types of ScoRNA_{Leu}

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

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**Figure 7.** The effects of ScoLeuRS-LSD in recognizing type I ScoRNA_{Leu}s. (A) Three-dimensional view of the ScoLeuRS model showing the LSD (red) and KMSKS motifs (yellow) in the aminoacylation state. (B) Aminoacylation of 1 nM ScoLeuRS, 1 nM SpurLeuRS, 10 nM SpurLeuRS-ScoLSD or no enzyme, respectively, for 5 μM ScoRNA_{Leu}(CAA). (C) Schematic demonstration of the detailed fusion sites of SpurLeuRS-ScoLSD. The definition of LSD domain was based on the crystal structure of EcLeuRS 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 SpurLRS-ScoLSD-WT, -L730, -L732A/G733A, -L732A, -L732I or no enzyme, respectively, for 5 μM ScoRNA_{Leu}(CAA) (D) or 5 μM ScoRNA_{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.
Figure 8. The aminoacylation of Sco tRNA\textsubscript{Leu}(CAA) by multiple alanine mutants of SpurLeuRS-ScoLSD. Schematic demonstration of detailed replacement sites of SpurLeuRS-ScoLSD multiple alanine mutants. The italic fonts indicate the original sequence of ScoLSD. Aminoacylation of each mutant for Sco tRNA\textsubscript{Leu}(CAA) and (UAA) is listed on the right compared with that of the wild-type.

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

The tertiary structure of tRNA associates the D/Tψ C-loop interaction with a compact core of seven base layers (39). Our results suggested that D/Tψ C-loop interaction plays different roles regarding different types of Sco tRNA\textsubscript{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\textsubscript{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 EcLeuRS-EctRNA\textsubscript{Leu}(UAA) implied an interaction between the CTD of EcLeuRS and the 47f-47i nucleotide of EctRNA (22); the complex of \textit{Thermus thermophilus} LeuRS (TtLeuRS) and Tt tRNA\textsubscript{Leu}(CAG) implied an interaction between R826 of TtLeuRS 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\textsubscript{Leu}(UAA). The residues that contacted with tRNA in EcLeuRS-EctRNA\textsubscript{Leu} system had a relatively low homology with those of ScoLeuRS (Supplementary Figure S10B); therefore, we suspected that there is no contact between CTD of ScoLeuRS and the variable loop of Sco tRNA\textsubscript{Leu}. The variable loop participates in the core of seven base layers in type I Sco tRNA\textsubscript{Leu}(CAA) more than it does in Sco tRNA\textsubscript{Leu}(UAA). However, altering the bases of the variable loop of Sco tRNA\textsubscript{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 ScoLeuRS to help to aminoacylate two types of tRNA\textsubscript{Leu}

LeuRS is a multi-domain class at AARS that comprises a main enzyme body (Rossmann-fold catalytic domain
and class Ia anticondon-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 ScoLeuRS, to another SpurLeuRS that could not leucylate type I ScoRNA\textsuperscript{Leu} to form three chimeric LeuRSs. Notably, aminoacylation assays of chimeric SpurLeuRS-ScoCP1 showed drastic loss of activity for both ScoRNA\textsuperscript{Leu}-(CAA) and -(UAA), indicating that the CP1 domain plays a crucial role in aminoacylation. Previous studies of EcLeuRS and TtLeuRS 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 inhibit the capacity for charging type I tRNALeu. However, how the CP1 domain from other species would inhibit the capacity for charging type I tRNALeu after substituting both L732 and G733, suggested to contact with C56 of tRNA\textsubscript{Leu}hmt and 47f to 47i of the variable loop of tRNA by crystal structure analysis (22). However, the results implied that the chimeric enzyme SpurLeuRS-ScoCTD did not charge this type I ScoRNA\textsuperscript{Leu}. Moreover, ScoLeuRS showed high activity for mutant ScoRNA\textsuperscript{Leu}-(CAA)\textsubscript{ΔU55/ΔC56} (Table 2). We concluded that the CTD does not specifically contribute to aminoacylation of ScoRNA\textsuperscript{Leu}. We then asked which domain of ScoLeuRS contributes to aminoacylation of type I ScoRNA\textsuperscript{Leu}? Our results showed that only the LSD help SpurLeuRS to gain the ability to charging type I ScoRNA\textsuperscript{Leu}. The crystal structure of EcLeuRS 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 ThrLeuRS showed its LSD exhibits five β-strands and two short α-helices (24). In comparison, the LSD of EcLeuRS 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 ScoLeuRS contributes the capacity for charging type I tRNA\textsuperscript{Leu}. However, how the LSD works when an armless tRNALeu binds with LeuRS is not clear. The biochemical experiments showed that a gross deletion of LSD abolished the aminoacylation activity of EcLeuRS (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\textsuperscript{Leu}. For type I tRNA\textsuperscript{Leu}, the LSD in ScoLeuRS 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 ScoLeuRS caused SpurLeuRS-ScoLSD to lose its charging capability of tRNA\textsuperscript{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\textsuperscript{Leu} further supported our original assumption that ScoLeuRS contains a regulatory peptide to adjust each domain to the right position to bind type I tRNA\textsuperscript{Leu}.

The coevolution of LeuRS and tRNA\textsuperscript{Leu}

While analyzing the genomes of Streptomyces strains, we noticed that some strains have LeuRSs highly similar to ScoLeuRS, but lack a type I tRNA\textsuperscript{Leu}. A model, SauLeuRS, showed its capability of charging ScoRNA\textsuperscript{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\textsuperscript{Leu}, such as ScoRNA\textsuperscript{Leu}(CAA) and hmtRNA\textsuperscript{Leu}(UUR), exist. The ancient tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{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 ScoRNA\textsuperscript{Leu}. The structural stability at the elbow region of the type I ScoRNA\textsuperscript{Leu}(CAA) is not so important to aminoacylation as it is for type II ScoRNA\textsuperscript{Leu}(UAA), which also illustrated the structural flexibility of type I tRNA\textsuperscript{Leu}, a feature of hmtRNA\textsuperscript{Leu}(UUR) showed in earlier studies (14,15).

Another subtle clue that these two types of ScoRNA\textsuperscript{Leu} probably function similarly came from previous in vivo studies related to secondary metabolism. ScoRNA\textsuperscript{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\textsuperscript{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\textsuperscript{Leu}(CAA) (50), given that there is a former report showing that this isoacceptor also reads UUA (51). Furthermore, previous data demonstrated that the modes of accumulation of ScoRNA\textsuperscript{Leu}(CAA) and ScoRNA\textsuperscript{Leu}(UAA) are similar (38). Most interestingly, ScoRNA\textsuperscript{Leu}(CAA) is the only isoacceptor that can replace ScoRNA\textsuperscript{Leu}(UAA) after altering its anticondon to UAA (38). Taken together, the results suggested that these two isoacceptors are related. However, ScoRNA\textsuperscript{Leu}(CAA), unlike ScoRNA\textsuperscript{Leu}(UAA), cannot be deleted from the S. coelicolor genome. Our effort to knockout the gene encoding ScoRNA\textsuperscript{Leu}(CAA) failed. This is probably related to the high codon usage of UUG (the codon deciphered by ScoRNA\textsuperscript{Leu}(CAA)). There are 6706 UUG codons in the
S. coelicolor genome, but only 145 UUA codons (the codon deciphered by Sco tRNA_{Leu}^{UA})). 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 (UA) were likely to have the same function until Sco tRNA_{Leu}^{(UA)} 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}^{(UA)}. 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 genome, but only 145 UUA codons (the codon deciphered by Sco tRNA_{Leu}^{UA}).

Supplementary Data are available at NAR Online.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**

1. Fujishima, K. and Kanai, A. (2014) tRNA gene diversity in the three domains of life. *Front. Genet.*, 5, 142.
2. Raina, M. and Ibbá, M. (2014) tRNAs as regulators of biological processes. *Front. Genet.*, 5, 171.
3. Brennan, T. and Sundaralingam, M. (1976) Structure of transfer RNA molecules containing the long variable loop. *Nucleic Acids Res.*, 3, 3235–3251.
4. Asahara, H., Himeno, H., Tamura, K., Hasegawa, T., Watanabe, K. and Shimizu, M. (1993) Recognition nucleotides of *Escherichia coli* tRNA^{Leu} and its elements facilitating discrimination from tRNA^{Ser} and tRNA^{Thr}. *J. Mol. Biol.*, 231, 219–229.
5. Tocchini-Valentini, G., Saks, M.E. and Abelson, J. (2000) tRNA leucine identity and recognition sets. *J. Mol. Biol.*, 298, 779–793.
6. Larkin, D.C., Williams, A.M., Martinis, S.A. and Fox, G.E. (2002) Identification of essential domains for *Escherichia coli* tRNA"Leu" aminoacylation and amino acid editing using minimalist RNA molecules. *Nucleic Acids Res.*, 30, 2103–2113.
7. Asahara, H., Nameki, N. and Hasegawa, T. (1998) In vitro selection of RNAs aminoacylated by *Escherichia coli* leucyl-tRNA synthetase. *J. Mol. Biol.*, 283, 605–618.
8. Du, X. and Wang, E.D. (2003) Tertiary structure base pairs between D- and Τα C-loops of *Escherichia coli* tRNA^{Leu} play important roles in both aminoacylation and editing. *Nucleic Acids Res.*, 31, 2865–2872.
9. Yao, P., Zhu, B., Jaeger, S., Eriani, G. and Wang, E.D. (2008) Recognition of tRNA^{Leu} by *Aquifex aeolicus* leucyl-tRNA synthetase during the aminoacylation and editing steps. *Nucleic Acids Res.*, 36, 2728–2738.
10. Metzger, A.U., Heckl, M., Willbold, D., Breitschopf, K., RajBhandary, U.L., Rösch, P. and Gross, H.J. (1997) Structural studies on tRNA acceptor stem microhelices: exchange of the discriminator base A73 for G in human tRNA^{Leu} switches the acceptor specificity from leucine to serine possibly by decreasing the stability of the variable domain. *Nucleic Acids Res.*, 25, 4551–4556.
11. Breitschopf, K. and Gross, H.J. (1996) The discriminator bases G73 in human tRNA^{Ser} and A73 in tRNA^{Leu} have significantly different roles in the recognition of aminoacyl-tRNA synthetases. *Nucleic Acids Res.*, 24, 405–410.
12. Breitschopf, K., Aehs, T., Busch, K. and Gross, H.J. (1995) Identity elements of human tRNA^{Leu}: structural requirements for converting human tRNA^{Ser} into a leucine acceptor in *vitro*. *Nucleic Acids Res.*, 23, 3633–3637.
13. Soma, A., Kumagai, R., Nishikawa, K. and Himeno, H. (1996) The anticodon loop is a major identity determinant of *Saccharomyces cerevisiae* tRNA^{Leu}. *J. Mol. Biol.*, 263, 707–714.
14. Sohn, B., Sissler, M., Park, H., King, M.P. and Florentz, C. (2004) Recognition of human mitochondrial tRNA^{Leu} (UUR) by its cognate leucyl-tRNA synthetase. *J. Mol. Biol.*, 339, 17–29.
15. Sohn, B., Frugier, M., Brulé, H., Olszak, K., Przykoroka, A. and Florentz, C. (2003) Towards understanding human mitochondrial leucine aminoacylation identity. *J. Mol. Biol.*, 328, 995–1010.
16. Eriani, G., Delarue, M., Poch, O., Gómez, J. and Moras, D. (1990) Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. *Nature*, 347, 203–206.
17. Ibba, M. and Soll, D. (2000) Aminoacyl-tRNA Synthetase. *Annu. Rev. Biochem.*, 69, 617–650.
18. Cusack, S., Yaremchuk, A. and Tukalo, M. (2000) The 2 Å crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-sildenafil analogue. *EMBO J.*, 19, 2351–2361.
19. Chen, J.F., Guo, N.N., Li, T., Wang, E.D. and Wang, Y.L. (2000) C1 domain in *Escherichia coli* leucyl-tRNA synthetase is crucial for its editing function. *Biochemistry*, 39, 6726–6731.
20. Fukunaga, R. and Yokoyama, S. (2005) Aminoacylation complex structures of leucyl-tRNA synthetase and tRNA^{Leu} reveal two modes of discriminator-base recognition. *Nat. Struct. Mol. Biol.*, 12, 915–922.
21. Vu, M.T. and Martinis, S.A. (2007) A unique insert of leucyl-tRNA synthetase is required for aminoacylation and not amino acid editing. *Biochemistry*, 46, 5170–5176.
22. Palencia, A., Crépin, T., Vu, M.T., Lincicum, T.L. Jr, Martinis, S.A. and Cusack, S. (2012) Structural dynamics of the aminoacylation and
proofreading functional cycle of bacterial leucyl-tRNA synthetase. Nat. Struct. Mol. Biol., 19, 677–684.

23. Yan, W., Tan, M., Eriani, G. and Wang, E.D. (2013) Leucine-specific domain modulates the aminoacylation and proofreading functional cycle of bacterial leucyl-tRNA synthetase. Nucleic Acids Res., 41, 4988–4998.

24. Takalo, M., Zarembuk, A., Fukunaga, R., Yokoyama, S. and Cusack, S. (2005) The crystal structure of leucyl-tRNA synthetase complexed with tRNALeu in the post-transfer-editing conformation. Nat. Struct. Mol. Biol., 12, 923–930.

25. Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D.J., Harris, D.E., Quail, M.A., Kieser, H., Harper, D. et al. (2002) Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature, 417, 141–147.

26. Lowe, T.M. and Eddy, S.R. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res., 25, 955–964.

27. Li, Y., Wang, E. and Wang, Y. (1999) A modified procedure for fast purification of T7 RNA polymerase. Protein Expr. Purif., 16, 355–358.

28. Kersey, P.J., Allen, J.E., Allot, A., Barba, M., Boddu, S., Bolt, B.J., Carvalho-Silva, D., Christensen, M., Davis, P., Grabmüller, C. et al. (2018) Ensemble Genomes 2018: an integrated omics infrastructure for non-vertebrate species. Nucleic Acids Res., 46, D802–D808.

29. Zhou, X.L., Du, D.H., Tan, M., Lei, H.Y., Ruan, L.L., Eriani, G. and Wang, E.D. (2014) A minimalist domain modulates the aminoacylation and proofreading functional cycle of bacterial leucyl-tRNA synthetase with tRNALeu isoacceptor specificity during proofreading. Nucleic Acids Res., 42, 5109–5124.

30. Yao, Y.N., Wang, L., Wu, X.F. and Wang, E.D. (2003) Human mitochondrial leucyl-tRNA synthetase with high activity produced from Escherichia coli. Protein Expr. Purif., 30, 112–116.

31. Fang, Z.P., Wang, M., Ruan, Z.R., Tan, M., Liu, R.J., Zhou, M.Z., Zhou, X.L. and Wang, E.D. (2014) Coexistence of bacterial leucyl-tRNA synthetases with archaeal tRNA binding domains that distinguish tRNALeu in the archaeal mode. Nucleic Acids Res., 42, 5109–5124.

32. Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem., 182, 319–326.

33. Zhou, X.L., Ruan, Z.R., Wang, M., Fang, Z.P., Wang, Y., Chen, Y., Liu, R.J., Eriani, G. and Wang, E.D. (2014) A minimalist mitochondrial threonyl-tRNA synthetase exhibits tRNA-isoacceptor specificity during proofreading. Nucleic Acids Res., 42, 13873–13886.

34. Hao, R., Yao, Y.N., Zheng, Y.G., Xu, M.G. and Wang, E.D. (2004) Reduction of mitochondrial tRNALeu(UUR) aminoacylation by some MELAS-associated mutations. FEBS Lett., 578, 135–139.

35. Hao, R., Zhao, M.W., Yao, Y.N., Chen, Y. and Wang, E.D. (2005) A T-stem slip in human mitochondrial tRNALeu(CUN) governs its charging capacity. Nucleic Acids Res., 33, 3606–3613.

36. Wang, M., Zhou, X.L., Liu, R.J., Fang, Z.P., Zhou, M., Eriani, G. and Wang, E.D. (2013) Multilevel functional and structural defects induced by two pathogenic mitochondrial tRNA mutations. Biochem. J., 453, 455–465.

37. Leskiw, B.K., Mah, R., Lawlor, E.J. and Chater, K.F. (1993) Accumulation of bldA-specified tRNA is temporally regulated in Streptomyces coelicolor A3(2). J. Bacteriol., 175, 1995–2005.

38. Pettersson, B.M. and Kirsebom, L.A. (2011) tRNA accumulation and suppression of the bldA phenotype during development in Streptomyces coelicolor. Mol. Microbiol., 79, 1602–1614.

39. Giege, R., Juhling, F., Putz, J., Stadler, P., Sauter, C. and Florentz, C. (2012) Structure of transfer RNAs: similarity and variability. WIREs RNA, 3, 37–61.

40. Lawlor, E.J., Baylis, H.A. and Chater, K.F. (1987) Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in Streptomyces coelicolor A3(2). Gene, 1, 1305–1310.

41. Leskiw, B.K. and Mah, R. (1995) The bldA-encoded tRNA is poorly expressed in the bldl mutant of Streptomyces coelicolor A3W. Microbiology, 141, 1921–1926.

42. Takano, E., Tao, M., Long, F., Bibb, M.J., Wang, L., Li, W., Buttnor, M.J., Bibb, M.J., Deng, Z.X. and Chater, K.F. (2003) A rare leucine codon in adpA is implicated in the morphological defect of bldA mutants of Streptomyces coelicolor. Mol. Microbiol., 50, 475–486.

43. Hackl, S. and Berchtold, A. (2015) The gene bldA, a regulator of morphological differentiation and antibiotic production in streptomycetes. Arch. Pharm. Res., 348, 455–462.

44. Bierman, M., Logan, R., O’Brien, K., Seno, E.T., Rao, R.N. and Schoner, B.E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene, 116, 43–49.

45. Blaby, I.K., Majumder, M., Chatterjee, K.J., Jana, S., Grosjean, H., de Crey-Lagard, V. and Gupta, R. (2011) Pseudouridine formation in archaean RNAs: The case of Haloferax volcanii. RNA, 17, 1367–1380.

46. Silvian, L.F., Wang, J. and Stitz, T.A. (1999) Insights into editing from an ile-tRNA synthetase structure with tRNAIle and mupirocin. Science, 285, 1074–1077.

47. Li, T., Guo, N.N., Xia, X., Wang, E.D. and Wang, Y.L. (1999) The peptide bond between E292-A293 of an ile-tRNA synthetase structure with tRNAIle and mupirocin. Biochemistry, 38, 13063–13069.

48. Tan, M., Zhu, B., Liu, R.J., Chen, X., Zhou, X.L. and Wang, E.D. (2013) Interdomain communication modulates the tRNA-dependent pre-transfer editing of leucyl-tRNA synthetase. Biochem. J., 449, 123–131.

49. Chater, K.F. and Chandra, G. (2008) The use of the rare UUA codon to define ‘expression space’ for genes involved in secondary metabolism, development and environmental adaptation in Streptomyces. J. Microbiol., 46, 1–11.

50. Trepanier, N.K., Jensen, S.E., Alexander, D.C. and Leskiw, B.K. (2002) The positive activator of cephalosporin C and clavulanic acid production in Streptomyces clavuligerus is mistranslated in a bldA mutant. Microbiology, 148, 643–656.

51. Takai, K., Horieap, N., Yamaizumib, Z., Nishimurab, S., Miyazawayta, T. and Yokoyama, S. (1994) Recognition of UUN codons by two leucine tRNA species from Escherichia coli. FEBS Lett., 344, 31–34.