Coexistence of bacterial leucyl-tRNA synthetases with archaeal tRNA binding domains that distinguish tRNA$^{\text{Leu}}$ in the archaeal mode

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ABSTRACT

Leucyl-tRNA (transfer RNA) synthetase (LeuRS) is a multi-domain enzyme, which is divided into bacterial and archaeal/eukaryotic types. In general, one specific LeuRS, the domains of which are of the same type, exists in a single cell compartment. However, some species, such as the haloalkaliphile Natrialba magadii, encode two cytoplasmic LeuRSs, NmLeuRS1 and NmLeuRS2, which are the first examples of naturally occurring chimeric enzymes with different domains of bacterial and archaeal types. Furthermore, N. magadii encodes typical archaeal tRNALeu. The tRNA recognition mode, aminoacylation and translational quality control activities of these two LeuRSs are interesting questions to be addressed. Herein, active NmLeuRS1 and NmLeuRS2 were successfully purified after gene expression in Escherichia coli. Under the optimized aminoacylation conditions, we discovered that they distinguished cognate NmtRNA$^{\text{Leu}}$ in the archaeal mode, whereas the N-terminal region was of the bacterial type. However, NmLeuRS1 exhibited much higher aminoacylation and editing activity than NmLeuRS2, suggesting that NmLeuRS1 is more likely to generate Leu-tRNALeu for protein biosynthesis. Moreover, using NmLeuRS1 as a model, we demonstrated misactivation of several non-cognate amino acids, and accuracy of protein synthesis was maintained mainly via post-transfer editing. This comprehensive study of the NmLeuRS/tRNALeu system provides a detailed understanding of the coevolution of aminoacyl-tRNA synthetases and tRNA.

INTRODUCTION

Protein biosynthesis is an essential step in all three domains of life (1). This complicated process requires a subset of proteins and nucleic acids. Aminoacyl-tRNA (transfer RNA) synthetases (aaRSs) are responsible for providing aminoacyl-tRNAs (aa-tRNAs) as the building blocks for protein biosynthesis by catalyzing the esterification of specific amino acids to their cognate tRNAs (2). This reaction (aminoacylation) is performed in two steps by most aaRSs; the amino acid is first activated with adenosine triphosphate (ATP) to form aminoacyl-adenylate (aa-AMP), which is then transferred to the 3'-terminus of the cognate tRNA to generate aa-tRNA for delivery to the ribosomes for protein biosynthesis. The aaRSs are divided into two classes (class I and class II) on the basis of conserved sequences and characteristic structural motifs (3,4). Leucyl-tRNA synthetase (LeuRS) belongs to class I aaRSs, which share a structurally similar Rossmann fold with two characteristic ‘HIGH’ and ‘KMSKS’ motifs in the catalytic domain.

Aminoacylation of tRNA requires precise regulation of the velocity of the aa-tRNA production for the ribosome and tight control of aberrant aa-tRNA synthesis (5). Various identity determinants and/or antideterminants are harbored by different tRNAs to facilitate the selection of cognate tRNA from a large set of tRNA species by specific aaRSs. However, the specificity of cognate amino acid activation is greatly challenged by the presence of various non-cognate amino acids and amino acid metabolites, which differ only in the side chain. Taking the mistranslation frequency (1/3000) in protein...
biosynthesis as the threshold (6), a proofreading (editing) mechanism is necessary for half of aaRSs to maintain accuracy during aa-tRNA synthesis. The editing activity can be divided further into the pre- and post-transfer editing. Pre-transfer editing refers to the hydrolysis of the misactivated aa-AMP either in the absence of cognate tRNA (tRNA-independent pre-transfer editing) or in the presence of cognate tRNA (tRNA-dependent pre-transfer editing), whereas post-transfer editing is performed by the editing domain [such as the connective peptide 1 (CP1) domain of class Ia synthetases (7), the N2 domain of class II threonyl-tRNA synthetase (8), the freestanding editing domain homologs (9)] after a misactivated amino acid has been loaded onto the cognate tRNA. The editing activity of an aaRS is of crucial importance in ensuring overall translational quality control. The impairment or loss of editing activity can lead to mistranslation and further ambiguity of the proteome with serious negative influences on the cellular function of most organisms (10–12). This is directly illustrated by the development of neurodegeneration in a mouse model with partial loss of the editing activity of alanyl-tRNA synthetase (13).

LeuRSs catalyze aminoacylation and editing reactions to synthesize Leu-tRNA^{Leu} and to prevent production of mischarged tRNA^{Leu}. Based on the primary sequence, relative domain position and orientation, LeuRS can be divided into bacterial (existing in bacteria and organelles) and archaeal/eukaryotic types (existing in eukaryotes and most archaea) (14,15). Both types of LeuRSs contain a Rossmann-fold domain (for amino acid activation and tRNA charging), a CP1 domain (for editing), an α-helix bundle domain (for tRNA binding) and a C-terminal domain (CTD, for tRNA binding) (16). Moreover, LeuRSs also contain some specific insertion domains, such as the leucine-specific domain to modulate their aminoacylation and editing activities (17,18). To date, all the domains identified in a single LeuRS polypeptide are of the same type, indicating that no gene (fragment) rearrangement or fusion between two LeuRS types has occurred.

LeuRSs use different tRNA^{Leu} discrimination modes besides recognition of the common specific discriminator base (A73) of tRNA^{Leu}. Biochemical (19,20) and 3D structural (21) data have revealed that the T-loop, D-loop and bases (47f–47i) on one side of the long variable stem in tRNA^{Leu} from bacteria (e.g. Escherichia coli) are recognized by the CTD in bacterial E. coli or Mycobacterium tuberculosis LeuRS. In the hyperthermophilic bacteria Aquifex aeolicus, which contains a heterodimeric LeuRS, the core structure of tRNA formed by the tertiary interactions (U8-A14, G18-U55 and G19-C56) and the orientation of the long variable stem are critical elements for tRNA^{Leu} aminoacylation, and the anticodon stem acts as an additional determinant for editing by LeuRS (22). In Homo sapiens, three base pairs (C3:G70, A4:U69 and G5:C68) in the acceptor stem, C20a in the D-loop, the long length of variable stem and the number of the unpaired residues in long variable loop are the identity elements of tRNA^{Leu} (23). However, Saccharomyces cerevisiae uses the anticodon loop of tRNA^{Leu} (A35 and G37) as the major identity determinant (24). The recognition mode of tRNA^{Leu} in archaea is highly conserved and differs from those of the previously mentioned species. The most obvious feature is the critical dependence on the two absolutely conserved bases (A47c, G47d) at the tip of the long variable loop in tRNA^{Leu}, as reported in halophilic Haloferax volcanii and thermophilic Pyrococcus horikoshii (25,26). The 3D structure of the P. horikoshii LeuRS (PhLeuRS) showed that its CTD, which contains five α-helices, antiparallel β-sheets and many flexible loops, is responsible for tRNA recognition, especially of Asp845, Ile849 and the last few conserved residues (Pro962, Ile964, Ile966 and Glu967) (26). Deletion of more than one residue in the CTD led to the total loss of aminoacylation activity of PhLeuRS. In contrast, deletion of the entire CTD had no effect on its editing activity (14).

The distribution of LeuRSs is diverse in the three domains of life. In bacteria (e.g. E. coli), only a bacterial LeuRS is encoded. Eukaryotes (e.g. H. sapiens) generally contain two LeuRSs: an archaeal/eukaryotic cytoplasmic form and a bacterial form in mitochondria and/or chloroplasts. A majority of archaeal (e.g. P. horikoshii) encode a LeuRS, which is evolutionarily close to the cytoplasmic form of the enzyme in eukaryotes. However, in a limited number of cases, such as the halobacteria Natriibla magadii, which thrives in alkaline and hypersaline conditions (pH 9.5, 3.5 M NaCl) (2 g/l KCl, 0.1 g/l MgSO_{4}.7H_{2}O and microscale of FeSO_{4} and MnCl_{2} are also needed in the culture medium) (27,28), the LeuRS/ tRNA^{Leu} system exhibits several striking features. First, the N. magadii genome encodes two LeuRS genes [designated as NmLeuRS1 (accession No. YP_003479843.1, 904-aa) and NmLeuRS2 (accession No. YP_003481407.1, 971-aa)] displaying 46% identity. Second, these two LeuRSs exhibit both bacterial and archaean characteristics. Specifically, the N-terminal region (including the Rossmann fold, the CP1 domain, the CP2 domain and the α-helix bundle domain) shares high homology with bacterial LeuRSs (Supplementary Figure S1A); however, the CTD is highly homologous to its archaeal LeuRS counterpart (Supplementary Figure S1B). Moreover, N. magadii expresses typical archaeal tRNA^{Leu}s in which the A47c and G47d bases in the long variable loop are absolutely conserved, whereas no bacterial tRNA^{Leu}s are expressed (Supplementary Figure S2). This raises the interesting questions of the mechanisms underlying the aminoacylation and editing activities, the active sites of which reside in the bacterial and archaean portion of two NmLeuRSs as well as the recognition mode for archaeal tRNA^{Leu}.

In the present study, genes encoding NmLeuRS1 and NmLeuRS2 were cloned and overexpressed in E. coli. Following successful purification, the aminoacylation, amino acid activation, tRNA recognition mode and editing activity of these NmLeuRSs were characterized. Our results provide an improved understanding of the activity and evolutionary pathway of tRNA synthetases from halobacteria.
MATERIALS AND METHODS

Materials

L-leucine (Leu), L-norvaline (Nva), α-amino butyric acid (ABA), L-isoleucine (Ile), L-methionine (Met), tetradsodium pyrophosphate, Tris–HCl, MgCl₂, NaCl, KCl and activated charcoal were purchased from Sigma (St Louis, MO, USA), [³H]Leu, [³²P]tetradsodium pyrophosphate and [γ-³²P]ATP were obtained from PerkinElmer (Waltham, MA, USA). Nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) mixtures were purchased from Sangon Biotech (China). The Pfu DNA polymerase, the DNA fragment rapid purification kit and plasmid extraction kit were purchased from Tiangen (Waltham, MA, USA). Nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) mixtures were purchased from Sangon Biotech (China). The Pfu DNA polymerase, the DNA fragment rapid purification kit and plasmid extraction kit were purchased from Tiangen Biotech (China). The KOD-plus mutagenesis kit was obtained from TOYOBO (Japan). T4 ligase, inorganic pyrophosphatase, protein ladder (#26614) and restriction endonucleases were obtained from Thermo Scientific (Waltham, MA, USA). The Ni²⁺-NTA (Ni²⁺-nitrilotriacetate) Superflow was purchased from Qiagen (Germany). Polyethyleneimine cellulose plates, Amicon Ultra-15 filter and nitrocellulose membranes (0.22 μm) were purchased from Merck (Germany). Oligonucleotide primers were synthesized by Life Technologies (Carlsbad, CA, USA). Competent E. coli BL21 (DE3) and Top10 cells were prepared in our laboratory. T7 RNA polymerase (29) and E. coli CCA-adding enzyme (30) were purified from an overproduction strain in our laboratory.

Sequence alignment of LeuRSs

For phylogenetic analysis of LeuRSs covering three domains, 63 protein sequences of various LeuRSs from representative species were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). Specific sequence alignments were carried out using the Clustal X2 program (31) and subsequent phylogenetic trees were constructed with the MEGA 5.2 program (32) using the minimal evolution algorithm. Bootstrap analysis was performed with 1000 replicates.

Gene cloning and protein purification

NmLeuRS1 and NmLeuRS2 genes were amplified from N. magadii ATCC 43099 genomic DNA and cloned into pET28a (pre-cleaved with NdeI/BamHI) to produce pET28a-NmLeuRS1 and pET28a-NmLeuRS2. The primers for gene amplification were as follows: NmLeuRS1 forward (5’ ccatatggaagttatacag3’), NmLeuRS1 reverse (5’ ccgatcctgctgctatg3’), NmLeuRS2 forward (5’ ccatatggaacacatgactatg3’) and NmLeuRS2 reverse (5’ ccgatcctgctgctatgaat3’).

Haloarcula hispanica LeuRS1 and LeuRS2 genes were amplified from H. hispanica ATCC 33960 genomic DNA and cloned into pET28a (pre-cleaved with NdeI/BamHI or NheI/BamHI) to produce pET28a-HhLeuRS1 and pET28a-HhLeuRS2. The primers for gene amplification were as follows: HhLeuRS1 forward (5’ ccgatcctgctgcaggtatg3’), HhLeuRS1 reverse (5’ ccgatcctgctgcaggtatg3’), HhLeuRS2 forward (5’ ccgatcctgctgcaggtatg3’), HhLeuRS2 reverse (5’ ccgatcctgctgcaggtatg3’).

Escherichia coli BL21 (DE3) cells were transformed with these constructs. A single transformant was selected and cultured in 11 of 2x Yeast extract and Tryptone (YT) medium (1% yeast extract, 1.6% tryptone and 0.5% NaCl) at 37°C. Cells were cultured to mid-log phase (OD₆₀₀, 0.6) and protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration, 200 μM). Cells were cultured at 22°C for a further 7 h before centrifugation at 3000g for 10 min at 4°C followed by washing with water. Purification was performed by Ni²⁺-NTA Superflow (Qiagen) chromatography according to the manufacturer’s protocol. The wet cells (~3.5 g) were lysed by ultrasonication on ice in 15 ml of buffer A [20 mM, pH 9.0, Tris–HCl, 1.2 M NaCl, 2 mM imidazole, 10% glycerol and 10 mM phenylmethylsulfonyl fluoride]. The lysates were centrifuged at 40000g for 60 min to remove the debris and insoluble fractions. The supernatant was applied to 2 ml of Ni²⁺-NTA resin mixed gently for 30 min at 4°C and then washed with 20 ml of buffer A and 20 ml of buffer B (20 mM, pH 9.0, Tris–HCl, 1.2 M NaCl, 25 mM imidazole and 10% glycerol) to remove nonspecific binding proteins. Binding proteins were eluted in 10 ml of buffer C (20 mM, pH 9.0, Tris–HCl, 1.2 M NaCl, 250 mM imidazole and 10% glycerol), and the eluted fractions were concentrated and dialyzed with buffer D (20 mM, pH 8.5, Tris–HCl, 3 M KCl, 0.1 M MgAc₂ and 5 mM β-mercaptoethanol) (33) using Amicon Ultra-15 filters (Millipore; cutoff 30 kDa). The resulting protein solution was concentrated to ~300 μl, mixed with glycerol to a final concentration of 40% (v/v) and stored at −20°C. Protein concentration was determined by active site titration (34).

Preparation of tRNA₅⁰⁶ transcript and [³²P] labeling at 3’ terminus of tRNA

The DNA sequence of the T7 promoter and the NmRNA₅⁰⁶(GAG) gene was obtained by ligating three chemically synthetized DNA fragments for each strand, which were then ligated into pTrc99b plasmid (pre-cleaved with EcoRI/PstI) to construct pTrc99b-T7-Nm tRNA₅⁰⁶(GAG). The forward primer (5’ ctaatacagctaatagctg3’) and reverse primer (5’ ttc gag ggg gac gtg cgt 3’) were used to amplify the template for in vitro transcription by Pfu polymerase (T7 promoter underlined). The PCR product was extracted by phenol/chloroform and precipitated in three volumes of ethanol and 0.3 M Sodium acetate (NaAc), pH 5.2. The in vitro transcription of NmRNA₅⁰⁶(GAG) was carried out in a reaction mixture containing 40 mM Tris–HCl, pH 8.0, 22 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol (DTT), 0.5% Triton X-100, 60 mg/ml tRNA template, 5 mM NTP (each), 1 U/μl ribonuclease inhibitor, 20 mM GMP, 500 U/μl T7 RNA polymerase and 1 U/ml pyrophosphatase for 3 h. Subsequently, 5 U/ml DNase I was added to digest the tDNA template for 1 h. The transcript was separated by 8 M urea −15% PAGE gel electrophoresis. The tRNA was excised from the gel, eluted with 0.5 M NaAc, pH 5.2, at room temperature three times, precipitated with three volumes of ethanol at −20°C and dissolved in 5 mM MgCl₂ after centrifugation (15000g, 30 min, 4°C) and drying. The tRNA was
annealed at 85°C for 10 min and cooled naturally in the bath of 11 l water to room temperature for correct folding. Nm tRNA\textsuperscript{Leu}(CAG), Ph\textsubscript{r} tRNA\textsuperscript{Leu}(GAG) and the corresponding mutants were prepared in the same way.

Nm tRNA\textsuperscript{Leu}(GAG) was labeled with [\(\alpha\)-\(\text{\textsuperscript{32}P}\)]ATP as reported previously (35). In detail, 750 pmol tRNA was added in a 50 μl of reaction mixture containing 60 mM Tris–HCl, pH 8.0, 12.5 mM MgCl\(_2\), 1 mM DTT, 50 μM Na\(_4\)PPi, 15 μM ATP, 0.666 μM [\(\alpha\)-\(\text{\textsuperscript{32}P}\)]ATP and 10 μM E. coli CCA-adding enzyme at 37°C for 5 min. Subsequently, 0.1 U pyrophosphatase was added for a further 5 min. The solution was extracted twice using phenol/chloroform and then precipitated in three volumes of ethanol. The ratio of [\(\text{\textsuperscript{32}P}\)]-labeled tRNA was determined by liquid scintillation counting of the sample washed with and without 5% trichloroacetic acid.

**Aminoacylation, misacylation and deacylation**

The aminoacylation assays of NmLeuRSs were carried out in a reaction mixture containing 20 mM Tris–HCl, pH 9.0, 3.5 M KCl, 30 mM MgCl\(_2\), 1 mM DTT, 4 mM ATP, 20 μM \([^{3} \text{H}]\)Leu (11 Ci/mM), various tRNAs and NmLeuRS1 (50–500 nM) or NmLeuRS2 (2 μM) unless otherwise stated at 40°C. Misacylation was performed under similar conditions with 8.80 μM Nm tRNA\textsuperscript{Leu} containing 3.67 nM [\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu}, 50 mM Nva or 170 mM ABA or 100 mM Ile or 100 mM Met, and 2 μM NmLeuRs or their mutants. The misacylated Nvm-[\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu} was obtained by using PhLeuRS1-D332A. Deacylation of Nva-[\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu} was carried out under similar conditions except pH 7.5 with 500 nM NmLeuRs or mutants were used.

To determine the aminoacylation level of [\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu}, the misacylation and deacylation samples were ethanol precipitated, digested by nuclelease S1 and then thin-layer chromatography (TLC) was performed to separate Nva-[\(\text{\textsuperscript{32}P}\)] adenosine monophosphate (AMP) (from Nva-[\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu}), [\(\text{\textsuperscript{32}P}\)]AMP (from [\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu} and [\(\text{\textsuperscript{32}P}\)]ATP in 0.1 M NH\(_4\)Ac and 5% acetic acid. The plates were visualized by autoradiography, and the data were analyzed using MultiGauge Version 3.0 software (FUJIFILM).

**Measurement of equilibrium dissociation constants for tRNA by filter binding assays**

Nitrocellulose filter binding assays were performed to detect the formation of NmLeuRSs/[\(\text{\textsuperscript{32}P}\)]-Nm tRNA\textsuperscript{Leu}(GAG) complexes at 4°C in a 50 μl of reaction mixture containing 20 mM Tris–HCl, pH 9.0, 3 M KCl, 30 mM MgCl\(_2\), 1 mM DTT, 75 nM Nm tRNA\textsuperscript{Leu}(GAG) (~13 000 counts per minute (CPM)/pmol, 1 nM [\(\text{\textsuperscript{32}P}\)]Nm tRNA\textsuperscript{Leu}(GAG)) and NmLeuRs (1–15 μM) for 30 min (36). The mixtures were then filtered through the nitrocellulose membrane (0.22 μm) [preequilibrated in washing buffer (50 mM potassium phosphate, pH 5.5, 50 mM MgCl\(_2\)) for at least 10 min] and washed twice with 0.3 ml of washing buffer. The membranes then dried before quantitation by radioactivity. Data were analyzed using GraphPad Prism 5 software.

**ATP-PPi exchange and AMP formation**

ATP-PPi exchange assays were performed at 40°C in a reaction mixture containing 20 mM Tris–HCl, pH 9.0, 3.5 M KCl, 30 mM MgCl\(_2\), 1 mM DTT, 4 mM ATP, 2 mM [\(\text{\textsuperscript{32}P}\)]tetrasodium pyrophosphate, Leu (0.5 μM–10 mM) or Nva (0.05–50 mM) or ABA (0.5–170 mM) and 1 μM NmLeuRS1 or 3 μM NmLeuRS2. Samples at specific time-points were added to 200 μl of quenching solution containing 2% activated charcoal, 3.5% HClO\(_4\) and 50 mM tetrasodium pyrophosphate.

AMP formation assays were carried out at 40°C in a reaction mixture containing 20 mM Tris–HCl, pH 9.0, 3.4 M KCl, 30 mM MgCl\(_2\), 1 mM DTT, 15 mM Nva, 4 mM [\(\text{\textsuperscript{32}P}\)]ATP, 2 μM NmLeuRS1 or NmLeuRS1-D354A mutant, in the presence or absence of 25 μM Nm tRNA\textsuperscript{Leu}(GAG). Samples were quenched in 200 mM NaAc (pH 5.2) and then spotted onto polyethyleneimine cellulose plates. TLC was performed as described previously.

**RESULTS**

*Natrialba magadii* encodes two chimeric LeuRSs and archaeal tRNA\textsuperscript{Leu}s

The expanded genome analysis of various species from all three domains of life significantly facilitated gene function investigations. Analysis of LeuRSs from bacteria, archaea and eukaryotes revealed that some halobacteria, such as *N. magadii*, *H. hispanica*, *Halorhabdus utahensis*, *Haloarcula marismortui*, *Halomicrobium mukohataei*, *Haloterrigena turkenica* and *Haladaptatus paucihalophilus*, and some sulfobolus (e.g. *Sulfobolus islandicus*) harbor two copies of *leuS*, encoding LeuRS. Sequence alignment showed that the N-terminal region of both NmLeuRSs (Met1-Pro756 of NmLeuRS1 and Met1-Pro788 of NmLeuRS2) displayed a high level of homology with the corresponding region of bacterial LeuRSs (e.g. Met1-Pro788 of *E. coli* LeuRS) (Supplementary Figure S1A), whereas the CTDs of both NmLeuRSs (Thr757-Asp904 of NmLeuRS1 and Ala789-Gln971 of NmLeuRS2) were highly homologous to their archaeal LeuRS counterparts (e.g. Glu822-Glu967 of PhLeuRS) (Supplementary Figure S1B). This observation was further supported by phylogenetic analysis of either the N-terminal regions or CTDs of LeuRSs from the three domains of life (Figure 1). In particular, the homology or identity of two LeuRSs in a single species was not high. For instance, the protein products NmLeuRS1 and NmLeuRS2 displayed 46.6% identity and 56.9% homology. Consistently, NmLeuRS1 and NmLeuRS2 were in separate branches of the phylogenetic tree, indicating the possibility of horizontal gene transfer, or gene duplication and subsequent divergence.

On the other hand, the *N. magadii* genome has five tRNA\textsuperscript{Leu} genes, including tRNA\textsuperscript{Leu}(GAG), tRNA\textsuperscript{Leu}(CAG), tRNA\textsuperscript{Leu}(UAG), tRNA\textsuperscript{Leu}(CAA) and tRNA\textsuperscript{Leu}(UAA) (37). Sequence analysis of tRNA\textsuperscript{Leu}s revealed that these tRNAs were all archaeal type, with absolutely conserved A47c and G47d bases in the
Figure 1. Phylogenetic analyses of LeuRSs from different species. (A) Phylogenetic analyses of the N-terminal regions (including Rossmann-fold, CP1, CP2 and \( \alpha \)-helix bundle domains) of various LeuRSs. (B) Phylogenetic analyses of the C-terminal domains (CTDs) of various LeuRSs. (C) Domain scheme of LeuRSs from bacteria/mitochondrion and archaea/eukaryote, respectively. N-terminal regions included Rossmann-fold, CP1, CP2 and \( \alpha \)-helix bundle domains.
long variable loop (Supplementary Figure S2). Above all, in halophiles such as *N. magadii*, two LeuRSs and tRNA^{Leu} exhibit chimeric and archaeal features, respectively (Figure 2). To our knowledge, this is the first description of a naturally occurring chimeric LeuRS.

**NmLeuRS activity requires high KCl and high pH**

To characterize the function of both *NmLeuRSs*, the genes encoding two *NmLeuRSs* were overexpressed in *E. coli*. Both *NmLeuRSs* were purified in Tris–HCl buffer, pH 9.0, by Ni²⁺-NTA affinity chromatography (see ‘Materials and Methods’ section). In accordance with previous reports (38,39), *NmLeuRS1* (102.2 kDa) and *NmLeuRS2* (107.6 kDa) migrated slowly in 8% SDS-PAGE gel (Figure 3A) because of the presence of more negative charges with more acidic (Asp, Glu) and less basic (Lys, Arg) amino acid residues in halophilic proteins compared with their mesophilic homologs.

To investigate the aminoacylation reaction, *NmLeuRS1* was used initially to determine an appropriate reaction system, followed by investigation of *NmLeuRS2*. As a halophilic protein, *NmLeuRS1* showed a linear increase in aminoacylation activity with increasing KCl concentration above the threshold of 2.5 M. It displayed the highest activity in the presence of 3.75 M KCl (higher KCl concentrations were not evaluated) (Figure 3B). To facilitate preparation of the reaction system, 3.5 M KCl was chosen for use in further assays. Surprisingly, no activity of *NmLeuRS1* was detected in NaCl solutions, even at concentrations as high as 4 M (Figure 3B). A similar phenomenon was reported for *H. volcanii* DNA ligase, in that K⁺ mediated stabilization and modulation of the enzyme activity, whereas Na⁺ did not (40). In contrast, the activity of mesophilic homologs such as *Ec* LeuRS and *Ph* LeuRS was obviously different from that of *NmLeuRS1*. Although the relative activity of *Ec* LeuRS increased from 20 to 100% after the addition of 0.1 M KCl, that lost in the presence of >0.8 M KCl (Figure 3B). The activity of *Ph* LeuRS was totally inversely correlated with the concentration of KCl, in >0.8 M KCl solution *Ph* LeuRS had no activity (Figure 3B). Similar to

**Figure 2.** Schema showing the different interaction modes of LeuRS/tRNA^{Leu} in bacteria, halobacteria (archaea) and eukaryotic types, respectively. CP, connective peptide domains; CTD, C-terminal domain.

**Figure 3.** Purification of *NmLeuRSs* and optimization of aminoacylation conditions. (A) A total of 8% SDS-PAGE analysis of the purified *NmLeuRSs* from *E. coli*. Lanes: 1, 2 and 3 are molecular markers (Thermo Scientific, #26614), *NmLeuRS1* and *NmLeuRS2*, respectively. (B) Relative aminoacylation activity of *NmLeuRS1*, *EcLeuRS* and *PhLeuRS* under different KCl or NaCl concentrations. The activities of *EcLeuRS* in 0.1 M KCl, *PhLeuRS* in 0 M KCl and *NmLeuRS1* in 3.75 M KCl were defined as 100%. (C) Relative aminoacylation activities of *NmLeuRS1* under different pH conditions in Tris–HCl (black circle), Bis-Tris-propane–HCl (black up-pointing triangle) and CHES–KOH (black square). The activity of *NmLeuRS1* in Tris–HCl, pH 9.0, was defined as 100%.


NmLeuRS1, the activity of NmLeuRS2 increased significantly from 2.0–3.1 M KCl; no obvious further stimulation of activity occurred at higher KCl concentrations (Supplementary Figure S3A).

The pH optimization for the aminoacylation activities of NmLeuRS1 was then studied. NmLeuRS1 exhibited the highest activity at pH 9.0 in Tris–HCl or Bis-Tris-propane–HCl buffer or at pH 9.5 in CHES-KOH buffer (Figure 3C). The activity decreased obviously at pH 8.0 or below. Similar results were obtained with NmLeuRS2 (Supplementary Figure S3B). High pH is optimal for the activity of many extracellular proteins of alkaliophiles (41,42). However, an almost neutral cytoplasmic pH is maintained by Na+/H+ antiporters (43,44) or cell wall macromolecules, which serve as a specific barrier to the flux of relevant ions (45). Our results suggest that the cytoplasmic proteins in N. magadii perform their physiological function in an alkaline solution through another mechanism. The genes necessary for survival in alkaline conditions have not been identified in the genome of N. magadii (46). The in vitro characteristics of recombinant NmLeuRSs may differ in the in vivo situation.

Distinct aminoacylation activities of the NmLeuRSs

NmLeuRSs have similar domain architecture, display 46% identity and coexist in a single cell compartment. It is possible that either one or both NmLeuRSs play an essential aminoacylation role in protein biosynthesis. The activities of the two NmLeuRSs were initially compared in ATP-PPi exchange assays. NmLeuRS1 displayed a much higher amino acid activation activity than NmLeuRS2, the $K_m$ value of NmLeuRS2 was nearly 105-fold greater than that of NmLeuRS1 ($K_m$: 891 ± 2 μM versus 8.43 ± 1.30 μM), and the activation rate of NmLeuRS1 was 58-fold greater than that of NmLeuRS2 [$k_{cat} / K_m$ (5.63 ± 0.12) × $10^{-1}$ s$^{-1}$ versus (9.61 ± 0.47) × $10^{-3}$ s$^{-1}$] (Table 1).

The aminoacylation assays showed that both NmLeuRSs were able to charge NmtRNA$^\text{Leu}$ (GAG), NmRNA$^\text{Leu}$ (CAA) and PhtRNA$^\text{Leu}$ (GAG) (the Leu accepting activity of the aforementioned tRNA$^\text{Leu}$ transcripts was 1350, 1468 and 1225 pmol/A$\text{260}$), although the catalytic efficiencies ($k_{cat}/K_m$) of NmLeuRS2 for different tRNA$^\text{Leu}$s were only 1/130 to 1/40 of those of NmLeuRS1 (Table 2). The data showed a distinct difference in the catalytic velocity ($k_{cat}$) but not the $K_m$ value for tRNA$^\text{Leu}$ during aminoacylation, which may be due to the much lower amino acid activation activity of NmLeuRS2. To further define the affinity between NmLeuRSs and NmtRNA$^\text{Leu}$ (GAG), filter binding assays were performed as reported previously (36). The equilibrium dissociation constants ($k_d$) of NmLeuRS1 and NmLeuRS2 for NmtRNA$^\text{Leu}$ (GAG) were 6.36 ± 1.17 μM and 3.36 ± 0.89 μM, respectively (Table 3). These results showed that NmLeuRS2 had obviously decreased aminoacylation activity compared with NmLeuRS1, whereas the affinity for cognate NmtRNA$^\text{Leu}$ was retained.

NmLeuRS1 showed the highest catalytic activity for NmtRNA$^\text{Leu}$ (GAG), which was used predominantly (4.93%) among all NmRNA$^\text{Leu}$ isoacceptors. The catalytic activity for NmRNA$^\text{Leu}$ (CAA) was only half of that for NmtRNA$^\text{Leu}$ (GAG). In addition, the catalytic activity for the heterologous PhtRNA$^\text{Leu}$ (GAG) was slightly lower than that for the homologous NmtRNA$^\text{Leu}$ (Table 2), implying that NmLeuRSs, despite the presence of the bacterial aminoacylation and editing domains, effectively distinguished archaeal tRNA$^\text{Leu}$.

To further explore the aminoacylation activities of other coexisted LeuRSs present in halobacteria, we then purified the protein of two leuS genes of H. hispanica after being overexpressed in E. coli [designated as HhLeuRS1 (accession NO. YP_004794828.1, 895-aa) and HhLeuRS2 (accession NO. YP_004797622.1, 893-aa)] (Supplementary Figure S4A) (47). Similar results with NmLeuRSs were obtained, that HhLeuRS2 had only little amino acid activation and aminoacylation activities, whereas HhLeuRS1 retained the normal activities (Supplementary Figure S4B and C). These results suggested that the coexistence of an active LeuRS and an inactive one might be a widespread phenomenon in halobacteria.

### Table 1. Leucine activation kinetics constant of NmLeuRSs at 40°C

<table>
<thead>
<tr>
<th>NmLeuRS1</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat} / K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.43 ± 1.30</td>
<td>(5.63 ± 0.12) × 10$^{-1}$</td>
<td>66.8</td>
<td>1$^b$</td>
</tr>
<tr>
<td>NmLeuRS2</td>
<td>891 ± 2</td>
<td>(9.61 ± 0.47) × 10$^{-3}$</td>
<td>0.0108</td>
<td>1/6185</td>
</tr>
</tbody>
</table>

$^a$The results are the average of three independent repeats with standard deviations indicated.

$^b$The activity of NmLeuRS1 for Leu is defined as 1.
by *NlLeuRS*1 was similar to that of *PhLeuRS*, both depending on the conserved Ile residue (Ile902 and Ile966 for *NlLeuRS*1 and *PhLeuRS*, respectively) (Figure 4A) (26). Moreover, complete deletion of the CTD (*NlLeuRS*1-ΔCTD) led to the total loss of aminoacylation activity (Figure 4B), indicating the importance of the archaeal CTD in the *NlLeuRS*1 aminoacylation reaction.

We then explored the recognition mode based on analysis of tRNA*Leu*. Sequence alignment of tRNA*Leu* showed that A47c and G47d at the tip of the long variable loop were absolutely conserved in archaea (Supplementary Figure S2). These residues have been identified as the sequence-specific determinants in archaeal *H. volcanii* tRNA*Leu* (*HvRNA*Leu) and *Ph tRNA*Leu* (25,26). Mutations of A47c and G47d to the other three nucleotides in *NlRNA*Leu(GAG) were performed to obtain *NlRNA*Leu(CAA), A47cU, A47cG, A47cU, -G47dA, -G47dC and -G47dU, respectively. None of the mutants were efficiently aminoacylated by *NlLeuRS*1 except *NlRNA*Leu(GAG)-A47cC, which retained 7% of the catalytic activity of the wild-type tRNA*Leu* (Figure 4C, Supplementary Table S1). Therefore, like archaeal *Ph tRNA*Leu* and *HvRNA*Leu, the A47c and G47d bases located in the tip of the long variable loop were of great importance in the recognition process.

These results showed that *NlLeuRS*1 distinguished *NlRNA*Leu via a similar mechanism to that used by archaeal *PhLeuRS* with the last few residues of enzyme (especially conserved Ile902) and the conserved A47c and G47d residues of tRNA*Leu* being crucial elements for recognition. As *NlLeuRS*1 also possesses bacterial aminoacylation and editing domains, the question of whether these two domains conferred the ability to recognize bacterial tRNA*Leu* on *NlLeuRS*1 remained unresolved. To address this issue, the aminoacylation activity of *NlLeuRS*1 for bacterial *EctRNA*Leu (the Leu accepting activity was 1600 pmol/A260) and archaeal *Ph tRNA*Leu was determined. *NlLeuRS*1 was able to charge archaeal *Ph tRNA*Leu but not bacterial *EctRNA*Leu (Table 2, Figure 4D), implying that the bacterial aminoacylation and editing domains contributed little to tRNA species specificity. On the other hand, *EcLeuRS* was only able to charge its cognate *EctRNA*Leu but not *NlRNA*Leu and *Ph tRNA*Leu (Figure 4E). Cross-activation of these tRNA*Leu* by *NlLeuRS*2 was also evaluated, and similar results were obtained (Figure 4F), suggesting that both *NlLeuRS*s distinguished tRNA*Leu* in the archaeal mode. In addition, we replaced the CTD (Thr759-Asp904) of *NlLeuRS*1 with its *EcLeuRS* counterpart (Asp791-Gly860) to generate *NlLeuRS*1-ΔCTD to mimic a complete bacterial *LeuRS*. However, *NlLeuRS*1-ΔCTD failed to aminoacylate *EctRNA*Leu, *Ph tRNA*Leu and *NlRNA*Leu (Supplementary Figure S5), indicating that the archaeal CTD plays a key role in archaeal tRNA*Leu* recognition and that fusion of a bacterial CTD with the basis of bacterial region of *NlLeuRS*1 failed to reestablish bacterial tRNA*Leu* aminoacylation capacity.

Taken together, these lines of evidence suggested that *NlLeuRS*1 distinguished *NlRNA*Leu in the archaean mode and indicated that the archaean CTD, but not the bacterial aminoacylation and editing domains of *NlLeuRS*1, contributed its archaeal tRNA recognition capacity.

### The N-terminal region of *NlLeuRS*1 is of bacterial type

To explore whether the N-terminal region of *NlLeuRS*s was of bacterial type, we constructed several mutations in amino acid residues of *NlLeuRS*1, only conserved in bacterial but not archaean/eukaryotic *LeuRS*s. Asp95 and Gln207 of *NlLeuRS*1 were equivalent to Asp80 and Gln190 in *EcLeuRS*, which, respectively, interacted with A76 and C74 of *EctRNA*Leu during aminoacylation reaction, as shown in its 3D structure (PDB: 4AQ7) (Figure 5A) (21), and conserved in only bacterial but not archaean/eukaryotic *LeuRS*s (Figure 5B), suggesting they might play a key role in the aminoacylation activity of bacterial *LeuRS*. We obtained *NlLeuRS*1-D95A and -Q207E mutants, which would disturb the interaction of *LeuRS* and tRNA*Leu*.

The results showed that the mutant *NlLeuRS*1-D95A abolished all the aminoacylation activity and *NlLeuRS*1-Q207E only retained half of the

### Table 2. Aminoacylation kinetics of *NlLeuRS*s at 40°C

<table>
<thead>
<tr>
<th>tRNA<em>Leu</em></th>
<th>LeuRS</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (mM⁻¹ s⁻¹)</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>N<em>ntRNA</em>Leu(GAG)*</td>
<td><em>NlLeuRS</em>1</td>
<td>7.73 ± 0.22</td>
<td>(6.40 ± 0.19) × 10⁻²</td>
<td>8.28</td>
<td>1/9</td>
</tr>
<tr>
<td></td>
<td><em>NlLeuRS</em>2</td>
<td>3.35 ± 0.21</td>
<td>(6.68 ± 0.22) × 10⁻⁴</td>
<td>0.199</td>
<td>1/42</td>
</tr>
<tr>
<td>N<em>ntRNA</em>Leu(CAA)*</td>
<td><em>NlLeuRS</em>1</td>
<td>11.01 ± 1.36</td>
<td>(4.64 ± 0.34) × 10⁻²</td>
<td>4.21</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td><em>NlLeuRS</em>2</td>
<td>13.13 ± 1.57</td>
<td>(4.18 ± 0.50) × 10⁻⁴</td>
<td>0.0318</td>
<td>1/260</td>
</tr>
<tr>
<td><em>Ph tRNA</em>Leu(GAG)*</td>
<td><em>NlLeuRS</em>1</td>
<td>12.51 ± 2.16</td>
<td>(4.18 ± 0.60) × 10⁻²</td>
<td>3.34</td>
<td>1/2.5</td>
</tr>
<tr>
<td></td>
<td><em>NlLeuRS</em>2</td>
<td>16.43 ± 0.62</td>
<td>(1.19 ± 0.08) × 10⁻³</td>
<td>0.0724</td>
<td>1/114</td>
</tr>
</tbody>
</table>

$^a$The results are the average of three independent repeats with standard deviations indicated.

$^b$The activity of *NlLeuRS*1 for *NlRNA*Leu(GAG) is defined as 1.

### Table 3. $k_d$ of *NntRNA*Leu(GAG)* in filter binding reaction at 4°C

<table>
<thead>
<tr>
<th>LeuRS</th>
<th>$k_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NlLeuRS</em>1</td>
<td>6.36 ± 1.17</td>
</tr>
<tr>
<td><em>NlLeuRS</em>2</td>
<td>3.36 ± 0.89</td>
</tr>
</tbody>
</table>

$^a$The results are the average of three independent repeats with standard deviations indicated.
aminoacylation activity compared with \textit{Nm}LeuRS1 (Figure 5C), suggesting the bacterial type of the N-terminal region of \textit{Nm}LeuRS1.

In addition, zinc binding domains (ZN domain) were conserved in many class I tRNA synthetase. However, besides the universal ZN-1 domain, only bacterial/mitochondrial but not archaeal/eukaryotic LeuRSs contained the ZN-2 domain (48). It has shown that mutations of conserved Cys residues in ZN domain obviously reduced the aaRS activities and the growth of cells (49,50).

Sequence analysis showed the ZN-2 domain existed in both \textit{Nm}LeuRSs as most bacterial LeuRSs (Figure 5B). Therefore, we performed the mutations of Cys to Ala at ZN-2 domain of \textit{Nm}LeuRS1 to produce double mutants \textit{Nm}LeuRS1-C448A/C452A and -C488A/C491A. The results showed both mutants totally lost their aminoacylation activities (Figure 5C), indicating the crucial role of bacterial LeuRS-specific ZN-2 domain and further supporting our suggestion that the N-terminal region of \textit{Nm}LeuRS1 was of bacterial type.
LeuRSs are characterized by the capacity to activate a wide range of amino acids that are structurally similar to cognate Leu (30,35). We determined the amino acid activation activity of NmLeuRS1 toward non-cognate Ile and Met as well as the non-proteinogenic amino acids Nva and ABA. Nva and ABA were misactivated by NmLeuRS1, whereas the kinetic constants of Ile and Met could not be determined because kinetic analysis at high concentrations of Ile and Met was not realized. The $k_{cat}$ of NmLeuRS1 for Nva (0.364 ± 0.008 s⁻¹) and ABA (0.518 ± 0.028 s⁻¹) activation were ~60 and 90% of that for cognate Leu (0.563 ± 0.012 s⁻¹), although the $K_m$ values for Nva (955 ± 22 mM) and ABA (37 820 ± 1630 μM) were much higher compared with that of cognate Leu (8.43 ± 1.3 μM) (Table 4). For ABA, the discrimination factor (DF) was 1/4912, which was lower than the mistranslation frequency (1/3000) of protein biosynthesis (6). In contrast, NmLeuRS1 has to clear the misacylated Nva (DF: 1/175) by its editing activity. Because of the extremely low amino acid activation activity of NmLeuRS2 even toward cognate Leu, accurate evaluation of the kinetics of misactivation for non-cognate amino acids was not achieved.

**NmLeuRS1 exhibits obvious post-transfer editing activity to prevent generation of mischarged tRNA<sub>Leu</sub>**

NmLeuRS1 has the bacterial Rossmann fold aminoaclylation and editing domains, which means that the orientation and the insertion point of the CP1 editing domain into the Rossmann fold is totally different from its archaeal counterparts, such as the CP1 domain of PhLeuRS (14). However, it has the normal archaeal CTD for tRNA<sub>Leu</sub> binding. Therefore, whether the editing activity of the bacterial CP1 domain and the tRNA<sub>Leu</sub> binding capacity of the archaeal CTD coordinate to mediate post-transfer editing is an interesting question. To confirm the editing activity of NmLeuRS1, the post-transfer editing essential Asp residue (Asp354 of NmLeuRS1), as revealed in various LeuRS systems (14,30,35,51,52), was mutated to Ala residue to generate NmLeuRS1-D354A. To monitor the post-transfer editing directly, misacylated Nva-[³²P]NmtRNA<sub>Leu</sub> was produced by PhLeuRS1-D332A (14). The hydrolysis of Nva-Nm tRNA<sub>Leu</sub> showed that only NmLeuRS1 was able to deacylate the misacylated product (Figure 6). In contrast, NmLeuRS1-D354A lost its editing ability (Figure 6A and B). We also analyzed the editing activity of NmLeuRS2 in a similar manner; however, both wild-type NmLeuRS2 and the mutant of the conserved Asp390 (NmLeuRS2-D390A)
exhibited no editing capacity (Figure 6B and C), suggesting the post-transfer editing of \( Nm \) LeuRS2 was lost or too weak to be detected in deacylation assays. Whether \( Hh \) LeuRSs exhibited similar results with \( Nm \) LeuRSs in deacylation activities was then explored. Our data revealed that although both \( Hh \) LeuRSs could deacylate mischarged Nva-\( Hh \)tRNALeu, but \( Hh \)LeuRS2 showed weaker activity than \( Hh \)LeuRS1. These further confirmed the phenomena that an active LeuRS and an inactive (or slightly active) one coexisted in halobacteria (Supplementary Figure S4D and E).

Previous work also showed that the editing activity of \( Ph \)LeuRS was not altered by deletion of the CTD (14). Therefore, to investigate the editing function of the archaean CTD of \( Nm \)LeuRS1, the \( Nm \)LeuRS1-\( \Delta \)CTD mutant was generated and shown to exhibit a complete loss of deacylation activity (Figure 6B and C). This suggested that the archaean CTD of \( Nm \)LeuRS1, unlike that of \( Ph \)LeuRS, coordinated with its CP1 domain to play a key role in post-transfer editing.

We then determined the misacylation activity of \( Nm \)LeuRSs. The result showed no Ile and little Met could be misloaded to \( Nm \)tRNALeu by \( Nm \)LeuRSs (Supplementary Figure S6). In addition, \( Nm \)LeuRS1-D354A produced significantly more (\( \sim 3 \)-fold) Nva-\( Nm \)tRNA\(_{\text{Leu}}^{\text{Leu}} \) and 30% more ABA-\( Nm \)tRNA\(_{\text{Leu}}^{\text{Leu}} \) compared with wild-type \( Nm \)LeuRS1, suggesting the essential editing role of \( Nm \)LeuRS1 in preventing the synthesis of mischarged tRNA\(_{\text{Leu}}^{\text{Leu}} \). However, the amount of generated Nva-\( Nm \)tRNA\(_{\text{Leu}}^{\text{Leu}} \) was comparable with wild-type \( Nm \)LeuRS2 and mutant \( Nm \)LeuRS2-D390A (Figure 7C and D), which is consistent with the observation that post-transfer editing was lost or significantly impaired in both enzymes (Figure 6B and C). In addition, it is notable that wide-type \( Nm \)LeuRSs were able to synthesize trace amount of Nva-tRNA\(_{\text{Leu}}^{\text{Leu}} \) and an obvious amount of ABA-tRNA\(_{\text{Leu}}^{\text{Leu}} \). This is also the case for Candida albicans LeuRS (CaLeuRS) (30), suggesting that editing capacity is insufficient for proofreading non-cognate amino acids at saturated concentrations. It can be speculated that the paradox between ABA misaminoacylation and charging accuracy can be accounted for by fine discrimination against ABA at the aminoacylation active site (1/4912) as proposed for \( Ca \)LeuRS (30).

Editing characteristics of \( Nm \)LeuRS1

On the basis of the efficient editing activity of \( Nm \)LeuRS1, we further investigated the editing characteristics of \( Nm \)LeuRS1 in TLC-based AMP formation assays on the basis that a single AMP molecule is released by either the hydrolysis of misactivated aa-AMP by pre-transfer editing or in the cycle of misacylation and post-transfer editing; therefore, measurement of the released AMP in the TLC assay can be used to quantify the editing capacity (53).

In the presence of \( Nm \)tRNA\(_{\text{Leu}}^{\text{Leu}} \), the observed rate constant \( k_{\text{obs}} \) of AMP formation by \( Nm \)LeuRS1 was \( 8.74 \pm 0.36 \times 10^{-2} \text{s}^{-1} \) (Figure 8A, Table 5), which represented the overall editing activity. In the absence of \( Nm \)tRNA\(_{\text{Leu}}^{\text{Leu}} \), the AMP formation rate was \( 1.00 \pm 0.13 \times 10^{-2} \text{s}^{-1} \), \( \sim 11.4\% \) of that in the presence of
Nm tRNA_{Leu}^\text{L} \left(1.00/8.74\right). This result suggested that tRNA-independent pre-transfer editing contributed little to the total editing activity. Theoretically, the enhanced AMP formation activity, as a result of tRNA addition, may be derived from tRNA-dependent pre-transfer editing and/or post-transfer editing. To distinguish these two pathways, we analyzed AMP formation using NmLeuRS1-D354A. The $k_{\text{obs}}$ of NmLeuRS1-D354A in the presence of tRNA was $\left(3.40 \pm 0.19\right) \times 10^{-2}$ s$^{-1}$ (Figure 8B, Table 5), indicating that tRNA-dependent pre-transfer editing contributed $\sim27.5\%$ to the total editing activity $\left[\left(3.40 - 1.00\right)/8.74\right]$. Taken together, these results demonstrate that post-transfer editing contributed more than half (61.1\%) of the Nva editing activity $\left[\left(8.74 - 3.40\right)/8.74\right]$.

In addition, if the editing activities failed to clear all the misproducts, Nva-AMP should be detected in the TLC assays. In the presence of Nm tRNA_{Leu}, the NmLeuRS1-D354A mutant still failed to clear all the misproducts (with obvious accumulation of Nva-AMP) (Figure 8B) in contrast to NmLeuRS1 with tRNA_{Leu} (without obvious accumulation of Nva-AMP), suggesting that pre-transfer editing is not sufficient for maintaining the catalytic fidelity. Only the wild-type NmLeuRS1, which has post-transfer editing in the presence of Nm tRNA_{Leu}, efficiently prevented the accumulation of Nva-AMP (Figure 8A).

**DISCUSSION**

**Unique LeuRS models identified to date**

There are several interesting examples of LeuRSs with unique features with regard to primary or tertiary structure. For example, in *A. aeolicus*, LeuRS is no longer a single polypeptide but rather a heterodimeric enzyme ($\alpha$/$\beta$-LeuRS), although no advantage associated with this structure has been identified (34). However, both $\alpha$ and $\beta$ subunits are of bacterial origin. In *Mycoplasma mobile*, LeuRS is naturally devoid of the CP1 editing domain (54). This is the first example of a truncated LeuRS, leading to an error-prone synthetase. Consequently, *M. mobile* exhibits genetic code ambiguity in its proteome, which might be advantageous for avoiding host immune defense (55). In addition, human mitochondrial LeuRS contains a naturally degenerated CP1 domain (56), which suggests a degenerate editing activity. However, mitochondrial LeuRS exhibited a fine discrimination capacity toward cognate Leu and non-cognate amino acids in the amino activation step. The reason for this degeneration has not been definitely identified. This work describes a unique example of a LeuRS/tRNA_{Leu} system, in which two chimeric LeuRSs coexist in a single cell compartment. Furthermore, we show that NmLeuRSs distinguish Nm tRNA_{Leu} in the archaeal mode.

![Figure 7. NmLeuRSs misacylate Nm tRNA_{Leu} with Nva and ABA. (A) Representative image showing the misacylation activities of 500 nM NmLeuRS1 and 500 nM NmLeuRS1-D354A for Nva and ABA based on TLC assay. A 2-fold diluted [\(\alpha\)-\(\text{\textsuperscript{32}}\text{P}\)ATP for quantification (initial, 52.8 pmol) is also shown. (B) Quantification of the misacylation activities of NmLeuRS1 [for Nva (black circle), for ABA (black square)] and NmLeuRS1-D354A [for Nva (white circle), for ABA (white square)] in A. (C) Representative image showing the misacylation activities of 2 mM NmLeuRS2 and 2 mM NmLeuRS2-D390A for Nva and ABA based on TLC assay. (D) Quantification of the misacylation activities of NmLeuRS2 [for Nva (black circle), for ABA (black square)] and NmLeuRS2-D390A [for Nva (black square), for ABA (white square)] in C.](https://academic.oup.com/nar/article-abstract/42/8/5109/1066036)
Coexistence of two aaRSs in a single compartment

There are sporadic examples of the existence of dual aaRS activities in a single cell compartment. In human cytoplasm, two arginyl-tRNA synthetases (ArgRSs) are derived from two translational initiation sites by a single messenger RNA (57). The short ArgRS is proposed to participate in modification at the N-terminus of proteins targeted for degradation by providing the substrate for arginyl-tRNA transferase (58). Moreover, higher eukaryotes encode two threonyl-tRNA synthetases. Sequence analysis and genetic studies have shown that the short form generates Thr-tRNA\textsuperscript{Thr} for human cytoplasmic protein biosynthesis, whereas the significance of the longer isoform remains to be clarified (53). The coexistence of two types of glycyll-tRNA synthetase (GlyRS, ScGlyRS1 and ScGlyRS2) has been reported in \textit{S. cerevisiae}. ScGlyRS1 is responsible for both cytoplasmic and mitochondrial activities, whereas GlyRS2 is dysfunctional and not essential for growth (59). Many bacterial species, such as \textit{Helicobacter pylori}, also contain two duplicated glutamyl-tRNA synthetase (GluRS, HpGluRS1 and HpGluRS2). It has been revealed that HpGluRS1 acylated only tRNA\textsuperscript{Glu}; whereas HpGluRS2 was specific solely for tRNA\textsuperscript{Gin} (60). In addition, two types of cytoplasmic LeuRSs have been reported in \textit{Agrobacterium radiobacter} K84 (61), in which a second non-essential LeuRS (AgnB2) is encoded by the pAgK84 plasmid, in addition to the essential genomic form. The activity of the genomic form can be inhibited by TM 84, which is synthesized to kill \textit{Agrobacterium tumefaciens}, which causes crown gall tumors in plants by specific inhibition of LeuRS activity. AgnB2 LeuRS, which is non-sensitive to TM 84, works as a self-protective copy.

Considering the obviously high aminoacylation and editing activity of \textit{NmLeuRS1}, we propose that \textit{NmLeuRS1} is responsible for Leu-tRNALeu generation. However, both the aminoacylation and editing activities of \textit{NmLeuRS2} were significantly lower than those of \textit{NmLeuRS1}; therefore, \textit{NmLeuRS2} may not be essential for protein biosynthesis. Compared with \textit{NmLeuRS1} and other bacterial LeuRSs, there are several substitutions at key amino acids (such as Ser631 instead of Lys in the catalysis-essential KMSKS motif) or insertion in the Rossmann-fold amino acid activation domain in \textit{NmLeuRS2} such as a 38-aa insertion (Glu174-Thr211), Supplementary Figure S1A). These changes may, individually or collectively, have some direct or indirect influence on the activity of \textit{NmLeuRS2}. Unfortunately, substitutions of these conserved residues with the corresponding sites in \textit{EcLeuRS} (such as Ser631 to Lys631 to regenerate the KMSKS motif) or deletion of the 38-aa insertion in \textit{NmLeuRS2} failed to restore its amino acid activation and aminoacylation activities (data not shown). Therefore, the detailed mechanism underlying the loss of \textit{NmLeuRS2} activity requires further investigation.

In contrast, \textit{NmLeuRS2} retained the affinity for NmtRNA\textsuperscript{Leu}, indicating \textit{NmLeuRS2} mediates other functions in a tRNA-dependent manner. Many aaRS-like proteins are responsible for tRNA-dependent trans-editing activities rather than the \textit{cis}-editing domains, such as human ProX for Ala-tRNAPro, AlaXp for Ser-tRNA\textsuperscript{Ala} and Gly-tRNAla\textsuperscript{Ala}, ThrXp for Ser-tRNA\textsuperscript{Thr} and Ybak for Cys-tRNA\textsuperscript{Pro} (9). However, the undetectable editing activity of \textit{NmLeuRS2} may exclude such a possibility. It is also possible that the editing function of \textit{NmLeuRS2} relies on other cofactors. Other studies revealed that a lysyl-tRNA synthetase-like protein PoxA modifies elongation factor-P with (R)-\textbeta-lysine and that a SerRS-like insect mitochondrial protein is also a tRNA-binding protein without aminoacylation activity but with an essential mitochondrial function (62,63). Therefore, it can be speculated that \textit{NmLeuRS2} functions in other pathways besides tRNA aminoacylation.

\textbf{Table 5.} The $k_{\text{obs}}$ of NmLeuRS1 and NmLeuRS1-D354A in AMP formation with Nva

<table>
<thead>
<tr>
<th>NmtRNA\textsuperscript{Leu}(GAG)</th>
<th>$k_{\text{obs}}$ (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NmLeuRS1</td>
<td>(1.00 ± 0.13) × 10\textsuperscript{-2}</td>
</tr>
<tr>
<td>NmLeuRS1+</td>
<td>(8.74 ± 0.36) × 10\textsuperscript{-2}</td>
</tr>
<tr>
<td>NmLeuRS1-D354A+</td>
<td>(3.40 ± 0.19) × 10\textsuperscript{-2}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The results are the average of three independent repeats with standard deviations indicated.
Chimeric characteristics of NmLeuRSs

Most archaea, such as *P. horikoshii*, harbor only one archaeal LeuRS gene. However, *N. magadii*, *H. paucihalophilus* and *S. islandicus* are extremophilic archaea, all of which contain two *leuS* genes. Sequence alignment revealed that the CTD in these LeuRSs are all of the archaeal type (Supplemental Figure S1B), suggesting the same archaeal recognition mode. However, the N-terminal region (including the Rossmann-fold, the CP1 domain and the α-helix bundle domain) is different. Specifically, the N-terminal regions of both *NmLeuRS* are bacterial type (Supplemental Figure S1A), but the counterparts [Met1-Pro781 in the LeuRS (accession No. YP_002829589.1)] of both *S. islandicus* LeuRSs are archaeal type. In contrast, the N-terminal region [Met1-Pro746 in the LeuRS (accession No. WP_007982262.1)] of one *H. paucihalophilus* LeuRS (HpLeuRS) is bacterial type, whereas the other [Met1-Pro790 in another HpLeuRS (accession No. WP_007982263.1)] is archaeal type. This phenomenon indicates the complex distribution pattern of LeuRSs in the archaeal world. The functional detail of both *S. islandicus* LeuRSs and HpLeuRSs requires investigation.

Based on the complicated distribution of LeuRS in these species, we propose that the ancestor of most archaea species encoded only one archaeal LeuRS. However, an ancient horizontal gene transfer of bacterial LeuRS occurred from an organism related to the bacterial ancestor to the haloarchaea. In some cases, such as in *N. magadii*, gene fusion may have occurred between the original archaeal LeuRS and the transferred bacterial LeuRS, leading to the presence of a chimeric LeuRS with the N-terminal region replaced by the bacterial counterpart. This new isoform coexists with the original archaeal copy. The bacterial form of LeuRS (or the newly generated chimeric LeuRS) further underwent gene duplications (64) and/or horizontal gene transfers (65) within the haloarchaea. It is possible that this new bacterial or chimeric LeuRS provided advantages in participating in the translational machinery in those species and thus, the original archaeal LeuRS copy was lost subsequently.

Despite the divergence of the N-terminal region of archaeal LeuRSs, all archaeal LeuRSs contain a similar archaeal CTD, suggesting the essential role of this domain in recognizing archaeal tRNA<sub>Leu</sub>s. The specific NmLeuRS/tRNA<sub>Leu</sub> interaction model strongly suggests that tRNA synthetases are able to evolve to adapt to the tRNAs and thus provides a striking example of coevolution between tRNA synthetase/tRNA. We showed that the CTD of NmLeuRS1 was essential for both aminoacylation and editing reactions, confirming its crucial role in Leu-tRNA<sub>Leu</sub> synthesis and translational quality control. Furthermore, substitution of archaeal CTD with bacterial CTD totally abolished aminoacylation (Supplemental Figure S5) and editing activities (data not shown), which is consistent with its maintenance of the archaeal CTD. Considering that EcLeuRS lost its aminoacylation activity in high KCl solution (Figure 3B), we cannot rule out the potential possibility that the bacterial CTD in swapping mutant could not fold correctly in reaction buffer. For NmLeuRS1, it is also possible that the archaeal CTD prevents miscytation of tRNA<sub>Leu</sub> as revealed in the PhLeuRS system (66).

In summary, our data reveal the haloalkaliphilic nature of *NmLeuRS* and further elucidate the aminoacylation and editing activities of *NmLeuRS* as well as the function of CTD in both the recognition and editing of NmRNA<sub>Leu</sub>. Thus, this study provides a comprehensive characterization of the aaRS/tRNA system in halobacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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