Supporting Information

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SI Materials and Methods

Synthesis of tRNA Analogs. Terminal A₇₆ in Mycoplasma mobile tRNA^{Leu} was replaced with 2'-deoxyadenosine (2'dA) or dideoxyadenosine (ddA) using an adapted protocol (1). Tran-scribed *M. mobile* tRNA^{Leu} (~400 A_{260} units) was incubated in 1 mL 50 mM NaOAc (pH 5.2) containing 2.5 mM NaIO₄ at 37 °C for 2 h in the dark. Excess NaIO₄ was reduced at 37 °C for 30 min by incubation with 2 mM glucose. Periodate-oxidized tRNA was ethanol-precipitated and resuspended in 563 µL of water. The terminal adenosine was β -eliminated by incubating periodate-oxidized tRNA with 187 µL of 1 M lysine (pH 9.0) at 25 °C for 2.5 h, followed by desalting using a CHROMA SPIN-10 column (Clontech). The tRNA residual phosphate group was removed by incubation in 1 mL of 100 mM Tris (pH 8.0), 10 mM MgSO₄, and 10 U of calf intestinal alkaline phosphatase for 1 h at 37 °C. The solution was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (125:24:1, pH 5.2). The organic phase-containing protein was removed and one-half volume of 4.6 M NH₄OAc (pH 5.0) was added. The tRNA was recovered by ethanol precipitation.

Modified tRNA was incubated with 100 mM Tris (pH 9.0), 100 mM KCl, 2 mM ATP, 2'-dATP or ddATP, 10 mM MgCl₂, 0.5 mM DTT, and 16 μ M *Escherichia coli* CCA-adding enzyme at 32 °C for 3 h. Protein was extracted and tRNA recovered as described above. The tRNA concentration was measured optically at A₂₆₀ (ϵ = 840,700 M⁻¹·cm⁻¹).

Biological Sample Preparation. The *Mycoplasma synoviae* leucyltRNA synthetase (LeuRS) gene was synthesized and cloned into pET14b plasmid with TGA codons mutated into TGG (Geneart). A SpeI restriction site was introduced into the plasmid p14LiMmoLeuRS (2) at the site encoding Glu229 and Gly232 within the *M. mobile* LeuRS gene. The resulting plasmid p14bMBMmLeuRSSpeI was linearized and ligated with PCRamplified DNA sequence of the *M. synoviae* LeuRS connective polypeptide 1 (CP1) domain using In-Fusion Advantage PCR cloning kit (Clontech), yielding plasmid p14bMBMmLeuRS/ MsLeuCP1. Protein was expressed in *E. coli* BL21 (DE3) and isolated using Affinity Gel His-Select Nickel resin (Sigma) fol-

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lowed by a HiTrap 16/60 size-exclusion Superdex 75 purification column (GE Healthcare) (3).

M. mobile LeuRS expression was induced overnight at 25 °C using 1 mM isopropyl β-D-1-thiogalactopyranoside using E. coli BL21(pLysS)Plus transformed with plasmid p14LiMmoLeuRS (2). Cells were harvested by centrifugation and resuspended in 10 mM Tris (pH 8.0), 200 mM NaCl, 5 mM NaH₂PO₄, and 5% (vol/vol) glycerol, followed by flash-freezing in liquid nitrogen and storage at -80 °C. The cells were lysed by sonication on ice for 2 min with 2-s intervals at 35% sonicator capacity (Vibra Cell; Midwest Scientific), followed by centrifugation at $23,000 \times g$ for 30 min. The supernatant was applied to a HiTrap Chelating HP column and washed with 10 mM Tris-base (pH 8.0), 1 M NaCl, 5 mM NaH₂PO₄, and 5% glycerol. A gradient of 10-75 mM imidazole in this buffer was applied before eluting LeuRS with 250 mM imidazole. Subsequent purification on a Superdex 200 column was carried out in 10 mM bis-Tris (pH 6.5), 500 mM NaCl, 2 mM NaH₂PO₄, 20 µM ZnCl₂, and 1% glycerol. Protein was concentrated to 30 mg/mL and flash-frozen in liquid nitrogen for storage at -80 °C.

Aminoacylation Assays. Leucylation assays with *M. mobile*, *M. synoviae*, or hybrid LeuRS enzymes were carried out at 25 °C in a 36- μ L reaction mixture containing 60 mM Tris (pH 7.5), 10 mM MgCl₂, 2 mM ATP, 1 mM DTT, 21 μ M [³H]leucine (318 Ci/mmol), 4 μ M tRNA, and 100 nM LeuRS enzyme as described (4). To test for isoleucine mischarging, 40 μ M [³H]isoleucine (166 Ci/mmol) was used and 1 μ M enzyme introduced. LeuRS hydrolytic activity was measured using a reaction mixture consisting of 60 mM Tris (pH 7.5), 10 mM MgCl₂, and about 6.5 μ M [³H]Ile-tRNA^{Leu} that was prepared as previously described (2).

Manual Model Building of *M. mobile* LeuRS X-Ray Structure. Manual model rebuilding was carried out using COOT (5) and was interspersed with a round of structure refinement using both PHENIX (6) and REFMAC (7). Cross validation, using 5% of the data for the calculation of the free R factor, was used throughout the model-building process to monitor building bias (8).

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Threonine-rich

			region	
Ec	LRS	222	NWIGRSEGVETTFNVNDYDNT	
Bs	LRS	220		
Tt	LRS	222		
Mm	LRS	223	NWIGKEE	
Ms	LRS	220		
Ma	LRS	222		
Ec	IRS	202	YYDKTSPSIDVAFQAVDQDALKAKFAVSNVNGPISLVIWTTTPWTLPANRAISIAPDFDY	
Bs	IRS	201	YQDKRSASIYVAFGVKDGKGVLE · · · · · · NG · ERIIIWTTTPWTIPANLGISVHPDLEY	
Tt	IRS	197	YKEIQDPSVYVRFPLKEPKKLGLEK · · · · · · ASLLIWTTTPWTLPGNVAAAVHPEYTY	
Ec	VRS	190	NRESKGSMWHIRYPLADGAKTADGK · · · · · · DYLVVA TTRPET LLGDTGVAVNPEDPR	
Bs	VRS	196	YKDVQGAFYHMSYPLADGSGS · · · · · · · · · IEIA TTRPET MLGDTAVAVHPEDER	
Tt	VRS	190	TEPTPGKLYTLRYEVEGGG · · · · · · · · · · · FIEIA TVRPET VFADQAIAVHPEDER	
Ec	LRS	268	AQ KAAENNPELAAFIDECRNTKVAEAEMATMEKKGVDTGFKAVHPLT GEEIPVW	
Bs	LRS	266	VENITTAEQKEAVEAYIKEIQSKSDLERTDLAKTKTGVFTGAYAINPVN GEKLPIW	
Tt	LRS	268	TLELAAPEKREEVLAYVEAAKRKTEIERQAEGREKTGVFLGAYALNPAT GERIPIW	
Mm	LRS	232	VLDLIKEAKLRE-SDLEQ	
Ms	LRS	264		
Ma	LRS	270		
Ec	IRS	262	ALVQIDGQAVILAKDLVESVMQRIGVTDYTILGTVKGAELELLRFTHPFMGFDVPAI	
Bs	IRS	253	SVIAVGEDRFVVASALVENVASACGFDQYEVTRTVKGKDLENIIAEHPLYGRDSLVM	
Tt	IRS	249	AAFQVGDEALILEEGLGRKLLGEGTPVLKTFPGKALEGLPYTPPYPQALEKGYFVV	
Ec	VRS	242	YKDLIGKYVILPLVNR······RIPIV	
Bs	VRS	242	YKHLIGKTVILPIVNR······EIPIV	
Tt	VRS	235	YRHLLGKRARIPLTEV····································	
Ec	LRS	322		
Bs Tt		322 324 232	IADYVLASY <mark>GTG</mark> AVMAVPGHDER <mark>D</mark> FBFAKTFGLPVKEVVKG-GN TADYVLFGY <mark>GTG</mark> AIMAVPAHDQR <mark>D</mark> YEFARKFGLPIKKVIERPGEPL	
Ms	LRS	285	DKAYNNKDFSKKYLINLNLKANLPN · QNLNLDLVVVD · · · · · · · · · · · · · · · · · ·	
Ma	LRS	291		
Ec	IRS	319	LGDHVTLDA GTG AVHTAPGHGPD D VVIGQKYGLETANPVGPDGTVLDGTY	
Bs	IRS	310	LGEHVTTDA GTG CVHTAPGHGEDDFIIGQKYGLDVICPVDEKG·VMTSEA	
Tt	IRS	305	LADVVSQED <mark>GTG</mark> IVHQAPAFGAE <mark>D</mark> LETARVYGLPLIKTVDEEGKLLVEP	
Ec	VRS	263	GDEHADMEK <mark>GTG</mark> CVKITPAHDFNDYEVGKRHALPMINILTFDGDIRESAQVFDTKGNESD	
Bs	VRS	263	GDDYVDMEFGSGAVKITPAHDPNDFELGNRHNLERILVMNEDGTNNENA	
Tt	VRS	256	ADPAVEKDF <mark>GTG</mark> ALKVTPAHDPLDVEIGERHGLKPVSVINLEGRMEGER	
Ec Bs Tt Mm	LRS LRS LRS LRS	368 365 370 232	PDLSQQALTEKGVLFNSGEFNGLDHEAAFNAIADKLTAM VEEAAYTGDGEHVNSDFLNGLHKQEAIEKVIAWLEET PEPLERAYEEPGIMVNSGFFDGTESEEGKRKVIAWLEEK	
Ms	LRS	321	FLSNNSNVDSQLFNLENEKHKSYFDLFN - LDKSLANNSFD	
Ma	LRS	328	FASLGTKNNALIINTKKLASYEKFALANKIDLSTSRSNFD	
Ec	IRS	369	FTLDGVNVFKANDIVVALLCEKGALLHVEKMQHSYPCCWRHKTPIIFRATPQW	
Bs	IRS	359	FGFEGMFYDDANKAITQQLDEKGALVKLEFITHSYPHDWRTKKPTIFRATAQW	
Tt	IRS	354	FKGLYFREANRAILRDLRGRGLLFKEESYLHSYPHCWRCSTPLMYYATESW	
Ec	VRS	323	VYSSEIPAEFQKLERFAARKAVVAAVDALGLLEEIKPHDLTVPYGDRGGVVTEPMLTDQW	
Bs	VRS	312	LQYQGMDRFECRKKLVKDLGEAGVLFKIEDHMHSVGHSERSGAVVEPYLSTQW	
Tt	VRS	305	VPEALRGLDRFEARRKAVELFREAGHLVKEEDYTIALATCSRCGTPIEYAIFPQW	
Ec Bs Tt Mm Ms Ma	LRS LRS LRS LRS LRS LRS LRS	406 401 408 232 360 368	GVGERKVNYRL RDWGVSR 	
Ec	IRS	422	FVSMD-QKGLRAQSLKEIKGVQWIP-DWGQARIESMVANRPDWCISR	
Bs	IRS	412	FASIKDFRSDLLDAIKETKWVP-EWGEQRLHNMVRDRGDWCISR	
Tt	IRS	405	FIKNTLFKDELIRKNQEIHWVPPHIKEGRYGEWLKNLV <mark>DW</mark> ALSR	
Ec	VRS	383	YVRADVLAKPAVEAVENGD - IQFVP - KQYENMYFSWMRDIQDWCISR	
Bs	VRS	365	FVRMQPLADAAIELQKKEEKVNFVP - DRF <u>E</u> KTYLHWMENIRDWCISR	
Tt	VRS	360	WLRMRPLAEEVLKGLRRGD - IAFVP - ERWKKVNMDWLENVKDWNISR	

Fig. S1. Sequence alignment of CP1 domains. The CP1 domains from representative LeuRS (black), IleRS (blue), and VaIRS (orange) were aligned using ClustalW, manually curated, and shaded using Biology Workbench (http://workbench.sdsc.edu). Shaded and black boxes denote homologous and conserved residues, respectively. Key motifs and residues of editing are highlighted: threonine-rich region (green), GTG motif (purple), and the universally conserved aspartic acid (red). Organism abbreviations are as follows: Bs, *Bacillus subtilus*; Ec, *Escherichia coli*, Ma, *Mycoplasma agalactiae*; Mm, *Mycoplasma mobile*; Ms, *Mycoplasma synoviae*; Tt, *Thermus thermophilus*.

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Fig. S2. *M. synoviae* LeuRS exhibits pretransfer editing activity. Reaction mixtures contained 10 μ M *M. mobile* tRNA^{Leu} or a tRNA analog with 2'-deoxyadenosine substituted for the A₇₆ nucleoside (2'dA-tRNA), 1 μ M *M. synoviae* LeuRS, and 0.5 U/mL inorganic pyrophosphatase. Amino acid-dependent AMP formation is measured by TLC of reaction aliquots from ATPase reactions with 5 mM isoleucine (A) or 5 mM leucine (B). Aminoacyl-adenylate was measured with 5 mM isoleucine (C) or 5 mM leucine (D). Abbreviations are as follows: **a**, isoleucine only (Ile); **a**, isoleucine and tRNA (Ile+tRNA); **v**, isoleucine and 2'dA-tRNA (Ile+2'dA); **e**, leucine only (Leu); **e**, leucine and tRNA (Leu+tRNA); **r**, leucine and 2'dA-tRNA (Leu+2'dA); and x, no amino acid control (No AA). Error bars represent SDs derived from triplicated reactions.



Fig. S3. Structural comparison indicates conformational changes for tRNA binding. (*A*) Structural alignment of *M. mobile* LeuRS (MmLeuRS, yellow) with *T. thermophilus apo* enzyme (TtLeuRS, PDB ID code 1H3N). The main body of TtLeuRS is colored in gray, the CP1 domain in cyan, and the leucine-specific domain in blue. The C-terminal domain of TtLeuRS was not resolved. (*B*) The aminoacylation site of MmLeuRS (gray) is structurally aligned with TtLeuRS (blue). The bound leucyl adenylate analog, Leu-AMS, is shown in green for MmLeuRS and orange for TtLeuRS. Key residues interacting with Leu-AMS are shown in licorice and labeled. (*C*) Structural alignment of MmLeuRS (yellow) with *E. coli* LeuRS aminoacylation complex (EcLeuRS, PDB ID code 4AQ7). The EcLeuRS is colored similarly to the TtLeuRS, except its C-terminal domain is shown in orange and its Zn-1 domain in purple. The major domain movement to accommodate tRNA binding is indicated by black arrows. (*D*) Structural alignment of MmLeuRS (gray) with EcLeuRS (blue). The binding of A₇₆ of tRNA (yellow) induces the side chain of Tyr43 in EcLeuRS to rotate about 120° (indicated by a black arrow). The leucyl adenylate analog, Leu-AMS, is shown in green for MmLeuRS and orange for EcLeuRS. Key residues interacting with Leu-AMS are shown in stick model and labeled.



Fig. 54. tRNA-dependent pretransfer editing for *M. mobile* LeuRS is specific for nonleucine amino acids. Reaction mixture contained 1 μ M enzymes, 10 μ M tRNA^{Leu}, or tRNA^{Leu} with A₇₆ replaced by dideoxyadenosine analog (ddA-tRNA), 18.1 μ M [α -³²P]ATP (40 μ Ci/mL), and 2.5 mM leucine. The AMP formation activities are measured for *M. mobile* LeuRS (A), MmLeuRS/CP1^{Leu} (B), MmLeuRS/CP1^{lle} (C), MmLeuRS/CP1^{Val} (D), and MmLeuRS/MSCP1^{Leu} (E). Symbols used are as follows: \blacklozenge , reaction without amino acid present (no AA); \blacktriangle , leucine (Leu); \blacktriangledown , leucine with tRNA (Leu+tRNA); and \blacksquare , leucine with ddA-tRNA (Leu+ddA). Error bars represent the SD values based on three separate experiments.

	M. mobile LeuRS + Leu-AMS*
Data collection	
Space group	R32
Cell dimensions	
a, b, c (Å)	202.9, 202.9, 213.9
α, β, γ (°)	90, 90, 120
Resolution (Å)	12–2.07 (2.07–2.12)
R _{merge}	0.227 (0.993)
//σ/	18.45 (2.56)
Completeness (%)	99.95 (99.20)
Redundancy	12.4 (11.8)
Refinement	
Resolution (Å)	2.07
No. reflections (work/free)	96,445/5,079
R _{work} /R _{free}	0.159/0.199
No. of atoms	
Protein	5,164 [A] [†] + 4,844 [B]
Ligand	31 [E] + 31 [F] (Leu-AMS)
Water/other	1,022/10 glycerol
B-factors average	
Protein	28.4 [A] [†] /22.7 [B]
Ligand	12.6 [E]/12.7 [F]
Water	33.2
Rms deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.509

Table S1. Data collection and refinement statistics

*Values in parentheses are for highest-resolution shell. $^{\rm t}$ Values are for each molecule in the asymmetric unit, with chain indicator given in square brackets.

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