Supporting Information

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SI Methods

Molecular Biology. The coding region of the *E. coli dusC* gene (EcoGene accession no. EG12022; UniProtKB accession no. P33371) was amplified from *E. coli* genomic DNA by PCR and inserted into the vector pET28a (Merck Biosciences). Mutagenesis of Cys98 to Ala was performed using overlap extension PCR (1). DusC (wild-type and C98A mutant) was expressed in Rosetta pLysS (DE3) cells. LB medium was inoculated with cells from a starter culture and incubated at 37 °C until OD_{600 nm} ~0.6. At this point isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 1 mM and the cells were incubated at 16 °C for 16 h before harvesting by centrifugation. Selenomethionine DusC was produced in a similar manner using methionine auxotrophic B834 (DE3) cells grown in a minimal medium containing L-selenomethionine (Acros Organics) as detailed in ref. 2.

Protein Purification. Cells were resuspended in buffer A (500 mM NaCl, 20 mM Tris•HCl pH 7.5, 20 mM imidazole, and 5 mM DTT) supplemented with a complete EDTA-free protease inhibitor tablet (Roche) and lysed by sonication. The lysate was clarified by centrifugation, and the soluble fraction was loaded onto a 5-mL HisTrap HP column (GE Healthcare) equilibrated with buffer A. Protein was eluted with a gradient of imidazole (20-500 mM), and fractions containing DusC were pooled and concentrated before dilution with 20 mM Tris•HCl pH 7.5. The pool was loaded onto a 5-mL MonoQ column (GE Healthcare) equilibrated with 50 mM NaCl, 20 mM Tris•HCl pH 7.5, and 5 mM DTT. Bound protein was eluted with a gradient of NaCl (50-1,000 mM). Fractions containing DusC without nucleic acid contaminants were pooled and concentrated. These fractions were loaded onto a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with buffer B (100 mM NaCl, 20 mM Tris•HCl pH 7.5, and 5 mM DTT) for SEC. The resulting protein was concentrated to 10–15 mg•mL⁻¹. For biochemical and cocrystallization experiments with tRNA^{Phe} and tRNA^{Trp}, the hexahistidine tag was removed after the anion-exchange chromatography step. Thrombin was added to DusC at a ratio of four units of thrombin per milligram of DusC, and the mixture was incubated overnight at 4 °C. Digested DusC was isolated by passing the protein through a HisTrap HP column and then further purified with a Superdex 75 column as described above.

SEC and Multiangle Light Scattering. Samples of purified DusC (2 mg•mL⁻¹) were loaded onto a Biosep SEC S3000 column (Phenomenex) preequilibrated with buffer B connected to a Dawn Helios II 18-angle light-scattering detector (Wyatt Technology). The eluting species was detected by measuring the UV absorbance at 280 nm, the concentration of the species was determined using an Optilab rEX refractometer (Wyatt Technology), and a refractive index increment of 0.18 mL•g⁻¹ was used for calculation of the molecular weight.

In Vitro tRNA^{Phe} Dihydrouridylation and Dihydrouridine Detection. Unmodified tRNA produced as described previously (3) was refolded by heating and cooling before reaction with enzyme. We incubated 2 μ M tRNA^{Phe} without enzyme, or with 1 μ M of purified DusC^{WT} or DusC^{O8A} in 100 mM Tris•HCl pH 8, 100 mM NH₄Ac, 5 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, 1 mM β -NADH, 1 mM NADPH, and 250 μ M flavin mononucleotide (FMN) in a total volume of 100 μ L for 1 h at 37 °C. DusC was removed using phenol extraction and tRNA^{Phe} isolated by ethanol precipitation. Treated tRNA^{Phe} was then resuspended in 9 μ L of diethyl pyrocarbonate-treated water.

Dihydrouridine residues incorporated following DusC treatment were subjected to alkaline hydrolysis causing ring-opening to form β -ureidopropionic acid. β -ureidopropionic acid is unable to form base pairs and consequently inhibits elongation by reverse transcriptase (4). Alkaline hydrolysis was performed by addition of 100 mM KOH and incubation for 20 min at 37 °C. The mixture was neutralized by addition of 125 mM Tris•HCl pH 8, 75 mM NaCl, and 25 mM DTT. Treated tRNA was used as a template for reverse transcriptase using a fluorescein (FAM)-labeled primer specific for the 3' end of tRNA^{Phe}. We used 0.6 μ g of tRNA and 5 nM of FAM-labeled primer complementary to tRNA^{Phe} (5' FAM-GCCCGGACTCGGAATCGAAC 3') in a reverse transcription primer extension reaction as per the manufacturer's instruction (Invitrogen SuperScript III Reverse Transcriptase). A reverse transcription enrichment step was included to enhance product concentration (5). Reverse transcript fragments were purified by ethanol precipitation, and a dilution series was prepared and separated by capillary electrophoresis. Diluted fragments were mixed with 0.3 µL of Genescan 600 LIZ size standards before loading on an AB3130XL instrument (Applied Biosystems) using default parameters. Results were analyzed using Genemapper software v4.0 (Applied Biosystems) default amplified fragment-length polymorphism analysis in the size range 25-70, normalized to total fluorescence. Capillary electrophoresis achieved separation of full-length and termination products with single nucleotide resolution. Enhanced termination of primer extension at position n of the tRNA indicates that extension beyond this point was prevented due to the presence of β -ureidopropionic acid (and therefore dihydrouridine) at position n - 1with respect to the 5'-3' sequence of the tRNA. The data presented are representative of replicate experiments that identified the same reverse transcript termination products.

Electrophoretic Mobility Shift Assays. Unmodified tRNA^{Phe} was refolded by heating and cooling before mixing with enzyme. tRNA^{Phe} (8.3 μ M) was incubated at 37 °C for 30 min in the absence or presence of a 1:1, 1:2, or 1:4 molar ratio of DusC^{WT} or DusC^{C98A} in buffer comprising 50 mM Tris pH 7.5, 20 mM NaCl, 5 mM MgCl₂, 8% glycerol, and 5 mM DTT. A 1.5-mm 10% (wt/vol) native PAGE gel was prepared with Tris/Borate buffer at pH 8.5 with the addition of 2% glycerol and 5 mM MgCl₂. The gel was prerun at 80 V for 30 min at 4 °C. tRNA^{Phe}, DusC^{WT}, DusC^{C98A}, and the mixed ratios described above were loaded and electrophoresed at 80 V for 150 min at 4 °C. The gel was stained using SYBR-Gold nucleic acid gel stain (Invitrogen) diluted in running buffer 1/10,000 with rocking at room temperature for 20 min. The gel was destained in running buffer for 20 min at room temperature and UV imaged before Coomassie staining by standard methods.

Analytical SEC. Deacylated unmodified tRNA^{Trp} (135 pmol) or tRNA^{Cys} (190 pmol) was mixed with an excess of DusC^{C98A} (250 pmol) or DusC^{WT} (500 pmol) in 50 mM Tris•HCl buffer (pH 7.5) containing 7 mM MgCl₂, 50 mM KCl, and 70 mM NH₄Cl. After 10 min of incubation at 25 °C, 200 µL of sample was injected and the complex was analyzed by SEC using a Biosuite 250 5 µm HR 7.8 × 300 column on a Waters Alliance HPLC system. Nucleic acid was detected by absorbance at 260 nm, and protein was detected by tryptophan fluorescence ($\lambda_{ex} = 280$ nm and $\lambda_{em} = 320$ nm) using UV/V is 2487 and Fluorescence 2475 HPLC detectors (Waters).

Crystallization of DusC. DusC was crystallized in MRC Crystallization Plates (Swissci AG) at 20 °C. Protein (150 nL) at a concentration of

10–15 mg•mL⁻¹ in 100 mM NaCl, 20 mM Tris-HCl pH 7.5, and 5 mM DTT was mixed with 150 nL of the crystallization condition and equilibrated against a 56- μ L reservoir of the crystallization condition. Crystals of native DusC grew over a period of 8 wk in condition C10 [10% (wt/vol) PEG 8000, 20% (vol/vol) ethylene glycol, 0.1 M Bicine/Tris pH 8.5, and 30 mM each of NaNO₃, Na₂HPO₄, and (NH₄)₂SO₄] of the Morpheus screen (6). Crystals of the selenomethionine derivative grew in 3 d in condition D3 [10% (wt/vol) PEG 4000, 20% (wt/vol) glycerol, 0.1 M Mes/imidazole pH 6.5, and 20 mM each of 1,6-hexanediol, 1-butanol, (RS)-1,2-propanediol, 2-propanol, 1,4-butanediol, and 1,3-propanediol] of the Morpheus screen. All crystals were flash-cooled in liquid nitrogen straight from the drop.

Crystallization of DusC^{C98A}-tRNA^{Phe} **Complex.** The DusC^{C98A}–tRNA^{Phe} complex was formed by mixing DusC^{C98A} and tRNA^{Phe} in a 1:1 molar ratio, following buffer exchange of the protein into a solution containing 100 mM MgCl₂ and 10 mM Hepes-NaOH pH 7.0 using a 0.5-mL Vivaspin 10 kDa MWCO concentrator. Protein concentration in the final complex was 7.5 mg•mL⁻¹. Complex (1 μ L) was mixed with 1 μ L of the reservoir solution containing 100 mM Hepes pH 7.0, 200 mM MgCl₂, 10 mM MnCl₂, and 11% (wt/vol) PEG 6K and equilibrated over 1 mL of reservoir. Crystals grew over the course of 3 d at 20 °C. Before vitrification, crystals were transferred into a solution containing 100 mM Hepes pH 7.0, 150 mM MgCl₂, 10 mM MnCl₂, 18% (wt/vol) PEG 6K, and 15% (vol/vol) glycerol.

Crystallization of DusC^{C98A}-tRNA^{Trp} **Complex.** *E. coli* tRNA^{Trp} transcript in 20 mM Hepes pH 7.5, 5 mM MgCl₂ was refolded by heating to 60 °C for 10 min followed by cooling to room temperature at a rate of 0.1 °C/s. DusC^{C98A} was then added in a 1:1 molar ratio. Crystals grew after ~6 mo in sitting drops (0.5 μ L) equilibrated against 0.1 M Bis•Tris pH 6.5, 0.2 M Li₂SO₄, and 22–28% (wt/vol) PEG monomethyl ether 2,000. Crystals were cryoprotected by addition of a solution containing 0.1 M Bis•Tris pH 6.5, 0.2 M Li₂SO₄, 30% (wt/vol) PEG monomethyl ether 2,000, and 10% (vol/vol) glycerol.

Structure Determination. X-ray data were collected at Diamond Light Source (Didcot) beamlines I02, I04, and I24. Two datasets were collected from crystals of DusC alone: a dataset from an orthorhombic crystal of native DusC (crystal 1) and a dataset from a tetragonal crystal of selenomethionine DusC (crystal 2) at the peak wavelength of 0.9808 Å. Two datasets were collected from a single crystal of the DusC-tRNA^{Phe} complex (crystal 3) at two different wavelengths: 0.9795 Å data for structure refinement and longer wavelength data (1.300 Å) for identification of Mn^{2+} ion positions. A single dataset was collected from a single crystal of the DusCtRNA^{Trp} complex (crystal 4). After integration with iMosflm (7) (crystals 1 and 2) or XDS (8) (crystals 3 and 4), the data were imported into the CCP4 suite (9). The data were analyzed with Pointless, scaled and merged with Scala (10) (crystals 1 and 2) or merged with Aimless (11) (crystals 3 and 4), and the resulting intensities were converted to amplitudes with cTruncate (Table 1).

The structure of selenomethionine DusC (crystal 2) was determined by single wavelength anomalous diffraction. The selenium positions were located by SHELXC and SHELXD (12), refined by Phaser (13), and the resulting phases were improved by density modification with Parrot (14). The single molecule of selenomethionine DusC present in the asymmetric unit was automatically built by Buccaneer (15). The model was further improved through alternate cycles of manual rebuilding with Coot (16) and refinement with Refmac5 (17) using an isotropic B factor for each atom and two TLS (18) groups as suggested by the TLSMD server (19). Restraints for FMN were created with GRADE (20). The FMN cofactor and all residues of DusC (including three preceding vector-derived residues) are clearly defined in the electron density maps. The final model contains one molecule of selenomethionine DusC, one molecule of FMN, and 104 water molecules (Table 1).

The structure of native DusC (crystal 1) was determined by molecular replacement by Phaser (13) using the structure of selenomethionine DusC as a search model. The model was refined as above, except with the use of anisotropic atomic *B* factors. The final model contains two molecules of DusC, two molecules of FMN, and 611 water molecules. The structure of the DusC^{C98A}-tRNA^{Phe} complex (crystal 3)

was determined by molecular replacement with Phaser (13) using the structures of DusC [Protein Data Bank (PDB) ID code 4bfa] with the FMN cofactor removed and the structure of tRNA^{Phe} transcript (PDB ID code 310u). Three molecules each of DusC^{C98A} and tRNA^{Phe} were found in the asymmetric unit, and these were rebuilt and refined using isotropic atomic B factors and 21 TLS groups identified by TLSMD (19). FMN molecules were built into clear positive density in the mF_o -DF_c electron density map that corresponded to the FMN cofactor in the structure of DusC. Toward the end of model building and refinement, water molecules were added using ARP/wARP (21), and tRNA geometry was improved using RCrane (22). Mg^{2+} ions were identified as peaks above 6 σ in the mF_o - DF_c difference electron density maps, where coordinating distances were in agreement with those expected for Mg²⁺ ions. To identify Mn²⁺ ion positions, the data collected at a wavelength of 1.300 Å were analyzed by ANODE (23), and inspection of the resulting anomalous difference map revealed the locations of three Mn²⁺ ions, one per molecule of tRNA. The final model contains three DusC^{C98A}-tRNA^{Phe} complexes, three molecules of FMN, 25 Mg²⁺ ions, three Mn²⁺ ions, and 614 water molecules. The DusC^{C98A} and tRNA^{Phe} models are complete, except for amino acids 101-104 (chains A-C), 313-315 (chain B), and 315 (chains A and C) and nucleotides 75-76 (chain D), 1-2 and 70-76 (chain E), and 72-76 (chain F).

The structure of the DusC^{C98A}–tRNA^{Trp} complex (crystal 4) was determined by molecular replacement with Phaser (13) using the structure of DusC (PDB ID code 4bfa) with the FMN cofactor removed. Clear density for tRNA^{Trp} enabled manual building of the majority of the RNA. The density for the anticodon loop, the end of the acceptor stem, and two nucleotides in the variable loop was very poor, and so these regions were not modeled. The RNA geometry was improved using ERRASER (24). The structure was refined using isotropic atomic *B* factors and nine TLS groups identified by TLSMD (19). The final model contains one DusC^{C98A}–tRNA^{Trp} complex, one molecule of FMN, one Mg²⁺ ion, two SO₄ ions, and 52 water molecules. The DusC^{C98A} and tRNA^{Trp} models are complete, except for amino acids 100–106 and nucleotides 1–4, 33–35, 45, 47, and 70–76.

Molecular interfaces were analyzed with PISA (25). The geometry of all models was assessed by MolProbity (26). Figures were created with ccp4mg (27) and PyMol (28). Models were prepared for electrostatic calculations using PDB2PQR (29), and calculations were performed using APBS (30). Multiple sequence alignments were created with Clustal Omega (31) and ESPript (32).

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Α

Mature E. coli tRNAs with D at position 16

	D-loop								T stem loop																									
Position	10	11	12	13	14	15	16	17	18	19	20	20A	20B	21	22	23	24	50	51	52	бω	54	თ თ	56	57	58	59	60	61	62	63	64	65	66
Asn_QUU Asp_QUC Gly_GCC His_QUG Leu_BAA Leu_CAG Leu_GAG Leu_HAA Lys_SUU Met_MAU Phe_GAA Thr_GGU Thr_GGU Thr_GGU Thr_CCA Val_GAC Val_GAC		000000000000000000000000000000000000000	U U U G G G U U U U U U U U U U U	CCCAGGCCCCCCC	A A A A A A A A A A A A A A A A	G G G A A A G G G G A G G	ם ם ם ם ם ם ם ם ם ם	С С D D C D D C D D C D D C D D C D D C D	0000####0#00000		0 0 0 0 0 0 0 0 0 0 0 0 0	- D - A A A A - D - D D D		A A G G G A A A A A A A A	G G G A A A G G G G G G G	A A C C C C A A A A A A A A	A G G A G G G G G G G G G G	C C D C G C C C D G C G D D	U G G C G G G A A U C C G U U	G G A G G G G G G G A A G G		T T T T T T T T T T T T T T T T T T T		$\begin{array}{c} \circ \circ$	G G G G A A G G G G G G G G	A A A A A A A A A A A A A A A A A	G G A G G G A A U A C G G G	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	000000000000000000000000000000000000000	000000000000000000000000000000000000000	A C C C C C C C C C C C C C C C C C C C	G G G A G C G G G A C G U C A	U P U U C U C C U G C C U U	C C U U C C C U A C U U C C U
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Fig. S1. Dihydrouridine locations in *E. coli* tRNAs. (*A*) Secondary structure-based sequence alignment of mature *E. coli* tRNAs containing dihydrouridine at position 16. DusC forms sequence-specific interactions with positions 16, 17, and 56 (highlighted in dark green) and sequence-independent interactions with positions 13–15, 20, 21, 23–24, and 55 (highlighted in pale green). The consensus row contains the most frequently represented nucleoside at each position. Noncanonical nucleosides are denoted as follows: D, dihydrouridine; *P*, pseudouridine; *#*, 2'-O-methylguanosine. (*B*) Location of U16 and U20 in unmodified *E. coli* tRNA^{Phe} (PDB ID code 310u).



Fig. S2. Active site of DusC. (*A*) The enzyme comprises an N-terminal triosephosphate isomerase barrel domain (1–245, light blue) and C-terminal recognition domain (246–315, blue). FMN cofactor (green sticks) is bound at the center of the TIM barrel. Proximal to this is the active site loop (red) containing the catalytically important cysteine C98 (green). (*B*) Variation in the active site loop positions in structures of DusC (red and yellow), DusC^{C98A}:tRNA^{Phe} complex (blue), and DusC^{C98A}:tRNA^{Trp} complex (cyan).



Fig. S3. Schematic of residues hydrogen-bonded to tRNA through direct (solid lines) and water-mediated (dashed lines) contacts in the two DusC:tRNA complexes.



Fig. 54. Difference electron density maps for the active sites of DusC and *Tt*Dus. Positive (green) and negative (red) $mF_o - DF_c$ difference density maps, calculated by PHENIX.MAPS, are contoured at 4 σ . Maps are not clipped around any of the displayed atoms. (*A* and *B*) Structure of DusC (this work, PDB ID code 4bfa; chains A and B). (C) Structure of DusC in complex with tRNA^{Phe} (this work). Map is calculated after omitting active site solvent molecules (PDB ID code 4yco; chains A and D). (*D*) Structure of *Tt*Dus in complex with a fragment of tRNA^{Phe} (1) (PDB ID code 3b0u; chains A and X). (*E* and *F*) Structure of DusC from Chen et al. (2) (PDB ID code 3w9z; chain A). Maps were contoured at 4 σ (*E*) and 2.5 σ (*F*). Shape and positional differences in the additional density previously ascribed to an unknown ligand (1, 2) indicate that the additional density may instead correspond to disordered solvent molecules, alternative conformations of the active site loop (residues 96–100), or partially occupied ions and/or polar crystallization ingredients that were brought to the active site by Coulombic attraction.

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Fig. S5. tRNA binding and modification specificity of *Ec*DusC. (*A*) Reverse transcription assay for dihydrouridine incorporation. (*B*) Capillary fragment analysis of reverse transcript products of unmodified tRNA^{Phe} (black), incubated with 1 μ M DusC^{VT} (red), or 1 μ M DusC^{C98A} (blue). (*C*) EMSA analysis of DusC^{WT}:tRNA^{Phe} and DusC^{C98A}:tRNA^{Phe} complex formation. (*D*–*G*) SEC analysis of DusC interactions with unmodified *E. coli* tRNAs. Unmodified tRNA^{Trp} was incubated with DusC^{WT} (*D*) or DusC^{C98A} (*E*), and the DusC–tRNA^{Trp} complex was separated by gel filtration. tRNA was detected by A₂₆₀ (black), and the protein was detected by Trp fluorescence (red). tRNA alone (A₂₆₀, black dotted line) and DusC alone (fluorescence, red dotted line) controls were separated under identical conditions. Complex formation of tRNA^{Cys} with DusC^{WT} (*F*) or DusC^{C98A} (*G*) was studied in the same way.



Fig. S6. Sequence alignment of DusC subfamily enzymes with 30–50% sequence identity. Residues that are proposed to define tRNA docking (binding signatures) are highlighted by blue stars. The secondary structure of *Ec*DusC is shown above the sequence alignment. The flexible loop is indicated with blue arrows.

T.thermophilus	1	$\xrightarrow{\beta_1}$	α1 200000000 20	β2 →	$ \stackrel{\beta 3}{\longrightarrow} $
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae		DPRLSVAPMVDR SGRFSVAPMLDW RGRFAVAPMLDW SRRFSVAPMLDW DRTFSIAPMLDW SLRLSVAPMMDW SLRLSVAPMLDW SRRFSVAPMLDW	IDRHFRFLVRC TDRHCRYFLRI ITRHCRYFHRC TDRHCRYFMRI IDRHYRYFARI IDRHCRVFHRI IDRHCRYFHRI IDRHCRFFLRC IDRHCRYFHRI	VSLCVRLYTEI LSRNTLLYTEI FSQQALLYTEI MSANALLYTE LAPSARLYTEI LTKQALLYTEI LSRHTLLYTEI LSAQTLLYTEI	TVDQAVLR VTTGAIIH ITAPAILH VTTGAILH VTTGAILH VTTGAILH VTTGAILH VTTGAILH
T.thermophilus	α3 η1 β ⁴ 2000 200 60	4 <u>000000</u> 70	(4 0000000 80	<u>β5</u> 90	α5 20000 2 100
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	GNERLIAREELH FAL GK.GDYLAYSEEHFAU AK.YDLEYDPAFOVAL GRERFLANDETEFFI GRORLIGHDAVEHFAL GRORLIGHDAVEHFAL GRORLIGHDAVEHFAL GRORLIGHDAVEHFAL GR.GDFLAYNOEEHPAL	QLAGSDPKSLAE QLGGSDPAALAQ QLGGSDPTQLAN QLGGSTAAGLAA QLGGSNPVELAR QLGGSDPALLAQ QLGGSDPALLAQ QLGGSDPQALAH QLGGSVPAELAA QFGGSNPKDLAH	AARIGEAFGYI CAKLAEARGYI CAKLAEARGYI CAKLAEAAGYI CAKLAEARGYI CAKLAEQRGYN CAKLAEQRGYN CAKLAQERGYI CAKLAQERGYI	ETNLNLGCPS EINLNVGCPS EINLNVGCPS EVNLNVGCPS EVNLNVGCPS EINLNCGCPS EINLNVGCPS EUNLNVGCPS EINLNVGCPS	EKAQEGGYG DRVQNGMFG DRVQNGMFG DRVQNGRFG DRVQNGRFG DRVQNGRFG DRVQNGRFG DRVQNGRFG DRVQNGRFG
T.thermophilus	η2 α6 20200 20202020200 110 120	$ \underset{130}{\overset{\beta6}{\longrightarrow}} $	TT 200 140	α7 0000000000 150	β7 160
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	A CLLLDLARVREILKAMG A CLMGNAQLVADCVKAMR A CLMANDUVADCXKAMQ A CLMANDUVADCXKAMQ A CLMAHPQLVADCVKAMR A CLMAEPELVACVDAMK A CLMGEADLVADCVKAML A CLMGEADLVADCVKAML A CLMGEPALVADCVKAML	EAVRVPVTVKMR DVVSIPVTVKTR DVVDIPVTVKTR QVVDIPVTVKHR QVVDIPVTVKTR AATALPVTVKCR DAVAIPVTVVKTR DAVEIAVTVKHR AVVDIPVTVKTR	LGLEGKETYRC IGIDDQDSYEF IGIDTIDSYAF IGIDEQDSYAF IGIDEQDSYAF LGVDDDDDYAV IGIDQLDSYEF IGINGRDSYAF IGIDQDDSYEF	L A Q S VE AMA E L C DF INT VS G L C DF INT VS G L C DF VG VG V L C DF VG V VG L THF IDT VMAI FAGF ID Q V VG L C EF VG Q VAE L C DF VG Q VRE L T Q F IAT VAE	A.GVKVFVV KGECEMFII Y.SQDFIV A.GCQSFTV A.GCQSFTV K.GCGEFII A.GAAMVVV RGECEIFTI A.GCRSFTV KGGCEQFTI
T.thermophilus	► 170 180	α8 <u>0000000</u> 190	εε <u>β8</u> 200	α9 <u>00000</u> 210	<u>β</u> 220
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	HARSALL ALSTKANRET HARKAWLGLSPKENRET HARKAWLGLSPKENRET HARKAWLGLSPKENRDT HARKAWLGLSPKENRET HARKAWLGLSPKENREV HARKAWLSCLSPKENREV HARKAWLSCLSPKENREV HARKAWLSCLSPKENREV HARKAWLGLSPKENRET	PPLRHDWVHRLK PPLDYPRVYQLK PPLDYPRVYQLK PPLDYDVVAQLK PPLRYDVVAQLK PPLRYDVVAQLK PPLRYDWAYRLK PPLDYERVYQLK PPLRYEVAAQLK PPLRYEVAAQLK	SDFPOLTFVTN RDFPHLTMSIN RDFPOLNISIN RDFPELEIVLN RDYPALNISIN QERPALPVVLN RDFPALTININ RDFPHLTIAVN	GGIRSLEEAL GGIKSLEEAK GGIKTIDEIK GGIKTLEQCS GGITSLEQAQ GGISLEAK GGVKTLAEAK GGVKSLEEAK	FHLKRVDGV AHLQHMDGV HLQTFVDGV FHLQHLDGV AHLQHTDGV HLQHTDGV EHLKHLDGV HLQFDGV HLQHLDGV
T.thermophilus	$\rightarrow \begin{array}{ccc} \alpha 10 & \eta 3 & \alpha 11 \\ \bullet & 0 0 0 0 & 0 0 0 & 0 0 \\ 2 3 0 & 2 4 0 & 0 \end{array}$	د 250	α12 2020202000 260	270	α13 2020 280
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	$\label{eq:model} \begin{split} \mathbf{M}_{\mathbf{G}}^{\mathbf{G}} \mathbf{C} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{V} \mathbf{D} \mathbf{P} \mathbf{F}^{\mathbf{V}} \mathbf{L} \mathbf{E} \mathbf{E} \mathbf{A} \mathbf{D} \mathbf{R} \\ \mathbf{M}^{\mathbf{V}} \mathbf{C} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{O} \mathbf{R} \mathbf{G} \mathbf{L} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{D} \mathbf{R} \\ \mathbf{M}^{\mathbf{V}} \mathbf{C} \mathbf{R} \mathbf{E} \mathbf{A}^{\mathbf{V}} \mathbf{O} \mathbf{N} \mathbf{P} \mathbf{A} \mathbf{L} \mathbf{L} \mathbf{G} \mathbf{E} \mathbf{I} \mathbf{D} \mathbf{Q} \\ \mathbf{N}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{E} \mathbf{A}^{\mathbf{V}} \mathbf{O} \mathbf{N} \mathbf{P} \mathbf{Y} \mathbf{L} \mathbf{L} \mathbf{A} \mathbf{Q}^{\mathbf{V}} \mathbf{D} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{V} \mathbf{N} \mathbf{P} \mathbf{Y} \mathbf{L} \mathbf{L} \mathbf{A} \mathbf{Q}^{\mathbf{V}} \mathbf{D} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{V} \mathbf{D} \mathbf{P} \mathbf{Y} \mathbf{V} \mathbf{L} \mathbf{H} \mathbf{O} \mathbf{L} \mathbf{A} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{V} \mathbf{D} \mathbf{P} \mathbf{Y} \mathbf{V} \mathbf{L} \mathbf{U} \mathbf{D} \mathbf{L} \mathbf{A} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{V} \mathbf{N} \mathbf{P} \mathbf{Y} \mathbf{V} \mathbf{L} \mathbf{U} \mathbf{A} \mathbf{L} \mathbf{V} \mathbf{D} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{N} \mathbf{N} \mathbf{P} \mathbf{Y} \mathbf{V} \mathbf{L} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{D} \mathbf{S} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{V}} \mathbf{N} \mathbf{N} \mathbf{P} \mathbf{Y} \mathbf{U} \mathbf{L} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{D} \mathbf{S} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{U}} \mathbf{N} \mathbf{N} \mathbf{V} \mathbf{N} \mathbf{V} \mathbf{U} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{D} \mathbf{S} \\ \mathbf{M}^{\mathbf{U}} \mathbf{G} \mathbf{R} \mathbf{A}^{\mathbf{U}} \mathbf{N} \mathbf{N} \mathbf{V} \mathbf{Y} \mathbf{U} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{U} \mathbf{S} \\ \mathbf{M} \mathbf{H} \mathbf{G} \mathbf{R} \mathbf{R} \mathbf{A} \mathbf{V} \mathbf{N} \mathbf{N} \mathbf{N} \mathbf{V} \mathbf{Y} \mathbf{U} \mathbf{A} \mathbf{A} \mathbf{V} \mathbf{V} \mathbf{S} \end{aligned}$	RVFGLPRR.PSR EIFGSSDTDADP QLFDQNQPLITA QLFGSVAPVISR VLCGSTKAVMSR ALSGR.PERAR ELFDPNAPVVDS QLFGSEAPPLSR QIFGLETPVKKR	LEVARRMRAYI VAVVRAMYPYI RIAVENLPYI HAALESMRPYI EAVIEAMLPYI ADLLQAYQPYV VKAIEALYPYI SEALLRLRPYI SQVIHEMMPYI	EEEVLKGTPP ERELSQGTYL EQQLSKGVYL AAHIASGGNM EAHLQVGGRL QAQLDQGLAL EQELSQGAYL ERHQAEGGAM ERELSQGTHL	WAVLRHMLN 3HITRHMLG HIVRHMLG HVTRHMLG VHITRHMIG VHITRHMIG SHITRHILG 3HMTRHMLG
T.thermophilus	α14 <u>0000000000</u> 290 30	• eeeee	α15 0000000000 310	320	330
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	LFRGRPKGRLWRRLLSEG LFOGIPGROWRRLSEN AFONCKGROWRRHLSEN LGLGFPGARRFROLLSVD LFOGLPGRAWRRYLSEN LFHG0PGRVFR0VLSEG IFOGIPGARQWRRHLSEN LAQGFPGRRFROLLSVD LF00MPGAROWRHLSEN	RSLQALD AHKAGADINVLE ACKQGAGIEVVKE IHKAENPLLLLD AHKNGAGIEVVK AHRPGAGWELVE AHKPGAGVSVVE VHKAADPLRVFD AHKPGAGLEVVE	RALRLMEEEVC HALKLVADKR QALRFVTE LAYQSVQTDLV QASQRTDDQAF EALALVSPSYY QALELLAGR QALAKIPYQEI	AQAQAQAQAQAQAQ.	RGQREAAPG
T.thermophilus	340				
T.thermophilus E.coli H.ducreyi P.syringae S.oneidensis X.campestris Y.pestis P.aeruginosa V.cholerae	PAREGV				

Fig. S7. Sequence alignment of DusA subfamily enzymes with 30–50% sequence identity. Residues that are proposed to define tRNA docking (binding signatures) are highlighted by red stars. The conserved eight-residue insertion, preceding Lys175, is highlighted by green triangles.

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		1	10	20	30	40		50	60	70	80	90
T.thermophilus_Dus E.coli_DusA H.ducreyi_DusA P.syringac_DusA S.oneidensis_DusA X.campestris_DusA Y.pestis_DusA P.aeruginosa_DusA V.cholerae_DusA E.coli_DusB H.inflüenzae_DusB E.coli_DusC S.typhi_DusC R.solanacearum_DusC N.meningitidis_DusC	MPEK MOPDIAPNPHS MLKNNIN MTLPPP MHEAQTFSSTPAT MRPETN. MRPETN. MRI MRI MRI		$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} P & W \\ \end{array} \end{array} \\ \end{array} \\ \begin{array}{c} \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} P & W \\ \end{array} \\$	HF ^C FLVRO ¹ HCRYFFLRO HCRYFFLRO HCRYFFLRO HCRYFFRO HCRYFFRO HCRYFFLRO HCRYFFLRO HCRYFFLRO FFRO FFRO LOR FFRO LOR ELLTE LVRELLTES VMRDLLTR VMRDLLTR	$ \begin{array}{l} & \forall S \perp G \forall R \perp y T \\ F S \subseteq Q A \perp L \ y T \\ F S \subseteq Q A \perp L \ y T \\ L S \in Q A \perp L \ y T \\ L S \in Q A \perp L \ y T \\ L S \in Q A \perp L \ y T \\ L S \in Q A \perp L \ y T \\ L S = A R \perp y T \\ L S = A R \perp y T \\ L S = A R \perp y T \\ L S = A R \perp Y T \\ L S = A R \perp Y T \\ L S = A R \perp Y T \\ L S = A R \perp Y T \\ L S = A R \perp Y T \\ L S = A R \perp Y T \\ M G = A C \perp T Y S \\ L G = A C \perp T Y S \\ L G = A C \perp T Y S \\ L G = A C \perp T Y \\ S = A C \perp Y T \\ M G = A C \perp T Y \\ M G = A C \perp T Y \\ M G = A C \perp Y T \\ M G = A C \perp Y \\ M G = $	EMTVDOAV EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EMUTTGAI EFVRVVDQ EFVRVVDQ EFVRVVDQ EFVRVVDQ EFVRVVDQ EFVRVVDQ EFVRVVDQ	DECNEE. HIGK.G HIGK.Y. HIGR.G. HIGR.G. HIGR.G. HIGR.G. 	. RLLAFRP . DYLAYSE . DYLAYSE . DYLYDP . RFLRHDT . DYLYNOC . RLIGFDA . DYLAYSE . FLRYDE . DFLAYNOE RLRA. HSE LHRA. HSEE LHRA. CHASRTP ELLARSRTP ELLARSRTP ELLARSRTP ELLARSRTP ELLARSRTP ELLARSRTP ELANSRTP ELANSRTP	$\begin{array}{c} \mathbf{E} \mbox{ H} \mathbf{P} \ \ \mathbf{A} \ \mathbf{L} \ \mathbf{C} \ \ \mathbf{C} \ \mathbf{C} \ \mathbf{B} \ \ \mathbf{P} \ \ \mathbf{A} \ \mathbf{A} \ \ \mathbf{C} \ \ \mathbf{C} \ \mathbf{C} \ \ $	SDPKSTAE SDPTQLAN SDPTQLAN STAAGLAA SNPVELAR SDPQALAQ SDPQALAQ SDPQALAQ SNPVELAR SNPKDLAN SDPLEMAQ QFPQWLAE QFPQWLAE SDPEWLAR SDPEVLAR SDPVCLAE	AMR TÜR AFÜ CAKLAPARU CAKLAPARU CAKLAPARU CAKLAPARU CAKLAPARU CAKLAPARU CAKLAPARU CARLAPERU AARTINVESGU AARTINVESGU AARTINVESGU NAARAVELU NAARAVELU NAARAVELU NAARAVELU NAARAVELU NAAPATVESU	V DE TINTINE CE PSE V DE TINTINE VIGE PSD VT ELINIE VIGE PSD VT ELINIE VIGE PSD V DE VINIE VIGE VIGE VIGE VIGE VIGE VIGE VIGE VI
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T.thermophilus_Dus E.coli_DusA H.ducreyi_DusA S.oneidensis_DusA X.campestris_DusA Y.pestis_DusA Y.estis_DusA P.aeruginosa_DusA E.coli_DusB H.influenza_DusB X.campestris_DusB E.coli_DusC S.typhi_DusC N.meningitidis_DusC	320 330 EEGEKEKPGPRGQ 	34 Q REAAPGPAREG T. AAA	v									

Fig. S8. Sequence alignment of Dus enzymes from subfamilies DusA, DusB, and DusC showing conservation (red background, white letters) and similarity (red letters). Residues constituting the proposed DusC and DusA binding signatures are highlighted by blue and red stars, respectively. The flexible loop of DusC is indicated by blue arrows. The conserved eight-residue sequence insertion of DusA is highlighted by green triangles.

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Fig. 59. tRNA binding in reverse orientations. (*A*) DusC. (*B*) *Tt*Dus. Images on the *Left* show proteins' electrostatic isosurfaces contoured at ± 1 kTe⁻¹ (blue/red). Protein–tRNA complex formation (*Center* and *Right*) is shown with U16 (orange) and U20 (pink) highlighted on tRNA surfaces. tRNA (*Right*) is rotated by 180° with respect to the center image.