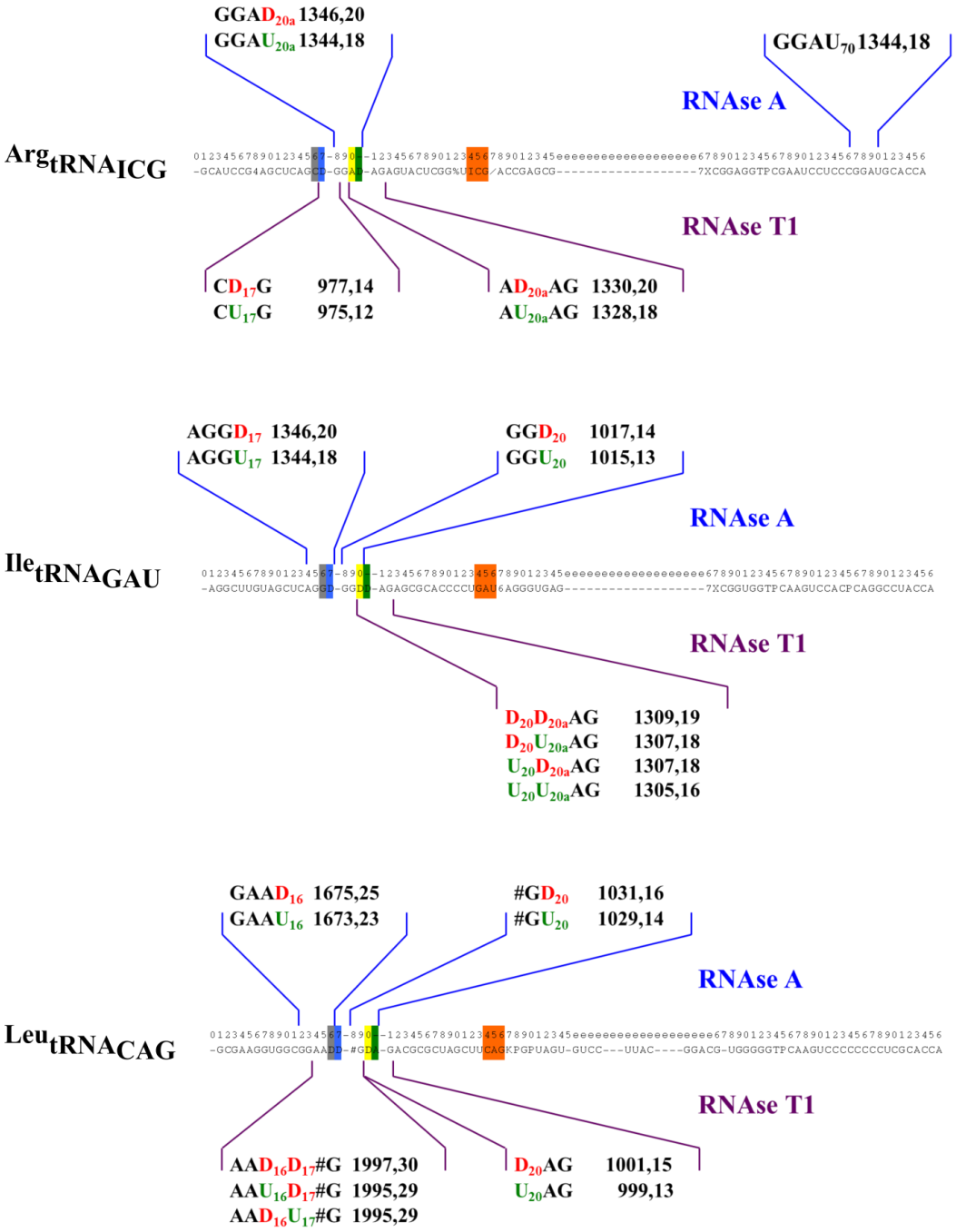
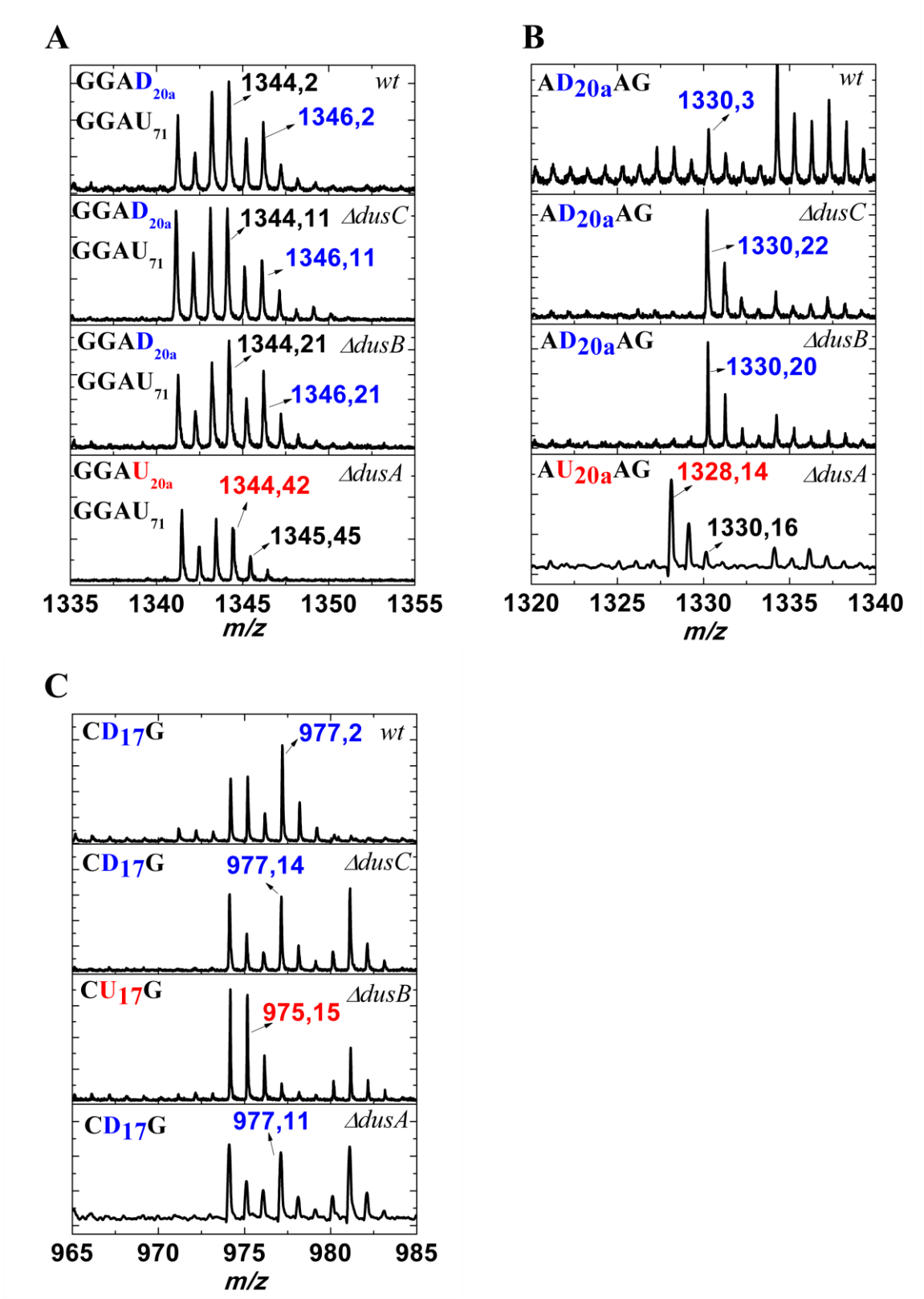
Supplementary Information

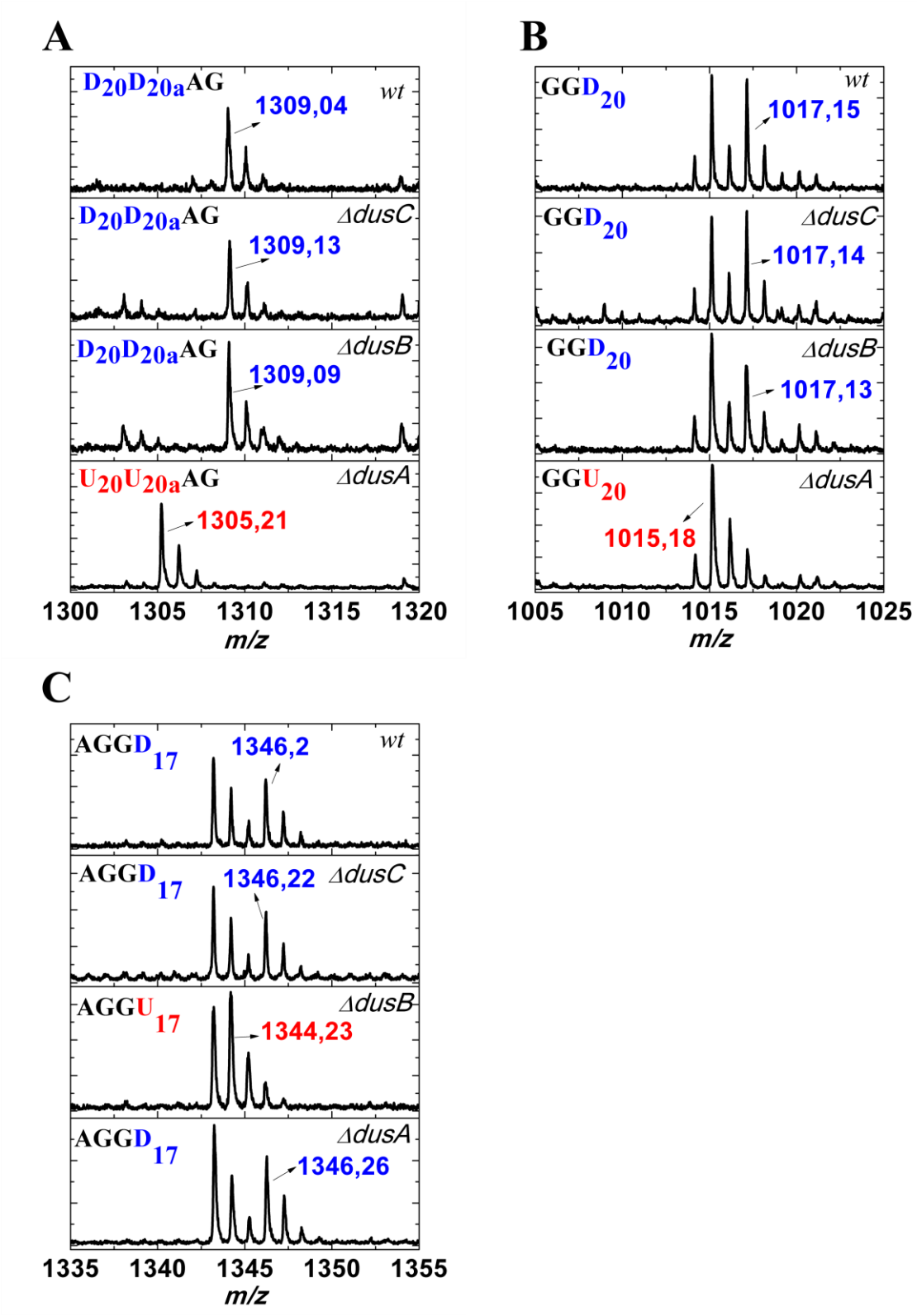


Supplementary Figure 1. Sequence of the three tRNA used to determine *E. coli* Dus specificities.

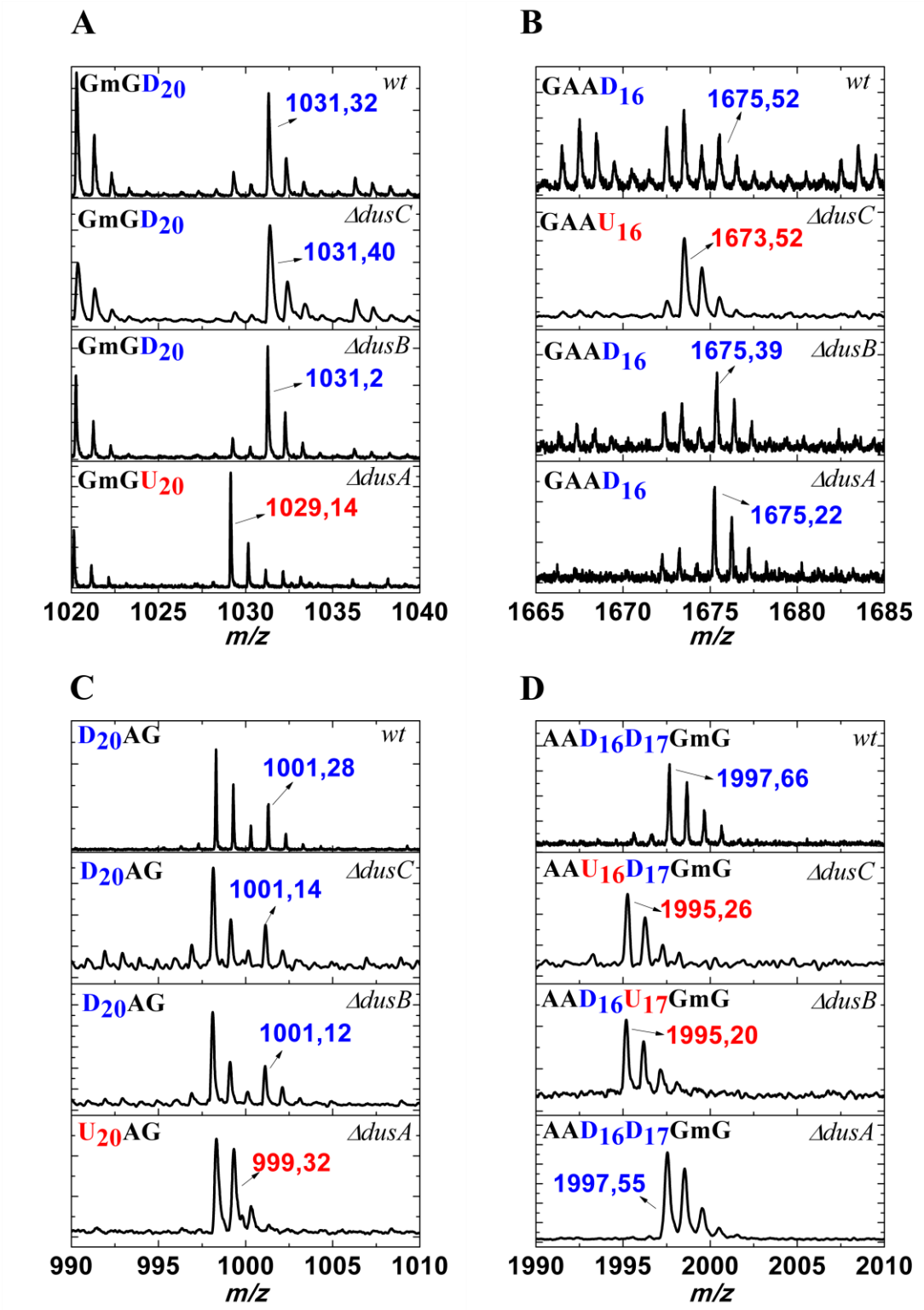
E. coli $^{Arg}tRNA_{ICG}$, $^{Ile}tRNA_{GAU}$ or $^{Leu}tRNA_{CAG}$ sequences with emphasis on the D-containing fragments issued from a theoretical digest by RNAse A or T1. In orange is highlighted the anticodon. In blue arrows are the fragments of interest upon digestion with RNAse A while in purple are those generated by RNAse T1. The expected *m/z* for protonated fragments are indicated next to each D-containing fragment of interest. The symbol # represents the 2'-O-methylguanosine. For the nomenclature of other modified nucleosides present in the three tRNA sequences, refer to http://modomics.genesilico.pl/modifications.



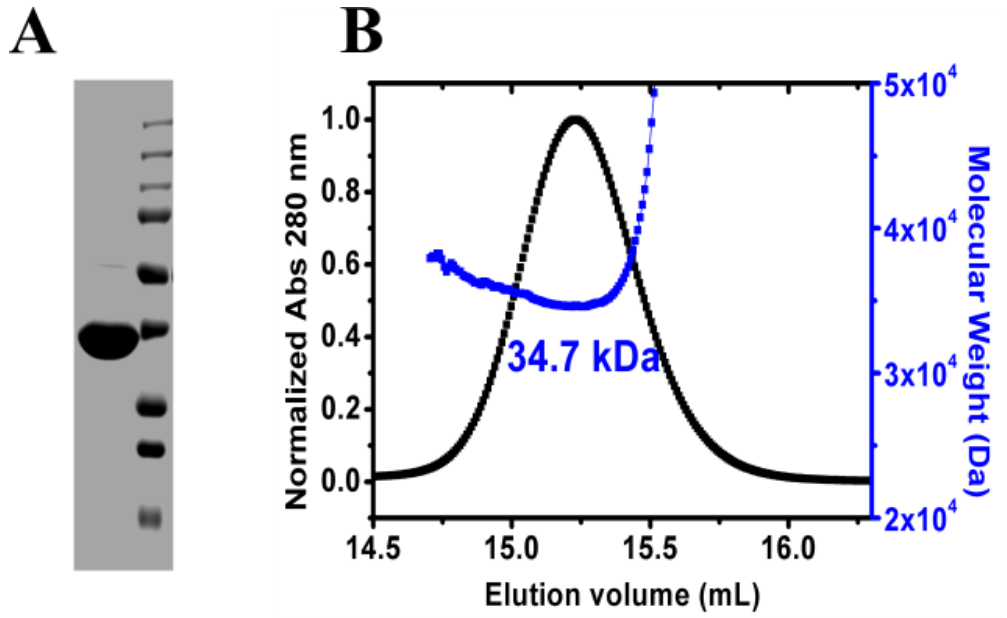
Supplementary Figure 2. MALDI-TOF analysis of ^{Arg}tRNA$_{ICG}$ extracted from wild-type and the different single *E.coli dus* knockout strains. **(A)** Represent a zoom in the spectral region of GGAU$_{20a}$ after digestion with RNAse A. **(B)** and **(C)** are zooms in spectral regions of AU$_{20a}$AG and CU$_{17}$G, respectively, obtained after RNAse T1 digestion. The strains are shown in italic while the sequence and *m/z* of the fragments are in red and blue for absence of dihydrouridine or presence, respectively.



Supplementary Figure 3. MALDI-TOF analysis of $^{Ile}tRNA_{GAU}$ extracted from wild-type and the different single *E.coli dus* knockout strains. **(A)** Represent a zoom in the spectral region of $U_{20}U_{20a}AG$ after digestion with RNAse T1. **(B)** and **(C)** are zooms in spectral regions of GGU_{20} and $AGGU_{17}$, respectively, obtained after RNAse A digestion. The strains are shown in italic while the sequence and *m/z* of the fragments are in red and blue for absence of dihydrouridine or presence respectively.

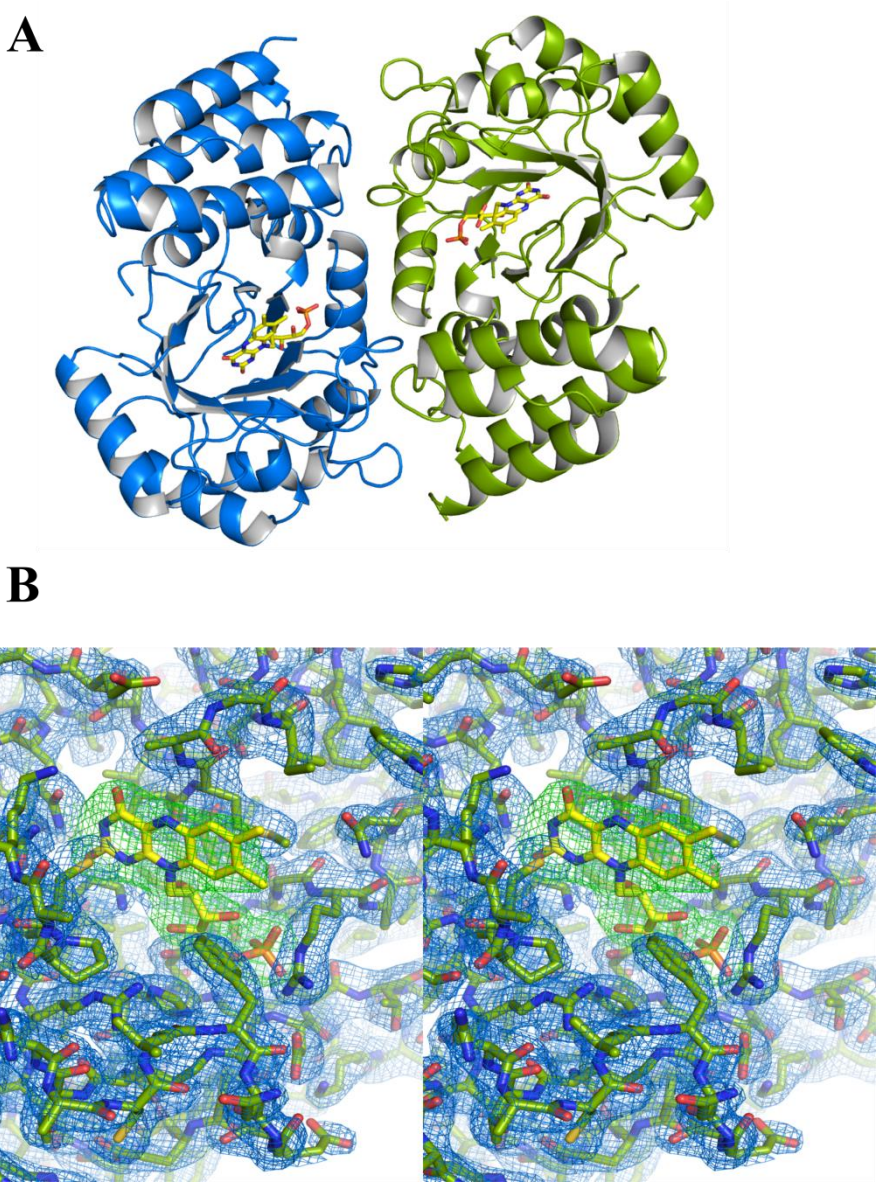


Supplementary Figure 4. MALDI-TOF analysis of ^{Leu}tRNA$_{CAG}$ extracted from wild-type and the different single *E.coli dus* knockout strains. **(A)** and **(B)** represent a zoom in the spectral region of GmGU$_{20}$ and GAAU$_{16}$, respectively, after digestion with RNAse A. **(C)** and **(D)** are zooms in spectral regions of U$_{20a}$AG and AAU$_{16}$U$_{17}$GmG respectively obtained after RNAse T1 digestion. The strains are shown in italic while the sequence and *m/z* of the fragments are in red and blue for absence of dihydrouridine or presence respectively. Gm is the abbreviation for 2'-O-methylguanosine.

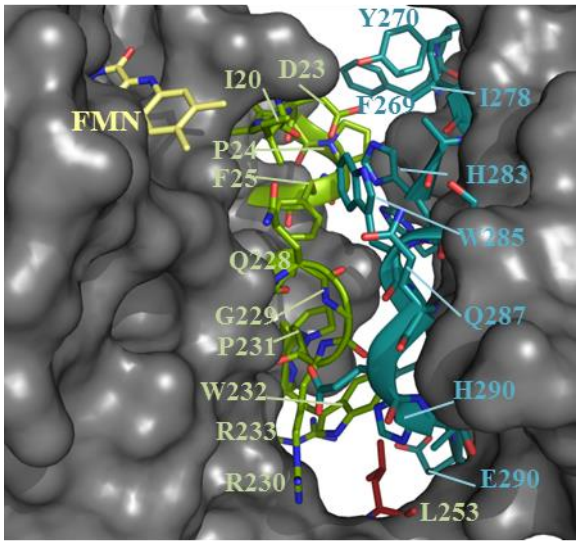


Supplementary Figure 5. Characterization of recombinant *E. coli DusB*.

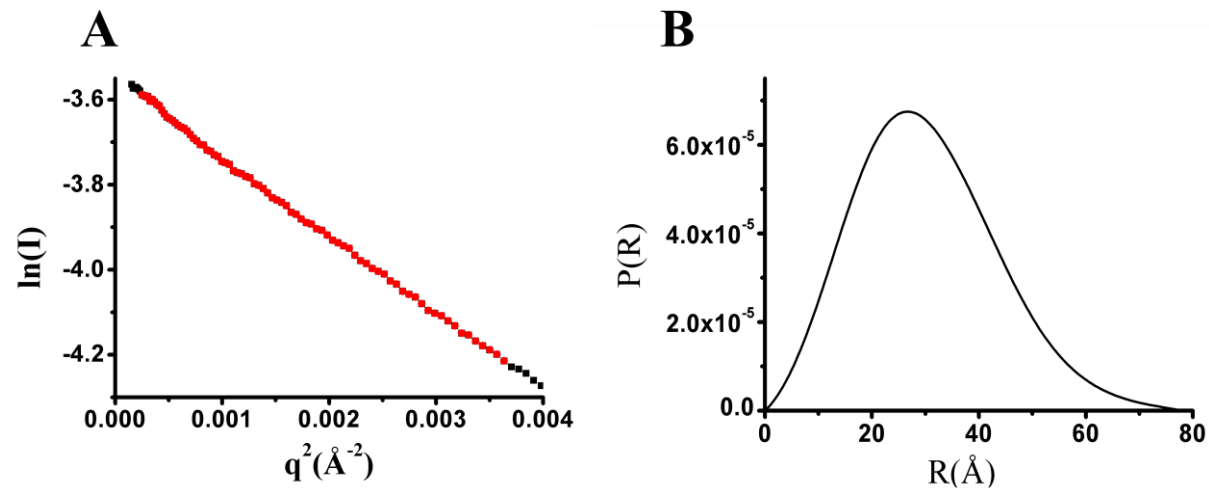
(A) 14 % SDS-PAGE of purified *E. coli* DusB (left) and standard molecular ladders (right) showing a single band migrating at ~36 kDa. (B) SEC-MALLS analysis of *E. coli* DusB.



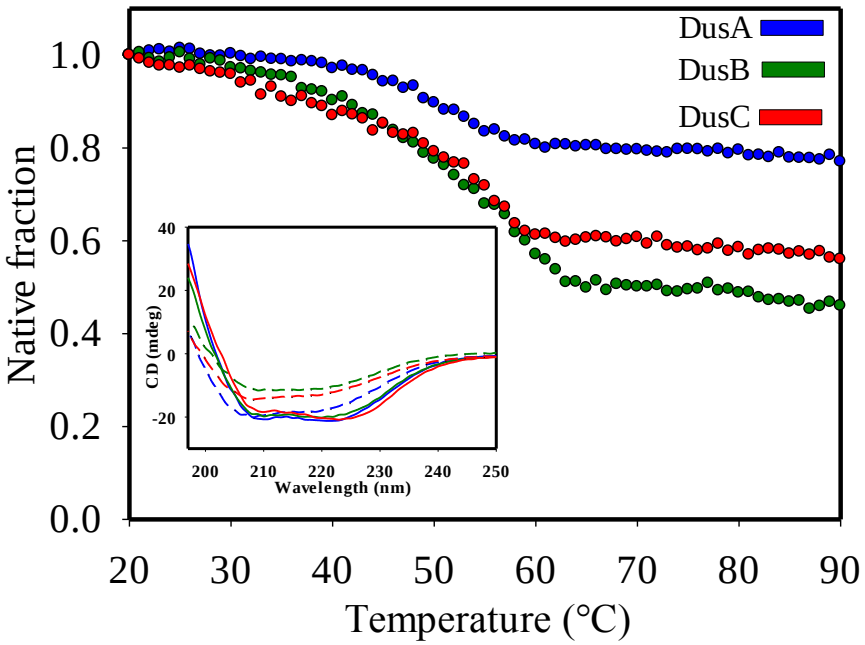
Supplementary Figure 6. (A) Crystallographic unit cell showing two molecules of *E. coli* DusB in blue and green cartoon with the FMN cofactor in yellow sticks. **(B)** Stereoview of a 2Fo-Fc σA-weighted electron density map of the FMN binding site contoured at 1 sigma. In green is showed the density when omitting FMN represented in yellow sticks while in blue is the density of the protein with its residues represented in green sticks.



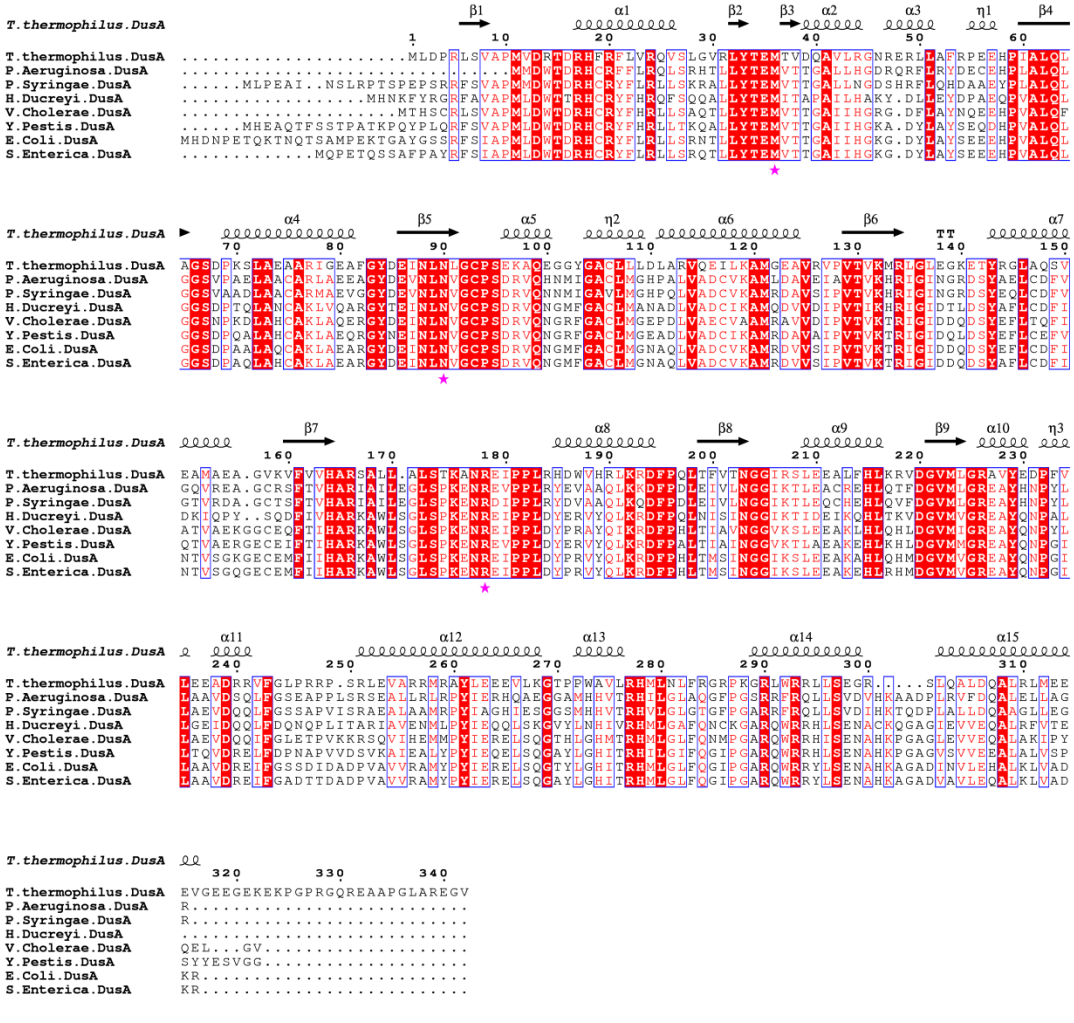
Supplementary Figure 7. Structure of the interface between the TIM barrel (residues shown in green) and HD (in blue) in *E. coli* DusB. The FMN is represented in yellow sticks and the protein surface is in grey surface.



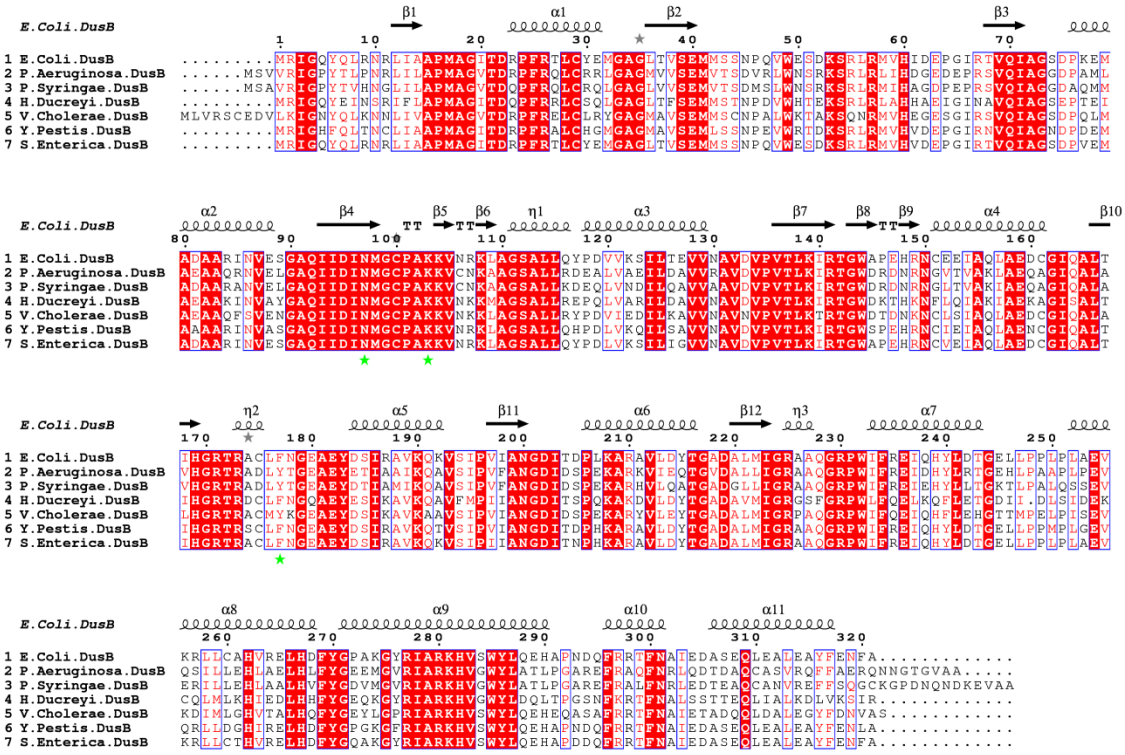
Supplementary Figure 8. (A) Guinier plot of *E. coli* DusB. **(B)** Electron-electron pair distribution of *E. coli* DusB derived from its SAXS curve using Primus.



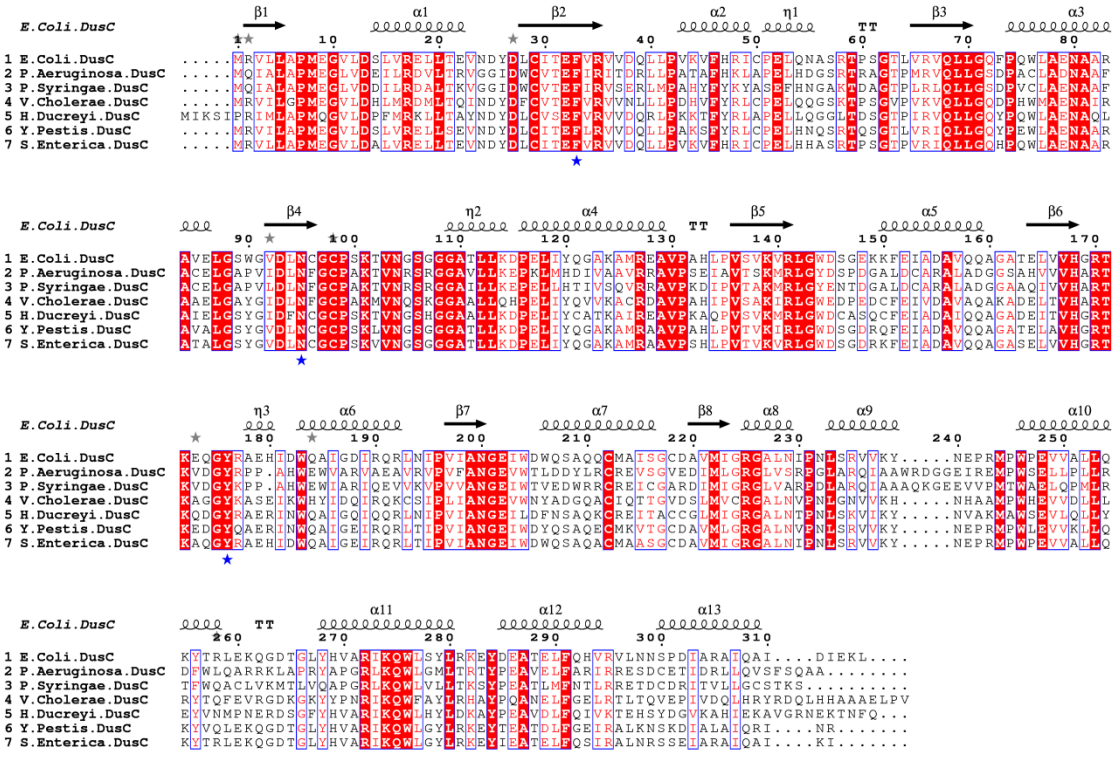
Supplementary Figure 9. Thermal stability of *E.coli* Dus enzymes. The dotted curves represent the normalized CD signal at 222 nm as function of temperature. The inset figure is the CD spectra of the respective *E. coli* Dus recorded at 20°C (solid lines) and at 90°C (short dash lines). The curves corresponding to DusA, B and C are colored in blue, green and red, respectively.



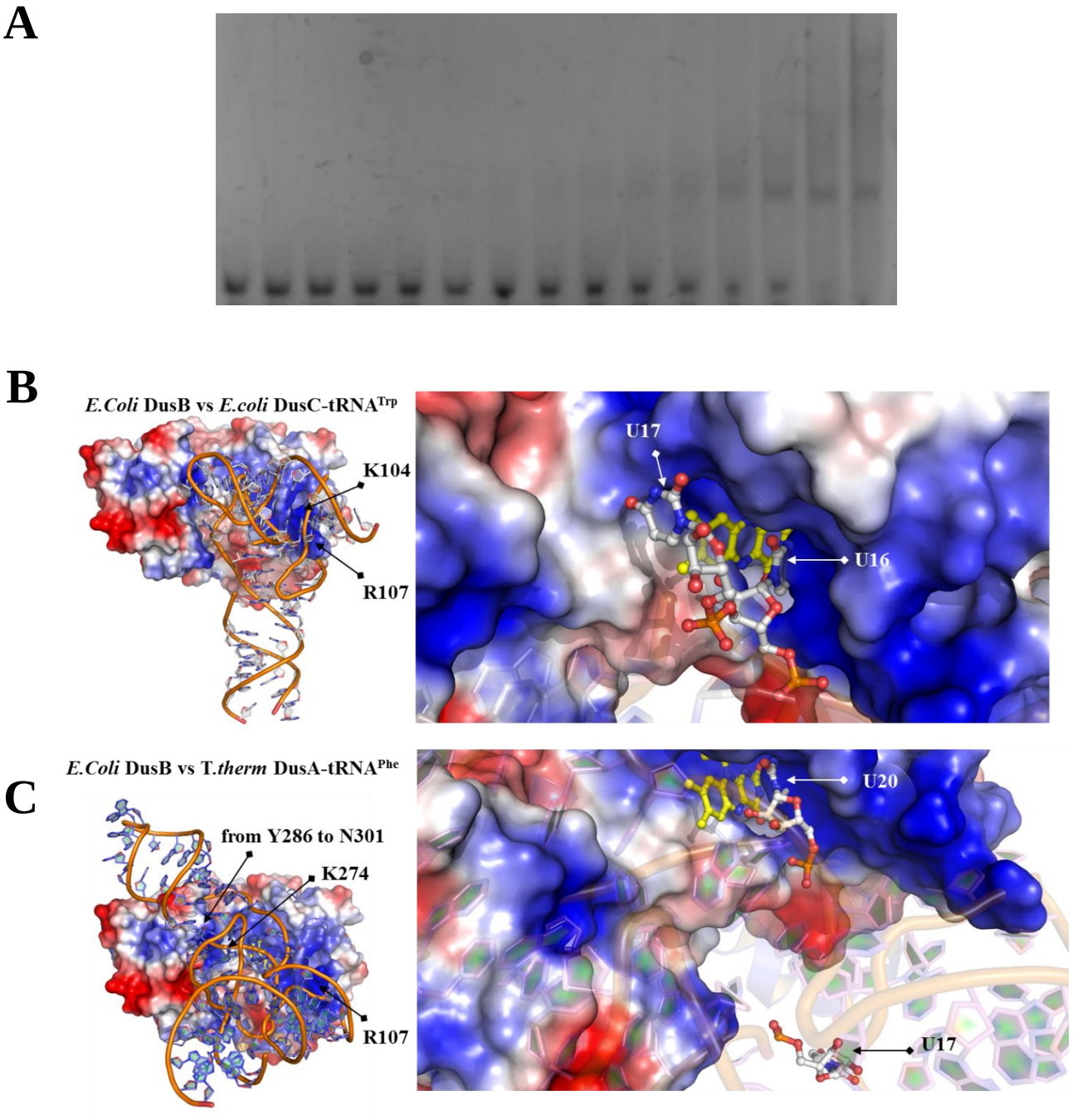
Supplementary Figure 10. Sequence alignment of DusA subfamily showing conserved residues (highlighted in red) and similarities (red letters). Residues implicated in stabilization of the target uridine are indicated with a magenta star. The secondary structure corresponding to the primary sequence is indicated above the alignment.



Supplementary Figure 11. Sequence alignment of DusB subfamily showing conserved residues (highlighted in red) and similarities (red letters). Residues implicated in stabilization of the target uridine are indicated with a green star. The secondary structure corresponding to the primary sequence is indicated above the alignment.



Supplementary Figure 12. Sequence alignment of DusC subfamily showing conserved residues (highlighted in red) and similarities (red letters). Residues implicated in stabilization of the target uridine are indicated with a blue star. The secondary structure corresponding to the primary sequence is indicated above the alignment.



Supplementary Figure 13. (A) Electrophoretic mobility shift assay of *E. coli* DusB with bulk tRNA extracted from *E. coli ΔDusB778::kan*. **(B)** Structural overlay of *E. coli* DusB on *E. coli* DusC in complex with *E. coli* $tRNA^{Trp}$ (4YCP). **(C)** Structural overlay of *E. coli* DusB on *T. thermophilus* DusA in complex with *T. thermophilus* $tRNA^{Phe}$ (3B0V). Insets are zooms near the active sites with FMN shown in yellow sticks while the target uridines are indicated with an arrow.

Supplementary Table 1- Summary of data collection and refinement statistics

	Escherichia coli DusB PDB 6EI9
Data collection	
Space group	$P4_3$
Cell dimensions	
a, *b*, *c* (Å)	108.48, 108.48, 70.31
α, β, γ (°)	90, 90, 90
Resolution (Å)	42.95-2.55 (2.641-2.55)
R_{sym} or R_{merge}	0.05516 (1.753)
I / σI	18.96 (0.84)
Completeness (%)	99.93 (100)
CC(1/2)	1 (0.439)
Redundancy	7.8 (8.1)
Refinement	
Resolution (Å)	42.95-2.55
No. reflections	26834 (2675)
R_{work} / R_{free}	0.19/0.24
No. atoms	4851
Protein	4604
Ligand/ion	129
Water	118
B-factors	
Protein	91.68
Ligand/ion	114.05
Water	74.52
R.m.s. deviations	
Bond lengths (Å)	0.015
Bond angles (°)	1.88

The data set was collected from a single crystal