

Supplementary online material

Distinctive contributions of the ribosomal P-site elements m²G966, m⁵C967 and the C-terminal tail of the S9 protein in the fidelity of initiation of translation in *Escherichia coli*

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Supplementary Methods:

Growth curve analysis: Overnight cultures (four replicates/colonies of each strain) were grown in test tubes in LB (2 ml) with the desired antibiotic(s) at 37 °C to saturation. A 200 µl volume of a thousand fold dilution (10^{-3}) of these cultures was taken in honeycomb plates with a capacity of 400 µl per well and placed in automated Bioscreen C growth readers (Oy Growth, Helsinki, Finland). O.D.₆₀₀ was measured every hour. Mean values and standard deviation were calculated and plotted with time on X-axis against O.D.₆₀₀ on Y-axis using Graph Pad Prism™ software.

Generation of the S9 tail deletion (S9Δ3:kan) strain: The Kan^R cassette was amplified from pKD4 by PCR using S9del3fp and S9del3rp (Table S3) and inserted at the C-terminus of the S9 protein gene in *E. coli* to delete the last three amino acids of the protein (37). The PCR conditions (using 100 ng pKD4 DNA) were 95 °C for 5 min, 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min 30 s, 72 °C for 10 min using Phusion DNA polymerase (2 U µl⁻¹, Finnzymes). The PCR mix was treated with DpnI, the amplicon eluted following agarose gel (0.8%) electrophoresis and electroporated into *E. coli* DY330 at 1.8 kV, 25 µF and 200 Ω. The cells were recovered at 33 °C for 4-6 h and plated on Kan plates (35 µg ml⁻¹) at 33 °C. The strain was verified by PCR, cold sensitive phenotype and DNA sequence analysis.

Generation of the ΔrsmB, ΔrsmD, ΔrsmB ΔrsmD and S9Δ3 ΔrsmD strains: P1 mediated transductions (38) were used to mobilize ΔrsmB::kan and ΔrsmD::kan alleles. The donor strain (ΔrsmD::kan) for ΔrsmD knockout was obtained from *E. coli* genetic stock center (39) and rsmB knockout (ΔrsmB::kan) was generated in the laboratory (14). To construct double knockout of rsmB and rsmD, marker-less ΔrsmD strain was generated by curing the kan marker using pCP20 (37). Kan^S colony was used as recipient for transduction with P1 raised on *E. coli* DY330

ΔrsmB::kan. For generation of the S9Δ3 *ΔrsmD* strain, P1 raised on *E. coli* DY330 S9Δ3:*kan* was used for transduction on pCP20 cured *ΔrsmD* strain. The strains were verified by PCR.

Verification of the S9 tail deletion strain (S9Δ3:*kan*): The strain was verified by PCR using a flanking primer S9_fp and S9del3rp (Table S3). The PCR conditions were an initial denaturation at 95 °C for 5 min, followed by 20 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min 30 s, 72 °C for 10 min using Taq DNA polymerase. A PCR product of ~2 kb confirmed the deletion. Further validation was done by checking for its cold-sensitive phenotype and by DNA sequencing.

Verification of the *rsmD* knockout by PCR: *rsmD* knockouts were verified by *YhhF*_upfp and *YhhF*_dnrp (Table S3) as forward and reverse primers, respectively. The PCR condition were an initial denaturation at 94 °C of 4 min, followed by 20 cycles of 94 °C for 1 min, 54 °C for 1 min, 70 °C for 2 min and a final extension of 10 min at 70 °C using Taq DNA polymerase. A 1.6 kb band visualized on a 1% agarose gel confirmed the knockout.

Verification of the *rsmB*, *rsmD* and (*rsmB rsmD*) knockouts: These knockouts were verified by *rsmB*_up fp: and *rsmB*_dnrp (Table S3). The PCR condition were initial denaturation at 94 °C of 4 min followed by 20 cycles at 94 °C for 1 min, 55 °C for 1 min, an extension of 2 min 45 s at 70 °C and a final extension of 10 min at 70 °C using Taq DNA polymerase. A 2.8 kb band on a 1% agarose gel confirmed the knockouts.

Acid urea PAGE and Northern blot analysis: A 6.5% acid urea PAGE with 8 M urea followed by a Northern blot was performed as described earlier (40). The 5'- end labeled DNA oligomer probes, UV5G or His_ini_rp (Table S3) were prepared for Figs. S8 or S4, respectively, and hybridized to the membrane at 42 °C.

Purification of tRNA encoded by *metY*_{GUG}: Total tRNA was isolated from *E. coli* BW $\Delta metY::cm$ transformed with pCAT_{CAC}*metY*_{GUG} (Q⁺) and SA $\Delta metY::cm$ transformed with pCAT_{CAC}*metY*_{GUG} (Q⁻) by extraction with water-saturated phenol (40). About 100 O.D.₂₆₀ units of this preparation were loaded onto a 45 cm long 15% native PAGE of 1.33 mm thickness and run at 300 V till both of the dyes (bromophenol blue and xylene cyanol) ran out. The tRNA^{fMet} band was visualized by UV shadowing, excised and diced into small cubes. The tRNA was eluted in 30 ml elution buffer (50 mM Tris-HCl, pH 8.0, 5 mM Na₂EDTA, 0.1 M LiCl) twice by incubating overnight at 37 °C with gentle agitation. The eluents were pooled, filtered through glass wool, and passed through a 1 ml DEAE cellulose column equilibrated with 50 mM Tris-HCl, pH 8.0 and 0.1 M LiCl. The column was washed with 6-10 volumes of wash buffer (50 mM Tris-HCl, pH 8.0 and 0.1 M LiCl) and the tRNA was eluted in 0.5 ml fractions using elution buffer (50 mM Tris-HCl, pH 8.0 and 1 M LiCl). The fractions containing RNA (as determined by A₂₆₀) were pooled and precipitated with 3 volumes of ethanol, recovered by centrifugation in an SS-34 rotor (17,000 rpm, 1 h, 4 °C) and resuspended in RNase free water.

Preparation of the tRNA hydrolysate: About 10 µg of *metY*_{GUG} encoded tRNA (in a volume of 25 µl water) was heated at 90 °C for 3 min, snap chilled on ice and used for digestion. The reaction mix consisting of 10 µg tRNA, 5 µl nuclease P1 (2 mg ml⁻¹ in 50 mM sodium acetate buffer, pH 5.3), 2 µl of 20 mM ZnSO₄ and 0.5 µl calf intestinal alkaline phosphatase (10 U µl⁻¹) was incubated at 37 °C for 1 h followed by addition of 15 µl of 1 M Tris-HCl, pH 7.9 and further incubation for 1 h. The hydrolysate was used directly for HPLC.

Separation of nucleosides by HPLC: HPLC was done on a Dionex UltiMate 3000 machine using a Dionex Acclaim 120 4.6 mm x 150 mm C-18 reverse phase column. The separation of nucleosides was done using a multistep gradient of 5 mM ammonium acetate and 40%

acetonitrile as described previously (41). The UV spectrum was acquired at 260 nm using Chromeleon software provided by the manufacturer. Fractions (0.3 ml) were collected from 5 independent runs for each sample and the peaks corresponding to adenosine, guanosine, cytidine and uridine were identified (41). The fractions between guanosine and adenosine peaks were pooled, dried by vacuum evaporation, resuspended in water and used for spotting MALDI plates.

MALDI analysis: RNA hydrolysate (1 μ l) was mixed with 1 μ l alpha-cyano-4-hydroxycinnamic acid matrix (CCA, 10 mg ml⁻¹ prepared in 50% acetonitrile + 0.1% TFA) and spotted onto MALDI plates. The samples were allowed to air dry and spectra were acquired using a Bruker Daltonics AutoFlex III Smart Beam Mass Spectrometer in reflectron mode. Data acquisition was done in positive ion mode using a 337 nm laser with 30% laser intensity and mass range 100 Da to 500 Da. Data was analyzed using Bruker Daltonics Flex Analysis software.

Supplementary Results and Discussion:

Initiation by tRNA_{GUG}^{fMet} and tRNA_{QUG}^{fMet}: The initiation activity of tRNA^{fMet} with GUG anticodon (tRNA_{GUG}^{fMet}, encoded by *metY*_{GUG}) was comparable in *E. coli* BW and *E. coli* SA strains (Fig. S2). This initiator tRNA mutant was constructed based on the earlier research findings (42). Although the tRNAs with GUN anticodon are modified by queuosine at position 34 (27), its presence in the background of initiator tRNAs was unknown. Hence, the presence of this modification was confirmed by MALDI mass spectrometric analysis (Fig. S3). An acid urea PAGE (Fig. S4) followed by Northern blot using the His_ini_rp probe was done to check whether the tRNA^{fMet} was aminoacylated/formylated (Fig. S4A and S4B). The tRNA^{fMet} (GUG anticodon) isolated from *E. coli* SA separated into deacylated and formylated forms when expressed from a moderate copy plasmid, pCAT_{CAC}*metY*_{GUG} (Fig. S4A, lane 2) or a high copy plasmid,

pTZ $metY_{GUG}$ (Fig S4A, lane 4). However, when the tRNA isolated from *E. coli* BW (harboring pCAT $_{CAC}metY_{GUG}$) was used, in place of the formylated tRNA band a smear was seen which indicated heterogeneous population (Fig. S4B, lane 2). Further, when the tRNA was isolated from *E. coli* BW harboring pTZ $metY_{GUG}$, the queuosine modification appeared partial (Fig. S4B, lanes 3, 4). A band in Fig. S4B (lanes 3 and 4) migrating faster than the deacylated band in lane 1 corresponds to unmodified and deacylated tRNA. This Northern blot was done with the aim to see whether there are differences in aminoacylation of the $metY_{GUG}$ encoded tRNA with and without queuosine modification. We observed that while these tRNA fMet with either GUG or QUG anticodons were reasonably aminoacylated/formylated for the present study, the queuosine containing tRNA fMet species could be a heterogeneous population because the pathway of modification involves multiple steps and a non-native tRNA may not be competently modified. Hence, for the simplicity of interpretations, we used the *E. coli* SA strain.

Growth curve analysis of SA and its $\Delta rsmB$, $\Delta rsmD$, $\Delta rsmB \Delta rsmD$ derivatives: In agreement with the published data (32), the strains knocked out for the methyltransferases grew as well as the parent strain, *E. coli* SA at 37 °C (Fig. S5A).

Growth curve analysis of SA and its $\Delta rsmD$, S9 Δ 3 and S9 Δ 3 $\Delta rsmD$ derivatives: The S9 Δ 3 strain and its derivatives grew normally at 37 °C (Fig. S5B). The S9 Δ 3 strain is reported to grow normally at 37 °C (8).

Initiation with CAC and CAU initiation codons as assessed by immunoblotting shows that the methylated nucleosides disfavored tRNA $^{fMet}_{GUG}$: Figs. 2A and 2B show that the methylated nucleosides disfavored tRNA $^{fMet}_{GUG}$. This was validated by immunoblotting for CAT and β -lactamase proteins, where the latter acted as a plasmid copy number control. As shown in Fig. S6, the copy number of the plasmid did not change as shown by β -lactamase protein levels while

the CAT protein levels changed with both the codons, CAC and CAU in the methyltransferase knockout strains. Nonetheless, all the CAT activities were normalized to β -lactamase activities to avoid errors due to any minor changes in the plasmid copy number.

Verification of S9 Δ 3 strain: The strain was validated by checking for its cold sensitivity phenotype (Fig. S7A), followed by PCR (using one flank primer, S9_fp and one deletion primer, S9del3rp, Fig S7B) and sequence analysis of the amplicon (~ 2 kb) obtained by a primer 415 bp upstream of the deletion (S9_fp) and an internal primer, 80 bp upstream of deletion (S9int_fp, Fig. S7C).

Formylation status of tRNA^{fMet}_{GUA} and tRNA^{fMet}_{GAU}: The tRNA^{fMet}_{GUA} (encoded by *metY*_{GUA}) which decodes UAC, and tRNA^{fMet}_{GAU} (encoded by *metY*_{GAU}) which decodes AUC were also examined for formylation levels (Fig. S8). The tRNA^{fMet}_{GAU} (*metY*_{GAU}) gets formylated and shows initiation [Fig. S8, (26)]. The formylated form of tRNA^{fMet}_{GUA} encoded by *metY*_{GUA} was undetectable on acid urea PAGE followed by Northern blot (Fig. S8).

Table S1: List of strains used in the study

<i>E. coli</i> strain	Genotype	Reference
BW25113 (or BW)	<i>lacI</i> ^q <i>rrnB</i> T14 <i>lacZ</i> WJ16 <i>hsdR</i> 514 <i>araB</i> ADAH33 Δ <i>rha</i> BADLD78	Nara Inst., Japan.
SA	BW Δ <i>tgt</i> :: <i>kan</i> , <i>tgt</i> deficient strain renamed as SA for convenience	Nara Inst., Japan.
DY330	W3110 Δ <i>lacU</i> 169 <i>gal</i> 490 [λ c1857 Δ (<i>cro</i> - <i>bioA</i>)]	(43)
SA Δ <i>rsmB</i> :: <i>kan</i> or SA Δ <i>rsmB</i>	SA strain derivative with <i>rsmB</i> deletion	This study
SA Δ <i>rsmD</i> :: <i>kan</i> or SA Δ <i>rsmD</i>	SA derivative with <i>rsmD</i> deletion	This study
SA Δ <i>rsmB</i> Δ <i>rsmD</i> :: <i>kan</i> or SA Δ <i>rsmB</i> Δ <i>rsmD</i>	SA strain derivative with deletion of <i>rsmB</i> and <i>rsmD</i>	This study
SA S9 Δ 3: <i>kan</i> or SA S9 Δ 3	SA strain derivative with C-terminus of S9 tail deleted	This study
SA S9 Δ 3 Δ <i>rsmB</i> :: <i>kan</i> or SA S9 Δ 3 Δ <i>rsmB</i>	SA strain derivative with C-terminus of S9 tail deleted along with <i>rsmB</i>	This study
SA S9 Δ 3 Δ <i>rsmD</i> :: <i>kan</i> or SA S9 Δ 3 Δ <i>rsmD</i>	SA strain derivative with C-terminus of S9 tail deleted along with <i>rsmD</i>	This study
SA S9 Δ 3 Δ <i>rsmB</i> Δ <i>rsmD</i> :: <i>kan</i> or SA S9 Δ 3 Δ <i>rsmB</i> Δ <i>rsmD</i>	SA strain derivative with C-terminus of S9 tail deleted along with <i>rsmD</i> and <i>rsmB</i>	This study
SA Δ <i>metY</i> :: <i>cm</i> or SA Δ <i>metY</i>	SA strain derivative harboring a deletion of an initiator tRNA gene - <i>metY</i>	This study
BW Δ <i>metY</i> :: <i>cm</i> or BW Δ <i>metY</i>	BW strain derivative harboring a deletion of <i>metY</i> gene	This study

Table S2: List of plasmid used in the study

Plasmid	Relevant Feature	Source/Reference
pCAT _{am1}	Renamed from pRSVCAT _{am1.2.5} . A pBR322 derivative harboring CAT reporter gene with UAG initiation codon.	(19)
pCAT _{am1} <i>metY</i> _{CUA}	Renamed from pRSVCAT _{am1.2.5} trnfMU35A36. A pBR322 derivative harboring CAT reporter gene with UAG initiation codon and expressing tRNA ^{fMet} with CUA anticodon.	(19)
pCAT _{am1} <i>metY</i> _{CUA/3GC}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutations at positions 29:41, 30:40 and 31:39 (from G:C, G:C and G:C to U:A, C:G and A:U).	(31)
pCAT _{am1} <i>metY</i> _{CUA/AUGU}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutation at positions 29:41 and 31:39 (from G:C base pairs to A:U and G:U base pairs, respectively).	(44)
pCAT _{am1} <i>metY</i> _{CUA/1GC(AU)}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutation at positions 29:41 from G:C to A:U.	(44)
pCAT _{am1} <i>metY</i> _{CUA/1GC(GU)}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutation at positions 29:41 from G:C to G:U.	(44)
pKD4	Amp ^R , Kan ^R , <i>kan</i> marker is flanked by FRT sequences.	(37)
pCAT _{AUG}	Renamed from pRSVCAT _{2.5} plasmid with AUG as initiation codon	(19)
pCAT _{UUG}	AUG initiation codon of pCAT _{AUG} mutated to UUG	This study
pCAT _{GUG}	AUG initiation codon of pCAT _{AUG} mutated to GUG	This study
pCAT _{CUG}	AUG initiation codon of pCAT _{AUG} mutated to CUG	This study
pCAT _{AUU}	AUG initiation codon of pCAT _{AUG} mutated to AUU	This study
pCAT _{AUA}	AUG initiation codon of pCAT _{AUG} mutated to AUA	This study
pCAT _{ACG}	AUG initiation codon of pCAT _{AUG} mutated to ACG	This study
pCAT _{CAC} <i>metY</i> _{GUG}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} wherein UAG initiation codon of CAT was mutated to CAC and anticodon of <i>metY</i> was changed from CUA to GUG	This study
pCAT _{CAU} <i>metY</i> _{GUG}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with UAG initiation codon of CAT was mutated to CAU and anticodon of <i>metY</i> was changed from CUA to GUG	This study
pCAT _{UAC} <i>metY</i> _{GUA}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with UAG initiation codon of CAT mutated to UAC and anticodon of <i>metY</i> changed from CUA to GUA	This study
pCAT _{AUC} <i>metY</i> _{GAU}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with UAG initiation codon of CAT mutated to AUC and anticodon of <i>metY</i> changed from CUA to GAU	This study
pCAT _{AUA} <i>metY</i> _{GAU}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with UAG initiation codon of CAT mutated to AUA and anticodon of <i>metY</i> changed from CUA to GAU	This study
pCAT _{AUU} <i>metY</i> _{GAU}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with UAG initiation codon of CAT mutated to AUU and anticodon of <i>metY</i> changed from CUA to GAU	This study
pTZ <i>metY</i> _{GUG}	<i>metY</i> _{GUG} gene cloned onto a high copy pTZ19R plasmid	This study
pCP20	An Amp ^R and Cm ^R plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis	(45)

Table S3: List of oligonucleotides used in the study

Oligonucleotide	Sequence (5' to 3')
<i>rsmB</i> fp	ctctcgtcgggaatggtttg
<i>rsmB</i> rp	ctgctctggtgcgatcagat
S9del3fp	gtcggctcgcgtaaagcacgctcgtcctccagttctagtaagcttgttaggctggagctgcttcg
S9del3rp	ttttcgaaaattgtttctgccggagcagaagccaacatatgaatatcctcctta
S9int_fp	gctggcttcgttactcg
S9_fp	gtaacgagcacaaccacgcg
<i>YhhF</i> _upfp	ttcatggcacagcgtaaacg
<i>YhhF</i> _dnrp	aacgaagatattcagcgggc
<i>rsmB</i> _up fp	ctctcgggaatggtttg
<i>rsmB</i> _dnrp	ctgctctggtgcgatcagat
<i>Kan</i> rp	actaacaggcggacatcttcgctccaagagtgactccgctgaggctgctcctcg
pCAT _{2.5GUG} fp	ctaaggaagctaaagtggacaaaaaaccac
pCAT _{2.5GUG} rp	gtggtttttgtccacttttagcttccttag
pCAT _{2.5UUG} fp	ctaaggaagctaaattggacaaaaaaccac
pCAT _{2.5UUG} rp	gtggtttttgtccaatttagcttccttag
pCAT _{2.5CUG} fp	ctaaggaagctaaactggacaaaaaaccac
pCAT _{2.5CUG} rp	gtggtttttgtccagtttagcttccttag
pCAT _{2.5AUA} fp	taaggaagctaaaatagacaaaaaaccac
pCAT _{2.5AUA} rp	gtggtttttgtctatttttagcttccttag
pCAT _{2.5AUU} fp	ctaaggaagctaaaattgacaaaaaaccac
pCAT _{2.5AUU} rp	gtggtttttgtcaatttttagcttccttag
pCAT _{2.5AUC} fp	ctaaggaagctaaaatcgacaaaaaaccac
pCAT _{2.5AUC} rp	gtggtttttgtcgatttttagcttccttag
pCAT _{2.5ACG} fp	ctaaggaagctaaaacggacaaaaaaccac
pCAT _{2.5ACG} rp	gtggtttttgtccgttttagcttccttag
UV5G	gaaccgacgatcttcgg
His_ini_rp	cgatctcgggttcacagcccgcagagcta

Table S4: List of oligonucleotides used for mutagenesis of CAT constructs

Plasmid	Primer Fp for codon mutation (5'-3')	Primer Fp for anticodon mutation (5'-3')
pCAT _{CAC} <i>metY</i> _{GUG}	ctaaggaagctaaacacgacaaaaaaccac	tagctcgtcgggctgtgaacccgaagatcg
pCAT _{CAU} <i>metY</i> _{GUG}	ctaaggaagctaaacaugacaaaaaaccac	tagctcgtcgggctgtgaacccgaagatcg
pCAT _{UAC} <i>metY</i> _{GUA}	ctaaggaagctaaauacgacaaaaaaccac	tagctcgtcgggctgtaaacccgaagatcg
pCAT _{UAU} <i>metY</i> _{GUA}	ctaaggaagctaaatatgacaaaaaaccac	tagctcgtcgggctgtaaacccgaagatcg
pCAT _{AUC} <i>metY</i> _{GAU}	ctaaggaagctaaaatcgacaaaaaaccac	tagctcgtcgggctgataacccgaagatcg
pCAT _{AUA} <i>metY</i> _{GAU}	ctaaggaagctaaaatagacaaaaaaccac	tagctcgtcgggctgataacccgaagatcg
pCAT _{AUU} <i>metY</i> _{GAU}	ctaaggaagctaaaattgacaaaaaaccac	tagctcgtcgggctgataacccgaagatcg

Table S5: Codons initiating with the native tRNA^{fMet}_{CAU}

Codon	Anticodon	Amino acid
AUG	CAU	Methionine
GUG	CAU	Methionine
UUG	CAU	Methionine
AUU	CAU	Methionine
AUA	CAU	Methionine
AUC	CAU	Methionine
ACG	CAU	Methionine

Table S6: List of codons decoded by the respective tRNA^{fMet} mutants

Codon	tRNA^{fMet} anticodon	Amino acid
CAC	GUG	Not known
CAU	GUG	Not known
UAC	GUA	Not known
AUC	GAU	Isoleucine
UAG	CUA	Glutamine

Table S7: Detailed summary of the dynamically stable H-bonds

Detailed summary of the dynamically stable H-bonds (present in more than 40% of the trajectories) obtained for all the four pairs of models in the absence and presence of the S9 tail. The H-bonds reported are between the residues (both amino acids and nucleotides) of interest, namely between the anticodon:codon base pairs and those between the tRNA and S9 protein. The % of occurrence of each H-bond in the MD ensemble is also indicated.

AUG:CAU - S9 (WT)			AUG:CAU + S9 (WT)		
N4 RC	34 -> O6 RG	82 : 98.44	N4 RC	34 -> O6 RG	82 : 98.89
N1 RG	82 -> N3 RC	34 : 99.78	N1 RG	82 -> N3 RC	34 : 100.00
N2 RG	82 -> O2 RC	34 : 99.22	N2 RG	82 -> O2 RC	34 : 99.44
N6 RA	35 -> O4 RU	81 : 82.87	N6 RA	35 -> O4 RU	81 : 90.55
N3 RU	81 -> N1 RA	35 : 99.11	N3 RU	81 -> N1 RA	35 : 99.00
N3 RU	36 -> N1 RA	80 : 99.33	N3 RU	36 -> N1 RA	80 : 99.67
N6 RA	80 -> O4 RU	36 : 93.77	N6 RA	80 -> O4 RU	36 : 93.66
			Contact with S9:		
			NE ARG	213 -> O1P RA	35 : 57.29
			NH2 ARG	213 -> O1P RA	35 : 72.19
CAC:GUG - S9			CAC:GUG + S9		
N1 RG	34 -> N3 RC	82 : 99.33	N1 RG	34 -> N3 RC	82 : 100.00
N2 RG	34 -> O2 RC	82 : 99.56	N2 RG	34 -> O2 RC	82 : 99.89
N4 RC	82 -> O6 RG	34 : 96.11	N4 RC	82 -> O6 RG	34 : 99.44
N3 RU	35 -> N1 RA	81 : 99.44	N3 RU	35 -> N1 RA	81 : 99.89
N6 RA	81 -> O4 RU	35 : 93.99	N6 RA	81 -> O4 RU	35 : 98.44
N1 RG	36 -> N3 RC	80 : 99.56	N2 RG	36 -> O2 RC	80 : 100.00
N2 RG	36 -> O2 RC	80 : 99.89	N4 RC	80 -> O6 RG	36 : 95.22
N4 RC	80 -> O6 RG	36 : 98.00	N1 RG	36 -> N3 RC	80 : 98.00
			Contact with S9:		
			NE ARG	213 -> O2P RG	34 : 80.98
			NH2 ARG	213 -> O2P RG	34 : 95.66
			NH1 ARG	213 -> O2P RU	33 : 69.41
			NH2 ARG	213 -> O2P RU	33 : 86.21
			NZ LYS	203 -> O1P RG	30 : 76.97
			NZ LYS	203 -> O2P RG	31 : 86.87

CAU:GUG – S9

N2 RG 34 -> O4 RU 83 : 56.10
 N3 RU 82 -> O6 RG 34 : 74.13
 N3 RU 35 -> N1 RA 81 : 84.88
 N6 RA 81 -> O4 RU 35 : 78.72
 N1 RG 36 -> N3 RC 80 : 80.29
 N2 RG 36 -> O2 RC 80 : 81.97
 N4 RC 80 -> O6 RG 36 : 69.99

CAU:GUG + S9

N1 RG 34 -> O2 RU 82 : 63.14
 N3 RU 35 -> N1 RA 81 : 97.44
 N6 RA 81 -> O4 RU 35 : 68.49
 N1 RG 36 -> N3 RC 80 : 99.89
 N2 RG 36 -> O2 RC 80 : 99.78
 N4 RC 80 -> O6 RG 36 : 99.00
 O2' RU 82 -> O5' RU 83 : 80.18

Contact with S9:

NZ LYS 212 -> O2P RG 34 : 77.62
 NZ LYS 212 -> O2P RU 35 : 52.00
 NH1 ARG 213 -> O1P RG 31 : 58.91
 NH2 ARG 213 -> O1P RG 31 : 72.38

AUC:GAU – S9

N1 RG 34 -> N3 RC 82 : 99.89
 N2 RG 34 -> O2 RC 82 : 99.56
 N4 RC 82 -> O6 RG 34 : 98.44
 N6 RA 35 -> O4 RU 81 : 93.88
 N3 RU 81 -> N1 RA 35 : 99.89
 N3 RU 36 -> N1 RA 80 : 99.89
 N6 RA 80 -> O4 RU 36 : 92.99

AUC:GAU + S9

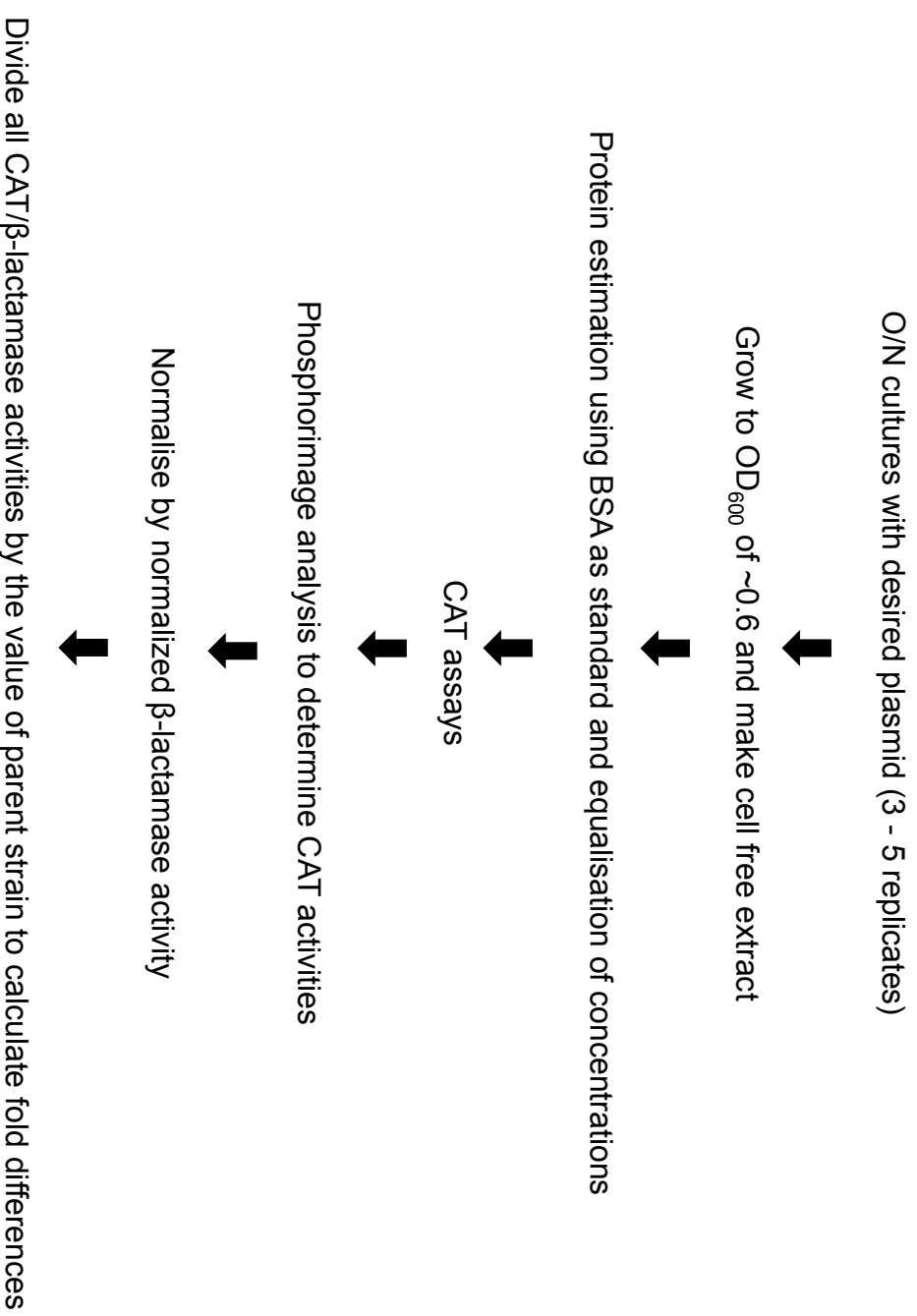
N1 RG 34 -> N3 RC 82 : 99.89
 N2 RG 34 -> O2 RC 82 : 99.44
 N4 RC 82 -> O6 RG 34 : 94.43
 N6 RA 35 -> O4 RU 81 : 71.94
 N3 RU 81 -> N1 RA 35 : 92.43

Contact with S9:

NZ LYS 212 -> O2P RG 34 : 73.39
 NZ LYS 212 -> O2P RA 35 : 80.07

References:

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Schematic of calculation of fold difference in CAT activity

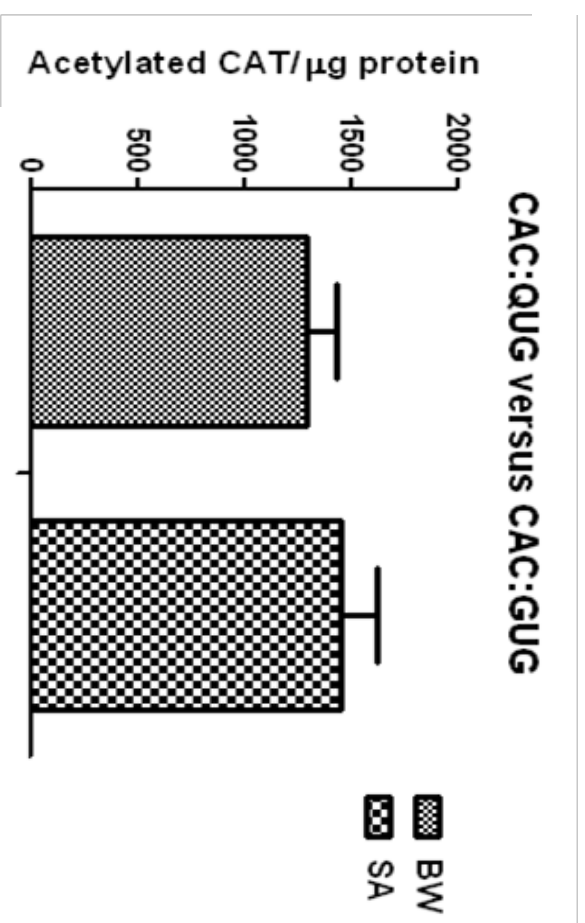


Figure S2: Relative CAT activities of *E. coli* BW (quenosine proficient, parent strain of SA) and *E. coli* SA harboring pCAT_{CAC^{metY}GUG}. Initiation with tRNA from *metY*_{GUG} (in SA) converted 1462 ± 374 pmol of Cm into acetylated form while initiation with tRNA with QUG anticodon [a mixed population of tRNAs with QUG and GUG anticodons] converted 1305 ± 294 pmol of Cm into its acetylated forms.

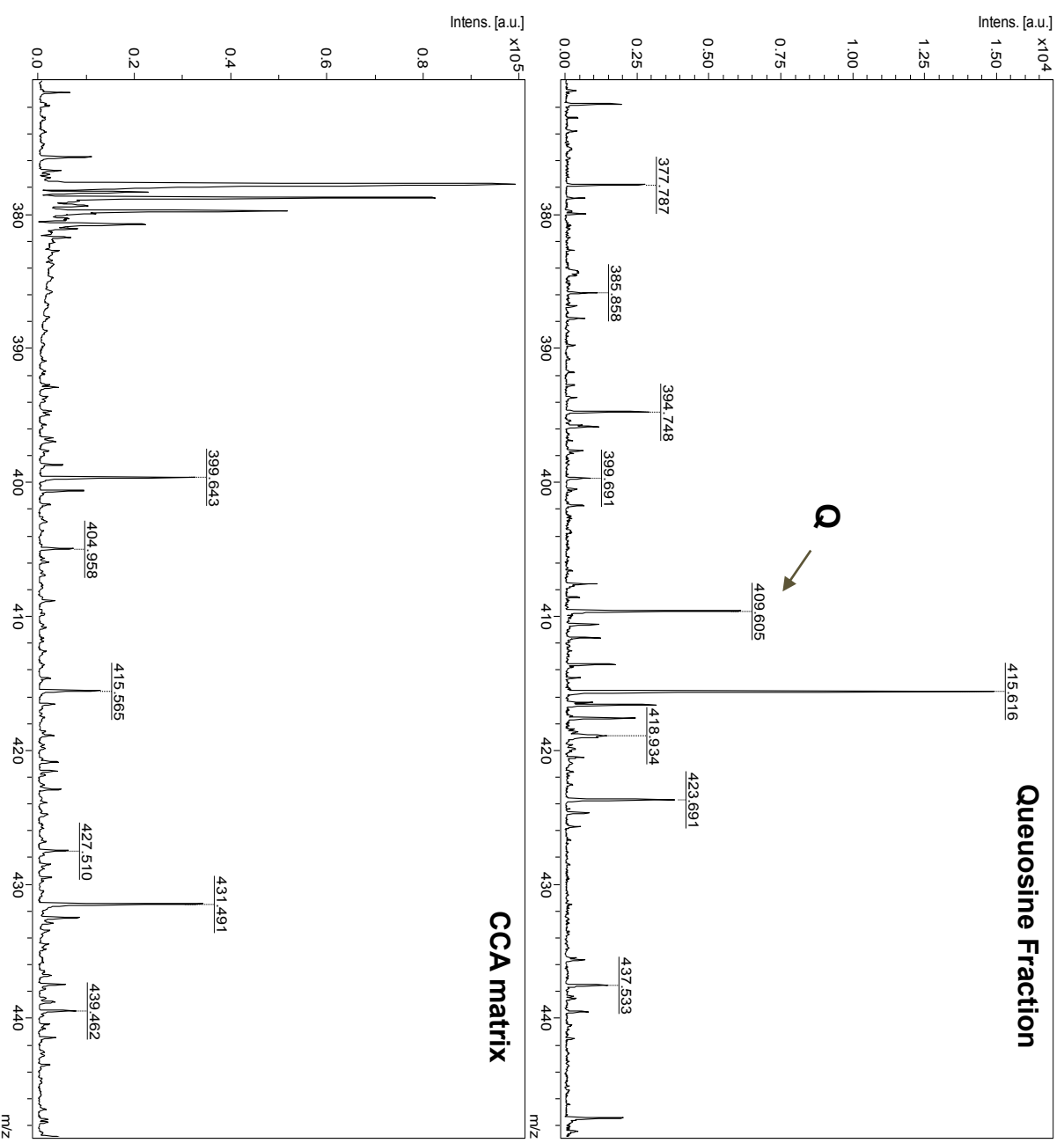


Figure S3: Mass spectrometric analysis to detect queuosine modification of in tRNA^{Met} mutant in *E. coli* BW. A peak at 409.5 Da (**Top panel**) absent in CCA matrix alone spectra (**Bottom panel**) showed the presence of queuosine modification.

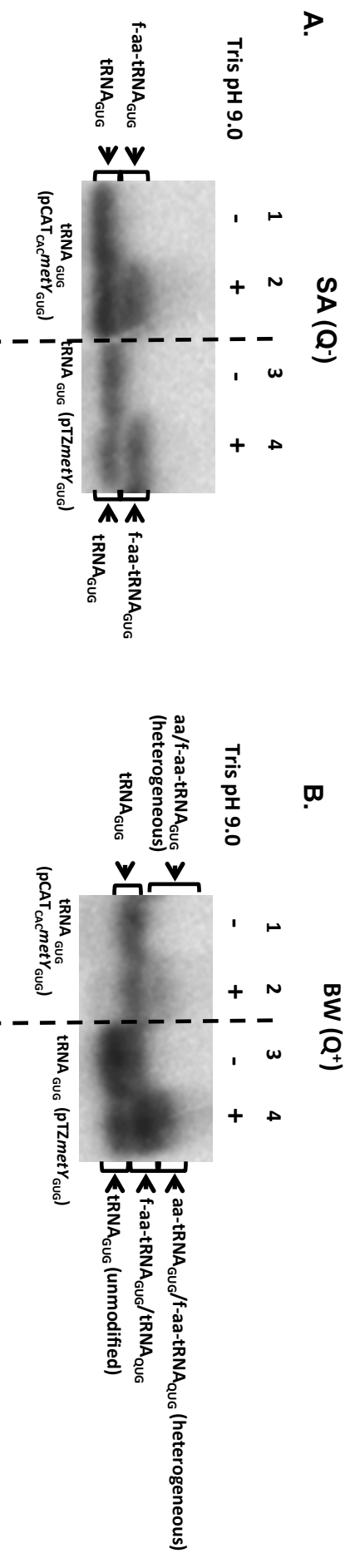


Figure S4: Analysis of the aminoacylation and formylation status of *metY*_{GUG} encoded tRNA from *E. coli* SA (panel A) and *E. coli* BW (panel B) using acid urea PAGE and Northern blot analysis. In lane 2 (panel B), smear in place of aminoacylated/formylated band, and in lanes 3, 4, a band migrating faster than that seen in lane 1 shows that upon over expression of tRNA^{Met} from pTZ*metY*_{GUG}, there is incomplete modification.

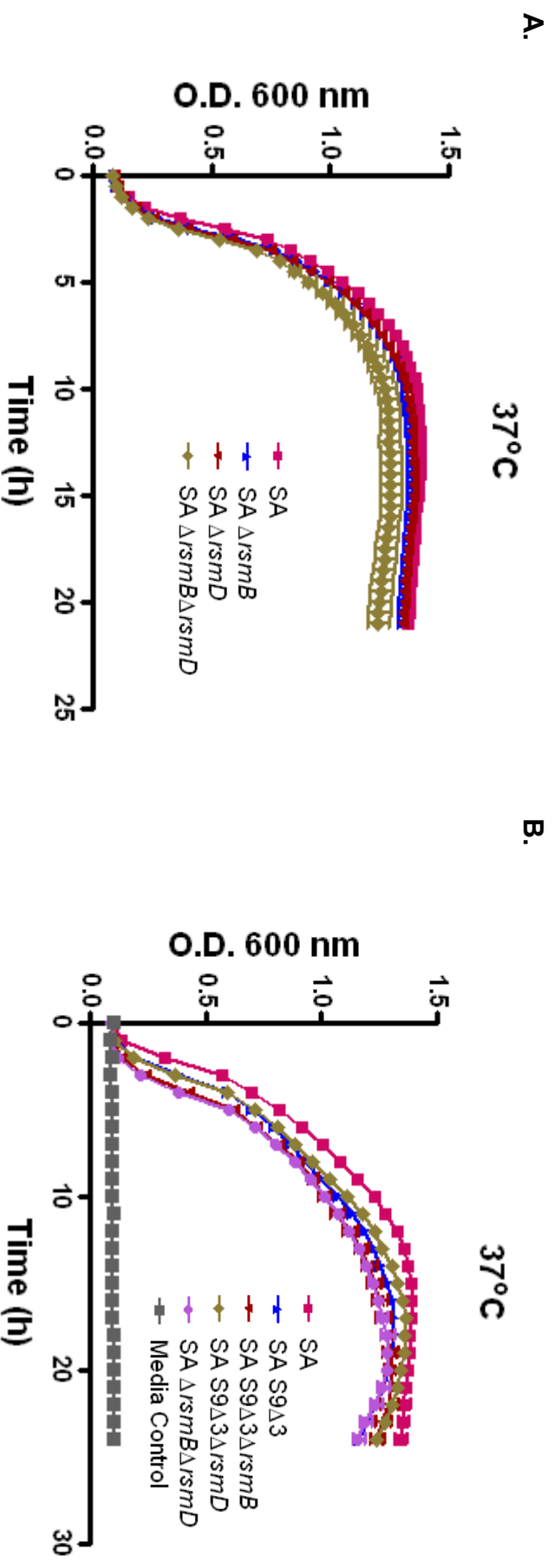


Figure S5: Growth analysis of knock out strains at 37 °C. **A.** Growth curve analysis of *E. coli* SA and its $\Delta rsmB$, $\Delta rsmD$, $\Delta rsmB\Delta rsmD$ derivatives. **B.** Growth curve analysis of *E. coli* SA, and its $\Delta rsmD$, S9 $\Delta 3$ and S9 $\Delta 3\Delta rsmD$ derivatives.

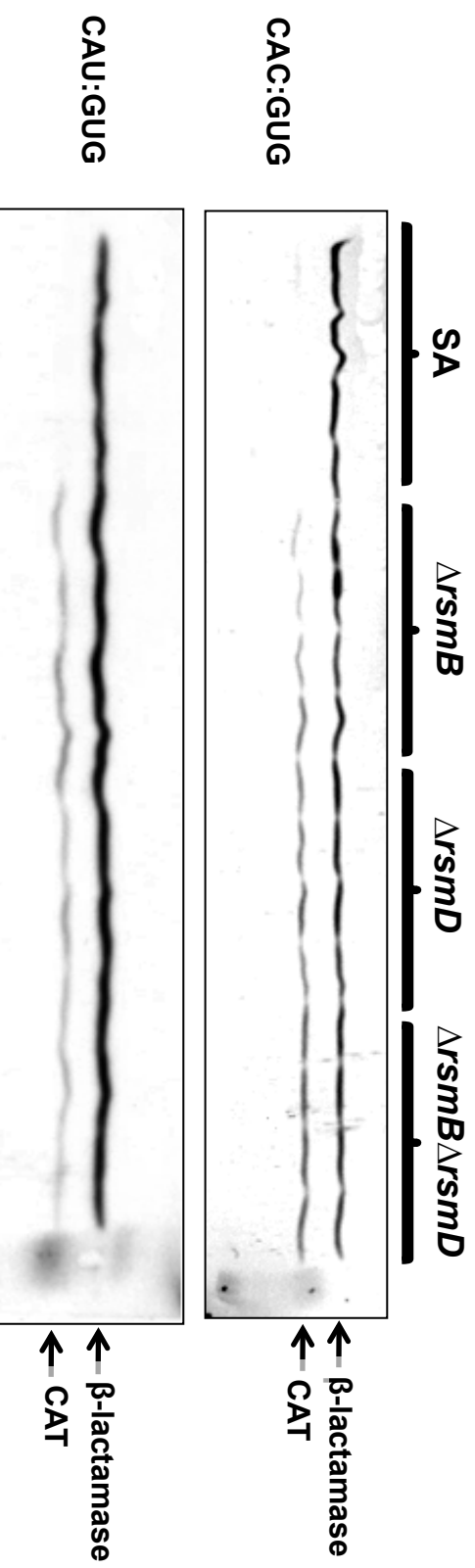


Figure S6: Initiation with CAC and CAU initiation codons with tRNA^{Met} (encoded by *metY*_{GUG}) as assessed by immunoblotting in the presence (SA) and absence of methyltransferases *rsmB*, *rsmD* or both. CAT protein levels are higher in methyltransferase deficient strains while levels of β -lactamase (control for plasmid copy number) remained the same in all the strains. Note: The lower levels of CAT protein produced in the SA background were not detected in this assay.

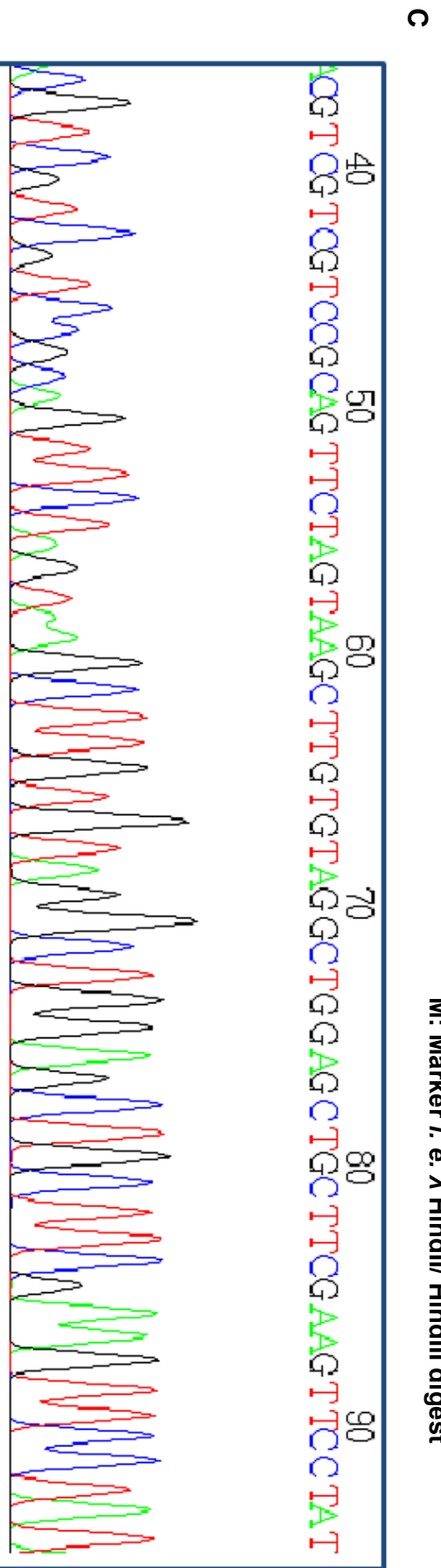
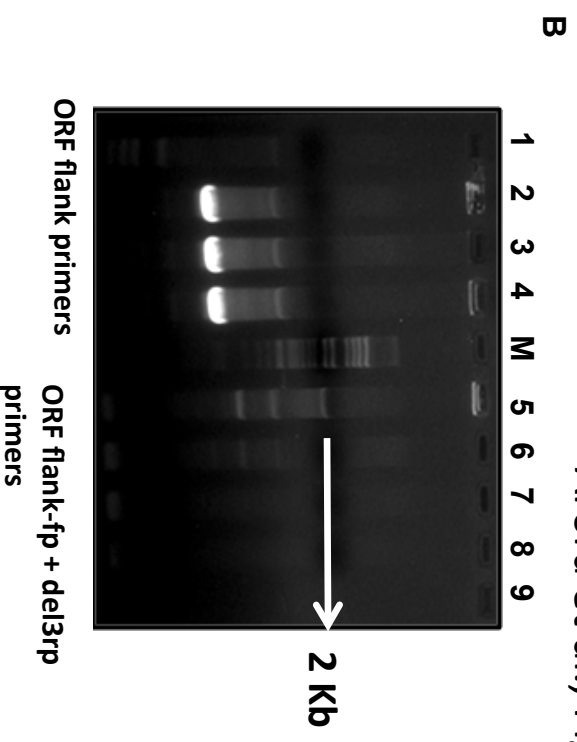
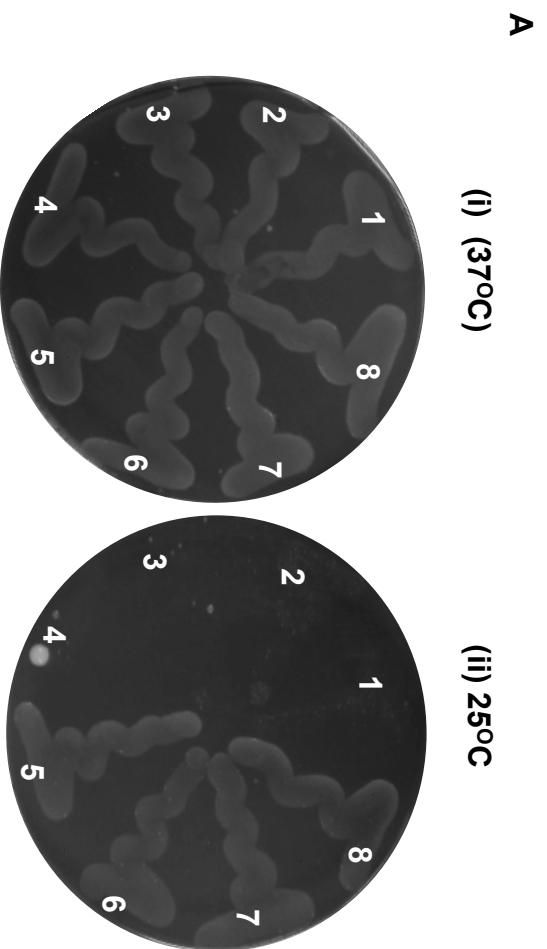


Figure S7: Confirmation of *E. coli* SA S9Δ3 (S9Δ3) strain. **A.** The S9Δ3 strain shows cold sensitivity compare sectors 1, 2, 3 and 4 (containing S9Δ3) with sector 5 (SA), 6, 8 (SA *ArsmD*) and 8 (SA *ArsmD*; markerless). **B.** Amplicon of ~ 2 kb confirmed the S9Δ3 knockout; M: λ HindIII/HindIII digest. **C.** Sequencing analysis of S9Δ3 ~ 2 kb PCR product using internal primer ~ 80 nucleotide upstream primer. Stop codons TAG and TAA follow after TTC codon between 50-60 position confirming the knockout.

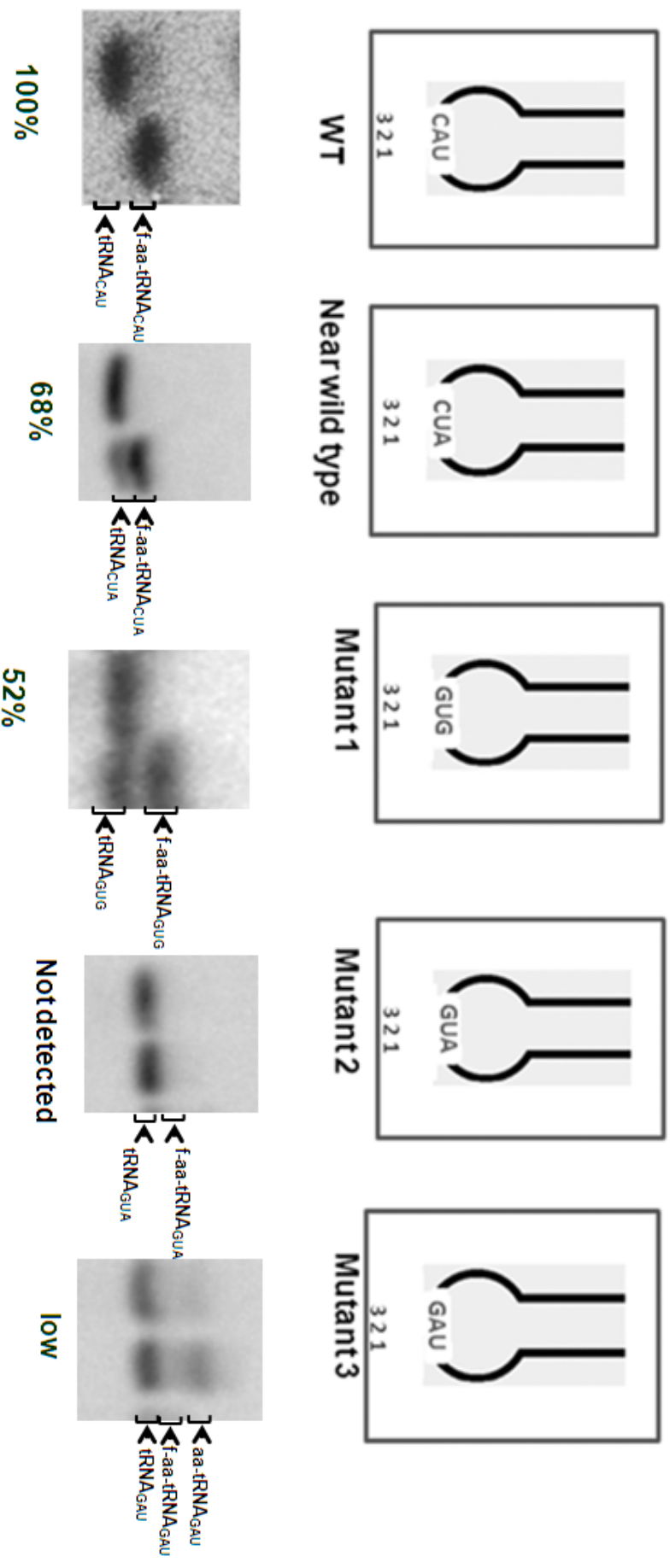


Figure S8: An acid urea PAGE followed by Northern blot analysis to check for aminoacylation/formylation status of the tRNAs. Level of aminoacylated and formylated forms is depicted below in the corresponding blot. Identity of the bands is as marked. Steady state accumulation of the aminoacylated and formylated forms of the GUA anticodon tRNA were not detected.