Cell Reports, Volume *41*

Supplemental information

tRNA methylation resolves codon usage bias at the limit of cell viability

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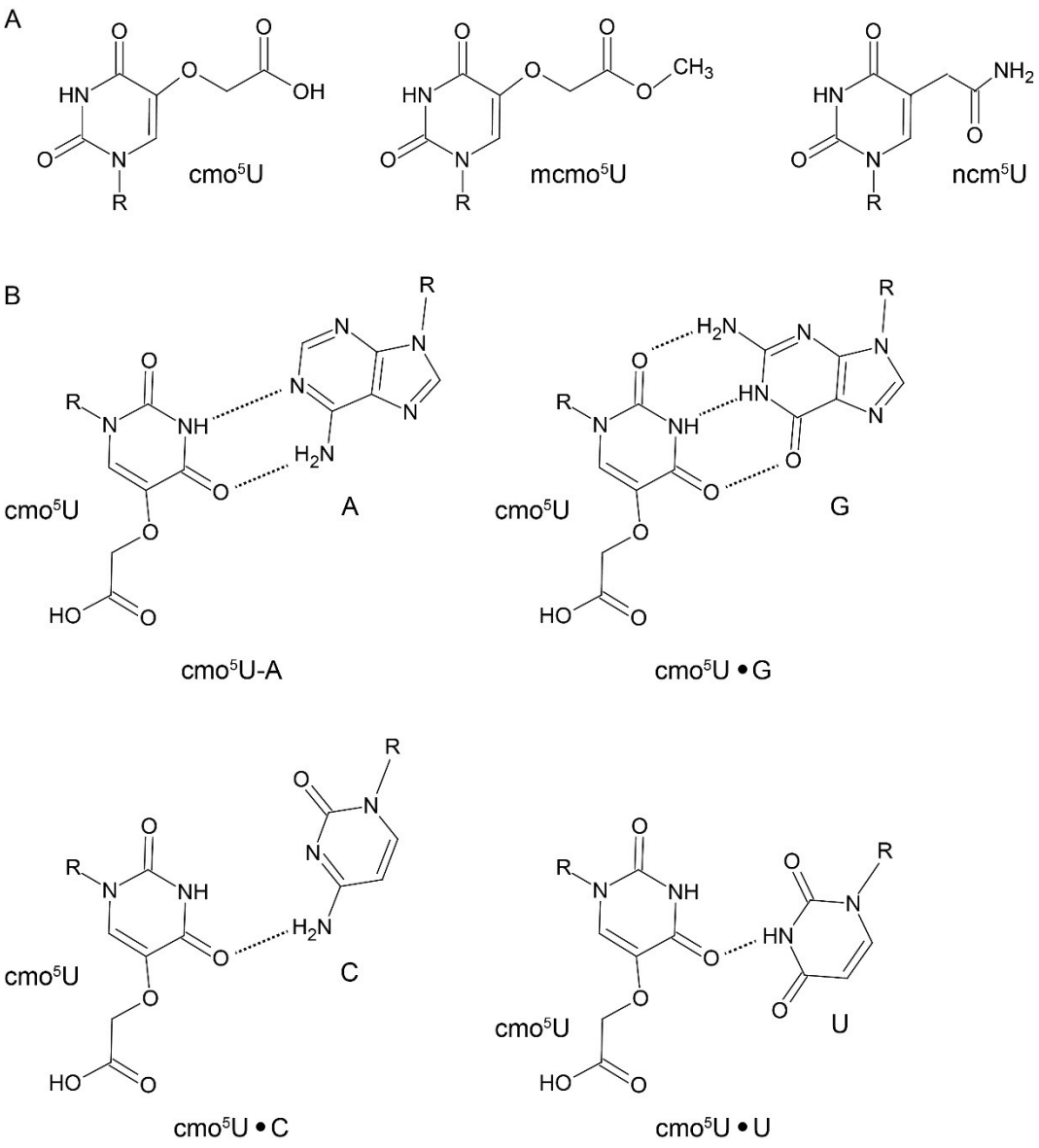

Figure S1 (Related to Figure 1). The cmo^{5}U34 modification expands the decoding capacity using various wobble base-pairing structures.

(A) Chemical structures of cmo^{5}U, mcmo^{5}U, and ncm^{5}U. **(B)** Expanded base pairings of cmo^{5}U with A, G, C, and U as observed in crystal structures (Weixlbaumer et al., 2007). The hydrogen bonds are shown by dotted lines.

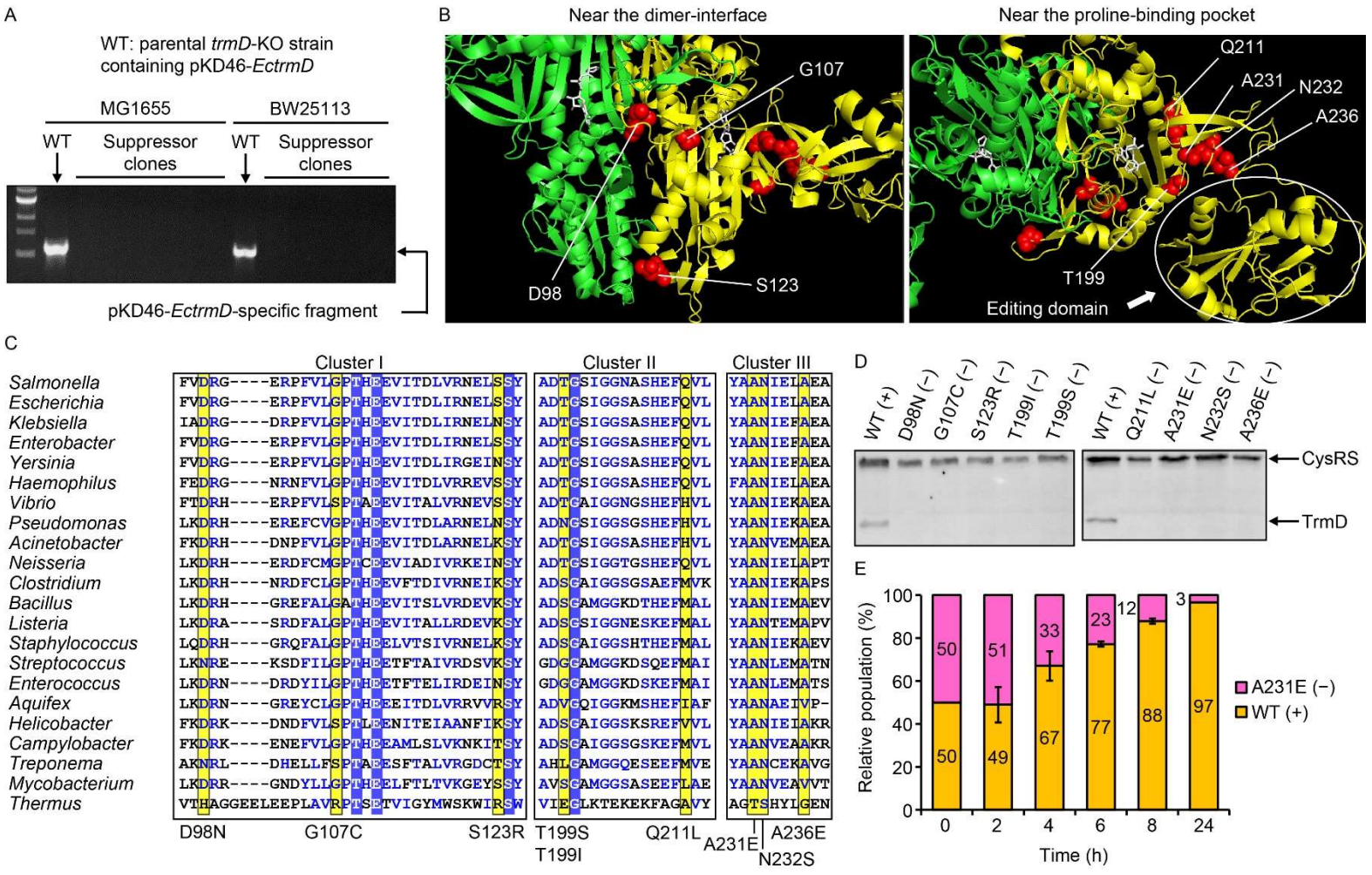

Figure S2 (Related to Figures 1 and 2). The isolated *proS* suppressor mutants are viable without *trmD* but less fit than the WT expressing *trmD*.

(A) Absence of the pKD46-*EctrmD* maintenance plasmid from suppressors with the *trmD*-KO allele. Suppressors were analyzed for the 5 colonies isolated from MG1655 and the 4 colonies isolated from BW25113. Absence of the plasmid was analyzed by PCR using primers specific to pKD46-*EctrmD*. The WT sample was isolated from the parental strain containing pKD46-*EctrmD*. **(B)** Mapping of *proS* suppressor mutations to the structure of *E. faecalis proS* enzyme as in Figure 1D. The images are blown up for Cluster I mutations near the monomer-monomer interphase (left) and for Clusters II and III mutations near the proline-binding pocket adjacent to the proof-reading domain (right). **(C)** A multi-sequence alignment of the ProRS enzyme as in Figure 1E, with an expanded set of species. **(D)** Absence of TrmD in the reconstructed *proS* suppressors in Western blots. Whole-cell lysates of early-log phase cultures were run on a 10% SDS-PAGE gel. Proteins transferred to a PVDF membrane were probed by anti-TrmD and anti-CysRS antibodies. **(E)** The *proS*-A231E (−) suppressor is out-competed by the WT (+) strain in a cell fitness assay as in Figure 2C. The bar graph shows the relative abundance of the two strains in a 1:1 mixture over the time course of 24 h.

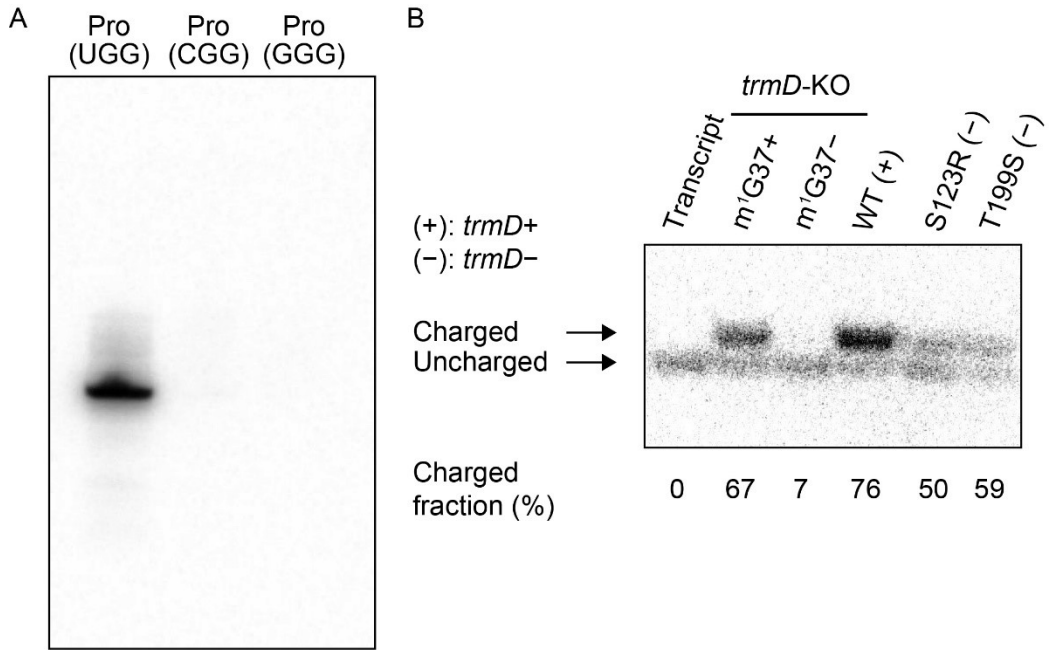

Figure S3 (Related to Figure 3). Acid-urea denaturing gel analysis of charged and uncharged Pro(UGG).

(A) Northern blot analysis showing that the probe for *E. coli* Pro(UGG) is specific to the tRNA and does not hybridize to the other two isoacceptors Pro(CGG) and Pro(GGG). Each tRNA in this analysis is an unmodified transcript, run on a 12% PAGE/7M urea gel, and probed with the [^{32}P]-labeled probe. **(B)** Acid-urea gel analysis of the *proS* suppressors S123R (–) and T199S (–), showing prolyl-aminoacylation level of 50% and 59%, respectively. An uncharged transcript of Pro(UGG) is shown in lane 1, and a positive control sample from an MG1655 *trmD-KO* strain expressing a plasmid-borne *trmD* grown in the Ara (+) condition and a negative control collected in the Ara (–) condition are shown in lanes 2 and 3. The sample from the WT MG1655 is shown in lane 4.

A Pro(UGG)

ProRS	State of G37	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m ($\mu M^{-1} s^{-1}$)	Fold change in k_{cat} / K_m Relative to G37-state
WT	m^1G37	0.96 ± 0.15	4.92 ± 0.78	5.14 ± 0.09	20.7
	G37	11.8 ± 2.24	2.91 ± 1.71	0.25 ± 0.12	1.0
D98N	m^1G37	0.29 ± 0.08	0.04 ± 0.003	0.13 ± 0.03	2.9
	G37	1.59 ± 0.41	0.07 ± 0.005	0.05 ± 0.1	1.0
T199I	m^1G37	0.61 ± 0.19	0.02 ± 0.007	0.03 ± 0.003	3.0
	G37	4.50 ± 1.90	0.05 ± 0.03	0.01 ± 0.006	1.0
N232S	m^1G37	0.25 ± 0.04	0.03 ± 0.01	0.13 ± 0.05	2.5
	G37	1.71 ± 0.88	0.07 ± 0.005	0.05 ± 0.03	1.0

B Pro(GGG)

ProRS	State of G37	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m ($\mu M^{-1} s^{-1}$)	Fold change in k_{cat} / K_m Relative to G37-state
WT	m^1G37	0.70 ± 0.25	1.97 ± 0.62	3.12 ± 1.14	20.5
	G37	9.10 ± 2.97	1.28 ± 0.19	0.15 ± 0.03	1.0
D98N	m^1G37	0.26 ± 0.08	0.04 ± 0.007	0.14 ± 0.04	4.4
	G37	2.15 ± 0.47	0.07 ± 0.007	0.03 ± 0.008	1.0

C Pro(CGG)

ProRS	State of G37	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m ($\mu M^{-1} s^{-1}$)	Fold change in k_{cat} / K_m Relative to G37-state
WT	m^1G37	8.33 ± 3.27	3.59 ± 1.15	0.45 ± 0.14	18.8
	G37	14.8 ± 1.27	0.35 ± 0.07	0.02 ± 0.005	1.0
D98N	m^1G37	0.53 ± 0.29	0.02 ± 0.003	0.07 ± 0.05	3.4
	G37	4.71 ± 0.47	0.10 ± 0.03	0.02 ± 0.006	1.0

Figure S4 (Related to Figure 3). Kinetic parameters of prolyl-aminoacylation.

(A) Kinetic parameters of prolyl-aminoacylation of Pro(UGG) shown in Figure 3B. For each enzyme, K_m (μM) for the tRNA substrate, k_{cat} (s^{-1}), k_{cat}/K_m ($\mu M^{-1}s^{-1}$), and the fold-change of k_{cat}/K_m of the G37-state relative to the m^1G37-state are shown. **(B)** Kinetic parameters of prolyl-aminoacylation of Pro(GGG) shown in Figure 3C. **(C)** Kinetic parameters of prolyl-aminoacylation of Pro(CGG) shown in Figure 3D.

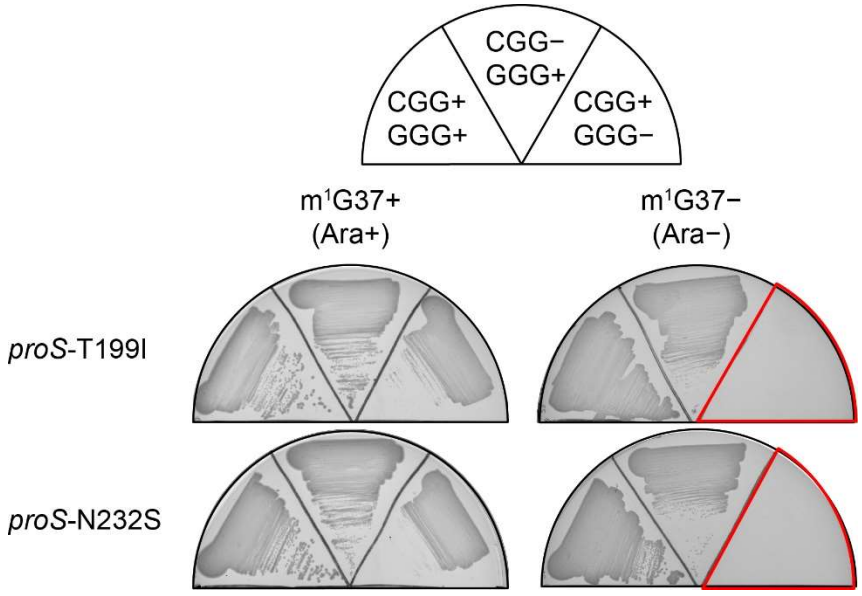

Figure S5 (Related to Figure 4). Requirement of Pro(GGG) for cell viability of *proS*-T199I and *proS*-N232S mutants in m^{1}G37− condition.

Pro(CGG) or Pro(GGG) was removed from *proS*-T199I and *proS*-N232S mutants lacking *trmD* and cells were grown in m^{1}G37+ or m^{1}G37− condition as in Figure 4C. No growth was observed when both m^{1}G37 and Pro(GGG) were absent.

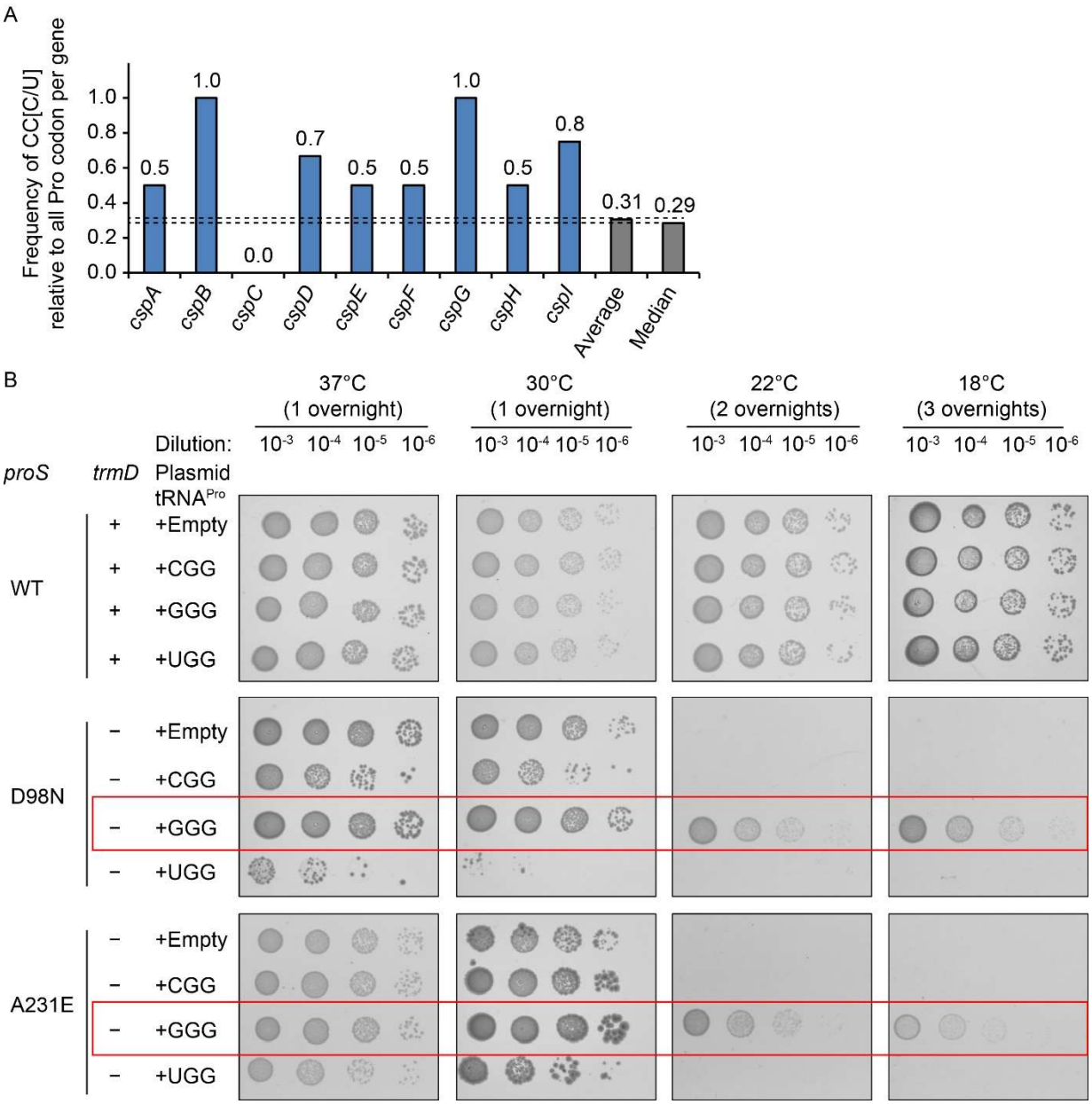

Figure S6 (Related to Figure 5). High frequency of CC[C/U] codons in *E. coli csp* genes.

(A) The frequencies of CC[C/U] codons are shown for selected *csp* genes, showing higher frequencies than the average and median (0.31 and 0.29, respectively) for most of these genes. **(B)** Rescue of cold sensitivity by expression of Pro(GGG) in *proS*-D98N and *proS*-A231E suppressors as in Figure 5D. The *proS*-A231E mutant was incubated at 30°C for 2 overnights due to a slower growth phenotype.

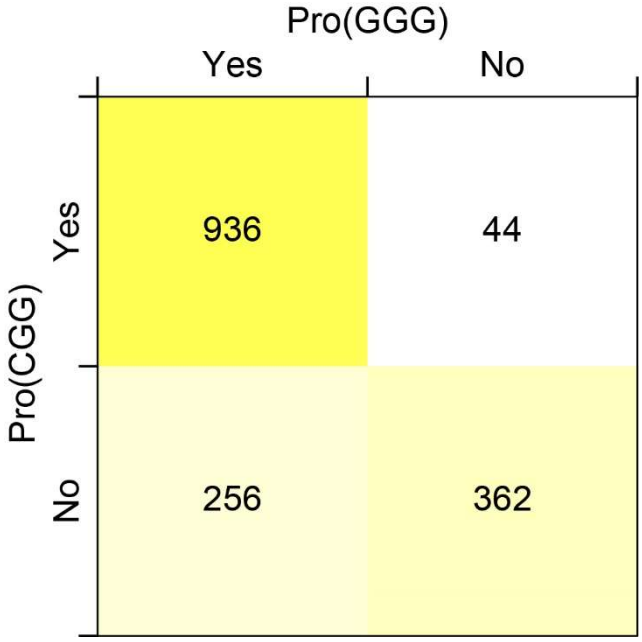

Figure S7 (Related to Figure 6). Co-occurrence of Pro(GGG) and Pro(CGG) across bacterial species.

Heat-map table showing the number of co-occurrence of Pro(GGG) and Pro(CGG) across all bacterial species that are available in GtRNAdb. Counts are species within GtRNAdb beyond the curated set used in Figure 6A. The greatest majority of bacterial species have both Pro(GGG) and Pro(CGG), followed by a majority lacking both tRNAs, whereas few have Pro(CGG) but lack Pro(GGG).

Table S1 (Related to Figure 1). Comparison between this study by Masuda *et al*. and the study by Clifton *et al* (Clifton *et al*., 2021).

	Masuda *et al*. (2022)	Clifton *et al*. (2021)									
Chromosomal *trmD*	KO	Y86*	R154A	S165L	G117N	R154A	S165L	Y86*	Y115A	G141A	D169A
Mutation description	Complete deletion	Y86 changed to a stop codon	Activity largely abolished	Colony size reduced by 90%	Colony size reduced by 90%	Activity largely abolished	Colony size reduced by 90%	Y86 changes to a stop codon	Catalytically compromised	Catalytically compromised	Activity largely abolished
Level of growth defect	Severe	Severe			Intermediate	Severe		Severe	Little	Little	Severe
Growth condition	LB, 43°C	LB, 37°C									
Days of cell culture	2 to 3	9			15	22		30	–	–	–
proS mutations	D98N G107C S123R T199I T199S Q211L A231E N232S A236E	A231E	E19K A36V Y89N G107C G200S A231E E514V	D98N G200S G200A A231E L235Q M441I		E19K G200S A231E E514V	G200S E514K	V195L G200A A231E			
trmD coding region mutation (Yes or No)	N.A.	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes (A169E)
rimM-trmD intergenic region mutation (Yes or No)	N.A.			Yes	Yes		Yes				
$tRNA^{Arg}$ gene mutations						*argQ* *argW* *argY* *argZ*	*argV*	*argQ* *argV* *argY* *argZ*			
Other gene mutations including intergenic region				*acrD* *fecA* *cfa* *nirB* *ulaG*		*spoT* *yfbM-yfbN* intergenic *yifK-argX* intergenic		*insH1* intergenic *lrhA-alaA* intergenic *rpoC* *spoT* *yifK-argX* intergenic *ytfK*			

Shown in red are *proS* suppressor mutations in common between the two studies.

Table S2 (Related to STAR Methods). Primers used in this study.

Category	Sequence (5'-3')	Purpose
Gene knockout		
E. coli proK-KO	AGGGCGTATCTGCGCAGTAAGATGCGCCCCGCATTGTGTAGGCTGGAGCTGCTTC	Forward to amplify *Kan* from pKD4 with homologous extension
	AAAAAGCCTGCTCGTTGAGCAGGCTTTTCGAATTATGGGAATTAGCCATGGTCC	Reverse to amplify *Kan* from pKD4 with homologous extension
CRISPR mutation		
E. coli proS	GTGCTCAGTATCTCTATCACTGA	Reverse to amplify pKDsgRNA
	CTGCTGCGTTTTGTTGACCGGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for D98N
	TGAGTTGGGCCGAGTACGAAGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for G107C
	AGCTAAGCTCGTTACGAATCGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for creating a new PAM site to make S123R
	TGTAAGAGCTAAGCTCGTTAGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for S123R
	CAAGCCGACACCGGTTCTATGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for T199I and T199S
	CTCTCACGAATTCCAGGTGCGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for Q211L
	CTATGCAGCGAACATTGAACGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for A231E and N232S
	CTATGCAGCGAACATTGAACGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA for creating a new PAM site for making A236E
	AGCTTCTGCCAGTTCAATATGTTTTAGAGCTAGAAATAGCAAG	Forward to amplify pKDsgRNA or A236E
	GGGAACAGTACGGTCCGGAACTGCTGCGTTTTGTGAATCGTGGCGAGCGTCCGTTCGTACTCGGCCCAACTCATGAAGAA	Mutation donor for making D98N
	CTGCGTTTTGTTGACCGTGGCGAGCGTCCGTTCGTACTGTGTCCAACTCATGAAGAAGTTATCACTGACCTGATTCGTAA	Mutation donor for making G107C
	GTACTCGGCCCAACTCATGAAGAAGTTATCACTGACCTTATCCGTAACGAGCTTAGCTCTTACAAACAGCTGCCGCTGAA	Mutation donor for making a new PAM site for making S123R
	CTCATGAAGAAGTTATCACTGACCTTATCCGTAACGAGTTGCGCTCTTACAAACAGCTGCCGCTGAACTTCTATCAGATC	Mutation donor for making S123R
	CCGCATGGGGCTGGATTTCCGCGCCGTACAAGCCGATATTGGTTCTATCGGCGGCAGCGCCTCTCACGAATTCCAGGTGC	Mutation donor for making T199I
	CCGCATGGGGCTGGATTTCCGCGCCGTACAAGCCGATTCTGGTTCTATCGGCGGCAGCGCCTCTCACGAATTCCAGGTGC	Mutation donor for making T199S
	CAAGCCGACACCGGTTCTATCGGCGGCAGCGCCTCTCACGAATTTCTTGTGCTGGCGCAGAGCGGTGAAGACGATGTGGT	Mutation donor for making Q211L
	GACGATGTGGTCTTCTCCGACACCTCTGACTATGCAGAAAATATTGAACTGGCAGAAGCTATCGCGCCGAAAGAACCGCG	Mutation donor for making A231E

	GACGATGTGGTCTTCTCCGACACCTCTGACTATGC AGCCAGTATTGAACTGGCAGAAGCTATCGCGCCGA AAGAACCGCG	Mutation donor for making N232S
	GACGATGTGGTCTTCTCCGACACCTCTGACTATGC AGCCAATATTGAACTGGCAGAAGCTATCGCGCCGA AAGAACCGCG	Mutation donor for making a new PAM site for making A236E
	TCTCCGACACCTCTGACTATGCAGCCAATATTGAAT TAGAAGAAGCTATCGCGCCGAAAGAACCGCGCGCT GCTGCTACC	Mutation donor for making A236E
Probe for Northern blotting		
E. coli Pro(UGG) tRNA	CCAAACCAGTTGCGCTACCA	Annealing to the tRNA region from the D-loop to the anticodon stem-loop
Plasmid construction		
pKD46-*EctrmD*	GCCATTCTCGAGAATGGCGATGACGCATCCTCAC	Forward to amplify pKD46 with an XhoI site
	AAGCGTCAGGTAGGATCCGCTAATCTTATGG	Reverse to amplify pKD46 with a BamHI site
pET22b-*EcproS* mutant	AAGGAGATATACATATGCGTACTAGCCAATACCTGC TCTC	Forward to amplify *proS* with an NdeI site
	GGTGGTGGTGCTCGAGGCCTTTAATCTGTTTCACC AGATAT	Reverse to amplify *proS* with an XhoI site
pKK223-3-*proK*	TCACCGGAATTCCGGTGATTGGCGCAGCCTGGTAG CGCACTTCGTTCGGGACGAAG	Forward to construct *proK* with EcoRI site
	CGACCACTGCAGTGGTCGGTGATAGAGGATTCGAA CCTCCGACCCCTTCGTCCCGAACGAAG	Reverse to construct *proK* with PstI site
pKK223-3-N-luc	AAGCTTGGCTGTTTTGGCGGATG	Forward to amplify the pKK223-3 backbone
	GAATTCTGTTTCCTGTGTGAAATTGTTATC	Reverse to amplify the pKK223-3 backbone
	CACACAGGAAACAGAATTCATGCCCGGTGTCTTCA CACTCGAAGATTTCG	Forward to amplify nLuc with a CCC at the second codon position
	CACACAGGAAACAGAATTCATGCCCCGGTGTCTTC ACACTCGAAGATTTCG	Forward to amplify nLuc with a CCC-C codon at the second position
	CGCCAAAACAGCCAAGCTTTTACGCCAGAATGCGT TCGCACAGC	Reverse to amplify nLuc
Confirmation of plasmid curing		
pKD46-*EctrmD*	CATATTGCATCAGACATTGCCG	Forward to amplify a partial fragment of pKD46-*EctrmD*
	CTATGTGCCATCTCGATACTCG	Reverse to amplify a partial fragment of pKD46-*EctrmD*