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Are bacterial processes dependent on global ribosome pausing affected by tRNA modification defects?

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Abstract

By integrating a literature review with transcriptomic, proteomic, and phenotypic data from two model bacteria, *Escherichia coli* and *Vibrio cholerae*, we put forward the hypothesis that defects in tRNA modification broadly impact processes that are evolutionarily tuned to be sensitive to translation speed. These include the translation of regulatory proteins associated with motility, iron homeostasis, and leader peptide-driven attenuation mechanisms. Some of these translation speed-dependent processes are influenced by the absence of a single modification, while others are affected by the absence of multiple modifications. Although further experiments are needed to clarify the mechanisms involved in each case, this work provides a foundational framework to guide future research.

Keywords

tRNA modification, leader peptide, iron homeostasis, motility, *Escherichia coli*, *Vibrio cholerae*, translation

Introduction

It is now well established that the pace at which the translation machinery decodes mRNAs to produce proteins is not uniform and greatly affects protein expression levels and folding patterns [1,2]. The rapid expansion of genomic data facilitating global codon usage bias analyses, the development of ribosome profiling techniques to measure in vivo ribosome pausing, and experimental advancements enabling the systematic study of synonymous mutations on protein function have collectively advanced our ability to dissect the diverse forces influencing mRNA translation speed [3–6].

As molecular adaptors linking mRNA codons to their corresponding amino acids during ribosomal elongation, tRNA molecules play a crucial role in regulating translation dynamics [1,2,7]. More than half [12 in the model *Escherichia coli* (Fig. 1)] of the 20 canonical amino acids are encoded by synonymous codons that different tRNAs can decode. The intracellular concentrations of different tRNA pools vary and correspond to the codon usage frequency of their cognate aminoacylated tRNAs [8,9]. Furthermore, highly expressed genes tend to utilize the most frequent codons [9]. Based on this, it has been proposed that the size of aminoacylated-tRNA isoacceptor pools influences ribosome pausing at specific codons by increasing the likelihood that cognate tRNAs are efficiently sampled at the A site, thereby facilitating the next step in elongation. [10,11]. However, recent studies indicate that variations in cognate tRNA concentrations have only a minimal impact on protein expression under physiological conditions [12–15]. These observations suggest that less abundant tRNAs primarily impact translation when their aminoacylated pools become depleted. Additionally, under physiological conditions, they function as a reserve of aminoacylated tRNAs during amino acid starvation [16].

For amino acids encoded by codons read by a single tRNA [8 in *E. coli* (Fig. 1)], codon choice can influence the strength of the codon-anticodon interaction. This is determined by the presence of Watson-Crick base pairs (GC and AU) at the third codon position rather than weaker GU wobble pairs [7,17]. Synonymous codon usage can be subject to selective pressure both between and within genes [2], although some controversy remains on this issue [18]. Several studies have shown that, particularly in organisms with low growth rates, highly expressed genes are enriched in abundant codons [19], while the first 30-50 bases of coding sequences are enriched in rare

codons [20]. The presence of rare codons might allow a ramping up of translation speed [20], but it seems that this effect is mainly attributable to base composition because these codons are enriched in A/U versus G/C [14,21,22]. Codon usage in mRNAs encoding membrane proteins appears to be specifically optimized for the coupled translation and secretion process, with alternating regions of slow and fast-translating codons playing a crucial role in ensuring proper protein secretion [2,4].

Beyond its impact on translation dynamics, ribosome pausing has distinct consequences in eukaryotes and bacteria, particularly in relation to mRNA degradation and ribosome quality control mechanisms that are crucial for managing extreme pausing events, such as ribosome stalling and collisions [1,23–25]. Some of these stalling events are caused by combinations of amino acids, which induce conformational stress within the ribosome peptidyl transferase center and the nascent peptide exit tunnel of the ribosome (NPET). The best-characterized case is the polyproline stretches that induce ribosome stalling and prevent the formation of the peptidyl bond. Another example is the repeat of acidic residues (Glutamate and Aspartate) early in the encoding sequence that can provoke pausing of the ribosome that can lead to ribosome stalling or abortive synthesis [26–29]. In both cases specialized factors, EF-P/eIF-5A for polyproline [30–32] and EttA for repeat of acidic residues [29] can resolve the pausing and restore elongation.

Gene expression can be regulated by mechanisms that depend on ribosome stalling. Indeed, because transcription and translation are coupled in some bacteria, sophisticated regulatory mechanisms have evolved to monitor translation stalling using a leader peptide, a small coding sequence that encodes a short peptide prone to ribosome stalling [33] (Fig. 2). When stalling occurs during the synthesis of the leader peptide, it prevents premature transcription termination by inhibiting the formation of a transcriptional terminator (Fig. 2A) or blocking access to the Rho termination site (Fig. 2B), both of which overlap with the ORF of the leader peptide [34]. Ribosomal stalling can be induced by uncharged tRNA, as seen in many amino acid synthesis operons where the leader peptide is enriched with corresponding codons, such as the TrpL or HisL leader peptides [35]. Stalling can also be induced by metabolites that bind between the NPET of the ribosome and the nascent peptide [36]. For example, the regulation of the *tnaAB* operon by the leader peptide TnaC (Fig. 2B) occurs when intracellular concentrations of tryptophan (Trp) increase. A single Trp molecule binds

within the NPET of the ribosome by interacting with the TnaC nascent peptide. This interaction prevents the termination of TnaC peptide translation, leading to ribosome stalling. Ribosome stalling, in turn, prevents Rho-dependent termination of transcription and allows transcription of the *tnaAB* operon [37]. This type of regulation by metabolites is frequently observed in the regulation of antibiotic resistance genes [38]. In some regulatory elements, the leader peptide can directly regulate the translation of downstream genes by using a translational attenuator, which, like a transcriptional terminator, is inactivated by ribosome stalling on the leader peptide, as seen in ErmC, SecM, and VemP [39–41] (Fig. 2C). Interestingly, the stimulus sensed by SecM and VemP is the mechanical force exerted on the leader peptide as it exits the NPET of the ribosome [40,41]. Another regulatory mechanism similar to the leader peptide uses overlapping genes, where the translation of the first gene dictates the expression of the downstream one (Fig. 2D).

By design, leader peptides are hence excellent sensors of translational stress, monitoring translation dynamics within the cell. For example, HisL has been used to study the elongation dynamics of synonymous codons [42], and the SecM sequence has been employed for its ribosome-stalling ability in genetic screens [25]. In eukaryotes, similar regulatory systems use small ORFs, called upstream open reading frames (uORFs), to regulate downstream genes. The regulation mainly occurs through the uORF's ability to titrate ribosomes, which can initiate translation of the downstream ORF [43] (Fig. 2D). This difference in regulation arises from the distinct mechanisms of translation initiation between eukaryotes and prokaryotes.

tRNAs are heavily post-transcriptionally modified molecules with any given tRNA harboring 5 to 10 modifications [44]. Modifications affect many aspects of tRNA functions including recognition by tRNA-synthetase or other components of the translation apparatus and provide quality control mechanisms to ensure correct structure and stability [45,46]. The most complex modifications are found in the anticodon-stem-loop (ASL) involved in decoding [17]. These ASL modifications can expand or restrict the decoding capacity of tRNAs and can play crucial roles in harmonizing the speed of decoding of different synonymous codons [47]. The absence of tRNA modifications can lead to changes in the translation speed of a given codon directly resulting in the misfolding of the translated protein but can also lead to the indirect misfolding of other proteins by overwhelming the protein homeostasis

machinery [48–50]. The final concentrations of translated proteins can also be affected by the absence/presence of modifications even if they are correctly folded, hence the phenotypes of mutations affecting tRNA modifications can be very pleiotropic and difficult to pin down to unique molecular mechanisms [51]. For example, the absence of t⁶A37, a universal modification located just upstream of the anticodon/codon first base pair, can lead to cell death in some organisms [52], high levels of protein aggregation in others [50], and multiple cellular phenotypes including genetic disease in humans [53]. On the other end of the spectrum, the absence of many conserved modifications does not lead to any specific growth defects under standard growth conditions and phenotypes can sometimes be revealed under specific stresses or growth conditions [54]. For example, it was postulated for over 40 years that the absence of Queuosine (Q), a wobble base modification found in tRNAs with GUN anticodons led to no dramatic growth phenotypes in *E. coli* beyond a fitness cost in stationary phase [55] until metal-related growth phenotypes and biofilm defects were reported in the same organism [56,57].

Some organisms use tRNA modifications to regulate the translation of specific genes [51,58]. Codon usage analysis focusing on modification-dependent codons can identify groups of genes whose translation is affected by modification levels [58]. Modification tunable transcripts (MOTTs) involved in regulatory cycles have been identified in both eukaryotes and prokaryotes [51,58]. Bacterial examples are found in the areas of Mg²⁺ homeostasis, oxidative stress resistance [59] or iron homeostasis [60] but as more studies integrating codon usage, ribosome profiling (RiboSeq), and proteomics analysis of tRNA modification deficient mutants are published, other examples of regulatory roles should emerge.

In the work described here, we compared the whole genome expression data generated for ten *E. coli* and three *Vibrio cholerae* mutants as well as proteomics data from of four *V. cholerae* mutants to their WT controls. Extracting the commonalities and the differences between these expression profiles led us to propose that the processes that are built to sense translation speed are particularly sensitive to tRNA modification defects.

Material and methods

Media, Strains and Plasmids

Luria-Bertani (LB) broth and agar (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, Fisher Scientific BP1426-2 and BP1425-2) were routinely used for growth of *E. coli* cells at 37°C from frozen stocks. When required, 50 µg/mL of kanamycin (Kan), 100 µg/mL Ampicillin or Arabinose (0.02%) were added to the medium. M9 minimal medium (12.8 g/L Na₂HPO₄·7H₂O, 3.0 g/L KH₂PO₄, 0.5 g/L NaCl, 1.0 g/L NH₄Cl, 2.0 mM MgSO₄, 0.1 mM CaCl₂) was used in the *E. coli* motility assays, with 0.3% agar.

V. cholerae cells were grown in LB or Mueller-Hinton (MH) (Sigma, Catalog number 70192) and 0.2 µg/mL of tobramycin (20% of Minimum Inhibitory concentrations (MIC)) was sometimes added. The strains used in this study are listed in Table S1. Some of the *E. coli* strains used here were constructed by transferring the Kan^R allele from the Keio collection strains [61] to a fresh background by P1 transduction [62]. The initial Keio strains and the transductants were checked by PCR for the presence of correct mutant allele as described in Bacusmo et al [63]. Deletions of *V. cholerae* tRNA modifications genes were performed as previously described [64]. Details are given in Table S1 and Table S2.

Motility assay

For *E. coli*, overnight cultures were prepared in LB at 37°C with agitation. The OD(A600nm) was normalized to 10, and 2 µL of cells was plated on the surface of 0.3 % agar plate (M9). Motility diameters were measured for biological triplicates after 22 hours of incubation at 37°C. For *V. cholerae*, overnight cultures were prepared in LB at 37°C with agitation. 5 µL of overnight cultures were deposited in the center of a 0.3 % agar plate (LB). Motility diameters were measured for biological triplicates after 6 to 12 hours incubation at 37°C and compared to WT *V. cholerae* N16961 *hapR*⁺.

Data mining

E. coli TnSeq data was accessed from <http://fit.genomics.lbl.gov> [65]. Information on *E. coli* genes was extracted from EcoCyc [66] and the resources of NCBI including Pubmed [67]. Mapping of identifiers was performed at EcoCyc for *E. coli* and BioCyc [68] for *V. cholerae* biovar El Tor str. N16961. Transmembrane helices were predicted by TMHMM2.0 [69].

RNA seq of *E. coli* strains

RNA was isolated from each of the different strains at grown in LB at 37°C up to mid- exponential phase (OD(A600nm) ~0.6) in three biological replicates and were extracted and purified as previously described [70]. Samples were sequenced using an Illumina HiSeq2000 Platform without depletion of rRNA prior to sequencing.

RNA-seq analysis was carried out using the KBase platform transcriptomics and expression analysis tools [71]. Paired-end reads were assembled from corresponding FASTQ files, trimmed of their adaptor sequences using Trimmomatic (v0.36). Quality of the paired-end reads was assessed using FastQC (v0.11.9) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) for each sample and analysis confirmed that all samples had uniform sequence quality scores and GC content, ranging from 39.36-39.77% and 51.0-52.0% across all paired-end reads, respectively and deposited in Kbase [72].

The wildtype *E. coli* BW25113 genome assembly (GCA_000750555.1/ASM75055v1) was annotated using RASTtk (v1.073) to give “BW25113_Annotated” as the reference genome for transcriptome analysis. Trimmed reads were aligned to the reference genome using HISAT2 (v2.1.0)[73] and aligned reads were assembled using StringTie (v2.1.5)[74]. Differential expression matrices were created comparing each mutant strain to the reference genome as well as each other using DESeq2 (v1.20.0)[75]. Up- and down-regulated feature sets were then created, filtering for genes with significant differential expression between each mutant and the wildtype reference as well as each mutant versus *dusB*, using a false discovery rate (FDR; q-value) of < 0.05 and an absolute log₂ fold change greater than 1 as significance cutoffs. Total differential expression data with no significance filtering was also compiled for quality control and spot-checking. The results of the comparison of each strain vs the WT and the *dusB* mutant are available in Supplementary Data Set 1 and Supplementary Data Set 2 respectively.

RNA seq of *V. cholerae* strains

Overnight cultures of the different *V. vibrio* strains were diluted 1:1000 in mild stress conditions in MH + tobramycin 0.2 µg/ml medium (20% of the MIC) and grown with agitation at 37°C until an OD(A600nm) of 0.3-0.4 (exponential phase). 0.5 mL of these cultures were centrifuged, and the supernatant removed. Pellets were homogenized by resuspension with 1.5 mL of room temperature TRIzol Reagent. Next, 300 µL chloroform were added to the samples following mix by vortexing. Samples were then centrifuged at 4°C for 10 minutes. Upper (aqueous) phase was transferred to a new 2mL tube and mixed with 1 volume of 70% ethanol. From this point, the homogenate was loaded into a RNeasy Mini kit (Qiagen) column and RNA purification proceeded according to the manufacturer's instructions. Samples were then subjected to DNase treatment using TURBO DNA-free Kit (Ambion) according to the manufacturer's instructions. The libraries were constructed using the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero Plus.

For Sequencing, quality control was performed on iSeq100 and the production runs were performed on NextSeq 2000 yielding 5M reads/sample. The RNA-seq analysis was performed with Sequana 0.16.11 [76]. We used the RNA-seq pipeline 0.19.2 (https://github.com/sequana/sequana_rnaseq) built on top of Snakemake 7.32.4 [77] Briefly, reads were trimmed from adapters using Fastp 0.22.0 [78] then mapped to the *Vibrio cholerae*_N16961 genome assembly (accession numbers: chromosome 1, [NC 002505](#), chromosome 2, [NC 002506](#) using bowtie2 2.4.5 [79]. FeatureCounts 2.0.1 [80] was used to produce the count matrix. Quality control statistics were summarized using MultiQC 1.17 [81]. Statistical analysis on the count matrix was performed to identify differentially regulated genes. The clustering of transcriptomic profiles was assessed using a Principal Component Analysis (PCA). Differential expression testing was conducted using DESeq2 library 1.34.0 [75] scripts indicating the significance (Benjamini-Hochberg adjusted p-values, false discovery rate FDR < 0.05) and the effect size (fold-change) for each comparison. The results of comparing each strain vs the WT are available in Supplementary Data Set 3.

Proteomics analyses

Overnight cultures of *V. cholerae* were diluted 1:100 in MH medium and grown under the same conditions as the RNAseq experiment until an OD 600 nm of 0.3 (exponential phase). 50 mL of these cultures were centrifuged for 10 min at 4°C and

supernatant removed. Lysis was achieved by incubating cells in the presence of lysis buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton 100X) supplemented with 0.1 mg/mL lysozyme and complete EDTA-free Protease Inhibitor Cocktail (Roche) for 1 hour on ice. Resuspensions were sonicated 3x50 sec (power: 6, pulser: 90%), centrifuged for 1 h at 4°C at 5000 rpm and supernatants were quantified using Pierce™ BCA Protein Assay Kit (Cat. No 23225) following the manufacturer's instructions. Proteins were then stored at -80°C. Proteomics MS and analysis were performed as described [59]. The results of the comparison of each strain vs the WT are available in Supplementary Data Set 4.

GO enrichment analysis

Gene Ontology (GO) enrichment was analyzed using R packages clusterProfiler[82]. Briefly, the GO-annotation of genes of *E. coli* and *Vibrio cholerae* O1 biovar El Tor str. N16961 were retrieved from the EcoCyc/BioCyc databases (biocyc.org)[66,68] The GO class description was retrieved from the GO Consortium (geneontology.org)[83]. Genes with an absolute log₂-fold change greater than 1 and adjusted p-values less than 0.05 were selected for the clusterProfiler enricher function. The enriched GO terms with p-values less than 0.05 and Benjamini-Hochberg adjusted p-values less than 0.2 were plotted. Heatmaps were generated using R package ComplexHeatmap [84]. The differentially expressed genes (DEGs) with their GO terms in the different *E. coli* tRNA modification mutants compared with the WT and the *dusB* mutant are listed in Supplementary Data Set 5A and Set 5B, respectively. The DEGs with GO terms in the *V. cholerae dusB*, *tgt* and *truA* strains are listed in Supplementary Data Set 5C. The enriched GO terms in each comparison of *E. coli* and *V. cholerae* are listed in Supplementary Data Set 5D.

Synonymous codon usage and correlation analyses

First, every codon in every *V. cholerae* CDS was counted excluding the start and stop codon (Supplementary Data Set 6A and Set 6B). Synonymous codons were hypothesized to follow a binomial distribution with the number of a synonymous codon in a CDS as the number of successful outcomes and the frequency of the codon within its synonymous codons of all proteins as the probability of success. Then, the lower

and upper bounds of the binomial distribution with 95% confidence interval (CI) for each codon in each CDS were calculated using the BINO.INV function. Finally, whether a codon lies within the 95% confidence interval for a binomial distribution was summarized (Supplementary Data Set 6C). When the hypothesis was rejected, the significance of biased synonymous codon usage was indicated by the difference of the observation of a synonymous codon to the bound of 95% CI normalized $\left(\frac{\text{observed occurrence of a codon} - \text{expected occurrence with 95\% CI}}{\text{expected occurrence with 95\% CI}}\right)$ (Supplementary Data Set 6C and 9C). The codon usage data was combined with other features for the *V. cholerae* *tgt*, *truA* and *dusB* mutants (Supplementary Data Set 7), and Pearson correlation between any two features was calculated using the *cor* function in R (Supplementary Data Set 8). The codon usage for every *E. coli* CDS was analyzed in similar fashion (Supplementary Data Set 9A-C). The transcription factors (TFs) that contain a helix-turn-helix (HTH) domain in *E. coli* and *V. cholerae* were predicted/retrieved from pantherdb.org[85]. The synonymous codon usage of TFs was listed in Supplementary Data Set 10.

Results and discussion

Motility can be increased or decreased by the absence of tRNA modifications in *E. coli* and *V. cholerae*

When conducting a synthetic lethal screen combining deletions in 20 different tRNA modifications genes in *E. coli*, we identified several mutations that when combined gave rise to growth defects (Fig. 2 of [63]). We performed an RNASeq analysis of 9 of these mutants (*dusB*, *truA*, *truD*, *thil*, *miaA*, *rlmN* *mnmA*, *mnmC*, *mnmG*) as described in the method section. When analyzing the differentially expressed genes in these mutant strains, we realized that the WT BW25113 laboratory collection strain used in the RNAseq study was not isogenic with the Keio collection strains [61] (Table S1). The main difference was the repression of motility genes, such as flagellar synthesis genes, in the laboratory WT strain (Table S3 and Fig. S2 and S3), suggesting that the WT strain used was non-motile. This hypothesis was confirmed in a motility assay (Fig. S1B). This phenomenon had already been reported as resting strains of *E. coli* can accumulate mutations that increase motility overnight and Keio collection strains vary widely in their motility phenotypes [86]. The *dusB* data was therefore used as a

reference for comparisons and we found that the expression of flagella synthesis genes still varied greatly between strains (Fig. 3 and Fig. S4). As these phenotypes could still be due to suppressor mutations, we constructed an isogenic set by transducing all the mutant alleles in a novel WT BW2113 obtained from the Coli Genetic Stock Center (CGSC) (<https://ecgrc.net/>) that was motile (Fig. S1B). As shown in Fig. 4A and 4B, correlations between the expression data and the mobility phenotypes were observed with some deletions reducing motility (*dusB*, *miaA*, *tgt* and *mnmA*) and others increasing motility (*thil*, *truA*).

Reduced motility phenotypes could also be caused by growth rate defects, but previous analysis had shown that *tgt* mutants grow like WT in most standard conditions [56]. In addition, we had previously conducted a comprehensive proteomics and transcriptomic analysis of *tgt* strain lacking the wobble base modification Q and found a global downregulation of genes involved in flagella synthesis and chemotaxis (see Fig.3 of [56]). Analysis of *E. coli* data from a Tn-seq fitness study [87] revealed that genes with similar profiles (high cofitness scores) to the *tgt* gene were related to Q biosynthesis as expected but also to motility (Table S3). We therefore tested if the motility phenotype of the *tgt* could be complemented. As shown in Fig. S1A, the *tgt* mutant was slightly less motile than the WT strain. Although subtle, this difference was significant and consistently observed in repeated experiments. This phenotype was complemented upon overexpression of the *tgt* gene, confirming the *tgt* gene absence as the cause of the observed motility phenotype (Fig. S1).

We had previously shown that mutations in different types of tRNA modification genes led to decreased (such as *tgt* or *miaA* deletions) or increased (such as *dusB*, *rlmN* or *truA* deletions) fitness of *V. cholerae* cells grown in the presence of sub-MIC concentrations of tobramycin [64]. This led us to perform RNAseq analyses of the *tgt*, *truA*, *dusB* strains as well as proteomic studies of the *tgt*, *truA*, *dusB* and *rlmN* strains as described in the methods section. Analysis of the *V. cholerae* RNAseq and proteomics data also showed a downregulation of the expression of genes involved in the synthesis of flagella in the *truA* mutant and to a lesser extent in *tgt* strain whereas expression of these genes was not affected in the *dusB* strain (Fig. S5 and Table S4). The changes in flagella gene expression correlated with the reduced motility of the *V. cholerae* *truA* and *tgt* mutants and the absence of any motility phenotype of the *dusB* strain (Fig. 4C). We then surveyed 19 other tRNA modifications genes deletion strains and found that several others were less motile. The most severely affected were the

truD, *mnmE* and *trmJ* mutants (Fig. 4C). If the absence of tRNA modifications led to motility phenotypes in both *E. coli* and *V. cholerae*, the phenotypes hardly overlapped except for the *tgt* mutants that were less motile in both organisms (Fig.4A and C).

Motility is regulated by multiple input signals and regulatory networks, including the master regulator FlhDC, which integrates with metabolic pathways and cell envelope systems [88]. Several mechanisms can be invoked to explain how the translation defects caused by the absence of tRNA modifications might impact motility. Of course, levels of key regulators such as FlhD or FlgM could be affected [89]. Flagellar biosynthesis genes are often organized in operons with translationally coupled genes [89,90] where tRNA modifications may influence translation efficiency, thereby affecting the proper stoichiometry required for flagellar assembly [91,92]. Additionally, the transcription of flagellar genes is tightly regulated by feedback mechanisms that respond to the state of flagellar assembly [88,90].

In *Salmonella enterica* serovar Typhimurium, synonymous codon changes significantly alter the translation of *flgM*, a key regulator of flagellar synthesis [93], suggesting that tRNA modifications may also influence flagellar biogenesis through codon-dependent translation control. Furthermore, the absence of certain tRNA modifications can lead to mistranslation, which has been linked to reduced motility via increased expression of the small RNA DsrA, though the underlying mechanism remains unknown [94].

Further studies will be required to elucidate the specific roles of tRNA modifications in motility. However, this analysis reinforces the observation that motility is highly sensitive to perturbations in the translation machinery. For *V. cholerae*, with the availability of proteomics datasets, we further explored the correlation between protein expression and codon usage, which is analyzed in the final section of this report.

Genome wide transcriptomic and/or proteomic analyses of 9 *E. coli* and 4 *V. cholerae* tRNA modification deficient strains reveal common cellular responses

In addition to the dysregulation of motility genes observed in the absence of the majority of tRNA modification genes tested both in *E. coli* and *V. cholerae*, several other global changes were observed in several tRNA modification deficient mutants, and a few were consistent between the two species as detailed below.

First, in *E. coli*, a partial clustering of the genome-wide expression profiles of strains lacking modifications of the ASL from those lacking modifications in the bodies of tRNAs was observed as the *miaA*, *truA*, *rlmN* and *mnmG* formed a clear cluster (Figs. S6 and S7). Second, in addition to genes involved in motility, other physiological areas were enriched in both the *E. coli* and *V. cholerae* tRNA modification mutants analyzed (Fig. 3, Fig. S4 and Fig. 5). Two processes were constitutively enriched: amino acid (particularly histidine) metabolism, and iron homeostasis. Investigating the potential molecular mechanisms underlying these similar responses across different bacterial species revealed a unifying theme. These were process processes that are regulated by ribosome pausing or translation speed, as elaborated in the following sections.

'Ribosome-mediated' transcription attenuation systems are affected by the absence of modifications

A historical example of the importance of tRNA modifications in ribosome-mediated transcription attenuation systems is the identification by Bruce Ames of *truA*[95]. The corresponding enzyme inserts Ψ at position 38-40 in the ASL of around half the bacterial tRNAs including those decoding His codons as *hisT* [95]. Mutations in *hisT/truA* increase the expression of the *his* operon genes. Like fully modified uncharged tRNAs, charged tRNAs lacking Ψ ³⁸⁻⁴⁰ increase ribosome pausing at His codons when translating the *hisL* leader peptide mRNA, not allowing the attenuator structure to form (as shown Fig. 2B), thus allowing the transcription of the downstream *his* operon genes. RNAseq analysis of a *truA* mutant reproduces in *E. coli* K12 the initial observation made in *S. Typhimurium* LT2, with a log₂ fold change >3 of the *his* synthesis genes occurring in the mutant (Fig. 6A and Fig. S4). A similar activation was observed with RNA-seq and proteomic analyses in *V. cholerae* (Fig. 6C and Fig. 5B). Two other modifications are present in the tRNA^{His}GUG ASL, m²A and Queuosine (Q) (Fig. 1). In *E. coli* ([56] and Fig.6A) and partly in *V. cholerae* (Fig. 6C), the absence of Q (*tgt* mutants) and m²A (*rlmN* mutants) also leads to the activation of *his* operon genes but less than in the *truA* strain.

The Ames group also showed that mutations in *hisT/truA* increased the expression of the *ilv* and *leu* operons in *S. Typhimurium*, two other systems regulated

by ribosome-mediated transcription attenuation [96]. We reproduced these findings in *E. coli* and *V. cholerae* for the *leu* operon (Fig. 6B and 6D). In *E. coli*, expression of the *ile* biosynthesis genes is unexpectedly down in the *truA* strain and most other mutants (Fig. S8A), and no trend was seen in *V. cholerae* (Fig. S8B). These variations might reflect previously reported differences between species and/or tRNA sequences on how Ψ_{38-40} deficiency affects reading efficiency, reading fidelity, and frameshifting [97,98]. In addition, the organization of the *ile* biosynthesis genes in *E. coli* is very complex with multiple regulators[99]. In addition, the llvL peptide sequence contains a di-proline (PP) that might influence the role of modifications in complex ways because of the increased stalling that requires the specialized EF-P factor [31].

Other amino acid biosynthesis genes/operons are known to be controlled by ‘ribosome-mediated’ transcription attenuation through leader peptides [34](Table 1). Several of these, including those under the control of *pheM*, *thrL* and *trpL*, are activated by modification defects (Table 1) as expected if these promote ribosome pausing. For example, we confirmed in *E. coli* the upregulation of the *trp* and *phe* genes in the absence of $\text{ms}^2\text{i}^6\text{A}$ (*miaA* mutant) previously reported in *Salmonella* [100]. Like in *E. coli*, two TrpE leader sequences (VC1175 and VC1176, Table 1) are found in front of the *V. cholerae* *trp* operon, but unexpectedly, Trp proteins (but not transcripts) are downregulated in *V. cholerae* *truA* and *rlmN* mutants (Supplementary Data Set 4). To a lesser extent, ThrB also appears to be down in *V. cholerae* *truA* the proteomic dataset (Supplementary Data Set 4). Increased ribosomal frameshifting has been observed in the absence of *truA* at leucine CUN codons [101]. When analyzing CUN/UUN ratios in *V. cholerae*, an increased usage of CUN codons is found in *thrB* and *trp* transcripts [in particular *trpA*, *trpB*, *trpE*, *trpD* and *trpG* (Table S5)]. This codon bias could explain the lower levels of proteins detected for these transcripts in the *truA* deletion mutant.

An elegant example of a homeostatic response that includes a tRNA modification is the regulation of the Mg^{2+} transporter encoded by *mgtA*. In this case, transcription attenuation is mediated by leader peptide (MgtL) sensing ribosome pausing at Pro codons that can be affected by the amount of $\text{m}^1\text{G}37$ in the tRNAs decoding Pro codons inserted by the TrmD enzyme that is dependent on Mg^{2+} [102].

Some of the major transcriptomic changes resulting from tRNA modification defects are not aligned with the expected outcomes, as illustrated by the *ilv* operon

example. These discrepancies may stem from indirect effects, as discussed in the conclusion section, but more broadly, they highlight the extensive unknowns regarding the fine-tuning of elongation speed [42,93]. For example, the poly-phenylalanine stretch in the PyrL leader peptide (encoded by TTTTCTT) is not regulated by ribosome-mediated transcription attenuation but by an RNA polymerase-dependent process dependent on a polyT track [103]. Hence, the PyrL regulated does not seem to be affected by the absence of MiaA (Table 1). However, the same stretch of Ts in PheM leader peptide seems to be affected by the absence of the corresponding modification (Table 1). In a seminal study using the HisL peptide linked to a reporter *lacZ* gene that was used as a “speedometer”, Chevance and Hughes showed that ribosome pausing at a given codon was greatly influenced by the neighboring codons proposing a triplet of triplet code [42,93]. This could explain how these same codons do not always elicit the same responses.

The TnaC leader peptide regulatory system that responds to ribosome pausing is sensitive to many tRNA modification defects

The pioneering work of the Yanofsky group dissected in the 1990’s the complex regulatory mechanisms of the tryptophanase (*tna*) operon of *E. coli* [104]. Not only is the expression of the *tnaABC* operon repressed by catabolite repression but it is induced by tryptophan-dependent transcription antitermination. Induction requires translation of the leader peptide (TnaC) that acts on the translating ribosome to stall in the presence of high tryptophan levels [37]. A similar regulation presumably also occurs in *V. cholerae* since the TnaC leader is also found in this species (Table 1). The absence of ⁱ⁶A in the *miaA* mutant had been shown to repress the expression of *tnaABC* already by Yanofsky [105]. We reproduced this finding but also showed that the absence of many other modifications, including mnm⁵s²U34 (*mnmAGC* mutants) and m²A37 (*rlmN* mutants), led to a strong repression of the *tnaABC* operon in *E. coli* (Fig. 7A and 7B). In *V. cholera* deleting *tgt* led to repression of the *tna* operon (Fig. 7C) but this was not seen in *E. coli* [56]. In summary, this data suggests that, at least in *E. coli*, the TnaC regulatory system is very sensitive to the absence of tRNA modifications.

Iron homeostasis is sensitive to tRNA modification defects

Over 15 years ago, Bläsi and colleagues showed that the translation of *fur*, the master regulator of iron homeostasis, was coupled to the translation of an upstream peptide (Uof, Upstream of *fur*) in a region that also binds the regulatory RNA RyhB [106] (Fig. 2D). They reported that the translation of a Ser codon in Uof was sensitive to the presence of the $ms^{2i^6}A37$ modification in the decoding tRNA inserted by MiaB, an iron-sulfur cluster enzyme. This led to a model for *fur* regulation partly dependent on the translation speed of *uof* (Fig. 2D). The same study proposed that other iron-dependent proteins harbor $ms^{2i^6}A37$ -dependent codons at the beginning of the coding sequence. Consistent with this model, it was reported that the levels of the enterobactin siderophore are increased twofold in a *miaA* mutant [107]. We did observe that genes repressed by Fur, the master regulation of iron homeostasis (compiled in [108]), were upregulated in the *miaA*, *rlmN* and *thil* backgrounds (Fig. 8 and Fig. S4). The molecular mechanisms underlying this regulation are not yet understood. However, it is tempting to propose that these could be caused by inefficient translation or pausing of the *uof* coding sequence, which leads to lower Fur levels. That said the same genes were downregulated in the *tgt* mutant [56] as well as the *mnmA* and *dusB* strains (Fig. 8), indicating that multiple mechanisms must be at play.

In *V. cholerae*, the Fur regulon has been characterized by Chip-seq [109]. We found that genes in the *V. cholerae* Fur regulon are downregulated in the *truA* and *tgt* deletion mutants (Fig. S9), particularly the vibriobactin/enterobactin genes (Fig. S9). No *uof* homolog is found upstream of *V. cholerae fur*, suggesting a different regulation mechanism from *E. coli*. One possibility involves reactive oxygen species (ROS) formation. In *V. cholerae*, proteomics data show that OxyR expression is induced in Δ *tgt*, indicating the presence of ROS [59]. The link between TGT and oxidative stress has also been observed in other species [56,110,111]. ROS can damage iron-sulfur clusters within proteins, leading to the release of free iron inside the cell. Fur expression and activity are regulated by iron. When intracellular levels of iron increase, Fur repressor is activated, which is consistent with decreased expression of iron transporters in Δ *tgt*.

Is the expression of membrane proteins sensitive to tRNA modification defects?

It has been proposed that the positions of slow or fast codons along the coding sequence could be important for the localization of membrane proteins [2]. Hence, the absence of tRNA modifications could lead to defects in membrane protein folding and localization by affecting translation speed. Indeed, a study from the Hou laboratory showed that levels of m¹G37 affect the expression of efflux pumps such as TolC due to the slow decoding of proline codons located at the beginning of the coding sequences [112]. Transcriptomic analyses are not adapted to monitor the integrity of membrane proteins, and proteomic analyses can be challenging because membrane proteins are often insoluble and, thus, not detected by Mass-Spectrometry. With this caveat in mind, we analyzed the available proteomic datasets of *V. cholerae* strains deficient in two ASL modifications (Q34, Ψ38-40) and one tRNA body modification (D20) to evaluate if membrane proteins are enriched in the down-regulated set. First, protein and transcript differential expression levels, fractions of transmembrane domains, amino acid, and codon usage frequencies were gathered for every CDS in the *V. cholerae* genome (Supplementary Data Set 7), and the correlations between all these features were analyzed (Supplementary Data Set 8). No correlation was observed between transmembrane domain fraction and protein expression but as expected, the fraction of transmembrane helices slightly correlated with the frequency of specific amino acids (Supplementary Data Set 8A), as more hydrophobic and less charged amino acids are enriched in transmembrane helices [113]. However, synonymous codons showed similar correlation coefficients: for example, F1 (UUC) and F2 (UUU) are positively correlated with the proportion of transmembrane helices with coefficients of 0.31 and 0.39 respectively (Supplementary Data Set 8A). Hence, in this analysis, differences in synonymous codon usage did not affect the translation of transmembrane helices.

Analyses of codon usage of motility genes in tRNA modification mutants of *V. cholerae*

To further investigate the mechanism how tRNA modifications affect the motility of *V. cholerae* (Fig. 4C), we calculated the correlation coefficient between the proteome and other features in three mutants, Δtgt , $\Delta truA$ and $\Delta dusB$ that showed severe, moderate, and no motility phenotype for motility related genes (Table S4). We found

that translation of two flagellar proteins, FlgP and FlgT, was significantly downregulated in the *tgt* mutant (at least 3-fold less and adjusted p-value <0.05). The levels of nine motility-related proteins were reduced by 2 to 4 folds in the *truA* mutant. None of those genes were downregulated at the transcriptional level (Table S4). To investigate whether the usage of NAU and NAC codons played a role in translation in the *tgt* mutant, we compared the distribution of NAU and NAC in each protein to that of the genome-wide NAU/NAC ratios. We did find that FlgT, which decreased in levels in the *tgt* and *truA* mutant, showed a weak bias towards CAC over CAU (Table S4). This could be explained by the slower decoding speed of NAC compared to NAU with the loss of Q [59].

Conclusions and perspectives

The field of tRNA modification has witnessed a renaissance in the last 10 years fueled by technical advances that have improved their detection [114] and by the characterization of the full set of tRNA modification genes in a few model organisms[44,51]. Many new researchers are being drawn into the field because of the wide array of phenotypes caused by the absence of tRNA modifications with some that can have great consequences for human health [115,116].

In this work, we emphasize that some of the common phenotypes observed in the absence of many different tRNA modifications are related to processes that are regulated by ribosome pausing such as regulation by leader peptides of amino acid biosynthesis genes (Fig. 2). In view of the known roles of tRNA modifications in affecting translation speed, this observation was not unexpected but had yet to be explicitly formulated. This proposed hypothesis can now be specifically tested using ribosome profiling even if it is still difficult to obtain single nucleotide resolution of ribosome pausing events in bacteria compared to eukaryotes [59,117].

Another difficulty in this field lies in interpreting the consequence of tRNA modifications in “multi-omics” data sets (transcriptomic, proteomic, metabolomic and more) because of compounding of direct and indirect effects. Indeed, tRNA modifications act as determinants or anti-determinants for many players of the translation machinery, including other tRNA modification enzymes and aminoacyl-tRNA synthetases[118,119]. For example, the upregulation of *his* operon genes in the *truA* deletion mutants of both *E. coli* and *V. cholerae* (Fig. 6) is due to ribosome pausing in the translation of the HisL leader peptide that triggers the antitermination and

subsequent transcription of the downstream *his* genes (Fig. 2B). However, it is not clear why defects in tRNA modifications that do not modify tRNA^{His} such as ms²i⁶A37 in the *miaA* strain or xm⁵s²U in the *mnmG* strain repressed the same *his* operon in *E. coli* (Fig. 6A). Were histidine pool levels higher in these mutants? Was there a global stress response that triggers a downregulation of amino acids synthesis? [56,59]. In summary, dissecting the molecular consequences of tRNA modification defects can take many years and requires combining different types of omics data and some serendipity as some phenotypes are revealed only in specific species or growth conditions as we recently showed when comparing the phenotypes of Queuosine deficient mutants in *E. coli* and *V. cholerae* [56,59]. That said, with the recent advancements in nanopore RNA modification detection methodologies and in light of the recent report from the US National Academies of Sciences [120], which underscores the importance of establishing “concerted efforts to deepen understanding of RNA modifications and their role in living systems”, research into dissecting the multiple and often subtle roles of tRNA modifications in bacterial physiology and stress adaptation is likely to increase in the coming years.

Supplementary material

Data Set 1. The differential expression analysis of *E. coli* *dusB*, *truA*, *truD*, *thil*, *miaA*, *rlmN* *mnmA*, *mnmC*, *mnmG* strain vs the WT.

Data Set 2. The differential expression analysis of *E. coli* *truA*, *truD*, *thil*, *miaA*, *rlmN* *mnmA*, *mnmC*, *mnmG* mutants and WT vs the *dusB* mutant.

Data Set 3. The differential expression analysis of the *V. cholerae* *tgt*, *truA* and *dusB* strains vs the WT.

Data Set 4. Proteomics analyses of *V. cholerae* WT, *tgt*, *truA*, *rlmN* with sub-MIC tobramycin (TOB) treatment or no treatment.

Data Set 5. GO enrichment of RNAseq data. (A) *E. coli* *dusB*, *truA*, *truD*, *thil*, *miaA*, *rlmN* *mnmA*, *mnmC*, *mnmG* mutants vs WT; (B) *E. coli* *truA*, *truD*, *thil*, *miaA*, *rlmN* *mnmA*, *mnmC*, *mnmG* mutants vs *dusB*; (C) *V. cholerae* *tgt*, *truA* and *dusB* mutants.

Data Set 6. Codon usage data for every coding sequence of *V. cholerae*.

Data Set 7. Proteome, transcriptome, transmembrane domain %, and codon usage for each protein in *tgt*, *dusB* and *truA* *Vibrio cholerae* mutants.

Data Set 8. Correlations between the codon frequency, the fraction of transmembrane helices and the proteome expression data.

Data Set 9. Codon usage data for every coding sequence of *E. coli*.

Data Set 10. Codon usage for transcription factors in *E. coli* and *V. cholerae*.

Supplemental tables

Table S1. Strains used in this study.

Table S2. Oligonucleotides used for *V. cholerae* strain constructions.

Table S3. Top cofit genes for *tgt* gene (b0406) from *Escherichia coli* BW25113.

Table S4. Differential Expression (RNASeq and proteomics) data of *V. cholerae* mobility genes in *dusB*, *tgt* and *truA* mutants and their corresponding usage of NAC and NAU codons.

Table S5. Codon usage data for *V. cholerae* *trp* operon genes.

Table S6. Sequenced of the leader peptides listed in Table 1.

Table S7. Q-related synonymous codons with significant codon usage biases.

Data statement

All mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD035297 and PXD057061. The raw reads of *E. coli* RNA-seq are available at <https://doi.org/10.2598/2/197146.10/2475943>. For the RNAseq of *V. cholerae*, the accession numbers are GSE275883 for the *dusB*, *truA* mutant datasets and GSE214520 for the *tgt* mutant datasets. The scripts for GO analysis and heatmap plot and GO annotation files are available at https://github.com/vdclab/published_scripts/tree/main/tRNAmod_speed.

Author contributions

V.d.C.-L., Conceptualization, Methodology, Data curation, Supervision, Writing – original draft, Writing – review and editing, Funding acquisition; Z.B., Methodology, Data curation, Supervision, Writing – original draft, Writing – review and editing, Funding acquisition; Y.Y., Formal analysis, Software, Data curation, Visualization, Writing – review and editing; G.B., Conceptualization, Writing – original draft, Writing – review and editing; J.B., Methodology, Formal analysis, Data curation, Investigation; J.M.B., Formal analysis, Data curation, Investigation; P.C.D, Supervision, Writing – review and editing; P.H.: Data analysis, Investigation; K.R.H., Investigation. Visualization; D.K., Supervision, Funding acquisition.

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Table 1. How the absence of tRNA modifications affects expression of genes regulated by leader peptide-dependent processes in *E. coli* and *V. cholerae*. The sequences of the leader peptides are given in Table S6. Analysis of expression data present in Supplemental data sets 1 and 3. Most Information was mined from EcoCyc and Pubmed.

Gene	Product	Effects of modification deficiencies	regulated genes	codon
<i>E. coli</i>				
<i>pyrL</i>	<i>Pyr</i> operon leader peptide	No effect	<i>pyrBI</i>	None, poly-T tract
<i>tnaC</i>	Tryptophanase operon leader peptide	Operon downregulated in any mutants	<i>tnaAB</i>	W
<i>pheM</i>	Phenylalanyl-tRNA synthetase (<i>pheST</i>) operon leader peptide	Operon upregulated in <i>miaA</i> and <i>dusB</i>	<i>pheST</i>	F
<i>leuL</i>	<i>Leu</i> operon leader peptide	Operon up-regulated only in <i>truA</i>	<i>leuABCD</i>	L
<i>ilvL</i>	<i>IlvGEDA</i> operon leader peptide	Operon downregulated in many mutants but not in expected direction	<i>ilvGEDA</i>	I, L, V
<i>ivbL</i>	<i>IivB</i> operon leader peptide	Gene downregulated in 7 out of the 10 mutants analyzed.	<i>ilvLBN</i>	L, V
<i>hisL</i>	<i>His</i> operon leader peptide	Operon upregulated in <i>truA</i> , <i>tgt</i> and <i>rlmN</i>	<i>hisGDCBHAFI</i>	H
<i>thrL</i>	<i>Thr</i> operon leader peptide	Operon upregulated in <i>truA</i>	<i>thrLABC</i>	T
<i>trpL</i>	<i>Trp</i> operon leader peptide	Operon upregulated in <i>miaA</i> as expected	<i>trpECBA</i>	W
<i>pheL</i>	Leader peptide of chorismate mutase-P-prephenate dehydratase	Operon upregulated in <i>dusB</i> but not <i>miaA</i> , downregulated in a few other mutants such as <i>mnmA</i>	<i>pheLA</i>	F
<i>mgtL</i>	MgtLA operon leader peptide	Operon upregulated when <i>trmD</i> is depleted [102] or EFP is depleted [121] and downregulated in <i>thil</i> .	<i>mgtLA</i>	P
<i>uof</i>	RyhB-regulated <i>fur</i> leader peptide	Many modifications activate Fur-dependent genes	<i>fur</i>	S
<i>V. cholerae</i>				
VC_1175	?	No data	trp operon?	
VC_1176	predicted TrpL	Operon downregulated in <i>truA</i> and <i>rlmN</i>	trp operon?	W
VC_1218	hypothetical PheM	Operon downregulated in <i>truA</i>	<i>pheST</i> VC_1219	
VC_1030	?	No data	possibly <i>guaB</i> VC_1031	P?
VC_1032	Hypothetical MgtL?	No data	possibly <i>mgtA/zntA</i> VC_1033	
VC_2509	<i>PyrL</i>	Operon downregulated in <i>truA</i>	<i>pyrB</i> VC_2510	None, poly-T tract
VC_A0162	<i>TnaC</i>	Operon upregulated in <i>truA</i> but down regulated in <i>tgt</i>	<i>tnaAB</i>	

Figure Legends

Figure 1. The tRNA decoding capacities and ASL modifications of *E. coli*. Letters from inside out indicate the codon's first, second, and third position (5'- to 3'- end) as the subscripts indicate. The circles outside of the wheel connected or not by a line represent one tRNA species. A filled circle indicates the capacity of that tRNA to base pair with the indicated codon by Watson-Crick pairing. An open (white) circle suggests base pairing by the wobble hypothesis [122]. The base at position 34 of the anticodon and the anticodon stem loop (ASL) modifications in positions 32 to 40 are shown next to each tRNA circle. Amino acids that are decoded by a single tRNA are highlighted in red. Data are compiled from references [122–125]. Discrepancies among references are shown in parentheses, suggesting partial modifications or modifications at certain conditions.

Figure 2: Gene regulation mediated by the translation efficiency of small ORFs.

In prokaryotes, three major mechanisms of regulation are known: (A) Rho-independent transcription termination, where translation stalling on the leader peptide alters the mRNA conformation to prevent the formation of a Rho-independent terminator; (B) Rho-dependent transcription termination, where translation stalling or ribosomal drop-off on the leader peptide alters the mRNA conformation to block Rho accessibility to the Rho utilization site (RUT); and (C) Transcriptional attenuation, where translation stalling on the leader peptide alters the mRNA conformation, allowing the initiation by the ribosome of the regulated gene. A to C, Dark blue and sky blue represent sequences that can form a stem-loop structure. (D) Proposed regulation of the *fur* gene by the small ORF *uof*; the two genes overlap, and a putative stalling or drop-off of the ribosome during translation of *uof* regulates *fur* expression. In eukaryotes (E), uORFs also mediate the expression of regulated genes, but the regulation occurs through the ability of uORFs to prevent ribosome scanning, thereby blocking the initiation of the regulated gene.

Figure 3. Differentially expressed genes in nine *E. coli* tRNA modification mutants. (A) Heatmap showing DEGs and their GO terms observed in three and more out of ten comparisons between the WT and nine tRNA modification mutants to the *dusB* mutant. The data is extracted from Supplementary Data Set 5B. The GO terms

associated with each gene are arbitrarily grouped. (B) The enriched GO terms with p-values less than 0.05 and Benjamini-Hochberg adjusted p-values less than 0.2 were shown in three of the mutants (the full analysis is shown in Fig. S4).

Figure 4. The absence of different tRNA modification genes can increase or decrease motility. Motility assays were performed as described in the method section for isogenic sets of (A) *E. coli* or (C) *V. cholerae* WT and deletion strains. Error bars represent the standard deviation of biological triplicates. (B) heatmap showing the differentially expressed motility genes in the *E. coli* tRNA modification mutants compared to the *dusB* strain. Data was extracted from Supplementary Data Set 5B.

Figure 5. DEGs in three *V. cholerae* tRNA modification mutants. (A) A heatmap showing DEGs and their GO terms in comparisons between the *dusB*, *tgt* and *truA* strains to the WT. The data is extracted from Supplementary Data Set 5C. (B) The enriched GO terms with p-values less than 0.05 and Benjamini-Hochberg adjusted p-values less than 0.2 were shown.

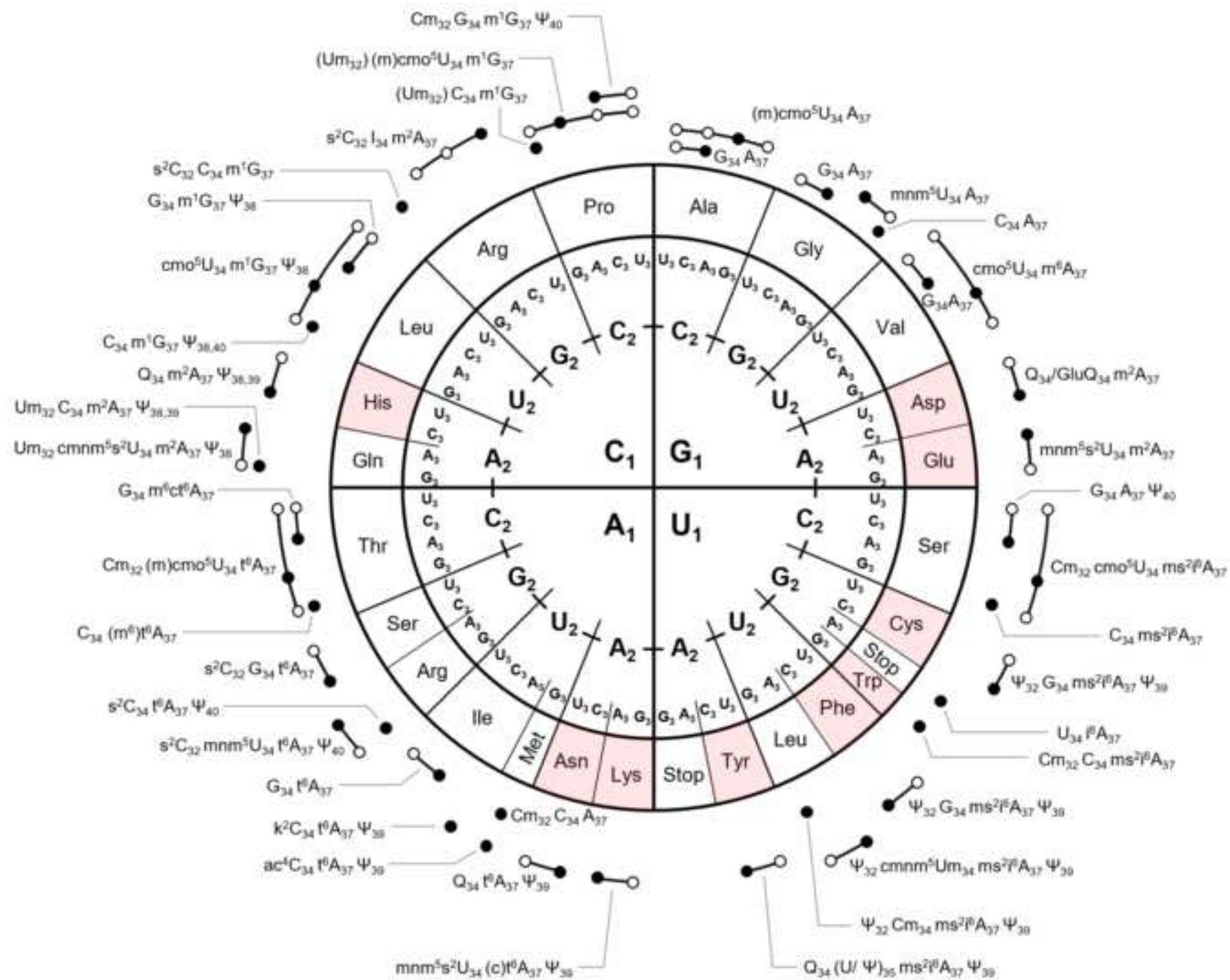
Figure 6. HisL and LeuL-regulated genes are upregulated specifically by the absence of Ψ 38-40. Log2 fold change of transcripts of HisL- (A) and LeuL- (B) regulated genes in *E. coli* deletion mutants compared to the WT. Data extracted from **Supplementary Data Set1**. Log2 fold change of transcripts and peptides of HisL- (C) and LeuL- (D) regulated genes in *V. cholerae* compared to the WT (light colors RNA seq, dark colors proteomics). The deletion mutants are indicated at the bottom in corresponding colors. Data extracted from Supplementary Data Sets 3 and 4.

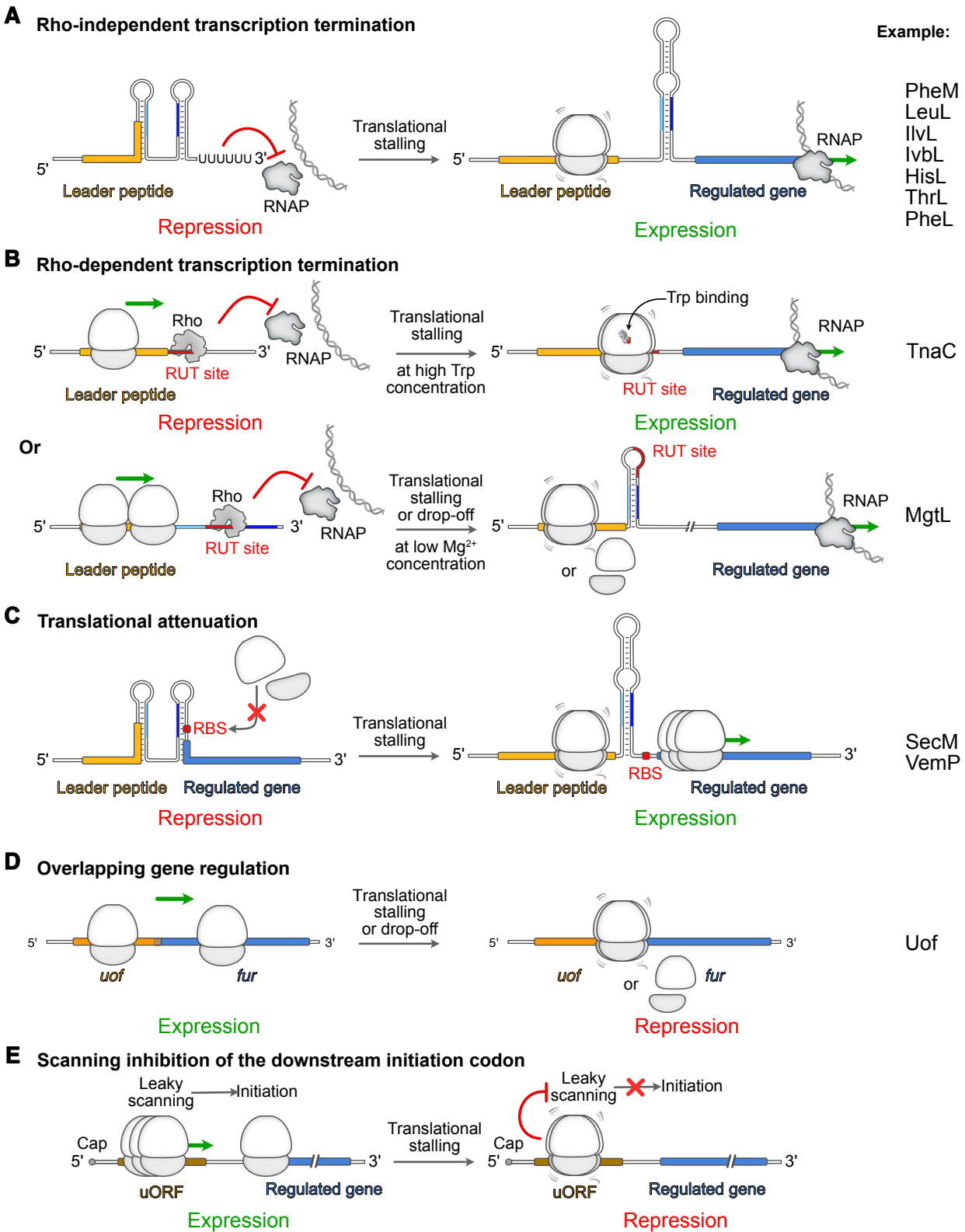
Figure 7. TnaC-regulated genes are greatly affected by tRNA modification deficiency. (A) and (B) Log2 fold change of transcripts of *tnaABC* genes in *E. coli* BW25113 deletion mutants compared to the WT. (A) Data extracted from **Supplementary Data Set1**. (B) Data extracted from NCBI Gene Expression Omnibus database (accession GSE237609). (C) Log2 fold change of transcripts of *tnaABC* genes and encoded peptides in *V. cholerae* (light colors RNA seq, dark colors proteomics). Data was extracted from Supplementary Data Sets 3 and 4. The deletion mutants are indicated at the bottom in corresponding colors.

Figure. 8. Iron homeostasis genes can be up or down-regulated in *E. coli* tRNA modification mutants. Log₂ fold changes of transcripts of iron homeostasis genes in *E. coli* deletion mutants compared to the WT strain. The deletion mutants are indicated at the bottom in corresponding colors. Data extracted from Supplementary Data Set 1.

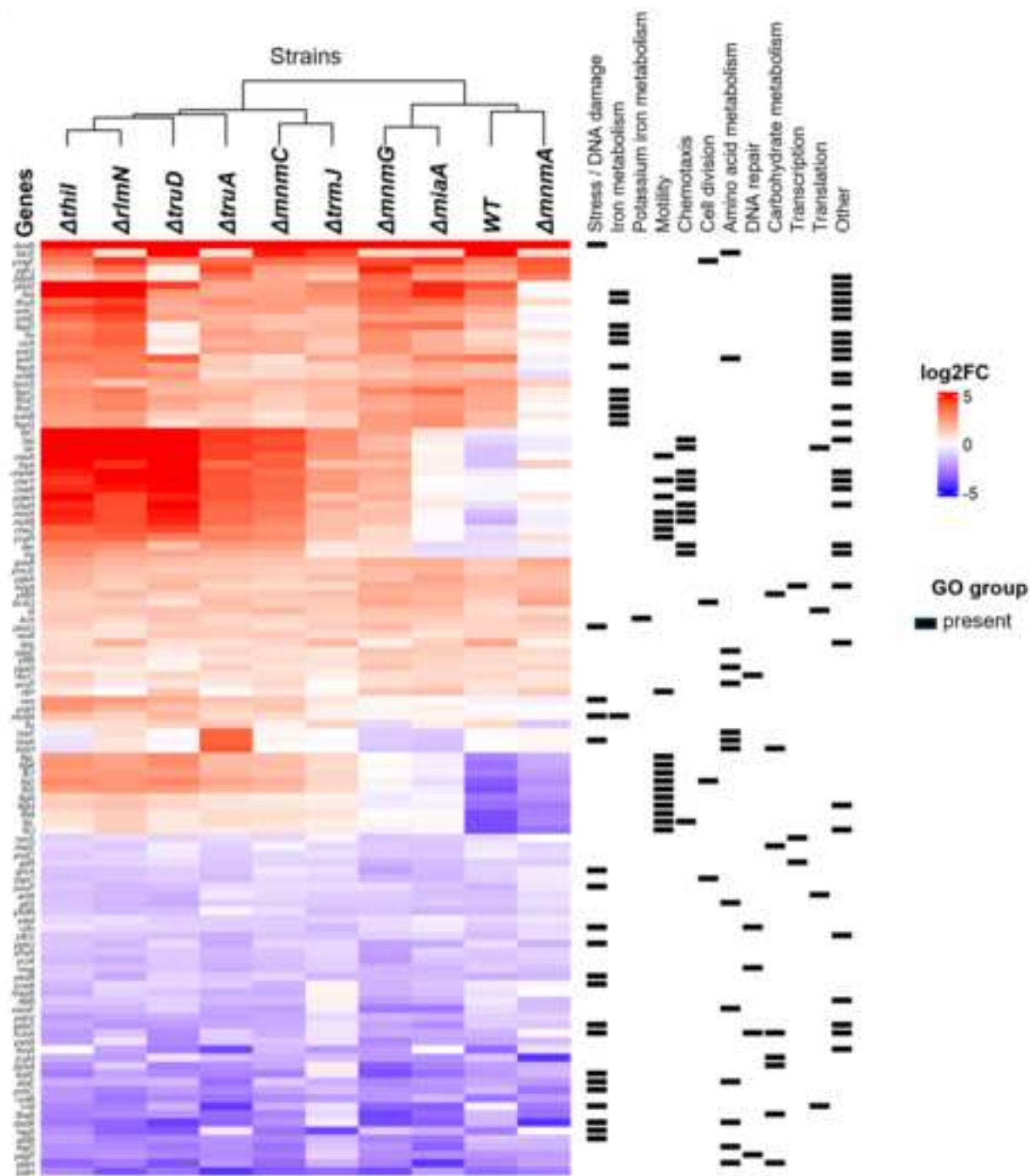
Figure 1

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A



B

