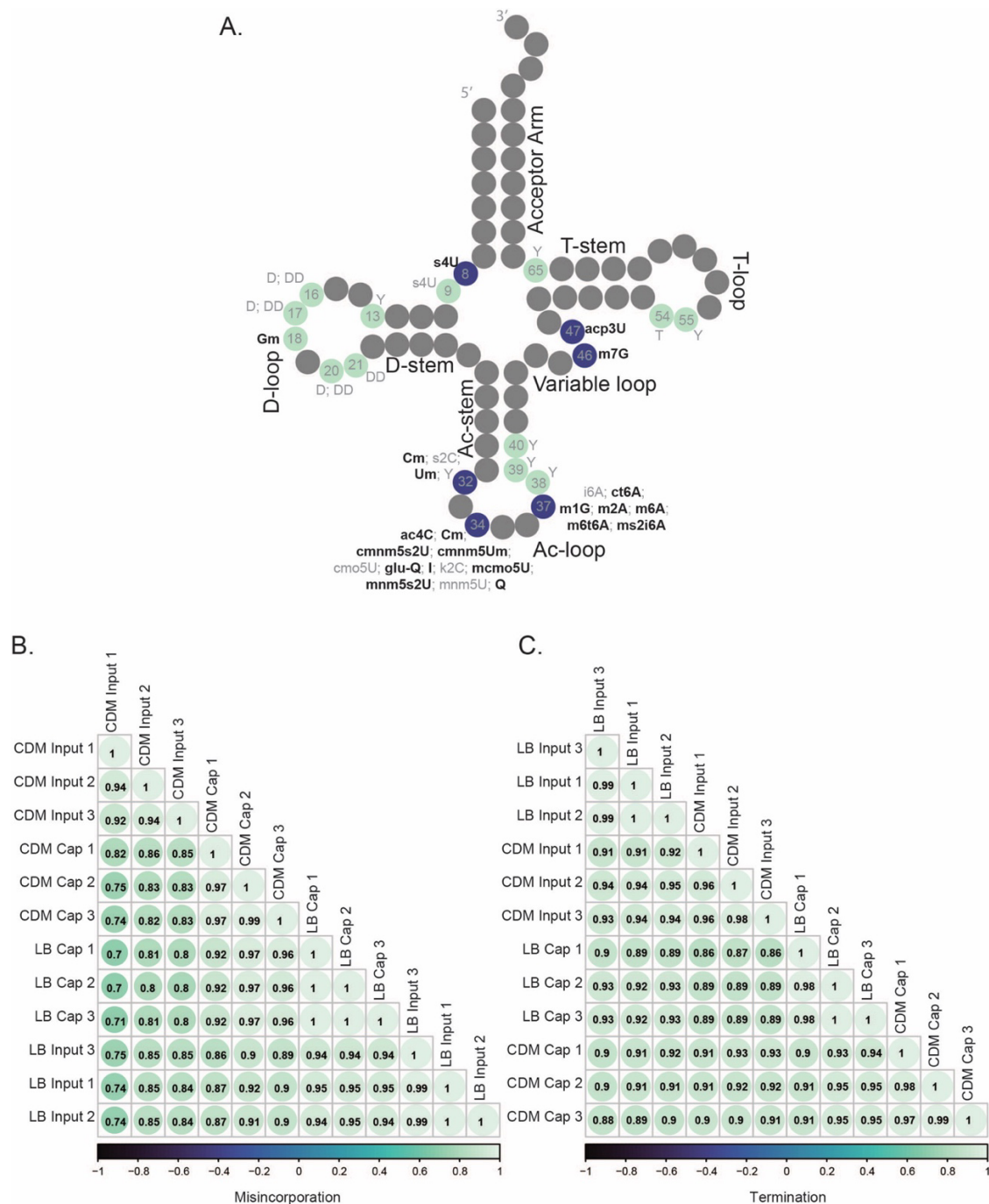




Supplemental Figure 1.

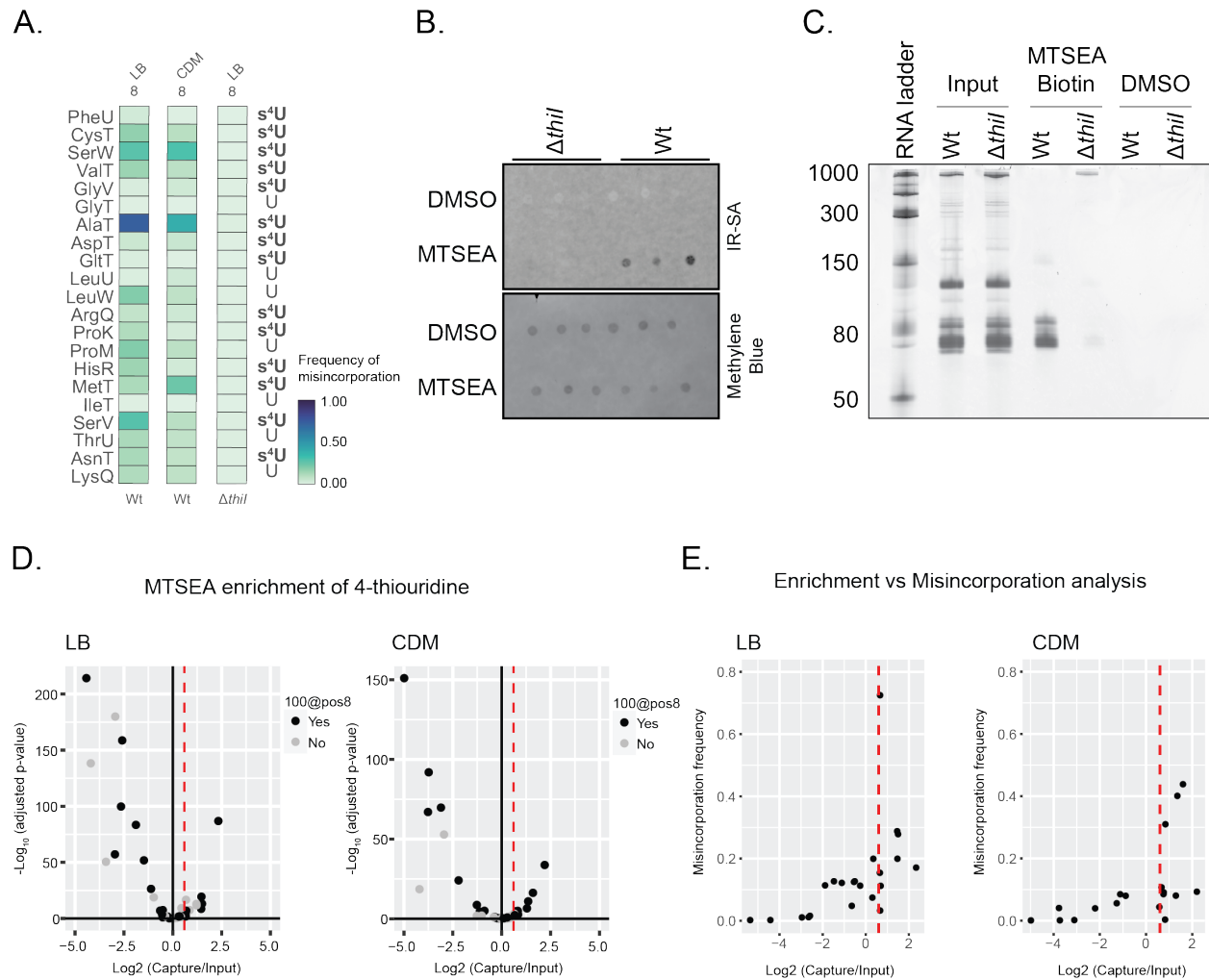
(A) Schematic representation of a mature tRNA. Positions known to be modified are indicated in green (positions where a cDNA mutational signature is not observed) or blue (positions where a mutational signature is observed). Modifications with a detectable mutational signature are represented in bold. **(B)** Pearson correlation coefficient plots for misincorporation and **(C)** termination between replicates for total RNA (input) and MTSEA-captured (Cap) samples in *E. coli* cultured in chemically defined media (CDM) and LB.

A.

E. coli CDMB. *E. coli* LB

Supplemental Figure 2.

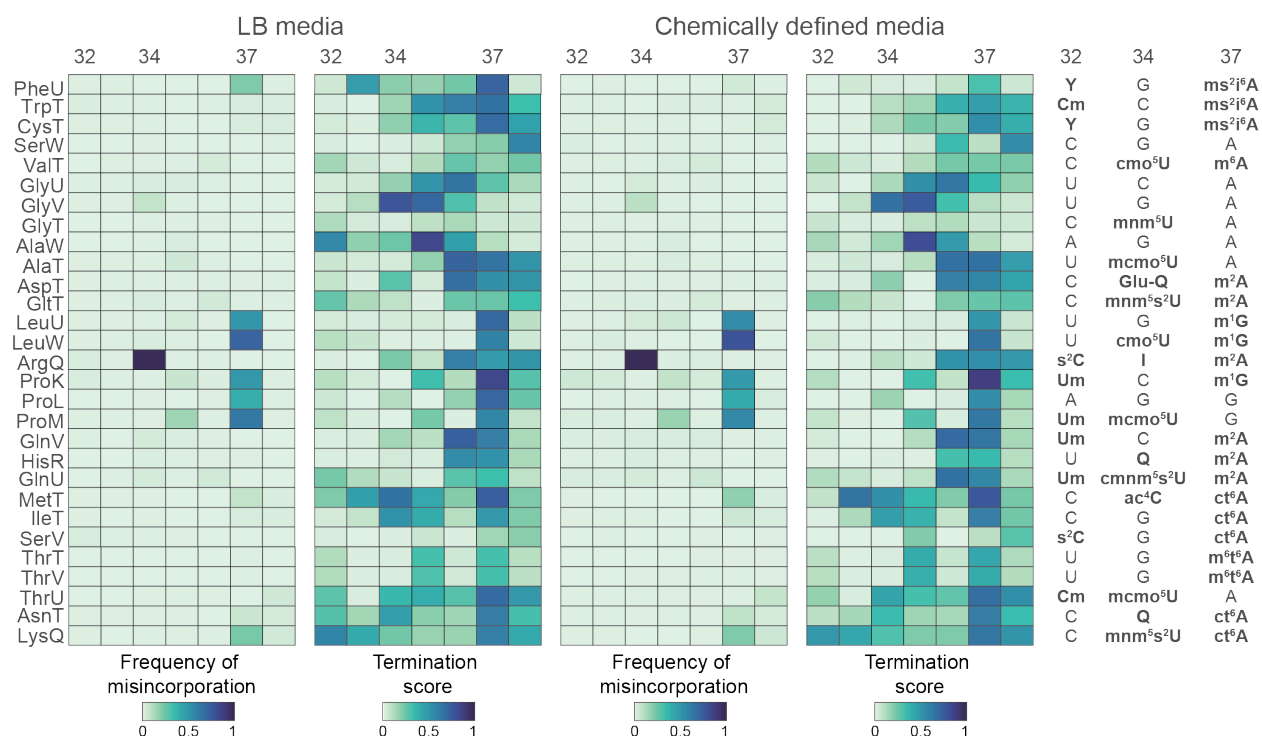
(A) Heatmap of average frequency (from three independent samples) of misincorporation (left) or termination score (right) at each position for *E. coli* tRNAs extracted from cells cultured in chemically modified media (CDM) and collected at $(OD)_{600}=0.6$. All tRNAs in the custom transcriptome are represented. The grey refers to regions of less than 100 reads per position. Different regions of the tRNA are represented at the bottom from the 5' (left) to the 3' end (right) of the tRNA. Darker lines indicate the loop in that region. **(B)** Heatmap of average frequency (from three independent samples) of misincorporation (left) or termination score (right) at each position for *E. coli* tRNAs extracted from cells cultured in LB and collected at $(OD)_{600}=0.6$. All tRNAs in the custom transcriptome are represented. The grey refers to regions of less than 100 reads per position. Different regions of the tRNA are represented at the bottom from the 5' (left) to the 3' end (right) of the tRNA. Darker lines indicate the loop in that region.



Supplemental Figure 4.

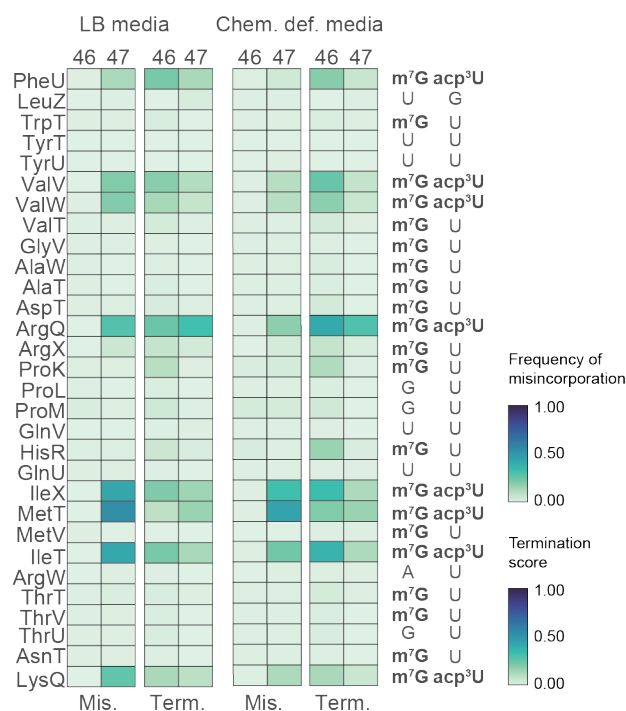
(A) Heatmap of average frequency (from three independent samples) of misincorporation at position 8 of tRNAs in *E. coli* wild type cells cultured in LB (left) or chemically defined media (CDM) (center) and $\Delta thil$ mutant cells cultured in LB media (right). All cells were collected at $(OD)_{600}=0.6$. Only tRNAs with read coverage > 100 at position 8 in all 3 experiments are represented. **(B)** Streptavidin (SA) dot blot was performed on RNA extracted from wild type (BW25113 strain) or $\Delta thil$ *E. coli* cells and treated with DMSO or MTSEA. Methylene blue was used as a loading control. **(C)** RNA extracted from wild type and $\Delta thil$ *E. coli* cells resolved on a 10% denaturing urea polyacrylamide gel. RNA was treated with MTSEA or DMSO, incubated with SA beads, and eluted RNA resolved in the gel. Total RNA from each bacterial strain is included. **(D)** Volcano plot representation of log 2-fold change in abundance after MTSEA capture of tRNA extracted from *E. coli* cells cultured in LB (left) or chemically defined media (right). Dotted red line indicates the threshold used to define a tRNA as enriched. Color indicates if read coverage at position 8 is

above 100. Log 2-fold change and adjusted p-value calculated with DESeq2. **(E)** Scatter plot representation of log 2-fold change in abundance after MTSEA capture and misincorporation frequency of tRNA from *E. coli* cells cultured in LB (left) or chemically defined media (right). Dotted red line indicates the threshold used to define a tRNA as enriched. Log 2-fold change and adjusted p-value were calculated with DESeq2.



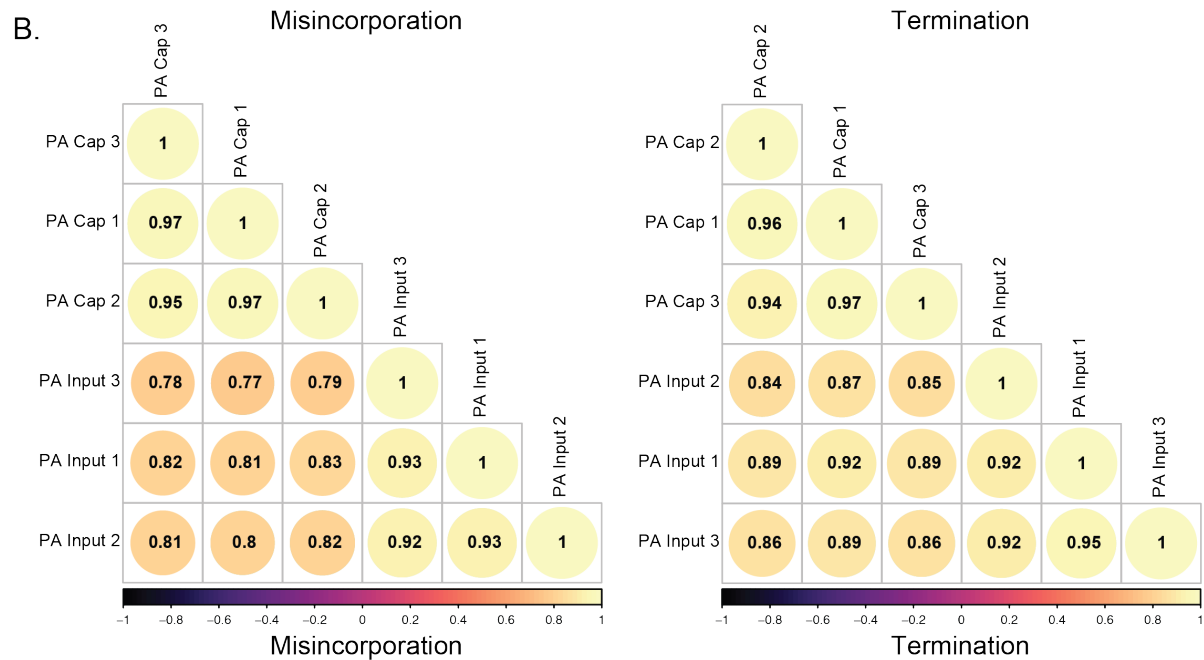
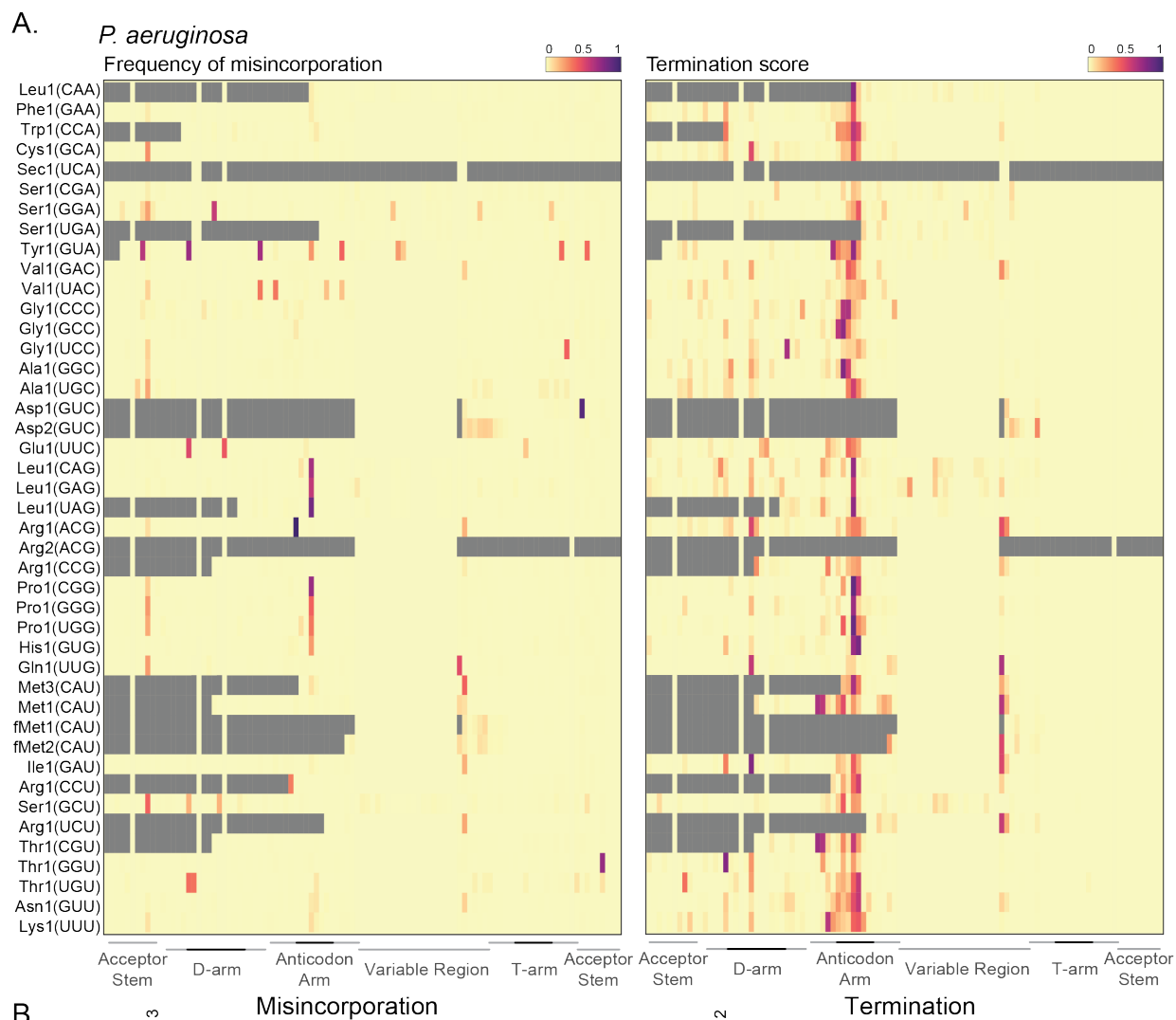
Supplemental Figure 5.

Heatmap representation of average (from three independent samples) frequency of misincorporation or termination score between positions 32 and 38 in tRNA from *E. coli* cultured in LB (left) or chemically defined media (right). Only tRNAs with read coverage > 100 in the anticodon loop in both experiments are represented. Positions 32, 34 and 37, known to be modified are annotated at the top of each heatmap. Known modifications for *E. coli* are represented on the right. The short name for each modification is used (Supplemental Table 11).



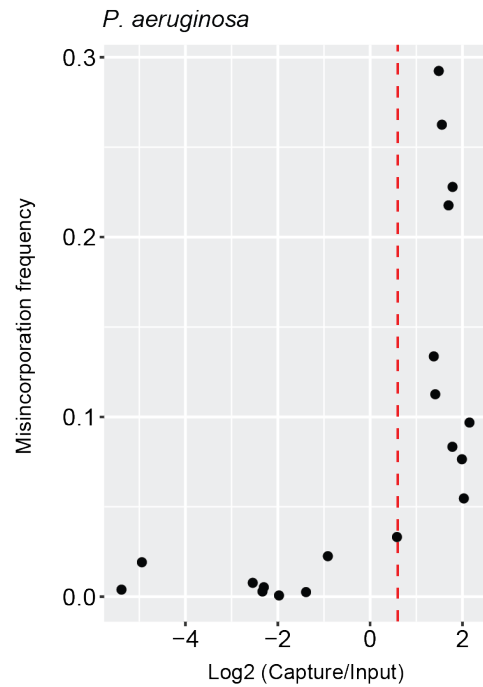
Supplemental Figure 6.

Heatmap representation of average (from three independent samples) frequency of misincorporation and termination score at positions 46 and 47 in tRNA from *E. coli* cultured in LB (left) or chemically modified media (right) and collected at $(OD)_{600}=0.6$. Only tRNAs with read coverage > 100 at position 46 in both experiments are represented. The annotated nucleotide is represented to the right side of the heatmap. The short name for each modification is used (Supplemental Table 11).



Supplemental Figure 7.

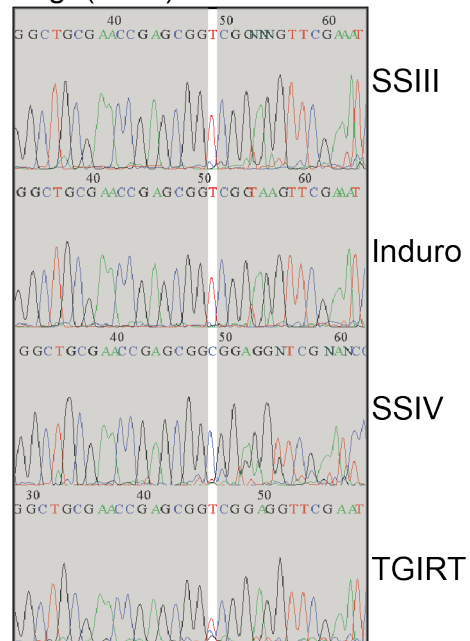
(A) Heatmap representation of average (from three independent samples) frequency of misincorporation (left) or termination score (right) at each position for *P. aeruginosa* tRNAs. All tRNAs in the custom transcriptome are represented. RNA was collected from cells cultured in chemically modified media and collected at $(OD)_{600}=0.6$. Different regions of the tRNA are represented at the bottom. Darker lines indicate the loop in that region. The GtRNAdb names are used for *P. aeruginosa* tRNAs. The anticodon is shown in superscript. **(B)** Pearson correlation coefficient plots for misincorporation and termination between replicates for total RNA and MTSEA-captured samples in *P. aeruginosa*.



Supplemental Figure 8.

Scatter plot representation of log 2-fold change in abundance after MTSEA capture and misincorporation frequency of tRNA from *P. aeruginosa* cells cultured in chemically defined media. Dotted red line indicates the threshold used for enrichment in the MTSEA enrichment experiment.

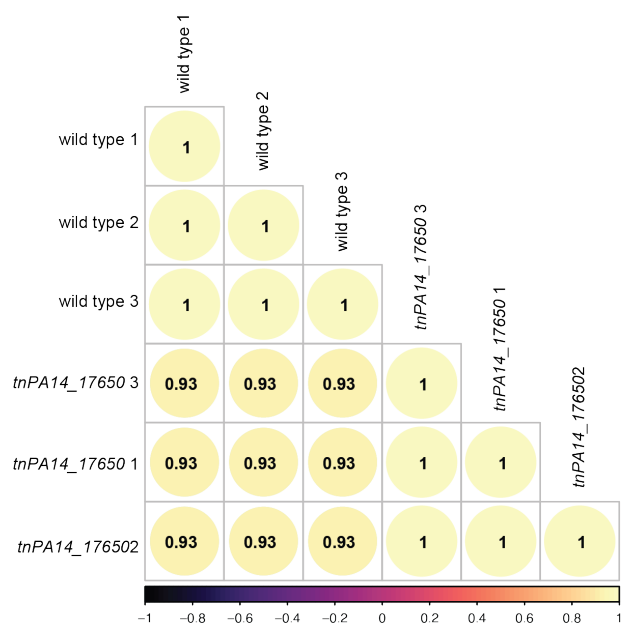
WT *P. aeruginosa*
Arg1(ACG)



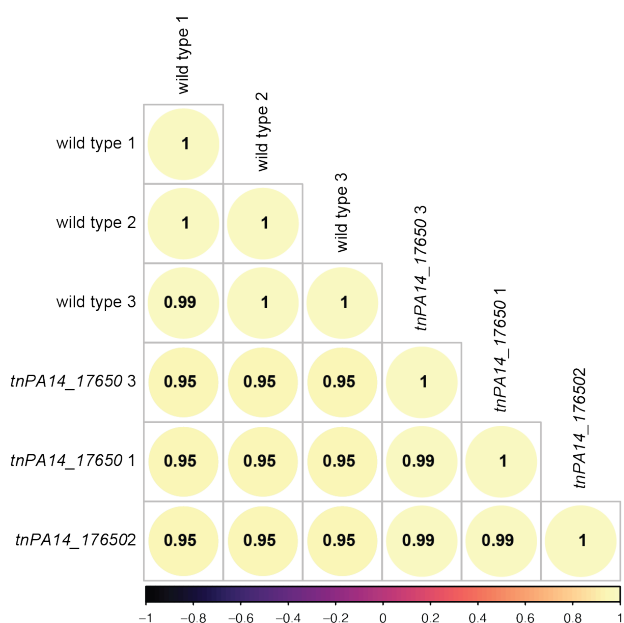
Supplemental Figure 9.

Sanger sequencing chromatograms of targeted RT-PCR to detect the acp³U signature observed at position 47 in tRNA^{Arg1(ACG)} from *P. aeruginosa*. Listed are four reverse transcriptase enzymes that were tested.

A. Misincorporation

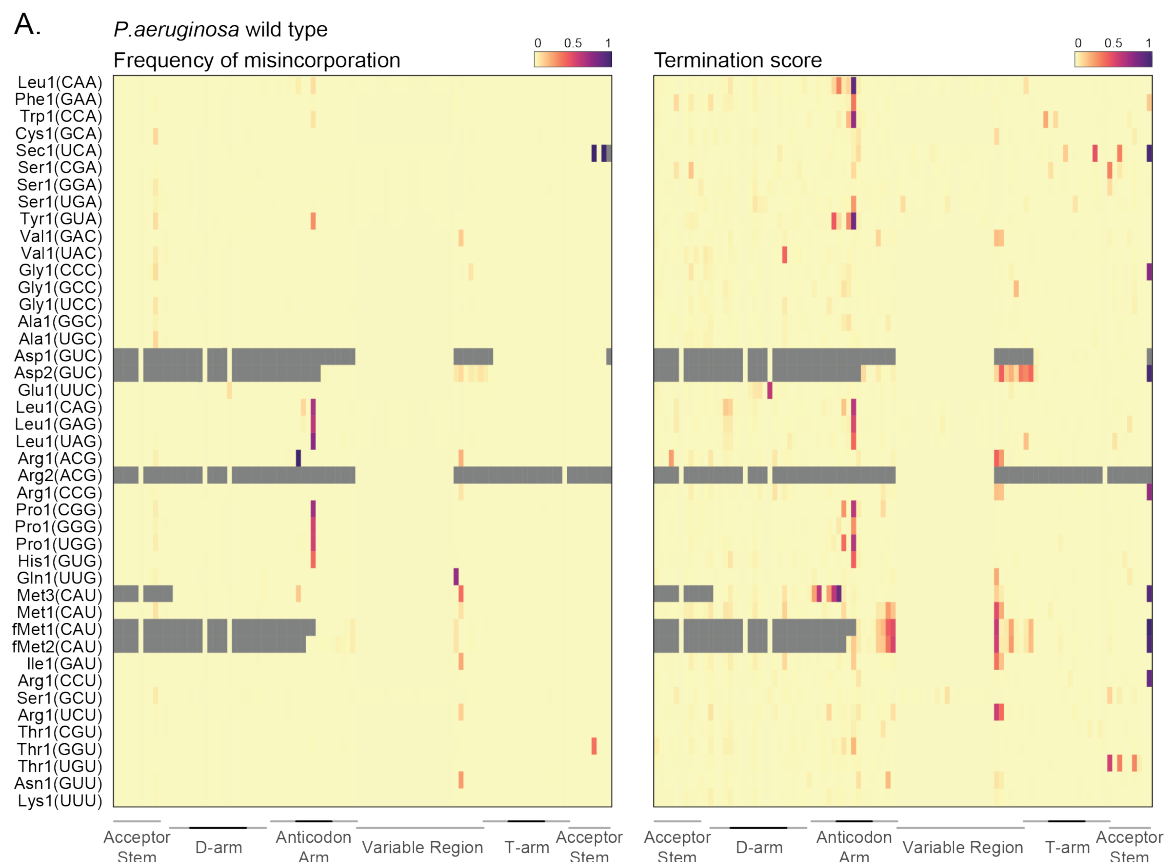


B. Termination



Supplemental Figure 10.

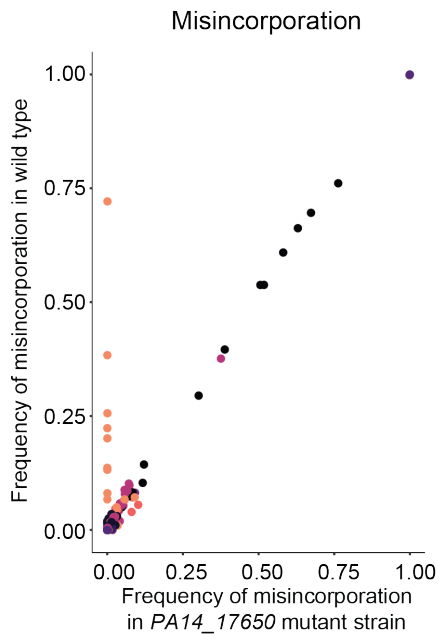
(A, B) Pearson correlation coefficient plots for misincorporation and termination between replicates for wild-type (Wt_PA) and PA14_17650 mutant (Mut_PA) from *P. aeruginosa* RNA sequencing.



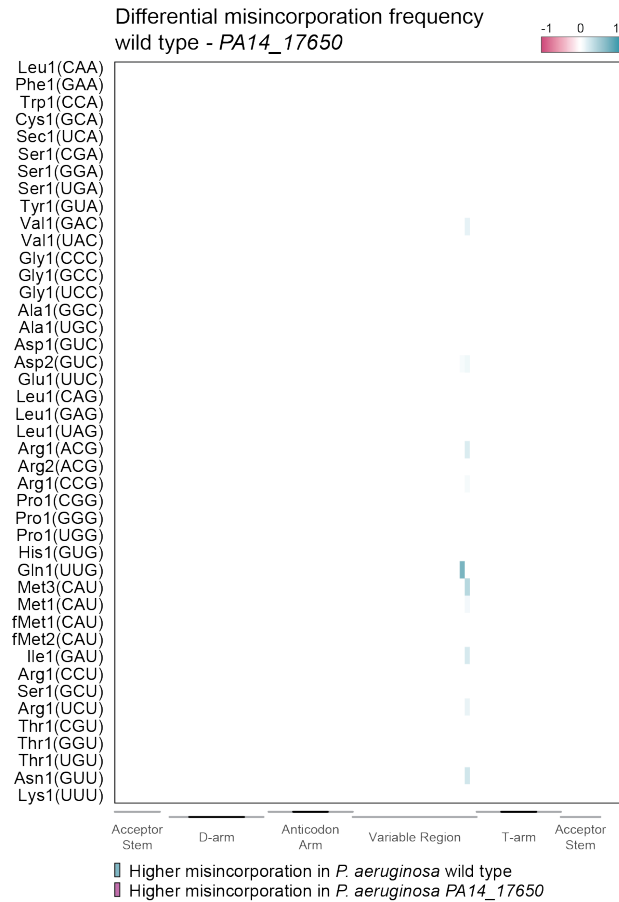
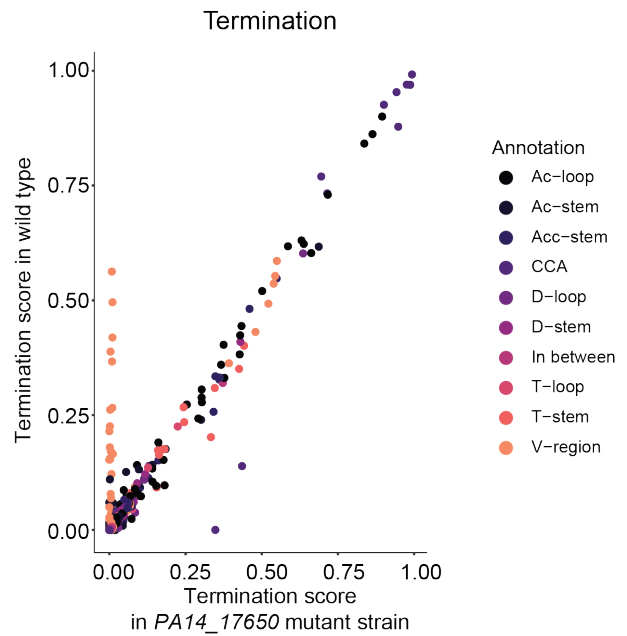
Supplemental Figure 11.

(A) Heatmap of average frequency (from three independent samples) of misincorporation (left) or termination score (right) at each position for wild-type (Wt) *P. aeruginosa* and *P. aeruginosa* PA14_17650 (Mut) mutant tRNAs extracted from cells cultured in chemically modified media (CDM) and collected at $(OD)_{600}=0.6$. All *P. aeruginosa* tRNAs are represented with the GtRNAb names, and the anticodons shown in parentheses. The grey refers to regions of less than 100 reads per position. Different regions of the tRNA are represented at the bottom from the 5' (left) to the 3' end (right) of the tRNA. Darker lines indicate the loop in that region. **(B)** Heatmap of average frequency (from three independent samples) of misincorporation (left) or termination score (right) at each position for PA14_17650 mutant (Mut) *P. aeruginosa* tRNAs extracted from cells cultured in LB and collected at $(OD)_{600}=0.6$. All *P. aeruginosa* tRNAs are represented with the GtRNAb names, and the anticodons shown in parentheses. The grey refers to regions of less than 100 reads per position. Different regions of the tRNA are represented at the bottom from the 5' (left) to the 3' end (right) of the tRNA. Darker lines indicate the loop in that region.

A.



B.



Supplemental Figure 12.

(A) Top: Scatter plot representing the level of misincorporation at all positions between wild-type *P. aeruginosa* or PA14_17650 mutant tRNA. Color coding at the right of each graph indicates the location of the tRNA positions within the annotated region of the tRNA. **Bottom:** Heatmap of differential misincorporation at each position for tRNAs. Values are calculated as signal in *P. aeruginosa* wild type subtracted of signal in PA14_17650 mutant strain. Sites with higher signal in *P. aeruginosa* are represented in blue gradient scale, while positions with higher signal in PA14_17650 mutant strain are represented in a purple gradient scale. **(B) Top:** Scatter plot representing the termination score at all positions between wild-type *P. aeruginosa* or PA14_17650 mutant tRNA. Color coding at the right of each graph indicates the location of the tRNA positions within the annotated region of the tRNA. **Bottom:** Heatmap of differential termination score at each position for tRNAs. Values are calculated as signal in *P. aeruginosa* wild type subtracted of signal in PA14_17650 mutant strain. Sites with higher signal in *P. aeruginosa* are represented in blue gradient scale, while positions with higher signal in PA14_17650 mutant strain are represented in a purple gradient scale.

Supplemental Table legends:

Supplemental Table 1. *E. coli* tRNAs are listed, including transcript ID, tRNA name, anticodon and tRNA sequences. tRNAs that contained 100% sequence identity were grouped together and named by the first tRNA in the group.

Supplemental Table 2. Master table of *E. coli* tRNA misincorporation and termination scores for each position from all tRNA of cultures grown in LB and CDM. Listed are the transcript ID, tRNA name, annotated position and corresponding region (a zero refers to locations in which regional information is not available), the reference nucleotide at each position, all annotated modifications, average coverage depth, and misincorporation (mis) and termination (ts) scores according to our sequencing data. Scores were calculated only for depths greater than 100.

Supplemental Table 3. DEseq2 analysis output of MTSEA-biotin captures versus inputs from *E. coli* grown in LB (3A) or CDM (3B). Listed are the transcript ID, tRNA name, base mean, log2fold change, lfc standard error value, the Wals statistic (stat), p values and adjusted p values (padj).

Supplemental Table 4. Table of *E. coli* tRNA misincorporation and termination scores for positions 32, 34 and 37 from all tRNA of cultures grown in LB and CDM. Listed are the transcript ID, tRNA name, annotated position and corresponding region, the reference nucleotide at each position, all annotated modifications, average coverage depths, and misincorporation (mis) and termination (ts) scores according to our sequencing data. Scores were calculated only for depths greater than 100.

Supplemental Table 5. Table of *E. coli* tRNA misincorporation and termination scores for positions 46 and 47 from all tRNA of cultures grown in LB and CDM. Listed are the transcript ID, tRNA name, annotated position and corresponding region, the reference nucleotide at each position, all annotated modifications (a zero refers to no annotated nucleotide at that position), average coverage depths, and misincorporation (mis) and termination (ts) scores according to our sequencing data. Scores were calculated only for depths greater than 100.

Supplemental Table 6. *P. aeruginosa* tRNA gene information pulled from Ensembl (6A). tRNAs transcript ID, *P. aeruginosa* (PA) gene name, and assigned tRNA nomenclature for each tRNA

(6B). Conserved *E. coli* and *P. aeruginosa* tRNAs were matched based on anticodons and by sequence similarity (6C).

Supplemental Table 7. Master table of matched *E. coli* and *P. aeruginosa* tRNAs. Listed are the transcript ID, tRNA name, annotated position and corresponding region (a zero refers to locations in which regional information is not available), the reference nucleotide at each position, all annotated modifications, average coverage depths, and misincorporation (mis) and termination (ts) scores according to our sequencing data. Scores were calculated only for depths greater than 100.

Supplemental Table 8. Deseq analysis output of MTSEA-biotin captures versus inputs of *P. aeruginosa*. Listed are the transcript ID, tRNA name, base mean, log2fold change, lfc standard error value, the Wals statistic (stat), p values and adjusted p values (padj).

Supplemental Table 9. Data used for the plot in Supplemental Figure 8 indicating a positive correlation between misincorporation score and log 2-fold enrichment by MTSEA-biotin. Listed are the transcript ID, tRNA name, misincorporation scores (mis) and log2fold values (l2f) for each tRNA that had 100 reads or more at position 8 and significant padj values (< 0.05).

Supplemental Table 10. Data from the correlation plot in Figure 2C comparing log2fold enrichment values between each matched tRNA of *E. coli* (Ec) and *P. aeruginosa* (PA) MTSEA-biotin capture experiments. Listed are each anticodon and the subsequent log2fold values.

Supplemental Table 11. Listed are the all the different modifications found in tRNA and their corresponding abbreviations or short names, based on literature and the Modomics RNA modification database [8,36].

Supplemental Table 12. List of tRNA modifying enzymes from *E. coli* and *P. aeruginosa* and their homology BLAST scores.

Supplemental Table 13. Master table of *P. aeruginosa* tRNAs from wild-type (Wt) and PA14_17650 (*tapT*) mutant (Mut) sequencing data. Listed are the transcript ID, tRNA name, reference base, annotated position and corresponding region (a zero refers to locations in which regional information is not available), the anticodon (AC), and the average misincorporation (mis),

depths, and termination scores according to the sequencing data. Scores were calculated only for depths greater than 100, otherwise listed as NA.

Supplemental Table 14. Oligo sequences used to make sequencing libraries and for targeted RT-PCR and sanger sequencing.