Supplementary Information

Mechanism of aminoacyl-tRNA acetylation by an aminoacyl-tRNA acetyltransferase AtaT from enterohemorrhagic *E. coli*

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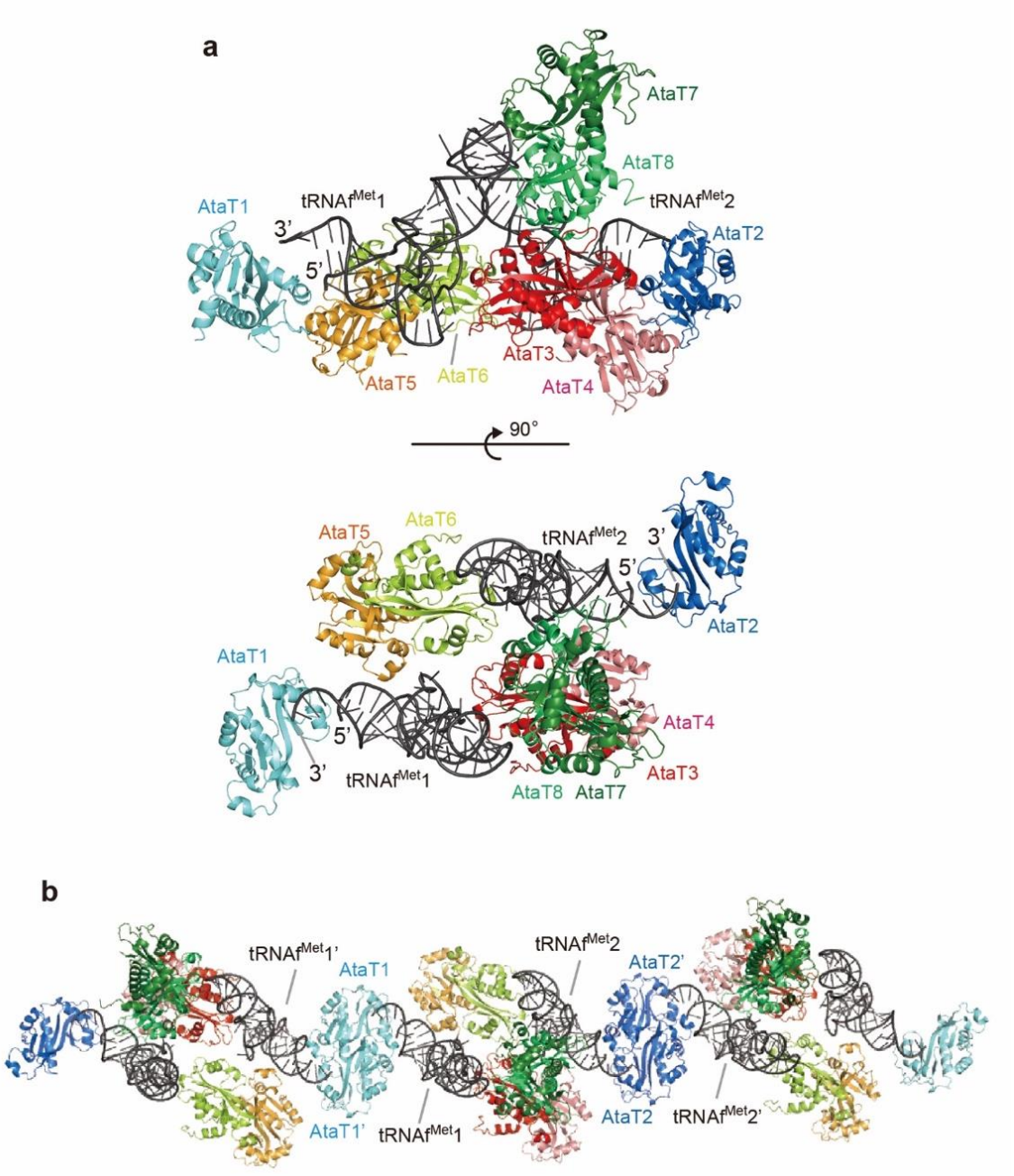
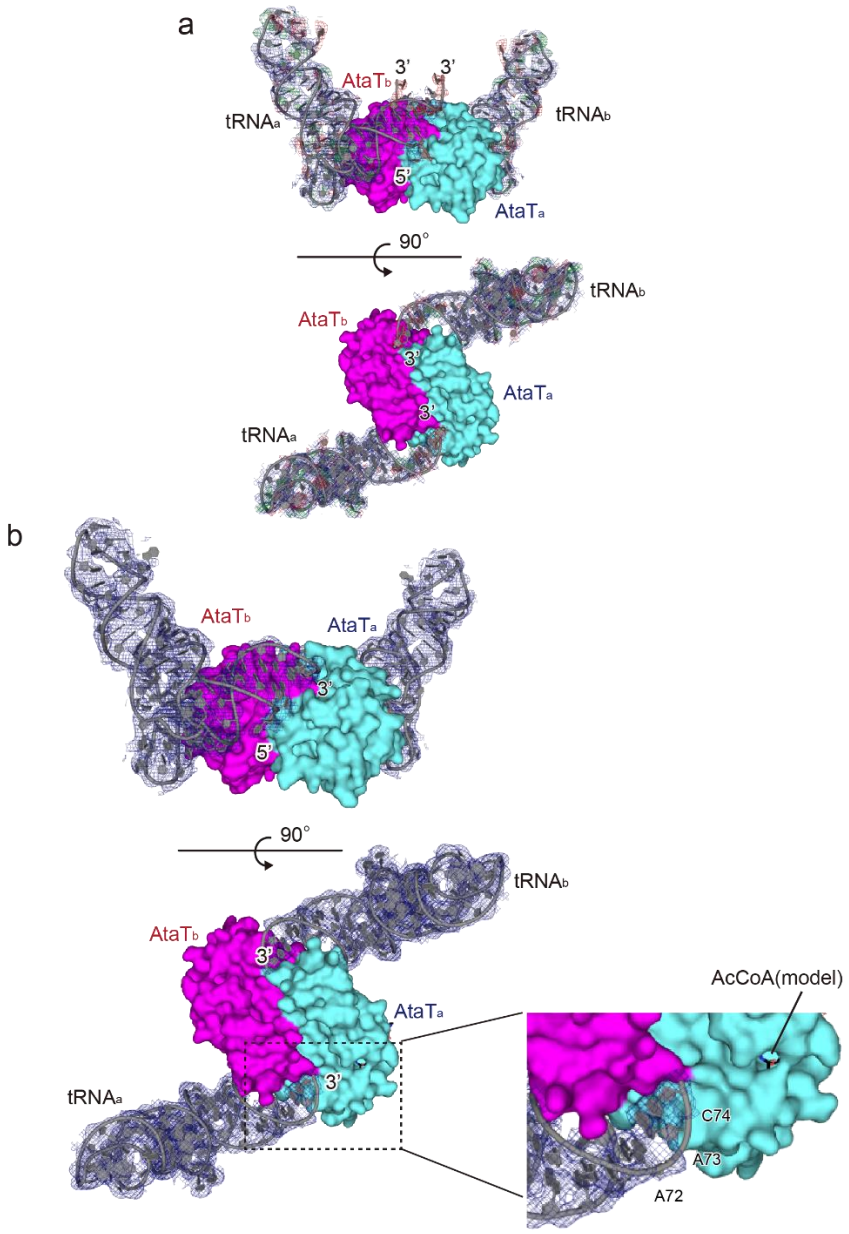
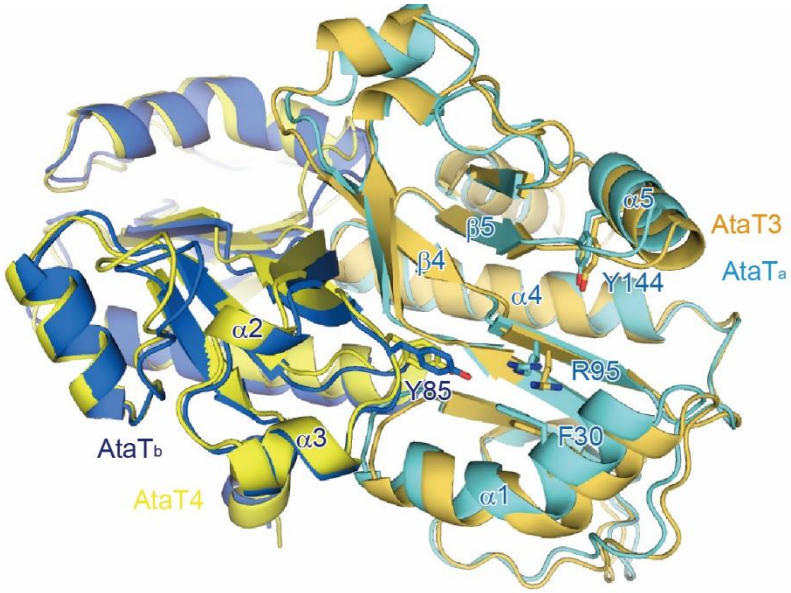


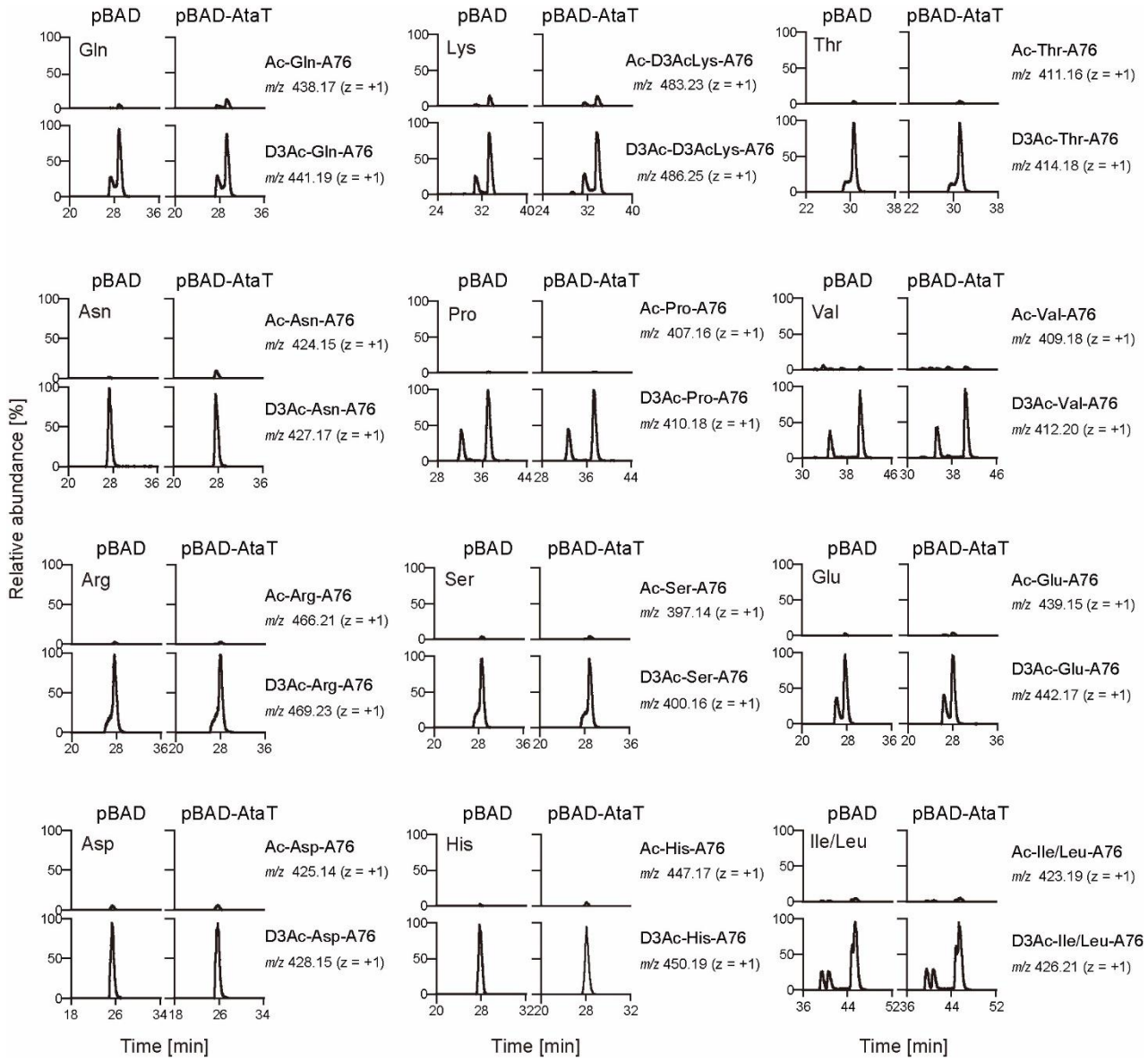
Supplementary Fig. 1: Structure of the AtaT-acMet-tRNAf^{Met} complex in the asymmetric unit cell. (**a**) The structure of the AtaT-acMet-tRNAf^{Met} complex in the asymmetric unit (ASU) of the crystal is shown in a ribbon representation. The ASU contains eight AtaT (AtaT1 - 8) molecules and two acMet-tRNAf^{Met} molecules (acMet-tRNAf^{Met}1 and acMet-tRNAf^{Met}2). The 3' single-stranded regions of tRNA^{fMet}1 and tRNA^{fMet}2 interact with the regions proximal to the catalytic sites of AtaT1 and AtaT2, respectively. The final models of AtaT1, AtaT2, AtaT3, AtaT4, AtaT5, AtaT6, AtaT7 and AtaT8 contain residues 3-172, 3-182, 2-180, 3-172, 5-175, 5-176, 5-172 and 5-172, respectively. (**b**) Ribbon representation of AtaT-acMet-tRNAf^{Met} in the three neighboring ASUs of the crystal. AtaT1 and AtaT2 form dimers with AtaT1' and AtaT2', respectively, through the crystallographic two-fold rotation axis.



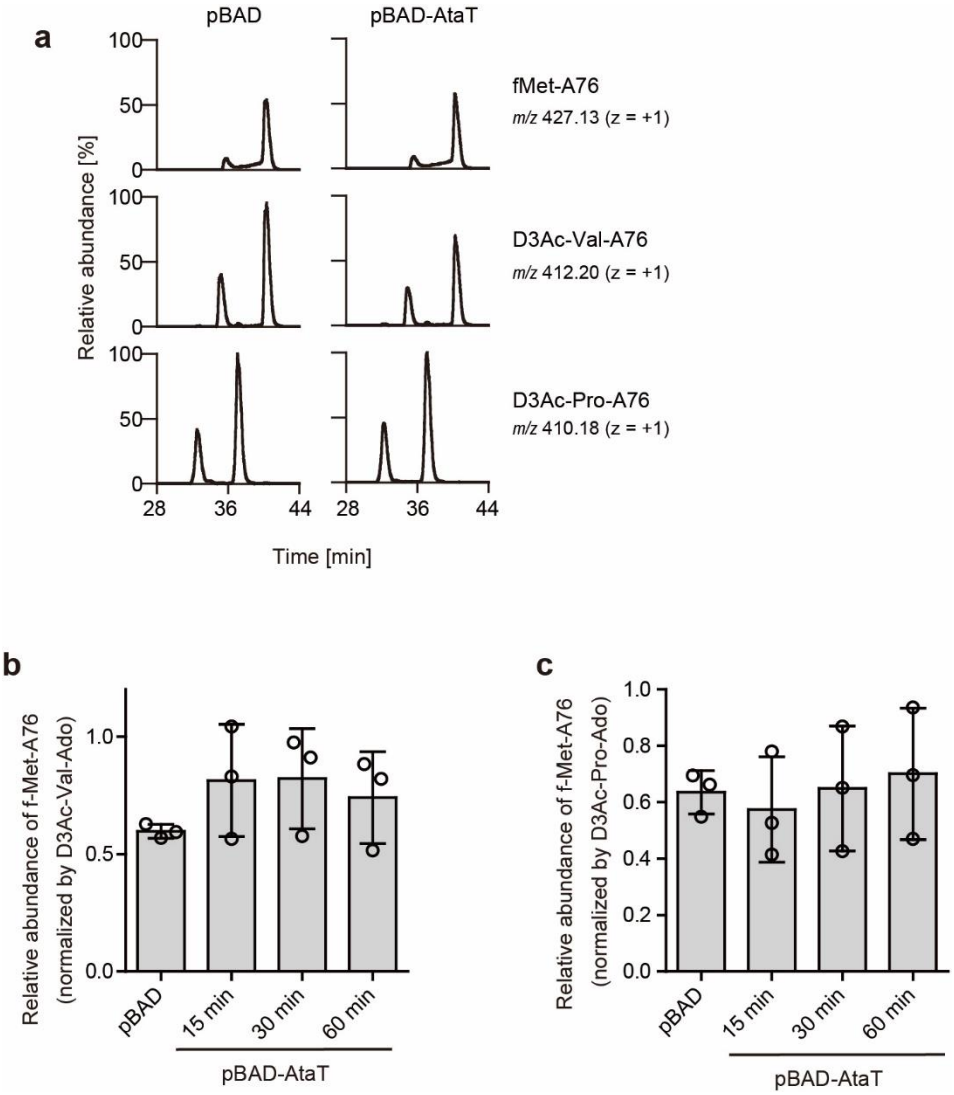
Supplementary Fig. 2: **Electron density maps of acMet-tRNAf^{Met} in the AtaT-acMet-tRNAf^{Met} complex** (**a**) Electron density maps of acMet-tRNAf^{Met} in the AtaT-acMet-tRNAf^{Met} complex after the molecular replacement calculation, using fMet-tRNAf^{Met} (PDB ID: 2FMT) as the search model. The 2*F*o-*F*c electron density map (blue mesh) contoured at 1.0 σ, and the *F*o-*F*c maps contoured at 1.0 and -1.0 σ (green and red meshes, respectively) of acMet-tRNAf^{Met} (tRNAa and tRNAb) in the AtaT-acMet-tRNAf^{Met} complex are shown. (**b**) Composite simulated annealing omit map of acMet-tRNAf^{Met} (tRNAa and tRNAb) in the AtaT-acMet-tRNAf^{Met} complex, contoured at 1.0 σ. Ac-CoA molecules are modeled in the active site of AtaT and shown as sticks. The electron densities corresponding to 3'-C75A76-acMet were not visible in the present structure.



Supplementary Fig. 3: Comparison of the structures of AtaTa-AtaTb in the AtaT- acMet-tRNAf^{Met} complex with AtaT3-AtaT4 in the asymmetric unit cell. Superimposition of the AtaTa-AtaTb structure in the $(AtaT)_2$(ac-Met-tRNAf^{Met})$_2$ complex and AtaT3-AtaT4 in the asymmetric unit cell in Supplementary Fig. 1. AtaTa, AtaTb, AtaT3, and AtaT4 are colored cyan, blue, orange, and yellow, respectively.



Supplementary Fig. 4: LC/MS analysis of RNase I-digested fragments of acetyl-aminoacyl-tRNAs. LC/MS analysis of RNase I-digested fragments of acetyl-aminoacyl-tRNAs after 15 min of AtaT expression induction. Extracted ion chromatograms (XIC) of the proton adducts corresponding to Ac-aminoacyl-A76 and D_3-aminoacyl-A76 are shown. The subtle peaks corresponding to Ac-aminoacyl-A76 detected in RNA samples from *E. coli* harboring the empty pBAD vector originated from the small fraction of non-deuterated acetic anhydride in the stable isotopic acetic anhydride-D_6. According to the manufacturer's information, the isotopic purity of the stable isotopic acetic anhydride-D_6 was 99.7 %. The molecular mass corresponding to Ac-Cys-A76, derived from Cys-tRNA^{Cys}, was not detected, probably due to the high reactivity of the thiol group, which leads to disulfide bond formation or oxidation by air oxygen during preparation. Cys-tRNA^{Cys} was not efficiently acetylated by AtaT *in vitro.*



Supplementary Fig. 5: Identification of *N*-formyl-Met-tRNAf^{Met} by LC/MS analysis. (**a**) LC/MS analysis of RNase I-digested fragments of acetyl-aminoacyl-tRNAs after 15 min of AtaT expression induction. Extracted ion chromatograms (XIC) of the proton adducts corresponding to f-Met-A76 (*N*-formyl methionyl-adenosine, *m/z* = 427.13) in RNA preparations from *E. coli* with or without AtaT induction. The intensity of f-Met-A76, D_3Ac-Val-A76, or D_3Ac-Pro-A76 (control) in each sample is expressed relative to the intensity of D_3Ac-Pro-A76. (**b**), (**c**) Relative quantification of fMet-A76. The intensity of f-Met-A76 in RNA preparations from *E. coli*, with or without AtaT induction for the indicated times, was normalized by the intensity of D_3Ac-Val-A76 (**b**) or D_3Ac-Pro-A76 (**c**). The bars in the graphs are SD of three independent experiments. The amounts of fMet-tRNAf^{Met} in the cells are not significantly altered by the AtaT expression. The bars in the graph are SD of three independent (n=3) experiments, and the data are presented as mean values ± SD.

a

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tRNA        Acc-stem     D-stem   D-loop   D-stem   Ac-stem  Ac-loop  Ac-stem        V-region          T-stem   T-loop   T-stem   Acc-stem
            1  456   8  10   14          22  26 27   32       39   44                     49   53       61   66     73 74
Gly(CCC)    -GCGGGCG TA GTTC AAT--GGT--A GAAC G AGAGC TTCCCAA GCTCT AT------------------A-C GAGGG TTCGATT CCCTT CGCCCGC T CCA
Gly(GCC)    -GCGGGAA TA GCTC AGTT-GGT--A GAGC A CGACC TTGCCAA GGTCG GG------------------GTC GCGAG TTCGAGT CTCGT TTCCCGC T CCA
Gly(TCC)    -GCGGGCA TC GTAT AAT--GGCT-A TTAC C TCAGC CTTCCAA GCTGA TG------------------A-T GCGGG TTCGATT CCCGC TGCCCGC T CCA
Ini(CAT)    -CGCGGGG TG GAGC AGCCTGGT--A GCTC G TCGGG CTCATAA CCCGA AG------------------ATC GTCGG TTCAAAT CCGGC CCCCGCA A CCA
Ini(CAT)    -CGCGGGG TG GAGC AGCCTGGT--A GCTC G TCGGG CTCATAA CCCGA AG------------------GTC GTCGG TTCAAAT CCGGC CCCCGCA A CCA
Phe(GAA)    -GCCCGGA TA GCTC AGTC-GGT--A GAGC A GGGGA TTGAAAA TCCCC GT------------------GTC CTTGG TTCGATT CCGAG TCCGGGC A CCA
Trp(CCA)    -AGGGGCG TA GTTC AATT-GGT--A GAGC A CCGGT CTCCAAA ACCGG GT------------------GTT GGGAG TTCGAGT CTCTC CGCCCCT G CCA
Tyr(GTA)    -GGTGGGG TT CCCG AGC--GGCCAA AGGG A GCAGA CTGTAAA TCTGC CGTC-----ATCG-----ACTTC GAAGG TTCGAAT CCTTC CCCCACC A CCA
Tyr(GTA)    -GGTGGGG TT CCCG AGC--GGCCAA AGGG A GCAGA CTGTAAA TCTGC CGTC-----ACAG-----ACTTC GAAGG TTCGAAT CCTTC CCCCACC A CCA
Gln(CTG)    -TGGGGTA TC GCCA AGC--GGT--A AGGC A CCGGA TTCTGAT TCCGG CA------------------TTC CGAGG TTCGAAT CCTCG TACCCCA G CCA
Gln(TTG)    -TGGGGTA TC GCCA AGC--GGT--A AGGC A CCGGT TTTTGAT ACCGG CA------------------TTC CCTGG TTCGAAT CCAGG TACCCCA G CCA
Asn(GTT)    -TCCTCTG TA GTTC AGTC-GGT--A GAAC G GCGGA CTGTTAA TCCGT AT------------------GTC ACTGG TTCGAGT CCAGT CAGAGGA G CCA

Ala(GGC)    -GGGGCTA TA GCTC AGCT-GGG--A GAGC G CTTGC ATGGCAT GCAAG AG------------------GTC AGCGG TTCGATC CCGCT TAGCTCC A CCA
Ala(TGC)    -GGGGCTA TA GCTC AGCT-GGG--A GAGC G CCTGC TTTGCAC GCAGG AG------------------GTC TGCGG TTCGATC CCGCA TAGCTCC A CCA
Arg(ACG)    -GCATCCG TA GCTC AGCT-GGAT-A GAGT A CTCGG CTACGAA CCGAG CG------------------GTC GGAGG TTCGAAT CCTCC CGGATGC A CCA
Arg(CCG)    -GCGCCCG TA GCTC AGCT-GGAT-A GAGC G CTGCC CTCCGGA GGCAG AG------------------GTC TCAGG TTCGAAT CCTGT CGGGCGC G CCA
Arg(CCT)    -GTCCTCT TA GTTA AAT--GGAT-A TAAC G AGCCC CTCCTAA GGGCT AA------------------T-T GCAGG TTCGATT CCTGC AGGGGAC A CCA
Arg(TCT)    -GCGCCCT TA GCTC AGTT-GGAT-A GAGC A ACGAC CTTCTAA GTCGT GG------------------GCC GCAGG TTCGAAT CCTGC AGGGCGC G CCA
Asp(GTC)    -GGAGCGG TA GTTC AGTC-GGTT-A GAAT A CCTGC CTGTCAC GCAGG GG------------------GTC GCGGG TTCGAGT CCCGT CCGTTCC G CCA
Cys(GCA)    -GGCGCGT TA ACAA AGC--GGT--T ATGT A GCGGA TTGCAAA TCCGT CT------------------A-G TCCGG TTCGACT CCGGA ACGCGCC T CCA
Glu(TTC)    -GTCCCCT TC GTCT AGA--GGCCCA GGAC A CCGCC CTTTCAC GGCGG TA------------------A-C AGGGG TTCGAAT CCCCT AGGGGAC G CCA
His(GTG)    GGTGGCTA TA GCTC AGTT-GGT--A GAGC C CTGGA TTGTGAT TCCAG TT------------------GTC GTGGG TTCGAAT CCCAT TAGCCAC C CCA
Leu(CAA)    -GCCGAAG TG GCGA AATC-GGTA-G ACGC A GTTGA TTCAAAA TCAAC CGTA-----GAAA-----TACGT GCCGG TTCGAGT CCGGC CTTCGGC A CCA
Leu(CAG)    -GCGAAGG TG GCGG AATT-GGTA-G ACGC G CTAGC TTCAGGT GTTAG TGTTC----TTAC----GGACGT GGGGG TTCAAGT CCCCC CCCTCGC A CCA
Leu(CAG)    -GCGAAGG TG GCGG AATT-GGTA-G ACGC G CTAGC TTCAGGT GTTAG TGTCC----TTAC----GGACGT GGGGG TTCAAGT CCCCC CCCTCGC A CCA
Leu(GAG)    -GCCGAGG TG GTGG AATT-GGTA-G ACAC G CTACC TTGAGGT GGTAG TGCCC----AATA----GGGCTT ACGGG TTCAAGT CCCGT CCTCGGT A CCA
Leu(TAA)    -GCCCGGA TG GTGG AATC-GGTA-G ACAC A AGGGA TTTAAAA TCCCT CGGCG----TTCG----CGCTGT GCGGG TTCAAGT CCCGC TCCGGGT A CCA
Leu(TAG)    -GCGGGAG TG GCGA AATT-GGTA-G ACGC A CCAGA TTTAGGT TCTGG CGCC-----GCAA-----GGTGT GCGAG TTCAAGT CTCGC CTCCCGC A CCA
Lys(TTT)    -GGGTCGT TA GCTC AGTT-GGT--A GAGC A GTTGA CTTTTAA TCAAT TG------------------GTC GCAGG TTCGAAT CCTGC ACGACCC A CCA
Ile(GAT)    -AGGCTTG TA GCTC AGGT-GGTT-A GAGC G CACCC CTGATAA GGGTG AG------------------GTC GGTGG TTCAAGT CCACT CAGGCCT A CCA
Ile(CAT)    -GGCCCCT TA GCTC AGT--GGTT-A GAGC A GGCGA CTCATAA TCGCT TG------------------GTC GCTGG TTCAAGT CCAGC AGGGGCC A CCA
Ile(CAT)    -GGCCCTT TA GCTC AGT--GGTT-A GAGC A GGCGA CTCATAA TCGCT TG------------------GTC GCTGG TTCAAGT CCAGC AAGGGCC A CCA
Met(CAT)    -GGCTACG TA GCTC AGTT-GGTT-A GAGC A CATCA CTCATAA TGATG GG------------------GTC ACAGG TTCGAAT CCCGT CGTAGCC A CCA
Pro(CGG)    -CGGTGAT TG GCGC AGCCTGGT--A GCGC A CTTCG TTCGGGA CGAAG GG------------------GTC GGAGG TTCGAAT CCTCT ATCACCG A CCA
Pro(GGG)    -CGGCACG TA GCGC AGCCTGGT--A GCGC A CCGTC ATGGGGT GTCGG GG------------------GTC GGAGG TTCAAAT CCTCT CGTGCCG A CCA
Pro(TGG)    -CGGCGAG TA GCGC AGCTTGGT--A GCGC A ACTGG TTTGGGA CCAGT GG------------------GTC GGAGG TTCGAAT CCTCT CTCGCCG A CCA
Sec(TCA)    GGAAGATC GT CGTC TCC--GGTG-A GGCG G CTGGA CTTCAAA TCCAG TTGGGGCCGCCAGCGGTCCCG-G GCAGG TTCGACT CCTGT GATCTTC - ---
Ser(CGA)    -GGAGAGA TG CCGG AGC--GGCTGA ACGG A CCGGT CTCGAAA ACCGG AGTAGGG--GCAA--CTCTAC-C GGGGG TTCAAAT CCCCC TCTCTCC G CCA
Ser(GCT)    -GGTGAGG TG GCCG AGA--GGCTGA AGGC G CTCCC CTGCTAA GGGAG TATGCGGTCAAA-AGCTGCAT-C CGGGG TTCGAAT CCCCG CCTCACC G CCA
Ser(GGA)    -GGTGAGG TG TCCG AGT--GGCTGA AGGA G CACGC CTGGAAA GTGTG TATACG---GCAA---CGTAT-C GGGGG TTCGAAT CCCCC CCTCACC G CCA
Ser(TGA)    -GGAAGTG TG GCCG AGC--GGTTGA AGGC A CCGGT CTTGAAA ACCGG CGACCC---GAAA---GGGTT-C CAGAG TTCGAAT CTCTG CGCTTCC G CCA
Thr(CGT)    -GCTCAAG TA GTTA AAAA-TGCA-T TAAC A TCGCA TTCGTAA TGCGA AG------------------GTC GTAGG TTCGACT CCTAT TATCGGC A CCA
Thr(CGT)    -GCCGATA TA GCTC AGTT-GGT--A GAGC A GCGCA TTCGTAA TGCGA AG------------------GTC GTAGG TTCGACT CCTAT TATCGGC A CCA
Thr(GGT)    -GCTGATA TG GCTC AGTT-GGT--A GAGC G CACCC TTGGTAA GGGTG AG------------------GTC CCCAG TTCGACT CTGGG TATCAGC A CCA
Thr(GGT)    -GCTGATA TA GCTC AGTT-GGT--A GAGC G CACCC TTGGTAA GGGTG AG------------------GTC GGCAG TTCGAAT CTGCC TATCAGC A CCA
Thr(TGT)    -GCCGACT TA GCTC AGTA-GGT--A GAGC A ACTGA CTTGTAA TCAGT AG------------------GTC ACCAG TTCGATT CCGGT AGTCGGC A CCA
Val(GAC)    -GCGTTCA TA GCTC AGTT-GGTT-A GAGC A CCACC TTGACAT GGTGG GG------------------GTC GTTGG TTCGAGT CCAAT TGAACGC A CCA
Val(GAC)    -GCGTCCG TA GCTC AGTT-GGTT-A GAGC A CCACC TTGACAT GGTGG GG------------------GTC GGTGG TTCGAGT CCACT CGGACGC A CCA
Val(TAC)    -GGGTGAT TA GCTC AGCT-GGG--A GAGC A CCTCC CTTACAA GGAGG GG------------------GTC GGCGG TTCGATC CCGTC ATCACCC A CCA
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Supplementary Fig. 6: Sequence alignment of *E. coli* tRNA genes. (**a**) *E. coli* K12 and (**b**) *E. coli* O157: H7 tRNA genes are shown. tRNA isoacceptors used for the *in vitro* acetylation assay in this study are colored red.

b

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tRNA        Acc-stem     D-stem   D-loop    D-stem   Ac-stem  Ac-loop  Ac-stem        V-region          T-stem   T-loop   T-stem   Acc-stem
             1  456  8  10   14            22  26  27       32       39  44                            49       53       61       66     73 74
Gly(CCC)  -GCGGGCG TA GTTC AAT--GGT--A GAAC G AGAGC TTCCCAA GCTCT AT------------------A-C GAGGG TTCGATT CCCTT CGCCCGC T CCA
Gly(GCC)  -GCGGGAA TA GCTC AGTT-GGT--A GAGC A CGACC TTGCCAA GGTCG GG------------------GTC GCGAG TTCGAGT CTCGT TTCCCGC T CCA
Gly(TCC)  -GCGGGCA TC GTAT AAT--GGCT-A TTAC C TCAGC CTTCCAA GCTGA TG------------------A-T GCGGG TTCGATT CCCGC TGCCCGC T CCA
Ini(CAT)  -CGCGGGG TG GAGC AGCCTGGT--A GCTC G TCGGG CTCATAA CCCGA AG------------------GTC GTCGG TTCAAAT CCGGC CCCCGCA A CCA
Phe(GAA)  -GCCCGGA TA GCTC AGTC-GGT--A GAGC A GGGGA TTGAAAA TCCCC GT------------------GTC CTTGG TTCAATT CCGAG TCCGGGC A CCA
Phe(GAA)  -GCCCGGA TA GCTC AGTC-GGT--A GAGC A GGGGA TTGAAAA TCCCC GT------------------GTC CTTGG TTCGATT CCGAG TCCGGGC A CCA
Trp(CCA)  -AGGGGCG TA GTTC AATT-GGT--A GAGC A CCGGT CTCCAAA ACCGG GT------------------GTT GGGAG TTCGAGT CTCTC CGCCCCT G CCA
Tyr(GTA)  -GGTGGGG TT CCCG AGC--GGCCAA AGGG A GCAGA CTGTAAA TCTGC CGTC-----ATCG-----ACTTC GAAGG TTCGAAT CCTTC CCCCACC A CCA
Tyr(GTA)  -GGTGGGG TT CCCG AGC--GGCCAA AGGG A GCAGA CTGTAAA TCTGC CGTC-----ACAG-----ACTTC GAAGG TTCGAAT CCTTC CCCCACC A CCA
Gln(CTG)  -TGGGGTA TC GCCA AGC--GGT--A AGGC A CCGGA TTCTGAT TCCGG CA------------------TTC CGAGG TTCGAAT CCTCG TACCCCA G CCA
Gln(TTG)  -TGGGGTA TC GCCA AGC--GGT--A AGGC A CCGGT TTTTGAT ACCGG CA------------------TTC CCTGG TTCGAAT CCAGG TACCCCA G CCA
Asn(GTT)  -TCCTCTG TA GTTC AGTC-GGT--A GAAC G GCGGA CTGTTAA TCCGT AT------------------GTC ACTGG TTCGAGT CCAGT CAGAGGA G CCA

Ala(GGC)  -GGGGCTA TA GCTC AGCT-GGG--A GAGC G CTTGC ATGGCAT GCAAG AG------------------GTC AGCGG TTCGATC CCGCT TAGCTCC A CCA
Ala(TGC)  -GGGGCTA TA GCTC AGCT-GGG--A GAGC G CCTGC TTTGCAC GCAGG AG------------------GTC TGCGG TTCGATC CCGCA TAGCTCC A CCA
Arg(ACG)  -GCATCCG TA GCTC AGCT-GGAT-A GAGT A CTCGG CTACGAA CCGAG CG------------------GTC GGAGG TTCGAAT CCTCC CGGATGC A CCA
Arg(CCG)  -GCGCCCG TA GCTC AGCT-GGAT-A GAGC G CTGCC CTCCGGA GGCAG AG------------------GTC TCAGG TTCGAAT CCTGT CGGGCGC G CCA
Arg(CCT)  -GTCCTCT TA GTTA AAT--GGAT-A TAAC G AGCCC CTCCTAA GGGCT AA------------------T-T GCAGG TTCGATT CCTGC AGGGGAC A CCA
Arg(TCG)  -CCGCCAT TA GCTC ATC--GGGATA GAAC G CCAGC CTTCGAA GCTGG TT------------------TCG CGGGG TTCGAGT CTCCG ATGGCGG T CCA
Arg(TCG)  -CCGCCAT TA GCTC ATC--GGGACA GAGC G CCAGC CTTCGAA GCTGG CT------------------GCG CGGGG TTCGAGT CCTCG ATGGCGG T CCA
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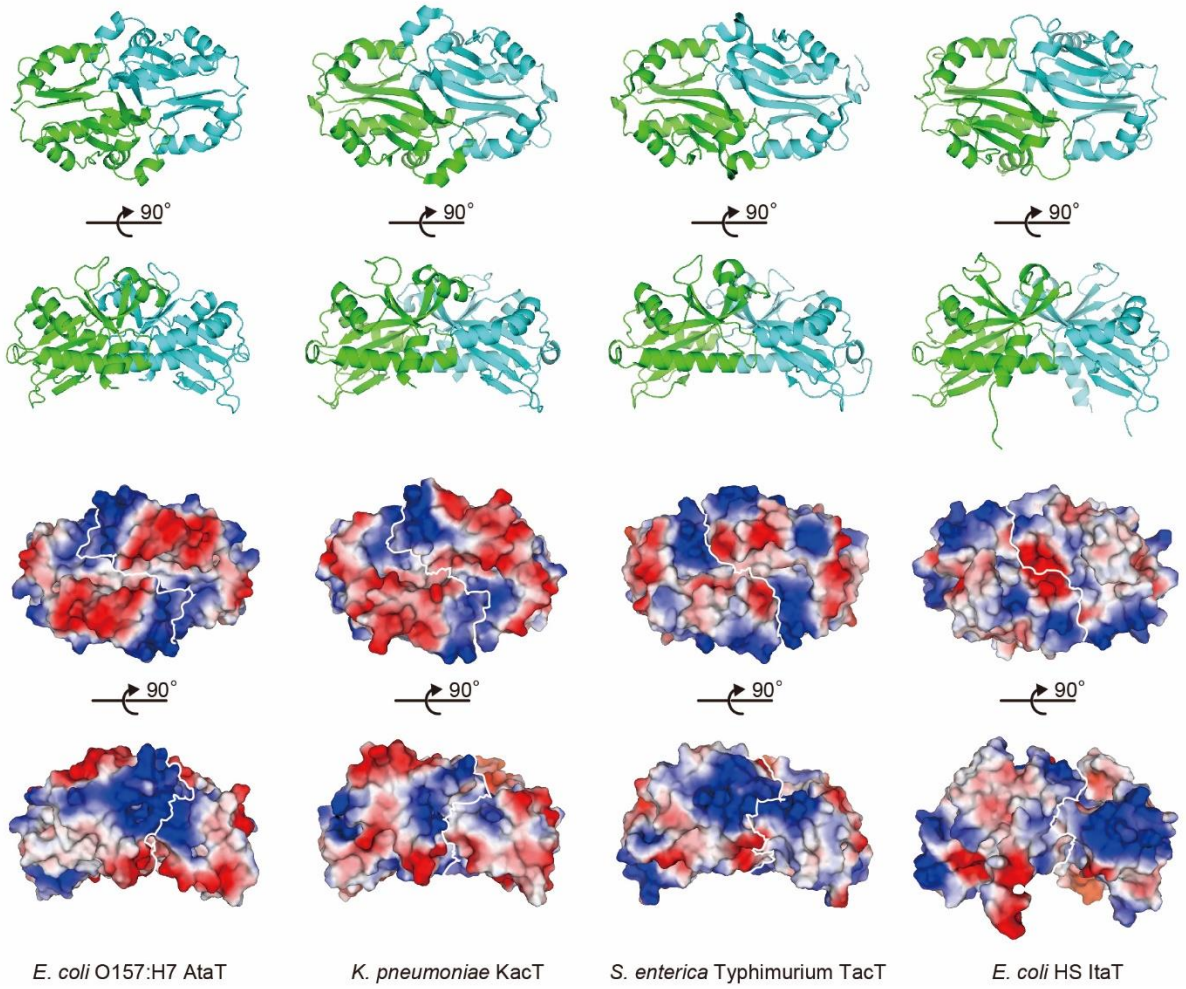
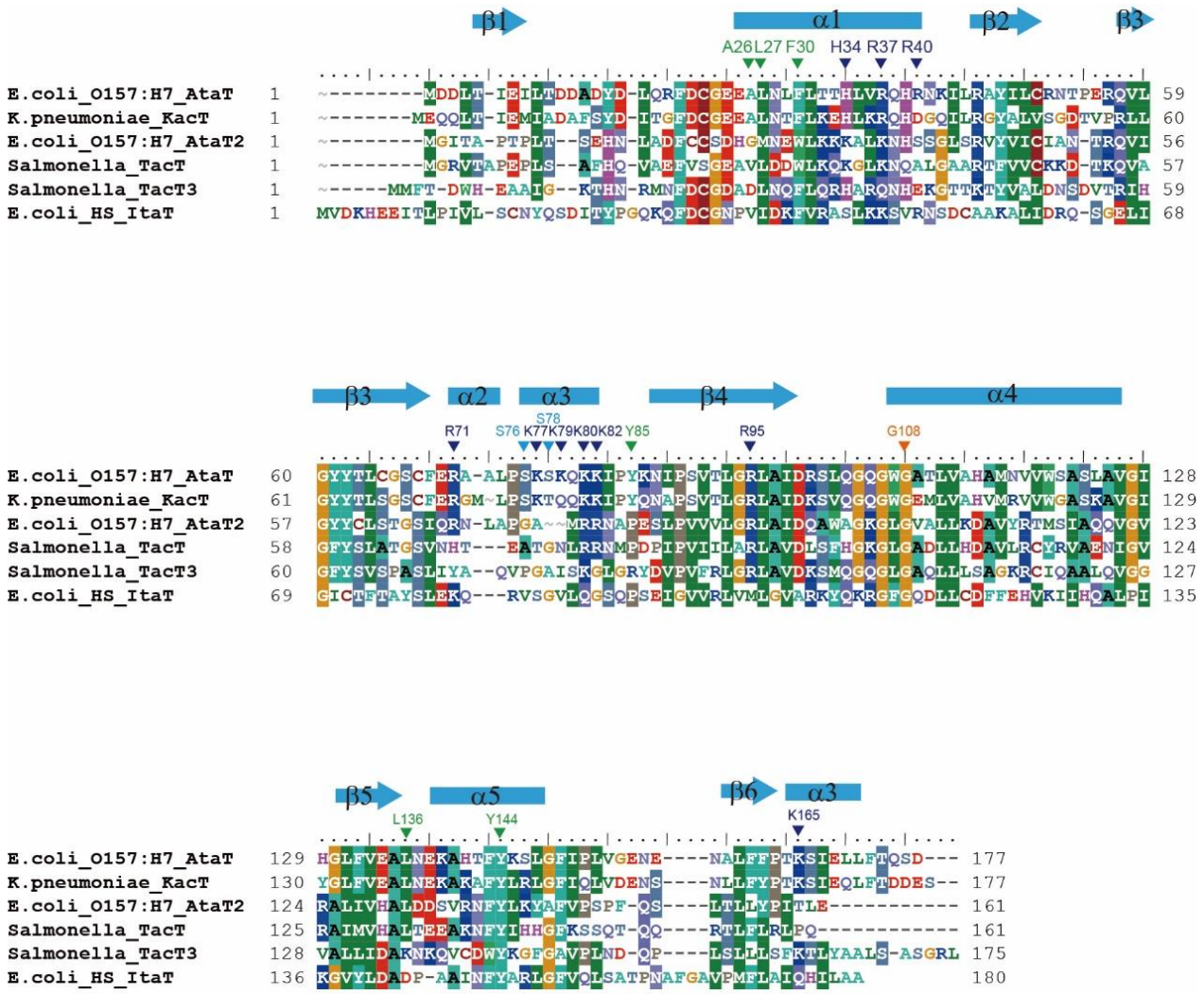
Arg(TCT)  -GCGTTGT TA GCTC AGCC-GGAC-A GAGC A ATTGC CTTCTAA GCAAT CG------------------GTC ACTGG TTCGAAT CCAGT ACAACGC A CCA
Arg(TCT)  -GCGCCCT TA GCTC AGTT-GGAT-A GAGC A ACGAC CTTCTAA GTCGT GG------------------GCC GCAGG TTCGAAT CCTGC AGGGCGC G CCA
Asp(GTC)  -GGAGCGG TA GTTC AGTC-GGTT-A GAAT A CCTGC CTGTCAC GCAGG GG------------------T-C GCGGG TTCGAGT CCCGT CCGTTCC G CCA
Asp(GTC)  -GGAGCGG TA GTTC AGTC-GGTT-A GAAT A CCTGC CTGTCAC GCAGG GG------------------GTC GCGGG TTCGAGT CCCGT CCGTTCC G CCA
Cys(GCA)  -GGCGCGT TA ACAA AGC--GGT--T ATGT A GCGGA TTGCAAA TCCGT CT------------------A-G TCCGG TTCGACT CCGGA ACGCGCC T CCA
Glu(TTC)  -GTCCCCT TC GTCT AGA--GGCCCA GGAC A CCGCC CTTTCAC GGCGG TA------------------A-C AGGGG TTCGAAT CCCCT AGGGGAC G CCA
His(GTG)  GGTGGCTA TA GCTC AGTT-GGT--A GAGC C CTGGA TTGTGAT TCCAG TT------------------GTC GTGGG TTCGAAT CCCAT TAGCCAC C CCA
Leu(CAA)  -GCCGAAG TG GCGA AATC-GGTA-G ACGC A GTTGA TTCAAAA TCAAC CGTA-----GAAA-----TACGT GCCGG TTCGAGT CCGGC CTTCGGC A CCA
Leu(CAG)  -GCGAAGG TG GCGG AATT-GGTA-G ACGC G CTAGC TTCAGGT GTTAG TGTCC----TTAC----GGACGT GGGGG TTCAAGT CCCCC CCCTCGC A CCA
Leu(GAG)  -GCCGAGG TG GTGG AATT-GGTA-G ACAC G CTACC TTGAGGT GGTAG TGCCC----AATA----GGGCTT ACGGG TTCAAGT CCCGT CCTCGGT A CCA
Leu(TAA)  -GCCCGGA TG GTGG AATC-GGTA-G ACAC A AGGGA TTTAAAA TCCCT CGGCG----TTCG----CGCTGT GCGGG TTCAAGT CCCGC TCCGGGT A CCA
Leu(TAG)  -GCGGGAG TG GCGA AATT-GGTA-G ACGC A CCAGA TTTAGGT TCTGG CGCC-----GCAA-----GGTGT GCGAG TTCAAGT CTCGC CTCCCGC A CCA
Lys(TTT)  -GGGTCGT TA GCTC AGTT-GGT--A GAGC A GTTGA CTTTTAA TCAAT TG------------------GTC GCAGG TTCGAAT CCTGC ACGACCC A CCA
Ile(GAT)  -AGGCTTG TA GCTC AGGT-GGTT-A GAGC G CACCC CTGATAA GGGTG AG------------------GTC GGTGG TTCAAGT CCACT CAGGCCT A CCA
Ile(CAT)  -GGCCCTT TA GCTC AGT--GGTG-A GAGC G AGCGA CTCATAA TCGCC AG------------------GCC GCTGG TTCAAAT CCAGC AAGGGCC A CCA
Ile(CAT)  -GGCCCTT TA GCTC AGC--GGTG-A GAGC G AGCGA CTCATAA TCGCC AG------------------GTC GCTGG TTCAAAT CCAGC AAGGGCC A CCA
Ile(CAT)  -GGCCCTT TA GCTC AGT--GGTG-A GAGC G AGCGA CTCATAA TCGCC AG------------------GTC GCTGG TTCAAAT CCAGC AAGGGCC A CCA
Ile(CAT)  -GGCCCTT TA GCTC AGT--GGTT-A GAGC A GGCGA CTCATAA TCGTC CG------------------GTC GCTGG TTCAAGT CCAGC AAGGGCC A CCA
Ile(CAT)  -GGCCCCT TA GCTC AGT--GGTT-A GAGC A GGCGA CTCATAA TCGCT TG------------------GTC GCTGG TTCAAGT CCAGC AGGGGCC A CCA
Ile(CAT)  -AGCCCTT TA GCTC AGT--GGTG-A GAGC G AGCGA CTCATAA TCGCC AG------------------GTC GCTGG TTCAAAT CCAGC AAGGGCC A CCA
Met(CAT)  -GGCTACG TA GCTC AGTT-GGTT-A GAGC A CATCA CTCATAA TGATG GG------------------GTC ACAGG TTCGAAT CCCGT CGTAGCC A CCA
Pro(CGG)  -CGGTGAT TG GCGC AGCCTGGT--A GCGC A CTTCG TTCGGGA CGAAG GG------------------GTC GGAGG TTCGAAT CCTCT ATCACCG A CCA
Pro(GGG)  -CGGCACG TA GCGC AGCCTGGT--A GCGC A CCGTC ATGGGGT GTCGG GG------------------GTC GGAGG TTCAAAT CCTCT CGTGCCG A CCA
Pro(TGG)  -CGGCGAG TA GCGC AGCTTGGT--A GCGC A ACTGG TTTGGGA CCAGT GG------------------GTC GGAGG TTCGAAT CCCAT TAGCCAC C CCA
Pro(TGG)  -CGGCGAG TA GCGC ACTT-GGT--A GCGC A ACTGG TTTGGGA CCAGT GG------------------GTC GGAGG TTCGAAT CCTCT CTCGCCG A CCA
Sec(TCA)  GGAAGATC GT CGTC TCC--GGTG-A GGCG G CTGGA CTTCAAA TCCAG TTGGGGCCGCCAGCGGTCCCG-G GCAGG TTCGACT CCTGT GATCTTC - ---
Ser(CGA)  -GGAGAGA TG CCGG AGC--GGCTGA ACGG A CCGGT CTCGAAA ACCGG AGTGGGG--GCAA--CTCCAC-C GGGGG TTCAAAT CCCCC TCTCTCC G CCA
Ser(GCT)  -GGTGAGG TG GCCG AGA--GGCTGA AGGC G CTCCC CTGCTAA GGGAG TATGCGGTCAAA-AGCTGCAT-C CGGGG TTCGAAT CCCCG CCTCACC G CCA
Ser(GGA)  -GGTGAGG TG TCCG AGT--GGCTGA AGGA G CACGC CTGGAAA GTGTG TATACG---GCAA---CGTAT-C GGGGG TTCGAAT CCCCC CCTCACC G CCA
Ser(TGA)  -GGAAGTG TG GCCG AGC--GGTTGA AGGC A CCGGT CTTGAAA ACCGG CGACCC---GAAA---GGGTT-C CAGAG TTCGAAT CTCTG CGCTTCC G CCA
Thr(CGT)  -GCCGATA TA GCTC AGTT-GGT--A GAGC A GCGCA TTCGTAA TGCGA AG------------------GTC GTAGG TTCGACT CCTAT TATCGGC A CCA
Thr(GGT)  -GCTGATA TG GCTC AGTT-GGT--A GAGC G CACCC TTGGTAA GGGTG AG------------------GTC CCCAG TTCGACT CTGGG TATCAGC A CCA
Thr(GGT)  -GCTGATA TA GCTC AGTT-GGT--A GAGC G CACCC TTGGTAA GGGTG AG------------------GTC GGCAG TTCGAAT CTGCC TATCAGC A CCA
Thr(TGT)  -GCCGACT TA GCTC AGTA-GGT--A GAGC A ACTGA CTTGTAA TCAGT AG------------------GTC ACCAG TTCGATT CCGGT AGTCGGC A CCA
Val(GAC)  -GCGTTCA TA GCTC AGTT-GGTT-A GAGC A CCACC TTGACAT GGTGG GG------------------GTC GTTGG TTCGAGT CCAAT TGAACGC A CCA
Val(GAC)  -GCGTCCG TA GCTC AGTT-GGTT-A GAGC A CCACC TTGACAT GGTGG GG------------------GTC GGTGG TTCGAGT CCACT CGGACGC A CCA
Val(TAC)  -GGGTGAT TA GCTC AGCT-GGG--A GAGC A CCTCC CTTACAA GGAGG GG------------------GGC GGCGG TTCGATC CCGTC ATCACCC A CCA
Val(TAC)  -GGGTGAT TA GCTC AGCT-GGG--A GAGC A CCTCC CTTACAA GGAGG GG------------------GTC GGCGG TTCGATC CCGTC ATCACCC A CCA
```

Supplementary Fig. 6: continued



Supplementary Fig. 7: Comparison of the structures of GNAT toxins. Ribbon representations and surface electrostatic potentials of *E. coli* O157: H7 AtaT in the AtaT-acMet-tRNAf^{Met} complex (this study), *K. pneumoniae* KacT (PDB ID: 5XUN) [1], *S. enterica* Typhimurium TacT (PDB ID: 5FVJ) [2], and *E. coli* HS ItaT (PDB ID: 7BYY) [3] are shown. Positively and negatively charged areas are colored blue and red, respectively.



Supplementary Fig. 8: Sequence alignment of GNAT toxins targeting aminoacyl-tRNAs. Amino acid sequences of *E. coli* O157: H7 AtaT [4] and AtaT2 [5], *S. enterica* Typhimurium TacT [2] and TacT3 [6], *K. pneumoniae* KacT [7], and *E. coli* HS ItaT [8] are aligned. The secondary structure elements (α1 - α5 and β1 - β6) of AtaT are depicted above the alignment.

Supplementary Table 1: Synthetic DNA primers used in this study

ataT_F30A_F	GCG CTG ACG ACA CAT CTC GTT CGT C
ataT_F30_R	GAGATTTAACGCTTCCTCGCC
ataT_H34R37R40A_F	CAA CAT GCG AAC AAA ATT CTG CGA GCG TAT ATC
ataT_H34R37R40A_R	CGCAACGAGCGCTGTCGTCAGAAAGAGATTTAACG
ataT_K77K79K81K82A_F	CAG GCG GCG ATT CCC TAC AAA AAT ATT CCC AGC
ataT_K77K79K81K82A_R	CGCCGACGCCGAGGGCAATGCGGCTCG
ataT_Y85A_F	GCG AAA AAT ATT CCC AGC GTT ACT CTT GG
ataT_Y85_R	GGGAATTTTTTTCTGTTTCGATTTCGAGG
ataT_R95A_F	GCG CTG GCA ATT GAT CGT TCA TTA CAG
ataT_R95_R	CCCAAGAGTAACGCTGGGAATATTTTTG
ataT_K165A_F	GCG TCC ATT GAA CTG CTT TTT ACA CAG AGC
ataT_K164_R	GGTTGGGAAAAATAACGCATTTTCG
ataT_S76A_F	GCG AAA TCG AAA CAG AAA AAA ATT CCC TAC
ataT_S78A_F	TCG AAA GCG AAA CAG AAA AAA ATT CCC TAC
ataT_S76,78F_R	GGGCAATGCGGCTCGTTCAAAACAAC
ataT_R71A_F	GCG GCC GCA TTG CCC TCG AAA TCG AAA CAG
ataT_R71_R	TTCAAAACAACTGCCGCATAATGTGTAATATCCC
ataT_Y144F_F	TTT AAA TCG CTG GGC TTT ATC CCT TTA GTC GG
ataT_Y144_R	AAACGTATGGGCTTTTTCATTCAGCGCCTCGAC
AtaT_A26E_F	gaa TTA AAT CTC TTT CTG ACG ACA CAT CTC
AtaT_A26_R	TTCCTCGCCGCAGTCGAATCGCTG
AtaT_L27E_F	gaa AAT CTC TTT CTG ACG ACA CAT CTC GTT CGT C
AtaT_L27_R	CGCTTCCTCGCCGCAGTCGAATCG
AtaT_L136E_F	gaa AAT GAA AAA GCC CAT ACG TTT TAT AAA TCG C
AtaT_L136_R	CGCCTCGACAAAAAGACCGTGAATACC
GlnRS_Fw	agctagctcatATGAGTGAGGCAGAAGCCCGCCC
GlnRS_Rv	agctctcgagCTCGCCTACTTTCGCCCAGGTATC

Supplementary Table 2: Synthetic DNA sequences used in this study

***rnpA* gene:** CATATGGTTAAGCTCGCATTTCCCAGGGAGTTACGCTTGTTAACTCCCAGTCAATTCACAT TCGTCTTCCAGCAGCCACAACGGGCTGGCACGCCGCAAATTACCATTCTCGGCCGCCTGA ATTCGCTGGGGCATCCCCGTATCGGTCTTACAGTCGCCAAGAAAAACGTTCGACGCGCCC ATGAACGCAATCGGATTAAACGTCTGACGCGTGAAAGCTTCCGTCTGCGCCAACATGAAC TCCCGGCTATGGATTTCGTGGTGGTGGCGAAAAAAGGGGTTGCCGACCTCGATAACCGTG CTCTCTCGGAAGCGTTGGAAAAATTATGGCGCCGCCACTGTCGCCTGGCTCGCGGGTCCC TCGAG
***rnp*B gene (M1 RNA) template:** GAATTCTAATACGACTCACTATAGGGAAGCTGACCAGACAGTCGCCGCTTCGTCGTCGTC CTCTTCGGGGGAGACGGGCGGAGGGGAGGAAAGTCCGGGCTCCATAGGGCAGGGTGCC AGGTAACGCCTGGGGGGGAAACCCACGACCAGTGCAACAGAGAGCAAACCGCCGATGG CCCGCGCAAGCGGGATCAGGTAAGGGTGAAAGGGTGCGGTAAGAGCGCACCGCGCGGC TGGTAACAGTCCGTGGCACGGTAAACTCCACCCGGAGCAAGGCCAAATAGGGGTTCATA AGGTACGGCCCGTACTGAACCCGGGTAGGCTGCTTGAGCCAGTGAGCGATTGCTGGCCT AGATGAATGACTGTCCACGACAGAACCCGGCTTATCGGTCAGTTTCACCTGGCTAGCTAC ATCCAAGCTT
tRNAf^{Met} WT template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCGGGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCCC GCAACCAGGCTAGCTACATCCAAGCTT
tRNAm^{Met} WT template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAGGCTACGTAG CTCAGTTGGTTAGAGCACATCACTCATAATGATGGGGTCACAGGTTCGAATCCCGTCGTA GCCACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} A1:C72 template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAGCGGGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCCC GCCACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} C1:G72 template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCGGGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCCC GCGACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} G1:C72 template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAGGCGGGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCCC GCCACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} U4A5C6:G67U68A69 template: gaattcTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCTACGTGGAG CAGCCtGGTAGCTCGTCGGGCTCATAACCCGAAGGtCGTCGGTTCAAATCCGGCCGTAGCA ACCAggctagctaCATCCAAGCTT

Supplementary Table 2: Synthetic DNA sequences used in this study**,** continued

tRNAf^{Met} C4C5C6:G67G68G69 template: gaattcTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCCCCGTGGAG CAGCCtGGTAGCTCGTCGGGCTCATAACCCGAAGGtCGTCGGTTCAAATCCGGCCGGGGC AACCAggctagctaCATCCAAGCTT
tRNAm^{Met} fMet acceptor stem template: GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCGGGGTAG CTCAGTTGGTTAGAGCACATCACTCATAATGATGGGGTCACAGGTTCGAATCCCGTCCCC GCAACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} (C4-G69) template GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCCGGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCC GGCAACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} (C5-G68) template GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCGCGGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCCG CGCAACCAGGCTAGCTACATCCAAGCTT
tRNAf^{Met} (C6-G67) template GAATTCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGACGCGGCGTGG AGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGGTCGTCGGTTCAAATCCGGCCGC CGCAACCAGGCTAGCTACATCCAAGCTT
tRNA^{Gly} 2 (GCC) gene: GCGGGAATAGCTCAGTTGGTAGAGCACGACCTTGCCAAGGTCGGGGTCGCGAGTTCGAG TCTCGTTTCCCGCTCCA
tRNA^{Trp} gene: AGGGGCGTAGTTCAATTGGTAGAGCACCGGTCTCCAAAACCGGGTGTTGGGAGTTCGAG TCTCTCCGCCCCTGCCA
tRNA^{Tyr} gene: GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTGTAAATCTGCCGTCATCGACTTCGA AGGTTCGAATCCTTCCCCCACCACCA
tRNA^{Gln} 2 gene: TGGGGTATCGCCAAGCGGTAAGGCACCGGATTCTGATTCCGGCATTCCGAGGTTCGAATC CTCGTACCCCAGCCA

References

1 Qian, H. *et al.* Toxin-antitoxin operon kacAT of Klebsiella pneumoniae is regulated by conditional cooperativity via a W-shaped KacA-KacT complex. *Nucleic acids research* **47**, 7690-7702, doi:10.1093/nar/gkz563 (2019).

2 Cheverton, A. M. *et al.* A Salmonella Toxin Promotes Persister Formation through Acetylation of tRNA. *Molecular Cell* **63**, 86-96, doi:10.1016/j.molcel.2016.05.002 (2016).

3 Zhang, C., Yashiro, Y., Sakaguchi, Y., Suzuki, T. & Tomita, K. Substrate specificities of Escherichia coli ItaT that acetylates aminoacyl-tRNAs. *Nucleic acids research*, doi:10.1093/nar/gkaa487 (2020).

4 Jurenas, D. *et al.* AtaT blocks translation initiation by N-acetylation of the initiator tRNA(fMet). *Nature Chemical Biology* **13**, 640-646, doi:10.1038/nchembio.2346 (2017).

5 Jurenas, D., Garcia-Pino, A. & Van Melderen, L. Novel toxins from type II toxin-antitoxin systems with acetyltransferase activity. *Plasmid* **93**, 30-35, doi:10.1016/j.plasmid.2017.08.005 (2017).

6 Rycroft, J. A. *et al.* Activity of acetyltransferase toxins involved in Salmonella persister formation during macrophage infection. *Nature Communications* **9**, 11, doi:10.1038/s41467-018-04472-6 (2018).

7 Qian, H. L. *et al.* Identification and characterization of acetyltransferase-type toxin-antitoxin locus in Klebsiella pneumoniae. *Molecular Microbiology* **108**, 336-349, doi:10.1111/mmi.13934 (2018).

8 Wilcox, B. *et al.* Escherichia coli ItaT is a type II toxin that inhibits translation by acetylating isoleucyl-tRNAIle. *Nucleic acids research* **46**, 7873-7885, doi:10.1093/nar/gky560 (2018).