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Supplemental Information

Retapamulin-Assisted Ribosome Profiling

Reveals the Alternative Bacterial Proteome

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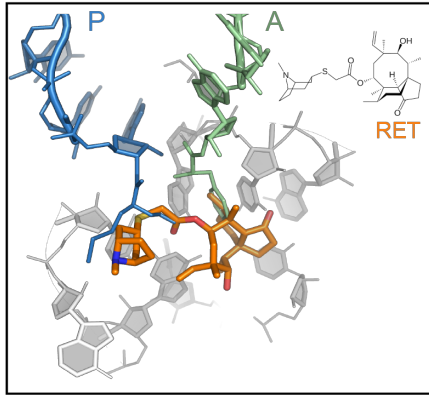
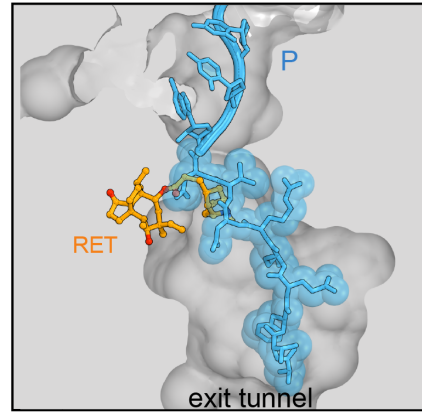
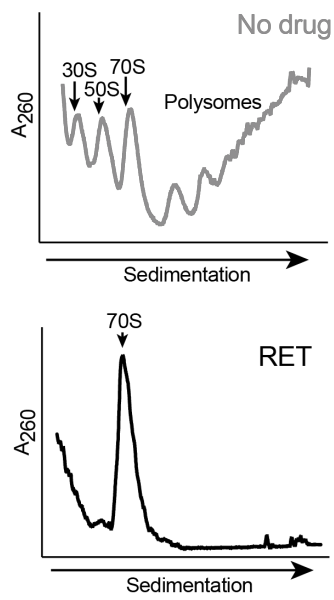
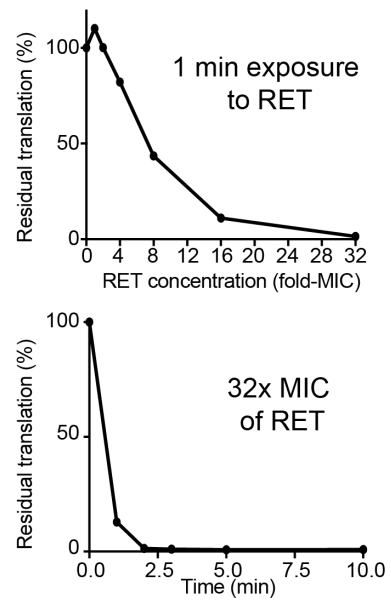
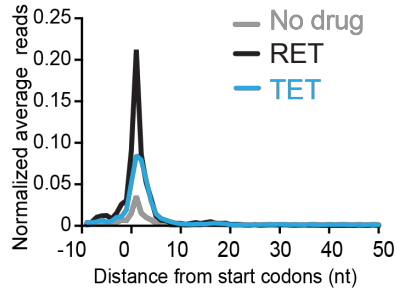
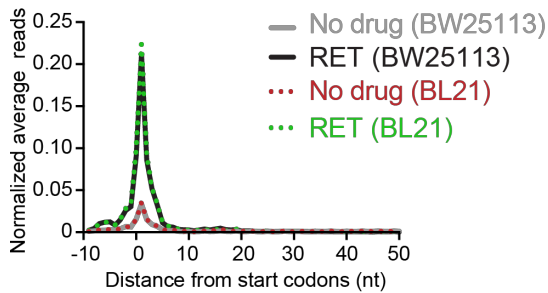
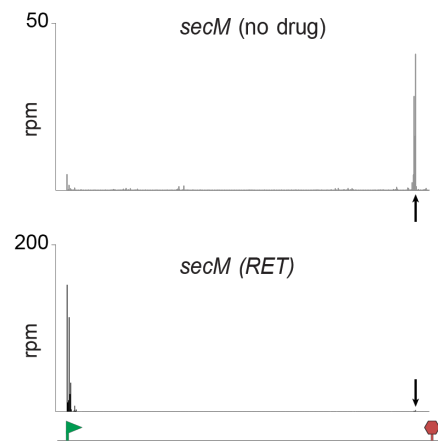
A**B****C****D****E****F****G**

Figure S1 Retapamulin arrests ribosomes at initiation, Related to Figure 1

(A) The chemical structure of the pleuromutilin antibiotic retapamulin (RET) bound at the PTC active site of the bacterial ribosome. The model is based on the structural alignment of the 50S ribosomal subunit of *Deinococcus radiodurans* (*Dr*) ribosomes in complex with RET (PDB 2OGO) (Davidovich et al., 2007) and *Thermus thermophilus* 70S ribosomes with fMet-tRNA bound in the P site and Phe-tRNA in the A site (PDB 1VY4) (Polikanov et al., 2014). Note that in the 70S initiation complex, the fMet moiety of the initiator tRNA has to be displaced from the PTC active site to allow for RET binding.

(B) RET cannot coexist with a nascent protein in the ribosome. Alignment of the structures of the *Dr* 50S RET complex with the *E. coli* 70S ribosome carrying ErmBL nascent peptide that esterifies P-site tRNA (PDB 5JTE) (Arenz et al., 2016).

(C) Sucrose gradient analysis of polysome preparation from *E. coli* BW25113 $\Delta toIC$ cells untreated (top) or treated for 5 min with 12.5 $\mu\text{g}/\text{mL}$ (100X MIC) RET. The shown profiles represent cryo-lyzed preparations used in Ribo-seq experiments. Qualitatively similar results have been obtained in analytical experiments with the samples prepared by freezing-thawing (see STAR Methods).

(D) Residual protein synthesis in *E. coli* BL21 $\Delta toIC$ cells treated with RET, as estimated by incorporation of [^{35}S]-methionine into the TCA-insoluble protein fraction, after 1 min exposure to increasing concentrations of RET (top) or treated with 2 $\mu\text{g}/\text{mL}$ of RET (32-fold MIC) for the indicated periods of time (bottom).

(E) Metagene plots comparing the normalized average relative density of ribosomal footprints in *E. coli* BW25113 $\Delta toIC$ cells untreated (gray trace) or treated 12.5 $\mu\text{g}/\text{mL}$ (100X MIC) of RET (black trace). Blue trace represents similar analysis of the publicly-available Ribo-seq data obtained with *E. coli* BW25113 $\Delta smpB$ cells exposed to tetracycline (TET) [the average of two replicates of Ribo-seq experiments reported in (Nakahigashi et al., 2016)].

(F) Metagene plots comparing the normalized average relative density of ribosomal footprints in the *E. coli* strains BW25113 $\Delta toIC$ cells or *E. coli* BL21 $\Delta toIC$ untreated or treated with RET.

(G) Snapshot of ribosomal footprints density in the *secM* gene of *E. coli* BW25113 $\Delta toIC$ cells untreated or treated with RET. The pTIS and stop codon of the gene are indicated by a green flag and red stop sign, respectively. The black arrow indicates the known site of translation arrest at the codon 165 of the 170-codon *secM* ORF (Nakatogawa and Ito, 2002).

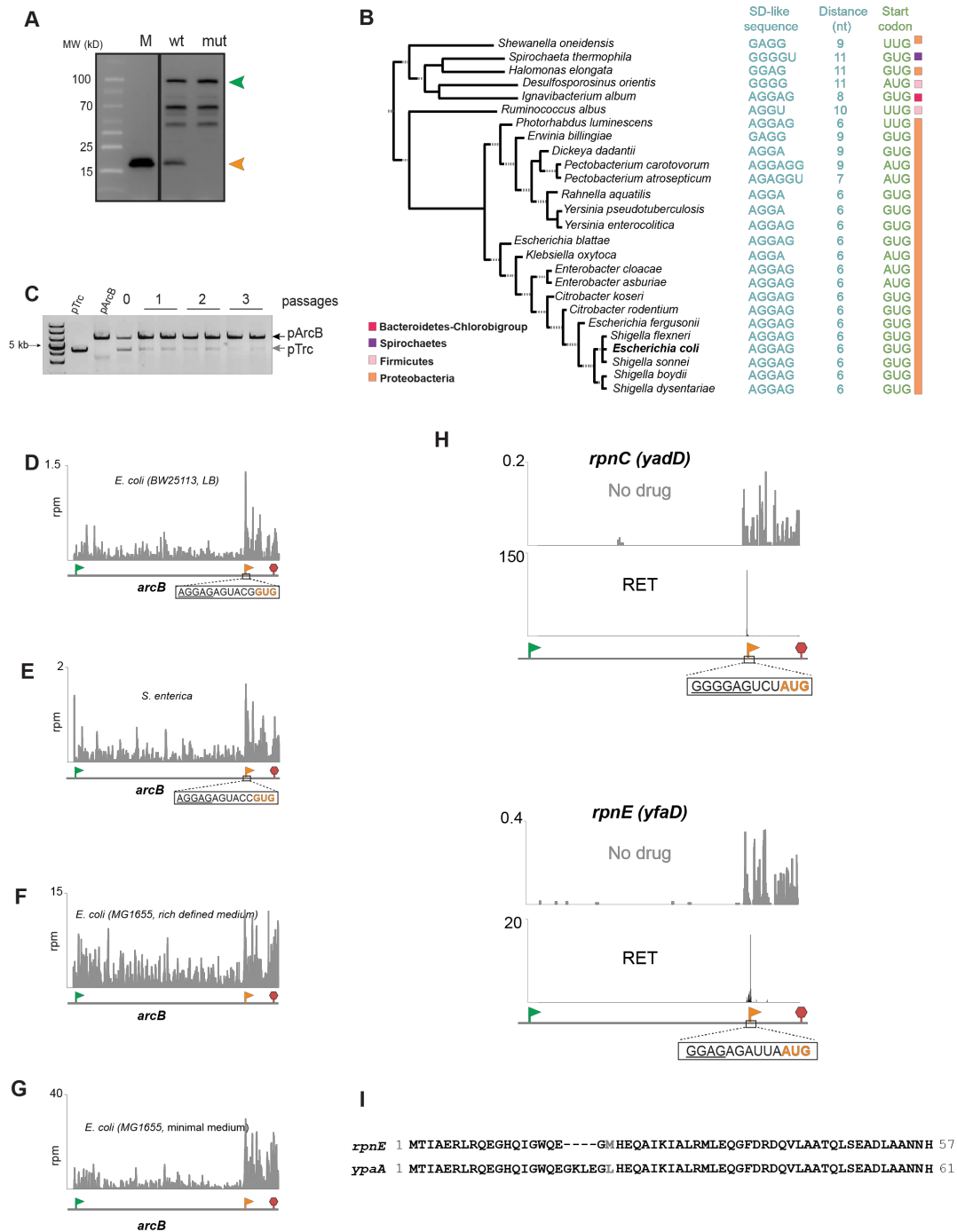


Figure S2 The utilization of an in-frame iTIS within the *arcB* gene leads to production of an alternative protein ArcB-C with a potential role in cell physiology, Related to Figure 3

(A) The uncropped image of the immunoblot shown in Figure 3E, representing the bands corresponding to full-length ArcB-3X FLAG and internal initiation product ArcB-C-3XFLAG (marked with arrow heads). Protein size markers are shown. The origin of the bands marked with dots is unknown.

(B) The iTIS that directs translation of the ArcB-C protein is conserved in the *arcB* gene of diverse bacterial species. The putative start codons and the SD-like sequences are shown.

(C) Presence of *arcB* facilitates *E. coli* growth under low oxygen conditions. BW25113 Δ *arcB* *E. coli* cells carrying the empty vector pTrc99a or pArcB were co-grown in low oxygen conditions. Gel shows the HindIII-linearized plasmids, isolated from the co-growth cultures to determine fraction of cells with or without *arcB* in the mixture (see Start Methods for details). The '0' sample represents plasmids from the initial mixture containing equal number of pTrc99A and pArcB cells.

(D-G) The upshift of ribosomal footprints in the *arcB* segment encoding ArcB-C observed in the Ribo-seq profiles of untreated *E. coli* or *Salmonella enterica* cells (Baek et al., 2017; Kannan et al., 2014; Li et al., 2014). The pTIS and iTIS of *arcB* are marked with green and orange flags, respectively, and the stop codon is indicated by a red stop sign.

(G) Representative examples of Ribo-RET and Ribo-seq profiles of two out of five *E. coli* *rpn* genes.

(H) Alignment of the amino acid sequence of the RpnE-C protein, translated from the iTIS within the *rpnE* gene and the protein encoded in an independent gene *ypaA*.

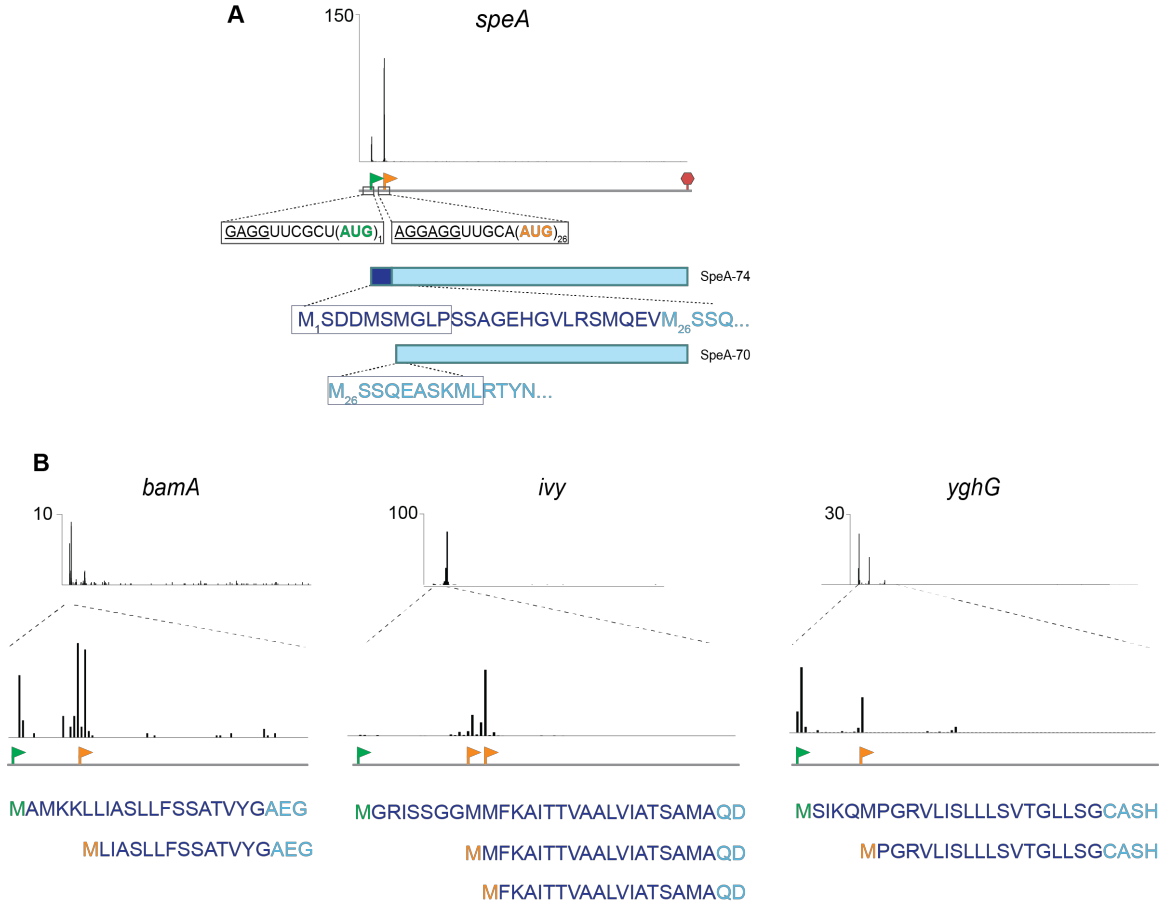


Figure S3. Initiation at the 5'-end proximal iTISs could produce alternative products with incomplete N-terminal signal sequences, Related to Figure 4

(A) Ribo-RET profile of the *speA* gene, showing peaks corresponding to pTIS (green flag) and iTIS (orange flag). The stop codon is indicated by a red stop sign. The putative signal sequence (indicated by dark blue letters) of SpeA-74 (Buch and Boyle, 1985) is lacking in the alternative product SpeA-70 whose translation is initiated at the iTIS. The SpeA isoforms, whose translation is initiated at the pTIS or the iTIS are expected to have different cellular localization. The peptides detected by N-terminomics are boxed (Bienvenut et al., 2015).

(B) Ribo-RET profiles of *bamA*, *ivy* and *yghG* genes. The N-terminal amino acid sequences of the primary and predicted alternative proteins are indicated. The reported signal sequences are shown in dark blue. The pTISs of the genes are marked by green flags; iTISs are indicated with orange flags.

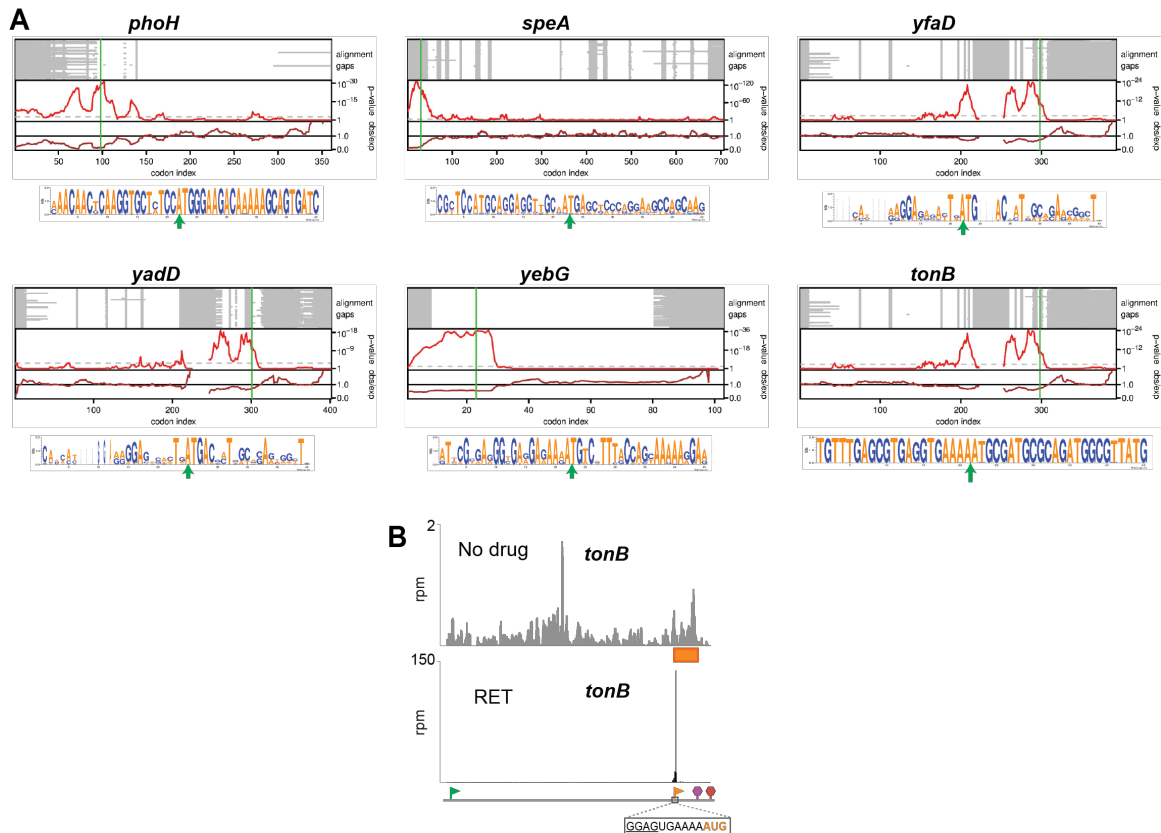


Figure S4. Synonymous site conservation for selected iTISs, Related to Figures 3-5

(A) Synonymous site conservation plots and weblogos for genes with in-frame iTISs (*phoH*, *speA*, *yfaD*, *yadD*, *yebG*) and for the *tonB* gene with an OOF iTIS. Alignment gaps in each sequence are indicated in grey. The two panels show the synonymous substitution rate in a 15-codon sliding window, relative to the CDS average (observed/expected; brown line) and the corresponding statistical significance (p -value; red line). The horizontal dashed grey line indicates a p -value of $0.05 / (\text{CDS length}/\text{window size})$ – an approximate correction for multiple testing within a single CDS.

(B) An upshift in the local density of ribosome footprints within the alternative frame defined by the *tonB* OOF iTIS (orange rectangle) in cells not exposed to antibiotic. Start codons of the pTIS and OOF iTIS are marked with green and orange flags, respectively, while the respective stop codons are indicated with red and purple stop signs. The start codon and SD-like sequence of the iTIS are shown.

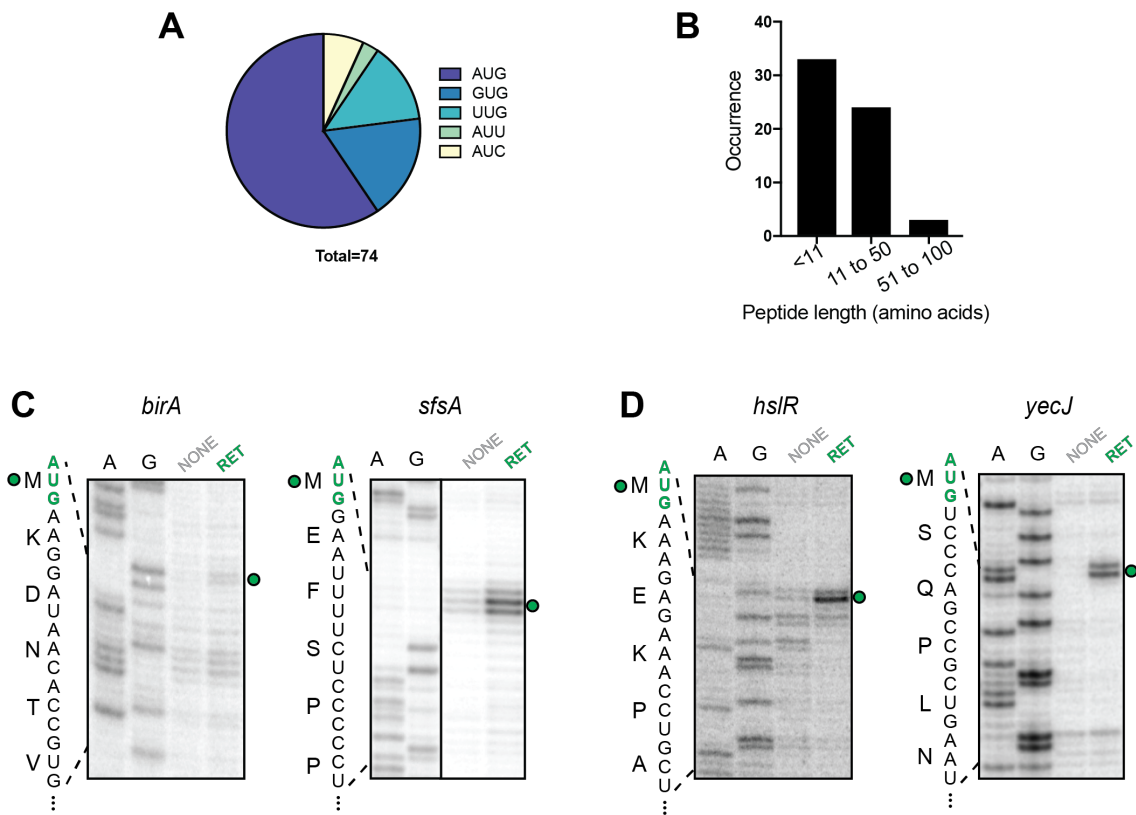


Figure S5 Ribo-RET reveals OOF iTISs, Related to Figures 4 and 5

(A) The distribution of start codons associated with OOF iTISs revealed by Ribo-RET.

(B) The length distribution of the putative alternative proteins whose translation is initiated at OOF iTISs.

(C) and (D) Toe-printing gels showing RET-induced ribosome stalling at the pTISs of *birA* and *sfsA* (shown in Figure 4) and *hsIR* and *yecJ* (shown in Figure 5) genes. Samples analyzed in the lanes marked NONE contained no antibiotics. Start codons of the pTISs are indicated in green. Sequencing lanes are shown.

Table S3. List of primers and synthetic DNA fragments used in this study, Related to STAR methods

	Sequence (5' to 3')	Purpose
P1	TGTCCTGGCACTAATAGTGA	Forward primer for amplification of <i>tol::kan</i> cassette
P2	ACGATGCGTGGCGTATGG	Reverse primer for amplification of <i>tol::kan</i> cassette
P3	TTGTGAGCGGATAACAATTTACACAGGAAA CAGACCATGGTGGGTATTATTGGGGCAGG	Forward primer for amplification of <i>arcB</i> -PCR 1-wt
P4	ACATAATACTGCGCCAGC	Reverse primer for amplification of <i>arcB</i> -PCR 1-wt
P5	AAGCAAATTCGTCTGCTGG	Forward primer for amplification of <i>arcB</i> -PCR 2-wt
P6	TGGGAATATCGAGCAATGCTT	Reverse primer for amplification of <i>arcB</i> -PCR 2-wt
P7	GAAGAGAACAGTAAATCAGAAGCATTG	Forward primer for amplification of <i>arcB</i> -PCR 3
P8	TCAGGCTGAAAATCTTCTCTCATCCGCCAAA ACAGCCAAGCTTTCACCTTGTGCATCGTCAT	Reverse primer for amplification of <i>arcB</i> -PCR 3
P9	TCCTGGGTATCCCAGAATTC	Reverse primer for amplification of <i>arcB</i> -PCR 1-mutant
P10	CGCTAACCGCGATGATCAAGAAATTCTGGGA TACCCAGGATGATGAAGAAAGTACGGTCACG ACAGAAGAG	Forward primer for amplification of <i>arcB</i> -PCR 2-mutant
P11	TGGGAATATCGAGCAATGCTTCTGATTTACT GTTCTCTTCTGTCGTGACCGTACTTTCTTCAT CATCCTGGGTATCCCA	Reverse primer for amplification of <i>arcB</i> -PCR 2-mutant
#12	AACAGACCATGGTACCCAGGATGATGAGGA GAGTACGGTGACGACAGAAGAGAACAGTAAA TCAGAAGCATTGCTCGATATTCCCATGCTGG AACAGTATCTCGAACTTGTAGGACCGAAGCT GATCACCGACGGGTTAGCGGTGTTTGAGAA GATGATGCCGGGCTATGTCAGCGTGCTGGA GTCGAATCTGACGGCGCAGGATAAAAAAGG CATTGTTGAGGAAGGACATAAAATTAAGGT GCGGCGGGGTGAGTGGGGTTACGCCATCTG CAACAGCTGGGTCAGCAAATTCAGTCTCCTG ACCTTCCGGCCTGGGAAGATAACGTCGGTG AATGGATTGAAGAGATGAAAGAAGAGTGGCG TCACGACGTAGAAGTGCTGAAAGCGTGGGT GGCAAAGCCACTAAAAAAGACTACAAAGAC CATGACGGTGATTATAAAGATCATGACATCG ATTACAAGGATGACGATGACAAGTGAAAGCT TGGCTGTT	gBlock for <i>arcB</i> -marker insert
P13	TAATACGACTCACTATAGGGCTGTAATTAACA ACAAAGGGT	Forward primer for amplification of <i>atpB</i>
P14	GGTTATAATGAATTTTGCTTATTAACCGAGAA TGTACGCAGTTAGTCCAGCTGAAGGTT	Reverse primer for amplification of <i>atpB</i>

P15	TAATACGACTCACTATAGGGACTAAAAGTAA GGCATTAAAC	Forward primer for amplification of <i>mgo</i>
P16	GGTTATAATGAATTTTGCTTATTAACCTGCTC CTCGGACGCTTATTTGCTTTTGCCGCC	Reverse primer for amplification of <i>mgo</i>
P17	GGTTATAATGAATTTTGCTTATTAAC	Reverse primer for toeprinting of <i>atpB</i> and <i>mgo</i>
P18	TAATACGACTCACTATAGGGAGCGCAGTGG GACA	Forward primer for amplification of <i>birA</i>
P19	CTACGCAAATAATTTGCAGGG	Reverse primer for amplification of <i>birA</i>
P20	TTTCACCCAACCTGCTC	Reverse primer for toeprinting of primary site of <i>birA</i>
P21	AATACTCCCCTTTCTTATTTTT	Reverse primer for toeprinting of internal site of <i>birA</i>
P22	TAATACGACTCACTATAGGGCAATAACAAGG ATTGTCGCAATG	Forward primer for amplification of <i>sfsA</i> -PCR 1
P23	GCCGTATTTTACTTCGCTTTCTAGCGAGCTAT AGCCTGACAG	Reverse primer for amplification of <i>sfsA</i> -PCR 1
P24	CTGTCAGGCTATAGCTCGCTAGAAAGCGAAG TAAAATACGGC	Forward primer for amplification of <i>sfsA</i> -PCR 2
P25	CTACAATGTAACCGGCAGTG	Reverse primer for amplification of <i>sfsA</i> -PCR 2
P26	TAATACGACTCACTATAGGG	Forward primer for amplification of <i>sfsA</i> -g321a, a322g
P27	CTACAATGTAACCGGCAGTG	Reverse primer for amplification of <i>sfsA</i> -g321a, a322g
P28	CATCGGGTGTGATCAC	Reverse primer for toeprinting of primary site of <i>sfsA</i>
P29	CGATTTCACTTCAATATA	Reverse primer for toeprinting of internal site of <i>sfsA</i>
P30	TAATACGACTCACTATAGGGGCTAATGTGAA GGAGACGC	Forward primer for amplification of <i>hslR</i>
P31	TTATTTCACTGTCGCCGTG	Reverse primer for amplification of <i>hslR</i>
P32	GGGCCAGCGCGC	Reverse primer for toeprinting of primary site of <i>hslR</i>
P33	TTGTCCGGGCGTCCG	Reverse primer for toeprinting of internal site of <i>hslR</i>
P34	TAATACGACTCACTATAGGGAATGCTATCAG GAGTTTACGATG	Forward primer for amplification of <i>yecJ</i>
P35	TTAATGGGATTCACCCTGTGGG	Reverse primer for amplification of <i>yecJ</i>
P36	CATCCAGAATTTGTTTGATAAC	Reverse primer for toeprinting of primary site of <i>yecJ</i>
P37	GCGGCGGCGGGATGG	Reverse primer for toeprinting of internal site of <i>yecJ</i>
P38	GTGAGCGGATAACAATTTACACAGAATTCA TTAAAGAGGAGAAATTAACCTATGGCT	Forward primer for amplification of <i>RFP</i>
P39	ATATCTCCTTCTTAAAGTTAAACAACCTAGTCT ATTCGCCAGAACCAGC	Reverse primer for amplification of <i>RFP</i>
P40	TTTAAGAAGGAGATATACATATGACTAGTGCA TCCAAGGGCGA	Forward primer for amplification of <i>GFP</i>

P41	TCAGCTAATTAAGCTTGGCTGCAGGTCGACC CGGGGTACCGAG	Reverse primer for amplification of <i>GFP</i>
P42	TCCGCTGCTGGTTCTGGCGAATAGACTAGTC AATAACAAGGATTGTCGCAATG	Forward primer for amplification of insert for pRXGSM-sfsA-wt
P43	AAAGAGCTCCTCGCCCTTGGATGCACTAGTG CGAGCTATAGCCTGAC	Reverse primer for amplification of insert for pRXGSM-sfsA-wt
P44	AAAGAGCTCCTCGCCCTTGGATGCACTAGTG CGAGCTATAGCCTGACAGTTCTGAAATTGAT TCGATAAGGATAGCCT	Reverse primer for amplification of insert for pRXGSM-sfsA-mutant
P45	GTTCTGGCGAATAGACTAGTAAATGCTATCA GGAGTTTACG	Forward primer for amplification of insert for pRXGSM-yecJ derivatives
P46	AGCTCCTCGCCCTTGGATGCACTAGTCATCG AGAACATCCAGAATTTG	Reverse primer for amplification of insert for pRXGSM-yecJ-iTIS-wt
P47	AGCTCCTCGCCCTTGGATGCACTAGTCGTGG AGAACATCCAGAATTTG	Reverse primer for amplification of insert for pRXGSM-yecJ-iTIS(-)
P48	AGAGCTCCTCGCCCTTGGATGCACTAGTTAC ATCGAGAACATCCAGAATTTG	Reverse primer for amplification of insert for pRXGSM-yecJ-pTIS-wt
P49	CTGCTGGTTCTGGCGAATAGACTAGTAATGC TATCAAAGTTTACGTCTCCAGCCGCT	Primer for site directed mutagenesis to generate pRXGSM- yecJ-pTIS(-)
P50	AGCTCCTCGCCCTTGGATGCACTAGTTACGT CGAGAACATCCAGAATTTG	Reverse primer for amplification of insert for pRXGSM-yecJ-pTIS- iTIS(-)
P51	AGCTCCTCGCCCTTGGATGCACTAGTGACAT CGAGAACATCCAGAATTTG	Primer for site directed mutagenesis to generate pRXGSM- yecJ-pTIS-iStop(-)
P52	GGCCTTAACCGCTAACGT	Direct primer for sequencing the iTIS region in the <i>arcB</i> gene
P53	TTTAATCTGTATCAGGCTGAAAATCTT	Reverse primer for sequencing the iTIS region in the <i>arcB</i> gene