

# Appendix: Environmental perturbations lift the degeneracy of the genetic code to regulate protein levels in bacteria

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## Bacterial strains

All strains used in this study were obtained from the *E. coli* Genetic Stock Center (CGSC), Yale University. For amino acid limitation experiments, standard auxotrophic strains (Table S5) were used depending on the amino acid that was limiting in the growth medium, unless mentioned otherwise. Strain CP78 was used for experiments involving leucine and arginine limitation. This strain has been used extensively in previous amino acid limitation studies [1, 2, 3, 4, 5, 6] and its multiple auxotrophy makes it a convenient choice for experiments involving limitation for several amino acids. The auxotrophic strains corresponding to the remaining amino acids are from the Keio-knockout collection [7], and are the commonly used auxotrophic strains for that amino acid (<http://cgsc.biology.yale.edu/Auxotrophs.php>).

For the growth lag measurements in Fig. 4A, the prototrophic strain MG1655 (Table S5) was used as the wild-type background. This background strain was tagged with *yfp* or *rfp* at the *attB $\lambda$*  locus (this tagging was a remnant from earlier experiments not related to this work, and has no relevance to any results presented here). Site-directed mutagenesis was used to create the synonymous mutant coding sequences for *leuA*, *leuC*, *leuD*, *carA*, *argA* and *argF* using the protocol described in the section on gene synthesis and cloning. Then to insert these mutant ORFs into their native locus without any additional markers, a two-step strategy based on  $\lambda$  Red-mediated homologous recombination [8] was used: In the first step, the respective wild-type ORF was replaced by a *kan* resistance gene, and in the second step the *kan* gene was replaced by the mutant ORF without any additional markers by selecting on M9-glucose plates for prototrophy of the respective amino acid. Plasmid *pSIM5* [9] was used as the helper plasmid and a previously published recombining protocol [9] was used without any modifications.

For RT-qPCR (Fig. 4B), a leucine auxotroph of MG1655 was created by deleting the *leuB* gene using the  $\lambda$  Red-mediated homologous recombination protocol outlined above. For Western blots (Fig. S1), the auxotrophic strains in Table S5 were further modified by insertion of the *tet* repressor gene at the *attB $\lambda$*  site using a previous method based on  $\lambda$  integrase-mediated site-specific recombination [10]. The presence of Tet repressor enabled inducible control of YFP expression. The Western blots for leucine and arginine *yfp* variants were performed in an MG1655 auxotroph strain background instead of the CP78 strain. The CP78 strain has lower transformation efficiency which prevented integration of the *tet* repressor gene into the chromosome. Strains were stored as 20%

glycerol stocks at -80°C either in 1ml cryo-vials or in 96-well plates (3799, Costar). In addition, for experiments involving over 25 strains, a temporary 20% glycerol stock was stored at -20°C in 96-well PCR plates.

## Plasmids

The *pZ* series of plasmids [10] were used for extra-chromosomal expression of genes. General features of the plasmid backbones are described here. Specific gene constructs that were cloned into these backbones is described in the section on gene synthesis and cloning. A low-copy plasmid, *pZS\*11* [*SC101\** ori (3-4 copies/cell), AmpR (*bla* gene) and a constitutive *P<sub>LtetO1</sub>* promoter] was used for expression of all fluorescent reporter genes and their fusions. The synthetic ribosome binding site (RBS) in the original *pZS\*11* backbone was replaced by a modified *T7*-based RBS that resulted in efficient protein expression from most coding sequences. A medium-copy plasmid, *pZA $\beta$ 2* [*p15A* ori (10-12 copies/cell), ChlR (*cat* gene) and *P<sub>LlacO1</sub>* promoter] was used for expression of all tRNA genes. Strains with *pZA $\beta$ 2* plasmids were grown with 1mM IPTG to ensure constitutive expression of all tRNA genes. Standard plasmids *pUC18* and *pUC19* (Invitrogen) were used as intermediate cloning vectors for site-directed mutagenesis. Plasmid *pSIM5* (13) was used as the helper plasmid expressing the  $\lambda$ -Red system for all chromosomal modifications in this project (except for Tet repressor insertion mentioned in the previous section).

## Growth and fluorescence measurements

Overnight cultures were inoculated either from freshly grown single colonies or, in experiments involving more than 25 strains, from temporary glycerol stocks stored at -20°C. Overnight cultures were grown in a modified MOPS rich-defined medium [11] made with the following recipe: 10X MOPS rich buffer, 10X ACGU nucleobase stock and 100X 0.132M K<sub>2</sub>HPO<sub>4</sub> (Teknova, Cat. No. M2105) were used at 1X final concentration as in the original recipe. In addition, the overnight growth medium contained 0.5% glucose as carbon source, 10<sup>-4</sup>% thiamine and 800 $\mu$ M of 19 amino acids and 10mM of serine. pH was adjusted to 7.4 using 1M NaOH and appropriate selective antibiotics (50 $\mu$ g/ml ampicillin and/or 20 $\mu$ g/ml chloramphenicol) were added. Amino acids, glucose, thiamine and antibiotics were purchased from Sigma. 1ml overnight cultures were grown in 2ml deep 96-well plates (40002-014, VWR) at 30°C with shaking at 1350rpm (Titramax 100 shaker) for 12 to 16 hours.

For amino acid limitation experiments, overnight cultures were diluted 1:1000 into 1ml of the same MOPS rich-defined medium as the overnight cultures. However the amino acid whose limitation was to be induced was added at a reduced concentration and supplemented with its methyl ester analog (Table S6). Amino acid methyl esters are analogs of the corresponding amino acids and have been previously used for steady growth of *E. coli* under amino acid limiting conditions [12, 13] (see Figs. S15 and S16 for the effect of methyl ester on growth and robustness of YFP synthesis). Addition of the methyl esters results in a steady but limiting supply of the amino acid due to slow hydrolysis of the ester (see Note 1). Concentrations of the amino acid and its methyl ester were chosen such that the cultures consumed the limiting amino acid and entered amino acid-limited growth at an  $OD_{600}$  of 0.6-0.7 (corresponding to an  $OD_{600}$  value of 0.2-0.25 in our 96-well plate reader). Slight variations in the initial concentration of either the limiting amino acid or its methyl ester shift the transition to a higher or lower cell density without appreciable changes in growth rate (see Note 2). Except for a single limiting amino acid, the remaining 19 amino acids were present at the overnight culture concentrations during the amino acid limitation experiments. For proline limitation, no proline was necessary in the growth medium since proline methyl ester supported growth at the same rate as proline until the  $OD_{600}$  reached around 0.6.

Diluted overnight cultures were grown in 2ml deep 96-well plates for 3 hours at 30°C with shaking at 1350rpm (Titramax 100 shaker). After this time interval, 3 aliquots of 150 $\mu$ l from each culture was pipetted into 3 wells of 3 different 96-well plates (3799, Costar). Wallac Victor2 plate reader (PerkinElmer) was used to monitor cell density (absorbance at 600nm) and YFP synthesis (fluorescence, excitation 504nm and emission 540nm). Each plate was read every 15 min using a robotic system (Caliper Life Sciences) and shaken in between readings (Variomag Teleshake shaker) for a total period of 6-10 hours. Temperature of 30°C and relative humidity of 60% was maintained throughout the experiment.

In the case of experiments without methyl ester (Figs. S15 and S16), the same protocol mentioned above was followed but the methyl esters were not added to the growth medium. For the RT-qPCR measurements shown in Fig. 4B, overnights cultures were diluted 1:1000 into the same medium. Then when the  $OD_{600}$  reached 0.5, the cells were spun down at 3000g for 5 min and then re-suspended in the same medium but either with or without leucine. Total RNA was extracted (see protocol below) after 30 min of shaking at 30°C, 200rpm.

For the growth lag measurements shown in Fig. 4A, overnight cultures of prototrophic strains were diluted 1:200 into medium either with or without one of leucine and arginine. Growth lag was measured as the difference in time taken to reach  $OD_{600}$  of 0.3 between two cultures of the same strain – one growing in the presence of either leucine or arginine and another growing in its absence.

## Gene synthesis and cloning

All gene sequences constructed for this study are provided in the gene\_sequences.fasta file. Plasmid backbone sequences are provided in the plasmid\_sequences.genbank file. Primer sequences used for cloning will be provided upon request. For all primers, 18 to 22bp homologies without any special primer design criteria were sufficient for successful PCR amplification with Phusion High-Fidelity DNA polymerase (NEB).

**Initial *yfp* construct.** All *yfp* variants used in this study were modified starting from a single yellow fluorescent protein gene sequence (called *yfp0* in the sequence file and plasmid map). This *yfp0* sequence encoded the fast-maturing ‘Venus’ variant of YFP [14]. All 238 codons of *yfp0* were chosen such that they were decoded by abundant tRNA isoacceptors for each amino acid. Such a choice of codons ensured that the native level of demand for each tRNA isoacceptor inside the cell was minimally perturbed by the low-copy expression of fluorescent reporter genes. The *yfp0* sequence was built de novo (synthesis by Genscript, USA). The synthesized *yfp0* sequence was cloned between the KpnI and HindIII restriction sites of the *pZS\*11* plasmid vector using standard molecular-biology techniques (19). The plasmid map of the resulting construct, *pZS\*11-yfp0* is shown in Fig. S17.

**Synonymous variants of *yfp*.** A subset of codons in *yfp0* corresponding to 7 amino acids (Leu, Arg, Ser, Pro, Ile, Gln, Phe) were mutated to create the initial 29 synonymous variants of *yfp* (*yfp1* – *yfp29* in the gene\_sequences.fasta file, sequences in the same order as shown in Fig. 1A). The 4 *yfp* variants corresponding to Pro (*yfp19-yfp22*) had all the Pro codons mutated to the most frequent CCG codon since the original *yfp0* sequence had a few CCA and CCT codons that are more sensitive to Pro limitation. Similarly, all the Phe codons in *yfp0* were mutated to the most abundant Phe codon TTT for the two Phe variants of *yfp0* (*yfp28-yfp29*). Both these groups of variants (6 total) had higher overall fluorescence during amino-acid rich conditions than the rest of the 23 variants. This higher fluorescence is likely due to changes in secondary structure near the ribosome binding region on the mRNA as a consequence of mutations near ATG. However, this change is common across all variants within the Pro and Phe synonymous codon groups and hence is not responsible for the differential response to cognate amino acid limitation measured within these synonymous codon groups.

For constructing the 29 *yfp* variants, *yfp0* from *pZS\*11-yfp0* was first cloned into a *pUC19* cloning vector between the KpnI and HindIII restriction sites. A commercial site-directed mutagenesis kit (Quickchange Lightning Multi, Applied Biosystems) was used to introduce the mutations corresponding to each of the 29 variants and the manufacturer’s protocol was followed. The resulting variants were verified by Sanger sequencing and then cloned into the *pZS\*11* expression vector backbone between the KpnI and HindIII sites. The 22 single CTA variants of *yfp* (Fig. S2) were constructed using the same procedure as above. The 29 *yfp* variants for Western blotting (Fig. S1) were created using the same procedure as above, but with the addition of a 22 codon sequence at the 5’ end that encoded a 3X-FLAG peptide recognized by a commercially available, anti-FLAG, antibody (Sigma). The 22-codon sequence is: GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAG.

**tRNA expression vectors.** The 5 distinct Leu tRNA isoacceptors encoded by the genes *leuQ*, *leuU*, *leuW*, *leuX* and *leuZ*, and the 4 distinct Arg tRNA isoacceptors encoded by the genes *argV*, *argX*, *argU* and *argW* were cloned between the EcoRI and HindIII sites of the *pZA32* expression vector (Fig. S18). These genes were amplified by PCR from the chromosome of *E. coli* strain MG1655. In addition to these native tRNA genes, a synthetic tRNA gene *arg2<sub>m</sub>* cognate to the CGA Arg codon was also created. Normally, the <sup>ACG</sup>Arg<sub>2</sub> tRNA with ICG anti-codon reads the CGA codon inefficiently through a purine-purine wobble pairing. Expressing a synthetic tRNA with an anticodon UCG restores efficient reading of this codon and is equivalent to increasing the supply

of the corresponding cognate tRNA isoacceptor. This synthetic tRNA isoacceptor was created from the *pZA32-argV* expression vector using overlap PCR to introduce the necessary single bp mutation in the anticodon of *argV*. The *pZA32* vectors with the tRNA genes were electroporated into strains already containing the YFP expression vectors.

**Library of *E. coli* ORF-*yfp* fusions.** 92 *E. coli* Open Reading Frames (ORFs) were selected for experimental validation of the Leu Codon Robustness Index (Leu CRI). These ORFs were chosen to span a wide range of predicted Leu CRI values and functional categories (Fig. S7 and Table S1). First, a modified *pZS\*11-yfp0* vector backbone was created in which the start codon of *yfp0* was replaced by a GGSGGS hexapeptide linker sequence: GGTGGATCCGGCGGTTCT containing a BamHI restriction site. Next, the 92 ORFs (without the stop codon) were amplified by PCR from the chromosome of *E. coli* strain MG1655 with 5'-KpnI and 3'-BamHI restriction site overhangs. These PCR fragments were cloned into the modified *pZS\*11-yfp0* vector backbone containing the BamHI restriction site. 13 of the 92 ORFs had either an internal KpnI or an internal BamHI site. In these cases, a larger fragment that included adjoining sections of the *pZS\*11-yfp0* vector was constructed by overlap PCR and then cloned using other restriction sites (EcoRI or HindIII). Thus the final constructs had one of the 92 *E. coli* ORFs connected through a hexapeptide linker with *yfp0*. All the cloned sequences were verified by PCR for inserts of right length and around 40 ORF constructs were verified by Sanger sequencing. Two biological replicates of each ORF construct were compared for their synthesis robustness values as measured during the amino acid limitation assay and these values showed a high degree of correlation (Pearson  $\rho = 0.93$ , Fig. S19).

For validating the Arg codon robustness index (Arg CRI), 56 *E. coli* ORFs that included a subset of the above 92 ORFs were chosen (Table S2). The cloning procedure was exactly analogous to the above 92 ORFs but with one difference: the *yfp0* part of the fusion construct was replaced by a synonymous variant of *yfp0* (*yfp7*) that had the Arg codon AGA instead of the CGT and CGC codons in the *yfp0* sequence. The codon AGA has the highest  $w_i$  value among the Arg codons (see Fig. 3B) and hence has a minimal effect on the measured robustness of the ORF fusions during Arg limitation.

**Co-expression of <sup>GAG</sup>Leu2-tRNA with *E. coli* ORF-*yfp* fusions.** Out of the 92 *E. coli* ORF-*yfp* fusions, 21 were chosen for co-expression with the <sup>GAG</sup>Leu2 tRNA that is cognate to the codons CTC and CTT. The 21 ORFs were chosen such that 11 of them had a lower Leu CRI prediction than their wild-type counterparts while the other 10 ORFs had a higher Leu CRI prediction than their wild-type counterparts (Table S3). This choice also corresponded respectively to either high frequency of the non-cognate TTA and TTG codons for <sup>GAG</sup>Leu2 or high frequency of the cognate codons CTC and CTT. The strains containing the 21 ORF fusions were each made electro-competent and then transformed with the *pZA32-leuU* plasmid that expresses <sup>GAG</sup>Leu2.

**Synonymous variants of *E. coli* ORF-*yfp* fusions.** Out of the 92 *E. coli* ORF-*yfp* fusions, 13 were selected for creating synonymous mutants (Table S2). These 13 ORFs had a high frequency of one or both of the Leu codons, TTA or TTG and these codons were mutated to the Leu codon, CTC. All these 3 codons, TTA, TTG and CTC occur at similar frequencies on average across the genome of *E. coli*. The 13 ORF-*yfp* fusions were amplified by PCR from the *pZS\*11* vectors between

the EcoRI and XbaI restriction sites (see Fig. S17). These fragments were cloned between EcoRI and XbaI sites of the *pUC19* cloning vector. A commercial site-directed mutagenesis kit (Quickchange Lightning Multi, Applied Biosystems) was used to introduce the TTA, TTG  $\rightarrow$  CTC mutations. A unique primer was designed for each of the TTG or TTA codons in the 13 ORFs, and these primers encoded the CTC mutation. All the primers corresponding to each ORF were mixed and then used in the mutagenesis reaction. This procedure resulted in mutant coding sequences with TTA, TTG  $\rightarrow$  CTC mutations at random locations. 10 colonies for each ORF were sequenced and each unique mutant sequence was then cloned into the *pZS\*11* expression vector. At the end of the procedure, a total of 63 constructs were created that each had between one and seven TTA, TTG  $\rightarrow$  CTC mutations (see *gene\_sequences.fasta* file for exact sequences).

### Total RNA extraction

Total RNA was extracted for two different experiments (Figs. 4B, S14). Phenol-chloroform extraction method was used to obtain total RNA. Briefly, 3ml of cells were quickly mixed with 5ml of ice-cold water and harvested by centrifugation at 3000g for 10min. Cell pellets were re-suspended in 500 $\mu$ l of 0.3M sodium acetate-10mM EDTA, pH 4.8 buffer. The re-suspended cells were mixed with 500 $\mu$ l of acetate-saturated phenol-chloroform at pH 4.8, 50 $\mu$ l of 20% SDS and 500 $\mu$ l of acid-washed glass beads (G1277, Sigma). The mixture was shaken in a vortexer for 20 min at 4C. The aqueous layer was extracted twice with acetate-saturated phenol-chloroform at pH 4.8 and once with chloroform. Total RNA was precipitated with an equal volume of isopropanol and washed with 70% ethanol-50mM sodium acetate pH 4.8 and finally re-suspended in 200 $\mu$ l of RNase-free water. 20 $\mu$ l of the total RNA was treated with DNase (EN0521, Fermentas) to remove residual DNA contamination (manufacturer's instructions were followed). The DNA-free RNA was re-suspended in 200 $\mu$ l of RNase-free water. Intact RNA was confirmed by observation of sharp rRNA bands in native agarose gel electrophoresis.

### RT-qPCR

Reverse transcription (RT) was performed using 4 $\mu$ l of the DNA-free RNA (100-200ng) and Maxima reverse transcription kit (K1641, Fermentas), used according to the manufacturer's instructions. Random hexamer primers were used for priming the RT reaction. At the end of the RT reaction, the 20 $\mu$ l reaction was diluted 100-fold and 10 $\mu$ l of this diluted sample was used for qPCR in the next step. qPCR was performed using Maxima SYBR-Green qPCR kit (K0221, Fermentas) and manufacturer's instructions were followed. qPCR was performed in triplicates for each RT reaction and appropriate negative RT controls were used to confirm the absence of DNA contamination. *gapA* mRNA was used as internal reference to normalize all other mRNA levels. Standard curves with 6 serial dilutions were used to optimize reaction conditions and ensure amplification efficiency of between 90-100% for the *yfp* and *gapA* amplicons.  $\Delta\Delta C_t$  method was used to obtain the change in mRNA levels due to amino acid limitation. The qPCR primer sequences are given in Table S8.

### Western blotting

Fresh colonies were used to inoculate overnight cultures. These overnight cultures were then diluted 1:100 into 1ml of rich-defined medium with all 20 amino acids (see section

on growth and fluorescence measurements for media composition). After approximately 3.5 hours of growth at 30°C when  $OD_{600}$  was  $\sim 0.4$ , cells were spun down at 9000g for 1 min, and then re-suspended in 1ml of rich-defined medium without the amino acid whose limitation was to be induced. This re-suspended culture was then split into two equal aliquots. The limiting amino acid was added to one aliquot (as a rich-medium control) while the other aliquot did not have the limiting amino acid. The re-suspended medium also contained 200ng/ml of anhydro-tetracycline in order to induce the  $p_{LtetO1}$  promoter that controls the 3XFLAG-*yfp* variants. After growth at 30°C for 60 min, cells were spun down at 12000g, 1 min and re-suspended in 40-400 $\mu$ l of CellLytic B buffer (Sigma, B7435). The buffer volume used was proportional to the  $OD_{600}$  measured at the time of harvesting the culture. The lysate was stored at -80°C. 10 $\mu$ l of the lysate was mixed with 2X Laemmli Buffer (Biorad) and then loaded onto each lane of a pre-cast polyacrylamide gel (Biorad) and SDS-PAGE was carried out at 100V for 120 min. Proteins were transferred to a nitrocellulose membrane by semi-dry blotting at 180mA for 60 min. The membrane was blocked in 2% skim-milk-TBST overnight, and then incubated with a 1:2000 dilution of an anti-FLAG antibody (F3165, Sigma) in 10ml of 2% skim-milk-TBST with shaking at room temperature for 90 min. After washing 4 times for 5 min with TBST, the membrane was incubated with 1:2000 dilution of a secondary HRP-conjugated antibody (7076, Cell Signaling) in 15ml of 2% skim-milk-TBST with shaking at room temperature for 60 min. After washing 4 times for 5 min with TBST, the membrane was treated with an HRP substrate (L00354, Genscript) for 5 min and exposed for 30s to a luminescence imager.

## Analyses

Matlab R2009b (Mathworks) was used for all analyses unless otherwise mentioned. All correlations and P-values reported in this work were calculated using the Matlab command ‘*corr*’ with the ‘*Type*’ option set to either ‘*Spearman*’ or ‘*Pearson*’ as appropriate.

**Growth and fluorescence analysis.** Background absorbance and fluorescence values (obtained from wells containing only growth media) were subtracted from the measured time series for each well. An exponential curve was fitted to the amino acid-rich growth regime for all data points located at least 50 min before the onset of amino acid limitation. A straight line was fitted to the amino acid-limited growth regime for all data points located at least 50 min after the onset time. These fits were performed using the Matlab command ‘*fit*’, and the in-built library options ‘*Exp1*’ and ‘*Poly1*’ respectively. To automatically identify the onset time, the intersection point between the two fitted curves was designated as the onset time of amino acid limitation. This inferred onset time coincided with the onset time identified through visual inspection of the growth curves.

To minimize noise in calculated protein synthesis rates, an exponential curve was fitted to the amino acid-rich regime of the fluorescence time-series and a straight line was fitted to the amino acid-limited regime of the fluorescence time-series. These fits were performed using the Matlab command ‘*fit*’, and the in-built library options ‘*Exp1*’ and ‘*Poly1*’ respectively. Protein synthesis rate,  $S$  was calculated as

$$\text{Protein synthesis rate } S = \frac{1}{\text{Absorbance}} \times \frac{d(\text{Fluorescence})}{d(\text{time})} \quad [1]$$

First, the above formula was evaluated at the onset time of amino acid limitation using the exponential fits for absorbance and fluorescence data in the amino acid rich growth regime. Next, the same formula was evaluated at the onset time using the linear fits for absorbance and fluorescence data in the amino acid limited growth regime. These two values correspond to the protein synthesis rates reported for the amino acid rich and amino acid limited growth regimes (such as the data in Fig. 1D). The protein synthesis rates were normalized within each synonymous codon family and for each growth condition. Robustness of protein synthesis to amino acid limitation was calculated as the ratio of normalized protein synthesis rates between the amino acid rich and amino acid limited growth regimes.

In the case of the experiment without methyl ester (Fig. S15 and Fig. S16), the onset time of amino acid limited growth was determined exactly as above. Then starvation robustness was calculated as the normalized ratio of total fluorescence increase after the onset of amino acid limited growth to the fluorescence increase before this onset. Total fluorescence increase rather than protein synthesis rate was used for this analysis since protein synthesis rates decreased continuously to zero after the onset of amino acid limited growth in the absence of methyl ester analogs.

**Calculation of CRI.** CRI for a protein coding sequence corresponding to a limiting amino acid was calculated by multiplying the  $w_i$  values for codons cognate to the limiting amino acid in that sequence.  $w_i$  values in Fig. 3B were calculated using the robustness of protein synthesis of the corresponding *yfp* variants during cognate amino acid limitation (Fig. 1D). Based on the non-cognate amino acid limitation experiment (Fig. S2), the  $w_i$  values for all codons other than those cognate to the limiting amino acid are set to be equal to 1. For illustration, we demonstrate the calculation of  $w_i$  for the six Leu codons below. The exact same procedure was followed for other synonymous codon families. Taking  $\log_2 w_i \equiv W_i$  for each codon, and  $\log_2(\text{robustness during amino acid limited growth}) \equiv SR$  for each *yfp* variant,

$$7 \times W_{CTA} + 15 \times W_{CTG} = SR_{yfp,CTA} \quad [2]$$

$$7 \times W_{CTC} + 15 \times W_{CTG} = SR_{yfp,CTC} \quad [3]$$

$$22 \times W_{CTG} = SR_{yfp,CTG} \quad [4]$$

$$7 \times W_{CTT} + 15 \times W_{CTG} = SR_{yfp,CTT} \quad [5]$$

$$7 \times W_{TTA} + 15 \times W_{CTG} = SR_{yfp,TTA} \quad [6]$$

$$7 \times W_{TTG} + 15 \times W_{CTG} = SR_{yfp,TTG} \quad [7]$$

The multiplicative factors on the LHS in front of  $W_i$  correspond to the frequency of the Leu codon  $i$  in the corresponding Leu variant of *yfp* (see Fig. 1A). The RHS is the measured ( $\log_2$ ) robustness of protein synthesis from the corresponding *yfp* variant during Leu limitation (see Fig. 1D). These equations were solved simultaneously to determine the  $w_i$  value for each Leu codon. Revised  $w_i$  values (Table S7) based on *yfp* measurements in the presence of  $^{GAG}\text{Leu2}$  tRNA (Fig. 2) were used for calculation of Leu CRI in the case of  $^{GAG}\text{Leu2}$  tRNA co-expression with *E. coli* ORFs (Fig. 3D).

**Leu and Arg CRI for *E. coli* ORFs.** 4300 *E. coli* ORF sequences were parsed out from the MG1655 genome sequence (NCBI website, Accession number: NC\_000913, downloaded on 14th Apr 2011). For each of these 4300 *E. coli* ORFs, Leu or Arg CRI was calculated by multiplying the  $w_i$  values for either all Leu or all Arg codons respectively in the ORF sequence. For the 63 synonymous variants of 13 ORFs (Fig.

S10), Leu CRI values were calculated using the same procedure as above after accounting for the synonymous mutations. For the 21 ORFs co-expressed with Leu2 tRNA (Fig. 3D), revised  $w_i$  values were first calculated using the method outlined in the previous section (Table S7), and using measurements on the 6 Leu variants of *yfp* complemented with <sup>GAG</sup>Leu2 tRNA (3rd column in Fig. 2A). These revised  $w_i$  values were then used to calculate Leu CRI under tRNA co-expression for the 21 tRNA co-expressed ORFs applying the same procedure as for the non co-expressed case.

**Z-score for CRI.** To quantify the deviation in CRI from its expected value for each of the 4300 ORFs in the *E. coli* genome, 1000 random coding sequences were generated for each ORF. Each random version preserved the original amino acid sequence, but the codons for a single amino acid were sampled randomly from a multinomial distribution based on the average frequency of codons for that amino acid in the genome. CRI values were calculated for each random version of the gene, and a distribution of CRI values was generated from the 1000 random trials. The average,  $\mu_{CRI}$  and standard deviation,  $\sigma_{CRI}$  of this CRI distribution was used to calculate the Z-score for CRI as follows:

$$Z_{CRI} = \frac{CRI_{observed} - \mu_{CRI}}{\sigma_{CRI}} \quad [8]$$

In the case of the Z-score for leucine shown in Fig. 4B, the leucine codon CTG was not randomized in the above calculation and only the remaining 5 leucine codons: CTA, CTC, CTT, TTA, and TTG were randomized. This step is important since CTG, which is read by an abundant tRNA isoacceptor, is enriched in highly-expressed genes, and such genes will show up falsely as perturbation-robust genes because CTG is also the codon that is most robust to leucine limitation in our experiments (see Fig. 1D).

**Codon-specific bioinformatic measures.** Codon usage in Fig. S4 was calculated as the average frequency of each codon across the genome of *E. coli* MG1655 (4300 ORFs). tRNA concentrations in Fig. S4 were taken from previous work (see Table 2 in [15]). Concentrations of all cognate tRNAs for each codon were summed together. The codon-tRNA adaptation index in Fig. S4 is taken from literature (see Table S2 in [16]). The tAI value for the CGA codon was revised from the unrealistically low value of 0.00005 to 0.1333 as explained previously [17]. For inferring codon elongation rates from charged tRNA fractions (Fig. S5), we used the formula for codon elongation rate from [18]:

$$\frac{1}{v_k} = \tau_0 + \frac{1}{\sum_i t_i \alpha_i k_{ik}}, \quad [9]$$

where  $v_k$  is the elongation rate of codon  $k$ ,  $\tau_0$  is the codon-independent elongation time across any codon,  $t_i$  is the concentration of tRNA isoacceptor  $i$  that is cognate to codon  $k$ ,  $k_{ik}$  is the second-order rate constant for binding of the ternary complex containing the charged isoacceptor  $i$  to the ribosome at codon  $k$ , and  $\alpha_i$  is the charged fraction of isoacceptor  $i$ . We calculated the codon elongation rates during amino acid limitation using the measured charged fractions from [6]. For amino acid rich conditions, we set the charged fraction to be equal to unity. We used  $\tau_0 = 0.05s^{-1}$ , and  $k_{ik} = 2 \times 10^7 M^{-1}s^{-1}$  similar to [18]. The ratio of codon elongation rates was then normalized within each codon family by the maximum value within that family.

**ORF-specific bioinformatic measures.** Codon Adaptation Index was calculated for each *E. coli* ORF using the method in [19]. This calculation was implemented using the CodonAdaptationIndex class in the CodonUsage module of BioPython (version 1.58). tRNA Adaptation Index was calculated for each *E. coli* ORF using the method in [20]. This calculation was implemented using the codonR package (<http://people.cryst.bbk.ac.uk/~fdosr01/tAI/index.html>, downloaded on 3rd Sep 2011). mRNA folding energy was calculated for the first 37nt of each *E. coli* ORF together with the 5 upstream nucleotides (GTACC) in the *pZS11* plasmid backbone. Calculation was implemented using the *hybrid-ss-min* command in UNAFold software v3.8 [21] with default parameter values for reaction conditions (NA = RNA, T = 37, [Na+] = 1, [Mg++] = 0, maxloop = 30).

## Supplementary notes

### Use of methyl esters in amino acid limitation experiments.

Before we settled on the methyl ester analog-based experiments, we tested two other amino acid limitation assays that are commonly used in the literature. The first assay is a spin → wash → resuspend in amino acid+ / amino acid- medium [6]. We did not pursue this assay for most experiments since it is logistically difficult to perform this assay when working simultaneously with more than a dozen strains. However, we used this assay for the Western blotting and RT-qPCR measurements on a few strains (Figs. S1, S14 and 4B).

The second assay involves starting with a low initial concentration of an amino acid and letting the bacterial cultures exhaust the amino acid in the medium through exponential growth [22]. The bacteria then enter the amino acid limited regime in mid-log phase without any intervention from the experimenter. However in the absence of exogenous sources of amino acid in the amino acid limited regime, protein synthesis occurs only transiently for less than an hour under these conditions and YFP synthesis rates from all *yfp* variants drop below measurable levels at the end of this time period (Fig. S15). More importantly, there is no extended steady state during which differential protein synthesis rates can be measured accurately. Nevertheless, we have confirmed that the measurements with and without methyl esters give qualitatively similar results (Fig. S16). In addition, Western blotting done in the absence of methyl ester reproduced the hierarchy in protein levels between synonymous variants of YFP during amino acid limitation (Fig. S1).

In contrast to the assay without methyl ester, presence of methyl ester analogs in the growth medium results in a quasi-steady state of amino acid limited growth due to hydrolysis of the ester, during which differential YFP expression can be measured easily (Fig. S15). Such partial amino acid limited growth is also likely to be the relevant scenario when prototrophic strains run out of amino acids in their growth media and have a limited supply of amino acids through protein degradation or partially up-regulated biosynthesis pathways.

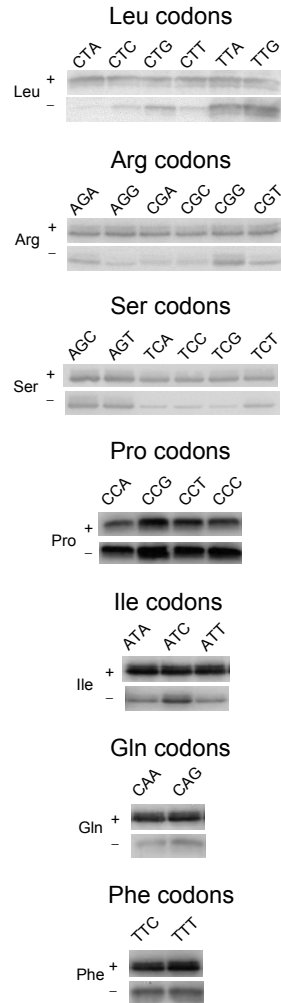
### Effect of varying the initial concentrations of amino acids and methyl esters.

Increasing the initial concentration of the amino acid or its methyl ester results in a higher cell density for the onset of amino acid limitation, and when the corresponding concentrations are decreased, this onset happens at a lower cell density. Importantly, the observed differential robustness of protein synthesis (such as the data shown in Fig. 1D) is qualitatively the same upon 2-fold changes to the initial concentration of either the amino acid or its methyl ester. As an extreme example, see Figs. S15 and S16 for comparison

between the cases with and without methyl ester analog in the growth medium.

### Supplementary references

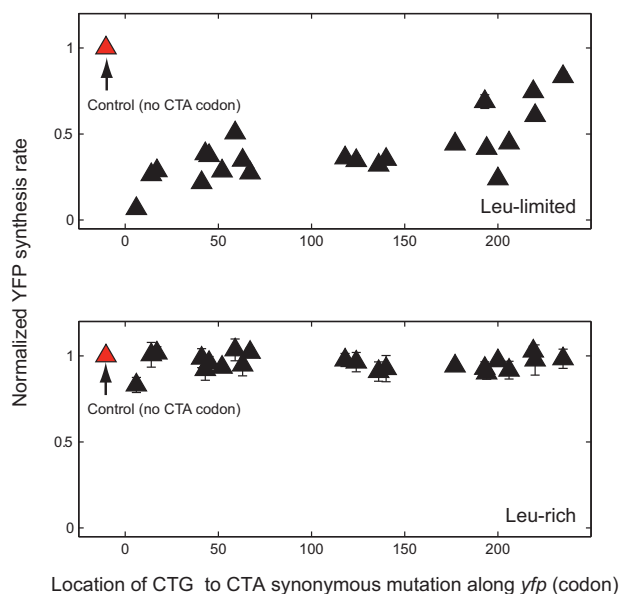
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**Fig. S1: Expression level of yfp variants quantified through Western blotting**

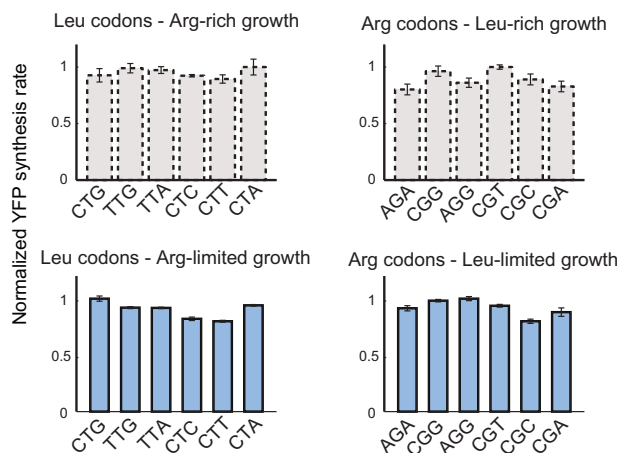
Modified versions of 29 *yfp* variants (Fig. 1A) were created that had a 3X-FLAG tag at the 5' end. These *yfp* variants were transformed into the respective *E. coli* auxotrophs in which YFP synthesis was repressed by the TetR protein. Cells were harvested at an  $OD_{600}$  of 0.4 and re-suspended in medium with or without the corresponding amino acid. Expression of YFP was induced using 200ng/ml anhydrotetracycline, and cells were harvested after 60 min. For each set of *yfp* variants under a specific growth condition, the same amount of total protein (as measured by  $OD_{600}$  before cell lysis) was used for Western blotting.





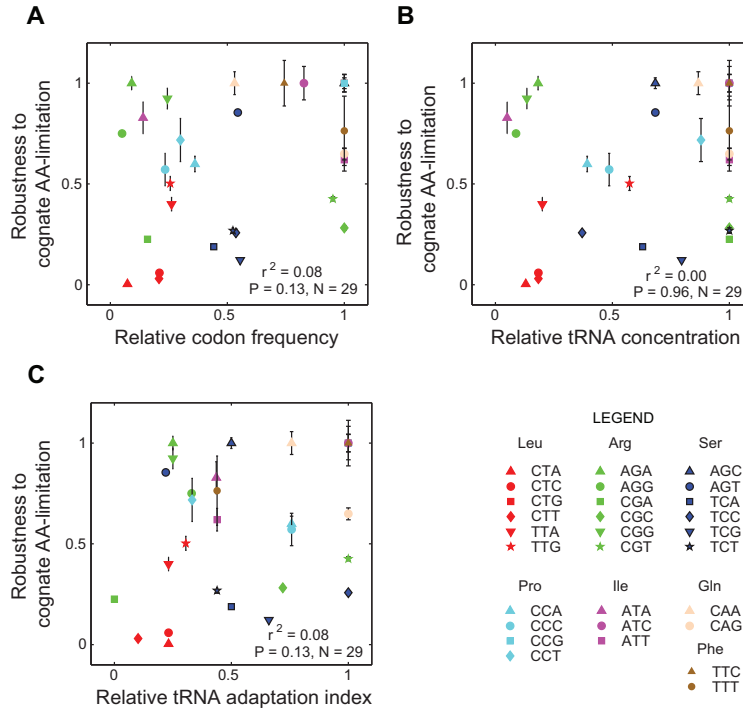
**Fig. S2: Effect of a single synonymous mutation on YFP synthesis rate**

22 variants of *yfp* were synthesized, each of which had a single CTA codon at one of the 22 leucine codon locations along *yfp*. The remaining leucine codons in each variant were the perturbation-robust CTG codon. The ‘control’ *yfp* variant did not have any CTA codon. Vertical axis refers to the YFP synthesis rate from the 22 variants normalized by that of the control variant, either during leucine limitation (top panel) or during leucine-rich growth (bottom panel). Horizontal axis indicates the location of the CTA codon along each *yfp* variant (ATG start codon = 1). Error bars show standard error over three replicate cultures.



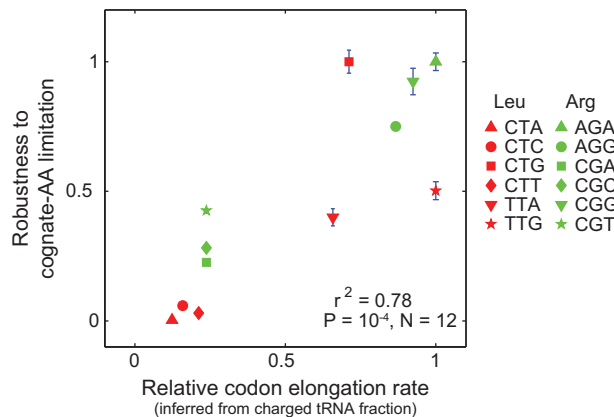
**Fig. S3: YFP synthesis rate during limitation for a non-cognate amino acid**

Leucine and arginine variants of *yfp* were expressed in an *E. coli* strain, CP78, that is auxotrophic for both leucine and arginine. Response of the 6 Leu variants to Arg limitation is determined by the Arg codons in *yfp* (CGT and CGC) that are common across all 6 Leu variants. Reciprocally, the response of the 6 Arg variants to Leu limitation is determined by the Leu codon that is common to the Arg variants of *yfp* (CTG). YFP synthesis rates are defined as in Fig. 1D. Error bars show standard error over three replicate cultures.



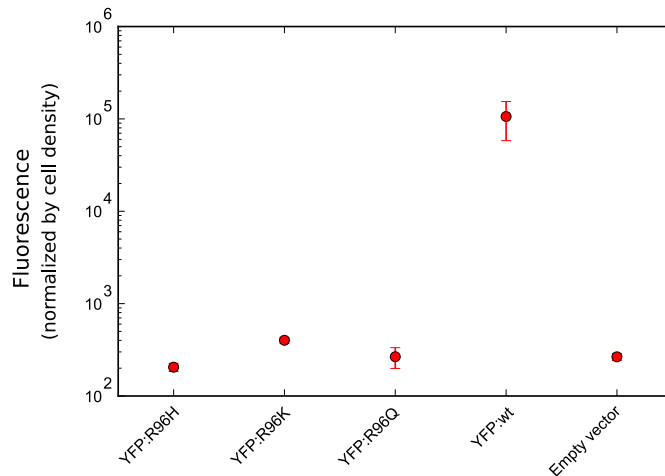
**Fig. S4: Comparison of synthesis rate robustness with codon usage and tRNA concentration**

(A) Codon usage was calculated as the average frequency of each codon across all protein coding sequences in *E. coli*. (B) tRNA concentration for each codon was calculated as the sum of tRNA concentrations for all cognate tRNAs [15]. (C) Since tRNAs can differ substantially in their affinity for their cognate codons, we also compared the measured robustness against the tRNA adaptation index for each codon [20]. This index accounts for different affinities of synonymous codons for the same tRNA isoacceptor. All three measures along the horizontal axes were normalized by the maximum value within each codon family. Robustness to amino acid limitation was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases. Error bars represent standard error over three replicate cultures. The data points that are not visible for a few codons overlap at the top right-hand corner of each plot.



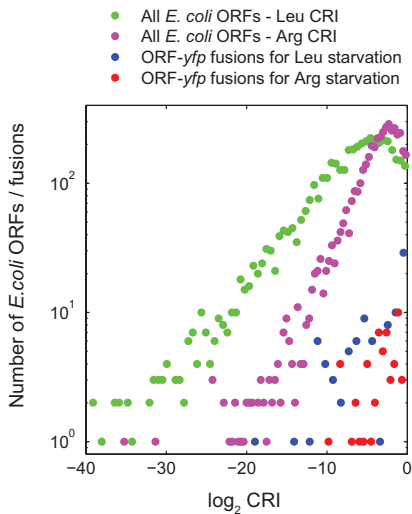
**Fig. S5: Comparison of synthesis rate robustness with charged tRNA fraction**

To compare YFP synthesis rates with charged tRNA fractions, the elongation rates for leucine and arginine codons were inferred from the measured charged fraction of leucine and arginine tRNA isoacceptors [6] (see section on codon specific bioinformatic measures). Previously assigned codon-tRNA assignments and kinetic parameters were used [18]. Note that charged tRNA fractions cannot be directly compared with synthesis rates of *yfp* variants due to overlapping and multiple codon assignments for several tRNA isoacceptors. Robustness to amino acid limitation was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases. Error bars represent standard error over three replicate cultures. Relative codon elongation rate is the ratio of codon elongation rates between amino acid starved and amino acid rich growth regimes, normalized to the maximum value within each synonymous codon family.



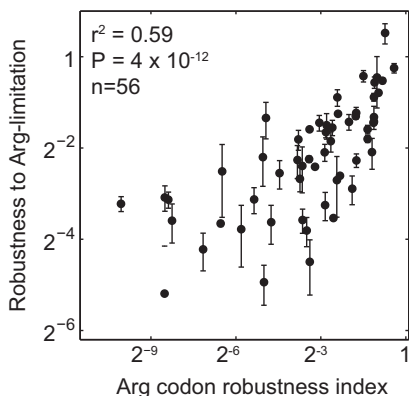
**Fig. S6: Miscoding of a single arginine residue in YFP causes loss of fluorescence**

To test whether mistranslation of arginine residues can underlie the high residual fluorescence of Arg *yfp* variant-Arg tRNA pairs (AGA: arg3, AGG: arg3, and CGG:arg4, arg5 in Fig. 2B), three YFP mutants were created that had one of three single point mutations at Arg96: R96H, R96K, and R96Q. The mutant and the ‘wild-type’ YFP proteins were expressed from a *pUC18* high-copy vector. Each of the three mutations at Arg96 to a chemically similar amino acid (H, K or Q) decreased YFP fluorescence to background level (that of an empty *pUC18* vector). Error bars denote standard deviation over five biological replicates.



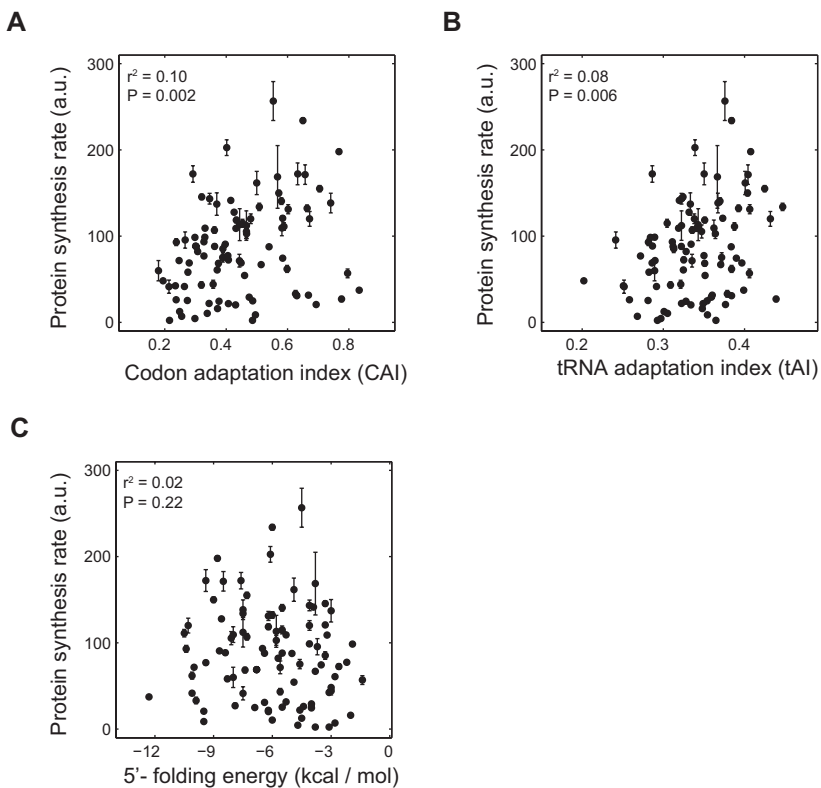
**Fig. S7: Histogram of CRI**

Green and purple data markers correspond to the Leu and Arg CRI values for 4300 ORFs in *E. coli*'s genome. Blue and red data markers correspond respectively to Leu and Arg CRI values for the *E. coli* ORF-*yfp* fusions that were used to experimentally validate CRI.



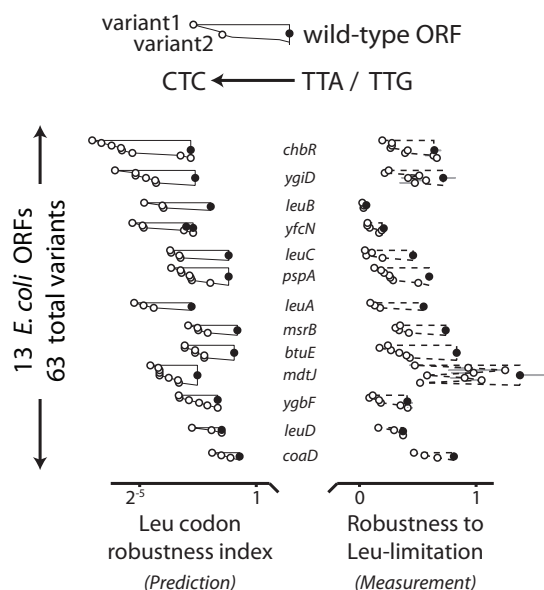
**Fig. S8: Correlation of Arg CRI with measured robustness of 56 *E. coli* ORF-yfp fusions**

The *yfp* sequence used for this experiment had the AGA codon at all Arg codon locations of *yfp* since AGA has the highest  $w_i$  value among arginine codons (see Fig. 3B). Correlation is reported as squared Spearman rank correlation. Error bars show standard error over three replicate cultures. Robustness to amino acid limitation was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases.



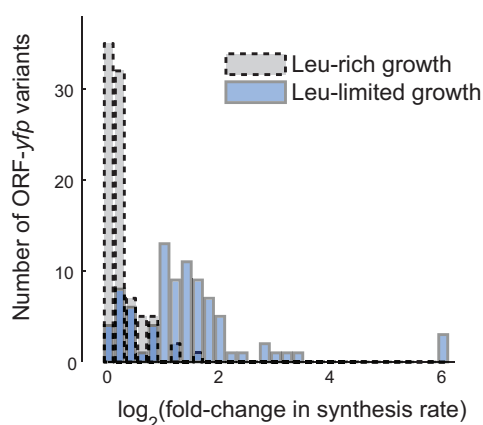
**Fig. S9: Correlation of protein synthesis rate during amino acid rich growth with measures of translation efficiency**

Protein synthesis rates from 92 *E. coli* ORF-*yfp* fusions during Leu-rich growth showed only a weak correlation with measures of codon adaptation, tRNA adaptation and 5' folding energy of mRNA. Folding energy was calculated from -5 to +37 nt of the ATG codon. Codon adaptation index (CAI) and tRNA adaptation index (tAI) were calculated using Biopython and codonR packages. Correlations are reported as squared Spearman rank-correlation coefficient.



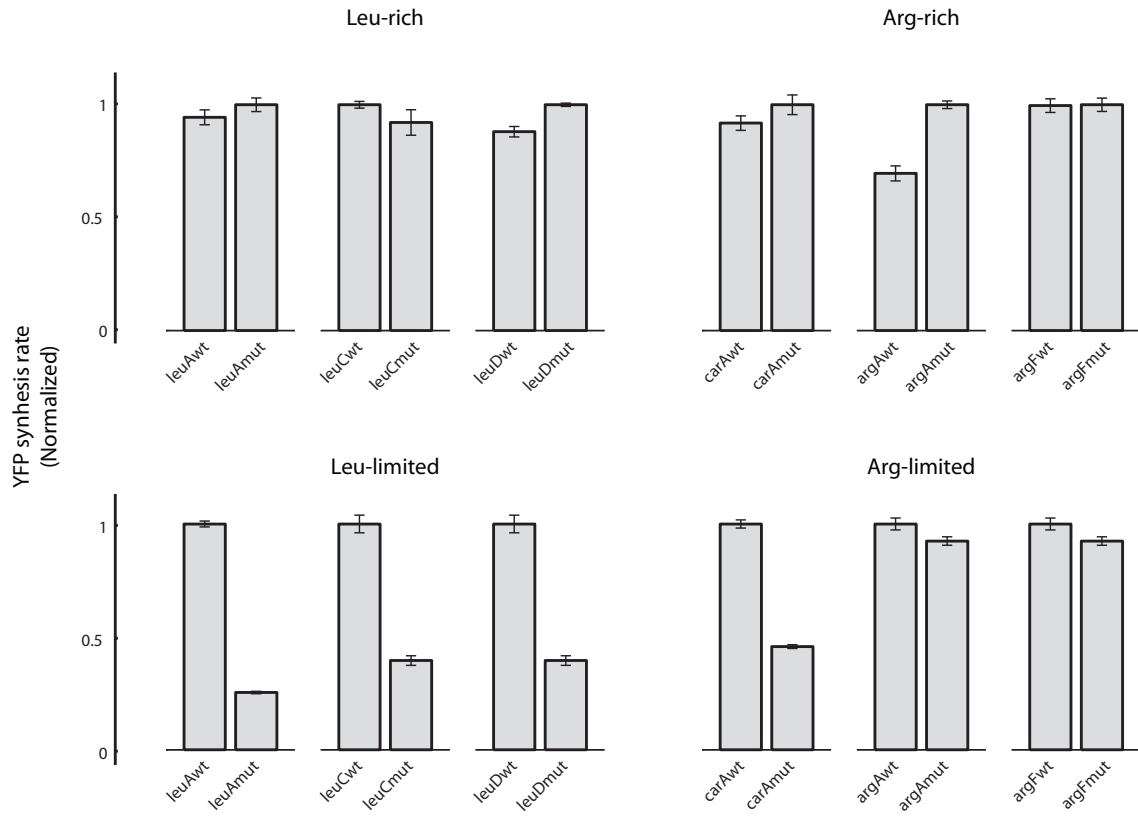
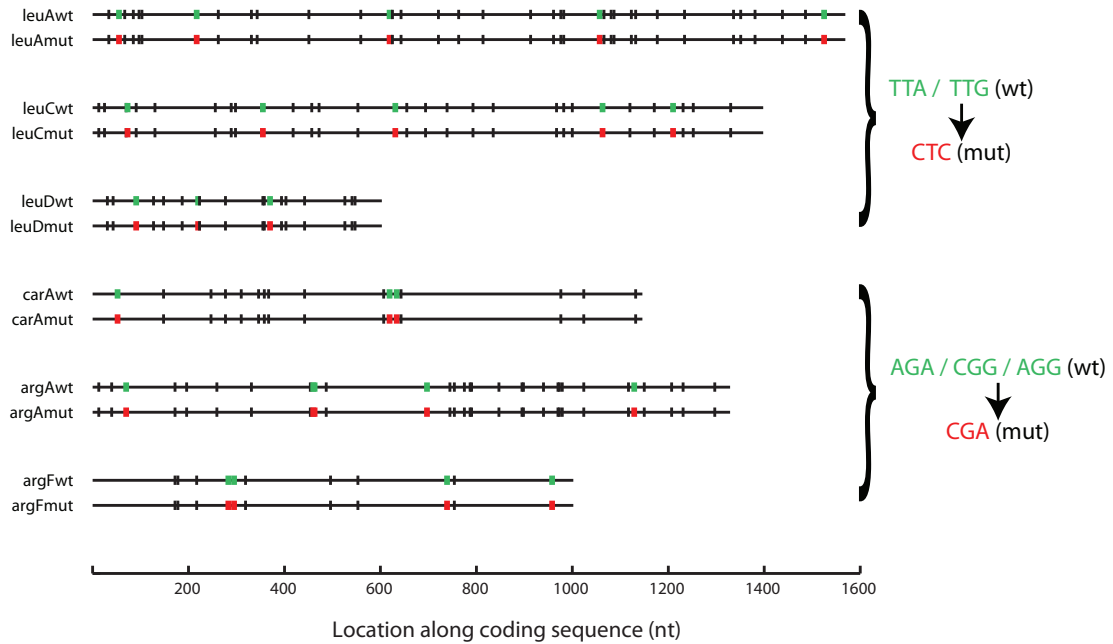
**Fig. S10: CRI predicts the change in robustness during amino acid limitation due to synonymous mutations**

Sixty three synonymous variants of 13 ORF-*yfp* fusions were constructed by mutating wild-type TTA or TTA codons in the ORF sequence to the codon CTC that causes sensitive protein synthesis rate under leucine limitation. The number of mutations was between 1 and 6 and the location of these mutations was random. 59 of the 63 variants displayed a decrease in their robustness during Leu limitation (dashed lines) that was predicted by Leu CRI (solid lines). In addition, magnitude of the changes in robustness during Leu limitation were positively correlated with magnitude of the changes in Leu CRI ( $r^2 = 0.19$ ,  $P = 10^{-4}$ ). Filled circles indicate values for ORF<sub>wild-type</sub> and open circles indicate values for ORF<sub>variant</sub>. Different open circles within a single polygon correspond to distinct ORF variants for the same wild-type ORF. Robustness to amino acid limitation was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases. Error bars show standard error over three replicate cultures. Most error bars are smaller than data markers. DNA sequences for variants are provided in *gene\_sequences.fasta* supplementary file.



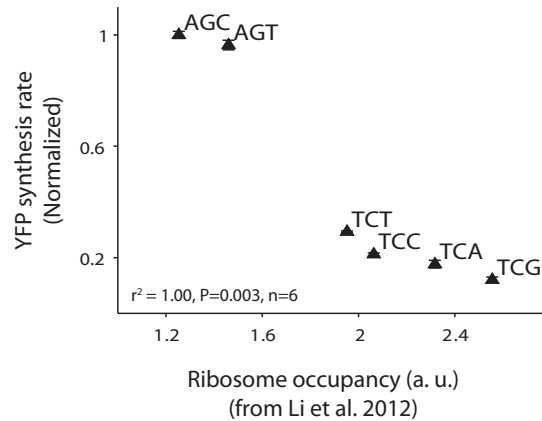
**Fig. S11: Effect of synonymous mutations and tRNA co-expression on synthesis rate from E. coli ORF-yfp fusions**

We analyzed the change in protein synthesis rates from the 21 ORF-*yfp* fusions co-expressed with <sup>GAG</sup>Leu2 (Fig. 3D) and the 63 different ORF-*yfp* variants with synonymous mutations (Fig. S10). Several of the <sup>GAG</sup>Leu2-coexpressed as well as the synonymously-mutated ORF-*yfp* variants (84 total variants) had significantly altered protein synthesis rates compared to their non-tRNA co-expressed or non-mutated counterparts (referred as wild type) during leucine limited growth (green histogram, median fold-change in protein synthesis rates = 2.37). By comparison, most of the 84 variants had similar protein synthesis rates to their wild-type counterparts during leucine rich growth (grey histogram, median fold-change in protein synthesis rates = 1.12). Protein synthesis rates were defined as in Fig. 1D.

**A****B**

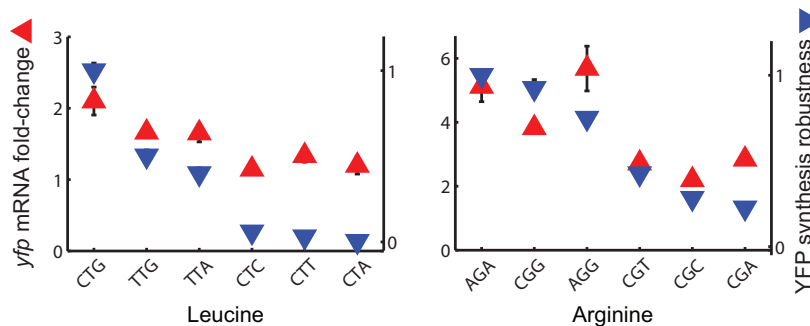
**Fig. S12: Effect of synonymous mutations on synthesis rate from amino acid biosynthesis genes**

(A) Synthesis rates from *leuA*, *leuC*, *leuD* and *carA*, *argA*, *argF* -*yfp* fusions encoding either wild-type or mutant ORF sequences during amino acid rich and amino acid limited growth. The synthesis rates were normalized for each pair of wild-type and mutant ORF-*yfp* fusions, and also for each growth condition. Error bars show standard error over six replicate cultures. (B) Position and identity of synonymous mutations in wild-type and mutant sequences used for the experiment in (A). The black vertical bars correspond to the non-mutated leucine codons in the case of *leuA*, *leuC* and *leuD*, and to the non-mutated arginine codons in the case of *carA*, *argA* and *argF*.



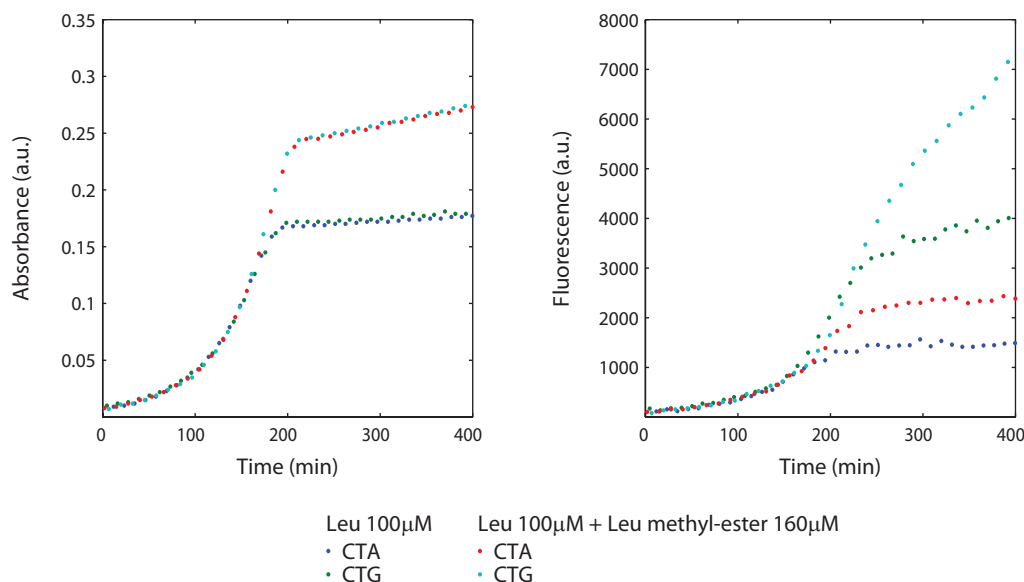
**Fig. S13: Correlation of protein synthesis rate with ribosome occupancy at serine codons during serine-limited growth**

Protein synthesis rate of serine synonymous variants of *yfp* during serine limitation (same data as in Fig. 1D, third panel) is negatively correlated with genome-wide ribosome occupancy at serine codons during serine-limited growth of *E. coli*. The increased occupancy at perturbation-sensitive serine codons is consistent with selective ribosome pausing at these codons. Ribosome occupancy data was taken from a recent ribosome profiling experiment in *E. coli* [23].



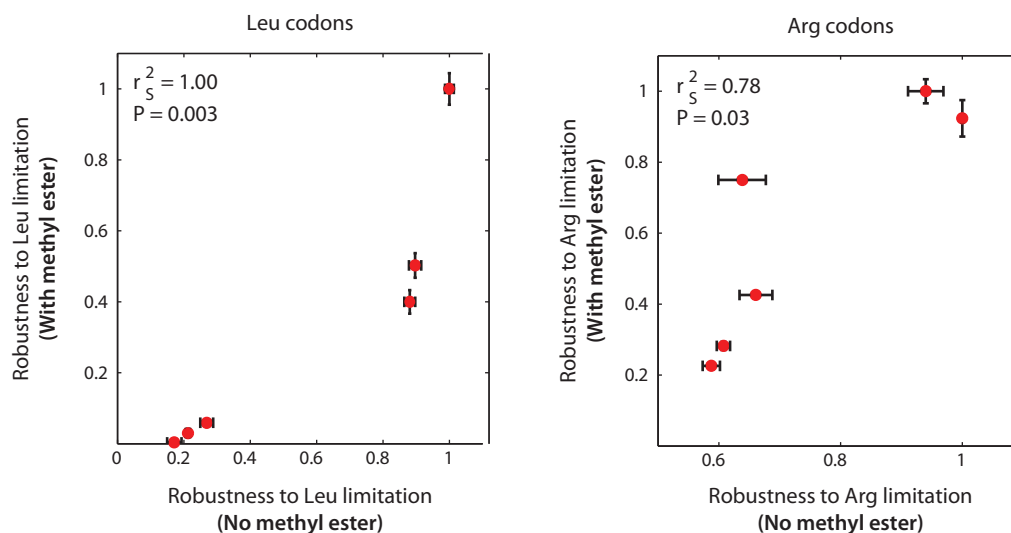
**Fig. S14: Change in mRNA level of *yfp* variants in response to cognate amino acid limitation**

We measured the change in mRNA levels of different *yfp* variants in response to amino acid limitation. Total RNA was extracted either during exponential amino acid rich growth or 60 min after amino acid limited growth in the presence of the amino acid methyl ester. mRNA levels were quantified by RT-qPCR relative to *gapA* mRNA. Error bars show standard error of triplicate qPCR measurements. Synthesis rate robustness to amino acid limitation was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases.



**Fig. S15: Raw absorbance and fluorescence curves with or without methyl ester analog of amino acids**

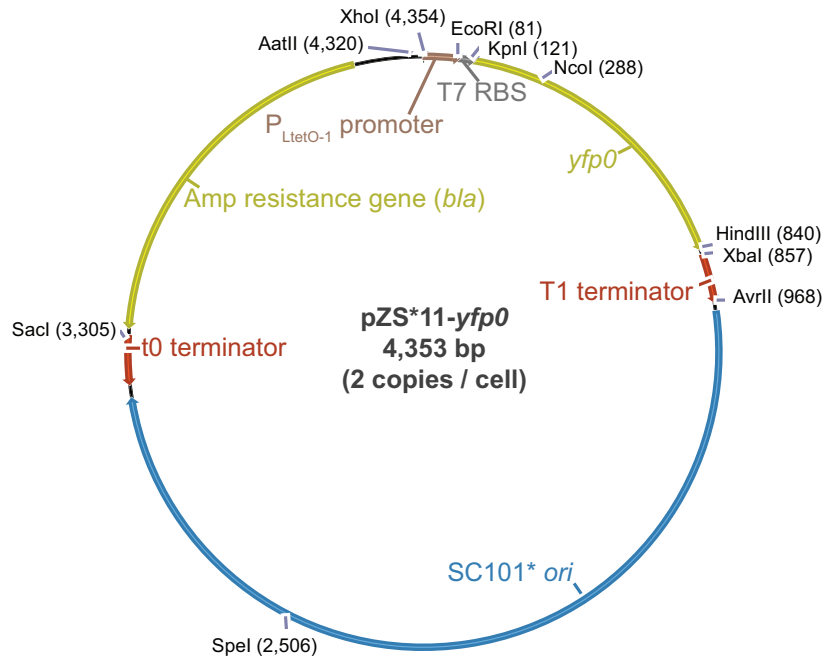
Growth and fluorescence curves for two *yfp* variants corresponding to CTA and CTG codons are shown here as representative examples for amino acid limited growth in the presence or absence of methyl ester analogs in the growth medium. Absorbance as measured using spectrometry is proportional to cell density. Presence of methyl ester analogs caused an increase in the time and cell density at which amino acid limited growth began. More importantly, inefficient metabolism of methyl ester analogs resulted in a slow but steady growth in amino acid limited regime. This residual growth ensured that YFP synthesis continued robustly from the CTG *yfp* variant under these conditions. By contrast, in the absence of methyl esters in the growth medium, YFP synthesis from all *yfp* variants eventually dropped to zero.



**Fig. S16: Synthesis rate robustness with or without methyl ester analog of amino acids**

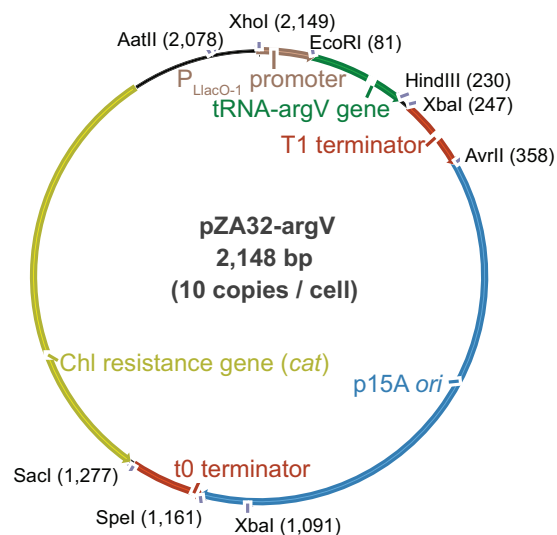
Robustness to amino acid limitation in the absence of methyl ester analogs was calculated as the ratio of fluorescence change between the amino acid limited growth phase and amino acid rich growth phase. This ratio was further normalized by the maximum value within each codon family. Robustness to amino acid limitation in the presence of methyl ester analogs was quantified as the ratio of normalized YFP synthesis rates between amino acid limited and amino acid rich growth phases. Error bars show standard error over three replicate cultures.





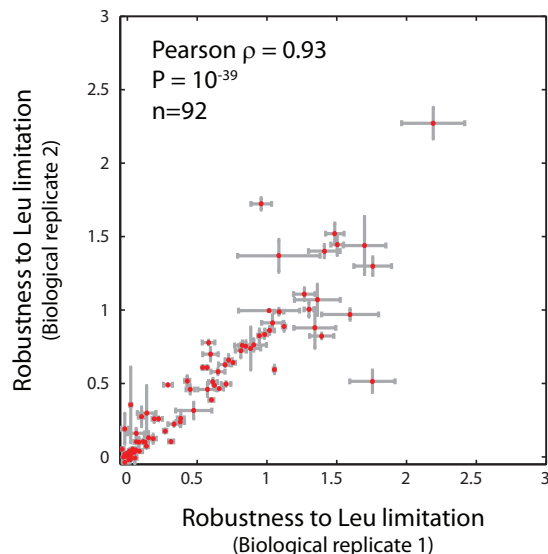
**Fig. S17: Plasmid map of expression vector for *yfp* and *E. coli* ORF-*yfp* fusions**

A specific plasmid construct with *yfp0* is shown here. In the case of ORF-*yfp* fusions, *yfp* was fused in-frame to the 3'-end of the ORF with a GGSGGS hexa-peptide linker sequence that encoded a BamHI restriction site and the resulting coding sequence of the fusion protein was cloned between the KpnI and HindIII restriction sites in the above vector.



**Fig. S18: Plasmid map of expression vector for tRNA genes**

A specific construct encoding an Arg tRNA is shown here.



**Fig. S19: Reproducibility of measurements between biological replicates of 92 *E. coli* ORF-yfp fusions**

Two different colonies were picked after cloning the 92 *E. coli* ORF-yfp fusions and the same leucine limitation assay that was used for the data in Fig. 3C was performed on these two biological replicates on two different days. None of the clones for replicate 2 were sequence-verified and hence the few outliers seen above could be the result of errors in the cloned sequences. The data reported in Fig. 3C is from replicate 1 for which about 40 constructs were sequence-verified. Robustness to Leu limitation was calculated as the ratio of normalized YFP synthesis rates between Leu limited and Leu rich growth phases.

**Table S1: 92 *E. coli* ORF-yfp fusions used for Leu CRI validation**

Genes are arranged by increasing values of Leu CRI.  $S_{Leu-rich}$  and  $S_{Leu-limited}$  refer to respective protein synthesis rates (a.u. per sec per cell). Robustness refers to the ratio between the two protein synthesis rates after normalization by the corresponding value for the CTG variant of *yfp* (which is the *yfp* tag in these ORF-yfp fusions).  $\pm$  refers to standard error of measurement.

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
1	polB	$10.4 \pm 0.3$	$0.6 \pm 0.6$	$0.063 \pm 0.062$	-19.45	DNA polymerase II
2	thiH	$69.7 \pm 1.1$	$-0.7 \pm 0.8$	$-0.012 \pm 0.013$	-14.52	tyrosine lyase, involved in thiamin-thiazolemoiety synthesis
3	aat	$26.7 \pm 0.8$	$1.3 \pm 0.3$	$0.054 \pm 0.015$	-12.52	leucyl/phenylalanyl-tRNA-protein transferase
4	gdhA	$20.4 \pm 0.3$	$-0.2 \pm 0.2$	$-0.013 \pm 0.009$	-11.62	glutamate dehydrogenase, NADP-specific
5	serC	$91.3 \pm 0.5$	$-1.7 \pm 0.6$	$-0.020 \pm 0.008$	-11.6	3-phosphoserine/phosphohydroxy threonine aminotransferase
6	gpsA	$111.8 \pm 3.3$	$1.9 \pm 1.9$	$0.020 \pm 0.018$	-11.38	glycerol-3-phosphate dehydrogenase (NAD <sup>+</sup> )
7	mlrA	$44.1 \pm 1.5$	$-0.7 \pm 0.8$	$-0.017 \pm 0.019$	-11.09	DNA-binding transcriptional regulator
8	ybeU	$41.5 \pm 7.6$	$0.5 \pm 0.3$	$0.017 \pm 0.013$	-11	conserved protein, DUF1266 family
9	argS	$67.6 \pm 1.9$	$1.0 \pm 0.6$	$0.017 \pm 0.010$	-10.93	arginyl-tRNA synthetase
10	ilvD	$59.2 \pm 5.6$	$3.4 \pm 0.9$	$0.064 \pm 0.017$	-10.39	dihydroxyacid dehydratase
11	ugpC	$88.5 \pm 2.7$	$1.9 \pm 0.9$	$0.023 \pm 0.011$	-10.33	glycerol-3-phosphate transporter subunit
12	argI	$44.5 \pm 4.5$	$5.3 \pm 2.6$	$0.138 \pm 0.067$	-10.24	ornithine carbamoyltransferase 1
13	ttdA	$86.1 \pm 0.2$	$-0.0 \pm 0.5$	$-0.000 \pm 0.006$	-10.06	L-tartrate dehydratase, alpha subunit
14	leuS	$143.4 \pm 3.7$	$-2.5 \pm 0.5$	$-0.019 \pm 0.003$	-9.46	leucyl-tRNA synthetase
15	hisB	$119.8 \pm 3.7$	$-0.3 \pm 3.0$	$-0.002 \pm 0.027$	-9.31	fusedhistidinol-phosphatase/imidazoleglycerol-phosphatedehydratase

**Table S1 (contd.): 92 E. coli ORF-yfp fusions used for Leu CRI validation**

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
16	rpoA	71.8 ± 7.5	0.0 ± 0.6	-0.001 ± 0.009	-9.05	RNA polymerase, alpha subunit
17	uvrY	173.2 ± 8.8	-5.2 ± 9.3	-0.027 ± 0.061	-8.36	DNA-binding response regulator in two-component regulatory system with BarA
18	rob	24.9 ± 0.7	0.5 ± 0.2	0.024 ± 0.007	-7.94	right oriC-binding transcriptional activator, AraC family
19	agaS	73.0 ± 3.3	1.3 ± 0.4	0.019 ± 0.005	-7.48	tagatose-6-phosphate ketose/aldehyde isomerase
20	lysS	111.8 ± 4.4	3.7 ± 2.1	0.035 ± 0.020	-7.17	lysine tRNA synthetase, constitutive
21	sdaB	121.0 ± 5.7	36.4 ± 4.1	0.333 ± 0.044	-7.14	L-serine deaminase II
22	purA	35.5 ± 2.2	-1.2 ± 0.4	-0.039 ± 0.015	-7.12	adenylosuccinate synthetase
23	rpoD	77.5 ± 2.2	2.3 ± 1.1	0.032 ± 0.014	-6.99	RNA polymerase, sigma 70 (sigma D) factor
24	melR	44.5 ± 3.3	15.1 ± 0.4	0.378 ± 0.031	-6.76	DNA-binding transcriptional dual regulator
25	kefF	99.2 ± 3.0	3.3 ± 0.5	0.038 ± 0.007	-6.52	flavoprotein subunit for the KefC potassium efflux system
26	uhpA	22.4 ± 1.5	0.4 ± 0.7	0.019 ± 0.034	-6.5	DNA-binding response regulator in two-component regulatory system with UhpB
27	ydcN	41.6 ± 2.7	1.9 ± 0.7	0.050 ± 0.015	-6.5	predicted DNA-binding transcriptional regulator
28	aspS	62.3 ± 4.2	10.9 ± 1.5	0.191 ± 0.014	-6.42	aspartyl-tRNA synthetase
29	relB	87.9 ± 2.3	0.8 ± 0.9	0.009 ± 0.010	-6.09	Qin prophage; bifunctional antitoxin of the RelE-RelB toxin-antitoxin system/ transcriptional repressor
30	guaA	122.6 ± 2.3	6.9 ± 2.2	0.061 ± 0.019	-5.81	GMP synthetase (glutamine aminotransferase)
31	phnM	203.7 ± 9.5	20.9 ± 1.2	0.113 ± 0.007	-5.81	carbon-phosphorus lyase complex subunit
32	tdcD	4.5 ± 0.4	7.1 ± 0.2	1.755 ± 0.163	-5.76	propionate kinase/acetate kinase C, anaerobic
33	ompR	114.2 ± 18.7	-1.4 ± 2.2	-0.019 ± 0.027	-5.74	DNA-binding response regulator in two-component regulatory system with EnvZ
34	phnL	75.7 ± 5.3	5.7 ± 1.1	0.082 ± 0.013	-5.51	carbon-phosphorus lyase complex subunit
35	purH	29.6 ± 1.4	10.2 ± 0.4	0.383 ± 0.025	-5.27	fused IMPcyclohydrolase / phosphoribosyl aminoimidazole carboxamide formyltransferase
36	argG	2.5 ± 0.2	3.0 ± 0.2	1.343 ± 0.150	-5.07	argininosuccinate synthetase
37	rimM	107.5 ± 3.7	13.3 ± 0.9	0.137 ± 0.014	-4.95	16S rRNA processing protein
38	ubiC	93.6 ± 3.9	7.5 ± 2.2	0.087 ± 0.022	-4.87	chorismate-pyruvate lyase
39	leuL	48.9 ± 2.3	0.7 ± 0.5	0.017 ± 0.011	-4.75	leu operon leader peptide
40	asnS	9.0 ± 0.4	7.2 ± 0.5	0.884 ± 0.071	-4.65	asparaginyl tRNA synthetase
41	ribB	107.6 ± 7.8	12.2 ± 1.0	0.127 ± 0.020	-4.49	3,4-dihydroxy-2-butanone-4-phosphate synthase
42	smpB	15.8 ± 0.8	1.5 ± 0.4	0.101 ± 0.023	-4.39	trans-translation protein
43	guaB	31.0 ± 0.3	5.2 ± 0.6	0.184 ± 0.022	-4.21	IMP dehydrogenase
44	proC	69.2 ± 3.1	9.4 ± 1.6	0.151 ± 0.030	-3.92	pyrroline-5-carboxylate reductase, NAD(P)-binding
45	pth	89.0 ± 2.6	25.2 ± 0.9	0.313 ± 0.018	-3.1	peptidyl-tRNA hydrolase
46	ivbL	1.8 ± 0.3	1.6 ± 0.1	1.085 ± 0.295	-2.89	ilvB operon leader peptide
47	chbR	25.6 ± 1.0	24.4 ± 0.8	1.054 ± 0.008	-2.81	rRepressor, chb operon for N,N'-diacetylchitobiose utilization
48	leuA	78.1 ± 2.2	53.6 ± 0.8	0.756 ± 0.019	-2.77	2-isopropylmalate synthase
49	argD	85.7 ± 4.4	51.0 ± 1.8	0.657 ± 0.031	-2.72	bifunctional acetylornithine aminotransferase/succinyl-diaminopimelate aminotransferase
50	yfcN	98.8 ± 2.1	24.2 ± 1.5	0.270 ± 0.011	-2.71	conserved protein

**Table S1 (contd.): 92 E. coli ORF-yfp fusions used for Leu CRI validation**

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
51	ygiD	$7.3 \pm 0.3$	$9.9 \pm 0.4$	$1.503 \pm 0.046$	-2.62	predicted dioxygenase, LigB family
52	mdtJ	$12.5 \pm 1.6$	$17.5 \pm 0.2$	$1.595 \pm 0.204$	-2.52	multidrug efflux system transporter
53	agaR	$80.6 \pm 2.5$	$31.4 \pm 2.0$	$0.428 \pm 0.018$	-2.39	DNA-binding transcriptional repressor of the aga regulon
54	hisA	$22.0 \pm 0.9$	$27.6 \pm 0.9$	$1.392 \pm 0.087$	-1.86	N-(5'-phospho-L-ribosylformimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarb oxamide isomerase
55	ygbF	$72.7 \pm 21.5$	$26.4 \pm 1.3$	$0.475 \pm 0.130$	-1.65	probable ssRNA endonuclease, CRISP-associated protein
56	rpoH	$89.2 \pm 1.8$	$50.5 \pm 0.9$	$0.623 \pm 0.005$	-1.53	RNA polymerase, sigma 32 (sigma H) factor
57	leuD	$128.4 \pm 2.7$	$70.0 \pm 0.6$	$0.601 \pm 0.018$	-1.48	3-isopropylmalate dehydratase small subunit
58	dinJ	$58.9 \pm 2.8$	$34.4 \pm 1.5$	$0.648 \pm 0.050$	-1.48	antitoxin of YafQ-DinJ toxin-antitoxin system
59	nuoI	$25.2 \pm 1.8$	$30.6 \pm 1.4$	$1.361 \pm 0.164$	-1.48	NADH:ubiquinone oxidoreductase, chain I
60	luxS	$109.5 \pm 0.7$	$70.3 \pm 3.9$	$0.706 \pm 0.035$	-1.34	S-ribosylhomocysteine lyase
61	leuC	$104.5 \pm 6.0$	$54.0 \pm 3.4$	$0.573 \pm 0.069$	-1.18	3-isopropylmalate dehydratase large subunit
62	pspA	$69.8 \pm 2.6$	$51.4 \pm 0.6$	$0.813 \pm 0.039$	-1.18	regulatory protein for phage-shock-protein operon
63	pyrI	$138.9 \pm 13.7$	$112.5 \pm 6.2$	$0.906 \pm 0.091$	-1.15	aspartate carbamoyltransferase, regulatory subunit
64	btuE	$149.1 \pm 4.6$	$132.9 \pm 3.6$	$0.982 \pm 0.020$	-0.95	glutathione peroxidase
65	msrB	$153.6 \pm 14.7$	$131.7 \pm 2.8$	$0.958 \pm 0.074$	-0.8	methionine sulfoxide reductase B
66	coaD	$94.4 \pm 0.3$	$96.2 \pm 0.8$	$1.122 \pm 0.012$	-0.71	pantetheine-phosphate adenylyltransferase
67	sfsB	$72.2 \pm 2.4$	$18.9 \pm 1.5$	$0.291 \pm 0.032$	-0.61	DNA-binding transcriptional activator of maltose metabolism
68	nirD	$113.2 \pm 17.8$	$22.1 \pm 1.5$	$0.223 \pm 0.027$	-0.52	nitrite reductase, NAD(P)H-binding, small subunit
69	fdnI	$62.4 \pm 1.6$	$57.8 \pm 3.4$	$1.019 \pm 0.034$	-0.43	formate dehydrogenase-N, cytochrome B556 (gamma) subunit, nitrate-inducible
70	greA	$172.5 \pm 12.4$	$90.0 \pm 1.2$	$0.581 \pm 0.045$	-0.38	transcript cleavage factor
71	hupB	$287.5 \pm 19.6$	$116.5 \pm 4.0$	$0.450 \pm 0.030$	-0.33	HU, DNA-binding transcriptional regulator, beta subunit
72	glpE	$144.2 \pm 2.0$	$142.0 \pm 2.6$	$1.086 \pm 0.035$	-0.33	thiosulfate: cyanide sulfurtransferase (rhodanese)
73	ogrK	$95.8 \pm 9.5$	$145.2 \pm 3.3$	$1.699 \pm 0.153$	-0.33	positive regulator of P2 growth (insertion of P2ogr gene into the chromosome)
74	rplD	$20.9 \pm 1.5$	$41.0 \pm 1.6$	$2.191 \pm 0.226$	-0.33	50S ribosomal subunit protein L4
75	dmsB	$116.2 \pm 5.3$	$156.3 \pm 5.1$	$1.486 \pm 0.067$	-0.28	dimethyl sulfoxide reductase, anaerobic, subunit B
76	rpsP	$241.7 \pm 3.7$	$118.5 \pm 0.3$	$0.540 \pm 0.007$	-0.19	30S ribosomal subunit protein S16
77	rplX	$150.6 \pm 3.2$	$83.7 \pm 3.5$	$0.613 \pm 0.031$	-0.19	50S ribosomal subunit protein L24
78	tpiA	$138.5 \pm 11.4$	$87.1 \pm 2.2$	$0.700 \pm 0.047$	-0.19	triosephosphate isomerase
79	gapA	$39.5 \pm 2.0$	$62.6 \pm 1.6$	$1.757 \pm 0.135$	-0.19	glyceraldehyde-3-phosphate dehydrogenase A
80	rpsT	$174.7 \pm 12.0$	$133.7 \pm 4.5$	$0.849 \pm 0.050$	-0.14	30S ribosomal subunit protein S20

**Table S1 (contd.): 92 E. coli ORF-yfp fusions used for Leu CRI validation**

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
81	rpsJ	170.2 ± 37.2	142.3 ± 1.6	1.015 ± 0.219	-0.14	30S ribosomal subunit protein S10
82	rplT	120.5 ± 8.5	112.6 ± 3.7	1.039 ± 0.077	-0.14	50S ribosomal subunit protein L20
83	ahpC	57.0 ± 5.0	64.9 ± 1.7	1.267 ± 0.078	-0.14	alkyl hydroperoxide reductase, C22 subunit
84	rpsK	109.9 ± 9.2	139.0 ± 3.5	1.411 ± 0.113	-0.14	30S ribosomal subunit protein S11
85	tsf	200.4 ± 2.8	103.8 ± 2.1	0.570 ± 0.008	0	protein chain elongation factor EF-Ts
86	rplU	173.3 ± 12.4	92.5 ± 4.9	0.595 ± 0.056	0	50S ribosomal subunit protein L21
87	rpmI	158.7 ± 4.0	104.2 ± 1.4	0.725 ± 0.025	0	50S ribosomal subunit protein L35
88	yjgF	131.9 ± 5.3	98.1 ± 3.0	0.824 ± 0.057	0	conserved protein, UPF0131 family
89	ppiB	133.6 ± 4.5	114.6 ± 2.7	0.945 ± 0.011	0	peptidyl-prolyl cis-trans isomerase B (rotamaseB)
90	yjbJ	134.6 ± 4.4	158.7 ± 1.1	1.300 ± 0.035	0	conserved protein, UPF0337 family
91	rpsI	27.5 ± 2.8	78.6 ± 6.0	3.198 ± 0.324	0	30S ribosomal subunit protein S9
92	rpsF	31.7 ± 1.6	97.1 ± 0.8	3.392 ± 0.183	0	30S ribosomal subunit protein S6

**Table S2: 56 E. coli ORF-yfp fusions used for Arg CRI validation**

Genes are arranged by increasing values of Arg CRI.  $S_{Arg-rich}$  and  $S_{Arg-limited}$  refer to respective protein synthesis rates (a.u. per sec per cell). Robustness refers to the ratio between the two protein synthesis rates after normalization by the corresponding value for the AGA variant of *yfp* (this AGA variant was also used as the *yfp* tag in these ORF-*yfp* fusions). ± refers to standard error of measurement.

Number	Gene	$S_{Arg-rich}$	$S_{Arg-limited}$	Robustness	$\log_2(\text{Arg CRI})$	Gene product
1	leuS	100.5 ± 2.6	16.1 ± 1.7	0.107 ± 0.012	-10.05	leucyl-tRNA synthetase
2	phoR	16.4 ± 3.2	0.7 ± 0.6	0.027 ± 0.029	-8.52	sensory histidine kinase in two-component regulatory system with PhoB
3	glnG	61.0 ± 3.4	10.8 ± 2.3	0.118 ± 0.022	-8.51	fused DNA-binding response regulator in two-component regulatory system with GlnL: response regulator/sigma54 interaction protein
4	asnS	15.5 ± 1.8	2.6 ± 0.1	0.114 ± 0.014	-8.38	asparaginyl tRNA synthetase
5	guaA	91.5 ± 4.2	11.7 ± 3.7	0.083 ± 0.024	-8.25	GMP synthetase (glutamine aminotransferase)
6	thiH	53.9 ± 1.1	4.4 ± 1.3	0.054 ± 0.015	-7.16	tyrosine lyase, involved in thiamin-thiazole moiety synthesis
7	fruR	44.7 ± 1.5	5.3 ± 0.1	0.079 ± 0.004	-6.54	DNA-binding transcriptional dual regulator
8	argG	10.0 ± 0.9	2.5 ± 1.1	0.175 ± 0.088	-6.49	argininosuccinate synthetase
9	gpsA	81.9 ± 3.9	9.1 ± 4.2	0.073 ± 0.032	-5.81	glycerol-3-phosphate dehydrogenase (NAD+)
10	rpoA	60.9 ± 1.6	10.5 ± 2.1	0.114 ± 0.022	-5.36	RNA polymerase, alpha subunit
11	yiiD	31.2 ± 1.8	9.8 ± 2.9	0.218 ± 0.078	-5.05	predicted acetyltransferase
12	agaR	53.2 ± 1.8	2.6 ± 0.8	0.032 ± 0.010	-5.01	DNA-binding transcriptional repressor of the agaregulon
13	rimK	17.9 ± 2.6	9.8 ± 1.5	0.393 ± 0.106	-4.94	ribosomal protein S6 modification protein

**Table S2 (contd.): 56 E. coli ORF-yfp fusions used for Arg CRI validation**

Number	Gene	$S_{Arg-rich}$	$S_{Arg-limited}$	Robustness	$\log_2(\text{Arg CRI})$	Gene product
14	rpoH	$75.2 \pm 2.8$	$9.3 \pm 2.9$	$0.081 \pm 0.023$	-4.76	RNA polymerase, sigma 32 (sigma H) factor
15	melR	$43.7 \pm 0.4$	$11.1 \pm 2.3$	$0.170 \pm 0.036$	-4.46	DNA-binding transcriptional dual regulator
16	tdcB	$32.1 \pm 2.0$	$10.2 \pm 2.9$	$0.208 \pm 0.051$	-3.83	catabolic threonine dehydratase, PLP-dependent
17	serC	$68.2 \pm 2.3$	$28.9 \pm 4.2$	$0.284 \pm 0.043$	-3.79	3-phosphoserine/phosphohydroxy threonine aminotransferase
18	rnc	$65.7 \pm 2.5$	$15.4 \pm 2.6$	$0.157 \pm 0.028$	-3.74	RNase III
19	chbR	$22.3 \pm 1.2$	$6.2 \pm 2.1$	$0.190 \pm 0.064$	-3.66	rRepressor, chb operon for N,N'-diacetylchitobiose utilization
20	rsuA	$100.3 \pm 5.9$	$12.8 \pm 2.8$	$0.084 \pm 0.015$	-3.65	16S rRNA U516 pseudouridine synthase
21	tauC	$70.1 \pm 3.2$	$7.4 \pm 1.3$	$0.071 \pm 0.016$	-3.5	taurine transporter subunit
22	smpB	$55.3 \pm 1.3$	$17.5 \pm 0.8$	$0.211 \pm 0.010$	-3.42	trans-translation protein
23	carA	$28.2 \pm 1.1$	$14.0 \pm 0.8$	$0.331 \pm 0.005$	-3.4	carbamoyl phosphate synthetase small subunit, glutamine amidotransferase
24	ubiC	$70.6 \pm 1.8$	$4.7 \pm 1.9$	$0.044 \pm 0.018$	-3.39	chorismate-pyruvate lyase
25	pth	$71.2 \pm 2.4$	$20.0 \pm 0.8$	$0.187 \pm 0.001$	-3.21	peptidyl-tRNA hydrolase
26	rpsF	$26.6 \pm 1.9$	$14.7 \pm 2.3$	$0.366 \pm 0.043$	-3.06	30S ribosomal subunit protein S6
27	gapA	$34.3 \pm 0.9$	$12.1 \pm 1.9$	$0.234 \pm 0.030$	-2.86	glyceraldehyde-3-phosphate dehydrogenase A
28	yihL	$43.2 \pm 3.4$	$6.7 \pm 1.1$	$0.105 \pm 0.022$	-2.85	predicted DNA-binding transcriptional regulator
29	allR	$36.0 \pm 1.5$	$17.1 \pm 0.8$	$0.318 \pm 0.026$	-2.82	DNA-binding transcriptional repressor for all(allantoin) and gcl (glyoxylate) operons; glyoxylate-induced
30	yfcN	$74.2 \pm 4.8$	$38.9 \pm 7.3$	$0.353 \pm 0.068$	-2.8	conserved protein
31	fdnI	$42.7 \pm 1.8$	$17.8 \pm 3.2$	$0.277 \pm 0.042$	-2.65	formate dehydrogenase-N, cytochrome B556 (gamma) subunit, nitrate-inducible
32	ruvA	$53.2 \pm 4.1$	$26.7 \pm 1.1$	$0.341 \pm 0.040$	-2.59	component of RuvABC resolvase, regulatory subunit
33	adiY	$47.5 \pm 8.2$	$6.1 \pm 0.9$	$0.086 \pm 0.003$	-2.55	DNA-binding transcriptional activator
34	holD	$15.1 \pm 3.6$	$2.9 \pm 1.3$	$0.153 \pm 0.066$	-2.43	DNA polymerase III, psi subunit
35	dmsB	$68.1 \pm 2.2$	$54.7 \pm 6.5$	$0.537 \pm 0.067$	-2.42	dimethyl sulfoxide reductase, anaerobic, subunit B
36	bglJ	$10.5 \pm 1.3$	$6.6 \pm 0.7$	$0.420 \pm 0.010$	-2.39	DNA-binding transcriptional activator for silent bgl operon, requires the bglJ4 allele to function; LuxR family
37	yfdT	$71.4 \pm 1.4$	$17.6 \pm 0.7$	$0.164 \pm 0.005$	-2.32	CPS-53 (KpLE1) prophage; predicted protein
38	argF	$64.9 \pm 2.8$	$35.7 \pm 2.5$	$0.371 \pm 0.044$	-2.01	ornithine carbamoyltransferase 2, chain F; CP4-6 prophage
39	rplU	$102.0 \pm 7.4$	$20.5 \pm 4.2$	$0.134 \pm 0.029$	-1.89	50S ribosomal subunit protein L21
40	luxS	$90.3 \pm 1.4$	$54.9 \pm 2.4$	$0.406 \pm 0.018$	-1.77	S-ribosylhomocysteine lyase
41	coaD	$70.9 \pm 1.5$	$45.3 \pm 3.4$	$0.426 \pm 0.036$	-1.75	pantetheine-phosphate adenylyltransferase
42	glnB	$199.0 \pm 6.0$	$61.9 \pm 7.2$	$0.207 \pm 0.021$	-1.75	regulatory protein P-II for glutamine synthetase
43	uidR	$32.2 \pm 1.7$	$35.7 \pm 1.5$	$0.745 \pm 0.064$	-1.5	DNA-binding transcriptional repressor

**Table S2 (contd.): 56 E. coli ORF-yfp fusions used for Arg CRI validation**

Number	Gene	$S_{Arg-rich}$	$S_{Arg-limited}$	Robustness	$\log_2(\text{Arg CRI})$	Gene product
44	relB	69.6 ± 4.3	29.8 ± 0.5	0.287 ± 0.016	-1.35	Qin prophage; bifunctional antitoxin of the RelE-RelB toxin-antitoxin system/ transcriptional repressor
45	btuE	106.1 ± 1.4	52.7 ± 4.0	0.331 ± 0.021	-1.35	glutathione peroxidase
46	argR	37.3 ± 3.4	13.7 ± 4.4	0.235 ± 0.054	-1.19	DNA-binding transcriptional dual regulator, L-arginine-binding
47	ogrK	53.3 ± 1.5	29.3 ± 0.9	0.367 ± 0.013	-1.14	positive regulator of P2 growth (insertion of P2ogr gene into the chromosome)
48	hupB	127.5 ± 3.6	71.2 ± 1.0	0.373 ± 0.008	-1.13	HU, DNA-binding transcriptional regulator, beta subunit
49	ppiB	95.0 ± 2.7	57.2 ± 10.5	0.401 ± 0.069	-1.13	peptidyl-prolyl cis-trans isomerase B (rotamase B)
50	yjgF	92.2 ± 4.7	74.4 ± 3.6	0.540 ± 0.032	-1.13	conserved protein, UPF0131 family
51	kefF	68.6 ± 2.5	69.7 ± 7.6	0.677 ± 0.058	-1.11	flavoprotein subunit for the KefC potassium efflux system
52	yjbJ	92.0 ± 5.1	96.6 ± 30.0	0.730 ± 0.272	-1.02	conserved protein, UPF0337 family
53	leuL	36.7 ± 1.4	31.8 ± 2.2	0.577 ± 0.023	-0.97	leu operon leader peptide
54	rimM	86.4 ± 1.7	89.9 ± 3.3	0.694 ± 0.029	-0.82	16S rRNA processing protein
55	ydjO	9.9 ± 0.9	20.7 ± 1.6	1.432 ± 0.219	-0.74	predicted protein
56	mdtJ	21.0 ± 0.4	26.4 ± 1.5	0.841 ± 0.060	-0.41	multidrug efflux system transporter

**Table S3: 21 E. coli ORF-yfp fusions co-expressed with <sup>GAG</sup>Leu2 tRNA**

Genes are arranged by increasing values of Leu CRI as calculated for <sup>GAG</sup>Leu2 tRNA co-expression.  $S_{Leu-rich}$  and  $S_{Leu-limited}$  refer to respective protein synthesis rates (a.u. per sec per cell) under <sup>GAG</sup>Leu2 tRNA co-expression. Robustness refers to the ratio between the two protein synthesis rates after normalization by the corresponding value for the CTG variant of *yfp* (see Fig. 1D). ± refers to standard error of measurement. Refer to Table S1 for corresponding values without <sup>GAG</sup>Leu2 tRNA co-expression.

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
1	ygiD	8.2 ± 0.6	3.8 ± 0.8	0.515 ± 0.103	-5.69	predicted dioxygenase, LigB family
2	chbR	26.5 ± 2.5	7.3 ± 1.5	0.299 ± 0.043	-5.68	rRepressor, chb operon for N,N'-diacetylchitobiose utilization
3	yfcN	92.7 ± 2.6	12.1 ± 2.9	0.142 ± 0.030	-5.47	conserved protein
4	mdtJ	12.7 ± 0.8	5.7 ± 1.7	0.513 ± 0.165	-4.46	multidrug efflux system transporter
5	ilvD	62.5 ± 1.5	23.6 ± 0.8	0.417 ± 0.022	-4.29	dihydroxyacid dehydratase
6	aspS	54.1 ± 2.0	18.5 ± 1.8	0.379 ± 0.046	-4.24	aspartyl-tRNA synthetase
7	lysS	106.3 ± 4.7	35.2 ± 0.8	0.367 ± 0.025	-4.22	lysine tRNA synthetase, constitutive
8	leuC	100.7 ± 0.5	19.0 ± 1.7	0.208 ± 0.019	-4.04	3-isopropylmalate dehydratase large subunit
9	ygbF	69.4 ± 3.4	8.1 ± 0.4	0.129 ± 0.009	-3.99	probable ssRNA endonuclease, CRISP-associated protein
10	pspA	65.1 ± 1.2	25.9 ± 2.0	0.437 ± 0.025	-3.91	regulatory protein for phage-shock-protein operon
11	guaA	122.0 ± 2.8	51.1 ± 1.9	0.462 ± 0.018	-3.27	GMP synthetase (glutamine aminotransferase)
12	btuE	137.7 ± 4.6	57.5 ± 3.9	0.462 ± 0.044	-3.23	glutathione peroxidase
13	purA	73.3 ± 1.8	34.8 ± 2.7	0.522 ± 0.035	-3.16	adenylosuccinate synthetase
14	ompR	116.4 ± 1.0	50.7 ± 3.2	0.479 ± 0.027	-2.86	DNA-binding response regulator in two-component regulatory system with EnvZ

**Table S3 (contd.): 21 E. coli ORF-yfp fusions co-expressed with <sup>GAG</sup>Leu2 tRNA**

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$	Gene product
15	phnM	$68.2 \pm 4.4$	$48.4 \pm 1.4$	$0.786 \pm 0.044$	-2.72	carbon-phosphorus lyase complex subunit
16	msrB	$113.4 \pm 3.1$	$37.9 \pm 2.1$	$0.370 \pm 0.028$	-2.68	methionine sulfoxide reductase B
17	purH	$71.4 \pm 1.3$	$7.9 \pm 2.0$	$0.120 \pm 0.028$	-2.61	fused IMPcyclohydro-lase/phosphoribosyl aminoimidazole carboxamide formyltransferase
18	coaD	$98.3 \pm 7.4$	$46.7 \pm 1.9$	$0.526 \pm 0.018$	-2.58	pantetheine-phosphate adenylyltransferase
19	rimM	$114.3 \pm 5.9$	$51.3 \pm 1.1$	$0.498 \pm 0.030$	-1.99	16S rRNA processing protein
20	relB	$79.9 \pm 3.0$	$66.6 \pm 3.3$	$0.923 \pm 0.077$	-1.73	Qin prophage; bifunctional antitoxin of the RelE-RelB toxin-antitoxin system/ transcriptional repressor
21	proC	$78.1 \pm 2.0$	$36.7 \pm 4.4$	$0.519 \pm 0.070$	-1.72	pyrroline-5-carboxylate reductase, NAD(P)-binding

**Table S4: 63 synonymous mutants of 13 E. coli ORF-yfp fusions**

$S_{Leu-rich}$  and  $S_{Leu-limited}$  refer to respective protein synthesis rates (a.u. per sec per cell) of mutant ORFs. Robustness refers to the ratio between the two protein synthesis rates after normalization by the corresponding value for the CTG variant of *yfp* (see Fig. 1D).  $\pm$  refers to standard error of measurement. Refer to Table S1 for corresponding values of wild-type ORFs. The DNA sequence of the mutant variants below is provided in the `gene_sequences.fasta` file. Three of the sequences below did not have any mutations compared to the wild-type ORF and were included as internal controls.

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$
1	btuE-yfp mutant 1	$137.0 \pm 5.3$	$50.1 \pm 1.1$	$0.403 \pm 0.007$	-2.22
2	btuE-yfp mutant 4	$131.5 \pm 3.5$	$20.5 \pm 0.5$	$0.172 \pm 0.009$	-3.06
3	btuE-yfp mutant 5	$138.9 \pm 1.8$	$34.1 \pm 0.1$	$0.270 \pm 0.003$	-2.62
4	btuE-yfp mutant 7	$123.4 \pm 4.9$	$48.2 \pm 1.8$	$0.433 \pm 0.032$	-2.22
5	btuE-yfp mutant 8	$130.7 \pm 5.2$	$28.8 \pm 0.9$	$0.243 \pm 0.015$	-3.06
6	btuE-yfp mutant 9	$128.9 \pm 0.9$	$40.0 \pm 0.6$	$0.342 \pm 0.003$	-2.62
7	chbR-yfp mutant 1	$29.5 \pm 1.7$	$17.7 \pm 0.3$	$0.663 \pm 0.028$	-2.81
8	chbR-yfp mutant 2	$26.7 \pm 2.2$	$15.1 \pm 0.4$	$0.634 \pm 0.072$	-3.25
9	chbR-yfp mutant 3	$31.7 \pm 0.9$	$11.3 \pm 0.6$	$0.393 \pm 0.029$	-5.31
10	chbR-yfp mutant 4	$36.0 \pm 2.5$	$9.0 \pm 0.3$	$0.279 \pm 0.027$	-6.2
11	chbR-yfp mutant 5	$28.9 \pm 2.4$	$10.7 \pm 0.2$	$0.414 \pm 0.033$	-5.76
12	chbR-yfp mutant 6	$32.6 \pm 2.7$	$5.7 \pm 0.5$	$0.197 \pm 0.021$	-7.03
13	chbR-yfp mutant 7	$32.1 \pm 1.2$	$7.7 \pm 0.5$	$0.267 \pm 0.023$	-5.8
14	chbR-yfp mutant 9	$31.3 \pm 0.9$	$7.9 \pm 0.5$	$0.278 \pm 0.026$	-6.64
15	coaD-yfp mutant 1	$92.3 \pm 1.3$	$39.1 \pm 1.3$	$0.467 \pm 0.021$	-1.89
16	coaD-yfp mutant 2	$91.7 \pm 4.1$	$55.5 \pm 0.8$	$0.669 \pm 0.034$	-1.1
17	coaD-yfp mutant 3	$94.0 \pm 2.4$	$47.7 \pm 2.3$	$0.558 \pm 0.014$	-1.5
18	leuA-yfp mutant 6	$70.5 \pm 2.1$	$11.3 \pm 0.3$	$0.177 \pm 0.010$	-4.4
19	leuA-yfp mutant 8	$77.9 \pm 2.2$	$6.6 \pm 1.4$	$0.093 \pm 0.020$	-5.23
20	leuA-yfp mutant 9	$70.0 \pm 2.1$	$8.4 \pm 0.2$	$0.132 \pm 0.007$	-4.84
21	leuB-yfp mutant 7	$273.5 \pm 8.7$	$6.1 \pm 0.9$	$0.025 \pm 0.003$	-4.81
22	leuB-yfp mutant 8	$273.3 \pm 10.6$	$9.6 \pm 1.0$	$0.039 \pm 0.005$	-4.02
23	leuB-yfp mutant 9	$274.9 \pm 8.1$	$8.4 \pm 1.7$	$0.034 \pm 0.007$	-3.97
24	leuC-yfp mutant 5	$86.8 \pm 0.6$	$8.5 \pm 1.7$	$0.108 \pm 0.021$	-3.64
25	leuC-yfp mutant 7	$95.6 \pm 1.9$	$5.1 \pm 1.7$	$0.059 \pm 0.019$	-3.3
26	leuC-yfp mutant 8	$100.5 \pm 1.7$	$4.3 \pm 0.9$	$0.047 \pm 0.009$	-3.69
27	leuC-yfp mutant 9	$80.0 \pm 4.9$	$14.3 \pm 0.8$	$0.198 \pm 0.013$	-3.25
28	leuD-yfp mutant 2	$123.6 \pm 0.9$	$18.3 \pm 1.2$	$0.163 \pm 0.011$	-2.76
29	leuD-yfp mutant 3	$123.1 \pm 3.7$	$39.5 \pm 1.0$	$0.355 \pm 0.020$	-1.48
30	leuD-yfp mutant 4	$122.0 \pm 1.0$	$32.9 \pm 0.3$	$0.297 \pm 0.002$	-1.92
31	mdtJ-yfp mutant 1	$17.3 \pm 0.6$	$9.1 \pm 0.4$	$0.580 \pm 0.020$	-4.15
32	mdtJ-yfp mutant 2	$12.2 \pm 0.5$	$5.8 \pm 0.3$	$0.520 \pm 0.008$	-3.31
33	mdtJ-yfp mutant 4	$10.0 \pm 0.9$	$4.3 \pm 0.1$	$0.475 \pm 0.047$	-4.54



**Table S4 (contd.): 63 synonymous mutants of 13 E. coli ORF-yfp fusions**

Number	Gene	$S_{Leu-rich}$	$S_{Leu-limited}$	Robustness	$\log_2(\text{Leu CRI})$
34	mdtJ-yfp mutant 5	13.6 ± 2.1	11.4 ± 0.5	0.979 ± 0.190	-4.15
35	mdtJ-yfp mutant 6	6.4 ± 0.9	5.1 ± 0.1	0.903 ± 0.125	-3.75
36	mdtJ-yfp mutant 7	5.9 ± 3.1	4.3 ± 0.3	1.251 ± 0.488	-4.15
37	mdtJ-yfp mutant 8	10.2 ± 1.5	8.3 ± 0.1	0.932 ± 0.117	-4.19
38	mdtJ-yfp mutant 9	10.3 ± 0.8	9.6 ± 0.2	1.049 ± 0.087	-3.36
39	msrB-yfp mutant 1	76.6 ± 1.9	23.7 ± 0.8	0.342 ± 0.014	-2.48
40	msrB-yfp mutant 2	65.3 ± 1.6	20.7 ± 0.8	0.348 ± 0.005	-2.92
41	msrB-yfp mutant 3	99.1 ± 2.7	27.8 ± 2.5	0.310 ± 0.031	-2.52
42	msrB-yfp mutant 4	94.0 ± 1.5	36.2 ± 0.4	0.424 ± 0.005	-2.08
43	pspA-yfp mutant 1	68.2 ± 1.9	16.3 ± 0.6	0.264 ± 0.006	-2.81
44	pspA-yfp mutant 2	64.2 ± 0.5	15.0 ± 0.7	0.257 ± 0.011	-2.85
45	pspA-yfp mutant 3	62.3 ± 1.5	28.5 ± 0.6	0.505 ± 0.016	-1.97
46	pspA-yfp mutant 5	73.3 ± 1.3	14.0 ± 0.9	0.211 ± 0.018	-3.2
47	pspA-yfp mutant 6	70.1 ± 3.0	18.3 ± 0.9	0.288 ± 0.013	-2.76
48	pspA-yfp mutant 7	73.5 ± 1.4	8.5 ± 0.2	0.127 ± 0.002	-3.64
49	pspA-yfp mutant 8	73.0 ± 1.2	12.3 ± 0.8	0.185 ± 0.012	-3.25
50	yfcN-yfp mutant 1	103.0 ± 3.5	8.3 ± 1.3	0.088 ± 0.012	-4.82
51	yfcN-yfp mutant 5	86.0 ± 3.6	14.9 ± 1.7	0.192 ± 0.022	-3.1
52	yfcN-yfp mutant 6	90.5 ± 1.6	5.6 ± 0.7	0.069 ± 0.009	-5.31
53	yfcN-yfp mutant 8	91.5 ± 4.1	14.1 ± 1.7	0.170 ± 0.017	-2.71
54	yfcN-yfp mutant 9	85.5 ± 1.1	5.2 ± 0.4	0.067 ± 0.005	-4.87
55	ygbF-yfp mutant 2	80.6 ± 2.6	6.2 ± 0.3	0.085 ± 0.006	-3.28
56	ygbF-yfp mutant 3	68.5 ± 3.9	25.4 ± 1.4	0.413 ± 0.044	-1.65
57	ygbF-yfp mutant 5	79.0 ± 4.1	25.1 ± 0.2	0.352 ± 0.017	-2.1
58	ygbF-yfp mutant 6	65.3 ± 2.9	10.8 ± 0.6	0.183 ± 0.019	-2.44
59	ygbF-yfp mutant 7	80.5 ± 4.0	8.3 ± 0.2	0.113 ± 0.004	-3.33
60	ygbF-yfp mutant 8	70.4 ± 3.1	10.8 ± 0.9	0.170 ± 0.019	-2.89
61	ygiD-yfp mutant 1	11.0 ± 1.0	2.1 ± 0.2	0.216 ± 0.009	-5.17
62	ygiD-yfp mutant 3	11.3 ± 0.9	4.2 ± 0.3	0.417 ± 0.056	-4.73
63	ygiD-yfp mutant 4	10.6 ± 0.5	5.4 ± 0.6	0.572 ± 0.090	-4.34
64	ygiD-yfp mutant 5	7.2 ± 1.1	3.3 ± 0.5	0.514 ± 0.041	-5.17
65	ygiD-yfp mutant 6	6.8 ± 1.2	2.7 ± 0.6	0.476 ± 0.131	-4.29
66	ygiD-yfp mutant 7	12.3 ± 2.5	2.7 ± 0.4	0.251 ± 0.016	-6.06

**Table S5: List of strains**

Limiting AA	Strain designation	CGSC number	Genotype
Leu, Arg	CP78	4695	W3110, <i>argH-leuB-thr-his-thi</i>
Ser	JW2880-1	10234	BW25113, $\Delta serA$
Pro	JW0233-2	8468	BW25113, $\Delta proA$
Ile	JW3745-2	10733	BW25113, $\Delta ilvA$
Gln	JW3841-1	10775	BW25113, $\Delta glnA$
Phe	JW2580-1	10048	BW25113, $\Delta pheA$
-	MG1655	6300	wild-type strain with known genome sequence

**Table S6: Concentration of amino acids and methyl esters used for amino acid limitation experiments**

Amino Acid	Amino acid concentration in overnight cultures ( $\mu M$ )	Amino acid concentration for amino acid limitation experiments ( $\mu M$ )	Amino acid methyl ester concentration for amino acid limitation experiments ( $\mu M$ )	Catalog number for amino acid methyl ester
Leu	800	100	160	L1002 (Sigma)
Arg	800	150	160	11030 (Sigma)
Ser	10000	5000	800	412201 (Sigma)
Pro	800	0	160	287067 (Sigma)
Ile	800	100	160	I0522 (VWR)

**Table S6 (contd.): Concentration of amino acids and methyl esters used for amino acid limitation experiments**

Amino Acid	Amino acid concentration in overnight cultures ( $\mu M$ )	Amino acid concentration for amino acid limitation experiments ( $\mu M$ )	Amino acid methyl ester concentration for amino acid limitation experiments ( $\mu M$ )	Catalog number for amino acid methyl ester
Gln	800	400	400	68604 (Astatech)
Phe	800	50	50	P17202 (Sigma)

**Table S7:  $w_i$  values for Leu codons under  $^{GAG}$ Leu2 co-expression**

Codon	$-\log w_i$ (with $^{GAG}$ Leu2 co-expression)
CTA	0.48
CTC	0.05
CTG	0.04
CTT	0.02
TTA	0.26
TTG	0.33

**Table S8: qPCR primer sequences**

Gene	Forward primer	Reverse Primer
<i>yfp</i>	TCATGCTGTTTCATGTGATC	AGGGTGATGCTACTTATGGC
<i>gapA</i>	GCTGAAGGCGAAATGAAAGG	GTACCAGGATAACCAGTTTCACG
<i>rpoD</i>	TGAAGCGAACTTACGTCTGG	AGAAGTTGTAACCACGGCG
<i>rpoS</i>	TCTCAACATACGCAACCTGG	AGCTTATGGGACAACCTCACG
<i>rpoH</i>	TCGTAATTATGCGGGCTATGG	CAGTGAACGGCGAAGGAG
<i>rpoE</i>	CCAGAAGGGAGATCAGAAAGC	TACCACATCGGGAACATCAC
<i>rpoN</i>	TGATCCAACCTCTCCCAATTTCG	TCGTGATTGGCTAACAGATCG
<i>fecI</i>	ACTACCACAGCTTCCTTAACG	TTTCGCTGACCATTACCCG
<i>fliA</i>	CGCTATGCTGGATGAACTTCG	CTAAACGTTCCGCTACCTCAG
<i>leuA</i>	GTCGCTAACTACAACGGTCG	GCACGCCAGATATTGTTTCAG
<i>ilvM</i>	GTTTCCACGTCTGCTCAATG	CTGACTAAACAGTAAGTCGACCG
<i>ilvB</i>	TGAGTTTCCGTGTCCAATCC	ATCTGATGCTGACCAACGTC

**Table S9: Codon-tRNA assignments**

tRNA	Unmodified Anticodon	Modified Anticodon (if known)	Cognate Codons	Reference
Leu1	CAG	-	CUG	[15]
Leu2	GAG	-	CUC, CUU	[15]
Leu3	UAG	cmo <sup>5</sup> UAG	CUA, CUG, CUU	[24]
Leu4	CAA	CmAA	UUA, UUG	[15, 25]
Leu5	UAA	cmnm <sup>5</sup> UmAA	UUA, UUG	[15, 25]
Arg2	ACG	ICG	CGU, CGC, CGA	[15, 25]
Arg3	CCG	-	CGG	[15]
Arg4	UCU	mm <sup>5</sup> UCU	AGA, AGG	[18, 25]
Arg5	CCU	-	AGG	[15]
Ser1	UGA	cmo <sup>5</sup> UGA	UCA, UCU, UCG	[15, 25]
Ser2	CGA	-	UCG	[15]
Ser3	GCU	-	AGC, AGU	[15]
Ser5	GGA	-	UCC, UCU	[15]
Pro1	CGG	-	CCG	[15]
Pro2	GGG	-	CCC, CCU	[15]
Pro3	UGG	cmo <sup>5</sup> UGG	CCA, CCU, CCG	[15, 25]
Ile1	GAU	-	AUC, AUU	[15]

**Table S9 (contd.): Codon–tRNA assignments**

tRNA	Unmodified Anticodon	Modified Anticodon (if known)	Cognate Codons	Reference
Ile2	CAU	k <sup>2</sup> CAU	AUA	[15, 25]
Gln1	UUG	mnm <sup>5</sup> s <sup>2</sup> UUG	CAA, CAG	[15, 25]
Gln2	CUG	-	CAG	[15]
Phe	GAA	-	UUC, UUU	[15]