1 ### *E. coli* translation strategies differ across nutrient conditions

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Abstract

16

17   For cells to grow faster they must increase their protein production rate. Microorganisms

18   have traditionally been thought to accomplish this increase by producing more ribosomes to

19   enhance protein synthesis capacity, leading to the linear relationship between ribosome level

20   and growth rate observed under most growth conditions previously examined. Past studies have

21   suggested that this linear relationship represents an optimal resource allocation strategy for

22   each growth rate, independent of any specific nutrient state. Here we investigate protein

23   production strategies in continuous cultures limited for carbon, nitrogen, and phosphate, which

24   differentially impact substrate supply for protein versus nucleic acid metabolism. Unexpectedly,

25   we find that at slow growth rates, *E. coli* achieves the same protein production rate using three

26   different strategies under the three different nutrient limitations. Upon phosphate (P) limitation,

27   translation is slow due to a particularly low abundance of ribosomes, which are RNA-rich and

28   thus particularly costly for phosphorous-limited cells. In nitrogen (N) limitation, translation is

29   slowed by limited glutamine and stalling at glutamine codons, resulting is slow elongation. In

30   carbon (C) limitation, translation is slowed by accumulation of inactive ribosomes not bound to

31   mRNA. These extra ribosomes enable rapid growth acceleration upon nutrient upshift. Thus,

32   bacteria tune ribosome usage across different limiting nutrients to enable balanced nutrient-

33   limited growth while also preparing for future nutrient upshifts.

Introduction

35   Resource allocation during growth is a fundamental challenge faced by all cells[1-4]. For

36   example, with a fixed resource budget, cells must balance production of the machinery that

37   makes proteins (ribosomes, tRNAs, translation factors) with the production of the proteins

38   themselves. This balance is generally represented by the RNA:protein ratio (R/P ratio)[5]. The

39   R/P ratio captures protein production capacity, as >95% of total RNA is devoted to translation

40   (rRNAs and tRNAs[5,6]). In single-cell organisms like *E. coli*, previous studies demonstrated that

41   there is a linear relationship between R/P ratio and growth rate, with faster growth rates

42   requiring more protein production capacity and therefore higher R/P ratios[5,7,8]. Production of

43   ribosomes is costly as each contains 52 protein subunits and three large rRNAs[9,10]; hence, it is

44   advantageous for the cell to saturate ribosomes with substrates. In this efficient ribosome

45   scenario, the ribosome level should be fixed and independent of nutrient conditions for any

46   growth rate, with the only way to increase protein synthesis rate being to increase the number of

47   ribosomes[1,5,11]. One surprise for such a seemingly optimized system is that multiple studies

48   have demonstrated that at slow growth rates *E. coli* accumulates inactive ribosomes[12]. There

49   are two possible explanations for the presence of inactive ribosomes. First, it is possible that *E.*

50   *coli* translation is constrained in such a way that it cannot function when ribosome levels drop

51   too low[12]. Alternatively, *E. coli* could regulate ribosome production independently of growth rate.

52   Here we settle this debate by showing that *E. coli* ribosome production and usage differ across

53   nutrient conditions.

54   **Results**

55   ***Phosphate-limited cells achieve the same growth rate with fewer ribosomes than Carbon-***

56   ***or Nitrogen-limited cells***

57   To determine the generality of the relationship between growth rate and ribosome

58   content, we examined how the R/P ratio changes as a function of growth upon different nutrient

59   limitations. We measured R/P ratios in *E. coli* under glucose- (C, carbon), ammonia- (N,

60   nitrogen), and phosphate- (P, phosphorous) limitations over a range of different growth rates in

61   chemostats (Figure 1A). Surprisingly, P-limited cells consistently exhibited lower R/P ratios than

62   C-limited or N-limited cells, with a roughly 2-fold difference at the slowest growth rate tested (0.1

63   h^{-1}, Figure 1B). Measured protein levels were similar in all cells regardless of growth rate or

64   nutrient limitation (Figure S1A-B), indicating that the changes in the R/P ratios reflect changes in

65   ribosome availability.

66   The finding that *E. coli* can grow at the same rate with a lower R/P ratio implies that P-

67   limited cells make the same amount of protein with fewer ribosomes, i.e. use ribosomes more

68   efficiently. Thus, C/N-limited *E. coli* cells do not use ribosomes with optimal efficiency and their

69   supply of "extra" ribosomes cannot be ascribed to requirements for productive translation. Since

70   RNA accounts for two-thirds of the mass of bacterial ribosomes[9,14], producing fewer ribosomes

71   upon phosphate-limitation makes sense as a way for cells to deal with a limitation that

72   preferentially reduces an elemental substrate needed to make RNA but not protein. This effect

73   may be a direct consequence of low phosphate resulting in limited nucleotide pools and thus

74   slow RNA synthesis, as deletion of phosphate sensing or storage systems, phoB[15] or ppk[16], did

75   not alter the R/P ratio (Figure S1C-D).

76   ***N-limited ribosomes translate slowly while C-limited cells accumulate more mRNA-free***

77   ***ribosomes***

78   Why do C/N-limited cells accumulate so many ribosomes if P-limited cells can achieve

79   the same protein synthesis rates with fewer ribosomes? One possibility is that the ribosomes in

80   these cells translate slowly. We thus used a *lacZ* induction assay to compare the translation

81   elongation rates of slow-growing C-, N-, and P-limited cells (0.1 h^{-1})[17,18]. We observed a reduced

82   elongation rate in N-limited cells compared to C- and P-limited cells but no difference between

83   C- and P-limited cells (Figure 1C). Thus, N-limited cells may need higher ribosome numbers to

84   compensate for their slow translation elongation, but something else must explain the elevated

85   ribosome numbers in C-limited cells.

86   To characterize ribosome pools we performed polysome profiling, which separates

87   ribosome species using a sucrose gradient[19]. Regardless of the growth condition, all cells

88   exhibited similar fractional pools of dissociated 30S and 50S subunits (Figure 1D). In contrast,

89   the fraction of 70S monosomes was significantly larger in C/N-limited cells than in P-limited cells

90   (Figure 1D). Since growth rate is proportional to protein synthesis rate, growth rate can be

91   estimated by the product of the number of active ribosomes and the translation elongation rate.

92   However, using the assumption that all 70S monosomes are active yielded very different growth

93   rate estimates for C-, N-, and P-limited cells (Figure 1F and SI), which is inconsistent with the

94   fact that these cells are growing at the same rate and have the same protein content. This

95   inconsistency suggested that a fraction of the 70S ribosomes may not be active.

96   The mass of a single mRNA is small relative to the mass of a ribosome, such that 70S

97   monosomes could represent either mRNAs with only one ribosome per transcript or inactive

98   "free" ribosomes that are not associated with an mRNA. To distinguish free and mRNA-bound

99   70S monosomes, we utilized their differential sensitivity to high potassium levels (170 mM)[20].

100   High potassium causes free ribosomes to shift to a lower density but does not shift the density

101   of mRNA-bound monosomes[20]. We thus designed a high-resolution "free-ribosome profiling"

102   method to resolve this density shift (Figure S2). As controls, we confirmed that our assay

103   detects the potassium-dependent shift of 70S monosomes induced by puromycin (Figure S2),

104   which releases elongating ribosomes from their associated mRNAs[21]. There was no potassium-

105   dependent shift detected in fast-growing cells that lack free ribosomes (Figure S2).

106   By combining traditional and free-ribosome polysome profiling, we quantified the relative

107   fractions of all ribosome species in slow-growing C-, N-, and P-limited *E. coli*. The fraction of

108   free monosomes was roughly 3-fold greater in the C-limited cells than in the P-limited cells,

109   while the fraction of mRNA-bound monosomes remained relatively constant across nutrient

110   limitations (Figure 1E). The accumulation of free 70S monosomes in C- and N-limited cells

111   appears to be independent of a previously-described RaiA-dependent mechanism for ribosome

112   storage as deletion of *raiA* had no impact on R/P ratios or polysome profiles (Figure S3).

113   Importantly, revising our protein synthesis rate estimates to account for the fraction of inactive

114   70S monosomes yielded similar values for all cells, regardless of nutrient limitation (Figure 1F).

115   These results both validate our experimental measurements and suggest that in different

116   nutrient states, *E. coli* differentially tune ribosome number, translation rate, and active fraction to

117   produce proteins at the same rates (Figure 2A).

118   ***Quantitative modeling describes three different strategies of ribosome dynamics to***

119   ***achieve the same protein production rate***

120   To better understand nutrient-dependent ribosome dynamics, we probed translation in

121   slow-growing C-, N-, and P-limited cells by ribosome profiling. Analysis of ribosome densities as

122   a function of distance from the start and stop codons revealed higher ribosome occupancy near

123   the start codon (Figure 2B). Ribosome density thereafter was similar, with no decrease in

124   ribosome density between the first and second halves of genes (Figures 2B and S4A),

125   suggesting that there is little to no aborted translation after the first few codons. We quantified

126   ribosome dynamics by building a simple macroscopic model (Figure 2C, S4B-E and details in

127   SI). In this model, unbound ribosomes (R_u) can bind to an mRNA with a free ribosomal binding

128   site (with rate constant k_f) to become initiating ribosomes (R_i). The initiating ribosomes can

129   proceed to elongation (with rate constant k_p) to become working ribosomes (R_w), or can abort

130   translation (with rate constant k_r). Working ribosomes elongate to finish translation and become

131   unbound (with rate constant k_{el}/N_{aa}), where N_{aa} is the length of an average protein in amino

132   acids. We defined the fraction of ribosomes bound to the first 10 codons as initiating ribosomes

133   since the ribosome footprint size is ~10 codons[22,23]. To validate our model we used it to

134   calculate elongation rates, which closely agreed with those we measured experimentally (Figure

135   S5A).

136   Analysis of our model indicated that the system can be characterized by two main

137   dimensionless parameters: the "relative proceeding rate", defined as the ratio between the rate

138   of ribosomes proceeding from initiation to elongation (k_p, s^{-1}) and the rate of elongation (k_{el}/N_{aa},

139   s^{-1}); and the "saturation parameter" ($R_t/(K_m+R_t)$), reflecting the degree of saturation of ribosome

140   binding sites on mRNAs, where R_t is the total ribosome number and $K_m = (k_r + k_p)/k_f$ (Figures

141   2D-E and S5B-C). Fitting the measured ribosome densities, translation elongation rates, and

142   pool sizes of ribosome species to the macroscopic model revealed that P-limited cells have the

143   highest relative proceeding rate while C-limited cells have the lowest saturation parameter, and

144   N-limited cells have intermediate values for both parameters (Figures 2D-E and S5B-H). Thus,

145   C-, N-, and P-limited cells produce proteins at the same rate using three different strategies: P-

146   limited cells have few ribosomes that are mostly active and elongate rapidly, N-limited cells

147   have more ribosomes but fewer are active and they elongate slowly, and C-limited cells have

148   many ribosomes, which elongate rapidly, but even fewer are bound to mRNA (Figure 2F and

149   S5I).

150   ***N-limited ribosomal regulation is mediated by RelA, the ppGpp alarmone synthase***

151   Insight into the molecular basis of nutrient-specific ribosome regulation came from

152   analysis of the codon occupancies in our ribosome profiling data. Codon-specific ribosome

153   stalling leads to increased codon occupancy and is a hallmark of insufficient pools of the

154   corresponding charged tRNAs. P-limited cells exhibited no elevated codon frequencies,

155   consistent with these cells' efficient ribosome usage (Figure 3A). In contrast, both C- and N-

156   limited cells exhibited significant codon-specific stalling. Under N-limitation, ribosomes stalled at

157   both of the two glutamine-encoding codons, which together account for 4.4% of all predicted

158   ORF codons in *E. coli*. This result is consistent with previous studies indicating that glutamine is

159   the most strongly-depleted amino acid pool upon N-limitation and serves as an intracellular

160   sensor for extracellular nitrogen levels[24-26]. Upon C-limitation, we observed elevated occupancy

161   of the Leu-CUA codon, which was surprising as there is no known intracellular carbon sensor

162   that controls translation. *E. coli* has six leucine codons decoded by five leucine tRNA

163   species[27,28]. Leu-CUA is the rarest Leu codon, accounting for only 0.4% of all predicted ORF

164   codons.

165   In addition to clarifying the link between metabolism and translation for N-limitation, the

166   observation that ribosomes stall at specific codons upon C/N-limitation suggested a molecular

167   mechanism for nutrient-specific translation regulation (Figure 3A). In bacteria, insufficient

168   charged tRNA pools activate the stringent response to induce accumulation of the cellular

169   alarmone, ppGpp[30,31]. ppGpp is known to regulate rRNA transcription *in vivo*. Its role in

170   translation is less well-understood but ppGpp has been shown *in vitro* to inhibit translation

171   factors such as EF-Tu and IF2 by competing with GTP[32,33]. To test how ppGpp accumulation

172   might affect translation *in vivo*, we induced ppGpp synthesis by treating batch-grown *E. coli* with

173   serine hydroxamate (SHX). SHX is a serine analog that competitively inhibits serine tRNA

174   synthetase to yield uncharged serine-tRNA and thereby activate the RelA ppGpp synthase[34].

175   SHX treatment increased the pool of mRNA-free 70S monosomes, and this effect was

176   completely dependent on *relA* (Figures S6A-B). Thus, inducing ppGpp by activating RelA alters

177   translation by increasing the fraction of inactive free 70S ribosomes.

178   RelA is primarily required for the accumulation of ppGpp upon N-limitation but not C-

179   limitation[31]. Consistently, deletion of *relA* had little impact on the accumulation of free ribosomes

180   upon C-limitation, but significantly reduced free ribosome pools upon N-limitation to levels

181   similar to those observed upon P-limitation (Figures 3B and S6C). We could not probe the role

182   of ppGpp in C-limited ribosome accumulation because unlike N-limitation, C-limitation elevates

183   ppGpp through *spoT*, which is essential[35]. N-limited *ΔrelA* cells also contained more polysomes

184   than wild type (Figure 3B), suggesting that these cells had a higher fraction of elongating

185   ribosomes. This result was initially confusing because the wild type and *ΔrelA* N-limited cells

186   were grown at the same growth rate and maintained the same R/P ratio (Figure S6D). We thus

187   measured the rate of translation elongation and found that while N-limited *ΔrelA* cells have

188   higher fractions of translating ribosomes, their ribosomes elongate more slowly (Figure 3C),

189   resulting in the same rate of protein production as wild type.

190   To understand how RelA influences ribosome dynamics we performed ribosome

191   profiling. N-limited *ΔrelA* cells exhibited more pronounced ribosome stalling at both glutamine

192   codons than wild type N-limited cells, while *relA* deletion had no effect on P- or C-limited cells

193   (Figures 3D and S7). Fitting the *ΔrelA* cells measurements to our ribosome dynamics model

194   revealed that *ΔrelA* cells specifically increase the relative proceeding rate under N-limitation, but

195   display no effect on relative proceeding rate under P- or C-limitation and no effect on the

196   saturation parameter in any condition (Figures 3E and S5B-C). These results suggest that,

197   consistent with its known effects on IF-2 *in vitro*, in N-limited cells RelA functions to restrict the

198   transition from translational initiation to elongation. Thus, in the absence of RelA, more

199   ribosomes attempt to elongate, which exacerbates the depletion of charged tRNA pools, leading

200   to increased stalling and a slower translational elongation rate.

201   ***Extra ribosomes may facilitate growth acceleration upon nutrient upshift***

202   While cells can modulate different aspects of ribosome dynamics to achieve the same

203   protein production rate, what benefits might be served by the inefficient translation system used

204   by C-limited cells where many ribosomes are inactive? Since free ribosomes accumulate the

205   most at the slowest growth rates, we hypothesized that our findings could reflect a trade-off

206   between steady-state growth rate and the ability to respond to a fluctuating environment. Such

207   transitions could include rapidly and safely slowing growth when nutrients become depleted and

208   rapidly increasing growth rate when nutrients are replenished. In this scenario, cells may benefit

209   more by optimizing their ability to rapidly utilize new nutrients, for example to outcompete their

210   neighbors or maximally utilize a transient pulse of nutrients, than by optimizing steady-state

211   growth rate when nutrient levels are low.

212   We built a mathematical model of cellular resource allocation to predict the growth

213   dynamics upon upshift. This model supported the hypothesis that the larger free ribosome pools

214   of C/N-limited cells should enable them to increase their growth rates more quickly than P-

215   limited cells upon nutrient upshift (Figures 4A, S8 and details in SI)[4,36]. We experimentally tested

216   this prediction by measuring the growth rates of slow-growing (0.1 h^{-1}) *E. coli* immediately after

217   being shifted to rich media (LB + 0.4% glucose) [37]. As predicted, C/N-limited cells increased

218   their growth rates significantly faster than P-limited cells (Figure 4B). Thus, the distinct

219   translation strategies employed under different nutrient conditions may represent nutrient-

220   specific adaptations, with P-limited cells optimizing for current steady-state growth under slow

221   growth conditions, and C- and N-limited cells favoring the ability to rapidly recover growth

222   (Figure 4C).

223   **Discussion**

224   In previous studies, *E. coli* were found to vary R/P ratio with growth rate independently of

225   the specific nutrient limitation used to produce a given growth rate[5,8]. Meanwhile, transcriptional

226   analysis in *Saccharomyces cerevisiae* suggested that the primary determinant of the response

227   to a wide range of stresses was the cellular growth rate rather than the specific stressor[38].

228   Together, these studies suggested that the primary regulator of microbial physiology is growth

229   rate. However, our findings demonstrate that at the same growth rate, *E. coli* exhibit significantly

230   different translation strategies across nutrient limitations; at the lowest growth rate tested, P-

231   limited cells produced the same amount of protein with roughly half as many ribosomes as C/N-

232   limited cells. P-limited cells also exhibited smaller inactive 70S monosome pools and higher

233   relative proceeding rates than C/N-limited cells. Furthermore, while C/N-limited cells have

234   similar ribosome numbers, they also display differences in free ribosome pools, translational

235   elongation rates, and sensitivity to the loss of the ppGpp synthase RelA. Thus, our results

236   suggest that the extra ribosomes of C/N-limited cells do not reflect essential constraints, but

237   rather reflect a selectively beneficial adaptation.

238   Our findings also implicate ppGpp as a key mediator of ribosome activity that specifically

239   modulates the transition from translation initiation to elongation. ppGpp is a well-characterized

240 cellular alarmone that senses translational activity and inhibits rRNA transcription[31,39]. However,

241 ppGpp can competitively inhibit GTP-dependent enzymes, including the translation initiation

242 factor IF-2 that is required for the transition to elongation[40]. Our data thus provide *in vivo* support

243 for a previous *in vitro* study demonstrating that ppGpp inhibits IF-2 function[33]. Since ppGpp can

244 also affect other GTP-binding proteins involved in translation[32,41], future studies on bacterial

245 translation will confirm exactly how ppGpp interferes with ribosome function.

246   In native environments such as the mammalian gut, bacteria such as *E. coli* are faced

247 with feast and famine cycles induced by feeding cycles[42]. Here we show that the linear

248 relationship between R/P ratio and growth rate in *E. coli* does not reflect the optimization of

249 steady-state growth rate but, instead, reflects the ability of cells with higher inactive ribosome

250 pools to rapidly accelerate growth upon nutrient repletion. These findings suggest that *E. coli*

251 may improve their fitness by sacrificing their maximal growth rate in nutrient-poor periods in

252 return for the ability to respond to a changing environment, including rapidly accelerating growth

253 in nutrient-rich periods. Our macroscopic model highlights how cells can tune total ribosome

254 number, the fraction of working ribosomes, and the rate of translational elongation to achieve

255 the same total protein production rate while balancing other constraints such as reduced amino

256 acid availability or the need to rapidly accelerate growth. Future studies will address the

257 consequences of adaptation strategies in dynamic conditions, for example the generality of

258 bacteria optimizing growth rate transitions at the expense of steady-state growth[43], but this

259 strategy could explain recent reports of the sub-optimality of protein allocation for *E. coli* in the

260 presence of poor carbon sources[44], as well as the sub-optimal expression levels of essential

261 genes in *B. subtilis*[45]*.

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263 **Author Contributions**

264 S.H.L., J.O.P., J.D.R., N.S.W, and Z.G. designed the experiments. S.H.L., J.O.P., and C.K.
265 performed experiments. Z.L. and N.S.W. constructed the mathematical models. S.H.L.

266 conducted computational analysis of sequencing data. S.H.L, Z.L., N.S.W, and Z.G. wrote the
267 paper with assistance from J.D.R.

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406

407 **Materials and Methods**

408 <u>Cell strains and growth conditions</u>

409   *Escherichia coli* strain NCM3722 was grown in batch or continuous cultures. To achieve
410 different growth rates, different carbon or nitrogen sources were provided in batch culture,
411 whereas dilution rates ranging from 0.1 h^{-1} to 0.7 h^{-1} were used in continuous (chemostat)

412   cultures. The chemostat (Sixfors, HT) volume was 300mL with oxygen and pH probes to
413   monitor the culture. pH was maintained at 7.2 +/- 0.1 and the aeration rate was set at 4.5 l/h. 40
414   mM MOPS media (M2120, Teknova) was used with glucose (0.4%, Sigma G8270), ammonia
415   (9.5 mM NH_4Cl, Sigma A9434) and phosphate (1.32 mM K_2HPO_4, Sigma P3786) added
416   separately. For carbon- and nitrogen- limiting media, glucose and ammonia concentrations were
417   reduced by 5-fold (0.08% and 1.9mM respectively). Phosphorus-limiting medium contains 0.132
418   mM K_2HPO_4. $\Delta relA$ mutant was generated by P1 transduction from the KEIO collection[46] into
419   *Escherichia coli* strain NCM3722

421   Nutrient upshift growth measurement
422   Cells from chemosats were mixed with 4X volumes of fresh pre-warmed media and grown
423   in flasks in a 37°C water bath. Cell growth was monitored every 5 minutes by checking
424   absorbance at 600nm in a quartz cuvette (Starna, 16.160-Q-10/Z8.5) using a
425   spectrophotometer (GENESYS™ 20, Thermo Scientific). The LB media (244610, BD) used for
426   upshift was supplemented with 0.4% glucose.

428   Total RNA measurement
429   The method for RNA measurement was adapted from You *et al.*[1]. 1.5 mL of cultures were
430   pelleted by centrifugation for 1 min at 13,000 X g. The pellet was frozen on dry ice and the
431   supernatant was taken to measure absorbance at 600 nm for cell loss. The pellet was then
432   washed twice with 0.6M $HClO_4$ and digested with 0.3M KOH for 1 hour at 37°C. The solution
433   was then precipitated with 3M $HClO_4$ and the supernatant was collected. The pellet was re-
434   extracted again with 0.5M $HClO_4$. The supernatants were combined and absorbance measured
435   at 260nm using NanoDrop (ND-1000, NanoDrop). Total RNA concentration was determined by
436   multiplying the A260 absorbance with 31 (μg RNA/mL) as the extinction coefficient.

438   Total protein measurement
439   The method for protein measurement is adapted from You *et al.*[1]. 1.5 mL of cell cultures were
440   pelleted by centrifugation for 1 min at 13,000 X g. Cells were washed with 1 mL MOPS buffer
441   once, re-suspended in 200 uL water and left on dry ice. All the supernatant was collected and
442   OD600 was measured for cell loss. To measure the protein content, samples were thawed,
443   100μL 3M NaOH were added, and the sample was heated for 5 min at 98°C. The samples were
444   cooled down to RT for 5 min before 300μL 0.1% $CuSO_4$ were added for biuret assay. The
445   samples were incubated at RT for 5 min and centrifuged at 13,000 X g for 1 min. The
446   supernatant was collected and absorbance was measured at 555nm for a 200μL sample
447   volume in a microplate reader (Synergy HT, BioTek) with software Gen 5.0. Proper dilution of
448   albumin (23209, Thermo) with known concentrations was used to infer the total protein
449   concentration in the cell.

451   Polysome profiling and quantification of ribosome fraction
452   200mL of cells were collected from cultures by filtration through 90mm cellulose acetate
453   membranes with a 0.2 μm pore size (CA029025, Strelitech) at 37°C, scratched with a clean and
454   pre-warmed stainless steel spatula, and snap-frozen in liquid nitrogen. The whole filtration
455   procedure did not take more than 2 minutes in order to maintain the original physiological state.
456   Cell pellets were mixed with 650μL lysis buffer frozen nuggets (20mM Tris-HCl pH 8.0, 10mM
457     $MgCl_2$, 100mM NH_4Cl, 0.4% Triton X-100, 0.1% NP-40, 1 mM Chloramphenicol, 100 U/mL
458   RNase-free DNase I (04716728001 Roche)) in a pre-chilled 10mL jar (014620331, Retsch).
459   Pulverization was done by cryomill (Retsch) at 15 Hz for 15 minutes. The thawed cell lysates
460   were quantified by NanoDrop and 200μL of lysates with RNA concentration ranging from 80μg
461   to 500μg were used. For overall polysome quantification, lysates were loaded to 10%-55%
462   linear sucrose gradients (20mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NH_4Cl, 300 μM

463 Chloramphenicol) made by GradientMaster (BioComp). The gradients were placed in a SW41Ti
464 bucket and centrifuged in Optima XE-100 Ultracentrifuge (Beckman Coulter) at 35,000 rpm for 2
465 hours at 4°C. Gradients were fractionated by BioComp Gradient Fractionator and the absorption
466 curves at 254nm were recorded by a UV monitor (EM-1, BioRad).
467   Quantification of the polysome profiles was done using customized MATLAB codes. First,
468 baselines were estimated using the readings where no peaks existed and the background was
469 subtracted. The background from the free nucleotides and tRNA was removed by fitting an
470 exponential decay function to the first peak representing the source of non-ribosome signals.
471 Each ribosome peak was picked and quantified by integrating the area underneath the curve.
472 To quantify different species of ribosomes in the 70S peak, 100mM NH_4Cl was replaced with
473 170mM KCl. Cell lysates were loaded onto 10%-30% linear gradient and centrifuged at 35,000
474 rpm at 4°C for 5 h. Because the two peaks for the free and mRNA-bound 70S ribosomes are
475 very close in mass and clean separation was not possible, the MATLAB file-exchange package,
476 *peakfit*, was used to fit the two overlapped peaks as two Gaussian distributions. For the free
477 ribosome control, cells were treated with 100μM puromycin for 5 minutes and collected. For
478 serine hydroxamate (SHX, Sigma S4503) treatment, cells were grown in MOPS glucose
479 minimal media until OD ~0.3 and treated with SHX with final concentration of 1 mg/mL for ten
480 minutes before collection.
481
482 *lacZ* induction and translational elongation rate measurement
483   The method was adapted from Zhu et al[47]. A final concentration of 5 mM IPTG (I2481C-25,
484 Gold Biotechnology) was added to cultures. At every 15 seconds, 1 mL of culture was collected
485 in a tube containing 10μL 100mM chloramphenicol, snap frozen in liquid nitrogen and stored at -
486 20°C before subsequent measurement. After cells were thawed, 400μL of the sample was
487 added to 100 μL 5x Z-buffer (0.3M $Na_2HPO_4.7H_2O$, 0.2M $NaH_2PO_4.H_2O$, 50mM KCl, 5mM
488 $MgSO_4$, 20mM β-mercaptoethanol) and incubated at 37°C for 10 minutes. 100μL of 4mg/mL
489 MUG (337210010, ACROS Organics) in DMSO was then added to each sample every 10
490 seconds to accurately control the reaction time. The samples were incubated at 37°C in a
491 thermomixer at 1,400 rpm mixing rate for 30 minutes to 2 hours, depending on the enzyme
492 expression level. The reaction was stopped by addition of 300μL of 1M Na_2CO_3. The tubes were
493 spun down at 16,000 X g for 3 minutes to sediment the cell debris. 200 μL of supernatant were
494 taken and measured fluorescence by a microplate reader (365 nm excitation and 450 nm
495 emission filter).
496   To infer translational elongation rate, the square root of the signal in excess of the signal at
497 time zero was plotted. A linear fit was performed on the points after signal began to increase.
498 The lag time is the x-intercept of the line. A previous study has shown that the initiation time
499 remains constant for about 10 seconds across a wide range of conditions tested[47]. Therefore,
500 we corrected the elongation times measured by subtracting 10 seconds in the lag times
501 measured.
502
503 Ribosome footprinting and total RNA extraction for RNA-Seq
504   The cell collection step was the same as for polysome profiling except that 1mM
505 chloramphenicol was used in the sucrose solution. The footprinting and library preparation steps
506 were adapted from Li *et al.*[2] After quantification of RNA concentration with NanoDrop, samples
507 with 500μg RNA were digested with 750U MNase (10107921001, Roche) for 1 hour at 25°C
508 before being quenched with 6mM EGTA. The lysates were then layered onto a 10%-55%
509 sucrose gradient and centrifuged. The monosome fraction was collected and snap frozen in
510 liquid nitrogen. No polysome peaks were observed, indicating a thorough digestion. The RNA
511 was isolated using hot phenol and size selected on 15% TBE-Urea PAGE gels run for 1 hour at
512 210V. Gels were stained with SYBR Gold and visualized using Dark Reader (Clare Chemical
513 Research). RNA fragments with size between 25-40 nt were extracted using isopropanol

514 precipitation. Total RNA was extracted with TRIZOL from the same pulverized cells used for
515 footprinting. After DNaseI (04716728001,Roche) treatment and cleanup using RNA clean &
516 concentrator 5 (R1016, Zymo Research), ribosomal RNA was subtracted using MicrobeExpress
517 (AM1905, Ambion). The recovered RNA was fragmented and size selected. RNA fragments
518 with size between 25-40nt were extracted using isopropanol precipitation.
519
520 Library preparation and sequencing
521   Fragments from footprints and total RNA were dephosphorylated at the 3' end by PNK
522 (M0201, NEB). The repaired fragments were linked to the Universal miRNA Cloning Linker
523 (S1315S, NEB), reverse transcribed (18080044, Thermo)and circularized (CL4111K, Epicentre).
524 The circularized samples were PCR amplified (M0531L, NEB) and size selected. High quality
525 PCR samples checked by Bioanalyzer high sensitive DNA chip. Deep sequencing was done by
526 Illumina HiSeq 2500 on Rapid flowcells with settings of single end and 75 nt-long read length.
527
528 Mapping and sequencing data analysis
529   Data manipulation including barcode splitting, linker trimming and mapping were done
530 using Galaxy. The processed reads were mapped to *Escherichia coli* genome
531 escherichia_coli_k12_nc_000913_3 from the NCBI database with the BWA short read mapping
532 algorithm. Only the reads between 20-45 nt that aligned to the coding region were used for
533 further analysis.
534   To infer the ribosome A-site position, python package Plastid[48] was used to align the 3' end
535 of reads to the stop and start codons[49], which are known to have higher ribosome densities. We
536 found that the offsets were 12 nt for stop codon and 15 nt for start codon. Therefore, we used
537 11nt for A site position and 14nt for P site. The counts were normalized to the total counts in the
538 coding region as reads per million (RPM) and transcripts per million (TPM). Further analysis
539 was done using customized Python and R codes with packages including Plastid, dplyr, tidyr
540 and ggplot2.
541
542 Analysis of ribosome profiling data
543   Transcripts per million (TPM) was used to identify highly expressed genes for codon
544 occupancy analysis. After assigning each mapped read to the A-site nucleotide, the raw counts
545 were first normalized by the length of their mapped coding region, to yield count density for each
546 gene. These count densities were then globally normalized to one million counts across all
547 genes within one sample. To determine codon occupancy, transcripts having total counts over
548 100 TPM and containing more than 200 codons were considered. Ribosome footprint counts for
549 the first and last 40 codons were removed to avoid possible effects from initiation and
550 termination, and counts per codon were recorded for the remaining counts. For each gene and
551 each codon type, the codon occupancy ratio is defined as the ratio of measured counts to
552 expected counts. Expected counts are simply proportional to the frequency of that particular
553 codon in the gene. The final reported codon occupancy ratio is the average of codon occupancy
554 ratios from all genes considered weighted equally.
555   We calculated ribosome counts along a transcript using reads per million (RPM). Only
556 transcripts having total counts over 10 TPM and containing more than 100 codons were
557 considered. Total counts after filtering were normalized to one million reads. The selected genes
558 account for more than 85% of the total counts. The ribosome counts are the sum of RPM at
559 each position.
560   To do the first and second halves comparison of ribosome counts of genes, the same set
561 of genes and trimming processing for codon occupancy were used. The RPM from positions
562 were summed up based on its assigned location (first or second half) on the transcript and plot
563 against each other.
564

565 Code availability

566 All the codes used for data analysis in this paper are available upon request.

567 Data availability

568 All the data used to reach the conclusion of this paper will be made available upon publication
569 on SRA BioProject with submission number SUB3256640.