

LETTER

EF4 knockout *E. coli* cells exhibit lower levels of cellular biosynthesis under acidic stress

Dear Editor,

Recently, a novel elongation factor, termed EF4, was identified in *E. coli* and shown to catalyze the back translocation of the E- and P-tRNAs (tRNA at E and P sites) to A- and P-sites (Qin et al., 2006). Despite a great number of recent biochemical studies, little remains known as to the precise physiological role of EF4 *in vivo*. In *E. coli*, 80% of EF4 molecules are associated with the cell membrane under optimal conditions, but when cells grow at pH 6 in media containing 100 mmol/L MgCl₂, this percentage is reduced to less than 20% (Pech et al., 2011). Furthermore, it was revealed that overexpression of EF4 in *E. coli* severely affected cell growth (Qin et al., 2006). However, EF4 knockout (Δ EF4, KO) cells exhibited no apparent phenotype in rich LB medium (Shoji et al., 2010). Under certain stresses, such as low temperature or high ionic stress, Δ EF4 cells were out-competed by wildtype (WT) cells within a short period of time (Shoji et al., 2010; Pech et al., 2011).

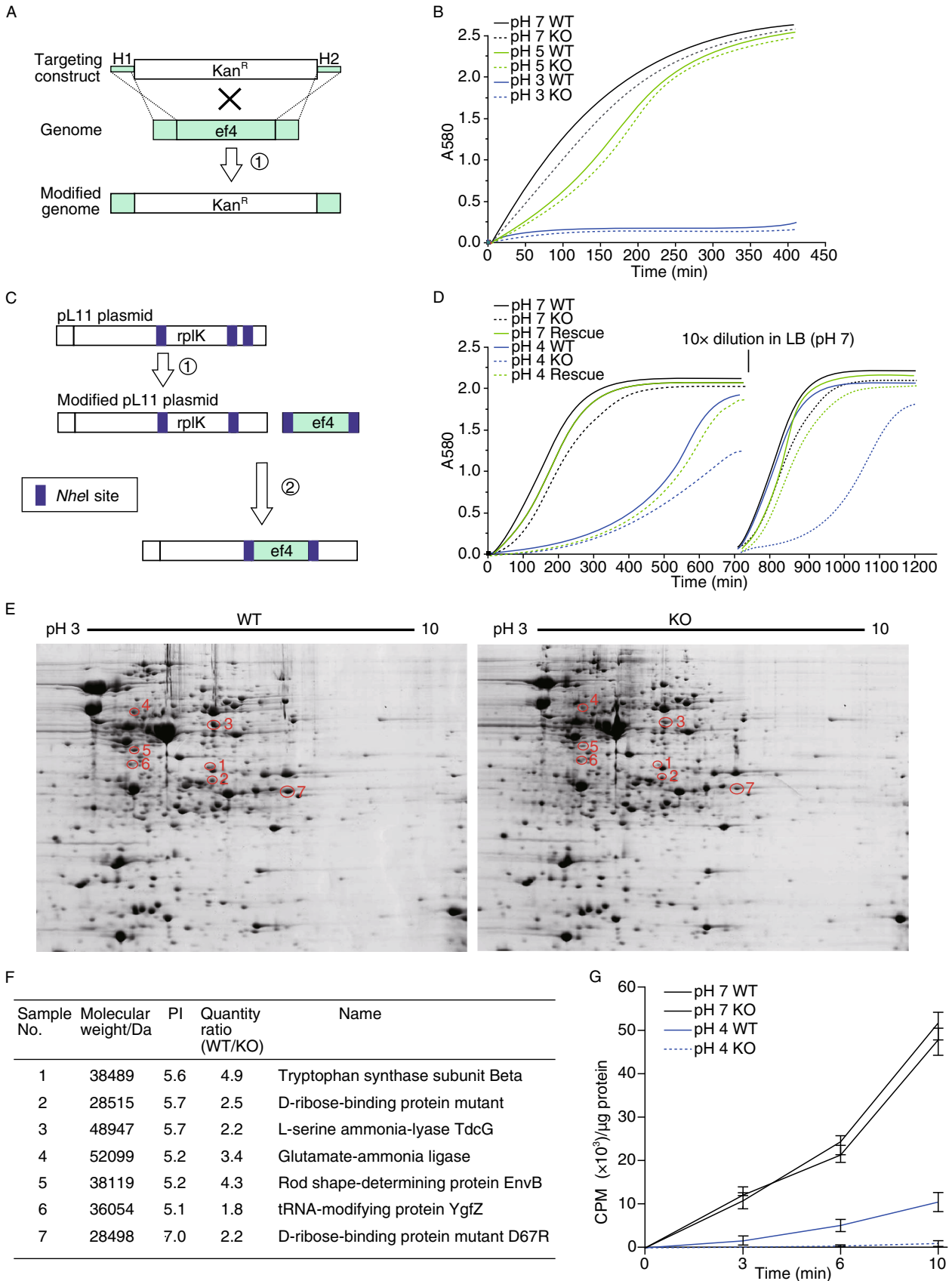
The only organism for which EF4 was shown to be essential is the gastritis pathogen *Helicobacter pylori*. A systematic knockout analysis in *H. pylori* revealed that EF4 was essential for cell survival at hostile pH levels close to the acidic conditions in the stomach (Bijlsma, 2000). We therefore wondered if low pH values represent a stress condition under which EF4 is required in *E. coli*. Because the *ef4* KO strain in Keio was not designed from the wildtype BW25113 (Karim, 2010), we deleted *ef4* in BW25113 strain by ourselves using the λ Red system (Datsenko and Wanner, 2000) (Fig. 1A).

In order to establish a relationship between EF4 dependency and pH conditions, we first prepared LB medium with a pH of 3, 5, or 7 and observed the growth of WT or Δ EF4 (KO) cells at each pH. As shown in Fig. 1B, KO cells grew as well as WT cells at both pH 7 and pH 5, but neither could survive at pH 3. We then found a difference in cell growth at pH 4 (Fig. 1D). WT cells grew vigorously after a relatively long lag phase, and finally reached saturation at an OD of 2 (A_{580}). In contrast, KO cells remained in lag phase for an extended period of time, to finally reach saturation at about OD₅₈₀ = 1. After a 10-fold dilution with pH 7 medium, WT cells regained their normal growth, comparable to that observed for cells grown continuously at pH 7. In contrast, the KO strain

underwent a relatively long lag phase even after increase of pH, and reached saturation at about OD₅₈₀ = 2, as observed for WT cells (Fig. 1D). We further found Δ EF4 cells with a complementary plasmid survived under pH 4 and regain similar growth curves to WT cells under the same conditions (Fig. 1C and 1D). These results clearly demonstrate that EF4 is required for the *E. coli* cell growth at pH 4.

To investigate the overall impact of EF4 deletion on the protein profile of WT or Δ EF4 cells, two-dimensional electrophoresis (2-DE) was employed. No protein changes were detected at pH 7 (Fig. S1). Intriguingly, despite the significant slow-down in growth, only six proteins were downregulated in KO cells at pH 4 (Fig. 1E and 1F). Spots 1–7 were identified as tryptophan synthase subunit β (TrpB), D-ribose-binding protein mutant, glutamate-ammonia ligase, L-serine ammonia-lyase (TdcG), rod shape-determining protein (MreB), tRNA-modifying protein (YgfZ), and D-ribose-binding protein mutant D67R, respectively. TrpB catalyzes the last step of the tryptophan biosynthesis (Lane and Kirschner, 1991). D-ribose-binding protein serves as the primary chemoreceptor for chemotaxis (Bjorkman et al., 1994). Glutamate-ammonia ligase catalyzes the single reaction in the glutamine biosynthesis pathway (Keseler et al., 2013). Three genes (*sdaA*, *sdaB*, *tdcG*) code for three L-serine deaminases in *E. coli*, and participate in glycine, serine, and threonine metabolism and cysteine metabolism, and null mutant cells for all three genes grow normally in glucose minimal medium. However, the mutant cells become very large, and many lyse upon subculturing in the same medium lacking tryptophan (Zhang and Newman, 2008). Deletion of *mreB* disrupts the typical shape of *E. coli*, enlarging them before finally lysing these cells (Kruse et al., 2005). YgfZ protein modifies tRNAs, and its deletion mutant grows slowly relative to WT, especially at low temperatures (Ote et al., 2006). In summary, these proteins are involved in protein biosynthesis, gluconeogenesis, and cell metabolism processes. Intriguingly, however, none of the six proteins are essential for *E. coli* growth. We speculate that KO cells do not display signs of physical damage, and can be rescued from growth arrest upon subculturing in medium of pH 7. Hence, when grown to the same A_{580} , the cells showed similar protein expression profiles.

Other conditions that lead to a slowing in growth upon *ef4* KO were shown to result in decreased translation (Pech



◀ **Figure 1. Effects of low pH on the growth and protein translation of *ef4*-deleted cells.** (A) Construction of a single-gene deletion mutant using the λ Red system. H1 and H2: homologous to chromosomal sequences of the deleted gene; Step ① indicates that the λ -mediated recombination. The targeting construct containing selection marker Kar^R was transferred to strain BW25113, which contained plasmids expressing the components of λ Red system. Then homologous recombination was performed, and the genomic *ef4* gene was replaced by Kar^R . Finally, the resistance gene was eliminated using a helper plasmid. (B) *E. coli* WT cells and $\Delta EF4$ cells (KO) were grown in LB medium at pH 3, 5 or 7. (C) Construction of the EF4 rescues plasmid. Step ① indicates the redundant *NheI* restriction site of pL11 plasmid containing tac promoter was mutated by PCR amplification. Step ② indicates the *ef4* gene amplified from *E. coli* genomic DNA and mutant pL11 digested by *NheI* enzyme before ligation to form the EF4 rescue plasmid. (D) *E. coli* WT cells, $\Delta EF4$ cells, and rescued cells were grown in LB medium at either pH 4 or pH 7. After cells reached stationary phase, each of the samples was diluted 10 times in LB medium (pH 7). (E) 2-DE images of total proteins in *E. coli* at pH 4. The number in red circle represents the number of proteins downregulated in $\Delta EF4$ cells. (F) Quantitative analysis of differentially expressed protein spots from (E). (G) Analysis of translation efficiency *in vivo* by ^{35}S -methionine incorporation. At times indicated, samples were taken from each culture, and the incorporation of radioactive material was determined.

et al., 2011). Similar phenotypes were observed in yeast, where EF4 was found to be located inside the mitochondria (Bauerschmitt et al., 2008). In yeast, loss of EF4 function caused growth defects under conditions of low temperature and starvation, and importantly, decreased mitochondrial translation (Bauerschmitt et al., 2008). In order to investigate whether translation was decreased in our EF4 KO strain at pH 4 we measured the ^{35}S -methionine incorporation rate to assay protein translation *in vivo*. Our analysis revealed that protein synthesis was inhibited at pH 4 even in WT cells, with levels of protein synthesis at about 20% relative to that at pH 7 (Fig. 1G). This result was consistent with the slower growth found in WT cells at pH 4. Under normal pH 7 conditions, loss of EF4 made no significant difference (Fig. 1G). Taken together, these findings suggested a translation defect present in the $\Delta EF4$ strain at pH 4. As a consequence, fewer amid acids and tRNAs are needed, which in turn induces a decrease in expression of proteins participating in these biosynthesis processes, such as TrpB, glutamate-ammonia ligase, TdcG, and YgfZ. This decrease in translation further impairs the response of *E. coli* cells to the acidic growth conditions, and the expression of proteins required directly or indirectly for adaption to this environment also changed (Fig. 1E and 1F). Collectively, our results suggested that the lack of EF4 affects translation and growth only in unfavorable conditions. Importantly, its high degree of sequence conservation throughout evolution indicates that EF4 may play

essential roles for the survival in unfavorable growth conditions other than extreme temperature, high metal concentrations, or low pH, and be in part an evolutionary response to temporary/transient unfavorable conditions.

Because of the association of EF4 with ribosomal movement, and the roles of EF4 in translational processes, we examined the polysome patterns of $\Delta EF4$ cells throughout the complete growth process, including lag phase ($A_{580} = 0.1$), log phase ($A_{580} = 0.4-0.6$), semi-log phase ($A_{580} = 0.8$), and stationary phase ($A_{580} = 1.0$). When cells grew at pH 7, the polysome profile of $\Delta EF4$ cells was similar to that of WT cells (Qin et al., 2006). Cells in the log phase ($A_{580} = 0.4-0.6$) had an active translation system and protein biosynthesis was efficient. All the classical peaks of ribosomes or ribosomal subunits were present under normal conditions pH 7 (Fig. 2A and 2C). When the pH was lowered to 4, WT cells showed the similar polysome pattern to that at pH 7 (Fig. 2A and 2B). However, the quantity of ribosomes in $\Delta EF4$ cells was reduced to 40% of that at $A_{580} = 0.1$, and some non-classical ribosomal subunits appeared as indicated with arrows in the top panel of Fig. 2D. Compared to the position of the 30S peak in Fig. 2C, the non-classical peak had a smaller sedimentation coefficient, indicating that the biogenesis of the 30S subunit was hindered. This situation became more profound when A_{580} reached 0.4. In addition to premature 30S subunits, there were premature 50S peaks, as shown in panel 2 of Fig. 2D. As A_{580} reached 0.6, there were no further non-classical peaks, but the total quantity of ribosomes did not increase relative to that at $A_{580} = 0.1$. In addition, the ratio between polysome and 70S ribosomes suggests that the translation efficiency was low. At $A_{580} \geq 0.8$, $\Delta EF4$ cells had the same polysome pattern, but exhibited lower levels of polysome and 70S ribosomes than that at pH 7, and the total quantity of ribosomes was much lower than the 4S fraction (Fig. 2D). We also noted that the total quantity of ribosomes in $\Delta EF4$ cells from different growth phases was always much lower than that of 4S, which represents the background absorbance of cell lysates at 260 nm (Fig. 2). Our results suggested that $\Delta EF4$ cells took 10 h to overcome subunit maturation defects, reflecting the inhibition of ribosomal biogenesis in $\Delta EF4$ cells at pH 4. In contrast, the WT strain succeeded in overcoming low pH-induced problems, even though the lag phase was prolonged to 6 h (Figs. 1 and 2). This data also provides a possible explanation for the null-phenotype observed by Shoji et al. (Shoji et al., 2010), who analyzed pH conditions ranging from 3.5 to 12. The difference observed here between the two strains only became apparent after 7 h of culture. In addition, the 70S ribosomes and the 30S/50S subunits had reached an equilibrium indicating that the translation machinery will not work efficiently (Nierhaus and Wilson, 2004). In addition, rescued $\Delta EF4$ cells showed classical polysome patterns (Fig. 2E and 2F).

In summary, our results showed slower growth of $\Delta EF4$ cells and a lower level of intracellular biosynthesis under

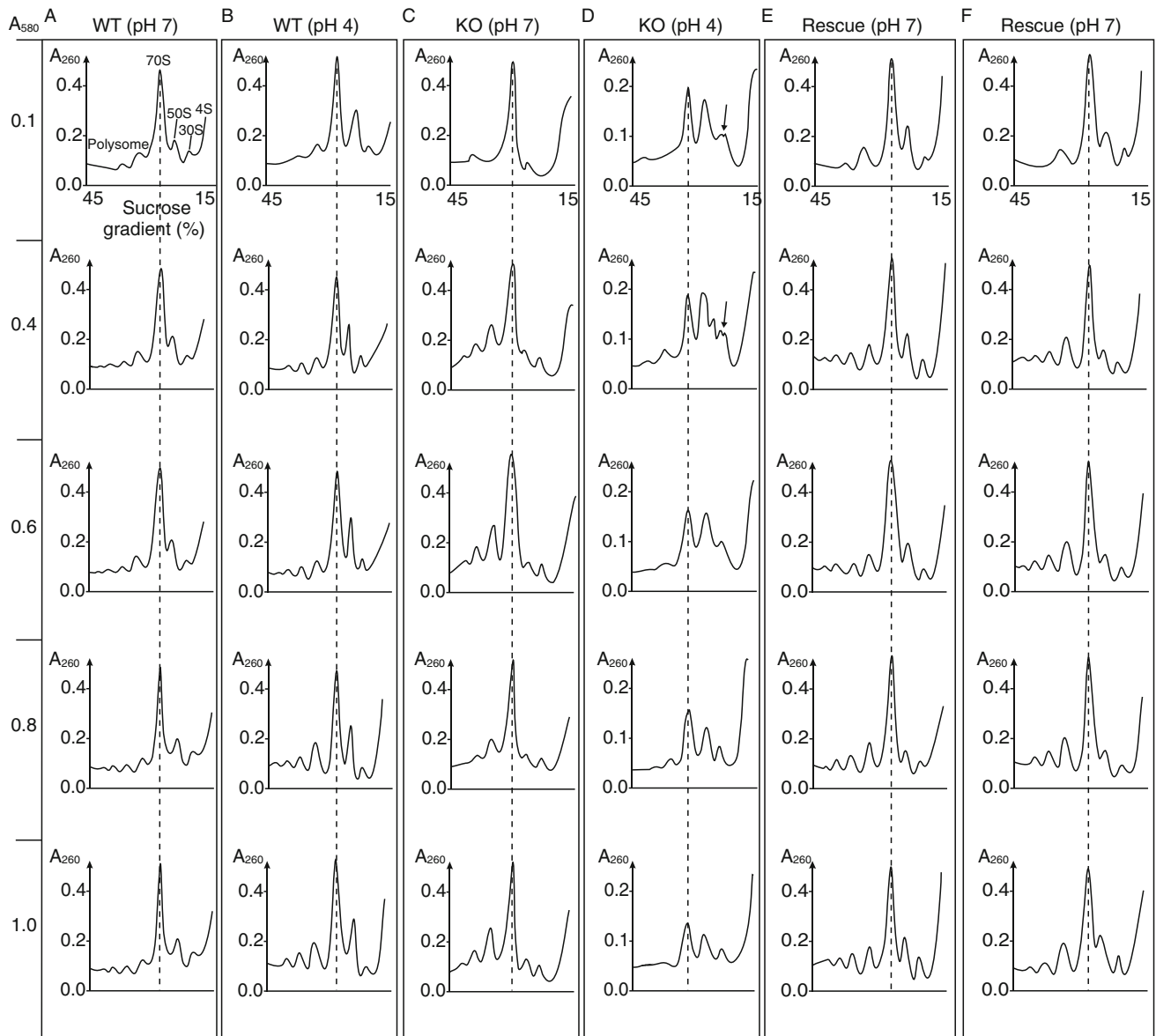


Figure 2. Polysome profile of Δ EF4 *E. coli* cells during different growth phases at pH 7 and pH 4. Cell extracts were prepared from Δ EF4 strains during different growth phases ($OD_{580} = 0.1, 0.4, 0.6, 0.8$ or 1.0). The gradient was fractionated while monitoring the absorbance at 260 nm (A_{260}). The dotted vertical line indicates the monomer peak. (A and B) Polysome profiles of WT cells grown at either pH 7 or pH 4. The ribosome peak-subunits (30S, 50S), monomer (70S) and polysome, and 4S peak are indicated. The polysome pattern of cells growing at pH 4 showed no obvious difference to those from cells growing under normal conditions. (C) Polysome profiles of Δ EF4 *E. coli* cells grown at pH 7. (D) Polysome profile of Δ EF4 *E. coli* cells grown at pH 4. Black arrows indicate non-classical ribosomal subunits. Note that units on the Y-axis for pH 4 samples are half of those for the pH 7 sample. The quantity of ribosomes in pH 4 samples was significantly reduced compared to that in pH 7 with only few polysomes detected. (E) Polysome profile of KO cells overexpressing EF4 grown at pH 7. The ribosome peaks showed nearly the same classical patterns as WT at pH 7. (F) Polysome profile of EF4-rescued KO cells grown at pH 4. The polysome quantity and patterns under this condition did not change significantly compared to those of cells grown at pH 7. However, the polysome quantity was higher than that in Δ EF4 *E. coli* cells at pH 4, with polysome patterns more classical.

acidic stress. In agreement with growth defects, the EF4-depleted strain exhibited a very slower protein translation at pH 4 *in vivo*. However, there was no difference between WT and Δ EF4 cells at normal condition pH 7 (Figs. 1 and 2). This

phenotype suggested that EF4 plays an essential and specific role in increasing the fitness of *E. coli* under unfavorable to extreme environmental conditions, where it ensures appropriate/adequate regulation of protein synthesis. Our

data presented here provide the first time evidence that the translation factor EF4, contribute to protein synthesis and cell growth at unfavorable conditions, explaining why no apparent role was described at optimal growth conditions before.

FOOTNOTES

This work was supported by grants to Y.Q. from the National Basic Research Program (973 Program) (Nos. 2012CB911001 and 2013CB531200) and the National Natural Science Foundation of China (Grant Nos. 31170756, 31322015, 31270847, and 31000596), CAS grant (KSZD-EW-Z-003), National Laboratory of Biomacromolecules in Institute of Biophysics. We thank Dejiu Zhang for help with the manuscript preparation, and Dr. Joy Fleming for critical reading of the manuscript. We thank Dr. Yong Tao of the Institute of Microbiology, CAS for his kind gifts of plasmids for *E. coli* λ Red-mediated homologous recombination.

Fang Yang, Zhikai Li, Jia Hao, and Yan Qin declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

Fang Yang^{1,2}, Zhikai Li¹, Jia Hao¹, Yan Qin¹✉

¹ Laboratory of Noncoding RNA, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

² University of Chinese Academy of Sciences, Beijing 100049, China

✉ Correspondence: qiny@ibp.ac.cn (Y. Qin)

OPEN ACCESS

This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

REFERENCES

- Bauerschmitt H, Funes S, Herrmann JM (2008) The membrane-bound GTPase Guf1 promotes mitochondrial protein synthesis under suboptimal conditions. *J Biol Chem* 283:17139–17146
- Bijlsma JJE (2000) Identification of loci essential for the growth of helicobacter pylori under acidic conditions. *J Infect Dis* 182:1566–1569

- Bjorkman AJ, Binnie RA, Zhang H, Cole LB, Hermodson MA, Mowbray SL (1994) Probing protein-protein interactions. The ribose-binding protein in bacterial transport and chemotaxis. *J Biol Chem* 269:30206–30211
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645
- Karim Z (2010) The importance of the ribosomal elongation factor 4 (LepA). PhD Dissertation, Freie Universität Berlin, Berlin
- Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, Bonavides-Martinez C, Fulcher C, Huerta AM, Kothari A, Krummenacker M et al (2013) EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res* 41:D605–D612
- Kruse T, Bork-Jensen J, Gerdes K (2005) The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. *Mol Microbiol* 55:78–89
- Lane AN, Kirschner K (1991) Mechanism of the physiological reaction catalyzed by tryptophan synthase from *Escherichia coli*. *Biochemistry* 30:479–484
- Nierhaus KH, Wilson DN (2004) Protein synthesis and ribosome structure: translating the genome (LibreDigital). Wiley, Weinheim
- Ote T, Hashimoto M, Ikeuchi Y, Su'etsugu M, Suzuki T, Katayama T, Kato J (2006) Involvement of the *Escherichia coli* folate-binding protein YgfZ in RNA modification and regulation of chromosomal replication initiation. *Mol Microbiol* 59:265–275
- Pech M, Karim Z, Yamamoto H, Kitakawa M, Qin Y, Nierhaus KH (2011) Elongation factor 4 (EF4/LepA) accelerates protein synthesis at increased Mg²⁺ concentrations. *Proc Natl Acad Sci USA* 108:3199–3203
- Qin Y, Polacek N, Vesper O, Staub E, Einfeldt E, Wilson DN, Nierhaus KH (2006) The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. *Cell* 127:721–733
- Shoji S, Janssen BD, Hayes CS, Fredrick K (2010) Translation factor LepA contributes to tellurite resistance in *Escherichia coli* but plays no apparent role in the fidelity of protein synthesis. *Biochimie* 92:157–163
- Zhang X, Newman E (2008) Deficiency in l-serine deaminase results in abnormal growth and cell division of *Escherichia coli* K-12. *Mol Microbiol* 69:870–881

Electronic supplementary material The online version of this article (doi:10.1007/s13238-014-0050-3) contains supplementary material, which is available to authorized users.