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Importance of the 5 S rRNA-binding Ribosomal Proteins for Cell Viability and Translation in *Escherichia coli*

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Summary

A specific complex of 5 S rRNA and several ribosomal proteins is an integral part of ribosomes in all living organisms. Here we studied the importance of *Escherichia coli* genes *rplE*, *rplR* and *rplY*, encoding 5 S rRNA-binding ribosomal proteins L5, L18 and L25, respectively, for cell growth, viability and translation. Using recombineering to create gene replacements in the *E. coli* chromosome, it was shown that *rplE* and *rplR* are essential for cell viability, whereas cells deleted for *rplY* are viable, but grow noticeably slower than the parental strain. The slow growth of these L25-defective cells can be stimulated by a plasmid expressing the *rplY* gene and also by a plasmid bearing the gene for homologous to L25 general stress protein CTC from *Bacillus subtilis*. The *rplY* mutant ribosomes are physically normal and contain all ribosomal proteins except L25. The ribosomes from L25-defective and parental cells translate *in vitro* at the same rate either poly(U) or natural mRNA. The difference observed was that the mutant ribosomes synthesized less natural polypeptide, compared to wild type ribosomes both *in vivo* and *in vitro*. We speculate that the defect is at the ribosome recycling step.

Keywords

5 S rRNA-binding proteins; ribosomal protein L25; ribosome; translation; *Escherichia coli*

INTRODUCTION

The 5 S rRNA-protein complex, which forms an autonomous structural domain of the ribosome, is essential for ribosome function. During the last 30 years a great body of experimental data has been accumulated on the importance of the complex and its formation as part of an active ribosomal particle. Thus, the total *in vitro* reconstitution of the 50 S ribosomal subunit performed in the absence of 5 S rRNA led to dramatic consequences:^{1,2} the ribosomes with such 50 S subunits were unable to synthesize a polypeptide chain and only retained EF-G-dependent GTPase activity. Removing more than one of the 5 S rRNA genes (out of eight) in *Escherichia coli* resulted in reduced cell growth.³ On the basis of data on

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cross-linking of the 5 S rRNA and the 23 S rRNA within the ribosome it was suggested that 5 S rRNA might link the peptidyl transferase and GTPase centres.⁴

Three 5 S rRNA-binding proteins have been found in the *E. coli* ribosome: L5, L18 and L25.^{5–7} Later, the *E. coli* 5S rRNA-binding ribosomal proteins L5 and L18 were shown to be important for incorporation of the 5S rRNA into the 50S ribosomal subunit *in vitro*.⁸ Many spontaneous *E. coli* mutants lacking one or two ribosomal proteins have been found and characterized (see for review).⁹ However, no mutant lacking any of the 5 S rRNA-binding proteins was found. This was indirect evidence that 5 S rRNA-binding proteins are important for ribosome functioning. Nevertheless, despite extensive *in vitro* studies, a specific role of the 5 S rRNA-protein complex in the ribosome remains unknown (see for review).^{10–12}

Experiments on the binding of ribosomal proteins to isolated 5 S rRNA have identified two to three 5 S rRNA-binding proteins in Bacteria (homologues of *E. coli* L5 and L18 are found in all cases), two in Archaea and one in Eukarya (see for review).¹³ Two archaeal 5 S rRNA-binding proteins appeared to be homologous to *E. coli* ribosomal proteins L5 and L18.¹⁴ A protein homologous to L18 was the only 5 S RNA-binding protein isolated from eukaryal ribosomes.¹⁵ However, a gene encoding a protein homologous to the bacterial ribosomal protein L5 was also found in eukaryotic genomes.¹⁶ Thus, all three domains of life have the two conserved ribosomal proteins, L5 and L18.

Genes encoding proteins homologous to *E. coli* L25 were found in Bacteria only.¹⁷ At present, the ribosomes having such a protein were isolated and studied from three bacteria: *E. coli* (protein L25), *Thermus thermophilus* (protein TL5) and *Deinococcus radiodurans* (protein DraCTC).^{5,18,19} These three proteins have homologues in different bacteria that belong to a so-called CTC-family because of their homology to the general stress protein CTC of *Bacillus subtilis* (BsuCTC).²⁰ The BsuCTC protein also specifically binds 5 S rRNA *in vitro*,²¹ it is accumulated in cells only under various stress conditions,²² and can be found in the ribosomal fraction.²³ However, in vegetative *B. subtilis* cells this protein is almost absent.^{22,24}

Although, the genome of another representative of Bacillaceae, *Bacillus stearothermophilus*, contains a gene encoding CTC protein,²⁵ the protein was not found in ribosomes.⁵ Moreover, genomes of other bacterial families such as Streptococcaceae, Mycoplasmataceae and also some species of Bacillaceae are seemed to contain no corresponding gene.^{17,25} This rises a question whether the proteins of the CTC family, including L25, are essential.

In the present work we studied the importance of each of the three *E. coli* 5 S rRNA-binding proteins, L5, L18 and L25, for cell viability and growth. Our data indicate that knockouts of L5 and L18 genes are lethal, whereas cells lacking the gene for L25 protein are viable, but grow more slowly than the parental strain. Detailed analysis revealed that ribosomes from the *rplY* knockout contain a full set of ribosomal proteins except L25 and that these ribosomes, although physically normal, were impaired for protein biosynthesis.

RESULTS AND DISCUSSION

Disruption of *rplE*, *rplR* and *rplY* genes in *E. coli* using recombineering

The *E. coli* 5 S RNA-binding protein genes *rplE*, *rplR*, and *rplY* (encoding ribosomal proteins L5, L18 and L25, correspondingly), were disrupted by recombineering.²⁶ This technique allows precise replacement of a target chromosomal gene open reading frame by a drug-resistance gene *orf* (Figure 1(a)) without nonspecific polar effects on the expression of genes flanking the replaced gene.^{27,28} Each of the three 5 S rRNA-binding protein genes has been inactivated by precisely replacing their *orfs* with the *orf* of chloramphenicol acetyltransferase cassette, *cat* (Figure 1). After recombination (described in Materials and Methods), two types of recombinants distinguished by the level of recombination were found. For one type, as

represented by the *rplY*<>*cat* replacement, a high recombination efficiency was observed ($\sim 10^4$ colonies/ 10^8 viable cells) similar to what is found for knocking out nonessential genes in *E. coli*.²⁸ The PCR analysis of the *rplY* chromosomal region in recombinant cells confirmed the *rplY*<>*cat* replacement in numerous slow-growing Cm^R colonies (Figure 1(b), Table 1). This recombinant phenotype features a growth-impaired gene knockout (see Materials and Methods).

For the second type, as represented by *rplR*<>*cat* and *rplE*<>*cat* replacements, only ~ 10 Cm^R colonies per 10^8 viable cells were observed. By PCR analysis, these recombinants carried the *rplR*<>*cat* and *rplE*<>*cat* replacements, but also contained the intact *rplR* or *rplE* allele, respectively (Figure 1(b), right panel). It is known that subpopulations within *E. coli* cultures contain cells with large regions of the chromosome duplicated.²⁹ Such partial diploid cells occur at a frequency $\sim 10^{-2}$ for any one region of the chromosome, and we have previously shown that essential genes in such diploid regions are targeted for gene replacement.^{27,28} Thus, two of the three 5 S rRNA-binding proteins, L5 and L18, appear to be essential.

By providing a cloned open reading frame of the *rplE* gene on an expression plasmid and repeating the recombination to replace the chromosomal *rplE* with *cat* (Figure 2), we found a higher recombination level and all recombinants tested by PCR analysis contained only the *cat*-replaced copy of *rplE* (Table 1). This confirmed that *rplE* was essential and also demonstrated that the *spc* operon control element,³⁰ which is located within the 5' proximal region of *rplE* gene (Figure 2(a)) is not essential.

It has been shown that ribosomes lacking 5 S rRNA are unable to synthesize polypeptide.^{1,2} Moreover, the *E. coli* 5 S rRNA-binding ribosomal proteins L5 and L18 were shown to be important for incorporation of the 5 S rRNA into the 50 S ribosomal subunit *in vitro*.⁸ Our finding that *E. coli* L5 and L18 are essential for cell viability is in good agreement with those *in vitro* experiments. Most likely, these two ribosomal proteins, which are found in all three domains of life, are indispensable for *in vivo* assembly of the active ribosome. The third *E. coli* 5 S rRNA-binding ribosomal protein, L25, and its homologues are found only in bacteria and not even in all bacteria.^{17,25} Our experiments show that L25 is dispensable for viability, but nevertheless is required for normal *E. coli* cell growth.

Growth of *rplY* knockout and *in vivo* analysis of L25 function

It was observed that the *rplY* knockout grew but more slowly than the parental W3110 cells over a wide range of temperatures, from 20 to 42°C. Under standard conditions of growth (LB broth, 37°C), the generation time for these L25-defective cells was approximately 72 minutes while it was 32 minutes for W3110 (Table 2).

Growth of the *rplY* knockout was complemented by the expression of the plasmid-cloned *rplY* gene (Figure 3, Table 2). This shows that the growth defect of the *rplY* knockout is specifically caused by the absence of L25 function, which for the most part can be restored by *in-trans* expression of L25.

Interestingly, we have also been able to partially complement the growth defect of the *rplY* knockout by *in-trans* expression of *B. subtilis* general stress protein CTC. It has been shown before, that the L25 protein is homologous to the N-terminal domain of CTC protein of *B. subtilis*.²⁰ The CTC protein specifically binds to bacterial 5 S rRNA²¹ and can be found in the ribosomal fraction of *B. subtilis* cells.²³ The expression of either the entire CTC protein or its N-terminal domain stimulated the growth of the knockout mutant, albeit less efficiently than the *E. coli* L25 protein (Figure 3, Table 2). These data show for the first time that the general stress protein CTC of *B. subtilis* is a functional homologue of *E. coli* ribosomal protein L25.

L25 is a ribosomal protein, which, due to its functional homology to the general stress protein CTC of *B. subtilis*, might also be involved with the stress response of the cell. Both L25 activities, as a ribosomal protein and a potential stress protein, have been analyzed *in vivo* in this work.

The potential stress functions of L25 have been analyzed by growing the *rplY* knockout under a variety of stressful conditions for cell growth: cold, heat shock and oxidative stress. As these stressful conditions were shown to induce the synthesis of the CTC protein in *B. subtilis* cells²² one might expect that *E. coli* L25-defective cells would become less resistant to such growth conditions compared to the parental strain. It turned out, that there were no significant differences between the *rplY* knockout and the parent cells in response to any of these stressful conditions. Hence, it is likely, that L25 is not involved with a cell stress response, at least in *E. coli*.

We measured the *in vivo* protein-synthesizing ability of the *rplY* knockout, using the standard β -galactosidase assay. The *rplY* knockout accumulated β -galactosidase approximately 5 times slower than the parent strain (Figure 4). At the same time as expected, there was no defect noticed in the *rplY* knockout regarding the major metabolic pathways: the basic metabolic microarray analysis³¹ did not reveal a difference between the mutant and the wild type cells (data not shown).

Thus, the lack of the ribosomal protein L25 decreases the protein-synthesizing capacity of the cell, which has been the only difference between L25 knockout and parental cells observed *in vivo* in our tests. We believe that this is the main reason for the slow growth of the L25-defective cells.

Analysis of L25-defective ribosomes

Ribosomes from the L25-defective strain were analyzed by sucrose density gradient centrifugation. The ribosomes retained compactness comparable to that of the wild-type ribosomes (Figure 5(a)). L25-defective 50 S ribosomal subunits were able to associate normally with 30 S ribosomal subunit to form a 70 S particle. Protein content of the mutant ribosomes was analyzed by two-dimensional gel-electrophoresis. Except for L25 protein, all other individual ribosomal proteins are present in 70 S ribosomes and found in similar amounts as in the wild-type ribosomes (Figure 5(b)). Thus, L25 is not an “assembly” protein and does not influence the incorporation of other proteins into the large ribosomal subunit. So, our data suggest that the ribosomes lacking L25 are physically normal, and L25 most probably is not required for either 50 S or 70 S ribosome formation.

Protein-synthetic activity of L25-defective ribosomes

Two approaches were applied to analyze the protein-synthesizing activity of L25-defective ribosomes *in vitro*. First, translation of poly(U) template, which measures the rate of elongation, and second, translation of a natural mRNA, which involves an initiation, termination and ribosome recycling, as well as the elongation step of translation.

Ribosomes from both the L25-defective and wild type cells were able to translate poly(U) template at virtually the same rate (Figure 6). The data show that both, L25 mutant and wild type ribosomes perform polypeptide chain elongation at the same efficiency. Next, we performed *in vitro* translation experiments using natural mRNAs for green fluorescent protein (GFP, Figure 7(a)) and luciferase (Figure 7(b)). In these experiments, the luciferase activity began to appear at the same time point (approximately 3 min) in reaction mixtures, containing either the L25-defective or wild type ribosomes. Since luciferase becomes active only if it is completely synthesized and released from the ribosome.³², we may conclude, that the entire

process of natural mRNA translation (*i.e.*, initiation, elongation, and termination) proceeds at the same rate with the wild type and L25 mutant ribosomes. The only difference observed between the wild type and the mutant ribosomes was in the amount of synthesized protein: L25-defective ribosomes produced 1.5 to 2 times lower levels of both the luciferase and GFP proteins than the wild type ribosomes. This result is in good agreement with our *in vivo* data comparing β -galactosidase translation levels (Figure 4).

One possible reason for the low yield of translation products in the L25-defective cells might be translation errors, leading to accumulation of inactive protein or abortive peptides. Monitoring the ratios of the active GFP (by fluorescence) to the total GFP polypeptide (by [^{14}C]Leu incorporation) during linear synthesis (6–30 min) shows that the ratios for the mutant and wild ribosomes coincided. This means that specific activity of GFP synthesized in both cases is the same. It is seen in Figure 7(a) (right panel) that the majority of synthesized polypeptide is represented by the full-length GFP and that the distribution of translation products synthesized by the mutant and wild type ribosomes is the same. The main conclusion from these *in vitro* translation tests is that L25-defective ribosomes are able to synthesize active full-length protein with a rate similar to that of the wild type ribosomes.

Is the ribosome recycling step defective in the L25 mutant?

It is interesting that results similar to ours were obtained for an *E. coli* mutant having a thermosensitive ribosome recycling factor, RRF.³³ When this RRF mutant is grown at non-permissive temperatures, decreasing the level of RRF, the protein synthesis *in vivo* is depressed and *in vitro* translation of natural mRNA by ribosomal fraction from such cells is reduced. However, poly(U) translation by the RRF mutant ribosomal fraction was unaltered; the poly (U) assay does not require ribosome recycling and only measures elongation rate.

It is commonly recognized that after protein release, 70S ribosomes normally dissociate into subunits and are freed from ligands (see for review).^{34, 35} This ribosome recycling provides the bacterial cell with 30S and 50S ribosomal subunits competent for initiating the synthesis of new protein molecules.³⁶ Thus, defective ribosome recycling should decrease the productivity of protein synthesis. At this point, we have no direct evidence that L25-defective ribosomes are impaired in the recycling process. However, on the basis of the above-mentioned data we hypothesize that the defect in protein synthesis in cells lacking L25 might be at ribosome recycling at the post-termination stage.

MATERIALS AND METHODS

Strains, plasmids and bacteriological techniques

The strains and plasmids used in this work are listed in table S1. Standard techniques for P1 transduction and β -galactosidase assay were used.³⁹ For β -galactosidase assay cells were grown to $\text{OD}_{600}=0.2$ and production of the enzyme was induced by adding isopropyl- β -D-thiogalactoside to 1 mM concentration. The enzyme accumulation in the culture was monitored using color reaction of 2-nitrophenyl- β -D-galactopyranoside conversion. Enzyme activity was then normalized to the cell mass (OD_{600}). Cells were grown in LB medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin (Kan) or 10 $\mu\text{g}/\text{ml}$ chloramphenicol (Cm) when needed.

Inactivation of *E. coli* *rplE*, *rplR* and *rplY* genes

The *E. coli* genes *rplE*, *rplR* and *rplY*, were inactivated by recombineering²⁸ (Figure 1(a)). Recombineering is based on the λ *red*-dependent recombination of DNA substrates into the chromosomes or plasmids of enteric bacteria⁴⁰ using short 40–50nt flanking homologies. Briefly, *E. coli* DY330 cells were induced for Red functions at $A_{600} \sim 0.5$ and prepared for electroporation with 300 ng of appropriate linear DNA cassette. The cells were recovered in

0.9 ml LB, grown for 2–3 h at 32°C, spread on LB agar plates supplemented with Cm and incubated for 1–3 days at 32–34°C to reveal Cm-resistant recombinants. The *cat* cassette for gene disruption was made by PCR amplification of *cat orf* (originated from pCR-Script Cam (Stratagene) using Platinum Taq DNA Polymerase High Fidelity Kit (Invitrogen) with primers (Table S2) that contained a 39–40nt sequence homologous to chromosomal regions surrounding the gene *orf* targeted for disruption followed by a 22–25nt priming sequence complementary to the 5' and 3' regions of the drug *orf*. The resulting cassettes should have a *cat orf* flanked by the 39–40nt regions of homology (Figure 1) that are needed for its recombination into chromosomal regions of the respective gene, so that the chromosomal *orf* is replaced with the drug *orf*.

For *rplE* replacement, the *cat* cassette was made using primers, *rplE-cat-F* and *rplE-cat-R*. For *rplR* replacement, the *cat* cassette was made using primers, *rplR-cat-F*, *rplR-cat-R*. To replace *rplY*, the *cat* cassette was made using primers, *rplY-cat-F* and *rplY-cat-R*.

Analysis of gene replacements

Recombinant colonies were purified on LB-Cm plates at 34°C. The individual colonies from the streaks were suspended in 30 µl of sterile water and 1 µl used as a template for PCR with two check primers flanking the target gene (Table S2, Figure 1(a)). For *rplE*<>*cat*, the verifying forward primer was *rplE-check-F*, and reverse primer was *rplE-check-R*; to verify *rplR*<>*cat* replacement the pair of primers used was *rplR-check-F* and *rplR-check-R*; for examination of *rplY*<>*cat* replacement those were *rplY-check-F* and *rplY-check-R*.

Cloning genes by gap repair recombination

The *E. coli rplE* and *rplY* genes were cloned by gene retrieval to a pCR-Blunt plasmid (Invitrogen) (generating pKAB122 and pKAB101 plasmids, respectively) from the chromosome using the gap repair⁴¹ version of recombineering.²⁸ The linear pCR plasmid, including *kan* and the origin, was amplified by PCR with primers bearing 39–40-nucleotide extensions homologous to the very 5'-and 3'-coding regions of the genes to be cloned (Table S2). This should allow the cloned *orfs* to start directly from the AUG initiation codon of the *lacZ* gene present in the pCR plasmid, thereby acquiring the promoter and translation elements of *lacZ*. Retrieval was carried out in *E. coli* strain DY330 by recombineering as described above. The desired plasmids were selected as Kan^R and were identified by restriction analysis of the insert and verified by DNA sequencing. The pairs of primers for *rplE* and *rplY* cloning were *rplE-pCR-F* with *rplE-pCR-R* and *rplY-pCR-F* with *rplY-pCR-R*, respectively.

We cloned the entire *ctc* gene, as well as its 5'-portion encoding N-terminal domain (1–94 amino acids) into pCR-Blunt by recombineering (plasmids pKAB110 and pKAB104, respectively). In this case, the linear pCR-Blunt was used directly from the company and coelectroporated with linear PCR products of the *ctc* gene fragments carrying flanking homologies to PCR-Blunt so as to allow end-joining of the linear vector in DY330. The pairs of primers for the entire *ctc* gene or its 5'-portion were *ctc-pCR-F* and *ctc-pCR-R* or *ctc-pCR-F* and *ctcN-pCR-R*, respectively. Cells were outgrown after electroporation in LB broth for 2 hrs at 30°C, spread on LB plates supplemented with Kan and incubated at 34°C.

Gene essentiality assay

Recombineering can be used for analysis of gene essentiality *via* direct selection²⁷ (Figure 1). Briefly, the assay is based on the high efficiency of recombineering that allows selection for gene knockouts when the essential gene region in the chromosome is duplicated.^{27,28} These duplications occur with a frequency $\sim 10^{-2}$ in a cell population.²⁹ When an essential gene is replaced during recombineering (Figure 1(b)), only these rare partial diploids survive, reflecting the lowered recombination frequency ($< 10^2/10^8$ viable cells). The duplication is

maintained in these cells by positively selecting for the drug marker in the replaced essential gene and the functional wild type allele, *gene orf*<>*drug orf/gene orf*+(Figure 1(b), right column). Nonessential genes are replaced with standard high recombination efficiency ($\sim 10^4/10^8$ viable cells) generating only haploid *gene orf*<>*drug orf* knockouts (Figure 1(b), left column). Consequently, three cell phenotypes can be detected featuring nonessential, essential and growth impaired gene knockouts. The essential and nonessential gene phenotypes are described above. They are characterized by the low and high recombination frequencies, as well as by the partial diploid and the haploid configurations of the replaced genes, respectively. The growth impaired cell gene knockout is represented by a few normally growing colonies amongst thousands of slow growing ones. The rarer normal colonies are duplicated for the replaced gene, whereas the small growth-impaired colonies contain just a single disrupted gene (Figure 1(b), left column).

To analyze the essentiality of the *rplE*, *rplR*, and *rplY* genes, their chromosomal *orfs* were replaced with a *cat* gene *orf* by recombineering, as described above and recombination frequency was scored. The diploid/haploid configuration of replaced genes was determined by the PCR analysis of the targeted chromosomal regions, as above.

Phenotypic analysis of *rplY* knockout

The *rplY*<>*cat* knockout was P1 transduced from KNB212 to W3110 selecting for Cm^R colonies at 37°C. The resulting KNB800 strain contains a precise *rplY orf* deletion in the otherwise wild type background. This was confirmed again by PCR analysis of the KNB800 cells followed by 2-D electrophoretic analysis of the ribosomal proteins.

The growth of the *rplY* knockout was analyzed on LB and M63 agar plates without antibiotic in the 30–42°C range of temperatures. The growth of cells under cold shock conditions was analyzed on LB plates at 15°C for 10 days. The heat shock growth of cells was analyzed at 46°C for 2 days. For oxidative stress, the cells were incubated in LB broth with 1–2 mM H₂O₂ at 37°C, measuring their growth rate at A₆₀₀, as recommended.³⁹ The phenotypic microarray analysis³¹ for major metabolic functions was done as recommended by the manufacturer (Biolog, Inc.), growing cells in the respective microtiter plates at 37°C. The parent wild type cells were always used as a control in these experiments.

Growth complementation of *rplY* knockout with plasmids expressing L25 or CTC

L25-defective cells were transformed by electroporation with plasmids expressing *E. coli* L25 (pKAB101), entire *B. subtilis* CTC protein or its N-terminal fragment (pKAB110 and pKAB104, respectively) and grown at 37°C on LB plates containing Kan. Individual colonies were streaked on the LB-Kan plates and incubated at 30, 37 and 42°C along with control cells, which were wild type and L25-defective strains carrying a blank pCR-Blunt vector (control plasmid). For estimating the growth rate, overnight cultures were obtained from individual colonies, diluted 1/50 into LB broth with Kan and grown at 37°C with shaking at 220 rpm.

Preparation of ribosomes

Ribosomes were obtained according to published procedure⁴² with modifications. *E. coli* cells were grown on LB medium at 37°C in an orbital shaker at 200 rpm to A₆₀₀ ~ 1 o.u., chilled on ice and collected by centrifugation at 14,000 x g and 4°C for 7 min. Cell pellet was resuspended in 1/4 of initial volume of cold buffer (10 mM Tris-HCl (pH 7.5), 200 mM NH₄Cl), centrifuged and kept at –70°C if needed. Cells were disrupted by grinding with Al₂O₃ using 1 ml of cold buffer A (10 mM Tris-HCl (pH 7.5), 200 mM NH₄Cl, 10 mM MgCl₂, 3 mM β-mercaptoethanol) and 2 g of Al₂O₃ per g of cells (wet weight) at 4°C. The ground paste was then suspended in buffer A (~ 2 ml for each gram of cell paste). The cell debris and alumina were removed by double centrifugation at 15,000 × g and 4°C for 30

minutes. The resulting supernatant was loaded over 30% (w/w) sucrose cushion made in buffer A, containing 500 mM NH₄Cl and centrifuged in a TL-100 ultracentrifuge (Beckman-Coulter, USA) in a TLA-100.3 rotor at 380,000 x g for 3 h at 4°C. Ribosomes were dissolved in cold buffer A containing 50 mM NH₄Cl plus 10% glycerol to a approximate concentration of 20 mg/ml, frozen in liquid nitrogen and kept at -70°C.

Analysis of ribosomes and ribosomal proteins

The distribution of ribosomes and ribosomal particles in logarithmically growing cells was analyzed by sucrose gradient centrifugation as follows: cells were grown and harvested as described above. Cell pellet was suspended in 1 volume of cold PS buffer (20 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl₂) and harvested again. Cells were then resuspended in 1/25 volume of cold PS buffer, containing 5 mM β-mercaptoethanol, 0.2 mM EDTA and 1 mg/ml lysozyme, put on ice for 10 minutes and then frozen at -70°C. After defrosting, debris was removed by centrifugation at 15,000 x g at 4°C for 10 min. 3 o.u. at 260 nm of supernatant (approximately 0.1 ml) were loaded onto a 15–30% (w/v) sucrose gradient in PS buffer, containing 5 mM β-mercaptoethanol, 0.2 mM EDTA. The gradients were centrifuged at 24900 rpm (Beckman SW-41 rotor) and 4°C for 14 h.

Proteins were isolated from 300 μg of 70 S ribosomes by acetic-acid extraction⁴³ and fractionated by two-dimensional gel electrophoresis⁴⁴ using acidic-acidic system IV. The gels were stained with Coomassie blue G250.

Preparation of ribosome-free extract and total enzyme fraction

To obtain the total enzyme fraction for cell-free translation of poly(U), the S100 extract was isolated from *E. coli* MRE600 strain and fractionated on DEAE-cellulose as described.⁴⁵

To obtain ribosome-free S100 extract *E. coli* W3110 cells were grown and collected. The cell pellet was suspended in 1/10 of original culture volume of cold buffer A, containing 150 mM NH₄Cl plus 0.1 mM EDTA, and centrifuged for another 20 min. The cells were resuspended in the same buffer (~1 ml for each gram of cells) and disrupted by ultrasonic treatment. Cell debris was removed by two centrifugations at 15,000 x g at 4°C for 15 min. The supernatant was supplemented with NH₄Cl to final concentration of 500 mM, loaded on a sucrose cushion and centrifuged for 1 h as described in “Preparation of ribosomes”. The ribosome-free extract was then dialyzed against buffer (10 mM Tris-acetate (pH 8.2), 100 mM potassium acetate, 10 mM magnesium acetate, 3 mM β-mercaptoethanol, 0.1 mM EDTA), frozen in liquid nitrogen and kept at -70°C.

Cell-free translation of poly(U) (polyuridylic acid) and natural mRNA

Cell-free poly(U)-dependent synthesis of polyphenylalanine was carried out as described.⁴⁶ 10 pmol of 70 S ribosomes, 80 μg of total enzyme fraction protein, 70 μg of poly(U) (Sigma), 50 μg of total tRNA *E. coli* MRE600 (Sigma), 3 mM ATP (Sigma), 0.4 mM GTP (Sigma) and 500 pmol of [¹⁴C]Phe (450 mCi/mmol, Amersham) were incubated in 100 μl of buffer (20 mM Tris- HCl (pH 7.5), 100 mM NH₄Cl, 10 mM MgCl₂, 3 mM β-mercaptoethanol, 0.1 mM EDTA) at 37°C. The reaction was terminated by applying the mixture to a 3MM filter paper (Whatman) treated with 5% trichloroacetic acid (TCA). The filters were boiled for 5 min in 5% TCA, washed with acetone, dried at room temperature and counted for ¹⁴C in Supersolve X scintillation liquid (Koch-Light LTD) in a scintillation counter Beckman LS 1801 (Beckman).

Aequorea victoria green fluorescent protein (GFP cycle 3 mutant⁴⁷) and *Photinus pyralis* luciferase mRNAs were synthesized *in vitro* using T7 RNA polymerase⁴⁸ from pMGFP³⁸ and pT7 luc³² plasmids, respectively. A published system³² was followed for *in vitro* translation of natural mRNAs.

30 pmol of 70 S ribosomes, 11 μ l of ribosome-free S100 extract, 25 μ g of total tRNA *E. coli* MRE600, 1.2 mM ATP, 0.8 mM GTP, 0.4 mM each L-amino acid (or except for Leu in case of GFP mRNA translation, when 0.225 mM Leu and 7.5 μ M [14 C]Leu were added), 0.1 mg/ml folinic acid, 0.02 mg/ml pyruvate kinase, 20 mM phosphoenolpyruvic acid and 20 mM acetyl phosphate were incubated in 50 μ l of buffer (100 mM HEPES-KOH (pH 7.5), 60 mM potassium acetate, 13 mM magnesium acetate, 2% PEG 8000, 2 mM DTT, 0.1 mM EDTA) at 30°C for 5 min, then 5 μ g of mRNA (and luciferin to 0.1 mM concentration in case of luciferase mRNA translation) were added at start point. The synthesis was carried out at 30°C.

Light emission by luciferase was measured in a thermostatic cell using a luminometer (Pribori Oy, Finland). In case of GFP mRNA translation, 3 μ l aliquots were taken from the reaction at each time point, 50 fold diluted with cold buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂) and incubated for 5 h at 0°C then fluorescence was measured using spectrofluorimeter (Shimadzu RF-5301PC, Japan). In parallel, 5 μ l of the reaction were taken for measuring of [14 C]Leu incorporation by TCA precipitation (as described above for poly (U) translation) and for analyzing protein size by SDS-PAGE with subsequent radio autography with MultiPurpose screen (MP) and CycloneTM phosphor imager (Packard Instruments, USA).

Supplementary Material

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Figure 1.

(a) Recombineering, the strategy applied for in frame deletion of *E. coli* chromosomal genes. Chromosomal gene *orf* is replaced by a drug-resistance PCR cassette during recombination utilizing short (39–40nt) homologies (shown with striped boxes). Open arrows indicate position of checking primers used to verify gene replacements by PCR. (b) Gene essentiality assay. Nonessential gene such as *rplY* can readily be replaced with a drug-resistance cassette using recombineering (see scheme, plate and gel on the left column). In the case of *rplY* knockout, a few normally growing *rplY* < cat/*rplY*⁺ diploids are seen among thousands of growth-impaired *rplY* < cat knockouts on the LB-Cm plate. Only *cat* insert-related PCR product is synthesized when *rplY* configuration is tested in *rplY* < cat Cm^R recombinants. At

the same time, only gene *orf*<>*cat orf*/gene *orf*+ gene partial diploids are viable when essential gene such as *rplE* or *rplR* is replaced (right column: scheme, plate and gels). In these cases only rare gene<>*cat*/gene+ diploids survive on the selective LB-Cm plate. Agarose gels below the plate show that in the case of *rplE* and *rplR* gene replacements both, *cat*-insert and wild type gene-related PCR products are synthesized.

Figure 2.

(a) The *E. coli* *spc* operon with *rplE* and *rplR* genes. The hairpin needed for feedback regulation of the operon is indicated with an asterisk. (b) The replacement of *rplE* orf with *cat* was made either in the absence or presence of an *rplE*-expressing plasmid. Only *rplE<=>cat/rplE+* diploids survive in the absence of the plasmid (left). The *rplE<=>cat* haploids survive if cells carry an *rplE*-expressing plasmid, showing that *rplE* is indeed essential while regulatory hairpin is not (right). (c) Agarose gel showing the configuration of *rplE* in viable Cm^R recombinants in the presence (+) or absence (-) of an *rplE*-expressing plasmid, as analyzed by PCR of *rplE* chromosomal region.

Figure 3.

Growth of wild type W3110 (WT) and L25-defective KNB800 (Δ L25) strains in the presence of plasmids providing expression of *E. coli rply* for L25 protein (pKAB101), *B. subtilis* gene *ctc* for CTC protein (pKAB110) and 5'-terminal portion of *ctc* encoding for N-terminal fragment (NfrCTC) of CTC protein (pKAB104). WT + control plasmid (1), Δ L25 + control plasmid (2), Δ L25 + L25-expressing plasmid (3), Δ L25 + NfrCTC-expressing plasmid (4), Δ L25 + CTC-expressing plasmid (5). Growth medium was supplemented with kanamycin. Incubation at 37°C for 16 hours.

Figure 4. Protein-synthesizing activity of wild type (●) and Δ L25 (▲) cells determined by standard β -galactosidase assay. The β -galactosidase activity is normalized to the cell mass (OD_{600}).

Figure 5.

(a) A sucrose gradient analysis of ribosomes from L25-defective ($\Delta L25$, left panel) and wild type (WT, right panel) cells. Crude cell lysates were fractionated by centrifugation on 15–30% sucrose gradients. (b) Two-dimensional polyacrylamide gel analysis of protein content of L25-defective (left panel) and wild type (right panel) ribosomes.

Figure 6. Poly(U)-dependent polyphenylalanine synthesis by wild type (●) and L25-defective (▲) ribosomes.

Figure 7.

Time course of GFP (a) and luciferase (b) mRNA translation in *E. coli* cell-free translation system containing wild type (WT, ●) and L25-defective (Δ L25, ▲) ribosomes. In case of GFP synthesis, an aliquot was taken at 30 min time point from the wild type and L25-defective systems and analyzed by SDS-PAGE with subsequent autoradiography (right panel of section (a)).

Table 1

Results of gene knockouts

Target	Type of knockout	Recombination efficiency ^a	Configuration of replaced gene ^b
<i>rplY</i>	<i>rplY<>cat</i>	~10 ⁴	<i>rplY<>cat</i>
<i>rplE</i>	<i>rplE<>cat</i>	~10	<i>rplE<>cat/rplE⁺</i>
<i>rplE^c</i>	<i>rplE<>cat</i>	~10 ²	<i>rplE<>cat</i>
<i>rplR</i>	<i>rplR<>cat</i>	~10	<i>rplR<>cat/rplR⁺</i>

^a Recombination efficiency was calculated as the number of Cm^R recombinant colonies per 10⁸ viable cells.

^b Determined by PCR analysis of chromosomal region with *cat*-replaced gene.

^c Recombination was done in the presence of the *rplE*-expressing plasmid.

Table 2

The growth rates of the parental strain (W3110) and *rplY* knockout (KNB800) alone and with plasmids constitutively expressing *E. coli* L25 (pKAB101), *B. subtilis* CTC (pKAB110) or N-terminal fragment of CTC protein (pKAB104)

Strain (plasmid)	Protein expressed from the plasmid	Generation time, min
W3110	-	32
KNB800	-	72
W3110 (control plasmid)	-	39
KNB800 (control plasmid)	-	120
KNB800 (pKAB101)	L25	46
KNB800 (pKAB104)	NfrCTC	54
KNB800 (pKAB110)	CTC	54

^a Measured in LB medium at 37°C by monitoring the optical density at 600 nm (cells transformed with plasmids were grown in the presence of kanamycin).