Involvement of ribosomal protein L6 in assembly of functional 50S ribosomal subunit in *Escherichia coli* cells[1]

Yuta Shigeno [a], Toshio Uchiumi [b], and Takaomi Nomura [a]*

[a] Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan

[b] Department of Biology, Faculty of Science, Niigata University, Niigata 950-2181, Japan

* Corresponding author. E-mail address: nomurat@shinshu-u.ac.jp

[1] **Abbreviations**: rRNA, ribosomal RNA; IF-2, translation initiation factor-2; EF-Tu, translation elongation factor-Tu; EF-G, translation elongation factor-G; RF-3, translation release factor-3; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electorophoresis

ABSTRACT

Ribosomal protein L6, an essential component of the large (50S) subunit, primarily binds to helix 97 of 23S rRNA and locates near the sarcin/ricin loop of helix 95 that directly interacts with GTPase translation factors. Although L6 is believed to play important roles in factor-dependent ribosomal function, crucial biochemical evidence for this hypothesis has not been obtained. We constructed and characterized an *Escherichia coli* mutant bearing a chromosomal L6 gene (*rplF*) disruption and carrying a plasmid with an arabinose-inducible L6 gene. Although this ΔL6 mutant grew more slowly than its wild-type parent, it proliferated in the presence of arabinose. Interestingly, cell growth in the absence of arabinose was biphasic. Early growth lasted only a few generations (LI-phase) and was followed by a suspension of growth for several hours (S-phase). This suspension was followed by a second growth phase (LII-phase). Cells harvested at both LI- and S-phases contained ribosomes with reduced factor-dependent GTPase activity and accumulated 50S subunit precursors (45S particles). The 45S particles completely lacked L6. Complete 50S subunits containing L6 were observed in all growth phases regardless of the L6-depleted condition, implying that the ΔL6 mutant escaped death because of a leaky expression of L6 from the complementing plasmid. We conclude that L6 is essential for the assembly of functional 50S subunits at the late stage. We thus established conditions for the isolation of L6-depleted 50S subunits, which are essential to study the role of L6 in translation.

Keywords

L6; ribosome assembly; immature 50S subunit; 45S particle; biphasic growth

1. Introduction

The ribosome is a universal molecular machine that translates the genetic information on messenger RNA into protein. Bacterial ribosomes, which are large RNA-protein complexes, have a sedimentation coefficient of 70S and are composed of a large (50S) and a small (30S) subunits. In *Escherichia coli*, the 50S subunit contains 33 ribosomal proteins (L1–L6, L9–L25, and L27–L36) and two rRNAs (23S and 5S rRNA), whereas the 30S subunit contains 21 ribosomal proteins (S1–S21) and one 16S rRNA [1–4]. It is widely recognized that the ribosomal RNAs play fundamental roles in ribosomal function [5,6]. In contrast, the detailed function of many ribosomal proteins remains ambiguous [1].

Ribosomal protein L6, an essential component of the large ribosomal subunit, is encoded by the *rplF* gene. This gene is located at the center in the chromosomal *spc* operon, which contains the genes for 11 ribosomal proteins (L14, L24, L5, S14, S8, L6, L18, S5, L30, L15, and L36) [7]. The L6 protein is noteworthy in its function. L6 primarily binds to helix 97 (nucleotides 2735–2769, *E. coli* numbering) of the 23S rRNA during a late stage of 50S subunit assembly [2–4,8,9] and seems to modulate the structure of this RNA, including the structure of the highly conserved sarcin/ricin loop (nucleotides 2653–2667 in helix 95) [10] which is directly involved in interactions with GTPase translation factors such as IF-2 [11], EF-Tu [12], EF-G [13], and RF-3 [14]. Recent structural studies of *Thermus thermophilus* ribosomes in complex with the translocase EF-G have revealed that L6 directly contacts EF-G, and that the manner of this interaction changes in different translocational states; that is, pre-translocation [15], intermediate [16] and post-translocation [17]. Interestingly, a gentamicin-resistant *E. coli* mutant (GE20-8/AB2834) with an L6 that lacks its C-terminal 18 amino acid residues exhibits a cold-sensitive growth defect due to a failure of 50S–30S subunit association [18–20]. Because L6 is unlikely to directly involved in the interaction of the 50S and 30S subunits, L6 may participate in structural modulations of the 50S subunit

that lead to a mature form able to associate with the 30S subunit. L6 is therefore likely to participate not only in functional interaction with GTPase translation factors within the intact 50S subunit but also in correct maturation of the 50S subunit in *E. coli* cells.

There is little biochemical evidence concerning L6 function. One of the main reasons for this is that the preparation of ribosomes lacking the essential protein L6 is technically difficult both *in vivo* and *in vitro*. In this study, we constructed a ΔL6 mutant of *E. coli* by disruption of the chromosomal L6 gene (*rplF*) using lambda-Red recombination [21] and P1 phage transduction [22]. This deletion was complemented by a plasmid that carries an arabinose-inducible copy of *rplF*. Depletion of L6 resulted in the arrest of cell proliferation and a decrease in ribosome/factor-dependent GTPase activity, owing to accumulation of 50S subunit precursors that sediment at 45S. This study is the first demonstration that L6 directly acts as a key player in the late stage of *E. coli* 50S subunit assembly.

2. Materials and Methods

2.1 Construction of plasmids and strains

All strains, plasmids, and primers used in this study are listed in Table S1. Construction of plasmid pJKspcL6 was performed as follows. The *spc* promoter and the *rplF* region of *E. coli* strain MG1655 (wild-type) was amplified by polymerase chain reaction (PCR) using primers 1–4, and the resultant two DNA fragments (P_{spc} fragment and *rplF* fragment) were linked by PCR and inserted between the *Eco*RI and *Bam*HI sites of plasmid pJK289 [23]. Plasmid pBADL6 was constructed by inserting the *rplF* region, which was PCR-amplified using primer 5 and 6, between the *Eco*RI and *Hin*dIII sites of plasmid pBAD24 [24]. The *E. coli* L6 deletion strain (ΔL6 mutant) was created using lambda-Red recombination [21] and P1 phage transduction [22] as follows (Fig. 1). The strain Red, prepared according to a previous

method [25], was transformed with plasmid pJKspcL6, and then the *rplF* region on the chromosome was replaced with the *rplF::cat* fragment to generate strain Red-ΔL6, which contained a chromosomal sequence flanking the *rplF* region joined to the ends of the *cat* gene that confers chloramphenicol resistance (Cm^R). The *rplF::cat* fragment, which replaced nucleotides 1–468 of *rplF* (full length, 534 nucleotides) with the *cat* gene to preserve the Shine–Dalgarno sequence for the downstream *rplR* gene (encoding L18), was constructed by overlap extension PCR using primers 9–14. Strain MG1655 transformed with plasmid pBADL6 was transduced with P1 phage grown on strain Red-ΔL6, and the obtained transductants were selected on LB agar supplemented with 50 μg/ml ampicillin, 17 μg/ml chloramphenicol, and 65 mM L-arabinose. PCR and sequence analysis of the chromosomal region around and within the *rplF* confirmed the *rplF::cat* replacement in a Cm^R colony (Fig. S1).

2.2 Growth analyses of ΔL6 mutant

The ΔL6 mutant was pre-cultured at 37°C in 5 ml of LB liquid medium supplemented with 50 μg/ml ampicillin, 17 μg/ml chloramphenicol, and 13 mM L-arabinose until the optical density at 600 nm (OD_{600nm}) reached 0.5. To remove the L-arabinose, the cells were collected and resuspended in 5 ml of LB liquid medium. After the washed cell cultures were serially diluted (10^1- to 10^4-fold) with the same medium, 2 μL of each dilution was spotted onto LB agar plates supplemented with 50 μg/ml ampicillin, 17 μg/ml chloramphenicol and 0 or 65 mM L-arabinose, and incubated at 37°C for 66 h. 100 μl of the same washed cultures were used to inoculate 200 ml of pre-warmed (37°C) LB liquid medium with the same supplementation, and cultivated at 37°C. When the growth of the ΔL6 mutant without L-arabinose reached a plateau, L-arabinose was added to a final concentration of 65 mM. Strain MG1655 was also analyzed in the same way, but without antibiotics.

2.3 Polysome profile analysis

The ΔL6 mutant was grown, without L-arabinose, to S-phase (suspended growth phase) or LII-phase (second log growth phase), while another portion of the ΔL6 mutant, with L-arabinose, and strain MG1655 were grown to the mid-log phase (approximately 0.8 OD_{600nm}) at 37°C in 200 mL of liquid LB medium. After chloramphenicol was added to a final concentration of 100 μg/ml to avoid polysome run-off, the cell cultures were incubated for 5 min. The harvested cells were then resuspended in 4 mL of Buffer A (10 mM $MgCl_2$, 60 mM KCl, and 10 mM Tris-HCl, pH 7.6) containing 0.4 mg/ml of lysozyme and 500 units/ml of RNasin ribonuclease inhibitor. After one freeze–thaw cycle, 1 ml of Buffer A containing 0.5% (v/v) Tween 20, 0.5% (w/v) deoxycholic acid, and 0.1 units/μl RNase-free DNase was added, and then the mixture was incubated on ice for 20 min. After cell lysates were clarified by centrifugation for 10 min at 18,000 × *g* and 4°C, 3 A_{260nm} (absorbance at 260 nm) units of lysates were overlaid on a 10–40% (w/v) sucrose gradient made in Buffer B (10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.6) and centrifuged for 2.5 h at 35,000 rpm and 4°C in a Hitachi RPS65T rotor. The gradient was pumped from the bottom of the tube using a peristaltic pump with simultaneous and continuous A_{260nm} monitoring.

2.4 Ribosome preparation and in vitro analyses

Cells of the ΔL6 mutant cultured in the absence of L-arabinose were harvested at three different growth phases: LI-phase (first log growth phase), S-phase, and LII-phase. Ribosomes were prepared and then their EF-G-dependent GTPase activity was analyzed according to a previously described method [26]. Total rRNA was obtained from the purified ribosomes via phenol extraction; 500 ng of rRNA samples were analyzed using 1% (w/v) agarose gel electrophoresis under native condition, and visualized with ethidium bromide

staining. The purified ribosomes (20 pmol) were overlaid on the top of a 10–28% (w/v) sucrose gradient made in 10 mM or 0.5 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.6. After centrifugation for 4 h at 35,000 rpm and 4°C in a Hitachi RPS65T rotor, the gradients were analyzed in the same manner as described for the polysome profile analysis. One drop (approximately 30 µl) of each peak fraction (50S, 45S, and 30S) was collected, analyzed by SDS-PAGE on a 16.5% (w/v) polyacrylamide gel, and visualized with silver staining (Silver Stain II Kit Wako).

3. Results and discussion

3.1 Construction of the ΔL6 mutant

To construct a system for studying the functional role of L6 in *E. coli*, we disrupted chromosomal L6 gene (*rplF*) and complemented the disruption using plasmid pBADL6, which allows arabinose-inducible expression of *rplF*. From the 13 potential candidates, one homologous recombinant (ΔL6 mutant) was screened by PCR and sequencing analyses (Fig. S1B and C). The ΔL6 mutant cells were grown in the presence of arabinose, collected by centrifugation, and suspended in LB medium to remove the arabinose. The resultant culture was serially diluted, spotted onto LB agar plates with (+) or without (–) arabinose, and cultivated at 37°C for 66 h (Fig. 2A). In the presence of arabinose (L6 expression), the ΔL6 mutant grew well. However, the strain showed remarkable growth delay in the absence of arabinose (L6 expression arrested), indicating that L6 plays an important role in cell growth. We thus succeeded in constructing a system for the *in vivo* study of L6 function.

3.2 In vivo functional characterization of the ΔL6 mutant

We first examined the growth characteristics of the ΔL6 mutant (Fig. 2B). In the presence of arabinose, the ΔL6 mutant showed logarithmic growth (black-filled circle), although it grew slower than the wild-type (WT) strain (black-filled triangle). In the absence of arabinose, the ΔL6 mutant grew in a biphasic manner; that is, following an initial log growth phase (LI-phase), suspension for approximately 12 h (S-phase), and a second log growth phase (LII-phase) (open circle). Implication of this abnormal growth behavior is discussed below. Cell growth resumed immediately after the addition of arabinose during S-phase (gray-filled circle), indicating that the growth delay and suspension were caused by L6 depletion. No difference in growth rate was observed in the wild-type strain in the presence (black-filled triangle) or absence (open triangle) of arabinose, indicating that arabinose itself has no adverse effect on the growth.

The unique growth caused by L6 depletion may be attributed to the protein's functions. We thus analyzed the polysome profile of the ΔL6 mutant (Fig. 2C). The wild-type strain showed a typical profile, which indicates that the ribosomal particles existed mostly as polysomes or 70S ribosomes, with minor amounts of 50S and 30S subunits (black dotted line). The ΔL6 mutant grown in the presence of arabinose contained considerable amounts of polysomes although the abundance was less than that in wild-type, and increased amounts of free subunits (black dashed line). When ΔL6 mutant was grown in the absence of arabinose, LII-phase cells exhibited a profile comparable to that of ΔL6 cells cultured in the presence of arabinose (gray line). In contrast, S-phase cells had no polysomes and a remarkably decreased amount of 70S ribosomes, which was accompanied with an increase in the broad peaks around the 50S and 30S subunits (black line). These results indicate that cell growth is correlated with the amounts of cellular polysomes and 70S ribosomes, and that L6 depletion causes a defect in the association of 50S subunits with 30S subunits.

3.3 In vitro functional characterization of the ΔL6 mutant

To investigate the effects of the L6 deletion on ribosomal particles, ribosome samples isolated from each growth phase of the ΔL6 mutant (Fig. 3A) were tested for EF-G-dependent GTPase activity (Fig. 3B). LII-phase ribosomes (orange bar) showed slightly reduced activity from that of the wild-type ribosomes (black bar). In contrast, the activities of the LI-phase (pink bar) and S-phase (red bar) ribosomes were approximately 40% of that of the wild-type ribosomes. Furthermore, we examined the ribosome profile using sucrose density gradient (SDG) analysis, in which 70S ribosomes and 50S/30S subunits were generally detectable when using 10 mM (high) and 0.5 mM (low) concentrations of Mg^{2+}, respectively (Fig. 3C and 3D; wild-type, black dotted-line). At high Mg^{2+} concentration (Fig. 3C), the LII-phase ribosomes showed significantly decreased amounts of the 70S fraction and higher amounts of dissociated subunits that sedimented at around 50S and 30S (orange line); a more pronounced tendency towards these effects was observed in both L1- (pink line) and S- (red line) phase ribosomes. Profiles of the same ribosome samples at low Mg^{2+} concentration (Fig. 3D) revealed that an ambiguous shoulder peak between 50S and 30S subunits in LII-phase ribosomes (orange line) became a distinct peak with a sedimentation coefficient of 45S in both LI- (pink line) and S- (red line) phase ribosomes. The amount of 45S fraction in S-phase ribosomes (red line) was slightly reduced from that in the LI-phase ribosomes (pink line), and in contrast, the amount of 50S fraction was increased. It is noteworthy that the amounts of 30S fraction were more or less similar in all samples. Therefore, the presence of a 45S fraction appears to correlate with a lower ability to form 70S ribosomes and also a lower ability to access the GTPase factor EF-G, suggesting that the 45S fraction contains translationally inactive 50S subunits. The 45S particle is generally recognized as a late stage precursor in the assembly of the 50S subunit [2,27]. In fact, along with the growth transition that characterizes the shifts from LI-phase to S-phase to LII-phase,

the amount of 45S particles tended to decline with an increase in 50S subunits (Fig. 3D), which indicates that 45S particles are 50S subunit precursors generated by disruption of the L6 gene, rather than dead-end particles. Furthermore, all rRNAs extracted from the ribosomes in each growth phase appear to remain intact, as observed using agarose gel electrophoresis (Fig. 4A), implying that the reduced function of LI- and S-phase ribosomes was not due to the fragmentation of their 23S and 16S rRNAs.

3.4 Protein Components of the ribosome from ΔL6 mutant

We proceeded to analyze the protein components in each peak fraction (50S, 45S and 30S) from SDG analysis under low Mg^{2+} concentration (Fig. 4B). The 45S particles of LI-phase (lane 3) and S-phase (lane 5) ribosomes completely lacked L6. In contrast, the 50S subunits from all growth phases contained the same amount of L6 as the wild-type (lane 1, 2, 4 and 6). These results lead to the conclusion that the depletion of L6 in *E. coli* cells is responsible for the appearance of the 45S particles, implying that L6 is an essential component in the late stage assembly of 50S subunits. The 30S subunits from all growth phases showed the same pattern as those from the wild-type (Fig. S2).

It must be mentioned that the LII-phase ribosomes were active in the EF-G-dependent GTPase assay (Fig. 3B) and their 50S subunits contained significant amount of L6 (Fig. 4B), even in the absence of arabinose. These suggest that L6 expression occurred from the complementing plasmid in an arabinose-independent manner. To confirm this, we assessed the expression of His-tagged L6 in wild-type *E. coli* transformed with plasmid pBADHisL6 in the absence of arabinose. The results showed very low levels of His-tagged L6 expression, relative to the levels seen in the presence of arabinose (Fig. S3). Based on these results, the biphasic growth (Fig. 2B, open circle) of the ΔL6 mutant without arabinose could be explained as follows. At the beginning of the experiment, the cells could divide a few times

because they could assemble active 50S subunits carrying L6, which were synthesized before removing arabinose (LI-phase). Cell growth was suspended upon accumulation of 45S particles, which results from a decrease in the L6 level (S-phase). At this point, ribosome assembly from 45S to 50S increased gradually due to the expression of L6 at a low rate from the complementing plasmid, and the cells could resume proliferation (LII-phase). The change of sedimentation coefficient from 50S to 45S could be a result of the decomposition of rRNA or the loss of ~10% (100–150 kDa) of the subunit mass. However, 23S and 16S rRNAs from the ribosomes present at each growth phase seem to be intact (Fig. 4A). Therefore, the 45S particles should be deficient in several ribosomal proteins in addition to L6 (~18 kDa). Mutant *E. coli* strains lacking L5 [28], L28 [29], L35, and L36 [30] accumulate 50S subunit precursors; most of which lack or have reduced levels of L16, L25, L27, L28, L31, L33, L35, and L36. We are now analyzing the detailed protein content of the 45S particles of ΔL6 mutant.

3.4 L6 function in ribosome assembly

Recently, accumulation of 45S particles was observed in an *E. coli* L36-deletion mutant. This report suggests that ribosomal protein L36 plays a pivotal role in late stage of the 50S subunit assembly [30]. Interestingly, L36 binds to helix 97, which is also the main site of L6 binding. The lack of L6 is likely to have a negative effect on L36 binding to ribosomes. Little has been reported about L6 function during ribosome assembly. The present study is the first to demonstrate that L6 acts directly as a key player in late stage assembly of *E. coli* 50S subunits. The field of ribosome biogenesis, including ribosome assembly factors, has recently gained momentum [2,27]. The GTPase RbgA is essential for late stage 50S subunit assembly in *Bacillus subtilis*, mutation or depletion of which results in the accumulation of 45S particles, and the suppressor mutations concentrate in the N-terminal region of L6 [31]. This

information suggests a functional correlation between L6 and GTPase factors in the late stage assembly of 50S subunit. Although this knowledge concerning the role of L6 in correct 50S subunit maturation is important, there is no RbgA orthologue in *E. coli*. Further analyses using isolated ΔL6 ribosomes, as well as ΔL6 mutant cells, might clarify the role of L6 in assembly of functional 50S subunit and also identify a factor that substitutes for *B. subtilis* RbgA in *E. coli.*

Acknowledgements

We are indebted to the Divisions of Gene Research and Instrumental Analysis, Research Center for Human and Environmental Sciences, Shinshu University, for providing facilities. This work was supported by JSPS KAKENHI Grant Numbers 25460707 and 25870287.

References

[1] R. Nikolay, D. van den Bruck, J. Achenbach, et al., Ribosomal proteins: role in ribosomal functions, In: eLS. John Wiley & Sons Ltd, Chichester (Jun 2015) doi: 10.1002/9780470015902.a0000687.pub4.

[2] Z. Shajani, M. T. Sykes, J. R. Williamson, Assembly of bacterial ribosomes, Annu. Rev. Biochem. 80 (2011) 501–526..

[3] K. H. Nierhaus, Bacterial ribosomes: assembly, In: eLS. John Wiley & Sons Ltd, Chichester (Jan 2006), doi: 10.1038/npg.els.0003947.

[4] M. Kaczanowska, M. Rydén-Aulin, Ribosome biogenesis and the translation process in *Escherichia coli*, Microbiol. Mol. Biol. Rev. 71 (2007) 477–494.

[5] M. Beringer, M. V. Rodnina, The ribosomal peptidyl transferase, Mol. Cell 26 (2007) 311–321.

[6] R. Green, H. F. Noller, Ribosomes and translation, Annu. Rev. Biochem. 66 (1997) 679–716.

[7] Y. Fu, K. Deiorio-Haggar, J. Anthony, et al., Most RNAs regulating ribosomal protein biosynthesis in *Escherichia coli* are narrowly distributed to Gammaproteobacteria, Nucleic Acids Res. 41 (2013) 3491–3503.

[8] M. Herold, K. H. Nierhaus, Incorporation of six additional proteins to complete the assembly map of the 50 S subunit from *Escherichia coli* ribosomes, J. Biol. Chem. 262 (1987) 8826–8833.

[9] K. H. Nierhaus, F. Dohme, Total reconstitution of functionally active 50S ribosomal subunits from *Escherichia coli*, Proc. Natl. Acad. Sci. USA. 71 (1974) 4713–4717.

[10] T. Uchiumi, N. Sato, A. Wada, et al., Interaction of the sarcin/ricin domain of 23 S ribosomal RNA with proteins L3 and L6, J. Biol. Chem. 274 (1999) 681–686.

[11] P. Julián, P. Milon, X. Agirrezabala, et al., The Cryo-EM structure of a complete 30S translation initiation complex from *Escherichia coli*, PLoS Biology 9 (2011) e1001095.

[12] N. Fischer, P. Neumann, A. L. Konevega, et al., Structure of the *E. coli* ribosome–EF-Tu complex at <3Å resolution by C_s-corrected cryo-EM, Nature 520 (2015) 567–573.

[13] A. Pulk, J. H. D. Cate, Control of ribosomal subunit rotation by elongation factor G, Nat. Struct. Science 340 (2013) 1235970.

[14] J. Zhou, L. Lancaster, S. Trakhanov, et al., Crystal structure of release factor RF3 trapped in the GTP state on a rotated conformation of the ribosome, RNA 18 (2012) 230–240.

[15] Y. Chen, S. Feng, V. Kumar, et al., Structure of EF-G–ribosome complex in a pretranslocation state, Nat. Struct. Mol. Biol. 20 (2013) 1077–1084.

[16] D. S. Tourigny, I. S. Fernández, A. C. Kelley, et al., Elongation factor G bound to the ribosome in an intermediate state of translocation, Science 340 (2013) 1235490.

[17] Y. G. Gao, M. Selmer, C. M. Dunham, et al., The structure of the ribosome with elongation factor G trapped in the posttranslocational state, Science 326 (2009) 694–699.

[18] C. Davies, D. E. Bussiere, B. L. Golden, et al., Ribosomal proteins S5 and L6: high-resolution crystal structures and roles in protein synthesis and antibiotic resistance, J Mol Biol. 279 (1998) 873–888.

[19] A. Bosl, A. Böck, Ribosomal mutation in *Escherichia coli* affecting membrane stability, Mol. Gen. Genet. 182 (1981) 358–360.

[20] P. Buckel, A. Buchberger, A. Böck, et al., Alteration of ribosomal protein L6 in mutants of *Escherichia coli* resistant to gentamicin, Mol. Gen. Genet. 158 (1977) 47–54.

[21] K. C. Murphy, Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*, J. Bacteriol, 180 (1998) 2063–2071.

[22] L. C. Thomason, N. Costantino, D. L. Court, *E. coli* genome manipulation by P1 transduction, Curr. Protoc. Mol. Biol. 79 (2007) 1.17.1-1.17.8..

[23] J. Kato, H. Ikeda, Construction of mini-F plasmid vectors for plasmid shuffling in *Escherichia coli*, Gene. 170 (1996) 141–142.

[24] L. M. Guzman, D. Belin, M. J. Carson, et al., Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter, J. Bacteriol. 177 (1995) 4121–4130.

[25] J. Kato, M. Hashimoto, Construction of long chromosomal deletion mutants of *Escherichia coli* and minimization of the genome, Methods Mol. Bio. 416 (2008) 279–293.

[26] T. Uchiumi, K. Hori, T. Nomura, et al., Replacement of L7/L12.L10 protein complex in *Escherichia coli* ribosomes with the eukaryotic counterpart changes the specificity of elongation factor binding, J. Biol. Chem. 274 (1999) 27578–27582.

[27] A. Dönhöfer, M. R. Sharma, P. P. Datta, et al., Factor-mediated ribosome assembly in bacteria, eLS 15 (2009) doi: 10.1002/9780470015902.a0021836.

[28] A. P. Korepanov, A. V. Korobeinikova, S. A. Shestakov, et al., Protein L5 is crucial for *in vivo* assembly of the bacterial 50S ribosomal subunit central protuberance, Nucleic Acids Res. 40 (2012) 9153–9159.

[29] B. A. Maguire, D. G. Wild, The roles of proteins L28 and L33 in the assembly and function of *Escherichia coli* ribosomes *in vivo*, Mol. Microbiol. 23 (1997) 237–245.

[30] T. Arai, K. Ishiguro, S. Kimura, et al., Single methylation of 23S rRNA triggers late steps of 50S ribosomal subunit assembly, Proc. Natl. Acad. Sci. USA. 112 (2015) E4707-4716.

[31] M. Gulati, N. Jain, J. H. Davis, et al., Functional interaction between ribosomal protein L6 and RbgA during ribosome assembly, PLoS Genet. 10 (2014) e1004694.

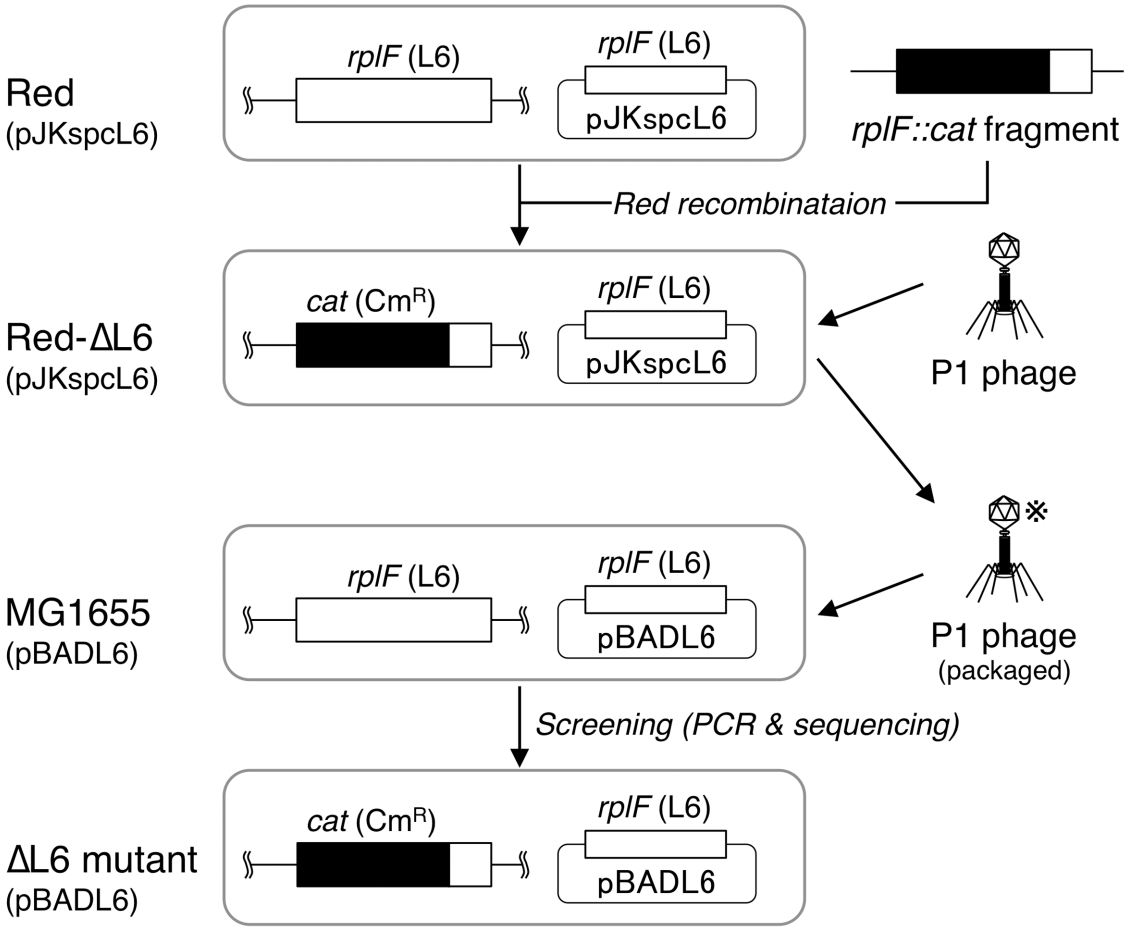
Figure legends

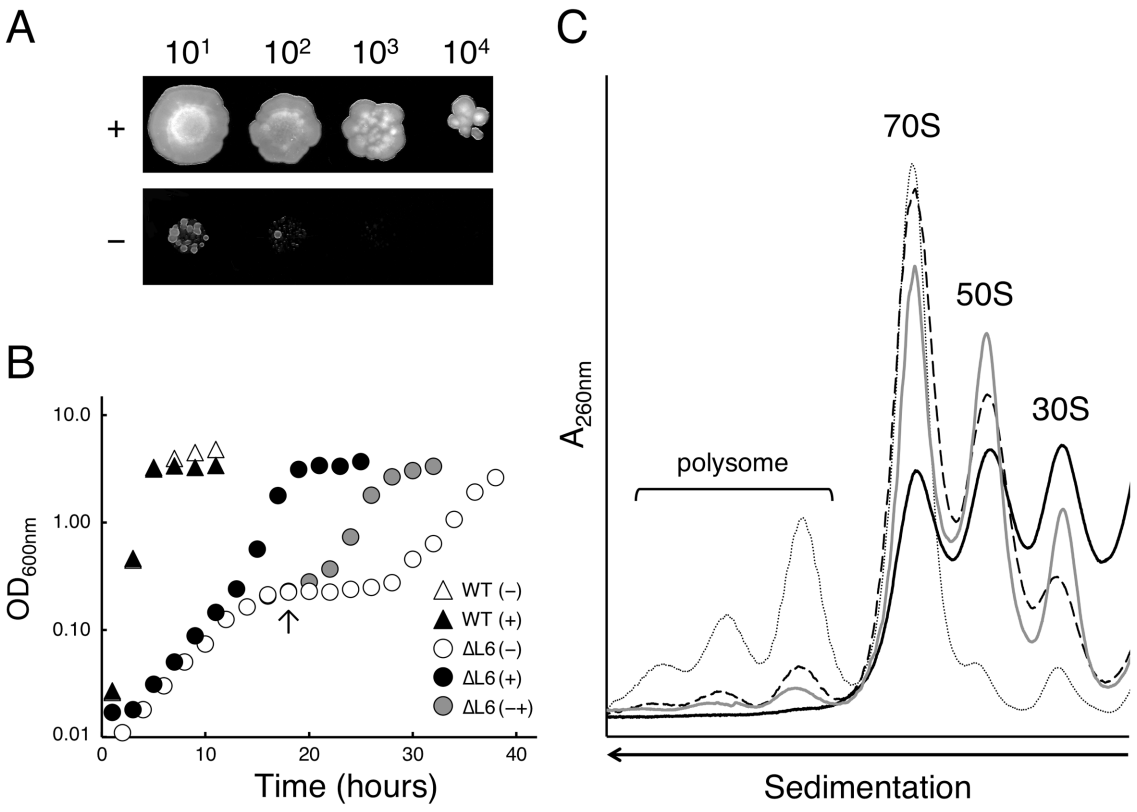
Fig. 1. ***Construction of the Escherichia coli ΔL6 mutant.*** The chromosomal L6 gene (*rplF*) was inactivated using lambda-Red recombination system, and the disrupted-*rplF* region was transduced, using P1 phage transduction, into wild-type strain MG1655 harboring plasmid pBADL6.

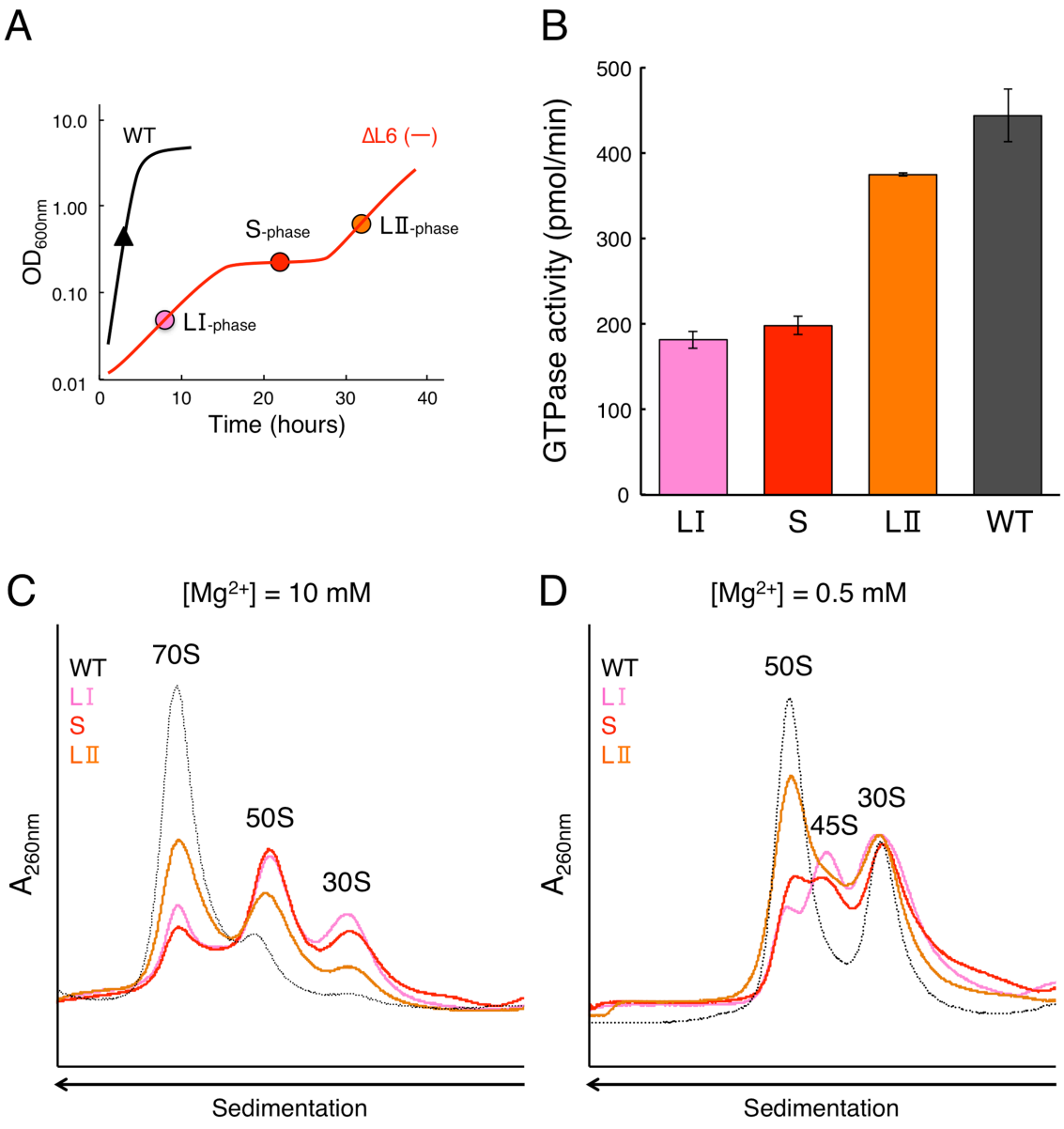
Fig. 2. ***In vivo characterization of the ΔL6 mutant.*** (A) The ΔL6 mutant was serially diluted (10^1- to 10^4-fold dilutions) and spotted onto LB ager plate in the presence (+) or absence (–) of arabinose. (B) The ΔL6 mutant (circle) and the wild-type (triangle) were cultivated in liquid LB medium with (black-filled) or without (open) arabinose. The arrow indicates the time that arabinose was added to the ΔL6 mutant culture without arabinose (gray-filled). (C) Cell extracts (3 A_{260nm} units) were sedimented in a 10–40% (w/v) sucrose gradient; A_{260nm} of the gradient was continuously measured from the bottom of the tube. Black and gray lines indicate the ΔL6 mutant at S-phase and LII-phase, respectively; the black dashed line indicates the ΔL6 mutant with arabinose and the black dotted line indicates the wild-type.

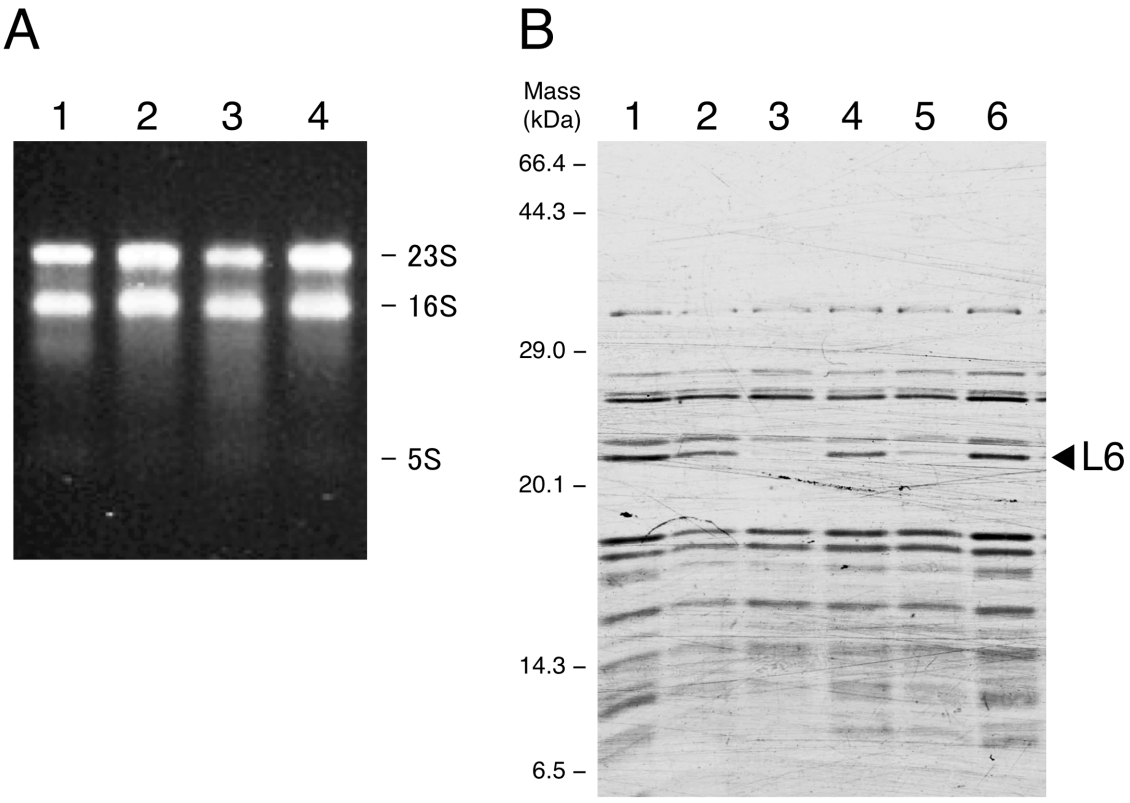
Fig. 3. ***In vitro characterization of the ΔL6 mutant.*** (A) The ΔL6 mutant without arabinose (circle) and the wild-type strain (triangle) were harvested at these points. (B) Ribosomes isolated from LI-phase (pink), S-phase (red), and LII-phase (orange) ΔL6 mutant, or from the exponential growth phase wild-type (black) were tested for EF-G-dependent GTP hydrolysis activity. (C and D) Ribosomes were sedimented in a 10–28% (w/v) sucrose gradient under high (C) or low (D) ionic strength conditions; A_{260mn} of the gradient was continuously measured from the bottom of the tube.

Fig. 4. ***Analysis of ribosomal components.*** (A) Total rRNAs (500 ng) extracted from the ribosomes of wild-type (lane 1), and ΔL6 mutant in LI-phase (lane 2), S-phase (lane 3), and LII-phase (lane 4), were separated on a 1% (w/v) agarose gel under native conditions; the gel was stained with ethidium bromide. (B) A sample from each peak containing the 50S subunit of wild-type ribosomes (lane 1); the 50S subunit of ΔL6 mutant ribosomes in LI- (lane 2), S- (lane 4), and LII-phase (lane 6); and the 45S particle of ΔL6 ribosomes in LI- (lane 3) and S-phase (lane 5) in Fig. 3D were analyzed by 16.5% (w/v) SDS-PAGE; the gel was stained with silver.


Red
(pJKspcL6)
rplF (L6)
rplF (L6)
pJKspcL6
rplF::cat fragment
Red recombinataion
Red-ΔL6
(pJKspcL6)
cat (CmR)
rplF (L6)
pJKspcL6
P1 phage
MG1655
(pBADL6)
rplF (L6)
rplF (L6)
pBADL6
P1 phage
(packaged)
Screening (PCR & sequencing)
ΔL6 mutant
(pBADL6)
cat (CmR)
rplF (L6)
pBADL6


A
10^1
10^2
10^3
10^4
+
–
B
OD600nm
10.0
1.00
0.10
0.01
0
10
20
30
40
Time (hours)
WT (–)
WT (+)
ΔL6 (–)
ΔL6 (+)
ΔL6 (–+)
C
A260nm
70S
50S
30S
polysome
Sedimentation


A
10.0
1.00
0.10
0.01
OD600nm
WT
ΔL6 (—)
S-phase
LII-phase
LI-phase
0
10
20
30
40
Time (hours)
B
500
400
300
200
100
0
GTPase activity (pmol/min)
LI
S
LII
WT
C
[Mg2+] = 10 mM
WT
LI
S
LII
70S
50S
30S
A260nm
Sedimentation
D
[Mg2+] = 0.5 mM
WT
LI
S
LII
50S
45S
30S
A260nm
Sedimentation


A
1
2
3
4
23S
16S
5S
B
Mass
(kDa)
1
2
3
4
5
6
66.4
44.3
29.0
20.1
14.3
6.5
L6

Supplementary Material

Involvement of ribosomal protein L6 in assembly of functional 50S ribosomal subunit in *Escherichia coli* cells

Yuta Shigeno [a], Toshio Uchiumi [b], and Takaomi Nomura [a]*

[a] Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan

[b] Department of Biology, Faculty of Science, Niigata University, Niigata 950-2181, Japan

* Corresponding author. E-mail address: nomurat@shinshu-u.ac.jp

Table S1.

All strains, plasmids and primers used in this study

Strains or plasmids or Primers	Genotype or charactaristic or Seaquancing
Strains	
MG1655	F⁻ λ⁻ *ilvG*⁻ *rfb-50 rph-1*
Red	MG1655 *red (TcR) hsdR:Ap* [25]
Red-ΔL6	MG1655 *red (TcR) hsdR:ApR rplF::cat* transformed with comlementing plasmid pJKspcL6
ΔL6 mutant	MG1655 *rplF::cat* transformed with comlementing plasmid pBADL6
Plasmids	
pJKsocL6	Ligated fragment of *rplF* under the *spc* promoter cloned into the plasmid pJK289 [23], which was low-copy-number (1–2 copies per cell) and conferred resistance to kanamycin
pBADL6	*rplF* cloned into the plasmid pBAD24 [24], which was carrying arabinose-inducible *rplF* and conferred resistance to ampicillin
pBADHisL6	N-terminal His-tagged L6 fusion gene cloned into the plasmid pBAD24, which was constructed by inverse-PCR method using the plasmid pBADL6 as a template
Primers (5'→3')	
Construction of pJKspcL6 plasimid	
1	GCGCGGATCCTTACTTCTTCTTAGCCTCTT
2	GGCGAAATTATCTGCTACGT
3	TTTTTTCCTCCGATTAGGCTACGTAGCAGATAATTTCGCCTTTAGTGCTCCGCTAATGTC
4	GCGCGAATTCCTCTGCAAGGTCGCGTTGTT
Construction of plasmid pBADL6	
5	CTCCATACCCGTTTTTTTGGGCTAGCAGGAG*GAATTC*ACCATGTCTCGTGTTGCTAAAGC
6	CATAACATCAAACATCGACCCACGGCGTAACGCG*AAGCTT*TTACTTCTTCTTAGCCTCTT
Construction of plasmid pBADHisL6	
7	ATGCATCATCATCATCATCATAGCAGCGGCTCTAGAATGTCTCGTGTTGCTAAAG
8	GGTGAATTCCTCCTGCTAGCCC
Construction of *rplF::cat* fragment	
9 (a)	CTTACTCTGAAGTATTTCCAGGG
10	TTTTTCCTCCGATTAGGCTA
11 (b)	TGGCGAAATTATCTGCTACGTAGCCTAATCGGAGGAAAAAATGGAGAAAAAAATCACTGG
12 (c)	GCACGACTTCGTCGGCGTAACGAACACCCTTGCCTTTATACACTTATTCAGGCGTAGCA
13	TATAAAGGCAAGGGTGTTCG
14	GTGAGCCATCTTACACCTCT
PCR analysis	
a (9)	CTTACTCTGAAGTATTTCCAGGG
b (11)	TGGCGAAATTATCTGCTACGTAGCCTAATCGGAGGAAAAAATGGAGAAAAAAATCACTGG
c (12)	GCACGACTTCGTCGGCGTAACGAACACCCTTGCCTTTATACACTTATTCAGGCGTAGCA
d	CCAACTACAGTCAGAGCTGTG
e	TGCAGATAATACCCTGACCTTCGGTCCGCG

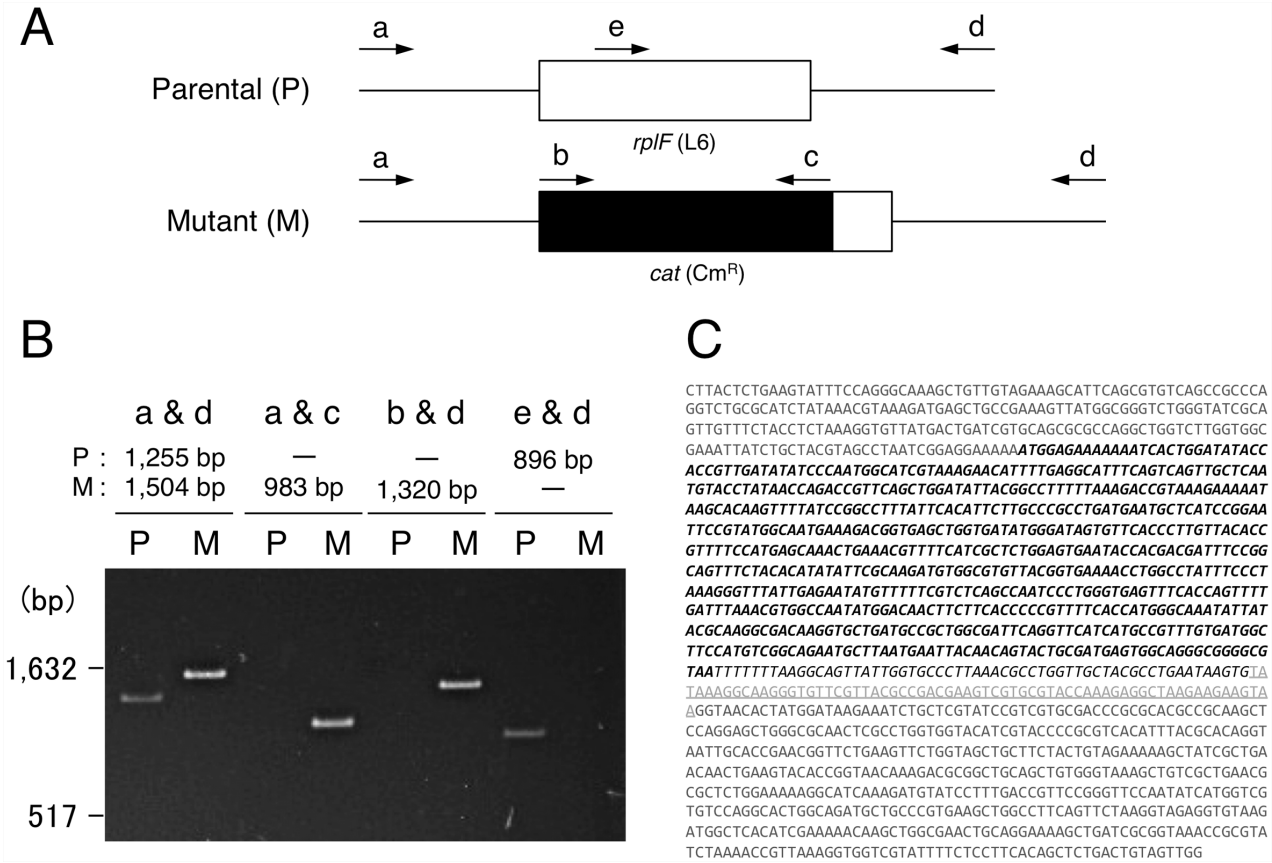


Fig. S1. ***Polymerase chain reaction (PCR) and sequencing screening of a homologous recombinant.*** (A) Design of primers to analyze for the desired homologous recombination. Black arrows represent primers used in this analysis. (B) Agarose gel electrophoresis of PCR products obtained with primer pairs presented in (A); M, ΔL6 mutant; P, MG1655 strain (control). (C) Nucleotide sequence near *rplF* in the ΔL6 mutant: Insert region, italic black; *cat*, bold italic black; a portion of *rplF*, underlined light gray; other regions, gray.

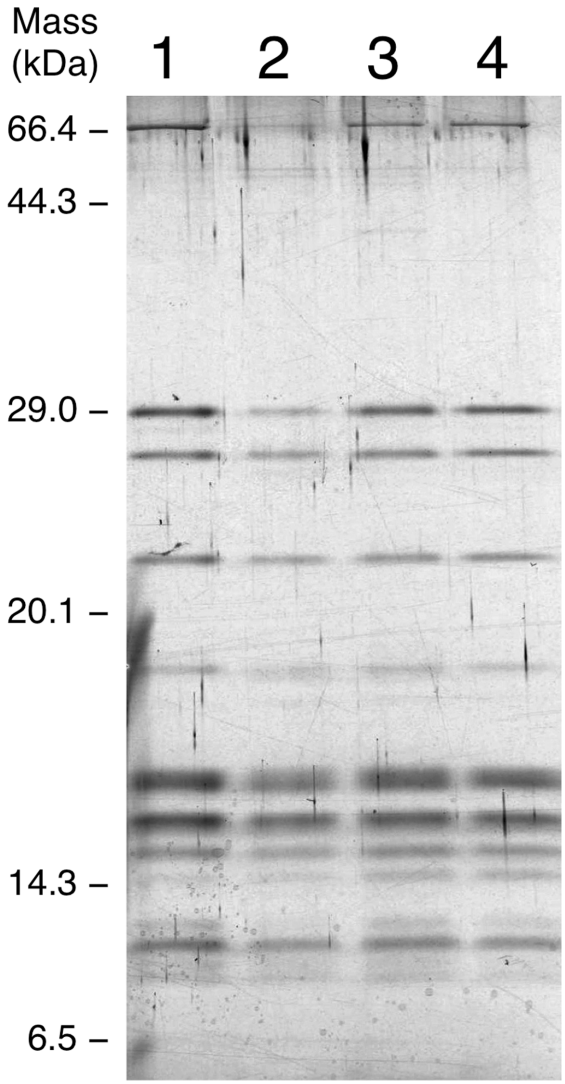


Fig. S2. ***Ribosomal protein components of the 30S subunits from each growth phase.*** Samples of the 30S subunit peaks from wild-type ribosomes (lane 1) and ΔL6 mutant ribosomes obtained at LI-phase (lane 2), S-phase (lane 3), and LII-phase (lane 4) (Fig. 3D) were analyzed by 16.5% (w/v) SDS-PAGE and the gel was stained with silver.

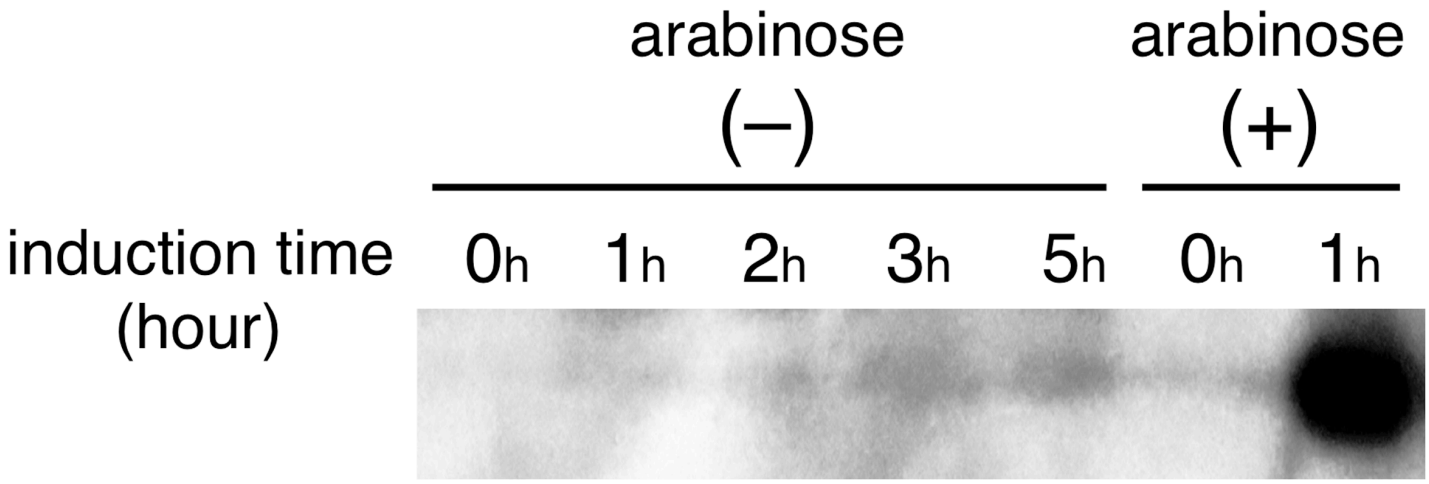


Fig. S3. ***Arabinose-induced L6 expression in Escherichia coli cells.*** The plasmid pBADHisL6, which directs the expression of the N-terminally His-tagged ribosomal protein L6, was constructed using inverse-polymerase chain reaction (PCR) with primer pair 13/14 and the plasmid pBADL6 as a template. Wild-type *E. coli* strain MG1655 transformed with plasmid pBADHisL6 was cultivated in liquid LB medium containing 50 μg/ml ampicillin until the OD_{600} reached 0.5. L-arabinose was added to a final concentration of 65 mM and the cells were cultured for an additional hour to express His-tagged L6. The same experiment was performed in the absence of arabinose, except that additional cultivation was carried out for 0, 1, 2, 3, and 5 h. The harvested cells were analyzed by 16.5% (w/v) SDS-PAGE, and the proteins separated on the gel were transferred to a PVDF membrane (Immobilon-P, Merck-Millipore, USA). The His-tagged proteins were detected by using Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore, USA) with HisProbe-HRP (Thermo Fisher Scientific, USA).