Genes to Cells

Ribosomal protein L31 in *Escherichia coli* contributes to ribosome subunit association and translation, whereas short L31 cleaved by protease 7 reduces both activities

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Ribosomes routinely prepared from *Escherichia coli* strain K12 contain intact (70 amino acids) and short (62 amino acids) forms of ribosomal protein L31. By contrast, ribosomes prepared from *ompT* mutant cells, which lack protease 7, contain only intact L31, suggesting that L31 is cleaved by protease 7 during ribosome preparation. We compared ribosomal subunit association in wild-type and *ompT*$^-$ strains. In sucrose density gradient centrifugation under low Mg^{2+}, 70S content was very high in *ompT*$^-$ ribosomes, but decreased in the wild-type ribosomes containing short L31. In addition, ribosomes lacking L31 failed to associate ribosomal subunits in low Mg^{2+}. Therefore, intact L31 is required for subunit association, and the eight C-terminal amino acids contribute to the association function. *In vitro* translation was assayed using three different systems. Translational activities of ribosomes lacking L31 were 40% lower than those of *ompT*$^-$ ribosomes with one copy of intact L31, indicating that L31 is involved in translation. Moreover, in the stationary phase, L31 was necessary for 100S formation. The strain lacking L31 grew very slowly. A structural analysis predicted that the L31 protein spans the 30S and 50S subunits, consistent with the functions of L31 in 70S association, 100S formation, and translation.

Introduction

The ribosome is an essential cellular complex that is widely conserved in all organisms and responsible for translating the genetic information from mRNAs into the amino acid sequences of proteins. In bacteria, the small (30S) and large (50S) ribosomal subunits associate to form the 70S particle. Early studies showed that, in the *Escherichia coli* ribosome, the small subunit consists of 16S rRNA and 21 ribosomal proteins (r-proteins) (S1–S21), whereas the large subunit contains 23S rRNA, 5S rRNA, and 34 r-proteins (L1–L34) (Kaltschmidt & Wittmann 1970). However, three proteins are excluded from this count: L7, the post-translationally acetylated form of L12; L8, a complex of L7/L12 and L10; and L26, which is identical to S20. L35 (*rpmI*) and L36 (*rpmJ*) were subsequently discovered using radical-free and highly reducing two-dimensional polyacrylamide gel electrophoresis (RFHR 2D-PAGE) (Wada 1986a,b; Wada & Sako 1987). Thus, 54 r-proteins are now recognized to constitute the *E. coli* ribosome: 21 in the small subunit and 33 in the large subunit.

It is important to investigate relationship between translation and r-proteins, but little is known about the specific functions of r-proteins in translation. To gain further insight into the role of individual

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r-proteins, isolation of mutants was tried. Nonessential mutants lacking any one of 16 of the 54 *E. coli* r-protein genes have been isolated (Dabbs 1991). Later, 22 r-protein genes containing 13 of Keio collection (Baba *et al.* 2006) could been individually deleted from the genome, and these proteins were nonessential (Shoji *et al.* 2011). From analysis of *rpsT* (S20) deletion mutant, 30S subunit lacking S20 indicated a poor capacity for the association with the 50S subunit, a defect at initiation step of translation, and poor growth (Götz *et al.* 1990).

Escherichia coli r-protein L31 is also nonessential in Keio collection and is conserved in high degree in bacteria including even smaller genome strains (e.g., *Mycoplasma*).

Escherichia coli r-protein L31 was initially reported to consist of 62 amino acids with a calculated molecular weight of 6967 Da and the C-terminal sequence -RFNK, as determined by amino acid sequencing of purified L31 (Brosius 1978). When r-proteins prepared from *E. coli* wild-type cells were analyzed by RFHR 2D-PAGE, another spot derived from L31, distinct from the 62-amino acid form, was detected; this new protein was more basic and larger than the previously characterized L31. It was named protein C or precursor L31 (Wada *et al.* 1990). The *rpmE* gene encoding L31, located at 88.5 min on the *E. coli* chromosome (Dabbs 1981), was sequenced (Plunkett *et al.* 1993). Unexpectedly, its translated product was predicted to contain 70 amino acids, eight amino acids longer than indicated by protein sequencing, with the C-terminal sequence -RFNIPGSK. We found that only precursor L31, but no 62-amino acid L31, was present in ribosomes prepared from cells harboring a deletion in *ompT* (Wada 1998). We designated the precursor as 'intact L31' and the 62-amino acid form as 'short L31'. The *ompT* gene encodes an outer membrane protein, protease 7, an endopeptidase that specifically cleaves between two consecutive basic residues (Arg/Lys)↓(Arg/Lys) (Dekker *et al.* 2001). In L31, protease 7 may cleave between the Lys62 and Arg63 residues of the C-terminal sequence -RFNK↓RFNIPGSK (Fig. S1A in Supporting Information). This evidence suggested that L31 is cleaved by protease 7 during grinding or disruption of cells, and that 'short L31' is an experimental artifact that should not be present in living cells. Later, another group carried out protein sequence analyses of L31 in the ribosomes of several strains of *E. coli* (MRE600, AT9, B, and D10) and obtained the same longer intact C-terminal sequences (Eistetter *et al.* 1999).

The ribosomal structures contributed to the proposed L31 functions (Fig. S1B in Supporting Information). In both the *E. coli* and *Thermus thermophilus* 70S ribosome structures, L31 was confused with L28 due to insufficient resolution, and it was incorrectly located adjacent to protein L9 in the root region of the L1 stalk (Jenner *et al.* 2005; Schuwirth *et al.* 2005). However, the higher resolution (2.8 Å) of *T. thermophilus* 70S ribosome structure showed the correct location of L31, adjacent to L5 near the central protuberance and to S13 near the head domain of the 30S subunit (Selmer *et al.* 2006). This location of L31 is consistent with a report that it formed a cross-linked dimer with L5 (Kenny & Traut 1979). Structural analyses of the *E. coli* ribosome by cryo-EM showed that *E. coli* L31 is in basically the same location as in the *T. thermophilus* ribosome (Agirrezabala *et al.* 2012; Fischer *et al.* 2015). L31 is the only r-protein that covers both the 50S and 30S subunits. In *E. coli* L31, the C-terminal 7 amino acids (Phe64-Lys70) within the C-tail are associated with helix-41 of the 16S rRNA, whereas Arg63, at the cleavage site that produces the short L31, hydrogen bonds with S14, which may confer protection against Protease 7 (Fig. S1B in Supporting Information). We therefore infer that L31 may be involved in the association of the 30S and 50S subunits to produce the 70S particle and also may function in translation itself. However, the physiological roles of L31 remain to be elucidated.

In the stationary phase of *E. coli* cells, pairs of 70S ribosomes form the 100S ribosomes via the binding of the ribosome modulation factor (RMF) and hibernation promoting factor (HPF), which are both expressed specifically under the stressful conditions associated with stationary phase (Wada 1998; Izutsu *et al.* 2001; Ueta *et al.* 2005; Yoshida & Wada 2014). Electron microscopy showed that the 100S ribosome consists of two 70S ribosomes bound to two 30S subunits in the order 50S-30S-30S-50S (Wada 1998; Yoshida *et al.* 2002). The 100S ribosome lacks translational activity because RMF binds near the peptidyl transferase center and the peptide exit tunnel (Wada *et al.* 1995; Yoshida *et al.* 2002, 2004); moreover, this complex lacks tRNA and mRNA (Kato *et al.* 2010). Based on these results, we proposed a new system for translational regulation by conversion of active 70S to inactive 100S ribosomes (Yoshida *et al.* 2004). Cryo-electron microscopy showed that the structure of the 100S ribosome relies on interactions between the S2, S3, and S5 proteins of the 30S subunit (Kato *et al.* 2010; Ortiz *et al.* 2010). Cryo-electron tomography

confirmed that native *E. coli* cells contain the 100S ribosome (Ortiz *et al.* 2010). The 100S ribosome is widely conserved in bacteria (Ueta *et al.* 2013) and probably negatively regulates translational activity via dimerization of active 70S. Previously, it was unclear how the absence or modification of L31 influences 100S formation.

In this study, we confirmed that the eight C-terminal amino acids of L31 were cleaved during ribosome preparation by the outer membrane protein protease 7, leading to formation of the short L31 protein. The 50S subunit containing short L31 reduced 70S association, 100S formation, and translation activities. Consistent with this, the deletion of L31 decreased subunit association, *in vitro* translation activity, and 100S formation.

Results

Ribosomes prepared from wild-type *E. coli* cell contain both intact and short L31

We prepared crude ribosomes (CRs) from the mid-exponential phase of wild-type K12 W3110 cells, and removed weakly bound proteins, aminoacyl-tRNAs, and fragmented mRNAs to yield high salt-washed ribosomes (HSRs). When we analyzed the r-proteins of HSRs by RFHR 2D-PAGE, we detected two spots corresponding to intact and short L31 [panel 1 upper and lower in Fig. 1]. Both proteins were also observed in CRs prepared from two other *E. coli* strains, W3350 and Q13 (Fig. S2 in Supporting Information).

Short L31 is an artificial product of intact L31 cleavage by protease 7 (OmpT)

To identify the protease responsible for production of short L31, we then compared the r-proteins of HSRs from W3110 cells and *ΔompT::Km* (YB1012) cells, which lack the outer membrane protein protease 7. In contrast to wild-type HSRs, in *ΔompT::Km* HSRs we exclusively detected intact L31 protein, but no short L31 [compare panel (2) with (1) in Fig. 1]. Likewise, no short L31 was observed in HSRs of another *ompT* mutant, AD202 (MC4100 *ΔompT::Km*) (Fig. S3A in Supporting Information). These observations suggest that protease 7 contacts ribosomes during and after cell disruption for ribosome preparation and cleaves intact L31 to yield short L31.

To test this idea, we investigated whether prolonged incubation of the disrupted cells would increase the content of short L31. After grinding wild-type and *ΔompT::Km* cells with quartz sand, we incubated the disrupted cells at 4 °C or 37 °C for 30 min before suspension in buffer I and then prepared HSRs for analysis of r-proteins by RFHR 2D-PAGE. In ribosomes from wild-type cells, the copy number of intact L31 decreased from 0.31 (no incubation) to 0.19 (4 °C incubation) or 0.12 (37 °C incubation). At the same time, the copy number of short L31 increased from 0.43 (no incubation) to 0.54 (4 °C incubation) or 0.69 (37 °C incubation) [panels 2–4 in Fig. 2A,B]. By contrast, in ribosomes from *ΔompT::Km* cells, no short L31 was detected in either nonincubated samples (copy number = 0.81) [panel 1 in Fig. 2A,B] or samples incubated at 37 °C (copy number = 0.60) (Fig. S4A in Supporting Information). However, when cell debris (CD) containing protease 7 was added to the *ΔompT::Km* cells during cell disruption, short L31 appeared in both nonincubated and 37 °C-incubated samples (Fig. S4B,C in Supporting Information). These observations show that short L31 is generated by protease 7 during routine ribosome preparation.

Intact L31 in purified 70S ribosomal particles is barely sensitive to protease 7, but L31 in free 50S subunits is highly sensitive

Next, we fractionated CRs from W3110 cells by routine preparative sucrose density gradient (SDG) centrifugation and analyzed the r-proteins in the resultant 50S and 70S fractions by RFHR 2D-PAGE. The majority of L31 in the 50S fraction was the short L31 but 70S fraction contained both intact L31 and short L31 (Fig. S5 in Supporting Information). In living cells, the concentration of intracellular free Mg^{2+} is estimated to be 1–2 mM (Alatossava *et al.* 1985; Froschauer *et al.* 2004). Therefore, the 70S fraction in this experiment might have re-associated with free 50S and 30S in buffer I (15 mM Mg^{2+}) during ribosome preparation. Therefore, we prepared 70S and 50S from wild-type cells by the routine method with modified buffer I (5 mM Mg^{2+}, nearer the concentration in living cells) instead of the usual buffer I (15 mM Mg^{2+}). Under the modified conditions, the 70S particles contained only intact L31, whereas the 50S subunits contained only short L31 (Fig. 3A).

To investigate this difference in the abundance of short L31, we prepared 50S subunits and 70S particles exclusively containing intact L31 from *ΔompT::Km* cells. CD obtained from a W3110 derivative harboring an IPTG-induced *ompT* over-expression plasmid (YB1017), which contains protease 7 (Fig. 3B), was

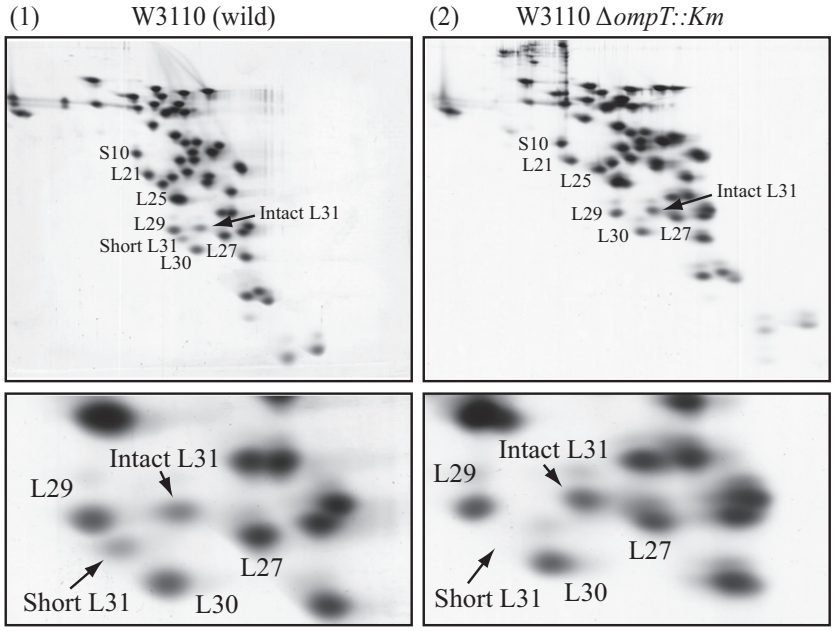

Figure 1 Ribosomes prepared from wild-type *Escherichia coli* cells contain both intact and short L31. W3110 wild-type (1) and W3110 *ΔompT*::*Km* (2) cells were grown at 37 °C in EP medium and collected in exponential growth phase (Klett units: 50), and high salt-washed ribosomes (HSRs) were prepared. HSR proteins were analyzed by radical-free and highly reducing two-dimensional polyacrylamide gel electrophoresis (RFHR 2D-PAGE). Gels were stained with CBB. (Upper) Spots corresponding to r-proteins S10, L21, L25, L27, L29, L30, intact L31, and short L31 are indicated. (Lower) An enlarged image of the L31 spot and surrounding area is shown.

mixed with the 70S and 50S fractions. After incubation at 37 °C for 30 min, we analyzed the proteins in the mixtures by RFHR 2D-PAGE. The 70S ribosomes contained approximately one copy each of intact L31 and trace levels of short L31 (Fig. 3C). By contrast, the 50S subunits contained large amounts of short L31 and virtually no intact L31, in stark contrast to samples not treated with CD (Fig. 3C). Thus, the C-terminal residues of L31 were cleaved more readily in the free 50S subunit than in the 70S ribosome, suggesting that the 30S subunit in the 70S ribosome protects or covers L31, preventing cleavage by protease 7.

HSRs containing short L31 or lacking L31 dissociate into 30S and 50S subunits during SDG centrifugation under low-Mg^{2+} conditions

We next examined the effect of truncation or absence of L31 on 70S ribosome stability. HSRs prepared as described in Fig. 2 were analyzed by SDG centrifugation in the presence of various concentrations of Mg^{2+} (2–15 mM). Irrespective of *ompT* status and incubation of disrupted cells, we observed only slight differences in 70S ribosomal profiles under 10 and 15 mM Mg^{2+}. When the Mg^{2+} concentration decreased from 15 mM to 10, 8, 6, or 5 mM, in the ribosome profile of wild-type cells containing intact L31 and short L31, the 70S fraction decreased whereas the 30S and 50S free subunit fractions increased proportional to the amount of the short L31 detected [panels 2–4 in Figs 2A,B and 4]. By contrast, the ribosome profile of *ΔompT*::*Km*, which has 0.8 copies per ribosome of intact L31 but no short L31, was characterized by a pronounced 70S peak and two very weak free subunit peaks, even under low-Mg^{2+} conditions (5–8 mM) [panel 1 in Figs 2A,B and 4], except at 3 mM Mg^{2+}, in which the 70S peak shifted to a lower S value. At 2 mM Mg^{2+}, even stable 70S ribosome from W3110 *ΔompT::Km* cells dissociated into 30S and 50S subunits [upper panel 1 in Fig. 4]. When disrupted cells of *ΔompT*::*Km* were incubated at 37 °C for 30 min, each ribosome contained 0.6 copies of intact L31, and the ribosome profile of SDG in 5 mM Mg^{2+} showed a strong 70S peak, as when the samples were not incubated (Fig. S4A in Supporting Information). However, when *ompT* mutant HSRs containing short

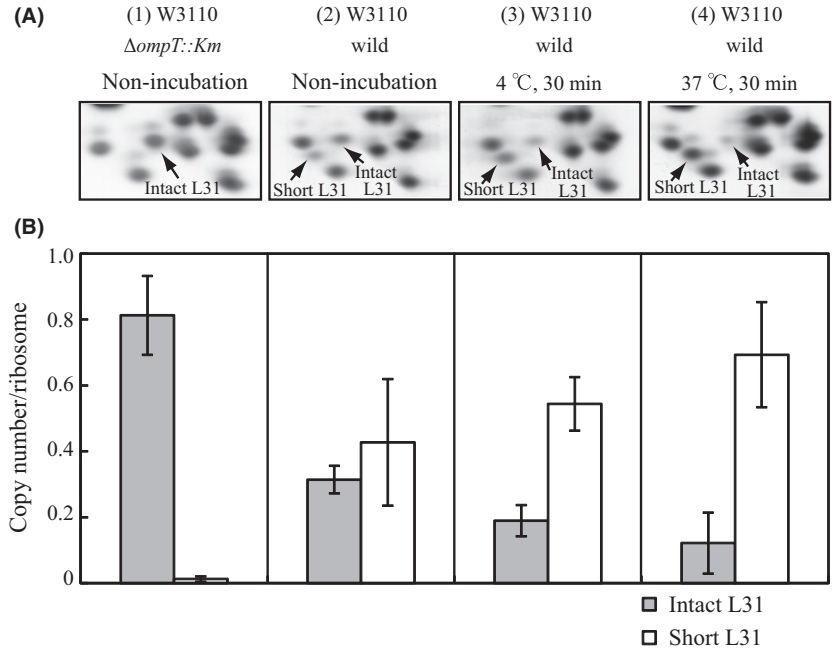

Figure 2 Intact L31 is cleaved by protease 7 during incubation of disrupted cells, yielding short L31. W3110 *ΔompT::Km* and W3110 wild-type cells were grown at 37 °C in EP medium and collected in exponential growth phase (Klett units: 50), and high salt-washed ribosomes (HSRs) were prepared. These samples were designated as 'nonincubation' (1), (2). To stimulate cleavage of the intact L31 C-terminal residues, disrupted W3110 cells were incubated at 4 °C (3) or 37 °C (4) for 30 min. (A) R-proteins of the four HSRs were analyzed by RFHR 2D-PAGE. Relevant gel areas are shown. Arrows indicate spots corresponding to intact L31 and short L31. (B) Protein copy number per ribosome was determined. Copy numbers of intact L31 or short L31 are shown as gray or white bars. The experiment was repeated at least three times. The bars and line bars show mean copy numbers of L31 and standard deviations, respectively.

L31 due to disruption with CD were analyzed by SDG centrifugation under 6 mM Mg^{2+}, 50S and 30S peaks were observed (Fig. S4B,C in Supporting Information). These results indicate that short L31-containing 70S ribosomes dissociate into 30S and 50S subunits during SDG centrifugation under low Mg^{2+} conditions and are less stable than 70S ribosomes containing only intact L31.

Next, we examined the association ability of HSRs lacking L31. For this purpose, we prepared *ΔrpmE::Km* HSRs lacking L31 and analyzed them by SDG centrifugation under various Mg^{2+} concentrations (2, 3, 5, 6, 8, 10, and 15 mM). Under 10 or 15 mM Mg^{2+}, the ribosomal profiles differed only slightly from those of *ompT* mutants and wild-type cells [panels 1,2,5 in Fig. 4], whereas, under 6 mM Mg^{2+}, the abundance of 70S ribosomes decreased dramatically and the S value shifted lower. Furthermore, under 3 mM Mg^{2+}, the 70S ribosome completely dissociated to 30S and 50S subunits [compare panel 5 with 1 in Fig. 4]. These results show that intact L31 is required for association of 30S and 50S subunits and the stability of the 70S ribosome.

Dissociated ribosomes containing short L31 cannot re-associate *in vitro*

We next investigated whether L31 is involved in the re-association of dissociated 30S and 50S subunits into 70S ribosomes *in vitro*. To this end, we prepared four HSR samples from W3110-derived strains: (i) *ΔompT::Km* cells, (ii) wild-type cells, (iii) the incubated wild-type cells (disrupted cells incubated at 37 °C for 30 min), and (iv) *ΔrpmE::Km* cells. L31 contents of these samples were as follows: 0.9 copies of intact L31 for *ΔompT::Km*, 0.5 copies of intact L31 and 0.2 copies of short L31 for the wild type, 0.2 copies of intact L31 and 0.5 copies of short L31 for the incubated wild type, and no L31 for *ΔrpmE::Km* [panels 1–4 in Fig. 5A]. These four types of HSRs were dissociated into 30S and 50S subunits by treatment with low-Mg^{2+} dissociation buffer [panels

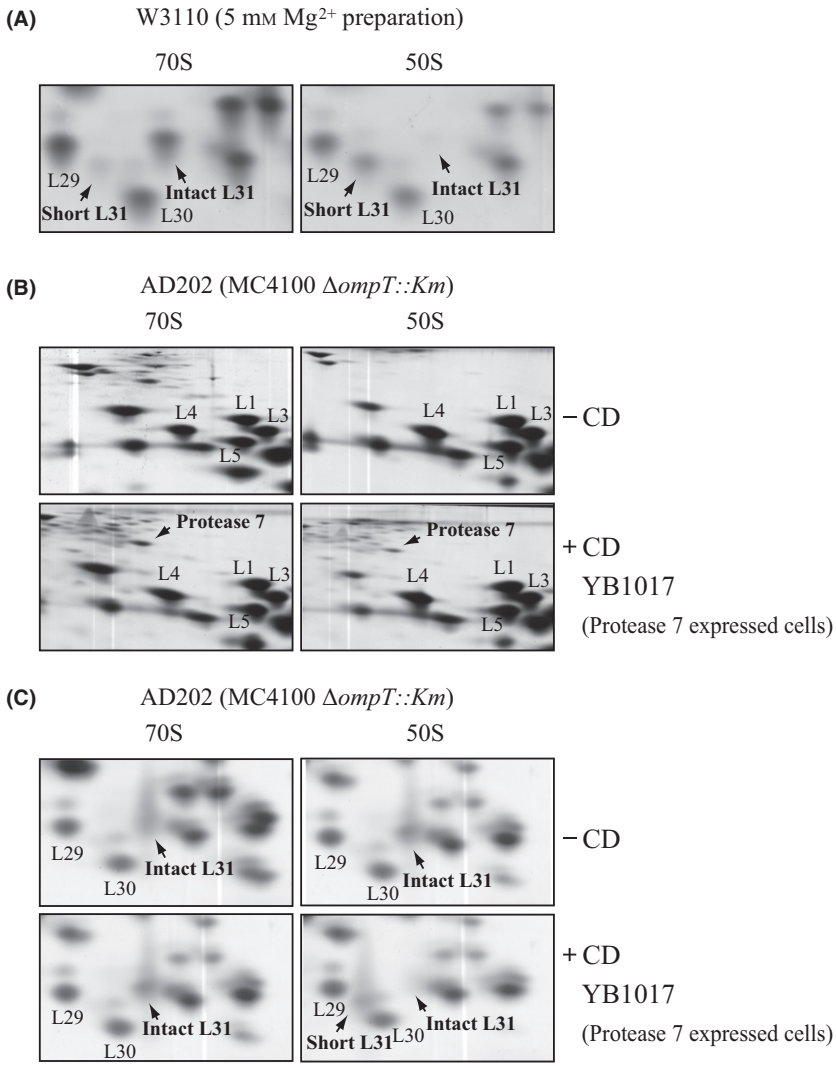

Figure 3 L31 is more accessible to cleavage by protease 7 when it is associated with the free 50S subunit, but not in the 70S complex. (A) 70S and 50S of W3110 cells prepared using buffer containing 5 mM Mg^{2+} contain only intact L31 and short L31, respectively. W3110 cells were grown at 37 °C in EP medium and collected in exponential growth phase (Klett units: 50). Crude ribosomes (CRs) were prepared by standard methods except that buffer I was modified to contain 5 mM Mg^{2+}. CRs were fractionated by 10%–40% SDG centrifugation under 5 mM Mg^{2+}, and 50S and 70S fractions were collected. Each fraction was analyzed by RFHR 2D-PAGE. Relevant gel areas of L31 are shown. (B, C) L31 is more accessible for cleavage by protease 7 in free 50S than in 70S. Cell debris (CD) was prepared from protease 7-overproducing W3110 (YB1017) cells and then added to 50S or 70S fractions containing intact L31 prepared from *ΔompT::Km* (AD202) cells. Each mixture (50S±CD or 70S±CD) was then incubated at 37 °C for 30 min. Subsequently, the proteins in the four samples were analyzed by RFHR 2D-PAGE. (B) Relevant gel areas of protease 7 are shown. Arrows indicate the spot corresponding to protease 7. (C) Relevant gel areas of L31 are shown. Spots corresponding to ribosomal proteins L29, L30, intact L31, and short L31 are indicated.

1–4 in Fig. 5B], and re-association to 70S ribosomes was carried out by incubation at 37 °C for 30 min. The re-associated samples were analyzed by SDG centrifugation under either 6 or 15 mM Mg^{2+}. In 15 mM Mg^{2+}, the 70S ribosome re-associated in all four samples [panels 1–4 in Fig. 5C]. *ΔompT::Km* formed 70S with similar efficiencies under 6 mM and 15 mM Mg^{2+} [panel 1 in Fig. 5C]. However, in the wild type, the 70S content in 6 mM Mg^{2+} was lower than that of *ΔompT::Km* [compare panel 2 with 1 in Fig. 5C]. In the incubated wild-type HSR, the 70S content in 6 mM Mg^{2+} was lower than in

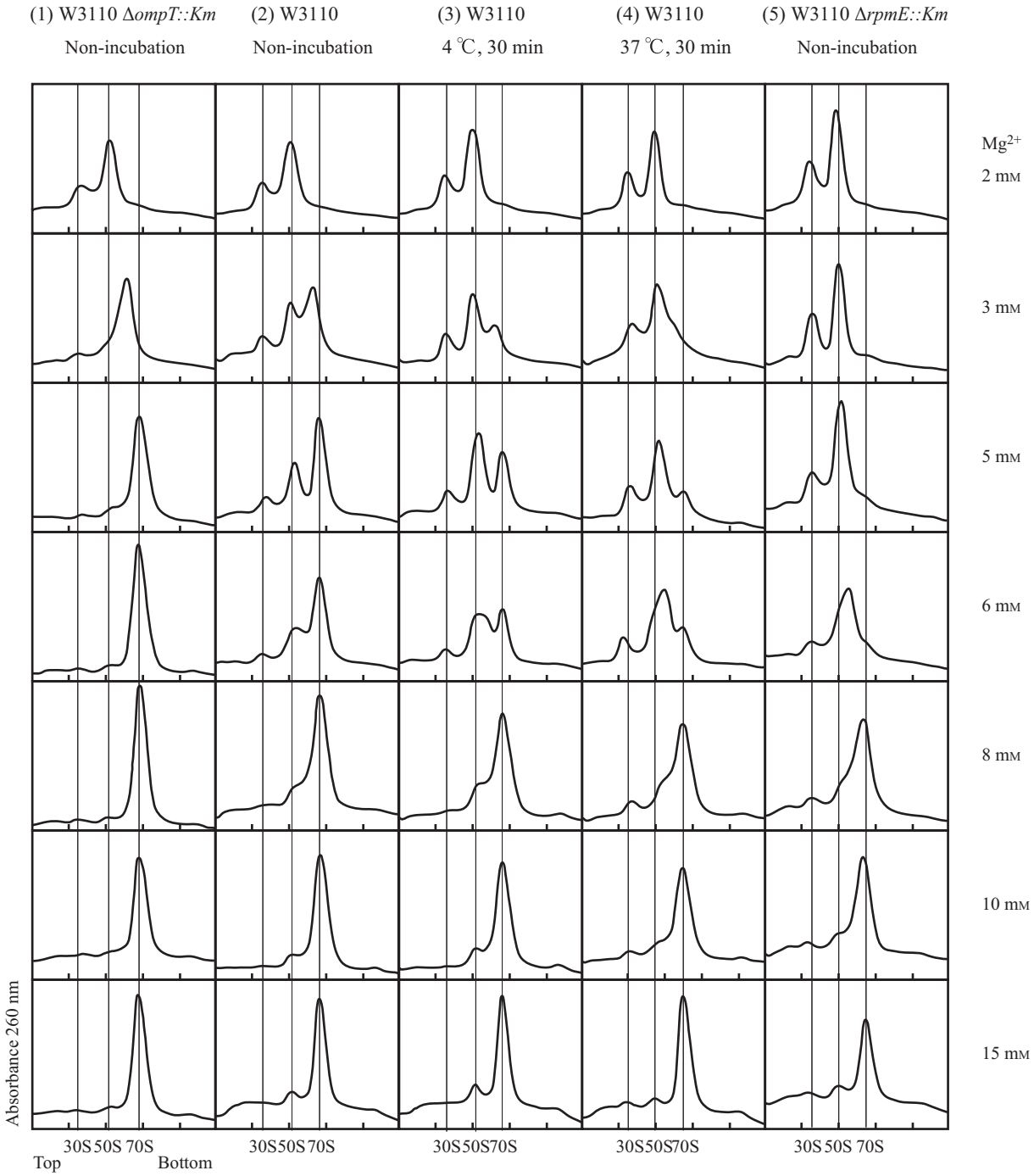

Figure 4 Short L31-containing or L31-lacking HSRs dissociate into 30S and 50S subunits during SDG centrifugation under low Mg^{2+} conditions. High salt-washed ribosomes (HSRs) of W3110 *ΔompT::Km* and W3110 cells were prepared as described in Fig. 2 and then analyzed by 5%–20% SDG centrifugation with the indicated Mg^{2+} concentrations. The resultant ribosome profiles are shown. (1) W3110 *ΔompT::Km* (nonincubation) and (2) W3110 (nonincubation). To stimulate the cleavage of the C-terminal residues of L31, disrupted W3110 cells were incubated at 4 °C (3) or 37 °C (4) for 30 min. HSRs (60 pmol) from (1) to (4) were subjected to SDG centrifugation. (5) High salt-washed ribosomes (HSRs) were prepared from W3110 *ΔrpmE::Km*, HSR samples (60 pmol) were analyzed by 5%–20% SDG centrifugation with the indicated Mg^{2+} concentrations, and the resultant ribosome profiles are shown.

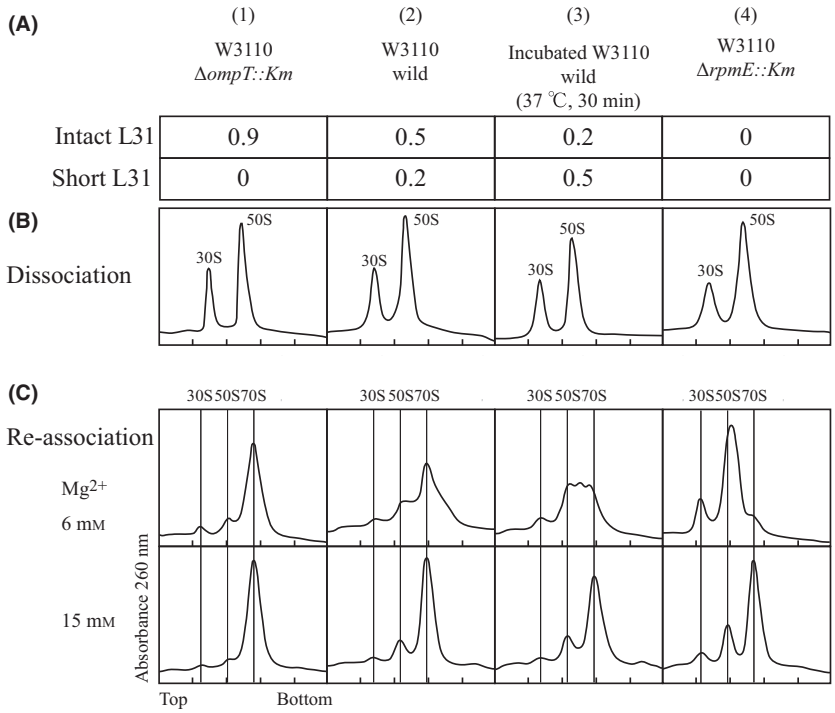

Figure 5 Dissociated ribosomes (50S+30S) containing short L31 or no L31 form unstable 70S *in vitro*. High salt-washed ribosomes (HSRs) were prepared from four types of cells: (1) W3110 *ΔompT::Km*, (2) W3110 wild type, (3) the incubated W3110, and (4) W3110 *ΔrpmE::Km*. Proteins from all four types of HSRs were analyzed by RFHR 2D-PAGE. (A) Copy numbers of L31 and short L31 in the four HSRs samples are shown in the panels. (B) HSRs of four types were dissociated to 30S and 50S subunits. The sample (60 pmol) was layered onto a 5%–20% SDG in dissociation buffer II and centrifuged. Ribosome profiles are shown in the dissociation panels. (C) Dissociated HSRs were incubated at 37 °C for 30 min to allow re-association. The samples (60 pmol) were analyzed by 5%–20% SDG centrifugation in 6 or 15 mM Mg^{2+}. The resultant ribosome profiles are shown in the re-association panels.

nonincubated W3110 [compare panel 3 with 2 in Fig. 5C]. In *ΔrpmE::Km*, which does not contain L31, little or no 70S was detected, and 70S dissociated to 30S and 50S under 6 mM Mg^{2+} [panel 4 in Fig. 5C]. Taken together, these results indicate that r-protein L31 is involved in re-association of the 30S and 50S subunits *in vitro*.

Short L31-containing 50S subunits do not form stable 70S by association with 30S subunits *in vitro*

Furthermore, to determine the importance of the C-terminal residues of L31 in ribosomal association, we examined *in vitro* the ability of the 50S subunit carrying intact L31 only or both intact and short L31 to form the 70S particle by association with the 30S subunit. We prepared HSRs from *ΔompT::Km* cells and the incubated wild-type cells, collected the free 30S and 50S subunit fractions, and analyzed r-proteins from all samples by RFHR 2D-PAGE [panels 1–4 in Fig. 6B]. *ΔompT::Km* 50S contained 0.9 copies/ribosome of intact L31, whereas the incubated wild-type 50S contained 0.7 copies/ribosome of short L31 and no intact L31 (Fig. 6A,B). Neither intact nor short L31 were present in 30S fractions of either strain (Fig. 6B, lower panels). The subunits were mixed in four combinations [(1)+(2), (1)+(4), (3)+(2), and (3)+(4)] and incubated at 37 °C for 30 min to enable re-association, and the mixtures were analyzed by SDG centrifugation under 6 or 15 mM Mg^{2+}. Re-association into 70S of the 50S fraction from the incubated wild-type cells, which contained a large amount of short L31 (Fig. 6A,B, right), was less efficient than that of the 50S fraction prepared from *ΔompT::Km* [Fig. 6C, compare (3)+(2) and (3)+(4) with (1)+(2) and (1)+(4)]. These tendencies were more pronounced during SDG centrifugation at a lower Mg^{2+} concentration (i.e., 6 mM), in

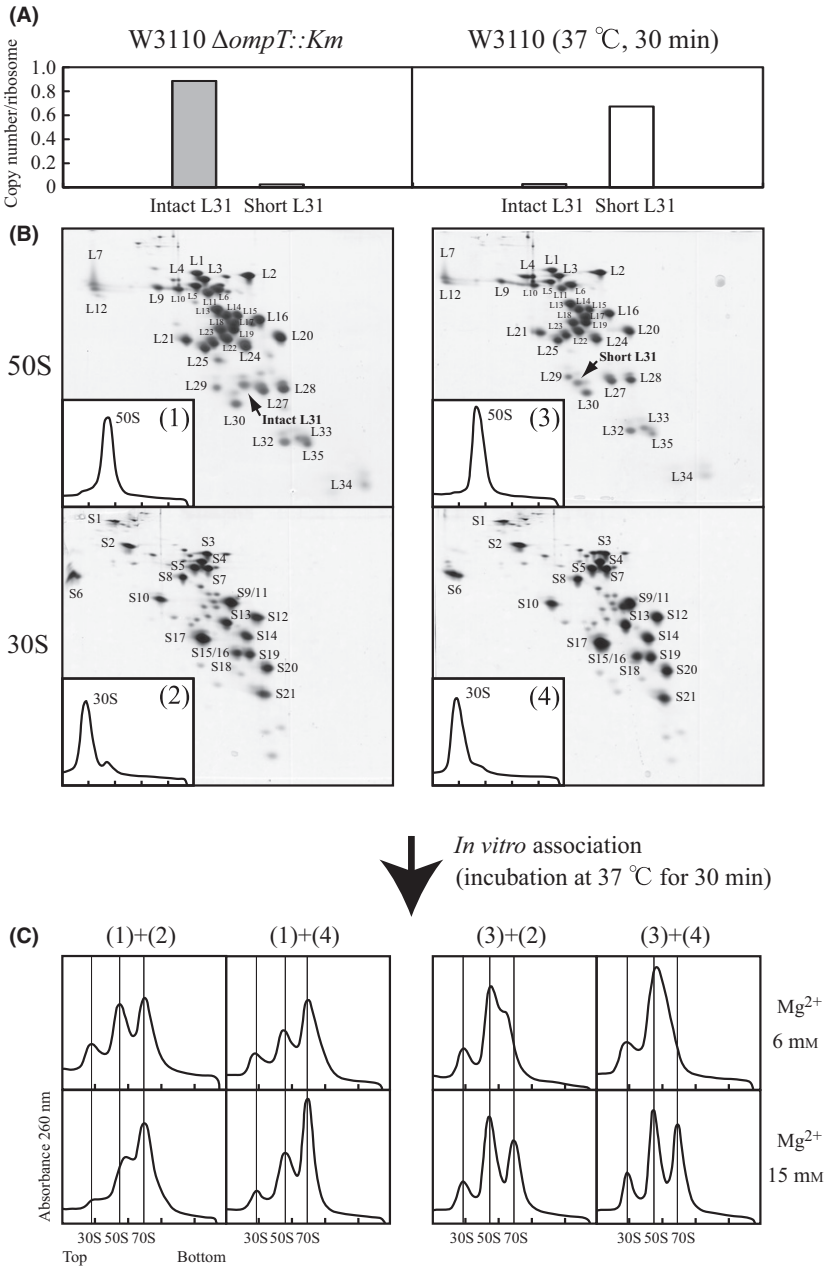

Figure 6 50S subunit containing short L31 does not form stable 70S by subunit association *in vitro*. 50S and 30S fractions from W3110 *ΔompT::Km* or the incubated W3110 were prepared [(1) 50S and (2) 30S for W3110 *ΔompT::Km*; and (3) 50S and (4) 30S for the incubated W3110]. Ribosomal proteins from the four samples were analyzed by RFHR 2D-PAGE. (A) 50S of W3110 *ΔompT::Km* and the incubated W3110 samples contains mainly intact L31 and short L31, respectively. Copy numbers of intact L31 and short L31 in 50S of W3110 *ΔompT::Km* and the incubated W3110 are shown by the bars. (B) 30S contains neither intact L31 nor short L31. Constituent proteins of 30S and 50S are shown as spots on gels. Both subunits from W3110 *ΔompT::Km* (1 & 2) and the incubated W3110 (3 & 4) were confirmed by 5%–20% SDG centrifugation in dissociation buffer I. (C) The 50S subunit containing short L31 does not form stable 70S. 50S (1 or 3) and 30S (2 or 4) subunits were mixed in four combinations (i.e., 1+2, 1+4, 3+2, and 3+4) and incubated at 37 °C for 30 min, after which the mixtures were analyzed by 5%–20% SDG centrifugation in 6 or 15 mM Mg^{2+}.

which the main fraction was 50S rather than 70S, and 70S was detectable only as a shoulder of the 50S peak [Fig. 6C, upper (3)+(2) and (3)+(4)]. By contrast, the 50S fraction of W3110 *ΔompT::Km* efficiently formed the 70S complex at 6 mM Mg^{2+} [Fig. 6C, upper (1)+(2) and (1)+(4)]. The 30S fraction, prepared from the incubated HSRs, formed stable 70S complexes with 50S particles prepared from *ΔompT::Km* [Fig. 6C, upper (1)+(4)].

These results show that intact L31 is required for stable 70S formation *in vitro*, whereas 50S containing short L31 or lacking L31 altogether form an unstable 70S complex that dissociates into 30S and 50S subunits during SDG centrifugation under 6 mM Mg^{2+}. These observations suggest that the eight C-terminal amino acids of L31 are important for subunit association.

HSRs defective in L31 exhibit reduced translation activity *in vitro*

To examine how HSRs with defects in L31 affect translational activity, we measured *in vitro* translation activities using HSRs prepared from W3110 (*ΔompT::Km*) and W3110 (*ΔrpmE::Km*) cells. Specifically, we examined synthesis of dihydrofolate reductase (DHFR) using a pure transcription/translation system, which is a commercial cell-free system reconstituted from purified components necessary for initiation, elongation, termination and ribosome-recycling steps of *E. coli* translation, T7 RNA polymerase, DHFR template DNA and ribosomes (Shimizu *et al.* 2005). The template DNA included in the kit contains a T7 promoter and ribosome-binding site (Shine-Dalgarno sequence) upstream of the gene of a complete DHFR (encoding 159 amino acid residues), the stop codon, and T7 terminator. As a template, the polymerase chain reaction (PCR) products of the DHFR (1–152 amino acid residues) gene derived from the template DHFR DNA were used. HSRs were prepared from disrupted cells incubated at 37 °C for 30 min, because this treatment increased translation activity. Pure transcription/translation reactions were incubated at 37 °C for 2 h and stopped by addition of SDS sample buffer on ice, and then electrophoresed on 10/20% SDS-PAGE gels. After the gels were stained with Coomassie Brilliant Blue (CBB), we quantified the density of the band corresponding to the DHFR (1–152) protein (Fig. S6 in Supporting Information). In samples with W3110 (*ΔrpmE::Km*) HSRs, the band intensity was approximately 38% lower than in samples with W3110 (*ΔompT::Km*) HSRs (Fig. 7A). W3110 (*ΔompT::Km*) HSRs used in the assay contained 0.85 copies/ribosome of intact L31, whereas W3110 (*ΔrpmE::Km*) HSRs contained neither intact nor short L31 (Fig. S7A,B in Supporting Information), indicating that intact L31 contributes to a ~40% increase in the protein synthesis *in vitro* in comparison with HSRs lacking L31.

Next, we investigated whether the decrease in translational activity resulted from inhibition of initiation or elongation. The AD202 (*ΔompT::Km*) HSRs used in the assay each contained one copy of intact L31 (Fig. S7C,D in Supporting Information). In contrast, the wild-type W3110 HSRs used contained 0.50 copies of intact L31, 0.20 copies of short L31, and no L31 (Fig. S6C,D in Supporting Information).

In the poly(U)-dependent L-[^{14}C] phenylalanine incorporation assay, 70S ribosomes bind to poly(U) without subunit dissociation, and elongation begins directly by binding of L-[^{14}C] phenylalanyl-tRNA to the P- and A-sites. Thus, this assay can measure only elongation activity, irrespective of initiation. The poly(U)-dependent L-[^{14}C] phenylalanine incorporation of wild-type ribosomes was 80% of that of *ΔompT::Km* ribosomes (Fig. 7B).

Next, using the same ribosomal preparations used in the preceding experiment, we measured translational activity in the MS2 mRNA-dependent L-[^{14}C] leucine incorporation assay, which requires a Shine-Dalgarno sequence, an initiation codon, initiation factors, and f-met tRNA, and is initiated by a natural translational process. In this assay, MS2 mRNA-dependent L-[^{14}C] leucine incorporation of wild-type ribosomes was 78%, relative to that of *ΔompT::Km* ribosomes (Fig. 7C). The elongation step is measured by both of the assay systems described above. Because the level of inhibition in the two assays was almost the same, we conclude that the elongation step rather than the initiation step was likely to have been affected by L31 modification.

Ribosomes carrying defective L31 cannot form stable 100S ribosomes

We then investigated the contribution of L31 to 70S dimerization. In the stationary phase, the majority of 70S ribosomes assemble into 100S ribosomes (70S dimer), which are translational inactive. Formation of these complexes is executed by RMF and facilitated by HPF (Wada *et al.* 1990; Wada 1998; Ueta *et al.* 2005; Yoshida & Wada 2014). We examined the involvement of L31 in the formation of 100S

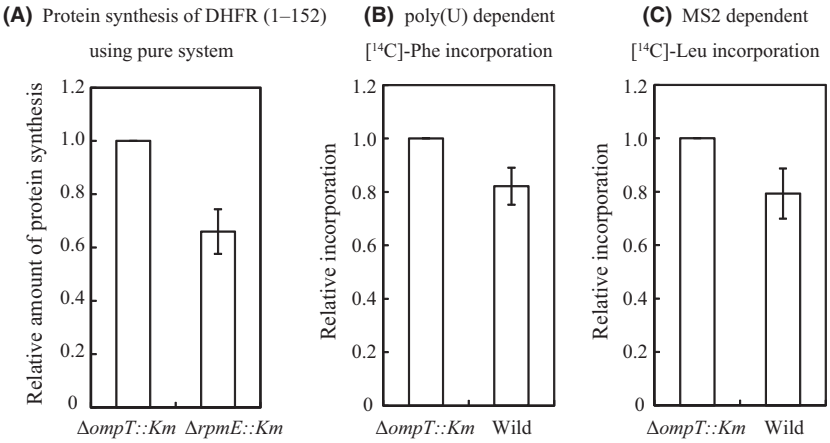

Figure 7 Translational activity of the ribosomes containing defective L31 is lower than that of ribosomes containing intact L31. (A) Synthesis of DHFR (1–152) is 40% lower in *ΔrpmE::Km* HSRs than in *ΔompT::Km* HSRs. Synthesis of DHFR (1–152) by the pure system was carried out *in vitro* using high salt-washed ribosomes (HSRs) containing exclusively intact L31 (from *ΔompT::Km* cells) or no L31 (from *ΔrpmE::Km* cells). After electrophoresis of reaction mixtures on 10/20% SDS-PAGE gels, the synthesized DHFR (1–152) was visualized by CBB (Fig. S5 in Supporting Information). The density of the DHFR (1–152) band was normalized against that of the r-protein band. Protein synthesis was normalized against synthesis by HSRs with only intact L31 (i.e., *ΔompT::Km*). The data shown are averages of four independent pure system reactions and two PAGE gels for each reaction (total of eight gels). (B, C) Translational activity *in vitro* is 20% lower in wild-type HSRs than in *ΔompT::Km* HSRs. (B) Poly(U)-dependent L-[U-^{14}C] phenylalanine incorporation and (C) MS2-dependent L-[U-^{14}C] leucine incorporation were carried out *in vitro* using HSRs containing either only intact L31 (*ΔompT::Km*) or both intact L31 and short L31 (wild type). Protein synthesis was normalized to that of HSRs containing only intact L31 (i.e., *ΔompT::Km*). Net incorporation of L-[U-^{14}C] Phe (B) and L-[U-^{14}C] Leu (C) by control *ΔompT::Km* HSRs was equivalent to 35.6 nmol and 1.0 nmol, respectively. The data represent the averages of three independent experiments.

ribosomes using three W3110 derivatives: (i) *ΔompT::Km*, (ii) wild type, and (iii) *ΔrpmE::Km*. The three strains were cultivated in EP medium at 37 °C for 3 days, and then CRs were prepared. Ribosome profiles were analyzed by SDG centrifugation in 6 or 15 mM Mg^{2+}, and the r-proteins were analyzed by RFHR 2D-PAGE. For (1) *ΔompT::Km*, the content of 100S ribosomes was 51% in 15 mM Mg^{2+} and 47% under 6 mM Mg^{2+} [panel 1 in Fig. 8C]. For (2) wild-type cells, the 100S contents were 59% and 50%, respectively [panel 2 in Fig. 8C]. In contrast, in *ΔrpmE::Km* cells, which lacked L31 but had 0.54 copies/ribosome of RMF [Fig. 8A,B, each (3)], 100S ribosomes were present at low levels (23%) in 15 mM Mg^{2+} and almost undetectable in 6 mM Mg^{2+} [panel 3 in Fig. 8C]. Furthermore, the 70S peak shifted to a smaller size, ~65S [panel 3 in Fig. 8C], suggesting that 70S ribosomes may undergo some sort of conformational changes upon L31 modification. These results suggested that the formation of stable 100S particles is suppressed in L31-deficient ribosomes, further implying that intact L31 contributes to the formation and stabilization of 100S.

Intact L31 is required for normal growth of *E. coli* cells

To further characterize the function of L31, we examined the effect of L31 strain status on bacterial growth. When the wild-type W3110 strain and the *ΔompT::Km* and *ΔrpmE::Km* deletion mutants were grown in the liquid EP medium, the exponential growth rate of *ΔrpmE::Km* [doubling time (DT), 35–40 min] was slower than that of W3110 (DT, 20–25 min) and *ΔompT::Km* (DT, 20 min). After 5 days of cultivation, the maximum culture turbidity of *ΔrpmE::Km* was also somewhat lower than that of the wild type and *ΔompT::Km* (Fig. 9A). The CFU value (colony-forming unit/mL culture) of *ΔrpmE::Km* cells after 5 days was half of that after 3 days, whereas wild-type and *ΔompT::Km* cells maintained high viability (Fig. 9B). When these strains were grown at 37 °C overnight on the LB solid medium, the colonies of *ΔrpmE::Km* were smaller than those of the wild type and *ΔompT::Km* (Fig. 9C). These data suggest that L31 is required for normal growth of *E. coli* and is involved in translational activities *in vivo* as well as *in vitro*.

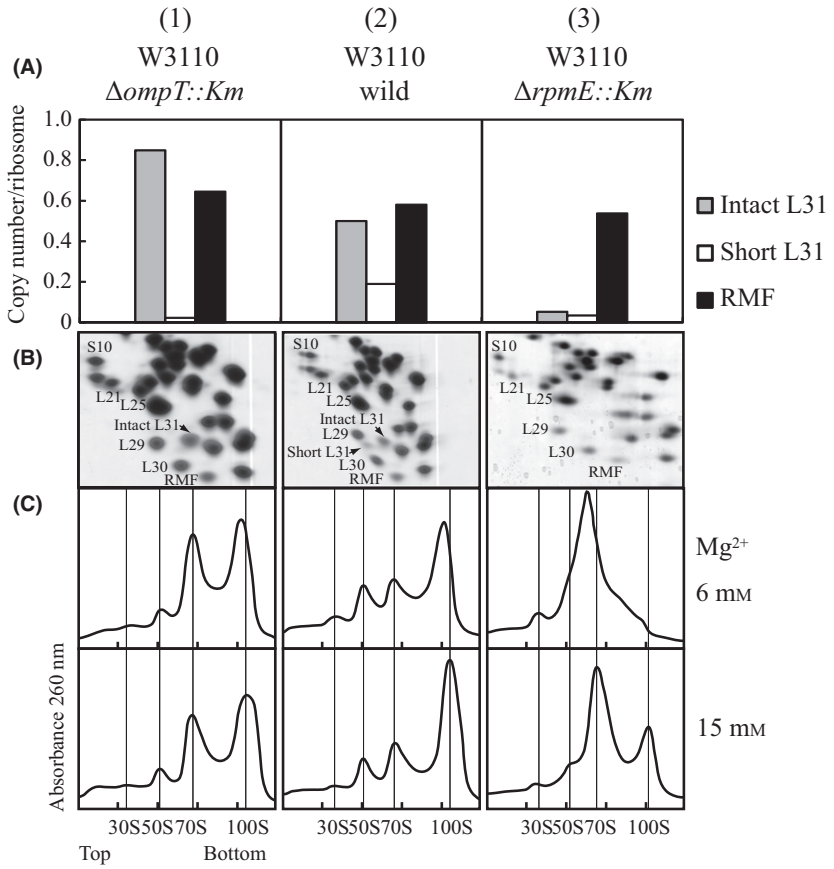

Figure 8 Ribosomes lacking L31 are defective in 100S formation in the stationary phase. (1) W3110 *ΔompT::Km*, (2) W3110, and (3) W3110 *ΔrpmE::Km* cells were grown at 37 °C for 3 days in EP medium, and then harvested. Crude ribosomes (CRs) were prepared and analyzed by RFHR 2D-PAGE and 5%–20% SDG centrifugation in 6 or 15 mM Mg^{2+}. (A) Copy numbers per ribosome of intact L31 (gray), short L31 (white), or RMF (black) in each CR sample are shown by bars. Copy numbers were calculated from gel spots shown in B. (B) Panels show CRs resolved on RFHR 2D-PAGE gels. Spots corresponding to S10, L21, L25, L29, L30, intact L31, short L31, and RMF proteins are indicated. (C) Ribosome profiles after 5%–20% SDG centrifugation in 6 or 15 mM Mg^{2+}. For each CR sample, 150 pmol was used.

Discussion

In this study, we showed that the *E. coli* r-protein L31 is cleaved by the outer membrane protein protease 7 during the routine preparation of ribosomes, yielding a short L31 protein lacking the eight C-terminal amino acids. Furthermore, the intact L31 performs important roles related to the formation and stability of the 70S ribosome and is also involved in translational activity; the deleted C-terminal amino acid residues contribute to these functions.

70S particles and free 50S subunits prepared from W3110 wild-type cells using modified buffer I (5 mM Mg^{2+}) mainly contained intact L31 and short L31, respectively (Fig. 3A). On the other hand, when 70S particles and 50S subunits prepared from *ΔompT::Km* HSRs, which exclusively contained intact L31, were incubated with protease 7 *in vitro*, intact L31 in 70S was barely sensitive to protease 7, whereas L31 in free 50S was readily cleaved (Fig. 3B,C). These results suggest that, in the 70S particle, the C-terminal region of intact L31 is protected by the 30S subunit.

In wild-type cells, the ratio of short L31 to intact L31 increased in proportion to incubation time after disruption [panels 2–4 in Fig. 2A,B], indicating that protease 7 acts on ribosomes in cytoplasmic solution flowing out of disrupted cell membranes. Intact cells have low free Mg^{2+} concentration in cytoplasmic solution (1–2 mM) (Alatossava *et al.* 1985; Froschauer *et al.* 2004). However, in addition to Mg^{2+}, various other components and molecules influence association

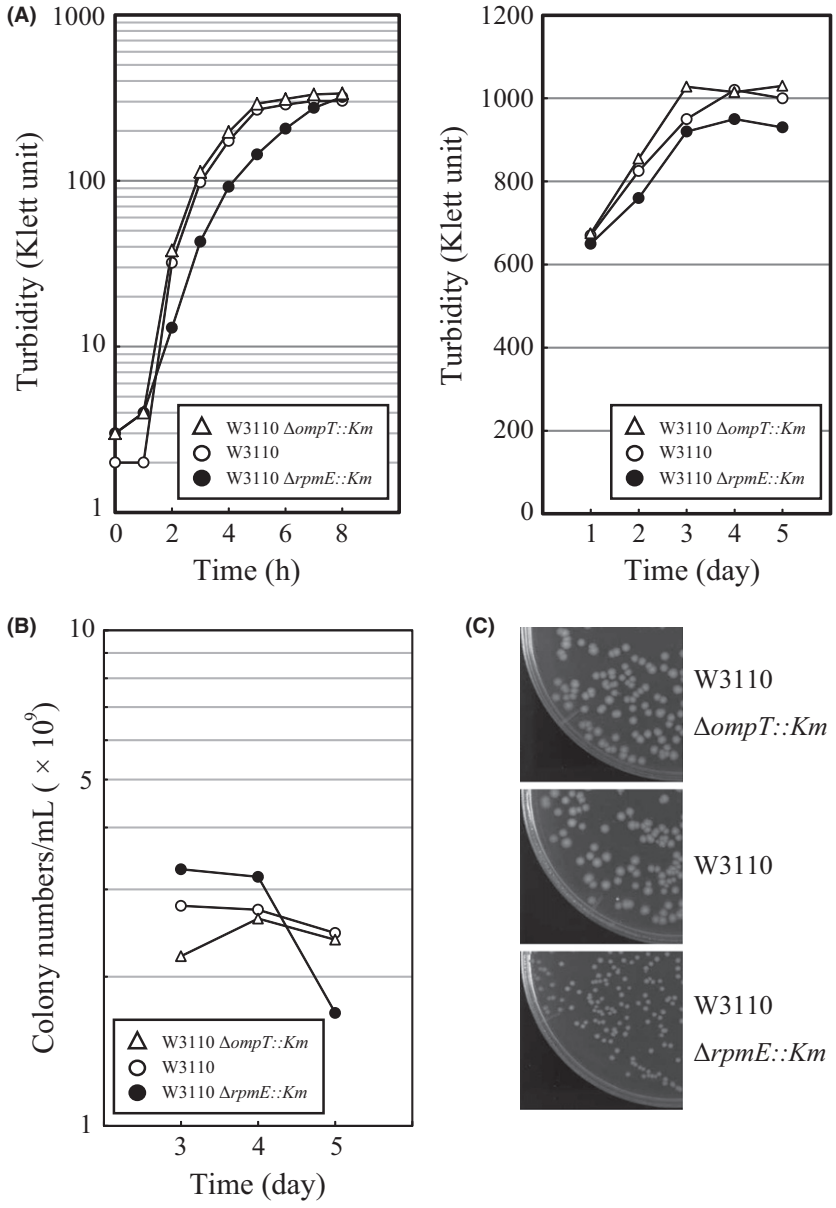

Figure 9 Depletion of L31 inhibits bacterial growth in both liquid EP and solid LB media. (A) (1) W3110 *ΔompT::Km* (open triangles), (2) W3110 (open circles), and (3) W3110 *ΔrpmE::Km* (closed circles) were cultured in EP medium at 37 °C. Cell growth was monitored by measuring Klett units. The time scales are 0–8 h (left panel) and 1–5 days (right panel). The vertical axis shows by normal logarithmic value for 0–8 h and by linear scale for 1–5 days. (B) Colony-forming unit (CFU) values of the three strains listed in (A). Cultured cells were harvested after 3, 4, or 5 days of cultivation, and the CFU value was measured. (C) Bacterial growth on LB solid medium. W3110 *ΔompT::Km*, W3110, and W3110 *ΔrpmE::Km* cells were incubated overnight (16–18 h) at 37 °C on solid LB medium, and resultant colony sizes are shown.

of ribosomes, and, in exponential phase, most ribosomes should be engaged with mRNAs. These mRNA-associated forms might be associated more loosely than *in vitro* 70S in association buffer (buffer I), and the C-terminus of L31 on the 30S may be easily attacked by protease 7 during ribosome preparation. To examine ribosome association in the disrupted cells, we attempted extraction of CRs with low Mg^{2+} near to free Mg^{2+} concentration in cells. After cell disruption, CRs were extracted with buffer

containing 5 mM Mg^{2+} and analyzed by SDG centrifugation in the same magnesium concentration. The CRs contained comparable molar ratios of 70S (intact L31 only) and 50S (short L31 and no intact L31) (Fig. 3A). By contrast, we also tried to strengthen these loosely associated forms by adding Mg^{2+} just before cell disruption. Ultimately, we set the concentration of Mg^{2+} at 15 mM, as in the association buffer. The ribosomes obtained under these conditions were mostly 70S, as determined by SDG centrifugation and ribosome profiling, and the strengthened 70S contained intact L31 exclusively (Fig. S8 in Supporting Information). This increase in 70S content reflects the looseness of ribosome association in disrupted cells.

The association and stability of the 70S particle *in vitro* strongly depend on the concentrations of various components including ribosomes, Mg^{2+}, monocations, and polyamines (Debey *et al.* 1975; Wishnia *et al.* 1975; Noll & Noll 1976; Rosano & Hurwitz 1977; Liiv & O'Connor 2006; Nierhaus 2014). However, in this study, to compare the association capacity of ribosomes, we simply exploited the Mg^{2+} dependency of association. In SDG centrifugation in 2 mM Mg^{2+}, even stable 70S particle from W3110 *ΔompT::Km* cells dissociated to 30S and 50S subunits (upper panel 1 in Fig. 4), whereas, in 3 mM Mg^{2+} ions, the S value shifted below 70S and in 5 mM Mg^{2+} ions, stable 70S particle was observed [panel 1 in Fig. 4]. Accordingly, we carried out the SDG centrifugation mainly in 5–15 mM Mg^{2+} to compare the stability of 70S or 100S ribosomes prepared from various cells.

Previous studies of *E. coli* (*ompT*$^{+}$) ribosomal profiles generated by routine SDG centrifugation reported that a certain proportion of ribosomes, 10% or more, exists as the free 30S and 50S subunits. However, these reports are incorrect, and are due to the use of ribosome samples containing short L31. Because protease 7 is an outer membrane protein, the C-terminal amino acids of L31 should not be cleaved *in vivo*, and thus, short L31 should never be present in living cells. From proteome analysis of W3110 cells, we confirmed that protease 7 was detected only in the cell debris (CD) fraction but not in all other fractions [the crude ribosome (CR) and postribosomal supernatant (PRS)] (Fig. S9; Table S2 in Supporting Information).

Our examination of *ΔompT::Km* cells showed that, during SDG centrifugation in association buffer, the majority of the ribosomes from exponential-phase cells existed in the associated state as 70S particles, and that the amounts of free 30S and 50S subunits obtained after SDG centrifugation were much smaller than expected based on numerous previous SDG profiles of *E. coli* ribosomes containing short L31.

We examined the effect of L31 on translation activity *in vitro* using three assay systems. When synthesis of truncated DHFR (1–152) was examined by the transcription/translation pure system, using HSRs prepared from *ΔompT::Km* and *ΔrpmE::Km* cells, the translational activity of *ΔrpmE::Km* HSRs was 40% lower than that of *ΔompT::Km* ribosomes (Fig. 7A). These observations suggest that HSRs lacking intact L31 have diminished translation activity *in vitro*.

The poly(U) mRNA-dependent ^{14}C-phenylalanine incorporation assay, which measures only the elongation step, and the MS2 mRNA-dependent ^{14}C-leucine incorporation assay, which measures both the initiation and elongation steps, were also used. In both assay systems, the translational activities of wild-type ribosomes (HSRs) were approximately 20% lower than those of *ΔompT::Km* HSRs (Fig. 7B,C). The common step in the two translational assays is not initiation, but elongation. Therefore, it is likeliest that short L31 reduces *in vitro* translation by affecting elongation. *ΔompT::Km* HSRs each contained one copy of intact L31, whereas half of wild-type HSRs retained intact L31 and the other half were modified by protease 7 (Fig. S7C,D in Supporting Information). Thus, of the 80% translational activity of the wild-type ribosomes, 50% is accounted for by ribosomes having intact L31, and the other 30% by ribosomes modified by protease 7, which includes both short L31 and the absence of L31. Therefore, the translational activity of ribosomes modified by protease 7 is 40% lower than that of ribosomes with intact L31.

The two ribosomal subunits are associated through 12 intersubunit bridges (B1a/b, B2a/b, c, B3, B4, B5, B6, B7a/b, B8). Five bridges are composed of contacts between 23S rRNA and 16S rRNA. B1b, the only protein–protein bridge, is formed by contacts between L5 and S13. Furthermore, another bridges are composed of contacts between 30S r-protein (S13, S15, or S19) and 23S rRNA, 50S r-protein (L2, L14, or L19) and 16S rRNA (Gao *et al.* 2003). In addition to intersubunit bridges, S20 is involved in the subunit association and translation initiation (Götz *et al.* 1990).

Structural evidence has indicated that the N-domain of L31 binds to the 50S subunit, whereas the C-domain extends to the 30S subunit (Selmer *et al.* 2006; Jenner *et al.* 2010; Shasmal *et al.* 2010; Fischer *et al.* 2015). Thus, by fastening the 30S and 50S subunits, L31 may contribute to increasing the

translational activity of the ribosome (Fig. 7A–C). The N-domain of L31 interacts with the 5S rRNA, L5 and part of S13 (Fanning & Traut 1981). On the other hand, the C-domain of L31 interacts with S13 and S19 and further extends toward S14, and the C-tail of L31 binds to helix-41 of the 16S rRNA (Fig. S1B,C in Supporting Information). Based on our results, when the 70S particle dissociates to subunits in the dissociation buffer, L31 was found only in the 50S subunit, showing the N-domain of L31 tightly binds to the 50S subunits whereas the C-domain of L31 can be dissociated from the 30S subunit under physiological conditions (Fig. 6B). In the 70S particle, although the C-terminal region of intact L31 is basically protected from protease 7 cleavage by associating with the 30S subunit (Fig. 3C), protease 7 might be able to cleave the C-tail of L31 when it temporarily and partially dissociates from the 30S subunit during the preparation of wild-type ribosome (panels 2–4 in Fig. 2). We showed that the C-tail of L31 is important for the translational activity, especially for the elongation step (Fig. 7B,C). In the EF-G-mediated ratcheted state, the L5-S13 associated B1b bridge, which connects the 50S and 30S subunits, opens and S13 dissociates from the N-domain of L31 (Fig. S1C in Supporting Information). L31 may play a significant role in defining the maximum amplitude of this motion (Shasmal *et al.* 2010). The shortening of L31 by protease 7 may perturb the ratcheting motion during the elongation step, especially in the translocation.

We also examined the contribution of L31 to 100S formation. *ΔompT::Km* CRs with intact L31 and wild-type CRs with both intact and short L31 formed large 100S peaks, but *ΔrpmE* CRs with no L31 formed only a small 100S peak (Fig. 8), indicating that L31 is involved in the formation of the 100S ribosome during the stationary phase. The proportion of 70S ribosomes in 100S particles was much higher in *ΔompT::Km* CRs than in CRs from wild-type cells [Fig. 8C each panel (1) (2)] This difference may be brought about by the increased tendency of 70S ribosomes in wild-type cells, which contain some short L31, to dissociate. In wild-type cells harvested in stationary phase, the ribosomes contained intact L31 more than HSR prepared from exponential-phase cells [compare panel 2 in Fig. 8A,B with panel 2 in Fig. 2A,B], suggesting that L31 in 100S is protected from cleavage by protease 7 during ribosome preparation. By contrast, in W3110 *ΔrpmE* cells, a smaller content (23%) of 100S ribosomes was observed in SDG centrifugation in 15 mM Mg^{2+}, and almost none in 6 mM Mg^{2+} [panel 3 in Fig. 8C]. This observation indicates that, in W3110 *ΔrpmE::Km*, the formation of stable 100S ribosome was impeded. In intact W3110 *ΔrpmE::Km* cells, RMF may bind to 70S to yield 100S, but the resultant 100S may be unstable due to the lack of L31. The early death observed in W3110 *ΔrpmE::Km* cells may have been caused by loss of 100S formation (Fig. 9B). Consistent with this, a mutant strain lacking *rmf*, which enables 100S formation, loses viability much more rapidly than the parental strain in the stationary phase (Yamagishi *et al.* 1993).

L31, a unique r-protein that bridges 50S and 30S directly via a single molecule, is conserved among bacteria. These homologues may also have bridging functions in other species as L31 has in *E. coli*. In eukaryotes, there are two similar intersubunit bridges formed by the 60S proteins L19e and L24e, which are bound to the 40S subunit by two arms on opposite sides (Ben-Shem *et al.* 2011). These proteins are eukaryote-specific, but functionally similar to the conserved bacterial L31 protein (Melnikov *et al.* 2012).

Over the years, numerous measurements of *E. coli* ribosomal translational activity have been carried out *in vitro*. However, it seems likely that the ribosomes used in previous experiments included some amount of short L31 resulting from cleavage by protease 7. The activities of the ribosomes used for those measurements probably decreased in response to varying degrees of L31 modification by this enzyme. Consequently, previous studies of the translational activity of *E. coli* ribosomes could only have yielded accurate data when *ΔompT* mutants were used.

Intact L31 is required for normal growth. Cells defective in L31 grew more slowly than the W3110 *ΔompT::Km* and W3110 wild-type cells, reflecting low translational activity. Future studies should seek to elucidate how ribosomal L31 contributes to translational activity in intact cells.

Experimental procedures

Bacterial strains and plasmids

All strains used in this study are listed in Table S1 in Supporting Information. The *rpmE::Km* and *ompT::Km* deletion mutants (BW25113-derived *E. coli* strains) were obtained from the Keio collection (Baba *et al.* 2006). P1vir phages were prepared using the Keio deletion mutant strains as donors. W3110 *ΔrpmE::Km* (YB1010) and W3110 *ΔompT::Km* (YB1012) were constructed by P1vir general transduction of the recipient strain W3110 (wild type). In each strain, replacement of *rpmE* or *ompT* by the kanamycin-resistance gene was confirmed by PCR. To generate YB1017 (W3110

pCA24N-*ompT*$^{+}$), W3110 was transformed with the pCA24N-*ompT*$^{+}$ (-GTP) plasmid from the ASKA library (Kitagawa *et al.* 2005).

Media and growth conditions

All strains were grown at 37 °C in medium E supplemented with 2% polypeptone and 0.5% glucose (EP medium) (Wada 1986a). Cell growth was measured by estimating the cell turbidity using a Klett–Summerson photoelectric colorimeter (Bel-Art Products, Wayne, NJ, USA) with a green filter (#54). Cells were harvested in exponential and/or stationary phases and stored at −80 °C until use. Cell growth on L-broth agar plates was determined by monitoring the colony size after an overnight incubation at 37 °C.

Preparation of CRs and CD

Crude ribosomes were prepared from cell extracts according to the method of Noll *et al.* (1973) with slight modifications (Horie *et al.* 1981). Harvested cells were ground with similar volumes of quartz sand (Wako, Osaka, Japan), and the disrupted cells were then extracted with buffer I [20 mM Tris–HCl (pH 7.6), 15 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol]. The homogenate was centrifuged at 9000 ***g*** for 15 min at 4 °C. The supernatant was saved, and the pellet was re-suspended in buffer I. The suspension was centrifuged again under the same conditions. The pellet was used as the CD fraction. The combined supernatants (cellular extract, CE) were layered onto a 30% sucrose cushion in buffer I and centrifuged in a 55.2 Ti rotor (Beckman, Fullerton, CA, USA) at 206 000 ***g*** for 3 h at 4 °C. The pellet was re-suspended in buffer I and used as the CR fraction, and the supernatant was used as the PRS.

Preparation of CD fraction from *ompT*-over-expressing cells

YB1017 (W3110 pCA24N-*ompT*$^{+}$) cells were grown in EP medium at 37 °C to mid-exponential growth phase (Klett units: 50). Expression of *ompT* was induced by addition of 0.1 mM IPTG (isopropylthiol-β-D-galactoside). After an additional 2 h of incubation, the cells were harvested. Cell pellets were ground with an approximately equal volume of quartz sand (Wako) and then extracted with buffer I. The homogenate was centrifuged at 9000 ***g*** for 15 min at 4 °C. The supernatant was saved, and the pellet was re-suspended in buffer I. The pellet suspension was centrifuged again under the same conditions, and the resultant pellet was used as the *ompT*-over-expressing CD fraction.

Preparation of HSRs

High salt-washed ribosomes were prepared as described by Horie *et al.* (1981). CRs were re-suspended in buffer II [20 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate, 1 M ammonium acetate, and 6 mM 2-mercaptoethanol]. After mixing at 4 °C for 1 h, the high salt-washed suspension (20 mL) was layered onto a 30% sucrose cushion in buffer II (10 mL) and centrifuged in a 55.2 Ti rotor (Beckman) at 206 000 ***g*** for 4 h at 4 °C. The pellet was re-suspended in buffer I and dialyzed against buffer I overnight. This suspension was used as HSRs. For HSRs used in translation assays *in vitro*, the high salt washing was repeated twice.

Preparation of 30S and 50S subunits

High salt-washed ribosomes were suspended in dissociation buffer I [20 mM Tris–HCl (pH 7.6), 1 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol] and dialyzed against the same buffer overnight. The sample was layered onto a 10%–40% SDG in dissociation buffer I and centrifuged in a 45 Ti rotor (Beckman) at 20 000 ***g*** for 19 h at 4 °C. The gradient was fractionated, and the absorbance of each fraction was measured with a UV-1700 spectrometer (Shimadzu, Kyoto, Japan) at 260 nm. The respective 30S and 50S fractions were collected, and each subunit was pelleted by centrifugation in a 55.2 Ti rotor (Beckman) at 206 000 ***g*** for 4 h at 4 °C. Each pellet was re-suspended in buffer I.

Analysis of ribosomes by SDG centrifugation

Each ribosome sample was layered onto a 5%–20% SDG (12 mL) in buffer I or modified Mg^{2+} buffer I [20 mM Tris–HCl (pH 7.6), 3–10 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol] and centrifuged in a SW 40 Ti rotor (Beckman) at 25 000 ***g*** for 20 h at 4 °C. The SDGs were prepared using a Gradient Mate 6T (BioComp Instruments, Fredericton, NB, Canada). The absorbance of each fraction was measured at 260 nm using a flow cell in an UV-1700 spectrometer (Shimadzu).

RFHR 2D-PAGE

Escherichia coli r-proteins were prepared using the previously described acetic acid method (Hardy *et al.* 1969). One-tenth volume of 1 M $MgCl_2$ and two volumes of acetic acid were added to the ribosomal solutions, and the mixture was stirred at 0 °C for 1 h. After centrifugation at 10 000 ***g*** for 10 min at 4 °C, the supernatant was dialyzed three times against 2% acetic acid (the volume of the dialysis buffer was 300-fold larger than the volume of the sample) for 24 h. The proteins were lyophilized and stored at −80 °C until use. The protein solution (2 mg of protein in 100 μL of 8 M urea containing 0.2 M 2-mercaptoethanol) was analyzed by RFHR 2D-PAGE, as described previously (Wada 1986a,b), with slight modification (Ueta *et al.* 2010). Sample charging electrophoresis was carried out at 100 constant volts (CV) for 15 min at room temperature (RT). Subsequently, one-dimensional electrophoresis was carried out at 170 CV for 8 h at RT, and

electrophoresis in the second dimension was carried out at 100 CV for 15 h at R.T. The 2D gels were stained with CBB G-250, and protein spots were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Determination of copy number of ribosomal proteins (intact and short L31) and protease 7 (OmpT)

'Copy number' refers to the molar ratio of a ribosome-binding protein to a single 70S ribosomal particle, the 30S subunit or the 50S subunit. The OD of a protein spot on an RFHR 2D-PAGE gel was determined by scanning the stained spot with a GS-800 calibrated densitometer. The molar amount of a protein was proportional to its OD value (OD/MW). OD/MW was calculated as a function of molecular weight, and the values for the intact L31, short L31, and RMF proteins were normalized against the OD/MW value for the r-proteins L27, L29, and L30 (Hardy 1975; Tal *et al*. 1990). The values for protease 7 were normalized against the r-proteins L1, L3, L4, and L5.

In vitro translation assays

In vitro translation assays were carried out using three methods: (A) Pure transcription–translation assay, (B) Poly(U) mRNA-dependent L-[U-^{14}C] phenylalanine incorporation assay, and (C) MS2 mRNA-dependent L-[U-^{14}C] leucine incorporation assay. (A) The pure *in vitro* transcription–translation protein synthesis system (Shimizu *et al.* 2005) was the PURE*frex* 1.0 kit (Gene Frontier Corporation, Kashiwa, Chiba, Japan). The ribosomes in kit component Solution III were replaced with HSRs prepared from W3110 Δ*ompT::Km* or W3110 Δ*rpmE::Km*. During ribosome preparation, disrupted cells were incubated at 37 °C for 30 min. To clearly separate synthesized protein from other proteins in SDS-PAGE analysis, the truncated cDNA of DHFR (1–152 a.a.) was generated as the template. The DNA was amplified using the GoTaq Green Master mixture (Promega, Madison, WI, USA), with DNA of intact DHFR (1–159 a.a.) as the template and primers 5′-gaaattaatacgactcactatagggagacc-3′ and 5′-gatagctcagctaattgcaatagctgtgagagtt-3′. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and used as template DNA in the pure system. The transcription/translation reactions were carried out at 37 °C for 2 h in a 20 μL volume containing 1 μM ribosomes, 20 ng of template DNA, and 10 units of RNase Inhibitor (Super) (Wako). The reaction mixtures were 13 mM Mg^{2+} concentration. After reaction, samples were analyzed by SDS-PAGE using Multi Gel II mini 10/20 (Cosmo Bio Inc., Koto, Tokyo, Japan). After CBB staining, gel bands were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories Inc.) to measure the density of the synthesized protein. For (B) and (C), S100 fractions and crude initiation factors were prepared from exponentially growing *E. coli* Q13 cells. The HSRs of W3110 and AD202 (MC4100 *ΔompT::Km*) were prepared as described above except that washes were carried out twice. The detailed methods for assays B and C are provided in previous paper (Ueta *et al.* 2008). Mg^{2+} concentration was 12 mM for poly (U) system and 8 mM for MS-2 system.

In vitro dissociation and re-association of the 70S subunits

High salt-washed ribosomes were prepared from CRs of mid-exponential cells and dialyzed overnight against dissociation buffer II [20 mM Tris–HCl (pH 7.6), 1 mM magnesium acetate, 30 mM ammonium acetate, and 6 mM 2-mercaptoethanol] to dissociate to the 30S and 50S subunits. To confirm dissociation, the solutions were subjected to 5%–20% SDG in dissociation buffer II and centrifuged in an SW 40 Ti rotor (Beckman) at 200 200 ***g*** for 2.5 h at 4 °C. Suspensions of dissociated ribosomal subunits were converted to buffer I by addition of magnesium acetate and ammonium acetate and then incubated at 37 °C for 30 min to re-associate the subunits into the 70S complex. The mixtures were analyzed by 5%–20% SDG centrifugation in the presence of 6 or 15 mM Mg^{2+}.

In vitro formation of 70S ribosomes from 30S and 50S subunits

The 30S and 50S subunits were prepared as described above from W3110 and W3110 *ΔompT::Km* cells in the mid-exponential growth phase. When the treated W3110 CR fraction was prepared, the suspension was incubated at 37 °C for 30 min after cell crushing by quartz sand grinding to facilitate cleavage of the C-terminal residues of L31. To re-associate the subunits, the 30S and 50S subunits (90 pmol each) were mixed and incubated at 37 °C for 30 min. The incubated mixture was then analyzed by 5%–20% SDG centrifugation.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 (A) Amino acid sequences of intact L31 and short L31. (B) The structural location of *E. coli* L31 on the 70S ribosome. (C) Schematic representation of the L31-involved ratcheting motion of the ribosome.

Figure S2 Crude ribosomes prepared from strains W3110, W3350, and Q13 contain intact and short L31.

Figure S3 (A) High salt–washed ribosomes (HSRs) from AD202 (*ΔompT::Km*) cells contain only intact L31 and no short L31. (B) AD202 (*ΔompT::Km*) HSRs mainly contain 70S in SDG centrifugation in 6 mM Mg^{2+}.

Figure S4 (A) When disrupted *ΔompT::Km* cells are incubated at 37 °C for 30 min, the HSRs contain only intact L31 and no short L31. (B, C) W3110 *ΔompT::Km* HSRs prepared after mixing with CD fraction of *ompT*-overexpressing cells contained intact L31 and short L31.

Figure S5 Intact L31 is contained only in 70S fraction and not in 50S fraction.

Figure S6 DHFR (1–152) synthesized by the pure transcription/translation assay system was detected by 10/20% SDS-PAGE.

Figure S7 Copy numbers and gel patterns of intact L31 or short L31 in HSRs used in the translation assay *in vitro*.

Figure S8 In the preparation of wild-type ribosomes, cell disruption in 15 mM Mg^{2+} yields 70S ribosomes containing mainly intact L31.

Figure S9 Proteomic analysis of *E. coli* W3110 cells by RFHR 2D-PAGE method.

Table S1 Bacterial strains used in this study

Table S2 Proteins of *Escherichia coli* W3110 separated by RFHR 2-D PAGE of Fig. S9 were identified by Matrix Assisted Laser Desorption/Ionization mass spectrometry, (MALDI-TOF MS) (Voyager DE-PRO, Applied Biosystems, California, USA)