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Biochemical Evidence for the Heptameric Complex L10(L12)$_6$ in the *Thermus thermophilus* Ribosome: *In Vitro* Analysis of its Molecular Assembly and Functional Properties

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The stalk protein L12 is the only multiple component in 50S ribosomal subunit. In *Escherichia coli*, two L12 dimers bind to the C-terminal domain of L10 to form a pentameric complex, L10[(L12)$_2$]$_2$, while the recent X-ray crystallographic study and tandem MS analyses revealed the presence of a heptameric complex, L10[(L12)$_2$]$_3$, in some thermophilic bacteria. We here characterized the complex of *Thermus thermophilus* (Tt-) L10 and Tt-L12 stalk proteins by biochemical approaches using C-terminally truncated variants of Tt-L10. The C-terminal 44-residues removal (Δ44) resulted in complete loss of interactions with Tt-L12. Quantitative analysis of Tt-L12 assembled onto *E. coli* 50S core particles, together with Tt-L10 variants, indicated that the wild-type, Δ13 and Δ23 variants bound three, two and one Tt-L12 dimers, respectively. The hybrid ribosomes that contained the *T. thermophilus* proteins were highly accessible to *E. coli* elongation factors. The progressive removal of Tt-L12 dimers caused a stepwise reduction of ribosomal activities, which suggested that each individual stalk dimer contributed to ribosomal function. Interestingly, the hybrid ribosomes showed higher EF-G-dependent GTPase activity than *E. coli* ribosomes, even when two or one Tt-L12 dimer. This result seems to be due to a structural characteristic of Tt-L12 dimer.

Key words: elongation factors, GTPase-associated centre, ribosomal stalk, ribosome, translation elongation.

Abbreviations: GST, glutathione S-transferase; WT, wild-type.

The ribosomal stalk protein L7/L12 (referred to here as L12 throughout) plays a crucial role in the interaction of the ribosome with elongation factors EF-G and EF-Tu, and in GTPase-associated events during bacterial translation (*1*, *2*). A number of biochemical studies have indicated that the *Escherichia coli* ribosome contains two L12 homodimers that bind to the C-terminal region of the anchor protein L10 (*3–7*). The L10•L12 pentameric protein complex binds to the 1070 region of 23S rRNA, together with the L11 protein, and constitutes a major part of the ribosomal GTPase-associated center (*8–10*). Because L12 is a highly flexible protein (*11–13*), the detailed structure of the L10•L12 complex within the ribosome has not been resolved by X-ray crystallography (*14–18*).

It had been long believed that a pentameric complex composed of four copies of L12-like proteins and one copy of an anchor protein was conserved in all ribosomes, including those from bacteria (*5–7*, *19*, *20*), eukarya (*21–24*) and archaea (*25*). Recent MS analysis, however, showed that ribosomes from the thermophilic bacteria *Thermus thermophilus* and *Thermatoga maritima* contain a heptameric complex that is composed of six copies of L12 and one copy of L10 (*26*). Furthermore, when full-length *T. maritima* L10 and the N-terminal domain of L12 were co-expressed, they formed the heptameric complex L10(L12)$_2$(L12)$_2$(L12)$_2$, as shown by X-ray crystallography (*27*). More recently, we demonstrated, by biochemical approaches, that the archaeal counterparts of bacterial L10 and L12 also form a heptameric protein complex (*28*). Thus, our understanding of the structure of the ribosomal stalk protein complex has been significantly modified. In the crystal structure of the *T. maritima* stalk protein complex, three L12 dimers bind side by side to the C-terminal helix α8 region of L10 (*27*).

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Comparison of the amino acid sequences of L10-like proteins shows that there is a short additional sequence within the C-terminal region of *T. maritima* L10, to which the third L12 dimer binds. The extra C-terminal sequence in L10-like proteins is observed in approximately half of bacterial species and all known archaeal species, but not in eukarya (*28*). This bioinformatics study suggested that the ribosomes of many organisms contain three L12-like stalk dimers, although the presence of three stalk dimers has been demonstrated in only a few species, so far.

In order to clarify the relationship between the extra C-terminal sequence in L10 and the presence of three L12 dimers in the ribosome, we performed a biochemical analysis of the *T. thermophilus* (Tt-) L10•L12 complex, which has been shown to be heptameric by MS analysis (*26*). The Tt-L10•Tt-L12 complex was reconstituted using various Tt-L10 mutants, whose C-terminal sequences were partially truncated to different extents. The reconstituted complexes were assembled onto *E. coli* 50S core particles. The resultant hybrid ribosomes had significant EF-G/EF-Tu-dependent ribosomal activity. Quantitative analysis of the Tt-L12 that was assembled onto the ribosome indicated that all three Tt-L12 dimers bound in tandem to the C-terminal region, and the extra sequence in Tt-L10 corresponded to an additional binding site for the L12 dimer. Each individual dimer appeared to contribute to ribosomal activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Protein Expression and Purification—DNA fragments that encoded full length Tt-L10 (WT, residues 1–173) or the C-terminally truncated variants, Δ13 (residues 1–160), Δ23 (residues 1–150), Δ30 (residues 1–143) and Δ44 (residues 1–129) (Fig. 1A), were amplified by PCR using *T. thermophilus* HB8 genomic DNA (*29*) as a template, and cloned into the *Bam*HI/*Eco*RI sites of an *E. coli* expression vector, pGEX-6p-1c (GE Healthcare). The proteins were expressed as GST-fusion proteins in *E. coli* BL21(DE3)pLysS (Novagen). The WT protein and the Δ13 and Δ23 mutants were obtained in the insoluble fraction. These were solubilized in Buffer A, which consisted of 7 M urea, 5 mM 2-mercaptoethanol and 20 mM Tris–HCl (pH 8.0). The Δ30 and Δ44 mutants were obtained in the S100 supernatant fraction and were dialysed against Buffer A. All the Tt-L10 GST-fusion proteins were then applied to a DEAE-cellulose column (Whatman) that had been equilibrated with Buffer A and each was recovered in a flow-through fraction. The proteins were further purified on Glutathione Sepharose 4B (GE Healthcare), as described by the manufacturer. The coding region of Tt-L12 was also amplified by PCR and inserted into the *Nde*I/*Bam*HI sites of another *E. coli* expression vector, pET-11c (Novagen), and the protein expressed in *E. coli* BL21(DE3)pLysS. The S100 fraction, which contained the expressed Tt-L12, was heated

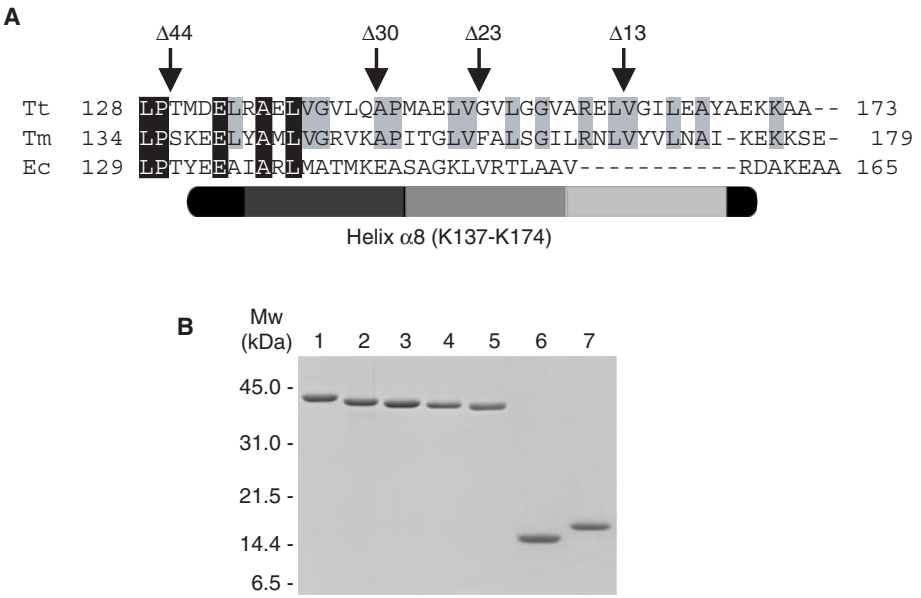

Fig. 1. **Preparation of truncated variants of *T. thermophilus* ribosomal protein Tt-L10.** (A) Comparison of amino acid sequences in the C-terminal region of L10 from *T. thermophilus* (Tt-L10, residues 128–173), *T. maritima* (Tm-L10, residues 134–179) and *E. coli* (Ec-L10, residues 129–165). The alignment is based on comparison of the amino acid sequences of L10-like proteins from 11 *Eukarya*, 10 *Archaea* and 28 *Bacteria*, as described by Maki *et al*. (*28*). The end-points of the C-terminal truncations constructed in the present study are indicated on the sequence of Tt-L10. The bar shown below the sequence of Ec-L10 denotes the position of helix α8 (Lys137–Lys174) of Tm-L10. The three binding sites for Tt-L12 correspond to the regions shown individually in dark grey, medium grey and light grey on the bar, according to the crystal structural data for Tm-L10 (*27*). (B) SDS–PAGE of purified Tt-L10 fused with GST. Samples (each 2 μg) of the WT (lane 1, 44.6 kDa), Δ13 (lane 2, 43.2 kDa), Δ23 (lane 3, 42.3 kDa), Δ30 (lane 4, 41.6 kDa) and Δ44 (lane 5, 40.0 kDa) Tt-L10 proteins, together with Tt-L12 (lane 6, 13.1 kDa) and Tt-L11 (lane 7, 15.5 kDa), were analysed by 18% (w/v) SDS–PAGE. The gel was stained with Coomassie Brilliant Blue R-250.

at 70°C for 30 min to denature host cell proteins. The soluble fraction, which contained Tt-L12, was dialysed overnight against Buffer B, which consisted of 6 M urea, 5 mM 2-mercaptoethanol and 20 mM Tris–HCl (pH 7.6), and then applied to a DEAE-cellulose column that had been equilibrated with Buffer B. The Tt-L12 protein was eluted in Buffer B containing 0.02 M LiCl. Site-directed mutagenesis was performed on the Tt-L12 expression construct, using the QuickChange Site-Directed Mutagenesis kit (Stratagene), to alter Leu63 to Cys (L63C). The L63C mutant was overexpressed and purified by the same procedures as the wild-type Tt-L12. The coding sequence for Tt-L11 was also amplified by PCR and inserted into the *Nde*I/*Bam*HI sites of pET-3a. Tt-L11 was expressed in the same way as Tt-L12. The Tt-L11-containing S100 fraction was dialysed overnight against Buffer C, which consisted of 7 M urea, 5 mM 2-mercaptoethanol and 20 mM sodium acetate (pH 4.5), and then applied to a CM-cellulose column (Whatman) that had been equilibrated with Buffer C. The Tt-L11 protein was eluted in Buffer C containing 0.05 M LiCl. The Tt-L11-containing fraction was dialysed overnight against Buffer D, which consisted of 6 M urea, 5 mM LiCl, 5 mM 2-mercaptoethanol and 20 mM sodium phosphate (pH 6.5), and further purified by HPLC on DEAE-5PW (Tosoh) using a linear gradient of 5–154 mM LiCl. The purity of the proteins was confirmed by SDS–PAGE (Fig. 1B).

Complex Formation Between Tt-L10 and Tt-L12—Each purified Tt-L10 variant fused with GST was mixed with Tt-L12 at a molar ratio of 1:10 in Buffer E, which contained 7 M urea, 300 mM KCl, 5 mM 2-mercaptoethanol and 20 mM Tris–HCl (pH 8.4), and dialysed against the same buffer without urea to form GST-Tt-L10•Tt-L12 complexes. The GST moiety in each complex was completely removed by digestion with PreScission Protease according to the manufacturer's instructions and heating at 70°C for 30 min. The formation of the Tt-L10•Tt-L12 complexes was confirmed by 6% (w/v) polyacrylamide (acrylamide/bisacrylamide ratio, 39:1) native gel electrophoresis at 6.5 V/cm with a buffer system that contained 100 mM KCl and 50 mM Tris–HCl (pH 8.0). The electrophoresis was performed for 10 h at constant voltage and 4°C with buffer recirculation. The gel was stained with Coomassie Brilliant Blue R-250.

In vitro *Transcription*—A DNA fragment that encoded residues 1086–1159 of *T. thermophilus* 23S rRNA (Supplementary Fig. S1) was ligated to an upstream T7 promoter (17 bp) and a downstream hammerhead ribozyme (48 bp) (*30*, *31*). This DNA fragment was amplified by PCR, and inserted into the *Bam*HI/*Hin*dIII sites of pUC119. The C1086-G1159 base pair was replaced with G1086-C1159 to increase the efficiency of T7 RNA transcription as well as cleavage by the hammerhead ribozyme. Synthesis and purification of ^{32}P-labelled RNA was performed as described previously (*32*), except that the template plasmid DNA was linearized with *Hin*dIII and T7 RNA polymerase was used instead of SP6 RNA polymerase.

Gel Retardation Assays—An aliquot (10 μl) that contained 5 pmol of the ^{32}P-labelled RNA fragment, 20 mM $MgCl_2$, 300 mM KCl and 30 mM Tris–HCl pH 8.0 was incubated at 40°C for 2 h to allow uniform cutting of the 3′-end by the linked hammerhead ribozyme. After cooling to 30°C, protein samples were added, as indicated in the figure legends, and the mixtures were further incubated at 30°C for 5 min. The samples were then assayed by 6% (w/v) polyacrylamide (acrylamide/bisacrylamide ratio, 39:1) native gel electrophoresis at 6.5 V/cm with a buffer system that contained 5 mM $MgCl_2$, 50 mM KCl and 50 mM Tris–HCl (pH 8.0) for 6 h, as described previously (*33*). The gel was subjected to autoradiography.

Ribosomes, 50S Core Particles, and Hybrid 50S Particles—Intact salt-washed ribosomes and 50S and 30S subunits were prepared from *E. coli* strain Q13, as described previously (*34*, *35*). L11-deficient *E. coli* ribosomes were also prepared from strain AM68 (*36*). *Escherichia coli* 50S subunit core particles that were deficient in both L10•L12 and L11 were prepared by treating 50S subunits with a solution that contained 50% ethanol and 0.5 M NH_4Cl at 0°C, as described previously (*35*). The *E. coli* 50S core particles were then mixed with the *T. thermophilus* Tt-L10•Tt-L12 complex and Tt-L11, as described in the figure legends, and incubated at 37°C for 10 min to form hybrid 50S particles. The formation of the hybrid 50S particles was confirmed by electrophoresis on an acrylamide/agarose composite gel that was composed of 3% (w/v) acrylamide (acrylamide/bisacrylamide ratio, 19:1) and 0.5% (w/v) agarose (*37*), as described previously (*35*). The gel was stained with Azur B.

Quantitative Analysis of Tt-L12 Incorporated into the Ribosome—The Tt-L12 L63C mutant was radiolabelled at its single cysteine residue with [^{14}C]iodoacetamide according to Griaznova and Traut (*7*), except that the reaction mixture contained 10 mM [^{14}C]iodoacetamide (10 mCi/mmol) and 100 mM Tris–HCl (pH 7.6). The specific radioactivity of the ^{14}C-labelled Tt-L12 was 21.15 cpm/pmol. The ^{14}C-labelled Tt-L12 was incubated with the different GST-Tt-L10 variants, and the GST moiety was removed from the complexes, as described above. In a typical quantitative assay, the *E. coli* 50S core (250 pmol) was incubated with a 3-fold molar excess of Tt-L10•[^{14}C]Tt-L12 complex and Tt-L11 in 100 μl of reconstitution buffer, which contained 10 mM $MgCl_2$, 60 mM NH_4Cl, 4 mM 2-mercaptoethanol and 20 mM Tris–HCl (pH 7.6), for 10 min at 37°C, and then layered onto a 20% (w/v) sucrose cushion in the same buffer. The hybrid 50S particles were recovered by ultracentrifugation for 2 h at 100,000*g* at 4°C and then resuspended in 40 μl of reconstitution buffer including 50% (v/v) glycerol. The recovered hybrid 50S particles (30 pmol) were filtrated through a nitrocellulose membrane (type HA, 0.45 mm pore size, 25 mm diameter; Millipore), and the amount of radioactivity present, in the form of [^{14}C]Tt-L12, was measured by using a liquid scintillation counter (LSC1000; Aloca). As a control for nonspecific binding of the ^{14}C-labelled Tt-L12 to the *E. coli* 50S core, the same assay was performed in the absence of Tt-L10. The control value obtained was subtracted from each assay. The number of Tt-L12 molecules bound to each 50S core was calculated using the specific activity of the ^{14}C-labelled Tt-L12.

Measurement of Elongation Factor-dependent GTPase Activity and Polyphenylalanine Synthesis—The *E. coli* elongation factors, EF-G and EF-Tu, were overexpressed in *E. coli* BL21(DE3)pLysS cells from plasmids that had been constructed by insertion of the appropriate coding sequences into the pET-3a vector (Novagen). The proteins were purified from cell extracts by DEAE-sephadex A-50 (GE Healthcare) column chromatography, as described by Arai *et al*. (*38*). The assays for ribosomal GTPase activity that was dependent on EF-G were carried out as described previously (*39*). The assay for ribosomal GTPase activity that was dependent on EF-Tu was performed as described (*35*), except that the reaction mixture contained 20 pmol of *E. coli* 30S subunits and EF-Tu was used instead of eEF-1α from pig liver. Polyphenylalanine synthesis by the hybrid ribosome was assayed using EF-Tu and EF-G, as described previously (*39*).

RESULTS

Preparation of C-terminally Truncated Variants of the T. thermophilus *Ribosomal L10 Protein*—The crystal structure of L10 (residues 1–179) from *T. maritima*, together with the N-terminal domain of L12, has been determined by Diaconu *et al*. (*27*) (also see Supplementary Fig. S2). In their structural model, three L12 dimers bind to the elongated C-terminal helix (α8; residues Lys137–Lys174) of L10 (*27*). The α8 helix is kinked twice, at residues Pro151 and Gly161, and is divided into three segments as shown in Fig. 1A. Each segment is responsible for the binding of one L12 dimer (*27*). This C-terminal region of L10, which comprises 46 amino acids, is highly conserved between *T. maritima* and *T. thermophilus* (43% sequence identity) (Fig. 1A), which suggests that the *T. thermophilus* Tt-L10 and Tt-L12 proteins interact in a similar manner to the *T. maritima* proteins. To confirm this hypothesis, we investigated the Tt-L10•Tt-L12 complex by biochemical approaches using four C-terminal truncation mutants of Tt-L10 (Δ13, Δ23, Δ30, and Δ44), (Fig. 1A). All the Tt-L10 variants were fused to the C-terminus of GST and expressed in *E. coli* cells, because expression of Tt-L10 in the absence of the tag was unsuccessful. Individual fusion proteins were isolated as described in the EXPERIMENTAL PROCEDURES section. Their purity was confirmed by SDS–PAGE (Fig. 1B). The apparent molecular masses of the fusion proteins were consistent with their predicted masses: GST-Tt-L10 (WT), 44.6 kDa (lane 1); GST-Δ13, 43.2 kDa (lane 2); GST-Δ23, 42.3 kDa (lane 3); GST-Δ30, 41.6 kDa (lane 4); GST-Δ44, 40.0 kDa (lane 5) (Fig. 1B). The homogeneity of purified Tt-L12 (lane 6) and Tt-L11 (lane 7) was also confirmed.

Effect of the C-terminal Truncation of Tt-L10 on Tt-L10•Tt-L12 Assembly—Individual C-terminally truncated variants of Tt-L10 fused with GST were mixed with a 10-fold molar excess of Tt-L12, and then the GST moiety was removed by digestion with PreScission Protease and heat treatment as described in the EXPERIMENTAL PROCEDURES section. The complexes of the Tt-L12 and the Tt-L10 variants were analysed by native PAGE (Fig. 2A). Two strong bands

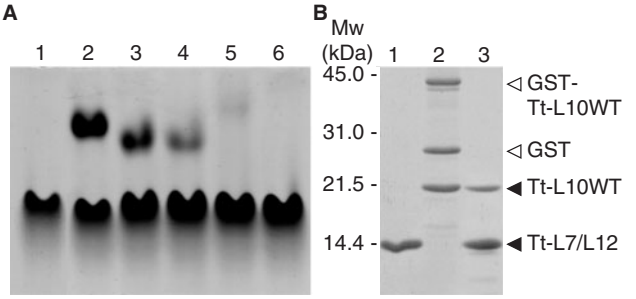

Fig. 2. **Effect of the C-terminal truncation of Tt-L10 on Tt-L10•L12 complex formation.** (A) The complex was formed by mixing each GST-Tt-L10 variant with a 10-fold excess of Tt-L12, and then the GST moiety was removed from the complex by digestion with PreScission protease and heat treatment, as described in the EXPERIMENTAL PROCEDURES section. The resultant Tt-L10•Tt-L12 complex samples that contained 100 pmol of WT (lane 2), Δ13 (lane 3), Δ23 (lane 4), Δ30 (lane 5) and Δ44 (lane 6) Tt-L10 protein were subjected to native gel electrophoresis. Purified Tt-L12 (400 pmol) was also analysed on the same gel (lane 1). The gel was stained with Coomassie Brilliant Blue R-250. (B) The shifted band in lane 2 of (A) was cut out of the gel, incubated in SDS sample solution and then subjected to SDS–PAGE (lane 3). Purified Tt-L12 (lane 1) and a sample of GST-Tt-L10 that had been partially digested with PreScission protease (lane 2: undigested GST-Tt-L10, 44.6 kDa; GST, 26.0 kDa; Tt-L10, 18.6 kDa) were also analysed on the same gel. The gel was stained with the fluorescent reagent SYPRO Orange (Molecular Probes, Invitrogen) and visualized with a STORM 860 PhosphorImager (GE Healthcare).

appeared in the sample mixture that contained WT Tt-L10 and Tt-L12 (Fig. 2A, lane 2). The band with higher mobility corresponded to free Tt-L12 (lane 1). Because free Tt-L10 did not enter the gel under the electrophoretic conditions used (data not shown), the shifted band with lower mobility appeared to be the Tt-L10•Tt-L12 complex. To confirm this, the shifted band in lane 2 of Fig. 2A was cut out of the gel and subjected to SDS–PAGE (Fig. 2B, lane 3). The protein components of the shifted band corresponded to Tt-L12 (Fig. 2B, lane 1) and WT Tt-L10 (lane 2). The shifted bands were also observed when two C-terminal truncation mutants of Tt-L10, Δ13 (Fig. 2A, lane 3) and Δ23 (Fig. 2A, lane 4), were used instead of WT Tt-L10. The mobility of these shifted bands was higher and the intensity of the bands was weaker than those in the sample that contained WT Tt-L10 (lane 2). A very weak and smeared higher mobility band was observed in the sample that contained the Δ30 mutant (Fig. 2A, lane 5). No complex formation was detected in the sample that contained the Δ44 mutant (lane 6).

The ability of the same proteins to bind to rRNA was tested by gel mobility shift assay using a small amount of a ^{32}P-labelled RNA fragment that corresponded to residues 1086–1159 of *T. thermophilus* 23S rRNA as a probe (Fig. 3A). All the proteins bound to the RNA (lanes 2–6), although the sample that contained the Δ30 mutant gave a weak smeared band (lane 5). The mobility of the complex that contained the Δ44 mutant was significantly higher than that of the other complexes (lane 6). Because the Δ44 Tt-L10 mutant failed to bind to Tt-L12, this high mobility complex may correspond to

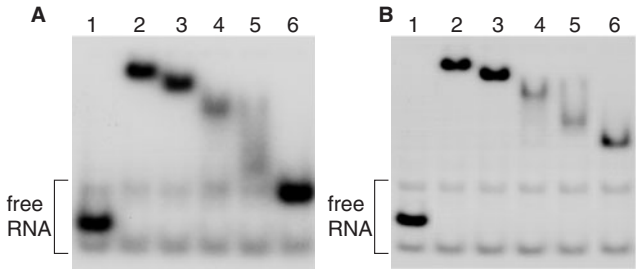

Fig. 3. **Effect of C-terminal truncation of Tt-L10 on the binding of the *T. thermophilus* ribosomal proteins to an rRNA fragment.** (A) Tt-L10•Tt-L12 complex samples that contained 20 pmol of WT (lane 2), Δ13 (lane 3), Δ23 (lane 4), Δ30 (lane 5) and Δ44 (lane 6) Tt-L10 protein were mixed with 5 pmol of a ^{32}P-labelled RNA fragment that corresponded to residues 1086–1159 of *T. thermophilus* 23S rRNA (lane 1, RNA alone), and separated by native gel electrophoresis. The gel was subjected to autoradiography. (B) The same experiments as in (A) were performed in the presence of 20 pmol of Tt-L11.

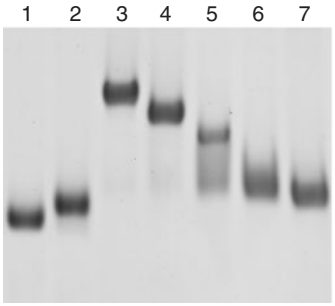

Fig. 4. **Binding of the *T. thermophilus* ribosomal proteins to *E. coli* 50S core particles deficient in L10•L12 and L11.** The *E. coli* 50S core particles (10 pmol each) were incubated with 30 pmol of Tt-L11 (lane 2) or the same amount of Tt-L11 together with 30 pmol of stalk complexes that contained WT (lane 3), Δ13 (lane 4), Δ23 (lane 5), Δ30 (lane 6), or Δ44 (lane 7) Tt-L10 protein, and then analysed by electrophoresis on an acrylamide-agarose composite gel, as described in the EXPERIMENTAL PROCEDURES section. The *E. coli* 50S core particles without protein samples were also analysed on the same gel (lane 1). The gel was stained with Azur B.

a complex of Δ44 and rRNA without the L12 dimer. These results indicate that the C-terminal 44 amino acids are responsible for binding all three Tt-L12 dimers, but are not involved in rRNA binding. The same rRNA binding experiments were repeated in the presence of an additional protein, Tt-L11 (Fig. 3B). After the addition of Tt-L11, a supershift was observed with all the samples, which implies that Tt-L11 can bind to the rRNA in the presence of either Tt-L10 alone or the Tt-L10•Tt-L12 complex.

Assembly of the T. thermophilus *Ribosomal Proteins onto the* E. coli *50S core*—We have established *in vitro* conditions for the preparation of hybrid ribosomal particles in which the L10•L12 complex and L11 of the *E. coli* 50S subunit are replaced with their eukaryotic or archaeal counterparts (*35*, *40*). This hybrid system is useful for characterizing the ribosomal proteins that constitute the GTPase-associated centre. Here, we used the hybrid system to investigate the assembly of the thermophilic ribosomal proteins Tt-L10 (or its mutants), Tt-L12 and Tt-L11 onto the ribosomal core particle. The formation of hybrid 50S particles that contained *T. thermophilus* proteins was analysed by acrylamide/agarose composite gel electrophoresis (Fig. 4). The gel mobility of the *E. coli* 50S core particles that were deficient in L10•L12 and L11 (lane 1) was shifted upwards slightly by the addition of Tt-L11 (lane 2), and markedly supershifted by the further addition of the Tt-L10 (WT)•Tt-L12 stalk complex (lane 3). When the stalk complexes that contained the Δ13 (lane 4) and Δ23 (lane 5) Tt-L10 mutants were added instead of the WT complex, smaller shifts were observed than with the WT complex. A single shifted band was clearly seen for the WT-, Δ13- and Δ23-containing samples (lanes 3–5), which indicated that each hybrid ribosome formed was homogeneous. When the samples that contained the Δ30 (lane 6) and Δ44 (lane 7) Tt-L10 variants were added, only very minor shifts were observed, which suggested that Tt-L12 failed to assemble stably onto the core particle in the presence of the Δ30 and Δ44 variants.

Table 1. **Quantification of Tt-L12 in hybrid 50S particles that were reconstituted with the Tt-L10 variants.**

Tt-L10 variant used	Copies of Tt-L12 per hybrid 50S particle[a]
WT	5.91 ± 0.39
Δ13	3.64 ± 0.02
Δ23	2.03 ± 0.03
Δ30	1.23 ± 0.01
Δ44	0.30 ± 0.01

[a]The values given show the number of Tt-L12 molecules that bound to a hybrid 50S particle that had been reconstituted with a particular Tt-L10 variant, and they are means of two to four independent experiments. The specific radioactivity of ^{14}C-labelled L63C Tt-L12, prepared as described in the EXPERIMENTAL section, was 21.15 cpm/pmol protein.

To quantify the number of Tt-L12 molecules that were bound to Tt-L10 or its variants in the *E. coli* 50S particles, a single cysteine was introduced into Tt-L12 at position 63 by site-directed mutagenesis to give the L63C mutant, which was then radiolabelled with [^{14}C]iodoacetoamide. The specific radioactivity of the ^{14}C-labelled L63C Tt-L12 was 21.15 cpm/pmol protein. The Tt-L10•[^{14}C]Tt-L12 complexes were reconstituted using the different Tt-L10 mutants, as described in the EXPERIMENTAL PROCEDURES section. An excess of each ^{14}C-labelled reconstituted complex, together with Tt-L11, was added to *E. coli* 50S core particles that were deficient in L10•L12 and L11, and then the hybrid 50S particles were recovered by centrifugation through a sucrose cushion. The results are summarized in Table 1. The amount of incorporation of [^{14}C]Tt-L12 was estimated to be 5.9, 3.6, 2.0 and 1.2 copies per hybrid 50S particle that contained WT, Δ13, Δ23 and Δ30 Tt-L10, respectively. Because Tt-L12 exists as a dimer, these results indicate that WT, Δ13 and Δ23 Tt-L10 bound three, two and one Tt-L12 dimers, respectively. It is likely that the binding of a single Tt-L12 dimer to the Δ30 mutant was unstable and the protein dimer was

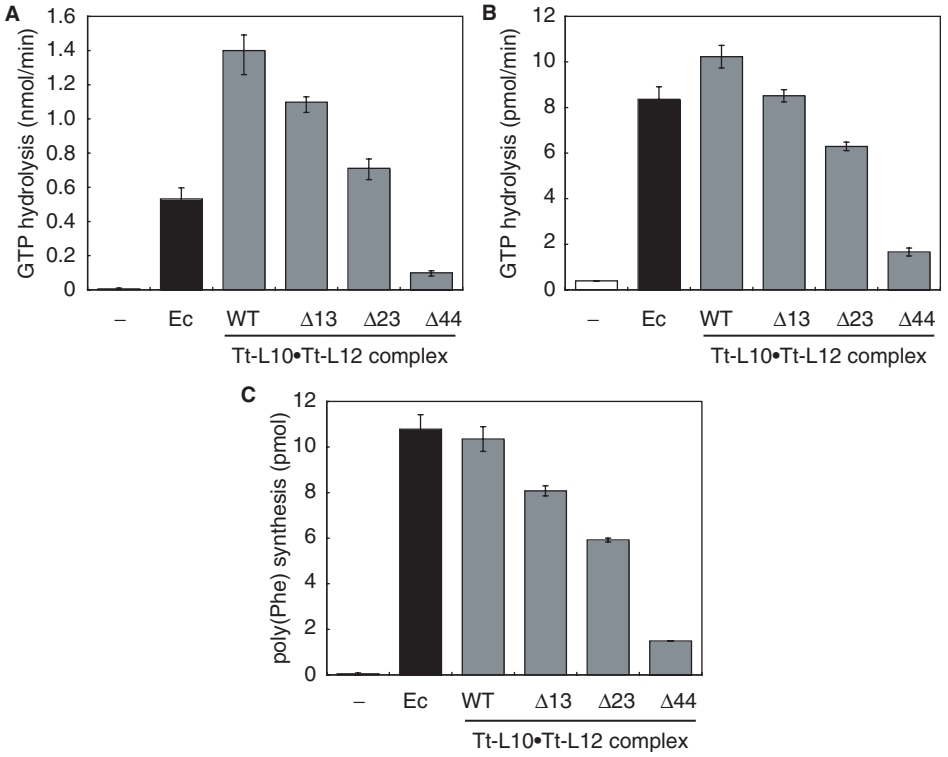

Fig. 5. **Functional effects of the removal of Tt-L12 dimers by the C-terminal truncation of Tt-L10.** (A) *Escherichia coli* 50S core particles (2.5 pmol) were incubated with 7.5 pmol of Tt-L11 and the same amount of stalk complexes that contained WT, Δ13, Δ23 and Δ44 Tt-L10 protein, as indicated below each column. The 50S cores were also incubated with the *E. coli* ribosomal proteins Ec-L10•Ec-L12 and Ec-L11 (Ec). The resultant 50S particles were assayed for EF-G-dependent GTPase activity in the presence of *E. coli* 30S subunits (5 pmol) at 37°C, as described in the EXPERIMENTAL PROCEDURES section. The same ribosome samples as in (A), except that the amounts of 50S core particles, each protein sample, and *E. coli* 30S subunits used were 10 pmol, 30 pmol and 20 pmol, respectively, were assayed for EF-Tu-dependent GTPase (B) and EF-G/EF-Tu-dependent polyphenylalanine synthetic activity (C).

partially released during centrifugation. No incorporation of Tt-L12 into 50S core particles that contained the Δ44 variant was observed. These quantitative data were consistent with the results of the gel mobility shift assays shown in Figs 3 and 4. Our results indicate that Tt-L10 has three binding sites for Tt-L12 dimers within the C-terminal 44 amino acids.

Activities of the Hybrid Ribosomes that Contained the Tt-L10 Variants—The hybrid 50S particles that contained the Tt-L10 variants and Tt-L12 were combined with *E. coli* 30S subunits, and the accessibility of the resulting ribosomes to the *E. coli* elongation factors EF-G and EF-Tu was tested at 37°C (Fig. 5), although *T. thermophilus* EF-G (Tt-EF-G) could also gain access to the hybrid ribosomes at this temperature (Supplementary Fig. S3). Unexpectedly, the hybrid ribosomes that contained WT Tt-L10 and three Tt-L12 dimers had 2.5-fold higher EF-G-dependent GTPase activity than the control *E. coli* ribosomes that were reconstituted with Ec-L10, Ec-L11 and Ec-L12 (Fig. 5A). The same hybrid ribosomes had only slightly higher EF-Tu-dependent GTPase activity than the reconstituted *E. coli* ribosomes (Fig. 5B). With the hybrid ribosomes that contained the Δ13 Tt-L10 mutant, to which only two Tt-L12 dimers bound, these activities decreased to 78–83% of the WT level. In the presence of the Δ23 mutant, to which only one Tt-L12 dimer bound, the activities decreased to 51–62%. An even greater reduction (7–16% of the WT levels) was seen with the Δ44 mutant, which did not bind Tt-L12 at all. Similar reductions in the levels of phenylalanine polymerization by the hybrid ribosomes were observed with the respective mutants (Fig. 5C). These functional data suggest that the level of elongation factor-dependent activity that is observed for ribosomes correlates with the number of Tt-L12 dimers that are bound to Tt-L10. It is noteworthy that, although the hybrid ribosomes that contain WT Tt-L10 and Tt-L12 possess very high GTPase activity, particularly that dependent on EF-G, these ribosomes show a very similar level of polyphenylalanine synthetic activity as the control reconstituted *E. coli* ribosomes (Fig. 5C).

DISCUSSION

The present study has focused on the ribosomal stalk protein complex from *T. thermophilus*. We have demonstrated by biochemical approaches that one copy of Tt-L10 associated with six copies of Tt-L12 and that all the Tt-L12 proteins bound side-by-side, presumably as stable homodimers, to the C-terminal 44 amino acids of Tt-L10. The present results confirm the previous mass spectrometric data on ribosomes from the same

species (*26*). The binding of Tt-L12 dimers to the C-terminal helix α8 region of Tt-L10 is consistent with that of the *T. maritima* orthologues, which was revealed by crystallographic analysis (Supplementary Fig. S2) (*27*). In contrast with these data, the results of a number of studies show that the ribosomes from many species contain a pentameric stalk protein complex, which is composed of one copy of L10-like protein and four copies of L12-like proteins (*5–7*, *19–25*). It is certainly of interest to determine how many organisms have ribosomes that contain the heptameric stalk complex, which comprises one copy of L10-like protein and six copies of L12-like proteins, and what is the functional significance of this stoichiometry. An extensive comparison of the amino acid sequences of L10-like proteins from 11 *Eukarya*, 10 *Archaea* and 28 *Bacteria* revealed that many species contain the expanded sequence in the C-terminal stalk binding region (*28*). The expanded sequence is present in all archaeal L10-like proteins but not in all eukaryotic proteins examined. In *Bacteria*, however, almost a half of known sequences were found to contain the expanded sequence in their C-terminal regions. It is therefore important to clarify the relationship between the presence of the expanded sequence in L10-like proteins and the presence of a third L12-like stalk dimer in the ribosome. The present study clearly shows that this correlation occurs in *T. thermophilus*. The C-terminal expanded sequence provides a good indication of the possible presence of a third stalk dimer in the ribosome.

The progressive removal of one to three Tt-L12 dimers caused a partial, and stepwise, reduction in EF-G-dependent (Fig. 5A) and EF-Tu-dependent (Fig. 5B) GTPase activities. Polyphenylalanine synthesis underwent a similar stepwise reduction upon the removal of the stalk dimers. These results indicated that each individual stalk dimer contributed to ribosomal function. It should be pointed out that hybrid ribosomes that contained the Δ23 L10 mutant, and thus only a single L12 dimer, still had high GTPase activities and levels of polyphenylalanine synthesis (51–62% of those with WT L10). This was not a surprising result, because it has been shown by Griaznova and Traut (*7*) that reconstituted mutant *E. coli* ribosomes that contain only a single L12 dimer retain similar activities to the WT ribosomes that contain two L12 dimers. They used a C-terminal truncation mutant of L10 (Δ10 mutant), in which the most C-terminal L12 dimer-binding site has been removed. It is therefore conceivable that the L12 dimer that is bound to the most N-terminal site of the two or three L12-binding sites on L10 makes the largest contribution to the ribosomal factor-dependent activity.

The hybrid ribosomes that contained the *T. thermophilus* proteins Tt-L10, Tt-L11 and Tt-L12 were highly accessible to the *E. coli* elongation factors (Fig. 5). This was an unexpected result, because these proteins are usually active at very high temperatures such as 70–75°C, which is the optimum temperature for *T. thermophilus* growth. In a previous study, we also observed that the equivalent ribosomal proteins from a thermophilic archaea, *Pyrococcus horikoshii*, whose optimum growing temperature is 95°C, efficiently interact with the translation factors from animal cells (*40*). From these results, we infer that the functional structural features of the stalk protein complexes are well conserved and that they can function over a wide range of temperatures. It is particularly interesting that the hybrid ribosome that contained Tt-L10 and Tt-L12 showed 2.5-fold higher EF-G-dependent GTPase activity than the WT *E. coli* ribosome at 37°C. Because the EF-Tu-dependent GTPase activity and the polypeptide synthetic activity of the same hybrid ribosome were not markedly different from those of the *E. coli* ribosome, this showed that the *T. thermophilus* ribosomal proteins had a specific effect on EF-G-dependent GTPase activity. The EF-G-dependent GTPase activity of the hybrid ribosomes that contained two Tt-L12 dimers (the Δ13 mutant) or even a single dimer (the Δ23 mutant) was also higher than that of the *E. coli* WT ribosome. It is therefore likely that the higher activity is related to a unique structural feature of each Tt-L12 dimer, not to the heptameric Tt-L10•Tt-L12 complex as a whole. In fact, our recent studies have shown that the characteristic amino acid sequence of the C-terminal domain of Tt-L12 is relevant to the high EF-G-dependent GTPase activity (T. Miyoshi *et al*., manuscript submitted for publication). Moreover, the physical contact of the CTD of L12 and EF-G has been demonstrated in recent analyses by cryo-EM (*41*) and NMR (*42*). These lines of evidence suggest that the presence of multiple copies of L12 in the ribosome might increase the frequency of contact between the CTD and the translation factors.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Hamel, E., Koka, M., and Nakamoto, T. (1972) Requirement of an Escherichia coli 50S ribosomal protein component for effective interaction of the ribosome with T and G factors and with guanosine triphosphate. *J. Biol. Chem.* **247**, 805–8147
2. Möller, W. (1974) The ribosomal components involved in EF-G- and EF-Tu-dependent GTP hydrolysis. in *Ribosomes* (Nomura, M., Tissieres, A., and Lengyel, P., eds), 711–731, Cold Spring Harbor Laboratory Press, New York
3. Pettersson, I., Hardy, S.J.S., and Liljas, A. (1976) The ribosomal protein L8 is a complex of L7/L12 and L10. *FEBS Lett.* **64**, 135–138

4. Österberg, B., Pettersson, I., Liljas, A., and Kurland, C.G. (1977) Small-angle X-ray scattering study of the protein complex of L7/L12 and L10 from Escherichia coli ribosomes. *FEBS Lett.* **73**, 22–24
5. Gudkov, A.T., Tumanova, L.G., Venyaminov, S.Y., and Khechinashvilli, N.N. (1978) Stoichiometry and properties of the complex between ribosomal proteins L7 and L10 in solution. *FEBS Lett.* **93**, 215–218
6. Gudkov, A.T., Tumanova, L.G., Gongadze, G.M., and Bushuev, V.N. (1980) Role of different regions of ribosomal proteins L7 and L12 in their complex formation and in the interaction with the ribosomal 50S subunit. *FEBS Lett.* **109**, 34–38
7. Griaznova, O. and Traut, R.R. (2000) Deletion of C-terminal residues of *Escherichia coli* ribosomal protein L10 causes the loss of binding of one L7/L12 dimer: ribosomes with one L7/L12 dimer are active. *Biochemistry* **39**, 4075–4081
8. Beauclerk, A.A.D., Cundliffe, E., and Dijk, J. (1984) The binding site for ribosomal protein complex L8 within 23S ribosomal RNA of *Escherichia coli*. *J. Biol. Chem.* **259**, 6559–6563
9. Egebjerg, J., Douthwaite, S.R., Liljas, A., and Garrett, R.A. (1990) Characterization of the binding sites of protein L11 and the L10.$(L12)_4$ pentameric complex in the GTPase domain of 23S ribosomal RNA from *Escherichia coli*. *J. Mol. Biol.* **213**, 275–288
10. Rosendahl, G. and Douthwaite, S. (1993) Ribosomal proteins L11 and L10.$(L12)_4$ and the antibiotic thiostrepton interact with overlapping regions of the 23S rRNA backbone in the ribosomal GTPase centre. *J. Mol. Biol.* **234**, 1013–1020
11. Traut, R.R., Lambert, J.M., and Kenny, J.W. (1983) Ribosomal protein L7/L12 cross-links to proteins in separate regions of the 50S ribosomal subunit of *Escherichia coli*. *J. Biol. Chem.* **258**, 14592–14598
12. Cowgill, C.A., Nichols, B.G., Kenny, J.W., Butler, P., Bradbury, E.M., and Traut, R.R. (1984) Mobile domains in ribosomes revealed by proton nuclear magnetic resonance. *J. Biol. Chem.* **259**, 15257–15263
13. Hamman, B.D., Oleinikov, A.V., Jokhadze, G.G., Traut, R.R., and Jameson, D.M. (1996) Rotational and conformational dynamics of *Escherichia coli* ribosomal protein L7/L12. *Biochemistry* **35**, 16672–16679
14. Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289**, 905–920
15. Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Gat, S., Agmon, I., Bartels, H., Franceschi, F., and Yonath, A. (2001) High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. *Cell* **107**, 679–688
16. Schuwirth, B.S., Borovinskaya, M.A., Hau, C.W., Zhang, W., Vila-Sanjurjo, A., Holton, J.M., and Cate, J.H. (2005) Structures of the bacterial ribosome at 3.5 Å resolution. *Science* **310**, 827–834
17. Berk, V., Zhang, W., Pai, R.D., and Cate, J.H.D. (2006) Structural basis for mRNA and tRNA positioning on the ribosome. *Proc. Natl Acad. Sci. USA* **103**, 15830–15834
18. Korostelev, A., Trakhanov, S., Laurberg, M., and Noller, H.F. (2006) Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell* **126**, 1065–1077
19. Hardy, S.J.S. (1975) The stoichiometry of the ribosomal proteins of *Escherichia coli*. *Mol. Gen. Genet.* **140**, 253–274
20. Subramanian, A.R. (1975) Copies of proteins L7 and L12 and heterogeneity of the large subunit of *Escherichia coli* ribosome. *J. Mol. Biol.* **95**, 1–8
21. Uchiumi, T., Whaba, A., and Traut, R.R. (1987) Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. *Proc. Natl Acad. Sci. USA* **84**, 5580–5584
22. Guarinos, E., Remacha, M., and Ballesta, J.P.G. (2001) Asymmetric interactions between the acidic P1 and P2 proteins in the *Saccharomyces cerevisiae* ribosomal stalk. *J. Biol. Chem.* **276**, 32474–32479
23. Hagiya, A., Naganuma, T., Maki, Y., Ohta, J., Tohkairin, Y., Shimizu, T., Nomura, T., Hachimori, A., and Uchiumi, T. (2005) A mode of assembly of P0, P1, and P2 proteins at the GTPase-associated center in animal ribosome: i*n vitro* analyses with P0 truncation mutants. *J. Biol. Chem.* **280**, 39193–39199
24. Krokowski, D., Boguszewska, A., Abramczyk, D., Liljas, A., Tchórzewski, M., and Grankowski, N. (2006) Yeast ribosomal P0 protein has two separate binding sites for P1/P2 proteins. *Mol. Microbiol.* **60**, 386–400
25. Casiano, C., Matheson, A.T., and Traut, R.R. (1990) Occurrence in the Archaebacterium *Sulfolobus solfataricus* of a ribosomal protein complex corresponding to *Escherichia coli* $(L7/L12)_4$•L10 and eukaryotic $(P1)_2/(P2)_2$•P0. *J. Biol. Chem.* **265**, 18757–18761
26. Ilag, L.L., Videler, H., Mckay, A., Sobott, F., Fucini, P., Nierhaus, K.H., and Robinson, C.V. (2005) Heptameric $(L12)_6$/L10 rather than canonical pentameric complexes are found by tandem MS of intact ribosomes from thermophilic bacteria. *Proc. Natl Acad. Sci. USA* **102**, 8192–8197
27. Diaconu, M., Kothe, U., Schlünzen, F., Fischer, N., Harms, J.M., Tonevitsky, A.G., Stark, H., Rodnina, M.V., and Wahl, M.C. (2005) Structural basis for the function of the ribosomal L7/L12 stalk in factor binding and GTPase activation. *Cell* **121**, 991–1004
28. Maki, Y., Hashimoto, T., Zhou, M., Naganuma, T., Ohta, J., Nomura, T., Robinson, C.V., and Uchiumi, T. (2007) Three binding sites for stalk protein dimers are generally present in ribosomes from archaeal organism. *J. Biol. Chem.* **282**, 32827–32833
29. Yokoyama, S., Hirota, H., Kigawa, T., Yabuki, T., Shirouzu, M., Terada, T., Ito, Y., Matsuo, Y, Kuroda, Y., Nishimura, Y., Kyogoku, Y., Miki, K., Masui, R., and Kuramitsu, S. (2000) Structural genomics projects in Japan. *Nat. Struct. Biol.* **7**, 943–945
30. Pley, H.W., Flaherty, K.M., and McKay, D.B. (1994) Three-dimensional structure of a hammerhead ribozyme. *Nature* **372**, 68–74
31. Price, S.R., Ito, N., Oubridge, C., Avis, J.M., and Nagai, K. (1995) Crystallization of RNA-protein complexes. I. Methods for the large-scale preparation of RNA suitable for crystallographic studies. *J. Mol. Biol.* **249**, 398–408
32. Uchiumi, T., Wada, A., and Kominami, R. (1995) A base substitution within the GTPase-associated domain of mammalian 28 S ribosomal RNA causes high thiostrepton accessibility. *J. Biol. Chem.* **270**, 29889–29893
33. Shimizu, T., Nakagaki, M., Nishi, Y., Kobayashi, Y., Hachimori, A., and Uchiumi, T. (2002) Interaction among silkworm ribosomal proteins P1, P2 and P0 required for functional protein binding to the GTPase-associated domain of 28S rRNA. *Nucleic Acids Res.* **30**, 2620–2627
34. Uchiumi, T., Hori, K., Nomura, T., and Hachimori, A. (1999) 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**, 27578–27582
35. Uchiumi, T., Honma, S., Nomura, T., Dabbs, E.R., and Hachimori, A. (2002) Translation elongation by a hybrid ribosome in which proteins at the GTPase center of the *Escherichia coli* ribosome are replaced with rat counterparts. *J. Biol. Chem.* **277**, 3857–3862
36. Dabbs, E.R. (1979) Selection for *Escherichia coli* mutants with proteins missing from the ribosome. *J. Bacteriol.* **140**, 734–737
37. Tokimatsu, H., Strycharz, W.A., and Dahlberg, A.E. (1981) Gel electrophoretic studies on ribosomal proteins L7/L12

and the *Escherichia coli* 50S subunit. *J. Mol. Biol.* **152**, 397–412
38. Arai, K., Kawakita, M., and Kaziro, Y. (1974) Studies on the polypeptide elongation factors from *E. coli*. IV. *J. Biochem.* **76**, 283–292
39. Nomura, T., Mochizuki, R., Dabbs, E.R., Shimizu, Y., Ueda, T., Hachimori, A., and Uchiumi, T. (2003) A point mutation in ribosomal protein L7/L12 reduces its ability to form a compact dimer structure and to assemble into the GTPase center. *Biochemistry* **42**, 4691–4698
40. Nomura, T., Nakano, K., Maki, Y., Naganuma, T., Nakashima, T., Tanaka, I., Kimura, M., Hachimori, A., and Uchiumi, T. (2006) *In vitro* reconstitution of the GTPase-associated centre of the archaebacterial ribosome: the functional features observed in a hybrid form with *Escherichia coli* 50S subunits. *Biochem. J.* **396**, 565–571
41. Datta, P.P., Sharma, M.R., Qi, L., Frank, J., and Agrawal, R.K. (2005) Interaction of the G' domain of elongation factor G and the C-terminal domain of ribosomal protein L7/L12 during translocation as revealed by Cryo-EM. *Mol. Cell* **20**, 723–731
42. Helgstrand, M., Mandava, C.S., Mulder, F.A.A., Liljas, A., Sanyal, S., and Akke, M. (2007) The ribosomal stalk binds to translation factors IF2, EF-Tu, EF-G and RF3 via a conserved region of the L12 C-terminal domain. *J. Mol. Biol.* **365**, 468–479