

In vitro reconstitution of the GTPase-associated centre of the archaeobacterial ribosome: the functional features observed in a hybrid form with *Escherichia coli* 50S subunits

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We cloned the genes encoding the ribosomal proteins Ph (*Pyrococcus horikoshii*)-P0, Ph-L12 and Ph-L11, which constitute the GTPase-associated centre of the archaeobacterium *Pyrococcus horikoshii*. These proteins are homologues of the eukaryotic P0, P1/P2 and eL12 proteins, and correspond to *Escherichia coli* L10, L7/L12 and L11 proteins respectively. The proteins and the truncation mutants of Ph-P0 were overexpressed in *E. coli* cells and used for *in vitro* assembly on to the conserved domain around position 1070 of 23S rRNA (*E. coli* numbering). Ph-L12 tightly associated as a homodimer and bound to the C-terminal half of Ph-P0. The Ph-P0•Ph-L12 complex and Ph-L11 bound to the 1070 rRNA fragments from the three biological kingdoms in the same manner as the equivalent proteins of eukaryotic and eubacterial ribosomes. The Ph-P0•Ph-L12 complex and Ph-

L11 could replace L10•L7/L12 and L11 respectively, on the *E. coli* 50S subunit *in vitro*. The resultant hybrid ribosome was accessible for eukaryotic, as well as archaeobacterial elongation factors, but not for prokaryotic elongation factors. The GTPase and polyphenylalanine-synthetic activity that is dependent on eukaryotic elongation factors was comparable with that of the hybrid ribosomes carrying the eukaryotic ribosomal proteins. The results suggest that the archaeobacterial proteins, including the Ph-L12 homodimer, are functionally accessible to eukaryotic translation factors.

Key words: archaeobacterium, elongation factor, ribosome, ribosomal protein, ribosomal stalk, translation elongation.

INTRODUCTION

The 'GTPase-associated centre' is a functional site in the large ribosomal subunit that is responsible for GTPase-related events, which are dependent upon translation factors in protein biosynthesis. Protein components of this site in most eubacterial ribosomes constitute a characteristic pentameric complex, L10(L7/L12)₂(L7/L12)₂ [1,2] in which two L7/L12 homodimers bind to the C-terminal region of L10 [3], although recent studies have discovered a heptameric form, L10(L7/L12)₂(L7/L12)₂(L7/L12)₂, in a few eubacterial species [4,5]. The L10•L7/L12 complex, together with another protein, L11, binds to the highly conserved domain around position 1070 (*Escherichia coli* numbering is used throughout) of 23S rRNA through interaction with the L10 moiety [6–8]. The protein complex constitutes a highly flexible and functionally important protuberance, the so-called 'stalk', in the ribosome [9]. Although the ribosomal stalk can be observed by cryo-electron microscopy [10], the detailed structure within the ribosome has not been resolved by X-ray crystallography [11–13].

The eukaryotic ribosomal phosphoproteins P0 and P1/P2 (P proteins) are counterparts of eubacterial L10 and L7/L12 respectively [14]. Unlike eubacterial, L7/L12, the eukaryotic stalk proteins, P1 and P2, are related, but are different proteins [15–17]. Previous biochemical and genetic studies indicate that P1 and P2 form a heterodimer [18–22], and that the interaction between the two proteins is important for their assembly on to P0 in order to constitute the pentameric complex [21,23], designated

as 'P0•P1-P2'. This complex not only binds to eukaryotic 28S rRNA, but also cross-binds to prokaryotic 23S rRNA [24] and determines the specificity of the ribosome for eukaryotic elongation factors (eEFs), eEF-1 α and eEF-2 [25].

Archaeobacteria constitute a third primary biological kingdom, separate from eubacteria and eukaryotes [26]. The archaeobacterial translocase, EF-2, functions only with ribosomes from the same kingdom [27], although the ribosomes from methanogens, but not from *Thermoplasma acidophilum* or *Sulfolobus solfataricus*, showed some accessibility to eEF-2 [27,28]. There is, however, limited information about the functional structure of the ribosomal GTPase-associated centre in archaeobacteria. Comparison of the components constituting the archaeobacterial active site with those in eubacteria and eukaryotes has shown that: (i) the secondary structure of the position 1070 domain of 23S/28S rRNA is highly conserved among the three kingdoms [28,29], although the base at position 1067, one of the EF-G (elongation factor-G)/eEF-2-binding sites [30,31], is adenine in archaeobacteria and eubacteria, but is guanine in eukaryotes [32,33] (see Supplementary Figures S1 and S2 at <http://www.BiochemJ.org/bj396/bj3960565add.htm>) and the amino acid sequences and molecular mass of L7/L12- and L10-like proteins in archaeobacteria show greater similarity with eukaryotic proteins than eubacterial proteins [34] (see Supplementary Figures S2–S4 at <http://www.BiochemJ.org/bj396/bj3960565.add.htm>).

The most inscrutable and interesting point is that only one species of archaeobacterial L7/L12-like protein has been detected,

Abbreviations used: eEF, eukaryotic elongation factor; Ph, *Pyrococcus horikoshii*.

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despite its structural similarity to eukaryotic P1 or P2 proteins. A chemical cross-linking study of *S. solfataricus* ribosomes has suggested that the L7/L12-like protein is present as a homodimer in the 50S subunit, and presumably two homodimers are anchored to the L10-like protein [35]. The truncation study of the *S. solfataricus* L7/L12-like protein has shown that the N-terminal region is important for its incorporation into the ribosome [36]. In a functional study, it was shown that *S. solfataricus* ribosomes that were treated with ethanol to remove the L7/L12-like proteins retained significant activity in polyphenylalanine synthesis [36].

To clarify the mode of assembly of the archaeobacterial ribosomal GTPase-associated centre and its function, we cloned and expressed the L7/L12-, L10- and L11-like proteins of *Pyrococcus horikoshii*, designated Ph (*Pyrococcus horikoshii*)-L12, Ph-P0 and Ph-L11 respectively, and performed *in vitro* reconstitution of the functional site, together with the position 1070 domain of 23S rRNA. The functionality of these *P. horikoshii* proteins was tested in a previously developed hybrid ribosome system [25], in which the *E. coli* L10•L7/L12 complex and L11 on the 50S subunit were replaced with the equivalent proteins from the other species. By using the hybrid ribosome system, the present study clearly shows that the *P. horikoshii* proteins, including the Ph-L12 homodimer, make the *E. coli* ribosomes accessible to eEFs, as well as archaeobacterial elongation factors, but not eubacterial elongation factors. The eukaryotic P1-P2 heterodimers associated with P0 appear to be compatible with the archaeobacterial stalk homodimers bound to the anchor protein, without significant loss of ribosomal function.

EXPERIMENTAL

Plasmid construction, protein expression and purification

Genomic DNA from the archaeobacterium *P. horikoshii* was prepared as described by Blin and Stafford [37]. The coding regions for ribosomal proteins Ph-L12, Ph-L11, Ph-P0 and the C-terminal truncation mutants of Ph-P0, C Δ 105 and C Δ 131 were amplified by PCR [38] and inserted into an *E. coli* expression vector, pET-3a or pET-28c (Novagen). Proteins expressed in *E. coli* cells were purified as follows. The expressed Ph-P0 was present in the insoluble fraction and was solubilized in Buffer A, which consisted of 20 mM sodium acetate (pH 5.5), 7 M urea and 5 mM 2-mercaptoethanol, dialysed against Buffer A, and was then applied to a column of CM-cellulose (Whatman) equilibrated with the same buffer. Ph-P0 was eluted in Buffer A containing 0.06 M LiCl. The Ph-P0 fraction was dialysed overnight against Buffer B, which consisted of 20 mM sodium phosphate (pH 6.5), 6 M urea, 100 mM LiCl and 5 mM 2-mercaptoethanol, and was further purified by HPLC on a DEAE-5PW column (Tosoh) in a linear gradient of 100–280 mM LiCl. The truncation mutants of Ph-P0 were purified by using an AKTA FPLC system with Resource S and Resource Q columns (Pharmacia) under similar conditions. Ph-L11 and Ph-L12 in the soluble fraction were enriched by incubation of the S100 fractions at 90 °C for 10 min followed by removal of insoluble material formed upon the heating by centrifugation. The soluble protein samples were dialysed overnight against Buffer C, which consisted of 20 mM sodium acetate (pH 4.5), 7 M urea and 5 mM 2-mercaptoethanol, and were then applied to a column of CM-cellulose equilibrated with the same buffer. Ph-L12 and Ph-L11 were eluted in Buffer C containing 0 and 0.06 M LiCl respectively. The Ph-L12 fraction was dialysed overnight against Buffer B and purified by HPLC on a DEAE-650S column in a linear gradient of 100–325 mM LiCl. The Ph-L11 fraction was dialysed overnight against Buffer D, which consisted of 20 mM sodium phosphate (pH 6.5), 6 M

urea, 5 mM LiCl and 5 mM 2-mercaptoethanol, and was purified by HPLC on a CM-5PW column (Tosoh) with a linear gradient of 5–204 mM LiCl. The silkworm (*Bombyx mori*) counterparts, P0, P1/P2 and eL12 (a eukaryotic homologue of prokaryotic L11) were also expressed in *E. coli* cells and purified as described recently [39]. Expression and purification of the *E. coli* equivalent proteins L10, L7/L12 and L11 were performed as described previously [40].

In vitro RNA synthesis

The *P. horikoshii* rDNA fragment corresponding to residues 1142–1240 of 23S rRNA, which is similar to residues 1030–1128 of *E. coli* 23S rRNA (designated here as the 1070 fragment), was amplified by PCR and inserted into the HindIII and BamHI sites of the pT7Blue T-Vector (Novagen). Synthesis and purification of the RNA fragment was performed as described previously [41] except that T7 RNA polymerase was used instead of SP-6 RNA polymerase. The RNA fragments containing residues 1029–1127 of *E. coli* 23S rRNA and the equivalent region (residues 1841–1939) in rat 28S rRNA were synthesized as described [41,42].

In vitro reconstitution of Ph-P0•Ph-L12•Ph-L11•rRNA

The putative 'stalk complex' corresponding to the eukaryotic P0•P1-P2 complex was reconstituted by mixing isolated Ph-P0 and Ph-L12 at a molar ratio of 1:6 in a buffer containing 7 M urea, 300 mM KCl, 5 mM 2-mercaptoethanol and 20 mM Tris/HCl (pH 7.6) and by dialysis against the same buffer without urea, as described previously [21]. This procedure using urea is essential for *in vitro* reconstitution of the functional eukaryotic protein complex, since isolated eukaryotic P0 is insoluble in the absence of urea. Although isolated Ph-P0 and Ph-L12 were soluble and bound to each other without urea, the Ph-P0•Ph-L12 complex formed by using urea showed slightly higher activity in factor accessibility than the complex formed without urea. Therefore, the stalk protein complexes used in the present study (Ph-P0•Ph-L12, as well as silkworm P0•P1-P2 and *E. coli* L10•L7/L12) were all reconstituted by the same procedure using urea [21]. The molecular assembly of Ph-P0•Ph-L12 and Ph-L11 on the 1070 rRNA fragment was carried out in a solution containing 20 mM MgCl₂, 300 mM KCl and 20 mM Tris/HCl (pH 7.6), as described in [21]. The complexes formed were assayed by 6% (w/v) polyacrylamide (acrylamide/bisacrylamide ratio 39:1) native gel electrophoresis at 6.5 V/cm with a buffer system containing 5 mM MgCl₂, 50 mM KCl and 50 mM Tris/HCl (pH 8.0). Samples were electrophoresed for 6 h at constant voltage and 4 °C with buffer recirculation. The gel was subjected to autoradiography.

Ribosomal subunits, 50S core particles and hybrid 50S particles

E. coli ribosomal subunits were prepared as described previously [24,25]. The 50S core, deficient in L10•L7/L12 and L11, was prepared by extraction of the 50S subunits from the L11-deficient *E. coli* mutant, AM68 [43], in a solution containing 50% ethanol and 0.5 M NH₄Cl at 0 °C, as described previously [25]. The *P. horikoshii*-*E. coli* or silkworm-*E. coli* hybrid 50S particle was prepared by mixing the *E. coli* 50S core with the *P. horikoshii* ribosomal proteins or silkworm proteins [25] as described in the Figure legends, followed by incubation at 37 °C for 5 min.

Acrylamide/agarose composite gel electrophoresis

The samples of hybrid 50S particles were analysed by electrophoresis on acrylamide/agarose composite gels composed of 3% (w/v) acrylamide (acrylamide/bisacrylamide, 19:1) and 0.5%

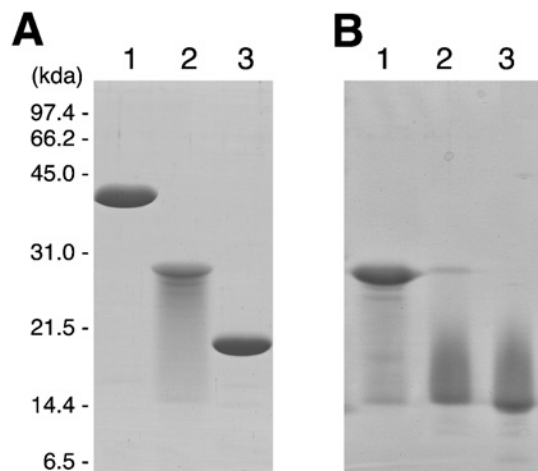


Figure 1 Isolated ribosomal proteins constituting the archaeobacterial GTPase-associated centre

(A) Isolated Ph-P0 (lane 1), Ph-L12 (lane 2) and Ph-L11 (lane 3) were subjected to SDS/gel electrophoresis (5 μ g of each). (B) Ph-L12 (5 μ g) was incubated with 0% (lane 1), 1% (lane 2) or 2% (lane 3) H_2O_2 at 30 °C for 1 h, and then subjected to SDS/PAGE. The gel was stained with Coomassie Brilliant Blue R-250.

(w/v) agarose [44], as described previously [25]. Gels were stained with 0.2% Azur B.

EFs and functional assays

eEF-1 α and eEF-2 were isolated from pig liver as described by Iwasaki and Kaziro [45]. Ph-EF-1 α and Ph-EF-2 from *P. horikoshii*, were expressed in *E. coli* cells using plasmids that were constructed by insertion of individual coding sequences into the pET-22b vector (Novagen). The S100 fractions of individual cells were heated at 90 °C for 10 min to remove *E. coli* proteins. The supernatant containing Ph-EF-1 α was applied to a column of CM-cellulose equilibrated with a buffer of 20 mM sodium phosphate (pH 6.8) and 5 mM 2-mercaptoethanol, and Ph-EF-1 α was eluted in the same buffer containing 0.02 M LiCl. Ph-EF-2 was isolated by elution from a DEAE-cellulose column equilibrated with a buffer of 20 mM Tris/HCl (pH 8.0) and 5 mM 2-mercaptoethanol. *E. coli* EF-Tu and EF-G were overexpressed in *E. coli* cells by using the pET-3a vector (Novagen), and purified from the cell extract by DEAE-sephadex A-50 column chromatography, as described in [46]. The assay for ribosomal GTPase activity dependent on eEF-2 and Ph-EF-2 was performed as described previously [21]. The assay for GTPase activity dependent on *E. coli* EF-G was described previously [40], except that the reaction mixture contained 7.5 pmol of the *E. coli* 30S subunit and 10 mM MgCl_2 . Polyphenylalanine synthesis by the hybrid ribosomes was assayed with eEF-1 α and eEF-2, as described previously [21].

RESULTS AND DISCUSSION

Expression and isolation of *P. horikoshii* ribosomal proteins Ph-P0, Ph-L11 and Ph-L12

P. horikoshii ribosomal proteins Ph-P0, Ph-L11 and Ph-L12 were expressed in *E. coli* cells and isolated by HPLC. SDS/PAGE of the isolated proteins (Figure 1A) showed that Ph-P0 (lane 1) and Ph-L11 (lane 3) appeared as single bands of 39.5 kDa and 18.4 kDa, close to the predicted molecular masses of 37.5 kDa and 17.7 kDa respectively. However, Ph-L12 appeared as a major

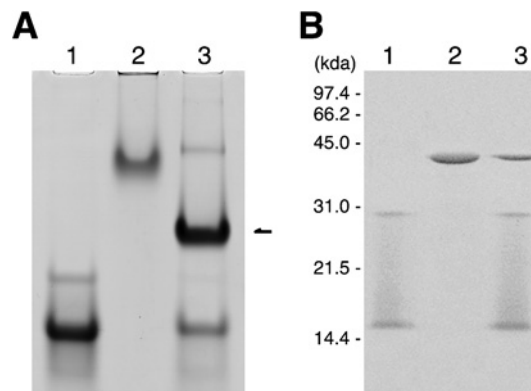


Figure 2 Formation of a complex between Ph-P0 and Ph-L12

(A) Isolated Ph-L12 (600 pmol, lane 1), Ph-P0 (100 pmol, lane 2) and the complex (lane 3) formed with both proteins was subjected to non-denaturing gel electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250. The band that appeared as a result of mixing the two proteins is indicated with an arrow. (B) Major bands that appeared in (A), lanes 1, 2 and 3 (arrow), were cut out of the gel, incubated in SDS sample solution and then used as samples for SDS/gel electrophoresis. The gel was stained with the fluorescent reagent SYPRO Orange (Molecular Probes, Invitrogen) and the pattern was visualized with a STORM 860 PhosphorImager (Amersham Biosciences).

band of 27.9 kDa with smearing (lane 2). This value is close to twice the predicted size of Ph-L12 (11.8 kDa), suggesting that Ph-L12 forms a homodimer, which is extremely stable even after heating at 95 °C in SDS solution. After treatment of Ph-L12 with 1–2% H_2O_2 and subsequently with the SDS sample solution, a smeared band of approx. 15 kDa appeared, corresponding to the monomer (Figure 1B, lanes 2 and 3). This tight association of the proteins was also observed in the N-terminal halves of Ph-L12 (T. Naganuma and T. Uchiumi, unpublished work), which implies that Ph-L12 monomers bind tightly to each other through interaction with the N-terminal halves. The destabilization of the stalk dimer by treatment with H_2O_2 has also been reported in *E. coli* L7/L12 [47,48]. The molecular details of the destabilization of the Ph-L12 homodimer with H_2O_2 remain to be clarified.

In vitro reconstitution of Ph-P0 • Ph-L12 and Ph-P0 • Ph-L12 • Ph-L11 • rRNA complexes

Ph-P0 was mixed with 6 M-fold amount of Ph-L12 and analysed by non-denaturing gel electrophoresis (Figure 2A). An intense band appeared (lane 3) with different mobility from those of isolated Ph-L12 (lane 1) and Ph-P0 (lane 2). To confirm that the new band in lane 3 of Figure 2(A) corresponded to the Ph-P0 • Ph-L12 complex, the band was cut out of the gel and subjected to SDS/gel electrophoresis (Figure 2B). The new band contained three protein components (lane 3) corresponding to Ph-P0 (lane 2), the Ph-L12 dimer, and its monomer dissociated presumably by oxidation of the dimer during native gel electrophoresis (lane 1). The results indicate that the expressed Ph-P0 can form a complex with Ph-L12 homodimers *in vitro*.

We recently analysed the binding sites for eukaryotic P1-P2 heterodimers within silkworm P0 [39]. The truncation mutant of silkworm P0 (Figure 3A), Δ 107, which lacks the C-terminal amino acid residues 210–316 failed to bind the two P1-P2 dimers, whereas the mutant lacking residues 236–316 (Δ 81) retained the ability to bind a single dimer. By using various truncation mutants including Δ 81 and Δ 107, we concluded that the region containing residues 210–235 in silkworm P0 is involved in binding one of the two P1-P2 dimers and the region spanning residues 236–261 is for binding another dimer. An alignment of sequences around the putative stalk binding sites of P0-like proteins shows

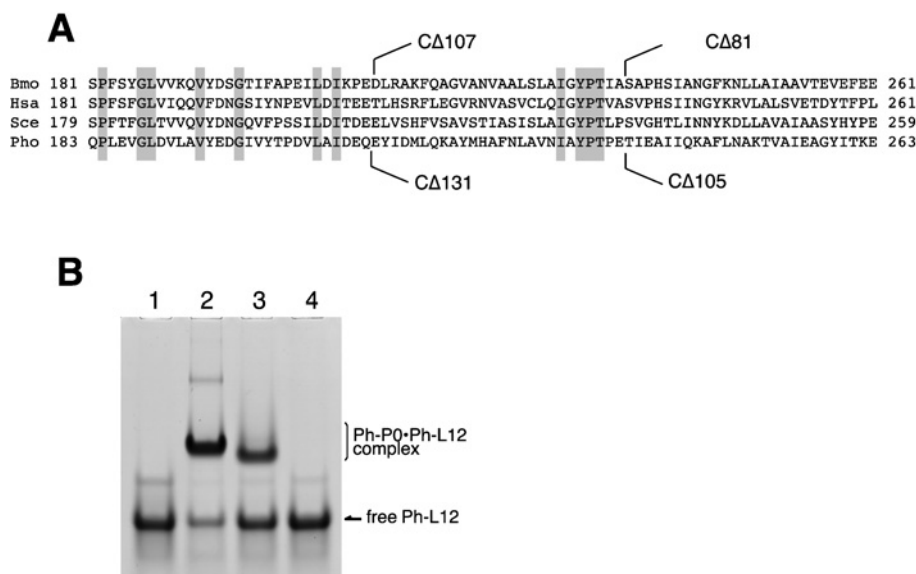


Figure 3 Effect of C-terminal truncation of Ph-P0 on Ph-P0•Ph-L12 complex formation

(A) Amino acid sequence alignment of the C-terminal parts of P0-like proteins from silkworm (*Bombyx mori*, Bmo), human (*Homo sapiens*, Hsa), yeast (*Saccharomyces cerevisiae*, Sce) and *P. horikoshii* (Pho). The sites of truncation in our previous study [39] are marked on the sequence of Bmo-P0. The truncation sites of *P. horikoshii* in the present study are also indicated below the Pho-P0 sequence. (B) The complexes were reconstituted by mixing 600 pmol of Ph-L12 with 100 pmol of wild-type Ph-P0 (lane 2), CΔ105 (lane 3) or CΔ131 (lane 4) as described in the Experimental section. The sample in lane 1 was Ph-L12 alone. Each sample was subjected to native gel electrophoresis. The gel was stained with Coomassie Brilliant Blue. Free proteins, CΔ105 and CΔ131, were not detected on the gel because of the failure of these proteins that were in the free state to run into the gel.

some similarity between eukaryotic and archaeobacterial proteins, although the sequence identity is low (Figure 3A).

To determine whether archaeobacterial Ph-P0 also has binding sites for Ph-L12 homodimers in this region, we constructed Ph-P0 mutants in which the C-terminal amino acids 238–342 (CΔ105) and 212–342 (CΔ131) were truncated (Figure 3A). The CΔ105 and CΔ131 truncation mutants of Ph-P0 correspond to the CΔ81 and CΔ107 truncation mutants of the silkworm P0, which could bind only one and no P1-P2 heterodimer respectively [39]. Binding of Ph-L12 homodimers to the truncated Ph-P0 mutants was tested by non-denaturing gel electrophoresis (Figure 3B). After mixing the CΔ105 Ph-P0 mutant with Ph-L12, a band that migrated slightly faster (lane 3) than that of the wild-type Ph-P0•L12 complex appeared (lane 2). However, the CΔ131 Ph-P0 mutant did not form a complex with Ph-L12 (lane 4), suggesting that binding sites for Ph-L12 homodimers are present within the C-terminal 131-amino-acid sequence of Ph-P0, and that there may be a conserved feature, at least in part, in binding to P0-like proteins between the archaeobacterial Ph-L12 homodimer and the eukaryotic P1-P2 heterodimer. Further biochemical, biophysical and crystal structure analyses will elucidate the details of the interaction between Ph-P0 and Ph-L12 dimers.

To test the ability of the expressed archaeobacterial ribosomal proteins to bind the 1070 domain of 23S rRNA, gel mobility-shift assays were performed using the [³²P]rRNA fragment (Figure 4). Ph-P0 (lane 2) and Ph-L11 (lane 4), but not Ph-L12 (lane 3), showed the ability to bind directly to the 1070 rRNA fragment. Supershifts of the complex bands were observed by mixing Ph-P0 with Ph-L11 (lane 5), Ph-P0 with Ph-L12 (lane 6), and Ph-P0, Ph-L11 and Ph-L12 together (lane 7), implying that all of the proteins assemble on to the 1070 rRNA region *in vitro*. Binding of the archaeobacterial proteins, Ph-P0•Ph-L12 and Ph-L11, to the 1070 rRNA fragment of *P. horikoshii* 23S rRNA (Figure 5A, lane 3) was compared with that of *E. coli* L10•L7/L12 and L11 (lane 2) and with that of silkworm P0•P1-P2 and

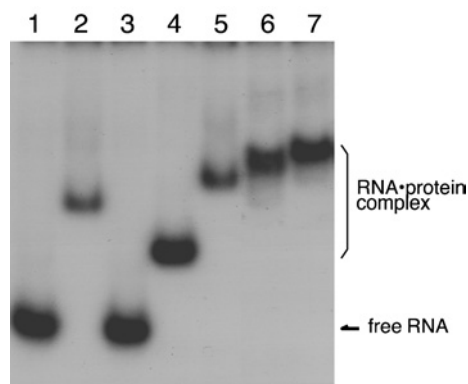


Figure 4 Binding ability of the *P. horikoshii* ribosomal proteins to the 1070 rRNA fragment

An aliquot (20 pmol) of each of isolated Ph-P0 (lane 2), Ph-L12 (lane 3), Ph-L11 (lane 4), Ph-P0 + Ph-L11 (lane 5), Ph-P0•Ph-L12 complex (lane 6), and Ph-P0•Ph-L12 + Ph-L11 (lane 7) was mixed with 5 pmol of the [³²P]rRNA fragment covering the 1070 domain of *P. horikoshii* 23S rRNA (lane 1, RNA alone), separated by native gel electrophoresis and then subjected to autoradiography.

eL12 (lane 4). These cross-bindings were also tested with the equivalent RNA fragments of rat 28S rRNA (Figure 5B) and *E. coli* 23S rRNA (Figure 5C). These protein homologues bound equally well to the 1070 rRNA fragments from members of the three kingdoms, suggesting that there are highly conserved protein–RNA interactions at the GTPase-associated centre among the three kingdoms.

Functional properties of the *P. horikoshii* ribosomal proteins assembled on to the *E. coli* 50S particles

We have established *in vitro* conditions under which to replace the *E. coli* ribosomal protein L11 and the L10•L7/L12 complex on

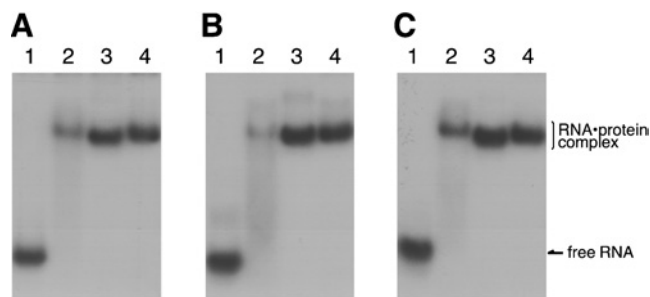


Figure 5 Conserved rRNA-protein binding property in the GTPase-associated centre

RNA fragments of the 1070 rRNA domain from *P. horikoshii* 23S (A), rat 28S (B) and *E. coli* 23S (C). rRNAs (lanes 1) were mixed with the *E. coli* L10•L7/L12 complex and L11 (lanes 2), the Ph-P0•Ph-L12 complex and Ph-L11 (lanes 3), and the silkworm P0•P1-P2 complex and eL12 (lanes 4), and were analysed as described in Figure 4.

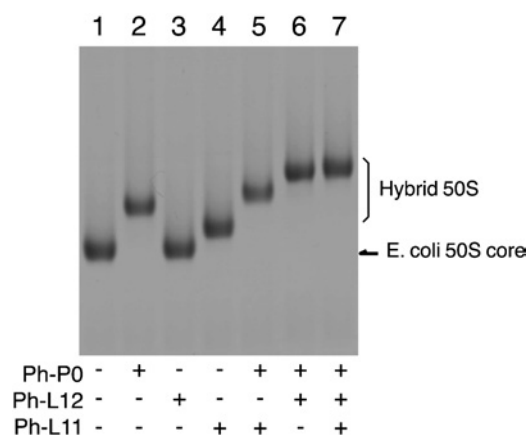


Figure 6 Assembly of *P. horikoshii* ribosomal proteins on to the GTPase-associated centre of *E. coli* 50S subunits

An aliquot (10 pmol) of the 50S core particles deficient in L10•L7/L12 and L11 (lane 1) was incubated with 20 pmol of Ph-P0 (lane 2), Ph-L12 (lane 3), Ph-L11 (lane 4), Ph-P0 + Ph-L11 (lane 5), the Ph-P0•Ph-L12 complex (lane 6), and Ph-P0•Ph-L12 + Ph-L11 (lane 7), and then analysed by acrylamide/agarose composite gel electrophoresis, as described in the Experimental section.

the 50S subunit with eukaryotic counterparts eL12 and P0•P1-P2 respectively [25]. The resultant hybrid ribosome showed polyphenylalanine synthesis, as well as GTPase activity, that was

dependent on eEF-1 α and eEF-2, but not on prokaryotic EF-Tu and EF-G [25]. This hybrid system is therefore useful for studying the functional role of the stalk protein complex at the GTPase-associated centre and also the structural determinants responsible for the kingdom-dependent specificity between ribosomes and translation factors. We attempted to examine the functional characteristics of the archaeobacterial stalk-constituting proteins, Ph-P0, Ph-L11 and Ph-L12, expressed in *E. coli* cells. The formation of the hybrid 50S particles that resulted from mixing *E. coli* 50S cores with archaeobacterial proteins was confirmed by acrylamide/agarose composite gel electrophoresis (Figure 6). The gel mobility of *E. coli* 50S cores (lane 1) was shifted upwards by the addition of recombinant Ph-P0 (lane 2) and Ph-L11 (lane 4), but not Ph-L12 (lane 3). Supershifts were observed after mixing Ph-P0 with Ph-L11 (lane 5), Ph-P0 with Ph-L12 (lane 6), or all three proteins (lane 7), implying that the three proteins are bound to *E. coli* 50S cores.

The hybrid 50S particles, together with *E. coli* 30S subunits, were tested for their function in enhancing accessibility to translocases from *P. horikoshii* (Figure 7A), pig liver (Figure 7B) and *E. coli* (Figure 7C). The GTPase activity of the hybrid ribosome carrying Ph-P0•Ph-L12 and Ph-L11 (Ph) was compared with that of the hybrid carrying silkworm P0•P1-P2 and eL12 (Bm), and also the *E. coli* ribosome reconstituted with L10•L7/L12 and L11 (Eco). The Ph-hybrid ribosome showed GTPase activity not only with *P. horikoshii* EF-2 (Figure 7A), but also with animal eEF-2 (Figure 7B). No activity of the Ph-hybrid was detected with *E. coli* EF-G (Figure 7C). Low *P. horikoshii* EF-2-dependent activity with the Ph-hybrid ribosome assayed at 37 °C could be predicted, considering that the optimum temperature for growth of *P. horikoshii* is extremely high (98 °C). Rather, it was unexpected that the Ph-hybrid showed high GTPase activity with animal eEF-2 at 37 °C, which was comparable with that of the silkworm hybrid ribosome carrying silkworm P0, P1/P2 and eL12 (Figure 7B). The functional accessibility of the hybrid ribosome carrying *P. horikoshii* proteins to eEFs was also tested in the polyphenylalanine synthesis assay, which is dependent on both eEF-1 α and eEF-2 (Figure 8). The hybrids carrying the L10-like and L7/L12-like proteins from *P. horikoshii* and silkworm had comparable activity, although the addition of Ph-L11 instead of *E. coli* L11 or the silkworm homologue eL12 slightly decreased the activity. These results imply that the archaeobacterial ribosomal proteins at the GTPase-associated centre have the potential to make the ribosome accessible to eEFs. The results also suggest that the L10-like and L7/L12-like proteins, but not the L11-like protein, are responsible for the specificity between

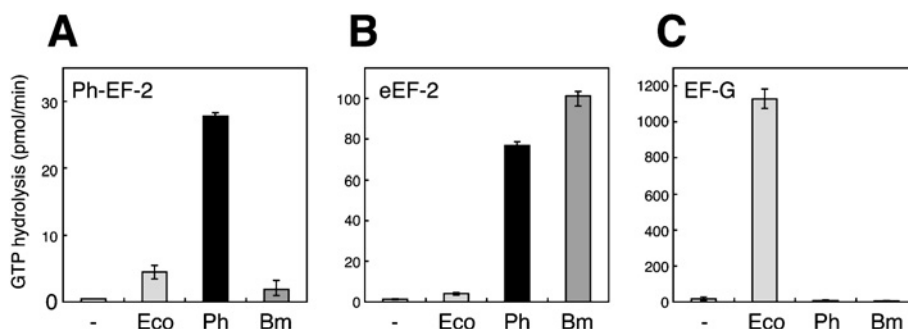


Figure 7 The GTPase activity of the hybrid ribosomes dependent on *P. horikoshii* EF-2 (A), pig eEF-2 (B) and *E. coli* EF-G (C)

E. coli 50S core particles (2.5 pmol) deficient in L10, L7/L12 and L11 were pre-incubated with 10 pmol of each of L10•L7/L12 and L11 (Eco). The 50S cores were also incubated with 10 pmol of each of Ph-P0•Ph-L12, and Ph-L11 (Ph) or silkworm P0•P1-P2 and eL12 (Bm). The resulting 50S particle samples were assayed for GTPase activity in the presence of *E. coli* 30S subunits (7.5 pmol).

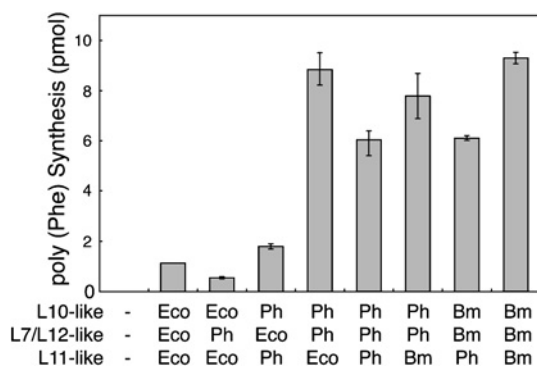


Figure 8 Polyphenylalanine synthesis of hybrid ribosomes dependent on eukaryotic elongation factors

The 50S core particles (10 pmol) were pre-incubated with L10- (20 pmol), L7/L12- (120 pmol) and L11-like proteins (20 pmol) from *E. coli* (Eco), *P. horikoshii* (Ph) or *Bombyx mori* (Bm, silkworm) in various combinations as indicated, and then assayed for polyphenylalanine synthesis dependent on pig eEF-1 α and eEF-2, in the presence of *E. coli* 30S subunits (50 pmol).

ribosomes and translation factors. This conclusion is consistent with previous data regarding direct contact between the stalk protein and EF-G [49], but is inconsistent with another report on direct L11–EF-G contact [10]. In the present study, it is particularly noteworthy that Ph-L12 homodimers are compatible with eukaryotic P1–P2 heterodimers. We infer that functionally important structural elements of the stalk are preserved from archaeobacterial homodimers to eukaryotic heterodimers.

Conclusions

The present study demonstrated the *in vitro* reconstitution of the *P. horikoshii* ribosomal GTPase-associated centre from purified components, which were cloned and expressed individually. The stalk protein, Ph-L12, was strongly bound as a dimer presumably through interactions between the N-terminal halves. The Ph-L12 homodimers bound to the C-terminal half of Ph-P0 in a similar manner to eukaryotic P1–P2 heterodimers [39], although the number of dimers bound to Ph-P0 has not been determined in this study. The Ph-P0•Ph-L12 complex and Ph-L11 bound to the 1070 rRNA domain from the three kingdoms in the same manner as the equivalent proteins from eukaryotes and eubacteria. The Ph-P0•Ph-L12 complex and Ph-L11 could replace the L10•L7/L12 complex and L11 respectively, on the *E. coli* 50S subunit. The *P. horikoshii*–*E. coli* hybrid ribosome showed functional accessibility for eEF-1 α and eEF-2. The levels of GTPase and polyphenylalanine synthesis activity were comparable with those of another hybrid ribosome carrying the eukaryotic P0•P1–P2 complex and eL12 instead of Ph-P0•Ph-L12 and Ph-L11. These results suggest that the archaeobacterial ribosomal proteins have the potential to function with eEFs. These results are reasonable, when considering that the amino acid sequences of the archaeobacterial proteins resemble eukaryotic rather than eubacterial counterparts [see Supplementary Figures S2–S4 at <http://www.BiochemJ.org/bj/396/bj3960565add.htm>]. On the other hand, the present evidence is not consistent with previous observations that archaeobacterial ribosomes (with the exception of those from methanogens) show no accessibility to eEFs [27,28]. This discrepancy may be explained as follows. Archaeobacteria grow in extremely hot, acidic or salty conditions. It is probable that the overall structure of the archaeobacterial ribosomal machinery does not work with eEFs that function under normal conditions.

However, some archaeobacterial ribosomal proteins may, in part, have functional compatibility with the equivalent eukaryotic ribosomal proteins. By using the hybrid system with *E. coli* ribosomes, we have demonstrated that the proteins constituting the archaeobacterial GTPase-associated centre are compatible with their eukaryotic counterparts, at least for translation elongation.

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REFERENCES

- 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
- Gudkov, A. T., Tumanova, L. G., Venyaminov, S. Y. and Khechinashvili, N. N. (1978) Stoichiometry and properties of the complex between ribosomal proteins L7 and L10 in solution. *FEBS Lett.* **93**, 215–218
- 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
- Ileg, L. L., Vldeler, H., McKay, A. R., 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. U.S.A.* **102**, 8192–8197
- 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
- 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
- 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
- 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
- Möller, W. and Maassen, J. A. (1986) *Structure, Function, and Genetics of Ribosomes* (Hardesty, B. and Kramer, G., eds.), pp. 309–325, Springer-Verlag, NY
- Agrawal, R. K., Linde, J., Sengupta, J., Nierhaus, K. H. and Frank, J. (2001) Localization of L11 protein on the ribosome and elucidation of its involvement in EF-G-independent translocation. *J. Mol. Biol.* **311**, 777–787
- Ban, N., Nissen, P., Hansen, J., Moore, P. B. and Steitz, A. T. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289**, 905–920
- Yusupov, M. M., Yusupova, G. Z., Baucom, A., Lieberman, K., Earnest, T., Cate, J. H. and Noller, H. F. (2001) Crystal structure of the ribosome at 5.5 Å resolution. *Science* **292**, 883–896
- Harms, J., Schlunzen, 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
- Newton, C. H., Shimmin, L. C., Yee, J. and Dennis, P. P. (1990) A family of genes encode the multiple forms of the *Saccharomyces cerevisiae* ribosomal proteins equivalent to the *Escherichia coli* L12 protein and a single form of the L10-equivalent ribosomal protein. *J. Bacteriol.* **172**, 579–588
- Maassen, J., Schop, E. N., Brands, J. H., van Hemert, F. J., Lenstra, J. A. and Möller, W. (1985) Molecular cloning and analysis of cDNA sequences for two ribosomal proteins from *Artemia*. The coordinate expression of genes for ribosomal proteins and elongation factor 1 during embryogenesis of *Artemia*. *Eur. J. Biochem.* **149**, 609–616
- Rich, B. E. and Steitz, J. A. (1987) Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, *in vitro* synthesis, and assembly. *Mol. Cell. Biol.* **7**, 4065–4074
- Wool, I. G., Chan, Y.-L. and Glöck, A. (1995) Structure and evolution of mammalian ribosomal proteins. *Biochem. Cell Biol.* **73**, 933–947
- Tchórzewski, M., Boldyreff, B., Issinger, O. G. and Grankowski, N. (2000) Analysis of the protein–protein interactions between the human acidic ribosomal P-proteins: evaluation by the two hybrid system. *Int. J. Biochem. Cell Biol.* **32**, 737–746

- 19 Garinos, 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
- 20 Gonzalo, P., Lavergne, J. P. and Reboud, J. P. (2001) Pivotal role of the P1 N-terminal domain in the assembly of the mammalian ribosomal stalk and in the proteosynthetic activity. *J. Biol. Chem.* **276**, 19762–19769
- 21 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
- 22 Tchórzewski, M., Krokowski, D., Boguszewska, A., Liljas, A. and Grankowski, N. (2003) Structural characterization of yeast acidic ribosomal P proteins forming the P1A–P2B heterocomplex. *Biochemistry* **42**, 3399–3408
- 23 Hanson, C. L., Videler, H., Santos, C., Juan, P. G., Ballesta, J. P. C., Carol, V. and Robinson, C. V. (2004) Mass spectrometry of ribosomes from *Saccharomyces cerevisiae*: implications for assembly of the stalk complex. *J. Biol. Chem.* **279**, 42750–42757
- 24 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
- 25 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**, 41401–41409
- 26 Woese, C. R. and Fox, G. E. (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5088–5090
- 27 Klink, F., Schümann, H. and Thomsen, A. (1983) Ribosome specificity of archaeobacterial elongation factor 2. *FEBS Lett.* **155**, 173–177
- 28 Beauclerk, A. A. D., Hummel, H., Holmes, D. J., Böck, A. and Cundliffe, E. (1985) Studies of the GTPase domain of archaeobacterial ribosomes. *Eur. J. Biochem.* **151**, 245–255
- 29 Gutell, R. R. and Fox, G. E. (1988) A compilation of large subunit RNA sequences presented in a structural format. *Nucleic Acids Res.* **16**, r175–r269
- 30 Sköld, S.-E. (1983) Chemical crosslinking of elongation factor G to the 23S RNA in 70S ribosomes from *Escherichia coli*. *Nucleic Acids Res.* **11**, 4923–4932
- 31 Moazed, D., Robertson, J. M. and Noller, H. F. (1988) Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S RNA. *Nature (London)* **334**, 362–364
- 32 Thompson, J., Cundliffe, E. and Dahlberg, A. E. (1988) Site-directed mutagenesis of *Escherichia coli* 23S ribosomal RNA at position 1067 within the GTP hydrolysis centre. *J. Mol. Biol.* **203**, 457–465
- 33 Uchiumi, T. and Kominami, R. (1994) A functional site of the GTPase-associated center within 28S ribosomal RNA probed with an anti-RNA autoantibody. *EMBO J.* **13**, 3389–3394
- 34 Shimmin, L. C., Ramirez, C., Matheson, A. T. and Dennis, P. P. (1989) Sequence alignment and evolutionary comparison of the L10 equivalent and L12 equivalent ribosomal proteins from Archaeobacteria, Eubacteria, and Eucaryotes. *J. Mol. Evol.* **29**, 448–462
- 35 Casiano, C. and Traut, R. R. (1991) Protein topography of *Sulfolobus solfataricus* ribosomes by cross-linking with 2-iminothiolane. *J. Biol. Chem.* **266**, 21578–21583
- 36 Köpke, A. K., Leggatt, P. A. and Matheson, A. T. (1992) Structure function relationships in the ribosomal stalk proteins of archaeobacteria. *J. Biol. Chem.* **267**, 1382–1390
- 37 Blin, N. and Stafford, D. W. (1976) A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.* **3**, 2303–2308
- 38 Saiki, R. K., Gelfand, D. H., Stoffel, S., Sharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491
- 39 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: *in vitro* analyses with P0 truncation mutants. *J. Biol. Chem.* **280**, 39193–39199
- 40 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
- 41 Uchiumi, T., Sato, N., Wada, A. and Hachimori, A. (1999) Interaction of the sarcin/ricin domain of 23S ribosomal RNA with proteins L3 and L6. *J. Biol. Chem.* **274**, 681–686
- 42 Uchiumi, T., Wada, A. and Kominami, Y. (1995) A base substitution within the GTPase-associated domain of mammalian 28S ribosomal RNA causes high thiostrepton. *J. Biol. Chem.* **270**, 29889–29893
- 43 Dabbs, E. R. (1979) Selection for *Escherichia coli* mutants with proteins missing from the ribosome. *J. Bacteriol.* **140**, 734–737
- 44 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
- 45 Iwasaki, K. and Kaziro, Y. (1979) Polypeptide chain elongation factors from pig liver. *Methods Enzymol.* **60**, 657–676
- 46 Arai, K., Kawakita, M. and Kaziro, Y. (1972) Studies on polypeptide elongation factors from *Escherichia coli*. II. *J. Biol. Chem.* **247**, 7029–7037
- 47 Caldwell, P., Luk, D. C., Weissbach, H. and Brot, N. (1978) Oxidation of the methionine residues of *Escherichia coli* ribosomal protein L12 decreases the protein's biological activity. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5349–5352
- 48 Gudkov, A. T. and Behlke, J. (1978) The N-Terminal sequence protein of L7/L12 is responsible for its dimerization. *Eur. J. Biochem.* **90**, 309–312
- 49 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

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