

Protein L5 is crucial for *in vivo* assembly of the bacterial 50S ribosomal subunit central protuberance

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ABSTRACT

In the present work, ribosomes assembled in bacterial cells in the absence of essential ribosomal protein L5 were obtained. After arresting L5 synthesis, *Escherichia coli* cells divide a limited number of times. During this time, accumulation of defective large ribosomal subunits occurs. These 45S particles lack most of the central protuberance (CP) components (5S rRNA and proteins L5, L16, L18, L25, L27, L31, L33 and L35) and are not able to associate with the small ribosomal subunit. At the same time, 5S rRNA is found in the cytoplasm in complex with ribosomal proteins L18 and L25 at quantities equal to the amount of ribosomes. Thus, it is the first demonstration that protein L5 plays a key role in formation of the CP during assembly of the large ribosomal subunit in the bacterial cell. A possible model for the CP assembly *in vivo* is discussed in view of the data obtained.

INTRODUCTION

Reconstruction of functionally active bacterial ribosomal subunits was first performed successfully over 40 years ago (1–3), and since then a great body of experimental data regarding ribosome assembly *in vitro* has been accumulated (4–7). Crystal structures of ribosomes as well as that of functional ribosomal complexes from several bacteria are now available, providing insights into spatial organization of the assembled ribosome (8–13).

At the same time, these studies cannot take into account all of the factors driving the ribosome assembly *in vivo* (14–18). The present work is aimed at studying the role of the ribosomal protein (r-protein) L5 in the assembly of

the large ribosomal subunit in the bacterial cell. The L5 protein is of particular interest for several reasons. First, it was shown earlier that two r-proteins, L5 and L18, are indispensable for the interaction between 5S rRNA and 23S rRNA (19,20). Recent crystallographic studies of bacterial ribosomes confirm the involvement of L5 in the conjunction of these rRNAs (8,10,12). Moreover, L5, which is conserved in Bacteria, Archaea and Eukarya, forms conserved intermolecular bonds with ribosomal RNAs (21–27). These observations may indicate that L5 performs a universal function in the formation of the ribosome. Second, protein L5 is known to be located on the ‘top’ of the central protuberance (CP) of the large ribosomal subunit (28) in the close vicinity of its functional centers (8,12,29–31). Crystallographic data show that L5 participates in formation of intersubunit bridge B1b and contacts the tRNA molecule in the ribosomal P-site (8–10,12). Taken together, these observations indicate the importance of protein L5 for the ribosome formation and functioning. However, the particular role of the protein in these processes still remains unknown. Earlier, we have shown that r-protein L5 is essential for the survival of *Escherichia coli* cells (32), but the reason for this has not been elucidated.

In this work we have studied the role of L5 in the ribosome assembly *in vivo*. We obtained an *E. coli* strain where the chromosomal gene encoding r-protein L5 (*rplE*) was inactivated in the presence of a complementing plasmid that provides inducible *rplE* expression. Thus, the synthesis of L5 could be stopped by removing the inducer from a growth medium. Analysis of the ribosomes from L5-depleted cells showed accumulation of large ribosomal subunits that lack most of the CP components. Our data demonstrate, for the first time, that r-protein L5 plays a key role in formation of this entire structural domain of the bacterial ribosome *in vivo*.

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MATERIALS AND METHODS

Strains, plasmids and bacteriological techniques

All the strains and plasmids mentioned in the present work are listed in Supplementary Table S1. Cells were cultivated at 37°C in LB broth, and LB-agar plates, supplemented (where indicated) with 100 µg/ml ampicillin, 20 µg/ml chloramphenicol and 0.2% (wt/vol) L-arabinose. To obtain an *rplE*-encoding complementary plasmid pNK12, the *NdeI-EcoRI* fragment of pKAB122, carrying *E. coli rplE* open reading frame, was cloned into the plasmid pBADET. The latter is a gift from A. Kaliman. It is a derivative of pBAD18 (33), see Supplementary Table S1 for more information. Thus, pNK12 carries *rplE* under control of an arabinose-inducible promoter and confers resistance to ampicillin. The deletion of chromosomal *rplE* gene was made by transferring the $\Delta rplE::cat$ allele from KNB219 to the wild-type strain W3110, transformed with pNK12, *via* generalized transduction by phage P1 according to (34). Transductants were selected on LB agar, supplemented with chloramphenicol and arabinose. For simplicity, we call the resulting strain MS01, meaning that it contains the plasmid pNK12 at all times.

Cell cultivation conditions

To obtain *E. coli* cells depleted for protein L5, strain MS01 was grown at 37°C in LB medium supplemented with ampicillin and arabinose until $A_{600} \sim 0.4$. Then, cells were washed and inoculated with 15–20 vol of pre-warmed at 37°C LB medium and cultivated until the growth curve reached a plateau (at $A_{600} \sim 0.8$ –1, within 6–8 h). During this time, aliquots of the cell culture were taken for the sedimentation analysis of ribosomes. The cells were collected, washed with buffer (30 mM Tris-HCl, pH 7.5, 200 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 3 mM 2-mercaptoethanol) and stored as described in (32).

The same strategy was applied to obtain the control cells. However, in this case the inducer was always present in the growth medium, providing *rplE* expression *in trans*. The cells were harvested at $A_{600} \sim 0.8$ –1.

Preparation and analysis of ribosomes and ribosomal subunits

Ribosomes were obtained according to (35) with modifications published in (32). To obtain ribosomal subunits, the ribosomes from the L5-depleted cells were loaded onto 5–20% (wt/wt) sucrose gradients prepared in buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂ and 3 mM 2-mercaptoethanol) and centrifuged at 64 000g and 4°C for 11–12 h. Under these ionic conditions intact 50S subunits remain associated with 30S subunits to form 70S ribosomes, which greatly facilitate purification of 45S particles. Ribosomal subunits from the control cells were prepared in the same manner but in the presence of 1 mM MgCl₂. Lowering magnesium concentration to 1 mM and below is a conventional approach allowing complete 70S ribosomes dissociation onto 50S and 30S subunits (35). Separated subunits were collected by

high-speed centrifugation, dissolved in cold buffer A with 10% (vol/vol) glycerol, frozen and stored at –70°C. Large ribosomal subunits from L5-depleted cells were additionally purified by a second centrifugation in 5–20% (wt/wt) sucrose gradients.

To analyze ribosome profiles, cells were lysed by freeze-thaw method with lysozyme as described earlier (32), with buffer (30 mM Tris-HCl, pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA and 3 mM 2-mercaptoethanol) being used at all the stages. Then, S₃₀ fractions were loaded onto 5–20% (wt/wt) sucrose gradients made in buffer A and centrifuged at 210 000g and 4°C for 1.5 h. Experiments on the association of ribosomal subunits were performed as follows. Equal amounts of small and large ribosomal subunits were mixed in buffer A with varying concentrations of MgCl₂ (5–20 mM) and incubated at 37°C for 20 min. Then, the mixtures were analyzed by centrifugation in 5–20% (wt/wt) sucrose gradients as described above. To determine the sedimentation coefficients, an Optima XL-I analytical ultracentrifuge (Beckman Coulter, USA) was used. Centrifugation was carried out in buffer A. Sedimentation data were analyzed with the program Sedfit (36). The integrity of high-molecular weight ribosomal RNA was tested using electrophoresis in composite 2.3% polyacrylamide/0.75% agarose gel as described (37). To estimate 5S rRNA distribution, low-molecular weight RNAs were extracted as described earlier (38) from equal amount (by A_{260}) of S₃₀ fractions from L5-depleted and control cells. Same volumes of the corresponding ribosome-free extracts were used for RNA extraction. An RNA content was analyzed by electrophoresis in 8% polyacrylamide gels containing 8 M urea (39). Band intensities were quantified using program ImageJ. In view of the fact that slow-growing L5-depleted cells contained reproducibly less ribosomes (per total A_{260}), 5S rRNA band was equally less intense when analyzing L5-depleted S₃₀ fraction as compared to the control. Ribosomal proteins were extracted from ribosomal subunits with acetic acid (40) and fractionated by 2D gel electrophoresis (41) using acidic-acidic system IV. A number of ribosomal proteins were eluted from the gel and identified by mass spectrometry on a Deca XP Plus ion trap mass spectrometer (Thermo Finnigan, USA).

Affinity purification of 5S rRNA from the cytoplasm of the L5-depleted cells

5S rRNA was isolated using affinity chromatography on L5-Sepharose. Ribosomal protein L5 was purified as previously described (42) and covalently attached to Sepharose-4B according to (43). Ribosome-free cytoplasmic fraction (S₁₀₀) was incubated with L5-Sepharose in buffer (10 mM Tris-HCl, pH 7.5, 50 mM NH₄Cl, 10 mM MgCl₂ and 3 mM 2-mercaptoethanol) at room temperature for 30 min. Then, the resin was washed sequentially with the same buffer containing 50 and 400 mM NH₄Cl. 5S rRNA and bound proteins were eluted with 3 M NaCl. RNA and protein content of the final wash was analyzed by electrophoresis in denaturing conditions, as described in (39) and (44), correspondingly. Protein bands were

quantified using ImageJ software. Two proteins possessing the electrophoretic mobility similar to that of 5S rRNA-binding proteins L18 and L25 were eluted from the gel and identified by mass spectrometry.

RESULTS AND DISCUSSION

Growth of the L5-depleted cells and the ribosome formation

As we have shown earlier, the chromosomal gene *rpLE* can be inactivated only in the presence of a complementing plasmid bearing the *rpLE* gene (32). To study the role of the protein L5 in large ribosomal subunit assembly, we inactivated the chromosomal *rpLE* gene in the presence of the plasmid, allowing arabinose-inducible expression of the *rpLE* gene (strain MS01). Thus, we were able to stop L5

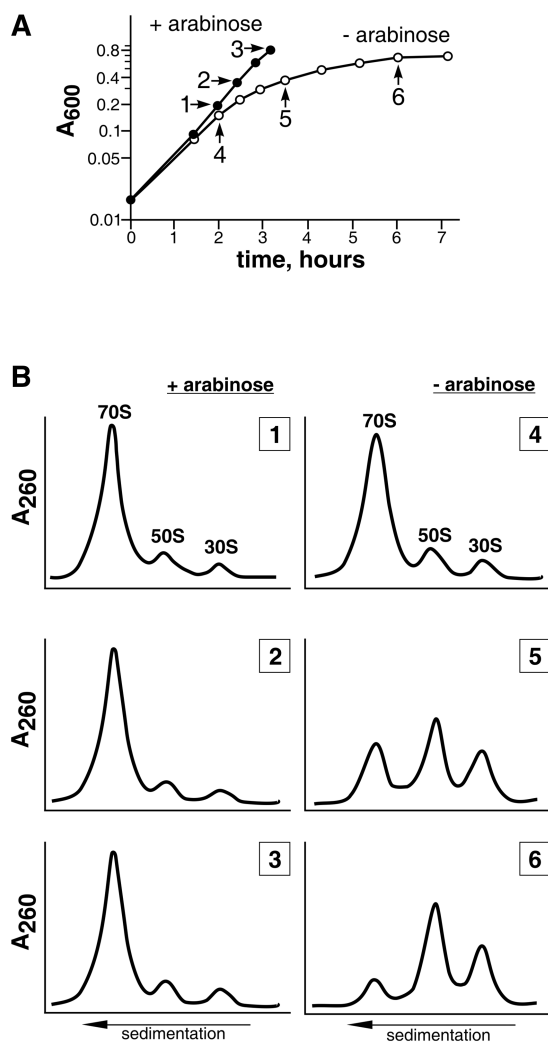


Figure 1. (A) Growth curves of MS01 cells in the medium with (filled circle) or without (unfilled circle) an inducer. Arrows indicate the time points when aliquots were taken for ribosome analysis. The numerals (1–6) correspond to the ribosomal profiles in Section B. (B) Sedimentation analysis of ribosomes from MS01 grown either in the presence (left panel) or absence (right panel) of the inducer. Components of cell cytoplasm were fractionated by centrifugation on a 5–20% (wt/wt) sucrose gradient.

production by removing the inducer from the growth medium. To do this, cells were first grown in a medium with arabinose, then they were spun down and suspended in the medium with the inducer (control cells) or without it (L5-depleted cells). Figure 1A shows, that after removal of arabinose, when synthesis of L5 is stopped, cells start growing linearly and after 6 h the culture reaches a plateau. During this time the cells are only able to divide four to five times. This confirms our previous observation that r-protein L5 is essential for cell survival (32). During the linear growth, cells were harvested at several time points to analyze the ribosomal profile. While ribosomes from the control cells demonstrate a typical sedimentation profile (most of the subunits are associated to form 70S ribosome; Figure 1B, left panel), accumulation of free subunits can be seen in the cells grown without arabinose (Figure 1B, right panel). By the time the L5-depleted culture reaches the plateau, there are only 10–15% associated subunits. The large ribosomal subunits from the control and L5-depleted cells were isolated. The particles from the L5-depleted cells were unable to reassociate with 30S subunits to form the 70S ribosomes. Thus, the large ribosomal subunits assembled in cells in the absence of L5 are not able to form functional 70S ribosomes, which is lethal for the cells.

Properties of large ribosomal subunits and the 5S rRNA–protein complex from L5-depleted cells

The sedimentation coefficient of large ribosomal subunits from the L5-depleted cells is 45S, which is less than that of intact subunits (50S) or even large subunits assembled *in vitro* in the absence of 5S rRNA (47S) (45,46). Such a change could be a result of a decompaction of rRNA or a loss of ~10% (100–150 kDa) of the subunit mass. We therefore analyzed the composition of the 45S particles. 23S rRNA from these particles remains intact (fragmentation of 23S rRNA could affect compactness and shape of the particle). At the same time, as could be expected in view of published data (19,20,45,46) the absence of L5 greatly reduced the ability of 5S rRNA to incorporate into large ribosomal subunits. Only trace amount (<10%) of 5S rRNA can be found in the 45S particles (Figure 2A, lanes 6 and 7). The presence of residual amounts of 5S rRNA and some proteins (see below) in preparations of the 45S particles is probably due to contamination with intact 50S subunits. As opposed to the control preparation (Figure 2A, lane 4), virtually all 5S rRNA is found in the ribosome-free cytoplasmic fraction of the L5-depleted cells (lane 5) in amounts corresponding to the amount of ribosomes. 5S rRNA from this fraction was purified using the affinity chromatography on L5-Sepharose. It was found that 5S rRNA copurifies with two proteins, whose electrophoretic mobility is similar to that of the 5S rRNA-binding proteins, L18 and L25 (Figure 2B). Subsequent mass spectrometry analysis has shown that the two major bands indeed correspond to the r-proteins L18 and L25 of *E. coli*. As a control, quadruple quantities of S_{100} from the control cells were treated with the affinity resin; neither 5S rRNA nor proteins L18 and L25 were detected in the

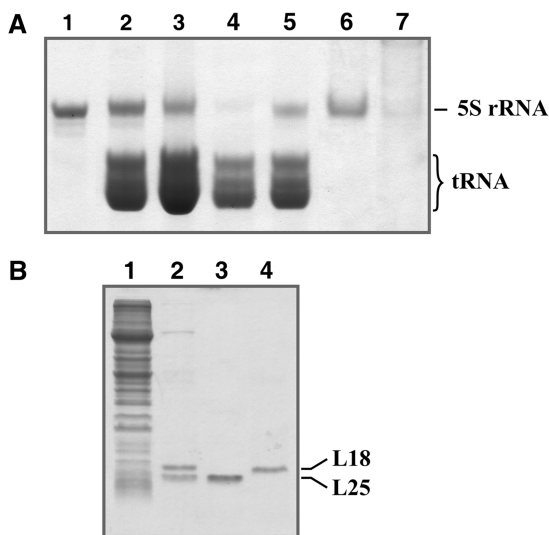


Figure 2. Distribution of 5S rRNA-protein complex components in L5-depleted cells. (A) Electrophoretic analysis of low-molecular weight RNAs (8% polyacrylamide gel, 8 M urea). 1—5S rRNA of *E. coli* (control); 2 and 3— S_{30} fraction from the control and L5-depleted cells, respectively; 4 and 5— S_{100} fraction from the control and L5-depleted cells, respectively; 6 and 7—large ribosomal subunits from the control and L5-depleted cells, respectively. Lanes 2 and 3 contain comparable amounts of the material and so do lanes 4 and 5. Lane 7 is loaded with twice as much material as lane 6. (B) Proteins that copurify with 5S rRNA by affinity chromatography on L5-Sepharose (15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis). 1— S_{100} fraction of the L5-depleted cells; 2—3 M salt wash; 3 and 4—*E. coli* r-proteins L25 and L18, respectively.

high-salt wash. Thus, the components of the 5S rRNA-protein complex that are not incorporated into the ribosome in the absence of protein L5 are found in the cell cytoplasm as a stoichiometric complex. It has been shown previously that L5, L18 and L25 bind specifically to 5S rRNA (47,48), forming the 5S rRNA-protein complex (5S rRNP). These and other data concerning 5S rRNP properties *in vitro* suggest that its formation should precede incorporation into the large ribosomal subunit. Here, we provide evidences that *in vivo* 5S rRNP is formed independently of its association with the large ribosomal subunit, and that such an association is promoted by the r-protein L5.

Participation of protein L5 in formation of the bacterial ribosome central protuberance

The protein composition of the 45S particles was determined using 2D gel electrophoresis with subsequent identification of some proteins by mass spectrometry. As seen in Figure 3B, the 45S particles are deprived almost completely of the 5S rRNA-binding proteins, L5, L18 and L25. The content of protein L16 is reduced to 10–15%. Furthermore, these particles contain only trace amounts (<10%) of several other r-proteins: L27, L31, L33 and L35. The proteins that partially overlap with proteins L27 and L33 on the gel have been identified as L28 and L32, respectively. These proteins (L28 and L32) are preserved in the 45S particles. The protein that migrates slower than L30 (Figure 3A) and which is missing from

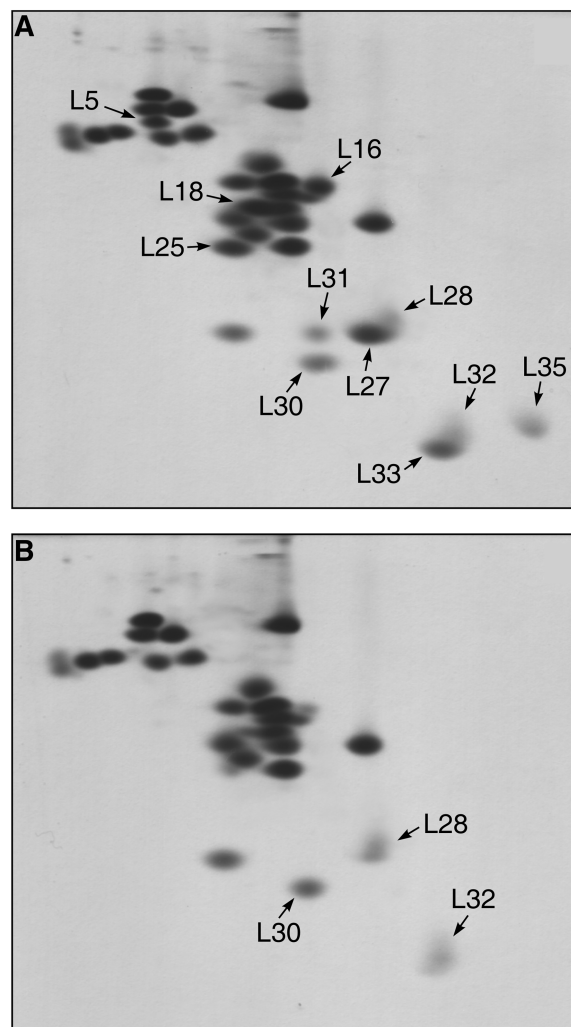


Figure 3. Protein composition of large ribosomal subunits from the control (A) and L5-depleted (B) cells by 2D gel electrophoresis. Arrows mark the positions of r-proteins that are discussed in the text. The gels contain all the proteins of large ribosomal subunits except for the proteins L34 and L36. Protein L34 was found in both control and 45S particles under different separation conditions. Protein L36 was not analyzed.

the 45S particles, has been identified by mass spectrometry as L31. This result is in agreement with the refined data on electrophoretic mobility of the full-length protein L31 (49). Thus, the 45S particles lose 5S rRNA and eight proteins: L5, L16, L18, L25, L27, L31, L33 and L35 (Figure 3B). The total mass of the components absent from the 45S particles in comparison with the intact 50S is ~125 kDa. This correlates well with the difference in the sedimentation coefficients of these particles. According to the recent crystallographic data, the CP of the *E. coli* large ribosomal subunit comprises 5S rRNP, elements of domains II and V of 23S rRNA, and proteins L16, L27, L30, L33 and L35 (10) (Figure 4A). The only protein from this list that remains in the 45S particles is L30. Thus, the neighboring CP components (mentioned above) do not affect the incorporation of L30 into the large ribosomal subunit. In accordance with 50S ribosomal subunit *in vitro* assembly map, the other CP proteins do not influence the

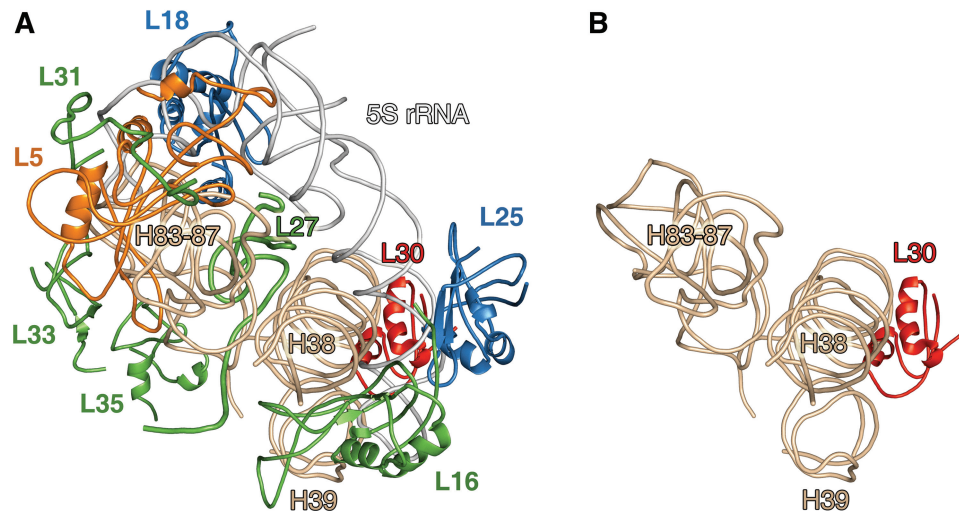


Figure 4. (A) Model of the *E. coli* ribosome CP structure. Position of protein L31 is modeled in the structure of the CP according to its position in the ribosome of *T. thermophilus*. Positions of ribosomal proteins, 5S rRNA and helices of 23S rRNA are indicated. (B) A proposed model for the structure of the CP region of bacterial ribosome formed in the absence of protein L5. The models were generated using the crystal structures of the *E. coli* and *T. thermophilus* ribosomes (PDB entries 2AW4 and 2J01, respectively). Model building and refinement were carried out using the program Coot; the picture was made using PyMol.

incorporation of protein L30 (5), which is consistent with our data. This allows to conclude that interaction of protein L30 with 23S rRNA occurs independently from the large ribosomal subunit CP formation. In the spatial structure of the *Thermus thermophilus* 70S ribosome (12) which was determined at a better resolution than that of the *E. coli* ribosome (10), the CP organization is similar to that of *E. coli* with only one exception. It contains r-protein L31, which is placed outside the CP in the *E. coli* ribosome model. It was suggested that L31 in the *E. coli* ribosome structure was confused with r-protein L28 due to insufficient resolution (12). Our data indicate that in the *E. coli* ribosome, protein L31 is most probably situated in the CP.

Taken together, crystallographic data and our results suggest that bacterial large ribosomal subunits assembled *in vivo* in the absence of protein L5 lose 5S rRNP and all proteins (except L30) of the CP (Figure 4B). Thus, r-protein L5 affects the formation and (or) stability of the entire structural domain of the ribosome in the bacterial cell. In early experiments it was demonstrated that large ribosomal subunits assembled *in vitro* in the absence of 5S rRNA are functionally inactive (45,50). These subunits contained all the r-proteins except for L5, L16, L18 and L25 (45,46,51). Differences in composition of these ribosomal subunits, reconstructed *in vitro*, and those obtained in the present work can be explained by different assembly conditions. Reconstitution of the 50S ribosomal subunits requires the conditions (high salt concentrations and prolonged incubation at elevated temperatures) that are far from physiological environment (3,5,17). Thereby, the results of the present work contribute significantly to a better understanding of the large ribosomal subunit assembly in bacteria. On the base of our data along with the data on structural organization of the CP (8,10,12,28,46,52) we suggest a model for the assembly of this 50S subunit structural domain in the

bacterial cell. Incorporation of the preformed 5S rRNP into the large ribosomal subunit takes place at the beginning of CP formation. According to our results, only r-protein L30 (of the CP proteins) may be already present in the subunit during this stage (Figure 4B). The position of the 5S rRNP in the ribosome is mainly determined by two conserved contacts with 23S rRNA (8,10,12). Firstly, 5S rRNA interacts directly with helix 38 of 23S rRNA domain II (A-site finger, ASF). Secondly, 5S rRNP contacts helices H83–H85 of 23S rRNA domain V through proteins L5 and L18. These intermolecular contacts probably induce local rearrangements in 23S rRNA that, in turn, allow incorporation of the other CP proteins. This model is in accordance with the data on protein composition of an early large ribosomal subunit precursor in *E. coli* (53). It was shown that these particles (p_150S) contain 5S rRNP but do not carry many r-proteins, *inter alia* L16, L31 and L33 (protein L35 was not analyzed).

Cooperativity and independence of the CP assembly suggests that it appears as a single structural unit not only during the ribosome assembly but also in ribosome functioning. On the basis of intermolecular cross-links in the ribosome, 5S rRNA was proposed to be a mediator between several ribosomal functional centers (31). According to the recent data on the ribosome structural organization (9–13), the CP components interact with each other, contacting with more than one component simultaneously (Figure 4A). Thereby, they form a complex self-contained system within the 50S subunit, where changes in one component can be transmitted to the others. Most of the components contact directly 5S rRNP, what explains its importance for CP formation and suggests that it plays a key role in CP function. At the same time, CP components contact directly tRNAs in all the three ribosomal sites (L16 and ASF in A-site, L5 in P-site and L33 in E-site); L27 protrudes into the 50S

subunit, reaching the peptidyltransferase center; L25 is situated in close vicinity of the GTPase-associated center. In view of this, we propose that the CP (as a whole) serves as a coordinator of the functioning of ribosomal active centers.

Considering the fact that 45S particles are not able to associate with 30S subunits a few words should be said about the role of the CP in the subunit association. As shown by recent crystallographic data, the ASF and the protein L5 form intersubunit bridges 1a and 1b, respectively, interacting with protein S13 of the small ribosomal subunit (8,10,12). Moreover, it has recently been shown that a knockout of the S13 chromosomal gene (*rpsM*) as well as certain mutations in the S13 or ASF, impair association of ribosomal subunits (54,55). Protein L5 is absent from the 45S particles, thereby, excluding the formation of bridge 1b. However, earlier it was demonstrated that large ribosomal subunits lacking 5S rRNP (including protein L5) but containing the other proteins of the CP were not completely incapable of associating with the small ribosomal subunit (45,46). The position of ASF, forming bridge 1a, in the ribosomal subunit is stabilized by the contacts with 5S rRNA and proteins L16, L27 and L30 (Figure 4A). One can suggest that the absence of most of the CP components in the 45S particles destabilizes position of the ASF and, as a consequence, impedes the formation of bridge 1a. Thereby, according to our data and results of (54) it seems that together these bridges contribute significantly to association of the ribosomal subunits.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1.

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