ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 2, pp. 156-163. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © A. V. Korobeinikova, G. M. Gongadze, A. P. Korepanov, B. D. Eliseev, M. V. Bazhenova, M. B. Garber, 2008, published in Biokhimiya, 2008, Vol. 73, No. 2, pp. 193-201.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-216, December 16, 2007.

5S rRNA-Recognition Module of CTC Family Proteins and Its Evolution

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Received June 27, 2007
Revision received September 28, 2007

Abstract—The effects of amino acid replacements in the RNA-binding sites of homologous ribosomal proteins TL5 and L25 (members of the CTC family) on ability of these proteins to form stable complexes with ribosomal 5S RNA were studied. It was shown that even three simultaneous replacements of non-conserved amino acid residues by alanine in the RNA-binding site of TL5 did not result in noticeable decrease in stability of the TL5–5S rRNA complex. However, any replacement among five conserved residues in the RNA-binding site of TL5, as well as of L25 resulted in serious destabilization or complete impossibility of complex formation. These five residues form an RNA-recognition module in TL5 and L25. These residues are strictly conserved in proteins of the CTC family. However, there are several cases of natural replacements of these residues in TL5 and L25 homologs in Bacilli and Cyanobacteria, which are accompanied by certain changes in the CTC-binding site of 5S rRNAs of the corresponding organisms. CTC proteins and specific fragments of 5S rRNA of *Enterococcus faecalis* and *Nostoc* sp. were isolated, and their ability to form specific complexes was tested. It was found that these proteins formed specific complexes only with 5S rRNA of the same organism. This is an example of coevolution of the structures of two interacting macromolecules.

DOI: 10.1134/S0006297908020065

Key words: bacterial 5S rRNA-binding proteins, CTC family proteins, ribosome

Earlier we discovered that ribosomal proteins L25 *Escherichia coli* and TL5 *Thermus thermophilus* are homologous to the general stress protein CTC *Bacillus subtilis* [1]. Then all known homologs of CTC *B. subtilis* were combined in the so-called CTC family [2]. Analyzing genomes from data banks [3, 4], we found that genes of CTC family proteins are present only in bacterial genomes. Proteins of this family such as L25 *E. coli*, TL5 *T. thermophilus*, and CTC *Deinococcus radiodurans* can be considered as true ribosomal proteins, because they were found in ribosomes of exponentially growing cells [5-7]. These proteins specifically bind to conserved site of 5S rRNA, so-called E-loop [7-9]. However, it was shown that some proteins of this family, namely CTC *B. subtilis* and *Listeria monocytogenes*, are produced by cells only under stress conditions [10-12]. The general stress protein CTC *B. subtilis* is able to specifically bind to 5S rRNA and is detected in the ribosomal fraction under various unfavorable cell growth conditions [13, 14]. This protein can be considered as only temporarily associated with ribosome. At the same time, the gene of CTC protein is absent from genomes of members of Streptococcaceae and Mycoplasmataceae bacterial families [3, 4]. Among CTC proteins, there are one-, two-, and even three-domain ones [2, 7, 15], and they all have a domain homologous to 5S rRNA-binding one-domain ribosomal protein L25 *E. coli*. The biological function of proteins of this family is unknown. Nonetheless, all of the known CTC proteins have a common feature: they are able to specifically interact with a certain site of ribosomal 5S RNA.

We discovered a two-domain ribosomal protein, TL5 *T. thermophilus*, and studied it in detail [1, 2, 6, 9, 16-18]. It was shown that an isolated N-terminal domain of TL5 (NfrTL5) protein possesses the same 5S rRNA-binding capacity as the full-sized protein [9]. Spatial structures of L25 *E. coli* and TL5 *T. thermophilus* proteins complexed with the same fragment of 5S rRNA *E. coli* were determined with high resolution [2, 19]. It was shown that in spite of low homology of primary structures of L25 and

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NfrTL5 proteins (18% identical residues), their spatial structures and modes of interaction with RNA are rather similar [2]. Amino acid residues of these proteins forming hydrogen bonds with RNA may be divided into two groups: nonconserved and conserved [18]. Residues of the first group (K14, S16, R20, R31, N34, R72, Q73, and N75 in TL5) are positioned peripherally on the protein surface contacting with RNA and form water-accessible intermolecular hydrogen bonds (Fig. 1). Residues of the second group (R10, R19, Y29, H85, and D87 in TL5) are positioned in the central part of the protein surface contacting with RNA and form water-inaccessible intermolecular hydrogen bonds.

Earlier we showed [18] that replacements of any residues of the second group in TL5 protein result in significant destabilization of RNA–protein complex or in impossibility of its formation. It was also shown that individual replacements of the first group residues in TL5 by alanine did not influence RNA-binding properties of the protein. The data indicate that water-inaccessible hydrogen bonds formed by conserved residues of TL5 and RNA are the main factor in intermolecular recognition. Cases of natural replacements of the second group residues in CTC proteins with simultaneous changes in E-loop nucleotides of 5S rRNA observed in Bacilli and Cyanobacteria suggest coevolution of the structures of these macromolecules.

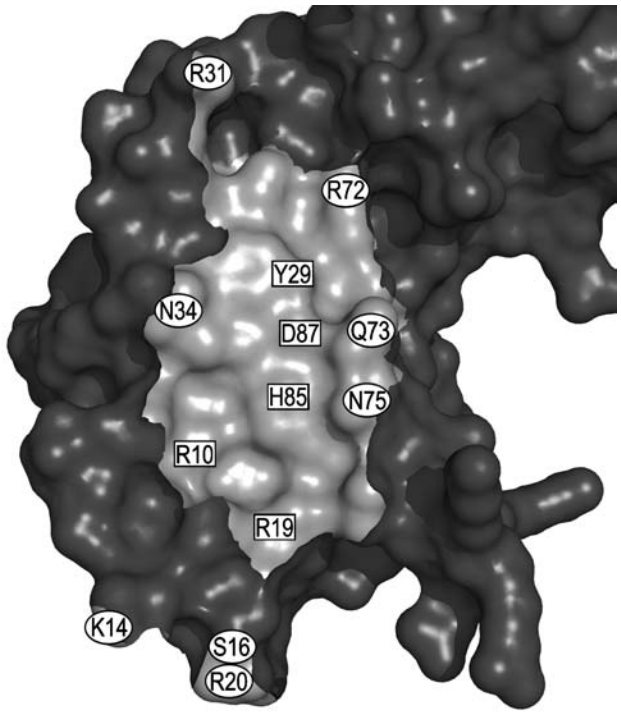

Fig. 1. Contact area of TL5 protein with 5S rRNA (gray colored on protein surface). Positions of conserved and nonconserved amino acid residues forming hydrogen bonds with RNA are marked by rectangles and ovals, respectively.

In this work, we analyzed in detail the contribution of amino acid residues of both groups in TL5 protein and contribution of the second group residues in L25 protein in formation of corresponding RNA–protein complexes. It is shown that in contrast to replacements of the second group residues, individual replacements of any first group residue of TL5 protein (excluding N34) and even simultaneous replacements of two or three residues of this group by alanine do not significantly influence RNA-binding properties of the protein. We also studied the contribution of intermolecular electrostatic interactions in formation and stability of TL5–5S rRNA complex. It is shown that electrostatic interactions do not contribute significantly in stability of the TL5–5S rRNA complex. We obtained stable homologous complexes of specific fragments of 5S rRNA with CTC *Enterococcus faecalis* and *Nostoc* sp. (representatives of Bacilli and Cyanobacteria, respectively). The data confirm our suggestion that simultaneous structural changes in CTC proteins and 5S rRNA of these organisms in the course of evolution are directed towards retention of the ability of these molecules to form stable complexes.

MATERIALS AND METHODS

Cloning of genes of recombinant proteins. Cloning a gene fragment of TL5 *T. thermophilus* (*ntl5*) encoding its N-terminal fragment (NfrTL5) as well as its certain mutant forms is described in [18]. Mutant *ntl5* were amplified by polymerase chain reaction (PCR) in two stages. On incorporation of individual replacements, pET11c/*ntl5* plasmid was used as a PCR template. On incorporation of double and triple replacements, plasmids encoding mutant NfrTL5 with one or two corresponding replacements were used. In the first stage, we obtained a small *ntl5* fragment (5′- or 3′-terminal depending on mutation position) using specific primers, one of which contained the necessary replacements. In the second stage, we obtained a full-sized *ntl5* bearing the desired mutations. If the mutation was at the beginning of the gene, the *ntl5* fragment obtained in the first stage was used as forward primer and T7Rev (5′-GCTAGTTATTGCTCAGCGG-3′) as reverse primer. If the mutation was at the end of the gene, T7For (5′-TAATACGACTCACTATAGGG-3′) was used as forward primer and the *ntl5* fragment obtained in the first stage was used as reverse primer. Mutant *ntl5* were cloned in pET11c vector (Novagen, USA) via *Nde* I and *Bam*H I sites. To obtain constructions encoding mutant forms of L25 *E. coli*, we followed QuickChange® Site-Directed Mutagenesis Kit protocol (Stratagene, USA). Plasmid pKAB101 [20] bearing L25 gene was used as a template in PCR. Genes of CTC *E. faecalis* and *Nostoc* sp. proteins were amplified by PCR from chromosomal DNA and cloned in pET11c vector via *Nde* I and *Bam*H I sites. 5′-GAATTAGGTTCATATGTCAGTACAATTA-

GAAGT-3′ was used as a forward primer for CTC *E. faecalis* gene and 5′-CTAAACCCATATGGCTCTGACAG-TCGAAC-3′ for CTC *Nostoc* sp. gene. 5′-GTGGGATC-CTTATTCGGCTAATTCTGGTTC-3′ was used as reverse primer for CTC *E. faecalis* gene and 5′-TGGGATCCCTTTAGCCTTTGGCAGTAGC-3′ for CTC *Nostoc* sp. gene.

Preparation of recombinant proteins in *E. coli* cells and their purification. Genes encoding L25 mutant forms were expressed in *E. coli* cells, KNB800 strain [20]. Cells were grown in 1 liter of LB medium containing 50 μg/ml kanamycin at 37°C to OD_{590} = 2.0-2.5. The Studier expression system [21] was used for expression of genes of other recombinant proteins. *Escherichia coli* cells, BL21(DE3) strain (Novagen), were transformed with corresponding plasmids. Cells were grown in 1 liter of LB medium containing 100 μg/ml ampicillin at 37°C to OD_{590} = 0.5-0.7. Expression of recombinant genes was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to the final concentration 1 mM. After induction, cells were grown for 3 h. Then the cells were precipitated by centrifugation and stored at –70°C. NfrTL5 and its mutant forms were purified as described earlier [18]. To isolate CTC *E. faecalis* or *Nostoc* sp. proteins, cells were suspended in 50 mM Tris-HCl, pH 7.5, containing 0.8 M NaCl, 20 mM $MgCl_2$, 0.5 mM EDTA, 5 mM β-mercaptoethanol and disrupted with ultrasonic treatment using a Sonic Dismembrator 550 from Fisher Scientific (USA). Then cell debris and ribosomes were sequentially precipitated by centrifugation. The supernatant was diluted 10-fold with 50 mM Tris-HCl, pH 7.5, containing 1.5 M $(NH_4)_2SO_4$ and subject to hydrophobic chromatography on Butyl-Sepharose FF from Pharmacia (Sweden). Protein was eluted by a gradient of $(NH_4)_2SO_4$ concentration from 1.5 to 0 M. Anion-exchange chromatography on DEAE-Sepharose FF from Pharmacia and cation-exchange chromatography on CM-Sepharose FF from Pharmacia were used for additional purification of CTC *E. faecalis* and CTC *Nostoc* sp. proteins, respectively. L25 *E. coli* protein was isolated as described in [22] but with some modifications. Cells were suspended in 20 mM Tris-HCl, pH 7.5, containing 200 mM NH_4Cl, 100 mM $MgCl_2$, 0.2 mM EDTA, 3 mM β-mercaptoethanol, two volumes of ice-cold acetic acid were added, and the mixture was stirred for 1 h at 4°C. Insoluble fraction was removed by centrifugation, and 4.5 volumes of acetone were added to the supernatant. The pellet was precipitated by centrifugation, dissolved in 70 mM sodium acetate buffer, pH 5.6, containing 6 M urea, 3 mM β-mercaptoethanol, 0.5 mM EDTA and chromatographed on CM-Sepharose FF from Pharmacia. Protein was eluted by a gradient of sodium acetate concentration from 100 to 300 mM. Protein-containing fractions were transferred by dialysis in 10 mM Tris-HCl, pH 7.5, containing 200 mM NH_4Cl, 0.2 mM EDTA, 1.2 M $(NH_4)_2SO_4$ and then chromatographed on Butyl-Toyopearl 650S from Toyo-Soda (Japan). Purity of protein preparations was evaluated by SDS-PAGE [23].

Isolation of 5S rRNA *E. coli*, preparation of 5S rRNA fragments, and incorporation of radioactive label into the 5′-end of RNA. 5S rRNA was isolated from *E. coli* cells as described earlier [24]. Gene fragments of 5S rRNA *E. coli*, *E. faecalis*, or *Nostoc* sp. were amplified by PCR and cloned into pUC18 vector. To obtain 5S rRNA *E. coli* gene fragments, forward 5′-CGCGGATCCTAATAC-GACTCACTATAGCCGATGGTAGTGTGGG-3′ and reverse 5′-CCTTAAGCATATGGGCGCCTACTCTCG-CATGGGGAGACC-3′ primers were used. To obtain 5S rRNA *E. faecalis* gene fragment, forward 5′-CGCG-GATCCTAATACGACTCACTATAGCCGATTGTAGT-GAAGGGTTTCCCT-3′ and reverse 5′-CCTTAAG-CATATGGGCGCCTACTCTCACAAAGGGAA-3′ primers were used. To obtain 5S rRNA *Nostoc* sp. gene fragment, forward 5′-GAAAAGCTTAATACGACTCAC-TATAGCGGCTACGATAGTCTAG-3′ and reverse 5′-ACCCCTAGGTGCGCAGCGATTGTGGCATAG-3′ primers were used. The resulting plasmids were linearized via the *Ege* I site in cases of 5S rRNA *E. coli* and *E. faecalis* gene fragments and via *Acc*16 I site in case of 5S rRNA *Nostoc* sp. gene fragment. 5S rRNA fragments were obtained by transcription *in vitro* using phage T7 RNA polymerase from corresponding linearized plasmids. Phage T4 polynucleotide kinase was used for the 5′-terminal labeling of RNA as described in [25]. RNA fragments after transcription and labeled RNA were purified by electrophoresis in polyacrylamide gel in the presence of 8 M urea.

Formation and analysis of RNA–protein complexes. Formation of RNA–protein complexes was monitored via change in RNA mobility on electrophoresis in polyacrylamide gel as described in [17, 18]. RNA and proteins were dialyzed in 20 mM Tris-HCl, pH 7.5, containing 170 mM KCl and 10 mM $MgCl_2$. 5S rRNA or its fragments were heated for 5 min at 60 or 75°C, respectively, and cooled to room temperature. RNA samples (2-4 μg) were incubated with corresponding amount of protein for 10 min at 37°C. The resulting complexes were analyzed by electrophoresis in 12% polyacrylamide gel using 90 mM Tris-acetate, pH 7.8, containing 10 mM $MgCl_2$ as electrode buffer. The dissociation constants of RNA–protein complexes were determined by sorption on nitrocellulose filters as described in [18, 26]. The data were used for plotting curves of the labeled RNA inclusion into the complex versus protein concentration in the incubation mixture. The apparent dissociation constant (K_d) was determined as the protein concentration required for 50% saturation.

RESULTS AND DISCUSSION

Peripheral and central regions of TL5 protein surface contacting with RNA are formed by two groups of amino

acid residues interacting with 5S rRNA: nonconserved and conserved (Fig. 1). Intermolecular hydrogen bonds formed by amino acid residues of the first group are accessible for solvent molecules [18]. Disruption of such RNA–protein hydrogen bonds is accompanied by their replacement for hydrogen bonds with solvent molecules; this does not result in uncompensated energy loss and does not affect stability of the RNA–protein complex. It was noted that interacting surfaces of L25 and 5S rRNA have regions with complementary positive and negative electrostatic potential [22]. The data suggest that intermolecular electrostatic interactions are crucial for formation of this specific RNA–protein complex. The presence of corresponding positively and negatively charged regions was detected on contacting surfaces of TL5 protein and 5S rRNA [2]. This type of intermolecular interaction is sensitive to concentration of ions in solution [27-29], so we used various KCl concentrations to evaluate how significant electrostatic interactions are for stability of the TL5–5S rRNA complex. Mutant forms of the N-terminal fragment of TL5 (NfrTL5) and L25 proteins were isolated from *E. coli* strain producers and purified as described earlier [18, 22]. 5S rRNA-binding properties of mutant forms of the abovementioned proteins were studied; the data are presented in the table.

As shown in our experiments, replacement of any amino acid residue of the first group (excluding N34) with alanine does not affect formation and stability of

5S rRNA-binding properties of mutant forms of L25 and NfrTL5 proteins

	Mutation	RNA binding[a]	K_d, μM[b]	Reference
Control	NfrTL5 wt	++	0.08 (0.10)[d]	this work
Replacements in the first group	K14A; S16A; R20A	++	0.13 (0.15)	[18]
	S16A/R20A	++	0.15	«
	K14A/Q73A	++	0.16 (0.25)	this work
	K14A/S16A/R20A	++	0.18 (0.25)	«
	S16A/R20A/R72A	++	0.18 (0.22)	«
	R31A	++	0.12	«
	N34A	++ [c]	0.60	«
	R72A	++	0.11	«
	Q73A	++	0.13	«
	N75A	++	0.11	«
Replacements in the second group	R10A; R19A; H85A	+ [c]	~1.30 (~5.00)	[18]
	D87E	+/– [c]	2.50	«
	H85F	–	NB[e]	«
	Y29F; D87S; D87N	–	NB	«
	R10A/R19A	–	NB	this work
Simultaneous replacements in the first and second groups	R19A/K14A	+/–	NB	this work
	R19A/R20A	+/–	NB	«
	R19A/Q73A	+/– [c]	~4.00 (NB)	«
	H85A/K14A	+/– [c]	~5.00 (NB)	«
Control	L25 wt	++	0.1	this work
Replacements in the second group	H88F	–	NB	this work
	Y31A	–	NB	«
	D90A	–	NB	«

Note: Symbol ";" is used when listing various mutant proteins, and "/" is used when listing mutations in one protein.

[a] Binding of these proteins to 5S rRNA was evaluated via change in RNA mobility on electrophoresis in polyacrylamide gel.

[b] Apparent dissociation constants (K_d) were determined by sorption of RNA–protein complexes on nitrocellulose filters (see "Materials and Methods").

[c] A band of this RNA–protein complex has free RNA tailing.

[d] K_d values obtained in experiments in solution with high ionic strength (500 mM KCl) are given in parentheses.

[e] NB, RNA binding is not detected at protein concentrations to 5.0 μM.

TL5–5S rRNA complex (table and Fig. 2). Even simultaneous replacement of two or three residues, which prevents formation of almost half of the intermolecular hydrogen bonds formed by residues of this group, had practically no effect on the RNA-binding properties of the protein. Increased ionic strength of solution (to 0.5 M KCl), which usually decreases electrostatic interactions between macromolecules significantly, did not markedly effect stability of the 5S rRNA complex with intact protein and its mutant forms mentioned above (table). Simultaneous exclusion of intermolecular electrostatic contacts and most peripheral hydrogen bonds was accompanied by only 2-3-fold change in K_d. At the same time, single replacement of a second-group residue (Fig. 1) in TL5 protein resulted in, at least, over 10-fold K_d increase (table). So, the data indicate that intermolecular hydrogen bonds formed by the first-group residues as well as electrostatic interactions between protein and RNA have almost no effect on formation of specific TL5–5S rRNA complex and its stability. Using the structure of 70S *Thermus thermophilus* ribosome [30], we analyzed intermolecular contacts of TL5 and 5S rRNA in the ribosome. It was found that the number of hydrogen bonds formed by the first-group residues is significantly (3 times) less than in the isolated RNA–protein complex. However, the central region of RNA–protein contact area did not change. These data support the conclusion that solvent-inaccessible hydrogen bonds formed by conserved amino acid residues with RNA play a key role in RNA–protein recognition.

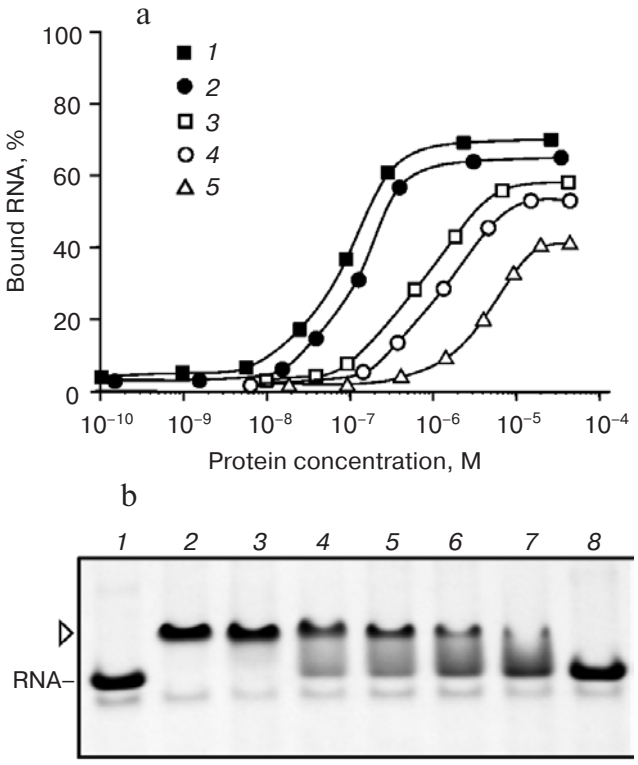

Fig. 2. 5S rRNA-binding properties of NfrTL5 and some of its mutant forms. a) Binding of ^{32}P-labeled 5S rRNA *E. coli* versus protein concentration. Curves: *1*) NfrTL5, 170 mM KCl; *2*) NfrTL5 S16A/R20A/R72A, 170 mM KCl; *3*) NfrTL5 N34A, 170 mM KCl; *4*) NfrTL5 R19A, 170 mM KCl; *5*) NfrTL5 R19A, 500 mM KCl. b) Electrophoregram of complexes of some NfrTL5 mutant forms with RNA (12% polyacrylamide gel, non-denaturing conditions). Lanes: *1*) 5S rRNA *E. coli*; *2*) + wild type NfrTL5 (1 : 1); *3*) + Nf TL5 S16A/R20A/R72A (1 : 1); *4*) + NfrTL5 N34A (1 : 3); *5*) + Nf TL5 R19A (1 : 3); *6*) + NfrTL5 R19A/Q73A (1 : 3); *7*) + NfrTL5 R19A/R20A (1 : 3); *8*) + NfrTL5 R10A/R19A (1 : 3). Molar ratios RNA/protein in the incubation mixture are given in parentheses. Position of RNA–protein complex is marked by an arrow.

N34 occupies a specific place among the first-group residues of TL5 protein. The effect of replacement of this residue in the protein is closer to that observed on replacement of the second-group residues (table and Fig. 2, lanes *4* and *5*). Not only comparable K_d increase (8- and 15-fold) is observed in these cases, but also free RNA tailing lower than the complex band; this tailing indicates that the complex dissociates during electrophoresis. The data indicate that this contact is important for stability of the RNA–protein complex. In L25 protein, this position is occupied by proline [19], which does not form hydrogen bonds with RNA (only weak van der Waals interactions were found between A73 5S rRNA and this proline residue). In more than a half of the known CTC family proteins, this position is occupied by proline [3, 4]. The fact that conformation of this part of polypeptide chain in TL5 and L25 proteins is almost identical [2] indicates that asparagine in this position in TL5 is an evolutionary gain increasing stability of RNA–protein complex in extremely thermophilic organisms. Replacement of this asparagine residue with alanine in TL5 results, on one hand, in the impossibility of formation of RNA–protein hydrogen bonds. On the other hand, this replacement may to a certain extent change conformation of the part of polypeptide chain that is in the immediate neighborhood of RNA–protein contact area inaccessible for solvent in wild type proteins. Such conformational change may cause decrease in stability of this RNA–protein complex.

Earlier we showed that amino acid residues of the second group crucially contribute to stability of specific TL5–5S rRNA complex (table). Single replacement of any residues of this group led either to drastic destabilization of RNA–protein complex or to impossibility of its formation [18]. In this work, we obtained identical results on the effect of replacements of the second-group residues on formation of complex with 5S rRNA for another CTC protein, ribosomal L25 *E. coli* (table). Accounting that amino acid residues of the second group in CTC proteins (more than 90%) and nucleotides in 5S rRNA E-loop (Fig. 3) are highly conserved, we suggest that solvent-inaccessible contact area of these two molecules did not change in the vast number of known bacteria in the course of evolution. The data indicate that these

five amino acid residues of the second group form an RNA-recognition module in proteins of this family. As shown earlier (table), single replacements of two residues of this RNA-recognition module, Y29 and D87, effect most significantly the ability of TL5 to interact with RNA. Any replacement of these two residues results in complete loss of RNA-binding properties of the protein. This drastic effect may be caused by the fact that unique orientation of side groups of these two residues on the protein surface is stabilized by a hydrogen bond between them [2]. Replacement of one of these residues seems to result in increased mobility of the side group of the second residue, and this may exclude possible formation of solvent-inaccessible hydrogen bonds with RNA with two residues at once. Although single replacements of any of the other three residues of the RNA-recognition module (R10, R19, or H85 in TL5 protein) significantly destabilize the complex (K_d increases more than 10 times), but do not completely cancel the ability of the protein to bind to 5S rRNA (Fig. 2, lane *5*). However, simultaneous replacement of these two residues in TL5 protein results in complete loss of its 5S rRNA-binding capacity (Fig. 2, lane *8* and table). Thus, we conclude that replacement of one of the residues forming solvent-inaccessible hydrogen bonds with RNA results in destabilization of the TL5–5S rRNA complex, and two such replacements completely cancel the ability of the protein to form a stable complex with RNA. Replacements of nonconserved residues forming hydrogen bonds with RNA (belonging to the first group) in addition to single replacements of R10, R19, or H85 residues also result in complete loss of RNA-binding capacity of the protein. As shown in the table and Fig. 2, a complex with single replacement of R10, R19, and H85 residues is at the boundary of stability. Additional replacement of the first-group residue in such protein (Fig. 2, lanes *6* and *7*) or shielding of intermolecular electrostatic interactions (increase in the ionic strength of solution) makes the formation of the complex impossible. These actions do not influence RNA binding to intact proteins, but markedly influence a protein with a damaged RNA-recognition module. The data can be rationalized by the effect of increased accessibility of the intermolecular contact area for solvent molecules. Thus, the function of the external contact area of two molecules (hydrogen bonds formed by the first-group residues and electrostatic interactions) seems to consist in protection of the internal contact area from the action of solvent. The data completely support our suggestion that the five conserved amino acid residues forming the central contact area inaccessible for solvent molecules play a key role in RNA recognition and formation of a stable complex.

As noted above, five residues of TL5 RNA-recognition module are strictly conserved among CTC family proteins. The nucleotide sequence of the E-loop with which proteins of this family interact is also highly conserved in most bacterial 5S rRNAs [31]. The data indicate

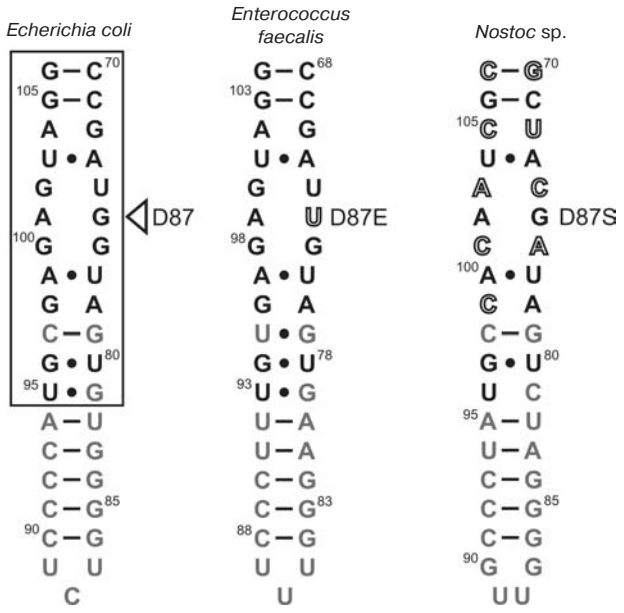

Fig. 3. Schemes of the secondary structure of bacterial 5S rRNA fragments (E-loop region) used in this work. The contact area of L25 and TL5 proteins with RNA is in a rectangular frame. Conserved nucleotides (>80% in bacterial 5S rRNA) are marked with black symbols and nonconserved nucleotides by gray symbols. Changes in conserved nucleotides are shown by open symbols. The nucleotide interacting with residue D87 of TL5 protein is shown by an arrow. Replacements of conserved aspartate in corresponding CTC proteins are shown to the right of the RNA.

that interaction between 5S rRNA and most CTC proteins is realized similarly to that in TL5–5S rRNA complex.

However, on analyzing the primary structures of more than 300 CTC proteins we found several exceptions: one of five residues of RNA-recognition module of protein was replaced. In several Bacilli representatives (e.g. *Enterococcus faecalis*), an asparagine residue was replaced by a glutamine residue (E90). It appeared that there is only one change in the E-loop of 5S rRNA of this organism (Fig. 3): G75 whose nitrogen base interacts with conserved aspartate in L25–RNA and TL5–RNA complexes is replaced with uracil. We suggested that this case is an example of coevolution of two interacting macromolecules [18]. In this work, we tested this suggestion: genes of CTC proteins were cloned from chromosome DNA of corresponding microorganisms and expressed in *E. coli* strain producer. Recombinant proteins were purified as described earlier [9, 18]. Fragments of 5S rRNA containing the E-loop were prepared by transcription *in vitro* and purified by electrophoresis in polyacrylamide gel under denaturing conditions.

It was found that CTC *E. faecalis* protein forms a stable complex with 5S rRNA of the same organism (Fig. 4, lane *5*) but not with 5S rRNA *E. coli* (data not presented here). The K_d of this complex was ~0.1 μM. This value is

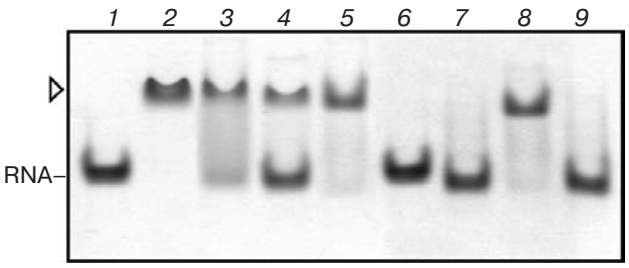

Fig. 4. 5S rRNA-binding properties of CTC family proteins from *Enterococcus faecalis* and *Nostoc* sp. (electrophoresis in 12% polyacrylamide gel, nondenaturing conditions). Lanes: *1*) 5S rRNA *E. coli* fragment; *2*) 5S rRNA *E. coli* fragment + wild type NfrTL5 (1 : 1); *3*) 5S rRNA *E. coli* fragment + NfrTL5 D87E (1 : 3); *4*) 5S rRNA *E. faecalis* fragment + NfrTL5 D87E (1 : 1); *5*) 5S rRNA *E. faecalis* fragment + CTC *E. faecalis* protein (1 : 1); *6*) 5S rRNA *E. coli* fragment + NfrTL5 D87S (1 : 2); *7*) 5S rRNA *Nostoc* sp. fragment + NfrTL5 D87S (1 : 2); *8*) 5S rRNA *Nostoc* sp. fragment + CTC *Nostoc* sp. protein (1 : 1); *9*) 5S rRNA *Nostoc* sp. fragment + wild type NfrTL5 (1 : 2). Designations are as in Fig. 2b.

very close to those for specific complexes of 5S rRNA with ribosomal proteins TL5 and L25 (table). The data support our suggestion about coevolution of interacting macromolecules.

We tried to check the effect of particularly single replacements of conserved residues in interacting regions of CTC protein (D/E) and 5S rRNA (G/U) on formation of a stable complex. For this, we compared the capacity of mutant TL5 protein to form a complex with specific fragments of 5S rRNA *E. coli* and *E. faecalis*. The only difference between these RNA fragments is replacement of G75 for U (Fig. 3). As shown in Fig. 4, NfrTL5 with replacement D87E forms a rather unstable complex with 5S rRNA *E. coli* fragment (lane *3*). This is supported by free RNA tailing on electrophoresis and more than 30-fold K_d increase (table). The same mutant protein forms a more stable hybrid complex with corresponding fragment of 5S rRNA *E. faecalis* (Fig. 4, lane *4*). RNA is quantitatively bound to the protein when the protein is in excess in the incubation mixture. The dissociation constant of this hybrid complex (0.45 μM) negligibly differs from K_d of homologous *E. coli* (table) and *E. faecalis* complexes. Thus, the data support our suggestion that these simultaneous replacements are compensatory.

Another case of natural replacement of conserved aspartate for serine or alanine was detected in cyanobacterial CTC proteins [18]. Such a replacement in TL5 or L25 proteins resulted in loss of 5S rRNA-binding capacity (table). However, we found that simultaneously with changes in RNA-recognition module of cyanobacterial CTC protein, numerous changes occurred in the E-loop of 5S rRNA of these organisms (Fig. 3). It appeared that NfrTL5 with replacement D87S does not form a complex with 5S rRNA *Nostoc* sp. fragment (Fig. 4, lane *7*) as well as with 5S rRNA *E. coli* (table and Fig. 4, lane *6*) fragment. Both NfrTL5 (Fig. 4, lane *9*) and L25 proteins (data not presented here) do not form complexes with 5S rRNA *Nostoc* sp. fragment. Only *Nostoc* sp. CTC protein forms a stable complex with RNA of the same organism (Fig. 4, lane *8*). The dissociation constant of *Nostoc* sp. RNA–protein complex (K_d = 0.1 μM) is comparable with the K_d values of *T. thermophilus* TL5–5S rRNA and *E. coli* L25–5S rRNA complexes. Thus, the data indicate that parallel changes in CTC protein and 5S rRNA *Nostoc* sp. are aimed at retention of specific RNA–protein complex. Accounting that all known CTC proteins and 5S rRNA Cyanobacteria have similar changes in the considered RNA–protein contact area, we suggest that the discovered changes were evolutionarily strengthened in this group of microorganisms.

Two main conclusions can be formulated. Firstly, 5S rRNA-recognition module formed by five strictly conserved amino acid residues of TL5 and L25 proteins forming solvent-inaccessible hydrogen bonds with RNA is typical of most CTC proteins. Secondly, simultaneous changes in contact area of CTC proteins and 5S rRNA, which occurred in these molecules in the course of evolution of some groups of bacteria, are aimed toward retention of their ability to form a stable complex.

The authors are grateful to S. V. Nikonov for valuable comments on the manuscript.

This work was financially supported by the Russian Academy of Sciences, the Russian Foundation for Basic Research, and the Council at the Russian Federation President (Grant for outstanding scientific schools 4913.2006.4). The research of M. B. Garber was supported by an International Research Scholar's award from the Howard Hughes Medical Institute (HHMI 55005609).

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