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General Stress Protein CTC from *Bacillus subtilis* Specifically Binds to Ribosomal 5S RNA

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Abstract—Two recombinant proteins of the CTC family were prepared: the general stress protein CTC from *Bacillus subtilis* and its homolog from *Aquifex aeolicus*. The general stress protein CTC from *B. subtilis* forms a specific complex with 5S rRNA and its stable fragment of 60 nucleotides, which contains internal loop E. The ribosomal protein TL5 from *Thermus thermophilus*, which binds with high affinity to 5S rRNA in the loop E region, was also shown to replace the CTC protein from *B. subtilis* in its complexes with 5S rRNA and its fragment. The findings suggest that the protein CTC from *B. subtilis* binds to the same site on 5S rRNA as the protein TL5. The protein CTC from *A. aeolicus*, which is 50 amino acid residues shorter from the N-terminus than the proteins TL5 from *T. thermophilus* and CTC from *B. subtilis*, does not interact with 5S rRNA.

Key words: general stress protein CTC, 5S rRNA-binding proteins, *Bacillus subtilis*, *Aquifex aeolicus*

Under stress conditions caused by various factors, *Bacillus subtilis* cells produce a set of proteins called general stress proteins (Gsps) [1, 2]. One of these proteins, Gsp10, is a product of the gene *ctc* (catabolic controlled), which is transcribed by RNA polymerase containing a minor σ^B-subunit [3]. Nothing is known about functions of the protein CTC produced by *B. subtilis* cells during stress.

In the mid-1990s, we found a pronounced homology of primary structures of the 5S rRNA-binding ribosomal protein TL5 from *Thermus thermophilus* and the general stress protein CTC from *B. subtilis* (BsuCTC) [4]. The two proteins are similar in size (about 200 amino acids) and are homologous along the whole length of their polypeptide chains. Moreover, the 5S rRNA-binding ribosomal protein L25 (94 amino acids) from *Escherichia coli* is homologous to the N-terminal region of these proteins. The number of known genes encoding proteins homologous to the protein BsuCTC has significantly increased due to the continuously growing number of decoded genomes. Our analysis of primary structures of these proteins has shown that several conservative amino acid residues in the ribosomal proteins L25 and TL5 especially important for interaction with 5S rRNA are invariant for proteins of this family [5]. Based on this finding, it is suggested that proteins of the CTC family should specifically bind to 5S rRNA.

Crystal structures of complexes of the ribosomal proteins TL5 and L25 with the specific fragment of 5S rRNA were recently determined [5, 6]. It was shown that structures of the protein L25 and of the N-terminal RNA-binding domain of TL5 protein were extremely alike and interacted similarly with 5S rRNA. It is not clear, whether these proteins execute extra-ribosomal functions in cells and if the stress protein BsuCTC can bind to 5S rRNA. Perhaps 5S rRNA is a target for BsuCTC produced by the cells under stress conditions.

Among proteins of the CTC family, two proteins have been found (from *Aquifex aeolicus* and *Burkholderia fungorum*) which are 50 amino acid residues shorter at the N-terminus. The question is whether such shortened CTC proteins can bind to 5S rRNA?

The purpose of the present work was to prepare recombinant proteins CTC from *Bacillus subtilis* and *Aquifex aeolicus* and investigate their RNA-binding properties. The protein BsuCTC was shown to specifically bind to 5S rRNA, whereas the protein CTC from *A. aeolicus* (AaeCTC), shortened and deprived of the full-size N-terminal domain, could not bind to 5S rRNA.

MATERIALS AND METHODS

Expression of genes of the proteins BsuCTC and AaeCTC in *E. coli* cells. Genes of the proteins BsuCTC and AaeCTC were amplified by polymerase chain reac-

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tion and cloned into the recognition sites of endonucleases *Nde*I and *Bam*HI of the vector pET11c (in the case of BsuCTC) and pET11c-joe (in the case of AaeCTC). The resulting constructions were called pET11c-BsuCTC and pET11c-joe-AaeCTC, respectively. Both plasmids provide for the resistance of *E. coli* to ampicillin and are different only in some recognition sites of endonucleases in the region of the polylinker. In both cases, the genes were inserted into the vector to be controlled by the promotor of RNA polymerase of bacteriophage T7. The recombinant genes were expressed using the expression system of Studier [7] by transforming the *E. coli* strain BL21(DE3) with the resulting plasmids. This strain additionally contained the plasmid pUBS520, which carried the gene of tRNA recognizing arginine codons AGG and AGA (which are rare for *E. coli*) and also the gene of resistance to kanamycin. The strain BL21(DE3) has in the chromosome the gene of RNA polymerase of bacteriophage T7 controlled by the inducible promotor lacUV5.

To build up the cell biomass, three or four colonies of transformants BL21(DE3) pUBS520/pET11c-BsuCTC or BL21(DE3) pUBS520/pET11c-joe-AaeCTC were inoculated into 30-50 ml of medium LB (1% tryptone, 0.5% yeast extract, 1% NaCl) containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) and grown overnight at 37°C with vigorous shaking. The next day the resulting liquid culture was inoculated into 400 ml of medium LB containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) and grown at 37°C with vigorous shaking until the absorption A_{590} reached 0.6-0.8. Then isopropyl β-D-thiogalactopyranoside (IPTG) was added to the concentration of 0.5 mM, and the cells were grown further for 3 h. The cells were harvested by centrifugation and stored at −70°C if required. The synthesis of the recombinant protein was tested by electrophoresis in the presence of SDS [8].

Purification of recombinant protein BsuCTC. To isolate the protein BsuCTC, 3-4 g of the overproducing cells were suspended in 20-25 ml of buffer A (0.05 M Tris-HCl, pH 7.5) supplemented with 0.02 M $MgCl_2$, 0.6 M NaCl, and 5 mM β-mercaptoethanol and disrupted with a Sonic Dismembrator 550 ultrasonic disintegrator (Fisher Scientific, USA). The debris was precipitated by centrifugation at 13,000*g* for 30 min. Then ribosomes were precipitated by centrifugation at 150,000*g* for 3 h. The supernatant was dialyzed against buffer A containing 0.05 M NaCl and 5 mM β-mercaptoethanol and applied onto a column with DEAE-Sepharose Fast Flow (Pharmacia, Sweden) equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl concentration (0.05-1.0 M) in buffer A. Fractions containing BsuCTC were diluted twofold in buffer A supplemented with 3 M $(NH_4)_2SO_4$ and applied onto a column with butyl-Toyopearl 650S (Toyo Soda, Japan) equilibrated with buffer A containing 1.5 M $(NH_4)_2SO_4$. The protein was eluted with a linear gradient of $(NH_4)_2SO_4$ concentration (1.5-0.0 M) in buffer A. The protein preparation was dialyzed against buffer containing 0.02 M sodium cacodylate (pH 7.5), 0.1 M KCl, and 0.01 M $MgCl_2$ and stored at −20°C.

Purification of recombinant protein AaeCTC. The cells were disrupted as described in the previous paragraph. The debris and ribosomes were precipitated by centrifugation (see above). The ribosome-free extract was heated at 60°C for 10 min. Aggregated proteins were precipitated by centrifugation for 30 min at 13,000*g*. The supernatant was dialyzed against buffer B (0.05 M sodium acetate, pH 5.5) supplemented with 0.05 M NaCl and 5 mM β-mercaptoethanol and applied onto a column with DEAE-Sepharose Fast Flow equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl concentration (0.05-1.0 M) in buffer B. Fractions containing AaeCTC were combined and supplemented with $(NH_4)_2SO_4$ to the concentration of 3 M. The aggregated protein was precipitated by centrifugation for 30 min at 13,000*g* and dissolved in buffer B containing 1.2 M $(NH_4)_2SO_4$ and 5 mM β-mercaptoethanol. This specimen was applied onto a column with butyl-Toyopearl 650S equilibrated with the same buffer. The protein was eluted with a linear gradient of $(NH_4)_2SO_4$ concentration (1.5-0.0 M) in buffer B supplemented with 5 mM β-mercaptoethanol. Fractions containing the pure AaeCTC were combined, supplemented with $(NH_4)_2SO_4$ to the concentration of 3 M, and stored at 4°C.

Preparation of 5S rRNA fragments and RNA complexes with proteins BsuCTC and AaeCTC. A limited hydrolysis of the 5S rRNA from *E. coli* was performed with ribonuclease A as described in [9, 10]. The hydrolysis was performed in buffer C (containing 20 mM sodium cacodylate, pH 7.4, 0.01 M $MgCl_2$, 0.1 M KCl, and 0.5 mM EDTA) in the presence of RNA (1 mg/ml) at the RNA/RNase ratio of 100 : 1 (w/w). To prepare RNA–protein complexes, samples of the proteins and RNA were dialyzed against buffer C (in the case of AaeCTC the buffer also contained 5 mM β-mercaptoethanol). RNA and the proteins were mixed and incubated at 40°C for 10-15 min [10, 11]. The presence of the complexes was determined by electrophoresis in 12% polyacrylamide gel in 90 mM Tris-acetate buffer (pH 7.8) containing 10 mM $MgCl_2$.

RESULTS AND DISCUSSION

Using the *E. coli* strain BL21(DE3), we prepared overproducing strains of proteins BsuCTC and AaeCTC, the protein content was no less than 5-10% of the total protein of the cell. A two-step procedure of chromatographic purification of these proteins was developed. This technique allowed us to isolate 5-10 mg CTC proteins of 95% purity from 1 g of cells of the producers (Fig. 1).

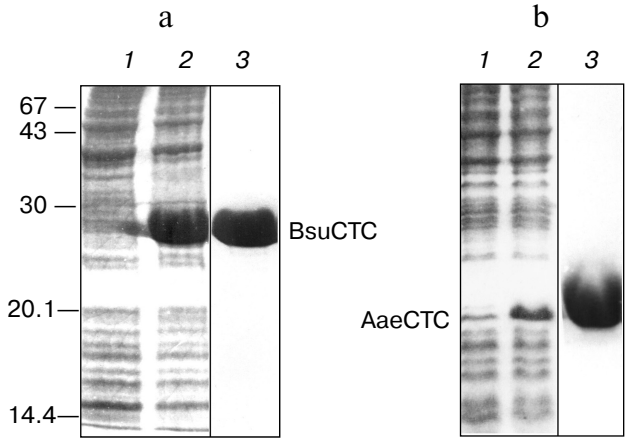

Fig. 1. Assessment of synthesis of the recombinant proteins and purity of isolated CTC proteins from *B. subtilis* (a) and *A. aeolicus* (b): *1*) the overproducing cells before the induction; *2*) the overproducing cells after the induction with IPTG; *3*) the protein preparation after chromatography on butyl-Toyopearl. To the left, positions of marker proteins and their molecular weights in kD are shown. Electrophoresis in 15% SDS-polyacrylamide gel.

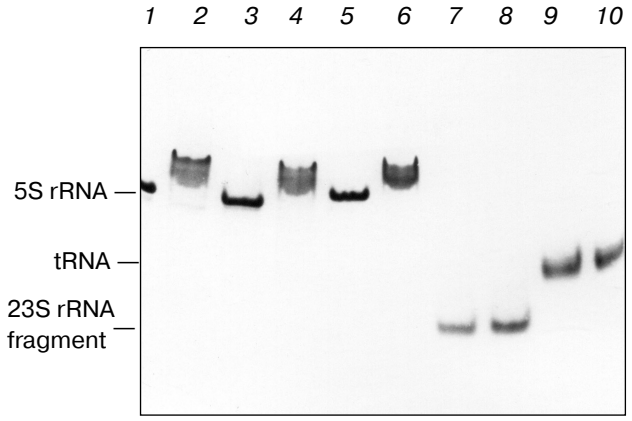

Fig. 2. Assessment of interaction of protein CTC from *B. subtilis* with RNA (electrophoresis in 12% polyacrylamide gel without denaturing): *1*, *3*, *5*) 5S rRNA (from *E. coli*, *B. stearothermophilus*, and *T. thermophilus*, respectively); *2*, *4*, *6*) 5S rRNA + CTC (RNA as in samples *1*, *3*, *5*); *7*) 23S rRNA fragment from *T. thermophilus* (55 nucleotides); *8*) 23S rRNA fragment from *T. thermophilus* + CTC; *9*) tRNA from *E. coli*; *10*) tRNA from *E. coli* + CTC. Molar ratios of RNA/CTC in the incubation mixture are 1 : 1.

The complexing of CTC proteins with RNA was determined by the gel shift method. This method is based on changes in the electrophoretic mobility of RNA during its complexing with protein. We found that under physiological values of pH and ionic strength the protein BsuCTC bound to 5S rRNA of various bacteria (Fig. 2, lanes *2*, *4*, *6*). In the experiments, the ratios of RNA/pro-

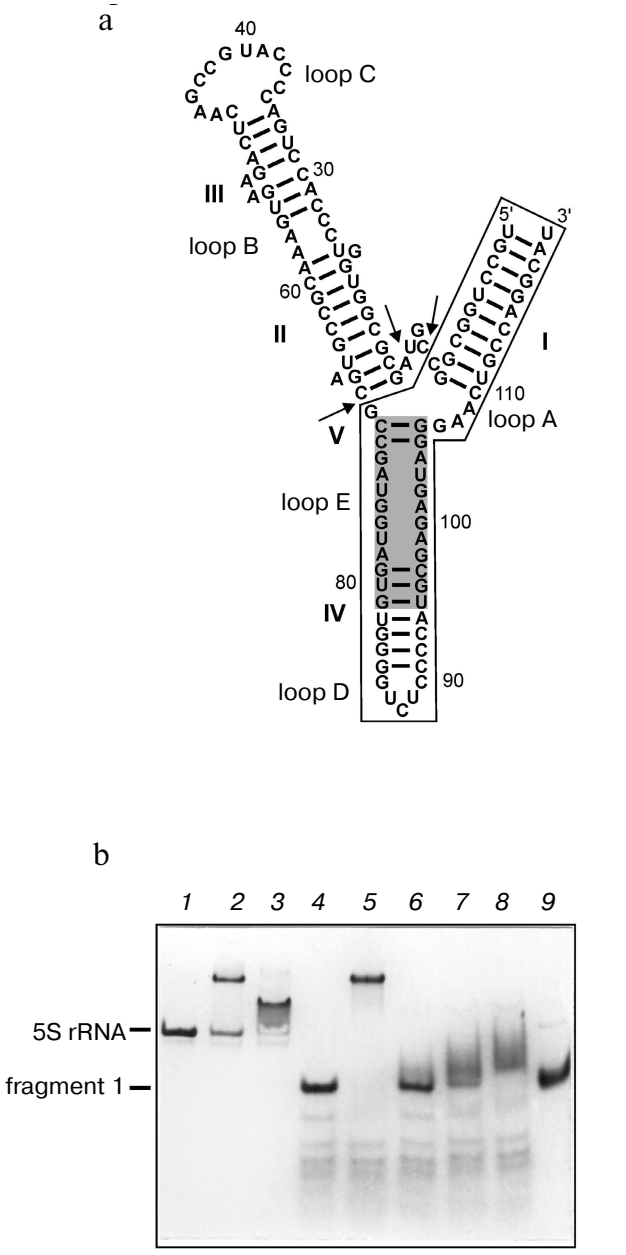

Fig. 3. Comparison of RNA-binding properties of proteins TL5 from *T. thermophilus* and CTC from *B. subtilis*. a) Scheme of the secondary structure of 5S rRNA from *E. coli*. The arrows indicate regions of hydrolysis with RNase A in the free 5S rRNA that results in two large fragments [9]. The region of binding the ribosomal proteins L25 from *E. coli* and TL5 from *T. thermophilus* is shown in gray. The stable fragment 1 is shown by the solid line. b) Assessment of RNA-binding properties of proteins TL5 and CTC (electrophoresis in 12% polyacrylamide gel without denaturing): *1*) 5S rRNA of *E. coli*; *2*) 5S rRNA of *E. coli* + TL5 (1 : 0.5); *3*) 5S rRNA of *E. coli* + CTC (1 : 1); *4*) 5S rRNA of *E. coli* treated with RNase A; *5*) hydrolyzed 5S rRNA + TL5 (1 : 1); *6*) hydrolyzed 5S rRNA + CTC (1 : 0.5); *7*) similarly to the lane *6* (1 : 1); *8*) similarly to the lane 6 (1 : 1.5); *9*) tRNA of *E. coli*. In parentheses, molar ratios of RNA/protein are shown.

tein in the incubation media were varied. Even at the RNA/protein ratio of 1 : 1, virtually all RNA was involved in complexing. This result suggested the equimolar complexing of the protein BsuCTC and 5S rRNA. However, under similar conditions the protein BsuCTC failed to bind to the 23S rRNA fragment from *T. thermophilus* and to tRNA from *E. coli* (Fig. 2, lanes *8* and *10*), which suggests the specificity of interaction of this protein with 5S rRNA. Thus, we have shown that the general stress protein BsuCTC specifically binds to 5S rRNA.

Then we attempted to locate the binding site of the protein BsuCTC on the 5S rRNA molecule. The ribosomal proteins TL5 from *T. thermophilus* and L25 from *E. coli* were earlier shown to bind to 5S rRNA in the region of loop E [11, 12]. Hydrolysis of 5S rRNA from *E. coli* with pancreatic ribonuclease A is also known to result in two stable fragments [9] (Fig. 3a). One of these RNA fragments (fragment 1) contains loop E and helices I, IV, and V and specifically binds only one of the bacterial 5S rRNA-binding proteins (protein L25 of *E. coli* and the homologous protein TL5 of *T. thermophilus*). Therefore, we tested the ability of the protein BsuCTC to bind to this fragment of 5S rRNA. Figure 3b shows that the stable fragment 1 (lane *4*) produced during the 5S rRNA hydrolysis binds the protein TL5 (lane *5*) and the protein BsuCTC (lanes *6*-*8*). Moreover, during competitive binding protein TL5 displaced protein BsuCTC from complexes with 5S rRNA or fragment 1 (data not presented).

All these findings suggest that binding sites for the proteins TL5 and BsuCTC on the 5S rRNA molecule are similar (the region of loop E) and confirm the specificity of interaction between the general stress protein BsuCTC and this rRNA. Therefore, we suggested that the 5S rRNA molecule should be a target for protein BsuCTC during its production in the cell under stress conditions. When the present article was in preparation for press, it was reported [13] that protein CTC had been detected in the ribosomal fraction of *B. subtilis* cells only under conditions of thermal stress. These data are in consistence with our findings. However, based on these data, the authors [13] concluded that BsuCTC should be a ribosomal protein. But it was earlier shown that in vegetative cells of *B. subtilis* protein CTC was synthesized very poorly and its production increased nearly tenfold only under stress conditions [1, 2]. BsuCTC is an acidic protein (p*I* 4.5) and no such protein has been found among ribosomal proteins of this microorganism [14, 15]. No such protein was also found among 5S rRNA-binding proteins of *Bacillus stearothermophilus*, another member of the Bacillaceae family [16]. Thus, if under stress conditions protein BsuCTC specifically binds to ribosome, this can be only a temporary association connected with the cell stress response.

Some proteins of the CTC family (*A. aeolicus* and *B. fungorum*) are approximately 50 amino acid residues shorter from the N-terminus than proteins TL5 and BsuCTC. These proteins contain the shortened N-terminal domain lacking three of five most conservative amino acid residues, which in proteins L25 and TL5 are involved in binding to 5S rRNA (Fig. 4). We were interested if these proteins retained the ability to bind 5S rRNA notwithstanding the "invalidity" of the N-terminal domain and attempted to determine the possible contribution to interaction with RNA of the most conservative amino acid residues in the N-terminal domains of other proteins of this family. The recombinant protein AaeCTC did not bind to 5S rRNA of *E. coli* (Fig. 5, lane *2*). Excess of the protein in the incubation mixture did not change

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L25 ECO    MFTINAEVRKEQGKGASRRLRAANKFPAIIYGGKEAPLAIELDHDKVMNMQAKAEFYSEVLTIVVDGKEIKV 72
TL5 TTH    MEYRLKAYYR-EGEKPSA--LRRAGKLPGLMYN-RHLNRKVYVDLVEFDKVFRQASIHHVIVLELPDGQSLPT 69
CTC BSU    MATLTAKERTDFTRSSLRNIRTSGHVPGIIYGKDTGNKPVSLDSVELIKTLRDEGKNAVITLEVS-GEKHSV 71
CTC AAE    ----------------------------------------------------MPHGEAFLIEAELDGETRVC 20
               + *  * +  + +   +* +   * +++        + +*               ++    **

L25 ECO    KAQDVQRHPYKPKLQHIDFVRA                                                  94
TL5 TTH    LVRQVNLDKRRRRPEHVDFFVLS-DEPVEMYVPLRFVGTPAGVRAGGVLQEIHRDILVKVSPRNIPEFIEVDV 141
CTC BSU    MVTDLQTDPLKNEITHADFQVVNMSEDIEVEVPIHLTGEAIGVKNGGVLQQPLYALTVKAKPKAIPQTIEADI 144
CTC AAE    LLKDIQFGWLGNNPIHIDLYDLSNVEEIEVEVPIEFVGTPAGVEAGGTFEPVMHTLTIKAPPAKIPEKIVVDV 93
              +++         * *       * +*+ **+  ++   **  **+ +     + +*  *  **+ *  *+

TL5 TTH    SGLEIGDSLHASDLKLPPGVELAVSPEETIAAVVPPEDVEKLAEEAAAEVAEPEVIKKGKEEEEE          206
CTC BSU    SSLDVNEVLTIADLPAGGDYSFNHESDEVVASILPPQQQE------AAEVDEEESADAQPEGENEQ          204
CTC AAE    SGLGLGEALHVRDITPPEGCMILDNPEETVAVVYEPEEEVIGEEETGAEETGETEATS                  151
           * * + + *   *+           +*+++ +  *++     **  **           * *+*
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Fig. 4. Comparison of the primary structures of the CTC family proteins from various bacteria. Identical amino acid residues are designated with asterisks. Conservative replacements (in accordance with the rule: A = S, V = T, L = V = I = M, K = R, D = Q = E = N [4]) are designated with "+". Invariant residues, which in the proteins L25 and TL5 are responsible for binding to 5S rRNA, are shown with white letters on black background. Abbreviations: ECO) *E. coli*; TTH) *T. thermophilus*; BSU) *B. subtilis*; AAE) *A. aeolicus*.

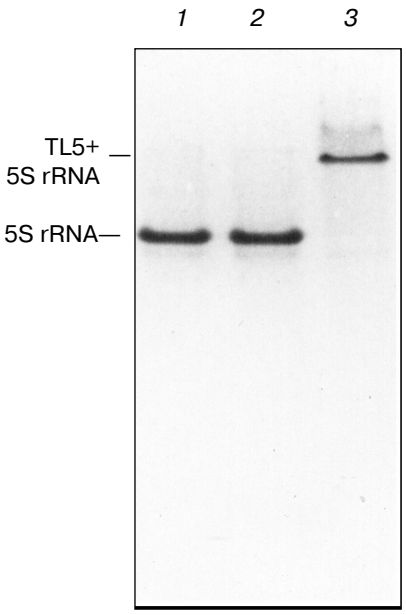

Fig. 5. Assessment of RNA-binding properties of CTC protein from *A. aeolicus* (electrophoresis in 12% polyacrylamide gel without denaturing): *1*) 5S rRNA from *E. coli*; *2*) 5S rRNA from *E. coli* + CTC; *3*) 5S rRNA from *E. coli* + TL5 from *T. thermophilus*. Molar ratio of RNA/protein in the incubation mixture is 1 : 1.

this picture (data not presented). Thus, we found that the CTC family protein lacking the full-size N-terminal domain was not able to bind to 5S rRNA. These findings confirm the earlier hypothesis [5] that only CTC family proteins with the N-terminal domain commensurate with the ribosomal protein L25 can bind to 5S rRNA. Thus, for now the family of CTC proteins seems to include at least three groups: true ribosomal proteins, proteins temporarily associated with ribosome, and proteins lacking the 5S rRNA-binding properties.

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