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EXPERIMENTAL ARTICLES

Investigation of the Functional Role of Ctp Proteins in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract—To understand the functional role of CtpB and CtpC proteins, which are similar to the C-terminal processing CtpA peptidase, the effect of the insertional inactivation of the *ctpB* and *ctpC* genes on the phenotypic characteristics of *Synechocystis* sp. PCC 6803 was studied. The inactivation of the *ctpC* gene was found to be lethal to the cyanobacterium, which indicates a vital role of the CtpC protein. The mutant with the inactivated *ctpB* gene had the same photosynthetic characteristics as the wild-type strain. The double mutant ΔctpAΔctpB with the two deleted genes was identical, in the phenotypic characteristics, to the mutant with a knock-out mutation in the *ctpA* gene, which was unable to grow photoautotrophically. The data obtained suggest that, in spite of the high similarity of the Ctp proteins, they serve different functions in *Synechocystis* sp. PCC 6803 cells and cannot compensate for each other.

Key words: cyanobacterium, insertion mutagenesis, photosynthesis, peptidase.

The carboxyl-terminal peptidase CtpA plays an important part in the biogenesis of photosystem II in cyanobacteria, processing the D1 protein [1]. Using the *ctpA* gene of *Synechocystis* sp. the probe [2], homologous genes in the nuclear genome of spinach and barley have been identified [3]. Investigations with the employment of site-directed mutagenesis showed that the active center of the CtpA protein is formed by two amino acid residues, Ser and Lys [4], in contrast to the active centers of other serine peptidases, which contain three amino acid residues. The activity of the CtpA peptidase is not inhibited by the known inhibitors of serine peptidases [4, 5], which allowed the Ctp peptidases to be classified into a separate group of serine peptidases [4].

In addition to the *ctpA* gene, the genome of *Synechocystis* sp. PCC 6803 contains two other genes of this family, *ctpB* [6] and *ctpC*, whose products are highly homologous to the CtpA protein (Fig. 1). These products are localized in the periplasm and are expressed under the normal growth conditions [7]. The physiological function of the CtpA and CtpC proteins is as yet unknown, although they are believed to be involved in the C-terminal processing of proteins.

The present work was undertaken to study the effect of the insertional inactivation of the *ctpB* and *ctpC* genes on the phenotypic characteristics of *Synechocystis* sp. PCC 6803 with the aim of elucidating the functional role of the CtpB and CtpC proteins and their possible involvement in photosynthetic processes.

MATERIALS AND METHODS

The wild-type and mutant *Synechocystis* sp. PCC 6803 strains were grown at 30°C in the liquid or solid BG11 medium [8]. For heterotrophic growth, the medium was supplemented with 5 mM glucose. The antibiotics kanamycin, erythromycin, and gentamycin were added to the solid BG11 medium at concentrations of 50, 100, and 15 μg/ml, respectively; while at twofold lower concentrations, they were added to the liquid BG11 medium. The strains were grown at an illumination of either 50 or 5 μE/(m^2 s). Growth was monitored by measuring the culture turbidity at 730 nm using a DW-2000 spectrophotometer (SLM–Aminco, United States). Cells were transformed by the standard procedure [9].

Molecular cloning and DNA isolation were carried out as described in the handbooks [10, 11]. Restriction endonucleases were purchased from New England Biolabs (United Kingdom) and MBI Fermentas (Lithuania). Deletion mutants were tested by the polymerase chain reaction (PCR) technique using the KlenTag1 polymerase and the following primers purchased from Life Technology: 5'-AAGTCCATGCTGTGGAAGCT (ctpA1 primer), 5'-GGATGCCTTTACTTATGGC (ctpA2 primer), 5'-CAAGTTCGCTGCTGACTTCT (ctpB1 primer), 5'-CAGCATTATTGATTTCACCG (ctpB2 primer), 5'-TGCCAAACCGCCTGGGGATA (ctpC1 primer), and 5'-CGCACTGGATGAATTTCAAT (ctpC2 primer).

To obtain deletions in the *ctpB* and *ctpC* genes, the fragments of the chromosomal DNA of *Synechocystis*

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CtpA --------MGKRTRRFWAL----AFSLLMGALIYLGNTPSALAFTEE------------ 35
CtpB MSPHLLCLRPLVAALVFGLGLGTALRPALSAPNVINPDSPTLATPKDKDSGVSDNVTLLE 60
CtpC -------MLKQKRSLILGT---TALLLTTVAVTGVG---LRLARSQG----------YLQ 37
                    . .     *:     *   :.     ** .:

CtpA ---QKLLLQSWRLVNQSYLDETFNHQNWWLLREKYVKRPLRNREETYTAIEEMLATLDEP 92
CtpB NSPKAVVDEVWQLVNQQFVDKDFNHSNWLSKRQELLGRNYQDNAEAYRQIGRILKDLNDP 120
CtpC DNPKELVDEVWQIVNRTYVDGTFNGEDWVAVRQDYLTRDYKNQEEAYTAIREMLEKLNDP 97
       : :: : *::**: ::*  ** .:*   *:. : *  ::. *:*  * .:*  *::*

CtpA FTRLLRPEQYGNLQVTTTGELSGVGLQININPETNQLEIMAPLAGSPAEEAGLQPHDQIL 152
CtpB YTRFLSPEEFAILSSQTSGEASGVGIRVLMDKRSSDLVVVDVMRGTPALKAGIRPGDRIV 180
CtpC YTRFMSPDEFQSMRIDTSGELTGVGIQITQDQDTKKIVVVAPIEDTPAYNAGILSKDVIT 157
     :**:: *:::  :   *:** :***:::  :  :..: ::  : .:** :**: . * *

CtpA AIDGVDTQTLSLDEAAARMRGPKNTKVSLEILSAGTEVPQEFTLTRQLISLSPVAAQLDD 212
CtpB RINGQPAALMSLEQATEAIQGEIGTELSLQLSRP-KSGVFSVTLKRENIEIDSVTYNVKE 239
CtpC KIDGKSTDGMEVDDAVKLIRGKPGTSVVLTIER--EGQAIEYPLTRTLIEIHPVRAQVED 215
      *:*  :  :.:::*.  ::*  .*.: * :       . .*.*  *.: .*  ::.:

CtpA SRPGQSVGYIRLSQFSANAYKEVAHALHQLEEQGADGYIL**DLRNNPGGLL**QAGIDIARLW 272
CtpB EG-ELRVGYIRLDEFSSHSAEQMEKAITELNKSRISGYVL**DLRGNPGGLL**LSSIDIARLW 298
CtpC IN-GARVGYIRLNQFSAQASEEMRQAVQKLEKENVVGYIF**DLRSNPGGLL**YSSVDIARIW 274
           ******.:**::: ::: :*: :*::.   **::***.****** :.:****:*

CtpA LPESTIVYTVNRQGTQESFTANGEAATDR**PLVVLVNQGTASASEILAGALQDNQRATLVG** 332
CtpB LNRGEIVSTIDRRGGDRHFSANGRSLTDL**PLVVLVNERSASASEILAGALKEQGRATVVG** 358
CtpC LDEGGIVSTVDRRGEVEQQSANKRQLSNR**PLVVLVDGGSASASEIVSGALQDNQRAVIVG** 334
     * .. ** *::*:*  .  :** .  :: ******:  :******::***::: **.:**

CtpA **EKTFGKG**LIQSLFELSDGAGIAVTVAKYETPQHHDIHKLGIMPDEVVEQP-------LIS 385
CtpB **TATYGKG**TVQSVNTLSDGSGLAVTIARYYPPSGTDINRKGISPDIHLDISNDTKLQFRND 418
CtpC **TKTFGKG**LVQSVRELGDGSGMAVTIAKYLTPNGRDINKHGIDPDVEVELTDAQRKELQQN 394
       *:*** :**:  *.**:*:***:*:* .*.  **:: ** **  :: .         .

CtpA FAEITSPADVQYQAALDLLTGGVAIAHKSSSTIPAMATAHKPN-- 427
CtpB PELMATEIDPQYQRAISVLRQHRHSLGLPPAKDLGIGLLEPSQL 462
CtpC REKVGTLEDPQFARAYEVLMQQVN---KTASK------------ 423
       : :  * *:  * .:*         ..:
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Fig. 1. Alignment of the amino acid sequences of the CtpA, CtpB, and CtpC proteins of *Synechocystis* sp. PCC 6803 with the aid of the ClastalW software (http://www2.ebi.ac.uk). Identical, conservative, and semiconservative amino acid residues are marked by the asterisks ("*"), colons (":"), and points ("."), respectively. The PDZ domain is underlined. The putative regions responsible for proteolytic activity [17] are given in bold letters.

sp. PCC 6803 containing these genes were cloned into plasmid pUC19. The DNA fragment with the *ctpC* gene was amplified using the ctpC1 and ctpC2 primers and cloned into plasmid pCR2.1 purchased from Invitrogen (United States). The recombinant plasmids thus obtained were digested with the respective restriction endonucleases (Fig. 2), and the nucleotide fragments containing the *ctp* genes were replaced with antibiotic resistance cassettes. The resultant plasmids pSL795, pPK2, pSL1155, and pSL1250 were used to transform the wild-type *Synechocystis* sp. PCC 6803 cells. The transformants were segregated by subcloning them sequentially on media with increasing concentrations of the antibiotics. The antibiotic-resistant transformants were analyzed further.

The double mutant was obtained by transforming the ΔctpB cells with the pSL795 plasmid. One of the Em^R transformants that was resistant to kanamycin, ΔctpAΔctpB, was used in further experiments.

The rates of oxygen uptake (or evolution) by cyanobacterial cells were measured at 30°C using a Clark-type oxygen electrode [12]. The concentration of cells in these measurements corresponded to a chlorophyll concentration of 5 μg/ml. The activity of photosystem II was measured in the presence of 1 mM $K_3Fe(CN)_6$ and 1 mM dichloro-*p*-benzoquinone. The photosystem I–mediated consumption of oxygen was measured in the presence of 1 mM ascorbic acid, 1 mM paraquat, 1 mM diaminodurene, and 10 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

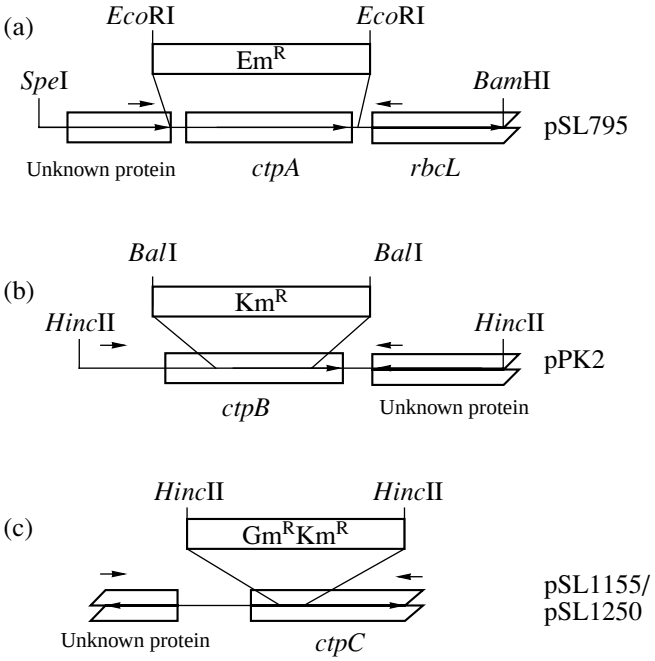

Fig. 2. Diagram showing the construction of the plasmids used to derive deletion mutants. The small arrows show the position of the primers used for the segregation studies of mutations. Em^R is the erythromycin resistance cassette from plasmid pRL271 [19]; Km^R is the kanamycin resistance cassette from plasmid pUC4K; and Gm^R is the gentamycin resistance cassette from plasmid pUCGM [20]. (a) Plasmid pSL795 was used to obtain mutant with a deletion in the *ctpA* gene; (b) plasmid pPK2 was used to obtain mutant with a deletion in the *ctpB* gene; and (c) plasmids pSL1155 (Gm^R) and pSL1250 (Km^r) were used to obtain mutant with a deletion in the *ctpC* gene.

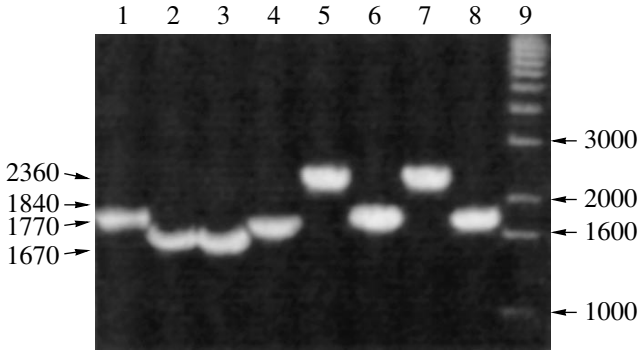

Fig. 3. The electrophoresis of PCR products, which demonstrates that the constructed *Synechocystis* sp. PCC 6803 strains are homozygous. The expected sizes of the PCR products are shown at the left. The sizes of DNA markers (lane 9 representing a 1-kb DNA ladder from New England Biolabs) are shown at the right. Lanes 1–4 correspond to the primers specific for the *ctpA* gene. Lanes 5–8 correspond to the primers specific for the *ctpB* gene. PCR amplifications were done using the chromosomal DNA isolated from (1 and 6) the ΔctpA mutant, (2 and 5) the ΔctpB mutant, (3 and 8) the wild-type strain, and (4 and 7) the double ΔctpAΔctpB mutant.

The concentration of chlorophyll *a* in methanol extracts and the content of phycobilins in cell suspensions were determined as described by Lichtenthaler [13]. The variable fluorescence (F_V) of chlorophyll *a* was determined as the difference between the maximum fluorescence of illuminated cells (F_M) and their dark fluorescence (F_0) [14]. The fluorescence of cells was measured using an FL-100 spectrophotometer (Photo Systems International, Czech Republic). The concentration of cells in these measurement corresponded to a chlorophyll concentration of 2 μg/ml.

RESULTS AND DISCUSSION

The functional role of the CtpB and CtpC proteins and their possible involvement in the biogenesis of photosystems was studied by the method of gene-targeted mutagenesis. The construction of the plasmids used for obtaining mutants with deletions in the *ctpA*, *ctpB* and *ctpC* genes is shown in Fig. 2. The ΔctpA mutant lacking functionally active photosystem II was used in further experiments as the control strain. The insertion mutagenesis approach allowed us to obtain the ΔctpB mutant with the deleted *ctpB* gene and the double ΔctpAΔctpB mutant with the two deleted genes. Thus, we deleted the entire *ctpA* gene or fragments of the *ctpB* and *ctpC* genes corresponding to the putative active centers of their protein products. The PCR analysis of chromosomal DNA confirmed the complete segregation of mutations in deletion mutants of *Synechocystis* sp. PCC 6803 (Fig. 3).

Our attempts to obtain homozygous mutants with the inactivated *ctpC* gene were unsuccessful, although we varied the conditions of the segregation of transformants (illumination with an intensity of 50 μE/(m^2 s) at 30 or 20°C) and used conditions promoting the growth of mutants defective in photosynthesis (i.e., illumination at an intensity as low as 5 μE/(m^2 s) and the glucose-containing growth media).

As can be seen from the table, the mutants ΔctpA and ΔctpAΔctpB were unable to grow autotrophically and virtually did not differ from the wild-type strain in the rate of growth on glucose under the conditions of low-level illumination. The ΔctpB mutant did not differ from the wild-type strain in the growth rate under photoautotrophic conditions and in the content of phycobilins and chlorophyll *a* (data not presented).

The measurement of the activity of photosystems I and II with the artificial electron acceptors showed that photosystem II of the ΔctpB mutant had the same activity as photosystem II of the wild-type strain, whereas photosystem II of the mutant with the deleted *ctpA* gene was blocked. The activity of photosystem I in all the mutants under consideration was the same as in the wild-type strain.

The estimation of the relative content of photosystem II in cyanobacterial cells from the intensity of vari-

Ability to grow photoautotrophically and some photosynthetic characteristics of the wild-type and mutant strains of *Synechocystis* sp. PCC 6803 with deletions in the *ctp* genes

Parameter	Strain			
	wild type	ΔctpA	ΔctpB	ΔctpAΔctpB
Photoautotrophic growth	+	–	+	–
Relative activity of electron transport through:				
photosystem I*	100	100	100	100
photosystem II*	100	0	100	0
Relative amount of photosystem II***	100	0	100	0

Note: All parameters are given as a percentage of the respective parameters of the wild-type cells. Experiments were carried out at least in triplicate. Wild-type cells consumed oxygen at a rate of 209 ± 57 μmol O_2/(mg chlorophyll h) and evolved it at a rate of 524 ± 89 μmol O_2/(mg chlorophyll h) (* and **, respectively). *** The maximal level of fluorescence measured in the presence of 40 μM DCMU was normalized to the level of dark fluorescence, $(F_m - F_0)/F_0$.

able fluorescence showed that photosystem II was inactive in the double mutant ΔctpAΔctpB but active in the ΔctpB mutant. Accordingly, the mutation of the *ctpB* gene does not induce any phenotypic changes in *Synechocystis* sp. PCC 6803 cells nor has it any additional influence on the manifestation of the mutation blocking the synthesis of the CtpA peptidase.

In the sequenced genome of *Synechocystis* sp. PCC 6803 (check the web site www.kazusa. or.jp/cyano/cyano.html), there are three *ctp* genes. Two of their protein products, CtpB and CtpC, show a high degree of homology (34.4 and 40.3%, respectively) to the CtpA peptidase, which plays a significant part in the biogenesis of photosystem II (Fig. 1).

The inactivation of the *ctpC* gene is lethal even when transformants are segregated in the presence of glucose and the illumination intensity is low. This suggests that the functional role of the CtpC protein is not related to biogenesis or the operation of the vitally important photosystems I and II and that the CtpA and CtpB proteins cannot functionally substitute for CtpC peptidase. The latter may be involved in the proteolysis of the aberrant translational products, thereby implementing the control of protein quality, as has been suggested for the Prc peptidase of *Escherichia coli* [15]. It should be noted in this regard that the Prc and CtpC peptidases are highly homologous and both are localized in the periplasm [7, 16]. On the other hand, in spite of the high homology of these peptidases in the regions responsible for their proteolytic activity [17], the low level of homology in other domains suggests that the physiological functions of the peptidases in *E. coli* and *Synechocystis* may be different.

The mutant of *Synechocystis* sp. PCC 6803 with the inactivated *ctpB* gene has the same photosynthetic characteristics as the wild-type strain. This implies that the CtpB peptidase is not involved in the biogenesis of the photosystems. The double mutant ΔctpAΔctpB with the two deleted genes is identical, in phenotypic characteristics, to the mutant with a knock-out mutation in the *ctpA* gene. The data obtained indicate that, in spite of the high similarity of the Ctp proteins, they serve different functions in *Synechocystis* sp. PCC 6803 cells and cannot compensate for each other. This may be explained by the different location of these proteins in cells. Indeed, the CtpA protein is localized in the thylakoid lumen [3], whereas the CtpB and CtpC proteins are localized in the periplasm [7]. As can be seen from their amino acid sequences (Fig. 1), the Ctp proteins show the lowest similarity in their *N*-terminal regions, where the signal peptide responsible for the transport of these proteins to the respective cell compartment or the small hydrophobic anchor domain responsible for their binding to membranes may occur. There are also some differences in the amino acid sequences of the Ctp proteins located between this hydrophobic domain and the active center. It is the differences in their N-terminal regions that may determine the specific functional roles of the CtpA, CtpB, and CtpC proteins in cyanobacterial cells. One of the conservative regions of the Ctp peptidases is the PDZ domain (Fig. 1), which is responsible for the interaction of some proteins with various membrane complexes [18]. Like CtpA peptidase, the CtpB and CtpC peptidases may be involved in the specific processing of proteins constituting these complexes.

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