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EXPERIMENTAL ARTICLES

The Insertional Inactivation of Genes Encoding Eukaryotic-Type Serine/Threonine Protein Kinases in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract—*Synechocystis* sp. PCC 6803 mutants, in which one of the eukaryotic-type serine/threonine protein kinase genes *pknD*, *pknE*, *pknG*, and *pknH* was inactivated, were obtained by insertion mutagenesis. None of these mutants differed phenotypically from the wild-type strain, indicating that the *pknD*, *pknE*, *pknG*, and *pknH* genes are not of crucial importance for the photoautotrophically grown cyanobacterium. The mutant with the inactivated *pknE* gene was resistant to L-methionine-D,L-sulfoximine and especially to methylamine. The resistance was due neither to the impaired transport of these compounds nor to the inhibition of the production of toxic γ-glutamylmethylamide from methylamine. The data presented suggest that resistance to methylamine may be associated with alterations in the regulation of the glutamine synthetase system and that the PknE protein kinase may be involved in the regulation of nitrogen metabolism in the cyanobacterium studied.

Key words: cyanobacteria, insertion mutagenesis, protein kinases, methylamine.

Protein kinases play a key role in the signal systems controlling gene expression. Prokaryotic cells contain a number of histidine protein kinases, which transduce signals by phosphorylating the regulatory proteins of two-component regulatory systems [1]. Zhang [2] and Shi *et al.* [3] showed that bacteria also have eukaryotic-type serine/threonine protein kinases. Such protein kinases may be involved in the regulation of cell differentiation and nitrogen metabolism in nitrogen-fixing filamentous cyanobacteria [4, 5].

The genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 contains seven genes putatively encoding a subfamily of the Pkn serine/threonine protein kinases, whose cellular functions are poorly understood [3, 6] except that the SpkA serine/threonine protein kinase is required for normal cell motility [7].

This study was undertaken (1) to obtain mutants of the cyanobacterium *Synechocystis* 6803 with insertion mutations in the four *pkn* genes (*pknD*, *pknE*, *pknG*, and *pknH*) encoding serine/threonine protein kinases, (2) to investigate the physiological and biochemical characteristics of these mutants, and (3) to analyze their phenotypic traits in order to reveal possible physiological functions of serine/threonine protein kinases in *Synechocystis* 6803 cells.

MATERIALS AND METHODS

The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) was obtained from the collection at the Department of Genetics, Moscow State University. The cyanobacterium was grown photoautotrophically at 30°C in BG11 medium [8] under illumination with an intensity of 20–50 μerg/(m^2 s). Solid BG11 medium contained 1% Difco agar. The *Escherichia coli* strains JM109 and TG1 were grown at 37°C in LB broth. *Synechocystis* 6803 mutants were selected using the solid BG11 medium with increasing concentrations of antibiotics (kanamycin at concentrations from 10 to 100 μg/ml with a step of 20 μg/ml; gentamicin at concentrations from 2 to 10 μg/ml with a step of 2 μg/ml; and chloramphenicol at concentrations from 7 to 35 μg/ml with a step of 7 μg/ml).

The resistance of the wild-type and mutant strains to methylamine and L-methionine-D,L-sulfoximine was determined by plating aliquots of cell suspensions (20 μl in volume) onto solid medium containing varied concentrations of these compounds. The results were expressed in the minimal concentrations of methylamine and L-methionine-D,L-sulfoximine inhibitory to growth. Bacterial growth in liquid media was assessed by measuring the optical density of cultures at 750 nm (OD_{750}).

The absorption spectra of cell suspensions were recorded using an Ultraspec spectrophotometer (LKB Biochrom).

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The activity of glutamine synthetase was determined in the γ-glutamyltransferase reaction [9].

Ammonium ions were analyzed with the Nessler reagent.

Synechocystis 6803 cells were transformed with plasmids by the standard procedure [10], selecting transformants resistant to antibiotics placed inside wells made at the edges of agar plates.

Molecular cloning was performed as described in the handbook of Maniatis *et al.* [11], using the plasmids pUC18 (ColE1Ap^r), pGEM®-T, pGEM®-TEasy (ColE1Ap^{r}Km^r), pUCGM (ColE1Ap^{r}Gm^r), pUC4K (ColE1Ap^{r}Km^r), and pSLCm (ColE1Ap^{r}Cm^r). The first three plasmids were purchased from Promega.

Particular genes were inactivated as follows: DNA fragments containing gene regions (determined from the Cyanobase database, available at http://www.kazusa.or.jp/cyano/cyano.html) were amplified by PCR. Specific forward and reverse primers were synthesized based on information about the unique restriction sites, into which cassettes with antibiotic resistance markers were inserted. Amplified fragments were cloned using vectors incapable of autonomous replication in the cyanobacterium (pUC18, pGEM®-T, and pGEM®-TEasy). Recombinant plasmids were introduced into *Synechocystis* 6803 cells by the transformation method, and the recombinants obtained were segregated by plating them onto solid medium with increasing concentrations of selective antibiotics. The plates were incubated under photoautotrophic conditions (see above). The completeness of segregation was checked by PCR.

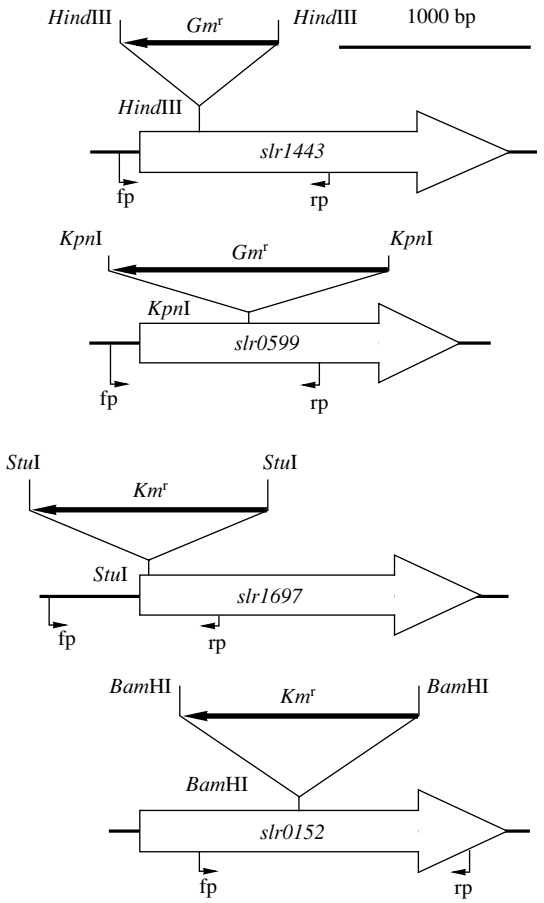

Fig. 1. Schematic representation of the insertional inactivation of genes encoding eukaryotic-type serine/threonine protein kinases in the cyanobacterium *Synechocystis* 6803. Open arrows indicate coding regions and the direction of gene transcription. Bold dark arrows indicate cassettes with the genes of resistance to gentamicin (*Gm^r*), chloramphenicol (*Gm^r*), and kanamycin (*Km^r*) and the direction of the transcription of these genes. Slender dark arrows show the position of forward and reverse primers (fp and rp, respectively).

RESULTS AND DISCUSSION

Insertion mutagenesis allowed us to obtain completely segregated *Synechocystis* 6803 mutants with the inactivated genes *pknD* (*slr0152*), *pknE* (*slr0599*), *pknG* (*slr1443*), and *pknH* (*slr1697*) (the *pkn* genes and the respective mutants were designated according to the Cyanomutants database, available at http://www.kazusa.or.jp/cyano/synechocystis/mutants). As can be seen from Fig. 1, cassettes with the marker genes of resistance to the antibiotics kanamycin, gentamicin, and chloramphenicol were inserted at convenient restriction sites located close to the starting termini or the centers of the genes in the orientation opposite to that of gene transcription. The mutants obtained were stable with respect to their antibiotic resistance and could not express the inactivated genes.

None of the mutants differed from the wild-type strain in visible phenotypic characteristics (size, color, and shape of colonies) when grown on the solid medium and in the growth rate when grown photoautotrophically in the liquid medium under illumination with an intensity of 40–50 μerg/(m^2 s). Furthermore, the mutant and wild-type cells did not differ in pigment composition, as is evident from the absorption spectra of cell suspensions. Taken together, these data suggest that the *pkn* genes either are not of crucial importance for cell viability or may perform similar functions and thereby can mutually compensate for each other, that the malfunction of the genes produces undetectable morpho- and physiological changes, or that the genes are not expressed under the cultivation conditions used (although they may be involved in the adaptive response of cells to various stress factors or considerable changes in cell metabolism).

Taking into account the data in the literature that eukaryotic-type serine/threonine protein kinases are involved in the regulation of nitrogen metabolism of the filamentous heterocystous cyanobacterium *Anabaena* PCC 7120 [4, 5], we investigated some relevant characteristics of *Synechocystis* 6803 mutants and found that, when grown in the liquid medium with ammonium or nitrate ions as the sole source of nitrogen, the mutants did not differ from the wild type in the growth kinetics.

Table 1. Resistance of the wild-type and mutant strains of *Synechocystis* 6803 to methylamine

Strain genotype	Minimal inhibitory concentration of methylamine, μg/ml	
	illumination, 20 μerg/(m^2 s)	illumination, 50 μerg/(m^2 s)
Wild type	0.5	0.5
pknE::Cm^r	50	8
pknD::Km^r	0.5	0.5
pknG::Gm^r	0.5	0.5
pknH::Km^r	0.5	0.5

Table 2. The effect of ammonium ions and methylamine on the activity of glutamine synthetase in the wild-type and mutant *Synechocystis* 6803 strains

Nitrogen source	Glutamine synthetase, units (%)	
	wild type	mutant *pknE::Cm^r*
Control	3.65 (100)	3.99 (100)
NH_4Cl	0.77 (21)	0.86 (21)
Methylamine	2.95 (81)	3.19(80)

Note: Glutamine synthetase activity is expressed in μmol γ-glutamylhydroxamate formed per min per mg protein. A 3-day-old culture was supplemented with methylamine or NH_4Cl at a final concentration of 1 mM and incubated for the next 24 h. The control activity refers to 3-day-old cells grown under standard conditions.

This finding indicates that insertion mutagenesis did not cause notable changes in the assimilation pathway of these nitrogen-containing compounds in *Synechocystis* 6803 grown under the standard photoautotrophic conditions.

At the same time, experiments showed that one of the mutants, *pknE*::*Cm*^{r}, differed from the wild-type strain and the other *Synechocystis* 6803 mutants in that it was highly tolerant to methylamine, especially under conditions of low-level illumination (Table 1). Namely, the threshold inhibitory concentration of methylamine for the *pknE*::*Cm*^{r} mutant was 15–100 times higher than it was for the wild-type strain and the other mutants.

It is known that protonated methylamine penetrates into cyanobacterial cells via the same permease systems as ammonium ions [12]. In these cells, methylamine transforms into γ-glutamylmethylamide in the reaction catalyzed by glutamine synthetase [13]. γ-Glutamylmethylamide is not metabolized further and accumulates in the cells. The wild-type strain *Synechocystis* 6803 cannot metabolize methylamine and γ-glutamylmethylamide as nitrogen sources [12], which suggests that the toxic action of methylamine on *Synechocystis* 6803 cells is due to its detrimental effect on photophosphorylation and/or to the accumulation of nonmetabolizable γ-glutamylmethylamide. The high tolerance of the *pknE*::*Cm*^{r} mutant to methylamine can be explained by alterations in the transport of this compound into the mutant cells and its further transformation, or the active metabolic detoxification of methylamine through its pumping-out from the cells or binding by specific proteins. It is also possible that the high tolerance of the mutant to methylamine is associated with a diminished sensitivity of the respective cellular target to this compound or to its conversion product γ-glutamylmethylamide.

To verify these suggestions, we investigated the growth of the wild-type and *pknE*::*Cm*^{r} cells in the medium with pH 7.2 and low concentrations of ammonium ions and found that these two kinds of cells differed neither in their growth characteristics nor in the dynamics of cell bleaching. This finding indicates that methylamine transport in this mutant is not impaired.

Likewise, the mutant and the wild-type strains virtually did not differ in the γ-glutamyltransferase activity of glutamine synthetase (Table 2). Therefore, the high tolerance of the *pknE*::*Cm*^{r} mutant to methylamine cannot be explained by a decreased production of γ-glutamylmethylamide either.

Singh and Singh [14] showed that spontaneous methylamine-resistant mutants of the nitrogen-fixing cyanobacterium *Nostoc muscorum* grow well in a medium with methylamine as the nitrogen source, although these researchers failed to reveal enzymatic systems responsible for methylamine utilization. Our experiments showed that the *pknE*::*Cm*^{r} mutant is unable to grow on methylamine as the sole nitrogen source. It can be assumed that this mutant cannot assimilate methylamine but possesses a system that transforms methylamine into a nontoxic product. This assumption requires further investigation.

The *pknE*::*Cm*^{r} mutant was somewhat more resistant to L-methionine-D,L-sulfoximine (MSX, an analogue of glutamine that irreversibly inhibits glutamine synthetase [15]) than was the wild-type strain (Fig. 2). This can be explained either by an impaired transport of MSX into the mutant cells or by an altered sensitivity of glutamine synthetase to MSX, as was found in some MSX-resistant mutants of the cyanobacteria *Anabaena variabilis* and *N. muscorum* [16, 17]. It is known that glutamine and MSX are transported into *Synechocystis* 6803 cells by a common permease system [18]. The finding that the *pknE*::*Cm*^{r} mutant grows in the medium with 0.25 mM glutamine no poorly than the wild-type strain does indicates that the transport of glutamine and MSX in this mutant is not impaired.

The inhibitory effect of MSX on the glutamine synthetase activity of the stationary-phase mutant cells was weaker than it was in the case of the wild-type cells (Fig. 3). The strong inhibition of glutamine synthetase by MSX in the wild-type cells grown in the medium with nitrate resulted in the accumulation of ammonium

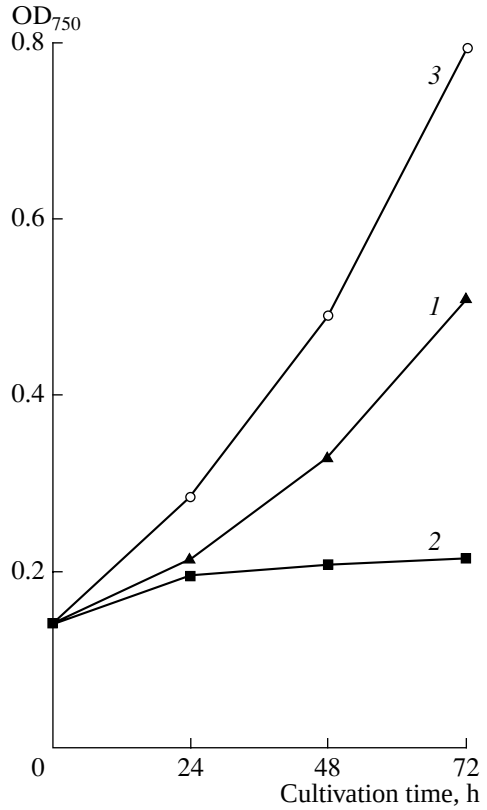

Fig. 2. The effect of L-methionine-D,L-sulfoximine on the wild-type *Synechocystis* sp. strain 6803 grown in the liquid medium with (*1*) 200 and (*2*) 400 μM MSX and (*3*) the *pknE*::*Cm*^{r} mutant grown in the same medium with 400 μM MSX.

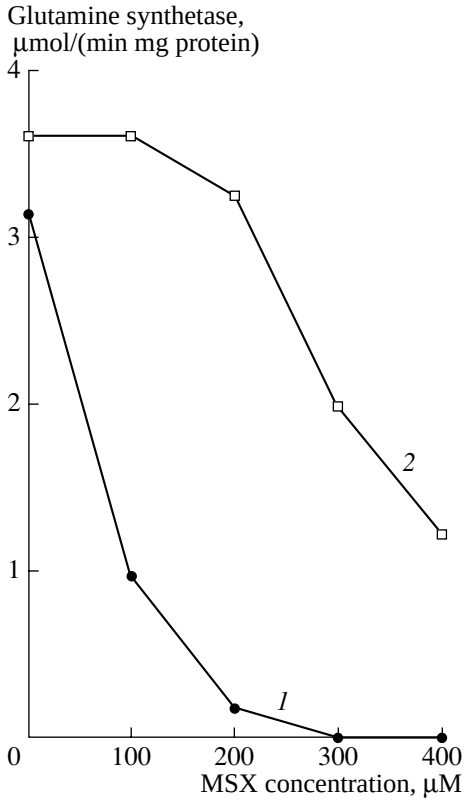

Fig. 3. The effect of L-methionine-D,L-sulfoximine on the activity of glutamine synthetase in (*1*) the wild-type *Synechocystis* 6803 and (*2*) the *pknE*::*Cm*^{r} mutant cells grown under the standard conditions for 5 days. Glutamine synthetase activity is expressed in μmol γ-glutamylhydroxamate formed per min per mg protein.

ions in these cells and the release of these ions into the medium (Fig. 4). In the case of the mutant, such a release was observed at higher MSX concentrations. These data are in agreement with the direct measurements of the effect of MSX on the glutamine synthetase activity of the wild-type and mutant *Synechocystis* 6803 cells (Fig. 3).

It should be noted that *Synechocystis* 6803 has two forms of glutamine synthetase [19], one of which (GlnA) functions when the content of ammonium or nitrate ions in the cultivation medium is normal, whereas the other (GlnN) operates under nitrogen starvation conditions. The sensitivity of these two glutamine synthetases to MSX may be different, which suggests that the high resistance of the *pknE*::*Cm*^{r} mutant to MSX may be associated with different ratios of the two glutamine synthetases in the wild-type and mutant cells [19, 20].

We attempted to gain deeper insight into the role of glutamine synthetase in the enhanced resistance of the *pknE*::*Cm*^{r} mutant to methylamine by investigating the combined effect of methylamine and MSX, the latter of which blocks the formation of γ-glutamylmethylamide and hence increases the intracellular concentration of methylamine. At a sublethal concentration of MSX (100 μM), the resistance of the wild-type cells to methylamine turned out to be drastically increased (Table 3). This can be explained by the fact that the toxic effect of methylamine decreases in the presence of MSX because of the inhibition of γ-glutamylmethylamide production. The different methylamine resistances of the wild-type and mutant cells result from the stronger inhibition of glutamine synthetase in the wild-type than in the mutant cells. At concentrations higher than 100 μM, the difference between the methylamine resistances of the wild-type and mutant cells diminishes due to the profound inhibitory effect of MSX itself.

Thus, the data presented allow the suggestions to be made that the high methylamine resistance of the *Synechocystis* 6803 mutant with the inactivated *pknE* gene is associated with changes in the assimilation pathway of methylamine via glutamine synthetase and that the serine/threonine protein kinase encoded by the *pknE* gene is involved in the regulation of nitrogen metabolism in *Synechocystis* 6803.

Further studies are intended to be directed toward elucidating the role of the *pknE* gene in the control of

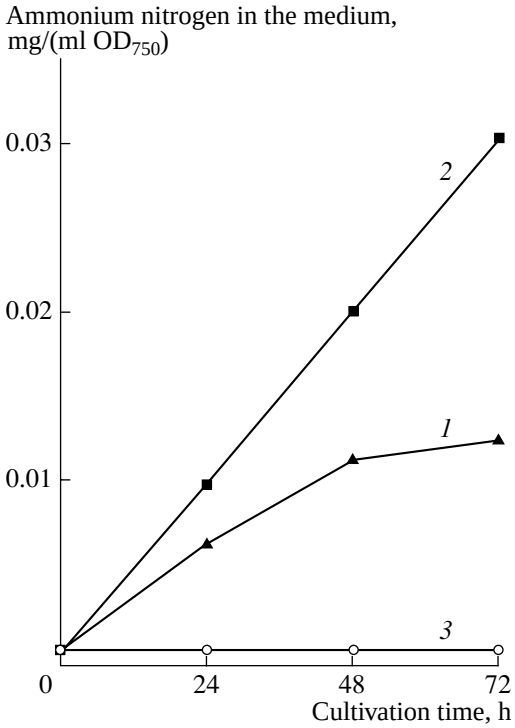

Fig. 4. The effect of L-methionine-D,L-sulfoximine on the release of ammonium ions from the wild-type *Synechocystis* 6803 cells grown in the liquid medium with (*1*) 200 and (*2*) 400 μM MSX and (*3*) the *pknE*::*Cm*^{r} mutant cells grown in the same medium with 400 μM MSX.

nitrogen metabolism and investigating the possibility of the interaction of different protein kinases in this control. These studies will employ the analysis of double mutants in which pairs of the eukaryotic-type serine/threonine protein kinase genes *pknD*, *pknE*, *pknA*, and *pknH* will be inactivated and the analysis of the expression of these genes in response to the action of different stress factors.

Table 3. Resistance of the wild-type and the *pknE*::*Cm*^{r} mutant strains of *Synechocystis* 6803 to methylamine when grown on the solid medium in the presence of the sublethal concentration (100 μM) of L-methionine-D,L-sulfoximine (MSX)

Strain genotype	Minimal inhibitory concentration of methylamine, μg/ml	
	control (no MSX)	MSX, 100 μM
Wild type	0.5	100
pknE::*Cm*^{r}	8	20

Note: The strains were grown under illumination with an intensity of 50 μerg/(m^2 s).

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