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NaCl enhances cellular cAMP and upregulates genes related to heterocyst development in the cyanobacterium, *Anabaena* sp. strain PCC 7120

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Abstract

Cellular cAMP was rapidly increased in the nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC 7120, by the addition of 200 mM NaCl to the culture medium. Other alkaline-metal chlorides such as KCl or LiCl caused a lesser increase. The increase in cellular cAMP was transient and diminished when an adenylate cyclase, CyaC, which contains the conserved domains of the bacterial two-component regulatory system, was disrupted. DNA microarray analysis showed that expression of a gene cluster containing *all5347* and *alr5351* (*hglE*) was upregulated by NaCl in the wild-type strain but not in the *cyaC* mutant. Primer extension analysis indicated that transcription levels of *all5347* and *hglE* were rapidly increased in response to the NaCl addition, and that these genes have NaCl-dependent transcription start sites. It was concluded that NaCl induced expression of genes related to heterocyst envelope formation in this cyanobacterium, possibly via a CyaC–cAMP signal transduction system.

Keywords: Cyanobacteria; *Anabaena*; cAMP; NaCl; Signal transduction; DNA microarray

1. Introduction

Salt stress induces many biological processes that assist organisms to survive in environments with high salt concentrations. The mechanisms responsible for tolerance to salt stress seem to be activated when cells are exposed to high salt concentrations. It has been reported that in *Synechocystis* sp. PCC 6803, the addition of NaCl to the medium enhanced expression of genes related to salt tolerance, such as those that express glucosylglycerol-phosphate synthase [1,2]. There must be a signal transduction pathway for environmental salt stress. However, the regulatory mechanism of gene expression has not yet been elucidated.

In the nitrogen-fixing filamentous cyanobacterium, *Anabaena cylindrica*, cellular cAMP levels exhibit dramatic changes in response to various environmental

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signals such as nitrogen depletion [3], light–dark, aerobic–anaerobic [4] and low pH–high pH [5]. cAMP is a ubiquitous signalling molecule well known as an intracellular second messenger that alters enzyme activities via phosphorylation in animal cells or regulates gene expression by binding to cAMP receptor protein (CRP) in bacteria [6,7]. Recently, we have found that blue light stimulates cell motility via a CRP-mediated cAMP signal-transduction system in the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 [8,9].

In *Anabaena* sp. strain PCC 7120 (hereafter, *Anabaena* PCC 7120), cAMP is synthesized from ATP by adenylate cyclases with various domain structures (CyaA, B1, B2, C, D and E) [10,11]. Of these, CyaC has a unique structure composed of the domains of a bacterial two-component regulatory system [12–14], and is considered to play a key role in the cAMP signalling pathway coupled with receptors for extracellular signals [10,15].

In this study, we found that NaCl increased the cellular cAMP in *Anabaena* PCC 7120. The effect of NaCl on gene expression was also determined using a DNA microarray. A comparison of genome-wide expression changes between wild-type and *cyaC* mutant suggested that the expression of genes for the glycolipid layer of the heterocyst envelope is enhanced via a CyaC–cAMP signal transduction system.

2. Materials and methods

2.1. Organisms and growth conditions

Anabaena PCC 7120 and its derivatives bearing inactivated *cyaA*, *cyaB1*, *cyaB2*, *cyaC* or *cyaD* genes with a spectinomycin and streptomycin-resistant cassette [10] were grown in 50 ml of modified Detmer's medium (MDM) [16] at 30 °C. Air containing 1% CO_2 was bubbled through the medium under continuous illumination at 50 μE m^{-2} s^{-1}. The culture medium contained 1.7 mM NaCl and 1.4 mM K_2HPO_4 as alkaline metal cation sources. Cells at the late log growth phase (optical density at 750 nm was about 0.8) were harvested and washed twice with nitrogen-free MDM (MDM_0). The cell suspension was diluted to an optical density of 0.2 at 750 nm with MDM_0 and incubated for one day under the same culture conditions before use.

2.2. Determination of cellular cAMP level

Cellular cAMP concentrations were determined according to the method of Ohmori [5]. One-millilitre aliquots of cell cultures were mixed with trichloroacetic acid to a final concentration of 5% (w/v) and centrifuged. Supernatants of the mixtures were collected, washed with 3 ml of ethyl ether six times to remove trichloroacetic acid and were then lyophilized. The amount of cAMP in each lyophilized sample was determined using an enzyme immunoassay kit (EIA system; Amersham Biosciences, NJ, USA) according to the manufacturer's protocol. The amount of cAMP in the medium was measured and found to be less than 5 nmol/mg chlorophyll, before and after the experiment. The change in the cAMP level of the lyophilized extract was thus considered to reflect the change in cellular cAMP level.

2.3. DNA microarray analysis

Cells were harvested from 25 ml of culture by centrifugation at 4000*g* for 5 min at 4 °C, frozen immediately in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated by the hot-phenol method of Mohamed and Jansson [17]. The crude total RNA was treated with 5 U of DNase I (Takara, Shiga, Japan) at 37 °C for 1 h to remove DNA contamination. The microarray of *Anabaena* PCC 7120 consisted of 2407 DNA segments of approximately 3 kb in size corresponding to about 90% of the entire genomic sequence, and each spot was duplicated [18]. Synthesis of Cy3-labelled and Cy5-labelled cDNA probes, hybridization with the cDNA probes and washing of the microarrays were performed as described previously [19]. For quantification of the fluorescent intensity of spots, microarrays were scanned with a laser fluorescent scanner (Scanarray 4000; GSI Lumonics, MA, USA), and raw data obtained with the optimum photo-multiplier gain were then analysed with Quantarray software (GSI Lumonics). The local background signal was subtracted, and the signals were normalized using the ratio of the total signal intensity, except for rRNA signals. Changes in the transcript, relative to the total amount of mRNA, were then calculated.

2.4. RT-PCR

One-microgram aliquots from the total RNA, isolated under the same culture conditions as the DNA microarray analysis, were used as the RNA template. Reverse transcription and the following PCR were performed with an RNA-PCR kit (Takara). The primers for RT-PCR are shown in Table 1. Each pair of primers was designed to produce PCR products approximately 300 bp in length. In the PCR amplification, 25 cycles, consisting of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, were applied. The whole process was performed three times using total RNAs purified from independent cell cultures to verify reproducibility.

2.5. Primer extension analysis

The amount and 5′-end of the mRNAs for *all5347* and *alr5351* (*hglE*) were analysed by the primer extension