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FurA influences heterocyst differentiation in *Anabaena* sp. PCC 7120

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ABSTRACT

In *Anabaena* sp. PCC 7120, FurA is a global transcriptional regulator whose expression is strongly induced by NtcA in proheterocysts and remains stably expressed in mature heterocysts. In the present study, overexpression of *furA* partially suppressed heterocyst differentiation by impairing morphogenesis at an early stage. Recombinant purified FurA specifically bound in vitro to the promoter regions of *ntcA*, while quantitative RT-PCR analyses indicated that *furA* overexpression strongly affected the transient increase of *ntcA* expression that occurs shortly after nitrogen step-down. Overall, the results suggest a connection between iron homeostasis and heterocyst differentiation via FurA, by modulating the expression of *ntcA*.

1. Introduction

Biological nitrogen fixation is performed by certain cyanobacteria in environments where inorganic nitrogen species (i.e., ammonium, urea, nitrate, nitrite) become depleted. Since the nitrogen-fixing enzyme nitrogenase is irreversibly inactivated when exposed to molecular oxygen, some filamentous diazotrophic cyanobacteria spatially separate photosynthesis and nitrogen fixation by differentiating highly specialized cells called heterocysts [1,2]. Heterocysts deposit glycolipid and polysaccharide layers outside of their cell walls to limit the entry of atmospheric oxygen. They also lack photosystem II activity and increase their respiration rate to consume O_2 that enters the cell, providing thereby a microoxic compartment for the expression of the oxygen-sensitive nitrogenase. In the filamentous heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, about 7–12% of the vegetative cells terminally differentiate into nitrogen-fixing heterocysts when grown in the absence of combined nitrogen. Single heterocysts are spaced at semiregular intervals of 10–15 vegetative cells along a filament composed of tens or even hundreds of individual cells, each surrounded by its own plasma membrane and a peptidoglycan layer, but sharing a continuous periplasm enclosed by a common outer membrane [3]. Heterocysts provide fixed nitrogen in the form of amino acids to the neighbouring vegetative cells, while vegetative cells supply heterocysts with fixed carbon produced by photosynthesis, thus creating a multicellular microorganism with two mutually dependent cell types [1].

Heterocyst development and its pattern formation are consequences of multiple external and internal signals, the action of several positive and negative regulators, the communication between cells in a filament, and the spatial–temporal regulation of gene expression and cellular processes [2,4,5]. Practically, the timeline of heterocyst development could be divided into three major stages. The early stage comprises the initiation of a complex gene regulatory cascade triggered by a metabolic signal, the accumulation of 2-oxoglutarate (2-OG) resulting from nitrogen limiting conditions. NtcA, a catabolite activator protein (CAP) family transcriptional regulator that functions as the global regulator of nitrogen metabolism [6], senses the increased levels of 2-OG and activates the expression of the master regulator of heterocyst differentiation HetR [7] via NrrA [8,9]. In a mutually dependent expression, the induction of *hetR* produces a transient increase of NtcA levels in a HetR-dependent manner [10], and this mutual up-regulation under a nitrogen deficiency status appears essential for the subsequent induction of other heterocyst development genes [11]. The early stage of heterocyst differentiation process is

Abbreviations: CAP, catabolite activator protein; Chl, chlorophyll *a*; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; Fur, ferric uptake regulator; *hep*, heterocyst envelope polysaccharide; *nif*, nitrogen fixation; nt, nucleotides; tsp, transcriptional start point; μE, microEinstein; qRT-PCR, quantitative reverse transcription PCR; 2-OG, 2-oxoglutarate

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also characterized by degradation of the nitrogen reserves due to inducible specific proteases in the committed cells, and these "early proheterocysts" begin to lose phycocyanin and oxygen-evolving capacity, becoming morphologically discernible [12,13]. The middle stage of differentiation to heterocysts is distinguished by structural and physiological changes [2]. These changes begin with morphogenesis of the heterocyst envelope by the deposition of the outer polysaccharide layer and the inner glycolipid layer by 12 h after nitrogen step-down [13]. Before this time, the process of differentiation is reversible if a source of combined nitrogen is added [2]. By the late stage, which occurs between 18 and 24 h after nitrogen deprivation and culminates with fully developed mature heterocysts, the nitrogen-fixation (*nif*) genes are expressed as a result of multiple DNA rearrangements due to developmentally programmed site-specific recombination events [2].

Acting downstream to NtcA/HetR induction, other players in the regulatory cascade have been identified, including PatS [14] and HetN [15], which respectively govern de novo pattern formation and pattern maintenance by suppressing the differentiation of vegetative cells near to the proheterocysts and mature heterocysts. In addition, expression of *hanA*[16], *hetC*[17], *devBCA*[18] or *devH*[19], among other genes [1,2,20], is essential for the early stage of differentiation as well as for maturation of heterocysts.

In nature, biological nitrogen fixation is also influenced by iron bioavailability [21,22]. Iron is one of the most important potentially limiting nutrients for photoautotrophic growth in diverse environments, from the open ocean to coastal regions and lakes [23,24]. Diazotrophy imposes additional iron demands for growth beyond those attributed to photosynthesis and respiration, since nitrogenase contains up to 36 iron atoms per enzyme complex, in addition to the iron-containing proteins required to supply sufficient reductant for nitrogen fixation. To maintain viability under iron plus nitrogen limitation, the marine cyanobacterium *Trichodesmium erythraeum* IMS101 selectively sacrifices nitrogen fixation to conserve iron for photosynthetic and respiratory electron transport [25,26], while nitrogen fixation and heterocyst differentiation are severely impaired in *Anabaena* sp. PCC 7120 grown under iron deficiency [27–29].

In previous studies using in vitro and in silico analyses, we have identified a variety of potentially overlapping transcriptional targets of NtcA and the essential ferric uptake regulator FurA [30]. In addition, *furA* appeared up-regulated after nitrogen deprivation specifically in proheterocysts and mature heterocysts. This induction depended on NtcA as it did not occur under a *ntcA* mutant background [31]. As the master regulator of iron homeostasis and sensor of iron availability, FurA could have a critical role in the modulation of heterocyst differentiation. In the present study, we present evidence that FurA influences heterocyst development in *Anabaena* sp. PCC 7120 by controlling the expression of NtcA.

2. Materials and methods

2.1. Strains and growth conditions

The heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 and its *furA*-overexpressing derivative strain AG2770FurA [32] were grown photoautotrophically at 30 °C in BG-11 medium [33] or BG-11_0 (BG-11 without $NaNO_3$) under continuous illumination with white light at 75 μE m^{-2} s^{-1}. All cultures were supplemented with 0.84 g of $NaHCO_3$ l^{-1} and bubbled with a mixture of CO_2 and air (1% v/v). For the strain AG2770FurA, cultures were supplemented with 50 μg ml^{-1} neomycin. Chlorophyll *a* (Chl) was determined in methanol extracts [34].

For heterocyst induction, exponentially growing filaments (3 μg Chl ml^{-1}) from standard BG-11 were collected by filtration, washed twice with BG-11_0 and resuspended in the same nitrogen-free medium. The cultures were further incubated for 72 h and the filaments were scored for heterocyst frequency at 24 h time intervals.

For RNA isolation, samples of 100 ml from cultures in BG-11_0 were collected at the number of hours after induction as indicated in the experiment. Filaments were harvested at room temperature and immediately frozen in liquid nitrogen for RNA isolation.

2.2. Heterocyst frequency, pattern spacing and microscopy

Filaments were treated with 0.05% alcian blue (Sigma) for 5 min to stain heterocysts. Heterocyst frequency was defined as the percentage of heterocysts in the total cyanobacterial cell population, and determined by counts of more than 1000 cyanobacterial cells per sample.

Pattern spacing was defined as the mean distance between differentiating cells, measured as the number of intervening vegetative cells, averaging 20 intervals per sample.

Photomicrographs were taken under a 40× dry objective lens on an Olympus BX60 microscope equipped with a DFC300 FX Leica digital camera.

2.3. Acetylene reduction assay

Nitrogenase activity was determined by the acetylene reduction assay at 24, 48 and 72 h after nitrogen step-down. Samples of 2 ml containing 10 μg Chl ml^{-1} in BG11_0 were incubated in an atmosphere of 14% acetylene in air, with shaking and standard illumination at 30 °C. Production of ethylene was determined by gas chromatography in samples taken after 0, 30 and 60 min of incubation.

2.4. Electrophoretic mobility shift assays

Recombinant *Anabaena* sp. PCC 7120 FurA protein was produced in *Escherichia coli* BL21 (DE3) using the expression vector pET28a (EMD Biosciences), and purified according to previously described methods [35]. The promoter regions of each gene of interest were obtained by PCR using the primers listed in Table S1. Electrophoretic mobility shift assays (EMSA) were performed as described previously [36]. Briefly, 100–120 ng of each DNA fragment were mixed with recombinant FurA protein at concentrations of 300, 500 and 700 nM in a 20 μl reaction volume containing 10 mM bis-Tris (pH 7.5), 40 mM potassium chloride, 0.1 mg ml^{-1} bovine serum albumin, 1 mM dithiothreitol (DTT), 100 μM manganese chloride, and 5% glycerol. In some experiments, EDTA was added to a final concentration of 200 μM. To ensure the specificity of EMSA, the promoter region of *Anabaena* sp. *nifJ* (*alr1911*) gene was included as non-specific competitor DNA in all assays. In addition, the promoter region of *furA* was included as positive control [32]. Mixtures were incubated at room temperature for 30 min, and subsequently separated on 4% non-denaturing polyacrylamide gels in running buffer (25 mM Tris, 190 mM glycine) at 90 V. Gels were stained with SYBR® Safe DNA gel stain (Invitrogen) and processed with a Gel Doc 2000 Image Analyzer (Bio-Rad).

2.5. DNase I footprinting analysis

DNase I protection assays were performed as previously described [9], with slight modifications. Briefly, protein-DNA complexes were formed in a final volume of 15 μl reaction buffer containing 10 mM bis-Tris (pH 7.5), 40 mM KCl, 100 μM $MnCl_2$, 1 mM DTT, 0.07 mg ml^{-1} bovine serum albumin, 0.07 mg ml^{-1} poly(dI–dC), 5% glycerol with 10 fmol of ^{32}P-end-labelled (with T4 polynucleotide kinase and [γ-^{32}P]ATP) DNA fragment, 1 mM