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Fatty alcohols can complement functions of heterocyst specific glycolipids in *Anabaena* sp. PCC 7120

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ABSTRACT

Heterocyst glycolipid synthase (HglT) catalyzes the final step of heterocyst glycolipid (Hgl) biosynthesis, in which a glucose is transferred to the aglycone (fatty alcohol). Here we describe the isolation of *hglT* null mutants. These mutants lacked Hgls under nitrogen-starved conditions and instead accumulated fatty alcohols. Differentiated heterocyst cells in the mutants were morphologically indistinguishable from those of the wild-type cells. Interestingly, the mutants grew under nitrogen starvation but fixed nitrogen with lower nitrogenase activity than did the wild-type. The mutants had a pale green phenotype with a decreased chlorophyll content, especially under nitrogen-starved conditions. These results suggest that the glucose moiety of the Hgls may be necessary for optimal protection against oxygen influx but is not essential and that aglycones can function as barriers against oxygen influx in the heterocyst cells.

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1. Introduction

Anabaena sp. PCC 7120 (hereafter denoted as *Anabaena*) is a representative filamentous cyanobacterium whose genomic sequence was determined more than a decade ago [1]. In the absence of fixed nitrogen, *Anabaena* differentiates its vegetative cells into heterocysts, which are specialized nitrogen-fixing cells [2–4]. These cells provide the microoxic environment necessary for the proper function of the oxygen-labile nitrogenase. Heterocysts have a thick envelope, consisting of an inner layer of heterocyst-specific glycolipids, Hgls, and an outer layer of heterocyst envelope polysaccharides, HEPs, which act as a barrier against the inward diffusion of oxygen [5–8]. The thick lipid layer consists of a hexose head group [9] and a fatty alcohol that has a very long chain of carbon atoms (26–32 carbons) with three or four oxygenated groups, most of which are hydroxyl groups [8]. In *Anabaena*, the structures of Hgls have been fully elucidated [10]. Hgls from *Anabaena* are comprised of 1-α-glucosyl-3,25-hexacosanediol as the major constituent and 1-α-glucosyl-3-keto-25-hexacosanol as the minor constituent [11].

A number of genes are involved in Hgls synthesis and deposition in *Anabaena*. The gene cluster containing *hglE*$_A$, *hglF*, *hglG*, *hglD*, *hglC*, *hglA*, *and hglB* is necessary for the synthesis of the fatty alcohol moiety (aglycones) of the Hgls [12], whereas *hglK* is required for the localization of glycolipids [13]. The *devBCA* gene cluster is necessary for glycolipid export [14], and this transporter interacts with a TolC protein in outer membranes [15]. The inactivation of any of these genes influences either the synthesis or localization of Hgls. In addition, the *devH* mutant forms heterocyst; however, they are incapable of fixing nitrogen in the presence of oxygen. An ultrastructural analysis showed that the absence of the Hgl layer from the heterocyst envelope was associated with such phenotypes [16] but the exact function of DevH protein is unknown.

The heterocyst glycolipid synthase (HglT), which is encoded by *hglT*, catalyzes the final step of Hgl biosynthesis, a reaction involving the transfer of glucose to the fatty alcohol. Partial knockout mutants of *hglT* were isolated and found to lack the Hgl layer in the heterocyst cells. These mutants showed retarded growth in a nitrogen-free medium. This may be due to the inability

Abbreviations: HEP, heterocyst envelope polysaccharide; HglT, heterocyst glycolipid synthase; Hgls, heterocyst glycolipids.

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of nitrogenases to fix nitrogen in the heterocysts, thus restricting the supply of fixed nitrogen to neighboring vegetative cells [17]. The mutants accumulated fatty alcohols instead of Hgls under nitrogen-starved conditions, implying that the sugar moiety of Hgls is important in maintaining the function of Hgls in the envelope. The mutants grew normally under nitrogen-replete conditions. However, we could not rule out the possibility that the partial knockout mutants underwent a recombination event that eliminated the mutation, which would indicate that the residual copy of *hglT* was necessary for the normal growth of *Anabaena*.

To clarify the function of the HglT protein under nitrogen-replete and -deprived conditions, we isolated null mutants of the gene. The growth of the null mutants was comparable to that of the wild-type under nitrogen-replete conditions. Unexpectedly, under nitrogen-deprived conditions, *hglT* mutants showed retarded, but abundant growth and were able to fix nitrogen. In this study, we show that the fatty alcohol can, at least in part, complement the function of Hgls in *Anabaena* heterocysts.

2. Materials and methods

2.1. Cyanobacterial strains and growth conditions

Anabaena sp. PCC 7120 and *hglT* mutant strains were grown in the liquid medium of BG11 (containing nitrate as a source of nitrogen) [18] at 30 °C in the light (50–80 μmol m^{-2} s^{-1}) on rotary shaker (120 rpm) as described previously [19]. For nitrogen starved condition experiments, cells were first grown in the BG11 medium to an optical density at 730 nm (OD_{730}) of 0.8–1.2 and washed three times with nitrogen-free medium ($BG11_0$: BG11 without nitrate) and then resuspended in $BG11_0$.

2.2. Isolation of null mutants of hglT

Knock out vector of *hglT* gene was constructed as follows. DNA fragments upstream and downstream of *hglT* gene were amplified by PCR using the primer pairs of Fw1 (ACTAGTGGATCCCCCTCTG ACAAATCCGACG) and Rv1 (GAATTCCTGCAGCCCGGGCGCAATGCGA AGCTTTG), and Fw2 (TACCGTCGACCTCGATTGGTCAGCCTGTATG) and Rv2 (CGGGCCCCCCCTCGAAGTTTTAGCCACAGTTC), respectively. The upstream fragment was cloned into *Sma*I site of pMobKm1 (see below) by In-Fusion HD Cloning Kit with Cloning Enhancer (Takara Bio., Shiga Japan), and then downstream fragment was cloned into *Apa*I site to construct the knock out vector, pMK1hglTKO. This plasmid vector was introduced into wild-type *Anabaena* by triparental mating by the method of Elhai and Wolk [20].

pMobKm1 was constructed with DNA fragments including oriVT from pRL271 (obtained from Dr. CP Wolk, Michigan State University) and SacB from pK18mobSacB (obtained from the National BioResource Project of Japan). The kanamycin resistance gene was from pRL161 and subcloned into *Hin*dIII site of pBluescript II SK+, then it was re-amplified with multi cloning sites and ligated with oriVT and SacB by the In-Fusion system.

Genomic DNA from wild-type and transformants were used for genotyping as templates for PCR with the primers described below and Hybripol DNA polymerase (Bioline). PCR-based confirmation of gene disruption was performed using primers Fw1 and Rv2 for amplification of full-length *hglT*, Fw3 (CCGCTTCCTTTAGCAGC) and Rv2 for insertion of the kanamycin resistance gene into *hglT*, and Fw1 and Rv3 (ACTACTGGAGTACCAGAG) for detection of deletion of the central part of *hglT*.

2.3. Microscopy

Anabaena sp. PCC 7120 wild-type and the mutant filaments were visualized with bright field and fluorescence microscope (BX53, Olympus). Heterocyst-containing culture were stained with 0.5% Alcian blue in a 50% ethanol solution prior to microscopy and incubated for 30 min before observation.

2.4. Chlorophyll content and cell spectrum

Cells of each strain were harvested from 1 mL culture with OD_{730} of 0.8–1.2. The pellet was resuspended in 90% methanol and measured by the method of Meeks and Castenholz [21]. Absorption spectra of cells were determined by harvesting 1 mL of the cells and resuspended in fresh BG11 medium prior to measurement. Then the cells were scanned from 350 to 800 nm by using a spectrophotometer UV-2450 (Shimadzu) with an integrating sphere.

2.5. Lipid analysis

Anabaena cultures ($OD_{730} \simeq 1.0$) were harvested at room temperature by centrifugation at 3000 rpm for 15 min. Lipids were extracted from those cell pellets by a modification of the Bligh and Dyer methods as described previously [17]. Then the lipids were separated by TLC using a solvent system of chloroform/methanol/acetic acid/water (85:15:10:3.7, v/v), and visualized with 50% sulfuric acid by spraying and heating at 120 °C for 10 min. Gas Chromatography (GC) was carried out using a Shimadzu QP2010SE equipped with a flame ionization detector on a capillary column (BPX5, 30 m × 0.25 mm, SGE Analytical Science). The column temperature was programmed at 240 °C. The injector and detector temperature were 200 °C and 240 °C, respectively. The flow rate of carrier gas (He) was 0.4 mL/min.

2.6. Acetylene reduction assays

The nitrogenase activity was determined by harvesting 1 mL culture (approximately 3–7 μg of chl a/mL) and transferred to 7.8 mL vials, and acetylene was added to a final concentration of 12% v/v in air. After 0.5–1 h of incubation under illumination, the concentration of ethylene was assayed as described previously [22].

2.7. Western blotting and immunodetection

Proteins extracts were electrophoretically resolved by 12% SDS–PAGE and electroblotted onto Hybond-P PVDF membrane (GE Healthcare). NifH was detected by an antibody, purchased from a company (Agrisera, Vännäs, Sweden), at a 1: 40,000 dilution. Blots were washed three times with blocking buffer (5% skim milk, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20) and incubated with secondary antibody (anti-hen IgY horse radish peroxidase conjugated, Abcam, Tokyo Japan) diluted to a 1: 40,000 for 1 h at room temperature. The protein was detected with an ECL Plus Western blotting detection reagents (GE Healthcare) according to the manufacturer's instruction and visualized using LAS-4000 Mini (GE Healthcare).

2.8. Reverse transcription and RT-PCR

Four hundred nanogram of purified RNA was used for cDNA synthesis with random hexamer and PrimeScript II Reverse Transcriptase (Takara) according to the manufacturer protocol. The generated cDNA was used as a template for RT-PCR with the primer pairs for nifH (ACCTCGTGACAACATCGTTC and TTGGTGTAGGAA TGGTGAGC) and rnpB (CCAGTTCCGCTATCAGAGAG and GAG-GAGAGAGTTGGTGGTAAG). The *rnpB*, encodes the RNA subunit of RNaseP, served as a loading control.

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