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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.

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.

3. Results

3.1. hglT is not essential in Anabaena

To clarify whether *hglT* (*all5341*) of *Anabaena* is required under both nitrogen-replete and -depleted conditions, we isolated a null mutant of *hglT* from *Anabaena*. A PCR analysis using primers external to *hglT* (Fig. 1A, Fw1 and Rv2; see Section 2) resulted in two PCR fragments of different sizes. The wild-type produced a 2976-bp fragment, and the mutant produced a larger 3259-bp fragment because of the presence of the kanamycin resistance cassette. PCR with a primer that annealed to the middle region of *hglT*, Rv3, produced an amplicon only from the wild-type genome, as the necessary region was not present in the mutant genome. PCR with primer Fw3, which anneals to the kanamycin resistance cassette, produced amplicons only from the mutant genome. These results indicated that no wild-type copies of *hglT* were present in the mutant cells. The mutants grew on BG11 plates, which use nitrate as a nitrogen source, indicating that *hglT* is not an essential gene, at least under nitrogen-replete conditions.

3.2. hglT mutants grow slowly but consistently under nitrogen-starved conditions

To evaluate whether the *hglT* mutants could grow under nitrogen-starved conditions, growth profiles of the wild-type and *hglT* mutant cells were compared. In the BG11 medium (Fig. 1B) and on agar plates (data not shown), the mutants did not show altered growth rates compared with the wild-type. Interestingly, the chlorophyll content of the null mutants under nitrogen-replete conditions was slightly decreased compared with the wild-type (Fig. 2A, Fig. S1). This phenomenon was also confirmed using a whole-cell absorbance spectrum normalized by cell density (Fig. 2B). Under the nitrogen-replete conditions, the absorbance peaks at ~438 and ~675 nm, both of which correspond to chlorophyll a, were decreased in the mutants, but there was no substantial change in the peak at ~625 nm, which corresponds to phycocyanin, as compared with the wild-type. However, under nitrogen-starved conditions, the mutants showed slightly retarded growth compared with the wild-type (Fig. 1B). In a previous report, a partial mutant of *hglT* that was transferred to a nitrogen-free medium for 3 days showed a pale green phenotype [17]. In accordance with that result, our *hglT* null mutants remained pale green with decreased chlorophyll

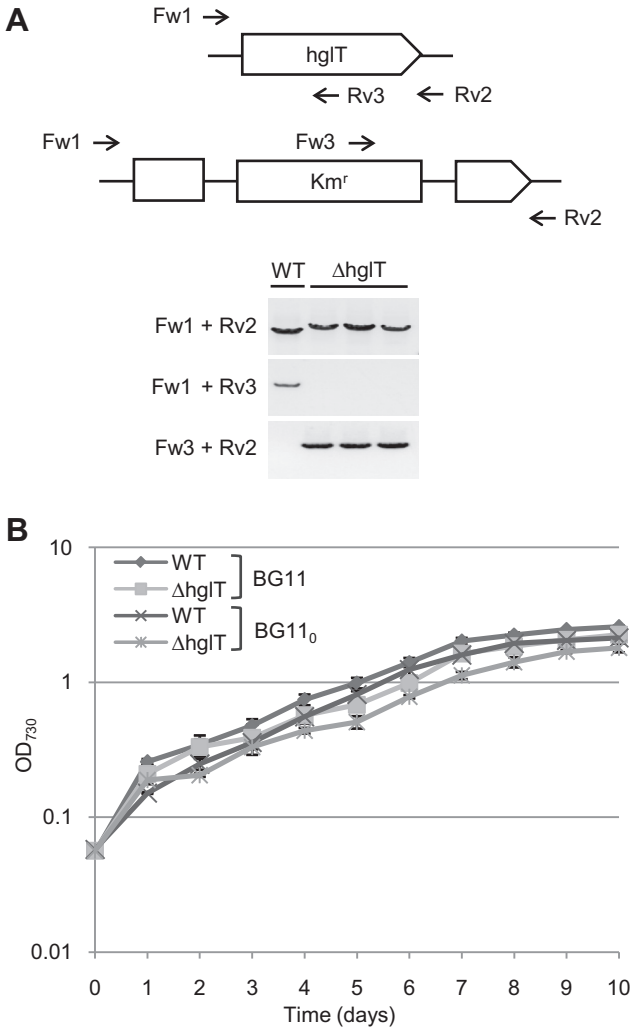

Fig. 1. Genotype and growth rate of the *hglT* mutant of *Anabaena* sp. PCC 7120. (A) The scheme shows the map of *hglT* gene disruptions and the primers used for genotyping. Km^r; kanamycin resistant gene, WT; *Anabaena* sp. PCC 7120 wild-type, ΔhglT; hglT mutant. (B) Growth rate of *Anabaena* wild-type and *hglT* mutant with nitrate as the nitrogen source (BG11 medium) or without combined nitrogen (BG11$_0$ medium). Cells were first grown in the BG11 medium to OD$_{730}$ = 0.8, washed and transferred to new medium of BG11 or BG11$_0$ at OD$_{730}$ = 0.05.

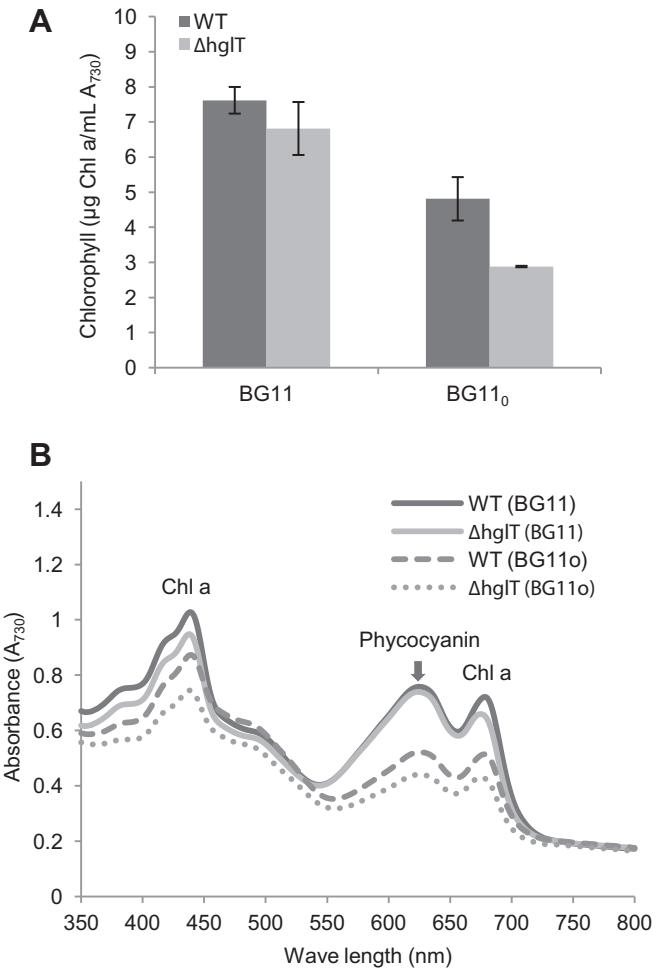

Fig. 2. Chlorophyll content and whole cell spectrum of the *Anabaena* wild-type (WT) and hglT mutant (ΔhglT). (A) Chlorophyll content in nitrogen replete (BG11) and deprived (BG11$_0$) conditions. Error bars indicate the SD based on three independent experiments. (B) Whole-cell spectrum. The intact cells spectra were measured in cell suspensions (OD$_{730}$ ≃ 0.8–1.0) and normalized to an OD$_{730}$ of 0.2. The peak at 637 nm is due to phycocyanin and the peaks at 683 nm and 439 nm chlorophyll a.

content under nitrogen-starved conditions (Fig. 2A, Fig. S1). A cell spectrum showed that all of the peaks, including that of phycocyanin, were decreased under the nitrogen-deprived conditions (Fig. 2B). Based on these results, we predicted that the decrease in chlorophyll and phycocyanin under the nitrogen-deprived conditions in *hglT* mutants was likely to result from a restricted nitrogen fixation activity in the heterocyst cells.

3.3. Nitrogenase is active in the hglT mutants

The ability of the *hglT* null mutant to fix nitrogen was measured under nitrogen-starved conditions. We first analyzed the morphology of the mutants and found that differentiated heterocysts from the mutant cells were indistinguishable from those of wild-type cells (Fig. S2). Heterocyst cells from both the mutants and wild-type stained with Alcian blue, which indicated that the cells were surrounded by the HEP layer (Fig. S3). We next evaluated the nitrogenase activity of the mutants under nitrogen-starved conditions. The nitrogenase activity of the *hglT* mutants was considerable, but the maximum levels were three times lower than that of the wild-type strain (Fig. 3A). The wild-type strain had its highest nitrogenase activity 24 h after the nitrogen step-down, whereas the mutants reached their maximum levels 48 h after the nitrogen step-down. We also evaluated the expression of *nifH*, which encodes a subunit of nitrogenase, at the mRNA and protein levels. According to Fig. 3B and C, the *hglT* mutants had similar amounts of NifH mRNA and protein compared with the wild-type, indicating that the retarded nitrogenase activities in the mutants could be due to the failure to maintain microoxic conditions in the heterocyst cells.

3.4. Fatty alcohol can complement the function of Hgls

Partial mutants of *hglT* do not contain detectable amount of Hgls under nitrogen-starved conditions [17]. To confirm that the null mutants also lacked Hgls, lipid profiles of the *hglT* mutants were analyzed by thin-layer chromatography. In the *hglT* mutants, neither the major nor minor Hgl was detected in the absence of combined nitrogen, and instead, there was an accumulation of the fatty alcohol (aglycone; Fig. 4A). This indicated that in the wild-type HglT transfer a glucose moiety to both the major and minor aglycone in *Anabaena*. A quantitative analysis of lipids showed that the aglycones in the mutant and the Hgls in the wild-type accumulated to similar levels (Fig. 4B), and the membrane lipid composition of the mutant was not significantly altered (Fig. 4B). These results suggested that the gradual increase in the nitrogenase activities of the mutants comes from fatty alcohols assuming a function that is, at least in part, complementary to that of the Hgls. Concomitantly, the rate of heterocyst formation increased in the mutants (Fig. S4), which may be due to their decreased ability to fix nitrogen.

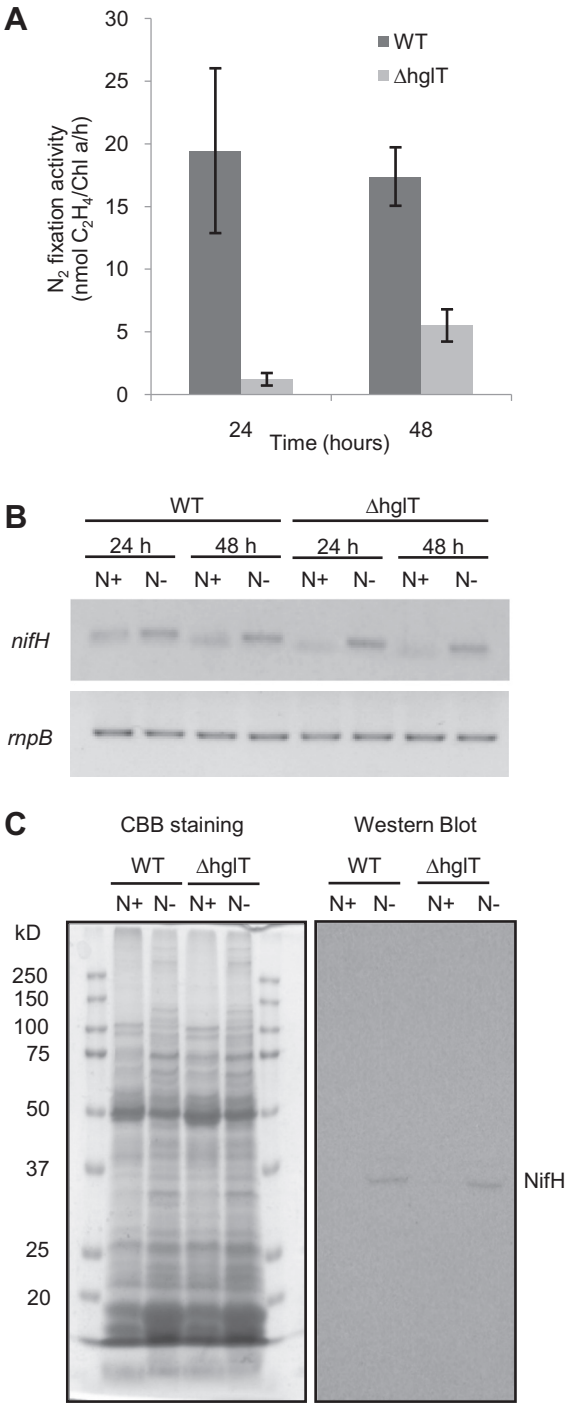

Fig. 3. Nitrogen fixation activity and expression of nifH in *Anabaena* wild-type (WT) and *hglT* mutant (ΔhglT). (A) Nitrogenase activity of WT and ΔhglT. Cells were first grown in BG11 and transfer to $BG11_0$ after three times of wash. Time indicates the hours after nitrogen depletion. Error bars indicate the SD based on three independent experiments. (B) RT-PCR of the gene for a subunit of nitrogenase, *nifH*. (C) Western blot analysis of NifH protein. Cells are deprived of fixed nitrogen for 48 h and proteins were extracted. Size of NifH is about 32.5 kDa. Size marker: Precision Plus Protein Kaleidoscope Standards (BioRad).

4. Discussion

4.1. Localization of the aglycones in the heterocysts of hglT mutants

We propose that heterocyst aglycones localize to the space between the outer membrane and the HEP layer, as do the Hgls in the wild-type. These aglycones are predicted to be synthesized by a polyketide synthase with the support of several enzymes, including a chain length factor, reductases and a dehydrogenase [12,17]. Among those enzymes, $HglE_A$ has a central role in the synthesis of Hgls, and knockout mutants of this enzyme cannot grow under nitrogen-deprived conditions. This mutant lacks neither Hgls nor its aglycones, indicating that Hgls are crucial for the function of the Hgl layer.

It has been speculated that in the partial knockout mutants of *hglT*, aglycones are not transported to the space between the outer membrane and HEP layer but instead are allowed to accumulate within the heterocyst cells [17]. However, the *hglT* null mutant

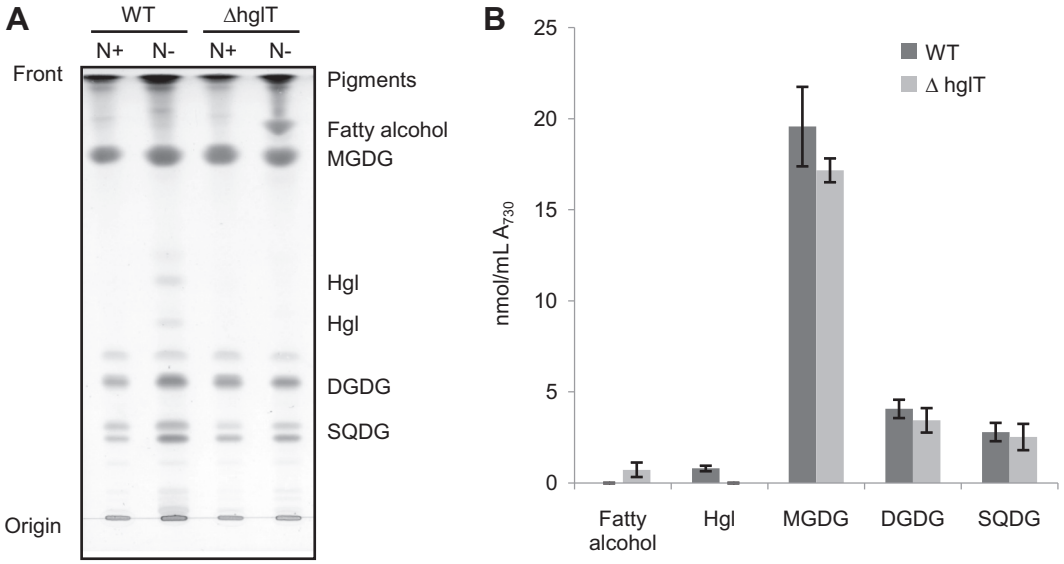

Fig. 4. Lipid composition of *Anabaena* sp. PCC 7120 wild-type (WT) and *hglT* mutant (ΔhglT). (A) Lipids profiles separated by TLC. *Anabaena* WT and ΔhglT were grown in BG11 or $BG11_0$ (48 h of nitrogen step down). (B) Lipid content per cell density. Lipids were quantified by GC. ΔhglT accumulated fatty alcohol and lacked Hgls. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

showed nitrogenase activity under nitrogen-deprived conditions without Hgls. This result indicates that the aglycones can complement, at least in part, the function of Hgls as a barriers against oxygen influx. Thus, the aglycones must form an envelope structure, creating a barrier for the heterocyst cells. In fact, in the *devBCA* mutant, Hgls accumulate in the heterocysts; however, this mutant does not have nitrogenase activity under nitrogen-starved conditions [23]. This indicates that the layered structure is required to protect the nitrogenase from oxygen influx. To visualize the accumulation of aglycones in the null mutants, we observed the heterocyst cells by electron microscopy but could not see the aglycones (data not shown). However, the space between the outer membrane and the HEP layer, especially at the connecting sites between heterocysts and vegetative cells, had gaps as previously reported [17]. It is likely that without the glucose head, the aglycones are barely stained by osmium and/or uranyl acetate. Other dyes that stain neutral lipids, such as Nile red, will be used in subsequent aglycone localization studies.

4.2. Other factor(s) involved in the formation of Hgl layers

It took over 6 months to isolate the *hglT* null mutants by segregation. One reason for the rarity of these mutants may be that *hglT* is essential under both nitrogen-replete and -deprived conditions, leading to the introduction of a mutation that suppresses the effect of an *hglT* deletion into the genomes of the null mutants. In fact, inactivation of *Anabaena hglT* reduced the chlorophyll content (Fig. 2) and bulk photosynthetic activity (data not shown) as compared with the wild-type, even under nitrogen-replete conditions. Winkenbach et al. reported that the isolated Hgl layer contains a very small amount of non-lipid organic material [24]. However, it is still possible that even this very small amount of material is pivotal for the formation of the glycolipid layer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.093.

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