SURE 静岡大学学術リポジトリ
Shizuoka University REpository

Physiological analysis of heterocyst specific glycolipid and production of its aglycone, fatty alcohol in Anabaena sp. PCC 7120

メタデータ	言語: en 出版者: Shizuoka University 公開日: 2015-04-24 キーワード (Ja): キーワード (En): 作成者: Heli, Siti Halimatul Munawaroh メールアドレス: 所属:
URL	https://doi.org/10.14945/00008274

THESIS

Physiological analysis of heterocyst specific glycolipid and production of its aglycone, fatty alcohol in *Anabaena* sp. PCC 7120

Heli Siti Halimatul Munawaroh

Graduate School of Science and Technology,
Educational division, Department of Bioscience
Shizuoka University

June 2014

THESIS

Physiological analysis of heterocyst specific glycolipid and production of its aglycone, fatty alcohol in *Anabaena* sp. PCC 7120

Anabaena sp. PCC 7120 におけるヘテロシスト

特異的糖脂質の生理学的解析とそのアグリコン

である脂肪アルコールの生産

Heli Siti Halimatul Munawaroh

静岡大学
大学院 自然科学系教育部
バイオサイエンス専攻

2014 年 6 月

Summary

Cyanobacteria is a large group of gram-negative prokaryotes that perform oxygenic photosynthesis. These microorganisms are widely distributed and occupy a wide range of environmental conditions because many of them are also have ability to convert nitrogen molecule to available form of ammonia using nitrogenases. However, photosynthesis and nitrogen fixation are incompatible each other because nitrogenase can be inactivated by oxygen. Cyanobacteria mainly use two mechanisms to separate these activities. Some cyanobacteria strictly separate those two processes temporally by expressing their nitrogenase only in dark when the photosynthesis is inactive and the intracellular oxygen pressure is low. Instead, the others, such as filamentous cyanobacteria, use a spatial separation by making a compartment to conduct nitrogen fixation in highly special cells, called heterocysts.

Anabaena sp. PCC 7120 (hereafter referred as *Anabaena*) is a multicellular cyanobacteria and has long filament consists of hundreds or more vegetative cells in the presence of combined nitrogen source in the medium. When the concentration of combined nitrogen is low in the environment, *Anabaena* the representative filamentous heterocystous cyanobacterium, develops heterocyst cells to separate oxygen-labile nitrogen fixation from oxygen-evolving photosynthesis in vegetative cells. These cells are surrounded by glycolipid layer (heterocyst specific glycolipid, Hgl) to protect nitrogenase from oxygen diffusion from outside of the cells. Heterocyst differentiation requires a number of genes involved in the different step of the developmental process. There are three group

of genes cluster that involved in heterocyst differentiation: (1) Sensory genes which perceive the signal of nitrogen starvation in the environment; (2) genes that are responsible for initiating the heterocyst differentiation; and (3) group of genes which play role in heterocyst morphogenesis and function. Among of these genes, *hglT*, encodes the heterocyst glycolipid synthase and involved in the maturation of heterocyst. Heterocyst glycolipid synthase, HglT, catalyzes the final step of the Hgls synthesis, a glucose transfer reaction to the aglycone (fatty alcohol).

To clarify the physiological function of HglT protein under nitrogen-replete and –depleted conditions, the author isolated the complete knock out mutants of *hglT* gene. The *hglT* mutants grew comparable to that of the wild type under nitrogen-replete conditions, indicating that *hglT* gene is not an essential gene at least in the nitrogen-replete conditions. *hglT* mutants formed morphologically indistinguishable heterocyst under nitrogen starved conditions. Alcian blue staining of heterocyst cells from both the mutants and wild type indicated that the cells were surrounded by heterocyst polysaccharide layer (Hep). The mutants, however lacked detectable amount of Hgls, and accumulated fatty alcohol (aglycone). Nitrogenase activity of the mutants reached to the maximum level but one fourth of those of the wild type 48 hours after nitrogen step-down. Protein accumulation and gene expression of a subunit of nitrogenase in the mutant was similar level to the wild type, indicating that the retarded nitrogenase activity is due to the failure of heterocyst to maintain the micro-oxic environment. These results suggest that the mutants can fix nitrogen without Hgls in the presence of oxygen and thus, fatty alcohol can complement the function of Hgls, not perfectly but sufficiently in the heterocyt cells.

To increase the amount of fatty alcohol accumulated in the *hglT* mutant, the author then combined mutation of *hglT* with *patS* and *hetN*. It is known that PatS and HetN are involved in the heterocyst development and maintain the pattern of heterocyst formation. Both of genes are necessary to prevent heterocyst differentiation, and knock out mutants of these two genes are known to form multiple heterocyst under nitrogen starvation. Similar to the *hglT* single mutant, the triple mutants differentiated heterocyst cells under nitrogen starved conditions. As expected the multiple heterocysts and high frequency of heterocysts formation were observed in the triple mutant compared to the *hglT* mutant and wild type. Interestingly, the triple mutant showed slight increase of growth and total chlorophyll content under nitrogen starved conditions compared with the *hglT* mutant. A plausible interpretation would be that the increased heterocyst frequency in the triple mutant might promote the recovery of chlorophyll biosynthesis and improve the energy flowing efficiency from phycocyanin to chlorophyll a. In addition, higher amount, almost two fold, of fatty alcohol was accumulated in the triple mutant. These fatty alcohols are probably substituting the function of Hgls as has been characterized in the *hglT* mutant. The increase of heterocyst frequency of the triple mutant, reached to three times higher compare with *hglT* mutants, resulted in accumulation of fatty alcohols. In addition, aeration to the culture increased the accumulation of Hgl in the wild type and fatty alcohol production in the single and triple mutant of *Anabaena*. These results suggest that filamentous cyanobacteria can be a good tool to provide fatty alcohol.

List of tables

List of figures

Contents

Abbreviations

Anabaena	*Anabaena* sp. PCC 7120
ATP	adenosine triphosphate
bp	base pair
Hep	heterocyst specific polysaccharide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hgl	heterocyst specific glycolipid
hglT	heterocyst glycolipid synthase
MS	mass spectrometry
MW	molecular weight
No.	number
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PSI	photosystem I
PSII	photosystem II
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
TLC	thin layer chromatography
v/v	volume per volume
WT	wild type
Δ	deletion

Chapter I

General Introduction

1.1. Cyanobacteria

Cyanobacteria, previously known as blue-green algae, constitute one of the largest taxa of eubacteria. The previous designation of "blue-green algae" comes from the high ratio of a blue pigment, phycocyanobilin, in addition to a green pigment, chlorophyll, in these organisms. Cyanobacteria are photoautothropic microorganisms, which are able to convert solar energy (sunlight) to metabolize carbon dioxide (CO_2) into organic compound (carbohydrate) and evolve oxygen molecule (O_2) via photosynthesis reaction, similar to land plants and algae. Cyanobacteria absorb light energy for photosynthesis by chlorophyll *a*, phycobiliproteins and other photosynthetic chemical compounds. They can perform oxygenic photosynthesis and respiration simultaneously in the same compartment, named thylakoid membranes [1,2].

Characteristics of cyanobacterial outer structure, including peptidoglycans and outer membrane, are different from other bacteria [3]. The outer membranes comprise lipopolysaccahrides (LPS) which contain small amount of phosphate, but mostly lack ketodeoxyoctanoate, common LPS components of gram-negative bacteria. The peptidoglycan layer of cyanobacteria contains unusual components present in gram-negative bacteria, namely carotenoid and fatty acid. According to these features, Hoiczyky and Hansel have discussed that cyanobacteria belong to

neither gram-negative nor gram-positive bacteria. Their cell wall structure has a thicker peptidoglycan layer than other gram-negative bacteria [3]. According to these results, it is hard to conclude whether cyanobacteria are gram-positive or negative. However, in general, cyanobacteria are classified as gram-negative bacteria.

1.2. Classification of cyanobacteria

In general, cyanobacteria can be divided into two groups, unicellular or multicellular type. Multicelluar type can be further divided into heterocystous and non-heterocystous cyanobacteria. Major revision of classification has distinguished cyanobacteria into five sections. Table 1.1 shows the classification of the five sections of cyanobacteria. Section I comprises unicellular strains such as *Synechocystis*, *Synechococcus*, *Prochlorococcus*, *Thermosynechococcus* and *Mycrocystis*. Cell reproduction of these strains are conducted by binary fission while cells of strains in section II, such as *Chroococcidiopsis* and *Pleurocapsa*, divide by budding and multiple fission. *Lyngbya*, *Leptolyngbya*, *Microcoleus*, *Oscillatoria*, *Phormidium*, and *Planktothrix* are classified into section III. These strains are filamentous and non heterocystous cyanobacteria that reproduce by trichome breakage and hormogonia. The filamentous and heterocystous cyanobacteria are placed into sections IV and V, which reproduce by trichome breakage, hormogonia and akinetes; and have the ability to form heterocyst [4,5]. The cyanobacteria strains in section IV and V groups show different pattern of

Table 1.1. Cyanobacteria classification

Section	Morphology	Reproduction	Family	Ilustrative genera
I	Unicelluar	Binary fission	*Chroococcalles*	*Aphanocapsa, Aphanothece, Gloeocapsa, Merismopedia, Microcystis, Synechococcus, Synechocystis*
II	Unicelluar	Multiple fission	*Peurocapsales*	*Chroococcidiopsis, Pleurocapsa*
III	Filamentous non differentiated (without heterocyst/ akinetes)	Trichome fragmentation, hormogonia	*Oscilattoriales*	*Lyngbya, Leptolyngbya, Microcoleus, Oscillatoria, Phormidium, Planktothrix*
IV	Filamentous and heterocystous (division in only one plane with heterocyst)	Trichome fragmentation, hormogonia, akinetes	*Nostocales*	*Anabaena, Aphanizomenon, Calothrix, Cylindrospermopsis, Nostoc, Scytonema, Tolypothrix*
V	Branched filamentous and heterocystous (division in more than one plane with heterocyst)	Trichome fragmentation, hormogonia, akinetes	*Stigonematales*	*Mastigocladus (Fischerella), Stigonema*

Source: [4,5]

their filament division. Section IV has ability to divide in only one plane, while section V can divide more than one plane.

1.3. Nitrogen fixation in cyanobacteria

Nitrogen is an essential nutrient for living organisms as a building block of all amino acids and proteins. Nitrogen molecule constitutes major gaseous components in the atmosphere. However this molecule is not readily available for assimilation by most organisms, except for diazotrophic microorganisms, including cyanobacteria. Cyanobacteria have ability to convert nitrogen molecule to available form by reducing dinitrogen into ammonia using nitrogenase, as following equation.

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

Therefore, cyanobacteria can survive and thrive under a wide range of environmental conditions.

The nitrogenase is a complex enzyme which consists of two major protein components, namely multi-subunit dinitrogenase (Mo-Fe-protein) encoded by *nifDK* and dinitrogenase reductase (Fe-protein) encoded by *nifH*. Both proteins contain iron-sulfur cluster and oxygen labile. Since nitrogenase is sensitive to oxygen, there are some varieties of strategies to cope the nitrogen fixation with oxygen-evolving photosynthesis. Some cyanobacteria strictly separate those two processes temporally. These groups are making their nitrogenase only in dark when the photosynthesis is inactive and the intracellular oxygen pressure is low. Instead, the others use a spatial separation by making a compartment to conduct

nitrogen fixation in special cells, heterocysts. These cells are morphologically and physiologically distinct from vegetative cells and differentiate regularly along with the filament [6].

1.4. *Anabaena* as a representative filamentous cyanobacteria

Anabaena sp. PCC 7120 (hereafter denoted as *Anabaena*) is a representative filamentous cyanobacteria. The entire nucleotide sequence of *Anabaena* has been fully elucidated [7]. The genome is composed of a 6.4 Mb chromosome and 6 plasmids (0.8 Mb), and contains more than 6,000 genes. *Anabaena* is a heterocystous cyanobacteria and performs spatial separation to conduct both photosynthesis and nitrogen fixation. This strain is multicellular and has long filament consists of hundreds or more vegetative cells in the presence of combined nitrogen source in the medium. When the nitrogen concentration is limited in the medium, *Anabaena* develops heterocyst cells (Fig. 1.1). In *Anabaena*, every single heterocyst cells are regularly separated by 10-20 vegetative cells [8] or 5-10% heterocyst cells, along each filament, are induced under nitrogen starved conditions [9]. The spatial compartment formation separates two incompatible processes, which are oxygen-evolving photosynthesis in vegetative cells and oxygen-labile nitrogen fixation in heterocysts.

Heterocyst cells are typically distinguishable from vegetative cells by their morphology such as larger and rounder shape, diminishment of pigments, thicker cell envelopes, and cyanophycin granules at poles adjacent to vegetative cells. The additional layer at the outer most of cell helps to keep microoxic conditions

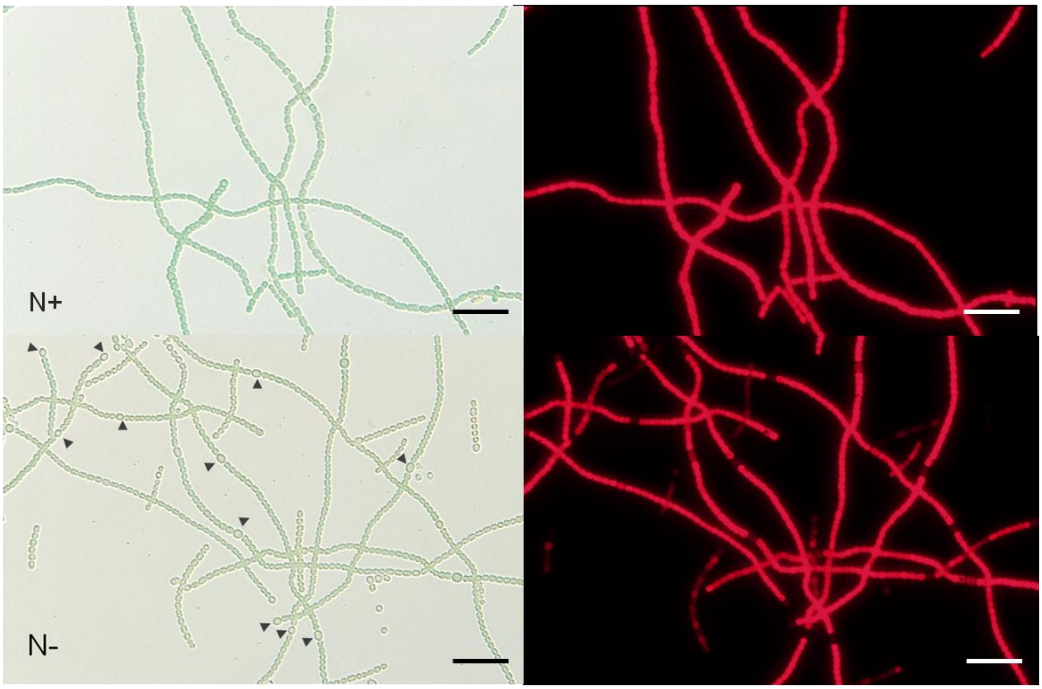


Figure 1.1. Light (left) and fluorescence (right) microscopy of *Anabaena***.** Long filaments of vegetative cells were observed when *Anabaena* was cultured in the medium contain combined nitrogen, BG11 (N+) (upper panel pictures). Two types of cells were found when cells were grown under nitrogen starved conditions, N- ($BG11_0$: BG11 without nitrogen source) (lower panel pictures). Heterocyst cells were indicated by black arrow head (▲, pictures on the left side) and lost auto-fluorescence of chlorophyll (pictures on the right side). Bars, 20 µm.

required for nitrogen fixation. In response to nitrogen starved conditions, heterocyst cells conduct three mechanisms: (i) switch off the photosystem II (PSII) in those cells. Therefore, the heterocyst cells does not evolve oxygen, (ii) increase the oxygen consumptions, and (iii) develop a thick layer of glycolipid to impede the oxygen entry into the cells [10].

Because heterocyst lacks PSII, these cells depend on vegetative cells for carbon source. This carbon source is used to supply energy and reductant, which are required for both respiration and nitrogen fixation. In return, heterocysts supply nitrogen source to vegetative cells in the form of glutamate or other amino acids [11]. Heterocyst cells are connected to adjacent vegetative cells through a pore, equipped with interconnecting structure, microplasmodesmata, which facilitate the exchange of organic material between those cells [12]. The scheme of heterocyst-vegetative cells mutual interdependency of metabolite exchange is shown in the Fig. 1.2.

1.5. Regulation mechanism for heterocyst formation

Heterocyst differentiation requires a number of genes involved in the different step of the developmental process. The heterocyst formation begins with the sensing of combined-nitrogen starvation. Heterocysts development is irreversibly complete 12-20 h after the combined nitrogen removal from the medium [13]. The process of differentiation is reversible when the source of combined nitrogen is added back to the medium by 9-12 h after nitrogen step-down [6]. Thus, availability of combined nitrogen is an inhibitor of the heterocyst

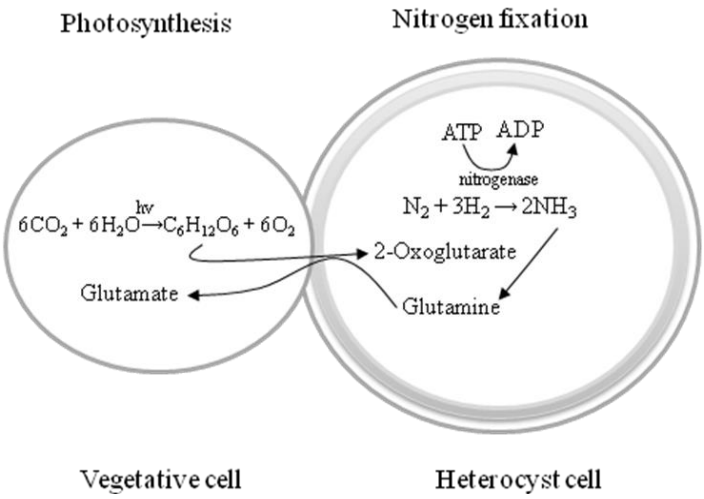


Figure 1.2. Important metabolic interaction between vegetative and heterocyst cells. Carbohydrate is produced in vegetative cells via photosynthesis then exported to heterocyst cells to supply the needs of carbon and energy sources. In contrast, vegetative cells import nitrogen source, which is generated by heterocyst cells through nitrogen fixation in the form of glutamate or other amino acids.

differentiation. Nitrogen limiting condition promotes the accumulation of 2-oxoglutarate (2-OG/2-ketoglutarate). This compound functions as a signal molecule of nitrogen starved conditions. In cyanobacteria, there is a protein which is known to interact with 2-OG, namely NtcA [12].

In *Anabaena*, NtcA and HetR are well known proteins, involved in the early stage of heterocyst formation. NtcA indirectly regulates the expression of *hetR* via NrrA to ensure more strict regulation of the hetR expression than the regulation by NtcA alone. This regulation mechanism is summarized in Fig. 1.3. The 2-OG accumulation induces the expression of *nrrA* via NtcA. Activated NtcA acts as a transcriptional activator for itself and variety of enzyme systems involved in the heterocyst formation [14]. Recent studies demonstrate that the presence of 2-OG increases the DNA binding activity of NtcA [15,16]. In addition, the study of signaling role of 2-OG showed that the addition of 2,2-difluoropentanedioic acid (DFPA), a structural analogue of 2-OG, triggers heterocyst differentiation and stimulates the DNA binding activity of NtcA under nitrogen starved conditions [15]. It was shown that accumulation of 2-OG up-regulates the expression of *nrrA* and the frequency of heterocyst and transcript level of *hetR* are increased in *Anabaena* which having extra copies number of *nrrA* [17]. An *nrrA* mutant shows a delay in heterocyst formation due to the delay in HetR accumulation [17]. These findings suggest that the NtcA, together with NrrA, is a key regulator to initiate expression of genes involved in the heterocyst formation as a primary response factor to the nitrogen deprivation [15].

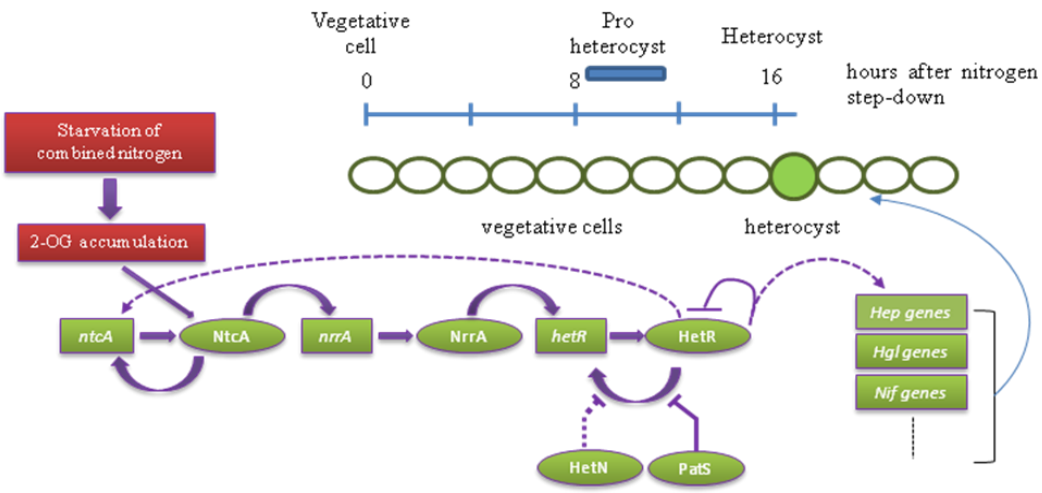


Figure 1.3. A model for regulatory cascade of heterocyst differentiation in *Anabaena*. Accumulation of 2-OG triggers the activation of NtcA that promote the constitutive expression of other genes. The figure shows only selected genes, proteins, and events. Closed green box represents the gene while the oval green is protein. HetN and PatS are known as proteins responsible for heterocyst differentiation [8,17]. Lines ending in arrows and bars refer to positive and negative interaction, respectively. Dashed lines represent indirect interaction.

It is known that PatS and HetN play a prominent role in the heterocyst development and maintain the pattern of heterocyst formation. The product of *patS* is proposed to control heterocyst formation through *hetR* regulation. The *patS* gene product is thought to function in cell-to-cell signaling to prevent the formation of multiple contiguous heterocysts. The formation of the heterocyst pattern possibly requires interaction between HetR and PatS. PatS diffuses laterally to inhibit the differentiation of the neighbouring cells by acting negatively on the DNA binding activity of HetR [9]. Another gene, *hetN*, is required for the maintenance of heterocyst pattern [18]. Both genes are necessary to prevent heterocyst differentiation in the presence of a combined nitrogen source. *hetN* and *patS* mutants are found to produce multiple heterocyst formation [19].

1.6. Chemical structure of heterocyst

Heterocyst is a specialized cell to provide the microoxic conditions for nitrogenase. As shown in the Fig. 1.2, vegetative and heterocyst cells are different in morphology and physiology. Mature heterocyst has two additional layers at the outside of outer membrane to protect nitrogenase from oxygen influx. The outermost envelope layer is made of polysaccharide (heterocyst polysaccharide, Hep) while the inner layer is made of glycolipid, which act as a main barrier for oxygen entry from outsides of the cells [4,5,10,20]. This lipid layer is found specific for heterocyst, called heterocyst glycolipid (Hgl). The Hep layer supports the compactness of this lipid layer. Hep from *Anabaena cylindrica* comprises a 1,3-manosyl-glucosyl-glucosyl-glucose tetrasaccharide backbone to which side

branches of mannose, glucose, and glucosyl glucose, galactose, and xylose are attached [21]. Similar structures of Hep are also found in *Anabaena varibilis*, *Anabaena sp*. PCC 7120, and *Cylindrospermum licheniforme* with minor changes in the side branches.

Structures of glycolipid from several classes of cyanobacteria were reported [22]. These lipids contain one unit of sugar which is glycosidically bound to long-chain polyhydroxy alcohol. The alkyl chain is even-carbon numbered and ranged from C_{26} to C_{32} (Fig. 1.4). The structure of heterocyst glycolipids in *Anabaena* have been fully elucidated [23]. Hgls from *Anabaena* comprise 1-α-glucosyl-3,25-hexacosanediol as a major constituent and 1-α-glucosyl-3-keto-25-hexacosanol as a minor [22]. Lipid analysis showed major Hgl migrates faster on the plate of thin-layer chromatography with the solvent system as described previously [20,24].

1.7. Biosynthesis of heterocyst polysaccharides (Heps) and glycolipids (Hgls)

There are numerous genes involved in the synthesis of heterocyst envelope layer. Cluster genes involved in the formation of polysaccharide layer are *hepA, hepB, hepC, hepK,* and *devR* genes [25]. *hepA, hepB* and *hepC* genes are involved in the accumulation of Hgls and encode proteins with similarity to ABC type transporter, glycosyltransferase and UDP-galactose-lipid carrier transferase, respectively [10]. *hetM* (*hglB*), *hglC,* hglD, hglE, *devBCA* and *hglK* are involved in the formation of the glycolipid layer [26] in *Anabaena*. Mutant of these genes produced immature heterocysts which are unable to fix nitrogen under

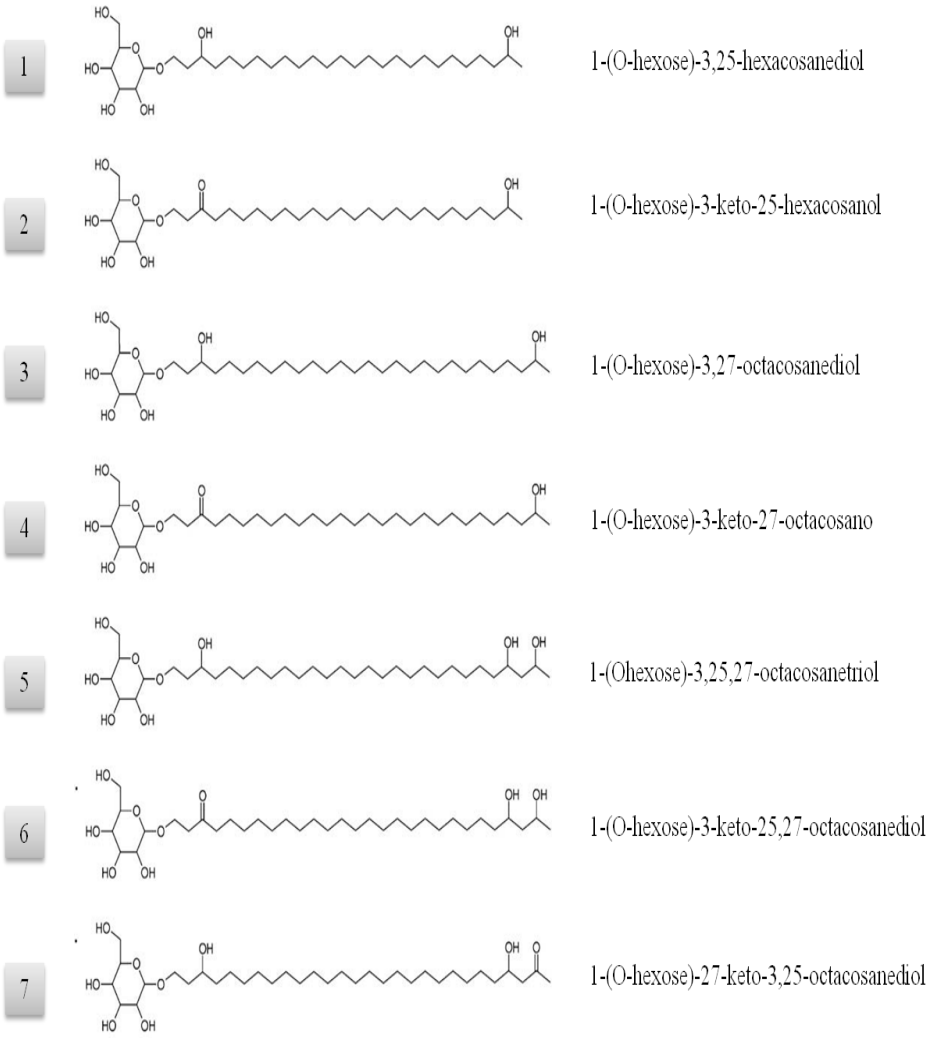


Figure 1.4. Structure of heterocyst glycolipids detected in cyanobacterial cultures. Detail structures of Hgl from several heterocystous cyanobacteria are shown. Number 1 and 2 indicate Hgls of *Anabaena* [23].

aerobic conditions. An ABC transporter, devBCA is also essential for deposition of glycolipid [27]. *hglB, hglC, hglD* and *hglE* genes encode enzymes for the synthesis of the heterocyst specific glycolipids. The protein product of *hglK* gene is also required for the glycolipid layer formation [26]. Recently several other genes involved in glycolipid layer formation, including *devB/devC* homologues, have been identified [24]. A glycosyl transferase was shown to be essential for glycosylation of the glycolipid [28]. The gene cluster involved in the Hgls synthesis is shown (Fig. 1.5).

1.8. Applied science using *Anabaena* cells

Some studies have reported the utilization of cyanobacteria in biofuel and other useful compounds production. For instance, production of alkane in genetically-modified *Synechocystis* sp. PCC 6803 [29], ethanol in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 [30,31], hydrophilic compounds and lactic acid in *Synechococcus* sp. PCC 7942 [32], and butanol in *Synechococcus* sp. PCC 7942 [33]. Cyanobacteria also contain numerous bioactive compounds that can be used for commercial applications. For instance, the filamentous and non heterocystous cyanobacterium *Spirulina plantesis* is widely used as food supplement of human and as an additional additives for animal feed. Genetically modified of cyanobacteria for production sustainable product is summarized in the Table 1.2.

Among cyanobacteria, heterocystous cyanobacteria species are recently attractive for the production of useful compounds, because of their ability to

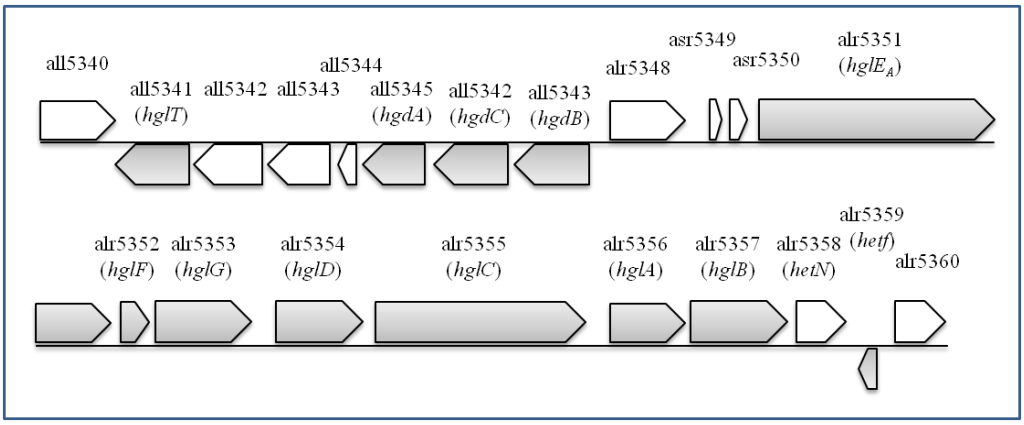


Figure 1.5. Map of "Hgl's gene island" in the chromosome of *Anabaena*. Genes highlighted by light gray gradient color are encoding proteins responsible for Hgl synthesis, transport, and deposition. Mutation in gene(s) above shows accumulation of immature Hgl and/or the inability to form Hgl layer [5].

Table 1.2. Various biochemicals produced in genetic-engineered cyanobacteria.

Organisms	Compounds
Synechocystis sp. PCC 6803	Acetone
Synechococcus elongatus sp. PCC 7942	2,3-butanediol
Synechococcus elongatus sp. PCC 7942	1-butanol
Synechocystis sp. PCC 6803	Ethanol
Synechocystis sp. PCC 6803	Ethylene
Synechocystis sp. PCC 6803	Fatty acids
Synechococcus elongatus sp. PCC 7942	Isobutanol
Synechococcus elongatus sp. PCC 7942	isobutyraldehyde
Synechocystis sp. PCC 6803	Isoprene
Synechococcus elongatus sp. PCC 7942	2-metil-1-butanol

Source: [34]

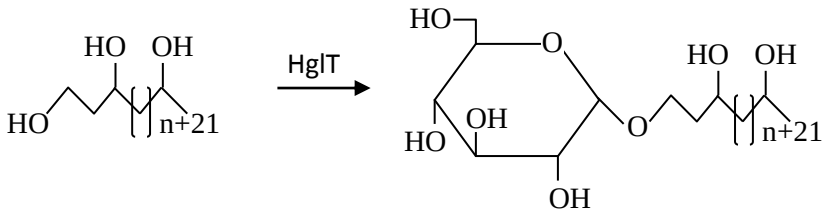
perform both photosynthesis and nitrogen fixation under aerobic conditions. This group uses gaseous dinitrogen in the atmosphere as the nitrogen sources. Lack of nitrogen source in the medium has economically positive effect because it will minimize the cost of nutrients and contamination of other microorganisms. The availability of the entire sequence and the facile technique for genetic manipulation of *Anabaena* has attracted researchers. Generally, *Anabaena* often use as a model to study the biological and chemical aspect of nitrogen fixation, pattern formation, and cell differentiation. However, there are few studies drawing out the genetic engineering of filamentous cyanobacteria for production of useful compounds.

1.9. Fatty alcohol synthesis in *Anabaena*

Fatty alcohol is one of the aliphatic compounds that are widely used as a detergent, emulsifier, lubricants, and cosmetics. The utilization of fatty alcohol has reached over than 1.3 million ton per year since 2006. Currently, fatty alcohols are produced through processing of natural oil (usually by hydrolysis and hydrogenation of triglycerides). The structure of fatty alcohols or n-alcohols is described in the Fig. 1.6.

Anabaena is known to accumulate fatty alcohol in glycosylated form (glycolipids, Hgl) under nitrogen starved conditions. These compounds are synthesized through a complex reaction by polyketide synthase like proteins (HglE). Synthesis of fatty alcohol in *Anabaena* starts from small molecule (Acetyl-coA). Fig. 1.7 showed the final reaction of Hgl synthesis in *Anabaena*.

Figure 1.6. General formulae of fatty alcohol. The total number of carbon needs to be greater than 8-10 ($n \geq 5$) to be a fatty alcohol. Shorter chain compounds have an appreciable water solubility.



Fatty alcohol (aglycone) Glycolipid

Figure 1.7. Proposed pathway for Hgl synthesis (glycosylation of fatty alcohol) in *Anabaena*. Glucose transfer to fatty alcohol (aglycone) is catalyzed by HglT protein which is encoded by *hglT* gene (*all5341*).

Nowadays, long chain fatty alcohols have gained importance as biodiesel fuels. Thus, fatty alcohol produced in *Anabaena* can be an alternative source to provide another precursor for further application in chemical industry or simply use for bioenergy.

1.10. Research objective

As described above, heterocyst glycolipid synthase (HglT) catalyzed the glycosylation reaction, which transferring glucose to the fatty alcohol. It was expected that the mutant of *hglT* accumulate fatty alcohol in *Anabaena* sp. PCC 7120. In accordance with this hypothesis, the author investigated as follows: in Chapter II, the author isolated mutants of knockout *hglT* (*all5341*) to see the accumulation of fatty alcohols and clarifying whether *hglT* gene is indispensable in both nitrogen replete and depleted conditions. The mutant was physiologically characterized. In chapter III, to enhance the amount of fatty alcohol, the author combined the *hglT* mutation with mutation of other genes involved in the regulation of heterocyst formation, *patS* and *hetN* genes. Knock out mutants of these two genes are known to form multiple heterocyst under nitrogen starvation. It was expected that the increased number of heterocyst will increase the ratio of heterocyst per filament. The more heterocyst formed the more accumulated glycolipid. The more glycolipid produced the more fatty alcohol expected. At Chapter IV, the author discussed physiological significance of Hgl and possible application of fatty alcohol for chemical industry.

References

[1] Vermaas, W.F.J. (2001) Photosynthesis and respiration in cyanobacteria. In Encyclopedia of life sciences ed.^eds), pp. 1-7. Macmillan Publishers Ltd, Nature Publishing Group, Arizona, USA.

[2] Lea-Smith, D.J., Ross, N., Zori, M., Bendall, D.S., Dennis, J.S., Scott, S.A., Smith, A.G. and Howe, C.J. (2013). Thylakoid terminal oxidases are esential for the cyanobacterium *Synechocystis* sp.PCC 6803 to survive rapidly changing light intensities. Plant Physiol 162, 484-495.

[3] Hoiczyk, E. and Hansel, A. (2000). Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J Bacteriol 182, 1191–1199.

[4] Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56, 340-73.

[5] Awai, K., Lechno-Yossef, S. and Wolk , C.P. (2009) Heterocyst envelope glycolipids. In Lipid in photosynthesis essential and regulatory functions (Wada, H. and Murata, N., ed.^eds), pp. 179-202. Springer, Netherlands.

[6] Golden, J.W. and Yoon, H.S. (2003). Heterocyst development in *Anabaena*. Curr Opin Microbiol 6, 557-63.

[7] Kaneko, T. et al. (2001). Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res 8, 205-213.

[8] Kumar, K., Mella-Herrera, R.A. and Golden, J.W. (2010). Cyanobacterial heterocysts. Csh Perspect Biol 2, 1-19.

[9] Zhang, C.C., Laurent, S., Sakr, S., Peng, L. and Bedu, S. (2006). Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol Microbiol 59, 367-375.

[10] Maldener, I., Hannus, S. and Kammerer, M. (2003). Description of five mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst differentiation and identification of the transposon-tagged genes. FEMS Microbiol Lett 224, 205-213.

[11] Cumino, A.C., Marcozzi, C., Barreiro, R. and Salerno, G.L. (2007). Carbon cycling in *Anabaena* sp. PCC 7120; Sucrose synthesis in the heterocysts and possible role in nitrogen fixation. Plant Physiol 143, 1385-1397.

[12] Meeks, J.C. and Elhai, J. (2002). Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66, 94-121.

[13] Adams, D.G. (2000). Heterocyst formation in cyanobacteria. Curr Opin Microbiol 3, 618–624.

[14] Tanigawa, R., Shirokane, M., Maeda, S., Omata, T., Tanaka, K. and Takahashi, H. (2002). Transcriptional activation of NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate in vitro. Proc Natl Acad Sci USA 99, 4251-4255.

[15] Laurent, S., Chen, H., Be`du, S., Ziarellis, F., Peng, L. and Zhang, C. (2005). Nonmetabolizable analogue of 2-oxoglutarate elicits heterocyst

differentiation under repressive conditions in *Anabaena* sp. PCC 7120. Proc Natl Acad Sci USA 102, 9907–9912.

[16] Va`zquez-Bermu`dez, M.F., Herrero, A. and Flores, E. (2002). 2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus* glnA promoter. FEBS Lett 512, 71-74.

[17] Ehira, S. and Ohmori, M. (2011). NrrA, a nitrogen-regulated response regulator protein, controls glycogen catabolism in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. J Biol Chem 286, 38109-38114.

[18] Callahan, S.M. and Buikema, W.J. (2001). The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. Mol Microbiol 40, 941-950.

[19] Borthakur, P.B., Orozco, C.C., Young-Robbins, S.S., Haselkorn, R. and Callahan, S.M. (2005). Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Mol Microbiol 57, 111-123.

[20] Huang, X., Dong, Y.Q. and Zhao, J.D. (2004). HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc Natl Acad Sci USA. 101, 4848-4853.

[21] Cardemil, L. and Wolk, C.P. (1979). The polysaccharides from heterocyst and spore envelopes of a blue-green alga. Structure of the basic repeating unit. J Biol Chem 254, 736-41.

[22] Bauersachs, T., Compaoré, J., Hopmans, E.C., Stal, L.J., Schouten, S. and Sinninghe Damsté, J.S. (2009). Distribution of heterocyst glycolipids in cyanobacteria. Phytochem 70, 2034-2039.

[23] Soriente, A., Sodano, G., Gambacorta, A. and Trincone, A. (1992). Structure of the heterocyst glycolipids of the marine cyanobacterium *Nodularia harveyana*. Tetrahedron 48, 5375-5384.

[24] Fan, Q., Huang, G.C., Lechno-Yossef, S., Wolk, C.P., Kaneko, T. and Tabata, S. (2005). Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120. Mol Microbiol 58, 227-243.

[25] Zhou, R.B. and Wolk, C.P. (2003). A two-component system mediates developmental regulation of biosynthesis of a heterocyst polysaccharide. J Biol Chem 278, 19939-19946.

[26] Black, K., Buikema, W.J. and Haselkorn, R. (1995). The *hglK* gene is required for localization of heterocyst-specific glycolipids in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 177, 6440-6448.

[27] Fiedler, G., Muro-Pastor, A.M., Flores, E. and Maldener, I. (2001). NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-

specific ATP-binding cassette transporter in *Anabaena* spp. J Bacteriol 183, 3795-3799.

[28] Awai, K. and Wolk, C.P. (2007). Identification of the glycosyl transferase required for synthesis of the principal glycolipid characteristic of heterocysts of *Anabaena* sp. strain PCC 7120. FEMS Microbiol Lett 266, 98-102.

[29] Wang, W.H., Liu, X.F. and Lu, X.F. (2013). Engineering cyanobacteria to improve photosynthetic production of alka(e)nes. Biotechnol Biofuels 6, 1-9.

[30] Dexter, J. and Fu, P.C. (2009). Metabolic engineering of cyanobacteria for ethanol production. Energ Environ Sci 2, 857-864.

[31] Ming, D. and Coleman, J.R. (1999). Ethanol synthesis by genetic engineering in cyanobacteria. Appl Environ Microbiol 65, 523-528.

[32] Niederholtmeyer, H., Wolfstadter, B.T., Savage, D.F., Silver, P.A. and Way, J.C. (2010). Engineering cyanobacteria to synthesize and export hydrophilic products. Appl Environ Microbiol 76, 3462-3466.

[33] Lan, E.I. and Liao, J.C. (2012). ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. Proc Natl Acad Sci USA 109, 6018-6023.

[34] Nicole, E. Nozzi, John, W.K., Oliver and Atsumi, S. (2013). Cyanobacteria as a platform for biofuel production. Front Bioeng Biotechnol 1, 1-6.

Chapter II

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

2.1. Introduction

In the absence of fixed nitrogen, *Anabaena* differentiates its vegetative cells into heterocysts, which are specialized nitrogen-fixing cells [1-3]. 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 [4-7]. The thick lipid layer consists of a hexose head group [8] 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 [7]. In *Anabaena*, the structures of Hgls have been fully elucidated [9]. 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 [10].

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 [11], whereas *hglK* is required for the localization of glycolipids [12]. The

devBCA gene cluster is necessary for glycolipid export [13], and this transporter interacts with a TolC protein in outer membranes [14]. 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 [15] 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 of nitrogenases to fix nitrogen in the heterocysts, thus restricting the supply of fixed nitrogen to neighboring vegetative cells [16]. 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, the author 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, the author 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, the author shows that the fatty alcohol can, at least in part, complement the function of Hgls in *Anabaena* heterocysts.

2.2. Material and Methods

2.2.1. 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) [17] at 30^{o}C in the light (50-80 µmol m^{-2} s^{-1}) on rotary shaker (120 rpm) as described previously [18]. 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 to 1.2 and washed three times with nitrogen-free medium ($BG11_0$: BG11 without nitrate) and then resuspended in $BG11_0$.

2.2.2. 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 (ACTAGTGGATCCCCCTCTGACAAATCCGACG) and Rv1 (GAATTCCTGCAGCCCGGGCGCAATGCGAAGCTTTG), 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 [19].

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, City, USA). 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*.

2.2.3. Microscopy

Anabaena sp. PCC 7120 wild type and the mutant filaments were visualized with bright field and fluorescence microscope (BX53, Olympus, City, Japan). 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.

2.2.4. Chlorophyll content and cell spectrum

Cells of each strain were harvested from 1 mL culture with OD_{730} of 0.8 to 1.2. The pellet was resuspended in 90% methanol and measured by the method of Meeks and Castenholz [20]. 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, Kyoto, Japan) with an integrating sphere.

2.2.5. Lipid analysis

Anabaena cultures (OD_{730}≃1.0) were harvested at room temperature by centrifugation at 3,000 rpm for 15 min. Lipids were extracted from those cell pellets by a modification of the Bligh and Dyer methods as described previously [16]. Then the lipids were separated by thin-layer chromatography (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^{o}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 x 0.25 mm, SGE Analytical Science, Perth, Australia). The column temperature was programmed at 240^{o}C. The injector and detector temperature were 200^{o}C and 240^{o}C, respectively. The flow rate of carrier gas (He) was 0.4 mL/min.

2.2.6. 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 to 1 h of incubation under illumination, the concentration of ethylene was assayed as described previously [21].

2.2.7. Western blotting and immunodetection

Proteins extracts were electrophoretically resolved by 12% SDS-PAGE and electroblotted onto Hybond-P PVDF membrane (GE Healthcare, Uppsala, Sweden). 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% Tween 20) and incubated with secondary antibody (anti-hen IgY horse radish peroxidase conjugated, Abcam, Tokyo, Japan) diluted to a 1: 40,000 for 1h 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).

2.2.8. 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 Bio) according to the manufacturer protocol. The generated cDNA was used as a template for RT-PCR with the primer pairs for nifH (ACCTCGTGACAACATCGTTC and TTGGTGTAGGAATGGTGAGC) and rnpB (CCAGTTCCGCTATCAGAGAG and GAGGAGAGAGTTGGTGGTAAG). The *rnpB*, encodes the RNA subunit of RNaseP, served as a loading control.

2.3. Results

2.3.1. *hglT* is not essential in *Anabaena*

To clarify whether *hglT* (*all5341*) of *Anabaena* is required under both nitrogen-replete and -depleted conditions, the author isolated a null mutant of *hglT* from *Anabaena*. A PCR analysis using primers external to *hglT* (Fig. 2.1A, Fw1 and Rv2; see Materials and Methods) resulted in two PCR fragments of different sizes. The wild type produced a 2,976-bp fragment, and the mutant produced a larger 3,259-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

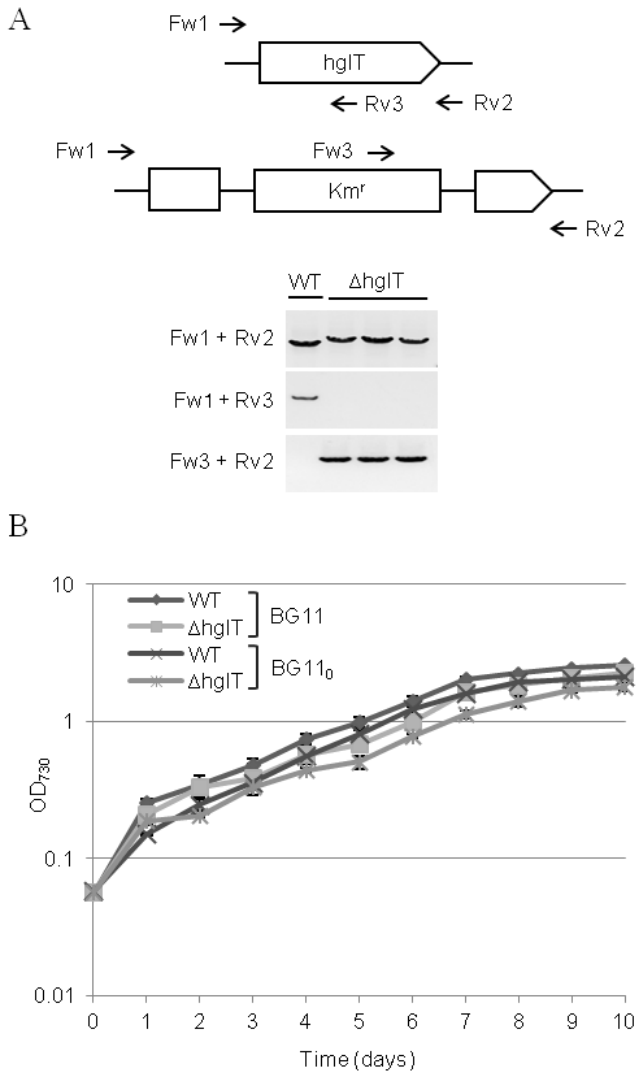


Figure 2.1. Genotype and growth rate of the *hglT* mutant of *Anabaena*. **A.** The scheme shows the map of *hglT* gene disruptions and the primers used for genotyping. Km^r: kanamycin resistant gene, WT: *Anabaena* sp. PCC 7120 wild type, ΔhglT: hglT mutant. **B.** Growth rate of *Anabaena* wild type 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.

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.

2.3.2. *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. 2.1B) 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. 2.2A, Fig. 2.3). This phenomenon was also confirmed using a whole-cell absorbance spectrum normalized by cell density (Fig. 2.2B). 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. 2.1B). 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 [16]. In accordance with that result, it was found *hglT* null mutants remained pale green with decreased chlorophyll content under nitrogen starved conditions (Fig. 2.2A, Fig. 2.3). A cell spectrum showed that all of the

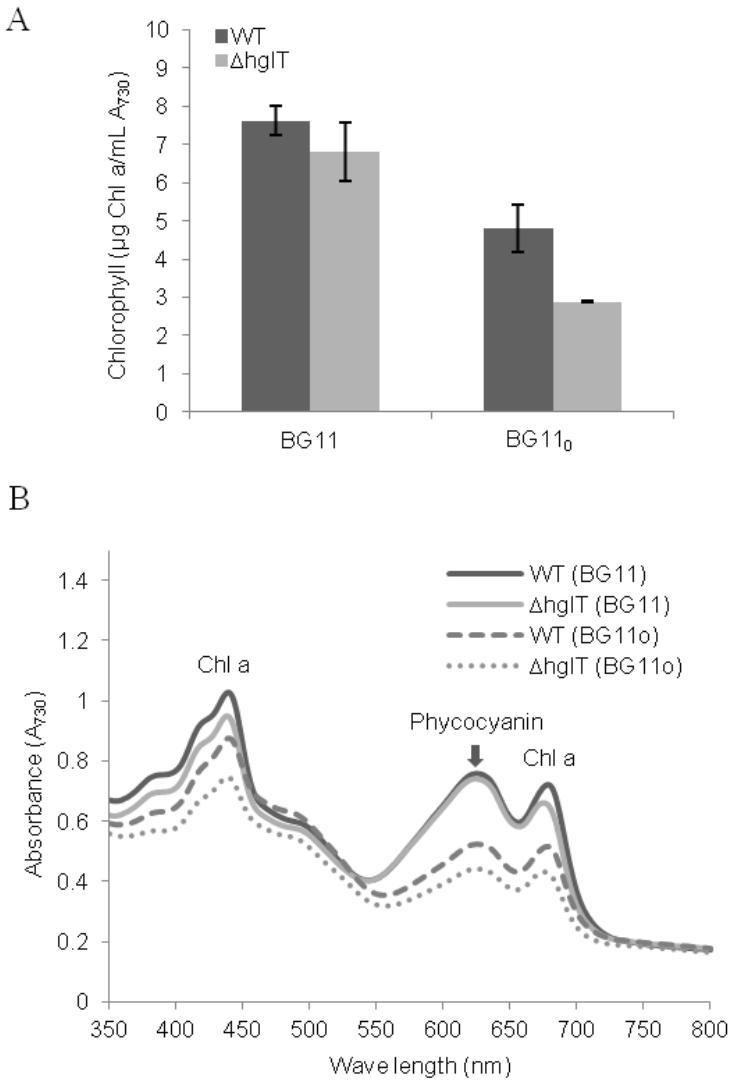


Figure 2.2. Chlorophyll content and whole-cell spectra of the *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). **(A)** Chlorophyll content in nitrogen replete (BG11) and deprived ($BG11_0$) conditions. Error bars indicate the SD based on three independent experiments. **(B)** Whole-cell spectra. 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*.

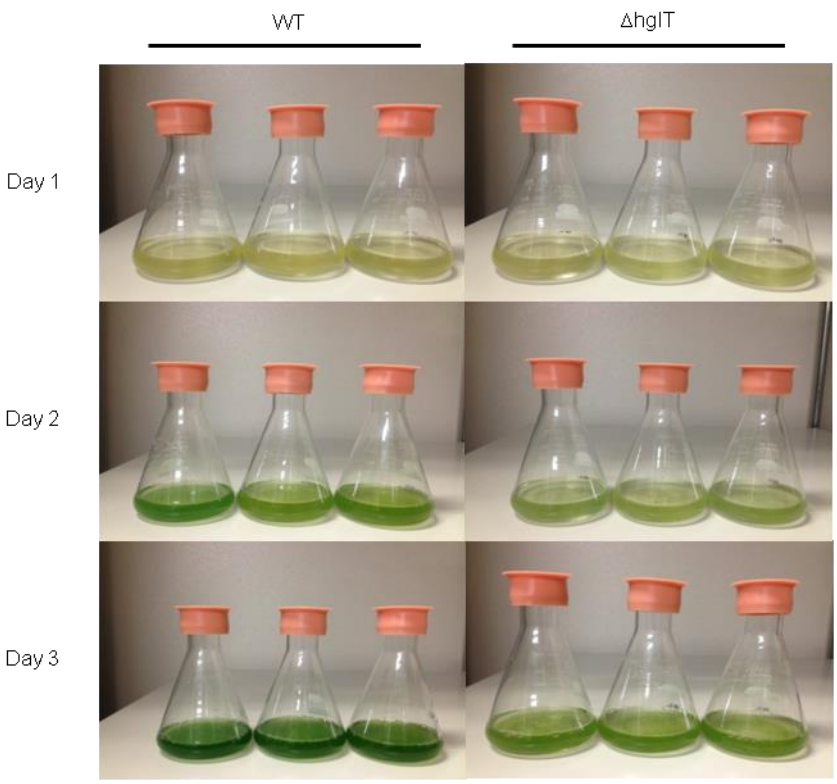


Figure 2.3. The growth of *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). Cells were grown in the BG11 medium to the OD_{730} of 0.8-1.2, washed three times by $BG11_0$ medium, then transferred to new medium.

peaks, including that of phycocyanin, were decreased under the nitrogen-deprived conditions (Fig. 2.2B). Based on these results, the author 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.

2.3.3. Nitrogenase is active in the *hglT* mutants

The ability of the *hglT* null mutant to fix nitrogen was measured under nitrogen starved conditions. The author 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. 2.4). 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. 2.5). The author 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. 2.6). 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. The author also evaluated the expression of *nifH*, which encodes a subunit of nitrogenase, at the mRNA and protein levels. According to Fig. 2.7A and B, 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.

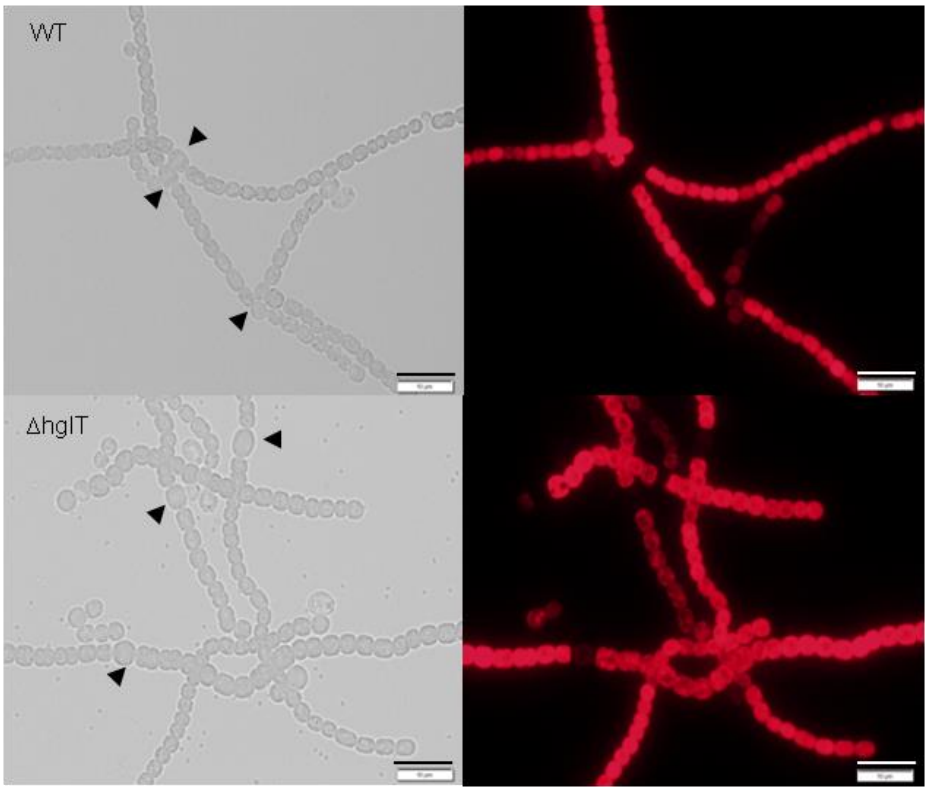


Figure 2.4. Light and flourescence microscopy of *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). Left panel, light field, right panel, fluorescence. The mutant differentiated heterocyst cells under nitrogen starved conditions. Heterocyst cells are indicated by triangles. These cells have diminished chlorophyll content therefore they lost fluorescence ability. Bar, 10 μm.

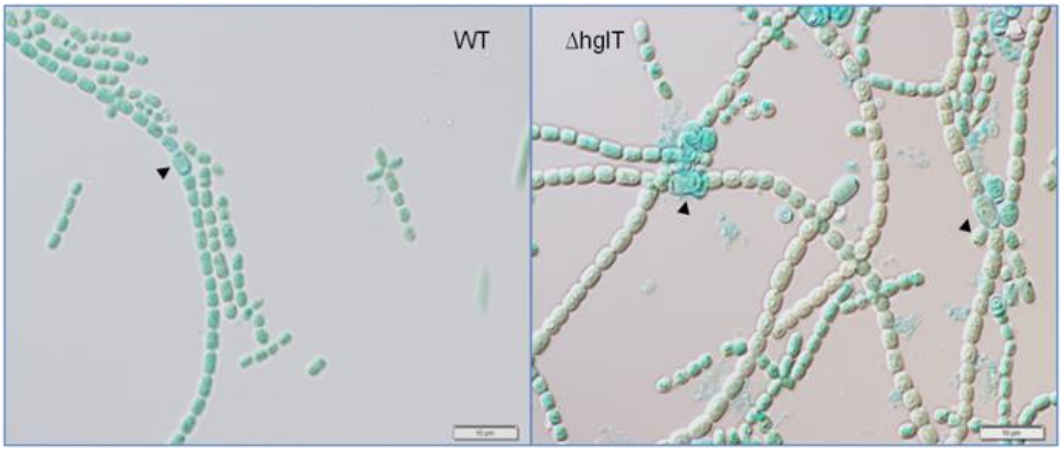


Figure 2.5. Heterocysts differentiation in the wild type (WT) and *hglT* mutant (ΔhglT). Cells were grown under nitrogen starved conditions then stained with 0.5% Alcian blue in a 50% ethanol solution for 30 min before observation. Heterocyst cells are pointed by black triangles. Bar, 10 µm.

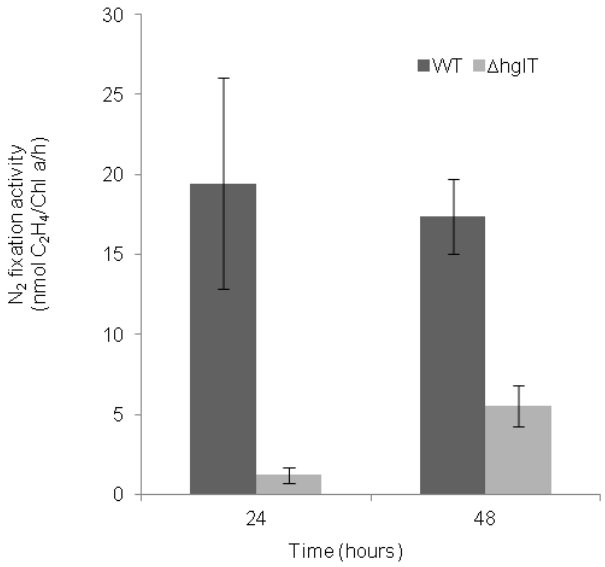


Figure 2.6. Nitrogen fixation activity of *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). Cells were first grown in BG11 and transferred to $BG11_0$ after three times of wash. Time indicates the h after nitrogen depletion. Error bars indicate the SD based on three independent experiments.

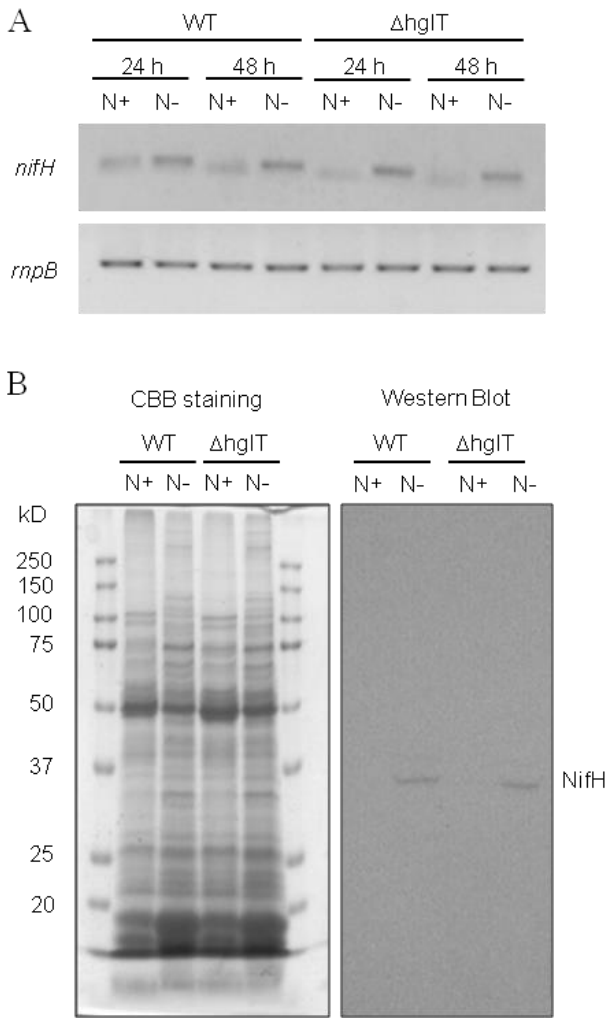


Figure 2.7. The expression of *nifH* in *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). **A.** RT-PCR of the gene for a subunit of nitrogenase, *nifH*. **B.** Western blot analysis of NifH protein. Cells are deprived of fixed nitrogen for 48 h and proteins were extracted. Size of NifH is about 32.5 kDa. Size marker: Precision Plus Protein Kaleidoscope Standards (BioRad).

2.3.4. Fatty alcohol can complement the function of Hgls

Partial mutants of *hglT* do not contain detectable amount of Hgls under nitrogen starved conditions [16]. To confirm that the null mutants also lacked Hgls, lipid profiles of the *hglT* mutants were analyzed by TLC. In the *hglT* mutants, neither the major nor minor Hgl was detected in the absence of combined nitrogen, instead, there was an accumulation of the fatty alcohol (aglycone) (Fig. 2.8A). This indicates 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. 2.8B), and the membrane lipid composition of the mutant was not significantly altered (Fig. 2.8B). These results suggest 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. 2.9), which may be due to their decreased ability to fix nitrogen.

2.4. Discussion

2.4.1. Localization of the aglycones in the heterocysts of *hglT* mutants

The author proposes 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 [11,16]. Among those enzymes, $HglE_A$ has a central role in the

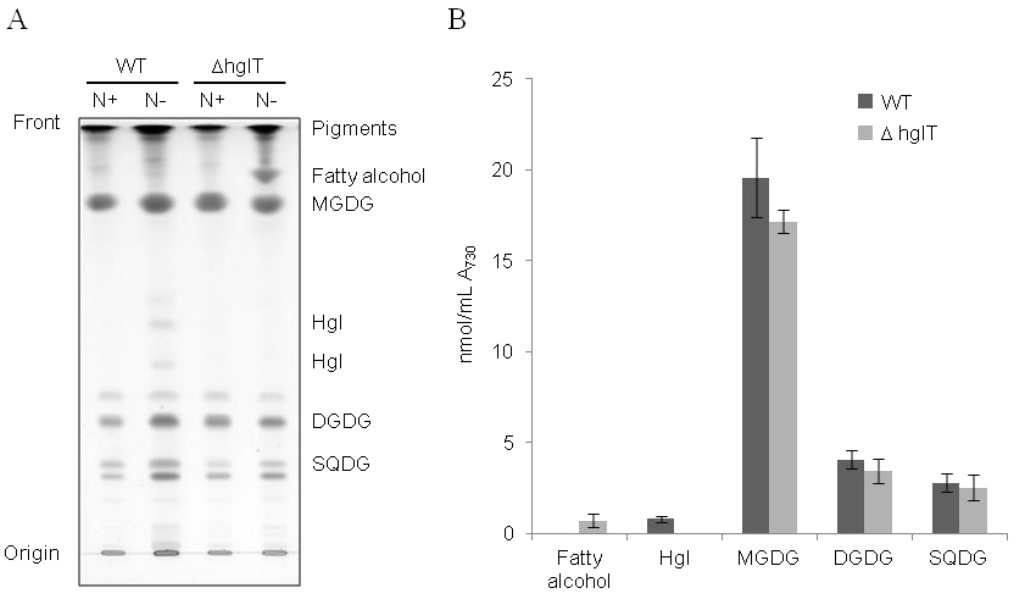


Figure 2.8. Lipid composition of *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). **A.** Lipids profiles separated by TLC. *Anabaena* WT and ΔhglT were grown in BG11 or $BG11_0$ (48 h of nitrogen step down). **B.** Lipid content per cell density. Lipids were quantified by GC. ΔhglT accumulated fatty alcohol and lacked Hgls. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

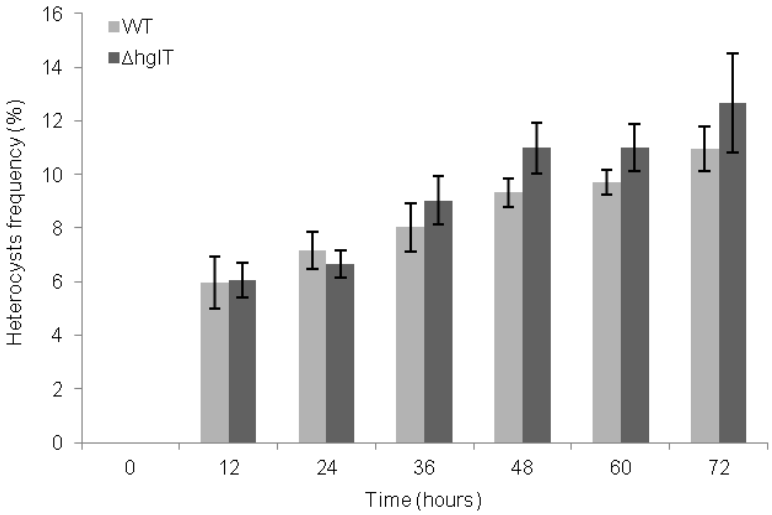


Figure 2.9. Heterocysts frequency in *Anabaena* wild type (WT) and *hglT* mutant (ΔhglT). Gray bars indicate the wild type and dark gray bars the mutant. Time after nitrogen step down is shown.

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 [16]. However, the *hglT* null mutant 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 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 [22]. 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, the author 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 [16]. 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.

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

It took over six 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 [23]. However, it is still possible that even this very small amount of material is pivotal for the formation of the glycolipid layer.

References

[1] Meeks, J.C. and Elhai, J. (2002). Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66, 94-121.

[2] Golden, J.W. and Yoon, H.S. (2003). Heterocyst development in *Anabaena*. Curr Opin Microbiol 6, 557-563.

[3] Shi, L., Li, J.H., Cheng, Y., Wang, L., Chen, W.L. and Zhang, C.C. (2007). Two genes encoding protein kinases of the HstK family are involved in synthesis of the minor heterocyst-specific glycolipid in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 189, 5075-5081.

[4] Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56, 340-373.

[5] Maldener, I., Hannus, S. and Kammerer, M. (2003). Description of five mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst differentiation and identification of the transposon-tagged genes. FEMS Microbiol Lett 224, 205-213.

[6] Huang, X., Dong, Y.Q. and Zhao, J.D. (2004). HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc Natl Acad Sci USA 101, 4848-4853.

[7] Awai, K., Lechno-Yossef, S. and Wolk , C.P. (2009) Heterocyst envelope glycolipids. In Lipid in photosynthesis essential and regulatory functions (Wada, H. and Murata, N., ed.^eds), pp. 179-202. Springer, Netherlands.

[8] Bryce, T.A., Welti, D., Walsby, A.E. and Nichols, B.W. (1972). Monohexoside derivatives of long-chain polyhydroxy alcohols; a novel class of glycolipid specific to heterocystous algae. Phytochem 11, 295-302.

[9] Soriente, A., Sodano, G., Gambacorta, A. and Trincone, A. (1992). Structure of the heterocyst glycolipids of the marine cyanobacterium *Nodularia harveyana*. Tetrahedron 48, 5375-5384.

[10] Gambacorta, A., Romano, I., Trincone, A., Soriente, A., Giordano, M. and Sodano, G. (1996). The heterocyst glycolipids of cyanobacteria. 5. Heterocyst glycolipids from five nitrogen-fixing cyanobacteria. Gazz Chim Ital 126, 653-656.

[11] Fan, Q., Huang, G.C., Lechno-Yossef, S., Wolk, C.P., Kaneko, T. and Tabata, S. (2005). Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120. Mol Microbiol 58, 227-243.

[12] Black, K., Buikema, W.J. and Haselkorn, R. (1995). The *hglK* gene is required for localization of heterocyst-specific glycolipids in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 177, 6440-6448.

[13] Fiedler, G., Muro-Pastor, A.M., Flores, E. and Maldener, I. (2001). NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-

specific ATP-binding cassette transporter in Anabaena spp. J Bacteriol 183, 3795-9.

[14] Staron, P., Forchhammer, K. and Maldener, I. (2011). Novel ATP-driven pathway of glycolipid export involving TolC protein. J Biol Chem 286, 38202-38210.

[15] Ramirez, M.E., Hebbar, P.B., Zhou, R.B., Wolk, C.P. and Curtis, S.E. (2005). *Anabaena* sp strain PCC 7120 gene devH is required for synthesis of the heterocyst glycolipid layer. J Bacteriol 187, 2326-2331.

[16] Awai, K. and Wolk, C.P. (2007). Identification of the glycosyl transferase required for synthesis of the principal glycolipid characteristic of heterocysts of *Anabaena* sp. strain PCC 7120. FEMS Microbiol Lett 266, 98-102.

[17] Stainer, R.Y., Kunisiwa, R., Mandel, M. and Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol Rev 35, 171-205.

[18] Awai, K., Watanabe, H., Benning, C. and Nishida, I. (2007). Digalactosyldiacylglycerol is required for better photosynthetic growth of *Synechocystis* sp. PCC 6803 under phosphate limitation. Plant Cell Physiol 48, 1517-1523.

[19] Elhai, J. and Wolk, C.P. (1988). Conjugal Transfer of DNA to Cyanobacteria. Method Enzymol 167, 747-754.

[20] Meeks, J.C. and Castenholz, R.W. (1971). Growth and photosynthesis in an extreme thermophile, *Synechococcus lividus* (Cyanophyta). Arch Mikrobiol 78, 25-41.

[21] Ehira, S. and Ohmori, M. (2011). NrrA, a nitrogen-regulated response regulator protein, controls glycogen catabolism in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. J Biol Chem 286, 38109-38114.

[22] Fiedler, G., Arnold, M., Hannus, S. and Maldener, I. (1998). The DevBCA exporter is essential for envelope formation in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol Microbiol 27, 1193-1202.

[23] Winkenbach, F., Wolk, C.P. and Jost, M. (1972). Lipids of membranes and of the cell envelope in heterocysts of a blue-green alga. Planta 107, 69-80.

Chapter III

Inactivation of *patS* and *hetN* genes enhanced production of fatty alcohol in *hglT* mutant of *Anabaena* sp. PCC 7120

3.1. Introduction

Anabaena sp. PCC 7120 (further referred as *Anabaena*) is a multicellular cyanobacterium and has long filament consists of hundreds or more vegetative cells in the presence of combined nitrogen source in the medium. When the concentration of combined nitrogen is low in the environment, *Anabaena* develops heterocyst cells to separate oxygen-labile nitrogen fixation from oxygen evolving photosynthesis in vegetative cells. These cells are surrounded by glycolipid layer to protect nitrogenase from oxygen diffusion from outside of the cells [1-4]. Numerous genes are involved in the development of heterocyst, including repressors.

Both PatS and HetN are known to be involved in the regulation of the heterocyst differentiation [5,6]. PatS plays role in the patterning of novel heterocysts formation and maintenance of it [7]. A *patS* null mutant found to form multiple contiguous heterocysts (Mch), abnormal patterning, and shortened intervals of vegetative cells under nitrogen limited conditions [7]. There are several stages in the development of heterocyst cells. The initial step of heterocyst formation requires interaction between PatS and HetR [4]. The *patS* product has

reported to cause the inhibition on HetR binding to the *hetR* promoter region [4] and inhibit heterocyst differentiation [7].

Similar to *patS*, *hetN* is reported to encode diffusible inhibitor of differentiation, the RGSGR pentapeptide, that affect the heterocyst formation and patterning. Unlike *patS* mutants, deletion of *hetN* gene in *Anabaena* shows no alteration in the heterocyst patterning and interval length of vegetative cells. However, this null mutant is also forming multiple heterocyst formation after normal pattern of heterocyst formation [8], indicating that HetN does not require for formation of the initial pattern of heterocysts in response to nitrogen straved conditions. Yet, this gene play important role in the maintenance of the heterocysts patterning [5].

Because mutants of *patS* and *hetN* showed different phenotypes, another question was raised related to the interdependence of regulation among these two genes. To examine whether both genes are independent systems or are depend on the other for its function, Borthakur et al [9] analyzed function of *patS* and *hetN* in heterocyst development. The results showed the pattern of heterocyst in *hetN* null mutant was initially same as the wild type, but then the multiple contiguous heterocysts are observed approximately 48 h after nitrogen step-down. In contrast, *hetN* mutant with extra copies of *patS* under the control of its native promoter prevent to form heterocyst under nitrogen starved condition. The failure in formation of heterocysts was also observed in the *patS* mutant overexpressing *hetN*. These results demonstrate that the product of *hetN* and *patS* has same function. However, it is reported that their expression pattern are timely and

spaciously different, which make physiological functions of both genes independent [9].

Another data also support PatS and HetN are independent. The double mutant of *patS* and *hetN* has significantly higher frequency of heterocyst than in the single mutant of *patS* or *hetN*, suggesting that *patS* and *hetN* are suppressing the heterocyst differentiation with an independent pathway. Surprisingly, inactivation of both genes leads to differentiation of almost all vegetative cells into heterocyst cells under nitrogen starved condition [9].

As characterized in Chapter II, null mutant of *hglT* differentiated heterocysts but fail to form heterocyst glycolipid layer (Hgls), and instead accumulated fatty alcohol, a substrate for final synthesis of Hgls. To increase the amount of fatty alcohol in the *hglT* mutant, the author combined mutation of *hglT* with *patS* and *hetN*. Inactivation of *patS* and *hetN* increased the heterocyst frequency in the filaments therefore it is expected to enhance indirectly the production fatty alcohol in the *hglT* mutant of *Anabaena*.

Long chain hydrocarbons including long chain fatty alcohol are nowadays drawing more attention due to its high energy density, low moisture absorption, low vitality, and compatibility with existing engines and transport facilities. However, fatty alcohol is mostly prepared from natural oil using trans-esterification and hydrogenation processes. These processes need harsh production environments, or bring harmful materials to the environment [10]. For those reasons, organisms efficiently produce fatty alcohol are awaited. Recently, production of fatty acid ethyl ester and fatty alcohols have been reported in

genetically-modified *Escherichia coli* [11]. However, this system requires addition of carbon source which will increase the cost of the production.

The single null mutant of *hglT* has physiologically characterized and found to accumulate fatty alcohol. To enhance the amount of fatty alcohol, the author isolated triple mutant of *hglT*, *patS*, and *hetN* genes (triple mutant). Knock out mutants of *patS* and *hetN* form multiple heterocysts under nitrogen starvation. As expected, the triple mutants accumulate higher amount of fatty alcohol compared to the single *hglT* mutant. In this chapter, the author demonstrated that increased ratio of heterocyst per filament in the triple mutant of *Anabaena* enhanced the accumulation of fatty alcohol.

3.2. Materials and Methods

3.2.1. Growth conditions of the wild type, single and triple mutant of *Anabaena*

Anabaena sp. PCC 7120 and the mutant strains were grown in the liquid medium as described in Chapter II (2.2.1)

3.2.2. Isolation of triple mutant of *Anabaena*

Knock out vector of *hglT* gene has constructed as described in Chapter II. Knock out vector of *hetN* gene was constructed as follows. DNA fragments upstream and downstream of *hetN* gene were amplified by PCR using the primer pairs of NFw1 (ACTAGTGGATCCCCCGATTTGTACCTCAAAG) and NRv1 (GAATTCCTGCAGCCCAAAGCACGAGCAATGTAG), and NFw2 (TCGACCTCGAGGGGGACAAATTTCTCCCACTTC) and NRv2

(GCGAATTGGGTACCGCTACCTTATCTAGTGAC), respectively. The upstream fragment was cloned into *Sma*I site of pMobΩ1 by In-Fusion HD Cloning Kit with Cloning Enhancer (Takara Bio), and then downstream fragment was cloned into *Apa*I site to construct the knock out vector, pMO1hetNKO.

Knock out vector of *patS* was also constructed by amplifying DNA upstream and downstream of *patS* gene. The upstream fragment was amplified by PCR using SFw1 (ACTAGTGGATCCCCCGACTCTGGGAGTAAATTG) and SRv1 (GAATTCCTGCAGCCCTGCCTCGACTATCGG), and the downstream fragment of *patS* was amplified using SFw2 (TCGACCTCGAGGGGGTGATATCTAGGAAGTTGG) and SRv2 (GCGAATTGGGTACCGCCTCGTGAAGCATG). The upstream fragment was cloned into *Sma*I site of pMobEm1 by In-Fusion HD Cloning Kit with Cloning Enhancer (Takara Bio), and then downstream fragment was cloned into *Apa*I site to construct the knock out vector, pMOE1patSKO. The constructed plasmid vectors were introduced consecutively into *Anabaena* and mutant strains by triparental mating by the method of Elhai and Wolk [12].

Genomic DNA from wild type and transformants were used as templates for PCR genotyping with the primers described below and Hybridpol DNA polymerase (Bioline). PCR-based confirmation of gene disruption was performed using external and internal primers to amplify the full length of *hglT, hetN, or patS*; to confirm the insertion of the antibiotics resistance gene into target gene; and for detection of deletion of the central part of *hglT, hetN, or patS.* Genotyping for *hglT* gene disruption was performed using primer TFw1

(ACTAGTGGATCCCCCTCTGACAAATCCGACG) and TRv2 (CGGGCCCCCCCTCGAAGTTTTAGCCACAGTTC) for amplification of full-length *hglT*, TFw4 (CCGCTTCCTTTAGCAGC) and TRv2 for insertion of the kanamycin resistance gene into *hglT*, and TFw1 and TRv3 (ACTACTGGAGTACCAGAG) for detection of deletion of the central part of *hglT*.

PCR-based confirmation for *hetN* gene deletion was conducted using primer NFw1 (ACTAGTGGATCCCCCGATTTGTACCTCAAAG) and NRv2 (GCGAATTGGGTACCGCTACCTTATCTAGTGAC) for amplification of full-length *hetN*, NFw4 (ATCACGGCACGATCATCGTG) and NRv2 for insertion of the spectinomycin/streptomycin resistance gene into *hetN*, and NFw1 and NRv3 (CCAGCAGGCTCAAGATATTG) for detection of deletion of the central part of *hetN*.

Genotyping for *patS* gene deletion was performed using primer SFw1 (ACTAGTGGATCCCCCGACTCTGGGAGTAAATTG) and SRv2 (GCGAATTGGGTACCGCCTCGTGAAGCATG) for amplification of full-length *patS*, SFw4 (AAGCTTCGCGTGCTATAATTATACTAA) and SRv2 for insertion of the erythromycin resistance gene into *hetN*, and SFw1 and SRv3 (CCGCGCTCATCACAGAAAT) for detection of deletion of the central part of *patS*.

3.2.3. Microscopy, chlorophyll content, cell spectrum, and lipid analysis

Cell morphology, chlorophyll content, cell spectrum, and lipid content of *hglT* single mutant and triple mutant were conducted as described in Chapter II.

3.2.4. Oxygen evolution rates

Bulk photosynthesis activities were determined by measuring the oxygen evolution rate at 25^{o}C with a Clark-type oxygen electrode (Hansatech instruments, Norfolk, England) using 1 mL cell suspension of the samples (approximately 5-7 µg Chl *a* mL^{-1}). The *Anabaena* cells were illuminated with a halogen lamp at light intensity of 0 (dark conditions) to1,600 µmol m^{-2} s^{-1}.

3.2.5. Glycogen content

Amount of intracellular glycogen was determined according to Forchhammer and Tandeau de Marsac [13]. Cells were suspended in 100 µl of 3.5% (v/v) sulfuric acid solution and boiled for 40 min. Glucose produced by acid hydrolysis was quantified with the use of *o*-toluidine solution according to the protocol recommended (Sigma, Dorset, UK).

3.2.6. RNA extraction, Reverse transcription and qRT-PCR

Total RNA was isolated from whole filaments according to the manufacturer's protocol (Promega, Wisconsin, USA) and treated with DNase I (Takara Bio, Shiga, Japan). Four hundred nanogram of purified RNA was used for cDNA synthesis with random hexamer and PrimeScript II Reverse Transcriptase

(Takara Bio) according to the manufacturer's protocol. The generated cDNA was used as a template for qRT-PCR. qRT-PCR was performed with Thermal Cycler Dice Real Time System (Takara Bio) in a 20 μl reaction mixture containing 10 μl of SYBR Premix Ex Taq (Takara Bio), 0.4 μM each of *nifH* gene-specific forward and reverse primers (ACCTCGTGACAACATCGTTC and TTGGTGTAGGAATGGTGAGC). Relative ratios were normalized with the values for *rnpB,* which encodes a subunit of RnaseP, using a primer set of rnpB forward and reverse (CCAGTTCCGCTATCAGAGAG and GAGGAGAGAGTTGGTGGTAAG). The relative quantities are represented as means of duplicate experiments.

3.3. Results

3.3.1. Triple mutant increased heterocyst frequency under nitrogen starved conditions

To increase the amount of fatty alcohol, the author combined the mutation of *hglT* with *hetN* and *patS,* genes which encode the repressors of the heterocyst formation. It was expected that the increased ratio of heterocyst per filament will increase the heterocyst specific glycolipids formation, and hence accumulate more fatty alcohol. To construct triple mutant, the author inactivated *hetN* and *patS* in the *hglT* mutant of *Anabaena* and isolated the triple mutant of *hglT*, *hetN*, and *patS* (hereafter denoted as triple mutant). PCR analysis with external primer of *hglT, hetN,* and *patS* genes showed the different sizes of PCR fragments between wild type and the mutant (Fig.3.1). Genotyping of the *hglT* gene (Fig. 3.1A, TFw1

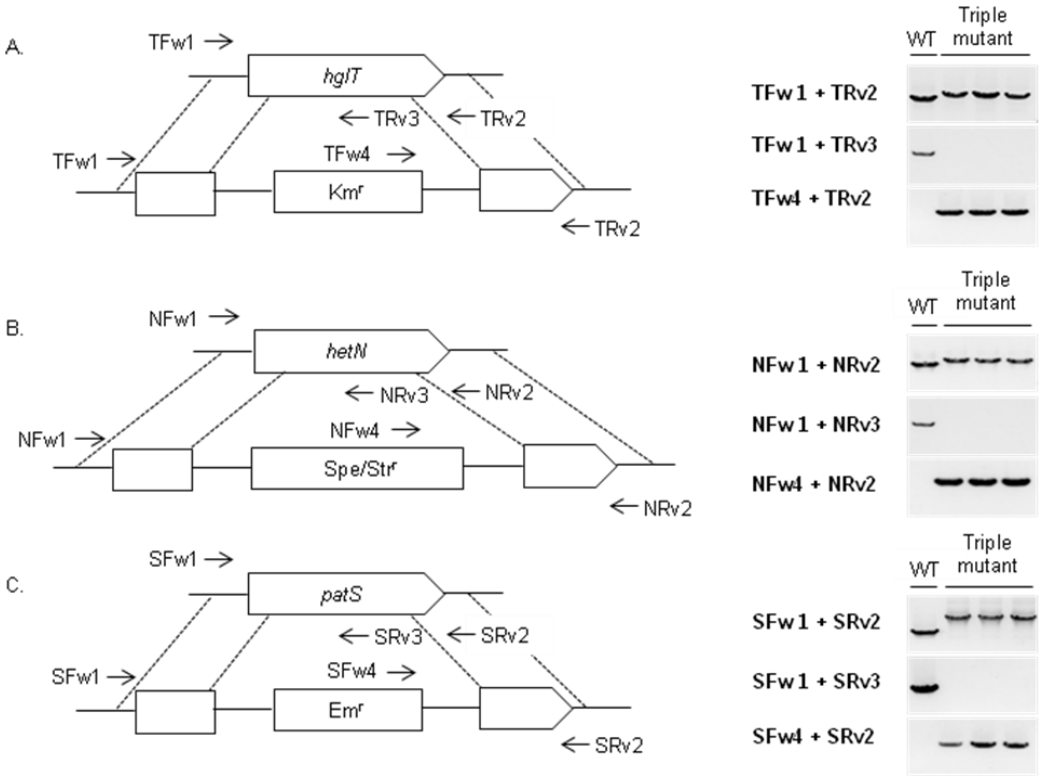


Figure 3.1. Genotype of the triple mutant**.** The schematic representations on the left side shows the map of gene disruptions and the primers used for genotyping of the triple mutant of *Anabaena*. **A.** *hglT* gene. **B.** *hetN* gene. **C.** *patS* gene. Km^r: kanamycin, Spe/Str^r: spectinomycin/streptomycin, Em^r: erythromycin resistance cassettes, WT: wild type. Arrows in each panel indicate the position and directions of primers for PCR.

and TRv2) showed that the wild type gave 2,976 bp and the size of the PCR fragment increased to 3,259 bp in the mutants because of the insertion of kanamycin resistance cassette. PCR with primer anneal to middle region of *hglT* gene (TRv3) gave amplicon only with the wild-type genome, since the region is deleted in the mutant genome. PCR with primer (TFw4), which anneal to kanamycin cassette, gave no signals in the wild-type, but showed strong signals in the mutant genomes. These results indicate that no wild type copies of the *hglT* gene were present in the mutant cells.

Genotyping of the *hetN* gene (Fig. 3.1B, NFw1 and NRv2) indicated that the wild-type gave 2,500 bp and the size of the PCR fragment increased to 3,500 bp in the mutants due to the insertion of spectinomycin/streptomycin resistance cassette. PCR with primer anneal to middle region of *hetN* gene (NRv3) and primer which anneal to kanamycin cassette (NFw4), showed complete replacement of all genomic copies of *hetN* gene. Genotyping of the *patS* gene (Fig. 3.1C, SFw1 and SRv2) revealed that the wild type gave 1,880 bp and the size of the PCR fragment increased to 2,655 bp in the mutants due to the insertion of erythromycin resistance cassette. PCR with internal primers which detected the middle region of *patS* gene (SRv3) gave amplicon only with the wild type genome due to the deletion of *patS* gene in the mutant genome. On the other hand, PCR with primer (SFw4), which anneal to erythromycin cassette, gave only signals only with the mutant genomes, indicating the replacement of all genomic copies of *patS* gene. Based on the results above, the triple mutants were isolated. The triple mutants grew both in the liquid and plate medium of BG11, which is

replete with nitrate as a nitrogen source. The author next examined the cell morphology of the triple mutant. Similar to the wild type and single mutant of *hglT*, the triple mutants differentiated heterocyst cells under nitrogen starved conditions (Fig. 3.2). In the triple mutants, the multicontiguous heterocyst cells were observed, which resulted in the increase heterocyst differentiation ratio in the triple mutant (Fig. 3.3 and 3.4).

3.3.2. Higher rate of heterocyst frequency improves the growth of the triple mutant under nitrogen-limited conditions

To evaluate whether the triple mutants can grow under nitrogen starved conditions, growth profiles of the wild-type, *hglT* single mutant (Δ*hglT*), and triple mutant cells were compared. As shown in Figure 3.5, inactivation *hglT*, *hetN*, and *patS* genes did not alter the growth rate in the medium replete with nitrogen, but decreased the growth rate under nitrogen starved conditions compared to the wild type. The retarded growth was almost the same as that of the *hglT* single mutant. Interestingly, total chlorophyll content of the triple mutant increased in nitrogen depleted conditions compared to the null mutant of *hglT* (Fig. 3.6A), as was confirmed by cell spectrum normalized by cell density (Fig. 3.6B) and photos of the cells (Fig. 3.7). The cell spectrum of the triple mutant cells also showed no significant alteration in the phycocyanin peak (625 nm) in the nitrogen replete conditions although the chlorophyll absorbance (438 and 675 nm) was reduced as compared to the wild type. This phenomenon was also seen in the *hglT* single mutant.

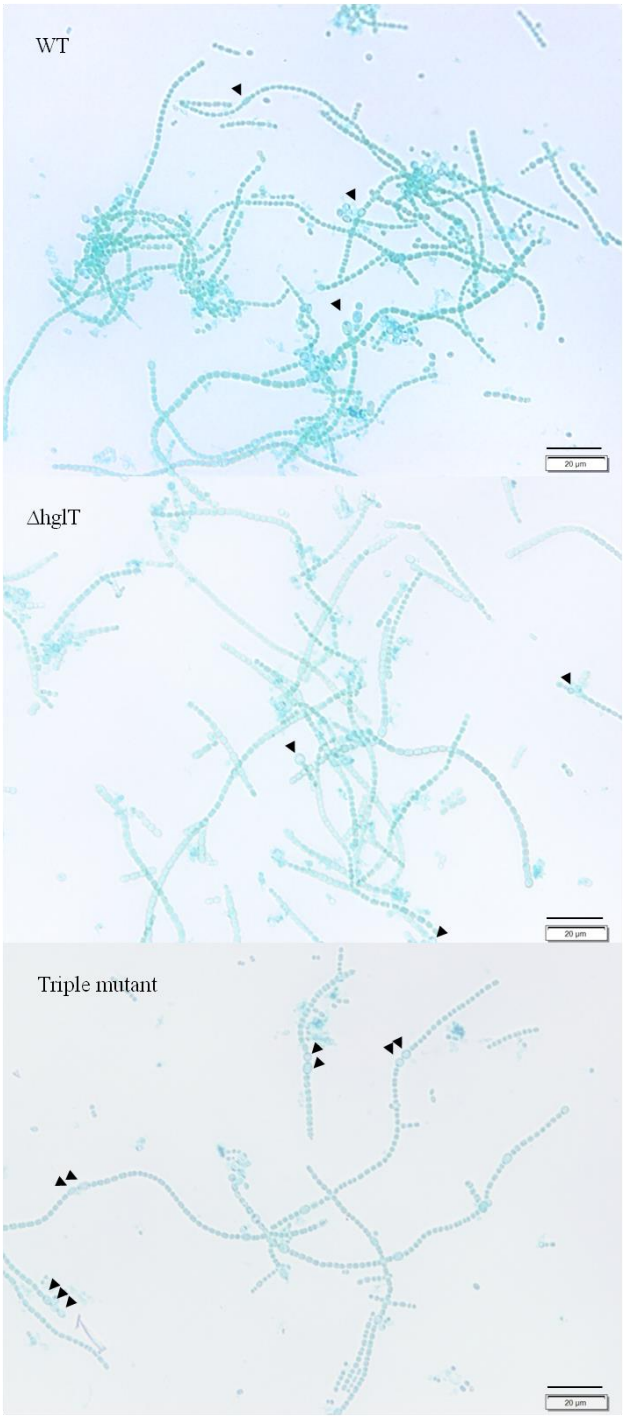


Figure 3.2. Heterocysts differentiation in the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. Cells were grown under nitrogen starved conditions then stained with 0.5% Alcian blue in a 50% ethanol solution for 30 min before observation. Heterocyst cells are pointed by black triangles. Bar, 20 µm.

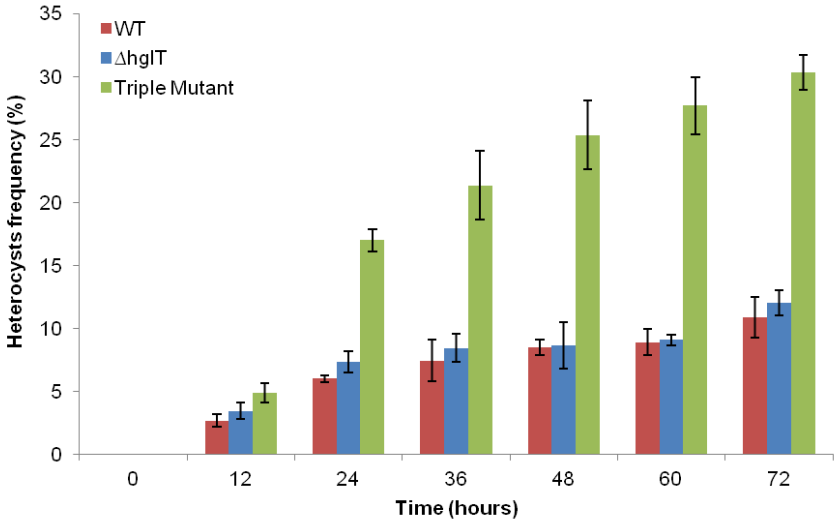


Figure 3.3. Heterocysts frequency in the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. Heterocyst frequencies were observed by growing cells under nitrogen starved conditions, then calculated the ratio of heterocyst cells at each indicated time. Error bars indicate the SD based on three independent experiments. Approximately 500 cells were counted per experiment.

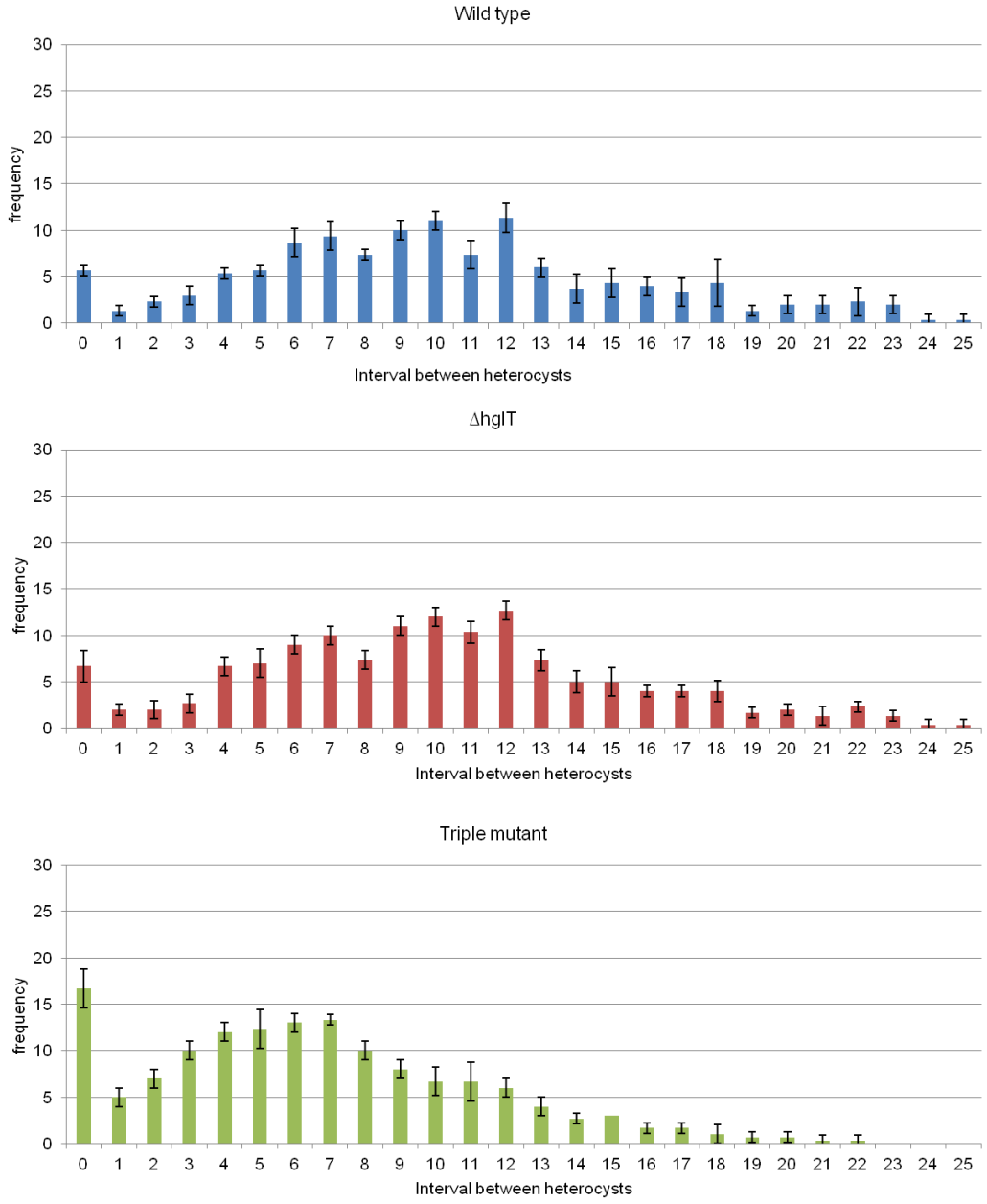


Figure 3.4. The frequency pattern of vegetative cells among heterocysts in filament of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. Cells were grown until mid-log phase then transferred to the medium deprived nitrogen for 48 h prior to heterocyst spacing determination. Error bars indicate the SD based on three independent experiments.

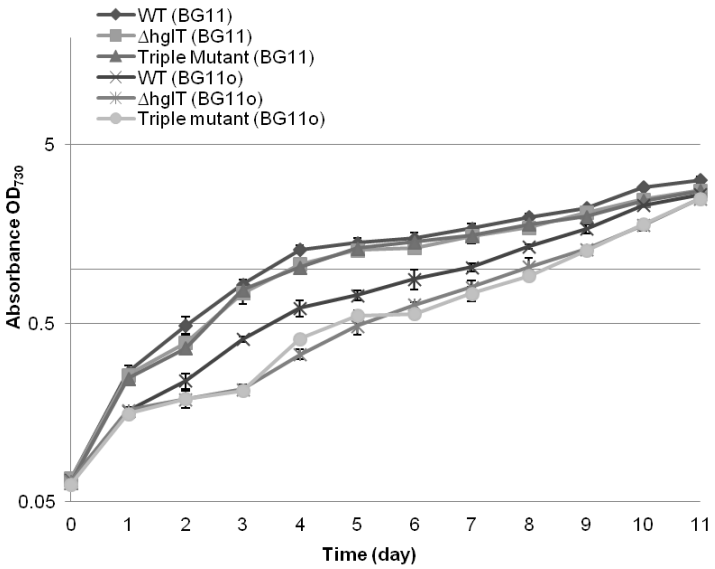


Figure 3.5. Growth rate of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant in the medium with (BG11 medium) or without combined nitrogen ($BG11_0$ medium). Cells were first grown in the BG11 medium to OD_{730} of 0.8, washed and transferred to new medium of BG11 or $BG11_0$ at OD_{730} of 0.05.

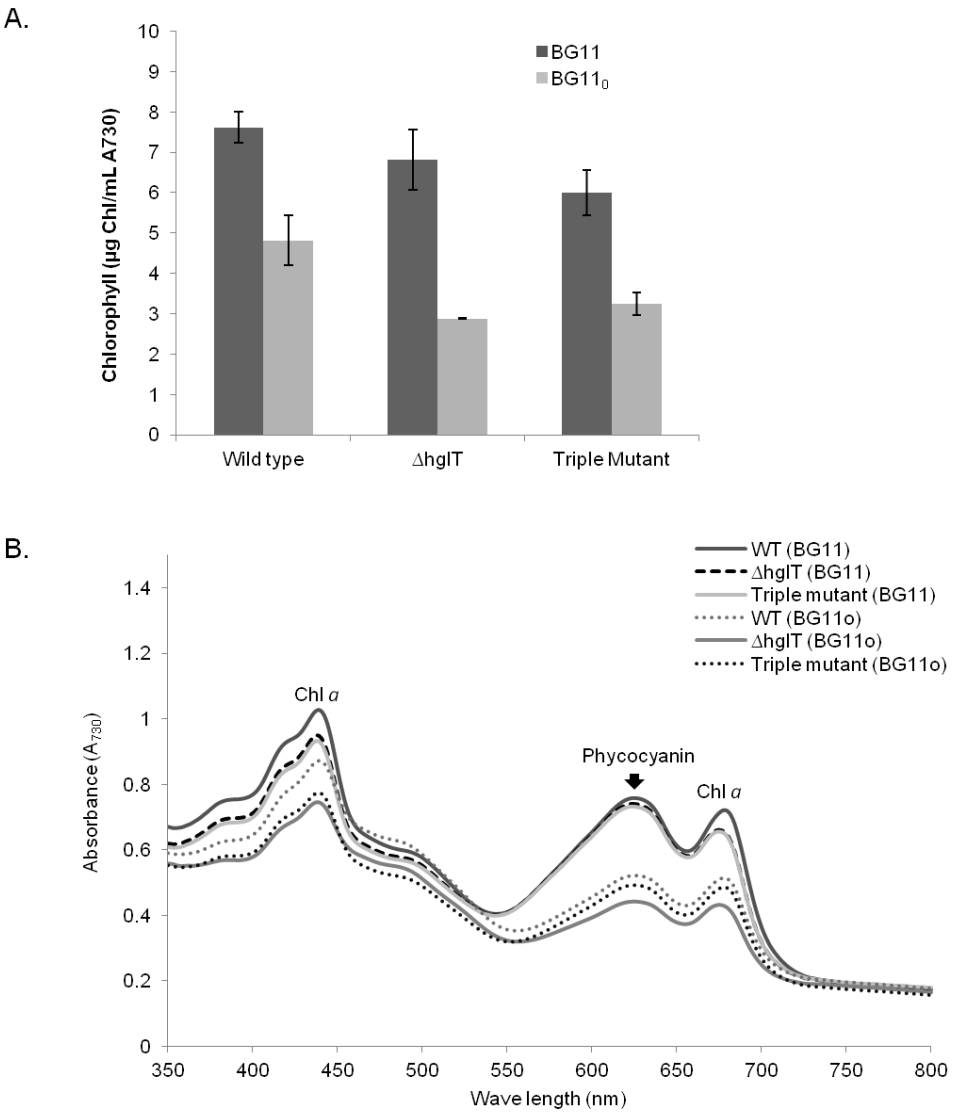


Figure 3.6. Chlorophyll content and whole-cell spectra of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. **A.** Chlorophyll content in nitrogen replete (BG11) and deprived ($BG11_0$) conditions. Error bars indicate the SD based on three independent experiments. **B.** Whole-cell spectra. The intact-cell spectra were measured with 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* (Chl *a*).

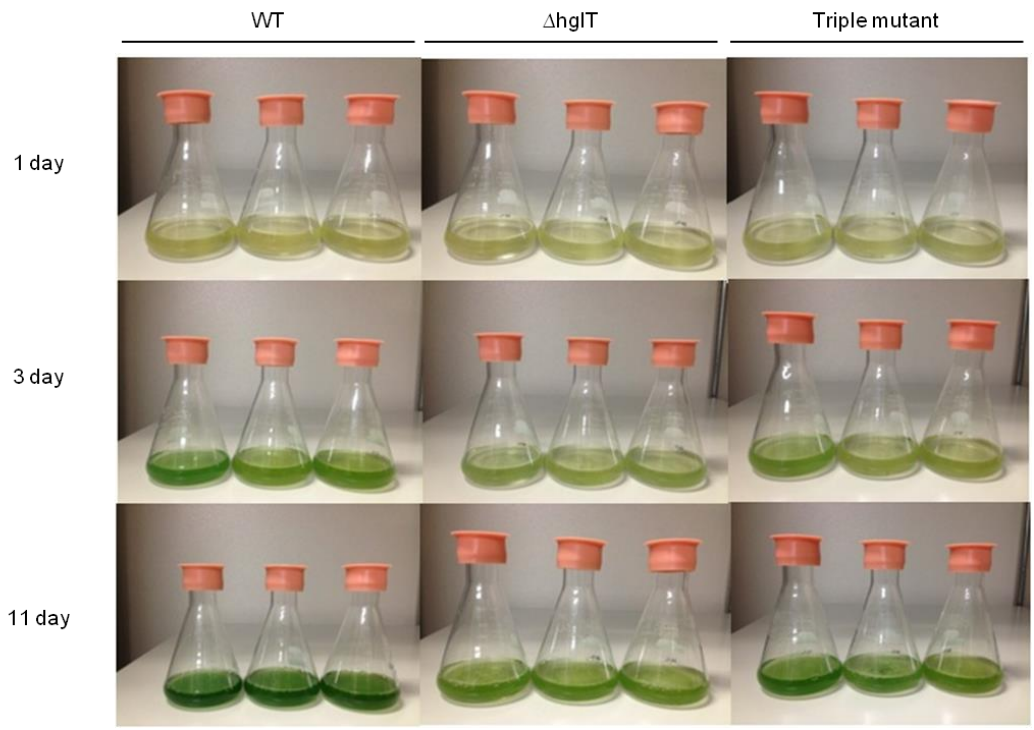


Figure 3.7. Growth of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant under nitrogen starved conditions. Cells were grown in the BG11 medium to the OD_{730} of 0.8-1.2, washed three times by $BG11_0$ medium, then transferred into new medium.

Because the chlorophyll content was different, photosynthetic activity of the wild type and mutant cells were compared (Fig. 3.8). The photosynthetic activity in the wild type was increasing gradually with light intensities up to 1,600 $\mu molm^{-2}s^{-1}$ and saturated at ~470 µmol mg Chl^{-1} h^{-1}. In contrast, the mutants showed saturated activity at ~270 µmol mg Chl^{-1} h^{-1}, less than 60% of the wild type. The author did not see any significant difference in the oxygen evolution rates between the single mutant and triple mutant cells, indicating that their retarded photosynthetic activity is due to inactivation of *hglT*, not *patS* and *hetN*.

3.3.3. Expression of *nifH* in the triple mutant was similar to that of the *hglT* single mutant

Since the triple mutants had increased rate of heterocyst cells, the author analyzed the expression level of *nifH*, the gene for a subunit of nitrogenase. As shown in the Figure 3.9, mRNA level of *nifH* was similar but slightly lower in the single *hglT* mutant and triple mutant compared to the wild type. This was also confirmed by RT-PCR (Fig. 3.10). It was shown that the expression level of *nif* gene in the wild type higher compared to hglT single and triple mutant after 24 h nitrogen step down, but then remain similar after more than 24 h incubation, indicating fatty alcohols were fail to provide the microoxic environment in the heterocyst cells of triple mutant as was in the *hglT* single mutant.

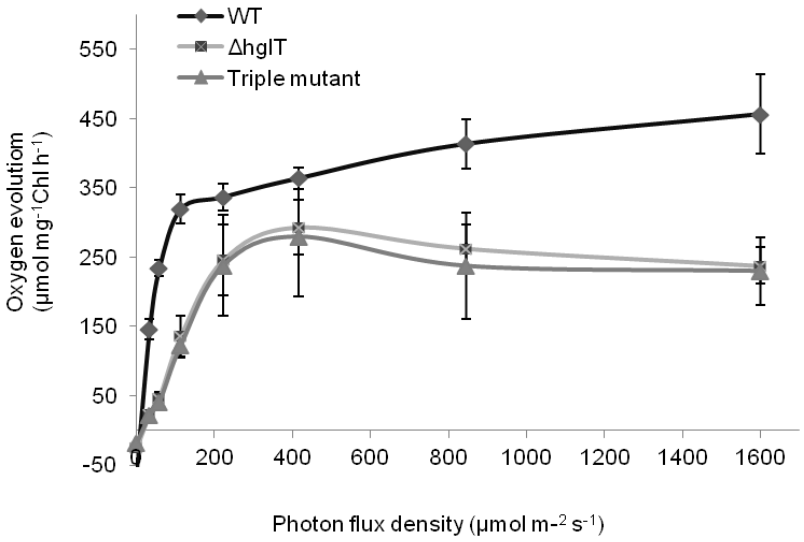


Figure 3.8. Oxygen evolution rate of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. Oxygen evolution rates were measured using cells of OD730=1.0.bError bars indicate the SD based on three independent experiments.

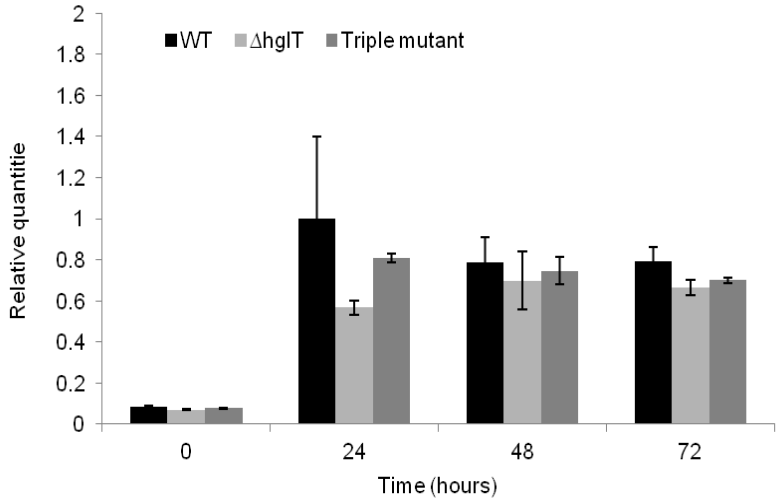


Figure 3.9. The transcript level of *nifH* under nitrogen deprivation. The relative quantities of *nifH* were determined by qRT-PCR in the wild type (WT) (black bars), *hglT* single mutant (ΔhglT) (light gray bars), and triple mutant (dark gray bars) for the indicated time.

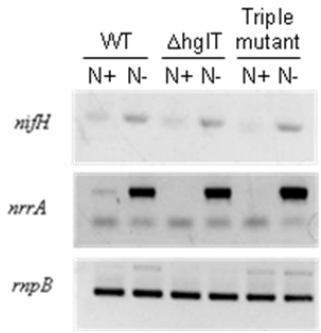


Figure 3.10. Transcript level of *nifH and nrrA* of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant under nitrogen deprivation. RT-PCR of the gene for a subunit of nitrogenase, *nifH, and nrrA gene*. Cells are deprived of fixed nitrogen for 48 h and RNA from cultures were extracted.

3.3.4. Triple mutant has higher expression of *nrrA*

It is reported that mutants with higher frequency of heterocysts had higher expression of *nrrA* gene and induced the degradation of intracellular glycogen [14]. To evaluate the expression level of *nrrA* in the triple mutant, the author did RT-PCR. As shown in Figure 3.10, expression level of *nrrA* seems slightly higher than the wild type and single *hglT* mutant. The author next analyzed accumulation of glycogen under nitrogen starved conditions (Fig. 3.11). It was found that the triple mutant has similar glycogen content with the *hglT* single mutant.

3.3.5. The triple mutant accumulated more fatty alcohol than *hglT* single mutant

To explore the effect of inactivation of *hetN* and *patS* in the *hglT* mutant on Hgl content, the author compared the lipid profile of both *hglT* single mutant and triple mutant. As expected, in nitrogen-limited conditions, the triple mutant lost Hgls similar to the *hglT* single mutant, and accumulated fatty alcohol, about higher amount than the single mutant of *hglT* (Fig. 3.12). Heterocyst frequency of the triple mutant was three times higher than the single mutant (Fig. 3.3), it might correspond to the higher production of fatty alcohol. On the other hand, there was no obvious change in membrane lipid content in the triple mutant.

In this study, the author used rotary shakers to grow cyanobacteria. It is well known that aeration to the medium enhance grow of cyanobacteria and it might also enhance production of Hgl or fatty alcohol. To study the effect of aeration on lipid production in the triple mutant, the author evaluated the lipid content of cynobacteria grown in the medium treated with and without aeration.

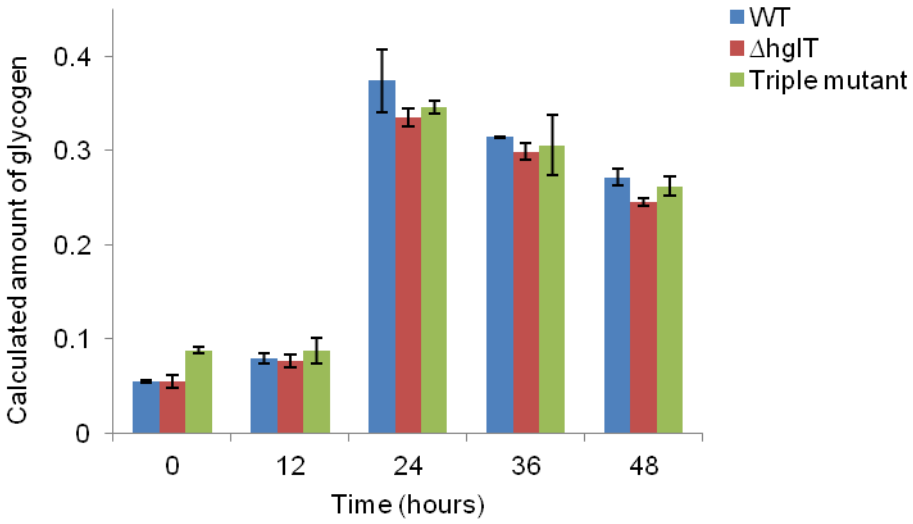


Figure 3.11. Intracellular glycogen content of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. The amount of glycogen in the indicated time after nitrogen step down was determined. Cells (OD_{730}=1.0) were resuspended and hydrolyzed by 3.5% H_2SO_4 at boiling temperature for 40 min.

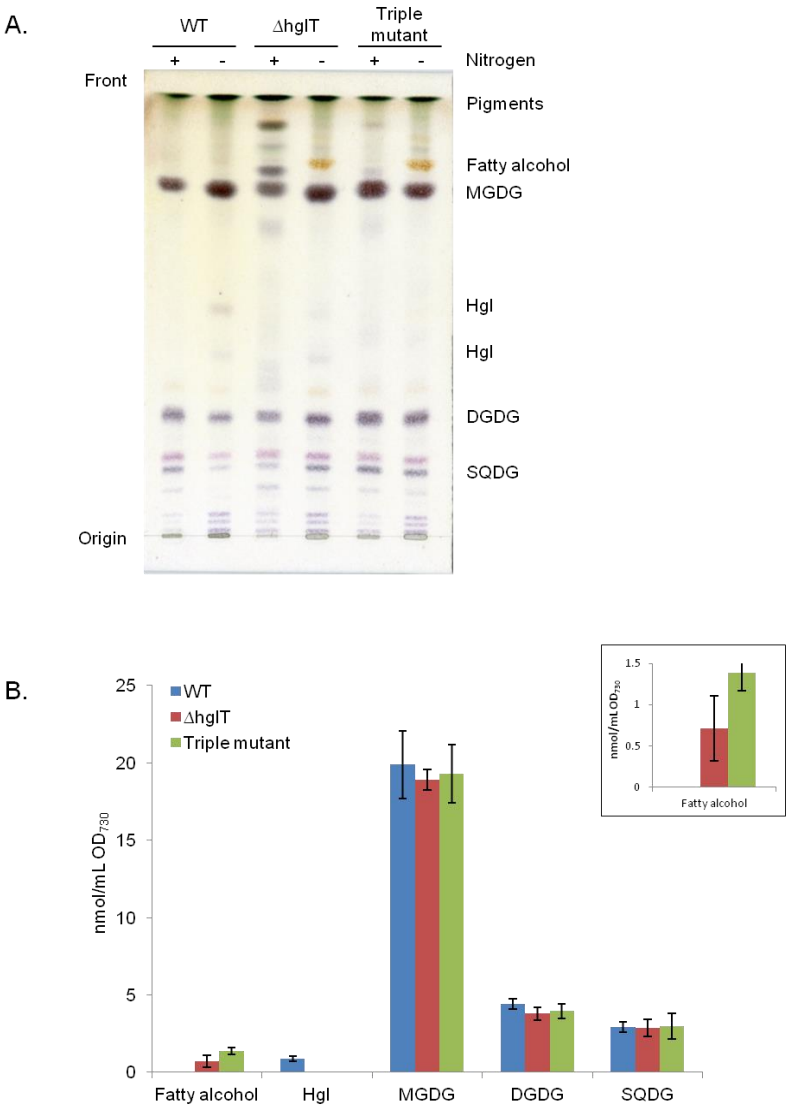


Figure 3.12. Lipid composition of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. **A.** Lipids separated by TLC. Lipids were extracted from the cells grown in BG11 or $BG11_0$ (48 h of nitrogen step down). **B.** Lipid content per cell density. Lipids were quantified by GC. Both the *hglT* mutants accumulated fatty alcohol and lacked Hgls. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol. Error bars indicate the SD based on three independent experiments. Inset refers to the magnification.

The mutants, with continually aeration of air accumulated fatty alcohol with a maximum production rate approximately two times higher than without the aeration (Fig. 3.13 and Fig. 3.14). In addition aeration also enhanced the glycolipid content in the wild type. Glycolipids contain polyunsaturated fatty acids attached to the glycerol backbone. Influx of CO_2 from air aeration increased the intracellular C/N ratio and triggered carbon conversion ratio of sugar to lipid. Thus treatment provided an efficient approach for increasing lipid [15].

3.4. Discussion

3.4.1. Extraction of fatty alcohol from the triple mutant

The triple mutant showed the increased heterocyst frequency and resulted in the higher accumulation of fatty alcohol. To extract this compound, it is very important to let the cells secrete the product into the growth medium because it minimizes the cost of harvest. In accordance with this, another possible modification on the triple mutant is to allow the secretion of fatty alcohol directly to the growth medium. The author proposed in Chapter II that heterocyst aglycone (fatty alcohol) localize to the space between the outer membrane and the Hep layer, as does the Hgls in the wild type. Disruption of Hep layer in the triple mutant may let the cells to secrete the fatty alcohol into the medium under nitrogen deprived conditions. This will be one of attractive future works.

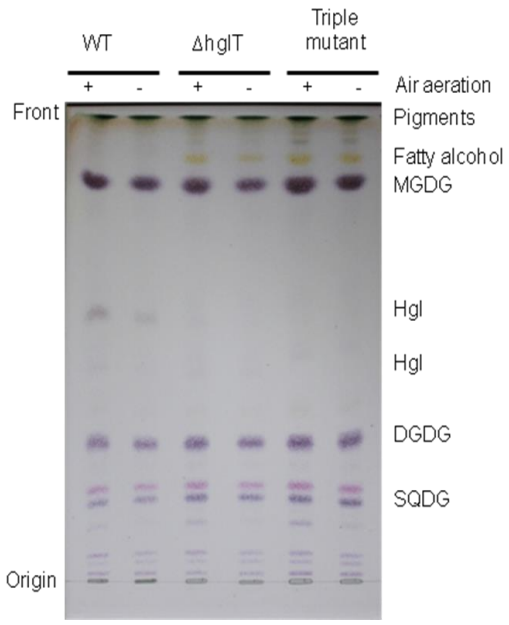


Figure 3.13. Effect of air aeration on lipid content of the wild type (WT), *hglT* single mutant (ΔhglT), and triple mutant. Lipids were separated by TLC using a solvent system of chloroform/methanol/acetic acid/water (85:15:10:3.7, v/v), and visualized with 50% sulfuric acid by staining and heating at 120^{o}C for 10 min. Cells were cultured in $BG11_0$ medium.

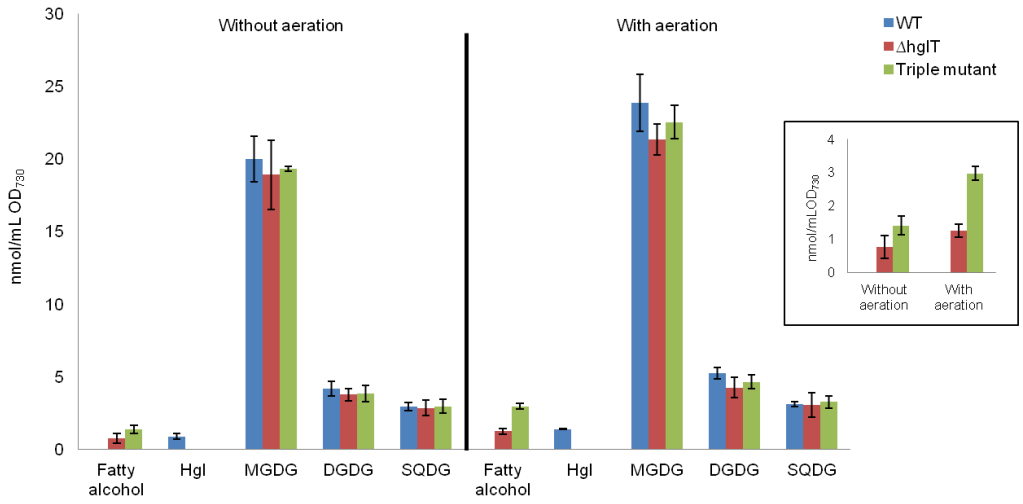


Figure 3.14. Lipid content of the wild type (WT), *hglT* single mutant (ΔhglT) and triple mutant. Cells were grown in $BG11_0$ medium with or without aeration. Lipids were quantified by GC-FID. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol. Error bars indicate the SD based on three independent experiments. Inset refers to the magnification.

3.4.2. Cell chlorosis of the single and triple mutants was slowly repaired under nitrogen starved condition

Nitrogen limitation induces chlorosis because of the degradation of phycobilisome and chlorophyll [16]. Both mutants and the wild type displayed similar rate of chlorosis 24 h after nitrogen step down (Fig. 3.6). After this point, the wild type and mutants differentiate mature heterocysts and start fixing nitrogen. The slower recovery of cell chlorosis was observed in both single and triple mutants compared with the wild type, probably because the mutant cells have less nitrogenase activity. However, the slight increased of chlorophyll content was also observed in the triple mutant compared with the *hglT* single mutant under nitrogen starved condition. The possible reasons to explain this phenomenon are as follows: First, the increased heterocyst frequency in the triple mutant triggered the partial recovery of the chlorophyll biosynthesis. Since the fatty alcohol produced in the triple mutant partially substitutes the function of Hgl as was in *hglT* single mutant, increased number of heterocyst may provide more nitrogen source to the vegetative cells. The other possibility is that the rate of chlorophyll degradation was slower in the triple mutant compared to the *hglT* single mutant. To confirm that, isotope labeling experiment will be required.

References

[1] Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56, 340-373.

[2] Awai, K., Lechno-Yossef, S. and Wolk , C.P. (2009) Heterocyst envelope glycolipids. In Lipid in photosynthesis essential and regulatory functions (Wada, H. and Murata, N., ed.^eds), pp. 179-202. Springer, Netherlands.

[3] Maldener, I., Hannus, S. and Kammerer, M. (2003). Description of five mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst differentiation and identification of the transposon-tagged genes. FEMS Microbiol Lett 224, 205-213.

[4] Huang, X., Dong, Y. and Zhao, J. (2004). HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc Natl Acad Sci USA 101, 4848–4853.

[5] Callahan, S.M. and Buikema, W.J. (2001). The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. Mol Microbiol 40, 941-950.

[6] Buikema, W.J. and Haselkorn, R. (1991). Characterization of a gene controlling heterocyst development in the cyanobacterium *Anabaena* 7120. Genes Dev 5, 321-330.

[7] Golden, J.W. and Yoon, H.S. (2003). Heterocyst development in *Anabaena*. Curr Opin Microbiol 6, 557-563.

[8] Fan, Q., Huang, G.C., Lechno-Yossef, S., Wolk, C.P., Kaneko, T. and Tabata, S. (2005). Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120. Mol Microbiol 58, 227-243.

[9] Borthakur, P.B., Orozco, C.C., Young-Robbins, S.S., Haselkorn, R. and Callahan, S.M. (2005). Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Mol Microbiol 57, 111-123.

[10] Zheng, Y.N. et al. (2012). Optimization of fatty alcohol biosynthesis pathway for selectively enhanced production of C12/14 and C16/18 fatty alcohols in engineered *Escherichia coli*. Microbial Cell Fact 11, 1-7.

[11] Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S.B. and Keasling, J.D. (2010). Microbial production of fatty acid-derived fuels and chemicals from plant biomass. Nature 463, 559-563.

[12] Elhai, J. and Wolk, C.P. (1988). Conjugal transfer of DNA to cyanobacteria. Methods Enzymol 167, 747-755.

[13] Forchhammer, K. and De-Marsac, N.T. (1995). Phosphorylation of the PII protein (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942: analysis of in vitro kinase activity. J Bacteriol 177, 5812–5817.

[14] Ehira, S. and Ohmori, M. (2011). NrrA, a nitrogen-regulated response regulator protein, controls glycogen catabolism in the nitrogen-fixing

cyanobacterium *Anabaena* sp. strain PCC 7120. J Biol Chem 286, 38109-38114.

[15] Xiong, W., Gao, C., Yan, D., Wu, C. and Wu, Q. (2010). Double CO_2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. Bioresour Technol 101, 2287-2293.

[16] Sauer, J., Gorl, M. and Forchhammer, K. (1999). Nitrogen starvation in *Synechococcus* PCC 7942: Involvement of glutamine synthetase and NtcA in phycobiliprotein degradation and survival. Arch Microbiol 172, 247-255.

Chapter IV

General Discussion

4.1. Maturation of heterocyst in *hglT* mutants

Anabaena develops special cell compartment, namely heterocyst, for nitrogen fixation in response to the nitrogen starved conditions [1-3]. In *Anabaena* every single heterocyst cells are particularly interrupted by 10-20 vegetative cells [4]. At least there are three groups of gene clusters that are involved in heterocyst differentiation: (1) Sensory genes which perceive the signals of nitrogen starvation in the environment; (2) genes that are responsible for initiating heterocyst differentiation; and (3) group of genes which play roles in heterocyst morphogenesis and function. All of these genes are expressed at each different stage of heterocyst formation and once the heterocyst differentiation and maturation have been completed, the expression of these genes returns to initial levels [5].

Heterocyst cell has special feature to facilitate nitrogen fixation. As described in Chapter I, heterocyst cells form specific glycolipid (Hgl) and polysaccharides (Hep) layers to impede oxygen influx and protect nitrogenase activity [6,7]. Walsby [8] reported that glycolipid layer is essential to prevent oxygen penetration, therefore mutation that affects the synthesis, transport, or deposition of Hgl could result the loss of the ability to fix nitrogen under aerobic conditions. It has been described that the mutants, which have missing or aberrant

heterocyst envelope layers, are unable to grow under nitrogen starved conditions [9]. Since this layer is hydrophobic, it may serve efficiently as a barrier to the migration of hydrophilic molecules and gases from the environment into the cells. Interestingly, the author found that *hglT* null mutants lacked Hgls and grew slower but significantly under nitrogen deprivation. This result implies that the mutants cannot fix enough nitrogen. The *hglT* mutant accumulated fatty alcohol (aglycone). If the fatty alcohol was not enough to substitute the function of Hgl, heterocyst cannot be matured and the mutant cells would have to keep synthesizing this lipid compound. Expression level of genes involved in Hgl synthesis should be analyzed in the mutant to answer this question.

4.2. Evolution of lipid layer in photosynthetic organisms

Heterocyst cells were reported to be one of the oldest forms of differentiated cells on earth, since they may have appeared in evolution between 2450 and 2100 million years ago [10]. As described in Chapter I, heterocystous and filamentous cyanobacteria, including *Anabaena*, develop heterocyst cells. These cells were differentiated from vegetative cells with distinct morphology and function. Heterocyst cells have glycolipid layer, made of heterocyst specific glycolipids (Hgls), to isolate nitrogenase from oxygen. This is similar to the function of wax, cutin and cuticle in the land plants, which protect plant cells from environmental stresses.

Hgl synthesis in cyanobacteria is catalyzed by polyketide synthase, while in the plant long chain fatty acid utilized for wax and cutin synthesis is catalyzed

by fatty acid elongase. Interestingly, these enzymes are in the same family and probably also have the same origin. In addition, ABC transporter is involved in the transport of both Hgls and wax from inside to the outside of the cells. A *devBCA* gene clusters are involved in Hgls export in cyanobacteria [11], while the ABCG11/12 are found to be implicated in cutin precursor and wax transport across the plasma membrane [12,13]. The similarities of the function, structures, and genes involved in the lipid layer synthesis and transport suggest that the layer in the land plants come from same origin with that of cyanobacteria or by convergent evolution.

4.3. Possible application of fatty alcohol from *Anabaena* in chemical industry

Fatty alcohols have many applications in chemical industry [14]. In fact, long chain fatty alcohols are used for precursor of applied products such as detergents, lubricants, cosmetic, emulsifier, pheromones, and potentially as biofuels. The increased demands of fatty alcohols attract the attention for the exploration of an alternative system for cost-effective and sustainable production of these compounds. Utilization of microorganisms, including *Anabaena*, would provide an alternative synthetic route to produce fatty alcohol in sustainable system. The author showed that knock out mutant of *hglT* gene can be an alternative tool for producing sustainable fatty alcohol. Both *hglT* single mutant and triple mutant accumulated fatty alcohol which has a very long chain of carbon atoms (26-32 carbons). This fatty alcohol may serve as a precursor compound for producing another derivatives, as described above, for further application in

chemical industry. By now, although the mutants describe above are not efficient enough to produce fatty alcohol to substitute fossil oil, these transformants can be a start point and modification the system will improve their ability to produce sustainable compounds in cyanobacteria.

References

[1] Meeks, J.C. and Elhai, J. (2002). Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66, 94-121.

[2] Golden, J.W. and Yoon, H.S. (2003). Heterocyst development in *Anabaena*. Curr Opin Microbiol 6, 557-63.

[3] Shi, L., Li, J.H., Cheng, Y., Wang, L., Chen, W.L. and Zhang, C.C. (2007). Two genes encoding protein kinases of the HstK family are involved in synthesis of the minor heterocyst-specific glycolipid in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 189, 5075-5081.

[4] Kumar, K., Mella-Herrera, R.A. and Golden, J.W. (2010). Cyanobacterial heterocysts. Csh Perspect Biol 2, 1-19.

[5] Ehira, S. and Ohmori, M. (2011). NrrA, a nitrogen-regulated response regulator protein, controls glycogen catabolism in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. J Biol Chem 286, 38109-38114.

[6] Awai, K., Lechno-Yossef, S. and Wolk , C.P. (2009) Heterocyst envelope glycolipids. In Lipid in photosynthesis essential and regulatory functions (Wada, H. and Murata, N., ed.^eds), pp. 179-202. Springer, Netherlands.

[7] Huang, X., Dong, Y. and Zhao, J. (2004). HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-

binding activity is inhibited by PatS. Proc Natl Acad Sci USA 101, 4848–4853.

[8] Walsby, A.E. (1985). The permeability of heterocysts to the gases nitrogen and oxygen. Proc R Soc Lond B 226, 345-366.

[9] Nicolaisen, K., Hahn, A. and Schleiff, E. (2009). The cell wall in heterocyst formation by *Anabaena* sp. PCC 7120. J Basic Microbiol Mol Biol Rev 49, 5-24.

[10] Tomitani, A., Knoll, A.H., Cavanaugh, C.M. and Ohno, T. (2006). The evolutionary diversification of cyanobacteria: Molecular-phylogenetic and paleontological perspectives. Proc Natl Acad Sci USA 103, 5442-5447

[11] Fiedler, G., Muro-Pastor, A.M., Flores, E. and Maldener, I. (2001). NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp. J Bacteriol 183, 3795-3799.

[12] Pighin, J.A., Zheng, H., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L. and Samuels, A.L. (2004). Plant cuticular lipid export requires an ABC transporter. Science 306, 702-704.

[13] McFarlane, H.E., Shin, J.J.H., Bird, D.A. and Samuels, A.L. (2010). *Arabidopsis* ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. Plant Cell 22, 3066-3075.

[14] Mudge, S.M., DeLeo, P.C. and Dyer, S.D. (2012). Quantifying the anthropogenic fraction of fatty alcohols in a terrestrial environment. Environ Toxicol Chem 31, 1209-1222.

Acknowledgments

I would like to express my deep gratitude to my supervisor, Assoc. Prof. Dr. Koichiro Awai for his guidance and continuous support along my study and research, for his motivation, encouragement, insightful comments for my thesis and article, (frequently) asked many hard questions, and the worth and meaningful knowledge over the years. He supported me during numerous hard time when I was thinking that such thing seem could not working out. He encouraged me to think further. Word seem would not be enough to express my deeply appreciation on his excellent guidance and be a great mentor and aspirator during my study.

I would like to thank you to Prof. Masahito Yamazaki for being my supervisor and my lecturer during my study in Shizuoka University.

I would also to sincerely thank you to my former supervisor, Prof. Yuzo Shioi, for his support and advice in the early first two years of my study and who is always pushed me up to beat my highest expectation and gave me strongly motivation to keep learning and exploring my research interest. Deeply appreciation also expressed for his meaningful input to my thesis.

I would like to thank you to Assoc. Prof. Shigeki Ehira for his help in measuring the nitrogen fixation activity of the wild type and *hglT* single mutant strains.

I would also very like and also Assoc. Prof. Rei Narikawa for very meaningful discussion, hardly questions and insightful comment related to my research.

I would very much like to thank you Prof. Nobuyoshi Shiojiri, Prof. Masakazu Hara, and Prof. Taketomo Fujiwara for reviewing my thesis.

A heartfelt thank you to all my lab mates in Awai laboratory for their support, stimulating discussion, immeasurable kind help, dependable friendship and making my experience I have had in the last three years enjoyable and unforgettable. Many thanks also to Keiji Fushimi for insightful discussion.

Last but not least, I would like to thank you to my parents, my lovely husband, brothers and sisters, for all their support, unrelenting love, and pride.