Full Paper

A polyketide synthase HglE_A, but not HglE2, synthesizes heterocyst specific glycolipids in *Anabaena* sp. PCC 7120

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Heterocysts are the specialized cells for nitrogen fixation in some filamentous cyanobacteria. To protect the oxygen labile nitrogen fixing enzyme, nitrogenase, heterocysts keep their inner environment microoxic by developing layers of barrier on the outside of their outer membranes. Heterocyst specific glycolipids (Hgl) are constituents of the layer of barrier and amphipathic compounds, synthesized from a very long chain fatty alcohol as a hydrophobic tail and a sugar as a polar head. In the model heterocystous cyanobacterium *Anabaena* sp. PCC 7120, Hgl is made of fatty alcohol with 26 carbons and a glucose, linked by an ether bond in alpha configuration. The fatty alcohol is synthesized via reactions of a polyketide synthase, HglE_A. In *Anabaena* sp. PCC 7120, another polyketide synthase HglE2 shared more than 50% identity in an amino acid sequence with HglE_A and is expected to be involved in Hgl synthesis. However, no direct evidence has been reported. Here, we experimentally show that HglE_A is the contributor of Hgl synthesis, and that HglE2 is not involved in the development of the heterocyst specific glycolipid layer.

Key Words: cyanobacteria; glycolipid; heterocyst; nitrogen fixation; polyketide synthase

Abbreviations: HEP, Heterocyst envelope polysaccharide; HGL, Heterocyst specific glycolipid layer; Hgl, Heterocyst specific glycolipid; Hgl-diol, 1- α -glucosyl-3; 25-hexacosanediol; Hgl-ketol, 1- α -glucosyl-3-keto-25-hexacosanol

Introduction

A heterocyst is a specialized cell for nitrogen fixation in some filamentous cyanobacteria and differentiates from a vegetative cell under nitrogen deficient conditions. Since the nitrogenase, which transforms gaseous dinitrogen into ammonium, is labile to oxygen, heterocysts have to keep their inner environment under microoxic conditions (Awai et al., 2009; Wolk et al., 1994). To achieve this, heterocysts suppress photosynthetic activity to decrease oxygen evolution, promote respiration to consume internal oxygen, and form additional outer envelope layers to prevent diffusion of oxygen from outside of the cell (Murry and Wolk, 1989).

The main components of the outer envelope layer are a heterocyst specific glycolipid layer (HGL) constituted by heterocyst specific glycolipids (Hgl), and a heterocyst envelope polysaccharide (HEP) layer. Construction of the outer envelope layer starts by deposition of the polysaccharides to the outside of heterocyst cells to form an HEP layer, followed by accumulation of Hgl between the HEP layer and the outer membrane (Awai et al., 2009; Nicolaisen et al., 2009). In the representative filamentous cyanobacteria *Anabaena* sp. PCC 7120 (hereinafter, *Anabaena*), Hgl is made of a glucose attached to a very long chain fatty alcohol consisting of 26 carbon atoms with hydroxyl groups (Hgl-diol) and/or a carbonyl group (Hgl-ketol) (Gambacorta et al., 1996) (Fig. 1A). Several genes encoding the enzymes responsible for Hgl synthesis have been identified. A Fox- mutant that can fix nitrogen under anaerobic conditions, but not aerobic conditions, was isolated in a filamentous cyanobacteria *Nostoc punctiforme* (hereinafter *Nostoc*), and found to be deficient in the synthesis of Hgl (Campbell et al., 1997). This indicates that Hgl is the major contributor to prevent diffusion of oxy-

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Fig. 1. Chemical structure of Hgls in *Anabaena* and a collapsed phylogenetic tree of polyketide synthases in cyanobacteria.

A. Chemical structure of Hgls, Hgl-diol and Hgl-ketol. **B.** Phylogenetic analysis of HglE_A and HglE₂. Amino acid sequence of PfaA of *Shewanella* sp. BR-2 was used as a query (and outgroup) of BLAST search against cyanobacterial amino acid sequences. HglE_A and HglE₂ sequences are highlighted by black triangles. Two sequences of HglE_A are NCBI sequence ID BAB77050.1 (upper one, used in this study) and RUR88086.1 (N663D amino acid change). Detail of the tree can be seen in Supplementary Fig. 1.

gen. The mutated gene encodes a protein similar to polyketide synthase and was designated *hglE*. Later, a Fox[−] mutant that did not accumulate Hgls under nitrogen-deficient conditions was isolated from *Anabaena*, and the mutated gene was found to encode a protein similar to HglE (Fan et al., 2005). HglE is classified into a type 1 polyketide synthase with functional domains enable the enzyme to synthesize polyketide alone (Jenke-Kodama et al., 2005), including a ketoacyl synthase (KS) domain, an acyltransferase (AT) domain, and two acyl carrier protein (ACP) domains (Awai et al., 2009; Fan et al., 2005). Type 1 polyketide synthases are also found in other species of cyanobacteria for the synthesis of toxic compounds, such as microcystin (Shishido et al., 2019), and swinholid (Humisto et al., 2018). PfaA, a type 1 polyketide synthase found in marine bacteria, is involved in the synthesis of very long chain polyunsaturated fatty acids, such as

docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Okuyama et al., 2007; Yazawa, 1996). The amino acid sequence of PfaA shared ~45% to that of HglE, leading to the prediction that HglE is responsible for the synthesis of the very long chain fatty alcohols of Hgls. *Anabaena* has two homologs of HglE: HglE_A and HglE₂. These polyketide synthases share more than 56% identity throughout their amino acid sequences (more than 1,000 residues, Expect = 0.0). Since the *hglE_A* (*alr5351*) gene, the orthologous gene of *Nostoc hglE*, forms a gene cluster, and mutants of the genes in this cluster were found to have a decreased amount of Hgls, it is considered that Hgls are synthesized by a protein group encoded by this *hglE_A* gene cluster (Fan et al., 2005). *hglE₂* (*all1646*) also forms a gene cluster similar to that of *hglE_A*, but its role is unknown. These two gene clusters are found in the filamentous and heterocyst forming cyanobacteria, such as *Anabaena variabilis*, *Nostoc* and *Nodularia spumigena*. Therefore, it seems likely that these gene clusters have roles in heterocyst development. Moreover, in *Anabaena*, two types of Hgl (Hgl-diol and Hgl-ketol) have been reported to construct HGL in about a 5:2 ratio (Gambacorta et al., 1996). These results suggest that one of the gene clusters is responsible for Hgl-diol, and the other for Hgl-ketol. However, the *hglE₂* gene cluster is also found in a non-heterocystous unicellular cyanobacteria, *Microcystis aeruginosa*, suggesting that HglE₂ is not directly involved in Hgls synthesis, namely heterocyst development (Awai et al., 2009).

In this research, we have tried to answer the question above by analyzing knock out mutants of the *hglE* genes in *Anabaena*, and found that HglE_A is the major contributor of Hgls synthesis and that HglE₂ is not involved in the Hgls synthesis, at least under the conditions we analyzed.

Materials and Methods

Cyanobacterial strains and growth conditions. *Anabaena* sp. PCC 7120 wild type and mutant strains were grown in a liquid medium of BG11 (containing nitrate as a source of nitrogen) (Stainer et al., 1971) at 30°C in the light (50–100 μ mol m^{−2} s^{−1}) on a rotary shaker (120 rpm) as described previously (Awai et al., 2007). For nitrogen deficient condition experiments, cells were first grown in the BG11 medium to an optical density at 730 nm (OD₇₃₀) of approx. 1.0 and washed three times with a nitrogen-free medium (BG11₀; BG11 without nitrate) and then resuspended in BG11₀. After this treatment, cells were grown in BG11 or BG11₀ media, and OD₇₃₀ was measured every 24 hours by Bio Spec-mini (Shimadzu, Japan).

Isolation of null mutants and transformants. The knock out vector of *hglE_A* (*alr5351*) gene was constructed as follows. DNA fragments upstream and downstream of *hglE_A* gene were amplified by PCR using the primers 1 and 2 (Supplementary Table 1), and 3 and 4, respectively. The upstream fragment was cloned into *Sma*I site of pMobQ1 by In-Fusion HD Cloning Kit with Cloning Enhancer (Takara Bio, Shiga Japan), and then the downstream fragment was cloned into the *Apa*I site to construct the knock out vector, pMO1hglE_AKO. pMobQ1, a derivative of the pMobKm1 (Halimatul et al., 2014) was constructed by

Fig. 2. Isolation and phenotype analysis of *hglE_A* mutants ($\Delta hglE_A$).

A. Schematic representation of *hglE_A* gene in the wild type and mutant genomes. Black arrows indicate position of primers used in (B). *Spe^r*: Spectinomycin resistant cassette. B. Genotype analysis of $\Delta hglE_A$. Each fragment was amplified by PCR with the primers indicated in (A). C. TLC analysis of lipids in the wild type (WT) and $\Delta hglE_A$. The gray arrow indicates the position of Hgl-ketol, and the black arrow Hgl-diol. D. Growth curve of wild type and $\Delta hglE_A$. Filled black circles: wild type (N+, nitrogen replete conditions); blank black circles: wild type (N-, nitrogen deprived conditions), filled gray triangles: $\Delta hglE_A$ (N+), blank gray triangles: $\Delta hglE_A$ (N-). Values are average $\pm$ standard deviation ($n = 6$).

replacing the kanamycin cassette with the omega cassette of pHP45 (Prentki and Krisch, 1984).

The knock out vector of *hglE2* (*all1646*) gene was constructed as follows. DNA fragments upstream and downstream of the *hglE2* gene were amplified by PCR using the primers 5 and 6, and 7 and 8, respectively. The upstream fragment was cloned into the *Sma*I site of pMobKm1 by the In-Fusion HD Cloning Kit, and then downstream fragment was cloned into the *Xho*I site to construct the knock out vector, pMK1hglE2KO.

The knock out vector of *all1643* gene was constructed as follows. DNA fragments upstream and downstream of *all1643* gene were amplified by PCR using the primers 9 and 10, and 11 and 12, respectively. The upstream fragment was cloned into the *Sma*I site of pMobΩ1 by the In-Fusion HD Cloning Kit, and then the downstream fragment was cloned into the *Xho*I site to construct the knock

Fig. 3. Isolation and phenotype analysis of *hglE2* mutants ($\Delta hglE2$).

A. Schematic representation of *hglE2* gene in the wild type and mutant genomes. Black arrows indicate position of primers used in (B). *Neo^r*: Neomycin resistant cassette. B. Genotype analysis of $\Delta hglE2$. Each fragment was amplified by PCR with the primers indicated in (A). C. TLC analysis of lipids in the wild type (WT) and $\Delta hglE2$. The gray arrow indicates the position of Hgl-ketol, and the black arrow Hgl-diol. D. Growth curve of wild type and $\Delta hglE2$. Filled black circles: wild type (N+, nitrogen replete conditions); blank black circles: wild type (N-, nitrogen deprived conditions), filled gray triangles: $\Delta hglE2$ (N+), blank gray triangles: $\Delta hglE2$ (N-). Values are average $\pm$ standard deviation ($n = 6$).

out vector, pMO1all1643KO.

The vector for replacing *hglE_A* into the *hglE2* gene was constructed as follows. Full length ORF fragments of *hglE2* gene was amplified by PCR using the primers 13 and 14, and cloned into the *Sma*I site of pMO1hglE_AKO by the In-Fusion HD Cloning Kit. The vector for the over expression of *hglE2* gene was constructed as follows. The promoter region of the *psbA* gene from *Amaranthus hybridus* (Elhai, 1993) and the *hglE2* gene fragment (+1 to 1,000 from the start codon) were amplified by PCR using the primers 15 and 16, and 17 and 18, respectively. These fragments were cloned together into the *Apa*I site of pMK1hglE2-5', which has only 5' upstream region of *hglE2* gene of pMK1hglE2KO, by the GeneArt Seamless Cloning and Assembly Kit (ThermoFisher). These vectors were introduced into wild-type *Anabaena* by triparental mating independently by the method of Elhai and Wolk

Fig. 4. Isolation and phenotype analysis of *all1643* mutants ($\Delta all1643$).

A. Molecular organization of proteins encoded in *hglE2* gene cluster. Abbreviation of domains are as follows: ACP: acyl carrier protein, AT: acyl transferase, CLF: chain length factor, DH: dehydrase, ER: enoyl reductase, KR: ketoacyl reductase, KS: ketoacyl synthase. B. Schematic representation of *all1643* gene in the wild type and mutant genomes. Black arrows indicate position of primers used in (C). *Spe^r*: Spectinomycin resistant cassette. C. Genotype analysis of $\Delta all1643$. Each fragment was amplified by PCR with the primers indicated in (B). D. TLC analysis of lipids in WT and $\Delta all1643$. Gray arrow indicates position of Hgl-ketol and black arrow Hgl-diol.

(1988). Genomic DNA from wild-type and transformants were used for genotyping as templates for PCR with the primers described in each figure and HybriPol DNA polymerase (Bioline).

Lipid analysis. Cyanobacterial cells were grown in BG11 or BG11₀ media until the OD₇₃₀ reached to 0.8–1.0. A fifty milliliter aliquot of culture was then centrifuged (1,630 g, 4°C) to harvest the cells and the pellet was resuspended in 500 μ L of BG11 or BG11₀ media. Then 3 ml of chloroform/methanol (1:2, v/v) was added and left at room temperature after vigorous mixing. Two milliliters of sodium chloride (0.9%, w/v) was added to the tube and centrifuged after vortexing. The resultant bottom layer was transferred to a new glass tube and washed again with the same volume of sodium chloride solution. After centrifugation, the bottom layer was collected into a new glass tube and stored at –30°C until use. Two hundred microliters of lipid samples were spotted on a TLC plate and developed with the solvent system: chloroform/methanol/acetic acid/H₂O (85/15/10/3.7, v/v) (Jang et al., 2007). Lipids were visualized by spraying 50% sulfuric acid followed by 10 min of heating at 120°C.

qRT-PCR. Total RNA was extracted according to Pinto et al. (2009) and treated with RQ1 RNase-Free DNase (Promega). Then the RNA was reverse transcribed to cDNA by PrimeScript II 1st strand Synthesis Kit (Takara Bio) and used as a template for qRT-PCR using SYBR Premix Ex Taq II (Takara Bio) and Thermal Cycler Dice TP800 (Takara Bio). For amplification of *rnpB* gene (as a

Fig. 5. Isolation and phenotype analysis of *hglE_A* mutant replaced with *hglE2* (*hglE_A::hglE2*).

A. Schematic representation of *hglE_A* gene in the wild type and transformant genomes. Black arrows indicate the position of primers used in (B). *Spe^r*: Spectinomycin resistant cassette. B. Genotype analysis of *hglE_A::hglE2*. Each fragment was amplified by PCR with the primers indicated in (A). C. TLC analysis of lipids in the wild type (WT) and *hglE_A::hglE2*. Gray arrow indicates position of Hgl-ketol and black arrow Hgl-diol. D. Growth curve of wild type and *hglE_A::hglE2*. Filled black circles: wild type (N+, nitrogen replete conditions); blank black circles: wild type (N–, nitrogen deprived conditions), filled gray triangles: *hglE_A::hglE2* (N+), blank gray triangles: *hglE_A::hglE2* (N–). Values are average $\pm$ standard deviation ($n = 6$).

standard) and *hglE2*, primers 19 and 20, and 21 and 22 were used, respectively.

Spot assay. Cyanobacterial cultures were grown in a BG11 medium until OD₇₃₀ attained around 0.8. The cultures were diluted to OD₇₃₀ = 0.1, and pelleted and resuspended 3 times with the same volume of medium used for each spot assay. Ten microliters of cultures were spotted onto the agar plate with a series of dilutions (1/1, 1/2, 1/10 and 1/100).

Results

Classification of two types of *hglE* in *Anabaena*

Anabaena has two homologs of *hglE* gene, *hglE_A* and *hglE2*, in its genome (Fan et al., 2005). To classify these paralogs, we conducted BLAST searches against

Fig. 6. Spot assay of $\Delta hglE2$ in different pH media.

Variation of pH was made by replacing the buffer in BG11 medium (0.1 M HEPES, pH 7.5) with 0.1 M MES (pH 6.2), 0.1 M MES (pH 6.5), 0.1 M Tris (pH 8.5), or 0.1 M CHES (pH 9.0). Dilution rates of spotted culture were 1/1, 1/2, 1/10, 1/100 (from left to right).

cyanobacterial sequences using full-length amino acid sequences of PfaA of *Shewanella* sp. BR-2 as an out group. The result showed that HglE_A is well conserved in filamentous heterocystous cyanobacteria, but not in the unicellular nitrogen-fixing cyanobacteria such as *Cyanothece*, supporting the previous reports that HglE_A is involved in the Hgls synthesis (Fig. 1B) (Campbell et al., 1997; Fan et al., 2005). On the other hand, HglE2 was found to be conserved only in several nitrogen-fixing cyanobacteria, such as *Nostoc commune*, *Scytonema hofmanii*, and *Calothrix* species. In addition, homologs of *hglE2* were also found in non-heterocystous cyanobacteria including *Moorea producens* and *Microcystis* species. These results suggest that HglE_A is involved in Hgls synthesis, but HglE2 might have function(s) not related to heterocyst.

HglE_A is a major contributor for Hgl synthesis in *Anabaena*

Previously reported *hglE_A* mutants in *Nostoc* and *Anabaena* were found by screening a Fox⁻ phenotype from the library of transposon-based random mutagenesis (Campbell et al., 1997; Fan et al., 2005). To revisit the *hglE_A* mutation by target mutagenesis, we first made knock out mutants of *hglE_A* in *Anabaena* ($\Delta hglE_A$). As shown in Fig. 2, genotyping analysis of the mutant strains revealed that the knock-out mutation by the insertion of an antibiotic resistant cassette were fully segregated in all genome

Fig. 7. Isolation and phenotype analysis of *hglE2* over expresser (*hglE2* o/x) in *Anabaena*.

A. Genotype analysis of *hglE2* o/x. PCR amplification was done by primers 15 and 22. Upper band shows single recombination and lower band double recombination. Only the double recombination yields over expression of *hglE2*. B. qRT-PCR analysis of *hglE2*. Relative amount to *rnpB* is described. Values are average of data from two independent strains. C. TLC analysis of lipids in the wild type (WT) and *hglE2* o/x.

copies, indicating that *hglE_A* can be knocked-out and is not an essential gene at least in optimal growth conditions. When the wild type cells were grown under nitrogen deficient conditions, accumulation of two types of Hgls (Hgl-diols and Hgl-ketols) were observed. On the other hand, $\Delta hglE_A$ mutants did not show accumulation of Hgls, neither Hgl-diols nor Hgl-ketols. Concomitantly, the growth of $\Delta hglE_A$ mutants was retarded in nitrogen deficient conditions. These results well matched previous reports and suggest that HglE_A is involved in the synthesis of both Hgl-diols and Hgl-ketols.

Knock-out mutation of *hglE2* did not affect the accumulation of Hgls under nitrogen deficient conditions

To identify whether HglE2 is involved in the Hgls synthesis, we knocked-out *hglE2*. Null mutants of *hglE2* ($\Delta hglE2$) were successfully segregated (Fig. 3), indicating that *hglE2* is also not essential, at least in optimal growth conditions. When the $\Delta hglE2$ strains were grown under nitrogen deficient conditions, both Hgls were accumulated comparable to that of the wild type. The growth rate of the mutant strains were also similar to that of the wild type in both nitrogen replete and deficient conditions. Taken together, HglE2 is most likely not involved in the Hgls synthesis.

Because *hglE2* is making a gene cluster similar to that of *hglE_A* (Fig. 4A), we also isolated a mutant of the gene in the cluster (*all1643*) to see whether the gene cluster is involved in the Hgls synthesis. *all1643* encodes a protein resemble to HglC in the *hglE_A* gene cluster with conserved CLF (chain length factor) and AT domains, and additional two DH (dehydrase) domains. Similar to $\Delta hglE2$, null mutants of *all1643* ($\Delta all1643$) accumulated both Hgls comparable to that of the wild type under nitrogen defi-

cient conditions. These results suggest that neither HglE2, nor the *hglE2* gene cluster, are involved in the Hgls synthesis.

HglE2 cannot complement the function of HglE_A

Because HglE2 and HglE_A share a high identity in their amino acid sequences with conserved KS, AT and ACP domains, we tried to complement the *hglE_A* mutation with *hglE2* (Fig. 5). *hglE2* was fused to the first 15 nucleotides of *hglE_A* ORF (corresponding to the first 5 amino acids from Met) under the native *hglE_A* promoter. The transformants showed a similar phenotype to Δ *hglE_A* mutants (no accumulation of Hgls and retarded growth under nitrogen deficient conditions), indicating that HglE2 cannot complement the function of HglE_A.

Physiological function of HglE2 in *Anabaena*

To determine the physiological role of *hglE2*, we conducted a spot assay using Δ *hglE2* mutants. As shown in Fig. 6, the mutants did not show any significant difference to the wild type under pH from 7.5 to 9.0. At the lower pH, such as 6.2 to 6.5, Δ *hglE2* showed slightly better growth compared with the wild type. We also tried iron deficient conditions [phosphate (P), iron (Fe) and copper (Cu)], high light condition (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$), and methylviologen oxidized condition (6 μM), but could not observe any difference compared with the wild type (data not shown). We next made over-expressers of *hglE2*. A promoter of *psbA* in the chloroplast genome of *Amaranthus hybridus*, a green amaranth, was used, since it is reported to be functional in *Anabaena* (Elhai, 1993). The transformants were not completely segregated but they were confirmed to accumulate 1.92 times more transcripts of *hglE2* compared with the wild type by qRT-PCR (Fig. 7). However, lipid analysis did not show any significant difference between the over-expressers and the wild type.

Discussion

In this study, we found that HglE_A is responsible for bulk synthesis of Hgls, but HglE2 is not involved in the Hgls synthesis in *Anabaena*. This conclusion is supported by the findings: (1) Δ *hglE_A* did not accumulate both Hgls under nitrogen deficient conditions. (2) Δ *hglE2* accumulated both Hgls under nitrogen deficient conditions. (3) Knock out mutants of a gene in *hglE2* gene cluster did not show any defect in the synthesis of Hgls. (4) *hglE_A* is conserved among heterocystous cyanobacteria, but not *hglE2*. (5) HglE2 could not complement HglE_A.

We further analyzed the co-expression profile of *hglE_A*, *hglE2*, and *all1643* using ALCodbCyano (<http://alcodb.jp/cyano/>). The results showed that *hglE_A* co-expressed with the genes known to be involved in Hgls synthesis, such as *alr5342*, *all5343*, *all5347* (*hgdB*), *alr5348*, *alr5352* (*hglF*), *alr5353* (*hglG*), *alr5354* (*hglD*), *alr5356* (*hglA*), *alr5357* (*hglB/hetM*) and *alr5358* (*hetN*). The genes involved in nitrogenase activity, such as *asr1408* (*nifZ*), *all1440* (*nifK*), *all1455* (*nifH*), *all1457* (*nifS*) and *all1517* (*nifB*) were also found in the top 50 co-expression list. On the other hand, *hglE2* and *all1643* did not show any co-expression profile with the gene involved in neither heterocyst development

nor nitrogen fixation. According to the previous report, in the *hglE2* gene cluster, only one gene, *all1645*, was found to be induced by nitrogen deficiency (Ehira and Ohmori, 2006). These results also support our conclusion that HglE2 is not involved in Hgls synthesis.

The synthetic mechanism of two types of Hgl is under question. We proposed in this report that HglE_A is responsible for synthesis of both types of Hgls, Hgl-diol and Hgl-ketol. We have also found that HglT is responsible for the glucose transfer reaction of both types of Hgls (Awai and Wolk, 2007; Halimatul et al., 2014). According to a proposed pathway for Hgls (Awai et al., 2009), Hgl-diol is predicted to be synthesized from Hgl-ketol by the reaction of ketoreductase (KR). In the *hglE_A* gene cluster, *hglG* encodes a protein with KR and DH domains, and is expected to be involved in the ketoreduction process. Recently, it has been reported that a ratio of Hgl-diol and Hgl-ketol is controlled by HgdA/B/C (Shvarev et al., 2018, 2019), which is crucial for proper heterocyst development. According to the co-expression profile described above, at least the expression of *hgdB* has a correlation with those of *hglE_A* and *hglG*. *hgdA* encodes UDP-galactose 4-epimerase and *hgdB/C* encodes subunits of an ABC transporter. Therefore, they might not be directly involved in the regulation of the Hgls ratio. How the ratio of Hgls is determined and why the ratio of Hgls has to be maintained, requires to be identified in the future.

The function of HglE2 is still unclear. We observed a slightly better growth of Δ *hglE2* under weak acidic conditions. At a low pH, the CO₂ concentration in the medium is limited and that affects the carbon fixation and cell growth (Mangan et al., 2016). It is possible that the product synthesized by HglE2 has an inhibitory effect for carbon fixation under those conditions. The other possibility is that HglE2 is simply involved in the synthesis of compounds that have inhibitory effects on the growth of *Anabaena* under weakly acidic conditions. Since HglE2 also share around 45% identity to PfaA throughout their sequences (more than 1100 amino acids. Expect = 0.0), it seems likely that HglE2 is also involved in the synthesis of fatty-acids-like compounds. *Microcystis aeruginosa* is known as a bloom forming cyanobacteria and it is reported to secrete linoleic acid to inhibit its competitor's growth (Song et al., 2017). Because Δ *hglE2* and *hglE2* o/x strains did not show any obvious phenotype, HglE2 might be involved in the synthesis of compounds related to allelopathic interactions. In either case, further study for the determination of the product is required to elucidate the physiological functions of HglE2.

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Supplementary Materials

Supplementary materials are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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