Title: Identification of the *chlE* gene encoding oxygen-independent Mg-protoporphyrin

IX monomethyl ester cyclase in cyanobacteria

Authors: Kaori Yamanashi, Kei Minamizaki, and Yuichi Fujita

Affiliation: Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho,
Chikusa-ku, Nagoya 464-8601, Japan

Corresponding Author: Yuichi Fujita, e-mail: fujita@agr.nagoya-u.ac.jp

Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku,
Nagoya 464-8601, Japan

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Abbreviations: BChl, bacteriochlorophyll *a*; Chl, chlorophyll *a*; MPE, Mg-protoporphyrin IX
monomethyl ester; Pchl_{id}, protochlorophyllide

Abstract

The fifth ring (E-ring) of chlorophyll (Chl) *a* is produced by Mg-protoporphyrin IX monomethyl ester (MPE) cyclase. There are two evolutionarily unrelated MPE cyclases: oxygen-independent (BchE) and oxygen-dependent (ChlA/AcsF) MPE cyclases. Although ChlA is the sole MPE cyclase in *Synechocystis* PCC 6803, it is yet unclear whether BchE exists in cyanobacteria. A BLAST search suggests that only few cyanobacteria possess *bchE*. Here, we report that two *bchE* candidate genes from *Cyanothece* strains PCC 7425 and PCC 7822 restore the photosynthetic growth and bacteriochlorophyll production in a *bchE*-lacking mutant of *Rhodobacter capsulatus*. We termed these cyanobacterial *bchE* orthologs “*chlE*.”

1. Introduction

Chlorophylls (Chls) are tetrapyrrole pigments that are essential for photosynthesis. The fifth ring (E-ring or isopentanone ring) of Chl is a unique structural feature different from other tetrapyrrole pigments such as heme and vitamin B₁₂. During the biosynthesis of Chl, the E-ring is formed by Mg-protoporphyrin IX monomethyl ester (MPE) cyclase, which converts MPE to 3,8-divinyl protochlorophyllide (Pchl_{id}). This reaction involves a six-electron oxidation and formation of an oxo group at C13¹ (Fig. 1). There are two evolutionarily unrelated MPE cyclases in photosynthetic organisms [1,2]. One is an oxygen-independent MPE cyclase, BchE, named after the gene *bchE* of *Rhodobacter capsulatus* [3]. The other is an oxygen-dependent MPE cyclase, known by different names depending on the source: AcsF (photosynthetic bacteria) [4], ChlA (cyanobacteria) [5], Crd1/Cth1 (*Chlamydomonas reinhardtii*) [6], CHL27 (*Arabidopsis thaliana*) [7], and XanL (barley) [8]. In this report, we use the term ChlA/AcsF. BchE belongs to the radical S-adenosylmethionine (SAM) superfamily and has an iron–sulfur cluster, which is extremely vulnerable to oxygen. BchE forms the C13¹ oxo group using oxygen from a water molecule. In contrast, ChlA/AcsF is a monooxygenase that incorporates an oxygen molecule (O₂) to form the C13¹ oxo group. BchE and ChlA/AcsF are mainly identified

in anoxygenic photosynthetic bacteria and plants, respectively. However, some photosynthetic bacteria have either both or one of the two enzymes [9,10].

The MPE cyclase reactions consist of three sequential two-electron oxidation steps in both systems. The BchE MPE cyclase has been assayed in the crude extract of *R. capsulatus*: the results show its dependence on vitamin B₁₂ [11]. However, no reconstituted system using purified BchE protein has been reported. The ChlA/AcsF MPE cyclase activity has been detected in lysed plastids and crude extracts of cyanobacteria, and the enzyme has been partially purified [12,13]. Other probable subunit proteins and a protein stimulating the activity have been identified [14-16]. However, this oxygen-dependent MPE cyclase has not been reconstituted using the purified proteins either. These MPE cyclases remain the least understood of the Chl biosynthesis enzymes [17].

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis in a manner similar to the plant photosynthesis. In our previous work, we have identified two homologous genes encoding two ChlA isoforms, ChlA_I (CycI) and ChlA_{II} (CycII), in the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) [5,18]. The *chlA_I* gene is constitutively expressed and essential for photoautotrophic growth under aerobic conditions, and the *chlA_{II}* gene is expressed only in low-oxygen conditions to complement the ChlA_I activity. There are

three *bchE*-like genes in *Synechocystis* 6803. We have isolated three mutants, each lacking one of the three *bchE*-like genes. However, no phenotype suggesting involvement of these *bchE*-like genes in Chl biosynthesis has been detected for any of the mutants. These observations strongly suggest that *Synechocystis* 6803 employs only ChlA (ChlA_I and ChlA_{II}) for MPE cyclase reaction rather than BchE for MPE cyclase reaction in Chl biosynthesis. Recently, Bandyopadhyay et al. [19] have reported putative genes encoding BchE in the genomes of two cyanobacteria *Cyanothece* strains PCC 7425 and PCC 7822. These BchE proteins show significantly higher similarity to BchE from *R. capsulatus* than those of *Synechocystis* 6803. This finding implies the presence of BchE in these *Cyanothece* strains [1].

In this study we investigated whether these two BchE-like proteins function as oxygen-independent MPE cyclases, using a *bchE*-lacking mutant of the purple sulfur bacterium *R. capsulatus*. Two transconjugants expressing the *bchE*-like genes restored the ability to produce bacteriochlorophyll *a* (BChl) and the photosynthetic function, suggesting that both *Cyanothece* BchE proteins are functional as MPE cyclases. Furthermore, we discuss the distribution of BchE, evolutionary aspects, and the physiological significance of this enzyme in cyanobacteria.

2. Materials and Methods

2.1. Bacterial strains

The *bchE*-lacking mutant DB575 of *R. capsulatus* [3] was used as the host to evaluate the MPE cyclase function encoded by cyanobacterial genes. *R. capsulatus* SB1003 was used as the wild-type strain.

2.2. Plasmid construction

Coding regions of two cyanobacterial *bchE* orthologous genes (2,028 bp, cyan7425_4778 and 2,235 bp, cyan7822_3999) were synthesized (GenScript, Piscataway, NJ). Three plasmids, pKAR7425, pKAR7822 and pKARE, were constructed to express cyan7425_4778, cyan7822_3999, and *bchE* from *R. capsulatus*, respectively, as described in the Supplementary Materials and Methods and Supplementary Tables 1 and 2. In addition, the other set of plasmids, pKNE1, pKNE2, pKNE3, and pKNbE were constructed to express Slr0905, Slr0309, Sll1242 and BchE, respectively, as described in the Supplementary Materials and Methods and Supplementary Tables 1 and 2.

2.3. Conjugative transfer of plasmids into *R. capsulatus*

The seven plasmids and two control vectors, pJN3 and pJN7, were transferred into DB575 of *R. capsulatus* by triparental conjugation as described previously [20]. For heterotrophic and photosynthetic growth, these transconjugants were grown on PY and RCV media, respectively. The media contained kanamycin ($10\ \mu\text{g ml}^{-1}$) and spectinomycin ($10\ \mu\text{g ml}^{-1}$). PY agar plates were incubated at 34°C in the dark. RCV agar plates were incubated in the light (100-W incandescent lamps, Panasonic, Osaka, Japan) at 30°C under anaerobic conditions using the GasPak system as described previously [20].

2.4. Pigment analysis

Concentrated suspension ($200\ \mu\text{l}$ at adjusted cell density OD_{660} of 3.5) of cells grown in RCV media was used for extraction with $800\ \mu\text{l}$ of acetone. Aliquots ($20\ \mu\text{l}$) of the supernatants were loaded onto a $4.6 \times 150\ \text{mm}$ Symmetry C8 $3.5\text{-}\mu\text{m}$ column and separated using an HPLC system as described previously [21,22].

3. Results

3.1. *bchE* orthologs in *Cyanothece*

In our previous work, we have concluded that three *bchE*-like genes, *slr0905*, *sll1242* and *slr0305*, of the cyanobacterium *Synechocystis* 6803 are not involved in MPE cyclase activity and that the ChlA isoforms are the sole MPE cyclases in this cyanobacterium. However, Bandyopadhyay et al. have recently reported probable cyanobacterial *bchE* genes in the genomes of *Cyanothece* sp. strains PCC 7425 and PCC 7822 [19]. As shown in Supplementary Figure 1, the amino acid sequences of Cyan7425_4778 and Cyan7822_3999 showed significantly higher similarity (59% and 58%, respectively) to the sequence of BchE than to the sequences of Slr0905, Slr0305 and Sll1242 (31%, 23%, and 27%, respectively). These results suggested that cyan7425_4778 and cyan7822_3999 are the *bchE* orthologs in cyanobacteria [1].

3.2. *Complementation analysis*

Further, we examined whether these *Cyanothece* genes encode functional oxygen-independent MPE cyclases. The entire coding sequences of cyan7425_4778 and cyan7822_3999 were

cloned into an expression vector pJN7 in *R. capsulatus* [23]. Both genes were placed under the control of the *puc* promoter in pJN7. In addition, these plasmids encoded fusion proteins with Strep-tag at their N-terminals. These recombinant plasmids were introduced into the *bchE*-lacking mutant DB575 by triparental mating. The growth of these isolated transconjugants DB575/7425 and DB575/7822 was examined under heterotrophic and photosynthetic conditions (Fig. 2). The transconjugants grew as well as the control strain under heterotrophic conditions (Fig. 2A). Both DB575/7425 and DB575/7822 transconjugants grew under photosynthetic conditions, although their growth was significantly slower than that of the positive control transconjugant DB575/BchE harboring *bchE* from *R. capsulatus* (Fig. 2B). This result suggested that the cyan7425_4778 and cyan7822_3999 genes encode the functional BchE proteins. We termed these cyanobacterial *bchE* orthologs “*chlE*.”

In addition, we performed another set of complementation analyses of transconjugants harboring the genes *slr0905*, *slr0305* and *sll1242* from *Synechocystis* 6803 (Fig. 2C and D). All the transconjugants grew well under heterotrophic conditions (Fig. 2C), However, none of them grew under photosynthetic conditions (Fig. 2D). This result indicated that the *bchE*-like genes from *Synechocystis* 6803 did not restore the *bchE* gene function in *R. capsulatus*. These data suggest that the *bchE*-like genes with low similarity (20–30%) have

functions other than Chl biosynthesis, which is consistent with the previous conclusion [5].

3.3. Pigment analysis of transconjugants

The analysis of the pigments from transconjugants, DB575/7425 and DB575/7822, was performed using HPLC (Fig. 3). The levels of BChl (eluted at 22 min) in DB575/7425 and DB575/7822 were approximately 33% and 42% of wild-type levels (WT/pJN7), respectively. The low level of BChl was consistent with the slow photosynthetic growth. In addition, significant amounts of MPE (eluted at 8.5 min), and 3,8-divinyl Pchlode (DV-Pchlode, eluted at 7 min) and monovinyl Pchlode (MV-Pchlode, eluted at 7.5 min) were identified in both transconjugants. These results indicate that these *Cyanothece* genes encode active MPE cyclases: however, the activities of the enzymes are not sufficient to convert all the MPE in DB575 cells.

3.4. Phylogenetic analysis of the cyanobacterial bchE orthologs

To examine the distribution of the *chlE* gene among the cyanobacteria, we performed a BLAST search using as a query the amino acid sequence of BchE of *R. capsulatus*, against non-redundant protein sequences of cyanobacteria (12 March, 2015). This search provided 26

hits with E-value of zero, including two ChlEs of *Cyanothece* strains PCC 7425 and PCC 7822, suggesting that these 26 proteins are the ChlE orthologs. In contrast, Slr0905 and Slr0309 showed lower similarity to BchE with E-values of $8e^{-51}$ (32nd highest) and $4e^{-18}$ (80th highest), respectively. Then, we constructed a phylogenetic tree including the 23 independent probable ChlE proteins, the nine BchE proteins from photosynthetic bacteria, and the three BchE-like proteins of *Synechocystis* 6803. BioB (Slr1364), a radical SAM enzyme with a different function (biotin biosynthesis), was included as an outgroup (Fig. 4A). The three BchE-like proteins were placed like as other outgroups similar to BioB. All ChlE proteins grouped as a sister clade of a subclade (*Chloroflexus aurantiacus* and *Heliobacillus mobilis*) of photosynthetic bacterial BchE proteins except for one ChlE from *Lyngbya confervoides*. This ChlE protein was included in the photosynthetic bacteria clade, suggesting that *L. confervoides* have obtained the *chlE* gene via a lateral gene transfer event from the purple bacteria lineage. The topological relationship among cyanobacterial ChlE proteins is largely consistent with the recently proposed cyanobacterial species relationship [24], suggesting that the *chlE/bchE* gene was first inherited by a cyanobacterial common ancestor. However, *chlE* has been lost in several lineages during evolution.

In addition, we constructed a phylogenetic tree of ChlA/AcsF for comparison among

cyanobacterial species (Fig. 4B). This tree includes the 23 species having ChlE and other representative cyanobacterial species (*Synechocystis* 6803, *Synechococcus elongatus* PCC 7942, *Nostoc* sp. PCC 7120 and *Gloeobacter violaceus* PCC 7421). Most cyanobacteria harbor two ChlA isoforms ChlA_I and ChlA_{II}: these isoforms form independent clades that have appeared after the divergence of the lineage of *G. violaceus* PCC 7421. Topological relationships in the ChlA_I and ChlA_{II} clades are largely similar to the cyanobacterial species relationship [24], while some subclades are duplicated in the ChlA_{II} clade. In addition, the topological relationship of ChlA clades is largely consistent with that of ChlE.

The phylogenetic relationships implied that both *chlE/bchE* and *chlA/acsF* genes had been the MPE cyclase genes inherited by a cyanobacterial common ancestor, and *chlE* has been later lost in several lineages. The *chlA* gene has persisted in all cyanobacterial lineages. A gene duplication of *chlA* to form *chlA_I* and *chlA_{II}* appears to have occurred in an early phase of evolution of cyanobacteria.

4. Discussion

4.1. Evolution of *ChlE*/*BchE*

The genome sequence analysis revealed that the species harboring *chlE* appeared to be randomly distributed in the cyanobacterial phylogenetic tree including 54 species [24]. They appeared in all subclades except for the subclades B3, C1, and G (Supplementary Table 3). The loss of the *chlE* gene seems to have occurred with high frequency.

The *nifH* gene is present in all genomes having *chlE* except for *Geitlerinema* sp. PCC 7105. The coexistence of *chlE* and *nifH* suggests a close relationship between Chl biosynthesis using ChlE and nitrogen fixation. ChlE/BchE is one of the radical SAM enzymes that contain an iron-sulfur cluster vulnerable to oxygen. Thus, the ChlE protein functions only under anaerobic conditions. ChlE contributes to the production of Chl in diazotrophic cells because nitrogen fixation also requires anaerobiosis. Some cyanobacteria (*Nostoc* sp. PCC 7524 and *Calothrix* sp. PCC 7103) have ChlE and three ChlA isoforms (one ChlA_I and two ChlA_{II} isoforms). Given that these cyanobacteria are heterocystous, ChlE and the second ChlA_{II} isoform might be important in Chl biosynthesis in heterocyst cells. However, some nitrogen-fixing cyanobacteria, such as *L. boryana* and *Nostoc* PCC 7120, do not have ChlE. In

these species, the activity of the low-oxygen type isoform ChlA_{II} is high enough for Chl biosynthesis under nitrogen-fixing conditions [25].

The distribution of BchE and AcsF in proteobacteria was systemically analyzed [10]. Some proteobacteria have both enzymes (e.g., *Rubrivivax gelatinosus*), and some have only one (e.g., *Rhodospirillum rubrum* has only BchE and *Ahrensia* sp. R2A130 has only AcsF). Although the phylogenetic tree of AcsF is consistent with that of 16S rRNA, the BchE tree is quite complex, suggesting extensive lateral gene transfer and loss events during the evolution of proteobacteria. However, during the evolution of cyanobacteria, simple loss events of *chlE* may have occurred in several cyanobacterial lineages. Only one example of lateral gene transfer of *bchE* was identified in case of *L. confervoides* (Fig. 4A).

4.2. MPE cyclase activity in the transconjugants

In the complementation experiment both transconjugants of DB575 (harboring *cyan7425_4781* or *cyan7822_3999*) produced BChl in amounts sufficient for photosynthetic growth. However, significant amounts of MPE and Pchl_{ide} were detected (Fig. 3). The accumulation of MPE suggests relatively low activity of these ChlE proteins. Interestingly, these proteins have long C-terminal extensions (120–180 amino acid residues) whose amino acid sequences show

significant similarity to those of LuxR-type transcriptional regulators (Supplementary Fig. 2).

The aspartate residues corresponding to phosphorylation sites in the transcriptional regulators are conserved in the ChlE proteins. This feature is identified in ChlEs of *Myxosarcina* sp. GI1, *Oscillatoria* sp. PCC 10802, *Leptolyngbya* sp. PCC 7375, and *Leptolyngbya* sp. Heron Island J, whereas it is not common to all cyanobacterial ChlE proteins. It would be intriguing to speculate that the C-terminal LuxR domain regulates the MPE cyclase activity.

The accumulation of Pchlide in these transconjugants is difficult to be explained by the low activity of MPE cyclase by ChlE. It is possible that there is an efficient transport of Pchlide from MPE cyclase (BchE) to Pchlide reductase (the next enzyme in BChl biosynthesis) and that the exogenous ChlE does not support this transport system, causing an accumulation of Pchlide.

4.3. The gene organization of the *chlE* gene

In the *Cyanothece* PCC 7425 genome, the *chlE* gene is located in a gene cluster, *chlA_{II}-ho2-hemN-chlE-desF* (Supplementary Fig. 3). The first three genes, *chlA_{II}*, *ho2*, and *hemN*, form a similar gene cluster in *Synechocystis* 6803. In addition, the *desF* gene is also located in the *chlA_{II}* gene cluster in *Synechococcus* sp. PCC 7002. In both strains, the

transcription of the *chlA_{II}* gene cluster is activated by ChlR, a MarR-type transcriptional activator, in response to low oxygen levels [26,27]. All genes in the gene clusters encode low-oxygen/anaerobic type enzymes, and their activities complement those of the corresponding aerobic enzymes under limited oxygen conditions [1]. In this context, the gene arrangement including *chlE* complements the oxygen-dependent activity of ChlA in *Cyanothece* PCC 7425 under anaerobic conditions. The probable *chlR* ortholog was found in a plasmid pP742501 of *Cyanothece* PCC 7425, suggesting that ChlR activates the gene cluster containing *chlE* in response to low-oxygen conditions, such as nitrogen-fixing conditions.

In *Cyanothece* PCC 7822, the *chlE* gene is located at some distance from the *chlA_{II}-ho2-hemN* gene cluster. A putative *chlR* gene was found in another locus in the genome (Supplementary Fig. 3); the ChlR protein would regulate the hypoxic expression of *chlE* and the *chlA_{II}* gene cluster in this cyanobacterium.

4.4. Perspectives

Cyanobacteria that produce oxygen via photosynthesis have adapted well to aerobic environments. The persistence of ChlA/AcsF as the main MPE cyclase is one of the indications of adaptive evolution of Chl biosynthesis in the aerobic environments. However, cyanobacteria

have to cope with darkness. During the night, the microenvironment becomes almost anaerobic because of high levels of respiration of neighboring heterotrophic bacteria [28,29]. In addition, anaerobic environments are also required for nitrogen fixation: the diazotrophic ability is important for cyanobacteria to survive in nitrogen-deficient environments. Thus, the use of ChlE in Chl biosynthesis would ensure continuous Chl supply under anaerobic conditions in cyanobacteria such as *Cyanothece*. Further study using *Cyanothece* strains is required to elucidate the physiological function of the *chlE* genes in cyanobacteria. In *Cyanothece* PCC 7822, a targeted gene disruption technique is now established [30]. Thus, this strain provides a promising system for the detailed physiological studies.

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Figure Legends

Figure 1. MPE cyclase reaction. The C13-methylpropionate in MPE and the E-ring of Pchl_a are highlighted in yellow. The oxygen atom of the oxo group at C13¹ is derived from water and oxygen in oxygen-independent (ChlE/BchE) and -dependent (ChlA/AcsF) MPE cyclase reactions, respectively.

Figure 2. Complementation of the *bchE*-lacking mutant DB575 by cyanobacterial genes. Transconjugants harboring the *chlE* genes from *Cyanothece* (A, B; Cy7425 and Cy7822) and the *bchE*-like genes from *Synechocystis* 6803 (C, D; *slr0905*, *slr0309* and *sll1242*) were cultivated under heterotrophic (A, C) and photosynthetic (B, D) conditions. Heterotrophic and photosynthetic agar plates were incubated for 2 and 5 days, respectively.

Figure 3. Analysis of the pigment from transconjugants. Aliquots (20 µl) of the pigments extracted from transconjugants WT/pJN7 (trace 1, black), DB575/pJN7 (trace 2, red), DB575/bchE7 (trace 3, blue), DB575/7822 (trace 4, green) and DB575/7425 (trace 5, orange), were analyzed by HPLC. The elution was monitored by measuring absorbance at 415 nm (1–18

min) and 730 nm (18–30 min). Peaks of BChl *a*, 3,8-divinyl Pchlode (DV-Pchlode), monovinyl Pchlode (MV-Pchlode), and MPE had been assigned in previous experiments [31].

Figure 4. Phylogenetic trees of ChlE/BchE (A) and ChlA/AcsF (B). Cyanobacteria and photosynthetic bacteria are shown in blue and dark red, respectively. A. The ChlE clade is highlighted in cyan box. The ChlA_I and ChlA_{II} clades are shown in cyan and light green boxes. B. The representative cyanobacterial without *chlE* are indicated by asterisks. The duplicated relationship in the ChlA_{II} clade is shown in dark cyan boxes. The phylogenetic distances of the outgroups are 2.95 and 2.85 in the ChlE/BchE and ChlA/AcsF trees, respectively. Multiple alignments and phylogenetic trees were constructed using CLUSTALW program (www.genome.jp/tools/clustalw) in the slow/accurate mode with default parameters, except for the weight matrix set to PAM.

Figure1

Figure2

Figure3

Figure4

A

B