Identification of Two Homologous Genes, chlAI, and chlAI′′, That Are Differentially Involved in Isocyclic Ring Formation of Chlorophyll a in the Cyanobacterium Synechocystis sp. PCC 6803

Kei Minamizaki1, Tedashi Mizoguchi2, Takeaki Goto, Hitoshi Tamiaki3, and Yuichi Fujita4,5

From the 4Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan and the 5Department of Bioscience and Biotechnology, Ritsumeikan University, Kusatsu 525-8577, Japan

The isocyclic ring (E-ring) is a common structural feature of chlorophylls. The E-ring is formed by two structurally unrelated Mg-protoporphyrin IX monomethyl ester (MPE) cyclase systems, oxygen-dependent (AcsF), and oxygen-independent (BchE) systems, which involve incorporation of an oxygen atom from molecular oxygen and water into the C-13 position of MPE, respectively. Which system operates in cyanobacteria that can thrive in a variety of anaerobic environments remains an open question. The cyanobacterium Synechocystis sp. PCC 6803 has two acsF-like genes, sll1214 (chlAI) and sll1874 (chlAI′), and three bchE-like genes, slr0905, sll1242, and slr0309. Five mutants lacking one of these genes were isolated. The ΔchlAI mutant failed to grow under aerobic conditions with anomalous accumulation of a pigment with fluorescence emission peak at 595 nm, which was identified as 3,8-divinyl MPE by high-performance liquid chromatography-mass spectrometry analysis. The growth defect of ΔchlAI was restored by the cultivation under oxygen-limited (micro-oxic) conditions. MPE accumulation was also detected in ΔchlAI′ grown under micro-oxic conditions, but not in any of the bchE mutants. The phenotype was consistent with the expression pattern of two chlAI genes: chlAI′′ was induced under micro-oxic conditions in contrast to the constitutive expression of chlAI. These findings suggested that ChlAI is the sole MPE cyclase system under aerobic conditions and that the induced ChlAI′′ operates together with ChlAI′ under micro-oxic conditions. In addition, the accumulation of 3,8-divinyl MPE in the ΔchlAI mutants suggested that the reduction of 8-vinyl group occurs after the formation of E-ring in Synechocystis sp. PCC 6803.

Chlorophylls (Chls)2 are a group of tetrapyrrole pigments involved in light reactions of photosynthesis. The isocyclic ring, E-ring, is a unique feature of all types of Chls, which distinguishes Chls from other tetrapyrroles such as hemes and vitamin B12. The E-ring is formed by an oxidative cyclization of C-13 methyl propionate of Mg-protoporphyrin IX monomethyl ester (MPE) to form 3,8-divinyl protochlorophyllide (Pchlide). The E-ring formation causes a 30-nm blue-shift of the Qy band of MPE (1) resulting in a dramatic color change from pink to green. The E-ring formation reaction involves a 6-electron oxidation of C-13 methylpropionate with incorporation of an oxygen atom to form oxo-group at C-13′ position (1). The 13′-oxo group of Chl a is derived from molecular oxygen in higher plants (2, 3) and green algae (4), suggesting that E-ring formation is catalyzed by an oxygenase. The acsF gene encoding a protein with a monooxygenase motif has been found to be involved in the oxygen-dependent E-ring formation (5). In contrast, water is the oxygen donor for the 13′-oxo group of bacteriochlorophyll a in a photosynthetic bacterium Rhodobacter sphaeroides (6), indicating that photosynthetic bacteria produce the E-ring by a hydratase. The bchE gene has been identified as the gene responsible for the oxygen-independent E-ring formation in purple bacteria (7). The AcsF protein shows no similarity to the BchE protein, suggesting that there are two structurally unrelated E-ring formation systems. The bchE gene is found in a variety of photosynthetic bacteria such as R. capsulatus (7), R. sphaeroides (8), Chlorobium tepidum (9), and Heliobacillus mobilis (10). The acsF gene is distributed among photosynthetic eukaryotes such as Chlamydomonas reinhardii (crl1 and ctl1, Ref. 11), Arabidopsis thaliana (chl27, Ref. 12), and barley (xantha-l, Ref. 13). Some purple bacteria such as Rubrivivax gelatinosus have both bchE and acsF genes (14). The distribution of acsF and bchE among extant photosynthetic organisms seems to be consistent with the oxygen levels of their natural habitats.

Cyanobacteria are prokaryotes performing oxygenic photosynthesis similar to plants. Ancient cyanobacteria are thought to have started to carry out oxygenic photosynthesis about 2.7 giga years ago (15, 16, 17), and later one lineage of cyanobacteria to have become endosymbionts of protoeukaryotic cells leading to chloroplasts of plants (18). Though current cyanobacteria
Chlorophyll E-ring Formation in Cyanobacteria

Alexander V. Voevodkin, Youngmann Lee, Masao Nakamura, and Fredric C. Neidhardt

are usually associated with aerobic environments, many strains thrive in environments with a variety of oxygen levels including anaerobic environments such as microbial mats, lake sediments, and soil (19, 20). In addition, most cyanobacteria face a diurnal light and dark cycle, and then the oxygen level in natural environments undergoes dynamic changes from anaerobic to aerobic throughout the day. Given the two structurally unrelated E-ring formation systems; the oxygen-dependent AcsF and the oxygen-independent BchE systems, it is a reasonable inference that oxygen is a key environmental factor by which Chl biosynthesis is regulated at the step of E-ring formation. However, little information is available regarding which E-ring formation system operates and how it is regulated under oxygen-fluctuated environments in cyanobacteria. Here we report the identification of two acsF-like genes, chlA and chlAI, in the cyanobacterium *Synechocystis* sp. PCC 6803. We found that ChlA is the sole E-ring formation system under aerobic conditions and that the second isoform ChlAI operates together with ChlA under oxygen-limited (micro-oxic) conditions in *Synechocystis* sp. PCC 6803.

**EXPERIMENTAL PROCEDURES**

**Cyanobacterial Strains and Growth Conditions—*Synechocystis* sp. PCC 6803** (*Synechocystis* 6803) and its derivative strains used in this study were cultivated in BG-11 medium (21) supplemented with 10 mM HEPES-KOH, pH 8.2 at 30 °C. Strains used in this study were cultivated in BG-11 medium (21) supplemented with 10 mM HEPES-KOH, pH 8.2 at 30 °C. For strains grown under aerobic and micro-oxic conditions on agar plates for 10 days. Because *sll1214* does not grow under aerobic conditions, it was grown under micro-oxic conditions for 3 days and incubated under aerobic conditions for 7 days to prepare the cells grown aerobically. Pigments were extracted in 90% methanol as described (24). Chl biosynthetic intermediates without phytol were extracted by mixing the methanol extract with a 3.5-volume of acetone and half volume of diethyl ether. The contaminating water was removed as ice after chilling at 2 °C, and cell pellets were frozen in liquid nitrogen after complete removal of culture media. Total RNA was prepared essentially as described (22). For complete replacement of the wild-type copy with the disrupted copy was examined by "colony PCR" (26).

**Pigment Extraction and Spectroscopic Analysis—**Pigments were extracted from cells grown under aerobic or micro-oxic conditions on agar plates for 10 days. Because *sll1214* does not grow under aerobic conditions, it was grown under micro-oxic conditions for 3 days and incubated under aerobic conditions for 7 days to prepare the cells grown aerobically. Pigments were extracted in 90% methanol as described (24). Chl biosynthetic intermediates without phytol were extracted by mixing the methanol extract with a 3.5-volume of acetone and half volume of water, and phase-partitioned with a 15-volume of hexane. Fluorescence spectra of the lower acetone-methanol phase were recorded with excitation at 435 nm (model FP777w, Jasco, Hachioji, Japan).

**Preparation of MPE—**MPE was prepared from culture medium of the *R. capsulatus* mutant DB575 (ΔbchE, 7) essentially as described for preparation of Pchlide (27). DB575 was grown in RCV-2/3 PY medium (80 ml in a 200-ml flask) containing 5 μg ml⁻¹ kanamycin at 34 °C in the dark with slow shaking at 130 rpm. The culture medium was collected by centrifugation at 13000 rpm for 30 min. The supernatant was mixed with the same volume of phenol-chloroform (23). The phenol-chloroform extraction was repeated five times, the nucleic acids were precipitated by ethanol. DNA in the nucleic acid fraction was digested with DNase I (0.1 units ml⁻¹; RNase-free grade, Takara, Ohtsu, Japan) in the presence of RNase inhibitor (0.8 units ml⁻¹; porcine liver; Takara) for 1.5 h at 37 °C. RNA was extracted by phenol-chloroform and concentrated by ethanol precipitation. RNA concentration was determined by absorbance at 260 nm (23). The isolated total RNA (2.5 μg) was used for the synthesis of cDNA with Superscript II (10 units μl⁻¹; Invitrogen Corp., Carlsbad, CA) and random primers according to the manufacturer's manual. After incubation at 25 °C for 10 min, at 42 °C for 50 min, and at 70 °C for 15 min, RNA was degraded by alkali treatment (0.23 x NaOH) followed by neutralization with HCl. Thus obtained cDNA fraction was used in 1:32-dilution as the template for PCR amplification with the specific primers (Supplemental Table S1).

**Construction of Plasmids for Gene Disruption and Transformation of *Synechocystis* 6803—**Plasmids for gene disruption were constructed by overlap extension PCR (Supplemental Table S1; Ref. 23). DNA fragments of the upstream (f1 and r1 primers) and downstream (f2 and r2 primers) regions of the target gene were amplified by PCR from genomic DNA of *Synechocystis* 6803 with a standard thermal cycle (KODplus DNA polymerase; Toyobo, Osaka, Japan). A kanamycin-resistant cartridge (neo) for gene disruption was also amplified with f3 and r3 primers (Supplemental Table S1) from pMC19 (24) as the template. A chimeric DNA fragment consisting of the upstream region of the target gene, neo and the downstream region of the target gene were amplified by overlap extension PCR and cloned into pUC118. *Synechocystis* 6803 was transformed with the plasmid constructed as above and the kanamycin-resistant colonies were segregated to isolate homozygous mutants (25). For the isolation of *sll1214*-disrupted mutant (Δsll1214), the kanamycin-resistant colonies that appeared on the first selective agar plates were picked up and cultivated under micro-oxic conditions. Complete replacement of the wild-type copy with the disrupted copy was examined by "colony PCR" (26).

**LC/MS Analysis of Pigments—**Cells grown on agar plates under aerobic (5 days) and micro-oxic (10 days) conditions were extracted with methanol. After washing with hexane, the
lower methanol-phase was corrected and evaporated to almost dryness by a stream of nitrogen. The extract thus obtained was dissolved in a small amount of Me$_2$SO for LC/MS analysis. The LC/MS was performed using a Shimadzu LCMS-2010EV system (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization probe as described previously (28). HPLC was performed using reverse-phase chromatography under the following conditions: column, Inertsil ODS-P (3.0 × 150 mm, GL Sciences, Tokyo, Japan); eluent, methanol/acetonitrile: 150 mm ammonium acetate (pH 5.25) = 400:50:100 (v/v/v); flow rate, 1.0 ml min$^{-1}$; and detection wavelength, 415 nm. The entire absorption spectra of MPE were also recorded, during elution, using a photodiode-array detector.

Preparation of Total Extracts and Immunoblot Analysis—Cells were grown under aerobic and micro-oxic conditions until OD$_{730}$ reached about 1.0. Harvested cells were disrupted by sonication as described (29). Proteins in the total extracts were separated by SDS-PAGE (a 5–20% acrylamide gradient gel, e-Pagel, ATTO, Tokyo, Japan) and electro-transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The polyvinylidene difluoride membrane was incubated with anti-CHL27 antiserum (Agrisera, Vännäs, Sweden) in a 1000 dilution, and then with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad). The specific protein signals were visualized by chemiluminescent substrate (ECL Western blotting Analysis System, GE Healthcare) with a lumino-image analyzer (LAS-3000mini, Fujifilm, Tokyo, Japan).

BchE from \textit{R. capsulatus}. BchE belongs to the radical SAM family, which uses an oxygen-sensitive [4Fe-4S] cluster and S-adenosylmethionine to catalyze diverse radical reactions such as biotin synthase (BioB), lipoyl synthase (LipA), and coproporphyrinogen III oxidase (HemN; 31, 32). They all conserve an Fe-S binding motif including three cysteine residues (CXXCXXX), which is found in all three BchE-like proteins (Fig. S1B).

Given the possible oxygen sensitivity of BchE (33), cyanobacterial BchE-like proteins may operate only under anaerobic conditions as in the case of HemN (34) or an oxygen-sensitive Pchlide reductase (29). The transcript levels of the five genes in wild-type cells grown photoautotrophically under aerobic and anaerobic conditions were semiquantified by RT-PCR (Fig. 1B). The “anaerobic” condition in this work means that the gas phase to incubate cyanobacterial cells is anaerobic (2% CO$_2$/N$_2$; see “Experimental Procedures”). Because cyanobacterial cells still evolve oxygen by photosynthesis under this condition, the environments of the cells is more properly referred to as “micro-oxic.” PCR products derived from \textit{slr1214} and \textit{slr0905} mRNAs were detected almost equally in both cDNA preparations from aerobic and micro-oxic conditions. In contrast, PCR products from \textit{slr1874}, \textit{slr1242}, and \textit{slr0309} mRNAs in cells grown under micro-oxic conditions were much more abundant than those in aerobically grown cells (Fig. 1B). This result suggests that \textit{slr1874}, \textit{slr1242}, and \textit{slr0309} genes are induced under micro-oxic conditions.

RESULTS
Two \textit{acsF}-like genes, \textit{slr1214} and \textit{slr1874}, were found in the genome of \textit{Synechocystis} sp. PCC 6803 (\textit{Synechocystis} 6803) by a BLAST search with the amino acid sequences of AcsF from \textit{R. gelatinosus} and CHL27 (AcsF-homolog) from \textit{A. thaliana}, as pointed out previously (5). \textit{Slr1214} and \textit{Slr1874} showed 42 and 39% identity, respectively, to AcsF from \textit{R. gelatinosus}, and 62 and 50% identity, respectively, to CHL27 from \textit{A. thaliana}. They conserved the two copies of a motif, (D/E)$_{X_{28-33}}$DEXRH, which are involved in binding of a binuclear-iron cluster for various monooxygenases (Supplemental Fig. S1A, and Ref. 30). Sequence identity between \textit{Slr1214} and \textit{Slr1874} was 57%.

Three \textit{bchE}-like genes, \textit{slr0905}, \textit{slr1242}, and \textit{slr0309}, were found in the genome by a BLAST search with the amino acid sequence of BchE from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The \textit{bchE}-like genes, \textit{slr0905}, \textit{slr1242}, and \textit{slr0309}, were found in the genome by a BLAST search with the amino acid sequence of BchE from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The \textit{bchE}-like genes, \textit{slr0905}, \textit{slr1242}, and \textit{slr0309}, were found in the genome by a BLAST search with the amino acid sequence of BchE from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of \textit{Slr0905}, \textit{Slr1242}, and \textit{Slr0309} show 29, 21, and 26% identity, respectively, to CHL27 from \textit{A. thaliana} with the amino acid sequences of \textit{BchE} from \textit{R. capsulatus}. The amino acid sequences of
while slr0309 and slr0905 are constitutively expressed. Two other genes, ho2 (slr1875) and hemN1 (slr1876), are present downstream of the slr1874 gene. The ho2 gene encodes heme oxygenase (HO) that catalyzes the oxidative cleavage of heme to form biliverdin IXα, the precursor for phycocyanobilin (35), and the hemN1 gene encodes coproporphyrinogen III oxidase (CPO) that catalyzes the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX in the biosynthetic pathway common to heme and Chl a. Thus, all of the contiguous three genes, slr1874-ho2-hemN1, are probably involved in tetrapyrrole biosynthesis. To confirm whether ho2 and hemN1 are also induced under micro-oxic conditions as well as slr1874, we also examined the transcript levels of ho2 and hemN1. As expected, the expression pattern of the two genes was similar to that of slr1874. There is no obvious sequence for transcriptional termination between the intergenic regions of the three genes. The expression pattern, and the sequence features suggest that the three genes form a transcriptional unit that is induced under micro-oxic conditions.

To identify which gene(s) encode MPE cyclase in Synechocystis 6803, we isolated five mutants lacking one of these genes (Fig. 1A). Four mutants lacking slr1874, slr0905, slr1242, and slr0309 were successfully isolated by the normal segregation procedure under aerobic conditions (Fig. 1C). However, kanamycin-resistant colonies isolated for slr1214-disruption under aerobic conditions were found to carry the wild-type copy accompanied with a small proportion of the mutant copy of slr1214 (Fig. 1C). However, the kanamycin-resistant colonies isolated under micro-oxic conditions contained no detectable wild-type copy (Fig. 1C). This suggests that slr1214 is a gene essential for growth under aerobic conditions but dispensable under micro-oxic conditions. Mutants lacking slr1214, slr1874, slr0905, slr1242, and slr0309 are called Δslr1214, Δsll1874, Δslr0905, Δslr1242, and Δslr0309, respectively, hereafter.

All mutants except for Δsll1214 grew photosynthetically on an agar plate under aerobic conditions in the light (70 μmol m⁻² s⁻¹; Fig. 2A). The mutant Δsll1214 did not grow in this condition as expected from the segregation process. In contrast, all mutants including Δsll1214 grew photosynthetically on an agar plate under micro-oxic conditions at the same light intensity (Fig. 2B). Slight growth retardation was observed in the Δsll1874 mutant. The mutant Δsll1242 grew somewhat more slowly than the wild-type showing a yellow-green color under both aerobic and micro-oxic conditions. In liquid cultures, essentially the same result was obtained (Fig. 2, panels C and D) except for Δsll1242. The mutant Δsll1242 did not grow in liquid culture irrespective of aerobic and micro-oxic conditions. The slight growth retardation of Δsll1874 observed on the agar plate was not so evident in liquid culture (Fig. 2D).

Chl contents of the five mutants grown on agar plates under aerobic and micro-oxic conditions were determined (Fig. 2, panels E and F). Because the Δsll1214 mutant did not grow under aerobic conditions, it was cultivated under the micro-oxic condition followed by aerobic cultivation. The
Chlorophyll E-ring Formation in Cyanobacteria

FIGURE 3. Accumulation of MPE in Δsll1214 and Δsll1874 mutants. Pigments were extracted in 90% methanol from cells (black, wild-type; red, Δsll1214; orange, Δsll1874; cyan, Δstr0905; green, Δsll1242; blue, Δstr0309; and yellow-green, an MPE standard) grown photosynthetically under aerobic (A) and micro-oxic (B) conditions. Chl a was removed by a phase-partitioning with hexane and the fluorescence emission spectra of the lower phase were recorded. The emission spectra were elicited by excitation at 435 nm. MPE shows a characteristic fluorescence emission peak at 595 nm by excitation at 435 nm (1). Only the extract from Δsll1214 of the five mutants grown aerobically exhibited a 595-nm emission peak, suggesting the accumulation of MPE in the Δsll1214 cells under the aerobic condition (Fig. 3A). MPE accumulation was also detected in Δsll1214 and Δsll1874 mutant cells grown under the micro-oxic conditions (Fig. 3B). This anomalous accumulation of MPE in Δsll1214 and Δsll1874 grown under micro-oxic conditions suggested that Chl biosynthesis is limited at the step of MPE cyclization, resulting in reduced Chl contents (Fig. 2F). The aerobically grown Δsll1874 mutant cells did not accumulate MPE, which is consistent with the result of RT-PCR. The expression level of sll1874 under aerobic conditions is much lower than that under micro-oxic conditions (Fig. 1B). No significant accumulation of MPE was detected in any three bchE-mutants including Δsll1242 under any conditions examined as well as the wild-type cells. No significant MPE accumulation was detected in aerobically grown Δsll1242 cells even though the Chl content was reduced significantly (Fig. 2, E and F).

Mg-protoporphyrin IX, the direct precursor of MPE, shows fluorescence spectra identical to MPE (1). To confirm that the pigment accumulated in Δsll1214 and Δsll1874 is MPE not Mg-protoporphyrin IX, a series of LC/MS analysis was carried out (Fig. 4). The standard MPE sample isolated from the R. capsulatus mutant contained two MPE-like pigments eluted at 18.6 min and 25.5 min (Fig. 4A, trace 2, peaks a and b, respectively). Their absorption spectra were very similar but the Soret peaks of the 18.6-min and 25.5-min pigments were 410 nm and 415 nm, respectively (Fig. 4C, traces 1 and 2). The m/z values of the 18.6-min and 25.5-min pigments were 600.3 and 598.3 (Fig. 4D, traces 1 and 2), which matched the calculated molecular mass of 3-vinyl 8-ethyl MPE (calculated for 600.26) and 3,8-divinyl MPE (calculated for 598.24), respectively. Detection of an adduct with methanol (633.3 and 631.2) originating from the eluent supported the identification of the pigments. Thus, we concluded that the 18.6-min and 25.5-min pigments are 3-vinyl 8-ethyl MPE and 3,8-divinyl MPE, respectively. The Δsll1214 cells grown in the aerobic and micro-oxic conditions and the Δsll1874 cells grown in the micro-oxic condition accumulated commonly the 25.5-min pigment (Fig. 4A). The 25.5-min pigment accumulated in Δsll1214 and Δsll1874 commonly showed characteristic absorption spectra (Fig. 4C) and the m/z values 598.1 (Fig. 4D), indicating that the accumulated pigment in these cells is 3,8-divinyl MPE not Mg-protoporphyrin IX. It should be noted that MPE was not detected in any of the three bchE mutants, confirming the result of the fluorescence emission spectra (Fig. 3). These results suggested that none of the bchE-like genes are involved in the E-ring formation.

To detect Sll1214 and Sll1874 proteins immunologically in cyanobacterial cells, we carried out immunoblot analysis using an antiserum against孤儿in C. thaliana (Fig. 5). Total extracts of wild-type, Δsll1214 and Δsll1874 cells grown under aerobic and micro-oxic conditions (except for Δsll1214 grown aerobically) were prepared and probed with the antiserum. The cross-reacting polypeptide with an apparent molecular mass of 38 kDa was specifically detected in wild-type and Δsll1874 cells grown under both conditions. Since the 38-kDa polypeptide was not detected in Δsll1214 grown under the micro-oxic condition (Fig. 5).
Chlorophyll E-ring Formation in Cyanobacteria

We identified two acsF-like genes involved in MPE cyclase by the phenotype and the accumulated pigment analyses of the mutants lacking sll1214 and sll1874 in *Synechocystis* 6803. The result of RT-PCR suggested that Sll1214 is substantially the sole MPE cyclase under aerobic conditions and that Sll1874 is induced in the micro-oxic conditions to operate together with Sll1214. The phenotype of Δsll1214, the growth defect with the operation of a di-iron monooxygenase ChlA₄ under micro-oxic conditions. ChlA₄ cyclase could use the endogenously evolved oxygen as the substrate for the MPE cyclase reaction. A yeast hemF (HEM13) gene encoding oxygen-dependent CPO, which is a monooxygenase belonging to the same family of ChlA, is induced under anaerobic conditions (36). The yeast cells were proposed to increase the concentration of HemF with a high affinity for oxygen (>0.1 μM) to maintain heme supply utilizing only a trace level of oxygen that appears to be contaminated in the cultures from the tubing. In the cyanobacterial cells, the chlA₄ gene could be induced to meet the increased requirement of ChlA proteins under low oxygen conditions. Otherwise, ChlA₄ may have some special enzymatic properties such as a higher affinity to oxygen than ChlA₉, which might be ascribed to the slight growth retardation of the Δsll1874 mutant. An oxygen-dependent MPE cyclase activity has been detected in the crude extracts of this cyanobacterium (37). This assay system and two mutants isolated in this study would be useful to confirm the possibility of higher affinity to oxygen of ChlA₄ than ChlA₉.

We found that the MPE pigment accumulated in cyanobacterial mutant cells as 3,8-divinyl MPE, providing valuable information about the substrate specificity of an 8-vinyl reductase that catalyzes the reduction of the vinyl group at the C-8 position. Mutant Δsll1214 and Δsll1874 cells accumulated 3,8-divinyl MPE, which is in contrast to the fact that both forms of anomalous accumulation of MPE, suggests that the function of Sll1214 is indispensable under aerobic conditions. Under micro-oxic conditions, both Δsll1214 and Δsll1874 mutants could grow photosynthetically despite the anomalous accumulation of MPE. It is suggested that the single operation of either Sll1214 or Sll1874 can supply a certain amount of Chl sufficient for photosynthetic growth under micro-oxic conditions. However, the slight growth retardation observed in Δsll1874 (Fig. 2) implies that the contribution of Sll1214 to the total MPE cyclase activity is somewhat larger than that of Sll1214 under micro-oxic conditions (Fig. 6). Here, we propose to call sll1214 and sll1874 chlA₉ and chlA₄, respectively.

Under the micro-oxic conditions we used in this study the oxygen evolved from the cells is quickly removed by the constant supply of anaerobic gas in the liquid cultures or by palladium catalysts in anaerobic jars in the agar plate cultures. Thus, the oxygen concentration in the cells is kept at a much lower level than in cells grown in aerobic conditions. One might question the micro-oxic condition and the apparent molecular mass is in good agreement with the calculated molecular mass 42.2 kDa, we concluded that the 38-kDa polypeptide corresponds to Sll1214 protein. However, no cross-reacting polypeptides corresponding to Sll1874 protein were detected, suggesting that this antiserum does not recognize Sll1874 protein because the sequence identity between CHL27 and Sll1874 (50%) is much less than that between CHL27 and Sll1214 (62%). This result indicated that Sll1214 is constitutively expressed irrespective of the environmental oxygen level in the wild-type cells, which is consistent with the RT-PCR result (Fig. 1).

Taken together, these results suggested that only sll1214 is involved in the MPE cyclase reaction under aerobic conditions, that sll1874 contributes to the MPE cyclase reaction in addition to sll1214 under micro-oxic conditions, and that none of the three bchE-like genes are involved in the MPE cyclase reaction under the conditions examined in this study.

**DISCUSSION**

FIGURE 4. LC/MS analysis of pigments from the five mutants and wild-type. Methanol extracted pigments from the cells of Δsll1214 (trace 3), Δsll1874 (trace 4), Δsll0905 (trace 5), Δsll1242 (trace 6), Δsll0309 (trace 7), and wild-type (trace 8) that were grown under aerobic (A) and micro-oxic (B) conditions were loaded onto an ODS column, and the elution of pigments was monitored by absorption at 415 nm. As controls, Pchlide (trace 1) and MPE (trace 2) prepared from the mutants ZY5 (26) and DB575 of *R. capsulatus* were loaded, respectively. Traces 1 and 2 in panels A and B are identical data. Values of absorbance at 415 nm were normalized. Absorption (C) and mass spectra (D) of the 18.6-min (trace 1) and 25.5-min (traces 2) pigments from the MPE standard and the 25.5-min pigments from aerobically grown Δsll1214 (trace 3), micro-oxically grown Δsll1214 (trace 4) and Δsll1874 (trace 5). The signal intensity of the MS spectra was normalized.

the micro-oxic condition and the apparent molecular mass is in good agreement with the calculated molecular mass 42.2 kDa, we concluded that the 38-kDa polypeptide corresponds to Sll1214 protein. However, no cross-reacting polypeptides corresponding to Sll1874 protein were detected, suggesting that this antiserum does not recognize Sll1874 protein because the sequence identity between CHL27 and Sll1874 (50%) is much less than that between CHL27 and Sll1214 (62%). This result indicated that Sll1214 is constitutively expressed irrespective of the environmental oxygen level in the wild-type cells, which is consistent with the RT-PCR result (Fig. 1).

Taken together, these results suggested that only sll1214 is involved in the MPE cyclase reaction under aerobic conditions, that sll1874 contributes to the MPE cyclase reaction in addition to sll1214 under micro-oxic conditions, and that none of the three bchE-like genes are involved in the MPE cyclase reaction under the conditions examined in this study.

**DISCUSSION**

We identified two acsF-like genes involved in MPE cyclase by the phenotype and the accumulated pigment analyses of the mutants lacking sll1214 and sll1874 in *Synechocystis* 6803. The result of RT-PCR suggested that Sll1214 is substantially the sole MPE cyclase under aerobic conditions and that Sll1874 is induced in the micro-oxic conditions to operate together with Sll1214. The phenotype of Δsll1214, the growth defect with the operation of a di-iron monooxygenase ChlA₄ under micro-oxic conditions. ChlA₄ cyclase could use the endogenously evolved oxygen as the substrate for the MPE cyclase reaction. A yeast hemF (HEM13) gene encoding oxygen-dependent CPO, which is a monooxygenase belonging to the same family of ChlA, is induced under anaerobic conditions (36). The yeast cells were proposed to increase the concentration of HemF with a high affinity for oxygen (>0.1 μM) to maintain heme supply utilizing only a trace level of oxygen that appears to be contaminated in the cultures from the tubing. In the cyanobacterial cells, the chlA₄ gene could be induced to meet the increased requirement of ChlA proteins under low oxygen conditions. Otherwise, ChlA₄ may have some special enzymatic properties such as a higher affinity to oxygen than ChlA₉, which might be ascribed to the slight growth retardation of the Δsll1874 mutant. An oxygen-dependent MPE cyclase activity has been detected in the crude extracts of this cyanobacterium (37). This assay system and two mutants isolated in this study would be useful to confirm the possibility of higher affinity to oxygen of ChlA₄ than ChlA₉.

We found that the MPE pigment accumulated in cyanobacterial mutant cells as 3,8-divinyl MPE, providing valuable information about the substrate specificity of an 8-vinyl reductase that catalyzes the reduction of the vinyl group at the C-8 position. Mutant Δsll1214 and Δsll1874 cells accumulated 3,8-divinyl MPE, which is in contrast to the fact that both forms of anomalous accumulation of MPE, suggests that the function of Sll1214 is indispensable under aerobic conditions. Under micro-oxic conditions, both Δsll1214 and Δsll1874 mutants could grow photosynthetically despite the anomalous accumulation of MPE. It is suggested that the single operation of either Sll1214 or Sll1874 can supply a certain amount of Chl sufficient for photosynthetic growth under micro-oxic conditions. However, the slight growth retardation observed in Δsll1874 (Fig. 2) implies that the contribution of Sll1214 to the total MPE cyclase activity is somewhat larger than that of Sll1214 under micro-oxic conditions (Fig. 6). Here, we propose to call sll1214 and sll1874 chlA₉ and chlA₄, respectively.

Under the micro-oxic conditions we used in this study the oxygen evolved from the cells is quickly removed by the constant supply of anaerobic gas in the liquid cultures or by palladium catalysts in anaerobic jars in the agar plate cultures. Thus, the oxygen concentration in the cells is kept at a much lower level than in cells grown in aerobic conditions. One might question the micro-oxic condition and the apparent molecular mass is in good agreement with the calculated molecular mass 42.2 kDa, we concluded that the 38-kDa polypeptide corresponds to Sll1214 protein. However, no cross-reacting polypeptides corresponding to Sll1874 protein were detected, suggesting that this antiserum does not recognize Sll1874 protein because the sequence identity between CHL27 and Sll1874 (50%) is much less than that between CHL27 and Sll1214 (62%). This result indicated that Sll1214 is constitutively expressed irrespective of the environmental oxygen level in the wild-type cells, which is consistent with the RT-PCR result (Fig. 1).

Taken together, these results suggested that only sll1214 is involved in the MPE cyclase reaction under aerobic conditions, that sll1874 contributes to the MPE cyclase reaction in addition to sll1214 under micro-oxic conditions, and that none of the three bchE-like genes are involved in the MPE cyclase reaction under the conditions examined in this study.

**DISCUSSION**

We identified two acsF-like genes involved in MPE cyclase by the phenotype and the accumulated pigment analyses of the mutants lacking sll1214 and sll1874 in *Synechocystis* 6803. The result of RT-PCR suggested that Sll1214 is substantially the sole MPE cyclase under aerobic conditions and that Sll1874 is induced in the micro-oxic conditions to operate together with Sll1214. The phenotype of Δsll1214, the growth defect with the
Chlorophyll E-ring Formation in Cyanobacteria

Pchlide, 3,8-divinyl Pchlide and 3-vinyl (8-ethyl) Pchlide, were accumulated in a ΔchlL mutant, in which the Chl biosynthesis in the dark was arrested at the Pchlide reduction, of Synechocystis 6803 (38). These observations suggested that the 8-vinyl reduction occurs after the E-ring formation. In addition, the exclusive accumulation of 3,8-divinyl MPE in the ΔchlA mutants is in contrast to the fact that the ΔbchE-mutant of R. capsulatus accumulated 3-vinyl 8-ethyl MPE accompanied with a small amount of 3,8-divinyl MPE (Fig. 4). Recently the dvr and bciA genes encoding a plant-type 8-vinyl reductase have been identified in A. thaliana and Chlorobium tepidum, respectively (39, 40), and a probable ortholog in R. capsulatus has been pointed out (40). However, there are no homologous genes in Synechocystis 6803, suggesting that the reduction of 8-vinyl group is catalyzed by an 8-vinyl reductase that is structurally unrelated to BciA. The difference of MPE in the C-8 position accumulated may reflect a difference in the substrate specificity of 8-vinyl reductases of Synechocystis 6803 and R. capsulatus. Namely, the 8-vinyl reductase of Synechocystis 6803 does not reduce the C-8 vinyl group of 3,8-divinyl MPE while that of R. capsulatus (BciA) can reduce the 8-vinyl group of 3,8-divinyl MPE.

We constructed a phylogenetic tree of 28 ChlA proteins from various photosynthetic organisms including 17 cyanobacterial species, a photosynthetic bacterium, a green alga and two higher plants (Fig. S2). ChlA proteins are clearly divided into two groups of ChlA1 and ChlAII. Except for some marine cyanobacterial strains, the ChlA1 is distributed ubiquitously among all photosynthetic organisms, and the ChlAII is found among a limited number of cyanobacteria that include Thermosynechococcus elongatus BP-1, Anabaena sp. PCC 79413, and Nostoc punctiforme ATCC 73102 in addition to Synechocystis 6803. Thus, most organisms including eukaryotic phototrophs have only the ChlA1.

Given the probable enzymatic properties and distribution of BchE and ChlA among extant phototrophs, the following evolutionary scenario can be depicted. The oxygen concentration was at a trace level (~10^-13 atm; Ref. 41) when the earliest form of photosynthesis evolved. At that time, an oxygen-independent and oxygen-sensitive enzyme BchE catalyzed the E-ring formation as the sole MPE cyclase in ancestral anoxygenic phototrophs. ChlA evolved from a superfamily of oxygenase after oxygenic photosynthesis had evolved and the oxygen level rose to allow the oxygen-dependent reaction. The newly evolved ChlA might co-exist with BchE for some time in protocyanobacteria, but later BchE appears to have been lost giving rise to the exclusive distribution of ChlA among modern oxygenic phototrophs. The ancestral undifferentiated ChlA group was divided into ChlA1 and ChlAII types. The ChlA1 was conserved ubiquitously in modern oxygenic phototrophs, while the ChlAII was conserved as an accessory isoform to

![Figure 5. Immunoblot analysis to detect ChlA proteins.](image)

![Figure 6. The reaction of E-ring formation and a working model of differential operation of two ChlA proteins for MPE cyclase reaction.](image)
Chlorophyll E-ring Formation in Cyanobacteria

adapt to oxygen-limited environments in some limited groups of cyanobacteria. Differential operation of two ChlA isoforms has also been found in a green alga C. reinhardtii (11, 42). The chlI (CHL27A) and crd1 (CHL27B) genes are expressed in a reciprocal manner in response to copper availability and oxygen levels in the environment. The CHL27A mRNA accumulates in cells grown in copper-sufficient or aerobic conditions, and the CHL27B mRNA accumulates in cells grown in copper-deficient or hypoxia conditions. CHL27A and CHL27B show 66% identity and both belong to the ChlA group, and no homolog of the ChlAα group has been found in C. reinhardtii. The regulatory mechanism to adapt to copper- and oxygen-deficient conditions seems to have evolved in the lineage of green algae, which is an independent event of the differentiation of two chlA isoforms in Synechocystis 6803. The differential operation of dual ChlA isoforms appears to provide selective advantages for some oxygenc phototrophs to thrive in natural environments where oxygen levels drastically fluctuate (29).

The chlAα gene appears to be co-transcribed with the two downstream genes, ho2 and hemNI, under micro-oxic conditions in Synechocystis 6803. The hemNI gene encodes oxygen-independent CPO. The CPO reaction is also catalyzed by the other type of CPO, namely oxygen-dependent CPO, encoded by hemF (sll1855) in Synechocystis 6803. Thus, the induction of oxygen-independent CPO under micro-oxic conditions appears to be another strategy to complement the function of oxygen-dependent CPO for the constant supply of heme and Chl under oxygen-limited environments. Similar regulation of dual CPOs, HemN and HemF, in response to the oxygen levels has recently been reported in a photosynthetic bacterium R. gelatinosus (43).

We did not obtain any evidence that bchE-like genes are involved in oxygen-independent MPE cyclase, though it has been speculated that Synechocystis 6803 has bchE-homologs in addition to chlA (1, 44). No significant accumulation of MPE was detected in any Δstr0905, Δsll1242, and Δstr0309 cells grown under conditions (Figs. 3 and 4) where their transcripts were detected by RT–PCR (Fig. 2B), suggesting that the three BchE-like proteins are not involved in the MPE cyclase reaction. We tried to isolate a double mutant of sll1214 and sll1874 from Δsll1874 as the parental strain to examine if a functional BchE system operates. However, the wild-type copy of sll1214 persisted even though the segregation process was carried out under micro-oxic conditions where Δsll1214 has been successfully isolated (data not shown). In another attempt, the three bchE-homologs were introduced into a ΔbchE mutant DB575 of R. capsulatus via a shuttle vector that was used for overexpression of bchl. (28, 45). None of the transconjugants restored the ability of bacteriochlorophyll biosynthesis though bchE of R. capsulatus fully complemented bacteriochlorophyll biosynthesis (data not shown). These results support the view that there is no functional bchE-ortholog in this cyanobacterium. These results suggest the possibility whether there are more than two bchE-orthologs. The characterization of double and triple mutants of the three bchE-homologs is needed.

The Δsll1242 mutant exhibited an interesting phenotype with growth retardation showing aberrant yellow-green color on agar plates (Fig. 2, C and D), slightly lower Chl contents (Fig. 2, E and F) and no growth in liquid culture. This suggests some important roles of the sll1242 gene for photoautotrophic growth irrespective of oxygen levels. However, no significant accumulation of MPE was observed in Δsll1242 cells. Thus, we excluded the possibility that sll1242 is involved in MPE cyclase. Interestingly, many dark–green colonies that look like the wild-type appeared after a long cultivation of Δsll1242 cells on agar plates. These colonies gave a PCR product corresponding to the mutant sll1242 copy, indicating that these pseudorevertants have mutations in some other gene loci. Elucidating the mutation sites would provide important clues for physiological roles of sll1242 gene. While no orthologous genes for str0905 and str0309 are found in the other cyanobacteria whose entire genome sequences are available, sll1242 homologs are ubiquitously distributed among known cyanobacteria. This distribution suggests some important roles of Sli1242 protein in cyanobacteria.

Characterization of MPE cyclase activity in lysed chloroplasts of xantha-I mutant of barley (Hordeum vulgare) suggested that the oxygen-dependent MPE cyclase reaction requires at least two other components, a soluble protein and a membrane-bound protein (Viridis–k), in addition to the membrane-bound protein ChlA (Xantha-I) (13). Given the similarity of ChlA to the oxygenase family, ChlA could function as the catalytic component in a probable MPE cyclase complex. The other components could serve as the oxidase component that interacts with NADPH.

This study demonstrates that cyanobacteria obviously provide a promising system to explore the molecular mechanism of E-ring formation and other reactions in Chl biosynthesis as well as Pchlide reduction (24, 29). The chlA mutants especially Δsll1214 would provide an interesting system to investigate the molecular mechanisms of photosystem biogenesis. The anaerobically grown Δsll1214 mutant stops producing Chl at the step of MPE cyclase reaction upon transfer to aerobic conditions.

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Chlorophyll E-ring Formation in Cyanobacteria

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