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Growth arrest of *Synechocystis* sp. PCC6803 by superoxide generated from heterologously expressed *Rhodobacter sphaeroides* chlorophyllide *a* reductase

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ABSTRACT

The photosynthetic growth of *Synechocystis* sp. PCC6803 ceased upon expression of *Rhodobacter sphaeroides* chlorophyllide *a* reductase (COR). However, an increase in cytosolic superoxide dismutase level in the recombinant *Synechocystis* sp. PCC6803 completely reversed the growth cessation. This demonstrates that COR generates superoxide in *Synechocystis* sp. PCC6803. Considering the dissolved oxygen (DO) level suitable for COR, the intracellular DO of this oxygenic photosynthetic cell appears to be low enough to support COR-mediated superoxide generation. The growth arrest of *Synechocystis* sp. PCC6803 by COR may give an insight into the evolutionary path from bacteriochlorophyll *a* biosynthetic pathway to chlorophyll *a*, which bypasses COR reaction.

1. Introduction

The biosynthetic pathways for bacteriochlorophyll *a* (Bchl *a*) and chlorophyll *a* (Chl *a*) share the Mg-chelated tetrapyrrole intermediates from protoporphyrin IX to chlorophyllide *a* (Chlide *a*) (Fig. 1). C_{20} geranylgeranyl moiety can be directly esterified to Chlide *a* by chlorophyll synthase (ChlG), which is subsequently reduced (at positions 6, 10, and 14) by geranylgeranyl-chlorophyll reductase (ChlP) to yield the phytol of Chl *a* [1,2]. Bchl *a* biosynthesis from Chlide *a* is initiated through its ring B reduction by chlorophyllide *a* reductase (COR) to form 3-vinylbacteriochlorophyllide *a*, whose C-3-vinyl group of ring A is subsequently converted into an acetyl group through the successive actions of hydratase (BchF) and dehydrogenase (BchC) to form bacteriochlorophyllide *a* (Bchlide *a*) [3]. Alternatively, BchF reaction may precede COR-mediated reduction (Fig. 1). Once Bchlide *a* is formed, C_{20} geranylgeranyl moiety is also esterified to the tetrapyrrole ring by bacteriochlorophyll synthase (BchG) and subsequently reduced by geranylgeranyl-bacteriochlorophyll reductase (BchP) to yield the phytol of Bchl *a* [4,5].

COR of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* is a nitrogenase-like enzyme which is composed of BchX, BchY, and BchZ [6,7]. The anaerobic reduction of Chlide *a* ring B by COR requires NADH as a reductant. Like NifH of nitrogenase, BchX also has an ATP-binding motif. COR accordingly requires ATP for its activity. BchX, which contains FMN, and BchY are iron–sulfur proteins, whereas BchZ is a hemoprotein subunit. Since BchZ, where Chlide *a* binds, is co-purified with BchY, BchY–BchZ complex provides a catalytic site. Thus, it appears that BchX, when its FMN and iron sulfur are reduced by NADH, reduces BchY–BchZ in an ATP-dependent way. Most strikingly, COR generates superoxide through the heme of BchZ at low O_2 (less than 5%) [6]. Superoxide generation from COR reaction is considered to be electron leakage through heme. This in turn rapidly diminishes COR activity at low O_2, which could result in keeping the newly synthesized Bchl *a* of *R. sphaeroides* at low levels under the semiaerobic conditions. *R. sphaeroides* is killed by the superoxide stress from COR reaction unless cytosolic Fe-containing superoxide dismutase (FeSOD) is expressed [6].

Xiong et al. have clarified that bacteriochlorophyll biosynthesis evolved before chlrorophyll biosynthesis based on the phylogenic analyses of the major groups of photosynthetic bacteria using the new sequence information for genes involved in photosynthesis [8]. Accordingly, the cyanobacteria-based Chl *a* biosynthesis was suggested as a recent development in the course of pigment evolution. Comparing the biosynthetic pathways of Chl *a* with that of Bchl *a*, a shortcut from Chlide *a* directly to the metabolic steps by ChlG and ChlP, which show significant similarity to BchG and

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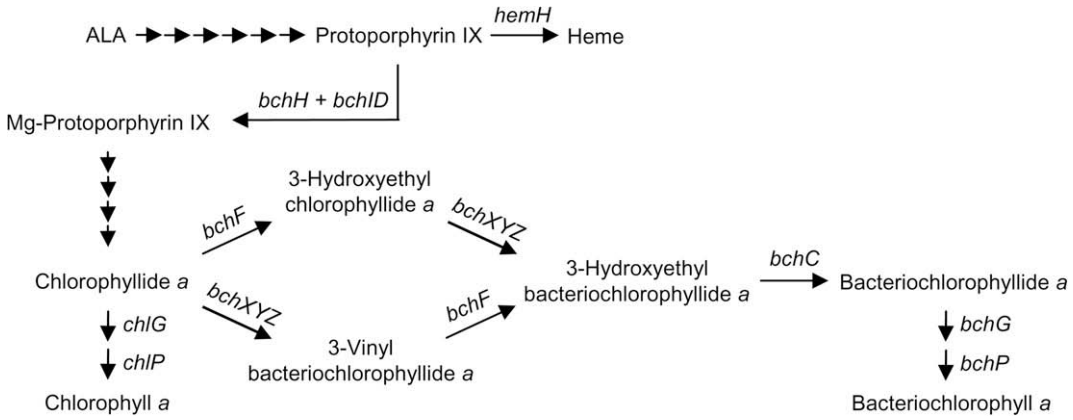

Fig. 1. Chl *a* and Bchl *a* biosynthetic pathways [1,3]. Protoporphyrin IX is synthesized from 5-aminolevulinate (ALA), and used either by ferrochelatase (*hemH*) to form heme or by Mg-chelatase (*bchH and bchID*) to form photosynthetic pigments of Chl *a* and Bchl *a*.

BchP, respectively, might have evolved to bypass superoxide-generating COR reaction during the rise in atmospheric oxygen level [6]. Accordingly, how COR of *R. sphaeroides* affected the photosynthetic growth of *Synechocystis* sp. PCC6803 was examined. We found that once COR was heterologously expressed in the photosynthetically-growing *Synechocystis* sp. PCC6803, it generates superoxide to cease cell growth. Analyses of COR reaction according to O_2 level further indicates the low dissolved oxygen (DO) level in *Synechocystis* sp. PCC6803 even though it performs oxygenic photosynthesis. The superoxide-generating COR reaction might reflect a selection pressure for the emergence of the Chl *a* biosynthetic pathway for an oxygenic photosynthetic organism.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Synechocystis sp. strain PCC6803 was cultivated at 30 °C in 250-ml Erlenmeyer flasks containing 100 ml of a BG11 medium [9] supplemented with 10 mM glucose, which was agitated on a rotary shaker at 130 rpm under 40 microEinsteins m^{-2} s^{-1} white light at 30 °C. *R. sphaeroides* 2.4.1 was grown at 28 °C in Sistrom's succinate-based minimal medium as described previously [10,11]. *Escherichia coli* was grown at 37 °C in Luria–Bertani (LB) medium. Kanamycin (Km), streptomycin (Sm), and spectinomycin (Sp) for *R. sphaeroides* and *E. coli* cultures were added as indicated previously [10]. Gentamycin (Gm) and Km were used at 30 and 10 μg/ml, respectively, for *Synechocystis* sp. strain PCC6803.

2.2. Construction of bchXYZ expression plasmids and their mobilization into Synechocystis sp. PCC6803

A 4.2-kb BalI–HincII DNA fragment containing *bchXYZ* was cloned into the SmaI site of pBluescript SK(−) (Stratagene, CA, USA), and was excised out as a SalI–XbaI DNA fragment. The *bchXYZ* DNA was subsequently cloned with 2.2-kb XbaI–HindIII Ω (Km^r) DNA into the XhoI–HindIII of a broad-host-range plasmid pSL1211 (Gm^r) [12] to generate pSLXYZ (Fig. 2), in which *bchXYZ* expression is under the control of the IPTG-inducible *trc* promoter (*Ptrc*). For constitutive expression of *R. sphaeroides* FeSOD, a 308-bp PstI–SmaI DNA of *R. sphaeroides rrnB* promoter [13] and a 1.1-kb SmaI–EcoRI DNA fragment containing *R. sphaeroides sodB* were ligated and cloned into the PstI–EcoRI of pBS (Stratagene, CA, USA) to generate pBS-rrnB-SodB. The recombinant 1.4-kb PstI–EcoRI DNA encompassing *PrrnB-sodB* was cloned into the NsiI–EcoRI of pSLXYZ to yield pSLXYZ-SodB, in which *PrrnB-sodB* is situated at the other side of Ω (Km^r) DNA from *bchXYZ* (Fig. 2). By observation, *sodB* expression of pSLXYZ-SodB is not influenced by *Ptrc*. Another plasmid pSL-Km was constructed and used as a control, in which a 2.2-kb BamHI–HindIII Ω (Km^r) DNA, which had been obtained after cloning of a 2.2-kb XbaI–HindIII Ω (Km^r) DNA into pBS, was cloned into the BamHI–HindIII of pSL1211.

Plasmids pSLXYZ, pSLXYZ-SodB, and pSL-Km were mobilized from *E. coli* S17-1 into *Synechocystis* sp. PCC6803 through conjugation as described previously [14]. Exponentially growing *E. coli* S17-1 and *Synechocystis* sp. PCC6803 were mixed and allowed to mate on a solid BG11 plate at 30 °C for 12 h under white light (20 microEinsteins m^{-2} s^{-1}). The plates were then overlaid with Km and Gm. Finally, exconjugants (Km^r and Gm^r) carrying pSLXYZQ, pSLXYZQ-SodB, and pSL-Km were selected in BG11 medium supplemented with Km and Gm.

2.3. Preparation of BchZ and Western immunoblot analysis

BchZ of *R. sphaeroides* was purified and used as an immunogen to raise an antiserum from mouse as described previously [15]. The preparation of cell-extracts, electrophoresis (SDS–PAGE, 12% polyacrylamide), and electro-blotting of proteins were also performed as described previously [16,17]. A blot treated with BchZ antibody was visualized using an ECL detection system supplied by Amersham Pharmacia after reaction with a 1/5000 dilution of goat anti-mouse IgG (Pierce) conjugated with horseradish peroxidase (HRP) as described previously [18]. Protein was determined by a modified Lowry method using bovine serum albumin (BSA, Sigma) as a standard [19].

2.4. Detection and determination of SOD activity

Preparation of soluble cell extracts, electrophoresis of a native polyacrylamide (13.5%) gel, staining of SOD activity, and the quantification of the relative SOD levels between samples were performed as described previously [16,20]. Cytosolic SOD activities in cell extracts were determined as described previously [21].

2.5. Purification and detection of Chlide a

Chlide *a* was extracted from the culture supernatant of *R. sphaeroides bchZ–bchF* mutant as described previously [6]. Chlide *a*, whose concentration was determined as described previously [7], was used as a substrate for COR reaction after dissolution in dimethyl sulfoxide.

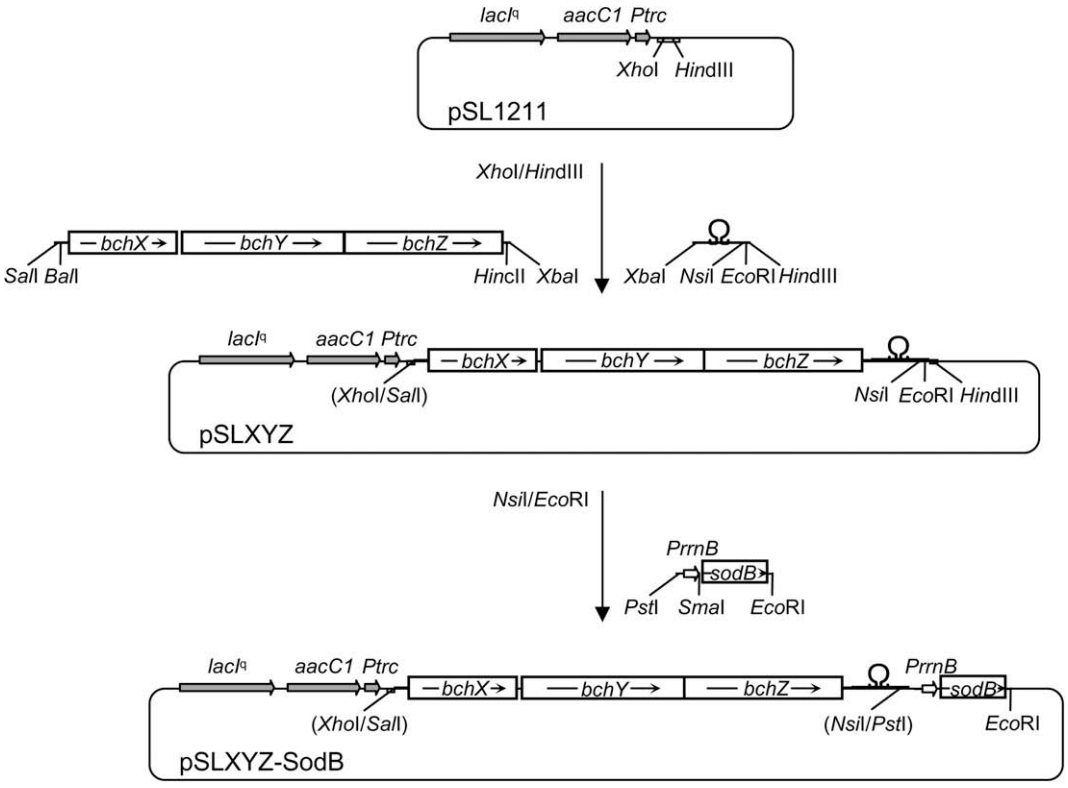

Fig. 2. Construction of plasmids for the expression of *bchXYZ* in *Synechocystis* sp. PCC6803. *R. sphaeroides bchXYZ* DNA was cloned with Ω (Km^r) DNA as a selection marker into a broad-host-range expression plasmid pSL1211 (Gm^r) [12] to generate pSLXYZ, in which *bchXYZ* is expressed by an IPTG-inducible *trc* promoter (*Ptrc*). A recombinant DNA of *R. sphaeroides rrnB* promoter [13]-*sodB* was cloned into the other side of Ω DNA from *bchXYZ* of pSLXYZ to yield pSLXYZ-SodB. *aacC1* codes for a Gm acetyltransferase gene conferring Gm^r. *lacI*^{q} codes for a *lac* repressor protein whose −35 site in promoter region is mutated for its overexpression.

2.6. COR reaction

COR reaction was performed with an equimolar (8.0 nM for each subunit) mixture of BchX, BchY, and BchZ in the presence of NADH and an ATP-generating system as described previously [6]. Reaction was initiated by adding Chlide *a* at 1–2 μM. The reaction mixture was analyzed for its product 3-vinylbacteriochlorophyllide *a* as described previously [7].

2.7. Superoxide determination by cytochrome c (Cyt c) reduction assay

COR reaction was performed with 20 μM Cyt *c* under the gas phase comprising from 0 to 20% O_2, in which the remaining gas phase was filled with N_2. The reduced Cyt *c* (550 nm) in reaction mixture was determined as described previously [21,22]. Superoxide generation was examined from the difference in the rates of Cyt *c* reduction in the absence and presence of *E. coli* MnSOD (10 units).

3. Results and discussion

3.1. Growth arrest of Synechocystis sp. PCC6803 by the superoxide stress from COR reaction

R. sphaeroides DNA encompassing *bchXYZ*, which codes for the three subunits of COR, was cloned at the downstream of IPTG-inducible *trc* promoter (*Ptrc*) to yield pSLXYZ (Fig. 2). The recombinant plasmid was mobilized into *Synechocystis* sp. PCC6803, and the cell growth under photoheterotrophic conditions was examined with or without IPTG (0.1 mM) treatment. The growth of the control cell, which carries pSL-Km, was not affected by IPTG (Fig. 3). *Synechocystis* sp. PCC6803 carrying pSLXYZ grew like the control cell in the absence of IPTG induction, whereas no growth ensued after IPTG treatment. The expression of the most distal gene *bchZ* was examined with total cell lysate by Western immunoblot analysis using BchZ antibody. The hybridization signal, which was not observed in the control cell, was detected from

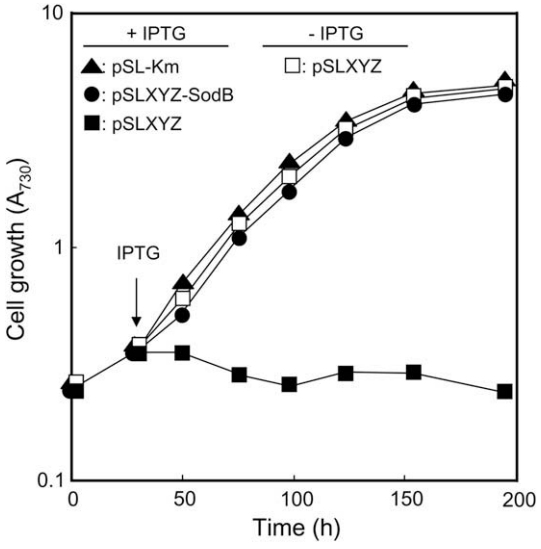

Fig. 3. Effect of *bchXYZ* expression on the growth of *Synechocystis* sp. PCC6803. Plasmids pSLXYZ, pSLXYZ-SodB, and pSL-Km were mobilized into *Synechocystis* sp. PCC6803 through conjugation, and the growth of the recombinant *Synechocystis* sp. PCC6803 was examined with (closed symbols) or without (open symbol) IPTG, which was treated at the time indicated by an arrow.

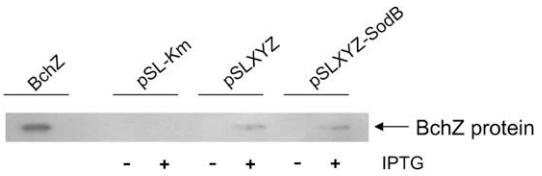

Fig. 4. Western immunoblot analysis of BchZ. The recombinant *Synechocystis* sp. PCC6803 containing pSLXYZ, pSLXYZ-SodB, and pSL-Km were grown with (+) or without (−) 0.1 mM IPTG. Cell lysates were prepared from exponentially growing cells (A_{730}, approximately 1.0) (Fig. 3). However, cell lysate from *Synechocystis* sp. PCC6803 containing pSLXYZ after IPTG induction, which showed no apparent growth (Fig. 3), was prepared from the cell 48 h after treatment. An aliquot (20 μg protein) of cell lysate was loaded per each lane for hybridization with BchZ antibody.

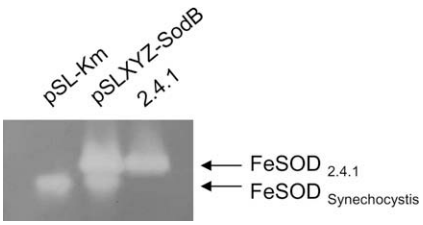

Fig. 5. FeSOD activity of the recombinant *Synechocystis* sp. PCC6803 containing pSLXYZ-SodB. Cell extracts of *Synechocystis* sp. PCC6803 containing pSLXYZ-SodB and pSL-Km were examined for FeSOD activity, and were compared with that of *R. sphaeroides* 2.4.1 grown exponentially under photoheterotrophic conditions at 10 W/m^2. The same amount of protein (50 μg) was loaded in each lane.

the cell carrying pSLXYZ after IPTG treatment only (Fig. 4), despite lack of any apparent growth (Fig. 3). Thus, *bchXYZ* of pSLXYZ is heterologously expressed after induction in *Synechocystis* sp. PCC6803.

COR generates superoxide from its reaction at low O_2 (less than 5%) [6], and Chlide *a* is an intermediate for Chl *a* biosynthesis. Accordingly, it was examined whether the growth arrest of *Synechocystis* sp. PCC6803 by COR expression was restored by the increase in SOD activity. *R. sphaeroides sodB*, which codes for FeSOD, was cloned at the downstream of the *R. sphaeroides rrnB* promoter (*PrrnB*) to generate the recombinant plasmid pSLXYZ-SodB (Fig. 2). Since *R. sphaeroides sodB* is expressed by the constitutive promoter *PrrnB*, *Synechocystis* sp. PCC6803 containing pSLXYZ-SodB expressed *R. sphaeroides* FeSOD irrespective of IPTG treatment (data not shown but only with IPTG was illustrated; Fig. 5). Measurement of total cytosolic SOD activities revealed approximately fourfold higher SOD activity in *Synechocystis* sp. PCC6803 carrying pSLXYZ-SodB compared with that of the control cell containing pSL-Km. *Synechocystis* sp. PCC6803 carrying pSLXYZ-SodB grew like the control cell even after IPTG treatment (Fig. 3). Thus, the increase of FeSOD activity reversed the growth cessation associated with COR expression. Although spectral analysis of the extracts from the recombinant cell carrying pSLXYZ-SodB did not tell COR reaction product 3-vinylbacteriochlorophyllide *a* from Chl *a* (data not shown) due to the masking by the large peak of the latter, COR expression after IPTG treatment was confirmed by Western hybridization analysis with BchZ antibody (Fig. 4) Taken together, the results clearly indicate that COR, once expressed in *Synechocystis* sp. PCC6803, generates superoxide to cease cell growth.

3.2. Chlide a reduction and superoxide generation by COR according to the varying levels of O_2

Oxygen is evolved significantly during photoheterotrophic growth of *Synechocystis* sp. PCC6803 in the presence of glucose [23]. Nonetheless, the generation of superoxide by COR, which is

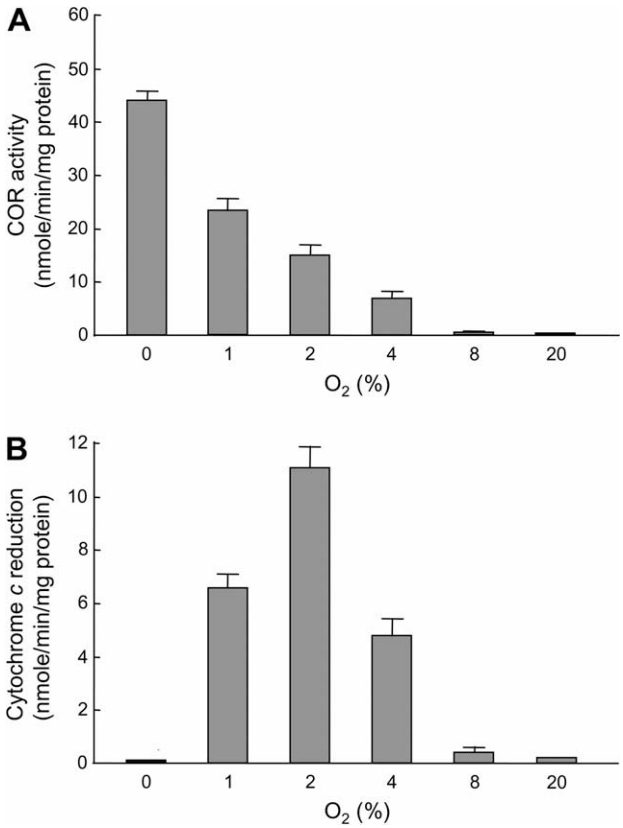

Fig. 6. Chlide *a* reduction (A) and superoxide generation (B) by COR at the varying levels of O_2 (0–20%). (A) Chlide *a* reduction activities were measured by determining the level of reaction product 3-vinylbacteriochlorophyllide *a* for 2 h. (B) Superoxide generation at varying levels of O_2 was measured by SOD-sensitive Cyt *c* reduction method. The error bars in both panels correspond to the standard deviations of the means.

observed at low O_2 [6], suggests the low O_2 environments in cell. Both Chlide *a* reduction and superoxide generation by COR were examined in vitro from 1% to 20% O_2, and compared with those measured in the absence of oxygen. As mentioned previously [6], Chlide *a* reduction by COR decreased gradually as O_2 level was increased (Fig. 6A). Interestingly, COR-mediated superoxide generation at 2% O_2 (DO, 31 ± 4 μM) was reproducibly higher than those measured at 1% (DO, 16 ± 2 μM) or 4% O_2 (DO, 55 ± 4 μM), and then sharply dropped at 8% (DO, 92 ± 4 μM) and 20% O_2 (DO, 207 ± 13 μM) (Fig. 6B). The lower superoxide generation at 1% O_2 may reflect either the limiting concentration of molecular oxygen in comparison with 2% O_2 or more electron flux to Chlide *a* at 1% O_2, as illustrated by higher Chlide *a* reduction activity. The detailed explanation for the maximal superoxide generation at 2% O_2 will have to await the elucidation of the COR reaction mechanism. The decrease in both Chlide *a* reduction and superoxide generation at O_2 higher than 4% may be explained by the decreasing stability of the iron–sulfur subunits BchX and BchY under the conditions, although the enzyme complex (BchXYZ) is fairly stable at 2% O_2 [6]. Thus, the O_2 level in photoheterotrophically-grown *Synechocystis* sp. PCC6803 appears to be low enough (∠8% O_2 [DO, 92 ± 4 μM]) in order to support superoxide generation by COR.

3.3. Evolutionary implication

There has been an argument that Chl *a*, which absorbs short-wavelength light compared with Bchl *a*, evolved for photosynthetic bacteria to get more energy to split water, one of the most abundant electron donors in nature. Both Chl *a* and Bchl *a*

pathways might be assumed to co-exist in the primitive lineage of photosynthetic bacteria. Although SOD, which is known to emerge as the advent of O_2 in the atmosphere [24], is assumed to be present in primitive anoxygenic photosynthetic bacteria, its abundance might not be sufficient to resolve superoxide stress. The relative SOD levels of various organisms are positively correlated with their oxygen exposure [25]. Accordingly, COR may be deselected due to its activity of superoxide generation as oxygen was liberated from the cleavage of water. Consequently, Bchl *a* biosynthetic pathway, which branches at the level of Chlide *a*, may be degenerated. Interestingly, *Chlorobium tepidum*, a green sulfur bacterium contains biosynthetic pathways for Chl *a* and Bchl *a* and *c* [26]. The evolution of Chl *a* biosynthesis is thought to accompany the emergence of ChlG and ChlP, possibly through the gene duplication of BchG and BchP. The predicted sequences of ChlG and ChlP of *Synechocystis* sp. PCC6803 bear 35% and 38% identity with those of *R. sphaeroides* BchG and BchP, respectively. Since cyanobacteria are believed to have emerged before the Proterozoic era (between 2500 and 543 million years go) [27], the evolution of a new shortcut Chl *a* pathway from Bchl *a* biosynthesis should be a preceding event.

In summary, we have expressed *R. sphaeroides* COR in *Synechocystis* sp. PCC6803, and found that the photoheterotrophic growth of recombinant *Synechocystis* sp. PCC6803 ceased unless the cytoplasmic FeSOD level was increased. Thus, COR generates superoxide in *Synechocystis* sp. PCC6803 to cease cell growth. The superoxide generation by COR according to the partial pressure of oxygen showed a bell-shaped curve with the maximal activity at 2% O_2, whereas Chlide *a* reduction decreased in inverse relation to O_2, reflecting the stability decrease of COR at high O_2. The superoxide generation by COR in *Synechocystis* sp. PCC6803 indicates its low intracellular DO even during oxygenic photosynthesis. The results shown in this work further provide an insight into the evolutionary path of the biosynthetic pathways from Bchl *a* to Chl *a*.

Acknowledgements

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