Essential roles of iron superoxide dismutase in photoautotrophic growth of *Synechocystis* sp. PCC 6803 and heterogeneous expression of marine *Synechococcus* sp. CC9311 copper/zinc superoxide dismutase within its *sodB* knockdown mutant

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*Synechocystis* sp. PCC 6803 possesses only one *sod* gene, *sodB*, encoding iron superoxide dismutase (FeSOD). It could not be knocked out completely by direct insertion of the kanamycin resistance cassette. When the promoter of *sodB* in WT *Synechocystis* was replaced with the copper-regulated promoter PpetE, a completely segregated PpetE–*sodB* strain could be obtained. When this strain was cultured in copper-starved BG11 medium, the chlorophyll a content was greatly reduced, growth was seriously inhibited and the strain was nearly dead during the 8 days of growth, whilst the WT strain grew well under the same growth conditions. These results indicated that *sodB* was essential for photoautotrophic growth of *Synechocystis*. The reduction of *sodB* gene copies in the *Synechocystis* genome rendered the cells more sensitive to oxidative stress produced by methyl viologen and norflurazon. *sodB* still could not be knocked out completely after active expression of *sodC* (encoding Cu/ZnSOD) from *Synechococcus* sp. CC9311 in the neutral site slr0168 under the control of the *psbAII* promoter, which means the function of FeSOD could not be complemented completely by Cu/ZnSOD. Heterogeneously expressed *sodC* increased the oxidation and photoinhibition tolerance of the *Synechocystis sodB* knockdown mutant. Membrane fractionation followed by immunoblotting revealed that FeSOD was localized in the cytoplasm, and Cu/ZnSOD was localized in the soluble and thylakoid membrane fractions of the transformed *Synechocystis*. Cu/ZnSOD has a predicted N-terminal signal peptide, so it is probably a lumen protein. The different subcellular localization of these two SODs may have resulted in the failure of substitution of *sodC* for *sodB*.

**INTRODUCTION**

Reactive oxygen species (ROS) are inevitable byproducts of aerobic metabolism and potent agents that cause oxidative damage. ROS usually include singlet-state oxygen (O2), the superoxide ion (O2−), hydrogen peroxide (H2O2) and the highly destructive hydroxyl radical (OH•). The reaction centres of photosystem (PS) I and II in thylakoids are the major sites of ROS generation (Asada, 2006). Within PSII, O2 is produced by energy input to oxygen from photo-sensitized chlorophyll, and univalent reduction of O2 using electrons from PSII generates O2− at the reducing side of PSI (Latifi *et al.*, 2009).

Low-concentration ROS play an important role in defence against infection, cell signalling and apoptosis (Valko *et al.*, 2007). However, when ROS are formed in excess, the oxidation of cellular biomolecules by ROS initiates oxidative damage of proteins, lipids and nucleic acids (Polle, 2001). Living organisms have developed various defences to protect against ROS damage (Imlay, 2013). Whilst some of these defences are non-enzymic (such as carotenoid, glutathione, vitamin A, C, E, etc.), others are enzymic [superoxide dismutases (SODs), catalases and peroxidases] (Apel & Hirt, 2004; Latifi *et al.*, 2009). SODs are the first line of defence to alleviate oxidative stress in virtually all living organisms that survive inoxic environments (McCord & Fridovich, 1969),

**Abbreviations:** MV, methyl viologen; NF, norflurazon; PS, photosystem; ROS, reactive oxygen species; SOD, superoxide dismutase.

Five supplementary figures are available with the online version of this paper.
and catalyse the transformation of $\text{O}_2^-$ to $\text{O}_2$ and $\text{H}_2\text{O}_2$. The scavenging of $\text{H}_2\text{O}_2$ differs between cyanobacteria and higher plant chloroplasts (Asada, 2006). Cyanobacteria do not contain an ascorbate peroxidase, and they use catalase and/or thioredoxin peroxidase (2-cysteine peroxiredoxin) activities to catalyse $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$ (Regelsberger et al., 2002).

To date, all SODs have been found to be metalloproteins with a redox-active metal. SOD isoforms are classified according to the redox metal within the active site, which includes iron, manganese, copper (with a structural zinc ion) and nickel. In addition, a cambialistic form that uses iron/manganese in its active site also exists (Lancaster et al., 2004). The existence of multiple SODs may result from the fact that cells are divided into compartments by internal membranes. Since $\text{O}_2^-$ ions are negatively charged and cannot readily cross a phospholipid bilayer, they are effectively trapped within the compartment where they were generated. This may have selected for the evolution of multiple SODs in compartmentalized cells (Takahashi & Asada, 1983). Herbert et al. (1992) carried out the first genetic analysis of SODs in a unicellular cyanobacterium (Synchococcus elongatus PCC 7942) and suggested that multiple SODs (FeSOD in soluble extracts and other SODs in membrane fractions) exist to protect the multiple cell compartments against oxidative stress. Cyanobacteria represent the first oxygenic photautotroph and they bear a particular risk of oxygen toxicity, because molecular $\text{O}_2$ can be photoreduced to $\text{O}_2^-$ by electrons from PSI (Mehler, 1951), and highly conserved A-type flavoproteins Flv1 and Flv3 are essential for this process in vivo (Helman et al., 2003). Therefore, cyanobacterial SODs play very important roles in preventing the oxidative damage associated with photosynthesis.

Among the four kinds of SODs, the structure, phylogenetic diversity, regulation and cellular location of FeSOD and MnSOD have been studied intensively in the past decades (Miller, 2004; Wolfe-Simon et al., 2005). However, there is rather limited information regarding Cu/ZnSOD and NiSOD in cyanobacteria. Cu/ZnSOD is rare among cyanobacteria, and genome sequence analyses of 64 cyanobacterial SODs confirmed its existence only in six Synechococcus and one Lyngbya strains, including Synechococcus sp. CC9311 (hereafter called Synchococcus 9311) (Priya et al., 2007). Cu/ZnSOD has different primary and tertiary structures from FeSOD and MnSOD, and almost certainly evolved independently (Wolfe-Simon et al., 2005). Chadd et al. (1996) first identified Cu/ZnSOD within the marine cyanobacterium Synechococcus sp. WH 7803 according to different sensitivities of in-gel activity staining bands to 5 mM $\text{H}_2\text{O}_2$, 2 mM cyanide or 2 mM copper chelator diethyldithiocarbamate. Two different signatures (G-F-H-[ILV]-H-x-[NGT]-[GPDA]-[SQK]-C and G-[GA]-G-[EAG]-[AG][EAG]-[AG]-C-G) were found in the cyanobacterial Cu/ZnSOD protein sequence (Priya et al., 2007), and protein sequences (GFHLHAGDQC and GGGGARIACG) of the Cu/ZnSOD putative encoding gene (sync_1771, sodC) in Synchococcus 9311 (Palenik et al., 2006) fitted well with them. Combined with in-gel activity staining and immunoblotting results, we concluded that sync_1771 in Synchococcus 9311 encoded Cu/ZnSOD (unpublished data).

Based on 64 cyanobacterial SODs available in public databases, an analysis found that cyanobacteria harbour either FeSOD or NiSOD alone, or a combination of FeSOD and MnSOD, FeSOD and Cu/ZnSOD or NiSOD and Cu/ZnSOD (Priya et al., 2007). All cyanobacteria have a cytosolic sod gene encoding either soluble FeSOD or soluble NiSOD, and these could be the housekeeping SODs (Regelsberger et al., 2004; Priya et al., 2007). Some cyanobacteria have additional SODs, e.g. filamentous species with nitrogenase activity appear to need one or more supplementary membrane-bound MnSODs to effectively lower $\text{O}_2^-$ levels (Regelsberger et al., 2004). When MnSOD was knocked out in nitrogen-fixing cyanobacterium Anabaena sp. PCC 7120, it was more susceptible to photoinhibition, and both PSI and II were damaged more severely by the photoinhibitory light, suggesting that MnSOD plays important roles in the protection of both PSs (Zhao et al., 2007). Furthermore, their protective roles during oxidative stress are dependent on nitrogen status: under nitrogen-fixing conditions, overexpression of MnSOD enhanced oxidative stress tolerance, whilst FeSOD overexpression was detrimental; under nitrogen-supplemented conditions, overexpression of either SOD protein, especially FeSOD, conferred significant tolerance against oxidative stress (Raghavan et al., 2011). Some Synechococcus and all Prochlorococcus genomes sequenced or assembled from metagenomic data contain a sodN gene coding for NiSOD (Palenik et al., 2003; Rusch et al., 2010; Dupont et al., 2012). Several attempts to inactivate insertionally the sole sod gene, sodN, in Synechococcus WH8102 were unsuccessful and its knock out appeared to be lethal (Dupont et al., 2012). When sodN of Synechococcus WH8102 was interrupted with sodB from Synechococcus sp. WH7803, the knockout strains exhibited an impaired ability to grow at low iron concentrations (Dupont et al., 2012). Except for NiSOD, Synechococcus sp. CC9311, CC9902 and CC9605 have an additional Cu/ZnSOD (Priya et al., 2007). However, attempts to interrupt their sodN genes were also unsuccessful (Dupont et al., 2012), suggesting that cytosolic NiSOD was necessary for the survival of these strains. Synechococcus elongatus PCC 7942 possesses only one kind of SOD (FeSOD), which is encoded by two genes (Priya et al., 2007). Knocking out one of them had no influence on photoautotrophic growth of Synechococcus elongatus PCC 7942, while double mutation was not conducted, which could be lethal (Herbert et al., 1992; Thomas et al., 1998).

So far, little information is available on whether different kinds of SODs from various cyanobacterial species function differently. Synechocystis sp. PCC 6803 (hereafter called Synechocystis 6803) is used widely to study fundamental processes of oxygenic photosynthesis. There is only one sod gene (slr1516, sodB, encoding FeSOD) in Synechocystis 6803 (Kaneko et al., 1996), which makes it
convenient for comparing the function of SODs derived from different species. In order to investigate whether Cu/ZnSOD from marine *Synechococcus* 9311 can substitute the function of FeSOD in freshwater *Synechocystis* 6803, we attempted to knock out sodB and heterologously express sodC in *Synechocystis* 6803. However, sodB was found to be essential for photoautotrophic growth of *Synechocystis* 6803 in this study. It could not be knocked out completely under antibiotic pressure even after heterologous expression of active sodC. Heterologous expression of sodC increased the tolerance of *Synechocystis* sodB− to oxidation and photo-inhibition stress. Different localizations of these two SODs may determine their inability to interchange in the transformed cyanobacterium.

**METHODS**

**Cyanobacterial strains, culture conditions and general methods.** A glucose-tolerant *Synechocystis* 6803 strain (hereafter referred to as WT) was cultured in BG11 medium at 30 °C under continuous illumination of 30 μmol photons m−2 s−1; for copper-regulated growth experiments, illumination of 45 μmol photons m−2 s−1 was used. BG11 plates were prepared by adding 1.4 % (w/v) agar, 8 mM TES (pH 8.2) and 0.3 % (w/v) Na2S2O3. According to culture requirements of the mutants or complemented strains, kanamycin (25 μg ml−1), spectinomycin (20 μg ml−1) or both were added into BG11 medium. *Synechococcus* 9311 was cultured in SN medium based on artificial seawater at 22 °C under continuous illumination of 15 μmol photons m−2 s−1. The preparation of SN medium was as described by Waterbury & Willey (1988). For complete segregation of the sodB− mutant, cultures were grown under conditions of decreased illumination of 5 μmol photons m−2 s−1 in the presence of 0.1 % (w/v) glucose, 10 mM NaHCO3, 100 μg BSA ml−1 and 20 μg leucine ml−1. For the analysis of *Synechocystis* 6803 growth in copper-starved medium, the cells were collected by centrifugation and washed twice before transferring and cultivating in fresh copper-starved BG11 medium. The strains grown in BG11 medium were inoculated into copper-starved BG11 medium for 3 times as above, the strains in the exponential phase were used for growth comparison experiments. Before transferring and cultivating in fresh copper-starved BG11 medium, the cells were collected by centrifugation and washed twice with fresh copper-starved medium. After transferring to copper-starved BG11 medium for three times as above, the strains in the exponential phase were used for growth comparison experiments. After transferring to copper-starved BG11 medium once as above, the strains in the exponential phase were used for whole-cell absorbance spectra comparison.

The growth of *Synechocystis* 6803 was monitored by turbidity (OD730) or chlorophyll a content from at least three parallel cultures. The specific growth rates (day−1) were calculated as follows: μ = (lnXt − lnX0)/t, where X0 and Xt are the OD730 values of the day 4 and initial inocula, respectively. A blank was prepared using growth medium. Chlorophyll a content was determined spectrophotometrically in 95% ethanol extracts (Lichtenthaler & Buschmann, 2001). A blank was prepared using 95% ethanol. Whole-cell absorbance spectra from 400 to 800 nm were recorded on a Cary 300 UV/visible spectrophotometer (Varian). A blank was prepared using BG11 medium.

**Construction of plasmids.** Molecular manipulations were performed following standard protocols. Enzymes were used according to the instructions provided by the manufacturers. Sticky ends of DNA fragments were blunted using T4 DNA polymerase (Promega). Restriction enzymes and T4 DNA ligase were purchased from Takara. Plasmids and primers used are listed in Table 1.

**Construction of pHS168 for inactivating sodB.** The DNA fragment containing the full-length sodB gene (slr1516) and its flanking sequences was generated by PCR using primers slr1516 up and slr1516 down with *Synechocystis* 6803 chromosome DNA as template, cloned into pMD18-T vector (Takara) (Yang et al., 2008), and confirmed by sequencing. The kanamycin-resistant cassette (C.K2) excised from pRL446 (GenBank accession no. EU346690) (Elhai & Wolk, 1988) by Psul was inserted into the Hpal site of the above-mentioned sequenced plasmid, resulting in pHS168 for the inactivation of sodB in *Synechocystis* 6803. The physical map of the *Synechocystis* 6803 genome region containing sodB, indicating the insertion position of C.K2 into sodB, is shown in Fig. 1(a). The resulting pHS168 was used to transform WT *Synechocystis* 6803, in which sodB was inactivated by insertion of C.K2.

**Construction of pHS203 for complementing the *Synechocystis* sodB− mutant.** The copper-regulated petE promoter (PpetE) (Zhang et al., 1992) fragment (436 bp, corresponding to −436 to −1, relative to the ATG of the petE gene) was generated by PCR using primers PpetE-1 and PpetE-2, with *Synechocystis* 6803 chromosome DNA as template, cloned into pMD18-T, and confirmed by sequencing, resulting in pHS179. A spectromycin resistance cassette (Omega) excised by Dral from pRL57 (Black et al., 1993) was inserted into the Xbal site of pHS179, resulting in pHS185. The DNA fragment containing the full-length sodB was generated by PCR using primers slr1516 exp-1 and slr1516 exp-2 with *Synechocystis* 6803 chromosome DNA as template, cloned into pMD18-T, and confirmed by sequencing, resulting in pHS193. The *Omega–PpetE* fragment was excised by Sall/BamHI from pHS185 and cloned into the Sall sites of pHS193, resulting in pHS194. The *Omega–PpetE–sodB* fragment was excised by Psrl/BamHI from pHS194 and cloned into the EcoRI sites of plasmid pKW1188 (Williams, 1988), resulting in pHS203. The main construction steps are shown in Fig. S1 (available in Microbiology Online). pKW1188 contained a slr0168 gene, which was regarded as a platform for inserting the exogenous gene by homologous recombination. The resulting pHS203 was used to transform the *Synechocystis* 6803 sodB− mutant, in which sodB was expressed under the control of the petE promoter.

**Construction of pHS198 for regulating the expression of FeSOD with the petE promoter in WT *Synechocystis* 6803.** The DNA fragment containing the upstream sequences of sodB (the putative promoter region, 700 bp, corresponding to −735 to −34, relative to the ATG of the sodB gene) was generated by PCR using primers slr1516 up and slr1516 down with *Synechocystis* 6803 chromosome DNA as template, cloned into pMD18-T, and confirmed by sequencing, resulting in pHS197. The *Omega–PpetE–sodB* fragment was excised by Psrl/BamHI from pHS194 and cloned into the Psrl sites of pHs197, resulting in pHS198. The main construction steps of pHS198 are shown in Fig. S2. The physical map of sodB in the *Synechocystis* 6803 genome with genetic modifications is shown in Fig. S3(a). pHS198 was used to transform WT *Synechocystis* 6803, in which sodB was expressed under the control of the petE promoter.

**Construction of pHS304 for expressing sodC (sync_1771, encoding Cu/ZnSOD) using a PsbAII expression platform vector within the *Synechocystis* sodB− mutant.** sodC (sync_1771) from *Synechococcus* 9311 genome was generated by PCR using primers sync_1771 up and sync_1771 down. The PCR products were inserted into the Ndel site (blunted with T4 DNA polymerase) of the expression platform plasmid pHs298 (Jiang et al., 2012), making the gene in the same orientation as the pbbAII promoter (PsbAII).
Construction of the mutant and complementation strains of Synechocystis 6803. Transformation of Synechocystis 6803 was performed as described by Williams (1988). Homologous double-cross-over recombinants were generated between the plasmid and genomic DNA under positive selection of corresponding antibiotics (Labarre et al., 1989). The degree of segregation of the sodB mutant was examined by PCR using the primer pair slr1516 up and slr1516 down with PCR of chromosomal DNA from WT Synechocystis 6803 and plasmid pHs168 as controls. The degree of segregation of the PpetE–sodB strain was examined by PCR using the primer pair slr1516 up and slr1516 exp-2 with PCR of chromosomal DNA from WT Synechocystis 6803 and plasmid pHs198 as controls. To complement the sodB mutant, pHs203 and pHs304 were introduced, respectively. During growth in BG11 medium with both 25 μg kanamycin ml⁻¹ and 20 μg spectinomycin ml⁻¹, sodB and sodC were expressed constitutively, respectively. Details of the generation of mutant and complementation strains are shown in Table 1, and Figs S1, S2 and S4.

Measurement of chlorophyll fluorescence. PSII activity was analysed in vivo with a WATER-PAM chlorophyll fluorometer (Walz). All samples were dark-adapted for 10 min before measurement. The maximum PSII quantum yield (Fᵥ/Fmᵥ) was determined with the saturation pulse method (Schreiber et al., 1995; Campbell et al., 1996).

Antibody production against FeSOD and Cu/ZnSOD. sodB and sodC genes from Synechocystis 6803 and Synechococcus 9311 were obtained by PCR amplification with their corresponding chromosomal DNA template in the presence of the high-fidelity enzyme Pfu (Promega), by using the primers sets: A 1516-1 and sync_1771 exp-1/sync_1771 exp-2, respectively. The amplified fragments were first cloned into pMD18-T vector and sequenced. The fragments obtained by digestion with Ndel and Xhol from the resultant sequenced plasmid were cloned into pET41a (Novagen) and transformed into Escherichia coli strain BL21 (DE3) for overexpression. The overexpressed proteins were purified by His-bind resin (Novagen) and then used to generate two kinds of polyclonal antibodies in rabbits that, respectively, recognized FeSOD and Cu/ZnSOD in WT Synechocystis 6803 and sodC-transformed Synechocystis 6803.

Membrane isolation and immunoblotting analysis. The transformed Synechocystis 6803 cells were collected by centrifugation at 6000 g, resuspended in 20 mM potassium phosphate (pH 7.8) with 100 μM PMSF and ruptured with glass beads by vortexing at 2500 r.p.m. for 30 s per time, with 30 s interval of cooling on ice. This process was repeated 10 times and the total time for breaking cells was 5 min. Observation under a microscope revealed most cells to be broken. The cell debris was removed by centrifugation at 4 °C and 4000 g for 10 min. The supernatant was then centrifuged at 4 °C and 100 000 g for 30 min to separate membrane proteins from soluble proteins; the pellet was the membrane fraction. The membranes were washed three times with 20 mM potassium phosphate (pH 7.8) and named crude membranes. Cell fractions containing the thylakoid membrane or the plasma membrane were prepared according to the procedures described by Huang et al. (2002) and Srivastava et al. (2005). Equal amounts of membrane and soluble proteins were loaded, separated by 12% SDS-PAGE, transferred to nitrocellulose filters (Millipore), detected with anti-FeSOD, anti-Cu/ZnSOD, anti-NrtA or anti-CP47 rabbit antisemum, and visualized with goat anti-rabbit alkaline phosphatase antibody (Invitrogen) with NBT and 5-bromo-4-chloro-3-indolylphosphate (Amresco) as substrates. SDS-PAGE and immunoblotting were performed using standard methods. Antibodies against NrtA and CP47 of Synechocystis 6803 (used at 1:6000 dilution) were provided kindly by Professor Xudong Xu (Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, PR China).

RESULTS

sodB is essential for photoautotrophic growth of Synechocystis 6803. sodB is the sole sod gene in the Synechococcus 6803 chromosome (see Cyanobase at http://genome.microbedb.jp/cyanobase/). We tried to inactivate it by insertion of a kanamycin resistance cassette. As cyanobacteria contain 10–20 copies of their chromosome in individual cells (Labarre et al., 1989), in order to segregate the mutant allele of sodB to a homozygous condition, they were selected under antibiotic pressure in solid or liquid BG11 medium many times. Many attempts to segregate the mutant allele of sodB to a homozygous condition were unsuccessful, even though the BG11 medium was supplemented with glucose (0.1%), NaHCO₃ (10 mM), BSA (100 μg ml⁻¹) and leucine (20 μg ml⁻¹), and the strains were cultured under a decreased illumination intensity of 5 μmol photons m⁻² s⁻¹, according to Herbert et al. (1992) and Nefedova et al. (2003). PCR analysis of transformant’s chromosomal DNA with gene-specific primers indicated that these colonies were still heterozygous (Fig. 1b). Although nearly all of the WT sodB copies in the Synechocystis genome were missed (Fig. 1b), FeSOD could still be detected at the protein level by immunoblotting and in-gel activity staining (Fig. 1c, d). These results indicated that sodB in Synechocystis 6803 could not be knocked out completely by insertion of the kanamycin resistance cassette directly. The Synechocystis 6803 sodB⁻ mutant was complemented with pHs203 carrying the WT sodB through homologous double-cross-over recombinants with the genome. In the complemented strain, sodB was expressed under the control of the PpetE and the growth was restored to the WT level (Fig. 2). Furthermore, a fragment containing Omega–PpetE was integrated upstream of sodB in the genome of WT
### Table 1. Cyanobacterial strains, plasmids, and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Derivation and/or relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>Synechocystis PCC 6803 strains</strong></td>
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<tr>
<td>WT</td>
<td>Glucose tolerant, without antibiotic resistance</td>
<td>This study</td>
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<td>sodB::C.CK2 (sodB&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, <em>Synechocystis</em> 6803 mutant</td>
<td>This study</td>
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<td>slr0168::Omega–PpetE–sodB (sodB&lt;sup&gt;−&lt;/sup&gt; com)</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, Omega–PpetE–sodB integrated into the EcoRI site of slr0168 in the genome of mutant <em>Synechocystis</em> sodB&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>Omega–PpetE–sodB (PpetE–sodB)</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, Omega–PpetE integrated upstream of sodB in the genome of <em>Synechocysts</em> WT</td>
<td>This study</td>
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<td>Omega–PpsbAII–sodC (sodB&lt;sup&gt;−&lt;/sup&gt;–sodC com)</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, Omega–PpsbAII–sodC integrated into slr0168 in the genome of mutant <em>Synechocystis</em> sodB&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pMD18-T</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, cloning vector</td>
<td>Yang et al. (2008)</td>
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<td>pHS168</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;, PCR fragment containing <em>sodB</em> and its flanking sequences cloned into pMD18-T and C.K2 inserted in its HpaI site</td>
<td>This study</td>
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<td>pHS179</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, PCR fragment containing the petE promoter region cloned into pMD18-T; the size of the petE promoter is 436 bp, corresponding to –436 to –1, relative to the ATG of the petE gene</td>
<td>This study</td>
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<td>pHS185</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, spectinomycin resistance cassette Omega from pRL57 inserted into pHS179 at the XhoI site</td>
<td>This study</td>
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<td>pHS193</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, PCR fragment containing full-length <em>sodB</em> cloned into pMD18-T</td>
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<td>pHS194</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, fragment Omega–PpetE from pHS185 which was excised by Sall/BamHI was inserted into Sall of pHS193</td>
<td>This study</td>
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<td>pHS197</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, PCR fragment containing upstream sequences of <em>sodB</em> (700 bp, corresponding to –735 to –34, relative to the ATG of <em>sodB</em>) cloned into pMD18-T</td>
<td>This study</td>
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<td>pHS198</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, fragment Omega–PpetE–sodB from pHS194 which was excised by PstI/BamHI was inserted into pHS197 at the PstI site</td>
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<td>pHS203</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, fragment Omega–PpetE–sodB from pHS194 which was excised by PstI/BamHI was inserted into pKW1188</td>
<td>This study</td>
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<td>pHS304</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, PCR fragment containing full-length <em>sodC</em> from Synechococcus cloned in the PpsbAII expression vector pHS298 at the NdeI site</td>
<td>This study</td>
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<td>pHS298</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;, PpsbAII expression vector, bearing an integrative platform slr0168 for <em>Synechocystis</em> 6803; the size of the ppsbAII promoter in pHS298 is 318 bp, corresponding to –331 to –1 (without the high-light-responsive region –30 to –16) relative to the ATG of the ppsbAII gene</td>
<td>Jiang et al. (2012)</td>
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<td>pKW1188</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;, plasmid bearing an integrative platform slr0168 for <em>Synechocystis</em> 6803</td>
<td>Williams (1988)</td>
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<td>pRL446</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, cloning vector with the kanamycin resistance cassette (C.K2)</td>
<td>Elhai &amp; Wolk (1988)</td>
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<td>pRL57</td>
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<td>Black et al. (1993)</td>
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<td><strong>Primers</strong></td>
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<td>PpetE-1</td>
<td>5′-AAGGATTGATGGAAGGTTGGC-3′</td>
<td>This study</td>
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<td>PpetE-2</td>
<td>5′-ACTTCTTGGGAGGTTGATC-3′</td>
<td>This study</td>
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<td>slr1516 up</td>
<td>5′-TACGGAGGACAAACAGGACT-3′</td>
<td>This study</td>
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<td>slr1516 down</td>
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Synechocystis 6803 through homologous recombination (Fig. S3a) to produce the PpetE–sodB strain. PCR analysis of transformant’s chromosomal DNA with gene-specific primers indicated that these colonies were homozygous (Fig. S3b). The Omega cassette upstream of the petE promoter has stem–loop structures at both ends to terminate background transcription. When the strain acclimated to copper starvation was transferred to BG11 medium containing the indicated amounts of supplementary copper, the growth photograph on day 4 showed clearly that the chlorophyll a content was regulated by the Cu2+ concentration (Fig. S5a). When the Cu2+ concentration was reduced to 9.9 nM (in normal BG11 medium with 316 nM Cu2+), no FeSOD signal was detected with immunoblotting (Fig. S5b) and trace FeSOD activity could be determined with in-gel activity staining (Fig. S5c). When the cells were cultured in copper-starved medium under illumination of 45 μmol photons m⁻² s⁻¹, the PpetE–sodB strain exhibited remarkable etiolation symptoms compared with that cultured in normal BG11 medium and WT cultured in copper-starved medium (Fig. 3a, b). Chlorophyll a content of the PpetE–sodB strain cultured in copper-starved medium remained constant during 8 days of growth (Fig. 3c) and the ratio of chlorophyll a to OD730 on day 4 was decreased significantly compared with the same strain cultured in normal BG11 medium (Fig. 3d). These results indicated that sodB was essential for photoautotrophic growth of Synechocystis 6803. The whole-cell absorption spectra of the PpetE–sodB strain and WT Synechocystis 6803 cultured in normal BG11 and copper-starved medium are shown in Fig. 3(e, f). The pigment contents showed no significant differences between WT Synechocystis 6803 cultured under normal and copper-starved BG11 medium conditions (Fig. 3e). For the PpetE–sodB strain, when it was cultured under copper starvation conditions, the ratio of carotenoid to chlorophyll a increased compared with that cultured in normal BG11 medium, and the ratio of phycobilin to chlorophyll a did not differ significantly between the two culture conditions (Fig. 3f).

**Table 1. cont.**

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**Synechococcus 9311 sodC transformed into the Synechocystis 6803 sodB⁻ mutant**

To investigate whether sodC from Synechococcus 9311 was functionally complementary to sodB, pHS304 for expressing sodC was transformed into the sodB⁻ mutant. In order to obtain homozygous mutants, the transformants were transferred many times on BG11 plates or in liquid BG11 medium supplemented with both kanamycin and spectinomycin. PCR analysis of these kanamycin- and spectinomycin-resistant cells showed that sodB was not disrupted fully by the kanamycin resistance cassette (Fig. 4a). Although Cu/ZnSOD was expressed actively, obvious FeSOD activity was still determined in the complemented

![Fig. 1](http://mic.sgmjournals.org)

**Fig. 1.** Construction and detection of the Synechocystis 6803 sodB⁻ mutant. (a) Physical map of the Synechocystis 6803 genome region containing sodB, with indications of the insertion position of the kanamycin resistance cassette (C.K2) into sodB at the Hpal site. (b) Detection of degree of segregation of the sodB⁻ mutant by PCR amplification. PCR was carried out using primers slr1516 up and slr1516 down with the genomic DNA of the WT (lane 1), plasmid pHS168 (lane 2) and mutants (three clones, lanes 3–5) as templates. The primer sequences are listed in Table 1. Molecular markers are shown on the left side of the gel, and represent 6000, 5000, 4000, 3500, 3000, 2500, 2000 and 1500 bp, respectively (from top to bottom). (c, d) Detection of the amount of FeSOD in the WT and sodB⁻ mutant by immunoblotting (c) and in-gel activity staining (d); 40 μg total protein was loaded in each lane for immunoblotting and 120 μg total protein was loaded in each lane for in-gel staining.
strain (Fig. 4b). It suggested that the function of FeSOD could not be substituted by Cu/ZnSOD. However, when sodC was introduced into the WT Synechocystis genome, sodB was disrupted in this background by insertion of a kanamycin resistance cassette, and a fully segregated mutant of sodB expressing actively Synechococcus 9311 sodC was attempted. Nevertheless, PCR analysis and SOD activity staining showed the same results as above (data not shown). These results indicated that sodC could not complement completely the function of sodB.

In order to study the function of Cu/ZnSOD in oxidative stress, the growth rates of three Synechocystis 6803 strains (WT, sodB− mutant and sodC complement strain) were compared in the presence or absence of methyl viologen (MV) or norflurazon (NF) (Fig. 5). MV is a cell-dependent O2−-producing agent and NF can promote the formation of 1O2 within the thylakoid membrane (Thomas et al., 1998). Upon 0.5 μM MV treatment, the relative growth rates of WT and the sodB− mutant decreased by 7.2 and 20.7%, respectively, whilst the sodC complement strain showed little effect, compared with their corresponding controls (Fig. 5a). Thus, the relative growth rate of the sodB− mutant was reduced significantly, whilst that of the sodC complement strain was increased significantly compared with the WT strain (P<0.05, Tukey multiple comparison) (Fig. 5a, b). Treatment with 40 μM NF caused the relative growth rates of the WT, sodB− mutant and sodC complement strains to decrease by 14.0, 22.2 and 12.2% respectively, compared with their corresponding controls (Fig. 5c). Thus, the relative growth rate of the sodC complement strain showed no difference from the WT, while that of the sodB− mutant was decreased significantly (P<0.05, Tukey multiple comparison) (Fig. 5c, d).

Photoinhibition of PSII by strong light has been proposed to occur by several hypothetical mechanisms, some of which include the role of O2− (Richter et al., 1990; Nishiyama et al., 2001; Tjus et al., 2001; Song et al., 2006).

To test whether FeSOD and Cu/ZnSOD were involved in protecting PSII against photoinhibition, samples of the WT, sodB− mutant and sodC complement strain were subjected to photoinhibitory conditions of 1000 μmol photons m−2 s−1 at 30 °C in the absence or presence of lincomycin (100 μg ml−1), an antibiotic that blocks the repair of PSII by inhibiting de novo protein synthesis. PSII activity was estimated by measuring Fv/Fm with the WATER-PAM chlorophyll fluorometer. In the presence of lincomycin, PSII activity of the three strains declined at the same rate with exposure to strong light (Fig. 6), which indicated Cu/ZnSOD and FeSOD were not involved in protecting PSII against photodamage. Upon exposure to a strong light in the absence of lincomycin, PSII activity of the sodC complement strain decreased slower than for the WT and sodB− mutant (Fig. 6). After 30 min strong light exposure, the Fv/Fm values of the WT, sodB− mutant and sodC complement strain were decreased to 16.2, 20.4 and 32.9% of the corresponding values at time zero, respectively. The sodB− mutant and WT strain had a similar response pattern to photoinhibition during 90 min exposure to strong light (Fig. 6), suggesting that FeSOD was not involved in protecting PSII against strong light. Synechocystis 6803 transformed with Cu/ZnSOD was more resistant to strong light than the WT and sodB− mutant, which indicated that Cu/ZnSOD played a role in alleviating photoinhibition. Combined with these results, it appeared that the expression of Cu/ZnSOD enhanced the repair of photodamaged PSII, but did not protect PSII from photodamage.

**Subcellular localization of FeSOD and Cu/ZnSOD in sodC-transformed Synechocystis 6803**

Analysis of the protein sequences of slr1516 (encoding FeSOD) and sync_1771 (encoding Cu/ZnSOD) with the SignalP program 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) predicted that no signal peptide existed in FeSOD, whilst an evident N-terminal signal peptide...
existed in Cu/ZnSOD. To examine the subcellular localization of these two SODs, plasma membrane, thylakoid membrane or soluble fractions were separated from cell extracts as described by Huang et al. (2002) and Srivastava et al. (2005). Detection of FeSOD and Cu/ZnSOD was performed with each subcellular fraction by immunoblotting.

Fig. 3. Growth characteristics and chlorophyll a content of the WT and PpetE–sodB strain grown in normal BG11 or copper-starved BG11 medium. (a, b) Photographs of the WT (a) and PpetE–sodB strain (b) grown in BG11 medium under normal copper concentrations (+Cu) or copper starvation conditions (–Cu). Three photographs are shown for each strain: 0 d, 4 d and 8 d represent the initial day, fourth day and eighth day of growth at the third transfer, respectively. (c) Growth curves (determined by the chlorophyll a content) of the WT and PpetE–sodB strain cultured in normal BG11 or copper-starved BG11 medium. (d) Chlorophyll a/OD730 ratios of the WT and PpetE–sodB strain on the fourth day of growth at the third transfer. Data are mean ± SD (n=3). Error bars indicate SD. (e, f) The whole-cell absorption spectra of the WT (e) and PpetE–sodB strain (f) after 4 days of cultivation in normal BG11 and copper-starved BG11 medium. The spectra were normalized to the chlorophyll a maximal absorption peak at 440 nm. Car, carotenoid; Chl, chlorophyll; PC, phycocyanin.
with their corresponding antibodies. FeSOD was only detected in the soluble fraction (Fig. 7a), whilst Cu/ZnSOD was detected in both the soluble fraction and thylakoid membrane fraction (Fig. 7b, c), and no detectable signal existed in the plasma membrane fraction (Fig. 7c). NrtA, a nitrate/nitrite transport system substrate-binding protein, was used as a marker for the plasma membrane. CP47, a chlorophyll-binding protein of PSII, was used as a marker for the thylakoid membrane. As expected, NrtA was found mainly in the plasma membrane fraction and CP47 was found almost exclusively in the thylakoid membrane fraction (Fig. 7d, e), indicating that these two membrane fractions were purified. These results indicated that FeSOD was localized in the soluble fraction, and Cu/ZnSOD was localized in both the soluble fraction and thylakoid membrane of transformed *Synechocystis* 6803.

![Fig. 4. Detection of degree of segregation of sodB in the sodC-complemented sodB− mutant by PCR amplification and in-gel activity staining. (a) PCR detection. PCR was carried out using the primers slr1516 up and slr1516 down with the plasmid pH5s168 (lane 1), and genomic DNA of the WT (lane 2), sodB− mutant (lane 3) and its sodC complement strain (lane 4) as templates. The primers sequences are listed in Table 1. Molecular markers are shown on the left side of the gel, and represent 4000, 3500, 3000, 2500, 2000 and 1500 bp, respectively (from top to bottom). (b) In-gel activity staining; 120 μg total protein was loaded in each lane for this detection. The additional SOD band in the complement strain compared with WT and mutant represented Cu/ZnSOD.](#)

![Fig. 5. Effects of 0.5 μM MV and 40 μM NF on the growth of the WT, sodB− mutant and its sodC-complemented strain, respectively. The WT, sodB− mutant and its sodC-complemented strain were grown under normal BG11 medium, and (a, b) 0.5 μM MV or (c, d) 40 μM NF was added into BG11 medium for 4 days. The growth rate of each strain grown in normal BG11 medium was used as control. All data are presented as the per cent of corresponding controls. Columns with different letters (a–c) on the error bars are significantly different (P<0.05, Tukey multiple comparison). Data are mean ± SD (n=3–6).](#)
DISCUSSION

Genome sequence data have shown that *Synechocystis* 6803 contains only one sod gene, *sodB* (Kaneko et al., 1996). This suggests that FeSOD in *Synechocystis* 6803 scavenges all O$_2^-$, in contrast to other cyanobacterial species containing multiple SODs (Kim & Suh, 2005). The expression of FeSOD in WT *Synechocystis* 6803 was light dependent and was more active in cultures incubated under continuous light (50 μmol photons m$^{-2}$ s$^{-1}$) compared with those incubated under continuous dark (Kim & Suh, 2005). Nefedova et al. (2003) reported that *sodB* of *Synechocystis* 6803 could be knocked out by insertion inactivation under decreased illumination conditions with the addition of 10 mM NaHCO$_3$, 100 U catalase ml$^{-1}$ (or 100 μg BSA ml$^{-1}$) and 20 μg leucine ml$^{-1}$, and catalase required for the growth of mutants could be substituted successfully with BSA. However, in the present study, the mutants were still heterozygous after transferring for >1 year under conditions of decreased illumination of 5 μmol photons m$^{-2}$ s$^{-1}$ in the presence of 0.1% glucose, 10 mM NaHCO$_3$, 100 μg BSA ml$^{-1}$ and 20 μg leucine ml$^{-1}$. Immunoblotting and in-gel activity staining results showed that these mutants were still knockdown strains and *sodB* could not be knocked out by insertion of an antibiotic cassette (Fig. 1). The discrepancy is probably due to the different strains used. The basic ROS level between the glucose-tolerant (in this study) and glucose-intolerant WT (Nefedova et al., 2003) *Synechocystis* 6803 cells could be distinct, which may lead to their different requirements of SOD.

When the PpetE-*sodB* strain was cultured in copper-starved medium under illumination of 45 μmol photons m$^{-2}$ s$^{-1}$, the chlorophyll a content decreased significantly and the strain nearly died during 8 days of growth (Fig. 3), and chlorophyll a content declined modestly when the culture light intensity was decreased to 30 μmol photons m$^{-2}$ s$^{-1}$ (data not shown). These results indicated that a decrease in *sodB* expression led to a remarkable decrease in chlorophyll a content and light tolerance. A previous study had reported that there are three FeSODs (FSD1, FSD2, and FSD3) in *Arabidopsis thaliana* (Myouga et al., 2008). A heteromeric protein complex formed by FSD2 and FSD3 defended chloroplast nucleoids against oxidative stress, and was essential for chloroplast development in *Arabidopsis* (Myouga et al., 2008). Hence, FeSOD could probably influence the synthesis or degradation of chlorophyll a, and this effect is regulated by light intensity in *Synechocystis* 6803. Most chlorophyll a in photosynthetic organisms serves as light-harvesting antennae, and some chlorophyll a in the PS...

![Fig. 6.](image-url) Effect of strong light (1000 μmol photons m$^{-2}$ s$^{-1}$) on F$_r$/F$_m$ of the WT, sodB$^-$ mutant and its sodC-complemented strain in the absence (open symbols) or presence (closed symbols) of lincomycin (Lin; 100 μg ml$^{-1}$). Exponential growth cultures with OD$$_{730}$ 0.4 were exposed to the strong light and withdrawn at the indicated times for F$_r$/F$_m$ measurement. The 100% values corresponded to the values at time zero. Data are mean ± SD (n=3–6). Error bars indicate SD.

![Fig. 7.](image-url) Subcellular localization of FeSOD and Cu/ZnSOD in the *Synechocystis* 6803 sodC-complemented sodB$^-$ mutant. (a, b) Total protein extract (T) from transformed *Synechocystis* 6803 was separated into soluble (S) and crude membrane (CM) fractions by ultracentrifugation, and detected using antibodies against FeSOD (a) and Cu/ZnSOD (b); 40 μg total protein was loaded in each lane. (c–e) The crude membrane fraction was separated into the plasma membrane (PM) fraction and the thylakoid membrane (TM) fraction by sucrose density gradient centrifugation in combination with aqueous polymer two-phase partitioning and examined by immunoblotting using antibodies against Cu/ZnSOD (c), NrtA (d) and CP47 (e); 6 μg total protein was loaded in each lane.
reaction centres is indispensable for energy transduction. When sodB was knocked down in *Synechocystis* 6803, the activity of PSI and II would be compromised, especially PSI, because most of chlorophyll *a* was bound with PSI (Shen *et al.*, 1993; Jordan *et al.*, 2001; Zouni *et al.*, 2001). The damage to PSI in the *Synechococcus* sp. PCC 7942 sodB*"* mutant was more sensitive to oxidative stress than that of PSII (Herbert *et al.*, 1992). In the present study, Fv/Fm values of the PpetE-sodB strain were also decreased significantly when it was cultured in low copper (9.9 nM) medium compared with the same strain cultured in normal BG11 or the WT strain cultured in low copper (9.9 nM) medium (data not shown). This suggested that FeSOD in *Synechocystis* 6803 is involved in protecting both PSs against oxidative stress, as with MnSOD in *Anabaena* sp. PCC 7120 (Zhao *et al.*, 2007). Carotenoids also act as light-harvesting pigments involved in photosynthesis, and can dissipate excess energy of excited chlorophyll and eliminate ROS. Thus, they are efficient ROS scavengers (Latifi *et al.*, 2009). In this study, the ratio of carotenoid to chlorophyll *a* was increased when the PpetE-sodB strain was transferred to the copper-starved BG11 medium for 4 days, compared with that cultured in normal BG11 medium. This increased ratio of carotenoid to chlorophyll *a* could be a strategy to cope with oxidative stress under conditions of decreased sodB expression.

MV is a very-low-potential redox compound that accepts primarily electrons from the FA and FB iron–sulfur clusters of PSI centres at the expense of ferredoxin reduction (Fujii *et al.*, 1990; Blot *et al.*, 2011). By reacting very quickly with molecular oxygen, the reduced MV⁺ radical is reoxidized spontaneously back to MV with the formation of the membrane-impermeable O₂⁻. The vast majority of O₂⁻ is generated at the FA and FB centres of PSI in photosynthetic organisms after MV addition in light (Fujii *et al.*, 1990). NF is a carotenoid synthesis inhibitor, which inhibits phytene desaturase specifically, and thus blocks the synthesis of β-carotene and other carotenoids (Ben-Aziz & Koren, 1974; Kümmel & Grimme, 1975). Phytene desaturase is a key enzyme in carotenoid biosynthesis, responsible for the conversion of phytene into β-carotene (Martínez-Férez & Vioque, 1992). Carotenoids (β-carotene in particular) normally quench O₂⁻ in the chlorophyll antenna and thus the addition of NF promotes the formation of O₂⁻ within the thylakoid membrane. Both FeSOD and heterologously expressed Cu/ZnSOD played important roles in protecting *Synechocystis* 6803 against oxidative stress induced by MV or NF (Fig. 5).

The different localization of MnSOD and FeSOD in *Anabaena* sp. PCC 7120 suggests that they have different roles in protecting cellular molecules against O₂⁻ damage (Li *et al.*, 2002; Regelsberger *et al.*, 2002, 2004). In *Arabidopsis thaliana*, chloroplastic Cu/ZnSOD is located mainly in the stromal face of thylakoid membranes where the PSI complex is located (Ogawa *et al.*, 1995). So far, Cu/ZnSOD found in cyanobacteria is encoded by only one sodC gene and is predicted to be membrane associated. Using isolated thylakoid membranes and plasma membranes, we demonstrated that Cu/ZnSOD was localized in the soluble fraction and thylakoid membrane fraction of sodC-transformed *Synechocystis* 6803, although in lower quantities in the latter (Fig. 7b, c). The presence of Cu/ZnSOD in the thylakoid membrane fraction cannot be due to cross-contamination of the soluble fraction, since total membranes were washed three times prior to isolating different membrane fractions. Cu/ZnSOD has a predicted evident N-terminal signal peptide as shown by using the SignalP program 4.1 server, so it is likely on the lumen side of thylakoid membrane. FeSOD was detected only in the soluble fraction (Fig. 7a) and it does not contain any predicted signal peptide or transmembrane region, so it should be in the cytoplasm. This spatial separation of Cu/ZnSOD and FeSOD in cells may result in the observed lack of complementation of sodB⁻ mutants by the heterologously expressed sodC.

Inhibition of the activity of PSII under strong light is referred to as photoinhibition. This phenomenon is due to the imbalance between the rate of photodamage to PSII and the rate of the repair of damaged PSII (Nishiyama *et al.*, 2006). Nishiyama and co-workers have suggested that the PSI repair cycle is sensitive to oxidative damage (Nishiyama *et al.*, 2004; Kojima *et al.*, 2007; Ejima *et al.*, 2012), but PSII damaging reaction is not sensitive to oxidative damage (Nishiyama *et al.*, 2001). In the present study, when cells were exposed to strong light in the presence of lincomycin, PSII activity of the WT, sodB⁻ mutant and sodC complement strain declined at the same rate (Fig. 6). This result indicated that Cu/ZnSOD and FeSOD were not involved in protecting PSII against photodamage, which corroborated the conclusion that PSII damage was not sensitive to oxidative stress (Nishiyama *et al.*, 2001). In the absence of lincomycin, the sodC complement strain was more tolerant against strong light than the WT and sodB⁻ mutant, whilst the WT and sodB⁻ mutant did not show significantly different tolerances against strong light (Fig. 6). Combined with the effects of strong light on PSII activity of the three strains in the presence and absence of lincomycin, we concluded that Cu/ZnSOD plays some role in PSII repair by alleviating oxidative damage produced by strong light, whilst FeSOD was not involved in the repair of photodamaged PSII. A previous study reported that FeSOD provided little protection in photoinhibition (Herbert *et al.*, 1992), which is consistent with the present results. The different roles played by FeSOD and Cu/ZnSOD in photoinhibition may result from their different subcellular localization in the transformed *Synechocystis* 6803. Under illumination of PSII, O₂⁻ is generated at the acceptor side by reduction of molecular oxygen (Ananyev *et al.*, 1994; Song *et al.*, 2006). This O₂⁻ generated on the stromal side of PSII may migrate to the luminal surface through the equilibrium between the charged O₂⁻ and the uncharged protonated radical HO₂ (Asada, 1999), resulting in O₂⁻ being conveniently scavenged by membrane-associated Cu/ZnSOD (Henmi *et al.*, 2004; Zhang *et al.*, 2011). This was similar to previous findings that the membrane-associated MnSOD protects the photosynthetic apparatus of *Anabaena* sp. PCC 7120 (Zhao *et al.*, 2007).

In conclusion, as the sole SOD, FeSOD located in the cytoplasm of *Synechocystis* 6803 has fluid characteristics
and should be involved in scavenging all $O_2^-$ generated within the whole cell. FeSOD is essential for photoautotrophic growth of *Synechocystis* 6803. However, membrane-associated Cu/ZnSOD has not been found to exist as the sole SOD in any cyanobacterial species so far and it may function as an auxiliary SOD with high efficiency to remove $O_2^-$ generated around the thylakoid membrane. Hence, the coexistence of multiple SODs within one cell is advantageous for organisms to cope with various stresses and adapt to their complex niches.

**ACKNOWLEDGEMENTS**

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