Title

Prerequisite for highly efficient isoprenoid production by cyanobacteria discovered through the over-expression of 1-deoxy-D-xylulose 5-phosphate synthase and carbon allocation analysis

Running title

Isoprenoid production by cyanobacteria

Authors

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ABSTRACT

Cyanobacteria have recently been receiving considerable attention owing to their potential as photosynthetic producers of biofuels and biomaterials. Here, we focused on the production of isoprenoids by cyanobacteria, and aimed to provide insight into metabolic engineering design. To this end, we examined the over-expression of a key enzyme in 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in the cyanobacterium *Synechocystis* sp. PCC6803. In the DXS-over-expression strain (Dxs_ox), the mRNA and protein levels of DXS were 4-times and 1.5-times the levels in the wild-type (WT) strain, respectively. The carotenoid content of the Dxs_ox strain (8.4 mg/g dry cell weight [DCW]) was also up to 1.5-times higher than that in the WT strain (5.6 mg/g DCW), whereas the glycogen content dramatically decreased to an undetectable level. These observations suggested that the carotenoid content in the Dxs_ox strain was increased by consuming glycogen, which is a C-storage compound in cyanobacteria. We also quantified the total sugar (145 and 104 mg/g DCW), total fatty acids (31 and 24 mg/g DCW) and total protein (200 and 240 mg/g DCW) content in the WT and Dxs_ox strains, respectively, which were much higher than the carotenoid content. In particular, approximately 54% of the proteins were phycobiliproteins. This study demonstrated the major destinations of carbon flux in cyanobacteria, and provided important insights into metabolic engineering. Target yield can be improved through optimization of gene expression, the DXS protein stabilization, cell propagation depression and restriction of storage compound synthesis.

INTRODUCTION

Cyanobacteria are not only widely used as model phototrophs for basic biological studies but are also attractive candidates for use in bioindustrial applications because of their high photosynthetic capability. Cyanobacteria can convert captured solar energy into biomass in the field at efficiencies that generally exceed those of terrestrial plant (3%–9% for cyanobacteria compared with <0.25%–3% for terrestrial plants) (1, 2). Many attempts have been made using cyanobacteria to produce valuable bioindustrial compounds from CO₂, for example, alcohols (3-7), aldehyde (8), fatty acids (and their derivatives) (9-13), hydrogen (14-18), formic acid (19), sugars (20-22), polyhydroxyalkanoates (and precursors) (23-32), and isoprenoids (33-35). However, the yields of the desired products are often much lower than the theoretical yield or the total biomass.

The strategies used in previous attempts can be largely divided into three categories: (i) optimization of cultivation conditions (5, 21-27, 29, 30, 32), (ii) reengineering of the light reaction (photosystems and light harvesting systems) (18, 36) and dark reaction (Calvin cycle, especially ribulose 1,5-bisphosphate carboxylase/oxygenase) (8, 37, 38), and (iii) rerouting of carbon flux (metabolic engineering). In many cases, metabolic engineering efforts have been directed towards basic studies for (iii), because naturally-occurring cyanobacteria are not optimized for the production of the desired product. Previous metabolic engineering studies have examined several aspects, including the design of artificial pathways for desired product (3-13, 19, 20, 22, 32-34), the optimization of expression levels of the enzymes required for synthesis (13, 21, 32-34), the enhancement of cofactor or reductants supplies (18, 20, 39), circumvention of feedback inhibition (10), the destruction of competing processes

(6, 10, 13, 14, 18, 22, 32), genome-wide searches of genetic targets affecting productivity or cell growth (28, 31), and protein engineering (5, 16, 19). However, little is known about how the overall carbon flux in cyanobacteria is influenced by such gene manipulations. Based on previous reports, Ducat et al. (40) estimated that even in the remarkable studies, only 5%–6% of the carbon fixed through photosynthesis is used for the synthesis of the desired products, suggesting that there is plenty of room for improvement.

Why is the synthesis of the desired products so restricted in cyanobacteria? In comparison, the efficiency of the conversion of glucose into lactic acid by engineered yeast is often 80%, and other metabolically engineered organisms operate at 25%-50% of the theoretical maximum efficiency (40). There are many possible causes, for example, rapid degradation of the products, rapid inactivation of the enzymes responsible for synthesis, strong competing pathways, not enough space to store the products in the cell, not surplus carbons to accumulate the products due to exhaustion the construction of cell architecture and photosynthesis apparatus, and so on. These possibilities should be examined before attempting to optimize a particular pathway. In this study, we focused on the cyanobacterial production of isoprenoids, and aimed to find strategies to design a robust production system by considering above possibilities.

Isoprenoids are derived from two common building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in bacteria and cyanobacteria (41-45). Isoprenoids are a diverse group of compounds that have extensive applications as biofuels, chemical precursors, pharmaceutical and in aromatherapy (46-48). Although isoprenoids are industrially produced mainly through

organic synthesis and extraction from plants, biosynthesis using genetically modified organisms is expected to be a more economical and environmentally friendly way to produce isoprenoids.

Pioneering studies have been conducted to improve the production of isoprenoids in *Escherichia coli* (*E. coli*) (46, 48-51). In the latest report, Zhao et al (52) successfully produced 2.1 g/L of β -carotene with a yield of 60 mg/g DCW after 50–60 hours of cultivation. They first introduced an exogenous β -carotene synthetic gene operon and increased the supply of IPP and DMAPP by optimizing the expression levels of two key genes in the MEP pathway, 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) and isopentenyl diphosphate isomerase (*idi*). Then the best yield (2.1 g/L of culture, 60 mg/g DCW) was achieved by modulating genes involved in ATP synthesis, the pentose phosphate pathway and the TCA cycle.

In cyanobacteria, carotenoids are vigorously produced and mainly accumulate in the thylakoid membranes where the light reactions of photosynthesis occur (45). Therefore, it has been suggested that these compounds perform important functions in photosynthesis. β -Carotene and several xanthophylls are the constituents of functional multiprotein complexes, such as photosystems I and II (PSI and PSII), cytochrome *b₆/f* complexes, and the light-harvesting complexes involved in photosynthetic electron transport. In addition, carotenoids are thought to play important roles in quenching reactive oxygen species, dissipating excess light energy, and maintaining proper cellular architecture, among others (53-55). Therefore, cyanobacteria are excellent isoprenoid producers and powerful and practical isoprenoid production may be realized by applying the findings from metabolic engineering studies. We prepared a recombinant *Synechocystis* sp. PCC6803 (*S. 6803*) strain, in which a rate-limiting enzyme in the

MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS), is over-expressed, and determined the differences in the carotenoids, total sugars, total fatty acids and total proteins contents. Based on the results, we discussed a strategy for the design of cyanobacterial isoprenoid production systems.

MATERIALS AND METHODS

Strains and growth conditions

S. 6803 was cultivated at 25°C in BG-11 medium (56) buffered with 5 mM TES-NaOH (pH 8.0), at a photon flux density of 20 or 200 $\mu\text{mol of photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and bubbled with air. BG-11 medium was supplemented with 20 $\mu\text{g/mL}$ of kanamycin for kanamycin-resistant strains. The optical density at 730 nm (OD_{730}) and UV-visible absorption spectra were measured using a UV-VIS spectrophotometer (model UV-1800; Shimadzu, Kyoto, Japan).

Plasmids

To generate a plasmid to replace *psbA2*, which encodes one of three photosystem II D1 homologs (PsbA1, PsbA2 and PsbA3) and can be safely used for an integration site, with *dxs*, three fragments were amplified from the *S. 6803* genomic DNA; a 500-bp fragment upstream of *psbA2*, a 500-bp fragment downstream of *psbA2*, and *dxs* using the primers shown in Table S1. These three DNA fragments, together with a kanamycin resistance cassette, were inserted into pUC19 (TaKaRa BIO, Otsu, Japan) linearized with *Hind*III and *Eco*RI in the following order: *psbA2* upstream fragment $\rightarrow$ *dxs* $\rightarrow$ kanamycin resistance cassette $\rightarrow$ *psbA2* downstream fragment using the In-fusion cloning kit (TaKaRa BIO). The resulting plasmid, pPSBA2_dxs, was used to

replace the *psbA2* gene in the *S. 6803* genome with the *dxs* gene via double homologous recombination. (Fig. 1).

To over-express a C-terminal His-tagged DXS protein, the *dxs* gene was cloned into pET22b (Merk, Whitehouse Station, NJ) as an *NdeI* and *NotI* fragment, to construct pET_dxs-his.

Transformation of *S. 6803*

S. 6803 was grown in liquid BG-11 medium until the cell density reached an OD₇₃₀ of approximately 1.0. The cells were concentrated 10-fold by centrifugation. The concentrated cell suspension (500 µL) was spread on cellulose mixed ester filters (0.45µm; Merk), which were placed on BG-11 agar plates. Then, 100 ng of plasmid DNA was spotted on the filter, and incubated at 25°C under low light (10 µmol of photons·m⁻²·s⁻¹) for 24 h. The filter was transferred to a selective BG-11 plate containing 20 µg/mL kanamycin and incubated at 25°C in the light (20 µmol of photons·m⁻²·s⁻¹). Single colonies were isolated after about 2 weeks, and these were inoculated into BG-11 liquid medium for analysis.

Dxs-His protein purification

E. coli BL21(DE3) cells (TaKaRa BIO) were transformed with pET_dxs-his to overproduce His-tagged DXS (DXS-His). Cells were grown in 700 mL of Luria-Bertani medium (Nacalai Tesque, Kyoto, Japan) at 37°C. Expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (Nacalai Tesque), and incubation was continued overnight at 30°C. The cells were then harvested by centrifugation at 8,000 rpm for 7 min at 4°C, resuspended in 25 mL of A buffer (50 mM Tris-HCl, pH 7.6, and 500 mM

NaCl), and lysed by sonication. To remove the cell debris, the cell lysate was centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was mixed with 2 mL of COSMOGEL His-Accept (Nacalai Tesque), equilibrated with A buffer, and incubated for 10 min at 4°C. The slurry was poured into a column (1 × φ3 cm), and the flowthrough was discarded. After washing with A buffer, DXS-His was eluted with B buffer (50 mM Tris-HCl, pH 7.6, 500 mM NaCl, and 500 mM imidazole). Fractions containing DXS-His were pooled, and analyzed by SDS-PAGE (Fig. S1). The buffer in the purified protein was exchanged for phosphate buffered saline by using an Amicon Ultra 15 (Merk). The DXS-His protein solution was then send to antibody production services company (ITM, Matsumoto, Japan) to generate anti-DXS polyclonal rabbit antibody.

Real-time quantitative PCR

Transcriptional levels of the *dxs* gene were determined by real-time quantitative PCR (qPCR). Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The cDNA was prepared by using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Otsu, Japan). The qPCR experiment was performed on a StepOne cycler (Applied Biosystems, Foster City, CA) using the KAPA SYBR FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems, Woburn, MA). The transcriptional level of the *rrn16S* gene was used as an internal standard to normalize the qPCR values. Table S1 shows the primers used for qPCR.

SDS-PAGE and western blot analysis

For the western blot analysis, *S. 6803* crude extracts were prepared by bead

beating the cells in Tris-NaCl buffer (50 mM Tris-HCl, pH 7.6, and 500 mM NaCl) with a Protease Inhibitor Cocktail (Nacalai Tesque). After bead-beating, the insoluble material was removed by centrifugation. Then, the soluble fractions were separated by 10% SDS-PAGE, blotted onto PVDF membranes (Merk), and probed sequentially with a primary specific polyclonal antibody and a goat anti-rabbit IgG-Horse radish Peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) as a secondary antibody. The protein bands were visualized by using Pierce Western Blotting Substrate (Thermo Scientific, Waltham, MA) following the manufacturer's instructions.

Carotenoid analysis

Carotenoids were extracted from the cells with an acetone:methanol solution (7:2, v/v). After centrifugation, the pellet was discarded and the supernatant was evaporated under nitrogen gas. The dried carotenoids were dissolved in ethanol and filtered using a MS PTFE syringe filter (0.22 μ m, Merk). Carotenoids were separated on a Xbridge C18 column (150 mm $\times$ ϕ 4.6 mm, 3.5 μ m, Waters, Milford, MA) by high-performance liquid chromatography (HPLC) (Prominence; Shimadzu), using a 15-min gradient of ethyl acetate (0%–100%) in acetonitrile:water:triethylamine (9:1:0.01, v/v/v) at a flow rate of 1.5 mL/min. The absorption data 440 nm of the eluate were recorded. Carotenoid species were identified by comparing to the chromatograms from a previous study (57). The content of each carotenoid was calculated by using a β -carotene standard curve and the molar extinction coefficients (58).

Total fatty acids analysis

Lipids were extracted from a lyophilized *S.* 6803 pellet with

chloroform:methanol (2:1), according to the Folch method (59). We added 3 mL of chloroform:methanol to the lyophilized sample and vortexed the mixture for 24 h at room temperature. Then, 100 µg of nonadecanoic acid (19:0) (Nacalai Tesque) was added to each sample as internal standard to normalize the yield in the subsequent procedures. After removing the insoluble matter by centrifugation, the solvent was removed with an evaporator, while maintaining the evaporation temperature below 40°C. The residue in the evaporator, containing lipids, was dissolved in 0.40 mL of toluene, and then 3.0 mL of methanol and 0.60 mL of an 8.0% HCl solution in methanol were added in this order. One third of the mixture was transferred to a screw top glass bottle and incubated at 45°C for at least 40 h. After incubation, 1 mL of hexane and 0.2 mL of water were added to extract the fatty acid methyl esters (FAMES) into hexane. The hexane layer was analyzed by a gas chromatograph (Shimadzu GC 2014) equipped with an Rxi-5ms capillary column (15 m × φ0.25 mm, 0.20 µm) and a flame ionization detector (FID). The operating conditions were as follows: split ratio 1:5; inject volume 2 µL; helium carrier gas with a constant linear velocity of 49.6 cm/s; H₂ 55 mL/min, air 40 mL/min, make up gas (helium) 80 mL/min; injector temperature 250°C; detector temperature 330°C; and initial oven temperature 140°C (for 1 min) increased at a rate of 4°C/min to 220°C. The peak assignments were determined by comparing to the retention times of each standard. The concentration of each fatty acid was determined based on the calibration curve prepared by using various concentrations of C16:0 standards.

Total sugar analysis

The total sugar content was measured by the phenol-sulfuric acid method using

a glucose standard (60). *S.6803* pellets (from 10-mL culture) were suspended in 200 μ L of water and 200 μ L of a 5% phenol aqueous solution (w/v) in a glass test tube, and then 1 mL of concentrated sulfuric acid was added. The suspension was vortexed and incubated at room temperature for 30 min. The absorbance at 490 nm was measured with a spectrophotometer (Nanophotometer, Implen GmbH, München, Germany).

Soluble sugar composition

We used TLC to analyze the composition of the soluble sugars. For the TLC analysis, the soluble fraction was extracted from the cell lysate obtained by bead beating, which was then divided into two; one half was treated with glucoamylase (1 unit/mL) at 60°C and for 60 min, and the other half was incubated in the same way without glucoamylase. To remove the proteins, the samples were heated at 95°C and for 5 min and then centrifuged. The supernatant was concentrated with an evaporator and then applied to a TLC plate (TLC aluminum sheets, 20 cm $\times$ 20 cm, Silica gel 60 F254; Merk). Sugars were separated by using a solvent system consisting of 1-butanol:2-propanol:ethanol:water (3:2:3:2). The sugar spots were visualized by spraying with sulfuric acid solution [4% (w/v) H₂SO₄ in methanol] and heating the plates at 100°C for 5 min.

Glycogen assay

Intracellular glycogen accumulation was measured by using a glucose assay with enzymatic coloration. *S. 6803* pellets (from a 10-mL culture) were suspended in methanol, homogenized by sonication, and centrifuged. The pellet was resuspended in water and sonicated again. The suspension was incubated at 100°C for 40 min. After a

second centrifugation, the supernatant was used as an intracellular sugar extract. The extract was divided into two 50- μ L aliquots. To one aliquot, 49 μ L of 50 mM acetate buffer (pH 4.5) and 1 μ L of glucoamylase (200 units/mL) were added and the mixture was incubated at 60°C for 60 min to promote the glycolytic reaction, which converts glycogen to glucose. Another aliquot was incubated in the same way except that glucoamylase was not added. Both aliquots were then incubated at 95°C for 2 min to inactivate the enzyme. Next, 50 μ L of glucose assay solution (5 μ L of glucose oxidase, 1 μ L of peroxidase, and 44 μ L of substrate solution [10 mM sodium citrate, 1.6 mM 3,3',5,5'-tetramethylbenzidine $\cdot$ 2 HCl, 20% N,N-dimethylformamide, and 1.25% polyethylene glycol 4,000]) was mixed with 50 μ L of the aliquot. The mixtures were incubated at 37°C for 60 min. The reaction was stopped by adding 50 μ L of 1.5 M phosphate solution. The absorbance at 450 nm of the reaction solution was measured by using a microplate reader (Model 680; Bio-Rad). The amount of glucose was determined using a glucose standard curve. The amount of glycogen was estimated by subtracting the amount of glucose in the control aliquot from that in the treated aliquot.

Protein assay

S.6803 pellets (from a 5-mL culture) were suspended in 2.5 mL water and sonicated on ice for 4 min, and then centrifuged at 15,000 rpm for 5 min. The soluble fraction was used for the subsequent protein assay directly, whereas the insoluble fraction was subjected to the assay after solubilization using B-PER Reagent (Thermo Scientific). The protein concentration was determined by using the Pierce BCA Protein Assay Kit Reagent (Thermo Scientific), according to the manufacturer's Enhanced Protocol. Extracted proteins were also separated on SuperSep™ Ace gradient

SDS-polyacrylamide gels (WAKO). The protein bands were visualized with the Rapid Stain CBB kit (Nacalai Tesque) and the Sil-Best Stain-Neo for Protein and Nucleic Acid/PAGE (Nacalai Tesque). The band quantification was performed using ImageJ software (<http://rsbweb.nih.gov/ij/index.html>).

Transmission electron microscopy

The samples were immersed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, postfixed in 1% osmium tetroxide (OsO₄) for 15 min, dehydrated through a graded series of ethanol and embedded in Plain Resin (Nisshin EM, Tokyo, Japan). After polymerization of the resin, ultra-thin sections (60 nm) were obtained using an ultramicrotome (Super Nova; Reichert-Jung, Vienna, Austria), mounted on 200-mesh copper grids (Nisshin EM), stained with 10% (v/v) TI Blue (Nisshin EM) and 2% (w/v) lead citrate and observed under a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan). The areas of the cell sections and granules were measured with Image J.

RESULTS

The DXS over-expressing strain

A DXS over-expressing strain of *S. 6803* was generated after homologous recombination, replacing the *psbA2* gene in the genome with the *dxs* gene and the kanamycin-resistance cassette (Fig. 1). This DXS-over-expressing strain of *S. 6803* (Dxs_ox) was isolated on selective solid medium. The Dxs_ox strain possesses two *dxs* genes, one is its normal location in the genome and a second, newly introduced *dxs* gene downstream of the *psbA2* promoter, a strong, light-dependent promoter. No band

corresponding to the original *psbA2* gene was observed in the Dxs_ox strain by PCR using primers upstream and downstream of *psbA2*, confirming the complete segregation in the Dxs_ox strain. The *dxs* gene fragment amplified from the genome of the Dxs_ox strain was confirmed by DNA sequencing.

The photo-autotrophic growth rates of the WT and the Dxs_ox strains were evaluated by measuring the optical density at 730 nm (OD_{730}). The strains were grown at two light intensities, 20 and 200 $\mu\text{mol of photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (referred to as LL and HL, respectively). The growth curves for the WT and Dxs_ox strains in both light conditions were nearly identical (Fig. S2), indicating that the additional *dxs* gene had no significant influence on growth.

Over-expression of the *dxs* gene

Real-time quantitative PCR (qPCR) was used to assess the mRNA expression of the *dxs* gene in the WT and Dxs_ox strains. The relative mRNA levels normalized to that the level in the WT strain at $OD_{730} = 0.4$ under LL conditions are shown in Fig. 2. In all conditions, the mRNA levels in the Dxs_ox strain were higher than those in the WT strain. However, we also observed that the mRNA levels in the Dxs_ox strain reached a peak at $OD_{730} = 0.4$, and then declined sharply as the OD_{730} increased from 0.4 to 0.8, and remained at a low level thereafter.

Western blotting was performed to assess DXS protein expression. The ratios of the protein levels in the Dxs_ox strain to those in WT strain under the same conditions are shown in Fig.3. At an OD_{730} of 0.4 and 0.8, the DXS levels in the Dxs_ox strain were 1.3–1.6-fold higher than those in the WT strain, whereas at an OD_{730} of 2.5, no significant differences were observed between the strains under either light

conditions.

Carotenoid accumulation

Interestingly, we found an apparent difference between the WT and Dxs_ox strains in the color of the cultures (Fig. S3). Indeed, as shown in Fig. S4, the absorbance at 450–500 nm of the Dxs_ox strain grown to a density (OD₇₃₀) of 0.4 or 0.8 under HL conditions also was significantly higher than the corresponding absorbance of the WT strain. The 450–500 nm range overlaps with the absorbance band for carotenoids, strongly suggesting that the carotenoid content in the Dxs_ox strain is significantly increased. We extracted the carotenoids from cells harvested at an OD₇₃₀ of 0.4 and 0.8 under HL, and analyzed the carotenoid content by HPLC (Fig. S5). The concentration of each carotenoid was determined by using the following equation:

$$C_{car} = \left(\frac{E_{\beta ca}}{E_{car}} \times A_{car} \right) \times 0.000311 - 0.758$$

where C_{car} is the concentration (μg/mL) of a particular carotenoid in a particular extraction; $E_{\beta ca}$ and E_{car} are the extinction coefficients at 440 nm of β-carotene and a particular carotenoid, respectively; A_{car} is the peak area of a particular carotenoid in the chromatogram, which was recorded at 440 nm. Their relationship was estimated from a standard curve prepared using a β-carotene standard. The content of each carotenoid per dry cell weight (DCW) are shown in Fig. 4A. All carotenoids we examined, except for myxoxanthophyll at OD₇₃₀ = 0.4, were significantly higher in the Dxs_ox strain than in the WT strain. Therefore, the total carotenoid content in the Dxs_ox strain at OD₇₃₀ = 0.8 (8.4 mg/g DCW) was 1.6-fold higher than that in the WT strain (5.6 mg/g DCW).

It is worthwhile to note that the shift in the carotenoid content in cells growth to OD₇₃₀ = 0.4 and 0.8 differ between the two strains. In the WT strain, increases in

echinenone and β -carotene were observed instead of the decrease in myxoxanthophyll, whereas, in the Dxs_ox strain, an increase in myxoxanthophyll and a decline in zeaxanthin were observed, resulting in a predominance of myxoxanthophylls in the Dxs_ox strain at $OD_{730} = 0.8$, the largest difference (2.6-fold) between the two strains.

Lipid and sugar analyses

To examine the carbon localizations, the total sugars and total fatty acids were analyzed in the WT and Dxs_ox strains at $OD_{730} = 0.4$ and 0.8 under HL. As described in the Material and Methods, total fatty acids were quantified by a GC assay using FAMES forms of the extracted lipids. Peaks corresponding to six fatty acids, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3, which were considered to be main composition of fatty acids in *S. 6803* (61), were identified by the retention time of each standard. The concentration of each fatty acid was determined based on a calibration curve prepared with various concentrations of C16:0 standards. The major fatty acids were C16:0 (~55% of the total fatty acids) in both strains (15 mg/g DCW) (Fig. S7). Other fatty acids present were C16:1 (7.7%), C18:0 (1.8%), C18:1 (6.0%), C18:2 (10%) and C18:3 (17.7%). The proportions of each fatty acid were similar in both strains under both light conditions. The total fatty acids content in the WT and Dxs_ox strains at $OD_{730} = 0.8$ were 31 and 24 mg/g DCW, respectively (Fig. 5 and S7). The concentration of all fatty acids decreased by 10% ~21% in both strains as the OD_{730} increased from 0.4 to 0.8.

The total sugar content was quantified as glucose equivalents by the phenol sulfuric acid method using a glucose standard curve. The total sugar of the Dxs_ox strain at $OD_{730} = 0.4$ (104 mg/g DCW) was approximately 30% lower than that of the WT strain (146 mg/g DCW) (Fig. 5). Next, the composition of soluble sugars was

analyzed by TLC. In both strains, the supernatant of the lysate that was not treated with glucoamylase did not move from the starting point, except for a very weak, unidentified band (Fig. S8). This observation is essentially the same as that with the glycogen standard. In contrast, the glucoamylase-treated samples spots of both strains did migrate, leaving only a trace spot at the starting point, similar to glycogen standard, strongly suggesting that a large portion of the soluble sugars in S. 6803 is glycogen.

Then, the intracellular glycogen content was quantified by performing a glucose assay using the glucoamylase-treated lysates. The glucose content in the treated and untreated lysates of the Dxs_ox strain grown to an $OD_{730} = 0.4$ and 0.8 was below the limit of detection (>0.05 mg/g DCW) (Fig. 4B). In contrast, the glucose content in the WT strain, treated with glucoamylase was estimated to be 0.21 and 0.37 mg/g DCW at $OD_{730} = 0.4$ and 0.8 , respectively (Fig. 4B). However, the content of the untreated lysates of the WT strain was also below the limit of detection. These observations indicate the following: (1) the concentration of free glucose was very low (>0.05 mg/g DCW) in our experimental conditions; (2) the WT strain accumulates glycogen in the cell and the content significantly increased when the OD_{730} increased from 0.4 to 0.8 ; (3) the Dxs_ox strain does not accumulate glycogen.

Protein analysis

Total proteins were extracted from the soluble and insoluble fractions and analyzed by BCA Protein Assay and SDS-PAGE. As shown in Fig. 5, the total protein contents (soluble and insoluble fractions) at $OD_{730}=0.4$ and 0.8 were 360 and 200 mg/g DCW, respectively, in the WT strain, and 280 and 240 mg/g DCW in the Dxs_ox strain. The ratios of the contents in the soluble and insoluble fractions were similar among all

samples, at approximately 4:1~5:1. The CBB-stained bands in the SDS-PAGE analysis were quantified, and the phycobiliproteins with molecular weights of 10–20 kDa, i.e. allophycocyanin and phycocyanin, are major proteins (50-55%) in both strains (Fig. 6A). A detailed image of the proteins in the lower molecular weight range was obtained by higher density SDS-PAGE and silver staining, which suggested that the intensity of the band at approximately 11 kDa, which corresponded to the phycocyanin α -subunit, in the Dxs_ox strain grown to an OD₇₃₀ of 0.4 seemed to be slightly weaker (by 25%) than that in the WT strain (Fig. 6B and 6C).

Morphological comparison between the WT and Dxs_ox strains

To observe the changes in the cell structure directly, we took transmission electron microscopy images of ultra-thin sections of the cells (Fig. 7). As shown in Fig. 7A and 7B, the S-layers were successfully observed, and their thicknesses were measured (an example is shown using two arrows in Fig. 7A and 7B). Consequently, the means of the thickness were 24 ± 0.4 and 20 ± 0.1 nm in the WT and Dxs_ox strains, respectively, indicating the significant phenotypic difference. The obvious difference was also observed in the cell section areas between the WT and Dxs_ox strains (Fig. 7C and 7D). The three micrographs (28 x 28 μm) were randomly selected, and the areas of 100-130 cells with an area of more than 1 μm^2 were determined (Fig. S9). The resulting mass average areas were 2.2 ± 0.4 and 1.6 ± 0.1 μm^2 in the WT and Dxs_ox strains, respectively, indicating the Dxs_ox strain has a significantly smaller cell size (~73%) compared with the WT strain. The low-electron density granules (arrowheads in Fig. 7C and 7D), which are assumed to be polyphosphate or cyanophycin, were evaluated by using the ratio of the total granules area to the total cells area in a micrograph. The

average of the randomly selected three micrographs was $\sim 1.5 \pm 0.1$ and $\sim 1.9 \pm 0.4$ % in the WT and Dxs_ox strains, respectively, suggesting the significant higher accumulation of the low-electron density components in the Dxs_ox strain. Interestingly, many unidentified high-electron density granules with a diameter of ~ 50 nm (arrowheads in Fig. 7B) were observed in the vicinity of the outer membrane in the Dxs_ox strain, but rarely in the WT strain.

DISCUSSION

To examine the over-expression of the DXS protein in *S. 6803*, we introduced an additional *dxs* gene downstream of the *psbA2* promoter, resulting in a strain (Dxs_ox) with two copies of the *dxs* gene, the original gene regulated by the *dxs* promoter and a newly introduced one regulated by the *psbA2* promoter. As shown in Fig. 2, the mRNA levels in the Dxs_ox strain (transcribed from the two promoters) was higher in the early growth stage ($OD_{730} = 0.4$), but decreased rapidly as the optical density of the culture increased. The mRNA levels in the WT strain (transcribed from the original *dxs* promoter) were $\sim 1/4$ that of the Dxs_ox strain throughout the cultivation period, indicating that transcription from the original *dxs* promoter in the Dxs_ox strain was negligible due to the much lower transcription activity of the *dxs* promoter. Because induction of transcription from the *psbA2* promoter is light-dependent (62, 63), it is most likely that the rapid decrease in the mRNA level in the Dxs_ox strain is caused by deactivation of transcription in response to the decrease in the average amount of light received by a cell at higher cell densities (the self-shielding effect). Therefore, the influence of the new *dxs* gene on metabolic phenomena would appear more clearly in samples taken from an early stage culture

(OD₇₃₀ = 0.4 and 0.8).

Interestingly, although the profile of the DXS protein expression was similar to that of the *dxs* mRNA transcription, the differences in the DXS protein levels between the WT and Dxs_ox strains were more modest (a maximum difference of 1.6 fold) (Fig. 3). The amount of a particular protein in the cell is determined not only by the mRNA level, but also by the stability of the protein itself in the cell (which is affected by the frequency of misfolding or aggregation, and its tendency to be a target of quality-control mechanisms) (64-67). We found that the *S. 6803* DXS protein purified from *E. coli* was significantly likely to aggregate *in vitro* (more than 90% of the protein was insoluble after an overnight incubation at 4°C). It is known that *in vitro* protein aggregation rates are strongly inversely correlated with the half-life of the protein *in vivo* (68). Therefore, we assumed that the DXS protein is easy to be aggregated or disposed of by quality-control mechanisms *in vivo*. Indeed, a significant DXS signal was observed in the insoluble fraction by western blotting (data not shown), supporting our assumption. If further enhancement of DXS protein levels is required, the *S. 6803* DXS protein would need to be improved by directed evolution methods or a heterologous DXS would need to be introduced into *S. 6803*.

Although this modest increase in the DXS protein levels may be problematic for the over-production of isoprenoids, it did not inhibit the growth of the Dxs_ox strain at all (Fig. S2), which makes it easy to assess the carbon flux changes. The carotenoid analysis showed that the total carotenoid content in the Dxs_ox strain was approximately 1.5-fold that of the WT strain (Fig. 4A). This successfully demonstrated that the carotenoid content can be enhanced by increasing the expression level of the *dxs* gene, and that DXS is the enzyme that catalyzes the rate-determining step of carotenoid

synthesis in *S. 6803*. However, because the ratio of the increase in the carotenoid content was nearly identical to that of the increase in DXS protein content, we cannot conclude whether or not DXS is still the rate-determining enzyme in the Dxs_ox strain.

Interestingly, the shift of the carotenoid composition in the Dxs_ox strain significantly differed from that in the WT strain (Fig. 4A). Among four carotenoids, myxoxanthophyll in the Dxs_ox strain at OD₇₃₀=0.8 was prominent, in comparison with the corresponding carotenoid in the WT strain. Myxoxanthophyll, which is a unique in its glycoside moiety, is known to be a strongest photoprotectant and synthesized at high light conditions (69). In fact, in the WT strain, the myxoxanthophyll content at OD₇₃₀=0.4 was higher than that at OD₇₃₀=0.8 (Fig. 4A), suggesting that the synthesis of myxoxanthophyll was stopped at OD₇₃₀=0.8 due to the reduced light transparency by the self-shielding effect. On the other hand, in the Dxs_ox strain, the content increased slightly as the OD₇₃₀ increased from 0.4 to 0.8. The increase in myxoxanthophyll was also observed in the *S.6803* strain deficient in echinenone synthesis gene and the strain over-expressing enzymes involving in the carotenoid biosynthesis (35). Because echinenone and myxoxanthophyll are synthesized *via* the same precursor γ -caroten, the deletion of echinenone synthesis gene would lead to the increase in carbon flow toward the myxoxanthophyll synthesis. Taken together, we assumed that under the low light intensity (or at high optical density), the myxoxanthophyll synthesis is inhibited, but is resumed preferentially in the presence of the enhanced carotenoid precursors.

How the metabolic balance was influenced by the DXS over-expression is of interest. Because the growth rate of the Dxs_ox strain was nearly identical to that of the WT strain, its influence on cell viability would be limited. Most intriguingly, the glycogen content of the Dxs_ox strain decreased dramatically (Fig. 4B). Because

glycogen is known to be a carbon storage compound and can be considered as an indicator of the excess carbon flux, the remarkably low level of glycogen suggests that there are almost no excess carbons in the Dxs_ox strain. In addition, the content of another major storage compound, phycocyanin, also decreased by ~25% in the Dxs_ox strain at OD₇₃₀ = 0.4 (Fig. 6B and 6C). It is assumed that the Dxs_ox strain enhances carotenoid productivity at the expense of almost all of the glycerol and a portion of the phycocyanin.

The total carotenoid content was estimated to be only 8.4~8.6 mg/g DCW (less than 1% of the biomass) in the Dxs_ox strain, which raises the question, *where have the remaining carbons gone?* Therefore, we analyzed the total sugars (104 mg/g DCW), total fatty acids (24 mg/g DCW), and total proteins (240 mg/g DCW) (Fig. 5), and showed that they were much higher (12-, 2.9- and 28-fold) than that of the total carotenoids. The total sugars include both soluble and insoluble forms. Based on the TLC analysis, the soluble sugars were found to be almost all glycogen (Fig. S8); however this was negligible compared to the total sugars content, indicating that almost all sugars exist in insoluble forms, perhaps as cell wall components, such as the S-layer (70, 71). The fatty acids are also mainly derived from membrane components (72). Taken together, these observations suggest that 15% or more of the cell biomass is part of the cell membrane structure. The main component, the protein, was further analyzed by SDS-PAGE, and the results indicate that the major proteins (50~55%) in both strains were phycobiliproteins, i.e. allophycocyanin and phycocyanin (Fig. 6A) and the percentage of phycobiliproteins is calculated to be ~13% of the biomass. Phycobiliproteins are known to be not only a critical component of the light-harvesting complex, but also a storage compound in cyanobacteria. PHB is also known to be a

storage compound in cyanobacteria; however, in our culture conditions (BG11), it did not accumulate significantly. Based on all these results, we can conclude that under our experimental conditions, the major destinations of carbon are cell structures and phycobiliproteins.

As shown in Fig. 5, the contents of total carbohydrate and total fatty acid in the Dxs_ox strain were lower than those in the WT strain (30~40% and 10~20%, respectively), suggesting the differences in the cell wall (S-layer) or membranes. In fact, transmission electron microscopy images demonstrated that the thickness of the S-layer was reduced by approximately 17% in the Dxs_ox strain, explaining the reduced total carbohydrate content. Interestingly, the cell size was also reduced by approximately 27%, and more high-electron density granules adjacent to the outer membrane were observed in the Dxs_ox strain. At present, although the relationship among the DXS protein over-expression and these phenotype changes is unclear, it is likely that the changes in phenotype on the Dxs_ox strain reflect the changes in the contents of total carbohydrate and total fatty acid.

Based on the above, we can answer some of the questions we raised in the Introduction: “Does the enzymes responsible for synthesis inactivate rapidly?” “yes”; “Is there a strong competing pathway?” “yes (cell membrane and phycobiliproteins)”; “Is there not enough carbons to accumulate the products because of exhaustion for the construction of cell architecture or photosynthesis?” “partially, yes (S.6803 has excess carbons to store as phycobiliproteins)”. In conclusion, we proposed a strategy to remarkably increase the yield of isoprenoids. First, the stability of the DXS protein needs to be addressed. To this end, a gene encoding a stable exogenous DXS protein would need to be introduced or the S.6803 DXS protein needs to be engineered for

greater stability. Second, the production should be carried out in a cell state, in which the construction of cell structures is stopped. For example, under nitrogen starvation conditions, the various cell activities, including cell division, protein synthesis and photosynthesis, are weakened. If in such a state, the genes responsible for the production of the desired products and involved in photosystems and calvin cycles are placed under the control of promoters that work under nitrogen starvation conditions, a much higher yield could be achieved. Finally, downsizing the light-harvesting complex, including phycobiliproteins would be essential. These improvements would be prerequisites for maximum utilization of the most attractive quality of cyanobacteria, the high ability of the photo-biomass conversion.

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Figure legends

FIG. 1. Generation of a DXS over-expressing strain by integrating the *dxs* gene in the *S. 6803* genome. The DXS over-expressing strain of *S. 6803* was constructed by using the *psbA2* gene as the integration site for the introduction of the *dxs* gene and a kanamycin resistance gene. This integrated construct allows the *dxs* gene to be controlled under the *psbA2* promoter.

FIG. 2. The mRNA levels of the *dxs* gene. The mRNA levels of the *dxs* gene in the WT and Dxs_ox strains under the low light (LL) (A) and high light (HL) (B) conditions were analyzed by qPCR. The relative mRNA levels were normalized to that in the WT strain at OD₇₃₀ = 0.4 and under LL conditions. The data are the mean ± SD of three

replicates ($n = 3$).

FIG. 3. DXS protein expression. The DXS protein expression levels in the WT and Dxs_ox strains were confirmed by western blotting with an anti-DXS antibody. The ratios of the protein levels in the Dxs_ox strain to that in the WT strain at the same condition are shown. The data are the mean $\pm$ SD of **three replicates**.

FIG. 4. Carotenoid and Glycogen analyses. (A) **Carotenoid contents.** Carotenoids extracted from cells harvested at $OD_{730} = 0.4$ and 0.8 under HL conditions. The extracts were analyzed by HPLC. The data are the mean $\pm$ SD of three replicates ($n = 3$). Myx, Myxoxanthophyll; Zea, Zeaxanthin; Ech, Echinenone; and Bcar, β -carotene. DCWs at $OD_{730} = 0.4$ and 0.8 are shown in Fig. S6. (B) **Glycogen contents.** The **intracellular glycogen was measured by a glucose assay for the sonicated and glucoamylase-treated cell samples.** The data are the mean $\pm$ SD of two replicates ($n = 2$). No significant signals were observed in the WT strain (are displayed as “ND”).

FIG. 5. Biomass components. The accumulation of total sugars, total fatty acids, total carotenoids, and total proteins in the WT and Dxs_ox strains at $OD_{730} = 0.4$ and 0.8 under HL conditions was compared.

FIG. 6. Protein analysis. The protein samples extracted from 5 ml culture pellets were analyzed by SDS-PAGE. Protein bands were visualized with CBB (A) or silver staining (B). The intensities of the bands of ~ 11 kDa corresponding to phycocyanin (indicated by arrow in **Fig. 6B**) were quantified with ImageJ, and the ratio of WT at $OD_{730} = 0.4$: WT

at $OD_{730} = 0.8$: Dxs_ox at $OD_{730} = 0.4$: Dxs_ox at $OD_{730} = 0.8$ was 1 : 1.5 : 0.75 : 1.4
(C).

FIG. 7. Transmission electron microscope Images. (A and B) High-magnification views of the WT and Dxs_ox strains. According to the previous reports (70, 71), the most outer layer of cell (between two arrows pointing in the same direction) was likely to be S-layer. In the Dxs_ox strain, many high-electron density granules (arrowheads) were appeared in the vicinity of the outer membrane. Bar = 500 nm. (C and D) Low-magnification views of the WT and Dxs_ox strains. A few low-electron density granules (arrowheads) were observed in the both cell. Bar = 5 μ m.

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