Journal of Bioscience and Bioengineering
VOL. 118 No. 1, 20–28, 2014

www.elsevier.com/locate/jbiosc

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

Kai Kudoh,[1] Yusuke Kawano,[1,2] Shingo Hotta,[1] Midori Sekine,[1] Takafumi Watanabe,[1] and Masaki Ihara[1,3,*]

Faculty of Agriculture, Shinshu University, 8403 Minamiminowa, Nagano 399-4511, Japan,[1] Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan,[2] and Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan[3]

Received 20 September 2013; accepted 21 December 2013
Available online 4 February 2014

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.

[**Key words:** Cyanobacteria; Isoprenoid; Carotenoid; Metabolic engineering; 1-Deoxy-D-xylulose 5-phosphate synthase; Carbon flux; 2-C-Methyl-D-erythritol 4-phosphate pathway]

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 CO_2, 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

* Corresponding author at: Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan. Tel./fax: +81 265 77 1518.
E-mail address: m_ihara@shinshu-u.ac.jp (M. Ihara).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.12.018

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* (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 h 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 b_6/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 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 Strain PCC6803 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 μmol of photons·m^{-2}·s^{-1}, and bubbled with air. BG-11 medium was supplemented with 20 μg/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 strain PCC6803 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 → *dxs* → kanamycin resistance cassette → *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 strain PCC6803 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 (Merck, Whitehouse Station, NJ, USA) as an *Nde*I and *Not*I fragment, to construct pET_dxs-his.

Transformation of strain PCC6803 Strain PCC6803 was grown in liquid BG-11 medium until the cell density reached an OD_{730} 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 (0.45 μm; Merck), 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 8000 rpm for 7 min at 4°C, resuspended in 25 mL of A

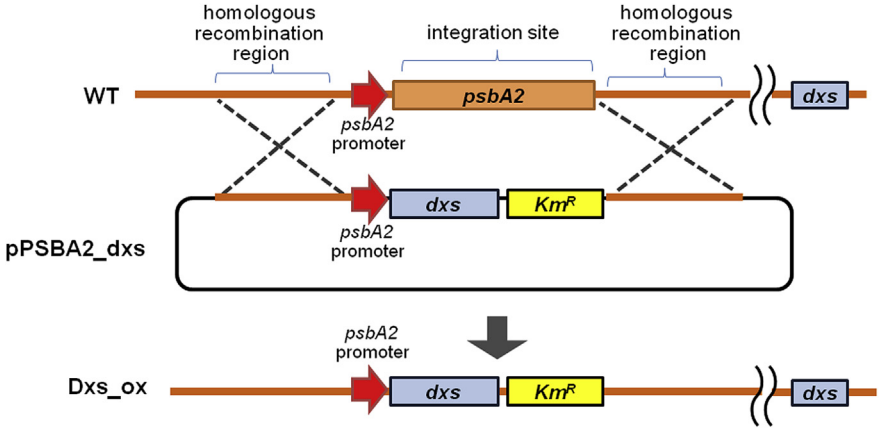

FIG. 1. Generation of a DXS over-expressing strain by integrating the *dxs* gene in the strain PCC6803 genome. The DXS over-expressing strain of PCC6803 was constructed by using the *psbA2* gene as the integration site for the introduction of the *dxs* gene and a kanamycin resistance gene. This integrated construct allows the *dxs* gene to be controlled under the *psbA2* promoter.