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Installing extra bicarbonate transporters in the cyanobacterium *Synechocystis* sp. PCC6803 enhances biomass production

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ABSTRACT

As a means to improve carbon uptake in the cyanobacterium *Synechocystis* sp. strain PCC6803, we engineered strains to contain additional inducible copies of the endogenous bicarbonate transporter BicA, an essential component of the CO_2-concentrating mechanism in cyanobacteria. When cultured under atmospheric CO_2 pressure, the strain expressing extra BicA transporters ($BicA^+$ strain) grew almost twice as fast and accumulated almost twice as much biomass as the control strain. When enriched with 0.5% or 5% CO_2, the $BicA^+$ strain grew slower than the control but still showed a superior biomass production. Introducing a point mutation in the large C-terminal cytosolic domain of the inserted BicA, at a site implicated in allosteric regulation of transport activity, resulted in a strain ($BicA^+_{(T485G)}$ strain) that exhibited pronounced cell aggregation and failed to grow at 5% CO_2. However, the bicarbonate uptake capacity of the induced $BicA^+_{(T485G)}$ was twice higher than for the wild-type strain. Metabolic analyses, including phenotyping by synchrotron-radiation Fourier transform Infrared spectromicroscopy, scanning electron microscopy, and lectin staining, suggest that the excess assimilated carbon in $BicA^+$ and $BicA^+_{(T485G)}$ cells was directed into production of saccharide-rich exopolymeric substances. We propose that the increased capacity for CO_2 uptake in the $BicA^+$ strain can be capitalized on by re-directing carbon flux from exopolymeric substances to other end products such as fuels or high-value chemicals.

1. Introduction

Some photoautotrophs including cyanobacteria have inducible mechanisms that allow the cells to raise the concentration of CO_2 at the carboxylation site of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco; EC 4.1.1.1.39) up to 1000-fold over that in the surrounding medium (Kaplan et al., 1980; Giordano et al., 2005). This capacity has evolved in response to decrease in the atmospheric CO_2 partial pressure (pCO_2) and increase in the pO_2 since emergence of cyanobacteria. Under the present-day ambient conditions, the slow turnover rate of rubisco and its ability to utilize not only CO_2 but also O_2 as substrate impair the carboxylation efficiency of photosynthesis (Eisenhut et al., 2008; Raven et al., 2008). For aquatic photoautotrophs such as cyanobacteria and algae, the situation is exacerbated by the low availability of CO_2 in water, where it has a diffusion rate four orders of magnitude slower than in air and where bicarbonate (HCO_3^-) is the predominant dissolved inorganic carbon (DIC) species at circumneutral or slightly alkaline pH (Price, 2011). Details of the CO_2-concentrating mechanism (CCM) in cyanobacteria differ between species but the salient features, depicted in Fig. 1, include a series of HCO_3^- and CO_2 transporters and the carboxysome, a protein-enclosed organelle-like micro-compartment that houses (most of) the rubisco protein and also contains different isoforms of the enzyme carbonic anhydrase (CA; EC 4.2.1.1) (Cannon et al., 2010; Yeates et al., 2010; Price, 2011; Rae et al., 2013). DIC enters the cells mainly via HCO_3^-/Na^+ symports but also through diffusion of CO_2. To limit seepage of CO_2 from the cell, CA-harboring NADPH dehydrogenase (NDH) complexes in the thylakoid and plasma membranes protonate incoming CO_2 to HCO_3^- according to the dissociation reaction:

$$CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-. \quad (1)$$

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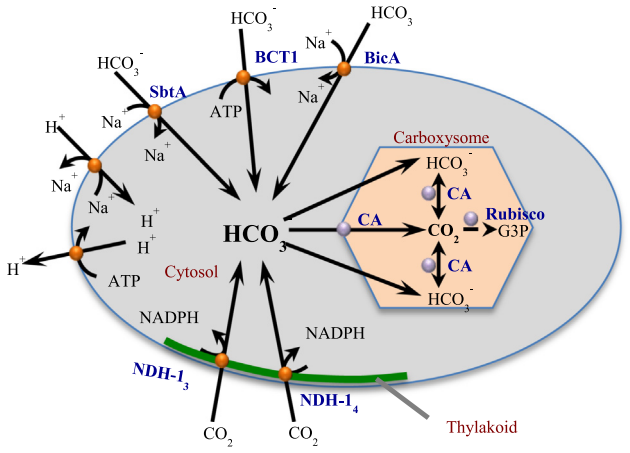

Fig. 1. The CCM in *Synechocystis* sp. PCC6803. The BicA, SbtA and BCT1 HCO_3^- transporters, and the redox-powered NDH-1_3 and NDH-1_4 CO_2 uptake systems are depicted. At low DIC levels, SbtA, BCT1 and NDH-1_3 are activated, allowing for efficient transport of HCO_3^- into the cytosol. Import of HCO_3^- into the carboxysome and its subsequent conversion to CO_2 by different carbonic anhydrase (CA) activities increases the local concentration of CO_2 around Rubisco. Na^+/H^+ antiports and H^+-ATPases are likely to be engaged to maintain intracellular Na^+ and pH homeostasis.

Under conditions of low DIC availability, e.g. in alkaline environments, when carbonate (CO_3^{2-}) species dominate over CO_2 and HCO_3^-, the CCM is activated, supporting effective flux of HCO_3^- into the cytosol (Fig. 1). The cytosolic HCO_3^- subsequently enters the carboxysome where it is converted by CA to CO_2 for the rubisco carboxylation (Reaction (1), Fig. 1). At non-limiting DIC concentrations, e.g. in a neutral pH environment or under $pCO_2 > 0.4\%$, the CCM is down-regulated to a basic, constitutive level, characterized by mainly CO_2 uptake (Price and Howitt, 2010; Ramanan et al., 2012). The cyanobacterium *Synechocystis* sp. strain PCC6803 (further *S.* 6803) harbors three different HCO_3^- transporters (Fig. 1). BicA (encoded by locus *sll0834*), a constitutive high-flux low-affinity Na^+/HCO_3^- symport, and SbtA (encoded by locus *slr1512*), an inducible low-flux high-affinity Na^+/HCO_3^- symport, are single-component transporters. BCT1 (encoded by the *cmpAB (porB)CD* operon; *slr0040-44*), an inducible high-affinity uniport, is a multi-component ATP-dependent transporter. The inducible SbtA and BCT1 transporters are strictly regulated at the transcriptional level by the CcmR transcription factor, which senses intracellular levels of α-ketoglutarate and $NADP^+$ (Daley et al., 2012).

Utilization of cyanobacteria for photosynthetic conversion of CO_2 to fuels and chemicals through introduction of heterologous (e.g. monoterpene biosynthesis), or enhancement of endogenous (e.g. alkane biosynthesis) pathways results in increased carbon (C) and electron sink demand (the "pull" end of photosynthetic metabolism), especially when targeting high-yield production. Since photosynthetic source strength (the "push" end of photosynthesis, i.e. primary photochemistry and Calvin–Benson cycle), is often controlled by sink demand (Paul et al., 2001; Iwaki et al., 2006; McCormick et al., 2006; Jansson et al., 2010; Ducat et al., 2012; Oliver et al., 2013), mostly via sugar signaling (Jansson, 2005; Rolland et al., 2006), an increase in sink strength may in itself be enough to enhance overall photosynthesis. However, to make full use of the potential for photosynthetic production in cyanobacteria it will be necessary to maximize both the pull and push end of photosynthesis, and restructure the metabolic link between them.

Increasing photosynthetic source strength in cyanobacteria entails enhancement of light and inorganic C (C_i) uptake and light utilization. In the present study we aimed at enhancing C_i uptake in *S.* 6803 by installing additional copies of the single-component and high-flux HCO_3-transporter BicA. To be able to control expression of the introduced transporter, we used the *nir* operon promoter *nirP* that is induced in absence of ammonium in the medium (Suzuki et al., 1993) and that has been shown to drive expression of transgenes in *Synechococcus* sp. PCC7942 (Price et al., 2004; Long et al., 2010) and *S.* 6803 (Qi et al., 2005). The *S.* 6803 strain endowed with extra BicA transporters ($BicA^+$ strain) was investigated for growth properties and photosynthetic and metabolic performance.

Although the BicA activity is not regulated at the transcriptional level (Wang et al., 2004), it is likely controlled allosterically, possibly through reversible phosphorylation in the cytosolic C-terminal Sulfate Transporter Anti-Sigma (STAS) domain (Aravind and Koonin, 2000; Shelden et al., 2010), with suppression imposed by cell exposure to high C_i concentrations (Karagouni et al., 1990; Bloye et al., 1992). In an attempt to retain high HCO_3^- influx in C_i-replete *S.* 6803, we generated a second strain containing a threonine-to-glycine substitution in the phosphorylatable and highly conserved T485 (*S.* 6803 numbering; T489 in *Synechococcus* sp. PCC7002 Shelden et al., 2010) in the installed BicA transporter. The properties of this strain (*S.* 6803 $BicA^+_{(T485G)}$ strain) were compared with those of *S.* 6803 $BicA^+$ and the wild type (WT).

2. Materials and methods

The glucose-intolerant *Synechocystis* sp. strain PCC6803, obtained from The Pasteur Culture Collection (www. pasteur.fr) was referred to as the WT strain and served as the control.

2.1. Plasmids and gene constructs

Plasmid *p*KRP13 (Reece and Phillips, 1995) was a kind gift from Gregory Phillips, Iowa State University. DNA constructs were designed using the MacVector software (www.macvector.com) and synthesized by GenScript (www.genscript.com).

We used the *psbAI* gene as an integrative platform in the *S.* 6803 genome. The *psbAI* gene encodes the photosystem II reaction center protein D1 isoform shown to be beneficial only under marginal growth conditions, e.g. in microanaerobic environment (Sicora et al., 2009) or with strong light and high ammonium concentrations (Dai et al., 2014). Under growth conditions used in this study, we considered the *psbA1* gene as dispensable (Jansson et al., 1987) and used it as a neutral integrative platform. A 226 base pairs (bp) fragment upstream of *nirA* containing the *nirP* in *Synechococcus* sp. strain PCC7942 was designed to terminate with a multiple cloning site and was flanked with the *psbAI* fragments. Insertion of this construct into the pUC56-simple (Hu et al., 2013) generated the *p*FUEL70 plasmid (Supplementary Fig. S1). Using Kpn*I* restriction sites, the streptomycin/spectinomycin (Str/Spc)-resistance cassette was transferred from *p*KRP13 to *p*FUEL70 to create *p*FUEL70b (Supplementary Fig. S1).

The *bicA* gene (Supplementary Fig. S2) was amplified by PCR from the genomic DNA of *S.* 6803 by using the primer pair bicNdeI-F (5′-GGGGATATTGACATATGCAAATAACTAAC AAAATTCATTTTAGG-3′) and bicSpeI-R (5′-CGCCGGGGCACTAGTCAGTATGTGG-3′). Underlined bases indicate Nde*I* and Spe*I* restriction sites that were incorporated into the primer sequences. PCR amplification, performed with Phusion high-fidelity DNA polymerase (NEB, www.neb.com) at annealing temperature of 56.5 °C, yielded a 1723-bp product. The amplicon was digested with Nde*I* and Spe*I* (NEB), gel-purified (Qiagen MinElute Gel Extraction kit, www.qiagen.com), and ligated into the Nde*I*- and Spe*I*-digested *p*FUEL70b (NEB Quick Ligation™ kit), resulting in the *p*FUEL70b::*bicA*.

To disable allosteric inhibition of the BicA transport activity, we used site-directed mutagenesis to alter regulatory phosphorylation switch previously mapped to a conserved loop of the C-terminal