Installing Extra Bicarbonate Transporters in the Cyanobacterium *Synechocystis* sp. PCC6803 Enhances Biomass Production

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1 ABSTRACT

2 As a means to improve carbon uptake in the cyanobacterium *Synechocystis* sp. strain PCC6803,
3 we engineered strains to contain additional inducible copies of the endogenous bicarbonate
4 transporter BicA, an essential component of the CO₂-concentrating mechanism in cyanobacteria.
5 When cultured under atmospheric CO₂ pressure, the strain expressing extra BicA transporters
6 (BicA⁺ strain) grew almost twice as fast and accumulated almost twice as much biomass as the
7 control strain. When enriched with 0.5% or 5% CO₂, the BicA⁺ strain grew slower than the
8 control but still showed a superior biomass production. Introducing a point mutation in the large
9 C-terminal cytosolic domain of the inserted BicA, at a site implicated in allosteric regulation of
10 transport activity, resulted in a strain (BicA⁺_(T485G) strain) that exhibited pronounced cell
11 aggregation and failed to grow at 5% CO₂. However, the bicarbonate uptake capacity of the
12 induced BicA⁺_(T485G) was twice higher than for the wild-type strain. Metabolic analyses,
13 including phenotyping by synchrotron-radiation Fourier transform Infrared spectromicroscopy,
14 scanning electron microscopy, and lectin staining, suggest that the excess assimilated carbon in
15 BicA⁺ and BicA⁺_(T485G) cells was directed into production of saccharide-rich exopolymeric
16 substances. We propose that the increased capacity for CO₂ uptake in the BicA⁺ strain can be
17 capitalized on by re-directing carbon flux from exopolymeric substances to other end products
18 such as fuels or high-value chemicals.

- 19 Keywords
- 20 CCM, cyanobacteria, bicarbonate transporter, engineering

INTRODUCTION

Some photoautotrophs including cyanobacteria have inducible mechanisms that allow the cells to raise the concentration of CO₂ 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 CO₂ partial pressure ($p\text{CO}_2$) and increase in the $p\text{O}_2$ since emergence of cyanobacteria. Under the present-day ambient conditions, the slow turnover rate of rubisco and its ability to utilize not only CO₂ but also O₂ 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 CO₂ in water, where it has a diffusion rate four orders of magnitude slower than in air and where bicarbonate (HCO₃⁻) is the predominant dissolved inorganic carbon (DIC) species at circumneutral or slightly alkaline pH (Price, 2011). Details of the CO₂-concentrating mechanism (CCM) in cyanobacteria differ between species but the salient features, depicted in Fig. 1, include a series of HCO₃⁻ and CO₂ 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 HCO₃⁻/Na⁺ symports but also through diffusion of CO₂. To limit seepage of CO₂ from the cell, CA-harboring NADPH dehydrogenase (NDH) complexes in the thylakoid and plasma membranes protonate incoming CO₂ to HCO₃⁻ according to the dissociation reaction:

Under conditions of low DIC availability, e.g. in alkaline environments, when carbonate (CO₃²⁻) species dominate over CO₂ and HCO₃⁻, the CCM is activated, supporting effective flux of HCO₃⁻ into the cytosol (Fig. 1). The cytosolic HCO₃⁻ subsequently enters the carboxysome where it is converted by CA to CO₂ for the rubisco carboxylation (Reaction 1, Fig. 1). At non-limiting DIC concentrations, e.g. in a neutral pH environment or under $p\text{CO}_2 > 0.4\%$, the CCM is down-regulated to a basic, constitutive level, characterized by mainly CO₂ uptake (Price and Howitt, 2010; Ramanan et al., 2012). The cyanobacterium *Synechocystis* sp. strain PCC6803 (further *S. 6803*) harbors three different HCO₃⁻ transporters (Fig. 1). BicA (encoded by locus *sll0834*), a constitutive high-flux low-affinity Na⁺/HCO₃⁻ symport, and SbtA (encoded by locus

slr1512), an inducible low-flux high-affinity $\text{Na}^+/\text{HCO}_3^-$ 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).

Figure 1. The CCM in *Synechocystis* sp. PCC6803. The BicA, SbtA and BCT1 HCO_3^- transporters, and the redox-powered NDH-1₃ and NDH-1₄ CO₂ uptake systems are depicted. At low DIC levels, SbtA, BCT1 and NDH-1₃ are activated, allowing for efficient transport of HCO_3^- into the cytosol. Import of HCO_3^- into the carboxysome and its subsequent conversion to CO₂ by different carbonic anhydrase (CA) activities increases the local concentration of CO₂ around Rubisco. Na⁺/H⁺ antiports and H⁺-ATPases are likely to be engaged to maintain intracellular Na⁺ and pH homeostasis.

Utilization of cyanobacteria for photosynthetic conversion of CO₂ 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).

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.

Plasmids and gene constructs

Plasmid *pKRP13* (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 *psbAI* 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 *pFUEL70* plasmid (Supplementary Fig. S1). Using *KpnI* restriction sites, the streptomycin/spectinomycin (Str/Spc)-resistance cassette was transferred from *pKRP13* to *pFUEL70* to create *pFUEL70b* (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' – GGGGATATTGACCATATGCAAATAACTAAC AAAATTCATTTTAGG – 3') and *bicSpeI-R* (5' - CGCCGGGGCACTAGTCAGTATGTGG – 3'). Underlined bases indicate *NdeI* and *SpeI* 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 *NdeI* and *SpeI* (NEB), gel-purified (Qiagen MinElute Gel Extraction kit, www.qiagen.com), and ligated into the *NdeI*- and *SpeI*-digested *pFUEL70b* (NEB Quick Ligation™ kit), resulting in the *pFUEL70b::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 STAS domain of BicA (Aravind and Koonin, 2000). In *Bacillus subtilis*, a serine-to-alanine substitution of the phosphorylatable serine (S58) located in conserved loop of the STAS-domain antisigma-factor antagonist SpoIIAA was detrimental to regulatory activity of the protein (Diederich et al., 1994). Based on multiple alignment of the conserved loop in STAS domains (Aravind and Koonin, 2000), threonine at the position 485 (T485) in BicA of *S. 6803* (T489 in *Synechococcus* sp. PCC7002 (Shelden et al., 2010)) was homologous to S58 and therefore could potentially be involved in reversible phosphorylation and impose regulation upon the transporter. To substitute T485 with non-phosphorylatable glycine (Supplementary Fig. S2), we used an internal primer, *bicA-Gly-F* (5' - CCATTGTTGGAGTAggCGCTTCCCTGG - 3') and its reverse complement sequence *bicA-Gly-R*. Underlined bases indicate the glycine-coding nucleotide triplet, with lower cases indicating the introduced mismatches. Two separate PCRs with the

bicNdeI-F/bicA-Gly-R and bicA-Gly-F/bicSpeI-R primers (Ta 72°C) yielded two *bicA* fragments (1480 and 270 bp) bearing the threonine-to-glycine substitution. Fusion PCR with the overlapping amplicons as a template resulted in amplification of the full-length *bicA* sequence bearing the substituted nucleotides (*bicA*(T485G)). The *bicA*(T485G) sequence was ligated into pFUEL70b as above, resulting in the formation of pFUEL70b::*bicA*(T485G).

Cyanobacterial strains and growth conditions

Transformation of *S. 6803* with the pFUEL70b::*bicA* and pFUEL70b::*bicA*(T485G) constructs and propagation of the transformants on selective media were performed as described (Eaton-Rye, 2010) and resulted in the *psbI550*::*bicA*⁺ and *psbI550*::*bicA*⁺(T485G) mutant strains, further referred to as BicA⁺ and BicA⁺(T485G).

For cell cultivations, we used liquid BG11 medium (Stanier et al., 1971), supplemented with 8.8 mM ammonium (BG11-NH₄⁺) and buffered with 10 mM N-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.2) to prevent medium acidification. Liquid cultures were placed in Innova 44 shaking incubator at 30°C with 160 rpm agitation under continuous illumination of ~50 μmol photons m⁻² s⁻¹. Mutant cultures were supplemented with 50 μg mL⁻¹ streptomycin. For investigation of growth properties, cells were cultivated in 30 mL tubes, purged with air or air-balance gas mixtures containing 0.5% or 5% CO₂ (v/v) at a rate of 1 L gas min⁻¹ L⁻¹, or with no aeration (ambient). The nitrate-based medium (BG11-NO₃⁻ containing 8.8 mM NaNO₃) was used to induce the BicA constructs during the cultures growth. For strong induction of the BicA construct, the cells were transferred to BG11₀ medium containing no N source (N source was substituted with 49.6 g L⁻¹ NaCl), purged with air, and starved for N shortly prior to the analyses.

Pigments determination

For quantitative determination of cell phycobilliprotein content, 2 mL of a mid-log culture were harvested by centrifugation. Cells were re-suspended in 1 mL of phosphate buffer saline (10 mM, pH7.2) and lysed with glass beads (0.1 mm) using a FastPrep®-24 beadbeater (MP Biomedicals, www.mpbio.com) with nine breaking cycles of 30 sec alternated with chilling the sample on ice. Subsequently, the sample was centrifuged, the supernatant containing extracted pigments was collected, and absorbance of the supernatant was measured at 562, 615, and 652 nm using a Beckman DU 640 Spectrophotometer (Beckman Coulter Inc.,

www.beckmancoulter.com). Phycobilliprotein concentrations were calculated after Bennett and Bogorad (1973).

Chl was extracted from cell pellets (1 mL; OD₇₅₀ of ~1.5) with 100% methanol, and the concentration was estimated spectrophotometrically (Lichtentahler, 1987).

Microscopy

Cell sizes were averaged from at least 10 cells visualized at the transmitted light bright field mode with 1000 x magnification using an up-right Axioskop 50 (Carl Zeiss Microscopy, www.zeiss.com/microscopy/). Images were captured with the MacroFire_C camera (Optronics, <http://optronics.com/>) and the PictureFrame (TM) App1.2.3 software.

For cell counts, cells were serially diluted, collected on a 0.2- μ m Anodisc® membrane filter (Whatman), and counted at 1000 x magnification using Axioskop 50 (Carl Zeiss Microscopy). At least 8 fields were counted for at least 3 dilutions.

For lectin stains, control or N-starved cells were pelleted by gentle centrifugation at 2000 x g for 1 min, re-suspended in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.5, and added to a final concentration of 5 μ g mL⁻¹ with fluorescein isothiocyanate-conjugated lectins (Vector Laboratories, Burlingame, CA) concanavalin A (ConA), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin I (RCA I), and/or Ulex Europaeus agglutinin I (UEA I), specifically binding mannose, N-acetylglucosamine, galactose and N-acetylgalactosamine, or fucose respectively. The cells were gently agitated for one hour in dark at room temperature, washed twice with HEPES buffer, mounted on Polysine adhesion slides (ThermoFisher Scientific, www.thermofisher.com), and imaged with Zen 2010 software and 710 Laser Scanning Confocal Microscope (Carl Zeiss Microscopy) using 488 nm (lectin conjugate fluorescence) and 594 nm (cyanobacterial autofluorescence) lines.

For SEM, control or N-starved cells incubated with 100 mM HCO₃⁻ for 1 h were fixed at +4°C overnight with glutaraldehyde (4% v/v final concentration), mounted on a silicon wafer, washed twice with DDW, and let dry under air. The cells were dehydrated by graded series of ethanol washes and sputtered with 136A gold film (Polaron SC7640, Quorum Technologies Ltd., www.quorumtech.com). The imaging was performed with an Ultra-55 Field Emission Scanning Electron Microscope (Carl Zeiss Microscopy), operated in a secondary electron mode at 5 kV.

Oxygen evolution

Cells were cultivated in BG11-NH₄⁺ or BG11-NO₃⁻ infused with air and harvested at a mid-log phase (OD₇₅₀ 0.4 – 0.7). For strong induction of the introduced BicA constructs, cultures from BG11-NO₃⁻ were transferred to BG11₀ medium, infused with air, and incubated under standard cultivation conditions for 3 hours prior to the analysis. For determination of the oxygen evolution, not induced (BG11-NH₄⁺), mildly induced (BG11-NO₃⁻), and strongly induced (BG11₀) cells were harvested by centrifugation at 5000 x g for 5 min at room temperature, washed, and resuspended to a final Chl *a* concentration of 7.5-10 µg mL⁻¹ in low-C_i BG11₀ buffered with 10 mM BisTrisPropane buffer to pH 9.3. The cells were supplemented with 15 mM NaCl and allowed to reach the CO₂ compensation point at a photon flux density of 100-150 µmol photons m⁻² h⁻¹ (Salon et al., 1996) shortly prior to the analysis. Oxygen evolution was measured using a standard Clark-type electrode (Hansatech Oxygraph with a DW-1 chamber), under a constant light of 400 µmol photons m⁻² h⁻¹. As the BicA transporter was shown to be slow to respond to added HCO₃⁻ (Price et al., 2004), the maximal value of O₂ evolution per minute measured during the first 5 min was recorded.

ζ-potential measurement

Cells were transferred to BG11₀ supplemented with 10 mM NaHCO₃ or 100 mM NaHCO₃ and incubated under standard cultivation conditions for 3 h. Then the cells were gently pelleted by centrifugation, washed once with TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) to remove cations, resuspended in sterile ice-cold distilled water, split to two tubes, and kept on ice. CaCl₂ was added to one tube to a final concentration of 10 mM. The pH in both tubes was adjusted to 8.0 using FE20-FiveEasy™ pH meter (Mettler Toledo International Inc., Columbus, OH). Electrophoretic mobility of the cells was measured at +25 °C using Zetasizer Nano ZS (Malvern Instruments Ltd, UK) and automatically converted to ζ-potential using the Smoluchowski's equation.

DIC determination

Cells were starved for N as described above, resuspended to a final OD₇₅₀ of ~0.6 with BG11₀ buffered with 10 mM BTP to pH 9.3, and supplemented with 15 mM of NaCl and 10 mM NaHCO₃. The 300-mL cultures were incubated in static hermetically sealed 0.5-L polystyrene tissue culture flasks (Corning, www.corning.com) at 30 °C with 275 µmol photons m⁻² s⁻¹. Samples for DIC determination were withdrawn at time zero and after 40, 80, and 150 min of

incubation. DIC was determined according to Friederich et al. (2002) except that the N₂ carrier gas was replaced with pure O₂. The DIC values were standardized against a Dickson standard (batch #131, Marine Physical Laboratory, Scripps Institution of Oceanography, La Jolla, CA).

Quantification of the extracellular carbohydrate

Cells were starved for N as described above, supplemented with HCO₃⁻ to 10 mM or 100 mM final concentrations, and incubated under standard conditions for 1 h. Following vigorous vortex, cultures were centrifuged at 8000 x g for 5 min to pellet the cells. The collected supernatant was filtered through a 0.45-μm polyethersulfone disk membrane (VWR) to remove the residual cells and carbohydrates were precipitated with 2 vol. of ice-cold pure ethanol at -20 °C overnight. The carbohydrate pellet, obtained by centrifugation at 16,000 x g for 30 min at +4°C, was washed with ice-cold ethanol (95% v/v), dried under air flow, and resuspended in 100 μl DDW. Carbohydrate concentrations were determined according to Masuko (2005) with SpectraMax® 340PC384 Absorbance Microplate Reader (Molecular Devices).

Infrared Spectromicroscopy

Synchrotron radiation-based Fourier transform infrared (SR-FTIR) metabolic fingerprinting of the WT *S. 6803* and mutant strains *BicA*⁺ and *BicA*^{+(T485G)} was performed at the infrared beamline of the Advanced Light Source at the Lawrence Berkeley National Laboratory (<http://infrared.als.lbl.gov>). Ten μl of cells in saline solution (0.9% w/v NaCl) was placed on Si-wafer chips and all the free-flowing solution was removed. All SR-FTIR absorption spectra were collected in transmission mode at a spectral resolution of 4 cm⁻¹ with 16 co-added scans and a peak position accuracy of 1/100 cm⁻¹, which were followed by baseline removal, vector normalization, and multivariate principal component-linear discriminant analysis (PC-LDA) using MathLab (7.0) as described in Hu et al. (2013). Spectral interpretation was guided by literature (Tipson, 1968; Cabaniss and McVey, 1995; Brandenburg and Seydel, 1996; Kačuráková et al., 2000; Naumann, 2000).

RESULTS

*Construction of the *S. 6803 BicA*⁺ and *BicA*^{+(T485G)} strains*

Constructs containing the *bicA* and *bicA(T485G)* genes were introduced into *S. 6803* for integration into the *psbA1 (slr1181)* locus (Jansson et al., 1987), yielding the *BicA*⁺ and

BicA⁺_(T485G) strains. To allow for controlled expression of the introduced genes, they were placed under control of the *nir* promoter (*nirP*) (Qi et al., 2005), which can be activated by shifting the nitrogen (N) source from ammonia to nitrate, or by N starvation (Suzuki et al., 1993; Qi et al., 2005). Plasmid maps and sequences are shown in Supplemental Fig. S1 and S2. SDS-PAGE of N-starved cells is shown in Supplemental Fig. S3.

Growth characteristics

Liquid stocks of WT and BicA⁺ had similar Chlorophyll (Chl) *a* contents when Chl *a* concentrations were normalized to culture turbidity (OD₇₅₀). However, BicA⁺ cells were twice the volume of WT cells and contained ~15% more Chl *a* per individual cell (Supplementary Table SI). Interestingly, BicA⁺_{T485G} cells were the smallest but contained the highest concentration of Chl *a* (Supplementary Table SI).

For growth experiments, WT and engineered strains were cultivated in BG11-NO₃⁻ to induce BicA constructs in the BicA⁺ and BicA⁺_(T485G) strains.

Figure 2. Analysis of growth performance of *S. 6803* WT, BicA⁺, and BicA⁺_(T485G) cultures. A, Specific growth rate, measured as doubling time in exponential phase; B, Maximal turbidity

measured as optical density; and C, Maximal cell concentrations reached at a stationary phase of the cultures without gas aeration (ambient), or infused with air or air-balanced gas mixtures containing 0.5% or 5% CO₂. Data used for bar graphs are presented in Supplemental Table SII. The insert in C exemplifies 0.5% CO₂ cultures at stationary phase. D, Formation of aggregates was a common feature of BicA⁺_(T485G) cells.

To assess the effect of extra HCO₃⁻ acquisition capacity on cellular growth properties, DIC was provided either 1) with air infusion (0.038% CO₂) to yield medium DIC availability; 2) sparged with 0.5% CO₂ to supply DIC just above the CCM activation threshold; 3) sparged with 5% CO₂ to maintain high DIC availability. Alternatively, cells were grown in static non-aerated cultures to represent severe DIC limitation. In liquid cultures infused with air, the BicA⁺ strain grew almost twice as fast as the WT and reached ~30% higher biomass, measured as turbidity at 750 nm or as cell concentration (Fig. 2 and S4, Table SII). In cultures infused with 0.5% CO₂, the growth rate for the WT strain was twice as fast as in air cultures, while that for the BicA⁺ strain increased by 25% only (Fig. 2A, Table SII). Under these conditions, both strains had similar growth rates, however, the accumulation of biomass in the BicA⁺ culture was 70-100% higher than in the WT culture (Fig. 2; insert in 2C). When the CO₂ fraction in the infused gas mixture was raised to 5%, the growth rate decreased for both strains, more so for BicA⁺ than for the WT (Fig. 2A). Despite its slower growth rate, the period of active growth for BicA⁺ was longer than for the WT, resulting in higher production of cellular biomass (Fig. 2 and S4). Severely DIC-limited cultures (ambient, Fig. 2 and S4) grew slowly and generated low biomass, with no significant difference between WT and BicA⁺ strains. The BicA⁺_(T485G) strain exhibited approximately the same growth rates as the WT, except when infused with 5% CO₂ when it failed to grow (Fig. 2 and S4). Compared to the WT, maximal turbidity of BicA⁺_(T485G) cultures was similar in the absence of gas infusion and when infused with air, and slightly higher when infused with 0.5% CO₂ (Fig. 2B and Table SII). We noted that BicA⁺_(T485G) cells, despite vigorous mixing of the culture before sampling, often formed clumps, which might compound calculations of growth rates (Fig. 2D).

Oxygen evolution

To assess differences in HCO_3^- uptake capacity between the *S. 6803* WT, BicA^+ , and $\text{BicA}^+_{(\text{T485G})}$ strains, we measured their photosynthetic oxygen (O_2) evolution. Control WT, BicA^+ , and $\text{BicA}^+_{(\text{T485G})}$ cultures were cultivated in BG11- NH_4^+ medium to a mid-log phase and, hence, the engineered strains were not induced for the BicA constructs. For induction, the cultures were cultivated in BG11- NO_3^- . Shortly prior to the analyses, the cells were transferred to BG11₀ without DIC supplement and buffered to pH 9.3. At this pH, HCO_3^- is the obligate source for C fixation since only 0.018% of the total C_i is found in a form of CO_2 under chemical equilibrium. Therefore we assumed that HCO_3^- uptake was at or close to a 1:1 ratio to photosynthetic O_2 evolution (Price et al., 2004) up to the saturation of the carbon fixation. The solution was supplemented with 15 mM NaCl to support Na^+ -dependent activity of the BicA transporter (Price et al., 2004). Increasing amounts of NaHCO_3 were added to cell aliquots and the maximal O_2 evolution at $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was determined for the following 5 min and plotted as a function of HCO_3^- concentration. WT, BicA^+ , and $\text{BicA}^+_{(\text{T485G})}$ cultures showed similar responses to increasing DIC concentrations, with the highest rate of $300\text{--}350 \mu\text{mol O}_2 \text{mg Chl } a^{-1} \text{h}^{-1}$ reached at DIC concentrations $\geq 125 \mu\text{M}$ (Fig. 3). Moderate increase in O_2 evolution was measured for BicA^+ and $\text{BicA}^+_{(\text{T485G})}$ strains induced by cultivation in BG11- NO_3^- (data not shown). In an attempt to increase the induction magnitude, we starved the *S. 6803* WT, BicA^+ , and $\text{BicA}^+_{(\text{T485G})}$ cells for N for 3 h prior to O_2 evolution measurements. This short starvation yielded the highest capacity for HCO_3^- uptake, presumably by strongly inducing the BicA constructs in BicA^+ and $\text{BicA}^+_{(\text{T485G})}$, while having no profound effect on the WT. Supplemented with 5 mM HCO_3^- , $\text{BicA}^+_{(\text{T485G})}$ showed an O_2 evolution rate twice that of the WT, but rates remained similar at DIC concentration $<100 \mu\text{M}$ (Fig. 3). In the following experiments, we used short N starvation prior to the analyses as a means to obtain strong induction for the BicA constructs.

Figure 3. Indirect assessment of HCO_3^- consumption rate. The rate of photosynthetic O_2 evolution, measured as a function of DIC for cultures of *S. 6803* WT, BicA^+ , or $\text{BicA}^+_{(\text{T485G})}$ strains non-induced (left) or strongly induced for the BicA constructs (right).

Rate of bicarbonate uptake

To directly assess the maximal rate of HCO_3^- uptake, we monitored the concentration of DIC in the medium of *S. 6803* WT, BicA^+ , and $\text{BicA}^+_{(\text{T485G})}$ cultures over time. Cells were collected from mid-log cultures and strongly induced for the BicA constructs, as described above. The induced cells were transferred to BG11₀ medium buffered to pH 9.3, to minimize the fraction of CO_2 that could potentially be utilized by cyanobacteria and to obviate the impact of photosynthetic CO_2 uptake on DIC concentrations. DIC was determined for the media samples collected at time zero and 40, 80, and 150 min after introduction of the cells into the medium. The results showed an uptake rate of 1.33 ± 0.12 and 2.25 ± 0.44 mg DIC h^{-1} (10^7 cells) $^{-1}$ for the BicA^+ and $\text{BicA}^+_{(\text{T485G})}$ strains, respectively, as compared to 1.17 ± 0.34 mg DIC h^{-1} (10^7 cells) $^{-1}$ for the WT (Fig. 4).

Figure 4. Direct measurement of HCO_3^- consumption rate. The rate of HCO_3^- consumption was measured for *S. 6803* WT, and BicA⁺ or BicA⁺_(T485G) strains strongly induced for the BicA constructs. *Denotes statistically significant difference as compared to the WT control (t-test, $p < 0.05$).

Structural analysis

Scanning electron micrographs of the WT control, BicA⁺, and BicA⁺_(T485G) cells, with no induction for the BicA constructs, revealed only minor morphological differences between the strains (Fig. 5). Short N starvation had no profound effect on the WT cells, even when they were supplemented with 100 mM HCO_3^- (Fig. 5). In contrast, BicA⁺ and particularly BicA⁺_(T485G) cultures strongly induced for the BicA constructs accumulated noticeable quantities of extracellular material after a short incubation with 100 mM HCO_3^- . BicA⁺_(T485G) cells appeared mainly in aggregates, cemented by the exudate. A zoom-in micrograph of the BicA⁺_(T485G) cells showed “pancake-like structures” of the exuding material spreading over the cell surface (Fig. 5). To investigate the nature of the organic material exuded by the BicA⁺ and BicA⁺_(T485G) strains, we probed cells with lectins specific to carbohydrates in exopolymeric substances (EPS) often

produced by cyanobacteria (Sengbusch and Müller, 1983; Neu and Lawrence, 1997). $BicA^+$ and particularly $BicA^+_{(T485G)}$ cells induced for the BicA constructs were associated with strong signals from fluorescent lectins (Fig. S5) indicative of the presence of glucose, mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and fucose on surface of the cells. Only occasional staining was observed for WT cells, or for the $BicA^+$ and $BicA^+_{(T485G)}$ strains not induced for the BicA construct (Fig. S5). The ζ -potential values for strongly induced $BicA^+$ and $BicA^+_{(T485G)}$ cells suspended in double distilled water (DDW) were 20% and 30% more negative (-64.1 ± 1.51 and -69.3 ± 1.29 , respectively) than the value for the WT (-52.8 ± 1.7 mV). In presence of Ca^{2+} , which is readily absorbed by negatively charged residues of acidic polysaccharides, the negative ζ -potential for all cell types was partially neutralized, converging at around -14.4 ± 1.1 (Fig. 6). The magnitude of neutralization is indicative of the relative amount of acidic extracellular polysaccharides for each type of the cells.

Not induced

WT

$BicA^+$

$BicA^+_{(T485G)}$

Induced

WT

$BicA^+$

$BicA^+_{(T485G)}$

368 **Figure 5.** Ultrastructural analysis of *S. 6803* WT and engineered cells. Scanning
 369 electromicrographs of *S. 6803* WT, Bica⁺, and Bica⁺_(T485G) cells not induced (upper panel) or
 370 strongly induced (lower panel) for the Bica constructs and cultivated with 100 mM CO₂ for 1 h.
 371 Note aggregation of Bica⁺_(T485G) cells compared to Bica⁺. Scale bar = 1 μm.

372 **Figure 6.** The ζ-potential (mV) measured as electrophoretic mobility for *S. 6803* WT, Bica⁺, and
 373 Bica⁺_(T485G) cells strongly induced for the Bica constructs. The cells were gently harvested and
 374 resuspended in either double distilled water (DDW) or 10 mM CaCl₂ solution. Electrophoretic
 375 mobility was determined for each of the treatments. Error bars represent standard deviation
 376 (n=5).

Figure 7. Quantification of extracellular carbohydrates. The amount of extracellular carbohydrates, expressed in μm mannose-equivalents $(10^7 \text{ cells})^{-1}$, generated by strongly induced cells cultivated with 10 mM or 100 mM HCO_3^- for 1 h.

* Denotes statistically significant difference as compared to the control (t-test, $p < 0.05$).

Quantification of extracellular carbohydrates

Concentrations of extracellular saccharides exuded by BicA⁺ and particularly by BicA⁺_(T485G) cells incubated with 10 mM HCO_3^- were significantly increased compared to the WT (t-test, $p < 0.05$). When the HCO_3^- concentration shifted from 10 to 100 mM, accumulation of extracellular saccharides in the BicA⁺_(T485G) strain became even more dramatic (Fig. 7).

SR-FTIR metabolic fingerprinting

As a direct assessment of the effect of HCO_3^- uptake on the metabolic features of the different strains, cells were incubated with 10 mM or 100 mM HCO_3^- and assayed by synchrotron-radiation Fourier transform infrared (SR-FTIR) phenotyping (Holman et al., 2010) (Supplemental Figure S4). The cluster vector plots of the biomolecular fingerprint region ($1800\text{--}900 \text{ cm}^{-1}$) of the SR-FTIR spectra revealed differences in the metabolic products of the strains, both in abundance and in composition relative to the WT (Fig. 8). When *S. 6803* WT and the

strongly induced BicA⁺ and BicA⁺_(T485G) strains were incubated with 10 mM or 100 mM HCO₃⁻, the BicA⁺_(T485G) cells (Fig. 8, red traces) increased production of EPS rich in carbohydrates containing units of galactose, glucose, mannose, xylose (Fig. 8; diagnostic features in the 1200-900 cm⁻¹ spectral region marked yellow), which possess the functional groups characterized by the C=O bands of carboxylated anions near 1630 to 1600 cm⁻¹; the C-H of alkyl and alicyclic carboxylate groups near 1460 to 1400 cm⁻¹, and sulfonyl functional groups RS(=O)₂R' near 1370 to 1305 cm⁻¹ (Fig. 8, diagnostics indicated by black arrows) (Tipson, 1968; Kačuráková et al., 2000). For BicA⁺ cells supplemented with 10 mM HCO₃⁻, the increase in EPS productions was less pronounced than for BicA⁺_(T485G) but became more evident with 100 mM HCO₃⁻ (Fig. 8, green trace). Compared with BicA⁺_(T485G) and the WT strain, the EPS of BicA⁺ at 100 mM HCO₃⁻ was enriched with glycopyranose and aldopyranose derivatives (near 960 cm⁻¹), propionic and higher ester (C-O) moieties (1200-1170 cm⁻¹), benzoic and fatty acid (C-O) moieties (1150-1100 cm⁻¹), phosphate (P=O and PO₂⁻) moieties (1275-1200 cm⁻¹), and proteinaceous components (Fig. 8, diagnostic features in areas marked purple).

Figure 8. Metabolic phenotyping by SR-FTIR, a pair-wise analysis. Cluster vector plots of FTIR spectra in the biomolecular fingerprint region (1800-900 cm⁻¹) for BicA⁺ versus *S. 6803* WT (green trace), or BicA⁺_(T485G) versus *S. 6803* WT (red trace). As a direct assessment of the effect of HCO₃⁻ uptake, cells were incubated with 10 mM or 100 mM HCO₃⁻. Features in the 1200-900 cm⁻¹ region arise mainly from the vibrational modes of different functional moieties of the EPS (yellow background). The 1690-1520 cm⁻¹ region, represent mainly the vibrational modes of the amide I and II groups of the peptide component (purple background). Diagnostic peaks indicated by black arrows (see text). The more complicated feature (both positive and negative intensities) in the protein amide I region (1610-1690 cm⁻¹) reflects a shifting in the population of the protein secondary structure and a change of the overall state of order of the proteins.

DISCUSSION

Growth dynamics and biomass accumulation measured for *S. 6803* (Fig. 2) demonstrate that, under our experimental conditions, cells were C_i limited when purged with air containing atmospheric level of CO₂. The WT strain accumulated higher biomass and grew almost twice as

fast in cultures enriched with 0.5% CO₂ as compared to air. A similar stimulation in growth rate and biomass accumulation was obtained for air cultures by endowing cells with extra copies of the HCO₃⁻ transporter BicA (strain BicA⁺). Even with extra BicA transporters, however, air-grown *S. 6803* was somewhat C_i limited, as growth rate and particularly biomass production of the BicA⁺ strain further increased when cultivated with 0.5% CO₂ as compared to air, possibly demonstrating the low-affinity nature of BicA. There was no obvious difference in growth rate between strains in static non-aerated cultures, which, again, may reflect the low affinity of the BicA transporter. Increasing the CO₂ concentration to 5% reduced the growth rate for WT and BicA⁺ strain compared to 0.5% CO₂ (Fig. 2A). Although cyanobacteria are commonly considered to be tolerant to high *p*CO₂, the response to increased CO₂ levels differs widely between cyanobacterial species (Miyachi et al., 2003), and *S. 6803* has been shown to exhibit only moderate tolerance to high CO₂ concentrations (Thomas et al., 2005). The reason(s) for high-CO₂ toxicity in many autotrophs, including plants, algae, and cyanobacteria, is not fully understood but is likely to be due to the impairment of the rubisco enzyme and the Calvin-Benson cycle by the acidic conditions generated by high CO₂ concentrations (Miyachi et al., 2003).

As opposed to the BicA⁺ strain, no increased growth rate over the WT was observed for the BicA^{+(T485G)} strain in air cultures (although we may have underestimated growth of the BicA^{+(T485G)} strain due to the flocculating nature of the culture). Reduced growth of BicA⁺ with 5% CO₂ as compared to the WT suggests that the presence of extra BicA transporters was detrimental under conditions of high C_i availability, and is in agreement with the notion that allosteric down-regulation of BicA does not obstruct its transport activity but rather decreases its affinity for the substrate ((Sültemeyer et al., 1998) and references therein). Failure of BicA^{+(T485G)} cells to grow under 5% CO₂ demonstrates that the threonine-to-glycine mutation of T485 in the C-terminal STAS domain of BicA is lethal under high-CO₂ conditions. Previous studies suggested that the C-terminal cytoplasmic STAS domain is involved in protein-protein interaction and contains a putative phosphorylation site (Shelden et al., 2010; Price, 2011). The potentially phosphorylatable threonine (T485) in BicA of *S. 6803* is homologous to serine (S58) in SpoIIAA of *Bacillus subtilis* (Aravind and Koonin, 2000), with the serine shown to undergo phosphorylation (Macek et al., 2007) and to play a crucial role in activity regulation of the anti-sigma factor SpoIIAA (Diederich et al., 1994). Additionally, fast decay of HCO₃⁻ uptake in *S.*

6803 shifted to high CO₂ conditions is accompanied by *in vivo* protein phosphorylation, suggesting specific inactivation of HCO₃⁻ transport activity and identifying phosphorylation as the regulatory switch of *p*CO₂-responsive HCO₃⁻ transport systems (Bloey et al., 1992). Altogether, previous knowledge suggests that STAS domain is involved in allosteric control of the BicA activity; under increased DIC levels it undergoes regulatory phosphorylation that suppresses HCO₃⁻ influx activity of BicA. Therefore, alteration of the regulatory phosphorylation switch in BicA disabled downregulation of its activity and the overexpressed BicA_(T485G) transporter presumably maintained high HCO₃⁻ influx even under elevated *p*CO₂, causing the lethal phenotype of BicA⁺_(T485G). As excess C_i can "leak" from a cell, we speculate that the lethal phenotype of BicA⁺_(T485G) was caused by excessive cellular uptake of Na⁺ rather than HCO₃⁻. Activity of the HCO₃⁻/Na⁺ symport, that is non-responsive to regulative feedback, dramatically increases intracellular Na⁺ concentration and may trigger a surge of H⁺ influx through Na⁺/H⁺ antiports. Genome annotation suggests the presence of five Na⁺/H⁺ antiports in *S. 6803* cells (Waditee et al., 2006), and Na⁺/H⁺ antiports are likely to be ancillary components of cyanobacterial CCMs and important for maintaining Na⁺ homeostasis (Fig. 1) (Waditee et al., 2006; Price, 2011). Superior performance of BicA⁺ over BicA⁺_(T485G) in air-infused cultures further implies that STAS domain-controlled regulation, in addition to being a *p*CO₂ gauge, may be responsive for intracellular Na⁺ and/or pH homeostasis. Further studies are required to elucidate the mechanism underlying failure of BicA⁺_(T485G) to grow under 5% CO₂ particularly and the detrimental effect of unregulated BicA activity in general.

When maximal rates of HCO₃⁻ consumption were assessed through the rate of O₂ evolution (Fig. 3), comparable values were measured for all strains when supplemented with <100 μM HCO₃⁻. A considerably higher capacity for HCO₃⁻ utilization was recorded for the strongly induced BicA⁺ and BicA⁺_(T485G) cells compared to the WT, when HCO₃⁻ concentrations were above the 100 μM threshold. These findings likely reflect the properties of the BicA transporter; under low to moderate HCO₃⁻ levels, the low-affinity activity of BicA is overshadowed by the activity of the high-affinity HCO₃⁻ transporters strongly induced under those conditions in all strains (BicA photosynthetic affinity K_{0.5}(HCO₃⁻) = 171 ± 15 μM (Price et al., 2004) as compared to ≤ 15 μM for higher affinity C_i transporters (Price and Howitt, 2010)), and under elevated HCO₃⁻ concentrations (>100 μM), the high-flux nature of the overexpressed BicA is manifested as a surplus photosynthetic C fixation. When the direct consumption of DIC from the growth medium

was measured in cultures strongly induced for BicA constructs (Fig. 4), BicA⁺_(T485G) demonstrated a statistically significant increase in DIC uptake rate compared to the WT, while the uptake for BicA⁺ was similar to the highly variable WT activity. We ascribe the high variability in DIC acquisition between biological replicates for the WT to stress caused by acute exposure to HCO₃⁻ (100 mM HCO₃⁻ is >500 times the C concentration in standard BG11) and potentially exaggerated by the effect of N starvation. This variable response of the WT strain is akin to the generally unstable growth in BicA⁺_(T485G) cultures (Supplemental Table SII), but here the exposure to excessive levels of HCO₃⁻ is likely caused by unregulated uptake of HCO₃⁻ by the BicA_(T485G) transporter. To the contrary, low variability in DIC acquisition between the BicA⁺ replicates implies metabolic preconditioning of BicA⁺ cells to increased C_i availability and demonstrates controlled DIC uptake at a rate above the WT average. Further studies including proteomic and metabolomic approaches are required to identify metabolic adjustments involved in the adaptation to increased C availability in *S. 6803*.

Increased C_i availability has been shown to trigger production of C-rich compounds such as glycogen (Stöckel et al., 2013) or EPS (De Philippis et al., 1996) in different strains of cyanobacteria. In agreement with their elevated photosynthetic HCO₃⁻ fixation, strongly induced BicA⁺ and BicA⁺_(T485G) cells supplemented with increased levels of DIC increased the accumulation of extracellular saccharides compared to the WT (Fig. 7). The extensive release of assimilated excess C to the external medium, which was particularly evident at the highest DIC levels (Fig. 7), is indicative of an “overflow metabolism” that has been well established in cyanobacteria under conditions of C surplus (De Philippis et al., 1996; Gordillo et al., 1998; Otero and Vincenzini, 2004; Cook et al., 2007).

Ultrastructural SEM and lectin stains analyses of induced BicA⁺_(T485G) cells confirmed the presence of copious EPS, which potentially contributed to formation of cell clumps in the culture (Fig. 2D, 5, and S5). For induced BicA⁺ and BicA⁺_(T485G), the increase in negative ζ-potential that could be neutralized by Ca²⁺ in a solute (Fig. 6) indicates that accumulation of negatively-charged EPS on the exterior cell surface was higher than for WT cells. The lack of aggregate formation in EPS-associated BicA⁺ cells (Fig. 5) may be indicative of differences in physicochemical properties of generated EPS compounds between BicA⁺ and BicA⁺_(T485G) cells. Based on molecular analysis of the FTIR cells spectra, we conclude with a 95% confidence (supplemental Fig. S6) that the EPS composition for BicA⁺ and BicA⁺_(T485G) strains differed

substantially; EPS formed by BicA⁺_(T485G) was more polyanionic in nature, with higher content of negatively charged residues, e.g., alkyl and alicyclic carboxylates and carboxylic acids, whereas a distinguishing feature of EPS in BicA⁺ cells was an abundance of phosphate moieties and inclusion of proteinaceous structures (Fig. 8). The more negative charge in BicA⁺_(T485G) EPS could conceivably enhance electrostatic interactions between EPS-associated particles and contribute to cell flocculation.

CONCLUSIONS

We show that the presence of extra BicA transporters in the *S. 6803* BicA⁺ and BicA⁺_(T485G) strains increased the capacity for HCO₃⁻ uptake. When cultured under atmospheric *p*CO₂, the BicA⁺ strain grew almost twice as fast as the control, and produced substantially more biomass. The BicA⁺ strain produced more biomass than the WT also at 0.5% and 5% CO₂ enrichment, although its growth rate deduced from the culture turbidity (Fig. S4) was comparable to that of the WT strain. The excess fixed C that was not assimilated into cellular biomass by the BicA⁺ and BicA⁺_(T485G) strains was funneled to the production of EPS. Phenotypic characterization of the BicA⁺_(T485G) strain was consistent with T485 in BicA's cytosolic STAS domain being involved in reversible phosphorylation as part of a feedback regulation of HCO₃⁻ transport activity by altering BicA's substrate affinity. The decreased overall fitness of the BicA⁺_(T485G) strain as compared to BicA⁺, demonstrates the importance of the endogenous control system that has evolved to fine-tune the CCM in cyanobacteria. Augmentation of physiological capacity of a cell through multiplication of its endogenous molecular components is a novel approach that enhances a desired trait of a harmonized metabolic system building upon its inherited resilience yet in a targeted manner. We advocate wider adoption of this approach in toolkit of metabolic engineering. Finally, we propose that the BicA⁺ strain, which exhibited increase in the photosynthetic source strength (the "push" end of photosynthesis), provides an ideal platform for engineering of the photosynthesis "pull" end to redirect the excess photosynthate from EPS to C-rich end products, e.g., photosynthetic fuel and chemicals.

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Figure 8**10 mM HCO_3^-** **BicA⁺ vs WT** **100 mM HCO_3^-** **BicA⁺ vs WT** **BicA⁺_(T485G) vs WT** **BicA⁺_(T485G) vs WT**